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Identification and hylogenetic analysis of Ficus species for acknowledgment and upkeep

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Abstract

***Ficus* L. is widely distributed in all the climatic stages and is of great diversity. Molecular marker is used for identification of genetic resources. Inter-simple Sequence Repeat (ISSR) markers was used to assess the identification of 23 important *Ficus* species / varieties and determination of the genetic relationships among these species. Out of twenty one ISSR primers tested, five primers produced 116 detectable fragments, out of which 106 were polymorphic across the species/varieties. Each of the five primers produced fingerprint profile unique to each of the species/variety studied and thus could be solely used for their identification. Thirteen unique bands specific to nine species were detected. These may be converted into species-specific probes for identification purposes. Genetic relationships among these species/varieties were evaluated by generating a similarity matrix based on the Dice coefficient and the Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendogram. The results showed a clear cut separation of the 23 *Ficus* varieties/species and were in broad agreement with the morphology. Both molecular and morphological markers will be useful for preservation of the *Ficus* germplasm.**

Keywords: Inter simple sequence repeat (ISSR), phylogeny, *Ficus* species, fingerprinting.

INTRODUCTION

Ficus Linn. (Moraceae) constitute one of the largest genera of flowering plants with about 800 species of free-standing trees, hemi-epiphytes and shrubs primarily occurring in subtropical and tropical regions world-wide. The genus is remarkable for the large variation in the habits of its species (Herre et al., 2008) . Many species are cultivated for shade and ornament in gardens. Some species are serves as good source of latex/rubber. The fig is a very nourishing food and is used as an industrial product. It is very energizing, rich in vitamin, mineral elements, water and fats. In India, the most important among them are; *Ficus bengalensis*, *Ficus carica* and *Ficus elastica*. It is propagated by seeds/cuttings. Thus, the *Ficus* germplasm is characterized by a great diversity since a high number of varieties/ accessions has been identified (Mars, 2003). Due to industrialization and deforestation, many of the species/varieties are currently threatening. As a consequence, a lack of landraces has occurred during recent years and this constituted a constrain in the improvement of the *Ficus* varieties. Apart from its morphological, physiological and agronomic traits, the genetic analysis through molecular marker is a pre-requisite to having a deep insight of the genome organization in the wild species. Moreover, the precise number of cultivars/species is still unknown since problem of mislabeling are often detected (Gao et al., 2006). Therefore, it is imperative to establish strategies for the preservation of *Ficus* germplasm.

Many DNA based markers are also available to identify the varieties / species. These markers can be effectively used to answer the phylogenetic relationship between *Ficus* varieties/species (Khadari et al., 2005: Salhi-Hannachi et al., 2006; Chatti et al., 2007). ISSR overcomes many of the limitations faced by different marker system and has a higher reproducibility (Guasmi et al., 2006). *Ficus* species are represented by a large number of varieties/accessions which are facing genetic erosion. To save these genetic resources, the present investigation is to study the dentification and

hylogenetic analysis of 23 species/varieties of *Ficus* through ISSR markers.

MATERIALS AND METHODS

Plant materials

Important *Ficus* cultivars (Species/varieties) were collected from Botanical garden of Regional Plant Resource Center, Bhubaneswar and Chandaka Reserve Forest, Bhubaneswar, India. Identification of the species based on their morphological characteristics was confirmed in our laboratory and doubtful samples were excluded from the analysis. In total, twenty-three varieties / species were collected for this study. All the species/varieties were given the accession number that is: F1, *Ficus religiosa*; F2, *Ficus microcarpa* "Microcarpa"; F3, *Ficus elastica* "Rubra"; F4, *Ficus repens*; F5, *Ficus krisnae*; F6, *Ficus elmira*; F7, *Ficus petiolaris*; F8, *Ficus* nervosa; F9, *Ficus microcarpa* "Varigatae"; F10, *Ficus benjamina* "Varigatae"; F11, *Ficus elastica* "Robusta"; F12, *Ficus elastica* "varigatae"; F13, *Ficus mollis*; F14, *Ficus callosa;* F15, *Ficus rumphii* "Varigatae"; F16, *Ficus benjimina* "Nuda"; F17, *Ficus glomerata*; F18*, Ficus virens "Glabella"*; F19, *Ficus arnottina*; F20, *Ficus bhengalensis;* F21, *Ficus amplissima*; F22, *Ficus virens* "Virens"; F23, *Ficus macrophylla*.

DNA PREPARATION

DNA was isolated using CTAB method following the protocol of Doyle and Doyle (1990), with minor modification. 1.0 - 1.5 g of young non-scenence leaves were ground in liquid nitrogen. Then they were incubated in CTAB buffer (3% w/v CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4M NaCl, 2% v/v -mercaptoethanol, 2% w/v polyvinyl pyrrolidine, pH 8.0) for 2 h at 65°C. The homogenate was then extracted with an equal volume mixture of chloroform: isoamylalcohol (24:1) and centrifuged at 9000 rpm for 10 min. The upper aqueous layer was recovered and precipitated with prechilled isopropanol. The pallet was suspended with Tris-EDTA buffer (pH 8.0) . The crude DNA was treated with RNase and incubated for 30 min at 37°C and again extracted with 1 volume phenol and subsequently with one volume of chloroform: isoamylalcohol (24:1). The supernatant were collected and precipitated with 3 M sodium acetate and prechilled ethanol. The DNA pellate was washed with 70% ethanol, dried and resuspended in TE buffer. The high molecular weight DNA was checked for quality and quantity electrophoretically using 0.8% agarose gel against a known amount of , DNA taken as standard. Twenty-one synthesized ISSR primers both 5'-anchored core motifs [$(AG)_8$ T, $(AG)_8$ C, $(AC)_8$ T, $(AC)_8$ G, $(GA)_8$ A, $(GA)_8$ T, $(CT)_8$ G, $(CT)_8$ A, $(TG)_8$ A, $(TG)_8$ C, $(GT)_8$ C, $(GT)_8$ A], 3'-anchored core motifs [$CAG(CA)_7$, $TGG(AC)_7$, $ACA(TG)_6$, $ACG(GT)_7$] and Non-Anchored core motifs [$(ATG)_6$, $(ACTG)_4$, $(GACA)_4$, $(ACAG)_4$, $(GACAGATA)_2$] were used for the experiment. To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded. A few well amplified fragments that were not reproducible across 2 replicate of DNA extraction were also discarded from analysis.

For ISSR study, the initial optimization of PCR was conducted including concentration of template DNA, primer, $MgCl_2$, number of PCR cycle and annealing temperature. The PCR reaction had a total volume of 25 µl containing 20 ng templates DNA, 100 mM each dNTPs, 20 ng of oligonucleotides synthesized primer (M/S Bangalore Genei, Bangalore, India), 2.5 mM $MgCl_2$, 1x Taq buffer [10 mM Tris-HCl) pH 9.0, 50 mM KCl, 0.01% gelatin] and 0.5 U Taq DNA polymerase (M/S Bangalore Genei, Bangalore, India).

DNA amplification was performed in a PTC -100 thermal cycler (M J Research, USA) programmed for a preliminary 5 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 20 s, annealing temperature depending on the primer (50 - 56°C) for 30 s and extension at 72°C for 45 s and finally at 72°C for 5 min. Amplification products were separated alongside a low range molecular weight marker (M/S Bangalore Genei, India) on a 2% (w/v) agarose gel electrophoresis in 1x TAE (Tris Acetate- EDTA) buffer stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Gel Doc. 2000, BioRad, California, USA) and the amplification product sizes were evaluated using the software Quantity one (BioRad, California, USA).

Clearly defined ISSR bands that behave as dominant markers were scored for the presence (1) or absence (0) for all the species/cultivars and entered into a data matrix. The genetic relationships among the species/cultivar were determined by calculating the Dice coefficient, estimated as $S = 2N_{AB} / N_A + N_B$ .Where N_{AB} is the number of amplified product common to both A and B. N_A and N_A corresponds to number of amplified product in A and B respectively. Diversity pattern were represented in the form of a dendrogram that was generated by subjecting the genetic similarity matrix to Unweighted Pair-group Method Arithmetic average (UPGMA) cluster analysis with software NTSYS-pc, Version 2.0 (Rohlf, 1995).

RESULTS AND DISCUSSION

The present investigation indicates that Twenty one ISSR primers were used for identification of genetic resources and to assess phylogenetic relationship among the *Ficus* species/ cultivars occurring in Orissa, India. Out of the 21 ISSR primers, nine primers generated clear multiplex banding profiles, among which five primers $(AG)_8$ C, $(GA)_8$ A, $(TG)_8$A, $(GT)_8$ C and ACA(TG) produced the best ISSR profiles. The results showed that the most of the primers based on GA/AG and GT/TG dinucleotides core repeats generated good banding profiles. The amplification of ISSR markers was consistent across two replicate DNA extractions from three samples, over with 98% of scorable fragments reproducible. A higher concentration of $MgCl_2$ (2.5 mM) gave best results. This may be due to non-specific amplification because of reduced enzyme fidelity (Hopkins and Hilton, 2001). The concentration of $MgCl_2$ affects the specificity and yield of reaction by increasing the stringency of primer annealing or has a direct effect on Taq Polymerase (Saiki, 1989). The five primers selected, produced highly polymorphic band profiles. Furthermore, each of these primers produced fingerprints profiles unique to each of the variety. Therefore, each primer can be used separately to identify these accessions in the future. ISSR amplification for all samples resulted in multiple band fingerprint profile for the five selected ISSR primers (Figure 1). The average number of scorable fragments per primer was 23, with a range from 17 to 34, while the average number of polymorphic fragments per primer was 21 with a range from 15 to 31. Out of the 116 scorable fragments, 106

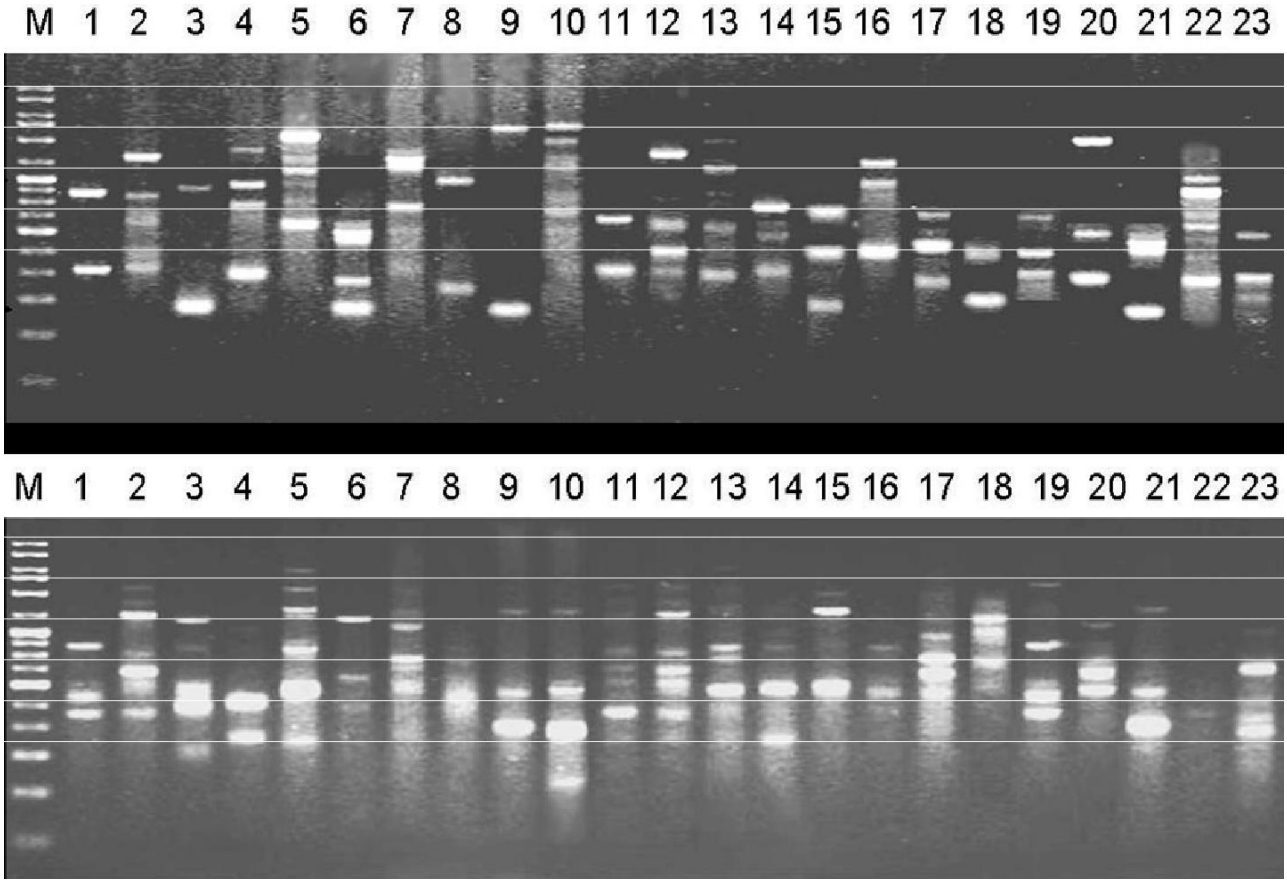

Figure 1. ISSR banding pattern of 23 species/ variety of *Ficus* using synthesis primer $(GA)_8$ A [A] and $(TG)_8$A [B]. M –low range DNA marker. **F1:** *Ficus religiosa,* **F2:** *Ficus microcarpa* "Microcarpa" , **F3:** *Ficus elastica* "Rubra", **F4:** *Ficus repens* , **F5:** *F. krisnae,* **F6:** *F. elmira,* **F7:** *F. petiolaris,* **F8:** F. nervosa, **F9:** *F. microcarpa* "Varigatae", **F10:** *F. benjamina* "Varigatae", **F11:** *F. elastica* "Robusta", **F12:** *F. elastica* "varigatae", **F13:** *F. mollis,* **F14***: F. callosa,* **F15:** *F. rumphii* "Varigatae", **F16:** F. benjimina "Nuda", **F17:** *F. glomerata,* **F18***: F. virens "Glabella",* **F19***: F. arnottina,* **F20***: F. bhengalensis,* **F21:** *F. amplissima* , **F22:** *F. virens* "Virens", **F23:** *F. macrophylla*.

were polymorphic revealing 91.3% polymorphism across the 23 varieties/species studied. Thirteen ISSR loci were recorded as germplasm specific as they occurred in only a single species/cultivars. These may be developed into cultivar specific probes for identification purposes. In the study primers based on GA/AG and GT/TG dinucleotides core repeats generated good profiles, which seem to indicate that the more frequent microsatellite in *Ficus* contains the repeated dineucleotides (AG/GA)n and (GT/TG)n. These results are in accordance with the SSR assay. Vignes et al. (2006), developed 13 microsatellite from *Ficus insipida* by screening CT and GT genomic library. All markers revealed a broad cross-species affinity when tested in 23 other *Ficus* species. Saddouda et al. (2005) studied the genetic diversity of Tunisian fig (*F. carica*) and cultivar characterization using microsatellite markers. Khadari et al. (2005) identified eight microsatellite loci in common fig (*F. ciarica*) by screening a TC and TG enriched genomic library. Present study indicates that there was a distant variation in DNA amplification of 23 species / varieties of *Ficus* collected from Orissa, India. The matrix calculated for all possible pair wise comparisons between accessions showed that the index value varied from a minimum of 0.15 between *F. repens* and *F. benjamina* "Variegata" to a maximum of 0.78 between *F bhengalensis "*Krishnae" and *F. bhengalensis "*Bhengalensis*"* with an average of 0.43 (Table 1). It seems to be the most divergent since they have presented the highest genetic distance value of 0.78. All the others ones have displayed different intermediate levels of similarity. Cluster analysis showing a dendrogram using Dice coefficient subjected to UPGMA method (Figure 2) were divided into four major clusters at 0.25 similarity level : A, B, C and D. Cluster-A had 17 accessions and cluster B, C and D, two species/accessions each. Cluster-D had two varieties/accessions of *F. virens* (Virens and Glabella) grouped together but with only 0.42 similarity. Similarly, two accessions of *F. benjamina (Varigatae* and Nuda) were also grouped together in Cluster-C with 0.41

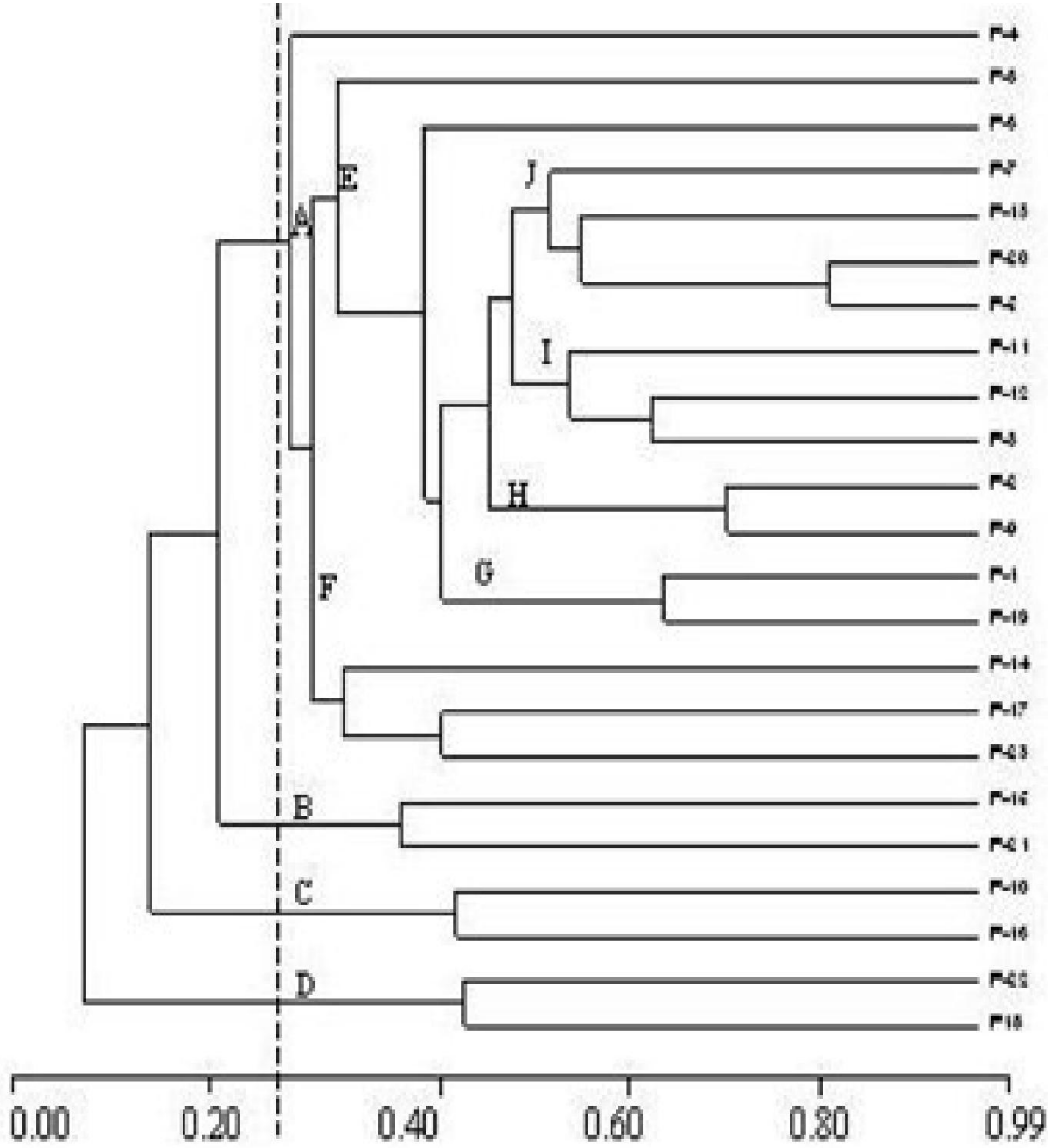

Figure 2. UPGMA dendrogram based on the similarity coefficient, showing the clustering pattern among the 23 *Ficus* species /variety F1 - F23 are the 23 *species*/variety.

similarities. *F. rumphii* "Varigata" had been grouped with *F. amplissima* at 0.36 similarity levels in Cluster B. In Cluster A, *F. repens* had isolated itself (0.27) as a unique cluster and the rest accessions had been further grouped into two sub-clusters-E and F at 0.30 levels. Sub-cluster-F had three species; *F. mollis*, *F. glomerata* and *F. callosa* whereas, sub-cluster- E had the rest 13 accessions. In sub-cluster -E, *F. nervosa* and *F. elmira* has separated themselves as single cluster at 0.32 and 0.39 similarity levels respectively. The rest 11 accessions in Cluster-E had been divided into four minor sub- clusters (G, H, I and J). *F. bhengalensis* "Bhengalensis" and *F. bhengalensis* "Krishnae" had been grouped together (0.78) in minor sub-cluster-J which was linked to *F. mollis*

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Table 1. Similarity coefficient among the 23 accession of *Ficus* obtained from ISSR markers. (F1-F23 is the 23 accessions of *Ficus* as in figure 1).

	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9	F-10	F-11	F-12	F-13	F-14	F-15	F-16	F-17	F18
F-1	1.00																	
F-2	0.61	1.00																
F-3	0.62	0.68	1.00															
F-4	0.25	0.33	0.38	1.00														
F-5	0.69	0.70	0.64	0.31	1.00													
F-6	0.44	0.45	0.55	0.32	0.52	1.00												
F-7	0.58	0.65	0.59	0.35	0.73	0.48	1.00											
F-8	0.36	0.38	0.45	0.38	0.48	0.52	0.44	1.00										
F-9	0.48	0.75	0.57	0.37	0.62	0.52	0.62	0.44	1.00									
F-10	0.33	0.32	0.35	0.15	0.39	0.40	0.45	0.33	0.42	1.00								
F-11	0.46	0.65	0.67	0.36	0.61	0.43	0.6	0.39	0.56	0.36	1.00							
F-12	0.58	0.68	0.72	0.37	0.66	0.48	0.71	0.51	0.62	0.38	0.77	1.00						
F-13	0.50	0.60	0.58	0.48	0.60	0.48	0.65	0.41	0.56	0.28	0.55	0.63	1.00					
F-14	0.37	0.57	0.48	0.35	0.55	0.44	0.54	0.26	0.52	0.4	0.63	0.51	0.60	1.00				
F-15	0.34	0.44	0.44	0.16	0.33	0.42	0.27	0.29	0.43	0.21	0.29	0.31	0.25	0.26	1.00			
F-16	0.26	0.33	0.36	0.20	0.30	0.42	0.31	0.29	0.35	0.43	0.42	0.35	0.36	0.58	0.31	1.00		
F-17	0.50	0.47	0.51	0.27	0.48	0.64	0.47	0.56	0.47	0.38	0.39	0.5	0.44	0.43	0.40	0.46	1.00	
F18	0.23	0.24	0.30	0.26	0.24	0.41	0.26	0.33	0.29	0.33	0.27	0.26	0.24	0.41	0.55	0.36	0.32	1.00
F-19	0.57	0.49	0.55	0.55	0.59	0.43	0.54	0.36	0.44	0.25	0.45	0.51	0.56	0.46	0.15	0.19	0.42	0.20
F-20	0.49	0.52	0.55	0.47	0.78	0.56	0.68	0.47	0.56	0.37	0.43	0.57	0.52	0.47	0.31	0.27	0.49	0.25
F-21	0.42	0.43	0.47	0.25	0.41	0.56	0.46	0.51	0.51	0.30	0.45	0.46	0.4	0.42	0.58	0.58	0.65	0.44
F-22	0.35	0.33	0.37	0.16	0.38	0.54	0.44	0.55	0.44	0.30	0.30	0.40	0.3	0.32	0.48	0.40	0.53	0.48
F-23	0.34	0.39	0.52	0.46	0.52	0.49	0.58	0.40	0.42	0.31	0.44	0.45	0.52	0.49	0.38	0.27	0.39	0.48

(0.56) and *F. petiolaris* (0.51) respectively. Similarly, *F. microcarpa* "Microcarpa" and *F. microcarpa "*Variegata" are grouped together with 75% similarity, owing to their morphological similarities with minor sub-cluster - H. All the three accessions of *F. elastica*, "Rubra", "Robusta" and "Variegata" are being grouped together in minor sub-cluster- I. Our results indicate that the genetic relationship among the *Ficus* accessions, inferred by ISSR markers, were in accordance with their morphological characters. *F. bhengalensis "*Krishnae" is morphologically similar to *F. bhengalensis* "Bhengalensis", but with most or all the leaves having the side lamina in its lower half reflexes and connate to form a cup. This modification may be due to bud-sport and most plants have been distributed in Orissa, India. Salhi-Hannachi et al (2005) assessed the genetic diversity in two Tunisian fig cultivars by using RAPD and ISSR markers. They compared two

molecular marke

In conclusion, mation for spec natural distributi with ISSR mar reproducible an phism to identify ISSR alleles dominant. This refined to includ

variety analysis for detailed characterization of *Ficus* taxa that would be essential for future breeding and tree improvement programme.

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