CHAPTER V

DISCUSSION

5.1 Molecular phylogenetic analyses of some Thai *Aeschynanthus* and implication for *Aeschynanthus* taxonomy

Molecular phylogenetic analyses of some *Aeschynanthus* in Thailand and other countries using ITS regions of nuclear ribosomal DNA genome in this MSc thesis revealed many interesting and new knowledges about evolutionary genetic relationship between *Aeschynanthus* species. The ITS sequences used as a target gene for this experiment have been popular in species-to-species relationship study. An overall rate of base substitution in these regions is fast enough to differentiate inter-specific relationship within a genus, much faster than that of a chloroplast genome (Wolfe and Sharp 1987), and then give higher resolution on the phylogenetic-tree results (Bruneau *et al.*, 2000). From all *Aeschynanthus* samples obtained and used for DNA extraction and PCR amplification, *A. superbus* was the only species of which the ITS regions could not be PCR amplified. This PCR problem may have caused by either low DNA concentration yielded from a difficultly in herbarium specimen DNA extraction or from nucleotide variations at the ITS primer sites. Newly-designed primers for more specific amplification on this species would be needed in the future.

From sequencing experiments, ITS sequences of almost all *Aeschynanthus* species provided by RBGE were virtually clear and not too difficult to align, although a few ambiguous positions were needed to be excluded before analysed. Indels (insertion and deletion) were additionally coded as missing data. Of the 31 newly additional species here, only *A. parviflorus* showed ITS length-polymorphism problem which resulted from a single 1-2 bp deletion between different intra-individual ITS copies. However, this sequence variation could be interpreted satisfactorily after comparing the forward and reverse sequences. This means that only 3% of *Aeschynanthus* species used in this research showed an evidence of intra-genomic polymorphism in their ITS sequences, very low ratio compared to 40% reported by Denduangboripant and Cronk (2000) (22% with severe polymorphism, and 15% with minor polymorphism). When all ITS sequences were aligned together, a matrix of 571 aligned-positions was prepared, having two-third of all characters as parsimony-informative characters.

Thirty-one *Aeschynanthus* species provided by RBGE used in this MSc experiment has confirmed that the genus *Aeschynanthus* is a natural group. All *Aeschynanthus* ITS sequences were grouped together as monophyletic clade, separating from the sequences of the two outgroups: *C. baillyi* and *L. forestii*. All phylogenetic trees resulted from these ITS sequence data revealed that the genus *Aeschynanthus* could be divided into two major clades. Clade I contains species mainly from the continental western area, whereas clade II species occur mainly in eastern Malesia, particularly on the Sunda shelf islands and east of Wallace's line. This new

finding confirms the previous study of Denduangboripant *et al.* (2001) and the positions of each *Aeschynanthus* species in this study also support their molecular study. Scanning electron microscope (SEM) studies of Mendum *et al.* (2001) on seed and appendage morphology suggested too that *Aeschynanthus* can be divided into two groups, A and B, by differences in testa cell orientation and appendage structure. Group A is essentially Malesian and contains sections *Microtrichium, Aeschynanthus* and *Haplotrichium* sens. str. while group B is largely confined to mainland South and Southeast Asia and contains sections *Polytrichium, Diplotrichium* and section *X.*

From phylogenetic trees in this study, *A. parviflorus* 19671067 was paired with *A.* aff. *paviflorus* 19672220 with high supporting-values. This could confirm that the plant 19272220 is truely *A. parviflorus*, not only by their similar morphology, but also by their close genetic relationship. Thus, the plant *A.* aff. *parviflorus* 19672220 should be placed in the section *Diplotricium* too, even though there is no seed sample available to study. This molecular phylogenetic approach to confirm a scientific name was not easy in the case of *A. viridiflorus* 20003332 and 20021227. Both parsimony and distance trees suggested that the additional *A. viridiflorus* 20003332 and 20021227 did not form a sister group with *A. viridiflorus* 20000228 from previous study. These two plants were placed at the polytomic node with *A.* sp. nov. 20000512, a pair of *A. longicaulis* and *A. fecandus*, and a group of *A. batakiorum*, *A. myrmecophilus*, *A.* sp. 00171, and *A. viridiflorus* 20000228. In fact, 50% majority rule consensus tree and neighbour-joining tree suggested that *A. viridiflorus* 20000228 is genetically closer to *A. batakiorum* than

the other two *A. viridiflous*. This raised the question of whether the plants 20000332 and 20021227 were the same species of *A. viridiflorus* 20000228 or any of these specimens once had been misidentified to be *A. viridiflorus*. Since a green colour flower is the unique character of this species, more investigations on reproductive parts of these plants are necessary to solve such problem.

Moreover, the major Clade I also contains the four Thai *Aeschynanthus* species which have a synonymous problem. According to the suggestion of Burtt (2001), *A. andersonii* and *A. humilis* and *A. hildebrandii* may be a synonymous species. *Aeschynanthus hildebrandii* and *A. persimilis* were also suggested by Mary Mendum, an *Aeschynanthus* expert of RBGE, to be the same plant. According to their positions on the phylogenetic tree, *A. andersonii* and *A. humilis* were more closely related to each other than to *A. hildebrandii*. This hypothesis was supported by an initial RAPD experiment. PCR-RAPD markers have proved being useful for species delimitation and population genetic analysis in plants (Fahima *et al.*, 1998). The RAPD results in this study showed that *A. andersonii* and *A. humilis* gave the same size and number of RAPD bands while those of *A. hildebrandii* were a bit different. Technically speaking, RAPD products from three RAPD primers did not show genomic DNA variation between *A. andersonii* and *A. humilis* at all. Since amount of their genomic DNA was limited, only three RAPD primers were used in this MSc study and more PCR-RAPD studies between *A. andersonii*, *A. humilis*, *A. hilderbrandii*, and probably also *A. gracilis* are necessary to confirm this hypothesis.

Considering the major clade II from the semistrict consensus tree (Fig. 30), *Aeschynanthus* sp. 20002051, *A.* sp. 20000557A, *A. tricolour* x *parvifolious*, *A. parvifolius*, *A. javanicus*, *A. chrysanthus* and *A. radicans* were clustered together as a group of members from the section *Aeschynanthus*. Their grouping was unresolved because of low amount of synapomorphic characters. This polytomic clade from the parsimony analyses did not show any clear phylogenetic relationship between these species. However, the distance method (Fig. 32) could give some suggestion on their relationship from amount of genetic distances. Up to date, *A.* sp. 20002051 and *A.* sp. 20000057A have not been classified to any section yet. But from their positions on the phylogenetic tree, these two specimens should be placed in the section *Aeschynanthus*. The same idea could be applied to *A. tricolor* X *parvifolius*, a supposingly hybrid plant long-time growing in RBGE. This plant was also clustered in the section *Aeschynanthus* subclade with a clear ITS sequence and very much identical to *A. parvifolius*, the species supposed to be its paternal side. The clear ITS sequence of *A. tricolor* x *parvifolius* raised the question whether this plant was actually a misidentified *A. parvifolius* or there was an unusual phenomenon of nuclear ribosomal DNA selection in a hybrid plant.

There are several other groupings of newly-added *Aeschynanthus* taxa which are quite interesting in the major clade II. For instance, *A.* aff. *siphonanthus* was grouped with the pair of *A. malulidii* 19980282 and *A. malulidii* 19980238, instead of *A. siphonanthus*. The coupling between *A. malulidii* 19980282 and *A. malulidii* 19980238

was expected because both of them may be the same species collected in the same expedition. However, *A.* aff. *siphonanthus* which should have similar morphology to *A. siphonanthus* was surprisingly clustered with *A. malulidii*. More closely study on morphology of *A.* aff. *siphonanthus*, especially on its reproductive parts, is needed for further discussion. Such unexpected position of some taxa in clade II also occurred with *A. burttii* 19980562. This plant was paired with *A.* sp. 00293 instead of sistered to *A. burttii* 19980562. The phylogenetic tree therefore suggested that an unidentified *A.* sp. 00293 would also be named as *A. burttii*. Further study on *A.* sp. 0029 morphology should be done in the future as the case of *A. siphonanthus*.

The phylogenetic tree of *Aeschynanthus* does not only have a collaborated trend to follow biogeography of the whole genus as found in the previous study (Denduangboripant *et al.*, 2001). But this phylogenetic tendency could also be applied to biogeography of *Aeschynanthus* species in Thailand. Almost all Thai *Aeschynanthus* were placed in the major clade I of the phylogenetic tree. These species are *A. flugens, A. macranthus, A. parviflorus, A.* aff. *parviflorus, A. lineatus, A. hossesuii, A. humilis, A. andersonii, A. hildebrandii, A. acuminatus, A. longicaulis, A. fecandus,* and *A. speciosus*. Majority of species in the clade I occur in mainland South and Southeast Asia. The long filiform seed-appendages possessed by most clade I species were proposed to be effective for wind dispersal in seasonally dry climates. More discussion about seed morphology and biogeography of some Thai *Aeschynanthus* in clade I are in the section 5.2. However, no information about seed appendage characteristics of *A.*

speciosus, *A.* aff. *parviflorus*, *A. hosseusii* and *A. hildebrandii* is available here and further SEM study to check their seed types is necessary to confirm or reject this phylogeography tendency. The two other Thai *Aeschynanthus* placed in Clade II, *A. parvifolius* and *A. radicans*, are well-known to be in the section *Aeschynanthus*. Members of this section were found only in the clade II and they occur mainly in eastern Malesia, particularly on the Sunda shelf islands and east of Wallace's line. *Aeschynanthus radicans* and *A. parvifolius* follow this biogeography trend. The two species are common in rain forests of southern part of Thailand. Climate of the South is so similar to that of Malesia area, especially to the Malay Peninsula. Moreover, seed of *A. radicans* and *A. parvifolius* could have crossed the Thailand-Malaysia border easily since long times ago or even recently. Again, more discussion about distribution patterns of these two Thai species could be found in the section 5.2.

5.2 Morphological and cytological characteristics of some *Aeschynanthus* in Thailand

Comparing seed and flower morphology to distribution areas of *Aeschynanthus* in Thailand revealed biogeographical trends of these plants. Flower morphology among Thai *Aeschynanthus* species is little different from each other depending on their habitat. Normally, *Aeschynanthus* colours are not too much different between species because the colour should be in a bright tone (red, orange or yellow) and attractive to avian

pollinators in the wild. On the other hand, seed appendages of each *Aeschynanthus* species could be very much different from each other regarding to its sectional classification. This seed appendage variation has been reported to relate to ecology of each species (Denduangboripant *et al.*, 2001).

Literature reviews and herbarium visiting indicated that *Aeschynanthus humilis* and *A. andersonii* have been found only in Chiangmai and Lumpang provinces in the North of Thailand. These two species were placed in the section *X* because of their single long seed-appendage at the hilar-end. Such seed appendage morphology agrees well with the distribution area of the plants since the long thin hairs could help its seeds to be carried away easily by wind. This wind dispersal mechanism should work well in a partially windy and seasonally humid climate of mountain areas in the northern part of Thailand. Another *Aeschynanthus* only found in the North is *A. lineatus*, also in Lumpang province. This species follows the same trend of having wind-dispersal mechanism as since it belongs to section *Diplotricium* with two long seed-appendages at the seed hilar end. If sectional classification could help predicting a possible ecology of *Aeschynanthus* species, *A. hookeri* which was reported to be in the section *Diplotricium* too but no information available about their locality in Thailand, would be found in a high latitude area of the North as well as *A. lineatus*.

In the case of *Aeschynanthus acuminatus* which belongs to the section *Haplotricium*, this species was found in Loei province at Phu kradueng national park. Although this province is in the Northeast which is the driest region of Thailand, Loei

locates in a high latitude and close to other northern provinces with similar climate to the North. Moreover, Phu kradueng national park is also in mountainous area and its climate is so similar to that of Chiangmai province. This upper northeastern ecology then still supports the distribution mechanism with seed appendage type of the section *Haplotricium* which are quite similar to the section *X* but shorter.

The wind dispersal mechanism should work best with *Aeschynanthus* in the section *Polytricium*. *Aeschynanthus* species in this section have seeds with many long appendages (coma) at the hilar end and this coma has greatest effectiveness in the wind dispersal. The wilder distribution of the section *Polytricium* could help explain why *A. longcaulis*, another *Polytricium* species, has been found both in an upper northeastern province like Lumpang and also in Chantaburi in the eastern part of country. The effectiveness of the coma-like organ on *A. longicaulis* seeds has expanded their dispersal range to reach Yala province at the most South of Thailand. Note that in contrary to the great distribution range of *A. longicaulis*, the distribution areas of *A. radicans* and *A. parvifolius* are limited to be only in the Southern part of Thailand. *Aeschynanthus parvifolius* was found only in Naratiwat province while *A. radicans* have been reported from Sungkhla, Nakhon Si Thammarat, Naratiwat, and Trung provinces, all in the South. Their localities are usually in shade areas along the streams or the rivers. These two species are in the section *Aeschynanthus* with short seed appendages at both ends and bubble-like cells on the seed surface. The bubble-like cell is a unique character of this *Aeschynanthus* section and it has been suggested to be another

important dispersal mechanism of the genus (Denduangboripant *et al.*, 2001) to help the seeds floating in riverine water.

Another important information to consider when study genetic relationship between species is cytogenetic information. Unfortunately, the cytogenetic study performed both in previous research and in this study revealed that chromosomes of most *Aeschynanthus* species are so small and then their chromosome numbers were not easy to be counted. This problem in chromosome size comes from the nature of chromosomes themselves, not from unoptimised cell treatment. Thus, karyotype preparation of *Aeschynanthus* was eventually believed to be impossible. Nevertheless, exact chromosome counting for some *Aeschynanthus* species had been performed in this study. The chromosomes numbers of six *Aeschynanthus* species (*A. andersonii*, *A. hildebrandii* (19991628), and *A. humilis* were counted and found 2n=28 in each cell wherease those of *A. radicans*, *A. obconicus* and *A.* sp. JJ_001 were 2n=32. Finding proper cells in metaphase stage of mitotic division was not easy since the only rough guide to that preferred mitotic stage was an external appearance and size of the root tips. The high chromosome number (2n=32) found in *A.* sp. JJ_001, *A. obconicus* and *A. radicans*. Among these six *Aeschynanthus* species there was only the chromosome number of *A. obconicus* which did not clear enough and the final number needed to follow previous reports. Chromosome sizes of these six *Aeschynanthus* species were very small, as tiny points when observed under normal light microscope even with highest magnification (1000X). This finding agrees very well with previous cytogenetic

investigations of Rashid *et al.* (2001) whom found that average *Aeschynanthus* chromosomes are smaller than 1.5 micron. In fact, having very small chromosomes is a common phenomenon in many plants and algae (Mutue and Marcelo 1997). This tiny size therefore prohibits cytologists to determine types of *Aeschynanthus* chromosome. Dr. Michael Moeller, a cytological expert in Gesneriaceae of RBGE also confirmed that it is very difficult to determine the *Aeschynanthus* chromosome types with classical cytogenetic methods either C-banding or G-banding technique. To resolve this problem, any cytologist working on *Aeschynanthus* chromosomes should observe the chromosomes under electron microscope or use advance molecular technique to locate a centromere of *Aeschynanthus* chromosome with Fluorescence *in situ* Hybridization (FISH) technique.

So far, the chromosome number information from this study suggested that *Aeschynanthus* species in Thailand, though vary in morphology, could be grouped cytologically into three. The first group is *Aeschynanthus* species having a chromosome number of 2n=28, then those with 2n=30, and the last group with 2n=32 or 64 (polyploidy). Interestingly, although *Aeschynanthus radicans* was clearly found in this experiment to have a chromosome number of 2n=32, previous studies reported a chromosome number for this species as 2n=30 or 32 (Table 9). This mosaic chromosome number is not unique to *A. radicans* since it also occurred in *A. parasiticus.* Eberle and Prentice (1964) suggested that this chromosome variation within a species may be a result of dysploid changing in the chromosome number.

Chromosome number aberration has become established in sections *X*, *Diplotricium*, *polytricium* and also the section *Aeschynanthus*. This presumably represents many independent dysploid evolutionary lines from 2n=32 to 2n=30 and eventually to 2n=28 in *A. gracilis* of the section X (Ratter, 1978). The chromosome number of *Aeschynanthus* does not vary within species only by one or two chromosomes, but some species could have higher polyploidy in the same species. For example, *A. ellipticus* has chromosome number equally to 2n=32, 64 (tetraploidy) and 96 (hexaploidy) resulted from a duplication of the basic chromosome number X=16 (Rashid *et al.*, 2001).

Usual chromosome numbers of *Aeschynanthus* are either 2n=30 or 32 which come from the basic chromosome number of the genus x=15,16 (Rashid *et al.*, 2001). However, an unusual chromosome number 2n=28 could be found specifically in the group of *A. humilis*, *A. hildebrandii*,(19991628), *A. andersonii* and *A. gracilis* (Rashid *et al.*, 2001). Such similarity in having a rare chromosome number of this group supports the molecular phylogenetic result which placed *A. humilis*, *A. hildebrandii* (19991628) *A. andersonii* and *A. gracilis* into the same subclade. The finding that the chromosome number of *Aeschynanthus* is more or less collaborate to ITS phylogeny could give an implication to sectional classification.

The recent seed morphological study had moved *A. andersonii* and *A. humilis* from section *Haplotricium* to section *X* (Rashid *et al.*, 2001). Their chromosome number 2n=28 was then suggested to be unique only for the section *X*. Although *A. longicaulis*, a species belongs to section *Polytricium*, once had been reported to have a

chromosome number of 2n=28 and 30, later studies (Roger 1954; Ratter and Prantice, 1964) suggested that it should be 2n=30. Thus, only members of the section *X* could have the rare chromosome number of 2n=28. Since *A. humilis*, *A. hildebrandii* (19991628) *A. andersonii* and *A. gracilis* were clustered as a subclade on the phylogenetic tree (Fig. 45) and all have that unusual chromosome number, the four species must have very close evolutionary genetic relationship. This suggestion supports the recommendation of moving *A. andersonii* and *A. humilis* to the section *X* and also could predict that *A. hildebrandii* should be placed in the section *X* too, even though its seed testa type has never been studied before.

The last *Aeschynanthus* species studied in this cytological investigation was *A.* sp. JJ_001, the strange *Aeschynanthus* cultivar bought from Jatujak market with pink flowers. The chromosome number of this plant was found equally to 32. Chromosome of *A.* sp. JJ_001 were prepared with two different methods. The first method used enzymes to lyse root cells before staining with an aceto-carmine dye while the other method uses a hematoxylin dye without cell lysis step. Both staining methods showed the same chromosome number for *A.* sp. JJ_001 but different in chromosome clarity on the slides. The cell-lysis method could separate the root cells from each other better than the hematoxylin method. However, reddish colour chromosomes stained with an aceto-carmine dye was not easily to observe, not so good as the blue colour from hematoxylin dye. The chromosome number (2n=32) of *A.* sp. JJ_001 confirmed the hypothesis suggested from the molecular phylogenetic tree that this plant should be placed in

section *Aeschynanthus*. Molecular classifying *A.* sp. JJ_001 into the section *Aeschynanthus* was also supported by its shallow-lobed calyx morphology, a unique character of the section, and then with this cytological evidence, i.e. all members of the section *Aeschynanthus* should have a chromosome number of 2n=30 or 32 (or 64) only.

Table 10 summary of *Aeschynanthus* chromosome numbers counted to date relative to sectional classification (all given as 2n for ease of comparison), modified from works of Rogers, 1954; Eberle, 1956; Ratter, 1963; Ratter and Prentice, 1967; Ratter and Milne, 1970; Milne, 1975; Hellmayr, 1989; Kiehn and Weber, 1997; Rashid *et al.*, 2001.

Type A seed group	2n	Type B seed group	2n
Section *Microtricium*		Section *X*	
A. buxifolius	32	*A. angustifolius*	30
A. ellipticus	32, 64, 96	*A. ceylanicus*	32
A. guttatus	32	*A. gracilis*	28
A. horsfieldii	32	*A. hosseussii*	32
A. rhododendron	32	*A. longiflorus*	30 (21, 28)
A. magnificus	32	*A. speciosus*	64
A. nummularius	64	*A. andersonii*	28*
A. vinaceus	32	*A. humilis*	28*
		A. hildebrandii	28*
Section *Aeschynanthus*		*Section Diplotricium*	
A. arctocalyx	32	*A. hookeri*	32
A. boschianus	64	*A. lineatus*	30
A. javanicus	64	*A. parasiticus*	30, 32
A. obconicus	32, 32*	*A. parviflorus*	32
A. obovatus	32	*A. sikkimensis*	32
A. parvifolius	32, 64	Section *Polytricium*	
A. praelongus	32	*A. albidus*	30
A. pulcher	60, 64	*A. fecundus*	32
A. radicans	30, 32, 32*	*A. longicaulis*	28, 30
A. tricolor	32	*A. myrmecophilus*	64
Section *Haplotricium* sens. str.			
A. bracteatus	32		

Numbers in brackets= polysomatic counts, * counts from this Msc study.

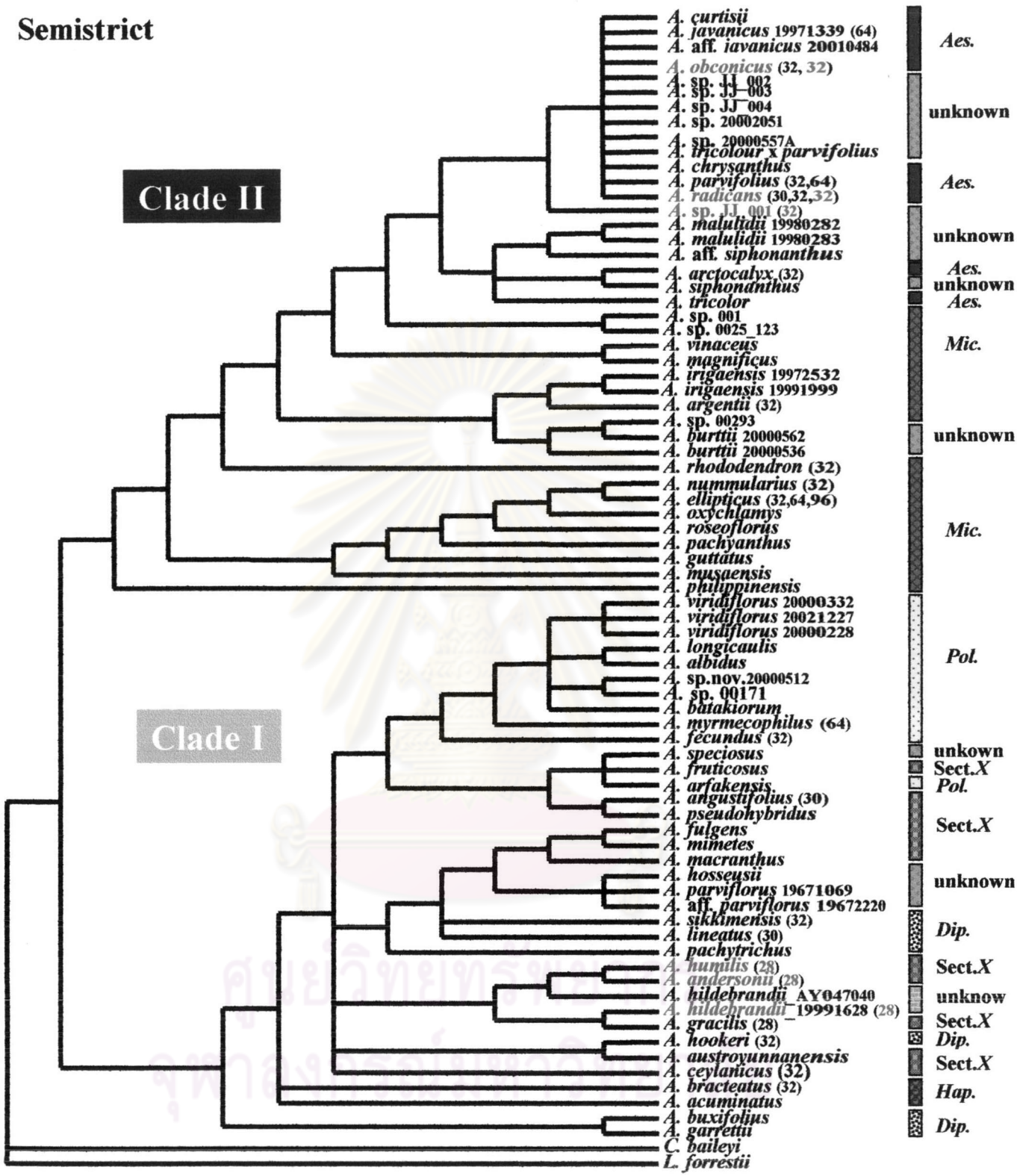

Fig. 44 A semistrict consensus tree of 203,700 most parsimonious trees (743 steps in length) for 77 *Aeschynanthus* taxa and two outgroup Gesneriaceae taxa (*Cytandra baileyi* and *Lysionotus forrestii*) based on parsimony analysis of the combined ITS1 and ITS2 sequence data without gap matrix. Taxon names in pink colour letters are *Aeschynanthus* samples used in cytogenetic experiments. Available chromosome numbers in red colour letter (Rogers, 1954; Eberle, 1956; Ratter, 1963; Ratter and Prentice, 1967; Ratter and Milne, 1970; Milne, 1975; Hellmayr, 1989; Kiehn and Weber, 1997; Rashid et al., 2001) are given in brackets following the species names (all as 2n for ease of comparison).

5.3 Molecular identification of cultivated and wild *Aeschynanthus* and a discovery of putatively new *Aeschynanthus* species

Now a day, advance technology has allowed researchers to identify specific names of organisms by using only a single cell. This molecular technology is based on gene amplification of DNA target organisms and the nucleotide sequence comparison of that multiplied gene with large nucleotide databases. The benefit of molecular identification has been increasing reported such as to identify bacteria and their catabolic genes in the environment (Widoda *et al.*, 2002) and to identify nontuberculous mycobacteria with faster and more accurate ability and also to discover a new species (Turene *et al.*, 2001). Using molecular analyses to name plant specimens is better than conventional identification methods when the plants they are not easily identified because of changing in colour and size. Morphology of many herbarium specimens could be modified by chemicals used in specimen preparation and some parts of the specimens would disappear after long time kept in the herbarium. Ideally, using both molecular data and morphological data retrieved from herbarium specimens should give the most accurate identification.

Using ITS sequences to perform molecular identification in both cultivated and wild *Aeschynanthus* sounds promising. Phylogenetic trees made from ITS sequences usually give a higher resolution power on species level than from other genes such as chloroplast *trn*-L intron. Moreover, conventional identification methods are also less effective when lack of important reproductive parts. This was also the case in

Aeschynanthus when collecting the plant samples either from Jatujak market or from Chiangmai on the second expedition. All of these plants had lost their flowers and then the molecular identification should be a better technique to rapidly identify the plants. DNA sequencing technique also has an advantage that it could be performed on most parts of the and the collector did not need much knowledge in taxonomy.

From the ITS sequences of 13 tested plants (six cultivars *Aeschynanthus* bought from Jatujak market and seven wild *Aeschynanthus* collected from Chiangmai), all but one were confirmed to be true members of the genus after Blast-searched against GenBank nucleotide database. The only wrong-named cultivars, *A.* sp. JJ_006 was found possibly belonging to the genus *Columnae* with the highest identify score when compared to *Columnae linaris* (Fig. 46). *Columnae* is a New World counterpart of *Aeschynanthus*, with similar flower characteristics, but found only in tropical areas of the Americas. This misidentification could happen if the local plant importer was misled about the correct name of this plant by its flowers similar to those of *Aeschynanthus* flowers. Apparently, comparison between *A.* sp. JJ_006 and *Columnae linaris* showed many differences in morphology especially in their petal colour and availability of thin hairs on leaf surfaces. *Columnae linaris* has red flowers without leaf hairs while *A.* sp. JJ_006 was found having yellow flowers with a lot of hairs under and above leaf surfaces. In fact, *A.* sp. JJ_006 should be morphologically identified as *Columnae argentea* (Fig. 47) because of their similar colour of flowers. Since ITS sequences of *Columnae argentea* had never been reported in GenBank, Blast-searching result of *A.*

sp. JJ_006 ITS sequence could match only to *Columnae linaris*. The ITS sequence of *Columnae argentea* therefore is needed to test this hypothesis whether *A.* sp. JJ_006 was actually *Columnae argentea*.

For the other twelve true *Aeschynanthus*, only the ITS sequences of *A.* sp. JJ_005 gave sequencing problems from an intra-individual sequence variation in the ITS regions. This phenomenon is common in *Aeschynanthus* and has been reported before in the previous study of Denduangboripant and Cronk (2000). They have shown that this intra-individual problem of ITS sequence could be solved by using molecular cloning method and then sequencing on each ITS clone. Both forward and reverse sequences of *A.* sp. JJ_005, though too awkward to be put together as a complete ITS sequence, were good enough to confirm that this plant was certainly *Aeschynanthus*. In the case of other four *Aeschynanthus* cultivars, their ITS sequences could be assembled and aligned nicely to the sequence data matrix of 73 *Aeschynanthus* taxa. The new *Aeschynanthus* phylogenetic tree was then reconstructed and it placed the cultivars *A.* sp. JJ_002, *A.* sp. JJ_003, and *A.* sp. JJ_004 (which were quite similar to each other) in the same group as *A. curtisii*, *A. javanicus*, *A. obconicus*, *A.* sp. 20002051, *A.* sp. 2000577A, *A. tricolor* X *parvifolius*, *A. chrysanthus*, *A. parvifolius*, and *A. radicans*. Most of these species are so far known as being in the section *Aeschynanthus* and therefore these four *Aeschynanthus* cultivars should be classified as members of the section *Aeschynanthus* too. After comparing genetic similarity among taxa with this group, *A.* sp.JJ_003 and *A.* sp. JJ_004 may be the same species and could be *A.* cf. *radicans*

(Fig. 48). *Aeschynanthus radicans* and *A. parvifolius* are the only two Thai species in this clade. But, the morphological characteristics of *A.* sp. JJ_003 and *A.* sp. JJ_004 were more similar to those of *A. radicans* than *A. parvifolius*. This conclusion agrees well with the seller's comments that the three cultivars were originally collected in Thailand. Another cultivated *Aeschynanthus*, *A.* sp. JJ_002, was also similar to *A. radicans* in DNA sequences but a little different in morphology. Some morphological differences between vegetative parts of *A.* sp. JJ_002 and *A. radicans* were observed. *Aeschynanthus* sp. JJ_002 had red stems and petioles instead of green colour as those of common *A. radicans*. Since this molecular data collecting project of all Thai *Aeschynanthus* species has not been completed yet, *A.* sp. JJ_002 cultivar might be identified as varience of *A. radicans*.

The most interesting point found from this experiment is the position of *Aeschynanthus* sp. JJ_001 on the phylogeny. This plant had a very unusual, pale-pinkish flowers (Fig. 49) which have never been observed before within the genus. The plant seller commented on an originality of this strange plant that this pink *Aeschynanthus* was a wild plant and imported from Sumatra, Indonesia. Two hypotheses could be drawn to explain this strange characteristics: *A.* sp. JJ_001 was either a cultivated hybrid plants or an undiscovered new species. If *A.* sp. JJ_001 was a true hybrid *Aeschynanthus*, the pink plant should have been resulted from a crossing between one *Aeschynanthus* species with red flower and another species with white colour flowers. Apparently, there is no reported about white *Aeschynanthus* except that

of *A. xanthanthos*, a rare species found only in China. Moreover, the ITS sequences of *A.* sp. JJ_001 was completely clear without any sign of intra-individual sequence variation. This also suggests that the plant did not originate from a hybridisation between two species but being a unique species.

On the other hand, the idea that *A.* sp. JJ_001 was an undiscovered new species agrees with an estimated evolutionary time of its speciation. Using molecular clock approach, a divergence of any species could be estimated by calculation from its sequence substitution rate. If the substitution rates of ITS sequences among plants are assumed to be more or less close to each other, such rates could be used to formalise an estimation of divergence time among species. There are some estimated ITS substitution rates reported for several plant groups, to be between 2.44×10^{-9} and 5.69×10^{-9} substitutions/site/year (s/s/y). On average, *Aeschynanthus* species have 38 ITS substitutions (in 542 sites) from the most recent common ancestor *Aeschynanthus* node to the tip of each branch. *Aeschynanthus* sp. JJ_001 has 5 base pair substitutions (in 572 site) as its autapomorphy (Fig. 29). Therefore, the estimated diversification time of *A.* sp. JJ_ 001 may be between 1.4 and 3.3 million years ago with a mean estimate of 2.34 mya. This ancient diversification time then suggests that *A.* sp. JJ_001 with pink flowers should have its own evolutionary history. More investigations are needed on its original collection area, flower morphology and seed characteristics, especially whether it has bubble-like cells on its seeds, an important character of the section *Aeschynanthus*. So far, from the ITS phylogenetic experiments, this pink *Aeschynanthus*

should be placed in the section *Aeschynanthus*. This molecular suggestion also agrees with the plant's shallow-lobed calyx character, another unique morphology of the section. A taxonomic expert on *Aeschynanthus* the genus, Mary Mendum of Royal Botanic Garden Edinburgh had been consulted. She also agreed that this plant could be recognised as a new undescribed species, even though this plant was commercially cultivated.

For molecular identification experiments of the other seven *Aeschynanthus* collected in the wild from Chiangmai province (*A.* sp. CM_007, *A.* sp. CM_009, *A.* sp. CM_013, *A.* sp. CM_022, *A.* sp. CM_026, *A.* sp. CM_030 and *A.* sp. CM_034) Blast-searching indicated that all of them had identical ITS sequences to those of *A. hildebrandii* AY047040, but not to *A. hildebrandii* 19991628. This finding is reasonable because *A. hidebrandii* is a common species of *Aeschynanthus* reported in Sutep Mountains. This identification from molecular data had been proved after revisiting the plant on flowering season. An interesting question came from the sequence similarity between Chiangmai *Aeschynanthus* and *A. hildebrandii* AY047040, not *A. hildebrandii* 19991628. The ITS sequence of *A. hildebrandii* AY047040 came from the works of Zimmer *et al.* (2002) on "Phylogenetic relationships in the Gesnerioideae (Gesneriaceae) based on nrDNA ITS and cpDNA *trn*L-F and *trn*E-T spacer region sequences" while *A. hildebrandii* 19991628 was given by Royal Botanic Garden Edinburgh. From all phylogenetic trees reconstructed in this MSc thesis, *A. hildebrandii* AY047040 always appeared to be close to *A. andersonii* and *A. humilis* – two other

synonymous species in question. However, *A. hildebrandii* 19991628 did not seem to be in this group of three, but usually paired with *A. gracilis.* Thus, a reasonable explanation for this problem is that *A. hildebrandii* is a correct name for the plant yeilded AY047040 ITS sequence of Zimmer *et al.* (2002) whereas the plant 19991628 of RBGE may have been misidentified. This error could happen from a similarity in vegetative morphology of these four *Aeschynanthus* taxa. Since the phylogenetic tree suggested that *A. hildebrandii* 19991628 was a sister taxon of *A. gracilis* and *Aeschynanthus* 19991628 may actually be *A. gracilis.* To clarify this skeptic *A. hildebrandii* 19991628 problem, reproductive parts (i.e. flower and seed) of this plant need to be investigated. At the mean time, this 19991628 plant will be called as *A. hildebrandii* through out this thesis.

Fig. 45 Flower morphology of *Columnae linaris* (photo by Ruth Zavitz, retrieved from http://www.gesneriads.ca/colum020.htm).

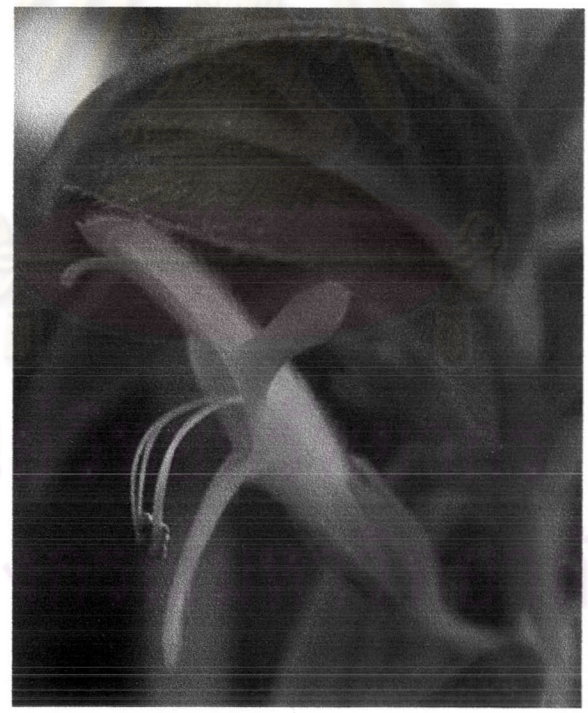

Fig. 46 Flower morphology of *Columnae argentea* (photo by Toshijiro Okuto, retrieved from http://www.gesneriads.ca/colum131.htm).

Fig. 47 Morphological characteristics of *Aeschynanthus radicans* (photo by Ron Myhr, retrieved from http://www.gesneriads.ca/aeschy20.htm).

Fig. 48 Flower characteristics of *Aeschynanthus* sp. JJ_001 with its unique pale pinkish