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Infraspecific variations and Molecular characterization by using ISSR Markers in *Curcuma* Species

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ABSTRACT: The genus *Curcuma* is one of the largest genera in the family Zingiberaceae comprising 120 species. Forty species are recorded from India, 8 species from Maharashtra and 4 species of Curcuma are reported from Melghat Forests Dist Amravati. Of these, *Curcuma inodora* Blatt., *Curcuma pseudomontana* J. Graham and *Curcuma decipiens* Dalzell are wild, while *Curcuma longa* L. is cultivated. *C. inodora* is widely distributed throughout Maharashtra and is very common in the Melghat Forests. Population of *C. inodora* display tremendous variations in aerial and underground parts. Major variations found are shape of root tubers, length of leaf stalk, position of spike, length of spike, shape, size and colour of bracts. Infraspecific variations are recently attracting attention of taxonomists. Creation of infraspecific variations is the main origin and storage of speciation and genetic divergence among populations of a species. Twelve variants of *C. inodora* and one accession each of *C. longa* and *C. pseudomontana* were collected for the present study. *C. decipiens* very rare could not be collected.

Genetic fingerprints of *Curcuma longa, Curcuma pseudomontana* and twelve variants of *Curcuma inodora* were developed using ISSR marker for genetic diversity analysis and relatedness among the species and within the species. Five Inter simple sequence repeat primers produced 305 bands out of which 299 were polymorphic bands. Dendrogram was constructed using MEGA software based on UPGMA. Cluster analysis on the basis of dendrogram placed 12 variants of *Curcuma inodora* and two species in two clusters and twelve variants of one cluster subdivided into four subcluster, indicating the relatedness and also the genetic distance pointing out clear polymorphism within the species.

Keywords: Infraspecific variations; MEGA; Inter Simple Sequence Repeat; Cluster analysis; *Curcuma longa* L; *Curcuma inodora* Blatt.; *Curcuma pseudomontana* J. Graham.

INTRODUCTION

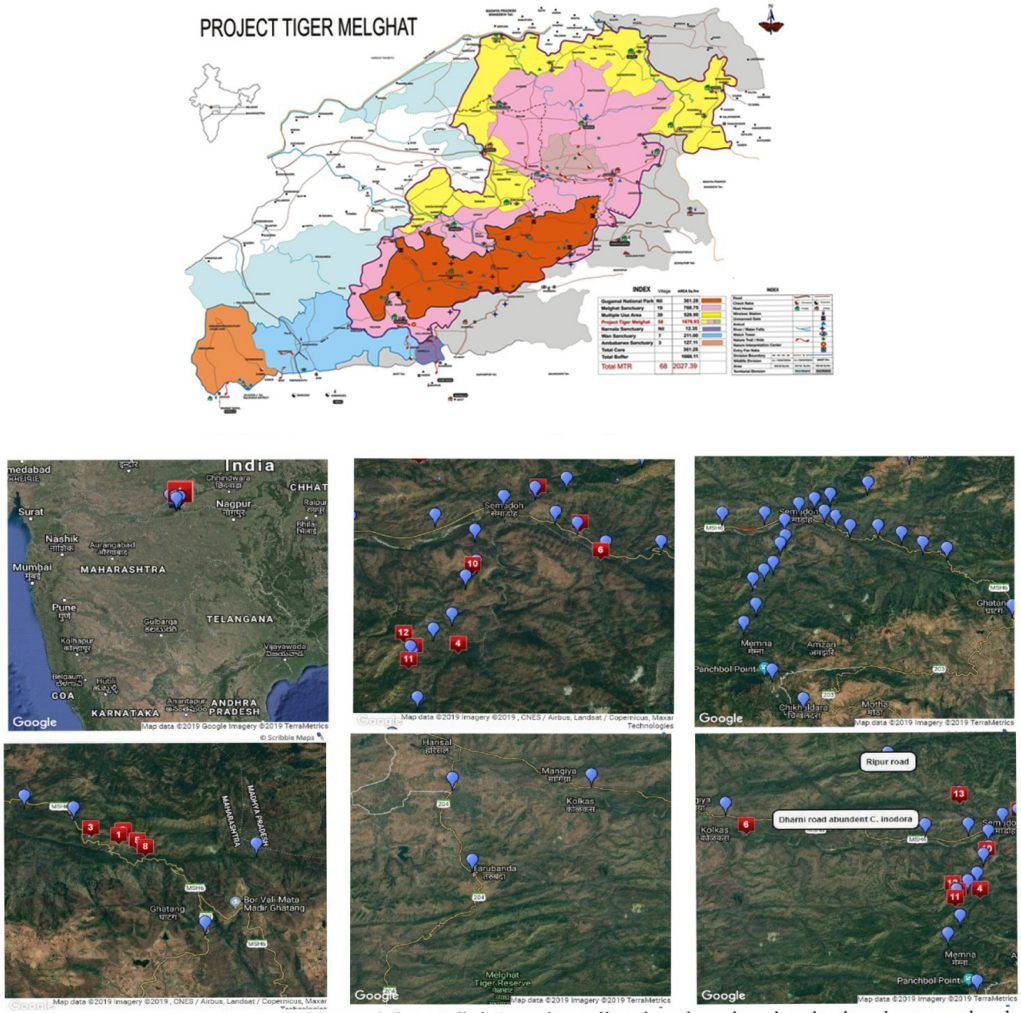
The genus *Curcuma* is one of the largest genera in the family Zingiberaceae, comprising of 120 species; widely used in spices, medicines, dyes as well as ornamentals (Skornickova *et al*., 2000). Among 120 species, 40 are from India: From Maharashtra, 8 species are reported (Sharma *et al*., 1966). Of these four are found in Melghat; *Curcuma longa* L. is cultivated, while *Curcuma pseudomontana* J. Graham, *Curcuma inodora* Blatt. and *Curcuma decipiens* Dalzell are wild. Melghat forest has a long history of conservation. Since 1860 it is enjoying the status of reserve forest. Melghat Tiger Reserve spreads over an area of 1618 sq.km. of which 361 sq.km. is core area. Melghat forests have one of the last few glorious and prestigious congregations of plant communities, which had survived the onslaught of the axe wielding man to a large extent so far. Forests of Melghat division are of deciduous nature and have been classified as dry deciduous.

Curcuma is easily recognized by its inflorescence, a spike with prominent spiral bracts and sterile terminal bracts forming 'comma'. *C. inodora* is widely distributed throughout Maharashtra and is very common and abundant in Melghat. It is commonly called 'Jangli halad' and used in traditional medicine by locals. Paste of root stock of *C. inodora* Blatt is applied in glandular diseases and piles (Dagawal and Bhogaonkar 2019; Mudaliar *et al*., 1987; Shah and Gopal, 1982). *C. pseudomontana* J. Graham is used in traditional medicine to cure jaundice and diabetes (Panal *et al*., 2012), body swellings and to increase lactation (Ramarao *et al.,* 2000). Fresh tubers are eaten as blood purifier (Acharya *et al*., 2012).

The identification of *Curcuma* has traditionally been achieved using morphological characters. However, *Curcuma* species show large morphological variations in both intra and inter species, but in some cases, especially early flowering groups show a very similar pattern of morphology between them which leads to confusion in their identification (Apavatjrut *et al*., 1999) . *C. inodoara* populations observed in Melghat display tremendous variation in morphological characters such as size of leaf , length and position of inflorescence, bract colour and shape, shape and notching of labellum etc. Infraspecific variations are

recently attracting attention of taxonomists. Genetic diversity is increasingly being based upon information at the DNA level by various molecular techniques such as Inter Simple Sequence Repeat, Randomly Amplified Polymorphic DNA, Amplified Fragment Length Polymorphism etc. ISSR provides tool for genetic mapping and assessment of genetic diversity between and within species as well (Davila *et al.*, 1998; Ghariani *et al.*, 2003; Moreno *et al.*, 1998). The DNA sequence-based markers are comparatively stable and not affected by diverse environment (Nadeem *et al.,* 2018). These are based on polymorphisms at the level of DNA sequence. Molecular characterization would help us not only to better understand the observed morphological diversity but also the real diversity at DNA level among them (Aswathi *et al.,* 2023). Present study was done to understand infraspecific and interspecific variations between the three species and twelve variants of *C. inodora* found in Melghat Forests.

Study Area



Satellite map showing GPS locations of the populations studied. Accessions collected are shown by red marks; they also are numbered.
Courtesy : https://www.scribblemaps.com and http://www.scribblemaps.com/maps/view/Rapid_survey/ykYiNqiWif

Photoplate-A

MATERIAL AND METHODS

Curcuma inodora Blatt 12 variants, *Curcuma pseudomontana* J. Graham and *Curcuma longa* L were selected for molecular characterization. *C. decipiens* is very rare and could not be collected. The leaf samples of selected species were collected from various locations in Melghat Forests Dist. Amravati Maharashtra (Photoplate-A). Species were identified using standard floras (Bhogaonkar and Devarkar 1999; Cooke, 1967; Dhore,1986; Hooker, 1927; Patel, 1968; Yadav and Sardesai 2002).

DNA Isolation. DNA is isolated by the C-Tab method (Murray and Thompson 1980). A total of 5 PCR reactions were performed using five primers for each sample, following Khaled *et al.* (2015).

ISSR data analysis. Amplified fragments were manually scored for presence as 1 and absence as 0 and binary matrix were subjected to statistical analysis and a dendrogram was drawn using MEGA Software

(Iruela *et al.*, 2002). The Similarity Matrix was calculated by Souframanien and Gopalakrishna (2004).

RESULTS AND DISCUSSION

Morphological profile. During a survey of *Curcuma* species in Melghat, it was found that in *Curcuma inodora* populations all the variations are continuous variations; no discontinuous variations were observed. However, for the study, distinct variations were considered as distinct variants. Variants of *C. Inodora* were coded as CI-1, CI-2, CI-3, CI-4, CI-5, CI-6, CI-7, CI-8, CI-9, CI-10, CI-11 and CI-12 while *C. pseudomontana* as CP-13 and *C. longa* as CL-14 presented in Photoplate-1. Fifty morphological characters were studied, of which 36 are qualitative and 14 quantitative as presented in Table 1.

Agarose Gel electrophoresis of the ISSR PCR. Agarose Gel product obtained with five primers produced a total of 305 distinct amplification products of which 299 bands were polymorphic, presented in Table 2 and Fig. 2 to 6.

Scored bands per primer ranged from 37 (UBC_812)) to 73 (UBC_811) with mean number of 61 per primer. The average number of polymorphic bands across the primers was 98.16 % ranging from 96.72 produced by UBC_824 ISSR primer to 100% obtained by UBC_812 ISSR primer.

The amplification of fourteen samples was found to be polymorphic with five different primers. Not a single primer has generated same profile of bands. Five primers have generated a total of 145 types (intervals) of different sizes of the fragments. A Total number of 305 DNA fragments were observed. All primers have shown polymorphic bands. UBC_811 and UBC_834 have shown two monomorphic bands in all samples, UBC_826 and UBC_835 have shown one monomorphic band in all samples, while UBC_812 primer has shown all polymorphic bands. Not a single primer has shown the same pattern of bands, also not a single primer has shown clear polymorphism amongst all the accessions / variants. Same was observed for *Arachis hypogea* varieties by Raina *et al*. (2001). Five ISSR primers are used for ISSR profile of 14 samples in present study, of which UBC_812 primer shows 100% polymorphism with 37 bands as compared to the polymorphic band produced in *C. alismatifolia* (Taheri *et al*., 2102) which is 62. 50 % that produced only 8 bands out of which only 5 bands are polymorphic. This indicates high genetic variability within the *C. inodora* populations studied.

Table 1: Qualitative and Quantitative morphological characters.

S. N.	Characters	CI-1	CI-2	CI-3	CI-4	CI-5	CI-6	CI-7	CI-8	CI-9	CI-10	CI-11	CI-12	CP-13	CL-14
1	**Plant height**														
a	At flowering stage	70 cm	46 cm	57 cm	72 cm	54 cm	55 cm	50 cm	52 cm	50 cm	90 cm	80 cm	50 cm	53 cm	105 cm
2	**Leaf stalk length**	30 cm	15 cm	24 cm	30 cm	22 cm	20 cm	22 cm	32 cm	28 cm	40 cm	23 cm	16 cm	22 cm	37 cm
3	**Lamina length :breadth**	30:11	26:12	30:11	30:16	28:13	20:13	26:11	24:12	20:08	40:30	32:15	28:8	26:13	42:15
4	**Leaf apex**	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute
5	**Leaf surface**														
a	Upper	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
		Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green	Dark Green
b	Lower	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
		Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green
6	**Spike position** lateral/Central	Central	Central	Central	Central	Central	Central	Central	Central	Central	Central	Central	Central	Central	Central
7	**Length of spike**	24 cm	22 cm	24 cm	28 cm	28 cm	24 cm	22 cm	26 cm	24 cm	30 cm	30 cm	26 cm	18 cm	35 cm
8	**Length of spike Stalk**	14cm	12 cm	14cm	16 cm	16 cm	14cm	11cm	13cm	15cm	20 cm	21 cm	15cm	12 cm	25 cm
9	**Sterile bract api**														
a	Pattern spread Horizontal/ Obliquely	Horizontal	Horizontal	Horizontal	Obliquely	Obliquely	Obliquely	Obliquely	Obliquely	Obliquely	Obliquely	Horizontal	Obliquely	Horizontal	Obliquely
b	Colour of bracts	Violet	White with purple tip	Violet	Magenta	Violet	Purple tip darker	White base with pink line	Light purple with dark tip	Faint purplewith dark purple tip	Whitish with purple tinge dark purple towards apex	Purplish white with dark middle strip	Light pinkish purple with dark pink apex	Purple pink	White
c	Tip- Acute/ mucronate/ obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Acute	Obtuse	Mucronate	Obtuse	Obtuse
d	Bract Margin- Straight Undulate	Undulate	Straight	Undulate	Straight	Straight	Undulate	Straight	Undulate	Straight	Undulate	Undulate	Undulate	Straight	Straight
e	Shape - ovate/obovate/linear	Linear	Linear	Linear	Ovate	Ovate	Linear lanceolate	Linear	Ovate	Linear	Linear	Linear	Linear	Linear	Ovate
10	**Fertile bract**														
a	Colour	Light Green	White green with purple tip	Light violet	Light green with violet tinge	Light violet	Pale green	Pale green	Pale yellowish green with purple tinge	White with green tip	Green with violet tip	Light green with violet tip	Green	Light green	Green
b	Shape- ovate/broadlyovate/Linear	BO	BO	BO	BO	Ovate	BO	BO	BO	BO	Ovate	Obovate	BO	Obovate	BO

c	Tip- obtuse/ acute/ mucronate	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse
d	Length in relation to flower Shorter / Longer / Equal	Shorter	Equal	Shorter	Shorter	Shorter	Shorter	Longer	Shorter	Shorter	Shorter	Shorter	Shorter	Shorter	Shorter
e	Spread of bractHorizontal/ Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique	Oblique
11	**Flower**														
a	Exerted / Inserted /Equal from bract	Exerted	Equal	Exerted	Exerted	Exerted	Exerted	Inserted	Exerted	Exerted	Exerted	Inserted	Exerted	Exerted	Inserted
12	**Calyx**														
a	Length and breadth	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm	1 cm
b	Colour	L. Pink	L. Pink	L. Pink	L. Pink	L. Pink	L. Pink	L. Pink	L. Pink	L. Pink	L. Pink	L. Pink	L. Pink	Yellow	White
c	Shape	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular	Tubular
13	**Corolla**														
a	Colour	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Yellow	White
b	Length	2m	2m	2m	2m	2m	2m	2m	2m	2m	2m	2m	2m	2m	2m
i	Tube length	2cm	2cm	2cm	2cm	2cm	2cm	2cm	2cm	2cm	2cm	2cm	2cm	2cm	2cm
ii	Lobe length	2.4	2	1.7	1.9	1.9	1.5	2.3	2.3	1.5	1.5	1.5	1.5	2	1.5
c	Shape of lobes	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Lanceol ate
d	Apex of lobes	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute	Acute
14	**Lip-Category as per Table no .7-B**	Cat-I	VII	IV	VII	V	V	VII	V	V	III	I	IV	III	III
15	Relative length of lip and staminodes	Equal	More	More	Equal	More	More	Equal	Less	More	More	More	More	Less	More
16 a	Stamen	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White Light pink base	White base	White Light pink base
b	Spur– Converging/ Diverging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging	Conver ging
c	Staminode colour	Reddish purple	Almost entire lip yellow	Purple with yellow blotch	Browni sh purple with yellow blotch	Purple with yellow blotch	Purple with yellow blotch	Browni sh purple with yellow blotch	Browni sh purple with yellow blotch	Purple with yellow blotch	Purple with yellow blotch	Upper half almost yellow	Purple with yellow blotch	Yellow	White with yellow blotch
17	Stigma fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed	Fringed
18	ovary	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy	Hairy
19	Seed	-	-	-	-	-	-	-	-	-	-	-	-	Arilate	-
20	**Tuber**														
a	Tuber Stalk length	8cm	8cm	8 cm	10cm	7cm	7cm	9 cm	8 cm	10cm	5 cm	4cm	5 cm	7	-
b	Long/ short	Long	Long	Long	Long	Long	Long	Long	Long	Long	Short	Short	Short	Long	Short
c	Tuber apical/ sub apical	Apical	Apical	Sub apical	Apical	Apical	Apical	Apical	Apical	Apical	Apical	Apical	Apical	Apical	Apical
d	Shape of tuber	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval
e	Colour of tuber from inside	White	White	White	White	White	White	White	White	White	White	White	White	White	Yellow

Table 2: Polymorphism generated by samples using different primers.

Sr. No.	Primer	Sequence of Primer	Total bands	Polymorphic bands	Monomorphic bands	Percent Polymorphism
1.	UBC_811	GAGAGAGAGAGAGAGAC	73	71	2	97.18
2.	UBC_812	GAGAGAGAGAGAGAGAA	37	37	0	100.00
3.	UBC_826	ACACACACACACACACC	69	68	1	98.53
4.	UBC_834	AGAGAGAGAGAGAGAGYT	63	61	2	96.72
5.	UBC_835	AGAGAGAGAGAGAGAGYC	63	62	1	98.39
	Total		**305**	**299**	**6**	**-**
	Mean		**61**	**-**	**Average**	**98.16**

Similarity Matrix. A similarity index of 12 variants and 2 species is presented in Table 3. Pair-wise estimates of similarity ranged from 93.14 to 100. Variants *C. inodora* CL-14 and CI-6 show the lowest similarity indices while CI-10 and CI- 12 show the highest similarity indices.

Table 3: Similarity Matrix.

0	CI-01	CI-02	CI-03	CI-04	CI-05	CI-06	CI-07	CI-08	CI-09	CI-10	CI-11	CI-12	CP-13	CL-14
CI-1	100													
CI-2	94.614	100												
CI-3	93.442	93.366	100											
CI-4	94.522	93.755	93.755	100										
CI-5	94.522	93.917	94.432	94	100									
CI-6	95.417	94.522	94	94.803	95	100								
CI-7	94.343	94.614	93.917	94.343	93.835	94.803	100							
CI-8	93.835	93.755	94.803	93.835	95.309	94.614	94.169	100						
CI-9	94.522	94.255	93.917	94.522	94.901	94.803	94.522	94.343	100					
CI-10	94.169	93.755	93.755	93.835	94	94.255	94	94.522	93.835	100				
CI-11	93.675	93.291	93.144	93.519	94	94.255	93.519	94.169	93.675	94.169	100			
CI-12	94.343	93.755	93.442	94.169	93.835	94.255	93.835	94.522	94	94.7085	93.835	100		
CP-13	94.169	93.291	92.318	93.835	93.366	93.917	93.835	93.519	93.217	94.3431	93.519	93.835	100	
CL-14	94.614	93.675	94.343	94.083	94.803	94.708	94.083	94.432	94.255	94.2554	93.755	93.917	93.442	100

Cluster Analysis

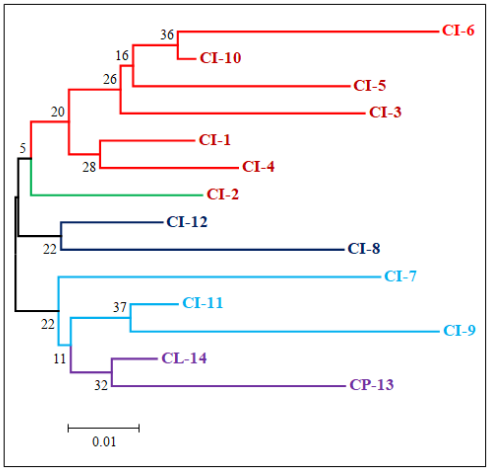


Fig. 1. Dendrogram (ISSR data).

Dendrogram Clusters

Dendrogram produces two distinct clusters.

Cluster- I

Sub cluster I- A -CP-13, CL-14

Subcluster I –B - CI-9, CI-11

Sub cluster I-C - CI-7

Cluster –II

Sub cluster II-A -CI-8 and CI-12

Subcluster II-B - CI-2

Sub cluster II-C -CI- 1, CI-3, CI-4, CI-5, CI-6 and CI-10

Dendrogram (Dendrogram Dig-1) produced on the basis of ISSR data shows two distinct clusters. Here also *C. longa* and *C. pseudomontana* both the species stand distinct still having much genetic distance. All variants of *C. inodora* form one cluster which further gets subdivided indicating the relatedness and also the genetic distance pointing out clear polymorphism within the species.

Photo plate 1: *Curcuma* accessions.

Agarose Gel Electrophoresis- ISSR PCR.

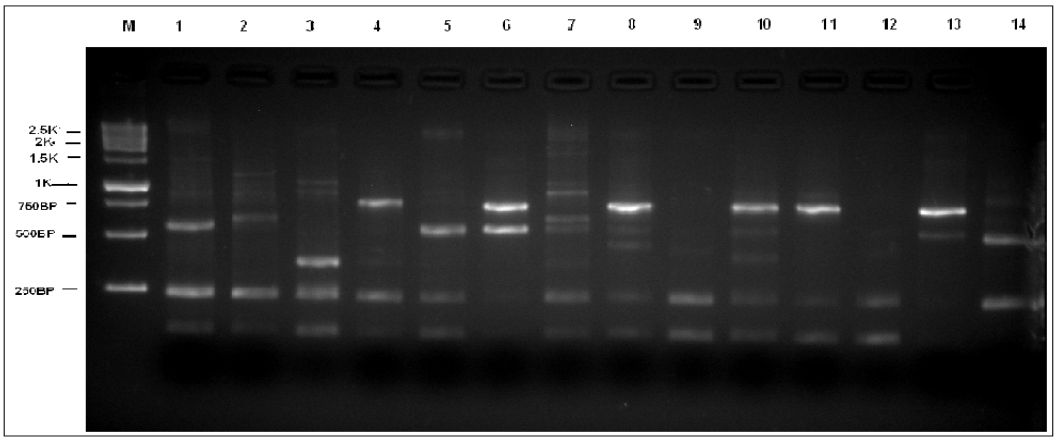


Fig. 2. Agarose (2% w/v) Gel Electrophoresis of ISSR PCR products for fourteen samples. Lane M: DNA size standard- Lane 1- CI-1; Lane 2-CI-2; Lane 3- CI-3; Lane 4- CI-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10 - CI-10 ; Lane 11- CI-11; Lane 12- CI-12; Lane 13-CP-13; Lane 14- CL-14 PCR products generated using primers UBC_811.

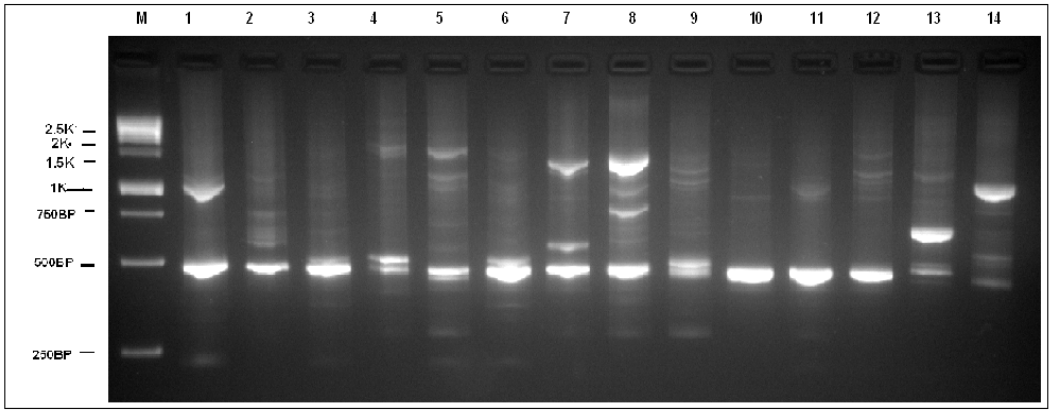


Fig. 3. Agarose (2% w/v) Gel Electrophoresis of ISSR PCR products for fourteen samples. Lane M: DNA size standard- Lane 1- CI-1; Lane 2-CI-2; Lane 3- CI-3; Lane 4- CI-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10 - CI-10; Lane 11- CI-11; Lane 12- CI-12; Lane 13-CP-13; Lane 14- CL-14 PCR products generated using primers UBC_812.

Agarose Gel Electrophoresis- ISSR PCR.

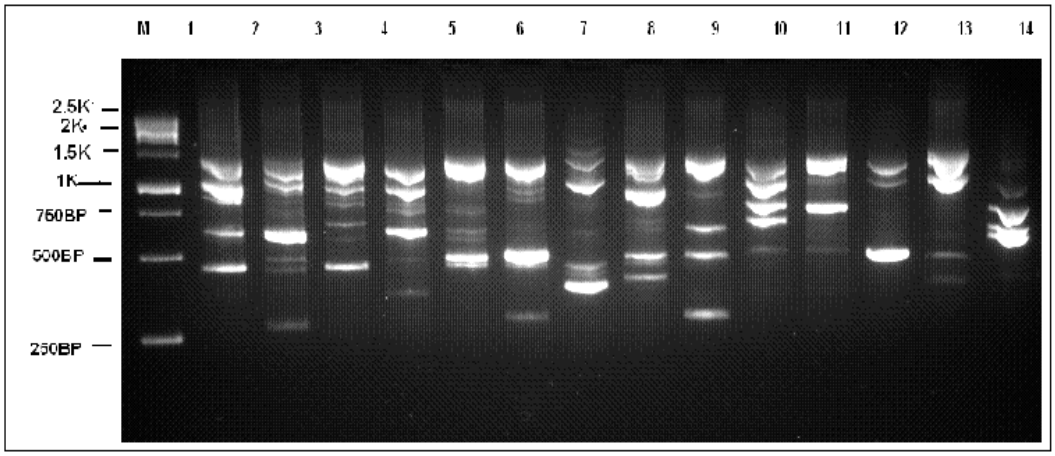


Fig. 4. Agarose (2% w/v) Gel Electrophoresis of ISSR PCR products for fourteen samples. Lane M: DNA size standard- Lane 1- CI-1; Lane 2-CI-2; Lane 3- CI-3; Lane 4- CI-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10 - CI-10 ; Lane 11- CI-11; Lane 12- CI-12; Lane 13-CP-13; Lane 14- CL-14 PCR products generated using primers UBC_826

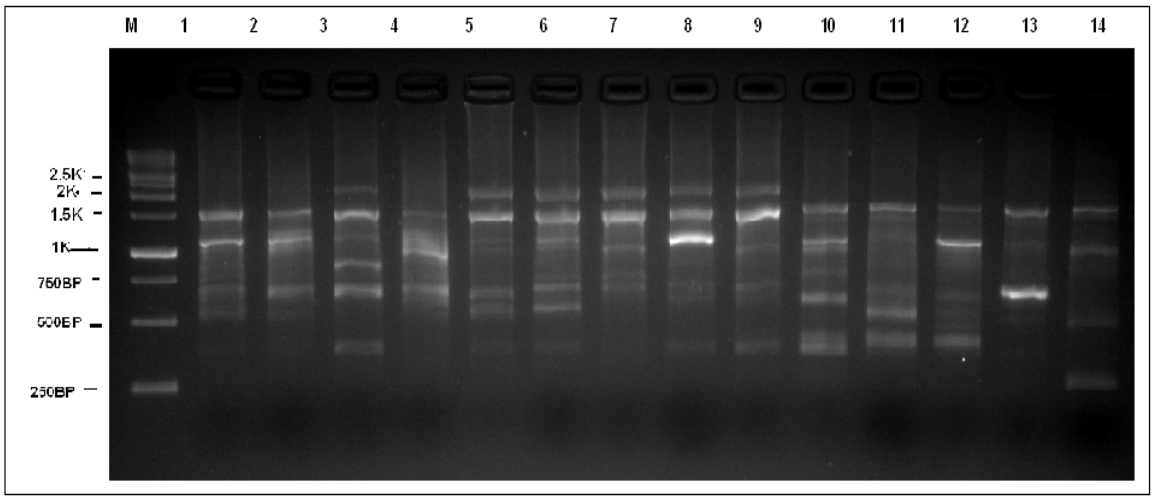


Fig. 5. Agarose (2% w/v) Gel Electrophoresis of ISSR PCR products for fourteen samples. Lane M: DNA size standard- Lane 1- CI-1; Lane 2-CI-2;Lane 3- CI-3; Lane 4- CI-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10 - CI-10; Lane 11- CI-11; Lane 12- CI-12; Lane 13-CP-13; Lane 14- CL-14 PCR products generated using primers UBC_834.

Agarose Gel Electrophoresis- ISSR PCR.

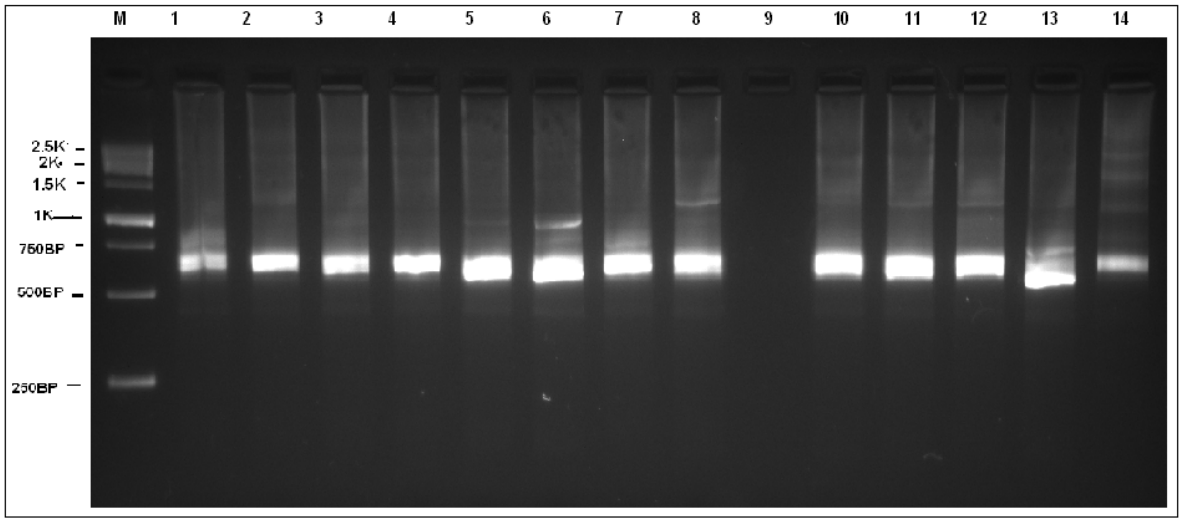


Fig. 6. Agarose (2% w/v) Gel Electrophoresis of ISSR PCR products for fourteen samples. Lane M: DNA size standard- Lane 1- CI-1; Lane 2-CI-2; Lane 3- CI-3; Lane 4- CI-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10 - CI-10; Lane 11- CI-11; Lane 12- CI-12; Lane 13-CP-13; Lane 14- CL-14 PCR products generated using primers UBC_835.

CONCLUSIONS

ISSR Primers produced almost 100% polymorphism attributing to genetic variability. Varied ecological niches and geographical conditions are expected to produce variations. However, the populations of *C. inodora* studied here grow in the same geographical and ecological niche, and still show great variations not only at morphological (Dagawal and Bhogaonkar 2019) but also at molecular level. This indicates the adaptive and evolutionary potential of *C. inodora* species.

FUTURE SCOPE

Great polymorphism indicates genetic diversity of *C. inodora*; surprisingly the species is rare and near threatened. It has a good potential to be introduced as ornamental; this will also help to increase the population of this limitedly distributed plant. *C. inodora* species populations studied show great variations indicates the evolutionary potential. No flora refereed mentioned such variations. It is therefore recommended to conserve the population with atmost care to redeem the future evolutionary lines.

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Conflict of Interest. None.

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