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Geographic Variation of Phenylethanoids and Flavonoids in the Leaves of *Plantago asiatica* in Japan

Yoshinori Murai[1]*, Seiko Takemura[2], Junichi Kitajima[3] and Tsukasa Iwashina[1,2,4]

[1] Laboratory of Plant Chemotaxonomy, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Saiwai-cho 3–5–8, Fuchu, Tokyo, 183–8509 Japan
[2] Laboratory of Plant Chemotaxonomy, Graduate School of Agriculture, Ibaraki University, Ami, Ibaraki, 300–0393 Japan
[3] Laboratory of Pharmacognosy, Showa Pharmaceutical University, Higashi-tamagawagakuen 3, Machida, Tokyo, 194–8543 Japan
[4] Department of Botany, National Museum of Nature and Science, Amakubo 4–1–1, Tsukuba, Ibaraki, 305–0005 Japan
* E-mail: murai@kahaku.go.jp

Abstract Phenylethanoids and flavonoids in the leaves of *Plantago asiatica* from 91 populations in Japan were surveyed. Two phenylethanoids, i.e. plantamajoside and acteoside, and twelve flavonoids, i.e. apigenin 7-*O*-glucoside, apigenin 7-*O*-glucuronide, hispidulin 7-*O*-glucoside, hispidulin 7-*O*-glucuronide, 6-hydroxyluteolin 7-*O*-glucoside, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, nepetin 7-*O*-glucoside, nepetin 7-*O*-glucuronide, scutellarein 7-*O*-glucoside, pedalitin and sorbifolin were isolated and identified. While plantamajoside, which is used as a chemical marker of *P. asiatica* in the Japanese Pharmacopoeia, was mainly distributed in 83 populations, acteoside was mainly present in only eight populaitons. Moreover, flavonoid composition was varied among their populations. Hispidulin 7-*O*-glucuronide was found in some populations. This flavonoid has been reported from *P. major* which is known as an adventive plant in Japan. Interfusion of *P. major* into Japan was suggested by the utilization of this flavonoid as a chemical marker.
Key words : Acteoside, Flavonoids, Phenylethanoids, *Plantago asiatica*, Plantamajoside.

Introduction

Plantago asiatica L. is widely distributed in East Asia. The aerial parts of the plant are used as Chinese medicine (‘Plantaginis Herba’) as a diuretic, an antiinflammatory and an antiasthmatic drug in China and Japan. Nine flavonoids, i.e. apigenin, apigenin 7-*O*-glucoside (cosmosiin), hispidulin 7-*O*-glucoside (homoplantaginin), 6-hydroxyluteolin, 6-hydroxyluteolin 7-*O*-glucoside, luteolin, luteolin 7-*O*-glucoside, scutellarein and scutellarein 7-*O*-glucoside (plantaginin); an iridoid glucoside, aucubin; and five phenylethanoid glycosides, plantamajoside, acteoside, hellicoside, isoplantamajoside, 3,4-dihydroxyphenethylalcohol-6-*O*-caffeoy-β-D-glucose, and some types of plantasioside have been isolated from *P. asiatica* (Nakaoki *et al.*, 1961; Ravn *et al.*, 1990; Miyase *et al.*, 1991; Nishibe, 2002). Nishibe (2002) summarized the bioactivities of these compounds. Acteoside and plantamajoside showed antibacterial and antioxidation activities, the former compound also provided analgesic activity. In addition, plantamajoside and plantaginin showed antiallergic activity. Moreover, three flavone aglycones, 6-hydroxyluteolin, scutellarein and luteolin showed antiHIV activity (Nishibe, 2002). However, geographic variation of these phenolics has not been reported, though altitudinal variation has been surveyed (Murai *et al.*, 2009). Recently, an alien species, *P. major* L., hardly morphologically distinguished with *P. asiatica*, has got into Japan and made hybrid plants with *P. asiatica* (Ishikawa *et al.*, 2005). In this study, the phenylethanoid and flavonoid composition of *P. asiatica* and their variation was surveyed in 91 populations in Japan.

Materials and Methods

Plant materials

Plantago asiatica L. were collected in 2003-2009 from 91 populations in Japan (Table 1). Voucher specimens are deposited in the Herbarium of National Museum of Nature and Science, Japan (TNS).

Isolation of compounds

In qualitative analysis, the fresh leaves of *P. asiatica* were collected from some of 91 populations in Japan and extracted with MeOH. The concentrated extracts were applied to preparative paper chromatography using solvent systems: BAW (*n*-BuOH/HOAc/H_2O=4 : 1 : 5, upper phase), 15% HOAc and then BEW (*n*-BuOH/EtOH/H_2O=4 : 1 : 2.2). The isolated flavonoids were purified by Sephadex LH-20 column chromatography using solvent system: 70% MeOH. The compounds were further purified by preparative HPLC using Pegasil ODS (I.D. 10×250 mm, Senshu Scientific Co. Ltd.), at flow-rate: 1.5 ml min^{-1}, detection: 350 nm, and eluent: MeCN/H_2O/HCOOH (23 : 77 : 1).

Quantitative HPLC analysis of phenylethanoids and flavonoids

Fresh leaves (0.2 g) of *P. asiatica* were extracted with 4 ml MeOH. After filtration with Maisyoridisc H-13-5 (Tosoh), the extracts were analyzed by HPLC using L-column2 ODS (I.D. 6.0×150 mm, Chemicals Evaluation and Research Institute), at flow-rate: 1.0 ml min^{-1}, detection: 190–700 nm, and eluent: MeCN/H_2O/H_3PO_4 (20 : 80 : 0.2).

Liquid chromatograph-mass spectra (LC-MS)

LC-MS were measured using a Pegasil-ODS column (I.D. 2.0×150 mm, Senshu Scientific Co. Ltd.), at a flow-rate of 0.2 ml min^{-1}, eluting with HCOOH/MeCN/H_2O (5 : 22 : 73), injection: 10 μl, ESI^+4.5 kV, ESI^- 3.5 kV, 250°C.

Identification of phenylethanoids and flavonoids

Two phenylethanoids from the leaves of *P. asiatica* were identified by LC-MS, ^1H and ^{13}C NMR and characterization of acid hydrolysates (in 12% HCl, 100°C, 30 min). Flavonoids were identified by UV spectroscopy according to Mabry *et al.* (1970), LC-MS, HPLC comparisons with authentic standards and characterization of acid hydrolysates. In cases of two flavonoids (**F11** and **F12**), their chemical structures were estimated by UV, LC-MS and ^1H and ^{13}C NMR. TLC, UV, HPLC, acid hydrolysis, LC-MS, ^1H and ^{13}C NMR data of the isolated compounds are as follows.

Plantamajoside (**P1**). Amorphous powder. TLC: Rf 0.45 (BAW), 0.49 (BEW), 0.76 (15% HOAc); UV–light blue, UV/NH_3–green fluorescence. UV: λmax (nm) MeOH 291, 330; +NaOMe 379 (inc.); +$AlCl_3$ 300, 360; +$AlCl_3$/HCl 291, 330; +NaOAc 290, 340; +NaOAc/H_3BO_3 294, 354. Acid hydrolysis: caffeic acid and glucose. HPLC: Rt 5.5 min. LC-MS: *m*/*z* 639 $[M-H]^-$ (each 1 mol 3,4-dihydroxyphenethyl alcohol and caffeic acid + 2 mol glucose). ^1H NMR (500 MHz, pyridine-d_5): δ 8.04 (1H, *d*, *J*= 15.9 Hz, H-7′), 6.83 (1H, *d*, *J*=15.9 Hz, H-8′), 5.31 (1H, *d*, *J*=7.6 Hz, glucosyl H-1), 4.88 (1H, *d*, *J*=7.9 Hz, *t*-glucosyl H-1), 3.9–4.6 (*m*, sugar protons), 2.96 (2H, *d*, *J*=7.0 Hz, H-7). ^{13}C NMR (125 MHz, pyridine-d_5): δ (3,4-dihydroxyphenethyl alcohol) 130.3 (C-1), 116.7 (C-2), 146.3 (C-3), 145.6 (C-4), 117.5 (C-5), 120.5 (C-6), 36.1 (C-7), 74.6 (C-8); δ (caffeic acid) 127.0 (C-1), 115.8 (C-2), 147.1 (C-3), 150.4 (C-4), 116.5 (C-5), 122.2 (C-6), 147.6 (C-7), 115.2 (C-8), 167.1 (C-9); δ (glucose) 104.0 (C-1), 76.3 (C-2), 84.8 (C-3), 71.5 (C-4), 76.2 (C-5), 62.7 (C-6); δ (*t*-glucose) 106.7 (C-1), 75.9 (C-2), 78.1 (C-3), 71.3 (C-4), 78.3 (C-5), 62.1 (C-6).

Acteoside (**P2**). Amorphous powder. TLC: Rf 0.61 (BAW), 0.61 (BEW), 0.82 (15%HOAc); UV–light blue, UV/NH_3–green fluorescence. UV: λmax (nm) MeOH 291, 332; +NaOMe 382 (inc.); +$AlCl_3$ 300, 360; +$AlCl_3$/HCl 290sh, 329; +NaOAc 290, 344; +NaOAc/H_3BO_3 295, 354. Acid hydrolysis: caffeic acid, glucose and rhamnose. HPLC: Rt 7.1 min. LC-MS: *m*/*z* 623 $[M-H]^-$ (each 1 mol 3,4-dihydroxyphenethyl alcohol, caffeic acid, glucose and rhamnose). ^1H

NMR (500 MHz, pyridine-d_5): δ 8.03 (1H, *d*, *J*= 15.9 Hz, H-7′), 7.21 (1H, *d*, *J*=17.7 Hz, H-8′), 5.69 (1H, *s*, rhamnosyl H-1), 4.82 (1H, *d*, *J*=8.2 Hz, glucosyl H-1), 3.8-4.6 (*m*, sugar protons), 2.92 (2H, *d*, *J*=7.0 Hz, H-7), 1.65 (3H, *d*, *J*=6.1 Hz, rhamnosyl Me). ^{13}C NMR (125 MHz, pyridine-d_5): δ (3,4-dihydroxyphenethyl alcohol) 130.4 (C-1), 116.5 (C-2), 146.8 (C-3), 145.6 (C-4), 117.5 (C-5), 120.4 (C-6), 36.0 (C-7), 71.3 (C-8); δ (caffeic acid) 126.9 (C-1), 115.8 (C-2), 147.1 (C-3), 150.6 (C-4), 116.7 (C-5), 122.3 (C-6), 147.7 (C-7), 114.7 (C-8), 167.1 (C-9); δ (glucose) 104.2 (C-1), 75.8 (C-2), 80.6 (C-3), 70.2 (C-4), 76.4 (C-5), 62.1 (C-6); δ (rhamnose) 103.1 (C-1), 72.6 (C-2), 72.6 (C-3), 74.0 (C-4), 70.3 (C-5), 19.2 (C-6).

6-Hydroxyluteolin 7-*O*-glucoside (**F1**). Pale yellow powder. TLC: Rf 0.19 (BAW), 0.20 (BEW), 0.06 (15%HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 255, 284, 350; +NaOMe 261, 306, 391 (inc.); +$AlCl_3$ 274, 301, 422; +$AlCl_3$/HCl 261, 296, 371; +NaOAc 263sh, 292sh, 392; +NaOAc/H_3BO_3 263, 287, 357. Acid hydrolysis: 6-hydroxyluteolin and glucose. HPLC: Rt 5.1 min. LC-MS: *m*/*z* 465 $[M+H]^+$, 463 $[M-H]^-$ (6-hydroxyluteolin+1 mol glucose) and 303 $[M-162+H]^+$, 301 $[M-162-H]^-$ (6-hydroxyluteolin).

Scutellarein 7-*O*-glucoside (plantaginin, **F2**). Pale yellow powder. TLC: Rf 0.74 (BAW), 0.70 (BEW), 0.05 (15%HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 285, 334; +NaOMe 268sh, 306sh, 337sh, 375 (inc.); +$AlCl_3$ 236, 304, 366; +$AlCl_3$/HCl 263sh, 289sh, 302, 360; +NaOAc 290, 381; +NaOAc/H_3BO_3 293, 331. Acid hydrolysis: scutellarein and glucose. HPLC: Rt 7.7 min. LC-MS: *m*/*z* 449 $[M+H]^+$, 447 $[M-H]^-$ (scutellarein + 1 mol glucose) and 288 $[M-162+H]^+$, 286 $[M-162-H]^-$ (scutellarein).

Luteolin 7-*O*-glucoside (**F3**). Pale yellow powder. TLC: Rf 0.34 (BAW), 0.37 (BEW), 0.05 (15%HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 255, 268, 287sh, 343; +NaOMe 266, 300sh, 385 (inc.); +$AlCl_3$ 273, 297sh, 375, 425; +$AlCl_3$/HCl 262sh, 275, 296, 360, 387sh; +NaOAc 260, 294sh, 400; +NaOAc/H_3BO_3 260, 290sh, 373. Acid hydrolysis: luteolin and glucose. HPLC: Rt 7.7 min. LC-MS: *m*/*z* 450 $[M+H]^+$, 448 $[M-H]^-$ (luteolin + 1 mol glucose) and 288 $[M-162+H]^+$, 286 $[M-162-H]^-$ (luteolin).

Luteolin 7-*O*-glucuronide (**F4**). TLC: Rf 0.26 (BAW), 0.28 (BEW), 0.07 (15% HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 255, 268sh, 347; +NaOMe 265, 387 (inc.); +$AlCl_3$ 274, 427; +$AlCl_3$/HCl 273, 294sh, 364, 385; +NaOAc 260, 402; +NaOAc/H_3BO_3 260, 372. Acid hydrolysis: luteolin and glucuronic acid. HPLC: Rt 8.4 min. LC-MS: *m*/*z* 464 $[M+H]^+$, 462 $[M-H]^-$ (luteolin + 1 mol glucuronic acid) and 288 $[M-176+H]^+$, 286 $[M-176-H]^-$ (luteolin).

Nepetin 7-*O*-glucoside (**F5**). TLC: Rf 0.40 (BAW), 0.52 (BEW), 0.12 (15%HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 270, 339; +NaOMe 270, 376 (inc.); +$AlCl_3$ 274, 299, 370, 416; +$AlCl_3$/HCl 278, 298, 360, 405sh; +NaOAc 265, 380; +NaOAc/H_3BO_3 267, 288, 336. Acid hydrolysis: nepetin and glucose. HPLC: Rt 8.9 min. LC-MS: *m*/*z* 479 $[M+H]^+$, 477 $[M-H]^-$ (nepetin+1 mol glucose) and 317 $[M-162+H]^+$, 315 $[M-162-H]^-$ (nepetin).

Nepetin 7-*O*-glucuronide. (**F6**) TLC: Rf 0.28 (BAW), 0.30 (BEW), 0.12 (15%HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 256, 271, 346; +NaOMe 276, 295sh, 427 (inc.); +$AlCl_3$ 275, 305sh, 428; +$AlCl_3$/HCl 262sh, 278, 296sh, 369; +NaOAc 265, 404; +NaOAc/H_3BO_3 263, 374. Acid hydrolysis: nepetin and glucunonic acid. HPLC: Rt 9.6 min. LC-MS: *m*/*z* 493 $[M+H]^+$, 491 $[M-H]^-$ (nepetin + 1 mol glucuronic acid) and 317 $[M-176+H]^+$, 315 $[M-176-H]^-$ (nepetin).

Apigenin 7-*O*-glucoside (cosmosiin, **F7**). TLC: Rf 0.57 (BAW), 0.56 (BEW), 0.13 (15% HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 268, 285sh, 332; +NaOMe 266sh, 274, 304, 347sh, 379 (inc.); +$AlCl_3$ 266sh, 275, 299, 349, 376sh; +$AlCl_3$/HCl 266sh, 275, 299, 341, 375sh; +NaOAc

Table 1. The distribution of phenylethanoids and flavonoids in the leaves of putative *P. asitatica*

Collection sites	Phenolic acids		Flavonoids												Collection date
	P1	P2	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	
Rukushi Pass, Saroma-cho, Hokkaido	++	t	t	t	+		++		t		+++		t	++	24 Aug. 2008
Lake Saroma, Saroma-cho, Hokkaido	++			t		+		++		t	t	++			24 Aug. 2008
Nakajima, Sapporo City, Hokkaido	++													t	24 Aug. 2008
Goryokaku, Hakodate City, Hokkaido	++			t			t	t	t	t	t	++			29 Sep. 2003
Oirase, Towada City, Aomori	++			t	t		t	t	t		t	+		t	2 Oct. 2003
Hakkoda, Towada City, Aomori	++							+				++			19 Aug. 2004
Aomori City, Aomori	++		t	++	+		++		t		++		t	t	19 Aug. 2004
Hirosaki station, Hirosaki City, Aomori	++		t		t				t	t	+	++		t	1 Oct. 2003
Nishi-Tsugaru-gun, Aomori	++	t	t	t		t	t	t	t		+	+	t	t	30 Sep. 2003
Yamato, Kurokawa-gun, Miyagi	++									t					7 Jun. 2005
Yumoto, Iwaki City, Fukushima	++													t	6 Nov. 2005
Nakasonedaira, Yama-gun, Fukushima	++									t					24 Aug. 2004
Hinoemata-mura, Minami-Aizu-gun, Fukushima	++					t									3 Sep. 2004
Minami Lake, Shirakawa, Fukushima	++									t					11 May 2009
Tsukuba, Tsukuba City, Ibaraki	++					t			t						19 Nov. 2005
Tsukuba Botanical Garden, Tsukuba City, Ibaraki	++									t					13 Sep. 2005
University of Tsukuba, Tsukuba City, Ibaraki	++			t		t				t					23 May 2005
Takeuchi-shinden, Haga-gun, Tochigi	++			t											11 Jun. 2005
Kumagaya City, Saitama	++					t				t					1 May 2006
Kinchakuda, Hidaka City, Saitama	++									t					4 Oct. 2005
Chichibu City, Saitama	++									t					21 Mar. 2006
Shimoshizu, Sakura City, Chiba	++									t					24 May 2009
Chiba City, Chiba	++									t					24 May 2009
Higashi-kurume, Higashi-kurume City, Tokyo	++									t					4 Oct. 2005
Nakanosawa, Hachioji City, Tokyo	++									t				t	11 Apr. 2006
Ikebukuro, Toshima-ku, Tokyo	++		t	t			+		t		++			t	5 Jun. 2006
Tsurumaki, Tama City, Tokyo	++							+	t		++				23 May 2009
Yamashita Park, Yokohama City, Kanagawa	++			t			+	+			+	++	t	t	26 Aug. 2005
Nakamura Park, Yokohama City, Kanagawa	++	t	+	+			++				+		t		8 May 2008
Daikoku Pier-1, Yokohama City, Kanagawa	++									t					26 Aug. 2005
Daikoku Pier-2, Yokohama City, Kanagawa	++			t		t				t			t	t	26 Aug. 2005
Seya-1, Yokohama City, Kanagawa	++				t				t	t					27 Apr. 2009
Seya-2, Yokohama City, Kanagawa	++		+	t		++	++			++		++			23 May. 2009
Yumegaoka, Yokahama City, Kanagawa	++	t								t					9 Jun. 2008
Yokosuka-1, Yokosuka City, Kanagawa	++			t				++				++			27 Aug. 2005
Yokosuka-2, Yokosuka City, Kanagawa	++							++		t		++	t		27 Aug. 2005
Seaside of Koajiro, Miura City, Kanagawa		++	t	+	+		+		t		++		t	t	8 Mar. 2006
Forest of Koajiro, Miura City, Kanagawa	+	++	t	+	+		++		t		++		t	t	8 Mar. 2006
Enoshima Island, Fujisawa City, Kanagawa	++	t		t							++			t	20 Aug. 2005
Kugenuma Beach, Fujisawa City, Kanagawa	++	t								t					20 Aug. 2005
Ninomiya-cho, Naka-gun, Kanagawa	t	++	+	t			+		t		++		t	t	10 Aug. 2004
Lake Ashinoko, Hakone-cho, Ashigara-gun, Kanagawa	++			t			+		t		+++		+	++	10 Aug. 2004
Mt. Kurodake, Susono City, Shizuoka	++		t	t	t		++		t		++		t	t	10 Aug. 2004
Kawazu, Gamou-gun, Shizuoka	t	++		t	t		++		t				t	t	21 Feb. 2006
Ugusu, Nishi-izu-cho, Gamou-gun, Shizuoka	t	++	t	t	+		+		t		++		t	+	8 Apr. 2007
Riverside of Nishi-izu-cho, Gamou-gun, Shizuoka	t	++	t	t	+		++		t		++		t	+	9 Apr. 2007
Seaside of Higashi-izu-cho, Gamou-gun, Shizuoka	t	++	t	t	+		+		t		++		t	+	9 Apr. 2007
Kakegawa City, Shizuoka	++		++	++	+				t		t		t	t	25 Apr. 2009

Table 1. -(Continued)-

Collection sites	Phenolic acids		Flavonoids												Collection date
	P1	P2	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	
Hamamatsu City, Shizuoka	++	t	++	++	+		++		t		++		+	t	26 Apr. 2009
Kitayama, Chino City, Nagano	++	t												t	23 May 2004
Nagawa-cho, Nagano	++		t	+	+				t				+	t	9 Oct. 2004
Takayama City, Gifu	t	++	+	++	+				t				+	++	23 Sep. 2005
Unazuki, Kurobe City, Toyama	++		t	t					t				t	t	25 Sep. 2005
Toyama City, Toyama	++	+		+									t	+	10 Aug. 2007
Tedori Valley, Hakusan City, Ishikawa	++				t				t				t	++	12 Nov. 2006
Nagoya City, Aichi	++	t	+	++			t		t		++			t	25 Sep. 2005
Odomari, Kumano City, Mie	++		+	++	++		++		+		++		t	t	5 May 2006
Nachi-katsuura-cho, Higashi-muro-gun, Wakayama	++		+	+	+		++		t		++		t	t	5 May 2006
Shugakuin, Kyoto City, Kyoto	t		+	+	++		+++		+		+++		t	t	1 Dec. 2005
Ohara, Kyoto City, Kyoto	++		+	t	++		++		t		++		+	t	1 Dec. 2005
Mitsu, Okayama City, Okayama	++			t			++		t		++		t	t	4 Nov. 2004
Kurotaki-1, Takehara City, Hiroshima	++			t			t		t		++			t	28 Apr. 2006
Kurotaki-2, Takehara City, Hiroshima	++		+	t	++				t				++	++	28 Apr. 2006
Ichinomiya, Kochi City, Kochi	t			t	t		++		t		++				23 May 2005
Rendai, Kochi City, Kochi	++		t	t			++		t		++		+	++	23 May 2005
Shimanto-cho, Takaoka-gun, Kochi	++		t	+			+		t		++		t	t	31 Aug. 2004
Ohkamiuchi, Hata-gun, Kochi	++			t			+		t		++			t	31 Aug. 2004
Kamegamori, Agawa-gun, Ehime	++		t	+			++		t		++		+	t	31 Aug. 2004
Fukuoka City, Fukuoka	++	+		+	+		++		t		++		++	t	22 Jun. 2005
Tsushima Island-1, Fukuoka	++		t	+	+		++		t		++		t	t	11 Apr. 2006
Tsushima Island-2, Fukuoka	++	t	t	+	+		++		t		++		t	t	12 Apr. 2006
Tsushima Island-3, Fukuoka	++	t	t	++	++		++		t		++		t	t	13 Apr. 2006
Kumatomo University, Kumamoto City, Kumamoto	+						+				++			t	15 Sep. 2006
Kumamoto City, Kumamoto	++			t			t				++			t	16 Sep. 2006
Arashida, Kunitomi-gun, Miyazaki	++			t	+		+		t		++		t	+	23 May 2007
Kagoshima City, Kagoshima	++	+	t	++			++		t		++		t	t	28 Nov. 2005
Amami Island-1, Kagoshima	++		t	t	t		++		t		++		t	+	13 Nov. 2004
Amami Island-2, Kagoshima	++		t	t			++		t		++		t	t	13 Nov. 2004
Amami Island-3, Kagoshima	++		t	+	t		++		t		++		t	t	13 Nov. 2004
Amami Island-4, Kagoshima	++		t	t			+		t		++		t	t	13 Nov. 2004
Izena Island-1, Shimajiri-gun, Okinawa	++		t	++									t	++	10 Jul. 2005
Izena Island-2, Shimajiri-gun, Okinawa	++		t	++					t				+	+++	10 Jul. 2005
Kunigami-son, Kunigami-gun, Okinawa	++		t	++					t				t	t	10 Feb. 2006
Ohgimi-son, Kunigami-gun, Okinawa	++			t			++				++		t		20 Aug. 2005
Nago City, Okinawa	++			t					t				t	+	26 Dec. 2004
Zamami Island, Shimajiri-gun, Okinawa	++		t	+	+				t				+	++	1 Feb. 2009
Kume Island-1, Shimajiri-gun, Okinawa	++		+	++			+++		t		+++	t	t	t	4 Oct. 2005
Kume Island-2, Shimajiri-gun, Okinawa	++								t				t	++	5 Dec. 2008
Mt. Omoto-dake, Ishigaki Island, Yaeyama-gun, Okinawa	++		t	t	t		++		t		++		+	t	10 Feb. 2006
Nagura, Ishigaki Island, Yaeyama-gun, Okinawa	++		+	+	+				t				++	t	10 Feb. 2006
Iriomote Island, Yaeyama-gun, Okinawa	++					++	t	+	t	+		+	t	t	12 Jun. 2005

P1: plantamajoside, **P2**: acteoside, **F1**: 6-hydorxyluteolin 7-glucoside, **F2**: scutellarein 7-glucoside, **F3**: luteolin 7-glucoside, **F4**: luteolin 7-glucuronide, **F5**: nepetin 7-glucoside, **F6**: nepetin 7-glucuronide, **F7**: apigenin 7-glucoside, **F8**: apigenin 7-glucuronide, **F9**: hispidulin 7-glucoside, **F10**: hispidulin 7-glucuronide, **F11**: pedalitin and **F12**: sorbifolin.

The contents of phenylethanoids and flavonoids are shown as ++>1,000,000, +>200,000 mAU, t > 100,000, and +++>1,000,000, ++>200,000 mAU, +>100,000 mAU, t<100,000 mAU, respectively, by peak areas at 340 nm of HPLC analysis.

255sh, 266, 284sh, 389; +NaOAc/H_3BO_3 268, 285sh, 340. Acid hydrolysis: apigenin and glucose. HPLC: Rt 12.4 min. LC-MS: *m/z* 433 $[M+H]^+$, 431 $[M-H]^-$ (apigenin + 1 mol glucose) and 271 $[M-162+H]^+$, 269 $[M-162-H]^-$ (apigenin).

Apigenin 7-*O*-glucuronide (**F8**). TLC: Rf 0.45 (BAW), 0.43 (BEW), 0.12 (15%HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 268, 334; +NaOMe 279, 277, 381 (inc.); +$AlCl_3$ 275, 298, 348, 381; +$AlCl_3$/HCl 276, 299, 343, 379sh; +NaOAc 267, 387; +NaOAc/H_3BO_3 268, 338. Acid hydrolysis: apigenin and glucuronic acid. HPLC: Rt 14.2 min. LC-MS: *m/z* 447 $[M+H]^+$, 445 $[M-H]^-$ (apigenin + 1 mol glucuronic acid) and 271 $[M-176+H]^+$, 269 $[M-176-H]^-$ (apigenin).

Hispidulin 7-*O*-glucoside (homoplantaginin, **F9**). TLC: Rf 0.64 (BAW), 0.63 (BEW), 0.23 (15%HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 275, 333; +NaOMe 277, 307, 356, 376sh (inc.); +$AlCl_3$ 284, 300, 360, 387sh; +$AlCl_3$/HCl 283sh, 299, 351, 385sh; +NaOAc 272, 389; +NaOAc/H_3BO_3 274, 336. Acid hydrolysis: hispidulin and glucose. HPLC: Rt 14.5 min. LC-MS: *m/z* 433 $[M+H]^+$, 431 $[M-H]^-$ (hispidulin + 1 mol glucose) and 271 $[M-162+H]^+$, 269 $[M-162-H]^-$ (hispidulin).

Hispidulin 7-*O*-glucuronide (**F10**). TLC: Rf 0.47 (BAW), 0.42 (BEW), 0.22 (15% HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 275, 333; +NaOMe 276, 305, 358, 378 (inc.); +$AlCl_3$ 284sh, 300, 360; +$AlCl_3$/HCl 286sh, 299, 353; +NaOAc 272, 390; +NaOAc/H_3BO_3 274, 335. Acid hydrolysis: hispidulin and glucuronic acid. HPLC: Rt 16.7 min. LC-MS: *m/z* 447 $[M+H]^+$, 445 $[M-H]^-$ (hispidulin+1 mol glucuronic acid) and 271 $[M-176+H]^+$, 269 $[M-176-H]^-$ (hispidulin).

Pedalitin (**F11**). Pale yellow powder. TLC: Rf 0.44 (BAW), 0.45 (BEW), 0.02 (15%HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 284, 345; +NaOMe 264sh, 390 (inc.); +$AlCl_3$ 273, 304, 421; +$AlCl_3$/HCl 257sh, 297, 368; +NaOAc 268, 286sh, 394; +NaOAc/H_3BO_3 260, 288, 357. HPLC: Rt 20.3 min. LC-MS: *m/z* 317 $[M+H]^+$, 315 $[M-H]^-$ (pedalitin). ^{1}H NMR (600 MHz, pyridine-d_5): δ 13.61 (1H, *s*, 5-OH), 7.99 (1H, *d*, *J*=2.2 Hz, H-2′), 7.59 (1H, *dd*, *J*=2.2 and 8.3 Hz, H-6′), 7.39 (1H, *d*, *J*=8.3 Hz, H-5′), 6.98 (1H, *s*, H-3), 6.79 (1H, *s*, H-8), 3.94 (3H, *s*, 7-*O*Me). ^{13}C NMR (150 MHz, pyridine-d_5): δ 165.0 (C-2), 132.1 (C-3), 183.2 (C-4), 151.7 (C-5), 103.6 (C-6), 155.1 (C-7), 91.3 (C-8), 147.8 (C-9), 106.5 (C-10), 123.4 (C-1′) 114.7 (C-2′), 146.0 (C-3′), 147.7 (C-4′), 116.9 (C-5′), 119.5 (C-6′), 56.4 (7-*O*Me).

Sorbifolin (**F12**). Pale yellow powder. TLC: Rf 0.75 (BAW), 0.70 (BEW), 0.02 (15% HOAc); UV–dark purple, UV/NH_3–bright yellow. UV: λmax (nm) MeOH 285, 334; +NaOMe 273sh, 286sh, 306sh, 374 (inc.); +$AlCl_3$ 264sh, 304, 366; +$AlCl_3$/HCl 262sh, 301, 360; +NaOAc 276sh, 287, 298sh, 380; +NaOAc/H_3BO_3 292, 331. HPLC: Rt 39.6 min. LC-MS: *m/z* 301 $[M+H]^+$, 299 $[M-H]^-$ (sorbifolin). ^{1}H NMR (600 MHz, pyridine-d_5): δ 13.59 (1H, *s*, 5-OH), 7.99 (2H, *d*, *J*=8.8 Hz, H-2′,6′), 7.33 (2H, *d*, *J*=8.8 Hz, H-3′,5′), 6.98 (1H, *s*, H-3), 6.93 (1H, *s*, H-8), 3.96 (3H, *s*, 7-*O*Me). ^{13}C NMR (150 MHz, pyridine-d_5): δ 164.6 (C-2), 132.1 (C-3), 183.3 (C-4), 155.1 (C-5), 103.5 (C-6), 162.7 (C-7), 91.5 (C-8), 150.0 (C-9), 106.5 (C-10), 122.3 (C-1′), 128.9 (C-2′,6′), 116.9 (C-3′,5′), 147.8 (C-4′), 56.4 (7-*O*Me).

Results and Discussion

Identification of phenylethanoids and flavonoids

In this survey, two phenylethanoids and twelve flavonoids were isolated from 91 populations of *P. asiatica*. Two phenylethanoids, **P1** and **P2** were obtained as amorphous white powder. The chemical properties of major phenylethanoids of *P. asiatica* were described in Ravn *et al.* (1990). UV spectral properties of these compounds were similar to each other and showed the existence of caffeic acid (λmax 291, 330 or 332 in MeOH). Their molecular ion peaks of LC-MS, *m/z* 639 and 623 showed the attachment of each 1 mol 3,4-dihydroxyphenethyl alcohol, caffeic acid,

glucose and rhamnose, and each 1 mol 3,4-dihydroxyphenethyl alcohol and caffeic acid and 2 mol glucose, respectively. ^{1}H and ^{13}C NMR data of these compounds were assigned and compared with those of Ravn *et al.* (1990).

On the other hand, the chemical structures of ten flavone glycosides (**F1**–**F10**) were deduced by UV spectrum, LC-MS, HPLC and TLC comparisons with authentic specimens and acid hydrolysates. Two flavone aglycones (**F11** and **F12**) were elucidated by UV spectral, LC-MS and NMR data. In the HMBC spectrum, correlations from methoxyl proton signal to C-7 of aglycone were observed in both compounds.

Murai *et al.* (2008a) listed up almost all flavonoids isolated from *Plantago* species. Sorbifolin and pedalitin was isolated from *Plantago* species for the first time. Sorbifolin (**F11**) and pedalitin (**F12**) have been reported from the leaves of *Sorbaria stellipila* Schneid. (Rosaceae) (Arisawa *et al.*, 1970) and *Sesamum indicum* L. (Pedaliaceae) (Harborne, 1967), respectively. Two flavone glycosides, luteolin and hispidulin 7-*O*-glucuronides, which have previously been reported from *P. major* (Lebedev-Kosov, 1976; Kawashty *et al.*, 1994), were isolated from *P. asiatica* for the first time. Moreover, apigenin and nepetin 7-*O*-glucuronides have been isolated from *P. lagopus* L. (Kawashty *et al.*, 1994) and *P. hakusanensis* Koidz. (Murai *et al.*, 2008a), respectively.

Phenylethanoid and flavonoid composition of P. asiatica

In a series of studies of phenylethanoids and flavonoids in *P. asiatica* (Nakaoki *et al.*, 1961; Ravn *et al.*, 1990; Miyase *et al.*, 1991; Nishibe, 2002), the chemical variation among populations has not been surveyed. In the present study, we surveyed 91 populations from Hokkaido to Okinawa in Japan, and two phenylethanoids and twelve flavonoids were isolated and identified (Figs. 1 and 2). Their flavonoid composition was different among their populations (Table 1). We also observed the variations of phenylethanoid composition among their populations (Table 1).

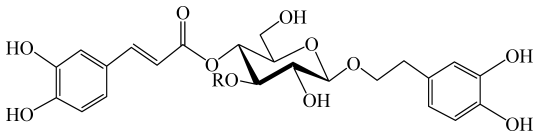

Fig. 1. The chemical structures of the isolated phenylethanoids from *Plantago asiatica.*
P1: plantamajoside (R=glucosyl), **P2**: acteoside (R=rhamnosyl).

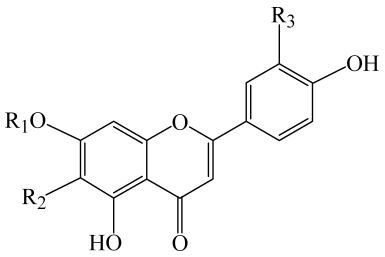

Fig. 2. The chemical structures of the isolated flavonoids from *Plantago asiatica*.
F1: 6-hydroxyluteolin 7-*O*-glucoside (R_1=glucosyl, R_2=R_3=OH), **F2**: scutellarein 7-*O*-glucoside (R_1=glucosyl, R_2=OH, R_3=H), **F3**: luteolin 7-*O*-glucoside (R_1=glucosyl, R_2=H, R_3=OH), **F4**: luteolin 7-*O*-glucuronide (R_1=glucuronyl, R_2=H, R_3=OH), **F5**: nepetin 7-*O*-glucoside (R_1=glucosyl, R_2=OCH_3, R_3=OH), **F6**: nepetin 7-*O*-glucuronide (R_1=glucuronyl, R_2=OCH_3, R_3=OH), **F7**: apigenin 7-*O*-glucoside (R_1=glucosyl, R_2=R_3=H), **F8**: apigenin 7-*O*-glucuronide (R_1=glucuronyl, R_2=R_3=H), **F9**: hispidulin 7-*O*-glucoside (R_1=glucosyl, R_2=OCH_3, R_3=H), **F10**: hispidulin 7-*O*-glucuronide (R_1=glucuronyl, R_2=OCH_3, R_3=H), **F11**: pedalitin (R_1=CH_3, R_2=R_3=OH), **F12**: sorbifolin (R_1=CH_3, R_2=OH, R_3=H).

Plantamajoside have been used as a chemical marker of *P. asiatica* for 'Plantaginis Herba' in the Japanese Pharmacopoeia (2006). However, it was shown by this survey that some populations did not contain plantamajoside, but they alternatively contained acteoside, e.g. Koajiro (Miura City, Kanagawa), Ninomiya (Naka-gun, Kanagawa) and Gamou-gun (Shizuoka) (Table 1). Flavonoids have often been used as chemotaxonomic markers in some plant species, e.g. *Cassytha* species (Lauraceae) (Murai *et al.*, 2008b), *Saussurea* and *Serratula* species (Asteraceae) (Kusano *et al.*, 2007), and so on. However,

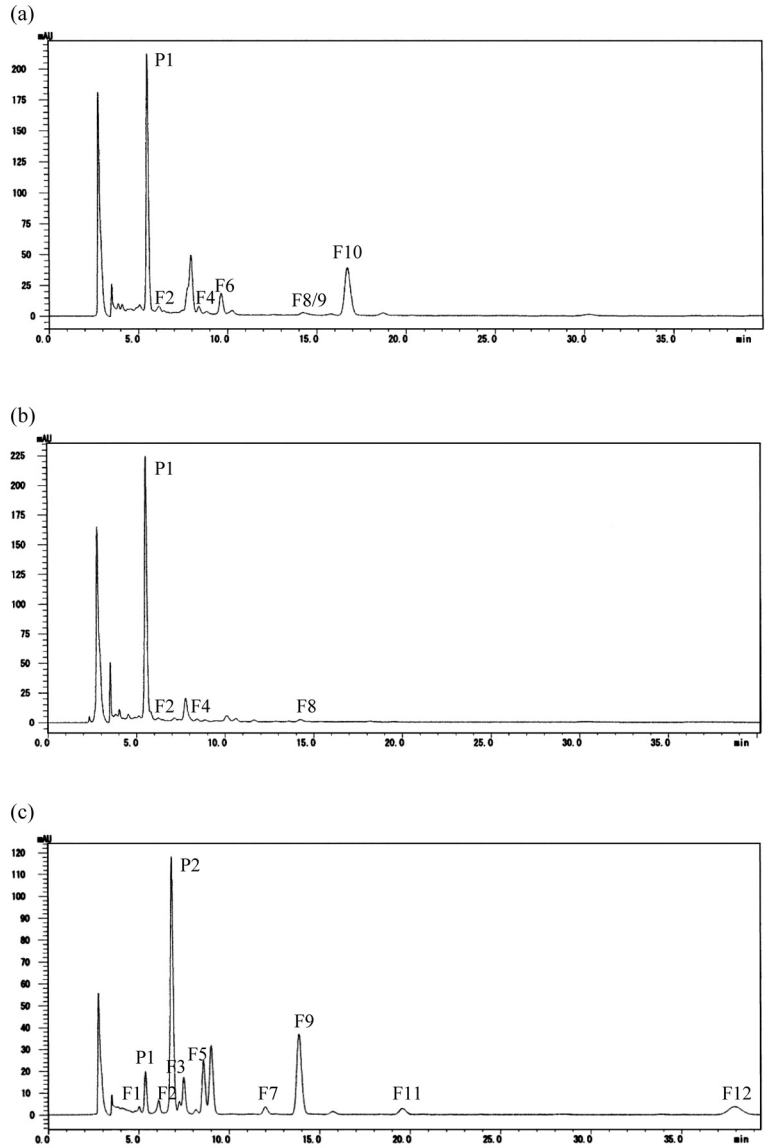

Fig. 3. HPLC patterns of MeOH extracts from putative *P. asiatica*.
(a): Lake Saroma, (b): University of Tsukuba, (c): Riverside of Nishi-izu-cho. **P1**: plantamajoside, **P2**: acteoside, **F1**: 6-hydroxyluteolin 7-*O*-glucoside, **F2**: scutellarein 7-*O*-glucoside, **F3**: luteolin 7-*O*-glucoside, **F4**: luteolin 7-*O*-glucuronide, **F5**: nepetin 7-*O*-glucoside, **F6**: nepetin 7-*O*-glucuronide, **F7**: apigenin 7-*O*-glucoside, **F8**: apigenin 7-*O*-glucuronide, **F9**: hispidulin 7-*O*-glucoside, **F10**: hispidulin 7-*O*-glucuronide, **F11**: pedalitin and **F12**: sorbifolin

our result suggests that *P. asiatica* in Japan shows the intraspecific variation of flavonoid composition.

Possibility of interfusion of P. major

P. asiatica is widely distributed in Asia region. On the other hand, European species, *P. major,* got into Asia region including Japan. The coexistence of the native *P. asiatica* and alien *P. major* led to make their hybrid (Ishikawa *et al.*, 2005; Sahin *et al.*, 2007). Flavonoid variation in *P. asiatica* may reflect not only geographic variation

but also introgressive hybridization. Some populations in Hokkaido, Kanagawa and Okinawa Prefectures contain hispidulin 7-*O*-glucuronide (Table 1). This flavonoid has been reported from *P. major* but not from *P. asiatica*. Interestingly, *P. major* have often been reported from these area. Two species have similar morphological characters, e.g. leaf, flower and root. Of their characters, the number of seeds in a capsule is comparatively different with each other (Ishikawa *et al.*, 2005). However, we could not count them of all populations, so there was the possibility that we investigated some *P. major* and/or hybrids between *P. asiatica* and *P. major* in this experiment. The number of seeds per capsule of *P. asiatica* and *P. major* is 4–6 and 7<, respectively. We observed that Seya-1 (Yokohama City, Kanagawa) had 5 seeds in a capsule. On the other hand, Seya-2 (Yokohama City, Kanagawa), which contained hispidulin 7-*O*-glucuronide, had 7 seeds per capsule. In addition, it has been suggested that the phylogeny among *Plantago* species has been highly complex (Ishikawa *et al.*, 2006). Flavonoid composition of *P. major* and other *Plantago* species in Japan and Asia also needs to be surveyed.

Flavonoid composition of putative polyploid P. asiatica

The chromosome numbers of *P. asiatica* in Japan have been reported as 2n=23, 24 and 36 (Iwatsubo *et al.*, 2000). Intraspecific ploidy levels often cause variation of plant size (Stebbins, 1971). Polyploid plants occasionally make their body big. In this study, the plant size of some populations, i.e. Shimoshizu (Sakura City, Chiba), Yumegaoka (Yokohama City, Kanagawa) and Daikoku Pier-1 (Yokohama City, Kanagawa) was at least twice larger than those of the other populations. We observed these samples barely contain flavonoids. Active vegetative growth makes plant body constitutively bigger and/or stronger. In this case, the bigger plants may not need to produce defensive substances such as the flavonoids. The study for revealing the relationships between chromosome number, plant size and flavonoid production in *P. asiatica* also needs to be performed.

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