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ACKNOWLEDGEMENTS. We thank Director, Space Applications Centre for the encouragement. The TRMM data from NASA/GSFC used in the study is acknowledged.

Received 10 October 2008; revised accepted 7 July 2009

Genetic diversity assessment in intra- and inter-populations of *Xylocarpus granatum* Koen.: a critically endangered and narrowly distributed species of Maharashtra

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The threatened mangrove species *Xylocarpus granatum* Koen. has been rediscovered after a period of nine decades from the West Coast of Maharashtra. On the basis of IUCN guidelines, the species is categorized as a critically endangered and narrowly distributed species of Maharashtra. When the inter- and intra-populations spread across the estuaries of Maharashtra Coast were studied, it was observed that the species shows considerable phenotypic variation with respect to leaflet size and shape, number of floral parts, number and size of fruits per inflorescence, and also dimorphism in leaves. Hence, populations of *X. granatum* from three different localities representing the above-mentioned phenotypic variations were selected and assessed at biochemical level by protein profiling and at genomic level using ISSR markers. The analyses showed that even though the variation exists at phenotypic level, the inter- and intra-populations showed similarity in total protein pattern and 92% similarity at genomic level. Thus, it seems that the phenotypic variations in the inter- and intra-populations of *X. granatum* are probably epigenetic. The low genetic variation in this species threatens its own existence and strongly demands immediate appropriate conservation measures.

Keywords: ISSR markers, intra- and inter-population variations, morphological variation, *Xylocarpus granatum* Koen.

THE mangrove genus *Xylocarpus*, belonging to the family Meliaceae, has three distinct species: *X. granatum* Koen., *X. moluccensis* Lamk. and *X. mekongenesis* Pierre., that are distributed in tropical tidal forests of Old World, typical mangrove habitat or in sandy or coastal habitats spread from Africa to Australia, including India and Malayan Archipelago[1]. In India, these three species were recorded from Andaman Islands[2] and Orissa Coast[3] whereas the two species, *X. granatum* Koen. and *X. mekongenesis* Pierre. were reported from Sundarbans[4], Tamil Nadu Coast[5] and Andhra Pradesh[6], and one species *X. granatum* Koen. from Maharashtra[7–9]. In Maharashtra, *X. granatum* Koen. has been reported by Cooke[10] and

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then by Blatter[11]. The subsequent reports did not record the species. However, after a long gap of more than 90 years, the species was rediscovered from Achara Estuary[7–9]. It is also newly recorded from Purnagarh, Jaitapur and Vijaydurg estuaries[12]. *X. granatum* Koen. is a moderately sized tree with a well-developed woody trunk, found on the borderline fringes of the estuaries of Ratnagiri and Sindhudurg districts of Maharashtra. The bark can be taken as a significant character for identification as it is thin and peels out into flakes or long patches[13]. The species shows smooth and pale trunk surface and presence of ribbon-like plank roots or plate roots or buttresses. The populations of *X. granatum* Koen. have been found isolated, fragmented and highly-disturbed due to its valuable timber and medicinally important seeds. Along the Achara Estuary, at the time of reporting in 1999 (ref. 8), there were 130 plants existing; the number has gone down to 100 in May 2008. According to the IUCN Redlist guidelines, the species comes under the category of critically endangered species of Maharashtra. When the populations of this species from the estuaries in Maharashtra were studied in detail, it was observed that the species shows enormous phenotypic variation in terms of leaflet size and shape, number of floral parts, number and size of fruits per inflorescence, and also dimorphism in leaves. Such phenotypic variations within and among populations can be confirmed at biochemical level through protein profiling and at genomic level using molecular markers. The molecular markers are phenotypically neutral having no epistatic and developmental effects. Moreover, they can detect the variation in both coding and non-coding regions of the genome. Among the several types of markers, inter simple sequence repeat (ISSR) markers have been successfully proved to distinguish the variations among the intra- and inter-populations of many mangrove species[14–17]. In India, during 2002–03, the National Bioresources Development Board (NBDB) had supported awareness building on the status of marine and coastal bioresources such as mangroves. The characterization of mangrove bioresources in selected locations was undertaken using molecular marker systems to assess the nature and extent of their genetic diversity in them. Species-specific genetic fingerprinting and intraspecific diversity have been studied in *Avicennia marina*, *Rhizophora mucronata* and *X. granatum* among the populations of Bhitarakanika[18]. But such studies are lacking in Maharashtra. Therefore, the present study has been undertaken to assess intra- and inter-population variations among the populations of *X. granatum* growing along the coast of Maharashtra, through protein profiling and ISSR markers.

The populations of *X. granatum* growing along different estuaries of Maharashtra were selected for the study. For protein profiling, accessions from two different localities having phenotypic variation with respect to leaf dimorphism and petal number were taken. Fifteen individuals from Achara and six from Purnagarh were used for extraction of proteins. For intra- and inter-population studies, 15 leaf accessions from three different sites were used for DNA isolation on the basis of morphological variations such as leaf size, shape and dimorphism, number of petals per flower and number of flowers with three petals per inflorescence, fruiting pattern with respect to its number per inflorescence and dimorphism.

Habit: Populations growing towards landward fringe/seaward side/on island.

Leaflet: Size – small (9.4 cm length × 5.2 cm breadth)/large (15 cm length × 9 cm breadth); shape of leaflets – elliptic/obovate.

Flower: Regular (tetramerous)/irregular (with three petals).

Flowering phenology: Yearly/biannually/once in three years.

Fruit: Size – large/medium/small (16.56 cm/12.64/7.07 cm in diameter); number – 2/3.

Fruiting pattern: 1 large + 1 small/1 large + 1 medium + 1 small/2 large + 1 small/1 large + 2 small.

The above-mentioned measurable variants were analysed statistically for test of significance using WASP 2.0 software from www.icargoa.res.in.

Total proteins were extracted by the method of Laemmli[19]. The loading sample was prepared by mixing 1.7 ml loading buffer (10 ml glycerol + 6.25 ml Tris-HCl buffer (1 M) pH-6.8 + 2 g SDS + 10 mg bromophenol blue + 12.05 ml DW) with 0.3 ml β-mercaptoethanol and 4 ml distilled water. The 30 µl of the sample was loaded in each well on SDS–PAGE gel. A mixture of proteins of molecular weight ranging from 10,000 to 100,000 Da (Genei, Bangalore) was used as standard marker.

Young leaves from individuals were collected for DNA isolation. DNA was extracted using CTAB method described by Doyle and Doyle[20] with some minor modifications such as the use of 2.4% polyvinyl pyrrolidone (PVP) instead of 1% in the extraction buffer. RNAse treatment at 37°C for one hour was given to remove RNA. Again the DNA was treated with equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) followed by two washes of chloroform : isoamyl alcohol (24 : 1) and the DNA pellet was obtained using 1/10 volume of sodium acetate. The pellet was dried after two washes of 70% alcohol and absolute alcohol. Finally, the pellet was dissolved in sterile distilled water. The quality and quantity of isolated DNA was checked on 1% agarose gel using λDNA as standard.

The DNA was diluted to the concentration of 5 µg/µl before using it in polymerase chain reaction. ISSR primers from UBC set 9 (Biotechnology Laboratory, The University of British Columbia, Canada) were used to amplify the DNA. A reaction mixture of 20 µl containing 1× *Taq* buffer, 2 mM of each dNTP, 25 mM $MgCl_2$, 1.5 µM primer, 30 ng DNA and 1 unit (0.2 µl) *Taq* DNA polymerase was prepared and amplifications were carried

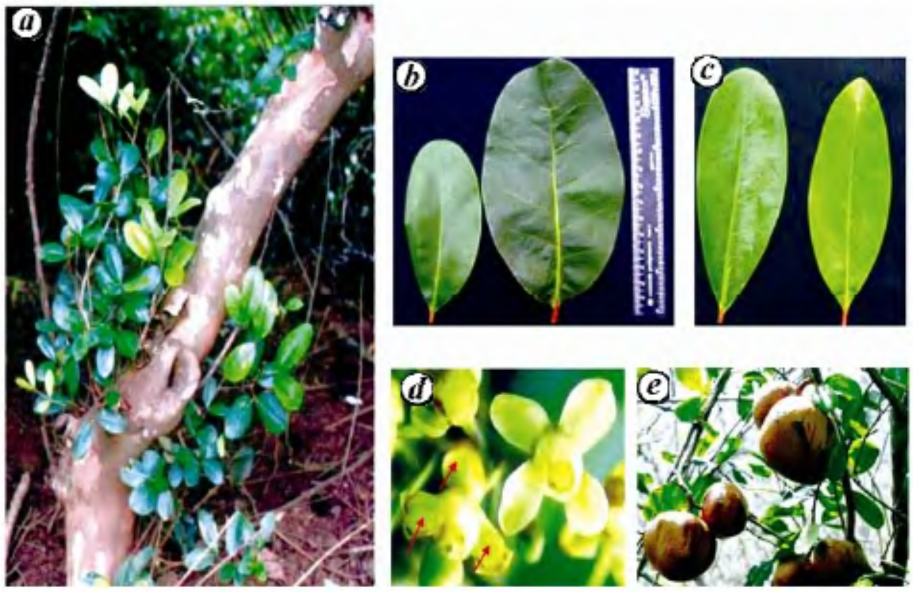

Figure 1. *Xylocarpus granatum* showing its morphological variation. ***a***, Habit; ***b*** and ***c***, Leaf size and shape variation; ***d***, Irregular flower with three petals (marked by arrows) and regular flower with four petals; ***e***, Fruit size variation.

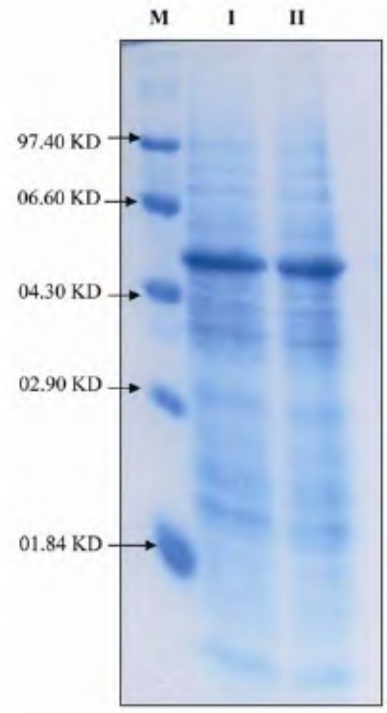

Figure 2. Total SDS–PAGE protein profile of *X. granatum* Koen. accessions collected from Achara (I) and Purnagarh (II) localities. M = Marker.

out in thermal cycler (Corbett Research, Australia) with an initial denaturation of 5 min at 94°C. The temperature profile of each cycle was 30 s denaturation at 94°C, 45 s annealing at 46°C and 120 s extension at 72°C. Reaction was of 35 cycles followed by 300 s final extension at 72°C. Amplified products were electrophoresed on 1.5% agarose gel with ΦX174 *Bsu*R1 (*Hae*III) digested DNA as size marker. The PCR products were stained with ethidium bromide and visualized using gel documentation system (Alpha Innotech, San Leandro, California, USA). DNA fragments amplified were scored as '1' for presence and '0' for absence of a band. Biodiversity Professional version 2.0 was used to estimate genetic diversity by Unweighted Pair Group Method with Arithmetic Mean Analysis (UPGMA) method and genetic distances were estimated according to Bray-Curtis Cluster Analysis method. The relationship among the populations and accessions was portrayed graphically in the form of a dendrogram. Genetic diversity was estimated using POPGENE software (version 1.31)[21].

The habit of *X. granatum* and the phenotypic variation in leaves, petals and fruits from the collected localities is shown in Figure 1.

Figure 2 shows the isolated total protein pattern of the *X. granatum* accessions collected from two different localities along the coast of Maharashtra. In case of both the localities, 17 bands ranging from 18.4 to 97.4 kDa were obtained. Thus, irrespective of localities and phenotypic variation, the profile was found to be the same, indicating zero correlation between the variation and total protein contents among the collected accessions.

In ISSR analysis, a total of 62 bands were obtained among 82 individuals belonging to three populations, of which 36 were polymorphic (58.06%; Figure 3). The percentage of polymorphism within population I (Achara) ranged from 17.14 (accessions 3 and 9) to 68.57 (accession 7). The percentage of polymorphism among all populations was 17.14 (accession 12 of Jaigarh, accessions 3 and 9 of Achara) to 68.57 (accession 7 of Achara) with

Table 1. ISSR primers and number of PCR amplified bands generated across 15 accessions of *X. granatum* Koen.

Primer number and sequence	Total no. of bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)
801(AT)8T	5	4	1	20
807(AG)8T	5	3	2	40
812(GA)8A	4	2	2	50
819(GT)8A	8	3	5	62.50
821(GT)8T	4	1	3	75
822(TC)8A	7	1	6	85.71
825(AC)8T	9	2	7	77.77
826(AC)8C	5	1	4	80
827(AC)8G	7	3	4	57.14
829(TG)8C	5	4	1	20
830(TG)8T	3	2	1	33.33
Total	62	26	36	58.06

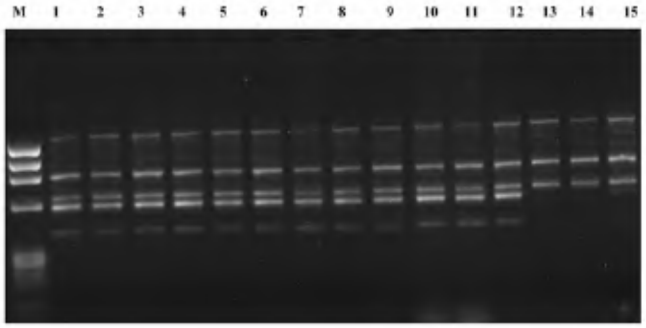

Figure 3. Inter simple sequence repeat (ISSR) electrophoretic assessment of genetic diversity in 15 accessions of *X. granatum* using ISSR primer 8258(AC)T. M, Marker ΦX174 *Bsu*R1; Accessions 1–11, Achara locality; Accession 12, Jaigarh locality; Accessions 13–15, Purnagarh locality.

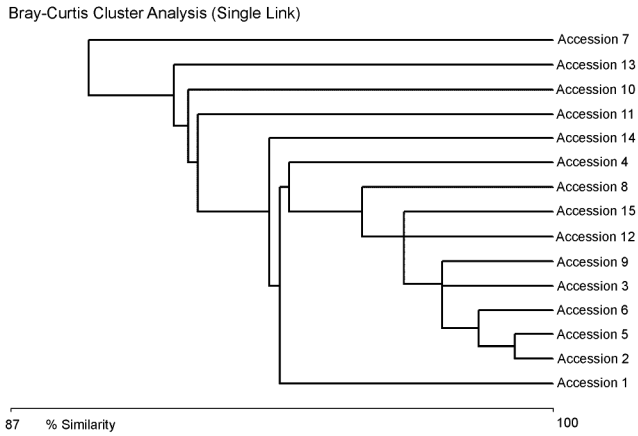

Figure 4. UPGMA dendrogram showing relationship among 15 accessions of *X. granatum* collected from West Coast of Maharashtra. Accessions 1–11, Achara locality; Accession 12, Jaigarh locality; Accessions 13–15, Purnagarh locality.

an average of 30.09. Each primer, generated 3–9 bands, with an average of 5.63 bands per primer. Out of these, average 3.3 bands were found polymorphic. The average polymorphic information content (PIC) per primer was 0.544, ranging from 0.2 to 0.85. The primers 801 and 829 recorded the lowest PIC values (0.20) and the highest value (0.857) was by 822 and six ISSR primers had intermediate PIC contents. The highest number of bands (9) was obtained with the primer UBC 825 whereas the lowest number (3) was obtained with the primer UBC 830.

All the accessions representing three populations showed 88.86% similarity amongst them (Figure 4). At 92% similarity, the accessions formed five major clusters. Cluster I, II, III and IV consisting of single accession each and cluster V with 11 accessions. The V cluster was further divided into two sub-clusters – A and B. Sub-cluster B consists of 10 accessions separating from each other at percentage of similarity ranging from 93.5 to 99 whereas, sub-cluster A is with single accession. Thus, overall the collected 15 accessions recorded 92% similarity amongst them. The sub-clustering based on 8% difference amongst them reveals the differences among the collected accessions. Clusters I–IV include all the accessions with leaf dimorphism whereas cluster V includes all the accessions without dimorphic leaves. The separation of the clusters I–IV can be correlated with phenotypic variation. Cluster I with the accession 7 had largest fruit size (16.56 cm in diameter) and with largest elliptic leaflets (15 cm length × 9 cm breadth), whereas others were with medium fruit size (12.64 cm in diameter). The cluster II including accession 13 showed flowering once in three years, whereas others flowered yearly and biannually. The clusters III and IV with accessions 10 and 11 showed biannual flowering but the accession 10 was with regular flowers (having four petals) and accession 11 was with irregular flowers (with three petals). The single accession 14 sub-clustered separately as sub-cluster A in cluster V, had polymorphic leaves (leaf having leaflets either elliptic or obovate shape, or both on the same leaf), whereas all other accessions in the sub-cluster B were without dimorphic leaves and dimorphic leaflets (leaves having either large elliptic or small obovate leaflets). In the sub-cluster B, out of 10 accessions, accession 1 was separated, which had three fruits per inflorescence (1 large + 1 medium +

Table 2. Genetic diversity in *X. granatum* Koen. populations estimated using ISSR marker data of 35 loci over three populations

Population	*N*	*n*	*P*%	*Ae*	*I*	*h*
Achara						
Accession 1	5	11	31.42			
Accession 2	6	8	22.85			
Accession 3	6	6	17.14			
Accession 4	6	7	20			
Accession 5	4	7	20			
Accession 6	6	5	14.28	1.675	0.541	0.375
Accession 7	1	24	68.57			
Accession 8	7	9	25.71			
Accession 9	7	6	17.14			
Accession 10	9	14	40			
Accession 11	17	19	54.28			
Jaigarh						
Accession 12	2	6	17.14	1.000	0.000	0.000
Purnagarh						
Accession 13	2	16	45.71			
Accession 14	2	13	37.14	1.321	0.249	0.174
Accession 15	2	7	20			
Average (population level)	5.46	10.53	30.092	1.666	0.566	0.384

N = sample size, *n* = number of polymorphic loci, *P*% = % band polymorphism, *Ae* = effective number of alleles, *I* = Shannon's Information index, *h* = Nei's gene diversity.

Table 3. Nei's (1972) original measures of genetic identity (above diagonal)) and genetic distance (below diagonal) in the three populations of *X. granatum*

Population ID	Achara	Jaigarh	Purnagarh
1	–	0.7280	0.7908
2	0.3174	–	0.7779
3	0.2347	0.2512	–

1 small size). Sub-cluster B with nine accessions showed mixed characters and hence could not be separated further. However, some of these which fall in different subclusters at 96.3% similarity had similar characters, viz. accessions 2, 5, 6 and 3 flowering annually, were with regular fruiting pattern and had similar habitat location.

The genetic variability (*h*) was found low (0.384) among the populations of *X. granatum* (Table 2). The average number of effective alleles for three populations (Ae_{pop}) was 1.666. It ranged from 1.00 (Jaigarh) to 1.675 (Achara). Nei's gene diversity (*h*) ranged from 0 (Jaigarh) to 0.375 (Achara) with the average heterozygosity at population level (Hs) being 0.183 and at species level (Ht) 0.316. Shannon's Information index (*I*), which is another parameter for determining heterozygosity, showed the same trend as Nei's gene diversity. It ranged from 0 (Jaigarh) to 0.541 (Achara) with an average value of 0.566 at population level.

In the mangrove species, assessed genetic variation has been using molecular markers. Ge and Sun[14] studied genetic variation within and among populations of *Aegiceras corniculatum* using allozyme and ISSR. They recorded very low allozyme variation and ISSR diversity with low gene differentiation among populations. Huang *et al.*[17] assessed interspecific and inter-population variation in three species of *Ceriops* using ISSR markers and recorded low-genetic diversity at population level. The conservation genetics of endangered species has now been studied with increased importance. It has become essential to study the species survival, its conservation and management. The primary step of conservation genetics is to estimate the level and distribution of genetic variation within and between populations. The genetic diversity in woody plant species is influenced by many factors such as geographic ranges, out crossing systems, seed dispersal, fecundity and phylogenetic history of the species[22]. The decrease in variation may represent decreasing ability of species to cope up with environmental changes and demographic fluctuations[23], resulting in an increased risk of extinction[24]. *X. granatum* is a critically endangered species of mangroves of not only Maharashtra but also from East Coast of India. It is disappearing from many locations and represented by very few individuals wherever it is present. The low genetic variation within inter- and intra-populations of *X. granatum* recorded here along the coast of Maharashtra threatens its own existence. Further, because of phenotypic variation, a great deal of confusion exists in identification of this species. Hence, it was necessary to study whether the

Table 4. Physicochemical parameters and microclimate of three sites of *X. granatum* Koen. from West Coast of Maharashtra

Physicochemical parameters of microclimate of *X. granatum*	Achara (Sindhudurg District) (Seasonal variation range)	Jaigarh (Ratnagiri District) (Seasonal variation range)	Purnagarh (Ratnagiri District) (Seasonal variation range)
Location			
Latitude	16°12′N–16°14′N	17°18′N–17°20′N	16°48′N–16°49′N
Longitude	73°25′E–73°30′E	73°12′E–73°29′E	73°18′E–73°28′E
Population size	105	2	9
Population structure	3 : 1, mature : young individuals	Two mature individuals	9 : 1, mature : young individuals
Number of individuals studied for DNA	82	2	6
pH			
Soil	4.66–8.1	5.2–7.6	6.34–6.60
Water	6–6.5	5.15–7.18	6–8
EC (mmhos/cm)			
Soil	1.98–21.24	1.45–2.98	2.98–19.68
Water	6.8–49.12	3.12–38.88	3.89–41.43
Pore water	16.89–59.11	10.88–46.00	11.88–48.23
Salinity			
Soil (%)	0.89–3.87	0.51–3. 32	0.19–4.88
Water (g/lit)	0.17–29.98	0.57–23.12	0.98–24.34
Minerals			
Soil (g/100 g)			
Na	1.423–1.669	0.954–1.072	0.823–1.897
K	0.073–0. 988	0.018–0. 781	0.031–0.811
Ca	0.191–0.292	0.093–1.112	0.121–1.124
Mg	0.090–0.543	0.038–0.354	0.074–0.478
Water (g/l)			
Na	0.059–12.68	0.071–9.121	0.389–9.811
K	0.053–0.542	0.002–1.319	0.004–1.417
Ca	0.095–0.498	0.044–4.192	0.053–4.689
Mg	0.063–1.065	0.048–1.143	0.049–1.635
Sulphates			
Soil (g/100 g dry soil)	0.092–4.12	0.017–1.829	0.028–2. 103
Water (g/lit)	0.25–8.81	0.02–3.98	0.02–4.67
Pore water (g/lit)	0.01–3.98	0.01–1.023	0.01–2.05
Organic matter of soil	6.206–9.309	2.921–5.172	3.678–7.240

Table 5. Type and extent of morphological variations (%) in the populations of *X. granatum*

Variation character	% of morphological variation in the populations from		
	Achara (11)	Jaigarh (1)	Purnagarh (3)
Leaf dimorphism*	33	0	33
Leaflet size and shape*	40	0	33
Flowering phenology*	54	0	33
Petals per flower**	54	0	33
No. of fruits/inflorescence**	36	0	0
Fruit size*	38	0	33

Figures in parentheses indicate number of accessions.
*Significant at 1%; **Significant at 5%.

morphological variations really have genetic parallels or they are epigenetic. Our studies have clearly shown that the morphological variations are epigenetic and the populations along the Maharashtra Coast represent a single species as *X. granatum*. Further autecological studies on this species are in progress.

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Received 7 July 2008; revised accepted 7 July 2009

Ediacaran megaplant fossils with Vaucheriacean affinity from the Jodhpur Sandstone, Marwar Supergroup, western Rajasthan

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The present study records the discovery of megaplant fossils from the middle part of the Ediacaran Jodhpur Sandstone, exposed in the mines around Sursagar area (GPS location 26°20′03″N; 72°59′72″E), Jodhpur, Rajasthan. This is the first record of the giant size noncarbonaceous plant fossils from the Precambrian sequences. The fossils show well-developed thallus, branching pattern, development of possible oogonia and zoospores, and antheridea. Showing morphological similarity with the extant Vaucheriacean plant, the thallus is about 140 times bigger in size. Preserved on the top of the bed, these are also associated with well-developed microbial mats. The same horizon has also yielded poorly preserved circular structures with medusoidal affinity which can be compared with the *Aspidella* sp.

Keywords: Ediacaran, Jodhpur Sandstone, Marwar Supergroup, plant fossils, Vaucheriacean affinity.

DOMINATED by the abundance of well-diversified microbial life, the Proterozoic Eon shows preservation of varied stromatolite morphologies in the carbonate rocks and microbial mats in the siliciclastic rocks. However, the occurrences of megascopic body fossils of plant affinity are rare and lack diversity. Generally, they are carbonaceous with simple morphological characters. The most common forms are carbonaceous circular discs and filaments[1,2]. However, the plants preserved as non-carbonaceous structures are rare and difficult to identify in the absence of definite clues. This communication reports well-preserved non-carbonaceous megaplant fossils from the middle part of the Ediacaran Jodhpur Sandstone of the Marwar Supergroup exposed in the mines of Sursagar (GPS location 26°20′03″N and 72°59′72″E), about 5 km NNW of Jodhpur city in western Rajasthan (Figures 1 and 2). Though the plants are megascopic, they have morphological characters comparable to the Vaucheriacean plant *Vaucheria* in thallus organization, branching pattern and in having oogonia and antheridia-like structures. However, the size of the plant is about 140 times larger than the extant forms[3]. It appears that the plant life attained large size during the Ediacaran period and may

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