Integrating tissue-direct PCR into genetic identification: An upgraded molecular ecology approach to survey fern gametophytes in the field

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Abstract
Premise: The gametophytes of different fern species collected in the field can be difficult to distinguish because of their morphological similarities. Nonetheless, emerging molecular ecology techniques are starting to be used to tackle such limitations. Here, using case studies and a detailed protocol, we demonstrate a convenient methodology, tissue-direct PCR (TD-PCR), that foregoes a traditional DNA extraction and facilitates the identification of fern gametophytes, as well as enabling the elucidation of their natural distribution.

Methods: Based on updated plastome information, we designed a universal primer set targeting the trnL–L–F region, which is effective across extant ferns. We used this primer set to perform TD-PCR on the case-studied populations of Taiwanese Lomariopsis gametophytes, using the generated sequences for their identification. In the case study concerning the microhabitat preference of Vaginularia junghuhnii, we designed and used a taxon-specific primer set.

Results: Compared with approaches requiring DNA extraction, the use of TD-PCR with either universal or taxon-specific primers could save significant time, money, labor, and research materials in the genetic identification of fern gametophytes.

Discussion: The use of modern genetic tools can aid in the identification of fern gametophytes. An updated TD-PCR strategy not only facilitates the DNA-based identification of gametophytes, but also promotes new avenues of research for investigating these plants in the field.

KEYWORDS
DNA-based identification, fern gametophyte, independent gametophytes, Lomariopsis, molecular ecology, tissue-direct PCR, tree fern specialist, Vaginularia

The discovery and investigation of the gametophyte generation of ferns, a free-living life stage with an inconspicuous size, has greatly enhanced our understanding of fern biology, particularly their reproduction (Raghavan, 1989; Hafler et al., 2016; Sessa et al., 2016). Our knowledge of fern gametophytes, however, relies mostly on observations or experimental results in labs, where gametophytes are cultured from identified sources (Farrar et al., 2008). For gametophytes in the field, it can be difficult to determine their source and identify them morphologically, even to a generic level (Momose, 1967; Nayar and Kaur, 1971; Atkinson, 1973; Chen et al., 2013). These challenges have therefore long impeded field investigation of fern gametophytes, as well as our understanding of fern ecology. Recent research has begun to tackle this issue with molecular tools. DNA characteristics,
e.g., DNA barcodes, perform tremendously well for the species identification of fern gametophytes, and even reveal hidden diversity, such as the discovery of independent fern gametophytes that reproduce via only gametophytic propagation without a spore-producing sporophyte (Ebihara et al., 2008, 2009, 2013; Li et al., 2009; Chen et al., 2013; Duffy et al., 2015; Pinson and Schuettpelz, 2016; Kuo et al., 2017a; Nitta et al., 2017; Lee et al., 2020; Park et al., 2020, 2021). Such methodologies use prevalent DNA barcodes, e.g., \textit{trnL}-L-F and \textit{rbcL}, and are now the major approach used to survey the field diversity of fern gametophytes (reviewed by Nitta and Chambers, 2022).

To enhance the value of the DNA-based identification of fern gametophytes, tissue-direct PCR (TD-PCR), a convenient molecular approach, has been developed (Li et al., 2010). This technique foregoes a traditional DNA extraction by performing a PCR on a solution containing very small amounts of dissolved tissue. When DNA-based identification is prioritized over the needs of other molecular studies, the use of TD-PCR is preferable due to its efficiency, convenience, and cost-saving benefits (Table 1). In comparison, a DNA-extraction approach for fern gametophytes carries the risk of failure if the tissue amount is insufficient (de Groot et al., 2011; Ebihara et al., 2013; Nitta et al., 2017), as well as the potential for contamination from other intermingled individuals/species (particularly for gametophyte colonies with ribbon-shaped or filamentous growth forms; Ebihara et al., 2009; Duffy et al., 2014). Furthermore, both the reagents and time required for a DNA extraction (e.g., using commercially available DNA extraction kits; Ebihara et al., 2009, 2013; Nitta et al., 2017) are costly compared with the TD-PCR protocol (Table 1).

In this study, we present an optimized TD-PCR protocol based on the newly designed universal primer set for the \textit{trnL}-L-F region of ferns, and the slightly modified procedure and buffers developed by Li et al. (2010). Using two empirical cases, we then demonstrate the proficiency of these DNA tools for ecological surveys of the natural distribution and microhabitats of fern gametophytes. We also highlight that the DNA identification of fern gametophytes can be accomplished through different TD-PCR strategies. In the first case, we surveyed the gametophyte populations of \textit{Lomariopsis Fée} (Lomariopsidaceae) around Taiwan to confirm whether there are any regionally undocumented independent gametophyte species. In this case, a TD-PCR protocol using the \textit{trnL}-L-F universal primer set was successful for the gametophyte samples. Based on the resulting DNA sequences, a phylogeny-guided approach was used to identify the species composition of the surveyed populations. For the second case, we investigated the gametophyte microhabitat of \textit{Vaginularia junghuhnii} Mett. (Vittarioideae, Pteridaceae), a tree fern specialist known to grow exclusively on tree fern trunks during its sporophyte generation (Knapp, 2011). We accessed vittarioid plastid DNA sequences and designed a specific primer set that only targets \textit{V. junghuhnii}. With both the taxon-specific and universal primer sets, we performed a TD-PCR for the gametophytes that were collected from various microhabitats on and around a tree fern trunk hosting established populations of \textit{V. junghuhnii} sporophytes. Based on these TD-PCR results, we verified the presence of \textit{V. junghuhnii} gametophytes among the samples, and therefore identified the microhabitat of its gametophyte.

### METHODS

**Field gametophyte sampling: Lomariopsis**

To find \textit{Lomariopsis} gametophyte populations in Taiwan, we first checked the historical herbarium records of their sporophytes and surveyed these documented sites and the adjoining areas. We collected ribbon-shaped gametophytes around 2–5 mm wide (Figure 1A), which fit the morphological characteristics of previously documented \textit{Lomariopsis} spp., found growing near a \textit{Lomariopsis} juvenile sporophyte. In total, we identified 16 populations around Taiwan, and for each gametophyte population, sampled 1–8 individuals (Appendix S1).

| Comparable factor | TD-PCR | PCR using extracted DNA$^a$ |
|-------------------|---------|-----------------------------|
| Input tissue (fresh weight per sample) | <0.006 mg | 20–100 mg |
| Time* | <20 min | ~60 min |
| Costs of consumable per sample$^b$ | <$0.2 | >$5 |
| Equipment | Tweezers, liquid nitrogen, ultrasonic cleaner, mini-centrifuge | Homogenizer, incubator, high-speed centrifuge, pipette |

$^a$Estimated by processing a batch of eight samples for use in the PCRs.

$^b$Costs are given in U.S. dollars.

$^c$Using a QIAGEN DNeasy Plant Mini Kit as an estimate.
Field gametophyte sampling: Vaginularia

To conduct the microhabitat survey of the *V. junghuhnii* gametophytes, we found one tree fern host, *Alsophila spinulosa* (Hook.) R. M. Tryon (Cyatheaceae), in Chinshuying (Taidong, Taiwan). We set plots on the trunk of the tree fern and the trunk of the closest angiosperm tree (*Litsea acuminata* (Blume) Sa. Kurata) with a similar diameter at breast height, as well as the surrounding terrestrial microhabitats within a 1 × 1 m area. For the tree microhabitats, the trunk surface was surveyed up to a height of 1.5 m. Like other vittarioids, *Vaginularia* gametophytes (Figure 1B) are only known to have a ribbon-shaped morphology (Farrar et al., 2008; Chen et al., 2013); therefore, we sampled all fern gametophytes with such a growth form. Additionally, we used a plastic net with square holes of 5 × 5 cm to assist our gametophyte investigation. When multiple patches of ribbon-shaped gametophytes were found in a single grid, we randomly sampled one individual from the patches within that grid.

Primer design

The primer sequences that were previously used to target the fern *trnL*-F region (including the intron-contained *trnL* gene and the *trnL*-trnF intergenic spacer) (Taberlet et al., 1991; Trewick et al., 2002; Li et al., 2010) were found to be mismatched at the binding sites in several fern lineages. To design a new universal primer set with improved affinity, we downloaded 250 fern plastomes from GenBank based on the "fern_plastome_list" updated by Paul G. Wolf (University of Alabama in Huntsville; https://paulwolflab.com/data-protocols/fern_plastome_list/ [accessed 24 September 2020]). A custom Python script was used to extract the 20–25-bp conserved priming regions within the *trnL* 5′ exon and *trnF* sequences of these plastome sequences. After preliminary PCR tests, we selected this pair as our universal *trnL*-F primer set: "FernL0725" (5′-ATGGCGRAAT-GGTAGACGC-3′) and "FernF4121" (5′-GGATTTCACGCTTGACGTA-3′).

To design a taxon-specific primer set for *V. junghuhnii*, we used the DNA alignments of Chen et al. (2017a), which include several plastid regions of all East Asian vittarioids. We first identified alignment segments with unique indels in *V. junghuhnii*, which can be a potential priming region, to avoid inadvertently amplifying other closely related taxa. We finally designed a *V. junghuhnii*-specific primer set targeting a small portion (~520 bp) of its *trnL*-F region: "Vj_trnL1f1" (5′-CTTITAAATAACCTGGAACCTATAATC-3′) and "Vj_trnL3r1" (5′-GCTATCCCAGCTTTAAAACGAG-3′). To double-check the specificity of this primer set, we also performed BLASTN analyses and preliminary PCR tests. We first BLASTed the primer sequences against the National Center for Biotechnology Information (NCBI) nucleotide collection and a conspecific plastome sequence (GenBank accession: MH173085). All BLAST results suggest that the primers have high specificity to *V. junghuhnii* *trnL*-F. Empirical PCR tests using this primer set were conducted on DNA of all Taiwanese vittarioid species, with the results further demonstrating that the ~0.5-kbp fragment was only amplified from *V. junghuhnii*.

Optimization of TD-PCR

Our optimized protocol of TD-PCR is outlined in Figure 2. After the gametophyte samples were cleaned with brushes under water, a piece of the healthiest green fern tissue (approximately 1 mm²) was sliced and detached using tweezers and placed into a PCR tube or an eight-strip PCR tube containing 20 μL of ddH₂O. The tweezers were cleaned with 75% alcohol and KIMTECH Kimwipes (Kimberly-Clark, Irving, Texas, USA) between the slicing of the different tissue samples. The tissue samples were then soaked in liquid nitrogen until frozen. Next, an ultrasonic cleaner (e.g., in this study, model UC-6108 [liayunda Technology, Shenzhen, China]) was used to dissolve the frozen samples, after which the liquified samples were spun down in a mini-centrifuge. If tissues were still visible in the PCR tubes, we repeated the freezing, dissolving, and spinning down for the crude tissue extractions. Three cycles were usually required to completely break down the gametophyte tissues.

For each TD-PCR reaction, 1 μL of the crude tissue solution was used as a DNA template and added to 12 μL of PCR mix, including 15 pM of each primer and SuperRed...
PCR Master Mix (Biotools, New Taipei City, Taiwan). The PCR was performed using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with an initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; with a final elongation at 72°C for 10 min for the \textit{trnL}–\textit{F} universal primer set. For the \textit{V. junghuhnii}–specific primer set, the conditions used were 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s; with a final elongation at 72°C for 10 min.

**Sequencing, reference database, and phylogeny**

The TD-PCR products generated from the \textit{Lomariopsis}-like gametophytes were cleaned using ExoSAP-IT (Thermo Fisher Scientific) prior to sequencing. Each (~10-µL) PCR product was combined with 4 µL of ExoSAP-IT, then incubated at 37°C for 4 min and 80°C for 1 min. An ABI 3730XL DNA Analyzer (Thermo Fisher Scientific) was then used for the Sanger sequencing of these PCR products, which was performed by Genomics BioSci & Tech (New Taipei City, Taiwan). The resulting sequences were then BLASTed (BLASTN) against the NCBI nucleotide collection. Only sequences yielding a high percentage of shared identity with the \textit{Lomariopsis} spp. sequences were included in the phylogenetic tree. We first aligned these \textit{Lomariopsis} gametophyte sequences using MUSCLE (Edgar, 2004) implemented within AliView (Larsson, 2014), along with other sequences generated from identified sporophytes (Appendices S1, S2), of which the African \textit{Lomariopsis} taxa were selected as the outgroups for Asian ones (Chen et al., 2017b). The resulting alignment was further divided into two partitions, the \textit{trnL} gene and the \textit{trnL}–\textit{trnF} intergenic spacer, then launched into the IQ-TREE web server (Trifinopoulos et al., 2016) to infer a maximum likelihood (ML) phylogeny. The ML phylogeny was performed with 1000 ultrafast bootstrap replicates using the appropriate substitution models inferred by ModelFinder (Kalyaanamoorthy et al., 2017) and based on the Bayesian information criterion.

For the gametophytes from the \textit{V. junghuhnii} plots, the taxon identity was determined by gel electrophoresis, rather than by sequencing; if a TD-PCR amplicon produced using the \textit{V. junghuhnii}–specific primer set had a ~0.5-kbp band, we identified this gametophyte sample as \textit{V. junghuhnii}.

**RESULTS**

**Cryptic \textit{Lomariopsis} species and their distribution**

Using TD-PCR, we generated \textit{trnL}–\textit{F} sequences from 84 samples among the 16 putative \textit{Lomariopsis} populations in Taiwan. Excluding the 15 samples found to belong to other genera, we identified 69 \textit{Lomariopsis} gametophytes in total (Appendix S1). The ML tree (Figure 3A) shows that these \textit{Lomariopsis} gametophytes from Taiwan belong to three clades, which correspond to three species: \textit{L. boninensis}...
Nakai, L. moorei L. Y. Kuo & Y. H. Wu, and an unknown species (L. sp.). The sporophyte morphologies of L. moorei and L. sp. are similar to L. boninensis, although there are slight differences between them (Wu et al., 2021). Among the three species, L. boninensis is the most widespread in Taiwan, and its gametophytes were found in 13 out of 16 populations (Figure 3, Appendix S1). In terms of their gametophytes, L. moorei is also widespread in Taiwan; we identified L. moorei gametophytes in the MX, LM, DH, LHC, ZW, Chu, and SK populations (see Appendix S1 for population names). By contrast, we discovered L. sp. gametophytes only in the SK population (Figure 3). A mature sporophyte producing spores has been found only in the ZW population, which is now identified as L. moorei (Wu et al., 2021).

Microhabitat survey of Vaginularia junghuhnii

In total, we collected 122 (ribbon-shaped) fern gametophytes from the tree fern trunk, and 84% (102/122) of them were identified as V. junghuhnii using its taxon-specific primer set. In addition, we also performed TD-PCR on all of the gametophytes using the universal primer trnL-L-F set, confirming that all pre-treated samples could be amplified well using this technique. This indicates that the samples were fresh enough and contained limited secondary metabolite concentrations, which can inhibit PCR reactions. No ribbon-shaped fern gametophytes were found in the terrestrial plot or on the angiosperm tree trunk, indicating that V. junghuhnii-like gametophytes were not detected in any microhabitats other than the tree fern trunk.

DISCUSSION

Factors affecting the performance of TD-PCR

In addition to the primer set, the performance of TD-PCR can be affected by the PCR buffer selection and the condition of the gametophyte tissue. Although we did not perform thorough tests on different PCR recipes, a commercial PCR premix containing normal Taq DNA polymerase worked well (yielding over a 90% success rate before any further adjustment). In our experience, neither so-called “tissue-direct” buffer nor additional PCR enhancement (e.g., betaine and dimethyl sulfoxide [DMSO]; Li et al., 2010) was necessary when a commercial PCR premix was used.
The success rate of TD-PCR seems much more sensitive to tissue condition, and we suggest using fresh gametophytes (i.e., as green as possible). It is therefore critical to prepare crude tissue solutions and/or freeze tissue materials soon after field collection when the gametophytes still look viable. In some cases, the adjustment of the input amount of crude tissue solution (e.g., by dilution) can improve TD-PCR performance and yield a 100% success rate. Even under storage at −20°C for three years, we found that tissue solutions were still suitable for TD-PCR (data not shown). It would be worthwhile to test whether adding a low concentration of Tris–ethylenediaminetetraacetic acid (TE) buffer to the initial raw tissue solution could further preserve the DNA template, while sustaining the success of TD-PCR performance.

The selection of the primer set is also critical for TD-PCR performance. For the goal of generating sequences from fern gametophytes collected in the field, comprising an unknown and potentially diverse species composition, primer universality is the most important consideration, but often limited by the target region itself. For some proposed DNA barcodes for plants, such as matK, conserved primer regions across the fern lineages are sparse (Kuo et al., 2011). With regard to primer universality, trnL–L–F, or solely the trnL–trnF intergenic spacer, has been the most successful among all proposed DNA barcodes for plants. In this study, we also present trnL–L–F as a new universal primer set for ferns, which appears to be more universal than the previous one designed by Li et al. (2010).

Independent fern gametophytes and their unexpected distribution

Floristic surveys of fern diversity have long been based on morphologically identifiable sporophytes; however, using DNA-based identification, recent studies aiming to survey the regional diversity of fern gametophytes have updated maps showing the natural distribution of ferns. In particular, independent gametophyte ferns have been identified, which are long lived and able to asexually propagate without the presence of a fertile sporophyte (Pinson et al., 2017). *Lomariopsis* is one of the most well-studied fern genera with such independent gametophytes (Pinson et al., 2017). In the current case, we also discovered independent gametophytes in this genus from most sites around Taiwan Island.

In total, three *Lomariopsis* species were revealed from our phylogeny (Figure 3). Among them, *L. moorei* is the only species known to have a fertile sporophyte population producing spores on Taiwan Island, but these are distributed in one locality (the ZW population [Figure 3B]; Wu et al., 2021). *Lomariopsis boninensis* has many fertile sporophyte populations (TPG, 2019, 2021), but on Taiwan Island it seems to produce only malformed sporangia and/or spores (L.-Y. Kuo and Y.-H. Wu, Institute of Molecular and Cellular Biology NTHU, personal observation). By contrast, *L. sp. very likely represents an undescribed cryptic species. Fertile sporophytes of *L. sp. can be found, but only on Orchid Island (Y.-H. Wu et al., unpublished data). Both *L. moorei* and *L. sp. produce obligate independent gametophytes, because their gametophyte populations are distributed outside those of spore-producing sporophytes, except for *L. moorei* in the ZW population. Similarly, *L. boninensis* gametophytes on Taiwan Island should also be considered as obligate independent gametophytes, because no spore-producing individual of this species has been found so far on the island. Based on our unpublished sequence data, these independent *Lomariopsis* gametophytes are also distinct from those found by Ebihara et al. (2013) in Iriomote, Japan. Interestingly, together with *L. boninensis*, these species were found to usually be sympatric (Appendix S1), and the SK population was found to be composed of all three species (Figure 3). Further studies using nuclear markers would be useful to clarify whether sterile *Lomariopsis* sporophytes in the Taiwanese populations are hybrids from these independent *Lomariopsis* gametophytes.

Microhabitat specificity of gametophytes of a tree fern specialist

Fern epiphytes are particularly known for their microhabitat specificity, and in some instances grow exclusively on root-mantle covered tree fern trunks (Wagner et al., 2015). However, our current understanding of these various life forms and their microhabitat specificity is restricted to the sporophyte generation of ferns. No previous study has documented microhabitat selection in both the sporophyte and gametophyte generation of a single fern species. Here, we demonstrate a case study of a tree fern specialist, *V. junghuhnii*, the sporophytes of which are known to exclusively grow on tree ferns.

Like its sporophytes, the gametophytes of *V. junghuhnii* were also exclusively found on the surveyed tree fern trunk. *Vaginularia junghuhnii*-like gametophytes (i.e., ribbon-shaped gametophytes) were completely absent in the other microhabitats surrounding the tree fern trunk, including terrestrial environments (soil and rock surface) and a nearby angiosperm tree trunk. Our study implies that these tree fern specialists might display a strong host/microhabitat specificity throughout its life cycle, even as early as the beginning gametophyte stages. Given that our study provides results from a single site, further investigation with additional sites and species are necessary to confirm this interesting phenomenon.

Cost-effective approaches to surveying and identifying fern gametophytes in the field

DNA barcodes in modern reference databases (e.g., GenBank and the Barcode of Life Data System [BOLD])
provide significant coverage of fern diversity, with plastome sequences available for a considerable proportion of genera. During this sequence-rich era, DNA-based identification to species level has become more feasible for fern gametophytes and, as demonstrated here, can be easily accomplished through PCR approaches using either universal or taxon-specific primers. Depending on the ecological or biogeographical issues addressed, primers with different purposes should be considered for a gametophyte TD-PCR approach. In the case of *Lomariopsis*, given that cryptic diversity might exist, obtaining sequence information of the gametophyte samples was essential to reveal their phylogenetic identities using reference sequences. We used the universal primer set for TD-PCR, and sequenced the PCR products of the *trnL*-L-F DNA barcodes. For cases when all sampled gametophytes are considered for DNA barcoding (e.g., for revealing the community composition of gametophyte populations), we additionally advocate for this TD-PCR approach. Notably, TD-PCR can be applied using indexed primers for a multiplexed amplicon (e.g., Guenay-Grenunke et al., 2021) using next-generation sequencing (NGS) (A. Quinlan and L. Y. Kuo et al., unpublished data). When a sampling size is extensive (e.g., >400 samples), costs can be greatly lowered using NGS amplicon sequencing (to <$1 per sample, by our estimation).

In the case of *Vaginularia* here, given that only one species was concerned, it was most cost effective to design a taxon-specific primer set to target this species, and apply the primer set to the TD-PCR protocol (also see Quinlan et al., 2022). Consequently, the TD-PCR results could be solely referenced for identification via a true-or-false verification. Most importantly, the entire identification procedure requires only a single step of TD-PCR, and completely bypasses a sequencing step and its costs. However, this type of “rapid-test” strategy requires a priori sequence information to develop a well-performing taxon-specific primer set. As one example, our taxon-specific primers were designed based on reference sequences with several plastid regions including all other closely related ferns (i.e., all Asian vittarioids; Chen et al., 2017a). We further predicted the specificity of our primers by BLASTing against the NCBI nucleotide collection. Moreover, to avoid errors, we recommend two additional experiments, which were also conducted in this study. First, a preliminary PCR test using the DNA of all sympatric and closely related ferns is useful to quickly evaluate the possibility of false positives. Second, TD-PCR using a universal primer set should be carried out at the same time. When a counterpart TD-PCR using a universal primer set fails, negative TD-PCR results using a taxon-specific primer set could be a true negative due to a poor tissue solution or a non-optimal PCR condition. For this situation, it is necessary to adjust the input amount of tissue solution and/or other PCR conditions until a TD-PCR using a universal primer set succeeds.

There are other potential uses for TD-PCR that can greatly encourage ecologists and pteridologists to study fern gametophytes within their topics of interest. First, because a very limited amount (1 mm² or less after dilution) of tissue is required for a single TD-PCR reaction (Table 1), it is not necessary to destroy the entire gametophyte individual, which can therefore be reserved for other experiments, such as tests of desiccation tolerance (Watkins et al., 2007; Nitta et al., 2021). Second, compared with a DNA extraction approach, the equipment required for TD-PCR is low tech, less expensive, smaller, and more portable, allowing for easy set-up outside the lab, including in the field. Alternative approaches to the mechanical destruction of the gametophyte tissue for the crude tissue solution preparation are available in case liquid nitrogen or an ultrasonic cleaner are not available. Bead vortexing, which has been applied to destroy fern spores (Kuo et al., 2017b), is a potential option, while simply squashing the gametophyte with tweezers is another. We believe that the straightforward protocol of TD-PCR can be easily performed by amateurs, such as high school students and teachers, who, from our experience, can yield a high success rate on their first attempt. In comparison, a DNA-extraction approach, e.g., using a commercial kit (Table 1), requires standard lab equipment, including a high-speed centrifuge, an incubator, and even a tissue homogenizer.

Finally, whether using a TD-PCR-incorporated or DNA-extraction approach, a basic understanding of the morphological diversity of fern gametophytes remains important. This knowledge not only enables us to initially locate fern gametophytes (Farrar et al., 2008), but also increases the efficiency of sampling efforts by narrowing targets to certain groups of interest. Prior sampling approaches adopting a morphological-based framework were also used in both study cases here. For the *Lomariopsis* sampling, we focused on only ribbon-shaped gametophytes, similar to those reported previously (Atkinson et al., 1973; Li et al., 2009; Ebihara et al., 2013; Watkins and Moran, 2019), although some samples were later found to be members of other fern groups that also produce ribbon-shaped gametophytes (Appendix S1). In the case of *Vaginularia*, we also sought only ribbon-shaped gametophytes because vittarioid species all grow in this form (Figure 1B).

**CONCLUDING REMARKS**

Using present-day DNA tools, the identification of fern gametophytes in the field is more accessible. To improve these tools, TD-PCR strategies can be incorporated not only to facilitate DNA-based identification, but also to explore new research directions for the investigation of fern gametophytes in the field. As the cases here have demonstrated, TD-PCR requires no DNA extraction and makes gametophyte identification possible using a single PCR step. Notably, this technique works well for the gametophytes of both ferns and bryophytes (C.-N. Yeh, Kaohsiung Medical University, personal communication).
Time, funding, labor, and research materials for gametophyte DNA identification can thus be directed to other advanced purposes. We are also looking forward to the further development of this molecular ecology technique and to improving the efficiency and reliability of the DNA-based identification of fern gametophytes. Most importantly, with advances in fern biology and ecology, this featured methodology reminds us that the mystery of fern gametophytes in the field is still waiting to be uncovered.

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AUTHOR CONTRIBUTIONS

Y.-H.W. and L.-Y.K. designed the experiments and prepared the manuscript. Y.-Y.C. and G.-J.W. prepared the Python scripts for the primer design. Y.-H.W., Y.-T.K., and L.-Y.K. carried out the sampling, lab experiments, and analyses. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The *trnL*-F sequences have been uploaded to the National Center for Biotechnology Information database (accession number: OL311961–2044).

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

**Appendix S1.** Lomariopsis sporophyte and gametophyte sampling in the gametophyte populations.

**Appendix S2.** Herbarium voucher information and Gen-Bank accession numbers for the Lomariopsis outgroup species sequences used for the phylogeny.

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