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Morphological, cytological, palynological and molecular characterization of certain *Mangifera* species

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The *Mangifera* genus has more than 60 species, mostly distributed in tropical Asia. The wild relatives of *Mangifera* are considered reservoirs of potential genes that can confer tolerance/resistance to biotic and abiotic stresses. The morphological, cytological and molecular characterization of eight species was done to study the diversity and phylogenetic relationship among different *Mangifera* species. In order to study the evolutionary relationship and polymorphism among the mango species, the ITS1/ITS4 gene and partial chloroplast *psbH-trnH* genes were sequenced. Phylogenetic analysis of the nuclear and chloroplast marker revealed that the *M. indica* L. is closely related to *M. griffithii* and *M. camptosperma*, which belong to subgenus *Mangifera*. Results indicate that the taxonomic position of *M. andamanica* should be reconsidered as this species is very close to *Bouea oppositifolia* which is evident from both ITS and *psbA-trnH* rDNA analysis. The morphological traits such as tree, leaf, flowers and fruits and palynological and cytology of the genus mango were used to distinguish the species and its phylogentic status. The morphological traits among various species indicate the high level of variability which were further confirmed with ITS sequences and cpDNA. Phylogenetic analysis illustrates that partial chloroplast *psbH-trnH* gene gave better polymorphism in mango species than nuclear ITS. The pollen morphology and chromosomal counts were also done in certain *Mangifera* species to study the phylogenetic relationship.

Keywords: Chromosome, ITS, mango, pollen grains, psbA-trnH and phylogenetic analysis.

MANGO (*Mangifera indica* L.), considered as 'King of fruits', belongs to the family Anacardiaceae. It is an important tropical fruit believed to have originated in the Himalayan hills of Indo-Myanmar region. There are 58 listed species of the genus *Mangifera* which are further classified into several sections, based on their flower morphologies[1]. Mango (*M. indica* L.) and some other species of this genus are diploid (2*x*) with somatic number (2*n*) of chromosomes 40 (refs 2, 3). The high chromosome number, secondary association of bivalents,

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regular pairing, absence of multivalent formation and good pollen fertility have led to the conclusion that mango is allopolyploid in nature[2]. Pollen morphology and chromosome numbers of the genus mango were used to distinguish the species in India and find the genetic variability. Several biotechnology tools have been used to determine the genotypic profiles of individuals and/or populations, of wild and cultivated plant species. DNA markers, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) are superior to morphological and isozyme markers due to their high-throughput capacities and their abilities provide greater differentiation of closely related cultivars besides being unaffected by environmental factors[4]. DNA-based methodologies are relatively quick and can be used to determine the inter-specific and intra-specific relationships[5,6]. The internally transcribed spacer (ITS) sequence mutations are suitable for resolving relationships between species and genera[7]. Their Angiosperm genomes have multiple copies of ITS, but these are generally homogenized by concerted evolution, and therefore can be treated as a single locus[8].

Studies on genetic variability within a cultivated crop have important consequences in plant breeding and germplasm management. Morphological, cytological, palynological and molecular characterization is required to identify the commercially important traits in wild relatives which could be used in hybridization to introgress the genes.

The present study aims at identifying and characterizing few *Mangifera* species as per the bioversity international descriptors, determine the cytological profile of *M. camptosperma* and *M. andamanica* and the palynology of *M. odorata*, *M. camptosperma* and *M. indica*. An attempt was also made to analyse the genetic diversity among eight mango species collected from different parts of India, based on nuclear ribosomal DNA internal transcribed spacer and the *psbA-trnH* spacer. The ITS1/ITS4 and psbA-trnH spacer regions were sequenced and submitted to the NCBI database.

Eight mango species, viz. *M. zeylanica*, *M. griffithii*, *M. odorata* and *M. indica* were collected from the field gene bank of ICAR-IIHR, Bengaluru (Karnataka). The *Mangifera pajang* leaves were collected from Kerala (where as *Bouea oppositifolia*) while *M. andamanica* and *M. camptosperma* were collected from the Andaman and Nicobar Islands.

Out of eight species, trees of six species were morphologically characterized according to the standard descriptor[9] while for the remaining two, leaves were obtained from Kerala and Andaman Islands.

The total genomic DNA of each species was isolated from newly sprouted leaves using modified CTAB method[10]. Leaves were ground to a fine powder and transferred to the pre-warmed extraction buffer and incubated at 65°C for 1 h. After incubation an equal amount of chloroform : isoamyl alcohol mix (24 : 1) was added. The mixture was gently inverted to ensure thorough mixing followed by centrifugation. The clear supernatant was transferred to a fresh tube and DNA was precipitated by the addition of three-fourths volume of isopropanol and then centrifuged. The resulting pellet was washed with 70% ethanol, dried and dissolved in 1× TE buffer. RNA was removed by RNase treatment, and the resultant DNA was quantified using an UV-absorption spectrophotometer.

Amplification of the ITS region was carried out using the universal primers ITS1 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)[11]. Each reaction mixture (15 µl volume) contained 1.5 µl of the reaction buffer A (pH 9.0, 10 mM *Tris* with 15 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin), 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 1.5 µl (5 pmol) of forward primer, 1.5 µl of reverse primer (5 pmol), 0.5 µl (3 U/µl) of *Taq* DNA polymerase, 3 µl of template DNA and 4.5 µl of nuclease-free water. The amplification was conducted in a Bioer Life Pro Thermal cycler (Bioer Technology, PR China), with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 sec at 94°C, primer annealing at 55°C for 30 sec, extension at 72°C for 1 min and final extension step at 72°C for 5 min. The amplified products were separated on a 2% agarose gel and documented.

Samples were collected from the freshly opened bisexual flowers and stored in liquid nitrogen at –196°C. These were subsequently used for pollen morphological studies. Anthers from flower fixed in ethanol were crushed on a slide. All the debris was removed and a small amount of alcohol was added again after slightly heating over a spirit flame for 10 sec. This was repeated to remove all fatty substances present in pollen grains. Small amount of glycerine jelly was added, covered with a cover slip and the diameter of 100 pollen grains was measured using BALETECSCD 005 sputtering device, imaging at 15 kV, using a JSM-6390 LV (JEOL, Tokyo, Japan) scanning electron microscope[12].

Shoot tip with small leaflets of 2–3 mm in length was excised and pre-treated with 0.003M 8 hydroxyquinone for 2 h at 14–16°C. It was rinsed in distilled water and fixed in Carnoy's-II fixative, viz. 6 : 3 : 1 of absolute alcohol : glacial acetic acid : chloroform and stored for 24 h. Later these were transferred to 70% alcohol after 24 h for long term storage (about 2–4 months). The stored shoot tips were again rinsed with distilled water and hydrolysed in a water bath with 1 N HCl at 60°C for 5 min or in 5 N HCl at room temperature for 30 min. The hydrolysed shoot tips were transferred to Schiff's reagent[13], also known as Feulgen stain, rinsed in distilled water and stored in the dark for 1½ to 2 h. Schiff's reagent stains the actively dividing meristematic tissue to deep magenta colour. The stained tips were squashed with 1%

Table 1. Fruit descriptions of six *Mangifera* species

Characters/species	*Mangifera odorata*	*Mangifera camptosperma*	*Mangifera zeylanica*	*Mangifera griffithii*	*Mangifera andamanica*	*Mangifera indica* (cv. Alphonso)
Fruit descriptors						
Fruit weight (g)	198.86	76.0	114.70	173.20	22.0	246.20
Fruit shape	Oblong	Flat	Round	Oblong	Round	Round
Shape of fruit apex	Acute	Round	Acute	Acute	Round	Acute
Fruit attractiveness	Average	Average	Average	Average	Good	Excellent
Fruit ground colour	Green	Green	Green	Green	Green	Green
Skin colour of ripe fruit	Green	Green	Greenish yellow	Orange	Yellow	Greenish yellow
Fruit blush	Green	Greenish yellow	Orange	Orange	Purple	Yellow
Fruit skin thickness (cm)	0.1	0.61	0.1	0.1	0.1	0.1
Fruit skin surface texture	Smooth	Rough	Smooth	Smooth	Smooth	Smooth
Density of lenticels on fruit	Intermediate	Spare	Intermediate	Dense	Intermediate	Dense
Fruit stalk insertion	Vertical	Vertical	Vertical	Vertical	Vertical	Vertical
Depth of fruit stalk cavity	Absent	Absent	Absent	Absent	Absent	Absent
Fruit stalk attachment	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate	Strong
Fruit neck prominence	Slightly prominent	Prominent	Slightly prominent	Prominent	Slightly prominent	Absent
Slope of fruit ventral shoulder	Ending and then round	Ending in a long curve	Ending and then round	Ending and then round	Ending and then round	Raising and then rounded
Fruit beak fruit	Perceptible	Pointed	Perceptible	Perceptible	Perceptible	Perceptible
Fruit sinus type	Absent	Shallow	Absent	Absent	Absent	Shallow
Fruit skin weakness	Waxy	Non- Waxy	Waxy	Waxy	Non waxy	Waxy
Skin colour of ripe fruit	Greenish yellow	Greenish	Yellow orange	Green with red blush	Yellow orange	Green with red blush
Pulp colour of ripe fruit	Yellow	Light yellow	Yellow orange	Dark orange	Yellow orange	Yellow orange
Pulp texture of ripe fruit	Firm	Intermediate	Soft	Soft	Soft	Firm
Adherence of fruit skin to pulp	Strong	Strong	Strong	Weak	Strong	Intermediate
Quantity of latex oozing from peduncle	Medium	Low	Medium	Medium	Medium	Medium
Fruit pulp thickness	*	*	*	*	*	*
Quantity of fibre in pulp	Medium	High	Low	Medium	High	Low
Adherence of fibre to skin	High	*	Low	Medium	Medium	Low
Fibre length in the pulp	Medium	*	Medium	Short	Medium	Medium
Pulp juiciness	Slightly juicy	Not juicy	Juicy	Juicy	Slightly juicy	Slightly Juicy
Pulp aroma	Strong	Mild	Mild	Mild	Mild	Strong
Presence of turpentine flavour	Strong	Mild	Absent	Mild	Mild	Absent
Type of embryony	Polyembryony	Monoembryony	Polyembryony	Monoembryony	Monoembryony	Monoembryony
Eating quality	Poor	Poor	Good	Poor	Poor	Excellent
Pulp TSS (^{0}Brix)	19.80	13.4	18.50	18.60	10.1	19.00
Pulp acidity (%)	0.256	0.16	0.384	0.384	0.38	0.32

aceto-carmine or orcein. The slides were sealed with wax and observed under a microscope on the same day or the next day. The slides were scanned for well spread metaphase chromosomes under 100× oil immersion objective using Olympus BX-51 research microscope.

Morphological characterization of six *Mangifera* species was done using the international descriptors. Huge variability was recorded with respect to tree characters, leaf characters, fruit characters and stone characters among the studied species (Table 1 and Figure 1). However, the fruit size, shape and colour of the peel are the critical characters to distinguish the species.

The ITS1/ITS4 primer amplified products were digested with *Taq* 1 and *Hinf* 1 restriction enzymes. The amplified product size of ITS1/ITS4 ranges from 300 to 750 bp whereas for chloroplast psbA-trnH rDNA ranges from 350 to 700 bp (Figures 2 and 3).

After sequencing, the generated nucleotide sequences were edited and manually adjusted to construct a phylogenetic tree using MEGA v7 (ref. 14). All sequences generated during this study have been accessioned at the National Centre for Biotechnological Information (NCBI), USA. The accession numbers MF444895–MF444902 pertain to ITS1/ITS4 and the accession numbers MF538520–MF538527 pertain to the *psbA-trnH* sequences (Table 2).

The phylogenetic analysis of ITS DNA and the sequences retrieved from the NCBI database for comparative studies reveal that there are genetic variations among the mango species.

Analysis with Mega 7.0 software resulted in a phylogram showing three main ITS clusters. The clade I has 7

Figure 1. Fruits of 6 different *Mangifera* species.

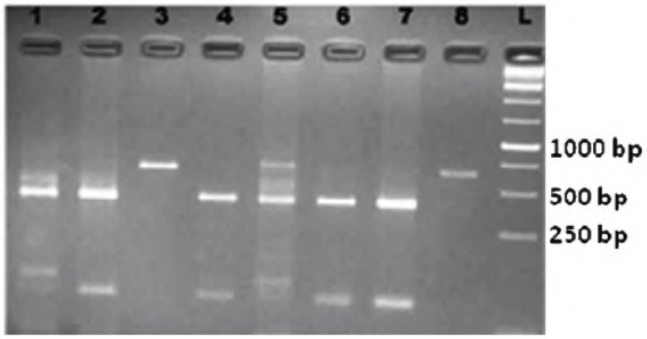

Figure 2. Restriction digestion of ITS primer amplified product with *Hinf*1 restriction enzyme.

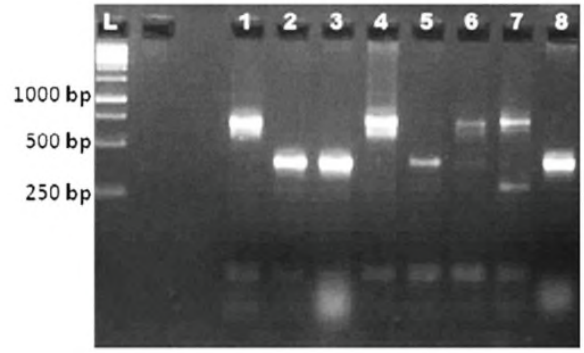

Figure 3. Chloroplast psbA-trnH primer amplified products.

species such as *M. sumatrana*, *M. torquenda*, *M griffithii*, *M. pajang*, *Bouea macrophylla*, *B. oppositifolia* and *M. andamanica*. The *B. oppositifolia, M. griffithii* and *M. andamanica* are endemic to Andaman and Nicobar Islands. About 88% similarity between *B. oppositifolia* and *M. andamanica* and 78% similarity with *B. macrophylla* were observed. *M. camptosperma* has been grouped in one sub-cluster leaving seven other species in group I. Group II has 2 species namely *M. zeylanica* and *M. gracillipes*. Group III has three species such as *M. indica*, *M. laurina* and *M. kemanga*. *M odorata* has been grouped in one sub-cluster leaving three other species in group III. This grouping clearly suggests that the *Mangifera* species are monophyletic in nature and have biogeographical origin in pan tropical Asia (Figure 4).

The phylogenetic analysis of partial chloroplast *psbA-trnH* rDNA sequences (ranging from 350 to 700 bp) reveals that there are genetic variations between the mango

Table 2. List of ITS and *trn*H–*psbA* sequences derived from different species of *Mangifera*

Taxonomy	Family	Genus	Species	Locality	Gene Bank accession no.	
					ITS	trnH-psbA
Eudicotyledons	Anacardiaceae	*Bouea*	*Bouea oppositifolia*	India	MF444895	MF538520
Eudicotyledons	Anacardiaceae	*Mangifera*	*Mangifera pajang*	India	MF444896	MF538521
Eudicotyledons	Anacardiaceae	*Mangifera*	*Mangifera andamanica*	India	MF444897	MF538522
Eudicotyledons	Anacardiaceae	*Mangifera*	*Mangifera zeylanica*	India	MF444898	MF538523
Eudicotyledons	Anacardiaceae	*Mangifera*	*Mangifera griffithii*	India	MF444899	MF538524
Eudicotyledons	Anacardiaceae	*Mangifera*	*Mangifera camptosperma*	India	MF444900	MF538525
Eudicotyledons	Anacardiaceae	*Mangifera*	*Mangifera odorata*	India	MF444901	MF538526
Eudicotyledons	Anacardiaceae	*Mangifera*	*Mangifera indica*	India	MF444902	MF538527
Eudicotyledons*	Anacardiaceae	*Bouea*	*Bouea macrophylla*	Thailand	AB071691.1	–
Eudicotyledons*	Anacardiaceae	*Mangifera*	*Mangifera kemanga*	Indonesia	KX347955.1	–
Eudicotyledons*	Anacardiaceae	*Mangifera*	*Mangifera sumatrana*	South Asia	KX347961.1	–
Eudicotyledons*	Anacardiaceae	*Mangifera*	*Mangifera torquenda*	South Asia	KX347958.1	–
Eudicotyledons*	Anacardiaceae	*Mangifera*	*Mangifera laurina*	Thailand	AB071687.1	
Eudicotyledons*	Anacardiaceae	*Mangifera*	*Mangifera gracilipes*	Thailand	AB071686.1	

*Six sequences are retrieved from the NCBI Database for comparative studies.

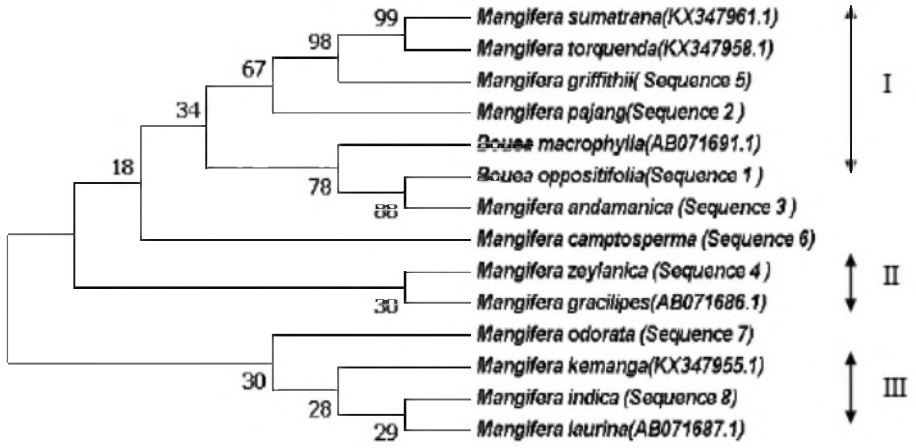

Figure 4. Phylogenetic tree constructed using sequences of the ITS regions of the species used in this study and members of the genus *Mangifera* available in the public domain. The accession numbers are depicted in parenthesis *Chloroplast marker* (*Psb-trnH*).

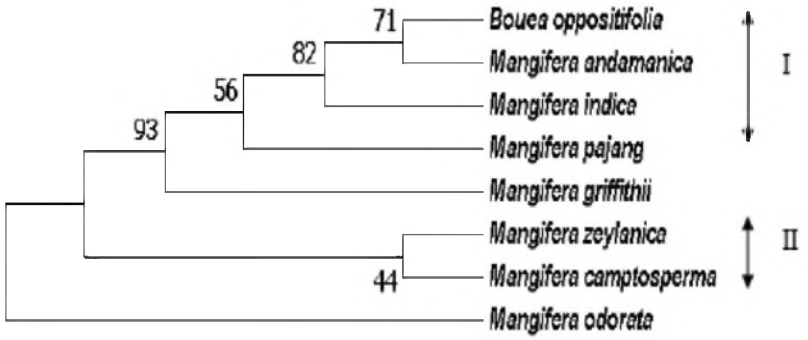

Figure 5. Phylogenetic tree constructed using sequences of Cp DNA of the species used in this study.

species. Group I has five mango species such as *B. oppositifolia*, *M. andamanica*, *M. indica* and *M. pajang* which are mostly distributed in South East Asia. *M. griffithii* has been grouped into one sub-cluster leaving four other species. Group I has *B. oppositifolia* and Psb-trnH which is endemic to Andaman and Nicobar Islands. Group II has

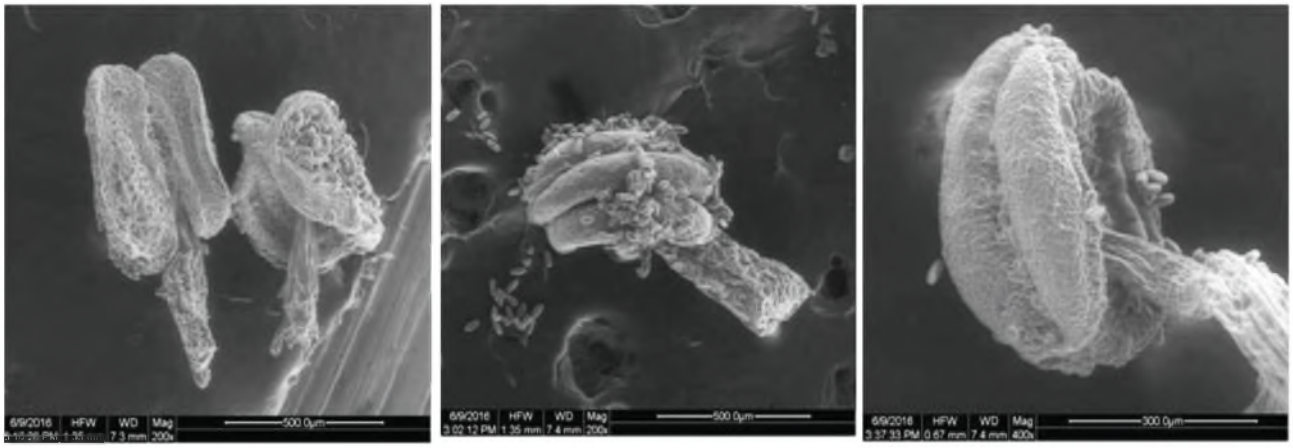

Figure 6. Filament attachment in the anther sacs of different *Mangifera* spp.

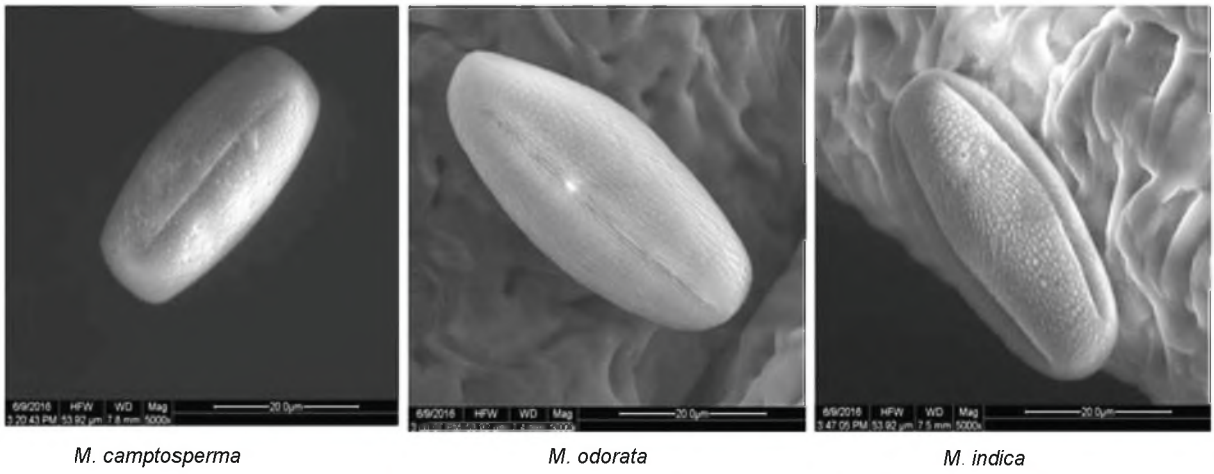

Figure 7. Individual view of pollen grains of different *Mangifera* spp.

M. zeylanica and *M. camptosperma* whereas *M. odorata* has been clustered into one sub-cluster leaving two other species (Figure 5).

The pollen grains of *M. odorata*, *M. camptosperma* and *M. indica* were examined under the scanning electron microscope (SEM) and differences were observed for filament attachment as well as pollen morphology (size, shape and exine structure) among the species (Figures 6 and 7).

Pollen grains from all examined species were elliptic to oblong in shape. *M. indica* has elliptic–oblong pollens with slightly flattened poles, the *M. odorata* pollens are elliptic and tapering towards poles, while *M. camptosperma* has oblong pollen grains. The pollen grains of *M. indica* were zono-tricolporate showing the presence of three colpi along the polar axis with a porate endoaperture. The exine structure is of semitectate type as the tectum is perforated. The exine sculpturing was striato-microreticulate. The polar axis (*P*) and breadth (*E*) were 35–40 μm and 15–20 μm respectively and the shape was concluded to be prolate. *M. odorata* has zono-tricolporate pollen with characteristic endoaperturate pores in its three colpi. The exine is semitectate and the exine ornamentation was clearly striato-reticulate. *P* and *E* were 40–45 μm and 15–20 μm respectively and the shape was concluded to be perprolate. *M. camptosperma* pollen did not have an endo aperture in its colpi and hence they are concluded to be of zono-tricolpate type. The exine structure is of semitectate type with reticulate exine sculpturing. *P* and *E* were 25–30 μm and 15–20 μm respectively and the shape was concluded to be perprolate. Pollen characters can also be applied along with morphological and anatomical characteristics and for solving controversial taxonomical and phylogenetical problems.

Cytological investigations revealed that *M andamanica*, *M. camptosperma*, and *M. odorata* had the similar somatic chromosome number ($2n = 40$) and could cross with each other (Figure 8).

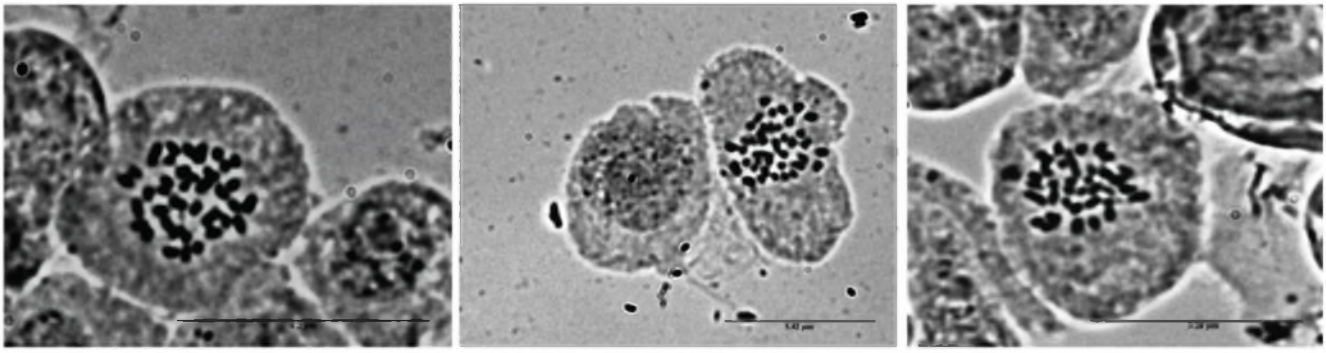

Figure 8. Cytological studies in various *Mangifera* species.

In an earlier study, the phylogenetic relationship among 41 species of *Mangifera* was studied based on morphological characteristics[15]. *Mangifera* was classified into 69 species, with 58 species grouped under the subgenus *Mangifera* under four sections. Genus *Limus* was assigned six sections[1]. According to this classification, *M. odorata* belongs to the section *Perennis* of the subgenus *Limus* while the other three species studied in the present work, were grouped into two separate sections of the subgenus *Mangifera*. While *M. indica* was accommodated under section *Mangifera*, *M. griffithii* and *M. andamanica* were accommodated under section Rawa. The position of *M. camptosperma* was not mentioned in this classification.

The results of this study agree with Kosterman's classification wherein *M. indica* and *M. griffithii* were accommodated in the subgenus *Mangifera*, unlike *M. odorata* which belongs to the subgenus *Limus*. The clustering of *M. camptosperma* with *M. indica* and *M. griffithii* suggests that they share a common ancestry, and hence, *M. camptosperma* has to be considered under the subgenus *Mangifera*. Phylogenectic analysis showed that *M. andamanica* clustered separately from other species of subgenus *Mangifera*, indicating that it does not belong to the same genus as reported earlier[1]. The fruits of *M. andamanica* are inferior in quality, highly fibrous and lack an edible pulp. There is no resemblance between the fruit shape of *M. andamanica* and other *M. indica* varieties. Results indicate that the taxonomic position of *M. andamanica* should be reconsidered as this species is very close to *B. oppositifolia* which is evident by both the ITS and *psbA-trnH* rDNA analysis. The chloroplast markers (*psbA-trnH*) and nuclear marker (ITS4) clearly show that *M. andamanica* is neither related to *M. indica* nor to *M. griffithii*, both of which belong to the subgenus *Mangifera*. This also re-confirms earlier objections about the taxonomical assignment of *M. andamanica* within the subgenus *Mangifera*[1]. This study also classifies *M. camptosperma*, whose position was not assigned earlier, under subgenus *Mangifera*. It is thus concluded that the classification of *M. andamanica* under genus *Mangifera* needs reconsideration and *M. camptosperma* has to be included in the subgenus *Mangifera*[16].

In the present study, the size of the amplified product of the partial chloroplast *psbA-trnH* gene ranged from 350 to 700 bp. Earlier reports[17] suggest that the lengths of trnH–psbA sequences in eudicotyledons, monocotyledons, gymnosperms, ferns and mosses range from 152 to 851 bp, from 151 to 905 bp, from 283 to 1006 bp, from 167 to 547 bp and from 103 to 265 bp respectively. The analysis of ITS sequences showed that genetic variations exist in mango species. Similar results were reported with ITS markers[18,19] and MatK[20]. It was observed that when *trnH–psbA* was combined with three other popular markers, viz. ITS2, *rbcL* and *matK*. The *trnH–psbA*+ITS2 combination performed the best among all locus combinations for the majority of taxa examined at the family or genus level[17]. Based on morphological tools, comparison of nucleotide evidence and phylogenetic analysis in *Mangifera* species affords a better way of understanding the evolutionary relationships among different groups of organism from different geographical area, habitat and other ecological factors.

Palynological studies[21–23] of cinnamon, *Acacia*, *Onobrychis*, etc. have also emphasized on the importance of the differentiating species using pollen characters. Therefore, further detailed studies on pollen apertural form, number, distribution and position, exine thickness, stratification, etc. could help differentiate *Mangifera* species. Significant variation was observed among three *Mangifera* species, viz. *M. indica*, *M. odorata* and *M. camptosperma* with respect to filament attachment, pollen shape, size and exine striae. The present result also confirms earlier results[24–26]. Cytological investigations revealed that *M. andamanica*, *M. camptosperma* and *M. odorata* have the same somatic chromosome number ($2n = 40$) (ref. 27). It is also observed that *M. indica* can cross with *M. camptosperma* and *M. odorata* due to similar chromosome numbers.

Six *Mangifera* species were characterized according to the Bioversity International descriptors which suggest the existence of variability among the species under genus

Mangifera. The somatic chromosome number of *M. andamanica* and *M. camptosperma* was determined as $2n = 40$. Analysis based on chloroplast markers *psbA-trnH* and ITS4 clearly shows that *M. andamanica* is not closely related to *M. indica* and *M. griffithii* which belong to subgenus *Mangifera*. Similarly, *M. camptosperma*, whose position was not known earlier, has been placed under subgenus *Mangifera* based on our analysis. It is concluded that classification of *M. andamanica* under genus *Mangifera* needs reconsideration and *M. camptosperma* has to be included in the subgenus *Mangifera*.

Palynological traits of *Mangifera* spp. revealed the taxonomic relationships among the species within the genera. The ITS and cpDNA sequences analysis of eight different *Mangifera* species shows that there exists a phylogenetic affinity and biogeographic distribution pattern amongst species that are endemic to the specific regions. Hence, it is necessary to combine the morphological, molecular, cytological and palynological data for phylogenetic classification and biogeographical locations of *Mangifera* species.

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