Hybrid Origin of the Apogamous Fern *Dryopteris hondoensis* (Dryopteridaceae)

Kiyotaka Hori1*, Yasuyuki Watano2 and Noriaki Murakami1

1 Makino Herbarium, Tokyo Metropolitan University, 1-1 Minamiosawa, Hachioji, Tokyo 192-0397, Japan.
2 hori-kiyotaka@ed.tmu.ac.jp (author for correspondence); 3 Department of Biology, Graduate School of Science, Chiba University, 1-33 Yayoi-cho, Inage, Chiba 263-8522, Japan

We report that the triploid apogamous species *Dryopteris hondoensis* Koidz. shares its sequences with the *D. erythrosora* complex (*D. erythrosora, D. caudipinna* and *D. koidzumiana*) and *D. chinensis* at two nuclear markers (*PgiC* and *GapCp*). This is the first report of reticulate evolution between subg. *Erythrovariae* sect. *Erythrovariae*, to which *D. erythrosora, D. caudipinna* and *D. koidzumiana* and *D. hondoensis* belong, and sect. *Aemulae* of subg. *Dryopteris*, to which *D. chinensis* belongs. Apogamous species of *Dryopteris* may hybridize with distantly related species congeners.

Key words: Dryopteridaceae, *Dryopteris chinensis, Dryopteris caudipinna, GapCp, PgiC, rbcL*, reticulate evolution

Apogamy, or agamosporic reproduction in ferns, is a type of asexual reproduction in which unreduced spores are formed. The resultant gametophytes produce sporophytes of the next generation without fertilization (Manton 1950). Apogamous reproduction is common in ferns. Approximately 10% of all species of ferns (Lovis 1977) and 13% of the taxa of Japanese pteridophytes with known mode of reproduction are reported to exhibit apogamous reproduction (Takamiya 1996). In apogamous species, all offspring from a parent are expected to be clonal with a small amount of genetic variation. Interestingly, Darnaedi *et al.* (1990) reported that *Dryopteris yakusilvicola* Sa. Kurata, a triploid apogamous species of recent hybrid origin, showed no allozyme variation within the 56 individuals they examined. They considered *D. yakusilvicola* to have originated from a single hybrid event between the tetraploid sexual species *D. sparsa* and the diploid sexual species *D. sabaei*.

This phenomenon, however, is not common. Despite the clonal nature of apogamous reproduction, many apogamous species of ferns exhibit extensive morphological and genetic variation and often form species complexes in which morphological species are difficult to distinguish because of their continuous variation (Watano & Iwatsuki 1988, Suzuki & Iwatsuki 1990, Lin *et al.* 1995, Grusz *et al.* 2009, Hori *et al.* 2014). This is because apogamous species of ferns can hybridize with other sexual species to produce apogamously reproducible offspring (Lin *et al.* 1995, Ebihara *et al.* 2012, Lee & Park 2013, Hori *et al.* 2014).

Lee and Park (2013) recently attempted to assess reticulate evolution in subg. *Erythrovariae* sect. *Variae* sensu Fraser-Jenkins (1986; the *Dryopteris varia* complex) in Korea. They reported that *D. sacrosancta* (subg. *Erythrovariae*, sect. *Variae*) and *D. chinensis* (subg. *Dryopteris*, sect. *Aemulae* Fraser-Jenks.) shared the same chloroplast DNA (cpDNA) haplotype. Subsequently, Hori *et al.* (2014) reported triploid apogamous individuals of the *D. varia* complex to have three *PgiC* sequences, which can be considered homoeologs, on homoeologous chromosomes, but not on homologous chromosomes. They reported that *D.
chinesis and Dryopteris sacrosancta and D. kobayashii, which Serizawa (2009) separated from D. sacrosancta s.l., shared the same nuclear PgiC sequences. The data suggest that apogamous D. sacrosancta s.s. and D. kobayashii are hybrids between the D. varia complex and D. chinensis, which are distantly related based on the cpDNA tree (Ebihara 2011, Hori et al. 2014).

Furthermore, Hori et al. (2014) reported that Dryopteris pacifica (subg. Erythrovariae, sect. Variae) and the D. erythrosora complex (subg. Erythrovariae, sect. Erythrovariae) also shared the same PgiC sequences, suggesting that hybridization with members of the D. erythrosora complex contributed to the diversification of the apogamous species of the D. varia complex. Thus, these results indicate that phylogenetically wider taxon sampling is necessary to understand reticulate evolution in apogamous ferns.

Dryopteris hondoensis Koidz is a triploid apogamous species of sect. Erythrovariae, distributed in China, Korea and Japan (Honshu, Shikoku, Kyushu). It is distinguished from other members section Erythrovariae by its yellowish green, dull lamina and mostly plane scales on the rachis of the pinnae (Iwatsuki 1995). In this study, we determined D. hondoensis to be composed of nuclear DNA sequences from the D. erythrosora complex (D. erythrosora, D. caudipinna and D. koidzumiana) of sect. Erythrovariae and those from D. chinensis of sect. Aemulae. This is the first report of reticulate evolution between subg. Erythrovariae sect. Erythrovariae and subg. Dryopteris sect. Aemulae.

Materials and Methods

Plant Materials

Leaf samples were collected from six individuals of Dryopteris hondoensis and from possibly related sexual species in Dryopteris sections Aemulae, Erythrovariae and Variae, mainly from our field surveys in nine prefectures in Japan (Appendix). Their ploidy levels and reproductive modes are shown in Table 1. The reproductive mode of several individuals in the samples was estimated by counting the number of spores per sporangium (32, apogamous; 64, sexual) according to Manton (1950). All voucher specimens are deposited in MAK and/or TNS.

Ploidy analyses

Ploidy analyses followed the methods of Hori et al. (2014). To determine ploidy level, the DNA content (2C value) of each nucleus extracted from fresh pinnae was measured once per sample by flow cytometry using a Cyflow Ploidy Analyzer PA-II (Partec, Munster, Germany) and a Cystain UV Precise P kit (Partec). Approximately 100 mm² of each pinna was torn into several pieces and finely chopped with a razor blade in 0.25 mL of nucleus extraction buffer from the kit. Then, 0.8 mL of staining solution from the kit was added to the chopped tissues. The crushed tissue and buffers were filtered through a 30-μm nylon mesh (Partec) before analysis. Approximately 25 mm² of fresh leaf tissues of Nicotiana tabacum L. (2C value = 11.71 pg., Narayan 1987) was used as an internal standard.

Molecular analyses

For molecular analyses, a small amount of leaf tissue was dried in plastic bags, 20 cm x 10 cm, using silica gel. Subsequently, total DNA was extracted from the dried leaves using cetyltrimethylammonium bromide solution according to the method of Doyle & Doyle (1987).

The plastid gene rbcL was used in this study as the cpDNA marker. Polymerase chain reaction (PCR) amplification of rbcL was performed using the primers aF and cR of Hasebe et al. (1994). PCR entailed an initial denaturation step at 95°C for 10 min; followed by 35 cycles of denaturation, annealing, and elongation steps at 98°C for 10 s, 50°C for 10 s, and 72°C for 7 s, respectively; and a final extension step at 72°C for 7 min.

The nuclear gene PgiC, including exons 14–16 and introns 14–15, and GapCp (short) exons 9–10 were chosen as the nuclear DNA (nrDNA) markers. The PgiC fragment was amplified using the primers 14F and 16R of Ishikawa et al. (2002). The GapCp fragment was amplified using the
primer 132F (5′-GTGCTTCCGGAGTTAAATGG-3′) and 488R (5′-CAACATCATCTTCGGTGATCC-3′), which were newly developed in this study. PCR entailed an initial denaturation step at 95°C for 3 min; followed by 40 cycles of denaturation, annealing, and elongation at 98°C for 10 s, 58°C for 5 s, and 72°C for 5 s, respectively; and a final extension step at 72°C for 1 min. PCR amplification was performed using PrimeSTAR Max DNA Polymerase (Takara, Kyoto, Japan) on a Model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

The nucleotide sequences of \( rbcL \) were determined by direct sequencing. For sequencing \( rbcL \), aF, aR, cR (Hasebe et al. 1994), and D. paci-bf (Hori et al. 2014) primers were used.

PCR-single-strand conformation polymorphism (SSCP) analysis was performed to examine allelic variation at the nuclear marker level for each individual, following the method of Hori et al. (2014). Electrophoresis was performed using MDE gel solution (Lonza) with 2% glycerol at 18°C for 16 h at 350 V for \( PgiC \) and 2% glycerol at 15°C for 9.5 h at 300 V for \( GapCp \). To sequence the bands separated on the SSCP gels, pieces of the DNA bands in the dried gel were peeled off and incubated in 50 μL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) at 37°C overnight. The supernatant solution was used as a template for further PCR amplification. Before sequencing, the PCR products were purified using Illustra ExoStar 1-Step (GE Healthcare, WI, USA) and used as templates for direct sequencing. The reaction mixtures for sequencing were prepared using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). The reaction mixtures were analyzed using an ABI 3130 Genetic Analyzer (Applied Biosystems).

All plant samples were classified based on their PCR-SSCP banding patterns. The genomic constitution of each band pattern was identified by determining the nucleotide sequences of each DNA band separated on the SSCP gel.

**Phylogenetic analyses**

For phylogenetic analyses, only one sequence representing each haplotype for cpDNA (\( rbcL \)
and each allele for nrDNA (GapCp, PgiC) was used in our datasets. The cpDNA and nrDNA sequences were aligned using MUSCLE Citation (Edgar 2004) and analyzed separately by neighbor-joining (NJ), maximum parsimony (MP), or maximum likelihood (ML) analyses using MEGA version 6 (Tamura et al. 2013). The NJ tree was obtained using the p-distance method (Nei & Kumar 2000). The data are expressed as the number of base differences per site. All ambiguous positions were removed from each sequence pair. The MP tree was obtained using the subtree-pruning-regrafting algorithm (Nei & Kumar 2000) at search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates). In the ML analysis, the best-fitting model of sequence evolution for each DNA region was selected using MEGA version 6 (Tamura et al. 2013). The GapCp tree was constructed using the T92+G model, the PgiC tree was constructed using the HKY model, and the rbcL tree was constructed using the K2+G model. The initial trees for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. The indels were treated as missing characters in the MP and ML analyses. The bootstrap method with 1000 replications was employed to estimate the confidence levels of monophyletic groups. Dryopteris sabaei (sect. Nephrocystis, Fraser-Jenk.), D. fragrans (quoted from DDBJ) and two species of Polystichum (quoted from DDBJ) were used as outgroups.

Morphological observation

The morphology of Dryopteris hondoensis, D. chinensis, D. erythrosora, D. caudipinna and D. koidzumiana was compared, particularly focusing on differences in the color and sheen of the lamina, shape of the lowest basiscopic pin- nule, and shape of the scales.

Results

Ploidy analyses

The genome size of each species in this study was estimated as follows: Dryopteris koidzumiana (diploid sexual species; Hirabayashi 1969, Mitui 1967, 1968), 17.14 pg (N = 1); D. caudipinna (diploid sexual species; Hirabayashi 1970), 18.04 ± 0.19 pg (N = 12); D. erythrosora (triploid apogamous species; Hirabayashi 1966, 1967, 1974, Kurita 1961, Mitui 1968), 23.48 ± 0.94 pg (N = 5); D. hondoensis (triploid apogamous species; Hirabayashi 1966, 1967, Mitui 1967, 1968), 21.44 ± 0.53 pg (N = 4); and D. kinkiensis (tetraploid sexual species; Hirabayashi 1967), 28.07 ± 0.92 pg (N = 3). The genome sizes of D. erythrosora and D. hondoensis (only the triploid apogamous cytotype has been reported for this species in Japan, Table 1) were less than two-thirds of those of D. caudipinna and D. koidzumiana (diploid sexual species), but they were larger and smaller than those of D. kinkiensis (tetraploid sexual species). Those samples were therefore estimated to be triploid apogamous.

Sequence variation of rbcL and molecular phylogenetic trees based on variation

In Dryopteris hondoensis and possibly related species, 12 types of rbcL sequences were found (Fig. 1, Appendix). The GenBank accession numbers of the obtained sequences are shown in Appendix. Among the 1205 sites, 105 (9%) were variable and 42 (3%) were parsimoniously informative. NJ, MP, and ML analyses uncovered phylogenetic trees with similar topology. The ML tree (highest log likelihood = −2451.5660) with bootstrap percentages (BPs) of NJ/MP/ML analyses are shown in Fig. 1. Dryopteris hondoensis, D. erythrosora, D. caudipinna, and D. koidzumiana displayed rbcL sequences belonging to the same clade.

Sequence variation of PgiC and phylogenetic trees base on variation

In each of the samples, several different sequences of nuclear PgiC were detected by SSCP
Fig. 1. ML tree (highest log likelihood = −2451.5660) based on sequence variation of plastid gene *rbcL* with BPs (>70) of NJ/MP/ML analyses.
Fig. 2. ML tree (highest log likelihood = −2061.8095) based on sequence variation of nuclear gene \( PgiC \) with BPs (>70) of NJ/MP/ML analyses is shown.
analyses. The number of different sequences was always the presumed ploidy level of the sample or less. Twenty seven different sequences of $PgiC$ were identified in the samples of $Dryopteris$ hon\-doensis and in other species of $Dryopteris$ analyzed in the present study. The length of the sequences varied from 607 to 661 bp. The data matrix for phylogenetic analyses included 672 characters after editing, of which 153 (23%) were variable and 68 (10%) were parsimoniously informative. The ML tree (highest log likelihood $= -2061.8095$) with BPs of NJ/MP/ML analyses is shown in Fig. 2. Two monophyletic groups (A and B) were recognized, each of which was supported by a high BP. Group A contained the $PgiC$ sequences of $D. erythrosora$, $D. caudipinna$, and $D. koidzumiana$, whereas Group B contained sequences of $D. chinensis$. $Dryopteris$ hondoensis contained both group A and group B sequences. Some samples of $D. hondoensis$ had two sequences of group A and one sequence of group B; others had one sequence of group A and one sequence of group B (Table 2).

Sequence variation of $GapCp$ and the phylogenetic tree based on the variation

In most of the samples, several different sequences of nuclear $GapCp$ were detected by SSCP analyses. In total, 21 different sequences were identified in the samples of $Dryopteris$ hondoensis and some $Dryopteris$ species analyzed in the present study. The length of the sequences varied from 301 to 350 bp. The data matrix for phylogenetic analyses included 360 characters after editing, of which 87 (24%) were variable and 48 (13%) were parsimoniously informative. The ML tree (highest log likelihood $= -1166.5740$) with BPs of NJ/MP/ML analyses is shown in Fig. 3. Four monophyletic groups (A, B, C, and D) were recognized, each of which was supported by a high BP. Group A contained the $GapCp$ sequences of $D. chinensis$, and Groups B–D contained those of $D. erythrosora$, $D. caudipinna$, and $D. koidzumiana$. $Dryopteris$ hondoensis had three sequences, one each from groups A, B, and D (Table 2). $D_2$ and $D_3$ were distinguished by the positions of indels, but they are not distinguished on the molecular tree because indels were excluded in the phylogenetic analyses.

The estimated genotype of each sample is summarized in Appendix. It must be noted that our current method cannot distinguish differences in gene dosage in polyploids; therefore, $A_1A_1C_1$, $A_1C_1$, and $A_1C_1C_1$ were not distinguishable and were thus indicated as $A_1C_1*$.

Morphological observation

The morphology of $Dryopteris$ hondoensis, $D. chinensis$, and species in the $D. erythrosora$ complex ($D. erythrosora$, $D. caudipinna$ and $D. koidzumiana$) is shown in Figs. 4–6 and Table 3. The adaxial lamina surface of $D. hondoensis$ is yellowish green and dull, fresh green and dull in $D. chinensis$, and dark-green and shiny in the $D. erythrosora$ complex (Fig. 4). The most basal basiscopic pinnule of the lowest pinnae of $D. hondoensis$ is slightly more shortened than the second-most basiscopic pinnule. Those of the $D. erythrosora$ complex is clearly shortened than the second most basiscopic pinnule. Those of $D. chinensis$ is more elongated than the second basiscopic pinnule. (Fig. 5). Iwatsuki (1995) described the scales of $D. hondoensis$ as being mostly plane, with bullate scales intermixed on the rachis of the pinna rachis. However, this is incorrect. These two types of scales are not intermixed on the pinna rachis. The scales of $D. hondoensis$ are bullate distally on the rachis of the pinnae (Fig. 6c), but plane on the basal portion of the pinnae (Fig. 6g).

Discussion

$Dryopteris$ hondoensis has never been considered to be closely related to $D. chinensis$. Fraser-Jenkins (1986) included $D. chinensis$ in $Dryopteris$ subgen. $Dryopteris$ sect. Aemulae, whereas he included $D. caudipinna$, $D. erythrosora$, $D. koidzumiana$, and $D. hondoensis$ in $Dryopteris$ subgen. Erythrovariae sect. Erythrovariae. Thus, he (Fraser-Jenkins, 1986) did not discuss the similarity in morphological characteristics between these species.

Our $PgiC$ and $GapCp$ analyses, however, sug-
Fig. 3. ML tree (highest log likelihood = −821.3017) based on sequence variation of nuclear gene GapCp with BPs (>70) of NJ/MP/ML analyses is shown.

D. sordidipes

A1(D. chinensis 1–6)

A2(D. hondoensis 1,3,5,6)

A3(D. hondoensis 2,4, D. chinensis 7–10)

D. saxifraga

D. varia

D. protobissetiana

D. sabaei

Dryopteris fragrans (JQ936905.1)

D. gymnophylla

D. kinkiensis

B1(D. caudipinna 1,4,5,10,11,13,14,16, D. koidzumiana 1,3,5, D. erythrosora 3, D. hondoensis 2,4)

B2(D. caudipinna 2,3,5–9,12,15, D. koidzumiana 4 D. erythrosora 1,2,4,5, D. hondoensis 1,3,5,6)

D. kinkiensis

C1(D. koidzumiana 2–4)

D1(D. erythrosora 2,4,5)

D2(D. caudipinna 1–3, D. erythrosora 1,3–5, D. hondoensis 1–6)

D3(D. erythrosora 2)

Polystichum munitum (JQ936887.1)
TABLE 2. Ploidy level, reproductive mode and constitution of nuclear makers (PgiC, GapCp).

<table>
<thead>
<tr>
<th>Species</th>
<th>PgiC type</th>
<th>GapCp type</th>
<th>Ploidy level, reproductive mode</th>
<th>number of samples</th>
<th>locality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. caudipinna</em> 1</td>
<td>A₁A₂</td>
<td>B₁D₂</td>
<td>2x / sexual</td>
<td>1</td>
<td>Hyogo pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 2,3</td>
<td>A₁A₂</td>
<td>B₂D₂</td>
<td>2x / sexual</td>
<td>2</td>
<td>Hyogo pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 4</td>
<td>A₁A₂</td>
<td>B₁B₁</td>
<td>2x / sexual</td>
<td>1</td>
<td>Kanagawa pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 5</td>
<td>A₁A₁</td>
<td>B₁B₂</td>
<td>2x / sexual</td>
<td>3</td>
<td>Kanagawa pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 6,7,12</td>
<td>A₁A₆</td>
<td>B₂B₃</td>
<td>2x / sexual</td>
<td>3</td>
<td>Kanagawa pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 9</td>
<td>A₁A₆</td>
<td>B₂D₂</td>
<td>2x / sexual</td>
<td>1</td>
<td>Kanagawa pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 10</td>
<td>A₁A₆</td>
<td>B₁B₁</td>
<td>2x / sexual</td>
<td>1</td>
<td>Kanagawa pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 11</td>
<td>A₁A₆</td>
<td>B₁B₁</td>
<td>2x / sexual</td>
<td>1</td>
<td>Kanagawa pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 13</td>
<td>A₁A₆</td>
<td>B₁B₁</td>
<td>2x / sexual</td>
<td>1</td>
<td>Kanagawa pref.</td>
</tr>
<tr>
<td><em>D. caudipinna</em> 14,16</td>
<td>A₁A₁</td>
<td>B₁B₁</td>
<td>2x / sexual</td>
<td>2</td>
<td>Kanagawa pref.</td>
</tr>
<tr>
<td><em>D. koidzumiana</em> 1</td>
<td>A₁A₈</td>
<td>B₁D₂</td>
<td>sexual</td>
<td>1</td>
<td>Kagoshima pref., Yakushima</td>
</tr>
<tr>
<td><em>D. koidzumiana</em> 2</td>
<td>A₁A₈</td>
<td>C₁C₁</td>
<td>2x / sexual</td>
<td>1</td>
<td>Kagoshima pref., Yakushima</td>
</tr>
<tr>
<td><em>D. koidzumiana</em> 3</td>
<td>A₁A₉</td>
<td>B₁C₁</td>
<td>sexual</td>
<td>1</td>
<td>Kagoshima pref., Yakushima</td>
</tr>
<tr>
<td><em>D. koidzumiana</em> 4</td>
<td>A₁A₉</td>
<td>B₂C₁</td>
<td>sexual</td>
<td>1</td>
<td>Kagoshima pref., Yakushima</td>
</tr>
<tr>
<td><em>D. koidzumiana</em> 5</td>
<td>A₁A₉</td>
<td>B₂D₁</td>
<td>sexual</td>
<td>1</td>
<td>Kagoshima pref., Yakushima</td>
</tr>
<tr>
<td><em>D. erythrosora</em> 1</td>
<td>A₁A₅</td>
<td>B₂D₂</td>
<td>3x</td>
<td>1</td>
<td>Miyazaki pref.</td>
</tr>
<tr>
<td><em>D. erythrosora</em> 2</td>
<td>A₁A₅</td>
<td>B₂D₂D₃</td>
<td>3x</td>
<td>1</td>
<td>Wakayama pref.</td>
</tr>
<tr>
<td><em>D. erythrosora</em> 3</td>
<td>A₁A₅</td>
<td>B₂D₂</td>
<td>3x</td>
<td>1</td>
<td>Wakayama pref.</td>
</tr>
<tr>
<td><em>D. erythrosora</em> 4</td>
<td>A₁A₅</td>
<td>B₂D₂</td>
<td>3x</td>
<td>1</td>
<td>Wakayama pref.</td>
</tr>
<tr>
<td><em>D. erythrosora</em> 5</td>
<td>A₁A₅</td>
<td>B₂D₂</td>
<td>3x / apogamous</td>
<td>1</td>
<td>Tokyo-to.</td>
</tr>
<tr>
<td><em>D. hondoensis</em> 1,5,6</td>
<td>A₁A₁B₁</td>
<td>A₁B₁D₂</td>
<td>3x / apogamous</td>
<td>3</td>
<td>Tokyo-to., Wakayama pref.</td>
</tr>
<tr>
<td><em>D. hondoensis</em> 2,4</td>
<td>A₁A₁B₁</td>
<td>A₁B₁D₂</td>
<td>3x</td>
<td>2</td>
<td>Tokyo-to.</td>
</tr>
<tr>
<td><em>D. hondoensis</em> 3</td>
<td>A₁B₁</td>
<td>A₁B₁D₂</td>
<td>3x</td>
<td>1</td>
<td>Miyazaki pref.</td>
</tr>
<tr>
<td><em>D. chinensis</em> 7</td>
<td>B₂B₂</td>
<td>A₁A₁</td>
<td>2x / sexual (Hori et al. 2015)</td>
<td>1</td>
<td>Miyazaki pref.</td>
</tr>
<tr>
<td><em>D. chinensis</em> 4-6</td>
<td>B₂B₂</td>
<td>A₁A₁</td>
<td>2x / sexual (Hori et al. 2015)</td>
<td>3</td>
<td>Miyazaki pref.</td>
</tr>
<tr>
<td><em>D. chinensis</em> 8-10</td>
<td>B₂B₂</td>
<td>A₁A₁</td>
<td>2x / sexual (Hori et al. 2015)</td>
<td>3</td>
<td>Miyazaki pref.</td>
</tr>
<tr>
<td><em>D. chinensis</em> 1-3</td>
<td>B₂B₂</td>
<td>A₁</td>
<td>apogamous</td>
<td>2</td>
<td>Tokyo-to., Wakayama pref.</td>
</tr>
</tbody>
</table>

TABLE 3. Morphological differences between *Dryopteris hondoensis* and its related species.

<table>
<thead>
<tr>
<th></th>
<th><em>D. hondoensis</em></th>
<th><em>D. chinensis</em></th>
<th><em>D. caudipinna</em></th>
<th><em>D. koidzumiana</em></th>
<th><em>D. erythrosora</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Color of lamina</td>
<td>yellowish green, dull</td>
<td>fresh green, dull</td>
<td>dark green, dull</td>
<td>dark green, shiny</td>
<td>dark green, shiny</td>
</tr>
<tr>
<td>The most basal basiscopic pinnule of the lowest pinna</td>
<td>elongate a little than the second one</td>
<td>elongate than the second one</td>
<td>shorten than the second one</td>
<td>shorten than the second one</td>
<td>shorten than the second one</td>
</tr>
<tr>
<td>Scales on distal positions of pinna rachis</td>
<td>bullate</td>
<td>flat</td>
<td>bullate</td>
<td>bullate</td>
<td>bullate</td>
</tr>
<tr>
<td>Scales on basal positions of pinna rachis</td>
<td>flat</td>
<td>flat</td>
<td>bullate</td>
<td>bullate</td>
<td>bullate</td>
</tr>
</tbody>
</table>
Fig. 4. Color of pinnule of the second most basal pinna. (a: D. caudipinna, b: D. erythrosora, c: D. hondoensis, d: D. chinensis; scale = 1 cm)

Fig. 5. Most basal basiscopic pinnule of lowest pinnae. (1: D. caudipinna, 2: D. erythrosora, 3: D. hondoensis, 4: D. chinensis; scale = 1 cm).
suggested that *Dryopteris hondoensis* is of hybrid origin between *Dryopteris caudipinna*, *D. koidzumiana*, *D. erythrosora* and *D. chinensis* because it contains DNA sequences matching the clades of those species in the molecular phylogenetic trees (Figs. 2 & 3). Furthermore, *D. hondoensis* possibly originated at least three times because *D. hondoensis* comprises three clones.

The results of the maternally inherited *rbcL* suggest that the maternal parent of *D. hondoensis* was *D. erythrosora* (triploid apogamous), *D. caudipinna* and *D. koidzumiana* (diploid sexual species). Hori et al. (2014) suggested that diploid apogamous offspring originating from triploid apogamous individuals through unequal meiosis can be both maternal and paternal species in the reticulate evolution of the *D. varia* complex. Therefore, we have two hypotheses about the origin of *D. hondoensis*. First, triploid apogamous *D. chinensis* (paternal) may result in unequal meiosis and then cross with diploid sexual *D. caudipinna* or *D. koidzumiana* (maternal). In this case, there may have been backcrossing to *D. caudipinna*, *D. koidzumiana* and *D. erythrosora*, because *D. hondoensis* shows two sequences belonging to the *D. erythrosora* complex clades in the trees. Second, triploid apogamous *D. erythrosora* (maternal) may have unequal meiosis and then cross with diploid sexual *D. chinensis* (paternal). Additional nuclear markers are needed to determine the more plausible hypothesis.

*Dryopteris hondoensis* and *D. chinensis* display clear ecological and morphological differentiation; specifically, *D. hondoensis* is evergreen and has ovate to broadly ovate–subdeltoid lamina, whereas *D. chinensis* is summer green and has pentagonal lamina. They have similar morphological characteristics, however, that coincide with the results from our DNA analyses that suggest a hybrid origin for *D. hondoensis*. *Dryopteris chinensis* has fresh green lamina and lanceolate scales on the stipes while *D. hondoensis* has yellowish green lamina and lanceolate scales on the stipes and basal portion of the rachis of the pinnae. Iwatsuki (1995) did not postulate a hybrid origin for *D. hondoensis* between the *D. erythrosora* complex and *D. chinensis*, even though he listed several natural hybrids as being in Japan.

In our study, we suggest the importance of phylogenetically wider sampling, including distantly related species belonging to other sections, to clarify the origin of apogamous species of ferns and to resolve the reticulate relationships in *Dryopteris*.

We are grateful to the following persons for their assistance in collecting plant materials: Dr. J. Yamashita (Okayama University); S. Kariyama (Kurashiki Museum of Natural History); and Mr. K. Akagi, Y. Inoue, T. Minamitani, K. Mizote, and K. Ohora (Nippon Fernist Club). This study was partly supported by a Grant-in-Aid for JSPS Fellows No. 26-1720 to K. H. and by a Grant-in-Aid for Scientific Research No. 25291089 to N. M.

**References**


Received November 11, 2015; accepted March 9, 2016
<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>DNA amount</th>
<th>Platyxylem</th>
<th>Repeatability score</th>
<th>rbcL type</th>
<th>Pgi type</th>
<th>GapCp type</th>
<th>Heterotaxia no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. hondoensis</td>
<td>Japan, Tokyo-to, Inagi-shi</td>
<td>LC094030</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
<tr>
<td>D. varia</td>
<td>Japan, Akita pref., Senboku-shi</td>
<td>LC094042</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
<tr>
<td>D. saxifraga</td>
<td>Japan, Tokyo-to, Inagi-shi</td>
<td>LC094042</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
<tr>
<td>D. protobissetiana</td>
<td>Japan, Tokyo-to, Inagi-shi</td>
<td>LC094042</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
<tr>
<td>D. sordidipes</td>
<td>Japan, Tokyo-to, Inagi-shi</td>
<td>LC094042</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
<tr>
<td>D. kinkiensis</td>
<td>Japan, Tokyo-to, Inagi-shi</td>
<td>LC094042</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
<tr>
<td>D. chinensis</td>
<td>Japan, Tokyo-to, Inagi-shi</td>
<td>LC094042</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
<tr>
<td>D. koidzumiana</td>
<td>Japan, Tokyo-to, Inagi-shi</td>
<td>LC094042</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
<tr>
<td>D. caudipinna</td>
<td>Japan, Tokyo-to, Inagi-shi</td>
<td>LC094042</td>
<td>2015a</td>
<td>***Quoted from Hori et al. (2015a).</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
<td>2015a</td>
</tr>
</tbody>
</table>

**APPENDIX.** Voucher specimens examined in this study. Genotypes (GapCp, PgiC) identified by sequencing are in bold, otherwise genotypes were deduced from comparison of band positions in SSCP gels. When ploidy is unknown, the genotype is in brackets. For samples with unknown genome dosage, unidentified genomes are in italic. *Quoted from Hori et al. (2014). **Quoted from Hori et al. (2015a). ***Quoted from Hori et al. (2015b).