Phylogenetic Relationship of Mangosteen (*Garcinia mangostana*) and Several Wild Relatives (*Garcinia* spp.) Revealed by ITS Sequence Data

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ABSTRACT. The phylogenetic relationships among 17 *Garcinia* species including *G. mangostana* (mangosteen) were analyzed by comparing sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA). Both parsimonious and neighbor joining (NJ) analyses revealed that *G. mangostana* is closely related to *G. malaccensis* believed to be a progenitor of mangosteen. Another suspected progenitor of mangosteen, *G. hombroniana*, was more distant from *G. mangostana* than *G. malaccensis* phylogenetically. *Garcinia hombroniana* formed a cluster with *G. rostrata*, *G. speciosa* and *G. sicygiifolia*, and this cluster was connected with a cluster of *G. mangostana* and *G. malaccensis*. The ITS sequence analysis showed that *G. atroviridis*, *G. cowa*, *G. dulcis*, *G. malaccensis*, *G. mangostana*, *G. rostrata* and *G. vilsianiana* have nucleotide additivity (two different nucleotides at the same nucleotide position) at several sites in the ITS region. The occurrence of these species might be related to hybridization with ancestors, but the genomic compositions, even chromosome numbers, of these species are still unknown.

Mangosteen (*Garcinia mangostana* L.) is a well-known tasty tropical fruit with attractive fruit shape and color. It is reported to have originated in southeast Asia and is famed as the ‘queen of tropical fruit’. Many visitors to southeast Asia enjoy this sweet and juicy fruit. In addition to its use as a dessert, the dried fruit rind, which contains tannin and xanthenes, is also used for treatment of inflammations, diarrhea, and dysentery in native medicine (Yapwattanaphun et al., 2002). Mangosteen is one of the most important economic fruit crops in Thailand, and the demand of mangosteen fruit usually exceeds the supply (Richard, 1990a; Yaacob and Tindall, 1995).

Mangosteen reproduction is very interesting because only female trees have been found. Since mangosteen is apomictic, all mangosteen trees are thought to be clones with the same genotype. Richard (1990b) reported that *G. mangostana* is allopolyploid and derived from the hybridization of *G. hombroniana* and *G. malaccensis*. He reported that 13 important morphological characteristics of *G. mangostana* are intermediate between *G. hombroniana* and *G. malaccensis*. However, his hypothesis has not been confirmed by analytical methods using molecular techniques. The origin of mangosteen is still unclear, although it is a very important fruit crop in southeast Asia.

Mangosteen belongs to the genus *Garcinia*, and many *Garcinia* species are distributed in southeast Asia. In the tropical forest in Thailand, there are 22 reported *Garcinia* species (Smitinand, 1980). Several of the species are important for commercial use such as timber, resin, or various natural products, in addition to the production of edible fruit. However, information on genetic diversity and phylogenetic relationships of these species to *G. mangostana* (mangosteen) is very limited. (Yaacob and Tindall, 1995; Yapwattanaphun et al., 2002).

Sequence analysis of internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) between the small subunit (18S) and the large subunit (26S) of nrDNA has been used as a source for analysis of phylogenetic relationships within genera and among closely related genera in many angiosperms (Baldwin et al., 1995). It frequently has been used to resolve phylogenetic relationships among many plant taxa, such as *Coffea* spp. (Lashermes et al., 1997), *Glycine* spp. (Kollipara et al., 1997), *Allium* spp. (Dubouzet and Shinoda, 1998), *Asarum* (Kelly, 1998), *Artemisia* (Kornkven et al., 1998), Loasaceae (Moody et al., 2001), and *Mangifera* (Yonemori et al., 2002). In this study we used a sequence analysis of ITS region of nrDNA between the small subunit and the large subunit of nrDNA to describe the relationship among *Garcinia* species to provide fundamental information for the breeder, because it will be important for genetic improvement of mangosteen in the future.

Materials and Methods

PLANT MATERIAL AND DNA EXTRACTION. Seventeen species of *Garcinia* and some accessions were collected from Thailand, Indonesia, Malaysia and Australia (Table 1). *Mammea siamensis* and *Calophyllum inophyllum* were selected as outgroup taxa. These two species belonged to the same family as *Garcinia*, the family Guttiferae, and *M. siamensis* was selected because it was easy to obtain in Thailand and *C. inophyllum* because it was morphologically close to *Garcinia*.

For DNA extraction, 5 g of leaf tissue were ground into a powder with liquid nitrogen in a mortar. The cetyltrimethyl ammonium bromide (CTAB) method reported by Doyle and Doyle (1987) was used for DNA isolation with a slight modification as described by Schnell et al. (1995). The isolated DNA was purified by precipitating with a polyethylene glycol (PEG) solution (Helig et al., 1992). Then, DNA was quantified by visual comparison with a DNA standard under UV light following electrophoresis on a 1% agarose gel and stained with 50 ppm of ethidiumbromide

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for 30 min. The DNA concentration was adjusted with Tris-EDTA (TE) buffer (pH8.0) to $\approx 50 \text{ ng} \cdot \mu\text{L}^{-1}$.

AMPLIFICATION AND SEQUENCING OF ITS REGION. The entire ITS region, including the 5.8S nrDNA coding region, was amplified by PCR using primers ITS-4 and ITS-5 (White et al., 1990). Reaction mixtures (100 μL) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 5% glycerol, 0.2 mM each dNTP, 25 pmol of each primer, 20 to 40 ng genomic DNA and 5 units of TaKaRa *rTaq* DNA polymerase (TaKaRa Shuzo Co. Ltd., Shiga, Japan). Amplification was carried out in a TaKaRa PCR Thermal Cycler MP (TP3000; TaKaRa Shuzo Co., Ltd., Shiga, Japan) for 5 min at 98 °C, followed by 35 cycles of 1 min at 97 °C, 30 s at 57 °C and 1.5 min at 72 °C, with a final run of 7 min at 72 °C. The amplified products were then purified through a spin column (SUPREC-02; TaKaRa Shuzo Co. Ltd., Shiga, Japan) for direct sequencing.

Forward and reverse sequencing reactions were performed using a Big Dye Terminator ver.3 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Perkin Elmer, Foster City, Calif.) using ITS-2 and ITS-5, and ITS-3 and ITS-4 (White et al., 1990) as sequencing primers for ITS1 and ITS2 regions, respectively. Sequencing reactions were done according to the standard protocol provided by Perkin Elmer with the kit. Sequence analysis was performed with an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Perkin Elmer, Foster City, Calif.) after the extra fluorescent ddNTPs were removed with Centri-Sep Spin Columns (Applied Biosystems, Perkin Elmer, Foster City, Calif.).

SEQUENCE ALIGNMENT. Sequence data of the ITS1, 5.8S, and ITS2 regions were first aligned by using GENETYX-MAC 7.3 (Software Development Co. Ltd., Tokyo, Japan), and then the alignment was checked and slight modifications made manually. The boundaries of ITS1 and ITS2 were determined by compar-

ing the aligned sequence with previously published sequences (Baldwin 1992; Kollipara et al., 1997; Lashermes et al., 1997). The 5.8S coding sequence was also considered for phylogenetic analysis, although only a few variations were found among the species examined.

PHYLOGENETIC ANALYSIS. The aligned sequence data matrix was analyzed by a PAUP 4.0b10 program (Swofford, 2002) by treating the gap as fifth base. Unordered parsimony analysis with accelerated transformation (ACCTRAN) optimization was performed by a heuristic search procedure with simple for stepwise-addition options, options of tree-bisection-reconnection (TBR) branch swapping, and saving the multiple trees (MulTrees). The most parsimonious trees were also found by a heuristic search procedure. In addition, bootstrap analysis with 250 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, two outgroup taxa were used to root the tree. The phylogenetic reconstruction was also performed by neighbor-joining (NJ) method (Saitou and Nei 1987) with PHYLIP (Felsenstein, 1993) after the distance matrices were calculated by the Kimura 2-parameter model of Dnadist in PHYLIP v.3.57 (Felsenstein, 1993). In the analysis, the polymorphic site from additivity having a nucleotide and a gap was treated as a gap at this site, since we could not define the site for this kind of additivity in PHYLIP. NJ tree was drawn by a Tree Draw Deck hacked into Hypercard (Gilbert, 1990) from Drawgram and Drawtree programs of the PHYLIP.

Results

ITS SEQUENCING. The entire ITS region in *Garcinia* species ranged from 616 to 621 bp (Table 2). The length of ITS1 region was 254 to 257 bp and of ITS2 was 199 to 202 bp. There is no

Table 1. List of *Garcinia* species and two outgroup taxa with their collected place.

Species	Collection place	Accession no.
<i>G. acuminata</i>	Chantaburi , Thailand	AB110796
<i>G. atroviridis</i>	Nakon Srithammarat, Thailand	AB110797
<i>G. costata</i>	Ubon Ratchathani, Thailand	AB110798
<i>G. cowa</i>	Chantaburi , Thailand	AB110799
<i>G. dulcis</i> accession 1	Ubon Ratchathani, Thailand	AB110800
<i>G. dulcis</i> accession 2	Bangkok, Thailand	AB110801
<i>G. hanburyi</i>	Chantaburi , Thailand	AB110802
<i>G. hombronaina</i> accession 1	Nakon Srithammarat, Thailand	AB110803
<i>G. hombronaina</i> accession 2	Bogor, Indonesia	AB110804
<i>G. malaccensis</i> accession 1	Bogor, Indonesia	AB110805
<i>G. malaccensis</i> accession 2	Bogor, Indonesia	AB110806
<i>G. mangostana</i> accession 1	Bogor, Indonesia	AB110807
<i>G. mangostana</i> accession 2	Nakon Srithammarat, Thailand	AB110809
<i>G. mangostana</i> accession 3	Narathiwat, Thailand	AB110810
<i>G. mangostana</i> accession 4	Trat, Thailand	AB110811
<i>G. mangostana</i> accession 5	Johore, Malaysia	AB110808
<i>G. porrecta</i>	Trat, Thailand	AB110812
<i>G. rostrata</i>	Ubon Ratchathani, Thailand	AB110813
<i>G. schomburgkiana</i>	Bangkok, Thailand	AB110814
<i>G. sicyiifolia</i>	Bogor, Indonesia	AB110815
<i>G. speciosa</i>	Bangkok, Thailand	AB110816
<i>G. tinctoria</i>	Brisbane, Australia	AB110817
<i>G. vilersiana</i>	Chantaburi, Thailand	AB110818
<i>G. xanthocymus</i>	Chiang Mai, Thailand	AB110819
<i>Calophyllum inophyllum</i>	Bangkok, Thailand	AB110820
<i>Mammea siamensis</i>	Bangkok, Thailand	AB110821

Table 2. The characteristic features of the ITS region among *Garcinia* species plus two outgroup taxa.

Species	Length range (nt)	Length mean (nt)	Aligned length (nt)	G+C content (%)	G+C mean (%)	No. of indels	No. of variable sites	No. of informative sites	No. of maximally parsimonious tree	Tree length	Consistency index (CI)	Retention index (RI)	Rescaled consistency index (RC)
<i>Garcinia</i>													
ITS1	254–257	255.41	261	47.65–52.94	51.38	7	93	81	128	132	0.8258	0.9475	0.7824
5.8S rDNA	163	163	163	50.31–52.15	51.48	0	3	3	3	3	1.0000	1.0000	1.0000
ITS2	199–202	199.70	202	49.50–52.76	51.15	4	83	68	127	127	0.8740	0.9498	0.8302
Entire sequence	616–621	618.12	626	49.59–52.51	51.33	11	179	152	263	264	0.8447	0.9466	0.7996
<i>Garcinia</i> + two outgroup taxa													
ITS1	236–259	254.80	261	47.66–73.30	52.45	27	216	138	357	357	0.8880	0.9279	0.8240
5.8S rDNA	163	163	163	50.31–57.67	51.81	0	14	8	16	16	1.0000	1.0000	1.0000
ITS2	172–202	198.42	202	49.50–73.71	52.36	36	177	127	329	336	0.8512	0.8879	0.7558
Entire sequence	593–621	616.23	626	49.59–69.13	52.27	63	407	273	708	708	0.8743	0.9125	0.7978

variation in length of 5.8S gene region, which had 163 bp (Table 2). In the two outgroups, the length of entire ITS region was 593 and 594 bp in *C. inophyllum* and *M. siamensis*, respectively. The lengths of ITS1 were 236 bp in *C. inophyllum*, much shorter than that in *Garcinia* species, and 259 bp in *M. siamensis*, almost the same as that in *Garcinia* species. The length of 5.8S in both outgroup taxa was the same as that of *Garcinia* species. The length of ITS2 in *C. inophyllum* was almost the same as that in *Garcinia* species (194 bp), but it was very short in *M. siamensis*, having the length of 172 bp. As a result, the entire length of ITS region in *C. inophyllum* and *M. siamensis* was shorter than in *Garcinia* species.

The percentage G+C content in the entire ITS region varied among the *Garcinia* species from 49.59% to 52.51%. However, the G+C content between ITS1 and ITS2 was fairly equivalent over the species (Table 2). The G+C content was higher in two outgroup species than in *Garcinia* species. The G+C content in entire ITS region was 69.13% in *C. inophyllum* and 57.74% in *M. siamensis*. The whole sequences obtained in the present study are accessible to DDBJ/EMBL/GenBank with accession code AB110796 to AB110821 (Table 1).

According to the entire sequence alignment among *Garcinia* species, only 11 indels (seven in ITS1, four in ITS 2) were assumed and it resulted in 626 bp in aligned length including 5.8S gene region. There were 93 and 83 polymorphisms in ITS1 and ITS2, respectively, while only three sites were polymorphic sites in 5.8 S gene region (Table 2).

Fig. 1. The consensus tree for unordered PAUP analysis from 250 bootstrap replications with option retaining groups with frequency >50% in TBR heuristic search. Numbers above branches indicate branch lengths drawn proportionally to the number of character-state changes. Numbers in parentheses show the percentage of occurrence of each monophyletic group from 250 bootstrap replication.

Fig. 2. Neighbor-joining tree obtained using the genetic distance of Kimura 2-parameter model of Dnadist in PHYLIP v.3.57 (1993).

However, when the sequence of two outgroup taxa were added to the alignment, the polymorphic sites were 216, 14, and 177 in ITS1, 5.8S, and ITS2, respectively. As a result, the polymorphic sites became 407 for the entire sequence in all species including outgroup taxa, and 273 sites among those were assumed to be informative for parsimonious analysis (Table 2).

In addition, we found nucleotide additivity in several species. Additivity, defined as having two different nucleotides at the same nucleotide position in the ITS region, was first used by Sang et al. (1995). The number of additivity sites was one in *G. atroviridis*, *G. vilersiana*, both accessions of *G. malaccensis*, and one accession of *G. mangostana*, and two in *G. rostrata* and both accessions of *G. dulcis*, and three in *G. cowa*.

However, despite the existence of additivity in some accessions, the sequence of the accessions of *G. dulcis*, *G. hombroniana*, or *G. mangostana* was completely the same, except for one accession of *G. mangostana* from Trat, which was different from the other four accessions showing additivity by having different nucleotides at only one position. Also, two accessions of *G. malaccensis* showed almost the same sequence between them, except for having different nucleotides in additivity site at two different positions.

PHYLOGENETIC ANALYSIS. The results of parsimonious analysis based on sequence data of ITS region are also summarized in

Table 2. Using the entire sequence of the ITS region, parsimonious analysis revealed a very close relationship between *G. mangostana* and *G. malaccensis* (Fig. 1). Five accessions of *G. mangostana* were included in the same group because of less variations in the sequences and constituted a clade with *G. malaccensis* (Fig. 1). This relationship was true even in the strict consensus tree constructed from 250 bootstrap analyses (data not shown).

According to parsimonious analysis, *G. hombroniana* formed a group together with *G. rostrata*, *G. speciosa*, and *G. sicyiifolia*, and this group formed a large group with the clade of *G. mangostana* and *G. malaccensis*. (Fig. 1). The group of *G. costata*, *G. vilersiana*, *G. dulcis*, *G. tinctoria* and *G. xanthochymus* formed a separate group with other *Garcinia* species tested in this study. As for outgroup taxa, *M. siamensis* was more distantly related to *G. mangostana* than *C. inophyllum*.

The result of neighbor-joining (NJ) analysis was similar to that of parsimonious analysis, showing a close relationship between *G. mangostana* and *G. malaccensis* and a little genetic distance between *G. mangostana* and *G. hombroniana* (Fig. 2). Furthermore, in the NJ tree, *G. costata*, *G. vilersiana*, *G. dulcis*, *G. tinctoria* and *G. xanthochymus* were separated by outgroup taxa from the other 12 *Garcinia* species as in the result of parsimonious analysis, showing a different evolutionary process from the other 12 species including *G. mangostana*.

Discussion

The most interesting feature of ITS sequence found in this study is the relationship among *G. mangostana*, *G. malaccensis* and *G. hombroniana*. *Garcinia mangostana* formed a cluster with *G. malaccensis*, and this cluster was joined with a cluster of *G. hombroniana*, *G. rostrata* and *G. speciosa*. According to the study on morphological characteristic of these species, *G. mangostana* seems to be derived from *G. malaccensis* and *G. hombroniana* as allopolyploid (Richard, 1990b). Among 13 main characters (flowering time, latex color, petal color, stigma, stigma surface, stigma lobes, stigma diameter, stamen mass, female flower, fruit shape, fruit surface, fruit color, and fruit flavor), four characters (latex color, petal color, sessile stigma, and fruit color) of *G. mangostana* are similar to those of *G. malaccensis* and four characters (smooth stigma surface, stamen/staminode mass clearly lobed, fruit globose, surface smooth) to those of *G. hombroniana*. Another five characters (flowering time, depth of stigma lobes, stigma diameter, presence of staminodes in the female flower, and fruit taste) fell between the two species. There are no characters of *G. mangostana* which fell outside the range of variability of *G. malaccensis* and *G. hombroniana*. Thus, Richard (1990b) proposed that morphologically, *G. mangostana* is a hybrid between *G. hombroniana* and *G. malaccensis*. He also suggested that the mother of the original *G. mangostana* was a cultivated *G. hombroniana* and the father was a wild *G. malaccensis* occurring nearby. The chromosome number of *G. mangostana* was almost double

that of *G. malaccensis* and *G. hombroniana* (Richard, 1990b), although the exact number of chromosome is still unidentified for *G. mangostana* and *G. malaccensis*. The ITS sequence in this study, however, showed that *G. hombroniana* was more distant from *G. mangostana* than *G. malaccensis* phylogenetically. It is possible that *G. hombroniana* is not a direct ancestor of *G. mangostana*. Since three other species, *G. rostrata*, *G. speciosa* and *G. sizygiifolia*, formed the same cluster as *G. hombroniana*, these species might be included in the origin of *G. mangostana*. The fruit of *G. hombroniana* is similar to that of *G. mangostana* and have a pleasant peach-like flavor (Ridley, 1922). The fruit of *G. rostrata* is also quite similar to that of *G. hombroniana*, but the latter is larger than the former and turns red when it ripens. Because *G. rostrata*, *G. speciosa*, and *G. sizygiifolia* are not economically fruit, important information on morphology and utilization of these species is very limited. We found *G. rostrata* in the eastern part of Thailand but it is not eaten as a fruit. *Garcinia speciosa* is a large tree with coriaceous and elliptic-lanceolate leaves. It has flowers with a pleasant smell and globose berry fruit 5 cm in diameter. The fruit skin turns bright red when ripe and the seeds are embedded in acidic pulp (Brandis, 1978). In Thailand, sweet-smelling flowers of *G. speciosa* are used as ornamental trees according to Thai's classical literature. Unfortunately, so far, there is no report on the relationship of these species to *G. mangostana*.

Another important result of our study is the finding of nucleotide additivity in ITS sequence among several species. This feature implies that there are two different templates with different base pairs at the same nucleotide position in the copies of ITS sequence in an organism, because we first used PCR amplicons as templates for direct sequencing. This allows us to assume the possibility of allopolyploidy by hybridization. Both parental sequences of the ITS region have been maintained in an allopolyploid species in *Krigia* (Kim et al., 1994), *Paeonia* (Sang et al., 1995) and *Arabidopsis suecica* (O'Kane et al., 1996) probably due to insufficient time for homogenizing parental ITS sequences by concerted evolution. We found additive sequences in *G. atroviridis*, *G. cowa*, *G. dulcis*, *G. malaccensis*, *G. mangostana*, *G. rostrata*, and *G. vilsersiana*. As for *G. mangostana*, additivity may be caused by the allopolyploidy of their origin. However, we found additivity in only one accession from Trat, in which there is a site having T and C in the ITS1 region. In our previous study on genetic variation in 23 accessions of *G. mangostana* by AFLP analysis, polymorphisms were detected in the same accession of *G. mangostana* from Trat (Yapwattanaphun et al., 2003). It is unclear why the additivity was found in this study. However, since the basic chromosome number of *Garcinia* species is not known and the ploidy of these species is not elucidated, there is a possibility that these species are of hybrid origin.

The relationship of relatively important species for commercial use was also shown in this study. *Garcinia atroviridis* and *G. schomburgkiana* are important species in Thailand. *Garcinia atroviridis* has a big fruit and is claimed to be efficacious in health promotion such as reducing cholesterol in blood, enlarging the blood vessel, and absorbing the excess fat. Dried fruit of *G. atroviridis* is used for improvement of blood circulation, as an expectorant, for treatment of coughs, and as a laxative (Yapwattanaphun et al., 2002). In Thailand, a lot of *G. atroviridis* products are sold in the market, such as tea, capsule and fruit's slice. *Garcinia schomburgkiana* also grows wild near streams and swamps in dry evergreen forests, and is sometimes cultivated in home gardens. The young leaf of *G. schomburgkiana* is served as

a vegetable accompaniment in many Thai dishes and can be eaten either raw or cooked. The fruit is rich in vitamin A and calcium and is eaten fresh, but it is very sour. It can be used in a sauce with shrimp paste and chili that is eaten with vegetables and fish. The fruit of *G. schomburgkiana* can also be processed as preserved fruit in syrup, pickled fruit, and dried fruit (Yapwattanaphun et al., 2002). Young leaves of *G. cowa* are used as a food additive in southeast Asia. In this study, these three species have formed a cluster with *G. porrecta*, which was apart from a large cluster involving *G. mangostana*. These commercially important species are not closely related to *G. mangostana*.

In addition, the young shoot and mature fruit of *G. xanthochymus*, which is native to the Philippines and Indonesia, are eaten as a vegetable and fruit in Indonesia, Malaysia, Thailand and the Philippines. *Garcinia dulcis* is sometimes sold as edible fruit in the local markets in Thailand and Malaysia. Fruit of *G. dulcis* is spheroid shaped and turns orange when ripe. The seeds are enveloped in edible pulp of a darker color than the skin and have a pleasant taste. *Garcinia xanthochymus* and *G. dulcis* formed a cluster with *G. costata*, *G. tinctoria*, and *G. vilsersiana*, and this cluster is clearly separated from *G. mangostana*, showing a different evolutionally process from *G. mangostana*. In this study, we used *G. tinctoria*, which was collected in Australia and has been said to be a synonym of *G. xanthochymus*. We found that these two species had the same ITS sequence, confirming that they were synonyms.

Overall, ITS sequence analysis of mangosteen and the several wild relatives used in this study gave us new information on the phylogenetic relationship among the species. This information will provide useful ideas for the improvement of the crop and further development and cultivation of it in the near future. Also, the usefulness of ITS sequence analysis for phylogenetic study was clearly demonstrated in this study.

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