CHAPTER 5

DISCUSSION

5.1 Molecular phylogenetic analyses of *Cassia* in Thailand

The phylogenetic analyses of Thai *Cassia* species with two DNA regions from different genomes (ITS regions of nuclear genome and *trn*L intron of chloroplast genome) revealed a nicely resolution for the long-time argument about relationships between Cassiinae species, both the taxa in Thailand and other Cassiinae throughout the world. From sequencing experiments, *trn*L intron sequences of all species were virtually clear and very easy to align, though a few ambiguous positions needed to be excluded before analysed. This *trn*L intron is in a chloroplast genome and so useful for studying genetic relationships among related genera (genus-to-genus) and then was suitable to use as a target gene for this study. The intron, however, may not be variable enough to differentiate interspecific relationship within a genus (as described in Bruneau *et al.*, 2000). On the other hand, ITS sequences are more variable than *trn*L intron sequences and then gave higher resolution on the phylogenetic-tree results. This is because ITS sequences are from a nuclear genome which an overall rate of base substitution across the genome is much faster than that of a chloroplast genome (Wolfe *et al.*, 1987). Such high evolutionary rate makes ITS sequences more suitable for species-to-species study than *trn*L intron.

Sixteen *Cassia* species in Thailand used in this M.Sc. experiment should be recognised as in a natural group (or monophyly). This suggestion came from phylogenetic analyses using their *trn*L intron sequences, ITS sequences and the combined sequence data. All analyses were performed by comparing with the sequences of two outgroups: *Gymnocladus dioica* of the tribe Caesalpinieae,

subtribe Gleditsia, and *Ceratonia siliqua* of the tribe Cassieae, subtribe Ceratoniinae; and additionally with other members of the subtribe Cassiinae in the case of *trn*L intron. Considering phylogenetic results from *trn*L intron sequence data, all consensus trees revealed that *Cassia* (*Senna*) *sophera*, *C.*(*S.*) *occidentalis* and *C.*(*S.*) *hirsuta* were clustered as a distinct clade having *C.*(*S.*) *sophera* paired to *C.*(*S.*) *occidentalis* before joined with *C.*(*S.*) *hirsuta*. The phylogenetic trees also suggested that *C.*(*S.*) *obtusifolia* was sistered to *C.*(*S.*) *tora* before grouped with *C.*(*S.*) *surattensis*. Another putative group suggested from phylogenetic analyses was the cluster of *C. javanica*, *C. bakeriana*, *C. grandis* and *C. fistula*. These three clades found in *trn*L data analyses were supported with the single phylogram of ITS sequence data and that of the combined data. Note that the phylogenetic trees found from the latter two analyses were a single most parsimonious tree probably because ITS sequence data gave much more informative characters than *trn*L intron sequences.

According to the suggestion in Irwin and Barneby (1981), these six *Cassia* (*C.*(*S.*) *sophera*, *C.*(*S.*) *occidentalis*, *C.*(*S.*) *hirsuta*, *C.*(*S.*) *obtusifolia*, *C.*(*S.*) *tora*, *C.*(*S.*) *surattensis*) should be moved from the genus *Cassia* to the genus *Senna* and the other four species (*C. grandis*, *C. fistula*, *C. bakeriana* and *C. javanica*) should remain *Cassia* species. Therefore, our findings from molecular phylogenetic analyses of these two DNA genomes (nuclear and chloroplast genomes) strongly confirmed the Irwin and Barneby (1981) recommendation and also supported previous investigations of these plants. Ghareeb *et al.* (1999) studied seed proteins, chromosome numbers and other morphological characters of some *Cassia* obtained from Egyptian botanical gardens and concluded that there should be separated into two groups. Group I contained *C. fistula*, *C. javanica* and *C. nodusa* while group II belonging to *C. occidentalis*, *C. sophera*, *C. siamea*, *C. didymobotrya*, *C. italica*, *C. Senna* and *C. surattensis*. Another M.Sc. thesis of Kidyue (2002), studied in anatomy

of 17 *Cassia* sensu lato in Thailand, suggested that these genus should be divided into four groups: *Cassia* (*C. bakeriana*, *C. fistula*, *C. grandis* and *C. javanica*), tree *Senna* (*C. garrettiana*, *C. spectabilis*, *C. timoriensis* and *C. siamea*), shrub *Senna* (*C. surattensis*, *C. alata*, *C. hirsuta*, *C. tora*, *C. obtusifolia*, *C. occidentalis* and *C. sophera*) and *Chamaecrista* (*C. leschenaultiana* and *C. pumila*). Moreover, another supporting experiment was done by Pechsri in 2003. It was a numerical taxonomic experiment by overall canonical discriminant analysis method based on non-discrete morphological data. The Study suggested that Thai *Cassia* should be split into three genera: *Cassia* (*C. fistula*, *C. javanica*, *C. grandis* and *C. bakeriana*), *Senna* (*C. alata*, *C. spectabilis*, *C. occidentalis*, *C. hirsuta*, *C. sophera*, *C. tora*, *C. obtusifolia*, *C. surattensis*, *C. timoriensis* and *C. garrettiana*), and *Chamaecrista* (*C. pumila* and *C. leschenaultiana*).

Not only the results from parsimony analyses that supported the Flora Malesiana, but a distance method also strongly suggested the same. Interestingly, *Ceratonia siliqua* was found in the NJ tree locating as a sister taxon to *Cassia* species which were moved to *Chamaecrista* in Flora Malesiana. This probably presented a good example of some difficulties occurring when analysing DNA sequence data with a distance method. Nucleotide sequence is well known to be more suitable for discrete methods like maximum parsimony (Page and Holmes, 1998). In fact, molecular phylogenetic study based on *rbc*L sequences and using parsimony analyses by Käss and Wink (1996) and Doyle *et al.* (1997) showed that *Gymnocladus dioica* (tribe Caesalpinieae) was strongly grouped with *Ceratonia siliqua* of the tribe Cassieae (subtribe Ceratoniinae) instead of pairing with *Cassia* (*Chamaecrista*) species, the same finding as in this M.Sc. thesis. Moreover, Polhill, Raven and Stirton (1981) considered *Ceratonia siliqua* to be a basal member of the natural group Cassieae and was joint to *Gymnocladus*. *trn*L intron analyses by Bruneau *et al.* (2000) also revealed that *Gymnocladus dioica* formed an unresolved clade to *Ceratonia siliqua* and located as a basal sister-clade of the subtribe

Cassiinae. These studies thus all supported the results from parsimony analyses in this M.Sc. experiments.

Cassia (*Senna*) siamea was the only species which could not be PCR amplified, both from *trn*L intron and ITS regions. This may cause by either any contaminant in the genomic DNA or its nucleotide variation particularly at the primer sites and might need more specific primers. Other three Thai *Cassia* (*C.*(*S.*) garretiana, *C.*(*Ch.*) *pumila* and *C.*(*Ch.*) *leschenaultiana*) could be amplified only at the *trn*L intron, but not ITS regions. This was probably because of either the same reasons above and/or GC rich problem in the ITS sequences. High level of GC ratio in a gene can lead to a PCR difficulty and the degree of encountered sequencing problem can then vary greatly from group to group (Soltis *et al.*, 1998).

From the *trn*L intron sequence analysis of 16 Thai *Cassia* with some other New-World Cassiinae members, retrieved from GenBank database, all consensus trees revealed that *C. grandis* (AF365092) from GenBank was paired with Thai *C. grandis* and this couple was then formed a group with other *Cassia* (*Cassia*) species (*C. fistula*, *C. javanica* and *C. bakeriana*). Nevertheless, this analysis also showed some differences to the results from *trn*L intron sequences of only Thai *Cassia*, (e.g. the position of *C.*(*S.*) *surattensis*). When analysed with only other Thai *Cassia*, *C.*(*S.*) *surattensis* was grouped with the pair of *C.*(*S.*) *obtusifolia* and *C.*(*S.*) *tora*. However, after phylogenetically compared with other New-World Cassiinae, *C.*(*S.*) *surattensis* moved to the base of the cluster of *C.*(*S.*) *obtusifolia*, *C.*(*S.*) *tora* and *S. bacillaris* from GenBank. Interestingly, this could suggest that *S. bacillaris* closer related to *C.*(*S.*) *tora*, *C.*(*S.*) *obtusifolia* and *C.*(*S.*) *surattensis*. More investigation on this species would be necessary to confirm such suggestion.

Among all Thai *Cassia* taxa used in the *trn*L analyses, two species not forming a resolved clade with others were *C.*(*S.*) *spectabilis* and *C.*(*S.*) *alata*. While *C.*(*S.*) *alata* could be grouped with *C.*(*S.*) *timoriensis* in ITS study, *C.*(*S.*) *spectabilis* was still left ungrouping with others and located at the most basal position of the

whole *Cassia*/*Senna* clade. *Cassia*.(*Senna*) *spectabilis* then may not have close relationships specifically with any particular Cassiinae species. Nevertheless, it could still be considered to a member of the genus *Senna* with other *Cassia* (*Senna*) species than being in the *Cassia* (*Cassia*) group because none of *trn*L and ITS analyses put *C.*(*S.*) *spectabilis* close to other *Cassia* (*Cassia*) species. Moreover, *C.*(*S.*) *alata* should also be a true member of the genus *Senna* with even more confidence than *C.*(*S.*) *timoriensis*. *Cassia* (*Cassia*) *alata* was found in ITS analysis pairing with *C.*(*S.*) *timoriensis* with low supporting-values. This suggested that *C.*(*S.*) *alata* would also be considered to be closely related to both *C.*(*S.*) *timoriensis* and *C.*(*S.*) *garrettiana*. By these criterias, *C.*(*S.*) *spectabilis* and *C.*(*S.*) *alata* should be moved to the genus *Senna* following Irwin and Barneby (1981), like other members of the *Cassia* (*Senna*) group. This recommendation got along well with the works of Larsen and Hou (1996), Kidyue (2002) and Pechsri (2003) but still disagree with previous seed-protein experiments and mitochondrial DNA-RFLP analysis (Mondal, Mondal and Mandal, 2000) suggesting that *C.*(*S.*) *alata* should be in the same cluster as *C. fistula*. The most noticed point of these two species is that these species probably imported into Thailand for a long time. Therefore, they could form the clade to another *Senna* from New-World in the phylogenetic trees from this study and form a discrete group while study in both numerical taxonomy (Pechsri, 2003) and anatomic study (Kidyue, 2002).

The position of Thai *Cassia* species in this study, both from *trn*L and ITS data sets, were somehow not supported by a molecular study based on *rbc*L sequences of Doyle *et al.* (1997) and Kajita *et al.* (2001). In their experiments, three species represented subtribe Casiinae (*C. fistula*, *Ch. fasciculata* and *S. alata*) suggested in their studies to be a basal group of other *Senna* while members of the genus *Senna* formed a sister clade to the genus *Chamaecrista*. Nevertheless, a study in chloroplast *trn*L intron sequences by Bruneau *et al.* (2000), which their sequences were shown and co-analysed in this M.Sc. thesis, supported the idea that *Cassia*

(*Cassia*) group should be a distinct group within the genus *Senna*, and also supported the cluster between Thai *Cassia* (*Chamaecrista*) to *Chamaecrista* species from GenBank, a distinct minor clade basal to the major clade of *Cassia*, *Cassia* (*Senna*) and New-World *Senna*. All of these were also supported with an ontogenetic study of Douglas and Tucker (1994).

5.2 Morphological and cytological characteristics of *Cassia* in Thailand

Molecular phylogenetics investigation of these Thai *Cassia* species investigation strongly suggested that several groupings of genetic related species should be recognised, i.e. a group of Thai *Cassia* moved to *Chamaecrista*, that of the species moved to *Senna*, and also a group of true *Cassia* within the *Senna* group. These phylogenetic groupings supposed to be very useful as a scraffold for other biological interpretations. For instance, morphological and cytogenetic data could be mapped to the *Cassia* phylogenetic trees (Fig. 51-53 and Fig. 54-56, based on *trn*L intron and ITS region sequence data, respectively). The cytological data incorporated to the trees were chromosome numbers of some plants, collected from several previous studies (Umpunjuntara, 1990 and Ghareeb *et al.*, 1999), whereas morphological characteristics (mostly discrete characters) were selected from taxonomic descriptions of those species.

The cytogenetic study performed in this M.Sc. thesis revealed that chromosome numbers of most *Cassia* species were not easy to be counted. This could be because of nature of the chromosomes themselves. Moreover, an external apprearance on the size of flower buds provide only a rough guide to the preferred stage of meiosis of the developing anthers inside (Jong, 1997). Cytogenetics studying from somatic cells normally should be easier but apparently could show only a rough guide for chromosome counting. The numbers of chromosomes of eight species (*C. occidentalis*, *C. surattensis*, *C. siamea*, *C. garrettiana*, *C. fistula*, *C.*

sophera, C. javanica, *C. spectabilis*, *C. timoriensis* and *C. tora*) were found in this study to be around 22 to 28 as 11 to 14 bivalent chromosome pairs were estimated from each cell. The highest chromosome numbers were found in C. surattensis and C. tora as 2n=56 inferred from 28 bivalent pairs. Comparing these chromosome numbers to previous cytogenetic investigations of Umpunjuntara (1990) and Ghareeb *et al.* (1999), chromosome sizes of these Thai *Cassia* species were confirmed to be very small, as same as small points in a normal light microscope. Therefore, the exact chromosome number in this M.Sc. thesis were difficult to tell straightfully except justifying from the closely number to the previous reports. Nevertheless, the chromosome numbers of *C. timoriensis* (2n=28) and *C. tora* (2n=56), through remain ambiguous, were the new reports for *Cassia* species in Thailand.

Up to date, chromosome numbers of Thai *Cassia* species investigated so far were reported to the same in most taxa, 2n=28 (Umpanjuntara, 1990 and Ghareeb *et al.*, 1999). The only exception is that of *C.*(*S.*) *surattensis* which was found to be 2n=56 by Umpunjuntara (1990) and Ghareeb *et al.* (1999) but contrary to 2n=28 in the experiment of Tandon *et al.* (in Moore, 1973). This disagreement in the chromosome number of *C. surattensis* would have come from an intraspecific variation in the chromosome number of such species and more cytogenetic investigation is needed for clarification. To understand a natural history of the chromosome doubling phenomenon, chromosome numbers of *C.*(*S.*) *obtusifolia* and *C.*(*S.*) *tora* should be counted to answer whether the phenomenon is a unique evolutionary event of *C.*(*S.*) *surattensis* or of the whole minor clade. More works on finding chromosome numbers of other *Cassia* species left unchecked are also appreciated. Moreover, *Cassia* was suggested to be allopolyploid genus, with a basic chromosome numbers equal to seven (x=7), after long-time breedings through their evolution (Umpunjuntara, 1990 and Ghareeb *et al.*, 1999). To prove this hypothesis, only chromosome numbers and other characteristics would not be enough and more advance cytogenetic techniques such as Karyotype banding, Fluorescense in situ

Hybridisation (FISH), or Genomic in situ Hybridisation (GISH), should be introduced to give more information on chromosomal evolutionary relationships among Thai Cassiinae taxa.

Morphological characters and the chromosome numbers of 16 Thai *Cassia* species were mapped onto the branches of the *trn*L phylognenetic trees (Fig. 51 to Fig. 53). The first major clade of Thai *Cassia* (*Chamaecrista*) species has some distinctive characters which are equal filaments and two bracteoles (Fig. 51). Another character separating this group from the *Cassia/Senna* group is their leaflets which are less than 5 mm broad while those of the *Cassia/Senna* are more than 5 mm broad (Fig. 52). Within the Thai *Cassia/Senna* group, character mapping was much more difficult to do because of its polytomic backbone occurred. Some specific characters suitable enough to use for subclade distinguishing are a presentation of foliar glands only on petiolar gland or on rachis between leaflets, a glabrous or pubescent (or hairy) surface of upper leaf, and pod shape. The first minor clade (*C.*(*S.*) *sophera*, *C.*(*S.*) *occidentalis* and *C.*(*S.*) *hirsuta*) could be further divided by shape of pod (glabrous or strigose) and foliar glands (subulate or ovoid) to specify species name. The clade II (*C.*(*S.*) *tora*, *C.*(*S.*) *obtusifolia* and *C.*(*S.*) *surattensis*) could be divided by present or absent of staminodes and then numbers of staminodes (Fig. 53). This morphological character-mapping were similar to what received from using the ITS phylogeny (Fig. 54-56). An unequal filament characteristics can be used to group all four *Cassia* (*Cassia*) species (*C. grandis* and *C. fistula*, *C. bakeriana* and *C. javanica*) together. All other Thai *Cassia*(*Senna*) however have equal filaments (Fig. 54). Moreover, almost all members of this major *Cassia* (*Cassia*) clade have pink or red flowers, except *C. fistula* flower which is yellow as same as other members in the subtribe Cassiinae. One problematic species for character mapping analyses was *C.*(*S.*) *timoriensis* which was paired with *C.*(*S.*) *garrettiana* in the semistrict consensus tree of *trn*L intron sequence data, but paired with *C.*(*S.*) *alata* in the ITS tree. One could say that these three taxa actually

should be put on the same clade (if ITS regions of *C.*(*S.*) *garrettiana* can be PCR amplified), having absent foliar gland as a unique character for the group. Anatomic characters of Kidyue's work (2002) were also mapped to these phylogenetic trees (Fig. 55). *Cassia*(*Senna*) *spectabilis* was at the basal position of the Thai *Cassia* (*Senna*) clade in the ITS tree. Radial xylem tissue of this plant is in uniseriate heterocellular type while other species in this *Cassia* (*Senna*) clade have multiseriate heterocellular type. This could suggest that the multiseriate heterocellular type of the radial xylem tissue is an advance character state and the uniseriate heterocellular type of the radial xylem tissue is probably more primitive. Other characters which shown in the *trn*L semistrict consensus tree (Fig. 53), were easier to map onto the ITS trees as shown in Fig. 56.

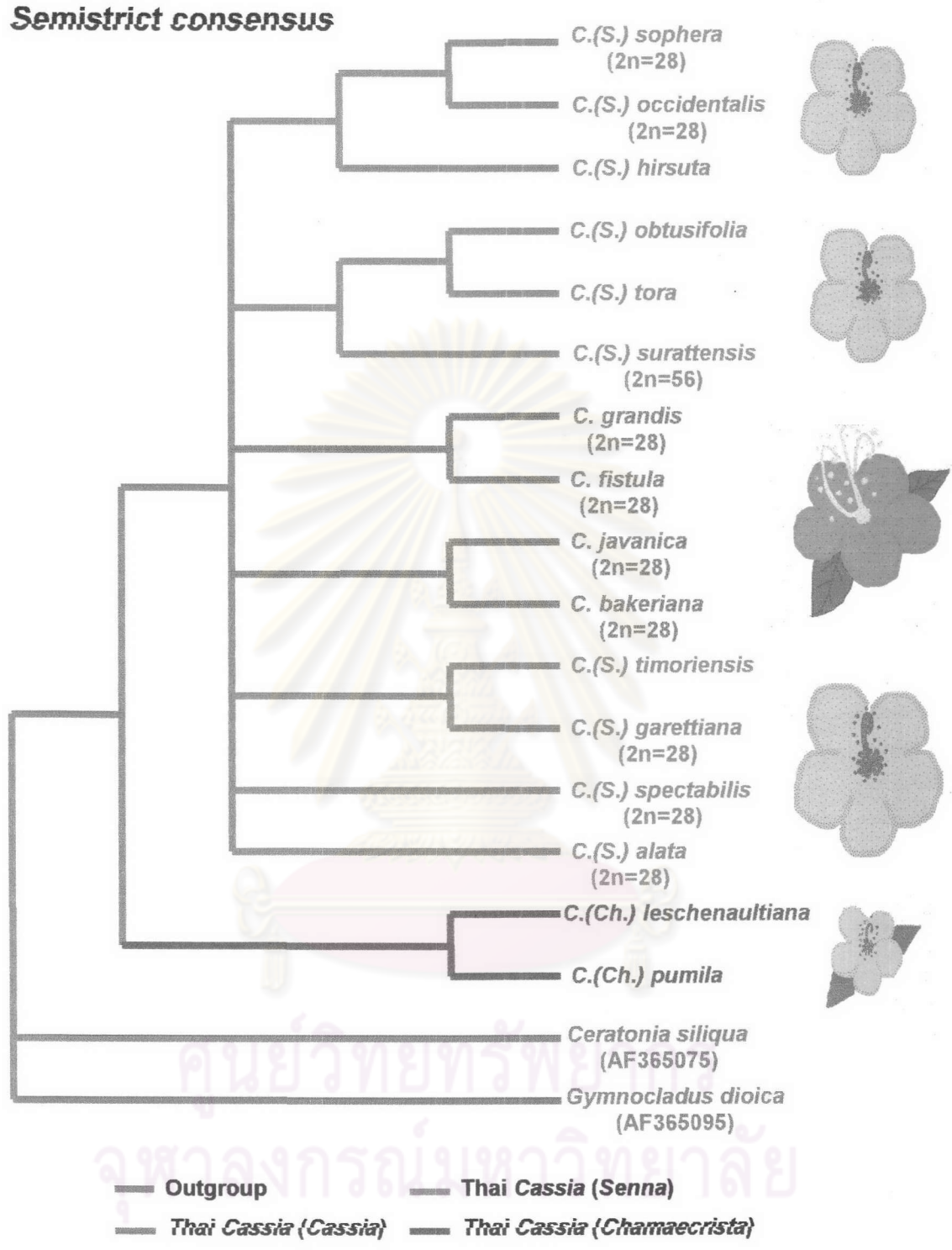

Fig. 51 mapping of morphological characters and chromosome numbers to the phylogenetic tree of 16 *Cassia* species in Thailand based on *trn*L intron sequences. Chromosome numbers follow previous work of Umpunjuntara (1990) and Ghareeb *et al.* (1999). Note that petal colour of most members of the *Cassia* (*Cassia*) subclade is pink to red while that of *C. fistula* and other taxa (*Cassia* (*Senna*) and *Cassia* (*Chamaecrista*)) is yellow.

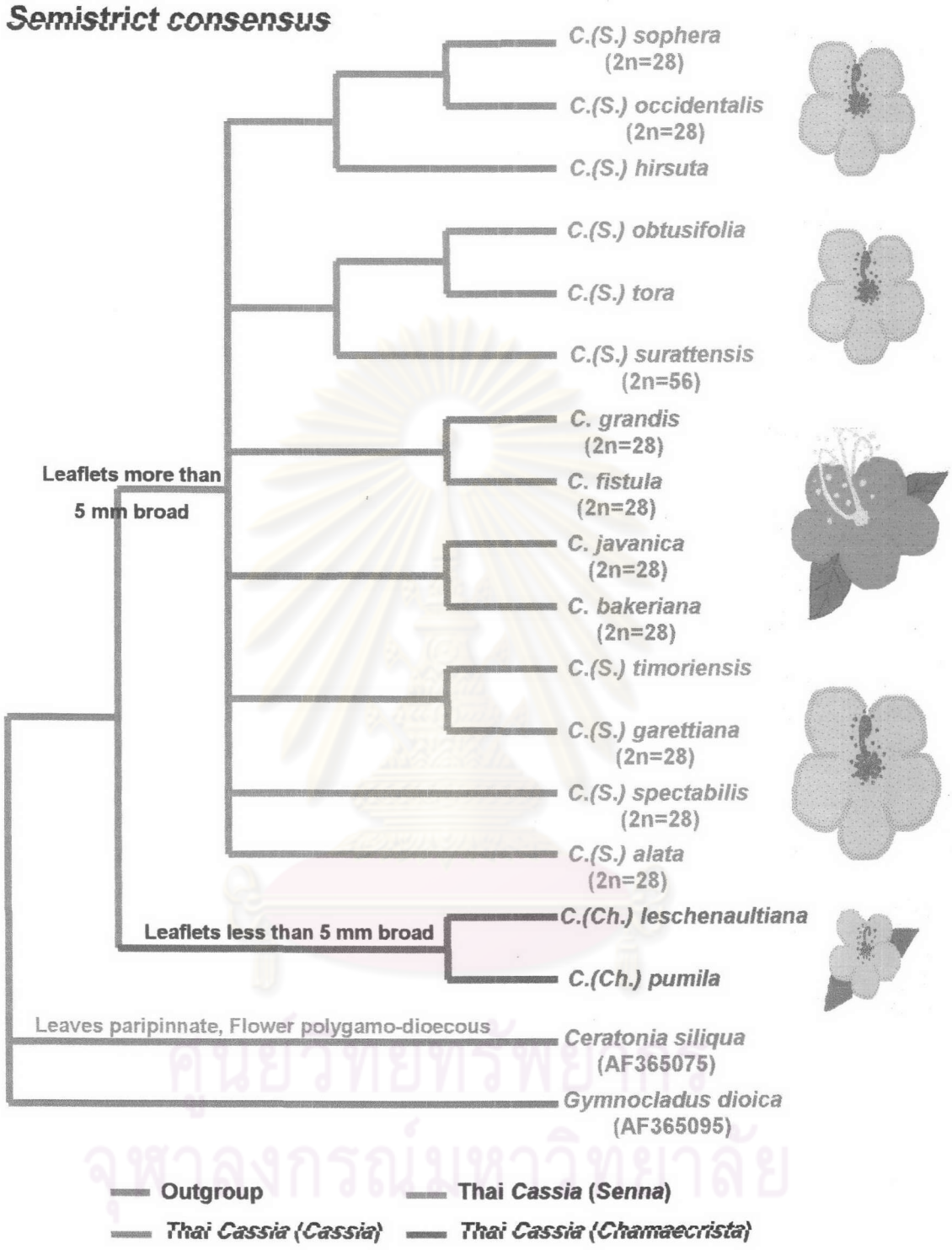

Fig. 52 mapping of morphological characters and chromosome numbers to the phylogenetic tree of 16 *Cassia* species in Thailand based on *trn*L intron sequences. Chromosome numbers follow previous work of Umpunjuntara (1990) and Ghareeb *et al.* (1999). Note that petal colour of most members of the *Cassia* (*Cassia*) subclade is pink to red while that of *C. fistula* and other taxa (*Cassia* (*Senna*) and *Cassia* (*Chamaecrista*)) is yellow.

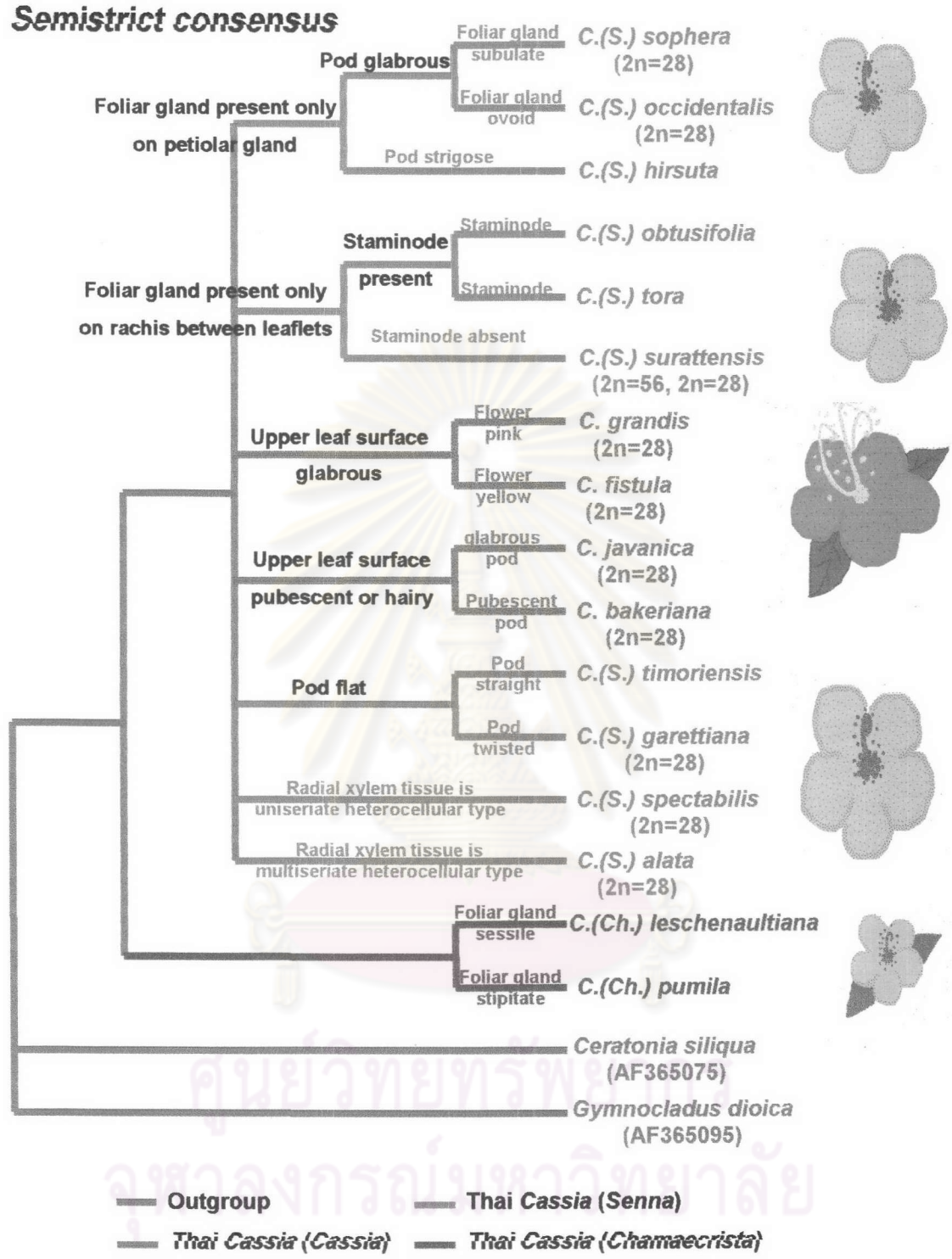

Fig. 53 mapping of morphological characters and chromosome numbers to the phylogenetic tree of 16 *Cassia* species in Thailand based on *trn*L intron sequences. Chromosome numbers follow previous work of Umpunjuntara (1990) and Ghareeb *et al.* (1999). Note that petal colour of most members of the *Cassia* (*Cassia*) subclade is pink to red while that of *C. fistula* and other taxa (*Cassia* (*Senna*) and *Cassia* (*Chamaecrista*)) is yellow.

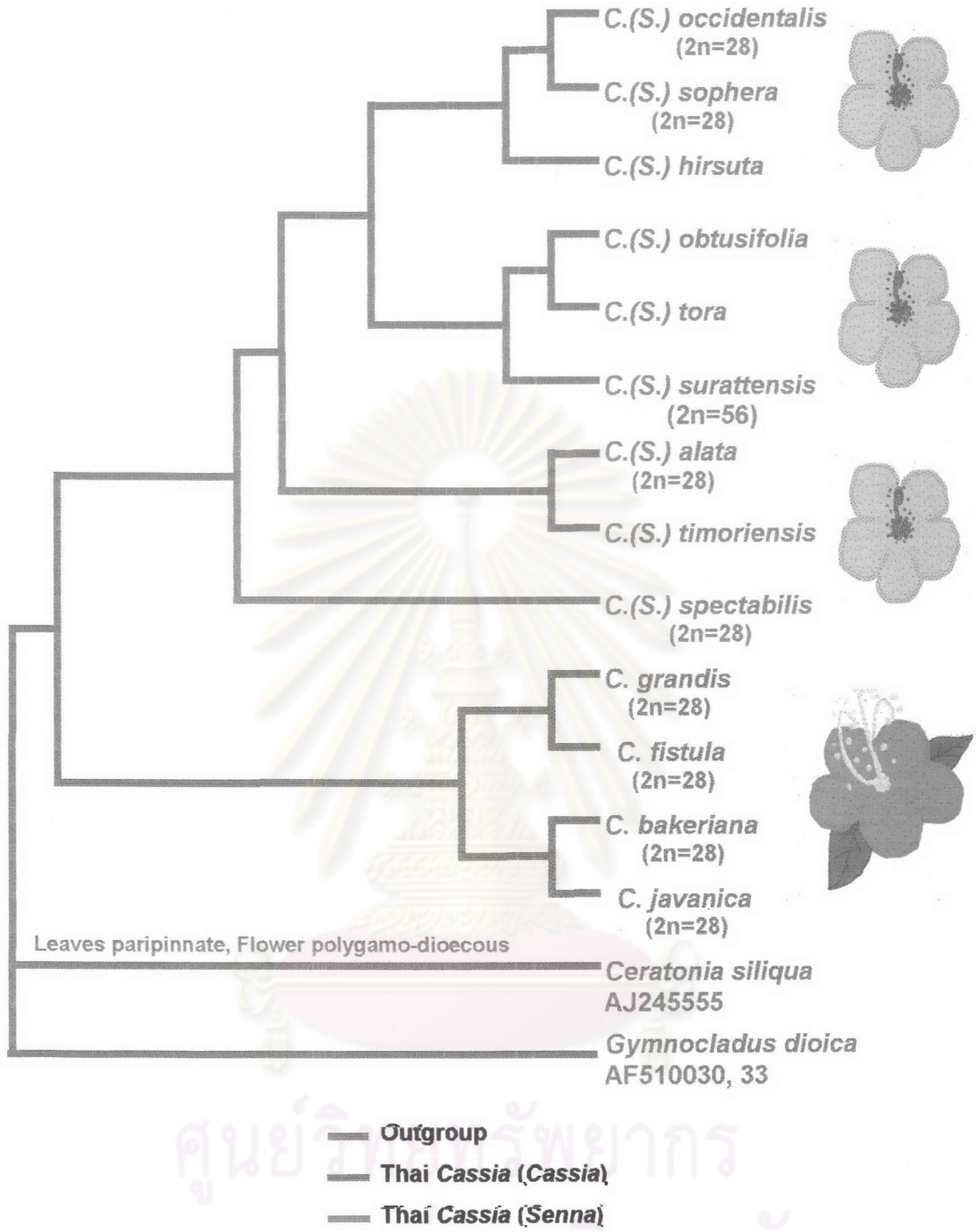

Fig. 54 mapping of morphological characters and chromosome numbers to the phylogenetic tree of 16 *Cassia* species in Thailand based on ITS regions sequences. Chromosome numbers follow previous work of Umpunjuntara (1990) and Ghareeb *et al.* (1999). Note that petal colour of most members of the *Cassia* (*Cassia*) subclade is pink to red while that of *C. fistula* and other taxa (*Cassia* (*Senna*) and *Cassia* (*Chamaecrista*)) is yellow.

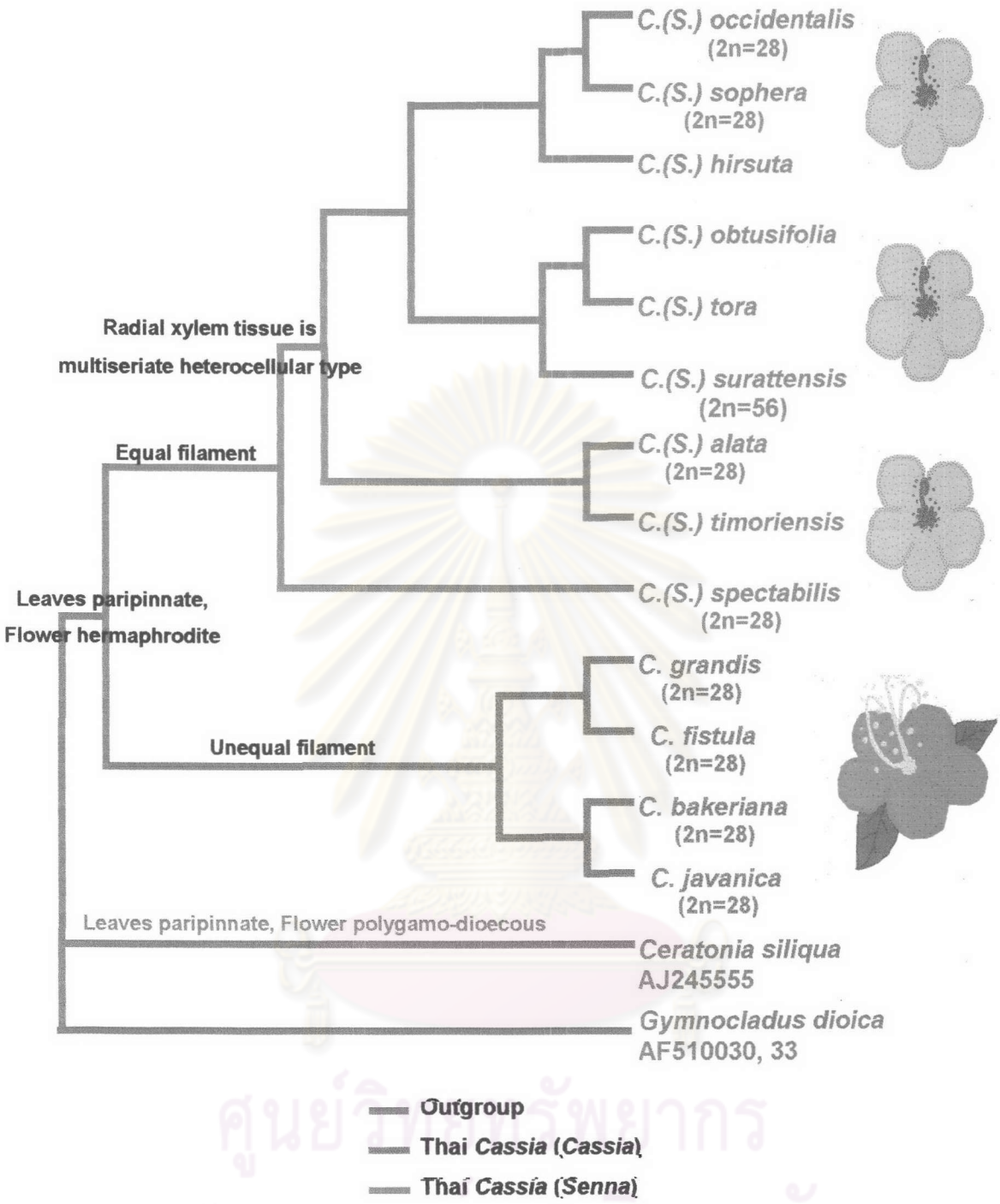

Fig. 55 mapping of morphological characters and chromosome numbers to the phylogenetic tree of 16 *Cassia* species in Thailand based on ITS regions sequences. Chromosome numbers follow previous work of Umpunjuntara (1990) and Ghareeb *et al.* (1999). Note that petal colour of most members of the *Cassia* (*Cassia*) subclade is pink to red while that of *C. fistula* and other taxa (*Cassia* (*Senna*) and *Cassia* (*Chamaecrista*)) is yellow.

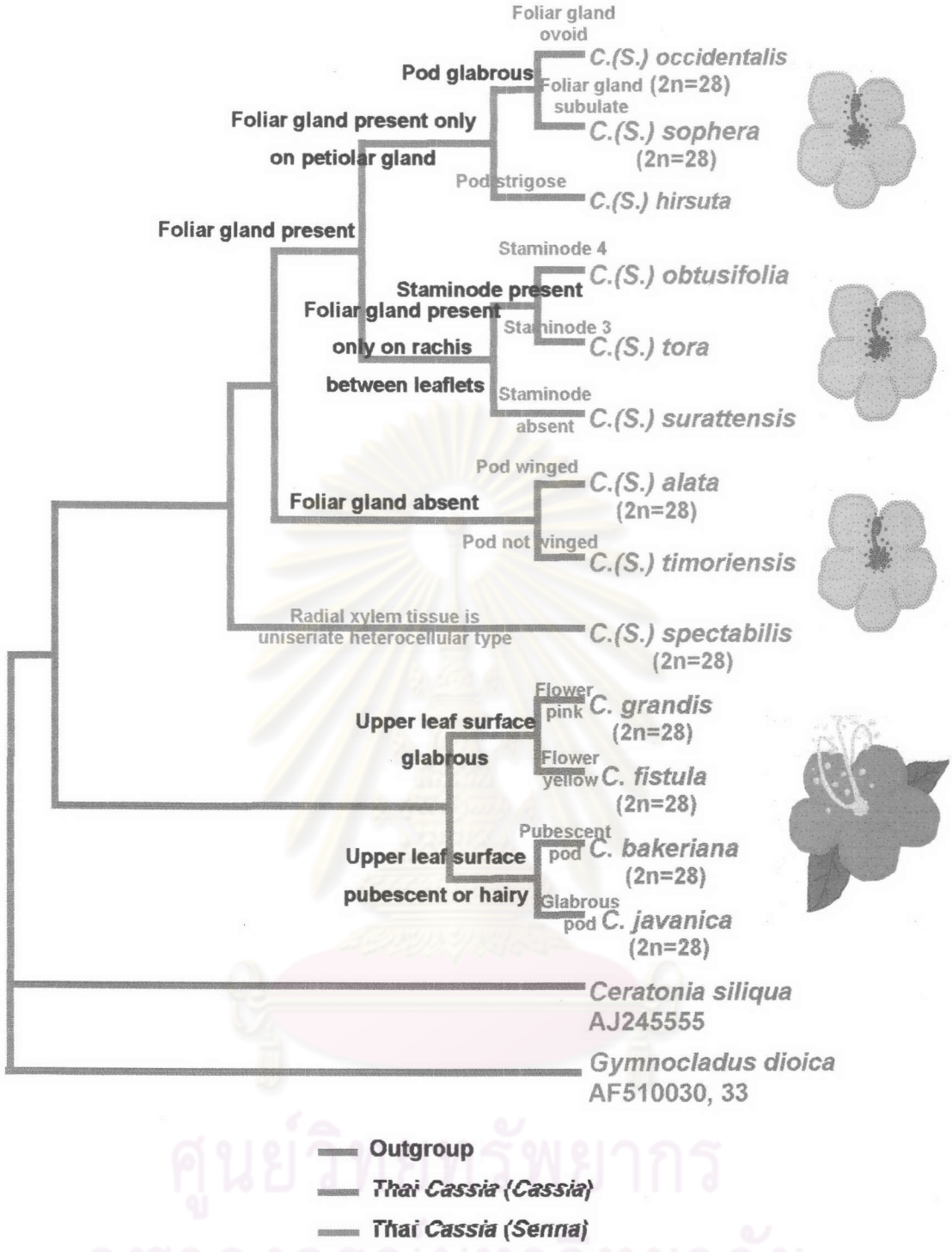

Fig. 56 mapping of morphological characters and chromosome numbers to the phylogenetic tree of 16 *Cassia* species in Thailand based on ITS regions sequences. Chromosome numbers follow previous work of Umpunjuntara (1990) and Ghareeb *et al.* (1999). Note that petal colour of most members of the *Cassia* (*Cassia*) subclade is pink to red while that of *C. fistula* and other taxa (*Cassia* (*Senna*) and *Cassia* (*Chamaecrista*)) is yellow.

5.3 Implications for taxonomy of *Cassia* in Thailand

Using molecular biology techniques such as DNA sequencing study has been proved to be useful in solving taxonomic and systematic problems of many organisms. The techniques can give accurate and fast answers for long time puzzles of very diverged lives on the planet. Comparing molecular phylogenetics to both shared and unique morphology of members of the interested group would lead to such answers. Key to species could be prepared after the comparison. In this phylogenetic study of *Cassia* in Thailand, phylogenies based on *trn*L intron sequences and ITS sequences were prepared, revealing some morphological characters which should be useful for species identification and subclade classification. The resulted phylogenetic key to species was provided below.

Since *C. fistula* is so important as being the national tree of Thailand and other species also have economic importance because of their herbal medicinal and timber usages, understanding an accurate systematic classification of the genus and allies should provide us precise background knowledge for other future works. The results from this molecular phylogenetic investigation could be an important platform for further taxonomic, evolutionary, breeding, developmental and pharmacological studies of the subtribe Cassiinae in Thailand and other tropical countries in the world.

Phylogenetic key to species following morphological and anatomical characters mapped on the most parsimonious trees of both *trn*L intron and ITS sequence data.

1a. Leaflets less than 5mm broad

2a. Foliar gland sessile..... **C.(*Ch.*) *leschenaultiana***

2b. Foliar gland stipitate..... ***C.(Ch.) pumila***

1b. Leaflets more than 5mm broad

3a. Unequal filament

4a. radial xylem tissue is multiseriate heterocellylar type

5a. Foliar gland present

6a. Foliar gland present on petiolar gland only

7a. Pod strigose.....***C.(S.) hirsuta***

7b. Pod glabrous

8a. Gland ovoid.....***C.(S.) occidentalis***

8b. Gland subulate.....***C.(S.) sophera***

6b. Foliar gland present on rachis between leaflets

9a. Staminode absent.....***C.(S.) surattensis***

9b. Staminode present

10a. Staminodes 4.....***C.(S.) obtusifolia***

10b. Staminodes 3.....***C.(S.) tora***

5b. Foliar gland absent

11a. Pod winged.....***C.(S.) alata***

11b. Pod not winged

12a. Pod straight.....***C.(S.) timoriensis***

12b. Pod twisted.....***C.(S.) garrettiana***

4b. radial xylem tissue is uniseriate heterocellylar type..***C.(S.) spectabilis***

3b. Unequal filament

13a. Upper leaf surface pubescent or hairy

14a. Pubescent pod.....***C. bakeriana***

14b. Glabrous pod.....***C. javanica***

13b. Upper leaf surface glabrous

15a. Flower pink.....***C. grandis***

15b. Flower yellow.....***C. fistula***