Chem. Pharm. Bull.

Regular Article

Identification of two phenanthrene derivatives from Australasian allied species in genus Dendrobium.

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Genus *Dendrobium* (Orchidaceae) contains numerous species. Phylogenetic analyses based on morphological characteristics and DNA sequences indicated that this genus is divided into two major groups: Asian and Australasian clades. On the other hand, little is known about the phytochemical differences and similarities among the species in each clade. In this study, we selected 18 *Dendrobium* species (11 from the Asian clade and 7 from the Australasian clade) and constructed HPLC profiles, arrays composed of relative intensity of the chromatographic peaks. Next, orthogonal partial least square discriminant analysis (OPLS-DA) was applied to the profile matrix to classify *Dendrobium* species into the Asian and Australasian clades in order to identify the peaks that significantly contribute to the class separation. In the end, two phenanthrenes, 4,9-dimethoxyphenanthrene-2,5-diol \(1\) and 1,5-dimethoxyphenanthrene-2,7-diol \(2\), which contributed to the class separation, were isolated from the HPLC peaks. The existence of \(2\) was limited to the genetically related Australasian species.

**Key words** *Dendrobium*; Metabolic profile; Orthogonal partial least square discriminant analysis; Phytochemical diversity
Introduction

Genus *Dendrobium* is comprised of around 1,100 species and is distributed in temperate or tropical regions of Asia and the Pacific.\(^1\) Yukawa *et al.* 1993\(^2\) demonstrated that *Dendrobium* is not monophyletic and comprises two major clades (Asian and Australasian clades: *sensu* Clements 2003\(^3\)). Several *Dendrobium* species in the Asian clade are used in traditional herbal medicines as herba dendrobii, and many compounds from medicinal species have been reported, including phenanthrenes, bibenzyl derivatives, and polysaccharides.\(^4-6\)

In contrast, the number of compounds isolated from the Australasian species is limited and compounds unique to the Australasian species have not been reported to date. For example, two phenanthrenes, 3,4-dimethoxyphenanthrene-2,7-diol (nudol) and 4-methoxyphenanthrene-2,5-diol (moscation), have been isolated from *D. antennatum*\(^7\) but they are not characteristic to the Australasian clade since they have been detected in some Asian species.\(^8\) However, we had previously detected several HPLC peaks that were characteristic of phylogenetically related species in the Australasian clade.\(^9\)

The identification of the characteristic compounds common in phylogenetically related species could lead to the revelation about biosynthetic pathways and gene coding biosynthetic enzymes. An effective strategy for detecting such compounds is to combine non-target metabolomics and data mining using multivariate analysis. This approach can provide an overview of secondary metabolites and be useful for biomarker characterization.\(^10\) For example, Nguyen *et al.* researched the relation between variations in secondary metabolites and the phylogenetic divergences of genus *Panax*.\(^11\) Orthogonal partial least square (OPLS) is a suitable method for these purposes. OPLS finds the relations between data $X$ and response $Y$ (known information about a sample). When working with discrete $Y$ variables, the method is called OPLS discriminant analysis (OPLS-DA). The advantage of OPLS-DA is that the between group variation (class separation) is seen in the first component, and the within group variation is seen in the orthogonal components.\(^12\)

In this study, we intended to identify the compounds that are possible indicators discriminating the Asian and Australasian clades. Thus, our strategy was threefold: (1) HPLC profiles of *Dendrobium* species were constructed and compiled in a matrix; (2) OPLS-DA was performed to determine the peaks that significantly contributed to the class separation of the two clades (the $Y$ variable); and (3) Isolation of compounds from the HPLC peaks and structure determination of the compounds was performed.
Experimental Materials

We have performed phylogenetic analysis of 210 species in the Asian clade\textsuperscript{13} and 139 species in the Australasian clade. We have taken the results of those phylogenetic analyses, morphological classification, and the availability of plant specimens into consideration, and selected 18 species (Table 1) for molecular phylogenetic analysis and phytochemical analysis based on HPLC profiling.

*Dendrobium* plants were grown in the greenhouse of Nihon University or of Tsukuba Botanical Garden. All species were identified by their morphological characteristics\textsuperscript{1} or DNA sequence analysis of nuclear ribosomal DNA internal transcribed spacer (ITS) regions\textsuperscript{13}. The infrageneric classification of the genus *Dendrobium* in this study followed the classification of Wood,\textsuperscript{1} which in turn is based on an earlier study by Schlechter.\textsuperscript{14}

Molecular phylogenetic analysis

Genomic DNA extracted from fresh leaves or flowers with a DNeasy Plant Mini kit (QIAGEN, Hamburg, Germany) was used as a template for PCR amplification. ITS1-5.8S-ITS2 regions were amplified and sequenced using the primer set 17SE (ACGAATTCATGGTCCGGTGAAGTGTTCG) and 26SE (GAATTCCCCGGTTCGCTCGCCGTTAC).\textsuperscript{15,16} PCR and sequencing reactions were performed as previously reported by Takamiya et al.\textsuperscript{13,17}

The DNA sequences of each specimen (Table 1) from the 18 species and outgroups (*D. oppositifolium* and *D. muricatum*) were aligned with ClustalW. Phylogenetic analysis of ITS sequences based on maximum parsimony was performed using PAUP\textsuperscript{*} version 4.0b10.\textsuperscript{18} Gaps were treated as missing data and all characters were equally weighted and unordered.\textsuperscript{19} Each data set was analyzed by a heuristic search method with tree bisection–reconnection branch swapping and the MULTREES option on 100 replications of a random addition sequence with stepwise addition, and each replicate after $1 \times 10^6$ rearrangements was assessed. Bootstrap support values were obtained from 1000 replicates using a full heuristic search method. The number of steps, CI, and RI were calculated using one of the most parsimonious trees in each analysis using the TREE SCORES command in PAUP\textsuperscript{*}.  

\textit{Chemical and Pharmaceutical Bulletin Advance Publication}
HPLC condition and profiling

We collected stems with fallen leaves from plants (Table 2) grown for more than one year. The stems were freeze-dried, ground to powder, and extracted with 80% methanol (20 mL/g) at 50° C for 30 min under ultrasonication. The solution was filtered and the residue was again extracted with 80% methanol as mentioned above. The extracts were passed through SPARTAN 30/0.2RC syringe filters (GE Healthcare, Buckinghamshire, UK) and evaporated in vacuo. Dried samples were suspended in 80% methanol at a final concentration of 0.10 g/mL. The solution was then centrifuged at 18,000 × g for 10 min. A 200 µL aliquot of the supernatant was diluted with 100 µL 80% methanol. Another 200 µL aliquot of the supernatant was diluted with 100 µL of p-pentylbenzoic acid (10 mg/mL, as the internal standard). HPLC analysis was conducted using a JASCO X-LC series (X−LC3185PU, X-LC3080DG, X-LC 3159AS, X-LC3180MX, X-LC 3067CO, and X-LC 3110MD). The ODS column (COSMOSIL 5C₁₈-PAQ, 4.6 mm I.D. × 250 mm, 5 µm, Nacalai Tesque, Inc., Japan) was kept at 40° C during the run. Chromatographic separation was carried out using a gradient elution of solvent A (acetonitrile with 0.1% trifluoroacetic acid) and solvent B (water with 0.1% trifluoroacetic acid) at a flow rate of 0.3 mL/min as follows: solvent A 10%→15% (0→30 min), solvent A 15%→40% (30→90 min), solvent A 40%→63.5% (90→130 min), solvent A 63.5%→90% (130→135 min), solvent A 90%→90% (135→160 min), solvent A 90%→10% (160→165 min), solvent A 10%→10% (165→195 min). A photodiode-array detector (PDA) was set at 280 nm with a 360 nm reference wavelength with full spectral scanning from 200 to 400 nm. HPLC peaks were identified by their retention times (Rt) and UV-visible absorption spectra.

Considering individual differences depending on environmental factors such as source and season, three independent specimens from each of the 18 species were collected and analyzed (Table 2). A peak detected in all three specimens was regarded as a reproducible peak in the species. In total, 48 peaks were determined as reproducible. The average relative amount of the reproducible peak was calculated and taken into the HPLC profiles.

The HPLC profiles were compiled in a matrix $X$, where, element $x_{ij}$ represents the average relative amount of the reproducible peak (chemical composition) $j$ of species $i$ (the $i$-th species). The index $i$ ranges from 1 to 18 and $j$ ranges from 1 to 48.
Multivariate analysis

OPLS-DA using SIMCA-P+12.0 (MKS, NJ) was performed to find the relationship between two data tables referred to as $X$, chemical compositions, and $Y$, a binary vector with the value 0 for the Asian clade and 1 for the Australasian clade. The values expressing the model quality were: $R^2_X$(cum)=0.88, $R^2_Y$(cum)=1, $Q^2$(cum)= 0.761. $R^2_X$(cum) is the sum of predictive and orthogonal variation in $X$ that is explained by the model, $R^2_Y$(cum) is the total sum of variation in $Y$ explained by the model, and $Q^2$(cum) is the goodness of prediction.

Extraction and isolation of compound 1

The dried and powdered stem of *D. speciosum* (140 g) that was grown in the greenhouse of Nihon University (Plant ID De156 and De259) was extracted with 80% methanol at 50° C for 30 min under ultrasonication three times consecutively for a total of about 4 L. After evaporation of the solvent, the residue (21.2 g) was resuspended in 0.5 L H$_2$O, and partitioned successively three times with hexane and ethyl acetate (hexane total 0.5 L, ethyl acetate total 1 L). The hexane fraction (3.6 g) was loaded onto a small silica gel column and eluted with hexane/ethyl acetate (graded increase of ethyl acetate). We checked the presence of 1 in the subfractions by HPLC analysis. The subfraction containing the target compound (dry weight 205 mg) was dissolved in 80% methanol, loaded into a COSMOSIL 5C$_{18}$-PAQ, 10 mm I.D. × 250 mm column (Nacalai Tesque, Tokyo, Japan), and eluted with acetonitrile/H$_2$O 55:45 at a flow rate of 3.0 mL/min. Compound 1 was eluted after about 13.5 min (0.29 mg, brownish gum).

NMR spectra of compound 1 were recorded on a JEOL ECX-500 spectrometer, operating at 500 MHz for $^1$H-NMR, and at 125 MHz for $^{13}$C-NMR, in CDCl$_3$ with tetramethylsilane as an internal standard. The spectroscopic parameters of $^1$H-NMR and $^{13}$C-NMR are given in Table 3. Correlations observed in HMQC, HMBC, COSY, and NOESY spectra were used for assignment. ESI-TOFMS $m/z$ 269 [M−H]; HR-ESI-TOFMS [M−H]$^+$ $m/z$: 269.0815 (Caled for C$_{16}$H$_{13}$O$_4$: 269.0814), UV $\lambda_{\text{max}}$ (MeOH) nm: 260, 283, 306,
Extraction and isolation of compound 2

The dried and powdered stem of *D. speciosum* (620 g) that was grown in the greenhouse of Nihon University (Plant ID De222) was extracted with 80% methanol at 50° C for 30 min under ultrasonication three times consecutively (total about 6 L). After evaporation of the solvent, the residue (46.5 g) was resuspended in H2O (0.5 L), and partitioned successively three times with hexane and ethyl acetate (hexane total 0.5 L, ethyl acetate total 1 L). The ethyl acetate fraction (3.3 g) was loaded onto a small silica gel column and eluted with hexane/ethyl acetate (graded increase of ethyl acetate). We checked the presence of 2 in the subfractions by HPLC analysis. The subfraction containing the target compound (dry weight 238 mg) was dissolved in 80% methanol, loaded into a COSMOSIL 5C18-PAQ 10 mm I.D. × 250 mm column, and eluted with acetonitrile/H2O (55:45) at a flow rate of 3.0 mL/min. The target compound was eluted at 8.1 min. The subfractions containing the target compound were combined and further subjected to a COSNOSIL Cholesterol 10 mm I.D. × 250 mm column (Nacalai Tesque), and eluted with acetonitrile/H2O (35:65) at a flow rate of 3.0 mL/min. The target compound was eluted at 17.5 min. Finally, the purified compound 2 was obtained as 2.43 mg of a magenta amorphous solid. The spectroscopic parameters of 1H-NMR and 13C-NMR are given in Table 4. EI-MS *m/z* 270 [M]+; HR-EI-MS [M]+ *m/z*: 270.0891 (Calcd for C16H14O4: 270.0892), UV *λ*max (MeOH) nm: 244, 262, 286, 296, and 309.

LC/MS analysis

Acquity I Class UPLC coupled with Xevo G2-S Q-TOF (Waters Co., UK) was used for the LC/MS analysis. Chromatographic separation was carried out using a gradient elution of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 0.2 mL/min as follows: solvent A 90%→85% (0→10.5 min), solvent A 85%→60% (10.5→30.5 min), solvent A 60%→36.5% (30.5→44.5 min), solvent A 36.5%→10% (44.5→46 min), solvent A 10%→10% (46→59 min), solvent A 10%→90% (59→65 min). The ACQUITY UPLC Peptide BEH C18 Column was heated at 40° C in a column oven. TOF data were collected between 100 and 1000 *m/z*, with desolvation gas 800 L/h at a temperature of 450° C, cone gas 50 L/h, and source temperature 120° C, and capillary and cone voltages of 1.5 kV and 40 V, respectively. The TOF mass spectrometer
was calibrated routinely in a positive electrospray ionization (ESI⁺) mode using a sodium formate solution. Data were centralized during acquisition using independent reference lock-mass ions via the LockSpray interface to ensure mass accuracy and reproducibility. Leucine enkephalin solution (Waters Co., UK) was used as lock-mass, with an [M+H]⁺ ion of m/z 556.2771. The accurate mass and composition for the precursor and fragment ions were calculated using the MassLynx 4.1 software incorporated in the instrument.

Results

HPLC profiling and OPLS-DA for determination of chemical compositions that contribute to the class separation between the Asian and Australasian clades.

We performed molecular phylogenetic analysis based on ITS sequences to clarify the genetic relationships of these 18 Dendrobium species. The strict consensus tree showed that the Asian and Australasian clades were genetically separate (Fig. 1), in agreement with the reports of Yukawa et al.,2,20-22) Yukawa,23) Clements,3,24) and Schuiteman.25) Figure 2 shows the HPLC chromatograms of D. speciosum (section Dendrocoryne, Fig. 2A), D. macrophyllum (section Latouria, Fig. 2B), D. nobile (section Dendrobium, Fig. 2C), and D. goldschmidtianum (section Pedilonum, Fig. 2D). When analyzing three specimens per species, there were peaks that weren’t detected in all of them. We considered that these irreproducible peaks were influenced by individual differences, and decided not to adopt them as descriptors for OPLS-DA.

Figure 3A shows an OPLS-DA score scatter plot. Diamonds indicate the species of the Asian clade, and squares indicate the species of the Australasian one. The between group variation (difference between the Asian and Australasian clades) is shown on the horizontal axis (first component, t[1]). The within group variation is shown on the longitudinal axis (first orthogonal component, to[1]). The diversity of the chemical composition in the Asian clade was larger than that in the Australasian clade.

Figure 3B is an S-plot from the OPLS-DA. Dots indicate chemical compositions (peaks). The S-plot shows the influence of chemical compositions in the model. The horizontal axis (p[1]) indicates the covariance between the X variables and the predictive score t[1]. The longitudinal axis (p(corr)[1]) indicates the correlation between the X variables and the predictive score t[1]. A high absolute value along the horizontal axis indicates that such chemical composition contributes more significantly to the class separation. Chemical compositions that show high absolute value along the longitudinal axis indicate high
reliability (lower uncertainty) as a putative biomarker. We then considered peaks 37 \((x_{37})\) and 40 \((x_{40})\), plotted in the upper right of the first quadrant, to be characteristic of the Australasian allied species.

**Isolation and identification of compounds 1 and 2 from Australasian species.**

Peak 40 was present in the HPLC chromatograms of *D. speciosum* (Fig. 2A, Rt 119.2 min), and in the Australasian allied species *D. macrophyllum* (Fig. 2B, Rt 119.4 min), *D. kingianum* (section *Dendrocoryne*, Rt 119.4 min), *D. shiraishii* (section *Latouria*, Rt 119.2 min), and *D. spectabile* (section *Latouria*, Rt 119.2 min). We isolated 1, 4,9-dimethoxyphenanthrene-2,5-diol, from peak 40 of *D. speciosum* and *D. spectabile*, respectively (Fig. 4, Table 3). We also confirmed the presence of 1 in *D. kingianum*, *D. macrophyllum*, and *D. shiraishii* by LC/MS. Compound 1 had been isolated from other Orchidaceae: Bulbophyllum vaginatum\(^{26}\) and Odontoglossum Harvengtense ‘Tutu’.\(^{27}\) Furthermore, the isolation of 1 from *D. chrysotoxum*,\(^ {28}\) *D. nobile*,\(^ {29}\) and *D. thyrsiflorum*\(^ {30}\) *D. chrysanthum*,\(^ {31}\) had been reported. Although three species other than *D. chrysanthum* were analyzed in this study, we couldn’t detect 1 in the three *Dendrobium* species. Compound 1 was detected in a single specimen in *D. fimbriatum*, *D. griffithianum*, and *D. taurinum* in this study. On the other hand, compound 1 was reproducibly detected in all three specimens of the Australasian species: *D. kingianum*, *D. macrophyllum*, *D. shiraishii*, *D. speciosum*, and *D. spectabile*.

We also isolated 2, 1,5-dimethoxyphenanthrene-2,7-diol, from peak 37 of *D. speciosum* and *D kingianum*, respectively (Fig. 4, Table 4). We also confirmed the presence of 2 in *D. macrophyllum*, and *D. shiraishii* by LC/MS. Compound 2 has not been reported from Asian species, and we didn’t detect it in the 11 Asian species. Although 2 has been identified from other Orchidaceae: Eulophia nuda\(^ {32}\) and closely related species *E. petersii*,\(^ {33}\) this study is the first report where 2 is contained in *Dendrobium* species.

**Discussion**

Phenanthenes in Orchidaceae are synthesized by the phenolic oxidative coupling reaction of the bibenzyl 4 which is synthesized from *m*-hydroxycinnamic acid (Fig. 5).\(^ {34,35}\) The regiospecific reaction of phenolic oxidative coupling produces orchinol 5 \((o, p'-coupling)\) and hircinol 6 \((o, o'-coupling)\). Orchinol contains oxygen atoms at the 2, 4, and 7 positions. On the other hand, hircinol contains oxygen atoms at the 2, 4, and 5 positions.
We searched for known phenanthrenes of *Dendrobium* (supplementary material). Fifty-six phenanthrenes of *Dendrobium* had been registered in Reaxys® (Elsevier, Amsterdam, Nederland) as of January 2018 (containing compound 1). Kovács *et al.* reviewed phenanthrenes isolated from plants. Two additional phenanthrenes of *Dendrobium* which were not included in Reaxys database were took from the review. We compared the structural features of compound 1 and 2 with the 57 phenanthrenes. Most of the 59 compounds can be classified into orchinol- or hircinol-types based on the position of oxygen atoms.

In the 59 phenanthrenes, there were 12 hircinol type compounds (oxygen atoms at the 2, 4 and 5-position). Compound 1 is a hircinol derivative containing oxygen atoms at the 2, 4, 5, and 9-position, and we designated this pattern as a 9-oxygenated hircinol type. There were eight 9-oxygenated hircinol type compounds (1, 12–18 in Fig. 6). In our experiments, the detection of compound 1 in the Asian species showed poor reproducibility. Compound 1 could be biosynthesized in various species of *Dendrobium*, but the yield of 1 might vary especially in Asian species depending on environmental factors. On the other hand, compound 1 is reproducibly detected in the closely related Australasian species (Fig. 1).

There were 10 orchinol type compounds (oxygen atoms at the 2, 4, and 7-position). Compound 2 is an orchinol derivative containing oxygen atoms at the 2, 4, 7, and 8-position, and we designated this pattern as an 8-oxygenated orchinol type. Compounds 2 and 7 (Fig. 5) were the only two 8-oxygenated orchinol type phenanthrenes in the 59 compounds. Although compounds 8–11 contain oxygen atoms at 2, 4, 7, and 8-position, they are further oxygenated than compounds 2 and 7 at the 3- or 1-positions (Fig. 5).

Compound 2 was reproducibly detected in four (*D. kingianum, D. macrophyllum, D. shiraishii, and D. speciosum*) of seven Australasian species in our experiments, and there has been no report on the isolation of compound 2 from an Asian species. It is thus considered that compound 2 is characteristic of several allied Australasian species. In Kovács *et al.*, twelve from 252 phenanthrenes were the 8-oxygenated orchinol type compounds (five from *E. nuda* (one is compound 2), one from *D. thyrsiflorum* (7 in Fig. 5), two from *Bletilla formosana*, and four from *Stemona collinsae, S. pierrei, S. tuberosa*).

The four *Dendrobium* species containing compound 2 are closely related in the Australasian clade, so that metabolic genes involved in oxidation at the 8 position of phenanthrene may be present in specific species. Although more detailed analysis is necessary, our results suggest that phylogenetic similarity correlates with the production of specific secondary metabolites in *Dendrobium*. Further studies are needed to investigate the
correlation between phylogenetic similarity and secondary metabolites such as bibenzyl derivatives.

**Acknowledgements**
We would like to thank K. Suzuki and K. Komura for plant cultivation, and Dr. S. Yoshikawa for advice on the multivariate analysis. We are also grateful to Dr. K. Metori, M. Kaneko, M. Kusanagi, T. Miyamoto, S. Yokoyama, S. Kikuchi, R. Shimizu, K. Yoshino for the LC/MS and HPLC analyses. We also wish to thank Suzuki S. for the structure analysis. This work was supported by JSPS KAKENHI (Grant Number 15K18889), and a grant for novel research project from the School of Pharmacy, Nihon University.

**Conflict of interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary material.
References

1) Wood H. P. “The Dendrobium,” A.R.G. Gantner Verlag Ruggell, Liechtenstein, 2006.
2) Yukawa T., Kurita S., Nishida M., Hasebe M., *Lindleyana*, **8**, 211–221 (1993).
3) Clements M. A., *Telopea*, **10**, 247–298 (2003).
4) Ng T. B., Liu J., Wong J. H., Ye X., Wing Sze S. C., Tong Y., Zhang K. Y., *Appl. Microbiol. Biotechnol.*, **93**, 1795–803 (2012).
5) Ito M., Matsuzaki K., Wang J., Daikonya A., Wang N. L., Yao X. S., Kitanaka S., *Chem. Pharm Bull.*, **58**, 628–633 (2010).
6) Kovács A., Vasas A., Hohmann J., *Phytochemistry*, **69**, 1084–1110 (2008).
7) Lin C. F., Lay H. L., Ni C. L., Chen C. C., *Chem. Nat. Compd.*, **49**, 520–522, (2013).
8) Zhou X. M., Zheng C. J., Gan L. S., Chen G. Y., Zhang X. P., Song X. P., Li G. N., Sun C. G., *J. Nat. prod.*, **79**, 1791–1797, (2016).
9) Takamiya T., Kitamura S., Suzuki S., Shioda N., Furukawa M., Makino M., Matsuzaki K., Kitanaka S., Yukawa T., Iijima H., *Proceedings of APOC11* Okinawa, Japan, 256–262 (2013).
10) Cox D.G, Oh J., Keasling A., Colson K. L., Hamann M. T., *Biochim. Biophys. Acta*, **1840**, 3460–3474, (2014).
11) Nguyen H. T., Lee D. K., Lee W. J., Lee G, Yoon S. J., Shin B. K., Nguyen M. D., Park J. H., Lee J., Kwon S.W., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **1012–1013**, 61–68 (2016).
12) Wiklund S., Johansson E., Sjöström L., Mellerowicz E. J., Edlund U., Shockcor J. P., Gottfries J., Moritz T., Trygg J., *Anal. Chem.*, **80**, 115–122 (2008).
13) Takamiya T., Wongsawad P., Sathapattayanon A., Tajima N., Suzuki S., Kitamura S., Shioda N., Handa T., Kitanaka S., Iijima H., Yukawa T., *AoB PLANTS*, **6**: plu045; doi:10.1093/aobpla/plu045 (2014).
14) Schlechter R., “Die Orchidaceen von Deutsch-Neu-Guinea, Repertorium Specierum Novarum Regni Vegetabilis, Beihefte 1,” Gebrüder Borntraeger, Berlin, 1914.
15) Sun Y., Skinner D. Z., Liang G. H., Hulbert S. H., *Theor. Appl. Genet.*, **89**, 26–32 (1994).
16) Hidayat T., Yukawa T., Ito M., *J. Plant Res.*, **118**, 271–284 (2005).
17) Takamiya T., Wongsawad P., Tajima N., Shioda N., Lu J. F., Wen C. L., Wu J. B., Handa T., Iijima H., Kitanaka S., Yukawa T., *Biol. Pharm. Bull.*, **34**, 779–782 (2011).
18) Swofford D. L., PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer Associates, Sunderland, 2002.
19) Fitch W. M., *Syst. Zool.*, **20**, 406–416 (1971).

20) Yukawa T., Ohba H., Cameron K. M., Chase M. W., *J. Plant Res.*, **109**, 169–176 (1996).

21) Yukawa T., Koga S., Handa T., *Proceedings of Third International Symposium on the Taxonomy of cultivated plants*, ed. by Andrews S., Leslie A., Alexander C., Kew: Royal Botanic Gardens, 351–354 (1999).

22) Yukawa T., Kita K., Handa T., “Monocots: systematics and evolution,” ed. by Wilson K. L., Morrison D. A., CSIRO, Melbourne, 2000, pp. 465–471.

23) Yukawa T., *Proceedings of APOC7*, Nagoya, Japan, 69–71 (2001).

24) Clements M. A., *Aliso*, **22**, 465–480 (2006).

25) Schuiteman A., *Gardens Bull. Singapore*, **63**, 245–257 (2011).

26) Leong Y. W., Kang C. C., Harrison L. J., Powell A. D., *Phytochemistry*, **44**, 157–165 (1997).

27) Suzuki R., Tanaka T., Yamamoto M., Sakagami H., Tomomura M., Tomomura A., Satoh K., Shirataki Y., *In Vivo*, **26**, 993–999 (2012).

28) Li Y. P., Qing C., Fang T. T., Liu Y., Chen Y. G., *Chem. Nat. Compd.*, 2009, **45**, 414–416.

29) Zhang X., Xu J. K., Wang N. L., Kurihara H., Yao X. S., *Journal of Chinese Pharmaceutical Sciences*, **17**, 314–318 (2008).

30) Li Y. P., Wang Y. J., Zhang J., *Chem. Nat. Compd.*, **52**, 880–882 (2016).

31) Li Y. P., Wang Y. J., Chen L. L., *Chem. Nat. Compd.*, **52**, 90–92 (2016).

32) Tuchinda P., Udchachon J., Khumtaveeporn K., Taylor W. C., Engelhardt L. M., White A. H., *Phytochemistry*, **27**, 3267–3271 (1988).

33) Blitzke T., Masaoud M., Schmidt J., *Fitoterapia*, **71**, 593–594 (2000).

34) Fritzemeier H. K., Kindl H., *Eur. J. Biochem.*, **133**, 545–550 (1983).

35) Preising-Müller R., Gnau P., Kindl H., *Arch. Biochem. Biophys.*, **317**, 201–207, (1995).
Figure legends

Fig. 1. Eighteen *Dendrobium* species analyzed in this study

Strict consensus tree derived from maximum parsimony analysis of ITS sequence shown with the detection of compounds 1 and 2. Maximum parsimony analysis generated four trees with a length of 730 steps, a consistency index (CI) of 0.6735 and retention index (RI) of 0.7030. Numbers above internodes indicate bootstrap values from 1000 replications. *D. oppositifolium* and *D. muricatum* are outgroup.

+: detected in three specimens, -: not detected, △: detected in one of the three specimens, ○: purification had been reported in literatures but not detected in our experiments.

Fig. 2. Reverse-phase HPLC elution chromatograms of 80% MeOH extracts from (A) *D. speciosum*, (B) *D. macrophyllum*, (C) *D. nobile*, (D) *D. goldschmidtianum*

The peak marked “S” represents the internal standard, *p*-pentylnbenzoic acid.

Fig. 3. OPLS-DA

(A) Score Scatter Plot of OPLS-DA between Asian and Australasian clades Diamonds and squares indicate species of the Asian and Australasian clades, respectively.

(B) S-plot of OPLS-DA between Asian and Australasian clades

Forty-eight chemical components from the HPLC chromatograms of the 18 *Dendrobium* species were plotted. Peaks 37 and 40 had potential as markers for the Australasian clade.

Fig. 4. Structures of phenanthrene derivatives isolated from Australasian species

1: 4,9-dimethoxyphenanthrene-2,5-diol, 2: 1,5-dimethoxyphenanthrene-2,7-diol.

Note that the numbering follows the IUPAC rules and is not linked with biosynthetic pathways.

Fig. 5. Biosynthetic route of phenanthrene skeleton

The production of orchinol and hircinol depends on whether oxidative coupling occurs at the *o*- or *p*-position of the right-side ring of compound 4.

Fig. 6. 9-oxygenated hircinol type phenanthrene isolated from *Dendrobium*
Figure 1. Eighteen *Dendrobium* species analyzed in this study

| Compound | 1 | 2 |
|----------|---|---|
| D. aphyllum (*Dendrobium*) | - | - |
| D. hercoglossum (*Breviflora*) | - | - |
| D. nobile (*Dendrobium*) | - | - |
| D. catenatum (*Dendrobium*) | - | - |
| D. chrysotoxum (*Densiflora*) | - | - |
| D. fimbriatum (*Holochrysa*) | Δ | - |
| D. moschatum (*Holochrysa*) | - | - |
| D. densiflorum (*Densiflora*) | - | - |
| D. griffithianum (*Densiflora*) | Δ | - |
| D. thyrsiflorum (*Densiflora*) | - | - |
| D. goldschmidtii (*Pedilionum*) | - | - |
| D. macrophyllum (*Latouria*) | + | + |
| D. shiraishii (*Laburia*) | + | + |
| D. speciosum (*Dendrocoryne*) | + | + |
| D. kingianum (*Dendrocoryne*) | + | + |
| D. amboinense (*Fugacia*) | - | - |
| D. spectabile (*Litouria*) | + | - |
| D. taurinum (*Spatulata*) | Δ | - |

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Figure 2. Reverse-phase HPLC elution chromatograms of 80% MeOH extracts from (A) *D. speciosum*, (B) *D. macrophyllum*, (C) *D. nobile*, (D) *D. goldschmidtianum*.
Figure 3A Score Scatter Plot of OPLS-DA between Asian and Australasian clades
Figure 3B. S-plot of OPLS-DA between Asian and Australasian clades
Figure 4. Structures of phenanthrene derivatives isolated from Australasian species

1: 4,9-dimethoxyphenanthrene-2,5-diol
2: 1,5-dimethoxyphenanthrene-2,7-diol
Figure 5. Biosynthetic route of phenanthrene skeleton

Chemical and Pharmaceutical Bulletin Advance Publication
Figure 6. 9-oxygenated hircinol type phenanthrene isolated from *Dendrobium*
Table 1. List of *Dendrobium* used in phylogenetic analysis

| Clade     | Species                   | Section       | ITS sequence | Plant ID   |
|-----------|---------------------------|---------------|--------------|------------|
| Asian     | *D. hercoglossum*         | Breviflores   | AB593580     | TBG118850  |
| Asian     | *D. aphyllum*             | Dendrobium    | AB593539     | TBG122508  |
| Asian     | *D. catenatum*            | Dendrobium    | AB593517     | TBG159450  |
| Asian     | *D. nobile*               | Dendrobium    | AB593619     | TBG128809  |
| Asian     | *D. chrysotoxum*          | Densiflora    | AB593533     | TBG118321  |
| Asian     | *D. densiflorum*          | Densiflora    | AB593546     | TBG159421  |
| Asian     | *D. griffithianum*        | Densiflora    | AB593573     | TBG138067  |
| Asian     | *D. thyrsiflorum*         | Densiflora    | AB593674     | TBG159453  |
| Asian     | *D. fimbriatum*           | Holochrysa    | AB593562     | TBG84386   |
| Asian     | *D. moschatum*            | Holochrysa    | AB593616     | TBG56113   |
| Asian     | *D. goldschmidtianum*     | Pedilonum     | AB593570     | TBG159434  |
| Australasian | *D. kingianum*         | Dendrocoryne  | AB894138     | MWC164     |
| Australasian | *D. speciosum*           | Dendrocoryne  | AB894143     | De156      |
| Australasian | *D. amboinense*         | Fugacia       | AB894133     | TBG134575  |
| Australasian | *D. macrophyllum*        | Latouria      | AB894131     | Tomiyama s.n. |
| Australasian | *D. shiraishii*          | Latouria      | AB894132     | TBG116119  |
| Australasian | *D. spectabile*          | Latouria      | AB894141     | TBG118865  |
| Australasian | *D. taurinum*            | Spatulata     | AB894142     | Shiraishi577 |
| Outgroup  | *D. oppositifolium*      | Tetrodon      | LC227998     | TBG140666  |
| Outgroup  | *D. muricatum*            | Inobulbon     | LC227999     | Yukawa97-2300 |
Table 2. Specimen information used in multivariate analysis

| Species                  | Plant 1 ID | Plant 2 ID | Plant 3 ID |
|--------------------------|------------|------------|------------|
| *D. hercoglossum*        | De183      | De248      | De345      |
| *D. aphyllum*            | De6        | De198      | De197      |
| *D. catenatum*           | De181      | De255      | KNY91      |
| *D. nobile*              | De78       | De75       | De286      |
| *D. chrysotoxum*         | De21       | De194      | De268      |
| *D. densiflorum*         | De125      | De29       | De379      |
| *D. griffithianum*       | De130      | De231      | TBG144119  |
| *D. thyrsiflorum*        | De158      | De94       | De93       |
| *D. fimbriatum*          | De35       | De36       | De350      |
| *D. moschatum*           | De67       | De209      | De340      |
| *D. goldschmidtianum*    | De39       | De62       | De230      |
| *D. kingianum*           | De134      | De365      | TBG145475  |
| *D. speciosum*           | De156      | De343      | T63        |
| *D. amboinense*          | De187      | De344      | TBG134575  |
| *D. macrophyllum*        | De164      | De138      | De229      |
| *D. shiraishii*          | De185      | T13        | De397      |
| *D. spectabile*          | De254      | De324      | De342      |
| *D. taurinum*            | De167      | De297      | De408      |
Table 3. $^1$H- and $^{13}$C-NMR data for 4,9-dimethoxyphenanthrene-2,5-diol (Compound 1) in CDCl$_3$

| Position | $\delta$H          | $\delta$C      |
|----------|--------------------|----------------|
| 1        | 6.88, $d$, $J$=2.5 Hz (6.88, $d$, $J$=2.6 Hz) | 106.3, d (106.3) |
| 2        |                    | 154.4, s (154.3) |
| 3        | 6.69, $d$, $J$=2.5 Hz (6.69, $d$, $J$=2.6 Hz) | 99.3, d (99.4) |
| 4        |                    | 155.4, s (155.4) |
| 4a       |                    | 110.7, s (110.5) |
| 4b       |                    | 119.8, s (119.9) |
| 5        |                    | 153.8, s (153.8) |
| 6        | 7.24, $dd$, $J$=8.0 Hz, 1.5 Hz (7.25, $dd$, $J$=7.9 Hz, 1.5 Hz) | 117.3, d (117.3) |
| 7        | 7.50, $t$, $J$=8.0 Hz (7.50, $t$, $J$=7.9 Hz) | 126.9, d (126.9) |
| 8        | 7.94, $dd$, $J$=8.0 Hz, 1.5 Hz (7.94, $dd$, $J$=7.9 Hz, 1.5 Hz) | 114.1, d (114.1) |
| 8a       |                    | 128.6, s (128.5) |
| 9        |                    | 154.6, s (154.6) |
| 10       | 6.72, s (6.73, s) | 101.5, d (101.5) |
| 10a      |                    | 136.8, s (136.8) |
| 2-OH     | 5.14, s (5.29, s) |                |
| 5-OH     | 9.50, s (9.50, s) |                |
| 4-OCH$_3$| 4.06, s (4.06, s) | 58.3, q (58.3) |
| 9-OCH$_3$| 4.04, s (4.04, s) | 55.5, q (55.5) |

Values in parentheses are those reported by Leong et al. $^{[26]}$. 

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Table 4. $^1$H- and $^{13}$C-NMR data for 1,5-dimethoxyphenanthrene-2,7-diol (Compound 2) in CDCl$_3$

| Position | δH                  | δC       |
|----------|----------------------|----------|
| 1        | 140.7, s             |          |
| 2        | 144.9, s             |          |
| 3        | 7.28, d, $J$=9.5 Hz (7.20, d, $J$=9.4 Hz) | 115.9, d |
| 4        | 9.25, d, $J$=9.5 Hz (9.19, dd, $J$=9.4 Hz, 0.8 Hz) | 125.0, d |
| 4a       |                      | 125.4, s |
| 4b       |                      | 115.7, s |
| 5        |                      | 159.6, s |
| 6        | 6.73, d, $J$=2.3 Hz (6.80, d, $J$=2.4 Hz) | 99.2, d  |
| 7        |                      | 153.6, s |
| 8        | 6.87, d, $J$=2.3 Hz (6.90, d, $J$=2.4 Hz) | 104.8, d |
| 8a       |                      | 134.2, s |
| 9        | 7.56, d, $J$=9.2 Hz (7.61, d, $J$=9.2) | 127.2, d |
| 10       | 7.91, d, $J$=9.2 Hz (7.96, dd, $J$=9.2 Hz, 0.8 Hz) | 120.6, d |
| 10a      |                      | 126.1, s |
| 1-OCH$_3$| 3.96, s (3.91, s)    | 62.0, q  |
| 5-OCH$_3$| 4.05, s (4.07, s)    | 55.7, q  |

Values in parentheses are those reported by Tuchinda et al. $^{[32]}$. 

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