Wild Ginseng Grows in Myanmar

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Ginseng, the underground parts of plants of *Panax* species, has been used in oriental traditional medicine for centuries. Unfortunately, because of extensive exploitation over thousands of years, the natural source of these species has been almost exhausted. Recently, we have found a wild ginseng growing in Myanmar. Here, by a combination of chemical composition study and gene sequence analysis, we unambiguously demonstrate that the wild ginseng is actually *P. zingiberensis*, commonly known as ginger ginseng. This ginseng was an indigenous to the southwestern China. However, now it is seriously threatened to brink of extinction and is put on the highest level of protection in China. Therefore, an appropriate protection measure is highly recommended to preserve this valuable resource, since this Myanmar ginseng might turn out to be the last *P. zingiberensis*, which could ever be seen in the planet.

Key words Myanmar ginseng; *Panax zingiberensis*; ginsenoside; liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS); 18S rRNA gene sequence; *mat*K gene sequence

Continued destruction and deterioration of ecosystems have led biodiversity conservation now to one of the most serious environmental problems in the world. More than 34000 plant species have been identified as threatened to extinction in the 1997 International Union for the Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Plants.[1] Ginseng is the dry underground parts of plants belonging to *Panax* species and it is worldwide well-known Chinese traditional medicine used to increase physical and mental performance, to provide resistance to stress and disease and to prevent exhaustion. These *Panax* species grow in the Northern Hemisphere, from the eastern Himalaya through China to Japan. However, because of continual harvest over thousands of years, the natural source of these species has been almost exhausted and wild plant is rarely available.[2]

In our continuing research project on Myanmar medicinal plants, we have found a colony of a *Panax* species at Par Moe Ne Water Spring area in Taung-gyi, Shan State, Myanmar, at an altitude of 1500 m above the sea level, and, therefore, tentatively call it Myanmar ginseng (MG). Here, we report that the *Panax* species found in Myanmar was unambiguously identified as *P. zingiberensis*, based on chemical constituent analysis by liquid chromatography (LC)-electrospray ionization (ESI)-mass spectrometry (MS) and gene sequence studies.

Results and Discussion

MG has many typical morphological characteristics for the *Panax* species. It is a perennial herb with erect stem of 30—50 cm in height, palmate leaves. In contrast to the *P. ginseng* that possesses carrot-like roots connected to a small rhizome, MG has a large horizontal rhizome with small roots (Fig. 1), being morphologically similar to the *Panax* species growing in eastern Himalaya and south-western China such as *P. japonicus*, *P. japonicus* var. *major*, *P. pseudo-ginseng* subsp. *himalaicus* and *P. zingiberensis*.[5]

Chemical constituents of the *Panax* species have been studied since the 1980s. It has been demonstrated that chemical compositions of plants of *Panax* species are related to their morphology, which can be tentatively divided into two groups.[6] The first group, consisting of *P. ginseng* and *P. quinquefolium*, which possess carrot-like roots with a small rhizome, contain dammarane triterpene saponins with minor amount of oleanolic acid saponins, whereas the second one, represented by *Panax* species growing in eastern Himalaya and south-western China such as *P. japonicus*, *P. japonicus* var. *major*, *P. pseudo-ginseng* subsp. *himalaicus* and *P. zingiberensis*, which possess large rhizomes and small roots, contain not only dammarane triterpene saponins but also contain a variety of oleanolic acid saponins in high contents.[5,6] The dried roots and rhizomes of MG were extracted with hot 60% EtOH to give EtOH extract. This extract was then subjected to a series of chromatographic separations on Diaion HP-20 column, followed by conventional silica gel column and preparative TLC. As a result, seven saponins, including three dammarane triterpene saponins, namely ginsenosides Rg_1 (**1**),[7] Rh_1 (**2**),[8] and Rb_1 (**3**)[9]; and four oleanolic acid saponins, ginsenoside Ro (**4**),[9] chikusetsusaponins

Fig. 1. Underground Part of Myanmar Ginseng

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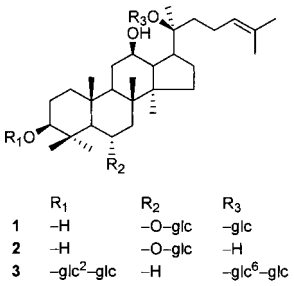
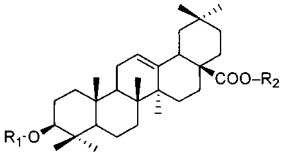


	R_1	R_2	R_3
1	–H	–O–glc	–glc
2	–H	–O–glc	–H
3	–glc^2–glc	–H	–glc^6–glc



	R_1	R_2
4	–glcUA^2–glc	–glc
5	–glcUA^4–ara(f)	–glc
6	–glcUA	–glc
7	–glcUA^2–glc	–H

glc : β-D-glucopyranosyl
glcUA : β-D-glucuronic acid
ara (f) : α-L-arabinofuranosyl

Chart 1. Structures of Saponins Isolated from MG

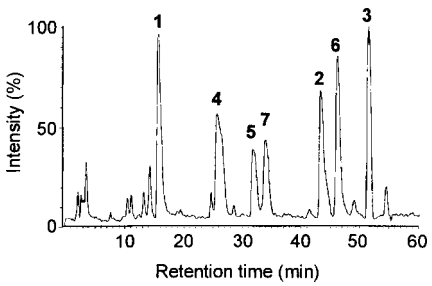


Fig. 2. LC-ESI-MS Chromatogram of the 60% Ethanol Extract of Myanmar Ginseng

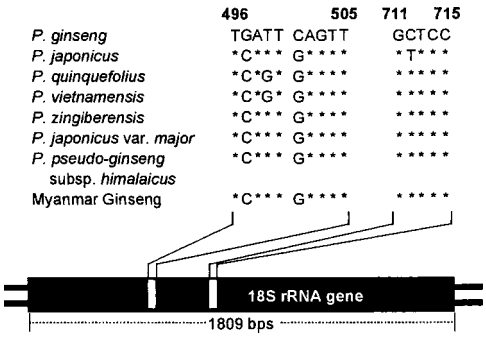


Fig. 3. Comparison of 18S rRNA Gene Sequences of Myanmar Ginseng with Those of Other Seven *Panax* Taxa

IV (**5**)[10] and IVa (**6**),[11] and zingibroside R_1 (**7**),[12] were isolated. Their structures were determined by comparison of their NMR spectroscopic data with those published in literature. These results further supported the suggestion that MG belongs to the second group of *Panax* species such as *P. japonicus*, *P. japonicus* var. *major*, *P. pseudo-ginseng* subsp. *himalaicus* and *P. zingiberensis*, which contain both dammarane triterpene and oleanolic acid saponins in high contents. In the other hand, to the best of our knowledge, the isolation of zingibroside R_1 (**7**) was reported only from *P. zingiberensis*.[7] This fact has firmly pointed the identity of MG to be *P. zingiberensis*.

In the last decade, LC-ESI-MS technique has been introduced as a highly sensitive and soft ionization technique for LC-MS analysis of thermo labile molecules for the study of natural products.[13] This useful tool has been applied for the study of ginseng saponins.[14,15] LC-ESI-MS was, therefore, conducted for the study of the chemical constituents of MG. Total ion chromatogram (TIC) of the 60% ethanol extract of MG showed the presence of seven major peaks (Fig. 2). By using the isolated compounds described above as standard samples, we were able to identify the seven major peaks to be ginsenosides Rg_1 (**1**), Rh_1 (**2**), Rb_1 (**3**), and Ro (**4**), chikusetsusaponins IV (**5**) and IVa (**6**), and zingibroside R_1 (**7**), by comparing their retention times and mass numbers. This result indicated that seven saponins isolated, actually, are major saponins present in the MG. Moreover, it is noteworthy that ginsenosides Rd and Re, the presence of which have been reported in most of the *Panax* species except for *P. zingiberensis*,[16] could not be found in the 60% ethanol extract of MG. All of these facts have indicated that MG is *P. zingiberensis*.

Recent studies have demonstrated that the sequence analysis of nuclear 18S ribosomal RNA gene, chloroplast *mat*K gene, *etc*. is a powerful tool for identifying the botanical origin of plant species.[17,18] To confirm the scientific name assigned for MG, 18S rRNA gene and *mat*K gene sequences of this plant were determined and compared to those sequences of other authentic *Panax* taxa, including *P. zingiberensis* collected from China. The 18S rRNA gene sequences of MG and *P. zingiberensis* were found to be 1809 base pairs in length, corresponding with other six taxa reported previously.[3] The nucleotide sequence of MG was identical to those of *P. zingiberensis*, *P. japonicus* var. *major* and *P. pseudoginseng* subsp. *himalaicus*, which showed two base substitutions at nucleotide positions 497 and 501 from upstream in comparison with that of *P. ginseng* and one base substitution at position 499 comparing with *P. quinquefolius* or *P. vietnamensis* (Fig. 3). The *mat*K gene is a long open reading frame located within the intron of the transfer RNA gene for lysine (*trn*K), which encodes a maturase, involved in intron splicing. The entire *mat*K gene sequence of all *Panax* taxa, including MG and *P. zingiberensis*, were found to be 1512 base pairs in length. The *mat*K gene sequence of MG was identical to that of *P. zingiberensis* and different from those of other six taxa (Fig. 4a). Although similar sequence was observed in *P. vietnamensis*, different nucleotide sites for *P. zingiberensis* were found at positions 104, 619 and 1043. Of the nucleotides at these three sites, cytosine at position 619 was specific to *P. zingiberensis*. The identity of the *mat*K gene sequence of *P. zingiberensis* reflected to the deduced amino acid sequence of maturase protein. The specific amino acid for *P. zingiberensis* was found to be leucine at position 207 (Fig. 4b). Above results, *P. zingiberensis* was specialized by *mat*K gene sequence. This gene sequence has been demonstrated to be stable in the same taxon of genus *Panax*.[3] Therefore, MG having the identical sequence to *P. zingiberensis* was determined to be this taxon.

Nearly all cultures, from ancient times to now, have used plants as a source of medicine. In many developing coun-

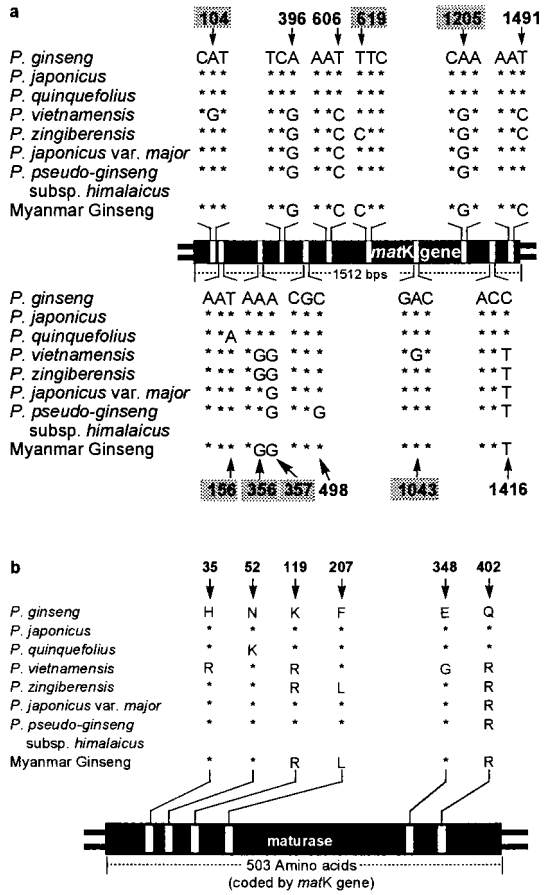


Fig. 4. Comparison of *mat*K Gene Sequences (a) and Encoded Maturase Amino Acid Sequences (b) of Myanmar Ginseng with Those of Other Seven *Panax* Taxa

Amino acid residues at positions 35, 52, 119, 207, 348 and 402 correspond to the nucleotide positions highlighted in *mat*K gene sequences.

tries, traditional medicine is still the mainstay of health care. In developed countries, many people are turning to herbal remedies, especially for minor ailments, and modern western medicine still depends on plants, and the knowledge gained from plants, for some essential drugs. However, little effort has been made to protect this valuable natural resource provided by medicinal plants. Today, many medicinal plant species face extinction. For most of the endangered species no conservation action has been taken, and for many countries there is not even a complete inventory of medicinal plants.

Panax zingiberensis C. Y. Wu *et* K. M. Feng, commonly known as ginger ginseng, extensively used in Chinese traditional medicine, wildly grows in the south-western region of China, mainly in Yunnan Province.[6] However, recently, *P. zingiberensis* was claimed to be threatened to the brink of extinction. It is listed in the 1997 IUCN Red List of Threatened Plants with an "endangered" status.[1] Therefore, now it has been put on the highest level of protection in China.[19] Here in this article we reported that a *Panax* species, growing wildly in Myanmar, has been identified by a combination of chemical composition and gene sequence analysis to be the seriously endangered *P. zingiberensis*. Therefore, it is highly recommended a full-scale inventory and resource assessment of the *P. zingiberensis* to be completed in Myanmar. This should be undertaken as soon as possible to allow appropriate protection plans. Otherwise, the fate of these Myanmar *P. zingiberensis* will undoubtedly be the same as that of its Chinese congener.

Experimental

Plant Materials Myanmar ginseng was collected in Taung-gyi, Shan State, Myanmar in December 2001. *P. zingiberensis* C. Y. Wu *et* K. M. Feng was obtained from Wenshan, Yunnan province of China in August 1999. The collection data of other *Panax* taxa were described in our previous report.[3] All specimens were stored in the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University (TMPW).

Extraction and Isolation Air-dried roots and rhizomes of MG (2.8 g) were extracted with hot 60% EtOH–H_2O to give 60% EtOH extract (0.9 g), which was subjected to column chromatography on Diaion HP-20, eluted successively with H_2O and MeOH, to give sugar (450 mg) and crude saponin (335 mg) fractions, respectively. A part of the crude saponin fraction (250 mg) was then chromatographed on silica gel with a $CHCl_3$–MeOH–H_2O (30 : 20 : 1) system to afford six fractions (frs. 1—6). Fraction 2 (36 mg) was found to be pure ginsenoside Rg_1 (**1**, 36 mg). Fraction 1 (16 mg) was purified by normal-phase preparative TLC (pTLC) with $CHCl_3$–MeOH–H_2O (60 : 10 : 1) to give ginsenoside Rh_1 (**2**, 2.1 mg). Normal-phase pTLC of fraction 3 with $CHCl_3$–MeOH–H_2O (60 : 10 : 1) gave chikusetsusaponin IV (**5**, 13 mg), whereas reversed-phase pTLC with H_2O–MeOH–MeCN (1 : 1 : 1) of fraction 4 (16.2 mg) gave ginsenoside Rb_1 (**3**, 2.4 mg) and zingibroside R_1 (**7**, 1 mg). Ginsenoside Ro (**4**, 6.5 mg) and chikusetsusaponin IVa (**6**, 13 mg) were obtained from fractions 5 (24.5 mg) and 6 (49 mg), respectively, by the same treatment.

LC-ESI-MS Analysis The crude saponin fraction and the isolated saponins were redissolved in HPLC grade methanol and filtered prior to LC-MS analysis. The isolated saponins were used as standard samples for LC-MS analysis by the same method as reported previously.[4] LC separation was accomplished using a Hewlett Packard HP-1100 system with a Waters Symmetry C_{18} column (5 μm, 2.1×150 mm) at column temperature of 40 °C. Mobile phase was a gradient system of (A) 8 mM NH_4OAc (pH 7.0) and (B) CH_3CN: 0—20 min, 80—75% A and 20—25% B; 20—45 min, 75—70% A and 25—30% B; 45—60 min, 70—65% A and 30—35% B; flow rate, 0.2 ml/min. Negative-ion ESI-MS were obtained by full range scan from 500—1300 unit in 20 s.

Isolation of Total DNA, PCR Amplification and Sequencing Reaction Total DNA was extracted from dried leaves or underground parts of the plant using the same method as described in our previous paper.[3] Complete 18S rRNA gene and *mat*K gene amplifications *via* the polymerase chain reaction (PCR) were performed using 10—100 ng total DNA as a template in 50 μl reaction mixture, consisting of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 mM of each primer, and 1.5 U Taq Polymerase (Promega, U.S.A). Two pairs of primers flanking 18S rRNA gene and *mat*K gene regions were as follows. 18S rRNA gene: 18S 5′F 5′-CAA CCT GGT TGA TCC TGC CAG T-3′, 18S 3′R 5′-CTG ATC CTT CTG CAG GTT CAC CTA C-3′; *mat*K gene: *trn*K 3914F 5′-TGG GTT GCT AAC TCA ATG G-3′, *trn*K 2R 5′-AAC TAG TCG GAT GGA GTA G-3′. PCR amplifications were carried out in a Thermal Controller PTC-100 (MJ Research Inc., U.S.A.) as cycling conditions shown below. 18S rRNA gene: 1 cycle consisting of 94 °C for 3 min, 65 °C for 8 min, followed by 30 cycles of 94 °C for 40 s and 65 °C for 8 min, and final extension at 72 °C for 30 min; *mat*K gene: Hot start at 94 °C for 1 min, followed by 35 cycles of 94 °C for 40 s, 45 °C for 40 s and 72 °C for 4 min, and final extension at 72 °C for 15 min. The 1/10 volume of resulting PCR product was detected by 1.0% agarose gel electrophoresis and then the remained part was purified using a QIA quick PCR purification Kit (QIAGEN, Germany). Sequencing reaction of the purified PCR products were carried out using a Thermo Sequenase Cycle Sequencing Kit (Amersham, U.S.A.) with a set of fluorescent-labeled sequencing primers.

Each sequence was determined directly by a 4000L DNA sequencer (LI-COR, U.S.A.) and analyzed using the BaseImagIR program (Version 4.0, LI-COR, U.S.A.). The obtained DNA sequences were assembled, and consensus sequences were constructed by the AutoAssemble program (Version1.3.0, Applied Biosystems, U.S.A.).

The nucleotide sequences of the 18S rRNA and *mat*K genes of *P. zingiberensis* were registered in DDBJ, EMBL and GenBank nucleotide sequence databases with accession numbers, AB085764 and AB085765, respectively. The accession numbers of other 6 *Panax* taxa were described in the previous report.[3]

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