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Genetic variability of *Curcuma* sp. accessions of North East Hill region of India using ISSR and RAPD markers

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Abstract
The ayurvedic herb turmeric (*Curcuma longa* L.), offers myriad benefits including anticancer, antioxidant and antifungal properties. Genetic variability study is an important aspect of crop improvement program and also for conservation of plant genetic resources. Considering these points, the present study was planned to assess the genetic diversity of 22 accessions of *Curcuma* belonging to three different species (*C. amada, C. longa* and *C. caesia*) collected from North East India and were analyzed using 42 ISSR and 10 RAPD markers. The study revealed high to moderate genetic variability, 78% and 76.7% with ISSR and RAPD markers respectively. Darwin software based dendrogram categorized accessions into three major clusters, at certain degree these clusters are congruent with classification based on morphological characters. The maximum genetic similarity was observed between *Curcuma amada* and *Curcuma longa* whereas, *Curcuma caseia* showed distinct variability.

Keywords: *Curcuma* spp., curcumin, genetic diversity, ISSR, RAPD, North East India

1. Introduction
Indian saffron or Turmeric (*Curcuma longa* L.) is a perennial vegetatively propagated spice crop native to Southeast Asia and Indian subcontinent. It is grown in warm, humid climate all over the world. It has various health benefits one being it's use as medicine in Ayurveda. Turmeric's journey started from traditional era to modern era as anciently it was popular for balancing three dosha to healthy equilibrium, today it has received much attention in clinical studies [2, 4, 3, 23]. One of the greatest discoveries pertaining to turmeric in recent times was its therapeutic properties to fight back cancer, which is one of major current health issues in the world industry [6, 24, 8]. Thus, new methods based on molecular markers are conducted to assess genetic relationships and diversity among genotypes. India is one of richest biodiversity country in which North Eastern part of India is recognized as one of the biodiversity hotspots. North Eastern states have been blessed with tremendous diversity in turmeric cultivars. Among the different regions of North Eastern states, major spices like chilies, turmeric, ginger, cardamom, bay leaf, and black pepper are grown [12]. Out of which, turmeric and ginger is being grown as cash crop and is prominently cultivated in jhum fields mostly. Apart from medicinal properties, turmeric also possesses prodigious ceremonial value as well as heritage in India. It is an economically salient supreme plant species as it is used in various industries such as food, spices, medicines and dyes [5, 7]. Polymorphism of any crop is related to its genetic variation, biodiversity and adaptive ability within the species of that crop. Therefore, knowledge of genetic variability is essential for crop improvement programs and for conservation of plant genetic resources. Genetic diversity assessment is helpful in management of species by selecting the high yielding variety in the given crop [25].
The major tool to reveal the polymorphism among the cultivars are molecular markers. Various study has been conducted using different molecular markers such as RAPD [18, 9]. ISSR markers have proven to be more reliable, inexpensive, reproducible, easy to generate that relies on repeat amplification of DNA strands using single primer [16]. ISSR markers overcomes limitations of other markers like low reproducibility in RAPD, high cost of AFLP, and prior flanking sequence information to develop SSR markers etc [17, 21, 13, 20, 16, 1]. Considering these observations, the present investigation was formulated in order to emphasize on genetic diversity in turmeric accession collected from the different locations of North East India region.

2. Materials and Methods

2.1 Plant material

The present study dealt with different *Curcuma* spp. A total of twenty-two accessions were collected from below different locations of North East India (Table 1). Eco-geographically, the locations were selected from three habitats such as hilly areas (Khasi and Garo hills), plain lands (Assam) and plateau lands (West Garo hills Ri-Bhoi, Tripura and Arunachal Pradesh, Mizoram, Manipur) of North-East India. The study on genetic diversity was carried out in the laboratory of plant molecular biology and biotechnology, School of crop Improvement, Central Agricultural University, Imphal. The plant accessions were grown in pots.

2.2 Genomic DNA extraction

Entirely opened fresh tender leaves were used for genomic DNA extraction by CTAB method [11] and quantified in 0.8% agarose according to standard DNA primers.

2.3 PCR condition and electrophoresis of PCR product

The ISSR and RAPD reactions was carried out in Thermal Cycler with the following program: Initial denaturation at 94° C for 5 min, followed by 36 cycles of 94° C for 50 sec, 30 to 40°C for 40 s (for ISSR primer) and 1 min (for RAPD primer), 72° C for 1.30 min, and finally at 72° C for 10 min. The range of DNA concentrations used in PCR was 30-50 ng. The amplified products were visualized by electrophoresing on 1.5% agarose gels at 80 V in a 1 X TBE buffer system. After completion of electrophoresis gels were photographed with a BioRad Geldoc 1000 computer system.

2.4 Data scoring and analysis

Binary data matrix prepared the presence of band was scored as “1” and absence of band scored as “0” for ISSR and RAPD primers. All statistical calculation analysis including polymorphic information content (PIC), marker index (MI), percentage of polymorphic band (PPB), polymorphic bands (PB), monomorphic bands (MB), total Number of scored bands (NSB) were done using respective data matrices. Binary data matrix subjected to analysis using Darwin software. Genetic diversity analyzed by dissimilarity present at genetic level.

3. Results

A total 22 turmeric accession were subjected to PCR amplification using 40 ISSR and 10 RAPD primers (Table no. 3) among which 23 ISSR and 5 RAPD primers got amplification.

A total of 149 amplicons of ISSR and 20 of RAPD were produced (fig no. 2-9). Average 6.47 amplicons were produced per primer with ISSR and 4 amplicons per primer with RAPD. The amplicon size ranged from 400bp–100bp for ISSR and in RAPD from 500bp to 1400bp (Table no.2) (fig no. 2 to9).

To find out efficiency of primer to distinguish turmeric individuals’ PIC value, polymorphic per cent and MI was calculated (Table no. 3). The maximum PIC value was calculated in primer P3 (0.497) followed by primer UBC-868 (0.474), UBC-808 (0.443), 814 (0.443), and UBC-848 (0.405). These high PIC value of primers indicated that primers are more efficient for diversity analysis in turmeric whereas, low PIC value calculated in UBC-852 (0.198) primer. Marker index shows the genetic variation among the genotype, it is observed that number of polymorphic bands is directly proportional to marker index. Further it is observed that there is no correlation between percentage of polymorphism and Marker Index. The highest MI is observed in UBC_848 (2.88) and lowest value of MI observed in OPH-03 (0.30). From the observation it can be suggested that UBC_848 marker is more favorable for genetic diversity analysis in turmeric accession.

3.1 Cluster analysis

Genetic diversity was analyzed by dissimilarity present at genetic level. Using Darwin software and the dissimilarity coefficient ranged between 0.218 to 0.848. The highest dissimilarity observed in T04 (Kedaram), T08 (Lakadong) and T07 (Megha). The lowest dissimilarity was observed in T20 (Thikur) and T07(Megha). Average dissimilarity coefficient found in 22 turmeric genotypes was 0.55. Clustering pattern obtain by dissimilarity coefficient based dendrogram (with combined molecular data of ISSR and RAPD primer) categorized all 22 genotypes into 3 major clusters (fig no.1). Obtained dendrogram showed diverse genetic profile of genotype Cluster I: First cluster was largest cluster with 12 genotypes included in it which are distributed in 2 sub-groups namely |A| and |B|. Group |A| again divided in 3 sub-groups namely a, b and c. Group a further divided into two groups one with 3 genotypes and second with 2 genotypes. Group b remain as solitary cluster and included 3 genotypes in it. Group c included 3 genotypes. Group |B| includes one genotype. Cluster II The second cluster included 9 genotypes and divided in 2 groups |C| and |D|. Group |C| and |D| further divided into 2 sub group Cluster III The third |E| cluster was made up of only one genotype The maximum (81%) bootstrap value was observed between T15 and T14. The bootstrap value ranged from 11% to 81%.

Table 1: List if turmeric genotype used for study

S. N.	Genotype	Given Designation	Collected From
1	Local III	T01	CoA, CAU, Imphal
2	Yai Heinouman	T02	CoA, CAU, Imphal
3	Yaimu	T03	CoA, CAU, Imphal
4	Kedaram	T04	CoA, CAU, Imphal
5	Tripura I	T05	AAU, Jorhat
6	Mizoram II	T06	AAU, Jorhat
7	Megha turmeric 1	T017	CoA, CAU, Imphal
8	Lakadong	T08	CoA, CAU, Imphal
9	Lachin	T09	Jaintia Hills
10	Local Jaintia hills	T010	Jaintia Hills
11	Tripura II	T011	AAU, Jorhat
12	Local II	T012	Byrnihat RiBhoi
13	Charmit	T013	Jaintia Hills

14	Yaingou	T014	CoA, CAU, Imphal
15	Local IV	T015	West Garo Hills
16	Black turmeric	T016	BSI, umiam
17	Local I	T07	CoA, CAU, Imphal
18	Local V	T018	BSI, umiam
19	Assam II	T019	AAU, Jorhat
20	Tikhur	T020	BSI, umiam
21	Arunachal Pradesh I	T021	AAU, Jorhat
22	Nirguli turmeric	T022	Papumpare Arunachal Pradesh

Table 2: ISSR and RAPD analysis of genomic DNA of turmeric

S. N.	Variable	ISSR Observations	RAPD Observations
1	Number of primers used	42	10
2	Number of amplified primers	23	6
3	Total Number of generated amplicons	149	20
4	Average of amplicon per primer	6.47	4
5	Polymorphic percentage	78%	76.7%
6	Size of amplified product	400-1100bp	500-1400bp

Table 3: Polymorphic markers used in study

S. N.	Marker Name	SEQ.	NSB	PB	MB	PPB	PIC	EMR	MI	TM
	ISSR									
1	807	AGAGAGAGAGAGAGAGT	4	4	0	100.0	0.438	4.00	1.75	50
2	UBC_808	AGAGAGAGAGAGAGAGC	5	5	0	100.0	0.443	5.00	2.22	52
3	UBC_848	CACACACACACACACARG	9	8	1	88.9	0.405	7.11	2.88	58.8
4	UBC_850	GTGTGTGTGTGTGTGTYC	8	7	1	87.5	0.348	6.13	2.13	58.8
5	825	ACACACACACACACACT	9	7	2	77.8	0.337	5.44	1.83	50
6	UBC_812	(GA)8A	11	9	2	81.8	0.315	7.36	2.32	54.8
7	814	CTCTCTCTCTCTCTCTA	4	2	2	50.0	0.443	1.00	0.44	50
8	816	CACACACACACACACAT	7	4	3	57.1	0.407	2.29	0.93	50
9	UBC_818	CACACACACACACACAG	8	7	1	87.5	0.394	6.13	2.41	57.2
10	UBC_835	AGAGAGAGAGAGAGAGYC	7	5	2	71.4	0.385	3.57	1.38	58.8
11	UBC_842	GAGAGAGAGAGAGAGAYG	5	4	1	80.0	0.401	3.20	1.28	58.8
12	844	CTCTCTCTCTCTCTCTAC	11	9	2	81.8	0.371	7.36	2.73	54
13	ISSR 05	(CT)7 TG	8	7	1	87.5	0.343	6.13	2.10	52
14	ISSR 16	(GAGAGA)2 GAGAT	3	2	1	66.7	0.318	1.33	0.42	50
15	ISSR 02	(CT) 8 AC	8	6	2	75.0	0.414	4.50	1.86	54
16	P3	AGAGAGAGAGAGAGAGTG	8	6	2	75.0	0.497	4.50	2.24	54
17	P6	CCACCACCACCACCA	7	5	2	71.4	0.416	3.57	1.48	50
18	UBC_852	TCTCTCTCTCTCTCTCCGA	5	4	1	80.0	0.198	3.20	0.63	58
19	UBC 873	GACAGACAGACAGACA	4	3	1	75.0	0.316	2.25	0.71	48
20	UBC_880	GGAGAGGAGAGGAGA	4	3	1	75.0	0.421	2.25	0.95	56.2
21	UBC_864	ATGATGATGATGATGATG	4	2	2	50.0	0.432	1.00	0.43	48
22	UBC_868	GAAGAAGAAGAAGAAGAA	5	5	0	100.0	0.474	5.00	2.37	48
23	811	GAGAGAGAGAGAGAGAC	5	4	1	80.0	0.336	3.20	1.07	52
	RAPD									
1	A-01	CAGGCCCTTC	3	2	1	66.7	0.289	1.33	0.39	36.4
2	OPA-18	AGGTGACCGT	5	5	0	100.0	0.399	5.00	2.00	36.2
3	OPP-8	ACATCGCCCA	6	5	1	83.3	0.397	4.17	1.65	37.6
4	OPQ-02	TCTGTCGGTC	3	2	1	66.7	0.343	1.33	0.46	33.4
5	OPH-03	AGACGTCCAC	3	2	1	66.7	0.227	1.33	0.30	33.9
Total			6.035	4.785	1.25	78.0	0.375	3.88	1.48	-

PB: Polymorphic bands, MB: Monomorphic bands, NSB: Total Number of scored bands, PPB: Percentage of polymorphic band, MI: marker index, PIC: Polymorphic information content.

4. Discussion

In present study, three clusters of diversity were formed using Darwin software and it is interesting to note that the accession falling in cluster-I belongs to separate geographical area and are having high curcumin content. In Meghalaya, Lakadong is one of the native turmeric variety which has highest curcumin content (6 to 7%) in the world. In our study Lakadong, Kedaram and Megha Turmeric-I, resemble same properties and fall under one subgroup of cluster which depict that kedaram and Megha turmeric-I may also have high curcumin content and can be useful for further breeding program. Singh *et al.* (2013) [26] examined curcumin percentage in 11 turmeric var. and showed Megha and Kedaram having 7.5 and 5.3% curcumin content respectively cognate to Lakadong. We can corelate the genetic diversity here with curcumin content, Lakadong having highest curcumin belongs to cluster having Kedaram and Megha Turmeric-I and other turmeric, indirectly it predicts curcumin content is higher in Kedaram, Megha Turmeric-I and other turmeric which is present in cluster-I. Basak *et al.* (2017) [3] reported genetic diversity by using ISSR and RAPD markers in assessment of 19 turmeric cultivars collected from North East India. 20 RAPD and 20

ISSR primers were used of which 9 RAPD and 7 ISSR showed distinct bands. Their finding showed highest polymorphic loci obtain from Meghalaya turmeric and lowest polymorphism from Manipur turmeric which is resembles to our study. It revealed Meghalaya has huge genetic variability in turmeric accessions. Paul *et al*. (2016) [19] observed that in North East India, genetic base of turmeric is broad. Their investigation revealed that high-quality turmeric cultivation is done in North East India especially in Meghalaya.

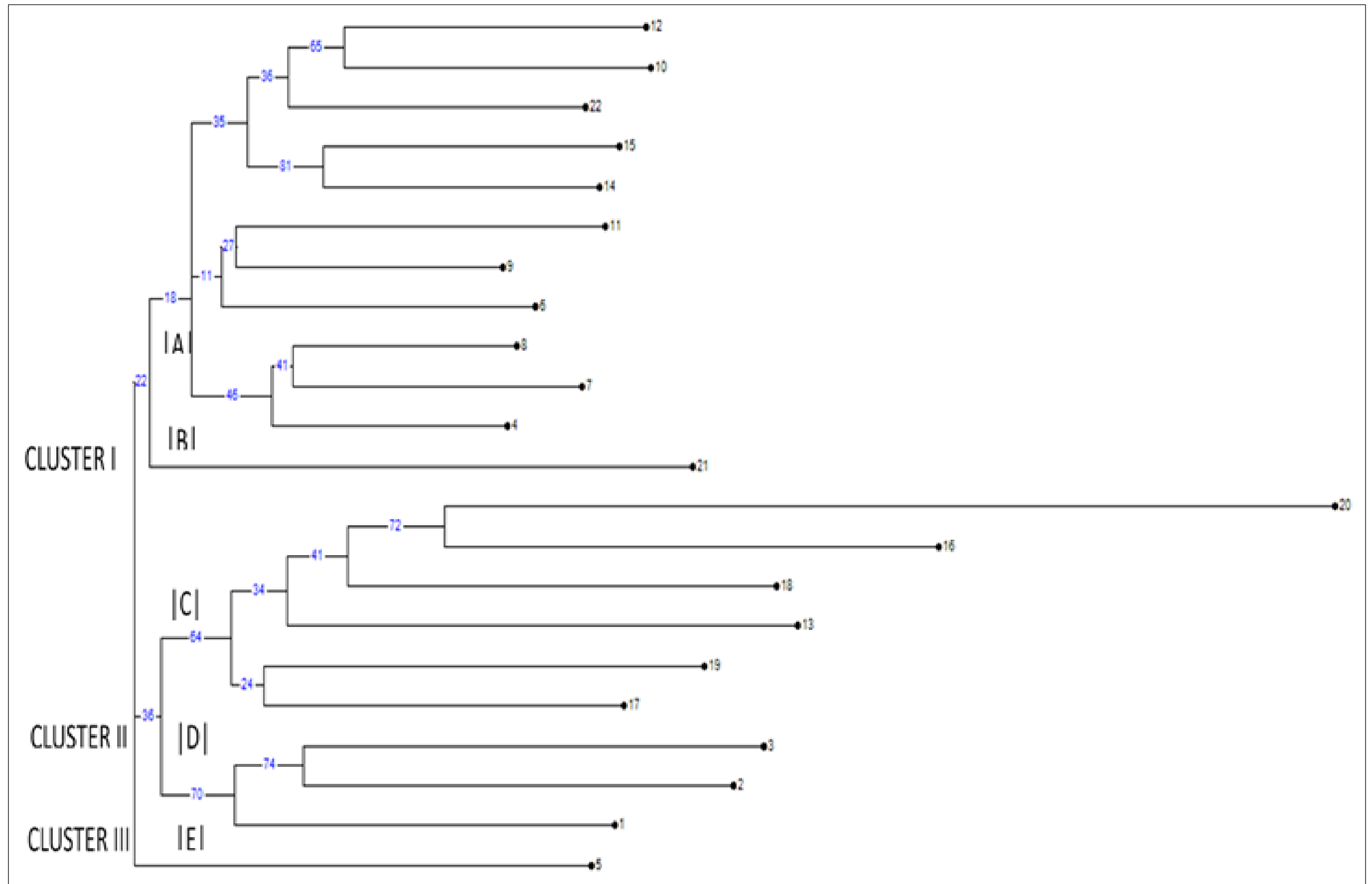

Fig 1: Dendrogram of 22 *Curcuma* accessions from North East India resulting from a UPGMA cluster analysis obtained from 23 ISSR and 5 RAPD primers.

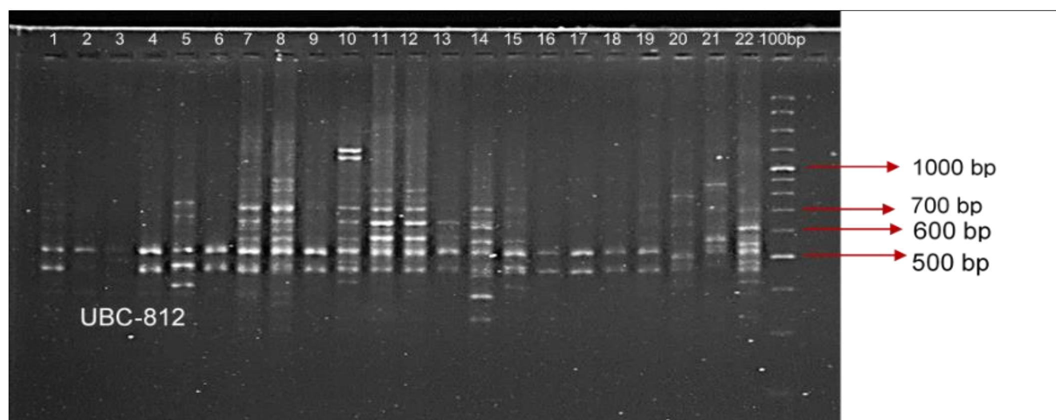

Fig 2: Electrophoretic banding pattern of PCR amplified product resolved on 2.5% agarose gel amplified product of UBC-814 primer 100bp = molecular size ladder, serial no. 1-22 represents genotypes as given in table no. 1.

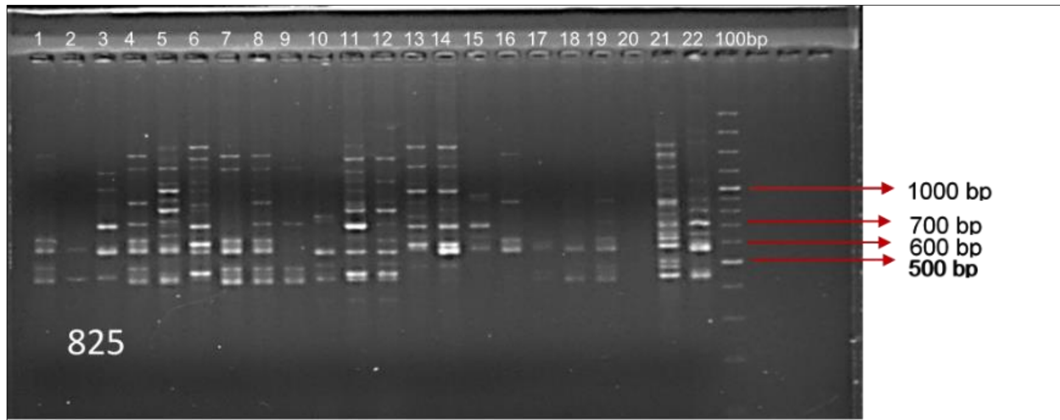

Fig 3: Amplified product of 825 primer 100bp = molecular size ladder

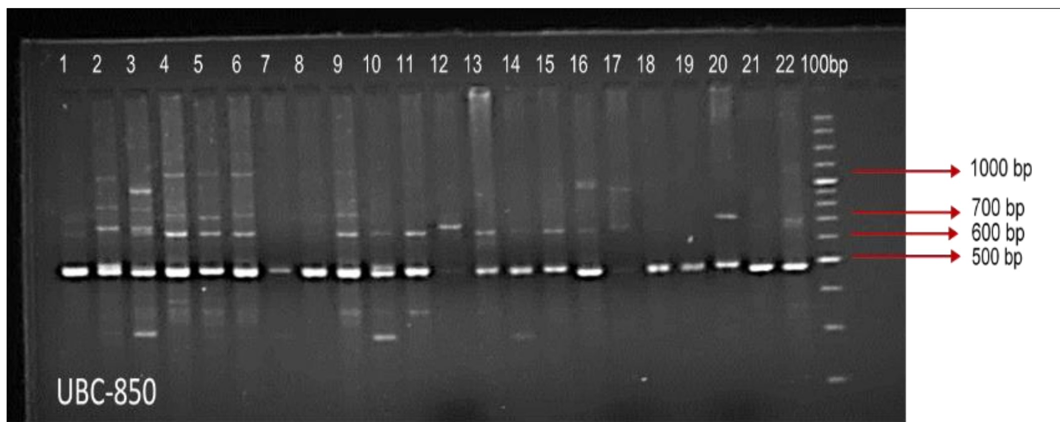

Fig 4: Amplified product of UBC-850 primer 100bp= molecular size ladder

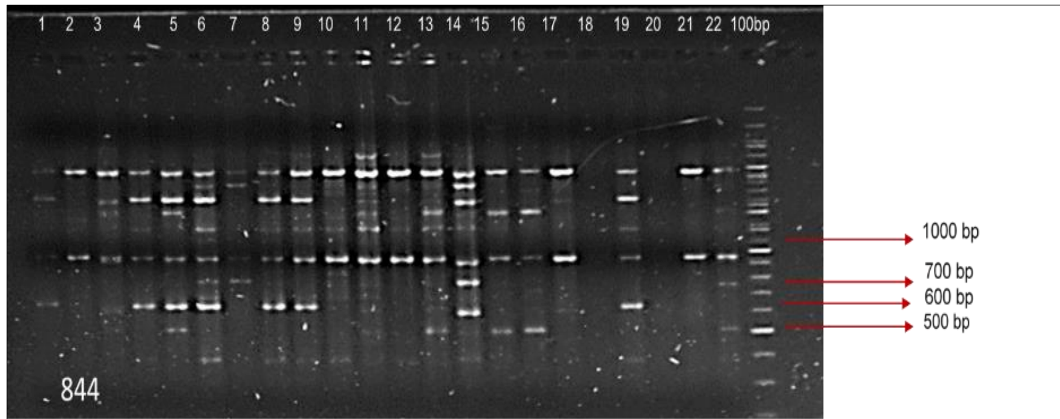

Fig 5: Amplified product of 844 primer 100bp= molecular size ladder

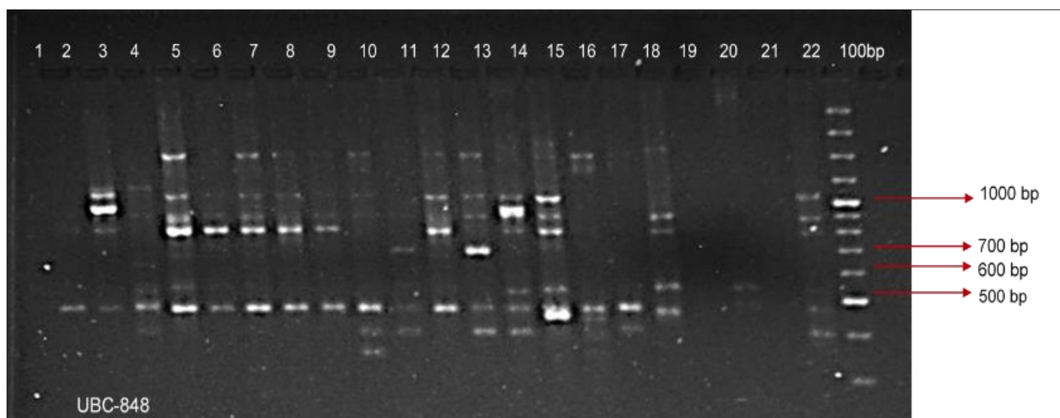

Fig 6: Amplified product of UBC-848 primer 100bp= molecular size ladder

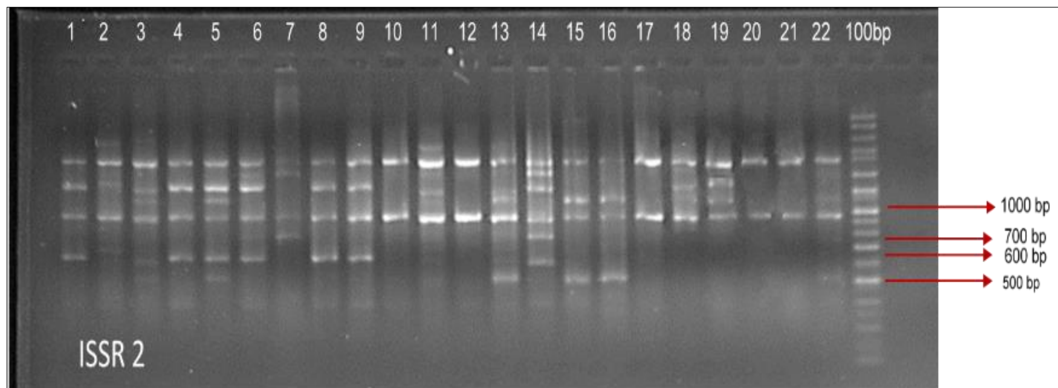

Fig 7: Amplified product of ISSR 2 primer 100bp= molecular size ladder

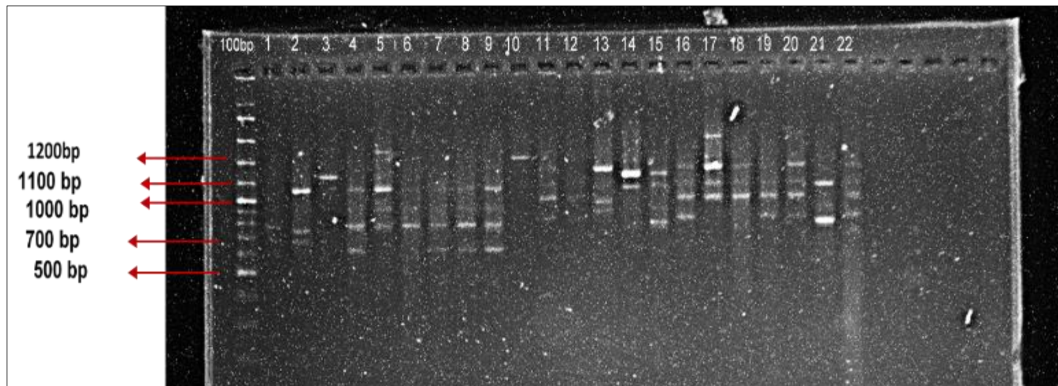

Fig 8: Amplified product of OPA 18 primer 100bp= molecular size ladder

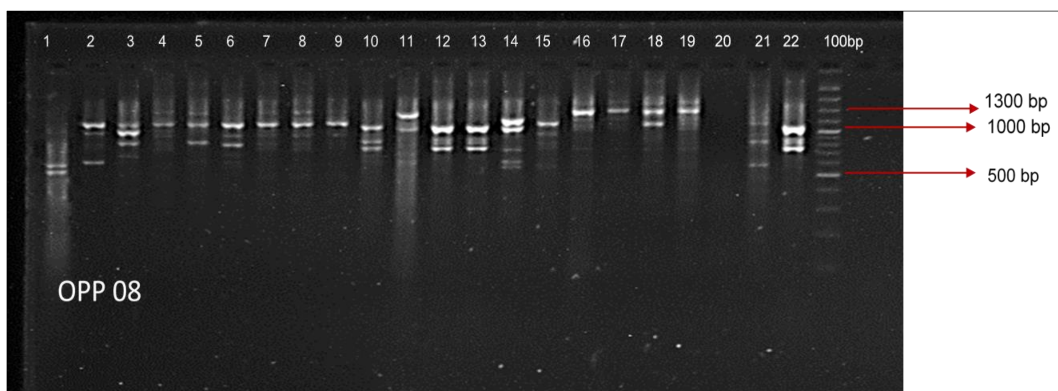

Fig 9: Amplified product of OPP 08 primer 100bp= molecular size ladder

It is one of the reasons that Megha, Lakadong like variety are cultivated more by farmers in this region, showing genetic polymorphism in accessions of North East India using RAPD primers. These accessions were in accordance with our findings. Findings of high polymorphism, M.I. and PIC of markers system used in present study are considerably significant. Large number of polymorphic bands showed that primers used in this study could be significant for genetic diversity analysis in turmeric genotypes. Saha *et al*. (2016) [22] successfully used ISSR markers to analyzed genetic diversity in *Curcuma* species in which UPGMA dendrogram obtained from 22 ISSR data placed 4 accessions into 2 different cluster, they obtained high PIC value in HB and UBC852 (0.40) whereas, in present study UBC-852 showed lowest PIC value (0.198). This may be due to different genotypes used in their study. kizhakkayil and Sasikumar (2010) [15] and Dash *et al*. (2019) [10] studied genetic diversity in species of *Curcuma*. Our results reflect similar findings as given in previous studies. In the present study overall, genetic distance was 0.510 within collected 22 accession and significant polymorphism obtained 78%. It is possibly due to sample collected from different geographical areas. Similar results were observed in investigation of Singh *et al*. (2012) [27].

The high genetic diversity among genotypes shows strong genetic structure present in between them and thus significant variation exists in genetic diversity in all the genotypes. Findings of other researchers like Basak *et al*. (2017) [3], Singh *et al*. (2012) [27], Saha *et al*. (2016) [22], Joshi *et al*. (2012) [14], Paul *et al*. (2016) [19] who reported genetic variation occurred in different genotypes of North East India are also in concordance of observations recorded during the present study. The molecular profiling of 22 turmeric accessions shared some similarity and dissimilarity which warrants that count on exclusively the floral, vegetative growth, rhizome feature like color, smell, taste for taxonomical characterization may lack clarity of diversity. In order to taxonomic revision of the genus ISSR/RAPD markers can be effectively used to resolve identity of closely related

accessions.

5. Conclusion
High to moderate variability was observed in collected 22 genotypes using molecular markers. The result obtained suggested that T07 (Megha Turmeric I) and T20 (Thikur) have different parental origin. As their genetic distance is high and could be used for further breeding crop improvement program. Closer affinity in the dendrogram grouping patterns of Lakadong, Kedaram and Megha, suggested a single parental origin, further reiterated by their similar levels of curcumin content.

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