

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

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(Received 8 May 2018; accepted 28 June 2018)

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 morpho-

logical 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/H₂O = 4:1:5, upper phase), 15% HOAc and BEW (*n*-BuOH/EtOH/H₂O = 4:1:2.2). After PC separation, each fraction was purified by Sephadex LH-20 column chromatography using MAW (MeOH/HOAc/H₂O = 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/H₂O (5:15:80) or HCOOH/MeCN/H₂O (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/H₂O/H₃PO₄ (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/H₂O/H₃PO₄ (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/H₂O (5:12:83) or HCOOH/MeCN/H₂O (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/H₂O = 5:1:3:3) and BTPW (*n*-BuOH/toluene/pyridine/H₂O = 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: λ_{\max} (nm) 0.1% HCl-MeOH 282, 310, 542; E_{440}/E_{\max} 10%; E_{acid}/E_{\max} 77%; + AlCl₃ 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: λ_{\max} (nm) 0.1% HCl-MeOH 281, 314, 543 nm; E_{440}/E_{\max} 11%; E_{acid}/E_{\max} 159%; + AlCl₃ 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/NH₃: dark yellow. HPLC: *t*R (min) 5.7 (Method 2). UV: λ_{\max} (nm) MeOH 273, 332; + NaOMe 282, 334, 399 (inc.); + AlCl₃ 260sh, 280, 305, 350, 385; + AlCl₃/HCl 260sh, 279, 304, 346, 380; + NaOAc 282, 387;

+ NaOAc/H₃BO₃ 286, 321, 355, 410sh; LC-MS: m/z 595 [M + H]⁺, 593 [M - H]⁻.

Swertijaponin hexoside (**CF2**). TLC: Rf 0.28 (BAW), 0.76 (15%HOAc), 0.42 (BEW); color UV (365 nm): deep purple, UV/NH₃: yellow. HPLC: *t*R (min) 8.4 (Method 2). UV: λ_{\max} (nm) MeOH 243, 255sh, 272, 347; + NaOMe 276, 310sh, 394 (inc.); + AlCl₃ 279, 300sh, 325, 422; + AlCl₃/HCl 265sh, 280, 300sh, 366, 390; + NaOAc 270, 310sh, 416; + NaOAc/H₃BO₃ 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/NH₃: yellow. HPLC: *t*R (min) 8.9 (Method 2). UV: λ_{\max} (nm) MeOH 255, 271, 351; + NaOMe 277, 325sh, 415 (inc.); + AlCl₃ 275, 300sh, 330, 421;

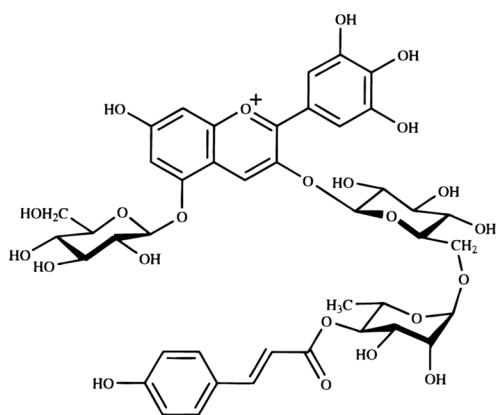


Fig. 1. Delphinidin 3-*O*-[(4''-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (delphanin, **An1**).

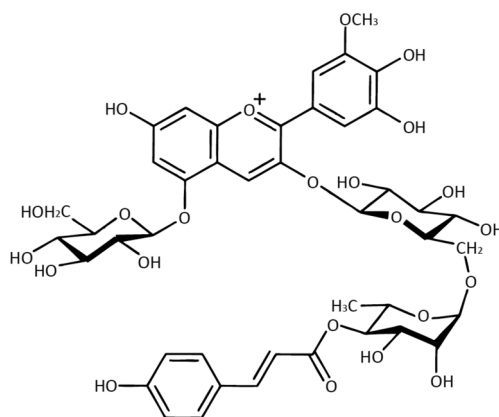


Fig. 2. Petunidin 3-*O*-[(4''-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (petanin, **An2**).

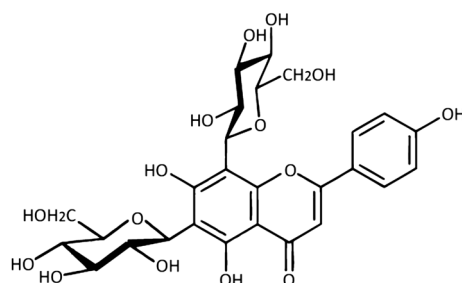


Fig. 3. Vicenin-2 (**CF1**).

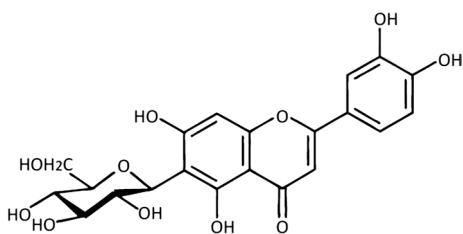


Fig. 4. Isoorientin (CF3).

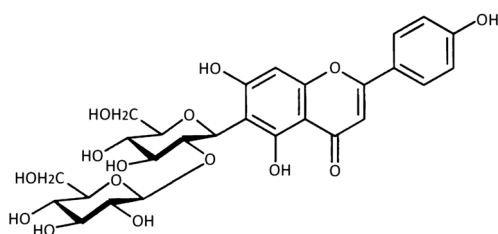


Fig. 6. Isovitexin 2''-O-glucoside (CF5).

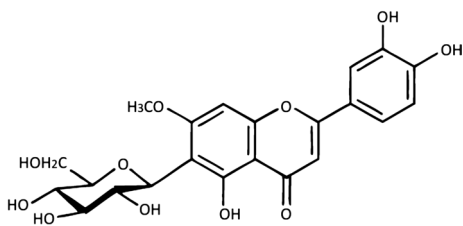


Fig. 5. Swertiajaponin (CF4).

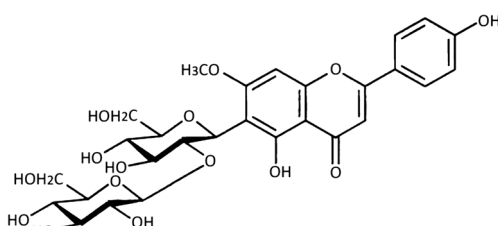


Fig. 7. Swertisin 2''-O-glucoside (flavoayamenin, CF6).

+ AlCl₃/HCl 265sh, 277, 295sh, 362, 385; + NaOAc 272, 320sh, 405; + NaOAc/H₃BO₃ 269, 382, 420sh; LC-MS: *m/z* 449 [M + H]⁺, 447 [M - H]⁻.

Swertiajaponin (CF4, Fig. 5). TLC: R_f 0.39 (BAW), 0.33 (15%HOAc), 0.46 (BEW); color UV (365 nm): deep purple, UV/NH₃: yellow. HPLC: *t*R (min) 10.1 (Method 2). UV: λ_{max} (nm) MeOH 244, 258sh, 270, 348; + NaOMe 270, 300sh, 401 (inc.); + AlCl₃ 277, 300sh, 328, 422; + AlCl₃/HCl 260sh, 277, 295sh, 363, 378; + NaOAc 268, 300sh, 405; + NaOAc/H₃BO₃ 265, 377, 420sh; LC-MS: *m/z* 463 [M + H]⁺, 461 [M - H]⁻.

Isovitexin 2''-O-glucoside (CF5, Fig. 6). TLC: R_f 0.32 (BAW), 0.72 (15%HOAc), 0.46 (BEW); color UV (365 nm): deep purple, UV/NH₃: dark yellow. HPLC: *t*R (min) 11.4 (Method 2). UV: λ_{max} (nm) MeOH 272, 335; + NaOMe 280, 330, 399 (inc.); + AlCl₃ 279, 304, 350, 385; + AlCl₃/HCl 280, 304, 346, 380; + NaOAc 279, 309, 333, 395; + NaOAc/H₃BO₃ 274, 327sh, 347, 395sh; LC-MS: *m/z* 595 [M + H]⁺, 593 [M - H]⁻.

Swertisin 2''-O-glucoside (flavoayamenin, CF6, Fig. 7). TLC: R_f 0.36 (BAW), 0.78 (15%HOAc), 0.48 (BEW); color UV (365 nm):

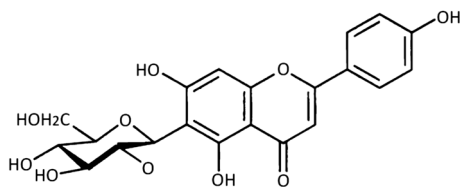


Fig. 8. Isovitexin (CF7).

deep purple, UV/NH₃: dark yellow. HPLC: *t*R (min) 14.4 (Method 2). UV: λ_{max} (nm) MeOH 273, 332; + NaOMe 274, 307, 385 (inc.); + AlCl₃ 260sh, 280, 302, 351, 380; + AlCl₃/HCl 260sh, 281, 302, 349, 380; + NaOAc 271, 330sh, 389; + NaOAc/H₃BO₃ 278, 337; LC-MS: *m/z* 609 [M + H]⁺, 607 [M - H]⁻.

Isovitexin (CF7, Fig. 8). TLC: R_f 0.61 (BAW), 0.33 (15%HOAc), 0.59 (BEW); color UV (365 nm): deep purple, UV/NH₃: dark yellow. HPLC: *t*R (min) 15.3 (Method 2). UV: λ_{max} (nm) MeOH 271, 335; + NaOMe 273, 330, 398 (inc.); + AlCl₃ 279, 300, 348, 385; + AlCl₃/HCl 280, 302, 348, 380; + NaOAc 278, 301, 391; + NaOAc/H₃BO₃ 272, 310sh, 345, 400sh; LC-MS: *m/z* 433 [M + H]⁺, 431 [M - H]⁻.

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

0.49 (15%HOAc), 0.67 (BEW); color UV (365 nm): deep purple, UV/NH₃: dark yellow. HPLC: *t*R (min) 18.3 (Method 2). UV: λ_{\max} (nm) MeOH 272, 333; + NaOMe 272, 308, 360sh, 386 (inc.); + AlCl₃ 260sh, 279, 301, 353, 370; + AlCl₃/HCl 260sh, 279, 301, 349, 380; + NaOAc 270, 335sh, 388; + NaOAc/H₃BO₃ 271, 339; LC-MS: *m/z* 447 [M+H]⁺, 445 [M-H]⁻.

Isoorientin 2''-*O*-glucoside (**CF9**, Fig. 10). TLC: R_f 0.26 (BAW), 0.65 (15%HOAc), 0.38 (BEW); color UV (365 nm): deep purple, UV/NH₃: yellow. HPLC: *t*R (min) 8.3 (Method 2). UV: λ_{\max} (nm) MeOH 255sh, 271, 352; + NaOMe 279, 330sh, 412 (inc.); + AlCl₃ 271, 305sh, 325, 421; + AlCl₃/HCl 262sh, 276, 295sh, 360, 390; + NaOAc 273, 325sh, 404; + NaOAc/H₃BO₃ 269, 385, 425sh; LC-MS: *m/z* 611 [M+H]⁺, 609 [M-H]⁻.

Acetylated swertisin 2''-*O*-glucoside (acetylated flavoayamenin, **CF10**). TLC: R_f 0.58 (BAW), 0.90 (15%HOAc), 0.64 (BEW); color UV (365 nm): deep purple, UV/NH₃: dark yellow. HPLC: *t*R (min) 19.2 (Method 2). UV: λ_{\max} (nm) MeOH 272, 334; + NaOMe 277, 310, 354sh, 383 (inc.); + AlCl₃ 260sh, 281, 302, 354,

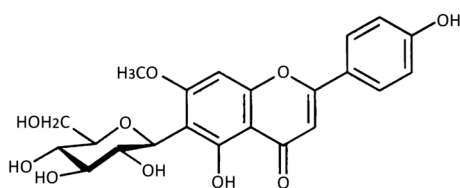


Fig. 9. Swertisin (**CF8**).

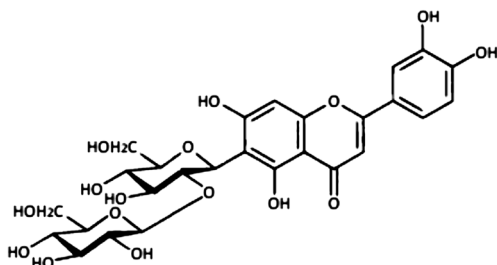


Fig. 10. Isoorientin 2''-*O*-glucoside (**CF9**).

385; + AlCl₃/HCl 260sh, 281, 303, 350, 380; + NaOAc 271, 295sh, 391; + NaOAc/H₃BO₃ 272, 340; LC-MS: *m/z* 651 [M+H]⁺, 649 [M-H]⁻.

Acetylated swertisin 2''-*O*-glucoside (acetylated flavoayamenin, **CF11**). TLC: R_f 0.49 (BAW), 0.83 (15%HOAc), 0.53 (BEW); color UV (365 nm): deep purple, UV/NH₃: dark yellow. HPLC: *t*R (min) 23.0 (Method 2). UV: λ_{\max} (nm) MeOH 272, 334; + NaOMe 278, 309, 355sh, 384 (inc.); + AlCl₃ 260sh, 281, 303, 354, 385; + AlCl₃/HCl 265sh, 280, 303, 350, 380; + NaOAc 272, 340sh, 391; + NaOAc/H₃BO₃ 272, 340; LC-MS: *m/z* 651 [M+H]⁺, 649 [M-H]⁻.

Acetylated swertisin (**CF12**). TLC: R_f 0.91 (BAW), 0.65 (15%HOAc), 0.93 (BEW); color UV (365 nm): deep purple, UV/NH₃: dark yellow. HPLC: *t*R (min) 44.0 (Method 2). UV: λ_{\max} (nm) MeOH 271, 333; + NaOMe 277, 305, 360sh, 384 (inc.); + AlCl₃ 260sh, 280, 302, 354, 380; + AlCl₃/HCl 260sh, 280, 301, 349, 380; + NaOAc 270, 325sh, 389; + NaOAc/H₃BO₃ 271, 340; LC-MS: *m/z* 489 [M+H]⁺, 487 [M-H]⁻.

Myricetin 3-*O*-rhamnoside (myricitrin, **FL1**, Fig. 11). TLC: R_f 0.57 (BAW), 0.39 (15%HOAc), 0.62 (BEW); color UV (365 nm): dark yellow, UV/NH₃: orange. HPLC: *t*R (min) 15.1 (Method 2). UV: λ_{\max} (nm) MeOH 257, 265sh, 300sh, 354; + NaOMe decomposition; + AlCl₃ 270, 314, 421; + AlCl₃/HCl 271, 310, 365sh, 398; + NaOAc 271, 323, 383; + NaOAc/

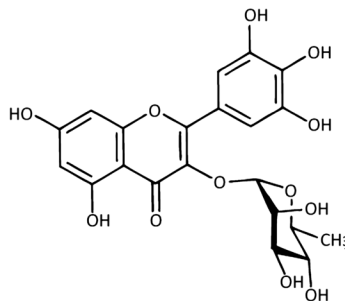


Fig. 11. Myricetin 3-*O*-rhamnoside (myricitrin, **FL1**).

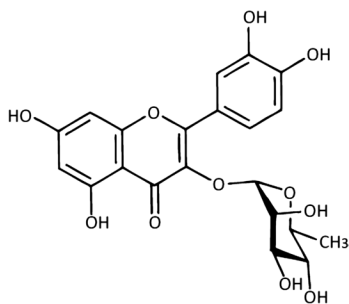


Fig. 12. Quercetin 3-*O*-rhamnoside (quercitrin, **FL2**).

H₃BO₃ 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: R_f 0.78 (BAW), 0.46 (15%HOAc), 0.77 (BEW); color UV (365 nm): deep purple, UV/NH₃: yellow. HPLC: *t*R (min) 30.0 (Method 2). UV: λ_{max} (nm) MeOH 257, 265sh, 352; + NaOMe 271, 326sh, 400; + AlCl₃ 274, 300sh, 360sh, 431; + AlCl₃/HCl 270, 300sh, 360, 395sh; + NaOAc 271, 320sh, 384; + NaOAc/H₃BO₃ 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°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 52–56°C for 30 sec and extension at 72°C for 2.5 min, and a final extension at 72°C 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°C, followed by the enzyme inactivation at 80°C 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, *Diets 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

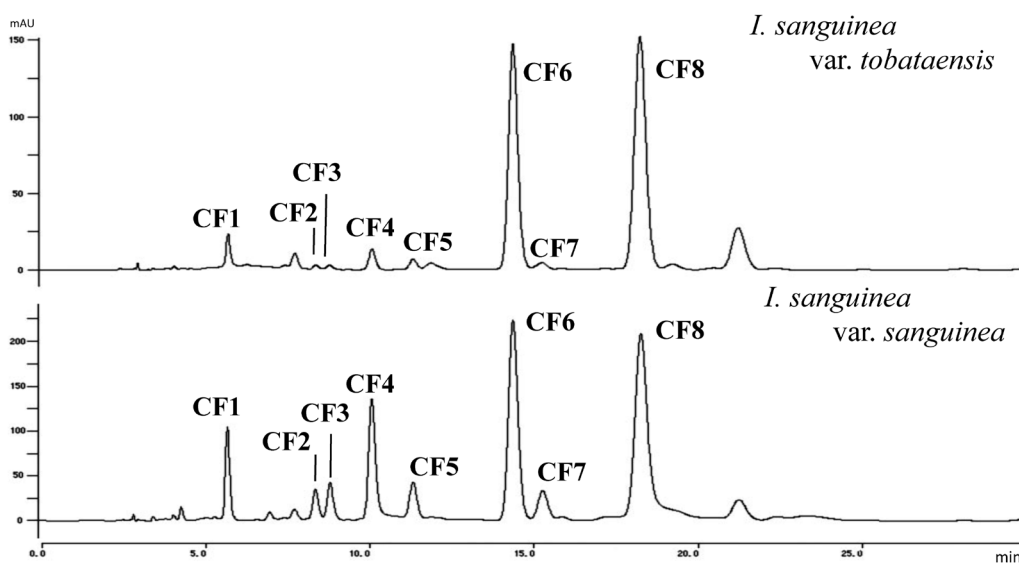


Fig. 13. HPLC comparisons of *C*-glycosylflavones from the flowers of *Iris sanguinea* var. *sanguinea* and var. *tobataensis*. **CF1** = vicenin-2, **CF2** = swertiajaponin *X''-O*-hexoside, **CF3** = isoorientin, **CF4** = swertiajaponin, **CF5** = isovitexin 2''-*O*-glucoside, **CF6** = swertisin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

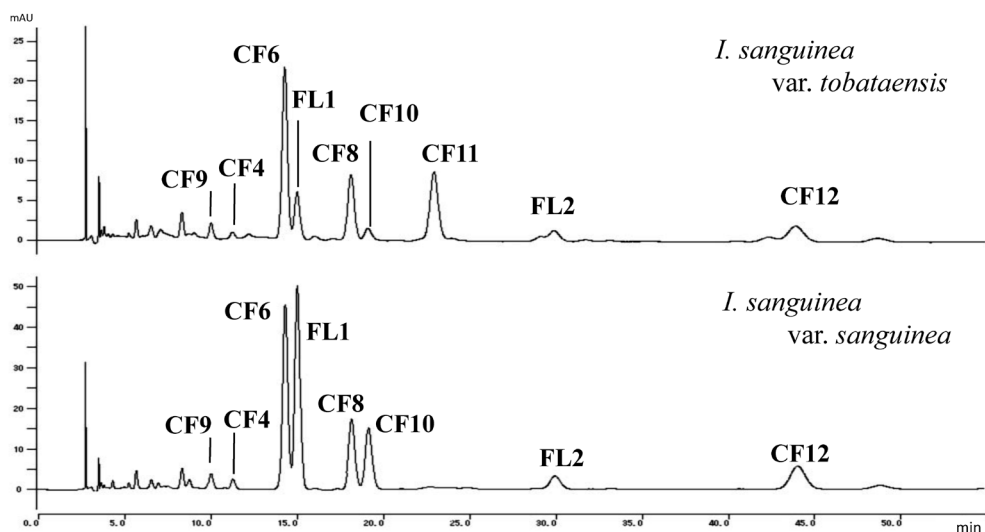


Fig. 14. HPLC comparisons of *C*-glycosylflavones and flavonols from the leaves of *Iris sanguinea* var. *sanguinea* and var. *tobataensis*. **CF4** = swertiajaponin, **CF6** = swertisin 2''-*O*-glucoside (flavoayamenin), **CF8** = swertisin, **CF9** = isoorientin 2''-*O*-glucoside, **CF10**, **11** = acetylated swertisin 2''-*O*-glucosides, **CF12** = acetylated swertisin, **FL1** = myricetin 3-*O*-rhamnoside, and **FL2** = quercetin 3-*O*-rhamnoside. Wave-length 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, delphinin, and seven *C*-glycosylflavones, isoorientin, swertiajaponin, isovitexin, swertisin, shaftoside, isoschaftoside and apigenin 6,8-di-*C*-arabino-side, 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.

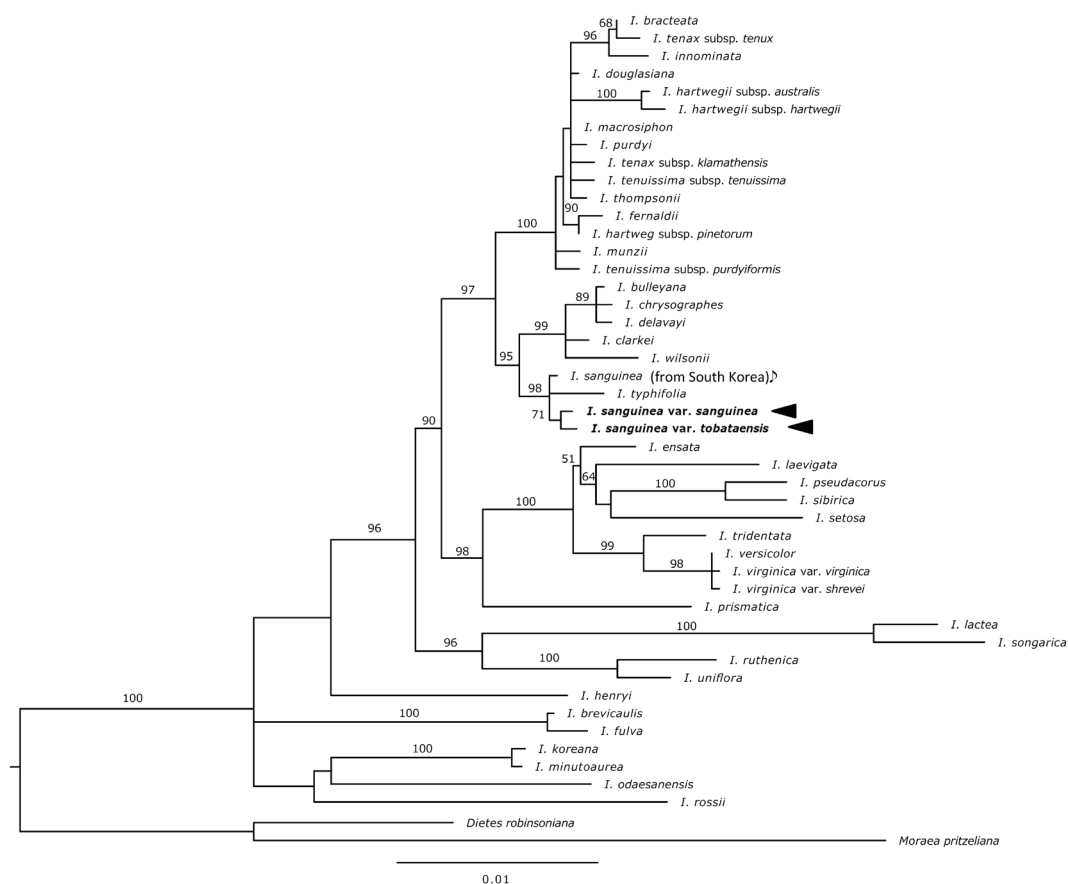


Fig. 15. A maximum likelihood (ML) tree based on chloroplast DNA sequences of *matK* and *trnL-F* regions. The black arrows are the samples newly added in this study. Others are already published by Wilson (2009) and Mizuno *et al.* (2012a). Numbers above branches are bootstrap supports.

Embigenin 2''-O-(2'''-acetylramnoside) has been isolated from the leaves of *I. lactea* Pallas (Pryakhina *et al.*, 1984). Irislactin A (acetin 6-C-[(6'''-acetylglucosyl)-(1→2)-glucoside]-7-O-(2''',4'''-diacetylramnoside) and irislactin B (apigenin 7,4'-dimethyl ether 6-C-[(2''',3'''-diacetylramnosyl)-(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'''-acetylramnosyl)-(1→2)-glucoside] has been isolated from the leaves of *I. tectorum* Maxim. (Ma *et al.*, 2012). Isovitexin and swertisin 2''-O-(2'''-acetylramnosides) 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'''-acetylramnosyl)-(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.

Acknowledgments

The authors are grateful to late Mr. Kazuaki Tsunemori and Tobata Ward Office, Kita-Kyushu City, Fukuoka-Pref., Japan for the opportunity to this study and the offer of plant materials.

References

- Akiyama, S. and Iwashina, T. 2009. *Tobata-ayame*: A new variety of *Iris sanguinea* Hornem. from northern Kyusyu, Japan. Bulletin of the National Museum of Nature and Science, Series B 35: 205–209.
- Doyle, J. and Doyle, J. L. 1987. Genomic plant DNA preparation from fresh tissue-CTAB method. Phytochemical Bulletin 19: 11–15.
- Galtier, N., Gouy, M. and Gautier, C. 1996. SEAVIEW and PHYLO_WIN: Two graphic tools for sequence alignment and molecular phylogeny. Bioinformatics 12: 543–548.
- Hattori, S. and Hayashi, K. 1931. Konstitution des Myricitrins. Acta Phytochimica 5: 213–218.
- Hayashi, K. 1940. Isolation of ensatin, an acylated anthocyanin from the flowers of Japanese *Iris*. Proceedings of the Imperial Academy, Tokyo 16: 478–481.
- Hayashi, K., Ootani, S. and Iwashina, T. 1989. Comparative analysis of the flavonoid and related compounds occurring in three varieties of *Iris setosa* in the flora of Japan. Science Report of the Research Institute of Evolutionary Biology 6: 30–60.
- Ishikura, N. and Yamamoto, E. 1978. Anthocyanins in the flowers of Japanese garden iris belonging to 'Higo' line. Kumamoto Journal of Science, Series B, Biology 14: 9–15.
- Iwashina, T., Matsumoto, S., Ozawa, K. and Akuzawa, K. 1990. Flavone glycosides from *Asplenium normale*. Phytochemistry 29: 3543–3546.
- Iwashina, T. and Ootani, S. 1998. Flavonoids of the genus *Iris*: Structures, distribution and function (review). Annals of the Tsukuba Botanical Garden 17: 147–183.
- Iwashina, T., Kitajima, J. and Matsumoto, S. 2006. Flavonoids in the species of *Cyrtomium* (Dryopteridaceae)

- and related genera. *Biochemical Systematics and Ecology* 34: 14–24.
- Ma, Y., Li, H., Lin, B., Wang, G. and Qin, M. 2012. *C*-Glycosylflavones from the leaves of *Iris tectorum* Maxim. *Acta Pharmaceutica Sinica B* 2: 598–601.
- Mabry, T. J., Markham, K. R. and Thomas, M. B. 1970. *The Systematic Identification of Flavonoids*. Springer, New York.
- Mizuno, T., Okuyama, Y. and Iwashina, T. 2012a. Phenolic compounds from *Iris rossii*, and their chemotaxonomic and systematic significance. *Biochemical Systematics and Ecology* 44: 157–160.
- Mizuno, T., Yabuya, T., Kitajima, J. and Iwashina, T. 2013. Identification of novel *C*-glycosylflavones and their contribution to flower colour of the Dutch iris cultivars. *Plant Physiology and Biochemistry* 72: 116–124.
- Mizuno, T., Yabuya, T., Sasaki, N. and Iwashina, T. 2012b. Phenolic compounds, including novel *C*-glycosylflavone, from the flowers of the tall bearded iris cultivar 'Victoria Falls'. *Natural Product Communications* 7: 1591–1594.
- Mizuno, T., Kamo, T., Sasaki, N., Yada, H., Murai, Y. and Iwashina, T. 2015. Novel *C*-xylosylflavones from the leaves and flowers of *Iris gracilipes*. *Natural Product Communications* 10: 441–444.
- Pryakhina, N. I., Sheichenko, V. I. and Blinova, K. F. 1984. Acylated *C*-glycosides of *Iris lactea*. *Chemistry of Natural Compounds* 20: 554–559.
- Reeves, G., Chase, M. W., Goldblatt, P., Rudall, P., Fay, M. F., Cox, A. V., Lejeune, B. and Souza-Chies, T. 2001. Molecular systematics of Iridaceae: Evidence from four plastid DNA regions. *American Journal of Botany* 88: 2074–2087.
- Shen, W. J., Qin, M. J., Shu, P. and Zhang, C. F. 2008. Two new *C*-glycosylflavones from the leaves of *Iris lactea* var. *chinensis*. *Chinese Chemical Letters* 19: 821–824.
- Stamatakis, A. 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313.
- Swofford, D. L. 2002. *Phylogenetic Analysis Using Parsimony*. Sunderland, MA: Sinauer Associates Inc, software.
- Wang, H., Cui, Y. and Zhao, C. 2010. Flavonoids of genus *Iris* (Iridaceae). *Mini-Reviews in Medicinal Chemistry* 10: 643–661.
- Wilson, C. A. 2004. Phylogeny of *Iris* based on chloroplast *matK* gene and *trnK* intron sequence data. *Molecular Phylogenetics and Evolution* 33: 402–412.
- Wilson, C. A. 2009. Phylogenetic relationships among the recognized series in *Iris* section *Limniris*. *Systematic Botany* 34: 277–284.
- Wilson, C. A. 2011. Subgeneric classification in *Iris* re-examined using chloroplast sequence data. *Taxon* 60: 27–35.
- Yabuya, T. 1991. High-performance liquid chromatographic analysis of anthocyanins in Japanese garden *Iris* and its wild forms. *Euphytica* 52: 215–219.