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GENETIC RELATIONSHIPS OF SEVERAL GARCINIA SPECIES (CLUSIACEAE)REVEALED BY ITS SEQUENCE DATA

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ABSTRACT

The genus *Garcinia* is known for their traditional uses such as edible fruit and ethno medicine. Genetic relationships of sixteen *Garciniaspecies* were determined using the sequence of the internal transcribed spacer region (ITS). Both Maximum likelihood and neighbor-joining method were used to analyze the sequences. The species distributed in China were clearly separated from the exotic species. *Garciniamangotana* and G. multiflora collected from different areas can be also clearly separated. It indicated that these two species have high genetic diversity in China. The topological structure of the phylogenetic tree is congruent with morphological classification. This study provided significant results to determine the phylogeny and relationship of *Garcinia* species for further development and cultivation.

KEYWORDS: *Garcinia*, ITS sequence, Maximum likelihood analysis

Introduction

*Garcinia*Linnaeus (1754: 526)is a tropical genus in Clusiaceae (or Guttiferae) with 450 species, distributed mostly in the south-east Asia, southern Africa and Polynesia. The species are evergreen trees and shrubs. In China there are 21 *Garcinia* species, and 13 of them are endemic.

The fruits of most *Garcinia* species are edible. Mangosteen (*G.mangotana* Linnaeus 1762: 635),for example, is a well-known tasty tropical fruit with attractive fruit shape and color.It is famed as the 'queen of tropical fruit' (Yapwattanaphun*et al*., 2004). The genus *Garcinia*can be used as medicine. It contains natural benzophenones which exhibit a range of biological activities including antifungal, anti-HIV, antimicrobial, antiviral and cytotoxic (Wu *et al*., 2014). Pharmacological investigations have shown some *Garcinia* species contain properties that have potential as treatment for HIV (Rukachaisirikul*et al*., 2003)or cancer (Nabandith*et al*., 2004). Burkill (1966) has recorded that*Garcinia* was used as post-childbirth medication, for menstrual problems, dysentery and fever. In China, *Garcinia*species are also used widely. Ethnic minorities in southern China such as Dai and Li use *G.xanthochymus*Hook f. ex T. Anders (1874: 269), *G.oblongifolia*Champ ex Benth (1851: 331)and *G.xipshuanbannaensis*Y. H. Li (1981: 497) as fruits and food seasoning for a long time. *G.paucinervis*Chun et How(1956: 12) is a precious timber species(Wang *et al*., 1994), to make furniture and handicrafts. As a traditional Chinese medicine, gamboge is most often extracted by tapping resin from various species of *Garcinia*. It can be used to treat swelling, bleeding, burn and some other diseases (Wang *et al*., 2003). In addition, gamboge is also used as dye for thousands of years in China. The oil extracted from seeds of *G. multiflora*Champ ex Benth (1851: 310)can be used to make soaps and lubricants. *Garcinia* species are also good ornamental plants, such as *G. xanthochymus*and *G. multiflora* (He & Wen, 2005, Zhang *et al*., 2015).

However, information on genetic diversity and genetic relationships of *Garcinia*in China is very limited.Yapwatannaphun*et al*. (2004) used ITS sequences to investigate the relationship between*G. mangostana*and its wild relatives.It had mainly found that *G. mangostana*is closely related to *G. malaccensis*. Nazer*et al*. (2007) studied phylogenetic relationships between cultivated *Garcinia* species distributed in Malaysia and some wild relatives.

Sequence analysis of internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) between the small subunit (18S) and the large subunit (28S) of nrDNA has been used as a source for analysis of genetic relationships within genera and among closely related genera in many angiosperms (Baldwin *et al*., 1995). It has been used frequently to resolve genetic relationships among many plant taxa(Yapwatannaphun*et al*., 2004) because it was proven to contain enough genetic information (Baldwin *et al*., 1995). In this study, we utilizedthe ITS region to analyze the relationship among *Garcinia* species andexpect to provide fundamental information ongenetic relationships of several *Garcinia* speciesfor further study.

Materials and Methods

Plant Material

Sixty-six samples belonged to sixteen species of *Garcinia* were collected from southern China. Fourteen species are distributed in Asia, one is distributed in America and one is distributed in Africa.Six of them (*G. bracteata*, *G.lancilimba*, *G.xipshuanbannaensis*, *G.paucinervis*, *G.yunnanensis*and *G.nujiangensis*)are endemic to China (Table 1). Additionally, *Mammeabrevipes* (Craib) Kosterm. from the family Clusiaceaewas selected as outgroup taxa, because it was used as an outgroup in a previous study (Nazer*et al*., 2007).

DNA Isolation

The CTAB method (Doyle and Doyle,1990)wasused for DNA isolation with a slight modifications as described by Kamiya*et al*. (1998).

PCR Amplification and DNA sequencing

The entire ITS sequence was amplified using the primers of "ITS-4" and "ITS-5" (5 pmol each) (White *et al*., 1990) in 25µl reaction mixtures. The amplified products were then sent to Boshang Biotechnology (Shanghai) Co., Ltd.for purification and sequencing.

Sequence Alignment

Sequence data of the ITS1, 5.8S, and ITS2 regions were first aligned using MEGA v6.0 (Tamura*et al*.,2013), and then the alignment was checked and slight modifications made manually. Compared the aligned sequence with other sequences in the GenBank by BLAST (Basic Local Alignment Search Tool)programme (Altschul*et al*., 1997)to determine the boundaries of ITS1 and ITS2, and confirm the correct sequences obtained. G+C contents of ITS1, 5.8S, and ITS2 regions were calculated by Editseq v7.1 (Liu *et al*.,2012). The 5.8S coding sequence was also considered for phylogenetic analysis, although only a few variations were found among the species examined.

Genetic Analysis

Maximum likelihood analyses were performed using MEGA v6.0. The program Model test v6.0 (Tamura*et al*.,2013) was utilized to find the best fit substitution model. The best sequence evolution model was Kimura 2-parameter+Gamma distribution. Pair wise distances between in groups and out group sequences were also calculated in MEGA v6.0. Because each species has more than one sequence, so first we should examine the intraspecific genetic distance. If the intraspecific genetic distance is very low, we can ignore the effects of interspecific variation.

In addition, bootstrap analysis (Felsenstein, 1985)with 1000 replications was conducted to assess the degree of support for each branch

with consensus tree option of retaining group with frequency >50%. In the analysis, one out group taxa was used to root the tree.

The genetic reconstruction was also performed by neighbor-joining (NJ) method (Saitou &Nei, 1987) using the same distance matrices calculated above.

RESULTS

Characterisation of ITS Sequences

The entire ITS region in *Garcinia* ranged from 615 to 623 base pairs (bp) but the length was longer for the outgroup taxa with 630 bp (Table 2). For all taxa studied, the aligned ITS1 region (253-259 bp in the ingroup and 250 bp in the outgroup) were longer than ITS2 (199-206 bp in the ingroup and 217 bp in the outgroup). There is no variation in length of 5.8S gene region, which had 163 bp (Table 2). As a result, the entire length of ITS region in *M. brevipes*was longer than those in *Garcinia* species.

Amongst the *Garcinia* species and outgroup, the ITS region was observed as a GC-rich region. In the *Garcinia* species, the values of G+C contents were varied, 47.84%-53.91% in ITS1, 50.00%-53.00% in ITS2 and 50.30-53.99% in 5.8S. However, the G+C contents of outgroup taxa was slightly higher in ITS1 (54.88%) and ITS2 (53.95%).

The alignment of all sequences resulted in a matrix of 630 characters including the 5.8S gene region. There were 129 and 109 polymorphisms in ITS1 and ITS2, respectively, while there were only 13 polymorphic sites in 5.8S gene region (Table 2). However, when the sequence of outgroup taxa was added to the alignment, the polymorphic sites were 161, 17, and 123 in ITS1, 5.8S, and ITS2, respectively. As a result, the polymorphic sites became 301for the entire sequence in all species including outgroup taxa, and 169 sites among those were assumed to be informative sites (Table 2).

Interspecific and intraspecific genetic distance

The intraspecific genetic distance ranged from 0-0.28% (Table 3). It's very low, and we can speculate that the intraspecific variation is very small, it can be ignored compared to the interspecific genetic distance. So, we can choose one ITS sequence per species to represent this species.

The sequence divergence between taxa ranged from 1.01% to 18.27% (Table 4). Among *Garcinia* species, the smallest divergence was found between *G. oblongifolia* and *G. cowa* Roxb. (1824: 622)(1.01%), while the largest was between *G. intermedia*(Pittier) Hammeland *G. paucinervis* (18.27%).

Genetic Analyses

Maximum likelihood analysis based on entire sequence data of ITS region, all the *Garcinia* species were separated into two major clades, Ⅰ and Ⅱ (Fig. 1).*G. xanthochymus* formed a group together with *G. dulcis* and *G. subelliptica*Merr. (1980: 261), and this group formed clade Ⅱ with *G. kola*and *G. intermedia*, the other eleven species formed clade Ⅰ (Fig. 1). The analysis revealed a very close relationship between *G. oblongifolia* and *G. cowa*, and these two species formed a small group together with *G. xipshuanbannaensis*.

The result of neighbor-joining analysis was similar to that of ML analysis, showed a close relationship between *G. oblongifolia* and *G. cowa*, and a little genetic distance with *G. xipshuanbannaensis* (Fig. 2). A big difference here was that *G. lancilimba*C. Y. Wu ex Y. H. Li (1981: 493) formed a major clade by itself. Furthermore, in the NJ tree, *G. mangostana*, *G. bracteata G. hanburyi*, *G. xipshuanbannaensis*, *G. cowa*, and *G. oblongifolia* were separated clearly from the other 9 *Garcinia* species as in the result of ML analysis, showing a different evolutionary process from the other 9 species.

Both ML analysis and NJ analysis were shown good results, the relationships among the species were well resolved, and all of them were found to be well separated into sister clades.

DISCUSSION

A total of 16 *Garcinia* species were successfully sequenced for ITS region. The result is in accordance with previous studies (Sari & Hanan, 2000) showing that ITS region could provide enough variation to infer phylogenetic information for *Garcinia*. The length of ITS sequence in this study is similar to the findings of Nazer's (2007)(617-624bp) and Yapwatannaphun's(2004) (616-621bp). Compared with other angiosperm, the length of ITS region for *Garcinia* found in this study was little shorter than those reported in Orobanchaceae (McNeal *et al*., 2013) and Brassicaceae (Crespo*et al*., 2000). As expected, both ITS1 and ITS2 regions show more variability than the 5.8S coding region, which is known to be a conserved region.

The interspecific genetic distance between *G. intermedia*and other *Garcinia*species are highly, most of them are more than 17.00%.This result can be attributedto their geographic distribution. *Garciniaintermedia*is distributed in tropical America, and far from other *Garcinia* species distribution areas (Asia and Africa), showing these two groups may have different evolutionary processes. In both ML and NJ analyses,the*G. mangostana* collected from Yunnanhad a little distance with those collected from Hainan. It is probably because of their collecting location differences. The same result appeared in *G. multiflora*.This indicated ITS sequence has good identification ability for our samples, and showed *G. mangostana*and *G. multiflora* have high genetic diversity in China. But *G. oblongifolia*collected from different areas don't exhibit variability, the genetic diversity of *G. oblongifolia* may low. Six endemic species (*G. bracteata*, *G.lancilimba*, *G.xipshuanbannaensis*, *G.paucinervis*, *G.yunnanensis*and *G.nujiangensis*)were clustered in clade Ⅰ, while all exotic species in clade Ⅱ. They were separated into two cladesclearly, it's consistent with their geographical distribution. The species in clade Ⅰ mainly distributed in southern China, India, Bengal, Vietnam and Thailand, these regions are in the north of the distribution areas of the species in clade Ⅱ (Indonesia, Papua New Guinea, Southern Africa, Philippines and Sri Lanka). So, the climate and temperature of these regions are different, it may trigger this different evolutionary process (Wang *et al*., 2012).

Interestingly,the result of this study on the phylogenetic relationship among *Garcinia*species based on ITS sequence datacorroborates with the classical classification system based on morphological data (Fig. 3) (Li, 1990). For example, in classical classification system, the *Garcinia* species distributed in China were separated into two major clades (Clade A and Clade B+C), the positions of *G. xanthochymus*and *G. subelliptica*(Clade A) were reasonably distant compared to other taxa. This is determined by their number of petals and sepals.*G. xanthochymus*and *G. subelliptica*have 5 petals and sepals while other species have 4 petals and sepals. But the inflorescence, fruit shape and sepalsize between these two species are different, so they are divided into two species. Similar topology structure was obtained in our study, according to the ML and NJ analyses, *G. xanthochymus*and *G. subelliptica*were also separated from other species existed in China (Fig. 1), but genetically, these two species are also distant. The sequence divergence between *G. xanthochymus*and *G. subelliptica*are 3.14%.

In addition, in morphological classification, *G. cowa* and *G. oblongifolia* formed a small cluster and then formed clade C with G. *xipshuanbannaensis*. In terms of morphological characters, *G. cowa* and *G. oblongifolia* were difficult to distinguish. But their shapes of fruit and number of locules are different. Molecular analysis also supported the closeness of these two species with the smallest 1.01% differences from their sequence divergence (Table 3). These results showed that the classical classification system is reliable in a certain degree.

Overall, ITS sequence analysis of *Garcinia* species gave us new information on the genetic relationships among the species. This information will give us basic and useful ideas for further development and cultivation of it in the near future.

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Table1. List of *Garcinia* species and out-group taxa and their sampling locations.

	Sample ID	Species	Locality Collected		Sample ID	Species	Locality Collected
1	ILCLbrY01	Garciniabracteata1	XTBG, Yunnan	31	ILCLpaY01	Garciniapaucinervis1	XTBG, Yunnan
2	ILCLbrY02	Garciniabracteata2	XTBG, Yunnan	32	ILCLpaY02	Garciniapaucinervis2	XTBG, Yunnan
3	ILCLbrY03	Garciniabracteata3	XTBG, Yunnan	33	ILCLpaY03	Garciniapaucinervis3	XTBG, Yunnan
4	ILCLcoY01	Garciniacowa1	XTBG, Yunnan	34	ILCLpaY04	Garciniapaucinervis4	XTBG, Yunnan
5	ILCLcoY02	Garciniacowa2	XTBG, Yunnan	35	ILCLsuY01	Garciniasubelliptica 1	XTBG, Yunnan
6	ILCLcoY03	Garciniacowa3	XTBG, Yunnan	36	ILCLsuY02	Garciniasubelliptica 2	XTBG, Yunnan
7	ILCLhaY01	Garciniahanburyi 1	XTBG, Yunnan	37	ILCLsuY03	Garciniasubelliptica 3	XTBG, Yunnan
8	ILCLhaY02	Garciniahanburyi 2	XTBG, Yunnan	38	ILCLsuY04	Garciniasubelliptica 4	XTBG, Yunnan
9	ILCLhaY03	Garciniahanburyi 3	XTBG, Yunnan	39	ILCLsuY05	Garciniasubelliptica 5	XTBG, Yunnan
10	ILCLinY01	Garciniaintermedia1	XTBG, Yunnan	40	ILCLxaY01	Garciniaxanthochymus1	XTBG, Yunnan
11	ILCLinY02	Garciniaintermedia2	XTBG, Yunnan	41	ILCLxaY02	Garciniaxanthochymus2	XTBG, Yunnan
12	ILCLinY03	Garciniaintermedia3	XTBG, Yunnan	42	ILCLxaY03	Garciniaxanthochymus3	XTBG, Yunnan
13	ILCLlaY01	Garcinialancilimba 1	XTBG, Yunnan	43	ILCLxaY04	Garciniaxanthochymus4	XTBG, Yunnan
14	ILCLlaY02	Garcinialancilimba 2	XTBG, Yunnan	44	ILCLxaY05	Garciniaxanthochymus5	XTBG, Yunnan
15	ILCLlaY03	Garcinialancilimba 3	XTBG, Yunnan	45	ILCLxaY06	Garciniaxanthochymus6	XTBG, Yunnan
16	ILCLmaY01	Garciniamangostana1	XTBG, Yunnan	46	ILCLyuY01	Garciniayunnanensis 1	XTBG, Yunnan
17	ILCLmaH01	Garciniamangostana2	Wuzhimontain, Hainan	47	ILCLyuY02	Garciniayunnanensis 2	XTBG, Yunnan
18	ILCLmaH02	Garciniamangostana3	Wuzhimontain, Hainan	48	ILCLyuY03	Garciniayunnanensis 3	XTBG, Yunnan
19	ILCLmaH03	Garciniamangostana4	Wuzhimontain, Hainan	49	ILCLnuY01	Garcinia nujiangensis 1	XTBG, Yunnan
20	ILCLmaH04	Garciniamangostana5	Wuzhimontain, Hainan	50	ILCLnuY02	Garcinia nujiangensis 2	XTBG, Yunnan
21	ILCLmaH05	Garciniamangostana6	Wuzhimontain, Hainan	51	ILCLnuY03	Garcinia nujiangensis 3	XTBG, Yunnan
22	ILCLmuY01	Garciniamultiflora1	XTBG, Yunnan	52	ILCLduY01	Garciniadulcis 1	XTBG, Yunnan
23	ILCLmuH01	Garciniamultiflora2	Wuzhimontain, Hainan	53	ILCLduY02	Garciniadulcis 2	XTBG, Yunnan
24	ILCLmuH02	Garciniamultiflora3	Wuzhimontain, Hainan	54	ILCLduY03	Garciniadulcis 3	XTBG, Yunnan
25	ILCLobY01	Garciniaoblongifolia 1	XTBG, Yunnan	55	ILCLkoY01	Garcinia kola 1	XTBG, Yunnan
26	ILCLobH01	Garciniaoblongifolia 2	FubaomontainHainan	56	ILCLkoY02	Garcinia kola 2	XTBG, Yunnan
27	ILCLobH02	Garciniaoblongifolia 3	FubaomontainHainan	57	ILCLkoY03	Garcinia kola 3	XTBG, Yunnan
28	ILCLxiY01	Garciniaxipshuanbannaensis1	XTBG, Yunnan	58	ILCLbrY01	Mammeabrevipes 1	XTBG, Yunnan
29	ILCLxiY02	Garciniaxipshuanbannaensis2	XTBG, Yunnan	59	ILCLbrY02	Mammeabrevipes 2	XTBG, Yunnan
30	ILCLxiY03	Garciniaxipshuanbannaensis3	XTBG, Yunnan	60	ILCLbrY03	Mammeabrevipes 3	XTBG, Yunnan

Table2. The characteristic features of the ITS region among Garcinia species plus out-group taxa.

Species	Length range (nt)	Length mean (nt)	Aligned length (nt)	G+C content (%)	G+C mean (%)	No.of variable sites	No.of informative sites	No.of conserved sites
Garcinia								
ITS1	253-259	255.28	260	47.84-53.91	51.23	129	76	131
5.8S rDNA	163-165	163.09	165	50.30-53.99	51.69	13	4	152
ITS2	199-206	200.72	205	50.00-53.00	51.40	109	70	96
Entire sequence	615-623	619.09	630	49.60-52.74	51.63	251	150	379
Garcinia+outgroup taxa								
ITS1	250-259	255.15	260	47.84-54.88	51.38	161	86	99
5.8S rDNA	163-165	163.09	165	50.30-53.99	51.80	17	6	148
ITS2	199-217	201.44	205	50.00-53.95	51.52	123	77	82
Entire sequence	615-628	619.69	630	49.60-54.31	51.74	301	169	329

Table 3.Intraspecific genetic distance about *Garcinia* species

No.	Species	intraspecific genetic distance	No.	Species	intraspecific genetic distance
1	*G. bracteata*	0.0011	10	*G. nujiangensis*	0
2	*G. cowa*	0.0006	11	*G. oblongifolia*	0
3	*G. dulicis*	0	12	*G. paucinervis*	0.0004
4	*G. hanburyi*	0	13	*G. subelliptica*	0.0005
5	*G. intermedia*	0.0006	14	*G. xanthochymus*	0
6	*G. kola*	0.0006	15	*G. xipshuanbannaensis*	0
7	*G. lancilimba*	0.0004	16	*G. yunnanensis*	0
8	*G. mangostana*	0.0005	17	*Mammeabrevipe*	0.0008
9	*G. multiflora*	0.0028			

Table4: Pairwise distance of the aligned ITS sequences of *Garcinia* species and outgroup species

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*G.dulcis*	—															
2	*G.kola*	0.1015	—														
3	*G.xipshuanbannaensis*	0.1618	0.1531	—													
4	*G.lancilimba*	0.1716	0.1548	0.1337	—												
5	*G.bracteata*	0.1590	0.1630	0.1119	0.1377	—											
6	*G.multiflora*	0.1239	0.1299	0.0838	0.1163	0.1222	—										
7	*G.subelliptica*	0.0370	0.1063	0.1542	0.1719	0.1514	0.1241	—									
8	*G.cowa*	0.1478	0.1519	0.0881	0.1393	0.1146	0.1266	0.1553	—								
9	*G.hanburyi*	0.1582	0.1613	0.1148	0.1310	0.1163	0.1182	0.1524	0.1201	—							
10	*G.paucinervis*	0.1519	0.1535	0.1209	0.1577	0.1475	0.1174	0.1470	0.1395	0.1491	—						
11	*G.oblongifolia*	0.1501	0.1522	0.0883	0.1370	0.1101	0.1244	0.1530	0.0101	0.1155	0.1397	—					
12	*G.intermedia*	0.1362	0.1393	0.1711	0.1802	0.1675	0.1542	0.1416	0.1744	0.1788	0.1827	0.1747	—				
13	*G.nujiangensis*	0.1445	0.1512	0.1068	0.1395	0.1303	0.0880	0.1397	0.1372	0.1334	0.0858	0.1305	0.1639	—			
14	*G.mangostana*	0.1517	0.1484	0.0900	0.1263	0.0741	0.1061	0.1365	0.1084	0.0894	0.1430	0.1040	0.1708	0.1332	—		
15	*G.xanthochymus*	0.0136	0.0947	0.1540	0.1634	0.1510	0.1191	0.0314	0.1453	0.1574	0.1442	0.1475	0.1312	0.1345	0.1439	—	
16	*G.yunnanensis*	0.1296	0.1310	0.1071	0.1241	0.1176	0.0821	0.1201	0.1239	0.1185	0.1097	0.1217	0.1545	0.1030	0.1017	0.1246	—
17	*Mammeabrevipes*	0.4084	0.4444	0.4106	0.3850	0.4405	0.4061	0.4232	0.4309	0.4396	0.4645	0.4223	0.4465	0.4251	0.4138	0.3965	0.4270

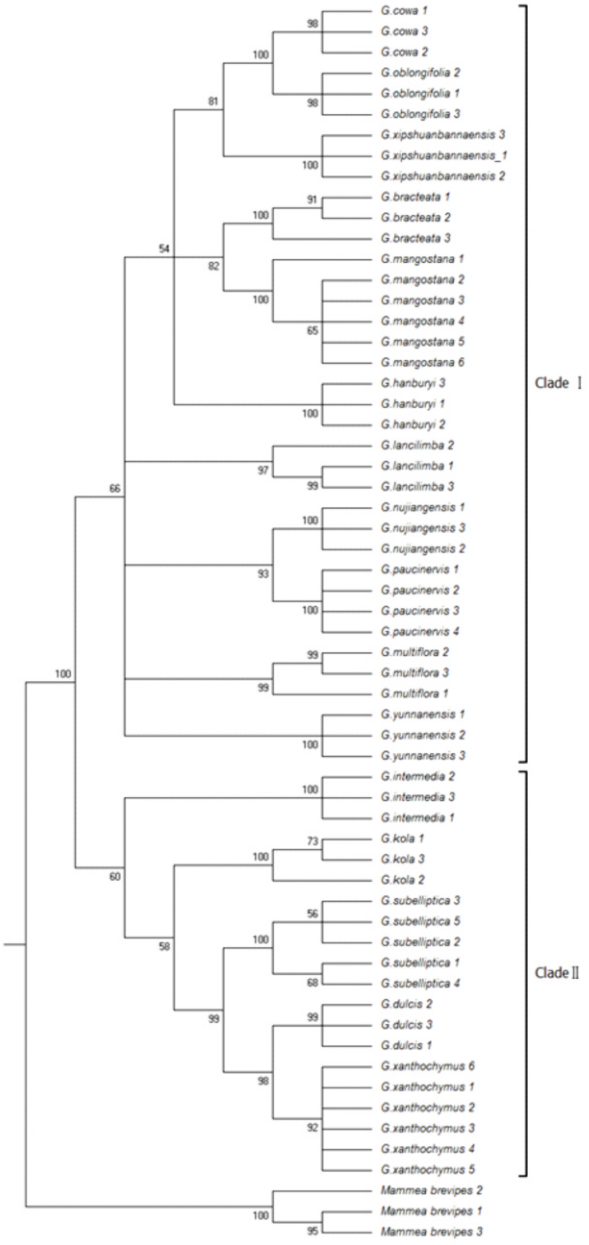

Figure 1. Maximum likelihood tree showing the relationships among the Garcinia species based on ITS sequences, -ln likelihood =3528.37, the bootstrap replications were 1000, and the ML bootstrap values (>50%) are shown above the branches.

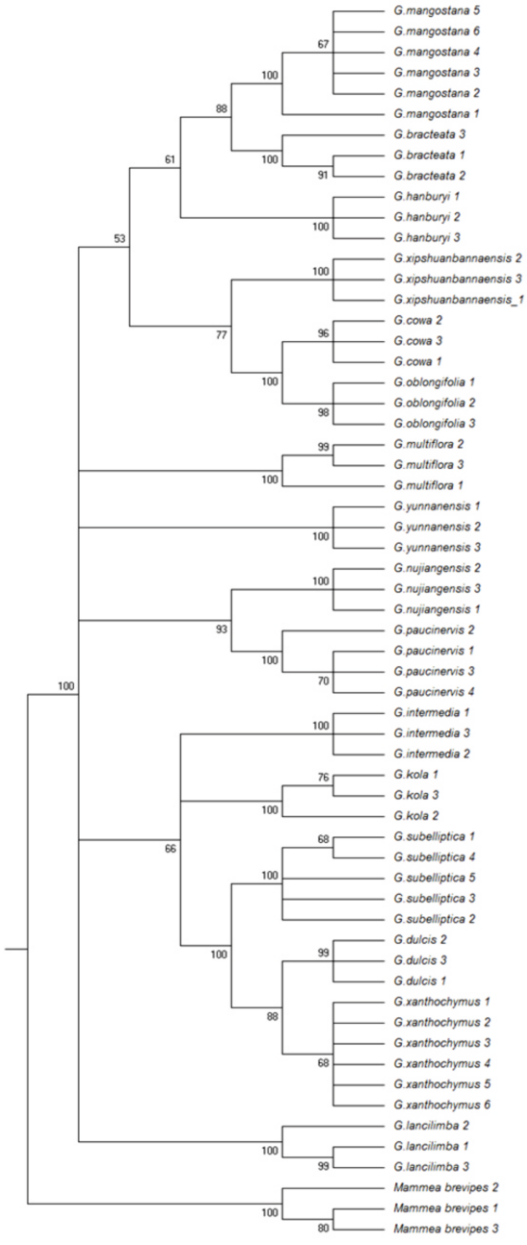

Figure 2. Neighbor-joining tree shown the relationships among the Garcinia species based on ITS sequences, SBL=1.1579, the bootstrap replications were 1000, and the NJ bootstrap values (>50%) are shown above the branches.

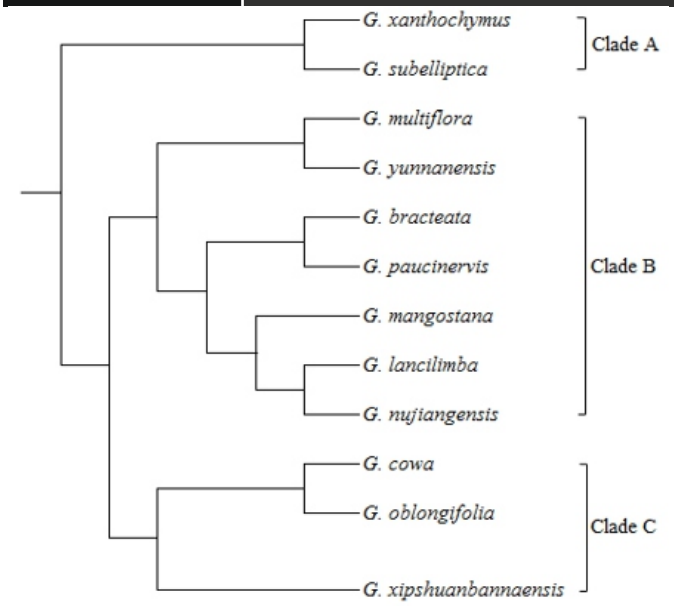

Figure 3. Classical classification system based on morphologic among the Garcinia species (*flora of China*).

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