Full Length Research Paper

Ribosomal DNA internal transcribed spacer 1 and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Zanthoxylum schinifolium*

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Molecular approaches are now being developed to provide a more rapid and objective identification compared to traditional phenotypic methods. Nuclear ribosomal DNA (nrDNA) targets, especially internal transcribed spacer 1 and 2 (ITS1 and ITS2), have been widely used for molecular identification of some plants and fungi. We therefore conducted an investigation in the identification of the fifth medically important *Zanthoxylum schinifolium* ecotypes using the common primers of the ITS region. About 620 bp fragments were obtained and the sequences of the polymerase chain reaction (PCR) products were tested. The sequence length, G+C content (%), DNA alignment and pariwise nucleotide comparisons demonstrated 98.8 to 100% sequence identities in the total ITS region, 98.3 to 100% identities in the ITS1 region and 99.5 to 100% in the ITS2 region. Comparative analysis using GenBank reference data showed that the exclusive reported data showed 100% identities with BEMR, CWDO, HCDC, JDGG and GJGD in the ITS1 region and 100% identities with thirteen ecotypes except BEMR and GRDG in the ITS2 region. The fifth different ecotypes were classified into five groups and the identification of medically important *Z. schinifolium* was highly improved due to the augmentation of our current ITS sequences.

Key words: *Zanthoxylum schinifolium*, molecular identification, phylogenetic relationship, ribosomal DNA, ITS1, ITS2.

INTRODUCTION

Zanthoxylum schinifolium is an aromatic plant, native to warm temperate and subtropical areas in the world, especially in Asia (Yang, 2008). Its pericarps and leaves are widely used as a pungent condiment and seasoning in some East Asian countries such as China, Korea and Japan (Paik et al., 2005). Its fruits have also been used as drugs in traditional Chinese medicine for epigastric pain (Yang, 2008) and invigorants for circulation of blood (Cui et al., 2009). Previous studies have reported that *Z. schinifolium* is rich in coumarins, alkaloids, triterpenoids, steroids and flavonoids (Cheng et al., 2002) and the essential components showed several biological activities such as antiplatelet aggregation (Chen et al., 1995), inhibitory activities (Jo et al., 2002), antioxidant and anti-cancer activities (Chon et al., 2009) as well as anti-inflammatory activities (Cao et al., 2009). However, to the best of our knowledge, there is little to investigate about the identification of this species, especially using molecular approaches.

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Abbreviations: nrDNA, Nuclear ribosomal DNA; **ITS,** internal transcribed spacer; **PCR,** polymerase chain reaction; **BLAST,** basic local alignment search tool; **NCBI,** National Center for Biotechnology Information; **SSR,** simple sequence repeat.

Table 1. Voucher information, abbreviation and GenBank accession numbers of the sequenced specimens.

Voucher collection	Abbreviation	Accession number
Boeun-Gun Maro-Myeon	BEMR	GU247226
Changwon-Si Dong-Eup	CWDO	GU247227
Eumseong-Gun Soi-Myeon	ESSI	GU247228
Geochang-Gun Namsang-Myeon	GCNS	GU247229
Gochang-Gun Sinlim	GCSL	GU247230
Goheung-Gun Doyang-Eup	GHDY	GU247231
Gangjin-Gun Gundong-Myeon	GJGD	GU247232
Gangneung-Si Gangdong-Myeon	GNGD	GU247233
Goryeong-Gun Deokgok-Myeon	GRDG	GU247234
Hapcheon-Gun Bongsan-Myeon	HCBS	GU247235
Hongcheon-Gun Duchon-Myeon	HCDC	GU247236
Jinan-Gun Bugwi-Myeon	JABG	GU247237
Jindo-Gun Gogun-Myeon	JDGG	GU247238
Wonju-Si Hojeo-Myeon	WJHJ	GU247239
Yeongdeok-Gun Changsu-Myeon	YDCS	GU247240

Traditional classification based on morphological charac-teristics may not distinguish some species to obstruct species identification and it require a long time (Klich, 2002). In addition, molecular approaches based on DNA sequences were found to provide more reliable and faster species identifications than traditional methods (Hinrikson et al., 2005). Therefore, traditional classification method has not met current identification need and rapid molecular approaches are required for development.

Various rRNA gene regions as targets for the molecular identification have been investigated (Iwen et al., 2002), including the ribosomal total internal transcribed spacer (ITS), ITS1 and ITS2 regions between the small- and large-subunit rRNA genes (White et al., 1990). Sequence diversity in the ribosomal regions has been investigated using polymerase chain reaction (PCR) amplification followed by fragment length analysis (Walsh et al., 1995; Turenne et al., 1999), DNA probe hybridization (Meletiadis et al., 2003), or DNA sequence analysis (Schmidt and Rath, 2003). However, molecular identification approaches especially using the ribosomal ITS1 and ITS2 regions as targets were widely used in fungi, and few studies were obtained from molecular identification in plants. There-fore, we investigated the classification of fifth different ecotypes using the ribosomal ITS1 and ITS2 regions as targets for the molecular identification. We acquired DNA sequence information concerning the ITS1 and ITS2 regions for each ecotype and conducted the sequence length, G+C content (%), DNA sequence alignments, pariwise nucleotide sequence analysis and comparative GenBank database searches.

MATERIALS AND METHODS

Plant materials

Fifth different ecotypes of *Z. schinifolium* were collected from various areas of South Korea. The voucher data for all ecotypes, abbre-viations and GenBank accession numbers are summarized in Table 1.

PCR amplification of the ribosomal ITS1 and ITS2 regions

Genomic DNAs were extracted using the modified sodium dodecyl sulfate (SDS) method (Möller et al., 1992). Common ITS primer sets ITS5, 5'-GAA AGT AAA AGT CGT AAC AAG G-3' and ITS2, 5'-GCT GCG TTC TTC ATC GAT GC-3' and ITS3, 5'-GCA TCG ATG AAG AAC GCA GC-3' and ITS4, 5'- TCC TCC GCT TAT TGA TAT GC-3' were used to amplify ribosomal ITS1 and ITS2, respectively (White et al., 1990). Common ITS primer sets ITS5 and ITS4 were used to amplify ribosomal total ITS region. PCR amplification was conducted using this set of primers with the following program: 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and a final extension step at 72°C for 1.5 min. All PCR products were purified before DNA sequence analysis using a QIAquick PCR Purification Kit (QIAGEN, Cat. No., 28104, Korea) according to the manufacturer's instructions. Purified PCR products were then sequenced at SolGent ASSA Service (Korea).

Sequence analysis

Analogue was detected with the basic local alignment search tool (BLAST) on the server on national center for biotechnology information (NCBI) (http://www.nncbi.nih.gov). The sequences of fifth different ecotypes were analyzed using DNAMAN 5.0.

Jaccard coefficients used to represent identity among the ecotypes were calculated by similarity coefficient [$S_j = a/(a+u°C)$]. In the total

Table 2. Symmetric matrix of Jaccard coefficients (% identity) in total ITS regions between fifth different ecotypes of medically important *Z. schinifolium.*

Fifth ecotypes	BEMR	CWDO	ESSI	HCBS	HCDC	JABG	JDGG	GCNS	GCSL	GHDY	GJGD	GNGD	GRDG	WJHJ	YDCS
BEMR	100														
CWDO	99.6	100													
ESSI	99.6	99.6	100												
HCBS	99.6	99.9	99.7	100											
HCDC	99.4	99.6	99.7	99.7	100										
JABG	99.6	99.9	100	100	99.9	100									
JDGG	99.6	99.1	99.3	99.3	99.3	100	100								
GCNS	99.4	99.4	99.9	99.6	99.9	99.9	99.1	100							
GCSL	99.4	98.8	98.8	99	99.3	99.9	99.6	99.1	100						
GHDY	99.6	99.1	99.6	99.3	99.3	100	99.1	99.4	98.8	100					
GJGD	99.4	99	99.1	99.1	99.3	99.9	99.9	99.1	99.7	99	100				
GNGD	99.4	99.7	99.6	99.9	99.9	99.9	99.1	99.7	99.1	99.1	99.1	100			
GRDG	99.6	99.6	99.3	99.4	99.3	99.6	99.1	99.1	98.8	98.8	99	99.3	100		
WJHJ	99.6	99.4	99.3	99.6	99.3	100	99.1	99.1	98.8	99.7	99	99.4	99	100	
YDCS	99.6	99.1	99.1	99.3	99.3	100	99.7	99.1	99.7	99.1	99.9	99.1	99.1	99.1	100

ITS region, ITS1 and ITS2 region, '1' was used for base variation and '0' was used for no variation; 'a' represents the number of the same bases and 'u' represents the number of different bases between the two ecotypes.

RESULTS

Symmetric matrix of Jaccard coefficients of the ribosomal ITS regions

The results of symmetric matrix of Jaccard co-efficients of total ITS regions showed 98.8 to 100% identity (Table 2), of which ITS1 ribosomal region played a more positive role than ITS2 ribosomal region. The greatest identity in nucleotide sequence appeared between *Z. schinifolium* JABG and ESSI, HCBS, JDGG, GHDY, WJHJ and YDCS (100% identity) and the sequence of JABG compared with that of CWDO, HCDC, GCSL, GJGD and GNGD was also very similar, showing 99.9% identity (Table 2). However, GCSL and CWDO, ESSI, GHDY, GRDG and WJHJ had the highest dissimilarity in the total ITS sequence, with 98.8% identity.

In ribosomal ITS1 region, symmetric matrix of Jaccard coefficients was found to have 98.3 to 100% sequence identity (Table 3). The most dis-similarity in ribosomal ITS1 region (98.3% identity) was found between GRDG and BEMR, CWDO, HCDC, JDGG and GJGD. Other ecotypes in *Z. schinifolium* such as ESSI, HCBS, JABG, GCNS, GCSL, GHDY, GNGD, WJHJ, YDCS and GRDG showed 100% similarity in the sequence of ribosomal ITS1 region and BEMR, CWDO, HCDC, JDGG and GJGD also showed very high similarity (100% identity) between each other but were likely to differ with ESSI and those parallel ecotypes.

On the other hand, symmetric matrix of Jaccard coefficients of the ribosomal ITS2 region showed relatively simple results (Table 4). Sequence identities among nearly all fifth different ecotypes showed absolutely single sequence, in represen-tative of the sequence of BEMR and GRDG. BEMR and GRDG had 99.5% sequence identity with all other ecotypes but 100% sequence identity with each other, indicating that the ITS2 region showed were highly conserved among *Z. schinifolium* and less advantageous in molecular identification than the ITS1 region.

Total ITS, ITS1 and ITS2 sequence length analysis and G+C content among *Z. schinifolium*

The total ITS region ranged in overall length from 618 to 620 bp (Table 5) and the difference was mainly affected by the ITS1 region. ITS2 ribosomal region showed absolutely identical sequence length,

Table 3. Symmetric matrix of Jaccard coefficients (% identity) in ITS1 regions between fifth different ecotypes of medically important *Z. schinifolium.*

Fifth ecotypes	BEMR	CWDO	ESSI	HCBS	HCDC	JABG	JDGG	GCNS	GCSL	GHDY	GJGD	GNGD	GRDG	WJHJ	YDCS
BEMR	100														
CWDO	100	100													
ESSI	98.7	98.7	100												
HCBS	98.7	98.7	100	100											
HCDC	100	100	98.7	98.7	100										
JABG	98.7	98.7	100	100	98.7	100									
JDGG	100	100	98.7	98.7	100	98.7	100								
GCNS	98.7	98.7	100	100	98.7	100	98.7	100							
GCSL	98.7	98.7	100	100	98.7	100	98.7	100	100						
GHDY	98.7	98.7	100	100	98.7	100	98.7	100	100	100					
GJGD	100	100	98.7	98.7	100	98.7	100	98.7	98.7	98.7	100				
GNGD	98.7	98.7	100	100	98.7	100	98.7	100	100	100	98.7	100			
GRDG	98.3	98.3	99.6	99.6	98.3	99.6	98.3	99.6	99.6	99.6	98.3	99.6	100		
WJHJ	98.7	98.7	100	100	98.7	100	98.7	100	100	100	98.7	100	99.6	100	
YDCS	98.7	98.7	100	100	98.7	100	98.7	100	100	100	98.7	100	99.6	100	100

Table 4. Symmetric matrix of Jaccard coefficients (% identity) in ITS2 regions between fifth different ecotypes of medically important *Z. schinifolium*

Fifth ecotypes	BEMR	CWDO	ESSI	HCBS	HCDC	JABG	JDGG	GCNS	GCSL	GHDY	GJGD	GNGD	GRDG	WJHJ	YDCS
BEMR	100														
CWDO	99.5	100													
ESSI	99.5	100	100												
HCBS	99.5	100	100	100											
HCDC	99.5	100	100	100	100										
JABG	99.5	100	100	100	100	100									
JDGG	99.5	100	100	100	100	100	100								
GCNS	99.5	100	100	100	100	100	100	100							
GCSL	99.5	100	100	100	100	100	100	100	100						
GHDY	99.5	100	100	100	100	100	100	100	100	100					
GJGD	99.5	100	100	100	100	100	100	100	100	100	100				
GNGD	99.5	100	100	100	100	100	100	100	100	100	100	100			
GRDG	100	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	100		
WJHJ	99.5	100	100	100	100	100	100	100	100	100	100	99.5	99.5	100	
YDCS	99.5	100	100	100	100	100	100	100	100	100	100	99.5	99.5	100	100

Table 5. Total ITS, ITS1 and ITS2 sequence length analysis among fifth different ecotypes of medically important *Z. schinifolium.*

Ecotypes	Size of total ITS region[a] (bp)	Size of ITS1 region (bp)	Size of ITS2 region (bp)
BEMR	620	236	219
CWDO	620	236	219
ESSI	619	235	219
HCBS	619	235	219
HCDC	620	236	219
JABG	619	235	219
JDGG	620	236	219
GCNS	619	235	219
GCSL	618	234	219
GHDY	619	235	219
GJGD	620	236	219
GNGD	619	235	219
GRDG	619	235	219
WJHJ	619	235	219
YDCS	619	235	219

[a] Total ITS regions of fifth different ecotypes included the ITS1 and ITS2 region and 5.8S rRNA gene that exhibited a conserved length of 165 bp between ITS1 and ITS2 region in all ecotypes.

Table 6. G+C content (%) of total ITS, ITS1, 5.8S rRNA and ITS2 region among fifth different ecotypes of medically important *Z. schinifolium.*

Ecotypes	G+C content (%)			
	Total ITS region (%)	ITS1 region (%)	5.8S rRNA (%)	ITS2 region (%)
BEMR	65.00	67.80	53.94	70.32
CWDO	65.16	67.80	53.94	70.78
ESSI	64.78	66.81	53.94	70.78
HCBS	64.78	66.81	53.94	70.78
HCDC	65.16	67.80	53.94	70.78
JABG	64.78	66.81	53.94	70.78
JDGG	65.16	67.80	53.94	70.78
GCNS	64.78	66.81	53.94	70.78
GCSL	65.05	67.52	53.94	70.78
GHDY	64.78	66.81	53.94	70.78
GJGD	65.16	67.80	53.94	70.78
GNGD	64.78	66.81	53.94	70.78
GRDG	64.62	66.81	53.94	70.32
WJHJ	64.78	66.81	53.94	70.78
YDCS	64.78	66.81	53.94	70.78

with 219 bp in all ecotypes (Table 5). The intervening part, 5.8S rRNA gene, exhibited a conserved length of 165 bp in all ecotypes investigated in this study (data to be shown in DNA alignment part). Therefore, ITS1 ribosomal region which showed less than 2 bp differences among all different ecotypes, mostly worked on the variant of the total ITS sequence length. Among them, GCSL had the shortest sequence length and BEMR, CWDO, HCDC, JDGG and GJGD had the longest sequence length.

The G+C content (%) in the total ITS region ranged from 64.62 to 65.16% (Table 6). The contents (%) of G +

C ranged from 66.81 to 67.80% and 70.32 to 70.78% in the ITS1 and ITS2 region, respectively. However, 5.8S rRNA showed the constant G + C content of 53.94% (Table 6). Among them, GRDG had the lowest G + C content (%) in the total ITS, ITS1 and ITS2 regions; CWDO, HCDC, JDGG and GJGD had the highest G + C content (%) in the total ITS, ITS1 and ITS2 regions.

DNA alignment of ITS1 and ITS2 ribosomal regions among *Z. schinifolium*

To discriminate the fifth different ecotypes properly, DNA alignments of the total ITS, ITS1 and ITS2 ribosomal regions were conducted to identify areas which displayed the dissimilarity in sequences. The 18S rRNA gene existing in the front of 5' end of the total ITS ribosomal region and the 28S rRNA gene existing in the back of 3' end of the ITS region were highly conversed among the fifth different ecotypes of *Z. schinifolium*, and the same results were obtained from the 5.8S rRNA gene intervening between the ITS1 and ITS2 ribosomal regions (Figure 1). The 5.8S rRNA gene started at a triplet code, AAC and ended at a triplet code, GCA, labeled with red fonts (Figure 1), showing 100% sequence identity in the total size of 165 bp among these ecotypes. The ITS1 ribosomal regions displayed the first nucleotide deletion at the 4th bp site among ESSI, HCBS, JABG, GCNS, GCSL, GHDY, GNGD, GRDG, WJHJ and YDCS ecotypes (Figure 2). And the second nucleotide deletion occurred successively at 15th bp site, but only GCSL showed this nucleotide deletion. In the ITS2 ribosomal region, the sequences had the same size of 219 bp, but a variety of sequence was obtained at 207th bp site (Figure 3). BEMR and GRDG exhibited nucleotide 'T' instead of 'C' in all other ecotypes.

Comparative GenBank analysis of total ITS, ITS1, and ITS2 ribosomal sequences

To determine the differences between our sequences and existing sequences in public database, comparative sequence analysis of the ITS1 and ITS2 ribosomal regions were conducted with BLAST searches of the NCBI GenBank database. For internal transcribed spacer sequences in *Z. schinifolium*, only two items, DQ225846 and DQ225861, were obtained and recited as ITS1 complete sequence and ITS2 partial sequence, respectively. Three ITS1 sequences showing diversity in our results (BEMR, GCSL and GRDG) were selected and compared with the existing ITS1 sequence (DQ225846), while two ITS2 sequences showing diversity in our results (BEMR and GCSL) were used to compare with the existing ITS2 sequence (DQ225861). The BEMR ecotype had the identical sequence in the ITS1 region compared with existing sequence (Figure 4), and GCSL and GRDG

also showed relatively high similarity in sequence, having 98.7 and 98.3% identity, respectively. Comparing with the existing sequence in the ITS2 region, the BEMR ecotype showed 99.5% sequence identity (Figure 5); GCSL had an absolutely identical sequence with the existing sequence (DQ225861), although these sequences in our results were shorter than the existing one.

DISCUSSION

Phylogenetic relationship in fungal pathogens and plants were mainly based on systematic studies, including morphology and molecular biology. Traditional methods have been found to have localization in species identification, and better methods are required to be exploited to meet the needs of more refined species confirmation. Several studies based on molecular identification have been investigated in some plant species such as *Sorghum* (Dillon et al., 2001; Dillon et al., 2004; Price et al., 2005). Dillon et al. (2001) had attempted to determine the phylogenetic relationships between 25 *Sorghum* species using the ribosomal ITS1 and ndhF, and obtained two distinct lineages. Price et al. (2005) combined sequence analysis of ITS1 and ndhF with chromosome number and 2C DNA content to evaluate the phylogenetic relationships between 25 sorghum species.

Simple sequence repeat (SSR) markers were used to characterize diversity in 28 Eritrean sorghum landraces and a high level of diversity was observed, indicating that SSR markers could be effective in species identification. In the present work, we investigated the identification among fifth different ecotypes of *Z. schinifolium* based on the sequence analysis of ITS1 and ITS2 regions. The ecotypes differ in their physiologies according to the ecological distributions (Yaun and KuÈpfer, 1995; Moore and Chisholm, 1999). Ecotypes also showed gene diversity due to the adaptability of environment and many years of natural evolution. The ITS region exhibits between highly conserved 18S and 28S rRNA genes which provides advantages for primer design and PCR amplification (Ebach and Holdrege, 2005). In addition, the ITS ribosomal region exhibits a great deal of length and sequence variation, it has frequently been used to identify species (Moore et al., 1998; Moritz and Cicero, 2004). Therefore, to better understand the phylogenetic relationships among these ecotypes, further molecular genetic research is necessary.

The emergence of sequence identification with a BLAST similarity search connected to public databases (Altschul et al., 1997) has resolved several experimental and taxonomic constraints. Thus, other molecular identification such as ndhF and EST should be done to explore the possibility of the multiple variety of sequence. To our knowledge, studies of molecular identification using the ITS region as markers in plants is far lesser than those in fungal and bacterial groups. This work not only provides

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BEMR          GTGACTGCGGAGGTCATTGTCGAAACCTCTGCAAGAGCAGAACGACCCGCGAA
CWDO          GTGACTGCGGAGGTCATTGTCGAAACCTCTGCAAGAGCAGAACGACCCGCGAA
ESSI          GTGACTGCGGAGGTCATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
HCBS          GTGACTGCGGAGGTCATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
HCDC          GTGACTGCGGAGGACATTGTCGAAACCTCTGCAAGAGCAGAACGACCCGCGAA
JABG          GTGACTGCGGAGGTCATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
JDGG          GNTGACTGCGGAGGTCATTGTCGAAACCTCTGCAAGAGCAGAACGACCCGCGAA
GCNS          GTGACTGCGGAGGACATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
GCSL          AGTGACTGCGGAGGACATTGTCGAA-CCTCTGCA-GAGCAGAACGACCCGCGAA
GHDY          GTGACTGCGGAGGTCATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
GJGD          GTGACTGCGGAGA-CATTGTCGAAACCTCTGCAAGAGCAGAACGACCCGCGAA
GNGD          GTGACTGCGGAGGACATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
GRDG          GTGACTGCGGAGGTCATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
WJHJ          GTGACTGCGGAGGTCATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
YDCS          AGTGACTGCGGAGGTCATTGTCGAA-CCTCTGCAAGAGCAGAACGACCCGCGAA
              **********  ********* ******* ******************

BEMR          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
CWDO          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
ESSI          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
HCBS          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
HCDC          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
JABG          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
JDGG          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
GCNS          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
GCSL          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
GHDY          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
GJGD          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
GNGD          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
GRDG          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
WJHJ          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
YDCS          CTCGTGATCACACTAGCGGGGGGCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTG
              ************************************************************

BEMR          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
CWDO          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
ESSI          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
HCBS          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
HCDC          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
JABG          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
JDGG          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
GCNS          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
GCSL          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
GHDY          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
GJGD          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
GNGD          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
GRDG          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
WJHJ          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
YDCS          TGGGACTCCTCCCGTTCCCCGCGGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAA
              ************************************************************

BEMR          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
CWDO          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
ESSI          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
HCBS          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
HCDC          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
JABG          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
JDGG          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
GCNS          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
GCSL          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
GHDY          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
GJGD          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
GNGD          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
GRDG          GGAAATCTAACGAGAGAGCACGCTCTCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
WJHJ          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
YDCS          GGAAATCTAACGAGAGAGCACGCTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGT
              ************************* *********************************
```

Figure 1. DNA alignment of the total ITS ribosomal region among fifth different ecotypes of medically important *Z. schinifolium*. To illustrate the sequence divergence, the boundary parts of 5.8S rRNA gene were labeled with red fonts.

```
BEMR    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
CWDO    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
ESSI    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
HCBS    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
HCDC    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
JABG    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
JDGG    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
GCNS    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
GCSL    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
GHDY    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
GJGD    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
GNGD    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
GRDG    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
WJHJ    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
YDCS    CGCCTTCTTTCACTCTATCTGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCG
        ************************************************************
BEMR    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
CWDO    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
ESSI    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
HCBS    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
HCDC    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
JABG    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
JDGG    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
GCNS    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
GCSL    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
GHDY    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
GJGD    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
GNGD    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
GRDG    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
WJHJ    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
YDCS    ATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGA
        ************************************************************
BEMR    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
CWDO    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
ESSI    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
HCBS    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
HCDC    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
JABG    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
JDGG    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
GCNS    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
GCSL    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
GHDY    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
GJGD    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
GNGD    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
GRDG    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
WJHJ    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
YDCS    GTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTC
        ************************************************************
BEMR    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
CWDO    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
ESSI    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
HCBS    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
HCDC    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
JABG    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
JDGG    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
GCNS    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
GCSL    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
GHDY    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
GJGD    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
GNGD    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
GRDG    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
WJHJ    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
YDCS    ACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGC
        ************************************************************
```

Figure 1. Continued.

```
BEMR        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
CWDO        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
ESSI        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
HCBS        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
HCDC        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
JABG        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
JDGG        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
GCNS        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
GCSL        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
GHDY        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
GJGD        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
GNGD        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
GRDG        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
WJHJ        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
YDCS        CTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGC
            ************************************************************
BEMR        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
CWDO        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
ESSI        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
HCBS        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
HCDC        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
JABG        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
JDGG        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
GCNS        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
GCSL        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
GHDY        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
GJGD        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
GNGD        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
GRDG        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
WJHJ        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
YDCS        GACGATCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTC
            ************************************************************
BEMR        GAGACTCAGGGACCCTGACGCTCCGCGCGAGTGGCGCTCGCATCGCGACCCCAGGTCAGG
CWDO        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
ESSI        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
HCBS        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
HCDC        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
JABG        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
JDGG        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
GCNS        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
GCSL        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
GHDY        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
GJGD        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
GNGD        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
GRDG        GAGACTCAGGGACCCTGACGCTCCGCGCGAGTGGCGCTCGCATCGCGACCCCAGGTCAGG
WJHJ        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
YDCS        GAGACTCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGG
            ******************************* **************************
BEMR        CGGGATTACCCGCTGAGTTTAAGCATACTA----------------
CWDO        CGGGATTACCCGCTGAGTTTAAGCATATCAAAAAGGCCGGAGGAA--
ESSI        CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGGCGGAGGA---
HCBS        CGGGATTACCCGCTGAGTTTAAGCATATCAATAGGCCGGAGGAAA-
HCDC        CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCCGGAGGA---
JABG        CGGGATTACCCGCTGAGTTTAAGCATATCAATA-------------
JDGG        CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA---
GCNS        CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGGCGGAGGAA--
GCSL        CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA---
GHDY        CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGGCGGGAGGAAA
GJGD        CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA---
GNGD        CGGGATTACCCGCTGAGTTTAAGCATATCAATAGNCCGGAGGAAA-
GRDG        CGGGATTACCCGCTGAGTTTAAGCATATCAAAANGCGGGAGGA---
WJHJ        CGGGATTACCCGCTGAGTTTAAGCATATCAATAGGCCGGGAGGAAA
YDCS        CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA---
            *************************   *
```

Figure 1. Continued.

```
BEMR    TCGAAACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
CWDO    TCGAAACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
ESSI    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
HCBS    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
HCDC    TCGAAACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
JABG    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
JDGG    TCGAAACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
GCNS    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
GCSL    TCG-AACCTCTGCA-GAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
GHDY    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
GJGD    TCGAAACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
GNGD    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
GRDG    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
WJHJ    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
YDCS    TCG-AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGGGCGC
        **********  *************************************************
BEMR    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
CWDO    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
ESSI    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
HCBS    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
HCDC    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
JABG    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
JDGG    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
GCNS    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
GCSL    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
GHDY    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
GJGD    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
GNGD    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
GRDG    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
WJHJ    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
YDCS    GCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGG
        ************************************************************
BEMR    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
CWDO    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
ESSI    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
HCBS    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
HCDC    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
JABG    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
JDGG    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
GCNS    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
GCSL    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
GHDY    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
GJGD    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
GNGD    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
GRDG    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCT
WJHJ    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
YDCS    GCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCC
        ************************************************************
BEMR    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
CWDO    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
ESSI    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
HCBS    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
HCDC    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
JABG    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
JDGG    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
GCWS    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
GCSL    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
GHDY    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
GJGD    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
GWGD    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
GRDG    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
WJHJ    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
YDCS    CGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
        ********************************************************
```

Figure 2. DNA alignment of the ribosomal ITS1 region among fifth different ecotypes of medically important *Z. schinifolium*. To illustrate the sequence divergence, the nonidentical parts in the ITS1 region were labeled with red fonts.

```
BEMR    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
CWDO    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
ESSI    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
HCBS    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
HCDC    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
JABG    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
JDGG    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
GCNS    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
GCSL    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
GHDY    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
GJGD    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
GNGD    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
GRDG    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
WJHJ    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
YDCS    TCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCC
        ************************************************************
BEMR    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
CWDO    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
ESSI    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
HCBS    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
HCDC    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
JABG    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
JDGG    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
GCNS    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
GCSL    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
GHDY    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
GJGD    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
GNGD    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
GRDG    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
WJHJ    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
YDCS    GTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGA
        ************************************************************
BEMR    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
CWDO    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
ESSI    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
HCBS    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
HCDC    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
JABG    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
JDGG    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
GCNS    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
GCSL    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
GHDY    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
GJGD    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
GNGD    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
GRDG    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
WJHJ    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
YDCS    TCGGTGGTGAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGAC
        ************************************************************
BEMR    TCAGGGACCCTGACGCTCCGCGCGAGTGGCGCTCGCATC
CWDO    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
ESSI    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
HCBS    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
HCDC    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
JABG    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
JDGG    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
GCNS    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
GCSL    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
GHDY    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
GJGD    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
GNGD    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
GRDG    TCAGGGACCCTGACGCTCCGCGCGAGTGGCGCTCGCATC
WJHJ    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
YDCS    TCAGGGACCCTGACGCTCCGCGCGAGCGGCGCTCGCATC
        ************************** ***********
```

Figure 3. DNA alignment of the ribosomal ITS2 region among fifth different ecotypes of medically important *Z. schinifolium*. To illustrate the sequence divergence, the nonidentical parts in the ITS1 region were labeled with red fonts.

```
ITS1          GGATCGCGGCGACGCGGGCGGTTCGCTGCCTGCGACGTCGCGAGAAGTCCACTGAACCTT
BEMR
GCSL
GRDG


ITS1          ATCATTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
BEMR
GCSL
GRDG


ITS1          ATTGTCGAAACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGG
BEMR             TCGAAACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGG
GCSL             TCG AACCTCTGCA-GAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGG
GRDG             TCG AACCTCTGCAAGAGCAGAACGACCCGCGAACTCGTGATCACACTAGCGGGGG
                 ********** ***********************************************
ITS1          GCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGC
BEMR          GCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGC
GCSL          GCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGC
GRDG          GCGCGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGC
              ************************************************************
ITS1          GGGGGCGGATAACGAACCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACG
BEMR          GGGGGCGGATAACGAACCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACG
GCSL          GGGGGCGGATAACGAACCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACG
GRDG          GGGGGCGGATAACGAACCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACG
              ***********************************************************
ITS1          CTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
BEMR          CTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
GCSL          CTCCCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
GRDG          CTCTCGGGGCCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGA
              *** ******************************************************
ITS1          AACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCG
BEMR          ------------------------------------------------------------
GCSL          ------------------------------------------------------------
GRDG          ------------------------------------------------------------


ITS1          ATACTTGGT
BEMR          ---------
GCSL          ---------
GRDG          ---------
```

Figure 4. Comparative GenBank analysis of the ITS1 ribosomal sequences with BEMR, GCSL and GRDG. ITS1 sequence investigated in this study is the existing ITS1 sequence of *Z. schinifolium* in GenBank, and accession no. is DQ225846.

```
ITS2          AGTTGCGCCCCAAGCCTTTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCATCGTTGCC
BEMR                                                                      TCGTTGCC
GCSL                                                                      TCGTTGCC
                                                                          ********
ITS2          CCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCCGTGCGCTC
BEMR          CCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCCGTGCGCTC
GCSL          CCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGAGGGCGGATAATGGCCTCCCGTGCGCTC
              ************************************************************
ITS2          CCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGATCGGTGGT
BEMR          CCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGATCGGTGGT
GCSL          CCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCGACCGGAGCCGCGACGATCGGTGGT
              ************************************************************
ITS2          GAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGACTCAGGGAC
BEMR          GAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGACTCAGGGAC
GCSL          GAAAACAAACCTCTCGAACACACGTCGCGTGCCCGGCTCTCCGTTTCGAGACTCAGGGAC
              ************************************************************
ITS2          CCTGACGCTCCGCGCGAGCGGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGC
BEMR          CCTGACGCTCCGCGCGAGTGGCGCTCGCATC-----------------------------
GCSL          CCTGACGCTCCGCGCGAGCGGCGCTCGCATC-----------------------------
              ****************** ************
ITS2          TGAGTTTAAGCATATCAATA
BEMR          --------------------
GCSL          --------------------
```

Figure 5. Comparative GenBank analysis of the ITS2 ribosomal sequences with BEMR and GCSL. ITS2 sequence investigated in this study is the existing ITS2 sequence of *Z. schinifolium* in GenBank, and accession no. is DQ225861.

more resources of ITS sequence in *Z. schinifolium*, but distinguishes five groups from the fifth different ecotypes, which makes it possible to elucidate the phylogenetic relationships of *Z. schinifolium* species.

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