Molecular Authentication of Chinese Herbs Derived from *Aristolochia*

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ABSTRACT

Aristolochia is a genus from Aristolochiaceae. Forty-five species are found in China including five species in Hong Kong. Plants derived from *Aristolochia* are noted to contain aristolochic acids (AA) which are nephrotoxic and carcinogenic and can induce renal failure and nephropathy after prolonged consumption. Some *Aristolochia* species are used in traditional Chinese medicine (TCM), for example, *A. fangchi* (廣防已 Guangfangji), *A. manshuriensis* (關木通 Guanmutong), *A. debilis* (青木香 Qingmuxiang) and *A. contorta* (馬兜鈴 Madouling). They are sometimes confused with or used as substitutes of other Chinese herbs such as *Stephania tetrandra* (防已 Fangji), *Akebia quinata* (木通 Mutong), *Aucklandia lappa* (木香 Muxiang) and *Lilium gigantum* (大百合).

In this research, chloroplast *trn*L-*trn*F and *psb*A-*trn*H gene spacers in the chloroplast DNA genome were used to distinguish six common Chinese herbs from their *Aristolochia* adulterants or alternatives by DNA sequences. They are Fangji (防已), Mutong (木通), Muxiang (木香), Madouling (馬兜鈴), Baiying (白英) and Zhushalian (朱砂蓮). The sequences of the genuine Chinese medicines and their *Aristolochia* adulterants or alternatives were aligned and analyzed. By comparing the aligned sequences, the herbs of *Aristolochia*-origin could be distinguished from the other alternative herbs.

Dendrograms were constructed using the aligned *trn*L-*trn*F and *psb*A-*trn*H sequences from the samples. The intraspecific percentage similarity among *Aristolochia* species and the interspecific percentage similarity beween genuine Chinese herbs and their adulterant or alternatives are calculated. The results showed that either gene regions could be used to discriminate the six Chinese herbs Baiying, Fangji, Madouling, Mutong, Muxiang and Zhushalian from their *Aristolochia* adulterants or alternatives. In addition, ISSR was attempted to find the sequence segments unique and specific to *Aristolochia*.

摘要

馬兜鈴屬是馬兜鈴科的一個屬。中國有四十五種馬兜鈴屬植物，而香港有其中五種。馬兜鈴屬植物含有可致癌和有腎毒性的馬兜鈴酸，長期使用含馬兜鈴酸植物會導致腎衰竭和腎病變。在傳統中醫藥中，馬兜鈴屬植物被用作藥材使用，包括廣防已 *A. fangchi* (藥材廣防已 Guangfangji)、木通馬兜鈴 *A. manshuriensis* (藥材關木通 Guanmutong)、馬兜鈴 *A. debilis* (藥材青木香 Qingmuxiang) 和北馬兜鈴 *A. contorta* (藥材馬兜鈴 Madouling)。馬兜鈴藥材有時會被混淆成其他藥材或用作其他藥材的代用品。例子有石蟾蜍 *Stephania tetrandra* (藥材防已 Fangji)、 木通 *Akebia quinata* (藥材木通 Mutong)、雲木香 *Aucklandia lappa* (藥材木香 Muxiang) 和大百合 *Lilium gigantum* (藥材百合果 Baiheguo)。

本研究運用基因測序技術，以葉綠體基因組中的 *trn*L-*trn*F 和 *psb*A-*trn*H 片段去識別六種常被馬兜鈴屬藥材混淆的藥材，包括白英 (Baiying)、防已 (Fangji)、馬兜鈴 (Madouling)、木通 (Mutong)、木香 (Muxiang)和朱砂蓮 (Zhushalian) 。這些正品藥材和其馬兜鈴藥材偽品的基因序列被對齊並作出分析。被對齊的基因序列經比對便能識別開馬兜鈴和其他藥材。

本研究利用對齊的 *trn*L-*trn*F 和 *psb*A-*trn*H 基因序列製造成樹狀圖，並計算出馬兜鈴屬植物之間的相似度和屬於不同科屬的正偽品之間的相似度。結果顯示研究用的兩個基因片段均能用作識別馬兜鈴屬藥材和以上六種藥材(白英、防已、馬兜鈴、木通、木香和朱砂蓮)。除用基因測序技術之外，本研究亦有用 ISSR 指紋圖譜摸索馬兜鈴屬的物種特異性基因片段。

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by maximum parsimony method using chloroplast *psbA-trnH* region of the authentic plant species and herb samples of Zhushalian. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.

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LIST OF ABBREVIATIONS

bp	Base pair
CTAB	Cetyltriethylammonium bromide
dH_2O	Distilled water
DNA	Deoxyribonuleic acid
dNTPs	Deoxytriphosphates (A, T, G, C)
EDTA	Ethylenediaminetetraacetate
HCl	Hydrochloric acid
IPTG	Isopropyl-ß-D-thiogalactopyranosid
ISSR	Inter- simple sequence repeat
$MgCl_2$	Magnesium Chloride
NaOAc	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rDNA	Ribosomal DNA
Tris	Tris [hydroxymethyl] aminomethane
UPGMA	Unweighted pair-group method using arithmetic averages

Chapter 1:   LITERATURE REVIEW

1.   *Aristolochia*

1.1   *Aristolochia*, as a plant

Aristolochia is a plant genus of the family Aristolochiaceae. It has about 450 species (Huang *et al.*, 2003). They are distributed mainly in tropical regions, but some species also inhabit subtropical and temperate habitats (Kelly and Gonzalea, 2003). According to the Flora of China, there are 45 species of *Aristolochia* in China (Huang *et al.*, 2003), including five in Hong Kong (Hong Kong Herbarium, 2004). They are mainly lianas, shrubs, or tuberous herbs. *Aristolochia* has very unique flowers with high levels of organ fusion (Fig. 1). The single whorl of perianth is fused to form a tubular structure that is bilaterally symmetrical. The stamens and pistil also are fused to form a gynostemium. These flowers are presumably adapted for fly pollination (Jaramillo and Kramer, 2004).

1.2   The chemicals in *Aristolochia*

Many chemical components have been reported from *Aristolochia*. The most noteworthy ones are the aristolochic acids (AAs) and aristololactams (ALs). AAs are structurally related nitrophenanthrene carboxylic acids, and ALs are the principal metabolites of AAs (Zhang *et al.*, 2006). The most common AAs are aristolochic acid I (AA-I) and aristolochic acid II (AA-II) (Arlt *et al.*, 2002) which differ from each other by a methoxy group (Chan *et al.*, 2007). AA-I and -II are present in all the species of *Aristolochia* and some *Asarum* species (also of the family Aristolochiaceae) such as *As. himalaicum* and *As. splendens* which were found to contain trace amounts of AA-I (Jong *et al.*, 2003; Martena *et al.*, 2007).
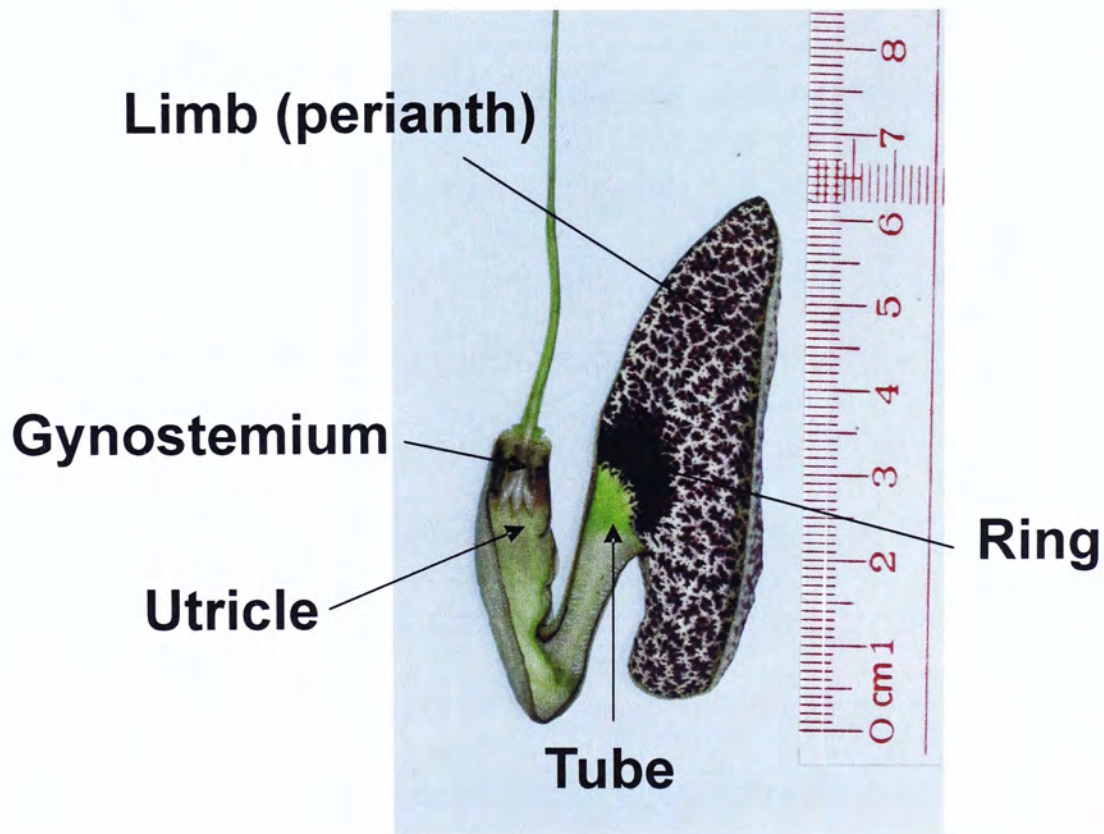
Figure 1. A flower of *Aristolochia elegans*: (A) lateral view; (B) front view; (C) longitudinal section.
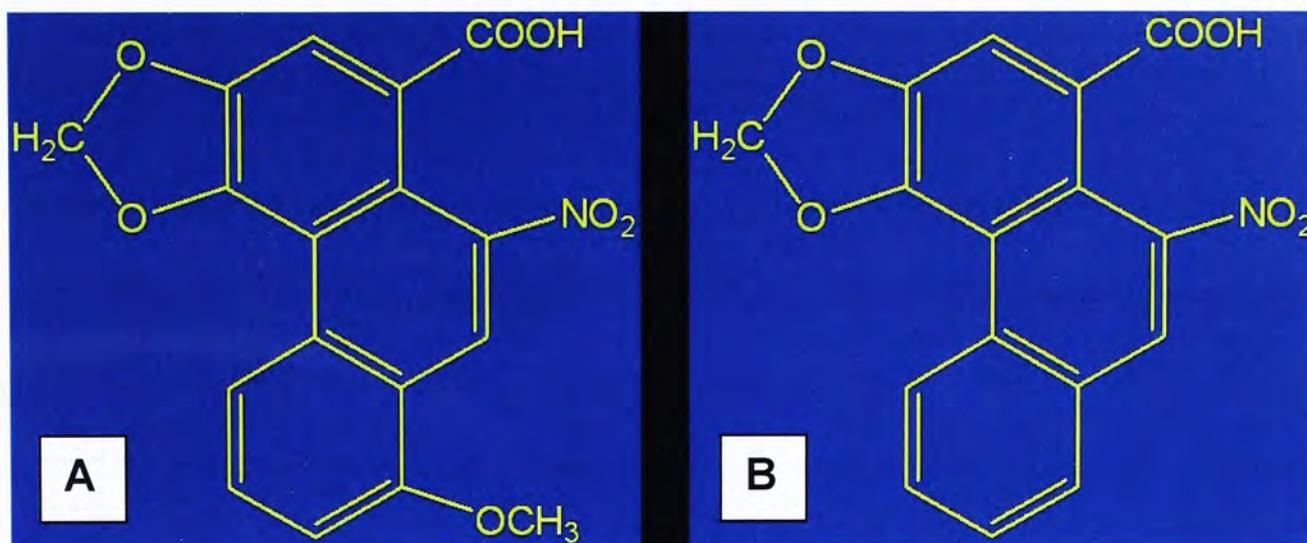
Figure 1.2   Chemical structure of (A) aristolochic acid–I and (B) aristolochic acid–II.

1.3   *Aristolochia*, as herbal remedies

Herbal drugs derived from *Aristolochia* have been used since antiquity worldwide (Arlt *et al.*, 2000; Martena *et al.*, 2007). In China, several *Aristolochia* species are often used as herbal remedies, e.g. *Aristolochia contorta, A. debilis, A. fangchi* and *A. manshuriensis*. The 2000 edition of the Pharmacopoeia of the People's Republic of China (Pharmacopoeia Commission, 2000) accepted five herbs derived from four *Aristolochia* species (Zhao *et al.*, 2003). They are used for various effects, including pain-relief by subduing hyperactivity of the liver, dispelling wind, reducing heat, inducing diuresis and relieving cough and asthma (Pharmacopoeia Commission, 2000). Besides these therapeutic purposes, *Aristolochia* herbs have also been applied in gynecopathy, snake bites, arthritis, gout, rheumatism and wounds (Arlt *et al.*, 2002; Zhang *et al.*, 2004; Martena *et al.*, 2007). The anti-inflammatory property of *Aristolochia* has once encouraged the development of pharmaceutical preparations in Germany (Arlt *et al.*, 2002).

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1.4 The *Aristolochia* poisoning cases

Herbs derived from Aristolochia species have been associated with adverse reactions in mainland China (But and Ma, 1995). The first confirmed case of herbal poisoning associated with *Aristolochia* herbs outside China happened in Belgium in 1991 (Arlt *et al.*, 2002). Some 80 patients who had followed a slimming regimen including Chinese herbs developed interstitial renal fibrosis. The slimming regimen included the introduction of a Chinese herb Fangji which should be *Stephenia tetrandra*. Vanherweghem *et al.* (1993) who first reported the Belgium episodes used the term Chinese Herbs Nephropathy (CHN) for this unique type of rapidly progressive renal fibrosis associated with the prolonged intake of Chinese herbs. But (1993) was prompt to point out that the herb incriminated actually was Guangfanji derived from *Aristolochia fangchi,* which contained AAs. It is believed that the adulteration was caused by the resemblance in names. *Stephenia tetrandra,* known as Hanfangji (漢防己), was inadvertently replaced by *Aristolochia fangchi* which is known as Guangfangji (廣防己) (Vanhaelen *et al.*, 1994; Balachandran *et al.*, 2005), apparently because both herbs share the same postfix 'Fangji'. Confusion in the delivery of the powders by the Chinese export companies was a possible reason for the substitution (Vanhaelen *et al.*, 1994).

After the 1993 report, more than 100 cases of rapidly progressive renal fibrosis associated with exposure to *Aristolochia* herbs were identified in Belgium and approximately 170 cases of CHN were described in other European countries, the USA and in Asia (Martena *et al.*, 2007). In substantial number of cases, urothelial carcinoma of the bladder was also detected several months after renal biopsy (Nortier *et al.*, 2002).

Aristolochia herbs poisoning cases have also been reported in Hong Kong.

From January 2000 to June 2005, the Department of Health received eight CHN reports from the Hospital Authority. Patients involved developed transient proteinuria, renal failure and carcinoma of the bladder. Investigation revealed that erroneous substitution of herbs was the cause of the cases. These cases involved either prolonged prescription of Baiying (白英), which was substituted by the *Aristolochia* herb Xungufeng (尋骨風) or replacement of Fangji with the *Aristolochia* herb Guangfangji (Sin and Chan, 2004; Wong and Chan, 2005).

Additionally, *Aristolochia* herbs have been suggested to play a role in the Balkan endemic nephropathy (BEN). BEN is a slowly progressive tubulointerstitial disease that presents with signs of uremia during the fifth or sixth decade of life, occurring almost exclusively in farmers, often affecting multiple family members in a given household. BEN was first recognized in the 1950s in geographically discrete areas along the Danube River and its tributaries in the Balkans (Batuman, 2006). The similarity of the morphological and clinical pattern raises the possibility of a common agent, aristolochic acids in BEN and CHN (Cosyns *et al.*, 1994). Both BEN and CHN cases are characterized by an aseptic leukocyturia and a mild tubular proteinuria (Cosyns *et al.*, 1994). Besides, aristolochic acids were found in flour contaminated with seeds of *Aristolochia clematis* in the Balkans regions for BEN (Vanherweghem *et al.*, 1993; Pfohl-Leszkowiczy *et al.*, 2002; Martena *et al.*, 2007) and AA-DNA adducts were also detected in BEN patients in Croatia. Therefore scientists proposed that *Aristolochia* might have contributed to the aetiology of BEN (Pfohl-Leszkowiczy *et al.*, 2002).

1.5　The mechanism of AAs in CHN

Aristolochic acids (AAs) and aristololactams (ALs) are genotoxic mutagens that form DNA adducts after metabolic activation through simple reduction of the nitro group on AAs (Arlt *et al.*, 2002). These adducts cause mutation in codon 61 in the H-*ras* mouse gene and human *p53* gene resulting in tumorigenesis. Therefore, these adducts are specific markers of exposure to AAs (Nortier *et al.*, 2000; Arlt *et al.*, 2002). AAs and ALs are also nephrotoxic and carcinogenic, causing impairment of kidney functions (Arlt *et al.*, 2002). In the early 1980s, AA was shown as potent carcinogen in rodents (Zhang *et al.*, 2004). The International Agency for Research on Cancer released a report in 2002 to highlight that herbal preparations with *Aristolochia* herbs are carcinogenic to humans and that naturally occurring mixtures of AAs are probably human carcinogens (Martena *et al.*, 2007). The AA-induced nephropathy is characterized by subacute renal failure, severe anemia, mild hypertension, and tubular proteinuria (Gillerot *et al.*, 2001). The severity of renal dysfunction of such nephropathy has proven related to the intensity of AA-exposure. Nortier *et al.* (2007) found a positive correlation between the estimated total dose of *Aristolochia* ingested by patients and the severity of renal dysfunction.

1.6 Renaming CHN to AAN

AA-induced nephropathy is not limited to Chinese herbs but actually the result of herbs or fruits from various *Aristolochia* species, including those which cause BEN in the Balkan region (Cosyns *et al.*, 1994; Pfohl-Leszkowiczy *et al.*, 2002). Gillerot *et al.* (2001) proposed to rename CHN to a more appropriate term, Aristolochic Acid Nephropathy (AAN) (Arlt *et al.*, 2002). This term, AAN, should be used only when the toxic role of AAs has been demonstrated. For other cases

associated with phytotherapy without definite identification of the aetiological chemicals, the term Phytotherapy-associated Interstitial Nephritis (PAIN) should be used (Gillerot *et al.*, 2001).

1.7 Banning *Aristolochia* herbs

Different countries regulate Chinese herbs in different ways. In the USA and the Netherlands, Chinese traditional herbal preparations (THPs) are regarded as food supplements, not drugs. In Dutch food law, THPs are regulated as herbal preparations in the Commodities Act "Herbal preparations" (Martena *et al.*, 2007).

After the outbreak of AAN, many countries reacted to it accordingly. France prohibited the selling and using of medicines or herbs containing AAs in 1994. In 2000, Singapore required all patent medicines which contain AAs to bear labels with warning. Herbs must be labeled with Chinese and Latin names for import into Singapore (Bao *et al.*, 2001). Since 2001, the use of Fangji, Madouling, Mutong and Qingmuxiang have been prohibited in UK and Malaysia (Bao *et al.*, 2001; Martena *et al.*, 2007). In the same year, several international food and drug authorities, including the United States Food and Drug Administration (FDA), have published safety information related to the presence of AAs in botanical products and dietary supplements and advised customers to stop using any herbal products containing or are suspected to contain AAs (Sun *et al.*, 2004; Martena *et al.*, 2007). The use of *Aristolochia* species in herbal medicine is currently no longer permitted in the U.S., Canada, Australia, as well as most European and Asian countries. Though banned in many countries, AA-containing herbs continue to be available on the internet in U.S. web sites for gastrointestinal symptoms, weight loss, cough, and immune stimulation (Chan *et al.*, 2007). The availability of aristolochic acid-containing products on the

web two years after an FDA alert revealed a serious flaw in the safety protection for the public, as the web is a marketing tool with low barriers to entry (Gold and Slone, 2003). In mainland China, traditional Chinese Medicine (TCMs) that are made from *Aristolochia* or contain AAs are banned since 2003 and had been removed from the 2005 edition of the Pharmacopoeia of People's Republic of China, except TCMs derived from *Aristolochia contorta, A. debilis, Asarum heterotropoides* var. *mandshuricum, As. sieboldii* and *As. sieboldii* var. *seoulense.* The fruits of *Aristolochia contorta* and *A. debilis* are used as Madouling (馬兜鈴) and their roots are used as Tianxianteng (天仙滕).

1.8 The possible cause of ANN

There are a number of possible reasons of ANN. Nortier *et al.* (2007) suggested three possible reasons: (1) the source plant is correctly identified but its toxicity is unknown or underestimated, (2) there has been an accidental or deliberate modification of the herb preparation by the addition of well-known, potentially toxic substances, and (3) the herb is incorrectly identified or substituted by another more toxic item. Martena *et al.* (2007) stated that TCM confusion occurs frequently and can result from similarities in appearance, mistakes in interpreting ancient herbals, counterfeits and, in many cases, ambiguous nomenclature.

Needless to say, misuse of Chinese medicines, substitution, complexities of the herbal nomenclature and adulteration are all relevant reasons for the continual re-surge of *Aristolochia*-poisoning cases.

1.8.1 Misuse of Chinese medicine

Misuse of Chinese medicines is one of the most common causes of herbal

poisoning. Misuse refers to the inappropriate use of Chinese medicines by either practitioners or consumers (Wong and Chan, 2005). In Chinese medicine practice, clinical management is based on differentiation of body states. Even with the same disease, patients of different constitutions (e.g. gender, age or health conditions) receive different prescriptions. Misuses happen when people take medicines which are not prescribed for them or take Chinese medicines without prior consultation (Sin and Chan, 2004). Such misuses enhance the risk of overdosing and frequently result in herbal poisoning.

1.8.2 Substitution

The selection of certain herbs may involve species-substitutions that are permissible under traditional or historical convention. For example Baiheguo (百合果) is used to substitute Madouling which is derived from *Aristolochia debilis* or *A. contorta* (Xu and Xu, 1994). In some cases, the substitution is erroneous by replacing the intended herb with a toxic herb (Sin and Chan, 2004). Such substitutions may not be intentional. One reason is that the appearance of some Chinese herbal medicines may look very similar, especially after processing through drying or grinding into powder.

1.8.3 The complexities of the herbal nomenclature

Contamination of THPs with AAs can often be traced back to confusion over nomenclature (Martena *et al.*, 2007). The Chinese herbal naming system is very complicated. But (1993) stated that there are four ways to name an herb: the English common name, transliteration of the herb name, the Latinised pharmaceutical name, and the scientific name. Scientific names are more specific and acceptable (But,

1993). Transliterations, pharmaceutical and common names are more loosely applied, making it difficult to trace them to the exact source species, especially when many herbs that re-exported from Hong Kong are derived from plant sources that are different from the ones referred to in major reference works (But, 1993). These names of plants are not very reliable for identification of the particular species as interpretation of common names can even differ in different geographical regions.

The nomenclature of Chinese herbs can be classified into three categories, (1) one to one, (2) multiple to one, and (3) one to multiple (Wu *et al,*, 2007).

(1) 'One to one' is defined as one plant part from one species corresponds to one herb. Yet, the herbs and the plant species may or may not share the same name. For example, the herb Honghua (紅花) refers to the flower of *Carthamus tinctorius* and the plant species is also called Honghua. But in the case of Baiguo (白果), the seed of *Ginkgo biloba*, the species is called Yinxing (銀杏) instead (Wu *et al.*, 2007).

(2) 'Multiple to One' is defined as different parts of one single plant can be treated as different herbs. In the case of *Aristolochia debilis*, its root is Qingmuxiang, while its fruit is called Madouling (Wu *et al.,* 2007). There is also the possibility that an herb has more than one common name, which can lead to confusion as well. For instance, *A. mollissima* is not only called Xungufeng (尋骨風) but also Baimaoteng (白毛藤). This last common name is also used for *Solanum lyratum*, which confusingly has an alternative name as well, namely, Baiying (白英). Adulteration of *S. lyratum* by *A. mollissima* can occur when only the common name Baimaoteng is used when the THP is prescribed, self-medicated or traded (Martena *et al.*, 2007).

(3) 'One to multiple' is defined as one herb name referring to multiple plant species. It is common for herbs derived from closely related species, as they share similar names. For instance, the herb Baibu (百部) is derived from three *Stemona* species, *S.*

japonica, S. sessilifolia and S. tuberosa. Their common names are Duiye Baibu (對葉百部), Zhili Baibu (直立百部) and Mansheng Baibu (蔓生百部), respectively; but they all are sold and dispensed as Baibu. In TCM several plant species share a Chinese common name with an *Aristolochia* plant, and this common name could be seen as a group name for the species concerned even though they are from different families. When a prefix is added to the group name, the common name refers to only one or two plant species of the group. In many cases, however, only the group name is used. The prefix can point to a region where the plant is grown; for example, the prefix "Chuan (川)" refers to Sichuan province. The group name Fangji refers to at least three plant species; but in combination with the prefix "Guang", it is exclusively used for the root of *A. fangchi*. Another example, Mutong (木通), Chuanmutong (川木通), and Guanutong (關木通), collectively grouped as Mutong (木通) but actually are from three different families i.e. Lardizabalaceae, Ranunculaceae and Aristolochiaceae, respectively. On top of the fact that they come from different families, each herb may be derived from more than one species. Mutong is derived from *Akebia quinata*, *A. trifolata* and *A. trifoliata* var. *australis*. Chuanmutong is derived from either *Clematis armandii* or *C. montana*. Guanmutong is derived from an *Aristolochia manshuriensis*.

1.8.4 Adulteration

Traditionally, herb identification heavily depends on experience. However most people are not professionals, and hence adulteration is common. AAN described in Belgium illustrated the drawbacks of using Chinese herbs by physicians who were not trained in traditional Chinese phytotherapy (Gillerot *et al.*, 2001). The adulterated materials are usually of lower medical value or economical value, or even poisonous.

In 1989, two people in Hong Kong suffered serious neuropathy and encephalopathy after consuming a broth from the root of Guijiu (鬼臼, *Podophyllum hexandrum*), a toxic herb disguised as Longdancao (龍膽草, *Gentiana rigescens*) (But *et al.*, 1996). Four cases of drowsiness were attributed to erroneous dispensing of Yangjinhua (洋金花) (the flower of *Datura metel*) as Lingxiaohua (凌霄花) which should have been the flower of *Campsis grandiflora* (But, 1994).

1.9 Methods for authentication

1.9.1 Traditional methods for authentication

Traditional methods for authenticating TCM involve inspecting external and organoleptic characters, such as size, shape, color, taste and texture. Such identification is carried out by observations and the accuracy heavily depends on the experience and judgments of the inspectors. Closely related species which share similar features would not be easily distinguished from one another. It is more difficult when herbs are in the form of slices, powder or extracts. They may be processed by various methods, e.g. drying, heating, honey-treating or grounding into powder. The morphological characters are lost during these treatments.

Anatomical and histological analyses may offer more authentication markers. The internal structure of herbs such as phloem, cambium or xylem are examined and compared under microscope. However, differentiating related species is very difficult as they often possess similar internal structures. Furthermore, the fine structures may often vary with geographical environment, growth period and storage conditions (Shaw *et al.*, 2002).

Chemical analyses involve techniques such as thin layer chromatography (TLC),

high-performance liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GC-MS). Specific chemical constituents are used as markers for authentication. Different combination of markers may produce specific chemical patterns that can be used as profiles for comparison. HPLC analysis was applied to test 17 brands of American ginseng tea and seven brands of ginseng tea available in Hong Kong (Lang *et al.*, 1993). But chemical analysis has it disadvantages. The chemical components of related species are very similar. The contents of active components may vary with geographical environment, growth stages and the time when they are harvested (Shaw *et al.*, 2002).

1.9.2 The advantage of using molecular methods

Unlike traditional methods, molecular methods are becoming more popular because of some obvious advantages. Generally, the DNA-based markers are less affected by physiological conditions of samples, environmental factors and age of the source plants. They are not tissue-specific and hence can be performed at any phase of organism development. Only a small amount of material is needed for analysis and the physical form of the sample is not restricted. The discrimination-capability of DNA-based markers is high so that very closely related varieties may be differentiated from one another (Shaw *et al.*, 2002).

1.9.2.1 DNA fingerprinting

DNA fingerprints make use of the different profiles of eletrophoresis gel bandings produced by different DNA fragments.

Inter simple sequence repeat (ISSR) is semiarbitrary makers amplified by PCR in the presence of one primer complementary to a target microsatellite. Such

amplification does not require genome sequence information and leads to multilocus and highly polymorphous patterns. Each band corresponds to a DNA sequence delimited by two inverted microsatellites. ISSR is quick and easy to handle (Bornet and Branchard, 2001). Vijayan et al. (2006) differentiated 44 mulberry (*Morus indica*) genotypes with 12 selected ISSR primers. Results showed significant differences between genotypes and the dendrogram generated clustered the genotypes into five groups and six isolates.

Random amplified polymorphic DNA (RAPD) is a PCR-based DNA fingerprint technique, using a single randomly chosen 10 bp oligonucleotide primer. The difference of band patterns among samples is used for differentiating samples (Shaw *et al.,* 2002). It can be used to differentiate closely related species and their relationship can be mapped from the band pattern developed from RAPD markers. This method has been used successfully in differentiating closely related *Akebia* species (Tian *et al.*, 2005), and revealed that *Akebia quinata* var. *polyphylla* was not closely related to *A. trifoliata, A. trifoliata* var. *australis* and *A. quinata.* RAPD markers are very quick and easy to develop but lack reproducibility (Bornet and Branchard, 2001).

Amplified fragment length polymorphism (AFLP) is a combination of RFLP and PCR techniques. The genomic DNA is first digested by restriction enzymes. The resultant fragments is then ligated to synthetic adaptors and amplified with specific primers which are complementary to a selective sequence on the adaptors. The separation of the fragments is performed on a highly resolving sequencing gel and visualized using autoradiography. The AFLP profile is dictated by the primers and restriction enzymes chosen and the composition of the genomic DNA. AFLP has been used for discriminating between *Panax quinquefolius* and *P. ginseng* and

between various cultivars of *P. quinquefolius* from different farms (Shaw *et al.*, 1998). AFLP has median reproducibility but is labor-intensive and has high operational and development costs.

1.9.2.2 DNA sequencing

DNA sequencing is another molecular method for authentication. This technique is applicable for samples that are in small amounts and the genomic DNA is partially broken, degraded or bearing few copies in the selected DNA regions which can be amplified by PCR for subsequent DNA sequencing. Authentication is achieved by comparing the sequence data of the selected DNA regions. Any insertions/deletions or species-specific based changes are used to differentiate the genuine TCM from the adulterants. However, the amplification process depends on the quality of DNA present in the sample. If the desired DNA region of the samples are degraded or broken, DNA sequencing is not applicable. Fungal contamination is another limitation for DNA sequencing. This problem can be solved by designing plant-specific primers. The specifically designed primers will only anneal on the plant DNA but not the fungal DNA.

1.10 Method selection rationale

DNA sequencing is successful in locating useful and distinct marker sites for authentication. Different genomes can be used in DNA sequencing analysis. Different genomes are present in plant or animal cells. Both cells contain mitochondria genomes and nucleus genomes and plant cells also bear chloroplast genome. The genome is made up of different gene regions and each gene evolved at a different rate. Fast evolving genes such as the ITS or *psbA-trnH* are good for

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differentiating samples at the species level. Slow evolving genes, such as *trnL-trnF*, are good for differentiating samples at the genus and family levels or above.

In this research two gene regions were used: *trnL-trnF* gene spacer and *psbA-trnH* gene spacer. They both come from the chloroplast genome.

The *trnL-trnF* region is located in the large single copy of the chloroplast DNA. It includes the *trnL* intron, *trnL* exon (UAA)3' exon, and *trnL-trnF* intergenic spacer. This region is highly variable and is suitable for differentiating samples at the genus and family levels. Neinhuis and co-authors (2005) applied *trnL-trnF* sequences to check the phylogenetic relationship in Aristolochiaceae, and their results supported morphological classification to divide Aristolochiaceae into two subfamilies: Asaroideae and Aristolochioideae. Asaroideae has two genera: *Asarum* and *Saruma*. Aristolochioideae houses the remaining genera in Aristolochiaceae (Neinhuis *et al.*, 2005).

The *psbA-trnH* region is located in the large single copy of chloroplast genome. It is a short intergenic spacer of about 450 bp. It is the most variable plastid region in angiosperms and is easily amplified across land plants (Kress *et al.*, 2005). It is recommended for its good primer sites, length, and interspecific variations. The *psbA-trnH* region is therefore suitable for species level study because of it has high amplification success rate and appropriate sequence length (Kress *et al.*, 2005).

Percentage similarities is an index used to differentiate samples and group them according to their likeliness. Intraspecific and interspecific similarities of *trnL-trnF* and *psbA-trnH* gene spacer among the adulterant/substitute and genuine genera are calculated. Before calculating the percentage similarities, the DNA sequences are aligned by using the online program ClustalW of the European Bioinformatics Institute and the computer program ClustalX version 1.83 (Thompson *et al.*, 1997).

Then the percentage similarity between each pair of sequences is calculated using BioEdit Sequence Alignment Editor (Hall 1999) as:

$$\text{Precentage similarity} = \frac{\text{number of identical bases}}{\text{size of the two aligned sequences}} \times 100\%$$

The relationship among samples can then be presented by dendrograms, constructed with the software Molecular Evolutionary Genetic Analysis (MEGA) version 2.1 (Kumar *et al.*, 2001), based on the sequence alignments. Two different dendrogram construction methods were used in this study, namely, unweighted pair-group method using arithmetic averages (UPGMA) and maximum parsimony. They are used in differentiating genuine samples from their corresponding adulterants and substitutes by clustering similar samples into distinct clades.

1.11 The need for molecular authentication of six medicinal herbs

The six medicinal herbs Mutong, Muxiang, Baiying, Fangji, Madouling and Zhushalian are common herbs used in Chinese medicine. The herbs are adulterated or substituted by *Aristolochia* species. Adulteration with *Aristolochia* species may lead to harmful adverse reactions.

1.11.1 The herb Mutong

Mutong is a commonly used TCM and is listed in Shennong Bencaojing (神農本草經) as a medium grade drug under the name of Tongcao (通草). Its large lacuna or large vessels giving an impression of see-through hole properties in both ends of the stem accounts for its name Mutong, meaning pierced wood (Hsu *et al.*, 1986).

There are five species used as Mutong. Three of them belong to the genus *Akebia* (family Lardizabalaceae). They are *Akebia quinata, A. trifoliata* and *A. trifoliata* var. *australis*. In Japan, only *A. quinata* is used as the official Mutong (Bao *et al.,* 2001; Liu *et al.,* 2003). Two species come from the family Ranunculaceae: *Clematis armandii* and *C. montana*. In the Chinese Pharmacopeia, the *Akebia* and *Clematis* species are used as genuine Mutong. The *Akebia* stems is listed as Mutong while the stems of *Clematis* species is listed as Chuanmutong. The stem of *Aristolochia manshuriensis* is known as Guanmutong.

Mutong and Chuanmutong share similar morphological characters. The herb Mutong is long cylindrical and slightly twisted, 0.5–2 cm in diameter, externally greyish-brown with longitudinal irregular furrows and ridges. It is light and the texture is firm and uneasily broken. The inner part has radial lines and scattered yellow dots (Pharmacopoeia Commission, 2005). The herb Chuanmutong is long cylindrical, slightly twisted, 2–3.5 cm in diameter, externally yellowish-brown with longitudinal furrows and ridges. Its texture is firm and uneasily broken, the slices 2–4 mm think, uneven along the margins (Pharmacopoeia Commission, 2000). The adulterant *Aristolochia* Guanmutong is long cylindrical, slightly twisted, 1–6 cm in diameter, externally greyish-yellow or brownish-yellow with shallow longitudinal grooves and remains of brown patches of course bark. Nodes slightly swollen with a branch scar. It gives off a smell like camphor on rubbing the remaining coarse bark (Pharmacopoeia of Commission, 2005).

AMutong from *Akebia quinata* has been used as an antiphologistic, diuretic and analgesic (Han *et al.,* 2005). Chuanmutong (derived from *Clematis*) is used in removing heat, inducing diuresis, stimulating menstrual discharge and promoting lactation.

1.11.1.1 The poisoning cases reported

Case of AAN was reported in Japan with the use of Mutong. Some Japanese patients took the extracts of Mutong, but some of these extracts were replaced by Guanmutong which is derived from *Aristolochia manshuriensis* (Liu *et al.*, 2003). In UK, two cases were reported of end-stage renal failure following the use of Mutong containing AA-I and AA-II (Martena *et al.*, 2007). Mutong was consumed by one patient as a tea for 6 years and the other patient used a preparation for two years in an undisclosed way (Martena *et al.*, 2007). They developed rapidly progressive interstitial nephritis after two years' treatment of eczema with an herbal preparation of Mutong which belong to *Aristolochia manshuriensis* (Cosyns, 2003).A patent pill preparation, Longdan Xieganwan (龍膽瀉肝丸), were found to contain *Aristolochia manshuriensis*, which contains aristolochic acid is an active ingredient (Laing *et al.*, 2006).

1.11.1.2 Other authentication studies of Mutong

Pyrosequencing has been applied to identify the *Akebia* and *Aristolochia* species (Han *et al.*, 2005). Pyrosequencing analysis is used to assess genetic variation and the results showed that the pattern of *Akebia quinata* was very different from that of *Aristolochia manshuriensis*. From the pyrosequencing profiles, in selected areas where single nucleotide polymorphisms take place, the height of selected peak is different in the two species. *Akebia quinata* has C nucleotide base but *Aristolochia manshuriensis* has A nucleotide base (Han *et al.*, 2005).

Three kinds of Mutong medicinal materials (*Akebia, Clematis, and Aristolochia*) were analyzed qualitatively by LC-MS (Ping *et al.*, 2004). The result showed *Akebia*

Mutong and *Clematis* Mutong have similar chemical constituents, and Guanmutong has fewer constituents. These results were consistent with their medicinal properties and could explain the similarity and difference in medicinal properties on chemical basis.

1.11.2 The herb Muxiang

Muxiang is listed in Shennong Bencaojing as a high grade drug by the name of mi-hsiang (honey flavor). Its honey favor accounts for its name. (Hsu *et al.*, 1986)

Five species have been used as Muxiang. They all come from Family Asteraceae. Muxiang, also known as Yunmuxiang (雲木香) or Guangmuxiang (廣木香) (Chang and But, 1986) is derived from dried root of *Aucklandia lappa* (= *Saussurea costus* or *S. lappa*). Chuanmuxiang (川木香) is derived from the dried root of either *Vladimiria souliei* (= *Dolomiaea souliei*) or *V. souliei* var. *cinerirea* (= *Dolomiaea souliei* var. *cinerirea*). Tumuxiang (土木香) is derived from either the dried roots of *Inula helenium* or *I. racemosa*. Qingmuxiang (青木香) is derived from the dried root of *Aristolochia debilis*.

Muxiang is cylindrical or semi cylindrical, about 0.5–5 cm in diameter. The external part is yellowish-brown or deep brown with distinct wrinkles, longitudinal furrows and lateral root scars. It is hard, and the fractures greyish-brown to dark brown, the outer layer greyish yellow or brownish-yellow, bearing radial lines and scattered brown dotted oil cavities (Pharmacopoeia Commission, 2000). Muxiang is used to relieve pain by promoting the flow of qi, and to improve digestion by reinforcing the spleen function as well as asthma, cough, diarrhea, vomit, indigestion, colic, cholecyctitis and hepatitis (Li *et al.*, 2005).

Chuanmuxiang is cylindrical or semi-cylindrical and longitudinally furrowed,

yellowish-brown or deep brown, longitudinally wrinkled, showing numerous fine veins in external. It is bitter in taste and sticky on chewing. Chuanmuxiang promotes the flow of qi and relieves pain. (Pharmacopoeia Commission, 2000).

Inula helenium and *I. racemosa* are both prescribed for invigorate the spleen, regulate the function of the stomach, relieve the depression of the liver qi, alleviate pain especially between the neck and the shoulders, and prevent abortion (Liu *et al.*, 2006).

1.11.2.1 Chemical profile

The effective components of Muxiang, *Aucklandia lappa*, are sesquiterpene lactones with costunolide and dehydrocostuslactone as the major components. Pharmacological tests revealed that these components not only have antibacterial, antalgic and antivirus activities, but could also dilate bronchus, improve stomach functions, depress blood pressure, and relieve the spasm of smooth muscles. The contents of costunolide and dehydrocostuslactone have been used as the standard index to appraise the quality of Muxiang and its products (Li *et al.*, 2005).

Inula helenium and *I. racemosa* have been demonstrated to contain alantolactone and isoalantolactone (Wang *et al.*, 2000). Isoalantolactone was disclosed to possess strong antifungal activities (Liu *et al.*, 2006). Furthermore, it exhibited repellent and toxic activities against rice weevil based on a food preference apparatus and a poisoned food technique. Isoalantolactone showed strong phytotoxic effects on seed germination and seedling growth of wheat (Liu *et al.*, 2006).

1.11.2.2 Other authentication studies of Muxiang

Shum *et al.* (2007) used GC-MS in differentiating 87 Muxiang and related

species samples. The GC-MS fingerprint profiles showed high degrees of separation and uniform peak distribution of chemical components in the tested samples. Based on the profile similarity and hierarchical clustering analysis, all the samples of *Aucklandia lappa* have similar chemical profiles and were clustered into one group while the samples derived from *Vladimiria*, *Inula* and *Aristolochia* were clustered into their own independent groups. Chen (2004) used ITS regions and 5S rRNA region in differentiating the variation of Muxiang and other related species by DNA fingerprininting. These two regions of the samples were amplified with PCR and identification is based on sequence similarity.

1.11.3 The herb Baiying

According to the Chinese Pharmacopoeia (Pharmacopoeia Commission, 1977), the genuine species of Baiying is derived from the aerial parts of *Solanum lyratum*. This herb is also called Baimaoteng. Baiying is not known to have any substitutes. However, an absurd case of mixing up this herb with Xungufeng has been reported in Hong Kong (Liang *et al.*, 2006).

The herb Baiying is hollow and cylindrical stems with branches of 2–7 mm in diameter. They are yellowish green or brownish green in color and are covered with white or grey hair in young branches. The stems are easily cracked and the fibrous section is white or pale yellow. The leaves are usually wrinkled and easily cracked. Fruits are dark green or dark red berries of 1.2 cm across. This herb is slightly pungent and bitter (Pharmacopoeia Commission, 1977). Baiying is used to remove damp-heat, remove toxic materials and promote the subsidence of swelling (Liang *et al.*, 2006). It is also used to treat flu, fever, headaches, convulsions in infants, chronic hepatitis, leukorrhea, rheumatic arthritis and cancer (Pharmacopoeia Commission,

1977).

The genus *Solanum* is known to produce allelopathic spirosolane glycoalkaloids such as solamargine and solasonine that can suppress the seedling growth of other plants (Ye *et al.*, 2000). *S. lyratum* grows wild in the southeastern part of Asia and is used to treat cancers, tumors and herpes in traditional Chinese medicine. Previous studies have resulted in the characterization of a series of solalyrantines A and B together with several furostanol, spirostanol and spirosolane glycosides (Wu and Sun, 2005).

1.11.3.1 The poisoning cases reported

One remarkable example of AAN in Hong Kong was reported in 2004. A 60-year-old man was diagnosed with renal failure and urethral cancer after he had erroneously been using *Aristolochia mollissima* instead of the desired *Solanum* species (Marttena *et al.*, 2007). He had been taking an herbal prescription that claimed to contain Baiying. Li (2005) revealed the Baiying, which should be derived from *Solanum lyratum*, was mistakenly replaced by Xungufeng derived from *Aristolochia mollissima*. As a result, the Department of Health in Hong Kong imposed a suspension on the use of Baimaoteng, Baiying and Xungufeng. Subsequently the Department also prohibited importation and sale of all Chinese herbs containing aristolochic acids, including Guanmutong (*Aristolochia manshuriensis*), Guanfangji (*Aristolochia fangchi*) and Huaitong (*Aristolochia kaempferi*) (Department of Health of Hong Kong, 2004). The resulted adulteration was partly due to the confusion with regard to the fact that two herbs share a common name Baimaoteng (Li, 2005; Liang *et al.*, 2006).

1.11.3.2 Other authentication studies of Baiying

Research has been conducted to distinguish the two species, *Solanum lyratum* and *Aristolochia mollissima*, with three criteria: macroscopic and microscopic features and chemical chromatographic analysis (Liang *et al.*, 2006).

Comparing the macroscopic features of the two species showed that they can be distinguished by the differences in color of stem, distribution of hair and the appearance of the stem. The stem of *Aristolochia mollissima* is marked with woolly while hair but the stem of *Solanum lyratum* is covered with grey-whitish hair. Microscopic analyses showed that the two species can be distinguished based on the types of vascular bundles, crystals of calcium oxalate, the presence of stone cells in pericyclic fibers and distinctiveness of xylary rays. The vascular bundles of *A. mollissima* are collateral type while *S. lyratum* are bicollateral type. Unlike *A. mollissima*, *S. lyratum* has no stone cells in the pericyclic fibers. Chromatographic analysis confirmed that only *Aristolochia mollissima* contains AAs but not *Solanum lyratum* in either water or methanol extracts (Liang *et al.*, 2006).

1.11.4 The herb Fangji

Fangji is listed in Shennong Bencaojing as a medium grade drug. The genuine Fangji is either derived from the dried root of *Stephania tetrandra* (Pharmacopoeia Commission, 2005) or *Cocculus orbiculatus* (Hsu *et al.*, 1986). The adulterant Guanfangji is the dried root of *Aristolochia fangchi*.

Fangji is regularly cylindrical, semi-cylindrical or nump-shaped, mostly tortuous, 1–5 cm in diameter. Externally, it is greyish-yellow, usually exhibiting deeply depressed transverse grooves. Its texture is heavy and compact, fracture even, greyish-white, starchy. Fangji is used to treat diuresis and to relieve rheumatic

conditions (Pharmacopoeia Commission, 2005).

1.11.4.1 Chemical profile

Tetrandrine and fangchinoline are two alkaloids that exist in *Stephania tetrandra*. They have long been known to show many pharmacological activities including anti-allergy, inhibiting the release of histamine, promoting phagocytosis, dilating coronary artery, decreasing myocardial oxygen consumption, inhibiting the growth of *Bacillus tuberculosis*, *Staphylococcus aureus* and *amoeba*, and anti-cancer activities (Liu *et al.*, 2005).

Fangchinoline attenuated morphine (SC)-induced antinociception in a dose-dependent manner with significant effect at doses of 30 and 60 mg/kg body weight (Fang *et al.*, 2005). Tetrandrine is a derivative of fangchinoline. Study was designed to examine therapeutic efficacy of the root extract of *Stephania tetrandra* for treatment of neovascularization of the retinal capillary in streptozotocin (STZ)-induced diabetic rats (STZ diabetic rats) in culture (Liang *et al.*, 2002). Administration of Fangji significantly suppressed neovascularization of the retinal capillary in both STZ diabetic rats and normal rats in a dose-dependent manner. It also suppressed neovascularization of the choroidal capillary in both STZ diabetic rats and normal rats. Through the direct administration of tetrandrine, similar neovascularization suppression occurs in both retinal capillary and choroidal capillary in STZ diabetic rats and normal rats in a dose-dependent manner. It was concluded that Fangji has a direct effect on the retinal capillary of posterior ocular region and suppresses neovascularization of retinal capillary in STZ diabetic rats through the activation of tetrandrine. These results suggested that STSM may prevent for delay the progression of retinopathy in diabetic patients (Liang *et al.*, 2002).

Tetrandrine and fangchinoline are not found in *Aristolochia* and, thus, can be used to distinguish herb samples derived from *Stephania* from those obtained from *Arisolochia* (Yang *et al.*, 1998; Koh *et al.*, 2006).

1.11.4.2 The poisoning cases reported

The earliest cases report of AAN concerning *Aristolochia fangchi* was in Belgium. Some 80 patients received a slimming regimen containing powdered extract of *Stephania tetrandra* which did not contain AAs. In June, 1992, three of twenty-five randomly selected patients who had followed the same regimen during at least 3 months from 1990 had impaired renal functions (Vanherweghem *et al.*, 1993). Three female users developed subacute interstitial fibrosis of the kidney and by 1995 approximately 100 female users had developed renal failure and 50% of them required renal transplant (Cheung *et al.*, 2006). In fact, the medicinal preparation of the capsules taken by patients had different alkaloid profiles from those expected in Chinese plants (Vanherweghem *et al.*, 1993). Phytochemical analyses of the pills revealed the presence of AAs instead of tetrandrine (active component of *Stephania tetrandra*), suggesting the substitution of *Stephania tetrandra* by *Aristolochia fangchi* which contains the nephrotoxic and carcinogenic AAs (Vanhaelen *et al.*, 1994; Nortier *et al.*, 2002).

1.11.5 The herb Madouling

The fruit of Madouling resembles the shape of the bells that hang on the necklace of a horse (Hsu *et al.*, 1986). The genuine Madouling is the dried ripe fruits of *Aristolochia contorta* or *A. debilis*. The substitute is the dried fruit of *Cardiocrinum giganteum* (=*Lilium giganteum*) or *Lilium longiflorum* (Xu and Xu,

1994). In Taiwan markets, the dried seed of *Lilium formosanum* or related species is used as a substitute (Hsu *et al.*, 1986).

Madouling is ovoid, about 3–7 cm long and 2–4 cm in diameter. The outer surface is yellow-green, grey-green or brown, with 12 longitudinal ribs, from which extend numerous horizontal parallel veinlets. The apex is flattened and obtuse, and the base with a slender fruit stalk. The pericarp is light and fragile, easily divided into six valves, and the fruit stalk would also divide into six splittings. The inner pericarp wall is smooth and lustrous, with dense transverse veins. Each fruit has six locules which contain many seeds, that are overlapping and arranged regularly. Seeds are flattened and thin, obtuse-triangular or fan shaped, 6–10mm long, 8–12 mm wide, winged all around, pale brown.

Madouling is used to remove heat from lung and relieve cough and asthma, and to remove heat from the large intestine for the treatment of hemorrhoid. Baihegao is used to nourish yin and moisten the lung and to tranquilize the mind (Pharmacopoeia Commission, 2005).

1.11.6 The herb Zhushalian

The genuine species of Zhushalian is derived from root of either *Aristolochia minutissima*, *A. kaempferi* or *A. cinnabarina* (Ma and Zhang, 2005). One of the adulterant herbs, Shugen (薯莨), is the ariel root of *Dioscorea cirrhosa* and another adulterant Honyaozi (紅藥子) is derived from aerial root of *Polygonum cillinerve* (= *Fallopia multifora var. ciliinervis*) (Yan, 1994).

Zhushalian is light brown on the outside and purple-brown or dark red inside. It has powdery texture and tastes bitter (Ma and Zhang, 2005). Zhushalian is used for releasing heat, relieving pain and treating snake bites (Ma and Zhang, 2005). One of

the AAN involving Zhushalian was in Guangzhou in 1999; a woman age 45 was diagnosed with kidney failure after she took 25 g Zhushalian powder for her chronic gastritis (Ma and Zhang, 2005)

1.12 *Aristolochia* specific markers

As mentioned, many *Aristolochia* derived Chinese herbs are involved in herbal poisoning. It is important to identify the presence of *Aristolochia* in Chinese medicine medications. With the help of DNA sequences obtained by molecular method, specific markers can be constructed to facilitate authentication.

Based on authenticated voucher species, databases can be constructed for different gene regions and for different plants (Techen *et al.*, 2004). From the known area of the DNA sequences in database, specific primers of target herbs are designed based on sequence divergence (Techen *et al.*, 2006). These primers are employed in the PCR of herbal mixture samples, and those with amplified products and of correct length indicate the presence of target herbs (Techen *et al.*, 2006). These DNA markers can be used in identifying plants that are poisonous to human or considered likely adulterants, and it has the advantage of detecting the presence of target herbs even only in trace amount. Various identifying markers have been discovered by using PCR method in plants and animals. The *psbA-trn*H spacer sequence of 21 *Ephedra* species and two *Ephedra* species, *Gnetum gnemon* and *Welwitschia mirabilis*, were amplified and sequenced (Techen *et al.*, 2006). Based on this spacer sequence, *Ephedra* species-specific primers were designed to identify *Ephedra* in plant mixtures. This method was successful in detecting the DNA in the mixture containing as little as 1/1000 part of *Ephedra* species (Techen *et al.*, 2006).

1.13 Significance of the research

Aristolochia poisoning is a serious problem worldwide. Among the reported cases, several *Aristolochia* species used in Chinese medicine were involved. Besides, some of the *Aristolochia,* such as *A. westlandii* and *A. championii* are listed as endangered species (Huang *et al.*, 2003). It is important to find a definite method in determining the exact species of *Aristolochia* involved in the poisoning cases and circulated in the herbal industry.

The advanced development of the molecular technology provides a more definite approach for the authentication of Chinese herbs. The DNA sequences to be generated in this research might help to distinguish the adulterants from genuine items of six different Chinese herbs by the sites of additions/deletions or substitutions in their sequences, which can then be used as markers for authentication. Furthermore, the sequences of the chloroplast *trnL-trnF* gene region and *psbA-trnH* region of the samples would enrich the sequence data bank for *Aristolochia* species. A detailed study on the molecular sequences of *Aristolochia* species is not only helpful for standardizing and harmonizing the use of Chinese medicines, but also for offering useful information for biodiversity conservation and plant barcoding

Chapter 2: OBJECTIVE

Herbal materials derived from *Aristolochia* are known to contain aristolochic acids (AAs) and aristololactams (ALs) which are nephrotoxic and carcinogenic and can induce renal failure and nephropathy after prolonged consumption. Some *Aristolochia* species are used in traditional Chinese medicine (TCM), for example, *A. contorta* (馬兜鈴 Madouling), *A. debilis* (青木香 Qingmuxiang), *A. fangchi* (廣防已 Guangfangji) and *A. manshuriensis* (關木通 Guanmutong). They are sometimes confused with or used as substitutes of other Chinese herbs such as *Lilium giganteum* (大百合), *Aucklandia lappa* (木香 Muxiang), *Stephania tetrandra* (防已 Fangji) and *Akebia quinata* (木通 Mutong). Such substitutions and adulterations can cause serious adverse reactions.

There are three objectives in this research:

1) to determine the sequences of two chloroplast gene regions (*trnL-trn*F and *psb*A-*trn*H) in *Aristolochia* species and correspondents

2) to examine the sequences potential values for use as molecular markers for the detection of the *Aristolochia* species in six TCM (Mutong, Muxiang, Baiying, Fangji, Madouling and Zhushalian) from genuine or alternative species; and

3) to explore ISSR for locating sequences that are specific to *Aristolochia*.

Chapter 3: MATERIALS AND METHODS

3.1 Samples source

There are totally 53 authentic specimens and plants used in the molecular authentication of the six herbs. Among them, 28 herbarium specimens were from Harvard University Herbaria; 19 fresh materials were collected from Hong Kong, mainland China and USA; and six authentic powder samples from the National Center for Natural Products Research, University of Mississippi. The detailed information of the samples is listed in Table 3.1.

Six samples of extracted DNA were obtained from the Royal Botanic Garden, Kew, U.K. Three seed samples were obtained from B&T Seed World and Radboud University Nijmegen, the Netherlands. Twenty samples were downloaded from Genbank, NCBI. Thirty-two herb samples were collected from herbshops in Hong Kong and mainland China. The detailed information of the samples is listed in Tables 3.2–3.5.

For the analyses of the ISSR of *Aristolochia*, a total of 17 fresh samples were collected. The detailed information of the samples is listed in Table 3.6.

For the fresh materials collected, small amount of either leaves, stems or fruits were dried and stored in silica gel for DNA extraction. The remaining parts were dried and stored either in the Herbarium, Department of Biology, or the Museum of Chinese Medicine, Institute of Chinese Medicine. The herb samples gathered were stored in the Museum of Chinese Medicine, Institute of Chinese Medicine, CUHK.

Table 3.1   Authentic specimens and plants included in this study

Sample	Sample code	Family	Voucher specimen
Akebia quinata (Houttuyn) Decaisne	13	Lardizabalaceae	Takahashi #1002, Shikoku Is., Japan (Harvard University Herbaria)
Akebia quinata (Houttuyn) Decaisne	42	Lardizabalaceae	Uyama and Yamaguchi #980176, Mt. Hikami, Japan (Harvard University Herbaria)
Akebia trifoliata (Thunberg) Koidzumi	10	Lardizabalaceae	Boufford, Ying, Zhang and Zhang #26485, Henan, China (A)
Akebia trifoliata (Thunberg) Koidzumi	14	Lardizabalaceae	Boufford, Ying, Zhang and Zhang #26457, Henan, China (A)
Akebia trifoliata var. australis (Diels) Rehd.	11	Lardizabalaceae	How #9 Hubei, China (A)
Akebia trifoliata var. australis (Diels) Rehd.	12	Lardizabalaceae	Boufford, Donoghue and Ree #27241, Sichuan, China (A)
Aristolochia cinnabarina C.Y.Cheng & J.L.Wu	Ms517	Aristolochiaceae	National Centre for Natural Product Research, Reference # 517 (NCNPR)
Aristolochia contorta Bunge	1	Aristolochiaceae	Smith #847, Harbin, China (A)
Aristolochia contorta Bunge	Acon1	Aristolochiaceae	Hilary Lam #8, Hong Kong, China (CUHK)
Aristolochia debilis Siebold & Zuccarini	3	Aristolochiaceae	Yonekura #97283, Kyushu, Japan , (A)
Aristolochia debilis Siebold & Zuccarini	4	Aristolochiaceae	Jutila and Yamazaki #693, Toyama, Japan (Harvard University Herbaria),
Aristolochia debilis Siebold & Zuccarini	Adeb3	Aristolochiaceae	Hilary Lam #7, Hong Kong, China (CUHK)
Aristolochia debilis Siebold & Zuccarini	Ms442	Aristolochiaceae	USA, National Centre for Natural Product Research (NCNPR), Reference # 442
Aristolochia fangchi Y. C. Wu ex L. D. Chow & S. M. Hwang	Ms460	Aristolochiaceae	USA, National Centre for Natural Product Research (NCNPR), Reference # 460
Aristolochia heterophylla Hemsl.	7	Aristolochiaceae	1980 Sino-Amer Exped #1121, Hubei, Shennongjia (A)
Aristolochia kaempferi Willd.	8	Aristolochiaceae	Muroi #158, Kobe, Japan (A)
Aristolochia elegans Mast	Alt-1	Aristolochiaceae	Mississippi, USA, which is cultivated in Greenhouse, NCNPR, UM
Aristolochia manshuriensis Komarov	Ams-1	Aristolochiaceae	Mississippi, USA, which is cultivated in Greenhouse, NCNPR, UM
Aristolochia manshuriensis Komarov	GMT1	Aristolochiaceae	National Institute for the Control of Pharmaceutical and Biological Products

			(NICPBP), # 930-9001, China
Aristolochia minutissima C.Y. Cheng	Ms459	Aristolochiaceae	USA, National Centre for Natural Product Research (NCNPR), Reference # 459
Aristolochia mollissima Hance	9	Aristolochiaceae	Fan and Li 77 (A)
Aristolochia mollissima Hance	Amo1	Aristolochiaceae	Ming Li #49, Hong Kong, China (CUHK)
Aristolochia mollissima Hance	Amo2	Aristolochiaceae	Ming Li #48, (CUHK) Hong Kong, China (CUHK)
Aristolochia mollissima Hance	Ms536	Aristolochiaceae	USA, National Centre for Natural Product Research (NCNPR), Reference # 536
Aristolochia trilobata L.	Atb-1	Aristolochiaceae	Mississippi, USA, which is cultivated in Greenhouse, NCNPR, UM
Aucklandia lappa Decne.	Ulp11	Asteraceae	Yunnan Institute of Materia Medica, Yuannan, China
Cardiocrinum cordatum (Thunb) Makino	24	Liliaceae	Tsugaru #19779, Kyoto, Japan (A)
Clematis armandii Franchet	16	Ranunculaceae	Liu #15361, Sichuan, China (A)
Clematis armandii Franchet	Cam-1	Ranunculaceae	Hilary Lam #44, Hong Kong, China (CUHK)
Clematis chinensis Osbeck	CCH	Ranunculaceae	Hilary Lam #18 (CUHK) Hong Kong, China (CUHK)
Clematis meyeniana Walpers	CME	Ranunculaceae	Hilary Lam #17 (CUHK) Hong Kong, China (CUHK)
Clematis montana Buchanan-Hamilton ex de Candolle	15	Ranunculaceae	1984 Sino-Amer Bot Exp #976, Yunnan, China (A)
Clematis uncinata champ	CUN	Ranunculaceae	Hilary Lam #16, Hong Kong, China (CUHK)
Cocculus orbiculatus (L.) DC.	Cor2	Menispermaceae	Hilary Lam, #24 (CUHK) Hong Kong, China
Cocculus orbiculatus (L.) DC.	Cor3	Menispermaceae	Hilary Lam #42, Hong Kong, China (CUHK)
Dioscorea cirrhosa Loureiro, Fl.	Dci-1	Dioscoreaceae	Hilary Lam #32, Hong Kong, China (CUHK)
Dolomiaea edulis (Franch.) Shih	35	Asteraceae	Wang, #71337, Yunnan, China (A)
Dolomiaea edulis (Franch.) Shih	38	Asteraceae	1984 Sino-Ame #1057 (A)
Dolomiaea forrestii (Diels) Shih	43	Asteraceae	Wang #69546, Yunnan, China (A),
Dolomiaea georgii (Anth.) Shih	29	Asteraceae	Rock #10841, Sichuan, China (GH)
Dolomiaea georgii (Anth.) Shih	30	Asteraceae	Schneider #2367, Sichuan, China (GH)
Dolomiaea platylepis (Hand.-Mazz.) Shih	32	Asteraceae	Schneider #3312, Yunnan , China (GH)
Dolomiaea souliei (Franch.) Shih	33	Asteraceae	Rock #16417, Sichuan, China (GH)
Dolomiaea souliei var. mirabilis (Anth.) Shih	31	Asteraceae	Rock #16600, Sichuan, China (A)
Inula helenium Linnaeus	17	Asteraceae	E.F. Williams S.N. , York, Maine (GH)

Inula racemosa Hook. f.	44	Asteraceae	Ching #676 (Harvard University Herbaria)
Lilium formosanum Wallace	22	Liliaceae	Boufford, Wood and Hsieh #19253, Hohunan-Shan, Taiwan (Harvard University Herbaria)
Polygonum multiflorum Thunb.	Pum-2	Polygonaceae	Hilary Lam #33, Hong Kong, China (CUHK)
Saussurea cordifolia Hemsl	39	Asteraceae	Sino-Amer Bot Exp #44, Guizhon (Harvard University Herbaria)
Solanum japonense Nakai	Sjap1	Solanaceae	Tsugam #7400 (Harvard University Herbaria)
Solanum jasminoides Paxt	Sjas1	Solanaceae	Museum of Chinese Medicine, Institute of Chinese Medicine, #2005-2673, Hong Kong, China
Solanum lyratum Thunb.	17L1	Solanaceae	Museum of Chinese Medicine, Institute of Chinese Medicine, #2005-2664, Hong Kong, China
Stephania tetrandra S. Moore	FFC-3	Menispermaceae	Hilary Lam #43, Hong Kong, China (CUHK)

Table 3.2   DNA extract used in this study

Sample	Sample code	Family	Source
Aristolochia californica Torrey	Aca	Aristolochiaceae	Chase #19176, (K)
Aristolochia macrophylla Lamarck	Ama	Aristolochiaceae	Qiu #91019
Cardiocrinum giganteum (Wallich) Makino	Cgi	Liliaceae	Chase #3689, (K)
Cardiocrinum giganteum var. yunnanense (Leichtlin ex Elwes) Stearn	Cyn	Liliaceae	Chase #935, (K)
Clematis integrifolia Linnaeus	Cit	Ranunculaceae	Chase#19236, (K)
Cocculus sarmentosus (Lour.) Diels	Csa	Menispermaceae	Chase #1319, (K)

Table 3.3   Seed samples used in this study

Sample	Sample code	Family	Source
Inula helenium Linnaeus	Ihe-1	Asteraceae	Reference #72427, Seed from B&T Seed World
Inula racemosa Hook. f.	Ira-1	Asteraceae	Reference #23086, Seed from B&T Seed World
Solanum dulcamara L.	Sol 2	Solanaceae	Reference #884750034, seed from Radboud University Nijmegen, the Netherlands

Table 3.4   Sequences from Genbank, NCBI, used in this study

Sample	Sample code	Family	Source
Akebia quinata (Houttuyn) Decaisne	AF335397	Lardizabalaceae	Accession # AF335397, Genbank, NCBI
Akebia trifoliata (Thunberg) Koidzumi	AF335294	Lardizabalaceae	Accession # AF335294, Genbank, NCBI
Aristolochia californica Torrey	AY689174	Aristolochiaceae	Accession # AY689174, Genbank, NCBI
Aristolochia kaempferi Willd	DQ532023	Aristolochiaceae	Accession # DQ532023, Genbank, NCBI
Aristolochia manshuriensis Komarov	AY689184	Aristolochiaceae	Accession # AY689184, Genbank, NCBI
Cocculus laurifolius DC.	AM397159	Menispermaceae	Accession # AM397159, Genbank, NCBI
Dioscorea alata Linnaeus	DQ841331	Dioscoreaceae	Accession # DQ841331, Genbank, NCBI
Dioscorea cirrhosa Loureiro	DQ841324	Dioscoreaceae	Accession # DQ841324, Genbank, NCBI
Dioscorea composita Hemsl	DQ841330	Dioscoreaceae	Accession # DQ841330, Genbank, NCBI
Dioscorea decipiens J. D. Hooker	DQ841329	Dioscoreaceae	Accession # DQ841329, Genbank, NCBI
Dioscorea panthaica Prain & Burkill	DQ124704	Dioscoreaceae	Accession # DQ124704, Genbank, NCBI
Dolomiaea edulis (Franch.) Shih	AY913839	Asteraceae	Accession # AY913839, Genbank, NCBI
Dolomiaea edulis (Franch.) Shih	AY914860	Asteraceae	Accession # AY914860, Genbank, NCBI
Lilium catesbaei Walter	AF303701	Liliaceae	Accession # AF303701, Genbank, NCBI
Saussurea forrestii Diels	DQ874338	Asteraceae	Accession # DQ874338, Genbank, NCBI
Saussurea forrestii Diels	DQ874339	Asteraceae	Accession # DQ874339, Genbank, NCBI
Solanum americanum Miller	AY727179	Solanaceae	Accession # AY727179, Genbank, NCBI
Solanum physalifolium Rusby	AY727180	Solanaceae	Accession # AY727180, Genbank, NCBI
Solanum ptychanthum Dunal	AY727181	Solanaceae	Accession # AY727181, Genbank, NCBI
Stephania delavayi Diels	AM397154	Menispermaceae	Accession # AM397154, Genbank, NCBI

Table 3.5   Herb samples used in this study

Herb sample	Sample code	Sample source	Accession number in the Museum of Chinese Medicine, Institute of Chinese Medicine, CUHK
Baiheguo	Csp	Taiwan, China	#2007-3102
Baiheguo	Lbr-2	Sichuan, China 972233	#2007-3106
Baiheguo	Lgi01	Yunnan, China 972233	#2007-3107
Baiyaozi	Bay-1	Bejing, China	#2007-3108
Baiying	SL2	Hong Kong, China	#2005-2676
Baiying	SL3	Hong Kong, China	#2005-2677
Baiying	SL4	Guangdong, China	#2005-2653
Chuanmutong	Clm-1	Sichuan, China	#2007-3094
Chuanmutong	Clm-3	Hong Kong, China	#840100
Fangji	FFC-1	Shenzhen, China	#2007-3101
Fangji	FFC-2	Guangzhou, China	#840516
Fangji	MFC-1	Hong Kong, China	#931860
Fangji	MFC-2	Hong Kong, China	#2007-3100
Guangfnagji	GFC-1	Guangzhou, China	#840517
Guangfnagji	M83	Hong Kong, China	#840177
Guanmutong	M82	Henan, China	#942062
Madouling	M5	Guangdong, China	#2007-3103
Madouling	M6	Guangdong, China	#2007-3104
Madouling	M92	Dunhuang, China	#2007-3105
Mutong	Atr1	Hong Kong, China	#992307
Mutong	Atr2	Hong Kong, China	#992308
Mutong	Atr3	Hong Kong, China	#901087
Mutong	MT-1	Guangzhou, China	#2007-3095
Qingmuxiang	M85	Hubei, China	#840040
Qingmuxiang	TMX-3	Hebei, China	#2007-3098
Xungufeng	AM1	Hong Kong, China	#2005-2657
Xungufeng	AM10	Shanxi, China	#2005-2680
Xungufeng	AM2	Hong Kong, China	#2005-2658
Xungufeng	AM7	Jiangxi, China	#2005-2678
Yunmuxiang	Ulp3	Yuannan, China	#2007-3096
Yunmuxiang	Ulp4	Yuannan, China	#2007-3097

Table 3.6   Authentic plants, specimens and DNA extracts used for ISSR

Sample	Sample code	Family	Source
Aristolochia anguicida Jacq.	Aan-2	Aristolochiaceae	Hilary Lam #5, Hong Kong, China (CUHK)
Aristolochia arborea Lindl.	Aab(1)	Aristolochiaceae	UM, NCCPR, USA
Aristolochia cucurbitifolia Hayata.	Acu-5	Aristolochiaceae	Hilary Lam #41, Hong Kong, China (CUHK)
Aristolochia debilis Siebold & Zuccarini	Adeb-4	Aristolochiaceae	Hilary Lam #6, Hong Kong, China (CUHK)
Aristolochia elegans Mast.	Alg1	Aristolochiaceae	Hilary Lam #11, Hong Kong, China (CUHK)
Aristolochia fimbriata Cham & Schltdl.	Afm-3	Aristolochiaceae	Hilary Lam #15, Hong Kong, China (CUHK)
Aristolochia grandiflora SW.	Agr-2	Aristolochiaceae	Hilary Lam #10, Hong Kong, China (CUHK)
Aristolochia heterophyll Hemsl.	Ahe-1	Aristolochiaceae	Hilary Lam #40, Hong Kong, China (CUHK)
Aristolochia elegans Mast.	Alt1	Aristolochiaceae	Mississippi, USA, which is cultivated in Greenhouse, NCNPR, UM
Aristolochia liukiuensis Hatusima	Alu-1	Aristolochiaceae	Hilary Lam #39, Hong Kong, China (CUHK)
Aristolochia manshuriensis Komarov	Ams1	Aristolochiaceae	Mississippi, USA, which is cultivated in Greenhouse, NCNPR, UM
Aristolochia mollissima Hance	Amo2	Aristolochiaceae	Ming Li #48, Hong Kong, China (CUHK)
Aristolochia serpentaria L.	Ase-1	Aristolochiaceae	Hilary Lam #9, Hong Kong, China (CUHK)
Aristolochia serpentaria L.	Ase-2	Aristolochiaceae	USA, which is cultivated in greenhouse, Department of Biology, CUHK
Aristolochia tagala Champ.	Ata-1	Aristolochiaceae	Fung Yuen Butterfly Reserve Fung Yuen Culture & education center, HK,
Aristolochia trilobata L.	Atb-1	Aristolochiaceae	Hilary Lam #36, Hong Kong, China (CUHK)
Aristolochia zollingeriana Miq.	Azo-3	Aristolochiaceae	Hilary Lam #35, Hong Kong, China (CUHK)

3.2 Total DNA extraction

3.2.1 Cetyltriethylammonium bromide extraction

Total DNA was isolated from fresh leaf and seed, herbarium specimens and herbal medicine samples by Cetyltriethylammonium bromide (CTAB) extraction method, suggested by Kang and co-author (1998). For fresh leaf that used either directly or dried by silicon gel beforehand, 0.01–0.1 g leaf was used. For herbarium specimens and herb samples, 0.05–0.5 g sample was used. The difference in amount of material used in these cases is according to the degree of preservation of DNA in the samples. Fresh and freshly dried leaf, they contain relatively good quality and quantity DNA, less amount of sample is needed. For herbarium specimens and herb samples, DNA degradation is more significant that smaller amount and poor quality DNA are found, hence a larger amount of sample is necessary. The samples were rinsed with 75% ethanol and distilled water to minimize contamination by removing dirt and fugi that might be present on sample surface. The samples were then air dried. After that they were placed in a 1.5 ml microtube which contained 400 µl extraction buffer and 10 µg proteinase K (Armersham Pharmacia Biotech. Code #E76230Y). Each sample was cut into small pieces using fine scissors and ground into powder by using a sterile plastic rod. The mixture were then incubated at 37°C for 60 min. CTAB

solution 400 μl was added and mixed with the sample by inversion. 800 μl of chloroform:isoamyl alcohol (24:1) with 5% phenol was then added, and the microtube was gently inverted for several times. The mixture was centrifuged at room temperature at 12,000 g for 10 min. The supernatant was transferred into a new 1.5ml microtube and mixed with 2/3 volume of isopropanol, followed by incubation at room temperature for 10 min. The solution was centrifuged at 10,000 g for 5 min. The supernatant was removed and the DNA pellet was washed by 70% ethanol twice. The samples were air dried by heat block at 60 °C. After it became air dried, the DNA was resuspended in 30–60 μl distilled water and stored in -20°C for further use. Quality of isolated total DNA was analyzed by gel electrophoresis.

3.2.2 Commercial kit extraction

Beside CTAB extraction method, a commercial kit, DNeasy® Plant Mini Kit (#69104) of Qiagen (USA), was used in isolating total DNA. Kit extraction provided an efficient and promising way to extract DNA especially in handling herbarium specimens and dried herbal materials, in which the total DNA were often much degraded. Kit extraction was applied on those samples that did not generate good quality and quantity DNA by modified CTAB extraction. About 0.02 g sample was cut into small pieces and placed into an microtube with 400 μl Buffer AP1 and 4 μl

RNase A. The sample was ground into powder using sterile plastic rod. It was then incubated at 65°C for 10 min with two to three times of inversion. 130 µl AP2 Buffer was added to the lysate, mixed and incubated for 5 min in ice followed by centrifuging at 12,000 g for 5 min. The supernatant was transferred to Mini Spin Column which was placed in a 2 ml collection tube and centrifuged at 12,000 g for 2 min. The flow-through fraction was transferred to a 1.5ml microtube without disturbing the cell-debris pellet. Then 1.5 volumes of buffer AP3/E were added to the cleared lysate and mixed by pipetting. All the mixture was applied to the DNeasy Mini Spin Column sitting in a 2 ml collection tube. It was centrifuged at 6,000 g for 1 min and the flow-through was discarded. The DNeasy Mini Spin Column was placed into a new 2 ml collection tube. 500 µl AW Buffer was added to the DNeasy Mini Spin Column followed by centrifugation at 6,000 g for 1 min. The flow-through was discarded and another 500 µl AW buffer was added to the column. It was centrifuged at 12,000 g for 2 min to dry the membrane. The DNeasy Spin Mini Column was transferred to a 1.5 ml microtube. 50 µl AE Buffer was added directly onto the DNeasy membrane and then incubated at room temperature for 5 min followed by centrifugation at 6,000 g for 2 min. This step was repeated twice. The DNA isolated was stored in -20°C for further use. The quality of isolated total DNA was analyzed by gel electrophoresis.

3.3 DNA amplification

The interested DNA region was amplified by polymerase chain reaction (PCR) using appropriate primers (Table 3.7). Reaction mixture of 17.3 μl distilled water, 2.5 μl 10X PCR buffer, 2 μl 25 mM $MgCl_2$, 1 μl 2.5 mM dNTPs, 0.5 μl 10mM forward primer, 0.5 μl 10 mM backward primer, 0.2 μl *Taq* polymerase and 1–2 μl total DNA were applied to an 200 μl microtube. It was placed in a thermocycler using the following PCR profile:

1. $95^{o}C$ for 5 min (denaturing)

2. $95^{o}C$ for 2 min (denaturing)

3. $57^{o}C$ for 40 sec (annealing)

4. $72^{o}C$ for 1.5 min (extension)

5. repeat steps 2-4 for 32-35 cycles

6. $72^{o}C$ for 5 min (extension)

7. $14^{o}C$ forever (temporary storage)

The PCR products were kept in $4^{o}C$ for storage. The quality of PCR products were analyzed by gel electrophoresis.

Table 3.7   Primers used in PCR for amplifying specific regions

Primer	Direction	Primer sequence	Region amplified	Annealing temperature
*psb*A-*trn*HF	Forward	5'- GGTATGCATGAACGTAATGCTC -3'	*psb*A-*trn*H	57°C
*psb*A-*trn*HR	Reverse	5'- CGCGCATGGTGGATTCACAAAT -3'	*psb*A-*trn*H	57°C
Tab C	Forward	5'- CGAAATCGGTAGACGCTACG -3'	*trnL* intron	57°C
Tab D	Reverse	5'- GGGGATAGAGGGACTTGAAC -3'	*trnL* intron	57°C
Tab E	Forward	5'- GGTTCAAGTCCCTCTATCCC-3'	*trnL-trnF* region	57°C
Tab F	Reverse	5'- ATTTGAACTGGTGACACGAG -3'	*trnL-trnF* region	57°C

3.4 DNA fingerprinting

3.4.1 DNA concentration determination

The concentration of total DNA was determined using ND-1000 UV-Vis Spectrometer manufactured by Nanodrop Techologies Ltd. 1 μl DNA samples was pipetted into sample retention pit for measuring the absorbance at wavelength 260 nm and then the concentration of double-stand DNA was calculated automatically. The DNA extracts of all the samples were diluted or concentrated, and the concentration

was standardized to 12.5 µg/ml for subsequent procedures.

3.4.2 ISSR fingerprinting

Inter-simple sequence repeat (ISSR) was used as the marker for DNA fingerprinting. 21 primers (UBC Primer Set #9 - Microsatellite, Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada) were used to perform single-primer PCR (Table 3.8).

Table 3.8 Primers used in ISSR fingerprinting

Primer	Primer sequence	Annealing temperature
807	$(AG)_8$-T	50 °C
808	$(AG)_8$-C	50 °C
810	$(GA)_8$-T	50 °C
811	$(GA)_8$-C	50 °C
812	$(GA)_8$-A	50 °C
817	$(CA)_8$-A	50 °C
818	$(CA)_8$-G	50 °C
825	$(AC)_8$-T	50 °C
826	$(AC)_8$-C	50 °C
834	$(AG)_8$-YT	50 °C
842	$(GA)_8$-YG	50 °C
845	$(CT)_8$-RG	50 °C
855	$(AC)_8$-YT	50 °C
856	$(AC)_8$-YA	50 °C
857	$(AC)_8$-YG	50 °C
867	$(GGC)_6$	50 °C
878	$(GGAT)_4$	50 °C
881	GGGT$(GGGGT)_2$-G	50 °C
885	BHB-$(GA)_7$	50 °C
887	DVD-$(TC)_7$	50 °C
890	VHV-$(GT)_7$	50 °C

Reaction mixture of 16.1 μl distilled water, 2.5 μl 10X PCR buffer, 2 μl 50 mM MgCl₂, 2 μl 10 mM dNTPs, 1 μl 50 mM primer, 0.4 μl 5 units/μl *Taq* polymerase and 1 μl 12.5 μg/ml total DNA were applied in an 200 μl microtube. It was placed in a thermocycler using the following PCR profile:

1. 96°C for 3 min (denaturing)

2. 96°C for 45 sec (denaturing)

3. 50 °C for 45 sec (annealing)

4. 72°C for 2:30 min (extension)

5. repeat steps 2-4 for 44 cycles

6. 72°C for 7 min (extension)

7. 4°C forever (temporary storage)

The ISSR fingerprinting products were kept in 4°C for storage. The results were analyzed by gel electrophoresis.

3.5 Agarose gel electrophoresis

Agarose gel 1%, 1.7% and 2% were used to analysis DNA extract, PCR product and ISSR fingerprinting results respectively. Agarose powder 1 g, 1.7 g or 2 g was dissolved per 100 ml TAE buffer by applying heat. Ethidium bromide 0.5 μg/ml was added before pouring the solution into a gel cassette and it was cooled and

solidified. It was then put in a gel tank filled with 1X TAE buffer. For DNA extract and PCR product, 6X loading dye was mixed with DNA extracts or PCR products to a final concentration of 1X. The mixture and a 100 bp DNA marker were loaded to the wells of the gel. It was kept constantly at 120 V for 20 min to 40 min. For ISSR fingerprinting, 6X loading dye (Orange G) was mixed with the ISSR product. The mixture and 1 kb plus DNA ladder were loaded to the wells of the gel. It was kept constant at 40 V for 15 hours. The results of DNA extraction, PCR and ISSR fingerprinting were examined using an UV illumination. Gel photos were taken by BIORAD Gel Documentation System 1000.

3.6 Purification of PCR product

Interested PCR product was purified from the PCR reaction mixture by using spin method of the Gel-M^{TM} Gel Extraction System (Cat # EG1002) from Viogene-Biotek Corp. After gel electrophoresis, the gel slice 50-200 mg containing the DNA fragment was excised using a clean razor under UV transillustrator, manufactured by Ultra-Violet Products Ltd, and placed into a sterile 1.5 ml microtube. 0.5 ml GEX buffer was added followed by 10 min incubation at 60°C. The mixture was inverted for every 1–2 min until all the gel was completely dissolved in the buffer. The solution was allowed to cool down to room temperature then loaded to the

Gel-M^{TM} column. It was centrifuged at 12,000 g for 1 min and the flow-through was

discarded. The column was washed once with 0.5 ml WF buffer and then with 0.7 ml

WS buffer by centrifuging at 12,000 g for 1 min and discarding the flow-through for

every wash. An additional 12,000 g centrifugation was performed for 3 min to dry the

membrane. The column was placed onto a new 1.5 ml microtube and 20-30 μl

distilled water was applied to the membrane. It stood for 5 min before centrifugation

at 12,000 g for 3 min to elute the DNA. The DNA elute was kept in -20°C for further

use.

3.7 Cloning of PCR product

Cloning of PCR product before DNA sequencing gives several advantages. First,

it is necessary to insert a single PCR product to a plasmid by cloning before DNA

sequencing. Sequences like *psb*A-*trn*H intergenic spacer, may be present in multiple

copies. Also, if samples are contaminated by fungi, the PCR products may contain

DNA of both the test sample and fungi. Second, DNA sequences obtain after cloning

are longer and better with PCR primers included.

3.7.1 Ligation

Ligation was performed using commercial ligation kit pGEM®–T Easy Vector

System I (Cat # A1360) from Promega Corp. The 5 µl ligation mixture include 2.5 µl

2X rapid ligation buffer, 0.25 µl pGEM®–T vector, 0.5 µl T4 DNA ligase and 1.75 µl

purified PCR product. It was incubated at 25°C for at least 2 hours.

3.7.2 Transformation

All the 5 µl ligation mixture was added to 200 µl thawed competent *Escherichia*

coli cells and stood in ice for 30 min. It was heat shocked by incubation at 42°C for 2

min and then stood in ice for another 2 min. LB 200 µl Pre-heated at 37°C was

applied to the cells which were then incubated for 30 min at 37°C for cell recovery.

X-gal 2% 50 µl and IPTG 0.4M 5µl were mixed with the competent cells and then

spread on a LBA plate. It was incubated at 37°C for 16 hours.

3.7.3 Cell cultivation

From the LBA plate, only the white colonies were picked. For the cloning of

trnL-trnF region, 1–2 clones were picked. For the cloning of *psbA-trnH* spacer, four

clones were picked as this region has multiple copies. Each colony was placed in a

sterile 1.5ml microtube with 1.0 ml LB and 50 µg/ml ampicillin. All the microtubes

were placed in a shaker at 250 rpm and 37°C for 16 hours.

3.7.4 Plasmid extraction

Plasmids from the cultured competent cells were extracted using the spin method of Mini-M™ Plasmid DNA Extraction System (Cat # GF1002) from Viogene-Biotek Corp. The medium in the 1.5 ml microtube was poured into a new sterile microtube. The cells were pelleted by centrifuging at 12,000 g for 10 sec and the supernatant was removed. 250 µl MX1 buffer was added to the cell pellet and resuspended by vortexing. 250 µl MX2 buffer was added with several gentle inversion and it was incubated at room temperature for 5 min. 350 µl MX3 buffer was added and then the solution was mixed immediately. It was centrifuged at 12,000 g for 10 min. The supernatant was transferred into the Mini-M™ column followed by centrifuging at 12,000 g for 1 min and the flow-through was discarded. The column was washed by 0.5 ml WF buffer once and 0.7 ml WS buffer once by centrifuging at 12,000 g for 1min each. The membrane was dried by centrifuged at 12,000 g for 3 min. The column was placed onto a new microtube. 40 µl distilled water was added to the membrane and it was stood at room temperature for 5 min. It was centrifuged at 12,000 g for 3 min to elute the plasmids which were then stored at -20°C.

3.7.5 Insert confirmation

Insert confirmation for the extracted plasmid was done by restriction digestion.

The 5 μl restriction digestion mixture included 1 μl sterile distilled water, 0.5 μl 10X

H Buffer (Amersham Pharmacia Biotech. Cat # E1040Y), 0.5 μl *EcoR*I (Amersham

Pharmacia Biotech. Cat # E1040Y) and 3 μl plasmid. It was incubated at 37°C for 1

hour. The results were analyzed by gel electrophoresis using 1.7% agarose gel.

3.8 DNA sequencing

3.8.1 Cycle sequencing

Cycle sequencing was performed using BigDye® Terminator v3.1 Cycle

Sequencing Kit (Cat # 4336917) from Applied Biosystems. The cycle sequencing

reaction mixture included 1 μl 2X sequencing buffer, 2 μl Big Dye reaction mix, 5-20

ng of purified PCR product (about 500-1000 bp), 1 μl 2 μM sequencing primers and

sterile distilled water adding up to a final volume of 10 μl. For direct sequencing of

PCR products, sequencing primers were the same as PCR primers listed in Table 3.7.

For sequencing plasmids, universal primers T7-promoter (5'- TAATA CGACT

CACTA TAGGG -3') and Sp6-promoter (5'- ATTTA GGTGA CACTA TAGAA T -3')

were used. The reaction mixture was put in a thermocycler using the following cycle

sequencing profile:

1.   96°C for 1 min

2.   96°C for 10 sec

3.   50°C for 5 sec

4.   60°C for 4 min

5.   repeat step 2-4 for 24 cycles

6.   4°C forever (temporary storage)

3.8.2   Purification of cycle sequencing product

For each reaction mixture, 1 µl 3 M pH 5.2 sodium acetate and 25 µl 95%

ethanol were added and placed in -20°C for 10 min. The mixture was centrifuged at

12,000 g for 30 min. The supernatant was removed carefully, and 180 µl 75% ethanol

was added and mixed by vortexing. It was centrifuged at 12,000 g for another 5 min

and the supernatant was removed as much as possible. The pellet was air dried and

stored in -20°C.

3.8.3   DNA analysis

The purified cycle sequencing product was dissolved in 12 µl Hi-diformamide

(Cat # 4311320) from Applied Biosystems. It was then loaded into a 96-well

sequencing plate. It was denatured at 95°C for 2 min followed by putting the

sequencing plate in ice immediately. DNA analysis was performed by using ABI prism 3100 Genetic Analyzer of Applied Biosystems.

3.9   Sequence analysis

The sequencing results were translated into readable DNA sequence by the ABI sequencing analyzer program. They were analyzed by the software Chromas of Technelysium Pty Ltd. Each sequence was blasted to the Genbank using Nucleotide BLAST (BLASTn) of the National Center of Biotechnology Information (NCBI). Different sequences were aligned using computer program ClustalX version 1.83 (Thompson *et al.*, 1997) and online program ClustalW of the European Bioinformatics Institute. Any substitutions and insertions/deletions were visually inspected and manual amendments were performed using BioEdit Sequence Alignment Editor (Hall, 1999). Percentage similarity was calculated using BioEdit Sequence Alignment Editor (Hall, 1999). After manual amendments, the dendrograms were constructed using the software Molecular Evolutionary Genetic Analysis (MEGA) version 2.1 (Kumar *et al.*, 2001).

Chapter 4: AUTHENICATION OF MUTONG

4.1 Results

The DNA was successfully extracted from (a) four samples of the herb Mutong, (b) two samples of Chuanmutong, (c) one sample of Guanmutong, (d) seven authentic samples of *Clematis*, (e) six authentic samples of *Akebia,* and (f) 14 authentic samples of *Aristolochia* species. The chloroplast *trn*L-*trn*F gene region and *psb*A-*trn*H region of these 34 samples were successfully sequenced. For *trn*L-*trn*F gene region analysis, two additional *Akebia* sequences and three additional *Aristolochia* sequences were downloaded from NCBI Genbank and used in sequence alignment and constructing dendrograms.

The lengths of the *trn*L-*trn*F region and *psb*A-*trn*H region among the three genera were different. In *trn*L-*trn*F region, *Akebia* and *Aristolochia* had similar length, about 970–1010 bp, whereas *Clematis* showed only 760–780 bp in length. In *psb*A-*trn*H region, the length among the three genera were different, 590–610 bp in *Akebia*, 270–340 bp in *Aristolochia*, and 430–460 bp in *Clematis*.
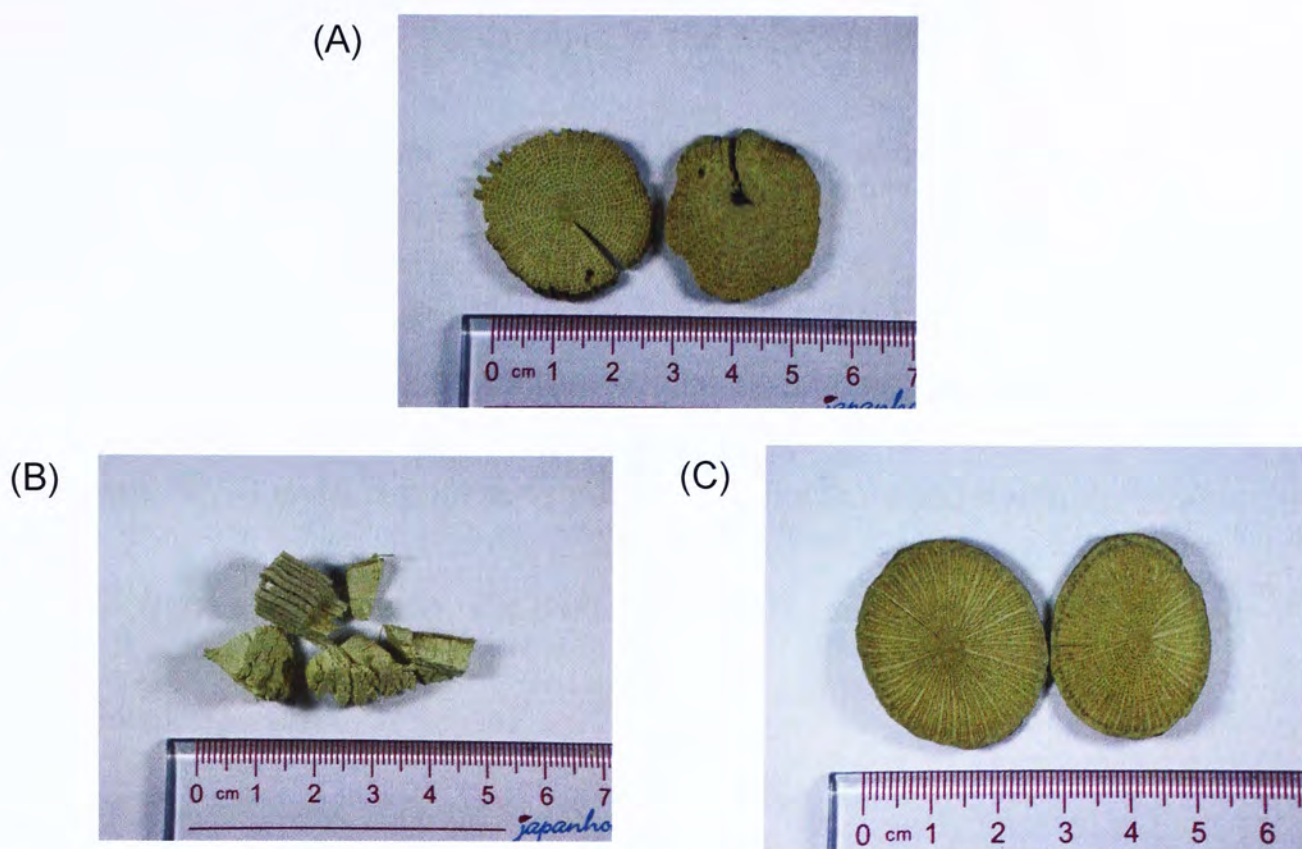
Figure 4.1 Morphological views of (A) Mutong (MT1), (B) Chuanmutong (Clm1) , and (C) Guanmutong (GMT1).

4.1.1   Sequence alignment

4.1.1.1 *trn*L-*trn*F sequences

The 39 sequences of *trn*L-*trn*F were aligned using the computer program ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were performed using BioEdit Sequence Alignment Editor (Hall, 1999). The aligned sequences are presented in Figure 4.2. Based on the sequence alignment of this gene region of the three genera *Aristolochia*, *Akebia* and *Clematis*, it became obvious that seven sites of insertions/deletions or base changes could be utilized to differentiate the herbs derived from these three genera. These sites are highlighted with a box in Figure

4.2 and presented in Table 4.1. For example, at site 770–780, all *Akebia* species share ATGTTATCCT, the *Aristolochia* species share AT-----CCT with a five bp deletion in the middle, while all *Clematis* species share AATTAATTCT.

The results of sequence alignment showed that the herb samples of Mutong (Atr1, Atr2, Atr3 and MT1) and Chuanmutong (Clm1 and Clm3) matched with the *Akebia* and *Clematis* species, respectively. The herb sample of Guanmutong (GMT1) matched with *Aristolochia* species.

4.1.1.2   *psb*A-*trn*H sequences

The 34 sequences of *psb*A-*trn*H were aligned using the computer program ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were performed using BioEdit Sequence Alignment Editor (Hall, 1999). The aligned sequences are presented in Figure 4.3. Based on the sequence alignment of this gene region of the genera *Aristolochia*, *Akebia* and *Clematis*, it became obvious that two sites of insertions/deletions or base changes could be utilized to differentiate the herbs derived from these three genera. These sites are highlighted with a box in Figure 4.3 and presented in Table 4.2. For example, at site 100–120, all *Akebia* species share T--TA-------------T with two significant deletions, the *Aristolochia* species share

Table 4.1 Sites of insertions/deletions or base changes for differentiating the herbs derived from *Aristolochia*, *Akebia* and *Clematis* from sequence alignment of *trn*L-*trn*F region in Figure 4.2. The gaps or base pairs labeled in red color indicate the insertions/deletions and base pairs labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes	
1	271–280	*Akebia*	CGTTCGTAGA
		Aristolochia	CATTGGTAGA
		Clematis	CGTTGATCGA
2	310–320	*Akebia*	ATGAAGGATGA
		Aristolochia	------GATGA
		Clematis	-------ATGA
3	400–410	*Akebia*	CGTTCGTAGA
		Aristolochia	CATTGGTAGA
		Clematis	CGTTGATCGA
4	581–590	*Akebia*	CG-----GAC
		Aristolochia	CGAGTGAGAC
		Clematis	TG-----GAC
5	770–780	*Akebia*	ATGTTATCCT
		Aristolochia	AT-----CCT
		Clematis	AATTAATTCT
6	888–910	*Akebia*	AAGTCAAGTCTTGTG-------A
		Aristolochia	-----------TGTGATAGATAT
		Clematis	-----------------------
7	956–980	*Akebia*	GGAATCCCCATTTGAATCATTTAAT
		Aristolochia	AGAATCTCCATT---ACGGAACCTT
		Clematis	-------------------------

Table 4.2 Sites of insertions/deletions or base changes for differentiating the herbs derived from *Aristolochia, Akebia* and *Clematis* from sequence alignment of the *psb*A-*trn*H region in Figure 4.3. The gaps or base pairs labeled in red color indicate the insertions/deletions and base pairs labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes
1	100–120	Akebia T--TA-------------T Aristolochia TCGT--------------- Clematis TCTTAGTGTATATGAGTCGT
2	182–197	Akebia TTCATTTAGTTTTAGT Aristolochia G---------CTCAAT Clematis TGCGTTTTGTTTTAAT

TCGT----------------, while all *Clematis* species share TCTTAGTGTATATGAGTCGT.

The results of sequence alignment showed that the herb samples of Mutong (Atr1, Atr2, Atr3 and MT1) and Chuanmutong (Clm1 and Clm3) matched with the *Akebia* and *Clematis* species, respectively. The herb sample of Guanmutong (GMT1) matched with *Aristolochia* species.

Figure 4.2   Sequence alignment of *trn*L-*trn*F region for herb materials of Mutong, Chuanmutong, Guanmutong and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Sites of insertions/deletions or base changes that could be utilized to differentiate the herbs derived from these three genera are highlighted in boxes.

Figure 4.2 (continued)

Sequence Name	Sequences

Figure 4.2 (continued)

Figure 4.2 (continued)

Sequence Name	Sequences

Akebia_trifoliata_AF335294
Akebia_quinata_AF335297
Akebia_quinata_42_S
Mutong_Atr3_TCM
Akebia_trifoliata_10_S
Akebia_trifoliata_14_S
Akebia_trifoliata_var.al2_S
Akebia_trifoliata_var.al1_S
Mutong_Atr1_TCM
Akebia_quinata_13_S
A.californica_Aca_S
A.californica_AY689174
A.kaempferi_DQ532023
A.manshuriensis_Ams1_S
A.manshuriensis_AY689184
Guanmutong_GMT1_TCM
A.macrophylla_Ama_S
A.mollissima_9_S
A.kaempferi_8_S
A.heterophylla_7_S
A.fangchi_Ms460_S
A.trilobata_Atb1_S
A.elegans_Alt1_S
A.cinnabarina_Ms517_S
A.contorta_1_S
A.debilis_4_S
A.debilis_Adeb3_S
A.contorta_Acon1_S
Clematis_armandii_6_S
Clematis_armandii_Cam-1_S
Mutong_MT1_TCM
Clematis_meyeniana_CME_S
Chuanmutong_Clm3_TCM
Mutong_Atr2_TCM
Clematis_integrifolia_Cit_S
Chuanmutong_Clm1_TCM
Clematis_montana_15_S
Clematis_uncinata_CUN_S
Clematis_chinensis_CCH_S
Clustal Consensus

Figure 4.2 (continued)

Sequence Name	Sequences
	1265
Akebia_trifoliata_AF335294	-----
Akebia_quinata_AF335297	-----
Akebia_quinata_42_S	CAAAT
Mutong_Atr3__TCM	CAAAT
Akebia_trifoliata_10__S_	CAAAT
Akebia_trifoliata_14__S_	CAAAT
Akebia_trifoliata_var.al2__S_	CAAAT
Akebia_trifoliata_var.al1__S_	CAAAT
Mutong_Atr1__TCM	CAAAT
Akebia_quinata_13__S_	CAAAT
A.californica_Aca__S_	CAAAT
A.californica_AY689174	-----
A.kaempferi_DQ532023	-----
A.manshuriensis_Ams1__S_	CAAAT
A.manshuriensis_AY689184	-----
Guanmutong_GMT1__TCM	CAAAT
A.macrophylla_Ama__S__	CAAAT
A.mollissima_9__S_	CAAAT
A.kaempferi_8__S_	CAAAT
A.heterophylla_7__S_	CAAAT
A.fangchi_Ms460__S_	CAAAT
A.trilobata_Atbl__S_	CAAAT
A.elegans__Alt1__S_	CAAAT
A.cinnabarina_Ms517__S_	CAAAT
A.contorta_1__S_	CAAAT
A.debilis_4__S_	CAAAT
A.debilis_Adeb3__S_	CAAAT
A.contorta_Acon1__S_	CAAAT
Clematis_armandiil_6__S_	CAAAT
Clematis_armandii_Cam-1__S_	CAAAT
Mutong_MT1__TCM	CAAAT
Clematis_meyeniana_CME__S_	CAAAT
Chuanmutong_Clm3__TCM	CAAAT
Mutong_Atr2__TCM	CAAAT
Clematis_integrifolia_Cit__S_	CAAAT
Chuanmutong_Clm1__TCM	CAAAT
Clematis_montana_15__S_	CAAAT
Clematis_uncinata_CUN__S_	CAAAT
Clematis_chinensis_CCH__S_	CAAAT
Clustal Consensus	
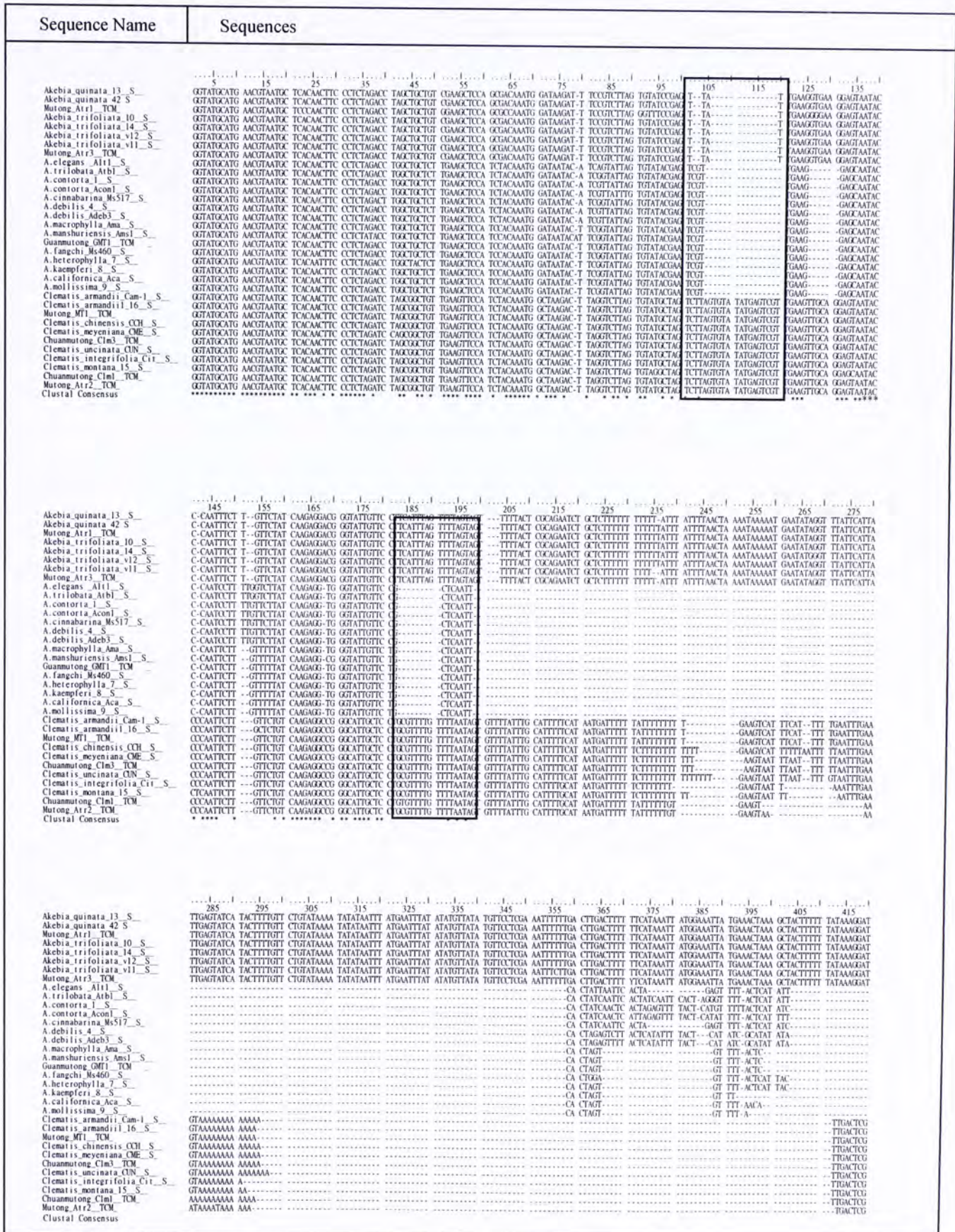
Figure 4.3 Sequence alignment of *psb*A-*trn*H region for herbal materials of Mutong, Chuanmutong, Guanmutong and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Sites of insertions/deletions or base changes that could be utilized to differentiate the herbs derived from these three genera are highlighted in boxes.

Figure 4.3 (continued)

Sequence Name	Sequences

(Sequence alignment, positions 425–555)

```
                                    425       435       445       455       465       475       485       495       505       515       525       535       545       555
Akebia_quinata_13_S_      TGGTCTTGCA TGCCCAATAT CTGTATCTTA GTATATCTTA GTATCAAGAA AAGTAAGAAC TACTCAA-TT AAAAACTCA ATTAAAAAT GAAAATAAC AAAAAAGAA ATTCAATTTC TACCATATGG TTTTTTTTT-
Akebia_quinata_42_S       TGGTCTTGCA TGCCCAATAT CTGTATCTTA GTATATCTTA GTATCAAGAA AAGTAAGAAC TACTCAA-TT AAAAACTCA ATTAAAAAT GAAAATAAC AAAAAAGAA ATTCAACTTC TACCATATGG TTTTTTTTTT
Mutong_Atr1_TCM           TGGTCTTGCA TGCCCAATAT CTGTATCTTA GTATATCTTA GTATCAAGAA AAGTAAGAAC TACTCAA-TT AAAAA---- ..........T GAAAATAAC AAAAAAGAA ATTCAATTTC TACCATATGG TTTTTTTTT
Akebia_trifoliata_10_S_   TGGTCTTGCA TGCCCAATAT CTGTATCTTA GTATATCTTA GTATCAAGAA AAGTAAGAAC TACTCAA-TT AAAAA---- ..........T GAAAATAAC AAAAAAGAA ATTCAATTTC TACCATATGG TTTTTTTTT
Akebia_trifoliata_14_S_   TGGTCTTGCA TGCCCAATAT CTGTATCTTA GTATATCTTA GTATCAAGAA AAGTAAGAAC TACTCAA-TT AAAAA---- ..........T GAAAATAAC AAAAAAGAA ATTCAATTTC TACCATATGG TTTTTTTTTT
Akebia_trifoliata_vl2_S_  TGGTCTTGCA TGCCCAATAT CTGTATCTTA GTATATCTTA GTATCAAGAA AAGTAAGAAC TACTCAA-TT AAAAA---- ..........T GAAAATAAC AAAAAATAA ATTCAATTTC TACCATATGG TTTTTTTTT-
Akebia_trifoliata_vl1_S_  TGGTCTTGCA TGCCCAATAT CTGTATCTTA GTATATCTTA GTATCAAGAA AAGTAAGAAC TACTCAA-TT AAAAA---- ..........T GAAAATAAC AAAAAAGAA ATTCAATTTC TACCATATGG TTTTTTTTT
Mutong_Atr3_TCM           TGGTCTTGCA TGCCCAATAT CTGTATCTTA GTATATCTTA GTATCAAGAA AAGTAAGAAC TACTCAA-TT AAAAA---- --TAAAAAT GAAAATAAC AAAAAAGAA ATTCAATTTC TACCATATGG TTTTTTTTT
A.elegans_Alt1_S_         .......... .......... .......... .......... .......... --GCATAA-A TAG-GCA-TT T----TATCT ATAT----- .......... .......... .......... .......... .........T
A.trilobata_Atb1_S_       .......... .......... .......... .......... .......... --GCATA-A TAG-GCA-TT T----TATCT ATAT----- .......... .......... .......... .......... .........T
A.contorta_1_S_           .......... .......... .......... .......... .......... --GCATAT-A TATTATA-TG TA---TATGT ATATGCATTT TATCCATAGT TTATGGTTAA AA------- .......... ..........
A.contorta_Acon1_S_       .......... .......... .......... .......... .......... -TACTCAT-A TCGCATA-TA TATTATATGT ATATGCATTT TATCCATAGT TTAAAGTTAA AAAAAAAA- .......... ..........
A.cinnabarina_Ms517_S_    .......... .......... .......... .......... .......... --GCATAA-A TAT-GCA-TT T----TCTCT AT---TTTTT AGTTTAAAGT TAAAAAAAA TT-------- .......... ..........
A.debilis_4_S_            .......... .......... .......... .......... .......... --ATATATTA TATGCTA-TG CAT--TTTAT CCATTTTTTT AGTTTAAAGT TAAAAAAAA AATGAAAAAG GA------- ..........
A.debilis_Adeb3_S_        .......... .......... .......... .......... .......... --ATATATTA TATGCTA-TG CAT--TTTAT CCATTTTTTT AGTTTAAAGT TAAAAAAAA AATGAAAAAG GA------- ..........
A.macrophylla_Ama_S_      .......... .......... .......... .......... .......... --GCGGA--T TAC-----TC C--------- .......... .......... .......... .......... ..........
A.manshuriensis_Ams1_S_   .......... .......... .......... .......... .......... --GCGGA--T TAC-----TC C--------- .......... .......... .......... .......... ..........
Guanmutong_GMT1_TCM       .......... .......... .......... .......... .......... --GCGGA--T TAC-----TC C--------- .......... .......... .......... .......... ..........
A.fangchi_Ms460_S_        .......... .......... .......... .......... .......... -AGCGGGA--T TAC-----TC C--------- .......... .......... .......... .......... ..........
A.heterophylla_7_S_       .......... .......... .......... .......... .......... -AGCGGGA--T TAC-----TC C--------- .......... .......... .......... .......... ..........
A.kaempferi_8_S_          .......... .......... .......... .......... .......... ........ TAC-----TC C--------- .......... .......... .......... .......... ..........
A.californica_Aca_S_      .......... .......... .......... .......... .......... --GCGGA--T TAC-----TC C--------- .......... .......... .......... .......... ..........
A.mollissima_9_S_         .......... .......... .......... .......... .......... ......... --C T------ .......... .......... .......... .......... .......... ..........
Clematis_armandii_Cam-1_S_  AATGGGGGAT TGGTTGTTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC ATATATAAAC CAATCCAATA CAATA-TGTT CCTAAAAATT TTGAAACAAT TTA--TTTGA GAAA----- ..........
Clematis_armandii_16_S_   AATGGGGGAT TGGTTGTTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC GTATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATT TTGAAACAAT TTA--TTTGA GAAA----- ..........
Mutong_MT1_TCM            AATGGGGGAT TGGTTGTTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATT TTGAAACAAT TTA--TTTGA GAAA----- ..........
Clematis_chinensis_CCH_S_ AATGGGGGAT TGGTTGTTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATT TTGAAACAAT TTA--TTTGA GAAA----- ..........
Clematis_meyeniana_CME_S_ AATGGGGGAT TGGTTGTTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATT TTGAAACAAT TTA--TTTGA GAAA----- ..........
Chuanmutong_Clm3_TCM      AATGGGGGAT TGGTTGTTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATT TTGAAACAAT TTA--TTTGA GAAA----- ..........
Clematis_uncinata_CUN_S_  AATGGGGGAT TGGTTGTTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATT TTGAAACAAT TTA--TTTGA GAAA----- ..........
Clematis_integrifolia_Cit_S_  AATGGAGGAT TGGTTGGTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATAGTTGTT CCTAAAAATT TGGAAACAAT TTA--TTTGA GAAA----- ..........
Clematis_montana_15_S_    AATGGAGGAT TGGTTGGTGA ATTCTTGATT ATCATACTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATT TGGAAACAAT TTA--TTTGA GAAA----- ..........
Chuanmutong_Clm1_TCM      AATGGAGGAT TGGTTGGTGA ATTCTTGATT ATCATCCTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATT TGGAAACAAT TTA--TTTGA GAAA----- ..........
Mutong_Atr2_TCM           AATGGAGGAT TGGTTGGTGA ATTCTTGATT ATCATCCTCT CGTTCTGTAC ATATATAAAC CAATCCAATA TAATA-TGTT CCTAAAAATA GTGAAACAAT TTA--TTTGA GAAA----- ..........
Clustal Consensus
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                                    565       575       585       595       605       615       625       635       645
Akebia_quinata_13_S_      -----ATTTT TTTGTGAGTT TGAGGGAGTA GGGGGGCGGA TGTAGCCAAG TGGCTCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Akebia_quinata_42_S       -----ATTTT TTTGTGAGTT TGAGGGAGTA GGGGGGCGGA TGTAGCCAAG TGGCTCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Mutong_Atr1_TCM           -----ATTTT TTTGTGAGTT TGAGGGAGTA GGGGGGCGGA TGTAGCCAAG TGGCTCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Akebia_trifoliata_10_S_   TTT--ATTTT TTTGTGAGTT TGAGGGAGTA GGGGGGCGGA TGTAGCCAAG TGGCTCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Akebia_trifoliata_14_S_   -----ATTTT TTTGTGAGTT TGAGGGAGTA GGGGGGCGGA TGTAGCCAAG TGGCTCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Akebia_trifoliata_vl2_S_  TT---ATTTT TTTGTGAGTT TGAGGGAGTA GGGGGGCGGA TGTAGCCAAG TGGCTCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Akebia_trifoliata_vl1_S_  TTT--ATTTT TTTGTGAGTT TGAGGGAGTA GGGGGGCGGA TGTAGCCAAG TGGCTCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Mutong_Atr3_TCM           TTTTTATTTT TTTGTGAGTT TGAGGGAGTA GGGGGGCGGA TGTAGCCAAG TGGCTCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.elegans_Alt1_S_         TTTTAGTTTA AAGTTAAAAA G--A---AGT CCAGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.trilobata_Atb1_S_       TTTTAGTTTA AAGTTAAAAA A--A---AGT CCAGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.contorta_1_S_           --------- -----AAAAG GAAA---GGC CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTG-ATTT GTGAATCCAC CATGCGCG
A.contorta_Acon1_S_       --------- -----AAAAG GAAA---GGC CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTG-ATTT GTGAATCCAC CATGCGCG
A.cinnabarina_Ms517_S_    --------- -----AAAAA GAAA---GGC CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTG-ATTT GTGAATCCAC CATGCGCG
A.debilis_4_S_            -----AAGG TTA--AAAAG GAAA---GGC CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.debilis_Adeb3_S_        ------AAGG TTA--AAAAG GAAA---GGC CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.macrophylla_Ama_S_      TTTTTTTTTT -AAGTTAAAG AAA---GGG CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.manshuriensis_Ams1_S_   TTTTTTTTTT -AAGTTAAAG AAA---GGG CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Guanmutong_GMT1_TCM       TTTTTTTTTT TAAGTTAAAG AAA---GGG CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.fangchi_Ms460_S_        TTTTTTTTT- -AAGTTAAAG AAA---GGG CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.heterophylla_7_S_       TTTTTTTTTT -AAGTTAAAG AAA---GGG CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.kaempferi_8_S_          TTTTTTTTT- -AAGTTAAAG AAA---GGG CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.californica_Aca_S_      TTTTTTTTTT TTAGTTAAAG AAA---GGG CAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
A.mollissima_9_S_         CCTTTTTTTT TTAGTTAAAG AAA---GGG CAGGGGCGGA TGTAGC-AAG TGGATCAAGG CAGTG-ATTT GTGAATCCAC CATGCGCG
Clematis_armandii_Cam-1_S_  --------- --------- -------GCG GAGGGGCGGA GGGAACCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Clematis_armandii_16_S_   --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Mutong_MT1_TCM            --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Clematis_chinensis_CCH_S_ --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Clematis_meyeniana_CME_S_ --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Chuanmutong_Clm3_TCM      --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Clematis_uncinata_CUN_S_  --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Clematis_integrifolia_Cit_S_  --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Clematis_montana_15_S_    --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Chuanmutong_Clm1_TCM      --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTGGATTT GTGAATCCAC CATGCGCG
Mutong_Atr2_TCM           --------- --------- -------GCG GAGGGGCGGA TGTAGCCAAG TGGATCAAGG CAGTG-ATTT GTGAATCCAC CATGCGCG
Clustal Consensus         ******* * * * *** *** ****** ***** **** ********* ********
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63

4.1.2   Percentage similarity analysis

The percentage similarities of the *trn*L-*trn*F region among all the herbal

materials of Mutong, Chuanmutong and Guanmutong and relevant authentic species

were calculated using BioEdit Sequencing Alignment Editor (Hall, 1999). The results

are summarized in Table 4.3. The interspecific similarities between *Akebia* and

Aristolochia varied from 60.9–75.4%, the average being 70.4%. The interspecific

similarities between *Clematis* and *Aristolochia* species ranged from 44.4–55.4%, the

average being 50.2%. The interspecific similarities between *Akebia* and *Clematis*

ranged from 49.1–60.9%, the average being 58%. The average intraspecific similarity

in *Aristolochia* is 88.4%, ranging from 74.9–99%.

The percentage similarities of the *psb*A-*trn*H region among all the herbal

materials of Mutong, Chuanmutong and Guanmutong and relevant authentic species

were also calculated using BioEdit Sequencing Alignment Editor (Hall, 1999). The

results are presented in Table 4.4. The interspecific similarities between *Akebia* and

Aristolochia varied from 33.0–37.6%, the average being 35.3%. The interspecific

similarities between *Clematis* and *Aristolochia* species ranged from 37.3–43.6%, the

average being 40.1%. The interspecific similarities *Akebia* to *Clematis* ranged from

42.6–45.7%, the average being 44.2%. The average intraspecific similarity in

Aristolochia is 79%, varying from 64.2–99.6%.

Table 4.3 Percentage similarities of *trn*L-*trn*F region among the plant and herb samples of Mutong, Chuanmutong and Guanmutong.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[31]	[32]	[33]	[34]	[35]	[36]	[37]	[38]	[39]
[1]	Akebia_trifoliata_AF335294	99.5	89.4	89.3	89.3	89.1	89.1	89	88.9	89.2	89.1	64.3	67.9	68.6	64.3	68.8	64.3	63.2	65.2	65	65.4	65.3	62.9	63.6	63.2	62.6	62.4	62.7	61.2	50.9	49.3	51	50.8	50.9	50.3	51.3	50.3	51.1	50.4
[2]	Akebia_quinata_AF335297		89.1	89	88.8	88.7	89	88.9	88.6	88.9	88.8	64	67.6	68.3	64	68.5	64	62.9	65	64.7	65.1	65	62.6	63.4	62.9	62.4	62.1	62.4	60.9	50.7	49.1	50.8	50.6	50.7	50.1	51.1	50.1	50.8	50.9
[3]	Akebia_quinata			99.7	99.6	99.4	99.3	99.3	99.2	99.5	99.3	73.8	65.8	66.4	73.9	64.7	73.9	72.6	75.1	75.2	74.7	75.2	72.1	73.1	72.9	71.9	71.9	72.2	70.2	60.4	58.8	60.5	60.3	60.4	59.8	60.9	59.9	60.5	60
[4]	Mutong_Atr3_TCM_				99.7	99.6	99.4	99.4	99.5	99.6	99.4	73.8	65.8	66.4	74	74.1	74	72.7	75.1	75.3	74.8	75.3	72.1	73.1	73.1	72	72	72.3	70.2	60.3	58.7	60.4	60.2	60.3	59.7	60.8	59.8	60.4	59.9
[5]	Akebia_trifoliata_10_S_					99.7	99.5	99.4	99.7	99.5	99.3	73.9	65.8	66.4	74.1	74.8	74.1	72.8	75.2	75.4	74.9	75.3	71.9	72.9	72.9	71.8	71.8	72.1	70.1	60.4	58.7	60.5	60.3	60.4	59.8	60.9	59.9	60.5	60
[6]	Akebia_trifoliata_14_S_						99.6	99.5	99.4	99.7	99.5	73.9	65.6	66.2	74.1	73.9	74.1	72.6	75	75.2	74.7	75.2	71.9	72.9	72.9	71.8	71.8	72.1	70.1	60.3	58.7	60.4	60.2	60.3	59.7	60.8	59.8	60.4	59.9
[7]	Akebia_trifoliata_var.a12_S_							99.3	99.2	99.5	99.7	73.7	65.6	66.2	73.9	64.6	73.9	72.6	75	75.2	74.7	75.2	71.9	72.9	72.9	71.8	71.8	72.1	70.1	60.3	58.7	60.4	60.2	60.3	59.7	60.8	59.8	60.6	59.9
[8]	Akebia_trifoliata_var.a11_S_								99.7	99.5	99.2	73.7	65.6	66.2	73.9	64.6	73.9	72.6	75	75.2	74.7	75.2	71.9	72.9	72.9	71.8	71.8	72.1	70.1	60.4	58.8	60.5	60.3	60.4	59.8	60.8	59.8	60.6	59.9
[9]	Mutong_Atr1_TCM_									99.4	99.1	73.7	65.6	66.2	73.9	64.6	73.9	72.6	75	75.2	74.7	75.2	71.9	72.9	72.9	71.8	71.8	72.1	70.1	60.3	58.7	60.4	60.3	60.3	59.6	60.7	59.7	60.5	59.8
[10]	Akebia_quinata_13_S_										99.4	73.9	65.8	66.2	74.1	64.8	74.1	72.8	75.2	75.4	74.9	75.4	72.1	73.1	72.9	71.9	71.9	72.3	70.3	60.2	58.6	60.3	60.2	60.3	59.6	60.8	59.7	60.6	59.9
[11]	A.californica_Aca_S_											73.7	65.6	66.2	73.9	64.7	73.9	72.6	75	75.1	74.7	75.1	71.9	72.9	72.9	71.8	71.8	72.1	70.1	60.2	58.6	60.3	60.2	60.3	59.6	60.8	59.7	60.3	60.5
[12]	A.californica_AY689174												91	86.5	93	83.4	92.9	91.7	95	94.6	95.2	95.2	86.5	87	91.1	86.2	86.3	86.6	82.7	54.2	52.5	54.1	54.2	54.2	53.2	54.2	53.2	54.2	53.4
[13]	A.kaempferi_DQ532023													94.1	84.4	90.7	84.3	83.2	86.2	85.8	86.4	86.4	78.5	78.8	82.4	78	78	78.4	74.9	46.2	44.4	45.9	46	46.1	45.2	46.1	45.2	46.1	45.3
[14]	A.manshuriensis_Ams1_S_														85.1	91.5	85	83.7	87.4	87	87	79	79.4	83.5	78.5	78.7	79	75.4	46.5	44.7	46.2	46.4	46.5	45.7	46.7	45.7	46.5	46.5	45.8
[15]	A.manshuriensis_AY689184															89.7	99.6	96.1	96.3	95.8	96.1	96.1	88.1	88.7	92.6	87.7	87.9	88.3	84.8	54.3	52.6	54	54.2	54.3	53.3	54.4	53.2	54.4	54.3	53.7
[16]	Guanmutong_GMT1_TCM_																89.6	86.3	86.2	85.9	86	86	88.8	79.4	82.8	78.6	78.9	78.9	75.8	45	45.1	45.2	44.2	44.3	45.2	44.2	45.3	45.1	44.6
[17]	A.macrophylla_Ama_S_																	96	96.2	95.7	96	96	88	88.6	92.5	87.6	87.8	88.2	84.7	54.3	52.6	54	54.2	54.3	53.2	54.4	53.2	54.4	54.3	53.7
[18]	A.mollissima_9_S_																		94.7	94.4	94.7	94.7	90.9	86.9	87.6	86.9	86.8	86.8	83	53.5	52.6	53.5	53.6	53.6	52.6	53.7	53.7	53.6	53.6	52.8
[19]	A.kaempferi_8_S_																			99	98.9	98.9	89.3	89.8	94.9	89.1	89.7	89.9	83.5	55.2	53.5	55.2	55.2	55.2	54.2	55.3	54.2	55.3	55.2	54.6
[20]	A.heterophylla_7_S_																				98.4	98.4	88.8	89.3	94.6	88.9	89.4	89.4	84.9	55	53.3	54.9	55	54	55	55.1	54	55.1	55	54.3
[21]	A.fangchi_Ms460_S_																					99.2	89.3	89.8	94.6	89	89.5	89.5	85.2	55.3	53.6	55.3	55.4	55.3	54.3	55.3	54.3	55.4	55.4	54.5
[22]	A.trilobata_Atb1_S_																						89.3	89.8	95.1	89	89.5	89.5	85.2	55.2	53.5	55.2	55.3	55.2	54.2	55.2	54.2	55.4	55.4	54.4
[23]	A.elegans_Alt1_S_																							95.8	87	87.6	87.9	87.9	84.4	54.1	53.2	54.1	54.1	53.2	54.1	53.1	54.1	54.1	54.1	53.5
[24]	A.cinnabarina_Ms517_S_																								87.5	90.1	87.6	89.8	86.3	54.6	52.9	54.5	54.6	53.6	54.5	53.5	54.5	54.6	54.6	53.9
[25]	A.contorta_1_S_																									88.7	92.8	89.6	89.8	54	52.3	54.3	54	53.1	54	53.1	54.5	54.1	54.1	53.4
[26]	A.debilis_4_S_																										88.9	89.2	84.9	54	53.8	54.5	54.2	53.2	54	53.1	53.6	53.1	54.2	53.5
[27]	A.debilis_Adeb3_S_																											90.8	91	91.9	53.9	53.3	53.9	54.2	54	54	53.1	54.6	54.9	54.6	54
[28]	A.contorta_Acon1_S_																												99.3	92.2	54.2	52.5	54.1	54.6	54.5	53.3	53.6	53.7	54.9	54.3	54
[29]	Clematis_armandii_6_S_																													92.4	54.3	52.6	54.2	54.3	53.4	54.3	53.3	54.6	54.3	54.3	53.7
[30]	Clematis_armandii_Cam-1_S_																														52.8	51.2	52.6	52.8	52	52.8	51.9	53	52.8	52.8	52.2
[31]	Mutong_MT1_TCM_																															96.4	98.4	98.2	97	98.3	96.9	98.2	98.5	98.2	97.1
[32]	Clematis_meyeniana_CME_S_																																96.6	96.1	95.2	96.7	94.8	96.9	96.5	96.9	95.6
[33]	Chuanmutong_Clm3_TCM_																																	98.1	97	98.4	96.4	98.5	98.2	98.5	97.4
[34]	Mutong_Atr2_TCM_																																		99.7	97.5	98.4	96.9	97.2	98.9	99	97.3
[35]	Clematis_integrifolia_Cit_S_																																			97.8	97.5	98.8	97.4	98.9	99.3	97.5
[36]	Chuanmutong_Clm1_TCM_																																				98.8	97.6	99.6	99.2	99.3	96.4
[37]	Clematis_montana_15_S_																																					98.1	97.8	97.9	98.2
[38]	Clematis_uncinata_CUN_S_																																						96	99	98.9
[39]	Clematis_chinensis_CCH_S_																																							97.6

Table 4.4 Percentage similarities of *psbA-trnH* region among the plant and herb samples of Mutong, Chuanmutong and Guanmutong.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[31]	[32]	[33]	[34]
[1]	Akebia_quinata_13_S_	99.5	96.6	97.1	97.4	97.2	96.6	97.9	35	36	36.7	37.1	36.7	37.5	37.6	34	34	34.2	34.5	34	33.2	34.2	33.1	44.2	44.2	44.7	45.6	45.5	45.5	45.5	44.2	44.4	43	43.5
[2]	Akebia_quinata		96.7	97.2	97.2	97.4	96.4	97.7	35.1	36.1	36.5	37	36.5	37.3	37.5	33.9	33.9	34.1	34.4	33.9	33.1	34.1	33	44.3	44.3	44.8	45.7	45.6	45.6	45.7	44.3	44.5	43.1	43.5
[3]	Mutong_Atr1_TCM_			98.5	98.5	98.6	97.7	96.7	34.1	35.1	35.3	35.7	35.3	36.5	36.7	34	33.9	34.2	34.5	34	33.2	34.2	33	42.6	42.6	43.1	44	43.9	43.9	44	42.6	42.8	41.4	41.8
[4]	Akebia_trifoliata_10_S_				0	99.8	99.1	98.2	35.3	36.2	35.7	36.2	35.7	35.7	37.2	35.1	35.1	35.3	35.6	35.1	34.3	35.3	33.8	43.2	43.2	43.7	44.6	44.4	44.4	44.6	43.2	43.3	41.9	42.4
[5]	Akebia_trifoliata_14_S_					0	98.1	97.2	34.6	35.6	36.1	36.6	36	37.2	37.4	34.7	34.6	34.9	35.2	34.7	33.9	34.9	33.7	43.6	43.6	44.1	44.9	44.9	44.9	45	43.6	43.8	42.4	42.8
[6]	Akebia_trifoliata_v12_S_						99.1	98	35.1	36.1	35.8	36.3	35.8	37.1	37.2	35	34.9	35.1	35.5	35	34.2	35.1	33.7	43.3	43.3	43.7	44.7	44.5	44.5	44.7	43.3	43.4	42	42.5
[7]	Akebia_trifoliata_v11_S_							99	35.2	36.2	35.7	36.2	35.7	36.8	37	35.1	35	35.2	35.6	35.1	34.3	35.1	33.8	42.7	42.7	43.2	44.1	44	44	44.1	42.7	42.9	41.5	41.9
[8]	Mutong_Atr3_TCM_								35.2	36.2	35.8	36.3	35.7	36.8	37	35.1	35	35.3	35.6	35.1	34.3	35.2	33.8	43.8	43.8	44.3	45.2	45.1	45.1	45.2	43.8	44	42.6	43.1
[9]	A.elegans_Alt_S_									95.2	77	73.5	82.3	70.4	37.1	35.1	35.1	35.3	35.6	35.1	34.3	35.3	33.8	43.8	38.3	38.7	38.3	38.4	38.6	38	39.7	39.4	40.3	40.1
[10]	A.triobata_Atb1_S_										78.7	75.2	79.7	71.9	72.2	77	76.1	77	76.8	76.8	75.7	75.5	37.9	37.5	38.1	37.7	37.8	37.9	38.6	39.1	39.7	38.8	39.6	39.4
[11]	A.contorta_1_S_											92.1	86.7	82.1	81.8	69.1	68.3	68.9	69.4	69.2	67	68.1	66.3	37.3	37.7	41.6	41.1	41.1	41.5	40.9	42.7	42.4	43.3	43
[12]	A.contorta_Acon1_S_												81.6	82.4	82.2	66.3	65.6	66.2	66.8	66.6	64.6	65.3	64.2	40.8	41	41.9	41.4	41.3	41.7	41.2	42.9	42.6	43.6	43
[13]	A.cinnabarina_Ms517_S_													80.8	81.1	72.4	71.6	72.2	72.7	72.5	70.2	71.2	69.4	41	40.9	41.4	40.9	40.9	41.3	40.9	42.5	42.4	43.5	43
[14]	A.debilis_4_S_														99.7	66.1	65.3	66.3	66.2	65.9	65.5	66.3	64.7	40.5	41	41.9	41.4	40.1	40.5	40.2	41.7	41.6	42.5	43
[15]	A.debilis_Adeb3_S_															66.1	65.3	66.3	66.2	65.9	65.5	66.3	64.7	40.2	40.3	41.6	40.2	40.1	40.5	40.2	41.6	41.6	42.5	41.9
[16]	A.macrophylla_Ama_S_																66.1	66.3	96.4	97.1	95.6	98.1	93	39.2	39.3	39.7	39.2	39	39.5	39	40.6	40.4	41.3	41.9
[17]	A.manshuriensis_Ams1_S_																	98.9	95.3	96	94.5	97	92	39.1	39.2	39.6	39.1	39	39.4	38.9	40.6	40.3	41.2	41.1
[18]	A.manshuriensis_GMT1_S_																		98.5	96	95.2	98.5	93.4	39.1	39.2	39.6	39.1	39	39.4	38.9	40.1	40.3	40.8	41
[19]	A.fangchi_Ms460_S_																			97.8	93.1	94.6	90	38.7	38.8	39.2	38.7	38.6	39	38.5	40.1	40.3	40.5	41
[20]	A.heterophylla_7_S_																				92.8	95.3	90.3	38.4	38.5	38.9	38.4	38.3	38.7	38.2	39.8	39.6	40.5	40.6
[21]	A.kaempferi_8_S_																					94.8	95	39.5	39.5	40	39.5	39.4	39.8	39.2	41	39.6	40.5	40.3
[22]	A.californica_Aca_S_																						93.7	39.1	39.2	39.6	39.1	39.4	39.8	39.2	40.7	40.7	41.7	41.4
[23]	A.mollissima_9_S_																							38.3	38.8	39.2	38.8	38.7	39.1	38.5	40.6	40.3	41.2	41
[24]	Clematis_armandii_Cam-1_S_																								98.9	98.9	96.9	97.1	97.6	95.9	95.9	40.2	40	40.9	40.7
[25]	Clematis_armandii_16_S_																									98.2	96.9	97.1	97.6	95.9	94.5	94.5	91.9	92.1
[26]	Mutong_MT1_TCM_																										99.3	97.1	97.6	96.5	94.9	94.9	92.3	92.5
[27]	Clematis_chinensis_CCH_S_																											97.6	98.2	97	95.6	95.6	92.9	93.2
[28]	Clematis_meyeniana_CME_S_																												98.2	97.4	94.3	94.8	91.3	91.5
[29]	Chuanmutong_Clm3_TCM_																													97.8	94.7	95.2	91.5	91.9
[30]	Clematis_uncinata_CUN_S_																														95.2	95.6	91.9	92.3
[31]	Clematis_integrifolia_Cit_S_																															94.2	91.4	91.4
[32]	Clematis_montana_15_S_																																94.2	95
[33]	Chuanmutong_Clm1_TCM_																																	94.4
[34]	Mutong_Atr2_TCM_																																	96.8

4.1.3　Dendrogram analysis

Dendrograms were constructed using the computer program MEGA version 3.1 (Kumar *et al.*, 2001) with two tree construction methods: unweighted pair-group methods using arithmetic averages (UPGMA; Figures 4.4 and 4.6) and maximum parsimony (Figures 4.5 and 4.7). Each method was tested by bootstrap tests with 1000 replications.

For the *trn*L-*trn*F region, the samples form three separate clades: *Aristolochia* clade, *Akebia* clade and *Clematis* clade with 100 bootstrap frequencies. In the *Akebia* clade, the herb samples of Mutong (Atr1 and Atr3) clustered with the authentic samples of genuine species *Akebia trifoliata, Akebia trifoliata* var. *australis* and *Akebia quinata*. This *Akebia* clade was supported by bootstrap frequencies of 100 in both bootstrap consensus trees of UPGMA and maximum parsimony. In the *Clematis* clade, the herb samples of Chuanmutong (Clm1 and Clm3) and two Mutong (MT1 and Atr2) clustered with the authentic samples of *Clematis* with Mutong (MT1) clustering with the genuine Mutong species *Clematis armandii*, while Chuanmutong (Clm1) clustered with the genuine species *Clematis montana*. This *Clematis* clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony. In the *Aristolochia* clade, the herb sample Guanmutong (GMT1) clustered with authentic

samples of *Aristolochia manshuriensis.* This *Aristolochia* clade was supported by bootstrap frequencies of 100 in the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony.

For the *psb*A-*trn*H region, the samples were distributed in three separate clades: *Aristolochia* clade, *Akebia* clade and *Clematis* clade with 99 bootstrap frequencies. In the *Akebia* clade, the herb samples of Mutong (Atr1 and Atr3) clustered with the authentic samples of *Akebia.* This clade was supported by bootstrap frequencies of 100 in both bootstrap consensus trees of UPGMA and maximum parsimony. In the *Clematis* clade, two herb samples of Chuanmutong (Clm1 and Clm3) and two samples of Mutong (MT1 and Atr2) clustered with the authentic samples of *Clematis.* This clade was supported by bootstrap frequencies of 100 in the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony. In the *Aristolochia* clade, the herb samples of Guanmutong (GMT1) clustered with the authentic samples of *Aristolochia.* This clade was supported by bootstrap frequencies of 99 in both bootstrap consensus trees of UPGMA and maximum parsimony.

Mutong Atr2 (TCM)
Clematis integrifolia Cit (S)
Chuanmutong Clm1 (TCM)
Clematis montana 15 (S)
Clematis uncinata CUN (S)
Clematis chinensis CCH (S)
Mutong MT1 (TCM)
Clematis armandii1 16 (S)
Clematis armandii Cam-1 (S)
Clematis meyeniana CME (S)
Chuanmutong Clm3 (TCM)

Clematis

Akebia quinata AF335297
Akebia trifoliata var. australis 12 (S)
Akebia trifoliata var. australis 11 (S)
Akebia quinata 13 (S)
Akebia trifoliata 14 (S)
Mutong Atr1 (TCM)
Mutong Atr3 (TCM)
Akebia trifoliata 10 (S)
Akebia trifoliata AF335294
Akebia quinata 42 (HS)

Akebia

A.debilis 4 (S)
A.debilis Adeb3 (S)
A.contorta Acon1 (S)
A.trilobata Atb1 (S)
A. elegans Alt 1 (S)
A.contorta 1 (S)
A.cinnabarina Ms517 (S)
A.kaempferi 8 (S)
A.manshuriensis Ams1 (S)
Guanmutong GMT1 (TCM)
A.manshuriensis AY689184
A.macrophylla Ama (S)
A.heterophylla 7 (S)
A.fangchi Ms460 (S)
A.mollissima 9 (S)
A.kaempferi DQ532023
A.californica Aca (S)
A.californica AY689174

Aristolochia

Bootstrap values: 100, 90, 64, 100, 56, 100, 99, 100, 98, 60, 64, 69, 68, 99, 93

Figure 4.4 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Mutong, Chuanmutong and Guanmutong. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
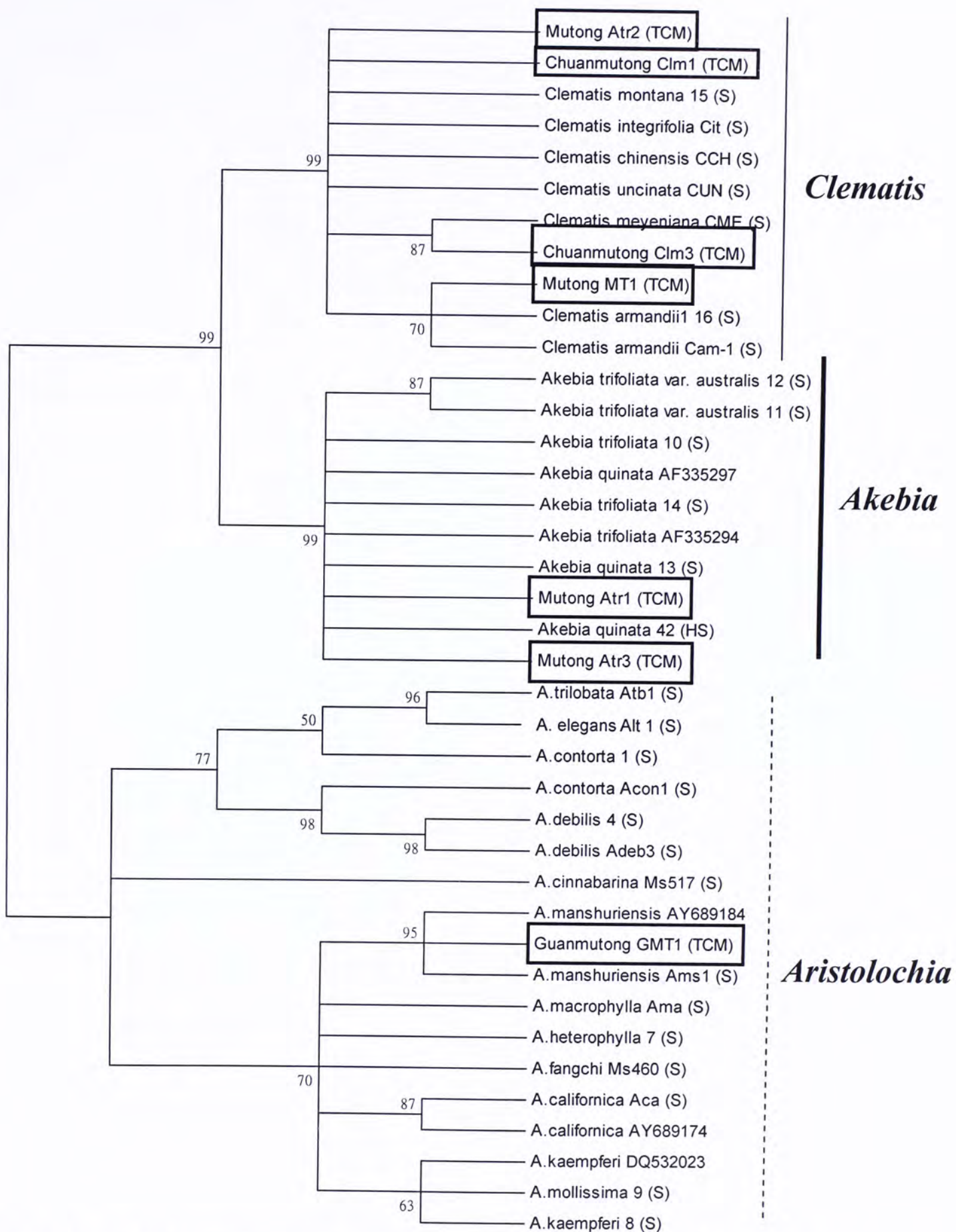
Figure 4.5 Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Mutong, Chuanmutong and Guanmutong. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
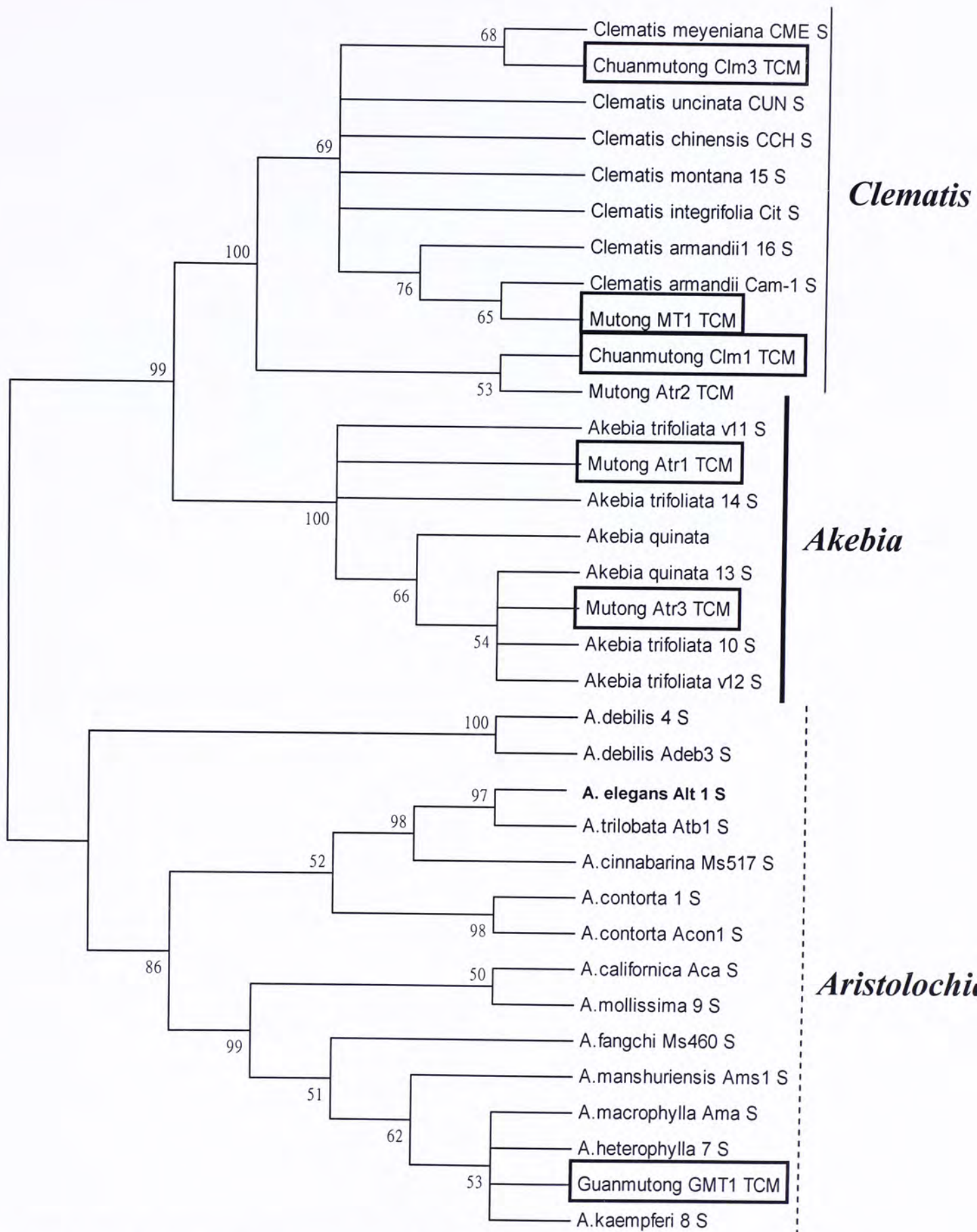
Figure 4.6 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Mutong, Chuanmutong and Guanmutong. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
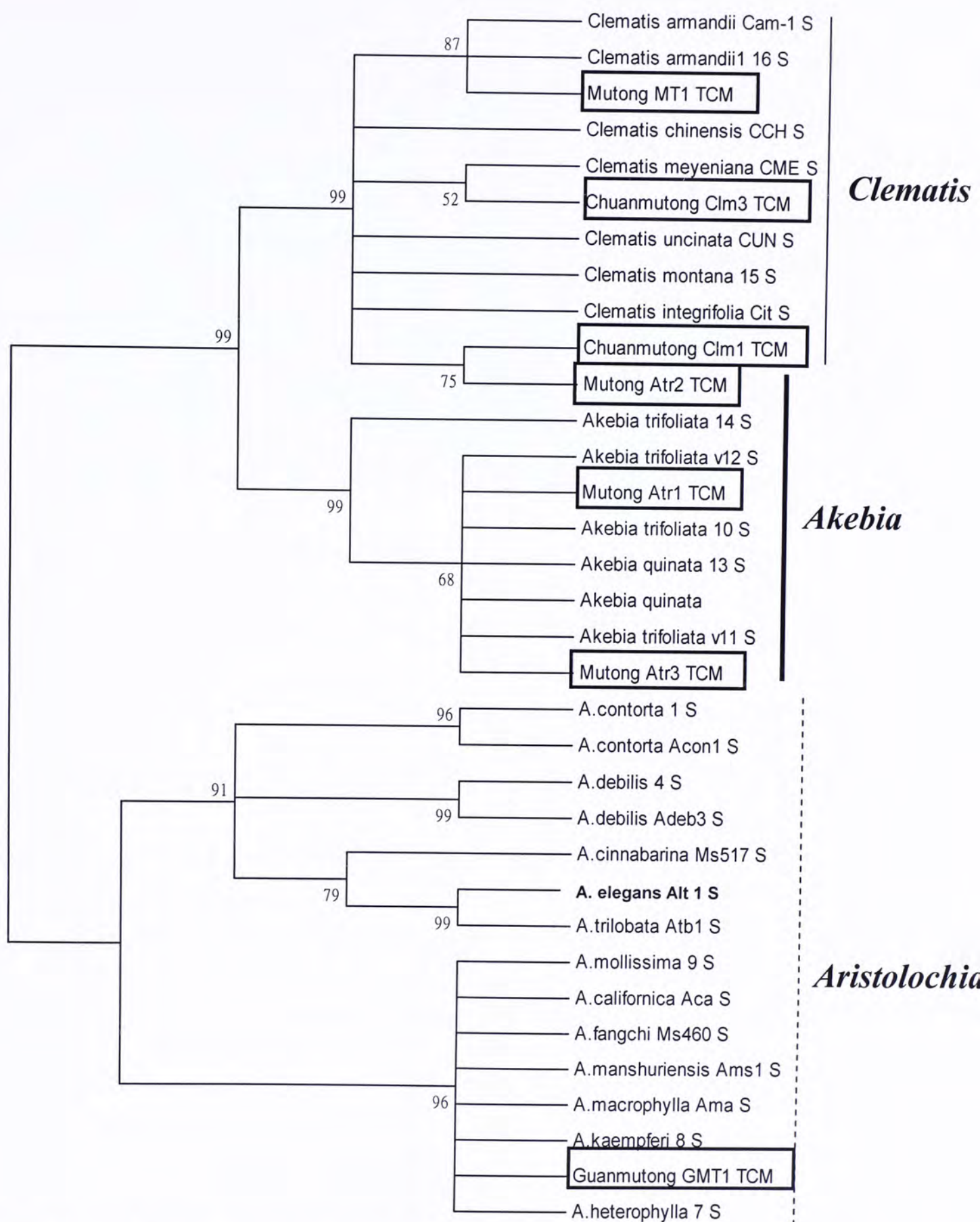
Figure 4.7 Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Mutong, Chuanmutong and Guanmutong. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.

4.2 Discussion

4.2.1 Evaluation of chloroplast *trn*L-*trn*F region in differentiation of Mutong

In this study, *trn*L-*trn*F region was analyzed to differentiate two types of genuine Mutong from the adulterant Guanmutong. The source species of Mutong are *Akebia quinata, Ak. trifoliata,* and *Ak. trifoliata* var. *australis,* whereas the source species of Chuanmutong are *Clematis armandii* and *C. montana.* The source species of Guanmutong is *Aristolochia manshuriensis.*

The intraspecific similarities among species in *Aristolochia* varied from 74.9–99% showing that this region is generally conserved in this genus (Table 4.1). The average interspecific similarities between *Aristolochia* and *Akebia*, and between *Aristolochia* and *Clematis* were 70.4 and 52.6 respectively, which were lower that the average intraspecific similarity within *Aristolochia.* This suggests that *trn*L-*trn*F can differentiate the genuine Mutong from the adulterant Guanmutong.

The dendrogram constructed by either UPGMA or maximum parsimony using this region can clearly separate the three genera into three clades with bootstrap frequency of 99–100. But the relationship within each clade (intraspecific relationship) was not well resolved. Although clades of replicate samples such as *Clematis armandii* (16 and Cam-1), *Akebia trifoliata* var. *australis* (12 and 11) and *Aristolochia*

californica (Aca and AY689174) clustered together, other species with only one sample form un-resolved clades. Obviously, the intraspecific similarities between samples of these three genera are high.

The *trnL-trnF* region is sufficiently variable among these three genera. Although it is quite conserved intraspecifically, differentiation of species is achieved by the dendrograms constructed. Nucleotide changes of insertions/deletions and base substitutions between samples can be taken as markers for the differentiation of genuine Mutong and Chuanmutong from Guanmutong (*Aristolochia manshuriensis*) (Figure 4.2). In conclusion, *trnL-trnF* region is able to differentiate the plants from different genera and thus can be utilized for differentiation of genuine and adulterant Mutong samples.

4.2.2 Evaluation of chloroplast *psbA-trn*H region in differentiation of Mutong

The chloroplast *psb*A-*trn*H is another region used to differentiate the genuine and adulterant Mutong. The average intraspecific similarity within *Aristolochia* is 79%, varying from 64.2–99.6%. This showed that the *psb*A-*trn*H region is variable within this genus. The average interspecific similarities between *Aristolochia* and *Akebia* and *Aristolochia* to *Clematis* are 35.3% and 40.1% respectively. They are much lower than

the intraspecific similarity of *Aristolochia* suggesting that this region is sufficient for differentiating herb samples originating from the three genera.

The dendrograms constructed by either UPGMA or maximum parsimony using this region can clearly separate the three genera into separate clades with bootstrap frequency of 99.

4.2.3 Evaluation of using DNA sequencing in differentiation of Mutong

Han et al. (2005) used pyrosequencing to identify *Akebia* and *Aristolochia* species (*Akebia quinata* and *Aristolochia manshuriensis*), using an automated system for pattern recognition software. This method is based on typing single-base genetic variations in DNA between species. The results showed that *Akebia* and *Aristolochia* species have different pyrosequencing patterns and can be distinguished from each others. Comparing the DNA sequencing used in this study with pyrosequencing conducted by Han et al. (2005), DNA sequencing provided more sites for authentication. These sites indicate distinct positions of base-changes, insertions/deletions and the sites are sequences of base pairs. Pyrosequencing analysis with number of possible single-base variations, but the DNA sequences alignments of *trn*L-*trn*F and *psb*A-*trn*H region provided significant markers. Furthermore,

pyrosequencing is not capable for reading long sequences. Almadian *et al.* (2006) compared pyrosequencing technology to DNA sequencing method under the effect of *E. coli* single-stranded DNA binding protein (SSB). The read length in pyrosequencing both with and without SSB was co-related to the PCR product length. The sequence quality decrease with the increase in PCR product length. The cause of such limitation on longer DNA templates (>600bp) is attributed to background disturbances such as primer mis-annealing (Almadian *et al.*, 2006). The DNA sequencing method used in this research does not have such limitations because, unlike pyrosequencing, the PCR products are further purified by ethanol precipitation. The background disturbance is reduced. Another shortcoming of pyrosequencing is that the sequence synthesis process depends on the enzyme performance. Insufficient enzyme activities would lead to frameshift or non-synchronized extension that increases the risk of misinterpreting the height of peak (Almadian *et al.*, 2006). As the intensity of the peaks detected during sequencing synthesis determines the number of nucleotides present in the DNA fragment, misinterpreting the height of peak may lead to different results. In DNA sequencing, the intensity of signaling peak only accounts for the presence but not the number of nucleotides.

4.3 Conclusion

The method adapted is successful, and both *trnL-trnF* region and *psbA-trnH* region are suitable for differentiating the genuine from the adulterant species involved in Mutong. In general, the interspecific similarities (i.e. the similarities between genuine and adulterants) of the *psbA-trnH* region are much lower than those of *trnL-trnF* region, suggesting that the former region is less conserved than the *trnL-trnF*. Though higher sequence variability was found in the *psbA-trnH* region, fewer significant sites were identified for herb differentiation. On the other hand, the more conserved *trnL-trnF* region provides more significant sites and thus more useful information for differentiation of the three genera studied in this project.

Chapter 5: AUTHENICATION OF MUXIANG

5.1 Results

The DNA was successfully extracted from (a) two samples of the herb Yunmuxiang, (b) two samples of Qingmuxiang, (c) four authentic samples of *Inula*, (d) eight samples of *Dolomaiea*, (e) one sample of *Aucklandia*, (f) one sample from *Saussurea*, and (g) 15 samples of *Aristolochia* species. The chloroplast *trn*L-*trn*F gene region and *psb*A-*trn*H gene region of these 33 samples were sequenced. For *trn*L-*trn*F gene region analysis, one additional *Dolomiaea* sequence, one additional *Saussurea* sequence and three additional *Aristolochia* sequence were downloaded from NCBI Genbank and used in sequence alignment and constructing dendrograms. For *psb*A-*trn*H region analysis, one additional *Dolomiaea* sequence and one additional *Saussurea* sequence were downloaded from NCBI.

There are no significant differences in the length of the *trn*L-*trn*F region among the five genera. The genera *Aucklandia, Dolomiaea, Inula* and *Saussurea* have similar length, about 920–960 bp. *Aristolochia* is relatively longer, about 970–1010 bp in length. There is difference in the number of bp in *psb*A-*trn*H region between the Asteraceae genera and *Aristolochia*. The length of this region in *Aristolochia* is from 260–300 bp but 480–515 bp in Asteraceae.
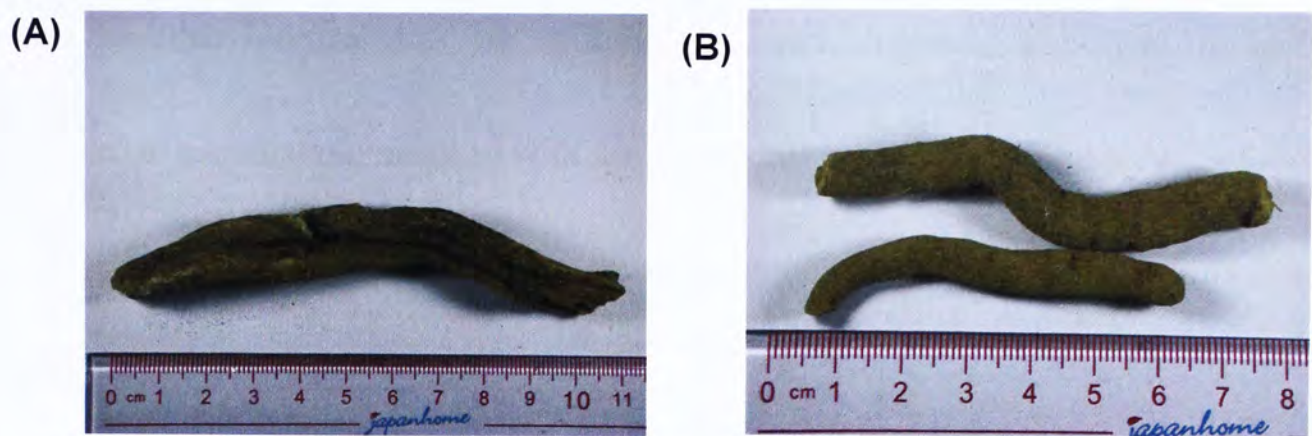
Figure 5.1 Morphological views of (A) Yunmuxiang (Ulp3), (B) Qingmuxiang (TMX3).

5.1.1 Sequence alignment

5.1.1.1 *trn*L-*trn*F sequences

The 38 sequences of *trn*L-*trn*F region were aligned using the computer program ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were performed using BioEdit sequence Alignment Editor (Hall, 1999). The aligned sequences are presented in Figure 5.2. Based on the sequence alignment of this gene region of the five genera *Aristolochia, Aucklandia, Dolomiaea, Inula* and *Saussurea*, four sites of insertions/deletions or base changes can be utilized to differentiate the herbs derived from these five genera. These sites are highlighted with a box in Figure 5.1 and presented in Table 5.1. For example at site 378–387, there was a CTGAA insertion in the samples of *Aristolochia* but not in *Aucklandia, Dolomiaea, Inula* and *Saussurea*. This region could be used to differentiate *Aristolochia* herbs

from the other four genera. The results of sequence alignment show that the herb samples of Yunmuxiang matched with the Asteraceae genera (*Aucklandia, Dolomiaea, Inula,* and *Saussurea*) and the herb samples Qingmuxiang matched with *Aristolochia* species.

Table 5.1 Sites of insertions/deletions or base changes for differentiating the herbs derived between *Aristolochia* and other four Asteraceae genera (*Aucklandia, Dolomiaea, Inula* and *Saussurea*) from sequence alignment of *trn*L-*trn*F region in Figure 5.2. The gaps or base pairs labeled in red color indicated the insertions/deletions and base pair labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes
1	209–213	*Aristolochia* CTCAA
		Other genus CTCGA
2	322–326	*Aristolochia* CCTTA
		Other genus A---G
3	378–387	*Aristolochia* TACTGAAATA
		Other genus TA-----ATA
4	389–396	*Aristolochia* CAAAGAT
		Other genus AGAAGAA

5.1.1.2 *psb*A-*trn*H sequences

The 34 sequences of *psb*A-*trn*H region were aligned using the computer program ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were performed using BioEdit sequence Alignment Editor (Hall, 1999). The aligned sequences are presented in Figure 5.2. Based on the sequence alignment of this gene

region of *Aristolochia, Aucklandia, Dolomiaea, Inula* and *Saussurea*, it became

obvious that two sites of insertions/deletions or base changes can be utilized to

differentiate the herbs derived from these five genera. These sites are highlighted in

Figure 5.3 and presented in Table 5.2. For example, at site 109–120, there was a 9 bp

deletion in *Aristolochia* when compared with samples of the other four genera

(*Aucklandia, Dolomiaea, Inula* and *Saussurea*).

Table 5.2 Sites of insertions/deletions or base changes for differentiating the herbs derived from *Aristolochia, Aucklandia, Dolomiaea, Inula* and *Saussurea* from sequence alignment of *psb*A-*trn*H region in Figure 5.3. The gaps or base pairs labeled in red color indicated the insertions/deletions and the base pairs labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes	
1	87–99	*Aristolochia*	TATTAGTGTATACG
		Other genera	TCTGATTGTATAGG
2	109–120	*Aristolochia*	A---------GG
		Other genera	ACTAAAAAA-GG
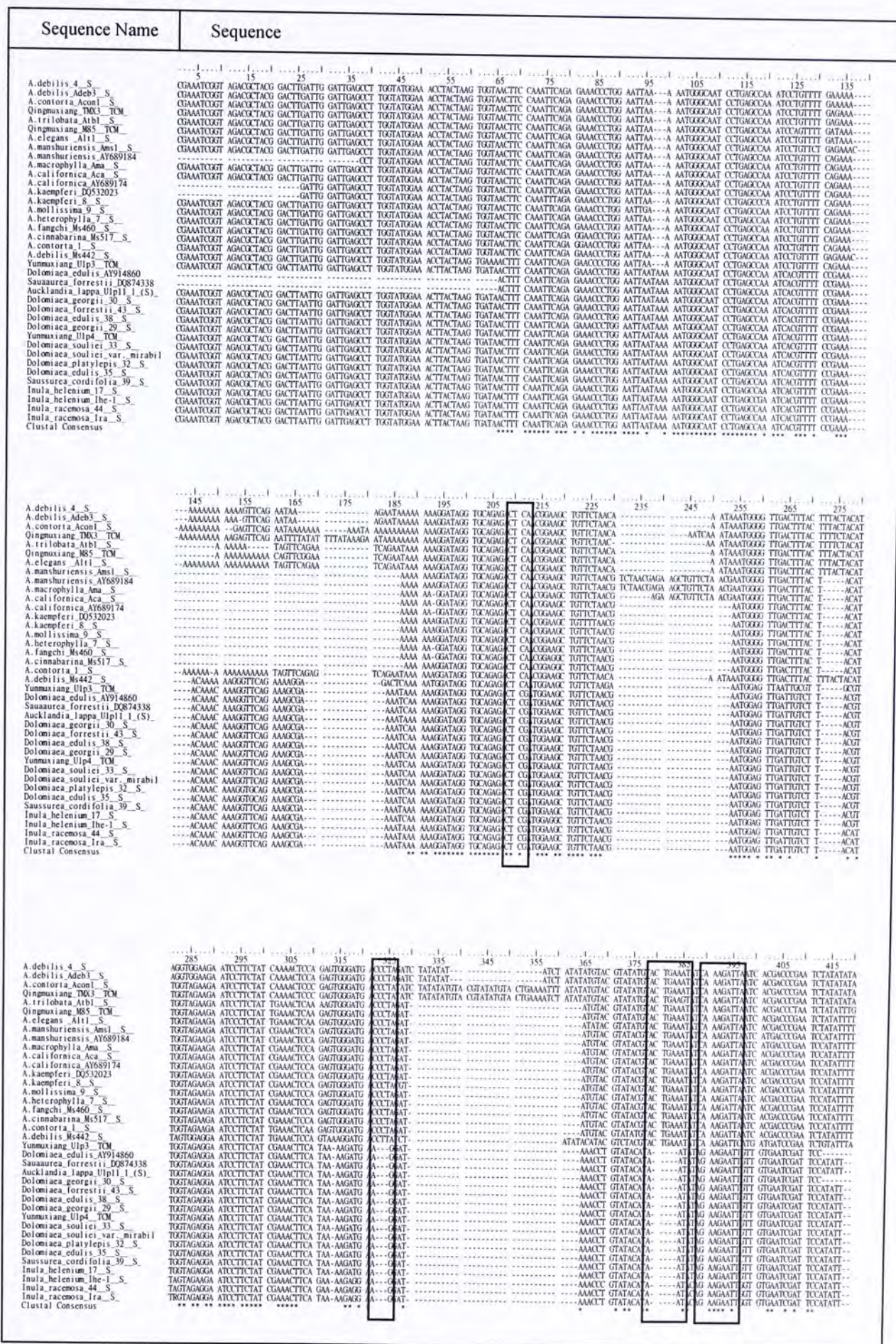
Figure 5.2 Sequence alignment of *trn*L-*trn*F region for herb materials of Yunmuxiang, Qingmuxiang and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Some of the nucleotide differences that are able to distinguish genuine Yunmuxiang from Qingmuxiang are highlighted in boxes.

Figure 5.2 (continued)

Sequence Name	Sequence

Figure 5.2 (continued)

83

Figure 5.2 (continued)

Sequence Name	Sequence

(Multiple sequence alignment figure — sequence names listed below with aligned nucleotide data)

A.debilis_4_S
A.debilis_Adeb3_S
A.contorta_Acon1_S
Qingmuxiang_TMX3_TCM
A.trilobata_Atb1_S
Qingmuxiang_M85_TCM
A.elegans_Alt1_S
A.manshuriensis_Ams1_S
A.manshuriensis_AY689184
A.macrophylla_Ama_S
A.californica_Aca_S
A.californica_AY689174
A.kaempferi_DQ532023
A.kaempferi_8_S
A.mollissima_9_S
A.heterophylla_7_S
A.fangchi_Ms460_S
A.cinnabarina_Ms517_S
A.contorta_1_S
A.debilis_Ms442_S
Yunmuxiang_Ulp3_TCM
Dolomiaea_edulis_AY914860
Sauaaurea_forrestii_DQ874338
Aucklandia_lappa_Ulp11_1_(S)
Dolomiaea_georgii_30_S
Dolomiaea_forrestii_43_S
Dolomiaea_edulis_38_S
Dolomiaea_georgii_29_S
Yunmuxiang_Ulp4_TCM
Dolomiaea_souliei_33_S
Dolomiaea_souliei_var._mirabil
Dolomiaea_platylepis_32_S
Dolomiaea_edulis_35_S
Saussurea_cordifolia_39_S
Inula_helenium_17_S
Inula_helenium_Ihe-1_S
Inula_racemosa_44_S
Inula_racemosa_Ira_S
Clustal Consensus

Figure 5.2 (continued)

Figure 5.2 (continued)

Sequence Name	Sequence
	I
	....I....
	1265
A.debilis_4_S_	AGTTCAAAT
A.debilis_Adeb3_S_	AGTTCAAAT
A.contorta_Acon1_S_	AGTTCAAAT
Qingmuxiang_TMX3_TCM_	AGTTCAAAT
A.trilobata_Atb1_S_	AGTTCAAAT
Qingmuxiang_M85_TCM_	AGTTCAAAT
A.elegans_Alt1_S_	AGTTCAAAT
A.manshuriensis_Ams1_S_	AGTTCAAAT
A.manshuriensis_AY689184	---------
A.macrophylla_Ama_S_	AGTTCAAAT
A.californica_Aca_S_	AGTTCAAAT
A.californica_AY689174	---------
A.kaempferi_DQ532023	---------
A.kaempferi_8_S_	AGTTCAAAT
A.mollissima_9_S_	AGTTCAAAT
A.heterophylla_7_S_	AGTTCAAAT
A.fangchi_Ms460_S_	AGTTCAAAT
A.cinnabarina_Ms517_S_	AGTTCAAAT
A.contorta_1_S_	AGTTCAAAT
A.debilis_Ms442_S_	AGTTCAAAT
Yunmuxiang_Ulp3_TCM_	AGTTCAAAT
Dolomiaea_edulis_AY914860	---------
Sauaaurea_forrestii_DQ874338	---------
Aucklandia_lappa_Ulp11_1_(S)_	AGTTCAAAT
Dolomiaea_georgii_30_S_	AGTTCAAAT
Dolomiaea_forrestii_43_S_	AGTTCAAAT
Dolomiaea_edulis_38_S_	AGTTCAAAT
Dolomiaea_georgii_29_S_	AGTTCAAAT
Yunmuxiang_Ulp4_TCM_	AGTTCAAAT
Dolomiaea_souliei_33_S_	AGTTCAAAT
Dolomiaea_souliei_var._mirabil	AGTTCAAAT
Dolomiaea_platylepis_32_S_	AGTTCAAAT
Dolomiaea_edulis_35_S_	AGTTCAAAT
Saussurea_cordifolia_39_S_	AGTTCAAAT
Inula_helenium_17_S_	AGTTCAAAT
Inula_helenium_Ihe-1_S_	AGTTCAAAT
Inula_racemosa_44_S_	AGTTCAAAT
Inula_racemosa_Ira_S_	AGTTCAAAT
Clustal Consensus	
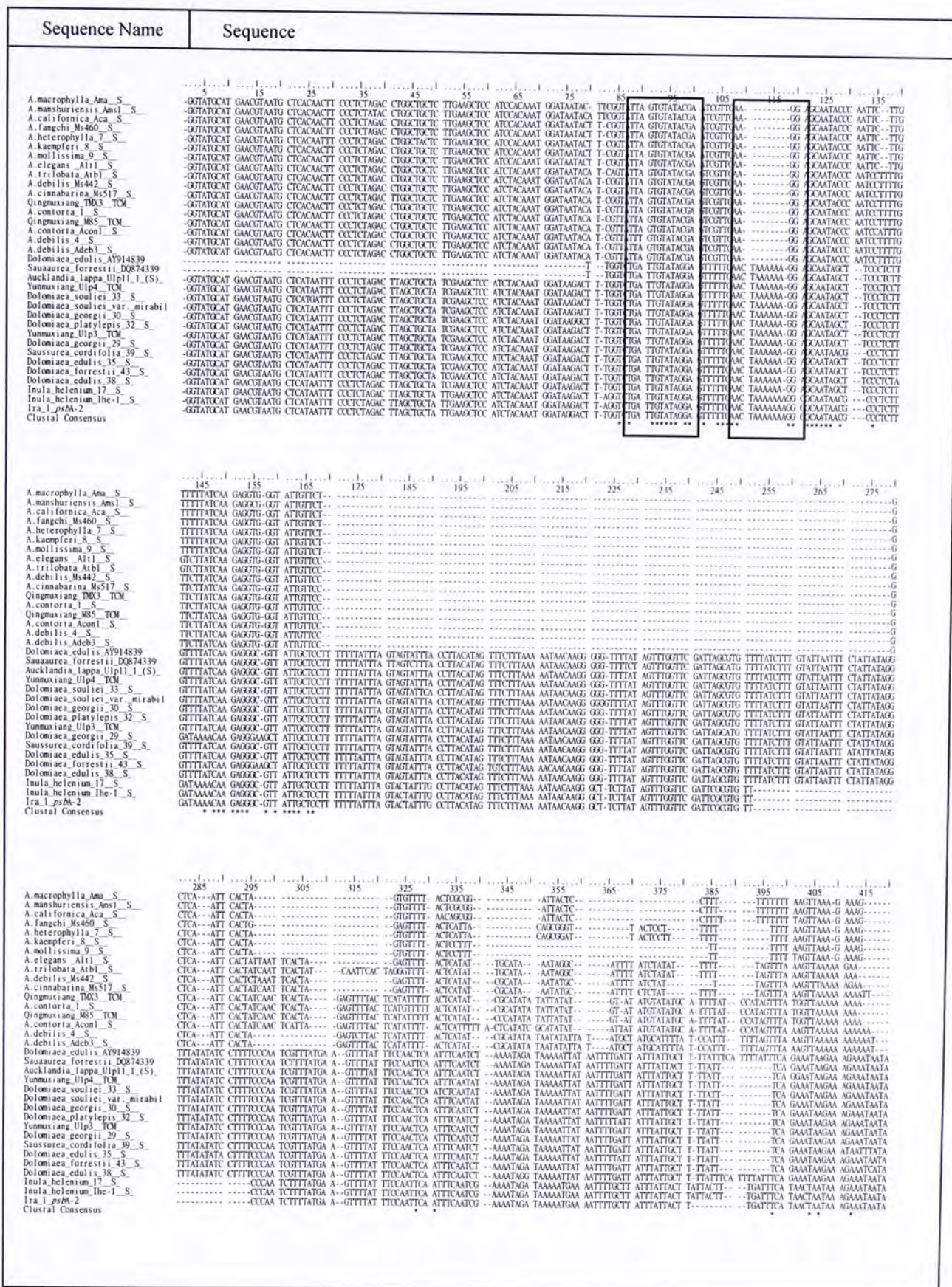
Figure 5.3 Sequence alignment of *psb*A-*trn*H region for herb materials of Yunmuxiang, Qingmuxiang and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Some of the nucleotide differences that are able to distinguish genuine Yunmuxiang from Qingmuxiang are highlighted in boxes.

Figure 5.3 (continued)

Figure 5.3 (continued)

Sequence Name	Sequence

5.1.2 Percentage similarity analysis

The percentage similarities of *trnL-trnF* region among all the herbal material of Yunmuxiang and Qingmuxiang and relevant authentic species were calculated using BioEdit Sequencing Alignment Editor (Hall, 1999). The results are summarized in Table 5.3. The interspecific similarities between the *Aristolochia* and *Aucklandia* samples varied from 58.6–65.8%, the average being 63.1%. The interspecific similarities between the *Aristolochia* and Asteraceae genus *Inula* varied from 58.7–68%, the average being 63.7%. The interspecific similarities between the *Aristolochia* and *Dolomiaea* varied from 60.2–82.3%, the average being 84.6%. The interspecific similarities between the *Aristolochia* and *Saussurea* varied from 59–67.7%, the average being 63.7%.

The percentage similarities of *psbA-trnH* region among all the herbal material of Yunmuxiang and Qingmuxiang and relevant authentic species were also calculated using BioEdit Sequencing Alignment Editor (Hall, 1999). The results are presented in Table 5.4. The interspecific similarities between *Aristolochia* and *Aucklandia* varied from 20.2–29.8%, the average being 25.1%. The interspecific similarities between the *Aristolochia* and *Inula* varied from 18.7–30%, the average being 23.8%. The interspecific similarities between the *Aristolochia* and *Dolomiaea* varied from 20.2–31.6%, the average being 25.3%, and the interspecific similarities between the *Aristolochia* and *Saussurea* varied from 20.2–30.9%, the average being 24.6%.

Table 5.3 Percentage similarities of *trnL-trnF* region among the plant and herb samples of Yunmuxiang and Qingmuxiang.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[31]	[32]	[33]	[34]	[35]	[36]	[37]	[38]
[1]	A.debilis_4_S_	99.2	91	90.3	85.8	88.7	87.7	86.6	86.5	85.3	85.3	84.9	87.7	87.9	88.1	87.9	87.9	87.9	88.8	86.1	77.5	60.3	61.5	62.2	62.9	62.8	62.8	62.7	62.7	61.5	62.7	63.6	63.9	64	62.1	62.5	64.8	61.6
[2]	A.debilis_Adeb3_S_		91.3	90.5	86.2	89.1	88.1	87.1	87.1	85.9	85.7	85.3	88.3	88.5	88.7	88.5	88.5	88.3	89.1	85.9	77.9	60.6	61.8	62.6	63.3	63.2	63.1	63.1	63.1	61.8	63.1	63.9	64.2	64.3	62.4	62.8	65.1	61.9
[3]	A.contorta_Acon1_S_			97	82.3	84.9	84.3	83.2	83.1	81.9	81.6	81.2	83.8	83.7	84	84	84	83.4	90.1	80.1	78.4	58.3	59.5	60.2	60.9	60.8	60.8	60.7	60.7	59.5	60.7	61.4	61.7	61.8	59.8	60.2	62.1	59.1
[4]	Qingmuxiang_TMX3_TCM_				0	84.2	83.7	81.7	81.7	80.4	80.5	80.1	82.6	82.7	83	82.9	82.8	82.7	90.1	79.3	78.5	57.9	59	59.8	60.4	60.3	60.3	60.2	60.2	59.1	60.2	61	61.3	61.4	59.4	59.8	61.7	58.7
[5]	A.trilobata_Atb1_S_					81.6	81.7	81.7	87.1	86.3	85.4	85	88.1	88	88.4	88.3	88.3	86.1	89.5	79	74.5	59.3	60.4	61.1	61.9	61.8	61.8	61.7	61.7	60.6	61.7	62.7	61.3	61.4	61	61.4	63.6	60.6
[6]	Qingmuxiang_M85_TCM_						95.5	95.4	89.7	88.8	87.9	87.5	90.7	90.6	91	90.9	90.9	88.7	92.9	81.7	76.7	61	62.2	62.8	63.7	63.5	63.5	63.4	63.4	62.3	63.4	64.5	64.7	64.7	62.8	63.2	65.6	62.5
[7]	A.elegans_Alt1_S_							96	87.8	89.6	85.8	85.4	88.5	88.4	88.8	88.7	88.7	86.5	92.2	80.9	75.9	60.5	61.6	62.3	63.1	63	63	62.9	62.9	61.8	62.9	63.9	64.1	64.1	62.3	62.7	65.3	62.1
[8]	A.manshuriensis_Ams1_S_								99.6	95.7	92.4	91.9	95.4	95.4	95.9	95.7	95.7	92.4	86.6	82	77.1	61.6	62.8	63.5	64.3	64.2	64.2	64.1	64.1	63	64.1	65.2	65.4	65.4	63.5	63.9	66.3	63.1
[9]	A.manshuriensis_AY689184									95.6	92.3	91.9	95.3	95.3	95.8	95.6	95.6	92.4	86.5	81.9	77	61.6	62.8	63.5	64.3	64.2	64.2	64.1	64.1	63	64.1	65.2	65.4	65.4	63.5	63.9	66.3	63.1
[10]	A.macrophylla_Ama_S_										90.9	90.5	93.7	93.9	94.1	94.1	94.1	90.9	85.1	80.6	75.7	60.3	61.4	62.1	62.9	62.8	62.8	62.7	62.7	61.6	62.7	63.7	63.9	63.9	62.1	62.5	64.9	61.7
[11]	A.californica_Aca_S_											99.5	94.4	94.2	94.6	94.8	94.8	91.2	84.9	80.8	76	60.6	61.8	62.8	63.6	63.5	63.5	63.4	63.4	62.3	63.4	64.5	64.7	64.7	62.8	63.2	65.5	62.3
[12]	A.californica_AY689174												93.9	93.7	94.2	94.4	94.4	90.7	84.5	80.4	75.6	60.9	62.1	62.5	63.3	63.2	63.2	63.1	63.1	62	63.1	64.1	64.3	64.3	62.6	63	65.3	62.1
[13]	A.kaempferi_DQ532023													98.3	98.8	99	98.6	94.9	87.5	83.5	79.1	63	64.3	64.9	65.7	65.6	65.6	65.5	65.5	64.3	65.5	66.7	66.6	66.9	65	65.3	67.7	64.4
[14]	A.kaempferi_8_S_														99	98.3	99	95.4	87.4	83.7	79.1	62.7	64	64.7	65.5	65.4	65.4	65.3	65.3	64.2	65.3	66.4	66.7	66.7	64.7	65.1	67.6	64.3
[15]	A.mollissima_9_S_															98.8	99	95.6	87.8	83.8	79.4	63.1	64.4	65.1	65.9	65.8	65.8	65.7	65.7	64.5	65.7	66.8	67.1	67.1	65.1	65.5	68	64.7
[16]	A.heterophylla_7_S_																99	95.2	87.8	83.8	79.4	63	64.3	64.9	65.7	65.6	65.6	65.5	65.5	64.3	65.5	66.7	66.9	66.9	64.9	65.3	67.7	64.4
[17]	A.fangchi_Ms460_S_																	96	87.7	83.7	79.2	62.9	64.2	64.8	65.7	65.6	65.6	65.5	65.5	64.3	65.5	66.8	66.8	66.8	65.1	65.3	67.7	64.3
[18]	A.cinnabarina_Ms17_S_																		87.3	83.2	79.1	62.4	63.6	64.3	65.2	65.1	65.1	65.1	65	63.8	65	66.6	66.2	66.3	64.7	65	67.6	64.2
[19]	A.contorta_1_S_																			81.7	82.3	61	62.2	62.9	63.7	63.6	63.6	63.5	63.5	62.3	63.5	64.4	64.7	64.7	62.6	63	64.7	61.5
[20]	A.debilis_Ms442_S_																				81.8	63.8	65.1	65.8	66.5	66.4	66.4	66.3	66.3	65.1	66.3	65.7	67.4	67.7	65.7	65.8	68.4	65.2
[21]	Yunmuxiang_Ulp3_TCM_																					73.8	74.7	77.6	76.9	76.8	76.8	76.7	76.7	75.3	76.7	77.8	77.8	78.1	75.9	74.7	77.3	74.2
[22]	Dolomiaea_edulis_AY914860																						96.7	95.3	96.7	96.6	96.6	96.4	96.4	93.9	96.1	93.7	93.7	93.6	89.6	88.2	76.6	73.5
[23]	Saussaurea_forrestii_DQ874338																							92.2	96.7	96.8	96.8	96.7	96.7	95.3	96.1	95.3	95.6	95.5	91.4	90.4	78.1	75
[24]	Aucklandia_lappa_Ulp11_S_																								98.6	98.4	98.4	98.2	98.2	96.3	98	95.6	95.6	95.5	91.4	90.4	78.1	75
[25]	Dolomiaea_georgii_30_S_																									98.4	98.4	98.2	98.2	96.3	98	95.6	95.6	95.7	91.5	90.2	78.7	75.6
[26]	Dolomiaea_forrestii_43_S_																										99.8	99.6	99.6	97.2	99.4	96.9	96.9	96.8	92.8	91.4	79.7	76.6
[27]	Dolomiaea_edulis_38_S_																											99.7	99.5	97.1	99.3	96.9	96.9	96.7	92.7	91.2	79.7	76.5
[28]	Dolomiaea_georgii_29_S_																												99.5	97.1	99.3	96.8	96.8	96.7	92.7	91.2	79.6	76.5
[29]	Yunmuxiang_Ulp4_TCM_																													96.9	99.3	96.6	96.6	96.5	92.5	91	79.6	76.5
[30]	Dolomiaea_souliei_33_S_																														97.3	94.2	94.2	94.1	90.1	88.6	78.1	74.9
[31]	Dolomiaea_souliei_var_mirabil																															97.1	96.5	96.4	92.5	91	79.5	76.4
[32]	Dolomiaea_platylepis_32_S_																																96.5	96.4	92.5	91	79.5	76.4
[33]	Dolomiaea_edulis_35_S_																																	99.2	94.6	93	80.9	77.7
[34]	Saussurea_cordifolia_39_S_																																		94.8	93.3	80.9	77.7
[35]	Inula_helenium_17_S_																																			93.5	81.2	78
[36]	Inula_helenium_Ihe-1_S_																																				97.4	77.8
[37]	Inula_racemosa_44_S_																																					95.7
[38]	Inula_racemosa_Ira_S_																																					

Table 5.4 Percentage similarities of *psbA-trnH* region among the plant and herb samples of Yunmuxiang and Qingmuxiang.

#	Species	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[31]	[32]	[33]	[34]
[1]	A.macrophylla_Ama_S_	98.6	95.2	83	83.7	87.5	86.8	64.4	61.8	63.3	62.6	59	59.5	59	56	55	55	20.4	20.2	20.2	20.8	20.5	20.7	20.5	20.7	20.7	19.4	21.1	20.7	20.7	21.1	18.8	18.8	18.7
[2]	A.manshuriensis_Ams1_S_		94.5	82.3	83.1	86.8	86.2	64.4	61.8	63.3	62.6	59	59.5	59	56	55	55	20.4	20.2	20.2	20.8	20.5	20.7	20.5	20.7	20.7	19.4	21.1	20.7	20.7	21.1	18.8	18.8	18.7
[3]	A.californica_Aca_S_			81	81.8	85.5	86.2	62.2	59.7	61.2	60.6	58.3	58.8	58.3	54.4	53.8	53.8	20.7	20.5	20.5	21.1	21.3	21	20.8	21	21	19.7	21.5	21	21	21.5	19.4	19.4	19
[4]	A.fangchi_Ms460_S_				97.3	85.9	85.2	66.6	63.4	65	63.7	59.8	60.2	59.8	57.7	55.7	55.7	21.2	21.7	21.7	22.3	21.9	22.1	21.9	22.2	22.2	20.9	22.7	22.2	22.1	22	19.6	19.6	19.3
[5]	A.heterophylla_7_S_					86.6	86	66.1	63.4	63.5	62.8	61.2	61.7	61.2	59.1	57.5	57.5	21.5	21.9	21.9	22.5	22.2	22.4	22.2	22.5	22.5	21.1	22.9	22.5	22.4	22.2	19.9	19.9	19.6
[6]	A.kaempferi_8_S_						99.2	62.6	60.1	62.7	60.9	57.3	57.8	57.3	55.8	53.8	53.8	19.7	20.2	20.2	20.7	20.4	20.6	20.4	20.7	20.7	19.3	21.1	20.7	20.6	20.5	18.3	18.3	18.6
[7]	A.mollissima_9_S_							62	59.5	62.2	60.4	57.8	58.3	57.8	55.3	53.4	53.4	19.7	20.2	20.2	20.7	20.4	20.6	20.4	20.7	20.7	19.3	21.1	20.7	20.6	20.5	18.6	18.6	18.9
[8]	A.elegans_Alt1_S_								92	90	87.8	72.3	73.2	71.4	71.4	62.6	62.6	25.7	25.6	25.6	26.1	25.8	26	25.8	26.3	26.1	24.8	26.6	26.3	26	26.5	24.4	24.4	24.1
[9]	A.trilobata_Atb1_S_									85.3	84	75.8	76.6	74.8	74.8	64.9	65.3	27.4	27.3	27.3	27.8	27.5	27.7	27.5	28.1	27.8	26.5	28.4	28.1	27.7	28.1	26.2	26.2	26
[10]	A.debilis_Ms442_S_										89.8	70.7	70.7	69.8	69.9	63.9	63.9	26	25.8	25.8	26.1	26.1	26.3	26.1	26.6	26.3	25	26.6	26.6	26.3	26.7	24.7	24.7	25.2
[11]	A.cinnabarina_Ms517_S_											70.2	70.2	69.3	70	65.3	65.3	27.5	27.4	27.4	27.6	27.6	27.8	27.6	28.1	27.9	26.5	28.7	28.1	27.8	28.2	26.3	26.3	26
[12]	Qingmuxiang_TMX3_TCM_												99	99	89.7	75.3	75.7	29.1	28.2	29	29	28.7	28.9	28.7	29.2	29	27.6	29.5	29.2	28.9	29.6	27.8	27.8	27.2
[13]	A.contorta_1_S_													99	88.7	74.4	74.8	28.6	27.7	28.5	28.5	28.2	28.4	28.2	28.7	28.5	27.1	29	28.7	28.4	29.1	27.3	27.3	26.7
[14]	Qingmuxiang_M85_TCM_														89.2	74.4	74.8	29.1	28.2	29	29	28.7	28.9	28.7	29.2	29	27.6	29.5	29.2	28.9	29.6	27.8	27.8	27.2
[15]	A.contorta_Acon1_S_															75.1	75.5	29.7	28.9	29.1	29.6	29.4	29.6	29.4	29.9	29	28.3	30	29.9	29.6	30.5	28	28	27.4
[16]	A.debilis_4_S_																99.5	30.8	29.3	29.5	29.8	29.8	29.9	29.8	30.2	30	28.7	30.6	30.2	29.9	31.3	29.7	29.7	29.1
[17]	A.debilis_Adeb3_S_																	31.1	29.5	29.8	30	30	30.2	30	30.5	30.2	28.9	30.9	30.5	30.2	31.6	30	29.7	29.1
[18]	Dolomiaea_edulis_AY914839																		95.1	95.9	95.9	95.7	95.9	96.4	96.2	96.2	93.9	93.4	95.7	94.7	98.7	75.5	75.5	75
[19]	Sauaaurea_forrestii_DQ874339																			96.1	96.1	95.8	96.1	96.6	96.3	96.9	94	93.5	95.8	94.8	93.9	74.3	74.3	73.7
[20]	Aucklandia_lappa_Ulp11_S_																				98.9	98.7	98.9	99.2	99.2	99.2	96.9	93.5	98.7	97.6	96.7	75.5	75.5	74.9
[21]	Yunmuxiang_Ulp4_TCM_																					99.2	99.4	99.2	99.2	98.9	96.9	96.3	98.7	97.6	96.7	75.5	75.5	74.9
[22]	Dolomiaea_souliei_33_S_																						99.2	98.9	98.9	99.2	96.6	96.1	98.4	97.4	96.4	75.3	75.3	74.6
[23]	Dolomiaea_souliei_var_mirabil																							99.2	99.2	98.9	96.9	96.4	98.7	97.6	96.7	75.6	75.6	75
[24]	Dolomiaea_georgii_30_S_																								99.2	98.9	96.9	96.6	98.9	97.9	96.9	75.7	75.7	75.1
[25]	Dolomiaea_platylepis_32_S_																									99.4	96.3	96.6	98.9	97.9	96.9	75.7	75.7	75.1
[26]	Yunmuxiang_Ulp3_TCM_																										99.4	96.6	98.9	97.9	96.9	75.7	75.7	75.1
[27]	Dolomiaea_georgii_29_S_																											97.1	99.4	97.1	94.7	77	77	76.4
[28]	Saussurea_cordifolia_39_S_																												94.3	95.3	94.2	77	77	76.4
[29]	Dolomiaea_edulis_35_S_																													96.3	96.4	74	74	73.3
[30]	Dolomiaea_forrestii_43_S_																														95.4	74.3	74.3	73.7
[31]	Dolomiaea_edulis_38_S_																															76.2	76.2	75.2
[32]	Inula_helenium_17_S_																																100	97.5
[33]	Inula_helenium_lhe-1_S_																																	97.5
[34]	Ira_1_psbA-2																																	

5.1.3 Dendrogram study

Dendrograms were constructed using the computer program MEGA version 3.1 (Kumar *et al.*, 2001) with two tree construction methods: unweighted pair-group methods using arithmetic averages (UPGMA; Figure 5.4 and 5.6) and maximum parsimony (Figure5.5 and 5.7). Each method was tested by bootstrap test with 1000 replications.

From the *trnL-trnF* region, the samples form two separate clades: *Aristolochia* clade and a clade including the from Asteraceae genera *Aucklandia, Dolomiaea, Inula* and *Saussurea*. In the clade consisting of *Aucklandia, Dolomiaea, Inula* and *Saussurea,* the herb samples Yunmuxiang (Ulp3 and Ulp4) clustered with the authentic samples of those for Asteraceae genera while the herb Yunmuxiang (Ulp4) clustered with *Dolomiaea souliei* var. *mirabilis*. This clade was supported by bootstrap frequencies of 66 in both bootstrap consensus trees of UPGMA and maximum parsimony. In the *Aristolochia* clade, the herb samples Qingmuxiang (TMX3 and M85) clustered with the authentic samples of *Aristolochia* while Qingmuxiang (M85) clustered with *A. contorta*. This clade was supported by bootstrap frequencies of 66 in both bootstrap consensus trees of UPGMA and maximum parsimony (Figure 5.4 and 5.5).

Based on the aligned sequences of the *psb*A-*trn*H region, the samples were distributed in two separate clades (Figure 5.6 and 5.7): *Aristolochia* clade and a clade including the Asteraceae genera *Aucklandia, Dolomiaea, Inula* and *Saussurea*. In the clade containing of *Aucklandia, Dolomiaea, Inula* and *Saussurea,* the herb samples Yunmuxiang (Ulp3 and Ulp4) clustered with the authentic samples of Asteraceae genera. This clade was supported by bootstrap frequencies of 100 in both bootstrap consensus trees of UPGMA and maximum parsimony. In the clade of *Aristolochia,* the herb samples Qingmuxiang (TMX3 and M85) clustered with the authentic samples of *Aristolochia contorta* (1 and Acon1). This *Aristolochia* clade was supported by bootstrap frequencies of 100 in both bootstrap consensus trees of UPGMA and maximum parsimony.
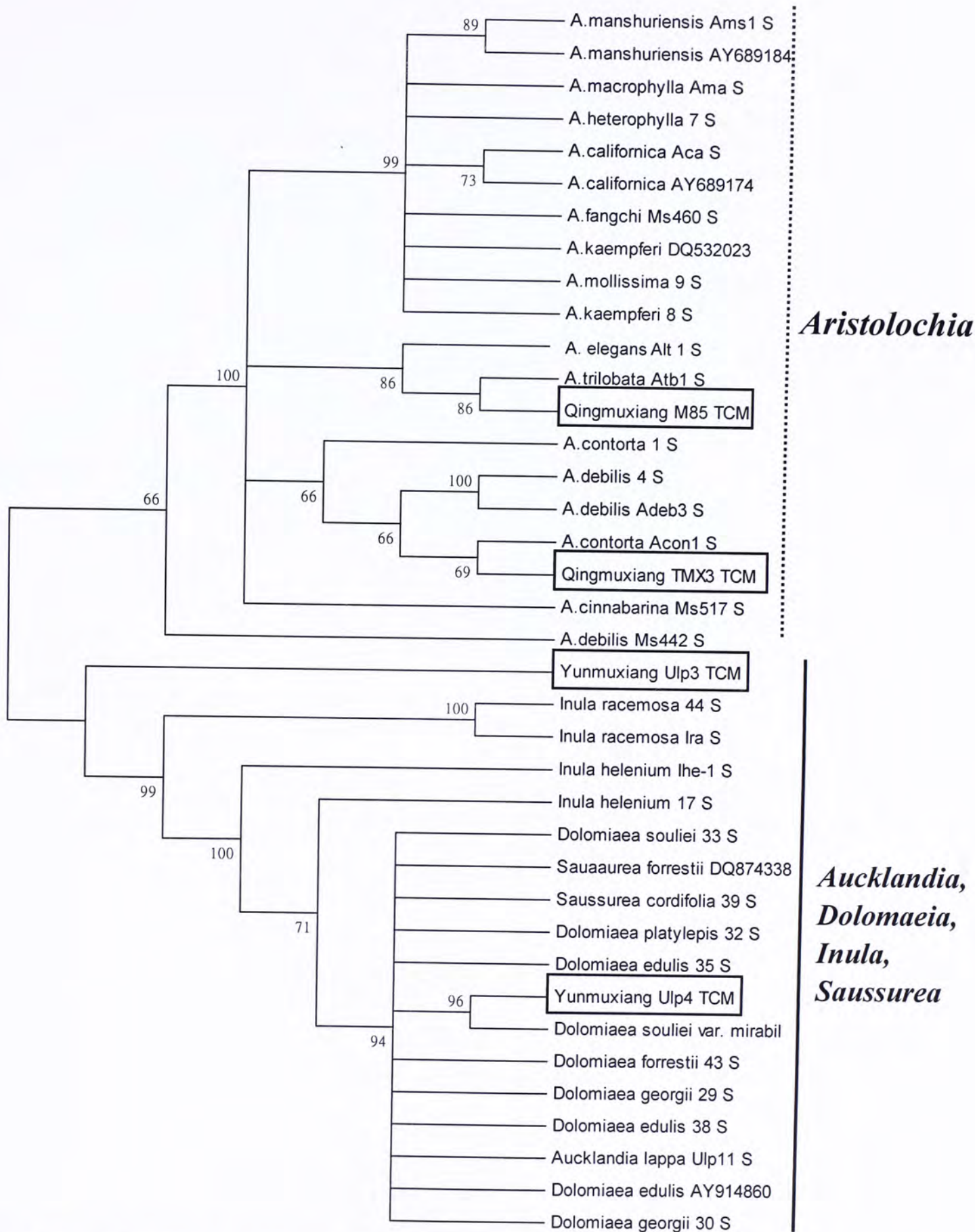
Figure 5.4 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Yunmuxiang and Qingmuxiang. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
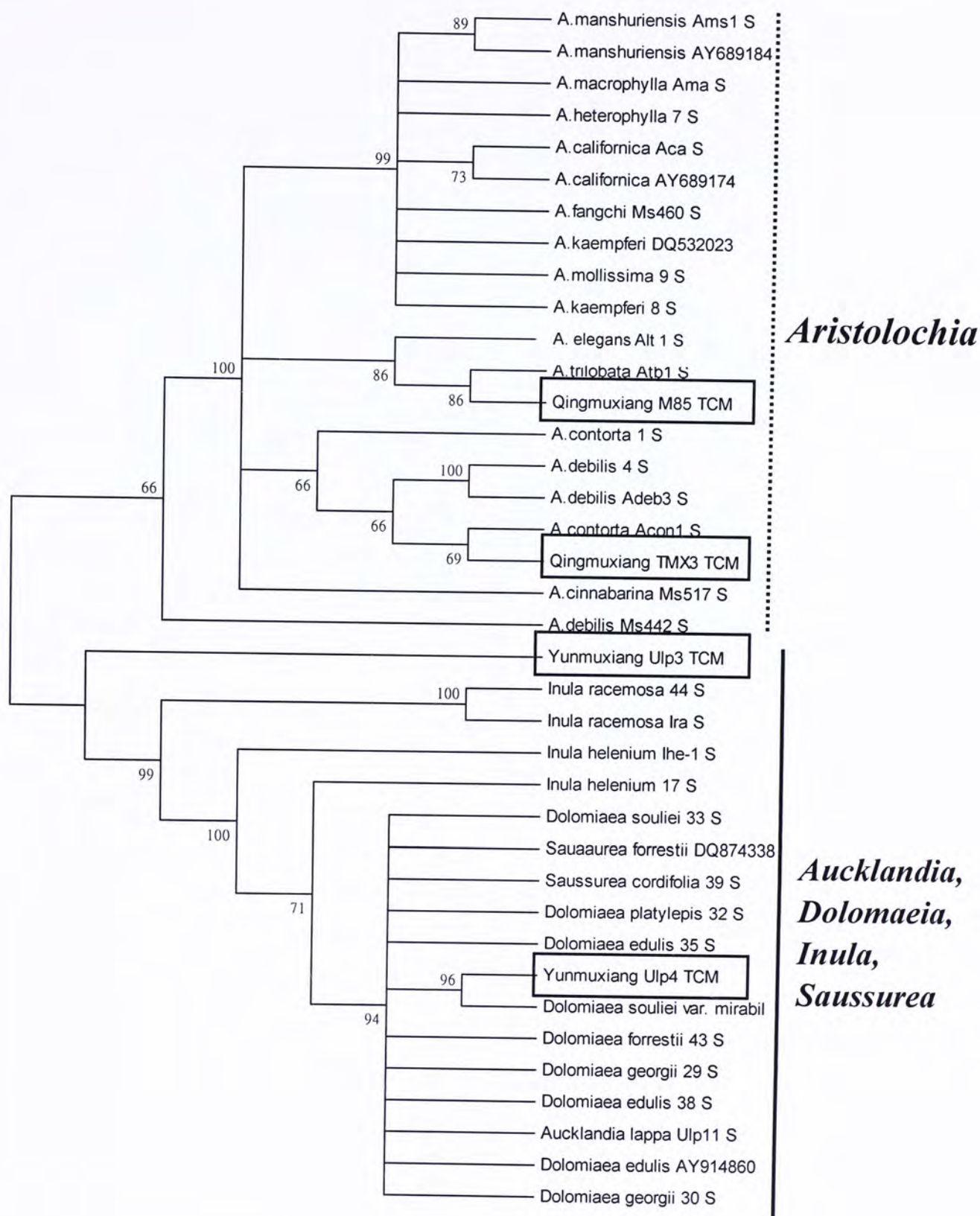
Figure 5.5 Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Yunmuxiang and Qingmuxiang. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
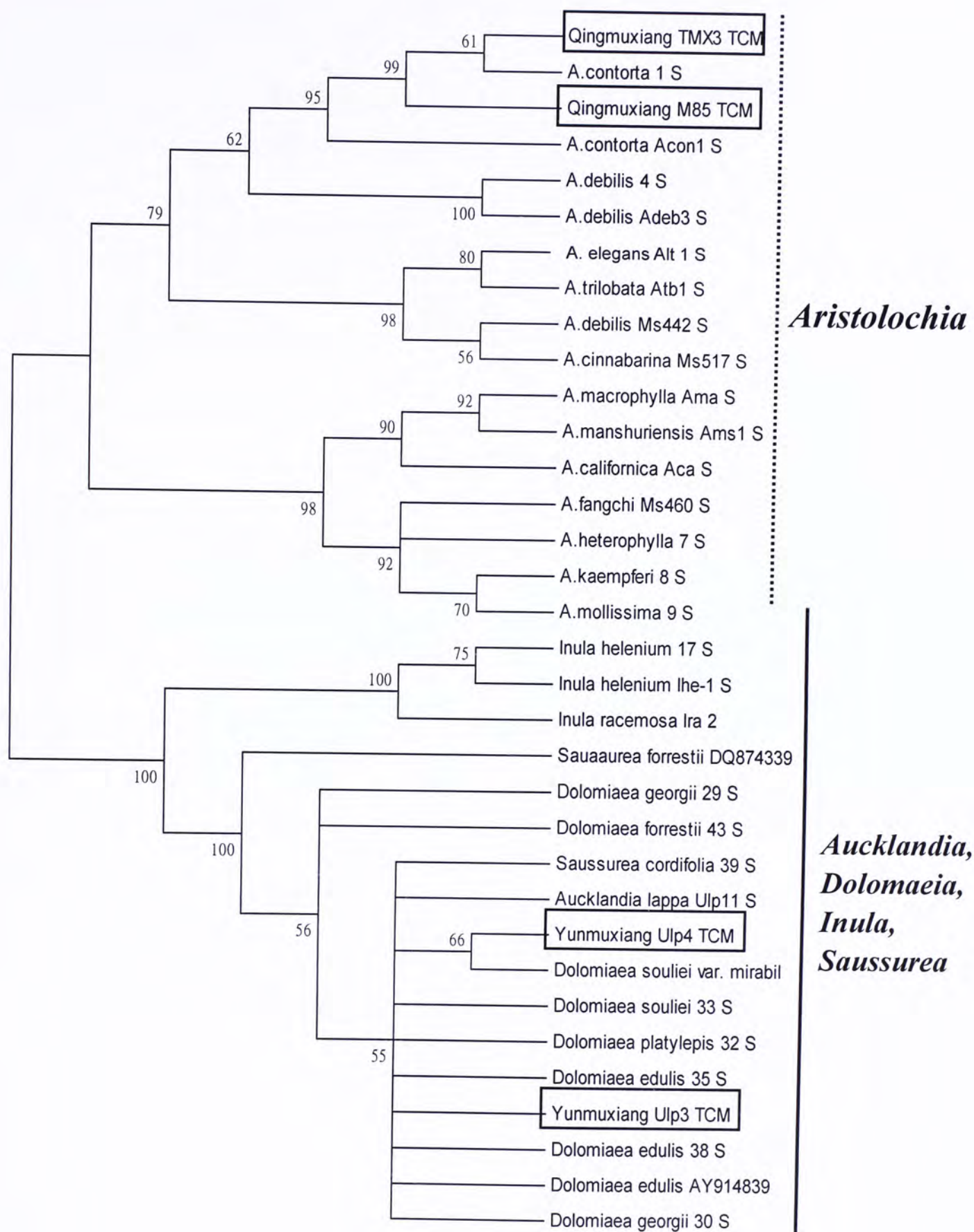
Figure 5.6 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Yunmuxiang and Qingmuxiang. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
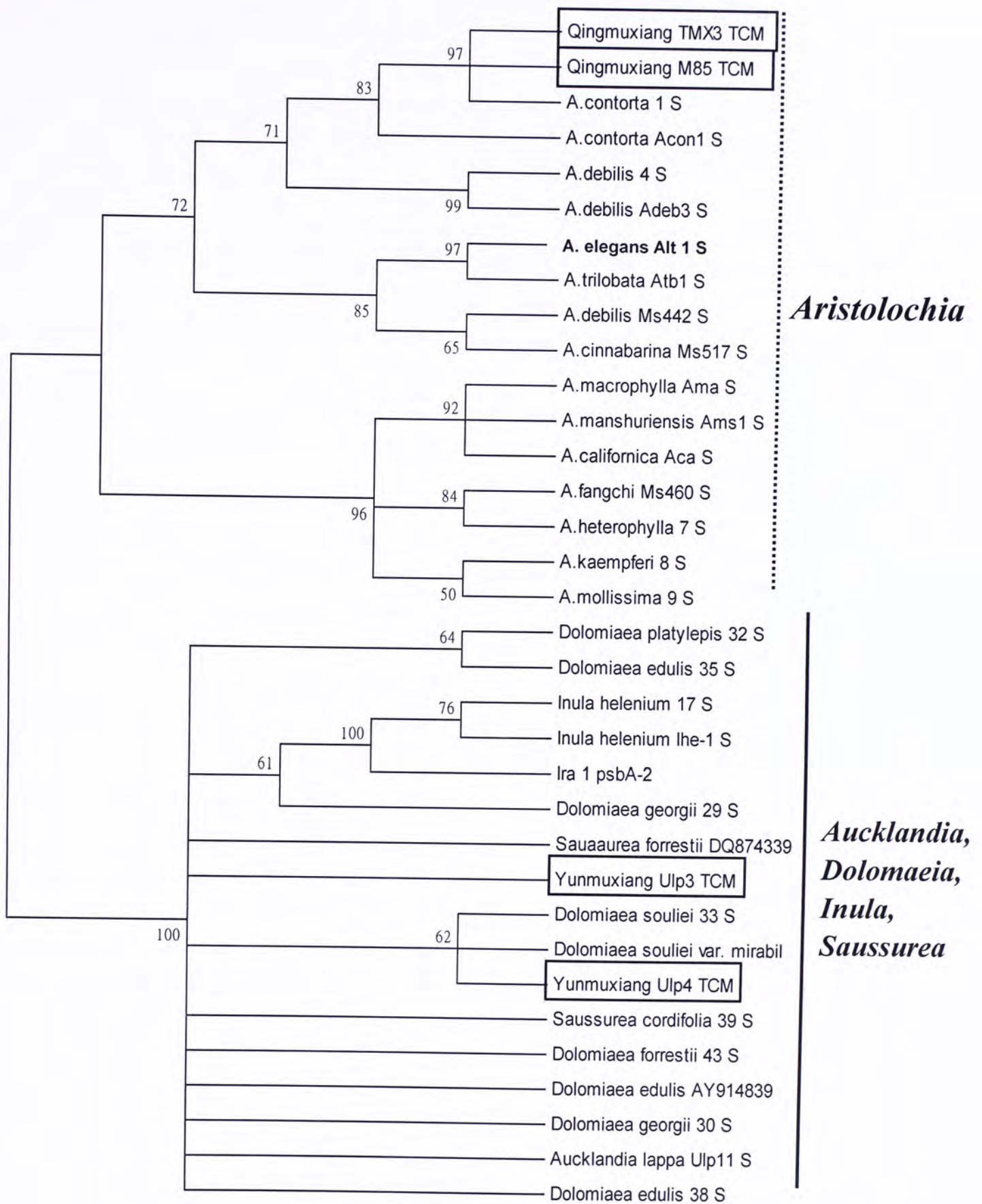
Figure 5.7 Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Yunmuxiang and Qingmuxiang. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.

5.2 Discussion

5.2.1 Evaluation of chloroplast *trn*L-*trn*F region in differentiation of Muxiang

In this study, *trn*L-*trn*F region was applied to differentiate genuine Muxiang from the adulterant Qingmuxiang. The source species of the genuine Muxiang are *Aucklandia lappa, Dolomiaea souliei, Inula helenium, Inula racemosa* and the adulterant is *Aristolochia debilis*, respectively.

The intraspecific similarity among species in *Aristolochia* is 88.4%, varying from 79–99.6%, showing that this region is conserved within the *Aristolochia* genus. (Table 5.3). The average interspecific similarities between *Aristolochia* and each of the four Asteraceae genera *Aucklandia*, and between *Aristolochia* and *Inula*, and between *Aristolochia* and *Dolomiaea* and *Saussurea* were 63.1%, 63.7%, 64.6% and 62.8% respectively, which were lower that the intraspecific similarity in *Aristolochia*. This suggests that *trn*L-*trn*F can differentiate the genuine Muxiang from the adulterant Qingmuxiang.

The dendrograms constructed by either UPGMA or maximum parsimony using the *trn*L-*trn*F region could clearly separate the five genera into two clades with bootstrap frequency of 66. The bootstrap value was quite low. The reason is the unsolved identity of one herb Yunmuxiang sample (Ulp3). The result of interspecific

similarities between the herb Yunmuxiang sample (Ulp3) and various authentic source species showed contradictions in the trn*L*-trn*F* and psb*A*-trn*H* regions (Table 5.5). The result of trn*L*-trn*F* did not match with psb*A*-trn*H* in Yunmuxiang sample (Ulp3). The result of trn*L*-trn*F* of Ulp3 show that it was 79% resemble *Aristolochia debilis* but not in psb*A*-trn*H* region. Furthermore, although trn*L*-trn*F* result indicates Ulp3 is more similar to *Aristolochia debilis* instead of an Asteraceae genera, all species showed 75–79% in the interspecific similarity with difference less than 4%. The trn*L*-trn*F* of Ulp3 sequence was blasted to NCBI Genbank and the search stated that is a *Saussurea* species with 0 E-value and 88% similarity. The identity of this sample remains questionable. Further analysis is needed to see if it was due to contamination of DNA with Yunmuxiang sample (Ulp3).

The relationship within each clade was not well resolved. Although clades of replicate samples such as *Inula racemosa* (44 and Ira) clustered together, the other species with only one sample form an un-resolved clade. Obviously the intraspecific similarities between samples of these three genera are high.

The *trnL-trnF* region is sufficiently variable among the five genera. Although it is quite conserved intraspecifically, differentiation of species is achieved by the dendrograms constructed. The differences in the nucleotide changes of insertions/deletions and base substitutions between samples can be taken as markers

for the differentiation of genuine Muxiang from Qingmuxiang. In conclusion, *trn*L-*trn*F region is able to differentiate the plants from different genera and thus can be utilized for differentiating genuine and adulterant Muxiang samples.

Table 5.5 Average percentage similarities of the chloroplast *trn*L-*trn*F region and *psb*A-*trn*H region among the authentic species of *Aucklandia, Dolomiaea, Inula* and *Aristolochia debilis* and herb samples of Yunmuxiang (Ulp3 and Ulp4).

Authentic species	*trn*L-*trn*F		*psb*A-*trn*H	
	Ulp3	Ulp4	Ulp3	Ulp4
Aucklandia lappa	77.6%	98.2%	99.2%	98.9%
Dolomiaea souliei	75.3%	97.3%	98.9%	99.2%
Inula helenium	75.8%	91.8%	75.7%	75.5%
Inula racemosa	75.7%	77.9%	75.1%	74.9%
Aristolochia debilis	79%	64%	28.8%	28.6%

5.2.2 Evaluation of chloroplast *psbA-trn*H region in differentiation of Muxiang

The chloroplast *psb*A-*trn*H was also used to differentiate the genuine and adulterant Muxiang. The average intraspecific similarity within *Aristolochia* is 69.5%, but the intraspecific similarities in the genus ranging from 53.8–99.5%. This showed

that the *psb*A-*trn*H region is variable within *Aristolochia*. The average interspecific similarities of *Aristolochia* to *Aucklandia*, to *Inula*, *to Dolomiaea*, and *Saussurea* were 25.1%, 23.8%, 25.3 % and 24.6%, respectively. They were much lower than the intraspecific similarity of *Aristolochia* suggesting that this region is sufficient to differentiate herb samples originating from *Aristolochia* from the Asteraceae genera.

The dendrograms constructed by either UPGMA or maximum parsimony using this region can clearly separate the five genera into two big clades with bootstrap frequency of 100. The relationship of *Aristolochia* clade is better resolved than the Asteraceae genera clade. The bootstrap value of the braches are generally over 50% in the *Aristolochia* clade. Most branches within the *Aristolochia* clade were higher than the cutoff value of 50% on bootstrap value. In the Asteraceae clade, the samples of *Aucklandia*, *Saussurea* and *Dolomiaea* all merged to form a clade, but their relationship is not well resolved.

5.3 Conclusion

The method adapted is successful, both *trn*L-*trn*F region and *psb*A-*trn*H region are suitable for differentiating the genuine from the adulterant species involved in Muxiang. One of the source species, *Vladimiria souliei* var. *cinerea*, was not available to this study as sample was collected but in poor condition and no DNA could be

amplified. More replicate samples and herb samples should be included further analysis.

In general, the interspecific similarities of the *psb*A-*trn*H region were much lower than those of the *trn*L-*trn*F region, suggesting that this region is less conserved than *trn*L-*trn*F.. Although *psb*A-*trn*H region is less conserved, fewer significant sites were identified for herbs differentiation than the *trn*L-*trn*F region. Therefore, it is concluded that *trn*L-*trn*F provide more useful information for herb differentiation.

Chapter 6:   AUTHENICATION OF BAIYING

6.1 Results

DNA was successfully extracted from (a) three samples of the herb Baiying, (b) four samples of Xungufeng, (c) four authentic samples of *Solanum*, and (d) 16 samples of *Aristolochia* species. The chloroplast *psb*A-*trn*H region of these 27 samples were successfully sequenced. Three additional *Solanum* sequences were downloaded from NCBI and used in sequence alignment and constructing dendrograms.
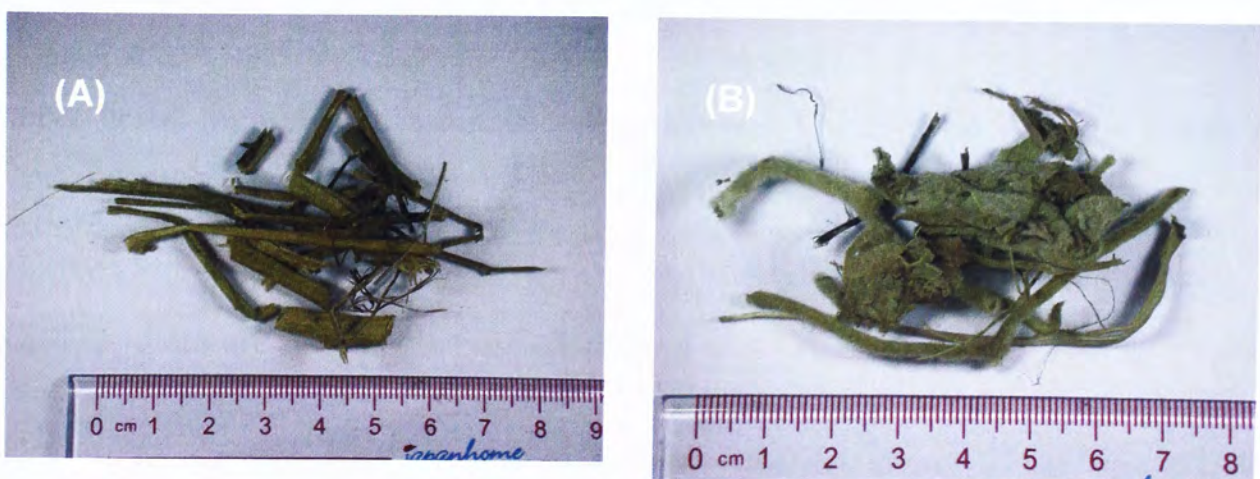
Figure 6.1   Morphological views of (A) Baiying (SL4), (B) Xungufeng (AM10).

The *psbA-trn*H region of the samples from these two genera show remarkable differences in length: 260–310 bp in *Aristolochia* and 530–570 bp in *Solanum*..

6.1.1 Sequence alignment

The 30 sequences of *psbA-trn*H region were aligned using the computer program ClustalX version 1.83 (Thompson *et al*., 1997). Additional manual amendments were performed using BioEdit Sequence Alignment Editor (Hall, 1999). The aligned sequences are presented in Figure 6.1. Based on the sequence alignment of this gene region of genera *Solanum* and *Aristolochia*, two sites of insertions/deletions or base changes could be utilized to differentiate the herbs derived from these two genera. These sites are highlighted with a box in Figure 6.2. The first site at bp 102–120, there was a 6 bp deletion in *Aristolochia* that could be used to differentiate the two different genera herbs. In addition, a 3-base difference was found at bp 102–104 where all *Aristolochia* species share TCG while all *Solanum* species share GTT. At second site 128–133, there are two single base changes at site 129 and site 132. At site 129, all *Aristolochia* species share A while *Solanum* species share T. At bp 132, all *Aristolochia* species share a C while *Solanum* species share a T. The result of sequence alignment showed that one sample of Baiying (SL4) and two samples of Xungufeng brought from Hong Kong (AM1 and AM2) were aligned with *Solanum*

species. On the other hand, another two Baiying samples brought from Hong Kong (SL2 and SL3) and two Xungufeng brought from mainland China (AM7 and AM10) aligned with the *Aristolochia* species.

Table 6.1 Sites of insertions/deletions or base changes for differentiating the herbs derived from *Aristolochia* and *Solanum* from sequence alignment of *psb*A-*trn*H region in Figure 6.2. The gaps or base pairs labeled in red color indicated the insertions/deletions and base pairs labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes	
1	102–120	*Aristolochia*	TCGTTGAA------GGAGC
		Solanum	GTTTTGAAAAGAAAGGAGC
2	128–133	*Aristolochia*	AATCCT
		Solanum	ATTTTC

Figure 6.2 Sequence alignment of the *psb*A-*trn*H region for herb sample materials of Baiying, Xungufeng and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Sites of insertions/deletions or base changes that could be utilized to differentiate the herbs derived from these two genera are highlighted in boxes.

Figure 6.2 (continued)

Sequence Name	Sequence

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                                   ...|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                                      425        435        445        455        465        475        485        495        505        515        525        535        545        555
A.debilis_4__S_                    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.debilis_Adeb3__S_                .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.trilobata_Atbl__S_               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ........GAAA GTCCAGGG-- CGGATGTAGC
A.elegans__Alt1__S_                .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ........GGAA GTCCAGGG-- CGGATGTAGC
A.cinnabarina_Ms517__S_            .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... GGCCAGGGG- CGGATGTAGC
A.contorta_1__S_                   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... GGCCAGGGG- CGGATGTAGC
A.macrophylla_Ama__S_              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.manshuriensis_Ams1__S_           .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
Xungufeng_Am10__TCM                .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
Xungufeng_AM7__TCM                 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.kaempferi_8__S_                  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.heterophylla_7__S_               .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.fangchi_Ms460__S_                .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.californica_Aca__S_              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.mollissima_9__S_                 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
Baiying_SL3__TCM                   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.mollissima_Ms536__S_             .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
A.mollissima_AMo2__S_              .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
Baiying_SL2__TCM                   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........G GGCCAGGGG- CGGATGTAGC
Solanum_ptychanthum_AY727181       .......... TTGAAATAAT AATCGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTGAATCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG AATGTAAGTA GGCGAGGGG- ..........
Solanum_americanum_AY727179        .......... TTGAAATAAT AATCGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTGAATCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG AATGTAAGTA GGCGAGGGG- ..........
Solanum_physalifolium_AY727180     ATTCTTATCT TTGAAATAAT AATCGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTGAATCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG AATGTAAGTA GGCGAGGGG- ..........
Solanum_japonense_Sjapl__S_        .........T TTGAAATAAT AATCGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTGAATCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG AATGTAAGTA GGCGAGGGG- ..........
Sol2_2_psbA                        .......... TTGAAATAAT AATCGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTTACTCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG ACTGTAAGTA GGCGAGGGGG CGGATGTAGC
Solanum_japonense_Sjasl__S_        .......... TTGAAATAAT AATCGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTTACTCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG ACTGTAAGTA GGCGAGGGGG CGGATGTAGC
Xungufeng_AM1__TCM                 GAAATAATAA ATATTCTTAT CTTTGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTTACTCTTT TGTTTTCGAA TTTCACTAAT GTCAAAATGG ACTGTAAGTA GTCGAGGGGG CGGATGTAGC
Solanum_lyratum_17L1__S_           GAAATAATAA ATATTCTTAT CTTTGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTTACTCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG ACTGTAAGTA GTCGAGGGGG CGGATGTAGC
Xungufeng_AM2__TCM                 GAAATAATAA ATATTCTTAT CTTTGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTTACTCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG ACTGTAAGTA GTCGAGGGGG CGGATGTAGC
Baiying_SL4__TCM                   GAAATAATAA ATATTCTTAT CTTTGAAATA ATAATATCAT TGAAATAAGA AAGAAGAGAA ATATTCGAAC TTTACTCTTT TGTTTTCGAA TTTAAATAAT GTAAAAATGG ACTGTAAGTA GTCGAGGGGG CGGATGTAGC
Clustal Consensus                                                                                                                                           *  ****
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                                   ...|....| ....|....| ....|....| ....|....| ..
                                      565        575        585        595
A.debilis_4__S_                    CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.debilis_Adeb3__S_                CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.trilobata_Atbl__S_               CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.elegans__Alt1__S_                CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.cinnabarina_Ms517__S_            CAAGTGGATC AAGGCAGTG- ATTTGTGAAT CCACCATGCG CG
A.contorta_Acon1__S_               CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.contorta_1__S_                   CAAGTGGATC AAGGCAGTG- ATTTGTGAAT CCACCATGCG CG
A.macrophylla_Ama__S_              CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.manshuriensis_Ams1__S_           CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Xungufeng_Am10__TCM                CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Xungufeng_AM7__TCM                 CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.kaempferi_8__S_                  CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.heterophylla_7__S_               CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.fangchi_Ms460__S_                CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.californica_Aca__S_              CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.mollissima_9__S_                 -AAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Baiying_SL3__TCM                   CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.mollissima_Ms536__S_             CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
A.mollissima_AMo2__S_              CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Baiying_SL2__TCM                   CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Solanum_ptychanthum_AY727181       .......... .......... .......... .......... ..
Solanum_americanum_AY727179        .......... .......... .......... .......... ..
Solanum_physalifolium_AY727180     .......... .......... .......... .......... ..
Solanum_japonense_Sjapl__S_        CAAGTGGATC AAGGCAGTG- ATTTGTGAAT CCACCATGCG CG
Sol2_2_psbA                        CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Solanum_japonense_Sjasl__S_        CAAGTGGATC AAGGCAGTG- ATTTGTGAAT CCACCATGCG CG
Xungufeng_AM1__TCM                 CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Solanum_lyratum_17L1__S_           CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Xungufeng_AM2__TCM                 CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Baiying_SL4__TCM                   CAAGTGGATC AAGGCAGTGG ATTTGTGAAT CCACCATGCG CG
Clustal Consensus
```

6.1.2 Percentage similarity analysis

The percentage similarities of the *psb*A-*trn*H region among all the herb material

of Baiying and Xungufeng and relevant authentic species were calculated using

BioEdit Sequencing Alignment Editor (Hall, 1999). The results are summarized in

Table 6.1. The interspecific similarity of *Solanum* to *Aristolochia* species varied from

15.3–24.6%, the average being 19.1%. The average intraspecific similarity of

*Aristolochia* is 73.6%, ranging from 51.3–100%.

6.1.3 Dendrogram analysis

Dendrograms were constructed using the computer program MEGA version 3.1

(Kumar *et al.*, 2001) with two tree construction methods: unweighted pair-group

methods using arithmetic averages (UPGMA; Figure 6.3) and maximum parsimony

(Figure 6.4). Each method was tested by bootstrap test with 1000 replications.

For the *psb*A-*trn*H region, the samples form two separate clades: *Aristolochia*

clade and the *Solanum* clade with 100 bootstrap frequencies. In clade of *Aristolochia*,

two herb samples of Baiying (SL2 and SL3) and two samples of Xungufeng samples

(Am7 and Am10) clustered with the authentic samples of *Aristolochia*. This clade was

supported by bootstrap frequencies of 100 in both bootstrap consensus trees of

UPGMA and of maximum parsimony. In *Solanum* clade, one herb sample of Baiying

Table 6.2 Percentage similarities of *psbA-trnH* region among the plant and herb samples of Baiying and Xungefung.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]
[1]	A.debilis_4_S_	99.5	64.8	68.1	75.1	77.6	76.6	57	56.3	54	54	54	57.9	57.9	55.3	54	54.4	54.4	55.3	54.4	24.2	24.2	23.5	24.4	24.2	15.9	21.9	21.9	21.9	21.9
[2]	A.debilis_Adeb3_S_		65.2	68.5	75.5	78	77	57.5	56.8	54.4	54.4	54.4	58.4	58.4	55.7	54.4	54.9	54.9	55.8	54.9	24.5	24.5	23.7	24.6	24.4	15.9	22.1	22.1	22.1	22.1
[3]	A.trilobata_Atb1_S_			90.1	78.4	67.6	72.1	60.7	59.8	58.7	58.7	58.7	60.6	59.5	59.6	58.2	58.7	58.2	59.7	58.7	23.4	23.4	22.8	23.6	23.4	19	20	20	20	19.8
[4]	A.elegans_Alt1_S_				84.1	64.6	68.4	63.9	63.1	61.9	61.9	61.9	62.6	61	62.9	61.4	61.9	61.4	63	63	23.3	23.3	22.6	23.2	23.1	18.3	19.9	20	19.9	19.7
[5]	A.cinnabarina_Ms517_S_					68.8	71.7	63.9	63	61.9	61.9	61.9	62.6	61.6	62.8	61.4	61.9	60.9	63	63	23.2	23.2	22.5	22.9	22.9	17.3	20	20	20	20
[6]	A.contorta_Acon1_S_						89	55.3	52.6	51.3	51.3	51.3	54.2	54.2	51.5	51.3	51.7	51.7	52.6	51.7	23.6	23.6	23.2	23.8	23.6	17	22.7	22.7	22.7	22.7
[7]	A.contorta_1_S_							55.3	54.6	53.2	53.2	53.2	56.2	56.2	54.4	54.2	53.7	53.7	54.6	53.7	24.1	24.1	23.6	24.2	24.1	17.5	22.9	22.9	22.9	22.9
[8]	A.macrophylla_Ama_S_								98.6	91.8	91.8	91.8	96.1	93.5	96.6	89.9	90.6	89.2	91.2	91.2	18.9	18.9	18.3	18.8	18.9	19.4	16.4	16.4	16.4	16.2
[9]	A.manshuriensis_Ams1_S_									90.6	90.6	90.6	94.8	92.2	95.3	88.6	89.3	88	89.9	89.9	18.9	18.9	18.3	18.8	18.8	19.3	16.4	16.4	16.4	16.1
[10]	Xungufeng_Am10_TCM_										100	100	88.3	87.5	90.6	94.9	95.6	93.5	94.9	94.9	17.8	17.8	17.3	17.7	18	20.1	15.6	15.6	15.6	15.3
[11]	Xungufeng_AM7_TCM_											100	88.3	87.5	90.6	94.9	95.6	93.5	94.9	94.9	17.8	17.8	17.3	17.7	18	20.1	15.6	15.6	15.6	15.3
[12]	A.kaempferi_8_S_												88.3	87.5	90.6	94.9	95.6	93.5	94.9	94.9	17.8	17.8	17.3	17.7	18	20.1	15.6	15.6	15.6	15.3
[13]	A.heterophylla_7_S_													97.4	92.9	86.4	87	85.8	87.6	87.6	19.6	19.6	19	19.5	19.5	19	16.8	16.8	16.8	16.6
[14]	A.fangchi_Ms460_S_														90.3	85.7	86.3	84.5	86.9	87.6	19.4	19.4	18.8	19.3	19.3	18.8	16.4	16.4	16.4	16.2
[15]	A.californica_Aca_S_															90	89.3	88.5	89.2	89.2	18.9	18.9	18.3	18.8	19.1	19.3	16.1	16.1	16.1	15.9
[16]	A.mollissima_9_S_																90	95.6	97	97	18.9	18.9	18.3	18.8	19.3	20.1	16.1	16.1	16.1	15.6
[17]	Baiying_SL3_TCM_																	99.2	98.3	97.8	18.3	18.3	17.7	18.2	18.4	20.1	15.8	15.8	15.8	15.6
[18]	A.mollissima_Ms536_S_																		96.3	97.8	18	18	17.5	18	18.2	20.2	15.8	15.8	15.8	15.6
[19]	A.mollissima_AMo2_S_																			98.5	17.8	17.8	17.3	17.7	18	20.1	15.8	15.8	15.8	15.6
[20]	Baiying_SL2_TCM_																				18.5	18.5	17.9	18.4	18.4	19.8	16.1	16.1	16.1	15.8
[21]	Solanum_ptychanthum_AY727181																					99.7	96.2	96.6	96.4	55.1	86.2	86.2	86.2	86
[22]	Solanum_americanum_AY727179																						96.5	96.4	96.6	55.1	86.4	86.4	86.4	86.2
[23]	Solanum_physalifolium_AY727180																							94.3	94.1	53.3	87.3	87.3	87.3	86.2
[24]	Solanum_japonense_Sjap1_S_																								97.5	55.7	87.9	87.9	87.9	87.1
[25]	Solanum_dulcamara_Sol2_(S)																									55.8	87.7	87.7	87.7	87.7
[26]	Solanum_japonense_Sjas1_S_																										54.8	54.8	54.8	54.6
[27]	Xungufeng_AM1_TCM_																											100	100	99.5
[28]	Solanum_lyratum_17L1_S_																												100	99.5
[29]	Xungufeng_AM2_TCM_																													99.5
[30]	Baiying_SL4_TCM_																													

(SL4) and two Xungufeng (AM1 and AM2) clustered with the authentic samples of

Solanum lyratum (17L1) to form a second clade. This *Solanum* clade was supported

by bootstrap frequencies of 100 in both bootstrap consensus trees of UPGMA and of

maximum parsimony.

6.2 Discussion

6.2.1 Evaluation of chloroplast *psb*A-*trn*H region in differentiation of

Solanum and *Aristolochia*

In this study, *psb*A-*trn*H region was analyzed to differentiate herb sample of

Baiying and Xungufeng. From the sequences alignment and dendrogram analysis, the

identities of the herb samples between Hong Kong and mainland China were revealed

and presented in Table 6.3. It was found that Baiying brought in Hong Kong was

actually derived from *A. mollissima* and Xungufeng was derived from *S. lyratum*. The

two herbs were swapped in Hong Kong but not in mainland China.

The average intraspecific similarity of the sequences among the *Aristolochia*

species in this study was 73.6%, varied from 51.3 to 100%, showing that this region is

quite variable in this genus. The average interspecific similarity between *Solanum* and

Aristolochia species was 19.1%, varied from 15.3 to 24.6%.
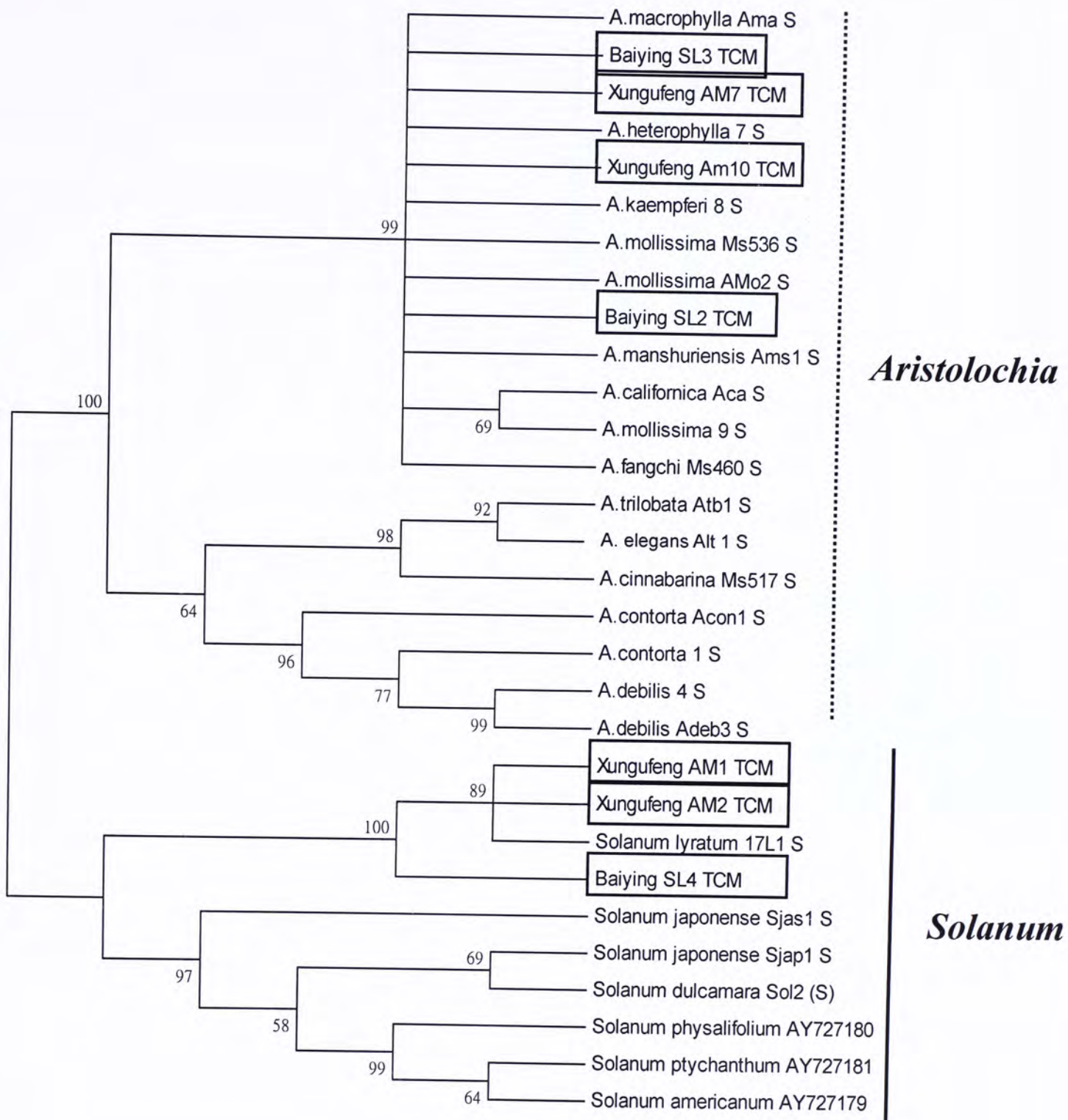
Figure 6.3 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Baiying and Xungufeng. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
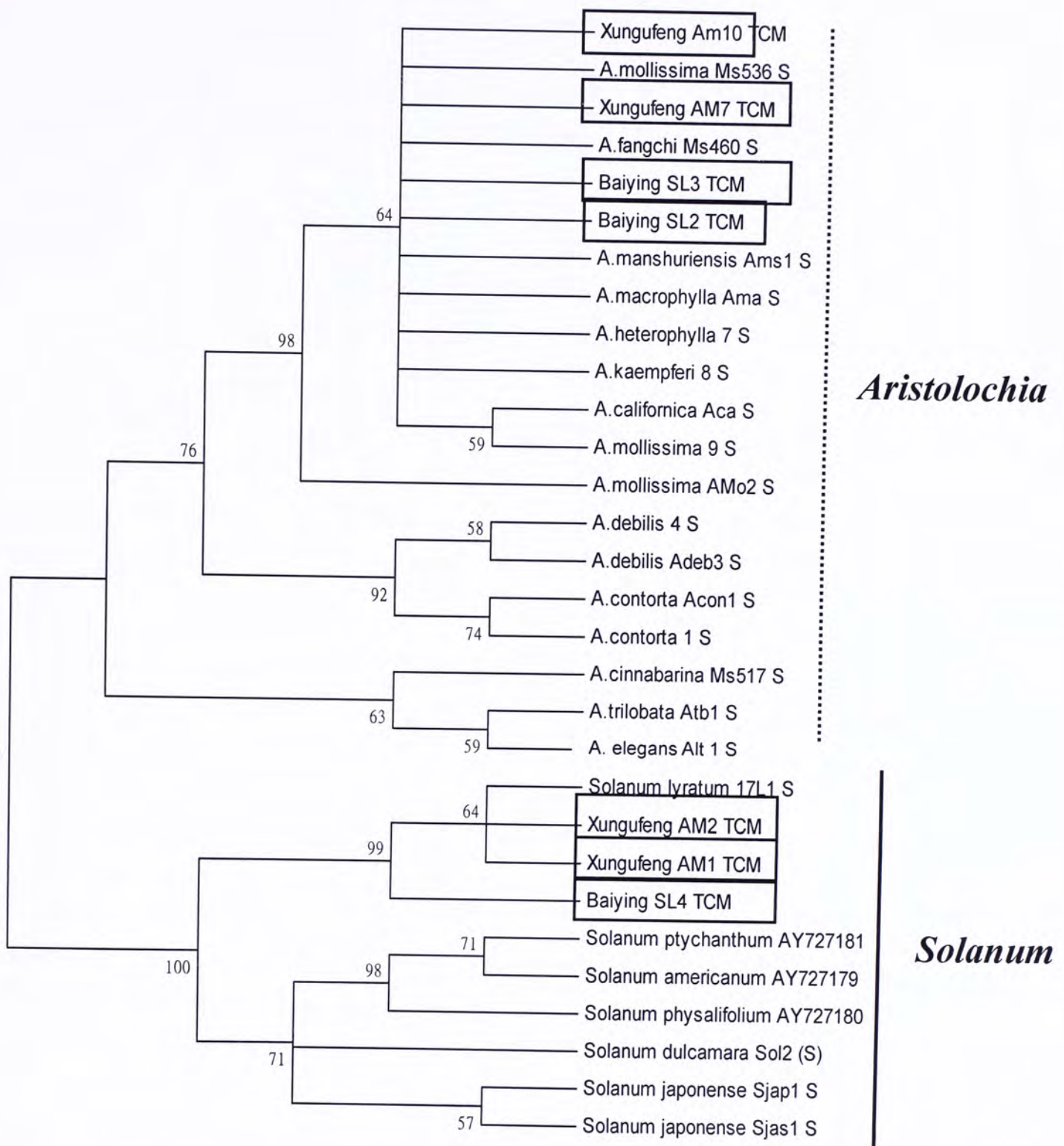
Figure 6.4 Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Baiying and Xungufeng. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.

Table 6.3 Summary of the identities of the herb samples of Baiying and Xungufeng brought from Hong Kong and mainland China.

Source of samples	Herbs	code	Identity
Hong Kong	Baiying	SL2	*A. mollissima*
Hong Kong	Baiying	SL3	*A. mollissima*
Hong Kong	Xungufeng	Am1	*S. lyratum*
Hong Kong	Xungufeng	Am2	*S. lyratum*
mainland China	Baiying	SL4	*S. lyratum*
mainland China	Xungufeng	Am7	*A. mollissima*
mainland China	Xungufeng	Am10	*A. mollissima*

In conclusion, *psbA-trn*H region is able to differentiate the plants from different genera and can be utilized for differentiating *S. lyratum* from *A. mollissima*. Nucleotide changes of insertions/deletions and base substitutions in *Solanum* and *Aristolochia* species can be taken as markers for the differentiation of *S. lyratum* from *A. mollissima*.

6.2.2 Molecular authentication of Baiying

The sequence alignment (Figure 4.2) showed that the *psbA-trn*H sequences of herb samples of Baiying from Guangdong (SL4) and of Xungufeng from Hong Kong (Am1 and Am2) matched with that of *S. lyratum*. In the two dendrograms constructed

by two different tree construction methods, samples (SL4, AM1 and AM2) all clustered with the clade of *S. lyratum*. On the other hand, the *psb*A-*trn*H sequences of two herb samples of Baiying from Hong Kong (SL2 and SL3) and a Xungufeng sample from Jiangxi (Am7) and a sample from Shanxi (Am10) matched with that of *A. mollissima* (Figure 6.2). They clustered to the clade of *A. mollissima* in the two dendrograms constructed by different tree construction methods. From the results, herb samples of Baiying from Guangdong (SL4) and of Xungufeng from Hong Kong (Am1 and Am2) should be derived from *S. lyratum*, while herb samples of Baiying from Hong Kong (SL2 and SL3), Shanxi (Am10) should be derived from *A. mollissima*. It matched the result pervious done by Li (2005). Baiying and Xungufeng retailed in Hong Kong were swapped.

6.3 Conclusion

The method adapted is successful. The authentic species of Baiying (*S. lyratum*) and Xungufeng (*A. mollissima*) were differentiated by *psb*A-*trn*H region. The results were the same with the pervious study by Li (2005) that herb samples of Baiying and Xungufeng retailed in Hong Kong were swapped. Two samples of Xungufeng from Guangdong were found to be Baiying. The other herb samples of Baiying and Xungufeng from other places in mainland China were determined as genuine items. The *psb*A-*trn*H region provides another gene region for differentiating the genuine Baiying from the adulterant Xungufeng.

Chapter 7:   AUTHENICATION OF FANGJI

7.1 Results

The DNA was successfully extracted from (a) four samples of the herb Fangji, (b) one sample of Guangfangji, (c) three authentic samples of *Cocculus*, (d) one authentic sample of *Stephania*, (e) and 14 authentic samples of *Aristolochia* species. The chloroplast *trn*L-*trn*F gene region and the *psb*A-*trn*H gene region of these 23 samples were successfully sequenced. For *trn*L-*trn*F gene region analysis, one additional *Cocculus* sequence, one additional *Stephania* sequence and three additional *Aristolochia* sequences were downloaded from NCBI Genbank and used in sequence alignment and constructing dendrograms.

There are differences in the length of the *trn*L-*trn*F region among the three genera. The genera *Stephania* and *Cocculus* had similar length, about 1010–1030 bp, whereas *Aristolochia* is relatively shorter, about 970–1000 bp in length. In *psb*A-*trn*H region, there are 260–300 bp in *Aristolochia* and 640–700 bp in *Stephania* and *Cocculus*.
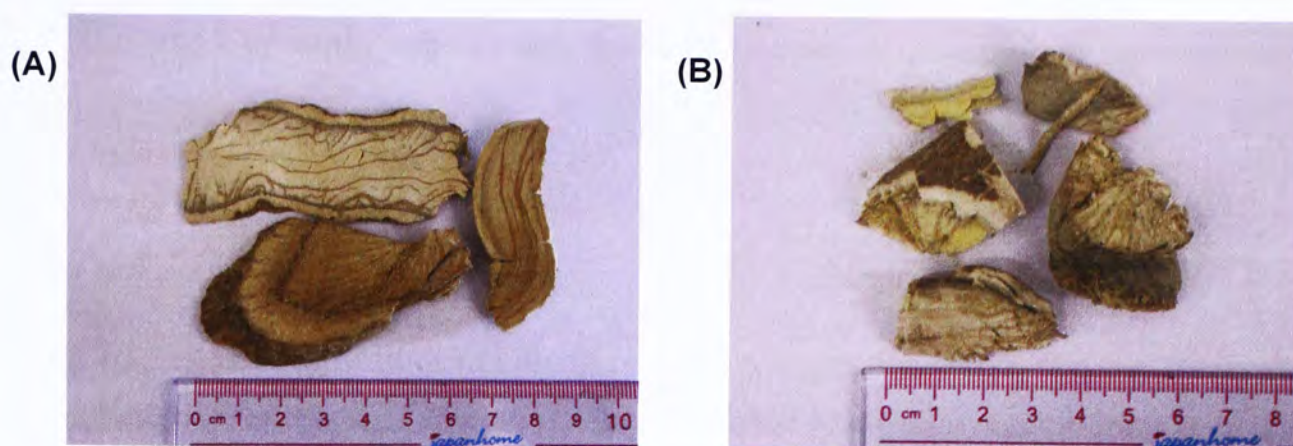
Figure 7.1   Morphological views of (A) Fangji (FFC1), (B) Guangfangji (GFC1)

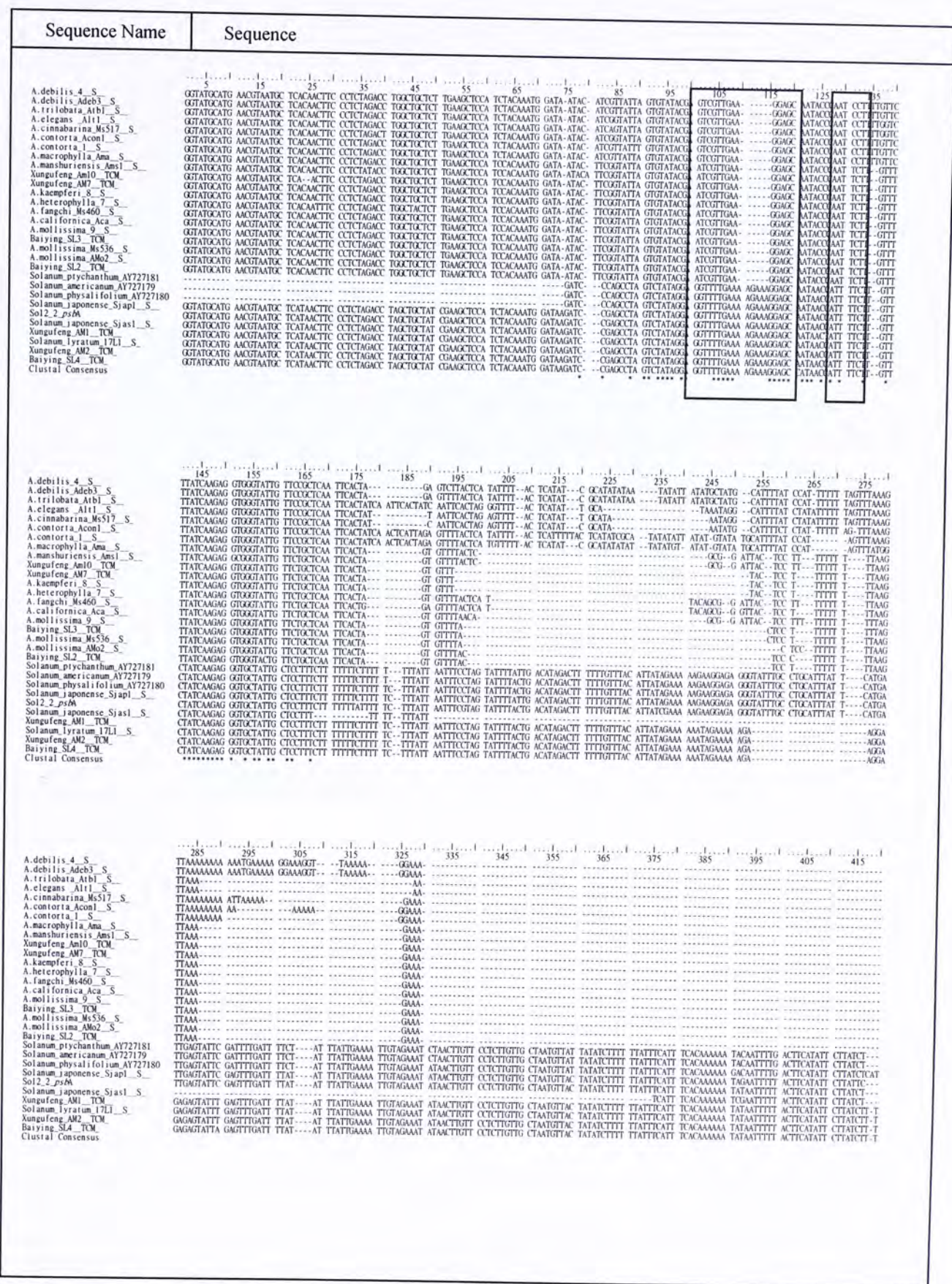## 7.1.1   Sequence alignment

7.1.1.1   *trn*L-*trn*F sequence

The 28 sequences of *trn*L-*trn*F region were aligned using the computer program

ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were

performed using BioEdit sequence Alignment Editor (Hall, 1999). The aligned

sequences are presented in Figure 7.2. Based on the sequence alignment of this gene

region of the genera *Aristolochia*, *Cocculus* and *Stephania*, six sites of

insertions/deletions or base changes can be utilized to differentiate the herbs derived

from these three genera. These sites are highlighted with a box in Figure 7.1 and listed

in Table 7.1. For example, at site 561–570, there is a 5 bp deletion in genera *Cocculus*

and *Stephania* that could be used to differentiate them from *Aristolochia*. The results

of sequence alignment showed that the herb sample of Fangji matched with the

Cocculus and *Stephania* species and the herb sample of Guangfangji matched with

Aristolochia species.

Table 7.1 Sites of insertions/deletions or base changes for differentiating the herbs derived from *Aristolochia, Cocculus* and *Stephania* from sequence alignment of the *trn*L-*trn*F region in Figure 7.2. The base pairs or gaps labeled in red color indicated the insertions/deletions and base pairs labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes	
1	70–72	*Aristolochia*	TCC
		Other genus	TTC
2	451–453	*Aristolochia*	GCA
		Other genus	GTT
3	470–472	*Aristolochia*	AGT
		Other genus	ATT
4	561–570	*Aristolochia*	G(G/A)G(C/T)GAGACG
		Other genus	G - - - --GACG
5	861–870	*Aristolochia*	AC------TG
		Other genus	ACAAGTATTG
6	936–940	*Aristolochia*	ACGG
		Other genus	T--G

7.1.1.2 *psb*A-*trn*H sequence

The 23 sequences of *psb*A-*trn*H region were aligned using the computer program

ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were

performed using BioEdit sequence Alignment Editor (Hall, 1999). The aligned

sequences are presented in Figure 7.2. Based on the sequence alignment of this gene

region of the genera *Aristolochia, Cocculus* and *Stephania,* three sites of insertions/deletions or base changes could be utilized to differentiate the herbs derived from these three genera. These sites are highlighted with a box in Figure 7.3 and listed in Table 7.2. For example, at site 104–110, there is a 6 bp deletion in *Aristolochia* that could be used to differentiate it from the two genera from Menispermaceae. The results of sequence alignment showed, similarly, that the herb samples of Fangji matched with the *Cocculus* and *Stephania* species and herb sample of Guangfangji matched with *Aristolochia* species.

Table 7.2 Sites of insertions/deletions or base changes for differentiating the herbs derived from *Aristolochia, Cocculus* and *Stephania* from sequence alignment of the *psb*A-*trn*H region in Figure 7.3. The gaps or base pair labeled in red color indicated the insertions/deletions and base pairs labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes
1	71–73	*Aristolochia* GAT
		Other genus GCT
2	104–110	*Aristolochia* T------
		Other genus TTGAAGT
3	139–145	*Aristolochia* CTTATCA
		Other genus CTGTCAA
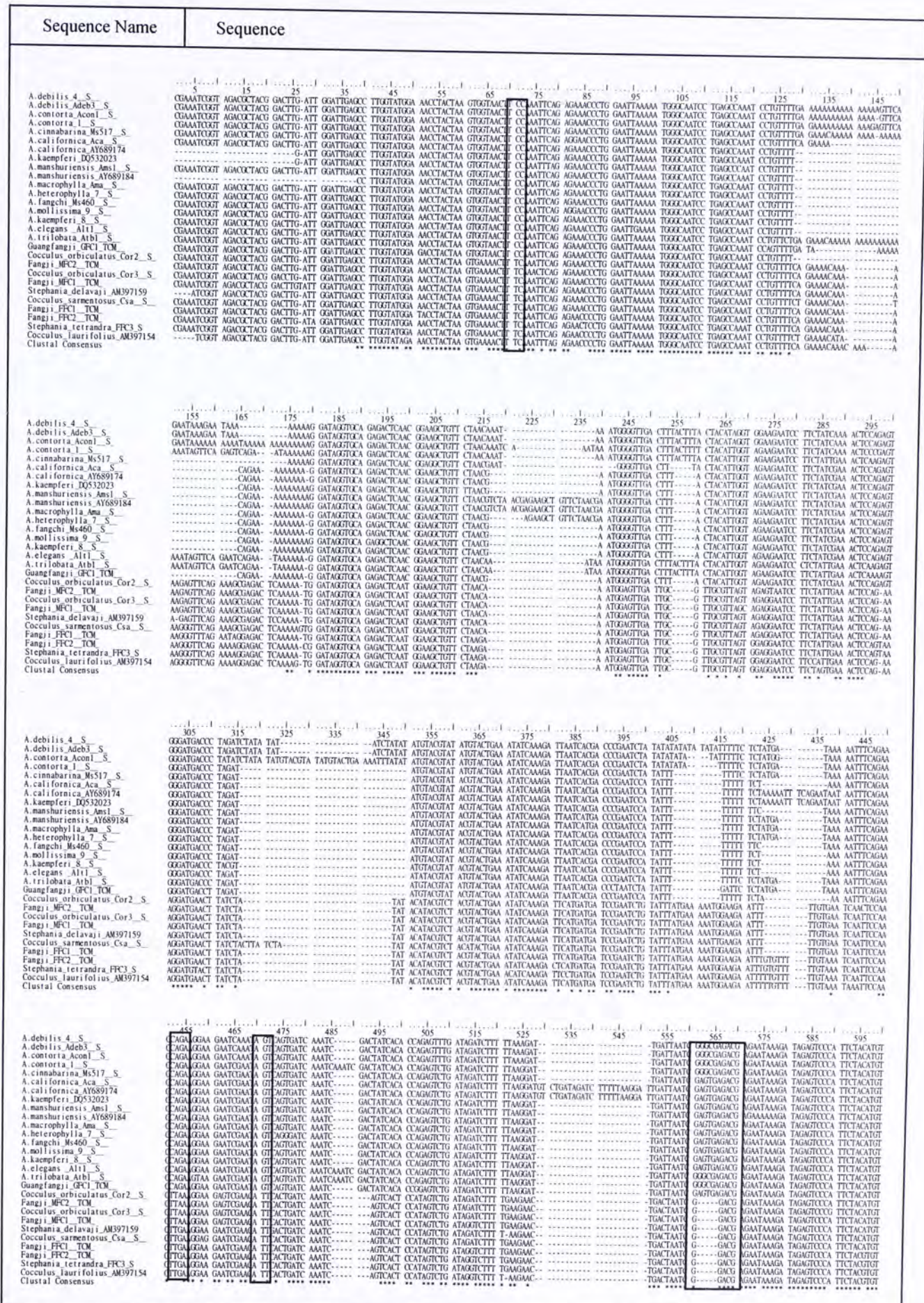
Figure 7.2 Sequence alignment of *trn*L-*trn*F region for herb materials of Fangji and Guanfangji and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Sites of insertions/deletions or base changes that could be utilized to differentiate the herbs derived from these three genera are highlighted in boxes.

Figure 7.2 (continued)

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Figure 7.2 (continued)

Sequence Name	Sequence

Figure 7.2 (continued)

119

Figure 7.2 (continued)

Sequence Name	Sequence
	....|....| ....|....| ....|....| ....|....| ....|....| ... 1205 1215 1225 1235 1245

A.debilis_4__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.debilis_Adeb3__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.contorta_Acon1__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.contorta_1__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.cinnabarina_Ms517__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.californica_Aca__S_   TAGCTCAGCT GGTAGAGCGG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.californica_AY689174   ....
A.kaempferi_DQ532023   ....
A.manshuriensis_Ams1__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.manshuriensis_AY689184   ....
A.macrophylla_Ama__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.heterophylla_7__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.fangchi_Ms460__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.mollissima_9__S_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.kaempferi_8__S_   TAGCTCAGCT GGTAGAGCTG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.elegans__Alt1__S_   TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
A.trilobata_Atb1__S_   TAGCTCAGTT GGTAGAGCAG AGGATTGAAA ATCCTCGTGT CACCAGTTCA AAT
Guangfangji_GFC1_TCM_   TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
Cocculus_orbiculatus_Cor2__S_ TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
Fangji_MFC2__TCM_   TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
Cocculus_orbiculatus_Cor3__S_ TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
Fangji_MFC1__TCM_   TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
Stephania_delavayi_AM397159 TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AA-
Cocculus_sarmentosus_Csa__S_   TAGCTCAGTT GGTAGAGCAG AGGACGGAAA ATCCTCGTGT CACCAGTTCA AAT
Fangji_FFC1__TCM_   TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATACTCGTGT CACCAGTTCA AAT
Fangji_FFC2__TCM_   TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
Stephania_tetrandra_FFC3_S TAGCTCAGTT GGTAGAGCAG AGGACTGAAA ATCCTCGTGT CACCAGTTCA AAT
Cocculus_laurifolius_AM397154 TAGCTCAGTT GGTAGAGCAG AGGGCTGAAA ATCCTCGTGT CACCAGTT-- ...
Clustal Consensus

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Figure 7.3 Sequence alignment of *psb*A-*trn*H region for herb materials of Fangji and Guanfangji and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Sites of insertions/deletions or base changes that could be utilized to differentiate the herbs derived from these three genera are highlighted in boxes.

Figure 7.3 (continued)

Figure 7.3 (continued)

Sequence Name	Sequence

```
                        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        565       575       585       595       605       615       625       635       645       655       665       675       685       695
A.contorta_Acon1__S_    .......... .......... .......... .......... .......... .......... ..........TA GTTTAAAGTT AAAAAAAAA A......... .......... .......AA AAGGAAAGGC C-AGGGGCCGG ATGTAGCCAA
A.contorta_1__S_        .......... .......... .......... .......... .......... .......... ..........TA GTTTATGGTT AAAAAAAA-- .......... .......... ......--OGAAAGGC C-AGGGGCCGG ATGTAGCCAA
A.elegans__Alt1__S_     .......... .......... .......... .......... .......... .......... ..........TA GTTTAAAGTT AAAAAGAA-- .......... .......... ......--GT C-CAGGGCCGG ATGTAGCCAA
A.trilobata_Atb1__S_    .......... .......... .......... .......... .......... .......... ..........TA GTTTAAAGTT AAAAAAAA-- .......... .......... ......--GT C-CAGGGCCGG ATGTAGCCAA
A.debilis_4__S_         .......... .......... .......... .......... .......... .......... ..........TA GTTTAAAGTT AAAAAAAAA ATGAAAAGG AAAGTTAAA AAGGAAAGGC C-AGGGGCCGG ATGTAGCCAA
A.debilis_Adeb3__S_     .......... .......... .......... .......... .......... .......... ..........TA GTTTAAAGTT AAAAAAAAAA ATGAAAAGG AAAGTTAAA AAGGAAAGGC C-AGGGGCCGG ATGTAGCCAA
A.cinnabarina_Ms517__S_ .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-AA AAAGGAAAGGC C-AGGGGCCGG ATGTAGCCAA
A.fangchi_Ms460__S_     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-GGC --AGGGGCCGG ATGTAGCCAA
Guangfangji_GFC1__TCM   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-GGC --AGGGGCCGG ATGTAGCCAA
A.heterophylla_7__S_    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-GGC --AGGGGCCGG ATGTAGCCAA
A.macrophylla_Ama__S_   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-GGC --AGGGGCCGG ATGTAGCCAA
A.manshuriensis_Ams1__S_.......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-GGC --AGGGGCCGG ATGTAGCCAA
A.californica_Aca__S_   .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-GGC --AGGGGCCGG ATGTAGCCAA
A.mollissima_9__S_      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-GGC --AGGGGCCGG ATGTAGCCAA
A.kaempferi_8__S_       .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......-GGC --AGGGGCCGG ATGTAGC--AA
Fangji_MFC1__TCM        TAAAGAGATG CGTAAGTTAA -----GAAG ACTTAACTTA AACATGTAAA A------GAA ATGAATACT- ------CATT CATAATAGAA -TTGAATGTT TT--GGGGG- .......... -TAAGGGCCGG ATGTGGCCA-
Fangji_MFC2__TCM        TAAAGAGATG CGTAAGTTAA -----GAAG ACTTAACTTA AACATGTAAA A------GAA ATGAATACT- ------CATT CATAATAGAA ATTGAATGTT TTTGGGGGT- .......... -TAAGGGCCGG ATGTGGCCAA
Cocculus_orbiculatus_Cor3__S TAAAGAGATG CGTAAGTTAA -----GAAG ACTTAACTTA AACATGTAAA A------GAA ATGAATACT- ------CATT CATAATAGAA ATTGAATGTT TTTGGGGGT- .......... -TAAGGGCCGG ATGTGGCCAA
Cocculus_orbiculatus_Cor2__S TAAAGAGATG CGTAAGTTAA CAAAGAAAAG ACTTAATTGA AACATGTAAA AAAG-CCGAA TGAAATACT- ------CATT CATAATCGAA ATTGAATCTT TTTGGGGGTA A-GGTAAAGT ATAAGGGCCGG ATGTAGCCAA
Fangji_FFC1__TCM        TAAAGAGATG CGTAAGTTAA CAAAGAAAAG ACTTAATTGA AACATGTAAA AAAG-CCGAA TGAAATACT- ------CATT CATAATCGAA ATTGAATCTT TTTGGGGGTA A-GGTAAAGT ATAAGGGCCGG ACGTGGCCAA
Fangji_FFC2__TCM        TAAAGAGATG CGTAAATTAA CAAGGAAAAG GCTTAATTGA AACATGTAAA AAAC-CCGAA TGAAATACTA AATCACCACT CATAATAGAA ATTGAATGTT TTTGGGGGTA A-GTTAAAGT ATAAGGGCCGG ATGTAGCCAA
Stephania_tetrandra_FFC3_S TAAAGAGATG CGTAAATTAA CAAGGAAAAG GCTTAATTGA AACATGTAAA AAAC-CCGAA TGAAATACTA AATCACCACT CATAATAGAA ATTGAATGTT TTTGGGGGTA A-GTTAAAGT ATAAGGGCCGG ATGTAGCCAA
Cocculus_sarmentosus_Csa__S TAAAGAGATG TGTAAGTTAA -----AAGG ACTTAACTTA AACATGTAAA AAAAACCGAA TTGAGTACT- ------CATT CATAATAGAA ATTGAATGTT TTTGGGGGCA AAGTTAAAGT ATAAGGGCCGG ATGTAGCCAA
Clustal Consensus                                                                                                                                                      ****** * ** ** *
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                        ....|....| ....|....| ....|....| ....|....|
                        705       715       725       735
A.contorta_Acon1__S_    GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.contorta_1__S_        GTGGATCAA- GGCAGTG-AT TTGTGAATCC ACCATGCGCG
A.elegans__Alt1__S_     GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.trilobata_Atb1__S_    GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.debilis_4__S_         GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.debilis_Adeb3__S_     GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.cinnabarina_Ms517__S_ GTGGATCAA- GGCAGTG-AT TTGTGAATCC ACCATGCGCG
A.fangchi_Ms460__S_     GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
Guangfangji_GFC1__TCM   GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.heterophylla_7__S_    GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.macrophylla_Ama__S_   GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.manshuriensis_Ams1__S_GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.californica_Aca__S_   GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.mollissima_9__S_      GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
A.kaempferi_8__S_       GTGGATCAA- GGCAGTGGAT TTGTGAATCC ACCATGCGCG
Fangji_MFC1__TCM        GTGGATTA-- -GCAGTG-AT T-GTGAATCC ACCATGCGCG
Fangji_MFC2__TCM        GTGGATTAAG -GCAGTGGAT TTGTGAATCC ACCATGCGCG
Cocculus_orbiculatus_Cor3__S GTGGATTAAG GGCAGTG-AT TTGTGAATCC ACCATGCGCG
Cocculus_orbiculatus_Cor2__S GTGGATTAAG -GCAGTGGAT TTGTGAATCC ACCATGCGCG
Fangji_FFC1__TCM        GTGGATTAAG -GCAATGGAT TTGTGAATCC ACCATGCGCG
Fangji_FFC2__TCM        GTGGATTAAG -GCAGTGGAT TTGTGAATCC ACCATGCGCG
Stephania_tetrandra_FFC3_S GTGGATTAAG -GCAGTGGAT TTGTGAATCC ACCATGCGCG
Cocculus_sarmentosus_Csa__S GTGGATTAAG -GCAGTG-AT TTGTGAATCC ACCATGCGCG
Clustal Consensus       ****** * *** ** ** *  ********* **********
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7.1.2 Percentage similarity analysis

The percentage similarities of the *trnL-trnF* region among all the herbal material

of Fangji and Guangfangji and relevant authentic species were calculated using

BioEdit Sequencing Alignment Editor (Hall, 1999). The results are summarized in

Table 7.3. The interspecific similarities between *Aristolochia* and *Cocculus* varied

from 66.3–70.8%, the average being 69%. The interspecific similarities between

Aristolochia and *Stephania* varied from 66.2–71.4%, the average being 69.2%. The

intraspecific similarity of *Aristolochia* varied from 80.9–99.6%, with an average of

90%.

The percentage similarities of *psbA-trnH* region among all herbal material of

Fangji and Guangfangji and relevant authentic species were also calculated using

BioEdit Sequencing Alignment Editor (Hall, 1999). The results are summarized in

Table 7.4. The interspecific similarities between *Aristolochia* and *Cocculus* varied

from 17.4–23%, the average being 19.7%. The interspecific similarities between

Aristolochia and *Stephania* varied from 14.3–18.8%, with an average of 16.4%. The

intraspecific similarity of *Aristolochia* vared from 40.1–99.5%, with an average of

63.3%.

Table 7.3 Percentage similarities of *trnL-trnF* region among the plant and herb samples of Fangji and Guangfangji.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]
[1]	A.debilis_4_S_	99.2	91.4	89.5	87.8	83.5	83.1	86	85.2	85.3	83.6	86.3	86.3	86.5	86.3	87.5	84.8	85.2	68.9	68.8	69.1	69.1	69.6	70.6	68.9	69.1	69	69.1
[2]	A.debilis_Adeb3_S_		91.7	89.9	88.1	83.9	83.5	86.6	85.6	85.7	84	86.8	86.8	87	86.8	87.7	85.2	85.7	68.7	68.6	68.9	68.9	69.4	70.3	68.7	69	68.9	69
[3]	A.contorta_Acon1_S_			91.3	83.4	80.9	80.5	83.3	83	83.1	81.1	83.5	83.5	83.5	83.2	85	82.5	82.5	66.3	66.2	66.5	66.5	66.8	67.9	66	66.3	66.2	66.8
[4]	A.contorta_1_S_				87.5	84.6	84.2	87.2	86.1	86.1	84.3	87.5	87.4	87.4	87	91	88.2	86.3	67.8	67.7	68	68	68.4	68.5	67.6	67.6	67.5	68.2
[5]	A.cinnabarina_Ms517_S_					88.4	87.9	91.9	89.9	90	88.1	92.2	93	92.6	92.4	85.2	84.3	90.9	69.3	69.2	69.5	69.5	70.3	69.9	69	69.2	68.9	68.5
[6]	A.californica_Aca_S_						99.5	94.4	92.4	92.3	90.9	94.8	94.8	94.6	94.2	85.8	85.5	93.4	68	68	68.2	68.2	69	68.8	68.1	68.3	68.3	67.7
[7]	A.californica_AY689174							93.9	91.9	91.9	90.5	94.4	94.4	94.2	93.7	85.4	85.1	93	67.8	67.8	68	68	68.7	68.5	67.8	68	68	67.4
[8]	A.kaempferi_DQ532023								95.4	95.3	93.7	99	98.6	98.8	98.3	88.3	88	96.8	70	69.9	70.1	70.1	71	70.8	69.4	69.6	69.7	69.3
[9]	A.manshuriensis_Ams1_S_									99.6	95.7	95.7	95.7	95.9	95.4	87.5	87	94.2	68.7	68.7	68.9	68.9	69.8	69.5	68.4	68.6	68.7	68.2
[10]	A.manshuriensis_AY689184										95.6	95.6	95.6	95.8	95.3	87.4	86.9	94.1	68.6	68.6	68.8	68.8	69.7	69.5	68.4	68.5	68.6	68.1
[11]	A.macrophylla_Ama_S_											94.1	94.1	94.1	93.9	86.8	86.3	93.4	67.6	67.6	67.8	67.8	68.7	68.5	67.6	67.6	67.6	67.1
[12]	A.heterophylla_7_S_												99	98.8	98.3	88.7	88.4	97.3	70.1	70.1	70.3	70.3	71.3	71	69.7	69.8	69.8	69.3
[13]	A.fangchi_Ms460_S_													99	98.3	88.7	88.4	97.5	69.9	70	70.3	70.3	71	70.8	69.4	69.6	69.6	69.1
[14]	A.mollissima_9_S_														99	88.6	88.3	97.3	70.1	69.9	70.3	70.1	71	70.8	69.7	69.8	69.9	69.3
[15]	A.kaempferi_8_S_															99	88.6	97.3	70.1	70.1	70.3	70.3	71.3	71	69.7	69.8	69.9	69.3
[16]	A.elegans_Alt1_S_																88.2	96.9	70.1	70.1	70.3	70.3	71.4	71	68.1	68.3	68.1	68.2
[17]	A.trilobata_Atb1_S_																	95.5	70.1	70.1	70.1	70.3	70.1	70	68.7	67	67	66.6
[18]	Guangfangji_GFC1_TCM_																		69	69	69.4	69.4	69.9	69.9	66.8	68.8	68.8	68.3
[19]	Cocculus_orbiculatus_Cor2_S_																			99.2	99.2	99.2	95.4	95.3	88.3	88.2	87.8	87.2
[20]	Fangji_MFC2_TCM_																				99.1	99.7	95.3	95.1	87.8	88.1	87.7	86.9
[21]	Cocculus_orbiculatus_Cor3_S_																					99.2	95.4	95.3	88	88.4	87.8	87
[22]	Fangji_MFC1_TCM_																						95.4	95.3	88.3	88.6	88	87.2
[23]	Stephania_delavaji_AM397159																							96.1	88.9	89.3	89	88.2
[24]	Cocculus_sarmentosus_Csa_S_																								89.4	89.8	89.3	88.4
[25]	Fangji_FFC1_TCM_																									98.5	96.1	93.8
[26]	Fangji_FFC2_TCM_																										96.4	94.4
[27]	Stephania_tetrandra_FFC3_S_																											94.4
[28]	Cocculus_laurifolius_AM397154																											

Table 7.4 Percentage similarities of *psbA-trnH* region among the plant and herb samples of Fangji and Guangfangji.

	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]
[1] A.contorta_Acon1_S_	88.8	71.4	74.8	75.2	74.7	65.7	43	43.2	43.3	44.4	43.8	44.4	45.5	45.3	20.3	20.5	20.5	19.6	19.6	19.1	18.4	23
[2] A.contorta_1_S_		75.1	78.6	72.4	72	66.8	45	44.5	45.2	46.4	45.8	46.4	47.7	47.4	19.7	19.7	19.7	19.2	18.2	18.1	17.3	21.5
[3] A. elegans_Alt1_S_			91.9	68	68.4	68.4	47.5	47.8	47.8	49.2	48.5	49.2	50.2	50.2	19.8	19.8	19.8	19.1	17	17	16.5	20.2
[4] A.trilobata_Atb1_S_				65.9	66.3	65.4	45.8	46	46	47.3	46.6	47.3	48.7	48	19.9	19.9	19.9	19.1	17.1	17	16.4	20.6
[5] A.debilis_4_S_					99.5	63.4	40.8	40.5	40.6	41.6	41	41.6	43	43.2	20.8	21.3	21.3	20.7	19.5	19.4	18.8	24
[6] A.debilis_Adeb3_S_						63.9	40.4	40.1	40.2	41.2	40.6	41.2	42.6	42.7	21	21.5	21.5	20.9	19.5	19.4	18.8	24.2
[7] A.cinnabarina_Ms517_S_							47.6	47.9	47.9	49.2	48.5	49.2	50.7	50.7	19.7	19.8	19.8	18.8	17.7	17.6	16.6	21.5
[8] A.fangchi_Ms460_S_								97.9	97.3	93.3	92	90	86	84.7	19.3	19.3	19.3	19.7	16.9	16.8	15.7	19.4
[9] Guangfangji_GFC1_TCM_									96.6	92.6	91.3	89.4	84.6	83.4	19.3	19.3	19.3	19.7	17.1	17	15.9	19.4
[10] A.heterophylla_7_S_										96	94.7	92.7	88	86.7	19.5	19.5	19.5	20.1	17.1	17	15.9	19.6
[11] A.macrophylla_Ama_S_											98.6	96.5	91.6	90.3	19.1	19.1	19.1	19.5	16.7	16.7	15.5	19.2
[12] A.manshuriensis_Ams1_S_												95.2	90.3	89	19.2	19.3	19.3	19.6	16.9	16.8	15.7	19.3
[13] A.californica_Aca_S_													91	90.3	18.3	18.3	18.3	18.7	16.2	16.1	15	18.6
[14] A.mollissima_9_S_														97	17.5	17.6	17.6	18	15.7	15.6	14.5	17.8
[15] A.kaempferi_8_S_															17.3	17.4	17.4	17.7	15.3	15.3	14.3	17.6
[16] Fangji_MFC1_TCM_																98.6	98.4	95.7	73.6	74	71.2	74.6
[17] Fangji_MFC2_TCM_																	99.4	96.7	74.5	74.9	71.8	75.4
[18] Cocculus_orbiculatus_Cor3_S_																		96.5	74.3	74.7	71.7	75.1
[19] Cocculus_orbiculatus_Cor2_S_																			73.6	74	70.8	74
[20] Fangji_FFC1_TCM_																				98.9	85.1	74.5
[21] Fangji_FFC2_TCM_																					85.7	74.6
[22] Stephania_tetrandra_FFC3_S_																						71.8
[23] Cocculus_sarmentosus_Csa_S_																						

7.1.3 Dendrogram study

Dendrograms were constructed using the computer program MEGA version 3.1 (Kumar *et al.*, 2001) with two tree construction methods: unweighted pair-group methods using arithmetic averages (UPGMA; Figure 7.4 and 7.6), and maximum parsimony (Figure 7.5 and 7.7). Each method was tested by bootstrap test with 1000 replications.

From the *trnL-trnF* region, the samples from two separate clades: *Aristolochia* clade and the clade of genera *Cocculus* and *Stephania* with 100 bootstrap frequencies. In the clade consist of *Cocculus* and *Stephania*, the herbs samples of Fangji (FFC1, FFC2, MFC1 and MFC2) clustered with the authentic sample of *Cocculus* and *Stephania*. Two Fangji (FFC1 and FFC2) clustered with genuine *Stephania tetrandra* (FFC3) while two Fangji (MFC1 and MFC2) clustered with genuine *Cocculus orbiculatus*. This clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony. In the *Aristolochia* clade, the herbal sample Guangfangji (GFC1) clustered with the authentic sample of *Aristolochia*. This clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony.

Base of the sequence of the *psb*A-*trn*H region, the samples were distributed in two separate clades: *Aristolochia* clade and the clade of genera *Cocculus* and *Stephania* with 100 bootstrap frequencies. In the clade consisting of *Cocculus* and *Stephania*, the herbal samples FFC1, FFC2, MFC1 and MFC2 clustered with the authentic samples of *Cocculus* and *Stephania*. This clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony. Two Fangji samples (FFC1 and FFC2) clustered together to form a single clade and supported by bootstrap frequencies of 100 in UPGMA and 99 in bootstrap consensus trees of maximum parsimony. Another two Fangji samples (MFC1 and MFC2) clustered with the authentic species of *Cocculus orbiculatus* (Cor2 and Cor2) and was supported by bootstrap frequencies of 99 in both UPGMA and bootstrap consensus trees of maximum parsimony. In the clade of *Aristolochia*, the herbal sample Guangfangji (GFC1) clustered with the authentic sample of *Aristolochia fangchi*. The *Aristolochia* clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony.
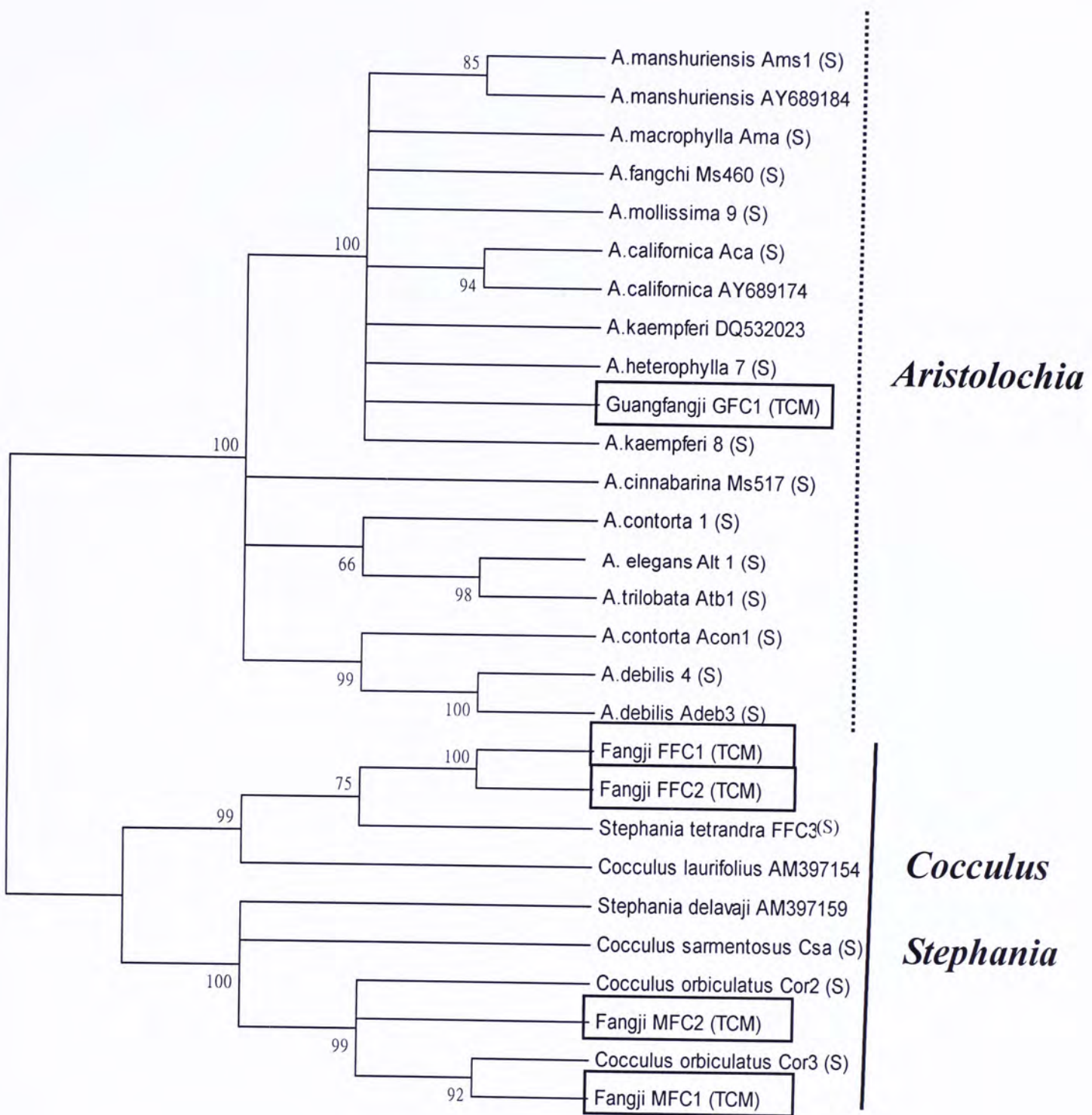
Figure 7.4 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Fangji and Guangfangji. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
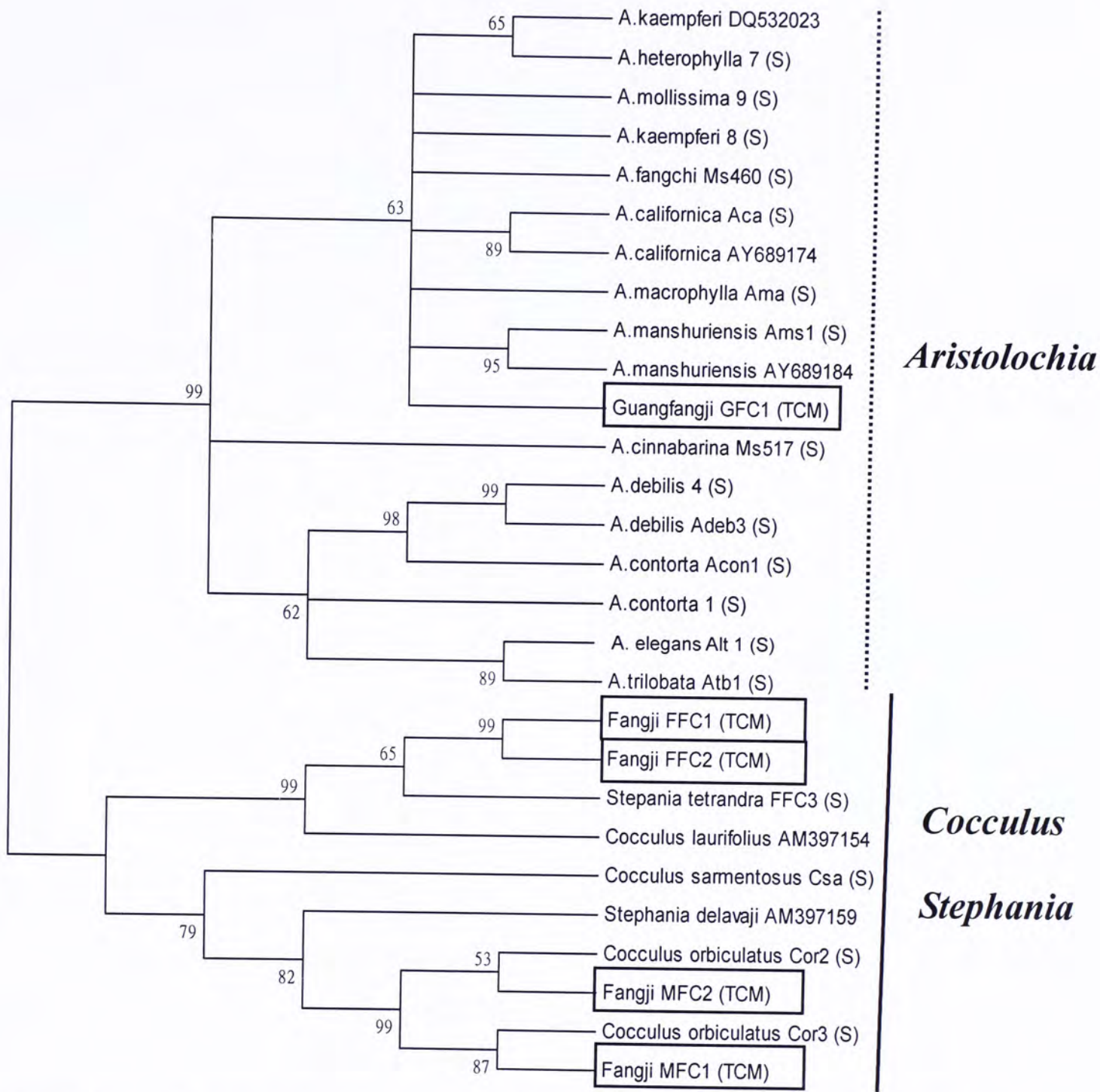
Figure 7.5   Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Fangji and Guangfangji. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
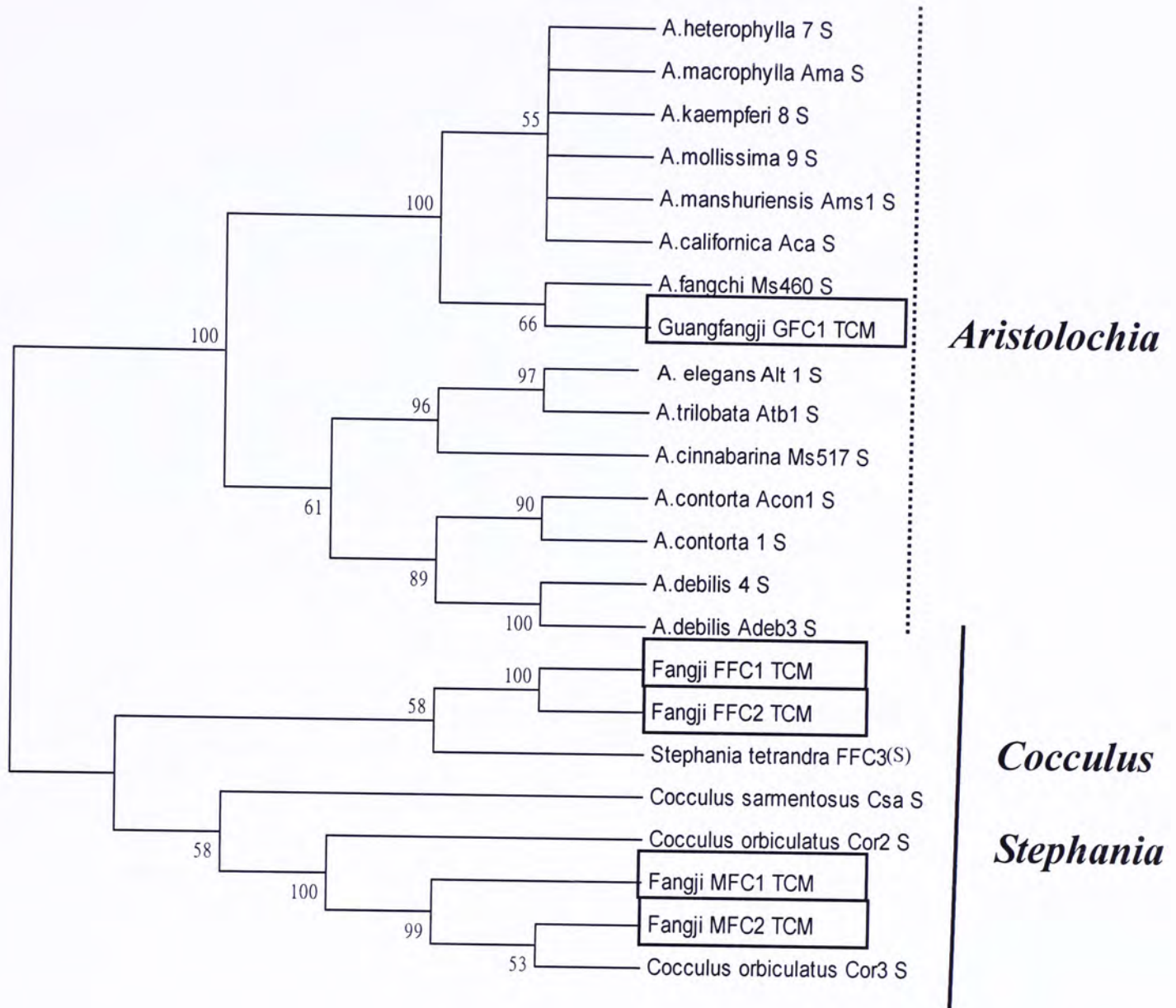
Figure 7.6 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Fangji and Guangfangji. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
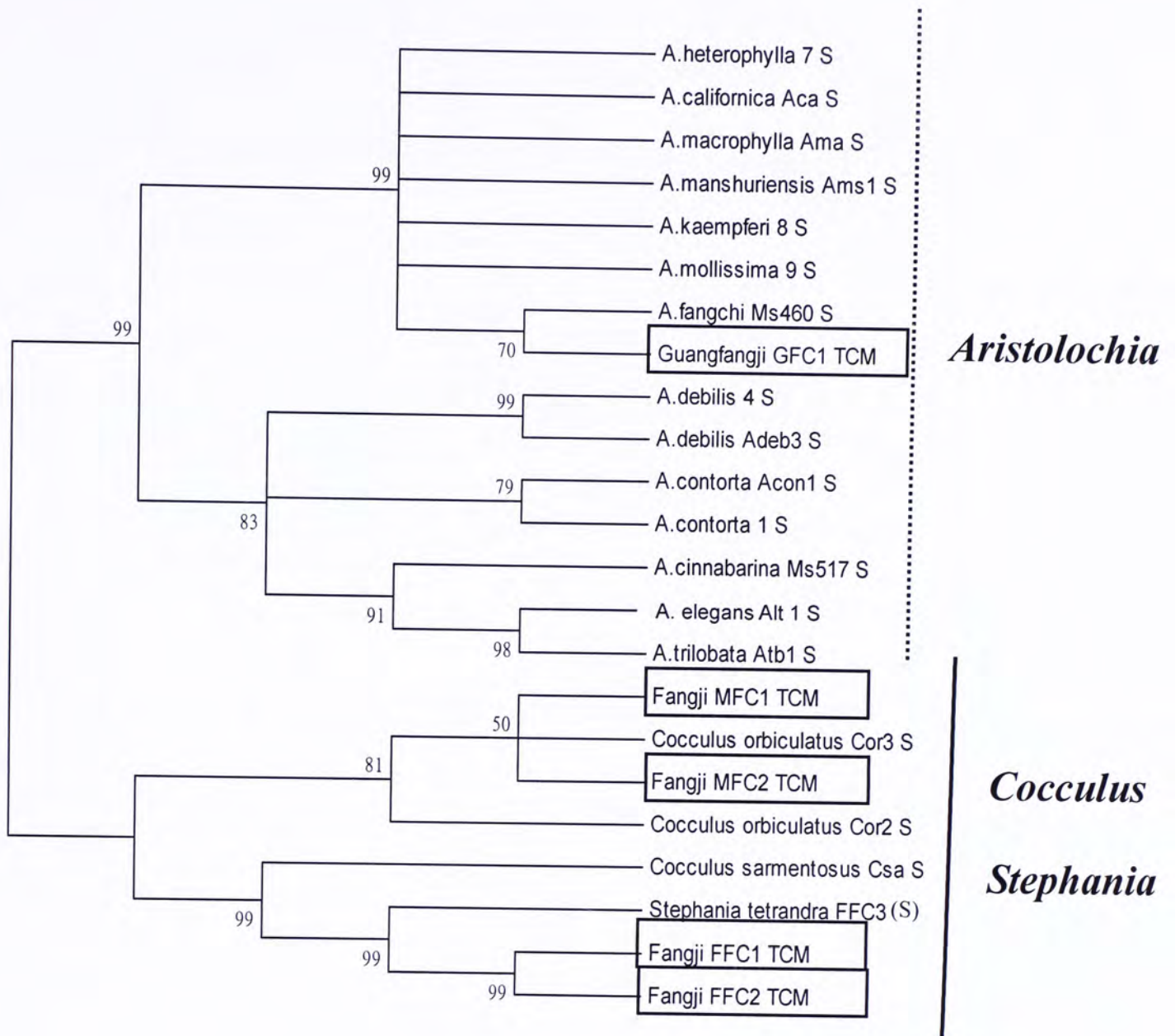
Figure 7.7   Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Fangji and Guangfangji. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.

7.2 Discussion

7.2.1 Evaluation of chloroplast *trn*L-*trn*F region in differentiation of Fangji

In this study, the *trn*L-*trn*F region was analyzed to differentiate genuine Fangji from the adulterant Guangfangji. The source species of Fangji are *Cocculus orbiculatus* and *Stephania tetrandra*. The source specie of Guangfangji is *Aristolochia fangchi*.

The average intraspecific similarity among species in *Aristolochia* is 90%, varying from 80.9–99.6%, showing that this region is conserved within the *Aristolochia* genus (Table 7.1). The mean interspecific similarities between *Aristolochia* and *Cocculus* and between *Aristolochia* and *Stephania* are 69 and 69.2% respectively, which are lower than the average intraspecific similarity of *Aristolochia*. This suggests that *trn*L-*trn*F can differentiate the genuine Fangji from Guangfangji.

The dendrograms constructed by UPGMA and maximum parsimony using this region can clearly separate the three genera into two clades with bootstrap frequencies 100 or 99. The intraspecific similarity within the three genera are high, the relationship is not well resolved.

Although *trn*L-*trn*F region is quite conserved, differentiation of species is achieved by the dendrogram construction. Nucleotide changes of insertions/deletions

and base substitutions between samples can be taken as markers for the differentiation

of genuine Fangji and adulterant Guangfangji. In conclusion, *trn*L-*trn*F region is able

to differentiate the plants from different genera and thus can be utilized for

differentiation genuine and adulterant Fangji.

7.2.2 Evaluation of chloroplast *psb*A-*trn*H region in differentiation of

Fangji

The chloroplast *psb*A-*trn*H is also suitable for differentiating the genuine from

adulterant Fangji. The average intraspecific similarity within *Aristolochia* is 63.3%,

ranging from 40.1–99.5%. This showed that the *psb*A-*trn*H region is very variable

within *Aristolochia*. The interspecific similarities between *Aristolochia* and *Cocculus*

and between *Aristolochia* and *Stephania* are 19.7% and 16.4% respectively. They are

are much lower than the intraspecific similarity of *Aristolochia* suggesting that the

region is sufficient to differentiate herb sample come from different genera.

7.3 Conclusion

The method adapted is successful. Both *trn*L-*trn*F region and *psb*A-*trn*H regions are

suitable for differentiating the genuine from the adulterant species sold as Fangji. In

general, the interspecific similarities of the *psb*A-*trn*H region are much lower than

those of *trn*L-*trn*F region, suggesting that the former region is less conserved than

*trn*L-*trn*F.

Chapter 8: AUTHENICATION OF MADOULING

8.1 Results

The DNA was successfully extracted from (a) two samples of the herb Madouling, (b) three samples of Baihegao, (c) four authentic samples of *Lilium*, and (d) 15 authentic samples of *Aristolochia* species. The chloroplast *trn*L-*trn*F gene region and *psb*A-*trn*H gene region of these 24 samples were successfully sequenced. For *trn*L-*trn*F gene region analysis, one additional *Lilium* sequence and three additional *Aristolochia* sequence were downloaded from NCBI and were used in sequence alignment and constructing dendrograms.

There are differences in the length of the *trn*L-*trn*F region between the two genera. The genus *Lilium* (or *Cardiocrinum*) is about 840–870 bp in length, 970–1000 bp in *Aristolochia*. In *psb*A-*trn*H region, 260–300 bp in *Aristolochia*, 420–490 bp in *Lilium*.
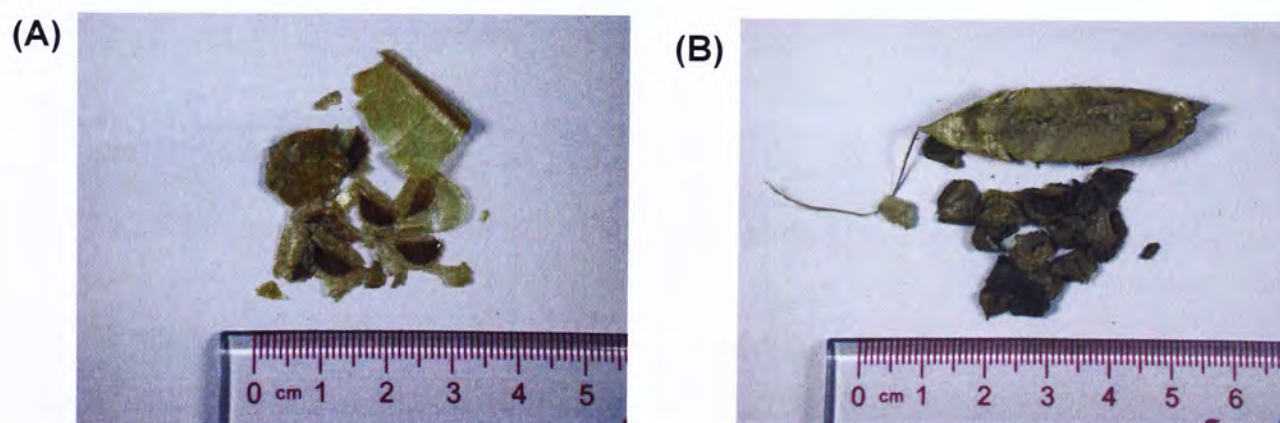
Figure 8.1   Morphological views of (A) Baiheguo (LGI01),(B) Madouling (M5).

8.1.1　Sequence alignment

8.1.1.1　*trn*L-*trn*F sequence

The 28 sequences of *trn*L-*trn*F region were aligned using the computer program

ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were

performed using BioEdit sequence Alignment Editor (Hall, 1999). The aligned

sequences are presented in Figure 8.2. Based on the sequence alignment of this gene

region of the genera *Lilium* and *Aristolochia*, seven sites of insertions/deletions or

base changes can be utilized to differentiate the herbs derived from these two genera.

These sites are highlighted with a box in Figure 8.2 and presented in Table 8.1. For

example at site 470–490, there was a 10 bp deletion in *Aristolochia* and 3 base

changes (at site 471, 484 and 490) that could be used to differentiate the two genera

herbs. The result of sequence alignment showed that the herbal sample of Madouling

were matched with the *Aristolochia* and the herbal sample of Baihegao matched with

Lilium species.

8.1.1.2　*psb*A-*trn*H sequence

The 24 sequences of *psb*A-*trn*H region were aligned using the computer program

ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were

Table 8.1 Sites of insertions/deletions or base changes for differentiating the herbs derived from *Aristolochia* and *Lilium* from sequence alignment of the *trnL-trnF* region in Figure 8.2. The gaps or base pairs labeled in red color indicated the insertions/deletions and base pairs labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes
1	309–321	*Aristolochia* GTGG--GATGAC
		Other genus GAGGATGCCCGC
2	390–396	*Aristolochia* A-----G
		Other genus ACGATTG
3	465–468	*Aristolochia* A--A
		Other genus TCTA
4	469–490	*Aristolochia* TTCA----------G A AGCAG
		Other genus TCCATATTCCAATCA(G/A)AGTTT
5	506–508	*Aristolochia* AGT
		Other genus ATT
6	530–537	*Aristolochia* TATCACAC
		Other genus ATTCATTT
7	598–604	*Aristolochia* GG(C/T)GA
		Other genus -- - --

performed using BioEdit sequence Alignment Editor (Hall, 1999). The aligned

sequences are presented in Figure 8.3. Based on the sequence alignment of this gene

region of genera *Lilium* and *Aristolochia*, three sites of insertions/deletions or base

changes could be utilized to differentiate the herbs derived from these two genera.

These sites are highlighted with a box in Figure 8.3 and presented in Table 8.2. For

example at site 80–84, there was a 4 bp deletion in *Aristolochia* that could be used to

differentiate the two different genera herbs. The result of sequence alignment showed

that the herbal samples of Madouling matched with *Aristolochia* species and herb

samples of Baihegao matched with *Lilium* species.

Table 8.2 Sites of insertions/deletions or base changes for differentiating the herbs derived from *Aristolochia* and *Lilium* from sequence alignment of the *psbA-trnH* region in Figure 8.3. The gaps or base pair labeled in red color indicated the insertions/deletions and base pairs labeled in blue color indicate base changes.

	Sites (bp)	insertions/deletions or base changes
1	80–83	*Aristolochia* - - - - Other genus TCCA
2	245–260	*Aristolochia* C- - - - - - - - - - - - -AC Other genus TTTGCCGATAAATGAT
3	424–444	*Aristolochia* A- - - - - - - - - - - - - - - - - -G Other genus AAAATCCTTTAGCTAGATAAG
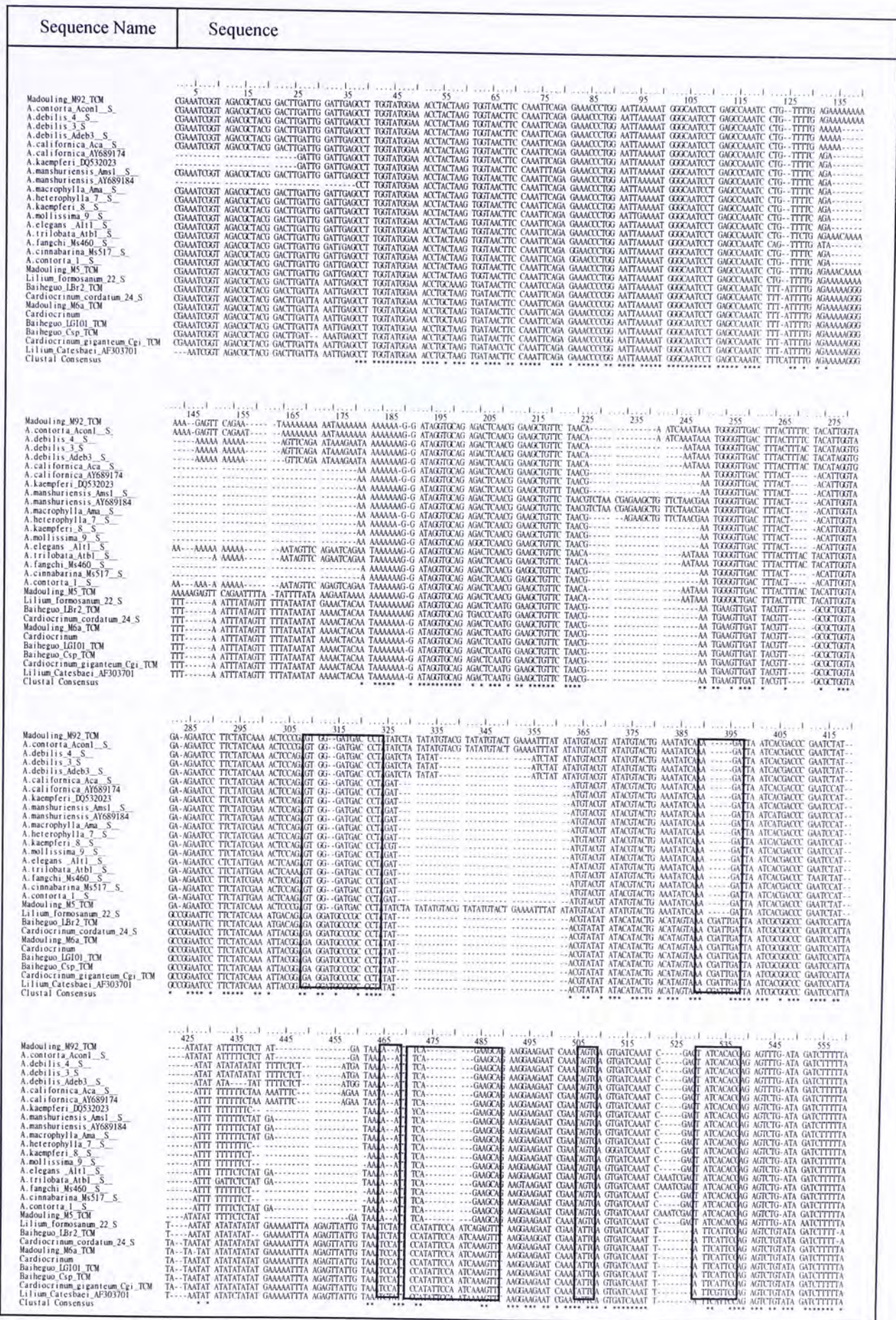
Figure 8.2 Sequence alignment of *trn*L-*trn*F region for herb materials of Madouling, Baihegao and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Sites of insertions/deletions or base changes that could be utilized to differentiate the herbs derived from these two genera are highlighted in boxes.

Figure 8.2 (continued)

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Figure 8.2 (continued)

Sequence Name	Sequence

Figure 8.2 (continued)

Figure 8.2 (continued)

Sequence Name	Sequence

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                                ...|...I ...|...I ...|...I ...|...I ...|...I ...|...I ...|...I ...|...I ...|...I ...|...I ...|...I ...|...I ...|.
                                1125      1135      1145      1155      1165      1175      1185      1195      1205      1215      1225      1235      1245      1255
Madouling_M92_TCM               ---------- ---------- ------CATA GACCCAAGCC TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.contorta_Acon1__S_            ---------- ---------- ------CATA GACCCAAGCC TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.debilis_4__S_                 ---------- ---------- ------CATA GACCCAAGCC TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.debilis_3_S                   ---------- ---------- ------CATA GACCCAAGCC TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.debilis_Adeb3__S_             ---------- ---------- ------CATA GACCCAAGCC TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.californica_Aca__S_           ---------- ---------- ------CATA GGCCCAAGCT TTCTTTTTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.californica_AY689174          ---------- ---------- ------CATA GGCCCAAGCT TTCTTTTTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CGGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.kaempferi_DQ532023            ---------- ---------- ------CATA GACCCAAGCT TT----TTCT TTTAGTAGGA TCATGCATCG AGAATG---- ---------- ---------- ---------- ---------- ---------- ------
A.manshuriensis_Ams1__S_        ---------- ---------- ------CATA GACCCAAGCT TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.manshuriensis_AY689184        ---------- ---------- ------CATA GACCCAAGCT TT----TTCT TTTAGTAGGA TCATGCATCG AGAATG---- ---------- ---------- ---------- ---------- ---------- ------
A.macrophylla_Ama__S_           ---------- ---------- ------CATA GACCCAAGCT TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.heterophylla_7__S_            ---------- ---------- ------CATA GACCCAAGCT TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.kaempferi_8__S_               ---------- ---------- ------CATA GACCCAAGCT TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CTGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.mollissima_9__S_              ---------- ---------- ------CATA GACCCAAGCT TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.elegans__Alt1__S_             ---------- -CGTCCCTTT CATTGACATA GACCCAAGCC TT----TTCT TTTAGTGGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.trilobata_Atb1__S_            CCTTTCATTG ACGTCCCTTT CATTGACATA GACCCAAGCC TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGATTG AAAATCCTCG TGTCACCAGT TCAAAT
A.fangchi_Ms460__S_             ---------- ---------- ------CATA GACCCAAGCT TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.cinnabarina_Ms517__S_         ---------- ---------- ------CATA TATCCAAGCC CT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
A.contorta_1__S_                ---------- ---------- ------CATA GACCCAAGCC TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Madouling_M5_TCM                ---------- ---------- ------CATA GACCCAAGCC TT----TTCT TTTAGTAGGA TCATGCATCG AGAATGGTCG GGATAGCTCA GCTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Lilium_formosanum_22_S          ---------- ---------- ------CATA GATAAAGTGC TCTAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGAT--CTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Baiheguo_LBr2_TCM               ---------- ---------- ------CATA GATAAAGTGC TCTAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATACTCG TGTCACCAGT TCAAAT
Cardiocrinum_cordatum_24_S      ---------- ---------- ------CATA GATAAAGTGC TCTAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Madouling_M6a_TCM               ---------- ---------- ------CATA GATAAAGTGC TCTAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Cardiocrinum                    ---------- ---------- ------CATA GATAAAGTGC TCTAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Baiheguo_LGI01_TCM              ---------- ---------- ------CATA GATAAAGTGC TCTAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Baiheguo_Csp_TCM                ---------- ---------- ------CATA GATAAAGTGC TCTAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Cardiocrinum_giganteum_Cgi_TCM  ---------- ---------- ------CATA GGTAAAGTGC TCTAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Lilium_Catesbaei_AF303701       ---------- ---------- ------CATA GATAAAGTGC TCCAC----- ----TAGGA TAATGCGCGA GAAAAGGTCG GGATAGCTCA GTTGGTAGAG CAGAGGACTG AAAATCCTCG TGTCACCAGT TCAAAT
Clustal Consensus               ---------- ---------- ------****  *            *       * *** * **** **          ** *
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Figure 8.3 Sequence alignment of *psb*A-*trn*H region for herb materials of Madouling, Baihegao and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. Sites of insertions/deletions or base changes that could be utilized to differentiate the herbs derived from these two genera are highlighted in boxes.

8.1.2 Percentage similarity analysis

The percentage similarities of the *trnL-trnF* region among all the herbal material

of Madouling and Baihegao and relevant authentic species were calculated using

BioEdit Sequencing Alignment Editor (Hall, 1999). The result is listed in Table 8.3.

The interspecific similarities between *Lilium* species and *Aristolochia* species varied

from 45.8–55.5%, average being 53.5%. The intraspecific similarity of *Aristolochia*

varied from 75.2–99.8%, average being 87.7%.

The percentage similarities of *psbA-trnH* region among all the herbal material of

Madouling and Baihegao and relevant authentic species were also calculated using

BioEdit Sequencing Alignment Editor (Hall, 1999). The results are listed in Table 8.2.

The interspecific similarities between *Lilium* species and *Aristolochia* species varied

from 43.9–54.7%, average being 48.5%. The intraspecific similarities of *Aristolochia*

varied from 43.9–54.7, average being 80.9%.

Table 8.3 Percentage similarities of *trnL-trnF* region among the plant and herb samples of Madouling and Baihegao.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]
[1]	Madouling_M92_TCM	99.4	90.3	90.3	90.3	90	83.1	75.2	84.8	75.8	83.8	85.5	85.2	85.5	85.9	83.8	85.3	84.9	91.4	97	53.4	53.4	54.1	54.2	54.2	54	54	53.9
[2]	A.contorta_Acon1_S_		90.2	90.2	89.9	90	83.1	75.2	84.8	75.8	83.5	85.4	85.1	85.4	86.1	84	85.4	84.8	91.6	97	53.4	53.3	54	54.2	54.2	54	53.9	53.8
[3]	A.debilis_4_S_			99.8	99.3	87.1	78.8	80.4	87.5	78.3	86.4	88.8	88.6	88.8	88.8	87.5	88.8	88.7	89.8	89.6	55.4	55.2	56	56.1	56.1	55.9	55.8	55.6
[4]	A.debilis_3_S				99.3	87.1	78.8	80.4	87.5	78.3	86.4	88.8	88.6	88.8	88.8	87.5	88.8	88.5	89.8	89.6	55.3	55.2	56	56.1	56.1	55.9	55.8	55.6
[5]	A.debilis_Adeb3_S_					87.1	78.7	80.3	87.5	78.3	86.3	88.7	88.5	88.8	88.6	87.3	88.7	88.5	89.6	89.4	55	54.8	55.6	55.8	55.8	55.5	55.5	55.4
[6]	A.californica_Aca_S_						87	86.4	93	83.5	91.7	95.2	94.6	95	86.9	86.5	95	91.9	86.2	82.3	54	53.8	54.2	54.4	54.4	54.2	54.3	54.4
[7]	A.californica_AY689174							78.7	91	93	94	93	94	95	86.9	86.5	95	87.6	78	74.5	46.3	46.1	46.4	46.5	46.5	46.3	46.4	46.8
[8]	A.kaempferi_DQ532023								86.4	84.4	83.2	86.4	85.8	86.2	78.7	78.4	86.2	83.2	80.5	76.8	47.3	47.2	47.3	47.4	47.4	47.2	47.2	47.7
[9]	A.manshuriensis_Ams1_S_									90.8	85.8	90.2	89.6	90	81.3	80.9	89.8	86.6	87.8	83.6	54.3	54	54.6	54.7	54.7	54.5	54.5	54.6
[10]	A.manshuriensis_AY689184										89.8	96.1	95.8	96.3	88.8	88.1	96.1	93.1	78.6	74.8	46.1	45.8	46.1	46.3	46.3	46.3	46	46.5
[11]	A.macrophylla_Ama_S_											86.4	86	86.3	79.6	79	86.1	83.4	86.5	82.5	53.6	53.3	53.8	54	54	53.8	53.7	53.9
[12]	A.heterophylla_7_S_												94.7	94.7	89.7	87.4	98.9	95.7	89	84.9	54.9	54.9	55.2	55.3	55.3	55.1	55.1	55.2
[13]	A.kaempferi_8_S_													98.4	89.3	88.8	98.6	95.7	88.5	84.7	54.9	54.9	55.2	55.3	55.3	55.1	55.1	55.2
[14]	A.mollissima_9_S_														99	89.8	99.1	96	89	85	55.1	55.1	55.4	55.5	55.5	55.3	55.2	55.4
[15]	A. elegans_Alt1_S_															95.8	89.7	89	85	85.5	54	53.9	54.4	54.5	54.5	54.3	54.3	54.5
[16]	A.trilobata_Atb1_S_																89.2	87.6	92.9	83.3	53.1	52.9	53.4	53.6	53.6	53.4	53.3	53.6
[17]	A.fangchi_Ms460_S_																	96.6	90.2	84.8	54.7	54.7	55	55.2	55.2	54.9	54.9	55
[18]	A.cinnabarina_Ms517_S_																		88.9	84.5	55.2	55.2	55.5	55.6	55.6	55.4	55.3	55.5
[19]	A.contorta_1_S_																			91.5	54.9	54.7	55.2	55.6	55.6	55.4	55.3	55.5
[20]	Madouling_M5_TCM																				53.4	53.2	54	54.3	54.3	54.1	54	53.9
[21]	Lilium_formosanum_22_S																					98.3	97.5	97.9	97.9	97.5	97.2	97.6
[22]	Baiheguo_LBr2_TCM																						97.3	97.7	97.7	97.2	97	97.4
[23]	Cardiocrinum_cordatum_24_S																							99.2	99.2	98.8	98.5	97.7
[24]	Cardiocrinum																								100	99.5	99.2	97.7
[25]	Baiheguo_LGI01_TCM																									99.5	99.2	98.2
[26]	Baiheguo_Csp_TCM																										99.2	98.2
[27]	Cardiocrinum_giganteum_Cgi_S																											97.5
[28]	Lilium_Catesbaei_AF303701																											

Table 8.4 Percentage similarities of *psbA-trnH* region among the plant and herb samples of Madouling and Baihegao.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]
[1]	A.californica_Aca_S_	94.8	95.3	94.6	98.1	97	72.4	72.7	72.5	70.7	70.2	70.7	72.8	77.2	80.6	78.8	93.7	45.4	45.5	45.1	47.7	45.5	45.7	45.7
[2]	A.kaempferi_8_S_		92.8	93.1	95.6	95.6	70.7	71	70.8	69.5	69	69.5	71	75.6	79	77.8	95.4	44.6	44.8	44.3	46.8	44.7	44.9	44.9
[3]	A.heterophylla_7_S_			97.8	97.1	96	73.1	73.4	73.2	71.9	71.4	71.9	73.3	77.9	82	80.1	91	46.6	46.5	46.3	49	46.7	46.9	46.9
[4]	A.fangchi_Ms460_S_				96.4	95.3	73.4	73.7	73.5	72.2	71.7	72.2	73.6	77.9	82.3	80.4	90.6	46	45.9	45.7	48.4	46.1	46.3	46.3
[5]	A.macrophylla_Ama_S_					98.9	73	73.3	73.1	71.8	71.3	71.8	73.3	78.1	81.6	80	93.7	45.8	45.7	45.5	48.1	45.9	46.1	46.1
[6]	A.manshuriensis_Ams1_S_						72.2	72.5	72.3	71	70.5	71	72.5	77.2	80.7	79.1	92.7	45.4	45.3	45.1	47.9	45.5	45.7	45.7
[7]	A.debilis_4_S_							99.7	99.4	85	85	85	84.6	77.6	75.9	79.5	70.2	51.1	51.2	51	54.5	51.4	51.6	51.6
[8]	A.debilis_Adeb3_S_								99.7	85.2	85.2	85.2	84.8	77.9	76.2	79.8	70.4	51.3	51.4	51.2	54.7	51.6	51.8	51.8
[9]	A.debilis_3_S									85.5	85.5	85.5	84.6	77.6	76	80.1	70.2	51.3	51.4	51.2	54.7	51.6	51.8	51.8
[10]	A.contorta_Acon1_S_										99.4	100	92.7	80.5	79.1	83.9	70.5	50.7	50.8	50.6	54	51	51.2	51.2
[11]	Madouling_M5_TCM											99.4	92.1	80	78.6	83.9	70	50.7	50.8	50.6	54.2	51	51.2	51.2
[12]	Madouling_M92_TCM												92.7	80.5	79.1	83.9	70.5	50.7	50.8	50.6	54	51	51.2	51.2
[13]	A.contorta_1_S_													83.5	80.8	83.1	72.6	48.6	48.7	48.5	51.8	48.9	49.1	49.1
[14]	A.trilobata_Atb1_S_														89.6	83.1	75.3	47.6	47.7	47.5	50.6	47.9	48.1	48.1
[15]	A. elegans_Alt1_S_															90.8	78.6	47.2	47.3	47.1	50.6	47.5	47.7	47.7
[16]	A.cinnabarina_Ms517_S_																77.8	49	49.1	49.1	52.2	49.3	49.5	49.5
[17]	A.mollissima_9_S_																	43.9	44.2	43.6	46.1	44.1	44.3	44.3
[18]	Lilium_formosanum_22_S_																		97.9	97.1	83.9	97.5	97.7	97.7
[19]	Baiheguo_LBr2_TCM																			97.3	83.8	97.5	97.7	97.7
[20]	Cardiocrinum_giganteum_Cgi_S_																				84.3	99.1	99.3	99.3
[21]	Cardiocrinum_cordatum_24_S_																					84.7	84.9	84.9
[22]	Cardiocrinum giganteum V. Yun Cyn S																						99.7	99.7
[23]	Baiheguo_LGl01_TCM																							99.7
[24]	Baiheguo_Csp_TCM																							100

8.1.3 Dendrogram study

Dendrograms were constructed using the computer program MEGA version 3.1 (Kumar *et al.*, 2001) with two tree construction methods: unweighted pair-group methods using arithmetic averages (UPGMA; Figure 8.3 and 8.5) and maximum parsimony (Figure 8.4 and 8.6). Each method is tested by bootstrap test with 1000 replications.

From the *trnL-trnF* region, the samples from two large clades: *Aristolochia* clade and the genus *Lilium* clade with 100 bootstrap frequencies. In the *Aristolochia* clade, the herbal samples of Madouling (M5 and M92) clustered with the authentic samples of *Aristolochia* This *Aristolochia* clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony. The Madouling samples (M5 and M92) clustered with the genuine species of Madouling, *Aristolochia contorta* (Acon1) and *Aristolochia debilis* (3, 4 and Adeb3), supported by bootstrap frequencies of 74 in UPGMA and 64 in bootstrap consensus trees of maximum parsimony. In the *Lilium* clade, the herbal samples of Baihegao (LBr2, Csp and LGI01) clustered with the authentic sample of *Lilium*. This clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony.

From the *psb*A-*trn*H region, the samples were distributed in two separate clades: *Aristolochia* clade and the *Lilium* clade with 100 bootstrap frequencies. In the *Aristolochia* clade, the herb sample Madouling (M5 and M92) clustered with the authentic sample of *Aristolochia*. This clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony. The Madouling samples (M5 and M92) clustered with the genuine species of Madouling, *A. contorta* (1 and Acon1), supported by bootstrap frequencies of 99 in both bootstrap consensus trees of UPGMA and maximum parsimony. In the *Lilium* clade, the herb samples of Baihegao (LBr2, Csp and LGI01) clustered with the authentic sample of *Lilium*. This clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony.
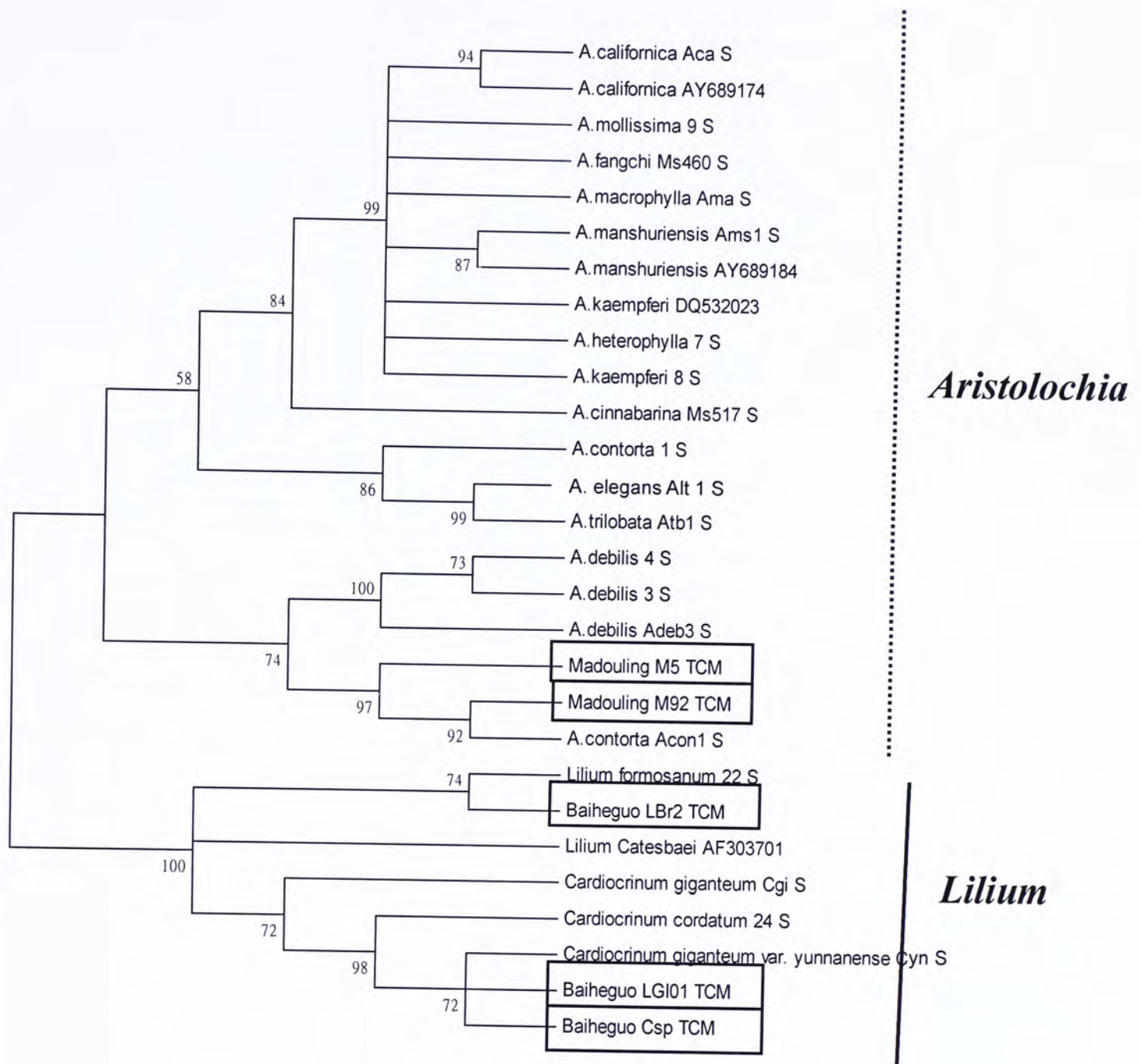
Figure 8.4   Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Madouling. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
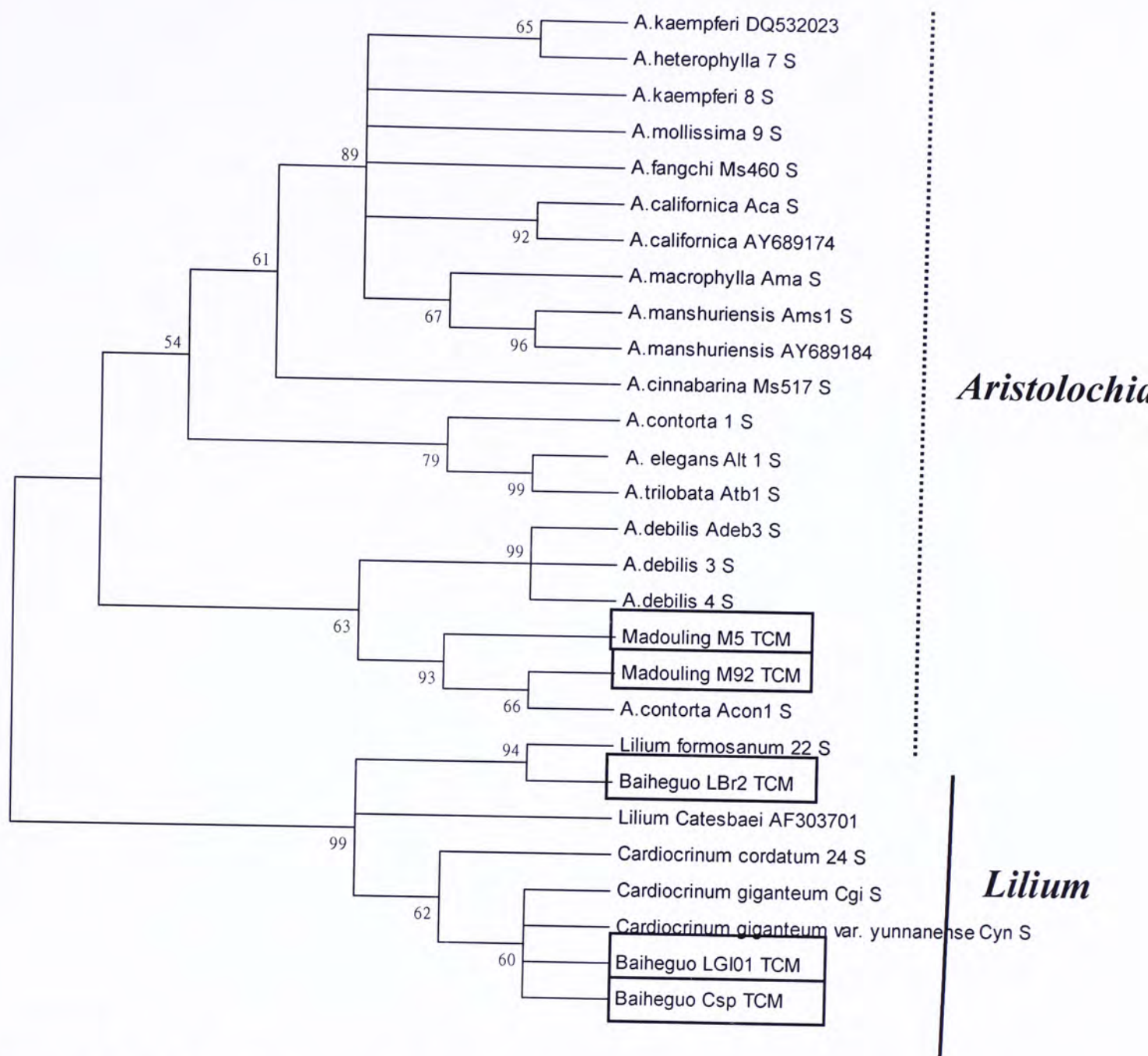
Figure 8.5 Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Madouling. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
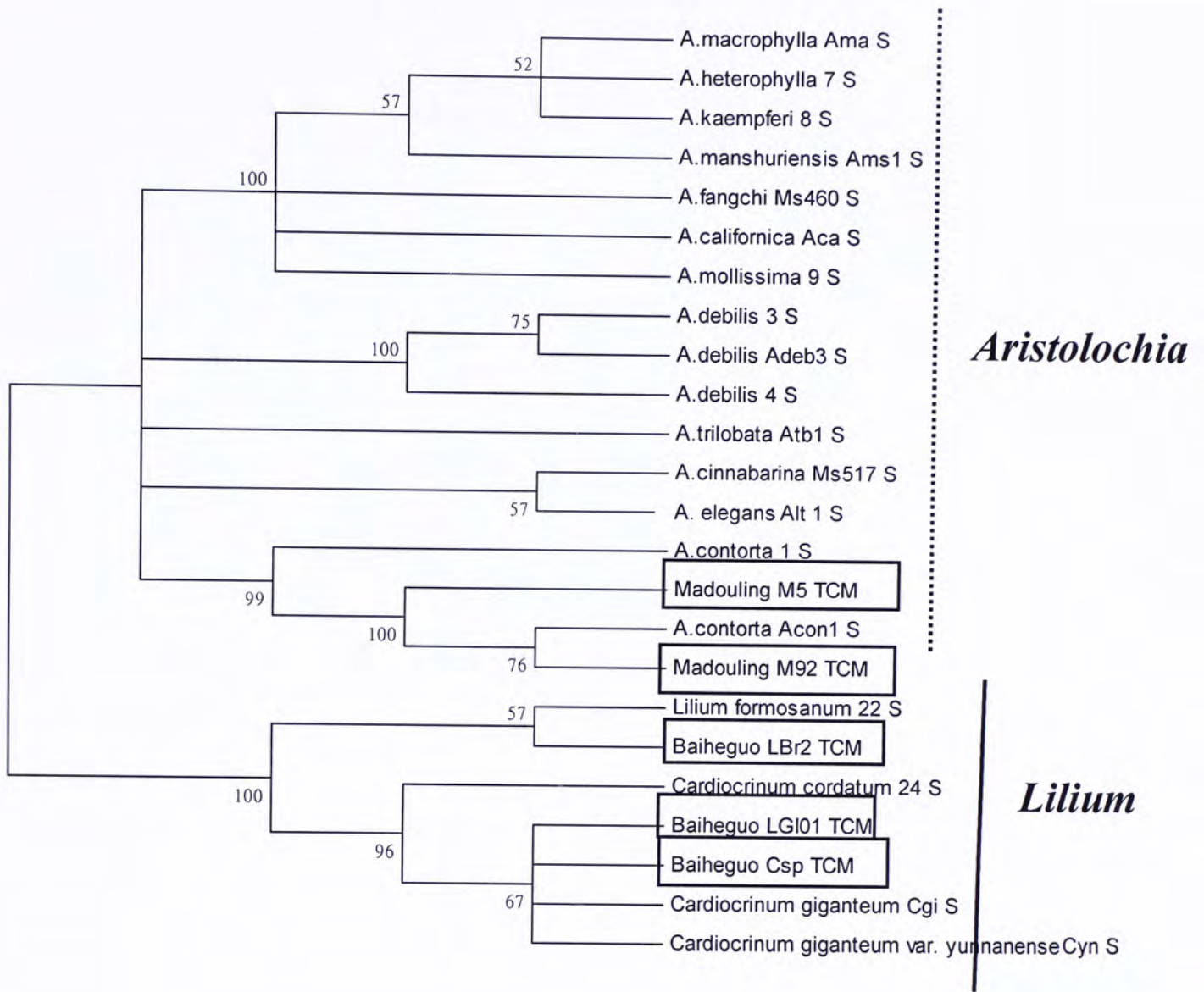
Figure 8.6 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Madouling. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
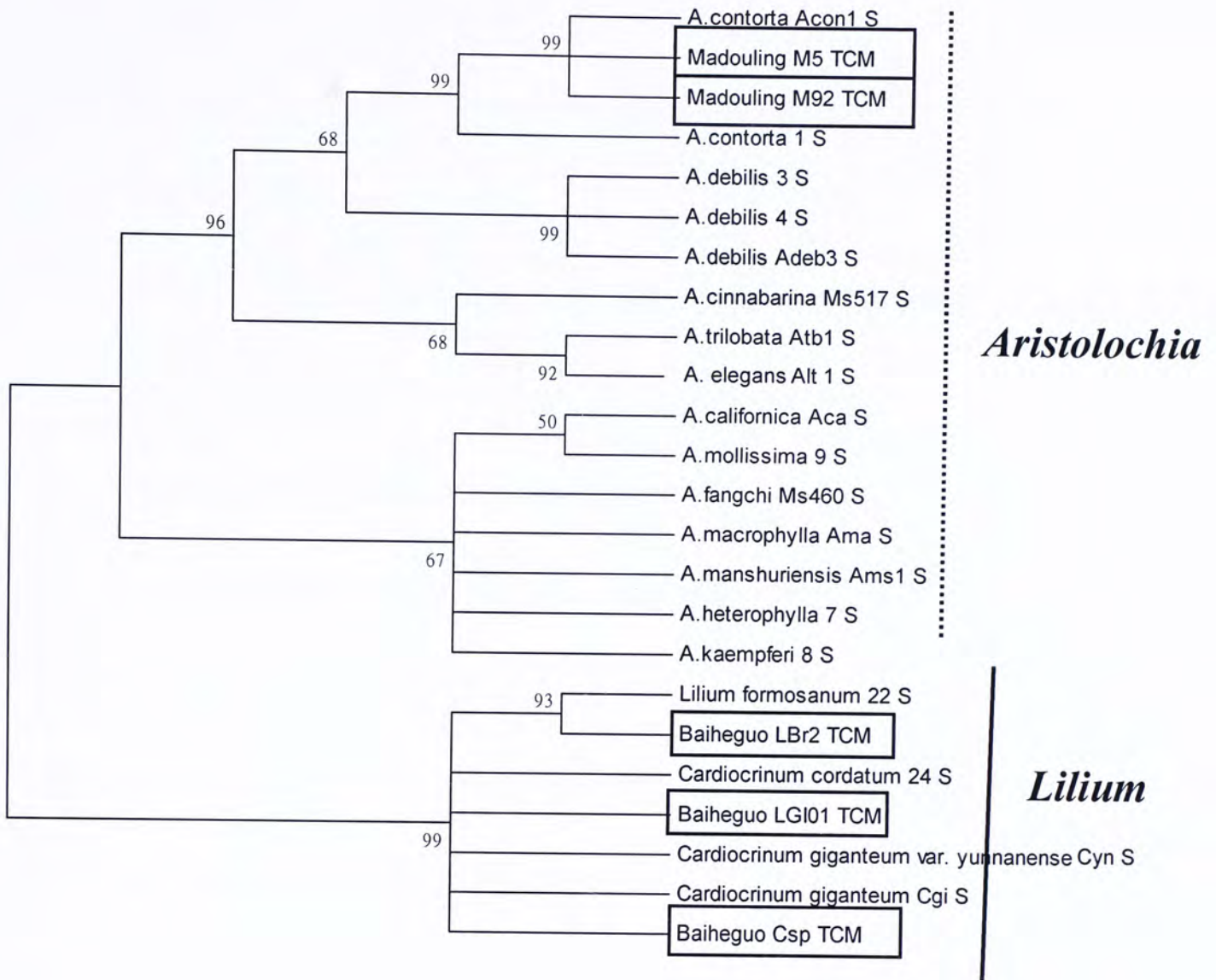
Figure 8.7 Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Madouling. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.

8.2 Discussion

8.2.1 Evaluation of chloroplast *trn*L-*trn*F region in differentiation of Madouling

In this study, *trn*L-*trn*F region was analyzed to differentiate genuine Madouling from the substitute Baihegao. The source species of these Madouling are *Aristolochia contorta* and *Aristolochia debilis*. The source species of Baihegao are *Cardiocrinum giganteum*, *Cardiocrinum giganteum* var. *yunnanens* and *Lilium longiflorum*, respectively.

The intraspecific similarity among species in *Aristolochia* is 87.7%, varying from 75.2–99.8%. The average interspecific similarity between *Aristolochia* and *Lilium* is 53.5% which are much lower that the intraspecific similarity of *Aristolochia*. This suggests that *trn*L-*trn*F can differentiate the genuine Madouling from the substitute Baihegao.

The dendrograms constructed by UPGMA and maximum parsimony using this region can clearly separate the two genera into two clades with bootstrap frequencies 100 or 99. The intraspecific similarity within the two genera are high, the relationship is not well resolved.

Although *trn*L-*trn*F region is quite conserved, differentiation of species is achieved by the dendrogram construction. Nucleotide changes of insertions/deletions

similarity of *psbA-trnH* region was much lower than that of *trnL-trnF* region, suggesting that the former region is less conserved than *trnL-trnF* and more variable for herb differentiating purposes. The *trnL-trnF* region is more conserved than *psbA-trnH* region and provides more sequence significant sites for herb differentiation.

Chapter 9: AUTHENICATION OF ZHUSHALIAN

9.1 Results

(A)

(B)

(C)
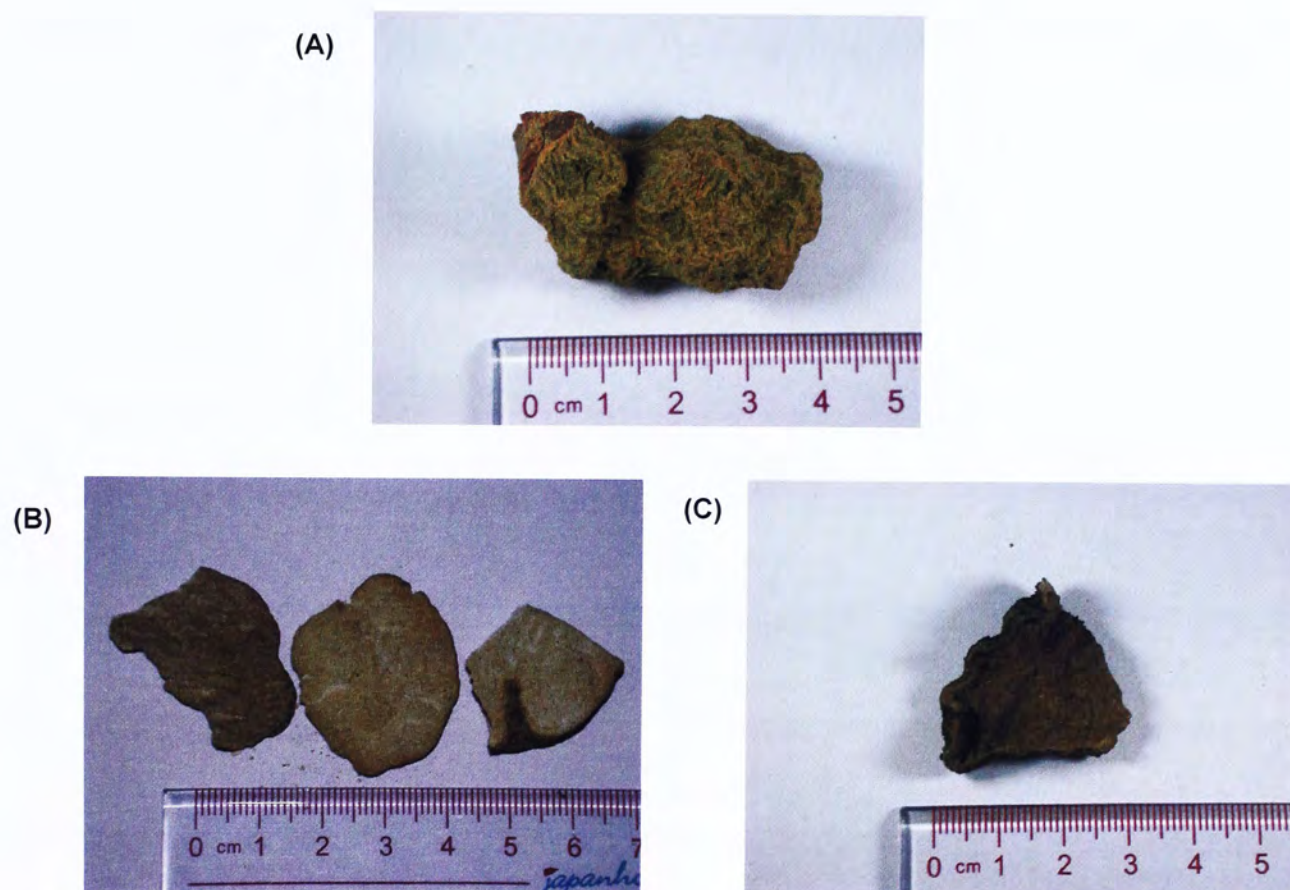
Figure 9.1 Morphological views of (A) Zhushalian , (B) Baiyaozi (Bay-1), (C)

Hongyaozi.

Due to the difficulties in amplifying the herb sample and the adulterant herb of

Zhushalian, this study focused on the differentiation of authentic species of only. The

DNA was successfully extracted from (a) one sample of adulterant herb Baiyaozi, (b)

one authentic sample of *Polygonum*, (c) one sample of *Dioscorea,* and (d) 15

authentic samples of *Aristolochia* species. The chloroplast *trn*L-*trn*F and *psb*A-*trn*H

gene regions of these 18 samples were sequenced. For *trn*L-*trn*F gene region analysis,

four additional *Dioscorea* sequences and three additional *Aristolochia* sequences were

downloaded from NCBI and were used in sequence alignment and constructing

dendrograms. For *psb*A-*trn*H region analysis, one additional *Dioscorea* sequence was

downloaded from NCBI.

9.1.1   Sequence alignment

9.1.1.1 *trn*L-*trn*F sequence

The 24 sequences of *trn*L-*trn*F region were aligned using the computer program

ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were

performed using BioEdit sequence Alignment Editor (Hall, 1999). The aligned

sequences are presented in Figure 9.2. Based on the sequence alignment of this gene

region of the genera *Aristolochia, Dioscorea* and *Polygonum*, only one site of

insertions/deletions or base changes can be utilized to differentiate the herbs derived

from these three genera. At site 695–700, there was a 5 bp insertion in *Aristolochia*

that could be used to differentiate the three different it from the other two.

9.1.1.2 *psb*A-*trn*H sequence

The 19 sequences of *psb*A-*trn*H region were aligned using the computer program

ClustalX version 1.83 (Thompson *et al.*, 1997). Additional manual amendments were

performed using BioEdit sequence Alignment Editor (Hall, 1999). The aligned

sequences are presented in Figure 9.3. From the sequence alignment of this gene

region of the genera *Aristolochia*, *Dioscorea* and *Polygonum*, due to insufficient data,

it is impossible to locate significant site of insertions/deletions or base changes can be

utilized to differentiate the samples.

9.1.2   Percentage similarity analysis

The percentage similarities of *trn*L-*trn*F region among the samples of relevant

authentic species of Zhushalian were calculated using BioEdit Sequencing Alignment

Editor (Hall, 1999). The result is listed in Table 9.1. The interspecific similarities

between *Aristolochia* and *Polygonum* varied from 58.4–62.5%, the average being

60.5%. The interspecific similarities between *Aristolochia* and *Dioscorea* varied from

45.2–48.4%, the average being 46.4%. The intraspecific similarities of *Aristolochia*

varied from 81.6–99.6%, the average being 89.96%,.

Sequence Name	Sequence

Sequence names (rows of the alignment):

- A.elegans _Alt1_S_
- A.trilobata_Atbl_S_
- A.manshuriensis_Ams1_S_
- A.manshuriensis_AY689184
- A.macrophylla_Ama_S_
- A.californica_Aca_S_
- A.californica_AY689174
- A.kaempferi_DQ532023
- A.heterophylla_7_S_
- A.kaempferi_8_S_
- A.mollissima_9_S_
- A.fangchi_Ms460_S_
- A.contorta_1_S_
- A.debilis_4_S_
- A.debilis_Adeb3_S_
- A.contorta_Acon1_S_
- A.cinnabarina_Ms517_S_
- Baiyaozi_Bay-1_TCM
- Polygonum_multiflorum_Pum-2_S
- Dioscorea_decipiens_DQ841329
- Dioscorea_alata_DQ841331
- Dioscorea_cirrhosa_DQ841324
- Dioscorea_cirrhosa_Dci-1_S
- Dioscorea_composita_DQ841330
- Clustal Consensus

Figure 9.2 Sequence alignment of *trn*L-*trn*F region for herb materials of Zhushalian and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5. One site of the nucleotide differences is highlighted in boxes.

Figure 9.2 (continued)

Figure 9.2 (continued)

Sequence Name	Sequence

A.elegans_Alt1_S_
A.trilobata_Atbl_S_
A.manshuriensis_Ams1_S_
A.manshuriensis_AY689184
A.macrophylla_Ama_S_
A.californica_Aca_S_
A.californica_AY689174
A.kaempferi_DQ532023
A.heterophylla_7_S_
A.kaempferi_8_S_
A.mollissima_9_S_
A.fangchi_Ms460_S_
A.contorta_4_S_
A.debilis_4_S_
A.debilis_Adeb3_S_
A.contorta_Acon1_S_
A.cinnabarina_Ms517_S_
Baiyaozi_Bay-1_TCM
Polygonum_multiflorum_Pum-2_S
Dioscorea_decipiens_DQ841329
Dioscorea_alata_DQ841331
Dioscorea_cirrhosa_DQ841324
Dioscorea_cirrhosa_Dci-1_S
Dioscorea_composita_DQ841330
Clustal Consensus

Figure 9.3 Sequence alignment of *psb*A-*trn*H region for herb materials of Zhushalian and the relevant authentic species. Details of the samples are presented in Tables 3.1–3.5.

Figure 9.3 (continued)

Sequence Name	Sequence

```
                                    ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                                    705        715        725        735        745        755        765        775        785        795        805        815        825        835
A.debilis_4__S_                     ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.debilis_Adeb3__S_                 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.contorta_Acon1__S_                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.contorta_1__S_                    ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.minutissima_Ms459_S               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTG- ATTTGTGAAT
A.cinnabarina_Ms517__S              ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTGGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.elegans__Alt1__S                  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTG- ATTTGTGAAT
A.trilobata_Atbl__S                 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.macrophylla_Ama__S_               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.manshuriensis_Ams1__S_            ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.heterophylla_7__S_                ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.fangchi_Ms460__S_                 ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.kaempferi_8__S_                   ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.californica_Aca__S_               ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
A.mollissima_9__S_                  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........-G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
Baiyaozi_Bay-1_TCM                  AATATCCTTA CTTCAGTATA TTCTAAAATG AAATACTCAT TCATCATTTA TAATAGAAAT TGAATGTTTT TGGGGGTAAG GTAAAGTATA AGG------G CGGATGTAGC -AAGTGGATC AAGGCAGTGG ATTTGTGAAT
Dioscorea_cirrhosa_Dci-1_S          ..........  ..........  .......-TC TCGATCTAAT AAGGTAAGAA TAAATTTAAA TACAATAATG ATGAATGGAA AAAGAGAAAA TCCTTTAGCT AGG------G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
Dioscorea_panthaica_DQ124704        ..........  ..........  .......-AAT AAATATAAAT ACAATAATGA TGAATGGAAA AAAGAGAAAA TCCTTTAGCT AGATAAGGGG CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATTTGTGAAT
Polygonum_multiflorum_Pum-2_S       TTTATAGT--  CTTCAAGAGA ----GAAAAA AAATGATGAA -----ATTTA TAAGTGTAAT TTGGACCATT TTCAAGTAAG GAACGAAAGA GGG------G CGGATGTAGC CAAGTGGATC AAGGCAGTGG ATT-GGAAT
Clustal Consensus                                                                                                                          *  *******  *  ********  *  *******  *** * ****
```

```
                                    ....|....| ...
                                    845
A.debilis_4__S_                     CCACCATGCG CG-
A.debilis_Adeb3__S_                 CCACCATGCG CG-
A.contorta_Acon1__S_                CCACCATGCG CG-
A.contorta_1__S_                    CCACCATGCG CG-
A.minutissima_Ms459_S               CCACCATGCG CG-
A.cinnabarina_Ms517__S              CCACCATGCG CG-
A.elegans__Alt1__S                  CCACCATGCG CG-
A.trilobata_Atbl__S                 CCACCATGCG CG-
A.macrophylla_Ama__S_               CCACCATGCG CG-
A.manshuriensis_Ams1__S_            CCACCATGCG CG-
A.heterophylla_7__S_                CCACCATGCG CG-
A.fangchi_Ms460__S_                 CCACCATGCG CG-
A.kaempferi_8__S_                   CCACCATGCG CG-
A.californica_Aca__S_               CCACCATGCG CG-
A.mollissima_9__S_                  CCACCATGCG CG-
Baiyaozi_Bay-1_TCM                  CCACCATGCG CG-
Dioscorea_cirrhosa_Dci-1_S          CCACCATGCG CG-
Dioscorea_panthaica_DQ124704        CCACCCTGCC CGG
Polygonum_multiflorum_Pum-2_S       CCACCATGCG CG-
Clustal Consensus                   ***** **   **
```

The percentage similarities of *psb*A-*trn*H region among samples of relevant

authentic species of Zhushalian were calculated using BioEdit Sequencing Alignment

Editor (Hall, 1999). The result is listed in Table 9.2. The interspecific similarities

between *Aristolochia* and *Polygonum* varied from 15.1–23.6%, the average being

19.6%. The interspecific similarities between *Aristolochia* and *Dioscorea* varied from

20.2–30.8%, the average being 24.4%. The intraspecific similarities of *Aristolochia* is

varying from 34.5–99.5%, the average being 59.1%.

9.1.3   Dendrogram study

Dendrograms were constructed using the computer program MEGA version 3.1

(Kumar *et al.*, 2001) with two tree construction methods: unweighted pair-group

methods using arithmetic averages (UPGMA; Figure 9.4 and 9.6) and maximum

parsimony (Figure 9.5 and 9.7). Each method was tested by bootstrap test with 1000

replications.

Table 9.1 Percentage similarities of *trn*L-*trn*F region among the plant and herb samples of Zhushalian.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]
[1]	A. elegans_Alt1_S_	95.5	87.8	87.8	87.7	86.9	85.4	88.5	88.7	88.7	88.4	88.8	91.5	87.5	87.5	85.4	85.5	64.6	58.6	47.2	43.2	47.8	47.5	47.6
[2]	A. trilobata_Atb1_S_		87.1	87.1	86.3	85.4	85	88.1	88.3	88.3	88	88.4	88.5	84.8	85.2	82.8	83.6	62.6	59.2	46	42.3	46.6	46.6	46.6
[3]	A. manshuriensis_Ams1_S_			99.6	95.7	92.4	91.9	95.4	95.7	95.7	95.4	95.9	86.4	86	86.5	83.5	89.4	64.8	61.2	47.7	43.9	48.3	48.3	48.4
[4]	A. manshuriensis_AY689184				95.6	92.3	91.9	95.3	95.6	95.6	95.3	95.8	86.3	85.9	86.4	83.4	89.3	64.7	61.3	47.7	43.9	48.3	48.3	48.4
[5]	A. macrophylla_Ama_S_					90.9	90.5	93.7	94.1	94.1	93.9	94.1	84.6	84.4	84.8	81.6	87.8	63.6	60.6	46.5	42.7	47	47	47.3
[6]	A. californica_Aca_S_						99.5	94.4	94.8	94.8	94.2	94.6	84.9	84.3	84.8	81.4	87.9	64.2	60.8	45.6	41.9	46.2	46.2	46.2
[7]	A. californica_AY689174							93.9	94.4	94.4	93.7	94.2	84.5	83.9	84.3	81	87.4	64	60.4	45.2	41.5	45.8	45.8	45.8
[8]	A. kaempferi_DQ532023								99	99	98.3	98.8	87.5	86.9	87.5	83.8	91.3	65.7	62.4	47	43.1	47.6	47.6	47.4
[9]	A. heterophylla_7_S_									99	98.3	99	87.8	87.1	87.7	84	91.8	65.9	62.5	47.2	43.3	47.8	47.8	47.6
[10]	A. kaempferi_8_S_										98.3	98.6	87.3	87.1	87.7	83.7	91.3	66	62.1	47.1	43.3	47.7	47.7	47.9
[11]	A. mollissima_9_S_											99	87.7	87.3	87.9	84	91.5	66	62.5	47.3	43.4	47.9	47.9	47.9
[12]	A. fangchi_Ms460_S_												87.7	87.1	87.7	84	92.2	65.7	62.3	47	43.2	47.6	47.6	47.5
[13]	A. contorta_1_S_													89.3	89.6	91.4	87	65	59	47.2	43.2	47.8	47.5	47.4
[14]	A. debilis_4_S_														99.2	91.4	87.7	65	59.5	47.6	43.6	48.1	48.1	48.3
[15]	A. debilis_Adeb3_S_															91.7	88	66.1	59.5	47.5	43.5	48.1	48.1	48.1
[16]	A. contorta_Acon1_S_																83.1	65.8	59.4	46.7	42.8	47.3	47	47.2
[17]	A. cinnabarina_Ms517_S_																	65.3	58.4	46.7	42.6	47.3	47	46.7
[18]	Baiyaozi_Bay-1_TCM																		56.3	43.6	39.9	44.2	44	44.1
[19]	Polygonum_multiflorum_Pum-2_S_																			42.4	39.6	42.9	42.9	43.2
[20]	Dioscorea_decipiens_DQ841329																				92.6	98.8	98.3	83.6
[21]	Dioscorea_alata_DQ841331																					92.2	92.7	79.2
[22]	Dioscorea_cirrhosa_DQ841324																						99.4	84.3
[23]	Dioscorea_cirrhosa_Dci-1_S_																							84.8
[24]	Dioscorea_composita_DQ841330																							

Table 9.2 Percentage similarities of *psbA-trnH* region among the plant and herb samples of Zhushalian.

		[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]
[1]	Polygonum_multiflorum_Pum-2_S	37.9	23.3	23.6	22.9	22.1	24.8	25.5	22.3	22.6	15.4	15.1	15.3	15.3	15.8	15.4	15.8	33.3	17.1
[2]	Baiyaozi_Bay-1_TCM		18.1	18.3	19.1	18.6	19.7	19.9	17.4	18	12.2	12	12.1	12.1	12.2	12	12.2	22.2	14.6
[3]	A.debilis_4_S_			99.5	79.8	77.9	71.2	70.3	64.8	67.2	36.1	35.6	36.3	36.4	36.7	34.8	36.2	27.8	17.2
[4]	A.debilis_Adeb3_S_				80.2	78.3	71.6	70.8	65.3	67.6	36.5	35.9	36.7	36.8	37.1	35.2	36.6	28.2	17.5
[5]	A.contorta_Acon1_S_					88.8	76.9	76	63.5	67.1	36.9	36.4	37.1	37.3	37.6	35.6	37	27.5	18.8
[6]	A.contorta_1_S_						75.2	74.3	64.7	66.8	38.8	38.3	39	39.2	39.6	37.5	39	27.1	19.6
[7]	A.minutissima_Ms459_S							97.8	69.7	70.2	41.9	41.3	42.1	42.3	42.9	40.5	42.3	31.5	20.4
[8]	A.cinnabarina_Ms517_S_								69.2	69.8	42.1	41.5	42.3	42.4	43.1	40.6	42.5	31.5	20.5
[9]	A.elegans_Alt1_S_									92	41.5	40.9	40.4	39.7	43	40.9	42.3	28.4	18.1
[10]	A.trilobata_Atb1_S_										39.5	38.9	38.8	38.6	40.9	38.9	40.2	28.3	18.2
[11]	A.macrophylla_Ama_S_											98.6	96	93.3	91.7	96.5	89.7	20.3	30.2
[12]	A.manshuriensis_Ams1_S_												94.7	92.1	90.4	95.2	88.4	19.9	29.7
[13]	A.heterophylla_7_S_													97.3	88	92.7	86.1	20.3	30.8
[14]	A.fangchi_Ms460_S_														87.3	90.1	85.4	20.4	31.2
[15]	A.kaempferi_8_S_															89.7	97.7	20.9	28.9
[16]	A.californica_Aca_S_																89.7	20.2	30.2
[17]	A.mollissima_9_S_																	20.9	28.5
[18]	Dioscorea_cirrhosa_Dci-1_S																		31.5
[19]	Dioscorea_panthaica_DQ124704																		

From the *trn*L-*trn*F region, the samples from two separate clades: *Aristolochia* clade and clade of genera *Polygonum* and *Dioscorea* with 100 bootstrap frequencies. In the clade consisting of *Polygonum* and *Dioscorea*, the herb sample Baiyaozi (Bay-1) clustered with the authentic sample of *Polygonum*. This clade was supported by bootstrap frequencies of 63 on the bootstrap consensus trees of UPGMA. *Dioscorea* samples clustered together to form a clade and was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony. The genuine species of Zhushalian, *A. kaempferi* and *A. cinnabarina,* clustered with other *Aristolochia* species to form a clade and was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 99 in bootstrap consensus trees of maximum parsimony.

From the *psb*A-*trn*H region, the samples are distributed into two separate clades: *Aristolochia* clade and clade of genera *Polygonum* and *Dioscorea* with 100 bootstrap frequencies. In the clade containing of *Polygonum* and *Dioscorea*, the herb sample Baiyaozi (Bay-1) clustered with the authentic sample of *Polygonum*. This clade was supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 95 in bootstrap consensus trees of maximum parsimony. *Dioscorea* samples clustered together to form a clade and is supported by bootstrap frequencies of 100 on the bootstrap consensus trees of UPGMA and 95 in bootstrap consensus

trees of maximum parsimony.

9.2 Discussion

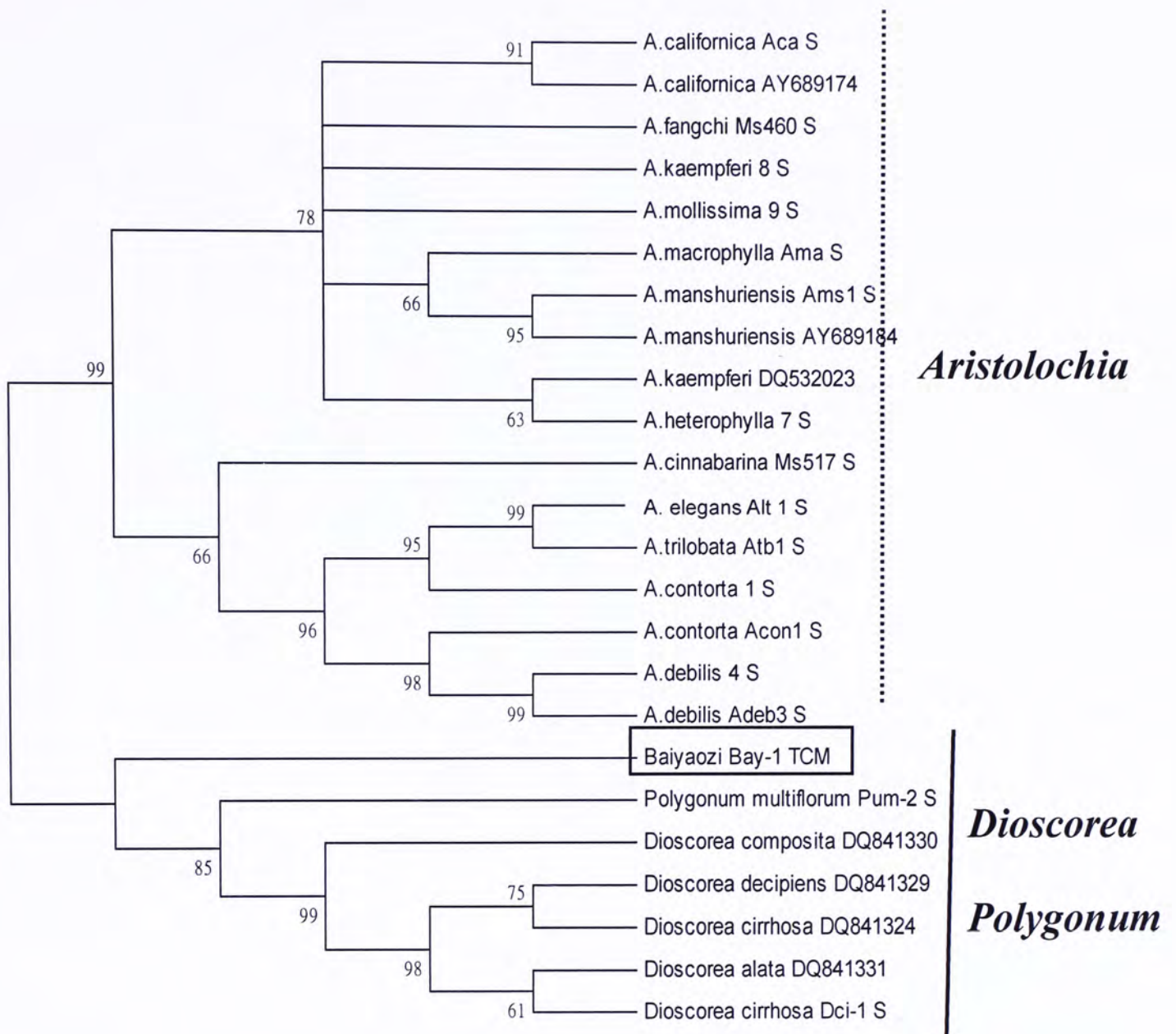### 9.2.1 Evaluation of chloroplast *trn*L-*trn*F region in differentiation of Zhushalian

In this study, *trn*L-*trn*F region was analyzed to differentiate genuine species of Zhushalian from the adulterant herb. The source species of genuine Zhushalian are *Aristolochia kaempferi, A. minutissima* and *A. cinnabarina.* The source species of adulterant herb are *Polygonum cillinerve and Dioscorea cirrhosa.*

The intraspecific similarities among species in *Aristolochia* is 90%, varying from 81.6–99.6%. The mean interspecific similarity between *Aristolochia* with *Polygonum* and *Dioscorea* is 51.1% which are much lower than the intraspecific similarities of *Aristolochia.* This suggests that *trn*L-*trn*F can differentiate the genuine species from the adulterant. The dendrograms construct by UPGMA and maximum parsimony using this region can clearly separate the clades with bootstrap frequency of 100 or 99.
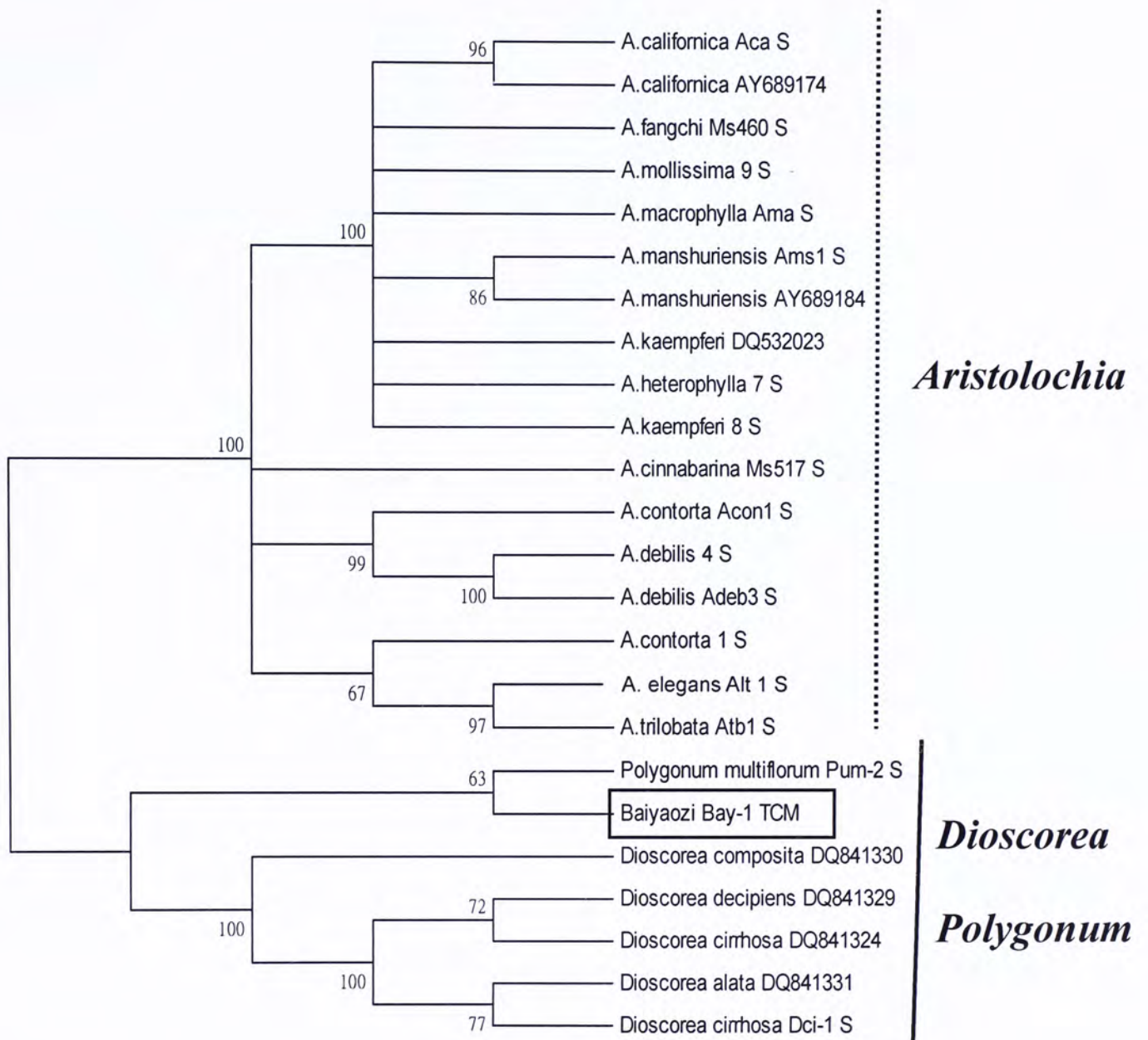
Figure 9.4 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Zhushalian. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.

Figure 9.5   Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *trn*L-*trn*F region of the authentic plant species and herb samples of Zhushalian. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
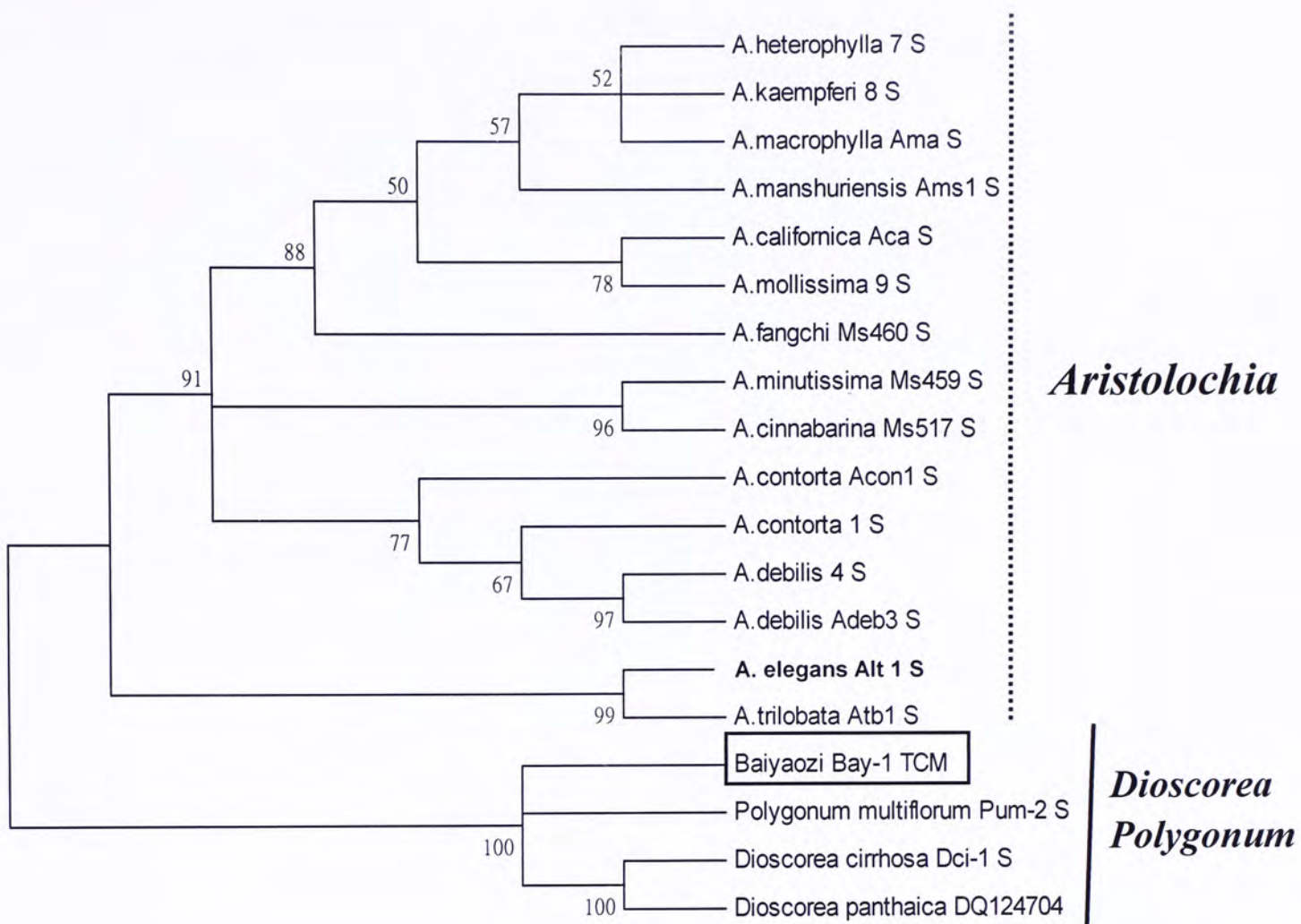
Figure 9.6 Bootstrap consensus tree (1000 replications) constructed by UPGMA method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Zhushalian. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.
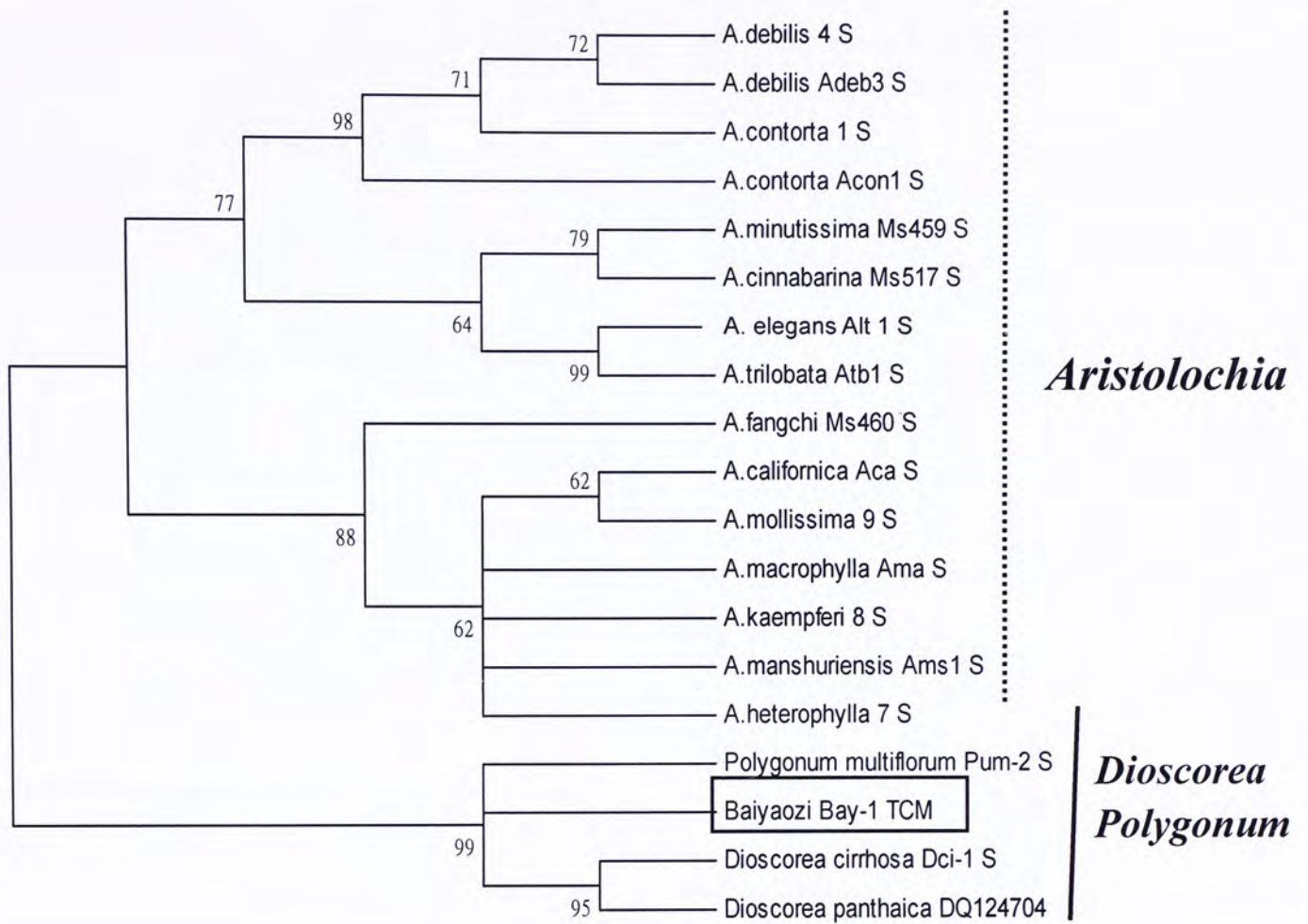
Figure 9.7 Bootstrap consensus tree (1000 replications) constructed by maximum parsimony method using chloroplast *psb*A-*trn*H region of the authentic plant species and herb samples of Zhushalian. Numbers above the branches are bootstrap frequencies with cutoff value of 50%. The abbreviations are sample labels. Details of the samples are presented in Tables 3.1–3.5.

9.2.2 Evaluation of chloroplast *psbA-trn*H region in differentiation of Zhushalian

The average intraspecific similarities within *Aristolochia* using *psb*A-*trn*H region is 59.1%, varying from 34.5–99.5%. This showed that the *psb*A-*trn*H region is variable within *Aristolochia*. The interspecific similarities between *Aristolochia* and *Polygonum* and *Dioscorea* is 21%. They are lower than the intraspecific similarities of *Aristolochia* suggesting that this region is sufficient to differentiate herb samples from different genera. But the data for *psb*A-*trn*H is not sufficient for differentiating the genuine and adulterants of Zhushalian.

9.3 Conclusion

Seven attempts were performed to extract the DNA from the herb samples of Zhushalian, using both CTAB and commercial kit extraction, but fail to obtain the genomic DNA. The results obtained from the research were used to construct a database in differentiate the genuine and adulterant Zhushalian. It is likely that, if the DNA of herb samples were successfully extracted, their would fall in the corresponding clades.

Chapter 10: *ARISTOLOCHIA* SPECIFIC MARKERS

10.1. ISSR fingerprinting*

The ultimate goal of this study was to identifying molecular markers for the rapid detection of *Aristolochia* material in herb samples. To explore for such markers, 17 fresh samples of *Aristolochia* were extracted and amplified with 21 inter-simple sequence repeat (ISSR) primers (UBC SSR Primer Set #9 - Microsatellite, Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada) were tested. They were 807 [(AG)$_8$-T], 808 [(AG)$_8$-C], 810 [(GA)$_8$-T], 811 [(GC)$_8$-C], 812 [(GA)$_8$-A], 817 [(CA)$_8$-A], 818 [(CA)$_8$-G], 825 [(AC)$_8$-T], 826 [(AC)$_8$-C], 834 [(AG)$_8$-YT], 842 [(GA)$_8$-YG], 845[(CT)$_8$-RG], 855 [(AC)$_8$-YT], 856[(AC)$_8$-YA], 857 [(AC)$_8$-Y], 867 [(GGC)$_6$], 878 [(GGAT)$_4$], 881 [GGGT(GGGGT)$_2$-G], 885 [BHB-(GA)$_7$], 887 [DVD-(TC)$_7$] and 890 [VHV-(GT)$_7$]. They are listed in Table 3.8.

In the first step for selecting useful primers, the DNA from a single fresh sample of *Aristolochia serpentaria* (ASE-1) was used to test the 21 ISSR primers.

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* Experiments of ISSR fingerprinting were conducted at the National Center for Natural Products Research, School of Pharmacy, University of Mississippi   172

The DNA of ASE-1 was extracted and amplified with the ISSR primers. They were resolved on agarose gel and different patterns of band combinations were observed. The ISSR fingerprints developed with the 21 primers are shown in Figure 10.1. Among the 21 primers, 10 primers (primers 807, 808, 811, 812, 817, 825, 826, 842, 856 and 857) which produced clear band patterns were selected. Three more *Aristolochia* species, *A. elegans* (Alt-1), *A. manshuriensis* (Ams-1) and *A. trilobata* (Atb-1), were used to test the value of developing genus-specific band patterns with the 10 selected primers. All 10 primers developed different band patterns among the four different *Aristolochia* species.
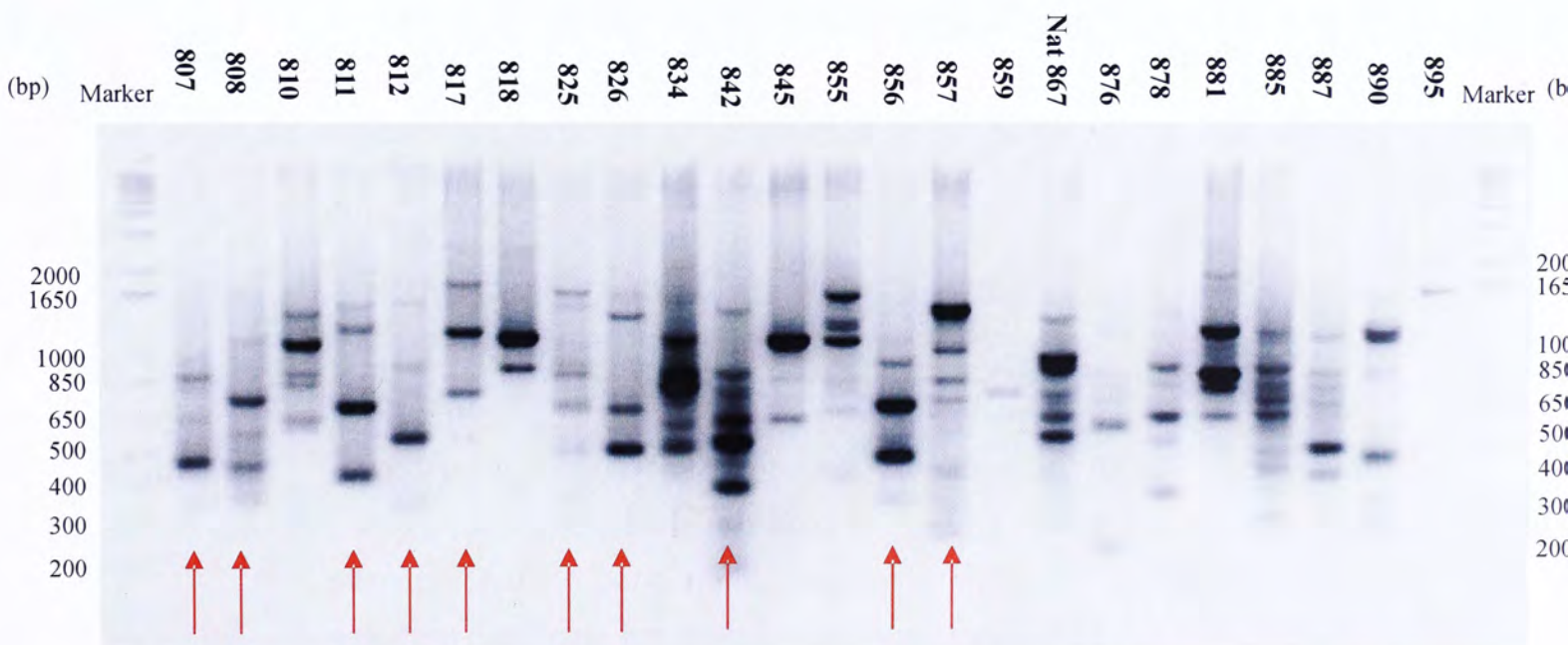
Figure 10.1 ISSR fingerprint of *Aristolochia serpentaria* (ASE-1) using primer 21 primers listed in Table 3.6.

From the band patterns developed by the 10 primers, five of them showed bands that were of similar molecular size among the four different *Aristolochia* samples. The bands that are highlighted by a bracket in Figures 10.2–10.6. could be potential candidates for identifying *Aristolochia* specific markers.
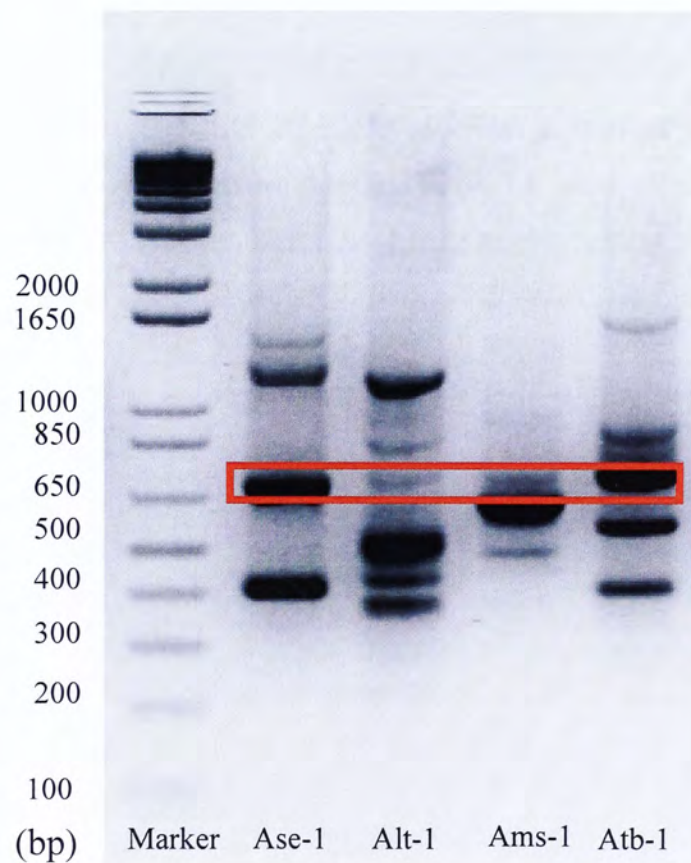
Figure 10.2 ISSR fingerprints of *Aristolochia serpentaria* (ASE-1), *A. elegans* (Alt-1), *A. manshuriensis* (Ams-1), and *A. trilobata* (Atb-1) using primer 811. The band highlighted in a bracket was regarded as is a potential candidate for *Aristolochia*-specific marker.
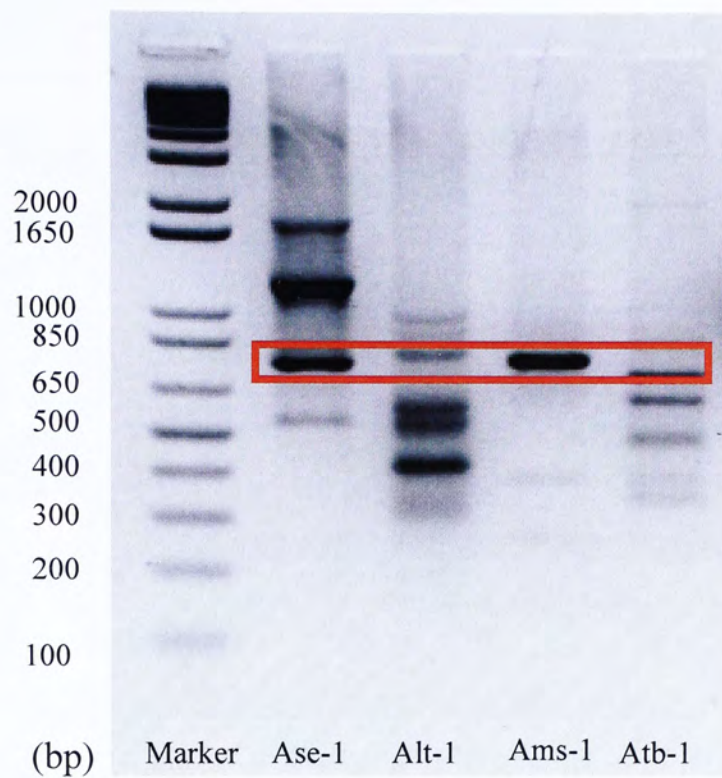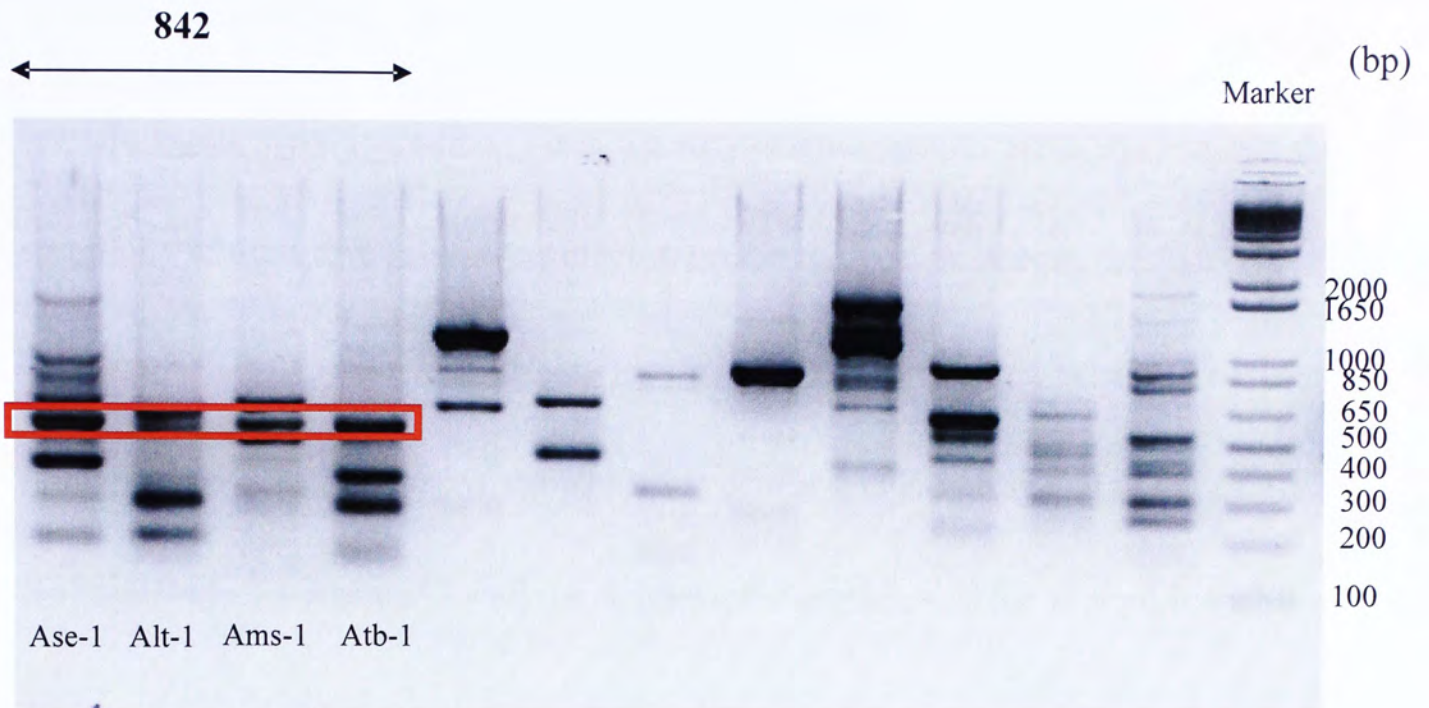
Figure 10.3 ISSR fingerprints of *Aristolochia serpentaria* (ASE-1), *A. elegans* (Alt-1), *A. manshuriensis* (Ams-1), and *A. trilobata* (Atb-1) using primer 817. The band highlighted in a bracket was regarded as is a potential candidate for *Aristolochia*-specific marker.
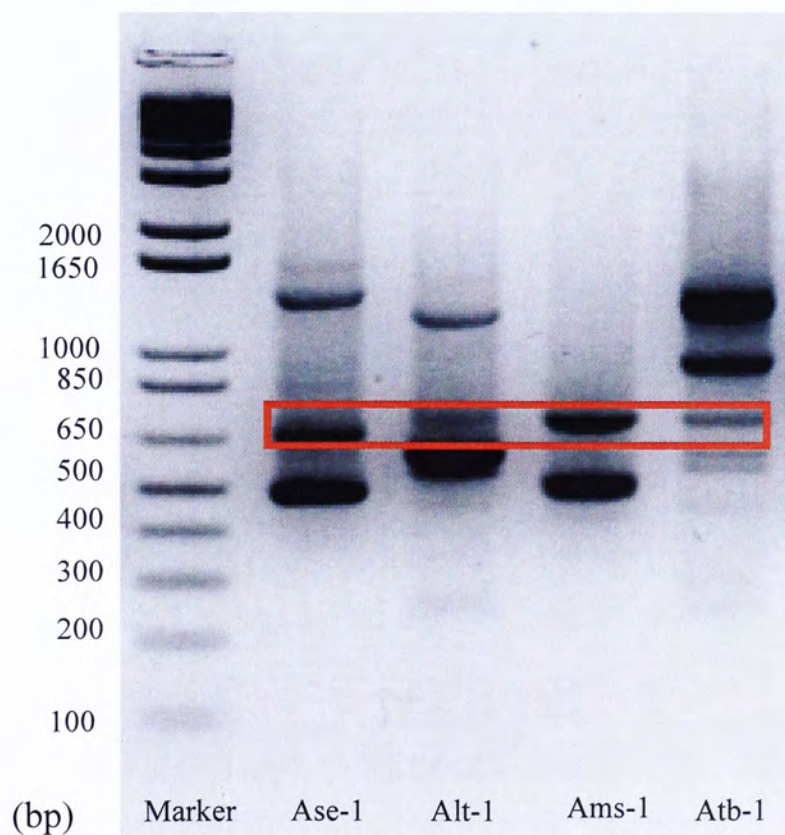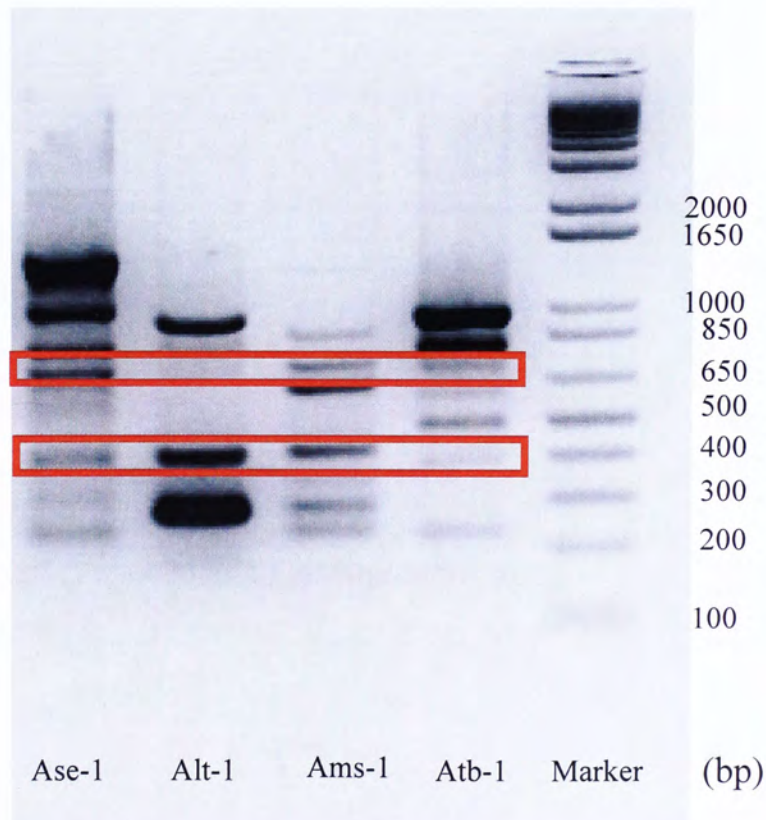

Figure 10.4 ISSR fingerprints of *Aristolochia serpentaria* (ASE-1), *A. elegans* (Alt-1), *A. manshuriensis* (Ams-1), and *A. trilobata* (Atb-1) using primer 826. The band highlighted in a bracket was regarded as is a potential candidate for *Aristolochia*-specific marker.
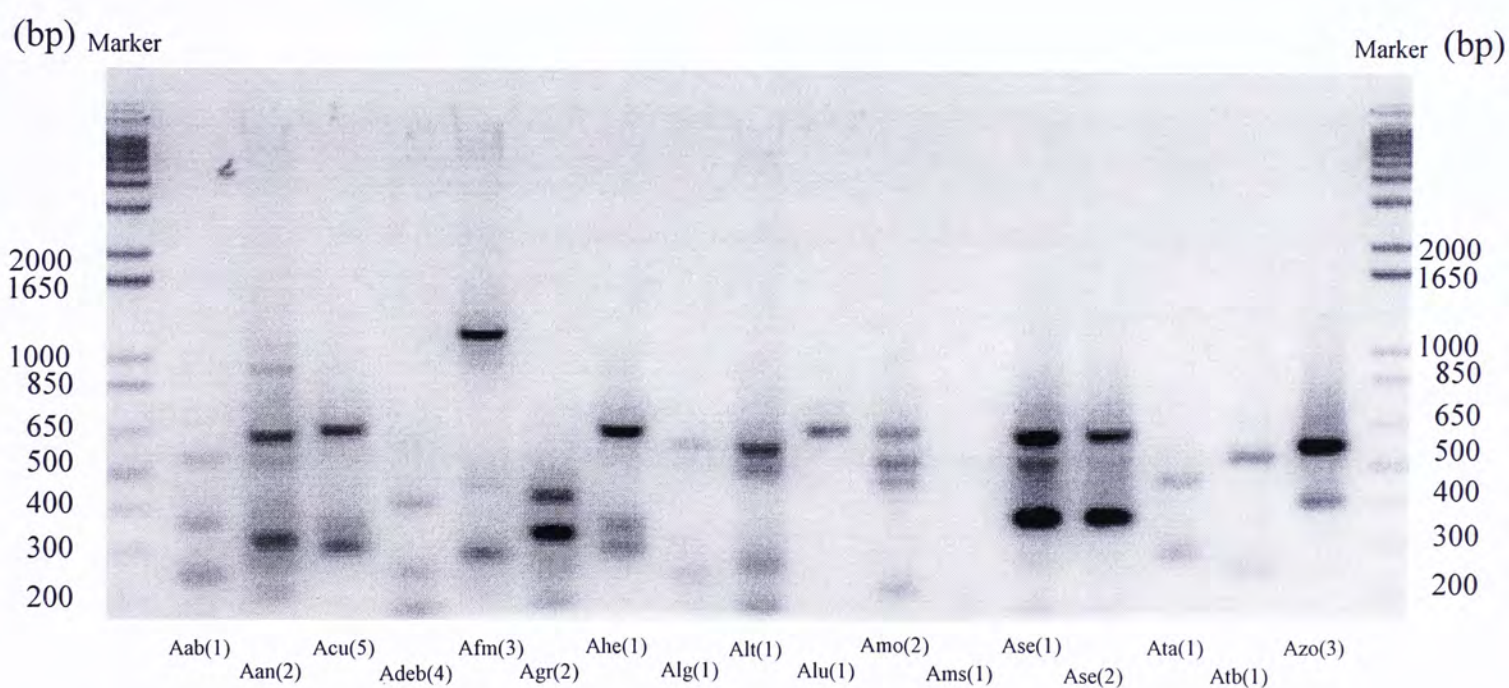
Figure 10.5 ISSR fingerprints of *Aristolochia serpentaria* (ASE-1), *A. elegans* (Alt-1), *A. manshuriensis* (Ams-1), and *A. trilobata* (Atb-1) using primer 842. The band highlighted in a bracket was regarded as is the potential candidate for *Aristolochia*-specific marker.

Figure 10.6 ISSR fingerprints of *Aristolochia serpentaria* (ASE-1), *A. elegans* (Alt-1), *A. manshuriensis* (Ams-1), and *A. trilobata* (Atb-1) using primer 857. The band highlighted in a bracket was regarded as is potential candidates for *Aristolochia*-specific marker.

Five primers (primers 811, 817, 826, 842 and 857) with potential *Aristolochia*-specific bands were identified. Then, 16 *Aristolochia* species were used to test the specificity of these five primers on other *Aristolochia* species. Among the five primers (primers 811, 817, 826, 842 and 857), primer 842 gave the best results. The ISSR fingerprints of 17 *Aristolochia* samples using primer 842 are presented in Figure 10.7. No band was found shared by all the *Aristolochia* species, and therefore this trial was not successful in locating potiential specific band.
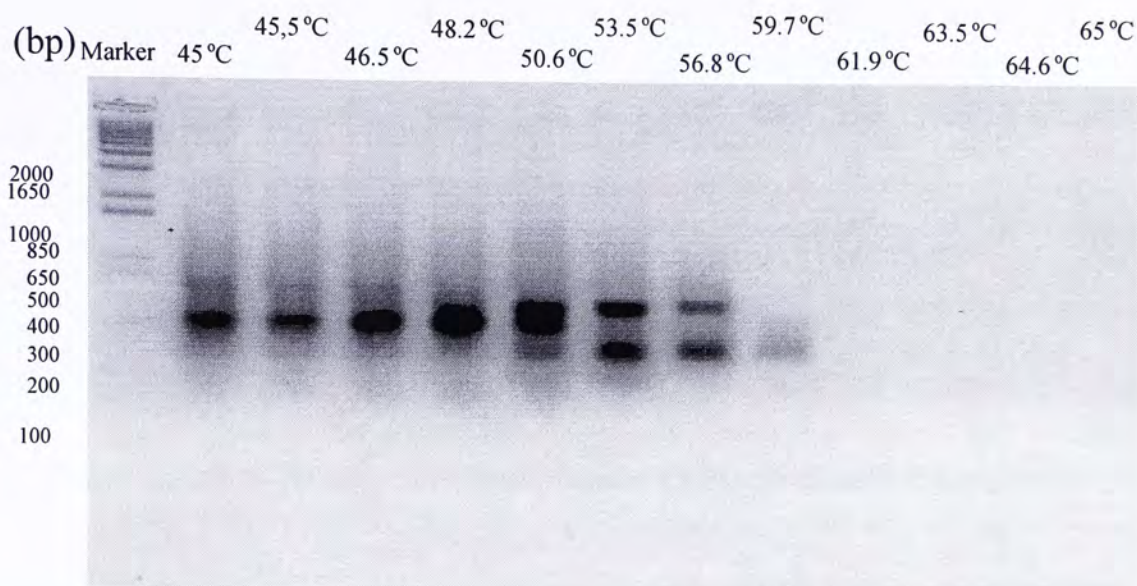

Figure 10.7   ISSR fingerprints of 17 *Aristolochia* samples (listed in Table 3.6) using primer 842.

10.2 Discussion

From the results, some of the ISSR primers (primers 811, 817, 826, 842 and 857) were more useful in locating *Aristolochia*-specific markers. But repeated tests did not reveal any specific bands that were universal to all *Aistolochia* Species.

The sample size of the *Aristolochia* species in locating the *Aristolochia*-specific band is small. Only 16 species were used in the ISSR screening. More *Aristolochia* species are needed to further test the value of the *Aristolochia*-specific markers. Only one ISSR condition was used thoughtout the experiments. Various factors such as annealing temperature could have affected the band patterns. Figure 10.8 showed the change of band patterns by annealing temperature in the ISSR of *Aristolochia serpentaria* (ASE-1), using primer 842. The bands gradually changed from one single thick band at 45°C to two separate bands above 50 °C. On the other hand, at temperatures higher than 61.9 °C, no bands were amplified.

Figure 10.8 Temperate-gradient ISSR fingerprints of *Aristolochia serpentaria* (ASE-1) using primer 842.

Some of the bands that were highlighted with a bracket in Figure 10.7 were weak. The experiment should be further repeated to obtain a clearer result. For future research, the band that may serve as the possible candidate for *Aristolochia*-specific marker can be extracted from the agarose gel and purified for DNA sequencing. These short sequences so obtained can then be tested on more *Aristolochia* samples, as well as on non-*Aristolochia* samples and herb mixtures to test the specificity of these selected bands in *Aristolochia*. Sequences that are specific to *Aristolochia* can be used as a primer or DNA tag in the detection of herb commodities derived from *Aristolochia*.

Chapter 11: CONCLUSION

Aristolochic acid nephropathy (AAN) has been a major issue of herbal poisoning since the 1990s. Numerous cases were reported worldwide including mainland China, Belgium, England and Japan. Researchers used different methods to differentiate Chinese herbs and their related *Aristolochia* herbs, for example by their morphology, macroscopic and microscopic features, chemical analyses, pyrosequencing and DNA fingerprinting.

In this study, molecular techniques of DNA sequencing *trn*L-*trn*F gene region and the *psb*A-*trn*H gene region were tested for differentiating six different Chinese herbs: Mutong, Muxiang, Baiying, Fangji, Madouling and Zhushalian, from their corresponding adulterants or substitutes derived from *Aristolochia* species.

Two different DNA regions were used in this research, *trn*L-*trn*F region and *psb*A-*trn*H region. The *psb*A-*trn*H region is more variable than the *trn*L-*trn*F region and was found to not only successfully in differentiate the six Chinese herbs from the adulterant/substitute genera, but also give more information on the dendrogram clade relationship. The chloroplast *trn*L-*trn*F region is relatively conserved, but more sequence significant sites were identified for herbs differentiation in *trn*L-*trn*F than *psb*A-*trn*H region. The sequences of either regions can be used for differentiating the

six Chinese herbs from the adulterant/substitute genera.

Other DNA regions had been applied to differentiate Chinese herbs and their related *Aristolochia* herbs. Li, (2005) successfully in using *trnL-trnF* region in differentiating Baiying and Xungufeng but failed in using ITS regions as the latter region was too variable for proper alignment. Chen (2004) and Shum et al. (2997) successfully used ITS regions and 5S spacer in differentiating 28 Muxiang and related species samples.

Regardless of the tools in differentiating the Chinese herbs, all these methods aim at a common goal: enhancing the safety and efficacy in using Chinese medicine. With the rising popularity of Chinese herbs in western countries, it is important to standardize TCM. No single method can be used solely; but DNA sequencing can definitely serve as a key approach to help safeguard the authenticity of TCM.

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APPENDIX – MATERIALS PREPARATION

Agarose gel loading buffer, 6X

Bromophenol blue, 0.25% (w/v)

Sucrose, 40% (w/v)

Ampicillin

Ampicillin, 50 mg/ml, in distilled water

CTAB solution, 2X

CTAB, 20 g/L

EDTA, 20 mM, pH 8.0

NaCl solution, 1.4 M

PVP, 1%

Tris–HCl, 100 mM, pH 8.0

Autoclaved at 121°C for 20 min

EDTA, 0.5M, pH 8.0

Ethylenediaminetetra – acetate $\cdot$ 2H$_2$O, 186.1 g/L

pH 8.0 was adjusted by adding solid NaOH

Autoclaved at 121°C for 20 min

Extraction buffer

EDTA, 25 mM

NaCl, 200 mM

SDS, 0.5%

Tris–HCl, 200 mM, pH 8.0

Autoclaved at 121°C for 20 min

IPTG

IPTG, 0.4 M, in distilled water

LB medium

Luria – Bertani powder (USB Cat # US75852), 20 g/L

Autoclaved at 121°C for 20 min

LBA plate

Luria agar (USB Cat # US75853), 37 g/L

Autoclaved at 121°C for 20 min

Ampicillin was then added to final concentration of 50 μg/ml

20ml solution was poured to sterile dish

MgCl$_2$ solution, 2M

MgCl$_2$.6H$_2$O, 406.6 g/L

Autoclaved at 121°C for 20 min

NaCl solution, 1M

NaCl, 23.376 g/L

Autoclaved at 121°C for 20 min

PCR buffer, 10X

(NH$_4$)$_2$SO$_4$, 200 mM

Tris – HCl, 750 mM, pH 8.8

Tween 20, 0.1%

Autoclaved at 121°C for 20 min

Sodium Acetate, 3M, pH 5.3

Sodium acetate, 246.09 g/L

pH 5.3 was adjusted by glacial acetic acid.

Autoclaved at 121°C for 20 min

TAE, 10X

Acetic acid, 11.4 ml

EDTA, 0.5 M, 20 ml/L, pH 8.0

Tris – base, 44.6 g/L

Autoclaved at 121°C for 20 min

TE buffer, pH 8.0

EDTA, 0.1 mM, pH 8.0

Tris – HCl, 10 mM, pH 8.0

Autoclaved at 121°C for 20 min

Tris – HCl, 1M, pH 8.0

Tris, 121 g/L

pH 8.0 was adjusted by HCl, 6 M

Autoclaved at 121°C for 20 min

X–gal, 5%

X–gal, 5%, in dimethyl formamide