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Full Length Research Paper

The internal transcribed spacer rDNA specific markers for identification of *Zanthoxylum piperitum*

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Genus *Zanthoxylum* which has significant medical importance belongs to the family Rutaceae. This investigation was aimed to identify total internal transcribed spacer (ITS) regions among the nuclear ribosomal DNA (nrDNA) to distinguish *Zanthoxylum piperitum* from *Zanthoxylum sichinifolium*. The nrDNA ITS sequence markers were developed by using universal ITS5/ITS4 primer pairs. Species-specific primers amplified the total ITS region sequence named ITS1-YL1 and ITS1-YL2. These were amplified efficiently when paired with universal primer ITS4 in *Z. piperitum*, but not in *Z. schinifolium*. ITS1-YL1/ITS4 or ITS1-YL2/ITS4 preferential amplification was shown to be particularly useful for detection and distribution of *Z. piperitum* from other plant species, especially *Z. schinifolium*. These primers are useful to study the structure of Rutaceae family. Such identifications will be helpful for phylogenetic analysis in intraspecies population of the genus *Zanthoxylum*.

Key words: *Zanthoxylum piperitum*, rDNA Int-sp markers, phylogenetic relationship, ribosomal DNA, internal transcribed spacer.

INTRODUCTION

The genus *Zanthoxylum* comprised of more than 200 species, such as *Zanthoxylum piperitum*, *Zanthoxylum schinifolium* and *Zanthoxylum bungeanum* (Yang, 2008). These plants are aromatic trees and shrubs, native to warm temperate and subtropical areas in the world, especially in Asia. The fruits, pericarps and leaves of these species have been widely used as a pungent condiment and seasoning in some East Asian countries such as China, Korea and Japan (Paik et al., 2005). Culinary purposes of *Zanthoxylum* species have not been found in America and Africa. The 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). Furthermore, *Zanthoxylum* is a complicated genus with many different, similar and not well-researched species. Literature often gives contradicting information about the local species used as spice. Therefore, rapid and accurate identification of the genus *Zanthoxylum* is required.

Traditional classification methods based on morphological characteristics may not distinctly distinguished closely related species (Klich, 2002). Such classification is time-consuming, unable to provide clear cut results and sometimes unreliable (Matsuki et al., 2002). Recent research has led to rapid advances in the application of molecular techniques based on rRNA gene sequences to study the diversity in ecosystems (Hinrikson et al., 2005). Various rRNA gene regions as targets for the molecular identification have been investigated (Iwen et al., 2002), including 16S, 23S rRNA gene sequences and the ribosomal internal transcribed spacer (ITS) region between the small- and large-subunit rRNA gene sequences (White et al., 1990). ITS region contains two variable non-coding regions that are nested within the rDNA repeat between the highly conserved small subunit,

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Abbreviations: ITS, Internal transcribed spacer; **PCR,** polymerase chain reaction; **SDS,** sodium dodecyl sulfate; **SSU,** small subunit; **LSU,** large subunit; **ETS,** external transcribed spacer.

Table 1. Geographical origin and accession numbers of 14 ecotypes *Z. piperitum* and 6 ecotypes *Z. schinifolium*.

Species	Geographical origin in South Korea	Abbreviation	GenBank Accession No.
Z. piperitum	Cheongdo-Gun Gangnam-Myeon	CDGN1	GU434276
	Cheongdo-Gun Gangnam-Myeon	CDGN2	GU434277
	Donghae-Si Cheongok-Dong	DHCG	GU434278
	Ganghwa-Gun Hwado-Myeon	GHHD	GU434279
	Goseong-Gun Maam-Myeon	GSMA	GU434280
	Gwangyang-Si Ongnyong-Myeon	GYON	GU434281
	Haenam-Gun Songji-Myeon	HNSJ	GU434282
	Jindo-Gun Gogun-Myeon	JDGG	GU434283
	Mungyeong-Si Sanbuk-Myeon	MGSB	GU434284
	Samcheok-Si Gyo-Dong	SCGY	GU434285
	Seosan-Si Haemi-Myeon	SSHM	GU434286
	Ulsan Ulju-Gun Beomseo-Eup	UJBS	GU434287
	Yangyang-Gun Hyeonbuk-Myeon	YYHB1	GU434288
	Yangyang-Gun Hyeonbuk-Myeon	YYHB2	GU434289
Z. schinifolium	Boeun-Gun Maro-Myeon	BEMR	GU247226
	Changwon-Si Dong-Eup	CWDO	GU247227
	Eumseong-Gun Soi-Myeon	ESSI	GU247228
	Gochang-Gun Sinlim	GCSL	GU247230
	Goryeong-Gun Deokgok-Myeon	GRDG	GU247234
	Jinan-Gun Bugwi-Myeon	JABG	GU247237

5.8S, and large subunit rRNA genes. Using ITS region for sequence diversity based on the polymerase chain reaction (PCR) amplification has been investigated followed by fragment length analysis (Walsh et al., 1995; Turenne et al., 1999), DNA probe hybridization (Meletiadis et al., 2003; Haugland et al., 2004) or DNA sequence analysis (Schmidt and Rath, 2003). One potential advantage of this method is that ITS primers designed according to the highly conserved rRNA gene sequences, could be highly applicable in a broad range of organisms including plants, fungi and animals (White et al., 1990). However, DNA from many plant species may not be amplified by the currently available primers, and thus species-specific ITS primers would be desirable.

In this study, we have designed two new primers that preferentially amplify the ITS region of *Z. piperitum*. Using newly designed primers paired with the universal primer ITS4, could efficiently amplify the total ITS region from *Z. piperitium*, but not from *Z. schinifolium*. This work provides a more rapid and accurate approach for the detection and identification of two related species.

MATERIALS AND METHODS

Plant materials

Twenty-one different ecotypes of *Z. piperitum*, including GSMA, MSJB, HCHC, CNGA, UJBS, MYDJ, GJSH, CGDM, GSCS, MGSB, GHHD, GYON, DHCG, HNSJ, SCGY, SSHM, JDGG, CDGN1, CDGN2, YYHB1 and YYHB2 were collected from Goseong-Gun Maam-Myeon (GSMA), Masan-Si Jinbuk-Myeon (MSJB), Hapcheon-Gun Hapcheon-Eup (HCHC), Changnyeong-Gun Goam-Myeon (CNGA), Ulsan Ulju-Gun Beomseo-Eup (ULBS), Miryang-Si Danjang-Myeon (MYDJ), Geoje-Si Sinhyeon-Eup (GJSH), Chigok-Gun Dongmyeong-Myeon (CGDM), Goesan-Gun Chiseong-Myeon (GSCS), Mungyeong-Si Sanbuk-Myeon (MGSB), Ganghwa-Gun Hwado-Myeon (GHHD), Gwangyang-Si Ongnyong-Myeon (GYON), Donghae-Si Cheongok-Dong (DHCG), Haenam-Gun Songji-Myeon (HNSJ), Samcheok-Si Gyo-Dong (SCGY), Seosan-Si Haemi-Myeon (SSHM), Jindo-Gun Gogun-Myeon (JDGG), Cheongdo-Gun Gangnam-Myeon (CDGN1), Cheongdo-Gun Gangnam-Myeon (CDGN2), Yangyang-Gun Hyeonbuk-Myeon (YYHB1) and Yangyang-Gun Hyeonbuk-Myeon (YYHB2) in Korea, respectively. The GenBank accession numbers of ITS region are shown partly in Table 1.

Six different ecotypes of *Z. schinifolium*, that is, BEMR, CWDO, ESSI, GCSL, GRDG and JABG were collected from Boeun-Gun Maro-Myeon (BEMR), Changwon-Si Dong-Eup (CWDO), Eumseong-Gun Soi-Myeon (ESSI), Gochang-Gun Sinlim-Myeon (GCSL), Goryeong- Gun Deokgok-Myeon (GRDG) and Jinan-Gun Bugwi-Myeon (JABG), respectively. The GenBank accession numbers of ITS region are shown in Table 1.

Design of species-specific primers

Two species-specific forward primers for ITS regions, that is, ITS1-YL1, 5'-ACT GAA CCT TAT CAT TTA GAG-3' and ITS1-YL2, 5'-AAG TCC ACT GAA CCT TAT CAT-3', were designed. The sequences used for comparison were obtained from NCBI GenBank, with Accession No. DQ225847, DQ225848, DQ225849 and DQ225850 for *Z. piperitium*, DQ225846 for *Z. schinifolium* and DQ143118, DQ225785, DQ225834, DQ225833, DQ225784, FJ593180 and FJ980442 for other plant species.

PCR amplification of the ribosomal ITS1 and ITS2 regions

Genomic DNA of each species was extracted by using the modified sodium dodecyl sulfate (SDS) method (Moller et al., 1992). The universal ITS primer pairs ITS5, 5'-GAAAGTAAAAGTCGTA ACAAGG-3'; ITS2, 5'-GCTGCG TTCTTCATCGATGC-3'; ITS3, 5'-GCATCGATGAAGAACGCAGC-3'; ITS4, 5'- TCCTCCGCTTATTGA TATGC-3', were used to amplify ribosomal ITS1 and ITS2, respectively (White et al., 1990). The universal ITS primer pairs ITS5 and ITS4, were used to amplify ribosomal total ITS region. For efficient amplification of the total ITS region in *Z. piperitum*, two newly designed species-specific primers, ITS1-YL1 and ITS1-YL2, were paired with the universal ITS primers, ITS2 or ITS4, to amplify ribosomal ITS1 or total ITS region, respectively. PCR amplification was conducted using these sets 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 Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) server (http://www.nncbi.nih.gov) (Altschul et al., 1997). The sequences of fifth different ecotypes were analyzed using DNAMAN 5.0.

RESULTS

DNA amplification in the ITS region

The universal ITS primer pairs, ITS5/ITS2, ITS3/ITS4 and ITS5/ITS4, were used to amplify DNA from *Z. piperitum* and *Z. schinifolium* (Figure 2A). The DNA from *Z. schinifolium* CWDO amplified efficiently with these primer sets, while DNAs from *Z. piperitum* GSMA and MSJB, resulted in a clear PCR band with ITS3/ITS4 primer pair and either no PCR product or an extremely faint product with other primer pairs. To determine whether the concentration and quality of DNAs used for reactions affected the occurrence of an efficient amplification, we tested various DNA concentrations of 5, 10 and 20 in a 20 µl volume for ITS amplification using the universal primer pairs. No efficient amplification was obtained from all cases with various DNA concentrations (Figure 2B). Based on these results, it was suggested that low efficiency in ITS amplification was not related with our DNA concentration and quality, and successful amplification of the ITS2 region implied well-matching of the universal primers, ITS3 and ITS4, with the DNA sequence in *Z. piperitum*. Thus, design of new species-specific primers applicable to *Z. piperitum*, especially the forward primer for ITS1 amplification, was desirable.

Design of species-specific ITS primers

It is known that the mismatches at the 3' end are critical for efficient amplification (Sommer and Tautz, 1989). However, based on the sequence analysis results, the universal primer, ITS5, used for amplification of the ITS1 or total ITS region, did not match well with the ITS1 region sequence from *Z. piperitum* (Figure 1). It resulted in the understanding of no DNA amplification in *Z. piperitum*, using the universal primer pairs, ITS2/ITS5 or ITS4/ITS5 (Figure 2A).

According to the rule of designing intended target sequences in the 5' end of the primers, ITS1-YL1 and ITS1-YL2 were designed and tested for the ability to amplify the ITS1 and total ITS regions when paired with ITS2 and ITS4, respectively (Figure 2C). In all cases, the *Z. piperitum* DNA was efficiently amplified to one single band in the proper size using ITS1-YL1 or ITS1-YL2 as the forward primer, while the *Z. schinifolium* DNA was amplified to multiple or vague bands, that did not meet the PCR and sequencing analysis's needs. Successful application of newly designed primers in *Z. piperitum* achieved preferential amplification of the total ITS region and effective design of species-specific primers in this species. To classify *Z. piperitum* and *Z. schinifolium*, amplification of the ITS1 region was investigated using the newly designed species-specific primer, ITS1-YL1, paired with the universal ITS primer, ITS2 in 21 ecotypes of *Z. piperitum*. We obtained a 322 bp PCR product of the ITS1 region, and the sizes of PCR product of 21 ecotypes of *Z. piperitum* were the same (data partly shown in Figure 3A). The ITS2 region was amplified from 21 ecotypes of *Z. piperitum* using the universal ITS3/ITS4 primer pair, and PCR products were about 389 bp in size (data partly shown in Figure 3B). To amplify the total ITS region of *Z. piperitum*, a length of 731 bp PCR product was obtained with no variation among 21 ecotypes using ITS1-YL1/ITS4 primer pair (data partly shown in Figure 3C).

Sequence analysis of the total ITS region in *Z. piperitum*

Fourteen PCR products of the total ITS region among 21 ecotypes of *Z. piperitum* were sequenced (Table 1), and highly conversed sequences including ITS1 and ITS2 rRNA gene sequences were obtained (Figure 4). Length analysis of these sequences suggested that no deletion occurred in the ITS1, 5.8S and ITS2 region, but only uncertain nucleotide denoted as 'N' in some sites (Table 2). However, the highly conversed 18S and 28S rRNA sequences in the 5' end of the ITS1 region and 3' end of the ITS2 region had some deletion. For example, 18S rRNA sequences of GSMA had a nucleotide deletion at 43 bp as compared with other sequences; CDGN2, DHCG, HNSJ, SCGY, SSHM, YYHB2 and JDGG had a nucleotide deletion in 28S rRNA sequence at 727 bp, while GSMA had a nucleotide deletion at 726 bp as compared with other sequences. Except for the sequence of CDGN2 which was absolutely read, other 13 sequences

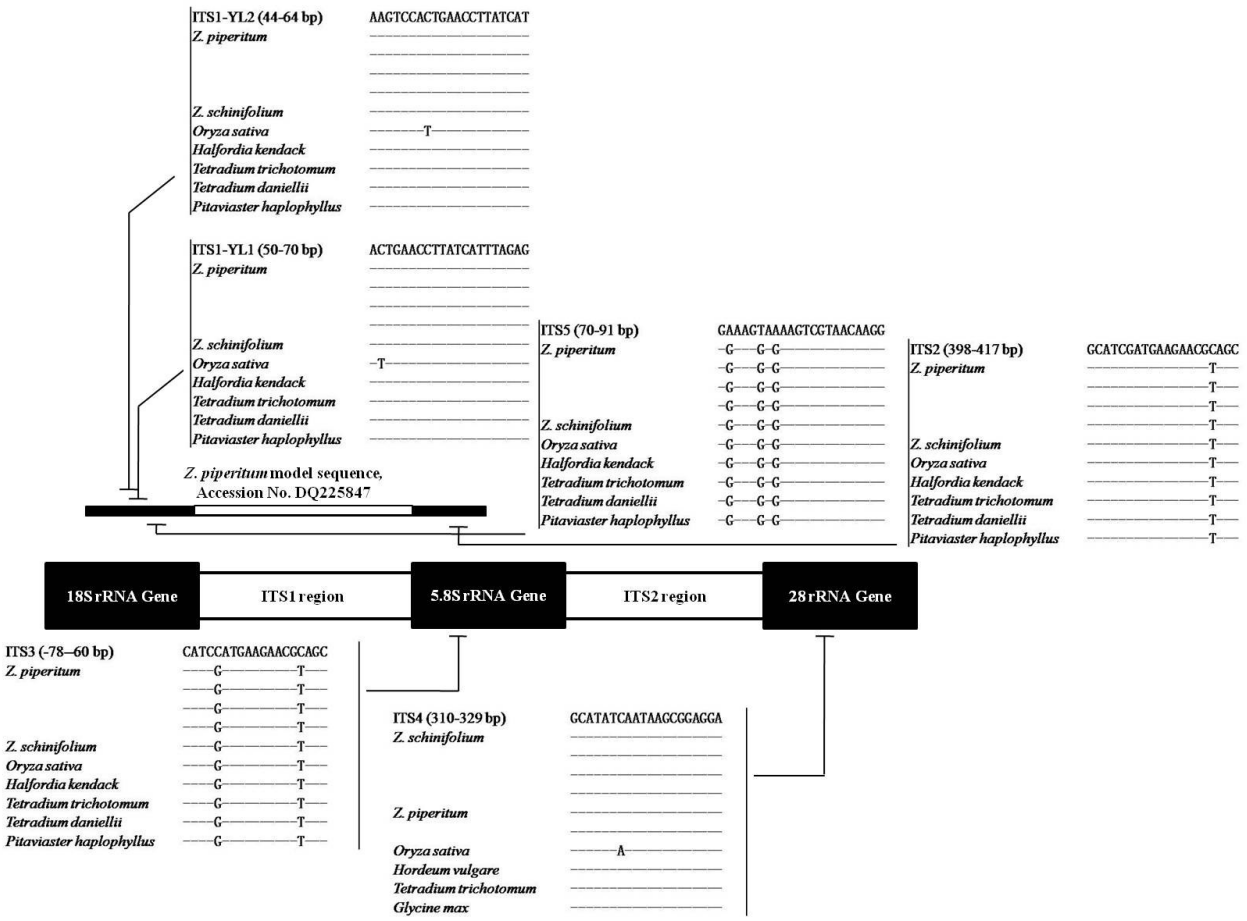

Figure 1. Species-specific primer design of *Z. piperitum* for the amplification of the total ITS region. The assured location of the universal ITS primers, ITS2, ITS3, ITS4 and ITS5 are shown according to the model sequences. Two newly designed species-specific forward primers, ITS1-YL1 and ITS1-YL2 are targeted in the '3 end of 18S rRNA gene sequence at 50 - 70 bp and 44 - 64 bp, respectively. The sequences used here were obtained from NCBI GenBank, with Accession No. DQ225847, DQ225848, DQ225849 and DQ225850 for *Z. piperitium*, DQ225846 for *Z. schinifolium* and DQ143118, DQ225785, DQ225834, DQ225833, DQ225784, FJ593180 and FJ980442 for other plant species. All primer sequences are listed here in the standard 5' to 3' direction.

had more or less uncertain nucleotide sites in the ITS1 region at 268 bp or the ITS2 region at 629, 630, 632, 633 and 661 bp.

To determine the diversity between our sequences and existing sequences in public database, comparative sequence analysis of the ITS1 and ITS2 regions were conducted in BLAST searches of the NCBI GenBank database. For the ITS region in *Z. piperitum*, four items, DQ225847-50, were obtained, but only recited as ITS1 complete sequence. Thus, complete sequences of the ITS2 and total ITS regions in 14 ecotypes of *Z. piperitum* were firstly reported here. And to determine the diversity in ITS1 region, CDGN2 as the model sequence was compared with the existing ITS1 region sequences (Figure 5). Thirteen variation nucleotide sites occurred in the ITS1 region among these sequences (Table 3). Compared with the existing sequences, our sequence, CDGN2, showed 100% identity with DQ225848, and 98.4, 97.6% identity with DQ225847, DQ225849 and DQ225850, respectively. Of them, CDGN2 compared with DQ225847 and DQ225848 had one nucleotide deletion at 119 bp.

Phylogenetic relationship between *Z. piperitum* and *Z. schinifolium*

The phylogenetic relationship analysis was investigatedbased on the total ITS region sequences of 14 ecotypes of *Z. piperitum* and 6 of *Z. schinifolium*. The homology tree showed two major clades (Figure 6), one formed with 14 *Z. piperitum* sequences and the other formed with 6 *Z. schinifolium* sequences. Our results made out the close evolution among 14 ecotypes of *Z. pipertium*, whereas in *Z. schinifolium* clade, there were three subclades, they showed 99% similarity with each other.

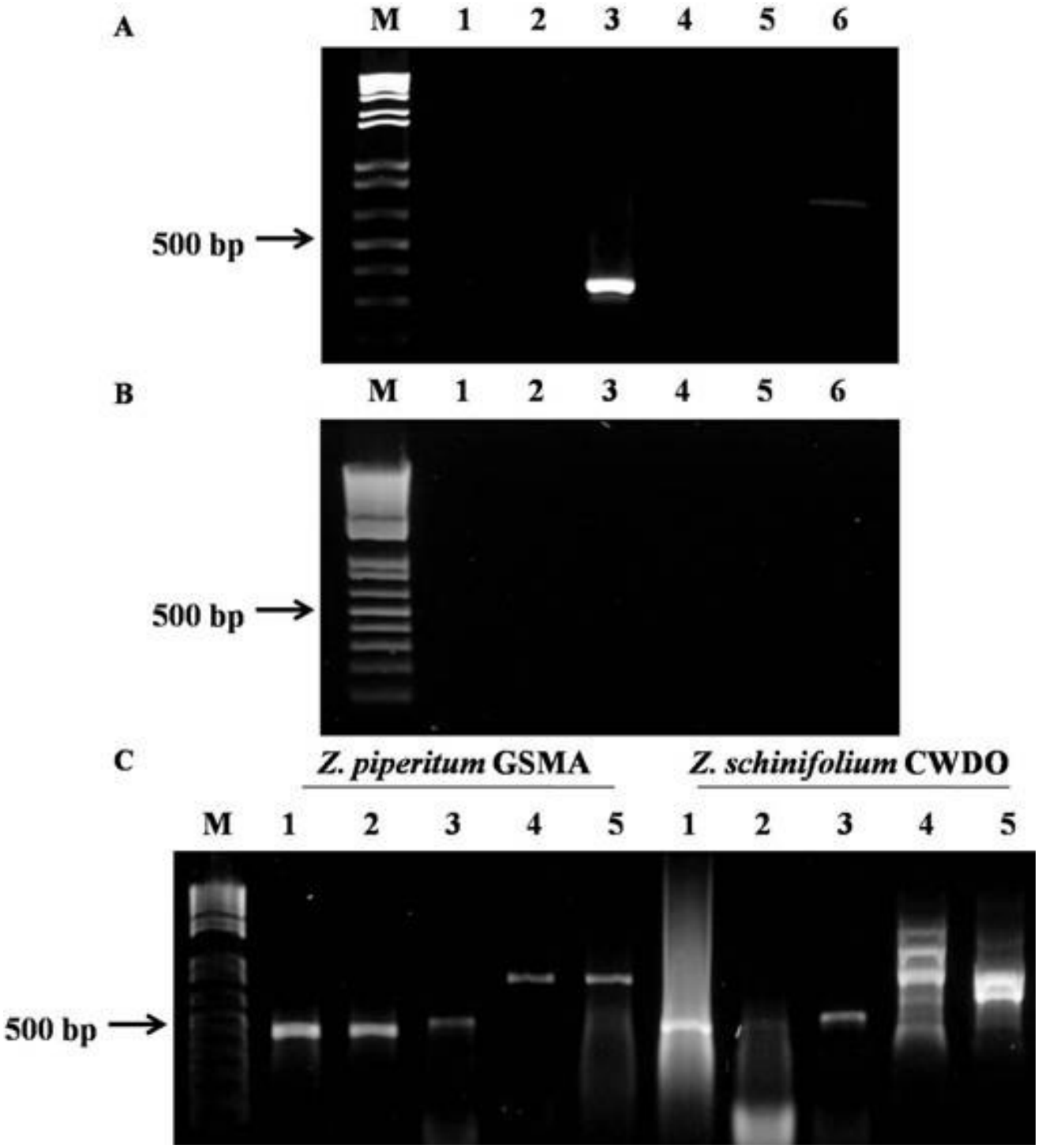

Figure 2. The ability of two species-specific primers in efficient amplification of ITS1, ITS2 and total ITS regions from *Z. piperitum* and *Z. schinifolium* DNAs. A. Reactions for the ITS1 (lane 1, 2 and 3) and total ITS region amplification (lane 4, 5 and 6) used ITS5/ITS2 and ITS5/ITS4 primers sets, respectively. DNAs were derived from *Z. piperitum* GYON (lane 1 and 4), *Z. piperitum* DHCG (lane 2 and 5) and *Z. schinifolium* CWDO (lane 3 and 6). B. All reactions were for the total ITS region amplification using ITS5/ITS4 primer set. DNAs were derived from *Z. piperitum* GYON (lane 1, 2 and 3 with 5, 10 and 20 µg in a 20 µl volume, respectively) and *Z. piperitum* DHCG (lane 4, 5 and 6 with 5, 10, 20 µg in a 20 µl volume, respectively). C. Reactions for the ITS1 region used ITS1-YL1/ITS2 and ITS1-YL2/ITS2 in lane 1 and 2, respectively. Reactions for the ITS2 region used ITS3/ITS4 in lane 3. Reactions for the total ITS region used ITS1-YL1/ITS4 and ITS1-YL2/ITS4 in lane 4 and 5, respectively. M, 1 kb Plus DNA ladder (Invitrogen, USA).

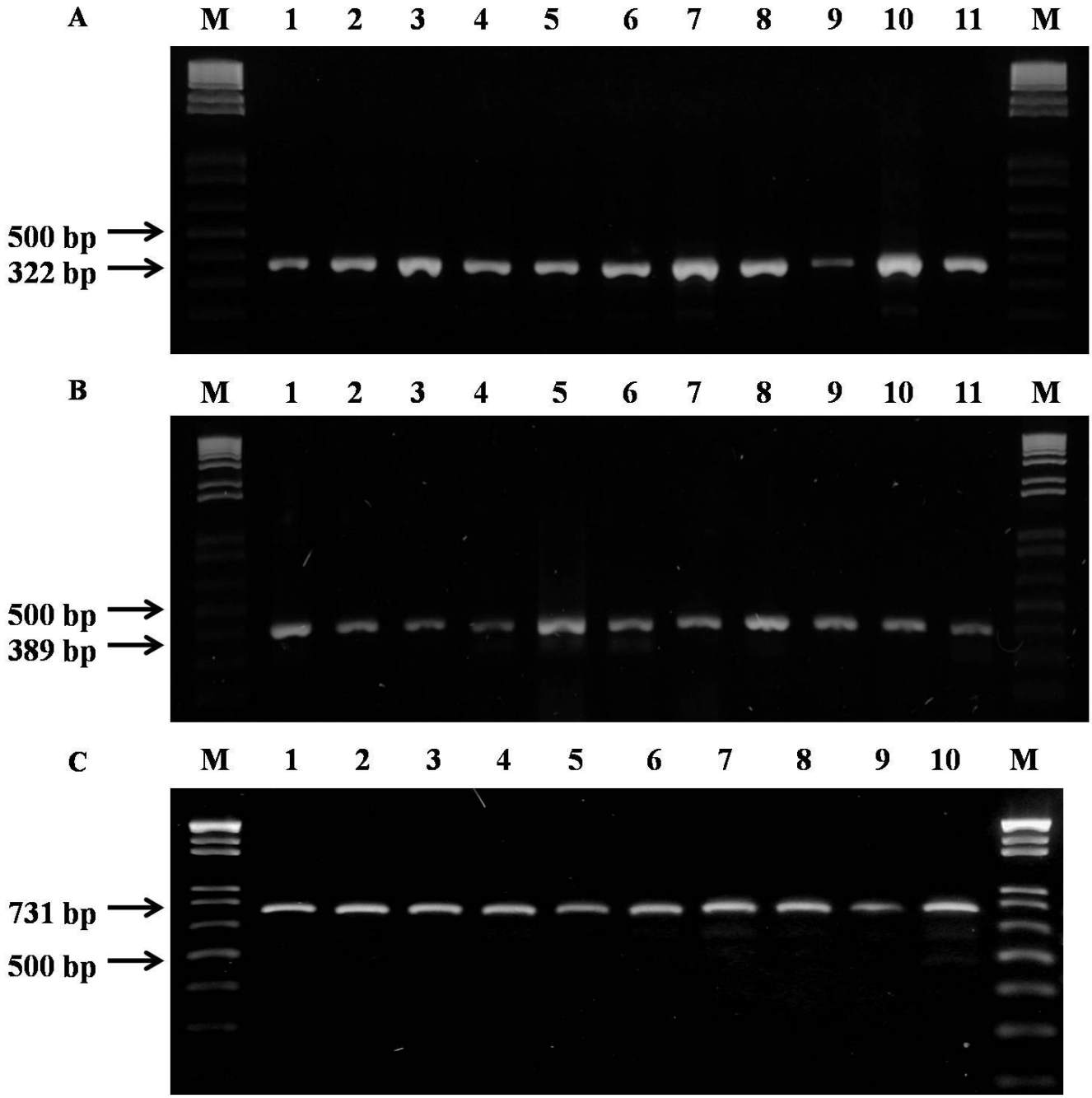

Figure 3. The ITS region amplification of *Z. piperitum*. A. DNAs were derived from *Z. piperitum* GSCS, GSMA, MSJB, HCHC, CNGA, UJBS, MYDJ, CDGN1, GJSH, CGDM, and MGSB in lane 1-11, respectively. Reactions used ITS1-F1/ITS2 primer set for the ITS1 region amplification. B. DNAs were derived from *Z. piperitum* GSCS, GSMA, MSJB, HCHC, CNGA, UJBS, MYDJ, CDGN1, GJSH, CGDM, and MGSB in lane 1-11, respectively. Reactions used ITS3/ITS4 primer set for the ITS2 region amplification. C. DNAs were derived from *Z. piperitum* GSCS, GSMA, MSJB, HCHC, CNGA, UJBS, MYDJ, CDGN1, GJSH, and CGDM in lane 1-10, respectively. Reactions used ITS1-F1/ITS4 primer set for the total ITS region amplification. M. 1kb Plus DNA ladder (Invitrogen, USA).

DISCUSSION

Systematic studies such as morphology and molecular biology have been mainly used for phylogenetic relationship analysis in fungal pathogens and plants. However, traditional methods based on morphological characteristics have been found to have localization in species identification, and better methods are therefore

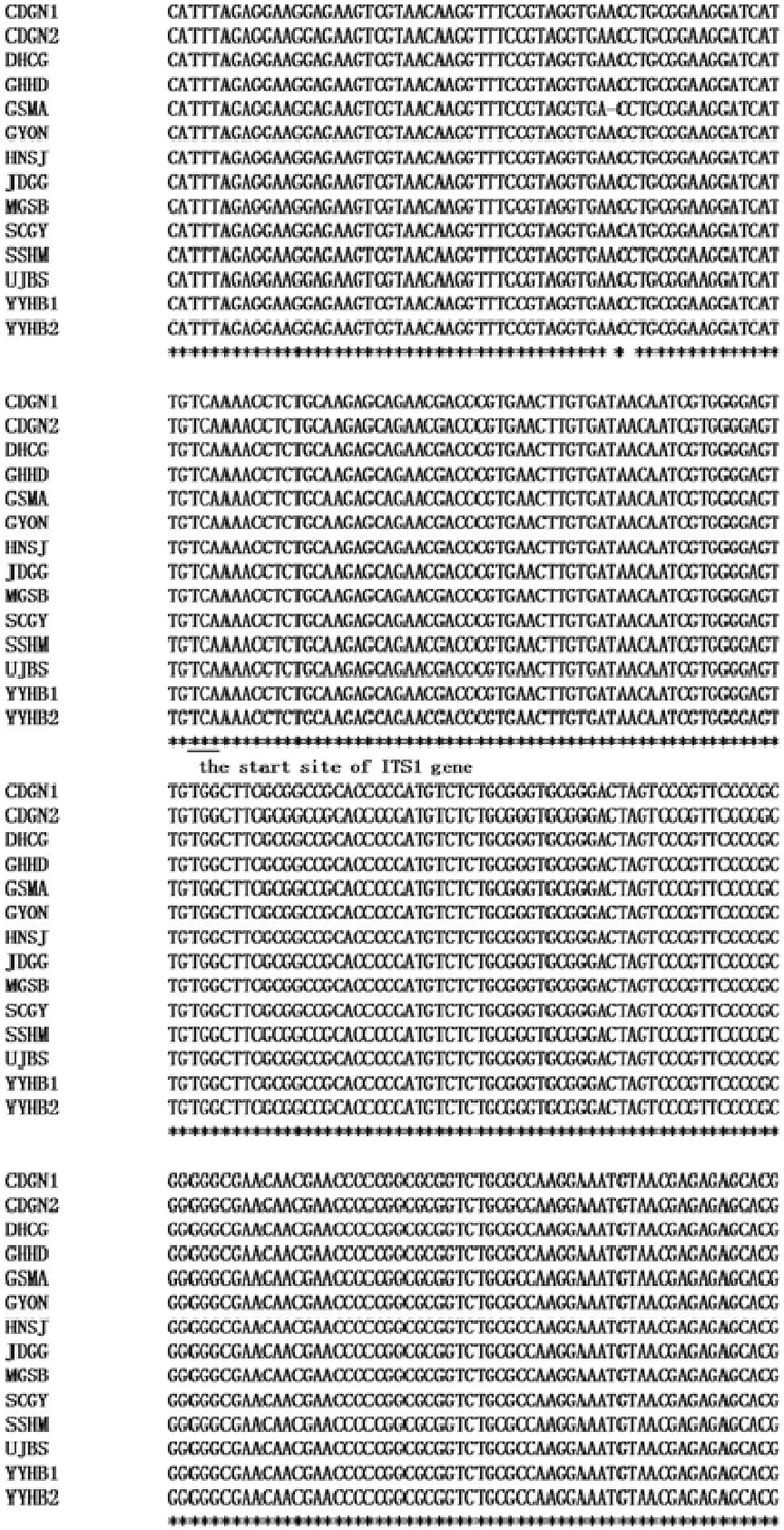

Figure 4. Legend below.

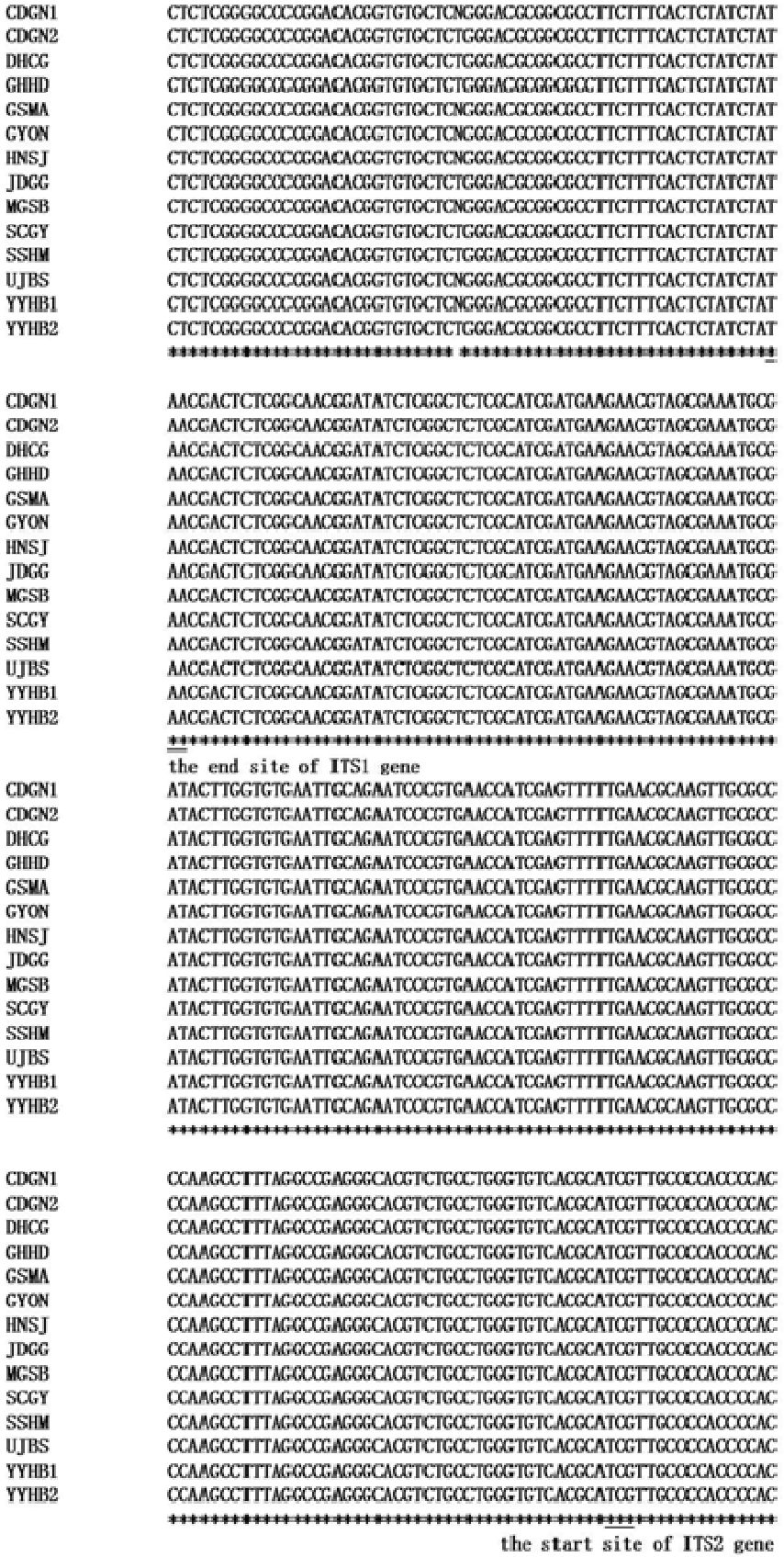

Figure 4. Contd.

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CDGN2           TCTCGAGCTCACGTCTCGTGCCCGGCCCCCTGTTACGGGACTCATGGACCCTGAAGCTCT
DHCG            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
GHHD            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
GSMA            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
GYON            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
HNSJ            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNNGNNACGGGACTCATGGACCCTGAAGCTCT
JDGG            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
MGSB            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
SCGY            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
SSHM            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
UJBS            TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
YYHB1           TCTCGAGCTCACGTCTCGTGCCCGGCCCCNTGTTACGGGACTCATGGACCCTGAAGCTCT
YYHB2           TCTCGAGCTCACGTCTCGTGCCCGGCCCCCTGTTACGGGACTCATGGACCCTGAAGCTCT
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CDGN1           GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
CDGN2           GTGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
DHCG            GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
GHHD            GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
GSMA            GCGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
GYON            GCGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
HNSJ            GCGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
JDGG            GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
MGSB            GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
SCGY            GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
SSHM            GCGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
UJBS            GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
YYHB1           GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAAGC
YYHB2           GNGCAAGCAGCGCTCGCATCGCGACCCCAGGTCANGCGGGATTACCCGCTGAGTTTAAGC
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                                the end site of ITS2 gene
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Figure 4. Contd.

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CDGN1          ATATCAATAA-
CDGN2          ATATCAAAA--
DHCG           ATATCAAAA--
GHHD           ATATCAATAAG
GSMA           ATATCATAAG-
GYON           ATATCAATAAG
HNSJ           ATATCAAAAG-
JDGG           ATATCAAAAG-
MGSB           ATATCAATAA-
SCGY           ATATCAAAAG-
SSHM           ATATCAAAAG-
UJBS           ATATCAATAA-
YYHB1          ATATCAATAAG
YYHB2          ATATCAAAAG-
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Figure 4. DNA alignment of the total ITS ribosomal region among 14 ecotypes of *Z. piperitum* (CDGN1, CDGN2, DHCG, GHHD, GSMA, GYON, HNSJ, JDGG, MGSB, SCGY, SSHM, UJBS, YYHB1 and YYHB2). To illustrate the sequence divergence, the start site and end site of ITS1 and ITS2 gene were labeled with underline. Nucleotide deletion was denoted as '-'.

required to be exploited for meeting higher needs of species confirmation. During the past decade, molecular identification methods which use various molecular data to infer the phylogenetic relationships among taxa have been rapidly developed and improved (Hwang and Kim, 1999). Due to the evolution of various molecular markers or gene regions with varying degrees of sequence conservation, appropriate molecular markers or gene regions should be selected with more accurate identification. Nuclear ribosomal DNA, which encodes rRNAs, has been commonly applied in phylogenetic approaches. Each nuclear ribosomal DNA repeat unit consists of genes coding for the nuclear small subunit (SSU), large subunit (LSU) and 5.8S rDNAs, and spacers separating the adjacent coding regions, such as ITS1 and ITS2 (Yaun et al., 1995; Hwang and Kim, 1999). Due to faster evolutionary rate of the ITS region as compared with the coding regions (Pamidimarri et al., 2009), we selected the ribosomal ITS1 and ITS2 regions as markers to enhance specificity among the genus Zanthoxylum.

Dillon et al. (2004) 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. The genus Ainsliaea in the Sino-Japanese region was divided into three major clades that correspond to species distributions by analyzing the sequences of ITS region combined with external transcribed spacer (ETS) region and ndhF (Mitsui et al., 2008).

The genus Zanthoxylum has more than 200 species. However, the genus has many different and not well-defined species; very little is known about their genetic relatedness and phylogenetics. In our work, we found the mismatch of the universal ITS primers with DNA sequences of *Z. piperitum* species. Thus, two new species-specific primers were designed for successfully isolating the ribosomal ITS region of *Z. piperitum*, whereas the newly designed primers that paired with the university primers were not well-matched with DNA sequences of *Z. schinifolium*, and this enhanced the identification between both closely related species. Fourteen different ecotypes of *Z. piperitum* which were used here were very highly conversed according to the ITS region sequences, with 100% sequence identity. Despite six different ecotypes of *Z. schinifolium* reported here with certain sequence variation, they could be distinguished from another species, with 92% sequence identity with other fourteen different ecotypes of *Z. piperitum*. Mismatch of two newly designed primers with DNA sequences of *Z. schinifolium*, but efficient amplification of DNA from very closely related species, indicated that the new primers were efficient and very species-specific.

This work not only provides more resources of ITS sequence in *Z. piperitum* using newly designed species-specific primers, but also distinguishes *Z. schinifolium*, which makes it possible to elucidate the phylogenetic relationships of the genus Zanthoxylum.

ACKNOWLEDGEMENT

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Table 2. Uncertain nucleotide sites in the ITS1, 5.8S and ITS2 region among 14 ecotypes of *Z. piperitum* (CDGN1, CDGN2, DHCG, GHHD, GSMA, GYON, HNSJ, JDGG, MGSB, SCGY, SSHM, UJBS, YYHB1 and YYHB2).

Ecotype name	268 bp	629 bp	630 bp	632 bp	633 bp	661 bp
CDGN2	T	C	T	T	T	T
YYHB2	T	C	T	T	T	N
GSMA, GYON	N	N	T	T	T	C
SSHM	T	N	T	T	T	C
HNSJ	N	N	N	N	N	C
GHHD, DHCG, SCGY, JDGG	T	N	T	T	T	N
UJBS, CDGN1, MGSB, YYHB1	N	N	T	T	T	N

Uncertain nucleotide was denoted as 'N'.

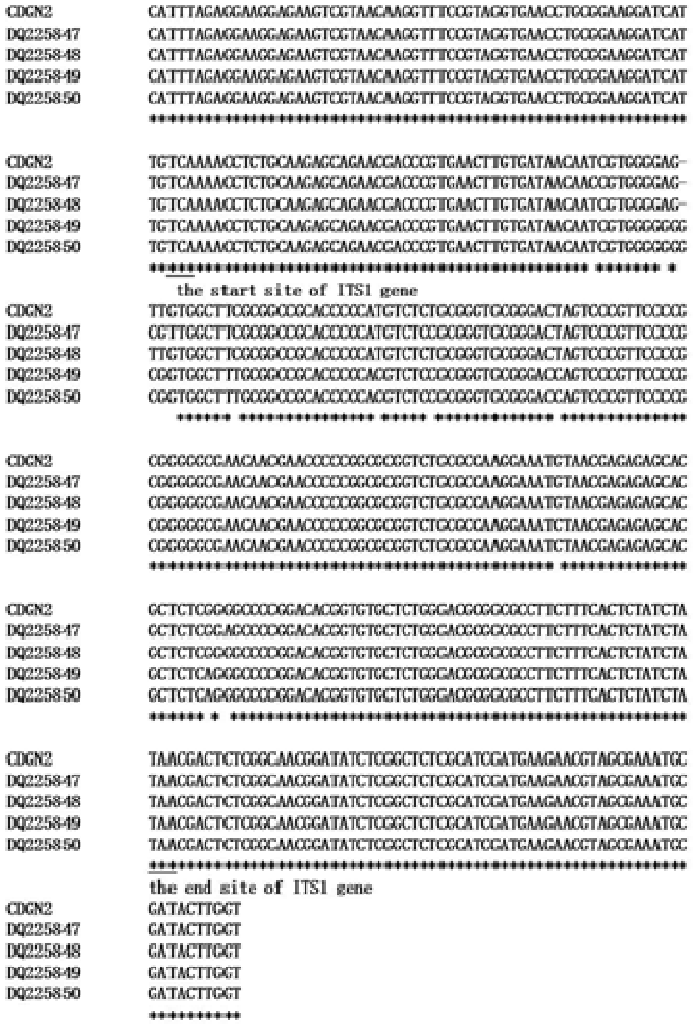

Figure 5. DNA alignment of the ITS1 region between one of our sequences (CDGN2) and 4 existing sequences (accession number DQ225847-50). To illustrate the sequence divergence, the start site and end site of ITS1 gene were labeled with underline. Nucleotide deletion was denoted as '-'.

Table 3. Nucleotide sites with variation in the ITS1 region between one of our sequences (CDGN2) and 4 existing sequences (accession number DQ225847-50).

Ecotype name	109 bp	117 bp	119 bp	120 bp	121 bp	122 bp	129 bp	145 bp	151 bp	165 bp	225 bp	246 bp	248 bp
CDGN2	T	A	-	T	T	G	C	T	T	T	G	G	G
DQ225047	C	A	-	C	G	T	C	T	C	T	G	G	A
DQ225848	T	A	-	T	T	G	C	T	T	T	G	G	G
DQ225849	T	G	G	C	G	G	T	C	C	C	C	A	G
DQ225850	T	G	G	C	G	G	T	C	C	C	C	A	G

Nucleotide deletion was denoted as -.

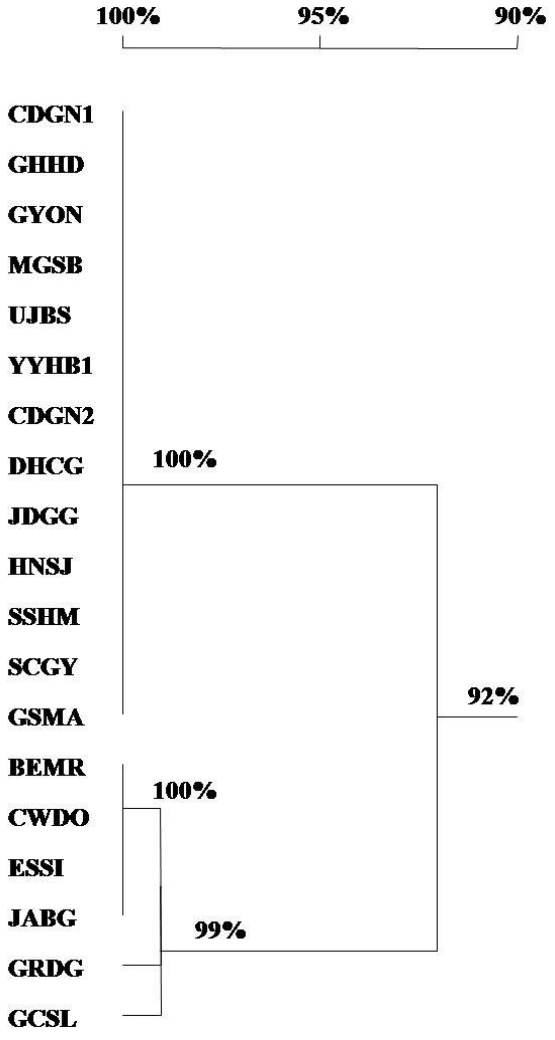

Figure 6. Homology tree among the total ITS regions of 14 ecotypes of *Z. piperitum* (CDGN1, CDGN2, DHCG, GHHD, GSMA, GYON, HNSJ, JDGG, MGSB, SCGY, SSHM, UJBS, YYHB1 and YYHB2) and 6 ecotypes of *Z. schinifolium* (BEMR, CWDO, ESSI, JABG, GRDG and GCSL).

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