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Genetic identification of *Sinomenium acutum* based on chloroplast gene *ndhF* sequences

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ABSTRACT

Objectives : This study was conducted to identify the original Sinomini Caulis et Rhizoma plant among *Stephania tetrandra, Cocculus trilobus, and Aristolochiae fangchi* to develop the genetic marker for Sinomini Caulis et Rhizoma.

Methods : *Sinomenium acutum* was identified by the classification and identification committee of the National Center for Standardization of Herbal Medicines. The chloroplast *ndhF* gene was amplified. We performed sequences alignment analysis of *Sinomenium acutum, Stephania tetrandra, C. trilobus*, and *A. fangchi* using BioEdit program. The SFR markers designed were consisted of SF01, SR04, and SR05 primers.

Results : Many variations of Sinomeni Caulis et Rhizoma are currently commercialized as herbal medicine. We compared the base sequences of the *ndhF* intergenic space of chloroplast DNA with *Sinomenium acutum, Stephania tetrandra, C. trilobus,* and *A. fangchi*. According to the results, it showed that the nucleotide variations were seen in 30 genes of four species. Phylogenetic analysis revealed that 4 species were classified into five groups based on an inter-group divergence in nucleotide sequence of 9%. We developed SFR marker nucleotides enough to authenticate respective species and confirmed its application on the band size at 419 base pair. These sequence differences at corresponding positions were available genetic markers to identity the Sinomeni Caulis et Rhizoma.

Conclusions : Base on these results, the *ndhF* region was effective in distinguishing Sinomini Caulis et Rhizoma The SFR genetic marker was useful for identifying Sinomini Caulis et Rhizoma with other species.

Key words : *Sinomenium acutum*, identification, *ndhF* gene, genetic marker

Introduction

Sinomenium acutum (Thunb.) Rehd. et Wils. called Bang Gi is a menispemaceaeous medicinal plant widely distributed in Korea. Sinomini Caulis et Rhizoma is originated from the climbing stem and rhizome of *Sinomenium acutum* in the Korea Pharmacopeia[1]. This plant species also produces chlorine containing alkaloids such as acutumine and acutumidine[2]. The stems of *Sinomenium acutum* have been used as a traditional medicine for treatment of various rheumatic and autoimmune diseases in East Asia[3] and its dried stem and rhizome have been used to treat obesity[4].

Many crude drugs such as *Stephania tetrandra*, *Cocculus trilobus*, and *Aristolochiae fangchi* are similar to *Sinomenium acutum* in Korea. At present, *Stephania tetrandra is* imported from China. A change in the marketplace has been observed; thus, it is difficult to say whether the correct medicinal drug is being selected. In particular, *A. fangchi* cannot be misused because of toxic components. The morphological identification among *Sinomenium acutum, Stephania tetrandra, C. trilobus,* and *A. fangchi* is limited to a few specialists. Also, there are no studies reported on possible identification method by *Sinomenium acutum* genes or components. Therefore, a technique to identify the original plant is important to ensure safe use of the crude drugs.

The chloroplast gene *ndhF* has about twice as many

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variable sites and is 〉 50% longer than those of rbcL; thus, it provides more parsimony-informative characters[5,6]. Many studies have used this gene to reconstruct phylogenies or as a molecular marker within medicinal plant families[7—13]. A study about Bang Gi was reported by Sano et al. that compared Boi (Sinomeni Caulis et Rhizoma) and Seifuto (Caulis Sinomenii) using internal transcribed spacer region sequence analysis[14].

In this study, we examined *ndhF* sequences from 30 samples to obtain phylogenetic information on the relationships among the species and developed a molecular marker for Sinomini Caulis et Rhizoma.

Materials and methods

1. Materials

The materials were collected from fresh leaf and purchased from a commercial supplier in Korea, China and Japan (Table 1). The samples were deposited in the herbarium of the Korea Institute of Oriental Medicine. The material was identified by the classification and identification committee of the National Center for Standardization of Herbal Medicines.

Table 1. Plant materials of *Sinomenium acutum*, *Stephania tetrandra*, *Stephania* species, *Cocculus trilobus*, and *Aristolochiae fangchi*

No.	Species	Plant tissue	Voucher No.	Locality	
1		Fresh leaf	112-1-1	Jeju	Korea
2		Fresh leaf	112-1-2	Jeju	Korea
3		Fresh leaf	112-1-8	Jeonnam	Korea
4		Dried stem	11B1001	Jeju	Korea
5	*Sinomenium acutum*	Dried stem	11B1002	Jeju	Korea
6		Dried stem	11B1003	Jeju	Korea
7		Dried stem	11B1004	Jeju	Korea
8		Dried stem	11B1009	Tokyo	Japan
9		Dried stem	11B1010	-	China
10		Dried stem	11B1011	-	China
11		Fresh leaf	35-3-10	Jiangsu	China
12		Fresh leaf	35-5-11	Jiangsu	China
13	*Stephania tetrandra*	Dried stem	11B1013	Jiangsu	China
14		Dried stem	11B1014	Jiangsu	China
15		Dried stem	11B1016	Zhejiang	China
16		Fresh leaf	35-3-12	Tokyo	Japan
17		Fresh leaf	35-3-6	Guangxi	China
18		Dried stem	11B1022	Jeju	Korea
19		Dried stem	11B1032	-	China
20	*Stephania species*	Fresh leaf	S1	-	China
21		Fresh leaf	S2	-	China
22		Fresh leaf	S3	-	China
23		Fresh leaf	S4	-	China
24		Fresh leaf	S6	Sichuan	China
25		Fresh leaf	35-5-2	Guangxi	China
26	*Cocculus trilobus*	Dried stem	11B1018	Gyeongju	Korea
27		Dried stem	11B1019	Gyeongju	Korea
28		Dried stem	11B1021	Jeju	Korea
29	*Aristolochiae fangchi*	Fresh leaf	35-6-1	Tokyo	Japan
30		Dried stem	G1	Sichuan	China

2. Genomic DNA extraction

Genomic DNA was extracted from each sample according to the manual for the DNeasy® Plant Mini kit (Qiagen, Valencia, CA, USA). DNA concentration and purity were determined by spectrophotometry (Nanodrop ND-1000, Nanodrop, Wilmington, DE, USA), followed by electrophoresis on a 1.5% agarose gel with DNA size markers (Takara, Shiga, Japan). The final concentration of each sample was diluted to approximately 20 ng/μl with diethyl pyrocarbonate-distilled water for polymerase chain reaction (PCR) amplification.

3. PCR amplification of the *ndhF* region, sequencing, and phylogenetic analysis

The chloroplast ndhF gene was amplified using the primers of Olmstead and Sweere (1994)[15] and Graham et al. (1998)[16]. The primers were *ndhF1* (5'-CGA AAT CGG TAG ACG CTA CG-3') and *ndhF972* (5'-ATT TGA ACT GGT GAC ACG AG-3'). The total 30 μl reaction mixture volume contained 10 pmol/μl of each primer, 2× premix (Takara) and 20 ng template. Reactions were carried out with a PTC-200 instrument (MJ Research, San Francisco, CA, USA). The temperature cycling parameters were programmed for a cycle of 5 min at 98° C, followed by 35 cycles of 30 sec at 98° C, 30 sec at 50° C, 1 min at 72° C, and a cycle of 4 min at 72° C. PCR products with LoadingSTAR (DyneBio, Seoul, Korea) were electrophoresed on a 1.5% agarose gel, with a 100 bp DNA size marker in Tris-borate EDTA buffer. The amplified DNA fragments were separated from the agarose gel using a Gel Extraction kit (Promega, Madison, WI, USA). The nucleotide sequences of the resulting PCR products were determined by an automatic DNA sequencer (ABI, 3730 Applied Biosystems, Foster City, CA, USA). Sequence alignment analysis was conducted using DNASTAR® Lasergene® 7.2 software and compared with data at the National Center for Biotechnology Information. Sequences were aligned with ClustalW using Bioedit software (http://www.mbio.ncsu.edu/ BioEdit/bioedit.html).

4. Development of genetic markers

We performed sequences alignment analysis of *Sinomenium acutum*, *Stephania tetrandra*, *C. trilobus*, and *A. fangchi*, using BioEdit program. The SFR markers designed were the SF01 primer (5'-ACG ACC AAT TGC GGC AAA T-3'), SR04 primer (5'-GAA GTC GAG CGA CAA GAA AAA T-3'), SR05 primer (5'-CGC CCC TAA TAG GAC TGT TA-3'). A 30 μl total volume of

reaction mixture contained 10 pmol/μl of each primer, 2× premix, and 20 ng template. Reactions were carried out with a PTC-200 instrument. The temperature cycling parameters were programmed for a cycle of 5 min at 98° C, followed by 30 cycles of 20 sec at 98° C, 30 sec at 58° C, 40 sec at 72° C, and a cycle of 5 min at 72° C. PCR products were electrophoresed with LoadingSTAR on a 1.5% agarose gel, with 100 bp DNA size markers in Tris-borate EDTA buffer.

Results

1. Analysis of the cpDNA *ndhF* region

The *ndhF* sequences consisted of 455 characters for each taxon. No indels were found during alignment of any taxon. Thirty *ndhF* sequences represented nine length-variable gene types, but we did not observe any sequence variation from any of the inner taxa (Fig. 1). As shown in Fig. 1, a comparison of the four taxa showed that 91% of their *ndhF* sequences were identical and 72 base variation conserved regions (72/455) were detected. In contrast, the intraspecific variation homology percentage observed at the sequence variation position was 100% in all except *Stephania* species. Intraspecific diversity of *Stephania* species had 96% homology (20/455). The results showed that the GC content of *Sinomenium acutum* was 41%, the GC content of *Stephania tetrandra* was 39%, the GC content of *C. trilobus* was 39%, and the GC content of *A. fangchi* was 39%. The optimal size fragment for sensitive base substitution detection in *Sinomenium acutum* by *ndhF* region analysis was approximately 119 bp, 141 bp, 188 bp 209 bp, 342 bp, and 419 bp (Fig. 1). Therefore, our results indicate that the *ndhF* sequence analysis was effective for identifying *Sinomenium acutum* from other species.

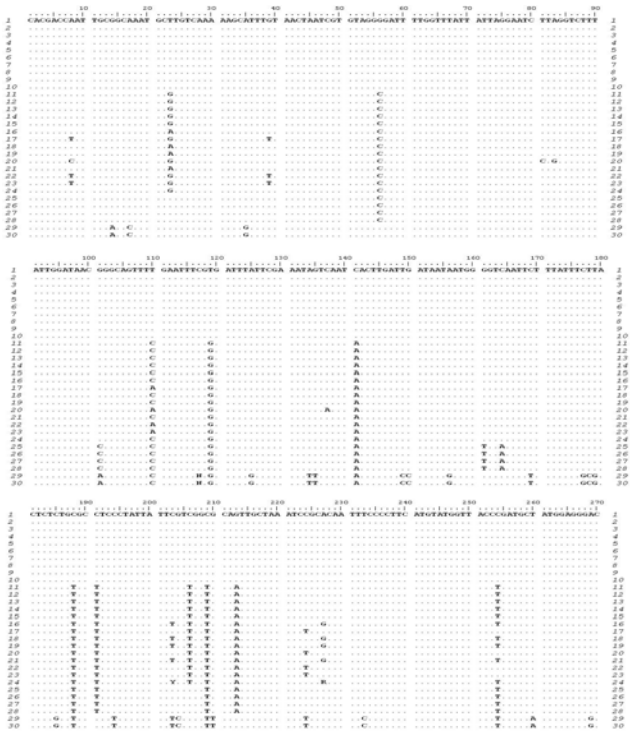

Fig. 1. Comparison of the *ndhF* region sequence of *Sinomenium acutum*, *Stephania tetrandra*, *Stephania* species, *Cocculus trilobus*, and *Aristolochiae fangchi*. Numbers are listed in Table 1.

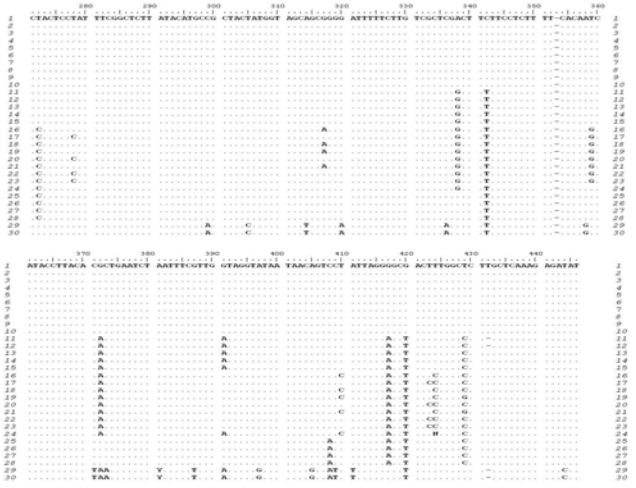

Fig. 1. (Continued)

2. Phylogenetic analysis of the *ndhF* sequences

A phylogenetic study was performed to evaluate the relationship among three taxa, including one outgroup of *A. fangchi* based on *ndhF* region sequences in chloroplast DNA. Thirty genes from the *ndhF* region, were classified into five groups based on a intergroup divergence in nucleotide sequence of 9%: group A (*Sinomenium acutum*), group B (*Stephania tetrandra*), group C (*Stephania species*), group D (*C. trilobus*), and group E (*A. fangchi*) (Fig. 2). Thus, the nine gene types were distributed into five groups with

different sequences. In particular, group B and group C *Stephania* species divided the gene into six subtypes. These results indicate that the genetic distance of the *Sinomenium acutum* (group A) clade was close to the *Stephania tetrandra* (group B) clade, whereas it was far from the *A. fangchi* (group E) clade outgroup.

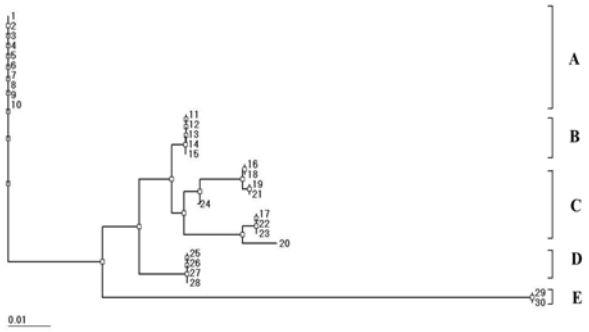

Fig. 2. The Phylogenetic tree obtained from the analysis of the *ndhF* region data. Bar length represent genetic distance value. A, B, C, D, and E represent clades. Numbers are listed in Table 1.

3. Development of a SFR genetic marker

A set of SFR primers was developed from six sequence variation positions of the *ndhF* gene (Fig. 3). Out of the designed 10 primer, eight primers failed to detect the polymorphism band of *Sinomenium acutum*. But the SF01 primer and SR05 primer were amplified the specific fragment of 416 bp for *Sinomenium acutum*. Then again, we detected that the SF01 primer and SR04 primer were the fragment of 342 bp for all four taxa. We were seeking way to use advanced technologies to optimize the primer concentration and PCR condition as follows. Each reaction contained 10 pmol/L SF01 primer, 5 pmol/L SR04 primer, 5pmol/L SR05 primer and 10 ng genomic DNA. The reaction mixture with no template DNA was used as a negative control. The thermal cycling conditions consisted of an initial denaturation at 98° C for 5 min, followed by 30 amplification cycles (98 ° C for 20 s, 57 ° C for 30 s, and 72 ° C for 40 s), and final extension step at 72 ° C for 5 min. These primers were designed to be *Sinomenium acutum* specific, based on the *ndhF* sequences, and were confirmed to effectively distinguish *Sinomenium acutum*. Additionally, a positive primer was designed to amplify all four taxa at 342 bp. Then, we monitored the 30 samples identified by this genetic marker. As a result, *Sinomenium acutum* (samples 1-10) were amplified at the specific 419 bp band size, and the other species (samples 11-30) were present at the 342 bp size product band (Fig. 4). Thus, our results show that the SFR primer was useful for identifying *Sinomenium acutu*m.

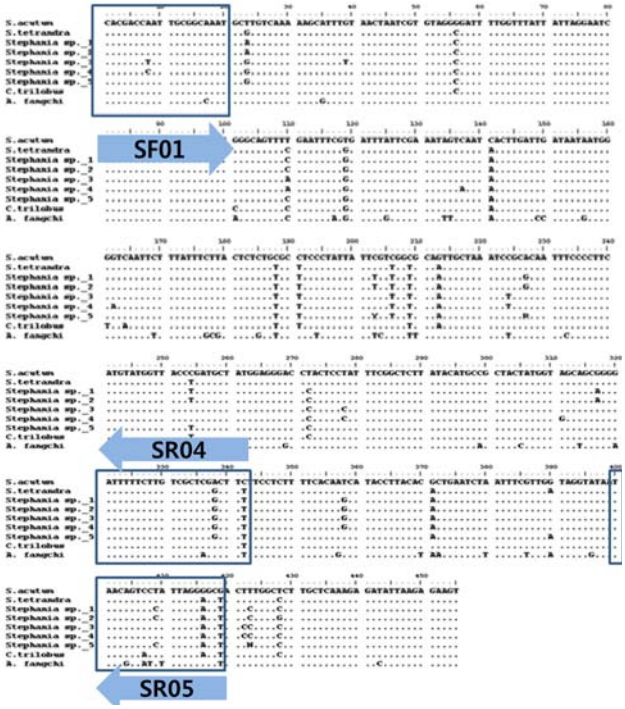

Fig. 3. Comparison of the *ndhF* region sequence of PCR products of *Sinomenium acutum*, *Stephania tetrandra*, *Stephania* species, Cocculus trilobus, and *Aristolochiae fangchi* by SFR primer. Dots represent identical nucleotides. Square boxes of nucleotides indicate the designed primers.

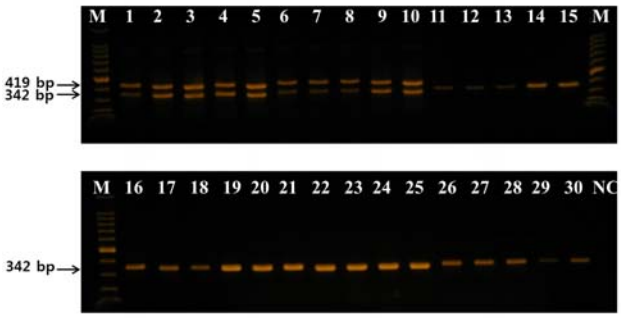

Fig. 4. PCR product amplified with SF01, SR04, and SR05 primers. Numbers are listed in Table 1; lane 1 –10, *Sinomenium acutum;* lane 11 –15, *Stephania tetrandra;* lane 16 –24, *Stephania* species; lane 25–28, *Cocculus trilobus;* lane 29–30, *Aristolochiae fangchi* ; NC; Negative control (No template control). M is DNA size marker.

Discussion

In the Korean Pharmacopoeia (KP), Sinomini Caulis et Rhizoma is defined as *Sinomenium acutum*. Now, other herbal medicines besides *Sinomenium acutum* are circulating. Thus, it is necessary for users to distinguish Bang Gi from other herbs. Boi and its original plant *Sinomenium acutum* from Japan were compared with Seifuto and its botanical origins from China in terms of their internal transcribed spacer sequences and major chemical components[14]. Based on

results from BLAST searches, our sequencing result was identified in the genome sequence of *Sinomenium acutum* (JN051718), *Stephania tetrandra* (JN051727), *C. trilobus* (JN051704) in GenBank accession number. A study has reported on a morphological classification of S*inomenium acutum*, *C. trilobus* and *A. fnagch*[17,18]. However, it is difficult to distinguish herbal medicine morphologically. Therefore, we developed a genetic marker to identify *Sinomenium acutum* at the DNA level.

Initial applications of *ndhF* sequences in systematic studies centered on relationships within families, making use of the greater length of the sequence [19–21]. A sequencing analysis of *ndhF* has proven to be a simple and effective technique for detecting single base variations. We used *ndhF* and *ndhF*972R to analyze 30 samples, and the amplified bands contained approximately 500 bp PCR products. Our results indicate that the type of base substitution mutation played a major role determining whether a mutation was detected by the *ndhF* analysis. Our results contribute to identify the exact herb medicine called Bang Gi when screening for polymorphisms without whole sequence analysis.

In the phylogenetic tree analysis, the gene sequences of other species were nearly 100% homology except the *Stephania* species (Clade C) in the *ndhF* trees. Clade C in particular, which divided five gene types, appears to be various genotypes. *Stephania* species are distributed in Asia and used in traditional medicine. However, Sinomini Caulis et Rhizoma is not originated from *Stephania* species. In order to report the misuse possibility in the herbal medicine market, we were used as compensated material.

In summary, we initially conducted *ndhF* sequences analyses to identify authentic Sinomini Caulis et Rhizoma. The pattern observed provided evidence that *Sinomenium acutum* could be distinguished from other species. The super fine resolution marker (SFR) provided an effective approach to distinguish Sinomini Caulis et Rhizoma.

Conclusion

We objectively identified *Sinomenium acutum* from other species using a genetic marker. The results are as follows.

1. The *ndhF* sequence analysis included 30 samples from four species and consisted of 455 characters for each taxon. Sequence alignments represented nine length variations of the gene type, whereas we did not observe any sequence variation from any inner taxon.

2. In the phylogenetic analysis, the genetic distance between the *Sinomenium acutum* (group A) clade was close to the *Stephania tetrandra* (group B) clade, whereas it was far from the *A. fangchi* (group E) outgrouped clade. *Stephania* species was divided into fix subtypes.

3. The SFR genetic marker designed in this study will be useful to confirm the identity of *Sinomenium acutum*.

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