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Flavonoids from *Iris sanguinea* var. *tobataensis* and Chemotaxonomic and Molecular Phylogenetic Comparisons with *Iris sanguinea* var. *sanguinea*

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Abstract *Iris sanguinea* var. *tobataensis* (Japanese name, "*Tobata-ayame*") is endemic to Tobata, Kita-Kyushu City, Fukuoka Pref., Japan, and has been extinct in the wild. To revalidate the taxonomic placement of this *Iris* plant, we performed chemotaxonomic and molecular phylogenetic comparisons of this variety with *I. sanguinea* var. *sanguinea*. Two anthocyanins, delphinidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (**An1**) and petunidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (**An2**), and twelve *C*-glycosylflavones, vicenin-2 (**CF1**), swertiajaponin X″-*O*-hexoside (**CF2**), isoorientin (**CF3**), swertiajaponin (**CF4**), isovitexin 2″-*O*-glucoside (**CF5**), swertisin 2″-*O*-glucoside (**CF6**), isovitexin (**CF7**), swertisin (**CF8**), isovitexin 2″-*O*-glucoside (**CF9**), acetylated flavoayamenins (**CF10** and **CF11**), and acetylated swertisin (**CF12**), and two flavonols, myricetin 3-*O*-rhamnoside (**FL1**), and quercetin 3-*O*-rhamnoside (**FL2**), were isolated and characterized from the flowers and leaves of two *I. sanguinea* varieties. It was shown that flavonoid composition of their varieties is essentially the same except for **CF11**, which was detected only in the leaves of *I. sanguinea* var. *tobataensis*. Furthermore, a phylogenetic analysis using chloroplast *matK* gene and *trnL-F* intron DNA sequence data of *Iris* species including these two *Iris sanguinea* varieties was performed. The resultant tree indicated that two *I. sanguinea* varieties were closely related. These results supported that the taxonomic position of var. *tobataensis* as a variety of *I. sanguinea* is appropriate.

Key words: acetylated *C*-glycosylflavone, anthocyanins, Iridaceae, *Iris sanguinea* var. *tobataensis*, *Iris sanguinea* var. *sanguinea*, phylogeny.

Introduction

Iris sanguinea Hornem. var. *tobataensis* S. Akiyama & Iwashina has been recorded as a new variety of *I. sanguinea* by Akiyama and Iwashina (2009). The variety is known as "*Tobata-Ayame*" in Tobata, Kita-kyushu City, Fukuoka Pref., Japan. In this area, although the variety is cultivated as an ornamental, the wild population has already been extinct (Akiyama and Iwashina, 2009). *I. sanguinea* var. *tobataensis* can be distinguished with other *Iris* species by the morphological characters. Its shorter stature, 10–15 cm tall, is easily distinguishable with *I. sanguinea* var. *sanguinea*. Furthermore, its bigger flower size, 5–7 cm across, is differ from the Japanese dwarf stature iris, *Iris rossii* Baker (Akiyama and Iwashina, 2009). However, chemotaxonomic and molecular phylogenetic features of *I. sanguinea* var. *tobataensis* have not been reported.

Flavonoid distribution including anthocyanins, flavones, flavonols, and isoflavones etc. in the genus *Iris* has been reviewed as an important resources and chemotaxonomic markers (Iwa-

shina and Ootani, 1998; Wang *et al*,. 2010). In this study, flavonoids were isolated and identified from the flowers and leaves of *I. sanguinea* var. *sanguinea* and var. *tobataensis* and compared between their varieties. Furthermore, we obtained the chloroplast *matK* gene and *trnL-F* intron DNA sequence data from the two *I. sanguinea* varieties to understand their phylogenetic relationship within the genus *Iris*. These data were added in the dataset for the previously reported phylogenetic tree of 43 species and varieties of section *Limniris* of the genus *Iris* (Wilson, 2009; Mizuno *et al.*, 2012a).

Materials and Methods

Plant materials

Iris sanguinea var. *tobataensis* and var. *sanguinea* were each collected from Tobata, Kitakyushu City, Fukuoka Pref., Japan, and Omachi City, Nagano Pref., Japan, respectively, and then cultivated in Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan. The voucher specimens of *I. sanguinea* var. *tobataensis*, and var. *sanguinea* were deposited in the herbarium of National Museum of Nature and Science (TNS), Japan.

Extraction and isolation

Fresh flowers (4.5 g and 96.0 g) and leaves (19.0 g and 696.4 g) of *I. sanguinea* var. *tobataensis* and var. *sanguinea* were extracted with HCOOH/MeOH (8 : 92) for flowers and MeOH for leaves. After filtration and concentration, the extracts were applied to preparative paper chromatography (PC) using solvent systems, BAW (*n*-BuOH/HOAc/H_2O = 4 : 1 : 5, upper phase), 15% HOAc and BEW (*n*-BuOH/EtOH/H_2O = 4 : 1 : 2.2). After PC separation, each fraction was purified by Sephadex LH-20 column chromatography using MAW (MeOH/HOAc/H_2O = 70 : 5 : 25 for anthocyanins) or 70% MeOH for other flavonoids. Moreover, preparative high performance liquid chromatography (HPLC) were performed with Tosoh HPLC systems using Inertsil ODS-4 (I.D. 10 × 250 mm, GL Science Inc., Japan) or *L*-column2 ODS (I.D. 10 × 250 mm, Chemicals Evaluation and Research Institute (CERI), Japan), at a flow-rate of 3.0 ml/min; injection of 300–350 μl; detection wavelength of 530 nm for anthocyanins and 340 nm for other flavonoids, and eluent of HCOOH/MeCN/H_2O (5 : 15 : 80) or HCOOH/MeCN/H_2O (1 : 18 : 81).

Analytical HPLC

HPLC analysis of anthocyanins and other flavonoids were performed using Shimadzu HPLC system with Inertsil ODS-4 column (I.D. 6.0 × 150 mm, GL Science Inc.), at flow-rate: 1.0 ml/min, detection: 530 nm and eluents: MeCN/HOAc/H_2O/H_3PO_4 (10 : 8 : 79 : 3) for anthocyanins (Method 1), and *L*-column2 ODS column (I.D. 6.0 × 150 mm, Chemical Evaluation and Research Institute), at flow-rate: 1.0 ml/min, detection: 340 nm and eluents: MeCN/H_2O/H_3PO_4 (15 : 85 : 0.2) for other flavonoids (Method 2).

Identification of flavonoids

Isolated flavonoids were identified by UV-Vis spectral survey (anthocyanins: 700–220 nm, flavones and flavonols: 500–220 nm) according to Mabry *et al.* (1970), LC-MS, alkaline and acid hydrolysis, and direct TLC (BAW, 15%HOAc and BEW) and HPLC comparisons with authentic samples.

Liquid chromatograph-mass spectra (LC-MS) was performed with Shimadzu LC-MS systems using Inertsil ODS-4 (I.D. 2.1 × 100 mm, GL Science Inc.) or *L*-column2 ODS (I.D. 2.1 × 100 mm, CERI), at a flow-rate of 0.2 ml/min, detection wavelength of 500 and 530 nm for anthocyanins or 350 and 300 nm for flavones and flavonols, and the eluent, HCOOH/MeCN/H_2O (5 : 12 : 83) or HCOOH/MeCN/H_2O (1 : 12 : 87), ESI^+ 4.5 kV and ESI^- 3.5 kV, 250°C.

Alkaline hydrolysis was performed in water, and a few drops of xylene. After neutralization and removal of xylene, diethyl ether was added to mother liquor. Organic acid and deacylated compounds were surveyed by HPLC. Acid

hydrolysis was performed in 12% aq. HCl for 30 min at 100°C. After cooling, the solution was shaken with diethyl ether. The hydrolysates, flavones and flavonol aglycones, were identified by HPLC. Glycosidic sugars were identified by PC with authentic sugars using solvent systems: BBPW (*n*-BuOH/benzene/pyridine/H_2O = 5 : 1 : 3 : 3) and BTPW (*n*-BuOH/toluene/pyridine/H_2O = 5 : 1 : 3 : 3). TLC, HPLC, UV, and LC-MS data of the isolated flavonoids were as followed.

Delphinidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (delphanin, **An1**, Fig. 1). UV–Vis: λmax (nm) 0.1% HCl-MeOH 282, 310, 542; E_{440}/E_{max} 10%; E_{acid}/E_{max} 77%; + $AlCl_3$ bathochromic shift; LC-MS: *m/z* 919 $[M]^+$; HPLC: *t*R (min) 17.2 (Method 1).

Petunidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (petanin, **An2**, Fig. 2). UV–Vis: λmax (nm) 0.1% HCl-MeOH 281, 314, 543 nm; E_{440}/E_{max} 11%; E_{acid}/E_{max} 159%; + $AlCl_3$ bathochromic shift; LC-MS: *m/z* 933 $[M]^+$; HPLC: *t*R (min) 29.4 (Method 1).

Vicenin-2 (**CF1**, Fig. 3). TLC: Rf 0.13 (BAW), 0.38 (15%HOAc), 0.33 (BEW); color UV (365 nm): deep purple, UV/NH_3: dark yellow. HPLC: *t*R (min) 5.7 (Method 2). UV: λmax (nm) MeOH 273, 332; + NaOMe 282, 334, 399 (inc.); + $AlCl_3$ 260sh, 280, 305, 350, 385; + $AlCl_3$/HCl 260sh, 279, 304, 346, 380; + NaOAc 282, 387; + NaOAc/H_3BO_3 286, 321, 355, 410sh; LC-MS: *m/z* 595 $[M + H]^+$, 593 $[M - H]^-$.

Swertiajaponin hexoside (**CF2**). TLC: Rf 0.28 (BAW), 0.76 (15%HOAc), 0.42 (BEW); color UV (365 nm): deep purple, UV/NH_3: yellow. HPLC: *t*R (min) 8.4 (Method 2). UV: λmax (nm) MeOH 243, 255sh, 272, 347; + NaOMe 276, 310sh, 394 (inc.); + $AlCl_3$ 279, 300sh, 325, 422; + $AlCl_3$/HCl 265sh, 280, 300sh, 366, 390; + NaOAc 270, 310sh, 416; + NaOAc/H_3BO_3 270, 381, 425sh; LC-MS: *m/z* 625 $[M + H]^+$, 623 $[M - H]^-$.

Isoorientin (**CF3**, Fig. 4). TLC: Rf 0.35 (BAW), 0.25 (15%HOAc), 0.44 (BEW); color UV (365 nm): deep purple, UV/NH_3: yellow. HPLC: *t*R (min) 8.9 (Method 2). UV: λmax (nm) MeOH 255, 271, 351; + NaOMe 277, 325sh, 415 (inc.); + $AlCl_3$ 275, 300sh, 330, 421;

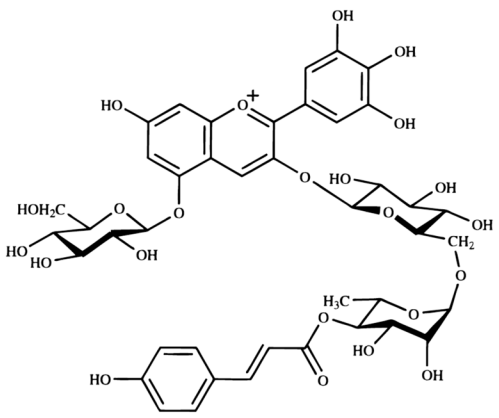

Fig. 1. Delphinidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (delphanin, **An1**).

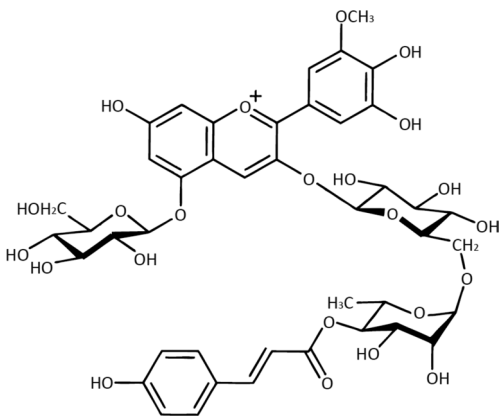

Fig. 2. Petunidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (petanin, **An2**) .

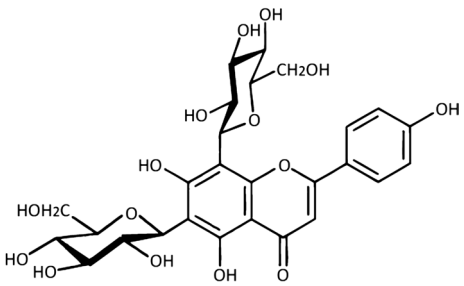

Fig. 3. Vicenin-2 (**CF1**).

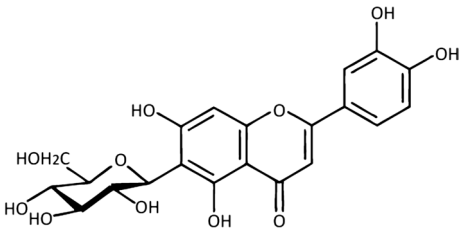

Fig. 4. Isoorientin (**CF3**).

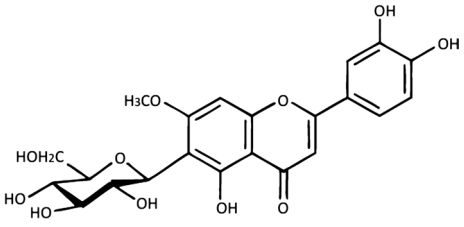

Fig. 5. Swertiajaponin (**CF4**).

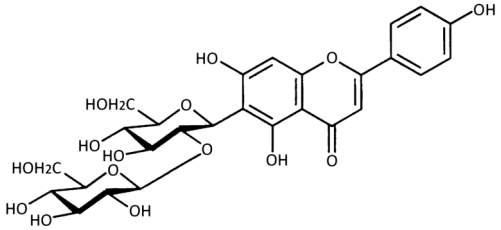

Fig. 6. Isovitexin 2″-*O*-glucoside (**CF5**).

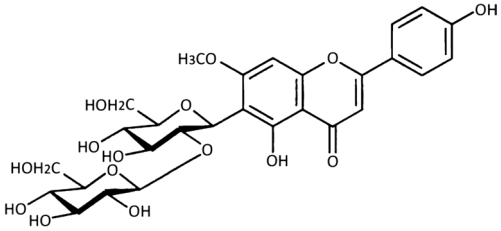

Fig. 7. Swertisin 2″-*O*-glucoside (flavoayamenin, **CF6**).

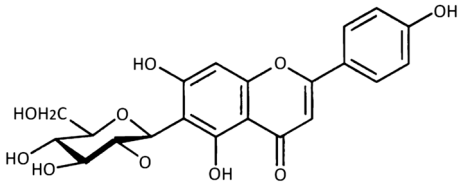

Fig. 8. Isovitexin (**CF7**).

+ $AlCl_3$/HCl 265sh, 277, 295sh, 362, 385; + NaOAc 272, 320sh, 405; + NaOAc/H_3BO_3 269, 382, 420sh; LC-MS: *m/z* 449 $[M+H]^+$, 447 $[M-H]^-$.

Swertiajaponin (**CF4**, Fig. 5). TLC: Rf 0.39 (BAW), 0.33 (15%HOAc), 0.46 (BEW); color UV (365 nm): deep purple, UV/NH_3: yellow. HPLC: *t*R (min) 10.1 (Method 2). UV: *λ*max (nm) MeOH 244, 258sh, 270, 348; + NaOMe 270, 300sh, 401 (inc.); + $AlCl_3$ 277, 300sh, 328, 422; + $AlCl_3$/HCl 260sh, 277, 295sh, 363, 378; + NaOAc 268, 300sh, 405; + NaOAc/H_3BO_3 265, 377, 420sh; LC-MS: *m/z* 463 $[M+H]^+$, 461 $[M-H]^-$.

Isovitexin 2″-*O*-glucoside (**CF5**, Fig. 6). TLC: Rf 0.32 (BAW), 0.72 (15%HOAc), 0.46 (BEW); color UV (365 nm): deep purple, UV/NH_3: dark yellow. HPLC: *t*R (min) 11.4 (Method 2). UV: *λ*max (nm) MeOH 272, 335; + NaOMe 280, 330, 399 (inc.); + $AlCl_3$ 279, 304, 350, 385; + $AlCl_3$/HCl 280, 304, 346, 380; + NaOAc 279, 309, 333, 395; + NaOAc/H_3BO_3 274, 327sh, 347, 395sh; LC-MS: *m/z* 595 $[M+H]^+$, 593 $[M-H]^-$.

Swertisin 2″-*O*-glucoside (flavoayamenin, **CF6**, Fig. 7). TLC: Rf 0.36 (BAW), 0.78 (15%HOAc), 0.48 (BEW); color UV (365 nm): deep purple, UV/NH_3: dark yellow. HPLC: *t*R (min) 14.4 (Method 2). UV: *λ*max (nm) MeOH 273, 332; + NaOMe 274, 307, 385 (inc.); + $AlCl_3$ 260sh, 280, 302, 351, 380; + $AlCl_3$/HCl 260sh, 281, 302, 349, 380; + NaOAc 271, 330sh, 389; + NaOAc/H_3BO_3 278, 337; LC-MS: *m/z* 609 $[M+H]^+$, 607 $[M-H]^-$.

Isovitexin (**CF7**, Fig. 8). TLC: Rf 0.61 (BAW), 0.33 (15%HOAc), 0.59 (BEW); color UV (365 nm): deep purple, UV/NH_3: dark yellow. HPLC: *t*R (min) 15.3 (Method 2). UV: *λ*max (nm) MeOH 271, 335; + NaOMe 273, 330, 398 (inc.); + $AlCl_3$ 279, 300, 348, 385; + $AlCl_3$/HCl 280, 302, 348, 380; + NaOAc 278, 301, 391; + NaOAc/H_3BO_3 272, 310sh, 345, 400sh; LC-MS: *m/z* 433 $[M+H]^+$, 431 $[M-H]^-$.

Swertisin (**CF8**, Fig. 9). TLC: Rf 0.64 (BAW),

0.49 (15%HOAc), 0.67 (BEW); color UV (365 nm): deep purple, UV/NH_3: dark yellow. HPLC: *t*R (min) 18.3 (Method 2). UV: λmax (nm) MeOH 272, 333; +NaOMe 272, 308, 360sh, 386 (inc.); +$AlCl_3$ 260sh, 279, 301, 353, 370; +$AlCl_3$/HCl 260sh, 279, 301, 349, 380; +NaOAc 270, 335sh, 388; +NaOAc/H_3BO_3 271, 339; LC-MS: *m/z* 447 $[M+H]^+$, 445 $[M-H]^-$.

Isoorientin 2″-*O*-glucoside (**CF9**, Fig. 10). TLC: Rf 0.26 (BAW), 0.65 (15%HOAc), 0.38 (BEW); color UV (365 nm): deep purple, UV/NH_3: yellow. HPLC: *t*R (min) 8.3 (Method 2). UV: λmax (nm) MeOH 255sh, 271, 352; +NaOMe 279, 330sh, 412 (inc.); +$AlCl_3$ 271, 305sh, 325, 421; +$AlCl_3$/HCl 262sh, 276, 295sh, 360, 390; +NaOAc 273, 325sh, 404; +NaOAc/H_3BO_3 269, 385, 425sh; LC-MS: *m/z* 611 $[M+H]^+$, 609 $[M-H]^-$.

Acetylated swertisin 2″-*O*-glucoside (acetylated flavoayamenin, **CF10**). TLC: Rf 0.58 (BAW), 0.90 (15%HOAc), 0.64 (BEW); color UV (365 nm): deep purple, UV/NH_3: dark yellow. HPLC: *t*R (min) 19.2 (Method 2). UV: λmax (nm) MeOH 272, 334; +NaOMe 277, 310, 354sh, 383 (inc.); +$AlCl_3$ 260sh, 281, 302, 354, 385; +$AlCl_3$/HCl 260sh, 281, 303, 350, 380; +NaOAc 271, 295sh, 391; +NaOAc/H_3BO_3 272, 340; LC-MS: *m/z* 651 $[M+H]^+$, 649 $[M-H]^-$.

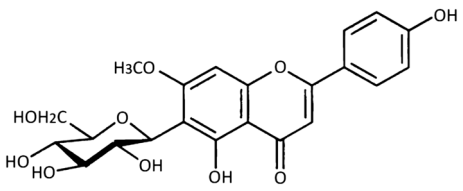

Fig. 9. Swertisin (**CF8**).

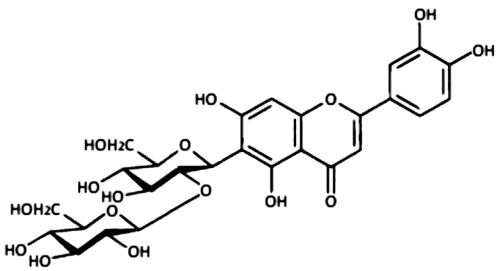

Fig. 10. Isoorientin 2″-*O*-glucoside (**CF9**).

Acetylated swertisin 2″-*O*-glucoside (acetylated flavoayamenin, **CF11**). TLC: Rf 0.49 (BAW), 0.83 (15%HOAc), 0.53 (BEW); color UV (365 nm): deep purple, UV/NH_3: dark yellow. HPLC: *t*R (min) 23.0 (Method 2). UV: λmax (nm) MeOH 272, 334; +NaOMe 278, 309, 355sh, 384 (inc.); +$AlCl_3$ 260sh, 281, 303, 354, 385; +$AlCl_3$/HCl 265sh, 280, 303, 350, 380; +NaOAc 272, 340sh, 391; +NaOAc/H_3BO_3 272, 340; LC-MS: *m/z* 651 $[M+H]^+$, 649 $[M-H]^-$.

Acetylated swertisin (**CF12**). TLC: Rf 0.91 (BAW), 0.65 (15%HOAc), 0.93 (BEW); color UV (365 nm): deep purple, UV/NH_3: dark yellow. HPLC: *t*R (min) 44.0 (Method 2). UV: λmax (nm) MeOH 271, 333; +NaOMe 277, 305, 360sh, 384 (inc.); +$AlCl_3$ 260sh, 280, 302, 354, 380; +$AlCl_3$/HCl 260sh, 280, 301, 349, 380; +NaOAc 270, 325sh, 389; +NaOAc/H_3BO_3 271, 340; LC-MS: *m/z* 489 $[M+H]^+$, 487 $[M-H]^-$.

Myricetin 3-*O*-rhamnoside (myricitrin, **FL1**, Fig. 11). TLC: Rf 0.57 (BAW), 0.39 (15%HOAc), 0.62 (BEW); color UV (365 nm): dark yellow, UV/NH_3: orange. HPLC: *t*R (min) 15.1 (Method 2). UV: λmax (nm) MeOH 257, 265sh, 300sh, 354; +NaOMe decomposition; +$AlCl_3$ 270, 314, 421; +$AlCl_3$/HCl 271, 310, 365sh, 398; +NaOAc 271, 323, 383; +NaOAc/

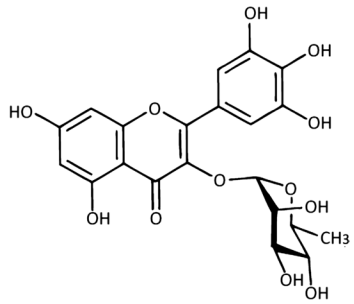

Fig. 11. Myricetin 3-*O*-rhamnoside (myricitrin, **FL1**).

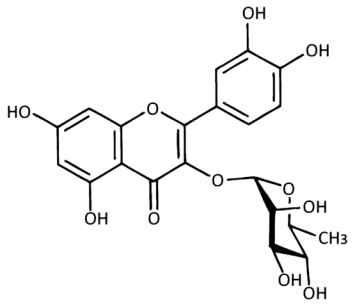

Fig. 12. Quercetin 3-*O*-rhamnoside (quercitrin, **FL2**).

H_3BO_3 259, 300sh, 374; LC-MS: *m/z* 465 $[M+H]^+$, 463 $[M-H]^-$, and 319 $[M-146+H]^+$.

Quercetin 3-*O*-rhamnoside (quercitrin, **FL2**, Fig. 12). TLC: Rf 0.78 (BAW), 0.46 (15%HOAc), 0.77 (BEW); color UV (365 nm): deep purple, UV/NH_3: yellow. HPLC: *t*R (min) 30.0 (Method 2). UV: λmax (nm) MeOH 257, 265sh, 352; +NaOMe 271, 326sh, 400; +$AlCl_3$ 274, 300sh, 360sh, 431; +$AlCl_3$/HCl 270, 300sh, 360, 395sh; +NaOAc 271, 320sh, 384; +NaOAc/H_3BO_3 260, 300sh, 370; LC-MS: *m/z* 447 $[M-H]^-$, 303 $[M-146+H]^+$

Authentic samples

Origins of the authentic samples were as follows: delphinidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside, and petunidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside from the flowers of *Iris ensata* Thunb. (Hayashi, 1940), vicenin-2 from the fronds of *Asplenium normale* D.Don (Aspleniaceae) (Iwashina *et al.*, 1990), isoorientin, swertiajaponin, isovitexin and swertisin from the flowers and leaves of *Iris rossii* (Mizuno *et al.*, 2012a), isovitexin 2″-*O*-glucoside and isoorientin 2″-*O*-glucoside from the fronds of *Cyrtomium falcatum* (L.f.) C.Presl (Dryopteridaceae) (Iwashina *et al.*, 2006), swertisin 2″-*O*-glucoside from the flowers of bearded iris (*Iris germanica* L.) cultivar 'Victoria Falls' (Mizuno *et al.*, 2012b), and myricetin 3-*O*-rhamnoside from the bark of *Myrica rubra* Sieb. et Zucc. (Myricaceae) (Hattori and Hayashi, 1931). Quercetin 3-*O*-rhamnoside from Extrasynthese (Genay, France).

DNA extraction, PCR and sequencing

Total genomic DNAs of two *Iris sanguinea* varieties were extracted from the fresh leaves using CTAB procedure (Doyle and Doyle, 1987). The protein-coding *matK* gene plus the flanking *trnK* introns, and *trnL-F* intergenic spacer region in the chloroplast DNA were sequenced. Specifically, the *matK*+*trnK* region was separated in two sections using the primer pairs 3914mF/1235R and 1176-iF/trnk2R (Wilson, 2004). The PCR amplifications were performed in a predenaturation step at 94℃ for 4 min, followed by 30 cycles of denaturation at 94℃ for 30 sec., annealing at 52–56℃ for 30 sec and extension at 72℃ for 2.5 min, and a final extension at 72℃ for 7 min. 2.5 µl of amplified PCR products were treated with 0.5 µl of ExoSAP-IT (USB corporation, USA) for 15 min at 37℃, followed by the enzyme inactivation at 80℃ for 15 min. The amplified DNA was then sequenced with BigDye Terminator v3.0 (Applied Biosystems, California) according to the manufacturer's protocol on a 3130xl Genetic Analyzer (Applied Biosystems).

Molecular phylogenetic analysis

In addition to sequenced DNA of two *Iris sanguinea* varieties, 90 (45+45) nucleotide sequences of corresponding chloroplast DNA regions of 43 species of the genus *Iris* subgenus *Limniris*, and other two species, *Dietes robinsoniana* (C.Moore & F.Muell.) Klatt, and *Moraea pritzeliana* Diels. were obtained from Genbank (Wilson, 2009, 2011; Reeves *et al.*, 2001). Alignment was conducted using ClustalW implemented in Seaview (Galtier *et al.*, 1996) and obvious errors were corrected manually. A maximum likelihood (ML) tree search was performed with PAUP*4.0a (build159) (Swofford, 2002) using a heuristic search with tree bisection–reconnection (TBR) branch swapping, 10 random-addition sequence replicates, and Multrees

option in effect. Topological uncertainty was assessed using non-parametric bootstrapping with 1000 replicates under the GTRCAT model as implemented in RaxML ver. 8.2.0 (Stamatakis, 2014).

Result and Discussion

It was shown by HPLC survey of flower extracts that anthocyanin composition between two *Iris sanguinea* varieties is the same. These anthocyanins were identified as delphinidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (**A1**), and petunidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (**A2**) by LC-MS, UV-Vis spectra, and direct HPLC comparisons with authentic samples. Ishikura and Yamamoto (1978) isolated their anthocyanins from the flowers of *I. sanguinea* var. *sanguinea* and identified by TLC. We reconfirmed anthocyanin composition of *I. sanguinea* var. *sanguinea* flowers by HPLC comparisons with authentic samples. On the other hand, anthocyanins in var. *tobataensis* flowers were reported for the first time in this paper. These anthocyanins were widely present in the flowers of many Japanese *Iris* species, e.g. *I. japonica* Thumb., *I. laevigata* Fisch., *I. ensata* Thumb., and three *I. setosa* varieties (Ishikura and Yamamoto, 1978; Yabuya, 1991; Hayashi *et al.*, 1989).

HPLC chromatograms of the flowers and leaves of two *I. sanguinea* varieties were shown in Figs. 13 and 14. Fourteen compounds (**CF1**–**12**, and **FL1**, **2**) were found and characterized. Of their compounds, three ones (**CF4**, **6** and **8)** were detected in the both flowers and leaves of two varieties. Other eleven compounds were detected in either flower or leaves. However, **CF11** was found in the leaves only of *I. sanguinea* var. *tobataensis*.

UV spectral properties showed that **CF1**, **5** and **7** are flavones having free 5-, 7- and 4′-hydroxyl groups (Mabry *et al.*, 1970). By LC-MS survey, molecular ion peaks, *m/z* 595 $[M+H]^+$ and 593 $[M–H]^-$ (**CF1** and **5**) and *m/z* 433 $[M+H]^+$ and 431 $[M-H]^-$ (**CF7**) appeared, showing that they are apigenin which attached 2

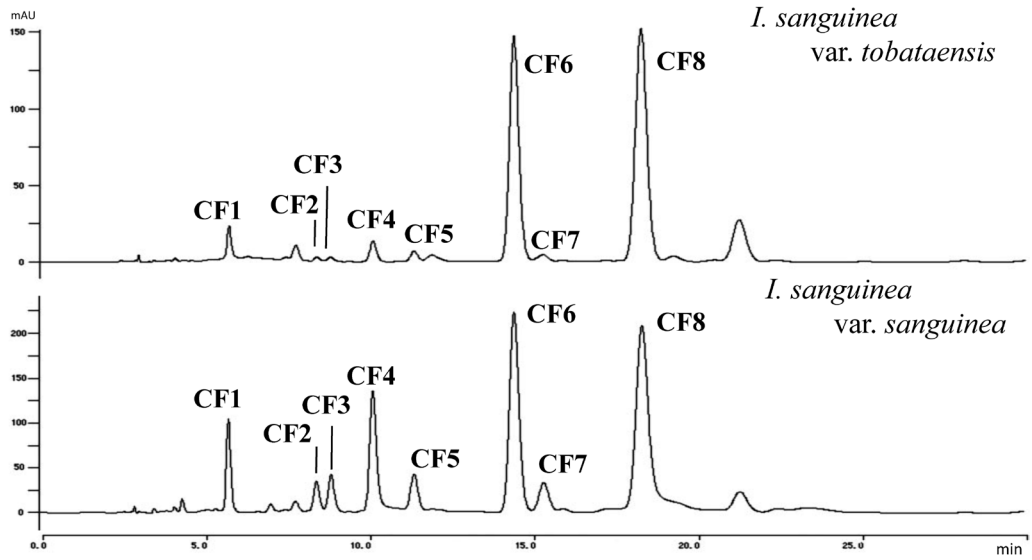

Fig. 13. HPLC comparisons of *C*-glycosylflavones from the flowers of *Iris sanguinea* var. *sanguinea* and var. *tobataensis*. **CF1** = vicenin-2, **CF2** = swertiajaponin X″-*O*-hexoside, **CF3** = isoorientin, **CF4** = swertiajaponin, **CF5** = isovitexin 2″-*O*-glucoside, **CF6** = swertisin 2″-*O*-glucoside (flavoayamenin), **CF7** = isovitexin, and **CF8** = swertisin. Wavelength is 340 nm.

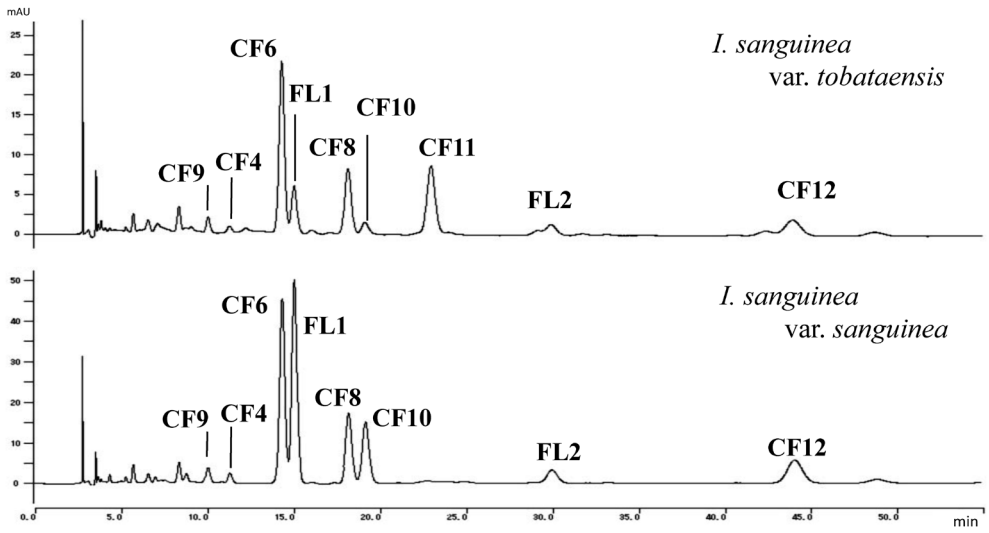

Fig. 14. HPLC comparisons of *C*-glycosylflavones and flavonols from the leaves of *Iris sanguinea* var. *sanguinea* and var. *tobataensis*. **CF4** = swertiajaponin, **CF6** = swertisin 2″-*O*-glucoside (flavoayamenin), **CF8** = swertisin, **CF9** = isoorientin 2″-*O*-glucoside, **CF10, 11** = acetylated swertisin 2″-*O*-glucosides, **CF12** = acetylated swertisin, **FL1** = myricetin 3-*O*-rhamnoside, and **FL2** = quercetin 3-*O*-rhamnoside. Wavelength is 340 nm.

and 1 mol hexose, respectively. Since **CF1** and **CF7** were unhydrolyzable by hot acid treatment, they are *C*-glycosylflavones. Retention time of hydrolysed **CF5** was the same with that of **CF7**. Finally, **CF1**, **5** and **7** were identified as vicenin-2, isovitexin 2″-*O*-glucoside and isovitexin by direct HPLC and TLC comparisons with authentic samples, respectively.

Compounds **CF2** and **CF4** were characterized as the flavones having free 5-, 3′- and 4′-hydroxyl and a substituted 7-hydroxyl groups by UV spectral survey. Finally, **CF4** was identified as swertiajaponin by HPLC and TLC comparison with authentic sample. **CF2** was partially characterized as swertiajaponin which attached 1 mol hexose by LC-MS.

UV spectral properties showed that **CF3** and **CF9** are 5,7,3′,4′-tetrahydroxylflavones (luteolin type). Molecular ion peaks, *m/z* 449 $[M+H]^+$ and 447 $[M-H]^-$ (**CF3**), and *m/z* 611 $[M+H]^+$ and 609 $[M-H]^-$ (**CF9**) appeared on LC-MS, showing that they are luteolin monohexoside and dihexoside, respectively. Compound **CF3** was unhydrolyzable. On the other hand, retention time of hydrolysed **CF9** is the same with that of **CF3**. Finally, **CF3** and **CF9** were identified as isoorientin and isoorientin 2″-*O*-glucoside by TLC and HPLC comparisons with authentic samples.

Compounds **CF6**, **8**, **10**–**12** had essentially the same UV spectral properties, i.e. flavones having free 5- and 4′-hydroxyl, and a substituted 7-hydroxyl groups. Compound **CF6** revealed molecular ion peaks, *m/z* 609 $[M+H]^+$ and 607 $[M-H]^-$ (dihydroxy-monomethoxyflavone which attached 2 mol hexose) and was identified as swertisin 2″-*O*-glucoside (flavoayamenin) by direct HPLC and TLC comparison with authentic sample. Compounds **CF10**–**12** were deacylated by alkaline saponification. LC-MS survey of deacylated compounds presented molecular ion peaks, *m/z* 609 $[M+H]^+$ and 607 $[M-H]^-$ (deacylated **CF10** and **11**), and *m/z* 447 $[M+H]^+$ and 445 $[M-H]^-$ (deacylated **CF12**), meaning that **CF10**–**12** are flavones which attached 1 mol acetic acid. Finally, **CF10** and **11** were partially identified as acetylated swertisin 2″-*O*-glucosides. On the other hand, **CF12** was

partially identified as acetylated swertisin.

UV spectral patterns of **FL1** and **FL2** were those of flavonols having a substituted 3-hydroxyl group. Their structures were determined as myricetin 3-*O*-rhamnoside (myricitrin) and quercetin 3-*O*-rhamnoside (quercitrin) by LC-MS, acid hydrolysis, TLC and HPLC comparisons with authentic samples.

In HPLC comparison of the flavonoids from two *I. sanguinea* varieties, they showed the difference of the foliar flavonoid composition, i.e. presence or absence of **CF10 (**acetylated swertisin 2″-*O*-glucoside). We presumed that **CF10** and **CF11** are isomers by their chemical properties.

Iris rossii, which is occasionally misidentified as *I. sanguinea* var. *tobataensis* because of its dwarf statue, has been reported that their floral and foliar flavonoids are anthocyanin, delphanin, and seven *C*-glycosylflavones, isoorientin, swertiajaponin, isovitexin, swertisin, schaftoside, isoschaftoside and apigenin 6,8-di-*C*-arabinoside, and *C*-glycosylxanthone, mangiferin (Mizuno *et al.*, 2012a). By contrast, in two *I. sanguinea* varieties, *C*-arabinosylflavones and xanthone were not found. As the results, it was confirmed that *I. sanguinea* var. *tobataensis* and *I. rossii* are chemotaxonomically different.

In this study, three acetylated *C*-glycosylflavones were isolated. There are some reports of acetylated *C*-glycosylflavones from *Iris* species.

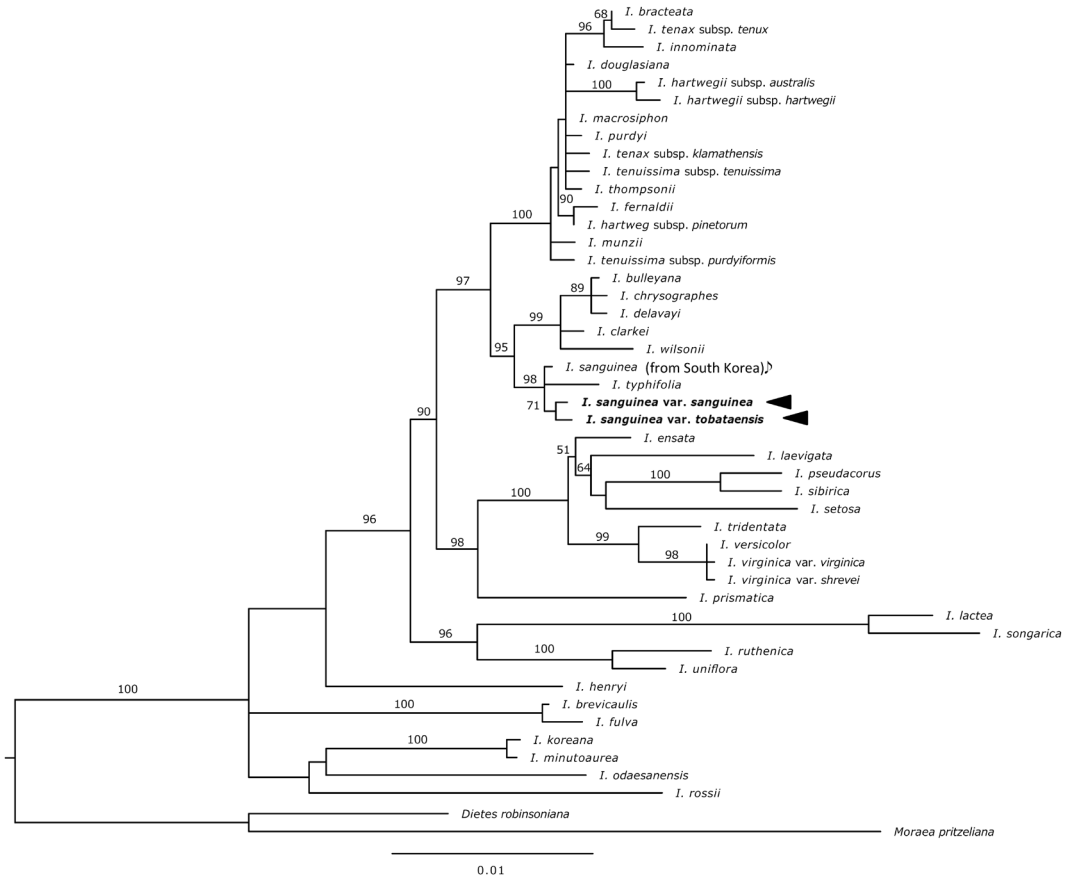

Fig. 15. A maximum likelihood (ML) tree based on chloroplast DNA sequences of *matK* and *trnL-F* regions. The black arrows are the samples newly added in this study. Others are already published by Wilson (2009) and Mizuno *et al.* (2012a). Numbers above branches are bootstrap supports.

Embigenin 2″-*O*-(2‴-acetylrhamnoside) has been isolated from the leaves of *I. lactea* Pallas (Pryakhina *et al.*, 1984). Irislactin A (acacetin 6-*C*-[(6‴-acetylglucosyl)-(1→2)-glucoside]-7-*O*-(2‴,4‴-diacetylrhamnoside) and irislactin B (apigenin 7,4′-dimethyl ether 6-*C*-[(2‴,3‴-diacetylrhamnosyl)-(1→2)-glucoside] have been isolated from the leaves of *I. lactea* var. *chinensis* (Fisch.) Koidz, (Shen *et al.*, 2008). Apigenin 7,4′-dimethyl ether 6-*C*-[(3‴-acetylrhamnosyl)-(1→2)-glucoside] has been isolated from the leaves of *I. tectorum* Maxim. (Ma *et al.*, 2012). Isovitexin and swertisin 2″-*O*-(2‴-acetylrhamnosides) and their 4′-*O*-glucosides have been isolated from the flowers of Dutch *Iris* cultivars (Mizuno *et al.*, 2013). Apigenin 7,4′-dimethyl ether 6-*C*-[(4‴-acetylrhamnosyl-(1→2)-xyloside) has been isolated from the leaves of *I. gracilipes* A.Gray (Mizuno *et al.*, 2015). Acetylated swertisin 2″-*O*-glucosides (**CF10** and **11**) have not been reported from *Iris* species until now. These results suggested that *I. sanguinea* var. *sanguinea* and var. *tobataensis* are closely related. However, they are chemotaxonomically different by the presence of **CF11** in the leaves of *I. sanguinea* var. *tobataensis*. Thus, it was presumed that acetylated swertisin 2″-*O*-glucoside (**CF11**) is chemotaxonomically useful to distinguish two *I. sanguinea* varieties.

In the present study, we also performed a molecular phylogenetic analysis, and four ML trees (-logL = 8959.6) were obtained using chloroplast DNA (*matK* gene and *trnL-F* region combined), of which 23 out of 44 internal branches were supported with ＞70% bootstrap replicates (Fig. 15). The resultant phylogenetic trees indicated that two *I. sanguinea* varieties were closely related, and it is concordant with the present taxonomic treatment. However, two *I. sanguinea* varieties analyzed in the present study were also shown to be closely related to *I. typhifolia* Kitag and *I. sanguinea* var. *sanguinea* collected from South Korea (Wilson, 2009).

Taken together, the evidences obtained from the present chemotaxonomic and molecular phylogenetic studies have verified that the taxonomic placement of *I. sanguinea* var. *tobataensis* as a variety of *I. sanguinea* would be appropriate, although some uncertainty remained concerning the taxonomic relationships of *I. sanguinea* with *I. typhifolia*. Further chemotaxonomic and phylogenetic evidences for *I. typhifolia* and other *I. sanguinea* varieties and populations would be necessary to solve this problem.

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