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Studies on the medicinal plant resources of the Himalayas(3), Random Amplified Polymorphic DNA Analysis and Saponin Contents of Himalayan Ginseng [*Panax pseudo-ginseng* Wall.]

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Relationship between the morphological variations and the saponin contents of *Panax pseudo-ginseng* Wall. (Araliaceae) plants (P.p-g) was investigated by using the pseudo-ginseng plants from thirteen habitats in Nepal-Himalayas and Japan. On the basis of the morphological characters and random amplified polymorphic DNA (RAPD) analysis, these P.p-g were classified into four types, Himalayan (H)-1, H-2, Japanese (J)-1 and J-2. On the phylogenic tree, H group strains and J group strains form distinct separate groups, and H-2 type forms a separate group with a large distance form the other three types. On the other hand, on the basis of the dammarane and oleanane saponin contents, P.p-g were grouped into three chemotypes, H-1, H-2 and J types. These results indicate that RAPD analysis in combination with the saponin content assay might be useful for the classification of P.p-g in different habitats.

Keywords: *Panax pseudo-ginseng*; Araliaceae; RAPD; HPLC; Saponins; Dkar-po chig-thub

Panax spp. is widly distributed in the Northern hemisphere from Central Himalaya through China, and Japan to North America.[1-2] *Panax pseudo-ginseng* Wall.(Araliaceae) plants, hereafter referred to as P.p-g, have been a most significant for natural medicine in Himalaya and Japan. However, its population is decreasing in the natural habitat and people of Nepal-Himalaya have very little knowledge about these species. The rhizomes of Himalayan P.p-g,[3] locally called "dkar-po chig-thub", are used as a Tibetan crude drug[4] in the prescriptions for anti-inflammatory, subduing plague effects, for preventing animalcules, etc. The *Panax* spp. with carrot-like root, characteristically contain a number of dammarane saponins along with a small amount of oleanane saponins,[5-7] whereas those with long bamboo-like rhizome such as *P. japonicus* C. A. Meyer (syn, P.p-g subsp. *japonicus* Hara)[1] contain a large amount of oleanane saponins along with a relatively small amount of dammarane saponins. However, P.p-g subsp. *pseudo-ginseng* Hara having carrot-like root contains only a trace of oleanane ones, and ocotillol-type saponins typically in the rhizomes and the leaves.[8-10] P.p-g, a perennial herb, 40-70 cm high, having morphological

variations in rhizomes within one population[3] grows in the upper temperate mixed broad-leaf forest with latitudinal range of 27° 20'-29° 60' and longitudinal range of 81° 48'-87° 42' in the area of altitude of 1680 m to 3350 m above sea level in Nepal.

Recently, DNA analysis techniques have been reported as effective means for the genetic analysis of the species, varieties, subspecies and even individual strains of plants.[11-13] However, molecular and genetic studies of P.p-g have been few reported,[14,15] In the present study, we assayed the correlation between the chemo-geographical variations and the genetic differences of P.p-g growing in 13 habitats by using the Random Amplified Polymorphic DNA (RAPD)[16,17] analysis and the saponin content analysis by HPLC.

MATERIALS and METHODS

Plant Materials Plant Materials from 13 different habitats (Table 1, Fig. 1) were transplanted to the nursery of Medicinal Plant Garden, School of Pharmaceutical Sciences, Kitasato University, Japan. These living plants were used for the experimental materials. Each specimen was deposited in National Herbarium, Dep. of Plant Resources, Nepal.

Preparation of genomic DNA Genomic DNA for the RAPD analysis was extracted from fresh rhizomes by using the modified Dellaporta's method.[18] Briefly, fresh rhizomes (*ca* 2.0 g) of each P.p-g, which had been stored at -80℃ were quickly frozen in liquid nitrogen after being cut into pieces of length *ca* 5 mm, ground to fine powder with a mortar and a pestle, and mixed with 20 ml of genomic DNA extraction buffer {100 mM Tris-hydrochloric acid, 50 mM ethylenediamine tetraacetic acid (EDTA), 500 mM NaCl, 10 mM β-mercaptoethanol; pH 8.0}. Then, 2 ml of 20 % SDS solution was added to the mixture, and the mixture was incubated at 65℃ for 10 min. After addition of 4 ml of 5 M potassium acetate, the mixture was kept on ice for 20 min and centrifuged at 8k rpm for 15 min at 4℃. The supernatant (sup.) was passed through a nylon cloth filter (No.508 mesh) into a clean tube containing 16 ml isopropanol, and the tube was gently shaken. After incubation at -20℃ for 30 min or longer, the mixture was centrifuged at 8k rpm for 15 min at 4℃. The precipitation (ppt.) dissolved in 2.8 ml distilled water was added to a mixture of 100 μl of 5 M ammonium acetate and 500 μl isopropanol, and mixture was centrifuged at 15k rpm for 15 min at 4 ℃. The ppt. was suspended in 70% EtOH, the suspension was centrifuged at 15k rpm for 5 min at 4 ℃ and then the ppt. was briefly dried. The DNA pellets

Table 1. Plant Materials examined

●, ▲, ■, ◆ Plant Species
Number and abbr. of locality; Locality
Experimental number; Collector

<< Nepalese *Panax* plants >>

● *Panax pseudo-ginseng* Wall. subsp. *himalaicus* Hara

① CHME: Chame; Manang Dist., the western foot of Mt. Annapurna, Nepal; Experimental number is P.p-g.NPKS-006.; Collector: T. Watanabe, N. Utsumi, S. Isoda, A. Takano and K. J. Malla

③ DMAN: Daman(Sim Bhanjang), Makawanpur Dist., Nepal ; Experimental number is P.p-g.NPKS-005(= P.p-g NPTW-3327).; Collector: T. Watanabe

④ LNTG: Langtang, Rasuwa Distr., Tangsep- Chyunama in Langtang Himalaya, Nepal; Experimental number is P.p-g.NPKS-002.; Collector: T. Watanabe, K. Shinozaki, A. Takano, H. Izumi and K.J. Malla

⑤ KARK: Kharka, Dolpa Distr., the south-east foot of Shey Phoksumdo National Park, Nepal; Experimental number is P.p-g. NPKS-003. ; Collector: T. Watanabe, H. Watanabe, K. Watanabe, K. Shinozaki, A. Takano and K.J. Malla

▲ *Panax pseudo-ginseng* Wall. subsp. *pseudo-ginseng* Hara

② BAGW: Bagdwar, Kathmandu Dist., Nepal; Experimental number is P.p-g.NPKS-001.; Collector: T. Watanabe and K.J. Malla

<< Japanese *Panax* plants >>

■ *Panax pseudo-ginseng* Wall. subsp. *japonicus* (Meyer) Hara = *Panax japonicus* C. A. Meyer

⑥ SHBR: Shiobara, Hiroshima Pref., Japan; Experimental number is P.p-g.JPKS-007(= P.p-g JPTW-3850).; Collector: T. Watanabe, H. Izumi and H. Kohda

⑦ BUNA: Bunao Pass, Hira Village, Higashitonami-gun, Toyama Pref., Japan; Experimental number is P.p-g.JPKS-001(= P.p-g JPTW-3502).; Collector: T. Watanabe, H. Fujino and H. Izumi

⑧ SAGA: Sasagamine, Myokoh highlands, Nagano Pref., Japan; Experimental number is P.p-g.JPKS-006(= P.p-g JPTW-3114).; Collector: T. Watanabe and M. Wakai

⑨ YAKE: Yakeyama, Towada, Aomori Pref., Japan; Experimental number is P.p-g.JPKS-011(= P.p-g JPTW-3791).; Collector: T. Watanabe and A. Takano

⑫ MYKO: Yatsusa, Miyakono-jyo, Miyazaki Pref., Japan; Experimental number is P.p-g.JPKS-009(= P.p-g JPTW-3867).; It was shared from Mr. S. Sakaguchi

⑬ YAGW: Mt. Gejyo, Yanogawa Pass, Mie Pref., Japan; Experimental number is P.p-g.JPKS-010(= P.p-g JPTW -3869).; Collector: T. Watanabe and H. Watanabe

◆ *Panax pseudo-ginseng* Wall. var. *angustatus* Hara

⑩ INOG: Inogashira Park, Yamanashi Pref., Japan; Experimental number is P.p-g.JPKS-008(= P.p-g JPTW-3866).; Collector: T. Watanabe and S. Isoda

⑪ MANJ: Manjiro Pass, Mt. Amagi, Shizuoka Pref., Japan; Experimental number is P.p-g.JPKS-005(= P.p-g JPTW-3099).; Collector: T. Watanabe and H. Izumi

It should not be announced about the localities in any publications because these plants are endangered species and prohibiting business on Washington Convention.

were suspended in 100 μl TE buffer. To obtain further purified genomic DNA, the DNA suspension was subjected to polyethylene glycol(PEG) precipitation. After addition of 60 μl of 20% PEG / 2M NaCl solution, the mixture was kept on ice for 1 hour and centrifuged at 15K rpm for 15 min at 4℃. The ppt. was rinsed with 70% EtOH and then beribly dried. The purified DNA thus prepared was dissolved in 100 μl TE buffer, and used for the RAPD analysis.

RAPD analysis Each genomic DNA sample was amplified by using eight different PCR primers (Table 2) in a 0.2 ml-microtube containing a PCR mixture (25 μl) consisting of *ca* 10 ng of genome DNA, 100 μM of each dNTP (dATP, dCTP, dGTP, and dTTP), 60 ng of 10 mer-arbitrary primer (Operon Technologies Inc., USA), 1.25 U of *rTaq* DNA polymerase, 10 x PCR buffer (Takara Shuzo Co., Ltd.; 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$). Thermal cycling was carried out for 45 cycles of 1 min at 94℃, 1 min at 36℃ and 2 min at 72℃, and then the mixture was incubated at 72℃ for 8 min. After the reaction, 8 μl of the solution containing the amplified DNA fragments was subjected to electrophoresis using 1.2 % agarose gel with ethidium bromide (0.5 μg / ml) as the stain, and the gel was photographed under UV light.

Table 2. 10-mer primers used in this experiment

(Operon Technologies Inc., USA)

code	5' to 3'
OPB-01	GTTTCGCTCC
OPB-02	TGATCCCTGG
OPB-04	GGACTGGAGT
OPB-05	TGCGCCCTTC
OPB-08	GTCCACACGG
OPB-12	CCTTGACGCA
OPB-17	AGGGAACGAG
OPB-19	ACCCCCGAAG

Analyses of dammarane and oleanane saponins by HPLC using the modified Kanazawa's method[19] Fresh rhizome (*ca* 10 g) was homogenized in MeOH in a Warning blender (Nihon Seiki, Co. Ltd., JPN), and the homogenate was kept for a week at room temperature. Then, MeOH was evaporated and the residue was dissolved in MeOH (40ml x 3) for 10 min with sonication. The combined MeOH solution was evaporated at 40℃ or below to dryness and dissolved in H_2O (20 ml). The H_2O solution was passed through a Sep-Pak C_{18} cartridge (Waters Corp., USA), and then the cartridge was washed with H_2O (10 ml), 30% MeOH in H_2O (15ml), and finally eluted with MeOH (5 ml x 3). The MeOH eluate was evaporated to give a saponin fraction. The saponin fraction was dissolved in MeOH (10ml), and this solution (20 μl) was analyzed on HPLC. Ginsenosides(G)-Rg_1, Re, Rb_1, Rb_2 and Rd, Chikusetsusaponins(C)- V, IV and III, Notoginsenosides(N)-R_1 and R_2, and Pseudo-ginsenoside(P)-RT_1 were used as the standard samples for qualitative and quantitative analyses. Analytical conditions: pump; L-6200 Intelligent Pump (Hitachi, Ltd., JPN), detector; Photal MCPD-3500 (detection; UV 203 nm, Otsuka Electronics, JPN), column; Cosmosil C_{18} (4.6 i.d. x 250 mm, Nacalai, JPN), mobile phase; flow programs[20] were (1) acetonitrile : water = 20 → 50 : 80 → 50 (v/v) in a linear gradient for 80 min to analyze dammarane saponins, and (2) acetonitrile : 1% phosphoric acid = 30 → 50 : 70 → 50 (v/v) in a linear gradient for 70 min to analyze oleanane saponins, flow rate: 1.0 ml / min at room temperature. Each analysis was made in triplicate.

TLC Analysis Each sample treated with Sep-Pak C_{18} was dissolved in 0.5ml of MeOH for the detection of ocotillol saponins (P-RT_2 and F_{11}, Majonosides(M)-R_1 and R_2). The conditions for TLC analysis: Plate; Kieselgel 60 F_{254} 10cm x 10cm (E. Merck, Art. 5629), Solvent system; $CHCl_3$: MeOH : H_2O = 60:33:7 (v/v/v). Detection; 10% H_2SO_4 and heating at 110℃ for a few minutes.

RESULTS AND DISCUSSION

Morphological variations As shown in Fig. 1, morphological variations of the aerial parts and rhizomes were observed among the P.p-g specimens from thirteen habitats in Nepal-Himalaya and Japan. Aerial part: 5-foliate; ① - ⑨, ⑫ and ⑬; 7-foliate; ⑩ and ⑪, rhizomes: long horizontally creeping rhizomes; ① and ③ - ⑤, carrot-

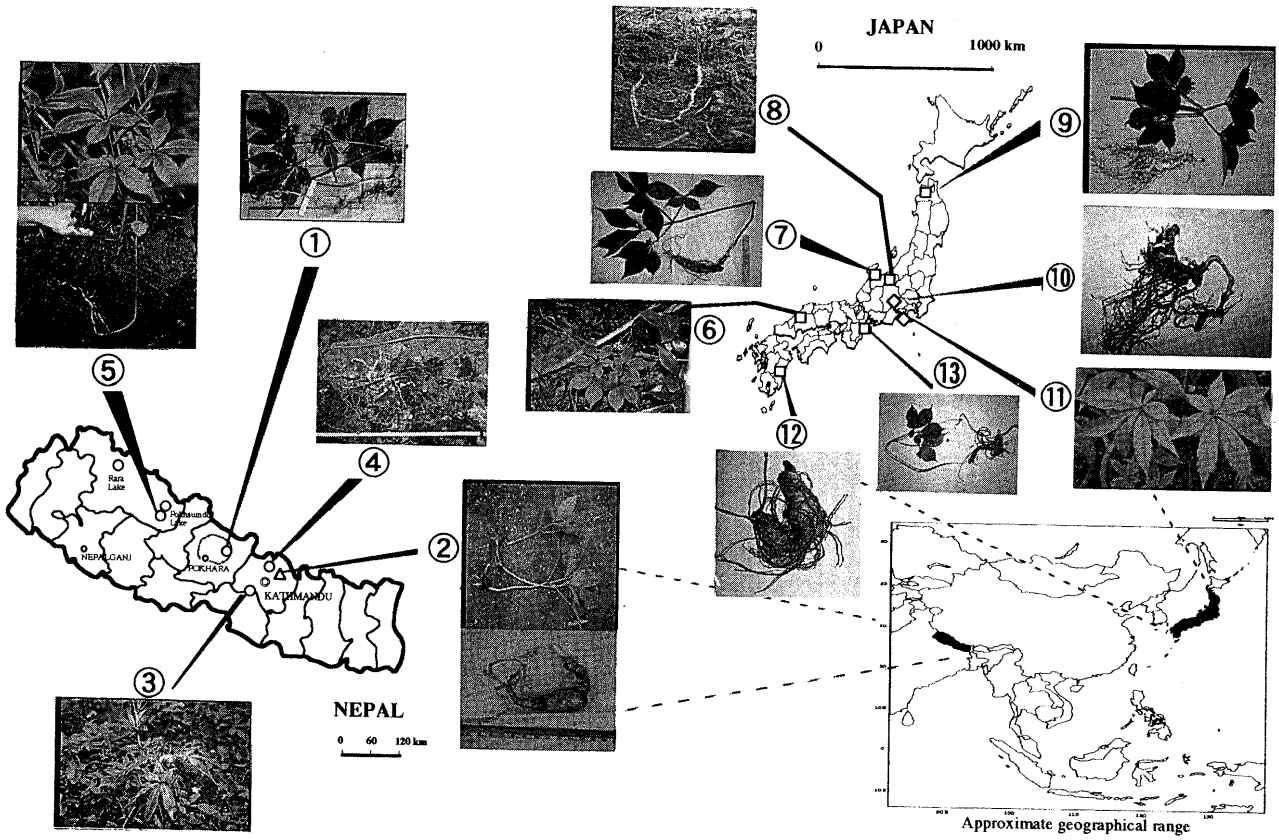


Fig. 1. Pictorial Map of Wild *Panax pseudo-ginseng* Plants from Nepal-Himalaya and Japan Examined:
Closed symbol, ○H-1 Type: P.p-g subsp. *himalaicus* Hara {①CHME, ③DMAN, ④LNTG, ⑤KARK}, △ H-2 Type: P.p-g subsp. *pseudo-ginseng* Hara {②BAGW: Carrot-like Root (= CR)}; ◇J-1 Type: P.p-g var. *angustatus* Hara {⑩INOG: CR, ⑪MAN J}, □J-2 Type: P.p-g subsp. *japonicus* (Meyer) Hara {⑥SHBR, ⑦BUNA, ⑧SAGA, ⑨YAKE, ⑫MYKO: CR, ⑬YAGW: CR} (See abbriviations of local names in Table 1.)

like roots; ②, ⑩, ⑫ and ⑬, long bamboo-like rhizomes; ⑥ - ⑨ and ⑪.

RAPD analysis In the preliminary RAPD analysis with 20 primers (Operon Technologies Inc., OPBs-01 to -20), 8 primers (Table 2) gave informative and reproducible bands with the genomic DNA of P.p-g. Therefore, these primers were used in the present study. Distinct bands on several lanes of RAPD patterns were obtained from the 13 genomic DNA samples (5 samples from Himalayan P.p-g, and 8 samples from Japanese P.p-g) by using the 8 primers, and each primer gave 13 to 43 common bands. Representative RAPD patterns obtained by using OPB-01, OPB-19 are shown in Fig. 2. For the construction of phylogenic tree, the estimated nucleotide diversities ($\underline{d}$) were calculated by the following formula. $\underline{d} = 1\text{-}2\,Nab / (Na + Nb)$, in which Nab is the number of common DNA-fragment bands detected in both of the two different strains a and b, and Na and Nb are the numbers of the total DNA-fragment bands of a nad b. A phylogenic tree was drawn on the basis of the unweighted pair-group method by using the arithmetic average (UPGMA)[21] and the calculated $\underline{d}$ values. The phylogenic tree indicated that these strains from 13 different habitats in Nepal-Himalaya and Japan, formed individual clusters (H-1; P.p-g subsp. *himalaicus* Hara, H-2; P.p-g subsp. *pseudo-ginseng* Hara, J-1; P.p-g var. *angustatus* Hara and J-2; P.p-g subsp. *japonicus* Hara) (Fig. 3). The genetic distance between the H group strains and the J group strains was large, and the H-2 type strains form a cluster with a large distance ($\underline{d} / 2 = 0.3309$) from the other types (H-1, J-1 and J-2). Therefore, the H-2 type plants having carrot-like root was considered to belong to a separate group. Thus, the RAPD analysis can distinguish between the Himalayan type P.p-gs and the Japanese type ones with intra-strain diversities.

Saponin contents As an example, HPLC profile of a H-1 type P.p-g rhizome is shown in Fig. 4-A, with the

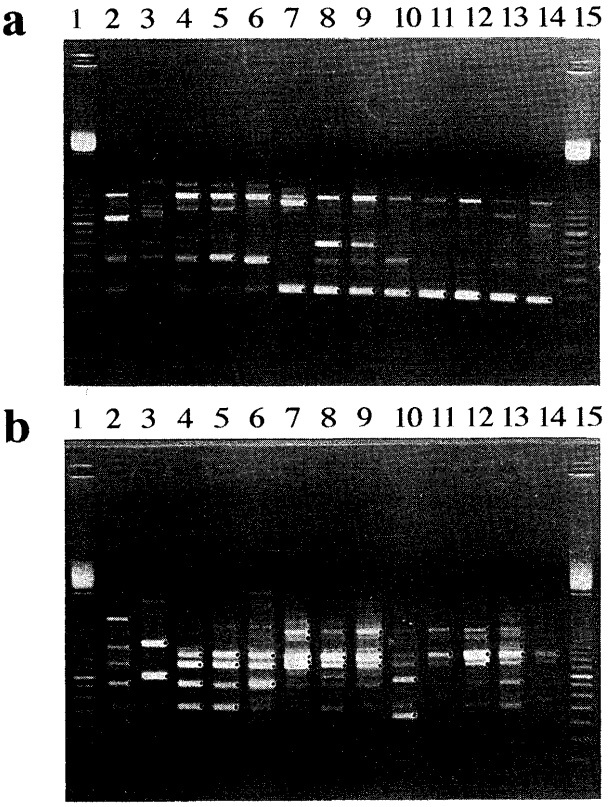


Fig. 2. Representative RAPD patterns obtained by PCR using primer OPB-01 (a: upper) and primer OPB-09 (b: lower) with genomic DNA from P.p-g.
Lanes 1 and 15 for 100 base-pair ladder marker ; lane 2 is equvalent to ①, lane 3 to ②, lane 4 to ③, lane 5 to ④, lane 6 to ⑤, lane 7 to ⑥, lane 8 to ⑦, lane 9 to ⑧, lane 10 to ⑨, lane 11 to ⑩, lane 12 to ⑪, lane 13 to ⑫, lane 14 to ⑬.
(① - ⑬ : shown in Table 1)

structures of these saponins. The HPLC profiles of those samples are schematically given in Fig. 4-B. According to the saponin contents, P.p-g was grouped into three chemotypes, H-1, H-2 and J types. H-1 type; ① and ③ - ⑤, no C-III (Fig. 4-A), and a large amount of dammarane saponins G-Rb_1 and G-Rg_1. H-2 type; ②, scarcely any dammarane saponins, and small amount of oleanane saponins. ⑥ - ⑬, larger amount of oleanane saponins, C- V and IV with small amount of dammarane saponins except C- III . The G-Rg_1 content was higher than the Re content in H-1 type sample, which is characteristic of H-1 type samples. The present HPLC patterns of H-1 type resembled those of Korean Ginseng [*P. ginseng* C. A. Meyer] (data not shown). In contrast, with relation between G-Rg_1 and Re in H-1 type, J types were reverse relation as there was a few quantities of G-Rg_1 comparing with Re relatively.

Relationship between saponin contents and genetic similarity of Himalayan and Japanese *Panax* plants

There are some reports on ocotillol-type saponins contained in Himalayan *Panax* plants.[5-7] The presence of ocotillol-type saponins in the two subspecies of P.p-g from different habitats (① - ⑤) was examined by using TLC. P-RT_2 (R_f 0.43), one of the ocotillol-type saponins, was detected only in the rhizomes of Himalayan P.p-g H-1 type collected at Chame (①): no other ocotillol-type saponins as P-F_{11}, M-R_1 and M-R_2 (R_f 0.35, 0.63 and 0.21) were detected. No ocotillol-type saponin was detected in the rhizomes of Japanese P.p-g. A phylogenic tree agreed with the results of this analysis.

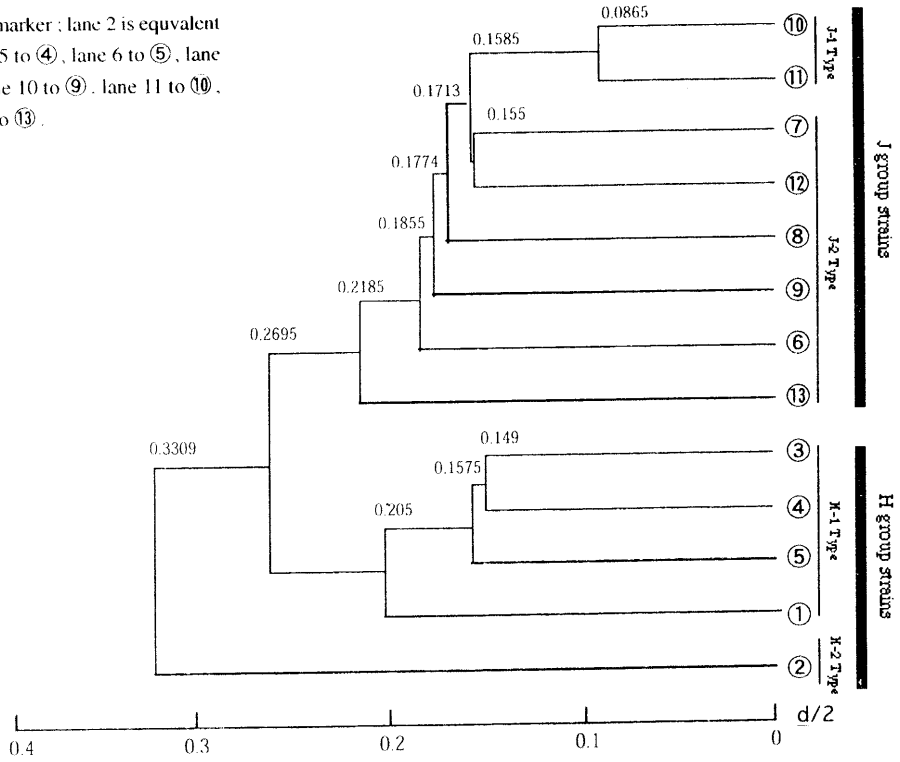
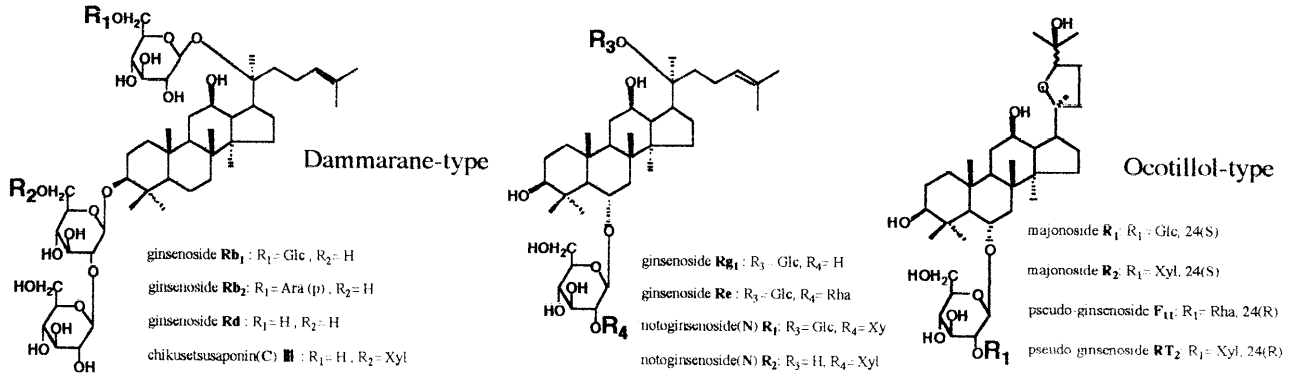
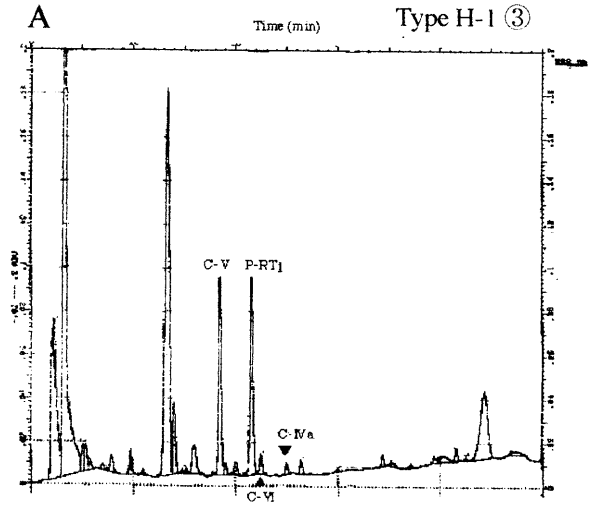
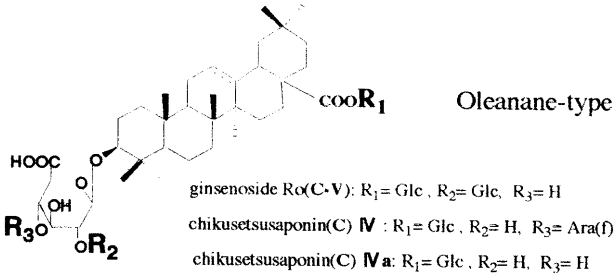


Fig. 3. A Phylogenic Tree of *Panax pseudo-ginseng* Plants by RAPD profiles
① to ⑬ are the numbers of sampling localities (See in Table 1)
The nucleotide diversity (d) was calculated from the RAPD profiles with eight-random primers (Operon Technologies Inc. USA, 10 mer-random primer)

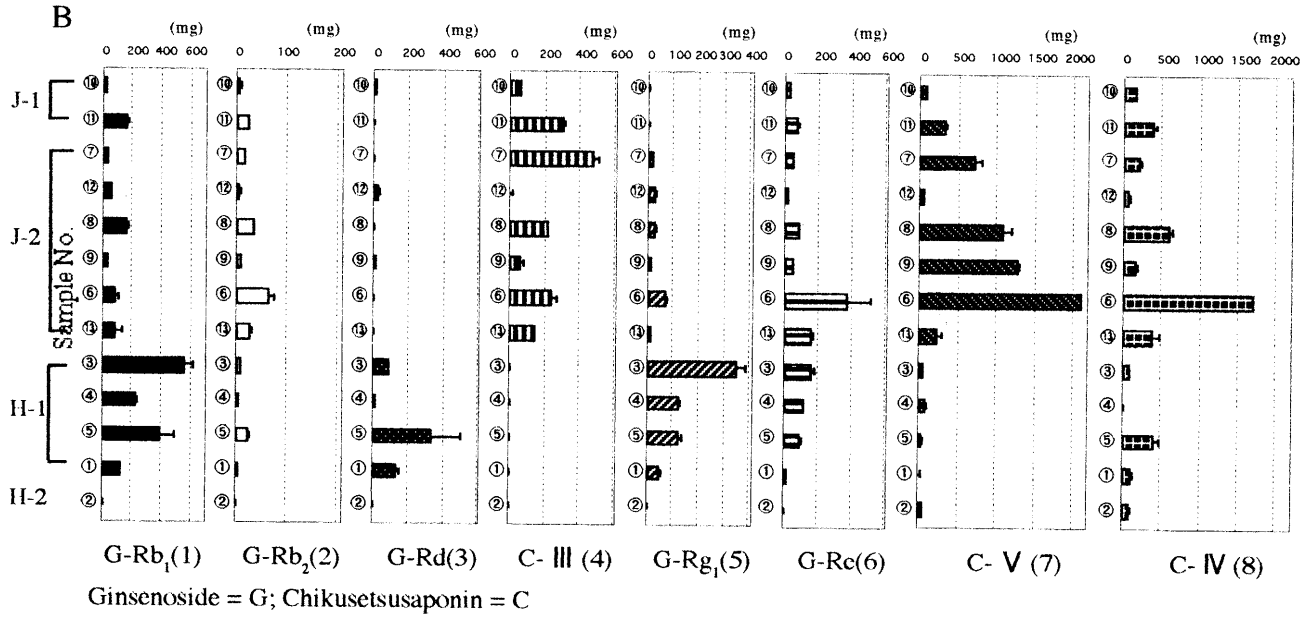




③ DMAN as shown in Table 1., eluent: Flow program (2)



Glc = β-D-glucopyranosyl; Rha= α-L-rhamnopyranosyl; Xyl = β-D-xylopyranosyl;
Ara (f) = α-L-arabinofuranosyl; Ara (p) = α-L-arabinopyranosyl;



Ginsenoside = G; Chikusetsusaponin = C

Fig. 4. Structures of saponins in rhizomes of *Panax pseudo-ginseng* plants, A; HPLC profiles of saponins of H-1 type rhizome (③ in Table 1), and B; Diagrams of saponin contents of wild *Panax pseudo-ginseng* plants from Nepal-Himalaya and Japan (mg / 100 gfr. wt). Each saponin fraction of the plant was identified by using eight standard samples: dammarane (1 - 6) and oleanane (7 and 8) types. Results represent arithmetic mean ± S.D. of three analyses.

P.p-g subsp. *pseudo-ginseng* Hara having a carrot-like root (②) and P.p-g var. *angustatus* Hara (called "hosobachikusetsu-ninjin" in Japanese ⑩ and ⑪), were genetically grouped into H-2 type and J-1 type, respectively, on phylogenic tree. It is interesting that chemo-geographical and genetical characters of each P.p-g are closely related to historical backgrounds such as the climatic and geographical changes of Central Nepal[22] in the Lesser Himalayan Range, and around the foot of Mt. Fuji in Japan.[23] The Himalayan *Panax* plants whose leaves are usually 5-foliate, rarely 3-foliate, with leaflets oblong-elliptic, doubly serrate, acuminate with scattered bristles on the upper surface, and Japanese P.p-g of the J-1 type whose leaves are usually 7-foliate, having no acuminate with scattered bristles on the upper surface, were closely related genetically, according to the RAPD analysis as shown in Fig. 3.

In this study, we classified P.p-g from 13 habitats into H-1, H-2, J-1 and J-2 types by the morphological characteristics and RAPD profiles, and H-1, H-2 and J types by their saponin contents. These results agree definitely to the morphological taxonomic study of *Panax pseudo-ginseng* plants by Hara.[1] We consider that RAPD analysis applied in combination with the analysis of saponin composition, is useful for distinguishing P.p-g plants.

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