TECHNICAL NOTE

Identifying fern gametophytes using DNA sequences

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Abstract

Identification of fern gametophytes is generally hampered by low morphological complexity. Here we explore an alternative: DNA-based identification. We obtained a plastid rbcL sequence from a sterile gametophyte of unknown origin (cultivated for more than 30 years) and employed BLAST to determine its affinities. Using this approach, we identified the gametophyte as Osmunda regalis. To evaluate the robustness of this determination, and the usefulness of rbcL in differentiating among species, we conducted a phylogenetic analysis of osmundaceous fern sequences. Based on our results, it is evident that DNA-based identification has considerable potential in exploring the ecology of fern gametophytes.

Keywords: DNA barcoding, DNA-based taxonomy, gametophytic generation, molecular ecology

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Homosporous ferns are vascular plants with two free-living generations. The gametophytic generation is distinct from the sporophytic generation in its cytology (haploid vs. diploid), usually shorter lifespan, smaller size and lower morphological complexity. Only the sporophyte shows differentiation into leaves, roots and shoots; the gametophyte lacks this separation, but usually shows a dorsiventral organization ranging from heart-shaped to ribbon-shaped or filamentous (Nayar & Kaur 1971). The extreme morphological disparity between the two independent generations is correlated with marked differences in their ecological preferences. Although sporophytes initially grow from where gametophytes grew, during their development they often creep from more shady and moist conditions preferred by the gametophytes into more exposed areas. Such a transition is especially pronounced in certain species of Pyrrosia in which gametophytes exhibit C3 photosynthesis, but sporophytes exhibit facultative CAM photosynthesis (Martin et al. 1995).

Most of our knowledge of fern distribution and ecology is based on observations of sporophytes, which are usually much larger and more easily studied. However, the results of the few field studies of gametophytes have shown that this generation plays an important role in the dispersal and reproductive biology of ferns (Farrar 1967; Dassler & Farrar 2001; Shorina 2001). More information about the gametophytic generation is required to develop a more comprehensive understanding of fern ecology and evolution.

Our limited knowledge of gametophytes is partly due to difficulty in collecting them from the wild because of their smaller size and seemingly more ephemeral growth habits, but the identification of collected gametophytes also poses problems, as existing fern floras only provide identification keys for sporophytes. Although the morphology of fern gametophytes is known for representatives of more than 90% of fern genera (Nayar & Kaur 1971), it is usually difficult to assign unknown fern gametophytes to genera based on morphology alone. Limited morphological differentiation and the occurrence of similar characters in distantly related taxa often preclude unambiguous identifications. Approaches using DNA sequences to determine taxonomic identity may provide a solution.

Recently, such DNA-based identification tools have become the subject of considerable discussion (Savolainen et al. 2005; Schindel & Miller 2005). DNA extraction, polymerase chain reaction (PCR) amplification and sequencing are standard protocols used in many laboratories worldwide. For ferns, several genomic regions are routinely sequenced, most frequently the plastid rbcL gene. Representative rbcL sequences can be found for all fern families and for more than 70% of all currently accepted genera. By sequencing this gene from unidentified fern gametophytes and comparing the recovered sequences to those deposited in
public databases, using for example blast (Altschul et al. 1997), we may be able to assign gametophytes to fern families, genera and, perhaps, even species.

To empirically explore the potential and pitfalls of DNA-based identification, we focused on a single gametophyte — of unknown origin — in cultivation at the Alter Botanischer Garten Göttingen. This ribbon-shaped gametophyte has been vegetatively propagated for more than 30 years. The cultures frequently produce archegonia, but never antheridia, and therefore sporophytes have never been produced. Based on morphological features, such as the relatively large cordate-thalloid shape, the thick midrib (more than 10 cells thick) covered by rhizoids, and the sunken and relatively large archegonia with straight necks that are restricted to lateral portions of the midrib, we were able to conclude that this gametophyte may well be a basal leptosporangiate fern. However, morphological features did not allow us to generate an unambiguous identification. In an attempt to obtain one, we extracted DNA from this gametophyte and generated an rbcL sequence for comparison to published sequences.

Genomic DNA was extracted with a DNeasy kit (QIAGEN). The rbcL gene was then amplified, purified and sequenced using established protocols and primers (Pryer et al. 2004), resulting in an rbcL sequence of 1309 base pairs (bp). To identify similar sequences in GenBank, we used nucleotide-nucleotide blast (blastn; Altschul et al. 1997), which located a 1227 bp rbcL sequence (AB076259) that was 100% identical to a 1227 bp subsequence of our gametophyte sequence (a larger rbcL fragment was generated from the gametophyte through the use of different PCR priming sites). This sequence and the second-best hit (99% identical; AB076258) were both identified as Osmunda regalis. All other blast hits above 95% were members of the fern family Osmundaceae, whereas other ferns had sequence similarities of 87% or less.

To further examine the affinities of the gametophyte sequence, and to test the power of rbcL sequence data to discriminate among species, we downloaded all rbcL sequences in GenBank attributed to Osmundaceae and reconstructed the phylogeny of these ferns. We aligned the sequences manually in macclade (Maddison & Maddison 2000) and identified the best-fitting model of sequence evolution (and parameter estimates) for this data set with modeltest (Posada & Crandall 1998). Using this model, we then conducted maximum likelihood and maximum-likelihood bootstrap analyses in paup* (Swofford 2002). Our analyses found six major well-supported clades within Osmundaceae (Fig. 1). The unidentified gametophyte is resolved as a member of the Osmunda subgenus Osmunda clade, which in turn comprises two subclades: one including Osmunda japonica, Osmunda lancea, and one of three available O. regalis rbcL sequences; the other including two other O. regalis sequences and the newly generated sequence of the gametophyte cultivated in Göttingen.

As demonstrated here, DNA sequences can be used to successfully identify fern gametophytes. Using blast, we were able to clearly establish that our gametophyte was a member of Osmundaceae, within the genus Osmunda, and likely assignable to the species O. regalis. Through a phylogenetic analysis, we were able to confirm, with considerable confidence, that our gametophyte was a

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**Fig. 1** Unrooted phylogram resulting from a maximum likelihood analysis of all rbcL sequences of Osmundaceae currently available in GenBank plus the sequence obtained from the gametophyte grown at the Alter Botanischer Garten Göttingen (O. reG). Some sequences are too similar to appear as terminal branches. Bootstrap values > 50% are shown. Osmunda species abbreviations: O. ba, O. banksiifolia; O. br, O. bromeliifolia; O. cin, O. cinnamomea; O. cl, O. claytoniana; O. jap, O. japonica; O. jav, O. javanica; O. la, O. lancea; O. re, O. regalis; O. ra, O. rachellii. Numbers are used to specify sequences if more than one exists per species.
shown that differences in gametophyte and sporophyte distribution using DNA sequences will improve our understanding of shaped gametophytes). Identification of fern gametophytes with unidentified gametophytes in the field (DNA can be problems by directly sequencing sporophytes co-occurring variations. Individual studies may also overcome these determinations, and explore inter- and intraspecific we will be better able to identify sequences with incorrect of available data is rapidly improving, and with more data sampling in GenBank. However, the quantity and quality of currently accepted fern genera have been deposited in GenBank, and this number continues to grow rapidly. Therefore, blast will likely be able to identify the correct family and perhaps even the genus for most unknown fern gametophytes. At the species level, however, more caution is warranted, as many genera are still insufficiently sampled. As an example, rbcL sequences exist only for about 10% of Asplenium species despite the fact that this genus has been studied extensively (Schneider et al. 2005). Furthermore, studies of Asplenium and other groups have shown that rbcL sequences are not always sufficient to discriminate among species (Janssen & Schneider 2005; Schneider et al. 2005). To circumvent this problem, more variable regions (e.g. intergenic spacers) are frequently used but fewer sequences currently exist.

To apply the suggested approach — identification of fern gametophytes using DNA sequences — broadly, one will have to overcome three problems outlined above: (i) the occurrence of misidentified or erroneous sequences in GenBank; (ii) the potential inability of rbcL to discriminate among species; and (iii) the problem of incomplete sampling in GenBank. However, the quantity and quality of available data is rapidly improving, and with more data we will be better able to identify sequences with incorrect determinations, and explore inter- and intraspecific variation. Individual studies may also overcome these problems by directly sequencing sporophytes co-occurring with unidentified gametophytes in the field (DNA can be successfully extracted and amplified from individual heart-shaped gametophytes). Identification of fern gametophytes using DNA sequences will improve our understanding of differences in gametophyte and sporophyte distribution and abundance, and probably result in a revolution in our understanding of fern ecology.

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References

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