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Full Length Research Paper

Assessing nrDNA ITS2 sequence based molecular signature of ginseng for potential use in quality control of drug

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***Panax* L. (Family: Araliaceae), which is commonly known as ginseng, is one of the most important medicinal herbs. The rhizome of the genus *Panax* is used as a source of medicine. Identification of raw or fresh samples of *Panax* species is challenging, owing to occurrence of high level of morphological variation within the genus as well as even within the populations too; therefore, the adulteration is common in its raw material trading which ultimately reduces the efficacy of the drug obtained from it. The internal transcribed spacer 2 (ITS2) sequence of nuclear ribosomal DNA is regarded as one of the important candidates for DNA barcoding. Cladistic analysis and ITS2 sequence variation (primary and secondary structure) among the 16 species of *Panax* using tools and techniques of molecular phylogenetics and bioinformatics were undertaken to demonstrate the assessment of ITS2 sequence of nrDNA based molecular signature of *Panax* for potential use by the pharmaceutical industries in the identification of *Panax* at species level, in order to ensure the quality of drug obtained from it. The ITS2 sequences were found successful in discriminating the *Panax* species.**

Key words: *Panax,* Ginseng, Araliaceae, internal transcribed spacer (ITS), nuclear ribosomal DNA (nrDNA), molecular signature.

INTRODUCTION

Panax L., a perennial rhizomatous herb which is commonly known as 'ginseng', consists of 18 species (Lee and Wen, 2004). The genus *Panax* is one of the important genera in the orient, where the rhizome of every species has been used as source of medicine. Three of the species of *Panax* namely *Panax ginseng, Panax quinquefolius* and *Panax notoginseng* (commonly known as ginseng, American ginseng and Sanchi, respectively), are highly regarded as medicinal and therefore being widely cultivated. Ginseng is renowned for improving physical and mental performance. The drug obtained from ginseng enhances the natural responses of the body to stress by increasing resistance to infections and improving energy metabolism (Zuo et al., 2011).

The adulteration of herbal material in its trading is a common problem; therefore, authentication of raw herbal material is one of the most important requirements needed by the pharmaceutical companies for quality control of the drug obtained from the medicinal plants. There are variety of methods which are based on morphological, biochemical or histological characteristics, employed for the accurate identification of medicinal plants in order to ensure the purity, quality and safety of the drugs. However, the results obtained from these method are not

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always reproducible because these characteristic changes under different environmental conditions. In contrast to aforesaid methods, the DNA-based methods for authentication of medicinal plants are considered to be more reliable for fresh as well as dried samples, particularly for those medicinal plants in which variation within species and the divergence among species is difficult to understand.

Identification of species of *Panax* is a major challenge due to occurrence of high level of morphological variation within the genus, as well as even within the population; hence, the adulteration is common in raw material trading of ginseng which ultimately reduces the efficacy of the drug obtained from it. Moreover, a reliable and practical method for species identification of *Panax* is lacking. Various techniques are in use or have been tried for the purpose of identification of *Panax* species such as metabolic chemicals profiling resolved by high performance liquid chromatography (Chan et al., 2000), molecular markers such as random amplified polymorphic DNA (RAPD) and microsatellite markers (Ngan et al., 1999; Hon et al., 2003), peptide nucleic acid microarray (Lee et al., 2010) and, pyrosequencing (Leem et al., 2005). However, the techniques used so far have suffered from low efficiency, reproducibility and reliability. Species identification based on DNA sequences is a method of high efficiency, reproducibility and reliability. The screening for candidate DNA barcoding loci in *Panax* demonstrated that the combination of *psb*A-*trn*H and internal transcribed spacer (ITS) is suitable for its identification (Zuo et al., 2011). Meanwhile, the success rates of *psb*A-*trn*H remain much lower at the species level (Chen et al., 2010). Therefore, there is need to explore the gene which can be easily amplified using universal primer and also can be used as molecular signature or typing for not only the genus *Panax*, but also for other taxa having medicinal properties.

Since the first report of the utility of the internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA) in plants (Baldwin, 1992), it is being extensively used for phylogenetic studies, molecular discrimination of raw drug material and DNA barcoding because it possesses a number of valuable characteristics such as the availability of conserved regions for designing universal primers, the ease of its amplification, short length and sufficient sequence variation which can easily distinguish even very closely related species (Chen et al., 2010; Yao et al., 2010). Moreover, a comprehensive analysis of ITS2 sequences of nrDNA for ability to discriminate species of *Panax* is lacking; hence, the main objective of this work is to evaluate the potential use of nrDNA ITS2 sequence for molecular signature of *Panax*.

MATERIALS AND METHODS

The leaf materials of *Panax assamicus, Panax japonicus, Panax pseudoginseng and Panax variabilis* were collected during plant explorations in the north eastern geographical region of India. Voucher specimens for all the collected materials were prepared for record and reference and deposited in the Herbarium of Tilka Manhji Bhagalpur University (BHAG), Bhagalpur, Bihar, India (Table 1).

DNA extraction and amplification

Leaves were dried in silica gel prior to DNA extraction. Total genomic DNA was extracted using DNeasy Plant Mini kit (Qiagen, Valencia, CA). ITS sequences of nrDNA were amplified using primers {ITS1F (5'-GTCCACTGAACCTTATCATTTAG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3')} of White et al. (1990) and AccuPower HF PCR PreMix (BIONEER, Daejeon, South Korea). The reaction condition for amplification was denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 1 min and extension at 72°C for 1 min, with a 5 min of final extension at 72°C.

DNA sequencing

Polymerase chain reaction (PCR) products were purified using SolGent PCR Purification Kit-Ultra (Solgent, Daejeon, South Korea) and sequenced using the same primers in 10 µL reactions using 2 µL BigDye, 1 µL primers (20 pmolar), template DNA and purified water to reach the reaction volume. Cycle sequencing used 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. sequencing product were visualized on an ABI Prism 377 automated DNA sequencer. Each sample was sequenced in both the sense and antisense direction. The sequences were analyzed using ABI sequence navigator software (Perkin-Elmer/Applied Biosystems). Nucleotide sequences of both the DNA strands were obtained and compared the forward and reverse sequence to ensure accuracy.

Data analysis

ITS sequences of nrDNA of 12 species of *Panax* (Table 1) were retrieved from GenBank database of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The boundaries between the ITS1, 5.8S, and ITS2 gene for the data set of all the 16 species of *Panax* (both the sequence generated for the present study and the sequences retrieved from the GenBank) were determined according to span mentioned in features of the nrDNA ITS sequences of *Panax* available in GenBank. ITS2 sequences were extracted from the complete set of the ITS sequence and used in the further analysis. Secondary structure of nrDNA ITS2 region for each *Panax* species was explored using the minimum free energy (MFE) program MFOLD (Zuker, 1989) in GCG version 8.1 (GCG, 1994) available at http://rna.tbi.univie.ac.at/. DNA sequence alignment was performed using ClustalX version 1.81 software (Thompson et al., 1997). The sequence alignment was subsequently adjusted manually using BioEdit (Hall, 1999). The ITS2 sequence based discrimination among the *Panax* species was inferred from MEGA4 (Tamura et al., 2007) using the neighbor-joining (Saitou and Nei, 1987), maximum composite likelihood method (Tamura et al., 2004) and Kimura 2-parameter model of base substitution (Kimura, 1980) which is generally accepted as the best model for species level analysis with low distances (Hebert et al., 2003b).

RESULTS AND DISCUSSION

The length of ITS2 sequences of nrDNA regions and percent GC content in the taxon included in the analysis ranges from 218 to 235 base pair and 54 to 70%,

Table 1. Voucher details including GenBank accessions numbers of taxon included in the analysis.

Taxon	Taxon abbreviation	Voucher	Geographic Location	GenBank accession no.
Panax assamicus Ban.	PASS	Ali and Pandey 7073 (BHAG)	West Bengal, India	FJ872555
Panax ginseng C.A. Meyer	PGIN	Wen 3127 (F)	Jilin, China	AY233326
Panax japonicus Meyer	PJAP	Ali and Pandey 7057 (BHAG)	Sikkim, India	FJ853616
Panax major Ting	PMAJ	Wen 1433 (CS)	Hubei, China	U41683
Panax notoginseng F.H. Chen ex C.Y.Wu & K.M. Feng	PNOT	Wen 1244 (F)	Guangdong, China	U41685
Panax omeiensis J. Wen	POME	Wen 1168 (CS)	Sichuan, China	U41686
Panax pseudoginseng Wall.	PPSE	Ali and Pandey 8057 (BHAG)	Nagaland, India	FJ853617
Panax quinquefolius L.	PQUI	Wen 1083 (A)	Ohio, USA	U41687
Panax shangianus J. Wen	PSHA	Wen 5075-8 (F)	Yunnan, China	AY233328
Panax sinensis J. Wen	PSIN	Wen 1204 (F)	Yunnan, China	U41696
Panax stipuleanatus H.T. Tsai & K. M. Feng	PSTI	Wen 1204 (F)	Yunnan, China	U41696
Panax trifolius L.	PTRI	Kramer and Kramer *s.n.* (CS)	Ohio, USA	U41690
Panax variabilis J. Wen	PVAR	Ali and Pandey 9605 (BHAG)	Nagaland, India	AY233329
Panax vietnamensis Ha. Grushv.	PVIE	Wen 5638-2(F)	China	AY271924
Panax wangianus S. C. Sun	PWAN	Wen 1174 (CS)	Sichuan, China	U41690
Panax zingiberensis C. Y. Wu & K. M. Feng	PZIN	Wen 1199 (CS)	Yunnan, China	U41699

respectively. Aligned sequence data matrix has total number of 235 characters (Figure 1). The ITS2 molecular signature in aligned format, nrDNA ITS2 secondary structure variation and the optimal NJ tree with the sum of branch length 0.2727 for reference framework are shown in Figures 1 to 3, respectively. The confidence probability (multiplied by 100) that the interior branch length was greater than 0, as estimated using the bootstrap test 1000 replicates (Dopazo, 1994; Rzhetsky and Nei, 1992) is shown next to the branches in NJ tree (Figure 3).

Since the first report of the utility of the cytochrome c oxidase subunit 1 (CO1) as a DNA barcode to identify animals, DNA barcoding has attracted worldwide attention (Hebert et al., 2003a, b). Many loci such as ITS (Chase et al., 2005; Kress et al., 2005), *rbc*L (Newmaster et al., 2006; Kress and Erickson, 2007), *psb*A-*trn*H (Kress and Erickson, 2007; Chase et al., 2007; Lahaye et al., 2008), *mat*K (Chase et al., 2007; Pennisi, 2007; Lahaye et al., 2008), and combination of *rbc*L and *mat*K (Hollingsworth et al., 2009) etc., have earlier been proposed for plant DNA barcode. Nevertheless, nuclear genes can provide more information than barcoding based on organellar DNA which is inherited from only one parent (Chase and Fay, 2009). It has been emphasized that an ideal barcode should possess sufficient sequence variation to discriminate the taxon at species level; however, it also need to have sufficiently conserved region so that there is less variability within species than between species (Kress and Erickson, 2007; Taberlet et al., 2007).

The ITS2 shows significant sequence variability at the species level or lower (Coleman, 2003, 2007, 2009; Schultz et al., 2005; 2006; Thornhill et al., 2007). The availability of structural information of ITS2 permits analysis even at higher taxonomic level too (Coleman, 2003, 2007, 2009; Aguilar and Sanchez, 2007; Schultz and Wolf, 2009; Keller et al., 2010). Chen et al. (2010) com-pared seven candidate DNA barcodes (*psb*A-*trn*H, *mat*K, *rbc*L, *rpo*C1, *ycf*5, ITS2, and ITS) and proposed that ITS2 has potential for use as a standard DNA barcode to identify medicinal plants. The ITS2 region has also been shown to be applicable in discriminating among a wide range of plants genera and families e.g. Asteraceae, Rutaceae, Rosaceae and Araliaceae (Gao et al., 2010; Liu et al., 2012a,b; Luo et al., 2010; Pang et al., 2011; Yao et al., 2010). Besides plants, the ITS2 sequence also has potential for use in barcoding of animals (Yao et al., 2010). The secondary structure of ITS2 are conserved and possesses sufficient variation in primary

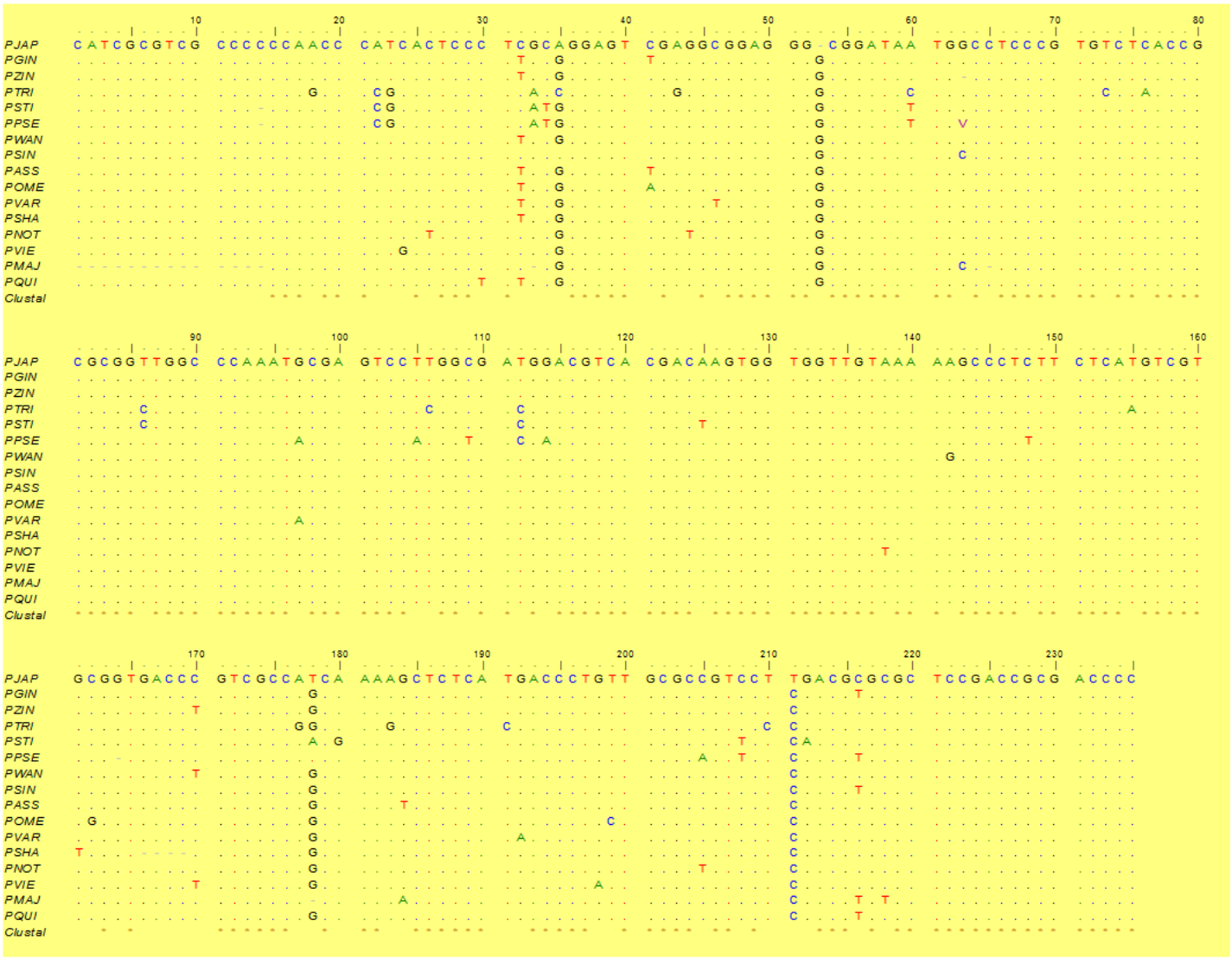

Figure 1. Aligned ITS2 sequences of nrDNA data matrix. Gaps in Clustal line indicate the variable position.

sequences as well as secondary structure, which also provides useful biological information for alignment; therefore, the ITS2 sequences is also used as molecular morphological characteristics for species identification (Coleman, 2007; Schultz et al., 2005; Koetschan et al., 2010).

Figures 1 and 2 illustrates specific nucleotide differences and variation of ITS2 secondary structure among various *Panax* species, respectively, which can be use as molecular signature. Figure 3 illustrates a reference framework for the genus *Panax* inferred from the analysis of the nrDNA ITS2 sequence. The resulted NJ tree (bootstrap support ranges from 43 to 95%) clearly discriminated the *Panax* species. The resulted NJ tree showed (a) *Panax trifolius* from eastern North America is sister to the clade consisting of all other *Panax* species; (b) *P. pseudoginseng- P. stipuleanatus* group: the Himalayan *P. pseudoginseng* is most closely related to *P. stipuleanatus* (bootstrap support 76%) of southwestern China; (c) *P. sinensis - P japonicus* group: *P. japonicus* showed close relationship with *P. sinensis* (bootstrap support 49%); (d) an independent *P. major* branch which showed close relationship (bootstrap support 51%) with an independent *P. notoginseng* branch and *P. ginseng*, *P. assamicus*, *P. omensis*, *P. quinquefolius*, *P. variabilis*, *P. shangianus*, *P. vietnamensis*, *P. zingiberensis*, *P. wangianus* group; and (e) the medicinally important *P. notoginseng* formed a clade (bootstrap support 43%) with the closely related *P. ginseng*, *P. assamicus*, *P. omensis*, *P. quinquefolius*, *P. variabilis*, *P. shangianus*, *P. vietnamensis*, *P. zingiberensis* and *P. wangianus*. This finding was largely congruent with the previous studies (Wen and Zimmer, 1996; Choi and Wen, 2000; Zhu et al., 2003; Lee and Wen, 2004; Zuo et al., 2011).

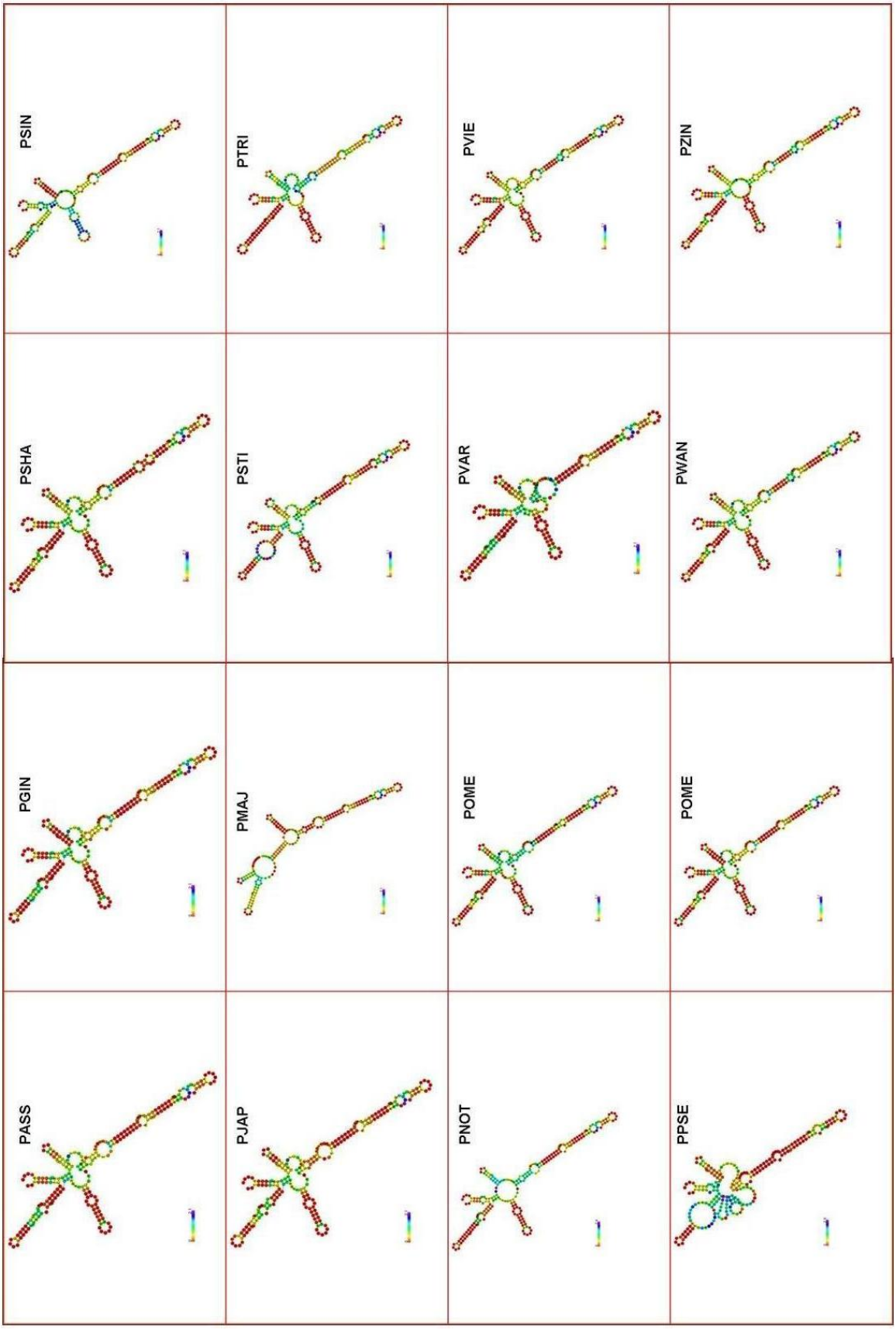

Figure 2. Variation among the ITS2 secondary structure of *Panax.*

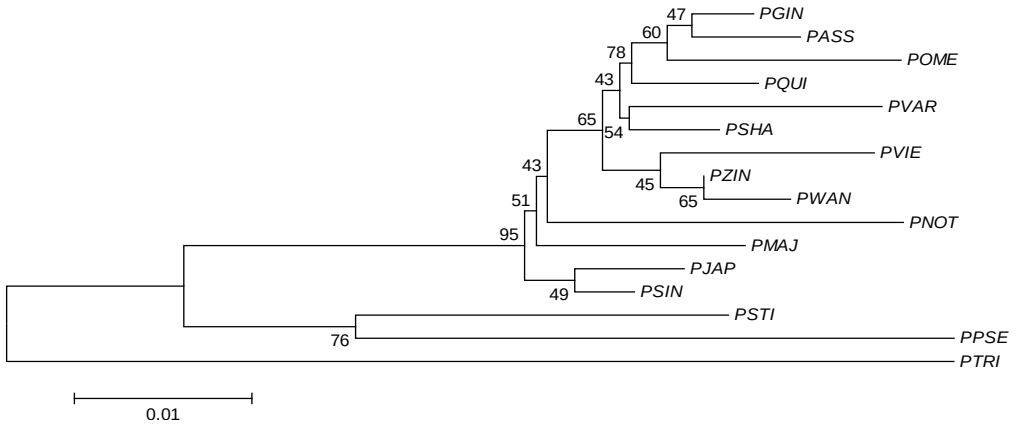

Figure 3. A neighbor-joining tree inferred from analysis of sequence data of ITS2 region of nrDNA.

In conclusion, the nrDNA ITS2 sequences of ginseng in particular has potential to act as molecular signature which can be use for the assessment of the ginseng samples by pharmaceutical industries to ensure the quality of drug obtained from it by discriminating the raw samples of ginseng species of interest from whatever its adulterants may be.

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