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Life cycle matters: DNA barcoding reveals contrasting community structure between fern sporophytes and gametophytes

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Abstract. Ferns are the only major lineage of vascular plants that have nutritionally independent sporophyte (diploid) and gametophyte (haploid) life stages. However, the implications of this unique life cycle for fern community ecology have rarely been considered. To compare patterns of community structure between fern sporophytes and gametophytes, we conducted a survey of the ferns of the islands of Moorea and Tahiti (French Polynesia). We first constructed a DNA barcode library (plastid *rbcL* and *trnH-psbA*) for the two island floras including 145 fern species. We then used these DNA barcodes to identify more than 1300 field-collected gametophytes from 25 plots spanning an elevational gradient from 200 to 2000 m. We found that species richness of fern sporophytes conforms to the well-known unimodal (i.e., mid-elevation peak) pattern, reaching a maximum at ~1000–1200 m. Moreover, we found that fern sporophyte communities become increasingly phylogenetically clustered at high elevations. In contrast, species richness of fern gametophytes was consistent across sites, and gametophytes showed no correlation of phylogenetic community structure with elevation. Turnover of sporophyte and gametophyte communities was closely linked with elevation at shallow phylogenetic levels, but not at deeper nodes in the tree. Finally, we found several species for which gametophytes had broader ranges than sporophytes, including a vittarioid fern with abundant gametophytes but extremely rare sporophytes. Our study highlights the importance of including diverse life history stages in surveys of community structure, and has implications for the possible impacts of climate change on the distribution of fern diversity.

Key words: DNA barcoding; elevational gradient; fern; gametophyte; Moorea; phylogenetic community ecology; Pteridophyte; rbcL; sporophyte; Tahiti; trnH-psbA; tropical islands.

Introduction

Ferns (monilophytes) are an ancient vascular plant lineage dating back ~300 million years (Ma) that has diversified into an astonishing array of ecological niches (Schneider et al. 2004). Ferns occupy habitats ranging from xeric to aquatic, and their morphologies span moss-like filmy ferns only a few cm in height to tree ferns reaching over 10 m. What unites this amazing diversity is a unique life cycle: the alteration of morphologically distinct diploid sporophyte and haploid gametophyte generations that are nutritionally and physically independent from each other for the majority of the life cycle. This contrasts with the life cycles of nearly all other land plants: in both bryophytes and seed plants, the two halves of the life cycle are completely overlapping due to nutritional dependence, and do not constitute distinct ecological entities. This independence of generations has important implications for fern evolution. For instance, it is possible for fern gametophytes to have broader distributions than their conspecific sporophytes, since not every gametophyte necessarily produces a sporophyte (Farrar 1990, Watkins et al. 2007*b*). Indeed, some fern species have reached an extreme degree of separation in this regard and no longer produce sporophytes at all, rather persisting solely in the haploid state via asexual reproduction (Farrar 1967, Raine et al. 1991, Ebihara et al. 2008, Pinson and Schuettpelz 2016).

Despite the potential for fern sporophytes and gametophytes to occur over partially disjunct ranges, there are few comprehensive studies of the ranges of gametophytes and sporophytes of the same species (Farrar et al. 2008). This is due to the cryptic nature of the fern gametophyte: these tiny (typically <1 cm) plants are easily overlooked in the field, and generally lack morphological characters for species-level identification (Nayar and Kaur 1971). Thus, the vast majority of fern field surveys include only the sporophyte generation, leaving a critical gap in our knowledge of fern distributions (e.g., Tuomisto and Poulsen 2000, Kessler 2001, Karst et al. 2005, but see Hamilton and Lloyd 1991, Watkins et al. 2007*b*). However, recently developed DNA-based identification techniques (i.e., DNA barcoding; Hebert et al. 2003) now allow for much more accurate identification of fern gametophytes, opening new avenues for investigations into their distribution,

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ecology, and evolution (Schneider and Schuettpelz 2006, Li et al. 2009, Ebihara et al. 2010, de Groot et al. 2011, Chen et al. 2013). Furthermore, the DNA sequences generated by such a study can also be used to infer phylogenetic trees, thus enabling a phylogenetically informed study of community ecology (Kress et al. 2009, Muscarella et al. 2014). Despite rapid advancements at the intersection of community ecology and phylogenetic studies, comparative phylogenetic methods are just beginning to be applied to fern ecology (Kluge and Kessler 2011, Hennequin et al. 2014, Lehtonen et al. 2015). However, there has been no community phylogenetic study of ferns to date investigating both gametophytes and sporophytes.

The goals of our study are to compare the ranges of fern gametophytes and sporophytes across a well-sampled island flora and infer processes of community assembly in ferns in a phylogenetic context. We first developed a DNA barcode system for identification of fern gametophytes by assembling a reference DNA library and verifying its species discrimination potential in the context of our study system (Moorea and Tahiti, French Polynesia). We then conducted community surveys of co-occurring fern gametophytes and sporophytes, and identified the gametophytes using our DNA barcode library. We investigated the following questions: (1) Do elevational ranges differ between fern sporophytes and gametophytes of the same species? (2) Are there any patterns in phylogenetic community structure along the elevational gradient, and do they differ between life stages? (3) What are the environmental factors that determine fern community composition and turnover, and do they differ between life stages?

Methods

Study site

Moorea and Tahiti (17.5–18.0° S, 149–150° W) belong to the Society Islands, a tropical oceanic archipelago located more than 5000 km from the nearest continental landmass (Fig. 1A). This distance acts as a strong barrier to dispersal (Carson and Clague 1995, Dassler and Farrar 2001), which combined with the young age of the islands (Moorea ~1.65 Ma [1 Ma = 10^6 yr]; Tahiti ~0.65 Ma; Dymond 1975), has led to a relatively small, yet phylogenetically diverse fern flora (~165 species; Florence, *in press*; 8 out of 11 orders sensu PPG I 2016). Although the two islands are only 17 km apart, Moorea is smaller (134 km^2) and reaches a maximum elevation of 1207 m, whereas Tahiti is much larger (1040 km^2) and has three peaks above 2000 m (Mt. Orohena at 2241 m, Mt. Pito Hiti at 2110 m, and Mt. Aorai at 2070 m). With the exception of a few endemic taxa, the flora of Moorea is generally a subset of the flora of Tahiti. The fern flora of Moorea has been the focus of several recent papers (Murdock and Smith 2003, Ranker et al. 2005, Nitta et al. 2011) and was surveyed as part of the Moorea Biocode Project (http://moorea biocode.org). The ferns of French Polynesia will be described in the next volume of the flora of French Polynesia (Florence, *in press*). We used these sources to compile a list of all ferns occurring on Moorea and Tahiti.

Community survey

Fern community diversity was sampled at a total of 25 sites established every ~200 m along elevational gradients from ~200 to 1200 m (Moorea) and 600 to 2000 m (Tahiti) during the Austral winters (June–August) of 2012–2014. Moorea sites were located mainly on three mountains, each with a trail to the summit: Mt. Rotui (899 m), Mt. Mouaputa (880 m), and Mt. Tohiea (1206 m). Tahiti sites were established on the only mountain with reliable trail access to the peak, Mt. Aorai (2066 m). Two to three sites were established for each elevation point (e.g., ~200, 400, 600 m, etc.) to 1200 m. Above 1200 m, replication and placement of sites was restricted because of extremely steep terrain; thus, single sites were established at ~1300, 1700, 1800, and 2000 m (Fig. 1A).

At each site, fern sporophytes (hereafter "sporophytes") were sampled in ~100-m^2 plots ("sporophyte plots"). In many cases, it was impossible to determine the exact number of sporophyte individuals because fronds growing close to each other may be either distinct individuals, or arise from a single underground rhizome. Furthermore, the complex, multilayered fern canopy including ~3 m tree ferns to tiny (<5 cm), colonial mat-forming filmy ferns also made assessing abundance by percent cover problematic. Therefore, we recorded presence/absence of each fern species in 2 × 2 m subplots. Plot design differed slightly between Moorea and Tahiti: on Moorea, each plot was 10 × 10 m and divided into a grid of 25 2 × 2 m subplots; on Tahiti, 24 2 × 2 m subplots were designated around the perimeter of a 14 × 14 m square. Presence/absence of species in subplots were summed to produce an abundance ranking, where each species' abundance ranged from 0 (not observed in the plot) to 24 or 25 (observed in all subplots). One voucher specimen was collected per species for each plot.

Sampling fern gametophyte (hereafter "gametophyte") diversity in the field is made difficult by two factors: most gametophytes are often tiny (typically <1 cm^2) and difficult to locate, and cannot generally be identified to species based on morphology. We therefore conducted initial surveys to locate microsites harboring gametophytes within each sporophyte plot, then sampled terrestrial gametophytes in 50 × 50 cm subplots, each consisting of a grid of 10 × 10 cm squares (Ebihara et al. 2013). A single gametophyte, when present, was sampled from each square. In the case multiple gametophytes were present in a single square, the one closest to the center was sampled; clonal individuals occurring continuously across two or more squares were only sampled once. Epiphytic gametophytes were sampled by morphotype (at least three individuals per morphotype observed) at ~2 m height on trees within each sporophyte plot. The final "gametophyte plot" included all terrestrial and epiphytic gametophytes collected from a given site. We

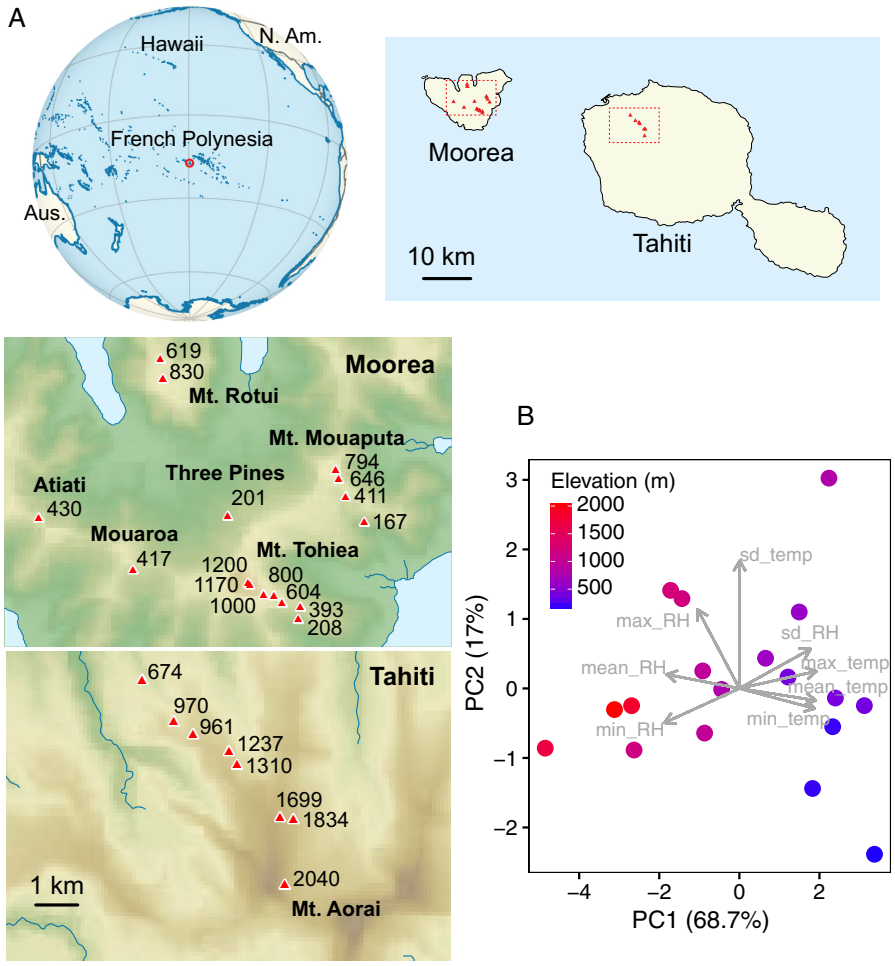

FIG. 1. Location and environmental characteristics of study sites. (A) Maps of study area. Location of Tahiti and Moorea, French Polynesia on world map indicated by circle. Dashed boxes show locations of insets for Moorea and Tahiti (both to same scale). Study sites indicated by triangles with elevation in meters. Maps adapted from Wikimedia Commons under Creative Commons License. (B) Principal component analysis of climatic variables (means of daily minimum, mean, maximum, and SD of temperature and relative humidity) from 12 November 2013 to 17 January 2014 for all sites with climatic data available (n = 18). Color gradient indicates elevation, from ~200 m (blue) to 2000 m (red).

sought to collect a minimum of 50 individuals from each site by repeating this sampling procedure as necessary, but the actual number varied due to local conditions and time restrictions. To test the effect of sampling effort on species richness, we sampled two sites ("Three Pines 201 m" and "Mouaputa 646 m") each for ~100 individuals, and compared species accumulation and sampling completeness curves between sites at similar elevations (see *Species richness*). Photographs of fresh gametophytes were taken using an Optio W60 camera (Pentax, Tokyo, Japan) mounted to a dissecting microscope. Each sampled gametophyte was cut in two; one half was kept as a herbarium voucher, and the other used for DNA extraction. DNA vouchers were kept in packets made from folded coffee filters, which were then placed in sealed plastic bags with silica gel. Herbarium vouchers were fixed in FAA (50% ethanol:formaldehyde:acetic acid, 90:5:5), then transferred to 70% ethanol in 1.5-mL Eppendorf tubes. Voucher specimens were deposited at UC, with duplicates at GH and PAP.

Environmental survey

Data loggers were used to record ambient relative humidity (RH) and temperature once every 30 min from 12 October 2013 to 5 July 2014 (Moorea) or once every hour from 18 July 2012 to 6 February 2015 (Tahiti) at 23 of the 25 sampling sites. Data were downloaded once every ~3–6 months. At each site, one data logger was mounted at ~2 m on a tree trunk or pole. Data loggers were protected with radiation shields to prevent heating due to direct solar radiation and buildup of moisture on the sensor tip. Data logger and radiation shield models differed between Moorea and Tahiti: on Moorea, we used Hobo Pro v2 data loggers with the RS3 Solar Radiation Shield (Onset Corporation, Bourne, Massachusetts,

USA), whereas on Tahiti we used RHTemp 1000 data loggers (MadgeTech, Warner, New Hampshire, USA) protected with custom radiation shields made from plastic circuit boxes (Taputuarai et al. 2014). We calculated daily maximum, mean, minimum, and SD for temperature and relative humidity from the raw data, then calculated the overall mean of each of these for each site. Days during which any of the data loggers malfunctioned and failed to record were excluded from the analysis.

DNA sequencing

To produce the DNA barcode library, we sought to collect at least one individual (sporophyte phase) of each fern species from Moorea and Tahiti. Our final sampling included 99% of the known species diversity of Moorea (~130 species) and 88% of the known species diversity of Tahiti (~165 species). Some of the specimens from Moorea were collected as part of the Moorea Biocode Project. Complete sampling of fern diversity at our site is made difficult by the complex topography of the islands, the rarity of some taxa, and the need for taxonomic revision in certain groups (e.g., Aspleniaceae, Hymenophyllaceae, Thelypteridaceae). However, the location of our transect on Tahiti (Mt. Aorai) has been historically well sampled, and we are confident that we have all the species known from that site, as well as nearly all of the species on Moorea, represented in our DNA barcode library. Leaf material was preserved on silica gel, and DNA extraction performed using the DNeasy Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions or CTAB (Doyle and Doyle 1987). Polymerase chain reaction (PCR) amplification of chloroplast *rbcL* and the *trnH-psbA* intergenic spacer was performed using primers and thermocycler protocols of Schuettpelz and Pryer (2007) and Tate and Simpson (2003), respectively. Raw PCR products were sent to Genewiz (South Plainfield, New Jersey, USA) for cleaning and sequencing. Forward and reverse PCR primers were used for sequencing, as well as internal primers ESRBCL654R and ESRBCL628F (Schuettpelz and Pryer 2007) for *rbcL*. ABI files were imported into Geneious v8 (Kearse et al. 2012), where they were assembled into contigs, which were then used to produce consensus sequences. All newly generated DNA sequences have been deposited in GenBank (accessions KY099742–KY100006; Data S1).

DNA extraction and sequencing were performed similarly for gametophytes, except that the CTAB protocol was modified to 96-well plate format for high-throughput preparation and sequencing due to the large number (>1500) of individuals (Beck et al. 2012). DNA extraction was not possible for some specimens that were lost or deemed too small to remove tissue and retain a morphological voucher. For gametophytes, forward primer ESRBCL1F and internal reverse primer ESRBCL654 were used, producing a ~650 bp amplicon (hereafter referred to as "*rbcL*-a"; Kress and Erickson 2007). Only the forward primer was used for sequencing, and *trnH-psbA* was only sequenced in the case that *rbcL*-a could not be obtained (see *Species identification of gametophytes*). ABI files were imported into Geneious and ends automatically trimmed with a 0.5% error cutoff. Due to the large number of redundant sequences, we did not deposit any gametophyte sequences in GenBank that were already represented in the DNA barcode library.

Phylogenetic analysis

Our goal here was to infer an ultrametric tree with branch lengths that are as accurate as possible to analyze phylogenetic diversity at our site and enable comparison across studies (Whitfeld et al. 2012). Therefore, rather than use only the taxon/gene sampling from Moorea and Tahiti, which would lack several clades and prevent the use of many available fossil calibration points for ferns, we first compiled a broad phylogenetic data set including additional species and genes from GenBank. Indels are common in *trnH-psbA* and render alignment impossible at this broad phylogenetic level, so we excluded *trnH-psbA* from phylogenetic analysis. We used the PHLAWD pipeline (Smith et al. 2009) to obtain one sequence per species for all fern species (NCBI taxon "Moniliformopses") in GenBank (release 210, October 2015) for *rbcL*, *atpA*, and *atpB*. We used our newly generated *rbcL* sequences for ferns from Moorea and Tahiti instead of the GenBank *rbcL* sequence for the same species whenever possible (144 out of 145 species). Outgroup taxa were selected to represent major lycophyte and embryophyte lineages and were manually downloaded from GenBank. Each gene region was aligned separately using MAFFT (Katoh et al. 2002), then concatenated into a single Nexus alignment using Phyutility (Smith and Dunn 2008). We removed 196 sequences that were exactly identical to others in the alignment prior to phylogenetic analysis. The final alignment (4778 bp) included 145 fern species from our study area, 3686 additional ferns, and nine outgroup species (lycophytes and seed plants; 3840 species total). We inferred phylogenetic trees using maximum likelihood (ML) as implemented in the parallel version of RAxML (Stamatakis 2006) using 16 threads (raxmlHPC-PTHREADS -T 16) run on the Odyssey cluster supported by the FAS Division of Science, Research Computing Group at Harvard University. We first did a search using the GTR + G model of sequence evolution on 20 distinct maximum parsimony starting trees, and saved the one with the best likelihood (-m GTRGAMMA -N 20). We performed bootstrap analysis with 100 replicates under the same model, and wrote the output to the branches of the best-likelihood tree. We used treePL (Smith and O'Meara 2012) to infer molecular divergence time estimates on the best-likelihood tree after trimming one of the outgroups (lycophytes) and 1059 other taxa identified by treePL as having extremely short branch lengths, which can interfere with divergence time estimation (Beaulieu and O'Meara 2016). We specified a fixed-age prior (i.e., minimum and maximum age the same) for the

root (euphyllophytes; 411 Ma; Magallón et al. 2013), minimum age priors for three outgroup clades (seed plants, conifers, and angiosperms), and minimum age priors for 26 internal fossil calibration points from Pryer et al. (2004), Schuettpelz and Pryer (2009), and Magallón et al. (2013; Appendix S2: Table S1). We used random subsample and replicate cross-validation (RSRCV) over values from 0.00001 to 100 in 10-fold increments to identify the best rate smoothing parameter. We conducted an initial search with the "prime" option to identify additional optimal parameter settings, which we then used in the final analysis with the selected smoothing value. After completion of dating analysis on the broad sampling data set, we pruned all taxa outside of our study area using the drop.tip function of the ape package (Paradis et al. 2004) in R (R Core Team 2015) to obtain a final ultrametric tree of species from Moorea and Tahiti for use in community phylogenetic analysis.

Species identification of gametophytes

First, we tested the ability of *rbcL*-a and *trnH-psbA* to discriminate between fern species in Moorea and Tahiti by conducting local BLAST searches using the sporophyte DNA library (Ebihara et al. 2010). Separate barcode libraries for *rbcL*-a and *trnH-psbA* were constructed using the makeblastdb command in BLAST (Altschul et al. 1997). During the test, all *rbcL*-a and *trnH-psbA* accessions (one per accession per species) were queried against the library. Species that matched only to themselves 100% over the entire sequence length and no other accessions were considered to be successful identifications; those that matched themselves and one or more other species 100% were considered unsuccessful. We also quantified the variability of each barcode marker using smallest interspecific genetic distances (number of substitutions per site; Meier et al. 2008, Srivathsan and Meier 2012). We found that *rbcL*-a alone could be reliably used as a barcode marker in this regional floristic context (139 out of 145 species, 95% identification success). Therefore, we used *rbcL*-a as a primary barcode marker and *trnH-psbA* as a backup marker only in the case that *rbcL*-a could not be successfully sequenced.

After confirming the utility of these two barcode regions, sequences of field-collected gametophytes were exported from Geneious as a FASTA file and queried against the local BLAST database using the "blastn" search, retaining the top five matches (i.e., subject IDs). To maximize confident identification while accounting for sequencing errors, we used a progressive match stringency test as follows (summarized in Table 1). First, any query failing to match at least a single subject ID at 99.0% similarity was counted as a failure. For queries matching at least one subject at ≥99.0%, the top two hits of each query were compared; in the case that the top subject ID matched the query ID at ≥99.0% similarity and the second best subject ID matched at <99.0% similarity, the query was identified as the top subject ID. If the top two subject IDs both matched at ≥99.0% similarity, the next most stringent test was applied. During this test, if the top subject ID matched at ≥99.5% similarity and the second best subject ID matched at <99.5% similarity, the query was identified as the top subject ID; if the top two subject IDs both matched at ≥99% similarity <99.5%, the query was counted as a failure; if the top two subject IDs both matched at ≥99.5% similarity, the next most stringent test was applied in the same way at 99.7%, and finally at 99.9% similarity levels. If the top two subjects each matched at 100%, the query was counted as a failure.

TABLE 1. Criteria used for identifying field-collected fern gametophytes during BLAST search.

Subject 1 (%)	Subject 2 (%)	Result
Round 1		
<99.0	<99.0	failure
≥99.0	<99.0	subject 1
≥99.0	≥99.0	go to round 2
Round 2		
≥99.0, <99.5	≥99.0, <99.5	failure
≥99.5	<99.5	subject 1
≥99.5	≥99.5	go to round 3
Round 3		
≥99.5, <99.7	≥99.5, <99.7	failure
≥99.7	<99.7	subject 1
≥99.7	≥99.7	go to round 4
Round 4		
≥99.7, <99.9	≥99.7, <99.9	failure
≥99.9	<99.9	subject 1
≥99.9	≥99.9	go to round 5
Round 5		
≥99.9	≥99.9	failure
=100	≥99.9, <100	subject 1
=100	=100	failure

Notes: The top two hits (subject 1 and subject 2) from a BLAST search querying an unknown gametophyte against the local barcode library are checked under conditions of increasing stringency (round 1 through round 5). Conditions (percent identity between query and subject 1 or subject 2) resulting in either a successful ID (query is identified as subject 1) or failure to identify the query are given for each round. See *Methods* for details.

We blasted unidentified sequences against the GenBank nr database to determine the cause of failure (e.g., query matches a fern species not in our reference database; query matches non-fern species thereby indicating contamination, etc.). We verified our putatively successful identifications with morphology: although fern gametophytes cannot typically be identified to species based on morphology alone, morphotypes (e.g., cordate, filamentous, and ribbon) are generally consistent within genera and/or other higher taxonomic groups (Nayar and Kaur 1971). We therefore compared observed morphotype with expected morphotype based on the barcode identification; individuals for which these did not match were excluded as possible cases of contamination.

Species richness

The number of individuals and sampling protocol varied across sites and generations (sporophytes vs. gametophytes), which may influence observed species richness. We compared estimated species richness between

sporophytes and gametophytes using first-order Hill numbers following Chao et al. (2014). We constructed individual-based species accumulation and sample coverage curves including bootstrap confidence intervals with a maximum sample size of 50 individuals using extrapolation/rarefaction in the iNEXT package (Chao et al. 2014) in R. For gametophytes, raw individual counts were used to construct the curves; for sporophytes, the abundance ranking (number of subplots present out of 24 or 25) was used as a proxy for number of individuals.

Phylogenetic community structure

We characterized the phylogenetic community structure of co-occurring fern sporophytes and gametophytes at each site separately using two metrics. We measured mean nearest taxon distance (MNTD), which is the average phylogenetic distance between all pairs of sister taxa occurring in a plot, and mean phylogenetic distance (MPD), which is the average phylogenetic distance between all possible pairwise combinations of taxa occurring in a plot (Webb 2000, Webb et al. 2002). High values of MPD or MNTD indicate phylogenetic overdispersion (i.e., greater phylogenetic diversity than expected under random community assembly), while low values indicate clustering (i.e., less phylogenetic diversity than expected under random community assembly). Mean phylogenetic distance reflects overall phylogenetic diversity, whereas MNTD is more sensitive to recent divergences. To assess statistical significance, we then compared these values to a null distribution of 1000 randomly simulated plots using the ses.mpd and ses.mntd functions in the picante package (Kembel et al. 2010) in R. We assume that ferns, which have tiny, light spores easily transported long distances by the wind, are not dispersal-limited at the scale of our study (<50 km maximum distance between plots) (Tryon 1970). We therefore defined the regional pool as all fern species from Moorea and Tahiti in our phylogenetic tree (145 species), and selected the phylogeny.pool null model, which creates null communities by randomly drawing from this regional pool. We report the standard effect sizes (SES) of MPD and MNTD, which are equivalent to the negative values of the Net Relatedness Index and Nearest Taxon Index of Webb (2000), respectively; we chose to keep the sign in the same direction between the observed value and standard effect size to make results easier to interpret. We measured the β-diversity analogs of MNTD and MPD between plots using the comdist and comdistnt functions in the R package picante (Kembel et al. 2010). We performed all analyses using presence/absence data only as well as weighted by relative abundances. To account for possible under-sampling of gametophyte communities that may drive differences between observed sporophyte and gametophyte community structure (see *Discussion*), we ran analyses on three different data sets: the "full" data set included all observed sporophytes and gametophytes (thus, it included some species that were only observed as sporophytes but not gametophytes and vice-versa); the "restricted" data set included only species that were observed in each generation at least once across all plots (but not necessarily both in the same plot); the "simulated" data set scored gametophytes as present if the sporophyte of that species was observed in the plot (even if the gametophyte was not actually observed; presence/absence only).

Correlation of phylogenetic community structure with environment

We calculated means of eight climatic variables for each site (minimum, mean, maximum, and SD of daily temperature and daily relative humidity) from the raw data logger output. We then log-transformed and scaled each variable to a mean of zero, and subjected them to a principle components analysis (PCA). We tested for spatial autocorrelation of climatic variables (PC axes 1 and 2) using Moran's I (Moran 1950) with the Moran.I function in the R package ape. We conducted linear and second-order polynomial regression for gametophytes and sporophytes separately with species richness, MPD, or MNTD as the response variable, and the first two environmental PC axes as explanatory variables for sites with environmental data available ($n = 18$), and with elevation as the single explanatory variable for all sites ($n = 25$). We compared models using the Akaike information criterion (Akaike 1973) to select the most likely model with the dredge function in the R package MuMIn (Bartoń 2011) for each combination of response and explanatory variables (Burnham and Anderson 2002).

We calculated turnover in community composition (i.e., β-diversity) using Bray-Curtis dissimilarities (species-level turnover), β-MNTD (phylogenetic turnover that is more sensitive to the tips of the tree), and β-MPD (phylogenetic turnover that is more sensitive to the overall tree) (Fine and Kembel 2011). We computed principal components of neighbor matrices (PCNM) using the latitude and longitude of each site (Borcard and Legendre 2002) using the function pcnm. This produces a series of orthogonal eigenvectors describing spatial relationships between sites that can be used to analyze the spatial component of β-diversity (Legendre 2008). We used linear regression to partition variation (adjusted r^2) of each measure of β-diversity in turn as the response variable in relation to environmental PC1, PC2, and the PCNM eigenvectors as explanatory variables with the "varpart" function for sites with environmental data available ($n = 18$). We tested for significance with 1000 permutations using the adonis function. All variance partitioning was done using the R package Vegan (Oksanen et al. 2015).

Results

Environmental survey

Of the 23 data loggers deployed, three failed to record for a portion of the survey time, and four failed completely. This is likely due to the extremely high humidity at many of the sites that may cause electronic devices to fail during

extended use. After excluding days that were missing data, the climatic data set included 67 days of data (12 November 2013–17 January 2014) for 16 sites. To obtain a maximally sampled data set, data that were available for these days from the next year (2014–2015) were used in place of missing data for the Tahiti 1834 and 2040 m sites ($n = 18$ sites in the final data set). There is a linear decrease in mean daily temperature with increasing elevation (0.0052°C per m; linear model, adjusted $r^2 = 0.98$, $P < 1.2 \times 10^{-14}$). The relationship between relative humidity (RH) and elevation is slightly different: RH increases and SD of RH decreases from ~200 to 1200 m; above 1200 m, the relationship reverses, and the air becomes drier with increasing elevation (Appendix S1: Fig. S1). The first two PC axes explained ~86% of the variation in environmental variables (minimum, mean, and maximum temperature and RH, and SD of temperature and RH; Fig. 1B). PC1 is positively correlated with minimum, mean, and maximum daily temperature and SD of RH, and negatively correlated with minimum and mean daily RH; PC2 is positively correlated with SD of temperature. PC1, but not PC2, is correlated with spatial distance ($P < 1 \times 10^{-6}$ and $P = 0.48$ respectively, Moran's *I*).

Evaluation of DNA barcode loci

trnH-psbA showed higher rates of interspecific variation than *rbcL*-a (Appendix S1: Fig. S2), but both performed similarly well in discriminatory power. In our BLAST test of *rbcL*-a and *trnH-psbA* as barcode markers, only six species were found that could not be successfully identified, i.e., matched 100% with another species, for one or both markers. These included *Asplenium gibberosum* (Forst.) Mett. and *Asplenium shuttleworthianum* Kunze (identical *rbcL*-a and *trnH-psbA*), *Microsorum* × *maximum* (Brack.) Copel. and *Microsorum grossum* (Langsd. & Fisch.) S. B. Andrews (identical *rbcL*-a and *trnH-psbA*), and *Prosaptia subnuda* (Mett. ex Kuhn) Copel. and *P. contigua* (G. Forst.) C. Presl (identical *rbcL*-a; identical *trnH-psbA* except for a 5 bp indel). *Asplenium shuttleworthianum* is an octoploid that may have *A. gibberosum* as a parent (Perrie and Brownsey 2005, Shepherd et al. 2008). The status of *M.* × *maximum* as a hybrid between *M. commutatum* (Blume) Copel. and *M. grossum* had been hypothesized on the basis of morphology but not previously tested with molecular data (Copeland 1932, Murdock and Smith 2003). This is the first evidence to our knowledge that the maternal parent of *M.* × *maximum* is *M. grossum*. *Prosaptia contigua* and *P. subnuda* are not known to be hybrids, and can be distinguished by location of sori (marginal on laminar protrusions in the former, on the abaxial laminar surface in the latter); it is possible that these are recently derived sister taxa. We treated each of the three species pairs with identical sequences as a single species for phylogenetic community analysis; since these are either hybrids or very closely related species, it is unlikely this treatment impacted phylogenetic diversity results. Five and four additional taxon pairs (15 species total) were >99.5% similar for *rbcL*-a and *trnH-psbA*, respectively (Table 2). Given that estimates of error rates for Sanger sequences range from 0.001% to 1% (Hoff 2009), we manually inspected any matches that were between 99.5% and 99.9% to verify if the different bases appeared to be due to sequence error (e.g., low read quality at an unexpected site) or interspecific variation (e.g., high read quality at a base pair known to differ between species).

DNA barcode identification of fern gametophytes

A total of 1667 fern gametophytes was collected from all sites (25 sites; mean 60.26 ± 17.34 per site, excluding Three Pines 201 m and Mouaputa 646 m sites, which received extra sampling), of which 1632 were used for DNA

TABLE 2. Species >99.5% similar at barcode markers.

Species 1	Species 2	Genetic distance (%)
rbcL-a		
Asplenium gibberosum	*Asplenium shuttleworthianum*	0
Microsorum grossum	*Microsorum* × *maximum*	0
Prosaptia contigua	*Prosaptia subnuda*	0
Nephrolepis biserrata	*Nephrolepis hirsutula*	0.18
Hypolepis dicksonioides	*Hypolepis tenuifolia*	0.33
Asplenium australasicum	*Asplenium nidus*	0.35
Plesioneuron attenuatum	*Plesioneuron* sp1	0.36
Hypolepis dicksonioides	*Hypolepis* sp1	0.49
trnH-psbA		
Asplenium gibberosum	*Asplenium shuttleworthianum*	0
Microsorum grossum	*Microsorum* × *maximum*	0
Prosaptia contigua	*Prosaptia subnuda*	0
Macrothelypteris polypodioides	*Macrothelypteris torresiana*	0.22
Plesioneuron attenuatum	*Plesioneuron* sp1	0.23
Asplenium affine	*Asplenium robustum*	0.24
Humata anderssonii	*Humata pectinata*	0.39

Note: Prosaptia contigua and *P. subnuda* differ in *trnH-psbA* by a single 5 bp indel, which is excluded from the calculation of genetic distance.

extraction (summarized in Table 3). We were able to successfully obtain at least one of the two barcode markers (*rbcL*-a and *trnH-psbA*) from 1526 individuals, and identify 1323 individuals to species using our reference library. The small size of many gametophytes (<0.01 mg) made DNA extraction difficult, and likely contributed to PCR failure. Thirty-nine individuals had apparently successful PCR (bands visible on an agarose gel), but could not be identified to fern species due to insufficient sequence quality. Fifty-two individuals blasted to species pairs in our reference library that were indistinguishable using *rbcL*-a, the majority of which were *P. contigua*/*P. subnuda*. Contamination by a non-fern species was detected in 46 individuals; contaminants included bryophytes (35 individuals), algae (three individuals), and seed plants (eight individuals). Upon careful inspection of morphology, it appears that 23 of the bryophyte sequences were indeed bryophytes collected by mistake. A smaller number of samples appeared to be contaminated by other fern species, as indicated by observed morphology that did not match morphology predicted by the DNA barcode identification (28 individuals) or multiple sequences from the same individual that matched to different fern species (five individuals). Finally, we recovered sequences from six fern species that were of high quality but were not represented in our reference library. These included three vittarioid ferns (*Antrophyum* sp., 14 individuals; *Haplopteris* sp., two individuals; and *Vaginularia paradoxa* (Fée) Miq. = *Monogramma paradoxa* (Fée) Bedd., 121 individuals), two bird's nest ferns (*Asplenium* sp1, two individuals and *Asplenium* sp2, one individual), and one species closely related to *Asplenium caudatum* G. Forst. (*Asplenium* sp3, 10 individuals). Although sporophytes of *V. paradoxa* from Moorea and Tahiti were not included in our reference library, we were able to identify these gametophytes to species by matching *rbcL* to an accession of *V. paradoxa* from Samoa on GenBank (accession EU024562; W. A. Sledge 1631 (L); 100% match except for a 297 bp region of missing data in the GenBank sequence).

Table 3. Gametophyte DNA barcoding results.

	Species	
	No.	%
Successes		
DNA extracted	1632	97.90†
Successful PCR		
rbcL-a only	1379	84.50‡
trnH-psbA only	110	6.74‡
Both	37	2.27‡
Total at least one sequence	1526	93.50‡
Successful ID		
rbcL-a only	1225	80.28§
trnH-psbA only	86	5.64§
Both	12	0.79§
Total at least one sequence	1323	86.70§
Failures		
DNA could not be extracted	35	2.10†
PCR failed	106	6.50‡
Non-fern contamination		
Bryophyte¶	35	2.29§
Algae	3	0.20§
Angiosperm	8	0.52§
Fern sequences		
Single sequence matches multiple fern species >99%	52	3.41§
Multiple sequences from same indiviudal match multiple fern species > 99%	5	0.33§
Morphology doesn't match sequence	28	1.83§
Low quality sequence	39	2.56§
High quality sequence but no match in database#	30	1.97§
Pseudogene	3	0.20§

† Out of 1667 individuals collected.
‡ Out of 1632 individuals extracted.
§ Out of 1526 individuals with successful PCR.
¶ Based on morphology, 23 are thought to be true bryophytes that were collected by mistake, and the rest contaminations.
# Possibly independent gametophytes (see *Discussion*); not including *Vaginularia paradoxa*, which was treated as a successful ID.

Phylogenetic analysis

The phylogeny inferred using ML analysis (Appendix S1: Fig. S3) was largely in agreement with previous fern plastid phylogenies (Pryer et al. 2001, 2004, Schuettpelz and Pryer 2007, Kuo et al. 2011, Lehtonen 2011), and was much better resolved than a phylogeny based solely on *rbcL* of species from our study area (results not shown). Our divergence times estimated by treePL were somewhat earlier than those of Schuettpelz and Pryer (2009); for example, we found that the polygrammoid lineage began diversifying ~90 Ma, whereas Schuettpelz and Pryer (2009) estimate ~55.8 Ma (Appendixes S1: Fig. S4 and S2: Table S2). Relationships between the four earliest diverging fern lineages (marattioid ferns, ophioglossoids + whisk ferns, horsetails, and leptosporangiate ferns) have been unclear, with recent molecular studies supporting horsetails sister to all other ferns (Knie et al. 2015, Rothfels et al. 2015). Our analysis recovered ophioglossoids + whisk ferns(marattioids(horsetails (leptosporangiates))), but with low support.

α-diversity

In total, 122 species of ferns were observed across the gradient, out of ~165 known to occur in Tahiti and Moorea (Florence, *in press*). Species richness differed between sporophytes and gametophytes: 116 species were observed in the sporophyte phase across all sites (mean 23.5 ± SD 6.9 species per site), whereas only 73 species were observed in the gametophyte phase across all sites (mean 11.0 ± SD 2.8 species per site). Sixty-seven species were observed as both sporophytes and gametophytes (Fig. 2). Sporophytes showed a mid-elevation peak in species richness at ~1000–1200 m (mean 28.2 ± SD 8.1 species per site at 1000–1200 m sites), with progressively less diverse communities above and below this elevation. However, no such mid-elevation peak was observed in gametophyte communities, which

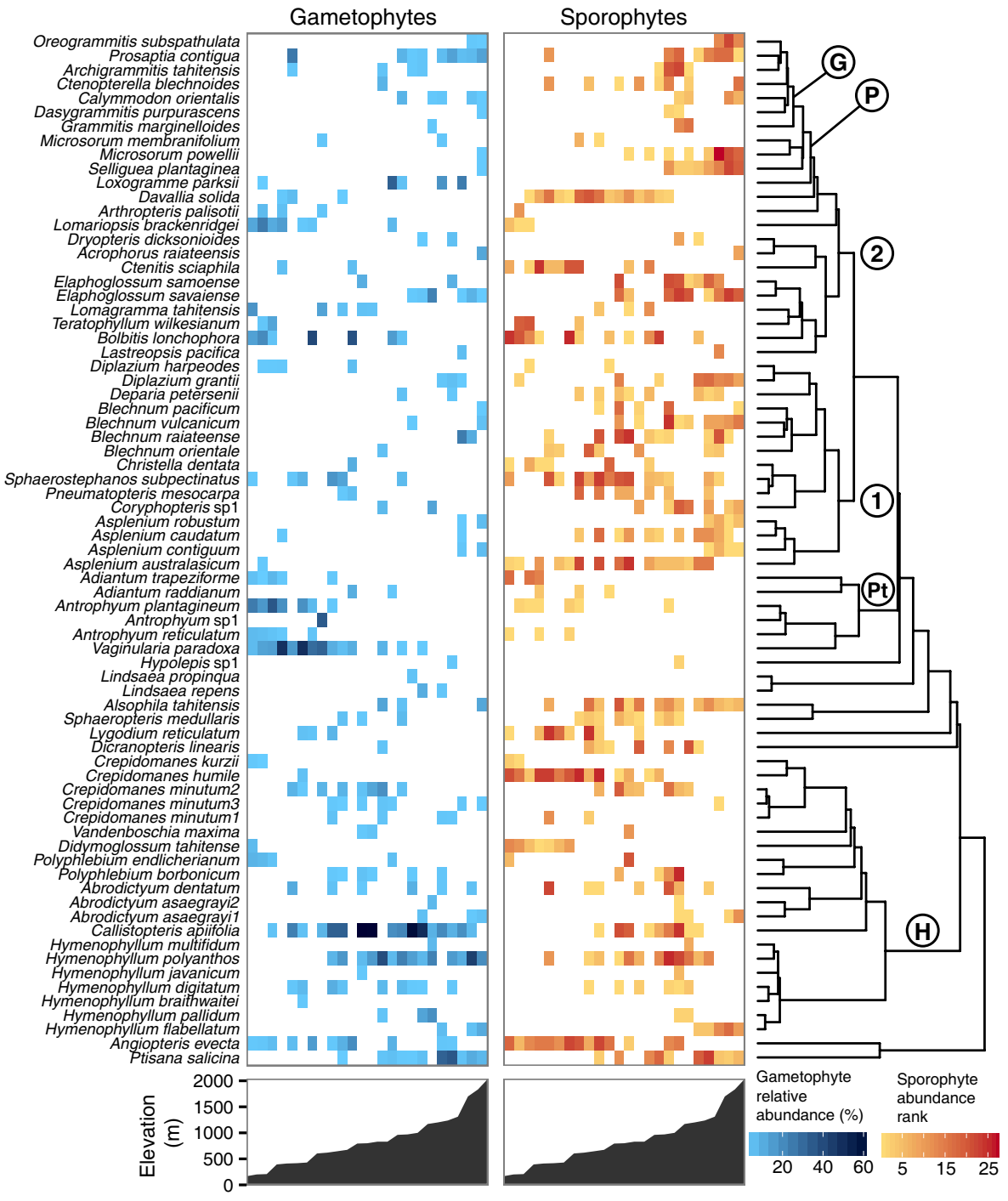

FIG. 2. Abundance of fern species by life stage (gametophytes, left; sporophytes, right) along an elevational gradient from ~200 to 2000 m on the islands of Moorea and Tahiti, French Polynesia. Each column represents one plot, with elevation shown by graph on bottom. Species not observed in a given plot in white; darker colors indicate greater abundance. Note different units for abundance of gametophytes and sporophytes. Phylogenetic tree inferred using maximum likelihood in RAxML. Letters and numbers in circles indicate major clades: H, Hymenophyllaceae; Pt, Pteridaceae; 1, Eupolypods I; 2, Eupolypods II; P, Polypodiaceae; G, grammitid ferns. Restricted data set including only species that were observed in both generations at least once across all plots, except for *Vaginularia pardoxa* and *Antrophyum* sp1, which were only observed as gametophytes.

tended to be ~10 species per site across the gradient, except for the highest site with 18 species (Figs. 2 and 3; Appendix S1: Fig. S5C). The sites selected for intensive sampling of gametophytes (Three Pines 201 m and Mouaputa 646 m) had slightly higher observed richness than the other sites at the same elevation, but estimated sampling completeness

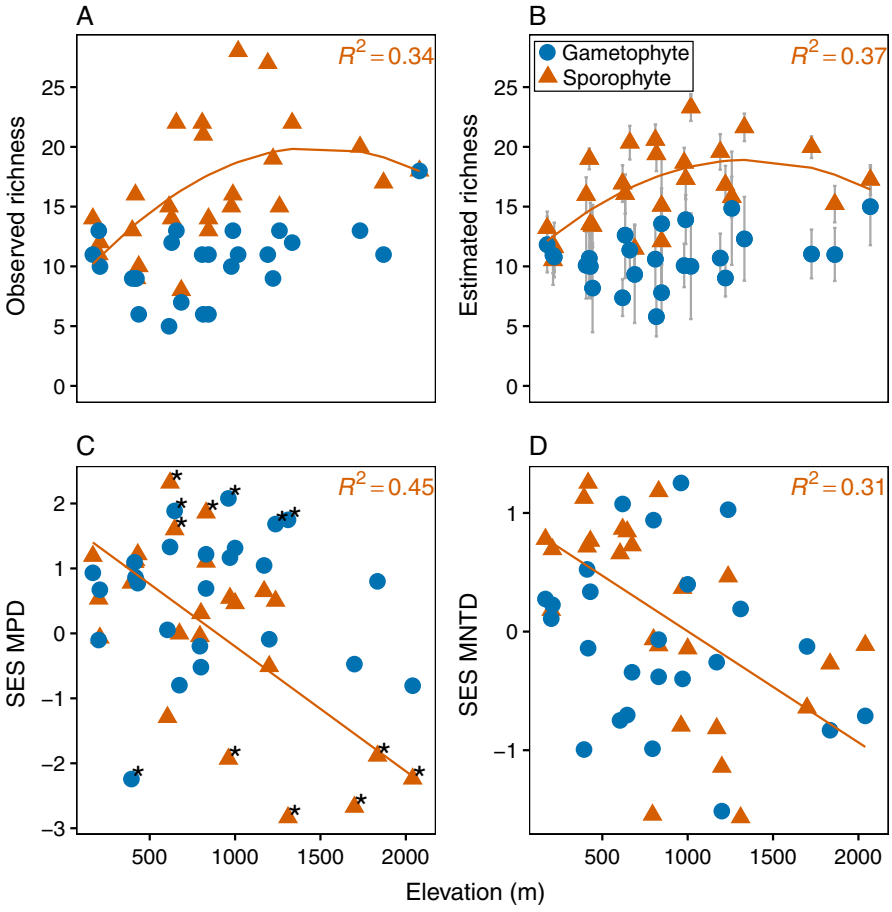

FIG. 3. Species richness and phylogenetic structure of fern gametophyte (blue circles) and sporophyte (red triangles) communities along an elevational gradient from ~200 to 2000 m on the islands of Moorea and Tahiti, French Polynesia. (A) Observed species richness (restricted data set). (B) Estimated species richness (zero-order Hill numbers); error bars are bootstrap 95% confidence intervals (estimated from full data set). (C) Standard effect size (SES) of non-abundance-weighted mean phylogenetic distance (MPD) (restricted data set). (D) Standard effect size of non-abundance-weighted mean nearest taxon distance (MNTD) (restricted data set). Trendlines indicate significant ($P < 0.05$) relationship between richness or phylogenetic community structure and elevation; linear or second-order polynomial regression selected using Akaike information criterion. For panels C and D, values greater than zero indicate phylogenetic overdispersion; values less than zero indicate phylogenetic clustering; asterisks indicate communities with significantly different phylogenetic structure from 999 randomly assembled null communities ($P < 0.05$, two-sided test). For results including other data sets and abundance weightings, see Supporting Information.

was similar for all sites above ~50 individuals (Fig. 4A–D). Comparison of the elevational ranges of the sporophytes and gametophytes of each species revealed that most (~71%) species had sporophyte ranges that equaled or exceeded that of conspecific gametophytes (Table 4). In ~11–15% of species, the range of gametophytes exceeded either the upper or lower elevational limit of sporophytes. Thirty-four species (27%) were observed growing in a plot that lacked conspecific sporophytes, and six species (5%) were observed for which a sporophyte match is completely lacking in our database. Hymenophyllaceae accounted for a disproportionately large number of species with gametophytes occurring beyond the range of sporophytes (Table 4, Fig. 2).

Neither collection curves for sporophytes nor gametophytes reached an asymptote, indicating that increasing the number of sites may recover additional species; however, the slope of the curve was steeper for sporophytes than gametophytes (Fig. 4E). Gametophyte communities tended to have a more skewed abundance distribution than sporophytes, and were often dominated by one or a few species: lower elevation sites (~200–400 m) were dominated by vittarioid ferns (*Antrophyum plantagineum* (Cav.) Kaulf. and *V. paradoxa*), whereas mid- to upper elevation sites were dominated by filmy ferns (Hymenophyllaceae), in particular *Callistopteris apiifolia* (Presl) Copel. (~600–1200 m) and *Hymenophyllum polyanthos* (Sw.) Sw. (~1800–2000 m; Appendix S1: Fig. S8A).

For phylogenetic community structure, we present results from the most conservative analysis (non-abundance weighted analysis of the restricted data set), and refer the reader to the Supporting Information for alternate analyses/data sets. There was no significant difference in mean phylogenetic community structure (MPD

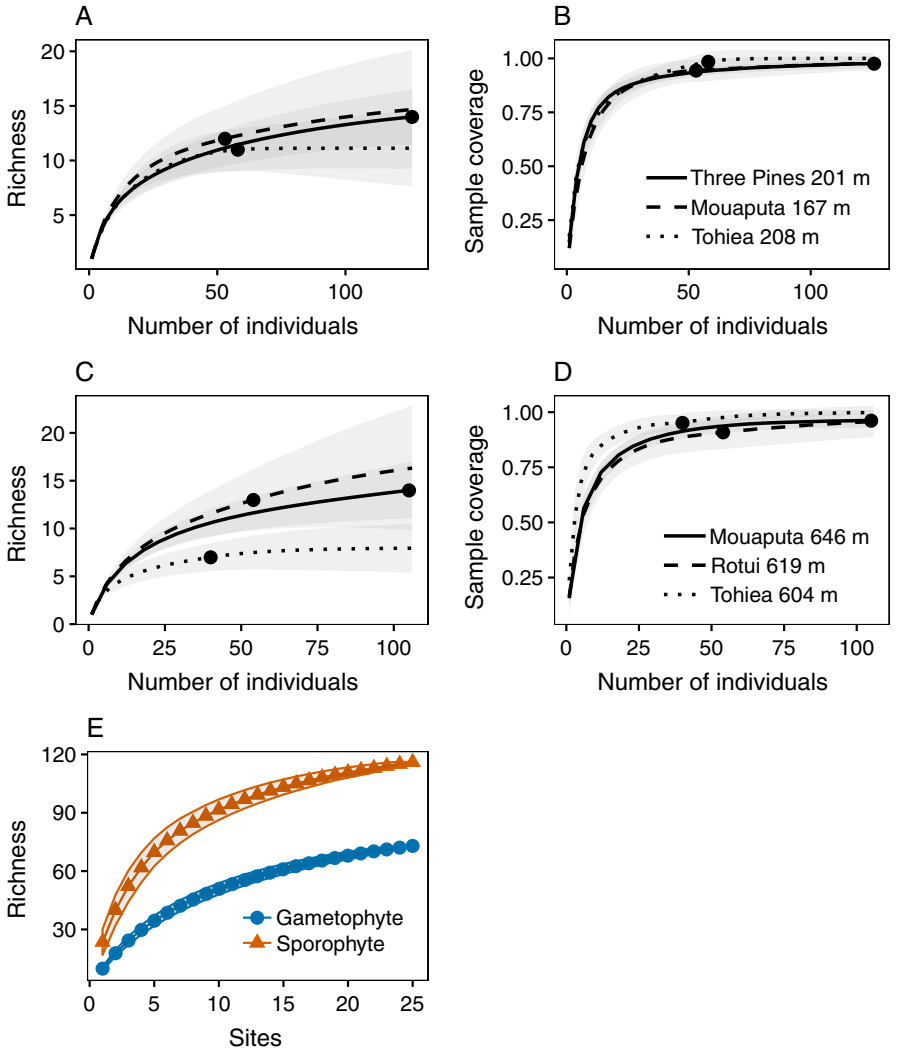

FIG. 4. Collection curves. (A, C) Individual-based rarefaction/extrapolation curves of fern gametophytes. (B, D) Individual-based sample completeness curves of fern gametophytes. Dots indicate observed values; portion of line before dot are rarefied values; portion of line after dot are extrapolated values; bands indicate ±SE. Panels divided by elevation; (A) and (B) are for ~200 m plots; (C) and (D) are for ~600 m plots. "Three Pines 201 m" and "Mouaputa 646 m" plots (solid lines) each received approximately twice the normal collection effort (~100 individuals instead of 50). (E) Site-based collection curve for fern gametophytes (blue circles) and sporophytes (red triangles) across all plots. Band indicates ±SE.

or MNTD) between sporophytes and gametophytes overall (paired t test, $P = 0.2007$ and 0.2794, respectively), and only a small number of communities showed phylogenetic structure that was significantly more clustered or overdispersed relative to the null model (Fig. 3; Appendix S1: Fig. S5A, B). However, the trend between phylogenetic community structure and elevation differed between the two generations: we found that MPD and MNTD tend to become more negative with increasing elevation in sporophytes, indicating that sporophyte communities become more phylogenetically clustered at high elevations. In contrast, phylogenetic community structure of gametophytes showed no correlation with elevation in most analyses (Fig. 3; Appendix S1: Fig. S5A, B), and a positive correlation only in the abundance-weighted MPD analysis of the full data set (Appendix S1: Fig. S5A). For sporophytes, increased abundance of species-rich clades such as grammitid ferns at high elevations contributed to clustering; for gametophytes, the widespread occurrence of early-diverging filmy ferns contributed to overdispersion (Fig. 2). The negative correlation between phylogenetic community structure and elevation in sporophytes was robust to data set or abundance weighting for MPD, but not significant across all data sets for MNTD (Appendix S1: Fig. S5A, B). Furthermore, for MPD, those sporophyte plots that were significantly overdispersed were at lower elevations, and those that were significantly clustered were at higher elevations (Fig. 3; Appendix S1: Fig. S5A). Similarly, estimated richness, MPD, and MNTD were correlated with environmental PC1 for sporophytes,

Table 4. Comparison of sporophyte and gametophyte elevational ranges by family (families not shown for species with sporophyte range ≥ gametophyte range).

Family	Species	
	No.	%
Sporophyte range ≥ gametophyte range		
Total	88	70.97
Gametophyte range > sporophyte range		
Hymenophyllaceae	4	3.23
Gametophyte lower range < sporophyte lower range		
Aspleniaceae	1	0.81
Athyriaceae	1	0.81
Dryopteridaceae	1	0.81
Hymenophyllaceae	8	6.45
Polypodiaceae	2	1.61
Pteridaceae	1	0.81
Total	14	11.29
Gametophyte upper range > sporophyte upper range		
Dennstaedtiaceae	1	0.81
Dryopteridaceae	1	0.81
Hymenophyllaceae	9	7.26
Lomariopsidaceae	1	0.81
Marattiaceae	1	0.81
Polypodiaceae	2	1.61
Pteridaceae	2	1.61
Tectariaceae	1	0.81
Total	18	14.52
Gametophyte without sporophyte in plot		
Aspleniaceae	1	0.81
Athyriaceae	2	1.61
Dennstaedtiaceae	1	0.81
Dryopteridaceae	3	2.42
Hymenophyllaceae	13	10.48
Lomariopsidaceae	1	0.81
Marattiaceae	2	1.61
Polypodiaceae	5	4.03
Pteridaceae	4	3.23
Tectariaceae	1	0.81
Thelypteridaceae	1	0.81
Total	34	27.42
Gametophyte without sporophyte in database		
Aspleniaceae	3	2.42
Pteridaceae	3	2.42
Total	6	4.84

Notes: The minimum observed elevation was subtracted from the maximum observed elevation to determine total elevational range of sporophytes and gametophytes separately for each species. Range sizes were compared on the basis of total elevational range, lowest elevation, highest elevation, occurrence of gametophyte without sporophyte in a given survey plot, and occurrence of gametophyte without a sporophyte match in the reference database.

but relationships between community structure and environment was weak (MNTD, estimated richness) or non-existent (MPD, observed richness) for gametophytes (Table 5; Appendix S2: Table S3).

β-Diversity

Sporophyte and gametophyte communities both show strong turnover with elevation (indicated by gradual change from red, or high elevation, to blue, or low elevation, across the NMDS plot from left to right) at the species (Bray-Curtis) and shallow phylogenetic (MNTD) level, but not at deeper phylogenetic levels (MPD; Fig. 5). Observed sporophyte and gametophyte communities differed strongly in species composition, largely due to the fact that many species were only observed growing as sporophytes. In the full data set and the abundance-weighted analysis of the restricted data set, sporophyte and gametophyte communities occupy distinct, non-overlapping portions of the Bray-Curtis NMDS plot (Appendix S1: Fig. S6A). In the non-abundance weighted analysis of the restricted data set, sporophyte and gametophyte communities overlap in the Bray-Curtis plot, but occupy partially non-overlapping areas in the MPD and MNTD plots (Fig. 5).

Table 5. Second-order polynomial and linear models of fern community composition (mean phylogenetic distance [MPD], mean nearest-taxon distance [MNTD], observed species richness, and estimated species richness) in relation to environment (axes 1 and 2 of environmental PCA) for all sites with environmental data available ($n = 18$).

Generation	Adjusted r^2	PC1	PC2
MPD			
G	0		
S	0.44	0.3†	
MNTD			
G	0.47	0.11*	−0.14*
S	0.52	0.23†	
Observed richness			
G	0		
S	0.18	−1.25 NS	
Estimated richness			
G	0.2		0.44 NS
S	0.28	−0.72*	

Notes: Both linear and squared forms of each environmental PC axis were included in the global model, but we only show those that were found to be significant in at least one model. G, gametophyte; S, sporophyte.

*$P < 0.05$; †$P < 0.005$; NS, not significant.

The amount of variance in β-diversity explained by environmental and spatial components varied for different measures of β-diversity (Bray-Curtis, MNTD, and MPD) and generation (gametophyte vs. sporophyte; Fig. 6; Appendix S1: Fig. S7). Very little of the variation in MPD could be explained by environment or space, whereas 25–50% of variation in Bray-Curtis and MNTD distances were attributed to environment, with environmental PC1 much greater than PC2. Spatial variables did not account for a significant portion of variance in β-diversity. Sporophytes were slightly more structured by environmental PC1 than gametophytes.

Discussion

Our study is the first to our knowledge to investigate processes of fern community assembly at a regional scale that includes both gametophytes and sporophytes identified to species. Investigations of gametophyte ecology prior to the advent of DNA sequencing were limited in scope due to

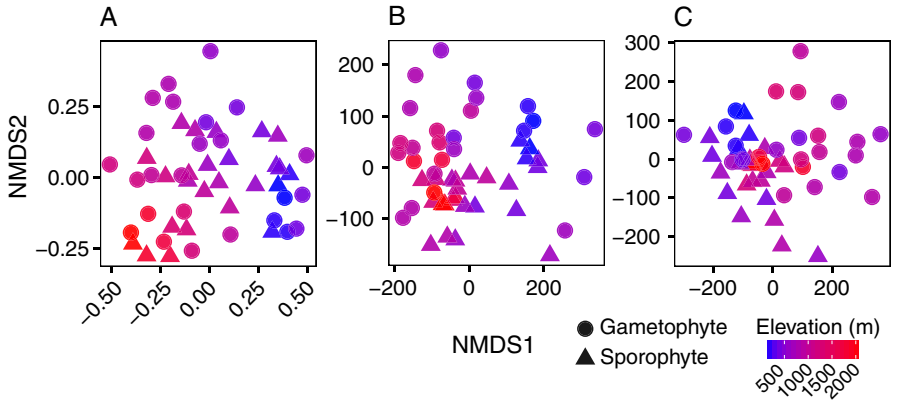

FIG. 5. Nonmetric multidimensional scaling (NMDS) of distances between fern gametophyte (circles) and sporophyte (triangles) plots. (A) Bray-Curtis (i.e., species-level) dissimilarities. (B) Mean nearest taxon distances (MNTD). (C) Mean phylogenetic distances (MPD). Non-abundance-weighted analysis of restricted data set. Color gradient indicates elevation, from ~200 m (blue) to 2000 m (red). For results including other data sets and abundance weightings, see Supporting Information.

insufficient ability to identify gametophytes to species, either relying on a few species with diagnostic characters, extrapolating from nearby juvenile sporophytes, or growing out sporophytes in the lab (Cousens et al. 1988, Peck et al. 1990, Watkins et al. 2007*b*). Recently, DNA barcoding has been increasingly applied to identify field-collected fern gametophytes, and has revealed that gametophytes do indeed occur outside of the range of sporophytes in some species (e.g., Chen et al. 2013, Ebihara et al. 2010, Kuo et al. 2016). Our study builds on these efforts by using DNA barcoding to compare patterns of community composition between fern gametophytes and sporophytes. We acknowledge that there are likely some sampling biases in our gametophyte survey due to the cryptic nature of this life stage, but we have accounted for potential artifacts whenever possible (see *Challenges of sampling a cryptic life stage*).

DNA barcoding and independent gametophytes

DNA barcoding has been the focus of interest in ecology recently because of its potential to vastly accelerate the rate of taxonomic identification in ecological surveys, especially in cases where morphological identification to species is extremely difficult (e.g., tropical insects; Gibson et al. 2014) or impossible (e.g., microorganisms, gut contents; Valentini et al. 2009). Although *rbcL*-a alone would probably not be sufficient to distinguish between closely related fern species in a global study, we found that it could distinguish between nearly all species in our study system (139/145 species = 95% identification success). This is likely because the ferns of the Society Islands contain few endemic sister species that evolved in situ, and rather are composed mostly of species that immigrated to the islands independently (cf. Hennequin et al. 2014), as has

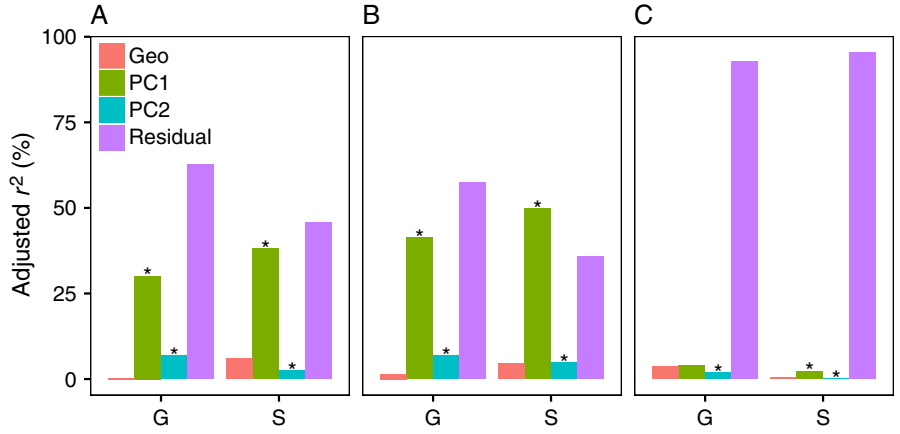

FIG. 6. Variance partitioning of environmental and spatial components that explain turnover in fern community composition (adjusted r^2) by generation (S, sporophyte; G, gametophyte). Spatial components (Geo) are eigenvectors produced by principal components of nearest neighbor matrices of latitude and longitude of each fern community; environmental components are the first two principal components axes (PC1 and PC2) of eight temperature and humidity metrics. β-diversity metrics include (A) Bray-Curtis (i.e., species-level) dissimilarities, (B) mean nearest taxon (MNTD) distances, and (C) mean phylogenetic distance (MPD) distances. Asterisk indicates significance at $P < 0.05$ (tested with 999 permutations using pseudo-F ratios). Non-abundance-weighted analysis of restricted data set. For results including other data sets and abundance weightings, see Supporting Information.

been shown for several angiosperm groups (Hembry and Balukjian 2016). The few cases of failure were mostly due to hybrid taxa that could not be differentiated from their progenitors on the basis of chloroplast sequences alone. For future studies, in the case that finer distinction was required, we recommend adding a low-copy nuclear marker such as *gapCp* (Schuettpelz et al. 2008). In a similar study of the pteridophyte flora of Japan, *rbcL*-a performed slightly worse (89% success rate for sexual diploids), probably due to the fact that the Japanese fern flora includes several endemic radiations for which discriminating close relatives is difficult (Ebihara et al. 2010). We found that *trnH-psbA* had similar discriminatory power as *rbcL*, and slightly higher rates of interspecific variation overall in the context of our local flora (Appendix S1: Fig. S2). However, *trnH-psbA* has been criticized as a barcode marker in ferns due to its low rates of variation in some lineages (e.g., core leptosporangiates; Li et al. 2011). Other fern barcoding studies have used different intergenic regions, such as *trnL-F*, with success (de Groot et al. 2011, Chen et al. 2013); this could be a useful marker in future community-level studies of fern gametophytes.

Once we established the utility of our DNA barcoding library, we identified fern gametophytes to species, and compared distribution patterns between sporophytes and gametophytes. We found that for the majority (71%) of species, fern gametophytes and sporophytes did not differ in their elevational range (i.e., gametophytes were not observed growing beyond the elevational range of sporophytes; Table 4). Despite this, there were several cases where gametophytes were observed growing without their conspecific sporophytes in a given plot, and a few cases where gametophytes were much more widespread than conspecific sporophytes (Table 4). Filmy ferns (Hymenophyllaceae) accounted for a large portion of species with gametophytes that exceeded the elevational range of sporophytes; other groups that tended to have widespread gametophytes included vittarioid ferns (Pteridaceae) and Polypodiaceae. All of these species have non-cordiform gametophytes, which is consistent with the results of Ebihara et al. (2013), who found that species with non-cordiform gametophytes tended to have ranges beyond sporophytes in Japan. One particularly abundant hymenophylloid gametophyte, *Callistoperis apiifolia*, exceeded the upper range of the sporophyte by 634 m and the lower range by 445 m; a similar situation has been documented in this species on Iriomote Island, Japan (Ebihara et al. 2013), as well in the closely related *Callistopteris baldwinii* (D.C. Eaton) Copel. on Hawaii (Dassler and Farrar 1997). In a more extreme case of spatial separation of life stages, the vittarioid fern *V. paradoxa* had some of the most abundant gametophytes observed in our survey (121 individuals from 14 sites), but was not observed as a sporophyte in any of our plots; indeed, sporophytes of this species are extremely rare on Tahiti (only 10 collections known; the most recent in 1982) and possibly no longer occur on Moorea (only three collections known; all from ca. 1850). Furthermore, we identified gametophytes of two additional vittarioid species (*Haplopteris* sp. and *Antrophyum* sp.) and three species of *Asplenium* that were not represented in our reference library. These must either have very rare sporophytes (as in the case of *V. paradoxa*) that we failed to sample, or are "independent gametophytes," i.e., gametophyte populations that persist via asexual reproduction without producing sporophytes (Farrar et al. 2008). Distinguishing between these two scenarios requires additional, thorough sampling of closely related sporophyte-producing ferns from possible source areas. The highest such sampling priorities include rare species known from Tahiti for which we were unable to obtain tissue samples, such as *Antrophyum subfalcatum* Brack. (Florence, *in press*), which may be a match for our *Antrophyum* sp. Similarly, *Haplopteris ensiformis* (Sw.) E. H. Crane is known from the Austral, Cook, and Pitcairn Islands (but not Tahiti; Florence, *in press*), and could be a match for our *Haplopteris* sp. Independent gametophytes are known from vittarioid ferns in North America (*Vittaria appalachiana*; Farrar and Mickel 1991) and Taiwan (Chen et al. 2013, Kuo et al. 2014), but have not yet been reported from the tropical Pacific to our knowledge. As with other epiphytic fern lineages, vittarioid ferns are most diverse in the tropics, and we may expect additional widespread or truly "independent" gametophytes to be found in these ferns as DNA barcoding is applied to other tropical fern floras.

Fern community structure differs between life stages

Despite the fact that gametophytes and sporophytes of most fern species occupy similar elevational ranges (Table 4), we detected different species composition between gametophyte and sporophyte communities (Bray-Curtis distances). This was clearest in the full data set, which includes all observed sporophytes and gametophytes, and quantitatively similar in the abundance-weighted analysis of the restricted data set, which includes only species observed at least once in both phases (Appendix S1: Fig. S6A). This indicates that the contrast in community composition between gametophytes and sporophytes is not simply an artifact driven by "missing species" that went unsampled during our gametophyte survey (see *Challenges of sampling a cryptic life stage*). It is likely driven by gametophytes that occur without sporophytes, as well as changes in abundance between gametophytes and sporophytes. Thus, it appears that the ability of gametophytes to grow independently of sporophytes does indeed impact community-level diversity of ferns.

We found that the relationship between community structure (α-diversity) and elevation varied across life stages in ferns at our site: although fern gametophyte communities show no correlation in phylogenetic community structure with elevation, fern sporophyte communities become more phylogenetically clustered at high elevations, and this clustering is stronger for deep

phylogenetic structure (MPD) than sister-level relationships (MNTD; Fig. 3; Appendix S1: Fig. S5). This contrasts with the results of Kluge and Kessler (2011), who found no overall trend in MPD or MNTD of fern sporophyte communities along an elevational gradient from 100 to 3400 m in Costa Rica. However, Kluge and Kessler (2011) used the number of nodes separating species in a taxonomy-based tree as a proxy for phylogenetic distance rather than branch lengths derived from a time-calibrated phylogeny, which may have diminished power to detect non-random phylogenetic structure (Kress et al. 2009). Lehtonen et al. (2015) detected a negative correlation between MPD and soil fertility (i.e., rich soils tended to have increased phylogenetic clustering) in tropical lowland fern communities using a similar tree-building approach as applied here (pruning from a globally sampled molecular phylogeny), supporting our observation that fern community structure changes along environmental gradients, at least in sporophytes.

Furthermore, we observed the well-known mid-elevation peak in species richness (Colwell et al. 2004) for sporophytes, but not gametophytes (Fig. 3A, B). A meta-analysis of 20 fern elevational transects found that a hump-shaped distribution of species richness is one of the most common patterns of fern diversity on mountains, especially in the tropics (Kessler et al. 2011); however, our study is the first to our knowledge to include gametophytes in an elevational fern transect survey. It is possible that including the gametophyte phase may also reveal different patterns from those observed in sporophytes in other transect studies. Pouteau et al. (2016) analyzed the same sporophyte plot data as well as island-wide diversity of fern sporophytes on Tahiti, and found that climate seems to explain the observed richness peak better than the mid-domain effect (i.e., species richness peak due to randomly distributed elevational ranges within a bounded space; Colwell and Lees 2000).

Assuming that the peak in species richness at mid-elevations is due to optimal environmental conditions and not the mid-domain effect, the negative trend of phylogenetic diversity with elevation in sporophytes that we observed may be due to differences in the evolutionary history of traits involved in filtering at either end of the gradient (Cavender-Bares et al. 2004). This would be expected if sporophytic cold-tolerance traits (relevant at higher, cooler elevations) are evolutionarily conserved, whereas sporophytic drought-tolerance traits (relevant at lower elevations with greater vapor pressure deficit) are evolutionarily labile. Gametophytes, which potentially have wider physiological tolerances than sporophytes, may be able to exist over a wider range of habitats, but only produce sporophytes in a portion of that range (Watkins et al. 2007*a*, Pittermann et al. 2013). Alternatively, gametophytes may have highly specific niche requirements, but are better able to exploit widely distributed, buffered microsites (e.g., crevices in rock and bark within the boundary layer) that are not available to sporophytes due to their larger size (Dassler and Farrar 1997). The importance of microsite availability for establishment of fern gametophytes has been supported by studies in the temperate zones (Cousens et al. 1988, Peck et al. 1990, Flinn 2007) and the tropics (Watkins et al. 2007*b*). Either scenario is consistent with the neutral pattern of phylogenetic community structure we observed in gametophytes relative to sporophytes. Results of desiccation tolerance experiments in one species at our site with particularly widespread gametophytes, *Callistopteris apiifolia*, suggest that both life stages of this species are sensitive to desiccation, and that its widespread gametophytes may be exploiting buffered microhabitats rather than relying on broader physiological tolerances (Nitta et al., *submitted*). We are unaware of any other studies that have tested for differences in phylogenetic community structure between gametophytes and sporophytes in ferns, but studies on ontogenic shifts in community phylogenetic diversity in tropical trees have found greater clustering in adult trees relative to seedlings, consistent with a scenario of environmental filtering acting over the course of development (Webb et al. 2006, Jin et al. 2015). Common garden experiments and tests of relevant physiological parameters on additional gametophyte–sporophyte pairs will help to provide insight into the relative roles of microhabitat tracking vs. physiological niche differences between life stages in ferns.

We found that β-diversity is more strongly correlated with elevation at shallower phylogenetic levels (species level and sister taxon level) and weakly correlated at deeper phylogenetic levels (MPD) (Fig. 5; Appendix S1: Fig. S6). This indicates that similar lineages occur throughout the elevational gradient, but within each lineage there is turnover of closely related species with elevation, and suggests that the ecological niche of ferns is at least somewhat labile (variation within lineage but not between closely related species pairs). Comparative phylogenetic analyses of ecologically relevant traits in fern gametophytes and sporophytes are needed to determine the degree of niche conservatism in specific traits. The lack of turnover explained by distance (Fig. 6; Appendix S1: Fig. S7) is unsurprising, given that our plots were located along a steep elevational gradient across small distances (median distance between plots 6.1 km, maximum distance between plots 40.3 km). Combined with the presumably high dispersal ability of ferns (Tryon 1970), it is likely that differences observed in β-diversity are driven by niche preferences rather than dispersal limitation. Although sporophyte and gametophyte communities both showed turnover with elevation, they occupied different portions of some of the NMDS plots, particularly MNTD and abundance-weighted Bray-Curtis (Fig. 5; Appendix S1: Fig. S6A). Furthermore, variance in β-diversity in sporophytes and gametophytes was partitioned similarly into spatial and environmental components (Fig. 6). Taken together, these results suggest that fern sporophyte and gametophyte communities at a given site may differ in composition, but that they change in similar ways with elevation.

Challenges of sampling a cryptic life stage

One important caveat needs to be mentioned when interpreting the results of this study. We found that total fern sporophyte species richness was higher than that of gametophytes (116 sporophyte species vs. 73 gametophyte species; $n = 25$ plots). This is counterintuitive, given that each sporophyte individual had to have been produced by a gametophyte in the past (except in the case of asexual sporophyte reproduction, which is only known in a few species from our study area). Thus, the sporophyte species richness should in theory represent minimum gametophyte richness, which could possibly be higher due to species that occur as gametophytes but do not produce sporophytes. There are several possible reasons why we did not observe this at our site. One explanation is that we did not sample across all seasons. Our surveys were conducted during the Austral winter (dry season), from June to August. It is possible that only a subset of species were sporulating and germinating as gametophytes at this time. A second explanation is that the lifespan of sporophytes vs. gametophytes can differ greatly. Although long-term demographic data for tropical fern species are few, fern sporophytes tend to be perennial, whereas fern gametophytes of most species have short life spans (<1 yr; but epiphytic gametophytes can be long-lived; Watkins et al. 2007*b*, Farrar et al. 2008). Thus, the sporophytes at a particular plot represent all species that successfully recruited there over a multiple year window, whereas the gametophytes growing at a given time point may change throughout the year. Finally, a third explanation is that our collection efforts were insufficient. It is relatively simple for an experienced botanist to visually check for presence of all fern sporophytes or seed plant species in a plot; however, this is impossible for fern gametophytes, as they generally cannot be identified to species by morphology (with the exception of a few species that have unique characters or character combinations). Gametophyte individuals must be collected and sequenced in order to determine species, which is time-consuming. Due to the limited amount of time available for fieldwork and the scope of this study that includes many plots over an elevational gradient, we could not sample each site exhaustively for all gametophyte individuals, and had to use a semi-randomized sampling approach. Furthermore, many plots were dominated by one or a few very abundant species, with the rest relatively rare (Appendix S1: Fig. S8A). This made random collection of rare species very difficult. Increasing sampling intensity by a factor of two (~100 individuals instead of 50) at the Three Pines 201 m and Mouaputa 646 m site resulted in an increase of species richness by two to three species relative to other sites at similar elevations; however, collection curves and sampling coverage were similar between sites regardless of sampling intensity, indicating that our target of 50 individuals per plot was sufficient (Fig. 4A–D). In a similar study comparing fern gametophyte and sporophyte composition carried out over a full year with exhaustive sampling of gametophytes every two months at a single plot in Taiwan, higher species richness was indeed found for gametophytes relative to sporophytes (Kuo et al. 2014).

Despite this caveat, we feel our data set can reveal patterns in β-diversity that are impossible to address in an exhaustive study confined to a single (or a few) sites such as Kuo et al. (2014). By sampling consistently during the Austral winter over three field seasons, we avoided confounding the effects of seasonality and elevation on species richness. Although rare species do contribute to community structure, we have captured the ecologically dominant species in each plot. Finally, we verified observed patterns whenever applicable with both a restricted data set including only those species that were observed both as sporophytes and gametophytes ($n = 66$ species), as well as a simulated data set that considered "missing" species observed as sporophytes but not gametophytes to be present in the gametophyte phase, thus controlling for the effect of under-sampling of gametophytes.

Conclusion

DNA barcoding of ferns is an efficient tool not only for identification of fern gametophytes, but also to produce DNA sequence data that can be used to infer phylogenetic trees and allow phylogenetically informed ecological analysis. Because of the local nature of our study, even a single marker provided enough resolution to distinguish species in 95% of cases. We found that, just as fern sporophytes and gametophytes may have partially non-overlapping ranges on a species basis (e.g., Chen et al. 2013, Ebihara et al. 2010, Kuo et al. 2016), sporophyte and gametophyte communities also differ in species composition and phylogenetic structure. On Moorea and Tahiti, fern sporophytes become more phylogenetically clustered at higher elevations, but fern gametophytes do not follow this trend. Above ~1200 m, the air became increasingly cool and dry, which may act as a filter to invasion by tropical species that evolved at lower, warmer elevations. Thus, our results are consistent with the hypothesis that fern sporophytes are more subject to environmental filtering than fern gametophytes, which have greater amplitude of ecological tolerances. It has been suggested that the ability of gametophytes to explore new habitats beyond the range of sporophytes may enable ferns to hedge against the impacts of climate change, especially in species with long-lived gametophytes (Farrar 2016). It should be possible to gain greater insight into the mechanisms structuring fern communities and how these will be affected by climate change by coupling community diversity surveys with field and greenhouse experiments on the physiological tolerances of fern sporophytes and gametophytes.

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Supporting Information

Additional supporting information may be found in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/ecm.1246/full

Data Availability

Data associated with this paper have been deposited in Dryad (https://doi.org/10.5061/dryad.df59g) and GenBank (accession numbers KY099742–KY100006).