



Metabolomic differentiation of *Goodyera* species across phylogenetic lineages and geographic regions based on LC-MS/MS and HPLC/PDA analyses

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Abstract The genus *Goodyera* exhibits considerable taxonomic complexity within Orchidaceae. However, its chemical composition and metabolomic characteristics remain insufficiently characterized. This study investigated whether evolutionary divergence and geographic isolation contribute to measurable metabolite differences among five species (*Goodyera schlechtendaliana*, *G. henryi*, *G. velutina*, *G. crassifolia*, and *G. biflora*) using a comparative metabolomics approach integrating liquid chromatography-tandem mass spectrometry-based qualitative profiling with high-performance liquid chromatography with photodiode array detection-based quantitative analysis. A total of twelve phenolic metabolites, including hydroxycinnamic acid derivatives, flavonol glycosides, and stilbenoids, were identified revealing pronounced chemical differentiation among phylogenetically distinct lineages and natural populations. Clear lineage-specific variation was detected between *G. schlechtendaliana* and *G. henryi*, as syringaldehyde, 3-feruloylquinic acid, and tamarixetin were exclusively present in *G. schlechtendaliana*, supporting their potential as chemotaxonomic markers. Distinct species-specific metabolite patterns were also observed, including marked enrichment of quercetin 3-*O*-robinobioside in *G. crassifolia* and preferential accumulation

of rhaponticin in *G. velutina*, reflecting biochemical diversification shaped by evolutionary history. In contrast, *G. biflora* displayed a chemically reduced profile, with most marker compounds being undetectable. Although quantitative differences were observed between populations from Jeju and Ulleung Islands, the qualitative consistency of metabolite composition indicates predominant genetic control rather than environmental influence. Collectively, these results provide the first chemotaxonomic framework for *Goodyera* species and demonstrate the strong discriminatory power of metabolomic signatures aligned with phylogenetic divergence.

Keywords Chemotaxonomy · *Goodyera* species · High-performance liquid chromatography with photodiode array detection · Liquid chromatography-tandem mass spectrometry · Metabolomics · Phenolic metabolites

Introduction

Orchids are highly prized ornamental plants that have undergone extensive diversification through both natural evolution and selective breeding, resulting in remarkable phenotypic diversity worldwide [1,2]. Within this large family, the genus *Goodyera*, commonly known as jewel orchids, is particularly valued for its unique ornamental foliage characterized by intricate reticulated leaf patterns, which has driven its widespread cultivation and horticultural interest [3]. The genus *Goodyera* belongs to the family Orchidaceae and exhibits especially high species diversity. Among the *Goodyera* species native to Korea, five have been formally recognized: *Goodyera schlechtendaliana* Rchb. f., *G. henryi* Rolfe, *G. velutina* Maxim. ex Regel, *G. crassifolia* H. J. Suh, S. W. Seo, S. H. Oh & T. Yukawa, and *G. biflora* (Lindl.) Hook. f. [4]. These species are widely distributed across East Asia, including Korea, Japan, and China, and display distinct morphological, ecological, and genetic differences in leaf venation patterns, flowering time,

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habitat elevation, microenvironmental preference, and chromosome number [5,6].

Recent plastome-based phylogenomic analyses revealed that *G. schlechtendaliana* possesses a unique plastome inversion and forms an independent lineage designated as Clade A, whereas *G. henryi*, *G. velutina*, and *G. biflora* constitute a closely related East Asian lineage referred to as Clade B, demonstrating a clear species-level divergence pattern [7]. Moreover, the recently described species *G. crassifolia* has been shown to have originated through polyploidization ($2n = 60$) and flowering-time separation, leading to rapid reproductive isolation [6], suggesting diverse evolutionary routes within these East Asian *Goodyera* species.

Despite advances in molecular phylogenetic research, studies on the chemical composition of *Goodyera* species remain extremely limited. Although several flavonol glycosides and simple phenolic compounds have been reported from *G. schlechtendaliana* [8], comprehensive metabolomic profiling and comparative analyses among closely related species such as *G. henryi*, *G. velutina*, *G. crassifolia*, and *G. biflora* remain largely unexplored. Recent advances in evolutionary metabolomics have demonstrated that the composition of secondary metabolites is not merely a product of environmental adaptation, but also an inherent biological trait that evolves alongside species diversification and reflects phylogenetic relationships [9,10]. Therefore, comparative metabolomic analyses among closely related species represent a powerful approach for elucidating biochemical divergence that cannot be fully resolved by morphological or genetic data alone [11].

In this context, *Goodyera* species provide an ideal model system for testing whether evolutionary differentiation is accompanied by chemical diversification. In the present study, *G. schlechtendaliana* and *G. henryi*, which represent the most pronounced phylogenetic contrast within the genus, were selected as a strategic comparative model for liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography with photodiode array detection (HPLC/PDA)-based metabolomic analysis. Notably, recent studies have shown that *G. henryi* forms natural hybrids with closely related East Asian species, including *G. velutina*, indicating strong genetic affinity and metabolic connectivity within Clade B [12]. This further suggests that *G. henryi* represents the characteristic biochemical features of the East Asian lineage, highlighting its suitability as a comparative counterpart to the phylogenetically isolated *G. schlechtendaliana*.

This study sought to determine whether genetic and evolutionary divergence is reflected in measurable differences in metabolite composition between these two representative lineages. Additionally, populations of *G. schlechtendaliana* and *G. henryi* collected from two geographically isolated regions, Jeju Island and Ulleung Island, were analyzed to evaluate whether the observed metabolic differences represent stable species-specific traits rather than environmentally-driven variation. The experimental design follows

a comparative model framework widely applied in evolutionary metabolomics and chemotaxonomy, integrating LC-MS/MS-based qualitative metabolite profiling with HPLC/PDA-based quantitative validation to assess chemical variation driven by phylogenetic lineage, evolutionary divergence, and geographical isolation. The results of this study establish the first chemotaxonomic baseline for *Goodyera* by defining the fundamental chemical characteristics of *G. schlechtendaliana* and *G. henryi*, enabling future expansion to *G. velutina*, *G. crassifolia*, and *G. biflora*, and providing both conceptual and analytical foundations for the development of a comprehensive chemotaxonomic framework for *Goodyera* orchids.

Materials and Methods

Plant materials

Total of seven individual plants from five *Goodyera* species were collected, with two species (*G. schlechtendaliana* and *G. henryi*) sampled from both Jeju and Ulleung Islands, and all specimens were authenticated by Dr. S.-Y. Jung (Forest Biodiversity Research Division, Korea National Arboretum, Pocheon, Republic of Korea). All specimens were collected from wild habitats and used for analytical studies immediately after sample processing. For all analyses, the whole plant was used as experimental material (Fig. 1).

Sampling sites

G. schlechtendaliana Rchb. f. from Jeju Island (GSJ) was collected from Georinsaseum-oreum, Jungmun-dong, Seogwipo-si, Jeju Island, Korea, whereas samples from Ulleung Island (GSU) were collected from Namseo-ri, Seomyeon, Ulleung-gun, Gyeongsangbuk-do, Korea. *G. henryi* Rolfe from Jeju Island (GHJ) was collected from Nohyeong-dong, Jeju-si, Jeju Island, Republic of Korea, and samples from Ulleung Island (GHU) were collected from Namseo-ri, Seomyeon, Ulleung-gun, Gyeongsangbuk-do, Korea. *G. velutina* Maxim. ex Regel (GVM) was collected from Georinsaseum-oreum, Jungmun-dong, Seogwipo-si, Jeju Island, Korea. *G. crassifolia* H. J. Suh, S. W. Seo, S. H. Oh & T. Yukawa (GCH) was collected from Sa-ri, Heuksan-myeon, Sinan-gun, Jeollanam-do, Korea. *G. biflora* (Lindl.) Hook. f. (GBH) was collected from Georinsaseum-oreum, Jungmun-dong, Seogwipo-si, Jeju Island, Republic of Korea (Fig. 2).

Chemicals and reagents

Ethanol was purchased from Samchun Pure Chemical (Pyeongtaek, Republic of Korea). HPLC-grade methanol (MeOH), acetonitrile (ACN), and water were obtained from J.T. Baker (Center Valley, PA, USA). Trifluoroacetic acid (TFA; HPLC grade, 99.5+%; Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. 044630.AY; CAS 76-05-1) and formic acid (FA; 99%, for analysis; Acros Organics,



Fig. 1 Experimental samples of GSJ (A), GSU (B), GHJ (C), GHU (D), GVM (E), GCH (F), and GBH (G)

Geel, Belgium; Cat. No. 270480010; CAS 64-18-6) were used as mobile-phase modifiers. 3-Feruloylquinic acid (**1**), *p*-coumaric acid (**2**), syringaldehyde (**3**), quercetin 3-*O*-robinobioside (**4**), ferulic acid (**5**), nicotiflorin (**6**), narcissoside (**7**), rhaponticin (**8**), rhapontigenin (**9**), and tamarixetin (**10**) were obtained from the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Republic of Korea (Fig. 3).

Instrumentation

Organic solvents were removed using a rotary vacuum evaporator

(OSB-2100, Eyela, Tokyo, Japan). LC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Q Exactive™ Orbitrap high-resolution LC-MS/MS spectrometer (Thermo Fisher Scientific), equipped with a CORTECS C18 column (150 mm×2.1 mm, 1.6 μm; Waters Corporation, Milford, MA, USA). HPLC analyses were conducted using an Alliance e2695 separations module equipped with a 2998 photodiode array detector (both Waters Corporation) and a YMC Pack Pro C18 column (4.6 mm×250 mm, 5 μm; YMC, Kyoto, Japan).

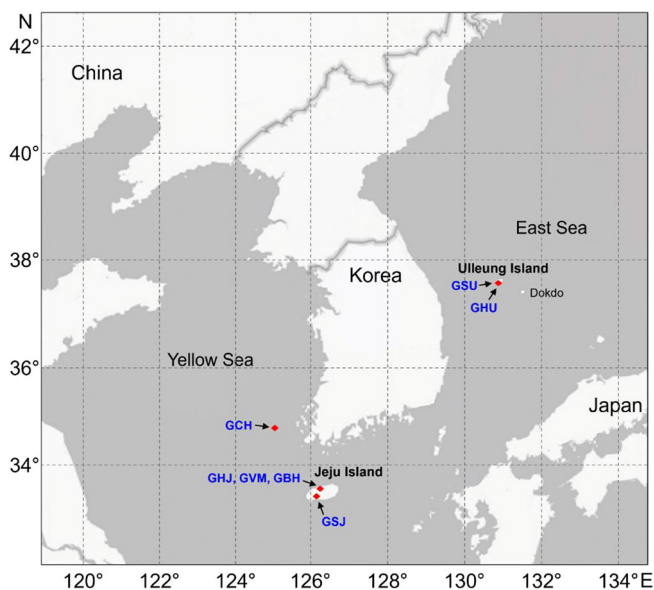


Fig. 2 Sampling sites for specimen collection

Extraction of plant samples

Fresh plant materials were thoroughly washed with distilled water, frozen at -80°C , and subsequently freeze-dried. The dried samples were finely pulverized using a mixer to facilitate solvent penetration. The powdered material (0.5 g per sample) was extracted three times with 300 mL of ethanol under reflux for 3 h per cycle. The combined extracts were filtered to remove insoluble residues and concentrated under reduced pressure using a rotary evaporator. The extraction yield was calculated using the following formula:

$$\text{Yield (\%)} = (\text{Weight of extract} / \text{Freeze-dried weight of plant material}) \times 100$$

LC-MS/MS conditions

The column temperature was maintained at 45°C with a flow rate of 0.30 mL/min. The mobile phases consisted of 0.1% FA in water (solvent A) and 0.1% FA in ACN (solvent B). The gradient program was as follows: 5% B at 0–0.1 min, 10% B at 10 min, linearly increased to 95% B by 50 min, held at 95% B until 55 min, and then returned to 5% B at 55.1 min and held until 60 min for column re-equilibration. The mass spectrometer was operated with a heated electrospray ionization source in both positive and negative ion modes under data-dependent acquisition. Full-scan MS spectra were acquired over an m/z range of 100–1500 at a resolving power of 70,000 at m/z 200, and MS/MS spectra were collected at a resolving power of 17,500 for the top 10 most intense precursor ions using stepped normalized collision energies of 10, 30, and 50%. The spray voltages were set to 3.5 kV in positive ion mode and 3.0 kV in negative ion mode. Sheath, auxiliary, and sweep gases were set to

50, 10, and 1 arbitrary units, respectively, and the ion transfer tube temperature was maintained at 320°C . Nitrogen was used as the nebulizing, auxiliary, and collision gas.

Preparation for HPLC/PDA analysis

Each sample extract (20 mg) was accurately weighed, dissolved in 1 mL of MeOH, and sonicated for 10 min. For standard solutions, compounds **1–10** (1 mg each) were also dissolved in 1 mL of MeOH and sonicated for 10 min. All sample and standard solutions prepared for chromatographic analyses were filtered through a polyvinylidene difluoride membrane filter (hydrophilic, $0.20\ \mu\text{m}$, 25 mm; Hyundai Micro Co., Ltd., Seoul, Republic of Korea; Cat. No. 101061, Model No. SV25P020NT) prior to injection.

HPLC/PDA conditions

Chromatographic separation was performed on the YMC Pack Pro C18 column maintained at 35°C . The mobile phases consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in ACN (solvent B). The gradient program was as follows: 10% B at 0–8 min, 20% B at 16 min, 30% B at 25 min, 50% B at 32 min, and 100% B at 40–48 min. The gradient was then returned to 10% B at 53 min and held until 56 min for column re-equilibration.

Calibration curves, limits of detection (LOD), limits of quantification (LOQ) and precision

Standard solutions were prepared by serial dilution to concentrations ranging from 0.3125 to 1000 $\mu\text{g/mL}$. Calibration curves were constructed by plotting peak area (Y, mAU) against concentration (X, $\mu\text{g/mL}$), and linearity was evaluated using the correlation coefficient (r^2). The entire procedure was performed in triplicate. Instrumental precision was assessed by repeated injections ($n=3$) of the reference standards and expressed as relative standard deviation (RSD, %) of the peak areas. The LOD and LOQ were calculated using the mean slope of the calibration curve (S) and the standard deviation of the y-intercept (σ) according to the following equations:

Statistical evaluation

To ensure data accuracy and reproducibility, all experiments were conducted in triplicate. All quantitative analyses were performed in technical triplicate ($n=3$) using extracts derived from single biological individuals. Results are presented as mean values with corresponding standard deviations (mean $\pm$ SD). Data normality was evaluated using the Shapiro-Wilk test prior to applying parametric statistical analyses. Data were analyzed using Student's *t*-test and one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. All statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software, Boston, MA, USA). A *p*-value <0.05 was considered statistically significant.

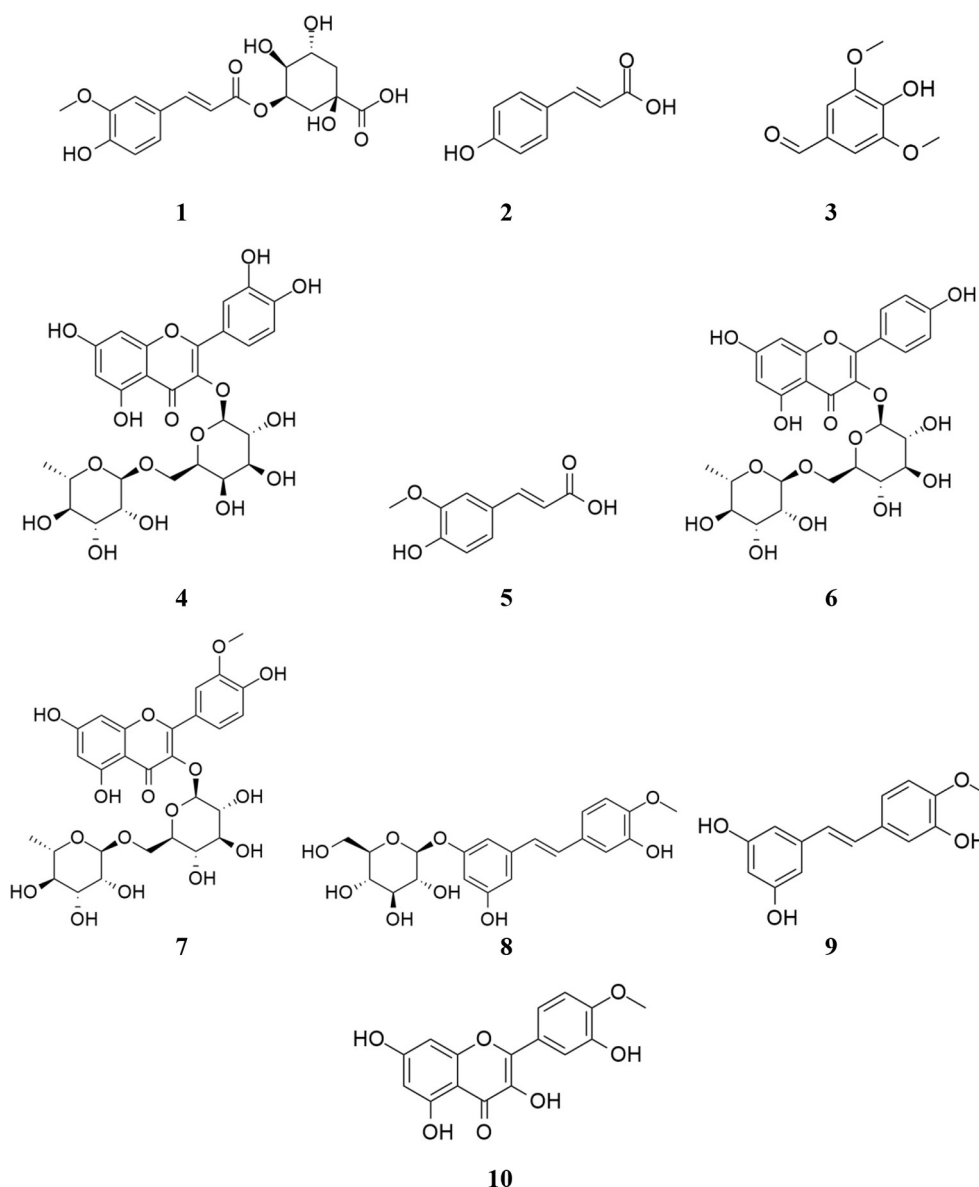


Fig. 3 Chemical structures of compounds 1-10. Compounds: 3-feruloylquinic acid (1), *p*-coumaric acid (2), syringaldehyde (3), quercetin 3-*O*-robinobioside (4), ferulic acid (5), narcissoside (6), narcissoside (7), rhaponticin (8), rhapontigenin (9), and tamarixetin (10)

Results and Discussion

LC-MS/MS based metabolite profiling and identification

To establish a chemical basis for chemotaxonomic comparison, LC-MS/MS profiling was performed on two representative samples, GSJ and GHJ, corresponding to the distinct phylogenetic lineages of Clade A and Clade B, respectively. Metabolite identification and data processing were conducted using Scaffold Elements. The raw LC-MS/MS data were processed using a minimum peak intensity threshold of 10,000 counts and a signal-to-noise ratio of 3 for peak picking. To compensate for chromatographic drifts, retention time alignment was performed with a tolerance of 0.2 min. For metabolite

identification, the mass tolerances for precursor and fragment ions were set to 10 ppm and 15 ppm, respectively. Feature identification was achieved by matching MS/MS spectra against the NIST 2020 and in-house spectral libraries, applying a minimum identification probability threshold of 90% and a precursor mass error tolerance of less than 10 ppm. Compounds were tentatively identified by matching observed precursor ions with theoretical exact masses and by evaluating candidate structures based on mass accuracy, isotopic patterns, and MS/MS spectral similarity [13].

Through this comprehensive approach, a total of 12 phenolic compounds were tentatively identified in both negative and positive ion modes (Fig. 4, Table 1). The identified metabolites

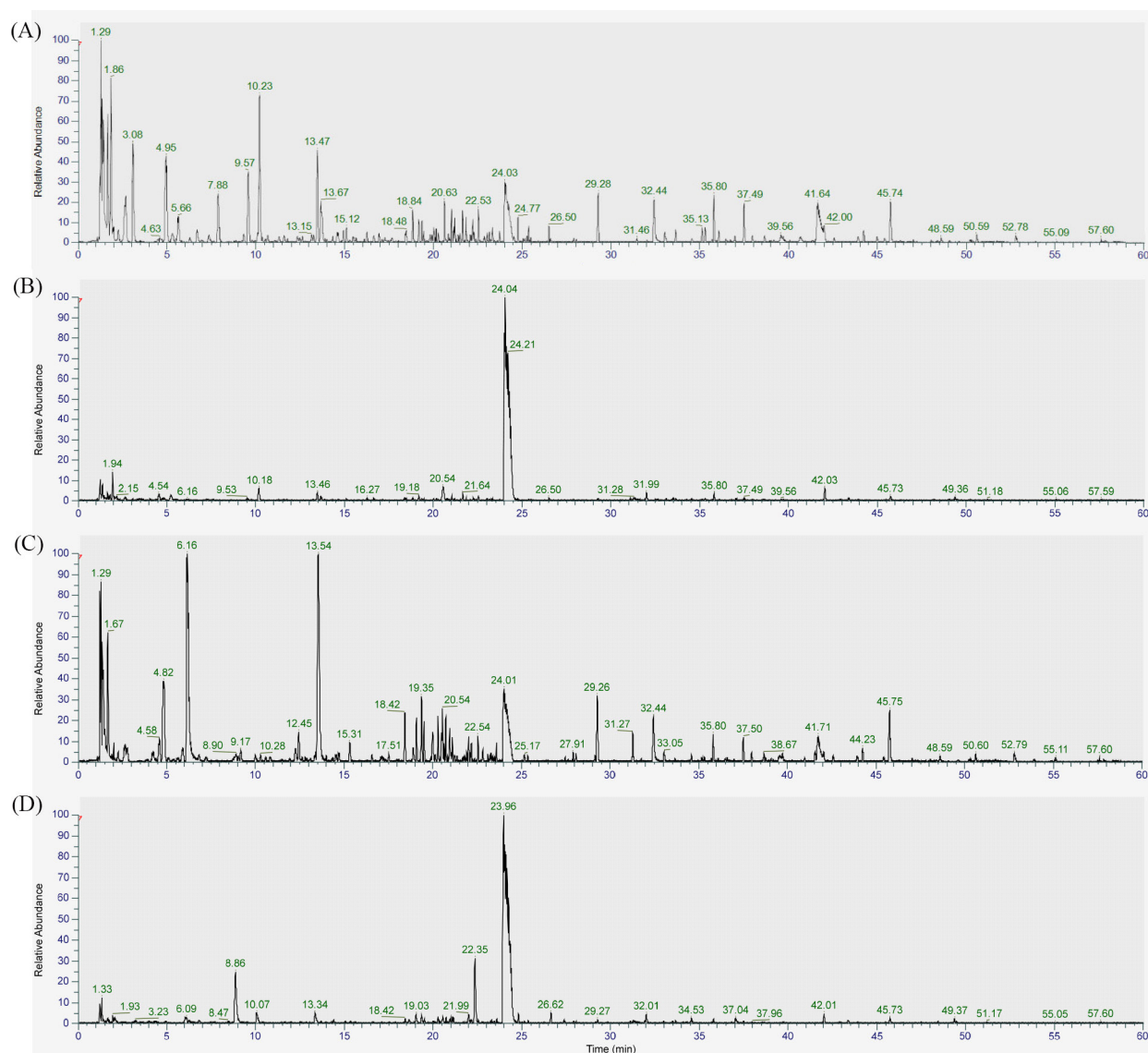


Fig. 4 Base peak chromatograms of GSJ analyzed in the negative (A) and positive (B) ion modes, and GHJ analyzed in the negative (C) and positive (D) ion modes

included hydroxycinnamic acid derivatives, such as *p*-coumaric acid glucoside, *p*-coumaric acid, ferulic acid, 3-feruloylquinic acid, and sinapic acid, as well as the phenolic aldehyde syringaldehyde. Several flavonol derivatives were also detected, including quercetin 3-*O*-robinobioside, nicotiflorin, narcissoside, and the methylated flavonol tamarixetin. In addition, the stilbenoids rhaponticin and rhapontigenin were identified.

Comparative LC-MS/MS profiling revealed clear qualitative differences between the two lineages. Of the twelve annotated metabolites, nine were commonly detected in both samples, whereas syringaldehyde, 3-feruloylquinic acid, and tamarixetin were absent in the GHJ extract. The absence of these compounds highlights the chemical distinction between the two lineages and supports their potential utility as chemotaxonomic markers. Although LC-

MS/MS-based metabolite profiling has been widely applied for geographic and cultivar discrimination in edible crops, vegetables, and medicinal plants [14-20], similar chemotaxonomic studies in ornamental species, particularly within Orchidaceae, remain extremely limited. Therefore, the application of LC-MS/MS to lineage-level metabolic comparison in *Goodyera* represents a rare and innovative approach, offering a valuable analytical framework for future chemotaxonomic assessment of ornamental orchids. These qualitative identification results also provided the foundation for subsequent quantitative analysis across all *Goodyera* species and populations using HPLC/PDA.

HPLC/PDA analysis

Although LC-MS/MS provides comprehensive structural information

Table 1 Tentatively identified compounds and their proposed structures in GSJ and GHJ extracts based on LC-MS/MS analysis in negative and positive ion modes

t_R (min)	Molecular weight	Adducts	Precursor (m/z)	Δ ppm	Tentative identification	
					GSJ	GHJ
10.50	326.1	[M-H] ⁻ [M+H] ⁺	325.0930 -	±0.34 -	<i>p</i> -Coumaric acid glucoside	<i>p</i> -Coumaric acid glucoside
15.49	164.0	[M-H] ⁻ [M+H] ⁺	163.0390 -	±6.81 -	<i>p</i> -Coumaric acid	<i>p</i> -Coumaric acid
15.56	182.1	[M-H] ⁻ [M+H] ⁺	181.0497 183.0650	±5.15 ±0.92	Syringaldehyde	-
17.09	194.1	[M-H] ⁻ [M+H] ⁺	193.0498 -	±4.26 -	Ferulic acid	Ferulic acid
17.33	368.1	[M+HCOO] ⁻ [M+HCOO] ⁺	413.1090 -	±0.25 -	3-Feruloylquinic acid	-
17.75	420.1	[M+HCOO] ⁻ [M+HCOO] ⁺	465.1407 -	±1.26 -	Rhaponticin	Rhaponticin
18.10	224.1	[M-H] ⁻ [M+H] ⁺	223.0607 -	±2.43 -	Sinapic acid	Sinapic acid
19.35	610.2	[M-H] ⁻ [M+H] ⁺	609.1463 611.1596	±0.32 ±1.68	Quercetin 3- <i>O</i> -robinobioside	Quercetin 3- <i>O</i> -robinobioside
20.63	594.2	[M-H] ⁻ [M+H] ⁺	593.1514 595.1653	±0.28 ±0.68	Nicotiflorin	Nicotiflorin
21.01	624.2	[M-H] ⁻ [M+H] ⁺	623.1620 625.1754	±0.31 ±1.44	Narcissoside	Narcissoside
21.92	258.1	[M-H] ⁻ [M+H] ⁺	257.0818 -	±0.54 -	Rhapontigenin	Rhapontigenin
26.41	316.1	[M-H] ⁻ [M+H] ⁺	315.0513 -	±0.85 -	Tamarixetin	-

Table 2 Calibration curves and validation parameters for compounds 1–10

Compounds	t_R (min)	Calibration equation	Correlation factor, (r^2)	LOD (μg/mL)	LOQ (μg/mL)	RSD (%)
1	19.209	Y = 16566090X + 1616	1.0000	0.20	0.50	0.36
2	20.695	Y = 73882052X – 52500	0.9998	1.21	3.66	0.30
3	21.186	Y = 31071981X – 17824	0.9998	1.03	3.11	0.29
4	21.351	Y = 5466806 X – 3799	0.9996	1.63	4.94	0.31
5	22.394	Y = 42891303 X – 27949	0.9998	1.14	3.47	0.30
6	23.390	Y = 11576075 X – 7084	0.9998	1.20	3.63	0.47
7	23.775	Y = 7347364 X – 4440	0.9997	1.27	3.84	0.52
8	24.039	Y = 27130334 X – 20734	0.9997	1.34	4.07	0.32
9	30.570	Y = 21634666 X – 16263	0.9997	1.33	4.03	0.39
10	34.308	Y = 15073761 X – 9188	0.9998	1.18	3.56	0.51

t_R , Retention time; Y, peak area; X, concentration of the standard (μg/mL); r^2 , correlation coefficient for six calibration data points ($n = 3$); RSD (%), relative standard deviation ($n = 3$). Compounds: 3-feruloylquinic acid (1), *p*-coumaric acid (2), syringaldehyde (3), quercetin 3-*O*-robinobioside (4), ferulic acid (5), nicotiflorin (6), narcissoside (7), rhaponticin (8), rhapontigenin (9), and tamarixetin (10)

for tentative compound identification, reliance on mass spectral data alone can lead to ambiguities, particularly when distinguishing isomeric compounds [21]. To rigorously confirm chemical identities and ensure accurate quantification, a validated HPLC/PDA analytical method was established using authentic standards.

The identity of each marker compound was unequivocally verified by comparing its retention time and UV-Vis absorption spectrum with those of the corresponding reference standards, and spike tests were additionally performed to confirm co-elution with authentic standards, thereby excluding potential matrix interference [22,23].

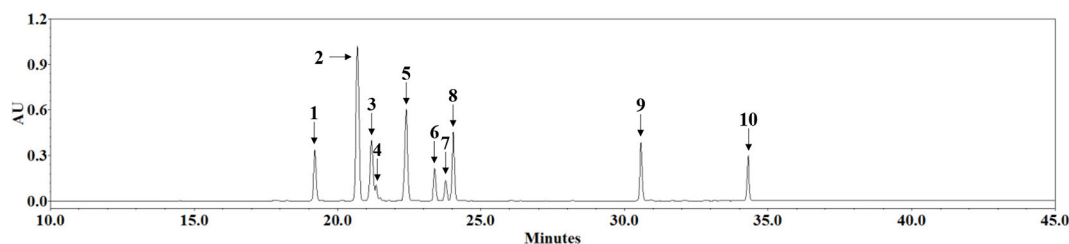


Fig. 5 HPLC/PDA chromatogram of compounds **1–10**. Compounds: 3-feruloylquinic acid (**1**), *p*-coumaric acid (**2**), syringaldehyde (**3**), quercetin 3-*O*-robinobioside (**4**), ferulic acid (**5**), nicotiflorin (**6**), narcissoside (**7**), rhaponticin (**8**), rhapontigenin (**9**), and tamarixetin (**10**)

The analytical method demonstrated high quantitative reliability, as evidenced by excellent linearity of the calibration curves for all marker compounds ($r^2=0.9996$ – 1.0000), LOD (0.20 – $1.63\text{ }\mu\text{g/mL}$), LOQ (0.50 – $4.94\text{ }\mu\text{g/mL}$), and instrumental precision with RSD values ranging from 0.29 to 0.52% (Table 2). Chromatographic profiles of the ten marker compounds obtained from authentic standards are shown in Fig. 5, while representative chromatograms of each *Goodyera* sample extract are presented in Fig. 6. The quantitative results for each compound across the seven populations are summarized in Table 3.

The quantitative analysis suggests the chemotaxonomic distinction between the two representative lineages, GSJ/GSU and GHJ/GHU. The GSJ and GSU samples consistently exhibited high levels of compound **7** (0.737 and 0.629 mg/g DW , respectively) and the presence of compound **10** (0.012 and 0.079 mg/g DW , respectively). In contrast, the GHJ and GHU samples displayed relatively simple profiles characterized by significantly lower amounts of compound **7** (0.170 and 0.383 mg/g DW , respectively) and the complete absence of compound **10**. This marked quantitative disparity reinforces the qualitative LC-MS/MS findings and supports compound **10** as a reliable chemotaxonomic marker distinguishing Clade A from Clade B.

Within the closely related species of Clade B (GHJ, GHU, GVM, and GBH), substantial chemical diversification was observed, reflecting their distinct evolutionary trajectories. GVM was chemically distinct, showing the highest concentration of compound **6** (0.545 mg/g DW) among all species and the unique accumulation of compound **8** (0.174 mg/g DW). The specific accumulation of compound **8** indicates that GVM possesses unique chemical characteristics associated with stilbenoid biosynthesis. This distinction suggests that GVM harbors metabolic pathways that differ from those of other species, serving as a key differentiating factor in chemotaxonomic classification.

Notably, GCH exhibited a remarkable accumulation of compound **4** (2.835 mg/g DW), reaching levels far exceeding those observed in other species in which this compound was not detected or was present only at trace levels. This distinctive chemical phenotype may be associated with the allopolyploid origin of GCH, as polyploidization is known to induce both qualitative

and quantitative changes in secondary metabolite profiles [24]. Polyploid genomes often alter metabolic output through gene dosage effects, increased copy number of biosynthetic enzymes, and regulatory rewiring of transcription factors governing secondary metabolism [25]. In allopolyploids, hybridization further introduces novel allelic combinations that can generate metabolite profiles not present in parental taxa [26]. In contrast, GBH displayed the most chemically impoverished profile, with most marker compounds being undetectable, suggesting a divergent metabolic strategy that may prioritize other, unmeasured compounds or physical traits. This pattern may arise partly from analytical limitations due to the current methods employed, specifically targeting phenolic and glycosylated compounds, but GBH may have accumulated alternative compounds such as alkaloids, terpenoids, or lipid-derived compounds that were not detected under the methods employed [27]. This may be due to ecological pressures such as reduced herbivory, a shift toward physical or resource allocation trade-offs that favor reproduction over chemical defense [28].

To evaluate whether these chemical differences represent stable species-specific traits or reflect environmental influences, geographically isolated populations from Jeju and Ulleung Islands were compared. A consistent geographic trend was observed, in which the Ulleung Island populations of GSU and GHU generally exhibited higher metabolite contents than the Jeju Island populations of GSJ and GHJ. For example, the levels of compounds **2** and **7** were approximately 1.5- to 3-fold higher in the Ulleung Island populations. This quantitative increase likely reflects a plastic response to environmental stressors specific to Ulleung Island, such as higher latitude or distinct climatic conditions. However, environmental effects cannot be completely excluded, as soil chemistry, microclimate, and biotic pressures are known to cause substantial quantitative shifts in plant metabolites across populations [29,30]. Several studies have demonstrated strong genotype-environment interactions in secondary metabolism, where qualitative profiles remain conserved but quantitative levels vary significantly with environmental conditions [31,32].

Crucially, however, the qualitative chemical profiles remained consistent regardless of geographic origin. The presence of compound **10** in GSJ/GSU and its absence in GHJ/GHU were

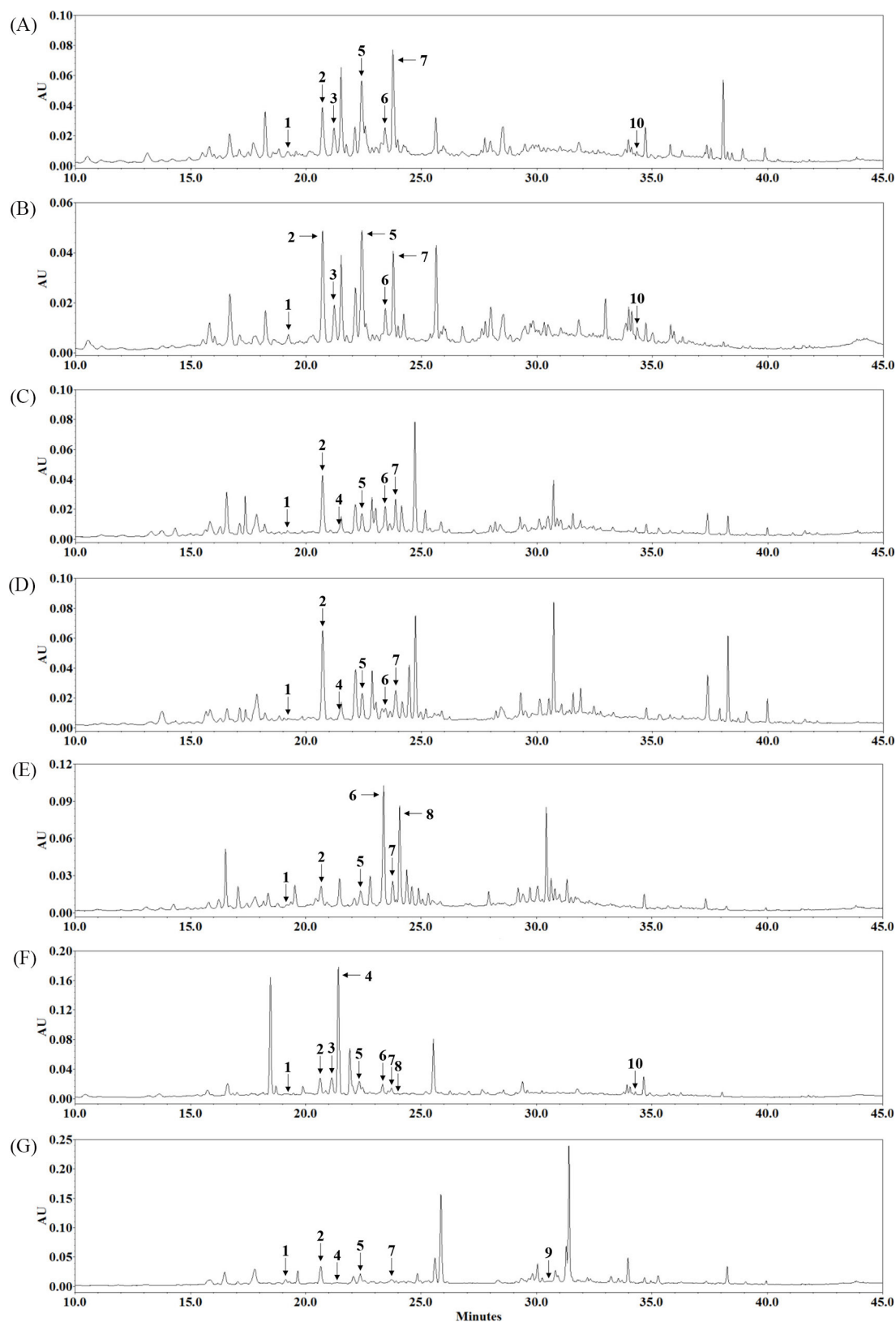


Fig. 6 HPLC/PDA chromatograms of GSJ (A), GSU (B), GHJ (C), GHU (D), GVM (E), GCH (F), and GBH (G) extracts. Compounds: 3-feruloylquinic acid (1), *p*-coumaric acid (2), syringaldehyde (3), quercetin 3-*O*-robinobioside (4), ferulic acid (5), nicotiflorin (6), narcissoside (7), rhaponticin (8), rhapontigenin (9), and tamarixetin (10)

Table 3 Quantification of 3-feruloylquinic acid (1), *p*-coumaric acid (2), syringaldehyde (3), quercetin 3-*O*-robinobioside (4), ferulic acid (5), nicotiflorin (6), narcissoside (7), rhaponticin (8), rhapontigenin (9), and tamarixetin (10)

Sample	Contents (mg/g DW)										Total
	1	2	3	4	5	6	7	8	9	10	
GSJ	0.016± 0.000 ^a	0.039± 0.000 ^a	0.047± 0.001 ^a	ND	0.117± 0.000 ^a	0.189± 0.001 ^a	0.737± 0.003 ^a	ND	ND	0.012± 0.000 ^a	1.157 ^a
GSU	0.030± 0.000 ^b	0.103± 0.002 ^b	0.080± 0.001 ^b	ND	0.199± 0.001 ^b	0.214± 0.011 ^b	0.629± 0.009 ^b	ND	ND	0.079± 0.001 ^b	1.255 ^b
GHJ	0.005± 0.000 ^c	0.044± 0.000 ^a	ND	tr	0.026± 0.000 ^c	0.090± 0.002 ^c	0.170± 0.003 ^c	ND	ND	ND	0.410 ^c
GHU	0.005± 0.000 ^c	0.129± 0.001 ^c	ND	tr	0.068± 0.000 ^d	0.114± 0.002 ^c	0.383± 0.016 ^d	ND	ND	ND	0.699 ^d
GVM	0.009± 0.000 ^d	0.027± 0.001 ^d	ND	ND	0.034± 0.002 ^c	0.545± 0.019 ^d	0.201± 0.014 ^c	0.174± 0.009 ^a	ND	ND	0.982 ^c
GCH	0.023± 0.002 ^c	0.065± 0.005 ^c	0.142± 0.010 ^c	2.835± 0.063	0.099± 0.000 ^f	0.191± 0.002 ^{ab}	0.193± 0.009 ^{ce}	0.021± 0.000 ^b	ND	0.065± 0.000 ^c	3.634 ^f
GBH	0.034± 0.001 ^f	0.036± 0.001 ^a	ND	tr	0.037± 0.001 ^g	ND	tr	ND	tr	ND	0.107 ^g

DW: dry weight; tr: trace amount; ND: not detected. Data are expressed as the mean ± SD (technical replicates, *n* = 3).

^{a–g}Different letters within the same column indicate statistically significant differences at *p* < 0.05, as determined by Tukey's post hoc test following one-way ANOVA or by unpaired *t*-tests, when appropriate

consistently observed in both regions. This demonstrates that, while environmental factors influence the overall accumulation of metabolites, reflecting phenotypic plasticity, the specific chemical composition is a genetically fixed trait that remains sufficiently stable to serve as a diagnostic character for species identification. These metabolite markers may therefore be applied to species identification, breeding material selection, and the authentication of *Goodyera* raw materials in horticultural and conservation programs.

In summary, the comparative HPLC/PDA profiling successfully established a chemotaxonomic framework for *Goodyera* species in Korea. To the best of our knowledge, this is the first report of compounds 1–10 in the genus *Goodyera*. These findings demonstrate that evolutionary divergence, ranging from lineage separation to polyploidization, is deeply reflected in the metabolomic architecture of these orchids.

This study presents the first comprehensive chemotaxonomic evaluation of five *Goodyera* species and seven natural populations native to Korea by integrating LC-MS/MS-based qualitative profiling with HPLC/PDA-based quantitative validation. Comparative metabolomic analysis revealed clear lineage-dependent metabolic divergence between Clade A and Clade B driven by evolutionary separation, and further demonstrated distinct chemical characteristics associated with polyploidization and species diversification within the genus. Although geographically isolated populations from Jeju and Ulleung Islands exhibited substantial quantitative variation in metabolite contents, their qualitative chemical profiles remained highly conserved, indicating that overall metabolite composition represents a genetically stable trait suitable for species-level authentication.

The identification of tamarixetin, syringaldehyde, rhaponticin, and quercetin 3-*O*-robinobioside as lineage-informative marker candidates highlights the strong potential of metabolomic signatures for resolving taxonomic relationships in *Goodyera*. Collectively, these findings establish a foundational chemical framework that can facilitate the development of reliable diagnostic markers, support molecular phylogenetic interpretations, and contribute to the conservation and management of native orchid resources. Future studies expanding to broader geographic ranges, additional *Goodyera* taxa, and complementary omics platforms, such as transcriptomics and genomics, will further clarify the evolutionary mechanisms shaping chemical diversity in Orchidaceae.

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