Identifying cryptic fern gametophytes using DNA barcoding: A review

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ABSTRACT

Ferns and lycophytes are unique among land plants for having sporophyte (diploid) and gametophyte (haploid) generations that can grow independently of each other. While most studies of fern ecology focus on the more obvious sporophytic stage, the gametophyte is critically important, as it is the sexual phase of the life cycle. Yet, fern gametophytes have long been neglected in field studies due to their small size and cryptic morphology. DNA barcoding is a powerful method that can be used to identify field-collected gametophytes to species and allow for detailed study of their ecology. Here, we review the state of DNA barcoding as applied to fern gametophytes. First, we trace the history of DNA barcoding and how it has come to be applied to fern gametophytes. Next, we summarize case studies that show how DNA barcoding has been used to better understand fern species distributions, gametophyte ecology, and community ecology. Finally, we propose avenues for future research using this powerful tool, including next-generation DNA sequencing for in-field identification of cryptic gametophytes.

Key words: DNA barcoding; fern; gametophyte; MinIon; *rbcL*

INTRODUCTION

Ferns and lycophytes (collectively known as pteridophytes) are unique among land plants for having sporophyte (diploid) and gametophyte (haploid) life cycle stages that can grow independently of each other, and therefore may occur separately in nature. This is not the case for the other major land plant groups: in bryophytes (mosses, liverworts, and hornworts), the sporophyte is nutritionally dependent upon and physically attached to the gametophyte, whereas in seed plants, the gametophyte is either contained within the flower (female gametophyte), or does not grow into a nutritionally independent organism (male gametophyte; pollen). For pteridophytes, however, sampling one life stage alone is not sufficient to understand the complete distribution of the organism. This has critical implications for understanding the biology of ferns and lycophytes.

As the sexual stage producing gametes and undergoing fertilization, gametophytes are particularly relevant. However, there are two qualities of pteridophyte gametophytes that make them difficult to study: their small size (typically < 1 cm) and cryptic morphology. While higher taxonomic groups such as family or genus often have defining characters, gametophytes typically lack morphological characters that can be used to differentiate species (Nayar and Kaur, 1971; Fig. 1). In some pteridophytes (most lycophytes; Ophioglossaceae; Psilotaceae; Schizaeaceae; *Stromatopteris*), the gametophytes are subterranean and achlorophyllous, making them even more cryptic and less likely to be noticed. For these reasons, there is a strong tendency among ecological studies of pteridophytes to only sample sporophytes. In contrast, field-based studies of pteridophyte gametophytes are rare, and most of the research on pteridophyte gametophytes has been based on cultivation from spores in the laboratory. These have yielded insights into morphology, development, and reproduction (i.e., mating systems). However, laboratory studies are inevitably limited with regards

to understanding natural genetic diversity, and in some cases rearing gametophytes on agar (a commonly used substrate) may result in artifacts that do not reflect natural processes (Ranker and Houston, 2002). Similarly, inferences of biodiversity and population structure based solely on the sporophyte stage may overlook "hidden" sources of diversity present in cryptic gametophytes (Ebihara et al., 2013; Duffy et al., 2015; Nitta et al., 2017; see 'Scaling up: Community ecology of fern gametophytes' below). Therefore, sampling gametophytes directly in the field is critical for understanding a range of topics related to the biology of pteridophytes.

DNA barcoding, the use of a standard, short region of DNA for species identification (Hebert, Cywinska, et al., 2003), is a powerful method to enable species identification of field-collected pteridophyte gametophytes. Since this method became available, it has revolutionized the study of pteridophyte ecology by enabling accurate identification of this cryptic sexual life stage over a wide taxonomic range. Here, we review the use of DNA barcoding as applied to fern gametophytes, starting with the development of the method, then discussing various case studies, and concluding with future directions. Although the concepts discussed here all apply equally to ferns and lycophytes, we are not aware of any studies using DNA barcoding of lycophytes, so our discussion will focus on ferns.

INITIAL STUDIES

The earliest examples of DNA barcoding applied to fern gametophytes were proof-of-concept studies that demonstrated the viability of the method. The basic approach taken by these studies is still used today: Sanger sequencing of one or more of the most frequently sequenced plastid genes in ferns (Fig. 2). Schneider and Schuettpelz (2006) were the first to do so, using *rbcL* to identify a gametophyte individual that had been in cultivation for > 30 years as *Osmunda regalis* L. Likewise, Li, Tan, et al. (2009) identified a single gametophyte individual kept in cultivation as a species of *Lomariopsis*. In their study, Li, Tan, et al. (2009) expanded gene sampling relative to Schneider and Schuettpelz (2006) to include more rapidly evolving intergenic regions in addition to the widely sequenced, but relatively more slowly evolving, *rbcL*. Despite this, they could not conclusively determine the species due to lack of sufficiently closely related reference sequences in GenBank. These two studies highlight some of the challenges facing DNA barcoding: 1. choice of barcode marker and 2. development of an appropriate reference library.

CHOOSING A BARCODE MARKER

The power of DNA barcoding lies in its universality: the ability to sequence a standard region of DNA and use this for identification of (theoretically) any unknown organism. The choice of mitochondrial *COI* as a universal barcode marker for animals was quickly adopted because this marker can be reliably sequenced across a broad phylogenetic range and tends to show high discrimination power at the species level (Hebert, Ratnasingham, et al., 2003; Hebert, Cywinska, et al., 2003). However, the quest for a similar universal barcode marker in plants has proven to be much more difficult (Hollingsworth et al., 2011). As a practical matter, *rbcL* was already the most commonly sequenced marker in plants, and an obvious strong candidate. However, *rbcL* has a relatively slow rate of evolution at the species level (particularly in angiosperms), and cannot be used to distinguish between species in many cases. From the period spanning the establishment of the Consortium of the Barcode of Life (CBOL) project in 2004 to the early 2010s, a series of studies debated various combinations of candidate barcodes to use across all plants (Chase et al., 2005, 2007; Kress et al., 2005; Newmaster et al., 2006; Kress and Erickson, 2007; Taberlet et al., 2007; Lahaye et al., 2008; Chen et al., 2010; Hollingsworth, 2011). A two-barcode system was eventually established based on *rbcL* and *matK* (Hollingsworth et al., 2009), but due to a rearrangement of the chloroplast in the ancestor of ferns, no universal primers are available that work for *matK* across

all ferns (although multiple *matK* primer sets have been developed that in total work across ferns; Kuo et al., 2011), and studies continued within ferns to find a set of suitable markers for this group of plants.

Ebihara et al. (2010) tested three regions based on sampling the fern flora of Japan, and suggested *rbcL* and the *trnH-psbA* intergenic spacer. Li et al. (2011) showed that *trnH-psbA* is a poor choice due to its decelerated rate of evolution in most groups of ferns (Li et al., 2016). Rather, Li et al. (2011) recommended *rbcL* and *matK* as a core set of fern barcodes, with the *trnLF* spacer, which also has high rates of evolution, as an alternate for *matK*. The *trnLF* spacer showed high performance in both amplification success and power to discriminate species in the ferns of Europe (de Groot et al., 2011) and vittarioid ferns in East Asia (Chen et al., 2013). Recently, Nitta, Ebihara, et al. (2020) compared rates of interspecific variation in *rbcL* in three fern floras (French Polynesia, Costa Rica, and Japan), and found that this marker had a higher identification failure rate in continental areas (Costa Rica and Japan) relative to the isolated islands of French Polynesia, which they attributed to the possibilty of higher rates of *in-situ* speciation in continental regions vs. long-distance dispersal in remote oceanic islands. This highlights the need to consider biogeography in DNA barcoding of fern gametophytes, at least when the provenance of specimens is known.

Although Li et al. (2011) demonstrated that *matK* is a strong candidate as a second DNA barcode marker in ferns, their recommendation was predicated on the development of additional primers for this region, but in the decade since, there has been no development of a single set of universal *matK* primers in ferns (Kuo et al., 2011). Rather, most studies have taken an ad-hoc approach, sampling genes and constructing reference libraries that make sense for the purpose of each study, while typically including *rbcL*. We thus consider DNA barcoding in ferns in the broad sense (use of DNA sequences to identify cryptic gametophytes), and do not limit our discussion to any particular "official" barcode.

BUILDING AND USING A REFERENCE LIBRARY

Equally important as choosing a barcode marker is constructing and validating the reference library. Typically for ferns, the reference library consists of sequences obtained from well identified sporophytes that are known to occur in the same region as the sampled gametophytes (Fig. 2). In some cases, species delimitation of sporophytes may be hampered by cryptic speciation and/or hybridization; such situations may require molecular species delimitation (e.g., Pons et al., 2006; Kekkonen and Hebert, 2014; Kapli et al., 2017) or molecular systematic analysis (e.g., Murakami et al., 1999; Ebihara et al., 2005, 2009; Hori et al., 2014; Hori et al., 2018), but a discussion of such methodologies is beyond the scope of this review. Prior to attempting DNA-based identification of unknown gametophytes, the barcode library should be evaluated to ensure that the selected markers are sufficiently divergent between species to allow discrimination. Ideally, markers should also show less variation within than between species, a disjunct known as the "barcode gap" (Meyer and Paulay, 2005). Results of the few studies that have analyzed the barcode gap in ferns vary by marker, taxonomic group, and geographic region; collectively, they do not support a clear barcode gap in ferns (Nitta, 2008; Wang et al., 2016; Liu et al., 2018; Trujillo-Argueta et al., 2021; but see de Groot et al., 2011). Rather, these indicate that DNA barcoding is unlikely to be 100% successful in all cases; therefore, it is important to quantify the expected failure rate and/or expected undistinguishable taxa for each study. As a practical matter, analyzing intraspecific variation increases the cost and effort of sequencing by multiple factors (as it requires sampling each species multiple times) and intraspecific variation is expected to be quite low in diploid, sexual taxa, especially for a slowly evolving marker like *rbcL* (Murakami et al., 2021). Therefore, previous studies including large numbers of taxa (e.g., > 100 species) typically have characterized failure rate using primar-

ily interspecific distances (Ebihara et al., 2010; Nitta et al., 2017), including multiple samples per taxon only in cases of known or putative species complexes (Nitta, Watkins, et al., 2020).

After assembly and validation of the reference library, these sequences are then used to guide identification of the unknown gametophyte sequence. Bioinformatic methods used for identification of fern gametophytes have included BLAST (Altschul et al., 1997) (e.g., Nitta et al., 2017), which provides metrics indicating the probability that the two sequences are identical by chance, and phylogenetic inference (e.g., de Groot et al., 2011). The choice of method depends on the degree of coverage of the reference library and quality of sequences. If the provenance of the gametophyte is unknown, it may be impossible to construct a reference library, and the only option is to conduct a BLAST search against all sequences on GenBank and/or BOLD (e.g., Li, Tan, et al., 2009). In such situations, insufficient sequencing of the world's extant ferns means that there may be no single exact match to the query. This is particularly expected in the case of long-lived sporophyteless gametophytes (see **Range expansions and long-lived sporophyteless gametophytes** below).

One challenge to identifying fern gametophytes with DNA barcoding, particularly when using chloroplast markers, is the high frequency of hybridization in ferns. The chloroplast is generally maternally inherited in ferns (Gastony and Yatskievych, 1992; Vogel et al., 1998; Kuo et al., 2018), so it cannot provide information on the differential parentage of hybrids. In particular, different hybrid taxa sharing a maternal progenitor will have identical cholorplast genomes, so these cannot be identified with plastid markers. A simple % identity or e-value cutoff in BLAST will not work in such situations—not only must the query sequence match the target above some threshold, but also it should not match multiple targets simultaneously. Nitta et al. (2017) implemented an algorithm that identified gametophytes using cutoff values that took such conditions into account, and this approach could be used in other regional studies that include a reference library (Fig. 2). Alternatively, bi-parentally inherited markers from the nucleus could be used instead, but these are both technically more challenging to generate sequence data (PCR is typically much more successful for plastid markers) and to bioinformatically process due to having multiple sequences per individual.

RANGE EXPANSIONS AND LONG-LIVED SPOROPHYTELESS GAMETOPHYTES

A major application of DNA barcoding in ferns has been documenting the distribution of disjunct populations of fern gametophytes relative to their sporophytic counterparts (reviewed in Pinson, Chambers, Nitta, et al., 2017). The ability of fern gametophytes to grow spatially separated from sporophytes reaches an extreme in long-lived sporophyteless species, whereby disjunct populations of gametophytes exist without sexual reproduction or recruitment from spores well beyond the geographic extent of sporophyte producing populations (obligate independent gametophytes *sensu* Kuo et al., 2017). This was first documented in temperate Eastern North American forests in otherwise typically tropical lineages, including vittarioid ferns and filmy ferns (Farrar, 1967). The gametophytes of these tropical lineages have distinct morphologies that allowed them to be distinguished to genus or similar higher taxonomic group.

The knowledge of these obligate independent gametophyte populations in the Eastern United States made them a natural target for study when DNA barcoding became available. Ebihara et al. (2008) used *rbcL* and *rps4-trnS* sequences to identify the obligate independent gametophyte of *Trichomanes* (*s.l.*) *intricatum* Farrar from the Eastern U.S. as being very closely related to *Crepidomanes schmidtianum* (Zenker ex Tasch.) K. Iwats. from Eastern Asia. Although *T. intricatum* was known to belong to the predominantly tropical Hymenophyllaceae, prior to DNA sequencing, there were no suggestions that it might be most closely related to an East-Asian species. Similarly, Pinson and Schuettpelz (2016) utilized four plastid genes (*atpA*, *chlN*, *rbcL*,

and *rpoA*) and one nuclear gene (*DET1*) to investigate the identity of another of the Eastern North American obligate independent gametophytes, *Vittaria appalachiana* Mickel and Farrar, and concluded it was embedded within *Vittaria graminifolia* Kaulf. from Central and South America. Another successful identification of North American independent gametophytes was on the Pacific North Coast, where DNA barcoding revealed that *Hymenophyllum wrightii* Bosch, a primarily East Asian species only occurring rarely as a sporophyte in British Columbia, Canada, actually has abundant populations of gametophytes as far south as Washington state and as far north as Alaska (Duffy et al., 2015). Finally, Pinson, Chambers and Sessa (2017) used *rbcL* and *rpoA* to identify independent gametophytes at a site in Broxton Rocks, GA as belonging to *Didymoglossum petersii* (A. Gray) Copel.

Unraveling the origins of *T. intricatum* and *H. wrightii* spurred on the search for additional populations of independent gametophytes in East Asia. Additional independent vittarioid gametophytes have been found in Taiwan (Kuo et al., 2017), Korea (Park et al., 2020, 2021), and Japan (Ebihara et al., 2013; Kuo et al., 2017). Lee et al. (2020) documented additional populations of *T. intricatum* and *H. wrightii* in Korea. Furthermore, the careful studies of Kuo et al. (2017) and Park et al. (2021), which included observations of gametangia, have helped to provide insights into the mechanisms suppressing functional sporophyte production in these populations.

Recently, Murakami et al. (2021) applied a new approach in the search for independent gametophytes in Japan: they specifically searched for and sampled gametophytes occurring in large, clonal mats based on the idea that these likely represent populations that have been reproducing asexually for long periods of time and are therefore good candidates for obligate independent gametophytes. The approach of Murakami et al. (2021) proved successful, revealing independent populations of *Hymenophyllum mikawanum* (Seriz.) Seriz., a rare and endangered species in Japan, *Haplopteris mediosora* (Hayata) X. C. Zhang, which was previously only known in Japan from a handful of sporophyte collections and whose existence was considered doubtful, and *Antrophyum plantagineum* (Cav.) Kaulf., which had never been previously observed in Japan at all.

Finally, the study of Chen et al. (2019) demonstrates the importance of the reference library in conducting DNA barcoding of fern gametophytes: although Kuo et al. (2017) could not confidently identify the obligate independent gametophyte population in Taiwan to species due to a lack of sufficiently similar reference sequences, Chen et al. (2019) later sequenced additional sporophytes and were able to confirm the identity of the gametophyte population as *Haplopteris capillaris* (Copel.) C. W. Chen, S. Linds. & K. T. Yong.

SCALING UP: COMMUNITY ECOLOGY OF FERN GAMETOPHYTES

Beyond identifying individual populations of gametophytes, some DNA barcoding studies have sought to compare community-level diversity between gametophytes and sporophytes. Since gametophytes have the ability to occur over wider ranges and only produce sporophytes within a portion of that range, it is possible that community structure may vary between the two life stages. Although DNA barcoding is a powerful tool to test this hypothesis, it has only been utilized a small number of times.

Ebihara et al. (2013) leveraged nearly complete sampling of *rbcL* of the ferns of Japan to conduct DNA barcoding surveys of gametophytes at multiple plots scattered throughout the country. Importantly, Ebihara et al. (2013) developed new methods for surveying communities of unidentified fern gametophytes. To quantify community-level diversity while maintaining sampling effort across sites, Ebihara et al. (2013) developed a quadrat system as follows: preliminary surveys were first conducted to identify habitats harboring gametophytes, then a square 0.25 m^2 quadrat

subdivided into 25 10 x 10 cm squares was established using a garden net. A single gametophyte individual, if present, was sampled from the point nearest the center of each square of the net, then cut in half. One half was kept as a voucher and the other used for DNA barcoding. Using this method Ebihara et al. (2013) showed that gametophytes with non-cordate morphologies, like those of the well-known North American independent gametophytes, tend to occur at large distances from sporophytes within the context of the Japanese fern flora. They also found three more taxa of independent gametophytes (two in *Hymenophyllum*, one in *Lomariopsis*) that lacked sporophyte counterparts in Japan.

Nitta et al. (2017) used the same quadrat sampling method as Ebihara et al. (2013) to sample fern gametophytes and compare the community structure of gametophytes and sporophytes along an elevational gradient from ca. 200 m to 2,000 m on the islands of Moorea and Tahiti, French Polynesia. They used *rbcL* as a primary barcode marker with *trnH-psbA* as a secondary marker. Although *trnH-psbA* has lower discrimination power than other markers, it is easy to amplify, and proved useful in some cases when amplification of *rbcL* failed. Interestingly, Nitta et al. (2017) highlighted a challenge to this type of community survey: they observed far fewer species as gametophytes than sporophytes, and the distribution of gametophyte individuals was dominated by a small number of very common species, with a long tail of rare species. This is in contradiction to the expectation that there should be at least as many species in the gametophyte phase as sporophytes, and may be due to skewed abundance distribution or seasonality in spore germination. Nitta et al. (2017) therefore used a reduced dataset only including species that had been observed at least once in each life stage across the elevational gradient to compare gametophyte and sporophyte community composition. This revealed that community structure does differ between fern sporophytes and gametophytes: sporophyte communities became increasingly phylogenetically clustered at high elevations, while gametophytes did not show any significant change in phylogenetic structure with elevation.

Indeed, the expectation of greater species richness in the gametophyte phase was borne out by the study of Kuo et al. (2014), who carried out exhaustive sampling of gametophytes throughout one year at a single site in Taiwan. They did this by establishing six subplots side-by-side and exhaustively sampling the subplots once every two months, then comparing the observed gametophyte diversity with the diversity of sporophytes at the site. This demonstrates that careful sampling is needed to accurately compare community-level diversity between fern sporophytes and gametophytes.

OTHER ECOLOGICAL STUDIES

Non-molecular studies of fern gametophyte ecology have relied on methods such as tracing the ontology from sporophyte back to gametophyte, or targeting taxa with unique morphological characters (e.g., presence of certain types of glandular hairs) that can be used for species-level identification at a particular site (e.g., Lagomarsino et al., 2012; Testo and Sundue, 2014; Watkins and Moran, 2019; Watts et al., 2019). Such methods, while simple and useful, are inherently limited in the number of species that can be studied. DNA barcoding of fern gametophytes clearly has the potential to enable study of a much broader range of taxa, and by extension, ecological phenomena. The morphological and ecological characteristics of the sampled individuals can be used to study various aspects of their biology once their taxonomic identity is known. As with community ecology, such studies are still limited in number, but demonstrate the great potential for this approach.

Nitta and Epps (2009) used DNA barcoding to confirm the identity of gametophytes of *Vandenboschia collariata* (Bosch) Ebihara & Dubuisson, a hemi-epiphytic filmy fern from Costa Rica. Hemi-

epiphytes are often poorly documented—typically, only a portion of the adult plant may be sampled from the host tree—so it is often unknown whether they are primary hemi-epiphytes, which start on the ground and climb up the tree, or secondary hemi-epiphytes, which start on the tree and send roots to the ground. Nitta and Epps (2009) sampled gametophytes and observed juvenile sporophytes at the base of host trees, which confirmed the status of *V. collariata* as a primary hemi-epiphyte. The overall prevalence of primary vs. secondary hemi-epiphytism is still unknown for ferns generally. Since epiphytes are a major component of fern diversity, accounting for ca. 30% of extant fern species, and hemi-epiphytism was likely a involved in the evolution of epiphytism, the approach of Nitta and Epps (2009) could help provide insights into the transition from terrestrial to epiphytic growth in ferns.

Ebihara et al. (2019) expanded on the results of Ebihara et al. (2013) to conduct long-term monitoring of a population of *Pleurosoriopsis makinoi* (Maxim. ex Makino) Fomin gametophytes in Japan. The survey of Ebihara et al. (2013) identified a population of *P. makinoi* gametophytes growing in a site without any sporophytes nearby. Ebihara et al. (2019) then monitored growth of the population for 5 years and compared microclimate of the gametophyte population with that of a site supporting sporophytes. They showed that the gemmae of the gametophytes have seasonal periods of growth, growing in size and number during the spring and summer, and detaching during the winter. The microclimate analysis suggested that a possible cause of sporophyte suppression was low light in winter due to the planting of evergreen conifers. Long-term monitoring of other populations with individuals indetified by DNA barcoding would greately contribute to our understanding of population dynamics and recruitment in ferns.

Other studies have also built upon previous DNA barcoding results to better understand fern ecology. Nitta, Watkins, et al. (2020) and Nitta et al. (2021) both leveraged the DNA barcoding results of fern gametophytes on the islands of Moorea and Tahiti by Nitta et al. (2017). Nitta, Watkins, et al. (2020) scored morphological traits including morphotype and presence of hairs, glands, and gemmae in the gametophytes surveyed by Nitta et al. (2017), then included these trait data in a comparison of the functional community structure of terrestrial vs. epiphytic fern communities. They found that epiphytic fern communities were more functionally diverse than terrestrial communities, despite the generally harsher (drier) conditions of the canopy. Additional studies of fern gametophyte morphological and physiological traits with species identified by DNA barcoding could provide even greater insight into the processes governing community assembly of epiphytic and terrestrial fern communities.

Nitta et al. (2021) took a unique approach which we are not aware has been used by any other study: in order to compare adaptations to water stress between terrestrial and epiphytic filmy ferns, they conducted desiccation tolerance assays on gametophytes of unknown species at the field station where they conducted their field survey, then later identified the individuals to species using DNA barcoding in the lab. This resulted in rather skewed sampling numbers for some species (e.g., the most common species, *Callistopteris apiifolia* (Presl) Copel. was sampled > 50 times, whereas rarer species were sampled only a few times or even just once each), but allowed them to sample a broad range of taxa without the need for cultivation of gametophytes from spores, which are often slow growing and may require > 1 year to attain enough material for physiological tests (Holloway, 1930). One of the most surprising results of this study was that the gametophytes of *C. apiifolia*, which had by far the broadest distribution compared to other species, were incapable of tolerating even slight amounts of desiccation. Rather, Nitta et al. (2021) hypothesize that *C. apiifolia* gametophytes exploit small, well-protected microhabitats at the bases of rocks and trees to avoid desiccation and thus attain a broader geographic range than conspecific sporophytes. This points

to the importance of microhabitat for understanding the distribution of fern gametophytes, which has also been supported by the finding of wide intraspecific variation in desiccation tolerance in vittarioid gametophytes mirroring variation in their environmental conditions (Stevens and Emery, 2015; Chambers and Emery, 2016; Chambers et al., 2017).

ADVANCES IN DNA SEQUENCING TECHNOLOGY

There have been no published studies applying next-generation DNA sequencing to identification of fern gametophytes, to our knowledge. Incorporating next-gen methods will offer two major benefits: the ability to generate much more data far more cheaply (in price per base) than Sanger sequencing, and the possibility to obtain DNA sequences while in the field.

The rapid development of next-generation sequencing platforms and technologies has resulted in an ability to sequence millions of base pairs at a time. In addition, bioinformatic and computational tools have made it possible to pool several samples into a single lane of sequencing, thereby greatly reducing overall sequencing costs relative to traditional Sanger methods. Unfortunately, the application of these technological advancements with respect to metabarcoding studies has been limited because these sequencing platforms generate reads between 300 and 500 bp. This is too short a fragment for barcoding research in ferns, as many of the utilized loci well exceed these limits. Furthermore, successful identification often requires sequencing more than one barcode locus, and Sanger sequencing does not scale well to large numbers of individuals or loci. Recently, Gostel et al. (2020) developed a metabarcoding methodology that overcomes some of these limitations. Utilizing a Fluidigm Access Array, they were able to conduct simultaneous PCR reactions on 96 samples spanning the four major land plant lineages, using 12 primer pairs to amplify four commonly used barcoding loci (*rbcL*, *matK*, *trnH-psbA*, and *ITS*). Reactions were then pooled, cleaned, and sequenced on an Illumina MiSeq, and the reads were trimmed, filtered, then mapped to barcode reference sequences spanning the plant tree of life. Of the four loci examined, *rbcL* and *trnH-psbA* had the highest number of reads per sample for ferns. This protocol may be especially useful when trying to identify fern gametophytes within a community using DNA barcoding as it ultimately saves time and money when a large number of individuals need to be sequenced. However, although microfluidic PCR enables high-throughput multi-locus sequence generation, this method alone cannot overcome the read-length limitations of Illumina platforms (currently ca. maximum of 300bp on MiSeq), and the sequences generated by Gostel et al. (2020) for long (>1000 bp) regions like *matK* included gaps in the middle. Platforms such as PacBio that can generate much longer reads have been used for amplicon sequencing of COI barcodes in insects (Hebert et al., 2018; D'Ercole et al., 2021) and disentangling multiple nuclear alleles in ferns (Rothfels et al., 2017); one promising future direction is to combine high-throughput PCR with long-read technologies to obtain full-length barcode sequences from multiple markers for thousands of individuals at once.

The future of DNA barcoding in ferns will incorporate technological advances to allow for greater sequencing efficiency and ability to conduct research while still in the field. Specifically, most of the previous studies have required the collection of gametophytes from the field, followed by transportation back to the lab for sequencing and subsequent identification via standard Sanger sequencing protocols. While useful, and clearly powerful, this approach may not be ideal given the time, budgetary, and logistical constraints associated with field travel, especially in international contexts. The remote nature of field work also complicates the feasibility of many studies, as many locations lack a field station with the necessary lab equipment and reagents.

A particularly exciting avenue for the future of DNA barcoding in ferns in the field involves the use of a relatively new portable sequencing system. The MinIon Mk1B (Oxford Nanopore Technologies) is

a powerful, portable sequencer that can successfully sequence entire communities (Mafune et al., 2020), and generate long-read sequences (Li and Harkess, 2018; Kuo and Li, 2019). Recent studies have taken advantage of the portability of this instrument by taking it into the field to effectively conduct real-time DNA barcoding studies in both tropical and temperate communities (Parker et al., 2017; Pomerantz et al., 2018). Srivathsan et al. (2018) found that roughly 500 barcodes can be accurately identified within about two hours of sequencing time, with an identification success rate ranging from 99-100% after the implementation of their barcoding strategy and analytical pipeline.

Although the MinIon sequencer is portable and powerful, DNA barcoding still requires the use of many lab instruments and reagents to extract DNA and amplify the targeted genomic region. Simplification of the workflow would enable more rapid identification. Fern gametophytes may be an ideal study system in this case, as PCR can be performed directly using the thallus tissue of fern gametophytes (TD-PCR). This technique, originally published by Li, Kuo, et al. (2009), involves the application of PCR reagents directly to tubes containing small amounts of thallus tissue. To determine the ideal protocol for amplification of the plastid *trnL-L-F* locus, the authors tested various chemical and mechanical methods to either increase PCR success or the lysis of cells. Their results indicated that chemical and mechanical manipulation increased PCR success among most of the examined fern gametophytes. They also found that amplifying each reaction twice (running a first round of PCR, followed by a second round using the first-round PCR product as template) greatly increased amplification success. Recently, Wu and Kuo (in this issue) further refined the protocol published by Li, Kuo, et al. (2009) by redesigning *trnL-L-F* primers to be universal across ferns and improving the tissue disruption step. They also used their improved TD-PCR protocol to reveal additional obligate independent gametophytes in Taiwan belonging to *Lomariopsis*. If tissue-direct methods could be combined with MinIon sequencing, it may become possible to conduct rapid, high-throughput DNA barcoding of fern gametophytes in the field, potentially providing much greater insight into the structure of fern gametophyte communities.

DISCUSSION

The study of fern ecology has long been hampered by the inability to investigate fern gametophytes in the field. While fern gametophytes were previously regarded as an “achilles heel” that limited dispersal due to the requirement of water for fertilization (Page, 2002), or as being too morphologically plastic to be useful for systematics (Bower, 1923), recently there is a much greater appreciation for both their ecological importance and status as a worthy topic of study. The potential for DNA barcoding to identify fern gametophytes to species has both fueled this interest and opened up previously inaccessible avenues for research. Since its inception, DNA barcoding has completely depended on Sanger sequencing of plastid markers. This has yielded important insights, including discovery of independent gametophyte populations and range expansions, as well as a better understanding of the role of gametophytes in community assembly and other aspects of fern ecology. Next-generation DNA sequencing methods are now poised to move the field beyond Sanger sequencing and enable high-throughput sequencing and identification of gametophytes in the field. The next phase of molecular ecology of fern gametophytes will undoubtedly bring new, exciting insights into these fascinating plants.

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AUTHOR CONTRIBUTIONS

J.H.N. and S.M.C. wrote the manuscript.

DATA AVAILABILITY

Code to generate the mansucript is available at https://github.com/joelnitta/gameto-barcode-review.

LITERATURE CITED

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25: 3389–3402.

Bower, F. 1923. The Ferns. Vol. III. University Press, Cambridge.

Chambers, S. M., and N. C. Emery. 2016. Population differentiation and countergradient variation throughout the geographic range in the fern gametophyte *Vittaria appalachiana*. *American Journal of Botany* 103: 1–13.

Chambers, S. M., J. E. Watkins, and E. B. Sessa. 2017. Differences in dehydration tolerance among populations of a gametophyte-only fern. *American Journal of Botany* 104: 598–607.

Chase, M., R. Cowan, P. Hollingsworth, C. van den Berg, S. Madrinan, G. Petersen, O. Seberg, et al. 2007. A proposal for a standardised protocol to barcode all land plants. *Taxon* 56: 295–299.

Chase, M., N. Salamin, M. Wilkinson, J. Dunwell, R. Kesanakurthi, N. Haidar, and V. Savolainen. 2005. Land plants and DNA barcodes: Short-term and long-term goals. *Philosophical Transactions of the Royal Society B: Biological Sciences* 360: 1889–1895.

Chen, C.-W., Y. Huang, L.-Y. Kuo, Q. Nguyen, H. Luu, J. Callado, D. R. Farrar, and W.-L. Chiou. 2013. *trnL-F* is a powerful marker for DNA identification of field vittarioid gametophytes (Pteridaceae). *Annals of Botany* 111: 663–673.

Chen, C.-W., S. Lindsay, K. T. Yong, A. M. A. Mustapeng, V. B. Amoroso, V. D. Dang, and Y.-M. Huang. 2019. Clarification of two poorly known vittarioid ferns (Pteridaceae): *Haplopteris angustissima* and *H. capillaris*. *Systematic Botany* 44: 483–493.

Chen, S., H. Yao, J. Han, C. Liu, J. Song, L. Shi, Y. Zhu, et al. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS ONE* 5: e8613.

D'Ercole, J., S. W. J. Prosser, and P. D. N. Hebert. 2021. A SMRT approach for targeted amplicon sequencing of museum specimens (Lepidoptera)—patterns of nucleotide misincorporation. *PeerJ* 9: e10420.

de Groot, G. A., H. J. During, J. W. Maas, H. Schneider, J. C. Vogel, and R. H. J. Erkens. 2011. Use of *rbcL* and *trnL-F* as a two-locus DNA barcode for identification of NW-European ferns: An ecological perspective. *PLoS ONE* 6: e16371.

Duffy, A. M., M. C. Stensvold, and D. R. Farrar. 2015. Independent gametophytes of *Hymenophyllum wrightii* in North America: Not as rare as we thought. *American Fern Journal* 105: 45–55.

Ebihara, A., D. R. Farrar, and M. Ito. 2008. The sporophyte-less filmy fern of eastern North America *Trichomanes intricatum* (Hymenophyllaceae) has the chloroplast genome of an Asian species. *American Journal of Botany* 95: 1645–1651.

Ebihara, A., H. Ishikawa, S. Matsumoto, and S.-J. Lin. 2005. Nuclear DNA, chloroplast DNA, and ploidy analysis clarified biological complexity of the *Vandenboschia radicans* complex (Hymenophyllaceae) in Japan and adjacent areas. *American Journal of Botany* 92: 1535–1547.

Ebihara, A., S. Matsumoto, and M. Ito. 2009. Taxonomy of the reticulate *Vandenboschia radicans* complex (Hymenophyllaceae) in Japan. *Acta Phytotaxonomica et Geobotanica* 60: 26–40.

Ebihara, A., J. H. Nitta, and M. Ito. 2010. Molecular species identification with rich floristic sampling: DNA barcoding the pteridophyte flora of Japan. *PLoS ONE* 5: e15136.

Ebihara, A., J. H. Nitta, Y. Matsumoto, Y. Fukazawa, M. Kurihara, H. Yokote, K. Sakuma, and O. Azakami. 2019. Growth dynamics of independent gametophytes of *Pleurosoriopsis makinoi* (Polypodiaceae). *Bulletin of the National Museum of Nature and Science, Series B (Botany), Tokyo* 45: 77–86.

Ebihara, A., A. Yamaoka, N. Mizukami, A. Sakoda, J. H. Nitta, and R. Imaichi. 2013. A survey of the fern gametophyte flora of Japan: Frequent independent occurrences of noncordiform gametophytes. *American Journal of Botany* 100: 735–743.

Farrar, D. R. 1967. Gametophytes of four tropical fern genera reproducing independently of their sporophytes in the southern Appalachians. *Science* 155: 1266–1267.

Gastony, G. J., and G. Yatskievych. 1992. Maternal inheritance of the chloroplast and mitochondrial genomes in cheilanthoid ferns. *American Journal of Botany* 79: 716–722.

Gostel, M. R., J. D. Zúñiga, W. J. Kress, V. A. Funk, and C. Puente-Lelievre. 2020. Microfluidic Enrichment Barcoding (MEBarcoding): A new method for high throughput plant DNA barcoding. *Scientific Reports* 10: 8701.

Hebert, P. D. N., T. W. A. Braukmann, S. W. J. Prosser, S. Ratnasingham, J. R. deWaard, N. V. Ivanova, D. H. Janzen, et al. 2018. A Sequel to Sanger: Amplicon sequencing that scales. *BMC Genomics* 19: 219.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B Biological Sciences* 270: 313–321.

Hebert, P., S. Ratnasingham, and J. DeWaard. 2003. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B Biological Sciences* 270: S96–S99.

Hollingsworth, P., S. Graham, and D. Little. 2011. Choosing and using a plant DNA barcode. *PLoS ONE* 6: e19254.

Hollingsworth, P. M. 2011. Refining the DNA barcode for land plants. *Proceedings of the National Academy of Sciences* 108: 19451–19452.

Hollingsworth, P. M., L. L. Forrest, J. L. Spouge, M. Hajibabaei, S. Ratnasingham, M. van der Bank, M. W. Chase, et al. 2009. A DNA barcode for land plants. *Proceedings of the National Academy of Sciences* 106: 12794–12797.

Holloway, J. E. 1930. The experimental cultivation of the gametophytes of *Hymenophyllum pulcherrimum*, Col. and of *Trichomanes reniforme*, Forst. f. *Annals of Botany* 44: 269–284.

Hori, K., A. Ebihara, and N. Murakami. 2018. Revised classification of the species within the *Dryopteris varia* complex (Dryopteridaceae) in Japan. *Acta Phytotaxonomica et Geobotanica* 69: 77–108.

Hori, K., A. Tono, K. Fujimoto, J. Kato, A. Ebihara, Y. Watano, and N. Murakami. 2014. Reticulate evolution in the apogamous *Dryopteris varia* complex (Dryopteridaceae, subg. *Erythrovariae*, sect. *Variae*) and its related sexual species in Japan. *Journal of Plant Research* 127: 661–684.

Kapli, P., S. Lutteropp, J. Zhang, K. Kobert, P. Pavlidis, A. Stamatakis, and T. Flouri. 2017. Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. *Bioinformatics* 33: 1630–1638.

Kekkonen, M., and P. D. N. Hebert. 2014. DNA barcode-based delineation of putative species: Efficient start for taxonomic workflows. *Molecular Ecology Resources* 14: 706–715.

Kress, W. J., and D. L. Erickson. 2007. A two-locus global DNA barcode for land plants: The coding *rbcL* gene complements the non-coding *trnH–psbA* spacer region. *PLoS ONE* 2: e508.

Kress, W. J., K. Wurdack, E. Zimmer, L. Weigt, and D. H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America* 102: 8369–8374.

Kuo, L.-Y., C.-W. Chen, W. Shinohara, A. Ebihara, H. Kudoh, H. Sato, Y. M. Huang, and W. L. Chiou. 2017. Not only in the temperate zone: Independent gametophytes of two vittarioid ferns (Pteridaceae, Polypodiales) in East Asian subtropics. *Journal of Plant Research* 130: 255–262.

Kuo, L.-Y., and F.-W. Li. 2019. A roadmap for fern genome sequencing. *American Fern Journal* 109: 212–223.

Kuo, L.-Y., F.-W. Li, W.-L. Chiou, and C.-N. Wang. 2011. First insights into fern *matK* phylogeny. *Molecular Phylogenetics and Evolution* 59: 556–566.

Kuo, L.-Y., D.-Y. Tang, Y.-M. Huang, C.-W. Chen, S.-W. Fan, C.-N. Wang, and W.-L. Chiou. 2014. Cryptic species diversity on field fern gametophyte flora in Fushan (Taiwan). The 6th Asian Fern Symposium, Bali, Indonesia.

Kuo, L.-Y., T.-Y. Tang, F.-W. Li, H.-J. Su, W.-L. Chiou, Y.-M. Huang, and C.-N. Wang. 2018. Organelle genome inheritance in *Deparia* ferns (Athyriaceae, Aspleniineae, Polypodiales). *Frontiers in Plant Science* 9: 486.

Lagomarsino, L. P., A. L. Grusz, and R. C. Moran. 2012. Primary hemiepiphytism and gametophyte morphology in *Elaphoglossum amygdalifolium* (Dryopteridaceae). *Brittonia* 64: 226–235.

Lahaye, R., M. Van Der Bank, D. Bogarin, J. Warner, F. Pupulin, G. Gigot, O. Maurin, et al. 2008. DNA barcoding the floras of biodiversity hotspots. *Proceedings of the National Academy of Sciences* 105: 2923.

Lee, C. S., K. Lee, and Y. Hwang. 2020. Identification of three independent fern gametophytes and *Hymenophyllum wrightii* f. *serratum* from Korea based on molecular data. *Korean Journal of Plant Taxonomy* 50: 403–412.

Li, F.-W., and A. Harkess. 2018. A guide to sequence your favorite plant genomes. *Applications in Plant Sciences* 6: e1030.

Li, F.-W., L.-Y. Kuo, Y.-M. Huang, W.-L. Chiou, and C.-N. Wang. 2009. Tissue direct PCR, a rapid and extraction free method for barcoding of ferns. *Molecular Ecology Resources* 10: 92–95.

Li, F.-W., L.-Y. Kuo, K. M. Pryer, and C. J. Rothfels. 2016. Genes translocated into the plastid inverted repeat show decelerated substitution rates and elevated GC content. *Genome biology and evolution* 8: 2452–2458.

Li, F.-W., L.-Y. Kuo, C. J. Rothfels, A. Ebihara, W.-L. Chiou, M. D. Windham, and K. M. Pryer. 2011. *rbcL* and *matK* earn two thumbs up as the core DNA barcode for ferns. *PLoS ONE* 6: e26597.

Li, F.-W., B. C. Tan, V. Buchbender, R. C. Moran, G. Rouhan, C.-N. Wang, and D. Quandt. 2009. Identifying a mysterious aquatic fern gametophyte. *Plant Systematics and Evolution* 281: 77–86.

Liu, H. M., S. R. Russell, J. Vogel, and H. Schneider. 2018. Inferring the potential of plastid DNA-based identification of derived ferns: A case study on the *Asplenium trichomanes* aggregate in Europe. *Plant Systematics and Evolution* 304: 1009–1022.

Mafune, K. K., B. J. Godfrey, D. J. Vogt, and K. A. Vogt. 2020. A rapid approach to profiling diverse fungal communities using the MinION™ nanopore sequencer. *BioTechniques* 68: 72–78.

Meyer, C., and G. Paulay. 2005. DNA barcoding: Error rates based on comprehensive sampling. *PLoS Biology* 3: e422.

Murakami, N., E. Sakai, and K. Yoneoka. 2021. Inventories of fern independent gametophytes in Japan using DNA barcoding based on nucleotide sequences of *rbcL* gene (in Japanese). *BSJ-Review* 12: 159–168.

Murakami, N., M. Watanabe, J. Yokoyama, Y. Yatabe, H. Iwasaki, and S. Serizawa. 1999. Molecular taxonomic study and revision of the three Japanese species of *Asplenium* sect. *Thamnopteris*. *Journal of Plant Research* 112: 15–25.

Nayar, B. K., and S. Kaur. 1971. Gametophytes of homosporous ferns. *The Botanical Review* 37: 295–396.

Newmaster, S., A. Fazekas, and S. Ragupathy. 2006. DNA barcoding in land plants: Evaluation of *rbcL* in a multigene tiered approach. *Canadian Journal of Botany* 84: 335–341.

Nitta, J. H. 2008. Exploring the utility of three plastid loci for biocoding the filmy ferns (Hymenophyllaceae) of Moorea. *Taxon* 57: 725–2E.

Nitta, J. H., A. Ebihara, and A. R. Smith. 2020. A taxonomic and molecular survey of the pteridophytes of the Nectandra Cloud Forest Reserve, Costa Rica. *PLOS ONE* 15: e0241231.

Nitta, J. H., and M. J. Epps. 2009. Hemi-epiphytism in *Vandenboschia collariata* (Hymenophyllaceae). *Brittonia* 61: 392–397.

Nitta, J. H., J.-Y. Meyer, R. Taputuarai, and C. C. Davis. 2017. Life cycle matters: DNA barcoding reveals contrasting community structure between fern sporophytes and gametophytes. *Ecological Monographs* 87: 278–296.

Nitta, J. H., J. E. Watkins, and C. C. Davis. 2020. Life in the canopy: Community trait assessments reveal substantial functional diversity among fern epiphytes. *New Phytologist* 227: 1885–1899.

Nitta, J. H., J. E. Watkins, N. M. Holbrook, T. W. Wang, and C. C. Davis. 2021. Ecophysiological differentiation between life stages in filmy ferns (Hymenophyllaceae). *Journal of Plant Research* 134: 971–988.

Page, C. 2002. Ecological strategies in fern evolution: A neopteridological overview. *Review of Palaeobotany and Palynology* 119: 1–33.

Park, S. H., J. S. Kim, and H. T. Kim. 2021. A small number of gametophytes with gametangia and stunted sporophytes of *Antrophyum obovatum* Baker (Pteridaceae): The suppression of functional sporophyte production by prezygotic and postzygotic sterility. *Plants* 10: 170.

Park, S. H., J. S. Kim, and H. T. Kim. 2020. Study of the independent gametophytes found on Jeju Island in South Korea and the first record of the obligate independent gametophyte of *Antrophyum obovatum* Baker. *Ecology and Evolution*: ece3.6510.

Parker, J., A. J. Helmstetter, D. Devey, T. Wilkinson, and A. S. T. Papadopulos. 2017. Field-based species identification of closely-related plants using real-time nanopore sequencing. *Scientific Reports* 7: 8345.

Pinson, J. B., S. M. Chambers, J. H. Nitta, L.-Y. Kuo, and E. B. Sessa. 2017. The separation of generations: Biology and biogeography of long-lived sporophyteless fern gametophytes. *International Journal of Plant Sciences* 178: 1–18.

Pinson, J. B., S. M. Chambers, and E. B. Sessa. 2017. *Vittaria graminifolia* (Pteridaceae) and *Didymoglossum petersii* (Hymenophyllaceae) in Broxton Rocks, GA. *American Fern Journal* 107: 257–264.

Pinson, J. B., and E. Schuettpelz. 2016. Unraveling the origin of the Appalachian gametophyte, *Vittaria appalachiana*. *American Journal of Botany* 103: 668–676.

Pomerantz, A., N. Peñafiel, A. Arteaga, L. Bustamante, F. Pichardo, L. A. Coloma, C. L. Barrio-Amorós, et al. 2018. Real-time DNA barcoding in a rainforest using nanopore sequencing: Opportunities for rapid biodiversity assessments and local capacity building. *GigaScience* 7.

Pons, J., T. Barraclough, J. Gomez-Zurita, A. Cardoso, D. Duran, S. Hazell, S. Kamoun, et al. 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* 55: 595–609.

Ranker, T. A., and H. Houston. 2002. Is gametophyte sexuality in the laboratory a good predictor of sexuality in nature? *American Fern Journal* 92: 112–118.

Rothfels, C. J., K. M. Pryer, and F.-W. Li. 2017. Next-generation polyploid phylogenetics: Rapid resolution of hybrid polyploid complexes using PacBio single-molecule sequencing. *New Phytologist* 213: 413–429.

Schneider, H., and E. Schuettpelz. 2006. Identifying fern gametophytes using DNA sequences. *Molecular Ecology Notes* 6: 989–991.

Srivathsan, A., B. Baloğlu, W. Wang, W. X. Tan, D. Bertrand, A. H. Q. Ng, E. J. H. Boey, et al. 2018. A MinION™-based pipeline for fast and cost-effective DNA barcoding. *Molecular Ecology Resources* 18: 1035–1049.

Stevens, S. M., and N. C. Emery. 2015. Dispersal limitation and population differentiation in performance beyond a northern range limit in an asexually reproducing fern. *Diversity and Distributions* 21: 1242–1253.

Taberlet, P., C. Eric, P. Francios, G. Ludovic, M. Christian, V. Alice, V. Thierry, et al. 2007. Power and limitations of the chloroplast *trn*L (UAA) intron for plant DNA barcoding. *Nucleic Acids Research* 35: e14.

Testo, W. L., and M. A. Sundue. 2014. Primary hemiepiphytism in *Colysis ampla* (Polypodiaceae) provides new insight into the evolution of growth habit in ferns. *International Journal of Plant Sciences* 175: 526–536.

Thiers, B. 2021. Index Herbariorum: A global directory of public herbaria and associated staff. Website http://sweetgum.nybg.org/science/ih/ [accessed 1 August 2021].

Trujillo-Argueta, S., R. F. del Castillo, D. Tejero-Diez, C. A. Matias-Cervantes, and A. Velasco-Murguía. 2021. DNA barcoding ferns in an unexplored tropical montane cloud forest area of southeast Oaxaca, Mexico. *Scientific Reports* 11: 22837.

Vogel, J. C., S. J. Russell, F. J. Rumsey, J. A. Barrett, and M. Gibby. 1998. Evidence for maternal transmission of chloroplast DNA in the genus *Asplenium* (Aspleniaceae, Pteridophyta). *Botanica Acta* 111: 247–249.

Wang, F.-H., J.-M. Lu, J. Wen, A. Ebihara, and D.-Z. Li. 2016. Applying DNA barcodes to identify closely related species of ferns: A case study of the Chinese *Adiantum* (Pteridaceae). *Plos One* 11: e0160611.

Watkins, J. E., and R. C. Moran. 2019. Gametophytes of the fern genera *Dracoglossum* and *Lomariopsis* (Lomariopsidaceae) and their phylogenetic significance. *International Journal of Plant Sciences* 180: 1004–1015.

Watts, J. L., R. C. Moran, and J. E. W. Jr. 2019. *Hymenasplenium volubile*: Documentation of its gametophytes and the first record of a hemiepiphyte in the Aspleniaceae. 124: 829–835.

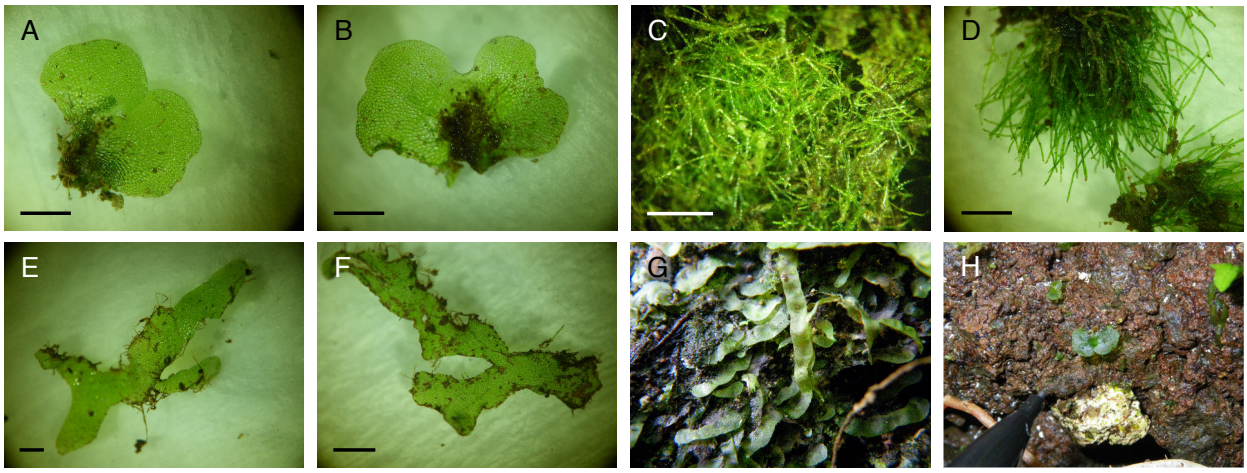

Figure 1. Examples of fern gametophytes identified using DNA barcoding (Nitta et al., 2017): (A–F) pairs of species with similar morphology that could not be distinguished without molecular data; (G, H) appearance of gametophytes in the field. Morphotype provides a hint to taxonomy but cannot be reliably used for species identification: the cordate (heart-shaped) morphotype (A, B, H) is the most common, especially in terrestrial species; the filamentous morphotype (C, D) is restricted to Hymenophyllaceae and Schizaeaceae; the ribbon morphotype (E–G) typically occurs in Hymenophyllaceae, Vittariaceae, and several lineages of polypod ferns. (A) *Sphaeropteris medullaris* Bernh. *Nitta 3454* (GH). (B) *Austroblechnum raiateense* (J.W.Moore) Gasper & V.A.O.Dittrich *Nitta 3637* (GH). (C) *Crepidomanes minutum* (Blume) K.Iwats. *Nitta 3938* (GH). (D) *Abrodictyum dentatum* (Bosch) Ebihara & K.Iwats. *Nitta 3487* (GH). (E) *Callistopteris apiifolia* (Presl) Copel. *Nitta 3450* (GH). (F) *Hymenophyllum polyanthos* (Sw.) Sw. *Nitta 3460* (GH). (G) *Arthropteris palisotii* (Desv.) Alston *Nitta 1104* (GH). (H) *Ptisana salicina* (Sm.) Murdock *Nitta 3507* (GH). Herbarium codes follow Thiers (2021). For (A–F) scalebars all 1 mm; photos taken under a dissecting microscope. All photos by J. H. Nitta.

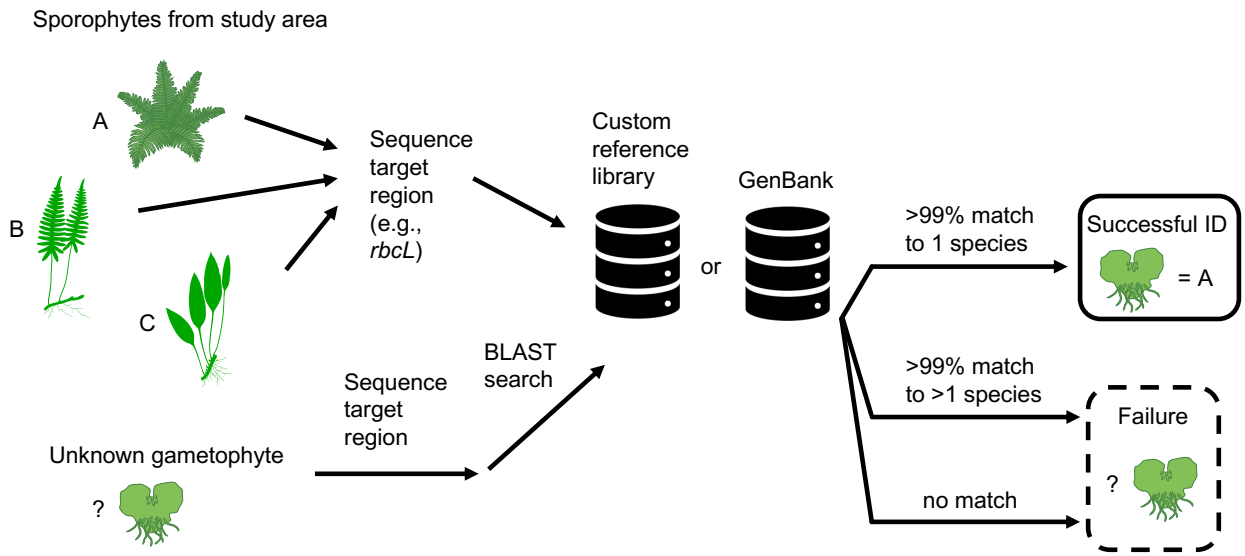

Figure 2. Generalized workflow for DNA barcoding of fern gametophytes. The first step is to assemble a reference library of DNA sequences for a marker of interest (e.g., *rbcL*) using well-identified sporophytes from the study area; or, if this is not possible (e.g., if the provenance of the gametophyte is unknown), public databases such as GenBank or BOLD may be used instead. Next, DNA sequences are obtained from the unknown gametophyte and queried against the reference library using BLAST. Finally, an algorithm is used to sort results into successful identifications or failures based on a percent-identity cutoff (the value used in the figure is an arbitrary example). Other approaches may be used to match the unknown gametophyte to a reference such as phylogenetic analysis (see **Building and using a reference library**). Gametophyte image and sporophyte silhouette (A) provided courtesy of Emily Sessa and used with permission. Sporophyte silhouettes (B, C) provided courtesy of Eric Schuettpelz and used with permission.