Molecular Authentication and Taxonomy of Radix Stemonae

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Abstract

Radix Stemonae is an antitussive drug for the treatment of respiratory diseases and it also possesses anthelmintic property. According to the Pharmacopoeia of the People's Republic of China (2000), Radix Stemonae is the dried root tuber of *Stemona japonica* (Blume) Miquel, *S. sessilifolia* (Miquel) Miquel or *S. tuberosa* Loureiro. However, *Asparagus filicinus* Ham. ex D. Don is a common adulterant in market. Authentication is necessary to differentiate the three *Stemona* species and the *Asparagus filicinus.*

In order to establish a solid basis for the development of molecular markers for the authentication of Radix Stemonae, a revision on the *Stemona* species of China was made based on live plant materials, voucher specimens and literature. Taxonomic study shows that *S. shandongensis* D. K. Zang conspecific with *S. sessilifolia*. A total of six species of *Stemona* were studied in this project.

A molecular authentication method of Radix Stemonae was developed. Using 5S rRNA spacer sequences, it is possible to differentiate *Stemona japonica, S. sessilifolia* (including *S. shandongensis*), *S. tuberosa*, *S. parviflora* and *Asparagus filicinus.* The size of the 5S rRNA spacer of *Stemona* is about 500 bp. The 300 bp-400 bp region of the spacer was the most variable region. The intraspecific percentage similarity among *Stemona* species were about 90-100%. The interspecific percentage similarity among species is about 70-80%. Radix Stemonae was also distinguished from the adulterant *Asparagus filicinus* by comparing 5S rRNA spacer and the *trn*L sequences. The 5S rRNA spacer sequences similarity between *Asparagus* and *Stemona* species is about 16% on average. And the *trn*L sequences

similarity between *Asparagus filicinus* and *Stemona* species is about 80% on average.

Molecular phylogenetic analysis based on *trn*L intron sequences shows that the genera *Croomia*, *Pentastemona*, *Stemona* and *Stichoneruron* should be settled in a single family Stemonaceae. The family Stemonaceae also shows close affinity to the order Pandanales.

摘要

中藥百部(Radix Stemonae)主要用於止咳及殺蟲。根據《中國藥典》2000 年版，其來源植物爲蔓生百部 *Stemona japonica* (Blume) Miquel，直立百部 *S. sessilifolia* (Miquel) Miquel 及對葉百部 *S. tuberosa* Loureiro。然而，有部分在市場上售賣的中藥百部實爲僞品羊齒天門冬 *Asparagus filicinus* Ham. ex D. Don。故此有需要對中藥百部進行鑑定, 以分別三種百部正品及僞品羊齒天門冬。

首先對中國的百部屬植物進行了形態比較，作爲分子生物學鑑定的基礎。經過對新鮮植物及臘葉標本的研究以及文獻考查，結論認爲山東百部 *S. shandongensis* D. K. Zang 實爲直立百部，故予歸併。

然後分析了四種百部和藥材的 DNA 序列，利用 5S 核糖體核酸基因之間的間隔區(5S rRNA spacer)的 DNA 序列，可以清楚區分蔓生百部、直立百部、對葉百部及細花百部 *Stemona parviflora* C. H. Wright。百部屬的 5S 核糖體核酸間隔序列約爲 500 bp，其中第 300 bp 至 400 bp 的種間差異最大。5S 核糖體核酸間隔序列的種內差異很小，相似度 90-100%，而種間差異比較大，不同品種只有 70-80% 的相似度。 5S 核糖體核酸間隔序列亦支持將山東百部歸併入直立百部的結論。利用 5S 核糖體核酸間隔及 *trn*L 基因的序列，亦可以分辨羊齒天門冬及百部。羊齒天門冬及百部的 5S 核糖體核酸間隔序列只有相似度 16%，而兩者的 *trn*L 基因序列只有相似度 80%。

至於 *trn*L 基因序列的分析結果顯示 *Croomia* Torrey, *Pentastemona* Van Steenis, and *Stichoneruron* Hooker 這三個屬的植物應該和百部屬一同收歸百部科 (Stemonaceae) 。分析結果亦顯示百部科應該歸入露兜樹目。

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Abbreviations

AA	Harvard University Herbaria
APG	Angiosperm Phylogeny Group
CTAB	Cetyltrimethylammonium Bromide
CNI	close-neighbor-interchange
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosates
EB	Ethidium Bromide
EBI	European Bioinformatics Institute (),
EDTA	Ethylenediaminetetraacetate
h	Hour
IGS	Intergenic spacer
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITS	Internal transcribed spacer
LB	Luria-Bertani
MEGA	Molecular Evolutionary Genetics Analysis software
min	Minute
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
rRNA	Ribrosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
sec	Second
SYS	Sun Yat-Sen University Herbarium

UPGMA	Unweighted Pair Group Method with Arithmatic Mean
US	The United States National Herbarium
X-gal	5-Bromo-4-chloro-3-indoly-β-D-galactopuyranoside

Chapter 1. Introduction

1.1 Background

1.1.1 Source of Radix Stemonae

Radix Stemonae (Baibu, 百部) is the dried root tuber of *Stemona.* Usage of Radix Stemonae was recorded in the Chinese herbal "Ming Yi Bie Lu" (名醫別錄) which appeared in about A.D. 220-450 According to the Pharmacopoeia of the People's Republic of China (2000), Radix Stemonae is the root tuber of *Stemona japonica, S. sessilifolia* or *S. tuberosa.*

The sources of Radix Stemonae is very confusing. The confusion may be because the name Baibu has also been used to describe plants from other genera. In the herbals "Tu Jing Ben Cao" (圖經本草) and "Ben Cao Gang Mu" (本草綱目), *Asparagus filicinus* was also called Baibu. People in Hubei, Hunan, Jiangxi and Shenxi also called *Asparagus* species as Baibu (Xu and Cong 1997). A review of herbals (Cong and Xu 1997) showed that some herbals usually referred *Stemona japonica, S. sessilifolia* or *S. tuberosa* and *Asparagus filicinus* as Baibu. Roots of *Asparagus,* are known to have used as adulterants of Radix Stemonae (Tsi 1978). The dried root tubers of different species of *Stemona* and the adulterant are very similar in appearance and thus it is difficult to differentiate them.

1.1.2 Medicinal usage of Radix Stemonae

Radix Stemonae is an antitussive drug to treat respiratory diseases. It is used as a traditional Chinese medicine to moisten the lung and relieve cough. It can also be used as an anthelmintic agent. Usually the root tuber is cut into slices and then dried

(Figure. 1.1). An alternative method is to briefly soak the root tuber in boiling water and then dried. Many alkaloids have been isolated from this plants, for example tuberostemonine and neotuberostemonine (Gotz and Strunz 1975, Jiang *et al.* 2002, Chung *et al.* 2003). These alkaloids were found to have antitussive effects (Chung *et al.* 2003).

Figure 1.1 Radix Stemonae purchased from commercial market.

1.1.3 Stemonaceae

Stemonaceae is a small monocotyledonous family with four genera: *Croomia*, *Pentastemona*, *Stemona* and *Stichoneuron*. The number of species of this family is about 37 (Kubitzki 1998). *Stemona* is the largest genus with about 25 (Dahlgren *et al.* 1985) to 30 (Rogers 1982) species. Van der Ham (1991) argued that *Stemona* contains less than 15 to 20 species. For *Croomia*, *Pentastemona* and *Stichoneuron*, each genus has about two species.

Distrubution of *Stemona* spreads from Sri Lanka and east India to Japan, and through Malaysia to northern Australia (Kubitzki 1998, Van der Ham 1991). *Stichoneuron* grows naturally in Bangladesh and Assam, near the border between Malaysia and Thailand (Van der Ham 1991). *Croomia* occurs in southern Japan, eastern China and southeastern USA (Rogers 1982, Dahlgren *et al.* 1985). *Pentastemona* is found in central west and north Sumatra (Duyfjes 1991).

1.1.4 Stemonaceae of China

In China, two genera in Stemonaceae are known, namely, *Stemona* and *Croomia*. According to *Florae Reipublicae Popularis Sinicae* (Ji 1997), five *Stemona* species (*Stemona japonica, S. mairei, S. parviflora, S. sessilifolia* and *S. tuberosa*) and one *Croomia* species (*Croomia japonica*) are found in China. Amendment was made when the Flora of China (English edition) was published (Ji and Duyfjes 2000). Two more *Stemona* species, *S. kerrii* and *S. shandongensis*, were added. *Stemona* in China mainly occurs in provinces along Changjiang and Hainan. *Croomia* occurs in Auhui, Fujian, Jiangxi and Zhejiang provinces.

1.1.5 Circumscriptions of Stemonaceae

The circumscription of Stemonaceae has been discussed by botanists for many years. Botanists have been trying to answer the questions based on morphological characters, anatomical characters, karyomorphology, pollen and seed structures and also recently with molecular data.

Nakai (1937) observed that *Croomia* and *Stichoneuron* have small flowers, short filaments, unappendaged anthers and apical placentation. These shared characters are

distinctive for *Croomia* and *Stichoneuron* and thus Nakai proposed to remove these two genera to a separate family Croomiaceae. Prof. Z.Y. Wu supported Nakai's point of view (Ji and Duyfjes 2000). Tomlinson and Ayensu (1968) concurred that the assemblage of *Croomia*, *Stemona* and *Stichoneuron* is not natural because the similarities among the three genera are few while the differences are many. Willis (1985) agreed that *Croomia* and *Stichoneuron* are closely related but argued that the segregation of Croomiaceae from Stemonaceae is not appropriate.

The most vigorous discussion is the debate on the segregation of *Pentastemona* from Stemonaceae. Van Steenis (1982) favoured housing it with the other three genera in a single family Stemonaceae. He pointed out that the four genera share similar "morphological and anatomical vegetative characters, anatropous ovules, one-celled ovary, and the striking similarity in the preculiar seed structure." Although Van Steenis admitted that the placentation of the four genera varies and *Pentastemona* has the most unique characters, he still kept them in a single family. He criticized Tomlinson and Aysensu (1968) as their conclusion was only based on vegetative anatomy and morphology.

Conover (1991) reported that the stomata are surrounded by four or more contact cells in *Stemona, Stichoneuron* and *Pentastemona*, and thus she supported Van Steenis's (1982) idea of including *Pentastemona* in Stemonaceae.

Based on phylogenetic analysis on 18S rRNA, *rbc*L and *atp*B sequences, and several morphological synapomorphies (seed morophological characters and petiolate leaves), Claddick *et al.* (2002) suggested *Pentastemona* has close affinities with the other three genera. He considered *Pentastemona* as a sister to the rest of

Stemonaceae and nested within Stemonaceae.

Although the author of *Pentastemona*, Van Stennis (1982), insisted to keep it in Stemonaceae, many botanists argued that it is not justified. Dahlgren *et al.* (1985) indicated that *Pentastemona* is "highly distinctive" and "worthy of family rank."

Duyfjes (1991) compared specimens of *Pentastemona* species and *Stemona* species. Based on differences in growth habit, morphological features, anatomical characters and chromosome number, she argued that *Pentatstemona* deserves a family rank of its own, which is Pentastemonaceae. She considered Van Steenis's reasons for including *Pentastemona* in Stemonaceae are "less strong than as supposed" and she believed that *Pentastemona* has more unique characters. She also disagreed with Van Steenis's (1982) idea on similarity in seed structure among the four genera. This is because the two *Pentastemona* species has a distinct "watery hyaline sarcotesta-like layer" as the exotesta. She (1993) thus segregated *Pentastemona* to Pentastemonaceae in Flora Malesiana.

Van der Ham (1991) surveyed on pollen morphology of the four genera. His results showed heterogeneity of pollen morphology among them. The family is eurypalynous, containing genera with diverse pollen morphology. Individual genera are natural assemblages, but the intergeneric relationship may not be very close. *Stemona* pollen has high infrageneric variation and thus is divided into five main types according to the exine ornamentation (Table 1.1). Van der Ham (1991) found that *Pentastemona* pollen is most distinct among the four genera. Its sexine consists of elements that resemble Ubisch bodies. He thus agreed that *Pentastemona* is worthy of family rank. Van Steenis (1982) also reported that reticulate exine structure

of *Croomia* and *Stichoneuron* is different from those of *Stemona* and *Pentastemona.*

Ornamentation	Species
1. microreticulate	*S. phyllantha, S. kerrii*
2. rugulate	*S. javanica, S. tuberosa*
3. scabrate	*S. parviflora, S. lucida, S. japonica, S. sessilifolia*
4. fossulate	*S. cochinchinensis, S. collinsae, S. curtisii*
5. psilate	*S. australiana, S. prostrata, S. wardii*

Table 1.1 Infrageneric variation of a number of pollen features in *Stemona* (Van der Ham 1991).

Studies by Bouman & Devente (1992) on ovules and seed structures also concluded in segregation of the family. They questioned Van Steenis's (1982) statement that "all four genera share a surprisingly similar seed structure with a characteristic aril." They commented the similarity was superficial. Although the ovules of both *Stemona* and *Pentastemona* are "anatropous, bitegmic and crassinucellate", these are plesiomorphic characters for angiosperms and common in monocotyledons. They also stated that the development and structure of ovules and seeds are different in *Stemona* and *Pentastemona*. *Stemona* ovule and seed are bigger than those of *Pentastemona*. *Stemona* seed has well-developed raphe and chalaza. The seed coat anatomy of both genera is also different.

Different botanists hold different ideas about the circumscriptions of Stemonaceae, while most of them agree that the four genera can be grouped into three groups: *Croomia/Stichoneuron* pair, *Stemona,* and *Pentastemona*. Van Steenis (1982) insisted

to keep the four genera in a single family, but he mentioned "three tribes for the four genera." It implied that he admitted *Croomia* is more closely related to *Stichoneuron* than the other two genera, although he thought establishing tribes within Stemonaceae was useless. The controversy on the circumscription of Stemonaceae remains unsettled.

1.1.6 Affinity of Stemonaceae

The affinity of Stemonaceae, is also debatable. Which families are the closest relatives of Stemonaceae? Which order should Stemonaceae belong to?

Stemonaceae was treated as a close relative of Liliaceae (Lacher-Sandoval 1892, Krause 1930). It was favoured by Cronquist (1981) and Stemonaceae was included in his Liliales. However, other botanists held different point of view.

Stemonaceae shares with Dioscoreales in having reticulate leaf venation, tuberous roots and prolongation of the anther connective, and was thus treated as a member of Dioscoreales by many authors. Hutchinson (1934) reported that Stemonaceae shared many characters with Dioscoreales, including prolongation of the connectives, the distinct pith in the stem, and tendencies towards an inferior position of the ovary. He thus included Stemonaceae in this order. Burkill (1960) suggested the "Proto-Liliales" near to Dioscoreaceae was an origin of Stemonaceae. Van Steenis (1982) agreed with Burkill and said that the suggestion was "vague" but "wise." Ayensu (1968) also aligned Stemonaceae with Dioscoreaceae based on anatomical characters. Huber (1969), on the other hand, grouped Stemonaceae and Trilliaceae into the order Stemonales. He also thought that the "Dioscorealean-Stemonalean families" were very close to the ancestor of the monocotyledons. Dahlgren *et al.*

(1985) considered Stemonaceae as one of the seven families included in Dioscoreales. However, Dahlgren *et al.* (1985) mentioned that Stemonaceae and Trillaceae deviated from the trimerous flowers condition found in the other five families.

Huber (1991) reported Stemonaceae differs in many aspects from other Dioscoralean plants, including different stem anatomy, articulated flowers, and absence of a defined endostesta. He thus proposed to place Stemonaceae in Asparagales.

Recent molecular phylogenetic studies provided new clues about the affinity of the family. The *rbc*L study by Chase *et al.* (1995) showed that Stemonaceae form a "weak clade" with Pandanaceae, Cyalanthaceae and Velloziaceae. Based on *rbc*L, *atp*B and 18S rRNA sequences and morphological data, Caddick *et al.* (2002) put Stemonaceae in Pandanales, together with Velloziaceae, Cyclanthaceae and Triuridaceae. This idea was also evident by shared occurrence of unilocular ovaries, parietal placentation, irregular stamen number and absence of septal nectarines in Stemonaceae and Pandanales (Caddick *et al.* 2002). Kubitzki (1998) disputed the position of Stemonaceae in Pandanales, based on the fact that the perianth in Stemonaceae are dimerous while Cyclanthaceae are tetramerous. The Angiosperm Phylogeny Group (2003) also put Stemonaceae in Pandanales. Thorne (2003), however, considered the proposal of Stemonaceae in Pandanales was "rather preposterous and without any morphological foundation."

	Dioscoreales	Liliales	Asparagales	Pandanales	Stemonales
Lindley 1853	✓				
Hutchinson 1959	✓				
Burkill 1960		✓ *Stemona* was originated from "proto-Liliales"			
Ayensu 1968, 1972	✓				
Huber 1969					✓
Cronquist 1981		✓			
Dahlgren *et al.* 1985	✓ (including *Pentastemona*)				
Takhtajan 1987	✓ (including *Pentastemona*)				
Huber 1991			✓		
Chase *et al*. 1995				✓	
Caddick *et al*. 2002				✓	
APG 2003				✓	

Table 1.2 Position of Stemonaceae in different orders suggested by different authors.

1.2 Molecular Markers for Authentication and Phylogenetic Studies

1.2.1 Choosing appropriate DNA region(s)

Molecular studies using DNA sequences have been widely applied to phylogenetic questions. A lot of molecular markers are available but they have different pros and cons. Choice of appropriate DNA region(s) for sequence comparison is the very first step of molecular phylogenetic studies. Different portions of the genome evolve at different rates. Fast evolving regions can be used to resolve relationships at lower taxonomic levels, such as species or genus levels (Soltis & Soltis 1998). Rates of evolution of different DNA regions may also vary among and within taxonomic groups (Doebley *et al.* 1990, Bousquet *et al.* 1992).

1.2.2 Chloroplast DNA markers

Chloroplast DNA is a circular molecule with size between 120 and 200 kb. The molecule is separated into a large and small single-copy region by two inverted repeat segments. (Figure 1.2) Most genes in the chloroplast are present in single-copy. Different regions of the chloroplast DNA may evolve at different rates. (Soltis & Soltis 1998) The phylogenetic studies of by the APG (2003), Caddick *et al.* (2002) and Chase *et al.* (1995) were based on two chloroplast regions, *rbc*L and *atp*B. These two coding regions are usually used for inferring relationships at or above family level. The results of *rbc*L and *atp*B studies support placing Stemonaceae in Pandanales. (Chase *et al.* 1995, Soltis *et al.* 2000, Caddick *et al.* 2002, APG 2003)

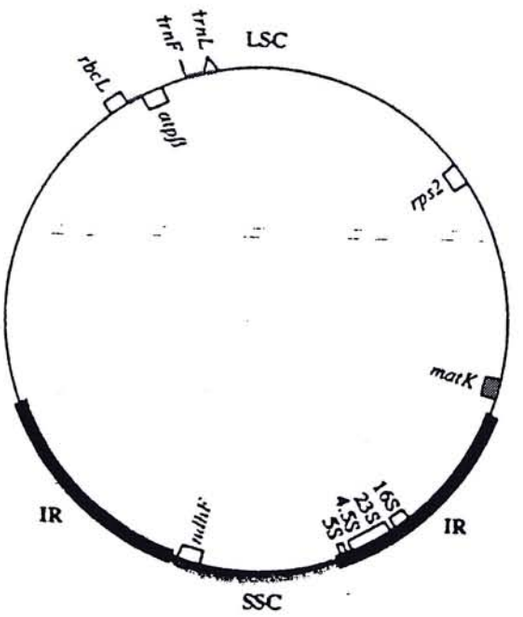

Figure 1.2. Illustration of Chloroplast genome. IR: inverted repeat segments; SSC: small single-copy region; LSC: large single-copy region. (Soltis & Soltis 1998)

No study has been made on *trn*L intron, *trn*L-F spacer and *trn*F gene sequences in Stemonaceae (Taberlet *et al.* 1991, Gielly & Taberlet 1996). The *trn*L and *trn*F are genes of transfer RNA of leucine and phenylalanine, respectively. They are located at the large single-copy region of chloroplast. This region includes *trn*L intron, intergenic spacer between *trn*L and *trn*F genes and *trn*F gene. The size of *trn*L intron is about 350 to 600 bp while that of *trn*L-F spacer is about 120-350 bp (Soltis & Soltis 1998). This non-coding region can be easily amplified and sequenced (Taberlet *et al.* 1991) and thus is useful for inferring interspecific relationship.

1.2.3 Nuclear sequences

In nuclear genome, many ribosomal RNA (rRNA) regions are used for phylogenetic studies. The studies of 18S rRNA sequences analysis of angiosperms suggested that Stemonaceae should be placed within Pandanales (APG 2003, Caddick *et al.* 2002). Ribosomal RNA regions (e.g. 18S and 26S rRNA) are usually very conserve and thus used at family level or above. However, the spacer between these ribosomal RNA

regions may be variable and useful for studying intergeneric or interspecific relationships.

The 5S rRNA genes occur in tandem arrays. The number of arrays in genome and the number of copies within an array vary. Between the 5S genes, there are nontranscribed spacer regions. Their size ranged from 100 to 700 bp (Sastri *et al.* 1992). The 5S spacers are highly variable and thus useful for resolving interspecific or intergeneric relationships. The 5S spacer sequences were used to identify Chinese medicine Beimu (Cai *et al.* 1999).

1.3 Objectives

Because of the confusion in source plants of Radix Stemonae, authentication of this herbal material is needed to avoid misuse of adulterant. Authentication also helps manufacturers to confirm the identity of material they use. In this thesis research, the trnL region and 5S rRNA spacer region were chosen as the molecular markers to authenticate Radix Stemonae.

Apart from authentication, there are a lot of discussions about circumscriptions and affinity of Stemonaceae. Several questions have not been resolved yet. Which genera should be included in Stemonaceae? Is *Pentastemona* worthy of a family rank? In which order should Stemonaceae be placed? Molecular phylogenetic analysis methods will be applied to analyse *trn*L and 5S rRNA spacer sequences, in order to answer these questions.

Based on the above questions, the objectives of this thesis project were:

1. To establish a molecular method to authenticate traditional Chinese medicine Radix Stemonae;
2. To revise the genus *Stemona* in China based on morphological and molecular characteristics;
3. To investigate the relationship among *Croomia, Pentastemona, Stemona*, and *Stichoneuron* based on DNA sequences;
4. To investigate the affinity of Stemonaceae with other families based on DNA sequences.

Chapter 2. Materials and Methods

2.1 Samples: Sources and Treatment

A total of six species of *Stemona*, two species of *Croomia*, one species of *Stichoneuron*, two species of *Pentastemona* and one species of *Asparagus* were used in this study.

2.1.1 Fresh Materials

Fresh samples included four collections of *Stemona japonica* (Blume) Miquel from the Institute of Botany, Chinese Academy of Sciences, Beijing (ICM 2004-2544), the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing (ICM 2004-2543), Anhui (Hu and But 24032), and the Nanjing Institute of Botany, Chinese Academy of Sciences (Hu & But 23971).

Three collections of *S. sessilifolia* (Miquel) Miquel were provided by the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing (Hu & But 23972), the China Phamaceutical University (Hu & Yung 606), and Prof. D.K. Zang (Zang 200401). One collection of *S. shandongensis* D.K. Zang was collected from Tai'an, Shandong (Zang 23974).

Four samples of *S. tuberosa* Loureiro were collected. One of them was provided by the South China Institute of Botany, Chinese Academy of Sciences (Chan200401). The other three collections were collected from Guangxi (Woo 23973), Hong Kong (Hu & But 23960) and Yunnan (ICM 20042541). One collection of *S. parviflora* C. H. Wright was collected from Hainan (Ma 9066, Hu and But 24034).

Fresh *Croomia* samples included two species. *C. japonica* Miquel was purchased from a nursery in USA (Hu & But 24033). A sample of *C. pauciflora* (Nuttall) Torrey & Gray was purchased from a nursery in USA (coded as Cp1) and another sample was provided by the United States National Botanical Garden (coded as Cp2).

Voucher specimens of these materials were deposited in the Herbarium, Department of Biology, the Chinese University of Hong Kong or in the Museum of Chinese Medicine, the Institute of Chinese Medicine, the Chinese University of Hong Kong.

Samples of *Stemona japonica*, *S. sessilifolia*, *S. shandongensis*, *S. tuberosa*, *S. parviflora* were cultivated in the green house of the Department of Biology, the Chinese University of Hong Kong for morphological study.

2.1.2 DNA Samples

Several DNA samples were ordered from the DNA bank of Royal Botanic Garden, Kew. This included one collection of *S. javanica* (Chase 2156 K), one sample of *S. tuberosa* (Wilkin 923K), one sample of *Croomia pauciflora* (Gholson 10360), two collections of *Stichoneuron caudatum* Ridley (Bygrave 50 K and Leiden B.G. 910654), one collection of *Pentastemona egregia* (Bogner 1724, 1985) and two samples of *Pentastemona sumatrana* (Duijfjes 21399 (8/1991) and Leiden B.G. 910375).

2.1.3 Dried Medicinal Material from Commerical Market

Totally, two samples of Radix Stemonae were collected from the commerical market. Sample ICM 2004-2540 was purchased from Beijing Tong Ren Tong (Figure 1.1).

Sample ICM 2004-2542 was collected from Yunnan. Both samples were deposited in the Museum of the Chinese Medicine, the Institute of Chinese Medicine, the Chinese University of Hong Kong. However, ICM 2004-2542 was subsequently found to be *Asparagus filicinus* by thin layer chromatography analysis (Xu 2004, personal communication).

Species	Sources	Voucher Specimens	Location of Deposition	Remarks
Croomia japonica	USA	Hu & But 24033	Herbarium, Chinese University of Hong Kong	Live plant
Croomia pauciflora	USA			Live plant, Coded CP1
Croomia pauciflora	United States National Botanic Garden			Live plant, Coded CP2
Croomia pauciflora	Royal Botanic Garden, Kew	Gholson 10360		DNA sample
Pentastemona egregia	Royal Botanic Garden, Kew	J. Bogner 1724, 1985		DNA sample
Pentastemona sumatrana	Royal Botanic Garden, Kew	Duijfjes 21399 (8/1991)		DNA sample
Pentastemona sumatrana	Royal Botanic Garden, Kew	Leiden B.G. 910375		DNA sample
Stemona japonica	Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing	ICM 2004-2543	Museum of Chinese Medicine, Institute of Chinese Medicine, Chinese University of Hong Kong	Dried material
Stemona japonica	Institute of Botany, Chinese Academy of Sciences, Beijing	ICM 2004-2544	Museum of Chinese Medicine, Institute of Chinese Medicine, Chinese University of Hong Kong	Dried material
Stemona japonica	Anhui	Hu and But 24032	Herbarium, Chinese University of Hong Kong	Live plant
Stemona japonica	Nanjing Institute of Botany, Chinese Academy of Sciences	Hu & But 23971	Herbarium, Chinese University of Hong Kong	Live plant
Stemona javanica	Royal Botanic Garden, Kew	Chase 2156 K		DNA sample
Stemona parviflora	Hainan	Ma 9066	Herbarium, Chinese University of Hong Kong	Live plant

Table 2.1 List of plant materials.

Species	Sources	Voucher Specimens	Location of Deposition	Remarks
Stemona parviflora	Hainan	Hu and But 24034	Herbarium, Chinese University of Hong Kong	Live plant
Stemona sessilifolia	Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing	Hu & But 23972	Herbarium, Chinese University of Hong Kong	Live plant
Stemona sessilifolia	China Phamaceutical University	Hu & Yung 606	Herbarium, Chinese University of Hong Kong	Live plant
Stemona sessilifolia	Shandong	Zang 200401	Museum of Chinese Medicine, Institute of Chinese Medicine, Chinese University of Hong Kong	Dried material
Stemona tuberosa	South China Institute of Botany, Academy of Sciences	Chan 200401	Herbarium, Chinese University of Hong Kong	Dried material
Stemona tuberosa	Guang Xi	Woo 23973	Herbarium, Chinese University of Hong Kong	Live plant
Stemona tuberosa	Yunan	ICM 20042541	Museum of Chinese Medicine, Institute of Chinese Medicine, Chinese University of Hong Kong	Live plant
Stemona tuberosa	Hong Kong	Hu & But 23960	Herbarium, Chinese University of Hong Kong	Live plant

Table 2.1 (continued) List of plant materials.

Species	Sources	Voucher Specimens	Location of Deposition	Remarks
Stemona shandongensis	Shandong	Zang 23974	Herbarium, Chinese University of Hong Kong	Live plant
Stemona tuberosa	Royal Botanic Garden, Kew	P. Wilkin 923K.		DNA sample
Stichoneuron caudatum	Royal Botanic Garden, Kew	P Bygrave 50 K		DNA sample
Stichoneuron caudatum	Royal Botanic Garden, Kew	Leiden B.G. 910654		DNA sample
Radix Stemonae (*Stemona tuberosa*)	Purchased from Tong ren tong, Beijing (originally cultivated in Guang Dong)	ICM 20042540	Museum of Chinese Medicine, Institute of Chinese Medicine, Chinese University of Hong Kong	Dried medicinal material
Radix Stemonae (*Asparagus filicinus*)	Yunnan	ICM 20042542	Museum of Chinese Medicine, Institute of Chinese Medicine, Chinese University of Hong Kong	Dried medicinal material

Table 2.1 (continued) List of plant materials.

2.2 DNA Isolation from Plant Materials

Leaves or root tubers of the plant materials were used as raw materials for DNA extraction. Several methods had been used for DNA extraction. Protocol of CTAB (cetyltrimethylammonium bromide) method was modified from Murray and Thompson (1980). Two commercial kits, DNeasy® Plant Mini Kit (Qigen) and GenElute Plant Genomic DNA Miniprep (Sigma) were also applied. For dried medicinal materials, the method of Kang *et al.* (1998) was used for extraction.

2.2.1 Reagents for DNA Isolation

1% CTAB (cetyltrimethylammonium bromide) Extraction Buffer

50 mM Tris-HCl (pH 8.0), 0.7 M NaCl, 10 mM EDTA, 1% (w/v) CTAB, 20 mM 2-mercaptoethanol

2% CTAB (cetyltrimethylammonium bromide) solution

100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 2% (w/v) CTAB, 1% PVP (polyvinylpyrrolidone) Mr 40000

SDS Extraction Buffer

200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA, 0.5% SDS

Chloroform/Isoamyl Alochol (24:1)

48 ml Chloroform, 2 ml Isoamyl Alochol

CTAB Precipitation Buffer

50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% (w/v) CTAB

10% CTAB solution

10% (w/v) CTAB, 0.7 M NaCl

1 M Sodium Chloride Solution

Proteinase K (10 µg/ µl),

DNeasy® Plant Mini Kit (Qigen, Cat. # 69104)

Buffer AP1

Buffer AP2

Buffer AP3/E

Buffer AW

Buffer AE

QIAshredder™ Spin Column

DNeasy Mini Spin Column

100 mg/ ml Rnase A

(Constituents of the reagents were manufacturer's proprietary formulation.)

GenElute Plant Genomic DNA Miniprep

Lysis Solution Part A

Lysis Solution Part B

Precipitation Solution

Binding Solution

Column Preparation Solution

GenElute™ Filtration Column

GenElute™ Nucleic Acid Binding column

(Constituents of the above reagents were manufacturer's proprietary formulation.)

Elution solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

1X TAE Buffer

40 mM Tris-acetate, 1 mM Na_2EDTA

6X Agarose Gel Loading Buffer

40% (w/v) Sucrose, 0.25% (w/v) Bromophenol Blue

1% Agarose Gel

1% Agarose (w/v), 40 mM Tris-acetate, 1 mM Na_2EDTA, 0.5 μg/ml Ethidium Bromide

Gel Documentation System

Gel Doc 1000 (BIO-RAD, cat.# 170-7552)

2.2.2 Procedures of DNA Isolation

2.2.2.1Treatments of Plant Materials

Before DNA extraction, the plant materials were treated to avoid fungal contamination. Fresh leaves were washed thoroughly with distilled water and then 75% ethanol to remove any contaminants. Soil particles were cleaned from root tubers by washing the tubers in distilled water. Then, the tubers were peeled off and the vascular bundle was cleared. The cortexes of root tubers were then rinsed with 75% ethanol. Remaining fresh materials were air dried for keeping in silica gel. Washed plant materials were ground into powder in liquid nitrogen by pestle and mortar. The

powders were kept at –80 °C until use.

2.2.2.2 CTAB (Cetyltrimethylammonium bromide) Method

About 50-100 mg grinded plants material was transferred into 1.5-ml eppendorf. The powder was suspended in 600 µl CTAB extraction buffer and then the suspension was incubated at 56 °C for 30 min. After addition of 600 µl chloroform/isoamyl alochol (24:1), the mixture was mixed gently. The eppendorf was centrifuged at 13000 rpm for 10 min. A layer of debris was formed between aqueous supernatant and the chloroform/isoamyl alcohol layer after centrifugation. About 300 µl supernatant was transferred into a new eppendorf without disturbing the debris layer. Then, 300 µl CTAB extraction buffer was compensated into the first eppendorf, and the tube was centrifuged again at 13000 rpm for 10 min. After centrifugation, another 300 µl supernatant was collected and mixed with the prior 300 µl supernatant. The resulting 600 µl supernatant was mixed with 0.1 volume 10% CTAB solution and equal volume of chloroform/isoamyl alochol (24:1). After centrifugation at 13000 rpm for 10 min, the supernatant was collected and transferred to a new eppendorf and then mixed with equal volume CTAB precipitation buffer. After keeping at room temperature for 30 min, the mixture was subjected to centrifugation at 13000 rpm for 10 min. The solution was then discarded. The pellet left in the eppendof was resuspended in 400 µl 1 M sodium chloride solution and then mixed with 800 µl 95% Ethanol. The mixture was kept at –20 °C for overnight or –70 °C for 30 min.

After centrifugation at 13000 rpm for 10 min, the solution was discarded. To wash the pellet, 1 ml 70% ethanol was added and then the eppendorf was centrifuged at 13000 rpm for 10 min. The solution was then discarded. The pellet was washed twice according to the above procedures. The eppendorf was then placed in a 60 °C heat

block to evaporate the ethanol. The dried DNA pellet was then dissolved in 50 µl autoclaved double distilled water. The resulting DNA extract was kept at –20 °C for storage.

2.2.2.3 DNeasy ® Plant Mini Kit

For DNeasy® Plant Mini Kit, 50–100 mg powder was mixed with 400 µl buffer AP1 and 4 µl 100 mg/ml RNase A. The mixture was incubated at 65 °C for 10 min. Then 130 µl buffer AP2 was mixed into the mixture. After incubated for on ice for 5 min, the mixtures were loaded to the QIAshredder™ spin column sitting in a 2-ml collection tube. After centrifugation at 13000 rpm for 2 min, the flow-through fraction collected at the collection tube was transferred to a new tube. The flow-through fraction was then mixed with 1.5 volume of buffer AP3/E. The resulting mixture was then loaded into a DNeasy mini spin column sitting in a 2-ml collection tube. The tube with column was then centrifuged at 9000 rpm for 1 min. After removal of the flow-through fraction, the column was placed into a new collection tube. The column was loaded with 500 µl buffer AW and then centrifuged at 9000 rpm for 1 min. This step was repeated after discarding the flow-through fraction in the collection tube. The column was then placed into a new tube. The column was loaded with 100 µl preheated (65 °C) buffer AE. After incubation at room temperature for 5 min, the column was centrifuged at 9000 rpm for 1 min to elute the DNA. Elution was repeated to collect several eluates. The eluates, which were the DNA extracts, were collected and stored at –20 °C.

2.2.2.4 GenElute Plant Genomic DNA Miniprep

About 50–100 mg powder was mixed with 350 µl Lysis solution part A and 50 µl Lysis solution part B and 50 units RNase. After vortexing, the mixture was incubated

at 65 °C for 10 min. Then 130 µl precipitation solution was mixed with the lysate by inversion and the mixture was incubated on ice for 5 min. Then the lysate was centrifuged at 13000 rpm for 5 min. The supernatant was loaded to a GenElute™ filtration column sitting on a 2-ml collection tube. The tube was centrifuged at 13000 rpm for 1 min. The flow-through was transferred to a new tube and then mixed with 700 µl binding solution. Before using the GenElute™ nucleic acid binding column, 500 µl of column preparation solution was added to the column. Then the tube was centrifuged at 13000 rpm for 1 min to remove the flow-through. The lysate was then loaded to the binging column sitting on a collection tube. The tube was centrifuged for at 13000 rpm for 1 min, and then the flow-through was discarded. This step was repeated if there were any reminding lysate. Wash solution (500 µl) was loaded to the binding column and then centrifuged at 13000 rpm for 3 min. Then the binding column was transferred to a new collection tube and loaded with 100 µl preheated (65 °C) Elution solution. The column was centrifuged 13000 rpm for one min to elute the DNA. Elution was repeated to collect several eluates. The eluates were the DNA extracts. DNA extracts were stored at –20 °C.

2.2.2.5 Extraction method of Kang *et. al* (1998)

Powdered sample was mixed with 400 µl of SDS extraction buffer and 50 µg proteinase K. The mixture was incubated at 37 °C for 1 h. Then, 400 µl of 2% CTAB solution was added and mixed well. The mixture was then mixed with chloroform:isoamyl alcohol (24:1) with 5% phenol and then centrifuged at 12,000 rpm for 10 min. The supernatant was mixed with 2/3 volume isopropanol and incubated at room temperature for 10 min. At last, the mixture was centrifuged at 12,000 rpm for 5 min. The pellet was washed with 70% ethanol and resuspened in 50 µl TE buffer.

2.2.2.6 Agarose Gel Electrophoresis of Genomic DNA

To determine the size of the DNA extracted, genomic DNA was separated by 1% TAE agarose gel. Five µl genomic DNA was mixed with 1 µl 6X Agarose Gel Loading Buffer. Ethidium bromine was added to the gel for DNA visualization. The samples were loaded into the wells of the gel immersed in a electrophoresis tank of 1X TAE buffer. Lambda HindIII DNA was used as marker. The system was run at 100 Volts for 20 min. The gel was then examined under ultraviolet light and recorded using Gel Doc 1000 (BIO-RAD).

2.3 Polymerase chain reaction (PCR)

Chloroplast DNA region *trn*L-F and genomic DNA repeats 5S ribosomal RNA spacer was amplified by PCR with appropriate primers. The amplified products were purified for further analysis.

2.3.1 Reagents

10X PCR Buffer

100 mM Tris-HCl (pH 8.8), 500 mM KCl

2.5 mM dNTP

2.5 mM 2'-Deoxyadenosine 5'-Triphosphate, 2.5 mM 2'-Deoxycytidine 5'-Triphosphate, 2.5 mM 2'-Deoxyguanosine 5'-Triphosphate, 2.5 mM 2'-Deoxythymidine 5'-Triphosphate

25 mM $MgCl_2$

5 u/μl Taq polymerase

Concert™ Gel Extraction Systems (InvitrogenTM, cat.# 11456)

Primers

For trnL-F region

c	5′- CGA AAT CGG TAG ACG CTA CG -3′	Taberlet *et al.* 1995
d	5′ - GGG GAT AGA GGG ACT TGA AC-3′	Taberlet *et al.* 1995
e	5′ - GGT TCA AGT CCC TCT ATC CC -3′	Taberlet *et al.* 1995
f	5′ - ATT TGA ACT GGT GAC ACG AG -3′	Taberlet *et al.* 1995

For 5S rRNA spacer

S-1	5′- GGA TCC GTG CTT GGG CGA GAG TAG TA -3′
AS-1	5′- GGA TCC TTA GTG CTG GTA TGA TCG CA -3′
5S2F	5′- GTG CTT GGG CGA GAG TAG TA-3′
5S2R	5′- TTA GTG CTG GTA TGA TCG CA -3′

2.3.2 Procedures

Primers c and f were used for amplification of *trn*L-F region while for PCR of 5S rRNA spacer, the primers combinations were either S-1, AS-1 pair or 5S2F, 5S2R pair. PCR was performed in a mixture containing 15.3 µl autoclaved double distilled water, 2.5 µl 10X PCR buffer, 2 µl 2.5 mM dNTP, 2 µl 25 mM $MgCl_2$, 1 u Taq polymerase, 1 µl (10 mM) of both primers and 1 µl template DNA. Thermal cycling was performed in a MJ-PTC100 thermocycler and carried out as follows: one cycle of 95 °C for 5 min; then 20 cycles of 95 °C for 20 sec, 56 °C for 30 sec and 72 °C for 1.5 min; and a final extension at 72 °C for 5 min.

After PCR, the PCR product was separated by 2% TAE agarose gel. Five µl PCR product was mixed with 1 µl 6X Agarose Gel Loading Buffer. The samples were

loaded into the wells of the gel immersed in a electrophoresis tank of 1X TAE buffer. 100bp DNA ladder was used as marker. The system was run at 100 Volts for 20 min. The gel was then examined under ultraviolet light and recorded using Gel Doc 1000 (BIO-RAD). The PCR product was then purified using Concert™ Gel Extraction Systems (Invitrogen™, cat.# 11456).

2.4 Ligation, Transformation and Bacterial Culture for 5S Ribosomal RNA Spacer Analysis

The 5S rRNA spacer, as a rule, is too variable and thus cloning of individual repeats is usually necessary (Soltis and Soltis, 1998). Instead of direct sequencing, purified PCR products were ligated into vectors to form circular DNA plasmids. The ligation was preformed using pGEM®-T Easy Vector Systems (Promega). The resulting plasmids were then transformed into *Escherichia coli* competent cells. Sequences of individual colony were analyzed.

2.4.1 Reagents

Luria-Bertani (LB) Medium

10 g/l Tryptone, 5 g/l Yeast Extract, 10 g/l NaCl

(The solution was sterilized by autoclaving at 121 °C for 20 min)

Luria-Bertani (LB) Agar

10 g/l Tryptone, 5 g/l Yeast Extract, 10 g/l NaCl, 1.5% (w/v) Lacto Agar

(The solution was autoclaved at 121 °C for 20 min. When the solution temperature dropped to about 50 °C, ampicillin was added to make ampicillin concentration 50 μg/ml. Then, 20 ml of solution was poured into petri dish. The plate was left at room temperature until the agar solidified and then kept at 4 °C until use.)

5% X-gal

5% 5-Bromo-4-chloro-3-indoly-β-D-galactopyranoside was dissolved in dimethyl formamide.

0.4 M IPTG

0.4 M Isopropyl-β-D-thiogalactopyranoside

pGEM®-T Easy Vector System I (Promega, Cat.# A1360)

2X Rapid Ligation Buffer, T4 DNA Ligase

60 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol (MW8000, ACS grade)

pGEM®-T Easy Vector

50 ng/µl pGEM®-T Easy Vector

T4 DNA Ligase

3 Weiss units/µl T4 DNA Ligase

pGEM®-T Easy Vector

50 ng/µl pGEM®-T Easy Vector

Escherichia coli (DH5α) competent cells

Stored at –80 °C

Concert™ Rapid Plasmid Miniprep (Invitrogen, Cat.# 11453)

Cell Suspension Buffer (G1)

50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 20 mg/ml RNase A

Cell Lysis Solution (G2)

200 mM NaOH, 1% SDS (w/v)

Neutralization Buffer (G3)

A proprietary formulation of manufacturer containing acetate and guanidine hydrochloride.

Wash Buffer (G4)

A proprietary formulation of manufacturer containing contains NaCl, EDTA and Tris-HCl (pH 8.0). 140 ml 95% Ethanol was added to 55 ml G4 Wash buffer before used.

Optional Wash Buffer (GX)

A proprietary formulation of manufacturer containing contains acetate, guanidine hydrochloride, EDTA, ethanol).

TE Buffer (TE)

10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA

Spin Cartridges

2.4.2 Procedures

2.4.2.1 Ligation

Ligation of PCR products as performed using pGEM®-T Easy Vector System I (Promega). The instruction of manufacturer was strictly followed except that the amount of reagents used was halved. Ligation mixture was prepared by mixing 1.5 µl purified PCR products, 2.5 µl 2X Rapid ligation buffer, 0.5 µl T4 DNA Ligase and 0.5 µl pGEM-T Easy Vector. 2X Rapid ligation buffer was vortexed before use. The ligation mixture was incubated at 25 °C for 3 h.

2.4.2.2 Transformation

An eppendorf of 100 µl *E. coli* competent cells was taken out from –80 °C and kept on ice to thaw the cells. When the cells were just thawed, all 5µl ligation mixture was added into the cells. The competent cells were left on ice for 30 min and then heat-shocked at 42 °C for 2 min. After heat-shock, the competent cells were kept on ice for further 2 min. Then 400 µl of 37 °C LB medium was added to the cells and the mixture was incubated at 37 °C for 50 min. After incubation, the eppendorf was centrifuged at 13000 rpm for 10 sec so that the cells formed a pellet at the bottom of the tube. The LB medium was poured out. Then 20 µl 5% X-gal and 5 µl 0.4 M IPTG were added to the cells. The cells pellet was resuspended. The culture were spread on a LBA plate and incubated at 37 °C overnight.

2.4.2.3 Blue-White Screening

After incubation, two kinds of colonies, blue and white colonies, were formed on the plate. For each plate, several white colonies were selected. Each single white colony was inoculated into 5 ml LB medium containing 50 µg/ml ampicillin. The cultures were incubated at 37 °C overnight with continuous shaking.

2.4.2.4 Plasmid Isolation

Plasmid isolation was preformed using Concert™ Rapid Plasmid Miniprep (Invitrogen, Cat.# 11453). First, the cells were harvested by centrifugation at 13000 rpm for 30 sec. The LB medium was carefully pipetted out. The cell pellet was resuspended and homogenized in 250 µl Cell Suspension Buffer (G1). Then 250 µl of Cell Lysis Solution (G2) was mixed with the cell suspension by inverting the eppendrof five times. Vortex was avoided. After incubation at room temperature for 5 min, 350 µl of Neutralization Buffer (G3) was added into the mixture. Again, it was mixed gently by inverting the tube five times. The eppendrof was then centrifuged at 12,000 rpm for 10 min. The supernatant was transferred in to spin cartridge sitting on a 2-ml collecting tube.

The spin cartridge and collecting tube were centrifuged at 13000 rpm for one min. The flow-through collected in the collecting tube was discarded. To wash the cartridge, 500 µl Optional Wash Buffer (GX) was loaded into the spin cartridge. After 1 min incubation at room temerature, the spin cartridge and collecting tube were centrifuged at 13000 rpm for 1 min. The flow-through collected in the collecting tube was discarded. Then, 700 µl of Wash Buffer (G4) was loaded onto the spin cartridge. The spin cartridge and collecting tube were centrifuged at 13000 rpm for 1 min. After discarding the flow-through in the collecting tube, the spin cartridge and collecting tube were further centrifuged at 13000 rpm for 1 min to remove the residual wash buffer. Then, the spin cartridge was placed in a 1.5 ml recovery tube and 75 µl of warm autoclaved doubled deionized water was added to it. After incubation at room temperature for 1 min, the spin cartridge and recovery tube were centrifuge at 13000 rpm for 2 min. The plasmid eluted was collected and stored at 20 °C.

2.4.2.5 Screening of plasmid DNA by PCR

In order to confirm the presence of insert in the plasmid DNA, PCR was performed using plasmid DNA as template. One µl of plasmid DNA was amplified by PCR using primers 5S2F (5′ GTG CTT GGG CGA GAG TAG TA 3′) and 5S2R (5′ TTA GTG CTG GTA TGA TCG CA 3′). PCR reaction was performed in a mixture containing 15.3 µl autoclaved doubled deionized water, 2.5 µl PCR buffer, 2 µl 2.5 mM dNTP, 2 µl 25 mM $MgCl_2$, 1 u Taq polymerase, 1 µl 10 uM primer 5S2F, 1 µl primer 5S2F and 1 µl plasmid DNA. Thermal cycling was performed in a MJ-PTC100 thermocycler and carried out as follows: one cycle of 95 °C for 5 min; then 30 cycles of 95 °C for 20 sec, 56 °C for 30 sec and 72 °C for 1.5 min; and a final extension at 72 °C for 5 min.

After PCR, the DNA amplified was separated by 1% agarose gel electrophoresis. The gel was examined under ultraviolet light after electrophoresis.

2.5 Cycle Sequencing and Electrophoresis

BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, PN 4337455) was used for cycle sequencing of the purified PCR products or plasmid DNA. To analyze *trn*L-F sequences, purified PCR products were used for sequencing directly while plasmids with PCR product inserted were used for 5S rRNA spacer sequences analysis. The products of cycle sequencing were purified by ethanol precipitation and then resuspended in HiDi Formamide (Applied Biosystems, PN 4311320). ABI PRISM® 3100 Genetic Analyzer was then used for electrophoresis of the samples.

2.5.1 Instruments and Reagents

ABI PRISM® 3100 Genetic Analyzer

Sequencing Run Configuration:

- Capillary: 80-cm Capillary Array (Applied Biosystems, PN 4319899)
- Matrix: 3100 POP-4 Polymer (Applied Biosystems, PN 4316355)
- Dye Set: Z
- Mobility File: DT3100POP4{BDv3}v1.mob
- BioLIMS Project: 3100_Project1
- Run Module: LongSeq80_POP4DefaultModule
- Analysis Module: BC-3100POP4_80cm_SeqOffFtOff.saz

BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, PN 4337455)

Ready Reaction Mix

(A proprietary formulation of the manufacturer.)

BigDye Terminator v1.1/3.1 Sequencing Buffer (5X) (Applied Biosystems, PN

4305605)

(A proprietary formulation of manufacturer, containg Tris-HCl and $MgCl_2$ buffer)

3M Sodium Acetate, pH5.2

Hi-Di™ Formamide Sample Resuspension Solution (Applied Biosystems, PN 4311320)

MicroAmp Optical 96-well plate (Applied Biosystems, PN N801-0560)

96-well plate septum (Applied Biosystems, PN 4315933)

2.5.2 Procedures of Cycle Sequencing and Electrophoresis

2.7.2.1 Cycle sequencing

Cycle sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit. Manufacturer's instruction was strictly followed except that the amounts of reagents added were halved. Primers c or d (5′ GGG GAT AGA GGG ACT TGA AC 3′) was used for sequencing of trnL-F sequences in 5′ and 3′ direction respectively. For 5S rRNA spacer sequences, primer M13 Forward (5′- GTA AAA CGA CGG CCA GT -3′) or primer M13 Reverse (5′- AAC AGC TAT GAC CAT G -3′) was used.

For each cycle sequencing reaction, 5–20 ng purified PCR product or 0.5–1.0 μg plasmid DNA was mixed with 2 μl Ready Reaction Premix, 1 μl 5X BigDye Terminator v1.1/3.1 Sequencing Buffer, 1.6 pmol primer. Water was added to make

the final volume 10 µl. Then the mixture was subjected to thermal cycling in MJ-PTC100 thermocycler. Twenty-five cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min was performed and the temperature was held at 4 °C until ready to purify.

2.5.2.2 Ethanol Precipitation

Each cycle sequencing product was mixed with 1 µl 3M sodium acetate, pH 5.2 and 25 µl 95% ethanol. The mixture was transferred into a 1.5 ml eppendrof and then vortexed briefly. The tube was kept at –20 °C for 10 min. After centrifugation at 13000 rpm for 30 min, the supernatant was removed. Then, 200 µl 75% ethanol was added. The tube was vortexed. After centrifugation at 13000 rpm for 5 min, the supernatant was removed. The pellet was air dried and stored at –20 °C until use.

2.5.2.3 Electrophoresis

The purified cycle sequencing product was resuspended in 12 µl Hi-Di™ Formamide. After vortexed, the suspension was loaded into a well of MicroAmp Optical 96-well plate with 96-well plate septum. The plate with septum was put on the MJ-PTC100 thermocycler to denature the suspension at 95 °C for 3 min. The plate was placed on ice after denatured.

The plate with samples was put into the ABI PRISM® 3100 Genetic Analyzer for electrophoresis. The configuration of electrophoresis has been mentioned in section "2.7.1 Instruments and Reagents". ABI PRISM® 3100 Genetic Analyzer Data Collection Software – version 1.0.1 was used to control the electrophoresis process. After electrophoresis, the data was analyzed using ABI PRISM® Sequencing Analysis 3.7 and two files were generated for each sample. The file type ".seq" can

be opened by Microsoft notepad while ".abi" file outputted can be opened by program Chromas 1.45.

2.6 Sequence Analysis

Using the program Clustalw of the European Bioinformatics Institute (EBI), the sequences were aligned and the percentage similarity among sequences were calculated. After alignment, Molecular Evolutionary Genetics Analysis software (MEGA) version 2.1 (Kumar *et al.* 2001) was used for phylogenetic analysis. Unweighted Pair Group Method with Arithmatic Mean (UPGMA), Neighbor Joining and Maximum Parsimony trees were constructed. For Neighbor Joining trees and UPGMA trees, the distances were calculated using the algorithum Kimura 2-parameter. For parsimony analysis, parsimonious trees were searched using close-neighbor-interchange (CNI) method. Bootstrap test was applied for 500 replications.

Chapter 3. Taxonomic Study of Chinese *Stemona* species

In order to establish a solid basis for the development of molecular markers for the authentication of Radix Stemonae, it was necessary to first understand the taxonomic status of all the *Stemona* species found in China.

3.1 History of the Genus *Stemona*

The genus *Stemona* was first described by Loureiro (1790). The synonym *Roxburghia* had also been used to name the same genus (Lindley 1853, Hutchinson 1959). *Stemona* has about 15 to 30 species (Rogers 1982, Dahlgren *et al.* 1985, Van der Ham 1991) spreading from Sri Lanka and eastern India to Japan, and through Malaysia to northern Australia (Kubitzki 1998, Van der Ham 1991).

According to the *Florae Reipublicae Popularis Sinicae* (Ji 1997), five *Stemona* species (*S. japonica, S. mairei, S. parviflora, S. sessilifolia* and *S. tuberosa*) are found in China. Ji and Duyfjes (2000) accept *S. kerrii* and *S. shandongensis* also occur in China. Thus there are seven reported *Stemona* species in China. Cong and Xu (1997) have also mentioned two new taxa, namely, *S. jinshajiangensis* and *S. jinshajiangensis* var. *dianbeiensis*. However, they have not been validly and formally published. These names are *nomen nudum*, without taxonomic status.

In this thesis project, five species of *Stemona* were planted in the greenhouse of the Biology Department, the Chinese University of Hong Kong. Morphological study was based on living specimens except *S. mairei* and S. *kerrii*. Voucher specimens deposited in Harvard University Herbaria (AA), the United States National Herbarium (US), Sun Yat-Sen University Herbarium (SYS) and the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing were also studied for comparison.

3.2 Characteristics in Genus *Stemona*

Subshrubs or vines, perennial. Roots tufted, tuberous, fusiform, fleshy (Figure 3.4J). Stems erect or climbing. Leaves whorled, opposite, or alternate, shining, petiolate or sessile; main veins 3 or more, transverse veinlets numerous. Inflorescences racemes or cymes, 1- to few flowered; peduncle axillary or borne on petiole or leaf midvein (Figure 3.2D, 3.3A); pedicel with articulation (Figure 3.1C) and bracts (Figure 3.3C).

Flowers bisexual, actinomorphic. Perianth segments 4, in 2 whorls, subequal or segments of inner whorl slightly larger, lanceolate, many veined, free. Stamens 4, in single whorl, subhypogynous, opposite to tepals, fleshy; filaments free or basally connate in a ring, short; anthers embedded on the expanded connective, erect, linear, introrse. In all the species investigated, the anther apex connected into a yellow or pale green sterile apical appendage (Figure 3.1C); the 4 sterile apical appendages pressed together, forming a crown-like structure. Connective attenuated into a fleshy connective extension, extending beyond the anther regions (Figure 3.1A). The connective also bearing a keel-like fleshy outgrowth on the adaxial surface between the anthers (Figure 3.1B). Ovary superior, 1-loculed; ovules 2 or more, basally attached to placenta. Stigma sessile, small. Capsule ovoid to oblong, slightly compressed, 2-valved.

Seeds 1-several, albuminous, arillate, the aril white, beard-like (Figure 3.1F); testa leathery, grooved.

The peculiar structures found in the stamens have attracted attention. Different authors used different descriptive terms. The sterile apical appendage of anther was also described as "adaxial sterile synangial peaks" (Van Heel 1992), sterile appendix (Duyfjes 1993), prolongation of endothecium (Swamy 1964) or free endothecium (Hooker 1890). However, it was said that this structure was absent in some *Stemona* species, for examples *S. australiana* (Telford 1986). The connective extension was described as subulate apical appendage of connective (Telford 1986), or tepal-like appendage of connective (Duyfjes 1993). The keel-like outgrowth on the connective between the

anthers was also described as lamellate outgrowth of connective (Ji and Duyfjes 2000) or median sterile longitudinal ridge (Van Heel 1992, Duyfjes 1993, Swamy 1964).

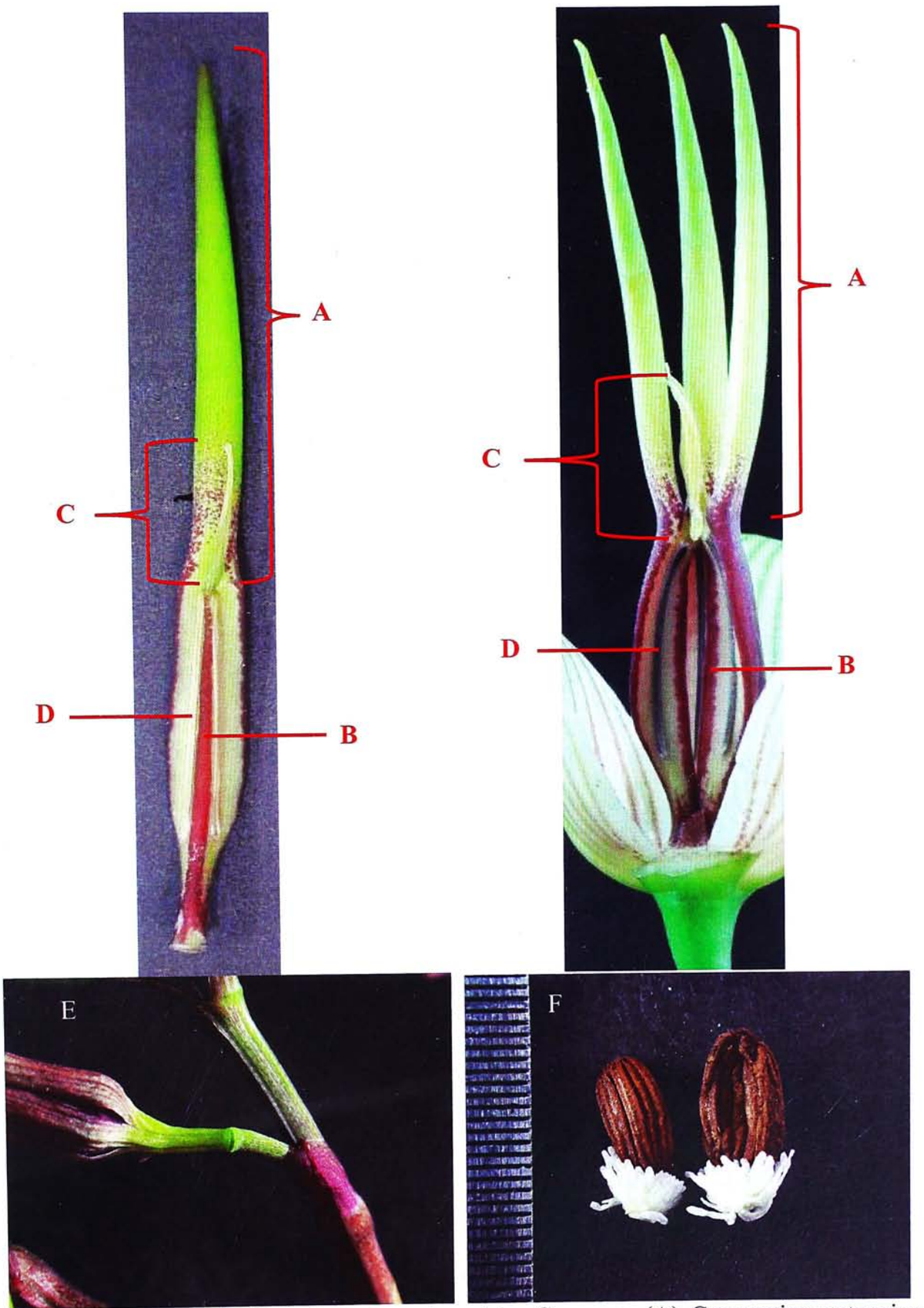

Figure 3.1 Morphological characters shared by *Stemona*. (A) Connective extension; (B) Keel-like outgrowth of connective;; (C) sterile apical appendage of anther; (D) Anther; (E) Articulate at pedicel; (F) Seeds.

3.3 Characteristics of *Stemona sessilifolia* (Miquel) Miquel (including *S. shandongensis* D. K. Zang)

The live *Stemona sessilifolia* specimens studied were originally planted in the Institute of Medicinal Plants, Beijing and the China Phamaceutical University, and the voucher specimens were coded as Hu & But 23972 and Hu & Yung 606. The live *S. shandongensis* specimens studied were collected from Shandong by D.K. Zang, the author of this species. The specimen collected was then planted in our greenhouse. The voucher specimen was coded as Zang 23974.

This plant is a subshrub or somewhat climbing vine. Root tubers tufted, spindle-shaped, 1–1.5 cm thick, 5–15 cm long (fig. 3.2A). Stems erect, simple, 30–70 cm. Leaves 2–5 whorled, shortly petiolate or sessile, entire, obovate- or ovate-elliptic or ovate-lanceolate, 3.5–6 ×1.5–4 cm, the base cuneate, the apex shortly acute (Figure 3.1B); venation bowed, 5 veins.

Inflorescences usually borne in scale axils at base of stem, and at leaf axils also; 1-flowered (Figure 3.2C-D); pedicel 1–1.5 cm, articulate near or above the middle; bracts scalelike (Figure 3.2F). Zang (1996) reported the basal 1/3 to 1/2 of the pedicel of *S. shandongensis* is adnate to the leaf base.

Perianth segments 4, in 2 whorls, ovate-lanceolate, abaxial surface pale green with purple at the edges (Figure 3.2I), the adaxial surface purple at the base and pale green at the tip (Figure 3.2J), 10–15 ×2–4 mm, segments of inner whorl slightly larger than the outer.

Stamens basifixed, introrse, purple, slightly shorter than the perianth; the filaments free, 2–4 mm, stout; the connective extensions fleshy, purple and flat; the anthers yellow, 3.5 mm; the sterile apical appendages yellow (Figure 3.2K-L).

Ovary 1 mm, bearing 1-5 ovules. Stigma sessile, short (Figure 3.2M). Capsules ovoid, 7–9 ×4–6 mm, 1–several seeded. Flower in March–May; fruit from June to July. However flowering in November was also observed in our green house.

The morphological characteristics of *S. sessilifolia* (Figure 3.2) and *S. shandongensis* (Figure 3.3) are overlapping, except the epifoliate pedicel found in the type specimen of the latter. The two taxa are here grouped together into *S. sessilifolia*. Our molecular data presented in Chapter 4 also support this merge.

Figure 3.2 Morphology of *Stemona sessilifolia*: (A) root tubers; (B) Leaves arranged in whorled; (C, D) Inflorescences borne in scale axils at base of stem or axillary; (E) Flower; (F) articulate pedicel.

Figure 3.2 (Continued) Morphology of *Stemona sessilifolia*: (G) Flower with one perianth segment and one anther removed, showing the yellow appendages; (H) Flower with all perianth segments removed. (I) Abaxial side of perianth segment, the two segments in the middle are the inner whorls; (J) Adaxial side of perianth segment, the two segments in the middle are the inner whorls; (K) Adaxial side of stamen; (L) Sideview of stamen.

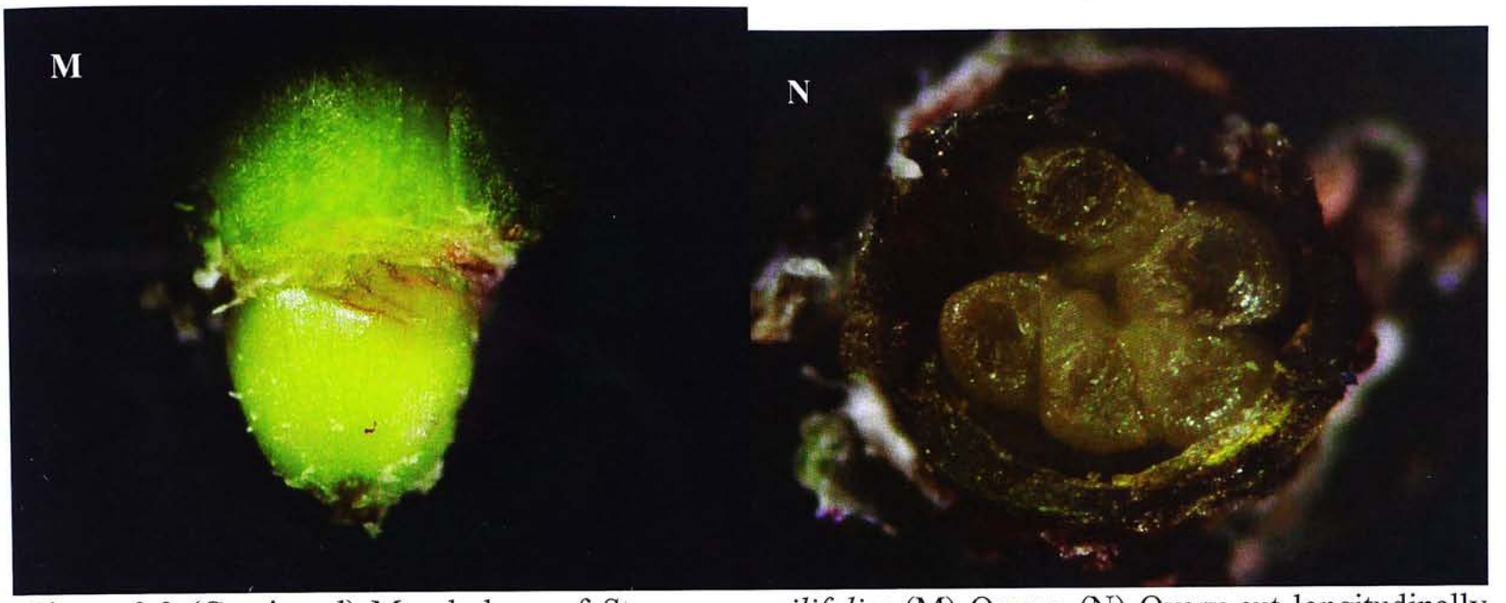

Figure 3.2 (Continued) Morphology of *Stemona sessilifolia*: (M) Ovary; (N) Ovary cut longitudinally, with five ovules inside.

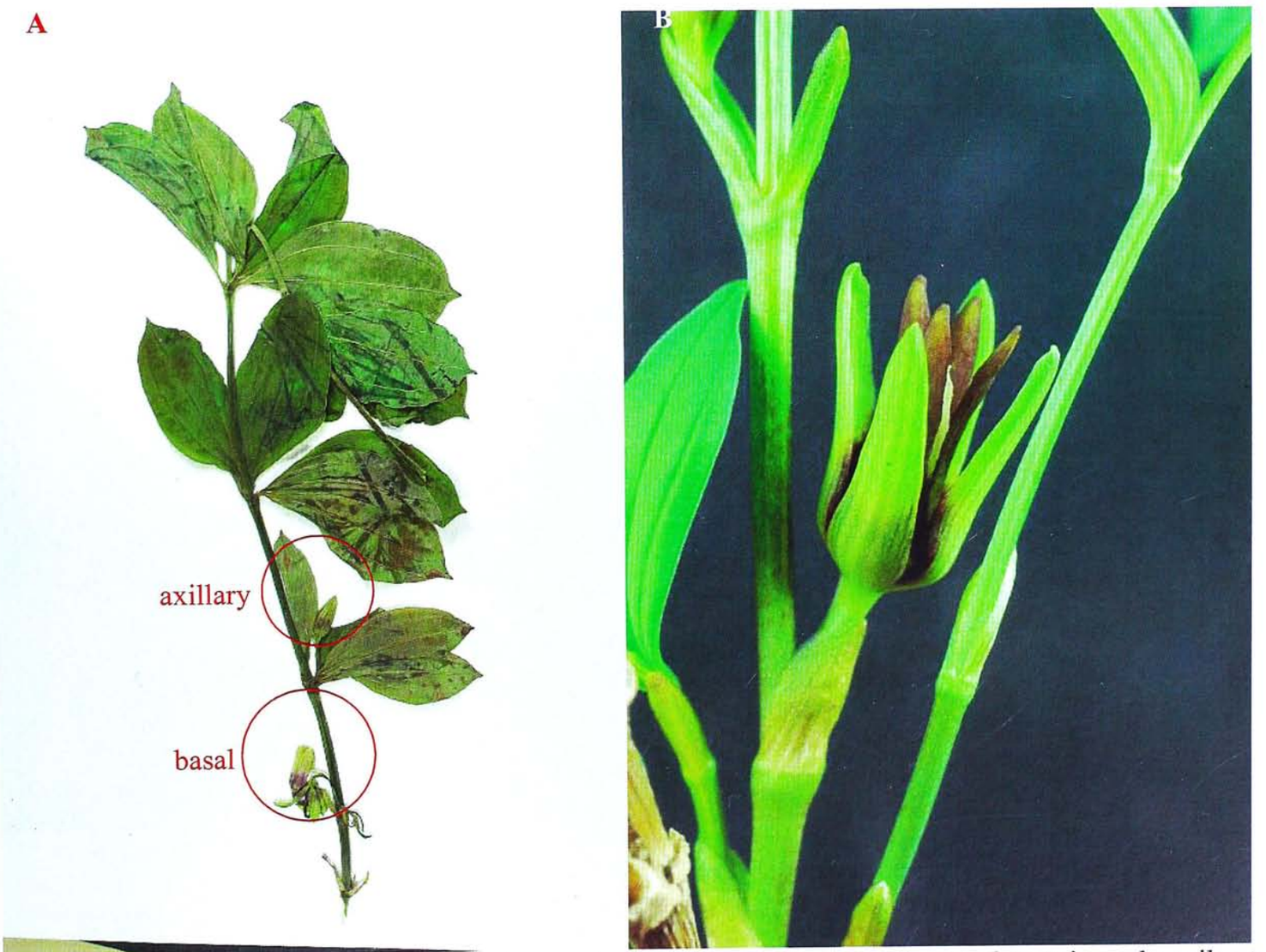

Figure 3.3 Morphology of *Stemona shandongensis*: (A) Inflorescences axillary or borne in scale axils at base of stem, 1-flowered ; (B) Flower.

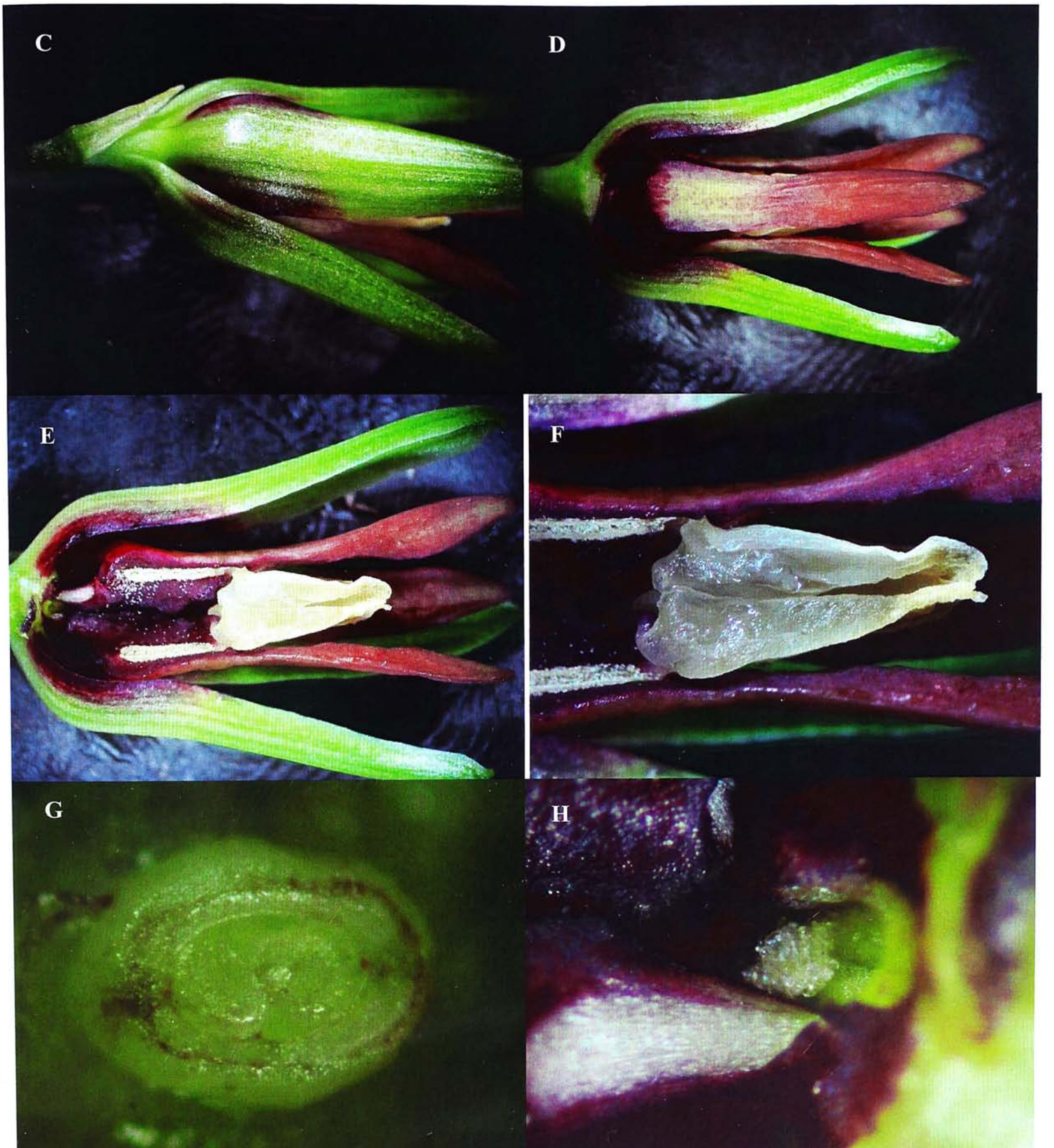

Figure 3.3 (Continued) Morphology of *Stemona shandongensis*: (C) Flower; Pedicel with a bract and articulate; (D) Flower with one tepal removed; (E-F) Flower with one tepal and one stamen removed, showing the anther and the sterile apical appendages; (G) Ovary; (H) Stigma.

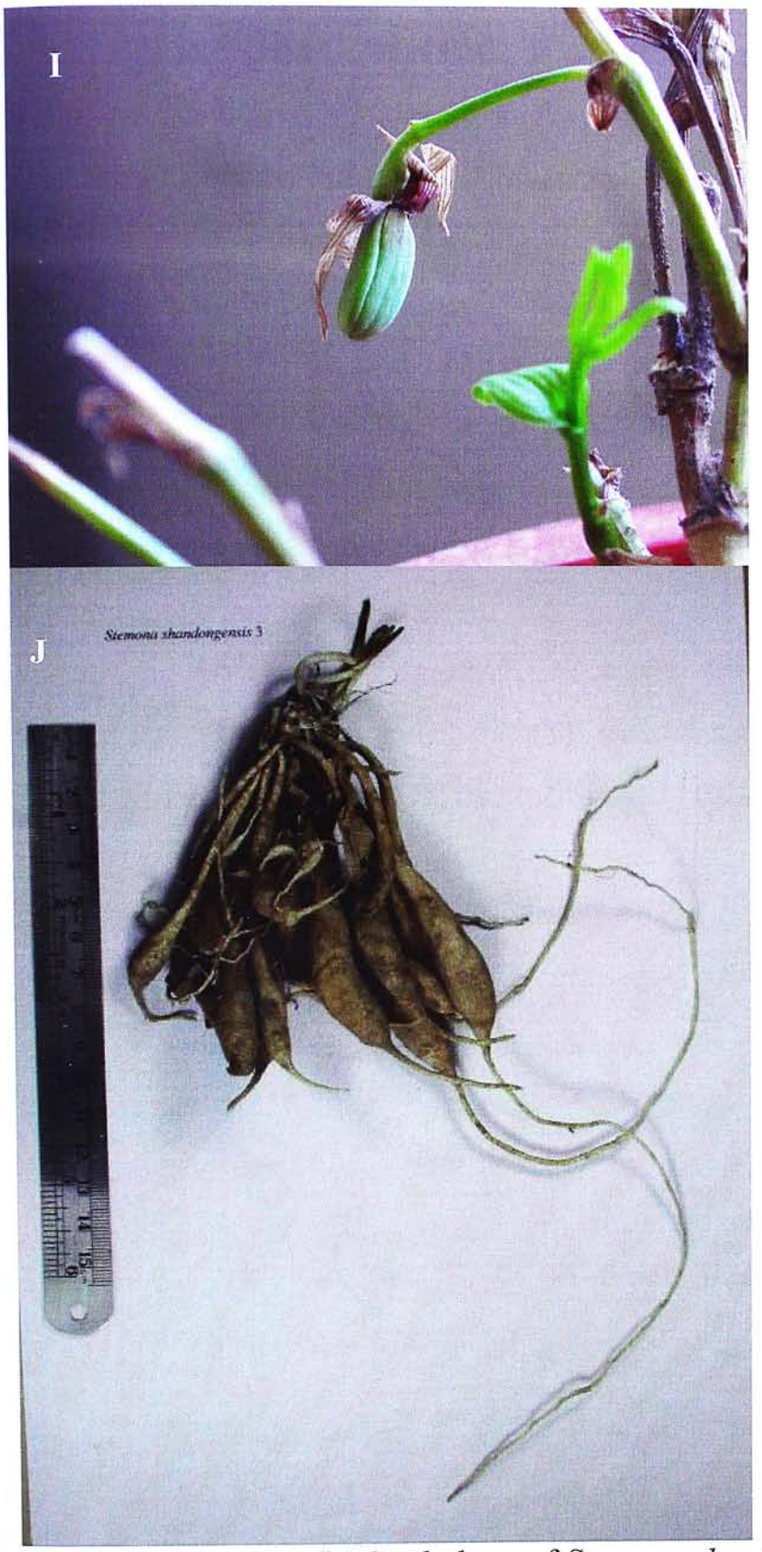

Figure 3.3 (Continued) Morphology of *Stemona shandongensis*: (I) Capsule; (J) Root tubers.

3.4 Characteristics of *Stemona japonica* (Blume) Miquel

The live specimens studied were originally from the Institute of Botany, Beijing, Chinese Academy of Sciences and from Anhui. The specimens were coded as Hu and But 24032 and ICM 2004-2544, respectively.

Plants vine, perennial. Root tubers 1–1.5 cm thick. Leaves 2-5 whorled; petiole 1–4 cm, slender; leaf blade ovate, ovatelanceolate, or ovate-oblong, 4–9 ×1.5–4.5 cm, veins 5 or more, base subtruncate to rounded, rarely rounded-cordate or cuneate, the margin entire or slightly undulate, the apex acuminate.

Inflorescences cymes, 1- to several flowered; Peduncle borne on leaf midvein (Figure 3.4A, L), 0.5–4 cm, slender; pedicel articulate about 5 mm below the flower; the bracts near the base of peduncle or pedicel narrowly lanceolate, about 3 mm (Figure 3.4A, D).

Perianth segments green, lanceolate, 10–15 ×2–3 mm. (Figure 3.4C, D). Stamens pale green and purplish in color, slightly shorter than perianth; the abaxial surface and the connective extension pale green while the adaxial side purple; filaments 1 mm; anthers 2–2.5 mm, yellow; the sterile apical appendage green, bearing silky hairs. (Figure 3.4E-I)

Capsules oblong, 10–14 ×4–8 mm, often 2- or 3-seeded. Seeds, arillate (Figure 3.4K). Flower from May to July.

Figure 3.4 Morphology of *Stemona japonica*: (A) Pedicel borne on leaf midvein; (B-C) Flower with articulate on pedicel; (D-E) Dissected flower, showing the stamens; (F) Stamen.

Figure 3.4 (continued) Morphology of *Stemona japonica*: (G) Adaxial side of stamen; (H) side view of stamen; (I) Sterile apical appendage of anther, with silky hairs borne on either side; (J) Dried fruit; (K) Seeds, with white aril; (L) Peduncle borne on leaf midvein.

3.5 Characteristics of *Stemona tuberosa* Loureiro

One specimen was collected from Guang Xi and then planted in the greenhouse in our campus. Another is a local specimen planted in the herb garden on our campus. The specimens were coded as Woo 23973 and Hu & But 23960, respectively.

Plants vine, perennial. Root tubers 9–13 long, 1–2 cm across. Stems often branched, base woody.

Leaves opposite, rarely alternate, sometimes both phyllotaxy appearing on the same plant.

Petiole 3–10 cm; leaf blade ovate to ovate-lanceolate, 6–24 ×5–17 cm, veins 7–13, the base cordate, the margins entire, slightly undulate, the apex acuminate.

Inflorescences racemes, 1–3 or more flowered; peduncle or pedicel axillary (Figure 3.5D), usually 2.5–5 (–10) cm; the pedicel articulate; the bract lanceolate, 5–10 mm (Figure 3.5E).

Appearance of perianth segments very variable. For the local sample, tepals pale green on the abaxial surface with purple at the edge, purple on the adaxial surface, linear lanceolate, 4.5 ×0.5–0.8 cm (Figures 3.5 A, B, F). For Guangxi samples, pale green on both abaxial and adaxial surfaces are, the base obtuse, the apex acuminate, 3.5–7.5 × 1–1.5 cm (Figure 3.5 C,D,G,H).

Stamens 4, slightly shorter than perianth (Figure 3.5 I-L); the connective extension pale green; the keel-like connective outgrowth purple; filaments stout, 2–5 mm; anthers linear, yellow or black, 10 mm; sterile apical appendages yellow (Figure 3.5 K,L).

Ovary 3 mm, 1-loculed, many ovules (Figure 3.5 N-Q). Stigma sessile, small (Figure 3.5

O-Q).

Capsules ovoid-oblong, 2.5–6 ×1–3 cm (Figure 3.5 R, S). Seeds several, grooved, arillate (Figure 3.5 T). Flower from April to July; fruits from June to August.

Ji and Duyfjes (2000) mentioned the peduncle or pedicel rarely borne on petiole, however, this is not observed in our samples.

Figure 3.5 Morphology of *Stemona tuberosa*: (A, B) Flower of *S. tuberosa* in Hong Kong; (C-D) Flower of *S. tuberosa* from Guang Xi; (E) Inflorescence, articulate and bract; (F) Dissected flower of *S. tuberosa* in Hong Kong (from left to right: abaxial side of outer whorl tepla, abaxial side of inner whorl tepal, stamens and ovary, adaxial side of inner whorl tepal, adaxial side of outer whorl tepal).

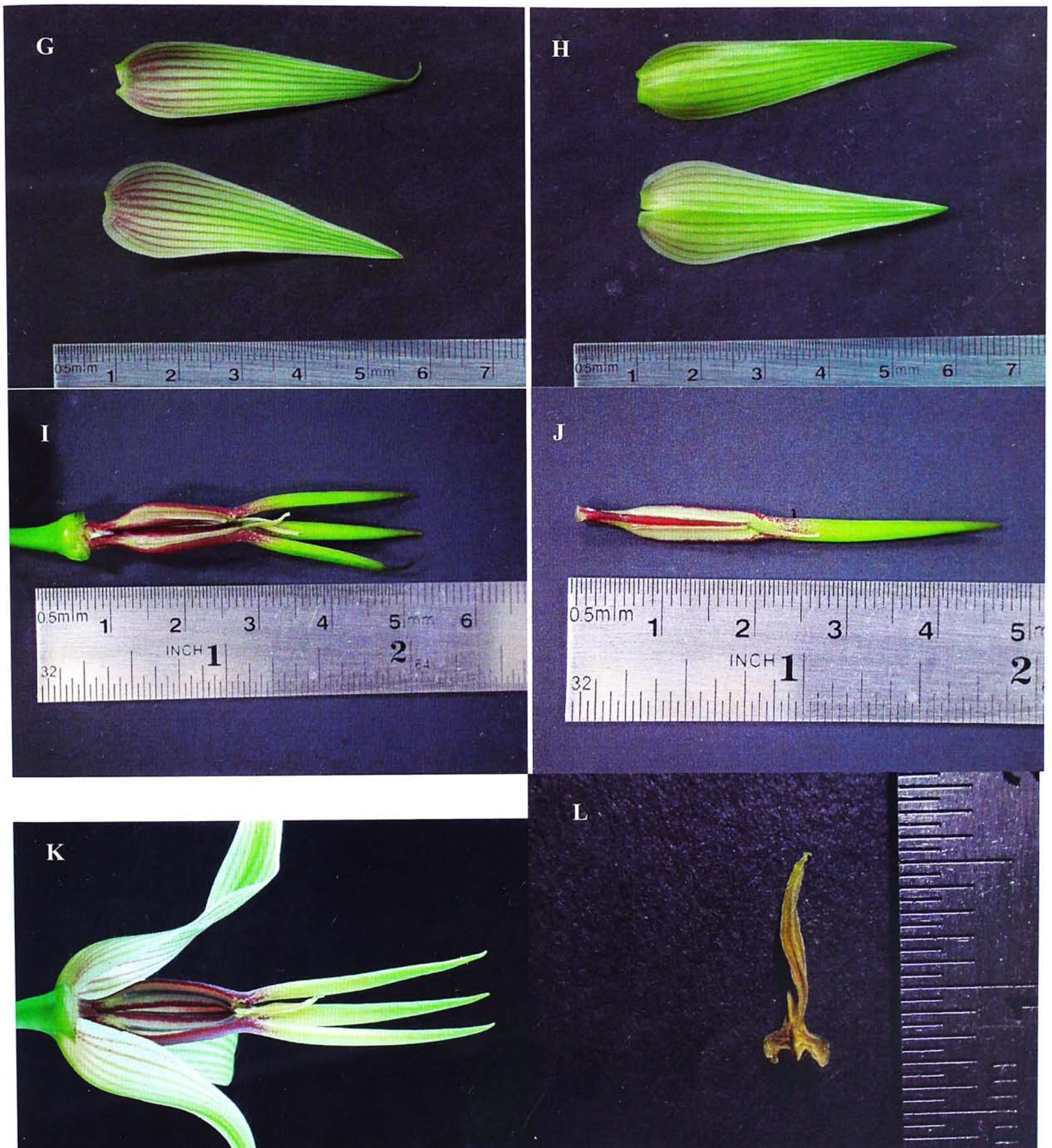

Figure 3.5 (continued) Morphology of *Stemona tuberosa*: (G) Adaxial side of tepals (upper: outer whorl tepal; lower inner whorl tepal); (H) Abaxial side of tepals (upper: outer whorl tepal; lower inner whorl tepal); (I-J) Stamens, with yellow anther; (K) Dissected flower with black anther; (L) yellow sterile apical appendages of anthers, 4 appendages fused.

Figure 3.5 (continued) Morphology of *Stemona tuberosa*: (M) Cross-section of fertile part of stamen, with adaxial side downward; (N) Cross-section of ovary; (O-Q) Ovary; (R) Fruit.

Figure 3.5 (continued) Morphology of *Stemona tuberosa*: (S) Dehiscence capsule; (T) Seed.

3.6 Characteristics of *Stemona parviflora* C. H. Wright

The specimens studied were collected from Hainan and planted in the greenhouse on our campus. The voucher specimens were coded as Ma 9066 and Hu and But 24034.

Plant vine, perennial. Stems many branched, usually 40–150 cm long. Leaves alternate, the petiole 1–3.5 cm, the leaf blade lanceolate, 5–10 ×1–3 cm, veins 5-7, the base subrounded to cuneate or cordate, margin slightly undulate, apex acuminate to caudate-acuminate.

Inflorescences axillary, racemes, 2–6 flowered (Figure 3.6A), the pedicel 0.5-1 cm, slender, articulate at the middle (Figure 3.6B); the bracts on pedicel subulate, small.

Perianth segments 4, in 2 whorls, ovate-lanceolate. Tepals of outer whorl 0.45 ×1cm, purple, pale green on abaxial side (Figure 3.6D), tepals of inner whorl 0.3 ×1.2 cm, pale green with purple at base of adaxial side (Figure 3.6E).

Stamens purple, slightly shorter than perianth; the connective extensions fleshy, purple, flat; the filaments 1 mm, slender; anthers yellow, 2 mm; sterile apical appendages (Figure 3.6 F-J).

Ovaries ovoid 1.5 ×1 mm (Figure 3.6K). Fruit 1.2–2 ×1 cm , seeds about 5 (Figure 3.6L). Flower from April to July.

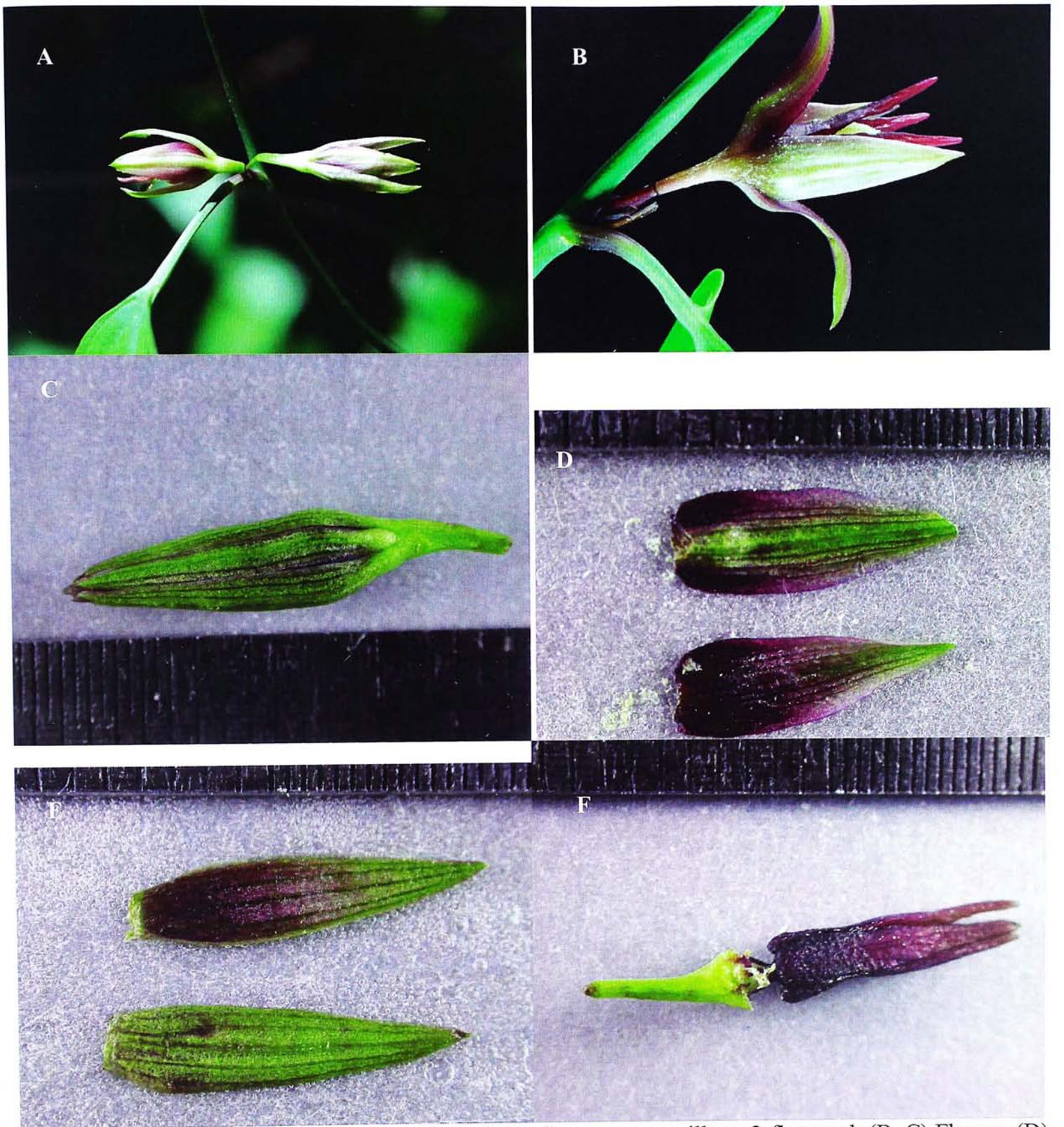

Figure 3.6 Morphology of *Stemona parviflora*: (A) Inflorescences axillary, 2-flowered; (B, C) Flower; (D) Tepals of outer whorl (upper: abaxial side; lower adaxial); (E) Tepals of inner whorl (upper: adaxial side; lower abaxial); (F) Flower with tepals removed.

Figure 3.6 (Continued) Morphology of *Stemona parviflora*: (G-J) Dissected flower, showing yellow stamens and sterile apical appendages; (K) Ovary; (L) Capsule.

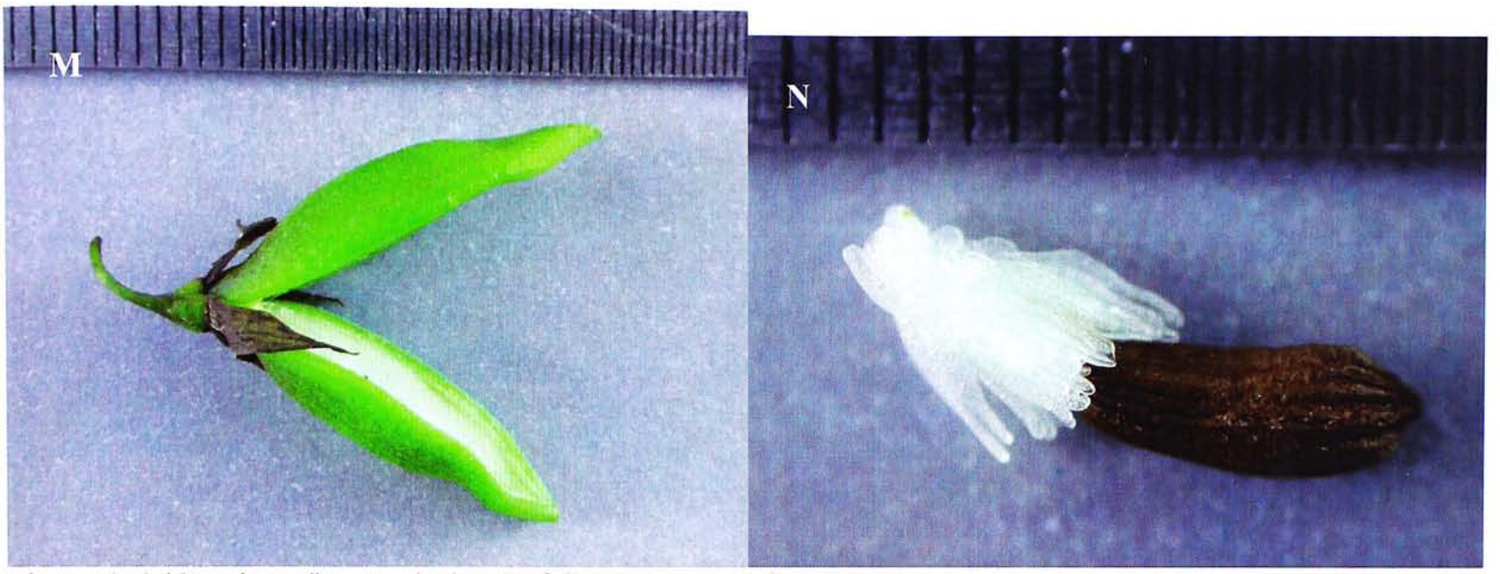

Figure 3.6 (Continued) Morphology of *Stemona parviflora*: (M) Dehiscence capsule; (N) Seed.

3.7 Characteristics of *Stemona mairei* (H. Léveillé) K. Krause

The description here was modified from Ji and Duyfjes (2000) based on the observation of voucher specimens deposited in Harvard University Herbaria (AA) and the United States National Herbarium (US). Representative specimens are Handel-Mazzetti 4391 (AA), Herbier E. Dake 376 (AA) and Rock 5144 (US).

Plants vine, perennial. Root tubers ovoid-oblong. Stems sometimes branched, 20–100 cm. Leaves opposite or 4-whorled, subsessile; leaf blade narrowly ovate to linear, 1.5–7 ×0.2–1.2 cm, veins 3–5, the base rounded to cuneate, the apex acute.

Inflorescences axillary or adnate to base of leaf midvein at 1 cm from the leaf base (Figure 3.7A), erect, racemes, 1-2 flowered; peduncle 1–3 cm; bracts on peduncle setaceous, 3 mm.

Perianth segments white tinged with pink, 2–2.5 ×0.5–0.8 cm, apex acute. Stamens shorter than perianth; the filaments very short; the anthers 6 mm; the connective extension 5 mm, obtuse; the sterile apical appendage 2 mm. (Figure 3.7 B,C)

Ovaries ovoid, small; ovules 6. Capsules globose-ovoid, 8 ×7 mm, 5-seeded (Figure 3.7D). Flower from April to July.

Figure 3.7 Morphology of *Stemona mairei*: (A) Inflorescences adnate to base of leaf midvein; (B, C) Flower; (D) Fruit.

3.8 Characteristics of *Stemona kerrii* Craib

The descriptions here were modified from Ji and Duyfjes (2000) based on the observation of voucher specimens deposited in Harvard University Herbaria (AA) and the United States National Herbarium (US) and also the photos of live plant in Kaltenegger *et al.* (2003).

Plants vine, perennial, shortly hairy. Root tubers 4–7 mm thick. Stems to 70 cm, woody at base. Leaves alternate; the petiole 0.2–7 cm, slender; leaf blade ovate to broadly ovate, 7–10 ×3–6 cm, veins 11–13, the base deeply cordate, the apex acuminate.

Inflorescences axillary, racemes, few flowered; peduncle filiform, 1.5–2 cm; bracts 3 mm.

Perianth segments 4, pink, 1–1.5 ×0.3–0.4 cm, the margins of inner ones crenulate, the apex acute. Stamens purple, equaling or longer than perianth; filaments very short; anthers 5–6 mm; sterile apical appendage of anther yellow.

Capsules globose-ovoid, 8–10 × 6–9 mm, 1- or 2-seeded.

Figure 3.8 Morphology of *Stemona kerrii*: (A,B) Flower; (C) Fruit and seed. (Photos adopted from Kaltenegger *et al.* (2003).)

Chapter 4. DNA Sequence Analysis for Authentication and Systematics

According to the Pharmacopoeia of the People's Republic of China (2000), Radix Stemonae is the root of *Stemona japonica, S. sessilifolia* or *S. tuberosa*. The dried root tubers of different *Stemona* species and adulterant are very similar in appearance and thus it is difficult to differentiate them from one another. In this thesis project, the *trn*L and 5S rDNA spacer regions were chosen to be the molecular markers to authenticate Radix Stemona. Apart from authentication, the molecular data were also used to solve some questions about systematics. Botanists have been debating on the circumscriptions and affinity of Stemonaceae. Which genera should be included in Stemonaceae? Is *Pentastemona* worthy of a family rank? Which order should Stemonaceae be placed in? Phylogenetic analysis of *trn*L and 5S rDNA spacer sequences would help to shed lights on these questions. Molecular phylogenetic analysis methods will be applied to answer these questions.

Totally 27 samples representing two *Croomia* species, two *Pentastemona* species, six *Stemona* species, one Stichoneuron species and one *Asparagus* species were collected. Among the 27 samples, eight of them were DNA supplied from Royal Botanic Garden, Kew. The other 19 samples are either fresh plants or dried plant materials. The DNA was extracted from the 19 plant material samples (part 4.1). The DNA extracts of 27 samples were then amplified by PCR (part 4.2) and then sequenced. The sequences were analysed for authentication (part 4.3) and for systematics study (part 4.4).

4.1 DNA Extraction

Genomic DNA were either extracted from plant materials collected or purchased from DNA bank of Royal Botanic Gareden, Kew. Totally, 19 samples representing eight species was extracted by the methods mentioned in chapter 2. The genomic DNA extracted was analyzed by gel electrophoresis in 1% agarose gel with EB (Figures 4.1, 4.2 and 4.3). DNA were visualized under UV. DNA samples purchased from Royal Botanic Gareden, Kew were not subjected to gel electrophoresis due to scarcity of samples.

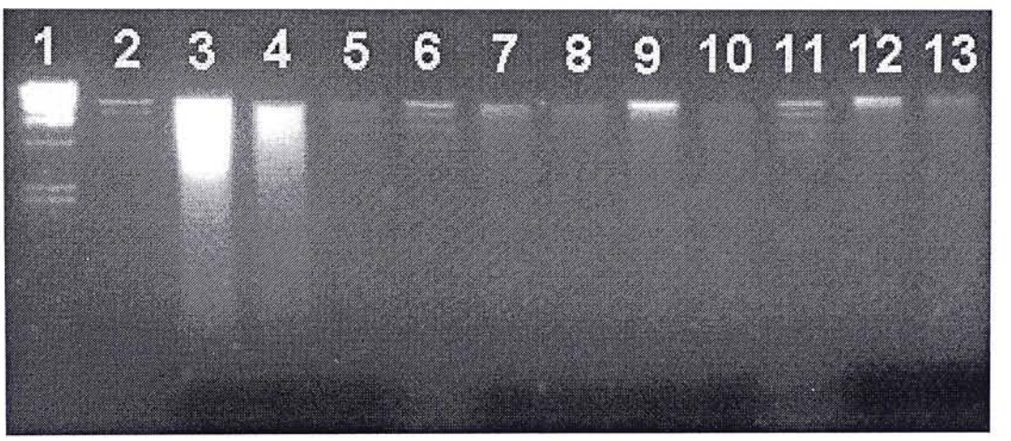

Figure 4.1 Agarose gel electrophoresis of total DNA. Lane 1 is Lambda DNA-*Hind* III Digest (84 μg). Lanes 2, 6 and 11 are Lambda DNA-*Hind* III Digest (8.4 μg). Lane 3 is *Croomia japonica* (Hu & But 24033). Lane 4 is *Croomia pauciflora* (code: CP1). Lane 5 is *Croomia pauciflora* (code: CP2). Lane 7 is *Stemona japonica* (ICM 2004-2543). Lane 8 is *Stemona japonica* (ICM 2004-2544). Lane 9 is *Stemona japonica* (specimen code: But 1) from Anhui providence. Lane 10 is *Stemona japonica* (Hu and But 24032). Lane 12 is *Stemona parviflora* (specimen code: Ma 9066). Lane 13 is *Stemona parviflora* (Hu and But 24034).

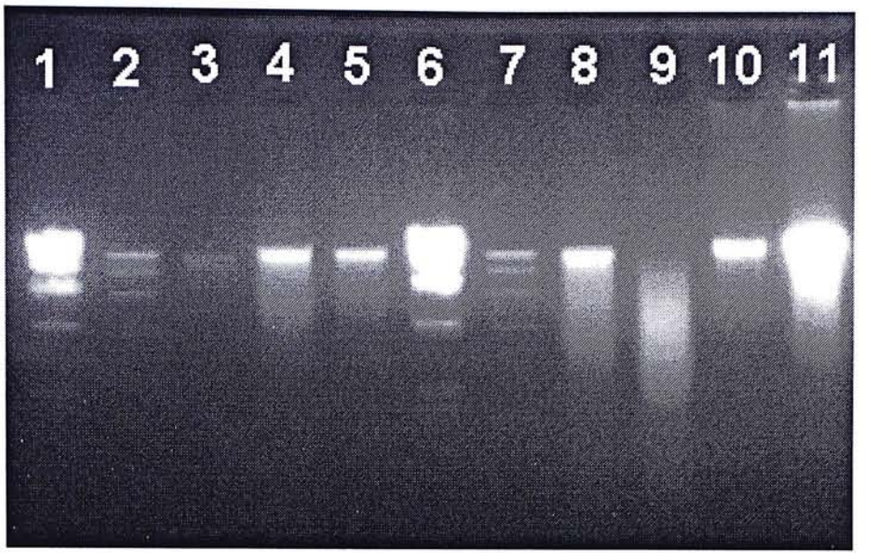

Figure 4.2 Agarose gel electrophoresis of total DNA. Lanes 1 and 6 are Lambda DNA-*Hind* III Digest (84 μg). Lanes 2 and 7 are Lambda DNA-*Hind* III Digest (8.4 μg). Lane 3 is *Stemona sessilifolia* (Hu & But 23972). Lane 4 is *Stemona sessilifolia* (Hu & Yung 606). Lane 5 is *Stemona sessilifolia* (code: SS3). Lanes 8 to 11 are *Stemona shandongensis* (specimen code: Zang 23974).

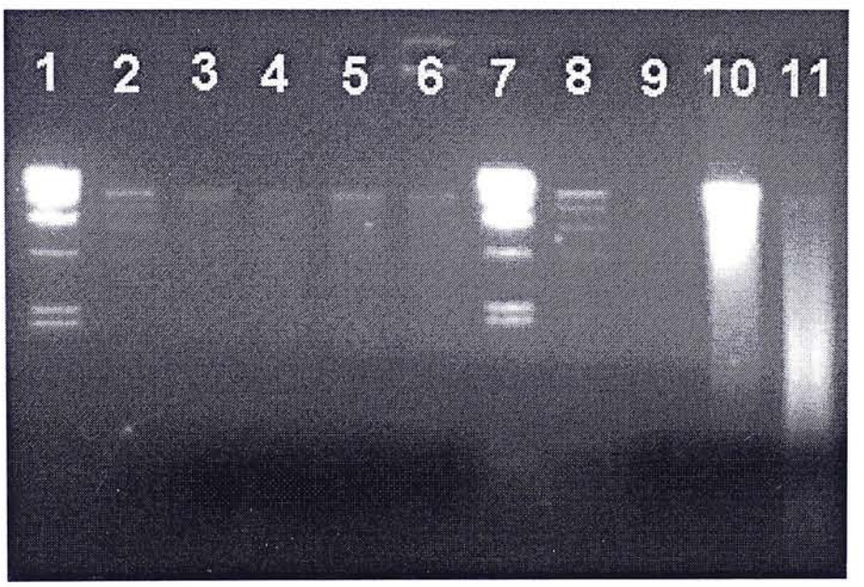

Figure 4.3. Agarose gel electrophoresis of total DNA. Lanes 1 and 7 are Lambda DNA-*Hind* III Digest (84 μg). Lanes 2 and 8 are Lambda DNA-*Hind* III Digest (8.4 μg). Lanes 3 to 6 are *Stemona tuberosa* from Guangxi (Woo 23973). Lane 9 is *Stemona tuberosa* provided by South China Institute of Botany, Academy of Sciences (Chan 200401). Lane 10 is *Stemona tuberosa* in Herb Garden of The Chinese University of Hong Kong (Hu & But 23960). Lanes 11 is *Stemona tuberosa* (ICM 20042541) from Yunnan.

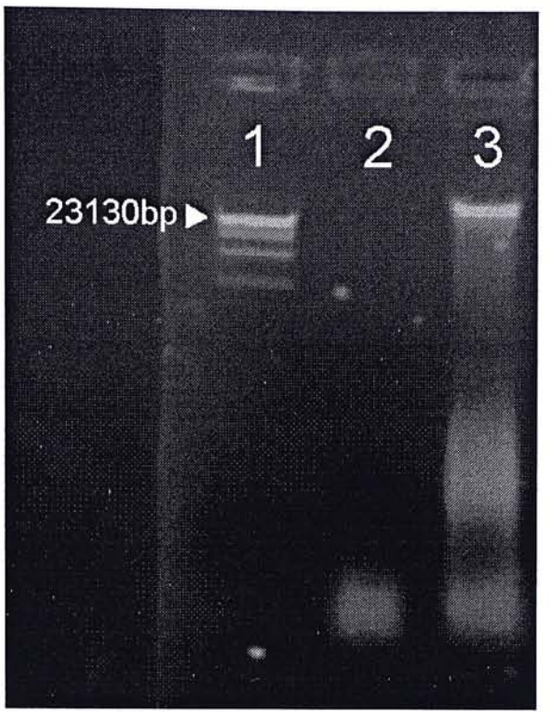

Figure 4.4 Agarose gel electrophoresis of total DNA extracted from dried material purchased from commercial market. Lane 1 is Lambda DNA-*Hind* III Digest (12 μg). Lanes 2 is Radix Stemonae (*Stemona tuberosa*) (ICM 2004-2540). Lane 3 is Radix Stemonae (*Asparagus filicinus*) (ICM 2004-2542).

In most of the samples, the DNA extracted contained a band of size larger than 23000bp. Most of DNA extracted were smeared. It was possible that the DNA was partially degraded. The lane representing *Stemona tuberosa* (Chan 200401) (Figure 4.3, lane 9) and Radix Stemonae (ICM 2004-2540) (Figure 4.4, lane 2) shows neither any band nor smear DNA. It was possible that the extracted DNA was at very low concentration that it could not be visualized by gel electrophoresis. However, these samples could be successfully amplified by PCR.

4.2 PCR

The DNA regions of interest were amplified by PCR. In this study, *trn*L-F region of chloroplast genome was amplified by primers Tab C and Tab F. Size of the PCR products were about 1000 bp to 1100 bp for all samples (Figures 4.5, 4.6, 4.7). The 5S rRNA spacers were amplified using primer S-1 and AS-1. Another pair of primers 5S2F and 5S2R were used if PCR was not successful with S-1 and AS-1. The two primer pairs anneal at the same region of the genome but the 5S2F and 5S2R are 6 bp shorter than S-1 and AS-1. The sizes of the PCR products obtained from 5S rRNA spacers varied among different taxa. The 5S rRNA spacer of *Croomia*, *Stichoneuron* and *Pentastemona* have about 300 bp (Figure 4.7), while all *Stemona* species have 5S rRNA spacer of about 500 bp long (Figures 4.8, 4.9). The 5S rRNA spacer in *Asparagus filicinus* is 600 bp (Figures 4.11).

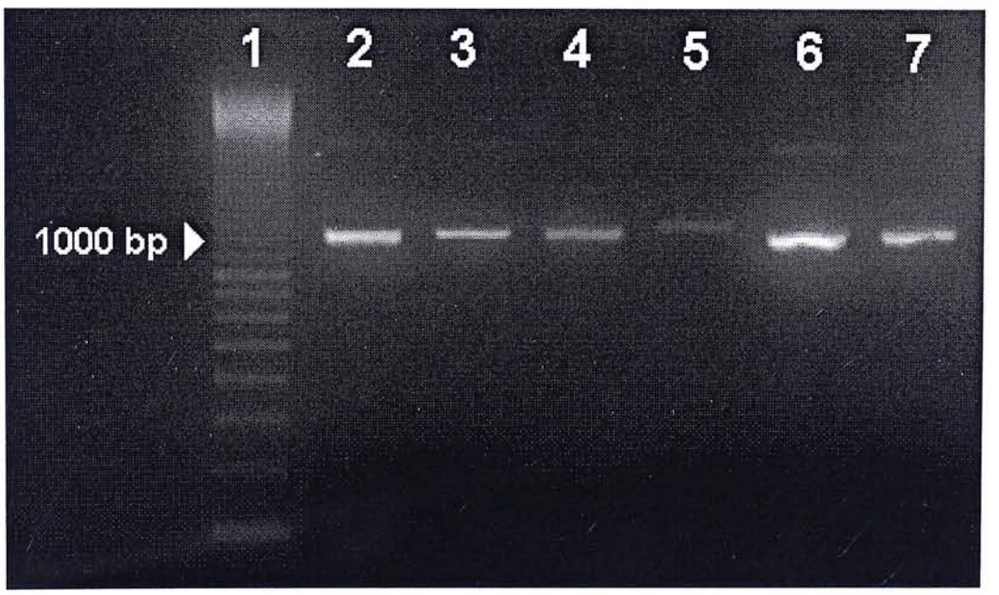

Figure 4.5. Agarose gel electrophoresis of the PCR products of the *trn*L-F region. Lane 1 is the 100 base pair marker. Lane 2 is *Stemona japonica* (ICM 2004-2543). Lane 3 is *Stemona japonica* (ICM 2004-2544). Lane 4 is *Stemona japonica* (Hu & But 23971). Lane 5 is *Stemona sessilifolia* (Hu & But 23972). Lane 6 is *Stemona sessilifolia* (Hu & Yung 606). Lane 7 is *Stemona sessilifolia* (code: SS3).

Figure 4.6. Agarose gel electrophoresis of the PCR products of the *trn*L-F region. Lanes 1, 6 and 12 are 100 base pair marker. Lanes 2, 3, 4 and 5 are *Stemona shandongensis* (Zang 23974). Lane 7 is *Pentastemona egregia* (J. Bogner 1724, 1985). Lane 8 is *Pentastemona sumatrana* (Duijfjes 21399 (8/1991)). Lane 9 is *Pentastemona sumatrana* (Leiden B.G. 910375). Lane 10 is *Stichoneuron caudatum* (Bygrave 50 K). Lane 11 is *Stichoneuron caudatum* (Leiden B.G. 910654). Lane 13 is *Croomia japonica* (Hu & But 24033). Lane 14 is *Croomia pauciflora* (Gholson 10360). Lane 15 is *Croomia pauciflora* (code: CP1). Lane 16 is *Croomia pauciflora* (code: CP2).

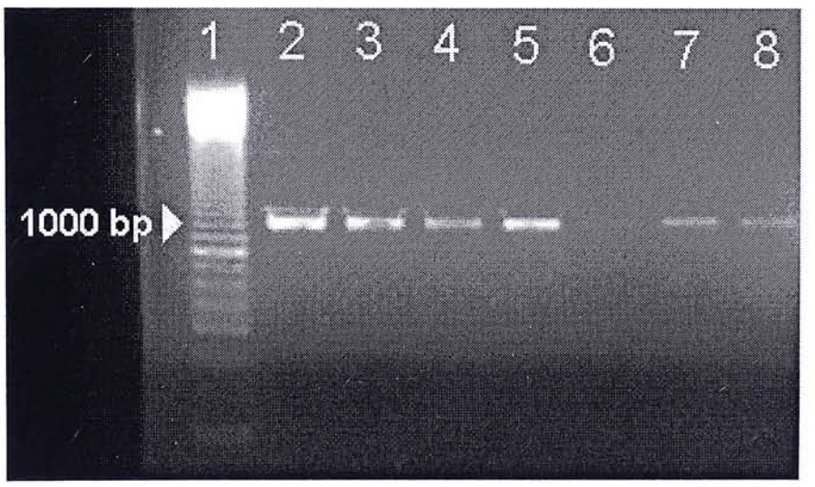

Figure 4.7. Agarose gel electrophoresis of the PCR products of the *trn*L-F region. Lane 1 is 100 base pair marker. Lanes 2, 3 and 4 are *Stemona tuberosa* (Woo 23973) from Guang Xi. Lane 5 is *Stemona tuberosa* (Hu & But 23960) cultivated in Herb Garden of the Chinese University of Hong Kong. Lane 6 is *Stemona tuberosa* (Chan200401) from the South China Institute of Botany. Lane 7 is *Stemona tuberosa* (ICM 2004-2541) from Yunnan. Lane 8 is *Stemona tuberosa* (specimen code: P. Wilkin 923K) from the Royal Botanic Garden, Kew.

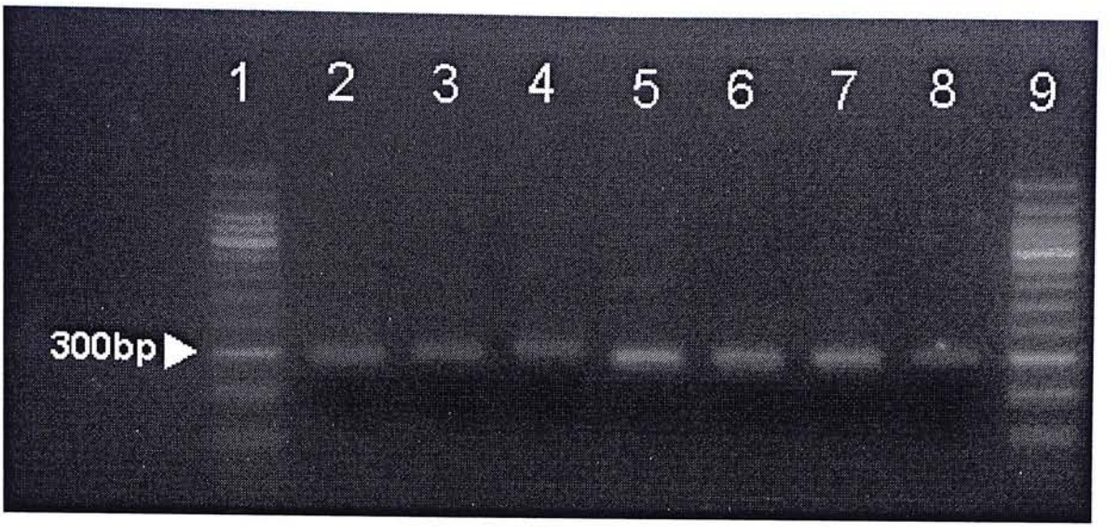

Figure 4.8. Agarose gel electrophoresis of the PCR products of the 5S rRNA spacer region. Lanes 1 and 9 are 100 base pair marker. Lane 2 is *Pentastemona egregia* (J. Bogner 1724, 1985). Lane 3 is *Stichoneuron caudatum* (P Bygrave 50 K). Lane 4 is *Stichoneuron caudatum* (Leiden B.G. 910654). Lane 5 is *Croomia japonica* (Hu & But 24033). Lane 6 is *Croomia pauciflora* (code: CP1). Lane 7 is *Croomia pauciflora* (code: CP2). Lane 8 is *Croomia pauciflora* (Gholson 10360).

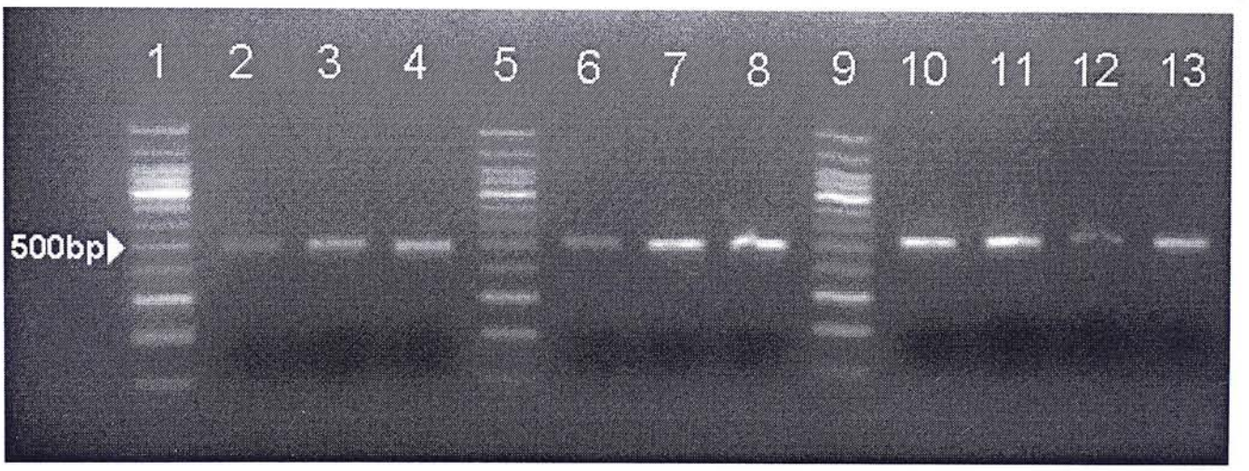

Figure 4.9. Agarose gel electrophoresis of the PCR products of the 5S rRNA spacer region. Lane 1, 5 and 9 are 100 base pair marker. Lane 2 is *Stemona japonica* (ICM 2004-2544). Lane 3 is *Stemona japonica* (Hu and But 24032). Lane 4 is *Stemona japonica* (Hu & But 23971). Lane 6 is *Stemona sessilifolia* (Hu & But 23972). Lane 7 is *Stemona sessilifolia* (Hu & Yung 606). Lane 8 is *Stemona sessilifolia* (code: SS3). Lanes 10 to 13 are *Stemona shandongensis* (Zang 23974) from Shandong.

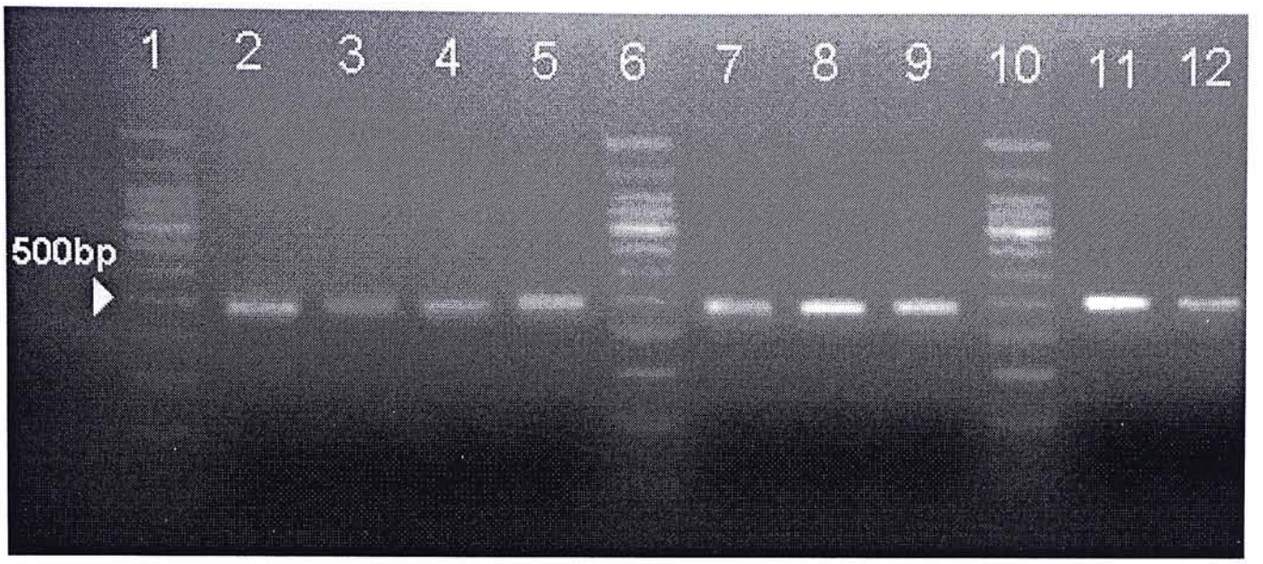

Figure 4.10. Agarose gel electrophoresis of the PCR products of the 5S rRNA spacer region. Lane 1, 6 and 10 are 100 base pair marker. Lane 2 is *Stemona tuberosa* (Chan200401) from the South China Institute of Botany. Lane 3 and 4 are *Stemona tuberosa* (ICM 20042541) from Yunnan. Lane 5 is *Stemona tuberosa* (Hu & But 23960) cultivated in Herb Garden of the Chinese University of Hong Kong. Lanes 7 to 9 are *Stemona tuberosa* (Woo 23973) from Guang Xi. Lanes 11 is *Stemona parviflora* (Ma 9066). Lane 12 is *Stemona parviflora* (Hu and But 24034).

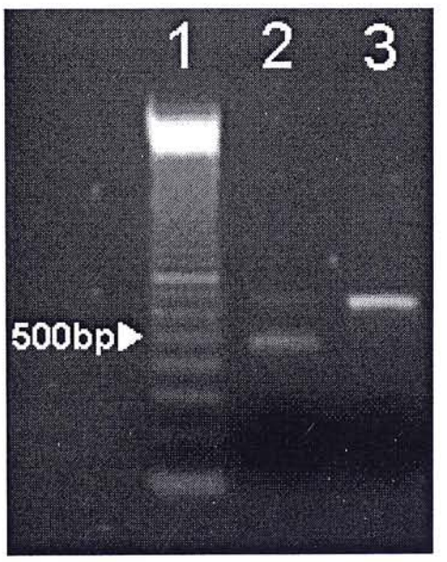

Figure 4.11. Agarose gel electrophoresis of PCR products of 5S rRNA spacer region. Lanes 1 is 100 base pair marker. Lanes 2 is Radix Stemonae (*Stemona tuberosa*) (ICM 2004-2540). Lane 3 is Radix Stemonae (*Asparagus filicinus*) (ICM 2004-2542).

4.3 DNA Authentication of *Radix Stemonae*

DNA sequences of *Stemona japonica, S.parviflora, S. sessilifolia* (including *S. shandongensis*), *S. tuberosa* and *Asparagus filicinus* were compared. Sequences having low intraspecific variation and high interspecific variation are favorable for authentication of Radix Stemonae. In this thesis project, both *trn*L region and 5S rRNA spacers sequences were obtained and analysed.

The sequences were aligned (Figure 4.12, 4.13) and the percentage similarity among them was calculated (Table 4.1, 4.2). Phylogenetic trees were constructed to visualize the relationship. MEGA 2.1 version was used for generating Unweighted Pair Group Method with Arithmatic Mean (UPGMA), Neighbor Joining and Maximum Parsimony trees. For Neighbor Joining trees and UPGMA trees, the distances were calculated using the algorithum Kimura 2-parameter. For parsimony analysis, parsimonious trees were searched using close-neighbor-interchange (CNI) method. Bootstrap test was applied for 500 replications.

4.3.1 *Trn*L intron sequences

*Trn*L intron and the intergenic spacer were amplified by PCR. However, only the *trn*L intron was sequenced. The sequences were aligned (Figure 4.12) and the percentage similarities among them were calculated (Table 4.1).

As shown in Table 4.1, the *trn*L intron sequences are not very variable among the five *Stemona* species. Both intraspecific and interspecific percentage similarities are over 96%. This variation is too small to differentiate the *Stemona* species. The *trn*L region is thus considered too conserve to infer intrageneric relationship of *Stemona*.

However, *Asparagus trn*L sequences were different from those of the *Stemona* species. The percentage similarity between *Asparagus* and the five *Stemona* species is about 80% on average., Thus, *Asparagus* can be easily distinguished from *Stemona* species by the *trn*L sequences.

	Stemona tuberosa	*Stemona japonica*	*Stemona sessilifolia*	*Stemona shandongensis*	*Stemona parviflora*	*Asparagus*
Stemona tuberosa	96-100% (98%)	97-99% (98%)	96-99% (97.5%)	98-100% (99%)	97-99% (98%)	78-79% (78.5%)
Stemona japonica		99-100% (99.5%)	97-99% (98%)	98-99% (98.5%)	98-99% (98.5%)	79-80% (79.5%)
Stemona sessilifolia			99-100% (99.5%)	99-100% (99.5%)	99%	80-81% (80.5%)
Stemona shandongensis				99%	98-99% (98.5%)	79-80% (79.5%)
Stemona parviflora					99-100% (99.5%)	81%

Table 4.1 Percentage similarity of *trn*L intron among five *Stemona* species (*S. japonica, S. parviflora, S. sessilifolia, S. shandongensis* and *S. tuberosa*) and *Asparagus filicinus.* (Average percentage in bracket).

```
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S_japonica_ICM20042544                -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_japonica_Hu&But24032                -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_parviflora_Ma9066                   -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_parviflora_Hu&But24034              GTATGGAA-CCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_sessilifolia_Hu&But23972            -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_sessilifolia_Hu&Yung606             -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_sessilifolia_Zang200401             -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_shandongensis_Zang23974_1           -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_shandongensis_Zang23974_2           GTATGGAAACCTGCTA-GTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_shandongensis_Zang23974_3           -TATGGAAACCTGCTAAGTGGTA-CTTCCAAATTCAGAGAAACCCTGGAA 48
S_shandongensis_Zang23974_4           -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_tuberosa_Woo23973_1                 -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_tuberosa_Woo23973_2                 -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_tuberosa_Woo23973_3                 -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_tuberosa_Woo23973_4                 -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_tuberosa_Hu&But23960                -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_tuberosa_ICM20042541                -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
S_tuberosa_Chan200401                 -TATGGAAACCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAA 49
A_falcatus                            -----GAAGMMTGCTAAGTGGAAACTTCCAAATTGAGAGAAACCCTGGAA 45
                                           ***   ***** **** * ********** ***************

S_japonica_ICM20042543                TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_japonica_ICM20042544                TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_japonica_Hu&But24032                TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_parviflora_Ma9066                   TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_parviflora_Hu&But24034              TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_sessilifolia_Hu&But23972            TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_sessilifolia_Hu&Yung606             TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
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S_shandongensis_Zang23974_2           TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_shandongensis_Zang23974_3           TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 95
S_shandongensis_Zang23974_4           TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_tuberosa_Woo23973_1                 TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_tuberosa_Woo23973_2                 TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_tuberosa_Woo23973_3                 TTAAAAATGGGCAATCCTGAGCCAATATCTTGAT-TTTGCGAAAACAAA- 97
S_tuberosa_Woo23973_4                 TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_tuberosa_Hu&But23960                TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_tuberosa_ICM20042541                TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
S_tuberosa_Chan200401                 TTAAAAATGGGCAATCCTGAGCCAA-ATCTTGAT-TTTGCGAAAACAAA- 96
A_falcatus                            CTAAAAATGGGCAATACCGAGCCAA-ATCTTTATGTTTAGAAAAACAAGG 94
                                       ************** * ******* ***** ** ***   *******
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Figure 4.12 Sequence alignment of *trn*L introns of five *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543              --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_japonica_ICM20042544              --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_japonica_Hu&But24032              --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_parviflora_Ma9066                 --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_parviflora_Hu&But24034            --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_sessilifolia_Hu&But23972          --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_sessilifolia_Hu&Yung606           --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_sessilifolia_Zang200401           --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_shandongensis_Zang23974_1         --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_shandongensis_Zang23974_2         --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_shandongensis_Zang23974_3         --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 132
S_shandongensis_Zang23974_4         --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
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S_tuberosa_Woo23973_3               --CTAGACTCAAAAA----------AAAGGATAGGTGCAGAGACTCGATG 135
S_tuberosa_Woo23973_4               --CTAGACTCAAAAA----------AAAGGATAGGTGCAGAGACTCGATG 134
S_tuberosa_Hu&But23960              --CTAGACTCAAAAA----------AAAGGATAGGTGCAGAGACTCGATG 134
S_tuberosa_ICM20042541              --CTAGACTCAAAAA----------AA-GGATAGGTGCAGAGACTCGATG 133
S_tuberosa_Chan200401               --CTAGACTCAAAAA----------AAAGGATAGGTGCAGAGACTCGATG 134
A_falcatus                          GTTTTAATTAAAAAACTATAAGATAAAGGGATAGGTGCACAGACTCAATG 144
                                       *  * * *****          ** ********** ****** ***

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S_japonica_ICM20042544              GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
S_japonica_Hu&But24032              GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
S_parviflora_Ma9066                 GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
S_parviflora_Hu&But24034            GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
S_sessilifolia_Hu&But23972          GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
S_sessilifolia_Hu&Yung606           GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
S_sessilifolia_Zang200401           GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
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S_shandongensis_Zang23974_2         GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
S_shandongensis_Zang23974_3         GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 182
S_shandongensis_Zang23974_4         GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
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S_tuberosa_Woo23973_4               GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 184
S_tuberosa_Hu&But23960              GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 184
S_tuberosa_ICM20042541              GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 183
S_tuberosa_Chan200401               GAAGCTGTTCTAACGAATGGAGTTGATTGCGTTACGTTGGTAGCTGGAAT 184
A_falcatus                          GAAGCTGTTCTAACGAATGGAGTTGACTATATTACGTTGGTAACCGGAAT 194
                                    ************************** *   ********** * *****
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Figure 4.12 (continued) Sequence alignment of *trn*L introns of five *Stemona* species and *Asparagus filicinus*

```
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S_japonica_ICM20042544              CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_japonica_Hu&But24032              CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_parviflora_Ma9066                 CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_parviflora_Hu&But24034            CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_sessilifolia_Hu&But23972          CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_sessilifolia_Hu&Yung606           CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_sessilifolia_Zang200401           CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_shandongensis_Zang23974_1         CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_shandongensis_Zang23974_2         CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_shandongensis_Zang23974_3         CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 227
S_shandongensis_Zang23974_4         CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_tuberosa_Woo23973_1               CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 229
S_tuberosa_Woo23973_2               CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 229
S_tuberosa_Woo23973_3               CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 230
S_tuberosa_Woo23973_4               CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 229
S_tuberosa_Hu&But23960              CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 229
S_tuberosa_ICM20042541              CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 228
S_tuberosa_Chan200401               CCCTCTATCGAAATTAGAGAAAGGATTGCCCTATATACCT-----GTACA 229
A_falcatus                          CCTTCTA----AATTAAAGAAAGGAT-GACCTATATATCTAATACGTACG 239
                                    ** ****    ***** ********* * ******** **     ****

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S_japonica_ICM20042544              TATACATACTGA----------CATAGCAAAAAATGCAAATTTATTATAT 268
S_japonica_Hu&But24032              TATACATACTGA----------CATAGCAAAAAATGCAAATTTATTATAT 268
S_parviflora_Ma9066                 TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 268
S_parviflora_Hu&But24034            TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 268
S_sessilifolia_Hu&But23972          TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 268
S_sessilifolia_Hu&Yung606           TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 268
S_sessilifolia_Zang200401           TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 268
S_shandongensis_Zang23974_1         TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 268
S_shandongensis_Zang23974_2         TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 268
S_shandongensis_Zang23974_3         TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 267
S_shandongensis_Zang23974_4         TATACATACTGA----------CATAGCAAAAAATTCAAATTTATTATAT 268
S_tuberosa_Woo23973_1               TATACATACTGATACATACTGACATAGCAAAAAATTCAAATTTATTATAT 279
S_tuberosa_Woo23973_2               TATACATACTGATACATACTGACATAGCAAAAAATTCAAATTTATTATAT 279
S_tuberosa_Woo23973_3               TATACATACTGATACATACTGACATAGCAAAAAATTCAAATTTATTATAT 280
S_tuberosa_Woo23973_4               TATACATACTGATACATACTGACATAGCAAAAAATTCAAATTTATTATAT 279
S_tuberosa_Hu&But23960              TATACATACTGATACATACTGACATAGCAAAAAATTCAAATTTATTATAT 279
S_tuberosa_ICM20042541              TATACATACTGATACATACTGACATAGCAAAAAATTCAAATTTATTATAT 278
S_tuberosa_Chan200401               TATACATACTGATACATACTGACATAGCAAAAAATTCAAATTTATTATAT 279
A_falcatus                          TATACATACTGG----------CATATCAAACGATTAATCACGACCCGAA 279
                                    **********           **** ****  **  *     *    *
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Figure 4.12 (continued) Sequence alignment of *trn*L introns of five *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543              TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_japonica_ICM20042544              TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_japonica_Hu&But24032              TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_parviflora_Ma9066                 TATATATATTATATGTATATGTAT---GTGTATATG-----------AA 303
S_parviflora_Hu&But24034            TATATATATTATATGTATATGTAT---GTGTATATG-----------AA 303
S_sessilifolia_Hu&But23972          TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_sessilifolia_Hu&Yung606           TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_sessilifolia_Zang200401           TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_shandongensis_Zang23974_1         TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_shandongensis_Zang23974_2         TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_shandongensis_Zang23974_3         TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 299
S_shandongensis_Zang23974_4         TATTTAT--TATAT-TATATGTAT---GTGTATATG-----------AA 300
S_tuberosa_Woo23973_1               TATTTAT--TATAT-TATTTATATTATATGTATATG-----------AA 314
S_tuberosa_Woo23973_2               TATTTAT--TATAT-TATTTATATTATATGTATATG-----------AA 314
S_tuberosa_Woo23973_3               TATTTAT--TATAT-TATTTATATTATATGTATATG-----------AA 315
S_tuberosa_Woo23973_4               TATTTAT--TATAT-TATTTATATTATATGTATATG-----------AA 314
S_tuberosa_Hu&But23960              TATTTAT--TATAT-TATTTATATTATATGTATATG-----------AA 314
S_tuberosa_ICM20042541              TATTTAT--TATAT-TATTTATATTATATGTATATGTATGTGTATATGAA 325
S_tuberosa_Chan200401               TATTTAT--TATAT-TATTTATATTATATGTATATG-----------AA 314
A_falcatus                          TCCATATTATATAT-AATATATGC----AAGACATG------------C 311
                                    *   ***  *****  ** * *         * ***

S_japonica_ICM20042543              AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_japonica_ICM20042544              AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_japonica_Hu&But24032              AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_parviflora_Ma9066                 AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 353
S_parviflora_Hu&But24034            AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 353
S_sessilifolia_Hu&But23972          AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_sessilifolia_Hu&Yung606           AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_sessilifolia_Zang200401           AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_shandongensis_Zang23974_1         AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_shandongensis_Zang23974_2         AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_shandongensis_Zang23974_3         AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 349
S_shandongensis_Zang23974_4         AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 350
S_tuberosa_Woo23973_1               AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 364
S_tuberosa_Woo23973_2               AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 364
S_tuberosa_Woo23973_3               AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 365
S_tuberosa_Woo23973_4               AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 364
S_tuberosa_Hu&But23960              AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 364
S_tuberosa_ICM20042541              AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 375
S_tuberosa_Chan200401               AAAATGAATAATTATTGTGAATTCACTTCAATCGAAATCGAAGTTGAAGT 364
A_falcatus                          AAAATTCAGAGTTATTATGGATCTATGCCAATA------GAAGTTGAAGG 355
                                    *****  * * ***** ** **  *   ****       **********
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Figure 4.12 (continued) Sequence alignment of *trn*L introns of five *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543              AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 399
S_japonica_ICM20042544              AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 399
S_japonica_Hu&But24032              AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 399
S_parviflora_Ma9066                 AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 402
S_parviflora_Hu&But24034            AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTTTGATAGA 402
S_sessilifolia_Hu&But23972          AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 399
S_sessilifolia_Hu&Yung606           AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 399
S_sessilifolia_Zang200401           AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 399
S_shandongensis_Zang23974_1         AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 399
S_shandongensis_Zang23974_2         AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 399
S_shandongensis_Zang23974_3         AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 398
S_shandongensis_Zang23974_4         AAGAATCGAATATTCATTGATCAAATCATTCATTCACAGAGTCTGATAGA 400
S_tuberosa_Woo23973_1               AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 413
S_tuberosa_Woo23973_2               AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 413
S_tuberosa_Woo23973_3               AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 414
S_tuberosa_Woo23973_4               AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 413
S_tuberosa_Hu&But23960              AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 413
S_tuberosa_ICM20042541              AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 424
S_tuberosa_Chan200401               AAGAATCGAATATTCATTGATCAAATCATTCATTCA-AGAGTCTGATAGA 413
A_falcatus                          AAGAATCGAATATTCAGTGATCAAATGATTCATTCC-AGAGTTTGATATA 404
                                    ********************* ********* ********  ***** ***** *

S_japonica_ICM20042543              TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 439
S_japonica_ICM20042544              TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 439
S_japonica_Hu&But24032              TCTTTTT---AAAAACGGATTC------AATCGGACGAGAATAAAGAGAG 440
S_parviflora_Ma9066                 TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 442
S_parviflora_Hu&But24034            TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 442
S_sessilifolia_Hu&But23972          TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 439
S_sessilifolia_Hu&Yung606           TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 439
S_sessilifolia_Zang200401           TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 439
S_shandongensis_Zang23974_1         TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 439
S_shandongensis_Zang23974_2         TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 439
S_shandongensis_Zang23974_3         TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 438
S_shandongensis_Zang23974_4         TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 440
S_tuberosa_Woo23973_1               TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 453
S_tuberosa_Woo23973_2               TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 453
S_tuberosa_Woo23973_3               TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 454
S_tuberosa_Woo23973_4               TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 453
S_tuberosa_Hu&But23960              TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 453
S_tuberosa_ICM20042541              TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 464
S_tuberosa_Chan200401               TCTTTTT---AAAAACGGATT-------AATCGGACGAGAATAAAGAGAG 453
A_falcatus                          CCTTTTTTTGAAAAATTGATTAATGATTAATCGGACGAGAATAAAGAGAG 454
                                     ******   *****  ****       **********************
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Figure 4.12 (continued) Sequence alignment of *trn*L introns of five *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543              AGTCCCGTTCCTCAC-ATGTCAATACC-GACAACAATGAAATTTATAGTA 487
S_japonica_ICM20042544              AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 485
S_japonica_Hu&But24032              AGTCCCGTTCCTCACTATGTCAATACC-GACAACAATGAAATTTATAGTA 489
S_parviflora_Ma9066                 AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 488
S_parviflora_Hu&But24034            AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 488
S_sessilifolia_Hu&But23972          AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 485
S_sessilifolia_Hu&Yung606           AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 485
S_sessilifolia_Zang200401           AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 485
S_shandongensis_Zang23974_1         AGTCCCGTTCCTCAC-ATGTCAATACCAGACAACAATGAAATTTATAGTA 488
S_shandongensis_Zang23974_2         AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 485
S_shandongensis_Zang23974_3         AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 484
S_shandongensis_Zang23974_4         AGTCCCGTTCTACAN---GTCAATACC-GACAACAATGAAATTTATAGTA 486
S_tuberosa_Woo23973_1               AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 499
S_tuberosa_Woo23973_2               AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 499
S_tuberosa_Woo23973_3               AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 500
S_tuberosa_Woo23973_4               AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 499
S_tuberosa_Hu&But23960              AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 499
S_tuberosa_ICM20042541              AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 510
S_tuberosa_Chan200401               AGTCCCGTTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 499
A_falcatus                          AGTCCCATTCTACAT---GTCAATACC-GACAACAATGAAATTTATAGTA 500
                                    ****** ***  **    ********* ********************

S_japonica_ICM20042543              AGAGG 492
S_japonica_ICM20042544              AGAGG 490
S_japonica_Hu&But24032              AGAGG 494
S_parviflora_Ma9066                 AGAGG 493
S_parviflora_Hu&But24034            AGAGG 493
S_sessilifolia_Hu&But23972          AGAGG 490
S_sessilifolia_Hu&Yung606           AGAGG 490
S_sessilifolia_Zang200401           AGAGG 490
S_shandongensis_Zang23974_1         AGAGG 493
S_shandongensis_Zang23974_2         AGAGG 490
S_shandongensis_Zang23974_3         AGAGG 489
S_shandongensis_Zang23974_4         AGAGG 491
S_tuberosa_Woo23973_1               AGAGG 504
S_tuberosa_Woo23973_2               AGAGG 504
S_tuberosa_Woo23973_3               AGAGG 505
S_tuberosa_Woo23973_4               AGAGG 504
S_tuberosa_Hu&But23960              AGAGG 504
S_tuberosa_ICM20042541              AGAGG 515
S_tuberosa_Chan200401               AGAGG 504
A_falcatus                          AAAGG 505
                                    * ***
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Figure 4.12 (continued) Sequence alignment of *trn*L introns of five *Stemona* species and *Asparagus filicinus*

4.3.2 5S rRNA spacer sequences

The 5S rRNA spacer sequences were sequenced. The sequences were aligned (Figure 4.13) and the percentage similarity among them was calculated (Table 4.2). The species examined have very conserved 5S rRNA sequences. However, the spacers between the 5S rRNA genes are highly variable. This variation is high enough to separate *Stemona* from its adulterant and also differentiate among different *Stemona* species.

5S rRNA spacer sequences in *Asparagus filicinus* were highly different from *Stemona* species. *Asparagus filicinus* can be easily distinguished from *Stemona* species according to the difference in size of the 5S rRNA spacers, the low percentage similarity and its position in the phylogenetic trees. The size of the PCR product from *Asparagus filicinus* is 600 bp, while that of *Stemona* is 500 bp only. The 5S rRNA spacer sequences of *Stemona* and *Asparagus* are too different for alignment. The percentage similarity between *Asparagus filicinus* and *Stemona* species is about 16% on average. In the UPGMA tree (Figure 4.14), Neighbour-Joining tree (Figure 4.15) and Maximum Parsimony tree (Figure 4.16) constructed, *Asparagus filicinus* does not group with the *Stemona* species.

The 5S rRNA spacer sequences of *Stemona* can also differentiate different *Stemona* species from one another. The result of sequence alignment shows that the 300 bp-400 bp region of the spacer was the most variable region (Figure 4.13). Within this variable region, each species has unique insertion and deletion sections or unique sequences. As is presented in Table 4.2, the intraspecific percentage similarity among *Stemona* species is about 90-100%. The interspecific percentage similarity among species is about 70-80%. The spacer sequences are very conserve within the same

species while the intraspecific variation is very large. Thus, 5S rRNA spacers is a very useful molecular marker for authenticating Radix Stemonae. In all the phylogenetic trees constructed based on 5S rRNA spacers, *S. tuberosa, S. japonica* and *S. parviflora,* and *S. sessilifolia* (including *S. shandongensis*) formed a clade distinct to *Asparagus filicinus.* The clade of *Stemona* further branches out to four smaller clades representing four *Stemona* species. These clades have bootstraps value of 100 in the bootstraps test, which means these clades are well supported.

As presented in Chapter 3, the morphological characteristics of *S. sessilifolia* and *S. shandongensis* are overlapping and indistinguishable. Molecular data also showed that the two taxa forming a single clade in the phylogenetic trees. They also have very similar insertion and deletion pattern. The percentage similarity between them is 98%. We thus concluded that this two taxa should be grouped under one single species.

```
S_japonica_ICM20042543a          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_japonica_ICM20042543b          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_japonica_Hu&But24032a          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_japonica_Hu&But23971a          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_japonica_Hu&But23971b          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_parviflora_Ma9066a             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_parviflora_Ma9066b             GGATCCGTGCTTGGTCGAGAGTAGTACTAGTATGGGTGACCTCCTGGGAA 50
S_parviflora_Ma9066c             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_parviflora_Ma200401            GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_sessilifolia_Hu&But23972a      GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_sessilifolia_Hu&But23972b      GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_sessilifolia_Hu&But23972c      GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_sessilifolia_Hu&Yung606a       GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_sessilifolia_Hu&Yung606b       GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_sessilifolia_Zang200401a       GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_sessilifolia_Zang200401b       GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_1a     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_1b     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_2a     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_2b     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_2c     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_3a     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_3b     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_3c     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_4a     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_shandongensis_Zang23974_4b     GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_ICM20042540a          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_ICM20042540b          GGATCCGTGCTCGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_ICM20042540c          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Woo23973a             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Woo23973b             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Woo23973c             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Woo23973d             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Woo23973e             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Woo23973f             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Woo23973g             GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_ICM20042541a          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_ICM20042541b          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Hu&But23960a          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Hu&But23960b          GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Chan200401a           GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
S_tuberosa_Chan200401b           GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
A_filicinus_ICM20042542a         GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
A_filicinus_ICM20042542b         GGATCCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCTCCTGGGAA 50
                                 ********** ** ********** ******************
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Figure 4.13 Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a                GTCCTCGTGTTGCACCCCTTTTCCTTTTTGAGCACGGGCATATTAAGTTT 100
S_japonica_ICM20042543b                GTCCTCGTGTTGCACCCCTTTTCCTTTTTGAGCACGGGCATATTAAGTTT 100
S_japonica_Hu&But24032a                GTCCTCGTGTTGCACCCCTTTTCCTTTTTGAGCACGGGCATATTAAGTTT 100
S_japonica_Hu&But23971a                GTCCTCGTGTTGCACCCCTTTTCCTTTTTGAGCACGGGCATATTAAGTTT 100
S_japonica_Hu&But23971b                GTCCTCGTGTTGCACCCCTTTTCCTTTTTGATCAAGGGCATATTAAGTTT 100
S_parviflora_Ma9066a                   GTCCTCGTGTTGCACCCCTTTTGTTTTTGGAGCACGCGAATTCCACGTTT 100
S_parviflora_Ma9066b                   GTCCTCGTGTTGCACCCCTTTTGTTTTTGGAGCACTCGAATTCCACGTTT 100
S_parviflora_Ma9066c                   GTCCTCGTGTTGCACCCCTTTTGTTTTTGGAGCACGCGAATTCCACGTTT 100
S_parviflora_Ma200401                  GTCCTCGTGTTGCACCCCTTTTGTTTTTGGAGCACTCGAATTCCACGTTT 100
S_sessilifolia_Hu&But23972a            GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACAGGAATATTAAGTTT 100
S_sessilifolia_Hu&But23972b            GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACAGGAATATTAAGTTT 100
S_sessilifolia_Hu&But23972c            GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACAGGAATATTAAGTTT 100
S_sessilifolia_Hu&Yung606a             GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACAGGAATATTAAGTTT 100
S_sessilifolia_Hu&Yung606b             GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACGGGAATATTAAGTTT 100
S_sessilifolia_Zang200401a             GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACGGGAATATTAAGTTT 100
S_sessilifolia_Zang200401b             GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACAGGAATATTAAGTTT 100
S_shandongensis_Zang23974_1a           GTCCTCGTGTTGCACCCCTTTTCCTTTTTTCGCACGGGAATATTAAGTTT 100
S_shandongensis_Zang23974_1b           GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACAGGAATATTAAGTTT 100
S_shandongensis_Zang23974_2a           GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACAGGAATATTAAGTTT 100
S_shandongensis_Zang23974_2b           GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACGGGAATATTAAGTTT 100
S_shandongensis_Zang23974_2c           GTCCTCGTGTTGCACCCCTTTTCCTTTTTTCGCACGGGAATATTAAGTTT 100
S_shandongensis_Zang23974_3a           GTCCTCGTGTTGCACCCCTTTTCCTTTTTTCGCACGGGAATATTAAGTTT 100
S_shandongensis_Zang23974_3b           GTCCTCGTGTTGCACCCCTTCTCCATTTTTCGCACGGGAATATTAAGTTT 100
S_shandongensis_Zang23974_3c           GTCCTCGTGTTGCACCCCTTTTCCTTTTTTCGCACGGGAATATTAAGTTT 100
S_shandongensis_Zang23974_4a           GTCCTCGTGTTGCACCCCTTCTCCTTTTTTCGCACAGGAATATTAAGTTT 100
S_shandongensis_Zang23974_4b           GTCCTCGTGTTGCACCCCTTTTCCTTTTTTCGCACGGGAATATTAAGTTT 100
S_tuberosa_ICM20042540a                GTCCTCGTGTTGCACCCCTTATCTTTTTGGGGCACGCGGATAATAAGTTT 100
S_tuberosa_ICM20042540b                GTCGTCGTGTTGCACCCCTTATCTTTTTGGGGCACGCGGATAATAAGTTT 100
S_tuberosa_ICM20042540c                GTCCTCGTGTTGCACCCCTTATCTTTTTGGGGCACGCGGATAATAAGTTT 100
S_tuberosa_Woo23973a                   GTCCTCGTGTTGCACCCCTTATCTTTTTGGTGCACGCGGATAATAAGTTT 100
S_tuberosa_Woo23973b                   GTCCTCGTGTTGCACCCCTTATCTTTTTGGTGCACGTGGATAATAAGTTT 100
S_tuberosa_Woo23973c                   GTCCTCGTGTTGCACCCCTTATCTTTTTGGTGCACGCGGATAATATGTTT 100
S_tuberosa_Woo23973d                   GTCCTCGTGTTGCACCCCTTATCTTTTTGGTGCACGCGGATAATAAGTTT 100
S_tuberosa_Woo23973e                   GTCCTCGTGTTGCACCCCTTATCTTTTTGGTGCACGCGGATAATAAGTTT 100
S_tuberosa_Woo23973f                   GTCCTCGTGTTGCACCCCTTATCTTTTTGGTGCACGCGGATAATANGTTT 100
S_tuberosa_Woo23973g                   GTCCTCGTGTTGCACCCCTTATCTTTTTGGTGCACGCGGATAATAAGTTT 100
S_tuberosa_ICM20042541a                GTCCTCGTGTTGCACCCCTTTTCTTTTTGGGGCACGCGGATAATGAGTTT 100
S_tuberosa_ICM20042541b                GTCCTCGTGTTGCACCCCTTTTCTTTTTGGGGCACGCGGATAATGAGTTT 100
S_tuberosa_Hu&But23960a                GTCCTCGTGTTGCACCCCTTATCTTTTTGCGGCTCGCGGATAATAAGTTT 100
S_tuberosa_Hu&But23960b                GTCCTCGTGTTGCACCCCTTATCTTTTTGCGGCTCGCGGATAATAAGTTT 100
S_tuberosa_Chan200401a                 GTCCTCGTGTTGCACCCCTTATCTTTTTGGGGCACGCGGATAATAAGTTT 100
S_tuberosa_Chan200401b                 GTCCTCGTGTTGCACCCCTTATCTTTTTGGGGCACGCGGATAATAAGTTT 100
A_filicinus_ICM20042542a               GTCCTCGTGTTGCACCCCTCCTTTTTGCCCGGCGCGCAAAT--TGCGACT 98
A_filicinus_ICM20042542b               GTCCTCGTGTTGCACCCCTCCTTTTTGCTCGGCGCGCAAAT--TACGACT 98
                                       *** ************** *  *     *     **    * *
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a              TGCAGAAAAATGGAGAGCG-GTTGCCGAAAA--CACGAATCCGTCGTCGT 147
S_japonica_ICM20042543b              TGCAGAAAAATGGAGCGCG-GTTGCCGAAAA--CACGAATCCGTCGTCGT 147
S_japonica_Hu&But24032a              TGCAGAAAAATGGAGAGCG-GTTGCCGAAAA--CACGAATCCGTCGTCGT 147
S_japonica_Hu&But23971a              TGCAGAAAAATGGAGAGCG-GCTGCCGAAAA--CACGAATCCGTCGTCGT 147
S_japonica_Hu&But23971b              TGCAGAAAAATGGAGAGCG-GTTGTCGAAAA--CACGAATCCGTCGTCGT 147
S_parviflora_Ma9066a                 CCAGGAACACAGTCGCGAG-GCATCCGGGAG--CACAAATCCGTCGATC- 146
S_parviflora_Ma9066b                 CGAGGAACACAGTCGCGAG-GCATCCGGGAA--CACAAATCCGTCGATC- 146
S_parviflora_Ma9066c                 CCAGGAACACAGTCGCGAG-GCATCCGGGAG--CACAAATCCGTCGATC- 146
S_parviflora_Ma200401                CGAGGAACACAGTCGCGAG-GCATCCGGGAA--CACAAATCCGTCGATC- 146
S_sessilifolia_Hu&But23972a          TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_sessilifolia_Hu&But23972b          TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_sessilifolia_Hu&But23972c          TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_sessilifolia_Hu&Yung606a           TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTTG- 146
S_sessilifolia_Hu&Yung606b           TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_sessilifolia_Zang200401a           TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_sessilifolia_Zang200401b           TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_1a         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_1b         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_2a         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_2b         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_2c         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_3a         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_3b         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_3c         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCATCG- 146
S_shandongensis_Zang23974_4a         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_shandongensis_Zang23974_4b         TGCAGAAAAATACAGAGCG-GTTGGCGAAAA--CAAGAATCCGTCGTCG- 146
S_tuberosa_ICM20042540a              TGGAGGAAACCGCGGGGAG-ACGGCCGAAAAAACACGAATCCGTCGATT- 148
S_tuberosa_ICM20042540b              TGGAGGAAACCGCGGGGAG-ACGGCCGAAAAAACACGAATCTGTCGATT- 148
S_tuberosa_ICM20042540c              TGGAGGAAACCGCGGAGAG-ACGGCCGAAAAAACACGAATCCGTCGATT- 148
S_tuberosa_Woo23973a                 TGGAGGAAACCGCGGAGAG-ACGGCCGAAAAATCACGAATCCGTCGATT- 148
S_tuberosa_Woo23973b                 TGGAGGAAACCGCGGAGAG-ACGGCCGAAAAATCACGAATCCGTCGATT- 148
S_tuberosa_Woo23973c                 TGGAGGAAACCGCGGAGAG-ACGGTCGAAAAATCACGAGTCCGTCGATT- 148
S_tuberosa_Woo23973d                 TGGAGGAAACCGCGGAGAG-ACGGCCGACAAATCACGAATCCATCGATT- 148
S_tuberosa_Woo23973e                 TGGAGGAAACCGCGGAGAG-ACGGCCGACAAATCACGAATCCATCGATT- 148
S_tuberosa_Woo23973f                 TGGAGGAAACCGCGGAGAG-ACGGCCGAAAAATCACGAATCCGTCGATT- 148
S_tuberosa_Woo23973g                 TGGAGGAAACCGCGGAGAG-ACGGCCGAAAAATCACGAATCCGTCGATT- 148
S_tuberosa_ICM20042541a              TGGAGGAAACCGCAGAGAG-ACGGCCGAAAAAACACGAATCCGTCGATT- 148
S_tuberosa_ICM20042541b              TGGAGGAAACCGCAGAGAG-ACGGCCGAAAAAACACGAATCCGTCGATT- 148
S_tuberosa_Hu&But23960a              TGGAGGAAAC-GCGGAGAG-ACGGCCGAAAAAACACGAATCCGTCTATT- 147
S_tuberosa_Hu&But23960b              TGGAGGAAACCGCGGAGAG-ACGGCCGAAAAAACACGAATCCGTCTATT- 148
S_tuberosa_Chan200401a               TGGAGGAAACCGCGGAGGGCACGGCCGAAAAAACACGAATCCGTCGATT- 149
S_tuberosa_Chan200401b               TGGAGGAAACCGCGGAGAG-ACGGCCGAAAAAACACGAATCCGTCGATT- 148
A_filicinus_ICM20042542a             GAGAGCACGTTTAATTTTA-TTTTATTATTTTCCGCCAATCGGCGGCTCC 147
A_filicinus_ICM20042542b             GAGAGCCCGTTTAATTTTA-TTTCATTATTTTCCGCCAATCGGCGGCTCC 147
                                        *                          *   * **
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          TCCGGACGGGTTAGAGATGGGATCTGAGGTAA-TCCTCGCCTCCGGCCGC 196
S_japonica_ICM20042543b          TCCGGACGGGTTAGAGATGAGATCTGAGGTAA-GCCTCGCCTCCGGCCGC 196
S_japonica_Hu&But24032a          TCCGGACGGGTTAGAGATGGGATCTGAGGTAA-TCCTCGCCTCCGGCCGC 196
S_japonica_Hu&But23971a          TCCGGACGGGTTAGAGATGGGATCCGAGGTAA-TCCTCGCCTCCGGCCGC 196
S_japonica_Hu&But23971b          TCCGGACAGGTTAGAGATGGGATTTGAGGTAA-TCCTCGCCTCCGGCCGC 196
S_parviflora_Ma9066a             --CGGACGTGTTAGGGATGGATTTTCTCGAAT-TTCTGGCCTCCGACGGC 193
S_parviflora_Ma9066b             --CGGACGTGTTAGGGATGGATTTTCATGAAA-TTCTGGCCTCCGGCGGC 193
S_parviflora_Ma9066c             --CGGACGTGTTAGGGATGGATTTTCTCGAAT-TTCTGGCCTCCGACGGC 193
S_parviflora_Ma200401            --GGGACGTGTTAGGGATGGATTTTCATGAAA-TTCTGGCCTCCGACGGC 193
S_sessilifolia_Hu&But23972a      --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_sessilifolia_Hu&But23972b      --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_sessilifolia_Hu&But23972c      --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_sessilifolia_Hu&Yung606a       --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_sessilifolia_Hu&Yung606b       --CGGACGCGTTAGGGATGGAATTTGATGAAA-TTCTCGTCTCCGGCCGC 193
S_sessilifolia_Zang200401a       --CAGACGCGTTAGAGATGGAATTTGATGAAA-TTCTCGTCTCCGGCCGC 193
S_sessilifolia_Zang200401b       --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_shandongensis_Zang23974_1a     --CGGACGCGTTAGAGATGGAATTTGATGAAA-TTCTCGTCTCCGGCCGC 193
S_shandongensis_Zang23974_1b     --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_shandongensis_Zang23974_2a     --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_shandongensis_Zang23974_2b     --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_shandongensis_Zang23974_2c     --CGGACGCGTTAGAGATGGAATTTGATGAAA-TTCTCGTCTCCGGCCGC 193
S_shandongensis_Zang23974_3a     --CGGACGCGTTAGAGATGGAATTTGATGAAA-TTCTCGTCTCCGGCCGC 193
S_shandongensis_Zang23974_3b     --CGGACGCGTTAGAGATGGAATTTGATGAAA-TTCTCGTCTCCGGCTGC 193
S_shandongensis_Zang23974_3c     --CGGACGCGTTAGAGATGGAATTTGATGAAA-TTCTCGTCTCCGGCCGC 193
S_shandongensis_Zang23974_4a     --CGGACGCGTTAGAGATGGAATTTGAGAAAAATTCTCGTCTCCGGCCGC 194
S_shandongensis_Zang23974_4b     --CGGACGCGTTAGAGATGGAATTTGATGAAA-TTCTCGTCTCCGGCCAC 193
S_tuberosa_ICM20042540a          --CGGACGTGTTAGAGTTGGAATTTGAGGACG-TAGTCGTCCCCGACGGC 195
S_tuberosa_ICM20042540b          --CGGACGTGTTAGAGTTGGAATTTGAGGACG-TAGTCGTCCCCGACGGC 195
S_tuberosa_ICM20042540c          --CGGACGTGTTAGAGTTGGAATTTGAGGACG-TAGTCGTCCCCGACGGC 195
S_tuberosa_Woo23973a             --CGGACGTGTTAGAGTTGGAATTTGAGGAAG-CAGTCGTCGCCGACGGC 195
S_tuberosa_Woo23973b             --CGGACGTGTTAGAGTTGGAATTTGAGGAAG-TAGTCATCGCCGACGGC 195
S_tuberosa_Woo23973c             --CGGACGTGCTAGAGTCAGAATTTGAGGAAG-TAGTCGTCGCGGACGGC 195
S_tuberosa_Woo23973d             --CGGACGTGTTAGAGTTGGAATTTGAGGAAG-TAGTCGTCGCCGACGGC 195
S_tuberosa_Woo23973e             --CGGACGTGTTAGAGTTGGAATTTGAGGAAG-TAGTCGTCGCCGACGGC 195
S_tuberosa_Woo23973f             --CGGACGTGTTAGAGTTGGAATTTGAGGAAG-CAGTCGTCGCCGACGGC 195
S_tuberosa_Woo23973g             --CGGACGTGTTAGAGTTGGAATTTGAGGAAG-CAGTCGTCGCCGACGGC 195
S_tuberosa_ICM20042541a          --CGGACGTGTTAGAGTTGGAATTTGAGGAAG-TAGTCATCCCCGACGGC 195
S_tuberosa_ICM20042541b          --CGGACGTGTTAGAGTTGGAATTTGAGGAAG-TAGTCATCCCCGACGGC 195
S_tuberosa_Hu&But23960a          --CGGACGTGTTAGAGTTGGAATTTGAGGACG-TAGTCATCCCCGACGGC 194
S_tuberosa_Hu&But23960b          --CGGACGTGTTAGAGTTGGAATTTGAGGACG-TAGTCATCCCCGACGGC 195
S_tuberosa_Chan200401a           --CGGACGTGTTAGAGTTGGAATTTGAGGACG-TAGTGATCCCCGACGGC 196
S_tuberosa_Chan200401b           --CGGACGTGTTAGAGTTGGAATTTGAGGACG-TAGTGATCCCCGACGGC 195
A_filicinus_ICM20042542a         TTTTTTTACCCCCTCATTTCCTCCCGCCGTCAGCGCGGACGTCTGGGAAT 197
A_filicinus_ICM20042542b         TTTTTTTACCCCCTCATTTCTTCCCGCCGTCAGGGCGGACGCCTGGGAAC 197
                                                      * *
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a                CTGCGGTCGAAGTCCAAGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 246
S_japonica_ICM20042543b                CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 246
S_japonica_Hu&But24032a                CTGCGGTCGAAGTCCAAGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 246
S_japonica_Hu&But23971a                CTGCGGTCGAAGTCCAAGTCGTAGGTCTCTGGATGGGCCTTCCGTCGGGA 246
S_japonica_Hu&But23971b                CTGCGGTCGAAGTCCACGTTGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 246
S_parviflora_Ma9066a                   CTGCGGCTGTCGGCCAAGTCGTAGATCTCCCGTTCCGCTTTCCGTTGACA 243
S_parviflora_Ma9066b                   CTGCGGCTGCCGGCCACGTCGTAGATCTCCCGTTCAGCTTTCCGTTGACA 243
S_parviflora_Ma9066c                   CTGCGGCTGTCGGCCACGTCGTAGATCTCCCGTTCCGCTTTCCGTTGACA 243
S_parviflora_Ma200401                  CTGCGGCTATCGGCCACGTCGTAGATCTCCCGTTCAGCTTTCCGTTGACA 243
S_sessilifolia_Hu&But23972a            CTGCGGTCGAAGTCCACGTCGTAGGTCACTGGATGGGCTTTCCGTCGGGA 244
S_sessilifolia_Hu&But23972b            CTGCGGTCGAAGTCCACGTCGTAGGTCACTGGATGGGCTTTCCGTCGGGA 244
S_sessilifolia_Hu&But23972c            CTGCGGTCGAAGTCCACGTCGTAGGTCACTGGATGGGCTTTCCGTCGGGA 244
S_sessilifolia_Hu&Yung606a             CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 244
S_sessilifolia_Hu&Yung606b             CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 243
S_sessilifolia_Zang200401a             CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 243
S_sessilifolia_Zang200401b             CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 244
S_shandongensis_Zang23974_1a           CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 243
S_shandongensis_Zang23974_1b           CTGCGGTCGAAGTCCACGTCGTAGGTCACTGGATGGGCTTTCCGTCGGGA 244
S_shandongensis_Zang23974_2a           CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 244
S_shandongensis_Zang23974_2b           CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 244
S_shandongensis_Zang23974_2c           CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 243
S_shandongensis_Zang23974_3a           CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 243
S_shandongensis_Zang23974_3b           CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 243
S_shandongensis_Zang23974_3c           CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 243
S_shandongensis_Zang23974_4a           CTGCGGTCGAAGTCCACGTCGTAGGTCACTGGATGGGCTTTCCGTCGGGA 244
S_shandongensis_Zang23974_4b           CTGCGGTCGAAGTCCACGTCGTAGGTCTCTGGATGGGCTTTCCGTCGGGA 243
S_tuberosa_ICM20042540a                CATCGGCCGTCGGGCACGCCGTAGGTCTCCCGATCGGCTTTCCGTGGACA 245
S_tuberosa_ICM20042540b                CATCGGCGGTCAGGCACGCCGTAGGTCTCCCGATCGGCTTTCGGTGGACA 245
S_tuberosa_ICM20042540c                CATCGGCGGTCGGGCATGCCGTAGGTCTCCCGATCGGCTTTCCGTGGACA 245
S_tuberosa_Woo23973a                   CGCCGGCTGTCGGCCACGTCGTAGGTCTCCCGATCGGCTTTCCGTGGACA 245
S_tuberosa_Woo23973b                   CTTTGGCTGTCGGCCACATCGTAGGTCTCCCGATCGGCTTTTCGTGGACA 245
S_tuberosa_Woo23973c                   CTTCGGCTGTCGGCCACGTCGTAGGTCTCCCGATCGGCTTTCCGTGGACA 245
S_tuberosa_Woo23973d                   CGTTGGCTGTCGGCCACGTCGTAGGTCTCCCGATCGGCTTTTCGTGGACA 245
S_tuberosa_Woo23973e                   CGTTGGCTGTCGGCCACGTCGTAGGTCTCCCGATCGGCTTTTCGTGGACA 245
S_tuberosa_Woo23973f                   CGCCGGCTGTCGGCCACGTCGTAGGGCTCCCGATCGGCTTTCCGTAGACA 245
S_tuberosa_Woo23973g                   CGCCGGCTGTCGGCCACGTCGTAGGTCTCCCGATCGGCTTTCCGTGGACA 245
S_tuberosa_ICM20042541a                CCCCGGCGGTCGGCCCCGTCGTACGTCTCCGGATCGGCTTTCCGTGGACA 245
S_tuberosa_ICM20042541b                CTCCGGCTGTCGGCCCCGTCGTACGTCTCCGGATCGGCTTTCCGTGGACA 245
S_tuberosa_Hu&But23960a                CTTTGGCCGTCGAGCACGCCGTAGGTCTCCCGATCGGCTTTCCGTGGACA 244
S_tuberosa_Hu&But23960b                CTTTGGCCGTCGAGCACGCCGTAGGTCTCCCGATCGGCTTTCCGTGGACA 245
S_tuberosa_Chan200401a                 CTCCGGCTGTCGGGCACGCCGTAGGACTCCCGATCGGCTTTCCGTGGACA 246
S_tuberosa_Chan200401b                 CTCCGGCTGTCGGGCACGCCGTAGGACTCCCGATCGGCTTTCCGTGGACA 245
A_filicinus_ICM20042542a               GAGAAGATGAGAAGCACGTCG--GGCTTCTCGTTGAACAAGGTTTCGGGG 245
A_filicinus_ICM20042542b               GAGAAGATGAGAAGCACGTCG--GGCTTCTCGTTGAACAAGGTTTCGCGG 245
                                             *       *   *       * * *   *      * *
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          GGTGATTCGTCCGAAACGAACAGCCATAGCTCCCGCGGCGCCGATTTTAG 296
S_japonica_ICM20042543b          GGTGATTCGTCCGAAAAGGACAGCCGTAGCTCCCGCGGCGCCGATTTTAG 296
S_japonica_Hu&But24032a          GGTGATTCGTCCGAAACGAACAGCCATAGCTCCCGCGGCGCCGATTTTAG 296
S_japonica_Hu&But23971a          GGTGATTCGTCCGAAACGAACAGCCATAGCTCCCGCGGCGCCGATTTTAG 296
S_japonica_Hu&But23971b          GGTGATTCGTCCGAAGCGGACAGCCGTAGCTCCCGCGGCGCCGATTTTAG 296
S_parviflora_Ma9066a             GGTAATTCGCACGAAACGGACACCCGTAGCTCCCGCTGCGTCGATTTTCG 293
S_parviflora_Ma9066b             GGTAATACGCACGAAACGGACACCCGTAGCTCCCGCGGCGTCGATTTTCG 293
S_parviflora_Ma9066c             TGTAATTCGCACGAAACGGACACCCGTAGCTCCCGCTGCGTCGATTTTCG 293
S_parviflora_Ma200401            GGTAATTCGCACGAAACGGACACCCGTAGCCCCCGCGGAGTCGATTTTCG 293
S_sessilifolia_Hu&But23972a      GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 294
S_sessilifolia_Hu&But23972b      GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 294
S_sessilifolia_Hu&But23972c      GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 294
S_sessilifolia_Hu&Yung606a       GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 294
S_sessilifolia_Hu&Yung606b       GGTAATTCGTCCGAAACGGACACACGTAGCCCCCGCGATGCCGATTTTCG 293
S_sessilifolia_Zang200401a       GGTAATTCGTCCGAAACGGACAACCGTAGCCCCCGCGACGCCGATTTTCG 293
S_sessilifolia_Zang200401b       GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 294
S_shandongensis_Zang23974_1a     GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 293
S_shandongensis_Zang23974_1b     GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATCTTCG 294
S_shandongensis_Zang23974_2a     GGTAATTCGCCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 294
S_shandongensis_Zang23974_2b     GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 294
S_shandongensis_Zang23974_2c     GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 293
S_shandongensis_Zang23974_3a     GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 293
S_shandongensis_Zang23974_3b     GGTAATTCGTCCGAAACGGACACCCGTAGCCTCCGCGACGCCGATTTTCG 293
S_shandongensis_Zang23974_3c     GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 293
S_shandongensis_Zang23974_4a     GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGACGCCGATTTTCG 294
S_shandongensis_Zang23974_4b     GGTAATTCGTCCGAAACGGACACACGTAGCCCCCGCGACGCCGATTTTCG 293
S_tuberosa_ICM20042540a          GGTAATTCGTCCGAAACGGACGCCCGTAGCCCCCGCGGCGACGATTTTCG 295
S_tuberosa_ICM20042540b          GGTAATTCGTCCGAAACGGACGCCCGTAGTCCCCGCAGCGACGATTTTCG 295
S_tuberosa_ICM20042540c          GGTAATTCGTCCGAAACGGACGCCCGTAGCCCCCGCGGCGACGATTTTCG 295
S_tuberosa_Woo23973a             GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGGCGACGATTTTCG 295
S_tuberosa_Woo23973b             GGTAATTCGTCCGAAACGGACACCCGCAGCCCCCGCGGCAAAGATTTTCG 295
S_tuberosa_Woo23973c             GGTAATTCGTCCGAAACGGACTCCCGTAGCACCCGCGGCGTCGATTTTCG 295
S_tuberosa_Woo23973d             GGTAATTCGTCCGAAACGGACACCCGCAGCCCCCGCGGCAAAGATTTTCG 295
S_tuberosa_Woo23973e             GGTAATTCGTCCGAAACGGACACCCGCAGCCCCCGCGGCAAAGATTTTCG 295
S_tuberosa_Woo23973f             GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGGCGACGATTTTCG 295
S_tuberosa_Woo23973g             GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGGCGACGATTTTCG 295
S_tuberosa_ICM20042541a          GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCGGCGTCGATTTTCG 295
S_tuberosa_ICM20042541b          GGTAATTCGTCCGAAACGGACACCCGTAGCCCCCGCAGCGTCGATTTTCG 295
S_tuberosa_Hu&But23960a          GGTAATTCGTCCGAAACGGACGCCCGTAGCCCCCGCGGCGACGATTTTCG 294
S_tuberosa_Hu&But23960b          GGTAATTCGTCCGAAACGGACGCCCGTAGCCCCCGCGGCGACGATTTTCG 295
S_tuberosa_Chan200401a           GGTAATTCGTCCGAAACGGACGCCCGTAGCCCCCGCGGCGACGATTTTCG 296
S_tuberosa_Chan200401b           GGTAATTCGTCCGAAACGGACGCCCGTAGCCCCCGCGGCGACGATTTTCG 295
A_filicinus_ICM20042542a         AGGGGGCTGACCGTCGGGCAACTTAG---CCCCCTCCGGATCGGAAGTTG 292
A_filicinus_ICM20042542b         AGGGGGCCGACCGTCGGGCAACTACGTCCCCCCCCCCGGATCGGAAGTTG 295
                                  *     *  **    * *           ** *     *    * *
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          TGGCGCT--------------AGTTTTATT--AT--TAGTATACTATTT 327
S_japonica_ICM20042543b          TGGCGCC--------------AGTTTTATT--AT--TAGTATACTATTT 327
S_japonica_Hu&But24032a          TGGCGCT--------------AGTTTTATT--AT--TAGTATACTATTT 327
S_japonica_Hu&But23971a          TGGCGCT--------------AGTTTTATT--AT--TAGTATACTATTT 327
S_japonica_Hu&But23971b          TGGCGCC--------------AGTTTTATT--AT--TAGTATACTATTT 327
S_parviflora_Ma9066a             CAGGGTCGCGC-----------GTCTCGGC--GTCTCATTGCAGCGACG 329
S_parviflora_Ma9066b             CGGGGTCGCCC-----------ATCTCGGC--GTCTCATTGCAGCGACG 329
S_parviflora_Ma9066c             CAGGGTCGCGC-----------GTCTCGGC--GTCTCATTGCAGCGACG 329
S_parviflora_Ma200401            CGGGGTCGCGC-----------ATCTCGGC--GTCTCATTGCAGCGACG 329
S_sessilifolia_Hu&But23972a      AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 342
S_sessilifolia_Hu&But23972b      AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 342
S_sessilifolia_Hu&But23972c      AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 342
S_sessilifolia_Hu&Yung606a       AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 342
S_sessilifolia_Hu&Yung606b       AGGCGTCGCGCAATCGTCGCCAAATTCTGCC--AGCGTGCCGTGCCTCTC 341
S_sessilifolia_Zang200401a       AGGCGTCGCGCAATCGTCGCCAAATTTCGCA--AGCGTGCCGTGCCTATC 341
S_sessilifolia_Zang200401b       AGGCGTCGCGCAATCGTCGCCAAATTCCGCC--AGCGTGCCGTGCCTATC 342
S_shandongensis_Zang23974_1a     AGGCGTCGCGCAATCGTCGCCAAATTCCGCC--AGCGTGCCGTGCCTATC 341
S_shandongensis_Zang23974_1b     AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 342
S_shandongensis_Zang23974_2a     AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 342
S_shandongensis_Zang23974_2b     AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 342
S_shandongensis_Zang23974_2c     AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 341
S_shandongensis_Zang23974_3a     AGGCATCGCGCAATCGTCGCCAAATTCCGCC--AGCGTGCCGTGCCTATC 341
S_shandongensis_Zang23974_3b     AGGCGTCGCGCAATCGTCGCCAAATTTCGCA--AGCGTGCCGTGCCTATC 341
S_shandongensis_Zang23974_3c     AGGCATCGCGCAATCGTCGCCAAATTCCGCC--AGCGTGCCGTGCCTATC 341
S_shandongensis_Zang23974_4a     AGGCGTCGCGCAATCGTCGCCAAATTCCGTC--AGCGTGCCGTGCCTCTC 342
S_shandongensis_Zang23974_4b     AGGTGTCGCGCAATCGTCGCCAAATTCCGCC--AGCGTGCCGTGCCTCAC 341
S_tuberosa_ICM20042540a          TGGCGTCGCGC-----------AATTCGGC--ACCAAACAGCAAGCACG 331
S_tuberosa_ICM20042540b          TGGCGTCGCGT-----------AATTCGAC--ACCAAACAGCAAGCACG 331
S_tuberosa_ICM20042540c          TGGCGTCGAGC-----------AATTCGGC--ACCAAACAGCAAGCACG 331
S_tuberosa_Woo23973a             TGGCGTCGCGC-----------AATTCGGC--ACCAAACAGCAAGCACG 331
S_tuberosa_Woo23973b             TGGCGTCGCGC-----------AATTCGGC--ACCAAACAGCAAGCACG 331
S_tuberosa_Woo23973c             TGGCGTCGCGC-----------AATTCGGC--ACCAAAGAGCAAGCACG 331
S_tuberosa_Woo23973d             TGGCGTCGCGC-----------AATTCGGC--ACCAAACAGCAAGCACG 331
S_tuberosa_Woo23973e             TGGCGTCGCGC-----------AATTCGGC--ACCAAACAGCAAGCACG 331
S_tuberosa_Woo23973f             TGGCGTCGCGC-----------AATTCGGC--ACCAAACAGCANGCACG 331
S_tuberosa_Woo23973g             TGGCGTCGCGC-----------AATTCGGC--ACCAAACAGCAAGCACG 331
S_tuberosa_ICM20042541a          TGGCGTCGCGC-----------AATTCGGC--AACAAATTGCAAGCACG 331
S_tuberosa_ICM20042541b          TGGCGTCGCGC-----------AATTCGGC--AACAAATTGCAAGCACG 331
S_tuberosa_Hu&But23960a          TGGCGTCGCGC-----------AATTCGGC--ACCTAACAGCAAGCACG 330
S_tuberosa_Hu&But23960b          TGGCGTCGCGC-----------AATTCGGC--ACCTAACAGCAAGCACG 331
S_tuberosa_Chan200401a           TGGCGTCGCGC-----------AATTCGGC--ACTAAACAGCAAGCACG 332
S_tuberosa_Chan200401b           TGGCGTCGCGC-----------AATTCGGC--ACCAAACAGCAAGCACG 331
A_filicinus_ICM20042542a         CGG-GTCGGGGAGGTCACGCGGAAGCCCGTCCGGCGGAATTGGAGTGTTT 341
A_filicinus_ICM20042542b         CGG-GTCGGGGAGGTCACGCGGAAGCCCGTCCGGCGGAATTGGAGTGTTT 344
                                    *
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          TTATTTTTATTTTTG-----------TTTTTATTTTTTTTT-----GGG 360
S_japonica_ICM20042543b          TTATTTTTATTTTTA-----------TTTTT--------TT-----GGG 352
S_japonica_Hu&But24032a          TTATTTTTATTTTTG-----------TTTTTATTTTTTTTT-----GGG 360
S_japonica_Hu&But23971a          TTATTTTTATTTTTG-----------TTTTTATTTTTTTTT-----GGG 360
S_japonica_Hu&But23971b          TTATTTTTATTTTTG-----------TTTTT-------TTT-----GGG 353
S_parviflora_Ma9066a             CCCCTCGCTTTCGTT-----------GTTGTCGGATTTTTCTTTTTTTT 367
S_parviflora_Ma9066b             CCCCTCGCTTTCGTT-----------GTTGTTG----TTTTTTTTTTT 363
S_parviflora_Ma9066c             CACCTCGCTTTCGTT-----------GTTGTCGGATTTTTCTTTTTTTT 367
S_parviflora_Ma200401            CCCCTCGCTTTCGTT-----------GTTGTTG----TTGTTTTTTTTT 363
S_sessilifolia_Hu&But23972a      CTATTATTACTAGAC-----------TATATATCCATTTTTTTAAAAAA 380
S_sessilifolia_Hu&But23972b      CTATTATTACTAGAC-----------TATATATCCATTTTTTTAAAAAA 380
S_sessilifolia_Hu&But23972c      CTATTATTACTAGAC-----------TATATATCCATTTTTTTAAAAAA 380
S_sessilifolia_Hu&Yung606a       CTATTATTACTAGAC-----------TATTTATCCATTTTTTTAAAAAA 380
S_sessilifolia_Hu&Yung606b       CTATTATTACTATAC-----------TATATATCCTTTTTT----AAAA 375
S_sessilifolia_Zang200401a       CTATTATTACTATAC-----------TATATATCCTTTTTTTT-----T 374
S_sessilifolia_Zang200401b       CTATTATTACTATAC-----------TATATATCCTTTTTTT-----AA 375
S_shandongensis_Zang23974_1a     CTATTATTACTATAC-----------TATATATCCTTTTTTT-----AA 374
S_shandongensis_Zang23974_1b     CTATTATTACTAGAC-----------TATATATCCATTTTTTTAAAAAA 380
S_shandongensis_Zang23974_2a     CTATTATTACTAGAC-----------TATATATCCATTTTTTTAAAAAA 380
S_shandongensis_Zang23974_2b     CTATTATTACTAGAC-----------TATATATCCATTTTTTTAAAAAA 380
S_shandongensis_Zang23974_2c     CTATTATTACTAGAC-----------TATATATCCATTTTTT-AAAAAA 378
S_shandongensis_Zang23974_3a     CTATTATTACTATAC-----------TATATATCCTTTTTTTT-----A 374
S_shandongensis_Zang23974_3b     CTATTATTACTATAC-----------TATATATCCTTTTTTTT-----T 374
S_shandongensis_Zang23974_3c     CTATTATTACTATAC-----------TATATATCCTTTTTTTT-----A 374
S_shandongensis_Zang23974_4a     CTATTATTACTAGAC-----------TATATATCCATTTTTTTAAAAAA 380
S_shandongensis_Zang23974_4b     CTATTATTACTATAC-----------TATATATCCTTTTTTTT----TA 375
S_tuberosa_ICM20042540a          TAGCGTGCATTTTTT-----------TTTTT-------CTT-------- 354
S_tuberosa_ICM20042540b          TAGCGTGCATTTTTT-----------TTTTT-------CTT-------- 354
S_tuberosa_ICM20042540c          TAGCGTGCATTTTTT-----------TTTT--------CTT-------- 353
S_tuberosa_Woo23973a             TAGCGTGCATTTTTT-----------TTTT--------TT--------- 352
S_tuberosa_Woo23973b             TAGCGTGCATTTTTT-----------TTT---------------------- 349
S_tuberosa_Woo23973c             TGGCGTGCATTTTTT-----------TTTT--------------------- 350
S_tuberosa_Woo23973d             TAGCGTGCATTTTTT-----------TTT---------------------- 349
S_tuberosa_Woo23973e             TAGCGTGCATTTTTT-----------TTT---------------------- 349
S_tuberosa_Woo23973f             TAGCGTGCATTTTTT-----------TTTT--------TTT-------- 353
S_tuberosa_Woo23973g             TAGCGTGCATTTTTT-----------TTTT--------TT--------- 352
S_tuberosa_ICM20042541a          CAGCGTGGTTTTCTA-----------CTTT--------TTT-------- 353
S_tuberosa_ICM20042541b          CAGCGTGGTTTTCTA-----------CGTT--------TTT-------- 353
S_tuberosa_Hu&But23960a          TAGCGTGCATTTTTT-----------TTT---------CTT-------- 351
S_tuberosa_Hu&But23960b          TAGCGTGCATTTTTT-----------TTTT--------CTT-------- 353
S_tuberosa_Chan200401a           TAGCGTGCATTTTTT-----------TTT---------CTT-------- 353
S_tuberosa_Chan200401b           TAGCGTGCATTTTTT-----------TTTT--------CTT-------- 353
A_filicinus_ICM20042542a         CCCCGCGCTCGGGGCAACGTCAGGGGGTCCGGGCCAGCGTTTCCCTTGTG 391
A_filicinus_ICM20042542b         CCCCGCGCTCGGGGCAACGGCTGGGGGTCCGGGCCAGCGTTTCCCTCGTA 394
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          GGGGGGGG-ATTTATGG-CCTCTCCCAGCCACTCGAGCGTCGTTCATGGA 408
S_japonica_ICM20042543b          GGG------ATTTATGG-CCTCTCCCAGCCACTCGAGCGTCGTTCATGGA 395
S_japonica_Hu&But24032a          GGGGGGGGGATTTATGG-CCTCTCCCAGCCACTCGAGCGTCGTTCATGGA 409
S_japonica_Hu&But23971a          GGGGGGGG-ATTTATGG-CCTCTCCCAGCCACTCGAGCGTCGTTCATGGA 408
S_japonica_Hu&But23971b          GGG------ATTTATGG-CCTCTCCCAGCCACTCGAGCGTCGTTCATGGA 396
S_parviflora_Ma9066a             TTTTTTGTGAATTG--ACCCCTCCCAGGCCTGTCGATCGTCGCTCAAGGC 415
S_parviflora_Ma9066b             TTTTTTGTGAATTA--ACCCCTCCCTGGCCAGTCGATCGTCGTTCAAGGC 411
S_parviflora_Ma9066c             TTTTT-GTGAATTG--ACCCCTCCCAGGCCAGTCGATCGTCGCTCAAGGC 414
S_parviflora_Ma200401            TTTT--GTGAATTA--ACCCCTCCCGGGCCAGTCGATCGTCGTTCAAGGC 409
S_sessilifolia_Hu&But23972a      AACAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 429
S_sessilifolia_Hu&But23972b      AACAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 429
S_sessilifolia_Hu&But23972c      AACAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 429
S_sessilifolia_Hu&Yung606a       AACAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 429
S_sessilifolia_Hu&Yung606b       AAAAAAAAATATTATGG-CCTCTCCCAGCCAGTCGGGCGTCATTGATGGA 424
S_sessilifolia_Zang200401a       TAAAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 423
S_sessilifolia_Zang200401b       AAAAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 424
S_shandongensis_Zang23974_1a     AAAAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 423
S_shandongensis_Zang23974_1b     AACAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 429
S_shandongensis_Zang23974_2a     AACAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 429
S_shandongensis_Zang23974_2b     AACAGAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 429
S_shandongensis_Zang23974_2c     AACAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 427
S_shandongensis_Zang23974_3a     AAAAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 423
S_shandongensis_Zang23974_3b     ---AAAAAAAATTATGGGCCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 421
S_shandongensis_Zang23974_3c     AAAAAAAAAA-TTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 422
S_shandongensis_Zang23974_4a     AACAAAAAAAATTATGG-CCTCTCCCAGCCAGTCGGGCGTCGTTGATGGA 429
S_shandongensis_Zang23974_4b     AAAAAAAAATATTATGG-CCTCTCCTATCCAGTCGGGCGTCGTTGATGGA 424
S_tuberosa_ICM20042540a          ----ACGGGATTTATGG-CCCACTTCGGCCAGTCGAGCGTCGCTGACGGA 399
S_tuberosa_ICM20042540b          ----ACGGGATTTATGG-CCCACTTCGGCCAGTCGAGCGTCGCTGACGGA 399
S_tuberosa_ICM20042540c          ----ACAGGATTTATGG-CCCACTTCGGCCAGTCGAGCGTCGCTGACGGA 398
S_tuberosa_Woo23973a             ----ATGGGATTTATGG-CCCACTTCGGCCACTCGAGCGTCGCTGACGGA 397
S_tuberosa_Woo23973b             -----TGGGATTTATGG-CCCACTTCGGCCACTCGAGCGTCGCTGACGTA 393
S_tuberosa_Woo23973c             ----ATGGGATTTCTGG-CCCACTTCGGCCACTCGAGCGTCGCTGAAGGA 395
S_tuberosa_Woo23973d             ----ATGGGATTTATGG-CCCACTTCGGCCACTCGAGCGTCGCTGACGTA 394
S_tuberosa_Woo23973e             ----ATGGGATTTATGG-CCCACTTCGGCCACTCGAGCGTCGCTGACGTA 394
S_tuberosa_Woo23973f             ----ATGGGATTTATGG-CCCACTTCGGCCACTCGAGCGTCGCTGACGGA 398
S_tuberosa_Woo23973g             ----ATGGGATTTATGG-CCCACTTCGGCCACTCGAGCGTCGCTGACGGA 397
S_tuberosa_ICM20042541a          ----ATGGGATTTATGG-CCAACTCCGGCCACTCGAGCGTCGTTGACGGA 398
S_tuberosa_ICM20042541b          ----ATGGGATTTATGG-CCAACTCCGGCCACTCGAGCGTCGTTGACGGA 398
S_tuberosa_Hu&But23960a          ----ACGGGATTTATGG-CCCACTTCGGCCAGTCGAGCGTCGCTGACGGA 396
S_tuberosa_Hu&But23960b          ----ACGGGATTTATGG-CCCACTTCGGCCAGTCGAGCGTCGCTGACGGA 398
S_tuberosa_Chan200401a           ----ACGGGATTTGTGG-CCCATTTCGGCCTGTCGAGCGTCGCTGACGGA 398
S_tuberosa_Chan200401b           ----ACGGGATTTGTGG-CCCATTTCGGCCTGTCGAGCGTCGCTGACGGA 398
A_filicinus_ICM20042542a         AGCAATAAAAATTGCCT-TTATCCCTGGCAGGTACAAAAACGCAGATATC 440
A_filicinus_ICM20042542b         GGCAATAAAAATTGCCT-TTATCCCTAGCAGAGACAAAAACGCGGATATC 443
                                              **          *         *   *
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          ATC-GGGATTGCGAATGGACAGGGTTAAGAGGGAAACGCGGTAGAAAAAT 457
S_japonica_ICM20042543b          ATC-GGGATTGCGAAGGGACAGTGTTAAGAGGGAAATGCGGTAGAAAAAT 444
S_japonica_Hu&But24032a          ATC-GGGATTGCGAATGGACAGGGTTAAGAGGGAAACGCGGTAGAAAAAT 458
S_japonica_Hu&But23971a          ATC-GGGATTGCGAATGGACAGGGTTAAGAGGGAAACGCGGTAGAAAAAT 457
S_japonica_Hu&But23971b          ATC-AGGATTGAGAAGGGACAGGGTTAAGAGGGAAACGCGGTAGAAAAAT 445
S_parviflora_Ma9066a             ATC-GGGAGGGCGGAAGAACATTGTTAAAAAGGAAAAGCAGCCAAGTAAG 464
S_parviflora_Ma9066b             ATC-GGGAGGGCGGAAGAACATTGTTAAAAGTGAAAAGCAGCCAAGTAAT 460
S_parviflora_Ma9066c             ATC-GGGAGGGCGGAAGAACATTGTTAAAAAGGAAAAGCAGCCAAGTAAG 463
S_parviflora_Ma200401            ATC-GGGAGGGCGGAAGAACATTGTTGAAAGTGAAAAGCAGCCAAGTAAT 458
S_sessilifolia_Hu&But23972a      ATC-GGGAGTGCGATACGTCAGGGTTAAGATGGAAACGCGGTAGAAAGAT 478
S_sessilifolia_Hu&But23972b      ATC-GGGAGTGCGATACGTCAGGGTTAAGATGGAAACGCGGTAGAAAGAT 478
S_sessilifolia_Hu&But23972c      ATC-GGGAGTGCGATAGGTCAGGGTTAAGATGGAAACGCGGTAGAAAGAT 478
S_sessilifolia_Hu&Yung606a       ATC-GGGAGTGCGATACGTCAGGGTTAAGATGGAAACGCGGTAGAAAGAT 478
S_sessilifolia_Hu&Yung606b       ATC-GGGAGTGCGATAGGACAGGGCTAAGATGGAAACGCGGTAGAAAGAT 473
S_sessilifolia_Zang200401a       ATC-GGGAGTGCGATAGGACGGGGTCAAGATGGAAACGCGGTAGAAAGAT 472
S_sessilifolia_Zang200401b       ATC-GGGAGTGCGATAGGATAGGGTTAAGATGGAAACGCGGTAGAAAGAT 473
S_shandongensis_Zang23974_1a     ATC-GGGAGTGCGATAGGACAGGGTTAAGATGGAAACGCGATAGAAAGAT 472
S_shandongensis_Zang23974_1b     ATC-GGGAGTGCGATACGTCAGGGTTAAGATGGAAACGCGGTAGAAAGAT 478
S_shandongensis_Zang23974_2a     ATC-GGGAGTGCGATACGTCAGGGTTAAGATGGAAACGCGGTAGAAAGAT 478
S_shandongensis_Zang23974_2b     ATC-GGGAGTGCGATAGGTCAGGGTTTAGATGGAAACGCGGTAGAAAGAT 478
S_shandongensis_Zang23974_2c     ATC-GGGAGTGCGATACGTCAGGGTTAAGATGGAAACGCGGTAGAAAGAT 476
S_shandongensis_Zang23974_3a     ATC-GGGAGTGCGATAGGACAGGGTTAAGATGGAAACGCGGTAGAAAGAT 472
S_shandongensis_Zang23974_3b     ATC-GGGAGTGCGATAGGACAGGGTTAAAATGGAAACGCGGTAAAAAGAT 470
S_shandongensis_Zang23974_3c     ATC-GGGAGTGCGATAGGACAGGGTTAAGATGGAAACGCGGTAGAAAGAT 471
S_shandongensis_Zang23974_4a     ATC-GGGAGTGCGATACGTCAGGGTTAAGATGGAAACGCGGTAGAAAGAT 478
S_shandongensis_Zang23974_4b     ATC-GGGAGTGCGATAGGACAGGGTTAAGATGGAAACGCGGTAGAAAGAT 473
S_tuberosa_ICM20042540a          ATC-GGGAGTGCGGGACGGCAGGGCTAAGAATTAAAAGCGGACGAAAGAT 448
S_tuberosa_ICM20042540b          ATC-GGGAGTGCGGGGCGGCAGGGCTAAGAAGTAAAAGCGGCAGAAAGAT 448
S_tuberosa_ICM20042540c          ATC-GGGAGTGCGGGACGGCAGGGCTAAGAAGTAAAAGCGGCAGAAAGAT 447
S_tuberosa_Woo23973a             ATC-GGGAGTGCGGGACAGCGGGTTTATGAAGTAAAAGCGGAAGAAAGAT 446
S_tuberosa_Woo23973b             ATC-GGGAGTGCGGGACAGCGGGTTTATGAAGTAAAAGCGGAAGAAAGAT 442
S_tuberosa_Woo23973c             ATC-GGGAGTGCGGGACAGCGGGTTTAAGAAGTAAAAGCGGAAGAAAGAT 444
S_tuberosa_Woo23973d             ATC-GGGAGTGCGGGACAGCGGGTTTATGAAGTAAAAGCGGAAGAAAGAT 443
S_tuberosa_Woo23973e             ATC-GGGAGTGCGGGACAGCGGGTTTATGAAGTAAAAGCGGAAGAAAGAT 443
S_tuberosa_Woo23973f             ATC-GGGAGTGCGGGACAGCGGGTTTATGAAGTAAAAGCGGAAGAAAGAT 447
S_tuberosa_Woo23973g             ATC-GGGAGTGCGGGACAGCGGGTTTATGAAGTAAAAGCGGAAGAAAGAT 446
S_tuberosa_ICM20042541a          ATC-GGGAGCGCGGGACTGCGGGGTTAAGAAGTAAAAGCGGAAGAAGGTT 447
S_tuberosa_ICM20042541b          AAC-GGGAGCGCGGGACTGCGGGGTTAAGAAGTAAAAGCGGAAGAAGGTT 447
S_tuberosa_Hu&But23960a          ATC-GGGAGTGCGGGACGGCGGGGCTGAGAAGTAAAAGCGGACGAAAGAT 445
S_tuberosa_Hu&But23960b          ATC-GGGAGTGCGGGACGGCGGGGCTGAGAAGTAAAAGCGGACGAAAGAT 447
S_tuberosa_Chan200401a           ATC-GGGAGTGCGGGACGGCGGGGTCAAGAAGTAAAAGCGGAAGAAGGAT 447
S_tuberosa_Chan200401b           ATC-GGGAGTGCGGGACGGCGGGGTCAAGAAGTAAAAGCGGAAGAAGGAT 447
A_filicinus_ICM20042542a         CCT-CAAACCGT-AAGGGATTTGGCGAAACGGAGGAAGCGAACGAACCCT 488
A_filicinus_ICM20042542b         CCT-CAAACCGT-AAGGGATTTGGCGAAACGGAGGAAGCGAACGAACCCT 491
                                       *  *                      * **     *
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          GTCGGGTGCGATCATACCAGCACTAA---------------------- 483
S_japonica_ICM20042543b          GTCGTGTGCGATCATACCAGCACTAA---------------------- 470
S_japonica_Hu&But24032a          GTCGGGTGCGATCATACCAGCACTAA---------------------- 484
S_japonica_Hu&But23971a          GTCGGGTGCGATCATACCAGCACTAA---------------------- 483
S_japonica_Hu&But23971b          GTCGGGTGCGATCATACCAGCACTAA---------------------- 471
S_parviflora_Ma9066a             GTCGGATGCGATCATACCAGCACTAA---------------------- 490
S_parviflora_Ma9066b             GTCGGATGCGATCATACCAGCACTAA---------------------- 486
S_parviflora_Ma9066c             GTCGGATGCGATCATACCAGCACTAA---------------------- 489
S_parviflora_Ma200401            GTCGGATGCGATCATACCAGCACTAA---------------------- 484
S_sessilifolia_Hu&But23972a      GTCGGGTGCGATCATACCAGCACTAA---------------------- 504
S_sessilifolia_Hu&But23972b      GTCGGGTGCGATCATACCAGCACTAA---------------------- 504
S_sessilifolia_Hu&But23972c      GTCGGGTGCGATCATACCAGCACTAA---------------------- 504
S_sessilifolia_Hu&Yung606a       GTCGGGTGCGATCATACCAGCACTAA---------------------- 504
S_sessilifolia_Hu&Yung606b       GTCGGGTGCGATCATACCAGCACTAA---------------------- 499
S_sessilifolia_Zang200401a       GTCGGGTGCGATCATACCAGCACTAA---------------------- 498
S_sessilifolia_Zang200401b       GTCGGGTGCGATCATACCAGCACTAA---------------------- 499
S_shandongensis_Zang23974_1a     GTCGGGTGCGATCATACCAGCACTAA---------------------- 498
S_shandongensis_Zang23974_1b     GTCGGGTGCGATCATACCAGC-CTAA---------------------- 503
S_shandongensis_Zang23974_2a     GTCGGGTGCGATCATACCAGCACTAA---------------------- 504
S_shandongensis_Zang23974_2b     GTCGGGTGCGATCATACCAGCACTAA---------------------- 504
S_shandongensis_Zang23974_2c     GTCGGGTGCGATCATACCAGCACTAA---------------------- 502
S_shandongensis_Zang23974_3a     GTCGGGTGCGATCATACCAGCACTAA---------------------- 498
S_shandongensis_Zang23974_3b     GTCGGGTGCGATCATACCAGCACTAA---------------------- 496
S_shandongensis_Zang23974_3c     GTCGGGTGCGATCATACCAGCACTAA---------------------- 497
S_shandongensis_Zang23974_4a     GTCGGGTGCGATCATACCAGCACTAA---------------------- 504
S_shandongensis_Zang23974_4b     GTCGGGTGCGATCATACCAGCACTAA---------------------- 499
S_tuberosa_ICM20042540a          GTCGGGTGCGATCATACCAGCACTAA---------------------- 474
S_tuberosa_ICM20042540b          GTCGGGTGCGATCATACCAGCACTAA---------------------- 474
S_tuberosa_ICM20042540c          GTCGGGTGCGATCATACCAGCACTAA---------------------- 473
S_tuberosa_Woo23973a             GTCGGGTGCGATCATACCAGCACTAA---------------------- 472
S_tuberosa_Woo23973b             GTTGGGTGCGATCATACCAGCACTAA---------------------- 468
S_tuberosa_Woo23973c             GTCGGGTGCGATCATACCAGCACTAA---------------------- 470
S_tuberosa_Woo23973d             GTCGGGTGCGATCA--CCAGCACTAA---------------------- 467
S_tuberosa_Woo23973e             GTCGGGTGCGATCA--CCAGCACTAA---------------------- 467
S_tuberosa_Woo23973f             GTCGGGTGCGATCATACCAGCACTAA---------------------- 473
S_tuberosa_Woo23973g             GTCGGGTGCGATCATACCAGCACTAA---------------------- 472
S_tuberosa_ICM20042541a          GTCGGGTGCGATCATACCAGCACTAA---------------------- 473
S_tuberosa_ICM20042541b          GTCGGGTGCGATCATACCAGCACTAA---------------------- 473
S_tuberosa_Hu&But23960a          GTCGGGTGCGATCATACCAGCACTAA---------------------- 471
S_tuberosa_Hu&But23960b          GTCGGGTGCGATCATACCAGCACTAA---------------------- 473
S_tuberosa_Chan200401a           GTCGGGTGCGATCATACCAGCACTAA---------------------- 473
S_tuberosa_Chan200401b           GTCGGGTGCGATCATACCAGCACTAA---------------------- 473
A_filicinus_ICM20042542a         -TCGGGTTCGTTCG-GTTGGCTCCGTCTCGCCGAGATAAGCGATTTTCAT 536
A_filicinus_ICM20042542b         -TCGGGTTAGTTCG-GTTGGCTCCGTCTCGTCGAGATAAGCGATTTTCGT 539
                                  * *  *  * **      ** *
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a                 ----------------------------------------------
S_japonica_ICM20042543b                 ----------------------------------------------
S_japonica_Hu&But24032a                 ----------------------------------------------
S_japonica_Hu&But23971a                 ----------------------------------------------
S_japonica_Hu&But23971b                 ----------------------------------------------
S_parviflora_Ma9066a                    ----------------------------------------------
S_parviflora_Ma9066b                    ----------------------------------------------
S_parviflora_Ma9066c                    ----------------------------------------------
S_parviflora_Ma200401                   ----------------------------------------------
S_sessilifolia_Hu&But23972a             ----------------------------------------------
S_sessilifolia_Hu&But23972b             ----------------------------------------------
S_sessilifolia_Hu&But23972c             ----------------------------------------------
S_sessilifolia_Hu&Yung606a              ----------------------------------------------
S_sessilifolia_Hu&Yung606b              ----------------------------------------------
S_sessilifolia_Zang200401a              ----------------------------------------------
S_sessilifolia_Zang200401b              ----------------------------------------------
S_shandongensis_Zang23974_1a            ----------------------------------------------
S_shandongensis_Zang23974_1b            ----------------------------------------------
S_shandongensis_Zang23974_2a            ----------------------------------------------
S_shandongensis_Zang23974_2b            ----------------------------------------------
S_shandongensis_Zang23974_2c            ----------------------------------------------
S_shandongensis_Zang23974_3a            ----------------------------------------------
S_shandongensis_Zang23974_3b            ----------------------------------------------
S_shandongensis_Zang23974_3c            ----------------------------------------------
S_shandongensis_Zang23974_4a            ----------------------------------------------
S_shandongensis_Zang23974_4b            ----------------------------------------------
S_tuberosa_ICM20042540a                 ----------------------------------------------
S_tuberosa_ICM20042540b                 ----------------------------------------------
S_tuberosa_ICM20042540c                 ----------------------------------------------
S_tuberosa_Woo23973a                    ----------------------------------------------
S_tuberosa_Woo23973b                    ----------------------------------------------
S_tuberosa_Woo23973c                    ----------------------------------------------
S_tuberosa_Woo23973d                    ----------------------------------------------
S_tuberosa_Woo23973e                    ----------------------------------------------
S_tuberosa_Woo23973f                    ----------------------------------------------
S_tuberosa_Woo23973g                    ----------------------------------------------
S_tuberosa_ICM20042541a                 ----------------------------------------------
S_tuberosa_ICM20042541b                 ----------------------------------------------
S_tuberosa_Hu&But23960a                 ----------------------------------------------
S_tuberosa_Hu&But23960b                 ----------------------------------------------
S_tuberosa_Chan200401a                  ----------------------------------------------
S_tuberosa_Chan200401b                  ----------------------------------------------
A_filicinus_ICM20042542a                ATATAATTCGTCCAATTCCGACTTACGGCTTGAAAGTTACGCCCTATTCT 586
A_filicinus_ICM20042542b                ATATAATTCGTCCAATTCCGACTTACGGTTTGAAAGTTACGCCCTATTCT 589
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Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          ----------------------------------------------
S_japonica_ICM20042543b          ----------------------------------------------
S_japonica_Hu&But24032a          ----------------------------------------------
S_japonica_Hu&But23971a          ----------------------------------------------
S_japonica_Hu&But23971b          ----------------------------------------------
S_parviflora_Ma9066a             ----------------------------------------------
S_parviflora_Ma9066b             ----------------------------------------------
S_parviflora_Ma9066c             ----------------------------------------------
S_parviflora_Ma200401            ----------------------------------------------
S_sessilifolia_Hu&But23972a      ----------------------------------------------
S_sessilifolia_Hu&But23972b      ----------------------------------------------
S_sessilifolia_Hu&But23972c      ----------------------------------------------
S_sessilifolia_Hu&Yung606a       ----------------------------------------------
S_sessilifolia_Hu&Yung606b       ----------------------------------------------
S_sessilifolia_Zang200401a       ----------------------------------------------
S_sessilifolia_Zang200401b       ----------------------------------------------
S_shandongensis_Zang23974_1a     ----------------------------------------------
S_shandongensis_Zang23974_1b     ----------------------------------------------
S_shandongensis_Zang23974_2a     ----------------------------------------------
S_shandongensis_Zang23974_2b     ----------------------------------------------
S_shandongensis_Zang23974_2c     ----------------------------------------------
S_shandongensis_Zang23974_3a     ----------------------------------------------
S_shandongensis_Zang23974_3b     ----------------------------------------------
S_shandongensis_Zang23974_3c     ----------------------------------------------
S_shandongensis_Zang23974_4a     ----------------------------------------------
S_shandongensis_Zang23974_4b     ----------------------------------------------
S_tuberosa_ICM20042540a          ----------------------------------------------
S_tuberosa_ICM20042540b          ----------------------------------------------
S_tuberosa_ICM20042540c          ----------------------------------------------
S_tuberosa_Woo23973a             ----------------------------------------------
S_tuberosa_Woo23973b             ----------------------------------------------
S_tuberosa_Woo23973c             ----------------------------------------------
S_tuberosa_Woo23973d             ----------------------------------------------
S_tuberosa_Woo23973e             ----------------------------------------------
S_tuberosa_Woo23973f             ----------------------------------------------
S_tuberosa_Woo23973g             ----------------------------------------------
S_tuberosa_ICM20042541a          ----------------------------------------------
S_tuberosa_ICM20042541b          ----------------------------------------------
S_tuberosa_Hu&But23960a          ----------------------------------------------
S_tuberosa_Hu&But23960b          ----------------------------------------------
S_tuberosa_Chan200401a           ----------------------------------------------
S_tuberosa_Chan200401b           ----------------------------------------------
A_filicinus_ICM20042542a         CCGATCTATGTTCAGAACCTCGCCTAAGGGGGGAAGGAGACGGCTAGTGG 636
A_filicinus_ICM20042542b         TCGATCTATGTTCGGAGCCTCGCCTAAGGGGGGAAGGAGACGGCGAGTGG 639
```

Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

```
S_japonica_ICM20042543a          ----------------------
S_japonica_ICM20042543b          ----------------------
S_japonica_Hu&But24032a          ----------------------
S_japonica_Hu&But23971a          ----------------------
S_japonica_Hu&But23971b          ----------------------
S_parviflora_Ma9066a             ----------------------
S_parviflora_Ma9066b             ----------------------
S_parviflora_Ma9066c             ----------------------
S_parviflora_Ma200401            ----------------------
S_sessilifolia_Hu&But23972a      ----------------------
S_sessilifolia_Hu&But23972b      ----------------------
S_sessilifolia_Hu&But23972c      ----------------------
S_sessilifolia_Hu&Yung606a       ----------------------
S_sessilifolia_Hu&Yung606b       ----------------------
S_sessilifolia_Zang200401a       ----------------------
S_sessilifolia_Zang200401b       ----------------------
S_shandongensis_Zang23974_1a     ----------------------
S_shandongensis_Zang23974_1b     ----------------------
S_shandongensis_Zang23974_2a     ----------------------
S_shandongensis_Zang23974_2b     ----------------------
S_shandongensis_Zang23974_2c     ----------------------
S_shandongensis_Zang23974_3a     ----------------------
S_shandongensis_Zang23974_3b     ----------------------
S_shandongensis_Zang23974_3c     ----------------------
S_shandongensis_Zang23974_4a     ----------------------
S_shandongensis_Zang23974_4b     ----------------------
S_tuberosa_ICM20042540a          ----------------------
S_tuberosa_ICM20042540b          ----------------------
S_tuberosa_ICM20042540c          ----------------------
S_tuberosa_Woo23973a             ----------------------
S_tuberosa_Woo23973b             ----------------------
S_tuberosa_Woo23973c             ----------------------
S_tuberosa_Woo23973d             ----------------------
S_tuberosa_Woo23973e             ----------------------
S_tuberosa_Woo23973f             ----------------------
S_tuberosa_Woo23973g             ----------------------
S_tuberosa_ICM20042541a          ----------------------
S_tuberosa_ICM20042541b          ----------------------
S_tuberosa_Hu&But23960a          ----------------------
S_tuberosa_Hu&But23960b          ----------------------
S_tuberosa_Chan200401a           ----------------------
S_tuberosa_Chan200401b           ----------------------
A_filicinus_ICM20042542a         ATGGGTGCGATCATACCAGCACTAA 661
A_filicinus_ICM20042542b         ATGGGTGCGATCATACCAGCACTAA 664
```

Figure 4.13 (continued) Sequence alignment of the 5S rRNA spacers of *Stemona* species and *Asparagus filicinus*

	Stemona tuberosa	*Stemona japonica*	*Stemona sessilifolia*	*Stemona shandongensis*	*Stemona parviflora*	*Asparagus filicinus*
Stemona tuberosa	89-100% (94.5%)	73-79% (76%)	76-81% (78.5%)	78-86% (82%)	75-78% (76.5%)	16-19 (17.5%)
Stemona japonica		96-100% (98%)	83-86% (84.5%)	83-86% (84.5%)	72-75% (73.5%)	16-18% (17%)
Stemona sessilifolia			96-100% (98%)	97-98% (97.5%)	71-74% (72.5%)	15-17% (16%)
Stemona shandongensis				95-99% (97%)	72-74% (73%)	15-17% (16%)
Stemona parviflora					94-98% (96%)	16%
Asparagus filicinus						95%

Table 4.2 Percentage similarity of 5S rRNA spacer sequences among five *Stemona* species (*S. japonica, S. parviflora, S. sessilifolia, S. shandongensis, S. tuberosa*) and one *Asparagus filicinus.* (Average percentage in bracket)

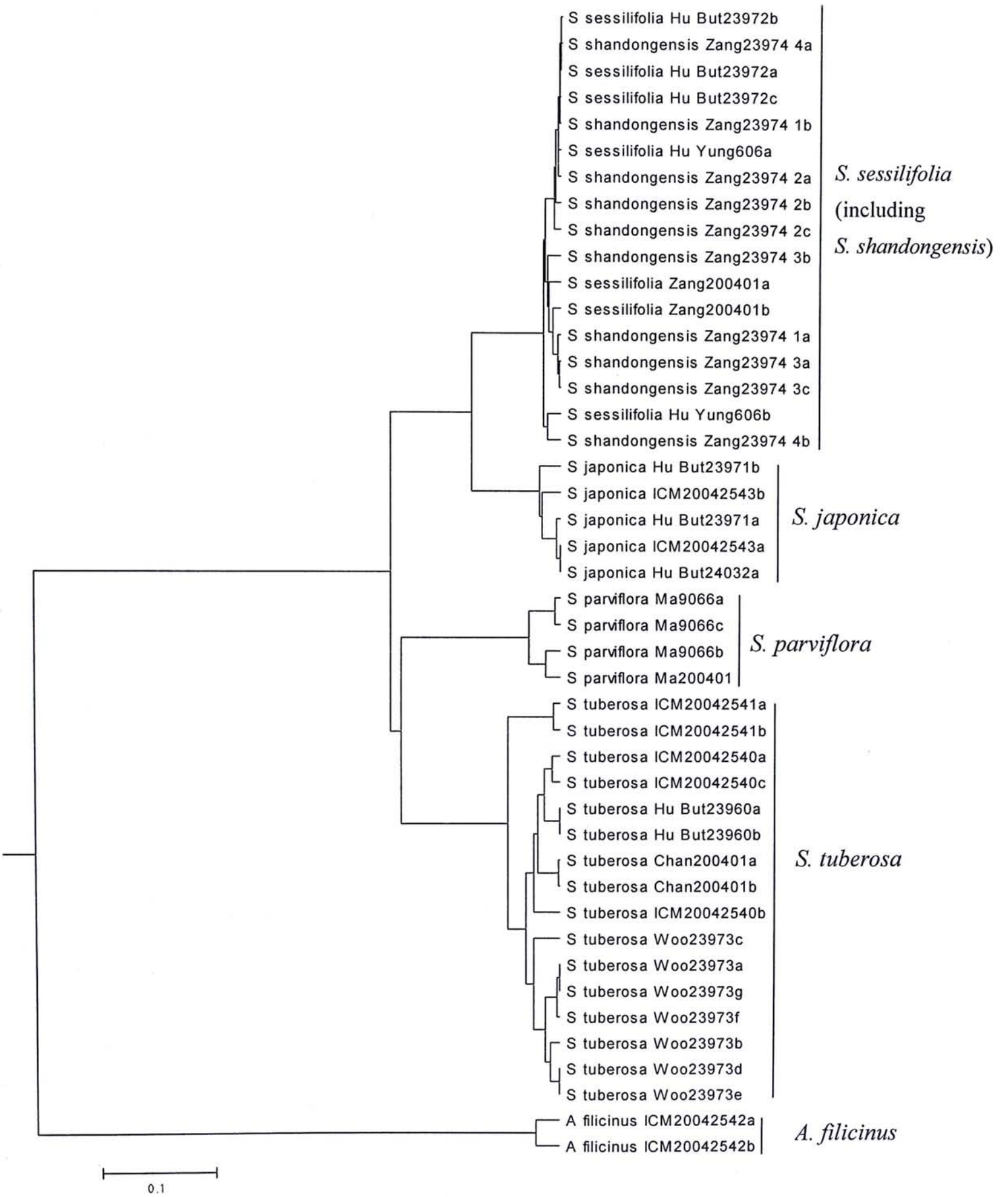

Figure 4.14. Phylogenetic tree generated by UPGMA tree construction method based on 5S rRNA spacer sequences.

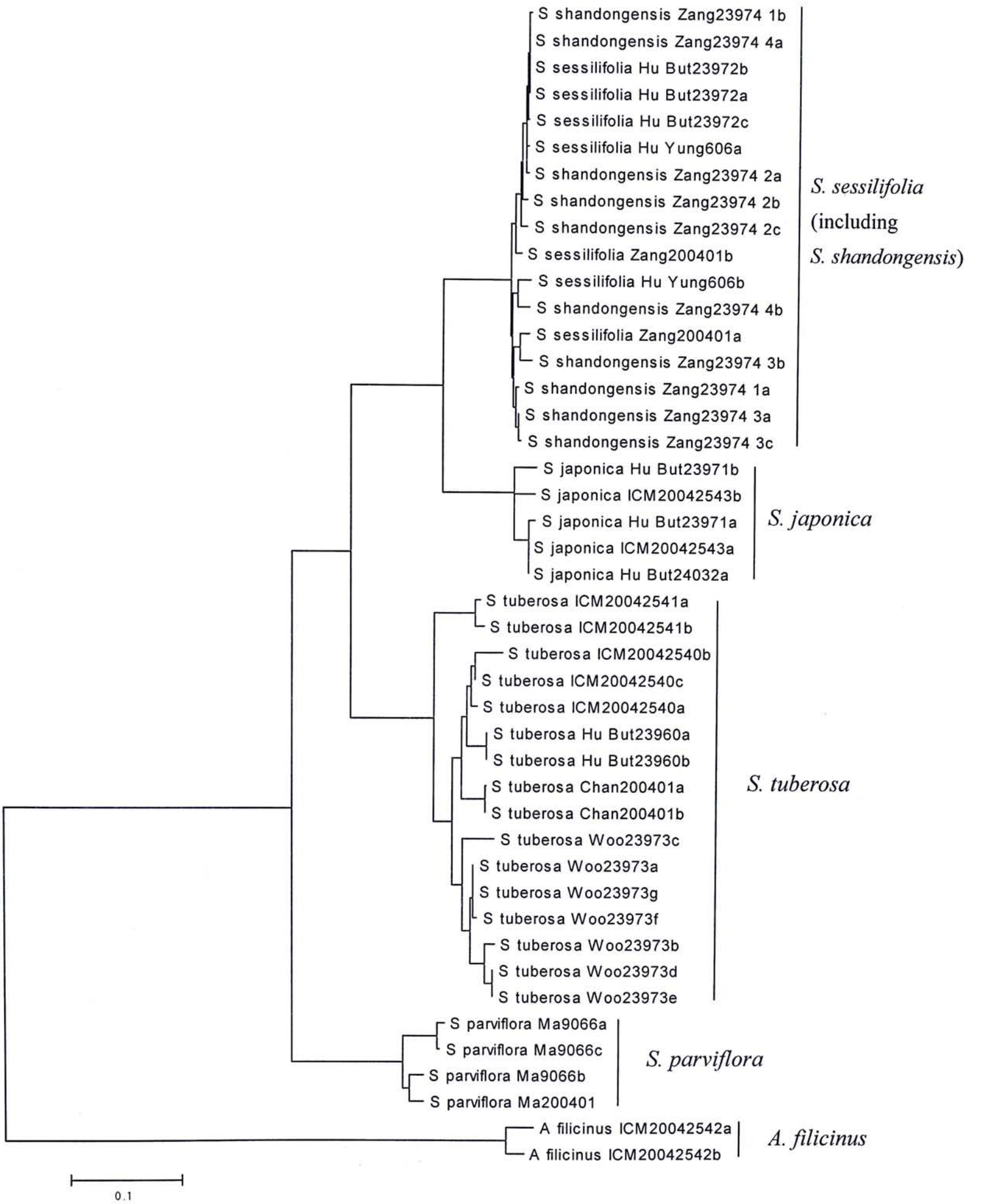

Figure 4.15. Phylogenetic tree generated by Neighbour-joining analysis based on 5S rRNA spacer sequences.

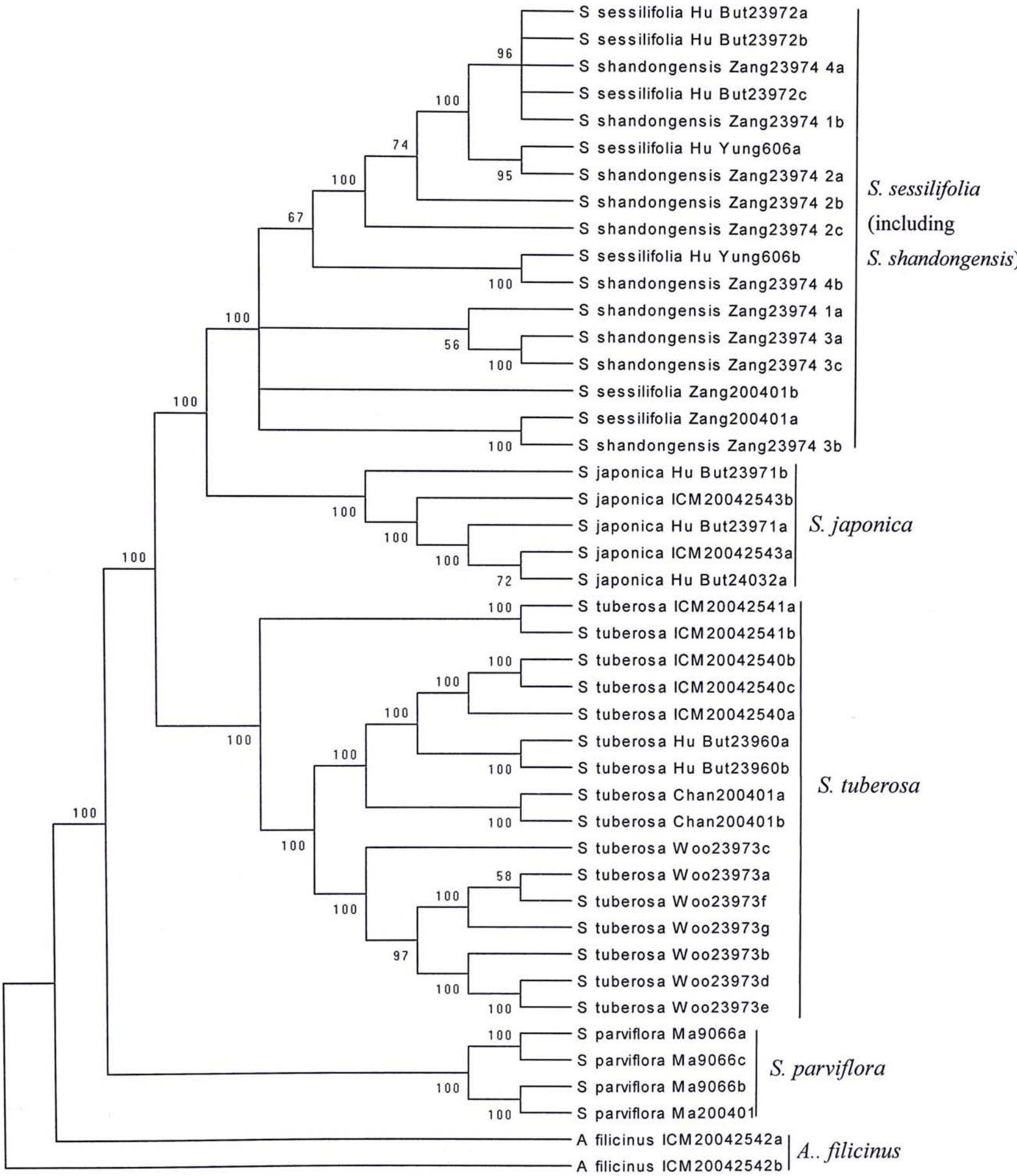

Figure 4.16. Strict consensus of 522 equally parsimonious trees generated based on 5S rRNA spacer sequence.

4.3.3 Conclusion of DNA Authentication

Using DNA techniques, the Chinese medicinal material Radix Stemonae can be authenticated from adulterants. It is also possible to find out the identity of herbal material of *Stemona* down to species level. Variation of *trn*L sequences is too small for differentiating different *Stemona* species. However, by analyzing the more variable 5S rRNA spacers, it is possible to separate the five *Stemona* species into four groups. The groups are *S. tuberosa* group, *S. japonica* group, *S. parviflora* group, and *S. sessilifolia* - *S. shandongensis* group. The two taxa in the last group have very similar morphological characteristics and 5S rRNA spacer sequences. Thus lending support to our conclusion to merge them together as one single species.

Radix Stemonae can also be distinguished from the adulterant, *Asparagus* by comparing the 5S rRNA spacer sequences and *trn*L sequences.

4.4 Molecular Systematics Analysis

Molecular phylogenetic analysis was performed in order to solve some questions about systematics of Stemonaceae. Should we include the *Croomia, Pentastemona, Stemona* and *Stichoneuron* in one single family Stemonaceae? Or should we further segregate the families Pentastemonaceae and Croomiaceae from Stemonaceae? Which order should Stemonaceae be placed in? What is the interspecific relationship between different *Stemona* species? Phylogenetic analysis of *trn*L and 5S rDNA spacer sequences would offer additional information to these questions.

To answer the questions, sequences of *trn*L intron and 5S rRNA spacer of *Croomia, Pentastemona, Stemona* and *Stichoneuron* were sequenced and analyzed. Apart from the sequences of the samples collected, sequences of other taxa were also collected from the database of the National Center for Biotechnology Information (NCBI) for analysis. These sequences included the taxa belonged to the orders Asparagales, Discoreales and Pandanales. These sequences were subjected to alignment using Clustalw and then phylogenetic analysis was performed using MEGA 2.1.

For *trn*L region, 33 sequences were collected from database (Table 4.3). A total of 55 sequences representing 43 taxa were analysed and 245 sites were compared. Two taxa of the dicot family Chloranthaceae, namely, *Chloranthus angustifolius* and *Ascarina polystachya,* were defined as outgroups and the trees were rooted at them. For Neighbor Joining trees and UPGMA trees, the distances were calculated using the algorithum Kimura 2-parameter. For parsimony analysis, parsimonious trees were searched using close-neighbor-interchange (CNI) method. Bootstrap test was applied

for 500 replications. The phylogenetic trees constructed are shown in Figures 4.17, 4.18 and 4.19.

The 5S rRNA spacer sequences of *Croomia*, *Pentastemona, Stemona* and *Stichoneuron* vary greatly. The 5S rRNA spacer sequences of *Stemona* are about 500 bp while the other three genera are about 300 bp. The sequences of *Stemona* failed to align with the other genera sequences and thus phylogenetic analysis among genera was impossible. As a result, the analysis about circumscription and affinity performed was only based on *trn*L intron sequences. The 5S rRNA spacer sequences were only used for inferring phylogenetic relationship among *Stemona* species.

4.4.1 Circumscription of Stemonaceae and its affinity to other monocots based on *trn*L intron sequences

The Neighbor Joining (Figure 4.17) and Maximum Parsimony trees (Figure 4.19) show similar results. They showed that *Croomia*, *Pentastemona*, *Stemona* and *Stichoneuron* formed a clade together. *Stemona* has closer relationship with *Pentastemona* than to the other two genera. *Croomia* and *Stichoneuron* form a clade themselves within the Stemonaceae clade. Families of Pandanales are closest sister group of Stemonaceae while Dioscoreales and Asparagales have comparatively more remote relationship with Stemonaceae. The only difference is the location of Liliales, but it is not the focus of this analysis. The UPGMA tree (Figure 4.18) is a bit different from the other two trees. Instead of forming a single group, the four genera of Stemonaceae were separated into two pairs. *Stemona* and *Pentastemona* formed a pair. *Croomia* and *Stichoneuron* form another pair, and this pair is sister to the Pandanales. The Liliales is neighbor to the group of Pandanales, *Croomia* and *Stichoneuron*. This

trees also suggests Dioscoreales and Asparagales are not close to Stemonacea.

Species	family	order	NCBI Accession no.
Agapanthus africanus	Agapanthaceae	Asparagales	AF508516
Anthericum liliago	Agavaceae	Asparagales	AF508513
Ascarina polystachya	Chloranthaceae	Chloranthales	AY237816
Asparagus acutifolius	Asparagaceae	Asparagales	AJ441168
Asparagus falcatus	Asparagaceae	Asparagales	AF508514
Asparagus officinalis	Asparagaceae	Asparagales	AJ441164
Bloomeria crocea	Alliaceae	Asparagales	AF508464
Camassia quamash	Agavaceae	Asparagales	AF508511
Carludovica palmata	Cyclanthaceae	Pandanales	AY337706
Chloranthus angustifolius	Chloranthaceae	Chloranthales	AF364600
Cyclanthus bipartitus	Cyclanthaceae	Pandanales	AY337705
Dioscorea balcanica	Dioscoreaceae	Dioscoreales	AJ441160
Dioscorea opposita	Dioscoreaceae	Dioscoreales	D89701
Dioscorea praehensilis	Dioscoreaceae	Dioscoreales	D89698
Dioscorea rotundata	Dioscoreaceae	Dioscoreales	D89695
Dioscorea trifida	Dioscoreaceae	Dioscoreales	D89682
Freycinetia cumingiana	Pandanaceae	Pandanales	AY337699
Freycinetia funicularis	Pandanaceae	Pandanales	AY337702
Hyacinthus_litwinowii	Hyacinthaceae	Asparagales	AJ508689
Jaimehintonia gypsophila	Amaryllidaceae	Asparagales	AF508481
Kabuyea hostifolia	Tecophilaeaceae	Asparagales	AJ290278
Lilium catesbaei	Liliaceae	Liliales	AF303701
Martellidendron masoalense	Pandanaceae	Pandanales	AY337709
Milla biflora	Alliaceae	Asparagales	AF508482
Muilla transmontana	Alliaceae	Asparagales	AF508487
Pandanus odoratissimus	Pandanaceae	Pandanales	AY337693
Pandanus veitchii	Pandanaceae	Pandanales	AF293104
Petronymphe decora	Amaryllidaceae	Asparagales	AF508488
Puschkinia scilloides	Hyacinthaceae	Asparagales	AJ232532.
Sararanga sinuosa	Pandanaceae	Pandanales	AY337704
Walleria mackenzii	Tecophilaeaceae	Asparagales	AJ290279
Xerophyllum asphodeloides	Melanthiaceae	Liliales	AF303668
Zigadenus glaberrimus	Melanthiaceae	Liliales	AF303699

Table 4.3 The *trn*L saequences collected from NCBI database.

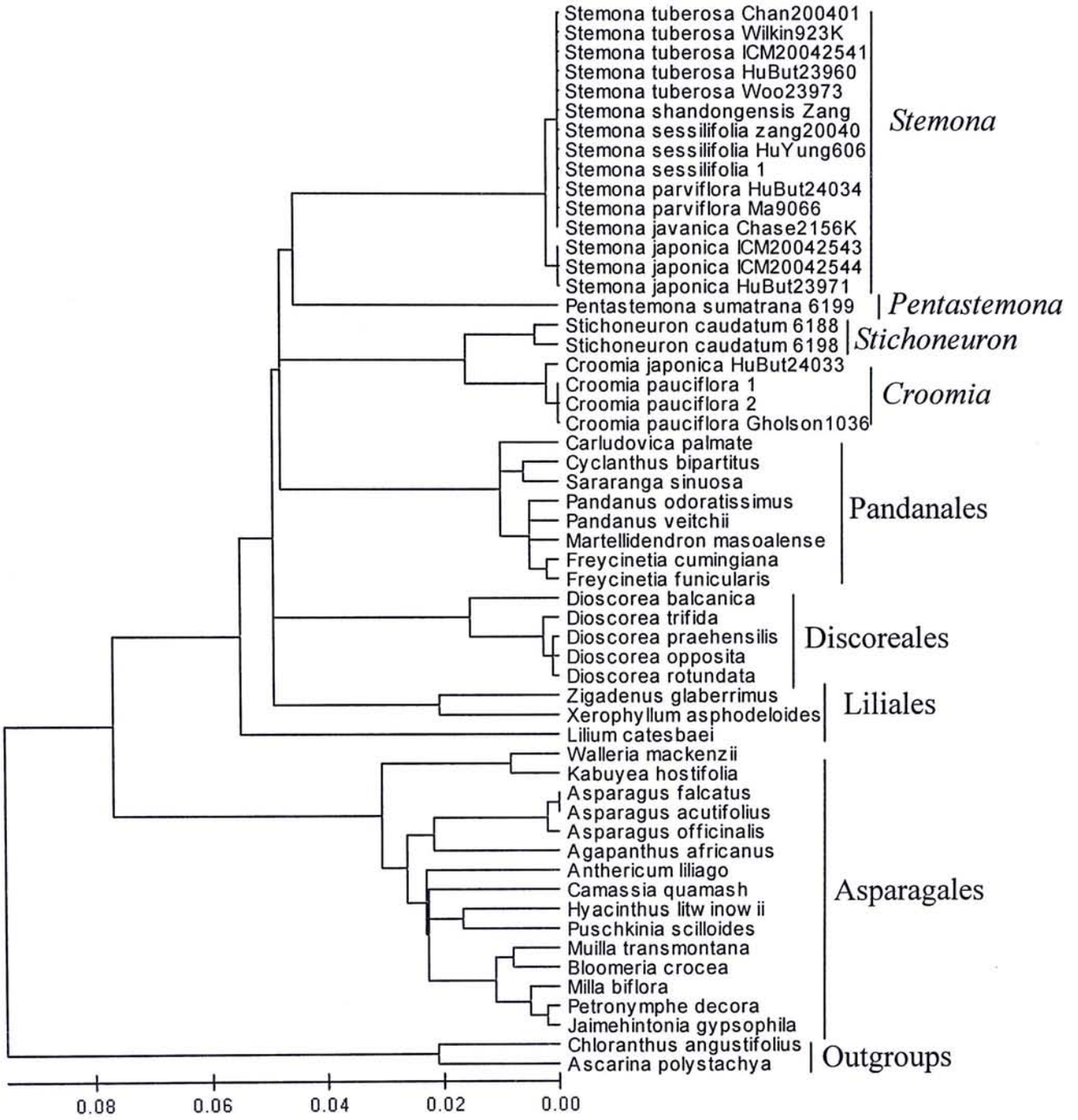

Figure 4.17. Phylogenetic tree generated by Neighbour-joining analysis based on *trn*L intron sequences.

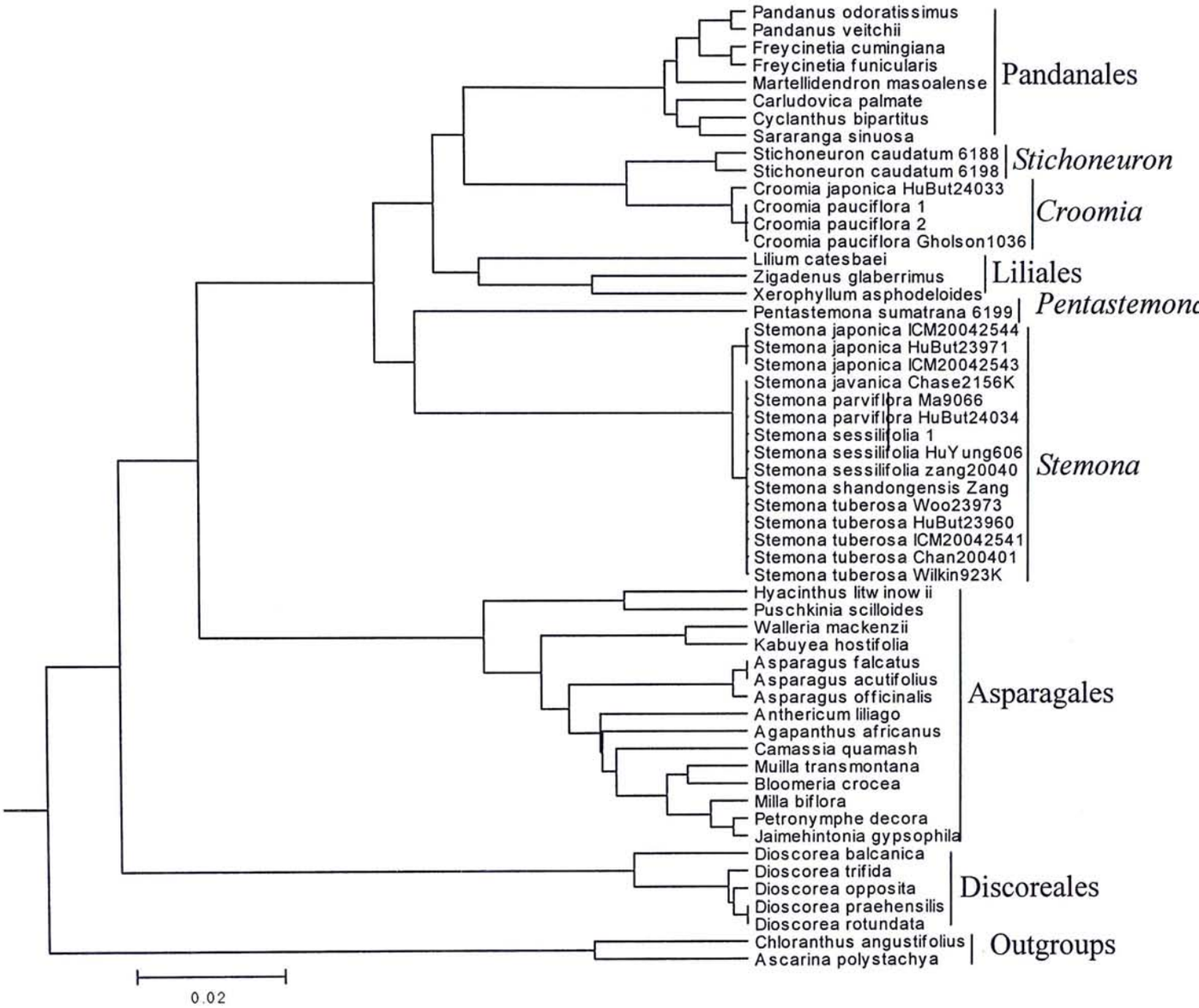

Figure 4.18. Phylogenetic tree generated by UPGMA analysis based on *trn*L intron sequences.

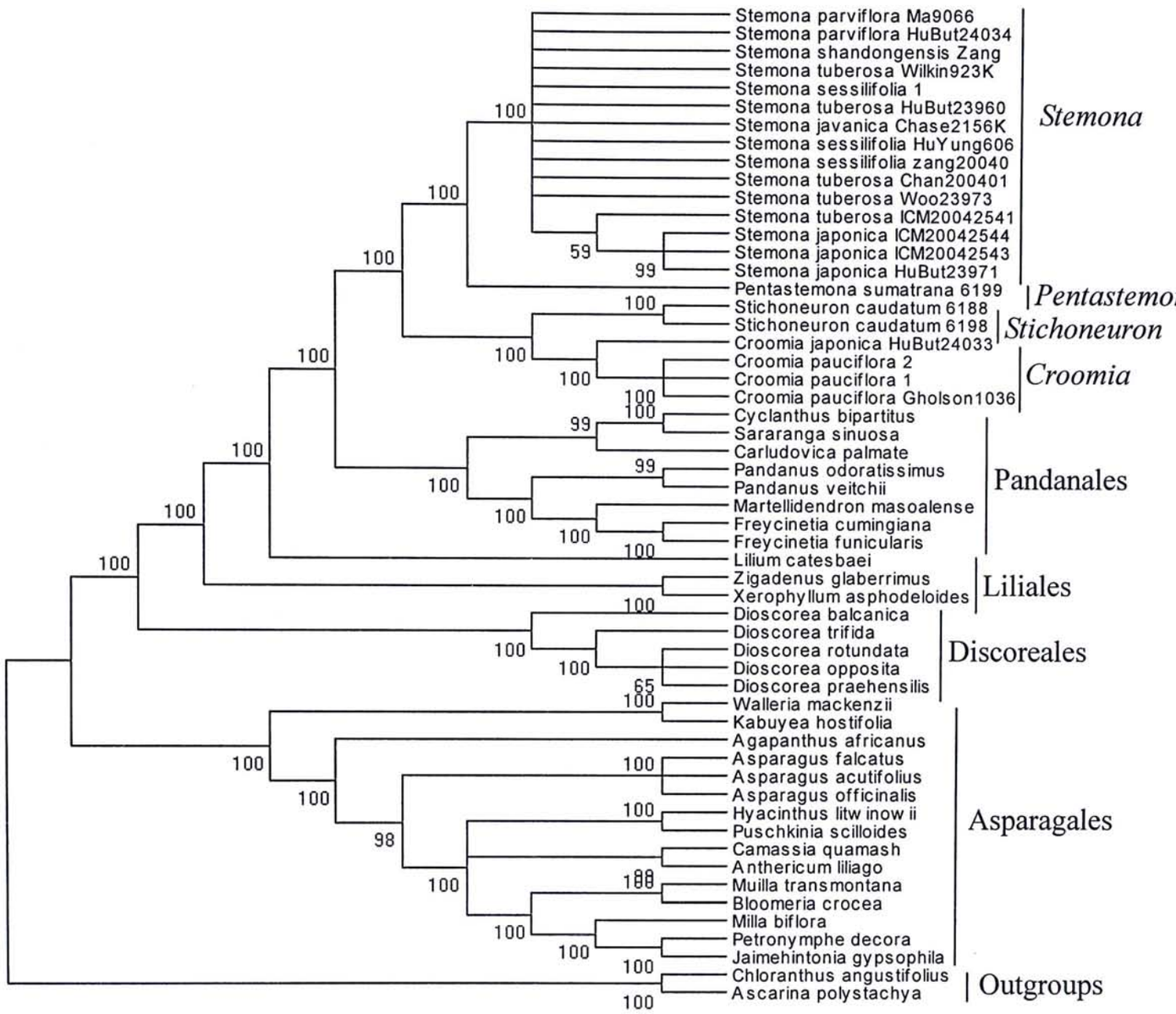

Figure 4.19. Strict consensus of 2634 equally parsimonious trees generated based on *trn*L intron sequences.

4.4.2 Interspecific relationship of Stemona

The analysis of interspecific relationship of *Stemona* was performed based on 5S rRNA spacer only. It is because the trees produced according to *trn*L sequences do not show a clear phylogenetic relationship among the different *Stemona* species.

The phylogenetic trees can be referred to Figures 4.14, 4.15 and 4.16. The three trees constructed by different methods show similar result. As it was mentioned before, the taxa *S. sessilifolia* and *S. shandongensis* formed a clade. Combining morphological

observations and molecular data, these two taxa should be grouped into one single species: *S. sessilifolia*. *S. japonica* is close to *S. sessilifolia*. The position of *S. parviflora* is different in different trees. In the Neighbour-Joining tree (Figures 4.15) and Maximum Parsimony tree (Figures 4.16), *S. parviflora* appears as a sister group of *S. tuberosa* and the whole genus is separated into two groups, one group with *S. sessilifolia* and *S. japonica* while the other group containing *S. tuberosa* and *S. parviflora*. And thus no conclusion can be made about the position of *S. parviflora*. Too few species were included in this project, and no conclusion could be drawn concerning the phylogenetic relationship among *Stemona* species.

Chapter 5. Discussion

5.1 Molecular Authentication of Radix Stemonae

Radix Stemonae, according to the Pharmacopoeia of the P.R. China, is the dried root tuber of *Stemona japonica, S. sessilifolia* or *S. tuberosa. S. parviflora* is said to be used as folk medicine. *Asparagus filicinus* is a common adulterant. In this thesis project, a molecular method based on 5S rRNA spacer sequences was developed to authenticate Radix Stemonae. The result shows that the 5S rRNA spacer sequences is variable enough to differentiate the four *Stemona* species. Radix Stemonae can also be distinguished from the adulterant *Asparagus filicinus*.

As Radix Stemonae is an antitussive drug, research on the phytochemistry of Radix Stemonae would result in the discovery of antitussive natural products. However, confusions or misuses of medicinal materials in such research may affect the accuracy and reproducibility of the experimental data, thus causing wastes of research effort. So, authenticated medicinal materials are important for such research. The molecular authentication method developed in this thesis project will be a foundation for studies of phytochemistry and pharmacognosy of Radix Stemonae.

To improve the molecular authentication method of Radix Stemonae, the method should expand to cover all *Stemona* species of China. Plant materials or DNA samples of *S. kerrii* and *S. mairei* should be collected. Apart from the 5S rRNA spacer, more molecular markers, for example ITS1 and ITS2, can also be applied to improve the accuracy.

5.2 Molecular Markers

In this research, two DNA regions were studied. It was found that the 5S rRNA spacer region is more variable than the trnL intron region. The variability of 5S rRNA region makes it a very powerful marker to differentiate different *Stemona* species from one another. However, the difference of the 5S rRNA spacer sequences is too large between genera and thus makes it unfavorable for phylogenetic analysis of intergeneric relationship.

The trnL region, on the other hand, is more conserve than 5S rRNA spacer. It is not possible to use this region to differentiate *Stemona* species. However, the region is variable enough to differentiate different genera and it was thus used for phylogenetic analysis of intergeneric relationship.

Furthermore, both regions can be easily amplified from DNA extracted. Successful amplification is also possible for DNA extracted from dried Chinese herbal material. DNA regions that can be easily amplified post an extra advantage in authentication or phylogenetic studies.

5.3 The Variation in *Stemona tuberosa*

It was found that the *S. tuberosa* sample in Hong Kong (Hu and But 23960) is different from that of Guangxi (Woo 23973) in perianth morphology. The Hong Kong sample has tepals with abaxial surface in pale green and purple, and the adaxial surface is purple, while the Guangxi samples have pale green tepals. The shape of the tepals for the Hong Kong sample is also more slender. In *Flora Reipublicae Popularis*

Sinicae and Flora of China, the descriptions of *S. tuberosa* are close to the Guangxi sample while the tepal pattern of Hong Kong sample was not reported.

Molecular analysis based on 5S rRNA spacer sequences also suggests the two morphologically different samples are actually very close phylogenetically. In the constructed phylogenetic trees (Figures 4.14, 4.15, 4.16), the Hong Kong sample and Guangxi sample are located in the same clade with the other *S. tuberosa* samples. It is concluded that both samples are of the same species, the *S. tuberosa,* however, show variation in perianth morphology.

It is also observed that the Hong Kong sample, together with the other two samples from Guangdong (ICM 2004-2540 and Chan 200401) form a clade distinct to Guangxi and Yunnan samples. The result shows that the 5S rRNA spacer sequences of *S. tuberosa* vary among samples of different providences, and thus, this marker may be able to differentiate the origin of Radix Stemonae. However, further investigation on more samples of *S. tuberosa* are needed to confirm whether the variation of the 5S rRNA spacer sequences is related to their origins.

5.4 Comparsion of *Stemona sessilifolia* and *S. shandongensis*

S. shandongensis is a new species published in 1996. However, it was found that this taxon is highly resemble to *S. sessilifolia*. The morphological features of *S. sessilifolia* and *S. shandongensis* are overlapping except the epifoliate pedicel found in the type specimen of the latter (Zang 1996, Ji and Duyfiles 2000). We do not consider such difference to be large enough to support a new species. Similar situation is found in *S. tuberosa*. Peduncle or pedicel of *S. tuberosa* are usually axillary and rarely borne on

petiole (Ji and Duyfiles 2000). However, the *S. tuberosa* is not segregated into further small taxa according to this variation.

Based on morphological comparison and molecular analysis, it is concluded that the two taxa should be grouped into one species. Yet, this conclusion is opened for further modification, as the type specimen of *S. shandongensis* is not examined in this study. There is still a possibility that the specimen being collected by D.K. Zang in 2003 is different to the type specimens. Analysis on the type specimens will provide a more reliable conclusion.

5.5 Circumscription of Stemonaceae

The circumscription of Stemonaceae has been debated by botanists for many years. Some botanists favoured housing all four genara *Croomia*, *Pentastemona*, *Stemona* and *Stichoneuron*, in a single family (Conover 1991, Van Steenis 1982). However some suggested *Pentastemona* is worthy of separate family rank (Dahlgren *et al.* 1985, Van der Ham 1991). *Croomia* and *Stichoneuron* are also segregated into a distinct family (Nakai 1937).

In this study, both 5S rRNA spacer and trnL sequences were studied. The trnL intron sequences appeared to be suitable for analysis on circumscription of Stemonaceae. The 5S rRNA spacer sequences are too variable among the four genera and thus are only suitable for authentication or for inferring intrageneric relationship.

Several phylogenetic trees were constructed based on the trnL sequences. (Figures 4.17, 4.18 and 4.19) Different tree building methods were tried and the results were

compared. The Maximum Parsimony and Neighbour-Joining analyses (Figures 4.17, 4.19) led to a similar tree. The four genera are clustered into a single group and this group can be further segregated into two groups. *Croomia* and *Stichoneuron* are closer to each other than the other two genera. The phylogenetic trees suggested that *Pentastemona* is a sister group of *Stemona* and the same conclusion was made by APG (2003).

UPGMA tree (Figure 4. 18) shows a bit different to the other two trees. Same as the other two trees, *Croomia* and *Stichoneuron* are grouped together. However, they cluster with members of Pandanales and Liliales rather than with *Pentastemona* and *Stemona*. Nevertheless, all three analyses have some things in common. The *Croomia* and *Stichoneuron* cluster together in all three trees, suggesting that they are closely related and this agrees with the observations on morphology (Van Steenis 1982, Willis 1985, Nakai 1937). *Pentastemona* also groups with *Stemona* in all the three trees being made.

By combining the phylogenetic analysis based on trnL sequences and the analyses based on 18S rDNA, *rbc*L and *atp*B sequences (Claddick et al. 2002), it becomes obvious that the four genera should be placed in a single group. The four genera of the family are more closely related to one another than to genera of the other families. Although the group can be further divided into smaller groups, it is not too meaningful to separate such a small family into further small tribes or families. It is thus concluded that the four genera should be settled in one single family Stemonaceae.

5.6 Affinity of Stemonaceae

Apart from the circumscription, botanists have also been discussing the affinity of Stemonaceae. Some botanists placed Stemonaceae in the orders Asparagales or Liliales (Burkill 1960, Cronquist 1981, Huber 1991) while another group of botanists support placing it in the order Dioscoreales (Lindley 1853, Hutchinson 1959, Ayensu 1968, Dahlgren *et al.* 1985 and Takhtajan 1987). Recent molecular phylogenetic analysis based on 18S rDNA, *rbc*L and *atp*B sequences concluded that Stemonaceae belongs to order Pandanales (Chase *et al.* 1995, Soltis 2000, Caddick *et al.* 2002, APG 2003), but it was criticized as "preposterous" and lacking morphological foundation (Thorne 2003).

The result of phylogenetic analysis based on *trn*L intron sequences matches with that of the 18S rDNA, *rbc*L and *atp*B sequences. In the constructed phylogenetic trees (Figures 4.15, 4.16, 4.17), the four genera of Stemonaceae are close to the other Pandanales species. It seems that molecular phylogenetic analyses results of different DNA regions coincide with one another. Based on the molecular data available now, it is concluded that Stemonaceae should be placed in the Pandanales. However, more study on the morphological or other aspects is needed to confirm it.

Chapter 6. Conclusion

In this thesis project, a revision on the *Stemona* species of China was made to establish a basis for the development of authentication method for Radix Stemonae. The result of taxonomic study showed that there are six *Stemona* species in China.

A molecular authentication method of Radix Stemonae was developed. Based on the 5S rRNA spacer sequences, we can differentiate *Stemona japonica, S. sessilifolia* (including *S. shandongensis*), *S. tuberosa*, *S. parviflora* and *Asparagus filicinus* from one another. Radix Stemonae can also be distinguished from the adulterant *Asparagus filicinus* by the *trn*L sequences.

Molecular phylogenetic analysis based on *trn*L introns sequences was also performed. The results showed that the genera *Croomia*, *Pentastemona*, *Stemona* and *Stichoneruron* should be settled in a single family Stemonaceae. The family Stemonaceae also showed close affinity to the order Pandanales and this coincides with the molecular phylogenetic study of Chase *et al.* (1995), Soltis *et al.* (2000), Caddick *et al.* (2002) and APG (2003). However, because too few species were included in this project, no conclusion could be drawn concerning the phylogenetic relationship among *Stemona* species in China.

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