

DNA BARCODING

DNA barcoding exposes a case of mistaken identity in the fern horticultural trade

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Abstract

Using cheilanthoid ferns, we provide an example of how DNA barcoding approaches can be useful to the horticultural community for keeping plants in the trade accurately identified. We use plastid *rbcL*, *atpA*, and *trnG-R* sequence data to demonstrate that a fern marketed as *Cheilanthes wrightii* (endemic to the southwestern USA and northern Mexico) in the horticultural trade is, in fact, *Cheilanthes distans* (endemic to Australia and adjacent islands). Public and private (accessible with permission) databases contain a wealth of DNA sequence data that are linked to vouchered plant material. These data have uses beyond those for which they were originally generated, and they provide an important resource for fostering collaborations between the academic and horticultural communities. We strongly advocate the barcoding approach as a valuable new technology available to the horticulture industry to help correct plant identification errors in the international trade.

Keywords: *Cheilanthes*, cheilanthoids, cultivated, DNA barcoding, ferns, horticultural trade, lip ferns

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Introduction

Gardening with ferns was an extraordinarily popular activity during the Victorian era (Allen 1969; Moran 2004) and has recently enjoyed renewed popularity due, in part, to the increasing availability of ferns originating from Asia, Australia, and New Zealand (Dancer 2009). The resurgence of fern gardening has been facilitated by the recent publication of several authoritative manuals on fern cultivation (Hoshizaki & Moran 2001; Mickel 2003; Olsen 2007). However, because the number of species introduced into the fern horticultural trade has greatly proliferated, it has become increasingly difficult to determine exactly which species are being propagated and sold. Ferns are especially difficult to monitor in the trade; without the advantage of flowers, fruits, or seeds, one has only relatively cryptic sporangial and vegetative features to distinguish between them. Furthermore, with

their spore dispersal capabilities and often long-creeping rhizomes, ferns in close quarters (such as in a greenhouse) can readily colonize other nearby pots, and eventually take up exclusive residency far from where they were originally 'potted'. Keeping track of the true identities of these ferns and their original provenance data is therefore a significant challenge.

Rock gardens are especially amenable to growing a diversity of ferns, and xeric-adapted members of the Pteridaceae (including the cryptogramroids, adiantoids, and cheilanthoids; see Schuettpelz *et al.* 2007) are perennial horticultural favourites. In nature, these ferns tolerate high temperatures, bright light, and alkaline conditions, making them well adapted to many landscape situations (Sealy & Bostic 1993). Ferns in the genus *Cheilanthes* usually occur in semiarid habitats and have evolved several adaptive strategies for surviving intense sunshine and long periods of drought (Olsen 2007). Commonly known as 'lip ferns', these plants have finely divided leaves that are usually <12 inches long, with leaf margins that curl to enclose the sori, and lower surfaces

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often densely covered with hairs and/or scales. Morphological convergence in these features makes cheilanthoids particularly prone to misidentification.

Cheilanthes wrightii Hook. (Wright's Lip Fern) is native to the desert areas of Arizona, New Mexico, Texas, and adjacent Mexico and is remarkable among members of this genus for its lack of protective hairs or scales (Mickel & Smith 2004). We recently recognized that a species marketed in the horticultural trade as *C. wrightii* did not, in fact, resemble *C. wrightii* as observed in its native habitat. Attempts to identify the cultivated plants using the *Cheilanthes* treatment in the *Flora of North America* (Windham & Rabe 1993) further revealed that there are no known North American species morphologically similar to the marketed plant. With no knowledge of the original geographical origin of this fern and without a worldwide key to the very diverse (and polyphyletic) genus *Cheilanthes*, it would seem impossible to determine the true identity of the taxon masquerading in the trade as *C. wrightii*.

However, our ongoing molecular phylogenetic studies of ferns, especially within Pteridaceae (Schuettelpelz *et al.* 2007; Rothfels *et al.* 2008; Grusz *et al.* 2009b; Windham *et al.* 2009), have allowed us to build up an extensive database of fern DNA sequences (<http://www.pryerlab.net>). We have plastid *rbcl*, *atpA*, and *trnG-R* (including the *trnG* intron and the *trnG-trnR* intergenic spacer) sequences for more than half of the world's cheilanthoid fern species. These three maternally inherited regions, either alone or in various combinations, have proven to be very useful for resolving phylogenetic relationships within ferns (Schuettelpelz *et al.* 2006; Korall *et al.* 2007; Nagalingum *et al.* 2007) and are very easy to amplify from small amounts of DNA extracted from fresh, silica-dried, or herbarium material.

Recently, a DNA sequence-based identification system has been advanced to provide rapid and accurate recognition of unidentified plants whose 'DNA barcodes' may already be registered in a sequence library (Kress *et al.* 2005; Kress & Erickson 2007; Pennisi 2007; Ledford 2008; Nitta 2008; Fazekas *et al.* 2009). The sequence data of the taxon in question are compared to a library of reference sequences from known species, and a match is considered to be an accurate identification. Here, we experiment with these DNA barcoding techniques to determine whether they are able to resolve the morphological disparity we observed between *C. wrightii*—as we know it from the field—and the '*C. wrightii*' encountered in the horticultural trade.

Materials and methods

Our study focused on a plant marketed as *Cheilanthes wrightii* in the horticultural trade that was obtained from

Juniper Level Botanic Garden (JLBG) in Raleigh, North Carolina. DNA sequences from this plant were compared to published sequences and to sequences from other wild and cultivated individuals (Table 1). Our protocols for isolating genomic DNA and for the amplification and sequencing of the plastid *rbcl* and *atpA* regions follow Schuettelpelz & Pryer (2007). The plastid region *trnG-R* was amplified and sequenced following Korall *et al.* (2007) and Nagalingum *et al.* (2007). These three regions are referred to above in order of increasing sequence divergence—and discriminating power—among fern species.

The basic local alignment search tool (BLAST) at the National Center for Biotechnology Information Web site (<http://www.ncbi.nlm.nih.gov/>) finds regions of local similarity between DNA sequences and calculates the statistical significance of matches (Altschul *et al.* 1997). BLAST was used here to compare the nucleotide sequences (*rbcl*, *atpA*, and *trnG-R*) that we obtained from the JLBG plant to those available in public DNA sequence databases. We also compared the sequence data against our own extensive (nearly 7000 records) database of vouchered, soon-to-be published sequences (<http://www.pryerlab.net>). Sequence data for species pair comparisons were aligned using SEQUENCHER 4.5 (Gene Codes Corporation), nonoverlapping bases at the 5' and 3' ends were removed, and those parts of the alignment where gaps had been introduced in one or the other sequence were excluded. The total number of nucleotides compared was then recorded, along with the number of nucleotide differences observed. Each pairwise alignment was then imported into PAUP* version 4 (Swofford 2001) where pairwise distances (sequence percent-divergence scores) were calculated.

Results

We successfully obtained DNA sequence data for all three plastid regions from our horticultural specimen (Schuettelpelz 320, DUKE, Fern DNA database number 2965, Table 1). BLAST searches using each of these sequences found the top match to be from a specimen of *Cheilanthes distans* (R. Br.) Mett. (Bristly Cloak Fern) that was field-collected in Australia (Nagalingum 23, DUKE, Fern DNA database number 3894, Table 1). There were no differences between the query sequences and the accessioned sequences across 1309 nucleotide sites for *rbcl*, one difference across 1739 sites for *atpA*, and one difference across 1091 sites for *trnG-R*; all told, the sequences from the cultivated plant differed from Australian *C. distans* by <0.05% (Fig. 1).

Because of our ongoing molecular phylogenetic studies of cheilanthoid ferns, we also had sequence data available for these same three plastid regions for

Table 1 Voucher information and GenBank accession numbers for cheilanthoid ferns (*Cheilanthes*) examined in this study

Fern DNA database number*	Original identification	Final identification	Voucher collection site	Voucher information	Original source location	GenBank Acc. Nos.
2965	<i>Cheilanthes wrightii</i> Hook.	<i>Cheilanthes distans</i> (R. Br.) Mett.	USA, cultivated at Juniper Level Botanic Garden, NC	Schuettpelz 320 (DUKE)	USA, cultivated at Casa Flora, Inc., TX	<i>rbcL</i> : HM003029 <i>atpA</i> : HM003025 <i>trnG-R</i> : HM003033
3148	<i>C. wrightii</i> Hook.	<i>C. wrightii</i> Hook.	USA, AZ, Yavapai Co., BLM, tributary of Black Canyon	Schuettpelz <i>et al.</i> 441 (DUKE)	NA	<i>rbcL</i> : HM003030 <i>atpA</i> : HM003026 <i>trnG-R</i> : HM003034
3209	<i>Cheilanthes pringlei</i> Davenport	<i>C. pringlei</i> Davenport	USA, AZ, Pima Co., Tucson Mountain County Park	Schuettpelz <i>et al.</i> 502 (DUKE)	NA	<i>rbcL</i> : HM003031 <i>atpA</i> : HM003027 <i>trnG-R</i> : HM003035
3894	<i>C. distans</i> (R. Br.) Mett.	<i>C. distans</i> (R. Br.) Mett.	Australia, NSW, SE of Sydney, Casula	Nagalingum 23 (DUKE)	NA	<i>rbcL</i> : EU268783 <i>atpA</i> : EU268734 <i>trnG-R</i> : EU268680
3905	<i>Cheilanthes lasiophylla</i> Pic. Serm.	<i>C. lasiophylla</i> Pic. Serm.	Australia NSW, Buckeroo Mountain	Greuter 20743 (NSW)	NA	<i>rbcL</i> : HM003032 <i>atpA</i> : HM003028 <i>trnG-R</i> : HM003036

NA, not applicable.

*<http://www.pryerlab.net/>

field-collected *Cheilanthes wrightii* from Arizona (Schuettpelz 441, DUKE, Fern DNA database number 3148, Table 1). When we compared the sequences from the horticultural specimen to those from true *C. wrightii*, we observed 64 differences across 1309 nucleotide sites for *rbcL*, 118 differences across 1823 sites for *atpA*, and 134 differences across 1100 sites for *trnG-R*; in all, the sequence data from the cultivated plant diverged from true *C. wrightii* by 7.5% (Fig. 1).

Discussion

Plastid *rbcL* is the most commonly sequenced gene for phylogenetic studies of plants (Schuettpelz *et al.* 2006). Previous studies (e.g. Gadek & Quinn 1993; Les *et al.* 1997; Chen *et al.* 1999) have reported *rbcL* sequence percent-divergence values as low as 0.4–0.9% between genera of seed plants, translating to an expectation of about 4–9 nucleotide differences over a stretch of 1000 base pairs. In ferns, however, *rbcL* percent-divergence values between species within the same genus have been reported to range from 0.2% to 1.8% (Wolf *et al.* 1994; Haufler & Ranker 1995; Hauk 1995; Gastony & Ungerer 1997; Yatabe *et al.* 1998; Smith *et al.* 2001). The ability of *rbcL* to discriminate between fern species indicates that the recent proposal to use this locus as part of an official multilocus plant DNA barcode (CBOL Plant Working Group 2009; Thomas 2009) may be especially well suited

to ferns. The *rbcL* gene has already been shown to be effective in identifying unknown fern gametophytes to the species level (Schneider & Schuettpelz 2006). The other proposed (and approved) plant barcode is the plastid *matK* gene (CBOL Plant Working Group 2009; <http://barcoding.si.edu/PDF/PlantWG/CBOL%20Decision%20-%20Plant%20Barcode%20Regions.pdf>). Although very useful in angiosperms, the lack of conserved priming sites in most ferns (which do not have the typical *trnK/matK* arrangement) has rendered the amplification of *matK* challenging (Duffy *et al.* 2009). The recent development of novel primers aimed at the *matK*-coding region should soon improve the utility of this gene in ferns for DNA barcoding (Kuo *et al.* 2008, 2009; Duffy *et al.* 2009).

Our finding here that *Cheilanthes 'wrightii'* from the horticultural trade has an *rbcL* sequence that is 100% identical to field-collected *Cheilanthes distans* from Australia (Fig. 1) strongly suggests that this cultivated individual is, in fact, *C. distans*. This conclusion is further supported by there being only a single nucleotide difference observed between the cultivated plant and *C. distans* for both *atpA* and *trnG-R* (Fig. 1), two plastid gene regions with a much greater sequence percent-divergence expectation between fern species than *rbcL* (Schuettpelz *et al.* 2006; Korall *et al.* 2007; Nagalingum *et al.* 2007).

Our ongoing molecular phylogenetic studies of cheilanthoids (Windham *et al.* 2009; Windham MD,

		<i>C. 'wrightii'</i> (hort trade) #2965	<i>C. pringlei</i> (Arizona) #3209	<i>C. wrightii</i> (Arizona) #3148	<i>C. lasiophylla</i> (Australia) #3905	<i>C. distans</i> (Australia) #3894
<i>C. distans</i> (Australia) #3894	<i>rbcL</i>	0 (1309) 0.00%	78 (1309) 5.96%	64 (1309) 4.89%	11 (1309) 0.84%	—
	<i>atpA</i>	1 (1739) 0.06%	97 (1732) 5.60%	106 (1731) 6.12%	6 (1717) 0.35%	
	<i>trnG-R</i>	1 (1091) 0.09%	126 (1058) 11.95%	126 (1085) 11.65%	6 (1081) 0.56%	
<i>C. lasiophylla</i> (Australia) #3905	<i>rbcL</i>	11 (1309) 0.84%	71 (1309) 5.42%	65 (1309) 4.97%	—	
	<i>atpA</i>	7 (1811) 0.39%	105 (1804) 5.82%	117 (1801) 6.50%		
	<i>trnG-R</i>	9 (1112) 0.81%	119 (1080) 11.02%	128 (1102) 11.62%		
<i>C. wrightii</i> (Arizona) #3148	<i>rbcL</i>	64 (1309) 4.89%	21 (1309) 1.60%	—		
	<i>atpA</i>	118 (1823) 6.47%	23 (1834) 1.25%			
	<i>trnG-R</i>	134 (1100) 12.18%	26 (1086) 2.40%			
<i>C. pringlei</i> (Arizona) #3209	<i>rbcL</i>	78 (1309) 5.96%	—			
	<i>atpA</i>	106 (1826) 5.81%				
	<i>trnG-R</i>	129 (1083) 11.91%				
<i>C. 'wrightii'</i> (hort trade) #2965	<i>rbcL</i>	—				
	<i>atpA</i>					
	<i>trnG-R</i>					

Fig. 1 Dissimilarity matrix indicating the number of nucleotide differences observed in *rbcL*, *atpA*, and *trnG-R* sequence comparisons between *Cheilanthes distans*, *Cheilanthes lasiophylla*, *Cheilanthes wrightii*, *Cheilanthes pringlei*, and cultivated *C. 'wrightii'* (= *C. distans*; see Table 1). Numbers in parentheses indicate the total number of base pairs compared; sequence percent-divergence is also indicated for each comparison.

Schuettpelz E, Huiet L, Grusz AL, Rothfels CJ, Pryer KM (unpublished) robustly place *C. wrightii* and *C. distans* in two distantly related lineages. *Cheilanthes wrightii* is a member of the well-supported myriopterid lineage—a primarily North American group (Fig. 2). Within the myriopterids, *C. wrightii* is the earliest diverging member of the 'alabamesis' clade (Grusz *et al.* 2009a; Windham *et al.* unpublished), which includes the closely related *Cheilanthes pringlei* Davenport. As expected by their close phylogenetic proximity, the sequence percent-divergence comparisons between *C. wrightii* and *C. pringlei* across all three plastid regions are low, ranging from 1.25% to 2.4% (Fig. 1). *Cheilanthes distans*, on the other hand, is a member of the hemionitid lineage (Fig. 2)—the largest and most diverse clade of cheilanthoids (about 67% of cheilanthoid diversity). Within the hemionitids, there is a primarily Australian clade where *C. distans* is strongly supported as sister to *Cheilanthes lasiophylla* Pic. Serm. (Windham *et al.* unpublished). As expected by their robust sister relationship, the sequence

percent-divergence comparisons between *C. distans* and *C. lasiophylla* across all three plastid regions are also very low, ranging from 0.35% to 0.84% (Fig. 1).

The sequence comparison statistics in Fig. 1 show that *C. 'wrightii'* from the horticultural trade is, in fact, very distinct from true *C. wrightii* and *C. pringlei*, members of the myriopterid clade. It appears that some, or probably all, individuals marketed as *C. wrightii* in the horticultural trade are actually *C. distans*. This is confirmed by the identical *rbcL* and near-identical *atpA* and *trnG-R* sequences of the horticultural unknown to *C. distans*, as well as by its close sequence similarity to the sister taxon to *C. distans*, *C. lasiophylla* (Fig. 1). At some point in time, either a labelling error was made, or perhaps a pot that once housed *C. wrightii* was colonized by *C. distans*. Indeed, *C. distans* is known to be an aggressive self-sower in greenhouses and rock gardens (D. Schwartz, personal observation). Misidentifications such as this one in the horticultural trade are not uncommon, nor are they by any means restricted to any particular nursery. Once we

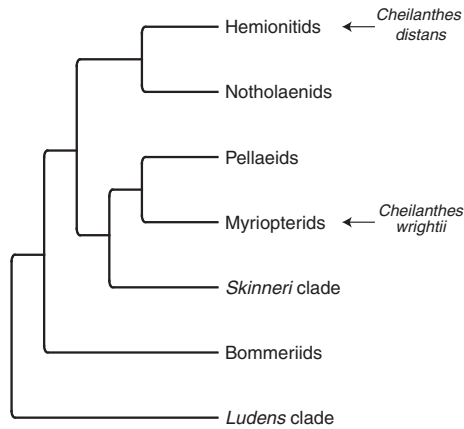


Fig. 2 Summary of phylogenetic relationships for major lineages of cheilanthoid ferns based on maximum likelihood analyses of *rbcL*, *atpA*, and *trnG-R* plastid sequence data for 157 species (*sensu* Windham *et al.* 2009). All seven clades are well supported, and the robust lineage placements of *Cheilanthes distans* and *Cheilanthes wrightii* are indicated (Windham *et al.* unpublished).

resolved this *C. wrightii/C. distans* identification problem, we realized that it extended to other nurseries and botanic gardens from across the country (e.g. in NC, TX, CA). Several other species misidentifications in the fern trade were also revealed (not discussed here).

Fortunately, the sporophytes of *C. wrightii* and *C. distans* can be distinguished morphologically (DNA sequencing is not essential to tell them apart). Most obviously, the leaf blades of *C. wrightii* are not scaly or hairy, whereas in *C. distans* they are covered by a combination of hairs and scales (Table 2). Another clear morphological difference is that *C. distans* has conspicuous vein endings that are usually visible on the adaxial leaf surface, forming a row of shallow indentations parallel to the leaf margins (Fig. 3; Table 2). These vein endings appear to be absent from all members of the myriopterid clade, including *C. wrightii* (Mickel & Smith 2004). Finally,

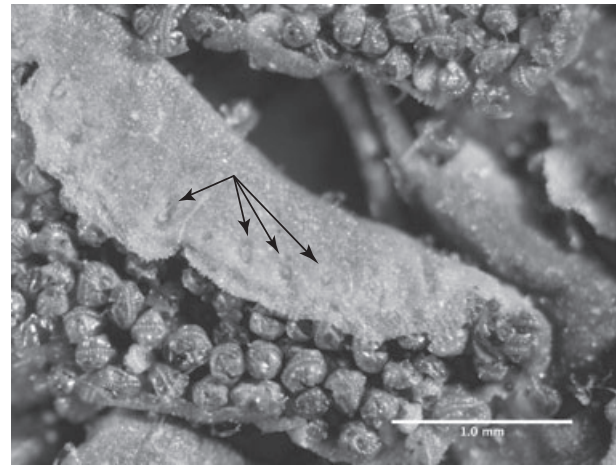


Fig. 3 Light micrograph of adaxial (upper) leaf surface of cultivated *Cheilanthes distans* (Schuettpelez 320, DUKE). Arrows point to conspicuous vein endings (hydathodes; Table 2).

fertile specimens of the two species are also readily distinguished: sporangia of *C. wrightii* each carry 64 spores (Windham & Rabe 1993), whereas the sporangia of *C. distans* contain only 16 spores (McCarthy 1998).

In highlighting this case of mistaken identity in cheilanthoid ferns, we hope to foster further collaboration between academic researchers amassing databases of molecular data as part of their systematic research studies and those in the horticultural community responsible for maintaining accurate identifications and provenance records on plants in the trade. Considerable data are available in both public and unpublished (but accessible with permission) databases, and relatively little cost and effort is required for the two communities to partner and work together to resolve these issues. We strongly advocate the barcoding approach as a valuable new technology available to the horticulture industry—not only to correct persistent errors in fern identification in the international trade, but also to protect against their overexploitation.

Table 2 Morphological characters useful in distinguishing *Cheilanthes distans* from *Cheilanthes wrightii*. Based on Windham & Rabe (1993), McCarthy (1998), and personal observations of herbarium and field material

Characters	<i>C. distans</i> (R. Br.) Mett. Bristly Cloak Fern	<i>C. wrightii</i> Hook. Wright's Lip Fern
Rachis	Rounded on upper (adaxial) surface; densely scaly	Grooved on upper (adaxial) surface; not scaly (rarely with a few hair-like scales)
Abaxial (lower) leaf surface	Sparsely to densely scaly, very sparsely hairy	Not scaly or hairy
Adaxial (upper) leaf surface	Very sparsely scaly, sparsely to densely hairy (slender white hairs)	Not scaly or hairy
Vein endings (hydathodes)	Generally visible adaxially; sometimes pronounced and sunken	Not visible adaxially
Spore number per sporangium	16 spores	64 spores
Native distribution	Australia; New Zealand; some Pacific Islands	Arizona, New Mexico, Texas; northern Mexico

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