

**A SURVEY OF THE FERN GAMETOPHYTE FLORA OF JAPAN:  
FREQUENT INDEPENDENT OCCURRENCES OF NONCORDIFORM  
GAMETOPHYTES<sup>1</sup>**

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- *Premise of the study:* Ferns and lycophytes are the only extant land plants with two free-living generations (sporophytes and gametophytes); hence, a single species may have two different distributions. The distribution of the gametophytes of most fern species, which are much smaller in size than sporophytes, are almost unknown due to the difficulty of identifying gametophytes using morphological characters.
- *Methods:* Twelve quadrats (1 m<sup>2</sup> or 0.25 m<sup>2</sup>), each subdivided into a grid of 100 (10 × 10) or 25 (5 × 5) squares, were used to survey gametophytes in the Japanese Archipelago, where distribution data of sporophytes and “DNA barcodes” for identification of gametophytes have fully been established in previous studies. Collected gametophytes were identified using the plastid *rbcL*-a region.
- *Key results:* In total, gametophytes of 38 species in two broad morphological categories (28 cordiform and 10 noncordiform species) were identified among 407 collections. The cordiform gametophytes discovered are without exception accompanied by their conspecific sporophytes at the periphery of the quadrats. On the other hand, the sporophytic counterparts of the noncordiform gametophytes are often not found or are rare around the sites.
- *Conclusions:* This study demonstrates with a regional flora that fern gametophytes do not always co-occur with sporophytes of the same species. In particular, noncordiform gametophytes tended to occur independently of conspecific sporophytes. This pattern may be due to the capability for indeterminate growth and vegetative reproduction by gemmae in noncordiform gametophytes.

**Key words:** distribution; DNA barcoding; fern; gametophyte; sporophyte.

Large amounts of distribution data of vascular plants now constitute an important part of global biodiversity information (e.g., 69 345 264 occurrence data points in the Global Biodiversity Information Facility [GIBF] data portal at <http://data.gbif.org>, 25 August 2012) owing to the ease of identification based on gross morphology. These data are being used for many purposes, including conservation planning (e.g., compiling Red Lists) and evolutionary studies (e.g., Biffin et al., 2012). However, in organisms with life cycles consisting of multiple, independent-living generations, the distribution of each generation may not necessarily overlap. Ideally, our discussions of species distributions should always be based on “true” distributions that include all stages of the life cycle.

Ferns and lycophytes are characterized by sporophytes and gametophytes that can occur independently of each other. Furthermore, the gametophytes and sporophytes of these plants contrast strongly in morphology: gametophytes are much

smaller (often <1 cm<sup>2</sup>) and usually lack obvious morphological differences among congeneric species (Momose, 1967). This habit may yield a cryptic distribution of gametophytes that is different from that of sporophytes. Indeed, gametophytes of some fern species are known to grow at great distances from their conspecific sporophytes (Ebihara et al., 2008) or do not produce sporophytes at all (Farrar, 1967); these are often referred to as independent gametophytes. Despite this, our knowledge of the distribution of fern and lycophyte species is almost solely based on sporophytic data. Although a few studies under special situations (e.g., local distribution of a limited number of species; widespread but morphologically distinct species) have succeeded in identifying gametophytes based solely on morphology (Rumsey et al., 1990; Farrar and Mickel, 1991; Raine et al., 1991; Farrar, 1992), identification of field-collected gametophytes is only possible for most fern species with DNA sequencing. Despite the increasing availability of molecular data, however, it is still unrealistic to explore the gametophyte distribution of morphologically cryptic species over a wide geographical area. At present, the only way to draw gametophyte distribution maps for most fern species is to connect many occurrence records generated by surveys of the gametophyte flora at a number of widely distributed sites.

Since no standard method has been established for the investigation of gametophyte flora of ferns and lycophytes, there is a need to discuss “effective” methods. We still have little knowledge about the biology of gametophytes in the wild, including

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Fig. 1. Locations of survey sites of fern gametophyte flora in the Japanese archipelago.

their distribution, morphology, and phenology because most of the studies on fern gametophytes have been ex situ, relying on spores collected from sporophytes identified to species.

Japan is one of the most information-rich areas in the world regarding ferns and lycophytes, and its fern and lycophyte flora, comprising more than 650 species (Iwatsuki et al., 1995), is

TABLE 1. List of survey sites for “fern gametophyte flora” in the present study.

Site	Location	Substrate	Habitat	Date of survey
OKT-A	Tokyo Pref., Nishitama-gun, Okutama-machi, Unazawa, 320 m a.s.l.	Rock	North-east facing cliff on the roadside, in a <i>Cryptomeria</i> plantation.	13 June 2007
OKT-B	Ibid.	Rock	Ibid.	3 Nov. 2007
CCB	Saitama Pref., Chichibu-shi, Nakatsugawa, 830 m a.s.l.	Rock	West-facing cliff (partly overhanging) eroded by a river, more than 10 m in height, in deciduous broadleaf forest.	5 Oct. 2007
TDR	Tokyo Pref., Setagaya-ku, Todoroki Valley, 20 m a.s.l.	Soil	West-facing slope along the walking path on a reinforced riverbank, under evergreen broadleaf trees.	30 May 2008
NIT	Kanagawa Pref., Kawasaki-shi, Nishi-ikuta Campus of Japan Women's University, 60 m a.s.l.	Soil	Southeast-facing cliff on the roadside, in evergreen broadleaf forest.	18 July 2008
KST1	Gunma Pref., Agatsuma-gun, Kusatsu-machi, Kusatsu, Yoshigataira, 1260 m a.s.l.	Soil	South-facing near-vertical exposure of earth by a path, in a <i>Cryptomeria</i> plantation.	2 Sept. 2008
KST2	Gunma Pref., Agatsuma-gun, Kusatsu-machi, Kusatsu, Ousen-no-taki Trail, 850 m a.s.l.	Soil	North-facing near-vertical exposure of earth by a path, in deciduous broadleaf forest with bamboo grass growing on the floor.	3 Sept. 2008
IOT1	Okinawa Pref., Yaeyama-gun, Taketomi-cho, Iriomote Isl., Maarei River, 10 m a.s.l.	Rock	North-facing cliff more than 3 m away from a watercourse, in evergreen broadleaf forest.	14 Oct. 2008
IOT2	Okinawa Pref., Yaeyama-gun, Taketomi-cho, Iriomote Isl., Yutsun River, 110 m a.s.l.	Rock	North face of a rock several meters in height by a river current, in evergreen broadleaf forest.	16 Oct. 2008
IOT3	Okinawa Pref., Yaeyama-gun, Taketomi-cho, Iriomote Isl., Yutsun River, 80 m a.s.l.	Rock	East-facing cliff 5 m away from a river current, in evergreen broadleaf forest.	16 Oct. 2008
ISG	Okinawa Pref., Ishigaki-shi, foot of Mt. Omoto-dake, 210 m a.s.l.	Soil	Near-vertical exposure of earth (loam soil) facing north on the side of a path in evergreen broadleaf forest.	17 Oct. 2008
H CJ1	Tokyo Pref., Hachijo-machi, Nakanogo, near Urami-ga-taki Fall, 110 m a.s.l.	Soil	Near-vertical exposure of earth facing south on the side of a path along a stream, in evergreen broadleaf forest.	19 Dec. 2008
H CJ2	Tokyo Pref., Hachijo-machi, Mt. Hachijo-fuji, 800 m a.s.l.	Rock	Northeast face of a volcanic rock several meters in height, in evergreen shrubs.	20 Dec. 2008



Fig. 2. An example of a 1-m<sup>2</sup> quadrat for fern gametophyte flora with a grid of 25 squares, 10 × 10 cm each.

ideal for a feasibility study of gametophyte flora survey methods. First, the sporophyte distribution identified for nearly every species in a 10-km geographical grid (“the secondary geographical grid” of Japan) (Kurata and Nakaïke, 1979, 1981, 1983, 1985, 1987, 1990, 1994, 1997) will enable us to compare distributions of the two generations. Second, a DNA barcode library (1200 bp of chloroplast *rbcL*, Ebihara et al., 2010) of richly sampled (ca. 95%) taxa will enable precise identifications of most gametophytes. It is not technically difficult to obtain DNA barcode sequences from any gametophyte collected in any site in the world, but this does not assure precise identification because GenBank currently holds partial DNA sequences of no more than 3000 fern and lycophyte species, approximately one-fifth of extant species (Lehtonen, 2011). Therefore, in a large part of the world, there is a high chance that simply querying sequences of field-collected gametophytes will not yield a match in GenBank. Gametophyte identification will be successful only when a rich data set for DNA barcoding is available for comparison with query sequences obtained from unidentified gametophytes. Japan is one of the exceptional areas that already has such a data set.

The goal of this study was to obtain perspectives on fern gametophyte flora based on a feasibility study at 12 field sites in Japan. We excluded lycophytes from our study, since their gametophytes are nongreen, and there is little possibility of collecting them by the same sampling method as ferns, even though they share a similar life cycle.

## MATERIALS AND METHODS

**Sites for gametophyte collection**—We selected 12 sites that were known to harbor native gametophytes representing several different vegetation types (Fig. 1, Table 1) from subtropical evergreen forests (IOT and ISG) to cool temperate deciduous forests (CCB and KST). Some of the sites were located in well-preserved native forests (IOT, ISG, and HCJ2), and others were in patchy conserved forests in urban areas (TDR and NIT). To examine the seasonal transition within a local gametophyte flora, we surveyed gametophytes twice at the same location at the OKT site, in July (OKT-A) and in November (OKT-B) 2007.

**Gametophyte collection**—We placed quadrats consisting of a grid of 10-cm squares on rock faces or on slopes using garden nets (Fig. 2). For two sites,

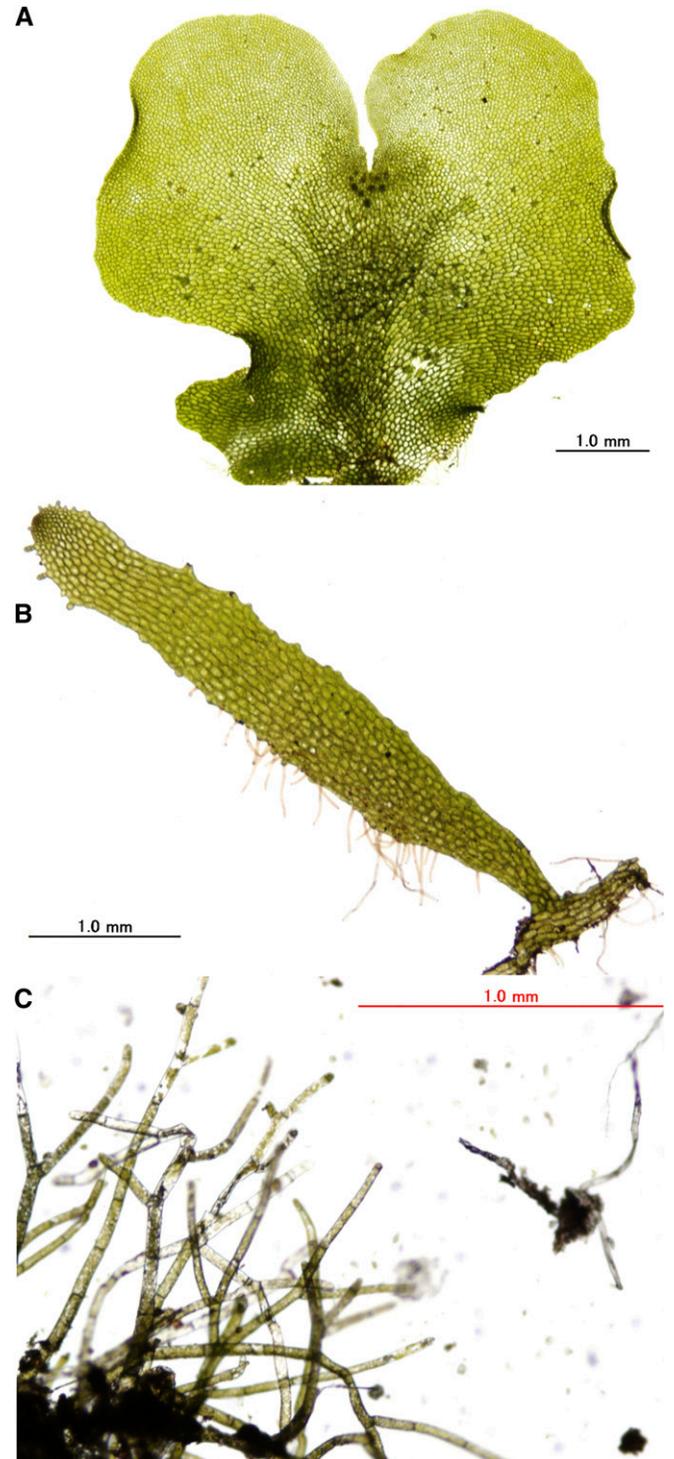


Fig. 3. Variation of gametophyte morphotypes collected in present study. (A) Cordiform gametophyte of *Polystichum tripterum* (Kunze) C.Presl (Dryopteridaceae), (B) ribbon-like gametophyte of *Pleurosoriopsis makinoi* (Maxim. ex Makino) Fomin (Polypodiaceae), (C) filamentous gametophyte of *Abrodictyum obscurum* (Blume) Ebihara et K.Iwats. (Hy-menophyllaceae). Bars = 1.0 mm.

OKT and CCB, which were investigated at an earlier stage of this study, the quadrat was 1 m<sup>2</sup> and was subdivided into a grid of 100 (10 × 10) squares. For the remaining sites, the size of the quadrat was reduced to 0.25 m<sup>2</sup> and was

TABLE 2. A summary of number of collected gametophytes and number of identified species at the survey sites of fern gametophyte flora.

Site	No. of grid squares/site	No. of squares with gametophytes	No. of squares without gametophytes	Percentage of squares with gametophytes	No. of species
OKT-A	100	91	9	91	7
OKT-B	100	87	13	87	5
CCB	100	60	40	60	6
TDR	25	23	2	92	2
NIT	25	22	3	88	5
KST1	25	16	9	64	2
KST2	25	10	15	40	3
IOT1	25	20	5	80	4
IOT2	25	25	0	100	4
IOT3	25	24	1	96	6
ISG	25	21	4	84	5
H CJ1	25	12	13	48	5
H CJ2	25	17	8	68	3
Total	550	428	122	77.82	

subdivided into a grid of 25 (5 × 5) squares, reflecting the survey results of the first two sites (see Discussion). One gametophyte was collected from each grid square. Gametophytes were sorted into two broad morphotypes, cordiform and noncordiform (either strap-like, ribbon-like, or filamentous; Fig. 3; Farrar et al., 2008). If both cordiform and noncordiform gametophytes were growing in a single grid square, we collected the cordiform gametophytes because the noncordiform can often form large colonies by clonal growth (Farrar et al., 2008); thus one noncordiform individual can occupy multiple grid squares. If multiple gametophytes were growing in a single grid square, we collected the one closest to its center. Collected gametophytes were cleaned with water, and their morphology was examined and imaged using a stereomicroscope (LEICA M420 with Nikon E995 digital camera) and a light microscope (OLYMPUS BX51 with DP70 CCD camera). They were divided into two, approximately equal parts with a razor blade. One of the two halves was kept in FAA solution (formalin-acetic acid-50% ethanol, 5:5:90) for further morphological observation as voucher specimens, and the other half was kept in API DNA extraction buffer (Qiagen, Hilden, Germany) at -20°C. Voucher specimens in liquid, numbered by the name of survey sites (Table 1) and the position in the grids, were deposited in the herbarium of the National Museum of Nature and Science (TNS).

**Selection of DNA barcode**—Our DNA barcode data set of Japanese ferns (Ebihara et al., 2010) is quite useful but not ideal for gametophyte identification, because it does not yet cover infraspecific genetic variation. In addition, in cases where identical barcode sequences are shared by multiple taxa, identification to a single species is not possible. Considering this situation, we have selected the *rbcL*-a region (ca. 700 bp of the 5' end of *rbcL*) because of its relatively high rates of successful amplification and discriminating power (Ebihara et al., 2010); *rbcL* has been repeatedly suggested as a barcode marker in studies focusing on ferns and across all plants (Kress and Erickson, 2007; CBOL Plant Working Group, 2009; Li et al., 2011). We considered ease of amplification to be especially important in marker selection since the concentration of template DNA extracted from tiny fern gametophyte fragments is expected to be extremely low.

**DNA analysis**—Gametophyte tissue was crushed with zirconia beads in a TissueLyser (Retsch, Haan, Germany) at a motion frequency of 28 times/s, and

subsequent DNA extraction processes using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) followed the protocol provided by the manufacturer. Extracted DNA was diluted to 50 µL in water. Chloroplast *rbcL*-a was amplified using a combination of primers (*rbcL* 1-1 and *rbcL*-HIR1 [Ebihara et al., 2003]), Ex-taq (Takara Bio, Ohtsu, Japan), and Ampdirect Plus (Shimadzu, Kyoto, Japan) under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, and 72°C for 5 min. Cycle sequencing reactions were performed using BigDye Terminator ver. 3.0 (Life Technologies, Grand Island, New York, USA) with the primer *rbcL* 1-1.

**Identification**—If an obtained *rbcL*-a sequence with ambiguous bases excluded was found to match that of a single taxon in the sequence database of Ebihara et al. (2010), we identified the gametophyte as the matching taxon (Appendix S1, see Supplemental Data with the online version of this article). If it matched multiple taxa, the identification remained ambiguous. If the query sequence did not match any sequence in the database, we calculated the distance (K2P) from the nearest match. Query sequences that differed less than 1% from the nearest taxon in the database were considered to match that taxon (i.e., show intraspecific variation); those greater than 1% were considered to lack a conspecific taxon in Japan. The GenBank accession numbers of the obtained sequences are listed in Appendix S1.

**Survey of sporophyte flora**—We surveyed mature (i.e., morphologically identifiable) fern sporophytes at three levels: (1) species growing inside the quadrats (1 m<sup>2</sup> or 0.25 m<sup>2</sup>), (2) species growing outside the quadrats, at a distance of 10 m or less (for the OKT and CCB sites) or 5 m or less (for the remaining sites) from the outer frame of the quadrats, and (3) species recorded in 10 km geographical grid squares (the secondary geographical grid of Japan) that include the survey sites, based on the distribution data of Kurata and Nakaïke (1979, 1981, 1983, 1985, 1987, 1990, 1994, 1997) and the specimen database of the National Museum of Nature and Science (TNS). The voucher specimens for (1) and (2) were deposited in TNS.

## RESULTS

Gametophytes were collected in 77.8% (428/550) of the grid squares at the survey sites in total (maximum 100% at site IOT2, minimum 40% at site KST2, Table 2). Cordiform gametophytes were collected at all sites and noncordiform gametophytes were collected at sites OKT(-A/B), CCB, KST2, IOT1, IOT3, ISG, and H CJ2. We obtained *rbcL*-a sequences from 97.7% (418/428) of the samples, with lengths ranging from 456 bp to 693 bp after deleting ambiguous bases. Of the collected gametophytes, 50.2% (210/418) could be identified to species (i.e., obtained sequence completely matched a single taxon in the DNA barcode library of Ebihara et al. [2010] or matched only one taxon with more than 99.0% sequence similarity), and 49.8% (208/418) were ambiguously identified. Further sequencing of another proposed plant barcode marker, the *trnH-psbA* intergenic spacer (CBOL Plant Working Group, 2009), failed to improve resolution in cases where the gametophyte *rbcL*-a sequence matched multiple species in the database of Ebihara et al. (2010) (results not shown). Results of identification for each quadrat by grid square are shown in Fig. 4, and the number of species in each quadrat is summarized in Table 3.

Fig. 4. Schematic maps of identification results of gametophytes in the survey sites. Grid squares without gametophytes are indicated with diagonal crosses. (A) OKT-A; (B) OKT-B; (C) CCB; (D) TDR; (E) NIT; (F) KST1; (G) KST2; (H) IOT1; (I) IOT2; (J) IOT3; (K) ISG; (L) H CJ1; (M) H CJ2. Abr-1, *Abrodictyum obscurum*; Agp-1, *Angiopteris lygodiiifolia*; Ath-1, *Athyrium niponicum*, Ath-2, *A. yokoscense*; Asp-1, *Asplenium tenuicaule*, Blc-1, *Blechnum amabile*; Blc-2, *B. orientale*; Clp-1, *Callistopteris apiifolia*; Ctn-1, *Ctenitis subglandulosa*; Cya-1, *Cyathea podophylla*; Cya-2, *C. lepifera*; Dpl-1, *Diplazium wichuriae*; Dep-1, *Deparia japonica*; Dep-2, *D. lancea*; Dpr-3, *D. petersenii*; Drp-1, *Dryopteris bissetiana*; Drp-2, *D. erythrosora*; Drp-3, *D. saxifragivaria*; Drp-4, *D. sordidipes*; Hym-1, *Hymenophyllum barbatum*; Hym-2, *H. badium*; Hym-3, *Hymenophyllaceae* sp.; Hym-4, *Hymenophyllum* sp.; Hym-5, *H. wrightii*; Lds-1, *Lindsaea merrillii* subsp. *yaeyamensis*; Lds-2, *L. chienii* group; Lmp-1, *Lomariopsis* sp.; Lxg-1, *Loxogramme grammitoides*; Osm-1, *Osmunda banksiifolia*; Psp-1, *Pleurosoriopsis makinoi*; Pst-1, *Polystichum tripterum*; Pst-2, *P. polyblepharon*; Spm-1, *Sphenomeris gracilis*; Spm-2, *S. chinensis*; Tlp-1, *Thelypteris decursivepinnata*; Tlp-2, *T. luikiuensis*; Wds-1, *Woodsia manchuriensis*; N/A, not yet identified.

**A**

OKT-A	1	2	3	4	5	6	7	8	9	10
A	Hym-1	Drp-1		Pst-1			Pst-2	N/A	Pst-1	Pst-1
B	Hym-1	Drp-1	Asp-1	Pst-1	Pst-1	Pst-1	Pst-1	Pst-1	Drp-1	Pst-2
C		Hym-1		Pst-1	Pst-1	Pst-1	Pst-1	Pst-1	Drp-1	Pst-2
D		Hym-1	Pst-1		Pst-1	Hym-1	Pst-1	Hym-1	Psp-1	Wds-1
E	Pst-1									
F	Psp-1	Hym-1	Psp-1	Pst-1	Pst-1	Psp-1	Pst-1	Wds-1	Wds-1	Pst-1
G	Asp-1	Psp-1	Psp-1	Pst-1	Psp-1	Pst-1	Pst-1	Psp-1	Pst-1	Asp-1
H	Psp-1	Pst-1	Psp-1	Pst-1	Psp-1	Asp-1	Pst-1	Pst-1	Psp-1	Pst-1
I	Psp-1	Psp-1	Psp-1	Psp-1	Psp-1	Pst-1	Psp-1	Psp-1	Psp-1	
J	Pst-1	Pst-1	Pst-1	Pst-1	Psp-1	Psp-1	Psp-1	Psp-1		Pst-1

**B**

OKT-B	1	2	3	4	5	6	7	8	9	10
A	Hym-1	Drp-1		Pst-1				Pst-1		Pst-1
B	Hym-1	Drp-1	Hym-1	Pst-1	Pst-1	Pst-1	Pst-1	Pst-1	Pst-1	Psp-1
C		Pst-1								
D			Pst-1	Pst-1	Psp-1		Pst-1	Pst-1	Hym-1	Pst-1
E	Psp-1	Pst-1	Pst-1	Pst-1	Pst-1	Psp-1	Pst-1	Pst-1	Pst-1	Pst-1
F	Pst-1	Psp-1	Pst-1	Pst-1	Pst-1	Psp-1	Pst-1	Pst-1	Pst-1	Pst-1
G	Pst-1	Psp-1	Psp-1	Psp-1	Wds-1	Asp-1	Pst-1	Pst-1	Pst-1	Pst-1
H	Psp-1	Pst-1	Psp-1	Psp-1						
I	Pst-1	Pst-1	Pst-1		Psp-1	Pst-1	Pst-1	Pst-1	Psp-1	Psp-1
J	Psp-1	Psp-1	Pst-1	Pst-1	Psp-1	Psp-1	Psp-1			Psp-1

**C**

CCB	1	2	3	4	5	6	7	8	9	10
A	Asp-1	Hym-2	Pst-1	Pst-1	Wds-1	Hym-2		Hym-2	Hym-2	Asp-1
B	Hym-2	Lxg-1	Hym-2	Pst-1	Wds-1	Hym-2			Pst-1	
C	Hym-2		Pst-1	Lxg-1						
D	Pst-1			Asp-1						
E	Pst-1			Asp-1						
F							Hym-2			
G							Pst-1			
H			Pst-1	Pst-1		Pst-1				
I			Pst-1	Pst-1	Pst-1	Pst-1				Pst-1
J	Pst-1	Asp-1	Pst-1	Pst-1	Pst-1			Pst-1		Psp-1

**D**

TDR	1	2	3	4	5
A	Drp-2	Drp-2	Drp-2	Drp-2	Tlp-1
B	Drp-2	Drp-2	Drp-2	Drp-2	Drp-2
C	Drp-2	Drp-2	Drp-2	Drp-2	Drp-2
D	Drp-2	Drp-2	Drp-2		Drp-2
E	Drp-2		Drp-2	Drp-2	Drp-2

**E**

NIT	1	2	3	4	5
A	Drp-1	Drp-1	Drp-1	Drp-1	Ath-1
B	Drp-1	Drp-3	Drp-1	Drp-1	Drp-3
C	Drp-3	Drp-1	Drp-1		Drp-1
D	Drp-1	Dep-1		N/A	Drp-3
E	Drp-1		Drp-3	N/A	Drp-1

**F**

KST1	1	2	3	4	5
A	Ath-2	Ath-2	Ath-2	Ath-2	
B	Drp-1		Ath-2	Ath-2	Ath-2
C	Ath-2	Ath-2	Ath-2		Ath-2
D	Ath-2				
E	Ath-2			Ath-2	Ath-2

**G**

KST2	1	2	3	4	5
A		Ath-2		Ath-2	Ath-2
B	N/A	N/A			Ble-1
C				Ath-2	Ble-1
D		Ath-2			
E				Ath-2	

**H**

IOT1	1	2	3	4	5
A	Osm-1	Osm-1	Osm-1	Osm-1	Spm-2
B		Spm-1	Osm-1	Osm-1	Osm-1
C	Osm-1	Osm-1		Osm-1	Spm-1
D	Hym-3		Hym-3	N/A	Osm-1
E	N/A			N/A	Osm-1

**I**

IOT2	1	2	3	4	5
A	Osm-1	Osm-1	Osm-1	Osm-1	Osm-1
B	Osm-1	Osm-1	Osm-1	Osm-1	Osm-1
C	Osm-1	Osm-1	Osm-1	Osm-1	Osm-1
D	Osm-1	Osm-1	Osm-1	Osm-1	Agp-1
E	Osm-1	Tlp-2	Osm-1	Tlp-2	Lds-1

**J**

IOT3	1	2	3	4	5
A	Lmp-1	Lmp-1	Lmp-1	Clp-1	Lmp-1
B	Clp-1	Clp-1	Lds-1	Lmp-1	Hym-3
C	Abr-1		Lds-1	Lmp-1	Lmp-1
D	Lmp-1	Lmp-1	Lmp-1	Lds-1	Hym-3
E	Agp-1	Abr-1	Lmp-1	Hym-4	Lmp-1

**K**

ISG	1	2	3	4	5
A	Lds-2	Lds-2	Cya-1		Drp-4
B	Lds-2	Drp-4	Cya-2	Drp-4	Cya-2
C	Lds-2	Cya-1	Drp-4	Cya-2	Drp-4
D	Ble-2	Cya-1	Drp-4	Lds-2	Drp-4
E			Lds-2	Cya-2	

**L**

HCJ1	1	2	3	4	5
A		Dep-2			
B		Ctn-1			
C		Agp-1	Dpl-1		
D	Dep-3	Dep-2	Ctn-1	Dep-2	
E		Dep-2	Dep-3	Ctn-1	N/A

**M**

HCJ2	1	2	3	4	5
A				Pst-1	Hym-1
B	Hym-5	Hym-5	Pst-1	Hym-5	Pst-1
C			Pst-1	Pst-1	N/A
D	Pst-1			Pst-1	Hym-5
E	Pst-1			Pst-1	Pst-1

TABLE 3. Number of recorded species for gametophytes and sporophytes at the survey sites of fern gametophyte flora. For gametophytes, number of species indicates those collected inside the quadrat. For sporophytes, number of species was counted for three different ranges.

Site	Substrate	Gametophyte			Sporophytes		
		Cordiform	Noncordiform	Total	Range 1	Range 2	Range 3
OKT-A	Rock	5	2	7	4	15	101
OKT-B	Rock	4	2	6	4	15	101
CCB	Rock	3	3	6	3	5	90
TDR	Soil	2	0	2	0	2	37
NIT	Soil	4	0	4	3	8	60
KST1	Soil	2	0	2	1	2	32
KST2	Soil	2	0	2	1	2	32
IOT1	Rock	3	1	4	3	10	127
IOT2	Rock	4	0	4	3	9	114
IOT3	Rock	2	5	7	4	15	114
ISG	Soil	5	0	5	3	9	132
HCJ1	Soil	5	0	5	3	11	35
HCJ2	Rock	1	2	3	2	8	93

Notes: Range 1, inside the quadrat; Range 2, at a distance of 10 m (for OKT and CCB) or 5 m (for the remaining sites) or less from the quadrat; Range 3, 10 km geographical grid square including the site.

## DISCUSSION

We found only minor differences between the gametophyte flora at the OKT site surveyed during the same year in June (OKT-A) and November (OKT-B). One species, *Polystichum polyblepharon*, whose gametophytes were present at low frequency in June, was absent in November. This result could possibly reflect seasonal differences in this species, but the major part of the gametophyte flora at this site, including cordiform gametophytes, was constant over the sampling period. This constancy suggests that seasonal changes in the gametophyte flora has no significant effect on the results of the present survey and that most gametophytes observed are either long-lived (more than half a year) or are continuously produced from spore banks; evidence for being long-lived has been demonstrated by Farrar and Gooch (1975) and Peck et al. (1990). After obtaining the results of OKT and CCB sites using 1-m<sup>2</sup> quadrats (100 grid squares), we decided to reduce the size of the quadrat because 25 grid squares seemed sufficient to fully illustrate the gametophyte diversity at a given site.

The conspecific sporophytes of all cordiform gametophytes, without exception, were found at our survey sites or at least had been recorded within 10 km (Table 4), whereas noncordiform gametophytes were often found unaccompanied by their conspecific sporophytes. In particular, gametophytes of *Hymenophyllum badium* Hook. & Grev. at the CCB site seemed to be growing approximately 100 km from the nearest recorded sporophytes of the same species (Kurata and Nakaike, 1979, 1981, 1983, 1985, 1987, 1990, 1994, 1997). Ribbon-like gametophytes of *Callistopteris apiifolia* (C. Presl) Copel. were found at site IOT3 of Iriomote Island, but collection records of sporophytes of this species are quite scarce on the island, and the species is considered to be severely endangered. Our results suggest that the present assessment as rare or endangered based on sporophyte occurrences may not reflect the true abundance of this species when gametophytes are taken into account. Furthermore, the disjunct distribution of *Callistopteris* gametophytes and sporophytes has also been reported from other Pacific areas, including Hawaii (Dassler and Farrar, 1997) and French Polynesia (J. H. Nitta, Harvard University, unpublished data). In extreme cases, gametophytes of three taxa, Hym-3,

Hym-4 and Lmp-1, were found for species that apparently do not occur in Japan.

The independent occurrence of gametophytes from conspecific sporophytes has so far been observed only in species producing noncordiform gametophytes and is likely related to vegetative reproduction by gemmae and indeterminate growth in noncordiform gametophytes, which enables long-term survival of gametophyte populations. Several studies suggest that gametophytes may be able to tolerate more severe environmental conditions (e.g., low light [Farrar, 1998; Rumsey and Sheffield, 1996], low temperature [Farrar, 1998; Sato and Sakai, 1981], and desiccation [Watkins et al., 2007]) than conspecific sporophytes. Independent gametophytes might persist by vegetative reproduction if they successfully developed from spores dispersed over long distances to the frontier of their distribution range where the environmental conditions are too harsh for sporophytes to survive. Alternatively, under long-term climate change such as glaciation or warming, sporophytes might disappear and leave only gametophytes capable of tolerating the more severe environment. Independent gametophyte species known in North America are hypothesized to have originated from sporophyte populations that went extinct due to southward glaciation during the Pleistocene (Farrar, 1990, 1998; M. Stensvold, USDA Forest Service, personal communication).

Spores of species producing cordiform gametophytes also may disperse to the frontier of their range, but unlike noncordiform gametophytes, lack gemmae, indeterminate growth or branching, and therefore cannot persist vegetatively. As a result, we hypothesize that the probability of detecting cordiform gametophytes that grow independently at the frontier of a range is expected to be very low. An alternative hypothesis is that species that produce cordiform gametophytes also have low dispersal capabilities, thus limiting the chances for gametophytes to occur significantly beyond the range of sporophytes. However, empirical data do not seem to support this. For example, the fern flora of the Hawaiian Islands, one of the most isolated island chains in the world, does not have a significantly lower percentage of species with cordiform gametophytes relative to those with noncordiform gametophytes (Dassler and Farrar, 2001), indicating that species with cordiform gametophytes are not compromised in dispersal ability.

TABLE 4. Comparisons between identification results of gametophytes and sympatric sporophyte flora at the survey sites of fern gametophyte flora.

Site	Identification	Gametophyte morphotype	No. individuals identified	Sporophyte				
				Sporophyte species for multiple matches	Range 1	Range 2	Range 3	
OKT-A	<i>Polystichum</i> sp.	Cordiform	44	* <i>P. tripteron</i> <i>P. hancockii</i> (Hance) Diels	Y N	— N	Y N	
	<i>Pleurosoriopsis makinoi</i>	Ribbon-like	25	N/A	N	N	Y	
	<i>Hymenophyllum barbatum</i> (Bosch) Baker	Ribbon-like	8	N/A	Y	—	Y	
	<i>Asplenium tenuicaule</i> Hayata	Cordiform	4	N/A	Y	—	Y	
	<i>Dryopteris</i> sp.	Cordiform	4	* <i>D. bissetiana</i> (Baker) C.Chr. <i>D. pacifica</i> (Nakai) Tagawa	N N	N N	Y Y	
	<i>P. polyblepharon</i> (Roem. ex Kunze) C.Presl	Cordiform	3	N/A	Y	—	Y	
	<i>Woodsia manchuriensis</i> Hook. Sequence not obtained	Cordiform	3 1	N/A	Y	—	Y	
OKT-B	<i>Polystichum</i> sp.	Cordate	52	* <i>P. tripteron</i> <i>P. hancockii</i>	Y N	— N	Y N	
	<i>Pleurosoriopsis makinoi</i>	Ribbon-like	27	N/A	N	N	Y	
	<i>Hymenophyllum barbatum</i>	Ribbon-like	4	N/A	Y	—	Y	
	<i>Dryopteris</i> sp.	Cordate	2	* <i>D. bissetiana</i> <i>D. pacifica</i>	N N	N N	Y Y	
	<i>Asplenium tenuicaule</i>	Cordate	1	N/A	N	N	Y	
CCB	<i>Woodsia manchuriensis</i>	Cordate	1	N/A	Y	—	Y	
	<i>Polystichum</i> sp.	Cordiform	21	* <i>P. tripteron</i> <i>P. hancockii</i>	N N	Y N	Y N	
TDR	<i>Hymenophyllum badium</i> Hook. & Grev.	Ribbon-like	9	N/A	N	N	N	
	<i>Asplenium tenuicaule</i>	Cordiform	5	N/A	N	N	Y	
	<i>Woodsia manchuriensis</i>	Cordiform	2	N/A	N	Y	Y	
	<i>Loxogramme grammitoides</i> (Baker) C.Chr.	Ribbon-like	2	N/A	N	N	N	
	<i>Pleurosoriopsis makinoi</i>	Ribbon-like	1	N/A	N	N	Y	
	<i>Dryopteris</i> sp.	Cordiform	22	* <i>D. erythrosora</i> <i>D. hondoensis</i> Koidz.	N N	Y N	Y Y	
NIT	<i>Thelypteris decursivepinnata</i> (H. C. Hall) Ching	Cordiform	1	N/A	N	N	Y	
	<i>Dryopteris</i> sp.	Cordiform	13	* <i>D. bissetiana</i> <i>D. pacifica</i>	Y N	— N	Y Y	
	<i>Dryopteris</i> sp.	Cordiform	5	* <i>D. saxifragivaria</i> Nakai <i>D. saxifraga</i> H. Itô	N N	N N	Y N	
	<i>Deparia</i> sp.	Cordiform	1	* <i>D. japonica</i> (Thunb.) M. Kato <i>D. petersenii</i> (Kunze) M.Kato <i>D. longipes</i> (Ching) Shinohara <i>D. conilii</i> (Franch. & Sav.) M.Kato <i>D. pseudoconilii</i> (Seriz.) Seriz. <i>D. dimorphophylla</i> (Koidz.) M.Kato	Y N N N N N N	— N N N N N N	Y N N Y N N N	
	<i>Athyrium niponicum</i> (Mett.) Hance	Cordiform	1	N/A	N	Y	Y	
	Sequence not obtained		2					
	KST1	<i>Athyrium yokoscense</i> (Franch. & Sav.) H.Christ	Cordiform	15	N/A	N	Y	Y
		<i>Dryopteris</i> sp.	Cordiform	1	* <i>D. saxifraga</i> <i>D. saxifragivaria</i>	N N	Y N	Y N
	KST2	<i>Athyrium yokoscense</i>	Cordiform	6	N/A	Y	—	Y
		<i>Blechnum</i> sp.	Cordiform	2	* <i>B. niponicum</i> (Kunze) Makino <i>B. hancockii</i> Hance	N N	Y N	Y N
IOT1	Sequence not obtained		2		N	N	N	
	<i>Osmunda banksiiifolia</i> (C. Presl) Kuhn	Cordiform	12	N/A	Y	—	Y	
	<i>Sphenomeris</i> sp.	Cordiform	2	* <i>S. gracilis</i> (Tagawa) Sa. Kurata <i>S. chinensis</i> (L.) Maxon <i>S. minutula</i> Sa. Kurata	Y N N	— N N	Y Y N	

TABLE 4. Continued.

Site	Identification	Gametophyte morphotype	No. individuals identified	Sporophyte			
				Sporophyte species for multiple matches	Range 1	Range 2	Range 3
IOT2	Hymenophyllaceae sp.	Ribbon-like + filamentous	2	N/A	N	N	N
	<i>Sphenomeris biflora</i> (Kaulf.) Tagawa	Cordiform	1	N/A	N	N	Y
	Sequence not obtained		3	N/A			
	<i>Osmunda banksiifolia</i>	Cordiform	21	N/A	Y	—	Y
	<i>Thelypteris liukuensis</i> (H. Christ ex Matsum.) K. Iwats.	Cordiform	2	N/A	N	N	Y
	<i>Lindsaea merrillii</i> Copel. subsp. <i>yaeyamensis</i> (Tagawa) K. U. Kramer	Cordiform	1	N/A	N	Y	Y
IOT3	<i>Angiopteris</i> sp.	Cordiform	1	* <i>A. lygodiifolia</i> Rosenst. <i>A. fokiensis</i> Hieron.	N N	Y N	Y N
	<i>Lomariopsis</i> sp.	Strap-like	12	N/A	N	N	N
	<i>Lindsaea merrillii</i> subsp. <i>yaeyamensis</i>	Cordiform	3	N/A	Y	—	Y
	<i>Callistopteris apitifolia</i> (C. Presl) Copel.	Ribbon-like	3	N/A	N	N	N
	Hymenophyllaceae sp.	Ribbon-like + filamentous	2	N/A	N	N	N
	<i>Abrodictyum obscurum</i>	Filamentous	2	N/A	Y	—	Y
ISG	<i>Angiopteris</i> sp.	Cordiform	1	* <i>A. lygodiifolia</i> <i>A. fokiensis</i>	N N	Y N	Y N
	<i>Hymenophyllum</i> sp.	Ribbon-like	1	N/A	N	N	N
	<i>Dryopteris sordidipes</i> Tagawa	Cordiform	6	N/A	Y	—	Y
	<i>Lindsaea</i> sp.	Cordiform	6	* <i>L. orbiculata</i> (Lam.) Mett. ex Kuhn var. <i>commixta</i> (Tagawa) K. U. Kramer <i>L. orbiculata</i> var. <i>orbiculata</i> <i>L. cambodgensis</i> H. Christ <i>L. kawabatae</i> Sa. Kurata <i>L. heterophylla</i> Dryand.	N N N N N	Y N N N N	Y N N Y N
	<i>Cyathea podophylla</i> (Hook.) Copel.	Cordiform	5	N/A	Y	—	Y
	<i>Cyathea lepifera</i> (J.Sm. ex Hook.) Copel.	Cordiform	3	N/A	N	N	Y
HCJ1	<i>Blechnum orientale</i> L.	Cordiform	1	N/A	N	Y	Y
	<i>Deparia lancea</i> (Thunb.) Fraser-Jenk.	Cordiform	4	N/A	Y	—	Y
	<i>Ctenitis subglandulosa</i> (Hance) Ching	Cordiform	3	N/A	N	Y	Y
	<i>Deparia</i> sp.	Cordiform	2	* <i>D. petersenii</i> <i>D. japonica</i> <i>D. longipes</i> <i>D. conilii</i> <i>D. pseudoconilii</i> <i>D. dimorphophylla</i>	N N N N N N	Y N N N N N	Y Y N N N N
	<i>Angiopteris</i> sp.	Cordiform	1	* <i>A. lygodiifolia</i> <i>A. fokiensis</i>	N N	Y N	Y N
	<i>Diplazium</i> sp.	Cordiform	1	* <i>D. wichurae</i> (Mett.) Diels <i>D. okudairae</i> Makino	Y N	— N	Y N
HCJ2	Sequence not obtained		1				
	<i>Polystichum</i> sp.	Cordiform	10	* <i>P. tripterum</i> <i>P. hancockii</i>	Y N	— N	Y N
	<i>Hymenophyllum</i> sp.	Ribbon-like	4	* <i>H. wrightii</i> Bosch <i>H. polyanthos</i> (Sw.) Sw. <i>H. coreanum</i> Nakai <i>H. mikawanum</i> (Seriz.) Seriz.	N N N N	N N N N	Y N N N
	<i>H. barbatum</i>	Ribbon-like	1		Y	—	Y
	Sequence not obtained		1				

Notes: Range 1, inside the quadrat; Range 2, at a distance of 10 m (for OKT and CCB) or 5 m (for the remaining sites) or less from the quadrat; Range 3, 10 km geographical grid square including the site. Y = present; N = absent, — = not applicable (species that were present as sporophytes in range 1 were not surveyed in range 2). Asterisks indicate possible correspondences in case of ambiguously identified gametophytes.

Our study did not reveal any clear patterns between species diversity at the sporophyte and gametophyte level: although the number of species recorded as sporophytes was higher in the subtropical sites (IOT 1–3 and ISG), this was not reflected in the number of gametophyte species observed (Table 3). The inability to detect such a relationship should it exist is likely due to the small sample size of the present study. However, we did observe that noncordiform gametophytes occurred more often on rocky substrates (Tables 1, 3), a habitat preference that has been noted in previous studies (Farrar et al., 2008). Furthermore, despite the limited number of sites, gametophytes growing distantly from conspecific sporophytes were discovered multiple times, especially in species producing noncordiform gametophytes. This result suggests that information on the distribution of gametophytes is essential for a fundamental understanding of fern floras. In fact, Ebihara et al. (2009) reported the formation of hybrid sporophytes by gametes likely produced by independently growing gametophytes. Independent gametophytes may play an important role in reticulate speciation processes. Further development of gametophyte survey methods is expected to clarify fern species distributions and contribute to our understanding of fern evolution.

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