DNA Authentication of Plantago Herb Based on Nucleotide Sequences of 18S–28S rRNA Internal Transcribed Spacer Region

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Internal transcribed spacer (ITS) regions of nuclear ribosomal RNA gene were amplified from 23 plant- and herbarium specimens belonging to eight *Plantago* species (*P. asiatica*, *P. depressa*, *P. major*, *P. erosa*, *P. hostifolia*, *P. camtschatica*, *P. virginica* and *P. lanceolata*). Sequence comparison indicated that these *Plantago* species could be identified based on the sequence type of the ITS locus. Sequence analysis of the ITS regions amplified from the crude drug Plantago Herb obtained in the markets indicated that all the drugs from Japan were derived from *P. asiatica* whereas the samples obtained in China were originated from various *Plantago* species including *P. asiatica*, *P. depressa*, *P. major* and *P. erosa*.

Key words Plantago Herb; DNA authentication; internal transcribed spacer; internal transcribed spacer (ITS); ribosomal DNA

Some *Plantago* species (Plantaginaceae) have long been traditionally used as a diuretic, anti-inflammatory and anti-asthmatic drug both in Asia and Europe.[3)] Plantago Herb is defined as dried whole plants of *Plantago asiatica* by the Japanese Pharmacopoeia[4)] while both *P. asiatica* and *P. depressa* are defined as original species of Plantago Herb in the Chinese Pharmacopoeia.[5)] In addition, *P. major*, *P. hostifolia* and *P. erosa* also have been used as crude drugs in China and are distributed in the Chinese crude drug market.[6)] *P. lanceolata* has been used as a herb originated in Europe.[7)] Therefore, it is important to discriminate *P. asiatica*-derived crude drugs from those originated from other *Plantago* species.

Morphological characteristics have been used as markers for identification of *P. asiatica*-derived crude drugs.[8—10)] Chemical identification of the *P. asiatica*-derived crude drugs based on the TLC- and HPLC-profiles of phenylethanoid glycosides such as plantamajoside and acteoside was also made.[11,12)] However, these phenotypic traits vary depending on environmental factors, developmental stage or the intraspecific variation of the plants, which sometimes makes unambiguous identification of the crude drug difficult.

DNA-based polymorphic assay has several advantages over phenotype analysis for correct identification of biological materials because genotypes are directly assayed and, therefore, the results are not affected by the environmental factors. Thus, the most immediate practical application of molecular biology to plants is in plant identification.[13)] Ribosomal RNA gene is an attractive target for molecular analysis, since (1) a large number of copies of these genes are present in the plant genome and (2) the regions encoding 18S-, 5.8S- and 28S rRNA are highly conserved, whereas two internal transcribed spacers (ITS1 and ITS2) between the ribosomal RNA genes are variable and useful as possible sources of polymorphisms for plant identification.[14)]

In the present investigation we compared the nucleotide sequences of the internal trancribed spacer region including ITS1, 5.8S rDNA and ITS2 of nuclear ribosomal DNA amplified from 23 plants belonging to eight *Plantago* species and showed that several polymorphic sites may be used as DNA markers for identification of this species, particularly *P. asiatica*. We applied the DNA sequencing method to the crude drugs in the market, showing that the DNA-based assay is reliable for identification of the crude drug, Plantago Herb.

MATERIALS AND METHODS

Materials Either field-grown plants or herbarium specimens of eight *Plantago* species were used as materials and are listed in Table 1. For DNA authentication, 29 crude drug samples were obtained in either Chinese or Japanese markets during the period between 1993 and 2005 (Table 4). The voucher samples of the herbarium specimens were stored in Gifu Pharmaceutical University and those of the crude drugs in the Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University and in the Department of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences.

Preparation of DNA Plant materials and crude drug samples were pulverized in liquid nitrogen. Total DNA was prepared from the powdered samples (about 100 mg for the fresh plant samples and 20 to 80 mg for the dried specimens and crude drugs) using a DNeasy Plant Mini Kit (Qiagen) according to the protocol supplied by the manufacturer. The DNA preparation from dried samples was further purified with a High Pure PCR Product Purification Kit (Roche). DNA content in the preparation was estimated with a DyNA Quant 200 Fluorometer (GE Healthcare Bio-Sciences) using calf thymus DNA (Sigma) as a standard.

Amplification of ITS Region PCR primers (Pla-rDNA

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# These authors contributed equally to this work.

Table 1. List of Plant Samples Used in the Present Investigation

No.	Plant species	Sample type[a)]	Voucher ID	Place of collection	Date of collection[b)]
1	*Plantago asiatica*	fp		Gifu Pharmaceutical University	2005.4
2	*P. asiatica*	fp		Nagoya City University	2005.4
3	*P. asiatica*	fp		Nagoya City University	2005.4
4	*P. asiatica*	fp		Gifu	2005.5
5	*P. asiatica*	hs	T88-108	Gifu	nd
6	*P. asiatica*	hs	T88-91	Sapporo	1987.1
7	*P. asiatica*	hs	T89-037	Akita	1989.8
8	*P. asiatica*	hs	T83-061	Kochi	1989.9
9	*P. asiatica*	hs	T89-048	Taiwan	1989.9
10	*P. asiatica*	fp		Jiangxi	2006.12
11	*Plantago major*	hs	T88-92	Sapporo	1987.1
12	*P. major*	hs	2047-3	Liaoning	1986.7
13	*P. major*	hs	T88-90	Sapporo	1987.9
14	*Plantago depressa*	hs		Shanxi	nd
15	*P. depressa*	hs	615-1	Shanxi	1983.5
16	*P. depressa* var. *montana*	hs	2030-1007	Inner Mongolia	1987.9
17	*P. depressa*	hs	2033	Liaoning	1988.6
18	*Plantago hostifolia*	hs	2065-1	Hebei	1988.8
19	*Plantago erosa*	hs	000835	Yunnan	1984.9
20	*Plantago camtschatica*	fp		Gifu Pharmaceutical University	2005.5
21	*Plantago virginica*	hs	059	Zhejiang	1988.5
22	*Plantago lanceolata*	fp		Gifu Pharmaceutical University	2005.5
23	*P. lanceolata*	hs	2006	Liaoning	1987.6

a) fp=field plant: hs=herbarium specimen; *b*) nd=no data.

1F and 1R) were designed based on the rRNA gene sequence of *P. major* retrieved from the DDBJ/EMBL/Genbank Nucleotide Sequence Database (accession no. AY101861). Pla-rDNA 1F and Pla-rDNA 1R were designed to anneal to the 3′-end of 18S rRNA gene and 5′-end of 28S rRNA gene, respectively. For some samples it was difficult to amplify the whole ITS region. In such cases internal primers Pla-rDNA 2F and Pla-rDNA 2R annealing to 5′-end and 3′-end, respectively, of 5.8S rRNA gene were used for PCR. Annealing positions of these PCR primers are shown in Fig. 1. Nucleotide sequences of the primers were as follows: Pla-rDNA 1F=5′-GTTTTCCCAGTCACGACCAAGGTTTCCGTAGGTGAAC, Pla-rDNA 1R=5′-ATTTAGGTGACACTATAGAATACGATATGCTTAAACTCAGCGG, Pla-rDNA 2F=5′-GTTTTCCCAGTCACGACCGTAGCGAAATGCGATAC, Pla-rDNA 2R=5′-ATTTAGGTGACACTATAGAATACGGGCGCAACTTGCGTTCAAA. The underlined sequence in the forward and reverse primers shows the M13 universal forward primer and SP6 primer sequence, respectively, for direct sequencing of the PCR products.

PCR amplification was carried out in a 25 μl reaction mixture consisting of 10 mM Tris–HCl (pH 8.8), 25 mM potassium chloride, 5 mM ammonium sulfate, 2 mM magnesium sulfate, 0.2 mM each dNTP, 0.4 mM of each primer, 0.4 ng/μl template DNA and 0.02 unit/μl *Taq* DNA Polymerase (Roche). Amplification was carried out under the following conditions: precycling at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min, and then final elongation at 72 °C for 5 min. PCR products were detected by ethidium bromide staining following 2% agarose gel electrophoresis using 5 μl of the PCR mixture.

Sequencing of the PCR Products Usually a 10 μl aliquot of the PCR mixture was incubated with 5 μl of ExoSAP-IT solution (GE Healthcare Bio-Sciences) at 37 °C for 15 min and then at 80 °C for 15 min. A 7 μl aliquot of the resulting mixture was used for sequencing reaction using Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare Bio-Sciences) and the nucleotide sequence was determined for both strands with DSQ-2000L (Shimadzu).

Phylogenetic Analysis DNA sequences of the ITS locus were aligned using the Clustal X program.[15] The genetic distances were computed by Kimura's two-parameter method,[16] and the phylogenetic tree was constructed by the Neighbor–Joining method in the PHYLIP software package (Felsenstein J., Phylogeny Inference Package, version 3.5C, University of Washington, Seattle, U.S.A.). The tree was evaluated using the bootstrap test[17] based on 1000 resamplings.

TLC Analysis Powdered crude drug (about 2 g) was briefly refluxed in 10 ml methanol and filtered. A 10 μl aliquot of the filtrate was applied to a silica gel plate which was developed with *n*-butanol–water–acetic acid (7 : 2 : 1) and plantamajoside was detected by spraying 9% ferric chloride solution.

HPLC Analysis Powdered crude drug (about 100 mg) was sonicated in 5 ml of 70% methanol for 30 min at room temperature and filtered. A 10 μl aliquot of the filtrate was applied to HPLC column (Unisil QC18, 5 μm, 4.6×250 mm, GL Sciences) with isocratic elution by methanol–2% acetic acid (35 : 65). The flow rate was 1.5 ml/min and the elution was monitored at 330 nm. The HPLC conditions are identical with those reported in ref. 12.

RESULTS AND DISCUSSION

Sequence Analysis of the ITS Region Amplified from Medicinal *Plantago* Species PCR products containing ITS1, 5.8S rDNA and ITS2 were amplified from all the samples of fresh plants and some herbarium specimens using a PCR primer set (Pla-rDNA 1F and Pla-rDNA 1R) designed

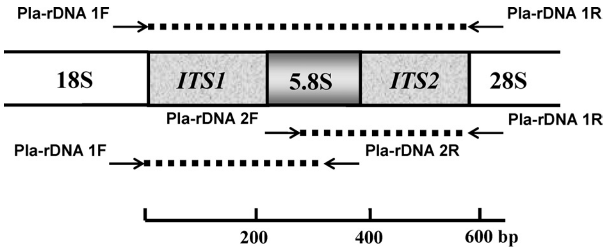

Fig. 1. General Structure of a Nuclear Ribosomal RNA Gene

ITS1 and ITS2 are internal transcribed spacer regions. Annealing positions of PCR primers used in the present investigation are shown by arrows.

based on the rDNA sequence of *Plantago major* retrieved from the DNA databank, and their nucleotide sequences were determined. In some herbarium samples, DNA was highly degraded into small fragments and did not give a PCR product when DNA preparations from these samples were used as templates. In such cases ITS1 and ITS2 were separately amplified using internal primers annealing to 5′- and 3′-end of 5.8S rDNA as shown in Fig. 1. The PCR products were directly applied to sequence reaction following treatment with endonuclease/alkaline phosphatase and their nucleotide sequences were determined.

The size of the ITS1 and ITS2 region was about 220 bp and 190 bp, respectively, varying depending on the species. The size of the 5.8S rDNA was 163 bp, irrespective of species. The nucleotide sequence of the ITS regions of *P. lanceolata* was quite different from other *Plantago* species examined in the present investigation. Among 21 plants belonging to seven other *Plantago* species, 44 nucleotide substitutions and four insertions/deletions were recognized. The variable positions are summarized in Table 2. Based on differences in these sites 11 sequence types were recognized. All the plant samples of *P. asiatica* collected in Japan exhibited type A1 whereas two *P. asiatica* plants collected in Taiwan and China revealed type A2. The nucleotide sequence of type A1 is completely identical with the sequence of *P. asiatica* var. *densiuscula* collected from various habitats in Japan.[18] The A2 sequence is consistent with that reported for *P. asiatica* collected in Southeast Asia.[19] Two plant samples of *P. major* exhibited genotype M1 and one *P. major* sample from China genotype M2. Three different genotypes were recognized among the *P. depressa* samples (Table 3).

The phylogenetic tree based on the approximately 0.6 kb sequences of the ITS locus showed that *P. asiatica*, *P. hostifolia*, *P. major* and *P. erosa* are closely related and *P. depressa* and *P. camtschatica* form another cluster (Fig. 2).

ITS-Sequence Based Authentication of Plantago Herb Obtained in the Market We have amplified the ITS regions from the crude drugs obtained in the Chinese and Japanese markets and determined their nucleotide sequences (Table 4). Of 29 samples of plantago herb, 10 specimens were from Japan and 19 were from China. All of the crude drugs obtained in Japan exhibited type A1 sequence and were, therefore, identified as *P. asiatica*. Of the crude drugs obtained in Chinese markets, one sample (ShaToc 21) showed type A1 and seven type A2; these were also identified to be *P. asiatica*. Three samples were estimated to be *P. deprssea* with type D1 sequence. There were also two samples having type M2 (*P. major*) sequence. This is consistent

Table 2. Sequence Comparison of the ITS1-5.5S rRNA-ITS2 Region of the Nuclear Ribosomal Gene from Seven *Plantago* Species

Sequence type	9	49	52	54	55	62	64	68	82	84	87	88	94	95	98	104	117	141	154	156	160	164	165	166	177
Type A1	T	T	C	T	C	C	G	C	T	A	C	C	T	G	C	T	G	C	T	T	A	T	A	G	C
Type A2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	G	-	-	-	*
Type A3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	G	-	-	-	*
Type H	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	A	A	G	-	-	-	*
Type M1	*	*	*	*	*	*	*	T	*	G	T	*	*	*	*	*	*	*	A	A	G	-	-	-	*
Type M2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	G	-	-	-	*
Type E	A	*	T	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	A	A	G	-	-	-	*
Type D1	*	G	*	*	*	T	A	*	G	*	*	T	*	A	T	A	*	*	A	A	G	-	-	-	*
Type D2	*	G	*	*	*	T	A	*	G	*	*	T	*	A	T	*	*	*	A	A	G	-	-	-	*
Type D3	*	G	*	*	*	T	A	*	G	*	*	T	*	A	T	*	*	*	A	A	G	-	-	-	T
Type C	*	G	*	*	*	T	A	*	G	*	*	T	*	A	T	*	*	T	A	A	G	-	-	-	*
Type V	*	G	*	C	T	*	*	*	G	*	*	*	*	*	*	A	*	*	A	A	G	-	-	-	*

Sequence type	178	195	201	209	260	261	391	395	407	409	417	422	428	448	453	479	482	520	521	530	537	547	560	583
Type A1	G	T	T	T	C	T	G	T	T	C	T	A	-	-	C	C	T	C	C	T	C	T	C	T
Type A2	*	*	*	*	*	*	*	C	*	*	*	*	-	-	*	*	*	*	*	*	*	*	*	C
Type A3	*	*	*	*	*	*	*	C	*	*	*	C	-	C	*	*	C	*	*	*	*	C	*	C
Type H	*	*	*	*	*	*	*	C	*	*	*	C	-	-	*	*	*	*	*	*	*	*	*	C
Type M1	*	*	*	*	*	*	*	C	*	*	*	C	-	-	*	*	C	*	*	*	*	*	*	C
Type M2	*	*	*	*	*	*	*	C	*	*	*	C	-	-	*	*	C	*	*	*	T	*	*	C
Type E	*	*	*	*	*	*	*	C	*	*	*	C	-	C	*	*	C	*	*	*	*	*	*	C
Type D1	*	*	*	A	*	*	*	C	*	T	*	C	G	-	T	*	C	T	T	A	T	*	*	C
Type D2	*	*	*	A	G	A	*	C	*	T	*	C	G	-	T	T	C	T	T	A	T	*	*	C
Type D3	*	*	*	A	*	*	*	C	*	T	*	C	G	-	T	*	C	T	T	A	T	*	*	C
Type C	T	A	*	A	*	*	T	C	*	T	*	C	G	-	T	*	C	T	T	A	T	*	T	C
Type V	*	*	C	A	*	*	*	C	C	*	C	C	-	-	*	*	C	*	*	*	*	*	*	C

An asterisk indicates the base same asin Type A1 and a hyphon reveals an deletion. The ITS sequence of Type L is quite different from other sequence types and, therefore, not included in the table.

Table 3. ITS Sequence Type of Each Plant Sample

Type	Sample name	DDBJ/EMBL/GenBank accession number
A1	*Plantago asiatica*	AB281164
	1. (Gifu Pharamaceutical University)	
	2. 3. (Nagoya City University)	
	4. 5. (Gifu); 6. (Sapporo); 7. (Akita)	
	8. (Kochi)	
A2	9. (Taiwan); 11. (Jiangxi)	AB296068
M1	*Plantago major*	AB281165
	12. (Sapporo); 14. (Sapporo)	
M2	13. (Liaoning)	AB296071
D1	*Plantago depressa*	AB281168
	15. (Shanxi); 18. (Liaoning)	
D2	16. (Shangxi)	AB296069
D3	*Plantago depressa* var. *montana*	AB296070
	17. (Inner Mongolia)	
H	*Plantago hostifolia*	AB281166
	19. (Hebei)	
E	*Plantago erosa*	AB281167
	20. (Yunnan)	
C	*Plantago camtschatica*	AB281169
	21. (Gifu Pharmaceutical University)	
V	*Plantago virginica*	AB281170
	22. (Zhejiang)	
L	*Plantago lanceolata*	AB281171
	23. (Gifu Pharmaceutical University)	
	24. (Liaoning)	

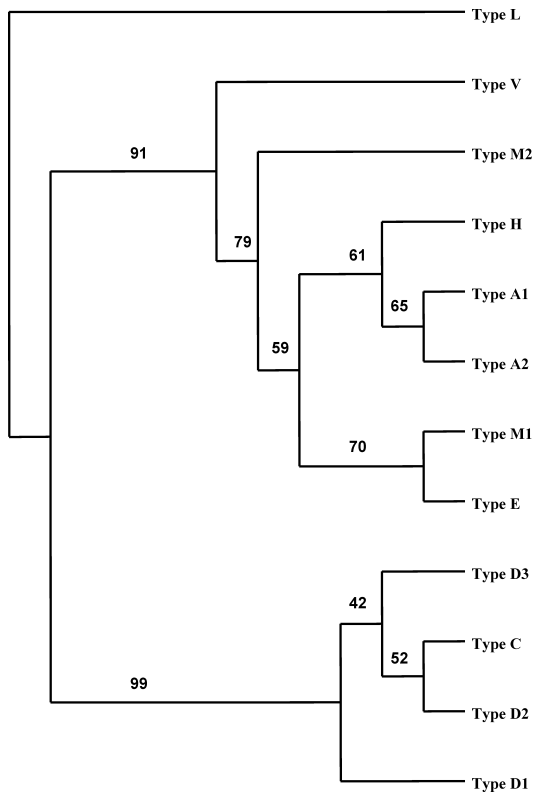

Fig. 2. Phylogenetic Tree of 12 Genotypes of *Plantago* Species Based on the Nucletide Sequence of the ITS Locus

The tree was constructed by Neighbor–Joining method based on the genetic distances calculated using Kimura's two-parameter method. Applying other tree-constructing algorithms such as UPGMA and parsimony methods gave essentially the same topology. The branch length does not reflect genetic difference between two genotypes. The tree is rooted using the genotype L as the outgroup. Values above the branches indicate percent of the bootstrap confidence level from 1000 resamplings.

Table 4. DNA Authentication of Plantago Herb Obtained in the Market

Sample No.	Voucher ID	Place of production or habitat	Date of collection (year.month)	Sequence type	Estimated plant origin
1	ShaUch1	Nagano, Japan	2000.9	A1	*P. asiatica*
2	ShaUch2	Miyazaki, Japan	2003.7	A1	*P. asiatica*
3	ShaUch3	Nagano, Japan	2004.4	A1	*P. asiatica*
4	ShaUch4	Jiangxi	2004.10	A2	*P. asiatica*
5	ShaToc5	Sichuan	2002.6	E	*P. erosa*
6	ShaToc6	Sichuan	2003.5	A2	*P. asiatica*
7	ShaToc7	Miyazaki, Japan	2004.2	A1	*P. asiatica*
8	ShaToc8	Shaanxi	2004.12	A2/M2	*P. asiatica*/*major*
9	ShaToc9	Sichuan	2004.12	E	*P. erosa*
10	ShaUch10	Gunma, Japan	2005.9	A1	*P. asiatica*
11	ShaUch11	Nagano, Japan	2005.9	A1	*P. asiatica*
12	ShaUch12	Niigata, Japan	2005.9	A1	*P. asiatica*
13	ShaUch13	Niigata, Japan	2005.9	A1	*P. asiatica*
14	ShaUch14	Sichuan	2005.9	A2	*P. asiatica*
15	ShaToc15	Miyazaki, Japan	2005.1	A1	*P. asiatica*
16	ShaToc16	Sichuan	2005.12	E	*P. erosa*
17	ShaToc17	Sichuan	2005.12	E	*P. erosa*
18	ShaToc18	Guizhou	2005.12	E	*P. erosa*
19	ShaToc19	Yunnan	2005.12	E	*P. erosa*
20	ShaToc20	Gansu	2005.12	D1	*P. depressa*
21	ShaToc21	Sichuan	2005.12	A1	*P. asiatica*
22	ShaToc22	Shandong	2005.12	D1	*P. depressa*
23	ShaToc23	Hubei	2005.12	M2	*P. major*
24	ShaToc24	Hunan	2005.12	A2	*P. asiatica*
25	ShaToc25	Guangdong	2005.12	A2	*P. asiatica*
26	ShaToc26	Shaanxi	2005.12	D1	*P. depressa*
27	ShaToc27	Zhejiang	2005.12	A2	*P. asiatica*
28	ShaToc28	Anhui	2005.12	M2	*P. major*
29	ShaToc29	Miyazaki, Japan	1999.11	A1	*P. asiatica*

with the result that a herbarium specimen of *P. major* collected in Japan exhibited type M1 while two *P. major* specimens collected in China type M2 (Table 3). Finally six crude drug samples showed type E (*P. erosa*) sequence. It is interesting to note that in one sample from Shaanxi (ShaToc8) two signals with similar intensities were detected at all the plolymorphic sites between type A2 and type M2 (68CT, 84AG, 87CT, 537CT), and this sample was assumed to be derived from a hybrid plant between *P. asiatica* and *P. major*. This is the first result suggesting the presence of the hybrid-derived Plantago Herb in the markets.

Phenotypic Evaluation of the Crude Drugs Twenty crude drug samples were subjected to morphological and chemical evaluation and the results were compared with those obtained by DNA sequencing (Table 5). It has been reported that Plantago Herb could be identified based on various morphological and anatomical characteristics such as presence of tap roots,[8] number of midveins,[9] presence of collenchyma cells in the leaf margin[9] and of non-glandular hair.[10] However, we found no correlation between these anatomical traits and original species of the crude drug estimated by DNA authentication (data not shown) except that all the *P. depressa*-derived crude drug samples (ShaToc20, 22, 26) had tap roots. Detection of a spot corresponding to plantamajoside by TLC has been used as an identification test for Plantago Herb in the Japanese Pharmacopoeia.[4] However, two crude drug samples identified as *P. erosa* (ShaToc5, 19) gave a positive result by this test while no plantamajoside spot could be detected in one of the *P. asiatica*-derived samples with genotype A2 (ShaToc25).

Noro *et al.*[12] analyzed composition of phenylethanoid glycosides in *Plantago* species by HPLC and described that those species with fibrous roots such as *P. asiatica*, *P. major* and *P. hostifolia* contained plantamajoside as a major phenylethanoid while those having tap roots including *P. depressa*, *P. camtschatica* and *P. lanceolata* contained mainly acteoside; these two groups could be discrimiated by the HPLC profile. As shown in Fig. 3, all the *P. depressa*-derived crude drug samples (ShaToc20, 22, 26) revealed a typical HPLC pattern with a major peak corresponding to acteoside (profile III) whereas *P. asiatica*-derived crude drugs with the sequence type A1 (ShaToc7, 15, 21, 29) and two *P. major*-derived crude drug samples (ShaToc23, 28) exhibited a typical profile (Ia) with a main peak corresponding to plantamajoside. These results are consistent with those reported previouly.[12] Of five samples identified as *P. erosa*, three (ShaToc5, 18, 19) exhibited an intermediate HPLC profile (profile II) in which both plantamajoside and acteoside are detected in a similar ratio and one (ShaToc16) profile III. Another *P. erosa* sample (ShaToc17) contained a very low amount of plantamajoside and its HPLC pattern (Profile Ib) is quite similar to that of one *P. asiatica*-derived crude drug with the sequence type A2 (ShaToc25).

These results indicate that unambiguous discrimination of *P. asiatica*-derived crude drugs from those of *P. major* or *P. erosa* origin is difficult based on morphological and chemical evaluation, although identification of Plantago Herb of *P. depressa* origin is possible based on the presence of tap roots and the HPLC profile with a major peak corresponding to acteoside.

In conclusion, the present investigation shows that Plantago Herb obtained in the Chinese markets are derived not only from *P. asiatica* or *P. depressa* but also from *P. major* or

Table 5. Comparison of DNA Identification and Chemical Charcteristics of Plantag

Voucher ID	Estimated plant origin based on sequence type	Tap roots[a)]	TLC detection of plantamajoside	HPLC profile[b)]
ShaToc5	*P. erosa*	NA	+	II
ShaToc6	*P. asiatica* (A2)	NA	+	Ia
ShaToc7	*P. asiatica* (A1)	NA	+	Ia
ShaToc8	*P. asiatica* (A2)/*major*	NA	±	II
ShaToc9	*P. erosa*	NA	−	II
ShaToc15	*P. asiatica* (A1)	NA	+	Ia
ShaToc16	*P. erosa*	NA	±	III
ShaToc17	*P. erosa*	−	−	Ib
ShaToc18	*P. erosa*	−	−	II
ShaToc19	*P. erosa*	−	+	II
ShaToc20	*P. depressa*	+	−	III
ShaToc21	*P. asiatica* (A1)	NA	+	Ia
ShaToc22	*P. depressa*	+	−	III
ShaToc23	*P. major*	−	±	Ia
ShaToc24	*P. asiatica* (A2)	−	+	Ia
ShaToc25	*P. asiatica* (A2)	−	−	Ib
ShaToc26	*P. depressa*	+	−	III
ShaToc27	*P. asiatica* (A2)	NA	±	Ia
ShaToc28	*P. major*	−	±	Ia
ShaToc29	*P. asiatica* (A1)	NA	+	Ia

a) NA=data not available because crude drug sample is either cut into small pieces or lack in underground parts. *b*) Ia=platomajoside was detected as a major peak; Ib=similar to A-I but plantamajoside peak is very small; II=plantmajoside and acteoside are detected in similar ratio; III=acteoside is detected as a major peak.

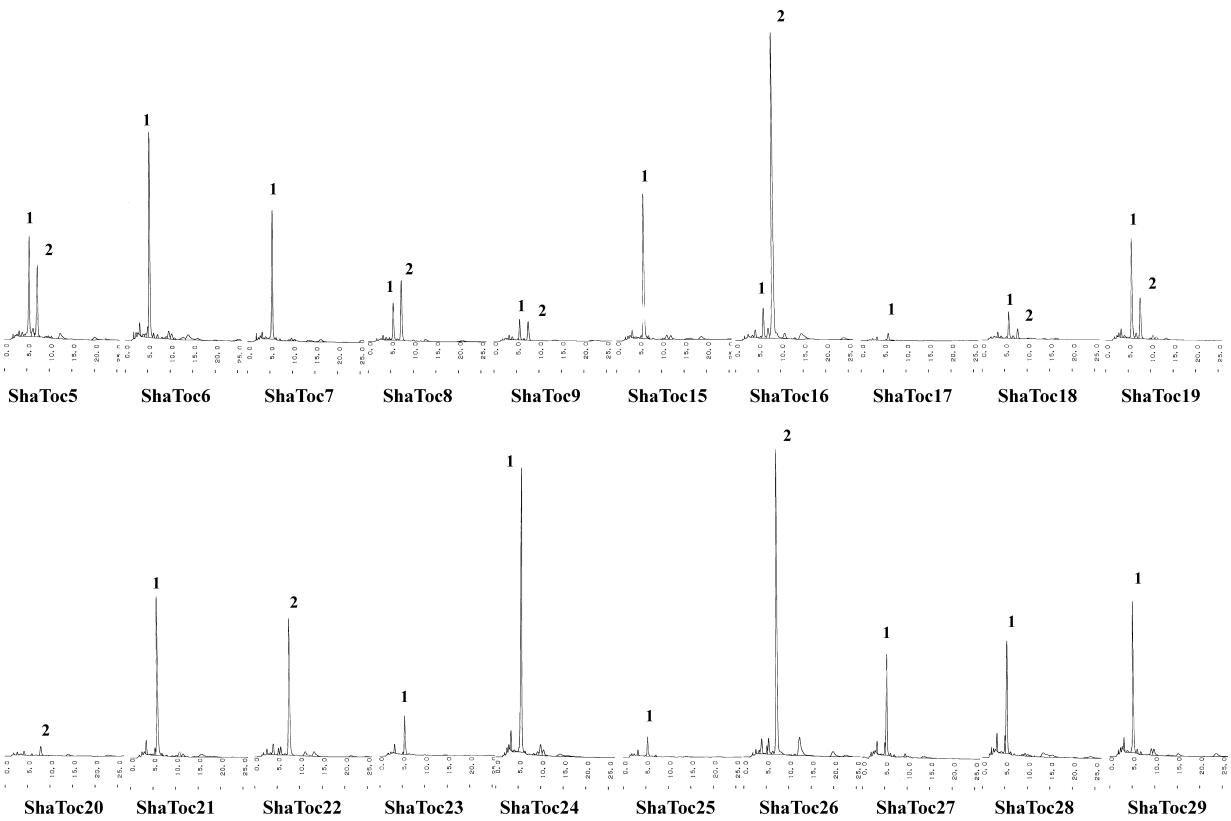

Fig. 3. HPLC Profiles of the Methanol Extract Prepared from 20 Plantago Herb Samples Obtained in the Markets

Peak 2 was identified as acteoside based on Rt values of an authentic sample of acteoside (our laboratory stock). Peak 1 was estimated as plantamajoside according to the previous paper.[12] For the sample IDs see Table 5.

P. erosa. Plantago Herb from these species is sometimes difficult to discriminate from *P. asiatica*-derived crude drugs based on morphological and/or chemical traits. DNA authentication, however, provides us with an efficient tool for identification of the plant origin of the crude drug, Plantago Herb. The method is essentially applicable to authentication of Plantago Seed (seeds of *P. asiatica*), once an efficient protocol for DNA preparation from the crude drugs is established.

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