Regular Article

Survey on the Original Plant Species of Crude Drugs Distributed as Cynanchi Wilfordii Radix and Its Related Crude Drugs in the Korean and Chinese Markets

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Cynanchi Wilfordii Radix (CWR) is used in Korea as a substitute for Polygoni Multiflori Radix (PMR), which is a crude drug traditionally used in East Asian countries. Recently, the use of Cynanchi Auriculati Radix (CAR) in place of PMR and CWR has emerged a major concern in the Korean market. In Japan, PMR is permitted to be distributed as a pharmaceutical regulated by the Japanese Pharmacopoeia 17th edition (JP17). Although CWR and CAR have not traditionally been used as medicines, CWR was recently introduced as a health food. The distribution of unfamiliar CWR-containing products could lead to the misuse of original species for PMR and CWR like in Korea. To prevent this situation, the original species of plant products distributed as PMR, CWR, and CAR in the Korean and Chinese markets were surveyed and identified by their genes and components. The results revealed that all two PMR in the Korean market were misapplied as CAR, and that CAR was incorrectly used in eight of thirteen products distributed as CWR in both markets. As PMR is strictly controlled by JP17, the risk of mistaking PMR for CWR and CAR would be low in Japan. In contrast, the less stringent regulation of health food products and the present situation of misidentification of CWR in the Korean and Chinese markets could lead to unexpected health hazards. To ensure the quality and safety of crude drugs, it is important to use the information about the genes and components of these crude drugs.

Key words *Cynanchum wilfordii*; *Cynanchum auriculatum*; *Polygonum multiflorum*; high-performance thin-layer chromatography analysis; genetic polymorphism

Cynanchi Wilfordii Radix (CWR, 白首烏) is defined as the root of *Cynanchum wilfordii* Hemsley (Asclepiadaceae) in the Korean Herbal Pharmacopoeia (KHP)[1]; in Korea, CWR has been used as a substitute for Polygoni Multiflori Radix (PMR, 何首烏). PMR, a root derived from *Polygonum multiflorum* Thunberg (Polygonaceae), has been traditionally used in East Asian countries for restorative effects, detoxification, and as a laxative. It is listed in the Japanese Pharmacopoeia 17th edition (JP17), the Korean Pharmacopoeia (KP), and the Chinese Pharmacopoeia (CP).

CWR is one of the most commonly used ingredients in health foods in Korea and various CWR-containing products exist for the treatment of menopausal symptoms.[2] In April 2015, an investigation into CWR-containing products by the Korea Food and Drug Administration revealed that *Cynanchum auriculatum* Royle ex Wight (Asclepiadaceae) was illegally used for 65% of these products.[3,4] The roots of *C. auriculatum* have been traditionally used as Cynanchi Auriculati Radix (CAR, 耳葉牛皮消) in China, but CAR is now listed as a toxic plant by the U.S. Food and Drug Administration (FDA) because of its ability to initiate miscarriage in female pigs.[5] Because CAR is similar to CWR, the misuse of CWR and CAR is a well-established problem. To assist in the prevention of this misuse, a PCR method for distinguishing CWR from CAR has been reported[6,7] and is prescribed in the KHP.[8] In addition to this problem, CWR is also sometimes misused as PMR because of the similarity in their Korean names of "*Baek Ha Su O*" and "*Ha Su O*," respectively.[9] Thus, the misuse of PMR, CWR, and CAR is a major concern in Korea.

In Japan, PMR is only permitted for use in pharmaceuticals, while classifications of CWR and CAR into pharmaceuticals or non-pharmaceuticals have not been carried out, yet. Therefore, CWR and CAR can be introduced as food materials in the Japanese market although they do not have sufficient history of use in traditional medicines or foods. Indeed, some CWR-containing health foods have already been released in the U.S. market and are likely to be released in the Japanese market in the near future. For example, there is a strong possibility that a clinical study of Japanese people using CWR-containing health foods was for the purpose of obtaining permission to sell these products as "Foods with Function Claims."[10] At present, there have been no reported cases of the misuse of PMR, CWR, or CAR in Japan, an increased transaction volume of CWR in the Japanese health food market could lead to the risks of the misuse of PMR and CAR that have been seen in Korea. As the abuse of PMR in health foods is illegal and

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misidentified CWR could cause unexpected health hazards, it is necessary to develop analytical methods to discriminate between PMR, CWR, and CAR.

To ensure the quality of pharmaceuticals containing natural products, the correct original plant species must be used. The importance of quality control at the raw material stage has been indicated not only for pharmaceuticals, but also for health foods containing natural products. In many countries, it has been reported that some health foods appear to contain natural products that are different from the indicated materials.[11–17] Considering the possibility of the distribution of health foods containing CWR, an unfamiliar material in Japan, it is necessary to investigate the original plant species of CWR distributed in foreign markets. In this study, we collected crude drugs from the Korean and Chinese markets that have been presented as PMR, CWR, and CAR, analyzed their chemical composition and DNA sequences, and identified their original plant species.

MATERIALS AND METHODS

Materials The market samples presented as PMR, CWR, and CAR are shown in Table 1. Products Ko1–7 were purchased at crude drug stores in the Gyeongdong market (Seoul, Korea), and products Ch1–19 were collected from a Chinese market and provided by Japanese crude drug wholesalers. The herbarium specimens of *C. wilfordii* and *C. auriculatum* were provided by the herbarium of The Kochi Prefectural Makino Botanical Garden (MBK) and the vascular plants herbarium, Department of Botany, National Museum of Nature and Science (TNS), respectively (Table 2). The herbarium acronym follows the Index Herbariorum.[18]

Fresh leaves of *P. multiflorum* were supplied by the Takeda Garden for Medicinal Plant Conservation, Kyoto (Takeda Pharmaceutical Co., Osaka, Japan) and the Yamashina Botanical Garden (Nippon Shinyaku Co., Kyoto, Japan) (Table 2). In addition, fresh whole plants were harvested from two sites in Kyushu, Japan for DNA analysis. These plants were morphologically dentified as *C. wilfordii* by Dr. Toshiyuki Atsumi, Kyushu University of Health and Welfare, and Mr. Masato Watanabe, Kumamoto University (Table 2).

DNA Sequence Analysis

Extraction of Total DNA

The weight of the ground roots was measured, and a 20–30-mg portion of tissue was used for DNA extraction. The total DNA of the market products was extracted using a DNeasy® Plant Mini Kit (Qiagen, Valencia, CA, U.S.A.) and QIAcube™ (Qiagen).

Table 1. The Korean and Chinese Market Samples Used in This Study

Product No.	Labeled name	Locality	Market
Ko1	CWR（白首烏）	Unknown	Korea
Ko2	CWR（白首烏）	Korea	
Ko3	CWR（白首烏）	Korea	
Ko4	CWR（白首烏）	Yeongcheon	
Ko5	CWR（白首烏）	Yeongcheon	
Ko6	PMR（何首烏）	Korea	
Ko7	PMR（何首烏）	Korea	
Ch1	PMR（何首烏）	Sichuan	China
Ch2	PMR（何首烏）	Sichuan	
Ch3	CWR（白首烏）	Jiangsu	
Ch4	CAR（異様牛皮消）	Guangxi	
Ch5	PMR（何首烏）	Sichuan	
Ch6	PMR（何首烏）	Sichuan	
Ch7	PMR（何首烏）	Sichuan	
Ch8	CWR（白首烏）	Guangxi	
Ch9	CWR（白首烏）	Jiangsu	
Ch10	CWR（白首烏）	Jiangsu	
Ch11	CWR（白首烏）	Jiangsu	
Ch12	CWR（白首烏）	Jiangsu	
Ch13	CWR（白首烏）	Jiangsu	
Ch14	CWR（白首烏）	Jiangsu	
Ch15	PMR（何首烏）	Guangxi	
Ch16	PMR（何首烏）	Sichuan	
Ch17	PMR（何首烏）	Sichuan	
Ch18	CAR（耳葉牛皮消）	Jiangsu	
Ch19	CAR（耳葉牛皮消）	Jiangsu	

Table 2. Authentic Plant Samples Used in This Study*

Voucher No.	Identified original species	Source	Sample form	GenBank accession no.		
				ITS	*trn*L-*trn*F	*trn*H-*psb*A
NIHS-DPP-40001	*P. multiflorum*	Takeda Garden for Medicinal Plant Conservation, Kyoto (Takeda Pharmaceutical)	Fresh leaves	KY610502	KY610503	—
NIHS-DPP-40002	*P. multiflorum*	Yamashina Botanical Garden (Nippon Shinyaku)	Fresh leaves	LC217191	LC217192	—
NIHS-DPP-10001	*C. wilfordii*	Wild (Miyazaki Prefecture, Japan)	Whole plant	LC217193	LC217197	LC217195
NIHS-DPP-10002	*C. wilfordii*	Wild (Kumamoto Prefecture, Japan)	Whole plant	LC217194	LC217198	LC217196
MBK0147750	*C. wilfordii*	Herbarium of The Kochi Prefectural Makino Botanical Garden (MBK)	Herbarium Specimen	LC217897	LC217909	LC217903
MBK0147752	*C. wilfordii*			LC217898	LC217910	LC217904
MBK0124851	*C. wilfordii*			LC217899	LC217911	LC217905
MBK0098266	*C. wilfordii*			LC217900	LC217912	LC217906
MBK0104147	*C. wilfordii*			LC217901	LC217913	LC217907
MBK0106808	*C. wilfordii*			LC217902	LC217914	LC217908
TNS601490	*C. auriculatum*	Herbarium, Department of Botany, National Museum of Nature and Science (TNS)	Herbarium Specimen	LC217915	LC217917	—
TNS727275	*C. auriculatum*			LC217916	LC217918	—

* The plant species were initially identified based on their morphological characteristics.

Amplification of the Internal Transcribed Spacer (ITS), *trn*L-*trn*F, and *trn*H-*psb*A Regions

We amplified the internal transcribed spacer (ITS) region from the nuclear DNA using ITS5a (5′-CCT TAT CAT TTA GAG GAA GGA G-3′) as the forward primer and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) as the reverse primer.[19,20] Two other primers, *trn*LF-c (5′-CGA AAT CGG TAG ACG CTA-3′) and *trn*LF-f (5′-ATT TGA ACT GGT GAC ACG AG-3′), were used for the amplification of the *trn*L (UAA)-*trn*F (GAA) intergenic spacer (*trn*L-*trn*F) region from chloroplast DNA.[21] The primer pair of TrnHf_05 (5′-CGC GCA TGG TGG ATT CAC AAT CC-3′) and PsbA3_f (5′-GTT ATG CAT GAA CGT AAT GCT C-3′) was also used to amplify the *trn*H-*psb*A intergenic spacer (*trn*H-*psb*A) region from chloroplast DNA.[22,23]

The PCR for the amplification of the ITS, *trn*L-*trn*F and *trn*H-*psb*A regions was performed in a reaction mixture of 25 μL composed of KOD FX Neo (0.5 U; Toyobo, Osaka, Japan), 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 0.2 μM forward and reverse primers, and DNA template (0.5 μL). The temperature cycling program for PCR was as follows: initial denaturation at 94°C for 120 s; followed by 31–40 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 70 s; and a final elongation at 68°C for 70 s. The amplicon size was confirmed by microchip electrophoresis using a MCE202 MultiNA system (Shimadzu, Kyoto, Japan).

Sequence Analysis of PCR Amplicons

Amplified PCR products were purified with the use of a MiniElute® PCR Purification Kit (Qiagen). Purified DNA sequences were determined by Eurofins Genomics (Tokyo, Japan). The obtained DNA sequences from the herbarium specimens and fresh plants were submitted to GenBank (Table 2). The sequences obtained from the crude drugs were used as queries, and identical sequences were confirmed by analysis with the BLAST algorithm from the GenBank database. We also compared the sequences with those of known plant species, in order to determine their original plant species.

High-Performance Thin-Layer Chromatography (HPTLC) Analysis

Chemicals and Apparatus

The HPTLC plates used in this study were HPTLC Silica gel 60 F254 glass plates (20×10 cm; Merck, Darmstadt, Germany). HPTLC was performed with Camag HPTLC equipment (Camag, Muttenx, Switzerland) including a TLC sample applicator, Linomat V (Camag), for spraying samples on the plates, and a TLC Visualizer documentation system (Camag) for capturing the HPTLC images. Compounds were detected by UV irradiation (either 254 or 366 nm) and stained by dilute sulfuric acid (10%, prepared with as described in JP17). 2,3,5,4′-Tetrahydroxystilbene 2-*O*-β-D-glucoside was purchased from Sigma-Aldrich.

Extraction of the Crude Drugs for HPTLC

Each purchased product was ground, dissolved in methanol (0.5 g ground powder in 5.0 mL solvent), and sonicated for 5 min. The extract was filtered, evaporated to dryness, and then redissolved in 1 mL methanol for HPTLC analysis.

Chromatographic Conditions and Detection

Aliquots (3 or 5 μL) of each extract were applied on the HPTLC plates as 8-mm wide bands, which were separated from their neighboring bands by a distance of 2 mm. Plates were developed in a TLC chamber saturated with a mobile phase of ethyl acetate–water–methanol–acetic acid (200 : 10 : 10 : 3). The development length was 7 cm. For the detection of the compounds, HPTLC plates were illuminated with UV 254 and 366 nm, sprayed with dilute sulfuric acid reagent, and heated at 105°C.

RESULTS

Identification of the Original Plant Species of Korean and Chinese Market Products Based on the ITS, *trn*H-*psb*A, and *trn*L-*trn*F Regions Before we analyzed the ITS, *trn*H-*psb*A, and *trn*L-*trn*F regions of the market products, we investigated these regions in authentic plant samples of species that were first identified based on their morphological characteristics (Table 2). The ITS regions of the two authentic plants of *P. multiflorum* Thunberg consisted of 548 nucleotides and showed the highest similarity with that of *P. multiflorum* (GenBank accession no. KR537762) at 99.5–99.8% identity. The *trn*L-*trn*F regions of these authentic plants consisted of 362 nucleotides and were identical to that of *P. multiflorum* (GenBank accession nos. KJ887075 and EU402461). In the case of the eight authentic plant samples morphologically identified as *C. wilfordii* Hemsley, their ITS regions each consisted of 632 nucleotides and showed the highest similarity with that of *C. wilfordii* (GenBank accession no. AY548207), with 99.5–99.8% identity. The *trn*H-*psb*A and *trn*L-*trn*F regions of these eight samples consisted of 380 and 322 nucleotides, respectively, and they were identical to those of *C. wilfordii* (GenBank accession nos. KT220733 and JX028243, respectively). Moreover, the ITS and *trn*L-*trn*F regions of the two authentic plants identified as *C. auriculatum* Royle ex Wight consisted of 632 and 322 nucleotides, and they were identical to that of *C. auriculatum* (GenBank accession nos. EU580717 and JX028242, respectively). However, it was difficult to analyze the DNA sequences of the *trn*H-*psb*A regions derived from authentic plants of *P. multiflorum* and *C. auriculatum*.

Our comparison of the same regions among *P. multiflorum*, *C. wilfordii*, and *C. auriculatum* revealed that the regions of *P. multiflorum* were significantly different from those of the other two species. Although the nucleotide identity between *C. wilfordii* and *C. auriculatum* was 99.1% for the ITS and *trn*L-*trn*F regions, the *trn*H-*psb*A region of *C. wilfordii* was clearly distinguishable from that of *C. auriculatum* by the size and arrangement of the nucleotides (Supplementary Fig. S1).

The results indicated that these three species could be distinguished by the analysis of their ITS, *trn*H-*psb*A, and *trn*L-*trn*F regions. In this study, the original plant species of the Korean and Chinese market products were predicted from the comparisons of their DNA sequences with those from authentic plants (Table 2). However, as *trn*H-*psb*A regions derived from *P. multiflorum* and *C. auriculatum* could not be obtained, those regions of the market products were compared with the sequences submitted to GenBank.

We first performed DNA sequence analysis on seven products presented as PMR and CWR in the Korean market. Their ITS and *trn*L-*trn*F and *trn*H-*psb*A sequences were compared with those of the authentic plant samples to distinguish their original species (Table 3). The results indicated that all regions derived from products Ko6 and Ko7, labelled as PMR, were identical to those from *C. auriculatum* (GenBank accession nos. LC217915, KT220734, and LC217917). The original

plant species of PMR is defined as *P. multiflorum* in the KP and the JP17, which indicated that other species have been mistaken for PMR, such as products Ko6 and Ko7.

Among the Korean market products presented as CWR, products Ko2–5 were thought to be correctly derived from *C. wilfordii*, because their ITS regions shared 99.7–100% identity with the ITS region of *C. wilfordii* (GenBank accession no. LC217897), and their *trn*H-*psb*A and *trn*L-*trn*F regions were identical to those of *C. wilfordii* (GenBank accession nos. LC217903 and LC217909, respectively). However, when we randomly selected four blocks from a package of product Ko1 (the other CWR product purchased in the Korean market) for the DNA analysis, the sequences from three of the blocks were similar to those from *C. wilfordii*, and those from the other block were similar to those from *C. auriculatum*, with each sharing 99.7–100% identity. The results suggest that product Ko1, which was labelled as CWR, was contaminated with CAR.

Subsequently, we performed a DNA sequence analysis of 19 products presented as PMR, CWR, and CAR in the Chinese market (Table 3). Several types of ITS and *trn*L-*trn*F regions were obtained from eight products distributed as PMR (products Ch1, Ch2, Ch5–7, and Ch15–17) and we used the sequences of these products as queries against the GenBank database using the BLAST algorithm in addition to the comparison of the sequences with those of authentic plants (NIHS-DPP-40001 and -40002).

Our findings revealed that all of the ITS and *trn*L-*trn*F regions derived from the eight market products showed the highest similarity (99.1–100% identity) with those from *P. multiflorum*. In the case of *P. multiflorum*, several types of DNA sequences have been submitted to GenBank and the nucleotide identity between the two sequences obtained from authentic the *P. multiflorum* used in this study was approximately 99.3%. These results implied that intraspecific mutations were more likely to occur in *P. multiflorum*. We thus concluded that these market products, presented as PMR in China (*i.e.*, products Ch1, Ch2, Ch5–7, and Ch15–17), are derived from the root of *P. multiflorum*, which is defined as the original plant species of PMR.

Among the Chinese market products presented as CWR, the sequences of products Ch3 and Ch8–14 were identical to those of *C. auriculatum*, the original plant species of CAR in the ITS, *trn*H-*psb*A, and *trn*L-*trn*F regions. In contrast, the sequences of the ITS, *trn*H-*psb*A, and *trn*L-*trn*F regions of product Ch13 (presented as CWR) showed the highest similarity with those of *C. wallichii* (GenBank accession nos. LN896989, LN896868, and LN896761, respectively), with 99.4, 99.7, and 100% identity. Products Ch4 and Ch19, which were distributed as CAR, harbored the same sequences as product Ch13. These results suggest that products Ch4, Ch13, and Ch19 were derived from *Cynanchum* spp. other than *C. wilfordii* and *C. auriculatum*.

The sequences of product Ch18 (presented as CAR) were identical to those of *C. auriculatum*. Our results revealed that no Chinese market product distributed as CWR used in this study was derived from the root of *C. wilfordii*, which is defined as the original plant species of CWR in Korea. DNA

Table 3. The Korean and Chinese Market Products and Their Identified Original Species Based on Their Genetic Sequences

Product No.	Labeled name	Scientific name predicted by labeled name	Identified species based on the genetic sequences
Ko1	CWR（白首烏）	*C. wilfordii*	*C. wilfordii*, *C. auriculatum*
Ko2	CWR（白首烏）	*C. wilfordii*	*C. wilfordii*
Ko3	CWR（白首烏）	*C. wilfordii*	*C. wilfordii*
Ko4	CWR（白首烏）	*C. wilfordii*	*C. wilfordii*
Ko5	CWR（白首烏）	*C. wilfordii*	*C. wilfordii*
Ko6	PMR（何首烏）	*P. multiflorum*	*C. auriculatum*
Ko7	PMR（何首烏）	*P. multiflorum*	*C. auriculatum*
Ch1	PMR（何首烏）	*P. multiflorum*	*P. multiflorum*
Ch2	PMR（何首烏）	*P. multiflorum*	*P. multiflorum*
Ch3	CWR（白首烏）	*C. wilfordii*	*C. auriculatum*
Ch4	CAR（異様牛皮消）	*C. auriculatum*	*Cynanchum* spp.
Ch5	PMR（何首烏）	*P. multiflorum*	*P. multiflorum*
Ch6	PMR（何首烏）	*P. multiflorum*	*P. multiflorum*
Ch7	PMR（何首烏）	*P. multiflorum*	*P. multiflorum*
Ch8	CWR（白首烏）	*C. wilfordii*	—[a)]
Ch9	CWR（白首烏）	*C. wilfordii*	*C. auriculatum*
Ch10	CWR（白首烏）	*C. wilfordii*	*C. auriculatum*
Ch11	CWR（白首烏）	*C. wilfordii*	*C. auriculatum*
Ch12	CWR（白首烏）	*C. wilfordii*	*C. auriculatum*
Ch13	CWR（白首烏）	*C. wilfordii*	*Cynanchum* spp.
Ch14	CWR（白首烏）	*C. wilfordii*	*C. auriculatum*
Ch15	PMR（何首烏）	*P. multiflorum*	*P. multiflorum*
Ch16	PMR（何首烏）	*P. multiflorum*	*P. multiflorum*
Ch17	PMR（何首烏）	*P. multiflorum*	*P. multiflorum*
Ch18	CAR（耳葉牛皮消）	*C. auriculatum*	*C. auriculatum*
Ch19	CAR（耳葉牛皮消）	*C. auriculatum*	*Cynanchum* spp.

Products in which the original species identified by genetic sequences were inconsistent with the species predicted by their labeled names are indicated by a gray background.
a) DNA was not extracted.

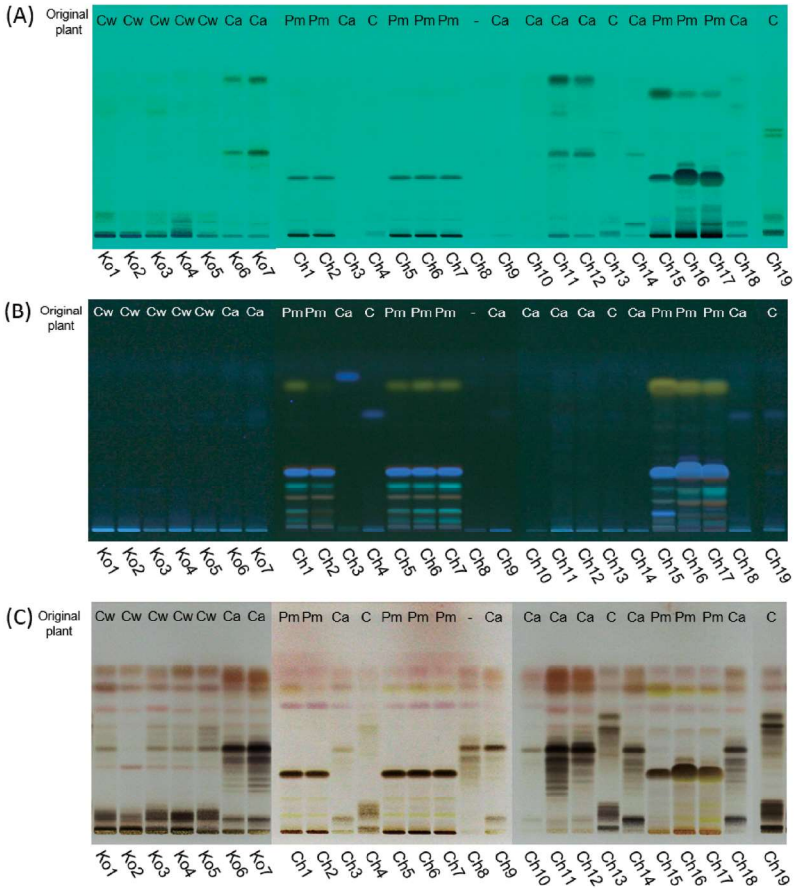

Fig. 1. HPTLC Chromatogram of the Crude Drug Products Available in the Korean and Chinese Markets

HPTLC plates illuminated with UV 254 nm (A) and UV 366 nm (B), sprayed with a dilute sulfuric acid reagent, and heated at 105°C (C). Pm: *P. multiflorum*, Cw: *C. wilfordii*, Ca: *C. auriculatum*, C: unknown species of genus *Cynanchum*. The extract of *C. wilfordii* was classed as product Ko1, which is a mixture of *C. wilfordi* and *C. auriculatum*.

could not be extracted from product Ch8.

Results of the HPTLC Analysis of the Market Products The results of the HPTLC analysis of the market products, in which the original plant species were identified by their DNA sequences, are shown in Fig. 1. Products Ch1, Ch2, Ch5–7, and Ch15–17, the original species of which were presumed to be *P. multiflorum*, showed very similar HPTLC separation patterns. Their HPTLC patterns were clearly distinguished from those of other market products derived from *Cynanchum*.

In the identification test for PMR listed in the JP17, a fluorescent bluish white spot detected under ultraviolet light (the main wavelength is 365 nm) at an *Rf* value of approximately 0.3 is defined as a characteristic marker; this spot is known to be 2,3,5,4′-tetrahydroxystilbene 2-*O*-*β*-D-glucoside.[24,25] This spot was detected in all products predicted to be derived from *P. multiflorum* (Figs. 1, 2), but was not detected in products Ko6 or Ko7, which were identified as derivatives from the root of *C. auriculatum* by their DNA sequences, despite their label of PMR (Fig. 1).

Similar to *P. multiflorum*, the separation patterns of the products derived from *C. wilfordii* (products Ko1–5) were similar to each other in the HPTLC analysis, and they did not correspond to those of *C. auriculatum* (products Ko6, Ko7, Ch3, Ch9–12, Ch14, and Ch18). Further investigations are in progress to identify compounds detected as spots that are specific to each original plant species.

The HPTLC separation patterns of products Ch4, Ch13, and Ch19, whose species were presumed to be *Cynanchum* spp. other than *C. wilfordii* and *C. auriculatum*, were different from those of the products derived from *C. wilfordii* and *C. auriculatum*. This result supported our DNA analysis that

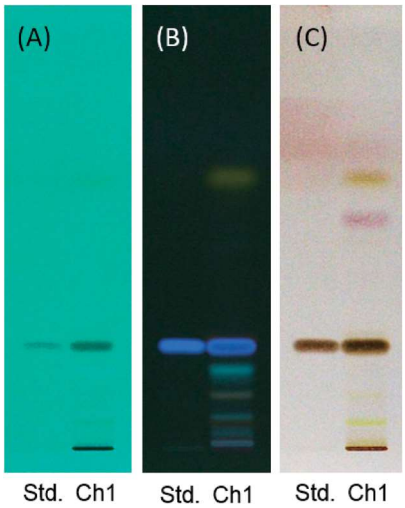

Fig. 2. HPTLC Chromatogram of the Crude Drug Derived from *P. multiflorum* (Product No. Ch1) and an Identification Marker Compound of JP17 Polygoni Multiflori Radix

An HPTLC plate was illuminated with UV 254 nm (A) or UV 366 nm (B), sprayed with a dilute sulfuric acid reagent, and heated at 105°C (C). Std.: 2,3,5,4′-Tetrahydroxystilbene 2-*O*-*β*-D-glucoside, a marker compound for identification of JP17 Polygoni Multiflori Radix.

Table 4. Comparison of Crude Drug Names in Japan, Korea, and China

Scientific names of original plant species	Names of crude drugs		
	Japan	Korea	China
P. multiflorum	Polygoni Multiflori Radix（何首烏）[a]	Polygoni Multiflori Radix（何首烏）[b]	Polygoni Multiflori Radix（何首烏）[c]
C. wilfordii		Cynanchi Wilfordii Radix（白首烏）[d]	Gé shān xiāo（隔山消）[e] Gé shān niú pí xiāo（隔山牛皮消）[e] Bai shou wu（白首烏）[e]
C. auriculatum		I yeob u pi so（耳葉牛皮消）[6] (Cynanchi Auriculati Radix)	Bai shou wu（白首烏）[e] Gé shān xiāo（隔山消）[e]

a) Regulated by the Japanese Pharmacopoeia (JP) 17th edition. *b*) Regulated by the Korean Pharmacopoeia (KP). *c*) Regulated by the Chinese Pharmacopoeia (CP). *d*) Regulated by the Korean Herbal Pharmacopoeia (KHP). *e*) Described in the Dictionary of the Chinese Traditional Medicines.[26,27] Crude drugs listed in JP, KP, KHP, and CP are indicated by a gray background.

confirmed that the original species of products Ch4, Ch13, and Ch19 were neither *C. wilfordii* nor *C. auriculatum*. In this study, the HPTLC separation patterns were different depending on the original species of the products and similar patterns were observed among products derived from the same species. These results indicated that the original species of PMR, CWR, and CAR could be distinguished by their chemical compositions.

DISCUSSION

Our study revealed that some Korean and Chinese market products distributed as CWR were contaminated by roots of *C. auriculatum* and other *Cynanchum* spp., which indicated that the analytical method for discriminating *Cynanchum* species is necessary to prevent the misuse of CWR in Japan. As these species are apparently similar to *C. wilfordii*, a high degree of experience in handling CWR would be required to identify market products by only their morphological features. Most of the previous studies on the quality control of CWR have reported methods for the discrimination of *C. wilfordii* and *C. auriculatum*, the respective original plant species of CWR and CAR. However, in this study, roots of *Cynanchum* spp. that were neither *C. wilfordii* nor *C. auriculatum*, were newly identified in CWR products distributed in the Chinese market (products Ch4, Ch13, and Ch19). More studies are needed to discriminate *C. wilfordii* from other *Cynanchum* spp. grown naturally in China.

One of the reasons why this contamination of the original species for crude drugs occurs is likely to be the ambiguous definitions of CWR and CAR. Unlike *P. multiflorum*, which is defined as the original species for PMR in the JP17, KP, and CP, the corresponding crude drugs of *C. wilfordii* and *C. auriculatum* differ between countries and even between scientific reports. In Korea, CWR is officially defined as the root of *C. wilfordii* by the KHP, whereas the term "白首烏 [bai-shou-wu (CWR)]" can be found in the Dictionary of the Chinese Traditional Medicines, a book that lists crude drugs used in China. This dictionary indicates that the original plant species for 白首烏 is *C. auriculatum* and that "隔山消 [ge-shan-xiao]" is a synonym for 白首烏.[26] The roots of *C. wilfordii* are described as "隔山消 [ge-shan-xiao]" and 白首烏 is listed as a synonym for 隔山消 in the same dictionary[27] (Table 4). Thus, the distinction between CWR and CAR is unclear in China. Indeed, one scientific study from China reported that both *C. wilfordii* and *C. auriculatum* were the original plant species of 白首烏 [bai-shou-wu].[28] Another report stated that all Chinese 白首烏 products were derived from the roots of *C. auriculatum*.[9] Therefore, it is possible that the quality and the original species of 白首烏 vary by the country of production in Korea or China. In addition, as approximately 30% of the CWR used in Korea are Chinese products,[9] our present findings revealed that information about the market from which 白首烏 was purchased cannot be used to predict its original plant species.

Unlike CWR and CAR, PMR is permitted only to be as a pharmaceutical in Japan, and its quality is strictly controlled under the JP17. In the present study, a characteristic marker for the identification of PMR listed in the JP17 was detected only in the market products correctly derived from *P. multiflorum* (Fig. 1B). As the crude drugs derived from *Cynanchum* spp. (*e.g.*, products Ko6 and Ko7) are incompatible with the identification of the test criteria for PMR, the distribution of spurious PMR-containing products would be unlikely to occur while the production of PMR is controlled under the JP17.

The establishment of Good Manufacturing Practices for crude materials is desirable for not only pharmaceuticals, but also for health foods containing natural products, in order to ensure the quality and safety of these products under the less stringent regulation.[29] When crude drugs with limited circulation in the Japanese market (such as CWR) are used as materials for health foods, it is important to confirm the existence of homonymous drugs and to use information on the genes and components of original plant material in addition to their morphological information.

The discrimination of the original plant species by HPTLC and DNA sequence analysis shown in this study was an effective tool to ensure the quality and safety of products containing crude drugs. Further investigations are in progress to identify the compounds detected as spots that were specific to each original plant species in the HPTLC analyses, and to explore characteristic marker components for a liquid chromatography-mass spectrometry analysis for the determination of the plant species. Additionally, microscopic observation is also underway to identify plant species in our research.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

Supplementary Fig. S1. Alignments of the nucleotide sequences derived from *C. wilfordii* and *C. auriculatum*. (A) ITS region from *C. wilfordii* (Cw, GenBank accession no. LC217897) and *C. auriculatum* (Ca, GenBank accession no. LC217915), (B) *trn*H-*psb*A region from *C. wilfordii* (Cw, GenBank accession no. LC217903) and *C. auriculatum* (Ca, GenBank accession no. KT220734), (C) *trn*L-*trn*F region from *C. wilfordii* (Cw, GenBank accession no. LC217909) and *C. auriculatum* (Ca, GenBank accession no. LC217917).

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