Molecular Evolution of Nuclear Ribosomal DNA in *Aeschynanthus* and *Streptocarpus* (Gesneriaceae)

Jessada Denduangboripant

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I declare that this thesis has been composed by myself and the work contained within, unless otherwise stated, is my own.

ABSTRACT

During the past few years, the multicopy nuclear ribosomal gene (rDNA) and associated spacer regions from many organisms have been sequenced, aligned, and analysed and been found useful for a range of evolutionary applications. However, to date, the detailed mechanism of rDNA evolution remains poorly known. This thesis presents an investigation of rDNA inheritance and evolution, using two genera of the plant family Gesneriaceae, namely *Streptocarpus* and *Aeschynanthus*. Molecular phylogenetics using internal transcribed spacer (ITS) sequences of 50 *Aeschynanthus* species was carried out using PCR cloning and RNA secondary-structure analysis techniques to help solve ITS intra-individual sequence variation problems in this genus. It is suggested that intra-individual variation in *Aeschynanthus* is the result of low molecular drive and the clone variation, although persistent, is found not to predate the divergence of *Aeschynanthus* species. RNA secondary-structure analysis reveals an insertion/deletion hotspot in the ITS2 sequences that is difficult to align, especially on the terminal part of arm 1 which is probably functionally superfluous. By contrast the base of the arm is relatively conserved and may function as an exonuclease recognition site. The phylogeny of *Aeschynanthus* reveals two major clades with different but overlapping geographic distributions: one occurs primarily in mainland SE Asia and the other in Malesia. This pattern is interpreted as indicating an ancient vicariance event followed by dispersal and plate fusion. The phylogeny also reflects the classification based on seed testa and seed appendage morphology; that is, clade I has some species of section *Microtrichium* forming a basal group with sections *Haplotrichium* sens. str., a putative section *X*,

Diplotrichium, and *Polytrichium* being polyphyletic or paraphyletic, and clade II has section *Aeschynanthus* nested within the paraphyletic, basal *Microtrichium*.

In *Streptocarpus*, a length difference between ITS2 of *S. dunnii* and *S. rexii* rDNA was used as a genetic marker to study the inheritance of rDNA in hybrids between the two species. The *Streptocarpus* rDNA gene is found to have additive inheritance without any detected recombination and there are apparently two rDNA loci. A PCR silent allele was detected as a band-intensity difference in *S. dunnii* ITS2. This phenomenon was reproducible and persistent in backcrosses. I suggest that an rDNA pseudogene is possibly present as an allele at one locus of *S. dunnii*.

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CHAPTER ONE: INTRODUCTION

1.1. Family Gesneriaceae: *Aeschynanthus* and *Streptocarpus*

1.1.1. Gesneriaceae

Gesneriaceae Dum. is a large plant family of about 147 genera and over 3700 species (Burtt and Wiehler, 1995). Plants are usually herbs or shrubs, sometimes vines, and rarely trees. Many members (20%) of the family are epiphytic (Kress, 1986). Most species occur in tropical or subtropical regions, with some minor exceptions (e.g. alpines in the Himalayas, Pyrenees, southern Chile, Japan, etc.). The family is distributed world-wide from the Americas (Mexico to Chile), to Southern Europe, Africa (East, West, and South Africa and Madagascar), the Indian subcontinent, China, East and Southeast Asia, Australasia, and Polynesia. Over 60% of the family are Old World with a few Tertiary relics in temperate Eurasia (Mabberley, 1997). The family contains many important horticultural plants, such as the African Violet (*Saintpaulia*), Gloxinia (*Sinningia*), and the Cape Primrose (*Streptocarpus*), three internationally popular houseplants. Some species have been used as locally important medicine.

Gesneriads are dicotyledonous plants with opposite (or rarely whorled or spiral) leaves. The leaves are usually simple, mostly entire or toothed (rarely pinnatifid), and without stipules (Mabberley, 1997). The plants are non-succulent. Their underground parts may be fibrous, woody tubers or scaly rhizomes, or they may have aerial stolons (Heywood, 1993). Gesneriad flowers are hermaphrodite,

usually with entomophilous or ornithophilous pollination. The flowers are mostly medium-sized to large, zygomorphic, solitary or borne in cymose (usually) or racemose inflorescences. The inflorescences are terminal, axillary or epiphyllous. There are five sepals that are free, or fused for part or all of their length. The five petals are fused into a basal tube with the free ends oblique (i.e. a five-lobed corolla), usually two-lipped or rarely rotate (Heywood, 1993). The two or four stamens (rarely five) are attached to the corolla tube; staminodes are often present. The anthers are all connivent or cohere in pairs and release pollen by longitudinal slits. The ovary is superior or inferior, and usually has one locule containing numerous ovules, or is two-locular through meeting of the two parietal placentas (e.g. *Monophyllaea*). The style is single, terminal and crowned with a two-lobed or mouth-shaped stigma. An annular, lobed or one-sided nectary-disk lies between the ovary and petals (Mabberley, 1997). The fruit is usually a rounded or elongated capsule or less often a berry. The capsule is loculicidal or septicidal and contains numerous small seeds. The seeds have straight embryos with oily endosperm (Gesnerioideae) or without endosperm (Cyrtandroideae), and with two cotyledons (Heywood, 1993; Mabberley, 1997). The basic chromosome numbers are x = 4-17+ (Mabberley, 1997).

Flowers have played an important role in the evolution of Gesneriaceae, partly by co-adaptation with pollinators. Many Gesneriads have a bird-specific flower, which is two-lipped and often red. Other pollinators such as bees, bats, butterflies, moths and flies have also been found to be involved in Gesneriad pollination. Many genera, especially in the New World, show transitions from insect- to bird-pollination. The genus *Columnea* of the New World is paralleled by *Aeschynanthus* of the Old World in being bird-pollinated epiphytes (Mabberley,

1997). Extra-floral attraction also exists in some species, such as strikingly coloured hairs of leaf and sepal, leaf pigmentation with stained-glass-like optical properties when viewed against the light, extrafloral nectaries for example in *Codonanthe*, epiphytic on nests of ants, or elaiophores attractive to bees in *Drymonia* (Heywood, 1993; Mabberley, 1997). It has also been suggested that many of the morphologically diverse features of Gesneriaceae are adaptations to the diverse habits of the family, especially the epiphytic habit (Ackerman, 1986).

Although Gesneriaceae was recognised as a family some 170 years ago (by Dumortier in 1822), it has been considered to be difficult, but intriguing, to classify (Burtt and Wiehler, 1995). Methods used by temperate zone botanists to order the family were unsuccessful and failed to reflect natural groupings. Classification of the family began in Europe soon after species and genera from the freshly explored tropical countries arrived there. The De Candolles (1839, 1845) worked in Paris; Bentham (1876) classified in London; Clarke (1883) published in Paris; and Fritsch (1893) in Graz and Vienna. Floral characters, especially ovary position and corolla shape, were largely used in these classification schemes. The major problem with these classifications was that, because of the possession of superior ovaries, the New World tribes Beslerieae and Episcieae were placed with the Old World Gesneriaceae.

A later more powerful classification system for Gesneriaceae, based on studies of seedling-morphology, gave a boost to the taxonomy of the family. The studies begun at Graz by Fritsch (1904) were completed in Edinburgh by Burtt (1963). He proposed the Old World subfamily Cyrtandroideae, containing all the genera that have unequal size cotyledons soon after germination (anisocotylous), and the New World subfamily Gesnerioideae (including the tribes Beslerieae and

Episcieae) and southern Pacific genera (e.g. *Coronanthera*, *Mitraria*, etc.) having equal cotyledons (isocotylous). Seedling morphology thus divides the family into clear geographical units, and is much more satisfactory than ovary position. Later intergeneric hybridisation work at Cornell University (Wiehler, 1983) reduced the tribal system of Fritsch and realigned the genera of the neotropical Gesneriaceae. He separated the southern Pacific group as a new subfamily, the Coronantheroideae. Since then, other changes have been suggested for the family. For instance, several new genera have been added, the tree genus *Sanango* was placed in the subfamily Gesnerioideae (Wiehler, 1994), and a new tribe Titanotricheae has been added to the subfamily Cyrtandroideae (Wang, 1992).

Although other recent sources (e.g. Mabberley, 1997) still recognise only two subfamilies, this thesis follows Burtt and Wiehler's classification of the family into three subfamilies as follows.

Classification of the family Gesneriaceae (Burtt and Wiehler, 1995):

3 subfamilies, 11 tribes, about 147 genera, and over 3700 species.

I. Subfamily Gesnerioideae – 5 tribes, 56 genera, over 1800 species; distribution: Neotropics.

1. Tribe Gloxinieae Fritsch:

Gloxinia L'Hér; *Monopyle* Benth.; *Kohleria* Regel; *Anodiscus* Benth.; *Koellikeria* Regel; *Pearcea* Regel; *Parakohleria* Wiehler; *Heppiella* Regel; *Diastema* Benth.; *Capanea* Planch.; *Paliavana* Vand.; *Vanhouttea* Lem.; *Sinningia* Nees; *Lembocarpus* Leeuwenb.; *Goyazia* Taub.; *Bellonia* L.; *Phinaea* Benth.;

Niphaea Lindl.; *Smithiantha* Kuntze; *Eucodonia* Hanst.; *Achimenes* Pers.; *Moussonia* Regel; and *Solenophora* Benth..

2. Tribe Episcieae Endl.:

Episcia Mart.; *Nautilocalyx* Hanst.; *Chrysothemis* Decne.; *Corytoplectus* Oerst.; *Alloplectus* Mart.; *Cobananthus* Wiehler; *Rhoogeton* Leeuwenb.; *Drymonia* Mart.; *Paradrymonia* Hanst.; *Alsobia* Hanst.; *Neomortonia* Wiehler; *Rufodorsia* Wiehler; *Oerstedina* Wiehler; *Pentadenia* (Planch) Hanst.; *Dalbergaria* Tussac; *Trichantha* Hook.; *Columnea* L.; *Bucinellina* Wiehler; *Codonanthopsis* Manst.; *Codonanthe* (Mart.) Hanst.; *Nematanthus* Schrad..

3. Tribe Beslerieae Bartl. & Wendl.:

Besleria L.; *Gasteranthus* Benth.; *Cremosperma* Benth.; *Reldia* Wiehler; *Resia* H.E.Moore; *Tylopsacas* Leeuwenb.; *Cubitanthus* Barringer; *Anetanthus* Hiern ex Benth..

4. Tribe Napeantheae Wiehler:

Napeanthus Gardner.

5. Tribe Gesnerieae:

Gesneria L.; *Sanango* Bunting & Duke.

II. Subfamily Coronantheroideae Wiehler – 1 tribe, 9 genera, 20 species; distribution: southern Chile, South Pacific Islands, Australia.

6. Tribe Coronanthereae Fritsch:

Mitraria Cav.; *Sarmienta* Ruiz & Pav.; *Asteranthera* Hanst.; *Fieldia* A.Cunn.; *Lenbrassia* G.W.Gillett; *Coronathera* Vieill. ex C.B.Clarke; *Negria* F.Muell.; *Rhabdothamnus* A.Cunn.; *Depanthus* S.Moore.

III. Subfamily Cyrtandroideae Endl. – 5 tribes; 82 genera; over 1900 species; distribution: Old World; chiefly in tropics; but 1 species in Neotropics.

7. Tribe Klugieae Fritsch:

Rhynchoglossum Blume; *Epithema* Blume; *Monophyllaea* R.Br.; *Whytockia* W.W.Smith; *Loxonia* Jack; *Gyrogyne* W.T. Wang; *Stauranthera* Benth.

8. Tribe Didymocarpeae Endl.:

Acanthonema Hook.; *Allocheilos* W.T.Wang; *Allostigma* W.T.Wang; *Ancylostemon* Craib; *Anna* Pellegr.; *Beccarinda* Kuntze; *Boea* Lam.; *Boeica* C.B.Clarke; *Bournea* Oliv.; *Briggsia* Craib; *Briggsiopsis* K.Y.Pan; *Calcareoboea* H.W.Li; *Cathayanthe* Chun; *Championia* Gardner; *Chirita* D.Don; *Chiritopsis* W.T.Wang; *Colpogyne* B.L.Burtt; *Conandron* Siebold & Zucc.; *Corallodiscus* Batalin; *Dayaoshania* W.T.Wang; *Deinocheilos* W.T.Wang; *Deinostigma* W.T.Wang ex Z.Y.Li; *Didissandra* C.B.Clarke; *Didymocarpus* Wall.; *Didymostigma* W.T.Wang; *Dolicholoma* D.Fang & W.T.Wang; *Gyrocheilos* W.T.Wang; *Haberlea* Friv.; *Hemiboea* C.B.Clarke; *Hemiboeopsis* W.T.Wang; *Hexatheca* C.B.Clarke; *Isometrum* Craib; *Jancaea* Boiss.; *Lagarosolen* W.T.Wang; *Leptoboea* Benth.; *Linnaeopsis* Engl.; *Loxocarpus* R.Br.; *Metabriggsia* W.T.Wang; *Metapetrocosmea* W.T.Wang; *Nodonema* B.L.Burtt; *Opithandra* B.L.Burtt; *Orchadocarpa* Ridl.; *Oreocharis* Benth.; *Ornithoboea* C.B.Clarke; *Paraboea* (C.B.Clarke) Ridl.; *Petrocodon* Hance; *Petrocosmea* Oliv.; *Phylloboea* Benth.; *Platyadenia* B.L.Burtt; *Platystemma* Wall.; *Primulina* Hance; *Pseudochirita* W.T.Wang; *Ramonda* Rich.; *Raphiocarpus* Chun; *Rhabdothamnopsis* Hemsl.; *Saintpaulia* H.Wendl.; *Schizoboea* (Fritsch) B.L.Burtt; *Streptocarpus* Lindl.; *Tengia* Chun; *Tetraphyllum* C.B.Clarke;

monophyletic subfamily, or within Gesnerioideae. The tribal classification system of the family is, however, largely incongruent with the results. The traditionally recognised tribes Trichosporeae and Didymocarpeae are not monophyletic in these analyses. The tribe Klugieae (Cyrtandroideae) appears to be separate and sister to the remainder of the family when analysed with either morphological or sequence data alone but, in combined-data analysis, to be in its traditional place as a member of the subfamily Cyrtandroideae. Tribal relationships within Gesneriaceae still require further investigation.

1.1.2. *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 the Himalayas to Southern China and throughout Indo-China, and from Southern India and Sri Lanka, throughout Southeast Asia to New Guinea and the Solomon Islands (Fig. 1.1).

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

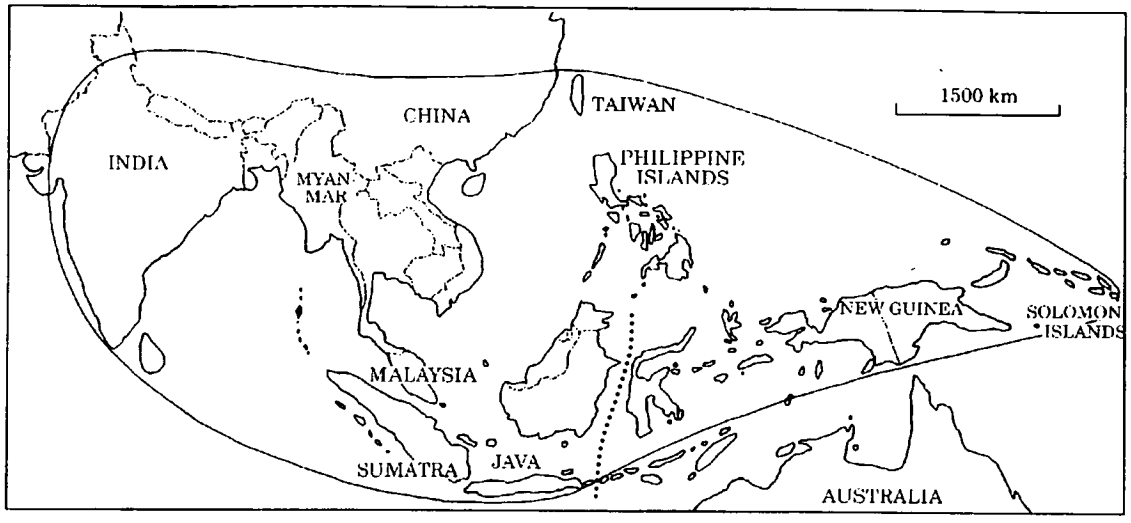


Fig. 1.1 The geographical distribution of *Aeschynanthus*, indicated by a solid line. Wallace's line is indicated out by a dotted line. (After Mendum et al., 2001)

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; Weber and Mendum, in prep.). 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-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).

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*) (Weber and Mendum, in prep.). Although *Aeschynanthus* is widespread, there is a high degree of endemism at the species level (Mendum et al., 2001).

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 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. 1.2.

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. 1.2.

- 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. 1.3.

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. 1.3.

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. 1.3.

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

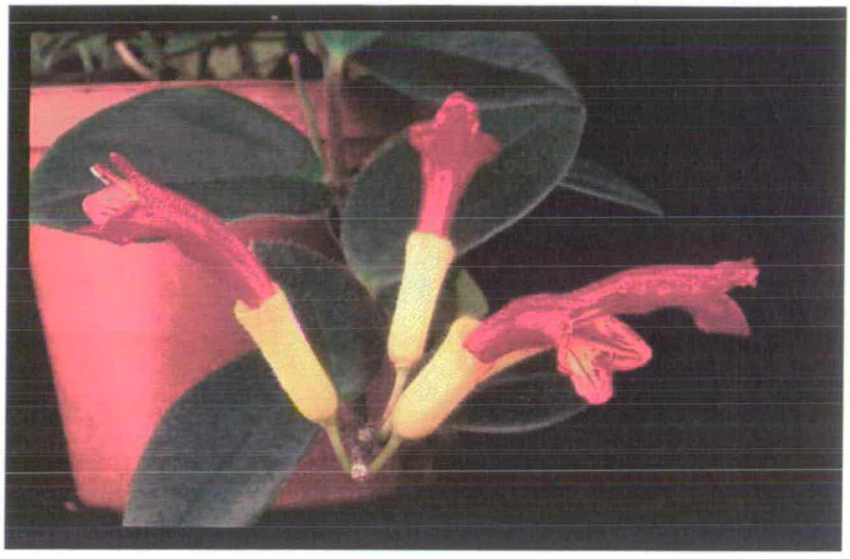


Fig. 1.2 Examples of flower morphology of *Aeschynanthus*: from top to bottom; *A. siphonanthus* (section *Aeschynanthus*) - species in this section have a distinctive shallow-lobed calyx, and *A. bracteatus* (*Haplotrichium*). Photos are by courtesy of the Royal Botanic Garden Edinburgh.

Fig. 1.3 Examples of flower morphology of *Aeschynanthus* (continued): from top to bottom; *A. lineatus* (*Diplotrichium*), *A. longicaulis* (section *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 have been undertaken by Mary Mendum and are concentrating mostly on the Malaysian, Philippine, and New Guinea species (for instance, Mendum, 1995, 1998, 1999; Mendum and Madulid, 1995; Mendum and Woods, 1997). Her recent studies (Mendum et al., 2001) on seed and appendage morphology identified two major groups (type A and B) within the genus (Fig. 1.4). 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 a 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.

Based on these findings a revised key to the sections (Mendum et al., 2001) is as follows (see also Table 1.1 and Fig. 1.5).

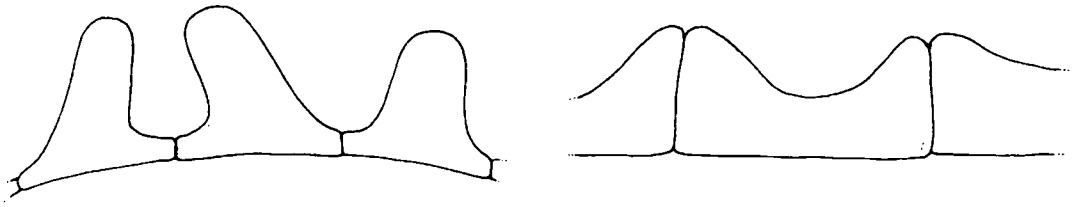


Fig. 1.4 Comparison of papillae of type A seed with single-celled papillae (left, of *A. parvifolius*) and type B seed with two-celled papillae (right, of *A. albidus*). (After Mendum et al., 2001)

Revised key to the sections

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* s.s.

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 1.1. Revised sectional classification of *Aeschynanthus* and characteristics of the sections.

Section	Section morphology	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 sens. str.	seed hilar appendage single, to 3.5 mm, slender to base; testa cell orientation a clockwise spiral; inflorescences long pedunculate; bracts persistent; calyx deeply divided, without abscission layer at base; stamens exserted.	*A. bracteatus* DC	c. 8	India, Indo-China and China
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.

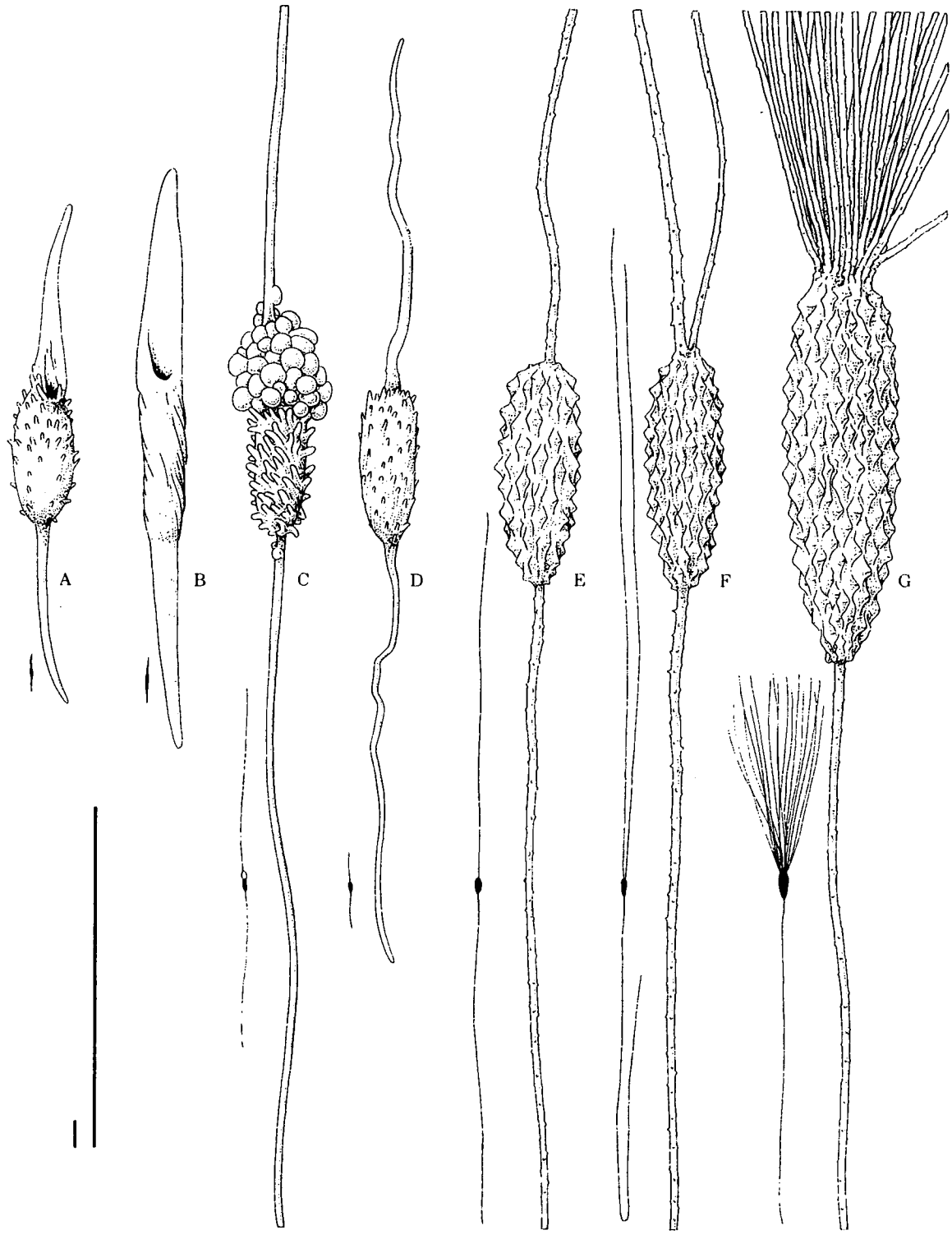


Fig. 1.5 *Aeschynanthus* seed: A. & B. section *Microtrichium* (*A. garrettii* and *A. musaensis* respectively), C. section *Aeschynanthus* (*A. curtisii*), D. section *Haplotrichium* sens. str. (*A. bracteatus*), E. section *X* (*A. longiflorus*), F. section *Diplotrichium* (*A. sikkimensis*), G. section *Polytrichium* (*A. arfakensis*). Scale bars = 2 mm. (After Mendum et al., 2001)

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 section, 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.

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).

Molecular phylogenetic studies utilising internal transcribed spacer (ITS) regions of *Aeschynanthus* nuclear ribosomal DNA (nrDNA) will help clarify the classification of the genus and, moreover, evaluate ideas about plant evolution and dispersal in a tectonically complex region like Southeast Asia. However, many of the sequenced results were found to be unreadable or poor, apparently because of ITS polymorphism. To circumvent this problem a PCR cloning technique has been adopted which successfully solved the sequencing problems.

1.1.3. *Streptocarpus*

Species in the genus *Streptocarpus* Lindl. (Gesneriaceae, subfamily Cyrtandroideae, tribe Didymocarpeae) are often found in tropical rain forests (sometimes as epiphytes) and on damp banks or in grassland. The genus contains about 130 species of herbs and subshrubs, annuals and perennials, some monocarpic (Christopher, 1996). The familiar horticultural Cape Primroses belong to this genus. *Streptocarpus* occurs in Africa, Madagascar, and the Comoro Islands, with four species from Southwest China, Burma, Thailand, and Sumatra (each with a single species) (Fig. 1.6) (Hilliard and Burtt, 1971). These Asiatic species are remote from the rest of the genus and not even closely related amongst themselves. Their streptocarpy (possession of twisted fruit) is merely a parallel development (Hilliard and Burtt, 1971).

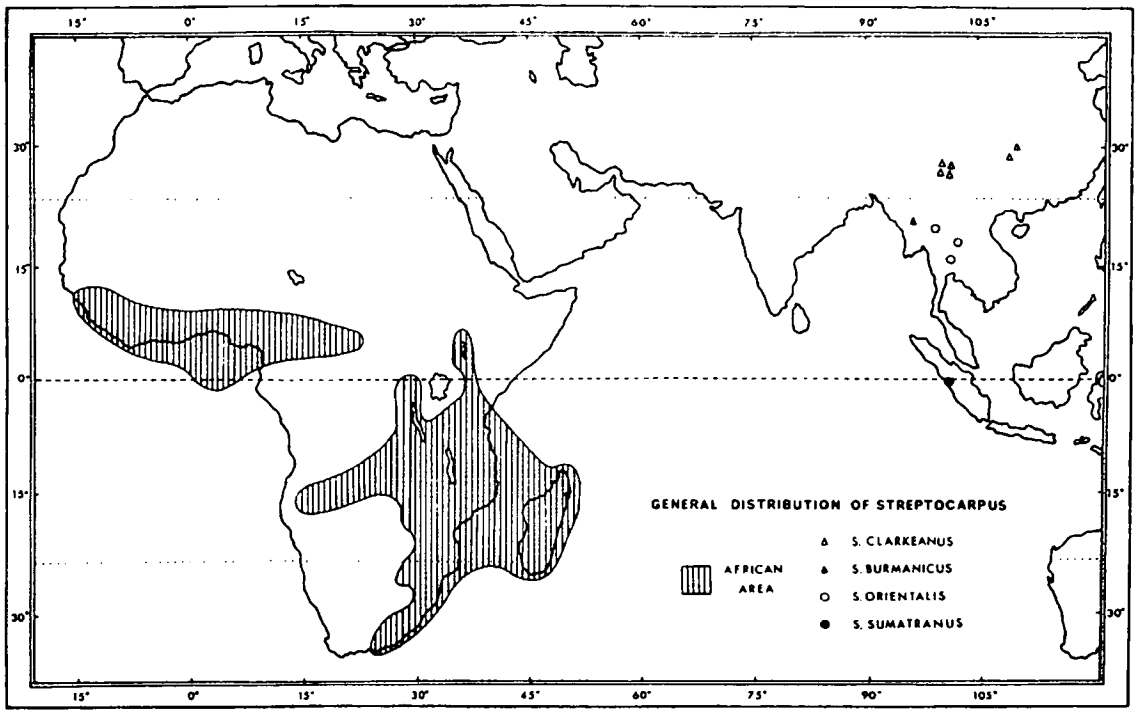


Fig. 1.6 The geographical distribution of *Streptocarpus*. (After Hilliard and Burtt, 1971)

As with all other members of the subfamily Cyrtandroideae, *Streptocarpus* cotyledons grow unequally after germination. One cotyledon continues to grow larger (= macrocotyledon), while the other remains very small and eventually withers. *Streptocarpus* leaves are linear to rounded, hairy, often mid-green, veined and wrinkled. Adventitious roots are produced from the lower part of the hypocotyl (Christopher, 1996; Mabberley, 1997). The plants may be propagated from small fragments of the leaf (Mabberley, 1997). The flowers are always borne in a cymose inflorescence. The inflorescences are axillary or borne from the leaf rosettes at the junction of hypocotyl and midrib. The calyx is usually divided to the base into five segments, or more rarely has a distinct tube and five teeth (Hilliard and Burtt, 1971). The corollas are typically tubular, zygomorphic, 2-lipped with five spreading lobes (Christopher, 1996). The corolla-form is diverse within the genus, depending on the specific pollinator (Fig. 1.7). For instance, many species have corollas with a narrow

slit-like opening, a shape associated with long-tongued hovering insects like hawk moths, whereas red corollas suggestive of bird-pollination are found in two species: *S. dunnii* and *S. myoporoides* (Hilliard and Burtt, 1971). The androecium is basically very uniform with two fertile anterior stamens and three posterior ones represented by staminodes. The ovary is usually unilocular (sometimes bilocular by fusion of the T-shaped intrusive placentae) and often narrowed gradually into the style; the stigma is variable (Hilliard and Burtt, 1971).

After pollination, *Streptocarpus* develops a spirally twisted fruit, the outstanding single feature which gives the genus its name. The fruits are variable in length and thickness (Hilliard and Burtt, 1971).

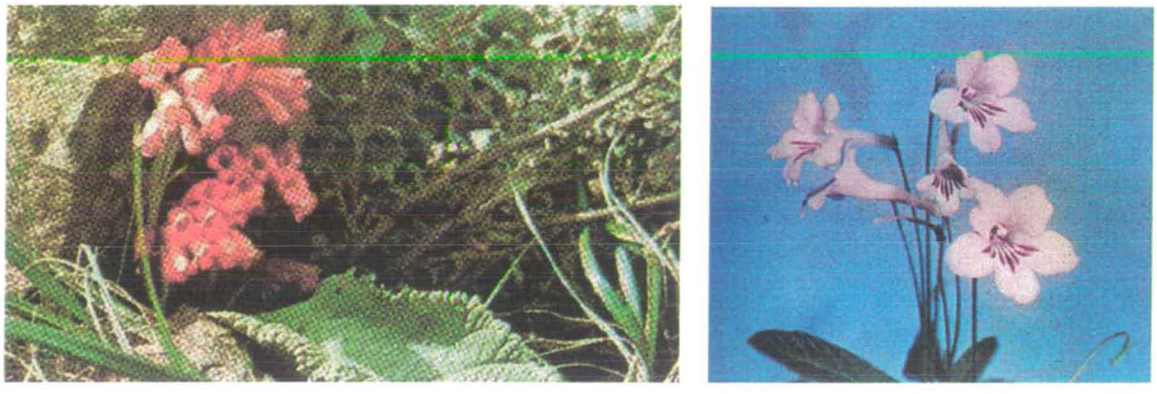


Fig. 1.7 Comparison between bird-pollinated and insect-pollinated *Streptocarpus* flowers, respectively. (Left: *S. dunnii*, right: *S. rexii*) (After Hilliard and Burtt, 1971)

The vegetative morphology of *Streptocarpus* is tremendously diverse, ranging from highly unusual acaulescent types to more or less caulescent. These differences in morphology can be used to divide *Streptocarpus* species into three basic growth forms: caulescent, rosulate, and unifoliate (Fig. 1.8). The caulescent species typically have aerial shoots with decussate leaves and axillary buds; the stem forms by the

forms by the hypocotyl increasing in thickness. The unifoliate habit consists of only a single unusually enlarged cotyledon (up to 76 cm in some species; Mabberley, 1997), with inflorescences arising from the base of the lamina. The rosulate species form a loose rosette of leaves. The enlarged cotyledon is known as a phyllomorph (Jong, 1970, 1973, 1978; Jong and Burtt, 1975), possessing mixed characteristics of shoot and leaf. The unifoliate species thus consist of a single phyllomorph, while the rosulate plant is an assemblage of many phyllomorphs (Jong and Möller, 2000). By supplying gibberellic acid or inhibiting auxin transport phyllomorph development can be suppressed and caulescent growth can be induced (Mabberley, 1997).

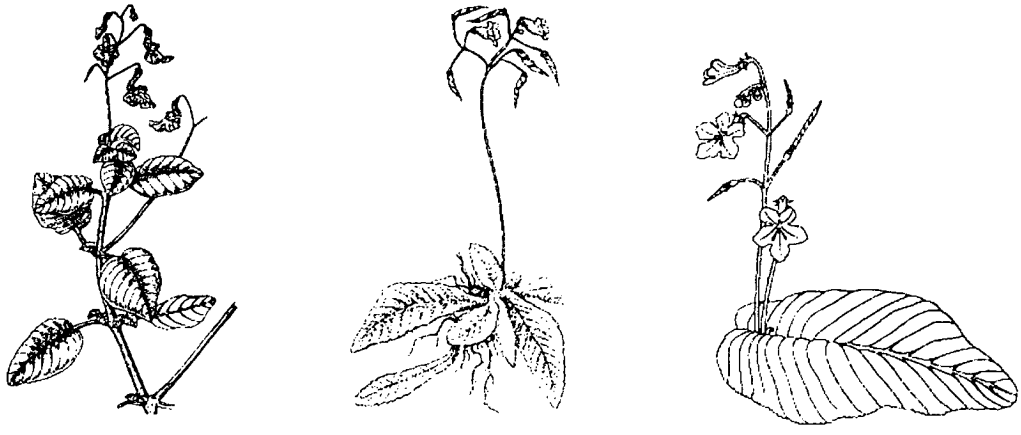


Fig. 1.8 Three different basic growth forms of *Streptocarpus*: from left to right; caulescent, rosulate, and unifoliate (After Möller and Cronk, 2001).

The immense diversity of growth forms in the genus poses a challenge to the taxonomist. The extraordinarily unequal growth of *Streptocarpus* has been recognised for over a hundred years. (Crocker, 1860; Hielscher, 1883). In 1883 C. B. Clarke first published an account of 17 *Streptocarpus* species and since then the total number of species has risen steadily to 132 (Hilliard and Burtt, 1971). Two

subgenera were recognised by B. L. Burtt in 1958, based on morphological diversity and cytological evidence: subgenus *Streptocarpus* containing unifoliate and rosulate species, and subgenus *Streptocarpella* containing the caulescent species. Subgenus *Streptocarpus* is found in southern, central and eastern Africa, from southern Ethiopia to Madagascar. Subgenus *Streptocarpella*, by contrast, ranges across tropical Africa from Sierra Leone to Madagascar, and in Asia. This subgeneric division therefore is almost clear cut amongst mainland African species, but more complicated amongst those from Madagascar. Some of these, moreover, cannot be easily placed in either of the two subgenera because of their unusual morphology (Hilliard and Burtt, 1971).

Phylogenetic relationships among *Streptocarpus* species, and also *Saintpaulia* (the African Violet), were examined by Möller and Cronk, (1997a, 1997b, 2001) using comparative internal transcribed spacers and the 5.8S region of nuclear ribosomal DNA. They found that *Streptocarpus* can be divided into two major clades congruent with morphological classification. One of these broadly corresponds to the caulescent group as subgenus *Streptocarpella* with a basic chromosome number x = 15, whereas the other is mainly composed of acaulescent species as subgenus *Streptocarpus* with x = 16. The results have confirmed the independent origin of the two subgenera from a common ancestor. This knowledge of the origin of the genus has important implications for horticulture as well as for evolutionary studies.

From their study, the ITS2 regions of *S. dunnii* Hook. f. and *S. rexii* Lindl. (both in subgenus *Streptocarpus*) were found to differ in length by 44 basepairs. *Streptocarpus dunnii* is a monocarpic and unifoliate species (rarely several-leaved

and perennial). All parts except the upper surface of the phyllomorph are spattered with red granules. The flowers are distinctively red, tubular in shape, and open together to confront a pollinator, thought to be a bird (Fig. 1.7 left). It is distributed in Transvaal and Swaziland (Hilliard and Burtt, 1971). *Streptocarpus rexii*, on the other hand, is a perennial with rosulate growth form. It was the first *Streptocarpus* to be discovered and became the type species and the best-known cultivated species in the genus. The flowers are butterfly/moth pollinated, narrowly tubular, and white faintly tinted with seven violet stripes (Fig. 1.7 right). The species is distributed in the Cape Province of South Africa eastward to southernmost Natal. (Hilliard and Burtt, 1971). F_1 hybrids between *S. dunnii* and *S. rexii* have been experimentally produced at the Royal Botanic Garden Edinburgh, and showed intermediate leaf- and flower-morphologies i.e. having pinkish flowers (from red of *S. dunnii* and white of *S. rexii*) and having both unifoliate and rosulate characters in one plant (Fig. 1.9).

This ITS2 length difference can be used as a good marker for the study of inheritance and a number of loci of *Streptocarpus* rDNA. The polymerase chain reaction (PCR) technique was performed to characterise ITS2 copies in F_1 hybrids and backcrosses of these two species. Moreover, the PCR-silencing phenomenon, reported previously in some other *Streptocarpus* species by Möller (2000) was also found in this research and some experiments were done to further investigate this phenomenon.

(A)

(B)

Fig. 1.9 Morphological characters of *S. rexii*, *S. dunnii*, and their F_1 hybrids. (A) comparison of vegetative growth form of *S. rexii* with rosulate form, F_1 hybrid, and *S. dunnii* with unifoliate form, respectively. (B) comparison of flower colour and shape. Photos taken by Debbie White, RBGE.

1.2. Plant nuclear ribosomal DNA

1.2.1. Structure of 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, 1976), 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, 1986). 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. 1.10.

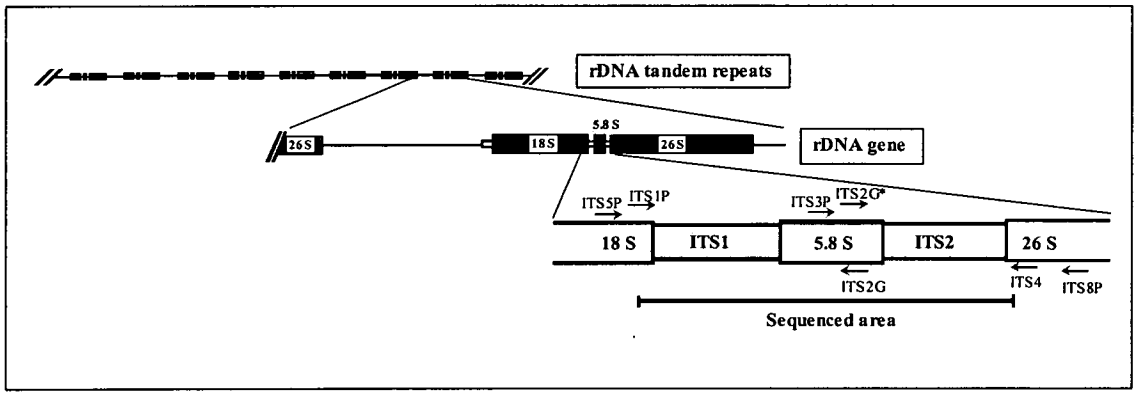


Fig. 1.10. Repeat units of the nuclear ribosomal DNA and the organisation of the internal transcribed space (ITS) region (modified from White et al., 1990). Arrows indicate orientation and approximate position of primer sites.

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)

1.2.2. 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).

1.3. Internal transcribed spacer (ITS) regions and plant phylogenetics

1.3.1. 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).

1.3.2 Intra-individual variation in plant ITS regions.

ITS variation within species is unusual in angiosperms (Baldwin et al., 1995). However, ITS variations have been reported recently in conifers (Karvonen and Savolainen, 1993), Winteraceae (Suh et al., 1993), peonies (Sang et al., 1995), *Zea* (Buckler and Holtsford, 1996), and *Amelanchier* (Campbell et al., 1997). Variability within any multigene families depends upon number of gene copies, rates of mutation and speciation, number and chromosomal location, and proportion of sexual and asexual reproduction. Variation of rDNA within species therefore may occur if concerted evolution is not fast enough to homogenise rDNA repeats, when there are high rates of mutation and/or recent interspecific hybridisation.

Many reasons for variation of rDNA within species have been proposed in many ways, such as in transition stages of concerted evolution (Strachan et al., 1985), when mutation rate exceeds the rate of concerted evolution as in length variants in the intergenic spacer (Crease and Lynch, 1991), as a result of interspecific hybridisation (Crease and Lynch, 1991; Rieseberg, 1991; Soltis and Soltis, 1991; Kim and Jansen, 1994; Sang et al., 1995), when pseudogenes evolve (Buckler and Holtsford, 1996), or when location of rDNA loci on nonhomologous chromosomes potentially disrupts concerted evolution (Karvonen and Savolainen, 1993; Suh et al., 1993; Jellen et al., 1994). A long generation time (Sang et al., 1995) and loss of sexual recombination (Campbell et al., 1997) was also suggested as a mechanism that might retard concerted evolution.

Most reported within-species ITS variations are associated with allopolyploidy (and/or agamospermy) or multiple nucleolar organising regions (NORs). Variation related to high levels of polyploidy was demonstrated by Suh et al. (1993) whereas variation relating to the number of NORs is well demonstrated in conifers (Karvonen and Savolainen, 1993). One exception is the case of *Zea* rDNA pseudogenes (Buckler and Holtsford, 1996). This intra-individual variation in ITS sequence indicates that care is required when using sequences of the region in phylogenetic studies, and more studies on this phenomenon need to be done.

1.4. Research objectives

: To investigate ITS intra-individual variation in *Aeschynanthus*.

: To reconstruct *Aeschynanthus* phylogenetic trees from ITS sequences.

: To study ribosomal DNA inheritance of *Streptocarpus* from *Streptocarpus dunnii* and *S. rexii* hybrids.

: To investigate the PCR-silencing phenomenon in ITS2 of *S. dunnii*.

CHAPTER TWO: Intra-individual variation in ITS sequences in *Aeschynanthus* (Gesneriaceae)

(Materials in this chapter have been published in 'Denduangboripant, J., and Q. C. B. Cronk. 2000. High intra-individual variation in ITS sequences in *Aeschynanthus* (Gesneriaceae): implications for phylogenetics. *Proceedings of the Royal Society of London, Series B* 267: 1407-1415.')

2.1. Introduction

The internal transcribed spacers of nuclear ribosomal DNA are frequently used for phylogenetic analysis at the species level (Baldwin et al., 1995). Although nuclear ribosomal DNA occurs in long arrays of repeats, it is still useful for phylogenetics as the copies are usually highly homogenous, due to concerted evolution. However, there is sometimes persistent intra-individual variation in the rDNA repeats (eg. Campbell et al., 1997): a phenomenon I report here for the genus *Aeschynanthus*.

As *Aeschynanthus* is an important component of the Southeast Asian epiphyte community and spread over phytogeographically contrasting areas, it is an interesting genus for evaluating ideas about the evolution of plants in a tectonically complex region, and in particular the relevance of Wallace's line (Fig. 1.1) to the distribution of wind dispersed epiphytes. I therefore wished to produce a phylogenetic analysis of the genus using sequences of the ITS of nrDNA, which have proved successful in phylogenetic analyses of other genera in the Gesneriaceae such as *Streptocarpus* of Africa and Madagascar (Möller and Cronk, 1997a) and *Saintpaulia* (Möller and Cronk, 1997b). For this reason I attempted direct sequencing

of PCR products as described previously by Möller and Cronk (1997a). However, many of the resulting *Aeschynanthus* ITS sequences were unreadable or poor, apparently because of significantly more extensive polymorphism at the template DNA level in *Aeschynanthus* than in *Streptocarpus*. To circumvent this problem I then introduced a PCR cloning technique to separate individual sequences. PCR products were cloned and two clones of each sample were sequenced.

2.2. Materials and methods

2.2.1. Ingroup and outgroup selection

As an outgroup I selected *Lysionotus* D.Don of the tribe Trichosporeae to which *Aeschynanthus* belongs. I also used a member of another tribe (Cyrtandreae), *Cyrtandra*, as a check, in the unlikely event that *Aeschynanthus* proved to be paraphyletic with respect to *Lysionotus*. Initially, I also sequenced a third outgroup species, *Dichrotrichum* (*Agalmyla*) *biflorum* Elmer (this species has yet to be combined under the preferred generic name *Agalmyla*). *Agalmyla* is another genus of Trichosporeae (like *Aeschynanthus*, typically climbing epiphytes with scarlet, presumably bird-pollinated flowers) which has been considered closely related to *Aeschynanthus* as discussed in Chapter One. However I had difficulty aligning ITS sequences of *Agalmyla* and *Aeschynanthus*, which may indicate that these two genera are not closely related but whose morphologies have converged in similar habitats. This idea was tentatively suggested by Rosser and Burtt (1969) but rejected by these authors on the basis of similarities in vegetative anatomy. Smith et al. (1997) have also suggested that *Agalmyla* and *Aeschynanthus* are not closely related. Because of

this uncertainty, and the alignment difficulties, I rejected *Agalmyla* as an outgroup for this study.

For the ingroup, I chose 23 species of *Aeschynanthus* representing all sections (except *Xanthanthos*) and geographical areas (see Fig. 2.1). Material of section *Xanthanthos* was not available for study. All samples came from the living collections at the Royal Botanic Garden Edinburgh. Details of their accession numbers and distribution are in Table 2.2 and photographs of the majority of the species are shown in Appendix D. Voucher herbarium specimens of all these accessions have been prepared and deposited at E. Although these species represent a relatively small sample, the choice of taxa is designed to encompass the full range of morphological variation in the genus.

2.2.2. DNA extraction, PCR, cloning and DNA sequencing

Details of DNA extraction, PCR, sequencing and alignment are given in Appendix A. Briefly, a leaf sample was taken from a single individual plant of each species. Forward and reverse sequencing reactions were performed for sequence confirmation. Sequencing products were analysed on an ABI 377 prism automatic DNA sequencer (PE Biosystems, Inc., Warrington, UK). ITS RNA secondary structures, generated by the program RNAdraw version 1.1 (Matzura and Wennborg 1996), were used to interpret insertions in *Aeschynanthus buxifolius* ITS1 (see Results and Fig. 2.2) and to optimise alignment in arm 1 of the ITS2 region (see Chapter Three). To clarify the intra-individual variation found in *Aeschynanthus* ITS sequences, and solve the problem of reading the PCR consensus sequences, a PCR

Table 2.1. Accessions of *Aeschynanthus*, *Cyrtandra baileyi*, and *Lysionotus forrestii* examined for ITS1 and ITS2 sequence variation.

Taxon	Locality collected (Distribution)	section	Genbank accession No. (ITS1 & ITS2)	RBGE accession No.
Cyrtandra baileyi F.Muell.	Australia: North Queensland	-	AF349151 & AF349232	Cronk T118[a]
Lysionotus forrestii W.W.Sm.	China: Yunnan	-	AF349152 & AF349271	19962269
(1) *Aeschynanthus albidus* (Blume) Steud.	Brunei Darussalam (Malaysia: Borneo, Sabah, Sarawak; Indonesia: Java, Sumatra)	*Polytrichium*	AF349190 & AF349271 (clone A) AF349191 & AF349272 (clone B)	19912436
(2) *Aeschynanthus angustifolius* (Blume) Steud.	Indonesia: Sumatra (Indonesia: Java; Malaysia: Malaya, Borneo)	*X*	AF349194 & AF349275 (clone A) AF349195 & AF349276 (clone B)	19881452
(3) *Aeschynanthus arctocalyx* Mendum & Madulid	Philippines: Palawan	*Aeschynanthus*	AF349172 & AF349253 (clone A) AF349173 & AF349254 (clone B)	19922776
(4) *Aeschynanthus argentii* Mendum	Malaysia: Sabah	*Microtrichium*	AF349180 & AF349261 (clone A) AF 349181 & AF349262 (clone B)	19801419
(5) *Aeschynanthus bracteatus* Wall. ex A. DC.	Viet Nam (China: Guangxi, Xizang, Yunnan; India: Assam, Sikkim, Bengal; Bhutan; North Burma; Tibet)	*Haplotrichium* sens. str.	AF349202 & AF349283 (clone A) AF349203 & AF349284 (clone B)	19970165
(6) *Aeschynanthus buxifolius* Hemsl.	Viet Nam: Lào Cai Province (China: Guangxi, Guizhou, Yunnan)	*Microtrichium*	AF349198 & AF349279 (clone A) AF349199 & AF349280 (clone B)	19970178

(16) *Aeschynanthus macranthus* Pellegr.	Thailand (Laos; North Vietnam; China: Yunnan)	*X*	AF349208 & AF349289 (clone A) AF349209 & AF349290 (clone B)	19801140
(17) *Aeschynanthus magnificus* Stapf	Malaysia: Sabah (Malaysia: Sarawak)	*Microtrichium*	AF349182 & AF349263 (clone A) AF349183 & AF349264 (clone B)	19812958
(18) *Aeschynanthus fulgens* Wall.	Cultivated (China: Xizang, Yunnan; India: Assam; Burma)	*X*	AF349212 & AF349293 (clone A) AF349213 & AF349294 (clone B)	19900384
(19) *Aeschynanthus pachyanthus* Schltr.	Papua New Guinea	*Microtrichium*	AF349186 & AF349267 (clone A) AF349187 & AF349268 (clone B)	19623299
(20) *Aeschynanthus parvifolius* R.Br.	Indonesia: Sumatra (Malaysia: Malaya, Sarawak, Sabah; Indonesia: Sumatra, Java, Kalimantan; Singapore)	*Aeschynanthus*	AF349176 & AF349257 (clone A) AF349177 & AF349258 (clone B)	19881451
(21) *Aeschynanthus radicans* Jack	Malaysia: Sarawak (Thailand; Malaysia: Malaya Sabah; Indonesia: Kalimantan)	*Aeschynanthus*	AF349178 & AF349259 (clone A) AF349179 & AF349260 (clone B)	19622826
(22) *Aeschynanthus sikkimensis* (C.B.Clarke) Stapf	Cultivated (Nepal; Bhutan; India: Assam, Bengal, Sikkim)	*Diplotrichium*	AF349214 & AF349295 (clone A) AF349215 & AF349296 (clone B)	19611984
(23) *Aeschynanthus tricolor* Hook.	Malaysia: Sabah (Malaysia: Sarawak)	*Aeschynanthus*	AF349170 & AF349251 AF349171 & AF349252	19812968

[a] From material cultivated at Canberra Botanic Garden, Australia

method was introduced. I sequenced two clones per species. PCR-amplified DNA of the whole ITS region was gel extracted and purified before cloning. The Prime PCR Cloner cloning system (5 Prime-3 Prime, Inc., Boulder, CO, USA) was used to clone the purified PCR products into the pN_0TA/T_7 cloning vector supplied, following the manufacturer's instructions (see Appendix A). The isolated plasmids were digested with an *Xba*I restriction enzyme (Promega, Co., Madison, WI, USA) to determine the size of the subcloned PCR fragment in the clones, which could then be compared with the original PCR product. Clones with the correct size insert (c. 700 bp) were sequenced using normal cycle sequencing methods as described in Appendix A. Both ITS1 and ITS2 were sequenced from each clone. For the outgroups, consensus sequences were obtained without cloning. All sequences have been submitted to the Genbank database.

2.2.3. Phylogenetic analysis

Phylogenetic trees were reconstructed from the combined ITS1 and ITS2 sequence data using parsimony as implemented in the program PAUP* (Swofford, 1998) version 4.0b. Details of tree reconstruction are available in Appendix A. Ambiguous regions in the aligned matrix were excluded from phylogenetic analysis. Indels (gaps) were treated as missing data and scored as separate presence/absence characters. Analyses with and without the gap matrix were carried out. Character state changes were weighted equally. Because the matrix is moderately large (more than 20 taxa), I performed heuristic searches instead of branch and bound searches (suitable only for under 20 taxa) to find the most parsimonious trees by using

SIMPLE addition sequence with TBR (tree bisection-reconnection) swapping, with options MULTREES, steepest descent, COLLAPSE, and ACCTRAN optimisation. Replication of searches using 1000 RANDOM addition sequences with TBR swapping for island searching strategy failed to find further shortest trees. Bootstrap analysis (full heuristic) (Felsenstein, 1985) and jackknife analysis (Lanyon, 1985) with 50% deletion ("fast" stepwise-addition) were used as indications of branch support for individual clades. Bootstrap and jackknife values were calculated using 100 replicates and 10000 replicates, respectively. The consistency index (CI) and retention index (RI) were calculated. The other sequence and tree statistics, namely G+C content, sequence divergence, and transition-transversion ratios were determined as described previously by Möller and Cronk (1997a). To test for possible effects of the outgroups on the analysis, the searches were re-run without outgroups. Midpoint-rooting was then employed. As the topology of the resulting tree and the position of the root were identical, these results are not reported further.

2.3. Results

2.3.1. Types of consensus sequencing problem

As mentioned in the introduction to this chapter, the preliminary direct sequencing of ITS PCR products of several *Aeschynanthus* taxa showed a difficulty in sequence interpretation. By contrast, introducing cloning prior to routine sequencing solved these consensus sequencing problems. It revealed the real sequences hiding behind the ambiguous regions in the consensus sequences. An

example of the sequence variation is shown in Fig. 2.1. There are three kinds of intra-individual variation found in *Aeschynanthus* ITS sequences:

1. Single-basepair sequence polymorphisms, showing in consensus sequencing a double-peak signal of two approximately equal nucleotide signals at a single base position (these are distributed apparently randomly through the sequences). This variation, in both ITS1 and ITS2 regions, was found in clone pairs of all taxa except *A. ceylanicus* and *A. hookeri*. In some cases the signal from one nucleotide was about half the magnitude of the other signal.

2. Single-basepair indel (= insertion/deletion) polymorphisms, showing a clear consensus sequence up to the indel position, at which point the sequence showed double-peak nucleotide signals to the end. As such sequences are the result of a frameshift, the underlying sequences cannot be easily reconstructed from the consensus or from reverse sequences. Such indels are found in ITS1 of four *Aeschynanthus* taxa, namely *A. guttatus*, *A. bracteatus*, *A. buxifolius*, and *A. parvifolius* and in ITS2 of three taxa, namely *A. guttatus*, *A. gracilis*, and *A. fulgens*.

3. Multiple-basepair indel polymorphisms. Consensus sequencing results are similar to the single-basepair indel polymorphism, but the ambiguous region is impossible to interpret from the consensus or reverse sequencing. I detected this phenomenon in only one species: it occurred in two places in the ITS1 of the *A. buxifolius* consensus sequence. Cloned sequences revealed two four-basepair insertions in these regions (see Fig. 2.1). RNA secondary structure analysis reveals that these two insertions are complementary insertions in a stem structure (Fig. 2.2).

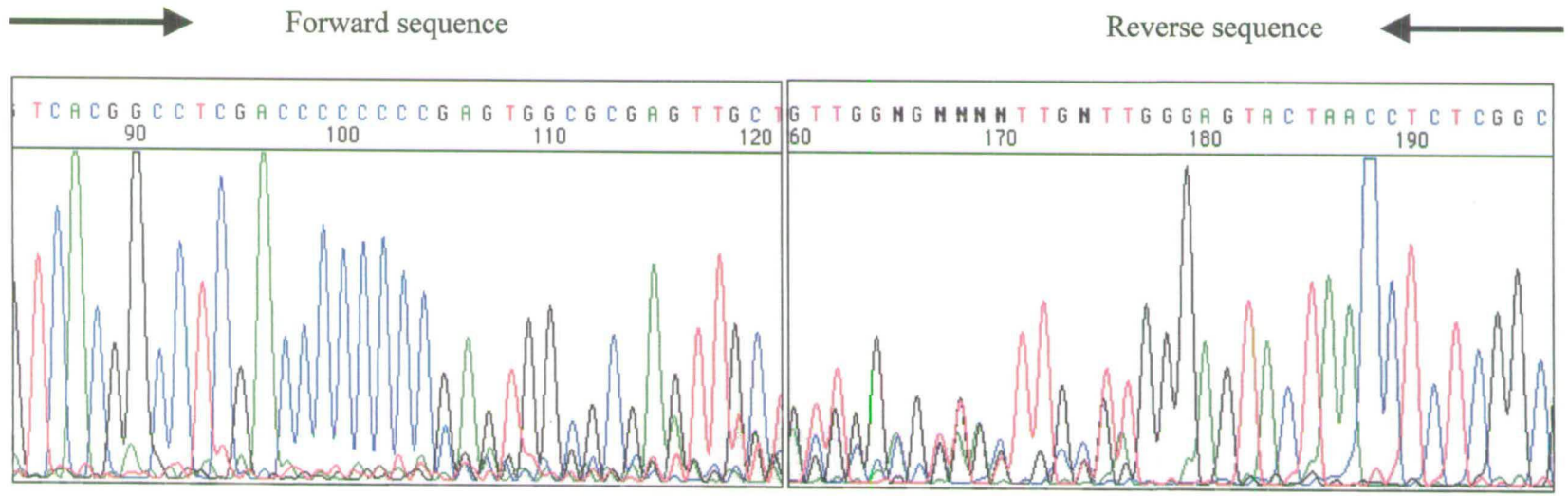


Figure 2.1. Electropherograms of an ITS1 consensus sequence of *Aeschynanthus buxifolius*, compared to the same region sequenced in two clones. When sequenced with primer 5P (left picture), the consensus electropherogram is easily readable until position 104, whereas sequencing with primer 2G for the reverse sequence (right picture) gives a reverse sequence readable to position 176. A comparison of the clones' sequences reveals two four-basepair indels in this region (clone A). A secondary structure analysis (Fig. 2.2) reveals that these indels are complementary insertions in a stem structure.

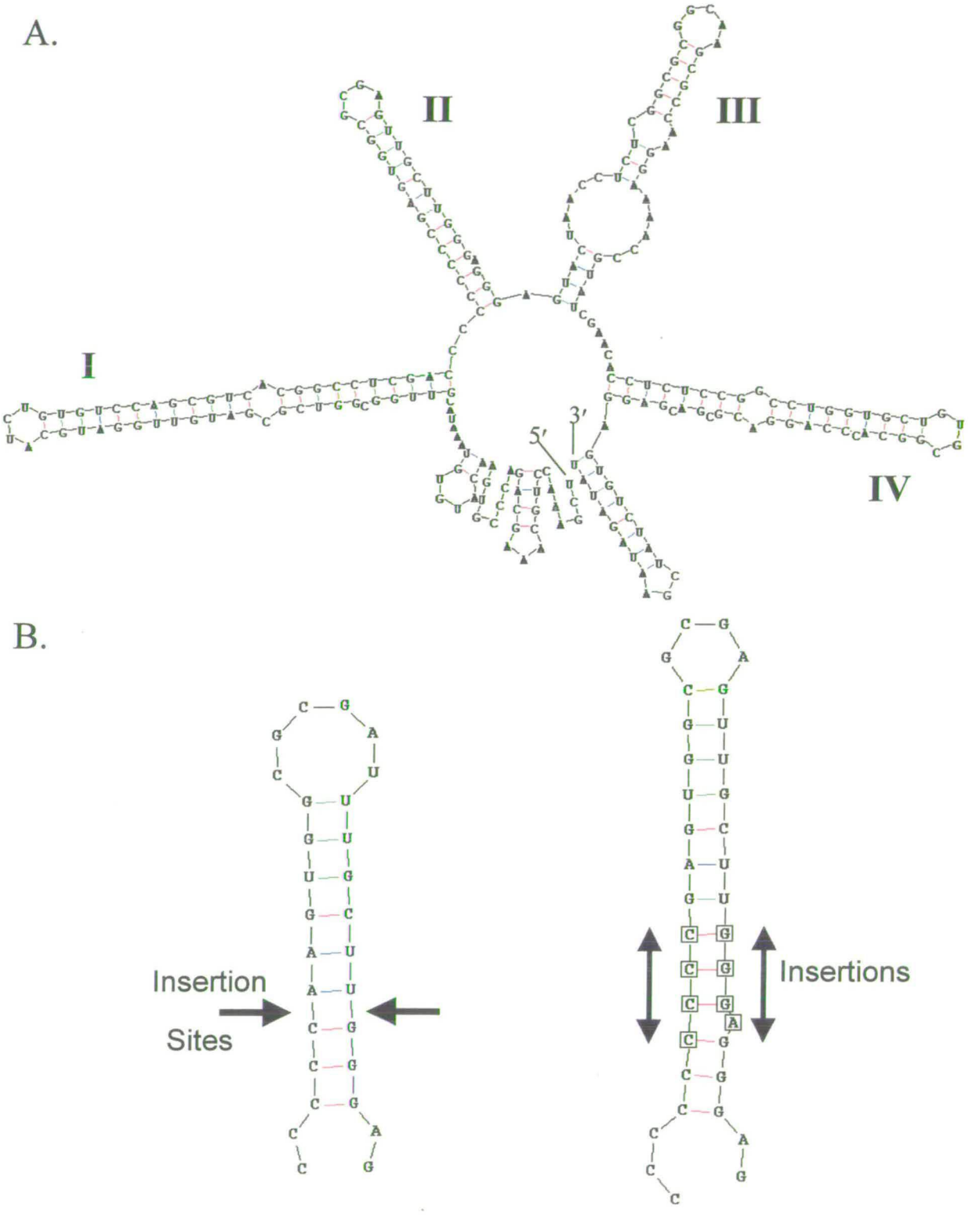


Figure 2.2. (A) Minimum free energy configuration of ITS1 of *A. buxifolius* clone B, dG = -117 kcal, at 20°C; (B) Minimum free energy configuration of arm 2 of ITS1 of *A. buxifolius* showing (right) clone A with complementary 4 basepair deletions and (left) clone B with the complementary insert sequence

2.3.2. Sequence characteristics: intra-individual clone variation and between species variation

The 581 basepair aligned ITS sequence matrix of *Aeschynanthus* and outgroup taxa (see the sequence matrix in Appendix B) was analysed with 31 ambiguous basepairs excluded. Five of the excluded basepair positions show inter-clonal differences. Each clone could be aligned with its clone pair without adding any gaps (indels), with the exception of five species (*A. guttatus*, *A. bracteatus*, *A. buxifolius*, *A. parvifolius*, and *A. fulgens*). However, the alignment between species required numerous gaps. Most of the added indels were found to be shared by both clones and may thus be species-specific markers. Although the sequences were aligned without much difficulty, the region corresponding to arm 1 of the ITS2 secondary structure (bases 278 ~ 385) was particularly variable. This region showed considerably different indel characteristics between clade I and clade II (see below in 2.3.3). Furthermore, two large deletions occurred in this region in *A. buxifolius* and *A. argentii* (28 and 61 basepairs, respectively). These deletions occur in both clones of each species and make alignment difficult. Although a secondary structure analysis was used to improve the alignment (see Chapter Three), an ambiguous 18 basepair region was excluded from the analysis at this location (Table 2.2; number of excluded sites in ITS2). The intra-individual clone divergences range from no difference to 5.01 % between *A. guttatus* clones, which is higher than the sequence divergence between the most closely related species. Approximately 50% of the *Aeschynanthus* clone pairs have low character divergences, from zero to two changes

Table 2.2. Sequence characteristics of ITS1 and ITS2 regions of 48 taxa of Gesneriaceae. Characteristics of the aligned matrix exclude ambiguous sequence sites.

Parameter	ITS1	ITS2	ITS1 and ITS2
Length range (bp) – Ingroup + outgroup	217 ~ 237	206 ~ 254	430 ~ 491
- Ingroup	217 ~ 229	206 ~ 246	430 ~ 491
- Outgroup	225 ~ 237	243 ~ 254	468 ~ 491
Length mean (bp) - Ingroup + outgroup	225.2	239.9	465.1
- Ingroup	225.0	239.5	464.5
- Outgroup	231.0	248.5	479.5
Aligned length (bp)	277	304	581
G + C content range (%)	49.34 ~ 59.32	49.39 ~ 57.43	49.37 ~ 57.92
G + C content mean (%)	54.01	54.28	54.15
Sequence divergence (%) - Ingroup to outgroup	13.87 ~ 20.18	16.41 ~ 23.91	15.96 ~ 21.00
- Ingroup (between spp.)	0.45 ~ 15.26	0.41 ~ 15.57	0.44 ~ 14.16
- Ingroup (within spp.) [a]	0.00 ~ 4.48	0.00 ~ 5.52	0.00 ~ 5.01
Number of indels - Ingroup + outgroup	26	47	73
- Ingroup (total)	20	33	53
- Ingroup (within spp.)	3	4	7
Size of indels (bp) - Ingroup + outgroup	1 ~ 4	1 ~ 6	1 ~ 6
- Ingroup (total)	1 ~ 4	1 ~ 6	1 ~ 6
- Ingroup (within spp.)	1 & 4	1	1 & 4
Number of excluded sites	13	18	31
Number of sites after exclusion	264	286	550
Number of variable sites	111	139	250
Number of constant sites (%)	153 (58.0)	147 (51.4)	300 (54.5)
Number of potentially informative sites (%)	84 (31.8)	96 (33.6)	180 (32.7)
Number of autapomorphic sites (%)	27 (10.2)	43 (15.0)	70 (12.7)
Transitions on trees (unambiguous)	79	89	205
Transversions on trees (unambiguous)	41	61	109
Transitions/Transversions	1.93	1.46	1.88

[a] Divergence between clone pairs

(Fig. 2.4 and 2.5). However, *A. guttatus* was remarkable in having 23 character changes (substitutions) between the two clones sampled.

2.3.3. Phylogenetic analysis

A heuristic search using the 46 *Aeschynanthus* sequences (23 species; two clones per species) with two outgroups yielded 18 equally most parsimonious trees of 550 steps in length when the gap matrix was included. The strict consensus tree is shown in Fig. 2.3. From the tree, all 23 *Aeschynanthus* species form a monophyletic group with two well-supported major clades. None of the clone pairs are separated from each other on different clades and mostly they occur as sister sequences. When the gap matrix was excluded from the analysis, 96 maximally parsimonious trees were obtained with a tree length of 452 steps and lower resolution. However, the branch lengths shown in Fig. 2.4 are calculated using only substitutions (no gap matrix) for simplicity. Bootstrap and jackknife support values were similar although the jackknife percentages were lower, giving less than 50% values to those nodes which collapse in the analysis without the gap matrix (indicated by arrows in Fig. 2.3).

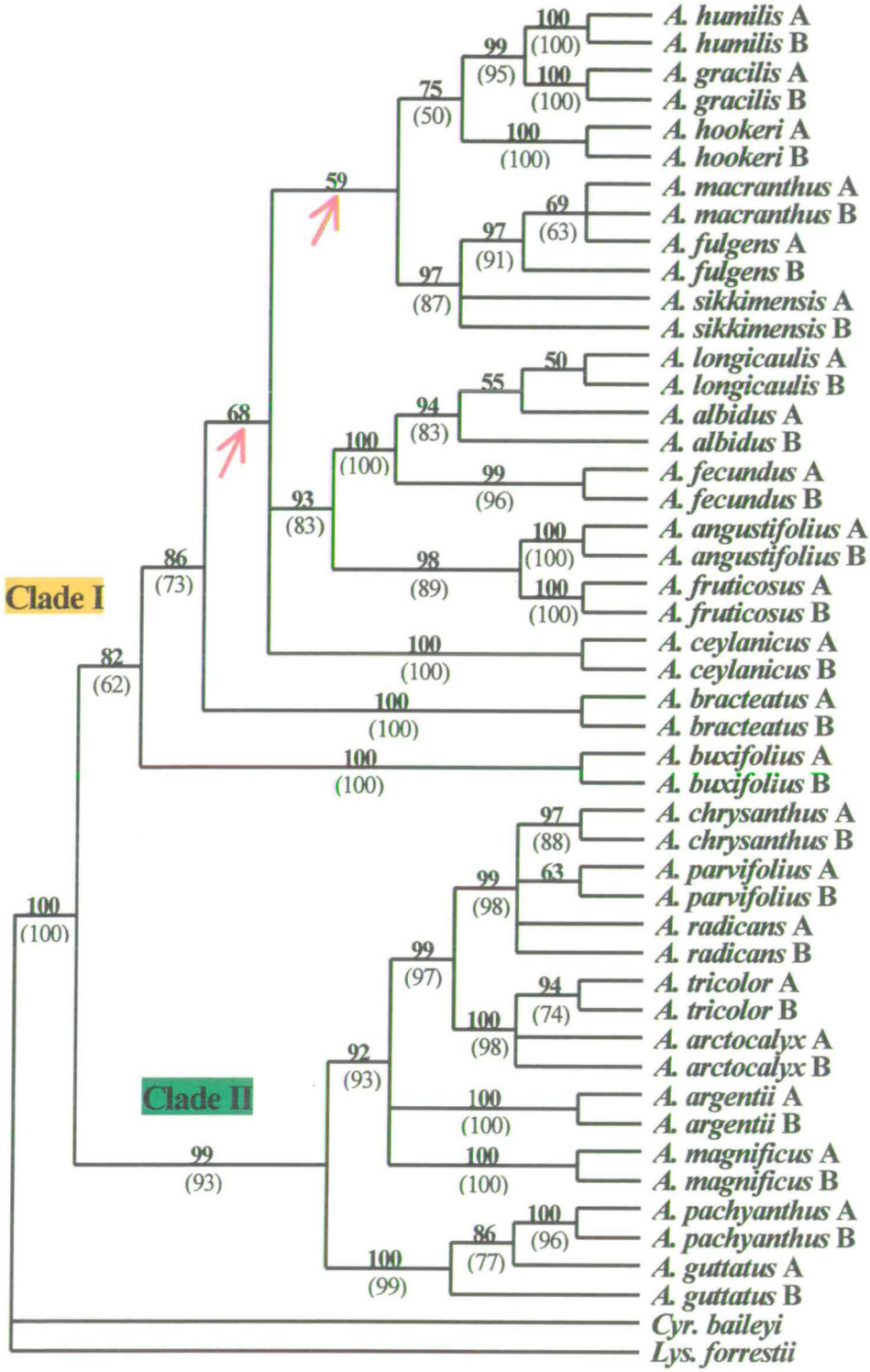


Fig. 2.3. Strict consensus tree based on 18 most parsimonious trees for 23 *Aeschynanthus* species (two clones per species) and two outgroup Gesneriaceae taxa of 550 steps in length, based on parsimony analysis of the combined ITS1 and ITS2 sequence data plus the alignment gap matrix. Upper numbers (in bold type) are full heuristic bootstrap percentages of 100 replicates. Lower numbers (in parentheses) are jackknife percentages ("fast" stepwise-addition) of 10000 replicates. The two arrows indicate branches that collapse when the gap matrix is excluded and the analysis rerun. [CI = 0.72, RI = 0.88, RC = 0.63].

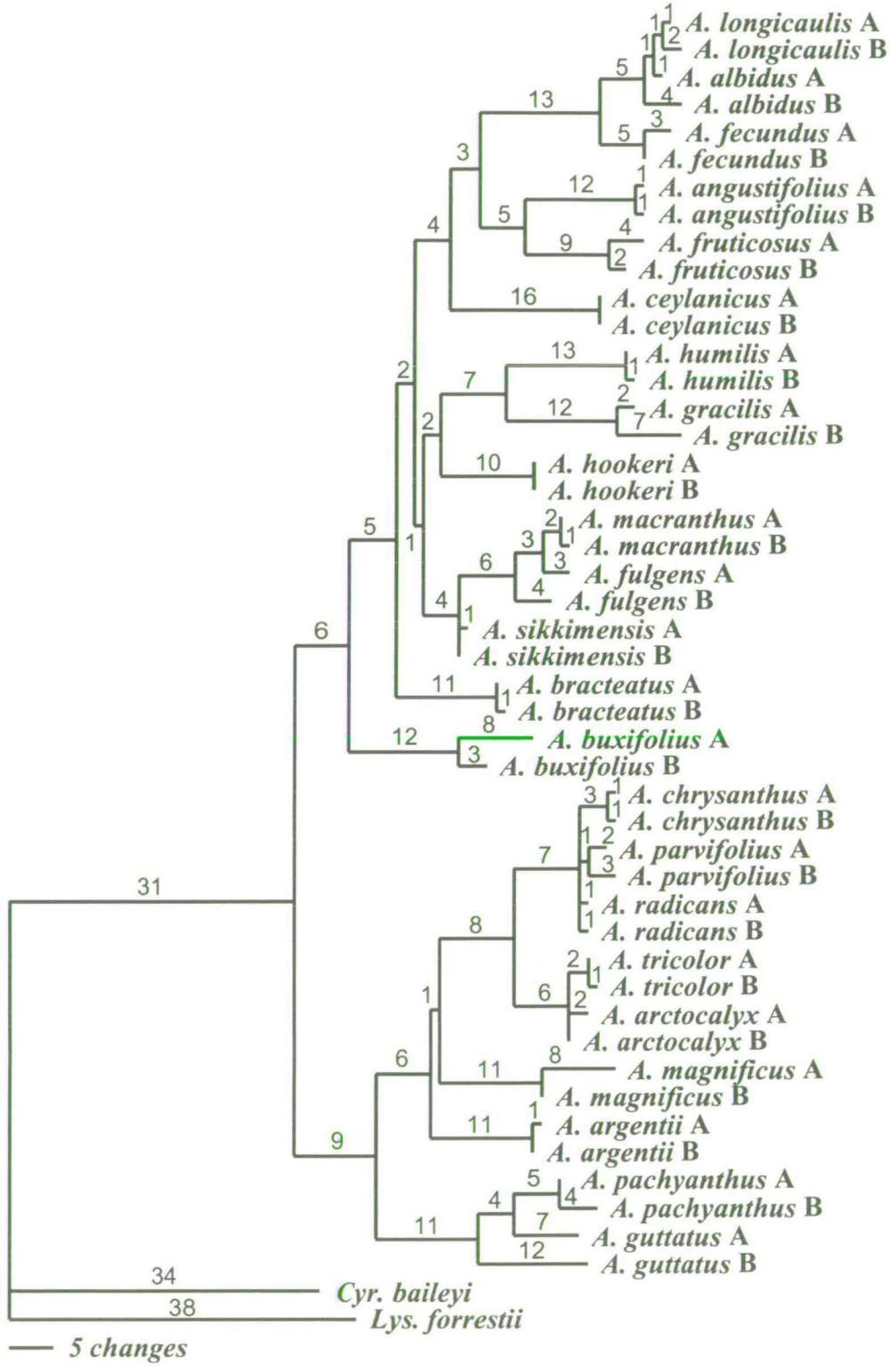


Figure 2.4. Phylogram of one of 96 most parsimonious trees for 23 *Aeschynanthus* species (two clones per species) and two outgroup Gesneriaceae taxa, of 452 steps in length, based on parsimony analysis of the combined ITS1 and ITS2 sequence data without the gap matrix. Numbers along branches indicate the number of character changes shared among taxa (branch length), including autapomorphic changes. [CI = 0.708, RI = 0.869, RC = 0.615].

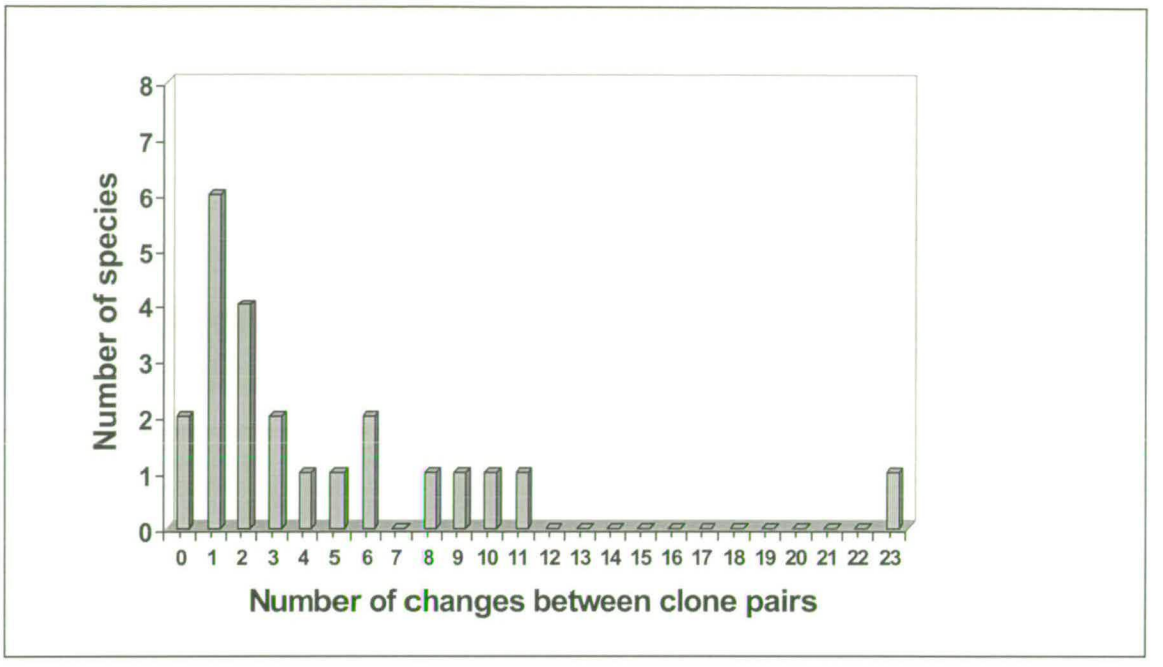


Figure 2.5. Pairwise clone divergence comparison between clones of 23 *Aeschynanthus* species. Divergence values are numbers of substitutions calculated over the tree topology shown in Fig. 2.4. Clones of *A. guttatus* show the highest divergence (23 changes), whereas those of both *A. ceylanicus* and *A. hookeri* are identical.

2.4. DISCUSSION

2.4.1. Failure of molecular drive in *Aeschynanthus*

Significant ITS polymorphism within individuals is generally considered to be unusual in plants. Nevertheless, intra-individual variation has been reported extensively in the ITS region (see Chapter One). Most reported ITS polymorphisms are associated with high ploidy levels and/or multiple nucleolar organizing regions (NORs), which may retard molecular drive or allow different rDNA arrays to evolve independently. However, in *Aeschynanthus* intra-individual variation of ITS appears, at least in my sample, not to be associated with polyploidy. For instance, ITS sequences of tetraploids, such as *A. parvifolius* (2n = 64; Ratter, 1975) are not more

polymorphic than those of the diploid *A. albidus* (2n = 30; Ratter, 1975). Furthermore, *A. guttatus* which has the highest divergence between clones is diploid (2n = 32; Milne, 1975). Further investigation of the cytology of *Aeschynanthus*, especially the number and position of rDNA loci, would be interesting in this context.

2.4.2. Use of clones for phylogenetic analysis

Theoretically, high intra-individual ITS clone variation could reduce the phylogenetic utility of this region. However, in *Aeschynanthus* the clones, even the most divergent, are always associated on the phylogenetic trees: usually as sister pairs. Phylogenetic analysis using clones is therefore straightforward. In some cases the clones of one species are paraphyletic with respect to the clones of another (e.g. the clones of *A. albidus* are paraphyletic with respect to those of *A. longicaulis*), but the species relationships are still clear. Although considerable variation can be found within individuals, it is therefore still possible to use these data to reconstruct species phylogeny, indicating that clone variation in *Aeschynanthus*, while persistent, does not significantly predate the divergence of species.

In my analysis, the sequence divergence between clones within *Aeschynanthus* taxa ranges from 0 - 5.01 % (0 - 0.025 substitutions per site). This variation is similar to that of *Amelanchier* (Campbell et al., 1997), in which within-individual divergence ranges from 0 - 4.2 % (0 – 0.021 substitutions per site). By contrast, highly divergent clones have been found in Winteraceae (Suh et al., 1993), in which the sequence divergence is 4.7 – 7.0 % (0.024 – 0.035 substitutions per

site). This is higher than some intergeneric divergence values, and might be the result of polyploidisation. In *Cucurbita*, intraspecific sequence variation of three cultivated species and three putative progenitors was found to range between 7 and 10 % (0.035 – 0.05 substitutions per site) (Jobst, King and Hemleben, 1998). In that case, the polymorphism may be due to introgression during domestication. Clone variation as high as that found in Winteraceae and *Cucurbita* reduces the utility of ITS for phylogenetic analysis at the species level.

CHAPTER THREE: Evolution and alignment of the hypervariable arm 1 of *Aeschynanthus* ITS2

(Materials in this chapter have been published in 'Denduangboripant, J., and Q. C. B. Cronk. 2001. Evolution and alignment of the hypervariable arm1 of *Aeschynanthus* (Gesneriaceae) ITS2 nuclear ribosomal DNA. *Molecular Phylogenetics and Evolution*. 20: 163-172.')

3.1. Introduction

In Chapter Two of this thesis I reported success in reconstructing the phylogenetic tree of *Aeschynanthus* with the aid of a PCR-cloning technique. However, I also found 70 aligned positions near the beginning of ITS2 that would conventionally be excluded from the initial analysis because the alignment in this region was ambiguous, the result of a large deletion in one species (*Aeschynanthus argentii*). In studies by Möller and Cronk (1997a; 1997b) 50 aligned base positions in the same area (arm 1 region of ITS2 sequences) of the plant genera *Streptocarpus* and *Saintpaulia* (Gesneriaceae) had to be excluded from the analysis because of alignment uncertainty. To study the nature of this variable area, I then analysed ITS2 arm 1 structures in *Aeschynanthus* species. I particularly wished to determine whether the structure of this region would offer any functional explanations for the hypervariability, and whether structural analysis would help with the alignment of this otherwise intractable region.

In the case of angiosperms, ITS2 sequences have been found to be moderately conserved. Hershkovitz and Zimmer (1996) studied 75 angiosperm species, and found that the region from the base (3' side) of arm 1 to the loop end (5' side) of arm 3, c. 40% of the total ITS2 sequence of these species, was fairly well alignable except for two small variable regions. Among eudicots, about 47% of the total ITS2 sequence was found to be conserved. Most of the conserved regions are inherently G/C rich, but a conserved region corresponding to the single-stranded core of the putative secondary structure is A/G (purine)-rich (Mai and Coleman, 1997). The arm 1 region of ITS2 was found to be highly variable in both length and sequence between taxa. In angiosperms this arm shows a C- to G-rich transition from 5' to 3' and high length variability of c. 26-54 basepairs (Hershkovitz and Zimmer, 1996). Even at the species level, sister taxa may show sequence and length variation in the arm 1 region.

The ITS2 region has been shown to be necessary for rRNA processing (van der Sande et al., 1992; Hadjiolova et al., 1994; van Nues et al., 1995). It participates in guiding endonucleases to the correct rRNA processing sites. Recent studies of rRNA processing in *Saccharomyces cerevisiae* (Lafontaine and Tollervey, 1995; Venema and Tollervey, 1995) show that rDNA transcription first produces a pre-rRNA which contains all three rRNAs found in the ribosome, namely 18S small subunit (SSU) rRNA, 25S large subunit (LSU) rRNA, and 5.8S rRNA. Cleavages of the pre-rRNA occur initially at the 5' external transcribed spacer (ETS), followed by the 5' end of the 18S rRNA and in the ITS1 to generate a mature SSU rRNA. Finally, cleavage occurs in the ITS2 and the 3' ETS to generate mature 5.8S and LSU

rRNAs. The pre-rRNA processing of plants appears to follow this pathway, but their rRNA processing sites have not yet been fully analysed (Brown and Shaw, 1998).

To assist in the study of ITS2 function, secondary structure folding patterns of the sequence have been examined in many organisms. To date, only yeast ITS2 structure has been unambiguously resolved, by a combination of minimum free energy calculations, chemical and enzymatic probing, and mutation and insertion/deletion experiments (Yeh and Lee, 1990; van der Sande et al., 1992; van Nues et al., 1995). The yeast cross-like structure contains two long central stems and three short branches. The structure common to other organisms, such as *Drosophila* (Schlötterer et al., 1994), trematodes (Michot et al., 1993), monogeneans (Morgan and Blair, 1998), green algae (Mai and Coleman, 1997), and flowering plants (Hershkovitz and Zimmer, 1996; Mai and Coleman, 1997), appears to be based on a four-arm model, in which the third arm (sometimes called the central stem) is longest. Secondary structure comparison of green algal, moss, and angiosperm ITS2 sequences reveals another two highly conserved characteristics: a pyrimidine mismatch loophole in arm 2 and a GGU triplet (UGGU in angiosperms) on the 5' end of arm 3 (Lie and Schardl, 1994; Mai and Coleman, 1997). The only significant difference between yeast ITS2 structure and that of other organisms is in this long central stem, which is branched in yeast with a conserved pairing region between the first and second arms (branches) of the yeast structure. However, there are no clearly conserved ITS2 sequence motifs shared between yeast and either green algae or flowering plants (Hershkovitz and Lewis, 1996). It should also be noted that the 3' sequence of ITS2 is conserved in yeast, while it is highly variable in angiosperms.

3.2. Materials and Methods

3.2.1. Plant materials

Selection of plant materials was treated as in Chapter Two except that only 12 *Aeschynanthus* species were chosen for this study. These 12 species represent both of the two main clades described in the previous chapter, namely *A. albidus*, *A. buxifolius*, *A. ceylanicus*, *A. humilis*, *A. hookeri*, *A. fulgens*, and *A. sikkimensis* in clade I; and *A. tricolor*, *A. chrysanthus*, *A. argentii*, *A. guttatus*, and *A. pachyanthus* in clade II. For comparison, GENBANK ITS2 sequences from a range of families in the Asteridae were examined including two other members of the family Gesneriaceae, four members of Scrophulariaceae, three Solanaceae, and two Compositae. The scientific names and Genbank accession numbers of these plants are also shown in Table 3.1.

Table 3.1. Representatives of the order Asteridae used for RNA secondary structure analysis, compared with Gesneriaceae, with accession numbers and length of the arm 1 region. Gesneriaceae have exceptionally large arm 1 lengths, except for *A. argentii* which has a deleted top of arm 1.

Taxon	Family	Genbank Accession Number	Base position of arm 1	Length of arm 1 (bp)
Other Asteridae				
Chionohebe densifolia	Scrophulariaceae	AF 037375	6 ~ 47	42
Derwentia nivea	Scrophulariaceae	AF 037382	6 ~ 47	42
Hebe tetragona	Scrophulariaceae	AF 069467	6 ~ 47	42
Veronicastrum sibiricum	Scrophulariaceae	AF 037398	6 ~ 46	41

Atropa belladonna	Solanaceae	AB019948	7 ~ 59	53
Lycopersicon esculentum	Solanaceae	X52265	3 ~ 48	46
Nicotiana tabacum	Solanaceae	AJ012367	7 ~ 60	54
Artemisia sublessingiana	Compositae	AF061394	7 ~ 52	46
Dahlia scapigeroides	Compositae	AF165845	2 ~ 54	52
Gesneriaceae				
Anna mollifolia	Gesneriaceae	AF055051	6 ~ 80	75
Chirita crassifolia	Gesneriaceae	AF055055	1 ~ 80	80
Cyrtandra baileyi	Gesneriaceae	AF349232	1 ~ 80	80
Lysionotus forrestii	Gesneriaceae	AF349271	6 ~ 79	74
Aeschynanthus tricolor	Gesneriaceae	AF349251	1 ~ 88	88
Aeschynanthus chrysanthus	Gesneriaceae	AF349255	1 ~ 87	87
Aeschynanthus argentii	Gesneriaceae	AF349261	1 ~ 46	46
Aeschynanthus guttatus	Gesneriaceae	AF349265	1 ~ 92	92
Aeschynanthus pachyanthus	Gesneriaceae	AF349297	1 ~ 89	89
Aeschynanthus albidus	Gesneriaceae	AF349271	8 ~ 86	83
Aeschynanthus buxifolius	Gesneriaceae	AF349289	1 ~ 78	78
Aeschynanthus ceylanicus	Gesneriaceae	AF349281	1 ~ 92	92
Aeschynanthus humilis	Gesneriaceae	AF349285	4 ~ 94	91
Aeschynanthus hookeri	Gesneriaceae	AF349289	1 ~ 95	95
Aeschynanthus fulgens	Gesneriaceae	AF349293	1 ~ 99	99
Aeschynanthus sikkimensis	Gesneriaceae	AF349295	1 ~ 98	98

3.2.2. Experimental strategy

DNA extraction, PCR amplification, PCR cloning, and DNA sequencing techniques were the same as in the previous chapter. Details of the strategies used are provided in Appendix A.

3.2.3. Secondary structure analysis and sequence alignment

The ITS2 sequences were first aligned using the sequence alignment program CLUSTAL X (EMBL, Heidelberg, Germany), with "Do Complete Alignment" and

"Slow/Accurate Pairwise Alignment" options, without changing any of the default parameters. A manual alignment was also performed. CLUSTAL X is equivalent to the CLUSTAL W program of Thompson, Higgins and Gibson (1994) but with a graphic user interface. RNA secondary structure analysis was introduced to optimize the initial alignment of the first c. 100-aligned positions. First, the secondary structure study was performed for the whole ITS2 sequences by the RNA folding program RNAdraw version 1.1 (Matzura and Wennborg, 1996). RNAdraw uses the energy structure prediction algorithm MFOLD based on the work of Zuker and Stiegler (1981). The temperature parameter for folding was 20°C, as in the work on other flowering plants (Mai and Coleman, 1997), and yielded a minimum energy structure. All the complete ITS2 sequences were first examined for a common secondary structure, and then the analyses were repeated using partial sequences (arm 1 only). The sequence alignment was then optimised using the secondary structure results. The optimised matrix was compared to the primary CLUSTAL and manual alignments.

3.2.4. Search for conserved and variable sites in ITS2

To understand the characteristics of the highly variable region of *Aeschynanthus* ITS2 sequences, the complete ITS2 alignment of 23 *Aeschynanthus* species (two clones per species) and two outgroups was searched for conserved and variable sites along the sequences. The character conservation was determined by using the Chart option of MacClade version 3.01 (Maddison and Maddison, 1992). Steps of character changes in each site were calculated over one of the most

parsimonious trees, with the ambiguous area in arm 1 still retained, and outgroups excluded from the analysis. The secondary structure of *A. fulgens*, which has the longest ITS2 sequence, was chosen as the template onto which to plot the step change values. Indel events found in the *Aeschynanthus* matrix were also marked on the structure in three categories: insertions found in *A. fulgens*, insertions found in other species, and deletions found in other species.

3.3. Results

3.3.1. Nature of hypervariable region in ITS2 sequence

An alignment of ITS2 sequences of 14 taxa analysed revealed an ambiguous region that was difficult to align. This area ranges approximately from position 30 to position 90 in the primary alignment suggested by CLUSTAL (Fig. 3.1 upper). The CLUSTAL alignment of this region clearly detected a very large deletion in the sequence of *A. argentii* and another single large deletion in that of *A. buxifolius*. The manual alignment (Fig. 3.1 lower) clearly suggests two groups of species with different alignments between positions 40 and 73 (clade I species and clade II species from Chapter 2). This area of 33 aligned positions contains almost the same proportion of purine (A/G) and pyrimidine (C/T) bases, while the whole 100-position aligned region shows a transition from C/T-rich to A/G-rich. The two groups of species are not unambiguously alignable, which might suggest that this 70 position ambiguous region be excluded for the purposes of phylogenetic analysis. For this reason, confirmation of the alignment using secondary structure was sought.

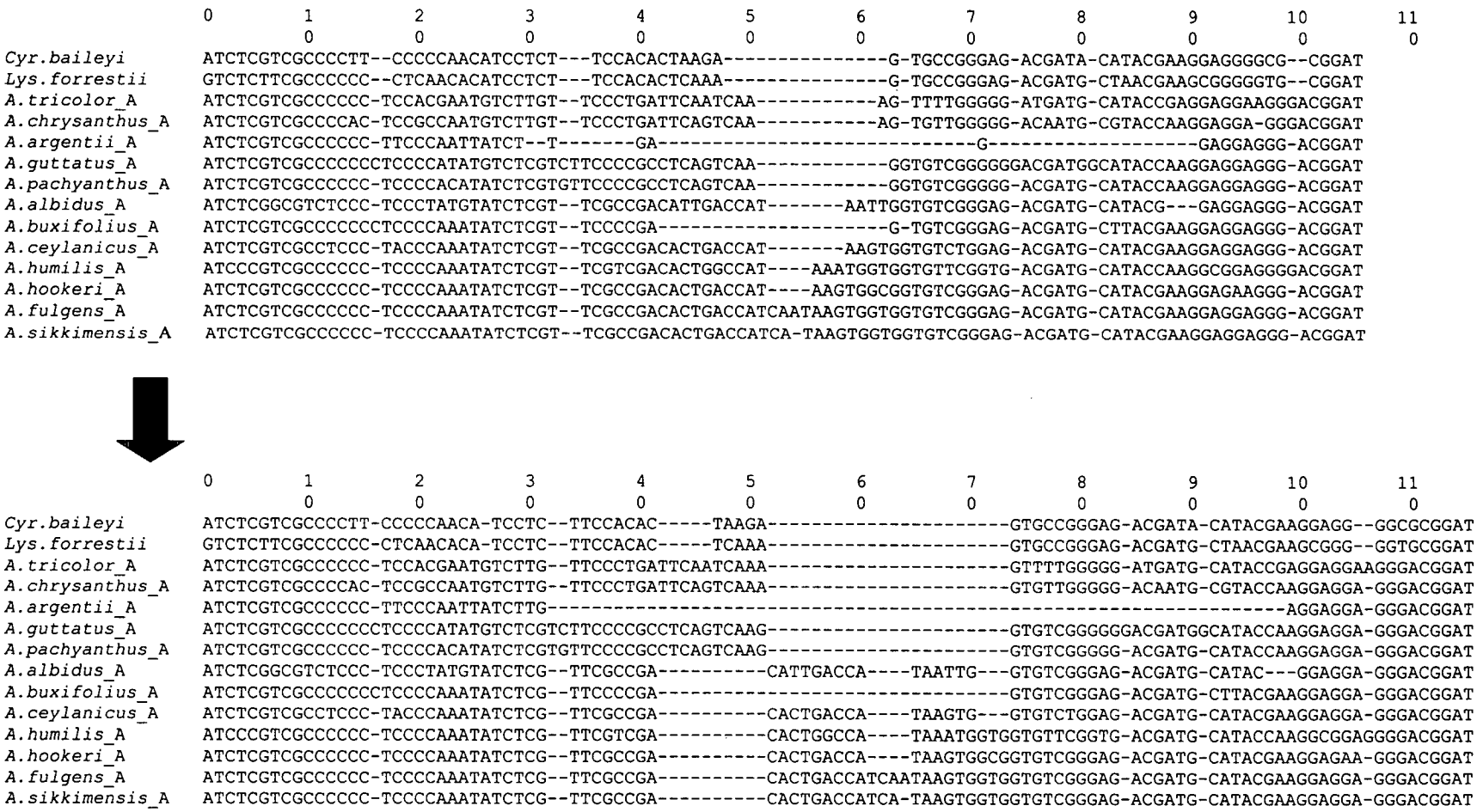


Figure 3.1. Alignment of 12 *Aeschynanthus* taxa and two outgroups using CLUSTAL X with default parameters (upper alignment), compared with the manual alignment (lower alignment).

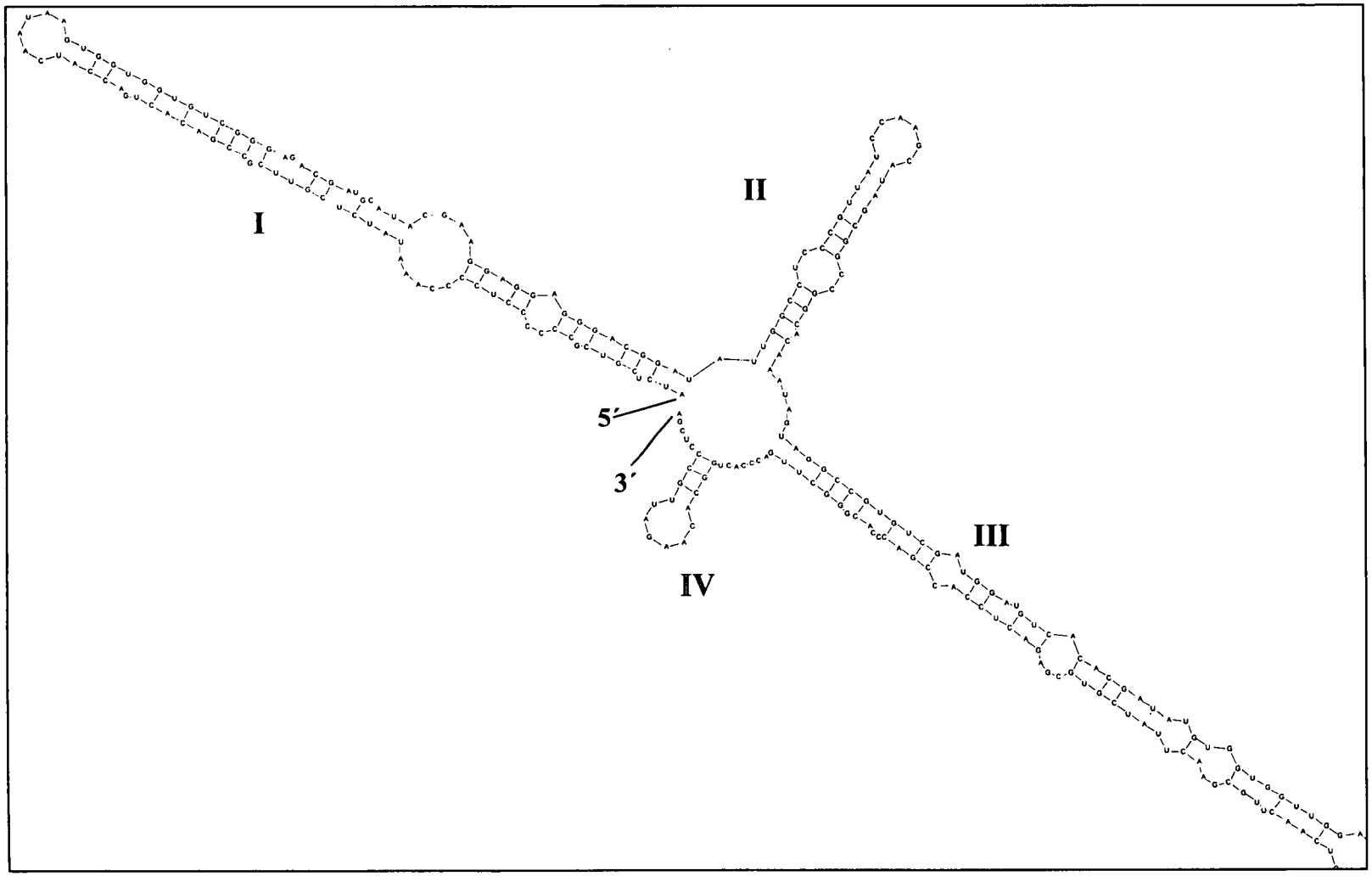


Figure 3.2. RNA secondary structure of the ITS2 sequence of *A. fulgens* clone A (20°C, -117.89 kcal), showing a four-arm model.

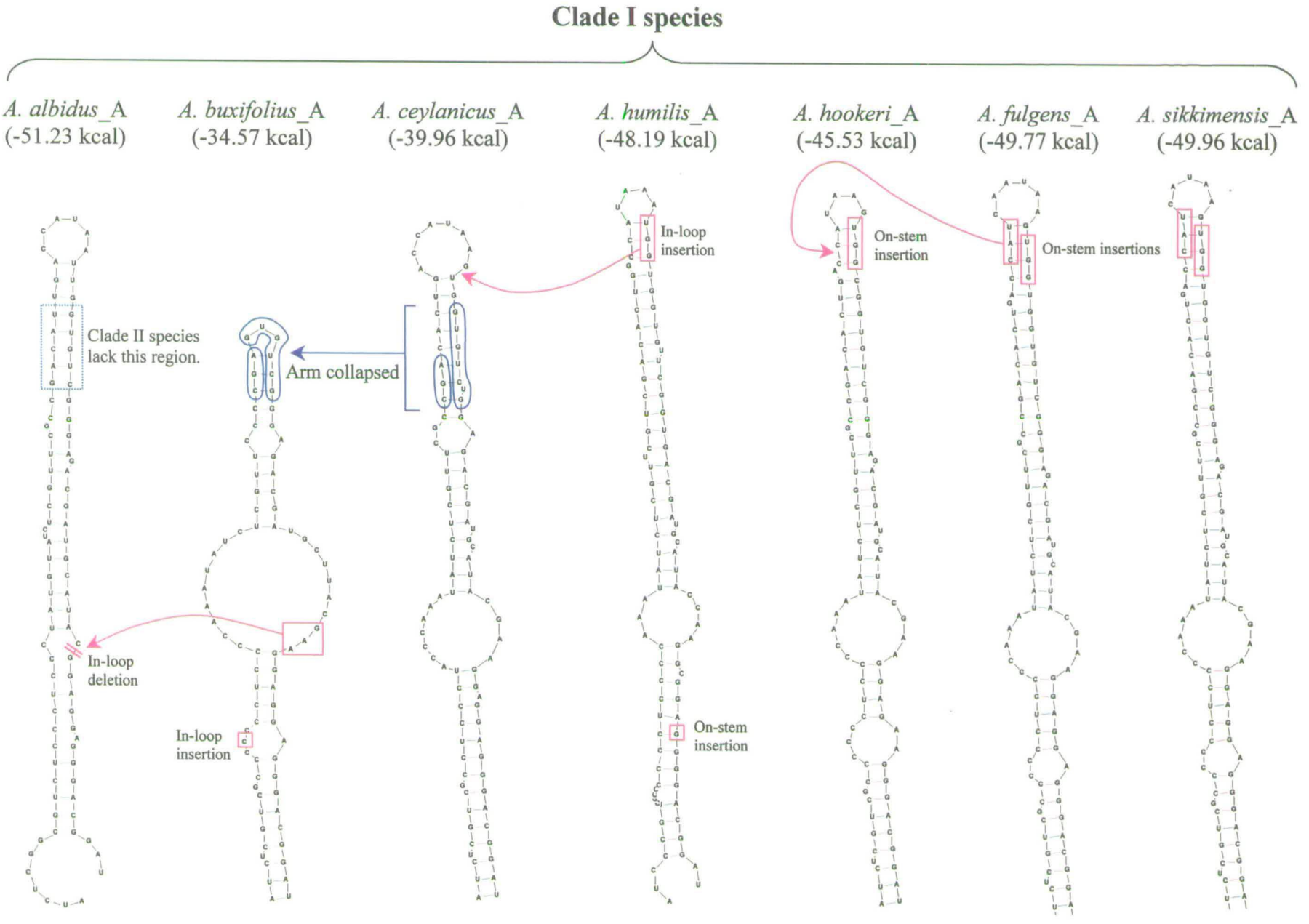


Figure 3.3. RNA secondary structures of the arm 1 region for ITS2 sequences of 12 *Aeschynanthus* species and two outgroup taxa.

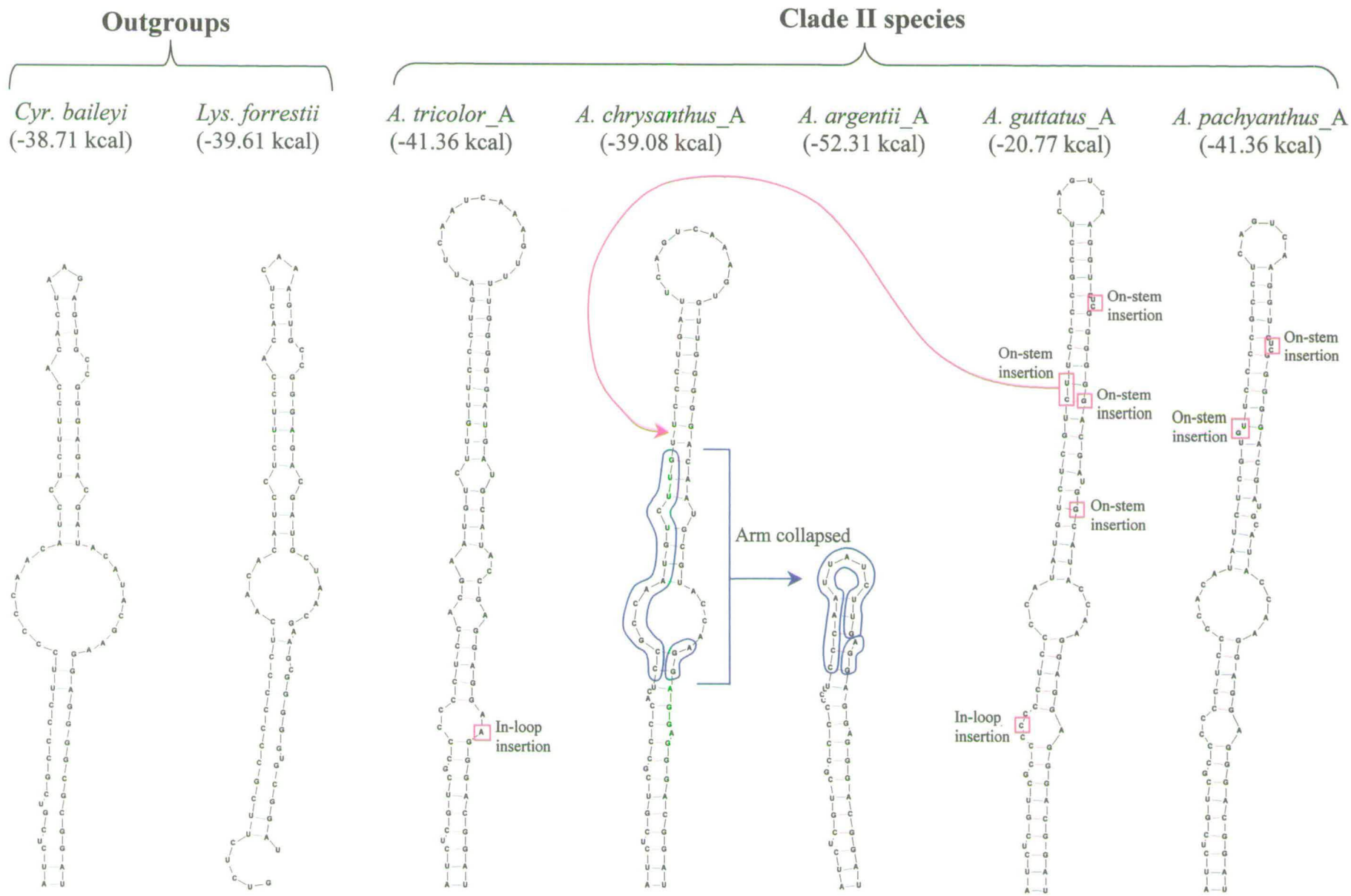


Figure 3.3. (Continued).

3.3.3. Arm 1 sequence realignment

Positioning of indels found from arm 1 secondary structure comparison was adopted to improve the previous sequence alignment, both manual and CLUSTAL alignments. This new alignment allowed detection of the five-basepair complementary deletion, which causes the main difference between the two *Aeschynanthus* clades. As well as the five-basepair deletions, the alignment also suggests a greater number of shorter indels in this region than predicted by the manual or CLUSTAL alignments: one-basepair insertions (one site in *A. tricolor*, *A. buxifolius*, *A. humilis*; three sites in *A. guttatus*), two-basepair insertions (two sites in *A. guttatus* and *A. pachyanthus*), and three-basepair indels (one deletion site in *A. albidus*; one insertion site in *A. humilis* and *A. hookeri*; two complementary insertions in *A. fulgens* and *A. sikkimensis*). This in turn helps to combine these two sets of superficially dissimilar sequences. The new sequence alignment (Fig. 3.4 upper) decreases the size of the ambiguous region from about 33 aligned positions to 18 aligned positions. This remaining ambiguous region is the loop-end region of the arm 1 structure, apparently composed of non-homologous sequences in the two groups of species.

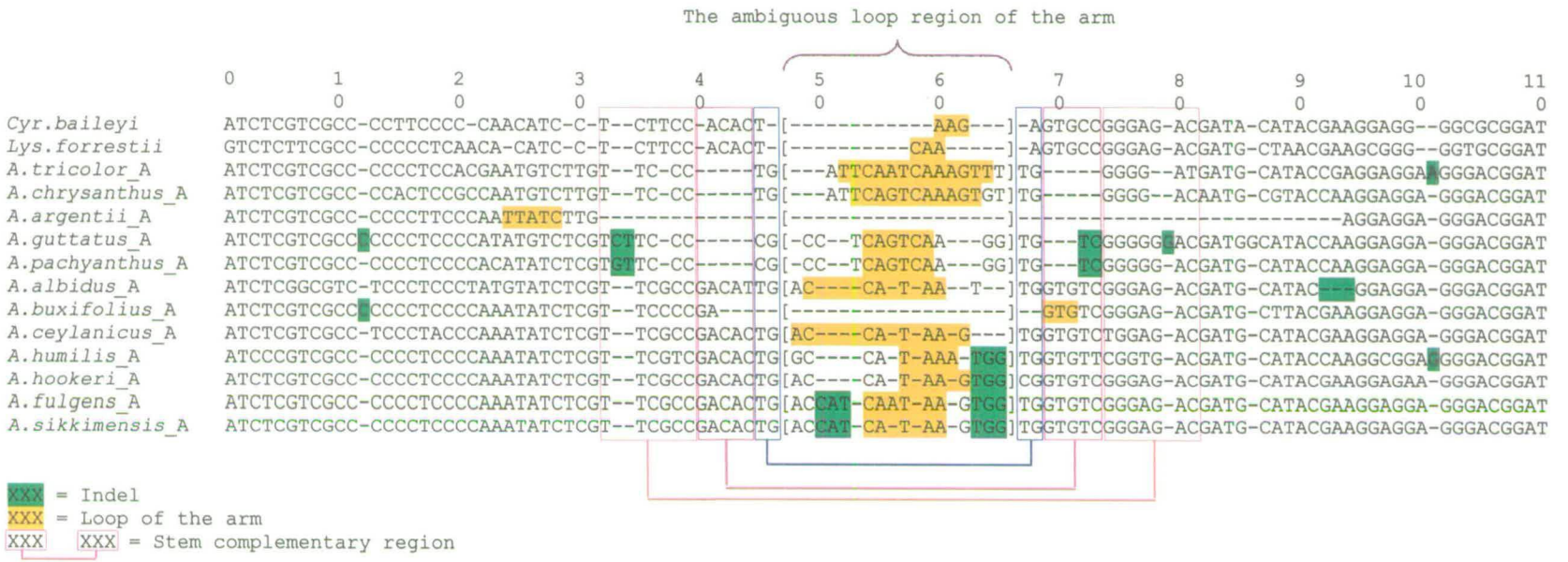


Figure 3.4. New alignment suggested by secondary structure analysis.

3.3.4. Superfluous arm 1 extension in *Aeschynanthus*

Nucleotide changes over the phylogenetic tree (Fig. 2.4) were calculated for each aligned position and mapped onto the secondary structure of *A. fulgens*. The position of indels was also mapped onto the structure (Fig. 3.5). Most of the indels, not surprisingly, were located on the arm 1 extension. Some other indels were found along arm 3 and arm 4, and on the basal part of the arm 1, but no indel is located on arm 2. ITS2 sequences of *Aeschynanthus* are highly conserved on arm 2 and in the central core of the structure. To investigate whether the variable arm 1 extension is a unique character of the family Gesneriaceae, secondary structures were determined for some other representatives of the class Asteridae, which have ITS2 sequences available in Genbank. The extension was found only in the family Gesneriaceae. Six genera of the family Scrophulariaceae (a family allied to Gesneriaceae) do not have the arm 1 extension. Examples from the Solanaceae (three genera) and Compositae (two genera) also revealed the same lack of arm 1 extension. Table 3.1 shows the comparison of the arm 1 length from each of the taxa analysed. Gesneriaceae arm 1 sequences are almost twice as long as those of other plants.

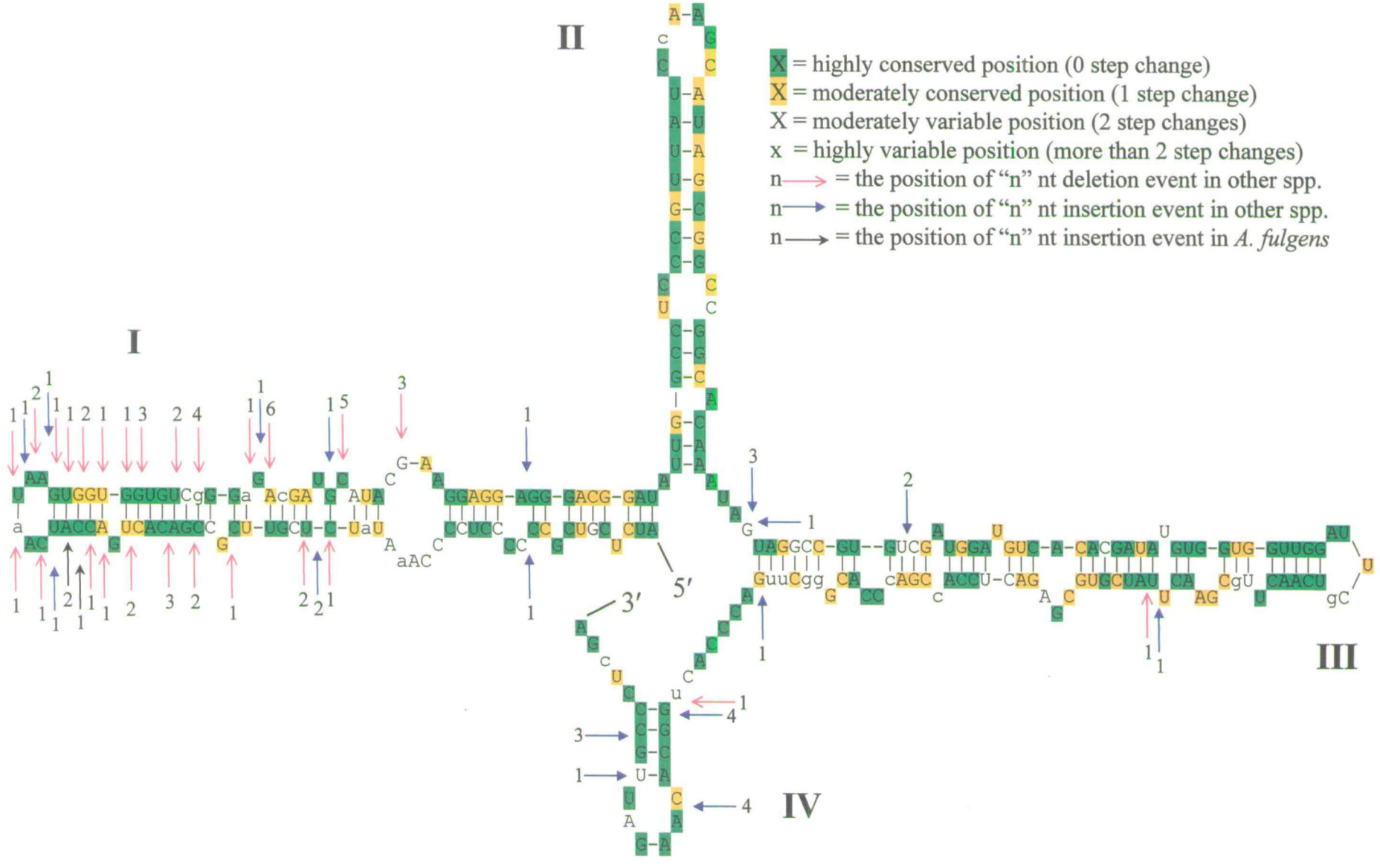


Figure 3.5. Conserved and variable sites and indels among *Aeschynanthus* ITS2 sequences, mapped on the predicted secondary structure of the ITS2 sequence of *A. fulgens*.

3.4. Discussion

3.4.1. Functional significance of conserved sites versus the superfluous Gesneriaceae arm extension

From this *Aeschynanthus* ITS2 sequence study, I propose that approximately 50 basepairs at the terminal part of arm 1 in the sequence are superfluous as they are an indel hot-spot and have been completely lost in *A. argentii*. The arm extension cannot therefore be important in ribosomal RNA processing, which is the main role of the ITS2 region (Hadjiolova et al., 1994; van Nues et al., 1995). Conversely the conservation of arm 2 and the central core suggest possible functional significance. *Aeschynanthus* ITS2 (as in Fig. 3.5) shows a pyrimidine mismatch loophole in arm 2 and a UGGU triplet at the 5' end of arm 3, reported as characteristic of flowering plants (Liu and Schardl, 1994; Mai and Coleman, 1997). I therefore suggest that ITS2 processing in angiosperms may occur in an analogous way to yeast exonuclease ITS2 processing (Mitchell, Petfalski, and Tollervey, 1996). To remove the ITS2 sequence from 5.8S-26S premature RNA, exonuclease enzymes in plant cells will bind to the UGGU binding site on the arm 3 of the ITS2 sequence, and have 3'→5' activity to digest the sequence. By analogy with yeast, the exoribonuclease activity probably degrades RNA in two steps: initial rapid 3'→5' exonuclease processing from the end of the arm to the central core, and then slower trimming activity. It would then stop the processing when it reaches the recognition sites at the basal part of arm 1 and arm 2. A similar mechanism has also been suggested for mammalian processing by Michot et al. (1999).

It is interesting to consider why only the family Gesneriaceae is known to have this superfluous arm 1 extension. It is most likely that a Gesneriaceae ancestor either gradually extended the terminal region by complementary insertions, or duplicated a long stretch of sequence which by chance could pair to form an arm twice as long. As this extension did not interfere with the ITS2 RNA processing, it has been retained. However, in some species the extension has been diminished by deletions, particularly by the large deletions found in *A. argentii* and *A. buxifolius.*

3.4.2. Use of RNA secondary structure for DNA sequence alignment

My study of *Aeschynanthus* ITS2 sequence alignment shows that CLUSTAL X with default parameters is fairly reliable, but does not detect the complementary indels suggested by the secondary structure alignment. Where primary homology assessment based only on sequence similarity is ambiguous, then it is reasonable to take secondary structure as an alternative guide. My secondary structure realigned matrix is advantageous in allowing the unambiguous combination of two very different sequence types (clade I and clade II), thereby reducing the size of the ambiguous region which might otherwise be excluded from phylogenetic analysis. This allows further hypotheses of homology to be tested by congruence with other characters.

CHAPTER FOUR: Evolution of *Aeschynanthus* based on ITS sequences

(Materials in this chapter have been accepted for publication: 'Denduangboripant, J., M. Mendum, and Q. C. B. Cronk. 2001. Evolution in *Aeschynanthus* (Gesneriaceae) inferred from ITS sequences. *Plant Systematics and Evolution* (In press).')

4.1. Introduction

In Chapter Two, I showed that many species of *Aeschynanthus* have an unusually high level of ITS sequence variation and I resolved this kind of sequencing problem using a PCR cloning technique. Two clones per species were sufficient and the clones were all found to cluster together on the tree. Cloned sequences therefore can be used satisfactorily for phylogenetic reconstruction and the first *Aeschynanthus* phylogenetic tree was then successfully reconstructed.

Then, in Chapter Three, I reported that the sequence alignment of a short region of the ITS2 corresponding to arm 1 of the predicted ITS secondary structure is not as straightforward as other regions. This arm is long in Gesneriaceae and the top of the arm 1 in particular appears to be superfluous. Secondary structure analysis was introduced and successfully guided the alignment of this problematic area.

Despite these unusual features of ITS evolution in *Aeschynanthus*, ITS appears to have robust phylogenetic utility in the genus. After those two crucial problems were solved, I was therefore ready for a major study of the genus. I have now more than doubled the sampling and in this chapter can present a much more

detailed analysis. A new approach introduced here is to combine cloned sequences with consensus PCR sequences where the latter are not problematic.

A preliminary study based on limited sampling, described in Chapter Two, suggested that the genus was divided into two main clades. As mentioned earlier in Chapter One (Introduction), recent scanning electron microscope (SEM) studies of seed and appendage morphology of 99 taxa (Mendum et al., 2001) also found that the genus can be divided into two groups, A and B, by differences in testa cell orientation and papilla and appendage structure. Group A (testa cell orientation spiral or rarely straight, papillae formed from single cells, hilar appendage one, short and smooth) is essentially Malesian and contains sections *Microtrichium*, *Aeschynanthus* and *Haplotrichium* sens. str. Group B (testa cell orientation always straight, papillae formed from the junction of two cells, hilar appendages one or more, long and papillose) is largely confined to mainland South and Southeast Asia and contains sections *Polytrichium*, *Diplotrichium* and a section not yet circumscribed, here referred to as section *X*. This finding raised a question whether the genus can be divided into smaller natural groups, recognised at sectional rank and characterised by their seed morphology. My detailed analysis of the *Aeschynanthus* phylogeny should be able to confirm or reject this hypothesis.

Aeschynanthus occurs throughout Southeast Asia and shows a high degree of endemism so the phylogenetic relationships of these species might be expected to have some relevance to the geological evolution of the area. Southeast Asia has a complex geological history, resulting from an intricate pattern of geotectonic movements (De Boer and Duffells, 1996; Hall, 1998; Metcalfe, 1998). Many islands in the region, for instance Sulawesi and New Guinea, are geological composites

formed of microcontinents and fragments of island arcs. The evolution and geographical distribution of fauna and flora has been greatly influenced by the geological complexity, and this may go some way towards explaining the high level of biodiversity in Southeast Asia (Gaston et al., 1995; Taylor et al., 1999; Myers et al., 2000). The region has long been attractive to biogeographers. Wallace's Line (Fig. 1.1) refers to the boundary proposed by Alfred Russel Wallace in 1860, running between Borneo and Sulawesi, and separating the Asian from the Australasian faunistic region, and marking the point at which the two biotas collided after having been separated since the break-up of Gondwana in the mid-Mesozoic. This boundary divides Bali and Borneo from Lombok and Sulawesi, and passes southeast of the Philippines (Wallace, 1860). However, many other biogeographic boundaries are evident in Southeast Asia and other lines have been proposed (Wallace, 1863; Huxley, 1868; Weber, 1904; Wallace, 1910). Study of the phylogenetic patterns of widespread groups of organisms of different evolutionary ages should reveal patterns explicable as a result of vicariance (geotectonic separation of land masses) and dispersal (geotectonic fusion of land masses or inter-island dispersal) (Nelson and Platnick 1980; Nelson and Platnick 1981). *Aeschynanthus* is another plant group thought to originate in Laurasia and its current distribution could be the result of biogeographical processes in Southeast Asia. Therefore, the analysis of *Aeschynanthus* evolution described here should add to our understanding of biogeographical patterns in the region.

4.2. Materials and methods

4.2.1. Plant materials

For the study in this chapter, 27 further species (Table 4.1) were added to the 23 species already sequenced (Table 2.2) to give a total of 50 *Aeschynanthus* species, about one-third of the genus, representing all morphological variation and geographical distribution of *Aeschynanthus*. Fresh leaf material of one plant representing each species was taken from the RBGE living collection, except for five Sulawesi taxa which were sequenced from wild-collected leaf samples.

4.2.2. DNA extraction, PCR, cloning, and DNA sequencing

Details of DNA extraction, PCR amplification, PCR cloning, and DNA sequencing strategies are provided in Appendix A. The genomic DNA of the newly added 27 *Aeschynanthus* taxa was prepared and the ITS PCR products were sequenced using either the Amplitaq-FS dye terminator cycle-sequencing kit (Perkin Elmer Biosystems Inc., Warrington, UK) or Thermo Sequenase II (Amersham Pharmacia Biotech UK Limited, Bucks, England). When I found uninterpretable sequence electropherograms caused by ITS length intra-individual variation (Chapter Two), I used PCR cloning for that particular species. Otherwise the consensus sequences from forward and reverse reactions were obtained without cloning. For cloning, the PCR products were cloned using the Topo TA Cloning kit (Invitrogen Co., Carlsbad, CA, USA). At least two transformed clones were sequenced. ITS

Table 4.1. Accessions of 27 additional species of *Aeschynanthus* examined in this study. Geographical distribution of each species is available in Fig. 4.4 and 4.5. Taxon number continued from Table 2.1.

Taxon	Locality Collected	Section	Genbank accession No. (ITS1 & ITS2)	RBGE accession No.
(24) *Aeschynanthus acuminatus* Wall. Ex A. DC.	Taiwan	*Haplotrichium*	AF349226 & AF349307	19991496
(25) *Aeschynanthus andersonii* C.B. Clarke	Yunnan (China)	section *X*	AF349231 & AF349312	19970465
(26) *Aeschynanthus arfakensis* C.B. Clarke	Irian Jaya (Indonesia)	*Polytrichium*	AF349216 & AF349297	19972046
(27) *Aeschynanthus austroyunnanensis* W.T. Wang	Yunnan (China)	section *X*	AF349217 & AF349298 (clone A) AF349218 & AF349299 (clone B)	19951561
(28) *Aeschynanthus batakiorum* Mendum & Madulid	Palawan (Philippines)	*Polytrichium*	AF349223 & AF349304	19980285
(29) *Aeschynanthus curtisii* C.B. Clarke	Sarawak (Borneo)	*Aeschynanthus*	AF349153 & AF349234	19622237
(30) *Aeschynanthus ellipticus* Lauterb. & K. Schum.	Papua New Guinea	*Microtrichium*	AF349158 & AF349239	19972009A
(31) *Aeschynanthus garrettii* Craib	Thailand	*Microtrichium*	AF349224 & AF349305 (clone A) AF349225 & AF349306 (clone B)	19750205
(32) *Aeschynanthus irigaensis* (Merr.) B.L.Burtt & P.Woods	Luzon (Philippines)	*Microtrichium*	AF349162 & AF349243	19972532

(33) *Aeschynanthus javanicus* Hook.	Cultivated	*Aeschynanthus*	AF349155 & AF349236	19971339
(34) *Aeschynanthus lineatus* Craib	Yunnan (China)	*Diplotrichium*	AF349219 & AF349300	19970163
(35) *Aeschynanthus musaensis* P. Woods	Papua New Guinea	*Microtrichium*	AF349154 & AF349235	19750186
(36) *Aeschynanthus myrmecophilus* P. Woods	Peninsular Malaysia	*Polytrichium*	AF349227 & AF349308	19981953
(37) *Aeschynanthus nummularius* (Burkill & S. Moore) K. Schum.	Papua New Guinea	*Microtrichium*	AF349157 & AF349238	19932365
(38) *Aeschynanthus obconicus* C. B. Clarke	Sarawak (Borneo)	*Aeschynanthus*	AF349164 & AF349245	19622987
(39) *Aeschynanthus oxychlamys* Mendum	Irian Jaya (Indonesia)	*Microtrichium*	AF349159 & AF349240 (clone A) AF349160 & AF349241 (clone B)	19930953
(40) *Aeschynanthus pachytrichus* W.T. Wang	Yunnan (China)	*Diplotrichium*	AF349220 & AF349301 (clone A) AF349221 & AF349302 (clone B)	19970171
(41) *Aeschynanthus philippinensis* C.B. Clarke	Mindoro (Philippines)	*Microtrichium*	AF349163 & AF349244	19972491
(42) *Aeschynanthus pseudohybridus* Mendum	Sarawak (Borneo)	section *X*	AF349222 & AF349303	19971340
(43) *Aeschynanthus rhododendron* Ridl.	Peninsular Malaysia	*Microtrichium*	AF349166 & AF349247	20001550
(44) *Aeschynanthus roseoflorus* Mendum	Seram	*Microtrichium*	AF349161 & AF349242	19880263
(45) *Aeschynanthus* sp. (001)	Sulawesi	*Microtrichium*	AF349165 & AF349246	Mendum, Argent & Hendrian 001

sequence results were analysed and aligned with the preliminary DNA data matrix. The method of minimum free energy secondary structure analysis of arm 1 of ITS2 was again used to guide alignment in this study. However, even with this aid, an approximately 20 bp region of the aligned matrix in this region was considered ambiguously aligned and was excluded from the analysis. All sequences and the alignment have been submitted to GenBank.

4.2.3. Phylogenetic analysis

Phylogenetic analyses by parsimony, branch support analyses, and other sequence and tree statistics were performed as described in the previous chapters, (more details in Appendix A). Heuristic searches were used to find the most parsimonious trees by using RANDOM sequence addition with TBR swapping for 10000 replicates with Multrees and Steepest Descent options. Decay Indices (Bremer support values) were calculated using the program AutoDecay version 4.0 (Eriksson, 1998). Three methods of combining PCR consensus sequences and multiple clone sequences were used: 1) Clones analysed as separate individual sequences, plus the consensus PCR sequences; the problem here is the relatively large number of terminal items for analysis (80 items representing 52 species). 2) Clones combined as a consensus sequence, plus the consensus PCR sequences. Where clones differ by substitutions, the new consensus sequence is coded as both nucleotides (e.g. A and G coded R etc.); where they differ as an indel, the available sequence is used (gaps ignored). 3) Clones analysed sequentially in an elision matrix. Both clone sequences for each species are analysed in combination, while PCR consensus sequences are included twice to give a matrix of uniform length. Elision matrices are commonly

used in two gene studies to combine data sets, and the method is used in an analogous way here.

A reweighting parsimony analysis was also carried out by weighting characters according to mean values of their rescaled consistency indices (RC). Successive reweighting was carried out four times, at which point no further topological changes occurred. The results of parsimony analyses were compared to a maximum likelihood (ML) analysis (Appendix A). To find the optimum model for the likelihood analysis, the program Modeltest version 3.0 (Posada and Crandall, 1998) was first used to compare the likelihood score results and associated P-values between 56 ML DNA-evolution models. The program then provides a choice of the model that best fits the data by nested likelihood ratio tests and the Akaike information criterion (minimum theoretical information criterion, AIC; Akaike, 1974). The model selected here was TrN+G[1]. Appropriate substitution values, base frequency parameters and Gamma distribution shape parameter (1.0051) determined by Modeltest were then used for the maximum likelihood analysis in PAUP* with TBR swapping.

[1] TrN+G = TrN model (Tamura and Nei, 1993), suggesting varied equilibrium nucleotide frequencies (four different frequencies of A, G, T, and C) and rates of substitution (one transversional rate and two transitional rates), combined with shape parameter of the gamma distribution (G).

4.3. Results

4.3.1. ITS sequence characteristics

Of the 27 additional species sequenced here, six had to be cloned (*A. austroyunnanensis*, *A. garrettii*, *A. oxychlamys*, *A. pachytrichus*, *A. sp.* 0025, and *A.* cf. *viridiflorus*). A further four species (*A. curtisii*, *A. lineatus*, *A. arfakensis*, and *A. javanicus*) showed ITS length polymorphism resulting from single 1-2 bp deletions between different intra-individual ITS copies, but these could be interpreted satisfactorily by comparison of forward and reverse sequences, with the indel bases coded as missing data. This suggests that nearly 40% of *Aeschynanthus* species show some evidence of significant intragenomic polymorphism in their ITS sequences (22% with severe polymorphism, and 15% with minor polymorphism).

When all the sequences are aligned (see Appendix C), a matrix of 603 aligned positions results in 213 (37.4%) potentially informative sites (Table 4.2). In addition, 92 indels were coded of which 64 were informative. The cause of the ITS polymorphism was usually evident from an inspection of the differences between the respective cloned sequences. Most striking was *A. pachytrichus* in which the clones differ by a nine-basepair indel event. The highest intra-individual clone divergence yet recorded in *Aeschynanthus* is between the two highly divergent (7.68 %) clones of *A.* sp. (0025) from Sulawesi. However, when analysed separately, even these clones fall together on the tree.

Table 4.2. Sequence characteristics of ITS1 and ITS2 regions of 81 sequences (representing 52 species) of Gesneriaceae. Characteristics of the aligned matrix excluding ambiguous sequence sites.

Parameter	ITS1	ITS2	ITS1 and ITS2
Length range (bp) - Ingroup + outgroup	217 - 237	206 - 254	430 - 491
- Ingroup	217 - 233	206 - 247	430 - 477
- Outgroup only	225 - 237	243 - 254	468 - 491
Length mean (bp) - Ingroup + outgroup	225.7	239.6	465.0
- Ingroup	225.3	239.3	464.6
- Outgroup only	231.0	248.5	479.5
Aligned length (bp)	289	314	603
G + C content range (%)	48.12 - 59.66	49.57 - 59.51	48.90 - 59.58
G + C content mean (%)	54.71	55.24	54.98
Sequence divergence (%) - Ingroup to outgroup	13.87 - 22.36	16.00 - 24.38	15.96 - 23.38
- Ingroup (between spp.)	0.00 - 19.20	0.00 - 17.18	0.00 - 16.95
- Ingroup (within spp.) [a]	0.00 - 8.04	0.00 - 7.33	0.00 - 7.68
Number of indels - Ingroup + outgroup	34	58	92
- Ingroup (total)	29	46	75
- Ingroup (within spp.)	5	15	20
Size of indels (bp) - Ingroup + outgroup	1 - 4	1 - 9	1- 9
- Ingroup (total)	1 - 4	1 - 9	1 - 9
- Ingroup (within spp.)	1, 2, 4	1, 2, 9	1, 2, 4, 9
Number of excluded sites	15	18	33
Number of sites after exclusion	274	296	570
Number of variable sites	140	171	310
Number of constant sites (%)	134 (48.9 %)	125 (42.2 %)	260 (45.6 %)
Number of potentially informative sites (%)	94 (34.3 %)	119 (40.2 %)	213 (37.4 %)
Number of autapomorphic sites (%)	46 (17.1 %)	52 (17.6 %)	97 (17.0 %)
Transitions on tree (unambiguous)	124	173	314
Transversions on tree (unambiguous)	59	106	166
Transitions/Transversions	2.10	1.63	1.89

[a] Divergence between clone pairs

4.3.2. Phylogenetic analysis

Three different types of matrix were analysed (see methods): 1) the full matrix with all clones analysed as separate entities (81 terminals); 2) the clones combined as a consensus matrix; and 3) combined as an elision matrix. Strict consensus trees resulting from the three methods of matrix assembly have no conflict, being fully congruent at the species level. Minor differences in resolution are noticeable between trees. Between two and three nodes supported in one tree collapse in the other and vice-versa. I found that the elision matrix was fastest to run and gave fewest trees (1440 trees), whereas the full matrix and the consensus combination matrix gave more than 37600 trees (trees exceeded the computer memory when using PAUP*) and 10073 trees respectively. The results presented here use the elision matrix, but none of the conclusions reached is affected by the type of analysis.

4.3.3. Morphological and geographical correlates with the putative phylogeny

All phylogenetic trees produced in this study, by whatever method, confirm the division of *Aeschynanthus* into two major clades which have high bootstrap support, 97 % for clade I and 99 % for clade II (Fig. 4.1). Clade I comprises sections *Haplotrichium* sens. str., *Diplotrichium, Polytrichium*, and *X.* Clade II comprises sections *Aeschynanthus* and *Microtrichium.* There are only two species that deviate from this arrangement: *A. buxifolius* and *A. garrettii* (both in section *Microtrichium*) in clade II. The maximum likelihood tree (Fig. 4.2) does not show any incongruence

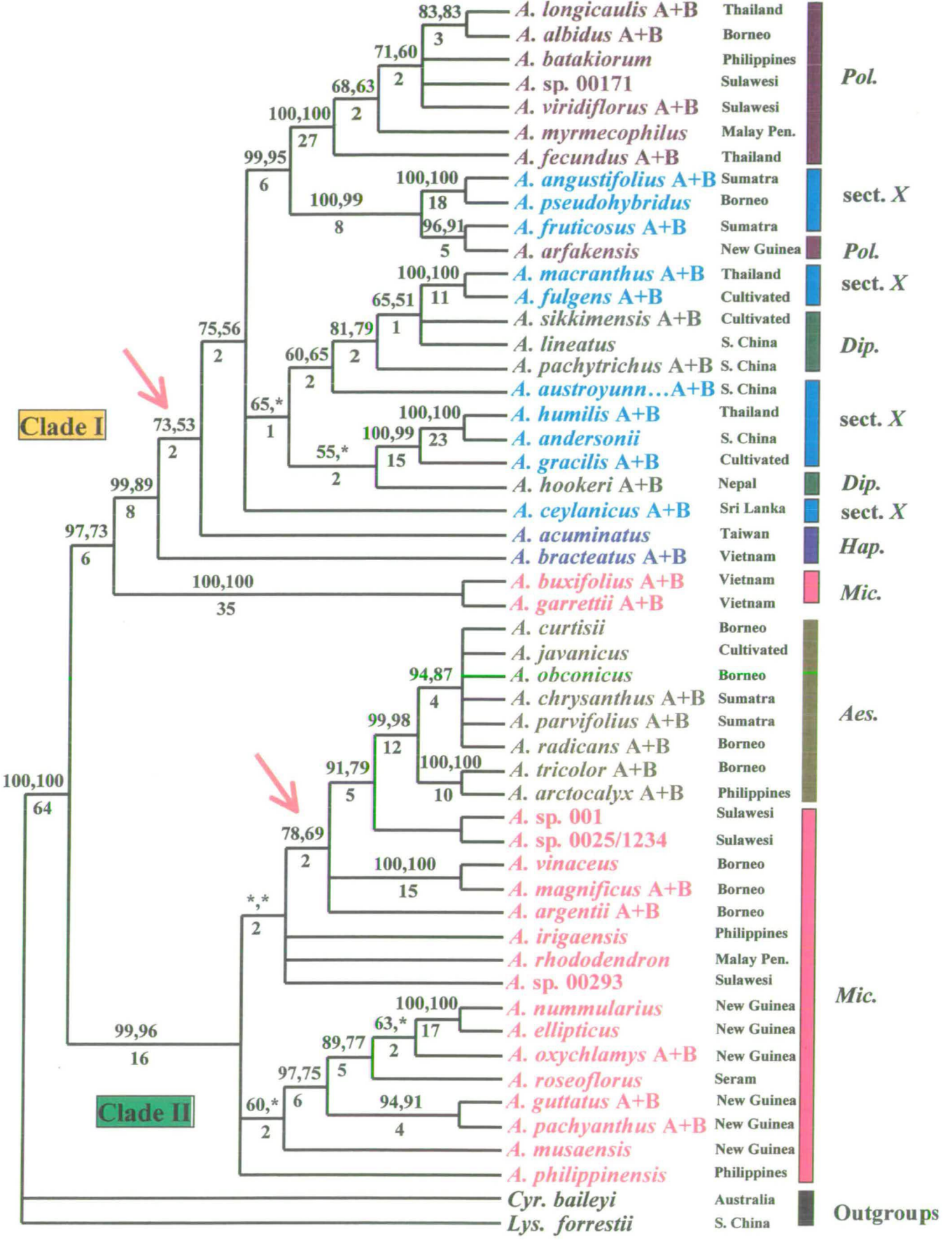


Fig. 4.1. Strict consensus of 1440 most parsimonious trees for 50 *Aeschynanthus* species and two outgroup Gesneriaceae taxa (1557 steps in length) based on parsimony analysis of an elision matrix of the combined ITS1 and ITS2 sequence data plus the alignment gap matrix. The values of upper numbers are full heuristic Bootstrap percentages of 100 replicates and 50% deletion Jackknife percentages ("fast" stepwise-addition) of 10000 replicates, respectively. Lower numbers are decay indices. The two arrows indicate branches that collapse when the gap matrix is excluded and the analysis rerun. The country of origin of the specimen is indicated. [CI = 0.62, RI = 0.79, RC = 0.49] The two clones per species are designated A and B, except for *Aeschynanthus sp.* 0025 for which the four clones are designated 1234.

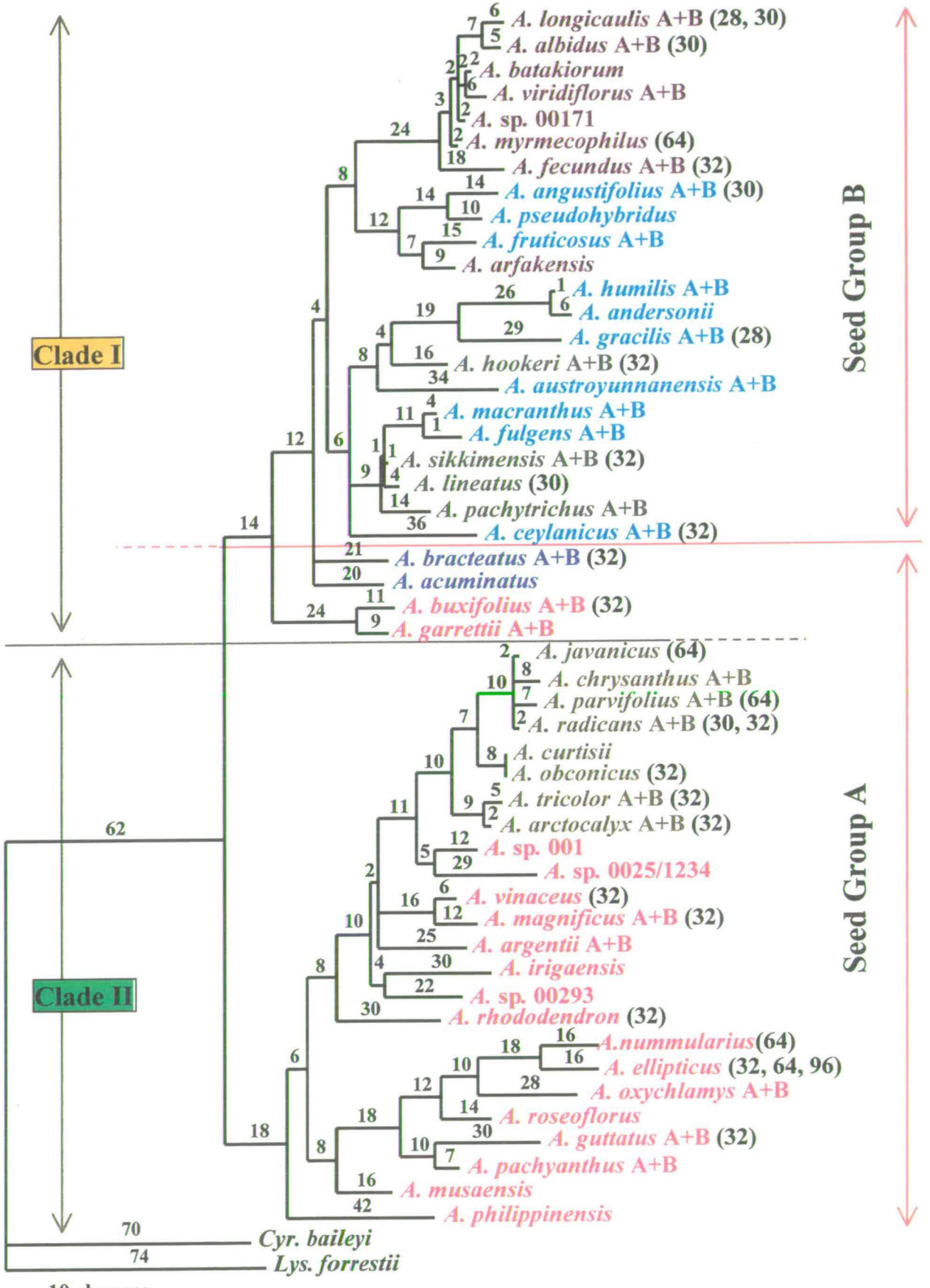


Fig. 4.2. Maximum likelihood (ML) tree for 50 *Aeschynanthus* species and two outgroup Gesneriaceae taxa (-ln likelihood = 8570.14) based on an analysis of the elision matrix of the combined ITS1 and ITS2 sequence data without the gap matrix. As the elision matrix is used, the branches are double their true length. Numbers along branches indicates the amount of character change (branch length). Available chromosome numbers (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). Vertical lines denote major seed types and the main clades, which are congruent except for the four basal clade I species. [CI = 0.62, RI = 0.79, RC = 0.49]

with the maximum parsimony strict consensus tree, and provides further support for the two-clade division of the genus. Many well-supported nodes on the MP tree allow me to conclude with a high degree of certainty that the existing sections, based largely on seed-appendage types, are paraphyletic or polyphyletic. An exception is section *Aeschynanthus* which is monophyletic, with 99% bootstrap support. In clade I, section *Microtrichium* is basal with the other sections polyphyletic or paraphyletic. In clade II most of the species belong to section *Microtrichium* and the monophyletic section *Aeschynanthus* is nested within it.

It is clear from inspection of Fig. 4.1 that there is considerable biogeographic pattern in the phylogeny. The two main clades show different but overlapping geographical distribution (Fig. 4.3). The majority of species in clade I occur in India, Indochina, South China and a few on islands of the Sunda shelf. By contrast species in clade II occur in New Guinea, Sulawesi, Philippines and Seram with some species also occurring on the Sunda shelf islands.

4.4. Discussion

4.4.1. *Aeschynanthus* biogeography: ancient vicariance and recent dispersal

The extended analysis of the phylogeny of *Aeschynanthus* described in this chapter confirms the division of the genus into two major clades differing in distribution. 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. Of the 26 clade I species, 17 occur on

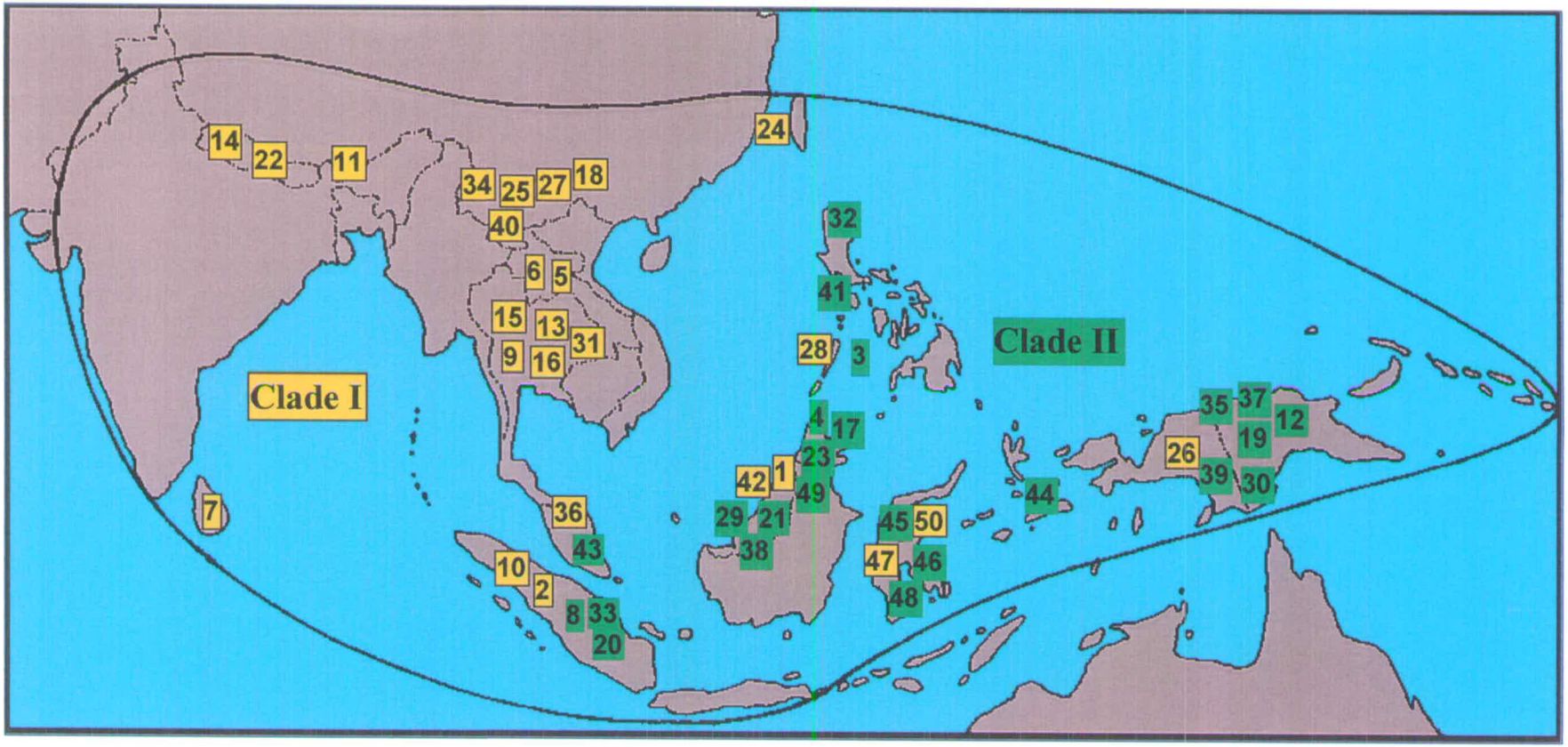


Figure 4.3. Approximate geographic distribution of the 50 *Aeschynanthus* species sequenced. The numbers refer to species as listed in Table 2.2 and Table 4.1. As can be seen from the map, Clade I species (yellow highlighted) are mainly continental in distribution, whereas Clade II species (green highlighted) occur only on Sunda Shelf islands and east of Wallace's Line (Fig. 1.1).

mainland SE Asia (including Taiwan) and only nine are Malesian (including Peninsular Malaysia). Six of the nine, including all clade I species east of Wallace's Line, are in section *Polytrichium*. The wider distribution of the latter (Fig. 4.4) may be due to the greater effectiveness of a coma of many hair-like appendages in wind dispersal. The long filiform appendages possessed by most clade I species are effective for wind dispersal in dry conditions, and the majority of these species occur in the seasonal climates of mainland Southeast Asia. The great majority of clade II species, with shorter, less elaborate appendages, occur in the more consistently wet forests of Malesia (Fig. 4.4 and Fig. 4.5). The 24 clade II species are all Malesian. The species basal to clade I are from Indo-China and Taiwan, implying a possible ancestral area for clade I in this region. Clade II (Fig. 4.5) on the other hand has Philippine and New Guinea species in basal position with *A. philippinensis* as most basal by ML and reweighting analyses (Fig. 4.2 and Fig. 4.6).

The geographical difference between the two major clades implies an ancient vicariance event at the time of the origin of the genus between Indo-China and the Philippines. De Boer and Duffells (1996) postulated identical vicariance patterns between the Asian mainland and eastern Malesia for two cicada groups some 20 million years ago. They suggested that the formation of a volcanic island chain at the western Pacific plate margin allowed separation of mainland and island arc clades. The island arc no longer exists, having migrated west as discrete terranes which now form parts of the Philippines, Sulawesi, and New Guinea. The subsequent coming together of the Australasian and Asian plates would result in an overlap of clades along the Sunda shelf margins. This vicariance postulation is supported by Hall's model of the geographic development of SE Asia (1998), using palaeomagnetic data

deduced from ocean floor magnetic anomalies. He found that in the early Miocene (20 mya) the Philippine sea plate rotated clockwise and necessitated a development of new subduction zones at the eastern edge of Eurasia and in the Southwest Pacific, resulting in widespread volcanic activity in the proto-South China Sea basin throughout the period. Both biogeographic and tectonic suggestions are consistent with the observed geographic patterns in *Aeschynanthus* (Fig. 4.7, more details presented in Fig. 4.4 and Fig. 4.5), which would then be the result of ancient vicariance overlain by recent dispersal and coalescence events, as summarised diagrammatically in Fig. 4.8.

An increase in seed appendage length and number (providing a favourable surface area to mass ratio) appears to be a "key innovation" allowing biogeographic transgression of the main clade areas. The *Microtrichium* type is basal in both clades and is probably the ancestral state. The only clade I species known to be present east of the dotted line in Fig. 4.7 are in section *Polytrichium*, whose derived morphology is extreme in appendage number.

4.4.2. Trangressor species: *A. buxifolius* and *A. garrettii*

On *Aeschynanthus* phylogeny *A. buxifolius* and *A. garrettii* appear to be in the "wrong" major clades for their sections. Both species, although *Microtrichium*, groups as sister to the *Haplotrichium/ Diplotrichium/ Polytrichium/ X* clade. Although they have large deletions in their ITS2 sequences corresponding to arm 1 of the RNA secondary structure, clear homology with other taxa in the rest of the

Species in clade I	S India [a]	N India [b]	E Tibet [c]	NE India [d]	Burma	S China [e]	Thai-land	Viet-nam	Laos	Tai-wan	Pen. Malay [f]	Suma-tra	Java	Borneo [g]	Phillip-pines [h]	Sula-wesi	Seram	New Guinea
A. ceylanicus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A. hookeri	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
A. acuminatus	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
A. fulgens	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
A. bracteatus	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0
A. sikkimensis	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
A. gracilis	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
A. andersonii	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
A. pachytrichus	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
A. austroyunnanensis	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
A. humilis	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
A. buxifolius	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
A. lineatus	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
A. macranthus	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
A. garrettii	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
A. longicaulis	0	0	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0
A. fecundus	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
A. myrmecophilus	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
A. albidus	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
A. angustifolius	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
A. fruticosus	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
A. pseudohybridus	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
A. batakiorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A. sp. 00171	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
A. viridiflorus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
A. arfakensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Total	1	2	3	6	7	12	11	8	2	1	5	3	2	3	1	2	0	1

[a] South India and Sri Lanka; [b] North India and Nepal; [c] East Tibet (China: Xizang province); [d] Northeast India (Sikkim, Assam, Bengal) and Bhutan; [e] South China (Yunnan, Guizhou, Guangdong, Guangxi); [f] Peninsular Malaysia and Singapore; [g] Borneo (Sabah, Sarawak, Kalimantan and Brunei); [h] the Philippines (Palawan, Mindanao, Luzon, Mindoro); [i] Papua New Guinea and Irian Jaya.

Fig. 4.4. Distribution areas of 26 *Aeschynanthus* species from clade I (0 = absence, 1 = presence).

Species in clade II	S India [a]	N India [b]	E Tibet [c]	NE India [d]	Burma	S China [e]	Thai -land	Viet- nam	Laos	Tai- wan	Pen. Malay [f]	Suma- tra	Java	Borneo [g]	Phillip- pines [h]	Sula- wesi	Seram	New Guinea
A. nummularius	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
A. ellipticus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
A. oxychlamys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
A. guttatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
A. pachyanthus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
A. musaensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
A. roseoflorus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
A. sp. 001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
A. sp. 0025	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
A. sp. 00293	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
A. arctocalyx	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A. irigaensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A. philippinensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A. vinaceus	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
A. magnificus	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
A. argentii	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
A. tricolor	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
A. curtisii	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
A. obconicus	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
A. javanicus	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
A. parvifolius	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
A. radicans	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0
A. chrysanthus	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
A. rhododendron	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	1	0	0	0	4	4	3	8	3	3	1	6

[a] South India and Sri Lanka; [b] North India and Nepal; [c] East Tibet (China: Xizang province); [d] Northeast India (Sikkim, Assam, Bengal) and Bhutan; [e] South China (Yunnan, Guizhou, Guangdong, Guangxi); [f] Peninsular Malaysia and Singapore; [g] Borneo (Sabah, Sarawak, Kalimantan and Brunei); [h] the Philippines (Palawan, Mindanao, Luzon, Mindoro); [i] Papua New Guinea and Irian Jaya.

Fig 4.5. Distribution areas of 24 *Aeschynanthus* species from clade II.

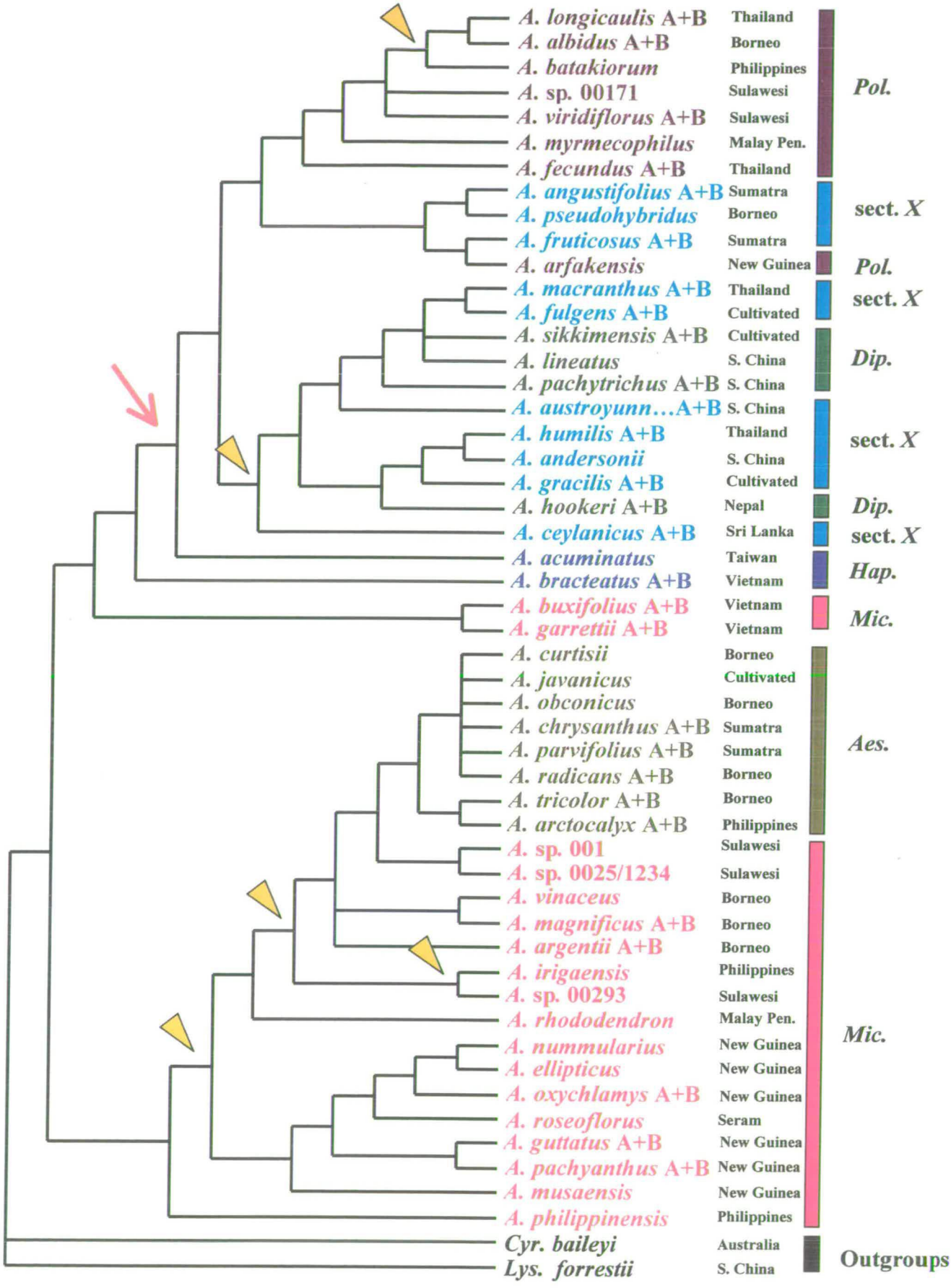


Fig. 4.6. Successive weighting strict consensus tree of 120 most parsimonious trees for 50 *Aeschynanthus* species and two outgroups (733 steps in length) based on parsimony analysis of an ITS elision matrix plus the alignment gap matrix. The 1440 most parsimonious trees of the original elision matrix were used to weight characters by their mean RC value and the analysis rerun. The arrow indicates a branch that collapses when the gap matrix is excluded and the analysis rerun. The five triangles show branches that collapse on the consensus tree of unweighted analysis. *Aeschynanthus philippinensis* is basal in clade I in this analysis as in the ML analysis (Fig. 4.2). [CI = 0.85, RI = 0.92, RC = 0.78].

sequences indicates that these deletions have not led to the species being misplaced in the phylogeny, but are merely coincidental. One possible explanation is that the *Microtrichium* seed appendage morphology is plesiomorphic and section *Microtrichium* is paraphyletic with respect to the other sections. Moreover, both species, unlike most members of the section, are from continental Asia, and their geographical distribution is in accord with their grouping with clade I.

4.4.3. Seed morphology reflects major clade structure in *Aeschynanthus*

Recent morphological studies (Mendum et al., 2001), recognising two major groups in *Aeschynanthus*, align well with these new molecular results. Clade I broadly corresponds to seed group B and clade II corresponds to seed group A (Fig. 4.2). The seed group A species that do fall into clade I are those that are basal. *Aeschynanthus buxifolius* and *A. garrettii* (section *Microtrichium*) are two of the only three *Microtrichium* species to have ± straight testa cell orientation (the others have an anticlockwise spiral); they are also the only ones that are not Malesian. *Aeschynanthus acuminatus* and *A. bracteatus* are two members of the very small section *Haplotrichium* which is not known to occur in Malesia. Clade I and clade II differ in the orientation of the testa cells and can therefore be defined morphologically. Clade I has straight or clockwise spiral orientation and clade II anticlockwise spiral orientation (Fig. 4.9).

Appendage numbers appear to be at least partly homoplastic. There is

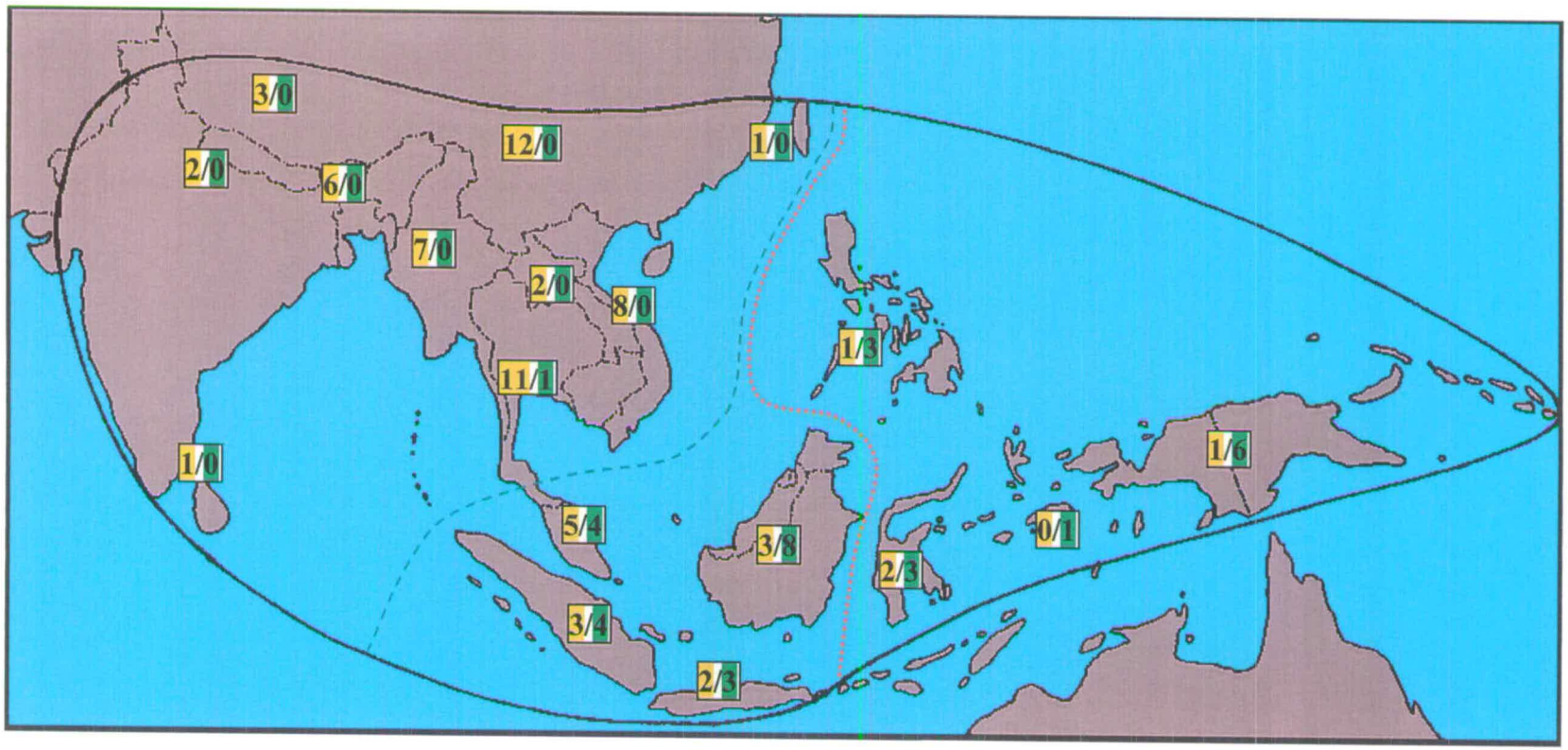


Fig. 4.7. Comparison between geographical distribution of clade I and clade II species. The first number in each ratio refers to the number of species from clade I. The second number refers to the number of species from clade II. The solid line shows the geographic distribution of the whole genus. The dashed line indicates an approximate western boundary of clade II species. Clade I species, with the exception of section *Polytrichium*, do not occur east of the dotted line. This line is similar to Huxley's line (Huxley, 1868) except that Palawan (Philippines) is to the east of the line.

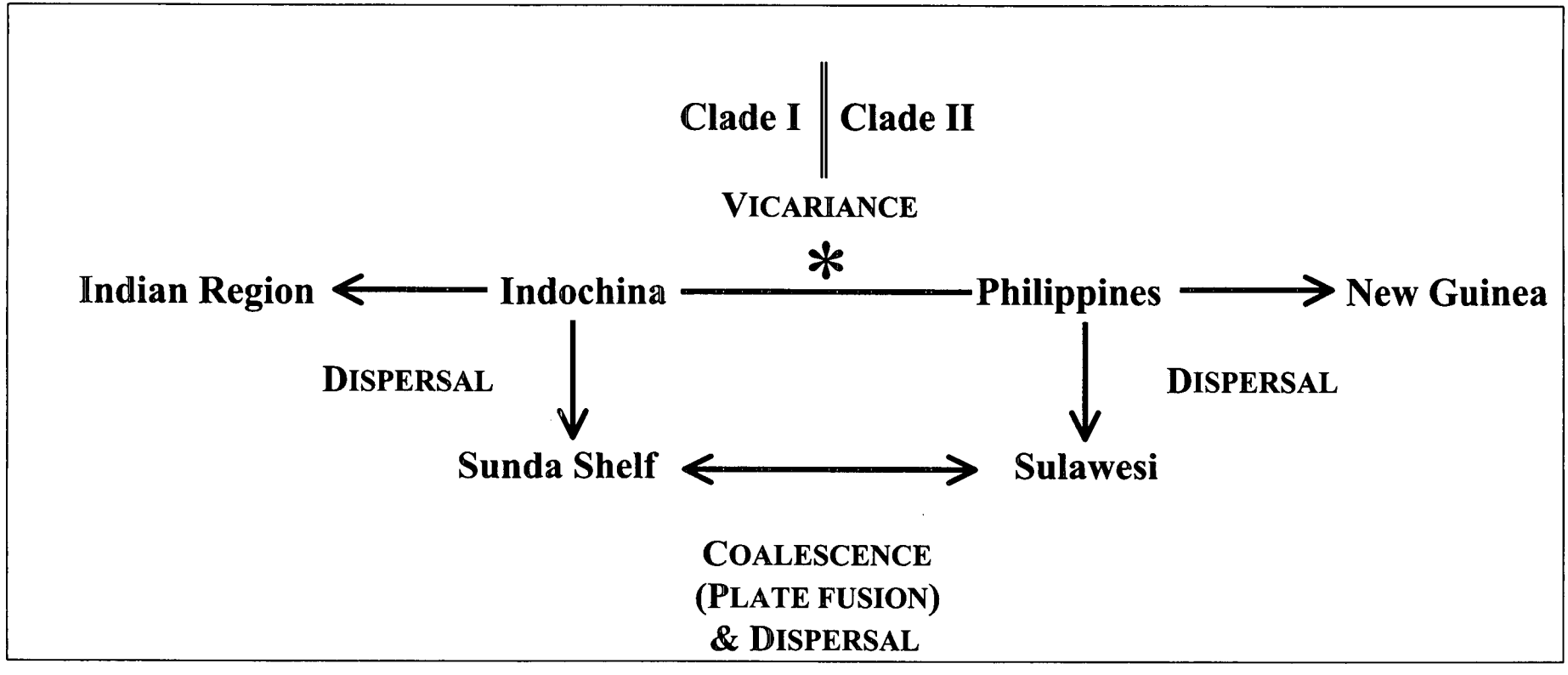


Fig. 4.8. A tentative model of the geographic pattern of *Aeschynanthus* evolution, consistent with the ITS phylogeny, suggesting a combination of ancient vicariance, recent dispersal and coalescence events in the regions. The asterisk indicates a proposed ancient origin of *Aeschynanthus* clades I and II by a vicariance event.

morphological evidence that the condition of more than one hilar appendage is derived. All other genera in tribe Trichosporeae are reported to have seed with a single hilar appendage, as do the majority of *Aeschynanthus* species including those basal to clade I in this study. Preliminary studies of *Aeschynanthus* seed ontogeny by Saueregger and Mühlbauer (unpublished) showed that, in section *Polytrichium*, one appendage develops a little before the others and remains somewhat longer and stouter. Current studies by Christie and Mendum (unpublished) confirm this and also show that in section *Diplotrichium* one appendage develops a little before the other. A small percentage of seeds of two section *X* species show development of a second appendage, and a few seeds of one collection of *A. parasiticus* (section *Diplotrichium*) show development of a short third appendage. Thus the clade I species with Type B seed morphology do appear to be a natural group, notwithstanding the differences in appendage number. The short smooth appendage type (section *Microtrichium*) is basal in both clades, and is thus paraphyletic. Section *Aeschynanthus*, with flexuous trailing habit and tubular or obconic calyx with abscission layer at the base, is a natural group.

The present sectional classification, based (with the exception of section *Xanthanthos*) on easily observable appendage characters, has proved to be of considerable practical taxonomic value and it is not my intention to revise it here to reflect the ITS data. However, the existence of two major clades in *Aeschynanthus*, differing in testa cell orientation and in geographical distribution patterns, raises the possibility of dividing the genus into two clearly defined natural subgenera.

4.4.4. Cytology and major clade structure

Available chromosome numbers (Rashid et al., 2001) show a possible slight trend towards dysploid reduction in clade I species, and polyploidy in clade II species. Counts are available for 23 of the sequenced species, 12 in clade I and 11 in clade II (Fig. 4.2). The commonest number is 2n = 32, but in clade I five aneuploids occur (2n = 30, 2n = 28), but only one polyploid, *A. myrmecophilus* with 2n = 64. In clade II by contrast, only one aneuploid occurs, but polyploids are more common. More investigation of *Aeschynanthus* chromosome numbers is required to confirm or reject this possibility.

4.4.5. Ecology of the two major clades and the evolution of *Aeschynanthus*

As noted above, the two major clades have eastern versus western geographical tendencies. The eastern Malesian forests tend to be consistently wet, whereas the western continental forests tend to have a marked dry season and monsoonal wet season (Riley and Spolton, 1974; Rudloff, 1981). The greater frequency of hydathodes in clade II (eastern) species (Rosser and Burtt, 1969) may result from this. The very thin stems found in some species of section *Microtrichium* (Rosser and Burtt, 1969) with little water conducting tissue may also reflect this. Other sections usually have stout stems (*Polytrichium/Diplotrichium*). A dry season provides ideal conditions for the dispersal of seeds with elaborate seed appendages and the greatest elaboration of seed appendages is found in the clade I species of sections *Haplotrichium* sens. str., *X*, *Diplotrichium*, and *Polytrichium*. In contrast the

small hairs of *Microtrichium* may be more important for substrate attachment than dispersal, consistent with the wet climate.

I propose a possible evolutionary pattern of seed appendage types in *Aeschynanthus*, suggested by the phylogenetic analysis, as in Fig. 4.9. An ancestral lineage is suggested with small seed appendages, as in section *Microtrichium*, occupying a central Malesian site without a pronounced dry season. The small seed-appendage type in the West (clade I) has given rise to types with long seed appendages as in sections *Haplotrichium* sens. str., *X*, and *Diplotrichium*. Further elaboration to the most complex *Polytrichium* types then appears to have occurred. In contrast, in east Malesia the *Microtrichium* seed morphology appears to have been retained and diversified, evolving only once into a long seed-appendage type: section *Aeschynanthus* with its characteristic bubble cells. Clade I appears to have spread westwards into seasonal forests in Sri Lanka and India, while clade II has achieved a wide distribution in eastern wet forests. Further species sampling will be needed to confirm this hypothesis.

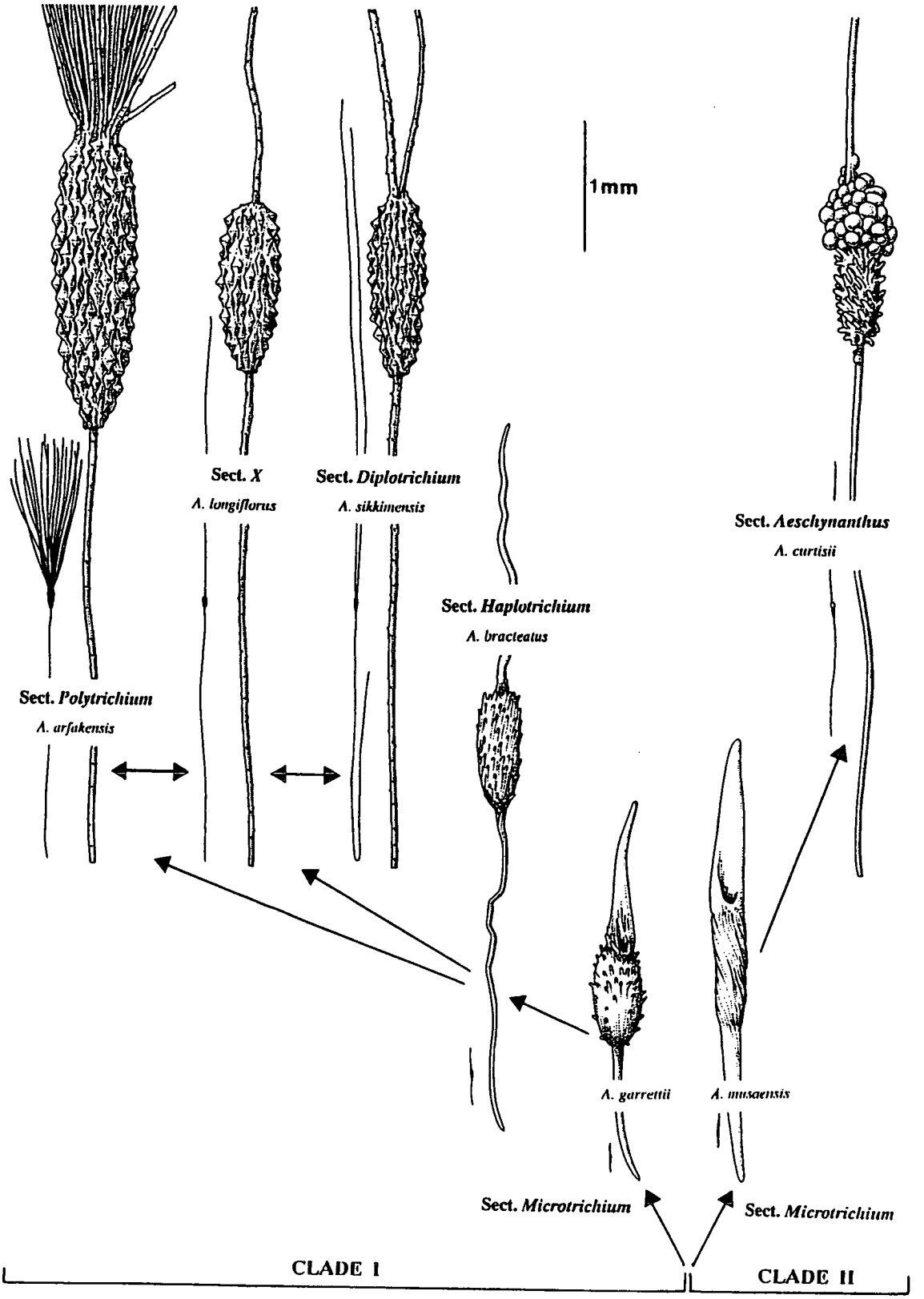


Fig. 4.9. Possible evolution of seed appendage types in Aeschynanthus as suggested by the phylogenetic analysis. The morphological types are represented by named exemplar species. The diagram illustrates suggested morphological transitions only, and does not imply transitions between these exemple species.

CHAPTER FIVE: Ribosomal DNA inheritance in *Streptocarpus* (Gesneriaceae) and its phylogenetic implications

5.1. Introduction

Sequences of multiple copies of nuclear ribosomal genes are homogenised rapidly by the molecular drive mechanism. The evolutionary population dynamics of molecular drive and concerted evolution remain largely unknown and more investigations on rDNA inheritance are strongly needed. No previous studies on this subject have been done in Gesneriaceae, and I was interested in using *Aeschynanthus* as a model for study. The phylogeny of the genus has been reconstructed and enabled a much clearer understanding of its classification and evolution. However, because of insufficient time for crossing experiments in *Aeschynanthus*, prepared hybrids of *Streptocarpus*, another genus in the Gesneriaceae collection of RBGE, were examined instead.

A molecular systematic study of *Streptocarpus* has been carried out by Möller and Cronk (1997a, 1997b and 2001) using ITS sequences. They found that ITS2 consensus sequences of some species showed an approximately 40 basepair deletion relative to other species. *Streptocarpus dunnii* and *S. wittei* differ in length from *S. rexii* by 44 basepairs, mostly as a result of a single 43-basepair large deletion near the 5′ end of the spacer, and another one basepair deletion elsewhere. This deletion in *Streptocarpus* is characteristic of a single clade (the "ITS2 deletion clade"

of Möller and Cronk, 2001). This provides a single marker system for the rDNA inheritance study: electrophoresis of PCR products was used to characterise ITS2 copies in F_1 hybrids between the species, and backcrosses to their parents. Knowledge gained from these studies (for instance, the number of rDNA loci in the *Streptocarpus* genome) should make possible an explanation of intra-individual ITS variation phenomena in *Aeschynanthus*.

Homogenisation of rDNA occurs effectively between copies within a locus on a single chromosome, between homologous chromosomes, and even between non-homologous chromosomes. It is likely that the number of rDNA loci in the genome and in the location within chromosomes have a strong influence on the rate of homogenisation of rDNA. In *Arabidopsis thaliana*, rDNA loci are located at two nucleolar organiser regions (NORs): one on the distal northern tip of chromosome 2 and the other on the distal northern tip of chromosome 4, both similar in size, approximately 3.6 - 3.7 Mbp (360 - 370 rDNA genes each) (Copenhaver and Pikaard, 1996). In this plant, homogenisation is more rapid within NOR than between the non-homologous chromosomes (Schlötterer and Tautz, 1994). However, the number and location of rDNA loci in plant genomes varies greatly. Scott and Bendich (1987) give an extensive review of plant rDNA locus variability. For example, in *Aloe* species there are 2-3 loci distributed both at telomeric and interstitial sites (Adams et al., 2000). These differences may explain the variation in the patterns of homogenisation found among hybrids. In higher plants, hybrids may have both parental sequences, for example, *Krigia* (Kim and Jansen, 1994), *Arabidopsis suecica* (O'Kane, Schaal and Al-Shehbaz, 1996), and *Cardamine* (Franzke and Mummenhoff, 1999), or may retain only one parental sequence as in

Gossypium (Wendel, Schnabel, and Seelaman, 1995a) and *Cardamine* (Franzke and Mummenhoff, 1999), or are a mixture of both parental sequences over consecutive generations, *Gossypium gossypiodes* (Wendel, Schnabel, and Seelaman, 1995b), *Microseris* (van Houten, Scarlet, and Bachmann, 1993), *Paeonia* (Song, Crowford, and Stuessy, 1995), and *Microthlaspi* (Mummenhoff, Franzke, and Koch, 1997). However, most studies of ITS inheritance have been carried out on natural hybrids of unknown or uncertain history. My investigation of rDNA inheritance in *Streptocarpus* would provide a better view under controlled conditions with artificially produced hybrids of known origin.

5.2. Materials and methods

5.2.1. Plant materials

Plant material was obtained from the living collections held at the Royal Botanic Garden Edinburgh (RBGE) with accession numbers as given in Table 5.1. Voucher herbarium specimens of the parental accessions analysed have been prepared and are lodged at E. Comparative morphology of *S. dunnii*, *S. rexii*, and F_1 hybrid is provided in Fig. 1.9 (Chapter One).

Table 5.1. Plant materials used in the study.

Plant accession no.	Plant definition	Number of plants
19941745	*Streptocarpus dunnii* Hook.	2 (A, D)
19870333	*Streptocarpus rexii* Lindl.	1

19972904	F_1 hybrid *S. rexii* 19870333 x *S. dunnii* 19941745A	16 [a]
19972905	F_1 hybrid *S. dunnii* 19941745A x *S. rexii* 19870333	19
19990104	Backcross F_1 19972904/no.4 [b] x *S. rexii* 19972904/no.6 [a]	38
19990107	Backcross F_1 19972904/no.13 [c] x *S. dunnii* 19941745D	22
19982610	Backcross F_1 of *S. rexii* x *S. wittei* (19870333 x 19871695B) x *S. wittei* De Wild.	19
19982595	Selfing progeny of *S. dunnii* 19941745A	20

[a] With four other F1 plants (no. 6, 12, 16 and 18) which were accidental selfings of *S. rexii*
[b] F_1 19972904/no.4 is in the strong-intensity group.
[c] F_1 19972904/no.13 is in the weak-intensity group.

5.2.2. Experimental strategy

Details of DNA extraction, PCR amplification, agarose gel electrophoresis, and DNA sequencing for ITS are provided in Appendix A. Briefly, genomic DNA of each individual was extracted using CTAB (Doyle & Doyle, 1987) and used as a template for PCR amplification, yielding the ITS2 region (mostly using primer combinations ITS3P & ITS4 or ITS2G* & ITS4). Primer ITS2G* is an alternative forward primer near the 3′ end of 5.8S rDNA and is the complement of primer ITS2G (Möller and Cronk, 1997a). Primer ITS2G* was also used in duplex PCR (see below) due to primer/primer incompatibility between ITS3P and the *trn*L primers. PCR products were cycle sequenced and analysed on an ABI 377 prism DNA sequencer (Perkin Elmer, Applied Biosystem Inc., Foster City, CA, USA) according to the manufacturer's recommendations.

The intron of the chloroplast gene *trn*L was used as an internal standard in duplex PCRs (Möller, 2000) amplified as a control using primers c + d (Taberlet et al., 1991). The PCR conditions used were the same as for ITS.

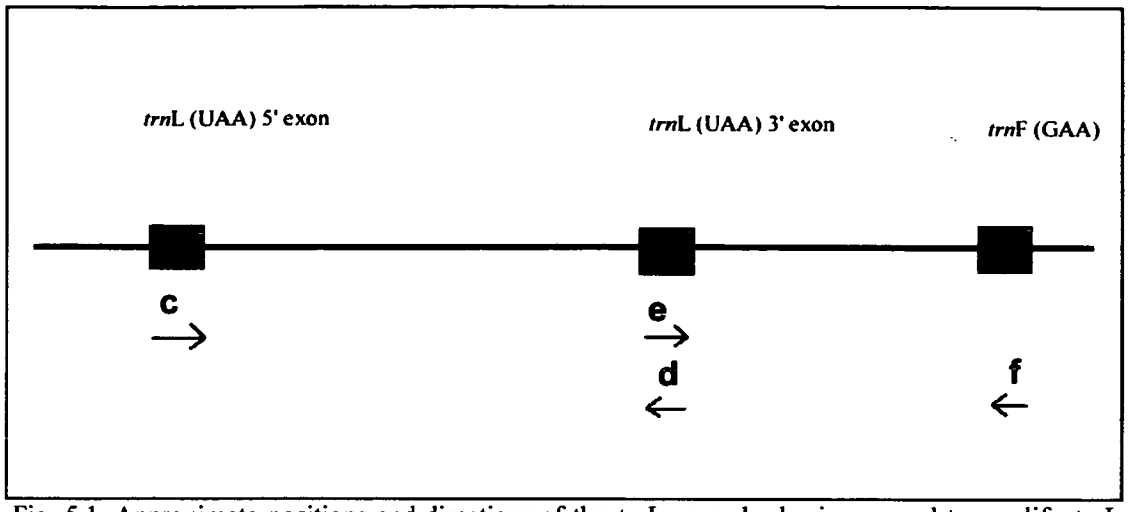


Fig. 5.1. Approximate positions and directions of the *trn*L –c and –d primers used to amplify *trn*L intron region (after Taberlet *et al.*, 1991).

In addition several different PCR conditions were tried in order to investigate the nature and reproducibility of the PCR-silencing phenomenon found in this study. Tested conditions include: different thermostable DNA polymerase enzymes - Dynazyme II (Finnzymes Oy, Espoo, Finland) and HotstarTaq (QIAgen, Ltd., Dorking, Surrey, UK); different primer sets - primers ITS3P & ITS4, ITS3P & ITS8P, ITS2G* & ITS8P, and ITS2G* & ITS4; different annealing temperatures – 48°C (low stringency) and 55°C (high stringency); and PCR reaction with either 10% DMSO (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) or Q-solution (QIAgen, Ltd., Dorking, Surrey, UK with HotstarTaq enzyme supplied), both supposed to reduce secondary structure formation which can prevent proper PCR amplification.

5.2.3. Secondary structure analysis

As mentioned previously the ITS2 region of *S. dunnii* has a large 43 bp deletion relative to that of *S. rexii* (Möller and Cronk, 1997a). To study this

difference between the ITS2 of both species, the PCR consensus sequence was first checked against cloned sequences to ensure that the sequence exists as single molecules. PCR products were cloned (see details in Appendix A). PCR products (primer ITS3P and ITS4) from one of the F_1 plants (individual no. 19 of cross 19972905) were purified and ligated into plasmid vectors using the Topo TA Cloning kit (Invitrogen Co., Carlsbad, CA, USA). Selected cloned ITS2 fragments were sequenced and analysed. I then generated minimum free energy RNA secondary structures of the *S. dunnii* and *S. rexii* ITS2 regions using the program RNAdraw version 1.1 (Matzura and Wennborg, 1996).

5.2.4. Band intensity analysis

Duplex PCR was performed using two primer pairs per reaction amplifying ITS2 and the *trn*L intron, simultaneously using the latter band as an internal standard. The ratio between ITS2 and the *trn*L intron band intensity for each individual plant was calculated: digital images of agarose gel results containing PCR samples were captured using a Kodak DC40 digital camera (Eastman Kodak Co., New Haven, CT, USA). The image was then analysed with an image analysis program, Kodak Digital Science 1D (Eastman Kodak Co., New Haven, CT, USA), using the Analysis and Profile functions. The width and height of each band-profile was manually adjusted to cover each PCR band. Net intensities (i.e. sum of background subtracted pixels within the band rectangle) of the bands were read and ratios of each ITS2/*trn*L pair calculated.

5.3. Results

5.3.1. *Streptocarpus dunnii* and *S. rexii* ITS2 variation

As expected, the F_1 hybrid plant (no. 19 of cross 19972905) contained clones with two different inserted-fragment sizes, corresponding to the consensus-sequence types of the parents. An alignment of the two size-different ITS2 clones sequenced is shown in Fig. 5.2 with an indication of the single large deletion in the *S. dunnii* sequence (position 23 to position 65) relative to the *S. rexii* sequence. These two cloned sequences were identical to the PCR consensus sequences of the parental species (in Möller and Cronk, 1997a), suggesting that *S. rexii* and *S. dunnii* copies are greatly homogenised.

5.3.2. The ITS2 deletion in relation to secondary structure

Minimum free energy analysis was then performed to predict RNA secondary structures of *S. dunnii* and *S. rexii* ITS2 sequences. Minimum free energy secondary structures of both ITS2 sequences showed a putative four-arm model, as illustrated in Fig. 5.3. The large deletion in *S. dunnii* resulted from the loss of the terminal part of the arm 1 of the ITS2 structure, as has already been shown for a similar phenomenon in *Aeschynanthus* (see Chapter Three).

```
[                    10         20         30         40         50         60         70]
[                    .          .          .          .          .          .          .]
                                ******** ********** ********** ********** *****
S. rexii      ATCTCGTCGC CACCCCCACA GACAGATCTT CGGTTCCCCA CCACAAGTGT CGGGAGCGGA AGCATGCTGG   [70]
S. dunnii     ATCTCGTCGC CACCCCCAAG AA-------- ---------- ---------- ---------- -----GGAGG   [27]

[                    80         90         100        110        120        130        140]
[                    .          .          .          .          .          .          .]

S. rexii      GGAGTGTGCG GATATTGGCC TCCCGTTACC TCGTGTAGCG GCTGGCCCAA ACAGGATACC GTGTCGATGG   [140]
S. dunnii     GGGGGGTGCG GATATTGGCC TCCCGTCACC TCGTGTAGCG GCTGGCCCAA ACAGGATACC GTGTCGATGG   [97]

[                    150        160        170        180        190        200        210]
[                    .          .          .          .          .          .          .]

S. rexii      ATGTCACACG ATACGTGGTG GTTGGATTCC ACAACTCTCG TAACTGTCGT GTGAGACCGC ATCAAGACAC   [210]
S. dunnii     ATGTCACACG ATACGTGGTG GTTGGATTCC ACAACTTTCG -AACTGTCGT GTGAGACCGC ATCGAGACAC   [166]

[                    220        230        240 ]
[                    .          .          .   ]

S. rexii      GAGCACGACC CCGTGGCACC GGCTGCCCTC GA   [242]
S. dunnii     GGGCACTACC CCATGGCACC GGTTGCCCTC GA   [198]
```

Fig. 5.2. Aligned sequence matrix of the ITS2 region of the *S. rexii* type and the *S. dunnii* type from clones of the F_1 hybrid plant no. 19 of cross 19972905. Hyphens denote alignment gaps and asterisks above nucleotide matrix indicate the 43 basepair deletion in the *S. dunnii* ITS2 type.

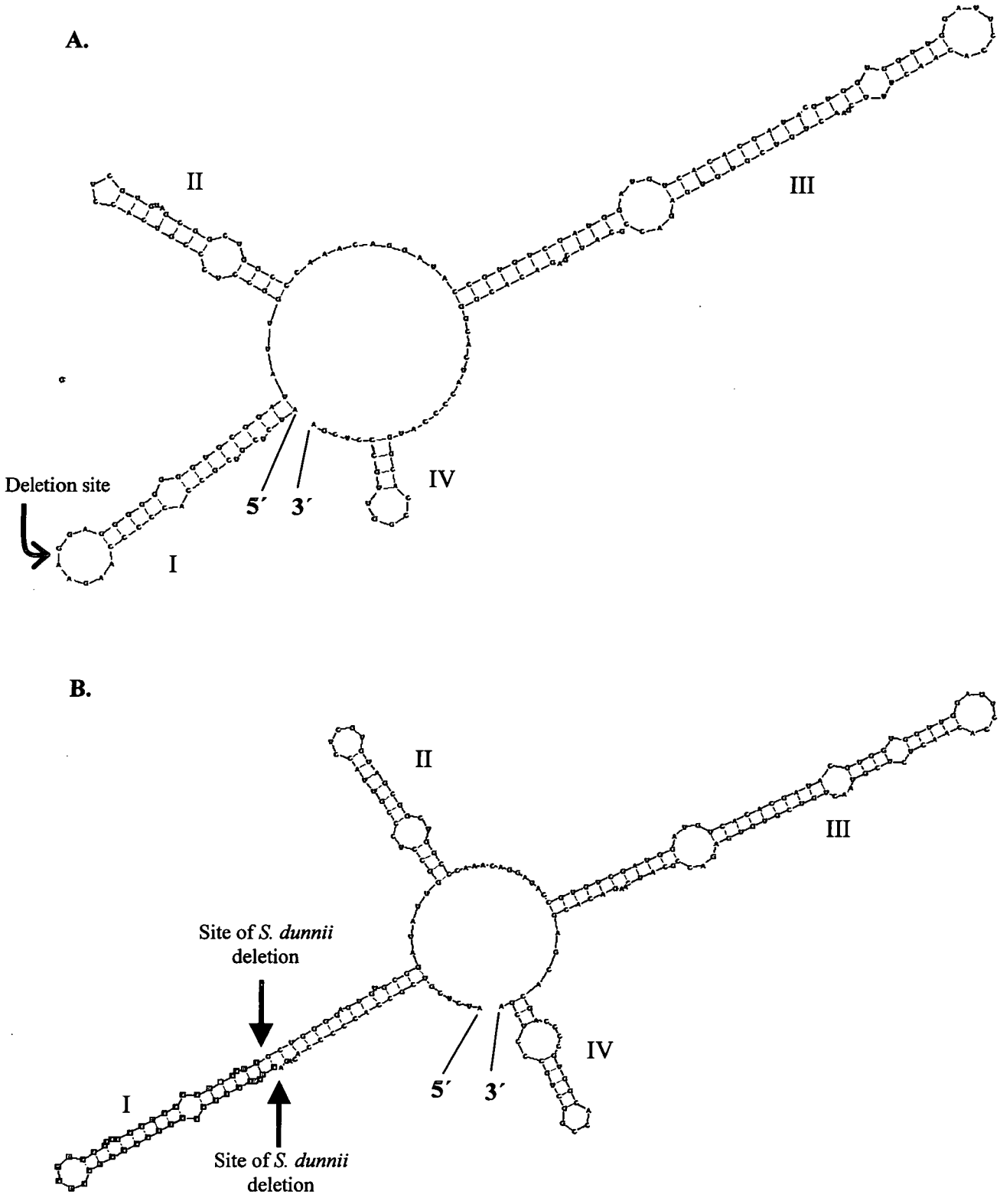
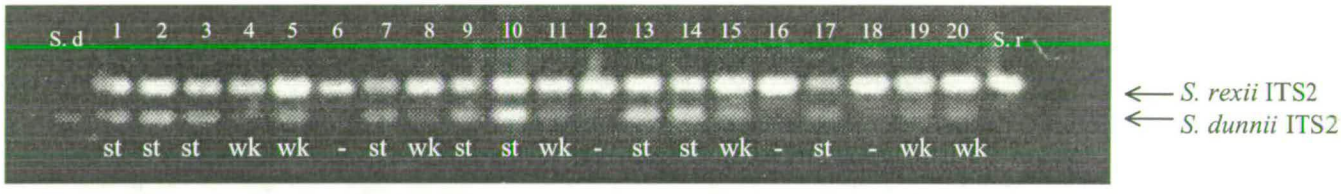


Fig. 5.3. Reconstruction of minimum free energy secondary structure of cloned ITS2. (A) *S. dunnii*, dG = -86.94 kcal, at 20°C. (B) *S. rexii*, dG = -104.96 kcal, at 20°C. The position of a 43 bp deletion in *S. dunnii* is indicated. A square box indicates each nucleotide in the deletion.

5.3.3. rDNA inheritance in F_1 hybrids between *S. dunnii* and *S. rexii* is additive

PCR amplification of all 35 F_1 hybrids (Table 5.1) showed the same result; each individual had two ITS2 bands (*S. rexii* allele and *S. dunnii* allele) (Fig 5.4a and 5.4b). I apply the word 'allele' in this thesis to the complete set of rDNA copies at one locus, a NOR, although this consists of multiples of up to several hundred individual tandem repeat units. It is interesting to note that the intensity of the *S. dunnii* band (Fig. 5.4a and 5.4b, lane 1) was always fainter in PCRs than that of *S. rexii*.

A.



B.

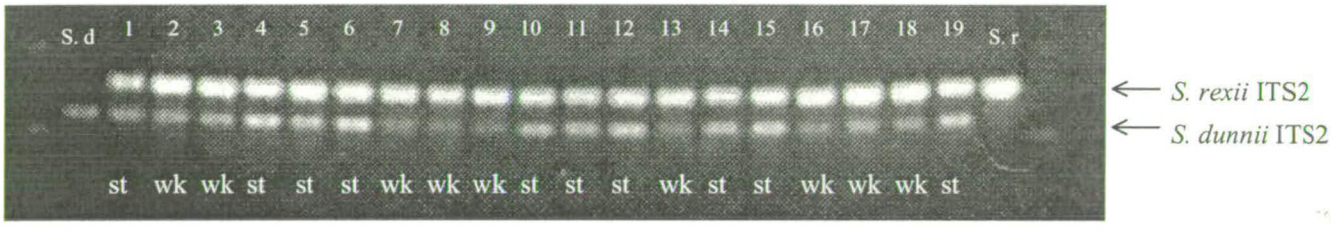


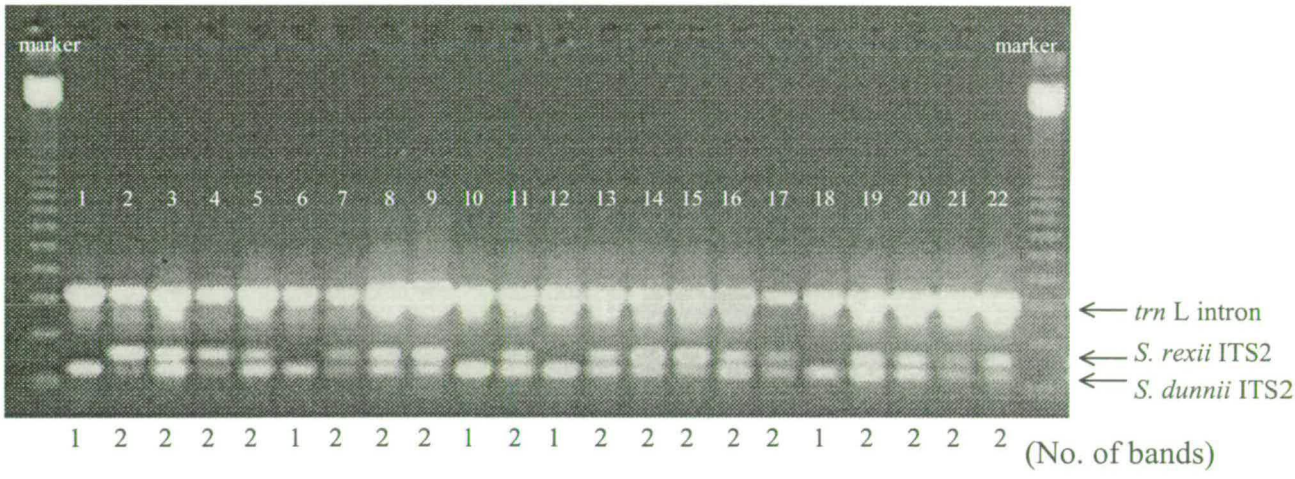
Fig. 5.4. Additive inheritance of ITS2. Amplified ITS2 bands (primers 3P & 4) of (A) F_1 19972904 *S. rexii* x *S. dunnii* (left to right: *S. dunnii*, no. 1-20 F_1 plants, *S. rexii*). F_1 19972904 no.6, 12, 16, 18 are accidental *S. rexii* selfings. (B) F_1 19972905 *S. dunnii* x *S. rexii* (left to right: *S. dunnii*, no. 1-19 F_1 plants, *S. rexii*). Band-intensity of the *S. dunnii* ITS2 band of each F_1 plant is shown as either "wk" (weak intensity) or "st" (strong intensity).

5.3.4. rDNA segregation in backcrosses is consistent with a two loci hypothesis

I wished to investigate the number of *Streptocarpus* nrDNA loci by studying the segregation of the marker. However, the F_1 was unfortunately found to be largely self-sterile. Backcrosses to the parents were fertile however. Backcross 19990107 [(*S. rexii* x *S. dunnii*) x *S. dunnii*] was used to study ITS2 segregation. Of 22 backcross plants, five had one ITS2 band (*S. dunnii*-type), the other 17 plants had two bands (*S. dunnii*- and *S. rexii*-type) (Fig. 5.5a). The observed one-band to two-band ratio was statistically tested against a one NOR locus hypothesis (1:1), a two NOR loci hypothesis (1:3) and a three NOR loci hypothesis (1:19). The one NOR locus hypothesis and the three NOR loci hypothesis were both rejected (Table 5.2), and the two NOR loci hypothesis was retained.

For further confirmation a parallel backcrossing experiment was performed with *S. wittei*, a species closely related to *S. dunnii* which also has a 43-basepair large deletion in ITS2 in comparison to *S. rexii*. Nineteen individuals of backcross 19982610 [(*S. rexii* x *S. wittei*) x *S. wittei*] were studied and shown to have a one-band to two-band ratio of 5:14 (Fig. 5.5b). This ratio is similarly consistent with a two loci hypothesis; the one locus and three loci hypotheses were rejected (Table 5.2).

A.



B.

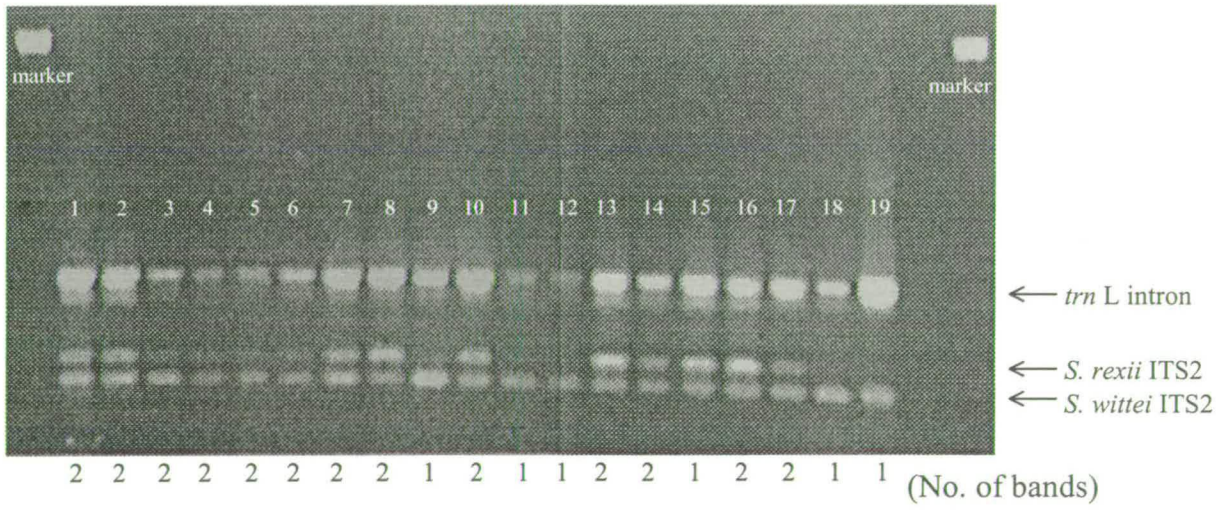


Fig. 5.5. Inheritance of ITS2 in backcrosses. Amplified *trn*L intron (primers c & d) and ITS2 bands (primers ITS 2G* & 4) of (A) backcross 19990107 (*S. rexii* x *S. dunnii*) x *S. dunnii* (left to right: 123 bp ladder, no. 1-22 backcross plants, 123 bp ladder). (B) backcross 19982610 (*S. rexii* x *S. wittei*) x *S. wittei* (left to right: 123 bp ladder, no. 1-19 backcross plants, 123 bp ladder).

Table 5.2. Chi-square test comparison between one-NOR, two-NOR, and three-NOR loci hypotheses of backcrosses 19990107 (*S. rexii* x *S. dunnii*) x *S. dunnii* and 19982610 (*S. rexii* x *S. wittei*) x *S. wittei*. R, D, and W indicate *S. rexii*, *S. dunnii*, and *S. wittei* respectively. (Degree of freedom = 1, n. s. d. = non significant difference).

Backcross	Total no. of plants	Observed value (No. of plants)		Expected value from one NOR locus hypothesis		Probability value & assumption	Expected value from two- NOR locus hypothesis		Probability value & assumption	Expected value from three- NOR locus hypothesis		Probability value & assumption
		1 band	2 bands	1 band	2 bands		1 band	2 bands		1 band	2 bands	
19990107 (RxD)xD	22	5	17	11	11	0.011 Hypothesis rejected ($P < 0.05$)	5.5	16.5	0.806 n. s. d.	1.1	20.9	0.000 Hypothesis rejected ($P < 0.05$)
19982610 (RxW)xW	19	5	44	9.5	9.5	0.036 Hypothesis rejected ($P < 0.05$)	4.75	14.25	0.895 n. s. d.	0.95	18.05	0.000 Hypothesis rejected ($P < 0.05$)

5.3.5. Variations in band intensity suggest that one rDNA allele in *S. dunnii* is PCR-silent

Band-intensity of the *S. dunnii* ITS2 band in *S. rexii* x *S. dunnii* crosses varied across the F_1 hybrid plants. Each hybrid could be classified into one of two groups: a weak intensity group and a strong intensity group, as compared to the *S. rexii* band (Fig. 5.4). This effect was highly reproducible, in a ratio of 16:19 in the 35 F_1 plants, suggestive of the segregation of a heterozygous locus in the *S. dunnii* parent plant (null hypothesis of a 1:1 ratio was not rejected; Chi-square P value = 0.612).

To test the consistency of this band-intensity phenomenon several different PCR conditions were tried on several hybrids (no. 10 and 19 of cross 19972904 and no. 6 and 7 of cross 19972905) representing those two different band-intensity groups (gel results not shown): First, using thermostable DNA polymerase enzymes from different companies, i.e. Dynazyme II and HotstarTaq, PCR reactions did not alter the phenomenon. Secondly, using various PCR primer combinations and different PCR annealing temperatures also had no effect on the band-intensity variation. Moreover, addition of DMSO and Q-solution, to exclude PCR problems of possible hairpin secondary-structure formation due to the relatively high GC content of ITS2, had no influence on the band-intensity difference between the two intensity groups. I therefore concluded that one ITS allele in the *S. dunnii* plant used as a pollen donor either had significantly fewer rDNA copies, or lacked all primer sites for the four primers used and may be an rDNA pseudogene.

5.3.6. Segregation of band intensity variation in hybrids

The hypothesis that the *S. dunnii* parent plant had one PCR-silent allele was tested in another backcross 19990104, [(*S. rexii* x *S. dunnii*) x *S. rexii*] from which we would expect a lower number of plants with a *S. dunnii* band than the 1:3 ratio expected under the two-locus hypothesis (Fig. 5.6). Of 38 plants, 21 individuals had one ITS2 band (*S. rexii*-type) while the other 17 plants had two ITS2 bands (Fig. 5.7). This is not significantly different from the expected 1:1 ratio under the two-locus hypothesis ($p = 0.516$) assuming one PCR-silent *S. dunnii* allele (Table 5.3).

A.

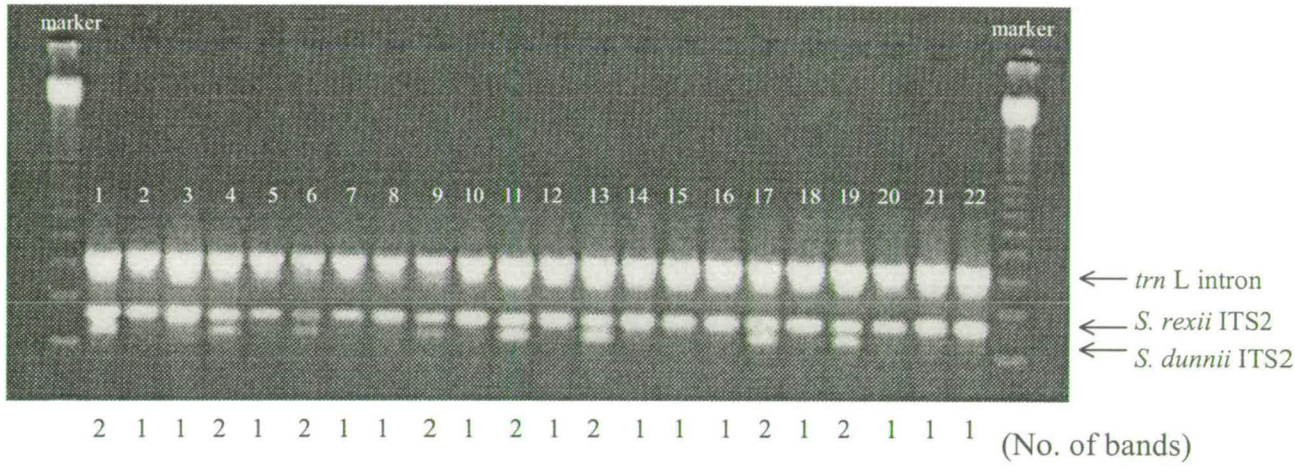
Parent;	RRRR (*S. rexii*)	x	DDDX (*S. dunnii*)
Gamete;	RR		DD, DX
F_1;	RRDD (F_1 strong-intensity group, 2 bands)		
Backcrossing;	RRDD (F_1 0107/13)	x	DDDD (*S. dunnii*)
Gamete;	RR, RD, RD, DD		DD
Backcrosses;	RRDD, RDDD, RDDD, DDDD		
	(Ratio 1 band:2 bands = 1:3)		

B.

Parent;	RRRR (*S. rexii*)	x	DDDX (*S. dunnii*)
Gamete;	RR		DD, DX
F_1;	RRDX (F_1 weak-intensity group, 2 bands)		
Backcrossing;	RRDX (F_1 0104/4)	x	RRRR (*S. rexii*)
Gamete;	RR, RD, RX, DX		RR
Backcrosses;	RRRR, RDRR, RXRR, DXRR		
	(Ratio 1 band:2 bands = 1:1)		

Fig. 5.6. Theoretical Mendelian-crossing diagram of backcrosses assuming two NOR loci and one PCR-silent rDNA allele in *S. dunnii* ("X"). (A) backcross 19990107 [(*S. rexii* x *S. dunnii*) x *S. dunnii*] with two-NOR locus hypothesis. (B) backcross 19990104 [(*S. rexii* x *S. dunnii*) x *S. rexii*] with two-NOR locus hypothesis and one PCR-silent rDNA allele of *S. dunnii* "X".

A.



B.

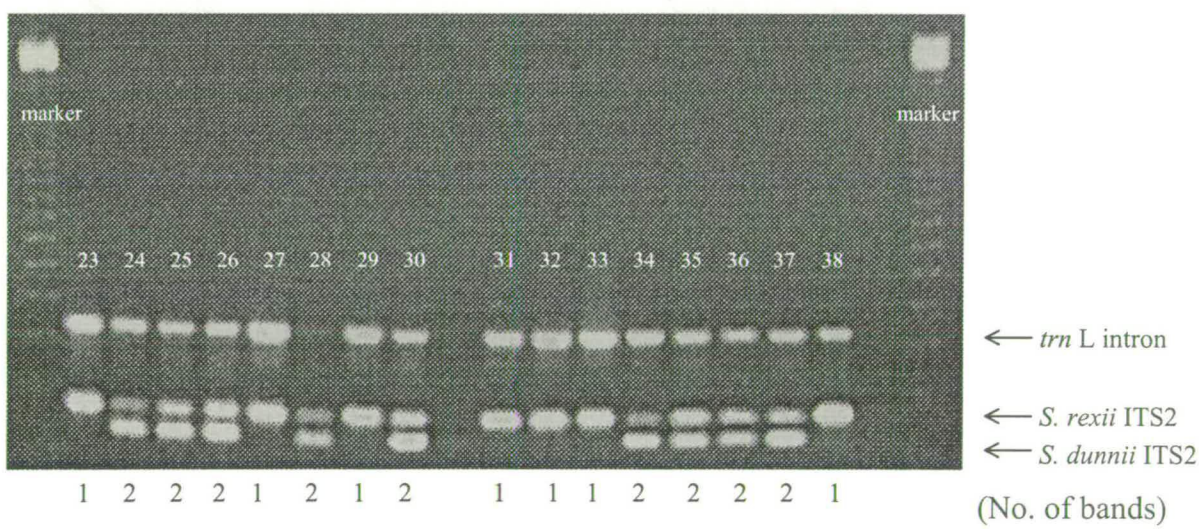


Fig. 5.7. Segregation of the PCR-silent *S. dunnii* rDNA allele. The figure shows amplified *trn*L intron bands (primers c & d) as a standard and ITS2 bands (primers ITS 2G* & 4) of backcross 19990104 (*S. rexii* x *S. dunnii*) x *S. rexii* (left and right: 123 bp ladder, no. 1-22 and 23-38 backcross plants). Top: plants no. 1-22; bottom: plants 23-38.

Table 5.3. Chi-square test comparison between two-NOR locus hypothesis with and without one *S. dunnii* PCR-silent allele of backcross 19990104 (*S. rexii* x *S. dunnii*) x *S. rexii*. R and D indicate *S. rexii* and *S. dunnii* respectively (Degree of freedom = 1, n. s. d. = non significant difference).

Backcross	Total no. of plants	Observed value (No. of plants)		Expected value from two-NOR locus hypothesis without *S. dunnii* PCR-silent allele		Probability value & assumption	Expected value from two-NOR locus hypothesis with one *S. dunnii* PCR-silent allele		Probability value & assumption
		1 band	2 bands	1 band	2 bands		1 band	2 bands	
19990104 (RxD)xR	38	21	17	9.5	28.5	0.000 Hypothesis rejected ($P < 0.05$)	19	19	0.516 n. s. d.

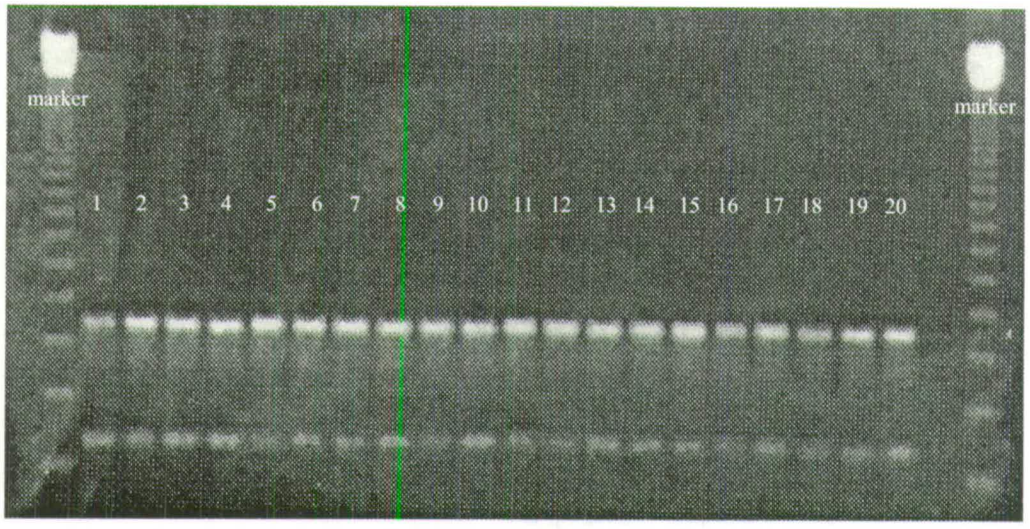
5.3.7. Segregation of band-intensity in *S. dunnii*

If it is the case that the *S. dunnii* plant used in my crossing experiments had one PCR-silent allele at one of the two loci, then this heterozygote should segregate on selfing to give a 1:2:1 ratio of plants with four, three and two ITS2 repeat arrays respectively. This might just be detectable as a binomial distribution of band intensities after PCR, which would give support to the PCR-silent allele hypothesis. In order to standardise the band intensities I used a chloroplast marker (*trn*L intron) as an internal standard. The selfed *S. dunnii* population (19982595), progeny of 19941745A, was assayed. Figure 5.8a shows band-intensity differences between the *S. dunnii* progeny. Although the distribution is continuous, the Chi-square statistic test after the ratios were grouped into three classes (5:12:3 plants, band ratio < 3 : 3 to 4 : >4) does not reject the hypothesis that it is a binomial distribution.

5.3.8 rDNA recombination in *Streptocarpus*

No additional PCR band or smear was observed in ITS2 electrophoresis profiles of any of the F_1 or backcross plants which might indicate a significant degree of recombination, and therefore there is no evidence of recombination. For a further check, the ITS2 PCR product from one randomly chosen plant of backcross accession 19990104 (no. 7; [(*S. rexii* x *S. dunnii*) x *S. rexii*]) which had only the *S. rexii* band, was sequenced. No change in the sequence from the parental consensus *S. rexii* sequence was detected.

A.



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.443	2.784	2.089	1.901	2.750	2.689	3.293	2.870	3.183	2.000	4.237	3.976	2.565	2.917	4.250	3.513	3.069	5.056	3.290	2.241

B.

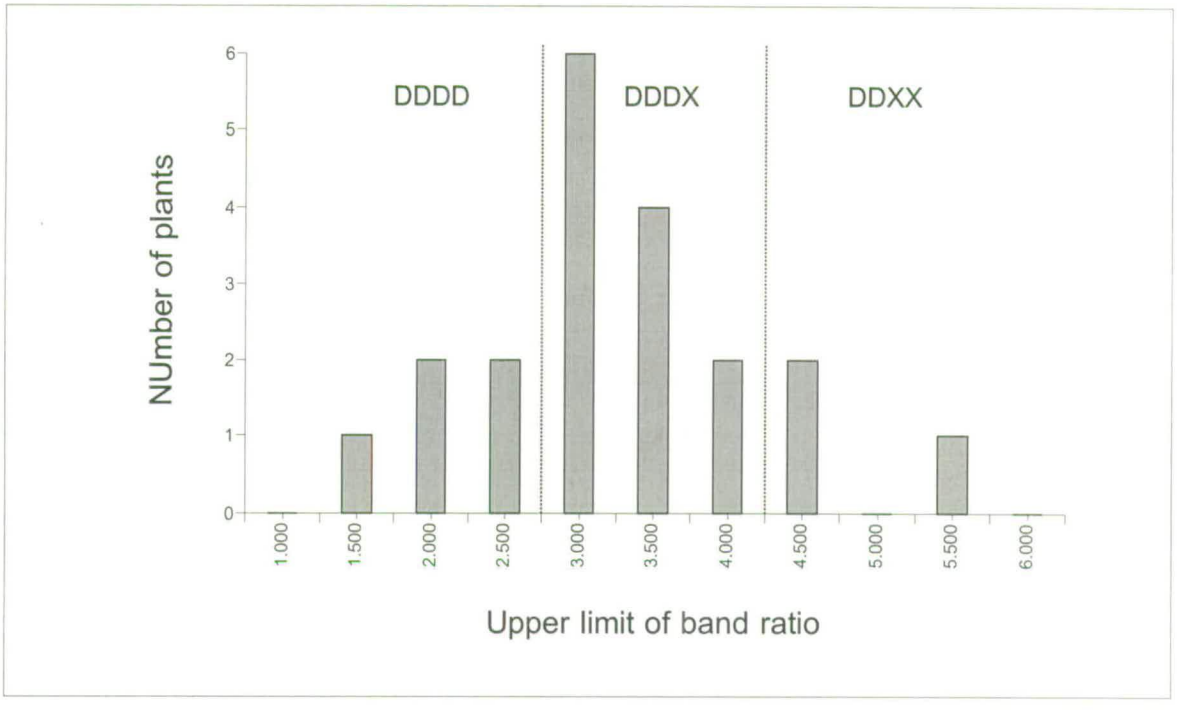


Fig. 5.8. Band-intensity variation in *S. dunnii* selfing offspring. (A) Amplified *trn*L intron (primers c & d) and ITS2 (primers 2G* & 4) bands of *S. dunnii* 19982595 (left to right: 123 bp ladder, no. 1-20 selfing plants, 123 bp ladder). The net intensity ratio between ITS2 and *trn*L of each plant, is shown below the gel. (B) Histogram comparison between selfing offspring of *S. dunnii* 19982595 and net-intensity ratios. Putative genotypes are suggested for the different band intensity ratios (DDDD, DDDX, and DDXX).

5.4. Discussion

5.4.1. Superfluous arm 1 of ITS2 in Gesneriaceae

The secondary structure comparison of the ITS2 sequences of *S. dunnii* and *S. rexii* showed that the single 43 basepair large deletion in *S. dunnii* is the result of a deletion of the terminal part of the first stem-helix (arm 1). This kind of arm shortening is also found in the Madagascan *Streptocarpus ibityensis* (Möller and Cronk, 2001) and in *Aeschynanthus*. Previous examination of numerous Gesneriaceae ITS2 sequences (see Chapter 3) suggests that a very long arm 1, almost twice as long as in related families, is a common feature of the family. I have concluded from my *Aeschynanthus* ITS2 studies that the long end of arm 1 is superfluous in Gesneriaceae and can be deleted without affecting ITS2 function. This is further evidence that conservation of the core of the secondary structure is more important than the stem regions for the endonuclease processing of the spacer, as indicated in ITS2 of yeast (Mitchell, Petfalski, and Tollervey, 1996) and mammal (Michot et al., 1999).

5.4.2. The two loci hypothesis for *Streptocarpus* rDNA

My finding that inheritance of *Streptocarpus* rDNA is additive and without apparent recombination is supported by the Mendelian segregation. This type of rDNA inheritance is similar to that previously reported for barley by Saghai-Maroof et al. (1984) who studied an IGS length difference in F_1 and F_2 hybrids between

cultivated barley (*Hordeum vulgare*) and its wild ancestor (*H. spontaneum*). Nevertheless, it is possible that the lack of detection of ITS2 recombination in my hybrids is a result of the PCR technique applied. Few recombined rDNA copies may be present which can only be detected by sequencing numerous clones.

I found strong evidence for two *Streptocarpus* rDNA loci, and in this respect *Streptocarpus* is similar to *Arabidopsis thaliana* (Copenhaver and Pikaard, 1996), *Pisum sativum* (Jorgensen, Cuellar, and Thompson, 1987), *Triticum dicoccoides* (wild wheat) (Flavell et al., 1986), and *Brassica nigra* (Maluszynska and Heslop-Harrison, 1993). Studies of rDNA locus numbers show that these can vary within a genus, e.g. *Brassica* (from two loci to six loci; Maluszynska and Heslop-Harrison, 1993), *Aloe* (two to three loci; Adams et. al., 2000), and *Nicotiana* (one major and one minor locus to four loci; Lim et. al., 2000). These plant genomes were examined by either cytological investigation (e.g. with the fluorescence in situ hybridisation (FISH) technique by Maluszynska and Heslop-Harrison, 1993), restriction mapping (e.g. Copenhaver and Pikaard, 1996) or DNA sequencing (The *Arabidopsis* Genome Initiative, 2000). My experiment is, however, one of the relatively few studies of plant rDNA locus number using segregation of a PCR genetic marker under controlled conditions. Further work by FISH, for example, is needed to confirm these findings. However this may be challenging in *Streptocarpus* because of the small size and relatively high number of chromosomes (Jong and Möller, 2000).

5.4.3. Selective rDNA PCR by "universal" primers

Evidence from band intensity, and from segregation patterns, suggests that

one of the *S. dunnii* rDNA loci has a "PCR silent" allele. PCR variability of rDNA has been reported before in other *Streptocarpus* species (Möller, 2000). PCR comparisons between *S. rexii* (high ITS PCR amplification) and *S. muscosus*, *S. levis*, and *S. tanala* (all low ITS PCR amplification) clearly reveal a difference of ITS1 band-intensity between both groups, while a control chloroplast gene in each reaction could be amplified equally well for all species. One possible explanation raised in that study was partial or selective amplification from the total number of ITS1 copies in the Madagascan *Streptocarpus*. Partial amplification can occur because of mutation in some primer-binding sites together with incomplete homogenization by concerted evolution. However, in my study none of four different combinations of four primers altered the observed patterns and neither did a lowering of the annealing-temperature or addition of denaturants (e.g. DMSO). It is therefore more likely that there are either significantly fewer rDNA copies or an rDNA pseudogene present in one rDNA allele of *S. dunnii*. Size differences between alleles could be tested elegantly by FISH using rDNA specific probes (e.g. pta 71 probe).

The PCR silent rDNA allele also raises the biological question of how this polymorphism is maintained in the wild population. The frequency of this non-amplifiable (probably non-functional) allele in natural populations is not known but if it were to affect plant survival rate, it would presumably be very low, as it would be selected against. However, under growing conditions used in RBGE, no difference in growth potential was observed between plants with four or two functional rDNA allele, I conclude that one functional rDNA locus is sufficient to sustain *S. dunnii* growth. In this case the PCR-silent allele would be selectively

neutral and its frequency in wild populations could be used for population structure analyses.

When analysing taxa having this PCR selective amplification phenomenon, caution must be exercised when considering the phylogenetic implications. Whenever PCR yield is low, only a few ITS copies from the whole gene pool are sampled, and whether the species is properly represented by the sequences obtained is questionable.

In conclusion, my observation of the PCR selective phenomenon is that it can be explained by the fact that the number of PCR-amplifiable rDNA alleles varies in *S. dunnii* and my experimental material represented an individual with one homozygous and one heterozygous locus.

CHAPTER SIX: Further discussion, conclusions and recommendations for further study

6.1 Aeschynanthus

Although cloning techniques solved the problem of sequence polymorphism found in the ITS region of *Aeschynanthus* nrDNA, and then led to success in reconstructing a primary phylogenetic tree of the genus, the exact reason why this phenomenon occurs is still unsolved. I have suggested that this high intra-individual sequence variation results from low molecular drive in ITS in the genus. Apart from plausible molecular factors like a very high mutation rate in *Aeschynanthus* ribosomal DNA, there are also many possibly physical reasons why this homogenisation mechanism may be retarded, for instance, ploidy level, generation time, reproductive patterns, etc. Among them, the factor which I suggest is the most interesting one to test in *Aeschynanthus* is the number of NORs, whether few or many, and the location of the NORs on the chromosomes, whether they are on homologous or non-homologous chromosomes. This suggested test will require further cytological investigation with an advantageous technique like FISH (fluorescence in situ hybridisation). This useful facility has recently been installed in the Royal Botanic Garden and should provide a better understanding of nuclear ribosomal evolution in *Aeschynanthus* and other taxa in the future.

Comparative secondary structure analyses of the ITS2 sequences of *Aeschynanthus*, other Gesneriaceae species, and some members of other families reveal that the extended terminal part of arm 1 in Gesneriaceae is functionally superfluous and so far unique to the family. Among *Aeschynanthus* species studied, *A. buxifolius, A. garretii*, and *A.argentii* show deletions in their arm 1 extension: small parts of the extensions of *A. buxifolius* and *A. garrettii*, and the whole extension of *A. argentii*. They are in section *Microtrichium* but in different phylogenetic clades. Further sampling of *Microtrichium* species should reveal the relationship between this particular trait and its phylogenetic implication: whether the arm 1 deletion is an autapomorphic character of several species across the whole genus or a synapomorphic character of a new unrecognised clade, expected to be a grouping of *Microtrichium* species in clade I.

Not only more ITS2 sequences sampled from other *Aeschynanthus* species, but also more samples from other plant taxa, both closely related and unrelated to Gesneriaceae, should be investigated. This future examination would answer the questions of how unique this phenomenon is to Gesneriaceae, and how the superfluous extension was added to arm 1 of the ITS2 of Gesneriaceae. Recent studies in *Begonia* (Laura Forrest, RBGE, pers. comm.) reported a similar arm 1 extension in one species in the genus which caused difficulty in sequence matrix alignment. This homoplastic finding indicates that there may be more examples of arm 1 extension in other plant species, though they are quite rare.

The main role so far known of the ITS2 region is to participate in rRNA processing. From sequence characteristic comparison of the ITS2 of *Aeschynanthus* and yeast, I propose that angiosperm ITS2 processing may occur in a similar way to that of

yeast: on the angiosperm ITS2 region there are conserved sites (at the end of arm 3 for a start site, and the central core for a stop site) which exonuclease enzymes will recognise to bind and remove the ITS2 sequence from 5.8S-26S premature RNA. To examine this hypothesis, more sophisticated methods, e.g. mutation experiments, enzymatic probing, and/or chemical probing, have to be adopted to study the plant ITS2 structure and function, as have been done in yeast. Future investigations require very close collaboration with molecular genetic experts.

My comparative analyses of *Aeschynanthus* ITS2 secondary structures illustrate that they can be helpful at the fine scale, when alignment is made difficult by large indels or indel hotspots. A computer alignment program based on RNA secondary structure information should be developed for better DNA alignment. This could be done using similar approach to that in some programs which use the secondary structure data of a peptide chain to align protein sequences. So far, there are several bioinformatic groups trying to write such alignment programs, although many algorithmic difficulties and conflicts have been found (Des Higgins, one of the CLUSTAL program authors, pers. comm.).

The latest phylogenetic analysis of *Aeschynanthus* confirms that the genus is divided into two clades. Clade I primarily occurrs in mainland SE Asia, and has straight or clockwise spiral orientation of the seed testa cells; it contains some species of section *Microtrichium* forming a basal group to sections *X*, *Haplotrichium* sens. str., *Diplotrichium*, and *Polytrichium*. Clade II occurs in Malesia, and has anticlockwise spiral orientation of the seed testa cells; it contains section *Aeschynanthus* nested within section *Microtrichium*. At present, phylogenetic classification fits well with the sectional

classification which has proved to be useful in taxonomic work, using easily observable characters like seed appendages. However, the existence of two major clades in *Aeschynanthus*, defined by testa cell orientation and differing in geographical distribution patterns, suggests that the genus may be divided into two clearly defined natural subgenera.

There are several aspects to be considered for further studies of *Aeschynanthus* phylogeny. First, the suggestion that the *Microtrichium* seed type is the ancestral character state and the origin of the genus is somewhere between Indo-China and the Philippines should be confirmed by adding more samples of *Microtrichium* species, especially those collected from each main island of the Philippines. Secondly, analysis is required of the sixth section *Xanthanthos*. Although this small section has only two known members, it will be worthwhile to investigate its position on the phylogenetic tree. If, as might be expected from seed morphology, it falls with members of section *X*, that would lead to the placement of section *X* species in section *Xanthanthos*, with consequent modification of the definition of that section. Living material of section *Xanthanthos* may become available from a future expedition to China, Vietnam, or Laos. Thirdly, further investigation of *Aeschynanthus* chromosome numbers is necessary to confirm or reject the apparent trend towards dysploid reduction in clade I species and polyploidy in clade II species. Fourthly, because section *Aeschynanthus* appears to be monophyletic, more examination of the relationships within this natural group could be performed by adding more members of the section, particularly from east of Wallace's line. Other molecules such as *trn*L-*trn*F and *mat*K genes should be sequenced to obtain more resolved branches within section *Aeschynanthus*.

Moreover, dating the divergence of the genus is important and needs to be done in the future. The time of the separation of the two major *Aeschynanthus* clades will indicate a possible time that an ancient vicariance event between Indo-China and the Philippines could have taken place. Basically, if we simply assume that substitution rates of ITS among plants are more or less close to each other, we could use such rates to formalise estimates of divergence times among species. Table 6.1 shows some ITS substitution rate estimates for a range of plant groups, 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. Using those cited rates, estimates of the diversification time are between 28.7 and 12.3 million years ago with a mean estimate of 19.5 mya. This is similar to 20 mya that De Boer and Duffells (1996) postulated from their cicada study.

To test whether there is a real molecular clock in the data set, a preliminary test on rate heterogeneity has recently been performed. The test was done on a small *Aeschynanthus* ITS matrix with 15 ingroups and two outgroups. ML analyses were performed. The hypothesis of rate constancy was evaluated using a likelihood ratio (LP) test (Felsenstein, 1981) that is twice the difference in log likelihood between a rate-constrained tree (forcing the molecular clock) and a tree that has no constraints on branch lengths. These log likelihoods were significantly different (clock not enforced vs clock enforced = -2629.33 vs -2645.17; P-value = 0.007; df. = 15), so the molecular clock hypothesis unfortunately has to be rejected. However, a nonparametic rate smoothing (NPRS) method (Sanderson, 1997) can be applied to produce constrained trees from unconstrained ML branch lengths having heterogeneous rates. These NPRS

trees will give dates for the diversification of *Aeschynanthus* when the data set incorporates sequences from other plants whose time of emergence on Earth is already known.

Table 6.1. Data from a range of other taxa, used to estimate the timing of divergence of *Aeschynanthus* based on average number of substitutions.

Rate source	Gene used to calibrate	Calibrated rate (s/s/y)	Average time of *Aeschynanthus* divergence (mya)
Phylica (Richardson et al., 2001)	ITS	2.44×10^{-9}	28.7
Silverswords (Baldwin & Sanderson, 1998)	ITS	3.00×10^{-9}	23.4
Lupinus (Käss & Wink, 1997)	ITS1/ITS2	$3.6 \times 10^{-9}/3.3 \times 10^{-9}$	19.4/21.2
Astragalus (Wojchiechowski et al., 1999)	ITS	3.5×10^{-9}	20.0
Dendroseris (Sang et al., 1994)	ITS	3.94×10^{-9}	17.8
Gossypium (Wendel et al., 1995)	ITS	5.3×10^{-9}	13.2
Aichryson (Mes et al., 1996)	ITS2	5.69×10^{-9}	12.3

Last but not least, studies of the importance of the coma of hair-like appendages of *Aeschynanthus* in wind dispersal would be valuable. In nature, there are many factors affecting the efficiency of seed dispersal, e.g. velocity and direction of the wind, and moisture in the air. However, sedimentation rates in the air seem to be the most

important factor, resulting from the ratio between surface area and mass of each object. The wide distribution of section *Polytrichium* may be due to the greater effectiveness of its coma of appendages. From preliminary experiments on seeds of three *Aeschynanthus* species (*A. irigaensis* (*Microtrichium*), *A. bracteatus* (*Haplotrichium* sens. str.), and *A. longicaulis* (*Polytrichium*)), the average weight of the *Polytrichium* seed could be 10 times greater than those of *Microtrichium* and *Haplotrichium* sens. str. However, the seed surface area of *A. longicaulis* seed is approximately 50 times greater than that of *A. irigaensis* and *A. bracteatus*, mainly because of number of appendages (c. 30-32 hilar-end appendages). This makes the surface area/mass ratio of *Polytrichium* seed to be five and ten times more than those of *Microtrichium* and *Haplotrichium* sens. str. seeds respectively. This preliminary result suggests that the coma of appendages on *Polytrichium* seeds is an evolutionary advantage, agreeing with the derived position of the section in *Aeschynanthus* phylogeny. The details of this preliminary study are shown in Table 6.2. More carefully-designed experiments need to be done to confirm this hypothesis.

6.2 Streptocarpus

Studies of ribosomal DNA inheritance in *Streptocarpus* by using a length difference between ITS2 of *S. dunnii* and *S. rexii* indicate that the inheritance of the gene is additive without any detected recombination and suggest that there are two rDNA loci in *Streptocarpus*. Fluorescence in situ hybridisation (FISH) with ITS or an rDNA probe

Table 6.2 Seed characteristics of three *Aeschynanthus* species representing three different sections within the genus. The surface area of seeds was calculated from a simplified ellipsoid surface area formula of Kamffer et al. (1989) and the surface area of seed appendages was calculated from a formula for a cylinder surface area, discounting the areas at the ends of the cylinder.

Species	Section	Average seed weight	Average seed length	Average seed radius
A. irigaensis	*Microtrichium*	0.9×10^{-5} g	0.8 mm	9.375×10^{-2} mm
A. bracteatus	*Haplotrichium* sens. str.	0.2×10^{-4} g	0.95 mm	1.145×10^{-1} mm
A. longicaulis	*Polytrichium*	0.132×10^{-3} g	1.85 mm	1.859×10^{-1} mm

Species	Average hilar-end appendage length	Average apical-end appendage length	Average hilar-end appendage width	Average apical-end appendage width	Total surface area	Surface area/weight ratio
A. irigaensis	2 mm	2.4 mm	7.8×10^{-2} mm	7.8×10^{-2} mm	1.916 mm^2	2128.89 mm^2/g
A. bracteatus	2.7 mm	2.45 mm	6.78×10^{-2} mm	6.78×10^{-2} mm	2.118 mm^2	1059.00 mm^2/g
A. longicaulis	15 mm	19 mm	3.75×10^{-2} mm	8.33×10^{-2} mm	131.245 mm^2	9942.80 mm^2/g

would be the best technique to confirm the proposed number of *Streptocarpus* NOR loci because of its advantageous visual image. Further work on F_2 hybrids would also give more support to the non-recombination rDNA inheritance finding, although there is difficulty in obtaining fertile F_2 seeds. Last but not least, the result from a band-intensity difference in the *S. dunnii* ITS2 PCR products, which supports the hypothesis of an rDNA pseudogene in *S. dunnii,* could be improved by the introduction of an advanced and costly technique like the quantitative PCR method, to give a more accurate measurement of PCR product quantity. Another technique to test the pseudogene hypothesis would be to set up a Southern blot hybridisation experiment. At different hybridisation temperatures, an ITS probe should detect a pseudogene by having less annealing ability to that pseudogene than to real ITS sequences, then showing different patterns of hybridised blots on a nitrocellular membrane.

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APPENDIX A: MOLECULAR TECHNIQUES FOR PLANT PHYLOGENETICS: DNA SEQUENCING

1. Plant genomic DNA extraction

1.1. CTAB method

Genomic DNA from all plants was extracted using the small scale DNA extraction based on that of Doyle & Doyle (1987). This method uses CTAB (Cetytrimethyl ammonium bromide) which is a cationic detergent that aids in the lysis of cell membranes and will form complexes with nucleic acids. The CTAB method gives the lowest level of enzyme inhibition compared with other methods (Scott & Bendich, 1994). The extraction protocol is shown below.

Healthy (i.e. no insect or fungal damage) and mature fresh leaves from each plant were taken. Cross contamination between samples must be avoided at all times. Leaf samples were harvested using a lid of a 1.5 ml Eppendorf tube. Two discs of leaf material were put into the microcentrifuge tube which then was frozen in liquid nitrogen for two to three minutes. While still frozen, the leaf discs were ground with a plastic pestle until the material was reduced to fine powder. (For long-term storage, other discs were put into a new tube with approximately one teaspoonful (around 1.2 grams) of self-indicating silica gel.) The Eppendorf tube was then kept in a freezer at -20 °C prior to extraction. 200 µl of pre-warmed 2x CTAB extraction buffer [2% w/v CTAB, 100 mM Tris HCl pH8, 20 mM EDTA, 1% PVP

(Polyvinyl pyrolidone), 1.4 M NaCl, and 0.2% v/v beta-mercaptoethanol (added prior to use)] was added to the leaf tissue which was macerated further with the pestle to make a homogeneous slurry. Another 800 µl CTAB buffer was then added and the contents were gently mixed. The homogenate should be green without large tissue fragments at this stage. The tube was incubated at 65 °C for 30 minutes in a heating block; thereafter, the tube was removed and allowed to cool to room temperature before adding 200 µl wet chloroform [chloroform:isoamylalcohol = 24:1]. The solution was mixed gently four or five times to obtain a single phase and centrifuged for two minutes at 13000 rpm. The aqueous (upper) phase was removed to a new tube and re-extracted with 200 µl wet chloroform. After mixing, the samples were centrifuged again. The aqueous layer was again transferred to a new tube with 600 µl cold isopropanol (normally kept at -20 °C). This was mixed gently and left for 10-15 minutes at room temperature. The chloroform waste (lower phase) from chloroform extractions was disposed in an appropriate bottle. After incubation, the pellet of nucleic acids was collected by centrifuging for two minutes at 13000 rpm. The supernatant was removed and 1 ml of wash buffer [76% ethanol, 10 mM ammonium acetate] was added. The tube was vigorously agitated to release the pellet from the bottom and left for at least 30 minutes to remove the CTAB buffer. After the tube was centrifuged, the supernatant was then removed as completely as possible and the pellet was dried completely in a spinning-drying oven for three minutes at the medium heat level. Finally, the pellet was dissolved in 50 µl of distilled water and stored in -20 °C freezer until required.

1.2. DNeasy plant mini kit

The QIAgen DNeasy Plant mini kit (QIAgen Ltd., Dorking, Surrey, UK) was also used in some experiments to give rapid and high quality DNA extraction. The kit uses a spin column to isolate very pure DNA free from inhibitory contaminants. A leaf powder ground under liquid nitrogen was added with 400 µl of AP1 buffer and 4 µl of 100 mg/ml RNase A stock solution. The tube was vortexed and then incubated at 65 °C for 10 minutes, also mixed several times by inverting the tube during incubation. 130 µl of buffer AP2 was added to the tube, mixed, and incubated for 5 minutes on ice. A lysate was applied to a QIAshredder spin-column set and centrifuged for 2 minutes. A flow-through fraction was transferred to a new tube and 1.5 volumes of AP3 added. 650 µl of the mixture was applied to the DNeasy mini spin-column set, centrifuged for one minute, and flow-through discarded. The remaining sample was added to the spin column and centrifuged for another minute. The column was placed in a new tube and 500 µl of buffer AW added. It was then centrifuged for another minute, another 500 µl of AW buffer added, and centrifuged for 2 minutes. The spin column was transferred to a new tube and 50 µl of 65 °C preheated buffer AE was pipetted onto the DNAeasy membrane. The column was incubated for 5 minutes at room temperature and then centrifuged for one minute. Another 50 µl of preheated buffer was added to elute the DNA. The centrifuged DNA was then stored in -20 °C freezer.

1.3. Agarose gel electrophoresis

Agarose gel electrophoresis was used to estimate the concentration of the DNA samples obtained from the DNA extraction. The horizontal type electrophoresis chamber set was routinely used. A 1.0 - 1.5 % agarose gel in 1x TBE buffer was generally used for DNA analysis. One µl of ethidium bromide solution was added to 30 ml of agarose gel solution before pouring into a gel-setting tray. Before loading DNA into each well of solidified agarose gel, an appropriate amount of DNA solution was mixed with a loading dye solution [50% glycerol, 0.1% bromophenol blue, 0.015 xylenecyanol, 0.25 M disodium-EDTA] (Sigma Chemicals, Poole, Dorset, UK). A 123 bp DNA marker (Sigma Chemicals, Poole, Dorset, UK) was used as a molecular weight marker to determine the size of DNA fragment. Electrophoresis was carried out at a constant voltage at room temperature, mostly at 80 Volt for one hour. The DNA fragments were visualised under an ultraviolet (UV) transilluminator. The concentration of the first band of the 123 bp ladder is approximately equal to 30ng/µl.

2. PCR Amplification

The polymerase chain reaction (PCR) technique described by Mullis and Faloona (1987) was used to make an in vitro amplification of an expected DNA segment. The principle of this method is separated into three steps as follows. The first process is termed "Denaturation" in which the reaction is heated to 94-96 °C in order to separate the two strands of the double stranded DNA template. Secondly, the

reaction is cooled to 40-60 °C, which lets two oligonucleotide primers anneal to the single stranded DNA. This step is called "Annealing". The reaction is then heated to about 72 °C which is the optimal temperature for the synthesis of new DNA by the thermostable DNA polymerase. This third process is termed "Extension". The cycle of denaturation, annealing and extension is repeated for the required number of cycles and DNA continues to accumulate exponentially.

2.1. Polymerase Chain Reaction (PCR)

Before use, the flow cabinet must be wiped down with 70% ethanol to prevent any cross-contamination. Double-stranded DNA of the ITS region was amplified using two primers (primers ITS 5P (forwards) and ITS 8P (reverse) in the case of complete ITS1-5.8s-ITS2 region; primers ITS 3P (forwards) and ITS 4 (reverse) for the ITS 2 region only; see Table A for the primer sequences). Each PCR reaction contained 5 µl of 10x Taq polymerase buffer, 1 µl of a 10mM dNTP mix (Sigma Chemicals, Poole, Dorset, UK), 5 µl each of 10 µM primer 1 and primer 2, about 2 µl of genomic DNA template, 1U of Taq polymerase, and topped up with sterile distilled water to reach a total of 50 µl. When a number of reactions was performed, a master mix comprising all the reagents except the target DNA was prepared and aliquoted to the reaction tubes. DNA was thereafter added as the last component before thermal cycling.

PCR amplification of the ITS region was carried out in 0.2 ml microcentrifuge tubes in a Perkin Elmer thermal cycler (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA). Each PCR reaction cycle proceeded at

follows: initial denaturation at 94 °C for 3 minutes; 30 Cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1.5 min.; and final extension at 72 °C for 5 min. The cycles were run until completion, which took around 3 hours. PCR amplification of *trn*L intron with primers 'c' and 'd' (Taberlet et al., 1991) was performed under the same condition as that described for the ITS. The PCR products were detected by gel electrophoresis with 1.5% agarose gel with 1x TBE buffer at 60 - 80 Volts for about 2 hours.

Table A. A list of the primers used in this study with their sequences (from Denduangboripant and Cronk, submitted; Möller and Cronk, 1997a; Taberlet et al., 1991). The location of each primer on rDNA gene and *trn*L is available in Fig. 1.10 (Chapter 1) and Fig. 5.1 (Chapter 5) respectively.

Primer name	Direction	Sequence (5′ to 3′)
ITS 5P	forwards	GGAAGGAGAAGTCGTAACAAGG
ITS 8P	reverse	CACGCTTCTCCAGACTACA
ITS 1P	forwards	TCCGTAGGTGAACCTGCGG
ITS 2G	reverse	GTGACGCCCAGGCAGACGT
ITS 2G*	forwards	ACGTCTGCCTGGGCGTCAC
ITS 3P	forwards	GCATCGATGAAGAACGTAGC
ITS 4	reverse	TCCTCCGCTTATTGATATGC
*trn*L -c	forwards	CGAAATCGGTAGACGCTACG
*trn*L -d	reverse	GGGGATAGAGGGACTTGAAC

2.2. Purification of the PCR products

Before sequencing the PCR products, any potential contaminants in the PCR mixture must be removed by purification. The purification was performed by using a QIAquick PCR purification kit (QIAgen Ltd., Dorking, Surrey, UK) as specified by the manufacturer. This kit is designed to separate either single- or double- stranded DNA ranging from 100 bp to 10 Kb from any contaminants, i.e. remaining primers, Taq DNA polymerase, unincorporated nucleotides and contaminated compounds introduced with the template DNA. Five volumes of buffer PB were mixed with one volume of the PCP product. The mixture was then applied to a QIAquick spin column placed in a 2-ml collection tube, and centrifuged for 1 minute at 13000 rpm. The flow-through was discarded. A 0.75 ml of buffer PE was added to the column which then was centrifuged for 1 minute at 13000 rpm. The flow-through was discarded again and the column was centrifuged for an additional 1 minute at 13000 rpm. The column was transferred to a new Eppendorf tube and 50 µl of buffer EB or sterile distilled water was added to the centre of the column, which was left standing for 1 minute before centrifuging for 1 min at 13000 rpm.

3. Automated cycle sequencing

The purified PCR products were sequenced with the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977) by using either Amplitaq-FS dye terminator cycle-sequencing kit (Perkin Elmer, Applied Biosystems Division, Warrington, UK) or Sequenase II (Amersham Pharmacia Biotech UK Limited, Bucks, England). This

kit allows a linear polymerase chain reaction to proceed using only one primer per reaction, not two as in normal PCR. A 20 μl sequencing reaction was performed in a 0.2 ml tube containing the following components: 9 μl of distilled water, 6 μl of sequencing mix (dNTPs, ddNTPs incorporated with fluorescence dyes, Taq polymerase enzyme), 1 μl of primer at 3.2 pM, and 4 μl of PCR products. The sequencing primers were identical to those used for normal PCR reactions. Either primer 5P, 1P, or 2G was used for ITS1 sequencing, whereas primer 3P, 4, or 8P was used for the ITS2. All primers were synthesized by and purchased from Oswel DNA Service, Southampton, UK. The PCR sequencing condition is: 25 cycles of denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds, and extension at 60 °C for 4 minutes.

Ethanol precipitation was performed to discard any unincorporated dye terminators after the thermal cycle had run. The entire 20 μl sequencing reaction was transferred into a 0.6 ml PCR tube containing 74 μl of ethanol-$MgCl_2$ solution (70% ethanol, 0.5 mM magnesium chloride). The resulting solution was then mixed and placed at room temperature for 15 minutes. After precipitation by centrifuging at 13000 rpm for 15 minutes, the supernatant was carefully removed as completely as possible. Finally, the pellet was dried in a vacuum dryer at medium temperature for 3 minutes. The sequencing samples were run on an ABI 377 Prism Automatic DNA Sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA). For each taxon, forward and reverse sequencing reactions were performed for sequence confirmation.

4. PCR cloning technique

4.1. Gel extraction

To solve the difficulty of reading the polymorphic sequences of *Aeschynanthus* ITS regions, a PCR cloning method has been introduced. PCR-amplified DNA of the whole ITS region was purified with the QIAquick gel extraction (QIAgen Ltd, Dorking, Surrey, UK) before cloning first to reduce the amount of primer-dimer artefacts. The PCR product was loaded into a combined-comb well of a 1.5% agarose gel and run for around 2 hours when the ITS band should be at the middle of the gel. The DNA fragment was then excised from the gel and weighed in a tube. Three volumes of buffer QG were added to one volume of the gel. The content was incubated at 50 °C for 10 minutes, vortexed several times during the incubation to help dissolve the gel completely. (There is no need to add isopropanol in this protocol) The sample was loaded into a QIAquick spin column which was then centrifuged for 1 minute at 13000 rpm. The flow-through was discarded and 0.5 ml of buffer QG was added to the column and centrifuged for 1 minute at 13000 rpm. 0.75 ml of buffer PE was added to the column and centrifuged again for 1 minute at 13000 rpm. The flow-through was discarded and the column was centrifuged for an additional 1 minute at 13000 rpm. The column was transferred to a new Eppendorf tube and 50 μl of buffer EB or sterile distilled water was added to the centre of the column, which was left standing for 1 minute before centrifuging for 1 min at 13000 rpm. The eluted DNA was checked by agarose gel electrophoresis to confirm the presence and amount of the product.

gently mixed by tapping the tube. The cells were incubated on ice for 20 minutes before heated-shock for 90 seconds at 42 °C. The tube was immediately placed on ice. 100 µl of IPTG solution, 40 µl of X-gal solution (20mg/ml X-gal dissolved in dimethylformamide), and 50 µl of the transformation reaction was spread on LB medium plates supplemented with 100 µg/ml ampicillin antibiotic. The transformed cells were incubated at 37 °C overnight. White *E. coli* colonies were then selected for extraction by a plasmid minipreparation.

Another PCR cloning kit that was used is the Topo TA Cloning kit (Invitrogen Co., Carlsbad, CA, USA). This reaction kit gives greater cloning efficiency in a rapid time by using the ligation activity of topoisomerase enzyme instead of ligase as in any other cloning kits. The cloning technique was performed as specified in the supplier's instructions. A 6 µl cloning mixture (2 µl of fresh PCR product, 1 µl of supplied salt solution, 2 µl of sterile water, and 1 µl of TOPO vector) was prepared and incubated for 5 minutes at room temperature. The cloning reaction was placed on ice ready for transforming to One Shot competent *E. coli* cells, supplied with the kit. A 2 µl of the reaction was added into a vial of the cells and mixed gently. The cells were incubated on ice for 30 minutes before heat-shock for 30 seconds at 42 °C. The vial was immediately transferred to ice and 250 µl of room temperature SOC medium was added. The tube was incubated, with shaking, at 37 °C for 1 hour. About 50 µl of the transformation reaction was then spread on a prewarmed selective plate and incubated overnight at 37 °C. Each reaction should produce hundreds of colonies for analysis. Note that although the blue-white selection system is not required for this cloning kit, I recommend selection to reduce

the number of false positive colonies. An addition of 3' A-overhangs post-amplification is also necessary if the PCR products are blunt-ended fragments.

4.3. Plasmid minipreparation

The plasmid miniprep method was used to determine whether the transformed plasmids from selected white colonies contained correctly subcloned PCR products. The minipreparation reaction kit mostly used was the QIAprep miniprep (QIAgen Ltd, Dorking, Surrey, UK) which is based on a standard alkaline lysis reaction of Sambrook et al. (1989) for preparing microgram quantities of purified plasmid DNA from 1-3 ml miniprep bacterial cultures. The technical details of this plasmid minipreparation are as described in the instructions provided by the manufacturer. A transformed bacterial colony was picked by a sterile toothpick and grown overnight in a 5 ml LB broth bottle supplied with 100 μg/ml ampicillin. The bacterial cells were pelleted in a 1.5 ml Eppendorf tube with 30 seconds centrifugation at 8000 rpm twice. The supernatant was removed without disturbing the bacterial pellet. The pelleted cells were resuspended in 250 μl of buffer P1 (already added with Rnase A) and transferred to an Eppendorf tube. 250 μl of buffer P2 was added and the tube was gently inverted 4-6 times to mix. 350 μl of buffer P3 was added and the tube was immediately but gently inverted 4-6 times. The tube was then centrifuged for 10 minutes. The supernatant was applied to a QIAprep spin column placed in a 2-ml collection tube and centrifuged for 1 minute at 13000 rpm. The spin column was washed by adding 0.75 ml of buffer PE and centrifuged for 1 minute. The flow-through was discarded and the column was centrifuged for an

additional 1 minute to remove residual wash buffer. The spin column was placed in a new Eppendorf tube and 50 µl of buffer EB or distilled water was added to the centre of the column. The column was then left standing for 1 minute and centrifuged for 1 minute at 13000 rpm to elute the DNA. A concentration of the isolated plasmid was estimated by an agarose gel electrophoresis.

4.4. Restriction enzyme digestion

After isolation, the subcloned plasmids were digested with *Xba*I (for the Prime PCR Cloner kit) or *Eco*RI (for the Topo TA Cloning kit) restriction enzymes to determine the size of the subcloned PCR fragment in the clones, compared with the original PCR product. The plasmid digestion was performed by using the general buffer, digestion conditions, and incubation time recommended by the manufacturer [10 µl of distilled water, 1 µl of 10x restriction enzyme buffer, 1 µl of 1 µg/µl DNA; with 0.5 µl of 10 U/µl restriction enzyme; incubated at 37 °C for an hour]. The size of the inserted PCR fragment was checked by an agarose gel electrophoresis. The clones that had an inserted fragment of the correct size were sequenced in almost the same way as the PCR products, using the same sequencing primers and the cycle sequencing method, but with large amount of the plasmid DNA to give better sequence results.

5. Sequence analysis

DNA sequences of each taxon were examined by comparison of their forward

and reverse sequences to find the position of the ITS region. The program Factura version 1.2Or6 (Perkin Elmer, Applied Biosystem Inc., Foster city, CA, USA) was used to handle the raw sequences. IUPAC codes were used for ambiguous nucleotides. Sequence boundaries of the ITS region were determined by comparison with published sequence data of *Streptocarpus* and *Saintpaulia* species (Möller and Cronk, 1997a). All ITS sequences were deposited in GenBank. The sequences were then initially aligned using the alignment program Sequence Navigator version 1.0.1 (Perkin Elmer, USA) with the CLUSTAL option. The alignment was subsequently adjusted by eye. The number and size of indel events were examined. Sequence characteristics such as the number of transitions and transversions, and their respective ratio were obtained using MacClade Version 3.0.1 (Maddison and Maddison, 1992). Sequence divergences among taxa and the G+C content were calculated using Base Frequencies and Show Pairwise Distance options in PAUP* (Swofford, 1998).

6. Phylogenetic analysis

Aligned ITS sequence matrices were analysed by the phylogenetic reconstruction program PAUP* version 4.0b (Swofford, 1998), with character states unordered and initially equally weighted. Polymorphic characters were treated as uncertain. Gaps were treated as missing values. Ambiguous regions in the aligned matrix were excluded from phylogenetic analysis. Indels were scored as a separate presence/absence character (0 was coded for an insertion and 1 for a deletion) and added to the sequence data matrix. Since the ITS analyses contained a large number

of taxa (more than 20), the use of exact algorithms (exhaustive and branch-and-bound methods) became limited by the excessive amount of computing time needed. Therefore, heuristic approaches which are considered to be approximate and relatively fast methods were employed. Heuristic searches were used to find the most parsimonious trees by using RANDOM sequence addition with TBR (Tree Bisection-Reconnection) branch swapping for at least 1000 replicates. The random sequence-addition option was used in order to detect any multiple islands of most parsimonious trees. MULPARS (save all equally parsimonious trees), COLLAPSE (collapse any zero-length branches) and STEEPEST DESCENT (not abandon a round of swapping until all input trees from the previous round have been examined by the swapping algorithm) options were selected. ACCTRAN (accelerated transformation) was chosen for character-state optimisation, i.e. to prefer reversals over parallelisms.

When more than one most parsimonious tree was generated, a strict consensus phylogenetic tree was computed. Separate parsimony analyses without gap matrices (indels scored as characters) were also performed in parallel. If needed, successive weighting searches were also performed, aimed to reduce the effect of homoplasious characters in the data. Reweighting parsimony analysis was carried out by weighting characters according to mean values of their rescaled consistency (RC; Swofford, 1993) indices. Successive reweighting was carried out four times, at which point the resulting tree length remained unchanged and no further topological changes occurred.

Bootstrap analysis (full heuristic) (Felsenstein, 1985) and jackknife analysis (Lanyon, 1985) with 50% deletion ("fast" stepwise-addition) were used as

indications of statistic-supports for internal branches. Bootstrap and jackknife values were calculated using PAUP* set to heuristic search option with TBR swapping, 100 replicates and 10000 replicates respectively. Another branch supporting value, Decay Indices (DI, Bremer support values; Bremer, 1988; Donoghue et al., 1992) were calculated using the program AutoDecay version 4.0 (Eriksson, 1998). Decay index provides an indication of the robustness of the data by determining which clades persist in a consensus tree as parsimony is relaxed. Descriptive statistics reflecting the amount of phylogenetic signal in the parsimony analyses were given by branch length, the consistency index (CI; CI =m/s, where m is the minimum possible length of a tree based on the number of variant characters, s is the actual tree length; Kluge and Farris, 1969), retention index (RI; RI = M-s/M-m, where M is the maximum possible tree length; Farris, 1989), and the rescaled consistency index (RC; RC = CI x RI; Farris, 1989). The higher the values of these indices, the more congruent the characters in the data set are with each other and with the tree.

Maximum Likelihood (ML) analysis was also performed using the program PAUP*. To find the optimum model for the likelihood analysis, the program Modeltest version 3.0 (Posada and Crandall, 1998) was first used to compare the likelihood score results and associated P-values between 56 ML DNA-evolution models. The program then selected the model that best fits the data by nested likelihood ratio tests (Huelsenbeck and Rannala, 1997) and the Akaike information criterion (minimum theoretical information criterion, AIC; Akaike, 1974). The selected model, appropriate substitution values, base frequency parameters and Gamma distribution shape parameter determined by Modeltest were then used for the

ML analysis with TBR branch swapping. The result of maximum likelihood analysis was compared to that of the parsimony analysis.

APPENDIX B

Sequence data matrix of aligned ITS1 and ITS2 regions of nuclear ribosomal DNA of 23 taxa of *Aeschynanthus* species (two clones per species) and two outgroup Gesneriaceae taxa. Nucleotide sequence displayed from 5' to 3'. ITS1 ranges from site 1 to 277 and ITS2 ranges from site 278 to 582. Hyphens denote alignment gaps; * = nucleotide sites excluded from part of the phylogenetics analyses; number in italic above nucleotide matrix, ranging from 1 to 73, indicate the number position of alignment gaps; number in square brackets at the end of sequences indicate the actual length of the combined region of ITS1 plus ITS2.

```
                          10        20        30        40        50        60        70        80
Taxon          ITS1       .         .         .         ********  .         .         .         .
                          .         .         .         .         .         .         .         .
                          .    1    .         .    2    .         .         .    3    .         .    4 5
Cyr.baileyi               TCGAAACCTGCAAAG-CAGACCCGTGAACTTGT-TAAA-AATA---TGCTTGCGGCCGCGATGT--CGGATGCATTT---
Lys.forrestii             TCGAAACCCGCAAAG-CAGACCCGTGAACATGTTTAAA--ATA-CACGCTTGCGTCCGCGATGT--TGGACGCCTCT---
A.tricolor A              TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGGGTCGTGAGGT--TGGATGCATTT---
A.tricolor B              TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGGGTCGTGAGGT--TGGATGCATTT---
A.arctocalyx A            TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGGGTCGTGAGGT--TGGATGCATTT---
A.arctocalyx B            TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGGGTCGTGAGGT--TGGATGCATTT---
A.chrysanthus A           TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGCGTCGTGAGGT--TGGATGCATTT---
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A.parvifolius A           TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGGGTCATGAGGT--TGGATGCATTT---
A.parvifolius B           TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGGGTCATGAGGT--TGGATGCATCT---
A.radicans A              TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGGGTCGTGAGGT--TGGATGCATTT---
A.radicans B              TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATCGGGGGTCGTGAGGT--TGGATGCATTT---
A.argentii A              TCGAAACCTGCAAAG-CAGACTGGTGAACATGTGTAAA---TA---CATTGGGGGTCGTGATGT--TGGATGCATTT---
A.argentii B              TCGAAACCTGCAAAG-CAGACTGGTGAACATGTGTAAA---TA---CATTGGGGGTCGTGATGT--TGGATGCATTT---
A.magnificus A            TCGAAACCTGCAAAA-CAGACTTGTGAACATGTGTAAA---TA---CATTGGGGGTCGTGATGTGTTGGATGCATTT---
A.magnificus B            TCGAAACCTGCAAAA-CAGACTTGTGAACATGTGTAAA---TA---CATTGGGGGTCGTGATGTGTTGGATGCATTT---
A.guttatus A              TCGAAACCTGCAAAG-CAGACCTGTGAACATGTGTATT---TA---CGTTGGGGGTCGTGATGT--CGGATGTGTTT---
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A.pachyanthus A           TCGAAACCTGCAAAG-CAGACCCGTGAACATGTGTAAA---TA---CGTTGGGGGTCGTGACGT--CGGATGCGTTT---
A.pachyanthus B           TCGAAACCTGCAAAG-CAGACCCGTGAACATGTGTAAA---TA---CGTTGGGGGTCGTGACGT--CGGATGCGTTT---
A.longicaulis A           TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTATT---TA---CGTTGGCGGCCGCGATGT--TGGATGCATTT---
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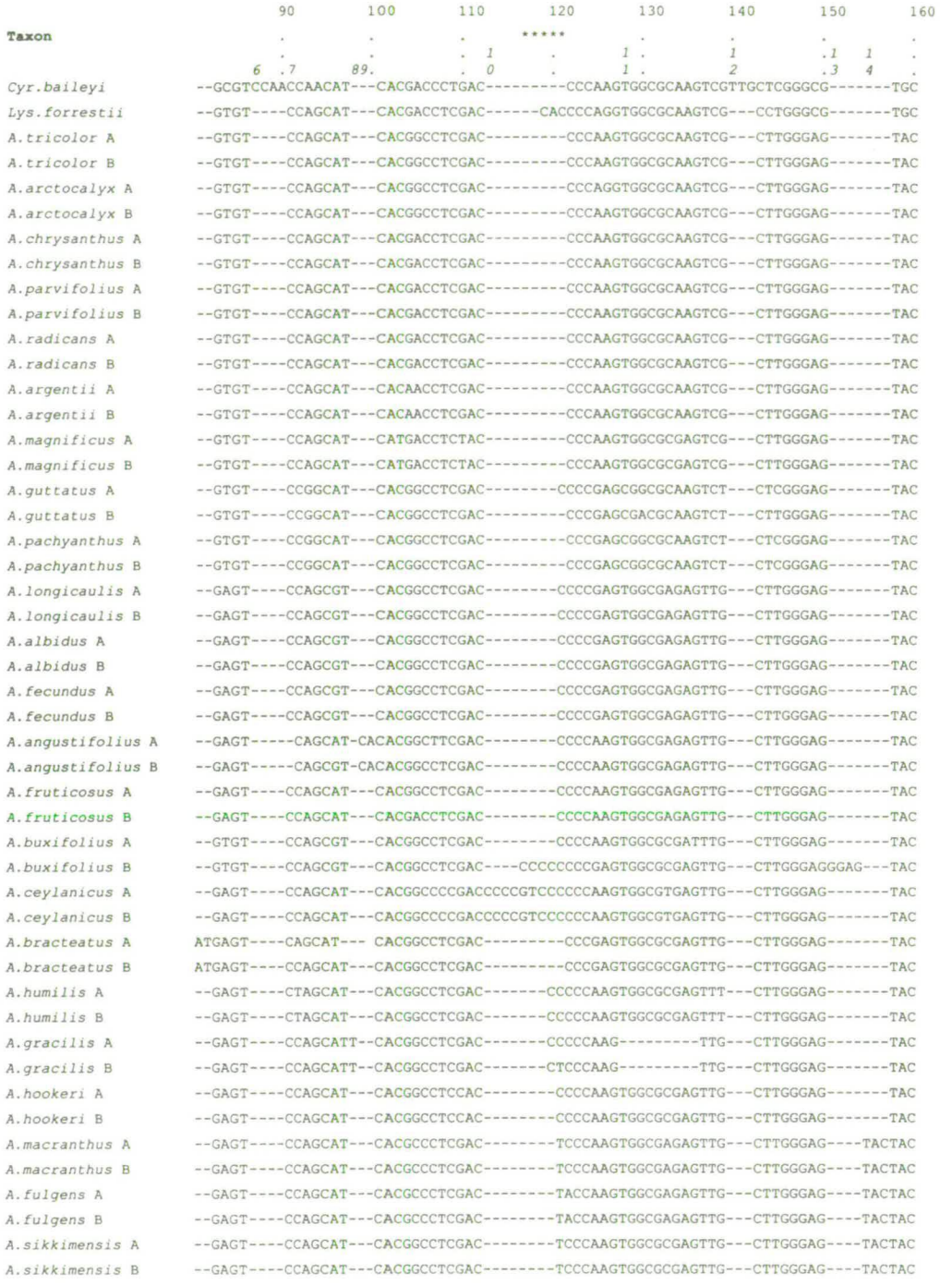
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A.radicans B
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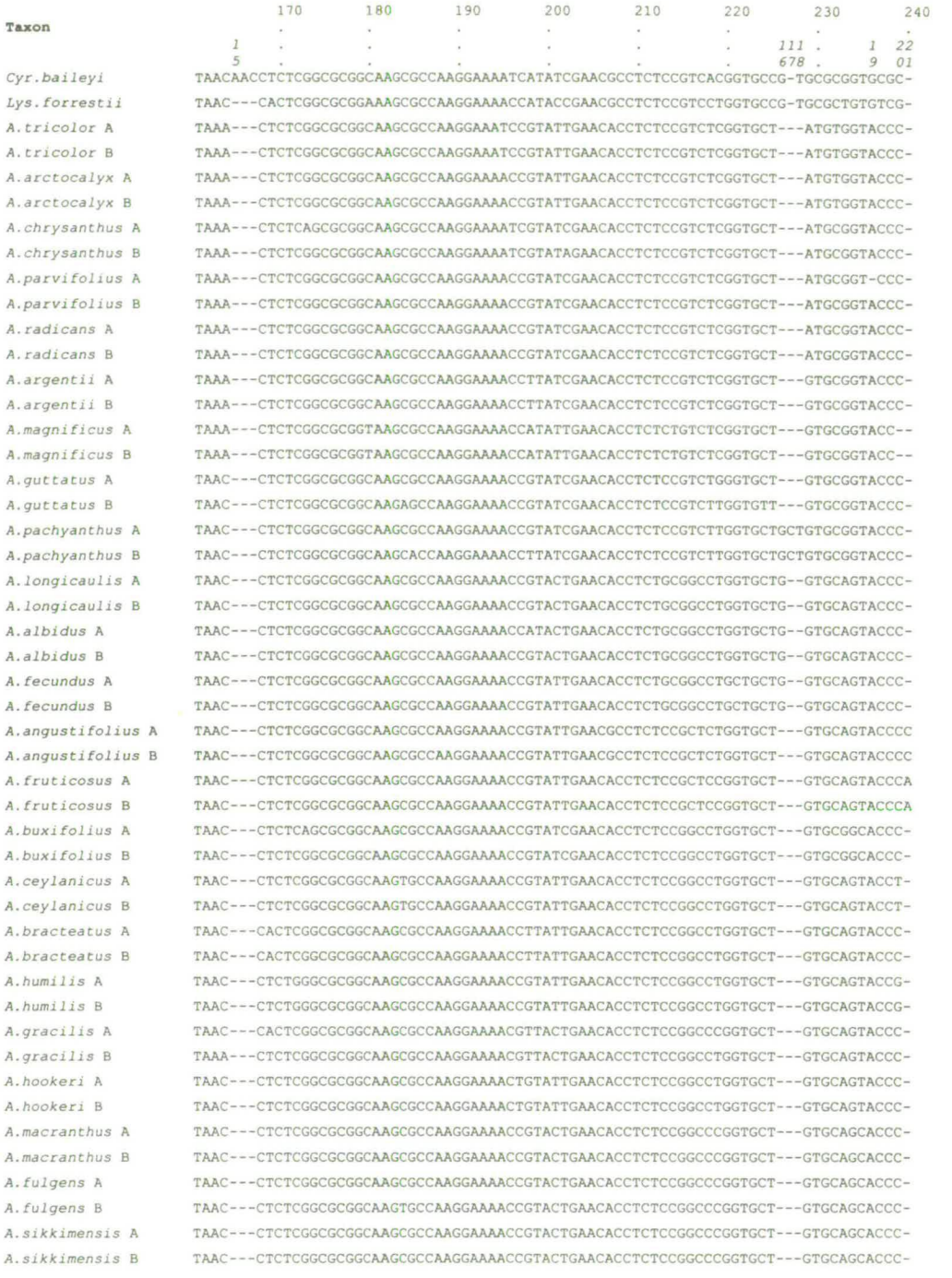
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Lys.forrestii TAAC---CACTCGGCGCGGAAAGCGCCAAGGAAAACCATACCGAACGCCTCTCCGTCCTGGTGCCG-TGCGCTGTGTCG-
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A.chrysanthus B TAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAATCGTATAGAACACCTCTCCGTCTCGGTGCT---ATGCGGTACCC-
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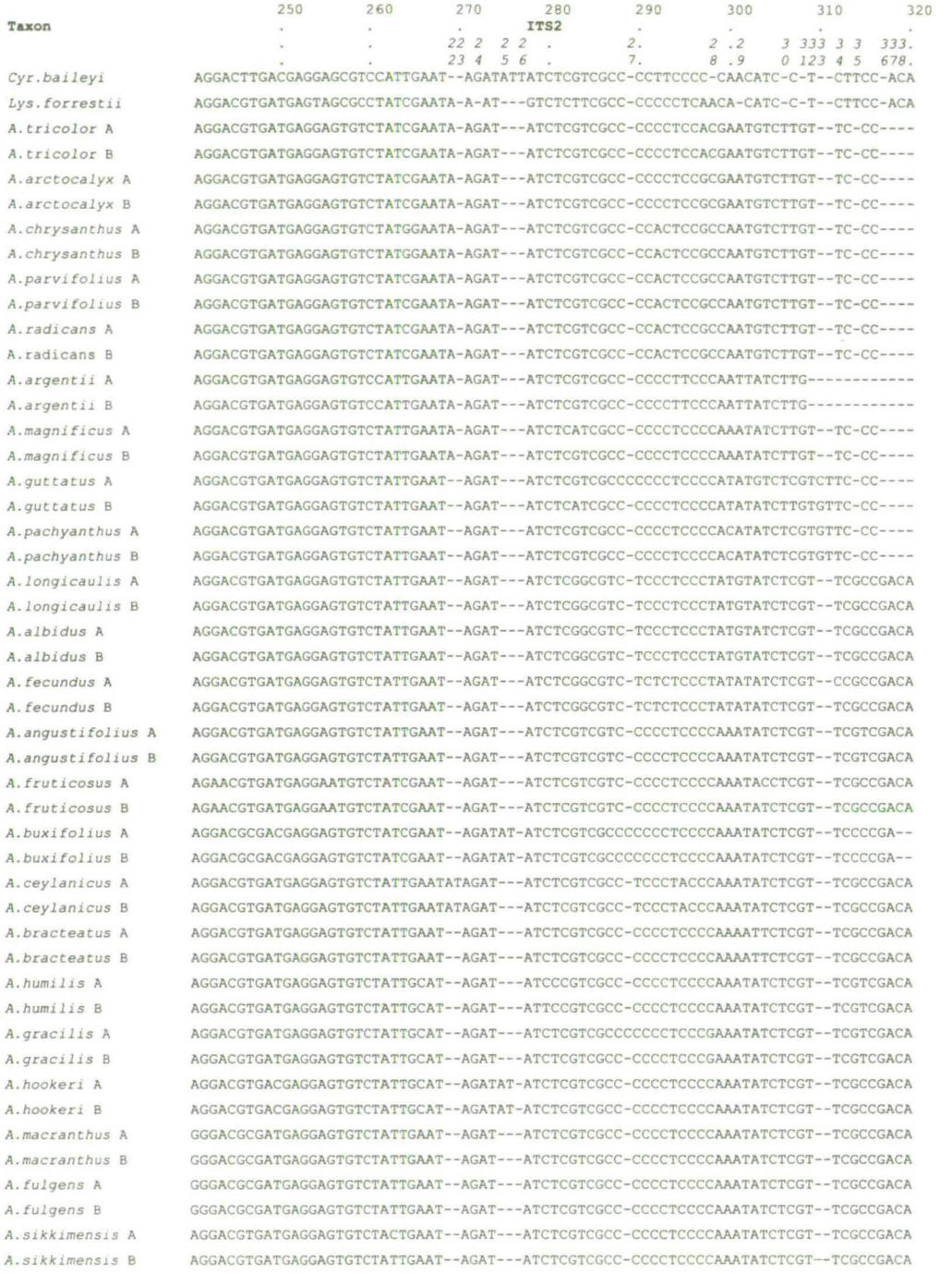
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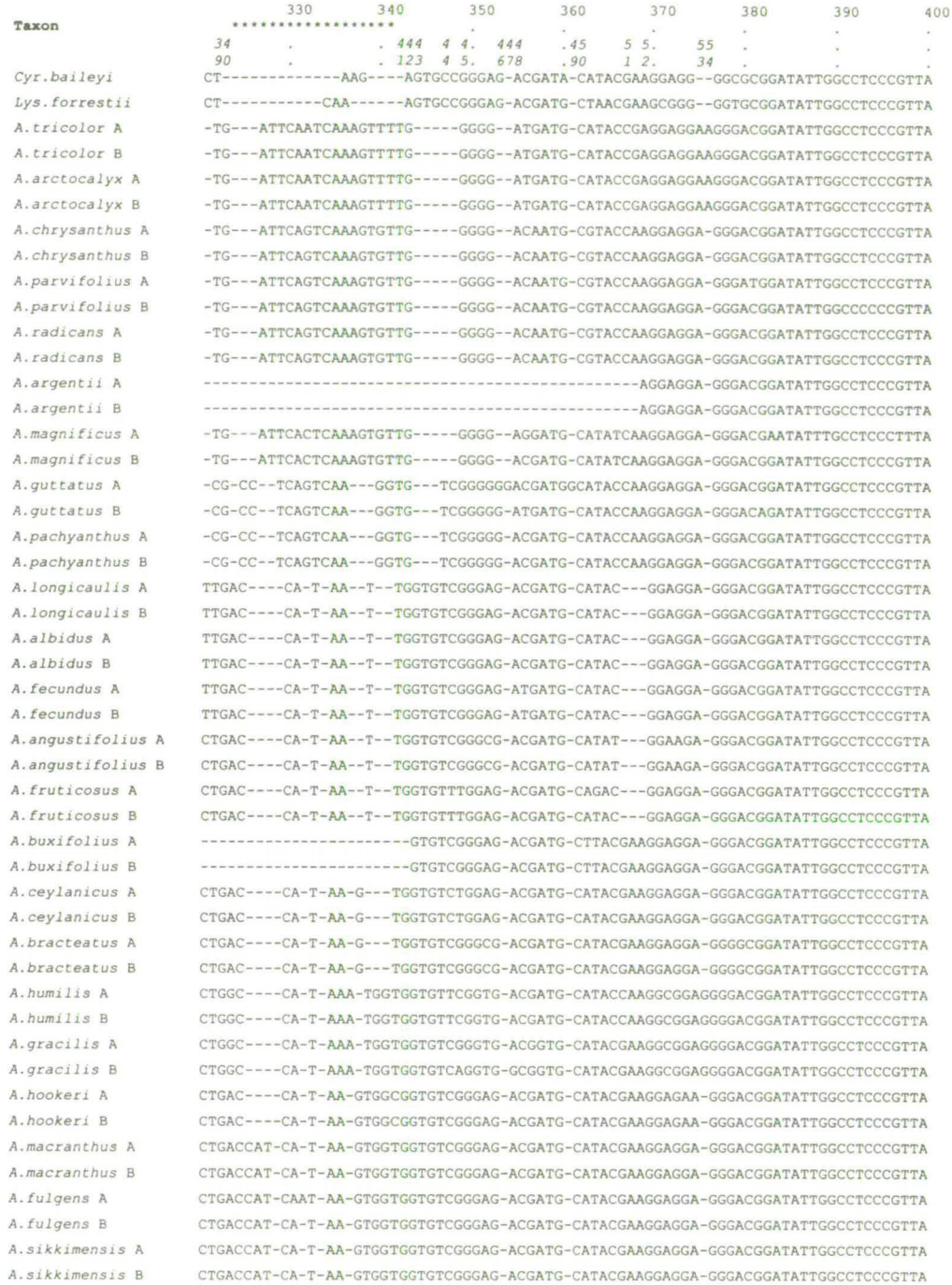
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A.arctocalyx B
A.chrysanthus A
A.chrysanthus B
A.parvifolius A
A.parvifolius B
A.radicans A
A.radicans B
A.argentii A
A.argentii B
A.magnificus A
A.magnificus B
A.guttatus A
A.guttatus B
A.pachyanthus A
A.pachyanthus B
A.longicaulis A
A.longicaulis B
A.albidus A
A.albidus B
A.fecundus A
A.fecundus B
A.angustifolius A
A.angustifolius B
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A.fruticosus B
A.buxifolius A
A.buxifolius B
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A.ceylanicus B
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A.humilis A
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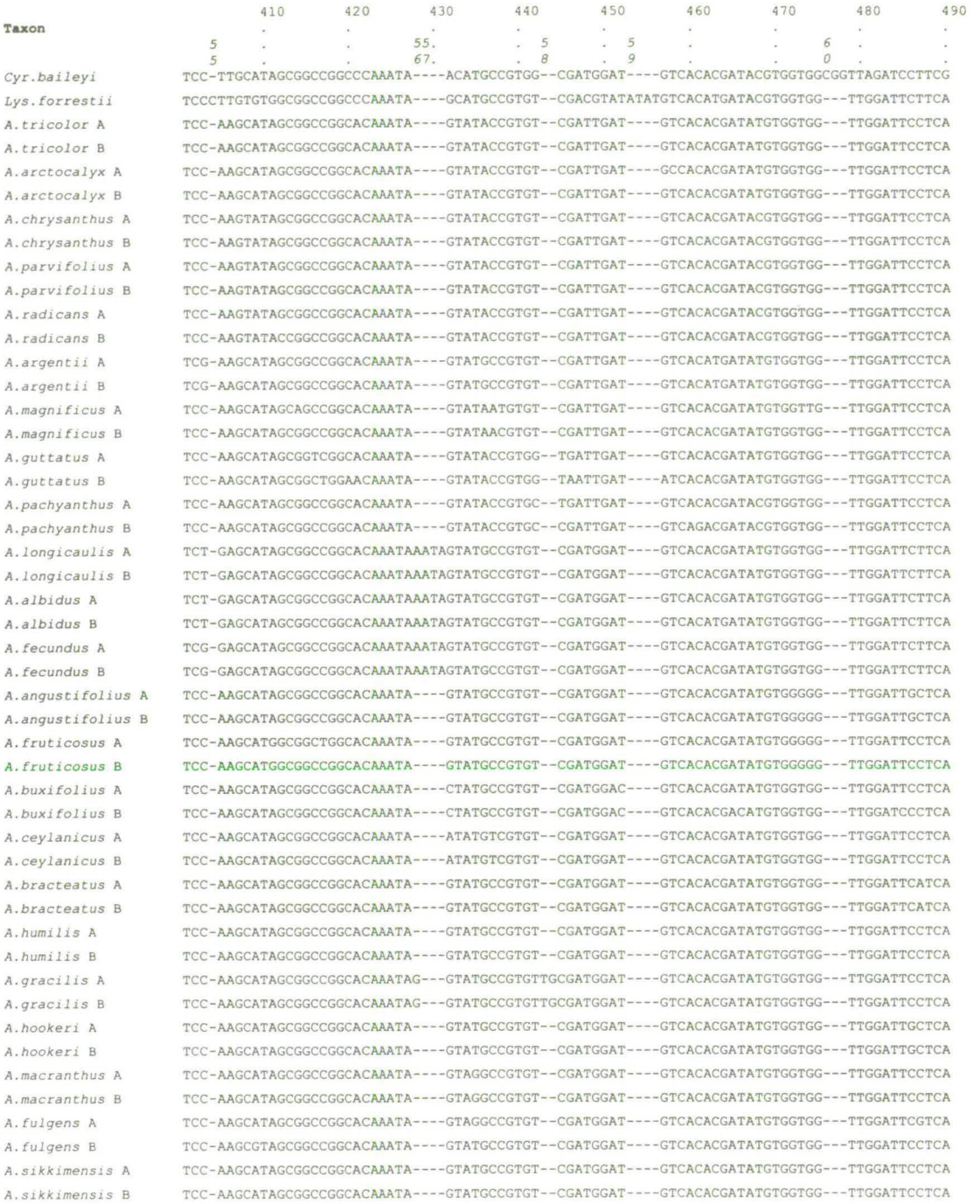
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Lys.forrestii TCCCTTGTGTGGCGGCCGGCCCAAATA----GCATGCCGTGT--CGACGTATATATGTCACATGATACGTGGTGG---TTGGATTCTTCA
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                         1  2    34.         .  56     .         . 7       .89    0  .     1  2. 3       .
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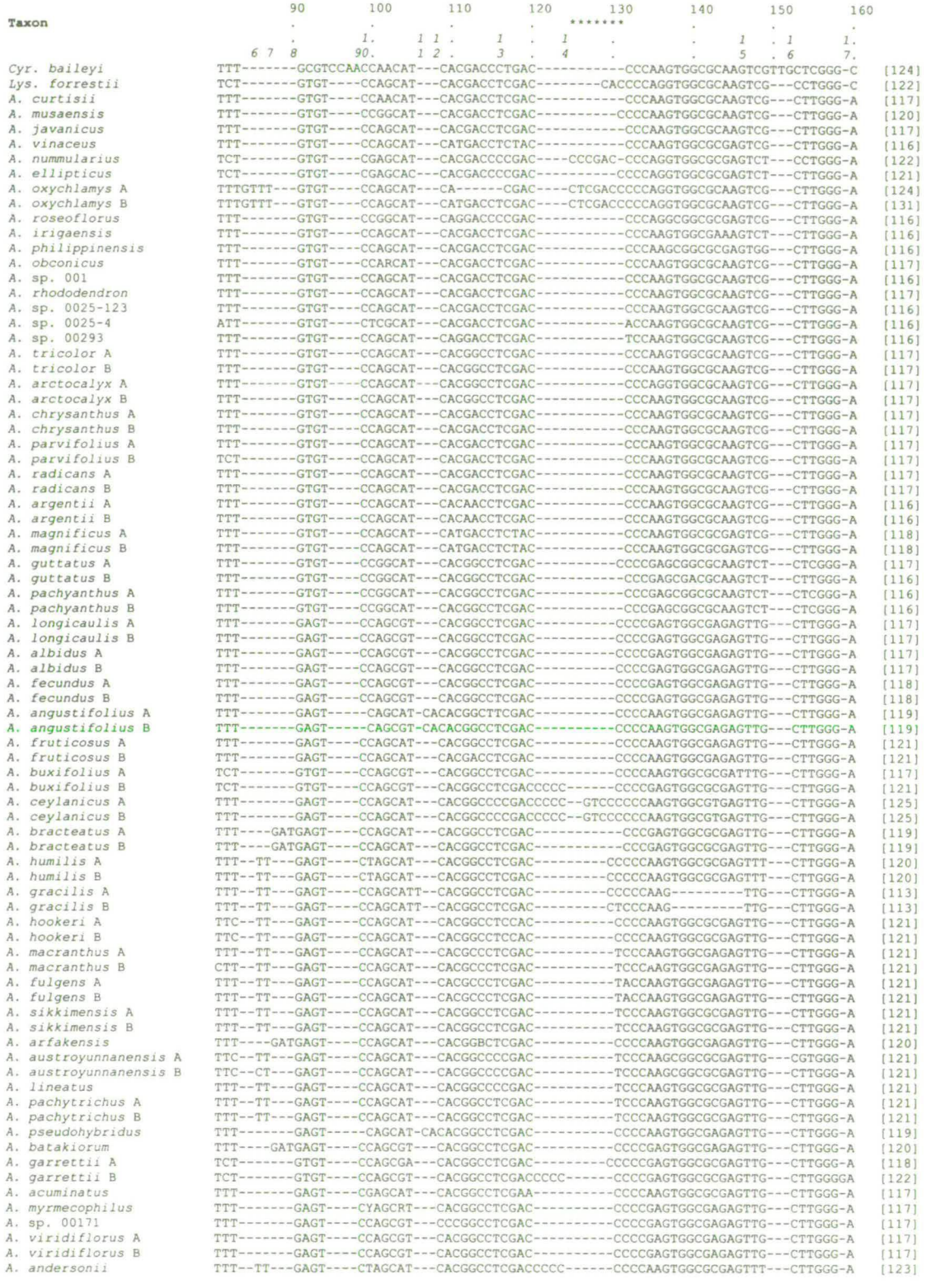
A.gracilis A             ACTTGCGTA-CT------TATCGTGCGGTACTC-CATCGACCCACGTGCTAGGACCCAGC----------AAGAT-CG------CCCTCGA [479]
A.gracilis B             ACTTGCGTA-CT------TATCGTGCGGTACTC-CATCGACCCACGTGCTG-GACCCAGC----------AAGTT-CG------CCCTCGA [477]
A.hookeri A              ACTTGCGAA-CT------TATCGTGCGGGACTC-CACCGAGCCACGTGCCC-GACCCAAT-GGCAC----AAGAT-TG------CCCTCGA [488]
A.hookeri B              ACTTGCGAA-CT------TATCGTGCGGGACTC-CACCGAGCCACGTGCCC-GACCCAAT-GGCAC----AAGAT-TG------CCCTCGA [488]
A.macranthus A           ACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCTT-GACCCAAT-GGCAC----AAGAT-TG------CCCTCGA [492]
A.macranthus B           ACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCTT-GACCCAAT-GGCAC----AAGAT-TG------CCCTCGA [492]
A.fulgens A              ACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCTT-GACCCACT-GGCAC----AAGAT-TG------CCCTCGA [493]
A.fulgens B              ACTTACGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGAGCCC-GACCCAAT-GGCAC----AAGAT-TG------CCCTCGA [492]
A.sikkimensis A          ACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCCC-GACCCAAT-GGCAC----AAGAT-TG------CCCTCGA [492]
A.sikkimensis B          ACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCCC-GACCCAAT-GGCAC----AAGAT-TG------CCCTCGA [492]
```

APPENDIX C

Sequence data matrix of aligned ITS1 and ITS2 regions of nuclear ribosomal DNA of 50 taxa of *Aeschynanthus* species and two outgroup Gesneriaceae taxa. Nucleotide sequence displayed from 5' to 3'. ITS1 and ITS2 range from site 1 to 289 and 290 to 603 respectively. Hyphens denote alignment gaps; * = nucleotide sites excluded from the analyses; italic numbers above matrix indicate the number and positions of alignment gaps; uncertain nucleotide states are coded as K = G/T, R = A/G, S = C/G, W = A/T, Y = C/T, H = A/C/T, B = C/G/T, V = A/C/G, D = A/G/T, and ? = unknown.

```
                                10        20        30        40        50        60        70        80
Taxon                     ITS1  .         .         .         ********  .         .         .         .
                                .  1      .         .  2      .         .  3      .         .  4      .  5
Cyr. baileyi              TCGAAACCTGCAAAG-CAGACCCGTGAACTTGT-TAAA-AATA---TGCT---TGCGGCCGCGATGT--CGGATGC---A  [66]
Lys. forrestii            TCGAAACCCGCAAAG-CAGACCCGTGAACATGTTTAAA--ATA-CACGCT---TGCGTCCGCGATGT--TGGACGC---C  [68]
A. curtisii               TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCGYGAGGT--TGGATGC---A  [66]
A. musaensis              TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TA---CGTT---GGGGGTCGTGATGT--TGGATGCATTA  [68]
A. javanicus              TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATT---GGGGGTCGTGAGGT--TGGATGC---A  [66]
A. vinaceus               TCGAAACCTGCAAAG-CAGACCTGTGAACATGTGTAAA---TA---CATT---GGGGGTCGTGATGT--TGGATGC---A  [65]
A. nummularius            TCGAAACCTGCAAAG-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGGGGTCGTGACGT--TGGACGC---A  [65]
A. ellipticus             TCGAAACCTGCAAAG-CAGACCCGCGAACATGTGTAAATACATA--CGTT---CGGGGTCGTGACGT--TGGATGC---A  [69]
A. oxychlamys A           TCGAAACCTGCAAAG-CAGACCTGTGAACATGTGTAAA---TA---CGTTGTTGGGGGTCGTGACGT--CGGATGC---A  [68]
A. oxychlamys B           TCGAAACCTGCAAAG-CAGACCTGTGAACATGTGTAAA--ATA---CGTTGTTGGGGGTCGTGACGT--CGGATGC---A  [69]
A. roseoflorus            TCGAAACCTGCAAGG-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGGGGTCGTGACGT--CGGATGC---A  [65]
A. irigaensis             TCGAAACCTGCAAAT-CAGACTCGTGAACATGTGTAAA---TA---CATT---GGGGGTCGTGATGT--TGGATGC---A  [65]
A. philippinensis         TCGAAACCTGCAAAG-CAGACCCGTGAACACGTGTAAA---TA---CTTT---GGGGGTCGTGATGT--TGGATGC---A  [65]
A. obconicus              TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCGTGAGGT--TGGATGC---A  [66]
A. sp. 001                TCGAAACCTGGAAAG-CAGACCTGTGAACATGTGCAAA---CA---CATT---GGGGGTCGTGATGT--CGGATGC---A  [65]
A. rhododendron           TCGAAACCTGCAAAG-CAGACCTGTGAACATGTGTAAA--ATA---TGTT---GGGGGTCATGATGT--TGGATGC---A  [66]
A. sp. 0025-123           TCGAAACCTGCAAAG-CAGACCTGTGAACATGTGCAAA---CA---CATT---GGGGGTCGTGATGT--CGGATGC---A  [65]
A. sp. 0025-4             TCGAAAACTGCAAAG-CAGACTTGTGAACATGTGTAAA---TA---CATT---GGGGGTCGTCATGT--TGGATGC---A  [65]
A. sp. 00293              TCGAAACCTGCAAAT-CAGACCTGTGAACATGTGTAAA---TA---CATT---GGGGGTCGTGATGT--CGGATGC---A  [65]
A. tricolor A             TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCGTGAGGT--TGGATGC---A  [66]
A. tricolor B             TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCGTGAGGT--TGGATGC---A  [66]
A. arctocalyx A           TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCGTGAGGT--TGGATGC---A  [66]
A. arctocalyx B           TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCGTGAGGT--TGGATGC---A  [66]
A. chrysanthus A          TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGCGTCGTGAGGT--TGGATGC---A  [66]
A. chrysanthus B          TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGCGTCGTGAGGT--TGGATGC---A  [66]
A. parvifolius A          TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCATGAGGT--TGGATGC---A  [66]
A. parvifolius B          TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCATGAGGT--TGGATGC---A  [66]
A. radicans A             TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCGTGAGGT--TGGATGC---A  [66]
A. radicans B             TCGAAACCTGCAAAG-CAGACTCGTGAACATGTGTAAA---TAA--CATC---GGGGGTCGTGAGGT--TGGATGC---A  [66]
A. argentii A             TCGAAACCTGCAAAG-CAGACTGGTGAACATGTGTAAA---TA---CATT---GGGGGTCGTGATGT--TGGATGC---A  [65]
A. argentii B             TCGAAACCTGCAAAG-CAGACTGGTGAACATGTGTAAA---TA---CATT---GGGGGTCGTGATGT--TGGATGC---A  [65]
A. magnificus A           TCGAAACCTGCAAAA-CAGACTTGTGAACATGTGTAAA---TA---CATT---GGGGGTCGTGATGTGTTGGATGC---A  [67]
A. magnificus B           TCGAAACCTGCAAAA-CAGACTTGTGAACATGTGTAAA---TA---CATT---GGGGGTCGTGATGTGTTGGATGC---A  [67]
A. guttatus A             TCGAAACCTGCAAAG-CAGACCTGTGAACATGTGTATT---TA---CGTT---GGGGGTCGTGATGT--CGGATGT---G  [65]
A. guttatus B             TCGAAACCTGCAAAG-AAGACCTGTGAACATGTGTAAA---TA---CGTT---GGGGGTCGTGATGT--CGGATGC---A  [65]
A. pachyanthus A          TCGAAACCTGCAAAG-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGGGGTCGTGACGT--CGGATGC---G  [65]
A. pachyanthus B          TCGAAACCTGCAAAG-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGGGGTCGTGACGT--CGGATGC---G  [65]
A. longicaulis A          TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTATT---TA---CGTT---GGCGGCCGCGATGT--TGGATGC---A  [65]
A. longicaulis B          TCGAAACCTGAAAAA-CAGACCTGCGAACATGTGTATT---TA---CGTT---GGCGACCGCGATGT--TGGATGC---A  [65]
A. albidus A              TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTATT---TA---CGTT---GGCGGCCGCGATGT--TGGATGC---A  [65]
A. albidus B              TCGAAACCTGCGAAA-CAGACCTACGAACATGTGTATT---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. fecundus A             TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTACT---T--TACGTT---GGCGGTCGCGGTGT--TGGATGC---A  [66]
A. fecundus B             TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTATT---T--TACGTT---GGCGGTCGCGATGT--TGGATGC---A  [66]
A. angustifolius A        TCGAAACCTGCAAAAACAGACCTGAGAATATGTGTAAT---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [66]
A. angustifolius B        TCGAAACCTGCAAAAACAGACCTGAGAATATGTGTAAT---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [66]
A. fruticosus A           TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTAATTAAT--TACGTT---GGCGGTCGCGATGT--TGGATGC---A  [69]
A. fruticosus B           TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTAATTAAT--TACGTT---GGCGGTCGCGATGT--TGGATGC---A  [69]
A. buxifolius A           TCGAAACCTACAAAG-CAGACCCATGAACATGCGTAAA---TA---CGTT---GGTGGTCACGATGT--TGGATGC---A  [65]
A. buxifolius B           TCGAAACCTGCAAAG-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. ceylanicus A           TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTAAA---TA---TGTC---GGTGGTCGCGATGT--TGGATGC---A  [65]
A. ceylanicus B           TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTAAA---TA---TGTC---GGTGGTCGCGATGT--TGGATGC---A  [65]
A. bracteatus A           TCGAAACCTGCAAAA-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. bracteatus B           TCGAAACCTGCAAAA-CAGACCCGTGAACATGTCTAAA---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. humilis A              TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTAAA---TA---CGTT---GGTGGTCGCGATGT--TGGATGC---A  [65]
A. humilis B              TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTAAA---TA---CGTT---GGTGGTCGCGATGT--TGGATGC---A  [65]
A. gracilis A             TCGAAACCTACAAAA-GAGACCCGCGAACATGTGTAAA--ATA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [66]
A. gracilis B             ACGAAACCTACAAAA-GTGACCCGCGAACATGTGTAAA--ATA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [66]
A. hookeri A              TCGAGACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCAGTCGCGATGT--TGGATGC---A  [67]
A. hookeri B              TCGAGACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCAGTCGCGATGT--TGGATGC---A  [67]
A. macranthus A           TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [67]
A. macranthus B           TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [67]
A. fulgens A              TCGAGACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCGGTCGTGATGT--TGGATGC---A  [67]
A. fulgens B              TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCGGTCGTGATGT--TGGATGC---A  [67]
A. sikkimensis A          TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [67]
A. sikkimensis B          TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [67]
A. arfakensis             TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTAAT---TA---CGTT---GGCGGTCGTGATGT--TGGATGC---A  [65]
A. austroyunnanensis A    TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-GATA---CGTT---GGCGGTCGCGACGT--CGGGCGC---A  [67]
A. austroyunnanensis B    TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-GATA---CGTT---GGCGGTCGCGACGT--CGGACGC---A  [67]
A. lineatus               TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAAA-AATA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [67]
A. pachytrichus A         TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAGA-AATA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [67]
A. pachytrichus B         TCGAAACCTGCAAAA-CAGACCCGCGAACATGTGTAGA-GATG---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [67]
A. pseudohybridus         TCGAAACCTGCAAAAACAGACCTGAGAATATGTGTAAT---TA---CGTT---GGCGGTCGTGATGT--TGGATGC---A  [66]
A. batakiorum             TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTATT---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. garrettii A            TTGAATCCTGCAAAG-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. garrettii B            TCGAAACCTGCAAAG-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. acuminatus             TCGAAACCTGCAAAA-CAGACCCGTGAACATGTGTAAA---TA---CGTT---GGAGGTCGCGATGT--TGGACGC---A  [65]
A. myrmecophilus          TCGAAACCTGCAAAA-CAGACYTGCGAACATGTRTATT---TA---YGTT---GRCGGTCGCGATGT--TGGATGC---A  [65]
A. sp. 00171              TCGAAACCTGCAAAA-CAGACCTGCGAACATGTGTATT---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. viridiflorus A         TCGAAACCTGCGAAA-CAGACCTGCGAACATGTGTATT---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. viridiflorus B         TCGAAACCTGCGAAA-CAGACCTGCGAACATGTGTATT---TA---CGTT---GGCGGTCGCGATGT--TGGATGC---A  [65]
A. andersonii             TCGAAACCTGCAAAA-CAGACCTGTGAACATGTGTAAA---TA---CGTT---GGTGGTCGTGATGT--TGGATGC---A  [65]
```


Taxon
90 100 110 120 130 140 150 160
Cyr. baileyi TTT-------GCGTCCAACCAACAT---CACGACCCTGAC----------CCCAAGTGGCGCAAGTCGTTGCTCGGG-C [124]
Lys. forrestii TCT-------GTGT----CCAGCAT---CACGACCTCGAC---------CACCCCAGGTGGCGCAAGTCG---CCTGGG-C [122]
A. curtisii TTT-------GTGT----CCAACAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. musaensis TTT-------GTGT----CCGGCAT---CACGACCTCGAC----------CCCCAAGTGGCGCAAGTCG---CTTGGG-A [120]
A. javanicus TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. vinaceus TTT-------GTGT----CCAGCAT---CATGACCTCTAC----------CCCAAGTGGCGCGAGTCG---CTTGGG-A [116]
A. nummularius TCT-------GTGT----CGAGCAT---CACGACCCCGAC----CCCGAC-CCCAGGTGGCGCGAGTCT---CCTGGG-A [122]
A. ellipticus TCT-------GTGT----CGAGCAC---CACGACCCCGAC----------CCCCAGGTGGCGCGAGTCT---CTTGGG-A [121]
A. oxychlamys A TTTGTTT---GTGT----CCAGCAT---CA------CGAC----CTCGACCCCCAGGTGGCGCAAGTCG---CTTGGG-A [124]
A. oxychlamys B TTTGTTT---GTGT----CCAGCAT---CATGACCTCGAC----CTCGACCCCCAGGTGGCGCAAGTCG---CTTGGG-A [131]
A. roseoflorus TTT-------GTGT----CCGGCAT---CAGGACCCCGAC----------CCCAGGCGGCGCGAGTCG---CTTGGG-A [116]
A. irigaensis TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGAAAGTCT---CTTGGG-A [116]
A. philippinensis TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGCGGCGCGAGTGG---CTTGGG-A [116]
A. obconicus TTT-------GTGT----CCARCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. sp. 001 TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [116]
A. rhododendron TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. sp. 0025-123 TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [116]
A. sp. 0025-4 ATT-------GTGT----CTCGCAT---CACGACCTCGAC----------ACCAAGTGGCGCAAGTCG---CTTGGG-A [116]
A. sp. 00293 TTT-------GTGT----CCAGCAT---CAGGACCTCGAC----------TCCAAGTGGCGCAAGTCG---CTTGGG-A [116]
A. tricolor A TTT-------GTGT----CCAGCAT---CACGGCCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. tricolor B TTT-------GTGT----CCAGCAT---CACGGCCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. arctocalyx A TTT-------GTGT----CCAGCAT---CACGGCCTCGAC----------CCCAGGTGGCGCAAGTCG---CTTGGG-A [117]
A. arctocalyx B TTT-------GTGT----CCAGCAT---CACGGCCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. chrysanthus A TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. chrysanthus B TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. parvifolius A TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. parvifolius B TCT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. radicans A TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. radicans B TTT-------GTGT----CCAGCAT---CACGACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [117]
A. argentii A TTT-------GTGT----CCAGCAT---CACAACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [116]
A. argentii B TTT-------GTGT----CCAGCAT---CACAACCTCGAC----------CCCAAGTGGCGCAAGTCG---CTTGGG-A [116]
A. magnificus A TTT-------GTGT----CCAGCAT---CATGACCTCTAC----------CCCAAGTGGCGCGAGTCG---CTTGGG-A [118]
A. magnificus B TTT-------GTGT----CCAGCAT---CATGACCTCTAC----------CCCAAGTGGCGCGAGTCG---CTTGGG-A [118]
A. guttatus A TTT-------GTGT----CCGGCAT---CACGGCCTCGAC----------CCCCGAGCGGCGCAAGTCT---CTCGGG-A [117]
A. guttatus B TTT-------GTGT----CCGGCAT---CACGGCCTCGAC----------CCCGAGCGACGCAAGTCT---CTTGGG-A [116]
A. pachyanthus A TTT-------GTGT----CCGGCAT---CACGGCCTCGAC----------CCCGAGCGGCGCAAGTCT---CTCGGG-A [116]
A. pachyanthus B TTT-------GTGT----CCGGCAT---CACGGCCTCGAC----------CCCGAGCGGCGCAAGTCT---CTCGGG-A [116]
A. longicaulis A TTT-------GAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [117]
A. longicaulis B TTT-------GAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [117]
A. albidus A TTT-------GAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [117]
A. albidus B TTT-------GAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [117]
A. fecundus A TTT-------GAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [118]
A. fecundus B TTT-------GAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [118]
A. angustifolius A TTT-------GAGT-----CAGCAT-CACACGGCTTCGAC----------CCCCAAGTGGCGAGAGTTG---CTTGGG-A [119]
A. angustifolius B TTT-------GAGT-----CAGCGT-CACACGGCCTCGAC----------CCCCAAGTGGCGAGAGTTG---CTTGGG-A [119]
A. fruticosus A TTT-------GAGT----CCAGCAT---CACGGCCTCGAC----------CCCCAAGTGGCGAGAGTTG---CTTGGG-A [121]
A. fruticosus B TTT-------GAGT----CCAGCAT---CACGACCTCGAC----------CCCCAAGTGGCGAGAGTTG---CTTGGG-A [121]
A. buxifolius A TCT-------GTGT----CCAGCGT---CACGGCCTCGAC----------CCCCAAGTGGCGCGATTTG---CTTGGG-A [117]
A. buxifolius B TCT-------GTGT----CCAGCGT---CACGGCCTCGACCCCC------CCCCGAGTGGCGCGAGTTG---CTTGGG-A [121]
A. ceylanicus A TTT-------GAGT----CCAGCAT---CACGGCCCCGACCCCC--GTCCCCCCAAGTGGCGTGAGTTG---CTTGGG-A [125]
A. ceylanicus B TTT-------GAGT----CCAGCAT---CACGGCCCCGACCCCC--GTCCCCCCAAGTGGCGTGAGTTG---CTTGGG-A [125]
A. bracteatus A TTT----GATGAGT----CCAGCAT---CACGGCCTCGAC----------CCCGAGTGGCGCGAGTTG---CTTGGG-A [119]
A. bracteatus B TTT----GATGAGT----CCAGCAT---CACGGCCTCGAC----------CCCGAGTGGCGCGAGTTG---CTTGGG-A [119]
A. humilis A TTT--TT---GAGT----CTAGCAT---CACGGCCTCGAC----------CCCCCAAGTGGCGCGAGTTT---CTTGGG-A [120]
A. humilis B TTT--TT---GAGT----CTAGCAT---CACGGCCTCGAC---------CCCCCAAGTGGCGCGAGTTT---CTTGGG-A [120]
A. gracilis A TTT--TT---GAGT----CCAGCATT--CACGGCCTCGAC---------CCCCCAAG---------TTG---CTTGGG-A [113]
A. gracilis B TTT--TT---GAGT----CCAGCATT--CACGGCCTCGAC---------CTCCCAAG---------TTG---CTTGGG-A [113]
A. hookeri A TTC--TT---GAGT----CCAGCAT---CACGGCCTCCAC----------CCCCAAGTGGCGCGAGTTG---CTTGGG-A [121]
A. hookeri B TTC--TT---GAGT----CCAGCAT---CACGGCCTCCAC----------CCCCAAGTGGCGCGAGTTG---CTTGGG-A [121]
A. macranthus A TTT--TT---GAGT----CCAGCAT---CACGCCCTCGAC----------TCCCAAGTGGCGAGAGTTG---CTTGGG-A [121]
A. macranthus B CTT--TT---GAGT----CCAGCAT---CACGCCCTCGAC----------TCCCAAGTGGCGAGAGTTG---CTTGGG-A [121]
A. fulgens A TTT--TT---GAGT----CCAGCAT---CACGCCCTCGAC----------TACCAAGTGGCGAGAGTTG---CTTGGG-A [121]
A. fulgens B TTT--TT---GAGT----CCAGCAT---CACGCCCTCGAC----------TACCAAGTGGCGAGAGTTG---CTTGGG-A [121]
A. sikkimensis A TTT--TT---GAGT----CCAGCAT---CACGGCCTCGAC----------TCCCAAGTGGCGCGAGTTG---CTTGGG-A [121]
A. sikkimensis B TTT--TT---GAGT----CCAGCAT---CACGGCCTCGAC----------TCCCAAGTGGCGCGAGTTG---CTTGGG-A [121]
A. arfakensis TTT----GATGAGT----CCAGCAT---CACGGBCTCGAC----------CCCCAAGTGGCGAGAGTTG---CTTGGG-A [120]
A. austroyunnanensis A TTC--TT---GAGT----CCAGCAT---CACGGCCCCGAC----------TCCCAAGCGGCGCGAGTTG---CGTGGG-A [121]
A. austroyunnanensis B TTC--CT---GAGT----CCAGCAT---CACGGCCCCGAC----------TCCCAAGCGGCGCGAGTTG---CTTGGG-A [121]
A. lineatus TTT--TT---GAGT----CCAGCAT---CACGGCCCCGAC----------TCCCAAGTGGCGCGAGTTG---CTTGGG-A [121]
A. pachytrichus A TTT--TT---GAGT----CCAGCAT---CACGGCCTCGAC----------TCCCAAGTGGCGCGAGTTG---CTTGGG-A [121]
A. pachytrichus B TTT--TT---GAGT----CCAGCAT---CACGGCCTCGAC----------TCCCAAGTGGCGCGAGTTG---CTTGGG-A [121]
A. pseudohybridus TTT-------GAGT-----CAGCAT-CACACGGCCTCGAC----------CCCCAAGTGGCGAGAGTTG---CTTGGG-A [119]
A. batakiorum TTT----GATGAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [120]
A. garrettii A TCT-------GTGT----CCAGCGA---CACGGCCTCGAC----------CCCCCGAGTGGCGCGAGTTG---CTTGGG-A [118]
A. garrettii B TCT-------GTGT----CCAGCGT---CACGGCCTCGACCCCC------CCCCGAGTGGCGCGAGTTG---CTTGGGGA [122]
A. acuminatus TTT-------GAGT----CGAGCAT---CACGGCCTCGAA----------CCCCAAGTGGCGCGAGTTG---CTTGGG-A [117]
A. myrmecophilus TTT-------GAGT----CYAGCRT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [117]
A. sp. 00171 TTT-------GAGT----CCAGCGT---CCCGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [117]
A. viridiflorus A TTT-------GAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [117]
A. viridiflorus B TTT-------GAGT----CCAGCGT---CACGGCCTCGAC----------CCCCGAGTGGCGAGAGTTG---CTTGGG-A [117]
A. andersonii TTT--TT---GAGT----CTAGCAT---CACGGCCTCGACCCCC------CCCCAAGTGGCGCGAGTTT---CTTGGG-A [123]

```
                                170       180       190       200       210       220       230       240
Taxon                           .         .         .         .         .         .         .         .
                           11 2 .       2 .         .         .         .         .         .      222.
                           89 0 .       1 .         .         .         .         .         .      234.
Cyr. baileyi               G-------TGCTAACAACCTCTCGGCGCGGCAAGCGCCAAGGAAAATCATATCGAACGCCTCTCCGTCACGGTGCCG-TG  [196]
Lys. forrestii             G-------TGCTAAC---CACTCGGCGCGGAAAGCGCCAAGGAAAACCATACCGAACGCCTCTCCGTCCTGGTGCCG-TG  [191]
A. curtisii                G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTAYTGAACACCTCTCCGTCTCRGTGCT---A  [184]
A. musaensis               G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCTGTCTTGGTGCT---G  [187]
A. javanicus               G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATBGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. vinaceus                G-------TACTAAA---CTCTCGGCGCGGTAAGCGCCAAGGAAAACCATATTGAACACCTCTCCGTCTCGGTGCT---G  [183]
A. nummularius             G-------TACTAAC---CTCACGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTCGGTGCT---G  [189]
A. ellipticus              G----T--TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTCGGTGCT---G  [189]
A. oxychlamys A            G-------TACTAAC---CACTCGGCGCGGCAAGCGCCAAGGAAAACTGCATCGAACACCTCTCCGTCTTGGTGCT---G  [191]
A. oxychlamys B            G-------TACTAAC---CACTCGGCGCGGCAAGCGCCAAGGAAAACCGCATCGAACACCTCTCCATCTTGGTGCT---G  [198]
A. roseoflorus             G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTTGGTGCT---G  [183]
A. irigaensis              G-------TTCTAAC---CTCTGGGCGCGGCAAGCGCCAAGGAAAACCATATTGAACACCTCTCCGTCTTGGTGCTGTTG  [186]
A. philippinensis          G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCCCGGTGCT---G  [183]
A. obconicus               G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. sp. 001                 G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCATATCGAACACCTCTCCGTCTCGGTGCT---A  [183]
A. rhododendron            G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCATACTGAACATCTCTCCGTCTTGGTGCT---G  [184]
A. sp. 0025-123            G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCATATCGAACACCTCTCCGTCTCGGTGCT---A  [183]
A. sp. 0025-4              G-------TACAAAA---CTCTCGCCGCAGCAAGCGCCAAGGAAAACCATATTGAACACCTCTCCGTCTCAATGAT---A  [183]
A. sp. 00293               G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACMTCTCCGTCCCGGTGCT---G  [183]
A. tricolor A              G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAATCCGTATTGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. tricolor B              G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAATCCGTATTGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. arctocalyx A            G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. arctocalyx B            G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. chrysanthus A           G-------TACTAAA---CTCTCAGCGCGGCAAGCGCCAAGGAAAATCGTATCGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. chrysanthus B           G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAATCGTATAGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. parvifolius A           G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. parvifolius B           G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. radicans A              G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. radicans B              G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTCGGTGCT---A  [184]
A. argentii A              G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCTTATCGAACACCTCTCCGTCTCGGTGCT---G  [183]
A. argentii B              G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCTTATCGAACACCTCTCCGTCTCGGTGCT---G  [183]
A. magnificus A            G-------TACTAAA---CTCTCGGCGCGGTAAGCGCCAAGGAAAACCATATTGAACACCTCTCTGTCTCGGTGCT---G  [185]
A. magnificus B            G-------TACTAAA---CTCTCGGCGCGGTAAGCGCCAAGGAAAACCATATTGAACACCTCTCTGTCTCGGTGCT---G  [185]
A. guttatus A              G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTGGGTGCT---G  [184]
A. guttatus B              G-------TACTAAC---CTCTCGGCGCGGCAAGAGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTTGGTGTT---G  [183]
A. pachyanthus A           G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGTCTTGGTGCTGCTG  [186]
A. pachyanthus B           G-------TACTAAC---CTCTCGGCGCGGCAAGCACCAAGGAAAACCTTATCGAACACCTCTCCGTCTTGGTGCTGCTG  [186]
A. longicaulis A           G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTGCGGCCTGGTGCTG--G  [185]
A. longicaulis B           G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTGCGGCCTGGTGCTG--G  [185]
A. albidus A               G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCATACTGAACACCTCTGCGGCCTGGTGCTG--G  [185]
A. albidus B               G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTGCGGCCTGGTGCTG--G  [185]
A. fecundus A              G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTGCGGCCTGCTGCTG--G  [186]
A. fecundus B              G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTGCGGCCTGCTGCTG--G  [186]
A. angustifolius A         G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACGCCTCTCCGCTCTGGTGCT---G  [186]
A. angustifolius B         G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACGCCTCTCCGCTCTGGTGCT---G  [186]
A. fruticosus A            G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGCTCCGGTGCT---G  [188]
A. fruticosus B            G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGCTCCGGTGCT---G  [188]
A. buxifolius A            G-------TACTAAC---CTCTCAGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGGCCTGGTGCT---G  [184]
A. buxifolius B            GGGAG---TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGGCCTGGTGCT---G  [192]
A. ceylanicus A            G-------TACTAAC---CTCTCGGCGCGGCAAGTGCCAAGGAAAACCGTATTGAACACCTCTCCGGCCTGGTGCT---G  [192]
A. ceylanicus B            G-------TACTAAC---CTCTCGGCGCGGCAAGTGCCAAGGAAAACCGTATTGAACACCTCTCCGGCCTGGTGCT---G  [192]
A. bracteatus A            G-------TACTAAC---CACTCGGCGCGGCAAGCGCCAAGGAAAACCTTATTGAACACCTCTCCGGCCTGGTGCT---G  [186]
A. bracteatus B            G-------TACTAAC---CACTCGGCGCGGCAAGCGCCAAGGAAAACCTTATTGAACACCTCTCCGGCCTGGTGCT---G  [186]
A. humilis A               G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGGCCTGGTGCT---G  [187]
A. humilis B               G-------TACTAAC---CTCTGGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGGCCTGGTGCT---G  [187]
A. gracilis A              G-------TACTAAC---CACTCGGCGCGGCAAGCGCCAAGGAAAACGTTACTGAACACCTCTCCGGCCCGGTGCT---G  [180]
A. gracilis B              G-------TACTAAA---CTCTCGGCGCGGCAAGCGCCAAGGAAAACGTTACTGAACACCTCTCCGGCCTGGTGCT---G  [180]
A. hookeri A               G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACTGTATTGAACACCTCTCCGGCCTGGTGCT---G  [188]
A. hookeri B               G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACTGTATTGAACACCTCTCCGGCCTGGTGCT---G  [188]
A. macranthus A            G----TACTACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGCT---G  [191]
A. macranthus B            G----TACTACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGCT---G  [191]
A. fulgens A               G----TACTACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGCT---G  [191]
A. fulgens B               G----TACTACTAAC---CTCTCGGCGCGGCAAGTGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGCT---G  [191]
A. sikkimensis A           G----TACTACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGCT---G  [191]
A. sikkimensis B           G----TACTACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGCT---G  [191]
A. arfakensis              G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAAYCGTATYGAACACCTCTCCGCTCCGGTGCT---G  [187]
A. austroyunnanensis A     G----TACCACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCTGGTGCT---G  [191]
A. austroyunnanensis B     G----TACCACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCTGGTGCT---G  [191]
A. lineatus                G----TACTACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGTT---G  [191]
A. pachytrichus A          G----TACCACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGCC---G  [191]
A. pachytrichus B          G----TACCACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTCCGGCCCGGTGCC---G  [191]
A. pseudohybridus          G-------TACTAAC---CTCTGGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACGCCTCTCCGCTCTGGTGCT---G  [186]
A. batakiorum              G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTGCGGCCTGGTGCTG--G  [188]
A. garrettii A             G-------TACTAAC---CTCTCGGCGCGGCAAGCGCTAAGGAAAACCGTATCGAACACCTCTCCGGCCTGGTGCT---G  [185]
A. garrettii B             G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATCGAACACCTCTCCGGCCTGGTGCT---G  [189]
A. acuminatus              G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGGCCTGGTGCT---G  [184]
A. myrmecophilus           G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTGCGGCCTGGTGCTG--G  [185]
A. sp. 00171               G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTGCGGCCTGGTGCTG--G  [185]
A. viridiflorus A          G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTGCGGCCTGGTGCTG--G  [185]
A. viridiflorus B          G-------TACTAAC---CTCTCGGCGCGGCAAGCGCCAAGGAAAACCGTACTGAACACCTCTGCGGCCTGGTGCTG--G  [185]
A. andersonii              G-------TACTAAC---CTCTGGGCGCGGCAAGCGCCAAGGAAAACCGTATTGAACACCTCTCCGGCCTGGTGCT---G  [190]
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Taxon                                  250       260       270       280       290       300       310       320
                                       .         .         .         .        ITS2       .         .         .
                                    2  22        .        22        .33 3    33.        .3        3  3      3 3
                                    5  67        .        89        .01 2    34.        .5        6  7      8 9
Cyr. baileyi                        CGCGGTGCGC-AGGACTTGACGAGGAGCG-TCCATTGAAT--AGATATTATCTCGTCGCC-CCTTCCCC-CAACATC-C-  [268]
Lys. forrestii                      CGCTGTGTCG-AGGACGTGATGAGTAGCG-CCTATCGAATA-A-AT---GTCTCTTCGCC-CCCCCTCAACA-CATC-C-  [260]
A. curtisii                         TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT??-ATCTCGTCGCC-CCAATCCGCCAATGTTTTG  [259]
A. musaensis                        TGAGGTACCC-AGGACGTGATGAGGAGTT-TCTATTGAAT--AGAT---ATCTCGTCGAC-CCCCTCCCCAAATATCTTG  [259]
A. javanicus                        TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCACTCCGCCAATGTCTTG  [257]
A. vinaceus                         TGCGGTACC--AGGACGTGATGAGGAGTG-TCTATTGAATA-AGAT---ATCTCGTCGCC-CCCCTCCCCGAATATCTTG  [255]
A. nummularius                      TGCGGTACTC-GGGACGTGACGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCATATATCTCG  [261]
A. ellipticus                       TGCGGTACCC-GGGACGTGACGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCATATATCTCA  [261]
A. oxychlamys A                     TGCGGTACCC-AGGACGCGACGAGGAGTG-TCTATTGAAT--AGATATTATCTCGTCGCC-CCCCTCCCCAAATATGTTG  [266]
A. oxychlamys B                     TGCGGTACCC-AGGACGCGACGAGGAGTG-TCTATTGAAT--AGATATTATCTCGTCGCC-CCCCTCCCCAAATATGTTG  [273]
A. roseoflorus                      TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [255]
A. irigaensis                       TGYGGTACCC-AGGATGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CTCCTCCCCAAATATCTCG  [258]
A. philippinensis                   TGCGGTGCCC-AGGACGTGATGAGGAGTGGTCTATCGAAT--AGAT---ATATCGTCGCC-CCCCTCCCCAAAACATCTC  [256]
A. obconicus                        TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCAMTCCGCCAATGTYTTG  [257]
A. sp. 001                          TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCCCTCCGCGAATATCTTG  [256]
A. rhododendron                     TGCGGTACCC-AGGATGTGATGAGGAA---TCTATTTAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAGATATCTT-  [253]
A. sp. 0025-123                     TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCCCTCCGCGAATATCTTG  [256]
A. sp. 0025-4                       TACGGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAATA-AGAT---ATCTTGTCGCC-CCTCTCCGCTAATATCTTG  [256]
A. sp. 00293                        TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTTGTCGCC?CCCCTCCCCAAATATCTTG  [256]
A. tricolor A                       TGTGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCCCTCCACGAATGTCTTG  [257]
A. tricolor B                       TGTGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCCCTCCACGAATGTCTTG  [257]
A. arctocalyx A                     TGTGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCCCTCCGCGAATGTCTTG  [257]
A. arctocalyx B                     TGTGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCCCTCCGCGAATGTCTTG  [257]
A. chrysanthus A                    TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATGGAATA-AGAT---ATCTCGTCGCC-CCACTCCGCCAATGTCTTG  [257]
A. chrysanthus B                    TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATGGAATA-AGAT---ATCTCGTCGCC-CCACTCCGCCAATGTCTTG  [257]
A. parvifolius A                    TGCGGT-CCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCACTCCGCCAATGTCTTG  [256]
A. parvifolius B                    TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCACTCCGCCAATGTCTTG  [257]
A. radicans A                       TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCACTCCGCCAATGTCTTG  [257]
A. radicans B                       TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATCGAATA-AGAT---ATCTCGTCGCC-CCACTCCGCCAATGTCTTG  [257]
A. argentii A                       TGCGGTACCC-AGGACGTGATGAGGAGTG-TCCATTGAATA-AGAT---ATCTCGTCGCC-CCCCTTCCCAATTATCTTG  [256]
A. argentii B                       TGCGGTACCC-AGGACGTGATGAGGAGTG-TCCATTGAATA-AGAT---ATCTCGTCGCC-CCCCTTCCCAATTATCTTG  [256]
A. magnificus A                     TGCGGTACC--AGGACGTGATGAGGAGTG-TCTATTGAATA-AGAT---ATCTCATCGCC-CCCCTCCCCAAATATCTTG  [257]
A. magnificus B                     TGCGGTACC--AGGACGTGATGAGGAGTG-TCTATTGAATA-AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTTG  [257]
A. guttatus A                       TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCCCCCCCTCCCCATATGTCTCG  [257]
A. guttatus B                       TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCATCGCC-CCCCTCCCCATATATCTTG  [255]
A. pachyanthus A                    TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCACATATCTCG  [258]
A. pachyanthus B                    TGCGGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCACATATCTCG  [258]
A. longicaulis A                    TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCCCTCCCTATGTATCTCG  [257]
A. longicaulis B                    TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCCCTCCCTATGTATCTCG  [257]
A. albidus A                        TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCCCTCCCTATGTATCTCG  [257]
A. albidus B                        TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCCCTCCCTATGTATCTCG  [257]
A. fecundus A                       TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCTCTCCCTATATATCTCG  [258]
A. fecundus B                       TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCTCTCCCTATATATCTCG  [258]
A. angustifolius A                  TGCAGTACCCCAGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGTC-CCCCTCCCCAAATATCTCG  [259]
A. angustifolius B                  TGCAGTACCCCAGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGTC-CCCCTCCCCAAATATCTCG  [259]
A. fruticosus A                     TGCAGTACCCAAGAACGTGATGAGGAATG-TCTATCGAAT--AGAT---ATCTCGTCGTC-CCCCTCCCCAAATACCTCG  [261]
A. fruticosus B                     TGCAGTACCCAAGAACGTGATGAGGAATG-TCTATCGAAT--AGAT---ATCTCGTCGTC-CCCCTCCCCAAATATCTCG  [261]
A. buxifolius A                     TGCGGCACCC-AGGACGCGACGAGGAGTG-TCTATCGAAT--AGATAT-ATCTCGTCGCCCCCCCTCCCCAAATATCTCG  [259]
A. buxifolius B                     TGCGGCACCC-AGGACGCGACGAGGAGTG-TCTATCGAAT--AGATAT-ATCTCGTCGCCCCCCCTCCCCAAATATCTCG  [267]
A. ceylanicus A                     TGCAGTACCT-AGGACGTGATGAGGAGTG-TCTATTGAATATAGAT---ATCTCGTCGCC-TCCCTACCCAAATATCTCG  [266]
A. ceylanicus B                     TGCAGTACCT-AGGACGTGATGAGGAGTG-TCTATTGAATATAGAT---ATCTCGTCGCC-TCCCTACCCAAATATCTCG  [266]
A. bracteatus A                     TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAAATTCTCG  [258]
A. bracteatus B                     TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAAATTCTCG  [258]
A. humilis A                        TGCAGTACCG-AGGACGTGATGAGGAGTG-TCTATTGCAT--AGAT---ATCCCGTCGCC-CCCCTCCCCAAATATCTCG  [259]
A. humilis B                        TGCAGTACCG-AGGACGTGATGAGGAGTG-TCTATTGCAT--AGAT---ATTCCGTCGCC-CCCCTCCCCAAATATCTCG  [259]
A. gracilis A                       TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGCAT--AGAT---ATCTCGTCGCCCCCCCCTCCCGAAATATCTCG  [253]
A. gracilis B                       TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGCAT--AGAT---ATCTCGTCGCC-CCCCTCCCGAAATATCTCG  [252]
A. hookeri A                        TGCAGTACCC-AGGACGTGACGAGGAGTG-TCTATTGCAT--AGATAT-ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [262]
A. hookeri B                        TGCAGTACCC-AGGACGTGACGAGGAGTG-TCTATTGCAT--AGATAT-ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [262]
A. macranthus A                     TGCAGCACCC-GGGACGCGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. macranthus B                     TGCAGCACCC-GGGACGCGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. fulgens A                        TGCAGCACCC-GGGACGCGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. fulgens B                        TGCAGCACCC-GGGACGCGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. sikkimensis A                    TGCAGCACCC-AGGACGTGATGAGGAGTG-TCTACTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. sikkimensis B                    TGCAGCACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. arfakensis                       TGCAGTACCCCAGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGTC-CCCCTCCTCAAATATCTCG  [260]
A. austroyunnanensis A              TGCAGTACCC-AGGACGTGACGAGGAGTG-TCTATCGAAT--AGAT---ATCTCGTCGCCCCCCCTCCCCAAGTATCTCG  [264]
A. austroyunnanensis B              TGCAGTACCC-AGGACGTGACGAGGAGTG-TCTATCGAAT--AGAT---ATCTCGTCGCCCCCCCTCCCCAAGTATCTCG  [264]
A. lineatus                         TGCAGCACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. pachytrichus A                   TGCAGCACCC-AGGACGTGATGAGGAGTG-TCTATTGAGT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. pachytrichus B                   TGCAGCACCC-AGGACGTGATGAGGAGTG-TCTATTGAGT--AGAT---ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [263]
A. pseudohybridus                   TGCAGTACCCAAGGACGTGATGAGGAGTG-TCTATTGAAT--AGATAT-ATCTCGTCGTC-CACCTCCCCAAATATCTCG  [261]
A. batakiorum                       TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCCCTCCCTATGTATCTCG  [260]
A. garrettii A                      TGCGGCACCC-AGGACGCGACGAGGAGTG-TCTATCGAAT--AGATAT-ATCTCGTCGCCCCCCCTCCCCAAATATCTCG  [260]
A. garrettii B                      TGCGGCACCC-AGGACGCGACGAGGAGTG-TCTATCGAAT--AGATAT-ATCTCGTCGCCCCCCCTCCCCAAATATCTCG  [264]
A. acuminatus                       TGCAGTACCC-AGGACGTGACGAGGAGTG-TCTATTGAAT--AGATAT-ATCTCGTCGCC-CCCCTCCCCAAATATCTCG  [258]
A. myrmecophilus                    TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCCCTCCCTATATATCTCG  [257]
A. sp. 00171                        TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCTCGGCGTC-TCCCTCCCTATATATCTCG  [257]
A. viridiflorus A                   TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCACGGCGTC-TCCCTCCCTATATATCTCG  [257]
A. viridiflorus B                   TGCAGTACCC-AGGACGTGATGAGGAGTG-TCTATTGAAT--AGAT---ATCACGGCGTC-TCCCTCCCTATATATCTCG  [257]
A. andersonii                       TGCAGTACCG-AGGACGTGATGAGGAGTG-TCTATTGCAT--AGATAT-ATCCCGTCGCC-CCCCTCCCCAAATATCTCG  [264]
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Taxon                                330       340       350       360       370       380       390       400
                                       .         .         .         .         .         .         .         .
                                     4 4 44 444  444    .         .   55  5 5   555    . 55  5    .6 66     66        .
                                     0 1 23 456  789    .         .   01  2 3   456    . 78  9    .0 12     34        .
Cyr. baileyi                         T--CTTCC-ACACT-------------AAG----AGTGCCGGGAG-ACGATA----CATACGAAGGAGG--GGCGCGGAT  [321]
Lys. forrestii                       T--CTTCC-ACACT-----------CAA------AGTGCCGGGAG-ACGATG----CTAACGAAGCGGG--GGTGCGGAT  [313]
A. curtisii                          T--TC-CC-----TR---ATTCAGTCAAAGTGTTG-----GGGG--ACGATG----CATACCAAGGAGGA-GGGACGGAC  [316]
A. musaensis                         T--TC-CC-----CG---ACTCGGTCAAGGTGTCG-----GGGG--ACGATG----CATACCAAGGAGGA-GGGACGGAT  [316]
A. javanicus                         T--TC-CC-----TG---ATTCAGTCAAAGTGTTG-----GGGG--ACAATG----CRTACCAAGGAGGA-GGGACGGAT  [314]
A. vinaceus                          T--TC-CT-----TG---ATTCACTCAAAGTGTTG-----GGGG--ACGATG----CATATCAAGGAGGA-GGGACGGAT  [312]
A. nummularius                       T--TC-CC-----CG-AC--TCGGTCAAA--GGTG---TCGGGGG-ACGATG----CATACGGAGGAGGA-GGGACGGAT  [319]
A. ellipticus                        T--TC-CC-----CGGC---TCGGTCAAAGG--TG---TCGGGGG-ACGATG----CATACGGAGGAGGA-GGGACGGAT  [319]
A. oxychlamys A                      T--TC-CC-----CGGC---CCAGTCAAAGG--TG---TCGGGGG-ACGATG----CATTCCGAGGAGGA-GGGACGGAT  [324]
A. oxychlamys B                      T--TC-CC-----CGGC---CCAGTCAAAGG--TG---TCGGGGG-ACGATG----CATTCCGAGGAGGA-GGGACGGAT  [331]
A. roseoflorus                       T--TC-CC-----CGAC---TCGGTCAAAGG--TG---TCGGGGG-ACGATG----CATACCAAGGAGGA-GGGACGGAT  [313]
A. irigaensis                        T--TC-CC-----CG-AT--TCAGTCAAAGTGTTG-----GGGG--ACGATG----CGTACCAAGGATGA-GGGACGGAT  [315]
A. philippinensis                    T--TC-CC-----TCG--ATTCAGTCAAAGTGTCG-----GGGG--ACGATG-CATCATACCAAGGAGGA-GGGACGGAT  [317]
A. obconicus                         T--TC-CC-----TG-AT--TCAGTCAAAGTGTTG-----GGGG--ACRATG----CATACCAAGGAGGA-GGGACGGAY  [314]
A. sp. 001                           T--TC-CC-----CG--ATTCAGTCAAAAGTGTTG-----GGGG--ATGATG----CATACCAAGGAGGA-GGGACGGAT  [314]
A. rhododendron                      C--TCCCC-----TG---ACTCGGTCAAAGTGTCG-----GGGG--ACGATG----CATAGCAAGGAGGA-GGGACGGAT  [311]
A. sp. 0025-123                      T--TC-CC-----TG--ATTCAGTCAAAAGTGTTG-----GGGG--ATGATG----CATACCAAGGAGGA-GGGACGGAT  [314]
A. sp. 0025-4                        T--TC-CC-----TG--ATTCAGTCAAA-GTGTTG-----GGGG--ATGATG----CATACCAAGGAGGA-GGGATAAAT  [313]
A. sp. 00293                         T--TC-CC-----CG--ATTAAGTCAAA-TTGTTG-----GGGG--ACGATG----CATACCAAGGAGGA-GGGACGGAT  [313]
A. tricolor A                        T--TC-CC-----TG---ATTCAATCAAAGTTTTG-----GGGG--ATGATG----CATACCGAGGAGGAAGGGACGGAT  [315]
A. tricolor B                        T--TC-CC-----TG---ATTCAATCAAAGTTTTG-----GGGG--ATGATG----CATACCGAGGAGGAAGGGACGGAT  [315]
A. arctocalyx A                      T--TC-CC-----TG---ATTCAATCAAAGTTTTG-----GGGG--ATGATG----CATACCGAGGAGGAAGGGACGGAT  [315]
A. arctocalyx B                      T--TC-CC-----TG---ATTCAATCAAAGTTTTG-----GGGG--ATGATG----CATACCGAGGAGGAAGGGACGGAT  [315]
A. chrysanthus A                     T--TC-CC-----TG---ATTCAGTCAAAGTGTTG-----GGGG--ACAATG----CGTACCAAGGAGGA-GGGACGGAT  [314]
A. chrysanthus B                     T--TC-CC-----TG---ATTCAGTCAAAGTGTTG-----GGGG--ACAATG----CGTACCAAGGAGGA-GGGACGGAT  [314]
A. parvifolius A                     T--TC-CC-----TG---ATTCAGTCAAAGTGTTG-----GGGG--ACAATG----CGTACCAAGGAGGA-GGGATGGAT  [313]
A. parvifolius B                     T--TC-CC-----TG---ATTCAGTCAAAGTGTTG-----GGGG--ACAATG----CGTACCAAGGAGGA-GGGACGGAT  [314]
A. radicans A                        T--TC-CC-----TG---ATTCAGTCAAAGTGTTG-----GGGG--ACAATG----CGTACCAAGGAGGA-GGGACGGAT  [314]
A. radicans B                        T--TC-CC-----TG---ATTCAGTCAAAGTGTTG-----GGGG--ACAATG----CGTACCAAGGAGGA-GGGACGGAT  [314]
A. argentii A                        ------------------------------------------------------------AGGAGGA-GGGACGGAT  [272]
A. argentii B                        ------------------------------------------------------------AGGAGGA-GGGACGGAT  [272]
A. magnificus A                      T--TC-CC-----TG---ATTCACTCAAAGTGTTG-----GGGG--AGGATG----CATATCAAGGAGGA-GGGACGAAT  [314]
A. magnificus B                      T--TC-CC-----TG---ATTCACTCAAAGTGTTG-----GGGG--ACGATG----CATATCAAGGAGGA-GGGACGGAT  [314]
A. guttatus A                        TCTTC-CC-----CG-CC--TCAGTCAA---GGTG---TCGGGGGGACGATGG---CATACCAAGGAGGA-GGGACGGAT  [318]
A. guttatus B                        TGTTC-CC-----CG-CC--TCAGTCAA---GGTG---TCGGGGG-ATGATG----CATACCAAGGAGGA-GGGACAGAT  [314]
A. pachyanthus A                     TGTTC-CC-----CG-CC--TCAGTCAA---GGTG---TCGGGGG-ACGATG----CATACCAAGGAGGA-GGGACGGAT  [317]
A. pachyanthus B                     TGTTC-CC-----CG-CC--TCAGTCAA---GGTG---TCGGGGG-ACGATG----CATACCAAGGAGGA-GGGACGGAT  [317]
A. longicaulis A                     T--TCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [316]
A. longicaulis B                     T--TCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [316]
A. albidus A                         T--TCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [316]
A. albidus B                         T--TCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [316]
A. fecundus A                        T--CCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ATGATG----CATAC---GGAGGA-GGGACGGAT  [317]
A. fecundus B                        T--TCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ATGATG----CATAC---GGAGGA-GGGACGGAT  [317]
A. angustifolius A                   T--TCGTCGACACTGAC----CA-T-AA--T--TGGTGTCGGGCG-ACGATG----CATAT---GGAAGA-GGGACGGAT  [318]
A. angustifolius B                   T--TCGTCGACACTGAC----CA-T-AA--T--TGGTGTCGGGCG-ACGATG----CATAT---GGAAGA-GGGACGGAT  [318]
A. fruticosus A                      T--TCGCCGACACTGAC----CA-T-AA--T--TGGTGTTTGGAG-ACGATG----CAGAC---GGAGGA-GGGACGGAT  [320]
A. fruticosus B                      T--TCGCCGACACTGAC----CA-T-AA--T--TGGTGTTTGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [320]
A. buxifolius A                      T--TCCCCGA------------------------GTGTCGGGAG-ACGATG----CTTACGAAGGAGGA-GGGACGGAT  [306]
A. buxifolius B                      T--TCCCCGA------------------------GTGTCGGGAG-ACGATG----CTTACGAAGGAGGA-GGGACGGAT  [314]
A. ceylanicus A                      T--TCGCCGACACTGAC----CA-T-AA-G---TGGTGTCTGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [328]
A. ceylanicus B                      T--TCGCCGACACTGAC----CA-T-AA-G---TGGTGTCTGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [328]
A. bracteatus A                      T--TCGCCGACACTGAC----CA-T-AA-G---TGGTGTCGGGCG-ACGATG----CATACGAAGGAGGA-GGGGCGGAT  [320]
A. bracteatus B                      T--TCGCCGACACTGAC----CA-T-AA-G---TGGTGTCGGGCG-ACGATG----CATACGAAGGAGGA-GGGGCGGAT  [320]
A. humilis A                         T--TCGTCGACACTGGC----CA-T-AAA-TGGTGGTGTTCGGTG-ACGATG----CATACCAAGGCGGAGGGGACGGAT  [325]
A. humilis B                         T--TCGTCGACACTGGC----CA-T-AAA-TGGTGGTGTTCGGTG-ACGATG----CATACCAAGGCGGAGGGGACGGAT  [325]
A. gracilis A                        T--TCGTCGACACTGGC----CA-T-AAA-TGGTGGTGTCGGGTG-ACGGTG----CATACGAAGGCGGAGGGGACGGAT  [319]
A. gracilis B                        T--TCGTCGACACTGGC----CA-T-AAA-TGGTGGTGTCAGGTG-GCGGTG----CATACGAAGGCGGAGGGGACGGAT  [318]
A. hookeri A                         T--TCGCCGACACTGAC----CA-T-AA-GTGGCGGTGTCGGGAG-ACGATG----CATACGAAGGAGAA-GGGACGGAT  [327]
A. hookeri B                         T--TCGCCGACACTGAC----CA-T-AA-GTGGCGGTGTCGGGAG-ACGATG----CATACGAAGGAGAA-GGGACGGAT  [327]
A. macranthus A                      T--TCGCCGACACTGACCAT-CA-T-AA-GTGGTGGTGTCGGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [331]
A. macranthus B                      T--TCGCCGACACTGACCAT-CA-T-AA-GTGGTGGTGTCGGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [331]
A. fulgens A                         T--TCGCCGACACTGACCAT-CAAT-AA-GTGGTGGTGTCGGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [332]
A. fulgens B                         T--TCGCCGACACTGACCAT-CA-T-AA-GTGGTGGTGTCGGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [331]
A. sikkimensis A                     T--TCGCCGACACTGACCAT-CA-T-AA-GTGGTGGTGTCGGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [331]
A. sikkimensis B                     T--TCGCCGACACTGACCAT-CA-T-AA-GTGGTGGTGTCGGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [331]
A. arfakensis                        T--TTGCCGACACTGAC----CA-T-AA--T?-TGGTGTTGGGAG-ACGATG----CATAT---GGAGGA-GGGACGGAT  [320]
A. austroyunnanensis A               T--TCGCCGACACTGAC----CA-T-AA-GTGGCGGTGTCGGGAG-ACGATG---CCATACGAAGGAGGA-GGGACGGAT  [330]
A. austroyunnanensis B               T--TCGCCGACACTGAC----CA-T-AA-GTGGCGGTGTCGGGAG-ACGATG---CCATACGAAGGAGGA-GGGACGGAT  [330]
A. lineatus                          T--TCGCCGACACTGACCAT-CA-T-AA-GTGGTGGTGTCGGGAG-ACGATG----CATACGAAGGAGGA?GGGACGGAT  [332]
A. pachytrichus A                    T--TCGCCGACACTGACCAT-CA-T-AA-GTGGTGGTGTCGGGAG-ACGATG----CATACGAAGGAGGAGGGGACGGAT  [332]
A. pachytrichus B                    C--TCGCCGACACTGACCAT-CA-T-AA-GTGGTGGTGCCGGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [331]
A. pseudohybridus                    T--TCGCCGACACTGAC----CA-T-AA--T--TGGTGTTGTGCG-ACGATG----CATAT---GGAGGA-GGGACGGAT  [320]
A. batakiorum                        T--TCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [319]
A. garrettii A                       T--TCCCCGA------------------------GTGTCGGGAG-ACGATG----CTTACGAAGGAGGA-GGGACGGAT  [307]
A. garrettii B                       T--TCCCCGA------------------------GTGTCGGGAG-ACGATG----CTTACGAAGGAGGA-GGGACGGAT  [311]
A. acuminatus                        T--CGGCCGACACTGAC----CA-T-AA-G---TGGTGTCGGGAG-ACGATG----CATACGAAGGAGGA-GGGACGGAT  [320]
A. myrmecophilus                     T--TCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [316]
A. sp. 00171                         T--TCGCCGACATTGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [316]
A. viridiflorus A                    T--TCGCCGACATCGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [316]
A. viridiflorus B                    T--TCGCCGACATCGAC----CA-T-AA--T--TGGTGTCGGGAG-ACGATG----CATAC---GGAGGA-GGGACGGAT  [316]
A. andersonii                        T--TCGTCGACACTGAC----CA-T-AAA-TGGTGGTGTTCGGTG-ACGATG----CATACCAAGGCGGAGGGGACGGAT  [330]
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Taxon                                410       420       430       440       450       460       470       480
                                     .         .         .         .         .         .         .         .
                                     .   6     6.        .         .   6   6 .         6.        .7        .
                                     .   5     6.        .         .   7   8 .         9.        .0        .
Cyr. baileyi                         ATTGGCCTCCCGTTATCC-TTGCATAGCGGCCGGCCCAAATA----ACATGCCGTGG--CGATGGAT----GTCACACGA   [390]
Lys. forrestii                       ATTGGCCTCCCGTTATCCCTTGTGTGGCGGCCGGCCCAAATA----GCATGCCGTGT--CGACGTATATATGTCACATGA   [387]
A. curtisii                          ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [385]
A. musaensis                         ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA-TTAGTATACCGTGC--CGATTGAT----GTCACACGA   [388]
A. javanicus                         ATTGGCCTCCCGTTATCC-AAGTATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [383]
A. vinaceus                          ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATAACGTGT--CGATTGAT----GTCACACGA   [381]
A. nummularius                       ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGG--TGATCGAT----GTCACACGT   [388]
A. ellipticus                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGC--CGATCGAT----GTCACACGA   [388]
A. oxychlamys A                      ATTGGCCTCCCGTTATCC-AGGCATAGCGGCCGGCACAAACA----GTACACCGTGC--TGATCGAT----GTCACACGA   [393]
A. oxychlamys B                      ATTGGCCTCCCGTTATCC-AGGCATAGCGGCCGGCACAAACA----GTACACCGTGC--TGATCGAT----GTCACACGA   [400]
A. roseoflorus                       ATTGGCCTCCCGTTATCC-AAGCGTAGCGGCCGGCACAAATA----GTATACCGTGC--TGATCGAT----GTCACGCGA   [382]
A. irigaensis                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [384]
A. philippinensis                    ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATG----GTACACCGTGT--CGATTGAT----GTCACACGA   [386]
A. obconicus                         ATTGGCCTCCCGTTATCC-AAGYATAGCGGCYGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [383]
A. sp. 001                           ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTGTACCGTGT--CGATTGAT----GTCACACGA   [383]
A. rhododendron                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGRCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [380]
A. sp. 0025-123                      ATTGGCCTCCCGTTATCC-AAGAATAGCGGCCGGCACAAATA----GTGTACCGTGT--CGATTGAT----GTCACACGA   [383]
A. sp. 0025-4                        TTTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----TTATACCATGT--CGATTGAT----GTCACACGA   [382]
A. sp. 00293                         ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [382]
A. tricolor A                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [384]
A. tricolor B                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [384]
A. arctocalyx A                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GCCACACGA   [384]
A. arctocalyx B                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [384]
A. chrysanthus A                     ATTGGCCTCCCGTTATCC-AAGTATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [383]
A. chrysanthus B                     ATTGGCCTCCCGTTATCC-AAGTATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [383]
A. parvifolius A                     ATTGGCCTCCCGTTATCC-AAGTATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [382]
A. parvifolius B                     ATTGGCCCCCCGTTATCC-AAGTATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [383]
A. radicans A                        ATTGGCCTCCCGTTATCC-AAGTATAGCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [383]
A. radicans B                        ATTGGCCTCCCGTTATCC-AAGTATACCGGCCGGCACAAATA----GTATACCGTGT--CGATTGAT----GTCACACGA   [383]
A. argentii A                        ATTGGCCTCCCGTTATCG-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATTGAT----GTCACATGA   [341]
A. argentii B                        ATTGGCCTCCCGTTATCG-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATTGAT----GTCACATGA   [341]
A. magnificus A                      ATTTGCCTCCCTTTATCC-AAGCATAGCAGCCGGCACAAATA----GTATAATGTGT--CGATTGAT----GTCACACGA   [383]
A. magnificus B                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATAACGTGT--CGATTGAT----GTCACACGA   [383]
A. guttatus A                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGTCGGCACAAATA----GTATACCGTGG--TGATTGAT----GTCACACGA   [387]
A. guttatus B                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCTGGAACAAATA----GTATACCGTGG--TAATTGAT----ATCACACGA   [383]
A. pachyanthus A                     ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGC--TGATTGAT----GTCACACGA   [386]
A. pachyanthus B                     ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATACCGTGC--CGATTGAT----GTCAGACGA   [386]
A. longicaulis A                     ATTGGCCTCCCGTTATCT-GAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. longicaulis B                     ATTGGCCTCCCGTTATCT-GAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. albidus A                         ATTGGCCTCCCGTTATCT-GAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. albidus B                         ATTGGCCTCCCGTTATCT-GAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACATGA   [389]
A. fecundus A                        ATTGGCCTCCCGTTATCG-GAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [390]
A. fecundus B                        ATTGGCCTCCCGTTATCG-GAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [390]
A. angustifolius A                   ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [387]
A. angustifolius B                   ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [387]
A. fruticosus A                      ATTGGCCTCCCGTTATCC-AAGCATGGCGGCTGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. fruticosus B                      ATTGGCCTCCCGTTATCC-AAGCATGGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. buxifolius A                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----CTATGCCGTGT--CGATGGAC----GTCACACGA   [375]
A. buxifolius B                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----CTATGCCGTGT--CGATGGAC----GTCACACGA   [383]
A. ceylanicus A                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----ATATGTCGTGT--CGATGGAT----GTCACACGA   [397]
A. ceylanicus B                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----ATATGTCGTGT--CGATGGAT----GTCACACGA   [397]
A. bracteatus A                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. bracteatus B                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. humilis A                         ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [394]
A. humilis B                         ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [394]
A. gracilis A                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATAG---GTATGCCGTGTTGCGATGGAT----GTCACACGA   [391]
A. gracilis B                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATAG---GTATGCCGTGTTGCGATGGAT----GTCACACGA   [390]
A. hookeri A                         ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [396]
A. hookeri B                         ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [396]
A. macranthus A                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTAGGCCGTGT--CGATGGAT----GTCACACGA   [400]
A. macranthus B                      ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTAGGCCGTGT--CGATGGAT----GTCACACGA   [400]
A. fulgens A                         ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTAGGCCGTGT--CGATGGAT----GTCACACGA   [401]
A. fulgens B                         ATTGGCCTCCCGTTATCC-AAGCGTAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [400]
A. sikkimensis A                     ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [400]
A. sikkimensis B                     ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [400]
A. arfakensis                        ATTGGCCTCCCGTTATCC-AAGCATGGCGGCTGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. austroyunnanensis A               ATTGGCCTCCCGTAATCC-AAGCATTGCGGCCGGCACAAATA----GTAAGCCGTGT--CGATGGAT----GTCACACGA   [399]
A. austroyunnanensis B               ATTGGCCTCCCGTAATCC-AAGCATTGCGGCCGGCACAAATA----GTAAGCCGTGT--CGATGGAT----GTCACACGA   [399]
A. lineatus                          ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [401]
A. pachytrichus A                    ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [401]
A. pachytrichus B                    ATTGG---------ATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACAAGA   [391]
A. pseudohybridus                    ATTGGCCTCCCGTTATCC-AATCATAGCGGCCGGCACAAATA----GTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. batakiorum                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [392]
A. garrettii A                       ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----CTATGCCGTGT--CGATGGAC----GTCACACGA   [376]
A. garrettii B                       ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----CTATGCCGTGT--CGATGGAC----GTCACACGA   [380]
A. acuminatus                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATTCCGTGT--CGATGGAT----GTCACACGA   [389]
A. myrmecophilus                     ATTGGCCTCCCGTTGTCC-AAGCATAGCGGCCGGCACAAATAAATAGTATGCYGTGT--CGATGGAT----GTCACACGA   [389]
A. sp. 00171                         ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. viridiflorus A                    ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. viridiflorus B                    ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATAAATAGTATGCCGTGT--CGATGGAT----GTCACACGA   [389]
A. andersonii                        ATTGGCCTCCCGTTATCC-AAGCATAGCGGCCGGCACAAATA----GTATGCTGTGT--CGATGGAT----GTCACACGA   [399]
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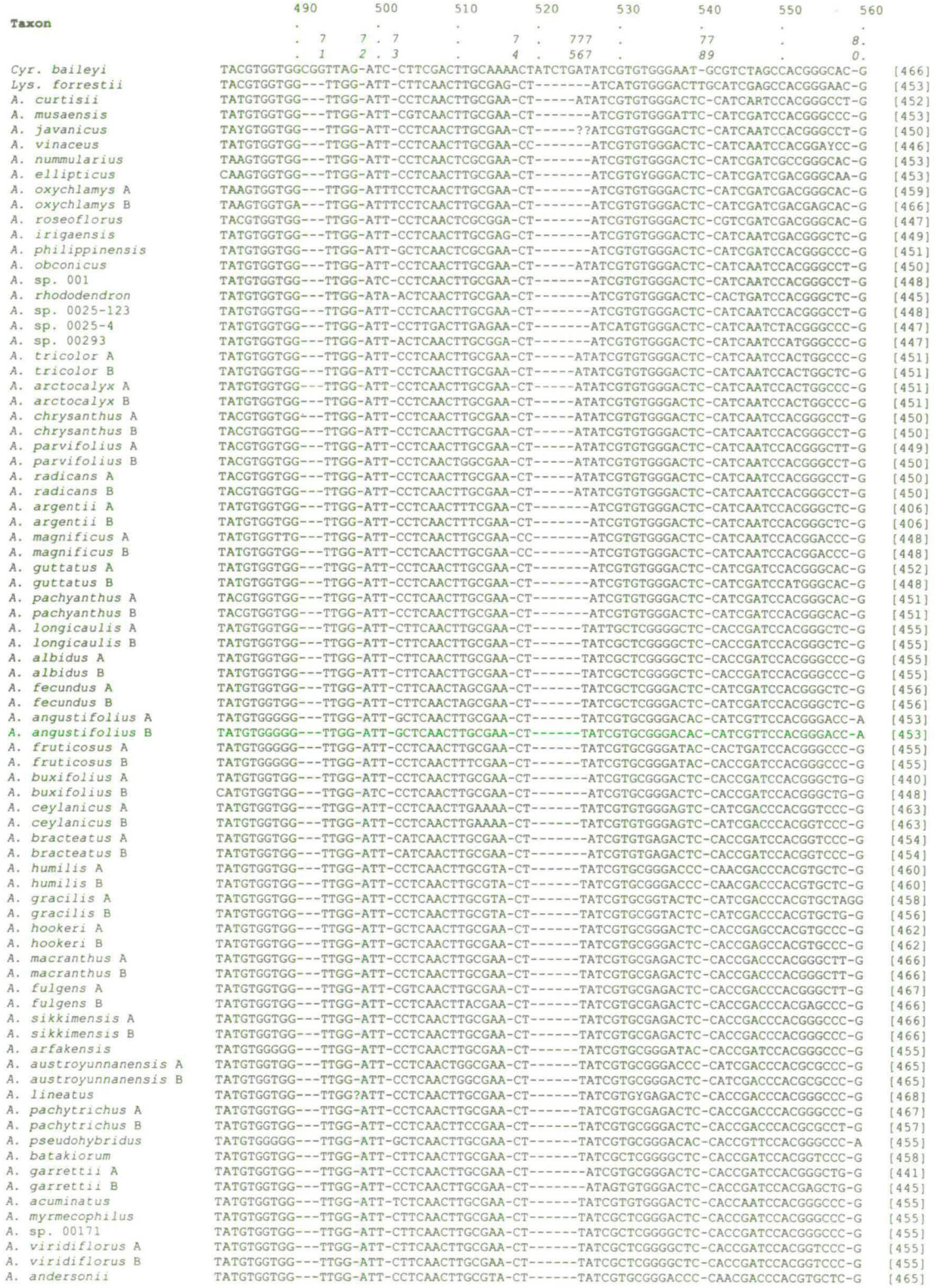
Taxon
490 500 510 520 530 540 550 560
Cyr. baileyi TACGTGGTGGCGGTTAG-ATC-CTTCGACTTGCAAAACTATCTGATATCGTGTGGGAAT-GCGTCTAGCCACGGGCAC-G [466]
Lys. forrestii TACGTGGTGG---TTGG-ATT-CTTCAACTTGCGAG-CT-------ATCATGTGGGACTTGCATCGAGCCACGGGAAC-G [453]
A. curtisii TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCARTCCACGGGCCT-G [452]
A. musaensis TATGTGGTGG---TTGG-ATT-CGTCAACTTGCGAA-CT-------ATCGTGTGGGATTC-CATCGATCCACGGGCCC-G [453]
A. javanicus TAYGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----??ATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [450]
A. vinaceus TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CC-------ATCGTGTGGGACTC-CATCAATCCACGGAYCC-G [446]
A. nummularius TAAGTGGTGG---TTGG-ATT-CCTCAACTCGCGAA-CT-------ATCGTGTGGGACTC-CATCGATCGCCGGGCAC-G [453]
A. ellipticus CAAGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATCGTGYGGGACTC-CATCGATCGACGGGCAA-G [453]
A. oxychlamys A TAAGTGGTGG---TTGG-ATTTCCTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CATCGATCGACGGGCAC-G [459]
A. oxychlamys B TAAGTGGTGA---TTGG-ATTTCCTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CATCGATCGACGAGCAC-G [466]
A. roseoflorus TACGTGGTGG---TTGG-ATT-CCTCAACTCGCGGA-CT-------ATCGTGTGGGACTC-CGTCGATCGACGGGCAC-G [447]
A. irigaensis TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAG-CT-------ATCGTGTGGGACTC-CATCAATCGACGGGCTC-G [449]
A. philippinensis TATGTGGTGG---TTGG-ATT-GCTCAACTCGCGAA-CT-------ATCGTGTGGGACTC-CATCGATCCACGGGCCC-G [451]
A. obconicus TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [450]
A. sp. 001 TATGTGGTGG---TTGG-ATC-CCTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [448]
A. rhododendron TATGTGGTGG---TTGG-ATA-ACTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CACTGATCCACGGGCTC-G [445]
A. sp. 0025-123 TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [448]
A. sp. 0025-4 TATGTGGTGG---TTGG-ATT-CCTTGACTTGAGAA-CT-------ATCATGTGGGACTC-CATCAATCTACGGGCCC-G [447]
A. sp. 00293 TATGTGGTGG---TTGG-ATT-ACTCAACTTGCGGA-CT-------ATCGTGTGGGACTC-CATCAATCCATGGGCCC-G [447]
A. tricolor A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACTGGCCC-G [451]
A. tricolor B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACTGGCTC-G [451]
A. arctocalyx A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACTGGCCC-G [451]
A. arctocalyx B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACTGGCCC-G [451]
A. chrysanthus A TACGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [450]
A. chrysanthus B TACGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [450]
A. parvifolius A TACGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACGGGCTT-G [449]
A. parvifolius B TACGTGGTGG---TTGG-ATT-CCTCAACTGGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [450]
A. radicans A TACGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [450]
A. radicans B TACGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-----ATATCGTGTGGGACTC-CATCAATCCACGGGCCT-G [450]
A. argentii A TATGTGGTGG---TTGG-ATT-CCTCAACTTTCGAA-CT-------ATCGTGTGGGACTC-CATCAATCCACGGGCTC-G [406]
A. argentii B TATGTGGTGG---TTGG-ATT-CCTCAACTTTCGAA-CT-------ATCGTGTGGGACTC-CATCAATCCACGGGCTC-G [406]
A. magnificus A TATGTGGTTG---TTGG-ATT-CCTCAACTTGCGAA-CC-------ATCGTGTGGGACTC-CATCAATCCACGGACCC-G [448]
A. magnificus B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CC-------ATCGTGTGGGACTC-CATCAATCCACGGACCC-G [448]
A. guttatus A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CATCGATCCACGGGCAC-G [452]
A. guttatus B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CATCGATCCATGGGCAC-G [448]
A. pachyanthus A TACGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CATCGATCCACGGGCAC-G [451]
A. pachyanthus B TACGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATCGTGTGGGACTC-CATCGATCCACGGGCAC-G [451]
A. longicaulis A TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATTGCTCGGGGCTC-CACCGATCCACGGGCTC-G [455]
A. longicaulis B TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATCGCTCGGGGCTC-CACCGATCCACGGGCTC-G [455]
A. albidus A TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATCGCTCGGGGCTC-CACCGATCCACGGGCCC-G [455]
A. albidus B TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATCGCTCGGGGCTC-CACCGATCCACGGGCCC-G [455]
A. fecundus A TATGTGGTGG---TTGG-ATT-CTTCAACTAGCGAA-CT------TATCGCTCGGGACTC-CATCGATCCACGGGCTC-G [456]
A. fecundus B TATGTGGTGG---TTGG-ATT-CTTCAACTAGCGAA-CT------TATCGCTCGGGACTC-CATCGATCCACGGGCTC-G [456]
A. angustifolius A TATGTGGGGG---TTGG-ATT-GCTCAACTTGCGAA-CT------TATCGTGCGGGACAC-CATCGTTCCACGGGACC-A [453]
A. angustifolius B TATGTGGGGG---TTGG-ATT-GCTCAACTTGCGAA-CT------TATCGTGCGGGACAC-CATCGTTCCACGGGACC-A [453]
A. fruticosus A TATGTGGGGG---TTGG-ATT-CCTCAACTTGCGAA-CT------TATCGTGCGGGATAC-CACTGATCCACGGGCCC-G [455]
A. fruticosus B TATGTGGGGG---TTGG-ATT-CCTCAACTTTCGAA-CT------TATCGTGCGGGATAC-CACCGATCCACGGGCCC-G [455]
A. buxifolius A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATCGTGCGGGACTC-CACCGATCCACGGGCTG-G [440]
A. buxifolius B CATGTGGTGG---TTGG-ATC-CCTCAACTTGCGAA-CT-------ATCGTGCGGGACTC-CACCGATCCACGGGCTG-G [448]
A. ceylanicus A TATGTGGTGG---TTGG-ATT-CCTCAACTTGAAAA-CT------TATCGTGTGGGAGTC-CATCGACCCACGGTCCC-G [463]
A. ceylanicus B TATGTGGTGG---TTGG-ATT-CCTCAACTTGAAAA-CT------TATCGTGTGGGAGTC-CATCGACCCACGGTCCC-G [463]
A. bracteatus A TATGTGGTGG---TTGG-ATT-CATCAACTTGCGAA-CT-------ATCGTGTGAGACTC-CACCGATCCACGGTCCC-G [454]
A. bracteatus B TATGTGGTGG---TTGG-ATT-CATCAACTTGCGAA-CT-------ATCGTGTGAGACTC-CACCGATCCACGGTCCC-G [454]
A. humilis A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGTA-CT------TATCGTGCGGGACCC-CAACGACCCACGTGCTC-G [460]
A. humilis B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGTA-CT------TATCGTGCGGGACCC-CAACGACCCACGTGCTC-G [460]
A. gracilis A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGTA-CT------TATCGTGCGGTACTC-CATCGACCCACGTGCTAGG [458]
A. gracilis B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGTA-CT------TATCGTGCGGTACTC-CATCGACCCACGTGCTG-G [456]
A. hookeri A TATGTGGTGG---TTGG-ATT-GCTCAACTTGCGAA-CT------TATCGTGCGGGACTC-CACCGAGCCACGTGCCC-G [462]
A. hookeri B TATGTGGTGG---TTGG-ATT-GCTCAACTTGCGAA-CT------TATCGTGCGGGACTC-CACCGAGCCACGTGCCC-G [462]
A. macranthus A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCTT-G [466]
A. macranthus B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCTT-G [466]
A. fulgens A TATGTGGTGG---TTGG-ATT-CGTCAACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCTT-G [467]
A. fulgens B TATGTGGTGG---TTGG-ATT-CCTCAACTTACGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGAGCCC-G [466]
A. sikkimensis A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCCC-G [466]
A. sikkimensis B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCCC-G [466]
A. arfakensis TATGTGGGGG---TTGG-ATT-CCTCAACTTGCGAA-CT------TATCGTGCGGGATAC-CACCGATCCACGGGCCC-G [455]
A. austroyunnanensis A TATGTGGTGG---TTGG-ATT-CCTCAACTGGCGAA-CT------TATCGTGCGGGACCC-CATCGACCCACGCGCCC-G [465]
A. austroyunnanensis B TATGTGGTGG---TTGG-ATT-CCTCAACTGGCGAA-CT------TATCGTGCGGGACTC-CATCGACCCACGCGCCC-G [465]
A. lineatus TATGTGGTGG---TTGG?ATT-CCTCAACTTGCGAA-CT------TATCGTGYGAGACTC-CACCGACCCACGGGCCC-G [468]
A. pachytrichus A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT------TATCGTGCGAGACTC-CACCGACCCACGGGCCC-G [467]
A. pachytrichus B TATGTGGTGG---TTGG-ATT-CCTCAACTTCCGAA-CT------TATCGTGCGGGACTC-CACCGACCCACGCGCCT-G [457]
A. pseudohybridus TATGTGGGGG---TTGG-ATT-GCTCAACTTGCGAA-CT------TATCGTGCGGGACAC-CACCGTTCCACGGGCCC-A [455]
A. batakiorum TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATCGCTCGGGGCTC-CACCGATCCACGGTCCC-G [458]
A. garrettii A TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATCGTGCGGGACTC-CACCGATCCACGGGCTG-G [441]
A. garrettii B TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGAA-CT-------ATAGTGTGGGACTC-CACCGATCCACGAGCTG-G [445]
A. acuminatus TATGTGGTGG---TTGG-ATT-TCTCAACTTGCGAA-CT------TATCGTGTGGGACTC-CACCAATCCACGGGCCC-G [455]
A. myrmecophilus TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATCGCTCGGGACTC-CACCGATCCACGGGCCC-G [455]
A. sp. 00171 TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATCGCTCGGGGCTC-CACCGATCCACGGGCCC-G [455]
A. viridiflorus A TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATCGCTCGGGGCTC-CACCGATCCACGGTCCC-G [455]
A. viridiflorus B TATGTGGTGG---TTGG-ATT-CTTCAACTTGCGAA-CT------TATCGCTCGGGGCTC-CACCGATCCACGGTCCC-G [455]
A. andersonii TATGTGGTGG---TTGG-ATT-CCTCAACTTGCGTA-CT------TATCGTGCGGGACCC-CAACGACCCACGTGCTC-G [465]

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                          570        580        590        600
Taxon                      .          .          .          .
                      888. 8    8 888  8  9 .  9  9   .
                      123. 4    5 678  9  0 .  1  2   .
Cyr. baileyi          ACCCTG-T-----GGCAG----CAGAT-TGGTGCTGCCTTCCA   [498]
Lys. forrestii        ACCCAA-C-----GGCAC----GAGAT-TG------CCCTCGA   [479]
A. curtisii           ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [478]
A. musaensis          ACCCAA-T-CAATGGCGC----AAGAT-CG------CCCTCGA   [483]
A. javanicus          ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [476]
A. vinaceus           ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [472]
A. nummularius        ACCCAAA------GGCAC----AAGAT-GG------CCCTCGA   [479]
A. ellipticus         ACCCAAA------GGCAC----AAGAT-CG------CCCTCGA   [479]
A. oxychlamys A       ACCCAA-C-----GGCAC----AAGAT-GG------CCCTCGA   [485]
A. oxychlamys B       ACCCAA-C-----GGCAC----AAGAT-GG------CCCTCGA   [492]
A. roseoflorus        ACCCAA-C-----GGCAC----AAGAT-GG------CCCTCGA   [473]
A. irigaensis         ACCCAA-T-----GGCAC----AAGAT-TG------CTCTCGA   [475]
A. philippinensis     ACCCAA-T-----GGCAC----GAGAT-TG------CTCTCGG   [477]
A. obconicus          ACCCAA-T-----GGCAC----MAGAT-TG------CCCTCGA   [476]
A. sp. 001            ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [474]
A. rhododendron       ACCCAAAT-----GGCAC----AAGAT-TG------CCCTCGA   [472]
A. sp. 0025-123       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [474]
A. sp. 0025-4         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [473]
A. sp. 00293          ACCCAA-T-----GGCAC----TAGAT-TG------CCCTCGA   [473]
A. tricolor A         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [477]
A. tricolor B         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [477]
A. arctocalyx A       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [477]
A. arctocalyx B       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [477]
A. chrysanthus A      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [476]
A. chrysanthus B      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [476]
A. parvifolius A      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [475]
A. parvifolius B      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [476]
A. radicans A         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTTGA   [476]
A. radicans B         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [476]
A. argentii A         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTTGA   [432]
A. argentii B         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [432]
A. magnificus A       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [474]
A. magnificus B       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [474]
A. guttatus A         ACCCAA-C-----GGCAC----AAGAT-CG------CCCTCGA   [478]
A. guttatus B         ACCCAA-T-----GGCAC----AAGAT-CG------CCCTCGA   [474]
A. pachyanthus A      ACCCAA-C-----GGCAC----AAGAT-CG------CCCTCGA   [477]
A. pachyanthus B      ACCCAA-C-----GGCAC----AAGAT-CG------CCCTCGA   [477]
A. longicaulis A      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. longicaulis B      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. albidus A          ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. albidus B          ACCCAA-T-----GGCAC----AAGTT-TG------CCCTCGA   [481]
A. fecundus A         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [482]
A. fecundus B         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [482]
A. angustifolius A    ACCCAA-T-----GGCAC----AAGAT-TG---CCGCCCTCGA   [482]
A. angustifolius B    ACCCAA-T-----GGCAC----AAGAT-TG---CCGCCCTCGA   [482]
A. fruticosus A       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. fruticosus B       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. buxifolius A       ACCCAA-TT----GGCATAAATAAGATCTG------CCCTTGA   [472]
A. buxifolius B       ACCCAA-TT----GGCATAAATAAGATCTG------CCCTCGA   [480]
A. ceylanicus A       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [489]
A. ceylanicus B       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [489]
A. bracteatus A       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [480]
A. bracteatus B       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [480]
A. humilis A          ACCCAAG---------------AAGAT-TG------CCCACGA   [481]
A. humilis B          ACCCAAG---------------AAGAT-TG------CCCACGA   [481]
A. gracilis A         ACCCAG-C--------------AAGAT-CG------CCCTCGA   [479]
A. gracilis B         ACCCAG-C--------------AAGTT-CG------CCCTCGA   [477]
A. hookeri A          ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [488]
A. hookeri B          ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [488]
A. macranthus A       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [492]
A. macranthus B       ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [492]
A. fulgens A          ACCCAC-T-----GGCAC----AAGAT-TG------CCCTCGA   [493]
A. fulgens B          ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [492]
A. sikkimensis A      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [492]
A. sikkimensis B      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [492]
A. arfakensis         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. austroyunnanensis A ACCCAA-T-----GGCAC----GAGAG-TG------CCCTCGA  [491]
A. austroyunnanensis B ACCCAA-T-----GGCAC----GAGAG-TG------CCCTCGA  [491]
A. lineatus           ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [494]
A. pachytrichus A     ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [493]
A. pachytrichus B     ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [483]
A. pseudohybridus     ACCCAA-T-----GGCAC----AAGAT-TG---CCGCCCTCGA   [484]
A. batakiorum         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [484]
A. garrettii A        ACCCAA-TT----GGCATAAATAAGATCTG------CCCTCGA   [473]
A. garrettii B        ACCCAA-C-----GGCATAA-TAA-AT-TG------CCCTCAA   [473]
A. acuminatus         ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. myrmecophilus      ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. sp. 00171          ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. viridiflorus A     ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. viridiflorus B     ACCCAA-T-----GGCAC----AAGAT-TG------CCCTCGA   [481]
A. andersonii         ACCCAA-C--------------AAGAT-TG------CCCACGA   [486]
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APPENDIX D: *AESCHYNANTHUS* COLOUR PLATES

Colour plates illustrating morphological characters and variation between sections of *Aeschynanthus*. All photos are by courtesy of the Royal Botanic Garden.

1. **Section *Aeschynanthus*:**

(1a) *A. curtisii*; (1b) *A. javanicus*; (1c) *A. obconicus*; (1d) *A. chrysanthus*; (1e) *A. parvifolius*; (1f) *A. radicans*; (1g) *A. tricolor*; (1h) *A. arctocalyx*

2. **Section *Microtrichium*:**

(2a) *A. vinaceus*; (2b) *A. magnificus*; (2c) *A. argentii*; (2d) *A. irigaensis*; (2e) *A. rhododendron*; (2f) *A. sp.* 00293; (2g) *A. nummularius*; (2h) *A. ellipticus*; (2i) *A. oxychlamys*; (2j) *A. roseoflorus*; (2k) *A. guttatus*; (2l) *A. pachyanthus*; (2m) *A. musaensis*; (2n) *A. philippinensis*; (2o) *A. buxifolius*; (2p) *A. garrettii*

3. **Section *Haplotrichium* sens. str.:**

(3a) *A. bracteatus*

4. **Section *X*:**

(4a) *A. angustifolius*; (4b) *A. pseudohybridus*; (4c) *A. fruticosus*; (4d) *A. fulgens*; (4e) *A. austroyannanensis*; (4f) *A. humilis*; (4g) *A. andersonii*; (4h) *A. ceylanicus*

5. **Section *Diplotrichium*:**

(5a) *A. sikkimensis*; (5b) *A. lineatus*; (5c) *A. hookeri*

6. **Section *Polytrichium*:**

(6a) *A. albidus*; (6b) *A. batakiorum*; (6c) *A. sp.* 00171; (6d) *A. viridiflorus*; (6e) *A. myrmecophilus*; (6f) *A. fecundus*; (6g) *A. arfakensis*

(1a)

(1b)

(1c)

(1d)

(1e)

(1f)

(1g)

(1h)

(2a)

(2b)

(2c)

(2d)

(2e)

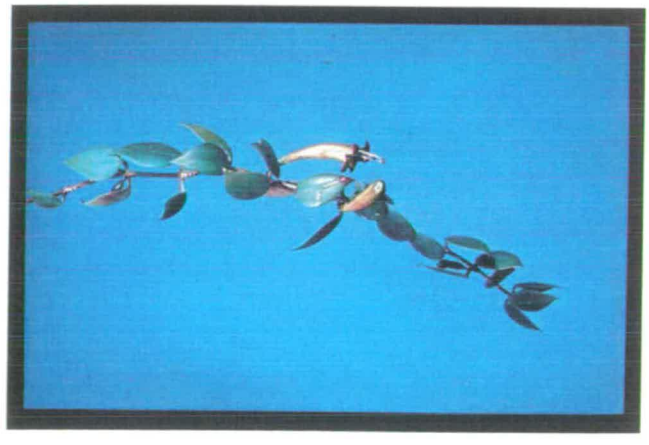
(2f)

(2g)

(2h)

(2i)

(2j)

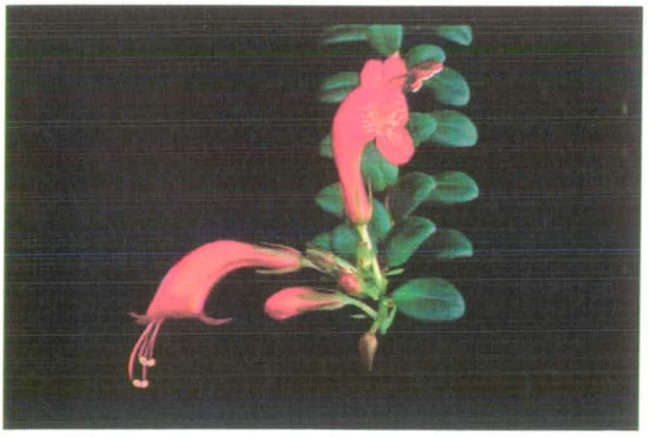


(2k)

(2l)

(2m)

(2n)

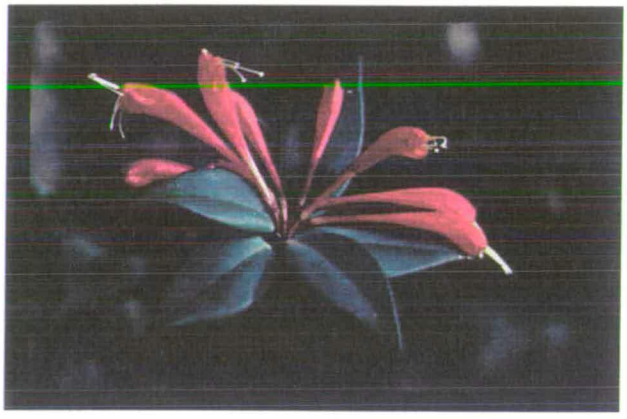


(2o)

(2p)

(3a)

(4a)



(4b)

(4c)

(4d)

(4e)

(4f)

(4g)

(4h)

(5a)

(5b)

(5c)

(6a)

(6b)

(6c)

(6d)

(6e)

(6f)

(6g)