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Arbuscular mycorrhizal colonization in field-collected terrestrial cordate gametophytes of pre-polypod leptosporangiate ferns (Osmundaceae, Gleicheniaceae, Plagiogyriaceae, Cyatheaceae)

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Abstract To determine the mycorrhizal status of pteridophyte gametophytes in diverse taxa, the mycorrhizal colonization of wild gametophytes was investigated in terrestrial cordate gametophytes of pre-polypod leptosporangiate ferns, i.e., one species of Osmundaceae (*Osmunda banksiifolia*), two species of Gleicheniaceae (*Diplopterygium glaucum*, *Dicranopteris linearis*), and four species of Cyatheales including tree ferns (Plagiogyriaceae: *Plagiogyria japonica*, *Plagiogyria euphlebia*; Cyatheaceae: *Cyathea podophylla*, *Cyathea lepifera*). Microscopic observations revealed that 58 to 97 % of gametophytes in all species were colonized with arbuscular mycorrhizal (AM) fungi. Fungal colonization was limited to the multilayered midrib (cushion) tissue in all gametophytes examined. Molecular identification using fungal SSU rDNA sequences indicated that the AM fungi in gametophytes primarily belonged to the Glomeraceae, but also included the Claroideoglomeraceae, Gigasporaceae, Acaulosporaceae, and Archaeosporales. This study provides the first evidence for AM fungal colonization of wild gametophytes in the Plagiogyriaceae and Cyatheaceae. Taxonomically divergent photosynthetic gametophytes are similarly colonized by AM fungi, suggesting that mycorrhizal associations with AM fungi could widely occur in terrestrial pteridophyte gametophytes.

Keywords Arbuscular mycorrhizal fungi · Cyatheales · Gametophyte · Gleicheniales · Osmundales · Pteridophyte

Introduction

Although recent studies have shown that most land plant groups, from bryophytes to angiosperms, are mycorrhizal (Wang and Qiu 2006), the status of mycorrhizal symbioses remains unknown in many plant species, especially in "lower" land plants (Read et al. 2000). For example, the mycorrhizal status of the gametophytes of pteridophytes (ferns and lycophytes) is largely unknown, as the simple structure of the tiny gametophytes makes it difficult to identify plant species using morphological features. In contrast, the mycorrhizal associations of pteridophyte sporophytes have been surveyed in many lineages worldwide (Cooper 1976; Gemma et al. 1992; Zhi-wei 2000; Lehnert et al. 2009; Kessler et al. 2010; Muthukumar and Prabha 2013; Muthuraja et al. 2014; Lara-Pérez et al. 2015). Since mycorrhizal associations influence plant nutrient uptake (Marschner and Dell 1994), plant responses to stress (Finlay et al. 2008), and the structure and composition of plant communities (van der Heijden et al. 2008), characterizing the mycorrhizal associations of pteridophytes is essential for a more thorough understanding of their ecology and evolution.

To date, the mycorrhizal fungi of pteridophyte gametophytes have been identified as being primarily arbuscular mycorrhizal (AM) fungi (Read et al. 2000), with a few

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exceptions, such as *Diphasiastrum alpinum* (Lycopodiaceae) associated with basidiomycetes (Horn et al. 2013). Gametophytes grow in divergent habitats ranging from underground, terrestrial on the ground, epilithic on rocks, and epiphytic on tree trunks (Farrar et al. 2008). The underground gametophytes of the Lycopodiaceae, Psilotaceae, and Ophioglossaceae are achlorophyllous and depend on mycorrhizal fungi for their carbon supply (Bierhorst 1971). The associations of such mycoheterotrophic gametophytes with AM fungi have been surveyed since the early twentieth century (Campbell 1908a; Boullard 1979; Schmid and Oberwinkler 1993; Winther and Friedman 2007, 2008, 2009). In contrast, the terrestrial and chlorophyllous gametophytes of ferns, which are generally cordate, have been considered devoid of mycorrhiza, with a few exceptions in the Marattiales, Osmundales, Gleicheniales (Campbell 1908a; Boullard 1979; Schmid and Oberwinkler 1995), and Polypodiales (Turnau et al. 2005, 2013). In a recent study, we found that more than 90 % of field-collected gametophytes were colonized by AM fungi in *Angiopteris lygodiifolia* (Marattiaceae) and *Osmunda japonica* (Osmundaceae) (Ogura-Tsujita et al. 2013). However, mycorrhizal colonization of gametophytes has only been documented in a few families, and the status of mycorrhizal associations remains largely unknown in pteridophyte gametophytes, especially in aboveground photosynthetic gametophytes.

The main objective of this study was to survey the mycorrhizal status of field-collected gametophytes of entire groups of leptosporangiate ferns. Phylogenetically, the leptosporangiate ferns comprise seven clades: osmundaceous, filmy, gleichenioid, schizaeoid, heterosporous, and tree- and polypod-ferns (sensu Pryer et al. 2004). The mycorrhizal status of the gametophytes of polypod ferns, the most derived and largest group of leptosporangiate ferns (Pryer et al. 2004), will be reported elsewhere since preliminary studies suggested that they exhibit a wide variation in fungal colonization levels. Among the "pre-polypod leptosporangiate ferns" (i.e. the leptostorangiate ferns excluding the polypod), filmy ferns and heterosporous ferns were not examined in this study because their gametophytes are non-cordate. Among the schizaeoid ferns, the Lygodiaceae have typical cordate gametophytes, but natural samples of lygodiaceous gametophytes unfortunately were not obtained at this time. For osmundaceous and gleichenioid ferns, the mycorrhizal status of *O. japonica* has been addressed elsewhere (Ogura-Tsujita et al. 2013) but other osmundaceous and gleichenioid ferns have not been analyzed using molecular identification techniques, although anatomical observations have been conducted in a few species (Campbell 1908a; Boullard 1979; Schmid and Oberwinkler 1995). Thus, in the present study, the mycorrhizal colonization status of the wild gametophytes of one species of osmundaceous fern (*Osmunda banksiifolia* (C. Presl) Kuhn), two species

of gleichenioid ferns (*Diplopterygium glaucum* (Houtt.) Nakai and *Dicranopteris linearis* (Burm. f.) Underw.), and four species of the tree-fern clade (*Plagiogyria japonica* Nakai and *Plagiogyria euphlebia* (Kunze) Mett. of the Plagiogyriaceae and *Cyathea podophylla* (Hook.) Copel. and *Cyathea lepifera* (J. Sm. ex Hook.) Copel. of the Cyatheaceae) were assessed using anatomical observations, and fungal lineages were identified by molecular methods.

Cordate gametophytes are composed of a multilayered midrib (cushion) and one-cell-thick wing portions (Bower 1923; Raghavan 1989). In a previous study, we documented that mycorrhizal colonization was limited to the cushion in both *A. lygodiifolia* and *O. japonica* gametophytes (Ogura-Tsujita et al. 2013), suggesting that the gametophyte cushion structure is key to associations with AM fungi. Therefore, anatomical observations to examine the site(s) of fungal colonization and cushion thickness in the gametophytes were also conducted.

Materials and methods

Sample collection

A recent survey of the natural distribution of fern gametophytes and sporophytes at a field site in Japan indicated that cordate gametophytes were without exception accompanied by their conspecific sporophytes at nearby sites, whereas the sporophytic counterparts of non-cordate (strap- or ribbon-like) gametophytes were often absent or rare near the sites (Ebihara et al. 2013). Therefore, field expeditions were conducted at locations where a large number of sporophytes of the seven studied fern species were growing. Wild gametophytes were collected from sites around which conspecific sporophytes were growing between May 2008 and November 2012 (Table 1). These study sites were located in subtropical evergreen forests (for Osmundaceae, Gleicheniaceae, and Cyatheaceae) or temperate deciduous forests (for Plagiogyriaceae). Most gametophytes were growing on the soil of slopes along trails.

All collected gametophytes were washed in water using a soft fine brush under a stereomicroscope, and images were taken using a digital camera (DP70, Olympus) attached to a light microscope (Olympus BX51). Immature gametophytes lacking archegonia were not used for examinations. Mature gametophytes were cut sagittally into two (right and left) pieces in the middle of the cushion using a razor blade. One piece was used for microscopic observations to confirm fungal colonization, and the other piece was used for DNA analysis to identify the species of both plant and AM fungi (Fig. S1). In addition, one gametophyte each of *C. podophylla*, *P. euphlebia*, and *Dip. glaucum* and two gametophytes of

Table 1 Samples of fern gametophytes used in this study

Taxa			Site	No. of population	No. of gametophytes
Osmundales					
	Osmundaceae	*Osmunda banksiifolia*	Marley River, Iriomote Island, Okinawa Pref.	1	3
			Urauchi River, Iriomote Island, Okinawa Pref.	3	17
			Yutsun River, Iriomote Island, Okinawa Pref.	3	8
			Kagoshima-shi, Kagoshima Pref.	2	4
Gleicheniales					
	Gleicheniaceae	*Diplopterygium glaucum*	Nago-shi, Okinawa Pref.	1	1
			Kumano-shi, Mie Pref.	1	2
			Nishiizu-cho, Shizuoka Pref.	4	8
			Shizuoka-shi, Shizuoka Pref.	1	1
		Dicranopteris linearis	Nago-shi, Okinawa Pref.	1	6
			Urauchi River, Iriomote Island, Okinawa Pref.	1	1
			Wakimoto, Aira-shi, Kagoshima Pref.	1	1
			Nishiizu-cho, Shizuoka Pref.	2	2
Cyatheales					
	Plagiogyriaceae	*Plagiogyria japonica*	Minamiise-cho, Mie Pref.	1	4
			Owashi-shi, Mie Pref.	2	3
			Taiki-cho, Mie Pref.	5	5
			Wakimoto, Aira-shi, Kagoshima Pref.	1	2
			Kamou-cho, Aira-shi, Kagoshima Pref.	2	5
			Nishiizu-cho, Shizuoka Pref.	1	4
			Shizuoka-shi, Shizuoka Pref.	3	8
		Plagiogyria euphlebia	Kyoto-shi, Kyoto Pref.	9	27
			Taiki-cho, Mie Pref.	2	3
			Nishiizu-cho, Shizuoka Pref.	6	20
			Shizuoka-shi, Shizuoka Pref.	3	5
	Cyatheaceae	*Cyathea podophylla*	Mt. Omoto, Ishigaki Island, Okinawa Pref.	3	16
			Marley River, Iriomote Island, Okinawa Pref.	4	5
			Urauchi River, Iriomote Island, Okinawa Pref.	4	7
			Yutsun River, Iriomote Island, Okinawa Pref.	3	3
			Kunigami-son, Okinawa Pref.	1	1
		Cyathea lepifera	Mt. Omoto, Ishigaki Island, Okinawa Pref.	1	2
			Nago-shi, Okinawa Pref.	1	1
			Marley River, Iriomote Island, Okinawa Pref.	1	4
			Yutsun River, Iriomote Island, Okinawa Pref.	1	1

O. banksiifolia were cut transversally into two pieces: the upper two thirds and basal one third for use in microscopic observations and DNA analysis, respectively.

Microscopic observations

Gametophyte pieces were fixed in FAA (50 % ethanol/formaldehyde/acetic acid, 90:5:5) for more than 24 h. Pieces were either stained using trypan blue or resin-sectioned (Table 2). For trypan blue staining, fixed pieces were cleared using 10 % KOH for 5 min and stained with acidic glycerol/trypan blue (0.05 %) (Koske and Gemma 1989). Materials stained using

trypan blue were dehydrated in a graded ethanol series and mounted with Permount (Fischer Scientific, Morris Plains, NJ, USA). For resin-sectioning, the fixed materials were dehydrated through a graded ethanol series, embedded in Technovit 7100 (Heraeus, Kulzer, Germany), cut longitudinally or rarely transversely into 3-μm-thick sections, and stained using modified Sharman's solution (Jernstedt et al. 1992). For the longitudinal and transverse sections, the thickest portion of the cushion was measured as the cushion thickness of individuals. Prepared slides of trypan blue-stained and resin-sectioned samples were observed under a light microscope (Olympus BX51). Colonization levels were

Table 2 Fungal colonization and cushion structure of fern gametophytes

Taxa	Fungal colonization		Cushion structure		
	Number of gametophytes	Fungal colonization (%)	Number of gametophytes	Number of cell layer	Thickness (µm)
Osmunda banksiifolia	32 (21[a]+11[b])	90.6	9	6.56±1.42	250.38±86.64
Diplopterygium glaucum	12 (7[a]+5[b])	58.3	3	8.33±1.15	259.68±32.28
Dicranopteris linearis	10 (2[a]+8[b])	80.0	5	8.40±1.14	305.57±67.40
Plagiogyria japonica	31 (9[a]+22[b])	96.8	20	6.00±1.72	252.33±78.71
Plagiogyria euphlebia	55 (44[a]+11[b])	81.8	10	6.40±1.07	252.30±46.31
Cyathea podophylla	32 (21[a]+11[b])	81.3	10	5.60±1.26	207.81±62.69
Cyathea lepifera	8 (8[a]+0[b])	62.5	–[c]	–	–

[a] Trypan blue staining

[b] Resin sections

[c] The gametophytes of *Cyathea lepifera* were not included in resin sectioning due to small sampling size

calculated as the total number of gametophytes infected by AM fungi divided by the total number of gametophytes investigated. All prepared slides were deposited at the herbarium of the National Museum of Nature and Science as voucher specimens.

Fungal and plant DNA analysis

DNA was extracted from fresh gametophyte tissues using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Gametophytes for which fungal colonization was confirmed via microscopic observation were used for fungal DNA analysis. The fungal small subunit (SSU) rDNA region was amplified and sequenced using a universal eukaryotic primer NS31 (Simon et al. 1992) and an AM fungal specific primer AML2 (Lee et al. 2008). PCR was performed using PrimeStarGXL DNA Polymerase (Takara-Bio, Shiga, Japan). PCR conditions and sequencing were conducted as described by Ogura-Tsujita et al. (2013). Most PCR products were cloned using the pGEM-T Vector System I (Promega, Madison, WI, USA), while a portion of samples was directly sequenced without cloning (see Supplementary information Table S1). Ten to 20 colonies were chosen from each PCR product for sequencing. Fungal sequences were assessed for similarities against the MaarjAM database (Öpik et al. 2010) and then assigned to either a virtual taxon (VT) based on sequence similarity of ≥97 % or an operational taxonomic unit (OTUs) for similarity <97 %. To avoid PCR-generated chimera, sequences from multiple individuals or the above three clones were assigned to OTUs. The DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) accession numbers of the fungal sequences determined in this study are given in Fig. 2.

The *rbcL*-a region or upper 700 bp of the chloroplast *rbcL* gene was amplified from all collected gametophytes to confirm the identity of the plant species, as described by Ebihara et al. (2008), using the primer combination *rbcL*1-1 (Hasebe et al. 1995) and *rbcL*HIR1 (Ebihara et al. 2002). Sequences were then compared to the DNA barcoding data set for Japanese pteridophytes (Ebihara et al. 2010).

Phylogenetic analysis

One representative sequence was selected for each VT or OTU for use in the phylogenetic analysis. Fungal SSU sequences from gametophytes were aligned manually against the data matrix in Schwarzott et al. (2001) using MacClade v. 4.06 (Maddison and Maddison 2003). Classification of Glomeromycota followed Schüßler and Walker (2010) and Krüger et al. (2012).

Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) employing the GTR+I+ G model of DNA substitution. Four Markov chain Monte Carlo chains were run for ten million generations with sampling every 1000 generations, and the first 2500 samples were discarded as burn-in. The bootstrap method (Felsenstein 1985) was used with 1000 replicates to assess the relative robustness of the branches. The SSU sequences of *Mortierella polycephala* (X89436) and *Endogone pisiformis* (X58724) were used as outgroup taxa.

Results

Gametophyte morphologies and fungal colonization

The wild gametophytes of the seven study species all exhibited the typical cordate shape with a deeply seated notch between nearly equally developed right and left wings (Fig. 1a, d, g, j, m, p; image of *C. lepifera* not shown), although the ratio between the width and length of the wings varied somewhat with age and individual, even within the same species. The cushion ran from the notch to the base. Archegonia
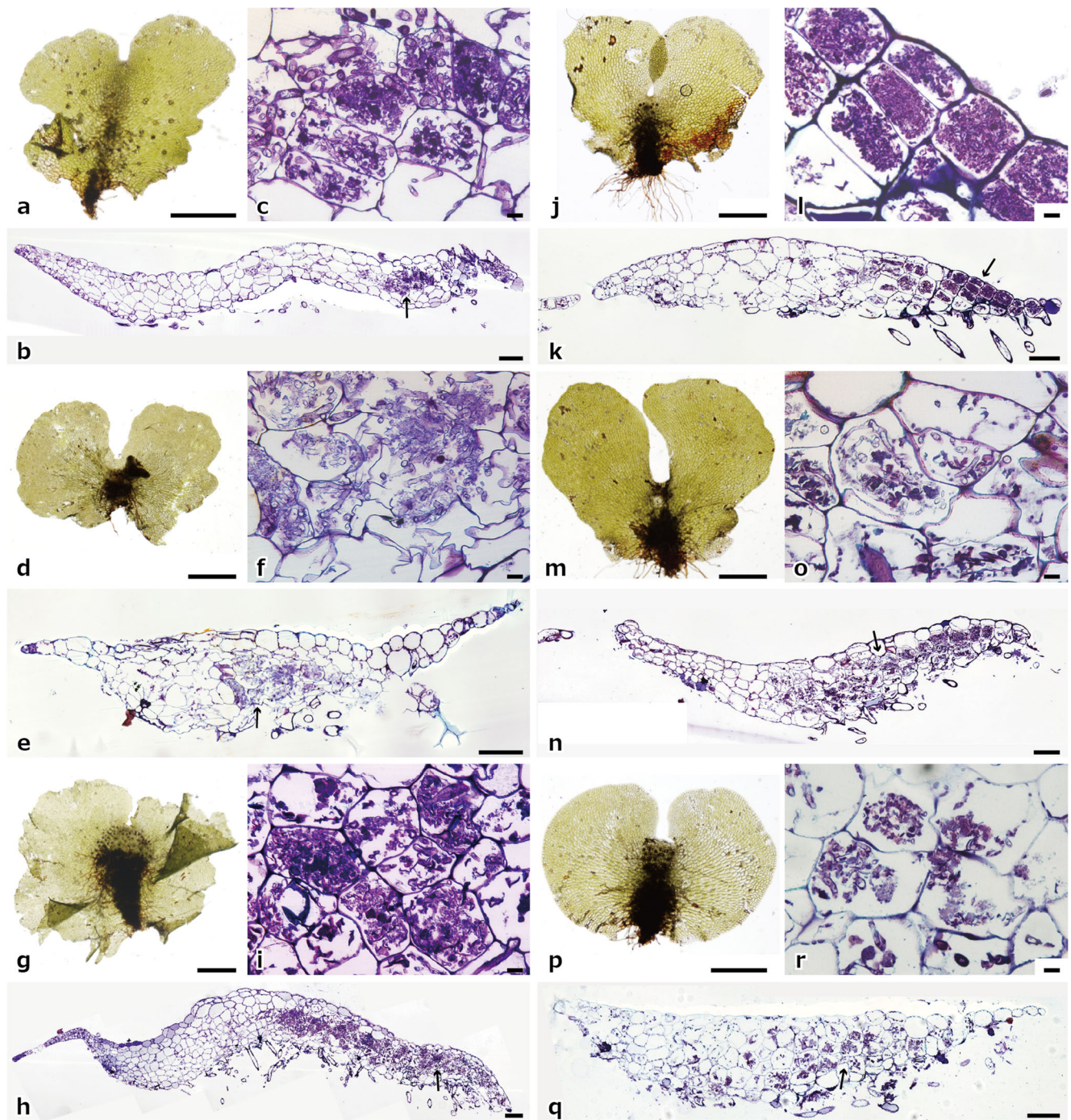
Fig. 1 Fern gametophytes. **a**–**c** *Osmunda banksiifolia*, **d**–**f** *Diplopterygium glaucum*, **g**–**i** *Dicranopteris linearis*, **j**–**l** *Plagiogyria japonica*, **m**–**o** *Plagiogyria euphlebia*, **p**–**r** *Cyathea podophylla*. **a**, **d**, **g**, **j**, **m**, **p** Microscope images of entire gametophytes, *bars*=1 mm; **b**, **e**, **h**, **k**, **n**, **q** resin sections in sagittal plane of the cushion, *bars*=100 μm; **c**, **f**, **i**, **l**, **o**, **r** enlarged figures of cells colonized by AM fungi, indicated by *arrows* in **b**, **e**, **h**, **k**, **n**, and **q**, *bars*=10 μm

occurred along both lateral sides of the cushion in *O. banksiifolia* (data not shown), whereas they arose from the entire surface of the cushion in the other species examined (e.g., small dots on the upper portion of the cushion of *Dic. linearis*, Fig. 1g). Antheridia were found on both wings in all species examined (data not shown).

Serial longitudinal sections of gametophytes in the sagittal plane revealed that fungal hyphae, if present, occupied the posterior (basal) portion of the cushion. The area of fungal colonization covered up to half, and sometimes two thirds, of the area from the base end of the gametophytes (e.g., Fig. 1e, h, n). Hyphae were absent from the anterior portion

Table 3 Virtual taxa (VT) of arbuscular mycorrhizal (AM) fungi and their frequencies in fern gametophytes

Plant species	No. of plants	Glo											
		VTX00069	VTX00070	VTX00080	VTX00083	VTX00084	VTX00103	VTX00113	VTX00122	VTX00124	VTX00126	VTX00135	VTX00159
Osmunda banksiifolia	24	1	2	1	4		14			3	1	1	1
Diplopterygium glaucum	5				1						2		
Dicranopteris linearis	7				5	2					2		
Plagiogyria japonica	24				8	2		3					1
Plagiogyria euphlebia	31	1			4	4				1	1		3
Cyathea podophylla	20	1	4		2				2	2	2		1
Cyathea lepifera	5	3		1	1	1							

Plant species	Glo												
	VTX00166	VTX00178	VTX00180	VTX00181	VTX00191	VTX00194	VTX00199	VTX00214	VTX00219	VTX00223	VTX00224	VTX00291	VTX00310
Osmunda banksiifolia	4	1	1	3				3	2	1	1		
Diplopterygium glaucum	1							1					
Dicranopteris linearis								1			1		
Plagiogyria japonica	1			6				9	5			1	
Plagiogyria euphlebia	10	1	1	15			1	9	2	1	3		1
Cyathea podophylla	2			4				9					
Cyathea lepifera	3					1		4					

Plant species	Glo					Cla			Aca	
	VTX00359	VTX00371	VTX00373	VTX00383	VTX00231	VTX00056	VTX00057	VTX00193	VTX00276	VTX00012
Osmunda banksiifolia	7	7	7			2	2	1		
Diplopterygium glaucum		1	1							
Dicranopteris linearis	1	2								
Plagiogyria japonica			1		1					
Plagiogyria euphlebia								1	1	
Cyathea podophylla				1					2	2
Cyathea lepifera				2	2					1

Plant species	Aca		Arc	Cla	Gig		OTU1	OTU2
	VTX00024	VTX00026	VTX00004	VTX00005	VTX00049			
Osmunda banksiifolia				1				2
Diplopterygium glaucum			2	1				
Dicranopteris linearis	1							
Plagiogyria japonica				3	1			

Table 3 (continued)

Plant species	Aca			Arc		Gig	OTU1	OTU2
	VTX00024	VTX00026	VTX00231	VTX00004	VTX00005	VTX00049		
Plagiogyria euphlebia		1		3	1			
Cyathea podophylla			2				2	
Cyathea lepifera				1				

Numbers show the number of gametophytes that had each VT sequence. The fungal sequences were assessed for the similarities against the MaarjAM database (Öpik et al. 2010) and assigned to the virtual taxa (VT) based on sequence similarity ≥97 %. The operational taxonomic units (OTUs) were assigned for the fugal sequences which had <97 % similarity at the database

Glo Glomeraceae, *Cla* Claroideoglomeraceae, *Aca* Acaulosporaceae, *Arc* Archaeosporaceae, *Gig* Gigasporaceae

just below the notch. Cells with fungal colonization were not always continuous but were sometimes found in separate island areas (Fig. 1b). In the typical case, fungi were not found in the dorsal surface cell layer but were primarily located in the inner tissue and ventral surface cells of the cushion (Fig. 1e, n). In some cases, however, the dorsal surface cells were also colonized by fungi (Fig. 1k). The fungal hyphae were sometimes found in rhizoids, which arose from the ventral surface cells. Serial transverse sections of gametophytes clearly indicated the absence of fungal colonization in the one-cell-thick wing portions (data not shown).

Fungal hyphae in the cushion tissue cells were aseptate and sometimes showed finely branched arbuscules (Fig. 1c, f, i, l, o, r), which are typical of AM fungi. Cells colonized by AM fungi contained very few plastids, whereas non-mycorrhizal cells had relatively large numbers of plastids (data not shown).

Fungal colonization levels and cushion thickness of gametophytes

Fungal colonization levels, which were calculated using the total number of trypan blue-stained and resin-sectioned preparations for each species, are summarized in Table 2. For all seven species examined, 58 to 97 % of gametophytes exhibited AM fungal colonization. High colonization (≥90 %) was observed in *O. banksiifolia* and *P. japonica*, whereas colonization was relatively low in *Dip. glaucum* (58 %) and *C. lepifera* (63 %).

Cushion thickness and the number of cushion cell layers were measured in samples that did not experience shrinkage in fixative solution. Cushion thickness ranged from 208 to 306 μm, and the number of cell layers ranged from 5.6 to 8.4 (Table 2). Cushion structure (either thickness or the number of cushion cell layers) did not appear to be correlated with the percentage of AM fungal colonization. Notably, resin-sectioned samples always contained fungi (i.e., 100 % colonization) for all species examined, whereas samples stained with trypan blue sometimes lacked fungi. In particular, when fungi were limited to the basal area with dense rhizoidal hairs, they were sometimes overlooked in trypan blue-stained samples but not in resin-sectioned samples.

DNA analysis

Fungal sequences obtained from the gametophytes were grouped into 40 VTs and two OTUs (Table 3). Individual gametophytes harbored 1–6 VTs or OTUs. Most VT sequences from the gametophytes belonged to the Glomeraceae (former *Glomus* group A), although Claroideoglomeraceae, Acaulosporaceae, Archaeosporaceae, and Gigasporaceae sequences were also identified. The most frequently observed VTs (≥20 instances) were VT 80, 166, 191, and 219; VT 80 and 219 were found in all of the fern taxa examined.

The phylogenetic analysis revealed that the Glomeromycota sequences isolated from gametophytes were widely divergent (Fig. 2). The OTU sequences, which shared less similarity (≤96 %) with VT sequences in the MaarjAM BLAST analysis, were included in the phylogenetic analysis, resulting in ambiguous phylogenetic positions for OTU1 and OTU2.

Discussion

Using microscopic and molecular techniques, the present study has demonstrated that field-collected gametophytes of the Osmundaceae, Gleicheniaceae, Plagiogyriaceae, and Cyatheaceae are colonized by AM fungi. To our knowledge, this study provides the first evidence of AM fungal colonization in wild gametophytes of the tree fern clade (sensu Pryer et al. 2004), i.e., the Plagiogyriaceae and Cyatheaceae where fungal colonization levels were high (63–97 %). Cooper (1976) sowed spores of several fern species, including *Cyathea*, into field soils and found that fungal colonization only sporadically occurred in gametophytes. Although Boullard (1979) reported that the gametophytes of the Cyatheaceae and Loxomataceae were non-mycorrhizal, the present study indicates that the Cyatheales include AM-associated gametophytes. Concerning the Osmundaceae and Gleicheniaceae, the gametophytes of *O. banksiifolia* and the two species *Dip. glaucum* and *Dic. linearis* were colonized by AM fungi at intermediate to high levels (58–91 %) suggesting that diverse taxa of the Osmundaceae and Gleicheniaceae harbor AM fungi in gametophytes. Mycorrhizal gametophytes have already been documented in *Osmunda* (Campbell 1908a; Boullard 1957) and *Todea* (Cooper 1976), and we have reported AM fungal colonization in 95 % of wild *O. japonica* gametophytes (Ogura-Tsujita et al. 2013). Endophytic fungal colonization of aboveground Gleicheniaceae gametophytes has been previously reported (Campbell 1908b; Boullard 1957), and Schmid and Oberwinkler (1995) observed intracellular hyphal coils with arbuscules and vesicles in field-collected gametophytes of the Gleicheniaceae using light and electron microscopy.

The molecular identification of AM fungi in the present study suggests that photosynthetic gametophytes of pteridophytes are associated with a broad range of fungi in the Glomeromycota, particularly the Glomeraceae. Pteridophyte sporophytes are well known to associate with a wide range of AM fungi (Zhang et al. 2004; West et al. 2009), whereas mycoheterotrophic gametophytes, such as *Botrychium* (Winther and Friedman 2007) and *Psilotum* (Winther and Friedman 2009), exhibit very specific associations with particular members of the Glomeraceae. In the present study, the most frequently observed (≥20 total instances) VTs were VT 80, 166, 191, and 219 of the Glomeraceae. VT 166 has been

Fig. 2 Bayesian phylogenetic tree based on nrSSU rDNA sequences of ▶ AM fungi obtained from fern gametophytes and GenBank. Bayesian posterior probabilities (only values exceeding 0.7 are given) are indicated *above branches*. The tree was rooted with *Endogone pisiformis* and *Mortierella polycephala*

recorded on six continents (Africa, North America, South America, Oceania, Europe, Asia), while VT 191 and 219 have been recorded on five continents (Öpik et al. 2010). These observations demonstrate that these VTs are globally distributed and extremely common taxa, accounting for their frequent occurrence among the gametophyte samples examined in the present study.

Colonization by AM fungi was limited to parts of the cushion in all observed gametophytes, similar to previous findings (Ogura-Tsujita et al. 2013). These results suggest that fungal colonization is limited to the cushion tissue in aboveground cordate gametophytes, as has been shown for liverwort thalli where associate AM fungi primarily colonize the inner parenchyma (Ligrone et al. 2007).

Among the gametophytes of the six species examined, AM fungal colonization levels did not appear to be correlated with cushion thickness or the number of cushion cell layers. The gametophytes of *P. japonica* exhibited highest colonization (97 %) but had the fourth thickest cushion (252 μm thick and 6.0 cell layers). Conversely, *Dic. linearis* (Gleicheniaceae) had the thickest cushion (306 μm thick and 8.4 cell layers) but exhibited only the third highest colonization level (80 %). However, *Angiopteris* and *Osmunda* gametophytes exhibited colonization levels of over 95 % and much thicker cushions compared to the gametophytes of Plagiogyriaceae, Gleicheniaceae, and Cyatheaceae: 807 μm thick and 14 cell layers for *A. lygodiifolia* and 470 μm thick and 10 cell layers for *O. japonica* (Ogura-Tsujita et al. 2013). In addition, preliminary examinations indicated that the terrestrial cordate gametophytes of some species of polypods, whose cushions are relatively thin, have low levels of AM fungal colonization. For example, *Polystichum tripteron* (Dryopteridaceae), with a thin cushion of 4.3 cell layers, exhibited only 16 % colonization (4/25 individuals) (Ayako Suzuki et al. unpublished data). Together, these findings suggest that the level of AM fungal colonization may indeed be related to cushion thickness. However, the data available for gametophytes of leptosporangiate ferns are quite limited. More extensive investigations across a wide spectrum of polypod groups (sensu Pryer et al. 2004) are needed to clarify the interrelationship between AM fungal colonization and cushion thickness,.

To our knowledge, the present study is the first report of the morphology of wild gametophytes in the Plagiogyriaceae and Cyatheaceae. Studies of the comparative morphology of fern gametophytes have typically used gametophytes cultured from spores, rather than wild gametophytes, because field-collected gametophytes are difficult to identify to the species,
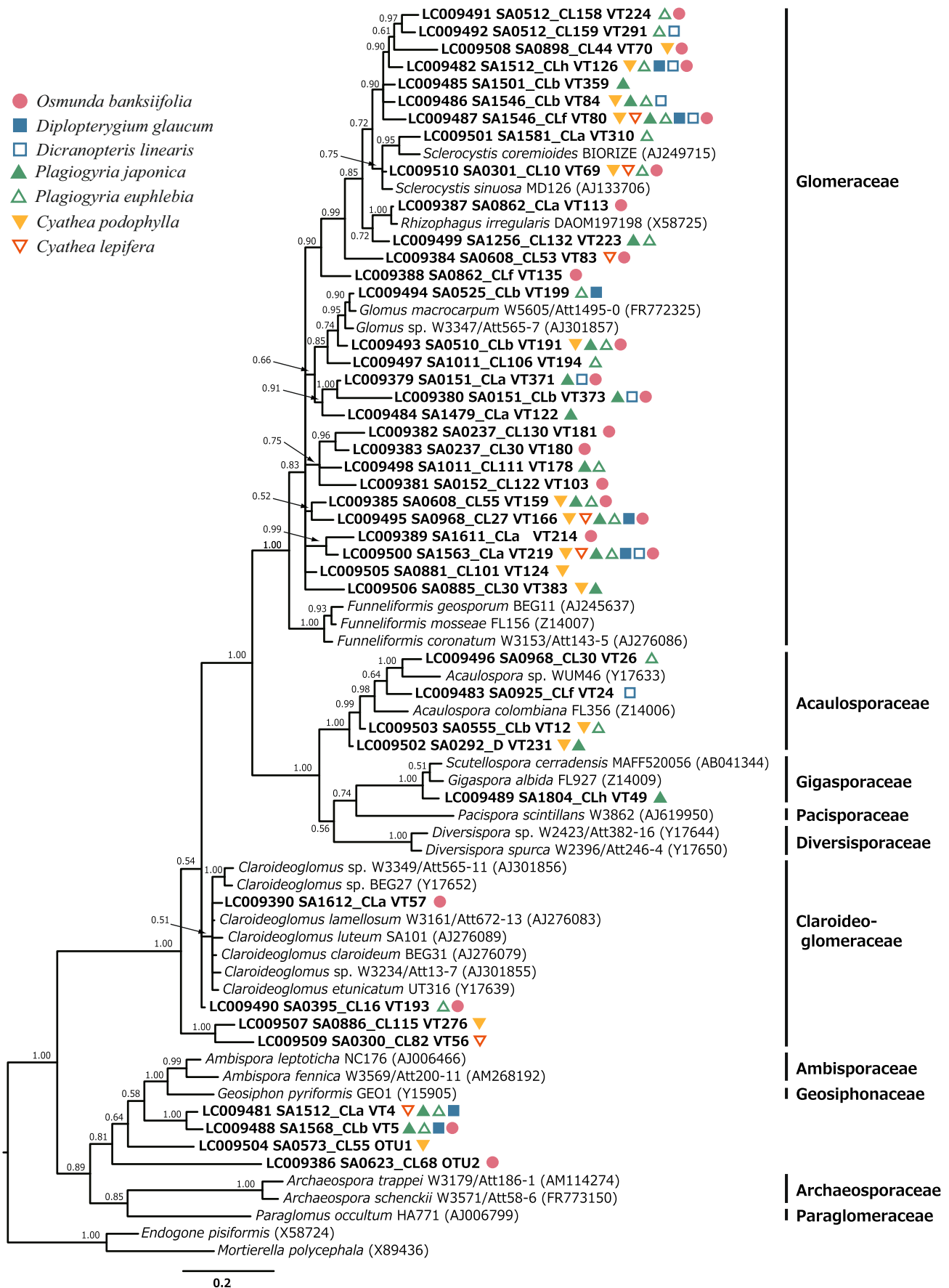
genus, or even family level due to insufficient morphological characters. However, recently established DNA bar-coding techniques have facilitated the identification of field-collected gametophytes (Ebihara et al. 2013). The present examination of wild gametophytes showed that their morphology is nearly identical to cultured gametophytes (Stokey and Atkinson 1956a for *O. banksiifolia*; Stokey 1950 for Gleicheniaceae; Stokey and Atkinson 1956b for *Plagiogyria*; Khare and Chandra 1995 for *Cyathea*). Cultured and wild gametophytes did not differ in the locations of reproductive organs, archegonia, or antheridia. Cushion thickness tended to be thinner in wild compared to cultured gametophytes: 7 vs. 18 cell layers for *O. banksiifolia*; 8 vs. 10–12 or 7–9 cell layers, respectively, for Gleicheniaceae; 6 vs. 4–7 or 6–7 cell layers for *Plagiogyria*; and 6 vs. 7–8 cell layers for *Cyathea* (Stokey and Atkinson 1956a for *O. banksiifolia*; Stokey 1950 for Gleicheniaceae; Stokey and Atkinson 1956b for *Plagiogyria*; Khare and Chandra 1995 for *Cyathea*).

In conclusion, previous mycorrhizal research on pteridophyte gametophytes has primarily focused on underground mycoheterotrophic gametophytes, such as the Lycopodiaceae and Ophioglossaceae (Duckett and Ligrone 1992; Schmid and Oberwinkler 1993; Winther and Friedman 2007), whereas the mycorrhizal associations of aboveground photosynthetic gametophytes remain largely unknown. The present study of five genera from four families of the Osmundales and Gleicheniales, and the Cyatheales, showed that taxonomically divergent photosynthetic gametophytes, which were mainly terrestrial and ground-dwelling, are colonized by AM fungi. These results suggest that mycorrhizal associations with AM fungi could widely occur among pteridophyte gametophytes, although more taxonomically and ecologically divergent species including epiphytic and aquatic gametophytes need to be examined. Because the tiny gametophytes exhibit simple rhizoid structures instead of root systems, AM fungal associations may play an important role in water and nutrient uptake in pteridophyte gametophytes.

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