Journal of Computer Science & Systems Biology - Open Access

Research Article JCSB/Vol.2 May-June 2009

Analysis of the Sequence of ITS1-5.8S-ITS2 Regions of the Three Species of *Fructus Evodiae* in Guizhou Province of China and Identification of Main Ingredients of Their Medicinal Chemistry

Liu-Zhongyu[1], Liu-Yang[2], Liu-Shouhan[1], Ding-Xianping[1], Yang-Yi[1]* and Huang-Hai[2]*

[1]College of Life Science, Sichuan University, China. 610041

[2]Department of Clinical Biochemistry, Guiyang Medical College China. 550004

*Corresponding authors: Yang-Yi, College of Life Science, Sichuan University, China. 610041, E-mail: zhongyujohn@gmail.com

Huang-Hai, Department of Clinical Biochemistry, Guiyang Medical College China. 550004, E-mail: hwanghai828@tom.com

Received May 07, 2009; Accepted June 23, 2009; Published June 24, 2009

Citation: Liu Z, Liu Y, Liu S, Ding X, Yang Y, et al. (2009) Analysis of the Sequence of ITS1-5.8S-ITS2 Regions of the Three Species of *Fructus Evodiae* in Guizhou Province of China and Identification of Main Ingredients of Their Medicinal Chemistry. J Comput Sci Syst Biol 2: 200-207. doi:10.4172/jcsb.1000032

Abstract

A total of fourteen specimens of three *Fructus Evodiae* species were collected from Zunyi and Tongren of Guizhou Province in China. The internal transcribed spacer (ITS) region (ITS1, ITS2 and 5.8S rDNA) of the nuclear ribosomal DNA (nrDNA) of the fourteen species were amplified and sequenced and another two sequences of *Evodia rutaecarpa (JUSS.) BENTH* species were obtained from Hunan Academy of Traditional Chinese Medicine, Changsha, Hunan Province of China. In addition to, the main ingredients of their medicinal chemistry of all specimens were identified by high performance liquid chromatography (HPLC). The three different morphological Fructus Evodiae species could not be distinctly classified by phylogenetic analysis of ITS combining the main ingredients of medicinal chemistry. This conclusion is inconsistent with early research results.

Keywords: *Fructus Evodiae*; ITS; Phylogenetic analysis; HPLC; Identification

Introduction

Fructus Evodiae (synonyms: *Tetradium ruticarpum*), which is the dried nearly ripe fruit of *Evodia rutaecarpa (JUSS.) BENTH (EB); Evodia rutaecarpa (JUSS.) BENTH. var. officinalis (DODE) HUANG (EBOH) or Evodia rutaecarpa (JUSS.) BENTH. var. bodinieri (DODE) HUANG (EBBH)*, are widely used in traditional Chinese medicines, which have about 150 species, distributed in Asia, eastern Africa and Oceania. In China there are about 20 species including 5 varieties (Zhengyi et al., 2007). *Fructus Evodiae* cultivation has a long history in China *Guizhou* province, mainly distributed in *Tongren, Zunyi* , and other places. With its first-class quality, the output ranks first in China main exporting to Southeast Asia as Chinese traditional commodities.

In Pharmacopoeia of P. R. China (Part I), *Fructus Evodiae* is classified into three groups, *EB; EBOH* or *EBBH* (Chi, 2000), however, the latter two are considered ered as the variants of the former group. So far, this taxonomy is more acceptable to most scholars. As a medicine product on the market, according to the size of the flower of plants, *Fructus Evodiae* is also grouped into two sections, big flowers referred to EB and small flowers referred to *EBOH* or *EBBH*. In general, all above classifications are based on the individual characters portrayed and macro-level observation. In practice, those classifications are facing challenges. For example, *Fructus Evodiae* is identified mainly relying on shapes of its leaves and fruit , but it has a strong seasonal limitations, for example, in winter and spring,

its breeding and screening of seedlings face the identification difficulties. In addition, many unscrupulous traders often use the fake and shoddy *Fructus Evodiae* to cheat consumers due to identification difficulties. Those require that people must find a new classification and identification methods of *Fructus Evodiae*.

Eukaryotic ribosomal RNA genes (known as ribosomal DNA or rDNA) are found as parts of repeat units that are arranged in tandem arrays, ITS (for internal transcribed spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. In the transcribed region, internal transcribed spacers (ITS) are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary. Universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 bp) enable easy amplification of ITS region due to high copy number (up to-30000 per cell (Dubouzet and Shinoda 1999) of rDNA repeats). This makes the ITS region an interesting subject for evolutionary/phylogenetic investigations (Baldwin et al., 1995; Hershkovitz et al., 1996; Hershkovitz et al., 1999) as well as biogeographic investigations (Sharma et al., 1993; Suh et al., 1993; Hsiao et al., 1994; Dubouzet et al., 1999). A molecular approach, using sequence data from the internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) were used in this study. The initial results of molecular phylogenetic analyses on taxa from the ITS region of *Fructus Evodiae* were showed in this paper. In addition, the content of *Evodiamine* and *Rutaecarpine*, both of which are major bioactive compounds in *Fructus Evodiae*, was determined by High Performance Liquid Chromatography (HPLC).

Material and Methods

Plant Materials

A total of fourteen specimens of three *Fructus Evodiae* species were collected from Zunyi and Tongren of Guizhou in China, including two vouchers of *EB*, six species each of *EBOH* and *EBBH,* and the sequences of another two species, code *EB.JL* and *EB.JS*, were obtained from the Provincial Key Laboratory of Drug Research, Hunan Academy of Traditional Chinese Medicine, Changsha, Hunan Province, 410013 (Dan et al., 2008). Due to the morphological variation of Petal and leaf of female *Fructus*

Specimens	Location (China)	No.	GenBank Accession	A	T	G	C	G+C (mol %)	Length(nt) analyzed dataset	E+R (%)
EB.ZY1	Zunyi,Guizhou	1	EU663538	18.5	18.7	29.4	33.3	62.7	615	0
EB.ZY2	Zunyi,Guizhou	2	EU663537	18.9	18.5	29.1	33.5	62.6	615	0.04
EB.JS*	Jiangsu	3	EF432817	18.7	18.5	29.3	33.6	62.9	611	0.65
EB.JL*	Jilin, Shangdong	4	EF432818	18.7	18.5	28.7	34.1	62.8	616	0.4
EBOH.TR1	Tongren,Guizhou	5	EU663542	18.2	18.7	29.2	33.9	63.1	616	0.2
EBOH.TR2	Tongren,Guizhou	6	EU663543	18.5	18.7	29.4	33.3	62.7	615	0.25
EBOH.TR3	Tongren,Guizhou	7	EU663544	18.5	18.7	29.4	33.3	62.7	615	0.11
EBOH.ZY1	Zunyi,Guizhou	8	EU663534	18.7	18.5	29.3	33.5	62.8	615	0.64
EBOH.ZY2	Zunyi,Guizhou	9	EU663535	18	17.9	29.8	34.3	64.1	615	0.54
EBOH.ZY3	Zunyi,Guizhou	10	EU663536	18.5	18.7	29.3	33.5	62.8	615	0.73
EBBH.TR1	Tongren,Guizhou	11	EU663545	18.7	18.5	29.3	33.5	62.8	615	1.34
EBBH.TR2	Tongren,Guizhou	12	EU663546	18.7	18.4	29.5	33.4	62.9	614	1.53
EBBH.TR3	Tongren,Guizhou	13	EU663533	18.7	18.9	29.3	33.2	62.5	615	1.41
EBBH.ZY1	Zunyi,Guizhou	14	EU663539	18.9	18.7	29.1	33.3	62.4	615	0.08
EBBH.ZY2	Zunyi,Guizhou	15	EU663540	18.7	18.5	29.3	33.5	62.8	615	0.22
EBBH.ZY3	Zunyi,Guizhou	16	EU663541	18	17.9	29.8	34.3	64.1	615	0.15
mean				18.6	18.5	29.3	33.6	62.92	614.81	0.59

Abbreviations: EB, Evodia rutaecarpa (JUSS.) BENTH; EBOH, Evodia rutaecarpa (JUSS.) BENTH. var. officinalis (DODE) HUANG; EBBH, Evodia rutaecarpa (JUSS.) BENTH. var. bodinieri (DODE) HUANG; TR, Tongren of Guizhou; ZY, Zunyi of Guizhou; JL, Jilin of Shangdong; JS, Jiangsu province; E, Evodiamine; R, Rutaecarpine.

*: EB.JS and EB.JL offered by Provincial Key Laboratory of Drug Research, Hunan Academy of Traditional Chinese Medicine, Changsha, Hunan Province,410013.

Table 1: Plant materials of three species used in this study and the accession of these species for ITS1-5.8S-ITS2 sequence in GenBank.

Evodiae, its specimens were grouped into 3 types, *EB* (shape-clustering and larger petals), *EBOH* (shape-dispersing and smaller petals, 13-18cm leaves with highly dense dots in diameter, commonly found in the limestone mountain), *EBOH* (shape-dispersing and smaller petals and about 10cm in diameter leaves with sparse dots in diameter, commonly found in relatively flat lands) (Zhengyi et al., 2007). All specimens were identified by Prof. Dr.Hesun.Zhi, Department of Clinical Biochemistry, Guiyang Medical College China 550004. Fresh leaves were collected, using ultra-low-temperature ice bags to conserve and keeping in the ultra-low-temperature refrigerator. Details of the plant materials are shown in Table 1.

DNA extraction, ITS amplification, cloning and sequencing

(a) DNA extraction and purification: Total cellular DNA was isolated using modified CTAB method based on the traditional one (Doyle et al., 1987). The isolated DNA was purified by RNase A treatment and chloroform: isoamyl alcohol extraction. The quality and quantity of DNA samples was checked on agarose gel using lambda DNA as marker.

(b) ITS amplification: ITS1-5.8S- ITS2 rDNA region was amplified using the following primer pair (White et al., 1990):

ITS-4 (5'-TCCTCCGCTTATTGATATGC-3')
ITS-5 (5'- GGAAGTAAAAGTCGTAACAAGG-3')

Amplifications were carried out in 50 µl reaction mixture containing 34.6µl sterile water , 5µl of 10x PCR buffer, 3µl of 25mM $MgCl_2$, 4µl of 10mM dNTPs, 1µl of each primer (20µM), 0.4µl (2 U) of *Taq* polymerase (TaKaRa Corp., China) and 1 µl (200ng) template DNA. Eppendorf DNA thermal cycles were used with the following PCR profile: an initial denaturation for 5 min at 95°C, 35 thermal cycles (1 min at 95 °C, 1min at 54°C and 1 min at72° C), and a final 10 min extension at 72 °C. The amplified DNA was purified using Tiangen QUICK PCR purification kit following manufacturer's instructions (Tiangen, China).

(c) Cloning and sequencing**:** Purified DNA was ligated in pMD18®-T Easy vector (TaKaRa Corp., China) overnight at 16°C. The ligated DNA was transformed in DH5α competent cells. The recombinant clones were identified through blue/white color selection and the presence of insert in the recombinant clones (white colonies) was confirmed following colony PCR. For sequencing, plasmid DNA was isolated following alkali lysis method (Sambrook et al., 1989). The insert DNA was sequenced on contract using automated sequencing facility at TaKaRa Corp., China.

(d) Sequence alignment**:** The sequences of ITS1-5.8S-ITS2 regions were manually aligned.

(e) Sequence submission: Sequences of clones were submitted directly to GenBank through Bankit (a World Wide Web sequence submission server available at NCBI home page). The sequences are available on line (http://www.ncbi.nlm.nih.gov) and can be located by accession numbers or GI numbers EU663533-663546.

An initial alignment of the ITS sequences was generated using CLUSTALW (Thompson et al., 1994; Higgins et al., 1996), and then made several minor adjustments manually. The sequences with those published on the ITS of Fructus Evodiae (Dan et al., 2008; Poon et al., 2007) were compared to determine boundaries of ITS1ans ITS2. A total of 616 nucleotides were included for the phylogenetic analyses under maximum-parsimony (MP) criteria. Gaps were coded as missing data. MP phylogenetic analyses were accomplished using PAUP* 4.0b10 (Swofford, 2000) on PC with windows platform. Pairwise evolutionary distances between accessions were generated under the Kimura 2-parameter model (Kimura, 1980). Each nucleotide position was treated as an independent, unordered, multistate character of equal weight. All characters have equal weight and the parameter setting with weights transversions 10 times transitions. A parsimony heuristic search was performed using addition sequence set at random, with 1000 repetitions, ACCTRAN character state optimization, tree bisection-reconnection (TBR) branch swapping, and MULTRREES on, trees are unrooted. The ÷2-test of paup*4.0b4a (David, 1999) was performed to obtain the information about the homogeneity of the nucleotide distribution. The robustness and stability of parsimony trees were estimated by using bootstrapping with 100 replicates.

High Performance Liquid Chromatography (HPLC) Analysis

The HPLC system consisted of Quat pumps (Model G1311A, Agilent, U.S.A.), a diode array detector (Model G1315B, Agilent, U.S.A.)". Chromatographic separation was carried out on a Agilent SB C18 column (250mm×4.6mm, 5µm; Elite, China), using a gradient solvent system comprised of (A) CH3CN and (B) H2O(with 0.05% H3PO4 and 0.04% Octane sulfuric acid sodium salt (Chaudhary, 2007). Gradient profile: 0—25 min: linear 95—65% of B; 25—40min: linear 65—60% of B; 40—55min: linear 60—58% of B; 55—65min: linear 58—25% of B; 65—100min: 10% of B; on-line UV spectra were

recorded with detection wavelength of 225nm, column temperature: 25°C. Data were stored on a personal computer.

Result

The base composition in the dataset was balanced with a mean G+C content of 62.9% for the aligned complete sequences. Table 1 shows the distribution of nucleotides as well as the G+C content in ITS1+5.8S+ITS2 complete sequences. After exclusion of highly variable sites to minimize the risk of a phylogenetic tree construction based on nucleotide distribution (Saitou et al., 1989; Hasegawa et al., 1993; Steel et al., 1993) the distribution pattern resulted in a chi-square-test with p=1.0000, allowing for phylogenetic reconstruction.

Internal Transcribed Spacer Sequence Diversity

16 complete ITS sequences were generated for the 4 EB and 6 EBBOH and 6 EBBH species. Each full sequence included complete sequences of ITS1, the 5.8S rRNA gene, and ITS2. All complete 5.8S sequences were 163 nucleotides in length, except for the two sequences from EB.JS and EB.JL, which were 182 nucleotides long. The ITS1 sequences varied in length from 220 to 232bp, whereas the ITS2 sequences varied from 196 to 220 bp. Alignment of the ITS1 sequences required the hypothesis of 11 insertion/deletion events of 1–2 bp each and resulted in an aligned length of 246 bp. Alignment of the ITS sequences required 3 indels of 1 bp each and 1 gap of 6 bp for a total aligned length of 617 bp. The guanine +cytosine (G+C) content averaged , respectively , 64.4%, 64.80% and 64.70% in ITS1, 54.62%, 54.09% and 53.89% in 5.8S, and 67.63%, 67.78% and 67.66% in the ITS in EB, EBBH and EBOH (shown in Table 2).

The studies show that some plant genomes harbor multiple, and in some cases, highly divergent ITS sequences, e.g., Buckler and Holtsford (1996), more than one clone for most studied accessions were sampled. When considering only substitution polymorphisms, the ITs sequence of EBBH.ZY1 was identical to EBOH.ZY2, EBBH.TR3 and EBBH were also identical as well because their evolutionary distance equals to zero (Table 3). Fourteen unique sequences among the 16 sequences included in the main phylogenetic analysis were selected in this study, 31 of 616 characters (5.03%) exhibited substitution polymorphisms, and 16 (2.3%) of those were parsimony-informative. These Informative sites were approximately equally distributed be-

S.No	Parameter	ITS1	5.8S	ITS2	Entire sequence
1	Length range (nt)	230-232 232 232	163-182 163 163	196-220 220 220	609-615 615 615
2	Length mean (nt)	231.0 232.0 232.0	175.67 163.0 163.0	205.67 220.0 220.0	612.33 615 615
3	Aligned length(nt)	234 232 232	182 163 163	220 220 220	617 615 615
4	G+C content, range (%)	63.91-64.94 64.22-66.38 64.22-66.38	53.99-54.99 53.99-54.60 53.37-53.99	67.27-68.16 67.27-68.64 66.82-69.09	62.70-62.89 62.60-64.07 62.44-64.07
5	G+C content, mean (%)	64.40 64.80 64.70	54.62 54.09 53.89	67.63 67.78 67.66	62.78 63.03 62.92
6	No. of variable sites (%)	14(5.98) 11(4.74) 7(3.02)	1(0.55) 3(1.84) 1(0.61)	6(2.73) 7(3.18) 7(3.18)	21(3.40) 21(3.41) 15(2.44)
7	No. of constant sites (%)	219(93.59) 221(95.26) 225(96.98)	181(99.45) 160(98.16) 162(99.39)	214(97.27) 213(96.82) 213(96.82)	595(96.43) 594(96.59) 600(97.56)
8	Transitions®	0-2 0-2 0-1	0-0 0-0 0-0	0-1 0-2 0-1	1-3 1-2 1-2
9	Transversions®	1-2 0-1 0-1	0-1 0-1 Onc	Onc-1 Onc-0 Onc-0	2-2 0-2 0-1

Note: In each box, upper value is for *EB*; middle value is for *EBBH* and lower values is for *EBOH*

Table 2: Sequence characteristics of ITS region of EB, EBBH and EBOH.

tween ITS1 and ITS2, with much less information present in the 5.8S gene (Table 2). Over all, in the entire ITS region, 15 (2.43%) autapomorphy sites (variation at a same site in one sequence) were detected, similarly, 24 (3.9%) synapomorphic sites (variation at the same site in more than one sequence) were detected (supplementary Fig 1 online).

Intraspecific Sequence Divergence in ITS1, ITS2 and 5.8S Regions

Of the three regions of the entire ITS, the sequence of 5.8S rRNA coding region, as expected, was conserved amongst EB.EBOH and EBBH species and this region exhibited only four substitutions (including three transitions and one transversion) and(supplementary Fig 1 online). It suggested that a similar level of intraspecific sequence divergence in 5.8S region in the three species.

Four EB (4 sequences), six EBBH (6 sequences) and six EBOH (6 sequences) accessions were used to investigate intraspecific ITS variation. In EBBH, 5 of 6 sequences were unique, sequence divergence (Kimura 2-parameter distances) ranged from 0.000 to 0.023 (mean =0.012) (Table 3); In EBOH, all six sequences were unique, sequence divergence ranged from 0.003 to 0.018 (mean =0.01); In EB, four sequences were unique, sequence divergence ranged from 0.005 to 0.027 (mean =0.0178). Therefore the three groups of species showed low within-group sequence divergence, it also clearly indicated that on the basis of sequence divergence, due to substitutions in the ITS region, the EB genotypes were more diverse than the EBBH and EBOH genotypes. The mean sequence divergence (Kimura 2-parameter distances, 0.0144) between EB and EBBH, (0.0185) between EB and EBOH were greater than average divergence (0.01) between EBBH and EBOH, which suggested that the diverseness between EB and EBBH and EBOH were more than that of between EBBH and EBOH.

Phylogenetic Relationship

Based on the ITS sequences of the three species determined in thisent study and two sequences obtained from GenBank (EB.JS, EF432817; EB.JL, EF432818), parsimony analysis produced two most parsimonious trees, Tree length = 153, with a Consistency index (CI) = 0.9673, Homoplasy index (HI) = 0.0327, CI excluding uninformative characters = 0.9231, HI excluding uninformative characters = 0.0769, Retention index (RI) = 0.9664 and Rescaled consistency index (RC) = 0.9349. As shown in Fig 1, bootstrap 50% majority-rule consensus tree divided the 16 Fructus Evodiae species into three main groups. The three EBOHs, three EBBHs and one EB species formed a biggest group with 54 bootstrap, which include two subgroups, EBOH.ZY1 and EBOH.TR3 with 65 bootstrap, EBBH.TR3 and EBBH.TR2 with 62 bootstrap; the second biggest group with 97 bootstrap comprised of two EBBHs, two EBs and one EBOH species, two EBs species, EB.JS and EB.JL (76 bootstrap)

Seq->	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
EB.ZY1	-	5	12	15	13	4	4	2	8	5	2	4	6	7	2	8
EB.ZY2	0.8	-	13	16	14	3	3	3	9	2	5	3	3	4	3	9
EB.JL	2	2.2	-	3	3	12	12	12	8	13	12	12	14	15	12	6
EB.JS	2.5	2.7	0.5	-	6	15	15	15	11	16	15	15	17	18	15	9
EBBH.TR1	2.2	2.3	0.5	1	-	13	13	13	9	14	13	13	15	16	13	7
EBBH.TR2	0.7	0.5	2	2.5	2.2	-	0	4	8	3	4	2	4	5	4	8
EBBH.TR3	0.7	0.5	2	2.5	2.2	0	-	4	8	3	4	2	4	5	4	8
EBBH.ZY1	0.3	0.5	2	2.5	2.2	0.7	0.7	-	8	3	2	4	4	5	0	8
EBBH.ZY2	1.3	1.5	1.3	1.8	1.5	1.3	1.3	1.3	-	9	8	8	10	11	8	2
EBBH.ZY3	0.8	0.3	2.2	2.7	2.3	0.5	0.5	0.5	1.5	-	5	3	3	4	3	9
EBOH.TR1	0.3	0.8	2	2.5	2.2	0.7	0.7	0.3	1.3	0.8	-	4	6	7	2	8
EBOH.TR2	0.7	0.5	2	2.5	2.2	0.3	0.3	0.7	1.3	0.5	0.7	-	4	5	4	8
EBOH.TR3	1	0.5	2.3	2.9	2.5	0.7	0.7	0.7	1.7	0.5	1	0.7	-	3	4	10
EBOH.ZY1	1.2	0.7	2.5	3	2.7	0.8	0.8	0.8	1.8	0.7	1.2	0.8	0.5	-	5	11
EBOH.ZY2	0.3	0.5	2	2.5	2.2	0.7	0.7	0	1.3	0.5	0.3	0.7	0.7	0.8	-	8
EBOH.ZY3	1.3	1.5	1	1.5	1.2	1.3	1.3	1.3	0.3	1.5	1.3	1.3	1.7	1.8	1.3	-

Note: Direct counts of transitions/transversions are shown above the diagonal; Percentage of sequence divergence distance, the true value = value * 10-2, is shown below the diagonal; Light grey area: *EB* cluster, middle grey area: *EBBH* cluster, thick grey area: *EBOH* cluster

Table 3: Pairwise nucleotide sequence divergence (%), based on substitutions, among 16 accessions of. Fructus Evodiae (Model: Nucleotide: Kimura 2-parameter ->Substitutions to Include: d: Transitions + Transversions) 31 Variable sites, 15 singleton sites and 16 parsimony-informative sites.

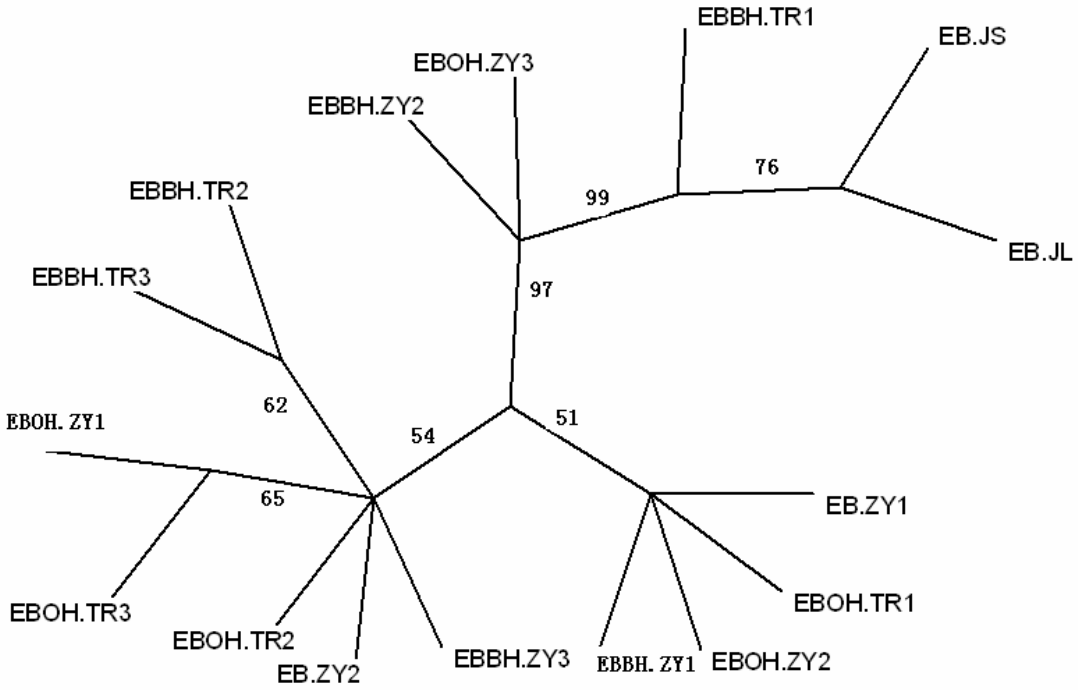

Figure 1: Bootstrap 50% majority-rule consensus tree of two Most Parsimonious Trees Reconstructed on the Basis of ITS Sequence from Fructus Evodiae Species Tree length = 153, with a Consistency index (CI) = 0.9673, Homoplasy index (HI) = 0.0327, CI excluding uninformative characters = 0.9231, HI excluding uninformative characters = 0.0769, Retention index (RI) = 0.9664 and Rescaled consistency index (RC) = 0.9349 number on line is the bootstrap value with 1000 replicates.

were a sister group to EBBH.TR1 with 99 bootstrap. In another two OTUs, EBBH.ZY2 and EBOH.ZY3, form a polyclade with 97 bootstrap; the remaining four species, EBOH.TR1, EBOH.ZY2, EBBH.ZY1 and *EB.ZY1* formed a polyclade in this phylogenetic tree as a individual branch.

Contents of Evodiamine and Rutavarpine from Fructus Evodiae

Evodiamine and rutavarpine, two of the main bioactive constituents in Fructus Evodiae, were selected as the reference standards for equalizing and evaluating the quality of the species (Dan et al., 2008). In our experimental chromatographic conditions, the retention time and peak area of Evodiamine were stable and reproducible. Therefore, Evodiamine was selected as the reference peak and marker for Fructus Evodiae in this study. The content of Evodiamine and Rutaecarpine in ten samples was also tested, according to the method of Committee for the Pharmacopoeia of P. R. China (Chi, 2000). The result is shown in the last column of Table 1.

Discussion

Dan et al., (2008) and Poon et al., (2007) tested the sequence of ITS of *Fructus Evodiae* samples from other districts, but GC contents,variation sequence, and intraspecific sequence divergence in ITS1, ITS1 and 5.8S regions of the entire ITS of *Fructus Evodiae* in their study were relatively few. Dan et al ., (2008) (30) reported that there was an ITS base sequence variation in length and similarity among different district *Fructus Evodiae*. The intraspecies identification of the three *Fructus Evodiae* species from *Guizhou, Jiangsu* and *Shandong* province in China was done in this study. When comparing to the former research results, the number of isolates was relatively high and the geography distribution was relatively narrow, which could contribute to the origin and evolution of *Fructus Evodiae* in Guizhou province of China. According to the ITS sequences of *Fructus Evodiae,* the results showed that there were variation sites on the rDNA ITS sequence in different isolates, and there was a genetic differentiation in various degrees. Therefore, it is concluded that the ITS sequence of *Fructus Evodiae* was very conservative. There was an ITS sequence variation among different *Fructus Evodiae* isolates, which showed that there was a tiny variation among *Fructus Evodiae* individuals of the same intraspecies.

The phylogenetic relationship of *Fructus Evodiae* isolates from Guizhou, Jiangsu and Shangdong was examined (Fig 1). In this phylogenetic tree, any clade almost includes three species of *Fructus Evodiae*, i.e. The different kinds

of *Fructus Evodiae* could not be effectively seperated and identify by using this method. . Furthermore, by combining with the results of HPLC test (in the last column of table 1), it is hardly possible to draw a similar conclusion which was deduced by Dan et al., (2008). The reason might be that Dan et al., (2008)'s conclusion is available to relatively wide samplings in geographic areas.

Conclusion

There are two conclusions may be drawn from this study. Firstly, in a relatively-narrow geographic area, the ITS sequence of *Fructus Evodiae* was very conservative, and there was no ITS sequence variation among different *Fructus Evodiae* species, therefore, ITS sequence analysis is considered as a non-effective measure for studying the phylogenetic evolution. Secondly, it is difficult to identify and distinguished the three *Fructus Evodiae* species in detail by using the ITS sequence analysis combined with High Performance Liquid Chromatography (HPLC), therefore a novel test method should be developed to differentiate the three *Fructus Evodiae species*.

Acknowledgments

The authors thank professor He-Xingjin from College of Life Science£¬Sichuan University to providing the paup4.0b.

Reference

1. Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, et al. (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Mo Bot Gard 82: 247-277. » CrossRef » Google Scholar
2. Buckler ES IV, Holtsford TP (1996) Zea systematics: ribosomal ITS evidence. Mol Bio Evol 13: 612-622. » CrossRef » Pubmed » Google Scholar
3. Chaudhary MI, Qing H, Xiao PG, Cheng Y (2007) A Traditional Chinese Herbal Medicine and Its Quality Control Using a High Performance Liquid Chromatography Technique. Biol Pharm Bull 30: 165-168.
4. Chi Z (2000) Committee for the Pharmacopoeia of P. R. China, "Pharmacopoeia of P. R. China, Part I," Beijing Chemical Industry Press. Beijing p118.
5. Dan H, Shun XL, Guang XC, Chun HY, Li JW, et al. (2008) Molecular Authentication and Quality Control Using a High Performance Liquid Chromatography Technique of Fructus Evodiae. Biol Pharm Bull 31: 312-315. » CrossRef » Pubmed » Google Scholar
6. David LS (1999) PAUP* 4.0 - Phylogenetic Analysis using parsimony and other methods. Version 4. Sinauer Associates. Sunderland MA USA.
7. Doyle JJ, Dotle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue [J]. Phytochem Bull 19: 11-15.
8. Dubouzet JG, Shinoda K (1999) Relationships among old and New world Alliums according to ITS DNA sequence analysis. Theor Appl Genet 98: 422-433. » CrossRef » Google Scholar
9. Hasegawa M, Hashimoto K (1993) Ribosomal RNA trees misleading?. Nature 361: 6407-6423. » CrossRef » Pubmed » Google Scholar
10. Hershkovitz MA, Zimmer EA (1996) Conservation patterns in angiosperm rDNA ITS2 sequences. Nucleic Acid Res 24: 2857-2867. » CrossRef » Pubmed » Google Scholar
11. Hershkovitz MA, Zimmer EA, Hahn WJ (1999) Ribosomal DNA sequences and angiosperm systematics. Taylor & Francis London pp268-326.» CrossRef » Google Scholar
12. Higgins DG, Thompson JD, Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. Methods in Enzymol 266: 383-402.» CrossRef» Pubmed» Google Scholar
13. Hsiao C, Chatterton NJ, Asay KH, Jensen KB (1994) Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. Genome 37: 112-120. » CrossRef » Pubmed » Google Scholar
14. Kimura, M (1980) A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120. » CrossRef » Pubmed » Google Scholar
15. Poon WS, Shaw PC, Simmons MP, Pui-Hay But P (2007) Congruence of Molecular, Morphological, and Biochemical Profiles in Rutaceae : a Cladistic Analysis of the Subfamilies Rutoideae and Toddalioideae. Systematic Botany 32: 837-846.» CrossRef » Google Scholar
16. Sharma S, Rustgi S, Balyan HS, Gupta PK (1993) Molecular phylogenetics of Calcydenia (Compositae) based on ITS sequences of nuclear ribosomal DNA: Chromosomal and morphological evolution reexamined. Am J Bot 80: 222-238.
17. Saitou N, Imanishi M (1989) Relative efficiencies of the Fitch-Margolis, maximum parsimony, maximum likelihood, minimum evolution and neighbor-joining methods of phylogenetic tree construction in obtaining the correct tree. J Mol Biol Evol 6: 514-525. » CrossRef » Google Scholar
18. Sambrook J, Fritech ES, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press. New York 16: 7-16.
19. Steel MA, Lockhart PJ, Penny D (1993) Confidence in

evolutionary trees from biological sequence data. Nature 364: 440-442.» CrossRef » Pubmed » Google Scholar

20. Suh Y, Thien LB, Reeve HE, Zimmer EA (1993) Molecular evolution and phylogenetic implications of ribosomal DNA in Winteraceae. Am J Bot 80: 1042-1055. » CrossRef » Google Scholar
21. Swofford, Swofford DL (2000) paup*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, MA: Sinauer Associates.
22. Taberlet PL, Geilly J, Patou G, Bouvert J (1991) Universal primers for amplification of three non coding regions of chloroplast DNA. Plant Mol Bio 17: 1105-1109. » CrossRef » Pubmed » Google Scholar
23. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.» CrossRef » Pubmed » Google Scholar
24. White, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Application [J]. San Diego, California: Academic Press 315-322.
25. Zhengyi W, Peter HR (2007) Flora of China 22: 56-58.