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Molecular Evidence in Identifying Parents of *Garcinia mangostana* L.

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ABSTRACT

The obligate apomicts *Garcinia mangostana* L. (Clusiaceae) was described from Malaysia and hypothesized to have originated from the natural hybridization between *G. malaccensis* and *G. hombroniana.* One of the parents, i.e. *G. malaccensis,* was believed to be endemic in the state of Melaka. However, this was determined only through a comparison of their chromosome number and morphological characteristics. There is still the possibility of other species within the same section of *G. mangotana* as the possible parents. Thus, investigations were carried out using molecular markers from three different regions of the internal spacer, chloroplast and micorsatellite. The objective of this study was to identify the possible parents of *G. mangostana* by comparing its relationship to other species within the same section, based on the genetic analysis of the internal spacer, chloroplast and microsatellite regions. Meanwhile, comparisons of allele sizes between *G. mangostana* with *G. malaccensis, G. opaca* and *G. hombroniana* using six polymorphic primers which had previously been developed were also performed. For phylogenetic analysis ITS, trnL and accD-psaL primers were used to determine the relationships between the four *Garcinia* species in the *Garcinia* section with two other sections and the genus *Clusia* as an outgroup. From the genetic analysis, it was found that *G. hombroniana* shares no common allele with the other species, while *G. opaca* has similar allele sizes with *G. mangostana* and *G. malaccensis*. The phylogenetic tree also showed that the closest relative to *G. mangostana* is *G. opaca* and *G. malaccensis*. This proves that *G. opaca* is more likely to be the other parent of *G. mangostana* rather than *G. hombroniana*.

Keywords: ***Chloroplast, phylogenetic, mangosteen, microsatellite***

INTRODUCTION

The origin of the apomictic and totally female *Garcinia mangostana* has been suggested to be from Malaysia. Based on the records by Whitmore (1973), one of the determined parents, i.e. *Garcinia malaccensis*, was only found in the state of Melaka. *G. mangostana* might have arisen sexually from the hybridization of this particular species with any other species that is from the same taxonomic section, i.e. Section *Garcinia*. According to Richards (1990b), *G. mangostana* might be a hybrid between *G. hombroniana* and *G. malaccensis.* The former species is sometimes cultivated, but *G. malaccensis* is only known as a wild plant. Richards (1990b) believed that only a single apomictic female arose after a hybridization event between the facultative apomicts, and that

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no sexual reproduction subsequently came after that. Consequently, no males have been reported in this species, apart from the single male which was last reported by Idris and Rukayah (1987) in Peninsular Malaysia. Although the tree and flower which were reported by Idris and Rukayah (1987) possess similar morphological characteristics to female *G. mangostana*, the flower has numerous stamens with pollen grains. Nonetheless, it was uncertain that the male tree was a hybrid of *G. hombroniana* with *G. malaccensis*. The possibility of *G. mangostana* back-crossing to either one of the parents should therefore be considered. However, further studies were not possible as the tree is no longer available. Individuals in the genus *Garcinia* are able to reproduce via apomixis, regardless of whether they are facultatively or obligately apomictic. Without the existing hybrids having been recorded, and having the means to reproduce through obligate apomixis, all *G. mangostana* are considered as the components of a clone containing no genetic variation within or between populations. This generates a negative impact towards *G. mangostana* as one of the economically important fruit as it lacks a number of varieties or cultivars as compared to other commercial fruit such as durian and rambutan. Another drawback that made *G. mangostana* the least planted fruit tree among the local growers is that they have a long juvenile period which usually takes ten years for a *G. mangostana* tree to mature and to bear fruit. By identifying the parents of *G. mangostana*, hybridization could be carried out once again to produce and screen for progeny with favourable traits, especially one with a shorter juvenile period. As suggested by Richards (1990b), sexual reproduction might have occurred between the diploid *G. hombroniana* and diploid *G. malaccensis* to produce the tetraploid *G. mangostana.* This was concluded from chromosome number, where *G. hombroniana* was n=24 (2n=48), as reported by Richards (1990a), and the chromosome count for *G. malaccensis* reported by Ha (1988) was 2n=42-43. The chromosome counts for *G. mangostana* have been speculative, as there is only one study, i.e. by Ha (1988), which has reported the chromosome count of *G. mangostana* as 2n=?90. This would be logical if the parents were *G. hombroniana* and *G. malaccensis.* Richards (1990b) also compared the morphological characteristics of the three species which gave strong support to the theory that *G. hombroniana* and *G. malaccensis* are the parents. However, the researcher did not compare the chromosome counts to other possible species within the same Garcinia section which could lead to the possibility of other species as being the possible parents. These include *G. penangiana* and *G. opaca* which have strong phenotypic resemblance to *G. hombroniana*. Phylogenetic studies, based on the Internal Transcribed Spacer (ITS) sequences of the nuclear ribosomal regions carried out by Nazre (2007), showed that *G. mangostana* is closely related to *G. hombroniana, G. malaccensis* and *G. opaca*, indicating that all three species have the possibility of being one of the parents. The methods for parentage analysis include exclusion, categorical and fractional likelihood, and genotyping reconstruction (Jones & Ardren, 2003). Although these methods may be an ideal and convincing way to determine the parents of *G. mangostana*, screening for the incompatibilities between the parents and offspring will consume a long period of time, especially for a long juvenile period species like *Garcinia* spp. Phylogenetic analysis which has always been the method of choice for determining the relationships among the species. Two DNA regions are commonly associated with phylogenetics, i.e. the internal transcribed spacer (ITS) and chloroplast regions. The nucleotide sequence variations found in each of the ITS sequences were often best suited for comparing species and closely related genera (Saar & Polans, 2000; Soltis & Soltis, 1998). The internal transcribed spacer region and the intergenic spacer of the nuclear rRNA repeat units evolve the fastest and may vary among the species within a genus or among the populations (White *et al*., 1990) and are more suitable for comparison of closely related taxa (Baldwin, 1995). The chloroplast (cp) genome has been shown to be maternally inherited in the majority

of angiosperms (Ennos *et al*., 1999; Palmer *et al*., 1988). According to Haruki *et al*. (1998), cpDNA markers have been successfully used for tracing the maternal parent in *Lilium* species. However, the cladistic analyses may not be able to completely resolve reticulate relationships and may not be useful for identifying hybrids or parental species (McCade, 1992). For most biological systems, the most powerful genetic tools for parentage analysis are the microsatellite markers (Jones & Ardren, 2003). The paternity of parentage analysis can be achieved by any type of genetic markers provided that it is sufficiently polymorphic, and for that reason, microsatellites are usually preferred (Gerber *et al*., 2000). Microsatellite markers proved to be a powerful tool to study the relationship of species, cultivars or varieties as they are inherited in a co-dominant Mendelian manner. The study was carried out to identify the possible parents of *G. mangostana* by comparing its relationship to other species within the same section based on the genetic analysis of the internal spacer, chloroplast and microsatellite regions.

MATERIALS AND METHODS

Four species from the section Garcinia, namely *G. mangostana, G. hombroniana, G. opaca* and *G. malaccensis*, were compared in the DNA fragment analysis and 11 other species were included for the phylogenetic analysis (Table 1). DNA was extracted from the leaf samples of 15 *Garcinia* species using Qiagen Plant Mini Kit and quantified by comparing it to two DNA ladders of 20µg and 40µg on 1.2% agarose gel. Microsatellite loci were isolated from *G.*

TABLE 1
A list of *Garcinia* species examined and their sampling locations

No.	Botanical name	Taxonomic Section	Location of the samples
1	*G. atroviridis*	Brindonia	FRIM
2	*G. bancana*	Brindonia	Pasoh Forest Reserve
3	*G. cowa*	Brindonia	Pasoh Forest Reserve
4	*G. dulcis*	Xanthochymus	Pasoh Forest Reserve
5	*G. grifithii*	Brindonia	Pasoh Forest Reserve
6	*G. hombroniana*	Garcinia	Rimba Ilmu, UM
			FRIM, Kepong
			Pangkor Island
7	*G. malaccensis*	Garcinia	Pasoh Forest Reserve
			Sg. Menyala Forest Reserve
8	*G. mangostana*	Garcinia	UPM-Puchong Mangosteen Orchard
			UPM- Farm 10
			Raub
			Miri
			Melaka
9	*G. nervosa*	Xanthochymus	Pasoh Forest Reserve
10	*G. nigrolineata*	Brindonia	Pasoh Forest Reserve
11	*G. opaca*	Garcinia	Taman Negara Forest Reserve
12	*G. opacaE*	Garcinia	Pasoh Forest Reserve
13	*G. opacaR*	Garcinia	Pasoh Forest Reserve
14	*G. parvifolia*	Brindonia	Pasoh Forest Reserve
15	*G. prainiana*	Xanthochymus	UPM Puchong Mangosteen Orchard
16	*G. pyrifera*	Xanthochymus	Pasoh Forest Reserve
17	*G. sp1*	Unknown	Pasoh Forest Reserve

mangostana and seven micosatellite primer pairs were developed following the hybridization and enrichment techniques (Edwards *et al*., 1996) to produce six polymorphic primer pairs which were later used for the fragment analysis (Table 2). The DNA fragments were amplified in 10µl reaction mix; 1X *Taq* Buffer (16mM $(NH_4)_2SO_4$, 67 mM Tris-HCL, 0.01% Tween-20), 2.0mM $MgCl_2$, 0.2mM dNTP each, 0.2µM of each of the microsatellite primer pairs, 0.33µM (F) dCTP, 0.5U *Taq* (Bioline) and 0.5µl template DNA. The primers were labelled with (F)dCTP dyes and the dyes used were R6G(dCTP) and R110(dCTP), visualised as 'green' and 'blue' peaks respectively on the ABI 310. The PCR was performed in a thermal cycler (PTC-100, MJ Research, Inc.) and it consisted of an initial denaturation of 12min at 95°C, followed by 20 cycles for 15s at 95°C, 15s at annealing temperature, and 15s at 72°C, 10 cycles for 15s at 89°C, 15s at annealing temperature, 15s at 72°C, with a final elongation of 30min at 72°C. Purification of the PCR products was carried out by mixing the PCR product with 1µl NaAc 3M (pH4.6) and 22µl 99% ethanol and precipitated at -20°C for 5min. Finally, 1µl of the fluorescently labelled reaction products were combined with 12µl deionised formamide and 0.5µl GeneScan ROX500 size standard. The mixture was heated three minutes at 95°C and transferred into ABI tubes, and fragment analysis was carried out on an ABI PRISM® 310 Genetic Analyzer. For the ITS and chloroplast DNA analysis, amplifications were carried out in 50µl reaction mixtures containing 1X PCR buffer, 2mM $MgCl_2$, 10µM dNTPs, 0.2pmol/µl of each primer (Table 2), 1U taq polymerase and 5µl template DNA. PCR cycles were programmed on a PTC-100 DNA Engine (MJ Research, Inc.) following the protocol for each primer. The protocol for amplification with ITS/trnL primers follows the protocol for ITS: 1 cycle

TABLE 2
A list of the primers and their sequences

Primer	Sequence (5' → 3')	Source
GM1	F: GAGCAATCCCAATGGCTAAA	*
	R: CCGAGCTAAATGAATTGTGGA	*
GM2	F: TATGGAGCCTTTCGAGCCTA	*
	R: CACCTCAGATTTAGGCCATCA	*
GM5	F: TGATGAGAAACATGCAGTTGA	*
	R: TGATTCTGCAGCAT GGAAC	*
GM8	F: GTTTTGTCCCGGTTAAGTT	*
	R: AAGGGTTTGCAATGAACAG	*
GM10	F: GGCAACTGCTCCAAGTTAG	*
	R: TTTATCGGCCAAGTTATCG	*
GM11	F: TTGTGCTCTCTTCGCTCTT	*
	R: ATGGCAGTTTATTGCTTGG	*
ITS4	TCCTCCGCTTATTGATATGC	White *et al*. (1990)
ITS5	GGAAGTAGAAGTCGTAACAAGG	White *et al*. (1990)
trnL-c	CGAAATCGGTAGACGCTACG	Taberlet *et al*. (1991)
trnL-d	GGGGATAGAGGGACTTGAAC	Taberlet *et al*. (1991)
accD-769F	GGAAGT TTGAGCTTTATGCAAATGG	Small *et al*. (1998)
psaL-75R	AGAAGCCATTGCAATTGCCGGAAA	Small *et al*. (1998)

* *Primers developed from G. mangostana DNA region following Edwards et al. (1996) hybridization and enrichment techniques*

of initial denaturing at 94ºC for 3min, 30 cycles of denaturing at 94ºC for 10s, annealing at 55ºC for 20s, elongation at 72ºC for 1min and 30s and final elongation of 72ºC for 4min. Amplification using the accD-psaL primer follows the same protocol with minor modification of 1 cycle of initial denaturing at 94ºC for 5min, 30 cycles of denaturing at 94ºC for 30s, annealing at 50ºC for 30s, elongation at 72ºC for 2min and final elongation of 72ºC for 4min. Successful PCR amplification which produced a single DNA band was checked on 1.4% agarose gel. The amplified DNA was purified using the QIAGEN PCR purification kit and the DNA concentration of the purified PCR product was estimated on the gel by comparing it to a ladder of known concentration. Sequence reactions were carried out in both directions for each purified double-stranded PCR product using the Applied Biosystems Big Dye Terminator Ver. 1.1, buffer and primers. The sequence analysis was done on the ABI Prism 310 and the sequences were viewed and edited on Chromas Lite 2.0. The sequences were aligned by using ClustalX (vers. 1.8) and edited using ProSequence (Filatov 2002). The phylogenetic relationship analyses were conducted with PAUP, Version 4.0b (Swofford, 1999). The most parsimonious tree was obtained using the heuristic search option involving 100 replications of random addition sequence and tree bisection-reconnection (TBR) branch swapping. All characters were specified as equally weighted. The analyses were based on nucleotide substitutions; therefore, all gaps in the sequence were treated as missing data. Strict consensus and bootstrap analyses were conducted to assess the reliability of the tree. For bootstrap, 1000 replications were calculated using the heuristic search option with TBR branch swapping. Four to six closely related species of *Clusia sp.* were chosen as the outgroups (Table 3).

RESULTS AND DISCUSSION

The parent of *G. mangostana* was determined by comparing the allele size for six microsatellite loci of three *Garcinia* species Garcinia species with support analysis using the ITS and chloroplast markers. *G. mangostana* DNA samples from five different locations were compared and the results showed that all five samples had the same allele size for each locus. This further confirmed that the *G. mangostana* plants are genetically similar and are possible clones. Since *G. mangostana* are obligate apomicts and without the existence of male tree for possible pollination and fertilization, all *G. mangostana* are believed to be carrying the same genetic constituents, and hence, having the exact allele size for each locus. Meanwhile, facultative apomicts, such as *G. hombroniana* and *G. opaca*, are able to propagate via apomixis and also through sexual reproduction. Thus, genetic variations among accessions and populations exist. In this study, three samples of *G. hombroniana* from three different locations showed differences in the allele sizes for three loci, i.e. GM8, GM10 and GM11 (Table 4). This was also observed in *G. malaccensis* as there were differences in the allele size from their samples taken from two different locations. This finding proved that there are some degree of genetic variations among the accessions of *G. hombroniana*, *G. malaccensis* and *G. opaca*. Five loci were heterozygous and one locus (GM2) was homozygous. Two of the primers were able to amplify four alleles per locus in tetraploid *G. mangostana*, namely GM8 and GM11. Table 4 shows that *G. mangostana* shares three similar sized alleles with *G. malaccensis* at locus GM1, GM2 and GM11, with the allele sizes of 235, 237bp, 210, 211bp, and 171,173 bp, respectively. When compared to *G. opaca*, *G. mangsotana* was found to share allele sizes at two loci, namely, GM5 (101bp) and GM11 (171, 173, 193,195bp). Sharing of similar allele sizes was also observed between *G. malaccensis* and *G. opaca* at loci GM10 (133,149bp) and GM11 (171,173bp). Meanwhile, sharing of alleles between *G. mangostana* and *G. malaccensis*, between *G. mangostana* and *G. opaca*, as well as between *G. malaccensis* with *G. opaca* directly linked the three species in a closely unique relationship. The similarity of allele sizes between *G. mangostana* and *G.*

TABLE 3
A list of the *Clusia* species used as the outgroup for phylogenetics according to DNA region extracted from Genbank

Region	Clusia Species	Source	GenBank Accession number
ITS	*C. rosea*	Gehrig *et al*. (2003)	AJ509230
	C. minor	Gehrig *et al*. (2003)	AJ509208
	C. major	Gustafsson *et al.* (2002)	AY145222
	C. ducu	Gustafsson *et al.* (2002)	AY145220
	C. lanceolata	Gustafsson *et al.* (2002)	AY145195
	C. multiflora	Vaasen *et al*. (2002)	AJ414719
trnL-trnF intergenic spacer region	*C. rosea*	Hale *et al*. (2004)	AY144094
	C. multiflora	Hale *et al*. (2004)	AY144091
	C. lanceolata	Hale *et al*. (2004)	AY144085
	C. minorA	Hale *et al*. (2004)	AY144087
	C. ducuA	Hale *et al*. (2004)	AY144076
accD and psaL genes	*C. rosea*	Hale *et al*. (2004)	AY144017
	C. multiflora	Hale *et al*. (2004)	AY144013
	C. lanceolata	Hale *et al*. (2004)	AY144007
	C. major	Hale *et al*. (2004)	AY144008

malaccensis supports the idea that *G. malaccensis* is one of the parents of *G. mangostana*, as suspected by Richards (1990b). Interestingly, *G. hombroniana* shared no allele size with any of the three species. Instead, all the possible loci strongly suggested that *G. hombroniana* was not involved in contributing allele to *G. mangostana*. Although none of the allele sizes of *G. malaccensis* from the Pasoh population was similar to *G. mangostana*, this does not exclude *G. malaccensis* as one of the possible parents. The fact that *G. malaccensis* does not have the same reproductive system as *G. mangostana* and is facultatively apomicts, the occurrence of sexual reproduction within the population causes them to become more genetically diverse than *G. mangostana,* and thus, some individuals may not share the same alleles as *G. mangostana*. It seems that obtaining the right sample which matches the progeny is crucial. Therefore, it is essential to have a large sample size and to obtain the sex ratio of a population before sampling to recognize the pattern of apomixis or sexual reproduction in that population. To further support the idea that *G. malaccensis* and *G. opaca* are the possible parents for *G. mangostana*, phylogenetics analysis was carried out on both nuclear and chloroplast regions. Both the regions compare the mutation rate of nucleotides for *G. mangostana* in relation to *G. hombroniana*, *G. malaccensis* and *G. opaca*. Incorporating other species from various sectionsis generally required in phylogenetics to ensure accuracy of the phylogentic tree as well as to observe the relationships among the species from different sections. In this case, the genus *Clusia* was used as it was linked with close resemblance to *Garcinia*. The ITS sequences of *Garcinia* ranged from 640bp to 683bp, with an aligned length of 860bp. The phylogenetic analysis resulted in 1578 most-parsimonious trees of tree length (L) = 609, consistency index (CI) = 0.6470 ($CI_{uninformative}$ = 0.5928), retention index (RI) = 0.8184 and rescaled consistency index (RC) = 0.5295. Of 666 characters, a total of 225 (33.8%) characters were phylogenetically informative,

TABLE 4
Fragment analysis using six polymorphic primer pairs showing the allele sizes (bp) of four *Garcinia* species

Species	Population	Allele size at 5 loci					
		GM1	GM2	GM5	GM8	GM10	GM11
G. mangostana	UPM-Farm 10	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
	UPM-Puchong	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
	Raub	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
	Miri	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
	Melaka	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
G. hombroniana	FRIM	n/a	217,219	n/a	209,209	313	183,183
	UM	n/a	217,219	n/a	207,209	314	181,183
	Pangkor	n/a	217,219	208	207,209	313	183,183
G. malaccensis	Pasoh	n/a	204	107,111	221	133,149	171,173
	Sg. Menyala	235,237	210,211	n/a	231	162	171,173, 193, 195
G. opaca	Rompin	n/a	n/a	149	n/a	133,149	n/a
	Pasoh	213	212,214	101	221	133,149	171,173, 193, 195

n/a – no amplification

71 variable characters are parsimony-uninformative and 370 (55.5%) characters were constant. The phylogenetic tree shown in *Fig. 1* indicates that all the *Garcinia* species were clustered accordingly to their respective section and the genus *Clusia* was the outgroup. The ITS data support three clades within *Garcinia*, with *G. bancana* not included in any one of them. Clade 1 (C1) contains the species from the section *Xanthochymus*, in clade 2 (C2) are the species from the section *Garcinia*, and clade 3 (C3) contains the species from section *Brindonia*. During leaf sampling in the Pasoh Forest Reserve, one tree was recorded as *G. eugeniaefolia* and eight trees were as *G. rostrata*. Both these species are from the section *Discostigma* (Jones, 1980). From the observations of leaf and flower morphologies, it was suggested that "*G. eugeniaefolia*" and "*G. rostrata*" are both of the same species with *G. opaca* (Nazre, pers.com). The BLAST inquiry from Genbank on the sequences of our collection, labelled as "*G. eugeniaefolia*" and "*G. rostrata*", was performed and shown as similar to *G. opaca* var *minor* with the E value of zero and the scores of 1106. The phylogram based on ITS sequences showed that "*G. eugeniaefolia*" designated as *G. opaca*E and "*G. rostrata*" designated as *G. opaca*R were in the same clade as *G. opaca* which is in section *Garcinia*. The results from the BLAST inquiry for *G. sp*1 showed a high similarity to *G. bancana*. Based on the phylogram illustrated in *Fig. 1*, *G. sp*1 is in the same clade as the species from section *Brindonia*, suggesting that G. *sp*1 is most probably *G. bancana.* Section *Garcinia*, which includes *G. mangostana*, *G. malaccensis*, *G. opaca* and *G. hombroniana*, showed that the number of changes in the branch length between *G. mangostana* with *G. malaccensis, G. opaca, G. opacaE, G. opacaR* and *G. hombroniana* was 19,17,9,5 and 21, respectively. This suggests that the closest species to *G. mangostana* are *G. opaca* and *G. malaccensis*. This also suggests that *G. opaca* and *G. malaccensis* are more likely to be the parents of *G. mangostana* than *G. hombroniana*, as suggested by Richards (1990b). The aligned trnL sequences were 707bp in length with the sequences varying from 640bp to 683bp. The phylogenetic analysis resulted in 1083 most-parsimonious trees of tree length (L) = 547, consistency index (CI) = 0.6216 ($CI_{uninformative}$ = 0.5651), retention index (RI) = 0.7214 and rescaled consistency index (RC) = 0.4484. One of the parsimonious trees is shown in *Fig. 2*. Of 671 characters, a total of 204 (30.4%) characters are phylogenetically informative, 62 (9.2%) variable characters are parsimony-uninformative and 405 (60.3%) characters are constant. Three major clades, which were recognized (C1-C4) with *G. atroviridis* and *G. bancana*, were not included in their expected group (*Fig. 2*). Clade 1 was section *Brindonia*, clade 2 section *Xanthochymus* and clade 3 section *Garcinia*. All the species in each clade otherwise agrees with Jones' (1980) classifications. The chloroplast data, like the previous ITS analyses, support that *G. opaca* and *G. malaccensis* is the closest sister to *G. mangostana.* The trnL chloroplast region of *Garcinia* contains mononucleotide repeats of the A nucleotide. The length of repeats ranged from 10 - 16 repeats at 113 – 132bp long. However, primers trnL-C and trnL-D were unable to amplify the DNA sample of *G. opaca*. It is important to note that the sequences used for this analysis might have been unreliable. This is because of the long A nucleotide repeats and the alignments of the sequences containing large gaps, notably from 387-480bp. Therefore, the phlygenetics analysis relies heavily on the other chloroplast region, i.e. the accD-psaL region. The length of accD-psaL DNA sequence ranged from 698bp to 753bp, with an aligned length of 798bp. The heuristic search yielded 401 trees length (L) =240, consistency index (CI) = 0.6125, homoplasy index (HI) = 0.3875, ($CI_{uniformative}$ = 0.4716), rescaled consistency index (RC) = 0.3882 and retention index (RI) = 0.6339. The phylogram can be divided into three clades (C1, C2 and C3) as shown in *Fig. 3*, and from the bootstrap analysis (*Fig. 4*), the monophyly of the ingroup was 100% supported. Of 681 characters, a total of 70 (10.3%) characters are phylogenetically informative, 61 (8.9%) variable characters are parsimony-unifnformative and 550 characters are constant. Clade 1 (76%

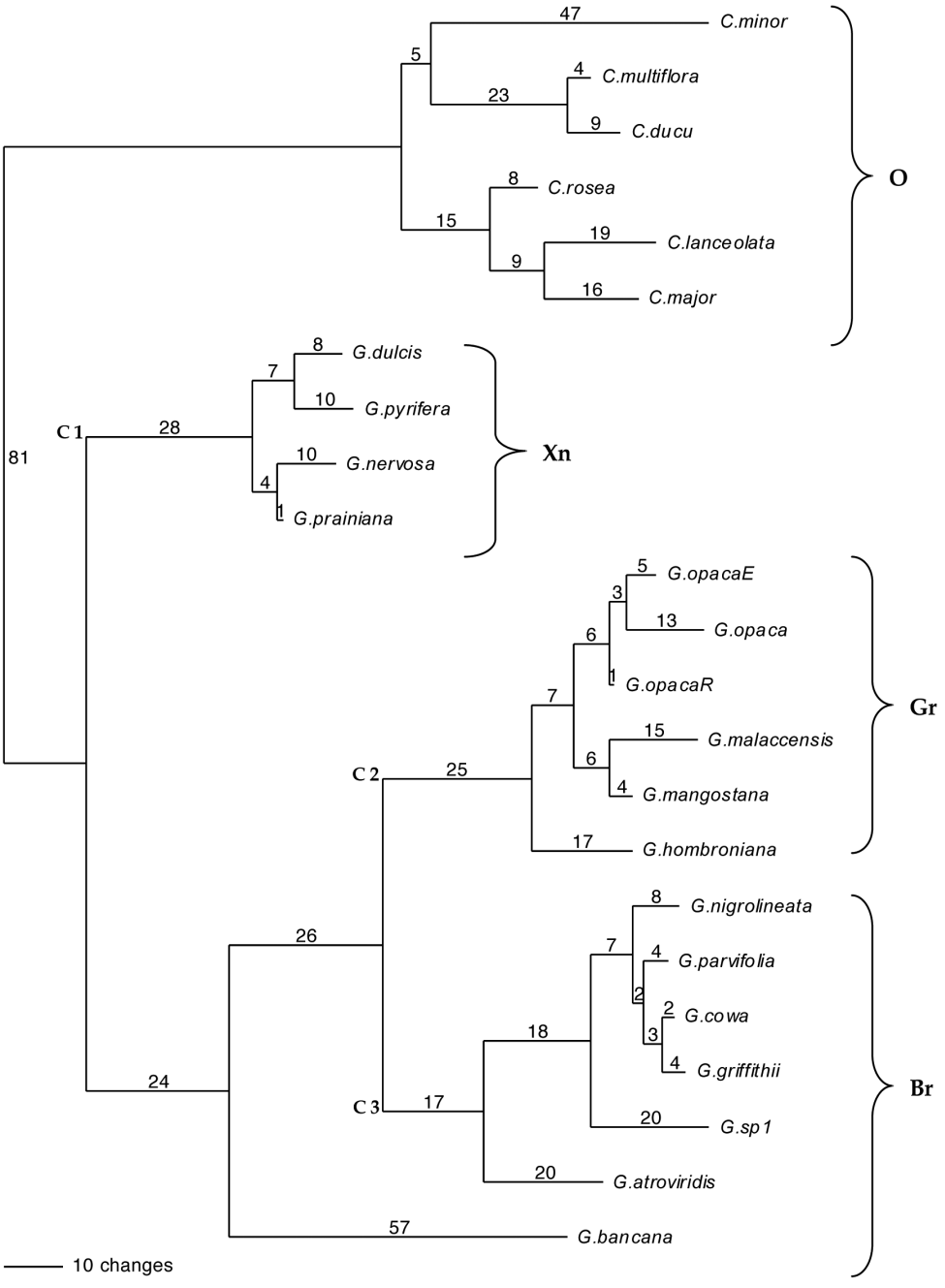

Fig. 1: One of most parsimonious trees based on the ITS sequence data from 15 species of Garcinia*. Figures are tree length based on the numbers of DNA substitution changes. Key: O-*Clusia *outgroup Xn-Section* Xanthochymus *Gr-Section* Garcinia *Br-Section* Brindonia *Ds-Section* Discostigma

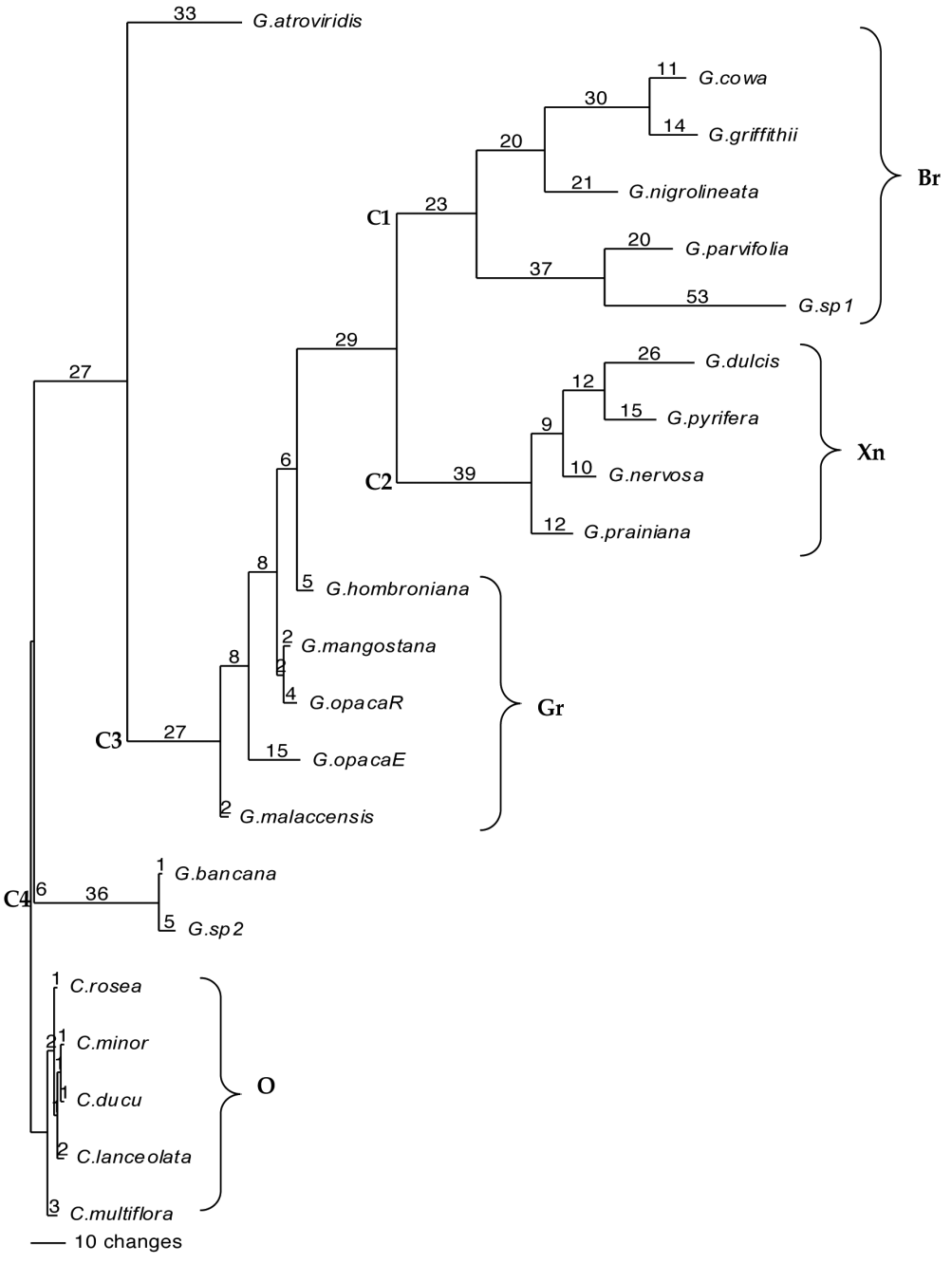

Fig.2: One of the most parsimonious trees based on the trnL sequence data from 17 species of Garcinia*. Figures are tree length based on numbers of DNA substitution changes. Key: O-*Clusia *outgroup Xn-Section* Xanthochymus *Gr-Section* Garcinia *Br-Section* Brindonia

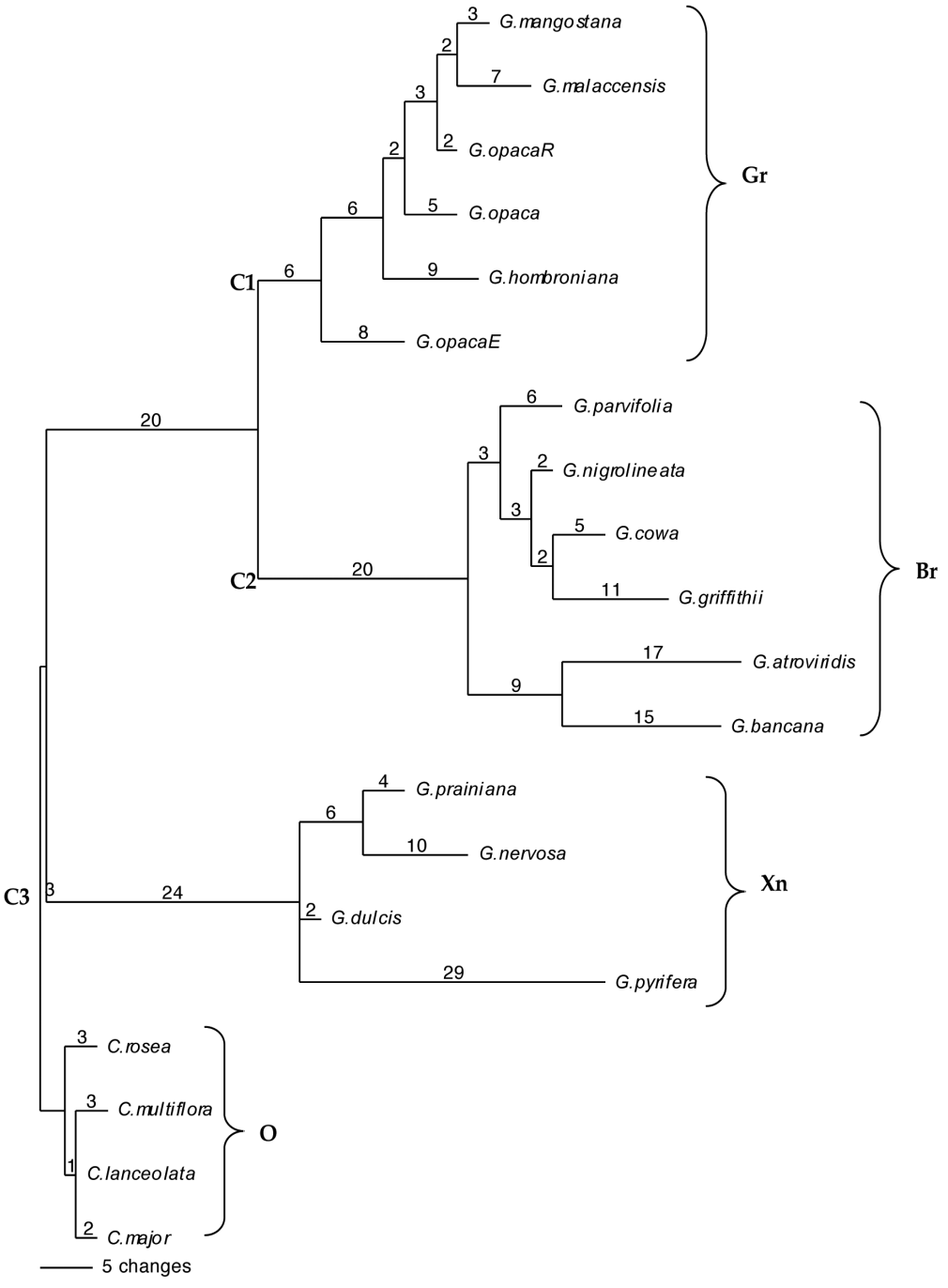

Fig. 3: One of the most parsimonious trees based on the accD-psaL sequence data from 14 species of Garcinia*. Figures are tree length based on the numbers of DNA substitution changes. Key: O-*Clusia *outgroup Xn-Section* Xanthochymus *Gr-Section* Garcinia *Br-Section* Brindonia

bootstrap supported) consists of the species from *Garcinia* section, clade 2 (85% BS supported) is from *Brindonia* section and clade 3 (52% bootstrap supported) is *Xanthochymus* section (*Fig. 3*). In the ITS phylogenetic analysis, *G. bancana* was slightly out of the *Brindonia* section, with 57 nucleotide changes from the main clade and in the trnL region; it is in the same clade with the rest of the members of the Brindonia section but the change of nucleotides was 53, showing that it has distant genetic relationships. In the accD-psaL region, however,

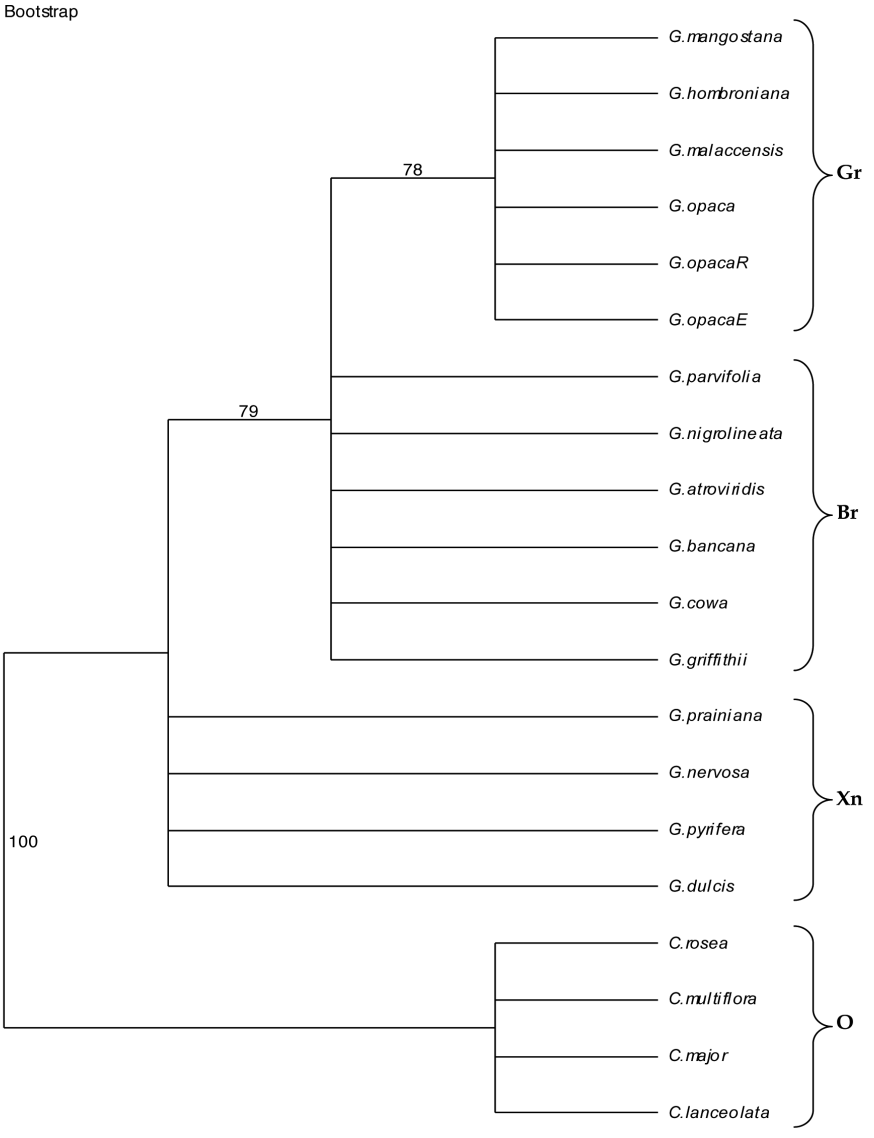

*Fig.4: Bootstrap support (50%) for various nodes (1000 replicates) with the values of more than 78%, based on accD-psaL region. Key: O-*Clusia *outgroup Xn-Section* Xanthochymus *Gr-Section* Garcinia *Br-Section* Brindonia

the changes of nucleotides are only 15 compared to *G. atroviridis*, and this agrees with Jones' (1980) *Garcinia* classification. The number of changes in the branch length between *G. mangostana* with *G. malaccensis, G. opaca, G. opacaE, G. opacaR* and *G. hombroniana* was 10, 8, 11, 5, and 12, respectively. The accD-psaL chloroplast region of *Garcinia* contains mononucleotide repeats of the A nucleotide but the length of the repeats was shorter than the ones in the trnL chloroplast region. The mononucleotides were at 460-470bp with 9–12 repeats. Meanwhile, the primers were able to amplify *G. opaca* DNA but not *Garcinia* sp.1. The number of nucleotide changes was more in the ITS region compared to the trnL, accD and psaL regions. This is typical as the rate of mutation in the internal transcribe spacer regions is much faster than that in the conserved chloroplast regions. Nevertheless, all the three regions showed similar results, whereby *G. mangostana* was found to be more closely related to *G. malaccensis* and *G. opaca* rather than to *G. hombroniana*.

CONCLUSIONS

The three molecular markers proved that *G. hombroniana*, which has previously been suggested as being one of the parents for *G. mangostana*, is unlikely to be so. The samples from three different geographical locations of *G. hombroniana* showed no similar sized microsatellite alleles to *G. mangostana* and *G. malaccensis*. On the other hand, *G. opaca* which was shown by three phylogenetic tree generated from the sequence of the ITS, trnL and accD-psaL regions showed close relationships to *G. mangostana* and *G. malaccensis*. *G. opaca* also shared similar sized alleles to the two species. In all the phylogenetic analyses, *G. hombroniana* has a distant relationship with *G. mangostana* compared *to G. malaccensis* and *G. opaca*. This strongly suggests that one of the parents for *G. mangostana* is *G. malaccensis* and another possible parent is *G. opaca*, but not *G. hombroniana*.

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