Phylogenetic Relationship of *Glycyrrhiza lepidota*, American Licorice, in Genus *Glycyrrhiza* Based on *rbcL* Sequences and Chemical Constituents

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Two known saponins, licorice-saponin H2 and macedonoside A, were isolated from the stolons of *Glycyrrhiza lepidota* (American licorice) as major saponins. Since licorice-saponin H2 and macedonoside A are minor saponins isolated from the three glycyrrhizin-producing species (*i.e. G. glabra*, *G. uralensis*, *G. inflata*) and the three macedonoside C-producing species (*i.e. G. macedonica*, *G. echinata*, *G. pallidiflora*), respectively, the present study suggests that *G. lepidota* is an intermediate of both glycyrrhizin-producing and macedonoside C-producing species. The phylogenetic tree constructed from the nucleotide sequences of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*) of these seven *Glycyrrhiza* plants indicated that *G. lepidota* was separated from the other six *Glycyrrhiza* species, and this phylogenetic relationship was in accordance with their saponin compositions.

Key words *Glycyrrhiza lepidota*; *rbcL*; licorice-saponin H2; macedonoside A

The roots and stolons of three *Glycyrrhiza* species, *i.e. Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* FISCH. and *Glycyrrhiza inflata* BATAL., contain large amounts of glycyrrhizin (**1**), an oleanane-type triterpene saponin, which is a well-recognized natural sweetener and pharmaceutical.[1,2] Three other *Glycyrrhiza* species, *i.e. Glycyrrhiza echinata* L., *Glycyrrhiza macedonica* BOISS. *et* ORPH. and *Glycyrrhiza pallidiflora* MAXIM., however, do not produce glycyrrhizin but produce macedonoside C (**2**) as a major triterpene saponin.[3,4] To elucidate the phylogenetic relationship of these six *Glycyrrhiza* plants, we determined the nucleotide sequences of a chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*).[4] Based on these *rbcL* sequences, the six *Glycyrrhiza* species were divided into two groups: the three glycyrrhizin-producing species: *G. glabra*, *G. uralensis*, and *G. inflata*, and the three macedonoside C-producing species: *G. echinata*, *G. macedonica* and *G. pallidiflora*, indicating that the phylogenetic relationship deduced from their *rbcL* sequences is in accordance with the similarity of their chemical constituents.[4]

Glycyrrhiza lepidota (NUTT.) PURSH, American licorice, is the only member of the genus native to North America,[5] and its aerial and underground parts were used by native North Americans medicinally.[5,6] It is reported that *G. lepidota* crosses in different combinations with species of the glycyrrhizin-producing species (*G. glabra* and *G. uralensis*) and macedonoside C-producing species (*G. echinata* and *G. pallidiflora*), suggesting that this American species occupies an intermediate position between the two groups.[7] Chemical constituents of the leaves of *G. lepidota* were elucidated to isolate flavonoids and stilbenoids,[8—10] including two flavanones, pinocembrin and glabranin,[8] which are species-specific constituents in the leaves of *G. glabra*.[11,12] In addition, there have been two preliminary reports about the isolation of saponins from the underground parts of *G. lepidota*,[13,14] but their data have not yet been published. Thus, we first attempted to characterize saponins from the stolons of this species, and then determined its *rbcL* sequence to elucidate the phylogenetic relationship of *G. lepidota* among the genus *Glycyrrhiza*.

RESULTS AND DISCUSSION

Isolation and Characterization of Saponins from Stolons of *G. lepidota* Air-dried stolons of *G. lepidota* collected in Canada were extracted with 70% ethanol, and the extract was subjected to a series of reverse-phase silica gel (ODS) column chromatography, Sephadex LH-20 column chromatography and preparative HPLC to afford two known compounds, **3** and **4**, and they were identified as macedonoside A (**3**)[3] and licorice saponin H2 (**4**)[15] by comparison of their spectral data with published data. The isolation and structural elucidation of compounds **3** and **4** was also preliminarily reported by Mizutani *et al.*,[13] but their data have not yet been published.

HPLC Analysis of Saponins in Stolons of *G. lepidota* Table 1 shows the contents of four saponins, glycyrrhizin (**1**), macedonoside C (**2**), macedonoside A (**3**) and licorice-saponin H2 (**4**) in the stolons ($n=4$) of *G. lepidota*, and Fig. 2 shows its HPLC profile. The saponin contents of **3** and **4** were quite high in the underground parts of *G. lepidota*. In addition, small peaks corresponding to glycyrrhizin (**1**) and macedonoside C (**2**) were also detected in the HPLC profiles of *G. lepidota*, and these peaks were identified by their characteristic UV spectra. It is noteworthy that **1** and **4** are saponins of the glycyrrhizin-producing species (*i.e. G. glabra*, *G. uralensis*, *G. inflata*), and that **2** and **3** are

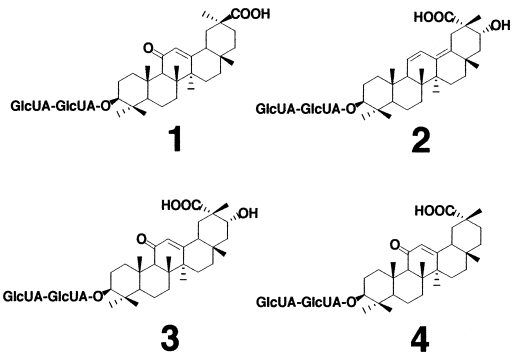

Fig. 1. Structures of Glycyrrhizin (**1**), Macedonoside C (**2**), Macedonoside A (**3**) and Licorice-Saponin H2 (**4**)

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Table 1. Contents of Glycyrrhizin (**1**), Macedonoside C (**2**), Macedonoside A (**3**) and Licorice-Saponin H2 (**4**) in Four Stolons of *G. lepidota* Collected in U.S.A. and Canada

Collection area	Root/Stolon	Diameter (mm)	Contents (% of dry weight) of			
			1	**2**	**3**	**4**
U.S.A.	Stolon	5.3	0.11	0.44	2.11	3.04
U.S.A.	Stolon	5.6	0.12	0.35	2.36	3.21
Canada	Stolon	8.8	0.05	0.33	0.75	0.45
Canada	Stolon	9.0	0.09	0.66	1.93	1.05

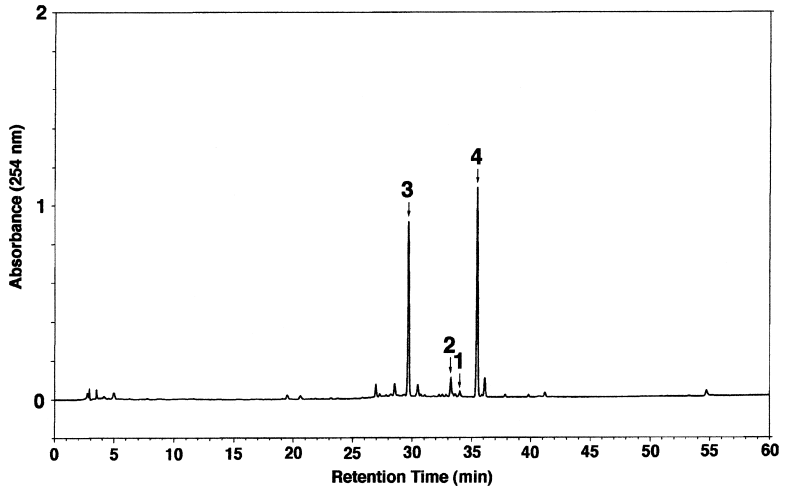

Fig. 2. HPLC Profile of Methanol Extracts of Stolons of *G. lepidota*

Absorbance at 254 nm. **1**, glycyrrhizin; **2**, macedonoside C; **3**, macedonoside A; **4**, licorice-saponin H2.

saponins of the macedonoside C-producing species (*i.e. G. macedonica*, *G. echinata*, *G. pallidiflora*). The present study shows that *G. lepidota* produces saponins isolated from both glycyrrhizin-producing and macedonoside C-producing groups, suggesting that this species is a chemotaxonomical intermediate of both groups.

Phylogenetic Relationship of *G. lepidota* and Six *Glycyrrhiza* Species Based on *rbcL* Sequences To elucidate the phylogenetic relationship of *G. lepidota* among genus *Glycyrrhiza* based on DNA sequences, its *rbcL* sequence was analyzed. Total DNA was isolated from the fresh leaves of a *G. lepidota* plant collected in the U.S.A. Using this DNA as a template, the 1374-bp DNA fragment covering most of the *rbcL* sequence was amplified by PCR and sequenced.[16] Table 3 shows the nucleotide substitutions in the 1324 nucleotides of *rbcL* genes of *G. lepidota* and six other *Glycyrrhiza* species. A phylogenetic tree (Fig. 3) constructed from the *rbcL* sequences, using Nei's unweighted pair-group method using arithmetic averages (UPGMA method),[17] indicated that the seven *Glycyrrhiza* species were divided into three groups: the three glycyrrhizin-producing species (*G. glabra*, *G. uralensis*, and *G. inflata*), the three macedonoside C-producing species (*G. echinata*, *G. macedonica* and *G. pallidiflora*) and *G. lepidota*. It is noteworthy that *G. lepidota* was separated from the other six *Glycyrrhiza* species based on their *rbcL* sequences, and this phylogenetic relationship is in accordance with their saponin compositions.

MATERIALS AND METHODS

General Methods ^{1}H- and ^{13}C-NMR spectra were recorded using an EX-400 (JEOL) spectrometer. Chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard. FAB-MS was measured on a JMS-DX300 (JEOL, Japan) spectrometer. ODS (100—200 mesh, Fuji Silysia Chemical, Japan) and Sephadex LH-20 (Amersham Biosciences) were used for column chromatography.

Plant Materials Stolons and leaves were collected from *G. lepidota* grown in the wild in North Dakota, U.S.A., and a collected stolon was cultivated outdoors in a pot in Gifu. The morphological identification of *G. lepidota* collected in North Dakota was undertaken based on phenotypic changes of the fruits and aerial parts.[5] Stolons of *G. lepidota* collected in Canada were gifts from Prof. W. G. Kurz of the Plant Biotechnology Institute, National Research Council of Canada.

Chemicals An authentic sample of glycyrrhizin was obtained from Maruzen Pharmaceuticals, Japan. Macedonoside C was a gift from Prof. emeritus G. Kusano of Osaka University of Pharmaceutical Sciences.

Isolation of Saponins from Stolons of *G. lepidota* Dried stolons (100 g) were extracted with 500 ml of 70% ethanol-water at 60 °C for 2 h, twice. The dried extract was chromatographed on a reverse-phase silica gel (ODS) (98 g) column with 50% ethanol-water containing 0.1% acetic acid in 12 ml fractions (frs. A1—95). Fractions A20—70 were applied on a Sephadex LH-20 (63 ml) column and eluted with 80% methanol containing 0.1% acetic acid in 12 ml fractions (frs. B1—20). Fractions B3—5 were repeatedly chromatographed on preparative HPLC to isolate licorice-saponin H2 (**4**, 227 mg). Conditions of preparative HPLC were as follows: column, Intersil PREP-ODS (20 mm I.D.×250 mm,

Table 2. Nucleotide Variation of *rbcL* Gene for *Glycyrrhiza* Species

Species	(Accession No.)	Nucleotide variation of *rbcL* sequences (Base substitutions have been presented from upstream.)																	
		156	165	192	259	267	433	537	582	639	672	729	732	738	762	1002	1008	1123	1125	1209	1278	1330
G. lepidota	(AB126685)	G	A	G	A	T	T	A	C	G	T	T	G	A	A	G	A	A	A	C	G	A
G. glabra	(AB012125)	A	A	G	C	C	A	G	C	T	A	C	A	T	T	A	A	C	G	T	A	G
G. uralensis	(AB012126)	A	A	G	C	C	A	G	C	T	A	C	G	T	A	A	A	C	G	T	A	G
G. inflata	(AB012127)	A	A	G	C	C	A	G	C	T	A	C	G	T	A	A	A	C	G	T	A	G
G. echinata	(AB012128)	G	A	G	C	T	A	G	C	T	A	T	G	A	A	G	G	C	G	T	A	A
G. pallidiflora	(AB012129)	G	T	T	C	T	A	A	C	T	A	T	G	A	A	G	G	C	G	T	A	A
G. macedonica	(AB032424)	G	A	G	C	T	A	G	T	T	A	T	G	A	A	G	A	C	G	T	A	A

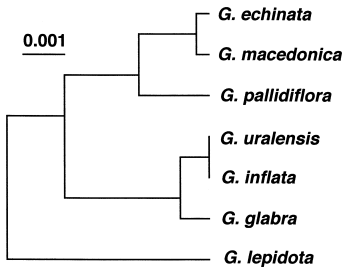

Fig. 3. Phylogenetic Tree Constructed from the Nucleotide Sequences of *rbcL* Genes of the Seven *Glycyrrhiza* Species by the UPGMA Method of Nei[17]

The phylogenetic tree was constructed by Genetyx-Mac Ver. 10 software (Genetyx Corporation, Japan).

Gasukuro Kogyo, Japan); solvent, 35% acetonitrile-water containing 0.1% formic acid; flow rate, 5 ml/min; column temp, room temperature; detector, UV 202 nm; retention time, 100.2 min (**4**).

Fractions A9—19 (15 g) were further chromatographed on another ODS (98 g) column with 40% ethanol-water containing 0.1% acetic acid in 12 ml fractions (frs. C1—64). Fractions C18—27 were applied on a Sephadex LH-20 column (63 ml) and eluted with 80% methanol containing 0.1% acetic acid in 12 ml fractions (frs. D1—20). Fractions D3—5 were repeatedly chromatographed on preparative HPLC to isolate macedonoside A[3] (**3**, 128 mg) under the same HPLC conditions mentioned above: retention time, 26.3 min (**3**).

HPLC Analysis of Stolons Dried stolons ($n=4$) were ground with a mortar and pestle, and then 40 mg of each powdered sample was extracted with 1 ml of 80% methanol at 60 °C for 6 h. An aliquot (10 μl) of the extract was analyzed by photodiode-array HPLC. Conditions of HPLC were as follows: column, Capcellpak C18 AG-120A (5 μm, 4.6 mm I.D.×250 mm, Shiseido); solvent, an acetonitrile-water (0.1% formic acid) gradient of 15% acetonitrile to 25% acetonitrile in 15 min, to 70% acetonitrile in another 35 min, then to 100% acetonitrile in 10 min; flow rate, 1 ml/min; column temp, 40 °C; detector, photodiode array SPD-M10$_{AVP}$ system (Shimadzu). Quantities of constituents were determined on the basis of the peak area of UV absorption at 254 nm (compounds **1**—**4**). Each constituent was identified by comparison of its retention time and UV spectrum with those of the authentic sample.

Amplification and Sequencing of *rbcL* Gene DNA was extracted from fresh leaves of a *G. lepidota* plant collected in the U.S.A. by DNeasy Plant Mini Kit (Qiagen). The 1374-bp DNA fragment covering most of the *rbcL* sequence was amplified by the polymerase chain reaction (PCR) using template DNA from the leaves, Taq-DNA polymerase (Takara), anti-Taq high (Toyobo) and two primers 5′-ATGTCACCACAAACAGAAACTAAAGC-3′ and 5′-AGCAGCAGCTAATTCAGGACTCCA-3′, as previously reported.[4] The amplified fragment was treated with ExoSAP-IT (Amersham Biosciences) to remove primers. The purified fragment was sequenced directly by the dideoxy chain termination method using a CEQ2000XL DNA sequencer (Beckman Coulter). Nine internal primers, 5′-ATGTCACCACAAACAGAAACTAAAGC-3′, 5′-GACCTTTTTGAAGAAGGTTCTG-3′, 5′-TTTATGCGTTGGAGAGACCG-3′, 5′-TCTGGTGGAGATCATATTCACGC-3′, 5′-TATTGATTTTCTTCTCCAGCAA-

C-3′, 5′-CGCGAAGACATTCATAAACTGC-3′, 5′-AAGTAGACCATTATCTCGGC-3′, 5′-CACCTGGTAAAGAAACCCAATCCTG-3′ and 5′-AGCAGCAGCTAATTCAGGACTCCA-3′, located at every 300—400 base of both strands, were used for sequencing.

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