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Discrimination of Xingren from Seeds of *Prunus* Sect. *Armeniaca* Species (*Rosaceae*) by Partial *rpl16* Intron Sequences of cpDNA, and the Botanical Origin of Xingrens in Markets in Japan

Hiroki YAMAJI[a], Kenji KONDO[a], Eiji MIKI[a], Hiroyuki IKETANI[b], Masami YAMAGUCHI[b] and Osami TAKEDA[a]

[a]Botanical Raw Materials Research Department, Botanical Raw Materials Division, Tsumura & Co., 3586, Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki, 300-1192 JAPAN;
[b]National Institute of Fruit Tree Science, Japan, National Agriculture and Food Research Organization, 2-1 Fujimoto, Tsukuba, Ibaraki, 305-8605 JAPAN

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To discriminate the botanical origin of Xingrens derived from seeds of *Prunus* sect. *Armeniaca* species (*P. armeniaca* var. *armeniaca*, *P. armeniaca* var. *ansu*, *P. sibirica*, and *P. mandshurica*) using genetic information, we sequenced a partial region of *rpl16* intron of cpDNA of these taxa, *P. mume*, and *P. persica*. Five genotypes were recognized from the total of 38 materials, all of the materials belonging to the same species had the same genotypes, and the species examined were discriminated from each other except *P. armeniaca* var. *armeniaca* and var. *ansu*. As a result, it was possible to discriminate reliably the botanical origin of Xingrens except the two varieties of *P. armeniaca*. Among 50 materials of Xingren from markets in Japan, 31 materials were identified with *P. armeniaca* var. *armeniaca* or var. *ansu*, 19 materials were identified with *P. sibirica*, and no materials had the other genotypes.

Key words: Markets in Japan, *Prunus* sect. *Armeniaca*, *Prunus armeniaca* var. *armeniaca*, *Prunus armeniaca* var. *ansu*, *Prunus sibirica*, *rpl16* intron, Xingren.

Xingren, a natural medicine prepared from seed of 'apricot' (the generic term of *Prunus* sect. *Armeniaca* species of *Rosaceae*) is used as a cough remedy and for correction of imbalances in body fluid in Kampo medicine (Namba 1993).

In the Japanese Pharmacopoeia (15th ed.; The Ministry of Health, Labor and Welfare 2006), only *Prunus armeniaca* L. var. *armeniaca*, and var. *ansu* Maxim. are prescribed as Xingren. On the other hand, in the Pharmacopoeia of the People's Republic of China (2005) Vol. I (The Pharmacopoeia Commission of People's Republic of China 2005), Xingren is prescribed as Kuxingren; *P. sibirica* L. and *P. mandshurica* (Maxim.) Koehne are also prescribed in addition to the species in the Japanese Pharmacopoeia (15th ed.).

Plants belonging to *Prunus armeniaca* var. *armeniaca* and var. *ansu* originated in central Asia, and are cultivated all over the world (Hormaza 2002). On the other hand, plants belonging to *P. sibirica* and *P. mandshurica* are wild ones, *P. sibirica* is distributed in northern China, eastern Mongolia, eastern Siberia, and Korea, *P. mandshurica* is distributed in northeastern China. These species, except the two varieties of *P. armeniaca*, are clearly distinguishable from each other by morphological characters, e.g., their leaves, fruits and drupes (Lu 1986, Lu

and Bartholomew 2003).

However, these species are hardly distinguishable by the appearance of their seeds only, which are the form of distribution and used for medicine.

Recently nucleotide sequence polymorphisms of coding and non-coding regions of cpDNA and nrDNA have been employed for discriminating the botanical origin of natural medicines so far: Baizhu and Cangzhu (*Atractylodes* spp.; Mizukami et al. 2000, Shiba et al. 2006.), Ginseng (*Panax* spp.; Zhu et al. 2003), Maidong (*Ophiopogon* and *Liriope* spp.; Shiba et al. 2004). Hence, this technique is expected to distinguish the botanical origin of Xingrens.

In *Prunus* species, Bortiri et al. (2001) applied *trnL-F* intergenic region of cpDNA and internal transcribed regions (ITS) of nuclear ribosomal DNA to estimate their phylogenetic relationship. In their study, the three species and one variety of the botanical origin of Xingren were reported to have unique sequences in both *trnL-F* intergenic and ITS regions, respectively. However, in our preliminary observation for these regions, the uniqueness for each taxon could not be reconfirmed.

In our preliminary observation using more than one material for each species, we found that a partial region of the *rpl16* intron of cpDNA was the most variable region among ca. 3600 bp sequences of introns and intergenic regions of cpDNA using the universal primer sets reported by Taberlet et al. (1991) and Nishizawa and Watano (2000). Its comparatively short length was expected to enable PCR amplification of this region with ease in examining comparatively damaged materials such as old or processed materials.

In this study, we obtained the partial *rpl16* intron sequences for the three species and one variety of the botanical origin of Xingren and its allied species to establish a reliable method for discriminating them, and examined the botanical origins of Xingrens distributed in markets in Japan.

Materials and Methods

Materials used in this study

In this study, 88 materials were examined (Tables 1, 2). Among them, 34 materials were possible to be identified taxonomically into the three species and one variety of the botanical origin of Xingren on the basis of morphological characters in Lu (1986) and Lu and Bartholomew (2003). In addition, one of the other species in sect. *Armeniaca*, *P. mume* (n = 2), and one of the species in the other section of *Prunus* (sect. *Amygdalus*), *Prunus persica* (L.) Batsch. (n = 2) were also examined for outgroups. The other three species in sect. *Armeniaca* were not examined because they are distributed or cultivated in limited local areas in China, and are unlikely to be distributed in markets in Japan as Xingren. The remaining 50 materials were ones of natural medicines (Xingrens) from markets in Japan (Table 2). To grasp the outline of variation for as many possible materials within Xingren, one seed from each material was used for analyses.

DNA sequencing

Total DNA was extracted using DNeasy Plant Mini Kit (QIAGEN) from fresh/dried leaf, or dried seed for each material. To detect the partial sequence of the *rpl16* intron, the universal primer set of Nishizawa and Watano (2000), rpl16/F (5'-GTT TCT TCT CAT CCA GCT CC-3') and rpl16/R (5'-GAA AGA GTC AAT ATT CGC CC-3') was used for PCR. The PCR mixture consisted of 10 × Gene Taq Buffer (Nippon Gene) 5 µl, dNTP mix (Nippon Gene) 4 µl, forward primer rpl16/F: 10 pmol/µl) 1 µl, reverse primer (rpl16/R: 10 pmol/µl) 1 µl, Gene Taq (Nippon Gene) 0.25 µl, DMSO 5 µl, D.D.W. 32.5 µl, and template DNA 1.25 µl. PCR cycling condition was as follows: (94ºC, 4 min) × 1 cycle, (94ºC, 1 min; 48

Table 1. Voucher, locality and *rpl16* intron genotype of *Prunus* sect. *Armeniaca* species and *P. persica*

Voucher	Locality	Taxon	Genotype
THS 75420	China, Neimenggu	*P. armeniaca* var. *armeniaca* (n = 13)	**Type 1** (n = 20)
THS 70727	China, Neimenggu		
THS 71036	China, Shaanxi		
THS 73220	China, Shaanxi		
THS 73842	China, Shaanxi		
THS 73573-1	China, Shaanxi		
THS 73220	China, Shaanxi		
THS 15110	China, Shandong		
THS 73841	China, Shanxi		
THS 70970	China, Sichuan		
THS 70947	China, Sichuan		
THS 70959	China, Sichuan		
THS 72196	China, Sichuan		
THS 72482	Japan, Tokyo Market	*P. armeniaca* var. *ansu* (n = 7)	
THS 75571	Japan, Ibaraki		
THS 75565	Japan, Nagano		
THS 72105	China, Heilongjiang		
THS 75399	China, Jilin		
THS 73573-2	China, Shaanxi		
THS 71944	China, Sichuan		
THS 75489	Japan, Ibaraki (cultivated)*	*P. sibirica* (n = 9)	**Type 2** (n = 9)
THS 71990	China, Heilongjiang		
THS 73794	China, Heilongjiang		
THS 73759	China, Heilongjiang		
THS 73760	China, Neimenggu		
THS 70734	China, Neimenggu		
THS 75437	China, Neimenggu		
THS 75426	China, Neimenggu		
THS 52894	China, a market in China		
THS 71987	China, Heilongjiang	*P. mandshurica* (n = 5)	**Type 3** (n = 5)
THS 71989	China, Heilongjiang		
THS 73755	China, Jilin		
THS 70706	China, Jilin		
THS 44092	China, Jilin		
THS 75643	Japan, Yamanashi (cultivated)	*P. mume* (n = 2)	**Type 4** (n = 2)
THS 75573	Japan, Ibaraki (cultivated)		
THS 75574	Japan, Ibaraki (cultivated)	*P. persica* (n = 2)	**Type 5** (n = 2)
THS 75543	Japan, Ibaraki (cultivated)		

* Material obtained from Resources in National Institute of Fruit Tree Science, National Agriculture and Food Research Organization, Japan. Its JP accession number of the NIAS GENEBANK is "JP 174951". (http://www.gene.affrc.go.jp/about_en.php).

Table 2. Voucher, locality and *rpl16* intron genotype in Japanese market materials of Xingren

Voucher	Date	Locality (Market)	DNA type
THS 51094	1966	**(Tokyo Market)**	**Type 1** (*P. armeniaca* var. *armeniaca* or var. *ansu*) (n = 31)
THS 52023	1969		
THS 54079	1970–1980		
THS 55031	1970–1980		
THS 55768	1970–1980		
THS 56106	1970–1980		
THS 56734	1970–1980		
THS 58750	1983		
THS 60155	1985		
THS 60204	1985		
THS 60220	1985		
THS 61172	1988		
THS 63175	1994		
THS 67752	2001		
THS 59910	1984		
THS 61015	1987	Japan, Nagano	
THS 75582	?	Japan	
THS 67419	2001	China, Hebei	
THS 72211	2002		
THS 58749	1983	China, Henan	
THS 72212	2002	China, Shaanxi	
THS 72209	2002	China, Guangxi	
THS 60972	1987	China, Guizhou	
THS 50835	1965	China	
THS 75580	?		
THS 55772	1970–1980	North Korea	
THS 50762	1965	South Korea	
THS 75276	1970–1980	**(Osaka Market)**	
THS 55117	1994		
THS 63249	2004		
THS 75275	2004		
THS 51490	1967	**(Tokyo Market)**	**Type 2** (*P. sibirica*) (n = 19)
THS 51666	1962		
THS 51939	1967		
THS 52022	1969		
THS 53127	1970–1980		
THS 53242	1970–1980		
THS 60154	1985		
THS 63516	1994		
THS 72210	2002		
THS 73221	2003		
THS 58699	1983	China, Hebei	
THS 64829	1996		
THS 72943	2003		
THS 72941	2003		
THS 72939	2003	China, Jilin	
THS 72942	2003		
THS 72938	2003	China, Neimenggu	
THS 72940	2003		
THS 72480	2002	**(Osaka Market)**	

ºC, 2 min; 72ºC, 3 min) × 30 cycles, and (72 ºC, 7 min) × 1 cycle. The PCR products were purified by electrophoresis in 1.0% TAE agarose gel stained with ethidium bromide, and GFX PCR DNA and Gel Band Purification Kit (Amersham biotech). We sequenced the purified PCR products using the BigDye Terminator Cycle Sequencing Kit ver.1.1 and Model 3100 automated sequencer (Applied Biosystems), following the manuf acturer's instructions. For sequencing, we used the same primers as those used for amplification. Sequences were aligned manually. All variant characters were compared in raw data of the automated sequencer.

Results and Discussions

Genotyping of the species examined in the partial *rpl16* intron sequence

Among the 38 materials identified taxonomically, the length of the partial *rpl16* intron region examined varied from 214–222 bp. Within the materials examined in this study, nucleotide substitutions and indels were recognized at five and two sites, respectively (Fig. 1). In combination of the states of the seven sites, five genotypes (types 1–5) were recognized (Table 1).

Type 1 was recognized in *P. armeniaca* var. *armeniaca* and var. *ansu* (GenBank Accession Number AB469163), type 2 was recognized in *P. sibirica* (AB469164), type 3 was recognized in *P. mandshurica* (AB 469165), type 4 was recognized in *P. mume* (AB469166), and type 5 was recognized in *P. persica* (AB469167). All of the materials belonging to the same species had the same genotypes, and the species examined were discriminated from each other. The two varieties of *P. armeniaca* were not discriminated.

Hence sequencing of the partial region of *rpl16* intron was expected to allow identification of the botanical origin of Xingren reliably except between the two varieties of *P. armeniaca*. It seemed to be difficult to discriminate the two varieties of *P. armeniaca*, because they are supposed to be cultivated varieties, and they would have been crossbred for selective breeding of 'apricot'.

Botanical origins of Xingrens in markets in Japan

Fifty materials of Xingren obtained from

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                                    10        20        30        40        50        60        70        80
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Type1 P.armeniaca*    1   TACACGGTGAATAATATACGGAATCGTGGTATTCTTTTAAATTTTATCTAATCTATATCTAT------TATAAATTTTAA 74
Type2 P.sibirica      1   ..............................................................................  74
Type3 P.mandshurica   1   ..............................................................................A..  74
Type4 P.mume          1   ..........................................................ATCTAT.........A..  80
Type5 P.persica       1   ........................A.....................................................  74

                                   90        100       110       120       130       140       150       160
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Type1 P.armeniaca     75  ATTTATTGTGTTTTAATATATAATTAAACATGTCTTCTATTTATAGATAATGTCGTTTTTATATAACGTTATCGTTATAA 154
Type2 P.sibirica      75  ....T.........................C.................................................  154
Type3 P.mandshurica   75  ....T.........................C.................................................  154
Type4 P.mume          81  ....T.........................C.................................................  160
Type5 P.persica       75  ....T.........................C.....................G.........................  154

                                   170       180       190       200       210       220
                          ....|....|....|....|....|....|....|....|....|....|....|....|...
Type1 P.armeniaca     155 TGAATCTTTATTTTCTTTTTCCTTTG--------TATTATCATATCGAATCGACTAAAATTTAGAAAT 214
Type2 P.sibirica      155 ..........................TATTATCA.................................... 222
Type3 P.mandshurica   155 ..........................TATTATCA.................................... 222
Type4 P.mume          161 ..............................................................................  220
Type5 P.persica       155 ..............................................................................  214
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Fig. 1. An alignment of the five *rpl16* intron genotypes recognized in the six taxa examined in this study.
*"*P. armeniaca*" includes *P. armeniaca* var. *armeniaca* and *P. armeniaca* var. *ansu*.

markets in Japan were classified into two DNA types in the partial *rpl16* intron sequences. 31 materials had type 1 sequence, and were identified as *P. armeniaca* var. *armeniaca* or var. *ansu*, 19 materials had type 2 sequence, and were identified as *P. sibirica*, and no materials had types 3–5 or the other sequences (Table 2). With respect to the localities of production, the Xingrens identified as *P. armeniaca* var. *armeniaca* or var. *ansu* were from Japan, Korea, and eastern, southeastern China, and the Xingrens identified as *P. sibirica* were from northeastern China.

Conclusion

In conclusion, a reliable discrimination among the botanical origin of Xingren except between *Prunus armeniaca* var. *armeniaca* and var. *ansu* is possible by the partial *rpl16* intron sequences. In markets in Japan, Xingrens identified as *P. sibirica* were also recognized in addition to those identified as *P. armeniaca* var. *armeniaca* or var. *ansu*, though *P. sibirica* is not prescribed in the Japanese Pharmacopoeia (15th ed.; The Ministry of Health, Labor and Welfare 2006).

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References

Bortiri E., Oh S., Jiang J., Baggett S., Granger A., Weeks C., Buckingham M., Potter D. and Parfitt D. E. 2001. Phylogeny and systematics of *Prunus* (*Rosaceae*) as determined by sequence analysis of ITS and the chloroplast *trnL-trnF* Spacer DNA. Syst. Bot. **26**: 797–807.

Hillis D. M. and Moritz C. 1990. Molecular Systematics. Sinauer Associates Inc., Massachusetts.

Hormaza J. I. 2002. Molecular characterization and similarity relationships among apricot (*Prunus armeniaca* L.) genotypes using simple sequence repeats. Theor. Appl. Genet. **104**: 321–328.

Lu L. 1986. *Armeniaca*. *In*: Yü T. (ed.), Flora Reipublicae Popularis Sinicae **38**: 24–33. Science Press, Beijing.

Lu L. and Bartholomew B. 2003. *Armeniaca*. *In*: Wu Z. Y., Raven P. H. and Hong D. Y. (eds.), Flora of China vol. 9. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Mizukami H., Okabe Y., Kohda H. and Hiraoka N. 2000. Identification of the crude drug atractylodes rhizome (Byaku-jutsu) and atractylodes lancea rhizome (So-jutsu) using chloroplast *trnK* sequence as a molecular marker. Biol. Pharm. Bull. **23**: 589–594.

Namba T. 1993. The Encyclopedia of Wakan-Yaku (Traditional Sino-Japanese medicines) with Color Pictures vol. I, New Completely Revised Edition. Hoikusha Publishing Co. Ltd., Tokyo (in Japanese).

Nishizawa T. and Watano Y. 2000. Primer pairs suitable for PCR-SSCP analysis of chloroplast DNA in angiosperms. J. Phytogeogr. Taxon. **48**: 63–66.

Shiba M., Yamaji H., Kondo K., Ichiki H., Sakakibara I., Terabayashi S., Amagaya S., Aburada M. and Miyamoto K. 2004. Discrimination of maidong derived from *Ophiopogon* and *Liriope* species by *rbcL* sequences, and their chemical components and tuber anatomy. Natural Medicines **58**: 15–21.

Shiba M., Kondo K., Miki E., Yamaji H., Morota T., Terabayashi S., Takeda S., Sasaki H., Miyamoto K. and Aburada M. 2006. Identification of medicinal *Atractylodes* based on ITS sequences of nrDNA. Biol. Pharm. Bull. **29**: 315–320.

Taberlet P., Gielly L., Pautou G. and Bouvet J. 1991. Universal primers for amplification of three noncoding regions of chloroplast DNA. Plant Mol. Biol. **17**: 1105–1109.

The Ministry of Health, Labor and Welfare 2006. Japanese Pharmacopoeia (15th ed.). Tokyo.

The Pharmacopoeia Commission of People's Republic of China 2005. Pharmacopoeia of the People's Republic of China (2005) I: 140. Chemical Industry Press, Beijing.

Zhu S., Fushimi H., Cai S. and Komatsu K. 2003. Phylogenetic relationship in the genus *Panax*: inferred from chloroplast *trnK* gene and nuclear 18S rRNA gene sequences. Planta Med. **69**: 647–653.

山路弘樹[a]，近藤健児[a]，三木栄二[a]，池谷祐幸[b]，山口正己[b]，武田修己[a]：**葉緑体 DNA 中の *rpl16* イントロン部分領域を用いたサクラ属アンズ節（バラ科）に由来する生薬「杏仁」の遺伝子鑑別技術確立と杏仁日本市場品の基原植物調査**

サクラ属アンズ節（ホンアンズ *Prunus armeniaca* var. *armeniaca*，アンズ var. *ansu*，モウコアンズ *P. sibirica* およびマンシュウアンズ *P. mandshurica*）の種子に由来する生薬「杏仁」の遺伝子情報に基づく基原鑑別技術の確立のために，上記 3 種 1 変種に加えてウメ，モモの葉緑体 DNA 中の *rpl16* イントロン部分領域を決定した．その結果38サンプルは 5 遺伝子型に分けられ，それぞれの分類群は同一の配列をもち，ホンアンズとアンズが同一だったのを除けば，互いに区別することが出来た．したがって，ホンアンズとアンズ間の区別が出来ないことを除けば，杏仁の基原鑑別が可能となった．杏仁日本市場品50サンプルのうち，31サンプルはホンアンズないしアンズと鑑別され，19サンプルはモウコアンズと鑑別された．その他の遺伝子型は見出されなかった．

（[a]株式会社ツムラ 生薬本部生薬研究部，[b]独立行政法人 農業・食品産業技術総合研究機構 果樹研究所）