Quality Assurance of Botanical Raw Materials Based on Species Identification Using Molecular Markers for Herbal Medicinal Product Manufacturing

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Abbreviations

API	Active pharmaceutical ingredient
ARMS	Amplification refractory mutation system
BRM	Botanical raw material
CAPA	Corrective action and preventive action
CBD	Convention on biological diversity
CITES	Conventions on international trade in endangered species of wild fauna and flora
cpDNA	Chloroplast DNA
EMA	European medicines agency
FDA	United States food and drug administration
GACP	Good agricultural and collection practice
GAP	Good agricultural practice
GDP	Good distribution practice
GFSI	Global food safety initiative
GHPP	Good herbal processing practice
GMP	Good manufacturing practice
GPSP	Good post-marketing study practice
GVP	Good vigilance practice
HPLC	High performance liquid chromatography
ICH	International conference on harmonization of technical requirements for registration of pharmaceuticals for human
ISO	International standards organization
ITS	Internal transcribed spacer on nuclear ribosomal DNA
JGAP	Japanese good agricultural practice
JKMA	Japan kampo medicines manufactures association
***mat*K**	maturase K gene for lysine on cpDNA
MHLW	Ministry of health, labour and welfare in Japan
NDA	New drug application on FDA
nrDNA	Nuclear ribosomal DNA
PCR	Polymerase chain reaction
PIC/S	Pharmaceutical inspection convention/Pharmaceutical inspection co-operation scheme
PQS	Pharmaceutical quality system
***psb*A**	protein D1 gene of Photosystem II
***rbc*L**	ribulose-1, 5- bisphosphate carboxylase/oxygenase gene
RFLP	Restriction fragment length polymorphism
***trn*H**	tRNA histidine gene
WHO	World health organization

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Chapter 1

General introduction

1.1 Herbal medicinal product

Herbal medicinal products are medicines consisting of medicinal plant materials, algae, macroscopic fungi, and combinations. They are sometimes called crude drug, botanical drugs, herbal drug, or herbal substance. The herbal medicinal products consisting of some kinds of plant materials are called as drug preparations or herbal preparations.

Kampo medicine is a typical herbal medicinal product in Japan. In 2014, the gross production amount of Kampo medicine in Japan is 158 billion Japanese yen which accounts for 2.3% of the gross production amount of all medicinal products made in Japan (Japan kampo medicines manufactures association; JKMA, 2017). Use of 148 kinds of Kampo medicines is covered as by the health insurance in Japan (JKMA, 2016). Kampo medicine is important for health of Japanese nation.

While, the United States food and drug administration (FDA) approved the only two botanical drugs Veregen and Fulyzaq in 2006 and 2012, respectively. Veregen is a proprietary extract of *Camellia sinensis* Kuntze and is use as a treatment for genital and perianal warts (Austin, 2006). Fulyzaq is a proprietary extract of the blood-red latex of *Croton lechlerii* Müll and is a novel indication for HIV-associated diarrhea (Austin, 2013). In use, the procedure of new drug application for herbal medicinal product is almost same as of chemical medicines (FDA, 2016). It is difficult to be approved because it would be difficult to manufacture herbal medicinal products assured their efficacy at the same level as chemical medicines.

1.2 Quality control of herbal medicinal product

International conference on harmonization of technical requirements for registration of pharmaceuticals for human (ICH) guides following four categories of quality, safety, efficacy, and multidisciplinary to medicinal manufacturing. Herbal medicinal product manufacturing complied with the guides of ICH would be required, too.

However, quality control of herbal medicinal products like Kampo medicines are difficult than chemical medicines because most of their raw materials of herbal medicinal products are made from natural products that are a part of plant, animal, fungi, or mineral. Qualities of natural products are affected by species used, cultivation, processing, storage, and/or shipping conditions and methods, *etc.*

Quality standards of over one hundred of crude drugs are provided by the Japanese Pharmacopoeia (Ministry of health, labour and welfare in Japan; MHLW, 2016a). The Japanese Pharmacopoeia prescribes origin (species, part, guiding component content), description,

identification (chemical test), purity, total ash, and acid-insoluble ash. Some drug companies use crude drugs complied with the Japanese Pharmacopoeia as ingredients of Kampo medicines in Japan.

Additionally, currently good agricultural and collection practice (GACP) is applicable as a production process control of botanical raw materials of herbal medicinal products.

1.3 Species identification of botanical raw materials

Medicinal use of correct species prescribed by national/regional regulations like the Japanese pharmacopoeia (MHLW, 2016a) is the most important. In 2004, the side effects caused by the misuse of the crude drugs with some resemblances of the crude drug names was informed (MHLW, 2014). *Aristolochia manshuriensis* was used instead of *Akebia quinata* or *A. trifoliata* as crude drug Mutong (木通, written in Chinese character), *Aristolochia fangchi* was used instead of *Sinomenium actum* as Fungji (防已, written in Chinese character), or *Aristolochia yunnanensis* was used instead of *Saussurea lappa* as Muxiang (木香, written in Chinese character). *Aristrolochia* species contain aristolochic acid causing the *Aristolochia* nephropathy. Herbal medicinal product produced from wrong species might have not only the medicinal effect but also have toxicity. Use of correct species as a raw material is the pharmaceutical company's responsibility.

To use correct species as a botanical raw material, accurate species identification is necessary. Until now, species of raw materials were identified mainly by their morphological and chemical characteristics. Accurate species identifications of raw materials are difficult because the raw materials usually made of a part of plant those are dried root, rhizome, or leaf. They have little useful information for species identification. It is hard to identify species from the root morphology or chemical constituents because related species may have the similar root morphology or chemical constituents.

1.4 Species identification using molecular markers for quality assurance of botanical raw materials

In the 1980s, the molecular phylogeny has progressed at rapid speed with DNA analysis technology. Coupled with that, the DNA sequence data of many organisms was accumulated and genetic polymorphisms were observed between species, variety, or cultivar that are minimum units of taxon. In these situation, it was considered that the accurate species identification would be possible comparing the species-specific DNA polymorphisms obtained by using molecular makers.

Therefore, in following 6 issues concerning the quality assurances of botanical raw materials, availabilities of the species identifications using molecular makers were evaluated.

Identification of scientific name of medicinal plant

Taxonomic position and scientific name of medicinal plant should be recognized accurately to understand medicinal properties of the medicinal plant.

Cnidium officinale is cultivated for medicinal uses in Japan and called "Senkyu (川芎, written in

Chinese character)" in Japanese. Their rhizomes are used for a crude drug for sedative and analgesic effects, *etc.* While, in China, *Ligusticum chuanxiong* called "Chuanxiong (川芎, written in Chinese character)" was used medicinally. *Cnidium officinale* is described only in the Japanese Pharmacopoeia (MHLW, 2016c), *Ligusticum chuanxiong* is only in the Chinese Pharmacopoeia (Chinese Pharmacopeia Commission, 2015a).

In taxonomic literatures, the medicinal plant "Senkyu" was first recognized as *Conioselinum* sp. by Yabe (1902). Makino (1908) described it as *Cnidium officinale* without stating any reason of classifying in the genus *Cnidium*. Hara (1954) suggested that *C. officinale* should be recombined to another genus but without mentioning about any appropriate genus. Kitagawa (1963) classified *C. officinale* in the genus *Ligusticum* because the gross morphology of *C. officinale* was similar to *Ligusticum jeholense* Nakai *et* Kitag. Suk *et al*. (1974) classified *C. officinale* to the genus *Conioselinum* based on their comparative anatomy of unripe fruit. Hatano *et al*. (1970) suggested that *C. officinale* was a diploid of hybrid origin between two allied species by their karyotype of the somatic chromosomes and meiosis in pollen mother-cells. Additionally, *C. officinale* and *L. chuanxiong* approximately had the same metabolic profiling (Kobayashi *et al.*, 2012). The discordance in taxonomic position of *Cnidium officinale* is mainly because no ripe fruit of *C. officinale* has been observed. Morphology of ripe fruit is the most important characters for delimiting genera of *Apiaceae*.

In this thesis, *rbc*L nucleotide sequences was used to infer taxonomic position of *Cnidium officinale*. Additionally, to identify the parent species of *C. officinale*, the multiple clones of internal transcribed spacer on nuclear ribosomal DNA (ITS) in an individual plant of *C. officinale* was obtained by cloning, sequenced, and compared with the ITS sequences of related species.

Species identification for botanical raw material consisting of multiple origins

In the Japanese Pharmacopoeia (MHLW, 2016a), many crude drugs consist of multiple origins. That means that some kinds of related species are possible to use as the same crude drug. Qualities of botanical raw materials would be different by species in detail.

Licorice（甘草） is one of them. Licorice consists of three species of *Glycyrrhiza uralensis*, *G. glabra*, and *G. inflate* (Chinese Pharmacopeia Commission, 2015c). Additionally, the various combinations of hybrids among *Glycyrrhiza* species have been reported (Ashurmetov, 1996). Although the root morphological studies of these *Glycyrrhiza* species have been reported (Fujita *et al.*, 1951; Zeng *et al.*, 1988), as a fact, it was difficult to identify species based on the morphologies of dried roots or rhizomes used as crude drugs.

In this thesis, to identify accurately original species of licorice, the four kinds of DNA regions of ITS, *rbc*L, maturase K gene for lysine (*mat*K), and *trn*H– *psb*A intergenic region (trnH: tRNA histidine gene, psbA: protein D1 code gene of Photosystem II) were researched. Species and hybrids were identified and mapped in China by the genotypes obtained from the research. Additionally,

relationships among medicinally used *Glycyrrhiza* species were presumed by their phylogenic analysis.

Confirmation of chemical properties of botanical raw materials identified by molecular markers

Understanding chemical properties of botanical raw materials by original species were essential for quality assurance of herbal medicinal product manufacturing. Then, for licorice, availability of chemical properties presumed by species identified using molecular markers for the quality assurance was evaluated.

Constituent properties of licorices have been reported about eleven kinds of constituent contents for the licorices on the markets (Yoneda *et al.*, 1990; Yoneda *et al.*, 1991), the high performance liquid chromatography (HPLC) patterns of licorices cultivated in botanical garden in Japan (Shibano *et al.*, 1996; Kitagawa *et al.*, 1998), and species-specific constituents of glycycoumarin, glabridin, or licochalcone A (Shibata and Saitoh, 1978; Hatano *et al.*, 1991; Hayashi *et al.*, 2000).

In this thesis, to confirm chemical properties of medicinally used *Glycyrrhiza* species identified from the above genotypes, six kinds of main constituent contents and three species-specific constituent contents of 205 licorices were researched by each original species.

Historical study of an important crude drug

The licorice root stored in Shosoin (正倉院, written in Chinese character) since 756AD is a national treasure. The Shosoin-licorice was investigated morphologically at the first scientific investigation of Shosoin-medicaments (Fujita *et al.*, 1955), and was assigned to *Glycyrrhiza glabra* var. *glandulifera*. As an appendix of the first investigation program of Shosoin-medicaments, one of the present authors performed the HPLC analysis of Shosoin-licorice to identify its characteristic species-specific flavonoid constituents (Shibata, 1991). However, the result of HPLC analysis was not so satisfactory, since the species-specific flavonoids in Shosoin-licorice were missing during the storage of more than 1250 years.

In this thesis, species identification of the Shosoin-licorice was attempted based on species identification and confirmation of chemical properties for licorice above.

Development of an easier discriminating method

To conduct species identification using molecular markers, high-priced device like DNA sequencer is used. To popularize species identification using molecular markers, an easier discrimination method without a high-priced DNA sequencer were required as a purity test to stop commingling of *Atractylodes lancea* rhizome (蒼朮, written in Chinese character) with *Atractylodes* rhizome (白朮, written in Chinese character) on the Japanese Pharmacopoeia (MHLW, 2016b).

Discrimination between *Atractylodes* rhizome and *Atractylodes lancea* rhizome using molecular markers of ITS or *trn*K have been reported (Cheng *et al.*, 1997; Mizukami *et al.*, 2000). This purity test based on the amplification refractory mutation system (ARMS) detected the differences of nucleotide sequences on ITS among the medicinal *Atractylodes* species (Guo *et al.*, 2006). The ARMS

required rigorous experimental condition comparatively because the ARMS detected the nucleotide substitution by existence or nonexistence of polymerase chain reaction (PCR) products using the specific mismatch primer.

In this thesis, to discriminate between *Atractylodes* rhizome and *Atractylodes lancea* rhizome, PCR restriction fragment length polymorphism (PCR-RFLP) as a simple, quick, and stable method was attempted.

Exclusion of fake raw material

Fake raw materials would be distributed on the market or mixed in a lot of raw materials (MHLW, 2014). Discrimination and exclusion of fake raw materials are essential for quality assurance of herbal medicinal manufacturing. Crude drugs of Tiannanxing (天南星, written in Chinese character) resembles Banxia (半夏, written in Chinese character) derived from *Araceae* species closely and sometimes distributes as Banxia or is mixed within Banxia on the markets.

In this thesis, discrimination between Banxia and Tiannanxing by their *rbc*L sequences and exclusion of Tiannanxing from Banxia were attempted.

1.5 Operating procedure of species identification on GACP or GMP

Species identification for botanical raw materials should be examined systematically at suitable timings on GACP or good manufacturing practice (GMP). Therefore, operating procedure of the species identifications mentioned above on GACP or GMP in herbal medicinal product manufacturing was considered. Especially, GACP as production process control is important because botanical raw materials are natural product and their qualities would be strongly affected from the production process. Additionally, Japanese pharmaceutical companies would have little GACP experiences but have many GMP experiences. They would be currently at the phase of thinking about a way to carry out GACP control.

In this thesis, the international guides concerning GACP were reviewed at first. Next, a suitable GACP was proposed based on the reviews. Finally, operation procedure of the species identification on the suitable GACP or GMP was proposed.

1.6 Objectives of this thesis

One of the purpose of this thesis is availability assessments for the molecular makers using species identification.

Next purpose is availability assessments for the species identification using molecular markers for herbal medicinal product manufacturing based on the studies of the following 6 issues. The 6 issues were; (1) identification of scientific name of medicinal plant, (2) species identification for botanical

raw material consisting of multiple origins, (3) confirmation of chemical properties of botanical raw materials identified by molecular markers, (4) historical study of an important crude drug, (5) development of an easier discriminating method, (6) exclusion of fake raw material.

Last purpose is reviewing of GACP and operation procedure of the species identification on GACP or GMP for herbal medicinal product manufacturing.

Chapter 2

Identification of scientific name of medicinal plant using molecular makers

Phylogenetic relationship of medicinally important *Cnidium officinale* and Japanese *Apiaceae* based on *rbc*L sequences and ITS cloning analysis

2.1 Introduction

Cnidium officinale Makino is cultivated for medicinal uses in Japan and called "Wa-Senkyu (和川芎, written in Chinese character)" in Japanese. Their rhizomes are used for a crude drug for sedative and analgesic effects, *etc.* Senkyu produced about 400 t every year in Japan and are important both medicinally and economically. Japanese botanists considered that *C. officinale* was introduced in Japan from China (Kimura *et al*., 1961). But, *C. officinale* is not listed in current Chinese flora (Chang, 1985). In China, a species resembled *C. officinale* is cultivated for medicinal uses, too. Those scientific name is *Ligusticum chuanxiong* Hort. ex Qiu *et al*. (Qiu *et al*., 1979) and called the same crude drug name "Chuanxiong (川芎, written in Chinese character)" in Chinese or "Kara-Senkyu (唐川芎, written in Chinese character)" in Japanese. As Senkyu or Chuanxiong (川芎, written in Chinese character), *Cnidium officinale* is described only in the Japanese Pharmacopoeia (MHLW, 2016c), *Ligusticum chuanxiong* is only in the Pharmacopoeia of the People's Republic of China (Chinese Pharmacopeia Commission, 2015a).

In taxonomic literatures, the medicinal plant "Senkyu" was first recognized as *Conioselinum* sp. by Yabe (1902). Makino (1908) described it as *Cnidium officinale* without stating any reason of classifying in the genus *Cnidium*. Hara (1954) suggested that *C. officinale* should be recombined to another genus but without mentioning about any appropriate genus. Kitagawa (1963) classified *C. officinale* in the genus *Ligusticum* because the gross morphology of *C. officinale* was similar to *Ligusticum jeholense* Nakai *et* Kitag. which was distributed in the northeast China but the two-species had the different morphologic characteristics in petal, involucral bract, and hair on leaflet. Suk *et al.* (1974) classified *C. officinale* to the genus *Conioselinum* based on their comparative anatomy of unripe fruit. Hatano *et al*. (1970) suggested that *C. officinale* was a diploid of hybrid origin between two allied species by their karyotype of the somatic chromosomes and meiosis in pollen mother-cells.

Accurate identification of the taxonomic position and the scientific name of a crude drug is essential to understand its pharmacological feature. About *Cnidium officinale*, two crude drugs having the same name "Senkyu (川芎, written in Chinese character)" and approximately the same metabolic profiling (Kobayashi *et al.* 2012) in Japan and China were classified into the two genera of *Cnidium*

or *Ligusticum*. The clarification of taxonomic position of medicinally important *C. officinale* and the taxonomic relationship of *C. officinale* and *L. chuanxiong* is important pharmacologically.

The discordance in taxonomic position of *Cnidium officinale* is mainly because no ripe fruit of *C. officinale* has been observed. Morphology of ripe fruit is the most important characters for delimiting genera of *Apiaceae*. To identify the taxonomic position of *Cnidium officinale*, it is necessary to explore any other taxonomic characters instead of the fruit morphology.

In the 1990s, nucleotide sequence polymorphisms of the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase gene (*rbc*L) have been employed for phylogenetic analyses in various plant taxa and proved to be useful in clarifying the phylogenetic relationships among genera (Soltis *et al*., 1993; Xiang *et al*., 1993; Morgan *et al*., 1994).

In this study, *rbc*L nucleotide sequences was used to infer taxonomic position of *Cnidium officinale* and phylogenetic relationships of genera of subfamily *Apioideae* where *C. officinale* might belong. The genera in *Apioideae* have been classified into the five tribes based on their fruit morphologies (Yamazaki, 1982). The *rbc*L sequences of representative genera in the five tribes of *Apioideae* including *Cnidium*, *Ligusticum*, and *Conioselinum* where *C. officinale* have been classified were determined and compared with the *rbc*L sequence of *C. officinale*.

Additionally, Liu *et al.* (2002) suggested that the scientific name of *Cnidium officinale* should be changed to *Ligusticum chuanxiong* in their phylogenetic study based on ITS and *mat*K. ITS from nuclear nrDNA was multicopy and inherited parentally (Álvarez *et al.*, 2003). *Cnidium officinale* presumed being a diploid hybrid (Hatano *et al.*, 1970) might have two kinds of the parental ITS in an individual plant. Therefore, in this study, to identify the parent species of *C. officinale*, the multiple ITS clones in an individual plant of *C. officinale* was obtained by cloning, sequenced, and compared with the ITS sequences of related species.

2.2 Materials and methods

*rbc*L *nucleotide sequencing*

The *rbc*L sequences of 30 species which represent the 24 genera of the three subfamilies; *Hydrocotyloideae*, *Saniculideae*, and *Apioideae* in *Apiacea* were determined (Table 2.1). *Apioideae* included the five tribes; *Scandiceae*, *Symrnieae*, *Amineae*, *Peucedanaeae*, and *Dauceae* recognized by Yamazaki (1982). In addition, the *rbc*L sequences of one species each of four genera in *Araliaceae* and one species each in *Pittosporaceae*, *Cornaceae*, and *Caprifoliaceae* were determined as outgroups based on the following considerations. Takhtajan (1980) and Cronquist (1988) considered that *Araliaceae* was a sister group of *Apiaceae*. Judd *et al*. (1994) pointed out the close relationship between *Pittosporaceae* and the *Araliaceae*/*Apiaceae* Complex. According to the phylogenetic

research using the *rbc*L sequence data by Chase *et al*. (1993), the genus *Corokia* in *Cornaceae* and *Viburnum* in *Caprifoliaceae* are clustered with *Apiaceae*. These *rbc*L sequences data of *Corokia cotoneaster* and *Viburnum acerifolia* deposited in DDBJ nucleotide sequence database were used. The other 35 *rbc*L sequences were newly determined in this study.

Total DNA was extracted from fresh leaves as described by Doyle *et al.* (1987). The two overlapping DNA fragments (A and B in Fig. 2.2) covering most of the *rbc*L gene were amplified by the polymerase chain reaction using the primers A-F and A-R for fragment A, and B-F and B-R for fragment B (Table 2.2). These fragments were sequenced using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and 373A DNA sequencer (Applied Biosystems Division, PERKIN ELMER). For the sequencing, the twelve internal primers were used (Fig. 2.2, Table 2.2). These primers were synthesized based on the *Nicotiana tabacum rbc*L gene (Shinozaki *et al.*, 1986) and were modified specifically for *Apiaceae*.

Phylogenetic analysis was performed on 1224 base pairs of the *rbc*L gene between 58 and 1281 from the start codon of *Nicotiana tabacum rbc*L gene using Phylogenetic analysis using parsimony (PAUP) version 3.1.1 (Swofford, 1993).

The phylogenetic analysis was conducted through a heuristic search with the tree bisection reconnection (TBR) branch swapping and MULPARS option. Multiple islands of equally most parsimonious trees were searched heuristically with 500 random sequence additions and a strict consensus tree of the most parsimonious trees obtained was constructed. The consistency index (Kluge *et al.*, 1969) and the retention index (Farris, 1989) for a strict consensus tree were calculated.

To evaluate confidences for clustering groups in a strict consensus tree, the bootstrap analysis (Felsenstein, 1985; Hillis *et al.*, 1993) and the decay analysis (Chase *et al*., 1993; Morgan *et al*., 1994) were conducted. The bootstrap and decay analyses were conducted for the representative 30 taxa because the taxa except these 30 taxa always clustered with the one of 30 taxa in the preliminary analyses. The bootstrap analysis was conducted through a heuristic search with TBR branch swapping, MULPARS, and 1,000 bootstrap resampling. The decay analysis was conducted through a heuristic search with TBR branch swapping, MULPARS, and 500 random sequence additions and confirmed trees having one or two steps longer variables than the most parsimonious trees.

ITS cloning analysis

Cnidium officinale, *Ligusticum chuanxiong*, and six species that referred to the relations with *C. officinale* or *L. chuanxiong* in the previous *rbc*L sequence research were used for the ITS cloning analysis (Table 2.3). Total DNA was extracted using the DNAeasy Plant Mini Kit (Qiagen) from the dried leaf of an herbarium specimen. Fourteen of cloned ITS were obtained respectively from *Cnidium officinale* or *Ligusticum chuanxiong* each by the pT7Blue Perfectly Blunt® Cloning Kit (Novagen). The cloned ITS of *C. officinale* or *L. chuanxiong* and the ITS of related species were amplified using

the universal primers by White *et al.* (1990). The PCR reaction mixture was: 10xEx-Buffer (TaKaRa) 5µl, dNTP mix (TaKaRa) 5µl, forward primer (10 pmol/µl), 1.0µl, reverse primer (10 pmol/µl) 1.0µl, template DNA 1.25µl, Ex-Taq (TaKaRa) 0. 25µl, DMSO 5µl, D.D.W. 31.5µl. The PCR cycle was: (94℃, 1 min; 48℃, 2 min; 72℃, 3 min) x 30 cycles, (72℃, 7 min) x 1 cycle. The amplified DNA was purified by the GFX® PCR DNA and Gel Band Purification Kit (Amersham biotech). The purified DNA was sequenced by the BigDye Terminator Cycle Sequencing Kit ver.2.0 and using the Model 3100 automated sequencer (Applied Bio Systems). Based on the ITS sequences obtained, a strict consensus tree of the most-parsimonious trees of equal length were computed after a heuristic search of trees using the TBR and MULPARS options. (Swofford, 1993).

2.3 Results

Phylogenetic relationships of Apiaceae species based on the rbcL sequences

Phylogenetic analysis resulted in 420 most parsimonious trees with 360 steps with relatively high values of the consistency index of 0.67 and the retention index of 0.75. The strict consensus tree of the 420 trees was illustrated in Fig. 2.3. The heuristic searches for the bootstrap and decay analyses resulted in 24 most parsimonious trees with 235 steps. For the decay analyses, 236 trees with 236 steps or 27,902 trees with 237 steps were found respectively. The bootstrap percentage (b) and decay index (d) were indicated in the strict consensus tree of the 24 most parsimonious trees with 235 steps (Fig. 2.4).

In the strict consensus trees (Figs. 2.3 and 2.4), subfamily *Apioideae* clustered to a monophyletic group. The monophyly of subfamily *Apioideae* were supported with the high confidence indices (b = 100, d >= 3). In the cluster of subfamily *Apioideae*, the three groups were recognized and were compared with the tribes classified by Yamazaki (1982). The first group in the subfamily *Apioideae* consisted of *Pleurospermum*. This group corresponded to the tribe *Smyrnieae sensu*. The second group consisted of *Bupleurum* species with the high confidence indices (b = 100, d >= 3). *Bupleurum* has simple leaves with parallel venations which is a unique characteristic in *Apiaceae*. *Bupleurum* was classified in tribe *Ammineae*. The last group consisted of the other *Apioideae* species with the low confidence indices (b = 54, d = 1).

In the last group of the subfamily *Apioideae*, the following three subgroups were recognized with the middle level of confidence indices (b = 65 or 53, d = 2). The first subgroup consisted of the four genera of *Cryptotaenia*, *Sium*, *Cicuta*, and *Oenanthe* were classified in tribe *Ammineae*. The second subgroup consisted of the eight genera classified in the four different tribes; (1) *Osmorhiza* and *Torilis* in tribe *Scandiceae*, (2) *Ostericum* in tribe *Peucedanaeae*, (3) *Pternopetalum*, *Spuriopimpinella*, *Tilingia*, and *Ligusticum scoticum* in tribe *Ammineae*, and (4) *Daucus* in tribe *Dauceae*. The last subgroup consisted of the 12 genera classified in the three different tribes; (1) *Heracleum*,

Conioselinum, *Peucedanum*, *Angelica*, *Glehnia*, and *Coelopleurum* in tribe *Peucedanaeae*, (2) *Foeniculum*, *Chamaele*, *Libanotis*, *Ligusticum sinense*, *Ligusticum jeholense* and *Cnidium officinale* in tribe *Ammineae*, and (3) *Anthriscus* in tribe *Scandiceae*.

The strict consensus trees based on the *rbc*L sequences supported monophyly of subfamily *Apioideae* (Drude, 1898). The tribal classification of East Asian *Apioideae* (Table 2.1) have been accepted by several taxonomists (Kitagawa, 1982; Yamazaki, 1982; Pimenov, 1987), except Shan (1979) who applied *Scandicineae* (De Candole, 1830) instead of *Smyrnieae* and *Scandiceae* (Koch 1824). In the monophyletic *Apioideae*, the clustering based on the *rbc*L sequences was discordant with the traditional tribal classification based mainly on their fruit morphologies and illustrated polyphylies of tribes *Scandiceae*, *Ammineae*, and *Peucedanaeae*.

Taxonomic position of Cnidium officinale based on the rbcL sequences

In the strict Consensus tree based on the *rbc*L sequences (Figs. 2.3 and 2.4), *Cnidium officinale* clustered with *Ligusticum chuanxiong*, *L. sinense* (藁本, written in Chinese character), and *L. jeholense* (遼藁本, written in Chinese character) distributed in China. *Cnidium officinale* had the same *rbc*L sequence of *L. chuanxiong* and there is only one base substitution of the *rbc*L sequence between *C. officinale* and *L. sinense* or *L. jeholense*. On the other hand, *Cnidium officinale* did not cluster with *Cnidium japonicum* or *Conioselinum* species where Suk *et al*. (1974) classified *C. officinale* based on their unripe fruit morphology.

The results suggested that *Cnidium officinale* had close relationships to the Chinese *Ligusticum* species, especially to *L. chuanxiong*. When it was assumed that *Cnidium officinale* belonged to *Cnidium* or *Conioselinum*, the length of the most parsimonious trees increased six or three steps, respectively. Hence it is difficult to accept that *Cnidium officinale* is classified in *Cnidium* or *Conioselinum*.

ITS cloning analysis of Cnidium officinale

The 14 of ITS clones obtained from an individual plant of *Cnidium officinale* or *Ligusticum chuanxiong* respectively, and ITS of the six kinds of related species were sequenced (Fig. 2.5). The ITS clones of *C. officinale* and *L. chuanxiong* showed approximately the same clustering feature and had mainly two kinds of genotypes in the strict consensus tree based on the ITS sequences (Fig. 2.6). The ITS clones of *C. officinale* or *L. chuanxiong* having the two genotypes divided into the two clusters by each genotype. One of them clustered with *Ligusticum jeholense*, the other clustered with *Ligusticum sinense*. Within the *L. jeholense* cluster, 0-2 substitutions in the ITS sequences were observed between *C. officinale* and *L. chuanxiong*, and 1-2 substitutions between *L. jeholense* and *C. officinale*, or between *L. jeholense* and *L. chuanxiong*. Within the *L. sinense* cluster, 0-2 substitutions in the ITS sequences were observed between *C. officinale* and *L. chuanxiong*, and 0-5 substitutions between *L. sinense* and *C. officinale*, or between *L. sinense* and *L. chuanxiong*.

Cnidium officinale or *L. chuanxiong* presumed that they were diploid hybrids (Hatano *et al.*, 1970). They had the two ITS genotypes each in an individual plant. One ITS genotype was similar to the ITS genotype of *L. jeholense*, the other was similar to *L. sinense*. It suggested that *C. officinale* or *L. chuanxiong* would be hybrid between *L. jeholense* and *L. sinense*, or their closely related species.

Ligusticum sinense and *L. jeholense* are distributed in China and used as the crude drugs "Gaoben (藁本, written in Chinese character)" and "Liao-Gaoben (遼藁本, written in Chinese character)", respectively (Fig. 2.1). *Cnidium officinale* or *Ligusticum chuanxiong* might be new medicinal plants generated by artificial hybridizations between the existing medicinal plants.

2.4 Discussion

Taxonomic position and Scientific name of Cnidium officinale

The strict consensus tree based on the *rbc*L sequences (Fig. 2.3) showed that *Cnidium officinale* related closely with *Ligusticum chuanxiong*, *L. sinense*, and *L. jeholense*. Additionally, the ITS cloning analysis of *C. officinale* or *L. chuanxiong* suggested that the two species would be hybrid between *L. jeholense* and *L. sinense*, or their closely related species. In these results, it was reconfirmed genetically that *C. officinale* related closely to *L. chuanxiong*. This conclusion agreed with Kitagawa (1963) who renamed *Cnidium officinale* Makino to *Ligusticum officinale* (Makino) Kitagawa, but neither with Yabe (1902) and Suk *et al.* (1974) who classified it in *Conioselinum*, nor with Makino (1908) who classified it in *Cnidium*.

However, Liu *et al.* (2002) suggested that the scientific name of *Cnidium officinale* should be changed to *Ligusticum chuanxiong*, *C. officinale* should be renamed *L. officinale* but not *L. chuanxiong* because some morphological, chemical, and genetically differences were observed between *C. officinale* and *L. chuanxiong*.

On the other hand, *Ligusticum scoticum* did not cluster with *Ligusticum chuanxiong*, *L. sinense*, *L. jeholense* distributed in China but with genus *Ostericum*, *Pternopetalum*, *Spuriopimpinella*, *Tilingia*, *Daucus*, and *Torilis* (Fig. 2.2). The genus *Ligusticum* was polyphyletic. It was considered that the genus name of *Ligusticum chuanxiong*, *L. sinense*, *L. jeholense* should be changed because *Ligusticum scoticum* is the type species of the genus *Ligusticum* (Linnaeus, 1753) and *L. scoticum* has taxonomical priority using the genus name of *Ligusticum*.

From the *rbc*L sequence and ITS cloning analyses, the aspects of medicinally important *Cnidium officinale* were defined that *C. officinale* did not belong to genus *Cnidium* but to genus *Ligusticum* and had close relationship to *L. chuanxiong* with the almost same hybridized origin. This definition should be referred when the crude drug Senkyu or Chuanxiong（川芎）were used in Japan or China.

Table 2.1. Plant materials for the *rbc*L sequencing. Subfamilial and tribal classifications of *Apiaceae* were based on Yamazaki (1982). The *rbc*L sequence data from D44552 to D44590 were deposited in the DDBJ, EMBL and NCBI nucleotide sequence databases.

species	source, voucher, and DNA accession number
Apiacea	
Hydrocotyloideae	
Centella asiatica (L.) Urban	Matsuzaki, Shizuoka, Japan; THS36331; D44559
Hydrocotyle sibthorpioides Lam.*	Naraha, Fukushima, Japan; THS38155; D44570
Saniculideae	
Sanicula chinensis Bunge	Mt. Yamizo, Ibaraki, Japan; THS36340, D44585
Apioideae	
Scandiceae	
Anthriscus aemula Schischkin*	Mt. Yamizo, Ibaraki, Japan; THS36339; D44554
Osmorhiza aristanata Makino *et* Yabe*	Matsuzaki, Shizuoka, Japan; THS36335; D44578
Torilis japonica (Houtt.) DC.	Shimazuma, Ibaraki, Japan; THS36337; D44590
Smyrnieae	
Pleurospermum camtschaticum Hoffm.*	Souya, Hokkaido, Japan; THS37755; D44583
Amineae	
Bupleurum fruticosum L.*	Cult. at Tsumura & Co.; THS38161; D44556
Bupleurum longeradiatum Turcz. subsp. *sachalinense* Kitag. var. *elatius* Kitag.*	Geibikei, Iwata, Japan; THS37853; D44557
Bupleurum scorzonerifolium Wild. var. *stenophyllum* Nakai*	Cult. at Tsumura & Co.; THS38157; D44558
Chamaele decumbens Makino*	Odaka, Fukushima, Japan; THS36352; D44560
Cicuta virosa L.*	Hanazono, Ibaraki, Japan; THS36355; D44561
Cnidium japonicum Miq.*	Hitachi, Ibaraki, Japan; THS36366; D44562
Cnidium officinale Makino*	Cult. at Tsumura & Co.; THS38159; D44586
Cryptotaenia japonica Hassk.*	Oshino, Yamanashi, Japan; THS36686; D44565
Foeniculum valgare Mill.*	Cult. at Tsumura & Co.; THS38158; D44567
Libanotis coreana Kitag. f. *ugoensis* Kitag.*	Kisakata, Akita, Japan; THS35370; D44573
Ligsticum chuanxiong Hort. *ex* Qiu *et al.*	Qingcheng, Sichuan, China; THS38154; D44572
Ligsticum jeholense Nakai *et* Kitag.	Nanjing Botanical Garden, China; D44574
Ligsticum scoticum L. subsp. *hultenii* Hulten*	Hirono, Fukushima, Japan; THS36105; D44575
Ligsticum sinese Oliv.*	Mt. Emei, Sichuan, China; THS3814; D44576
Oneanthe javanica DC.*	Shimozuma, Ibaraki, Japan; THS36337; D44577
Pternopetalum tanakae Hand.-Mazz.	Azumi, Nagano, Japan; THS36369; D44584
Sium serra (Franch. et Savat.) Kitag.*	Hanazono, Ibaraki, Japan; THS36357; D44587
Spuriopimpinella calycina (Maxim.) Kitag.	Okutama, Tokyo, Japan; THS37771; D44588
Tilingia ajanensis Regel	Nenakusa, Gunma, Japan; THS36690; D44589
Peucedanaeae	
Angelica dahurica (Fisch.) Benth. *et* Hook.*	Okutsu, Kumamato, Japan; THS36189, D44553
Coelopleurum gmelinii (DC.) Ledeb.*	Souya, Hokkaido, Japan; THS37757; D44563
Glehnia littoralis Fr. Schm. *ex* Miq.	Hanazono, Ibaraki, Japan; THS36372; D44579
Peucedanum japonicum Thunb. *ex* Murr.*	Matsuzaki, Shizuoka, Japan; THS36332; D44581
Dauceae	
Daucus carota L. var. *sativa* DC.	Matsuzaki, Shizuoka, Japan; THS36694; D44566
Araliaceae	
Aralieae	
Aralia cordata Thunb.*	Ookuma, Fukushima, Japan; THS36681; D44555
Panax japonicas C. A. Mayer*	Ookuma, Fukushima, Japan; THS36651; D44571
Schefflereae	
Acantopanax sciadophylloides Franch. et Savat.*	Hanazono, Ibaraki, Japan; THS36358; D44552
Kalopanax pictus (Thunb.) Nakai*	Odaka, Fukushima, Japan; THS36351; D44571
Caprifoliaceae	
Viburunum acerifolia L.	Olmstead *et al.*, 1992; L01959
Cornaceae	
Corokia cotoneaster Raoul	Xiang *et al.* 1993; L11221
Pittosporaceae	
Pittosporum tobira (Thunb. *ex* Murr.) Aiton	Matsuzaki, Shizuoka, Japan; THS36333; D44582

Abbreviations used: Cult. = cultivated, THS = Tsumura herbarium specimen. Asterisks indicate species used for the bootstrap and decay analyses.

Table 2.2. Locations and base compositions of the amplification and sequencing primers for *rbc*L gene.

Primer	Sequence (5' to 3')	Location
for the DNA fragments A and B amplification		
A-F	ATG TCA CCA CAA ACA GAG ACT AAA GC	1 - 26
A-R	CCC TTT TCA TCA TTT CTT CAC ATG TAC	760 - 734
B-F	TGA AAA CGT GAA TTC CCA ACC GTT TAT GCG	609 - 1360
B-R	CCA TAC TTC ACA AGC AGC AGC TAG TTC	1386 - 1360
for the DNA fragments A and B sequencing		
1F	AAG CAG GTG TTG GAT TCA CAG C	23 - 44
2F	CTT GAT CGT TAC AAA GGG CG	229 - 248
3F	TGG CAT CCA AGT TGA GAG AG	459 - 478
4F	TTC TTA TTT TGT GCC GAA GC	652 - 671
5F	GCA TGC AGT TAT TGA TAG AAC	891 - 910
6F	GTC TCT CTA CCA GGT GTT CT	1087 - 1106
1R	AGA TCA CGC CCC TCA TTA CG	1310 - 1291
2R	GGT GAA ATA AAT ACC GCG AC	1095 - 1076
3R	GAA GTA GGC CAT TAT CTC G	871 - 853
4R	CCC TTT TCA TCA TTT CTT CAC ATG TAC	760 - 734
5R	TTA GGT TTA ATA GTA CAT CCC AAC AG	530 - 505
6R	AGG TCT AAT GGG TAA GCT AC	320 - 301

The location numbers correspond to the location number of *Nicotiana tabacum rbc*L (Shinozaki *et al.*, 1986).

Table 2.3. Plant materials for the ITS cloning analysis. Sixteen ITS were cloned from *Cnidium officinale* or *Ligusticum chuanxiong*, respectively.

Scientific name	Locality	DNA No.
Angelica sinensis (Oliv.) Diels	China	1048
Cnidium officinale Makino (cloned, 01-14)	Hokkaido, Japan; THS897	1036
Conioselinum filicinum (H. Wolff) H. Hara	Fukushima, Japan; THS803	1037
Ligustichum sinensis Oliv. (1)	Sichuan, China; THS39680	1061
Ligustichum sinensis Oliv. (2)	China; THS61723	1125
Ligustichum sinensis Oliv. (3)	Sichuan, China; THS65104	1127
Ligusticum chuanxiong Hort. (cloned, 01-14)	Sichuan, China; THS70994	1047
Ligusticum jeholense (Nakai & Kitag.) Nakai & Kitag. (1)	Jiangsu BG., China; THS462	1039
Ligusticum jeholense (Nakai & Kitag.) Nakai & Kitag. (2)	Liaoning, China; THS63133	1128
Ligusticum pteridophyllum Franchet	Yunnan, China; THS63134	1121
Ligusticum tenuissimum (Nakai) Kitagawa	Liaoning, China; THS949	1031

THS meant Tsumura herbarium specimen. DNA No. was the managed by Dr. H. Yamaji in Tsumura & Co.

Cnidium officinale (和川芎)

Ligusticum chuanxiong (唐川芎)

Ligusticum sinense (藁本)

Ligusticum jeholense (遼藁本)

Figure 2.1. Dried rhizomes of medicinal parts and leaves of *Cniduum officinale*, *Ligusticum chuanxiong*, *L. sinense*, and *L. jeholense*. The plant names written in Chinese characters were indicated in parentheses.

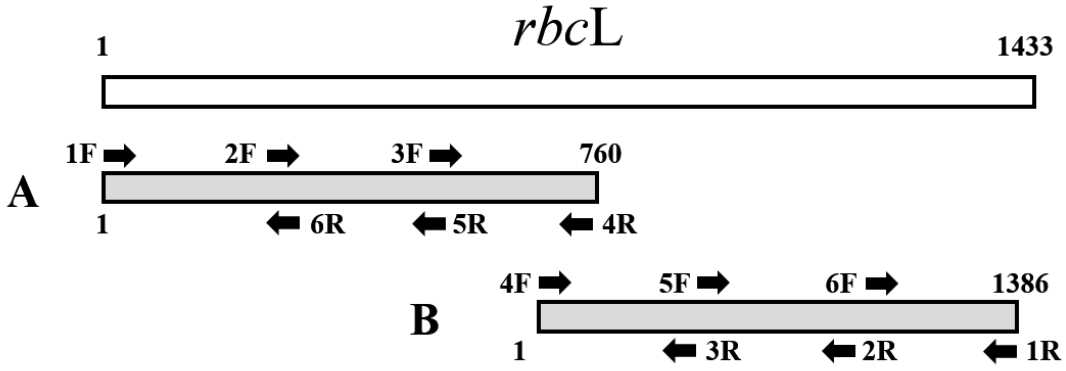


Figure 2.2. Relatevie positions of the twelve sequencing primers for the *rbc*L sequencing. A and B were DNA fragments amplificated for the *rbc*L sequencing. Arrows were the twelve sequencing primers and indicated the direction of strand synthesis.

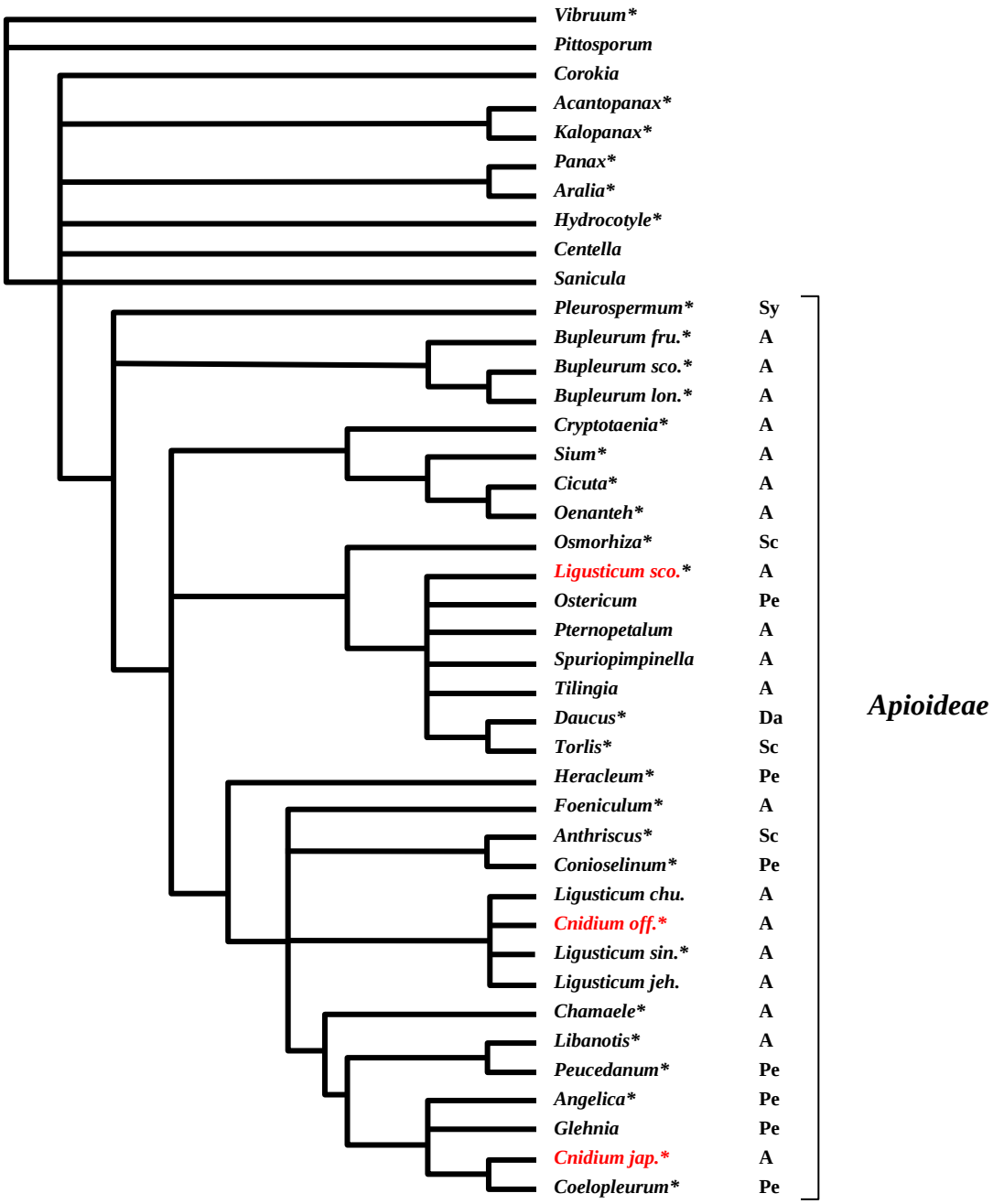


Figure 2.3. Strict consensus tree of 420 most parsimonious trees with 360 steps each resulting from the phylogenetic analyses of *rbc*L sequences. Asterisks indicate species used for bootstrap and decay analyses. The acronyms in right hand side show the tribes of *Apioideae* (Yamazaki, 1982): Sc = *Scandiceae*, Sy = *Symrnieae*, A = *Amminaeae*, Pe = *Peucedanaeae*, and Da = *Dauceae*.

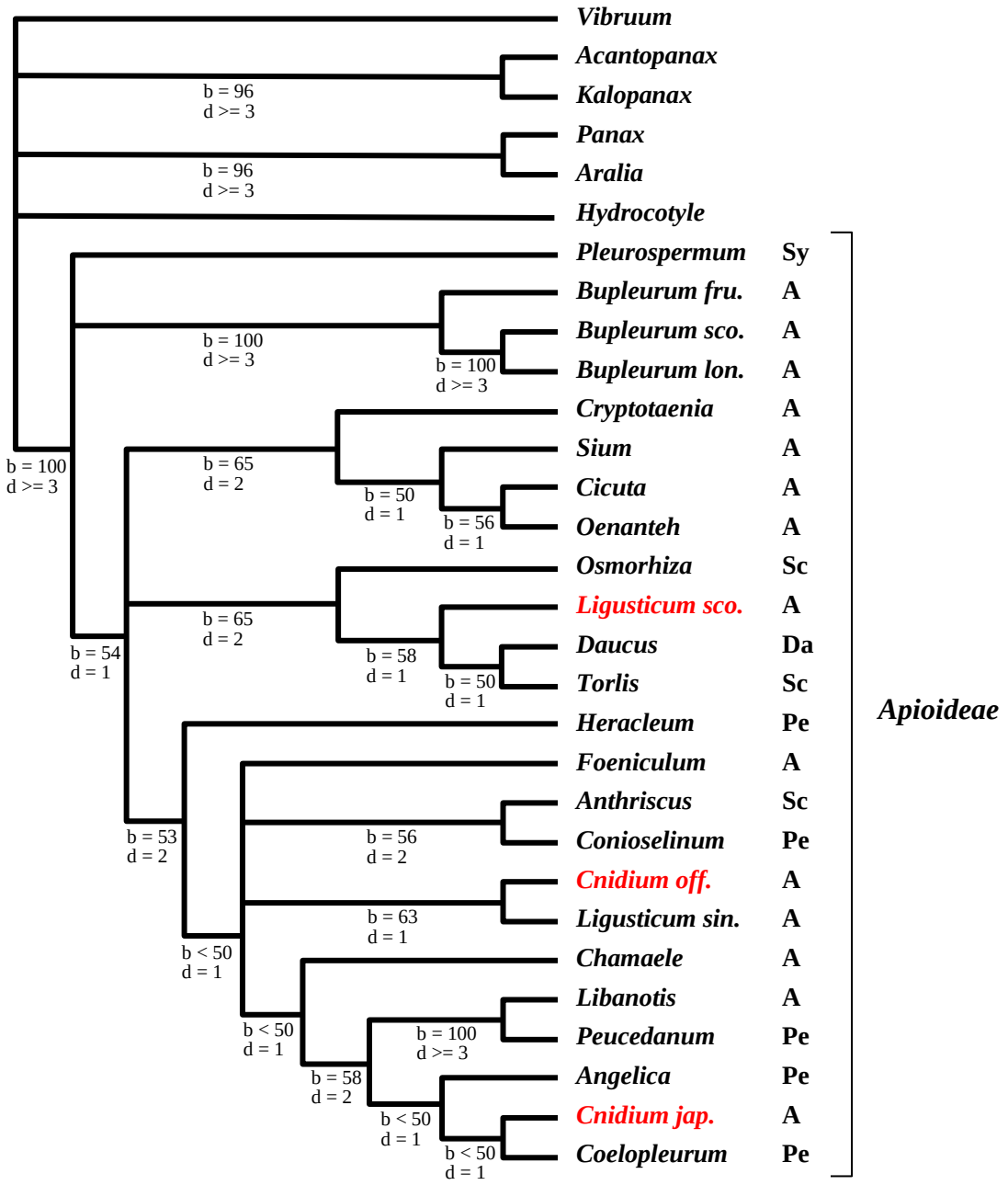


Figure 2.4. Strict consensus tree of 24 most parsimonious tree with 235 steps for 30 taxa. Bootstrap percentage (b) and decay index (d) were indicated below each branch. The acronyms in right hand side mean the tribes of *Apioideae* (Yamazaki, 1982): Sc = *Scandiceae*, Sy = *Symrnieae*, A = *Amminaeae*, Pe = *Peucedanaeae*, and Da = *Dauceae*.

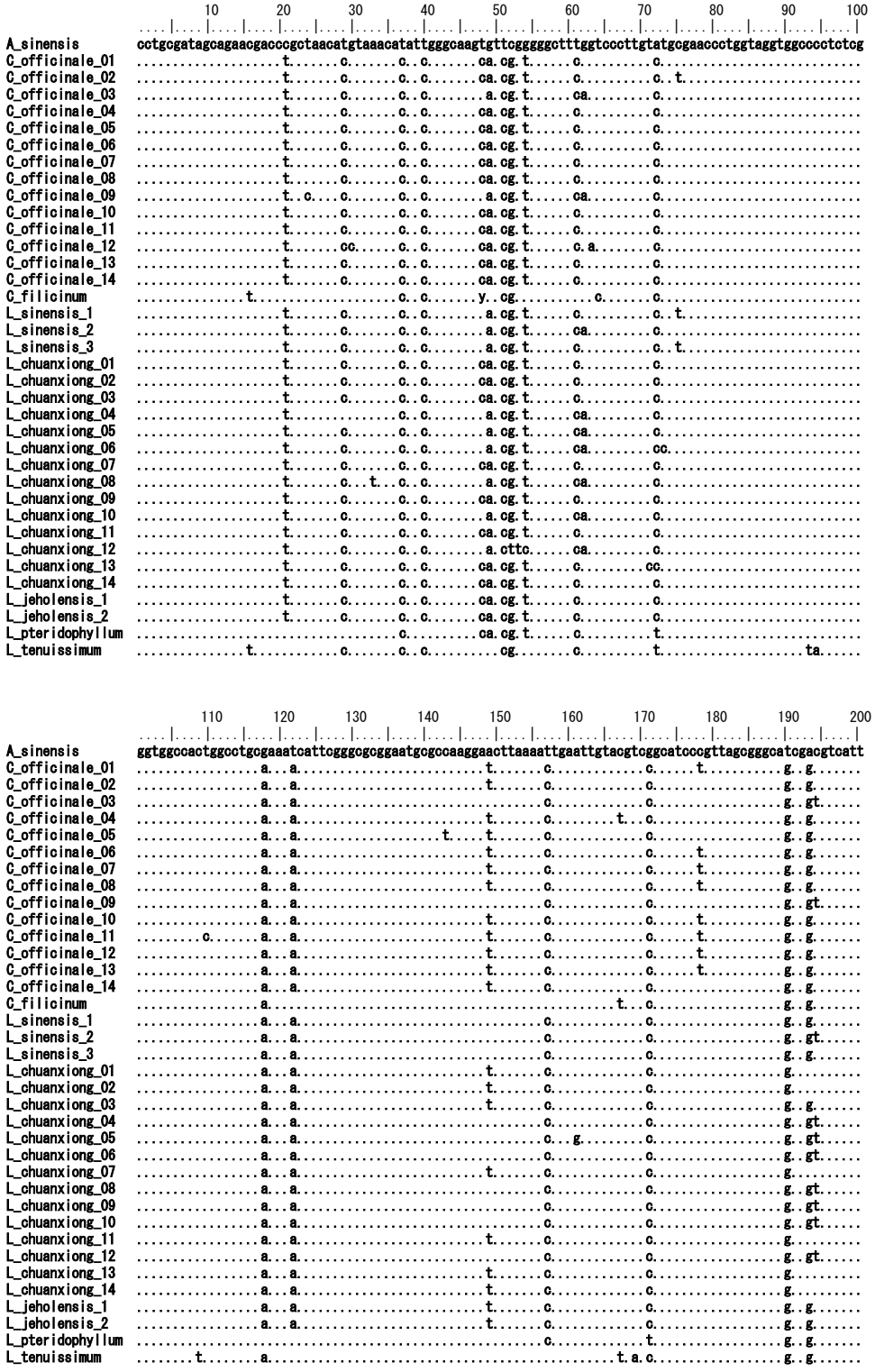


Figure 2.5. ITS sequences of *Cnidium officinale* and related species. The ITS sequences of *Cnidium officinale* (01-14) or *Ligusticum chuanxiong* (01-14) were cloned from the one individual plant, respectively.

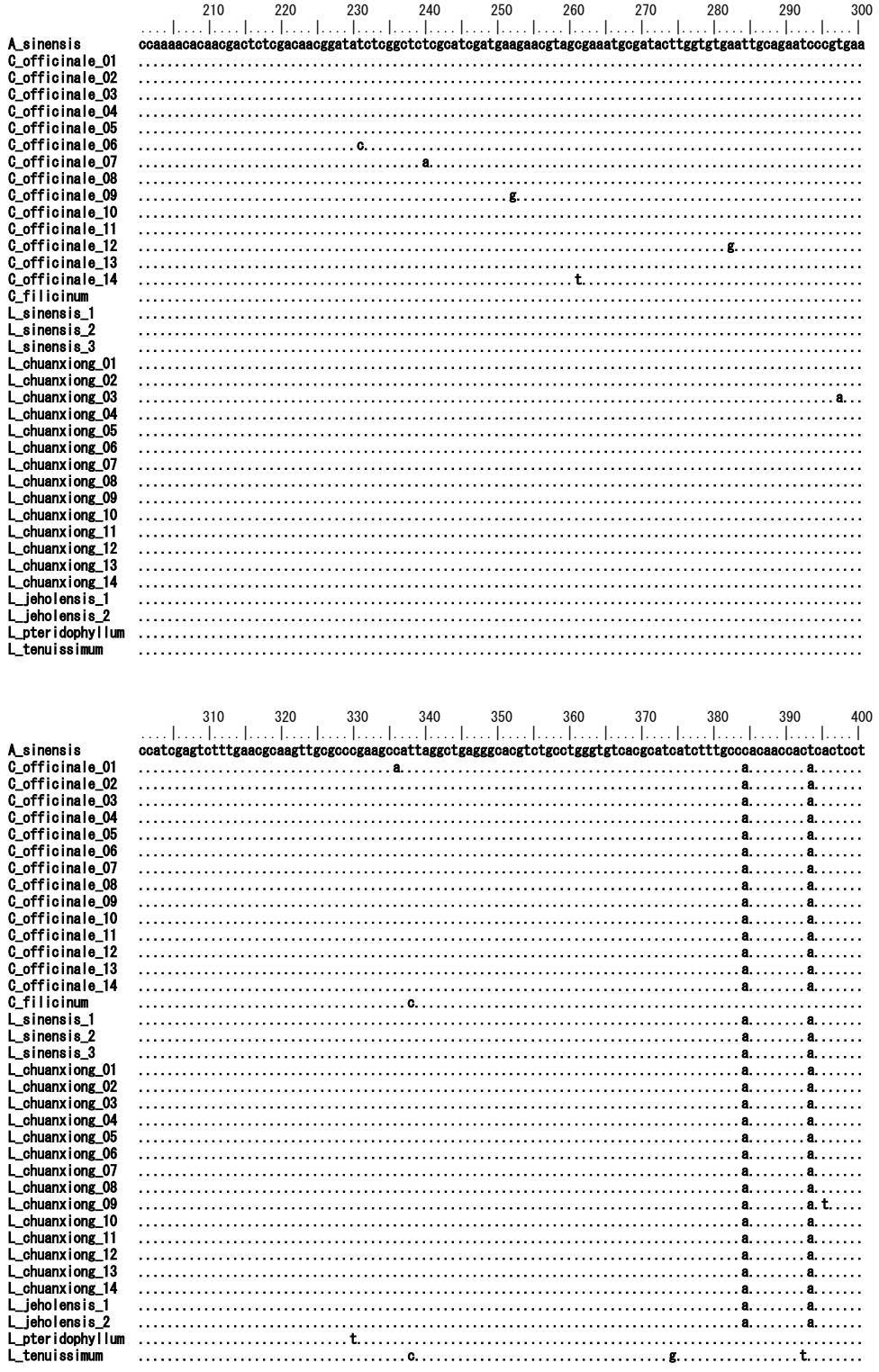


Figure 2.5. Continued.

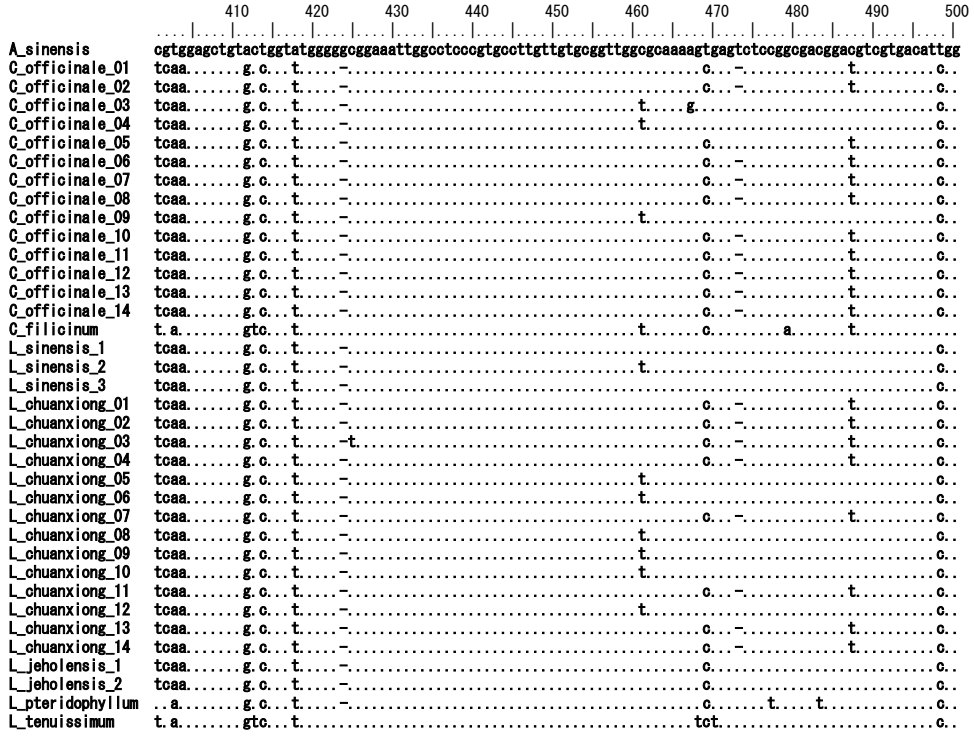


Figure 2.5. Continued.

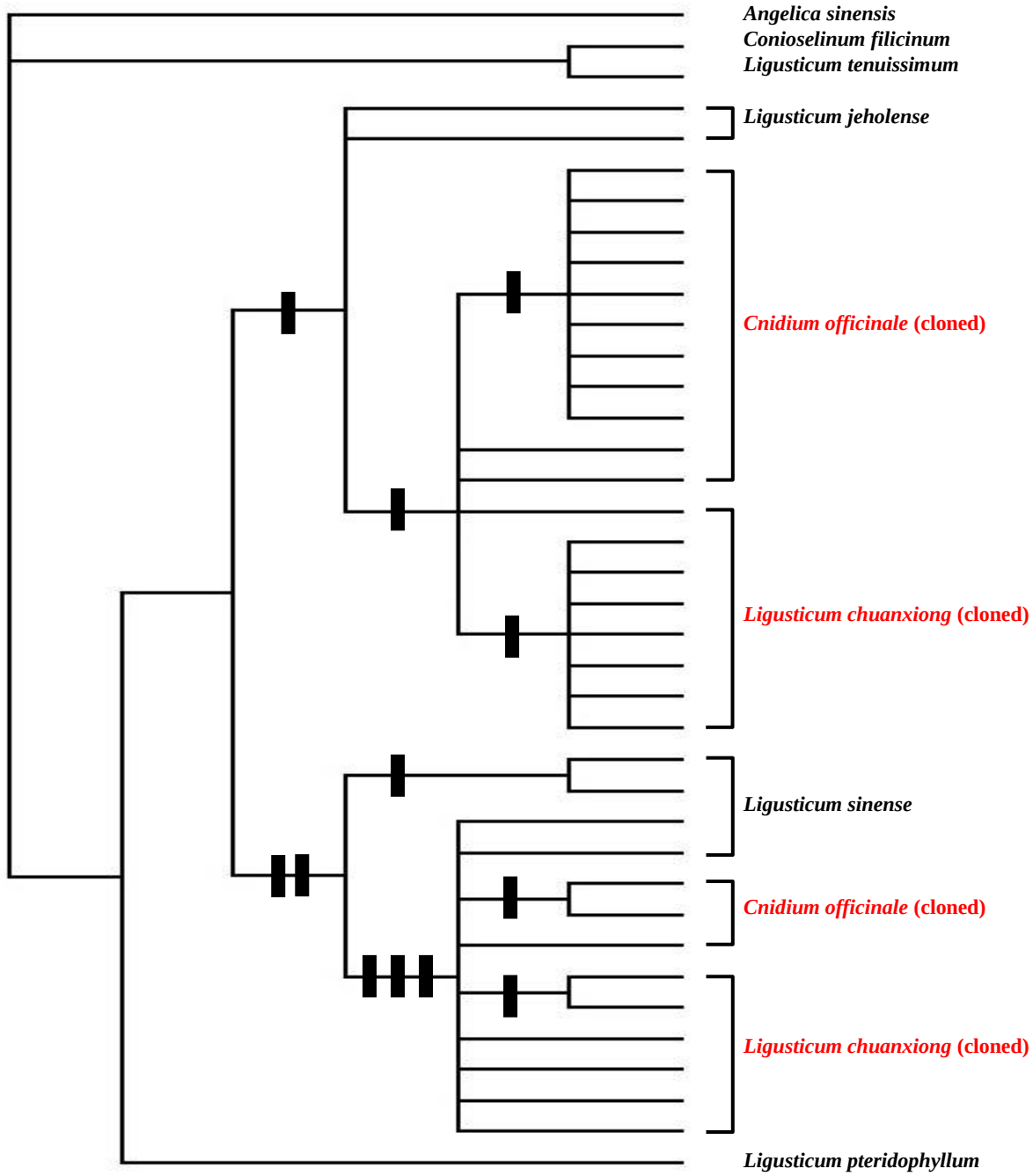


Figure 2.6. Strict consensus tree of the most parsimonious trees based on the ITS sequences of *Cnidium officinale* and related species. The ITS sequences of *Cnidium officinale* and *Ligusticum chuanxiong* were obtained from the one individual plant by cloning, respectively.

Chapter 3

Species identification using molecular makers for botanical raw materials consisting of multiple origins

Species identification of licorice using nuclear ribosomal DNA and Chloroplast DNA genetic markers

3.1 Introduction

Licorice is one of the most useful herbs in traditional Chinese medicine and Japanese Kampo medicine. In the Chinese Pharmacopoeia, three species of *Glycyrrhiza uralensis*, *G. glabra*, and *G. inflate* are prescribed as licorice: (Chinese Pharmacopeia Commission, 2015c). In the Japanese Pharmacopoeia, two species of *G. uralensis* and *G. glabra* are prescribed (MHLW, 2016d). Although these *Glycyrrhiza* species are identified based on the morphologic characteristics of their aerial part, especially on their leaf and fruit morphologies (Zhang, 1998; Fig. 3.1), it is difficult to identify species accurately based on the morphologies of dried roots or rhizomes using medicinally. The species-specific root morphologies of licorice (Fujita *et al.*, 1951; Zeng *et al.*, 1988), and component properties (Shibata *et al.*, 1978; Hatano *et al.*, 1991; Kitagawa *et al.*, 1998; Shebang *et al.*, 1996; Yonder *et al.*, 1990 and 1991) have been reported. Additionally, the various combinations of hybrids among *Glycyrrhiza* species have been reported (Ashurmetov, 1996). To stabilize pharmacological effect of herbal medicinal products including licorice, the accurate species identification is essential for the crude drug licorice consisting of multiple origins including hybrids because the species-specific component properties of licorice have been reported.

About species identifications for crude drugs, species identification using molecular markers have been evaluated as a higher-accuracy method (Goda *et al.*, 2003). The identifications of *Glycyrrhiza* species using RAPD, RFLP, or *rbc*L sequences on chloroplast DNA (cpDNA) have been reported (Yamazaki *et al.*, 1994; Kakutani *et al.*, 2001; Hayashi *et al.*, 1998 and 2000). The species identification using only genetic markers on cpDNA would lead to a misidentification because cpDNA generally inherited uniparentally (Mogensen, 1996). Since genetic markers vary in their rates of nucleotide substitution and since there are intra specific variations in their nucleotide sequences, a selection of genetic markers with a unique nucleotide sequence for each species is needed for highly accurate species identification (Goda *et al.*, 2003).

Therefore, in this study, to identify accurately original species of crude drug licorice, the four kinds of DNA regions were researched for 205 *Glycyrrhiza* specimens. One of the four kinds of DNA regions were the ITS on nuclear ribosomal DNA (nrDNA). Since ITS is inherited from both parents,

ITS sequences can detect genetic information on both parents and hybrids (Baldwin *et al.*, 1995). Three of the four kinds of DNA regions were the *rbc*L gene, the *mat*K gene, and the *trn*H–*psb*A intergenic region on cpDNA. It is generally considered that they have different rates of nucleotide substitution those are known as evolution rates. The evolution rate of the *rbc*L gene is slower than that of the *mat*K gene, or the *trnH–psbA* intergenic regions (Goda *et al.*, 2003). Suitable genetic markers for the species identification of licorice could be selected from the genetic information of those four kinds of DNA regions.

3.2 Materials and methods

Materials

The 205 herbarium specimens were identified based on their morphologic characteristics of the aerial part (Zhang *et al*., 1998). Three *Glycyrrhiza* species used as licorice (*G. glabra*: n = 51, *G. inflata*: n =18, and *G. uralensis*: n =128) and four *Glycyrrhiza* species as out-group (*Glycyrrhiza echinata*: n = 2, *Glycyrrhiza lepidota*: n = 1, *Glycyrrhiza macedonika*: n = 1, and *Glycyrrhiza pallidiflora*: n = 4) were included in the 205 specimens. The vouchers and localities of the 205 licorice specimens were listed (Table 3.1). The vouchers were deposited in the herbaria of Osaka University of pharmaceutical sciences (OY), Tsumura & Co. (THS), Tokyo University (TI), and Tohoku University (TUS) in Japan.

DNA Sequencing

Total DNA was extracted using the DNAeasy Plant Mini Kit (Qiagen) from a dried leaf on the herbarium specimen. The complete sequence of the ITS, the partial sequences of *rbc*L (168 bp), *mat*K (143bp), and *trn*H–*psb*A (239bp) including nucleotide polymorphisms among the *Glycyrrhiza* species examined were amplified with PCR under the following conditions. The following amplifying primers were used for the ITS: ITS5; GGA AGT AAA AGT CGT AAC AAG G and ITS4; TCC TCC GCT TAT TGA TAT GC (White *et al.*, 1990), for the *rbc*L: r662f; GTG CCG AAG CAA TTT ATA AAG C and r829r; TTG CAG TGA AAC CTC CAG TT, for the *mat*K: 1242f; CTT CGA CAC TGG GTG AAA GAT G and m1384r; AGG AAC AAG AAT AAT CTT GG, or the *trn*H–*psb*A: *trn*H-forward; ACG GGA ATT GAA CCC GCG CA (Demesure *et al.*, 1995) and Gly-trnHR1; CAT ATG ACT TCA CAA TGT AAA ATC. The PCR reaction mixture consisted of 10x Gene Taq Buffer 5µl (Nippon Gene), dNTP mix 4 µl (Nippon Gene), forward primer (10 pmol/ml) 2.5 µl, reverse primer (10 pmol/ml) 2.5 µl, Gene Taq 1 µl (Nippon Gene), DMSO 5 µl, D. D. W. 25.75 µl, and template DNA 5 µl (5 ng). For the PCR cycle, the step-down PCR was applied (Kawakami *et al.*, 1999).

Electrophoresis was performed for the amplified DNA fragments through 1.2% TAE agarose gel. The amplified DNA fragments were cut from the agarose gel and purified using the GFX PCR DNA

and Gel Band Purification Kit (Amersham Biotech). The purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit ver.2.0 and a Model 3100 automated sequencer following the manufacturer's instructions (Applied Biosystems). For sequencing the ITS, *mat*K, and *trn*H–*psb*A, their amplifying primers were used as sequencing primers. For sequencing the *rbc*L, r694f: ACT GGT GAA ATC AAA GGG C and r809r: AAG TAG TCA TGC ATT ACG AT were designed as sequencing primers.

Phylogenetic Analyses

The phylogenetic analyses were carried out using the PAUP software package 3.1.1 (Swofford, 1993). The most-parsimonious trees of equal length were computed after a heuristic search of trees using the TBR and MULPARS options.

3.3 Results

Variable nucleotide sites and genotypes of ITS, rbcL, matK, and trnH-psbA

In the sequencing results of the four kinds of DNA regions for the 205 *Glycyrrhiza* specimens, the following nucleotide substitutions and the genotypes were observed (Table 3.2). On the ITS sequence (accession number AB280738 in GenBank), two variable sites were observed at the 187th and 411-413th nucleotides. The ITS genotypes were defined by the combinations with the nucleotide substitutions on the two variable sites, and were named as I-1(T, TGC), I-2 (T, CAA), I-3 (C, TGC), and ADD (Y, YRM). Within the ADD of ITS genotype, two kinds of nucleotides were observed at every variable site: Y (= C + T) at the 187th and YRM (Y = C + T, R = A + G, and M = A + C) at the 411-413th nucleotides. The ADD genotype coincided with a combination of the I-2 (T, CAA) and I-3 (C, TGC) genotypes. On the *rbc*L sequence (AB012126), two variable sites were observed at the 706th and 736th. The *rbc*L genotypes were named as R-1 (A, T) and R-2 (G, A). On the *mat*K sequence (AB28074), a variable site was observed at the 568th-573rd. The *mat*K genotypes were named as M-1 (CTTATT) and M-2 (Deletion). On the *trn*H–*psb*A (AB280745), three variable sites were observed at the 72nd, 125th, and 171st. The *trn*H–*psb*A genotypes were named T-1 (C, A, T), T-2 (C, A, G), T-3 (C, A, G), and T-4 (T, A, T).

Total genotypes and their phylogenetic analysis

In the 205 *Glycyrrhiza* species examined, the ten total genotypes (TG-1–TG-9 and ADD) were recognized as combinations of the genotypes obtained from the ITS, *rbc*L, *mat*K, and *trn*H–*psb*A. The total genotypes of 205 samples described in Table 3.1. The combinations of the ITS, *rbc*L, *matK*, and *trnH–psbA* genotypes and frequencies of each species identified by the morphological characteristics for the total genotype were illustrated in Fig. 3.2.

In the 205 *Glycyrrhiza* species examined, all the out-group (n = 8) had the TG- 1 genotype.

Glycyrrhiza glabra identified by the morphologic characteristics had TG-2, TG-3, TG-7, TG-9, and ADD genotypes. The TG-3 genotype was the most observation in *G. glabra* (78% = 40/51). *Glycyrrhiza inflata* had the TG-2, TG-4, TG-5, and ADD genotypes. The TG-5 genotype was the most in *G. inflata* (78% = 14/18). *Glycyrrhiza uralensis* had the TG-4, TG-6, TG-7, TG-8, TG-9, and ADD genotypes. The TG-7, TG-8, and TG-9 genotypes dominated 91% (116/128) in *G. uralensis*.

To define phylogenetic relationships among the total genotypes, a phylogenetic analysis was performed. In this result, only one most-parsimonious phylogenetic tree of eight steps in length was calculated based on the sequences of ITS, *rbc*L, *mat*K, and *trn*H–*psb*A except samples having the ADD genotype (Fig. 3.2). The 17 samples having the ADD genotype were excluded from this phylogenetic analysis because they had two kinds of nucleotides at the same nucleotide substitution site. In the phylogenetic tree, two clades consisting of TG-2–TG-5 or TG-6–TG-9 were recognized. In the clade of TG-2–TG-5, TG-4 and TG-5 formed a sub-clade. On the other hand, in the clade of TG-6–TG-9, TG-8 and TG-9 formed a sub-clade.

Species identification by the total genotypes

Species-specific total genotypes were defined from the frequencies of species identified by morphological characteristics and from the phylogenetic relationships for the total genotypes (Fig. 3.2). The TG-1 genotype was presumed as a specific genotype of the out group examined. TG-2 was of *G. glabra* or *G. inflata*, and did not identify which was *G. glabra* or *G. inflata*. TG-3 was of *G. glabra*. TG-4 was of *G. glabra* or *G. inflata*, and did not identify which was *G. glabra* or *G. inflata* because observed TG-4 was too small in number. Additionally, TG-4 was not of *G. uralensis* which was in another clade. TG-5 was of *G. inflata*. On the other hand, TG-6–TG-9 clustering a clade in the phylogenetic tree were presumed as species-specific genotypes of *G. uralensis*.

Additionally, licorices having the ADD genotype were presumed as hybrids between *G. uralensis* and either *G. glabra* or *G. inflata* because the ADD genotype might be mixed the I-2 with I-3 genotypes of ITS (Table 3.2). The I-2 genotype was observed in the TG-2–TG-5 of total genotypes that were presumed as the species-specific genotypes of *G. glabra* or *G. inflata*, and the I-3 genotype was observed in the TG-6–TG-9 that were presumed as the species-specific genotypes of *G. uralensis*. In this study, the hybrid between *G. glabra* and *G. inflata* could not be detected because all of *G. glabra* and *G. inflata* examined had the same ITS genotype (I-2) and the mixtures of nucleotides on variable sites inferring the hybridization of the both species could not be observed in ITS.

The consistency of licorice species identification based on the total genotypes and the morphological characteristics was indicated about 96% (197/205) in this study. The species identification of three samples were discordant on the two methods and five samples with TG-2 of the total genotype could not be distinguished between *G. glabra* and *G. inflata*.

3.4 Discussions

Efficient molecular markers for species identification of Licorice

The species identification of licorice was possible from the total genotype obtained from the nucleotide sequences of ITS on nrDNA, *rbc*L, *mat*K, *and trn*H–*psb*A on cpDNA. Meanwhile, from this result, *G. uralensis*, *G. glabra*, or *G. inflata* was identified efficiently based on the genetic information only from ITS, *mat*K, *trn*H–*psb*A but not need *rbc*L. *G. uralensis* was identified by the I-3 genotype of ITS (Fig. 3.2). In the same, *G. glabra* or *G. inflata* was by I-2 genotype of ITS. Additionally, *G. glabra* was by the T-2 genotype of *trn*H–*psb*A. *G. inflata* was by the M-2 genotype of *mat*K.

Phylogenetic relationships among G. uralensis, G. glabra, and G. inflata

Hayashi *et al.* (1998) have reported that *G. uralensis* was more closely related to *G. inflata* than to *G. glabra* because *G. uralensis* and *G. inflata* had the same *rbc*L sequence. The same *rbc*L sequence of *G. uralensis* and *G. inflata* (Hayashi *et al.*, 1998) was equivalent to the R-2 genotype of *rbc*L in this study and the *rbc*L sequence of *G. glabra* was the R-1 genotype. On the phylogenetic tree in this study (Fig. 3.2), the R-1 genotype was observed in the clade of *G. uralensis*, *G. glabra*, or *G. inflata*, and the R-2 genotype was observed only in the clade of *G. uralensis*. Meanwhile, the phylogenetic tree based on the ITS, *rbc*L, *mat*K, and *trn*H–*psb*A sequences clearly showed the two clades in the three *Glycyrrhiza* species using medicinally (Fig. 3.2). One clade consisted of *G. glabra* and G. inflata, the other of *G. uralensis*. That was suggested that *G. glabra* was closely related to *G. inflata* than to *G. uralensis*.

In the clade of *G. glabra* and *G. inflata*, the TG-2 of total genotype was presumed as an ancestral genotype and remained in *G. glabra* and *G. inflata*. Later, *G. glabra* would acquire the nucleotide substitution from T to G at the 171th on *trn*H–*psb*A intergenic region (TG-3). In the same, *G. glabra* or *G. inflata* would acquire from T to G at the 72nd on *trn*H–*psb*A (TG-4). Additionally, G. inflata would acquire the deletion of the six nucleotides on *mat*K (TG-5). On the other hand, the TG-6 was presumed as an ancestral genotype in *G. uralensis*. *G. uralensis* would acquire the nucleotide substation from T to G at the 171st on *trn*H–*psb*A (TG-7), or from A and T to G and A at the 706 and 736th on *rbc*L respectively (TG-8) and from A to G at the 125th on *trn*H–*psb*A (TG-9).

Distribution and expansion of the total genotype in China

The 205 samples examined with the total genotypes were mapped for each province in China (Fig. 3.3). *G. glabra* or *G. inflata* with the TG-2–TG-5 of total genotypes distributed in northwestern China. On the other hand, *G. uralensis* with the TG-6–TG-9 genotypes distributed from northeastern to northwestern China. The hybrids between *G. uralensis* and either *G. glabra* or *G. inflata* with the ADD genotype were distributed in northwestern China where *G. uralensis*, *G. glabra*, and *G. inflata*

mix-distributed.

The ancestral TG-2 genotype in *G. glabra* or *G. inflata* was only observed with the most of *G. glabra* and *G. inflata* examined in Xinjiang province. In *G. uralensis*, the four intra-specific variations of the total genotypes (TG-6–TG-9) were recognized. The ancestral TG-6 genotype in *G. uralensis* was observed in Xinjiang province. *G. uralensis* with TG-7–TG-9 genotypes were observed in wide areas from northeastern to northwestern China. Frequencies of TG-8 were comparatively higher in western China, especially in Xinjiang province. The TG-9 were higher in eastern China. From the distribution pattern and frequencies of the genotype, it might be considered that *G. uralensis* have expanded its distribution from western China to eastern China because the phylogenetic ancestral genotypes (TG-6 and TG-8) were distributed mainly in western areas and the derivative genotype (TG-9) was in eastern areas in China.

Morphological phenotypes and maternal species of hybrids

In this study, the 17 samples with the ADD of the total genotype were observed. They were presumed hybrids between *G. uralensis* and either *G. glabra* or *G. inflata*. The morphological phenotypes or species identified by the morphological characteristics, and maternal species of these 17 samples with the ADD genotype were reconfirmed (Table 3.3). The maternal species of the hybrids were presumed from the combinations of the genotypes of *rbc*L, *mat*K, and *trn*H–*psb*A on cpDNA in the result of the phylogenetic analysis (Fig. 3.2).

In the 17 hybrids with the ADD genotype, ten, six, or one hybrids have been identified as *G. uralensis*, *G. glabra*, or *G. inflata* by their morphological characteristics, respectively. From their cpDNA information, the maternal species of three in ten hybrids identified as *G. uralensis*, five in the six hybrids identified as *G. glabra*, and one hybrid identified as *G. inflata* were different from the species identified by morphological characteristics. It was suggested that the hybrids among *G. uralensis*, *G. glabra,* and *G. inflata* would have no species-specific hazard on their hybridizations and the morphological phenotype of hybrid would have no linkage with the maternal inheritance.

Additionally, in *G. glabra* identified by morphological characteristics with the ADD genotype, four of *G. inflata* were recognized as the maternal species (THS498-1, 2, 3, and 4; Table 3.3). In this study, the hybrid between *G. glabra* and *G. inflata* could be not detected because *G. glabra* and *G. inflata* examined had the same ITS genotype (I-2). However, the four hybrids had the genotypes of *G. uralensis* and either *G. glabra* or *G. inflata* on ITS (ADD), *G. inflata* on *mat*K (M-2) and *trn*H–*psb*A (T-3), and the morphological phenotype of *G. glabra*. It was suggested that cross-hybridizations among *G. uralensis*, *G. glabra*, and *G. inflata* would have occurred.

Therefore, in the case of *G. uralensis*, *G. glabra*, and *G. inflata* are cultivated for medicinal use in the same field, a careful attention is required in the crude drug use because they would be easy to hybridize and difficult to identify their species or hybrids by their morphological phenotypes.

Table 3.1. Species identified by the morphologic feature of the aerial part, vouchers, locations and the total genotypes (TG-1−TG-9 and ADD).

OutGroup (n = 8)

voucher	species	locality	genotype
OY-7	G. echinata	clutivated in OY	TG- 1
OY	G. echinata	clutivated in OY	TG- 1
OY-25	G. lepidota	Canada	TG- 1
OY-18	G. macedonika	Italy	TG- 1
OY-2	G. pallidiflora	clutivated in OY	TG- 1
OY-3	G. pallidiflora	clutivated in OY	TG- 1
OY-19	G. pallidiflora	Hungary	TG- 1
OY-20	G. pallidiflora	Russia	TG- 1

G. glabra (n = 51)

voucher	locality	genotype
THS66496	Spain	TG- 2
THS66495	France	TG- 2
OY-24	Egypt	TG- 2
OY-10	Russia	TG- 3
OY	Russia	TG- 3
TI	Russia	TG- 3
TUS134993	Russia	TG- 3
THS758-1	Czechoslovakia	TG- 3
THS758-2	Czechoslovakia	TG- 3
THS758-3	Czechoslovakia	TG- 3
THS758-4	Czechoslovakia	TG- 3
THS758-5	Czechoslovakia	TG- 3
THS491	Spain	TG- 3
THS493-1	Spain	TG- 3
THS493-2	Spain	TG- 3
THS493-3	Spain	TG- 3
THS493-4	Spain	TG- 3
THS494-1	Spain	TG- 3
THS494-2	Spain	TG- 3
THS494-3	Spain	TG- 3
THS494-4	Spain	TG- 3
THS494-5	Spain	TG- 3
THS494-6	Spain	TG- 3
THS494-7	Spain	TG- 3
OY-22	Italy	TG- 3
THS41794	Turkey	TG- 3
THS31678	Turkey	TG- 3
THS496-1	Turkey	TG- 3
THS496-2	Turkey	TG- 3
OY-9	Turkey	TG- 3
THS72275	China, Xinjiang	TG- 3
THS72276	China, Xinjiang	TG- 3
THS40866	China, Xinjiang	TG- 3
THS72277	China, Xinjiang	TG- 3
THS72278	China, Xinjiang	TG- 3
THS72279	China, Xinjiang	TG- 3
OY-4	clutivated in OY	TG- 3
OY-5	clutivated in OY	TG- 3
OY-11	clutivated in OY	TG- 3
OY	clutivated in OY	TG- 3
OY	clutivated in OY	TG- 3
OY	clutivated in OY	TG- 3
THS35184	-	TG- 3
THS40627	China, Xinjiang	TG- 7
THS497-2	China, Xinjiang	TG- 9
THS31689	Turkey	ADD
THS497-1	China, Xinjiang	ADD
THS498-1	China, Xinjiang	ADD
THS498-2	China, Xinjiang	ADD
THS498-3	China, Xinjiang	ADD
THS498-4	China, Xinjiang	ADD

G. inflata (n = 18)

voucher	locality	genotype
THS72280	China, Xinjiang	TG- 2
THS71515	China, Xinjiang	TG- 2
THS66047-2	China, Xinjiang	TG- 4
THS72281	China, Xinjiang	TG- 5
THS72283	China, Xinjiang	TG- 5
THS72284	China, Xinjiang	TG- 5
THS40630	China, Xinjiang	TG- 5
THS40620	China, Xinjiang	TG- 5
THS502-1	China, Xinjiang	TG- 5
THS502-2	China, Xinjiang	TG- 5
THS503-3	China, Xinjiang	TG- 5
THS43490	China, Xinjiang	TG- 5
THS43491	China, Xinjiang	TG- 5
THS43492	China, Xinjiang	TG- 5
THS43493	China, Xinjiang	TG- 5
THS43494	China, Xinjiang	TG- 5
THS71518	China, Gansu	TG- 5
THS71521	China, Gansu	ADD

G. uralensis (n = 128)

voucher	locality	genotype
THS43478	China, Xinjiang	TG- 4
THS41797	China, Xinjiang	TG- 6
THS500-4	China, Xinjiang	TG- 7
THS500-5	China, Xinjiang	TG- 7
THS43478	China, Xinjiang	TG- 7
TUS231103	China, Qinghai	TG- 7
TUS231099	China, Qinghai	TG- 7
TUS231101	China, Qinghai	TG- 7
THS72314-02	China, Ningxia	TG- 7
THS72314-03	China, Ningxia	TG- 7
THS72314-07	China, Ningxia	TG- 7
THS72314-08	China, Ningxia	TG- 7
THS72316-06	China, Nei Monggol	TG- 7
THS72317-05	China, Nei Monggol	TG- 7
THS72319	China, Nei Monggol	TG- 7
THS770-2	China, Liaoning	TG- 7
THS72270	China, Xinjiang	TG- 8
THS72273	China, Xinjiang	TG- 8
THS72274	China, Xinjiang	TG- 8
THS72272	China, Xinjiang	TG- 8
THS40621	China, Xinjiang	TG- 8
THS500-6	China, Xinjiang	TG- 8
THS501-2	China, Xinjiang	TG- 8
THS43475	China, Xinjiang	TG- 8
THS43480	China, Xinjiang	TG- 8
THS43481	China, Xinjiang	TG- 8
THS43483	China, Xinjiang	TG- 8
THS44496	China, Xinjiang	TG- 8
THS44497	China, Xinjiang	TG- 8
THS44498	China, Xinjiang	TG- 8
THS44499	China, Xinjiang	TG- 8
THS44500	China, Xinjiang	TG- 8
THS44501	China, Xinjiang	TG- 8
THS44502	China, Xinjiang	TG- 8
THS44503	China, Xinjiang	TG- 8
THS44504	China, Xinjiang	TG- 8
THS44505	China, Xinjiang	TG- 8
THS44506	China, Xinjiang	TG- 8
THS44507	China, Xinjiang	TG- 8
THS44508	China, Xinjiang	TG- 8
THS44509	China, Xinjiang	TG- 8
THS44510	China, Xinjiang	TG- 8
THS44511	China, Xinjiang	TG- 8
THS44512	China, Xinjiang	TG- 8
THS44513	China, Xinjiang	TG- 8
THS44514	China, Xinjiang	TG- 8
THS44515	China, Xinjiang	TG- 8
THS44516	China, Xinjiang	TG- 8
THS44517	China, Xinjiang	TG- 8
THS44518	China, Xinjiang	TG- 8
THS44519	China, Xinjiang	TG- 8
THS44520	China, Xinjiang	TG- 8
THS44521	China, Xinjiang	TG- 8
THS44522	China, Xinjiang	TG- 8
THS44523	China, Xinjiang	TG- 8
THS44524	China, Xinjiang	TG- 8
THS44525	China, Xinjiang	TG- 8
THS40629	China, Xinjiang	TG- 8
THS42506	China, Xinjiang	TG- 8
THS42508	China, Xinjiang	TG- 8
THS72271	China, Xinjiang	TG- 8
THS71543	China, Gansu	TG- 8
THS42164	China, Nei Monggol	TG- 8
TUS133887	China, Nei Monggol	TG- 8
THS72316-04	China, Nei Monggol	TG- 8
THS72316-05	China, Nei Monggol	TG- 8
THS31538	China, Hebei	TG- 8
THS41989	China, Liaoning	TG- 8
OY-1	clutivated in OY	TG- 8
OY-8	clutivated in OY	TG- 8
OY-12	clutivated in OY	TG- 8
OY-14	clutivated in OY	TG- 8
OY-15	clutivated in OY	TG- 8
OY-17	clutivated in OY	TG- 8
OY	clutivated in OY	TG- 8
THS43132	Mongolia	TG- 9
THS43133	Mongolia	TG- 9
THS43134	Mongolia	TG- 9
THS43135	Mongolia	TG- 9
THS43136-1	Mongolia	TG- 9
THS43136-2	Mongolia	TG- 9
THS43137	Mongolia	TG- 9
THS43483	China, Xinjiang	TG- 9
THS71524	China, Gansu	TG- 9
THS71528	China, Gansu	TG- 9
THS71531	China, Gansu	TG- 9
THS71534	China, Gansu	TG- 9
THS71537	China, Gansu	TG- 9
THS71540	China, Gansu	TG- 9
THS72314-04	China, Ningxia	TG- 9
THS72314-05	China, Ningxia	TG- 9
THS72314-06	China, Ningxia	TG- 9
THS72315-01	China, Ningxia	TG- 9
THS72315-05	China, Ningxia	TG- 9
THS41885	China, Nei Monggol	TG- 9
TUS118100	China, Nei Monggol	TG- 9
TUS95951	China, Nei Monggol	TG- 9
TI	China, Nei Monggol	TG- 9
THS72317-01	China, Nei Monggol	TG- 9
THS72317-02	China, Nei Monggol	TG- 9
THS72317-03	China, Nei Monggol	TG- 9
THS72317-04	China, Nei Monggol	TG- 9
THS72317-06	China, Nei Monggol	TG- 9
KUN0615911	China, Shaanxi	TG- 9
THS42163	China, Shanxi	TG- 9
THS32115	China, Hebei	TG- 9
THS42161	China, Hebei	TG- 9
THS42162	China, Hebei	TG- 9
THS40393	China, Heilongjiang	TG- 9
THS40392	China, Heilongjiang	TG- 9
THS15811	China, Heilongjiang	TG- 9
THS41990	China, Liaoning	TG- 9
THS770-1	China, Liaoning	TG- 9
KUN43222	China, Liaoning	TG- 9
KUN43223	China, Liaoning	TG- 9
OY-16	clutivated in OY	TG- 9
OY	clutivated in OY	TG- 9
THS38110	-	TG- 9
THS40619	China, Xinjiang	ADD
THS40622	China, Xinjiang	ADD
THS40624	China, Xinjiang	ADD
THS43476	China, Xinjiang	ADD
THS43479	China, Xinjiang	ADD
THS500-1	China, Xinjiang	ADD
THS500-2	China, Xinjiang	ADD
THS500-3	China, Xinjiang	ADD
THS501-1	China, Xinjiang	ADD
OY-21	clutivated in OY	ADD

The herbarium abbreviations of the vouchers meant Osaka University of Pharmaceutical Sciences (OY), Tsumura & Co. (THS), University of Tokyo (TI), and Tohoku University (TUS).

Table 3.2. Variable sites and genotypes obtained from the nucleotide sequences of ITS, *rbc*L, *mat*K and *trn*H−*psb*A.

ITS (AB280738 = I-3)			*rbc*L (AB012126 = R-2)			*mat*K (AB28071 = M-1)		*trn*H – *psb*A (AB280745 = T-4)			
	site			site			site		Site		
	187	411-413		706	736		568-573		72	125	171
I-1	T	TGC	R-1	A	T	M-1	CTTATT	T-1	C	A	T
I-2	T	CAA	R-2	G	A	M-2	Deletion	T-2	C	A	G
I-3	C	TGC						T-3	T	A	T
ADD	Y	YRM						T-4	C	G	T

The variable sites were located on the sequence with the accession number. Gray backgrounds indicate nucleotide substitutions or a deletion compared with I-1, R-1, M-1, or T-1. IUPAC ambiguity symbols are adopted (Y = C + T, R = A + G, M = A + C).

Table 3.3. Species identified by morphological characteristics and maternal species presumed by cpDNA information of the hybrids with the ADD of the total genotype.

Species identified by morphologic characteristics	Voucher	Locality	Genotype				Maternal species presumed from cpDNA genotypes
			ITS	*rbc*L	*mat*K	*trn*H –*psb*A	
G. uralensis	THS40619	China, Xinjiang	**ADD**	R-1	M-2	T-3	***G. inflata***
	THS40622	China, Xinjiang	**ADD**	R-1	M-1	T-1	*G. uralensis* or *G. glabra* or *G. inflata*
	THS40624	China, Xinjiang	**ADD**	R-1	M-2	T-3	***G. inflata***
	THS43476	China, Xinjiang	**ADD**	R-1	M-1	T-3	***G. glabra*** or ***G. inflata***
	THS43479	China, Xinjiang	**ADD**	R-1	M-1	T-2	*G. uralensis* or *G. glabra*
	THS500-1	China, Xinjiang	**ADD**	R-2	M-1	T-4	*G. uralensis*
	THS500-2	China, Xinjiang	**ADD**	R-2	M-1	T-4	*G. uralensis*
	THS500-3	China, Xinjiang	**ADD**	R-2	M-1	T-4	*G. uralensis*
	THS501-1	China, Xinjiang	**ADD**	R-2	M-2	T-3	*G. uralensis* or *G. inflata*
	OY-21	Cultivated in OY	**ADD**	R-2	M-1	T-1	*G. uralensis*
G. glabra	THS31689	Turkey	**ADD**	R-1	M-1	T-2	*G. uralensis* or *G. glabra*
	THS497-1	China, Xinjiang	**ADD**	R-2	M-1	T-4	***G. uralensis***
	THS498-1	China, Xinjiang	**ADD**	R-1	M-2	T-3	***G. inflata***
	THS498-2	China, Xinjiang	**ADD**	R-1	M-2	T-3	***G. inflata***
	THS498-3	China, Xinjiang	**ADD**	R-1	M-2	T-3	***G. inflata***
	THS498-4	China, Xinjiang	**ADD**	R-1	M-2	T-3	***G. inflata***
G. inflata	THS71521	China, Gansu	**ADD**	R-2	M-1	T-1	***G. uralensis***

.

Licorice（甘草）

G. uralensis ***G. glabra*** ***G. inflata***

Figure 3.1. Crude drug of licorice and fruits of medicinal used *Glycyrrhiza* species. The clude drug name of licorice written in Chinese characters were indicated in parentheses.

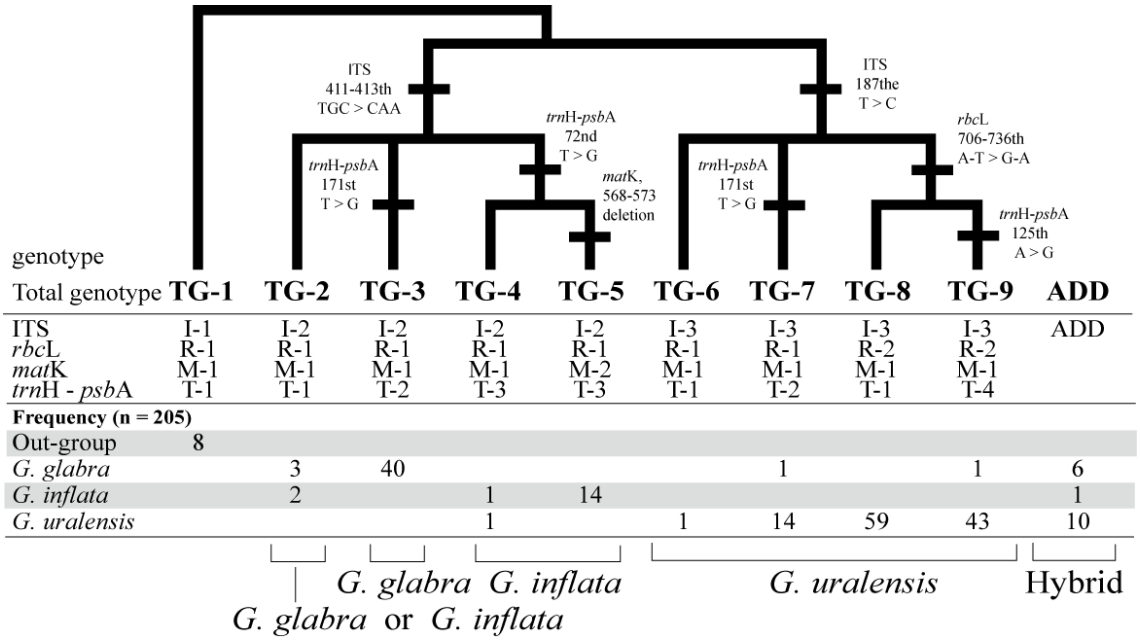


Figure 3.2. Total genotype (TG-1−TG-9 and ADD) consisted of the combination of the genotype (I-1−I-3, ADD, R-1−R-2, M-1−M-2, and T-1−T-4) obtained from the ITS, *rbc*L, *mat*K and *trn*H-*psb*A. Under the genotypes, the frequency of appearance of each species identified by aerial morphologic features is shown by each total genotype. Phylogenetic relationships among the total genotypes were described as the only most-parsimonious tree of eight steps long. The tree consistency index was 0.875.

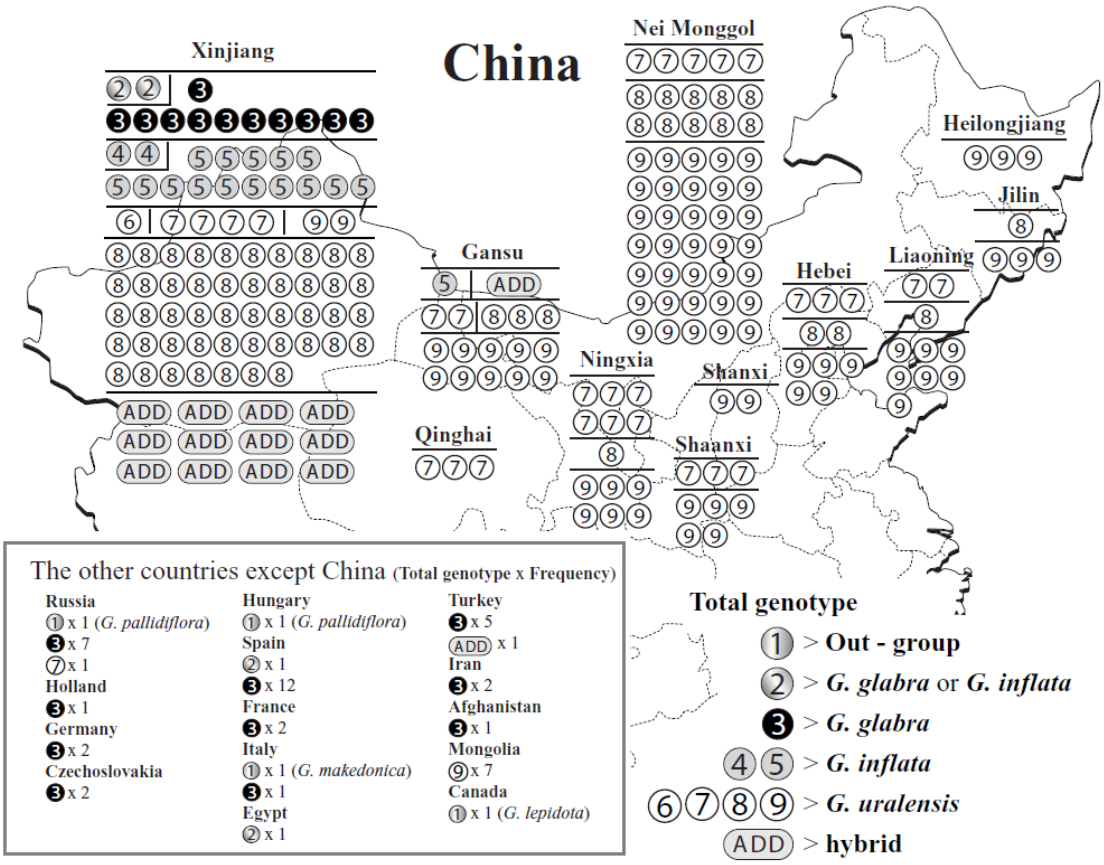


Figure 3.3. 205 samples examined with the total genotype mapped by each province in China.

Chapter 4

Chemical properties of botanical raw materials identified by molecular makers

Constituent properties of licorices derived from *Glycyrrhiza uralensis*, *G. glabra*, or *G. inflata* identified by molecular markers

4.1 Introduction

Licorice is one of the most useful herbs in traditional Chinese medicine and Japanese Kampo medicine. In the Chinese Pharmacopoeia, three species of *Glycyrrhiza uralensis*, *G. glabra*, and *G. inflate* are prescribed as licorice: (Chinese Pharmacopeia Commission, 2015c). In the Japanese Pharmacopoeia, two species of *G. uralensis* and *G. glabra* are prescribed (MHLW, 2016d). Constituent properties of licorices derived from the three species have been reported about eleven kinds of constituent contents (Yoneda *et al.*, 1990; Yoneda *et al.*, 1991), HPLC patterns (Shibano *et al.*, 1996; Kitagawa *et al.*, 1998), and species-specific constituents of glycycoumarin, glabridin, or licochalcone A (Shibata *et al.*, 1978; Hatano *et al.*, 1991; Hayashi *et al.*, 2000). However, it was difficult to clarify differences of constituent properties among licorices derived from the three species because the accurate species identification of licorice was difficult from their morphological features of roots or rhizomes those were medicinal parts, and existences of hybrids among *Glycyrrhiza* species have been reported, too (Ashurmetov, 1996; Hayashi *et al.*, 2003 and 2005). The method of species identification of licorice using four kinds of genetic markers; ITS on nuclear ribosomal DNA (nrDNA), *rbc*L gene, *mat*K gene, and *trn*H–*psb*A intergenic region on chloroplast DNA (cpDNA) have been reported (Kondo *et al.*, 2007b). This method would be able to detect the hybrid between *G. uralensis* and either *G. glabra* or *G. inflata* from the ITS sequence.

In this study, to define the differences of constituent properties among the licorices derived from the three species, at first, original species of 117 licorices were identified by their nucleotide sequences of the four kinds of DNA regions reported by Kondo *et al.* (2007b). Secondly, contents of six kinds of main constituents of licorice that were glycyrrhizin, liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside, and liquiritigenin, and three kinds of the species-specific constituents that were glycycoumarin, glabridin, and licochalcone A were measured in the 117 licorices. Consequently, the constituent properties by the licorice derived from the three *Glycyrrhiza* species used medicinally were reconfirmed.

4.2 Materials and methods

Materials

Vouchers and localities of 117 licorices were listed (Table 4.1). The vouchers were deposited in the herbarium of Tsumura & Co. in Japan.

DNA sequencing and species identification

The methods of the DNA sequencings of ITS, *rbc*L, *mat*K, and *trn*H–*psb*A, and of the species identification of licorices were followed the methods of the chapter 3 in this thesis or Kondo *et al.* (2007b).

Chemical Analysis

The powders of licorice (0.05 g) were extracted with 80% methanol (25 ml) under ultra-sonication for 30 min. This operation was repeated and scaled up to 50 ml. The 20 ml of this extract was submitted to HPLC analysis. HPLC equipped with a LC-10 system (Shimadzu) using a TSK gel ODS-80T_S column (250 x 4.6 mm) was used. The solvents were 0.05M $AcONH_4$ (pH 3.6) (A) and CH_3CN (B). A linear gradient of 100% (A) and 0% (B) changing over 60min. to 0% (A) and 100% (B) was used. The flow rate was 1.0 ml/min. The effluent from the column was monitored at from 200 nm to 400 nm.

Statistical Analysis

Significant differences and a principal component analysis for the constituent contents were calculated by the Excel statistics 2002 for windows software (Social Survey Research Information Co., Ltd., 2002).

4.3 Results

Species identification of 117 licorices based on the four kinds of molecular markers

Six kinds of the genotypes (TG-3, TG-5, TG-7, TG-8, TG-9, and ADD, chapter 3 in this thesis or Kondo *et al.*, 2007b) were recognized by the DNA sequencings of ITS, *rbc*L, *mat*K, and *trn*H–*psb*A for the 117 licorices (Table 4.1). Seventeen licorices located in Russia, France, Holland, Germany, Iran, Afghanistan, and Xinjiang in China had the TG-3 genotype and were identified as *G. glabra*. Eight licorices in Xinjiang and Gansu in China had the TG-5 genotype and were identified as *G. inflata*. Eighty seven licorices in Russia and a large north area from Jilin to Xinjiang province in China had the tg7 (n = 14), tg8 (n = 19), and tg9 (n = 54) genotypes and were identified as *G. uralensis*. Five licorices in Xinjiang and Gansu in China had the ADD genotype and were identified as hybrids between *G. uralensis* and either *G. glabra* or *G. inflata*.

Six kinds of main constituent contents

The contents of the six kinds of main constituents that were glycyrrhizin, liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside, and liquiritigenin in the 117 licorices examined were

indicated in Table 4.1 and Fig. 4.1. Comparing mean contents of the six main constituents by *G. uralensis*, *G. glabra*, or *G. inflata* identified from the genotypes, there was no significant difference among the three species in the contents of glycyrrhizin, liquiritin apioside, or isoliquiritin apioside. While, the mean contents of liquiritin, isoliquiritin, and liquilitigenin in *G. uralensis* were significantly higher than those of *G. glabra* or *G. inflata* (Fig. 4.1).

In the result of a principal component analysis for the six main constituent contents, the licorices derived from *G. glabra* and *G. inflata* were located together on the third quadrant of the scatter diagram mainly, while *G. uralensis* were scattered on all the quadrants (Fig. 4.2). It meant that the content property of the six main constituents of *G. glabra* resembles to *G. inflata*. On the other hand, the content property of the six main constituents of *G. uralensis* were distinguishable from *G. glabra* or *G. inflata*, and was characterized by a wider content variation than that of *G. glabra* or *G. inflata*.

In *G. uralensis*, the three kinds of genotypes (TG-7, TG-8, TG-9) were recognized as intra-specific variations. There is no difference of content properties among licorices having the three genotypes on the scatter diagram of the principal component analysis. Additionally, five licorices presumed as hybrids with the ADD genotype located at the intermediate area in the distributions of the three species on the scatter diagram (Fig. 4.2).

Species-specific constituents of licorice

Glycycoumarin, glabridin, or licochalcone A have been reported as the species-specific constituents of *G. uralensis*, *G. glabra*, or *G. inflata* respectively. Their constituent contents in the 117 licorices examined were indicated in Table 4.1 and Fig. 4.1. The detection frequencies of the three kinds of the species-specific constituents by the three species and hybrid identified from the genotypes were indicated in Table 4.2. In *G. glabra* (n = 17), 88% (n = 15) had glabridin and 12% (n = 2) had no species-specific constituents. In *G. inflata* (n = 8), 88% (n = 7) had licochalcone A and 12% (n = 1) had no species-specific constituent. In *G. uralensis* (n = 87), 95% (n = 83) had glycycoumarin and 5% (n = 4) had no species- specific constituent. In hybrids between *G. uralensis* and either *G. glabra* or *G. inflata* (n = 5), 40% (n = 2) had glycycoumarin and 60% (n = 3) had licochalcone A. From this result, it was reconfirmed that glabridin, licochalcon A, or glycycoumarin were species-specific constituents of *G. glabra*, *G. inflata*, or *G. uralensis* respectively because each constituent was recognized in only *G. glabra*, *G. inflata*, or *G. uralensis* identified by the genotype, respectively.

Meanwhile, in the hybrids identified by their genotypes, only one kind of species-specific constituent was detected from an individual plant but not two or more kinds of the species-specific constituents derived from their both parents of hybridization (Table 4.1).

4.4 Discussion

Species selection of licorice for the medicinal use

From the comparisons of the main and species-specific constituent contents of 117 licorices identified species using the molecular markers, it was revealed that the constituent property of *G. uralensis* was distinguishable from of *G. glabra* or *G. inflata*. The mean contents of liquiritin, isoliquiritin, and liquilitigenin in *G. uralensis* were significantly higher than those of *G. glabra* or *G. inflata*. Especially, liquiritin in *G. uralensis* had more than 1 % higher content than that of *G. glabra* or *G. inflata*.

Therefore, species should be selected according to the medicinal purpose however every *G. uralensis*, *G. glabra*, and *G. inflata* could be used as the crude drug licorice in the Chines pharmacopeia. For example, isoliquiritin had inhibitory effects on aldose reductase activity and granuloma angiogenesis (Aida *et al.*, 1989; Kobayashi *et al.*, 1995). Hence, licorice used for Byakko-ka-ninjin-to or Saiko-keishi-kankyo-to in the Japanese Kampo medicines that were often used as a diabetic medicine (Hujihira, 1982) should be selected under consideration of the amount of isoliquiritin in their prescriptions. Additionally, an antitussive effect of liquiritin apioside, liquiritigenin, and liquiritin, and a week estrogen-like activity of liquiritigenin have been reported (Kamei *et al.*, 2005; Nomura *et al.*, 2002).

Species identification of the species-specific constituents

Species identification of the three species-specific constituents of glycycoumarin, glabridin, or licochalcone A would be effective because those results were the almost same as the results of the species identification by the genotypes. However, there was no detection of the three species-specific constituents from 6% (= 7/117) of licorices examined. Additionally, the hybrids would not be detected from the species-specific constituents because the five putative hybrids with the ADD genotype observed in this study had only one kind of the species-specific constituent. It was not known exactly why the putative hybrid had only one kinds of the species-specific constituent and should be researched in detail.

Table 4.1. Vouchers, locations, species identified by the genotypes, the six kinds of main constituent contents of licorice, and the three kinds of species-specific constituent contents of 117 licorices examined.

Voucher	Locality	Geno-type	Species by genotype	Main constituent contents (%)						Species-specific constituent contents (%)		
				Glycyr-rhizin	Liquiri-tin	Liquiritin apioside	Iso-liquiritin	Iso-liquiritin apioside	Liquiriti-genin	Glycy-coumarin	Glabridin	Lico-chalconeA
THS60130	Russia	TG-3	*G. glabla*	5.860	0.112	2.131	n.d.	0.838	n.d.	n.d.	0.273	n.d.
THS61104	Russia	TG-3	*G. glabla*	3.787	0.097	1.217	n.d.	0.515	n.d.	n.d.	0.136	n.d.
THS61121	Russia	TG-3	*G. glabla*	7.007	0.232	1.612	n.d.	0.625	0.043	n.d.	0.045	n.d.
THS66496	Spain	TG-3	*G. glabla*	2.787	0.107	2.743	n.d.	1.021	0.021	n.d.	0.247	n.d.
THS66495	France	TG-3	*G. glabla*	2.666	0.078	2.674	n.d.	0.213	n.d.	n.d.	0.102	n.d.
THS66497	Holland	TG-3	*G. glabla*	4.206	0.144	1.802	n.d.	0.699	0.023	n.d.	0.239	n.d.
THS66219	Germany	TG-3	*G. glabla*	1.875	0.134	0.723	0.047	0.240	0.022	n.d.	0.055	n.d.
THS66221	Germany	TG-3	*G. glabla*	4.842	0.106	1.612	n.d.	0.616	n.d.	n.d.	0.026	n.d.
THS54514	Iran	TG-3	*G. glabla*	2.301	0.035	0.904	n.d.	0.339	n.d.	n.d.	0.056	n.d.
THS64261	Iran	TG-3	*G. glabla*	3.481	0.008	1.373	n.d.	0.455	0.010	n.d.	n.d.	n.d.
THS60101	Afghanistan	TG-3	*G. glabla*	5.384	0.096	1.683	n.d.	0.726	n.d.	n.d.	0.345	n.d.
THS71515	China, Xinjiang	TG-3	*G. glabla*	0.991	0.223	0.598	0.030	0.160	0.022	n.d.	n.d.	n.d.
THS72275	China, Xinjiang	TG-3	*G. glabla*	2.275	0.049	0.937	n.d.	0.383	0.070	n.d.	0.159	n.d.
THS72276	China, Xinjiang	TG-3	*G. glabla*	3.080	0.352	1.784	0.077	0.519	n.d.	n.d.	0.157	n.d.
THS72277	China, Xinjiang	TG-3	*G. glabla*	2.984	0.308	1.859	0.070	0.629	0.023	n.d.	0.099	n.d.
THS72278	China, Xinjiang	TG-3	*G. glabla*	1.766	0.148	0.845	n.d.	0.293	n.d.	n.d.	0.207	n.d.
THS72279	China, Xinjiang	TG-3	*G. glabla*	4.867	0.660	2.759	0.165	0.813	0.045	n.d.	0.259	n.d.
THS40620	China, Xinjiang	TG-5	*G. inflata*	2.401	0.363	0.806	0.068	0.280	0.038	n.d.	n.d.	0.686
THS40630	China, Xinjiang	TG-5	*G. inflata*	2.540	0.084	0.841	0.011	0.321	n.d.	n.d.	n.d.	1.820
THS60203	China, Xinjiang	TG-5	*G. inflata*	1.129	0.264	0.843	0.038	0.208	0.026	n.d.	n.d.	n.d.
THS72281	China, Xinjiang	TG-5	*G. inflata*	5.380	0.385	2.291	0.059	0.855	n.d.	n.d.	n.d.	0.634
THS72282	China, Xinjiang	TG-5	*G. inflata*	3.771	0.195	1.663	0.026	0.600	0.078	n.d.	n.d.	0.762
THS72283	China, Xinjiang	TG-5	*G. inflata*	5.320	0.465	2.445	0.065	0.866	n.d.	n.d.	n.d.	0.659
THS72284	China, Xinjiang	TG-5	*G. inflata*	4.143	0.296	1.947	0.037	0.648	n.d.	n.d.	n.d.	1.037
THS71518	China, Gansu	TG-5	*G. inflata*	1.453	0.119	0.959	0.020	0.297	0.021	n.d.	n.d.	0.338
THS58482	Russia	TG-7	*G. uralensis*	6.178	2.984	2.975	0.799	1.219	0.084	n.d.	n.d.	n.d.
THS40627	China, Xinjiang	TG-7	*G. uralensis*	0.850	0.282	0.557	0.050	0.157	0.008	n.d.	n.d.	n.d.
THS64165	China, Gansu	TG-7	*G. uralensis*	6.721	3.110	1.069	0.675	0.347	0.134	0.139	n.d.	n.d.
THS72299	China, Gansu	TG-7	*G. uralensis*	2.744	1.876	1.016	0.330	0.315	0.115	0.139	n.d.	n.d.
THS58610	China, Ningxia	TG-7	*G. uralensis*	1.847	0.449	1.859	0.069	0.604	0.061	0.070	n.d.	n.d.
THS72303	China, Ningxia	TG-7	*G. uralensis*	3.008	2.153	0.390	0.444	0.094	0.108	0.031	n.d.	n.d.
THS72305	China, Ningxia	TG-7	*G. uralensis*	2.658	0.659	1.418	0.136	0.607	0.029	0.078	n.d.	n.d.
THS58144	China, Shaanxi	TG-7	*G. uralensis*	4.519	1.371	1.646	0.261	0.704	0.068	0.033	n.d.	n.d.
THS58600	China, Shaanxi	TG-7	*G. uralensis*	2.475	1.773	0.699	0.329	0.160	0.138	0.055	n.d.	n.d.
THS59991	China, Shaanxi	TG-7	*G. uralensis*	1.966	1.303	1.118	0.184	0.277	0.063	0.101	n.d.	n.d.
THS72285	China, Nei Monggol	TG-7	*G. uralensis*	3.364	1.612	0.694	0.299	0.179	0.164	n.d.	n.d.	n.d.
THS72286	China, Nei Monggol	TG-7	*G. uralensis*	6.183	4.061	2.407	0.753	0.747	0.279	0.188	n.d.	n.d.
THS42161	China, Hebei	TG-7	*G. uralensis*	2.230	1.432	1.399	0.233	0.407	0.050	0.143	n.d.	n.d.
THS72290	China, Liaoning	TG-7	*G. uralensis*	2.010	1.593	1.331	0.302	0.416	0.072	0.025	n.d.	n.d.
THS40621	China, Xinjiang	TG-8	*G. uralensis*	3.030	1.002	0.994	0.252	0.248	0.130	0.049	n.d.	n.d.
THS40629	China, Xinjiang	TG-8	*G. uralensis*	2.222	1.146	1.162	0.216	0.286	0.054	0.055	n.d.	n.d.
THS56597	China, Xinjiang	TG-8	*G. uralensis*	1.996	0.076	0.857	0.013	0.281	0.070	n.d.	n.d.	n.d.
THS72270	China, Xinjiang	TG-8	*G. uralensis*	2.628	0.477	0.713	0.151	0.213	0.026	0.045	n.d.	n.d.
THS72271	China, Xinjiang	TG-8	*G. uralensis*	2.762	0.843	0.622	0.223	0.177	0.066	0.016	n.d.	n.d.
THS72273	China, Xinjiang	TG-8	*G. uralensis*	2.057	0.873	0.805	0.154	0.215	0.087	0.015	n.d.	n.d.
THS72274	China, Xinjiang	TG-8	*G. uralensis*	3.228	1.327	0.894	0.228	0.197	0.065	0.018	n.d.	n.d.
THS61198	China, Gansu	TG-8	*G. uralensis*	2.692	0.989	1.605	0.182	0.435	0.085	0.086	n.d.	n.d.
THS71543	China, Gansu	TG-8	*G. uralensis*	1.058	0.285	0.742	0.039	0.209	0.013	0.038	n.d.	n.d.
THS72297	China, Gansu	TG-8	*G. uralensis*	3.165	2.070	0.738	0.492	0.200	0.285	0.130	n.d.	n.d.
THS72302	China, Ningxia	TG-8	*G. uralensis*	4.085	1.512	1.424	0.307	0.585	0.047	0.074	n.d.	n.d.
THS42506	China, Nei Monggol	TG-8	*G. uralensis*	0.595	0.139	0.425	0.023	0.115	0.011	0.054	n.d.	n.d.
THS42508	China, Nei Monggol	TG-8	*G. uralensis*	1.130	0.635	0.384	0.136	0.120	0.099	0.073	n.d.	n.d.
THS58985	China, Nei Monggol	TG-8	*G. uralensis*	3.854	2.727	0.856	0.357	0.251	0.066	0.171	n.d.	n.d.
THS59002	China, Nei Monggol	TG-8	*G. uralensis*	4.514	2.060	4.216	0.329	1.260	0.120	0.064	n.d.	n.d.
THS72295	China, Nei Monggol	TG-8	*G. uralensis*	1.126	0.159	0.861	0.032	0.223	0.042	0.054	n.d.	n.d.
THS31537	China, Hebei	TG-8	*G. uralensis*	5.578	4.022	1.557	0.750	0.401	0.049	0.121	n.d.	n.d.
THS61625	China, Jilin	TG-8	*G. uralensis*	4.650	2.419	1.141	0.460	0.335	0.390	0.160	n.d.	n.d.
THS41989	China, Liaoning	TG-8	*G. uralensis*	1.924	1.333	0.585	0.312	0.158	0.074	0.031	n.d.	n.d.
THS72304	China, Qinghai	TG-9	*G. uralensis*	3.950	3.157	1.431	0.474	0.362	0.014	0.103	n.d.	n.d.
THS58707	China, Gansu	TG-9	*G. uralensis*	2.110	1.827	1.199	0.224	0.280	0.096	0.021	n.d.	n.d.
THS61197	China, Gansu	TG-9	*G. uralensis*	4.739	3.269	1.506	0.638	0.387	0.081	0.235	n.d.	n.d.
THS64188	China, Gansu	TG-9	*G. uralensis*	4.974	1.489	1.112	0.371	0.405	0.175	0.205	n.d.	n.d.
THS71524	China, Gansu	TG-9	*G. uralensis*	2.566	1.434	0.611	0.242	0.159	0.012	0.079	n.d.	n.d.
THS71528	China, Gansu	TG-9	*G. uralensis*	2.319	1.229	0.400	0.160	0.101	0.007	0.101	n.d.	n.d.
THS71531	China, Gansu	TG-9	*G. uralensis*	2.434	1.046	0.348	0.208	0.117	0.019	0.062	n.d.	n.d.
THS71534	China, Gansu	TG-9	*G. uralensis*	2.741	1.425	0.722	0.221	0.232	0.028	0.066	n.d.	n.d.
THS71537	China, Gansu	TG-9	*G. uralensis*	3.083	0.912	0.821	0.139	0.301	0.064	0.083	n.d.	n.d.
THS71540	China, Gansu	TG-9	*G. uralensis*	2.686	1.051	0.674	0.196	0.218	0.053	0.052	n.d.	n.d.
THS72300	China, Gansu	TG-9	*G. uralensis*	3.405	1.208	1.471	0.203	0.411	0.024	0.073	n.d.	n.d.
THS72306	China, Ningxia	TG-9	*G. uralensis*	4.421	2.666	2.677	0.395	0.782	0.064	0.092	n.d.	n.d.
THS58143	China, Shaanxi	TG-9	*G. uralensis*	2.090	0.302	1.133	0.081	0.407	0.073	0.039	n.d.	n.d.

Table 4.1. (continued)

Voucher	Locality	Geno-type	Species by genotype	Contents of the six main constituents of licorice (%)						Contents of the species-specific constituens (%)		
				Glycyr-rhizin	Liquiri-tin	Liquiritin apioside	Iso-liquiritin	Iso-liquiritin apioside	Liquiriti-genin	Glycy-coumarin	Glabridin	Lico-chalcone A
THS58171	China, Shaanxi	TG-9	*G. uralensis*	3.435	0.810	2.156	0.173	0.800	0.046	0.100	n.d.	n.d.
THS66460	China, Shanxi	TG-9	*G. uralensis*	2.810	1.996	1.586	0.342	0.483	0.051	0.079	n.d.	n.d.
THS42504	China, Nei Monggol	TG-9	*G. uralensis*	1.187	0.561	0.394	0.104	0.119	0.016	0.053	n.d.	n.d.
THS58601	China, Nei Monggol	TG-9	*G. uralensis*	7.671	3.900	1.910	0.834	0.531	0.136	0.057	n.d.	n.d.
THS58605	China, Nei Monggol	TG-9	*G. uralensis*	0.964	0.264	0.443	0.043	0.135	0.022	0.031	n.d.	n.d.
THS58743	China, Nei Monggol	TG-9	*G. uralensis*	4.147	1.441	1.546	0.268	0.518	0.480	0.087	n.d.	n.d.
THS58745	China, Nei Monggol	TG-9	*G. uralensis*	3.700	1.801	2.295	0.241	0.728	0.030	0.098	n.d.	n.d.
THS58747	China, Nei Monggol	TG-9	*G. uralensis*	5.524	2.123	1.637	0.406	0.627	0.083	0.193	n.d.	n.d.
THS58748	China, Nei Monggol	TG-9	*G. uralensis*	3.303	0.875	1.392	0.127	0.504	0.058	0.150	n.d.	n.d.
THS58966	China, Nei Monggol	TG-9	*G. uralensis*	5.244	3.236	1.824	0.568	0.619	0.057	0.094	n.d.	n.d.
THS58967	China, Nei Monggol	TG-9	*G. uralensis*	2.742	1.863	0.675	0.281	0.214	0.052	0.055	n.d.	n.d.
THS58968	China, Nei Monggol	TG-9	*G. uralensis*	4.915	3.719	0.895	0.666	0.240	0.050	0.083	n.d.	n.d.
THS58986	China, Nei Monggol	TG-9	*G. uralensis*	3.281	2.532	1.414	0.446	0.401	0.052	0.088	n.d.	n.d.
THS58987	China, Nei Monggol	TG-9	*G. uralensis*	5.510	2.934	2.669	0.475	0.916	0.092	0.118	n.d.	n.d.
THS60967	China, Nei Monggol	TG-9	*G. uralensis*	4.033	1.809	2.361	0.340	0.701	0.101	0.228	n.d.	n.d.
THS64185	China, Nei Monggol	TG-9	*G. uralensis*	3.533	1.832	0.748	0.342	0.250	0.067	0.145	n.d.	n.d.
THS65279	China, Nei Monggol	TG-9	*G. uralensis*	3.260	2.086	1.630	0.341	0.506	0.196	0.111	n.d.	n.d.
THS65898	China, Nei Monggol	TG-9	*G. uralensis*	5.474	4.356	1.495	1.256	0.406	0.407	0.080	n.d.	n.d.
THS72287	China, Nei Monggol	TG-9	*G. uralensis*	4.523	3.281	1.477	0.401	0.438	0.103	0.215	n.d.	n.d.
THS72288	China, Nei Monggol	TG-9	*G. uralensis*	0.259	0.136	0.502	0.015	0.099	0.011	0.085	n.d.	n.d.
THS72291	China, Nei Monggol	TG-9	*G. uralensis*	3.181	3.458	2.431	0.404	0.617	0.057	0.137	n.d.	n.d.
THS72292	China, Nei Monggol	TG-9	*G. uralensis*	5.858	1.048	1.287	0.248	0.673	0.156	0.053	n.d.	n.d.
THS72293	China, Nei Monggol	TG-9	*G. uralensis*	4.228	1.696	0.868	0.284	0.272	0.031	0.078	n.d.	n.d.
THS72294	China, Nei Monggol	TG-9	*G. uralensis*	4.439	3.132	2.441	0.524	0.673	0.115	0.158	n.d.	n.d.
THS72296	China, Nei Monggol	TG-9	*G. uralensis*	3.395	1.204	1.225	0.264	0.505	0.129	0.120	n.d.	n.d.
THS72307	China, Nei Monggol	TG-9	*G. uralensis*	7.009	3.234	1.827	0.764	0.637	0.637	0.044	n.d.	n.d.
THS64732	China, Hebei	TG-9	*G. uralensis*	8.310	3.935	3.571	0.836	1.470	0.284	0.165	n.d.	n.d.
THS64733	China, Hebei	TG-9	*G. uralensis*	3.878	1.262	1.140	0.265	0.561	0.155	0.045	n.d.	n.d.
THS64864	China, Hebei	TG-9	*G. uralensis*	2.962	2.457	0.783	0.467	0.202	0.210	0.077	n.d.	n.d.
THS65441	China, Hebei	TG-9	*G. uralensis*	3.429	1.388	1.110	0.259	0.358	0.148	0.157	n.d.	n.d.
THS72301	China, Hebei	TG-9	*G. uralensis*	3.166	1.625	1.083	0.233	0.253	0.054	0.079	n.d.	n.d.
THS72308	China, Hebei	TG-9	*G. uralensis*	1.927	1.811	0.560	0.277	0.125	0.041	0.057	n.d.	n.d.
THS41990	China, Liaoning	TG-9	*G. uralensis*	3.267	1.846	0.830	0.378	0.242	0.085	0.092	n.d.	n.d.
THS58141	China, Liaoning	TG-9	*G. uralensis*	3.381	1.011	1.356	0.211	0.451	0.161	0.137	n.d.	n.d.
THS72298	China, Heilongjiang	TG-9	*G. uralensis*	3.304	1.842	2.007	0.289	0.552	0.101	0.209	n.d.	n.d.
THS62089	China, Jilin	TG-9	*G. uralensis*	2.749	0.529	1.863	0.108	0.675	0.101	0.131	n.d.	n.d.
THS66783	China, Jilin	TG-9	*G. uralensis*	2.945	1.486	1.077	0.307	0.249	0.152	0.066	n.d.	n.d.
THS72309	China, Jilin	TG-9	*G. uralensis*	3.482	2.183	2.434	0.253	0.731	0.063	0.061	n.d.	n.d.
THS58598	China, Liaoning	TG-9	*G. uralensis*	3.396	1.932	0.578	0.451	0.153	0.469	0.080	n.d.	n.d.
THS65901	China, Liaoning	TG-9	*G. uralensis*	6.165	2.892	1.218	0.892	0.392	0.609	0.095	n.d.	n.d.
THS40622	China, Xinjiang	ADD	Hybrid	1.517	0.741	0.816	0.115	0.170	0.027	0.043	n.d.	n.d.
THS40624	China, Xinjiang	ADD	Hybrid	3.741	0.538	0.459	0.172	0.215	0.076	0.048	n.d.	n.d.
THS40626	China, Xinjiang	ADD	Hybrid	1.656	0.318	0.360	0.074	0.120	0.051	n.d.	n.d.	0.067
THS40628	China, Xinjiang	ADD	Hybrid	2.352	0.232	0.968	0.042	0.374	n.d.	n.d.	n.d.	0.813
THS71521	China, Gansu	ADD	Hybrid	1.977	0.550	0.920	0.042	0.234	0.025	n.d.	n.d.	0.361

The voucher abbreviation means Tsumura & Co. (THS). “n. d.” means no detected of the constituent contents.

Table 4.2. Detection frequencies of the three kinds of species-specific constituents by species identified from the genotypes for the 117 licorices examined.

Genotype	Species identified from genotype	Detection frequency of species specific constituents			
		Glycycoumarin	Glabridin	Licochalcone A	n. d.
TG-3	*G. glabra* n = 17	0	15 (88 %)	0	2 (12 %)
TG-5	*G. inflata* n = 8	0	0	7 (88 %)	1 (12 %)
TG-7, 8, 9	*G. uralensis* n = 87	83 (95 %)	0	0	4 (5 %)
ADD	Hybrid n = 5	2 (40 %)	0	3 (60 %)	0

'n. d.' means no detected of the three species-specific constituents.

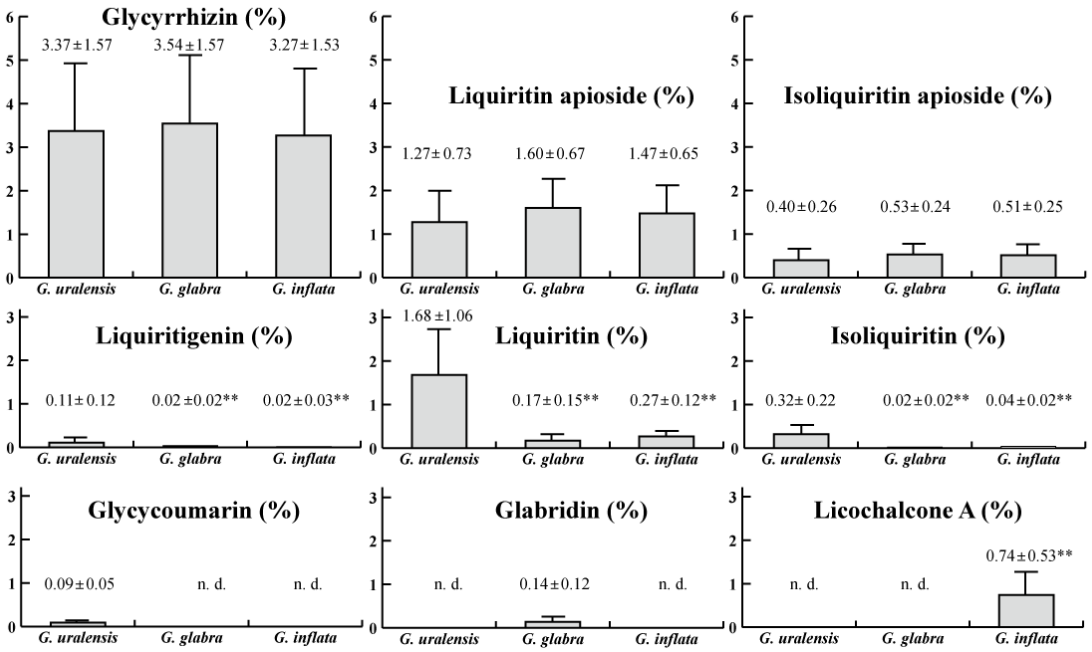


Figure 4.1. Contents of the six kinds of main constituents by *G. uralensis* (n = 87), *G. glabra* (n = 17) and *G. inflata* (n = 8). Values indicated means ± S.D. ** *P* <0 .01 vs. *G. uralensis*.

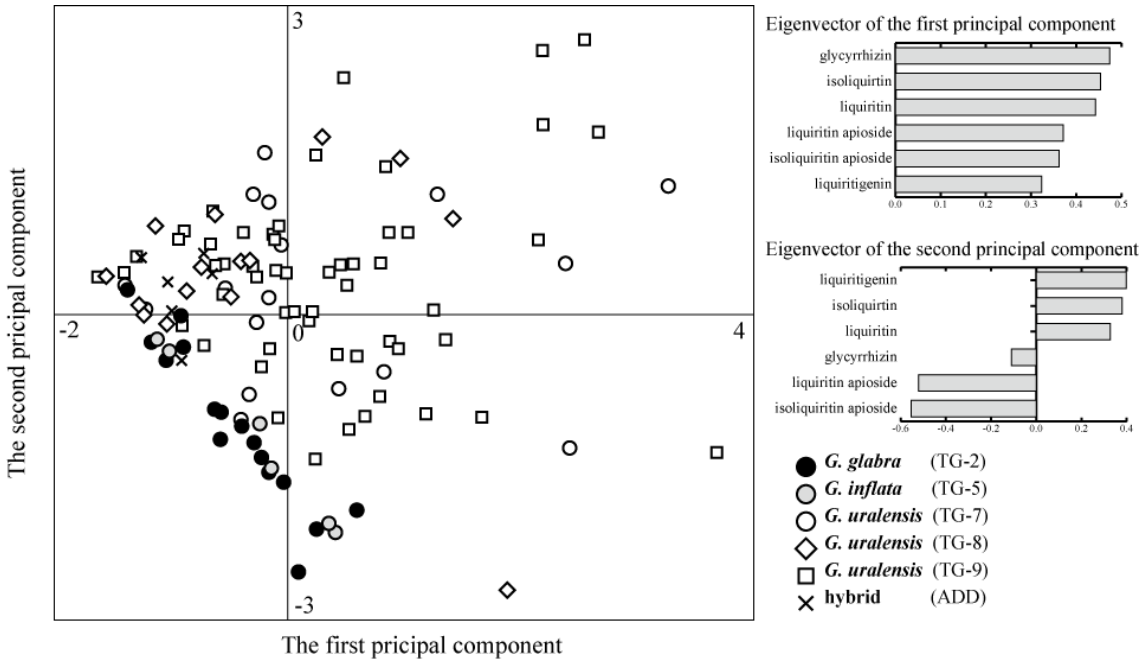


Figure 4.2. Scatter diagram of principal component analysis for the six kinds of the main constituents of Licorice derived from *G. uralensis* (n = 87), *G. glabra* (n = 17), *G. inflata* (n = 8) and *Hybrids* (n = 5).

Chapter 5

Historical study of an important crude drug using molecular markers

Identification of the licorice root stored in Shosoin (正倉院, written in Chinese character) based on the sequences of internal transcribed spacer (ITS) on nuclear ribosomal DNA and the chemotaxonomic consideration

5.1 Introduction

Licorice stored in Shosoin (coded ML-44, N-99, Fig. 5.1) since 756AD is a national treasure. It was investigated morphologically at the first scientific investigation of Shosoin-medicaments (1948-1949; Fujita *et al.*, 1955), and was assigned to *Glycyrrhiza glabra* var. *glandulifera* Waldet & Kit. The assignment was according to the comparative microscopic observation of the tissue section of a specimen named Fukushu-licorice (福州甘草, written in Chinese character) stored in the Pharmaceutical institute, University of Tokyo.

Licorice has well been known since ancient times in the East and West as a herb drug and a sweetening. According to the Shang-Han-Lun (傷寒論, written in Chinese character), the oldest medical book written in the 1st century during Tang Dynasty, licorice was involved in 80% of the 113 prescriptions cited there in. Among 60 kinds of medicaments stored in Shosoin, licorice (recorded with the quantity of 214 kg at the time of dedication, 756AD) was lost rapidly and only 10 kg remained after 100 years, indicating the high demand of this drug.

The original licorice plants, *Glycyrrhiza* spp. in *Leguminosae*, are widely distributed over the Eurasian continent. They occur in eastern Mongolia, northeastern and northwestern China including Xinjiang province, central Asian countries, Afghanistan, Pakistan, Iran, Iraq, Turkey, Southern part of Russia, and even in Italy and Spain, but not in Japan.

Therefore, Shosoin-licorice is apparently an imported material. The following species are the original licorice plants. *Glycyrrhiza uralensis* Fischer: northeastern China, far eastern Russia. *G. glabra*, L. var. *typica*, Reg. & Herd.: Spain, Italy. *G. glabra*, L. var. *violacea* Boiss: Turkey, Iran. *G. glabra* L. var. *glandulifera* Waldet & Kit: China, Russia, Central Asia. *G. inflata* Batalin: Xinjiang. *G. enuricarpa*, P. C. Li (= *G. korshinskyi* Grigori = *G. uralensis* x *G. inflata*): Xinjiang, Russia, Central Asia. *G. aspera* Pallas: Xinjiang. *G. echinata* L. and *G. pallidiflora* Maxim. are not in use for medical purpose.

The botanical classification of licorice has been mostly according to the morphological observation of the aerial parts of *Glycyrrhiza* plants. The macro- and microscopic examination of the

roots is not so effective for the classification. Therefore, the morphological evaluation of licorice without aerial parts as a drug material is rather difficult.

To get the more reliable identification of Shosoin-licorice, genetic analyses were applied to this problem. Previously, Kondo *et al*. (2007a) attempted several methods to determine the genetic identification of licorice species using ITS sequence on nuclear ribosomal DNA (nrDNA), the cording region of *rbc*L and *mat*K genes, and *trn*H–*psb*A intergenic region on chloroplast DNA (cpDNA). Therefore, in this study, the sequencing above the DNA regions of the Shosoin- licorice were attempted. In the preliminary analyses, only the partial ITS fragment of the Shosoin-licorice could be amplified. The other molecular markers on cpDNA could not be amplified in the Shosoin-licorice due to the aging effect.

Recently, the chemical principles of licorice extensively have been able to characterize. Apart from the well-established main saponin, glycyrrhizin, and its aglycone, glycyrrhetinic acid, a number of flavonoid compounds have been isolated and structurally established (Shibata *et al.*, 1978; Nomura *et al.*, 1998). The presence of some of these compounds was species-specific, so those could be applied to the chemotaxonomic survey and the species identification of licorice.

As an appendix of the first investigation program of Shosoin-medicaments, one of the present authors performed HPLC analysis of Shosoin-licorice (ML-44, N-99) to identify its characteristic species-specific flavonoid constituents (Shibata, 1991). However, the result of HPLC was not so satisfactory, since the species-specific flavonoids in Shosoin-licorice were missing during the storage of more than 1250 years.

In the present study as an appendix of the second scientific investigation of Shosoin-medicaments (1994-1995), the characteristic difference was researched between the Shosoin-licorice and the three representative species of licorice (*G. uralensis*, *G. glabra*, and *G. inflata*) in the ratio of occurrence of some common flavonoids (liquiritin, isoliquiritin, their apiosides and liquiritigenin). Furthermore, their characteristically differences were analyzed in a principal component analysis based on the above main flavonoids. This could be adoptable for the species identification of licorice roots.

5.2 Materials and methods

Genetic analysis

Total DNA of the Shosoin-licorice (ML-44, N99) was extracted by using DNAeasy® Plant Mini Kit (QIAGEN) from the dried roots of the Shosoin-licorice. The partial DNA fragment of ITS (379 bp) was amplified by polymerase chain reaction (Fig. 5.2). The amplifying primers for the ITS were as follows.

Forward primers: GCC ACG CAC TGT GTT CTC TCC T.

Reverse primers: GCA ATG CTC ACG GGA AGC CAA CA.

The composition of the reaction solution was 10 x Gene Tad Buffer (Nippon Gene) 5 µl, 2.5 mM dNTP mix (Nippon Gene) 4 µl, forward Primer (10 pmol/ml) 2.5 µl, reverse primer (10 pmol/ml) 2.5 µl, Gene Taq (Nippon Gene) 0.25 µl, DMSO 5 µl, D.D.W. 25.75 µl, template DNA 5 µl. For the PCR cycle, the step-down method was applied (Kawakami *et al.*, 1999).

The amplified fragments were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biotech). The purified PCR products were sequenced by using the Big Dye Terminator Cycle Sequencing Kit ver. 2.0 and Model 3100 automated sequencer (Applied Bio Systems), following the manufacturer's instructions. For DNA sequencing, the same primers used for the ITS fragment amplification were used as the sequencing primers.

Chemical analysis

The six kinds of main constituent contents of licorice that were glycyrrhizin, liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside, and liquiritigenin, and the three kinds of the species-specific constituents that were glycycoumarin, glabridin, and licochalcone A in the Shosoin-licorice were measured by following methods. 500 mg of the Shosoin-licorice was used for extracting with 80% methanol (25 ml) under ultra-sonication for 30 min. This operation was repeated and scaled up to 50 ml. I submitted 20 µl of this elution to HPLC analysis. HPLC was equipped with an LC-10 system (Shimadzu Co., Ltd.) using a TSK gel ODS-80Ts column (250 x 4.6 mm). The solvents were A (0.05 M $AcONH_4$ (pH 3.6)) and B (CH_3CN). A linear gradient of A (100%) and B (0%) changing over 60 min to A (0%) and B (100%) was used. The flow rate was 1.0 ml/min. The effluent from the column at from 200 nm to 400 nm was monitored.

In a principal component analysis based on the six main constituent contents using Excel Statistics 97 for Windows (Social Survey Research Information Co., Ltd. 2002), the constituent property of the Shosoin-licorice was compared with the species-specific constituent properties defined by the same research for the 117 of licorice (the chapter 4 in this thesis or Kondo *et al.*, 2007a).

5.3 Results

ITS sequence of the Shosoin-licorice

The partial ITS of the Shosoin-licorice stored since 756AD was amplified and sequenced (Fig. 5.3). The previous study found that *G. glabra* and *G. inflata* showed nucleotides T at the site 1 (187th) and CAA at the site 2 (411-413th), while *G. uralensis* gave nucleotide C at the site 1 and TGC at the site 2 in ITS on nrDNA (the chapter 3 in this thesis or Kondo *et al.*, 2007b). The ITS sequence of the Shosoin-licorice had the same nucleotides C at the site 1 and TGC at site 2 as that of *G. uralensis* (Fig. 5.3).

Constituent contents of the Shosoin-licorice

The contents of six principal constituents that were glycyrrhizin, liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside, liquiritigenin) of *G. uralensis*, *G. glabra* and *G. inflata* are tabulated in comparison with those of the Shosoin-licorice (Table 5.2). None of the species-specific flavonoids; glycycoumarin (*G. uralensis*), glabridin (*G. glabra*), and licochalcone A (*G. inflata*)) was detectable in the Shosoin-licorice probably due to aging destruction.

Scatter diagram of the principal component analysis for the six main constituent contents of licorices identified from genotype of nrDNA (n = 117) and the Shosoin-licorice was illustrated in Fig. 5.4. The Shosoin-licorice gave a spot in the region of *G. uralensis*. This result suggests that the constituent prosperity of the Shosoin-licorice resembled to of *G. uralensis*.

5.4 Conclusion

Based on the genetic and chemotaxonomic analyses, the botanical origin of Shosoin-licorice (ML-44, N-99) was identified as the root of *Glycyrrhiza uralensis* Fischer.

Table 5.1. The six principal constituent contents and the three species-specific constituent contents of the Shosoin-licorice and the three species of *Glycyrrhiza* identified from the genetic analyses of nrDNA and cpDNA. The data of three species referred in the chapter 4 in this thesis or Kondo *et al.* (2007a).

	main constituent contents (%)						Species-specific constituent contents (%)		
	Glycyr-rhizin	Liquiri-tin	Liquiritin apioside	Iso-liquiritin	Iso-liquiritin apioside	Liquiri-tigenin	Glabri-gin	Lico-chalcone A	Glycy-coumarin
Shosoin-licorice	5.05	0.64	0.11	0.64	0.08	0.16	n. d.	n. d.	n. d.
G. glabra n = 17	3.35 ± 1.62	0.17 ± 0.15	1.60 ± 0.69	0.08 ± 0.05	0.53 ± 0.24	0.03 ± 0.02	0.16 ± 0.10	n. d.	n. d.
G. inflate n = 8	3.27 ± 1.64	0.27 ± 0.13	1.47 ± 0.70	0.04 ± 0.02	0.51 ± 0.27	0.04 ± 0.03	n. d.	0.85 ± 0.48	n. d.
G. uralensis n = 87	3.43 ± 1.59	1.74 ± 1.06	1.30 ± 0.73	0.33 ± 0.23	0.41 ± 0.27	0.11 ± 0.12	n. d.	n. d.	0.09 ± 0.05

Values indicated mean ± S. D. and n. d. meant no detected.

Figure 5.1. Shosoin-licorice (Shosoin Medicament ML-44, N-99)

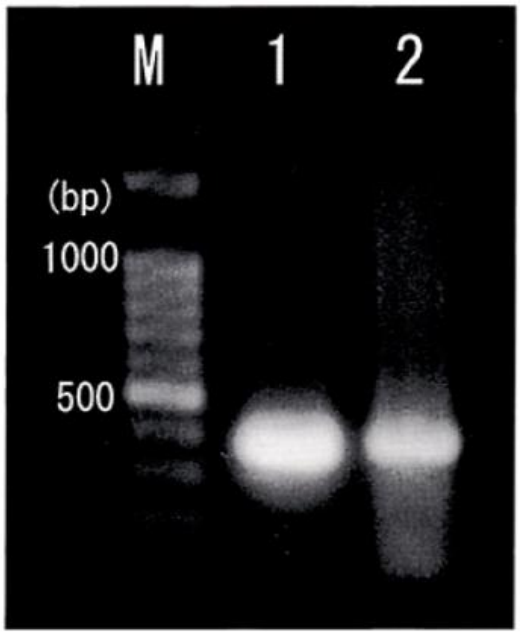


Figure 5.2. DNA fragments (379 bp) of partial ITS region amplified by PCR. M: 100 bp DNA ladder marker, 1: *G. uralensis* (THS41990), 2: Shosoin-licorice. THS meant Tsumura herbarium specimen.

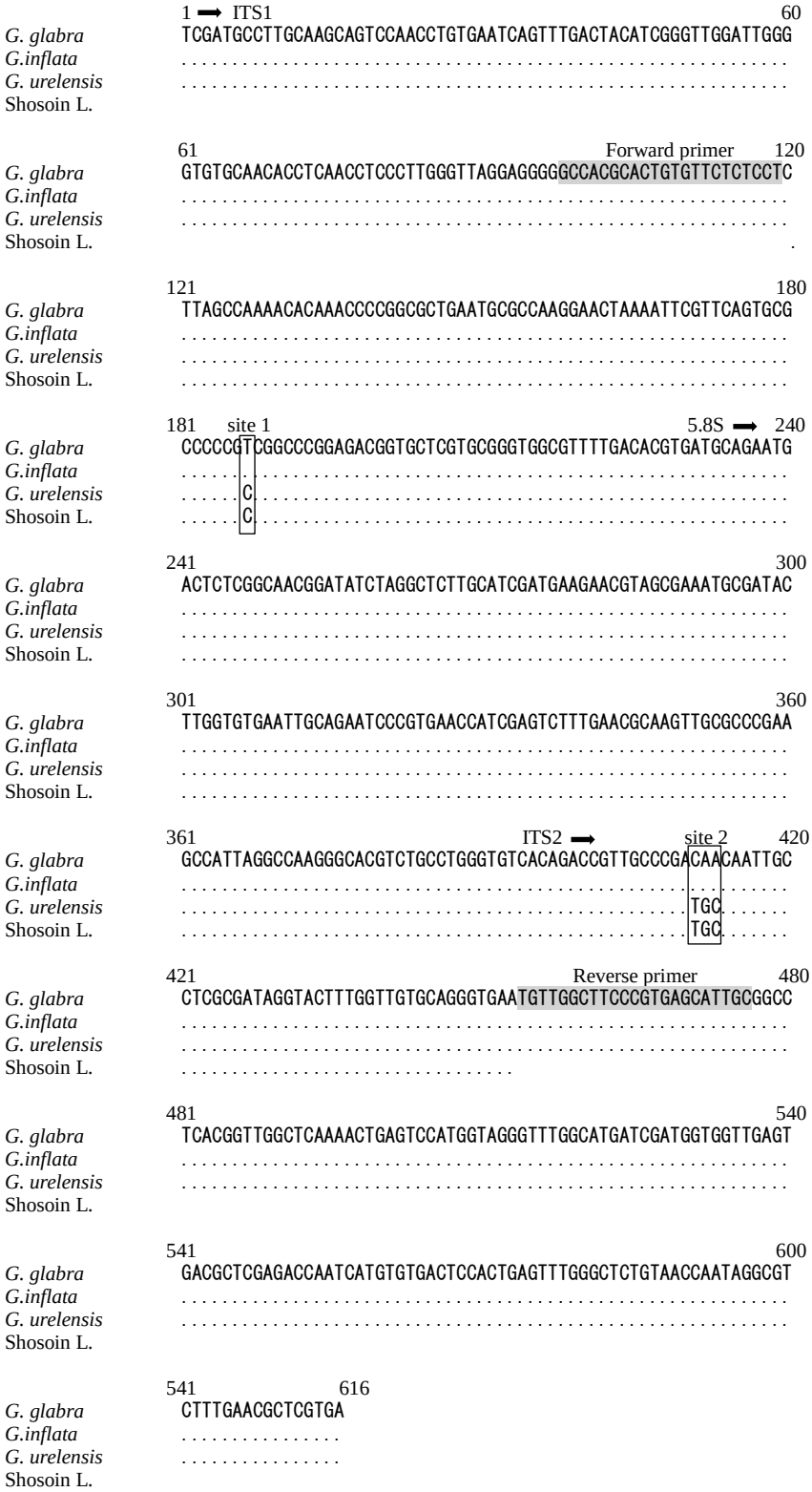


Figure 5.3. ITS sequences of *G. glabra*, *G. inflata*, *G. uralensis* and the Shosoin-Licorice.

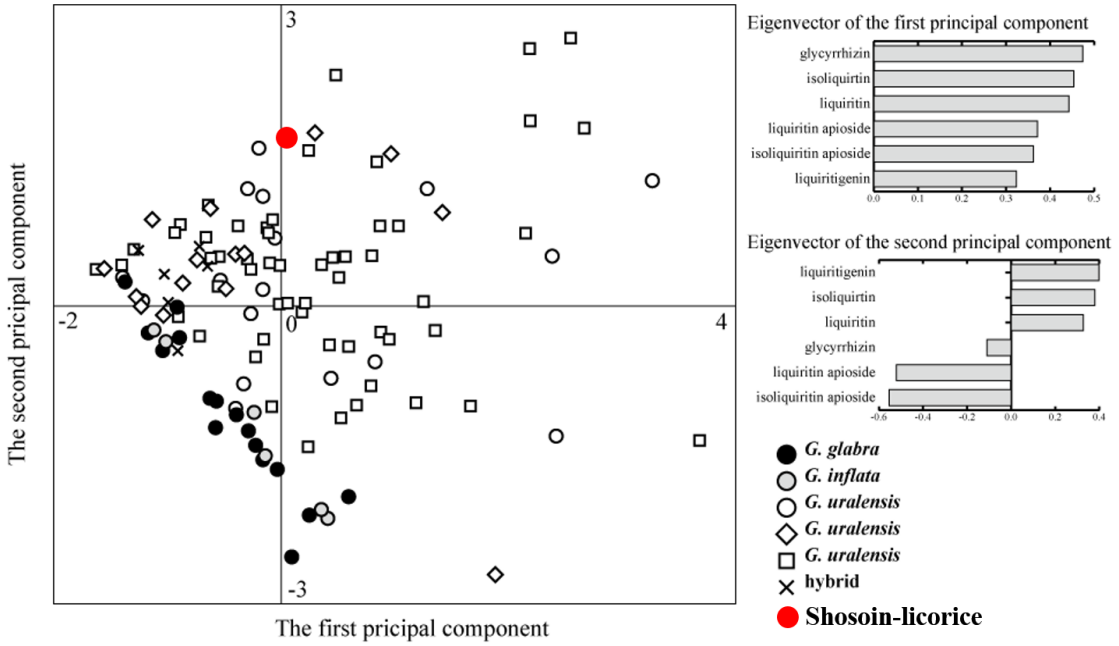


Figure 5.4. Scatter diagram of the principal component analysis for the six main constituent contents of licorices identified from the total genotype (the chapter 4 in this thesis or Kondo *et al.*, 2007a; n = 117) and the Shosoin-licorice.

Chapter 6

Development of an easier discriminating method of botanical raw materials without DNA sequencing on the Japanese pharmacopoeia

Discrimination between *Atractylodes* rhizome and *Atractylodes lancea* rhizome by the PCR-RFLP analysis of ITS region on nrDNA

6.1 Introduction

Dried rhizomes of *Atractylodes macrocephala* Koidz. (= *A. ovata* DC. in *Asteraceae*) and *A. japonica* Koidz. are used as the crude drug called *Atractylodes* rhizome (白朮, written in Chinese character), and *A. lancea* DC. and *A. chinensis* Koidz. called *Atractylodes lancea* rhizome (蒼朮, written in Chinese character) in the Japanese Pharmacopoeia (MHLW, 2016b). Discrimination between *Atractylodes* rhizome and *Atractylodes lancea* rhizome using molecular markers have been reported (Cheng *et al.*, 1997; Mizukami *et al.*, 2000).

In the general information on the Japanese Pharmacopoeia (MHLW, 2016f), the purity test for *Atractylodes* rhizome by molecular biological method was established to stop commingling of *Atractylodes lancea* rhizome with *Atractylodes* rhizome. This purity test based on the amplification refractory mutation system (ARMS) detected the differences of nucleotide sequences on ITS among the medicinal *Atractylodes* species (Guo *et al.*, 2006). The ARMS required rigorous experimental condition comparatively because the ARMS detected the nucleotide substitution by existence or nonexistence of PCR products using the specific mismatch primer. Therefore, to discriminate between *Atractylodes* rhizome and *Atractylodes lancea* rhizome, PCR restriction fragment length polymorphism (PCR-RFLP) as a simple, quick, and stable method was attempted.

6.2 Materials and methods

Materials

Dried rhizomes of *Atractylodes macrocephala* (THS 83160-1), *A. japonica* (THS 83160-22), *A. lancea* (THS 83160-18) and *A. chinensis* (THS 82810-1) identified by their ITS sequences were prepared. Additionally, the hybrid between *A. lancea* and *A. chinensis* (THS 82811-2) was added as

a material because many natural hybrids are distributed in China (Shiba *et al.*, 2006). THS meant Tsumura Herbarium specimen. The species identification by the ITS sequence followed in Shiba *et al.* (2006).

Target site for PCR-RFLP analysis

Based on the ITS sequences among the medicinal *Atractylodes* species (Shiba *et al.*, 2006), two nucleotide sites were selected as species specific nucleotide substitutions that were digestible by restriction enzymes. *Atractylodes lancea* only has nucleotide 'G' at the 75th site which can be cut by the restriction enzyme *Fau*I (recognition site: GCGGG, cut site: between the 64th and 65th), and *Atractylodes chinensis* only has nucleotide 'C' at the 94th site which can be cut by *Msp*I (recognition site: CCGG, cut site: between the 93rd and 94th; Fig. 6.1). Primers were designed to amplify the 137 bases of DNA fragment containing the two nucleotide sites. 5′-primer: 5′-GGC ACA ACA CGT GCC AAG GAA AA-3′, 3′-primer: 5′-CGA TGC GTG AGC CGA GAT ATC C-3′.

DNA extraction and amplification

For DNA extraction and amplification, Ampdirect® Plus enzyme kit (Shimadzu Biotech, 241-08890-92) were applied. Ampdirect® Plus enzyme kit with Ampdirect® Plus and Nova Taq™ Hot Start DNA Polymerase allow simple and quick DNA extraction, and stable DNA amplification from crude drugs. Shredded crude drug material (20 mg) within SNET buffer (400 μL) incubated at 55℃ for overnight (about 16– 18 hr). The SNET buffer contains Tris-HCl pH 8.0 (20 mM), EDTA (5 mM), NaCl (400 mM), SDS (0.3 %) and Protainase K (200 μg/mL). Following the incubation, DNA polymerase was inactivated at 95℃ for 5 min. After precipitating solids by centrifuge, the supernatant was used as template DNA solution. Amount of DNA within the template DNA solution is difficult to estimate accurately by $OD_{260\ nm}$ observation because the solution contains many foreign substances.

The PCR mixture contained 2× Ampdirect® Plus described with $MgCl_2$ and dNTP (10 μL), the avobe-descrived 5′- and 3′-primers (0.5 μM), Nova Taq™ Hot Start DNA Polymerase (0.5 units), the template DNA solution (0.5 μL), and added D. W. to 20 μL of total mixture volume. The PCR mixture was prepared under cool conditions.

DNA amplification by PCR was carried out under the following conditions: pre-cycling denaturation at 95℃ for 10 min, 40 cycles of denaturation at 95℃ for 30 seconds, annealing at 65℃ for 15 seconds, and elongation at 72℃ for 7 min; and stored at 4℃.

Digestion with the restriction enzyme

For the digestion of the PCR products by *FauI* (New England BioLabs Inc.), a 15 μL reaction containing 3 μL template DNA, one unit of enzyme and 1× SEBuffer B was incubated at 55℃ for 2 hr. For *MspI* (New England BioLabs Inc.), a 15 μL reaction containing 3 μL template DNA, 20 units of enzyme and 1× NEBuffer B was incubated at 37℃ for 2 hr. After the incubation, the enzymes were inactivated at 72℃ for 10 min. The digested DNA fragments were detected by electrophoresis using E-gel® 4% agarose (Invitrogen).

6.3 Results

The PCR products of the medicinal *Atractylodes* species digested by the restriction enzyme *Fau*I and *Msp*I were indicated on an electrophoretic profile (Fig. 6.2). All of the PCR products of *Atractylodes* rhizome derived from *A. macrocephala* and *A. japonica* were not digested by *Fau*I or *Msp*I, and were observed as one band (137 bp) on the electrophoretic profile. In *Atractylodes lancea* rhizome, the PCR products of *A. lancea* were digested by *Fau*I only and were observed as the three bands (137, 77, and 62 bp) on the electrophoretic profile. The PCR products of *A. chinensis* were digested by *Msp*I only and were observed as the three bands (137, 91, and 48 bp) on the electrophoretic profile. The PCR products of the hybrid between *A. lancea* and *A. chinensis* were digested by *Fau*I and *Msp*I, and were observed as three bands.

6.4 Discussion

Digestion of PCR products of *A. lancea* and *A. chinensis* by *Fau*I or *Msp*I expected that two bands would be observed on the electrophoretic profile. However, in this study, the PCR products of *A. lancea* and *A. chinensis* produced digestion of the three bands on the electrophoretic profile (Fig. 6.2). According to the expected length of digested band, the shorter two bands were derived from the digestion by *Fau*I or *Msp*I. The longest bands (137 bp) would be derived from the undigested PCR products. These undigested PCR products were not digested under different experimental conditions that the enzyme reaction times were set longer or amount of the enzymes were increased. It has been suggested that the ITS sequences in *A. macrocephala* and *A. japonica* is ancestral phylogenetically from *A. lancea* or *A. chinensis* (Shiba *et al.*, 2006). *Atractylodes lancea* and *A. chinensis* would retain the ancestral minor ITS copy which is indigestible by *Fau*I or *Msp*I.

Though remaining the undigested PCR products, the three bands derived from the digestion of

the PCR products by *Fau*I or *Msp*I were recognized as *A. lancea*, *A. chinensis* and their hybrid. Thus, the discrimination between *Atractylodes* rhizome derived from *A. macrocephala* and *A. japonica*, and *Atractylodes lancea* rhizome derived from *A. lancea* and *A. chinensis* was possible by the PCR-RFLP analysis using *Fau*I and *Msp*I. On the electrophoretic profile after the enzyme reaction by *Fau*I and *Msp*I for the PCR products, one band only was recognized with *Atractylodes* rhizome, 2 or 3 bands were recognized on the reaction of *Fau*I or *Msp*I with *Atractylodes lancea* rhizome.

Additionally, in this PCR-RFLP method, the hybrids between *Atractylodes* rhizome derived from *A. macrocephala* or *A. japonica* and *Atractylodes lancea* rhizome derived from *A. lancea* or *A. chinensis* would be difficult to identify because the electrophoretic profile of the hybrids would show the same three bands observed in *A. lancea* or *A. chinensis* mentioned above.

Consequently, this PCR-RFP method was described as a purity test on crude drugs using genetic information in the general information of Japanese pharmacopoeia (MHLW, 2016f).

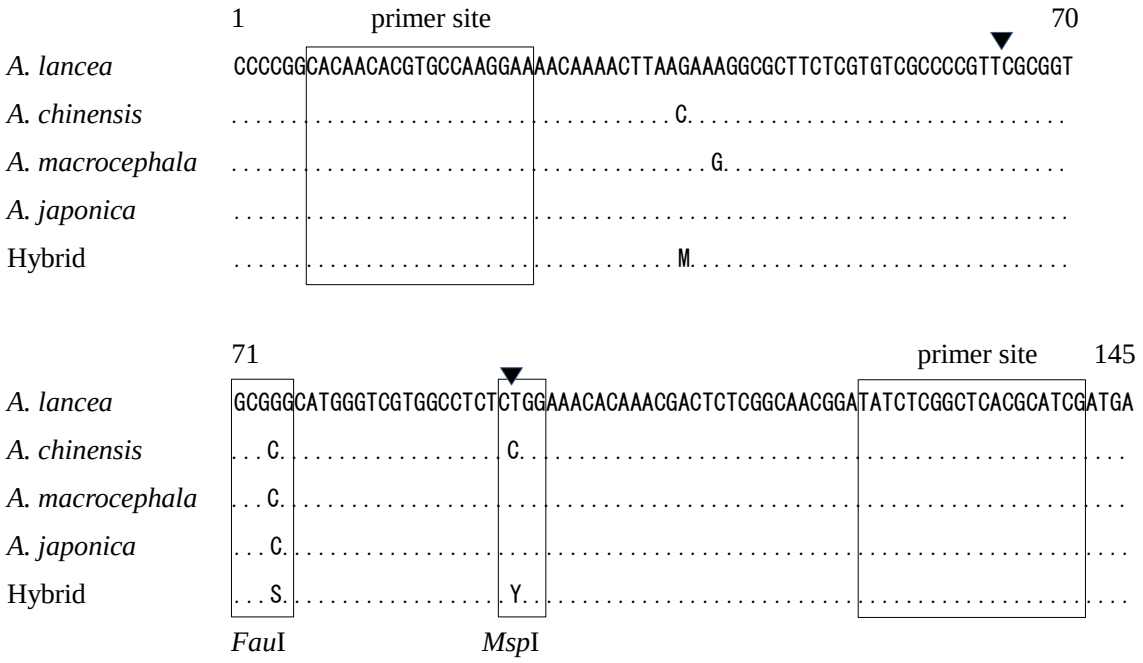


Figure 6.1. Amplified DNA region and recognition sites by *Fau*I and *Msp*I on the ITS sequences in the *Atractylodes* species. The triangle means cut sites by *Fau*I and *Msp*I. Hybrid was a derivative between *A. lancea* and *A. chinensis*.

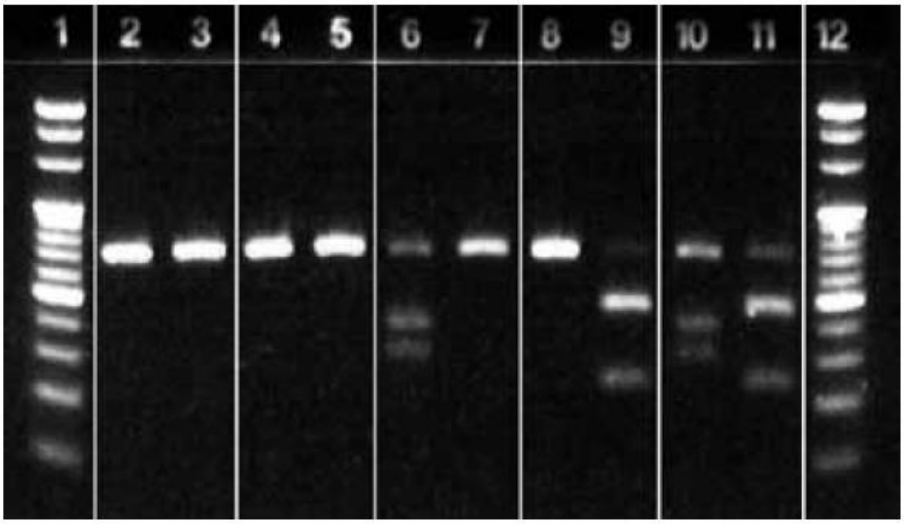


Figure 6.2. Electrophoretic pattern in the *Atractylodes* species. 1, 12: DNA ladder (20 bp). 2, 3: *A. macrocephala* (*Fau*I, *Msp*I). 4, 5: *A. japonica* (*Fau*I, *Msp*I). 6, 7: *A. lancea* (*Fau*I, *Msp*I). 8, 9: *A. chinensis* (*Fau*I, *Msp*I). 10, 11: Hybrids between *A. lancea* and *A. chinensis* (*Fau*I, *Msp*I).

Chapter 7

Exclusion of fake raw materials

Discrimination between Banxia and Tiannanxing based on *rbc*L sequences

7.1 Introduction

Crude drugs of Banxia（半夏, written in Chinese character）, Zhangyebanxia (掌葉半夏, written in Chinese character), Shuibanxia (水半夏, written in Chinese character), and Tiannanxing (天南星, written in Chinese character) are derived from the tubers of *Araceae* species. In the Japanese Pharmacopoeia, *Pinellia ternata* was prescribed as the original plant of Banxia (MHLW, 2016e; Chinese pharmacopeia commission, 2015d). Shuibanxia, Zhangyebanxia, and Tiannanxing have been regarded as tubers of *Typhonium* sp., *Pinellia pedatisecta, and Arisaema* sp., respectively (Jiangsu new medical college, 1977; Institute of materia medica, 1982; Chinese pharmacopeia commission, 2015b). Tiannanxing resembles Banxia very closely and sometimes distributes as Banxia or is mixed within Banxia on the markets. Therefore, in this study, discrimination between Banxia and Tiannanxing by a molecular marker was attempted. A part of *rbc*L sequence was applied as the molecular marker. The original plants of Banxia and Tiannanxing belong different genera and the *rbc*L is known as a useful gene for identification of the higher taxa than species (Soltis *et al*., 1990).

7.2 Materials and methods

Materials

The *rbc*L sequences of 27 plants and 30 crude drug samples relating to Banxia were assayed. The plants were identified from their morphological characters including the original plants of Banxia or Tiannanxing (Table 7.1). The crude drugs examined were Banxia, Zhangyebanxia, Shuibanxia, and Tiannanxing commercially obtained in the markets (Table 7.2). Those crude drugs were grouped into four types according to their appearance (Types A, B, C, and D; Fig. 7.1). Type A tubers were white spheroidal tubers of diameters less than 2 cm. Type B tubers were yellowish brown spheroidal tubers of diameters above 2 cm. Type B tubers usually had some small spheroidal tubers beside the main tubers. Type C tubers were light brown ellipsoidal tubers of diameters less than 2 cm. Type D tubers were light brown or white spheroidal tubers of diameters above 2 cm.

DNA extraction

DNA was extracted from fresh leaves or herbal specimens of about 4cm^2 and the crude drugs of about 1 g by grinding with 1 ml of CTAB buffer (2x). These mixtures were centrifuged at 15,000 rpm for 5 minutes and 500 µl of the supernatants were transferred to fresh tubes. These supernatants were mixed with 500 µl of isopropanol. After centrifugation at 15,000 rpm for 5 minutes, the pellets were

vacuum dried and the residue was dissolved in 20 µl TE buffer (Watanabe *et al*., 1989).

DNA amplification and sequencing

The 154 base pairs of the *rbc*L gene between 709 and 836 from the start codon of *Nicotiana tabacum* (Shinozaki *et al.*, 1986) were amplified by PCR. The amplified fragments were including inter-genus substitutions between *Pinellia* and *Arisaema.* PCR primers for the amplifying DNA were 5'-GGG CAT TAC TTG AAT GCT ACT G-3' and 5'-CCA TTG TCT CGG CAA TAA TGA GC-3'. The PCR was performed under the following condition: 94°C for 2 minutes, and 60°C for 3 minutes, for 35 cycles. The amplified DNA fragments were purified by MERmaid Spin Kit (BIO101) and sequenced by using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and 373A DNA sequencer (Applied Biosystems Division, PERKIN ELMER).

7.3 Results

Sequence types of rbcL recognized in plant and crude drug materials

Seven types of the *rbc*L sequences (Type 1–7; Fig. 7.2) were observe from the 57 samples examined.

In the plant materials (Table 7.1), sixteen plants of *Pinellia ternate* had the sequence type 1 and 2 of *rbc*L that would be intraspecific variations. *Pinellia cordata*, *P. pedatisecta*, and *P. tripartita* had the sequence type 3. *Typhonium divaricataum* had the sequence type 5. *Arisaema serratum* had the sequence type 6. Arisaema *amurense*, A. *erubescens*, A. *negishii*, and A. *thunbergii* var. *urashima* had the sequence type 7.

Meanwhile, in the crude drug materials (Table 7.2), eleven of Banxia with the appearance type A had the sequence type 1 and 2 of *rbc*L. Seven of Tiannanxing and a Zhangyebanxia with the appearance type B had the sequence type 3. A Shuibanxia with the appearance type C had the sequence type 4. Three of Banxia with the appearance type A and seven of Tiannanxing with the appearance type D had the sequence type 7.

Additionally, about the crude drugs except Banxia or Tiannanxing, a Shuibanxia with the appearance type C and sequence type 4 would be derived from *Typhonium* species. Seven of Tiannanxing and a Zhangyebanxia with the appearance type B and the sequence type 3 would be Zhangyebanxia derived from *Pinellia pedatisecta*.

Fake raw materials mixed within Banxia

From the results of the plant materials (Table 7.1), *Pinellia ternata* which is original plant of Banxia had the sequence type 1 or 2 of *rbc*L and *Arisaema amurense*, *A. erubescens*, and *A. heterophyllum* those are original plants of Tiannanxing had the sequence type 7. Therefore, in the crude drug materials (Table 7.2), the three crude drugs named Banxia with the appearance type A and the sequence type 7 were presumed that they were not Banxia derived from *Pinellia ternata* but Tiannanxing derived from *Arisaema* species.

In this research, it was recognized that the small sized tubers derived from *Arisaema* species closely resembled to Banxia and were distributed as Banxia (Fig. 7.3). They were produced in Anhui and Guizhou provinces in China. Guizhou province is known as a main producing district of Banxia and Tiannanxing (China Natural Corporation of Traditional Medicament, 1996).

Morphological differences between Small size Tiannanxing and Banxia

The appearances of the small sized Banxia derived from *Arisaema* species identified by their *rbc*L sequences were observed anew in detail. They had a big stem scar and one-lined big root scars (Fig. 7.3). While, Banxia derived from *Pinellia ternata* had comparatively a small stem scar and scatted small root scars.

7.4 Discussion

The discrimination between Banxia and Tiannanxing was possible by comparing their *rbc*L sequences. Consequently, it was revealed that small sized Tiannanxing were distributed as Banxia or mixed within Banxia because the appearance of small sized Tiannanxing resembled to Banxia. In the regions producing both of Banxia and Tiannanxing, careful attention is required to contaminations of Banxia and Tiannanxing each other.

Meanwhile, research of the *rbc*L sequence for all individuals in a crude drug lot is impossible because those are too many. In this case, at first, Banxia and small sized Tiannanxing were discriminated morphologically by reviewing the appearance of the crude drug distinguished by the genetic information. The new morphological characteristics for the discrimination between the both crude drugs were given to the sorting site of the crude drug producing company. Finally, the exclusion of the fake raw materials those were small sized Tiannanxing from Banxia became possible.

Exclusion of fake raw material required not only discrimination of the crude drugs by the genetic information but also translation from the discrimination information to a realistic exclude application on GACP or GMP.

Table 7.1. Sequence types of the *rbc*L gene of plants identified by their morphological features.

Sequences	Species	Locarity	Voucher*
Type 1	*Pinellia ternata* (Thunb.) Breit.	Japan	40251, F
	Pinellia ternata (Thunb.) Breit.	Japan	40252, F
	Pinellia ternata (Thunb.) Breit.	Japan	40253, F
	Pinellia ternata (Thunb.) Breit.	Japan, Ibaraki	39273, F
	Pinellia ternata (Thunb.) Breit.	Japan, Ibaraki	29281, F
	Pinellia ternata (Thunb.) Breit.	Japan, Ibaraki	40255, F
	Pinellia ternata (Thunb.) Breit.	Japan, Shizuoka	40254, F
	Pinellia ternata (Thunb.) Breit.	China, anhui	40257, F
	Pinellia ternata (Thunb.) Breit.	China, Fujian	39276, F
	Pinellia ternata (Thunb.) Breit.	China, Guizhou	40259, F
	Pinellia ternata (Thunb.) Breit.	China, Hubei	40260, F
	Pinellia ternata (Thunb.) Breit.	China, Sichuan	38727, H
	Pinellia ternata (Thunb.) Breit.	China, Sichuan	40258, F
Type 2	*Pinellia ternata* (Thunb.) Breit.	China, Henan	25270, H
	Pinellia ternata (Thunb.) Breit.	China, Yunnan	39274, F
	Pinellia ternata (Thunb.) Breit.	China, Yunnan	40256, F
Type 3	*Pinellia cordata* E. Br.	cultivated**	39280, F
	Pinellia pedatisecta Schott	China, Yunnan	40250, F
	Pinellia tripartita (Blume) Schott	cultivated**	40248, F
Type 5	*Typhonium divaricatum* (L.) Decne	cultivated**	40261, F
Type 6	*Arisaema serratum* (Thunb.) Schott	Japan, Fukushima	39269, F
	Arisaema serratum (Thunb.) Schott	Japan, Fukushima	40294, F
Type 7	*Arisaema amurense* Maxim. subsp. *sobustum* (Engler) Ohashi *et* J. Murata		
		Japan, Niigata	39278, F
	Arisaema erubescens (Wall.) Schott	China, Sichuan	25208, H
	Arisaema heterophyllum Blume	China, Jangxi	25207, H
	Arisaema negishii Makino	cultivated**	39270, F
	Arisaema thunbergii Blume subsp. *urashima* (Hara) Ohashi *et* J. Murata		
		Japan, Kanagawa	39279, F

* Referance number of herbarium specimen of Tsumura & Co. (THS). “F” and “H” represented the DNA extracted from fresh leaves or herbarium specimens, respectively.

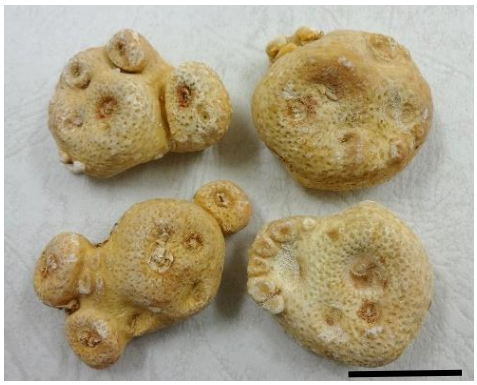
** Culutivated in Tsumura herb garden.

Table 7.2. Sequnce and appearance types of crude drug examined.

Sequence	Trade name	Market	Voucher*	Appearance
Type 1	Banxia	Japna, Iwate	13938	A
	Banxia	China, Gansu	15506	A
	Banxia	China, Hubei	15490	A
	Banxia	China, Shanghai	13536	A
	Banxia	China, Shanghai	15508	A
	Banxia	China, Yunnan	15139	A
Type 2	Banxia	China, Guizhou	15494	A
	Banxia	China, Shanghai	15503	A
	Banxia	China, Yunnan	15498	A
	Banxia	China, Yunnan	13849	A
	Banxia	China, Zhejiang	14542	A
Type 3	Tiannanxing	China, Hebei	13129	B
	Tiannanxing	China, Henan	8218	B
	Tiannanxing	China, Henan	14656	B
	Tiannanxing	China, Henan	15554	B
	Tiannanxing	China, Henan	6855	B
	Tiannanxing	China, Hongkong	14660	B
	Tiannanxing	China, Hongkong	15614	B
	Zhangyebanxia	China, Bejing	6890	B
Type 4	Shuibanxia	China, Guangxi	15252	C
Type 7	Banxia	China, Anhui	15550	A
	Banxia	China, Guizhou	8956	A
	Banxia	China, Guizhou	15553	A
	Tiannanxing	China, Guizhou	10367	D
	Tiannanxing	China, Guizhou	10368	D
	Tiannanxing	China, Guizhou	14657	D
	Tiannanxing	China, Guizhou	15557	D
	Tiannanxing	China, Hongkong	14661	D
	Tiannanxing	China, Sichuan	9707	D
	Tiannanxing	China, Yunnan	7740	D

* Reference number of Herbariu specimen of Tsumura & Co. (THS).

Type A



Type B

Type C

Type D

Figure 7.1. Appearace types of the crude drugs derive from *Araceae* speceis. Bars meant 1 cm.

Figure 7.2. Types of the *rbc*L sequences obtained from the 57 sample examied.

Tiannanxing Banxia

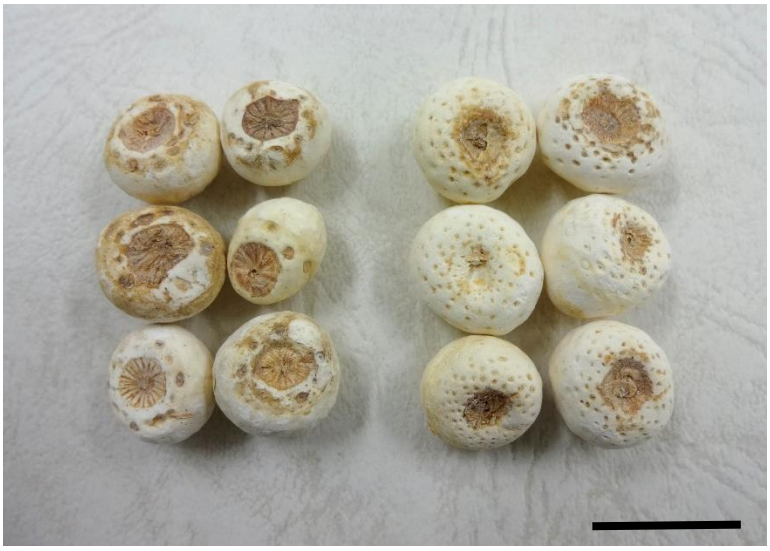


Figure 7.3. Appearaces of small sized Tiannanxing and Banxia. Bar meant 1 cm.

Chapter 8

Operation procedure of species identification on GACP or GMP

Operation procedure of species identification on a suitable GACP
for herbal medicinal product manufacturing

8.1 Introduction

In herbal medicinal product manufacturing, a quality control of a botanical raw material (BRM), including medicinal plants and herbal substances, is essential to ensure consistent quality of herbal medicinal products. In particular, agricultural BRM production processes strongly affect the quality of BRM and can influence the consistency of herbal medicinal products. Hence, implementation of good agricultural and collection practices (GACP) to BRM production is recommended as quality assurance systems (PIC/S, 2017a). GACP guidelines are available from the European medicines agency (EMA, 2006) and the World health organization (WHO, 2003), and from various other agencies.

BRM that are starting materials for herbal medicinal products should comply with good manufacturing practices (GMP). Thus, to confirm that the required controls are present in GACP for herbal medicinal products, I reviewed requirements for GACP in the GMP guidelines provided by the Pharmaceutical inspection convention/Pharmaceutical inspection co-operation scheme (PIC/S), the international council for harmonization of technical requirements for pharmaceuticals for human use (ICH), and the development guidance for herbal medicinal products from the U. S. Food and drug administration (FDA).

In this study, at first, international guides concerning GACP for herbal medicinal product manufacturing were reviewed and quality controls of the GMP guidance with those of the EMA and WHO GACP guidelines were compered. Consequently, a suitable GACP for herbal medicinal products was proposed. Additionally, in the suitable GACP, operation procedure of the species identification for botanical raw material was proposed.

8.2 Reviews of international guides concerning GACP

Requirements for GACP according to GMP guidelines for herbal medicinal products GMP from the PIC/S

Japan became the 45th PIC/S participating authority in July 2014 following special consideration of the globalization of pharmaceutical manufacturing. However, to apply for membership, Japan's GMP inspectorate needs to fulfill PIC/S GMP requirements (Katori, 2014). Moreover, the Japanese ministry of health, labor and welfare (MHLW) announced requirements for application of and compliance with the PIC/S GMP guidance as GMP ministerial ordinances (MHLW, 2012 and 2013).

In Annex 7 of the PIC/S GMP guidance for herbal medicinal products (PIC/S, 2017a), starting materials are defined as medicinal plants, herbal substances, or herbal preparations, and the requirements for GACP are described as follows: Application of GACP from EMA, WHO, or equivalent authorities is recommended, and GACP apply to cultivation, collection, harvesting, cutting, and drying of plants, algae, fungi, lichens, and exudates. In contrast, GMP apply to expression, extraction, purification, concentration, or fermentation of starting materials, and documentation of starting material specifications and processing instructions are required for processes, such as cleaning, drying, crushing, and sifting. Additionally, the manufacturing processes should verify, where appropriate, whether suppliers of starting materials are compliant with GACP.

Pharmaceutical quality systems of ICH and FDA

Pharmaceutical quality system (PQS) is provided by the ICH as quality guideline ICH-Q10 (ICH, 2008) and by the FDA as the guidance for industry (FDA, 2009). The ICH-Q10 section describes a comprehensive model for an effective PQS that is based on quality concepts of the International standards organization (ISO) and includes applicable GMP regulations. Implementation of PQS throughout medicinal product lifecycles should facilitate innovation and continual improvement, and may strengthen links between pharmaceutical development and manufacturing activities. In 2010, the Japanese MHLW announced that ICH-Q10 includes applicable GMP regulations and complements the pharmaceutical developments in ICH-Q8 and the quality risk management strategies in ICH-Q9 (MHLW, 2010; ICH, 2009; ICH, 2005b).

ICH-Q10 provides guidelines for implementation of PQS in frameworks for the following outcomes: (1) pharmaceutical quality system; (2) management responsibility; (3) continual improvement of process performance and product quality; and (4) continual improvement of PQS. Although ICH-Q10 does not specify GACP, the frameworks of PQS should be applicable to GACP for herbal medicinal products.

The botanical drug development guidance from the FDA

The FDA provides a botanical drug development guidance for industry (FDA, 2016) and describes current considerations of the center for drug evaluation and research regarding appropriate development plans for a botanical drugs with potential as new drug application (NDA). The guideline also provides specific recommendations for submission of investigational new drug in support of future NDA submissions for botanical drugs.

This FDA guidance states the requirements for BRMs in each phase of botanical drug development. In particular, quality control of botanical drug products starts with the raw materials and should be described in the NDA. Specific information for medicinal plants should be identified, such as that required for verification of authentic morphology, macroscopic, microscopic, and chemical analyses, and agricultural practices such as cultivation, harvesting, and storage conditions, geographic locations, and collection and processing methods. Accordingly, applicants are required to establish

GACP and summarize related procedures for each of the BRMs during submission of an NDA. Moreover, general GACP principles from the WHO (2003), EMA (2006), and the local regulatory body should be referenced.

Reviews of botanical drugs from the FDA

The FDA approved the botanical drugs Veregen and Fulyzaq in 2006 and 2012, respectively. Veregen is a proprietary extract of *Camellia sinensis* Kuntze and is use as a treatment for genital and perianal warts (Austin, 2006). Fulyzaq is a proprietary extract of the blood-red latex of *Croton lechlerii* Müll, and is a novel indication for HIV-associated diarrhea (Austin, 2013).

Prior to approval of Veregen, the FDA required that all cultivars of BRMs that were used in clinical studies should be identified, and BRMs for future batches should be limited to these cultivars and to the farms that provided the clinical trial material. Moreover, any changes in cultivars or farms of BRMs should be approved by the FDA first. These control measures are designed to reduce variability at the BRM level (Dou, 2006). Because plant growth and composition can be affected by soil, weather, seasonal variations, geographic location, and other agricultural practices, batch-to-batch inconsistency is a common problem. Hence, manufacturers could achieve adequate quality control of botanical starting materials by applying the principals outlined in the FDA botanical guidance and by applying GACP for starting materials of botanical origin (Chen *et al.* 2008).

Prior to approval of Fulyzaq, the botanical review of FDA required sufficient details (*Dou, 2012*) from the WHO GACP guidelines, field manuals on how to identify BRMs, collection procedures, pesticides/defoliant questionnaires, and details for training of field collectors and for storage and transportation of BRMs. In addition, the harvesting area of the BRM was restricted to specific eco-geographic regions (EGRs) to reduce variability at the BRM level. However, because BRMs of Fulyzaq were collected in the wild, more detailed information is required to better define the EGRs, and it was requested that these details include the global positioning system (GPS) coordinates and borders of collection sites (Lee, 2015).

8.3 Control comparison between PIC/S GMP and GACP guidelines

Representative GACP guidelines were provided from the EMA and the WHO in 2006 and 2003, respectively. However, the GMP guidance for medicinal products is updated annually in keeping with newer quality control approaches from the PIC/S and the ICH. Thus, the EMA and WHO GACP guidelines should be revised to ensure compliance with newer quality control approaches. The EMA GACP guideline states that to ensure appropriate and consistent quality of the BRM, GMP for active pharmaceutical ingredients (APIs) are also applicable. Accordingly, the manufacturing guidance for APIs is provided as Part II of the PIC/S GMP guidance (PIC/S, 2017b). Therefore, I compared controls in Part II of the PIC/S GMP guidance with those from EMA and WHO GACP guidelines (Table 8.1).

These comparisons revealed that the GACP guidelines lack eight controls from Part II of the

PIC/S GMP guidance (Table 8.1), including (1) laboratory controls, (2) validation, (3) change control, (4) rejection and re-use of materials, (5) complaints and recalls, (6) specific guidance for APIs manufactured by cell culture/fermentation, (7) agents, brokers, traders, distributors, repackers, and relabellers, and (8) APIs for use in clinical trials. In particular, GACP guidelines stipulate only production processes with minimal quality controls, and lack GMP level approaches to production process and quality controls. These risk management systems and PQS are required for APIs of herbal medicinal products, and relevant controls are detailed in ICH-Q7, Q9, and Q10 (ICH, 2005a, 2005b, and 2008) and should be introduced to GACP for herbal medicinal products.

In the absence of these eight controls, all but the specific guidance for APIs manufactured by cell culture/fermentation should be complementary to GACP controls. Cell culture/fermentation requirements are listed in the "manufacture of biological medicinal substances and products for human use" section of Annex 2 of the PIC/S GMP guidance, but are not listed for herbal medicinal products. Controls that are recommended for GACP in herbal medicine production are presented in Table 8.1.

In the introduction of Annex 7 from the PIC/S GMP guidance, the scope indicates that GACP is to be applied to cultivation, collection and harvesting, cutting, and drying, whereas GMP for APIs are applied to expression, distillation, fractionation, purification, concentration, and fermentation. GMP is applicable to further cutting and drying steps and manufacturers should ensure that these steps are performed in accordance with the marketing authorization and registration. Moreover, it is the responsibility of the manufacturer of the medicinal product to ensure that the appropriate GMP classification is applied.

Part II of the PIC/S GMP guidance includes sections regarding quality risk management, the responsibilities of quality units and production activities, and those of internal audits and product quality reviews. This quality management guideline also includes risk management (ICH-Q9) and PQS (ICH-Q10). In contrast, in the EMA and WHO GACP guidelines, quality agreements between producers and buyers of medicinal plants/herbal substances are the only quality assurances. Hence, risk management and PQS should be included in quality management procedures of GACP.

The EMA and WHO GACP guidelines require control of facilities and equipment for processing after harvesting of BRM to prevent contamination, quality deterioration, and pest damage, but do not provide guidance on computerized systems. Hence, GACP related computerized systems for traceability and other records should be validated as appropriate. In addition, EMA and WHO GACP guidelines require that collectors of wild species must be able to differentiate between collected species and botanically related and/or morphologically similar species to avoid risks to public health.

In terms of documentation and records requirements, all agricultural and collection activities relating to GACP must be recorded, and traceability of starting materials, production, and in-process batches must be recorded completely. Finally, traceability guidelines generally refer to GS1 global traceability standards for healthcare (GS1, 2013).

In materials management sections, particular attention should be paid to the management of seeds and propagation of starting materials for BRM production. Moreover, compliance is required with conventions on international trade in endangered species of wild fauna and flora (CITES) and with the convention on biological diversity (CBD). Finally, endangered medicinal plant species must be sourced only in accordance with national and/or regional legislation (WHO, 2003) and traceability of seeds and cultivars must be managed with approval of their receipt and derivation.

Production instructions should be prepared for production control, and these must include cultivation or collection areas and identify the cultivators and collectors. Accordingly, the FDA recommends using GPS information to identify cultivation or collection areas (Lee, 2015), and tight controls for pesticide use during cultivation are required to ensure the safety of BRM. Hence, the identified cultivators must correctly record pesticide uses.

Neither EMA nor WHO GACP includes guidelines for laboratory controls, validation, change control, rejection and re-use of materials, or complaints and recalls. To comply with appropriate PIC/S GMP guidance, these controls must complement GACP for herbal medicinal products. Moreover, laboratory controls should be applicable to all specifications, sampling plans, and test procedures that are scientifically sound and appropriate for ensuring quality of BRM. Specifically, validation procedures should be applicable to those determined as critical to the quality and purity of the BRM, change control procedures must evaluate all changes that may affect production and control of the BRM, and all rejections and re-uses of starting materials or intermediates of BRMs should be identified and recorded. All quality related complaints, whether received orally or in writing, should be recorded and investigated according to a written procedure. Specifically, the recall procedure must designate personnel to evaluate the information, decide how to initiate recall, who should be informed about the recall, and how the recalled material should be treated.

In terms of contract manufacturers, Annex 7 of the PIC/S GMP guidance stipulates that comprehensive audits of herbal starting material suppliers must be performed by or on behalf of the herbal medicinal product manufacturer, and that the resulting documentation should be made available. Audit trails for active substances are fundamental to the quality of starting materials. Moreover, good agricultural practice (GAP) include audits and certifications that are generally conducted by third parties. Hence, this third-party audit and certification system might be introduced into GACP.

EMA or WHO GACP guidelines do not control agents, brokers, traders, distributors, repackers, or relabellers, and all parties who trade and/or take possession, repack, relabel, manipulate, distribute, or store BRMs other than the original manufacturer should maintain complete traceability of all BRMs and intermediates that they distribute.

Part II of the PIC/S GMP guidance mentions that raw materials of APIs for use in clinical trials must be evaluated by testing, or must be received with a supplier’s analysis and subjected to identity testing. In some instances, the suitability of a raw material can be determined before use based on

acceptability in small-scale reactions.

8.4 A suitable GACP

Applicable GACP guidelines

The EMA or the WHO provide representative GACP guidelines for medicinal plants and other GACP guidelines include those from the Chinese general committee of the state administration of pharmaceutical supervision (2002) and those from the Japan Kampo manufacturing association (JKMA, 2014). The most recent draft of the WHO guidelines on good herbal processing practices (GHPP) for herbal medicines was announced in March 2017 (WHO, 2017), and states that GHPP are integral to GACP for medicinal plants, and to GMP quality assurance and control of herbal medicines. Thus, sections of the WHO GHPP guideline regarding processing of herbs into herbal materials and parts of herbal materials into herbal preparations should be referenced as an available GACP guideline for herbal medicinal products.

Concerning applications of GAP to vegetable and other agricultural products, the GLOBAL G.A.P. was approved by the Global food safety initiative (GFSI) and has become the de-facto standard. GLOBAL G.A.P. adopts an efficient risk management strategy based on the regulation of GFSI. Similarly, the Japanese good agricultural practice (JGAP) is a GAP certification that is specific for Japanese agriculture. However, none of these GAP documents have specific instructions for medicinal plants.

In accordance with GACP guidelines for herbal medicinal products, PIC/S participating authorities and applicants of FDA NDAs are instructed to comply with EMA or WHO GACP guidelines. However, the present comparisons indicate that EMA and WHO GACP guidelines lack some of the controls that are required by the PIC/S GMP guidance for the manufacture of APIs. Additionally, the WHO GHPP guideline was introduced as a newer processing guidance for herbal medicinal products. Therefore, in addition to EMA or WHO GACP guidelines, applicable GACP guidelines for herbal medicinal products should comprise Part II and Annex 7 of the PIC/S GMP guidance and the WHO GHPP guideline.

GACP compliance with PQS (ICH Q10)

In comparisons with the PIC/S GMP guidance, EMA and WHO GACP guidelines lack concepts of PQS. However, PQS are the most important quality control approach for medicinal product manufacturing and should be applied to GACP for herbal medicinal products.

PQS apply to the development and manufacture of APIs and herbal medicinal products throughout the product lifecycle, and ICH-Q10 provides a harmonized model for PQS (ICH, 2008). Hence, implementation of the ICH-Q10 model should result in product realization and establishment and maintenance of a state of control, and should facilitate continual improvement. Moreover, ICH-Q10 augments GMPs or GACPs by describing specific PQS elements and management

responsibilities. Thus, knowledge and quality risk management will enable companies to implement the ICH-Q10 model effectively and successfully. Elements of PQS include (1) process performance and product quality monitoring systems, (2) corrective action and preventive action (CAPA) systems, (3) change management systems, and (4) management review of process performance and product quality.

To assess process performance and product quality, a well-defined system should be applied to assure performance within a state of control, and to identify improvement areas. Accordingly, good process performance on GACP involves suitable conduct of all agricultural and collection activities based on production instructions. To ensure good process performance during production of BRM, the production records should be monitored at control points based on hazard analysis critical control point and quality checks of BRM specifications. Thus, BRM production companies should have systems for implementing CAPA following investigations of complaints, product rejections, non-conformances, recalls, deviations, audits, regulatory inspections and findings, and trends from process performance and quality monitoring of BRMs. Accordingly, the FDA guideline includes an entry-level instruction about CAPA under the heading “Corrective and preventive action basics” in GLOBAL G.A.P.

Change is driven by innovation, continual improvement, and the outputs of process performance, product quality monitoring, and CAPA. To evaluate, approve, and implement these changes properly, companies should have effective change management systems (ICH, 2008). In particular, when production methods and cultivation or collection areas are applied, quality risk management should be used to confirm that the changes do not influence BRM quality. Similarly, after implementation, changes should be assessed to confirm that the objectives were achieved and that BRM quality was maintained. Moreover, a management review should be provided to assure that process performance and product quality are managed throughout the lifecycle of the BRM. Accordingly, senior managers should establish management commitments, quality policies, and quality planning for GACP, and take responsibility for resource management. Finally, quality policies for GACP should be established with consideration of overall quality intentions and directions of the company.

Knowledge management is a systematic approach to acquiring, analyzing, storing, and disseminating information related to BRMs, production processes, and production components. Quality risk management is a proactive approach to identifying, scientifically evaluating, and controlling potential risks to quality. However, risk management strategies are not present in the EMA or WHO GACP guidelines. Thus, the GFSI emphasizes risk management as part of GAP, and advocates proactive application of risk management to suitable GACP for herbal medicinal products.

The EMA and the WHO provide representative guidelines for GACP. However, these GACP guidelines are limited to production processes with minimal quality control, and fail to subscribe GMP levels for production and quality control processes that contribute to risk management and PQS.

Therefore, to implement suitable GACP for herbal medicinal products, applicable GACP guidelines should comprise Part II and Annex 7 of the PIC/S GMP guidance, the EMA or WHO GACP guidelines, and the WHO GHPP guideline. Additionally, it is essential that PQS concepts are complementary to GACP, because they are the most important quality control approaches for medicinal product manufacturing.

The basically management system for the suitable GACP was illustrated in Fig. 8.1. PIC/S GMP part II was applicable as the GACP management policy. Factory, production company of raw materials, and farmer would realize the GACP management policy by using the PQS elements.

8.5 Operation procedure of the species identification in the suitable GACP or GMP

In the suitable GACP, species identification of raw material of herbal medicinal product should be performed in accordance with PQS. The four phases of (1) Pharmaceutical development, (2) Technology transfer, (3) Commercial manufacturing, and (4) Product discontinuation were defined as life cycle of herbal medicinal product in PQS (Fig.8.2). In the phase of pharmaceutical development, scientific name, chemical property, and methods of species identification and purity test for a raw material should be confirmed or configured. In the phase of commercial manufacturing, the species identification or purity test should be examined for raw materials of herbal medicinal products.

Especially, in the phase of commercial manufacturing, the herbal medical product manufacturing would be managed by GACP in famer and production company producing raw materials of herbal products to factory, by GMP in factory manufacturing herbal products, by good distribution practice (GDP), good vigilance practice (GVP), good post-marketing study practice (GPSP) after the herbal product delivery (Fig. 8.3). In GACP, species identification should be examined based on both morphological and genetic information at introduction time of seeds or seedlings in cultivation or determination time of collection site in wild plant collection. The first species identification would be the most effective for the botanical raw material of herbal medicinal product. Next species identification should be examined based on morphological information as quality test at the raw material delivery to factory. Morphological homogeneities of raw materials should be confirmed at the quality test in the production company when species cultivated or collected had been identified accurately at the introduction time. In factory manufacturing herbal medicinal product, species identification of raw materials should be examined as acceptance test based on morphological or as necessary genetic information that recommended by FDA (2016) and the United States pharmacopeia and the national formulary (United States pharmacopeial convention, 2017).

8.6 Discussion

Species identification of BRM using molecular markers is effective showing in the chapter 2–7 in this study. However, in international guide concerning herbal medicinal product manufacturing

reviewed, Botanical drug development guidance for industry (FDA, 2016) only recommended species identification using molecular markers named as DNA fingerprinting (Table 8.2). Organizations concerned herbal medicinal product manufacturing should apply species identification using molecular markers on their GACP or GMP to assure quality of herbal medicinal product.

Table 8.1. Comparisons of control strategies among the Part II of PIC/S GMP guidance, EMA GACP guideline, WHO GACP guideline and a suitable GACP recommended for herbal medicinal product manufacturing.

PIC/S GMP (Part II) (2017)	EMA GACP (2006)	WHO GACP (2003)	A suitable GACP
1. Introduction	1. Introduction 2. General	1. General introduction	Introduction
2. Quality management	3. Quality assurance	Including in 4	Quality management
3. Personnel	4. Personnel and education	Including in 2, 3, and 4	Personnel
4. Buildings and facilities	5. Buildings and Facilities	Including in 4	Buildings and Facilities
5. Process equipment	6. Equipment	Including in 4	Process equipment
6. Documentation and records	7. Documentation	Including in 4	Documentation and records
7. Materials management	8. Seed and Propagation material	Including in 2, 5	Materials management
8. Production and in-process controls	9. Cultivation 10. Collection 11. Harvest 12. Primary processing	2. Cultivation 3. Collection 4. Common technical aspect of GACP	Production and in-process controls 1) Cultivation 2) Collection 3) Harvest 4) Primary processing
9. Packaging and identification labeling of APIs and intermediates	13. Packing	Including in 4	Packaging and identification labeling of BRMs
10. Storage and distribution	14. Storage and distribution	Including in 4	Storage and distribution
11. Laboratory controls			Laboratory controls
12. Validation			Validation
13. Change control			Change control
14. Rejection and re-use of materials			Rejection and re-use of materials
15. Complaints and recalls			Complaints and recalls
16. Contract manufacturers		Including in 4	Contract manufactures
17. Agents, brokers, traders, distributors, repackers and relabellers			Agents, brokers, traders, distributors, repackers and relabellers
18. Specific guidance for APIs manufactured by cell culture/fermentation			
19. APIs for use in clinical trials			BRMs for use in clinical trials
20. Glossary	Glossary	Including in 1	Glossary

Table 8.2. Descriptions concerning species identification of botanical raw material in the international guidelines.

Guideline	Description
WHO GACP (2003)	The botanical identity – scientific name (genus, species, subspecies/variety, author, and family) – of each medicinal plant under cultivation should be verified and recorded.
EMA GACP (2006)	Seeds should be verified botanically, indicating genus, species, variety/cultivar/chemotype and origin and should be traceable.
FDA Botanical Drug Development Guidance for Industry (2016)	Verification of authenticity with morphology, macroscopic and microscopic analysis, and chemical analysis. **DNA fingerprinting** may be warranted in cases of complicated taxonomy and when identification issues related to the botanical raw material exist.
PIC/S GMP Part II for API (2017)	At least one test to verify the identity of each batch of material should be conducted.
PIC/S GMP Annex 7 for herbal medicinal products (2017)	Macro and microscopic examination. Suitable identification tests including, where appropriate, identification tests for constituents with known therapeutic activity, or markers.

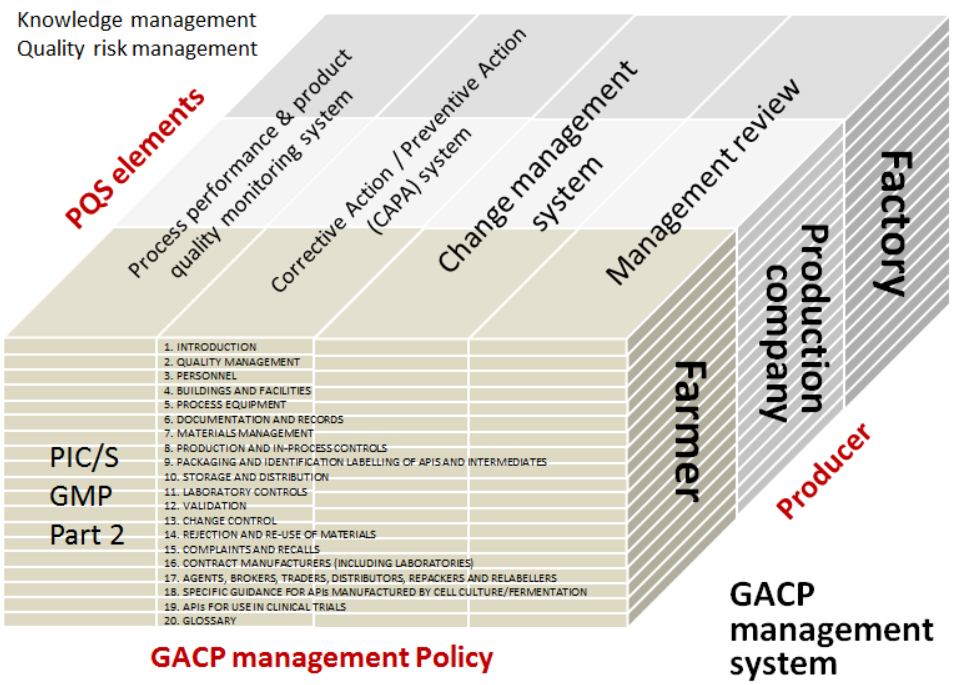


Figure 8.1. Concept of the basically management system for the suitable GACP.

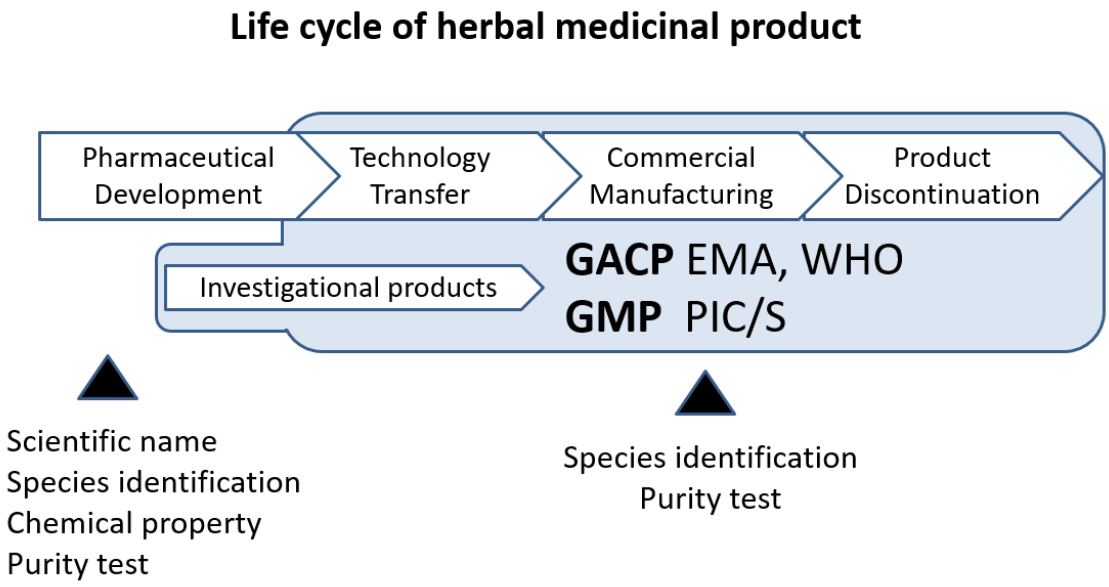


Figure 8.2. Life cycle of a herbal medicinal product and events concerning species identification of botanical raw materials.

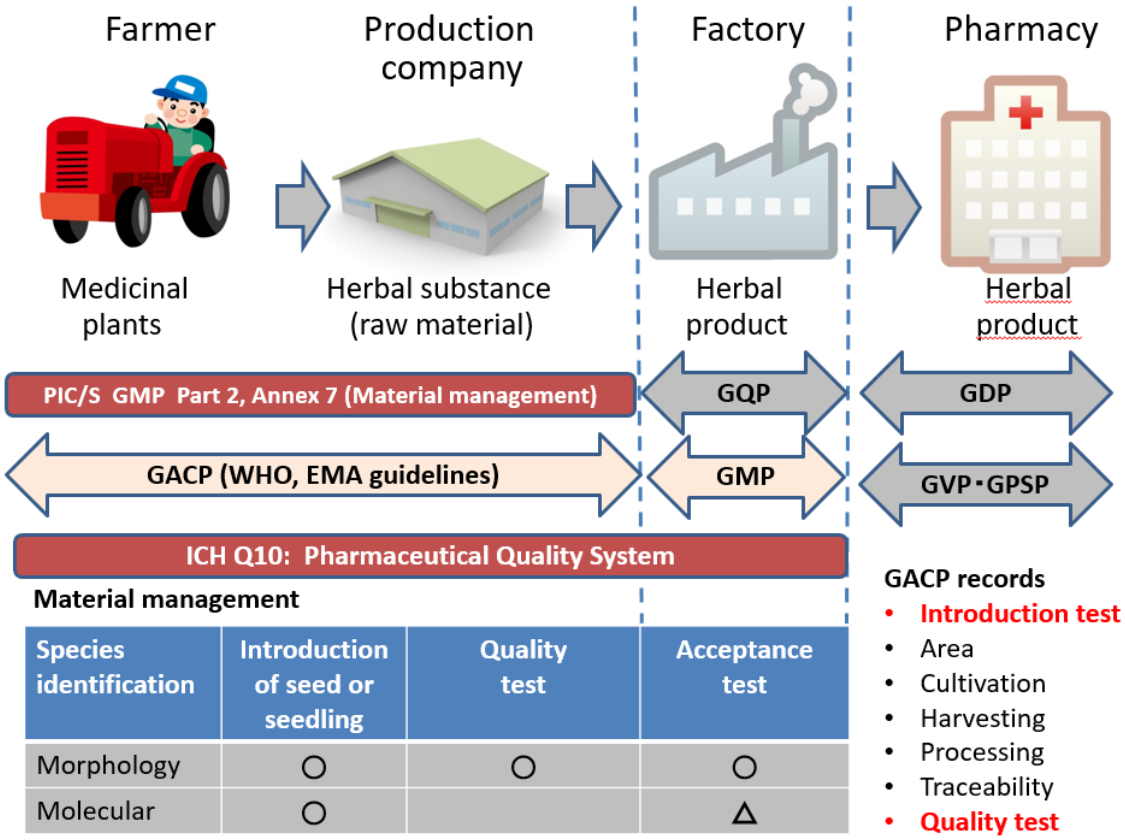


Figure 8.3. Timing of species identification on GACP or GMP.

Chapter 9

General consideration

9.1 Availability assessments for the molecular makers using species identification

Selection of molecular markers for the species identification of botanical raw materials is very important. The ITS sequence on nrDNA was very useful for the species identification of botanical raw materials because it is easy to amplify at many organism species, has relatively many nucleotide polymorphisms (Poczai *et al.*, 2010). In this thesis, ITS was applied to identify *Glycyrrhiza* species in the chapter 3, 4, and 5, or *Atractylodes* species in the chapter 6. ITS on nrDNA was marked by showing two-parental genetic information (Koch *et al.*, 2003). In this thesis, ITS was applied to the cloning analysis to identify parent species of *Cnidium officinale* in the chapter 2, and to detect hybrids within *Glycyrrhiza* species in the chapter 3 and 4, or to detect hybrids within *Atractylodes* species in the chapter 6. Additionally, the ITS sequence which is relatively short length of under 1,000 bp nucleotides and is multi copy could be amplified from the fragmented DNA within dried raw materials. In this thesis, only ITS could be amplified the step-down PCR (Kawakami *et al.*, 1999) from the Shosoin-licorice stored since 756AD but *rbc*L, *mat*K, or *trn*H–*psb*A on cpDNA could not be amplified in the chapter 5. On the other hand, in the chapter 3, *Glycyrrhiza glabra* and *G. inflata* could distinguish by *mat*K but not ITS sequences.

Meanwhile, in this thesis, *rbc*L was applied to detect inter-genus polymorphism within *Apiaceae* in the chapter 2, or within *Araceae* in the chapter 7. The *rbc*L sequences showed inter-genus polymorphism as predicted but showed little interspecific polymorphism. In the chapter 7, *Arisaema amurense*, *A. erubescens*, and *A. heterophyllum* of original plants of crude drug Tiannanxing could not be discriminated by the *rbc*L sequences.

In present consequence, ITS would be the first choice for the species identification of botanical raw materials. The botanical drug development guidance for industry by FDA and the general chapter <563> of United States pharmacopeia recommends ITS, ITS1, ITS2, *matK*, *rbc*L, *psb*A–*trn*H intergenic spacer, *cox*3, *CO*I (also known as *cox*1), external transcribed spacer, 18S, 5S, *trn*L–*trn*F intergenic spacer, and *trn*L intron as molecular markers for botanical identification (FDA, 2016; United States pharmacopeial convention, 2017).

Most importantly, to select molecular markers for species identifications, many individuals of the target and related species identified by their morphological or chemical characteristics and several molecular markers should be observed. Within one species, at least 10 individuals by main distribution

area should be researched because the species would have unexpected parallel intraspecific or interspecific variations such as showed the T-2 genotype of *psb*A–*trn*H in the *Glycyrrhiza* species in the chapter 3 (Fig. 3.2). Additionally, the species-specific like polymorphism of the molecular marker selected from the genetic research of a few individual would have existed within related species. When using molecular markers having high revolution rate like intergenic region, it should be paid attention in those case.

Additionally, the phylogenetic analysis was informative for the selection of species-specific genotype, the estimation of phylogenetic relationship among related species, and the detection ancestral character (Fig. 3.2). A phylogenetic analysis should be applied when a species identification using molecular markers is conducted.

At present, DNA sequencing technology is developing rapidly by the next-generation sequencing technology (NGS). A large amount of DNA sequence can be read in a short time and a low-cost by the next generation sequencers. NGS will make it possible to detect more than ten thousands of single nucleotide polymorphisms between target organisms. However, those numerous polymorphisms might confuse the species identification because the polymorphisms would include many paraphyletic variations that become noises for the species identification. Genes or intergenic regions those were already applied to taxonomic studies like recommended by above the general chapter <563> of United States pharmacopeia (United States pharmacopeial convention, 2017) should be used as molecular markers for the species identification. Because those genetic homogeneities in the target taxa and evolution rates were already known in those taxonomic studies. Meanwhile, the portable DNA sequencer MinION from Oxford Nanopore Technologies is compact and enables a real-time-on-site DNA sequencing in the field (Menogon *et al.*, 2017). It is expected that the species identification of raw materials of herbal medicinal product can be performed in the field by such as the portable DNA sequencer.

Additionally, a genetically detect technology of a few related or fake raw material in a lot of BRM would be needed because, in general, a quality test for BRM on GMP was performed to a extracted, triturated, and mixed BRM sample but not to individuals of BRM. The direct DNA sequencing by using the Sanger's method could not be detect polymorphisms of a few DNA in a mixture because the signal intensity of dNTP fluorescence depends on amounts of the PCR products. In these case, the PCR-RFLP showed in the chapter 6 might be effective as a detection method to confirm an existence of a few DNA in a mixture. At first, the DNA fragments which have a restriction site in prescribed species as BRM are amplified sufficiently by PCR. Next, the DNA fragments are digested by the

restriction enzyme. In the observed undigested DNA fragments by an electrophoresis, an existence of related or fake raw materials in a mixture are suspected. The undigested DNA fragments should be sequenced as appropriate.

9.2 Availability assessments for the species identification using molecular markers for herbal medicinal product manufacturing

Quality assurance of botanical raw material is essential for herbal medicinal product manufacturing because the quality of herbal medicinal product is greatly affected by the quality of the botanical raw material. The quality of botanical raw material for medicinal product manufacturing means the properties of raw material ensuring the medicinal efficacy expected and safety based on the ICH guide. The medicinal efficacy is ensured mainly by the kinds, amounts, and their homogeneities of the medicinal active constituents included in the raw material. The safety is ensured by toxic and contamination control. As a first step to ensure the efficacy and the safety, the accurate species identifications of raw materials are essential because at least species prescribed by a regulation such as Pharmacopoeia should be used. Therefore, in this thesis, the availability of species identification using molecular makers for the quality assurance of raw materials were assessed in some phase of botanical raw material production.

Taxonomic position and scientific name

In the chapter 2, the accurate taxonomic position of crude drug Senkyu (*Cnidium officinale*) was clarified by the *rbc*L sequence of cpDNA. In this result, it was revealed that the Japanese Senkyu belongs genus *Ligusticum* where includes Chinese Senkyu (*Ligusticum chuanxiong*) but not genus *Cnidium* in *Umbelliferae*. Additionally, it was presumed that Japanese and Chinese Senkyu would be hybrids derived from *Ligusticum* species used medicinally by their ITS cloning analysis. Japanese and Chinese Senkyu resemble each other in many morphological and chemical characteristics, too. Understanding the accurate taxonomic position and scientific name of the medicinal plant is very important to get greater understanding the medicinal property of the raw material. Additionally, the *rbc*L sequence data in each genus of *Umbelliferae* which includes many kinds of medicinal plants were accumulated as basic data for species identification.

Multiple origins

In the chapter 3, the species identification of the botanical raw material consisting of multiple origins including hybrid was examined. Licorice consists of *Glycyrrhiza uralensis*, *G. glabra*, and *G. inflata*, and their hybrids. The *Glycyrrhiza* species were identified by the phylogenetic analysis based on the genotypes of the DNA sequences obtained from the four kinds of molecular markers (ITS, *rbc*L,

*mat*K, and *trn*H−*psb*A). Those genotypes include interspecific and intraspecific variations. The phylogenetic analysis suggested that *G. inflata* was closely related with *G. glabra* than *G. uralensis*. The genotypes obtained were mapped and confirmed their distribution areas in China and their expand direction by the phylogenic analysis. The ITS sequence data were effective to identify hybrids because ITS on nrDNA showed two-parent genetic information. It was suggested that the species identification for the botanical raw materials consisting of multiple origins including hybrid could be possible by the combination use of multiple molecular markers.

Chemical properties

In the chapter 4, the nine kinds of constituent contents of 205 *licorice* were researched. Species of those 205 licorices were identified by the genotypes observed in the Chapter 4. In this result, the already-known species-specific constituents were recognized in the species identified by the genotype, too. Additionally, the significant differences of some constituent contents were recognized by the *Glycyrrhiza* species identified by the genotype. It suggested that species identified by the genotype and constituent were linked and genotype selection which means only use of medicinal plants having the specific genotype would lead to improvement of the medicinal efficacy ensured by the kinds, amounts, and their homogeneities of the medicinal active constituents.

Historical study

In the chapter 5, Shosoin-licorice stored in Shosoin since 756AD which is the national treasure. Species of the Shosoin-licorice was identified as *Glycyrrhiza uralensis* by the ITS sequences. ITS of the Shosoin-licorice stored over 1,200 year could be amplified by the step-down PCR (Kawakami *et al.*, 1999). It suggest that species identification using molecular markers would contribute to analyses for the historical important crude drugs represented by the Shosoin medicaments and give new insight into the crude drug having historical value.

Development of an easier discriminating method

In the chapter 6, a discrimination method was developed as a purity test to stop commingling of *Atractylodes lancea* rhizome with *Atractylodes* rhizome. This method applied the PCR-RFLP method without DNA sequencing without a high-priced DNA sequencer. This method is described as comparatively easier purity test using molecular markers in the general information of Japanese pharmacopoeia (MHLW, 2016f).

Exclusion of fake raw material

In the chapter 7, the exclusion of fake raw materials was demonstrated. The discrimination between Banxia and Tiannanxing was possible by comparing their *rbc*L sequences. Consequently, it was revealed that small sized Tiannanxing were distributed as Banxia or mixed within Banxia because the appearance of small sized Tiannanxing resembled to Banxia. Meanwhile, examination of the *rbc*L

sequence for all individuals in a crude drug lot is impossible because those are too many. In this case, appearances of small sized Tiannanxing identified by the *rbcL* sequences were given to the sorting site of the crude drug producing company. Finally, the small sized Tiannanxing were excluded in the sorting site. Exclusion of fake raw material required not only discrimination of the crude drugs by the genetic information but also translation from the discrimination information to a realistic exclude application on GACP or GMP for herbal medicinal product manufacturing.

9.3 Reviewing of GACP and operation procedure of the species identification on GACP or GMP for herbal medicinal product manufacturing

In the chapter 8, a suitable GACP for herbal medicinal product manufacturing was reviewed. In the review, it was suggested that the production control of raw materials based on the GMP for medicinal product part II by PIC/S (2017b) and the pharmaceutical quality system Q10 by ICH (2008) was needed on GACP for herbal medicinal product manufacturing, not but only the EMA (2006) or WHO (2003) guidelines.

In the suitable GACP, species identification of raw material of herbal medicinal product should be performed in accordance with PQS. The four phases of (1) Pharmaceutical development, (2) Technology transfer, (3) Commercial manufacturing, and (4) Product discontinuation were defined as life cycle of herbal medicinal product in PQS.

In the phase of pharmaceutical development, scientific name (the chapter 2 in this thesis), species identification method (the chapter 3 and 7), chemical property (the chapter 4), method of purity test (the chapter 6) for raw materials should be confirmed or developed. In the phase of commercial manufacturing, the species identification or purity test (the chapter 7) should be examined for raw materials of herbal medicinal products. Species identification in this phase should be managed on GACP or GMP complained with material management in PIC/S GMP Part II (2017b). The timings and methods of the species identification examined on GACP or GMA were illustrated in the chapter 8 (Fig. 8.2).

9.4 Conclusion

In quality assurance of botanical raw material, quality means safety and efficacy. Using genetic information obtained from suitable molecular markers, species of botanical raw material could be identified accurately than using morphological or chemical methods. Use of correct species and exclusion of fake raw materials by this species identification using molecular markers prevent toxic and contamination derived from botanical raw materials, and assure predicted kinds, amounts, or homogeneities of medicinal active constituents. Those means that species identification using

molecular makers could be contribute to quality assurance of botanical raw material. The examples were presented in this thesis form the chapter 2 to 7. Therefore, species identification using molecular marker should be applied systematically in suitable timing on life cycle of herbal medicinal product.

The suitable timing and operation procedure of species identification for botanical raw material were indicated in the chapter 8. The confirmation of scientific name or chemical property, and development of method of identification or purity test for a medicinal plant used as a botanical raw material performed out of GACP or GMP. Species identification test or purity test for botanical raw materials are examined on GACP or GMP. However, species identification using molecular marker for all individual in a lot is impossible. Then, the quality assurance of botanical raw material complemented by their production process control on GACP is essential.

Summary

Kampo medicine is a typical herbal medicinal product in Japan. Quality control of herbal medicinal product is difficult than medicinal chemicals because most of their raw materials are made from natural products that are a part of plant, animal, fungi, or mineral, and their qualities greatly affected by various producing environments of cultivation, harvesting, processing, storage, transportation, *etc.*

Quality of medicinal products is defined by safety and efficacy. In raw materials of herbal medicinal products, the safety is ensured by toxic and contamination control and the efficacy is ensured by their chemical properties presented by kinds, amounts, or homogeneities of medicinal active constituents. Quality standards of raw materials are provided by the Japanese Pharmacopoeia (MHLW, 2016a). The standards include origin (species, part, guiding component), description, identification (chemical test), purity, total ash, acid-insoluble ash, etc. In the standard items, species identifications of raw materials are the first order for quality assurance of raw materials. The compliant species must be used as raw materials of herbal medicinal products.

In the 1980s, the molecular phylogeny has progressed at rapid speed with DNA analysis technology. Coupled with that, DNA sequence data of many organisms were accumulated and genetic polymorphisms were observed between species, varieties, or cultivars. It was considered that the accurate species identification was possible by comparing the species-specific DNA polymorphisms of these molecular makers and was helpful for the quality assurance of botanical raw materials (BRMs) of herbal medicinal products.

Therefore, to develop accurate species identification methods using molecular markers and to assess their availability, followings were examined; identification of taxonomic position of *Cnidium* rhizome, species identification of *Glycyrrhiza* roots having multiple origins, chemical properties of *Glycyrrhiza* roots identified species from molecular markers, identification of *Glycyrrhiza* root stored in the Shosoin that is the national treasure in Japan as an application, removal of fake raw materials for Pinellia tuber, and development of an easier discrimination method without DNA sequencing for *Atractylodes* rhizome.

On the other hand, herbal medicinal product manufacturing is controlled on a good manufacturing practice (GMP). However, the most of quality controls of BRMs should be controlled on a good agricultural and collection practice (GACP) because BRMs qualities are affected greatly by their producing environments. Japanese pharmaceutical companies have little GACP experiences but have many GMP experiences. They are currently at the phase of thinking about a way to carry out GACP

control. Therefore, a suitable GACP for herbal medicinal product manufacturing was proposed by reviewing to current international regulations and guidance for herbal medicinal product manufacturing, and that when and how the species identification should be examined in the suitable GACP.

As these results, the accurate taxonomic position of *Cnidium* rhizome was clarified by *rbc*L sequence of cpDNA. The accurate taxonomic position gave correct knowledges concerning medicinal properties of *Cnidium* rhizome.

The three Glycyrrhiza species and their hybrids were distinguished by the genotypes defined from the DNA sequence data of four kinds of molecular markers (*rbc*L, *mat*K, *trn*H−*psb*A and ITS). The interspecific and intraspecific genotype variations of the *Glycyrrhiza* species were observed in China. Additionally, the ITS sequence data was effective to identify the hybrids because the ITS sequence of nrDNA showed the two-parent genetic information.

The nine kinds of constituent contents of *Glycyrrhiza* root were researched by the genotypes observed in the above research. In this result, the three already-known species specific constituents were recognized in the species identified by the sequence data, too. Additionally, the significant differences of the three constituent contents by the three species identified by the genotypes were observed. It suggested that a genotype selection which means only use of medicinal plants having the specific genotype will lead to improvement of the medicinal efficacy ensured by the kinds, amounts, or homogeneities of the medicinal active constituents. Additionally, the *Glycyrrhiza* root stored in the Shosoin was identified *G. uralensis* by the genetic and chemotaxonomic analyses.

The fake raw materials of *Pinellia* tuber could distinguish by the comparing their *rbc*L sequences. However, the species identification using the molecular marker for all botanical raw materials is impossible. In this case, the fake raw materials were exclusive by the feedback of the new species-specific morphological characteristics found by the species identification using the rbcL information to the sorting section of the raw material production site. The removal of fake raw materials will lead directly to improvement of the safety of botanical raw materials.

The specified method of Atractylodes roots was developed by the PCR-RFLP without a high-priced DNA sequencer. This method is described as purity tests on crude drugs using genetic information in the general information of Japanese pharmacopoeia (MHLW, 2016f).

On GACP, the species identification should be conducted to starting materials in cultivation. Contamination of fake raw materials should be prevented at the level of seeds, seedlings using the cultivation. Additionally, when new seeds or seedlings for the cultivation will be introduced, the

species identification must be conducted. About the collection of wild plants, the species identification of the wild plants in specified collection areas should be conducted at first. Collections of wild plants must be permitted only in the specified areas. Farmers or BRM production companies must cultivate or collect the medicinal plants only identified by authorities. Additionally, when BRM will be produced from new cultivation or collection areas, the species identification must be conducted.

On GMP, the species identification using molecular markers should be applied to the acceptance testing on GMP because fake raw materials that are undistinguishable from their morphology must be removed. However, the acceptance testing to all BRMs in a lot is impossible. The assurance of raw material origin complemented by their production process control on GACP is essential.

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