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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 characteristics 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. Therefore, 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, abbreviations 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℃ for 1 min, annealing at 55℃ for 1 min and a final extension step at 72℃ 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 [Sj = a/(a+u℃)]. 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 coefficients 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 dissimilarity 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 representative 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            CGGGATTACCCGCTGAGTTTAAGCATATCAAAAGGCCGGAGGAA--
ESSI            CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGGCGGAGGA---
HCBS            CGGGATTACCCGCTGAGTTTAAGCATATCAATAGGCCGGAGGAAA-
HCDC            CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCCGGAGGA---
JABG            CGGGATTACCCGCTGAGTTTAAGCATATCAATA-------------
JDGG            CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA---
GCNS            CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGGCGGAGGAA--
GCSL            CGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGAAGGAA---
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            GGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACG
BEMR            GGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACG
GCSL            GGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACG
GRDG            GGGGGCGGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACG
                 ************************************************************
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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