PERGAMON

Phytochemistry 54 (2000) 363–368

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Species identification of Radix Astragali (Huangqi) by DNA sequence of its 5S-rRNA spacer domain

X.Q. Ma[a,c], J.A. Duan[b], D.Y. Zhu[c], T.T.X. Dong[a], K.W.K. Tsim[a,*]

[a]*Department of Biology and Biotechnology Research Institute, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong*

[b]*Jiangsu Research Institute of Traditional Chinese Medicine, Maigaoqiao, Nanjing 210028, People's Republic of China*

[c]*State key Laboratory for New Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 294 Taiyuan Road, Shanghai 200031, People's Republic of China*

Received 17 November 1999; received in revised form 8 March 2000

Abstract

About 300 species and varieties of *Astragalus* are identified in China, making the identification of the origin of a particular *Astragalus* species on the consumer market difficult. A molecular genetic approach was developed to identify various species of *Astragalus*. Although the 5S-rRNA coding sequence is conserved in higher eukaryotes, the spacer domain of the 5S-rRNA gene has great diversity among different species. The 5S-rRNA spacer domain was amplified by polymerase chain reaction (PCR) from the isolated genomic DNA, and the PCR products (~300 bp) covering the 5S-rRNA spacer domain were sequenced. The nucleotide sequences of *Astragalus membranaceus*, *A. membranaceus var. mongholicus*, *A. lehmannianus*, *A. hoantchy*, and of one closely related species *Hedysarum polybotrys* (Hongqi), were determined. Diversity in DNA sequence and restriction enzyme mapping among various species was found in their 5S-rRNA spacer domains. This is the first report on the detection of 5S-rRNA spacer region sequence of *Astragalus*, and the results could be used for genetic identification of Huangqi.

Keywords: *Astragalus*; Fabaceae; DNA sequence; Species identification

1. Introduction

Huangqi (Radix Astragali, root of *Astragalus*) is a traditional Chinese medicine commonly used as a tonic and diuretic. Although *Astragalus* has a long history of medicinal use in Chinese herbal medicine, it is only recently that its pharmacological properties and clinical applications have been examined. *Astragalus* has a wide range of immunopotentiating effects, and has proven efficacious as an adjunct cancer therapy (Sinclair, 1998). A large quantity of Huangqi is needed throughout the world, particularly in Southeast Asia. The genus *Astragalus* L. is composed of 278 species, two subspecies, 35 varieties and two forma in China (Fu, 1998). However, only 12 of these carry the name Huangqi on the market of herbal medicine. The authentic identification of Huangqi is very difficult for consumers. The most commonly used Huangqi are *Astragalus membranaceus* (Fisch.) Bunge and *A. membranaceus* (Fisch.) Bunge *var. mongholicus* (Bunge) Hsiao. Both of these are recorded as the botanical sources of Huangqi in Chinese Pharmacopoeia (Chen, 1995). *Astragalus lehmannianus* Pall. and *A. hoantchy* Franch may be commonly used as adulterants of Huangqi. Another herbal medicine Hongqi (Radix Hedysaris, root of *Hedysarum polybotrys* Hand.-Mazz.) is a species closely related to *Astragalus*, and is commonly used as a replacement of

* Corresponding author. Tel.: +852-2358-7332; fax: +852-2358-1559.

E-mail address: botsim@usthk.ust.hk (K.W.K. Tsim).

0031-9422/00/$ - see front matter
PII: S0031-9422(00)00111-4

Huangqi. However, they have different efficacy. The close resemblance of different types of *Astragalus* greatly compromises the value of traditional medicines in the market place. Thus, the correct identification of Huangqi is very important for the modernization of traditional Chinese medicine.

Astragalus membranaceus and *A. membranaceus var. mongholicus* grow mainly in the north, the northeast and the northwest parts of China, while *H. polybotrys* is distributed mainly in the northwest. The natural resources of *Astragalus* are decreasing gradually due to the exhaustive exploitation. Indeed, Huangqi is currently supplied largely from farming. The general approaches to herbal identification are dependent on morphological, anatomical, and chemical analyses, but these characteristics are often affected by environmental and developmental factors during plant growth. Additionally, medicinal plants are processed for use as crude drugs, causing many morphological and anatomical characteristics as well as some chemical constituents to change. Therefore, it is difficult to determine the botanical origins of crude herbs through anatomical and chemotaxonomical studies. A DNA-based polymorphism assay may offer an alternative method for the identification of herbal medicines. Using a polymerase chain reaction (PCR) technology, nanogram quantities of DNA can be amplified to yield sufficient amounts of template DNA for molecular genetic analysis.

In higher eukaryotes, the 5S-rRNA gene is separated by simple spacers. The gene occurs as a tandem repeated unit (cistron) consisting of a ~120 bp coding region separated by few hundred base pairs of spacer. Although 5S-rRNA is highly conserved, the spacer domains are variable in different species domains (Gerlach and Dyer, 1980; Long and Dawid, 1980; Cai et al., 1999). Thus, the diversity of the spacer domain can be used as a molecular marker for species identification. Here, two primers complementary to the coding region of the 5S-rRNA were used to amplify the intergenic spacer domain by PCR. The amplified spacer regions of different species of *Astragalus* were sequenced and compared.

2. Results and discussion

Fig. 1 shows the quality of genomic DNA isolated from different sources of *Astragalus*. Although DNA degradation was observed in the crude drug preparation, PCR products amplified by using primers from regions flanking the spacer domain of 5S-rRNA were revealed from DNA isolated from fresh leaf, fresh root and crude drug. In different species of

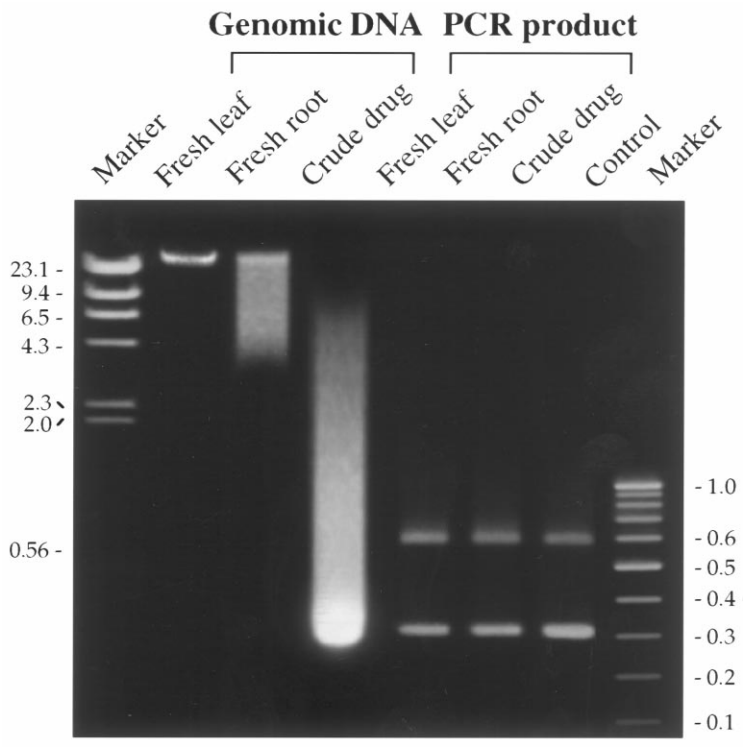

Fig. 1. Agarose gel electrophoresis of genomic DNAs and PCR products. Ten μg of genomic DNA was loaded per lane. Degradation was revealed in the DNA isolated from crude drug. PCR products (~1 μg) were generated by primers (P1 and P2) flanking the spacer domain of 5S-rRNA using DNA templates from various sources of *A. membranaceus*. The same size PCR products were obtained from several tested samples ($N = 4$). DNA markers (in kb) are indicated.

Astragalus, two PCR products having lengths of ~300 and ~600 bp were detected in agarose electrophoresis analysis, while *H. polybotrys* had ~200 and ~400 bp fragments (Fig. 2). The PCR products were subcloned and sequenced. Several individual clones of the same PCR product were sequenced to avoid mutation introduced by *Taq* polymerase. The higher molecular weight PCR product, ~400 bp in *H. polybotrys* or ≈600 bp in *Astragalus*, is a dimer of the smaller one, since the larger PCR product contained repeat sequences of the smaller one including the 5S-rRNA coding region. The respective lengths of the 5S-rRNA spacer domain in different species are: *A. membranaceus* 234 bp, *A. membranaceus var. mongholicus* 233 bp, *A. lehmannianus* 228 bp, *A. hoantchy* 226 bp and *H. polybotrys* 142 bp (Fig. 3). The DNA fragments were sequenced several times in both directions. Identical sequences were obtained from all the tested species when the template DNAs were isolated from either fresh leaf, fresh root, dry crude drug, or different regions of cultivation of the same *Astragalus* species (data not shown). The unique restriction enzyme sites on their 5S-rRNA spacer domain could easily discriminate between different species. Fig. 3(B) shows the restriction map of various *Astragalus* and of *H. polybotrys*, which could provide a rapid way for identification of *A. membranaceus* and *A. membranaceus var. mongholicus* from its adulterants. This criterion could also be used to identify the crude dry herb, therefore allowing for secure and effective usage of *Astragalus* in the future.

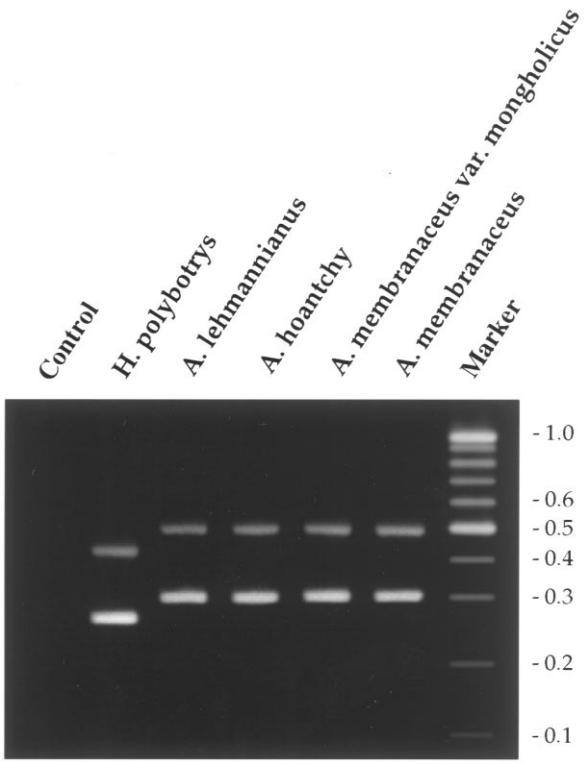

Fig. 2. PCR products generated by primers (P1 and P2) flanking the spacer domain of 5S-rRNA using DNAs from *A. membranaceus*, *A. membranaceus var. mongholicus*, *A. lehmannianus*, *A. hoantchy* and *H. polybotrys* as templates. The PCR products were separated using a 1.2% agarose gel. The same size PCR products were obtained from three tested samples ($N = 4$). DNA markers (in kb) are indicated.

The spacer domains among all *Astragalus* species are highly conserved. Fig. 4 shows the phylogenetic tree obtained by comparing the sequence identity from different *Astragalus* species. *Astragalus membranaceus* and *A. membranaceus var. mongholicus* have a closer phylogenetic relationship, sharing 97% DNA identity. This identity is the highest among the four species being compared. This sequence identity data agrees with the proposal raised by Prof. P. G. Hsiao, who classified *A. mongholicus* as a variety of *A. membranaceus* (Hsiao, 1964). However, the 5S-rRNA sequences of other members within the subgenus of *A. membranaceus* and *A. membranaceus var. mongholicus* are not yet determined, and their sequence data are required to draw a definitive conclusion. Besides the 5S-rRNA, it would be interesting to determine the homology of other gene sequences between *A. membranaceus* and *A. membranaceus var. mongholicus*. The sequence of *A. lehmannianus* is 73% identical to that of *A. membranaceus*. *Astragalus lehmannianus* belongs to subgenus *Calycophysa* while *A. membranaceus* is a member of the subgenus *Phaca*, which could explain the greater diversity of gene sequences. The spacer domain sequence of *A. hoantchy* is only 70% identical to that of *A. membranaceus*. *Astragalus hoantchy* belongs to subgenus *Pogononophace*. As expected, *H. polybotrys* (Hongqi) shows the greatest diversity in its 5S-rRNA, having less than 50% identity to *Astragalus*.

There are twelve species that could carry the name Huangqi in Southeast Asia. *Astragulus membranaceus* and *A. membranaceus var. mongholicus* are the species recognized as Huangqi (Chen, 1995). Other species could act as adulterants of Huangqi, indeed, *A. lehmannianus* and *A. hoantchy* are common in the market. Although Hongqi has been used in the past as a replacement for Huangqi, in present day traditional Chinese medication, Hongqi has a medical purpose distinct from that of Huangqi (Cui, 1990).

Precise identification of crude drugs is a prerequisite for chemical and pharmacological investigations of traditional Chinese medicine, as well as for their clinical applications. The molecular genetic method has several advantages over the classical morphological and chemical analysis. The genetic method is based on the genotype rather than the phenotype, and is therefore not dependent on environment. Only a small amount of drug is needed for DNA analysis, and the test is applicable to different forms of the drug. In Southeast

Asia, many traditional Chinese medicines are sold in the form of capsules containing ground powder of crude drug mixed with other supplements, making identification by classical methods difficult. The molecular genetic approach provides a rapid and reliable method for identification of traditional Chinese medicine; indeed, a similar approach has been developed to classify over 25 species of *Fritillaria* (Cai et al., 1999). We are in the process of collecting DNA sequence data from the most popular traditional Chinese medicines for further development.

3. Experimental

3.1. Acquisition of plant materials

Astragalus membranaceus was collected from different regions of China including Heilongjiang, Beijing and Shanxi; *A. membranaceus var. mongholicus* was from Neimeggu, Beijing and Shanxi; *A. lehmannianus* was from Xinjiang; *A. hoantchy* and *H. polybotrys* were from Ninxia and Gansu. Samples were kept in silica gel during field collection. The crude drugs were collected from the same regions as the fresh materials. Four to five different samples from different sources were tested in each *Astragalus* species. Voucher specimens were deposited in the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China.

3.2. Extraction of genomic DNA

The dried leaves were frozen with liquid nitrogen and ground into a powder. Genomic DNA was extracted from the ground powder using DNA extraction buffer consisting of 25 mM Tris–HCl pH 8.0, 50 mM EDTA, 0.5% SDS, 10 μg/ml RNAse, 0.2% β-mercaptoethanol. The mixed solution was incubated for 15 min at 58°C, then centrifuged. The supernatant was extracted by equal volumes of water saturated phenol:chloroform (1:1), mixed and

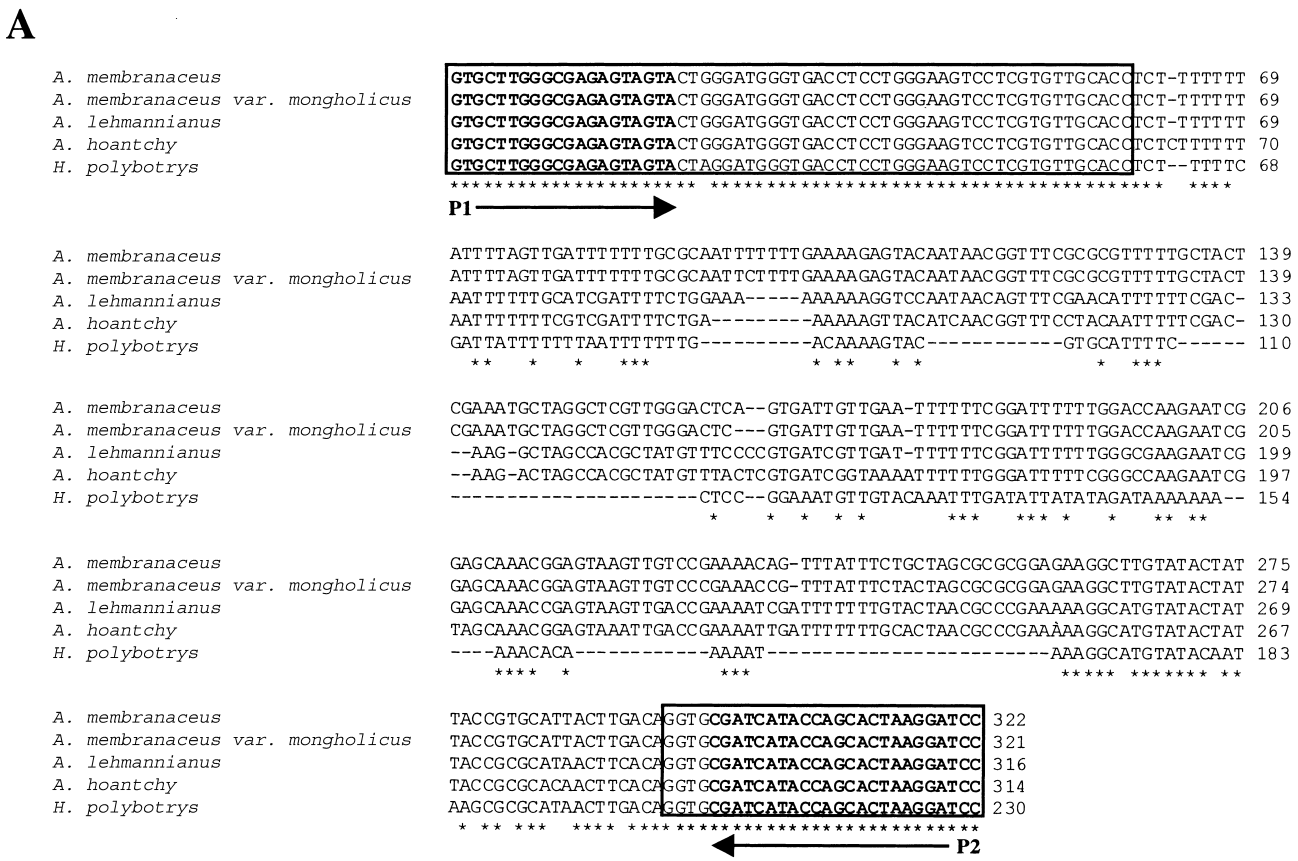

Fig. 3. Sequence analysis of the spacer domain of 5S-rRNA. (A): Sequence alignments of the spacer domains of 5S-rRNA genes from *A. membranaceus* (GenBank accession number AF239711), *A. membranaceus var. mongholicus* (AF239712), *A. lehmannianus* (AF239714), *A. hoantchy* (AF239713) and *H. polybotrys* (AF239715). The coding regions are boxed. Primer sequences (P1 and P2) used for amplification are indicated by arrows and in bold. Identical sequences are indicated by (∗). Gaps (-) are introduced for the best alignment. (B): Restriction enzyme mapping of the spacer domains of 5S-rRNA genes. *Apo I, Nhe I, Taq I* and *Tsp RI* are shown in the map. Arrows indicate the restriction sites. Only the distinct sites are shown here.

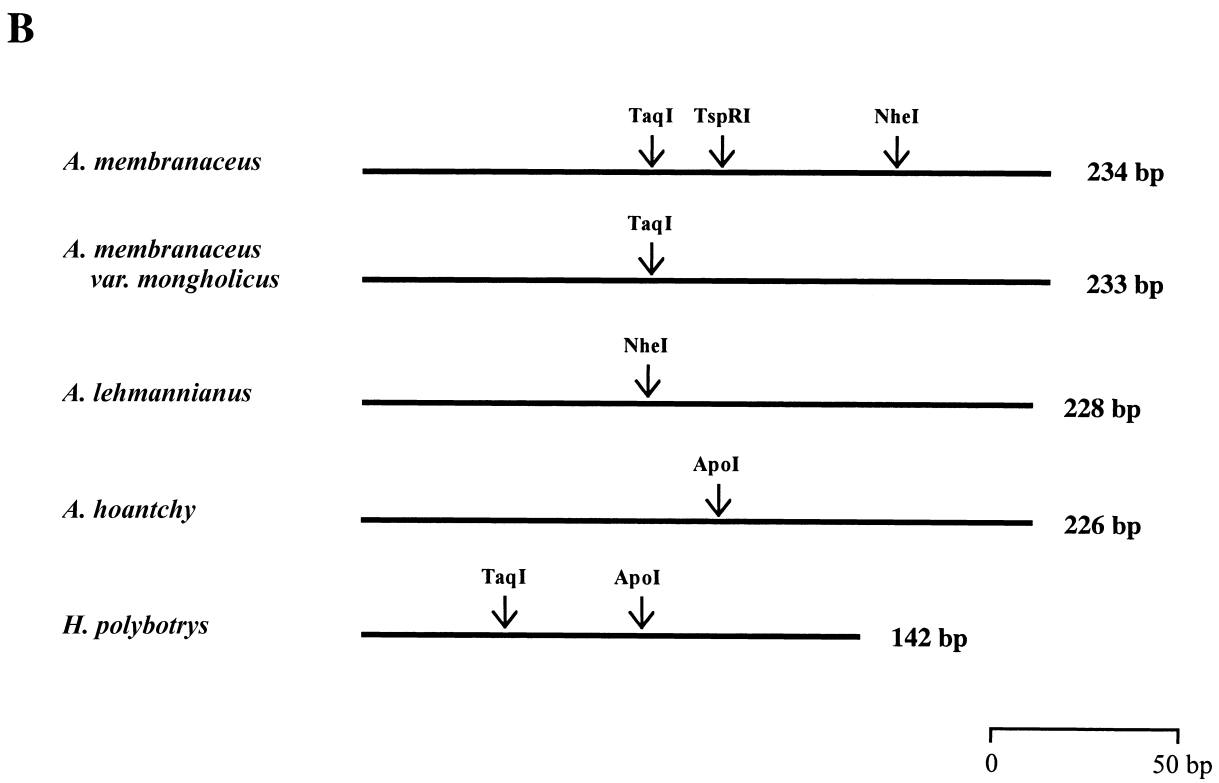

Fig. 3 (*continued*).

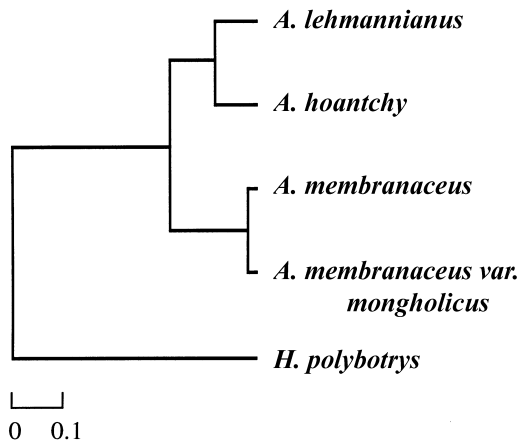

Fig. 4. Phylogenetic tree for the *Astragalus* species and *H. polybotrys* assessed by UPGMA method. The highest identity is between *A. membranaceus* and *A. membranaceus var. mongholicus*. *Hedysarum polybotrys* shows great diversity when compared to to *Astragalus*. The distance corresponding to 0.1 sequence divergence is indicated by the bar.

then centrifuged. The aqueous phase was collected and added to 0.1 volume of 3M sodium acetate and 2 volumes of 100% ethanol. The resulting pellet was collected after centrifugation and was dissolved in 10 mM Tris–HCl pH 8.0, 5 mM EDTA (Tris–EDTA).

DNA isolation from fresh roots (dried in silica gel) and the crude drug of *Astragalus* was difficult because of the high amounts of polysaccharides and secondary metabolites that formed insoluble complexes with nucleic acids during extraction (Pandey et al., 1996; Cai et al., 1999). Genomic DNA was extracted from the ground powder using CTAB extraction buffer (100 mM Tris–HCl, pH 8.0, 20 mM EDTA pH 8.0, 4% CTAB, 1.5M NaCl, 4% PVP-40, 0.5% ascorbic acid, 0.5% diethyldithiocarbamic and 10 mM β-mercaptoethanol). The mixed solution was incubated for 10 min at 65°C, then a 0.25 volume of cold 5 M potassium acetate was added and incubated at 0°C for 40 min and centrifuged for 10 min. The supernatant was added to 0.7 volume of cold isopropanol, mixed gently and incubated at −20°C for 10 min to precipitate genomic DNA. After centrifugation, the supernatant was discarded, and the pellet was suspended in 1.0 ml Tris–EDTA. Sodium acetate (3 M, 65 μl) and cold isopropanol (600 μl) were added and incubated at −20°C for 10 min. After spinning for 30 s, the pellet was washed carefully with 76% ethanol, and suspended in Tris–EDTA.

3.3. PCR amplification

A 50 μl PCR reaction mix consisted of 5 μl 10× reaction buffer, 1 μl each of 10 mM dNTPs stock, 2.5

μl containing 0.3 μg forward and reverse primers (synthesized by GIBCO-BRL, Grand Island, NY) and 1 unit of *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN). The primers used for amplification of 5S-rRNA were P1 forward primer (5′-GGA TTC GTG CTT GGG CGA GAG TAG TA-3′) and P2 reverse primer (5′-ACG CTA GTA TGG TCG TGA TTC CTA GG-3′). These primers flank the spacer domain of 5S-rRNA (Gerlach and Dyer, 1980; Long and Dawid, 1980; Cai et al., 1999). 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 conditions consisted of an initial 5 min at 94°C followed by 1 min denaturing at 94°C, 2 min annealing at 53°C and 3 min elongation at 72°C repeated for 30 cycles and with 10 min extension at 72°C. The PCR products were subjected to ~1% agarose gel electrophoresis and visualized by ethidium bromide staining under UV. DNA was purified using an agarose Gel DNA Extraction Kit (Boehringer Mannheim).

3.4. Subcloning and sequencing

The PCR products were subcloned into a TA cloning vector pTAg (R&D Systems, UK). Competent *E. coli* JM109 cells were transformed with the ligated products, and the colonies identified by color selection were picked and grown in 3 ml of Luria–Bertani (LB) liquid medium overnight. The mini-preparation of plasmid DNAs from the ligator cell was performed using alkaline lysis (Sambrook et al., 1989). The correct DNA inserts were verified by restriction analysis. The plasmid DNAs from verified colonies were also isolated with the Wizard™ Minipreps DNA Extraction system (Promega, Madison, WI). ABI Prism™, BigDye™ Terminator, cycle Sequencing Ready Reaction Kit were used for the sequence reaction with T7 or SP6 primers. Sequences were detected by an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Acknowledgements

We are grateful to Roy Choi from our laboratory for his expert technical assistance. Thanks are also due to Prof. C.Y. Yang for field assistance in Xinjiang, China. The research was supported by grants from the Biotechnology Research Institute at The Hong Kong University of Science and Technology (Chinachem Group, ISF AF-178-97 and AF-150-99 to K.W.K.T.) and the Foundation of Chinese Natural Sciences (#39730500 to J.A.D.).

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