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Karyological studies in thirteen species of Zingiberacaeae from Tripura, North East India

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Abstract. Tripura being a state in North East India belongs to Indo-Burma bio-diversity hotspot and is considered as a centre of origin of many species of Zingiberaceae. *Alpinia calcarata*, *Alpinia malaccensis*, *Alpinia nigra*, *Amomum aromaticum*, *Amomum koenigii*, *Amomum maximum*, *Curcuma amada*, *Curcuma caesia*, *Curcuma longa*, *Curcuma picta*, *Hedychium coccineum, Hedychium coronarium* and *Hedychium thyrsiforme* are found in wild state in different geographical locations of Tripura. Their karyotypes were analyzed both at interspecific and intraspecific levels. The somatic chromosome number of *Alpinia* spp. and *Amomum* spp. was found to be 2n = 4X = 48. The *Curcuma* spp. represented by *C. amada* had 2n = 42 chromosomes and three other species *viz*., *C. caesia*, *C. longa* and *C. picta* had 2n = 63 chromosomes having X=21, indicating that polyploidy is a common feature in this genus. The somatic chromosome number of *Hedychium* spp. was found to be 2n = 34 chromosomes having a basic no. X = 17. Chromosomal data based clustering pattern and their sub-grouping at intra-specific level validates the taxonomic status of these species. Gower's similarity matrix is an indicator of cryptic changes leading genus specific karyotype conservatism.

Keywords. Indo-Burma bio-diversity hotspot, Zingiberaceae, Karyotype, Polyploidy, Cryptic changes.

INTRODUCTION

The State of Tripura (20°56'-24°34' North latitude and 91°10'-92°21' East longitude) is situated in the Sub-Himalayan region of North East India and belongs to Indo-Burma biodiversity hotspot of the world (Myers *et al.* 2000; Mao *et al.* 2009). The diverse vegetation of this region includes a good number of plants, which are endemic either to the state or to the North-Eastern region of India (Deb 1981). In India, the Zingiberaceae family includes 22 genera and about 200 species which are distributed in Andaman and Nicobar Island, Western Ghats and Eastern Himalaya region including North-Eastern India (Jain and Prakash 1995; Sabu 2006). Many natural useful products such as food, dyes, medicines, spices and condiments are obtained from the members of this family (Nayak 2002; Arambewela *et al.* 2004; Sahoo *et al.* 2014; Rahman and Islam 2015). The local people of South Asian countries also use

several plant products of Zingiberaceae in traditional medicines (Jayaweera 1982; Prakash and Mehrotra 1996; Gupta *et al.* 1999; Kala 2005; Bhuiyan *et al.* 2010; Roy *et al.* 2012; Sarangthem *et al.* 2013; Rajkumari and Sanatombi 2018). Some species of Zingiberaceae are also highly valued as ornamental plants because of their showy scented flowers (Jatoi *et al.* 2007; Gao *et al.* 2008). Deb (1983) recorded nine genera having 24 different species of this family from this phyto-geographical region. However, *Alpinia galanga*, *Hedychium ellipticum* and *Hedychium marginatum* could not be found in specific localities as was earlier reported by Deb (1983). Probably, these species have eventually perished due to anthropological disturbances. In-spite of such habitat destruction, five more species *viz*., *Curcuma caesia*, *Curcuma picta*, *Hedychium coccineum*, *Amomum aromaticum* (Syn. *A. jainii* S. Tripati & Vedprakash), *Amomum koenigii* (Syn. *Amomum corynostachyum*. Wall.) and *Amomum maximum* are found in different locations which were not reported by Deb (1983). Thus, 13 different species belonging to four genera *viz., Alpinia*, *Amomum*, *Curcuma* and *Hedychium* of Zingiberaceae are now available in Tripura. These are *Alpinia calcarata* (Haw.) Roscoe, *Alpinia malaccensis* (Burm.f.) Roscoe, *Alpinia nigra* (Gaertn.) Burtt, *Amomum aromaticum* Roxb. (Syn. *Amomum jainii* S. Tripati and Vedprakash), *Amomum koenigii* J.F.Gmel. (Syn. *Amomum corynostachyum* Wall.), *Amomum maximum* Roxb., *Curcuma amada* Roxb., *Curcuma caesia* Roxb., *Curcuma longa* L., *Curcuma picta* Roxb. ex Skornick, *Hedychium coccineum* Buch.-Ham. ex Sm., *Hedychium coronarium* J. Koenig. and *Hedychium thyrsiforme* Sm. Morphologically these species are quite distinct in their rhizome, floral and fruit characters (Deb 1983; Vanchhawng and Lalramnghinglova 2016). Delineation of plant species based on morphological characters alone is not adequate (Larsen and Smith 1978) and so, for characterization of species at inter- and intra-specific levels cytological tools are now effectively used to understand the taxonomic relationship and evolutionary patterns between and within species (Yoshikane and Naohiro 1991; Joseph *et al.* 1999). Previously, cytological works on Zingiberaceae were mainly focussed on *Curcuma* spp. and various researchers from time to time (Raghavan and Venkatasubban 1943; Chakraborti 1948; Sato 1948; Sharma and Bhattacharya 1959; Sato 1960; Ramachandran 1961,1969; Fedorov 1969; Prana 1977; Prana *et al.* 1978; Nambiar 1979; Mandi 1990; Ardiyani 2002; Skornickova *et al.* 2007; Bhadra and Bandhyopadhyay 2015; Lamo and Rao 2017; Bhadra *et al.* 2018) reported the existence of different ploidy levels, ranging from diploid (2n = 42) to tetraploid (2n=84), having a basic number X=21. In addition, aneuploid cytotypes in *Curcuma* spp. had also been reported (Das *et al.* 1999, Sugiura (1931, 1936), Sato 1960). Cytological studies previously carried out in *Alpinia* spp. and *Amomum* spp. were mainly concentrated on the determination of somatic chromosome numbers having 2n = 4X= 48 chromosomes (Raghavan and Venkatasubban 1943; Chakravorti 1948; Sharma and Bhattacharya 1959; Ramachandran 1969). But, from the literature it is evident that the detailed karyotype analysis in different species of *Alpinia* and *Amomum* was very meagre (Chen *et al.* 1988; Joseph 1998). In *Hedychium* spp. the occurrence of different ploidy levels (2n=34, 51 and 68) was reported in various cytological studies (Sharma and Bhattacharyya 1959; Bhattacharyya 1968; Ramachandran 1969; Mukherjee 1970; Mahanty 1970; Khoshoo 1979; Gao *et al.* 2008). Tripura being a part of Indo- Burma hotspot is considered as a centre of origin of many Indian species of Zingiberaceae but till date, cytological investigation of these species have not been carried out from this region. The present study is the first attempt to assess the karyotypic relationship in different species of Zingiberaceae at inter- and intra- specific level from the sub – Himalayan region of Tripura and for providing additional information to the chromosomal database of Zingiberean plants of Indian origin.

MATERIALS AND METHODS

Plant materials

We examined two populations (described as Pop-I and Pop-II) of 13 different wild species of Zingiberaceae *viz*., *Alpinia calcarata* (Haw.) Roscoe, *Alpinia malaccensis* (Burm.f.) Roscoe, *Alpinia nigra* (Gaertn.) Burtt, *Amomum aromaticum* Roxb. (Syn. *Amomum jainii* S. Tripati and Vedprakash), *Amomum koenigii* J. F. Gmel. (Syn. *Amomum corynostachyum* Wall.), *Amomum maximum* Roxb., *Curcuma amada* Roxb., *Curcuma caesia* Roxb., *Curcuma longa* L., *Curcuma picta* Roxb. ex Skornick, *Hedychium coronarium* J. Koenig., *Hedychium coccineum* Buch.-Ham. ex Sm. and *Hedychium thyrsiforme* Sm. growing in different locations in Tripura. They were grown in the experimental garden, Department of Botany, Tripura University for future research. All the plant species were independently authenticated by the taxonomy experts. Herbarium of each species has been submitted in Tripura University Herbarium with respective voucher numbers (Table S1).

Preparation of somatic chromosomes

The somatic chromosome preparation was carried out with modified aceto-orcein staining technique

(Sharma and Sharma 1980). Young healthy root tips of each species were pre-treated in a mixture of saturated solution of para dichloro-benzene (p-DB) and 0.002M 8- hydroxyquinoline (1:1) at 12-15^{0}C for 6 hrs. The root tips were then washed with distilled water and kept in acidulated alcohol (mixture of 1NHCl and absolute ethyl alcohol in 1:1 ratio) for 1 hour. Thereafter, root tips were kept in 45% acetic acid for 20 minutes. After a thorough wash with distilled water, root tips were treated with 5% cellulase (Sigma Cat. No. 22178) and 5% pectinase (Sigma Cat. No. 17389) mixture (1:1) in citrate buffer (pH - 4.8) for 3 hours at 37^{0}C. Enzyme treated root tips were then washed with double distilled water and stained with 2% aceto-orcein : 1NHCl (9:1) mixture for overnight and finally squashed in 45% acetic acid. The well spread metaphase plate was captured using Zeiss make AXIO (Lab.A1) Microscope and Zen software was used for determining the length of short and long arm of individual chromosome of the species studied.

Study of nucleoli in somatic cells

Nucleolar staining was carried out following the technique of Fernandez-Gomez *et al*. (1969). Initially root tips were fixed in 10% Formol :1% Hydroquinone (1:1) solution for 2 hrs. These were thoroughly washed in distilled water and immersed in 2% silver nitrate solution at 60°C in dark for overnight. $AgNO_3$ treated root tips were again kept in Formol-Hydroquinone (1:1) solution for 1 hour and finally squashed in 45% acetic acid.

Data analysis

In preparing the numerical data of karyotype, three well spread metaphase plates of each species were compared. In cases, where the length and the arm ratio varied the mean was taken to calculate the value of centromeric Index (F%). The centromeric Index, TF%, Inter-chromosomal asymmetry index (A_2), Coefficient of variation of chromosome length (CV_{CL}), and Mean Centromeric Asymmetry (M_{CA}) were calculated by the following formulae:Centromeric Index (F%) = S/(L+S)×100 (Levan *et al*. 1964);TF% = (ΣS/ΣCL) × 100 (Huziwara 1962);Inter-chromosomal asymmetry index (A_2) = s_{CL}/X_{CL} (Zarco 1986); Degree of karyotype asymmetry (A) = [Σ (L-S)/(L+S)]/n (Watanabe *et al*. 1999); Coefficient of variation of chromosome length (CV_{CL}) = A_2×100 (Paszko 2006); Mean Centromeric Asymmetry (M_{CA}) = A×100 (Peruzzi and Eroglu 2013); (S = Length of short arm, L = Length of long arm, CL = Chromosome length, s_{CL}- Standard deviation of chromosome length, X_{CL}- Mean of chromosome length, n – haploid number of chromosome complement).

Along with Stebbins asymmetry indices, the inter- and intra-chromosomal asymmetry indices were measured statistically (Zarco 1986; Watanabe *et al*. 1999) and the relationship among the species was explained by means of bi-dimensional scattered plot (Peruzzi and Eroglu 2013).To determine the karyological relationship among taxa UPGMA mediated dendrogram was constructed using the software Past 3.03 (Hammer 2013). In addition, multivariate Principal Co-ordinate Analysis (PCA) was also performed using different parameters of numerical data of karyotypes (Table 1) of the respective species and their populations (Peruzzi and Altinordu 2014).

RESULTS

Due to difficulty in obtaining suitable metaphase chromosome spreads with the conventional aceto-orcein staining technique, a new protocol has been developed to determine the somatic chromosome number and to analyze the detailed karyotype of all the taxa studied. The somatic chromosomes of *Alpinia* spp., *Amomum* spp., *Curcuma* spp. and *Hedychium* spp. could be classified into three distinct morphological types.

Type A**:** Short chromosomes (1.33 µm – 2.66 µm) bear two constrictions, primary and secondary, one is nearly median (m) to sub-median (sm) and the other is terminal (t) in position.

Type B**:** Chromosomes are short in size and their length range from 0.96 µm – 2.79 µm. The position of centromere is sub-median (sm) to median (M) (F% >33.33%).

Type C**:** Chromosomes are short in size and their length range from 1.86 µm – 2.13µm. The position of centromere is sub-median (sm) (F% <33.33%).

Analysis of karyotypes

Except in *Alpinia nigra*, which had two pairs of C type of submetacentric chromosomes, karyograms (Figure S1) of rest of the 12 species showed the presence of different combinations of A and B types chromosomes as classified in the present study. The detailed analyses of the karyotypes of Pop-I and Pop-II of *Alpinia* spp., *Amomum* spp., *Curcuma* spp. and *Hedychium* spp. reveal the following data:

Alpinia calcarata-Pop-I:

Somatic chromosome number 2n=48 (Figure 1a); Number of chromosomes bearing secondary constric-

Table 1. Karyological parameters for cluster analysis and Gower's similarity coefficient matrix.

Name of the species and Population	SCN	HCL (µm)	BCN	TF%	M_{CA}	CV_{CL}
Alpinia calcarata, Pop-I	48	45.22	12	42.61	30.00	28.44
Alpinia calcarata, Pop-II	48	45.60	12	42.44	30.23	27.71
Alpinia malaccensis, Pop-I	48	41.05	12	43.57	26.00	18.17
Alpinia malaccensis, Pop-II	48	42.87	12	43.65	25.41	17.79
Alpinia nigra, Pop-I	48	44.06	12	40.29	38.83	23.00
Alpinia nigra, Pop-II	48	44.14	12	40.27	38.91	22.45
Amomum maximum, Pop-I	48	33.06	12	44.75	20.76	18.61
Amomum maximum, Pop-II	48	32.93	12	44.79	20.84	17.82
Amomum aromaticum, Pop-I	48	30.61	12	45.39	18.45	21.75
Amomum aromaticum, Pop-II	48	30.57	12	45.52	17.94	20.93
Amomum koenigii, Pop-I	48	39.50	12	44.56	23.67	26.46
Amomum koenigii, Pop-II	48	39.32	12	44.28	22.87	26.48
Hedychium coronarium, Pop-I	34	20.72	17	47.32	10.72	15.91
Hedychium coronarium, Pop-II	34	20.78	17	47.24	11.06	15.11
Hedychium coccineum, Pop-I	34	26.40	17	46.68	13.27	15.51
Hedychium coccineum, Pop-II	34	26.46	17	46.47	14.11	15.40
Hedychium thyrsiforme, Pop-I	34	19.13	17	46.60	13.56	15.24
Hedychium thyrsiforme, Pop-II	34	19.70	17	46.61	13.69	14.88
Curcuma amada, Pop-I	42	31.05	21	47.02	11.91	12.52
Curcuma amada, Pop-II	42	32.35	21	46.96	12.17	12.77
Curcuma caesia, Pop-I	63	34.29	21	47.98	12.05	12.08
Curcuma caesia, Pop-II	63	34.37	21	48.11	11.35	12.08
Curcuma picta, Pop-I	63	44.67	21	47.96	12.23	13.16
Curcuma picta, Pop-II	63	44.43	21	47.94	12.34	12.66
Curcuma longa, Pop-I	63	44.16	21	47.01	16.78	11.18
Curcuma longa, Pop-II	63	44.04	21	47.13	17.23	11.01

SCN – Somatic chromosome number; HCL-Total chromosome length of the haploid complement; BCN- Basic chromosome number; TF% - Total Form percentage; M_{CA} - Mean Centromeric Asymmetry; CV_{CL} - Coefficient of variation of chromosome length.

tion - 2; Range of chromosome length – (1.06 µm – 2.79 µm); Total chromosome length – 90.43 µm; Ratio of largest and smallest chromosome – 2.63:1; Mean arm ratio (L/S) –1.38; Karyotype formula - $A_{2\ (2m)}\ B_{46\ (6M+30m+10sm)}$; Stebbins category – 1B; TF% - 42.61; Coefficient of variation of chromosome length (CV_{CL}) – 28.44; Mean centromeric asymmetry (M_{CA}) – 30.00.

Alpinia calcarata-Pop-II:

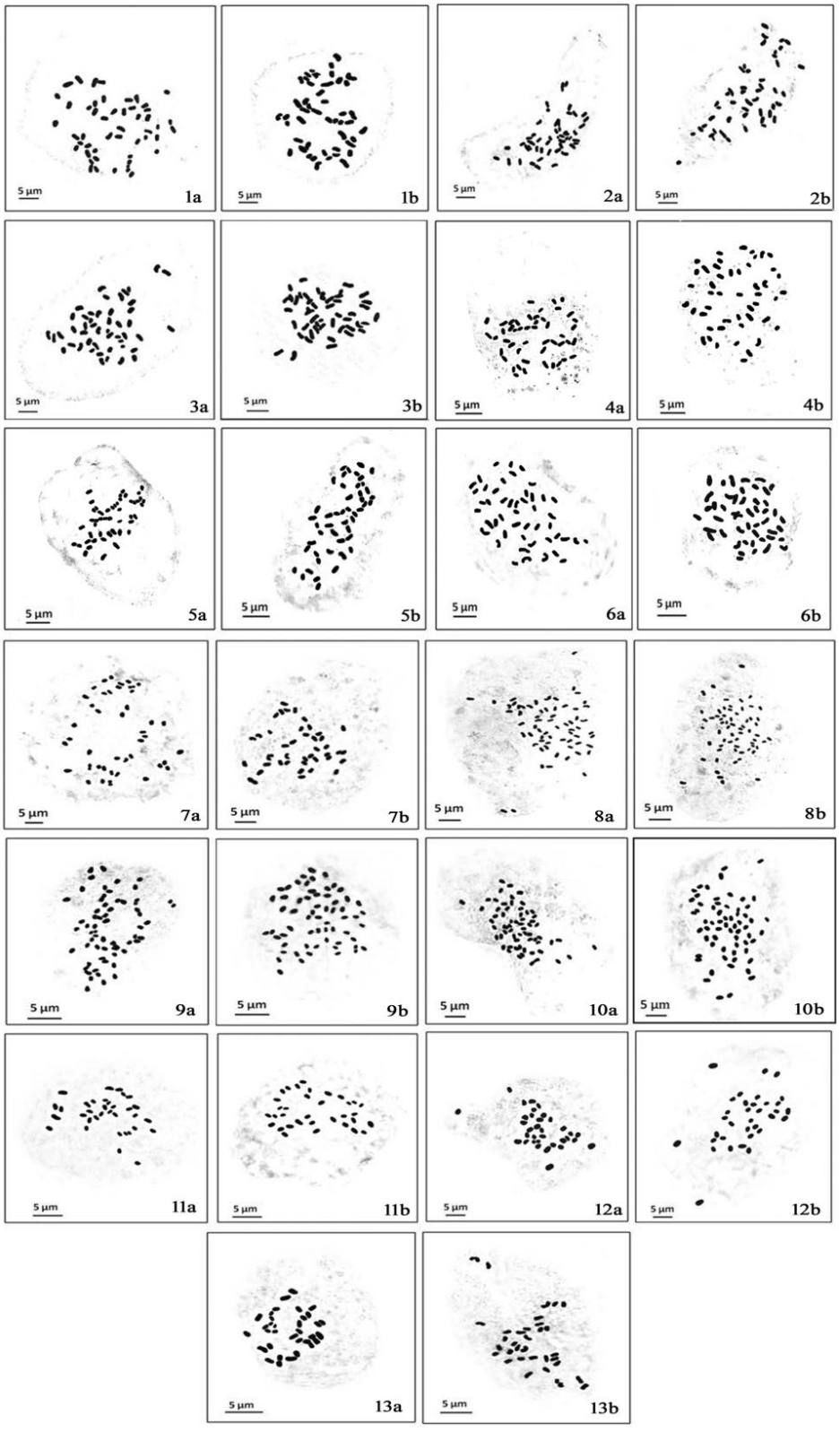
Somatic chromosome count 2n=48 (Figure 1b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 2.79 µm); Total chromosome length – 91.20 µm; Ratio of largest and smallest chromosome – 2.63:1; Mean arm ratio (L/S) –1.39; Karyotype formula - $A_{2\ (2m)}\ B_{46\ (6M+34m+6sm)}$ Stebbins category – 1B; TF% – 42.44; CV_{CL} – 27.71; M_{CA} – 30.23.

Alpinia malaccensis-Pop-I:

Somatic chromosome count 2n=48 (Figure 2a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.16 µm – 2.14 µm); Total chromosome length – 82.10 µm; Ratio of largest and smallest chromosome – 1.84:1; Mean arm ratio (L/S) – 1.33; Karyotype formula - $A_{2\ (2m)}\ B_{46\ (36m+10sm)}$; Stebbins category – 1A; TF% – 43.57; CV_{CL} – 18.17; M_{CA} – 26.00.

Alpinia malaccensis-Pop-II:

Somatic chromosome number 2n=48 (Figure 2b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.16 µm - 2.26 µm); Total chromosome length – 85.74µm; Ratio of largest and smallest chromosome – 1.95:1; Mean arm ratio (L/S) –1.33; Karyotype formula - $A_{2\ (2m)}\ B_{46\ (4M+36m+6sm)}$; Stebbins category – 1A; TF% – 43.65; CV_{CL} – 17.79; M_{CA} – 25.41.



Figures. 1a-13b. Mitotic metaphase chromosomes of thirteen Zingiberaceae species from Tripura. 1- *Alpinia calcarata*; 2- *A. malaccensis*; 3- *A. nigra*; 4- *Amomum maximum*; 5- *A. aromaticum*; 6- *A. koenigii*; 7- *Curcuma amada*; 8- *C. caesia*; 9- *C. picta*; 10- *C. longa*; 11- *H. coronarium*; 12- *H. coccineum*; 13- *H. thyrsiforme* (a – Pop - I; b – Pop - II); scale bars = 5µm.

Alpinia nigra-Pop-I:

Somatic chromosome number 2n=48 (Figure 3a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.09 µm – 2.80 µm); Total chromosome length – 88.12 µm; Ratio of largest and smallest chromosomes – 2.57:1; Mean arm ratio (L/S) – 1.57; Karyotype formula – $A_{2\ (2sm)}\ B_{42\ (4M+26m+12sm)}\ C_{4\ (4sm)}$; Stebbins category – 2B; TF% – 40.29; CV_{CL} – 23.00; M_{CA} – 38.83.

Alpinia nigra-Pop-II:

Somatic chromosome number 2n=48 (Figure 3b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.09 µm – 2.72 µm); Total chromosome length – 88.28 µm; Ratio of largest and smallest chromosome – 2.50:1; Mean arm ratio (L/S) – 1.57; Karyotype formula - $A_{2\ (2sm)}\ B_{42\ (4\ M+26\ m+\ 12\ sm)}\ C_{4\ (4\ sm)}$; Stebbins category – 2B; TF% – 40.27; CV_{CL} – 22.45; M_{CA} – 38.91.

Amomum maximum-Pop-I:

Somatic chromosome number 2n=48 (Figure 4a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 1.94 µm); Total chromosome length – 66.12 µm; Ratio of largest and smallest chromosome – 1.83:1; Mean arm ratio (L/S) –1.27; Karyotype formula - $A_{2\ (2m)}\ B_{46(14M+26m+6sm)}$; Stebbins category – 1A; TF% – 44.75; CV_{CL} – 18.61; M_{CA} – 20.76.

Amomum maximum-Pop-II:

Somatic chromosome number 2n=48 (Figure 4b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 1.94 µm); Total chromosome length – 65.86 µm; Ratio of largest and smallest chromosome – 1.83:1; Mean arm ratio (L/S) –1.26; Karyotype formula - $A_{2\ (2m)}\ B_{46(16M+24m+6sm)}$; Stebbins category – 1A; TF% – 44.79; CV_{CL} – 17.82; M_{CA} – 20.84.`

Amomum aromaticum-Pop-I:

Somatic chromosome number 2n=48 (Figure 5a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 2.10 µm); Total chromosome length – 61.22 µm; Ratio of largest and smallest chromosome – 1.98:1; Mean arm ratio (L/S) – 1.22; Karyotype formula - $A_{2\ (2m)}\ B_{46\ (16M+30m)}$; Stebbins category – 1A; TF% – 45.39; CV_{CL} – 21.75; M_{CA} – 18.45.

Amomum aromaticum-Pop-II:

Somatic chromosome number 2n=48 (Figure 5b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 2.10 µm); Total chromosome length – 61.14 µm; Ratio of largest and smallest chromosome – 1.98:1; Mean arm ratio (L/S) –1.21; Karyotype formula - $A_{2\ (2m)}\ B_{46(16M+30m)}$; Stebbins category – 1A; TF% – 45.52; CV_{CL} – 20.93; M_{CA} – 17.94.

Amomum koenigii-Pop-I:

Somatic chromosome number 2n=48 (Figure 6a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 2.39 µm); Total chromosome length – 79.00 µm; Ratio of largest and smallest chromosome – 2.25:1; Mean arm ratio (L/S) – 1.27; Karyotype formula - $A_{2\ (2m)}\ B_{46(12M+28m+6sm)}$; Stebbins categorization – 1B; TF% – 44.56; CV_{CL} – 26.46; M_{CA} – 23.67.

Amomum koenigii-Pop-II:

Somatic chromosome number 2n=48 (Figure 6b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 2.39 µm); Total chromosome length – 78.64 µm; Ratio of largest and smallest chromosome – 2.25:1; Mean arm ratio (L/S) –1.28; Karyotype formula - $A_{2\ (2m)}\ B_{46(8M+32m+6sm)}$; Stebbins category – 1B; TF% – 44.28; CV_{CL} – 26.48; M_{CA} – 22.87.

Curcuma amada-Pop-I:

Somatic chromosome number 2n=42 (Figure 7a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.35 µm – 1.91 µm); Total chromosome length – 62.10 µm; Ratio of largest and smallest chromosome – 1.41:1; Mean arm ratio (L/S) –1.13; Karyotype formula - $A_{2\ (2m)}\ B_{40\ (10M+30m)}$; Stebbins category – 1A; TF% – 47.02; CV_{CL} – 12.52; M_{CA} – 11.91.

Curcuma amada-Pop-II:

Somatic chromosome number 2n=42 (Figure 7b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.37 µm – 1.91 µm); Total chromosome length – 64.70 µm; Ratio of largest and smallest chromosome – 1.39:1; Mean arm ratio (L/S) – 1.14; Karyotype formula - $A_{2\ (2m)}\ B_{40(10M+30m)}$; Stebbins category – 1A; TF% – 46.96; CV_{CL} – 12.77; M_{CA} – 12.17.

Curcuma caesia-Pop-I:

Somatic chromosome number 2n=63 (Figure 8a); Number of chromosomes bearing secondary constriction - 3; Range of chromosome length – (0.96 µm –

1.33 µm); Total chromosome length – 68.58 µm; Ratio of largest and smallest chromosome – 1.39:1; Mean arm ratio (L/S) –1.09; Karyotype formula – $A_{3\ (3m)}$ $B_{60(30M+30m)}$; Stebbins category – 1A; TF% – 47.98; CV_{CL} – 12.08; M_{CA} – 12.05.

Curcuma caesia-Pop-II:

Somatic chromosome number 2n=63 (Figure 8b); Number of chromosomes bearing secondary constriction - 3; Range of chromosome length – (0.96 µm – 1.33 µm); Total chromosome length – 68.73 µm; Ratio of largest and smallest chromosome – 1.39:1; Mean arm ratio (L/S) – 1.08; Karyotype formula – $A_{3\ (3m)}$ $B_{60(33M+27m)}$; Stebbins category – 1A; TF% – 48.11; CV_{CL} – 12.08; M_{CA} – 11.35.

Curcuma picta-Pop-I:

Somatic chromosome number 2n=63 (Figure 9a); Number of chromosomes bearing secondary constriction - 3; Range of chromosome length – (1.10µm – 1.67 µm); Total chromosome length – 89.34 µm; Ratio of largest and smallest chromosome – 1.52:1; Mean arm ratio (L/S) – 1.10; Karyotype formula – $A_{3\ (3m)}$ $B_{60\ (42M+18m)}$; Stebbins category – 1A; TF% – 47.96; CV_{CL} – 13.16; M_{CA} – 12.23.

Curcuma picta-Pop-II:

Somatic chromosome number 2n=63 (Figure 9b); Number of chromosomes bearing secondary constriction - 3; Range of chromosome length – (1.10 µm – 1.63 µm); Total chromosome length – 88.86 µm; Ratio of largest and smallest chromosome – 1.48:1; Mean arm ratio (L/S) – 1.10; Karyotype formula – $A_{3\ (3m)}$ $B_{60\ (42M+18m)}$; Stebbins category – 1A; TF% – 47.94; CV_{CL} – 12.66; M_{CA} – 12.34.

Curcuma longa-Pop-I:

Somatic chromosome number 2n=63 (Figure 10a); Number of chromosomes bearing secondary constriction - 3; Range of chromosome length – (1.17 µm – 1.70 µm); Total chromosome length – 88.32 µm; Ratio of largest and smallest chromosome – 1.45:1; Mean arm ratio (L/S) –1.13; Karyotype formula – $A_{3\ (3m)}$ $B_{60\ (15M+45m)}$; Stebbins category – 1A; TF% – 47.01; CV_{CL} – 11.18; M_{CA} – 16.78.

Curcuma longa-Pop-II:

Somatic chromosome number 2n=63 (Figure 10b); Number of chromosomes bearing secondary constriction - 3; Range of chromosome length – (1.18 µm – 1.70 µm); Total chromosome length – 88.08 µm; Ratio of largest and smallest chromosome – 1.44:1; Mean arm ratio (L/S) – 1.13; Karyotype formula – $A_{3\ (3m)}$ $B_{60\ (15M+45m)}$; Stebbins category – 1A; TF% – 47.13; CV_{CL} – 11.01; M_{CA} – 17.23.

Hedychium coronarium-Pop-I:

Somatic chromosome number 2n=34 (Figure 11a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 1.65 µm); Total chromosome length – 41.14 µm; Ratio of largest and smallest chromosome – 1.56:1; Mean arm ratio (L/S) –1.18; Karyotype formula - $A_{2\ (2m)}$ $B_{32(18M+14m)}$; Stebbins category – 1A; TF% – 47.32; CV_{CL} – 15.91; M_{CA} – 10.72.

Hedychium coronarium-Pop-II:

Somatic chromosome number 2n=34 (Figure 11b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.06 µm – 1.60 µm); Total chromosome length – 41.56 µm; Ratio of largest and smallest chromosome – 1.51:1; Mean arm ratio (L/S) –1.18; Karyotype formula - $A_{2\ (2m)}$ $B_{32\ (16M+16m)}$; Stebbins category – 1A; TF% – 47.24; CV_{CL} – 15.11; M_{CA} – 11.06.

Hedychium coccineum-Pop-I:

Somatic chromosome number 2n=34 (Figure 12a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.32 µm – 2.08 µm); Total chromosome length – 52.80 µm; Ratio of largest and smallest chromosome – 1.58:1; Mean arm ratio (L/S) –1.22; Karyotype formula - $A_{2\ (2m)}$ $B_{32\ (18M+14m)}$; Stebbins category – 1A; TF% – 46.68; CV_{CL} – 15.51; M_{CA} – 13.27.

Hedychium coccineum-Pop-II:

Somatic chromosome number 2n=34 (Figure 12b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (1.32 µm – 2.08 µm); Total chromosome length – 52.92 µm; Ratio of largest and smallest chromosome – 1.58:1; Mean arm ratio (L/S) – 1.22; Karyotype formula - $A_{2\ (2m)}$ $B_{32\ (18M+14m)}$; Stebbins category – 1A; TF% – 46.47; CV_{CL} – 15.40; M_{CA} – 14.11.

Hedychium thyrsiforme-Pop-I:

Somatic chromosome number 2n=34 (Figure 13a); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (0.96 µm – 1.44 µm); Total chromosome length – 38.26 µm; Ratio of largest and smallest chromosome – 1.50:1; Mean arm ratio (L/S) –1.21; Karyotype formula - $A_{2\ (2m)}$ $B_{32\ (12M+20m)}$; Stebbins category – 1A; TF% – 46.60; CV_{CL} – 15.24; M_{CA} – 13.56.

Hedychium thyrsiforme-Pop-II:

Somatic chromosome number 2n=34 (Figure 13b); Number of chromosomes bearing secondary constriction - 2; Range of chromosome length – (0.96 µm – 1.44 µm); Total chromosome length – 39.40 µm; Ratio of largest and smallest chromosome – 1.50:1; Mean arm ratio (L/S) –1.21; Karyotype formula - $A_{2\ (2m)}\ B_{32\ (12M+20m)}$; Stebbins category – 1A; TF% – 46.61; CV_{CL} – 14.88; M_{CA} – 13.69.

DISCUSSION

Somatic chromosome number 2n=48 having a basic number X=12 was found to be constant in *Alpinia calcarata*, *Alpinia malaccensis* and *Alpinia nigra* as was reported by earlier researchers (Raghavan and Venkatasubban 1943; Chakravorti 1948, 1952; Ramachandran 1969; Chen 1988; Joseph 1998). The different proportions of metacentric and submetacentric chromosomes present in the somatic chromosome complements of these three species are in partial agreement with the reports of Joseph (1998). At intra-specific (Pop-I and Pop-II) level, the karyotype of each species is more or less homogeneous having one pair of chromosomes bearing secondary constriction, a characteristic of the karyotype itself. On the contrary, at inter-specific level, their karyotypes differ though the chromosomes are mostly metacentric and submetacentric in nature. According to Stebbins categorization, the karyotype of *A. malaccensis, A. calcarata* and *A. nigra* falls under category 1A, 1B and 2B, respectively. Therefore, the reduction in size of some of the chromosomes in relation to other has occurred in chromosome complements of *A. calcarata* and *A. nigra*. Moreover, the presence of two pairs of submetacentric chromosomes having F% < 33.33 in Pop-I and Pop-II of *A. nigra* indicates that their karyotypes are slightly asymmetric. Thus, in *Alpinia* spp. a tendency from symmetric to asymmetric karyotype is observed. The somatic chromosome count 2n=48 is the distinctive character of *Amomum* species which corroborates previous findings (Sharma and Bhattacharya 1959; Chen *et al*. 1988). Due to limited cytological studies in these three species, little information was available regarding the detailed chromosomal architecture. In general, chromosomes are short in size where one pair of metacentric chromosomes possessed secondary constriction. The karyotype of *A. aromaticum*, *A. koenigii* and *A. maximum* at inter-specific and intra-specific levels exhibit gross similarity in the types of chromosomes present, number of chromosomes bearing secondary constriction, TF%, total chromosome length and L/S arm ratio. The arm ratio L/S and TF% are the indicators of symmetric type of karyotype. According to Stebbins (1971) categorization, the karyotype of *A. aromaticum* and *A. maximum* falls under 1A while that of *A. koenigii* belonging to category 1B indicates minor deviation in the size of largest to smallest chromosome ratio. The identical karyotype formula A_2B_{46} without any pair of acrocentric or telocentric chromosomes having arm ratio L/S >2:1 suggests accentuated chromosomal homology. In *Curcuma* complex, the somatic chromosome number of *C. amada* was found to be 2n=42 with a basic number X=21 which corroborates previous reports (Sharma and Bhattacharya 1959; Ramachandran 1961, 1969; Islam 2004; Joseph 1998; Bhadra and Bandhyopadhyay 2015; Lamo and Rao 2017). At intra-specific level, the karyotype of Pop-I and Pop-II of *C. amada* is almost identical and symmetrical (1A) in nature indicating the absence of acrocentric or telocentric chromosomes. Based on asymmetry index value, Bhadra and Bandyopadhyay (2015, 2018) reported that the karyotype of *C. amada* falls under Stebbins category 1B which may be due to its occurrence in different eco-climatic zone. The somatic chromosome number 2n=63 of *C. caesia* and *C. longa* is in agreement with previous reports (Ramachandran 1961, 1969; Joseph *et al*. 1999; Nair and Sasikumar 2009; Nair *et al*. 2010; Lamo and Rao 2014; Bhadra and Bandyopadhyay 2015; Lamo and Rao 2016, 2017). The karyotype of these two species are identical both at inter- and intra-specific levels. According to Stebbins formula, their karyotypes belong to category 1A which is not in agreement with the report of Bhadra and Bandyopadhyay (2015, 2018). The somatic chromosome count of 2n=63 chromosomes of *C. picta* is a first report and the karyotypes of Pop-I and Pop-II of *C. picta* exhibit gross similarity in the types of chromosomes present, number of chromosomes with secondary constriction, TF%, total chromosome length and mean arm ratio (L/S). According to the degree of asymmetry, the karyotype of *C. picta* belongs to the category 1A. There are different views regarding the basic number of *Curcuma* spp. According to one school, the different species of *Curcuma* have a basic number X=21 (Sharma and Bhattacharya 1959; Ramachandran 1961, 1969; Islam 2004; Joseph 1998; Chen *et al*. 2013; Bhadra and Bandhyopadhyay 2015; Lamo *et al*. 2016) which has been derived from a combination of X=12 and X=9 (Ramachandran 1961; Lamo and Rao 2017). The other group (Sato 1960; Skornickova *et al*. 2007) proposed that the basic number of *Curcuma* is X=7 suggesting, thereby, all the species of *Curcuma* studied, having somatic chromosome number 2n=63 are nonaploid and *C. amada* with 2n=42 chromosomes is a hexaploid species. Apparently, there is no conflict between X=7 and X=21 in *Curcuma* complex. But the absence of cytotypes

in multiple of X such as 2X, 3X, 4X and 5X in natural populations and/or cultivars of *Curcuma* species suggests that the basic number X=21 is deep seated in *Curcuma* spp. from which the diploid and triploid species have eventually evolved in the due course of evolution. The occurrence of 2X, 3X, 4X, 5X and 6X cytotypes is not uncommon in flowering plants and in the monocot genus *Dioscorea*, valid cytotypes having 2X, 3X, 4X, 5X etc. are found in the natural population (Muthamia *et al.* 2014). In the light of this knowledge, the acceptance of X=7 in *Curcuma* complex is questionable. Moreover, if X= 7 is accepted as basic number in *Curcuma* spp., then their natural cytotypes having 9X, 12X and 15X chromosomes would have more copy number of genes. In such a situation, the regulation of dosage compensation having excess copy of genes in their cytotypes is beyond any elucidation. The somatic chromosome count 2n=34 chromosomes found in Pop-I and Pop-II of *Hedychium coccineum* and *H. coronarium* having a basic number X=17 supports previous findings (Mukherjee 1970; Mahanty 1970; Gao *et al.* 2008). Sharma and Bhattacharya (1959) reported a cytotype of *H. thyrsiforme* having 2n = 24 chromosomes with basic number X=12. In the present investigation, the presence of 34 chromosomes recorded in most of the somatic cells (modal number) of *H. thyrsiforme* indicates that the diploid chromosome number is, indeed 2n=34 as was reported by Mahanty (1970). This also suggests that all the three *Hedychium* species growing in this region are stabilized with a basic chromosome number X=17. It is imperative that the karyotypes of the six individuals studied are almost identical and have the same karyotype formula A_2B_{32}, but the absence of acrocentric and telocentric chromosomes infers the symmetric nature of karyotypes justifying their inclusion under Stebbins category 1A. Karyotype conservatism without any structural alteration leading to the formation of acrocentric or telocentric chromosomes in these species having a basic number X=17 reveals a karyotype stasis. Information regarding the number of chromosomes having secondary constriction in all the taxa of Zingiberaceae studied by previous researchers is minimum (Bhattacharyya 1957; Mandi 1990; Joseph *et al.* 1999; Islam 2004) and in some of the previous investigations there was no reference with regard to the number of chromosomes possessing secondary constriction. For resolving this problem, Ag-NOR study (Figure S2) has been carried out for the first time. There are different views regarding the maximum number of nucleoli per cell and the number of chromosomes bearing secondary constriction present in somatic chromosome complements of flowering plants (Sharma and Ghosh 1954; Sato 1980). However, the coincidence between the number of chromosomes possessing secondary constriction and the maximum number of nucleoli per cell indicates the relationship between the presence of secondary constriction and the ability of those chromosomes to form the maximum number of nucleoli in all the taxa studied. The presence of three nucleoli in triploid species of *Curcuma* is also an indicator of such a relationship. The inter- and intra chromosomal asymmetry based on Stebbins quali-quantitative method reveals that the karyotypes of most of the species are symmetrical in nature. A progressive asymmetry is, however, observed in *Amomum koenigii* (1B), *Alpinia calcarata* (1B) and *Alpinia nigra* (2B). Undoubtedly, Stebbins (1971) quali-quantitative method is the determinant of evaluation of the karyotype asymmetry index but based on this data alone, it would not be possible to ascertain the genetic relationship between different taxa of the same family. The bi-dimensional (Figure 14) scatter plot (Peruzzi and Eroglu 2013) drawn against Mean Centromeric Asymmetry (M_{CA}) and Coefficient of Variation of Chromosome Length (CV_{CL}) clearly indicates that the two tribes Alpinieae (represented by *Alpinia* spp. and *Amomum* spp.) and Zingibereae (represented by *Curcuma* spp. and *Hedychium* spp.) of Zingiberaceae (Angiosperm Phylogeny Group – 2009) have distinct karyotypes in which *Alpinia* spp. and *Amomum* spp. show comparatively high inter-chromosomal and intra-chromosomal asymmetry indices (Zarco 1986; Watanabe *et al.*1999; Paszko 2006) than those of *Curcuma* spp. and *Hedychium* spp. The dendrogram reveals that *Alpinia spp.*, *Amomum spp.*, *Curcuma spp.* and *Hedychium* spp. with their respective populations formed four separate clusters (Figure 15).

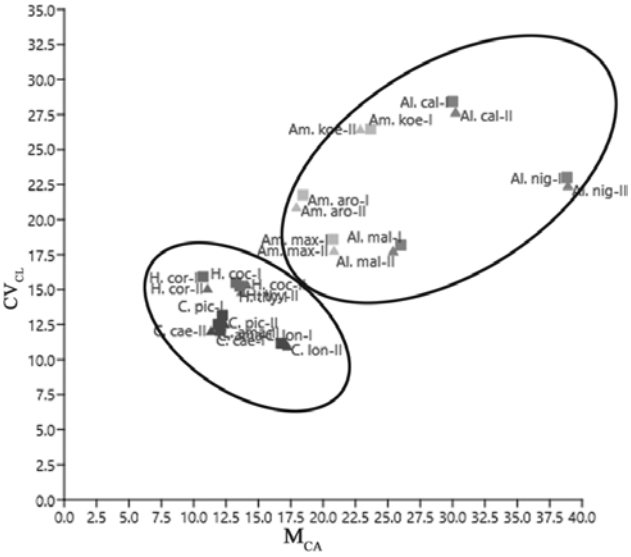


Figure 14. Bi-dimensional scattered plot against M_{CA} (x axis) and CV_{CL} (y axis).

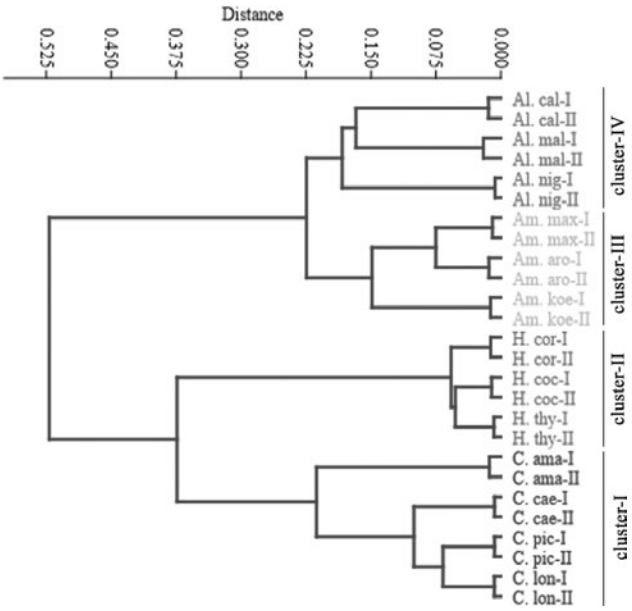


Figure 15. UPGMA dendrogram based on Gower's similarity matrix of six karyological parameters.

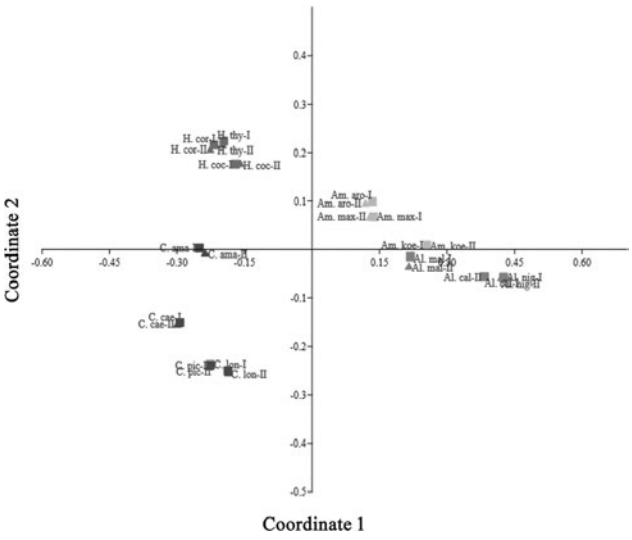


Figure 16: Principle Coordinate Analysis of selected species in 2D Plot.

The PCA illustrates no overlap and thus, the distinctive position of each genus with respect to other is evident (Figure 16). The karyotype data, therefore, validates the taxonomic position of *Alpinia* spp., *Amomum* spp., *Curcuma* spp. and *Hedychium* spp. The chromosomal data of *Alpinia* spp., *Amomum* spp., *Curcuma* spp., and *Hedychium* spp. suggest that they might have originated from a common ancestry but eventually through gradual changes, evolved as separate species in the due course of time. Gower's (1971) similarity matrix points towards a possible rearrangement of the chromosomal architectures in 13 different taxa studied. Such chromosomal rearrangements are possibly associated with cryptic changes maintaining a high syntenic value.

CONCLUSION

The present study has been focussed on the detailed karyotype analysis of two populations of *Alpinia calcarata*, *A. malaccensis*, *A. nigra*, *Amomum aromaticum*, *A. koenigii*, *A. maximum*, *Curcuma amada*, *C. caesia*, *C. longa*, *C. picta*, *Hedychium coccineum*, *H. coronarium*, and *H. thyrsiforme* of Zingiberaceae, collected from different geographical locations of Tripura. The somatic chromosome number of *Alpinia* and *Amomum* is found to be 2n = 48 having a basic number of X=12 chromosomes. In *Hedychium* spp. the diploid chromosome number is 2n=34 with basic number X=17 chromosomes. The present findings reveal that in majority of the somatic cells of *Curcuma caesia*, *Curcuma longa* and *Curcuma picta*, 63 chromosomes are present indicating their triploid nature. In contrast, diploid somatic chromosome number (2n=42) is recorded in *Curcuma amada*. Stebbins quali-quantitative analysis indicates that except in *Alpinia nigra*, the karyotype of other 12 species is symmetric in nature. The accentuated chromosome homology at intra-species level is the intrinsic characteristic of their karyotypes. The inter-chromosomal and intra-chromosomal asymmetry indices, based on statistically based method, suggest that *Alpinia* spp. and *Amomum* spp. have higher inter- and intra-chromosomal asymmetry in comparison to *Curcuma* spp. and *Hedychium* spp. The dendrogram and PCA derived data clearly validate the taxonomic position of these four genera investigated. Gower's similarity matrix endorses the cryptic changes leading to karyotype conservatism at species level of each genus.

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DISCLOSURE OF STATEMENT

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY FILE (APPENDICES)

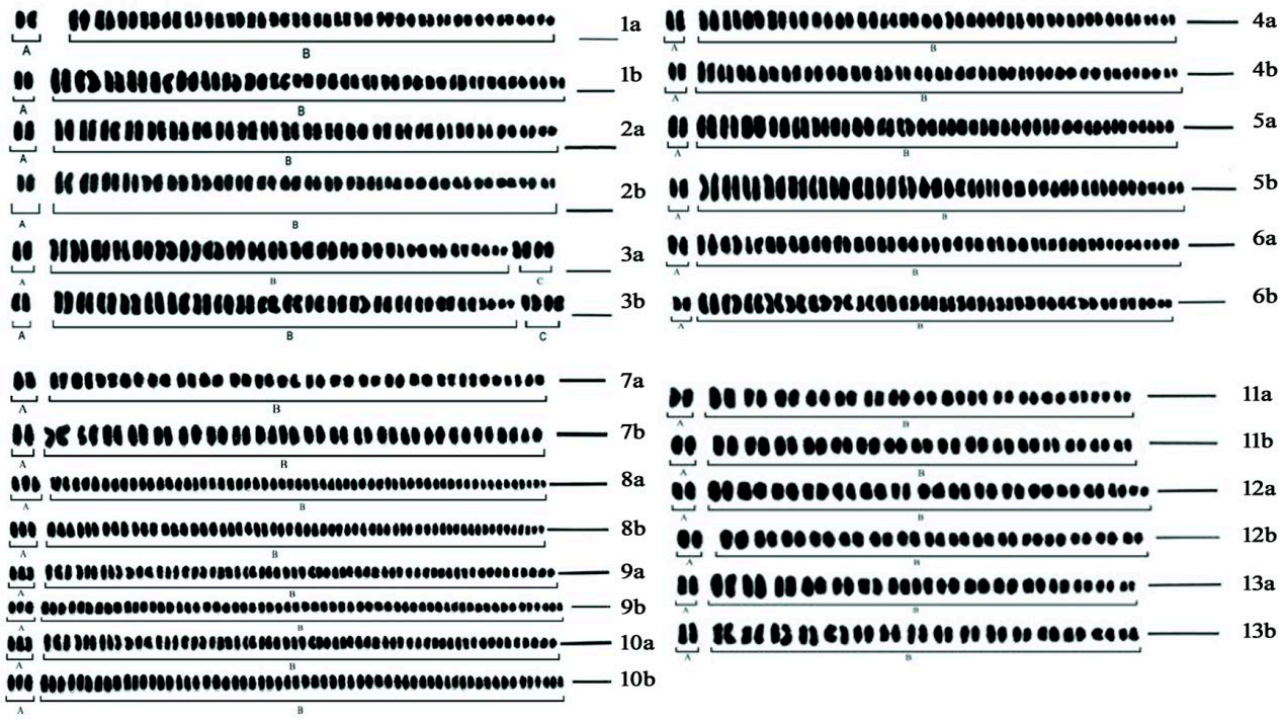

Figure S1. Karyogram of thirteen Zingiberaceae species from Tripura. 1- *Alpinia calcarata*; 2- *A. malaccensis*; 3- *A. nigra*; 4- *Amomum maximum*; 5- *A. aromaticum*; 6- *A. koenigii*; 7- *Curcuma amada*; 8- *C. caesia*; 9- *C. picta*; 10- *C. longa*; 11- *H. coronarium*; 12- *H. coccineum*; 13- *H. thyrsiforme* (a – Pop - I; b – Pop - II); scale bars = 5μm.

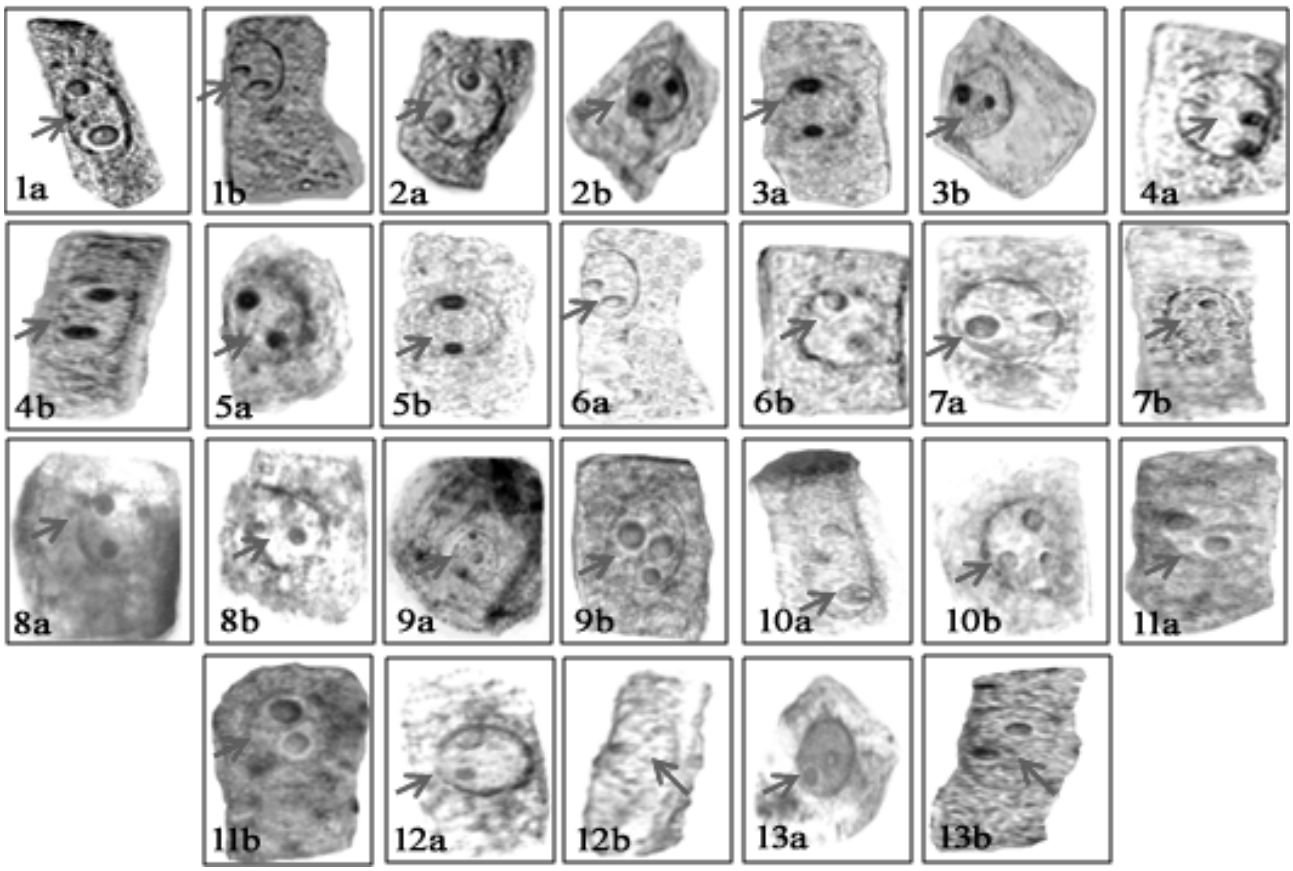

Figure S2. Ag- impregnated somatic cells of thirteen Zingiberaceae species. 1- *Alpinia calcarata*; 2- *A. malaccensis*; 3- *A. nigra*; 4- *Amomum maximum*; 5- *A. aromaticum*; 6- *A. koenigii*; 7- *Curcuma amada*; 8- *C. caesia*; 9- *C. picta*; 10- *C. longa*; 11- *H. coronarium*; 12- *H. coccineum*; 13- *H. thyrsiforme* (a – Pop - I; b – Pop - II).

Table S1. Details of plant samples collected from different geographical locations of Tripura.

Name of the species	Herbarium Voucher No.	Locations and altitudes
Alpinia calcarata	TUH – 460 (Pop-I)	Suryamaninagar, (34 mt.) 23045.0'44.74" N, 91015.0'54.29"E
	TUH – 2001 (Pop-II)	Baramura, (110 mt.) 23049.0'0.70" N, 91031.0'2.33"E
Alpinia malaccensis	TUH – 466 (Pop-I)	Debtamura, (48.0 mt.) 23033.0'2.50"N, 91038.0'5.40"E
	TUH – 2002 (Pop-II)	Atharamura, (134 mt.) 23029.0'54.10"N, 91037.0'34.30"E
Alpinia nigra	TUH – 2049 (Pop-I)	Mohanpur, (30.0 mt.) 23049.0'28.50"N, 91026.0'45.0"E
	TUH – 2003 (Pop-II)	Chabimura, (110 mt.) 23033.0'18.20"N, 91037.0'0.98"E
Amomum aromaticum	TUH – 2055 (Pop-I)	Tulamura,(46 mt.) 23023.0'11.3"N, 91026.0'10.7"E
	TUH – 2004 (Pop-II)	Unakoti, (200 mt.) 24023.0'11.3" N, 9204.0'13.0"E
Amomum maximum	TUH – 1407 (Pop-I)	Atharamura foot Hills, (45 mt.) 23053.0'2.10"N, 91042.0'12.5"E
	TUH – 2005 (Pop-II)	Jampui Hills, (191 mt.) 23059.0'4.63" N, 92017.0'50.6"E
Amomum koenigii	TUH- 470 (Pop-I)	Dhuptali, (40 mt.) 23023.0'11.3" N, 91026.0'15.1"E
	TUH- 2006 (Pop-II)	Baramura,(120 mt.) 23050.0'4.0" N, 91036.0'38.96"E
Curcuma amada	TUH – 465 (Pop-I)	Suryamaninagar, (21 mt.) 23045.0'44.27" N, 91015.0'53.38"E
	TUH – 2007 (Pop-II)	Howaibari,(105 mt.) 23049.0'0.60" N, 91034.0'04.61"E
Curcuma longa	TUH – 1407 (Pop-I)	Belkum Para, (48 mt.) 23053.0'2.1" N, 91043.0'13"E
	TUH – 2008 (Pop-II)	Jampui Hills, (163 mt.) 2402.0'30.21" N, 92016.0'42.34"E
Curcuma caesia	TUH- 466 (Pop-I)	Suryamaninagar, (30 mt.) 23045.0'43.13" N, 91015.0'52.17"E
	TUH- 2009 (Pop-II)	Sepahijala, (30 mt.) 23039.0'35.74" N, 91018.0'8.54"E
Curcuma picta	TUH – 459 (Pop-I)	Kamarikhala, (47 mt.) 23033.0'3.0" N, 91038.0'5.30"E
	TUH – 2010 (Pop-II)	Baramura foot Hills (102 mt.) 23048.0'41.24" N, 91030.0'54.24"E
Hedychium coronarium	TUH – 458 (Pop-I)	College Tilla, (25 mt.) 23049.0'46.72" N, 91017.0'36.28"E
	TUH – 2011 (Pop-II)	Baramura, (105 mt.) 23049.0'0.10" N, 91033.0'3.54"E
Hedychium coccineum	TUH – 461 (Pop-I)	Paikhola, (45 mt.) 23022.0'27.8" N, 91030.0'48.30"E
	TUH – 2012 (Pop-II)	Karbook, (106 mt.) 23021.0'38.90" N, 91042.0'12.15"E
Hedychium thyrsiforme	TUH- 507 (Pop-I)	Jampui Hills, (120 mt.) 23059.0'4.7" N, 92017.0'56.0"E
	TUH- 2013 (Pop-II)	Manpui, (290 mt.) 2408.0'5.9" N, 92016.0'39.0"E