JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Phylogeny of *Astragalus* in China: Molecular Evidence from the DNA Sequences of 5S rRNA Spacer, ITS, and 18S rRNA

TINA T. X. DONG, XIAO Q. MA, CLARLES CLARKE, ZONG H. SONG, ZHAO N. JI, CHUN K. LO, AND KARL W. K. TSIM*

Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong SAR, China

Radix Astragali (root of *Astragalus*; Huangqi) is a traditional Chinese medicine commonly used as an immunostimulant, hepatoprotective, diuretic, antidiabetic, analgesic, expectorant, and sedative drug. Although the species of Radix Astragali have been defined as *Astragalus membranaceus* and *A. membranaceus* var. *mongholicus* in Pharmacopoeia of China, their taxonomy remains controversial. The phylogenetic relationships among 10 *Astragalus* taxa, which are commonly found in China including *A. membranaceus*, *A. membranaceus* var. *mongholicus*, *Astragalus propinquus*, *Astragalus lepsensis*, *Astragalus aksuensis*, *Astragalus hoantchy*, *Astragalus hoantchy* subsp. *dshimensis*, *Astragalus lehmannianus*, *Astragalus sieversianus*, and *Astragalus austrosibiricus*, were determined using the DNA sequences of the 5S ribosomal RNA (5S rRNA) spacer, internal transcribed spacer region (ITS), and 18S rRNA coding region. The 5S rRNA spacer, ITS, and 18S rRNA, amplified by polymerase chain reaction from the isolated genomic DNAs, were sequenced. By using neighbor-joining and maximum parsimony analyses, phylogenetic trees were mapped by their sequence diversity. *A. membranaceus* and *A. membranaceus* var. *mongholicus* shared the greatest sequence homology. In addition, *A. propinquus* shared a closer relationship with *A. membranaceus* and *A. membranaceus* var. *mongholicus*, while other *Astragalus* species were less closely related. This is the first paper to show the phylogenetic relationship of *Astragalus* species related to Radix Astragali in China by the molecular genetic approach.

KEYWORDS: ***Astragalus*; DNA sequence; phylogeny; Radix Astragali**

INTRODUCTION

In traditional Chinese medication, when one has Qi deficiency and Yang weakness, one will be prescribed Radix Astragali (root of *Astragalus* species; Huangqi), which has been proven to be an immunostimulant, tonic (adaptogenic), hepatoprotective, diuretic, antidiabetic, analgesic, expectorant, and sedative drug (*1*−*3*). Although Radix Astragali has a long history of usage in Chinese herbal medicine, its pharmacological properties and clinical applications have not been studied until recently.

Astragalus L. (Leguminosae) is a large genus with over 2000 species worldwide and more than 250 sections in the angiosperm family Fabaceae (subfamily Papilionoideae). Distribution is mainly in cool arid continental regions of the Northern Hemisphere and South America, while *Astragalus* is especially diverse in southwestern Asia (ca. 1000−1500 spp.) (*4*). Of these, 278 species, two subspecies, 35 varieties, and two forms are recorded from China (*5*). The most commonly used Radix Astragali are roots of *Astragalus membranaceus* (Fisch.) Bunge and *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) (*3*, *6*, *7*); both are listed as the authentic botanical sources of Radix Astragali in Chinese Pharmacopoeia (*1*, *3*).

Although *A. membranaceus* and *A. membranaceus* var. *mongholicus* are very similar in morphology and chemical composition (*3*, *6*, *8*), their taxonomic status and, by implication, phylogenetic relationship are not clear. The original species of Radix Astragali were determined by Hsiao (*6*) to be *A. membranaceus*, *Astragalus mongholicus*, and *Hedysarum polybotrys* Hand.-Mazz. On the basis of the morphological characters, Hsiao (*6*) further reduced *A. mongholicus* to a variety of *A. membranaceus*. This interpretation was adopted by Qian (*9*) and Fu (*5*) but has not received broad support from scientists outside China (*10*−*12*). Another controversial taxonomic problem, *Astragalus propinquus* Schischk., which is presumed to be closely related to *A. membranaceus* and *A. membranaceus* var. *mongholicus*, was considered to be absent from China by Fu (*5*), even though it is known to occur in Xinjiang Province (*1*). Although the geographical distributions of *A. propinquus* and *A. membranaceus* (including *A. membranaceus* var. *mongholicus*) are distinct in China, the aforementioned factors frequently lead to taxonomic uncertainty among scientists and raise the possibility that adulterants of Radix Astragali could go undetected.

The DNA sequences of 5S ribosomal RNA (5S rRNA) spacer (*13*−*16*), internal transcribed spacer region (ITS; *4*, *17*), and

* To whom correspondence should be addressed. Tel: (852)2358-7332. Fax: (852)2358-1559. E-mail: botsim@ust.hk.

10.1021/jf034278x CCC: $25.00
Published on Web 10/01/2003

Table 1. Voucher Number, Respective Length, and GenBank Accession Number of the 5S rRNA Spacers, ITS, and 18S rRNA Coding Regions in the Ten *Astragalus* Taxa Examined

taxon[a]	voucher no.	5S rRNA spacer[b]		ITS[b]		18S rRNA[b]	
		length (bp)	accession no.	length (bp)	accession no.	length (bp)	accession no.
A. membranaceus	99-01-102	234	AF239711	619	AF359749	1784	AF359594
A. membranaceus var. *mongholicus*	99-01-089	233	AF239712	619	AF359750	1784	AF359595
A. propinquus	99-02-038	232	AF359580	618	AF359751	1786	AF359596
A. lepsensis	99-06-482	237	AF359581	616	AF359752	1784	AF359597
A. aksuensis	99-05-255	235	AF359582	617	AF359753	1784	AF359598
A. hoantchy	99-04-011	226	AF239713	618	AF359754	1785	AF359599
A. hoantchy subsp. dshimensis	99-04-438	216	AF359583	618	AF359755	1785	AF359600
A. lehmannianus	99-08-103	228	AF239714	612	AF359756	1791	AF359601
A. sieversianus	99-07-199	227	AF359584	618	AF359757	1786	AF359602
A. austrosibiricus	99-09-211	215	AF359585	611	AF359758	1786	AF359603

[a] Ten different *Astragalus* taxa were chosen for analysis. These *Astragalus* taxa are commonly found in China and could serve adulterants of Radix Astragali. Different populations of *Astragalus* were collected fresh and dried as described in the Materials and Methods. [b] About 15 individual samples from each taxon were analyzed. Specific primers for 5S rRNA spacer ITS and 18S rRNA are shown in **Figure 1A**.

18S rRNA coding region (*18*) have been used to determine the phyogenetic relationship of various genus. In addition, the genetic analysis on 5S rRNA spacers of *A. membranaceus* and *A. membranaceus* var. *mongholicus* has been reported (*19*). To investigate the phylogenetic relationships of Radix Astragali with other related species of *Astragalus*, the DNA sequences of 5S rRNA spacer, ITS, and 18S rRNA coding region were determined and compared among 10 *Astragalus* taxa that included *A. membranaceus*, *A. membranaceus* var. *mongholicus*, *A. propinquus*, *Astragalus lepsensis* Bunge, *Astragalus aksuensis* Bunge, *Astragalus hoantchy* Franch., *A. hoantchy* Franch subsp. *dshimensis* (Gontsch.), *Astragalus lehmannianus* Bunge, *Astragalus sieversianus* Pall., and *Astragalus autrosibiricus* Schischk. These taxa are thought to be closely related and are commonly found in northern parts of China. The phylogenetic relationships of these taxa are also discussed in a biogeograhical context.

MATERIALS AND METHODS

Selection of Taxa and Sources of Plant Materials. Ten taxa of *Astragalus* related to Radix Astragali were collected from China for investigation. *A. membranaceus* was obtained from Heilongjiang, Jilin, Neimenggu, and Shanxi; *A. membranaceus* var. *mongholicus* was from Neimenggu, Shanxi, Hebei, and Xinjiang; *A. hoantchy* was from Ninxia, Neimenggu, Gansu, and Qinhai; *A. austrosibiricus* was from Qinhai and Xinjiang. *A. propinquus*, *A. lepsensis*, *A. aksuensis*, *A. lehmannianus*, *A. sieversianus*, and *A. hoantchy* subsp. *dshimensis* were all collected from Xinjiang. They were kept in silica gel during the field collection. Samples of the tested species were stored dry for further identification; the storage time was less than 6 months before the DNA extraction. About 15 different samples collected from different geographical regions were tested in each species. At least three samples were collected from one geographical population, and they were collected in September to October, and they were all 3 year old plants (as described in *20*). A single plant was used for DNA extraction and analysis. Voucher specimens (**Table 1**; see also *20*) were deposited in the Department of Biology, The Hong Kong University of Science and Technology, Hong Kong, China.

Genomic DNA Extraction. Fresh leaves (desiccated by silica gel) were frozen with liquid nitrogen and ground into powder. Genomic DNA was extracted from the ground powder by using DNA extraction buffer consisting of 25 mM Tris-HCl, pH 8.0, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate, 10 μg/mL RNase, and 0.2% β-mercaptoethanol. The mixed solution was incubated for 15 min at 58 °C and then centrifuged at 12 000*g* for 10 min (*13*, *16*). The supernatant was extracted by equal volumes of water-saturated phenol:chloroform (1:1), mixed, and then centrifuged. The aqueous phase was collected and added to 0.1 volume of 3 M sodium acetate, and 2 volumes of 100% ethanol were added. The resulting pellet was collected after centrifugation at 12 000*g* for 15 min at 4 °C and dissolved in Tris-EDTA.

Polymerase Chain Reaction (PCR) Amplification and DNA Sequencing. A 50 μL PCR reaction mix consisted of 5 μL of 10X reaction buffer, 1 μL each of 10 mM dNTPs stock, 2.5 μL of forward and reverse primers (synthesized by Invitrogen Life Technologies, Carlsbad, CA), and 1 unit of *Taq* polymerase (Boehriger Mannheim, Indianapolis, IN). The primers used for amplification of the 5S rRNA spacer were 5S-1 forward primer (5′-GGA TCC GTG CTT GGG CGA GAG TAG TA-3′) and 5S-2 reverse primer (3′-GGA TCC TTA GTG CTG GTA TGA TCG CA-5′) (*13*, *16*, *19*). The primers for amplification of ITS were ITS-1 forward primer (5′-AGG AGA AGT CGT AAC AAG GT-3′) and ITS-2 reverse primer (3′-TGA TAT GCT TAA ACT CAG CG-5′) (*21*). Primers used for amplification of 18S rRNA were 18S-1 forward primer (5′-CTG GTT GAT CCT GCC AG-3′) and 18S-2 reverse primer (3′-CAC CTA CGG AAC CCT TG-5′) (*18*). The specific locations of these primers are shown in **Figure 1A**. Approximately 50 ng of genomic DNA was used as a template for the reaction. The reaction mix was overlaid with mineral oil and placed in a Robocycle Gradient 40 (Stratagene, La Jolla, CA). Cycling (30 cycles) condition was described previously (*13*, *16*, *19*). The PCR products were subjected to ~1% agarose gel electrophoresis and visualized by ethidium bromide staining under UV. DNAs were purified by agarose Gel DNA Extraction Kit (Boehringer Mannheim).

The PCR products were subcloned into a TA cloning vector pTAg (R&D Systems, U.K.) (*22*). The plasmids were purified for sequencing. ABI Prism, BigDye Terminator, and cycle Sequencing Ready Reaction Kit (all from Applied Biosystems, Foster City, CA) were used for sequence reaction with T7 or SP6 primers. The longer clones were sequenced by using oligonucleotides synthesized according to the sequences obtained by T7 or SP6 primers. The sequences were detected using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Several PCR products from one sample were sequenced several times in both directions.

Sequence Alignments and Polygenetic Trees. Comparisons of entire sequences of the tested samples were aligned by a software Clustal X having a gap opening of 10 and gap extension of 0.05. Polygenetic trees based on the hierarchical clustering of the alignments of the 5S rRNA spacer, ITS, and 18S rRNA were assessed by both neighbor-joining (UPGMA) and maximum parsimony analyses (PAUP). An initial parsimony analysis was performed on each set of sequences using a heuristic search with a random addition sequence of 10 replicates, tree−bisection−reconnection branch-swapping, with *A. austrosibiricus* as the out group. Gaps were treated as "missing". The preliminary analyses of the 5S rRNA spacer and ITS sequences revealed two main clades, but analysis of the 18S rRNA sequence was inconclusive; thus, further analyses were performed only on the 5S rRNA spacer and ITS sequences.

Additional parsimony analyses were performed using heuristic searches of the bootstrap method in PAUP. Because of probably rate heterogeneity between ITS and 5S rRNA spacer regions, separate analyses were performed for these sequences. Bootstrap values were calculated from 200 replicates of analyses using the random addition

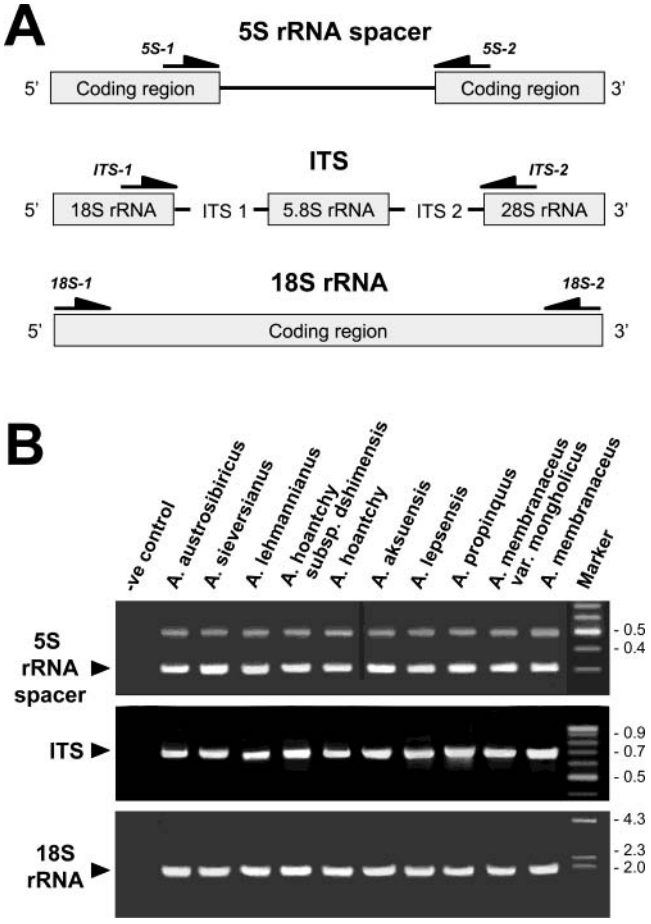

Figure 1. PCR of 5S rRNA spacer, ITS, and 18S rRNA from different *Astragalus* taxa. (**A**) Basic organizations of the repeating units in 5S rRNA spacer, ITS, and 18S rRNA gene clusters in higher plants. The coding regions (shaded) and primers flanking the regions are shown. (**B**) The PCR products were separated in a 1.2% agarose gel. Agarose gel electrophoresis of 5S rRNA spacer, ITS, and 18S rRNA. PCR products (~1 μg) were generated by primers flanking 5S rRNA spacer (5S-1 and 5S-2), ITS (ITS-1 and ITS-2), and 18S rRNA (18S-1 and 18S-2). Sizes of the PCR products are indicated by arrowheads. By using DNA templates from various sources of each species, the same size of PCR product was obtained from 15 tested samples ($N = 15$). DNA markers in kb are indicated. Negative control contained no genomic DNA.

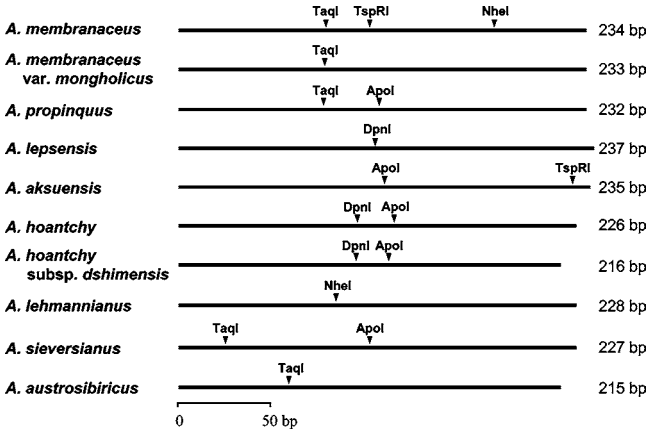

Figure 2. Restriction enzyme mapping of 5S rRNA spacers. The DNA sequences of 5S rRNA spacers from 10 taxa of *Astragalus* as shown in **Table 1** were compared. Restriction enzyme mapping of 5S rRNA spacers from various *Astragalus*. *Apo* I, *Dpn* I, *Nhe* I, *Taq* I, and *TspR* I are shown. Arrows indicate the restriction sites. Only the distinct sites are shown. Bar is 50 bp.

and heuristic search options. A neighbor-joining tree was constructed from the corrected sequence divergence using the NJ option in PAUP. Sequence divergence values were computed using the Kimura two parameter method (*23*). When the rate heterogeneity occurs among the ITS and 5S rRNA spacer regions, parsimony methods may perform poorly (*24*, *25*). Thus, maximum likelihood methods were also applied to the data using PAUP. Transition/transversion ratios were estimated to be 0.9339 and 1.3588 for the 5S rRNA spacer and ITS sequences, respectively.

RESULTS

PCR Products and Their DNA Sequence Analyses. Specific primers flanking the 5S rRNA spacer, ITS region, and 18S rRNA coding region were used in PCR analyses of genomic DNAs isolated from different *Astragalus* species (**Figure 1A**). The sizes of PCR products of the 5S rRNA spacer, ITS, and 18S rRNA derived from the genomic DNAs of different *Astragalus* species were determined (**Figure 1B**). Two PCR products having lengths of ~300 bp and ~600 bp were shown in amplification of the 5S rRNA spacer. After sequence analysis, the higher molecular weight PCR product (~600 bp) was identified as a dimer of the smaller one (~300 bp; data not shown). PCR products having sizes of ~700 bp and ~1800 bp were obtained in the ITS and 18S rRNA, respectively (**Figure 1B**). All of the PCR products were subcloned and sequenced. Several individual clones of the same PCR product were sequenced to avoid mutation introduced by *Taq* polymerase. In total, about 15 PCR products from a single taxon were sequenced. Identical sequences were obtained from the same taxon regardless of its geographical origin (data not shown). The sequenced DNAs were deposited in the GenBank with an accession number (**Table 1**).

Phylogenetic Analyses by DNA Sequences of 5S rRNA Spacer, ITS, and 18S rRNA. By sequence alignment of 5S rRNA spacer domain, the DNA sequence of *A. membranaceus* is 97.43 and 84.38% identical to that of *A. membranaceus* var. *mongholicus* and *A. propinquus*, respectively, while *A. membranaceus* has only 71.37% identity to *A. autrosibiricus*, which shows the least homology among all of the tested *Astragalus* species. An unique DNA sequence could be found within the tested *Astragalus*; these sequences could serve as markers in the authentication of different species. The restriction mapping of 5S rRNA spacer among *Astragalus* species was compared, which could provide a rapid distinction of *A. membranaceus* and *A. membranaceus* var. *mongholicus* from other species. Restriction enzymes such as *Taq* I, *TspR* I, and *Nhe* I could be used here for the distinction of *A. membranaceus* and *A. membranaceus* var. *mongholicus* from others (**Figure 2**).

Figure 3 shows the phylogenetic tree obtained by comparing the sequence identities of 5S rRNA spacers in different *Astragalus* taxa by UPGMA method. Two distinct clades are resolved. The first includes *A. membranaceus*, *A. membranaceus* var. *mongholicus*, *A. propinquus*, *A. lepsensis*, and *A. aksuensis* (hereinafter referred to as the "*A. membranaceus* clade"), while the second comprises *A. hoantchy*, *A. hoantchy* subsp. *dshimensis*, *A. lehmannianus*, *A. sieversianus*, and *A. austrosibiricus* (hereinafter referred to as the "*A. hoantchy* clade"). In *A. membranaceus* clade, *A. membranaceus* and *A. membranaceus* var. *mongholicus* are the most closely related pair of taxa. *A. propinquus* has a closer relationship to this pair than *A. lepsensis* and *A. aksuensis*. The members of *A. membranaceus* clade are in the same subgenus section (Subgen. *Monadalphia* Cheng *f. ex* P. C. Li & C. C. Ni; Sect. *Cenantrum* Koch.) as determined by Fu (*5*). In *A. hoantchy* clade, the relationship between *A.*

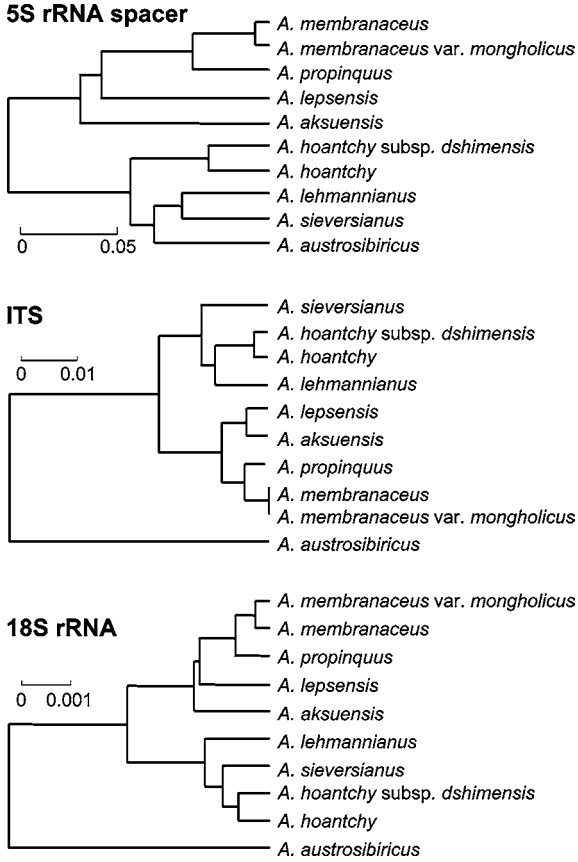

Figure 3. Phylogenetic trees of the *Astragalus* species including *A. membranaceus*, *A. membranaceus* var. *mongholicus*, *A. propinquus*, *A. lepsensis*, *A. aksuensis*, *A. hoantchy*, *A. hoantchy* subsp. *dshimensis*, *A. lehmannianus*, *A. sieversianus*, and *A. austrosibiricus* were constructed by UPGMA method in 5S rRNA spacer, ITS, and 18S rRNA. The distance corresponding to sequence divergence is indicated by the bar. The maximum parsimony analyses (PAUP) showed similar results in the DNA sequences of 5S rRNA spacer, ITS, and 18S rRNA.

hoantchy and *A. hoantchy* subsp. *dshimensis* is closer than for any other pairs of taxa.

The respective lengths of the ITS regions among the different *Astragalus* taxa are presented in **Table 1**. In comparison to the 5S rRNA spacer, the ITS sequences of *Astragalus* are highly conserved. *A. membranaceus* is 100 and 99.19% identical to *A. membranaceus* var. *mongholicus* and *A. propinquus*. The lowest homology (93.38%) is between *A. membranaceus* and *A. austrosibiricus*. In the phylogenetic tree (**Figure 3**), the 10 taxa were divided into three clades. *A. austrosibiricus* stands alone in a single clade, suggesting that it is most distantly related to the remaining taxa. *A. membranaceus*, *A. membranaceus* var. *mongholicus*, *A. propinquus*, *A. lepsensis*, and *A. aksuensis* are in the same clade, which contains the same taxa as the *A. membranaceus* clade in the 5S rRNA spacer analysis. *A. hoantchy*, *A. hoantchy* subsp. *dshimensis*, *A. lehmannianus*, and *A. sieversianus* are in the same clade, with *A. hoantchy* and *A. hoantchy* subsp. *dshimensis* showing the closest relationship among these taxa, which is similar to the *A. hoantchy* clade in 5S rRNA spacer except not including *A. austrosibiricus*.

The analysis of 18S rRNA was also determined. The respective lengths of the 18S rRNA coding region in different species are presented in **Table 1**. In the sequence alignment, *A. membranaceus* differs from *A. membranaceus* var. *mongholicus* and *A. propinquus* by just 1 and 3 bp, respectively. Even the most distantly related taxon, *A. austrosibiricus*, differs from *A. membranceus* by only 15 bp (99.13% identity). Again, three clades were resolved in the 18S rRNA phylogenetic tree, which is very similar to the phylogenetic tree derived from ITS (**Figure 3**).

Table 2. Descriptive Statistics for the Parsimony Analyses of the 5S rRNA Spacer and ITS Sequences[a]

statistic	5S rRNA spacer	ITS
no. of bases	215–237	611–619
total no. of characters	246	620
no. of constant characters	124	555
no. of parsimony uninformative characters	61	45
no. of parsimony informative sites	61	20
no. of MPTs	176	30
tree length of MPT (steps)	175	75
ci	0.840	0.947
ri	0.823	0.909
rc	0.691	0.861

[a] Parsimony analyses were performed using heuristic searches, using the bootstrap method in PAUP. Sequence divergence values were computed using the Kimura two parameter method (*23*).

The aforementioned DNA sequences were also analyzed by PAUP that resulted in parsimonious trees, except that the 18S rRNA that was too close among all tested *Astragalus* species to be analyzed. **Table 2** presents several descriptive statistics of the sequences and the results of the parsimony analyses. The strict consensus tree for the 5S rRNA spacer sequence is shown as that in **Figure 3**. As in the preliminary analyses, two main clades can be distinguished. *A. membranaceus* and *A. membranaceus* var. *mongholicus* are closely related to *A. propinquus*, *A. lepsensis*, and *A. aksuensis* as the "*A. membranaceus* clade", whereas *A. hoantchy*, *A. hoantchy* subsp. *dshimensis*, *A. lehmannianus*, and *A. sieversianus* form a clade as the "*A. hoantchy* clade". The neighbor-joining analysis produced an almost identical tree, as did the maximum likelihood analysis. The corresponding analyses of the ITS sequences revealed the same clades, although there were more unresolved polytomies in the *A. hoantchy* clade (**Figure 3**).

DISCUSSION

Our results support the interpretation of Hsiao (*6*) that *A. mongholicus* is a variety of *A. membranaceus*, and *A. propinquus* is closely related with these taxa. The analysis of the 5S rRNA spacer sequences shows that these sequences can be used to reliably distinguish between the 10 *Astragalus* taxa examined. We suggest that future taxonomic treatments of the genus *Astragalus* could benefit from the consideration of the molecular analyses of the 5S rRNA spacer, ITS, and 18S rRNA DNA sequences. Finally, we note that geographical variation among closely related *Astragalus* taxa may contribute to inter- and intraspecific variation in the DNA sequences examined, despite high levels of overall homology in these sequences.

Comparison of Relationships among Astragalus Taxa Inferred from DNA Sequences. The results of the analyses of 5S rRNA spacer, ITS, and 18S rRNA are remarkably similar by both methods of determination, suggesting that the patterns observed in **Figure 3** accurately reflect the phylogenetic relationships of the 10 *Astragalus* taxa examined. In particular, they support the interpretation of Hsiao (*6*), that *A. mongholicus* should be reduced as a variety of *A. membranaceus*. On the basis of the morphologically analysis, *A. membranaceus* differs to *A. mongholicus* in their ovary and pod as well as the shape and number of their leaves. However, Hsaio analyzed 115 specimens of Radix Astragali from 48 different habitats in China and proposed to reduce *A. mongholicus* as a variety of *A. membranaceus*. Hsaio's interpretation was supported by several

observations: (i) the habitats of *A. membranaceus* and *A. mongholicus* are too close to be considered for two independent taxa; (ii) they share a close similarity in sexual organs, root morphology, and their root vascularization; (iii) the morphology of *A. mongholicus* could be changed to *A. membranaceus*-like when the habitat has changed.

On the basis of the phylogenetic analysis, *A propinquus* appears to be closely related to these taxa, as do *A. lepsensis* and *A. aksuensis*. The main clades as determined by two clustering analyses, except *A. austrosibiricus*, are rather distinctive; therefore, it is well-supported that the species within the same clade are closely related. Of the remaining taxa, the analyses support the interpretation that *A. dshimensis* is best considered as a subspecies of *A. hoantchy*. The sequences of these taxa differ more than those of *A. membranaceus* and *A. membranaceus* var. *mongholicus* but considerably less than any other pairs examined here. *A. austrosibiricus* appears to be relatively distantly related to the other nine taxa and is placed as an out group in **Figure 3**.

Although our results support the taxonomic interpretations of Hsiao (*6*), Fu's (*5*) keys to *Astragalus* species in China are based largely on flower and leaf structure, characteristics that may be of little use in detecting adulterants, as it is the roots and stems of *A. membranaceus* and *A. membranaceus* var. *mongholicus* that are used in Radix Astragali. Of the taxa examined, *A. propinquus*, *A. lepsensis*, and *A. aksuensis* would appear to be the most difficult to distinguish from *A. membranaceus*, but variations in the 5S rRNA spacer sequences provide a reliable tool for overcoming this problem.

In higher eukaryotes, the 5S rRNA gene is separated by simple spacers and occurs as a tandem repeated unit (cistron) consisting of a ~120 bp coding region separated by a few hundred base pairs of spacer domains (*26*). Although 5S rRNA is highly conserved, the spacer is highly variable in different species (*14*, *15*). Therefore, the diversity of the spacer is very useful in the identification of traditional Chinese medicinal plants (*13*, *16*, *19*). In addition, DNA sequences from ITS and 18S rRNA could also be used for species distinction (*15*, *27*). Corresponding to 18S, 5.8S, and 28S rRNAs, each transcription unit consists of highly conserved coding sequences in higher plants. ITS between 18S and 5.8S (ITS1) and between 5.8S and 28S (ITS2) are highly variable among closely related species and have been demonstrated to be phylogenetically informative (*17*). In angiosperm family Fabaceae, phylogenetic analysis based on ITS has been determined extensively (*28*, *29*). In addition, the maximum parsimony and neighbor-joining analyses of ITS DNA sequences from 115 species of *Astragalus* have been determined (*4*), where *A. membranaceus* was also included in the matrix analysis; however, other *Astragalus* species commonly found in China were not included in their analysis. Our DNA sequence analysis suggests that the ITS (ITS1, 5.8S, and ITS2) and 18S rRNA coding regions are more conserved than that of the 5S rRNA spacer; therefore, the 5S rRNA spacer can provide more accurate phylogenetic information than ITS and 18S rRNA in the molecular identification of *Astragalus* species.

Biogeographical Distribution of Astragalus in China. **Figure 4** illustrates the geographical distributions of the 10 *Astragalus* taxa in China and the former USSR (*12*). The ranges of *A. membranaceus* and *A. membranaceus* var. *mongholicus* largely overlap in northeastern China, thereby reducing the likelihood of adaptive radiation due to physical isolation. However, *A. propinquus* is confined to northwestern China, is rarely sympatric with *A. membranaceus* var. *mongholicus*, and is never sympatric with *A. membranaceus*. Accordingly, the effects of physical isolation and differing environmental pressures may have led to the significant variations in DNA sequences between *A. propinquus* and *A. membranaceus*. *A. lepsensis* and *A. aksuensis* are similarly confined to northwestern China and adjacent parts of the former USSR.

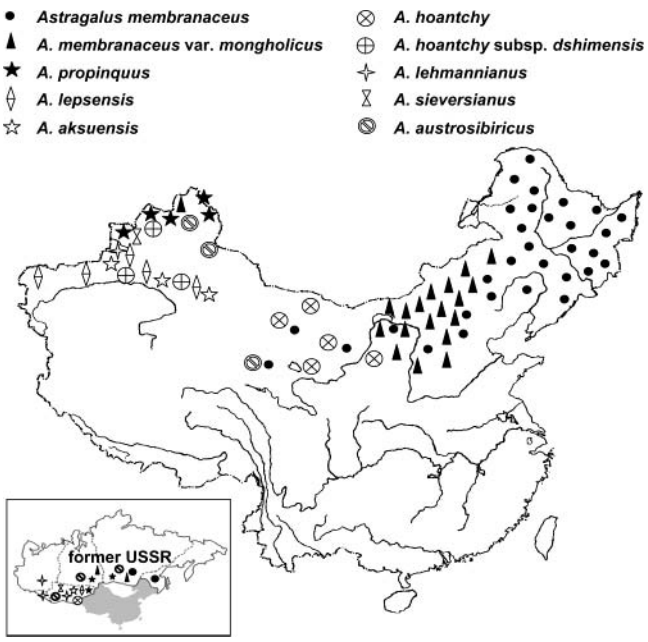

Figure 4. Geographic distribution of 10 *Astragalus* taxa in China and the former USSR (inset). The denotations of the different *Astragalus* taxa are shown. The number of the denotation also indicates the relative population density of the growth distribution.

Given this, the species of *Astragalus* that are most similar to *A. membranaceus* are unlikely to be encountered in samples of Radix Astragali, which have been collected from northeastern China. If the point of origin of Radix Astragali samples can be determined with certainty, the need to use DNA sequence analyses to detect adulterants is greatly diminished, unless such samples are known to be derived from *A. membranaceus* populations in northwestern China. In these cases (or when reliable locality data is unavailable), comparative analyses of DNA sequences may be the only means of determining the authenticity of the product.

Of the remaining taxa, populations of *A. hoantchy* and *A. hoantchy* subsp. *dshimensis* are separated by substantial geographical barriers in China. A possible explanation for the differences in DNA sequences between these taxa is that *A. hoantchy* subsp. *dshimensis* is evolving away from *A. hoantchy* under different selection pressures. *A. lehmannianus* and *A. sieversianus* are rare in China, being confined to a small region in the northwest, whereas *A. austrosibiricus* has a patchy distribution extending from the northwest to central northern regions. Furthermore, 12 different species in China carry the name of Radix Astragali on the market and act as adulterants; they show a close resemblance of morphological appearances; therefore, the quality control of Radix Astragali is a serious problem. Our findings show that through the use of information derived from geographical and DNA sequence analyses, the purity of commercial samples Radix Astragali can be determined accurately, expeditiously, and economically.

In a parallel study, chemical analyses were performed on 10 *Astragalus* taxa from 28 different geographical regions of China (*8*, *20*). Radix Astragali and its adulterants were tested, and levels of the main constituents (such as isoflavonoids and astragalosides) were determined by high-performance liquid chromatography. The results showed that the amounts of main constituents such as isoflavonoids and astragalosides varied in

different species. In distinction, *A. membranaceus* and *A. membranaceus* var. *mongolicus* contained higher amount of astragalosides I and IV. In addition, the main constituents of *A. membranaceus* var. *mongholicus* changed according to seasonal variation and the age of the plant. Except for *A. membranaceus* and *A. membranaceus* var. *mongholicus* (which contained higher content of isoflavonoids and astragalosides), the chemical composition of the other *Astragalus* taxa was very similar. In addition, the chemical composition of Radix Astragali could be varied greatly from their habitat, and Shanxi of China produced the best quality of herbs in terms of the contents of isoflavonoids and astragalosides (*20*). Thus, the analysis of chemical composition could only be used to determine the quality of Radix Astragali samples, rather than detecting adulterants. Clearly, variations in the 5S rRNA spacer, ITS, and 18S rRNA DNA sequences provide more reliable information for the latter purpose.

ACKNOWLEDGMENT

We thank Dr. Roy Choi from our laboratory for his expert technical assistance, Dr. J. A. Duan of Jiangsu Research Institute of TCM in Nanjing, and Prof. C. Y. Yang of Xinjiang Agricultural University in Urumqi for their assistance during the field collection.

LITERATURE CITED

(1) Zheng, X. Y. *Pharmacopoeia of the People's Republic of China*, English ed.; Zheng, X. Y., Ed.; Chemical Industry Press: Beijing, China, 2000; Vol. 1, pp 161−162.

(2) Sinclair, S. Chinese herbs: a clinical review of *Astragalus*, *Ligusticum*, and *Schizandrae*. *Altern. Med. Rev.* **1998**, *3*, 338−344.

(3) Wagner, H.; Bauer, R.; Xiao, P. G.; Chen, J. M.; Michler, H. Radix Astragali (Huang Qi). In *Chinese Drug Monographs and Analysis*; Staudinger, A., Eds.; Verlag für Ganzheitliche Medizin Dr. Erich Wühr GmbH, Kötzting/Bayer: Wald, Germany, 1997; Vol. 1, pp 1−17.

(4) Wojciechowski, M. F.; Sanderson, M. J.; Hu, J. M. Evidence on the monophyly of *Astragalus* (Fabaceae) and its major subgroups based on nuclear ribosomal DNA ITS and choroplast DNA *trn*L intron data. *Syst. Bot.* **1999**, *24*, 409−437.

(5) Fu, K. T. *Flora of Reipublicae Popularis Sinicae*; Science Press: Beijing, China, 1998; Vol. 42.

(6) Hsiao, P. K. Studies on the original plant and pharmacognosy of traditional Chinese medicine: Huangqi. *Acta Pharm. Sin.* **1964**, *11*, 117−123.

(7) Upton, R.; Petrone, C.; Swisher, D.; Siverly, C. *Astragalus* root. In *American Herbal Pharmacopoeia*; Upton, R., Ed.; Santa Cruz, CA, 1999.

(8) Ma, X. Q.; Duan, J. A.; Zhu, D. Y.; Dong, T. T. X.; Tsim, K. W. K. Chemical Comparison of Astragali Radix (Huangqi) from Different Regions of China. *Nat. Med.* **2000**, *54*, 213-218.

(9) Qian, X. Z. *Pharmacopoeia of the People's Republic of China*; The People's Public Health Press: Beijing, China, 1985.

(10) Anetai, M.; Katsura, E.; Katoh, Y.; Yamagishi, T. Chemical evaluation of Astragali Radix. *Nat. Med.* **1994**, *48*, 244−252.

(11) Anetai, M.; Aoyagi, M.; Shibata, T.; Iida, O.; Hatakeyama, Y. Preparation and chemical evaluation of Astragali Radix produced in Hokkaido. *Nat. Med.* **1998**, *52*, 10−13.

(12) Czerepanov, S. K. *Vascular Plants of Russia and Adjacent States (The Former USSR)*; Cambridge University Press: New York, U.S.A., 1995.

(13) Cai, Z. H.; Li, P.; Dong, T. T. X.; Tsim, K. W. K. Molecular diversity of 5S rRNA spacer domain in *Fritillaria* species revealed by PCR analysis. *Planta Med.* **1999**, *65*, 360−364.

(14) Gerlach, W. L.; Dyer, T. A. Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. *Nucleic Acids Res.* **1980**, *8*, 4851−4865.

(15) Long, E. O.; Dawid, B. Repeated genes in eukaryotes. *Annu. Rev. Biochem.* **1980**, *49*, 727−726.

(16) Zhao, K. J.; Dong, T. T. X.; Tu, P. F.; Song, Z. H.; Lo, C. K.; Tsim, K. W. K. Molecular genetic and chemical assessment of Radix Angelica (Danggui) in China. *J. Agric. Food Chem.* **2003**, *51*, 2576−2583.

(17) Baldwin, B. G.; Sanderson, M. J.; Porter, J. M.; Wojciechowski, M. F.; Campbell, C. S.; Donoghue, M. J. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Garden* **1995**, *82*, 247−277.

(18) Wada, H.; Satoh, N. Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequences of 18S rRNA. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 1801−1804.

(19) Ma, X. Q.; Duan, J. A.; Zhu, D. Y.; Dong, T. T. X.; Tsim, K. W. K. Species Identification of Huangqi (Radix Astragali) by DNA Sequence of its 5S rRNA Spacer Domain. *Phytochemistry* **2000**, *54*, 363−368.

(20) Ma, X. Q.; Shi, Q.; Duan, J. A.; Dong, T. T. X.; Tsim, K. W. K. Chemical analysis of Radix Astragali (Huangqi) in China: a comparison with its adulterants and seasonal variations. *J. Agric. Food Chem.* **2002**, *50*, 4861−4866.

(21) Wen, J.; Zimmer, E. A. Phylogeny and biogeography of *Panax* L. (the Ginseng genus, Araliaceae): inferences from ITS sequences of nuclear ribosomal DNA. *Mol. Phylogenet. Evol.* **1996**, *6*, 167−177.

(22) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual*, 3; Sambrook, J., Russell, D. W., Eds.; Cold Spring Harbor Laboratory Press: New York, U.S.A., 2001; Vol. 1.

(23) Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111−120.

(24) Gaut, B. S.; Weir, B. S. Detecting substitution-rate heterogeneity among regions of a nucleotide sequence. *Mol. Biol. Evol.* **1994**, *11*, 620−629.

(25) Kuhner, M. K.; Felsenstein, J. A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates. *Mol. Biol. Evol.* **1994**, *11*, 459−469.

(26) Peyretaillade, E.; Biderre, C.; Peyret, P.; Duffieux, F.; Metenier, G.; Gouy, M.; Michot, B.; Vivares, C. P. Microsporidian *Encephalitozoon cuniculi*, a univellular eukaryote with an unusual chromosomal dispersion of ribosomal genes and a LSU rRNA reduced to the universal core. *Nucleic Acids Res.* **1998**, *26*, 3513−3520.

(27) Nickrent, D. L.; Soltis, D. E. A comparison of angiosperm phylogenies based upon complete 18S rDNA and *rbc* L sequences. *Ann. Missouri Bot. Garden* **1995**, *82*, 208−234.

(28) Delgado-Salinas, A.; Turley, T.; Richman, A.; Lavin, M. Phylogenetic analysis of the cultivated and wild species of *Phaseolus* (Fabaceae). *Syst. Bot.* **1999**, *24*, 438−460.

(29) Nickrent, D. L.; Doyle, J. A molecular phylogeny of diploid *Glycine* (Fabaceae) based upon nuclear ribosomal ITS sequences. *Am. J. Bot.* **1995**, *82*, S153.

Received for review March 21, 2003. Revised manuscript received August 19, 2003. Accepted August 28, 2003. This work was supported by Area of Excellence Scheme established under the University Grants Committee of Hong Kong SAR, China (AoE/B-10/01), to K.W.K.T.

JF034278X