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Exploration, Characterization and Phylogenetic Studies in Wild *Mangifera indica* Relatives

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Abstract

Exploratory surveys were carried out in the Andamans and Nicobar group of islands during 2006 and 2014 to locate wild species viz. *Mangifera andamanica* King, *Mangifera camptosperma* Pierre and *Mangifera griffithii* Hook. Not much variation was observed for fruit shape and size for the species *Mangifera andamanica,* which was endemic to this region. The species *M. griffithii* has been reported to be only in Mt. Harriet. However, another plant of *M. griffithii* in the Shoalbay region was found during the second survey. The foliage & fruit characteristics of the two specimens were similar, with a slight difference in the morphological features, which could be attributed to their origin from seeds. The DNA finger printing carried out showed minor changes in the species. The phylogenetic relationships amongst five *Mangifera species* viz. *M. indica*, *M. griffithii*, *M. camptosperma*, *M. odorata* and *M. andamanica* were analyzed by employing chloroplast markers viz., petB-petD intergenic spacer, rps16 gene, trnL-trnF intergenic spacer and nuclear marker—External Transcribed Spacer (ETS). The nuclear markers and chloroplast markers based on phylogenetic analysis showed that the common mango *M. indica* L. was closely related to *M. griffithii and M. camptosperma*, which belonged to subgenus *Mangifera*. However, *M. odorata* that belonged to subgenus *Limus* was grouped separately along with *M. andamanica*. The above results are in congruent with the accepted classification of genus *Mangifera* reported by Kostermans and Bompard with the exception of *M. andamanica,* which has been earlier classified under subgenus *Mangifera*.

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Results clearly indicated that classification of *M. andamanica* under subgenus needed to be reconsidered.

Keywords

Characterization, Diversity, Exploration, ETS, Mango, petB-D, trnL-F, rps16, Phylogeny

1. Introduction

The genus *Mangifera* is one of the 68 genera in the family Anacardiaceae [1]. *Mangifera indica* L. is the most important species in this genus for commercial fruit production in tropical and sub-tropical regions of the world. The genus *Mangifera* is believed to have originated somewhere in Myanmar, Thailand, Indo-China and Malaya during the Eocene or an earlier period in the Cretaceous, and then the species have spread to India and Sri Lanka in the west, and to Eastern Malaysia and the Philippines in the east [2]. The center of diversity of this genus is thought to be South East Asia, with increased diversity in Peninsular Malaysia. The highest number of Mangifera species is now found in Borneo, Sumatra, Java and Malay Peninsula [3]. The most acceptable classification of the genus *Mangifera* is reported by Kostermans and Bompard [4] who described 69 species in the genus based on the morphological characteristics and classified 58 species into two subgenera *Mangifera* and *Limus* with several sections. The 11 remaining species were placed in an uncertain position in the classification. India is reported to be the home of four other species viz., *Mangifera andamanica*, *Mangifera khasiana*, *Mangifera sylvatica* and *Mangifera camptosperma* [5]. Two exploration surveys were carried out at Andamans, one in 2006 and another in 2014 to collect the species viz., *Mangifera andamanica*, *Mangifera camptosperma* and *Mangifera griffithii*. Hence, survey was undertaken to locate diversity and distribution of wild/related *Mangifera* species within the Andamans.

The phylogenetic relationships among *Mangifera* species have been described earlier by using genomic restriction fragment length polymorphisms (RFLPs) and amplification of chloroplast DNA (cpDNA) [6]. Additionally the sequence analysis of External Transcribed Spacer (ETS) has also been reported to be involved in the construction of phylogeny in plants [7]-[11]. For phylogenetic studies at lower taxonomic levels noncoding chloroplast regions have been used frequently and successfully [11]-[13]. The rationale behind using noncoding regions is the assumption that they are phylogenetically more informative because they are under less functional constraints [14].

In the present study, morphological (foliage & fruits) and molecular characterization (SSR, ETs and Chloroplast markers) was carried out to infer the evolutionary relationships among the Mangifera Species including *Mangifera indica*, *Mangifera griffithii*, *Mangifera camptosperma*, *Mangifera odorata* and *Mangifera andamanica*.

2. Plant Materials and Methods

An exploration was undertaken in greater Andaman Islands, India to study the genetic diversity of *Mangifera species* during 2006. Observations were made on the morphological characters of the tree, leaves and fruits wherever it was available. The fruits of the three species of *Mangifera* viz., *Mangifera andamanica*, *Mangifera camptosperma* and *Mangifera griffithii* were collected and the observations were recorded on fruit weight, fruit length, fruit breadth and total soluble solids. An exploratory mission was again undertaken during 2014 to survey Shoalbay, Chauldhari, Naya Shahar and Chidiyatapu regions besides Mount Harriet, of the Andaman Islands. Wild species like *Mangifera andamanica*, *Mangifera camptosperma* and *Mangifera griffithii* were found distributed in specific isolated pockets, which were identified based on taxonomic keys. Bud sticks and leaf samples were collected from voucher accessions and foliage characteristics were recorded. *In situ* evaluation of leaf characters for samples collected from these regions and molecular characterization was carried out using total genomic Deoxyribonucleic acid-DNA isolated by the Cetytrimethylammonium Bromide—CTAB method [15], including *Mangifera indica* cultivars namely Kurukkan, Muvandan, Olour, Alphonso, Raspuri and Langra

for phylogenesis; as DNA yields more phylogenetic information among the biomolecules.

PCR Amplification, Sequencing and Analysis

Fluorescence based PCR method [16] was used to amplify the microsatellites in a quick, accurate and efficient manner. Eight microsatellite markers of mango showing high PIC [17] were employed for amplification. SSR markers were amplified in 10 µl volume containing 25 - 50 ng mango DNA-3 µl, Taq Buffer 10× (pH 9.0, 10 mM Tris with 15 mM $MgCl_2$, 50 mM KCl, 0.01% Gelatin)-1.0 µl, 1mM dNTPs-1.0 µl, 25 mM $MgCl_2$-0.3 µl, forward labelled specific primer (5 µM)-0.1 µl each thus mixed four PCR products labelled with different fluorophores (FAM, VIC, NED and PET), reverse primer (5 µM)-0.1 µl each, nuclease free water 3.6 µl and 0.3 µl of Taq DNA polymerase (Bangalore Genei, India). PCR was performed on Life Pro Thermocycler (Bioer, Hangzhou, China) with the following temperature profile: 94˚C for 2 min followed by 35 cycles at 94˚C for 30 s, 30 s annealing temperature of 55˚C and 72˚C for 1 min. A final extension reaction was allowed to proceed at 72˚C for 5 min. Amplified products were initially separated on 3% agarose gel for confirmation of the amplification. These samples were separated on the automatic 96 capillary automated DNA Sequencer (ABI 3730) at M/s Eurofin Ltd. facility at Bengaluru. The molecular data was used to generate dendrogram with DARwin 5.0 (**Figure 1**) [18].

The phylogeny study comprised of five different *Mangifera species*, namely *M.indica*, *M. griffithii*, *M. camptosperma*, *M. odorata* and *M. andamanica*. Within *M. indica* a total of six cultivars; three polyembryonic cultivars viz *Kurukkan*, *Muvandan* and *Olour* and 3 monoembryonic cultivars viz *Alphonso*, *Raspuri* and *Langra*, were taken for the study.

The PCR reaction mixture compositions and amplification conditions varied among the markers (petB-petD; trnC-trnF; rps16 and ETS) taken for the study.

The rps16 intron was amplified with the rps16F and rps16R2 primers as described [19]. For the trnL-trnF intergenic spacer, we used the primers from [20]. The petD region was amplified with the forward primer PIpetB1365F and the reverse primer PIpetD738R [21]. The PCR reaction mixture and conditions were used according to [22] for rps16 and trnL-trnF, and [21] for petB-petD intergenic spacer. Amplification of ETS region

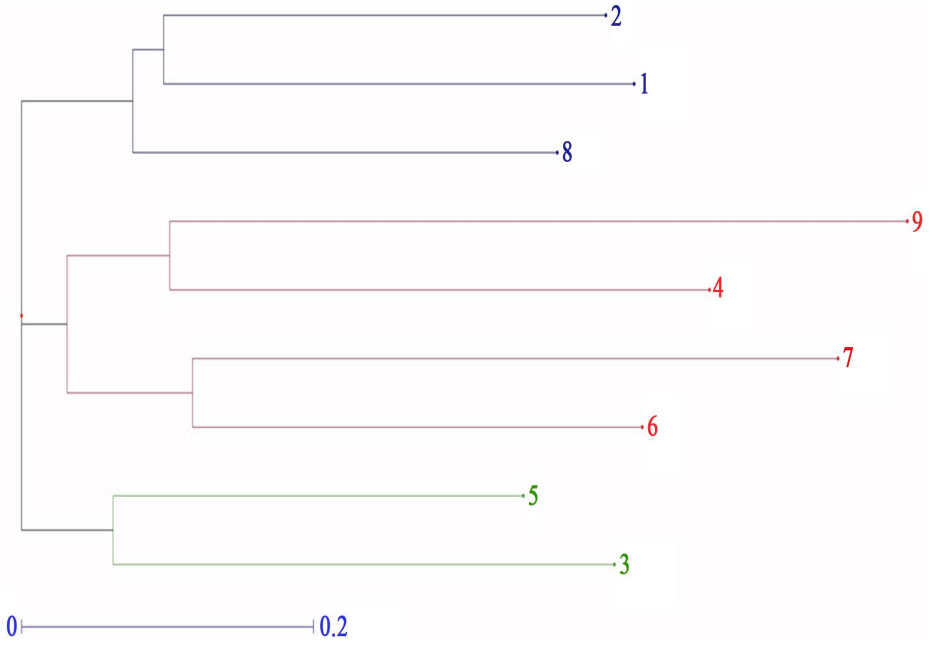


Figure 1. Dendrogram analysis of nine *Mangifera* species from Andaman by NJ method. 1 *Mangifera camptosperma*, 2 *Mangifera camptosperma* (Grafted), 3 *Mangifera andamanica* (Nayashahar), 4 *Mangifera griffithii* (Near School), 5 *Mangifera andamanica* (Biopark-1), 6 *Mangifera andamanica* (Biopark-2), 7 *Mangifera andamanica* (Biopark gate), 8 *Mangifera camptosperma* (Bio Park), 9 *Mangifera griffithii* (Mount Harriet).

was done according to [23]. All the PCR products were separated on 3% agarose gel electrophoresis to check for efficiency of amplification and to ensure that only a single product of the expected size was present, PCR products were purified by MinElute PCR Purification Kit (Qiagen). PCR cleanup products were then sequenced using the same primers as used in initial PCR amplification using in ABI 3730 XL automated sequencer through Big Dye terminator sequencing technology.

Sequence data of all the products were first confirmed through the homology search BLAST of NCBI database and aligned using ClustalW program [24] of BioEdit. Then these aligned files were further converted to. meg file, exported to the MEGA 4.0 software environment for further analysis to construct the phylograms.

The aligned sequence data matrix was analyzed using the Molecular Evolutionary Genetic Analysis (MEGA) 4.0 software program [25]. The phylogeny was analyzed using Maximum Parsimony (MP) method, which is a character based on computational approach. The bootstrap test of Phylogeny was computed for all the nuclear and chloroplast markers with 2000 iterations.

3. Results and Discussion

Identification of *Mangifera* species involves the observation of vegetative and fruit characteristics. The initial identification is carried out based on the growth habit of the tree, other morphological features and the vernacular names. One of the main problems in the identification is the variation in the morphological characters because of the seedling origin of the progenies and the in-built heterozygosity. The trees belonging to the *Mangifera* genus are generally tall growing. The phylogenetic taxonomy carried out has shown that the species are included under two sections, depending on the presence or absence of a prominent disc in between the stamen and the carpel [5].

3.1. Morphological Analysis

The first survey was carried out during 2006 in the South Andamans and Middle Andaman. Several *Mangifera indica* varieties were observed in these regions. The survey of Chauldhari and Jirkhatang regions resulted in locating one and two specimens of *M. andamanica* respectively. One tree of *Mangifera camptosperma* was located near the coastal region almost by the side of sea at Jirkhatang. In Chidiya Tapu region, one tree of *Mangifera griffithii* was located. The specimen was evaluated for leaf characteristics and passport data was recorded (**Table 1**; **Table 2**), these were further characterised by using microsatellitre markers (**Table 3**). And compared with other wild species *M. odorata* maintained in the field genebank.

Table 1. Leaf parameters of *M. griffithii.*

Sl. No	Species	Leaf length (cm)	Leaf width (cm)
1	*M griffith*ii (near school at Shoalbay)	3.4	2.7
2	*M griffithii* (Mt. Harriet)	3.9	3.8

Table 2. Passport data of the *Mangifera griffithii* located at Shoalbay and Mount Harriet.

Village	Shoalbay	Mt. Harriett
Block	Ferrargunj	Ferrargunj
District	Andamans	Andamans
State	Andaman & Nicobar Islands	Andaman & Nicobar Islands
Country	India	India
Continent	Asia	Asia
Latitude	11˚67'N	32˚43'N
Longitude	92˚76'E	152˚10'E

Table 3. Locus name and sequence of the eight SSR markers used in this study (Ravishankar *et al*., 2011).

Primer 5' - 3'	Primer name
F: GCTTGCTTCCAACTGAGACC R: GCAAAATGCTCGGAGAAGAC	MiIIHR17
F: TCTGACGTCACCTCCTTTCA R: ATACTCGTGCCTCGTCCTGT	MiIIHR18
F: TCTGACCCAACAAAGAACCA R: TCCTCCTCGTCCTCATCATC	MiIIHR23
F: GCGAAAGAGGAGAGTGCAAG R: TCTATAAGTGCCCCCTCACG	MiIIHR26
F: AGCTATCGCCACAGCAAATC R: GTCTTCTTCTGGCTGCCAAC	MiIIHR30
F: TTCTGTTAGTGGCGGTGTTG R: CACCTCCTCCTCCTCCTCTT	MiIIHR31
F: CTGAGTTTGGCAAGGGAGAG R: TTGATCCTTCACCACCATCA	MiIIHR34
F: TCTATAAGTGCCCCCTCACG R: ACTGCCACCGTGGAAAGTAG	MiIIHR36

***Mangifera andamanica* (Figure 3(a)):** It is a huge tree with oval shaped fruits borne in clusters. The peel of the fruits was observed to be thin, orange coloured. The pulp is fibrous and juicy with sweet taste (TSS: 22˚ Brix) and yellow in colour. The average weight of fruits was 11.57 g. The leaf tip was found to be acute and the leaf base was cuneate with flat margin. The leaf length and breadth were found to be 17.7 and 4.8 cm respectively. The fruit length and breadth were observed to be 3.15 cm and 2.07 cm respectively.

***Mangifera camptosperma* (Figure 3(b)):** It is a tall tree with sparse foliage and fruits were found to be totally flat and round in shape. The fruit pulp was found to be very fibrous and non-edible. The fruit weight was around 51.8 g. The fruit length and fruit breadth were around 9.86 g and 29.5 g respectively. The pulp recovery was 23% with hardly any edible pulp.

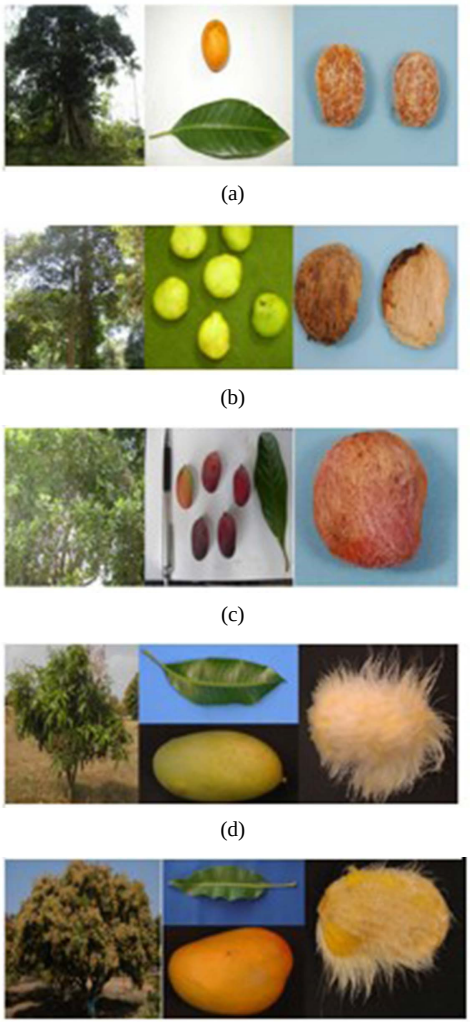
***Mangifera griffithii* (Figure 3(c)):** The species *Mangifera griffithii* was located on the hill top of Mt. Hariett. The tree was found to be moderately vigorous. The leaves resembled the leaves of *Anacardium occidentale* leaves with obtuse base and round tip. The leaves were also leathery, with a length of 13.5 cm and a breadth of 4.3 cm. The fruits were small oval shaped with attractive purple peel color. The fruit weight was observed to be 11.2 g and the pulp recovery was 12%. Although the fruits were observed to have very little pulp, they were juicy with sweet taste (22.6˚ Brix).

***Mangifera odorata* (Figure 3(d)):** The species *Mangifera odorata* is characterized by the distinct odour of the fruit. It possibly represents hybrid forms between *M. indica* and *M. foetida* (Ding Hou, 1978). The fruits on an average weigh about 200 g and the TSS is about 21.4˚ Brix. The pulp recovery is less than 55 per cent. The fruits on ripening have greenish purple peel colour and yellow pulp colour. The panicle is characterized by sparse flowers contrary to the dense flowers of *Mangifera indica*. *Mangifera odorata* is also polyembryonic in nature; compared to other wild species the edible quality in *M. odorata* is far superior, although, much inferior to *Mangifera indica.*

***Mangifera indica* (Figure 3(e)):** It is a native of the Indian Peninsula with a spreading tree of 20 - 45 m; all parts glabrous except inflorescence. *Leaves* were thinly coriaceous or membranaceous varied in size and shape. Fruit is *Drupe* large, oblong or subreniform; flesh thick with sweet juice; highly variable in size, shape and co-loration of the epicarp in the different cultivated varieties; stone fibrous, very hard, the fibrous are very long in the wild types and inferior cultivated types; cotyledons two, rarely many, unequal. *M. indica* is economically the most important species of the genus, as it bears one of the most delicious tropical fruits, the mangoes.

In the second exploratory survey **c**arried out in the Andaman Islands, resulted in locating the variants of *M. griffithii*, in Shoalbay region apart from Mt Harriet. The leaf and fruit characteristics of the two specimens were observed to be similar. The original specimen of *M. griffithii* from Mt. Harriet (**Figure 2** and **Figure 3**) had slightly longer, wider leaf than the ones observed near the Shoalbay School. The apex of the leaves (**Figure 3**) observed near the Shoalbay School was slightly more pointed compared to the Mt Harriet leaf sample. The fruits were observed to be similar in both the types but the tree near Shoalbay School was taller compared to the one at

Figure 2. *Mangifrera griffithii* tree, leaf and fruit found near Shoalbay School



(a)

(b)

(c)

(d)

(e)

Figure 3. Tree, leaf, fruit and stone characteristics of *Mangifera species* studied. (a) *Mangifera andamanica*; (b) *Mangifera camptosperma*; (c) *Mangifera griffithii*; (d) *Mangifera odorata*; (e) *Mangifera indica*.

Mt. Harriet. Survey carried out previously by several workers [26]-[28] in the diversity rich regions of *Mangifera indica* has resulted in several seedling selections in mango, which shows that there is every chance that seedling variants with desirable traits can be identified. The exploratory surveys help in the location of useful types be it wild species or varieties having desirable traits in the places of their diversity. They also help in conserving these types *in situ* as well as *ex situ.*

3.2. Molecular Analysis

The nucleotide sequence data generated from chloroplast and nuclear markers has been deposited in DDBJ with the accession numbers DDBJ: AB597999 - DDBJ: AB598008 for petB-petD intergenic spacer; DDBJ: AB598010 - DDBJ: AB598019 for trnL-trnF intergenic spacer; DDBJ: AB598021 - DDBJ: AB598030 for rps16 gene; and DDBJ: AB598032 - DDBJ: AB598041 for ETS region.

3.3. Chloroplast Markers

The Phylograms that are generated for all the 3 chloroplast makers, used in the present study are in congruent to each other by resulting in similar clustering of *Mangifera species.*

3.3.1. petB-petD

The phylogram of MP (**Figure 4**) method resulted into two clusters of which *M. odorata and M.andamanica* have been clustered into one group leaving the rest 3 species into another that have been further subgrouped into 2 subclusters. Here *M. camptosperma* has been grouped into one subcluster leaving, *M. griffithii* and *M. indica* into the other.

3.3.2. trnL-trnF

The phylogram of MP (**Figure 5**) method resulted into two clusters of which *M. andamanica* clustered into one leaving the rest 4 species into another that have been further subgrouped into 3 subclusters leaving *M. odorata* and *M. camptosperma* into two individual subclusters with *M. griffithii* and *M. indica* into the other.

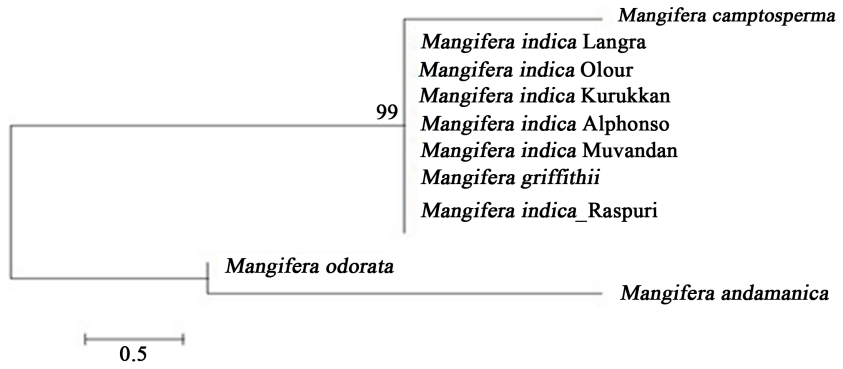


Figure 4. Phylogram of *Mangifera* species generated from petB-petD marker using Maximum Parsimony method, with a Bootstrap value of 2000 iterations, of Molecular Evolutionary Genetic Analysis (MEGA) 4.0 program.

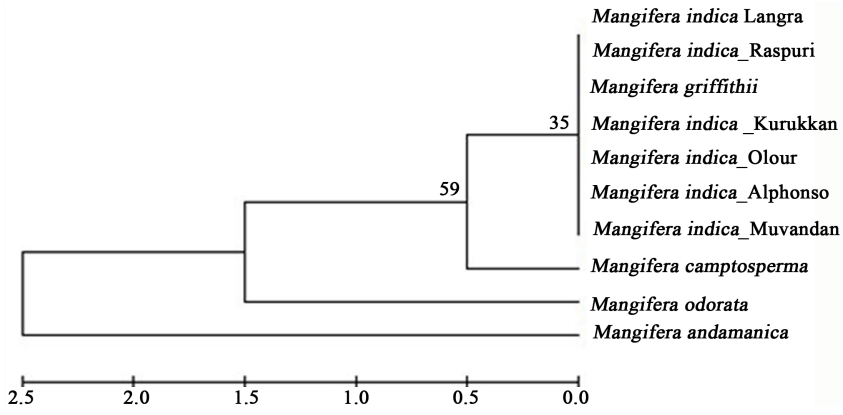


Figure 5. Phylogram of *Mangifera* species generated from trnL-trnF marker using Maximum Parsimony method, with a Bootstrap value of 2000 iterations, of Molecular Evolutionary Genetic Analysis (MEGA) 4.0 program.

3.3.3. rps16

The clustering pattern of rps16 is similar to that of trnL-trnF. The phylogram by MP (**Figure 6**) method resulted into two clusters of which *M. andamanica* clustered into separate group leaving the rest 4 species into another that have been further subgrouped into 3 subclusters leaving *M. odorata* and *M. camptosperma* into two individual subclusters with *M. griffithii* and *M. indica* into the other.

3.4. Nuclear Markers

3.4.1. ETS

The clustering pattern of ETS using MP (**Figure 7**) method resulted in the phylograms showing two main clusters containing *M. andamanica* in one cluster leaving the rest 4 species into another that have been further subgrouped into 2 subclusters leaving *M. odorata* into one subcluster and *M. camptosperma*, *M. griffithii* and *M. indica* into the other.

3.4.2. Phylogenetic Relationships among *Mangifera* Species

The phylogenetic relationships among *Mangifera spp* was earlier reported by [2] based on the morphology where he mentioned the existence of 41 valid species of *Mangifera* that had been classified into two different

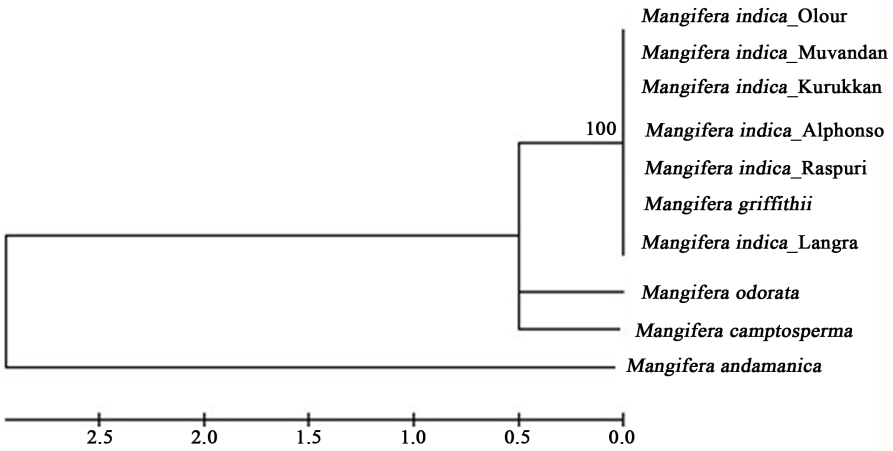


Figure 6. Phylogram of *Mangifera* species generated from rps16 marker using Maximum Parsimony method, with a Bootstrap value of 2000 iterations, of Molecular Evolutionary Genetic Analysis (MEGA) 4.0 program.

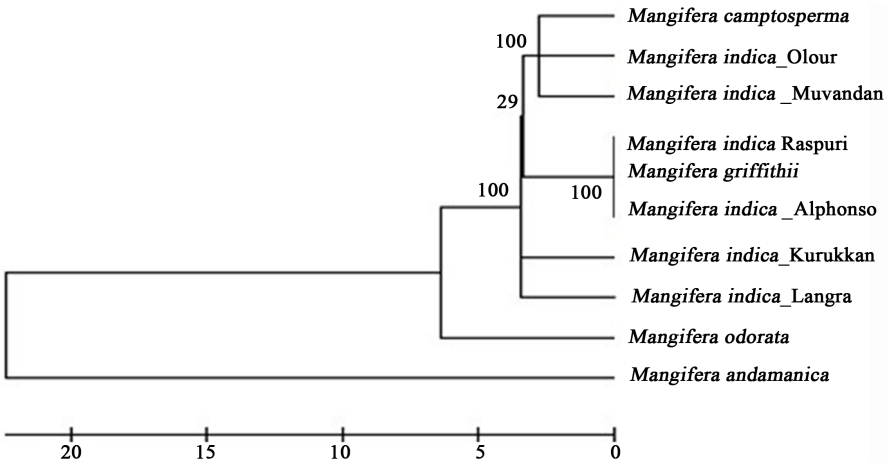


Figure 7. Phylogram of *Mangifera* species generated from ETS marker using Maximum Parsimony method, with a Bootstrap value of 2000 iterations, of Molecular Evolutionary Genetic Analysis (MEGA) 4.0 program.

sections viz section I and section II. Again in 1993 the most acceptable classification of Mangifera species has been described by [4], which includes 69 species of which 58 species have been classified into subgenera *Mangifera* and *Limus* with several sections *Marchandra*, *Euantherae*, *Rawa* and *Mangifera* under *Mangifera* subgenus and sections *Deciduae* and *Perennis* for the subgenus *Limus*. According to this classification *M.odorata* was classified as the section *Perennis* of the subgenus *Limus* leaving the other 3 species of the present study, which were classified into the two sections of the subgenus *Mangifera*; the section *Mangifera* for *M.indica* and the section *Rawa* for *M. griffithii* and *M. andamanica*. The remaining final *Mangifera species* taken in the study is *M.camptosperma* whose position was not mentioned in this classification.

The results of the present study are in congruence with Kosterman's classification by clustering *M. indica* and *M. griffithii*, belonging to the subgenus *Mangifera*, when compared to *M. odorata* which belongs to subgenus *Limus*. The clustering pattern of *M.camptosperma* with *M. indica* and *M. griffithii* shows that they share common ancestry and are evolutionary related hence the position of *M. camptosperma* has to be considered under the subgenus *Mangifera*. All the marker analysis in this study showed that *M. andamanica* grouped separately from the other *Mangifera species* of subgenus *Mangifera* showing that it doesn't belong to the genus *Mangifera* as earlier stated [29]. The fruits of *M. andamanica* are very inferior in quality without any pulp. The shape of the fruits does not resemble any of the *indica* varieties and fruits have no edible pulp, being juicy and fibrous. The present study results indicate that the taxonomic position of *M. andamanica* should be reconsidered.

4. Conclusion

Chloroplast markers (petB-petD; trnL-trnF; rps16) and nuclear marker (ETS) clearly show that *M. andamanica* is not closely related to *M. indica* and *M. griffithii* which belong to subgenus *Mangifera*. This re-confirms the earlier objections which were raised by Mukherjee [29] about *M. andamanica* taxonomical position in this subgenus. This study also classifies *M. camptosperma* under subgenus *Mangifera* based on our analysis, whose position was not assigned earlier. Thus, finally we conclude that classification of *M. andamanica* under genus *Mangifera* needs reconsideration and *M. camptosperma* has to be included in the subgenus *Mangifera*. The climatic requirement for these species is very specific. The surveys carried out have shown that the tree observed in Shoalbay region is also *M. griffithii*. Due to the propagation by seeds, there is difference in certain morphological features between the two specimens. The *in situ* evaluation, collection and *ex situ* conservation has to be taken up on priority; otherwise we may lose these species.

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