

## Taxonomic Reconsideration and Phylogeographic Implication for *Nertera yamashitae* (Rubiaceae)

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**Abstract** Anatomical and morphological characters and phylogenetic position of *Nertera yamashitae* were investigated with those of related species in the family Rubiaceae. In the anatomy and morphology, disk morphology and number of ovules in an ovary locule of *N. yamashitae* differed from those of other *Nertera* species, and were more similar to those of *Ophiorrhiza* species. In the sequence similarity search of ITS in nrDNA, and *atpB-rbcL* and *trnK/matK* in cpDNA, *N. yamashitae* showed the highest similarity to *Ophiorrhiza* species. In the MP phylogenetic analyses based on nrITS, *atpB-rbcL*, and *trnK/matK*, *N. yamashitae* was outside of a clade of the genus *Nertera* in tribe Anthospermeae, while it was included in a clade of the genus *Ophiorrhiza* in tribe Ophiorrhizeae. The present study, in taxonomy, suggests that *N. yamashitae* should be treated as a species in *Ophiorrhiza* as a new combination; and in phylogeography, implies that it could be a relict species lineage in Amami Island of the Ryukyu Archipelago.

**Key words** : dwarfism, *Nertera*, *Ophiorrhiza*, Rubiaceae, Ryukyus.

### Introduction

The genus *Nertera* Banks et Sol. ex Gaertn. is composed of ca. 16 (or six in a strict sense) species found in Malesia, Oceania and Americas; and is classified in tribe Anthospermeae (subfamily Rubioideae), being well supported by morphological and molecular data (Bremekamp, 1966; Bremer and Manen, 2000; Bremer, 2009). Although Heads (1996) included *Nertera* species in the genus *Coprosma*, disagreeing with most taxonomic treatments, he still recognized it as a taxon at section level, namely sect. *Nertera*. As the first species of the genus *Nertera* discovered in Japan, *Nertera yamashitae* T. Yamazaki (Fig. 1) studied herein was described based on a type specimen collected from Amami Island in the Ryukyu Archipelago (Yamazaki, 1998), because

it has small leaves, four stamens, and a four-lobed corolla, showing the generic-representative morphologies for *Nertera* (e.g. Lo, 1999; Liu and Yang, 1998). This species has been thought to be an insular endemic species of Amami Island, and only three populations have been found in two river systems in this island (Hotta, 2004). Therefore, it is treated as critically endangered by the Japanese Ministry of the Environment (2007). After Yamazaki's description (1998), although a few studies on cytology (Kokubugata *et al.*, 1998) and conservation biology (Hotta, 2004) have been carried out, further taxonomical reconsideration based on anatomical, morphological, and molecular data has never been applied to this species.

Dwarfism, a phenomenon in which body size is strongly reduced, is well-known in alpine

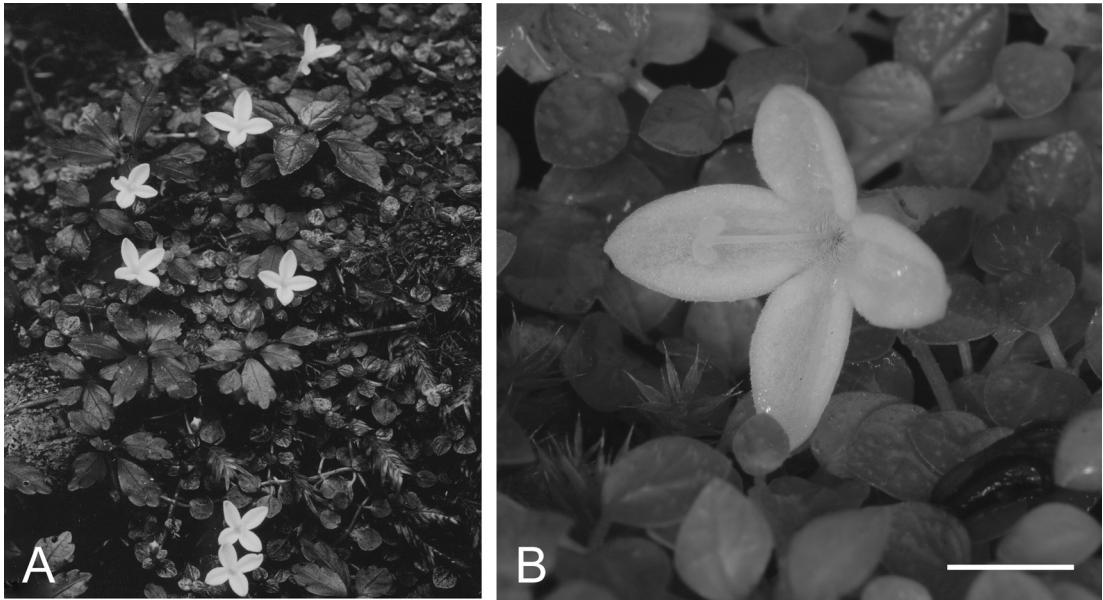


Fig. 1. *Nertera yamashitae*. (A) Habit (photographed by H. Yamashita); (B) flower in plants (GK11029) cultivated in Tsukuba Botanical Garden, National Museum of Nature and Science. Bar=5 mm.

ecosystems (e.g. Körner, 1999). In the Ryukyus, some leaf dwarfisms are known at elevations over 1,000 m on Yakushima Island in the northern Ryukyus, and phylogenetic and their evolutionary biology has been examined in these plants (e.g. Yokoyama *et al.*, 2003; Shinohara and Murakami, 2006; Tsukaya *et al.*, 2007). Also, certain dwarf leaf species are known at altitudes of less than 100 m on Amami Island, such as *N. yamashitae* studied herein, *Lysimachia liukiensis* Hatus. (Myrsinaceae), *Oxalis exilis* A. Cunn. (Oxalidaceae), *Rubus amamianus* Hatus. et Ohwi (Rosaceae), and *Sphenomeris minutula* Sa. Kurata (Lindsaeaceae) (Hatusima, 1975). In contrast with leaf dwarfism at high elevation on Yakushima Island, however, few phylogenetic studies have examined for the dwarf leaf species at low altitude on Amami Island without *Lysimachia liukiensis* (Kokubugata *et al.*, 2010).

In the present study, we investigated the floral anatomy and morphology of *N. yamashitae*; and its phylogenetic position among related species of subfamily Rubioideae based on three molecular loci to reconsider the taxonomic status, and to discuss the dwarfism of this species.

## Materials and Methods

### *Plant materials*

Plants of *Nertera yamashitae* were collected from two localities in two river systems in Amami Island (Table 1). Also, plants of *N. granadense* (Mutis ex L.f.) Druce following a strict taxonomic treatment of Liu and Yang (1998) in four Asian countries, and two *Ophiorrhiza* species in two Asian countries were collected and used in the present molecular phylogenetic analyses (Table 1). Voucher specimens were deposited in the herbarium of the National Museum of Nature and Science (TNS).

### *DNA extraction, polymerase chain reaction, and sequencing*

Fresh leaves were harvested from the plants cultivated in a greenhouse of the Tsukuba Botanical Garden for DNA extraction, which was conducted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocols. Total DNA samples isolated were deposited in the Molecular Biodiversity Research Center of the National Museum of Nature and Science.

Table 1. Plant materials of *Nertera* and *Ophiorrhiza* investigated in the present study, and their localities, voucher numbers, and ITS, *atpB-rbcL* and *trnK/matK* GenBank accession numbers

| Species                             | Locality                                      | Voucher <sup>a</sup> | Accession no. <sup>b</sup> |   |
|-------------------------------------|---|----------------------|----------------------------|---|
|                                     |   |                      | ITS (type)                 | <i>atpB-rbcL</i> (type) <i>trnK/matK</i> (type) |
| <i>Nertera yamashitae</i> T. Yamaz. | JAPAN, Ryukyus: Naze, Amami Is., Kagoshima    | GK9854               | AB559526 (A)               | AB559538 (a) AB559550(l)                        |
|                                     | JAPAN, Ryukyus: Sumiyo, Amami Is., Kagoshima  | GK11029              | AB559527 (A)               | AB559539 (a) AB559551(l)                        |
|                                     | TAIWAN: Nanao, Iilan                          | GK11028              | AB559529 (B)               | AB559540 (b) —                                  |
|                                     | TAIWAN: Taoyuan, Kaohsiung                    | GK8824               | AB559528 (B)               | AB559541 (c) —                                  |
|                                     | TAIWAN: Taoyuan, Kaohsiung                    | GK10821              | AB559530 (C)               | AB559542 (d) —                                  |
|                                     | PHILIPPINES: Bontoc, Mountain Province, Luzon | GK8301               | AB559531 (D)               | AB559543 (e) —                                  |
|                                     | PHILIPPINES: Trinidad, Benguet, Luzon Is.     | GK8254               | AB559532 (E)               | AB559544 (f) —                                  |
|                                     | PHILIPPINES: Banaue, Ifuga, Luzon Is.         | GK9647               | AB559533 (E)               | AB559545 (g) —                                  |
|                                     | MALAYSIA, Borneo: Kinabaru, Sabah             | GK7903               | AB559534 (F)               | AB559546 (h) —                                  |
|                                     | INDONESIA, West Java: Cibodas, Cianjur        | GK8559               | AB559535 (G)               | AB559547 (i) —                                  |
|                                     | INDONESIA, West Java: Cibodas, Cianjur        | GK8503               | AB559536                   | AB559548  |
| <i>Ophiorrhiza longiflora</i> Blume | THAILAND: Ang Kang, Chiang Mai                | GK12391              | AB559537                   | AB559549  |
| <i>Ophiorrhiza</i> sp.              |   |                      |                            | AB559552<br>AB559553                            |

<sup>a</sup> GK: G. Kokubugata (TNS). <sup>b</sup> Sequences investigated here and registered in the DDBJ/EMBL/GenBank database; letters in parentheses indicate the locus type.

In the present study, ITS1, 5.8S rDNA and ITS2 with part of the 18S rDNA (ITS) of nuclear ribosomal DNA (nrDNA), the intergenic spacer of *atpB* and *rbcL* genes (*atpB-rbcL*), the *maturaseK* gene and adjacent *trnK* introns (*trnK/matK*) of cpDNA were selected for the molecular phylogenetic analyses. In the polymerase chain reaction (PCR), we amplified ITS using the primer set AB101 and AB102 (Douzery *et al.*, 1999), *atpB-rbcL* using *atpB2F* and *rbcL2R* (Nakamura *et al.*, 2006), *trnK/matK* using *trnK-3914(D)F* (Johnson and Soltis, 1994) and *psbA5'R* (M. Nepokroeff, University of South Dakota, personal communication). The PCR amplifications were performed using Takara Ex Taq (Takara, Tokyo) with Ampdirect Plus (Shimadzu, Kyoto) on an iCycler (BioRad, Hercules, CA). The PCR profile comprised 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C for ITS, and 35 cycles of 30 s at 94°C, 30 s at 55°C, and 3 min at 72°C for *atpB-rbcL* and *trnK/matK*. After purification of PCR products using ExoSAP-IT (USB Corp., Cleveland, OH), direct sequencing was performed using the BigDye Terminator Cycle Sequencing Kit ver. 3 (ABI PRISM; Applied Biosystems, Foster City, CA), with the PCR primers AB101 and AB102, and two additional primers ITS2 and ITS3 (White *et al.*, 1990) for the ITS; the PCR primers *atpB2F* and *rbcL2R* for *atpB-rbcL*; and the PCR primers *trnK-3914 (D)* and *psbA5'R* and additional primers of *matK-300F* (Yamashita and Tamura, 2000), *trnK2621R* (Liston and Kadereit, 1995), *matK-AF*, *matK-8R* (Ooi *et al.*, 1995), and *matK-AR* (Kazempour Osaloo *et al.*, 1999) for *matK/trnK*. Automated sequencing was performed on a 3130xl Genetic Analyzer (Applied Biosystems). The sets of sequence data were assembled using ATGC ver. 4.01 (GENETYX Co., Tokyo). Sequence data of ITS, *atpB-rbcL*, and *trnK/matK* were deposited in the DDBJ/EMBL/GenBank international DNA database under the accession numbers shown in Table 1.

### *Sequence similarity searches and taxon sampling in DNA database*

Sequence similarity searches in the ITS, *atpB-rbcL*, and *matK/trnK* regions of *N. yamashitae* were conducted with the program Basic Local Alignment Search Tool (BLAST) ver. 2.2.18 (Altschul *et al.*, 1997) in the DDBJ/EMBL/GenBank database (<http://blast.ddbj.nig.ac.jp/top-j.html>) on 23 February 2010. Following the BLAST results, we selected tribe members that showed high similarity to *N. yamashitae* in the examined three regions, and their sequence data deposited in the DNA database were added to the present phylogenetic analyses under the accession numbers shown in Table 2.

### *Phylogenetic analysis*

DNA sequences were aligned using the program ClustalX 1.8 (Thompson *et al.*, 1994) and then manually adjusted. Phylogenetic analyses were conducted based on a maximum parsimony (MP) criterion using PAUP\* version 4.0b10 (Swofford, 2002). First, to verify the phylogenetic position of *N. yamashitae* at the tribe level, MP phylogenetic analyses based on ITS and *atpB-rbcL* were independently conducted with members of tribes Anthospermeae including *Nertera* species, Ophiorrhizeae including *Ophiorrhiza*, and Urophyllaeae, which is thought to be a sister group of tribe Ophiorrhizeae (Rydin *et al.*, 2009a, 2009b). Second, to elucidate the phylogenetic position of *N. yamashitae* among phylogenetically closely related species, MP phylogenetic analysis was conducted based on combined data of ITS, *atpB-rbcL*, and *trnK/matK*. The combinability of the two cpDNA regions was assessed using the incongruence length difference (ILD) test (Farris *et al.*, 1994), with the partition homogeneity test implemented in PAUP\* version 4.0b10 (Swofford, 2002). Indels were treated as missing data. Characters were treated as unordered, and character transformations were weighted equally. The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony search was performed with 200 replicates of random additions

of sequences, with the ACCTRAN character optimization, tree bisection–reconnection (TBR) branch swapping, MULTREES, and STEEPEST DESCENT options on. Statistical support for each clade was assessed by bootstrap analysis (Felsenstein, 1985). One thousand replicates of heuristic searches, with the TBR branch swapping and MULTREES options off, were performed to calculate bootstrap values. Strict consensus trees of the output tree files from PAUP were generated by TREEVIEW (Page, 1996).

### *Floral anatomical and morphological observations in Nertera yamashitae*

Corolla morphology of *N. yamashitae* was observed based on living plants and herbarium specimens (GK 9854 and 11029 in TNS). Number of ovules was counted on a mature flower (GK 11029) fixed in FAA solution after cultivation in the Tsukuba Botanical Garden of National Museum of Nature and Science. The ovary was torn using a pair of tweezers, the number of ovules counted, and their position observed and photographed under a light microscope.

## Results

### *Sequence similarity searches of ITS, atpB-rbcL, and matK/trnK*

In two plants of *Nertera yamashitae* collected from two different river systems on Amami Island, no intraspecific sequence variation was detected in the three sequences of ITS, *atpB-rbcL*, and *trnK/matK*. The results of the sequence similarity searches using BLAST are summarized in Table 3. The BLAST analyses indicated that ITS (684 bp) of *N. yamashitae* had highest similarity to 18 accessions of six *Ophiorrhiza* species in Japan and Taiwan. An accession of *N. dichondrifolia* Hook. f. (DQ501279; Wright *et al.*, 2006) was 91<sup>st</sup> in the BLAST list. The *atpB-rbcL* (1149 bp) of *N. yamashitae* had the highest similarity to 17 accessions of six *Ophiorrhiza* species in Japan and Taiwan. An accession for *atpB-rbcL* of *Nertera* sp. (DQ131755; J. F. Manen, unpubl. data) was 78<sup>th</sup> in the BLAST list. The *trnK/matK*

Table 2. Tribal classification of cited species and their ITS, *atpB-rbcL* and *trnK/matK* sequences according to the DDBJ/EMBL/GenBank database

| Tribe <sup>a</sup> | Species   | Voucher                     | Accession no. <sup>b</sup> |                             |                             |
|--------------------|---|-----------------------------|----------------------------|-----------------------------|-----------------------------|
|                    |   |                             | ITS (type)                 | <i>atpB-rbcL</i> (type)     | <i>trnK/matK</i> (type)     |
| Anthospermeae      | <i>Anthospermum herbaceum</i> L.f.  | Bremer 3039(UPS)            | EU145355 <sup>1</sup>      | AJ234028 <sup>1</sup>       | —                           |
|                    | <i>Nertera granadensis</i> (Mutis ex L.f.) Druce                            | —                           | AF257927 <sup>4</sup>      | —                           | —                           |
| Ophiorrhizeae      | <i>Normandia neocaledonia</i> Hook.f.                                       | Munzinger 532(MO)           | AF257930 <sup>4</sup>      | —                           | —                           |
|                    | <i>Lerchea bracteata</i> Valetou  | Axellus 343(S)              | EU145374 <sup>1</sup>      | AJ233997 <sup>2</sup>       | —                           |
|                    | <i>Neurocalyx championii</i> Benth. ex Thwaites                             | Thor 601(S)                 | EU145376 <sup>1</sup>      | —                           | —                           |
|                    | <i>Neurocalyx zeylanicus</i> Hook.  | B & Bremer 937(S)           | EU145375 <sup>1</sup>      | AJ233995 <sup>2</sup>       | —                           |
|                    | <i>Ophiorrhiza elmeri</i> Merr.   | Kjeldsen & Poulsen 233(AAU) | EU145378 <sup>1</sup>      | —                           | —                           |
|                    | <i>Ophiorrhiza mungos</i> L.  | Bremer 2201(UPS)            | EU145377 <sup>1</sup>      | —                           | —                           |
|                    | <i>Ophiorrhiza amamiana</i> (Hatus.) Koh Nakam., Denda, Kameshima et Yokota | NK061455(RYU)               | AB269875 <sup>5</sup> (H)  | AB269877 <sup>5</sup> (j)   | AB270601 <sup>5</sup> (II)  |
|                    |   | NK T0126(RYU)               | AB269875 <sup>5</sup> (H)  | AB269877 <sup>5</sup> (j)   | AB270601 <sup>5</sup> (II)  |
|                    |   | NK061456(RYU)               | AB269875 <sup>5</sup> (H)  | AB269877 <sup>5</sup> (j)   | AB270601 <sup>5</sup> (II)  |
|                    |   | NK T0128(RYU)               | AB269875 <sup>5</sup> (H)  | AB269877 <sup>5</sup> (j)   | AB270601 <sup>5</sup> (II)  |
|                    |   | NK T0129(RYU)               | AB269875 <sup>5</sup> (H)  | AB269878 <sup>5</sup> (k)   | AB270601 <sup>5</sup> (II)  |
|                    |   | NK061458(RYU)               | AB269875 <sup>5</sup> (H)  | AB269878 <sup>5</sup> (k)   | AB270601 <sup>5</sup> (II)  |
|                    |   | NK T0127(RYU)               | AB269876 <sup>5</sup> (l)  | AB269877 <sup>5</sup> (j)   | AB270601 <sup>5</sup> (II)  |
|                    |   | NK061457(RYU)               | AB269876 <sup>5</sup> (l)  | AB269877 <sup>5</sup> (j)   | AB270601 <sup>5</sup> (II)  |
|                    |   | NK T01015(RYU)              | AB247257 <sup>5</sup> (j)  | AB247238 <sup>5</sup> (l)   | AB247245 <sup>5</sup> (III) |
|                    |   | NK T0104(RYU)               | AB269874 <sup>5</sup> (k)  | AB247238 <sup>5</sup> (l)   | AB247121 <sup>5</sup> (IV)  |
|                    |   | NK T0107(RYU)               | AB269874 <sup>5</sup> (k)  | AB247238 <sup>5</sup> (l)   | AB247245 <sup>5</sup> (III) |
|                    |   | NK T0111(RYU)               | AB269874 <sup>5</sup> (k)  | AB257115 <sup>5</sup> (m)   | AB257122 <sup>5</sup> (V)   |
|                    |   | NK061419(RYU)               | AB269874 <sup>5</sup> (k)  | AB257115 <sup>5</sup> (m)   | AB257122 <sup>5</sup> (V)   |
|                    | NK050501(RYU)   | AB269874 <sup>5</sup> (k)   | AB257115 <sup>5</sup> (m)  | AB257122 <sup>5</sup> (V)   |                             |
|                    | NK T0113(RYU)   | AB269874 <sup>5</sup> (k)   | AB257115 <sup>5</sup> (m)  | AB257122 <sup>5</sup> (V)   |                             |
|                    | NK050790(RYU)   | AB269874 <sup>5</sup> (k)   | AB257115 <sup>5</sup> (m)  | AB257122 <sup>5</sup> (V)   |                             |
|                    | NK T0118(RYU)   | AB269874 <sup>5</sup> (k)   | AB247239 <sup>5</sup> (n)  | AB247246 <sup>5</sup> (VI)  |                             |
|                    | NK T0120(RYU)   | AB269874 <sup>5</sup> (k)   | AB247239 <sup>5</sup> (n)  | AB247246 <sup>5</sup> (VI)  |                             |
|                    | NK T0119(RYU)   | AB269874 <sup>5</sup> (k)   | AB247239 <sup>5</sup> (n)  | AB247246 <sup>5</sup> (VI)  |                             |
|                    | NK T0121(RYU)   | AB247258 <sup>5</sup> (l)   | AB247239 <sup>5</sup> (n)  | AB247246 <sup>5</sup> (VI)  |                             |
|                    | NK T0122(RYU)   | AB247258 <sup>5</sup> (l)   | AB247239 <sup>5</sup> (n)  | AB247246 <sup>5</sup> (VI)  |                             |
|                    | NK T0123(RYU)   | AB269874 <sup>5</sup> (k)   | AB247240 <sup>5</sup> (o)  | AB247248 <sup>5</sup> (VII) |                             |
|                    | NK T0124(RYU)   | AB269874 <sup>5</sup> (k)   | AB247241 <sup>5</sup> (p)  | AB247246 <sup>5</sup> (VI)  |                             |
|                    | GK3989(TNS)   | AB247259 <sup>5</sup> (M)   | AB247238 <sup>5</sup> (l)  | AB247250 <sup>5</sup> (IX)  |                             |
|                    | NK G001(RYU)  | AB247260 <sup>5</sup> (N)   | AB247238 <sup>5</sup> (l)  | AB247249 <sup>5</sup> (X)   |                             |
|                    | NK C001(RYU)  | AB247336 <sup>5</sup> (O)   | AB247243 <sup>5</sup> (q)  | AB247252 <sup>5</sup> (XI)  |                             |
|                    | NK C002(RYU)  | AB247339 <sup>5</sup> (P)   | AB247243 <sup>5</sup> (q)  | AB247253 <sup>5</sup> (XII) |                             |
|                    | NK C004(RYU)  | AB247339 <sup>5</sup> (P)   | AB247243 <sup>5</sup> (q)  | AB247253 <sup>5</sup> (XII) |                             |
|                    | <i>Ophiorrhiza hayatana</i> Masam.  | —                           | —                          | —                           |                             |

Table 2. (Continued)

| Tribe <sup>a</sup> | Species   | Voucher               | Accession no. <sup>b</sup> |                           |                              |
|--------------------|---|-----------------------|----------------------------|---------------------------|------------------------------|
|                    |   |                       | ITS (type)                 | <i>atpB-rbcL</i> (type)   | <i>trnK/matK</i> (type)      |
| Ophiorrhizeae      | <i>Ophiorrhiza hayatana</i> Masam.                  | NK C003(RYU)          | AB247337 <sup>1</sup> (Q)  | AB247243 <sup>5</sup> (q) | AB247254 <sup>5</sup> (XIII) |
|                    |   | NK C005(RYU)          | AB247338 <sup>1</sup> (R)  | AB247243 <sup>5</sup> (q) | AB247255 <sup>5</sup> (XIV)  |
|                    | <i>Ophiorrhiza kuroiwae</i> Ohwi                    | NK T0141(RYU)         | AB247341 <sup>1</sup> (S)  | AB247244 <sup>5</sup> (r) | AB247256 <sup>5</sup> (XV)   |
|                    |   | NK T0142(RYU)         | AB247342 <sup>1</sup> (T)  | AB247244 <sup>5</sup> (r) | AB247256 <sup>5</sup> (XV)   |
|                    | <i>Ophiorrhiza michelloides</i> (Masam.) H.S. Lo    | NK W001(RYU)          | AB247340 <sup>1</sup> (U)  | AB247242 <sup>5</sup> (s) | AB247251 <sup>5</sup> (XVI)  |
|                    | <i>Ophiorrhiza pumila</i> Champ. ex. Benth.         | NK H001(RYU)          | AB247273 <sup>1</sup> (V)  | AB247147 <sup>5</sup> (t) | AB247150 <sup>5</sup> (XVII) |
|                    |   | NK H002(RYU)          | AB247273 <sup>1</sup> (V)  | AB247147 <sup>5</sup> (t) | AB247150 <sup>5</sup> (XVII) |
|                    |   | NK T0137(RYU)         | AB247274 <sup>1</sup> (W)  | AB247147 <sup>5</sup> (t) | AB247150 <sup>5</sup> (XVII) |
|                    |   | GK 3979(TNS)          | AB247272 <sup>1</sup> (X)  | AB247147 <sup>5</sup> (t) | AB247150 <sup>5</sup> (XVII) |
|                    |   | J.B.H. 55(S)          | EU145379 <sup>1</sup>      | —                         | —                            |
| Urophyllaeae       | <i>Spiradictis bifida</i> Kurz                      | Axelius 316(S)        | EU145381 <sup>1</sup>      | EU145567 <sup>1</sup>     | —                            |
|                    | <i>Xanthophyllum borneense</i> (Valeton) Axelius    | Ridsdale 2473(L)      | EU145380 <sup>1</sup>      | AJ233996 <sup>2</sup>     | —                            |
|                    | <i>Xanthophyllum capitellatum</i> Ridl.             | Clark & Watt 736(UPS) | EU145383 <sup>1</sup>      | EU145337 <sup>1</sup>     | —                            |
|                    | <i>Amphidasya ambigua</i> (Standl.) Standl.         | Ridsdale 2471(L)      | EU145384 <sup>1</sup>      | —                         | —                            |
|                    | <i>Maschatacorymbus corymbosus</i> (Blume) Bremek.  | Lantz 123(UPS)        | EU145385 <sup>1</sup>      | AJ233998 <sup>2</sup>     | —                            |
|                    | <i>Pauridiantha paucinervis</i> (Hiern) Bremek.     | Bremer 2090(UPS)      | EU145386 <sup>1</sup>      | EU145338 <sup>1</sup>     | —                            |
|                    | <i>Pauridiantha symplocoides</i> (S. Moore) Bremek. | —                     | EU145387 <sup>1</sup>      | —                         | —                            |
|                    | <i>Pravavinta suberosa</i> (Merr.) Bremek.          | Beaman 7950(S)        | EU145388 <sup>1</sup>      | AJ234001 <sup>2</sup>     | —                            |
|                    | <i>Pravinaria leucocarpa</i> Bremek. Beaman         | Boeca 7887(S)         | —                          | DQ131793 <sup>3</sup>     | —                            |
|                    | <i>Urophyllum arboreum</i> (Reinw. ex Blume) Korth. | Lundqvist 11085(UPS)  | EU145389 <sup>1</sup>      | AJ234002 <sup>2</sup>     | —                            |

<sup>a</sup> Taxonomic treatment follows Bremer and Manen (2000).

<sup>b</sup> Sequences in the DDBJ/EMBL/GenBank database; letters in parentheses indicate the types of three loci; references are as follows: <sup>1</sup> Rydin *et al.* (2009a, 2009b); <sup>2</sup> Bremer and Manen (2000); <sup>3</sup> Manen (GenBank unpublished data); <sup>4</sup> CL Anderson *et al.* (GenBank unpublished data); <sup>5</sup> Niakamura *et al.* (2006).

Table 3. The top five accessions with high similarity to the sequences of *Nertera yamashitae* using BLAST and their localities

| Species                         | Locality  | Locus <sup>a</sup>           | Accession no. <sup>b</sup> | Score (bits) | Identities      |
|---------------------------------|---|------------------------------|----------------------------|--------------|-----------------|
| <b>ITS</b>                      |   |                              |                            |              |                 |
| <i>Ophiorrhiza amamiana</i>     | JAPAN (Okinawa Is.)   | ITS(I)                       | AB269876                   | 1144         | 659/684 (96%)   |
| <i>Ophiorrhiza amamiana</i>     | JAPAN (Amami, Tokunoshima, & Okinawa Isls. in the Ryukyus)                                      | ITS(H)                       | AB269875                   | 1136         | 658/684 (96%)   |
| <i>Ophiorrhiza japonica</i>     | TAIWAN  | ITS(N)                       | AB247260                   | 1128         | 657/684 (96%)   |
| <i>Ophiorrhiza japonica</i>     | JAPAN (Shikoku, Kyushu & Amami, Tokunoshima, Okinawa, Ishigaki & Iriomote Isls. in the Ryukyus) | ITS(K)                       | AB269874                   | 1120         | 656/684 (95%)   |
| <i>Ophiorrhiza japonica</i>     | TAIWAN  | ITS(M)                       | AB247259                   | 1112         | 655/684 (95%)   |
| <b>atpB-rbcL</b>                |   |                              |                            |              |                 |
| <i>Ophiorrhiza japonica</i>     | JAPAN (Tokunoshima & Okinawa Isls.)   | <i>atpB-rbcL</i> (m)         | AB257115                   | 1132         | 597/608 (98%)   |
| <i>Ophiorrhiza hayatana</i>     | TAIWAN  | <i>atpB-rbcL</i> (q)         | AB247243                   | 1132         | 597/608 (98%)   |
| <i>Ophiorrhiza michelloides</i> | TAIWAN  | <i>atpB-rbcL</i> (s)         | AB247242                   | 1132         | 597/608 (98%)   |
| <i>Ophiorrhiza japonica</i>     | JAPAN (Iriomote Is.); Taiwan  | <i>atpB-rbcL</i> (p, l)      | AB247241, AB247238         | 1132         | 597/608 (98%)   |
| <i>Ophiorrhiza amamiana</i>     | JAPAN (Amami, Tokunoshima, & Okinawa Isls. in the Ryukyus)                                      | <i>atpB-rbcL</i> (j)         | AB269877                   | 1124         | 596/608 (98%)   |
| <b>matK/trnK</b>                |   |                              |                            |              |                 |
| <i>Ophiorrhiza michelloides</i> | TAIWAN  | <i>matK/trnK</i> (XVI)       | AB247251                   | 4220         | 2276/2335 (97%) |
| <i>Ophiorrhiza japonica</i>     | JAPAN (Ishigaki & Iriomote Isls. in the Ryukyus)  | <i>matK/trnK</i> (VI)        | AB247246                   | 4209         | 2274/2334 (97%) |
| <i>Ophiorrhiza hayatana</i>     | TAIWAN  | <i>matK/trnK</i> (XIII, XIV) | AB247254, AB247255         | 4205         | 2274/2335 (97%) |
| <i>Ophiorrhiza japonica</i>     | JAPAN (Iriomote Is. in the Ryukyus)   | <i>matK/trnK</i> (VII)       | AB247248                   | 4201         | 2273/2334 (97%) |
| <i>Ophiorrhiza hayatana</i>     | TAIWAN  | <i>matK/trnK</i> (XI)        | AB247252                   | 4197         | 2273/2335 (97%) |

<sup>a</sup> Letters in parentheses indicate locus type in Table 2.

<sup>b</sup> Sequences in the DDBJ/EMBL/GenBank database.

locus (2328 bp) of *N. yamashitae* had highest similarity to 19 accessions of six *Ophiorrhiza* species in Japan and Taiwan. No accession for *trnK/matK* of *Nertera* species was found in the DDBJ/EMBL/GenBank database.

#### Phylogenetic position of *Nertera yamashitae* at the tribe level

In the MP analyses at tribe level based on the

ITS sequence (Fig. 2), 233 of the 313 variable characters were parsimony-informative, and 305 equally most parsimonious trees of 726 steps were obtained, with a consistency index (CI)=0.65, a retention index (RI)=0.87, and a rescaled consistency index (RC)=0.56. In the MP analyses at tribe level based on the *atpB-rbcL* sequences (Fig. 3), 110 of the 228 variable characters were parsimony-informative, and 216 equal-

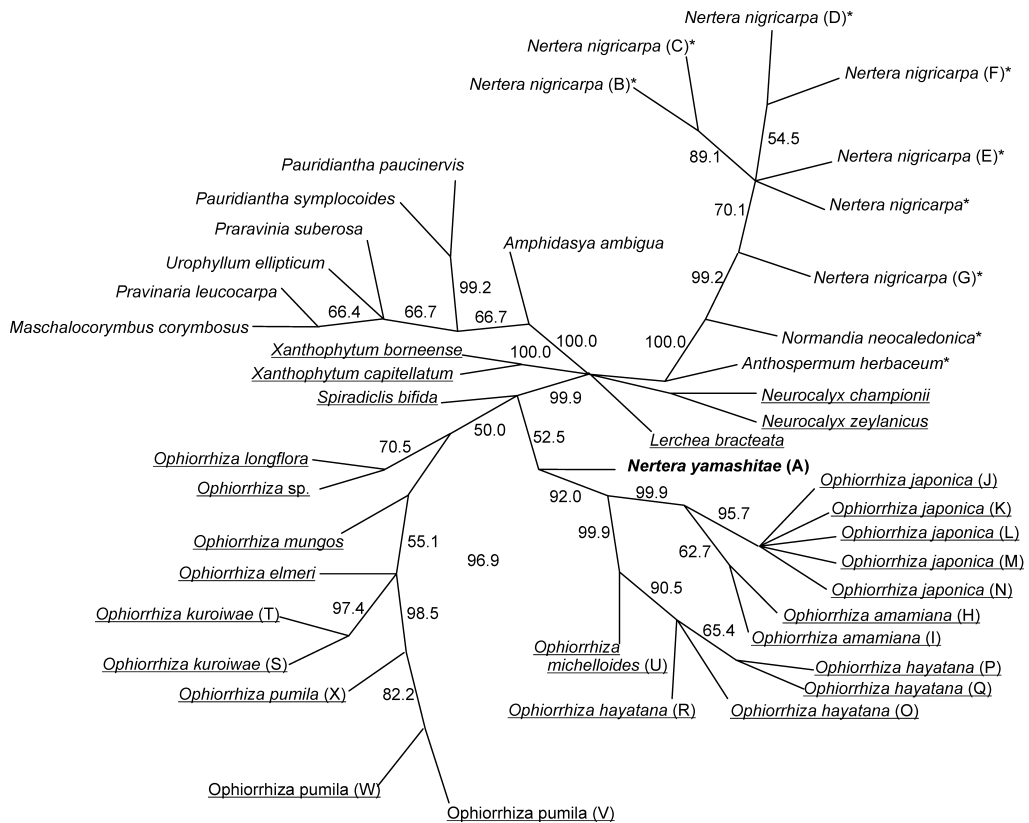


Fig. 2. Unrooted maximum parsimony, strict consensus tree to detect phylogenetic position of *Nertera yamashitae* in tribes Anthospermeae, Ophiorrhizeae, and Urophyllaeae based on the ITS locus. Species with underlines and asterisks are members of tribes Ophiorrhizeae and Anthospermeae, respectively (Bremer and Manen, 2000). Numerals above branches indicate bootstrap percentages ( $\geq 50\%$ ). Letters in parentheses indicate loci types in *N. yamashitae*, other *Nertera* species, and *Ophiorrhiza* species (Tables 1 and 2).

ly most parsimonious trees of 288 steps were obtained (CI=0.89, RI=0.95, and RC=0.85). Both of the unrooted strict consensus trees based on the ITS and *atpB-rbcL* loci indicated that *N. yamashitae* was not positioned in a clade with other *Nertera* species nor in a clade of Anthospermeae (Figs. 2 and 3). On the other hand, *N. yamashitae* was included in a clade with *Ophiorrhiza* species and *Spiradiclis bifida* (tribe Ophiorrhizeae) based on analysis of the ITS sequence (BP=99.9%; Fig. 2). Based on the *atpB-rbcL* sequence (Fig. 3), a clade consisting of *N. yamashitae* and *Ophiorrhiza* species had high bootstrap support (97.2%). In both of the unrooted strict consensus trees, *N. yamashitae* included in a subclade with four *Ophiorrhiza* species of *O. amamiana*

(Hatus.) Koh Nakam., Denda, Kameshima et Yokota, *O. japonica* Bl., *O. hayatana* Masam. and *O. michelloides* (Masam.) H.S. Lo.

#### Phylogenetic relationship of *Nertera yamashitae* to the other *Ophiorrhiza* species

To analyze the phylogenetic relationship within the subclade consisting of *N. yamashitae* and four *Ophiorrhiza* species (Figs. 2 and 3), the MP analysis was conducted based on the full set of ITS, *atpB-rbcL*, and *matK/trnK* loci with other four *Ophiorrhiza* species, namely *O. kuroiwae* Ohwi, *O. pumila* Champ. ex Benth., *O. longiflora* Blume and an unknown *Ophiorrhiza* species as an outgroup following the two independent analyses based on ITS (Fig. 2) and *atpB-rbcL*



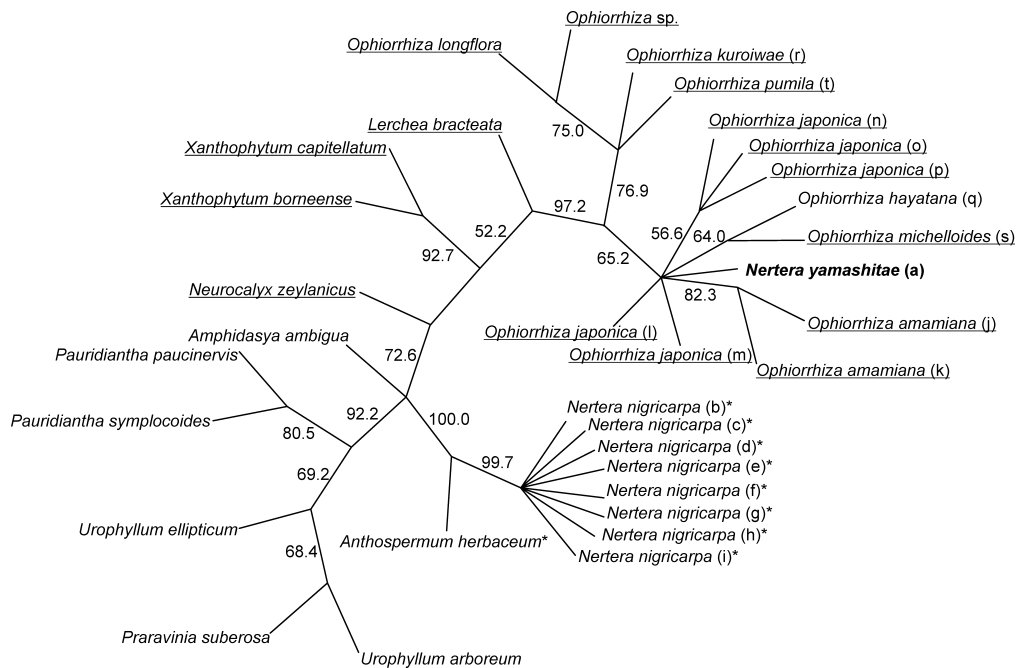


Fig. 3. Unrooted maximum parsimony, strict consensus tree to detect phylogenetic position of *Nertera yamashitae* in tribes Anthospermeae, Ophiorrhizeae, and Urophyllae based on the *atpB-rbcL* locus. Detail explanation given in Fig. 2.

(Fig. 3) loci. In the MP analysis, 124 of 188 variable characters were parsimony-informative, and six most parsimonious trees of 239 steps were obtained, with CI=0.86, RI=0.94, and RC=0.81 (Fig. 4).

The MP analysis (Fig. 4) indicated that *N. yamashitae* was connected to a basal position of a subclade consisting of *O. amamiana*, *O. japonica*, *O. hayatana*, and *O. michelloides* with high probability (BP=99.4%). Another dwarf leaf species of *O. michelloides* in Taiwan was not sister to *N. yamashitae*.

#### Floral anatomy and morphology of *Nertera yamashitae*

Flowers of *N. yamashitae* had a four-lobed and whitish corolla, a style with stigma bilobed at the top (Fig. 1) and four stamens (not shown). The ovary of *N. yamashitae* was inferior, bilocular, and had ca. 25 ovules in a locule, and the disk on the ovary was thick (Fig. 5).

## Discussion

### *Taxonomic reconsideration of Nertera yamashitae*

Following previous taxonomic studies, the genera *Nertera* and *Ophiorrhiza* commonly have a two-celled ovary, but the former has a single ovule in a locule, while the latter has many ovules in a locule (e.g. Verdcourt, 1958; Liu and Yang, 1998; Bremer and Manen, 2000). Furthermore, the former genus has an annular disk and a style with stigma bilobed at the base, or no disk (Puff, 1986), a four-lobed corolla, and four stamens; while the latter genus has a thick disk (Fukuoka, 1979), a style with stigma bilobed at the top, a five- or six-lobed corolla, and five or six stamens (Chen and Zhu, unpubl. data). Although *N. yamashitae* was similar to other *Nertera* species in the numbers of corolla lobes and stamens, it was similar to *Ophiorrhiza* species in the number of ovules, the morphologies of disk and stigma. Out of these anatomical and morpho-

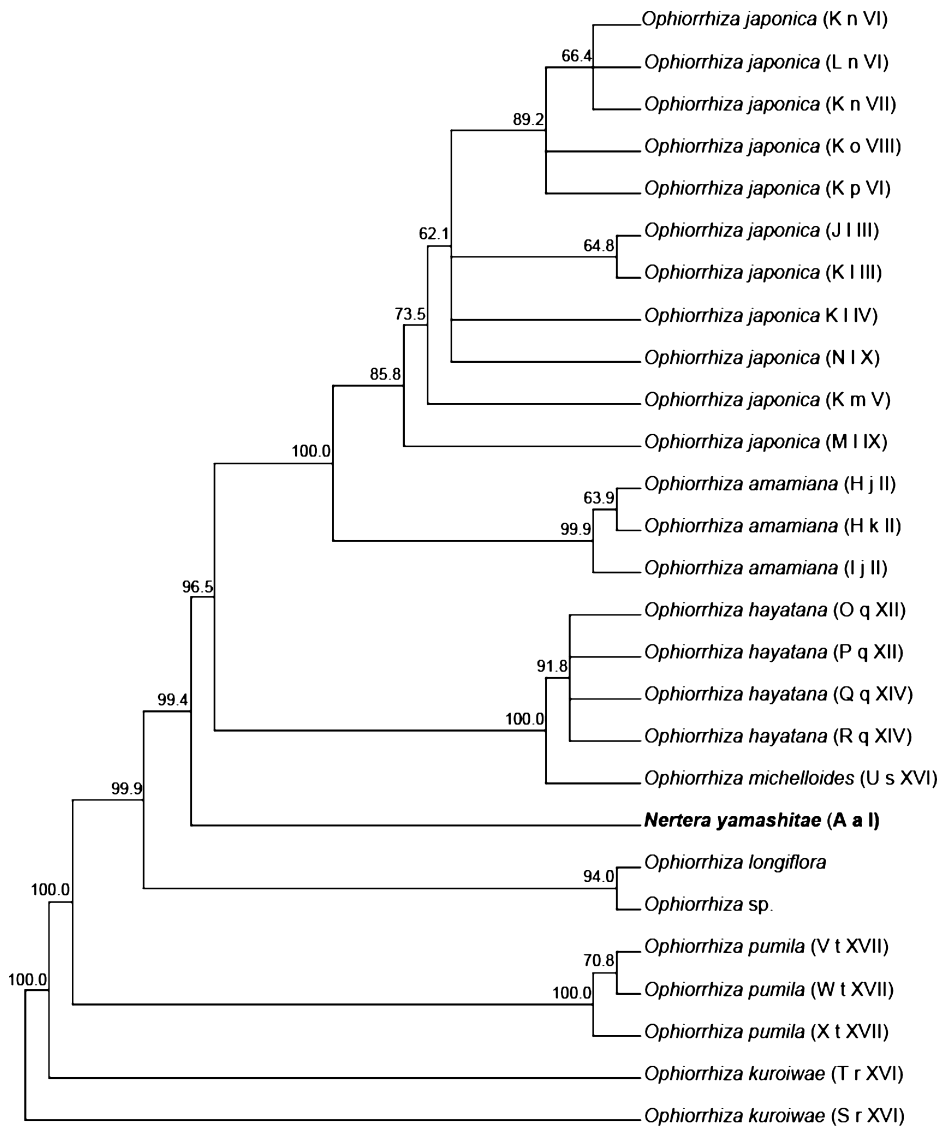


Fig. 4. Rooted maximum parsimony, strict consensus tree to detect phylogenetic position of *Nertera yamashitae* within the genus *Ophiorrhiza* based on the full dataset of ITS, *atpB-rbcL*, and *trnK/matK* loci. Numerals above branches indicate bootstrap percentages ( $\geq 50\%$ ). Letters in parentheses indicate the types of three loci in *N. yamashitae* and *Ophiorrhiza* species (Tables 1 and 2).

logical characters, the number of ovules in an ovary locule was thought to be an important character for delimitations at the tribes (Bremer and Manen, 2000) and generic level (e.g. Hooker, 1873; Bremekamp, 1966) in the family Rubiaceae. In terms of anatomy and morphology, *N. yamashitae* should not be treated as a species of the genus *Nertera*.

Previous phylogenetic studies at tribal or generic levels in the family Rubiaceae commonly verified that tribe Anthospermeae including *Nertera* spp., and tribe Ophiorrhizeae including *Ophiorrhiza* spp., branched at a basal position in a clade of subfamily Rubioideae and that they were obviously not sister groups (Bremer and Manen, 2000; Bremer, 2009; Rydin *et al.*, 2009a, 2009b).

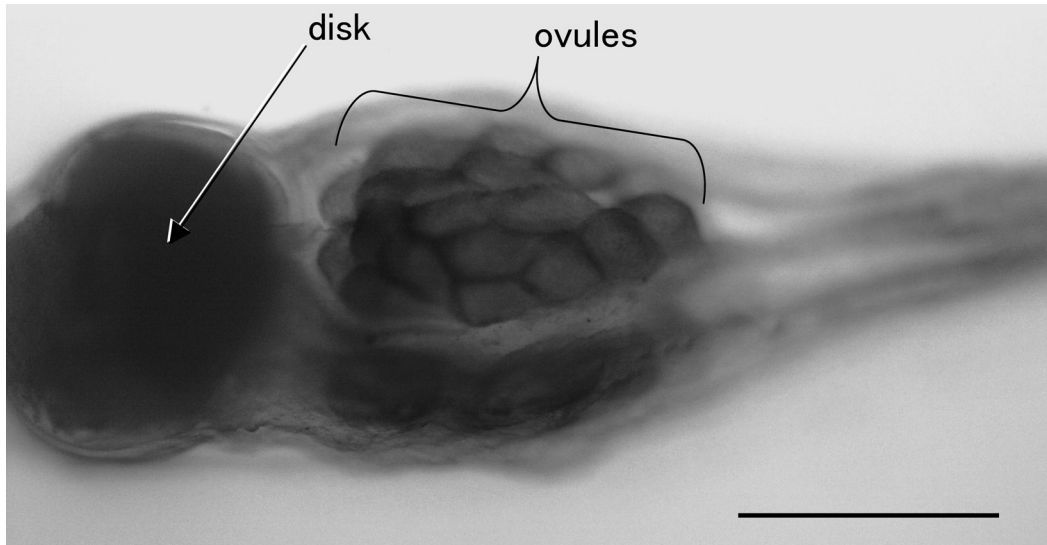


Fig. 5. Anatomical and morphological observations of the disk and ovules in *Nertera yamashitae*. Bar=500  $\mu\text{m}$ .

The present molecular analyses reveal that *N. yamashitae* is apparently included in the *Ophiorrhiza* clade in tribe Ophiorrhizeae but is distantly related to the *Nertera* clade in tribe Anthospermeae.

For taxonomy based on the anatomical, morphological and molecular results, we propose to treat *N. yamashitae* as a species of the genus *Ophiorrhiza*.

***Ophiorrhiza yamashitae*** (T. Yamaz.) Kokubugata, Koh Nakam. et Yokota, **comb. nov.** Basionym: *Nertera yamashitae* T. Yamazaki, J. Jpn. Bot. 73: 89–91, Figs. 1 and 2, 1998.

Fruit morphology is also a very valuable character to delineate the genera *Nertera* and *Ophiorrhiza*; the former has succulent (drupe) and the latter has dry (dehiscent) fruits (e.g. Liu and Yang 1998; Chen and Zhu, unpubl. data). However, no fruit of *O. yamashitae* has been observed in wild in spite of long-term efforts to find it (Yamazaki, 1998; Hotta, 2004), and thus this species is assumed to have lost ability to reproduce by means of intrapopulation crossing (Hotta, 2004). Artificial hand-pollination between plants from different populations in culti-

vation could be useful to investigate the fruit morphology of *O. yamashitae*.

#### *Phylogeography of Ophiorrhiza yamashitae*

In the present phylogenetic trees based on ITS, *atpB-rbcL*, and *trnK/matK* (Fig. 4) *O. yamashitae*, being an insular-endemic to Amami Island of the Ryukyus, was connected to a basal position of a clade consisting of four *Ophiorrhiza* species, namely *O. amamiana* being endemic to the central Ryukyus (Nakamura *et al.*, 2007); *O. japonica* widely distributing in China, Japan, Taiwan, and Vietnam (Yamazaki, 1993); and *O. hayatana* and *O. michelloides* being endemic to Taiwan (Liu and Yang, 1998; Nakamura *et al.*, 2006). Of four *Ophiorrhiza* species, *O. amamiana* and *O. japonica* presently occur on Amami Island (Hatusima and Amano, 1994). However, our phylogenetic analyses suggest that *O. yamashitae* is not derived from *O. amamiana* nor *O. japonica*. These results imply that the ancestral species of *O. yamashitae* has been extinct, and *O. yamashitae* could survived as a relict species lineage in Amami Island.

Previously, some species lineages assumed to be relicts are reported in the central part of the Ryukyu Archipelago, including Amami Group

and Okinawa Group for terrestrial plants (Hatusima, 1975; Hotta, 2004), amphibians and reptiles (Hikida *et al.*, 1989; Ota, 1998). Hikida and Ota (1997) suggested that these relict species lineages of amphibians and reptiles migrated to the central Ryukyus when the area was connected to surrounding landmasses by a landbridge via the northern and southern Ryukyus. After partial submergence of the landbridge during the Pliocene, the lineages were isolated in the central Ryukyus and have been extinct in other areas of this archipelago (Hikida and Ota, 1997). There is a possibility that *O. yamashitae* might have a similar phylogeographic background to the relict species lineages of the terrestrial amphibians and reptiles in the central part of the Ryukyu Archipelago.

*Parallel evolution and ecological aspects of dwarf leaf species*

In the *Ophiorrhiza* species in Japan and Taiwan, *O. michelloides* being endemic to Taiwan was regarded as a dwarf leaf species (Masamune, 1932; Liu and Yang, 1998; Nakamura *et al.*, 2006). Although *O. yamashitae* and *O. michelloides* commonly have dwarf leaves, they are distantly related in the present phylogenetic analyses. The results suggest that leaf dwarfism events have occurred independently in *O. yamashitae* and *O. michelloides*.

In habit environments, *O. yamashitae* is found along streams at low elevation on Amami Island (Yamazaki, 1998), while *O. michelloides* is found at altitudes above 1,000 m in the Central Mountain Range of Taiwan (Liu and Yang, 1998). However, they commonly occur on oligotrophic rocky slopes (data not shown). In evolutionally aspect of leaf dwarfism, Sugimoto (1957) suggested that oligotrophic environment is one of possible factors to lead to leaf dwarfism. We can hypothesize that leaf dwarfisms might be an adaptation to survive in oligotrophic environments on the rocky slopes as a common phenomenon between *O. yamashitae* and *O. michelloides*, but parallel events in Amami Island and Taiwan. Further studies including soil physico-

chemical investigations of these dwarf leaf species on Amami Island and in Taiwan are necessary to address these hypotheses.

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