Molecular Identification of “Chuanxiong” by Nucleotide Sequence and Multiplex Single Base Extension Analysis on Chloroplast *trnK* Gene

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Chloroplast *trnK* gene sequences of *Cnidium officinale* and *Ligusticum chuanxiong* were determined to establish an effective method for identifying Japanese Senkyu and Chinese Chuanxiong, the two which have the same drug name in Chinese characters, similar external feature, but different botanical origins. Three sites of nucleotide differences were found between these 2 species at positions 767, 924 and 964 from upstream in *trnK* gene sequence, allowing molecular identification of the two plants and crude drugs. Further, three kinds of specific primers of 14 mer, 23 mer and 30 mer long were designed to detect these 3 sites of marker nucleotides. By using multiplex single base extension (MSBE) analysis with the 3 specific primers, *C. officinale* and *L. chuanxiong* could be distinguished clearly by the electrophoretograms, where 3 peaks with different color of ddTMP, ddCMP and ddTTP were observed in case of *C. officinale* and those of ddGMP, ddAMP and ddGMP in *L. chuanxiong*. Moreover, *trnK* gene sequence of “Dongxiong,” a kind of Chuanxiong cultivated in Northeast China, suggested that its botanical origin was *C. officinale*.

Key words molecular identification; *Cnidium officinale*; *Ligusticum chuanxiong*; *trnK* gene sequence; multiplex single base extension (MSBE)

“Chuanxiong” has been used in traditional Chinese medicine for more than 2000 years. It was firstly described with the name “Xiongqiong” in the oldest Chinese herbal classic “Shen Nong Ben Cao Jing” (before A.D. 1st century) as a medium drug for activating “qi” (vital energy, functional activity), expelling wind, relieving pain, alleviating spasm and promoting blood circulation.¹⁾ It has been widely used nowadays in China and Japan for curing gynecological diseases, such as dysmenorrhea, amenorrhea, anemia and postpartum abdominal pain, etc.²⁾ However, the botanical sources of Chuanxiong are different in the two countries. “Senkyu” (Japanese pronunciation of “Chuanxiong”) has been prescribed to be the dried rhizome of *Cnidium officinale* MAKINO in Japanese Pharmacopoeia (2005 ed.),³⁾ whereas the dried rhizome of *Ligusticum chuanxiong* HORT. in Chinese Pharmacopoeia (2005 ed.).⁴⁾

The two plants, *C. officinale* and *L. chuanxiong* have been cultivated for medicinal purpose in Japan and China, respectively, for several hundred years. The longtime cultivation through vegetable propagation results in the both plants haven't ripe fruit which is one of the most important characteristics for delimitation of Umbelliferous plants, and the scientific name of both plants were given based on the cultivated ones.^{5,6)} *C. officinale* is believed to be transported from China during Edo era,²⁾ however the same plant could not be found in China as well as in Chinese botanical records nowadays. Due to the above reasons, the taxonomic position of *C. officinale* has been debated among Japanese botanists.⁷⁾ Inquiring of the relation between Japanese Senkyu and Chinese Chuanxiong has been an interested subject for a long time. So far, differences between these two in anatomical and cytogenetic characters,^{8–10)} chemical constituents,^{10–12)} pharmacological effects¹⁰⁾ have been widely investigated and the re-

sults suggested these two were closely related, but different taxa. However, previous molecular studies that tried by comparison of nucleotide sequences of nuclear 18S rRNA gene¹³⁾ and chloroplast *rbcL* gene⁷⁾ failed to discriminate these two plants since the sequences of both regions from *C. officinale* and *L. chuanxiong* were completely identical to each other. ITS sequence data suggested that *C. officinale* might be a hybrid of *L. sinense* and *L. jeholense* (or relatives of *L. jeholense*).¹⁴⁾ In the present study, sequence of *trnK* gene, one of chloroplast DNA regions with the highest rate of nucleotide substitution which has been demonstrated to be potential to discriminate species within the same genus,¹⁵⁾ was investigated in order to establish an objective and effective method for identifying the Japanese and Chinese “Chuanxiong” and to find out molecular inferences for elucidating their relation. Further to develop a convenient and rapid method for identification, multiplex single base extension (MSBE) analysis has been applied on the basis of the determined *trnK* gene sequence.

In addition, a kind of Chuanxiong locally called as “Dongxiong” or “Japanese Chuanxiong” has been cultivated in Jilin, Northeast China.¹⁶⁾ It is said that this plant was introduced to Northeast China from Japan during the 1930s.¹⁷⁾ As in *C. officinale*, there is no ripe fruit being observed in “Dongxiong” even during such several decades' cultivation.¹⁶⁾ In the present study, we analyzed the nucleotide sequences of chloroplast *trnK* gene from several samples of “Dongxiong” collected in this region to obtain molecular evidences for clarifying its botanical source and originating legend. Moreover, sequence of nuclear 18S rRNA gene was also determined and compared with the previous data¹³⁾ for confirmation.

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MATERIALS AND METHODS

Materials *Cnidium officinale* was obtained from Hokkaido (H. Fushimi 22), Japan. One specimen of *Ligusticum chuanxiong* was collected from Sichuan Prov., China (H. Fushimi 23) and 2 specimens (A. Takano Cl-1, Cl-4) cultivated in Showa Pharmaceutical University. Crude drug samples, Senkyu derived from *C. officinale* (TMPW No. 15103) was purchased in 1994 from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan), and Chuanxiong derived from *L. chuanxiong* (TMPW No. 20986) was purchased in 2001 from Hehuachi crude drug market of traditional Chinese medicine, Chendu, Sichuan Prov., China. Three plant specimens (T. Tsuchida 020001-A, 020001-B, 020001-C) and two drugs samples (TMPW No. 21978 obtained in 2001, No. 21979 in 2002) of “Dongxiong” were collected from Jilin Prov., China. All the materials used in this study are deposited in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama (TMPW).

Isolation of Total DNA, PCR Amplification and Sequencing Reaction Total DNA was extracted from dried leaf or rhizome of plant specimens and crude drug by using DNeasy™ plant Mini Kit (QIAGEN, Germany) with several modifications to the protocol provided by manufacturer (incubated sample with AP1 buffer at 65 °C for 4 h and then after adding AP2 buffer kept the mixture on ice for 1 h) and then purified by GeneClean 101 Kit (Qbiogene, Inc., U.S.A.). Amplifications of *trnK* gene via the polymerase chain reaction (PCR) were performed in 50 µl reaction mixture, consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.25 mM of each primer, 1.5 U *Taq* Polymerase (Promega, U.S.A.), and 10–100 ng total DNA as a template. A pair of primers flanking whole intron region of *trnK* gene were trnK3914F (5'-TGG GTT GCT AAC TCA ATG G-3') and trnK2R (5'-AAC TAG TCG GAT GGA GTA G-3'). PCR amplifications were carried out in a Thermal Controller PTC-100 (MJ Research Inc., U.S.A.) by the cycling condition of hot start at 94 °C for 2 min, followed by 35 cycles of 94 °C for 40 s, 52 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 20 min. The 18S rRNA gene sequences of the 3 specimens of “Dongxiong” from Jilin Prov., China were investigated by using the same method as in our previous report.¹⁸⁾ The 1/10 volume of resulting PCR product was detected by 1.0% agarose gel electrophoresis and then the remained part was purified using a QIA quick PCR purification Kit (QIAGEN, Germany). Sequencing reaction of the purified PCR products were carried out using a Thermo Sequenase Cycle Sequencing Kit (GE Healthcare Bioscience, U.K.) with a set of fluorescent-labeled sequencing primers (Table 1).

Sequencing Analysis Sequence was determined directly by a 4000L DNA sequencer (LI-COR, U.S.A.) and analyzed using the BaseImagIR program (Version 4.0, LI-COR, U.S.A.). The obtained DNA sequences were assembled, and consensus sequences were constructed by the AutoAssemble program (Version1.3.0, Applied Biosystems, U.S.A.). The boundary of the *matK* gene region and the deduced amino acid sequence of maturase were investigated by using the computer program GENETYX-SV/SC (version 7.0.5, Japan) and were also confirmed by comparison with the sequence of tobacco (*Nicotiana tabacum*)¹⁹⁾ and ginseng (*Panax*

Table 1. Fluorescently Labeled Primers of *trnK* Gene for Sequencing Reaction

Primer sequence (5' to 3')		
<i>trnK</i> gene	Forward primers	
	IRD-Rh- <i>trnK</i> 5'F	AGA GTA CTC GGC TTT TAA GTG C
	IRD-CX <i>trnK</i> 698F	CCT CGT TTT GAC TGT ATC GC
	IRD- <i>matK</i> 748F	AAG GAT CCT TTC ATG CAT TAT
	IRD- <i>trnK</i> PT2045F	TAT ACT TCG ACT TTC TTG TGC
	Reverse primers	
	IRD- <i>trnK</i> PT713R	TGA TAC ATA GTG CGA TAC AG
	IRD-CX- <i>trnK</i> 2R	ACC ATA GAC TGT CCT GAA AGC
	IRD-Chu- <i>trnK</i> 2R	CCA GTA GCG AAG AGT TTG AAC
	IRD- <i>matK</i> 1219R	GTC GAA GTA TAT ACT TTA TTC G

Table 2. Sequences of the Designed Primers for Identification of Chuanxiong Using Multiplex Single Base Extension (MSBE) Analysis

Primer name	Sequence (5' to 3')	Detecting position
CL14merF	CCT TAG TTC AAC TA	767
CL23merF	TTT TTT GTT GGA AAA TGC AGG TT	924
CL30merF	TTT TTT TTT TAT TGT GAA ACG TGC AAT TGA	964

ginseng).²⁰⁾ The nucleotide sequence data of the *trnK* gene was recorded in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers, AB262551 for *C. officinale* and AB262552 for *L. chuanxiong*.

Multiplex Single Base Extension (MSBE) Analysis A short fragment of *trnK* intron region was amplified as template for MSBE analysis using a pair of newly designed primers (Chu-*trnK* 493F: 5'-ATG TGT GTG TAG AAG AAA CAG-3', Chu-*trnK* 1217R: 5'-GGG TAT TAG TAT CTC TAA CAC-3'). PCR mixture was prepared as same as in the former part of amplifying the whole *trnK* gene region, and the PCR reaction was conducted by cycling condition of hot start at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 s, 50 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 10 min. Then, MSBE analysis was carried out with 3 specific primers of different length (Table 2) and using the purified PCR product as template. Total volume of 10 µl reaction mixture composed of the 3 primers (CL14merF, 0.4 µM; CL23merF, 0.2 µM; CL30merF, 0.2 µM), PCR product of partial *trnK* intron region (0.1–0.2 pmol), and SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems, U.S.A.) in which fluorescently-labeled ddNTP are contained. Thermal condition for MSBE analysis was a cycle at 96 °C for 1.5 min, followed by 25 cycles of 96 °C for 10 s, 40 °C for 5 s and 60 °C for 30 s. After reaction, 2.0 units of shrimp alkaline phosphatase (SAP, Sigma, U.S.A.) was added into the mixture, then incubated at 37 °C for 1 h and denatured at 75 °C for 15 min to eliminate unincorporated ddNTP. The resulted mixture was diluted with Hi-Di formamide and then run on ABI PRISM Avant-3100 Genetic Analyzer with 36 cm capillaries filled with POP-4 polymer and then analyzed by the software GeneScan (Applied Biosystems, U.S.A.).

Fig. 1. *trnK* Gene Sequence of *Cnidium officinale*

Two parts of exon of *trnK* gene are in shade box. The *matK* gene region of 1518 bp long (776th—2293th) is enclosed in line box and the deduced amino acid sequence of maturase is shown below the nucleotide sequence. Arrow heads indicate the positions of nucleotide difference presented between *Cnidium officinale* and *Ligusticum chuanxiong*. Arrows indicate sequences of primers used in PCR reaction.

RESULTS

trnK* Gene Sequences of *C. officinale* and *L. chuanxiong The chloroplast *trnK* gene, encoded tRNA^{Lys}^{UUU}, consists of only 70-bp exon and a *ca.* 2500 bp intron inside in the two parts of exon.¹⁹⁾ An open reading frame which encodes a maturase involved in intron-splicing, *matK* gene is located within the intron region.²¹⁾ The whole length of determined *trnK* region was of 2569 bp long in all samples of both *C. officinale* and *L. chuanxiong*. By comparing the obtained sequence with those of tobacco¹⁹⁾ and ginseng,²⁰⁾ the corresponding parts in the *trnK* gene sequence of *C. officinale* were determined as shown in Fig. 1. The 5'-exon was between positions 2 and 38, being 37 bp in length, 3'-exon (partial) from positions 2560 to 2569, and the *matK* gene region of 1518 bp was defined between positions 776 and 2293 for coding 505 amino acid sequence of maturase (Fig. 1). The completely same *trnK* gene sequences were obtained from plant specimens and corresponding crude drug samples

of *C. officinale* and *L. chuanxiong* collected from different locations, respectively, indicating the intraspecies-stability and specificity of *trnK* gene sequence in these two species. Three sites of nucleotide differences were found between *C. officinale* and *L. chuanxiong*, which enabled these two species to be clearly distinguished from each other by the *trnK* gene sequence. At nucleotide positions 767, 924 and 964 from upstream, *C. officinale* possessed sequences of thymine, cytosine and thymine, whereas *L. chuanxiong* possessed guanine, adenine and guanine, respectively (Fig. 2). The later two sites located within the *matK* gene region, thus the nucleotide differences resulted in variations in the deduced amino acid sequence of maturase. Amino acid sequence exhibited changes at positions 50 and 63, corresponding to the nucleotide differences at positions 924 and 964, from serine and aspartic acid in *C. officinale* to tyrosine and glutamic acid in *L. chuanxiong* (Fig. 2).

Multiplex Single Base Extension (MSBE) Analysis To further develop a convenient and rapid method for identifica-

tion of Japanese Senkyu and Chinese Chuanxiong, multiplex single base extension (MSBE) analysis was applied on the basis of the determined *trnK* gene sequence. MSBE analysis enables simultaneous detection of multi-points of single nucleotide polymorphisms in various loci of template DNA, which has been widely used in investigation of individual's response to medication,²²⁾ disease-related gene,²³⁾ and forensic DNA typing,²⁴⁾ etc. Based on the nucleotide differences in the *trnK* gene sequences between *C. officinale* and *L. chuanxiong*, we designed three kinds of forward primers with different lengths, CL14merF, CL23merF and CL30merF (Table 2), for detecting the nucleotides at positions 767, 924 and 964 (Fig. 2). Beyond a consideration that degraded DNA extracted from crude drug sample was difficult to produce long PCR fragment, a pair of primers (Chu-*trnK* 493F and Chu-*trnK* 1217R) were designed to amplify a short fragment including the 3 nucleotide substitution sites as template (Fig. 2). In subsequent MSBE analysis the specific primers, CL14merF, CL23merF and CL30merF annealed to the template DNA at corresponding regions, their 3' end binding to the nucleotides next to the substitution sites. Then in the presence of fluorescently labeled ddNTPs ([F]ddNTPs), primers were extended by adding only a specific [F]ddNMP to the 3'-end which was complementary to the substitution nucleotide of template. The single-base extended products of 15 mer, 24 mer and 31 mer were separated and detected by electrophoresis on a 3100-Avant Genetic Analyzer. As results, *C. officinale* and *L. chuanxiong* were distinguished

Fig. 2. Comparison of *trnK* Gene Sequences between *Cnidium officinale* and *Ligusticum chuanxiong*

Nucleotide differences presented at positions 767, 924 and 964 from upstream. Two sites of substitutions in *matK* gene region were nonsynonymous substitutions which induced amino acid alteration in translated protein (maturase). The corresponding alteration of amino acid residues are shown in the next frame. Two arrows in lower part indicate the annealing positions of a pair of primers (Chu-*trnK*493F, Chu-*trnK*1217R) which were designed to amplify a 745-bp fragment as template for subsequent multiplex single base extension (MSBE) analysis. The sequence of plant specimens "Dongxiong" collected from Jilin Prov., Northeast China was identical to that of *C. officinale*.

Fig. 3. Electrophoretograms of Multiplex Single Base Extension Analysis for *Cnidium officinale* and *Ligusticum chuanxiong*

Three peaks with different color resulted from multiplex single-base extension analysis by using the 3 specific primers of CL14merF, CL23merF and CL30merF. *C. officinale* and *L. chuanxiong* were distinguished clearly by the different electrophoretograms. Three peaks with red, black, red colors in order were observed in *C. officinale*, corresponding to the incorporated [F]ddTMP, [F]ddCMP and [F]ddTMP; while 3 peaks of blue, green, blue colors were observed in *L. chuanxiong*, corresponding to the incorporated [F]ddGMP, [F]ddAMP and [F]ddGMP.

clearly by the different electrophoretograms, where 3 peaks with red, black and red colors in order derived from the incorporated [F]ddTMP, [F]ddCMP and [F]ddTMP were observed in case of *C. officinale* and those of [F]ddGMP (blue), [F]ddAMP (green) and [F]ddGMP (blue) in *L. chuanxiong* (Fig. 3). In the electrophoretogram, the relative positions of the central peak, [F]ddCMP (black) in *C. officinale* and [F]ddAMP (green) in *L. chuanxiong*, showed a little bit different. That was due to the mobility of labeled single-base extended products in electrophoresis were affected by not only the length of primer but also the size of attached dye. In the case of *C. officinale* (Fig. 3, left), size of dye (dROXTM) labeled on ddTMP is bigger than that of dye (dTAMRATM) labeled on ddCMP, which resulted in relatively lower mobility of 15mer and 31mer products than that of 24mer product. The established method was tested for all the samples used in the present study and the unambiguous results were obtained either from plant specimens or from crude drug samples.

Nucleotide Sequences of *trnK* Gene and 18S rRNA Gene of "Dongxiong" The whole sequences of *trnK* gene as well as 18S rRNA gene from three specimens of "Dongxiong" collected from Jilin Prov., China were determined. The 3 samples possessed the same sequences in the both regions, respectively. The 18S rRNA gene sequence, as shown in our previous paper, is quite conservative and both *C. officinale* and *L. chuanxiong* possessed the same sequence.¹³⁾ Here, the 18S rRNA gene sequence of "Dongxiong" was of 1808 bp long and identical to that of *C. officinale* and *L. chuanxiong*. The *trnK* gene sequence of "Dongxiong" was completely same as that of *C. officinale*, differing from that of *L. chuanxiong* at the 3 nucleotide positions (767, 924, 964, in Fig. 2). The molecular results suggested that the botanical origin of "Dongxiong" was *C. officinale*, and also provided evidence supporting the legend that this plant was originated from Japan. The so far obtained chemical and pharmacological data of "Dongxiong" also supported our result.¹⁰⁾ Moreover, two crude drug samples (TMPW No. 21978, 21979) obtained from Jilin Prov., China were analyzed by the established MSBE analysis. Using total DNA extracted from dried rhizomes of the 2 samples, a 745 bp PCR fragment was easily amplified to use as template for MSBE analysis. Detection of the MSBE products revealed that these two samples had the same electrophoretogram patterns, in which three peaks of red ([F]ddTMP), black ([F]ddCMP), red ([F]ddTMP) colors in order were observed. Therefore, the 2

crude drugs were identified to be derived from *C. officinale*, as same as the plant materials collected in this region.

DISCUSSION

Compared with formerly reported chloroplast *rbcL* gene sequence,⁷⁾ the *trnK* gene sequence determined in the present study showed 3 sites of nucleotide difference between *C. officinale* and *L. chuanxiong*, which provided molecular marker to discriminate the two plants as well as their derived crude drugs Senkyu and Chuanxiong. Moreover, it has already been demonstrated that the substitution rate of *matK* gene (embed in *trnK* intron region) is about three times higher than that of *rbcL* gene.^{20,25)} We estimated that the *trnK* gene sequence was potential to be used in phylogenetic analysis within Umbelliferae family and might provide insight into the taxonomic treatment of both genera *Cnidium* and *Ligusticum*. Further investigation of *trnK* gene sequences from wide taxa in Umbelliferae is promising to obtain valuable information for constructing phylogenetic relationship within this family.

Cytogenetic study⁹⁾ suggested that *C. officinale* might be an allotriploid species because of unpaired J-shaped chromosomes and prevalent formation of abnormal types of sporad and micronucleus at pollen tetrad stage. On the other hand, sterility of *C. officinale* and vegetable propagation for long-time prevent allelic exchange and gene flow, therefore the intact characteristics of original parent species could be possibly preserved. Formerly, Kondo *et al.*¹⁴⁾ analyzed nuclear ITS sequence of these two and suggested they might be originated from the same parental plants. The *trnK* gene sequence determined in the present study which belongs to chloroplast DNA is mostly of maternal inheritance in angiosperm.²⁶⁾ Our results suggested that at least *C. officinale* and *L. chuanxiong* were not from the same maternal line. Morphology, cytogenetic characters^{9,10)} and chemical constitutions^{10–12)} revealed the 2 species were quite different. Meanwhile, the previous phylogenetic analysis based on *rbcL* gene sequence suggested that *C. officinale* was closely-related to *Ligusticum taxa*, but far from *Cnidium taxa*.⁷⁾ To further elucidation of their genesis and formational mechanism, simultaneous investigation based on biparentally-hereditary nuclear ITS sequences and maternal chloroplast *trnK* sequence from wide sampling of *Ligusticum* and *Cnidium* genera as well as other genera of Umbelliferae, is desired.

Based on *trnK* gene sequences, an objective method for identification of crude drugs of Senkyu and Chuanxiong has been established and convenient MSBE analysis was further developed, by which the origins of drugs could be identified obviously by the 3 peaks in the electrophoretograms. The MSBE analysis enables 16 samples to be analyzed simultaneously within 20 min, providing a rapid and effective method for guarantee the efficiency of crude drugs.

In conclusion, we elucidated the species-specific *trnK* gene sequences of *C. officinale* and *L. chuanxiong* and established an objective and convenient MSBE analysis for identification of crude drugs, Senkyu and Chuanxiong. Moreover, our molecular results suggested that the origin of “Dong-xiong” in Northeast China is *C. officinale*.

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