CHAPTER II

BACKGROUND

2.1 The genus *Aeschynanthus*

2.1.1 Characteristics of the genus *Aeschynanthus*

The genus *Aeschynanthus* Jack (Gesneriaceae, subfamily Cyrtandroideae, tribe Trichosporeae) contains about 160 species widely distributed in subtropical and tropical forests in the Indo-Malayan region, from Southern India and Sri Lanka, throughout the Himalayas to Southern China, Indo-China, Southeast Asia, New Guinea and the Solomon Islands (Fig. 1).

Aeschynanthus are evergreen subshrubs, climbers, or trailing perennials. They are usually epiphytes in the wild, growing on trunks and branches of forest trees, and a few species are semi-terrestrial. Several species are cultivated for their splendid flowers. They are attractive plants with twiggy, arching or flexuous, pendulous stems and numerous pairs of leaves, opposite or rarely in whorls (e.g. *A. angustifolius*). The leaves are ovate to lance-shaped, thick and leathery, and markedly dorsi-ventral in all species (Rosser and Burtt, 1969). In the leaf there is a thick hypodermis, which is characteristic of the genus, and associated with water storage function in epiphytic plants (Rosser and Burtt, 1969). The inflorescences are usually axillary and/or pseudo-terminal, though some variation is found within species. The flower colour is commonly brilliant red, more

rarely orange, yellow or greenish-brownish. The five sepals are either free, partially connate, or tubular or saucer-shaped with small blunt lobes. The calyx varies from green to red or purple and probably plays a part in the attraction system of the flower. The corollas are always tubular, widening upwards, usually curved and zygomorphic. The four stamens form two pairs, the anthers of each pair cohering at their tips and held either at the mouth of the corolla, or exserted. The ovary is stipitate, long and slender and bears a long style with a peltate or capitate stigma at the top (Christopher, 1996; (Mendum *et al.*, 2001).The pollen characters of *Aeschynanthus* and other Old World Gesneriaceae were studied by Luegmayr (1993). The fruits are long slender unilocular capsules containing many small seeds, each of which lies with its apical end pointing towards the base of the capsule. A single appendage develops from the apical end and one or more appendages develop from the hilar end of each ovule. The possession of these seed appendages defines the tribe *Trichosporeae* (*Aeschynanthus*, *Agalmyla* [*Dichrotrichium*], *Loxostigma*, and *Lysionotus*). Seed of the doubtful genus *Micraeschynanthus* is not known. When the capsules dry, they open loculicidally by two valves, releasing the wind-borne seeds. Dispersal is especially effective where the appendages form a coma (section *Polytrichium*) or have bubble-like cells at the base of the hilar appendage (most members of section *Aeschynanthus*). The appendages may also serve as anchorage devices for seeds landing on trees (Mendum *et al.*, 2001). *Aeschynanthus* develops unequal cotyledons soon after seedling germination, a character of the Old World subfamily Cyrtandroideae (Burtt and Woods, 1958).

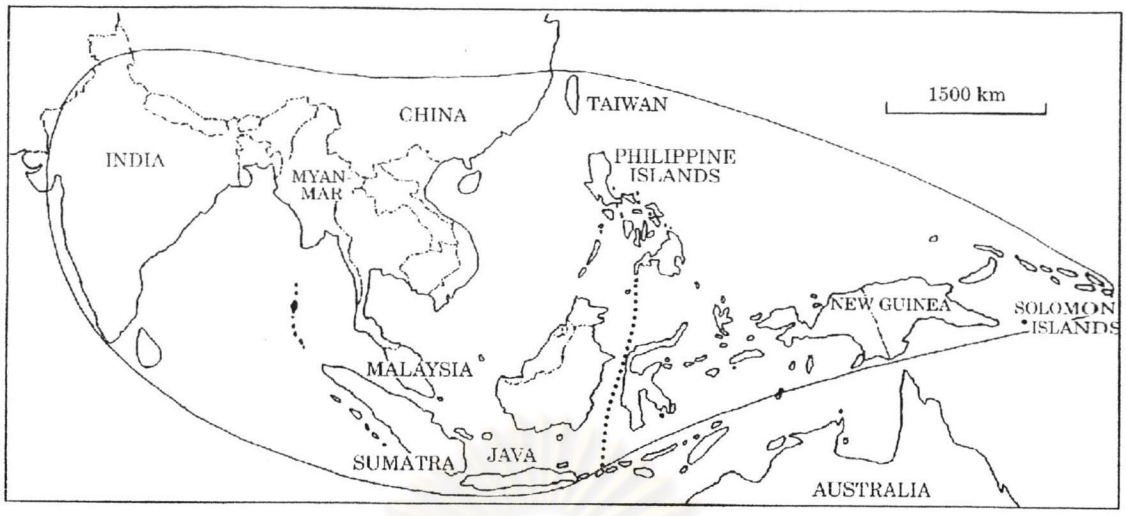

Fig. 1 Geographical distribution of *Aeschynanthus*, indicated by a solid line. Wallace's line is indicated out by a dotted line (after Mendum *et al.*, 2001).

The tubular corollas with strong protandry, and the production of copious nectar, suggest that *Aeschynanthus* are pollinated by birds (Nectariniidae). Although records of actual bird visits are very rare, the suggestion is supported by low-sucrose-content nectar, which is common in ornithophilous flowers. *Agalmyla* (Gesneriaceae, also in the tribe Trichosporeae) has the greatest floral similarity with *Aeschynanthus* because of an adaptation syndrome to bird pollination. However, some Chinese species in *Aeschynanthus* section *Xanthanthos* may be excepted, and the likelihood of bird pollination is less obvious for those species with greenish flowers (found mainly in section *Polytrichium*) (Mendum *et al.*, 2001). Although *Aeschynanthus* is widespread, there is a high degree of endemism at the species level (Mendum *et al.*, 2001).

2.1.2 Taxonomy of *Aeschynanthus*

The generic name *Aeschynanthus* was established by Jack in 1823, soon after Don (1822) had already published the generic name *Trichosporum*, a reference to the appendages on the seeds. The name *Aeschynanthus* was considered to be preferable to *Trichosporum* by other influential botanists such as Bentham (1876) and Clarke (1883). The later name was then conserved and the older name *Trichosporum* is reflected in the tribal name Trichosporeae, in which *Aeschynanthus* is the largest genus. Bentham (1876) first proposed a sectional classification based almost entirely on seed appendages, and recognised four sections, *Haplotrichium*, *Diplotrichium*, *Polytrichium*, and *Holocalyx* (later changed to *Aeschynanthus*). The fifth section, *Microtrichium*, was added by Clarke in 1883. Section *Anisocalyx* was proposed by Schlechter (1923), but was later subsumed under *Microtrichium* by Burtt and Woods (1975). Section *Xanthanthos*, a very small sixth section of two Chinese species, was created by Wang (1984) and based not on seed but on corolla characters. He also divided section *Haplotrichium* into two series: *Bracteati* and *Novograciles*. This resulted in the following classification:

1. Section *Aeschynanthus* (= section *Holocalyx* Benth.): type species *A. volubilis* Jack, seed with single appendage at hilar end, with a podium of bubble-like cells at the base; calyx with blunt shallow lobes; Fig. 2

2. Section *Haplotrichium* Benth.: type species *A. bracteatus* [Wall. ex] DC, seed with a single long appendage at each end; calyx with acute lobes or deeply divided to base; Fig. 2 Series *Bracteati* W.T.Wang; type species as for section

- Series *Novogracilis* W.T.Wang; type species *A. novogracilis* W.T.Wang (= *A. gracilis* [Parish ex] C.B.Clarke)

3. Section *Diplotrichium* Benth.: type species *A. parasiticus* (Roxb.) Wall, seed with two long appendages at the hilar end and a single appendage at the apical end; Fig. 3

4. Section *Polytrichium* Benth.: type species *A. longicaulis* [Wall. ex] R.Br., seed with few to many appendages at the hilar end and a single appendage at the apical end; Fig. 3.

5. Section *Microtrichium* C.B.Clarke (including section *Anisocalyx* Schltr.): type species *A. microtrichus* C.B.Clarke, seed with short, broad-based flat appendages at either end; Fig. 3

6. Section *Xanthanthos* W.T.Wang.: type species *A. denticuliger* W.T.Wang, habit trailing; corolla white or yellow; stamens not exserted.

Fig. 2 Examples of flower morphology of *Aeschynanthus*: from top to bottom; *A. siphonanthus* (section *Aeschynanthus*) - species in this section have a distinctive shallow-lobed calyx, and *A. bracteatus* (*Haplotrichium*). Photos are by courtesy of the Royal Botanic Garden Edinburgh.

Fig. 3 Examples of flower morphology of *Aeschynanthus* (continued): from top to bottom; *A. lineatus* (section *Diplotrichium*), *A. longicaulis* (*Polytrichium*) and *A. buxifolius* (*Microtrichium*).

The Royal Botanic Garden Edinburgh has an extensive living collection of *Aeschynanthus*. Current studies of this genus at the Botanic Garden had been undertaken by Mary Mendum before she passed away and were concentrating mostly on the Malaysian, Philippine, and New Guinea species (for instance, Mendum, 1995, 1999; Mendum and Madulid, 1995; Mendum and Woods, 1997). Her most recent studies (Mendum *et al.*, 2001) on seed and appendage morphology identified two major groups (type A and B) within the genus (Fig. 4). Type A seed has spiral testa cell orientation, papillae formed from a single cell and short smooth appendages. Type B is recognised by the straight orientation of the testa cells, combined with the presence of papillae formed form the raised ends of two adjacent cells on the long hair-like appendages and usually on the testa. The group of species with type A seeds encompasses sections *Microtrichium*, *Aeschynanthus*, and *Haplotrichium* sens. str. (with *A. bracteatus*, a sectional type species). Species with type B seeds comprise sections *Polytrichium*, *Diplotrichium*, and *Xanthanthos*. Many species previously placed in section *Haplotrichium* were found to have type B seeds. These species are currently placed in the section *X*, which cannot yet be circumscribed until more material of section *Xanthanthos* becomes available for study. They may belong in section *Xanthanthos*, but not as that section is currently described. Section *Haplotrichium* sens. str. is a very small section of about eight known members, confined to mainland South and East Asia.

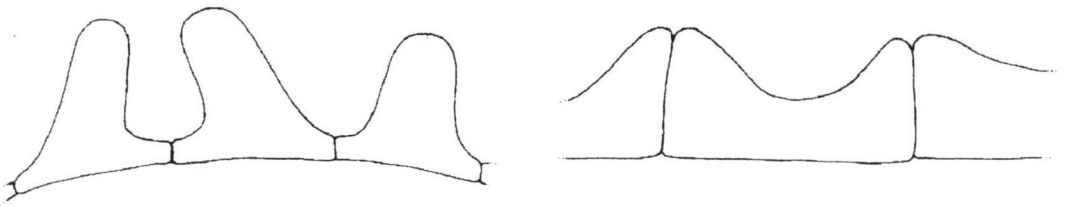

Fig. 4 Comparison of papillae of type A seed with single-celled papillae (left, of *A. parvifolius*) and type B seed with two-celled papillae (right, of *A. albidus*), after Mendum *et al.*, 2001.

Based on these findings a revised key to the sections (Mendum *et al.*, 2001) is as follows (see also Table 1 and Fig. 5).

Revised key to the sections of *Aeschynanthus*

1a. Seed with a single appendage at hilar end 2

1b. Seed with more than 1 appendage at hilar end 6

2a. Appendage short, not papillose.....3

2b. Appendage long, slender, papillose.....5

3a. Testa cell orientation anticlockwise, rarely straight; inflorescences rarely pedunculate; bracts small, not persistent 4

3b. Testa cell orientation clockwise; inflorescences long pedunculate; bracts large, usually persistent ..... Sect *Haplotrichium* Sens.Str.

4a. Appendages not slender to base; calyx without abscission layer at base, usually deeply divided, rarely tubular or spathaceous ..... Sect. *Microtrichium*

4b. Appendages slender to base, bubble cells often present; calyx with abscission layer at base, tubular or cup-shaped, lobes rounded, rarely pointed

..... Sect *Aeschynanthus*

5a. Corolla white or yellow, stamens not exserted ..... Sect. *Xanthanthos*

5b. Corolla orange, red or green, stamens usually exserted ..... Sect. *X*

6a. Seed with 2 appendages ..... Sect. *Diplotrichium*

6b. Seed with a coma of several to many appendages ..... Sect. *Polytrichium*

Table 1 Revised sectional classification of *Aeschynanthus* and characteristics of the sections.

Section	Morphological characthers	Type	number of species	Distribution
Aeschynanthus	seed hilar appendage single, to 15 mm, slender to base; often with a podium of bubble cells at the base; testa cell orientation an anticlockwise spiral; calyx tubular or campanulate with abscission layer at base; lobes almost always blunt; stamens not or slightly exerted.	*A. volubilis* Jack	c. 55	from South Thailand and throughout Malesia to New Guinea (centre of diversity is western Malesia).
Microtrichium	seed hilar appendage single, to 3.5 mm, not slender to base; testa cell orientation an anticlockwise spiral, rarely straight; calyx usually deeply divided, rarely tubular or spathaceous; without abscission layer at base; stamens sometimes exserted.	*A. microtrichus* C.B. Clarke	c. 50	from the Malay Peninsula to New Guinea (centre of diversity is New Guinea).
Haplotrichium	seed hilar appendage single, to 3.5 mm, slender	*A. bracteatus* DC	c. 8	India, Indo-China and China

sens. str.	to base; testa cell orientation a clockwise spiral; inflorescences long pedunculate; bracts persistent; calyx deeply divided, without abscission layer at base; stamens exserted.			
Section *X*	seed hilar appendage single, to 4.5 cm, testa cell orientation straight; calyx partly or deeply divided, lobes pointed; stamens usually exserted.	? Studies not complete	c. 20	from North and South India, Sri Lanka and mainland Southeast Asia, West Malesia to Sumatra and Borneo (centre of diversity is India and Indo-China to South China).
Diplotrichium	seed with two hilar appendages, to 3.5 cm, testa cell orientation straight; calyx partly or deeply divided, lobes pointed; stamens exserted.	*A. parasiticus* (Roxb.) Wall.	c. 10	from North India throughout Indo-China to China, not extending into Malesia.
Polytrichium	seed with few to many hilar appendages, to 3 cm, testa cell orientation straight; calyx partly or deeply divided, lobes pointed; stamens exserted.	*A. longicaulis* R.Br.	c. 11	from Burma and China throughout Malesia to New Guinea.
Xanthanthos	corolla white or yellow; calyx deeply divided; stamens not exserted; seed with a single hilar appendage as in section *X*.	*A. denticuliger* W.T. Wang	2	Bhutan to North India, Indo-China and China.

Fig. 5 *Aeschynanthus* seeds: A. & B. section *Microtrichium* (*A. garrettii* and *A. musaensis* respectively), C. section *Aeschynanthus* (*A. curtisii*), D. section *Haplotrichium* sens. str. (*A. bracteatus*), E. section *X* (*A. longiflorus*), F. section *Diplotrichium* (*A. sikkimensis*), G. section *Polytrichium* (*A. arfakensis*). Scale bars = 2mm. (After Mendum *et al.*, 2001)

The sectional classification of *Aeschynanthus* based on seed morphology presents some problems of species placement, especially when the seeds are unavailable and then their assignment to sections is only tentative. Other taxonomic characters are not always reliable and have resulted in some inaccurate placements in the past. For example, *A. arfakensis*, *A. leptocladus*, and *A. philippinensis* were placed by Clarke (1883) in section *Haplotrichium*, but are now known to be in section *Polytrichium* (*A. arfakensis*), and *Microtrichium* respectively (Mendum *et al.*, 2001).

2.1.3 Cytological study of the genus *Aeschynanthus*

Cytological investigations of *Aeschynanthus* have also been carried out, firstly by Rogers (1954). The basic chromosome numbers for the genus were found by Ratter (1975) to be x = 16 and x = 15. Rashid *et al.* (2001) found one species, *A. gracilis*, with x = 14. The chromosome size is very small, not exceeding 1.5 μm in mitotic metaphase (Kiehn and Weber, 1997). Polyploidy (mostly 4x) has been recorded from almost every sections, predominantly from *Aeschynanthus*, but not *Diplotrichium* and *Haplotrichium* sens. str. Cytological variants within a species have also been found, for instance, *A. longicaulis* was found by Eberle (1956) to have 2n = 28 (an unusual number for the genus; not in the pattern of x =16 or 15) while Rogers (1954) and Ratter and Prentice (1964) found 2n = 30 for the same species. Differentiation in ploidy numbers is also known in several species. Three different ploidy levels have been reported in *A. ellipticus* (Milne, 1975; Ratter, 1963; Ratter and Prentice, 1964).

Ratter (1975) suggested that the basic number x = 16 is ancestral and x = 15 derived through dysploid reduction; Rashid *et al.* (2001) supported this. *Agalmyla* and *Lysionotus*, two other genera in the tribe Trichosporeae, also have x = 16 (Fussell, 1958; Ratter, 1975; Kiehn and Weber, 1997) supporting the suggestion that x = 16 is the ancestral state.

2.1.4 *Aeschynanthus* in Thailand

Some species of the genus *Aeschynanthus* are native plants in Thailand. They have become important ornamental plants in now aday because of their brightly coloured flowers (usually red or orange) which make them a characteristic part of the epiphytic flora. From the descriptions on *Aeschynanthus* herbarium specimens of the Bangkok Foresty Department (BKF) their habitats in Thailand are partly shade places in slightly disturbed, along the streams, trails, or open areas in degraded. The ranging of their growing attitudes are between 500 – 1800 metres from the sea. Most of them grow on tree trunks in primary evergreen/seasonal hardwood forests which on granite bedrocks.

According to the herbarium collection of BKF upto 27 samples of *Aeschynanthus* have been collected and preserved in the herbarium. However, only 12 species were identified (Table 2) while the rests have not been named yet. There are still no researchers currently working seriously on Thai *Aechynanthus*. Only a few studies (Denduangboripant and Cronk, 2000 and 2001; Denduangboripant *et al.*, 2001) refer to some species of *Aeschynanthus* in Thailand. In 2001, BL. Burtt published a recent

checklist of Thai *Aeschynanthus* in Thai Forest Bulletin and in his report there are 14 species of *Aeschynanthus* found in Thailand, distributing in different areas of the country (Fig. 6). Burtt noted that more herbarium materials could be useful, especially from the eastern borders, where there are already indications that some species in neighboring countries were found on Thai territory. Their seed samples are also welcome for seedling study and possible works on DNA characteristics. Unfortunately, his Thai *Aeschynanthus* list has not completed yet, still having some synonymous species caused by uncompleted herbarium specimens. For instance, Burtt suggested that *A. andersonii* should be the correct name for *A. hildebrandii*, *A. hosseusianus*, *A. humilis*, and *A. persimilis* because of little specimens examined by previous taxonomists. The final revision of *Aeschynanthus* in Thailand has been preparing by taxonomists of Royal Botanic Garden Edinburgh and it is expected to be published soon.

Table 2 *Aeschynanthus* herbariums collected in BKF.

Name	Location
A. acuminatus	Loei (Phu Kradueng)
A. andersonii	Chiangmai (Doi Pui)
A. garrettii	Chiangmai (Doi Inthanon)
A. hosseusii	Chiangmai (Doi Sutep)
A. hildebrandii	Chiangmai (Doi Sutep), Lumpang
A. lineatus	Chiangmai (Doi Sutep)
A. longicualis	Chanthaburi (Kao Phra Bat), Yala (Batong), Loei (Phu Kradueng)
A. longiflorus	Yala
A. macranthus	Tak (Mae Sod), Kamphaeng Phet (Umphang)
A. parvifolius	Narathiwat (Kok Doun, Tak Bai)
A. radicans	Songkhla (Boripat falls), Nakhon Si Thammarat, Trung
A. superbus	Chiangmai (Doi Inthanon)

Fig. 6 The distribution areas of *Aeschynanthus* in many parts of Thailand

B.L. Burtt check list on *Aeschynanthus* in Thailand

1. *Aeschynanthus acuminatus* [Wall. ex] A. Dc.

Collecting locality: Northeast; Loey (Phukradung national park).

2. *Aeschynanthus andersonii* C.B. Clarke

Collecting locality: North; Chiangmai (Doi Inthanon national park, Doi Sutep); Lumpang*

3. *Aeschynanthus breviflorus* Ridl.

Collecting locality: unlocated

4. *Aeschynanthus fecundus* P. Wood.

Collecting locality: the type of this species is Ridley 13599 (K) from Pahang. The two collections from Thailand cited by Woods were both from cultivated material and unlocalised: Sorensen, Larsen & Hensen P. 1958/995 (C) and Larsen 103, cult.at E as C. 5838 (E)

5. *Aeschynanthus fulgens* [Wall. Ex] R. Br.

Collecting locality: North; Mae Hong Son.

Note This and *A. macranthus* (Meriil) Pellegr. May belong on the same species.

6. *Aeschynanthus garretii* Craib

Collecting locality: North; Chiangmai (Doi Inthanon national park), Prachinburi (Khoa-Yai national park), Nakorn Nayok (top area of Khoa-Yai national park), Nakorn Rachasima (Khau Rom, Khoa-Yai national park).

7. *Aeschynanthus gracilis* C.B. Clarke

Collecting locality: unlocated

8. *Aeschynanthus hildebrandii* Hemsl.

Collecting locality: North; Chiangmai (Doi Inthanon national park, Doi Sutep)

9. *Aeschynanthus hosseusii* Pellergr.

Collecting locality: North; Chiangmai (Doi sutep, Doi Inthanon national park), Lumpang (Jae Sewn national park)

10. *Aeschynanthus hosseusianus* Kr.

Collecting locality: unlocated

11. *Aeschynanthus humilis* Hemsl.

Collecting locality: North; Chiangmai (Doi Inthanon national park, Doi Sutep)

12. *Aeschynanthus parviflorus* sensu Ridl.

Collecting locality: unlocated

13. *Aeschynanthus persimilis* Craib.

Collecting locality: unlocated

14. *Aeschynanthus macanthus* (Merriil) Pellegr

Collecting locality: Tak (Mae sod), Kamphangphet (Umphang), Ranong

Identifying a scientific name of each *Aeschynanthus* species is not simple for Thai taxonomists because no key to species for the genus in Thailand is available. However, recently Mary Mendum, an expert in *Aeschynanthus* of the Royal Botanic

Garden of Edinburgh, UK, kindly gave a key to species especially for Thai *Aeschynanthus*. This key is based on morphological characters described in literatures and from large living collection of the garden. The key made by Mary Mendum is shown below.

The key to species of Thai *Aeschynanthus*

1a. Robust plant with conspicuous red floral bracts: infloresecence several-flowered, pedunculate; calyx 3 cm long, divided to base; cololla 7.5 cm, red, paler inside with dark mottling on the spreading lobes ..... *A. superbus*

1b.Bracts not conspicuous; calyx and corolla not combination 2

2a.Calyx tubular or campanulate to at least halfway, lobes not acute 3

2b.Calyxe partly fused or divided to base, lobes usually acute 7

3a.Creeping plant, rooting from nodes; leaves to 4 x 2 cm, ovate-elliptic, rounded to cordate at base, hairy below and sometimes above; calyx tubular, ca. 2 cm, hairy; corolla ca. 5 cm, red, hairy ..... *A. radicans*

3b. Plant not creeping; leaves > 6 cm, not rounded at base, glabrous; calyx campanulate or narrowly campunulate, glabrous 4

4a.Corolla < 4.5 cm 5

4b. Corolla >4.5cm 6

5a.Calyx 2 cm, lobes small, bluntly triangular, spreading; corolla 4 cm, bright crimson with dark lines, yellow at base and in throat ..... *A. flugen*

5b.Calyx 1.3 cm, lobes rounded oblong, not spreading; corolla 3 cm, scarlet to orange-scarlet Corolla < 4.5 cm t with dark lines ..... *A. hookeri*

6a.Calyx campanulate, to 1.6 cm; corolla red, to 6.5 cm, very narrow in lower half then abruptly flaring and curving; anthers 2-4 mm ..... *A. stenosiphonius*

6b.Calyx narrowly campanulate, to 2 cm; corolla not very narrow at base, light scarlet with dark stripes, paler inside, to 6.3 cm; anthers to 6 mm. .....

..... *A. macranthus*

7a.Corolla > 5 cm long 8

7b.Corolla < 5 cm long 10

8a.Calyx 4.5 cm, tubular to ca. 3 cm then with slender pointed lobes; corolla to 7 cm, red with purple lines ..... *A. hosseussii*

8b.Calyx < 2 cm long, divided to base; corolla slender, > 8 cm long 9

9a.Upper leaves in whorls of 3-8; inflorescences several-flowered; corolla greenish-yellow at base, shading to flame-red apical ..... *A. speciosus*

9b.Leaves opposite; corolla crimson shading to purple at the base ..... *A. longiflorus*

10a.Leaves with conspicuous whitish marbling above, purple-mottled below; calyx divided to base, lobes to 2 cm; corolla to 3.5 cm, greenish with crimson marks on lobes ..... *A. longicaulis*

10b.Leaves not marbled; corolla not as above 11

11a.Creeping, flexuous plants; leaves small 12

11b.Twiggy plants, stem not flexuous 13

12a.Leaves to 2 cm, ovate-lanceolate, acute, thick and fleshy; corolla 2.5 cm ..... *A. gracilis*

12b.Leaves to 1.5 cm, spathulate; corolla < 2 cm..... *A. monetarius*

13a.Small twiggy plants; leaves to 5 cm, narrowly spathulate; calyx to 5 mm, not divided to base; corolla to 3.5 cm 14

13b.Plants not twiggy, leaves > 5 cm 17

14a.Calyx divided almost to base; corolla quite broad towards base & internally with coarse upward-pointing hairs just above base; lower lobes strongly reflexed 15

14b. Calyx divided to about halfway; corolla > 2 cm, quite narrow towards base & internally with few coarse hairs near base, lower lobe not strongly reflexed 16

15a.Calyx < 3 mm; corolla < 2 cm, scarlet ..... *A. andersonii*

15b.Calyx c.5 mm, corolla 2.5 cm, orange-red with dark margins to lobes

..... *A. hildebrandii*

16a.Calyx 3.5 mm; corolla to 2.1 cm, internally with line of upward-pointing

..... *A. humilis*

16b.Calyx 6 mm; corolla to 3.1 cm, internally with few hairs ..... *A. persimilis*

17a.Stem transversely wrinkled and rather warty; calyx 1cm, devided to base; corolla scarlet, to 3.5 cm ..... *A. garrettii*

17b.Stem not wrinkled or warty 18

18a.Calyx to 1.1 cm, divided to base, lobes very slender, glabrous; corolla to 1.3 cm, hardly opening, yellow at base shading to dull dark red epically

..... *A. fecundus*

18b.Calyx to 5 mm; corolla >1.5 cm, opening normally 19

19a.Inflorescence pendunculate, bracts persistent; calyx to 4 mm, divided almost to base, lobes ovate-elliptic, blunt; corolla 1.7 cm, green (reported elsewhere to be red)

..... *A. acuminatus*

19b.Inflorescence not pedunculate, bracts not persistent 20

20a.Calyx to 6 mm, divided to base; corolla 2.5 cm, orange-red with dark lines

..... *A. lineatus*

20b.Calyx to 15 mm, tubular in lower; corolla 2.5-3 cm, orange shading to red apically with dark lines on lobes ..... *A. parviflorus*

2.2 Plant molecular phylogenetics

2.2.1 Principle of molecular phylogenetics

In 1950 a German entomologist Willi Hennig published a book named *Grundz ge einer Theorie der Phylogenetischen Systematik*. Hennig' s book contained five basic ideas which began a major revolution in systematics:

1. The relationships lending to the cohesion of living and extinct organisms are genealogical ("blood") relationships.

2. Such relationships exist for individuals within populations, between populations, and between species.

3. All other types of relationship (i.e., phenotypic and genetic) are phenomena correlated with genealogical descent and thus are best understood within the context of genealogical descent with modification (quite literally 'evolution').

4. The genealogical relationships among populations and species may be recovered (discovered) by searching for particular characters which document these

relationships.

5. The best general classification of organisms is one that exactly reflects the genealogical relationships among these organisms.

Wide discussion of phylogenetic methods came after the publication of Hennig's revised book (1966) in English-language. In fact, Hennig proposed many ideas other than the five basic points listed above. Some of these ideas are still used (e.g. monophyly) while others have been discarded. However, these five basic ideas with some modifications continue to provide the major theoretical concepts for 'phylogenetic systematics'.

Phylogenetic systematics or simply 'phylogenetics' is the approach that accomplishes those tasks above. The phylogenetic system can give baseline data for beginning investigations of other comparative biology disciplines. Phylogenetic systematic approaches attempt to recover phylogenetic (genealogical) relationship among groups of organisms and produces classifications that exactly reflect those genealogical relationships. By this concept, taxonomy therefore could be an alternative of phylogenetic systematics because it comprises the theory and practice of describing a diversity of organisms and ordering this diversity into a wording system that conveys information concerning the kind of relationship between organisms (Wiley, 1981).

The meaning of phylogenetics has been extended by Kitching *et al.* (1998) that phylogenetics is a method of classification that utilises the hypothesis of character transformation to hierarchically group taxa into nested sets, and then interprets these

relationships as a phylogenetic tree (i.e. an hypothesis of genealogical relationships among a group of taxa with specific ancestry and implied time axis).

There are several types of characters can be used for phylogenetic analyses. First, we can utilise morphological characters which are structural attributes of an organism. Morphological characters are the primary source of characters in most groups of organisms. To be a useful set of characters, they must vary between taxa but not depend on their environment. Morphological characters may be observed easily by sight or with simple optical aids such as a microscope. They may be simple or complex and have proven useful in distinguishing taxa at varying levels, from phyla to species. External characters are more predominant characters than internal characters because they are relatively easy to observe. Some of them can be quantified as a reference such as colour patterns, which may be measured by wavelength.

One of reliable characters is molecular character. DNA is the genetic material that a living descendant was inherited from their ancestor in the lineage. Data retrieved from molecular characters like DNA sequencing always give a robust confidence. Most phylogeneticists have been interested in finding new genes or non-coding regions to study about relationships among organisms. Nowaday, this type of knowledge and approach is known as molecular phylogenetics.

The task of molecular phylogenetics is to convert information in DNA and/or protein sequences into an evolutionary tree. There are two different major methods to build such phylogenetic trees based on how the data are treated. First is a 'distance

method', which converts aligned sequences into a pairwise distance matrix, then input that matrix into a tree building method, whereas, a 'discrete method' considers each nucleotide site directly. If the original data are in the form of genetic distances, such as those obtained from DNA hybridisation studies, the distance method then should be used. However, if we have the nucleotide or protein sequences, we should analyse them with a discrete method to avoid the loss information that occurs when sequences are converted into distances. The discrete methods are different from the distance methods as they operate directly on the sequences or on functions derived from the sequences rather than pairwise distances. Neighbour-joining is a popular distance method which seeks the tree whose sum of branch lengths is minimum, while the major discrete method is maximum parsimony which choses the tree (or trees) that require fewest evolutionary changes (Page and Holmes, 1988).

2.2.2 Molecular techniques for phylogenetic study in plants

2.2.2.1 PCR amplification on gene target

Deriving plant phylogenies from morphological data is a process opening to error because of our lack of understanding of the mode and tempo of the evolution of these characters. Molecular data is, however, more often used as it allows the derivation of phylogenies (or measures of relatedness or differences) which are based on quantifiable data and derive from the genetic core which underlies organismal fitness.

For phylogenetic studies in plants there are many steps to be done. First of all, choosing a gene target is a very important step because different genes have different mutation rates, making them suitable to different prospects of study. Analysis of non-coding regions permits assessment of phylogenetic relationships at lower taxonomic levels because they evolve more rapidly than coding regions which owe to accumulation of indels (insertions/deletions) at a rate at least equal to nucleotide substitions (Clegg and Zurawzki, 1992 ; Wolfe and Sharp,1987). For example, nuclear ribosomal DNA (nrDNA) data provide valuable information in phylogenetic study of plants. Internal transcribed spacer (ITS) regions of the nrDNA have been shown to be a valuable source of evidence to resolve phylogenetic relationships at different taxonomic levels, in particular at an intraspecific level because relatively rapid evolutionary rates of the ITS fragments.

Another example of gene targets for phylogenetic analysis is choroplast DNA which is popular and has proved to be well suited for evolutionary and phylogenetic studies. Chloroplast genome has been a focus of plant molecular evolution and systematic reserved. The modes of chloroplast DNA evolution (cpDNA) in plants usually conserve in terms of genome sizes, structures, gene contents and linear orders of genes among lineages of land plants (Palmer, 1987). This conservative mode suggest that any change in structure and content of chloroplast genome may have significant phylogenetic implications and therefore be useful for the study of phylogenetic relationships (Clegg and Zurawzki, 1992). The chloroplast genome evolves at a slower rate than the nuclear genome (Wolfe and Sharp, 1987). However, some regions change

either more rapidly or more slowly than the average (Plamer, 1987). Nucleotide substitution rates vary among plant lineages (Wolfe and Sharp,1987), therefore, cpDNA is useful for relationship studies, particularly at the inter-generic as well as the inter and intra-specific level.

After already chosen a suitable gene for phylogenetic study, specific primers will be used for polymerase chain reaction (PCR) to amplify the gene target. PCR uses a synthetic process to copy a specific target sequence over and over again. Mixtures of oligonucleotides, usually called primers, are uses in the reaction to initial DNA synthesis at specific place on the template. Two primers are designed to anneal close to the one another (within several thousand base pairs) but on different strands. These primers anneal in conserved flanking regions both sides of the target gene. The more conserved these regions flanking are, the higher the chance there is that the primers will work over a wider taxonomic range (Taberlet *et al.*, 1991). The basic protocal of PCR is simple ; (1) double strand DNA is denatured at high temperature to form single strand (template); (2) short oligonucleotide primers bind at a lower annealing temperature to the single strand complementary templates; (3) the temperature is raised for synthesis of targeting sequences by primer extension; (4) the newly synthesised double strand DNA target sequence are denatured at high temperature, and the cycle is repeated. The amplified target DNA can be increased exponentially as every cycle has the potential to double the amount of target DNA from the previous cycle if there is sufficient amount of polymerase, primers, and nucleotides in the solution. PCR is the powerful technique in

plant molecular systematics because sometime source of DNAs were from herbarium specimens ranged quality from normal to several degraded (Doyle *et al.* 1995). Savolainen *et al.* (1995) found that amplification of DNA from some herbarium samples may be difficult because oxidised material coprecipitates with the DNA, but the addition of certain additives can overcome the inhibiting activities of some herbarium extracts.

2.2.2.2 DNA sequencing

Recently, the number of gene and DNA regions used for phylogeny estimation has grown rapidly. In early 1990s, most plant molecular phylogenetic studies relied on *rbc*L sequence but in now a day, wildly-used sequences are nrDNA internal transcribed spacer (ITS), cpDNA *trn*-L, *trn*-F and mtDNA. One of the adventages frequently cited for the use of DNA sequence data in phylogeny reconstruction is that character evolution models based on our understanding of sequence evolution may be incorporated into the analysis. In the way the method for recovering the pattern of evolutionary divergence can be tried more closely to the processes that produce the variation (Clegg and Zurawski, 1992). Within the context of parsimony analysis, it has been assumed that the reliability of information for phylogeny reconstruction can be inferred from estimation of substitution rate for different sites, with the most slowly evolving sites being the most reliable.

Sequencing technology has developed rapidly over the past two decades. Although nucleic acid sequencing is a comparatively new approach for systematicss,

the power of the technique has ensured that DNA sequencing has become one of the most utilised molecular approaches for inferring phylogenetic history. The primary attractions of nucleic acid sequencing include the facts that (1) nucleotides are the basic units of information encoded in organism; (2) it is relatively easy to extract and incorporate information about molecular evolutionary processes into analysis; (3) sequence evolution is comparatively easy to model and (4) the potential sizes of informative data sets are immense.

2.2.2.3 PCR -RAPD

Random amplified polymorphic DNA (RAPD) is a technique that produces arbitrary fragment length polymorphisms. The RAPD technique utilises single, arbitrary, decamer DNA oligonucleotide primers to amplify regions of the genome using PCR (Welsh and McClelland, 1990; Williams *et al.*, 1990; Hadrys *et al.*, 1992; Newbury and Ford-Lloyd, 1993; Williams *et al.*, 1993). Priming sites are thought to be randomly distributed throughout a genome and the polymorphism in these priming regions results in differing amplification products. The methodology is simple and has been wildely used for the assessment of genetic diversity, investigation of hybridisation and introgression events, examining species relationships and investigating population structures and processes.

A maximally informative, RAPD marker would be expect to show (1) variation at a suitable taxonomic range; (2) no environmental or developmental influence; (3) simple

codominant inheritance; (4) independence of other markers; (5) reliability and reproducibility; (6) a random scattering across the genome; and (7) a state of similarlity attributable to common ancestry. Thus an ideal marker allows the possibility of unambiguously characterising taxa.

RAPD has a considerable appeal for surveys of genomic variation and the identification of DNA-base character systematics since they are relatively inexpensive, randomly sample a potentially large number of loci, and sequence information is not necessary for primer design (Hardy *et al.*, 1992; Huff *et al.*, 1993; Wiliums *et al.*, 1993; Weising *et al.*, 1995), thus the technique simplicity of the RAPD procedure has obscured the difficulties of understanding product banding patterns which have led to criticism of this approach (Hillis, 1994).

2.2.3 Plant nuclear ribosomal DNA, its ITS region, and their uses

2.2.3.1 Plant nuclear ribosomal DNA

Ribosomal DNA (rDNA) is the set of DNA sequences that code for the synthesis of ribosomal RNA (rRNA). In a plant genome, nuclear ribosomal DNA (nrDNA) exists in large arrays of tandem repeats of the transcription unit and nontranscribed spacer, ranging variously from 200 repeats (in *Linum usitatissimum*) to 2200 (in *Vicia faba*) (Rogers and Bendich, 1987). The long tandem arrays form a nucleolar organising region (NOR) at one or a few chromosomal loci (Long and Dawid, 1980). Although there is

variability among copies of rDNA within individuals, the rDNA repeat units are highly homogenous as a result of concerted evolution (Arnheim *et al.*, 1980). The gene arrays evolve together rapidly through processes such as gene conversion (Fogel and Mortimer, 1969; Ohta, 1984; Walsh, 1986; Hillis *et al.*, 1991), unequal crossing over (Smith, 1973), and repeat amplification. These processes have been described as molecular drive (Dover, 1982 and 1986) which can fix mutations that spread through a multigene family in a relatively short time, resulting in the overall sequence homogeneity of the rDNA repeats. This homogeneity makes rDNA more attractive for phylogeny reconstruction than other nuclear gene regions (Arnheim, 1983).

The structure of higher plant nrDNA is similar to that of other eukaryotes (Long and Dawid, 1980). The rDNA array is transcribed as several types of ribosomal RNA. There are three distinct rRNA types characterised in sedimentation velocity units (S, for Svedburg): 1) large subunit rRNA, which is around 28S (over 4,000 nucleotides) in plant nuclear genomes; 2) 5.8S rRNA, which is around 160 nucleotides; 3) small subunit rRNA, which is around 18S (1,800 nucleotides). Another rDNA type, which is not a member of the rDNA array but found elsewhere in the genome, is a 5S rRNA (around 120 nucleotides) (Gerbi, 1985). In plants and also other eukaryotes, two internal transcribed spacers (ITS1 and ITS2) separate the 18S, 5.8S, and 28S genes, and an external transcribed spacer (ETS) is located upstream of the 18S gene. An arrangement of rDNA gene is shown in Fig. 7.

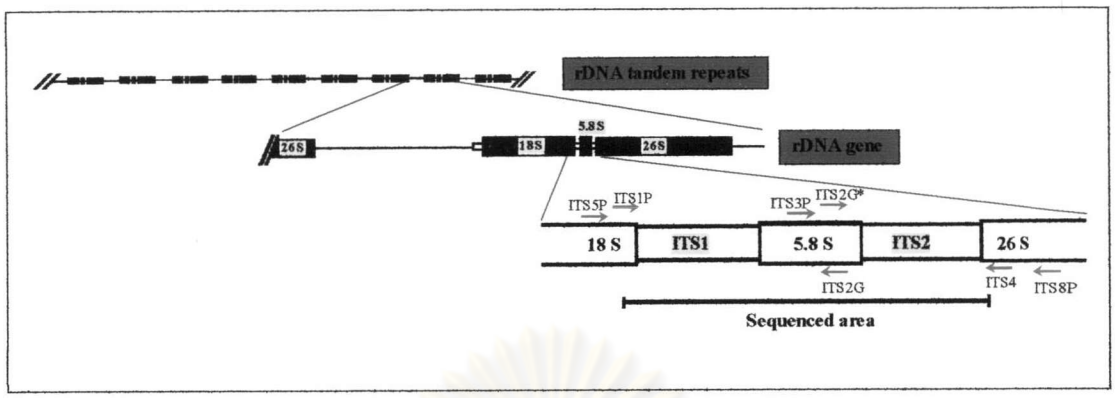

Fig. 7 Repeat units of the nuclear ribosomal DNA and the organisation of the internal transcribed space (ITS) region (modified from White *et al.*, 1990). Arrows indicate orientation and approximate position of primer sites.

The whole rDNA repeat unit is transcribed as a single large precursor rDNA which is processed subsequently by cleavages of ITS1 and ITS2, resulting in the mature ribosomal RNAs (Brown and Shaw, 1998). The rRNAs then combine with ribosomal proteins to form two major (large and small) subunits of ribosomes, the universal organelles that direct protein synthesis from messenger RNA. The transcribed spacers contain signals for processing the rRNA transcription. Adjacent copies of the rDNA repeat unit are separated by a nontranscribed spacer (NTS; also called an intergenic spacer, IGS), which ranges in length from one to eight kilobases in most plants (Jorgensen and Cluster, 1988). This IGS region contains subrepeating elements which vary interspecifically in length (Appels and Dvorak, 1982) and this length variation might have a role in evolution of the rDNA gene family (Federoff, 1979). The subrepeats in the IGS have been postulated as enhancers of rDNA transcription (Reeder, 1984). In

contrast to the IGS, the ITS region in angiosperms is relatively short and evolutionarily conserved in length (Baldwin *et al.*, 1995)

2.2.3.2 Phylogenetic implications from nuclear ribosomal DNA

Because protein synthesis is a prerequisite for life, rDNAs are present in every organism (Hillis and Dixon, 1991) and have proven to be a powerful phylogenetic tool (Hamby and Zimmer, 1991). The most extensively studied rDNA is the small subunit gene (Hillis and Dixon, 1991), which is one of the slowest evolving sequences, found in all living organisms, and has therefore been very useful for investigating ancient evolutionary events. The large subunit gene is useful for reconstructing more recent events, because it has many divergent domains or expansion segments (Hassouna *et al.*, 1984), and so the size of the gene varies considerably among phyla (Gutell and Fox, 1988). The 5.8S rDNA of eukaryotes is similar in variability to the small subunit gene, although the shortness of the sequence restricts its phylogenetic usefulness across great time scales (Hillis and Dixon, 1991). The transcribed and nontranscibed spacer regions of rDNA arrays have frequently been used for inferring phylogeny among closely related taxa. Variation in the spacer regions has also been used to identify species or strains, to study hybridisation, and as markers in population genetic studies (Saghai-Maroof *et al.*, 1984; Rogers *et al.*, 1986; Sites and Davis, 1989). Among the spacers, IGS evolves most rapidly (Hoshikawa *et al.*, 1983) and the transcribed spacers are more conserved (Appels and Dvorak, 1982).

2.2.3.3 The use of ITS regions in plant phylogenetics studies

While the large and small subunit rDNA regions have been used to address phylogenetic questions at the family level or higher taxonomic levels in plants (Zimmer *et al.*, 1989), the ITS sequences appear to be useful for assessing relationships at lower taxonomic levels. ITS regions have rates of sequence substitution useful for evaluating the generic and species level relationships (Hillis and Dixon, 1991). The regions evolve more rapidly than other coding regions in general (Brown *et al.*, 1972; Appels *et al.*, 1986) and are more variable as a result of mutagenic processes such as single-base substitutions and indels (Venkateswarlu and Nazar, 1991). Thus ITS increasingly became an important locus for molecular systematic studies of a wide diversity of organisms from fungi to flowering plants (White *et al*, 1990.). A number of ITS regions have been sequenced from different genera of many plant families, such as Adoxaceae, Apiaceae, Betulaceae, Canellaceae, Cucurbitaceae, Fagaceae, Onagraceae, Malvaceae, Poaceae, Polemoniaceae, Ranunculaceae, Rosaceae, Salicaceae, Saxifragaceae, Scrophulariaceae, Solanaceae, Styracaceae, Viscaceae, and Winteraceae (Baldwin *et al.*, 1995).

The ITS region is even more attractive for molecular phylogenetic studies because it can be amplified easily by a polymerase chain reaction (PCR) for DNA sequencing with universal primers from conserved flanking regions in the 18S, 5.8S, and 28S genes. Moreover, the length conservation of ITS sequences among closely related species helps their sequence alignment and phylogenetic analysis (Baldwin *et al.*,

1992). By combining the ITS sequence data with morphological data, ITS data have been playing a useful role in modern plant systematics and evolution research. To date, uses of many ITS sequences were reported from various agricultural crops, such as wheat (Chatterton *et al.*, 1992a and 1992b), rye (Chatterton *et al.*, 1992c), oats (Chatterton *et al.*, 1992d), barley (Chatterton *et al.*, 1992e), rice (Takaiwa *et al.*, 1985), and mung bean (Schiebel and Hemleben, 1989).

2.2.4 Phylogenetic tree reconstruction methods

2.2.4.1 DNA Data matrix preparation

Phylogenetic analysis of DNA sequence data begins with an alignment of two or more sequences that are hypothesised to be homologous. An alignment involves determining which positions along the DNA or protein sequence are derived from common ancestral positions. The alignment probably inserts gaps to increase or decrease the nucleotides in each DNA sequence and also to decrease nucleotide mismatching. A character-taxon data matrix is established whenever the sequence alignment is completed (Page and Holmes, 1998).

2.2.4.2 Maximum parsimony and Neighbour-joining

The next step after establishing the character-taxon data matrix is to calculate relationships among taxa. There are several methods (either discreat or distance) to

reconstruct a phylogenetic tree (or trees). One most popular method among discrete methods is maximum parsimony which choses the tree (or trees) that requires the fewest evolutionary changes (Page and Holmes, 1998). Optimal criteria for this method is a strategy to search the set of possible trees. Such strategy contain exact searching methods (i.e. exhaustive search and branch-and-bound search) and heuristic searching methods. The exhaustive search method guarantee to find one or all of the shortest cladograms. Every possible cladograms for all included taxa is examined and their lengths are calculated. A simple (phylogenetic trees) algorithm to perform an exhaustive search are that first three taxa are chosen and connected to form an unrooted, fully resolved cladogram for these taxa. The fourth taxon is then selected and joined to each of the tree branches of the first cladogram, yielding three possible networks for four taxa. The fifth taxon is then added to each of the five branches of the three cladograms. This procedure is continued until all possible cladograms have been constructed. Finally, the lengths of all these cladograms are calculated and the shortest tree is chosen as the most parsimonious phylogenetic trees. This method is however a practical solution for problems with a few number of taxa (approximately 10-12 taxa).

Another exact method but does not require every tree topology to be examined individually is branch-and-bound search method. This method begins with a heuristic calculation of the first cladogram. The length of this cladogram is retained as a reference length (or upper bound) for subsequent cladogram construction. The branch-and-bound method then proceeds in a similar manner to exhaustive search but the

lengths of the partial networks are calculated at each step and compared with that of the upper bound. If its length is equal to the upper bound, then this cladogram is retained as one of the set of optimal topologies and the branch-and-bound process continued. However, if the length is less than the upper bound, then this topology is an improvement and its length is substituted as the new upper bound. The attachment of additional taxa can serve only to increase the length further and the number of evaluated cladograms is greatly and quickly reduced. Once all possible paths have been examined, the set of optimal cladograms will have been found. Branch-and-bound applications employ algorithmic devices that reduce computation estimate of the upper bound. However, a branch-and-bound analysis is still time consuming to implement and should not generally be considered for data sets comprising large numbers of taxa.

Approximate or heuristic methods should be adopted in the case of having large members of taxa. This strategy generally uses 'hill climbing' techniques which are essentially trial-and-error and do not guarantee to find all of the minimum-length cladograms but can reduced computational time. Imagine a group of hill hikers aiming to climb to the top of mountain as fast as possible. In order to do that, the 'hikers' best strategy would be to walk up the mountain following the line of steepest ascent and will eventually reach the summit. However, if there is more than one peak to the mountain, then this approach might yield only a locally optimal result, in that the hikers will simply reach the peak nearest to their starting point. There may be a higher summit elsewhere. Such isolated peaks are referred to as 'islands' if such islands exist then one. This can

be translated directly into a search for minimum-length cladograms, the global optimum. The simplest computer algorithms for heuristic search methods make a single pass through the data and construct one tree topology. The resultant cladogram is likely to be only locally optimal unless having good fortune. More complex routines begin with that single topology, then seek to locate the global optimum by rearranging the cladogram in various ways such as 'branch-swapping' algorithm. If multiple islands of tree topology also do exist, then we can run several analyses, which individually start from topologically distinct cladograms.

To use distance methods for phylogenetic tree building, one widely used method is the neighbour-joining. It combines less computational speed with only one resultant tree given. Neighbour-joining technique is a clustering method rather than optimality method, and can not optimise a fitting criterion between tree and data. However, it is a good heuristic method for estimating a minimum evolution tree. One strategy for finding the minimum evolution tree is to first compute the neighbour-joining tree, then see if any local rearrangement of the neighbour-joinging tree produces an even shorter tree. Note that this strategy does not guarantee to find the minimum evolution tree (Page and Holmes, 1998).

2.2.4.3 Tree rooting and consensus tree analyses

When searching for phylogenetic tree, an unrooted tree is first reconstructed, then a rooted tree is further reconstructed. A particular node which is nearest to the

common ancestor of analysing taxa will be assigned to be a rooting point of the unrooted tree. Evolutionary polarity will then occur to character states of the ingroups after this process. Briefly, we generally choose one or more taxon to add into the data set as an outgroup for the analysis. Thereafter, most parsimonious tree will be calculated and the nodes connected to that outgroup will be used as the most proper rooting-point.

After searching with discrete methods, the most parsimonious tree may occur more than one tree. Consensus methods are then usually introduced to solve this problem. They are convenient means of summarising agreement and disagreement (or congruence and incongruence) between two or more phylogenetic trees. All methods of consensus analysis commonly construct a tree from any non contradictory components found among the set of cladograms generated from the initial analysis. Consensus trees can be considered to be indirect methods for resolving character conflicts. They reduce the number of fundamental cladograms produced by parsimony analysis to one tree showing their common components (Kitching *et al.*, 1998). Different consensus methods are suited to different tasks (Nixon and Carpenter, 1996). Here are three consensus methods used in this M.Sc. investigation.

- Strict consensus: a strict consensus tree includes only those groups that occur in all fundamental cladograms.

- Semistrict consensus: a semi-strict consensus tree is formed from all uncontradicted components from a set of fundamental cladograms.

- 50% majority-rule consensus: a majority-rule consensus tree includes only those components that occur in more than 50% of the fundamental cladograms.

2.2.4.4 Tree evaluation

The simplest supporting measurement for individual clades in the tree is 'branch length'. However, homoplasy (a character that specifies a different and overlapping group of taxa from another to character) makes difficulty to an interpretation of branch length as support. Other tree support approaches aim to circumvent the problem by assessing the number of extra steps that are required before the clade is lost from the consensus tree of near-minimum-length cladograms. Here are two tree evaluation methods used in this investigation.

- Bootstrap supporting value: The bootstrap analysis randomly samples characters with replacement to form a pseudoreplicate data-set of the same dimensions as the original. The effect is to delete some characters randomly and to reweight others randomly, with the constraint that the sum of the weights for all characters equals to the number of characters in the matrix. A large number of pseudoreplicates is generated, typically 1000 or more. The most parsimonious cladograms for each pseudoreplicate are then found and the degree of conflict among them assessed by a 50% majority-rule consenseus tree in which a particular group is found might be interpreted as a confidence level associated with that group.

- Jackknife supporting value: In contrast to the bootstrap, jackknife sampling is applied without replacement and hence the pseudoreplicate data sets are smaller than the original. Jackknifing aims to achieve better variance estimates from small samples. In first-order jackkniff, pseudoreplicates are constructed by randomly removing one observation (either taxon or character) from the data set. Hence, for a data set of T observations, T pseudoreplicates are possible, each comprising T-1 observations from the original sample. The variances of the T pseudoreplicates are then averaged to give the estimate of the parametric variance.

Current parsimony computer programs also utilise a number of other different statistics to assess the quality of cladograms. Standard measures are consistency index (CI), retention index (RI) and rescaled consistency index

- Consistency index: a measure of the amount of homoplasy in a character relative to a given cladogram. The consistency index is calculated as the ratio of m, the minimum number of steps a character can exhibit on any cladogram, to s, the minimum number of steps the same character can exhibit on the cladogram in question.

- Retention index (RI): a measure of the amount of similarity in a character that can be interpreted as synapomorphy (shared-derived character) on a given cladogram. The retention index is calculated as the ratio of (g-s) to (g-m), where g is the greatest number of steps that a character can exhibit on any cladogram.

- Rescaled consistency index (RC): the product of the consistency index and the retention index of a character.

2.3 The use of molecular phylogenetics

2.3.1 Using molecular phylogenetics to study plant systematics and evolution

Molecular phylogenetics is helpful in plant systematics and evolution. The result of phylogenetic analyses can be used for many distinct (but inter-related). Molecular phylogenetics are often most useful when there is conflict among the phylogenies constructed with different morphological character data set. Moreover, molecular phylogenetic data have help settle the question of plant systematic and evolution. For example,

1. To understand the relationship among extant and extinct plants.

2. To understand the effect of history and phylogeny on development, function, adaptation, ecology, molecular evolution, mating system, life cycle, speciation and biogeography.

3. To studies evolutionary path (Wang *et al.*, 2003).

4. To resolve taxonomic problem, molecular data from different from informative DNA region can address some of the controversies on generic and sectional limits and to evaluate the genetic distance between minor entities with molecular data included samples ascribed to different genera, sections and intraspecific taxa (Cubas *et al.*, 2002).

6. To provide a new tool to build up an accurate picture of the genetic relationship within complex group. However, a molecular phylogenetic tree is a scientific hypothesis that should be subjected to attempts of falsification (Nei and Kumar 2000).

7. To address fundamental questions of relationship in a family, including (1) the conformance of traditional classification system to phylogeny (2) the utility of character traditionally considered taxonomically important of intrafamily level, and (3) basic pattern of biogeography within and among the major clades of the family (Plunkett *et al.*, 2004).

2.3.2 Molecular identification

Molecular techniques provide attractive use for identification of an organism, especially when morphological characters are not practical for routine identification to species level. Genetic characters can be used to provide unambiguous taxonomic discrimination. A reliable routine method to identify species is important for establishing their horizontal and vertical ranges (Fleminger and Hulman 1997). Such method would also enable about the distribution and abandance of developmental stages to be answer. Moreover, the application of molecular identification is increasingly used and has provided useful information. Several recent developments in molecular identification also provide rapid, sensitive, and accurate methods. The technique has been used worldwide to identify organisms and genes, for examples :

(1) To identify bacteria and their catabolic genes in the environment and also to design an active biological containment system which could prevent spreading of microorganism (Widoda *et al.*, 2002).

(2) To identify Atlantic *Calunus* species which the analysis is reliable, reproductive and relatively simple, inexpensive, and performed on ethanol preserved samples which make it suitable for used on *Calanus* samples collected at the sea (Lindegus *et al.*, 1999).

(3) To determine ectomycorrhizal associates of *Pinus contorta* in soils (Colling and Marka, 2001)

(4) To identify nontuberculous mycobacteria with faster and more accurate ability in addition to contributing significantly in the discovery of new species (Terene *et al.*, 2001)

For *Aeschynanthus*, species identification and sectional classification of the genus rely mostly on their flower and seed morphology. *Aeschynanthus* capsule-like fruits contain many anatropous ovules, with one or more appendages (hair) at the hilar end. The number and type of these hilar appendages have been used to subdivide the genus in to sections This will then present great problems to taxonomists when the reproductive parts of the wild plant are unavailable, usually happening most of the time in the year. Moreover, amateur taxonomists and horticulturists could easily misidentify the plant to be a member of any other epiphytic genus, or vice versa. Using current molecular biological techniques should help clarify identification problem of

Aeschynanthus much quicker than a classical practice. Internal transcribed spacer 1 and 2 (ITS1 and ITS2) of the nuclear ribosomal DNA (nrDNA) have become important nuclear regions for molecular systematic studies of flowering plants (reviewed by Baldwin *et al.*, 1999). These regions have fast rates of substitution which are useful for evaluating relationship at generic and species levels. Therefore molecular identification was also performed on several different morphotypes of *Aeschynanthus* cultivars and those collected from the wild. ITS sequences of nrDNA of the plant samples ware obtained and aligned with a previously prepared data matrix of 54 *Aeschynanthus* species to reconstruct a phylogenetic tree for scientific name identification of these *Aeschynanthus*.