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Genetic Diversity of Black Turmeric (*Curcuma caesia* Roxb.) Genotypes Identified by SSR Markers

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Abstract

Black turmeric is widely distributed in Indian subcontinent and having high economic importance due to varied medicinal properties. Presently the species is considered as critically endangered and mainly propagated vegetatively through underground rhizomes. Urgent need is there to conserve this rare and unconventional medicinally important plant. Genetic diversity studies in *C. caesia* Roxb. still in primitive stage for selecting the better performing accessions. SSR finger print for 33 black turmeric accessions by using sixteen primers revealed a total of 77 scorable bands. Among which 43 were polymorphic with an average of 2.68 per primer. SSR-02 and SSR 07 primers produced highest polymorphism (100%), followed by SSR-01, SSR-03, SSR-04, SSR-05 (75.00%). Whereas, SSR 10 produced least polymorphism (37.50%) and SSR 13, SSR 23 had showed no polymorphism. Overall primers exhibited 55.84 per cent polymorphism and the PIC value ranged between 0.255 to 0.480, which is the range to predict moderate diversity among the population. All genotypes were grouped in to three genetically diverse clusters and nine subgroups. Dendrogram depicts majority of the genotypes intermingled with each other in different clusters irrespective of their geographical affiliations. Twelve sets of genotypes had showed 100 per cent genetic similarity, while fourteen sets had least (10%). The observed intraspecific variation may be associated with cultivation and targeted genotype selection of desirable characters in *C. caesia* as the species is economically important. These results are the first step towards a better knowledge of the germplasm available in Indian subcontinent and can help to guide conservation of the species.

Keywords : Black turmeric, Critically endangered, DNA, Simple sequence repeat (SSR), Primers, Polymorphism, Dendrogram, Clustering

Curcuma caesia Roxb. is an erect, perennial, medicinal rhizomatous herb commonly named as 'black turmeric' due to its bluish-black rhizome colour. It is an important, lesser known, non-conventional medicinal plant of Zingiberaceae family.

*Black turmeric*is widely distributed in India, Bangladesh, China, Nepal, Malaysia and Thailand, also reported from Java and Myanmar as cultivated species (Liu *et al.,*2013). In India black turmeric is found in West Bengal, Madhya Pradesh, Orissa, Chhattisgarh and Uttar Pradesh states. The species is native to northeast and central India, also sparsely found in papi hills of Godavari, root hills of the Himalayas and Northern hill forest of Sikkim (Anonymous, 2001). This herb is having a high economic importance owing to its putative medicinal properties. All parts of the plant *viz.,* leaves, roots, bulbs and rhizomes used in Ayurvedic, Unani and Siddhaherbal medicine system (Pandey and Chowdhary, 2003).

Traditionally rhizomes of *Curcuma caesia* Roxb. are used as home remedy for several ailments by tribal communities. Khamti tribe of Arunachal Pradesh use collar paste of plants to heal severe wounds and injuries and paste of fresh rhizomes in case of snake and scorpion bite (Hui *et al.*, 2007). In Manipur rhizome paste is applied in bruises, contusions and rheumatic pains (Sarangthem and Haokip, 2010). Baiga, Sahariya, Agariya, Gond and Korku tribes of Madhya Pradesh use rhizomes for

the treatment of pneumonia, cough, cold in children and for fever and asthma in adults (Paliwal *et al.,* 2011).

Pharmacologically blood purifying activity (Arulmozhi *et al.,* 2006), bronchodilating activity (Paliwal *et al.,* 2011), antioxidant activity (Mangla *et al.,* 2010), anxiolytic and CNS depressant activity, locomotor depressant, anticonvulsant (Karmakar *et al.,* 2011), anthelmintic activity (Gill *et al.,* 2011), anti-bacterial activity (Rajamma *et al.,* 2012), anti-ulcer activity (Das *et al.,* 2012) were reported by several workers.

Black turmeric is sterile triploid (2n=3x=42), which can be vegetatively propagated by using its underground rhizomes. Since hybridization is ineffective in most cases, genetic improvement is often limited to germplasm selection and mutation breeding. Even though germplasm collections represent the main source of variability for black turmeric genetic improvement, studies aimed at characterizing germplasm collections are scarce or nil. Presently this herb is considered as critically endangered (threatened) species, due to wide destruction of natural habitat through several human activities such as over exploitation for traditional medicine purposes, industrialization, urbanization etc. Urgent need is there to conserve this rare and unconventional medicinally important plant.

The important link between the conservation and utilization of plant genetic resources is collection and characterization of germplasm. Molecular techniques allow researchers to identify accessions at most accuracy and certainty, assess the relative diversity within and among species and locate diverse accessions for improvement purposes. Assessing the diversity or characterization of germplasm based on morphological or taxonomical descriptors is the common method which results in overlapping complex results. These morphological methods depend on the environmental conditions hence there is a chance for occurrence of variation at phenotypic expression level. Some species diverse at morphological level may not at molecular level and vice-versa. Therefore, there is a need to support morphological variability with molecular variability studies in selecting better performing genotypes.

The use of simple sequence repeats (SSR) markers is by considering the fact that they are highly reproducible due to their primer length and the high stringency achieved by the annealing temperature and provides highly polymorphic finger prints. Hence, the experiment designed to assess the genetic diversity and characterization of the black turmeric genotypes using SSR molecular markers. Molecular studies in *C. caesia* is still in a primitive stage and only a few studies are reported so far (Syamkumar & Sasikumar, 2007 and Das *et al*., 2011).

Material and Methods

Thirty three Black turmeric genotypes were collected from the provenance of the country (Table 1), other *Curcuma* species *viz.,* Black ginger, Turmeric, Ginger, Kasthuri turmeric, Mango ginger and unknown Zingiberaceae species also included in the study, mainly to confirm the black turmeric genotypes precisely and to analyze the diversity among the different species. Plants were grown at ICAR-KVK, Chamarajanagar. The SSR analysis was taken up from the DNA isolated from young leaves of the genotypes. Isolation of total genomic DNA of black turmeric genotypes was carried out according to Porebski *et al.* (l997) Saiki *et al.* (1988) using CTAB with some modifications, followed by polymerase chain reaction (PCR) by employing SSR primers.

DNA Extraction and Purity

Total DNA was extracted from fully grown fresh young leaves by the modified Cetyl Tri-methyl Ammonium Bromide (C-TAB) method. Surface sterilized leaf tissues (0.5 mg) were homogenized in 500 µl of CTAB Extraction Buffer (2% Cetyl tri-methylammonium bromide, one per cent Polyvinyl pyrrolidone, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA or CTAB Extraction Buffer). The homogenate was incubated at 60 °C for 30 minutes and followed by centrifugation (14,000 x g) for 10 minutes. An equal volume of chloroform / isoamyl alcohol (24:1) was

added, vortexed and then centrifuged (14,000 x g) for 10 minutes, to separate the phases. The aqueous upper phase was used to precipitate the DNA by adding 0.7 ml cold isopropanol and incubate at -20 °C for overnight. The pellets were pooled out and washed with ice cold 70 per cent ethanol. Ethanol was decanted and residual ethanol was removed by drying in a Speed Vac. DNA pellets were dried and dissolved in 30 µl of TE buffer and then treated with R Nase solution A in order to remove any possible contamination of RNA and incubated at 37 °C for 30 minutes. The quantity and purity of DNA was confirmed by electrophoresis, using agarose gel (0.8 %). DNA concentration was calculated based on 1 Kb standard ladder. Column purification was done using spin column-based tube (cat. No. 69702) with a solid phase of silica layer (0.45 µm pore size) to get rid of salt contamination. Amount of DNA was determined from the concentration of DNA in the eluate. It was measured by absorbance at 260 nm / 280 nm. DNA purity was determined by calculating the ratio of absorbance at 260 nm to absorbance of 280 nm (Fig.1). The isolated DNA was of good quality as it showinga reading between 1.7 - 2.14 is showing the purity of DNA (Table 1).

PCR Analysis and Gel Electrophoresis

A set of 25 SSR markers (Table 2) were used for polymorphism analysis. The PCR amplifications were performed in a Eppendorf Master cycler Gradient (Germany) and carried out in a final volume of 25 µl in a reaction mixture containing two µl (20- 40 ng) of template DNA, 10 pm of each primer set, 10 Mm dNTPs, 2.5 x PCR reaction buffer (containing 100 mM KCl, 20 mM Tris-HCl (pH 8), 1 mM DTT, 0.5 per cent Igepal, and 0.5 per cent Tween 20, 50 per cent glycerol, 100 mM $MgCl_2$) and 0.5 units of DNA Taq polymerase. The amplification condition was: Initial step of denaturation at 95 °C for five min. followed by 40 cycles of denaturation at 94 °C for 20 secs. primer annealing at 50 °C for 30 sec. and extension at 72 °C for 1 min, followed by an extended elongation step at 72 °C for 8 min. Samples of 10 µL PCR products were mixed with 3 µL of 6X

TABLE 1

Details of black turmeric genotypes and other *Zingiberaceae* species along DNA Quantification used for the study

Species	coding	Place of origin	Latitude	Longitude	Altitude (m)	State	Nucleic acidcon centration (ng/ìl)	260/280 ratio
Black turmeric	GKM-1	Mijar,	13° 4' 7.6764''N	74° 59' 36.9564'' E	147	Karnataka	38.8	1.76
Black turmeric	GKM-2	Mangalore	12°55'2.03''N	74°51'21.71''E	22	Karnataka	35.4	1.61
Black turmeric	GKB-3	Bangalore	12.9716° N	77.5946° E	920	Karnataka	22.8	1.57
Black turmeric	GKB-4	Sanjeevinivatika	13.0801° N	77.5785° E	924	Karnataka	152.8	1.79
Black turmeric	GKJ-5	Joida	15.1688° N	74.4848° E	532	Karnataka	123.0	1.86
Black turmeric	GMV-6	Vidarbha-Gadchirolli	21.1286° N	79.0964° E	1000	Maharashtra	125.5	1.62
Unknown species	UNK-7	Nagpur	21.1458⁰N	79.0882⁰E	310	Maharashtra	68.3	1.80
Black turmeric	GBS-8	Samastipur	25.8629679 N	85.7810263E	53	Bihar	100.6	1.37
Black turmeric	GBH-9	Hajipur	25.6858392 N	85.2145907E	56	Bihar	73.3	1.74
Black turmeric	GGR-10	Rajkote	22° 17' 30 N	70° 47' 36E	252	Gujarat	49.0	1.81

Species	coding	Place of origin	Latitude	Longitude	Altitude (m)	State	Nucleic acidcon centration (ng/il)	260/280 ratio
Black turmeric	GAB-11	BhokaGhat Forest	26.2006° N	92.9376°E	76	Assam	38.1	1.78
Black turmeric	GAB-12	Bijuli	28.0312°N	82.9555°E	97	Assam	51.4	1.61
Black turmeric	GAB-13	Bokoliya	26.0564°N	93.1955°E	600	Assam	53.5	1.55
Black turmeric	GAK-14	Killing Basti	26.8140°N	82.7630°E	680	Assam	212.5	1.56
Black turmeric	GMW-15	Wakhro	23° 43' 2.6256'' N	92° 43' 5.2212'' E	1619	Mizoram	58.0	1.73
Black turmeric	GMK16	Kolasib	24.2246° N	92.6760° E	722	Mizoram	88.5	1.84
Black turmeric	GMA-17	Aizwal	23.727106°N	92.717636°E	1132	Mizoram	47.6	1.75
Black turmeric	GOK-18	Khurda	20.1301° N	85.4788° E	75	Odisha	65.0	1.81
Black turmeric	GOK-19	Koraput	18.82°N	82.72°E	870	Odisha	101.3	1.82
Black turmeric	GAP-20	Pasighat Area	28.0619° N	95.3260° E	153	Arunachal Pradesh	61.7	1.76
Black turmeric	GMI-21	Manipur – Forest	24° 48' 50.2812'' N	93° 57' 1.0044'' E	900	Manipur	134.3	1.80
Black turmeric	GMT-22	Imphal	24.8170° N	93.9368° E	786	Manipur	224.8	1.82
Black Ginger	BG-1	Thoubal	24.63⁰N	94.02⁰E	765	Manipur	124.4	2.40
Black turmeric	GMS-24	Sagar	23.8388° N	78.7378° E	427	Madhya Pradesh	38.8	1.80
Black turmeric	GNK-25	Kohima	25.6751° N	94.1086° E	1444	Nagaland	46.7	1.96
Black turmeric	GNU-26	Uhkagoronga Hill	25° 54' 22.5612'' N	93° 43' 39.3312'' E	3827	Nagaland	612	1.77
Black turmeric	GNF-27	Nepal – Forest	27° 42' 2.7684'' N	85° 18' 0.5040'' E	330	Nepal	215.4	1.92
Black Ginger	BG-2	Mandalay	21.98⁰N	96.08⁰E	80	Burma	122.3	1.81
Black turmeric	GKT-29	Thrissur-Vellanikara	10.5452° N	76.2740° E	22	Kerala	113.4	1.82
Black turmeric	GKK-30	IISR Kozhikode	11.2588° N	75.7804° E	01	Kerala	397.2	1.83
Black turmeric	GMR-31	Ri-Bhoi	25.8432° N	91.9856° E	485	Meghalaya	82.2	1.64
Black turmeric	GAD-32	DolamoraBorpung	26° 14' 38.9616'' N	92° 32' 16.2312'' E	615	Assam	57.9	1.76
Black turmeric	GNP-33	Peren	25.5125° N	93.7391° E	1445	Nagaland	72.9	1.78
Black turmeric	GBC-34	Champaran	27.1543° N	84.3542° E	62	Bihar	95.4	1.81
Black turmeric	GJG-35	Godda	24.8255° N	87.2135° E	87	Jharkh	98.7	1.80
Black turmeric	GNP-36	Phek	25.6634° N	94.4703° E	1524	Nagaland	84.9	1.84
Turmeric	T-37	GKVK,Bangalore	13.0801° N	77.5785° E	924	Karnataka	163.1	1.81
Ginger	G-38	-do-	13.0801° N	77.5785° E	924	Karnataka	197.2	1.83
Kasthuri turmeric	KT-39	-do-	13.0801° N	77.5785° E	924	Karnataka	372.1	1.85
Mango ginger	MG-40	-do-	13.0801° N	77.5785° E	924	Karnataka	95.6	1.83

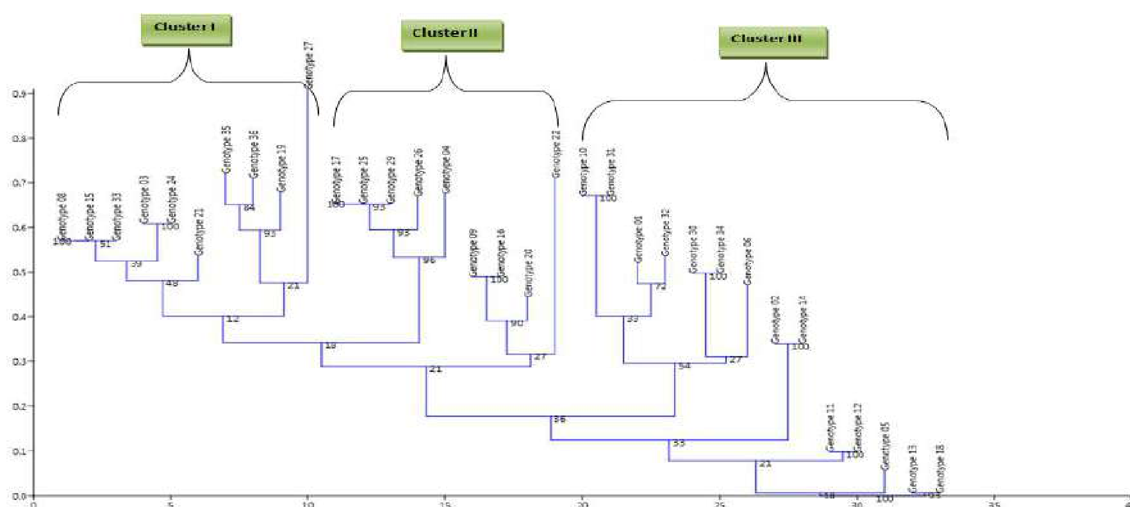


Fig. 1 : Dendrogram of black turmeric genotypes–Jackard co-efficient

TABLE 2
Details of SSRprimers used for the study

Name of primer	Forward Sequence (5'-3')	Reverse sequence (3'-5')	Annealing Temperature
SSR01	CATGCAAATGGAAATTGACAC	TGATAAATTGACACATGGCAGTC	50°C
SSR02	TTCAACTTCTCCTCGCTC	GCAAGGTCTGCATCTATT	47°C
SSR03	ATGTGGTTGAGGAATGAT	CTATTTCCCATAGCCCTT	45°C
SSR04	GTTCACAGCTTTAGCAGGGACAA	CTCCTCTCCATATTCTCCATCTCG	56°C
SSR05	TTGCCAGTGTGCTTGTTCTC	TTGAAGGGAACACTGAAGGG	52°C
SSR06	GGAGGAGGCAGTTGATTTGT	GCTTTGGTGGCTAGAGATGC	53°C
SSR 07	GGCACAACTTGCGTTCAAAG	GCATCGATGAAGAACGTAGC	52°C
SSR 08	GGAAGGAGAAGTCGTAACAAGG	CACGCTTCTCCAGACTACA	50°C
SSR 09	GGGGATAGAGGGACTTGAAC	GGTTCAAGTCCCTCTATCCC	54°C
SSR 10	CGAAATCGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	52°C
SSR 11	ATCAGCAGCCATGGCAGCGAC	AGGGGATCATGTGCCGAAGGC	58°C
SSR 12	ACCCTCTCCGCCTCGCCTCCTC	CTCCTCCTCCTGCGACCGCTCC	64°C
SSR 13	CTCTGTCTCCTCCCCCGCGTCG	TCAGCTTCTGGCCGGCCTCCTC	63°C
SSR 14	CAGCAGATTTTTGCTCCG	GTCGCGTTCGTGGAAAT	48°C
SSR 15	ACCGTAGCAAAGAAATAGGAC	AAGGTGGAAGGAAACTCG	48°C
SSR 16	TTCATTCGACGCAAACAGC	CGACGCAATAGTCGAAGGC	50°C
SSR 17	GCCAAAGAAAGAACTGACATCC	TTACAACCCTCCTCCCATTAGA	53°C
SSR 18	CGCAGCTGACACTTCTTCCT	AAGTCCGGGAGTTCTAAAGG	53°C
SSR 19	CTGCGGTCCAAGTACAAGATC	CTAGCTGGTGGCGGTGGT	55°C
SSR 20	CTTTTGGCTGATAAATGGAAGG	AAGAAAGAACTGACATCCTCCG	52°C
SSR 21	CATGCAAATGGAAATTGACAC	TGATAAATTGACACATGGCAGTC	50°C
SSR 22	AGAGCTTTCTGCACTTCCAC	GAAGTGTGCCCAAATAGCAC	52°C
SSR 23	CTGTCTTCATCCACTTTCCC	TAGGCACAGACACGGACATA	52°C
SSR 24	GTCGTGGGTGCGAGATTA	AATATGGGATCCACTCTCCC	52°C
SSR 25	ACTCACCGAGTCGGAAATAG	GTAGTGAGGCTTTGGCAGAT	52°C

loading buffer and spin briefly in a microcentrifuge before loading. The amplification products were analyzed on two per cent agarose gel in 1X TAE buffer running at 80v for 60 minutes followed by visualizing under UV light and photographing. Standard ladder used in electrophoresis was of 100bp and 50bp. Final gel image was used for construction of dendrogram and taxon sampling.

Primer Screening

Twenty-five simple sequence repeat (SSR) primers were initially screened to determine the suitability of

TABLE 3

Percent polymorphism as revealed by SSR primers in black turmeric genotypes

Primer code	Primer Forward sequence (5'-3') Reverse sequence (3'-5')	Total number of bands produced	No. of polymorphic bands	No. of monomorphic bands	Polymorphism (%)
SSR 01	F: CATGCAAATGGAAATTGACAC R: TGATAAATTGACACATGGCAGTC	4	3	1	75.00
SSR 02	F: TTCAACTTCTCCTCGCTC R: GCAAGGTCTGCATCTATT	2	2	0	100.00
SSR 03	F: ATGTGGTTGAGGAATGAT R: CTATTTCCCATAGCCCTT	8	6	2	75.00
SSR 04	F:GTTCACAGCTTTAGCAGGGACAA R: CTCCTCTCCATATTCTCCATCTCG	4	3	1	75.00
SSR 05	F: TTGCCAGTGTGCTTGTTCTC R: TTGAAGGGAACACTGAAGGG	4	3	1	75.00
SSR 07	F: GGCACAACTTGCGTTCAAAG R: GCATCGATGAAGAACGTAGC	1	1	0	100.00
SSR 08	F: GGAAGGAGAAGTCGTAACAAGG R: CACGCTTCTCCAGACTACA	3	2	1	66.66
SSR 09	F: GGGGATAGAGGGACTTGAAC R: GGTTCAAGTCCCTCTATCCC	7	4	3	57.14
SSR 10	F:CGAAATCGGTAGACGCTACG R: ATTTGAACTGGTGACACGAG	8	3	5	37.50
SSR 13	F:CTCTGTCTCCTCCCCCGCGTCG R: TCAGCTTCTGGCCGGCCTCCTC	4	0	4	0.00
SSR 14	F: CAGCAGATTTTTGCTCCG R: GTCGCGTTCGTGGAAAT	5	2	3	40.00
SSR15	F: ACCGTAGCAAAGAAATAGGAC R: AAGGTGGAAGGAAACTCG	4	2	2	50.00
SSR16	F: TTCATTCGACGCAAACAGC R: CGACGCAATAGTCGAAGGC	5	3	2	60.00
SSR20	F: CTTTTGGCTGATAAATGGAAGG R: AAGAAAGAACTGACATCCTCCG	8	4	4	50.00
SSR 23	F: CTGTCTTCATCCACTTTCCC R: TAGGCACAGACACGGACATA	2	0	2	0.00
SSR 25	F: ACTCACCGAGTCGGAAATAG R: GTAGTGAGGCTTTGGCAGAT	8	5	3	62.50
	Total	77	43	34	55.84

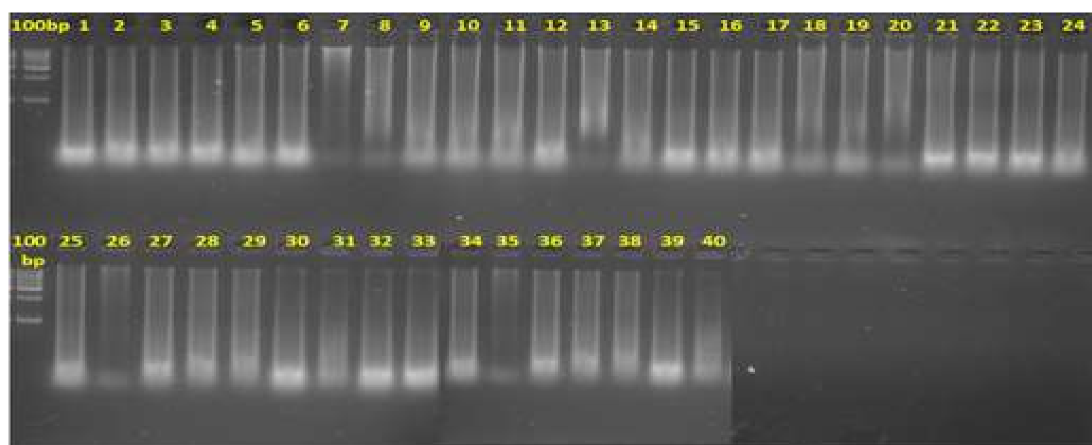


Plate 1: Genomic DNA of 33 black turmeric genotypes and other *Zingiberaceae* species

each primer for the study. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified within the genotypes. To ensure reproducibility, the primers generating no weak or complex patterns were discarded selected only 16 SSR primers for further study (Table 3).

Data Analysis

Scoring : Amplified DNA fragments detected after electrophoretic separation in each genotype was scored for the presence (1) or absence (0) of clear and unambiguous bands. The data matrix comprising of '1' and '0' was formed and this data matrix was subjected to further analysis.

Construction of Dendrogram : The tree was computed based on the information extracted from the gel image and the genetic distances were displayed on the branches. The distance matrix corresponding to the gel image was computed and saved using the software PyElph (Ana and Cristian, 2012). Further, the binary score value was used by PAST 325 software to construct the final dendrogram showing diversity among all species with all primers (Oyvind, 2019). The program used was cluster analysis joining (tree clustering) with raw input data of each population separately. The main parameter, which guided the joining process linkage rule, is unweighted pair group average (UPGMA) and the genetic distance was estimated from raw data. Genetic distance denotes the genetic divergence between species or between populations within a species. It is measured by a variety of factors; large genetic distances indicate relativeness whereas smaller genetic distances indicate a more detached genetic relationship.

Polymorphic Information Content : Polymorphic information content and heterozygosity values were determined by using PIC calculator by uploading allelic frequencies binary data. The polymorphic information content (PIC) value described by Botstein *et al.* (1980).

Cluster Analysis : Hierarchical cluster analysis is a multivariate procedure that can be used to detect patterns in ecological data. A binary data matrix obtained above for 33 Curcuma caesia Roxb. genotypes from 16 primer sets were entered in PAST 326b window. Before constructing dendrogram similarity and distance indices was calculated using Jaccard's matrix. Further using the algorithm UPMGA and Jaccard's similarity index the genetic tree was constructed to understand the evolutionary relationship among 33 genotypes. K-means is a distance-based algorithm, used to calculate the distances and clustering among the data group. Here k-means clustering is done to identify the subgroups among the genotypes.

Results and Discussion

DNA Quantification

The isolated DNA was analyzed quantitatively by using Nanodrop. DNA had an A260 / A280 ratio of

TABLE 4

PIC values revealed by SSR primers in black turmeric genotypes

Primer code	Primer Forward sequence (5'-3') Reverse sequence (3'-5')	PIC Value
SSR 01	F:CATGCAAATGGAAATTGACAC R: TGATAAATTGACACATGGCAGTC	0.375
SSR 02	F:TTCAACTTCTCCTCGCTC R: GCAAGGTCTGCATCTATT	0.381
SSR 03	F:ATGTGGTTGAGGAATGAT R: CTATTTCCCATAGCCCTT	0.398
SSR 04	F:GTTCACAGCTTTAGCAGGGACAA R: CTCCTCTCCATATTCTCCATCTCG	0.420
SSR 05	F:TTGCCAGTGTGCTTGTTCTC R: TTGAAGGGAACACTGAAGGG	0.368
SSR 07	F:GGCACAACTTGCGTTCAAAG R: GCATCGATGAAGAACGTAGC	0.288
SSR 08	F:GGAAGGAGAAGTCGTAACAAGG R:CACGCTTCTCCAGACTACA	0.294
SSR 09	F:GGGGATAGAGGGACTTGAAC R: GGTTCAAGTCCCTCTATCCC	0.348
SSR 10	F:CGAAATCGGTAGACGCTACG R: ATTTGAACTGGTGACACGAG	0.255
SSR 13	F:CTCTGTCTCCTCCCCCGCGTCG R: TCAGCTTCTGGCCGGCCTCCTC	0.480
SSR 14	F:CAGCAGATTTTTGCTCCG R: GTCGCGTTCGTGGAAAT	0.401
SSR15	F:ACCGTAGCAAAGAAATAGGAC R: AAGGTGGAAGGAAACTCG	0.420
SSR16	F:TTCATTCGACGCAAACAGC R: CGACGCAATAGTCGAAGGC	0.361
SSR20	F:CTTTTGGCTGATAAATGGAAGG R: AAGAAAGAACTGACATCCTCCG	0.375
SSR 23	F:CTGTCTTCATCCACTTTCCC R: TAGGCACAGACACGGACATA	0.381
SSR 25	F:ACTCACCGAGTCGGAAATAG R: GTAGTGAGGCTTTGGCAGAT	0.294

1.80 to 2.00 showing that it was relatively pure and free from impurities. The quality of DNA isolated was very good (Plate 1). The DNA yield for all the genotypes are presented in Table 1, DNA yield ranged from 22.8 ng/µl to 397.2 ng/µl. Genotypes GKK-30(397.2 ng/µl), GMI-22 (224.8 ng/µl), GNF-27 (215.4 ng/µl) and GAK-14 (212.5 ng/µl) gave highest yield of DNA and GKB-3 (22.8 ng/µl) had the lowest amount of DNA.

Other Zingiberaceae species black ginger (BG 23, BG 28), turmeric (T 37), ginger (G 38), Kasthuri turmeric (KT 39), mango ginger (MG 40) and unknown species (UKN 7) were also included in the study mainly to confirm the black turmeric species precisely and to analyse the diversity among the different Zingiberaceae species. Among other species Kasthuri turmeric (KT 39) gave highest yield of DNA (372.1 ng / µl) followed by ginger (197.2 ng / µl), turmeric (163.1 ng / µl), black ginger (122.3 to 124.4 ng / µl) and unknown species (68.3 ng / µl) (Table 1).

SSR Analysis

Primer Selection for SSR Analysis : After screening 25 SSR primers, sixteen primers that yielded maximum number of consistent and clear bands were selected for finger printing. The number of bands varied from 1-8 with an average of 2.68 bands per primer and the size ranged from 100 bp to 800 bp. The bands which are more than 100 bp were selected for scoring (Table 3).

SSR Profile Analysis : The SSR fingerprint of 33 black turmeric genotypes by using sixteen primers revealed a total of 77 scorable bands that were well defined, consistent, unambiguous, readable and reproducible polymorphic bands which were used to estimate genetic diversity.

A total of 77 bands were observed, among which 43 were polymorphic with an average of 2.68 polymorphic bands per primer. Among sixteen primers screened, SSR 03 scored maximum number of polymorphic bands (6 bands) followed by SSR 25 (5 bands), SSR 09, SSR 20 (4 bands), SSR 01, SSR 04, SSR 05, SSR 10, SSR 16 (3 bands), SSR 02, SSR 08, SSR 14, SSR 15 (2 bands) and SSR 7 (1 band) produced minimum number of bands. The primers SSR 02 and SSR 07 produced highest polymorphism of 100 per cent followed by SSR 01, SSR 03, SSR 04, SSR 05 of 75.00 per cent, where as primer SSR 10 produced least polymorphism of 37.50 per cent and SSR 13, SSR 23 had showed no polymorphism (Plate 2A & 2B).

Overall per cent polymorphism of 33 genotypes shows 55.84 per cent and the PIC value ranged

TABLE 5

K means clustering of black turmeric genotypes

Main cluster	Sub group	Genotypes	Number of geno - types
Cluster I	I	GKB-3, GBS-8, GMW-15, GMF-21, GMS-24 ,GNP-33	06
	II	GOK-19, GNF-27, GJG-35, GNP-36	04
Cluster II	I	GKB-4, GMA-17, GNK-25, GNU-26, GKT-29	05
	II	GBH-9, GMK-16, GAP-20	03
	III	GMI-22	01
Cluster III	I	GGR-10, GMR-31, GKK-30, GBC-34, GAB-11, GAB-12 GMK-02, GAK-14	08
	II	GKM-1,GAD-32	02
	III	GMV- 06	01
	IV	GKJ-5, GAB-13, GOK-18	03
		Total	33

between 0.255 to 0.480, which is the range to predict moderate diversity among the population (Table 4). Overall it is important to consider that this index depends on the number of accessions analyzed. This moderate polymorphism is probably due to the crop is mainly propagated through vegetative means by using underground rhizomes. Majority of the genotypes collected were belongs to N-E parts of India. The results are in congruence with the findings of Jain and Jain (2018) reported average PIC value of 0.43 for 25 black turmeric genotypes of Chatisgarh. Islam, 2004; Sigrist *et al.,* 2011; Singh *et al.,* 2012; Khan *et al.,* 2013 and Sahoo *et al.,* 2017, found similar moderate diversity among turmeric population. These results are the first step towards a better knowledge of the germplasm available in Indian subcontinent and can help to guide future studies of the crop.

Genetic Similarity Matrix and Cluster Analysis among Black Turmeric Genotypes : SSR bands were scored from the gel profile by assigning binary values '1' for the presence of band and '0' for the absence of band. Such binary data generated from all the gel profiles used for statistical analysis. The similarity matrix was computed using Jaccard's Co-efficient (J). The pooled SSR binary data was utilized for cluster analysis using the PAST 326b software for the 33 Black turmeric genotypes.

The dendrogram was constructed by UPGMA (Unweighted Pair Group Method with Arithmetical averages) method of clustering using Jaccard's co-efficient (J). All genotypes were grouped in to three genetically diverse major clusters (Fig. 1). Within each major cluster, there were further subgroups; basically the 33 genotypes are sub-divided into nine sub groups which inform the divergence of species (Table 5). The results are in line with the findings of Singh *et al.* (2015); Verma *et al.* (2015) and Singh *et al.* (2018) in turmeric.

The first major cluster included 10 genotypes is further divided into two sub groups. Sub group I consists of six genotypes namely GKB-3, GBS-8, GMW-15, GMF-21, GMS-24 and GNP-33 with similarity coefficient of 0.87. Whereas, Sub group II having 04 genotypes *viz.,* GOK-19, GNF-27, GJG-35 and GNP-36 which were similar at similarity coefficient matrix range of 0.77 to 0.82.

In dendrogram the major cluster II is divided into three sub groups. Sub group I is having GKB-4, GMA-17, GNK-25, GNU-26 and GKT-29 genotype. Similarity index among the members of this sub-group ranged from 0.69 (between GKB-4 and GNU-26) to 1.00 (between GMA-17 and GNK-25, GNK-25 and GKT-29). Sub group-II having GBH-9, GMK-16 and GAP-20 with a similar index ranges from 0.85 (between GBH-9 and GAP-20) and 1.00 (between GBH-9 and GAP-20) whereas, sub group III consists of GMI-22 genotype alone.

Cluster III is further divided into four sub groups to simplify their comparative study. Sub group I included 08 genotypes *viz.,* GGR-10, GMR-31, GKK-30, GBC-34, GAB-11, GAB-12 and GMK-02 & GAK-14 genotype. Similarity index among the members of this group is 1.00. Sub group-II having GKM-1 and GAD-32 with a similar index ranges

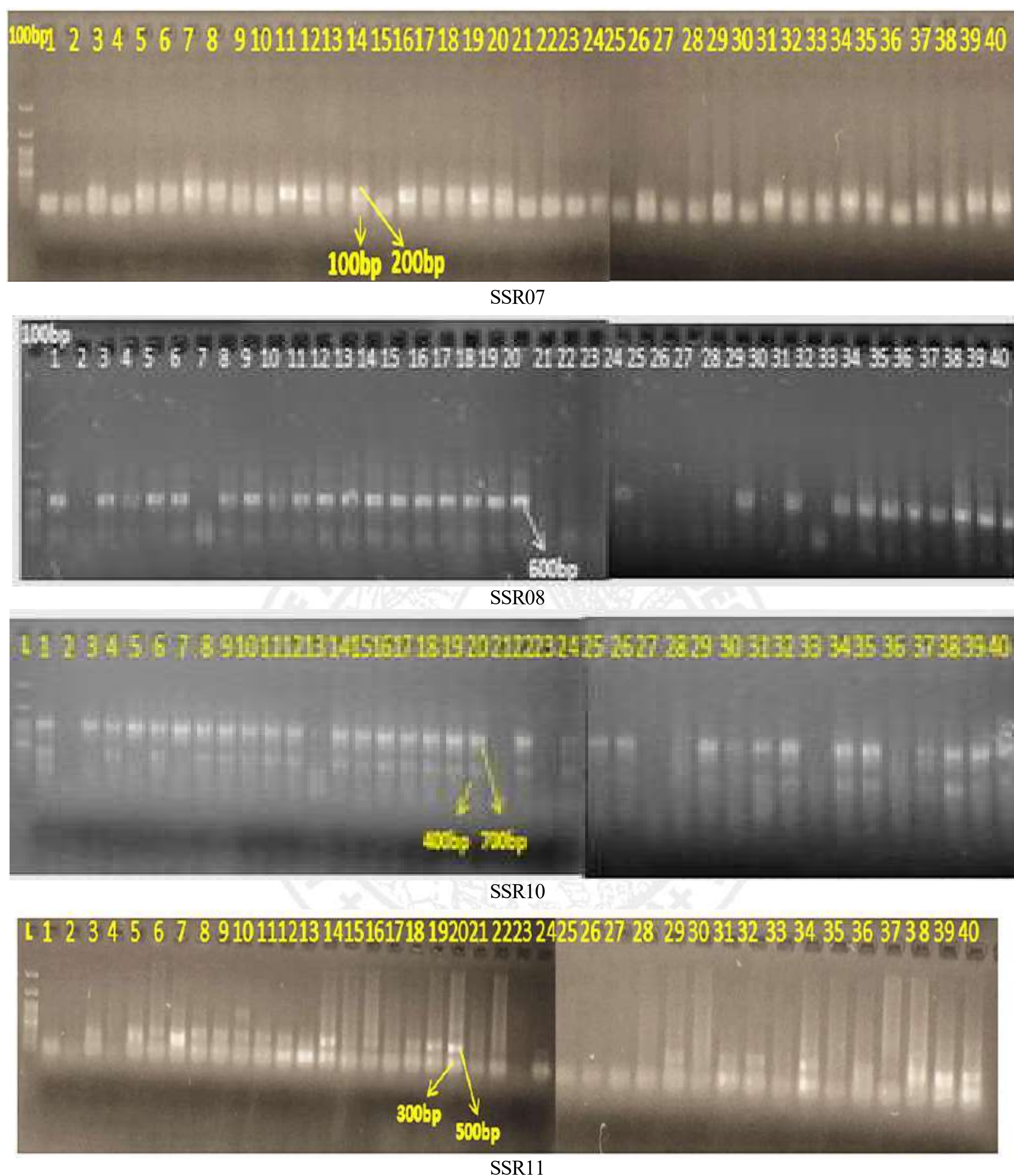


Plate 2A: SSR primers gel image of different black turmeric genotypes

from 0.89 whereas, sub group III consists of GMV-6 genotype alone. Sub group IV consists of GKJ-5, GAB-13 and GOK-18 genotypes, similar matrix of 0.94. Larger genetic distances indicate relativeness whereas smaller genetic distances indicate a more detached genetic relationship.

The overall idea of clustering of data during generation of a dendrogram is to recognize meaningful sub groups from the total data group taken for the study. The dendrogram clustering revealed that majority of the genotypes intermingled with each other in different clusters irrespective of

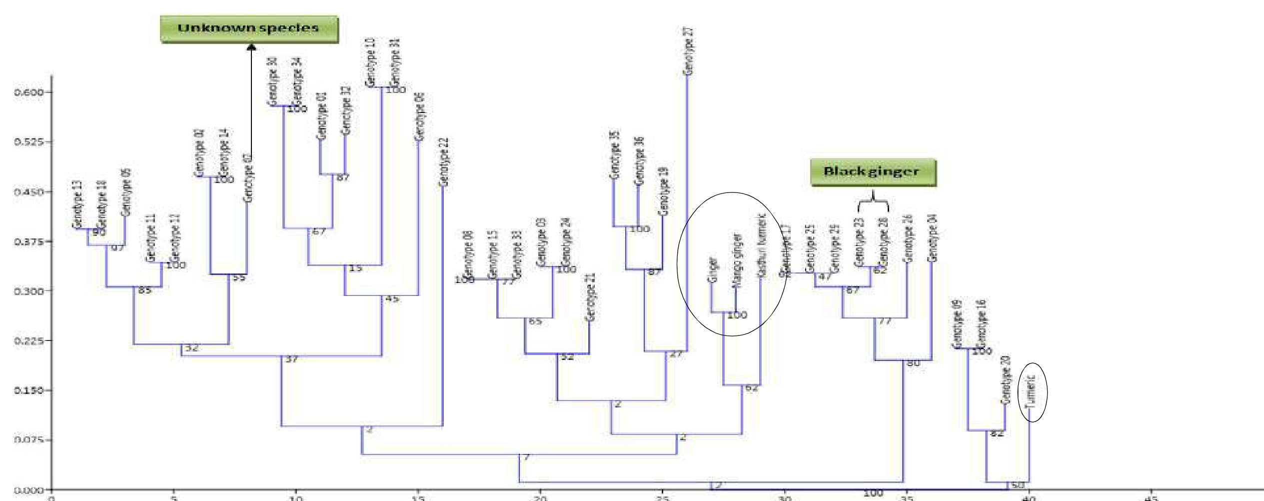


Fig. 2 : Dendrogram for Curcma caesiaRoxb. and other Zingeberaceae spp. - Jackard co-efficient

their geographical affiliations. Pioneers reported similar trend, where genetic distance was independent of geographical distance in population of *C. wenyujin* collected from various parts of China by Zheng *et al.,* 2015; Singh *et al.,* 2015 and Basak *et al.,* 2017 in turmeric; Ranemma and Reddy (2017) in black turmeric.

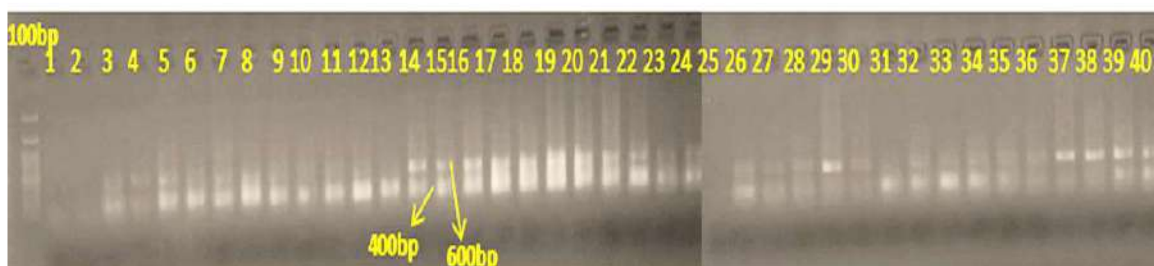
Clustering pattern in thirty-three accessions of *C. caesia* showed intraspecific variation as three major clusters and more sub groupings were observed in dendrogram. In SSR marker, genotype GMV-06 displayed the least similarity with rest of the genotypes and separated into a lone sub group in the dendrogram.

Although majority of the accessions were clustered irrespective of their place of collection, two genotypes *viz.,* GKT-29 and GKK-30 collected from Kerala grouped in seperate cluster in the dendrogram. Similar trend was observed for the genotypes collected from Karnataka, Manipur, Bihar, Mizoram and Odisha. The accessions belonging to the same geographical location did not always occupy the same cluster. Lack of location specificity in the clustering was also reported earlier in *C. longa* by Corcolon *et al.* (2015) and Gupta *et al.* (2016).

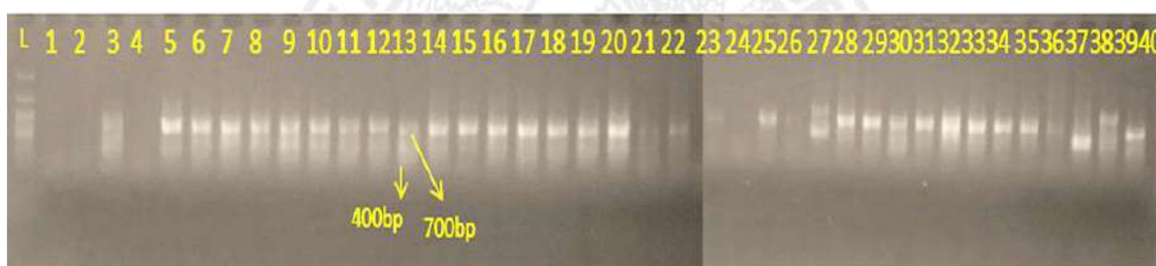
Molecular marker based dendrogram was quite different from the one constructed using morphological and biochemical traits. Morphologically similar accessions like GKM-2, GKB-3 and GKJ-5 (Mangalore, Bangalore and Joida of Karnataka) were found genetically distinct from each other. A similar incongruence between morphological and molecular marker based clustering pattern was earlier reported by Shegro *et al.,* 2013; Biabani *et al.,* 2013; Jain and Parihar, 2019 and Paw *et al.,* 2021. Moreover, all genetic differentiation need not result in morphological differentiation (Siva and Krishnamurthy, 2005). Jain and Jain (2018) also reported similar results in *Curcuma caesia* and *Curcuma longa* genotypes collected from Chatisgarh region.

The genotypes GGR-10, GAB-11, GAB-12, GAK-14 and GAD-32 are from Assam clustered in the same cluster (GAD-32 at different sub group). This could be probably because of lowest divergence among the population. Low genetic divergence may have ascribed to rapid germplasm movement across the state by the collectors / settlers which resulted in the wide spread distribution of same germplasm in various parts in course of time which are geographically closer. Further indicates the occurrence of gene flow between *C. caesia* genotypesis very limited. Basak *et al.* (2017) also reported similar gene flow among the populations of *C. longa* collected from four northeast states of India. The morphological similarity might be

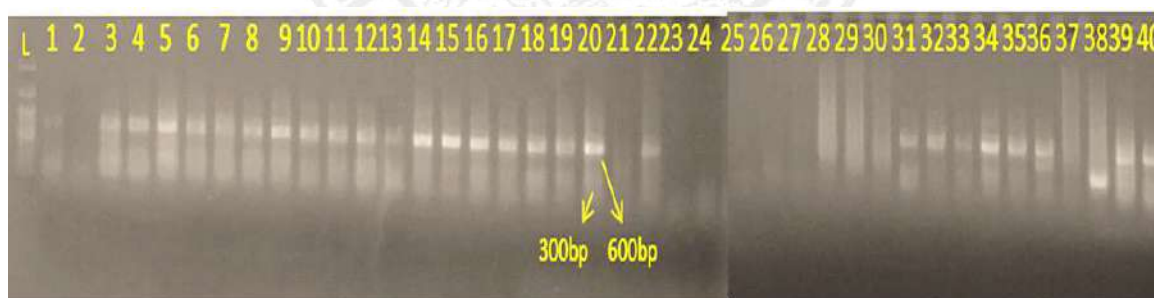
SSR12



SSR20



SSR24



SSR25

Plate 2B: SSR primers gel image of different black turmeric genotypes

attributed to the environmental conditions of same geographical location from which accessions were collected.

In light of the results from present and previous study, it can be concluded that the cluster analysis could successfully disclose intraspecific diversity with equal effectiveness. The observed intra specific variation in the genotypes of *Curcuma caesia* species at molecular level may be contributed by several factors; like genetic variation, including DNA mutation and chromosomal variation (particularly polyploidization), environmental factors, including phenotypic plasticity of the species and

TABLE 6

Similarity co-efficient matrix of black turmeric genotypes based on SSR marker – Jackard coefficent

G	1	2	3	4	5	6	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	24	25	26	27	29	30	31	32	33	34	35	36
1	1.00																																
2	0.27	1.00																															
3	0.44	0.22	1.00																														
4	0.10	0.47	0.41	1.00																													
5	0.50	0.60	0.42	0.25	1.00																												
6	0.57	0.39	0.28	0.20	0.51	1.00																											
8	0.44	0.24	0.87	0.29	0.47	0.19	1.00																										
9	0.23	0.30	0.61	0.38	0.58	0.23	0.70	1.00																									
10	0.56	0.41	0.16	0.08	0.24	0.38	0.18	0.10	1.00																								
11	0.50	0.76	0.43	0.38	0.55	0.53	0.44	0.51	0.31	1.00																							
12	0.48	0.74	0.41	0.36	0.85	0.63	0.45	0.56	0.33	0.64	1.00																						
13	0.53	0.64	0.44	0.27	0.94	0.54	0.49	0.62	0.25	0.43	0.90	1.00																					
14	0.27	1.00	0.22	0.47	0.60	0.39	0.24	0.30	0.41	0.52	0.74	0.60	1.00																				
15	0.44	0.24	0.87	0.29	0.47	0.19	1.00	0.70	0.18	0.48	0.45	0.49	0.24	1.00																			
16	0.23	0.30	0.61	0.38	0.58	0.23	0.70	1.00	0.10	0.49	0.56	0.62	0.30	0.70	1.00																		
17	0.10	0.54	0.29	0.77	0.28	0.19	0.33	0.45	0.19	0.38	0.40	0.30	0.54	0.33	0.45	1.00																	
18	0.53	0.64	0.44	0.27	0.94	0.54	0.49	0.62	0.25	0.60	0.90	1.00	0.64	0.49	0.62	0.30	1.00																
19	0.19	0.41	0.53	0.53	0.23	0.17	0.60	0.33	0.31	0.34	0.32	0.24	0.41	0.60	0.33	0.63	0.24	1.00															
20	0.21	0.40	0.55	0.51	0.53	0.32	0.62	0.85	0.17	0.51	0.66	0.56	0.40	0.62	0.85	0.61	0.56	0.44	1.00														
21	0.40	0.32	0.78	0.39	0.43	0.26	0.89	0.62	0.25	0.48	0.53	0.45	0.32	0.89	0.62	0.44	0.45	0.71	0.73	1.00													
22	0.27	0.10	0.25	0.13	0.24	0.43	0.28	0.38	0.19	0.39	0.35	0.25	0.10	0.28	0.38	0.15	0.25	0.16	0.53	0.39	1.00												
24	0.40	0.22	1.00	0.41	0.42	0.28	0.87	0.61	0.16	0.39	0.41	0.44	0.22	0.87	0.61	0.29	0.44	0.53	0.55	0.78	0.25	1.00											
25	0.10	0.54	0.29	0.77	0.28	0.19	0.33	0.45	0.19	0.42	0.40	0.30	0.54	0.33	0.45	1.00	0.30	0.63	0.61	0.44	0.15	0.29	1.00										
26	0.10	0.50	0.28	0.69	0.34	0.18	0.31	0.42	0.19	0.36	0.38	0.28	0.50	0.31	0.42	0.87	0.28	0.57	0.56	0.42	0.14	0.28	0.87	1.00									
27	0.21	0.19	0.32	0.24	0.30	0.18	0.22	0.10	0.35	0.22	0.17	0.20	0.19	0.22	0.20	0.18	0.20	0.41	0.29	0.31	0.15	0.20	0.26	0.21	1.00								
29	0.20	0.54	0.29	0.77	0.28	0.19	0.33	0.45	0.19	0.42	0.40	0.30	0.54	0.33	0.45	1.00	0.30	0.63	0.61	0.44	0.15	0.29	1.00	0.87	0.10	0.10							
30	0.54	0.31	0.19	0.10	0.57	0.65	0.21	0.26	0.42	0.52	0.55	0.60	0.31	0.21	0.26	0.20	0.60	0.22	0.24	0.19	0.32	0.19	0.20	0.20	0.20	0.20	1.00						
31	0.56	0.41	0.16	0.18	0.24	0.38	0.18	0.10	1.00	0.31	0.33	0.25	0.41	0.18	0.20	0.19	0.25	0.31	0.17	0.25	0.19	0.16	0.29	0.29	0.35	0.10	0.42	1.00					
32	0.89	0.25	0.37	0.12	0.46	0.52	0.40	0.21	0.65	0.47	0.45	0.48	0.25	0.40	0.21	0.20	0.48	0.18	0.19	0.37	0.25	0.37	0.22	0.22	0.20	0.20	0.74	0.65	1.00				
33	0.44	0.24	0.87	0.29	0.47	0.19	1.00	0.70	0.18	0.42	0.45	0.49	0.24	1.00	0.70	0.33	0.49	0.60	0.62	0.89	0.28	0.87	0.33	0.31	0.22	0.33	0.28	0.28	0.40	1.00			
34	0.64	0.31	0.19	0.10	0.57	0.65	0.21	0.26	0.42	0.58	0.55	0.60	0.31	0.21	0.26	0.20	0.60	0.20	0.24	0.19	0.32	0.19	0.20	0.20	0.20	0.20	1.00	0.42	0.74	0.21	1		
35	0.20	0.29	0.55	0.37	0.30	0.10	0.63	0.35	0.21	0.33	0.23	0.25	0.29	0.63	0.35	0.43	0.25	0.77	0.31	0.56	0.20	0.55	0.43	0.51	0.27	0.43	0.23	0.31	0.18	0.63	0.23	1	
36	0.21	0.30	0.57	0.38	0.25	0.10	0.66	0.37	0.22	0.32	0.24	0.26	0.30	0.66	0.37	0.45	0.25	0.82	0.83	0.59	0.20	0.57	0.45	0.42	0.29	0.45	0.24	0.27	0.19	0.66	0.30	0.87	1

germplasm exchange, as geographically separated plants tend to adapt to their growing environment by genetically modifying themselves. The observed variability within the species can be utilized for selecting high yielding plants with desirable traits.

Intraspecific Relations of Black Turmeric Genotypes : The similarity matrix coefficient ranged from 10 to 100 per cent (Table 6), suggesting a moderate genetic variation within black turmeric genotypes. The highest genetic similarity of 100 per cent was observed between twelve set of genotypes *viz.,* GKM-2 and GAK-14, GKB-3 and GMS-24, GBS-8 and GMW-15, GBS-8 and GNP-33, GBH-9 and GMK-16, GGR-10 and GMR-31, GAB-13 and GOK-18, GMW-15 and GNP-33, GMA-17 and GNK-25, GMA-17 and GKT-29, GNK-25 and GKT-29 and GKK-30 and GBC-34. These set of genotypes are genetically similar.

Similarly of >80 per cent also observed with eighteen sets of genotypes *viz.,* GKM-1 and GAD-32; GKB-3 and GBS-8; GKB-3 and GNP-33; GJG-35 and GNP-36; GNU-26 and GKT-29; GKN-25 and GNU-26; GMS-24 and GNP-33; GMF-21 and GNP-33; GOK-19 and GNP-36; GMA-17 and GNU-26; GMW15 and GMS-24; GAB-12 and GAB-13; GAB-12 and GOK-18; GBH-9 and GAP-20; GBS-8 and GMS-24; GKJ-5 and GAB-12; GKB-3 and GBS-8; GKB-3 and GMW-15.

While least (10 %) genetic similarity was observed between fourteen set of genotypes *viz.,* GKM-1 and GKB-4, GKM-1 and GMA-17, GKM-1 and GNK-25, GKM-1 and GNU-26, GKB-4 and GKK-30, GKB-4 and GBC-34, GMV-6 and GJG-35, GMV-6 and GNP-36, GBH-9 and GNF-27. GBH-9 and GMR-31, GGR-10 and GMK-16, GAK-14 and GMT-22, GNF-27 and GKT-29, GKT-29 and GMR-31 etc.

The results are in line with the findings of Susngi and Lasker (2015) in black turmeric; Singh *et al.* (2015); Basak *et al.* (2017) in turmeric. Singh *et al.* (2018) noted 100 per cent similarity between NVST-80 & Pratibha, NVST-55& GNT-2 and NVST 55 & NVST-53 turmeric genotypes.

The observed intraspecific variation may be associated with cultivation and targeted genotype selection of desirable characters in *C. caesia* as the species is economically important.

Interspecific Relations of Curcuma caesia Roxb. and other Zingiberaceae spp. *: Zingiberaceae* spp. exhibit significant morphological variations at both intra-specific and inter-species level, but the similarities of some species create problems during their identification. Various authors have reported false taxonomic identification of the specimen because of the similarities of some species like Curcuma caesia, Curcuma aureginosa etc. in this background to assess the relationship between black turmeric and other species of Zingiberaceae family, genetic diversity tree was constructed with the help of SSR markers.

Dendrogram showed genotype seven (unknown species) as separate branch and genotypes 23, genotype 28 shown to be similar which was very much expected as they belong to same species (Black ginger). We could also observe that the genotypes turmeric, ginger, kasthuri turmeric and mango ginger are shown in separate branches stressing the genetic diversity among them (Fig. 2). The similarity matrix coefficient ranged from 0.42 to 0.92 per cent suggesting a moderate inter species genetic variation between Zingiberaceae species *viz.,* black turmeric, unknown species, turemric, black ginger, turmeric, ginger, kasthuri turmeric, mango ginger. Results are in line with the observation of Islam (2004); Angel *et al.* (2008); Ahmed *et al.* (2009); Saha *et al.* (2016) and Sangin & Mongkholsathian (2017).

The genetic diversity documented in the present study provides a baseline data for optimization of conservation programme of the *Curcuma caesia* Roxb. species. Intra species divergence through 09 sub-groups reflects diversity of the species. Non-significant correlation between genetic distance and geographical distance and genotypes clustered irrespective of their geographical affiliations is evident. The similarity matrix coefficient exhibits moderate inter species genetic variation between Zingiberaceae species *viz.,* black turmeric, unknown species, turmeric, black ginger, ginger, kasthuri turmeric and mango ginger.

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