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Chemotaxonomy of Ferns: Triterpenoids and *rbc*L gene Sequences of *Polypodium, Polypodiodes* and *Goniophlebium*

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Triterpenoids obtained from the Polypodiaceous ferns of the genus *Polypodium* (*P. vulgare, P. sibiricum, P. fauriei* and *P. polypodioides*), the genus *Polypodiodes* (*P. niponica, P. formosana* and *P. amoena*), and *Goniophlebium mengtzeense* were compared. The *rbc*L gene sequences of typical species of each group were also analyzed. The chemotaxonomical classification of the *Polypodium, Polypodiodes* and *Goniophlebium* groups on the bases of chemical constituents (triterpenoids) was supported by the results of the gene analysis.

Keywords: chemotaxonomy; triterpenoids; *rbc*L gene sequences; fern; *Polypodium vulgare*; *Polypodiodes niponica* ; *Goniophlebium persicifolium.*

We have reported[1-6] various types of triterpenoids from fresh rhizomes of the Polypodiaceous ferns of the *Polypodium* and *Polypodiodes* groups, and showed that their triterpenoid constituents were useful for identification of these ferns in the genus level. Particularly, some of the triterpenoids are diagnostic chemotaxonomically, *viz.* a pentacyclic triterpenoid hydrocarbon, serratene for the *Polypodium,* e.g. *P. vulgare* L. (Oo-ezodenda in Japanese), *P. sibiricum* Sipliv. (Ezodenda), *P. fauriei* Christ, (Osyaguji-denda) and *P. polypodioides* L., and oleanene and migrated oleanenes for the genus *Polypodiodes,*[7] e.g. *P. niponica* (Mett.) Ching (=*Polypodium niponicum* Mett., Aone-kadzura), *P. formosana* (Bak.) Ching (=*Polypodium formosanum* Bak.; Taiwan-Aone-kadzura) and *P. amoena* (Wall.) Ching (=*Polypodium amoenum* Wall. ex Mett.; Alisan-denda). Recently, many studies of the *rbc*L gene sequences have been applied for the classification of ferns.[8] The gene of the large subunit of the riblose-biphosphate carboxylase (*rbc*L), located on the chloroplast genome, is an ideal choice for chemosystematics of ferns because of its slow synonymous substitution rate in comparison with nuclear genes and its functional constraint that reduces the evolutionary rate of nonsynonymous substitution.[9] Since *Polypodiodes niponica* and *P. formosana* are recently referred to *Goniophlebium niponicum* and *G. formosanum,*[10] it was considered necessary to compare their genomes with those of *Polypodium vulgare* L. (type of genus) and *Goniophlebium persicifolium* (Desv.) Bedd. (type of genus), to confirm their chemotaxonomic relations.

MATERIALS AND METHODS

Plant Materials for *rbc*L The fresh leaves (*ca.* 5 g) of *Polypodium vulgare* (Alsace, France), *Polypodiodes niponica* (Shizuoka city, Shizuoka) and *P. formosana* (Wulai, Taiwan) were collected from the plants cultivated in the green house of our University. Those of *Goniophlebium persicifolium* were collected from these

growing in the green house of Kobe Gakuin University by courtesy of Prof. Saiki. These voucher specimens have been deposited in the Herbarium of Showa Pharmaceutical University.

Preparation of Genomic DNA The leaves were powdered and the sample (*ca.* 1–2 mg), in a mortar with liquid N_2, was extracted with 2% cetyl trimethylammonium bromide (CTAB). After purification, the pure DNA was subjected to PCR.

DNA Fragment Amplification by PCR Genomic DNA was amplified with two designed primers as follows: 1-1[8a] (ATGTCACCACAAACAGAGACTAAAGC), TWNP1[8b] (TATCCCTTAGACCTCTTCGAAGAAGGTTC), N2-1[8a] (TGAAAACGTGAATTCCCAACCGTTTATGCG), F001[8c] (ATTCACCGCGCGATGCATGC) for forward (5′-3′) and NN3-2[8a] (GCAGCAGCTAGTTCCGGGCTCCA), TW2PR[8b] (CGTTCACCTTCTAGTTTACCTACAACAGT), R001[8c] (TCGTTGCCTATCGATCACAGCA) and 2R[8a] (CTTCTGCTACAAATAAGAATCGATCTCTCCA) for reverse.

Sequences of Amplified DNA The designed eight primers (1-1, TWNP1, N2-1, F001, NN3-2, TW2PR, R001 and 2R) labeled on the 5′ end with Texas red were employed for sequencing of the amplified DNA fragment, and the sequencing was performed. The *rbc*L nucleotide sequence data have been submitted to the DNA Data Bank Japan, National Institute of Genetic Center for Information Biology (Accession numbers: *Polypodium vulgare* AB044899, *Polipodiodes niponica* AB043098, *Polypodiodes formosana* AB043100, *Goniophlebium perscifolium* AB043099).

RESULT AND DISCUSSION

Triterpenoids of *Polypodium, Polypodiodes* and *Goniophlebium* The characteristic triterpenoids hitherto reported from our laboratory are summarized in Table 1 and Chart 1. The triterpenoids obtained from *Polypodium vulgare, P. sibiricum* and *P. fauriei* were very similar to each other and the characteristic compound was serratene.[11] Although various alcohols and ketones having serratane skeleton were reported in *Lycopodium* and *Pinus* species,[12] the hydrocarbon was isolated only from *Polypodium* species. The constituents of *P. vulgare* of various origins, *i.e.* of Japan (Oki and Aomori) and Europe (Sweden, Norway, England and France), were confirmed to contain similar triterpenoid constituents and the sweet substance, osladin.[13] *Polypodium sibiricum* was formerly called *P. virginianum* L.,[11] which was proved to be not suitable.[14] Prof. Haufler suggested that Japanese *P. vulgare* was an allotetraploid between *P. sibiricum* and *P. fauriei*.[14] We do not agree to this suggestion because the rhizomes of Japanese *P. vulgare* (Oki and Aomori) are sweet, but neither *P. sibiricum* nor *P. fauriei* contains the sweet substance.[15] Although the triterpenoids of *Polypodium polypodioides* L.[16] are similar to those of *P. vulgare*, including serratene, this fern has been recently referred to as *Pleopeltis polypodioides* (L.) E. G. Andrews & Windham.[17]

The characteristic features of triterpenoids obtained from *Polypodiodes* species, *P. niponica*, *P. formosana* and *P. amoena*[1-6] are: 1) They posses linear, mono-, bi-, tri-, tetra- and pentacyclic skeleta, many of which have new structures interesting from the biogenetic point of view. 2) Various pentacyclic triterpenoid hydrocarbons and alcohols belonging to the oleanane and migrated oleanane groups were found in a considerable amount, and this feature has not been known in other ferns studied including *Goniophlebium mengtzeense* (Christ) Rödl-Linder (=*Polypodium taiwanianum* Hayata; Taiwan-uraboshi).[18] 3) A very interesting compound, preoleanatetraene (**P**)[5] was obtained in a considerable amount, which might be supposed to be a precursor of the oleanane and migrated oleanane triterpenoids in *Polypodiodes*. As *Polypodiodes amoena* and *Goniophlebium mengtzeense* are found in the same place in Taiwan, the rhizomes were carefully collected in several locations and the difference in the triterpenoid constituents was carefully studied. We have concluded that the ferns belonging to the genus *Polypodiodes* have obvious chemotaxonomical characters to separate them from *Polypodium* and *Goniophlebium*. In the present study this conclusion has been strongly supported by the DNA studies.

Similarity of the DNA Genetic similarity of the *rbc*L gene among *Polypodiodes niponica* and *P. formosana, Goniophlebium persicifolium,* and *Polypodium vulgare* was calculated by using the Higgins method (Fig. 1). The *rbc*L genes of the two species of the genus Polypodiodes were very similar to each other (99.7%), but not very

Table 1 Characteristic Triterpenoids from Rhizomes of *Polypodium, Polypodiodes* and *Goniophlebium* Species

	L	A	B	T	P	D	E	H	O	S
Polypodium vulgare	−	−	±	±	−	+	+	++	−	+
Polypodium sibiricum	−	−	±	±	−	+	±	++	−	+
Polypodium fauriei	−	−	+	+	−	+	+	++	−	+
Polypodium polypodioides	−	−	±	±	−	−	−	++	−	+
Polypodiodes niponica	+	+	+	+	+	+	+	++	+	−
Polypodiodes formosana	+	+	+	+	+	+	+	++	+	−
Polypodiodes amoena	+	−	+	+	++	+	+	++	+	−
Goniophlebium mengtzeense	±	−	−	+	−	±	+	++	±	−

L: linear **A**: achillane **B**: bicycle **T**: tricyclic **P**: preoleanane **D**: dammarane **E**: euphane **H:** hopane
O: oleanane **S**: serratene ++: isolated in a considerable amount, +: isolated, ±: detected, −: not detected.

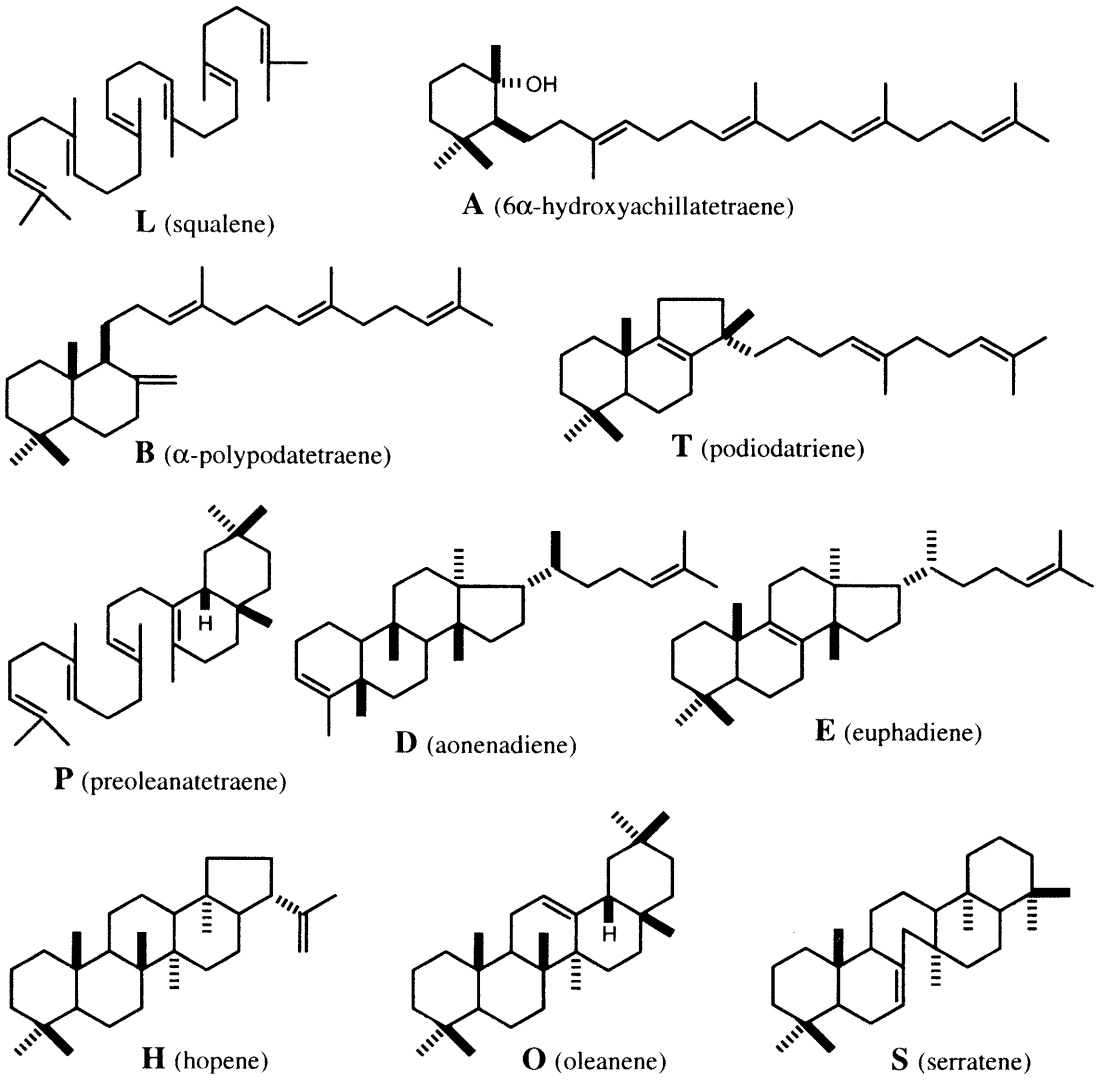

Chart 1 Chracteristic Compounds in *Polypodium*, *Polypodiodes* and *Goniophlebium* Species

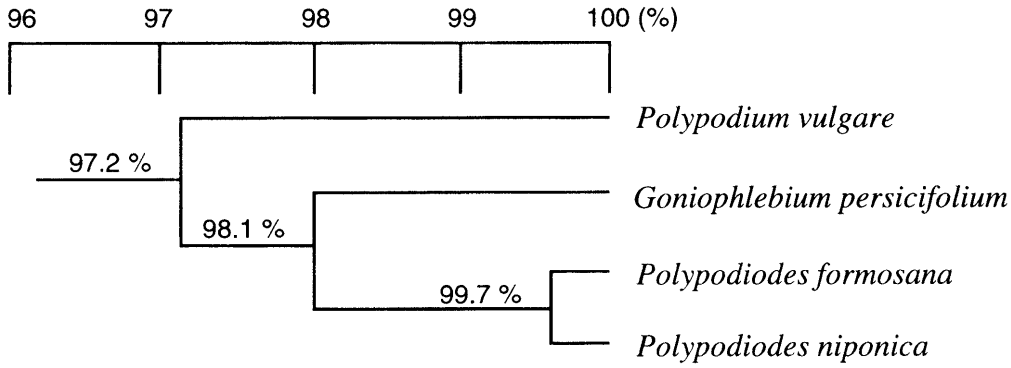

Fig. 1 Inferred Phylogenetic Tree from *rbc*L Gene Sequences

similar to those of *Goniophlebium perscifolium* (98.1 %) and *Polypodium vulgare* (97.2 %).

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