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Vittaria graminifolia (Pteridaceae) and *Didymoglossum petersii* (Hymenophyllaceae) in Broxton Rocks, GA

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ABSTRACT.—We report on the occurrence of independent gametophytes of *Didymoglossum petersii* and both gametophytes and sporophytes of *Vittaria graminifolia* in the Broxton Rocks Preserve of southern Georgia. This is the first time sporophytes of *V. graminifolia* have been observed in the United States. In order to unambiguously identify both taxa, we extracted DNA for each. In the case of *V. graminifolia*, we used BLAST to compare our results to sequences in GenBank for two plastid loci (*rbcL* and *rpoA*) to determine its affinities. Because there are no GenBank data for *D. petersii*, it was necessary to collect an additional specimen of this species for comparison in a phylogenetic analysis. Results confirm the identity of each specimen and provide insight into the biogeographic history of *D. petersii*.

KEY WORDS.—Filmy fern, shoestring fern, sporophyte, gametophyte, outcrops

Vittaria graminifolia Kaulf. and *Didymoglossum petersii* (A. Gray) Copel. are in two distantly related families of ferns, yet they share an enigmatic feature: the ability of their gametophytes to form long-lived populations absent of sporophytes (Bray, 1996; Farrar, 1993a). Archeologist Frankie Snow and botanist Carl Taylor recently noted small populations of two fern species in the Broxton Rocks Preserve of Southern Georgia (*pers. comm.*). One population contained gametophytes of *D. petersii*, which had been previously reported as growing in the preserve, while a second population had sporophytes and gametophytes of an unreported vittarioid species. Based on the ranges of vittarioid species near the area, the second population was thought to be either 1) a northern range extension for *V. lineata* (L.) Sm., which grows in Florida in the U.S., 2) a rare population of *V. graminifolia*, or 3) the first account of mature sporophytes of *V. appalachiana* Farrar and Mickel, which grows only as gametophytes throughout the Appalachian Mountains. Given that the spores from the specimen collected at Broxton rocks were trilete, a defining feature of *V. graminifolia*, as opposed to monolete, which is characteristic of *V.*

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lineata (Farrar, 1993a), we predicted that these sporophytes were likely *V. graminifolia* rather than *V. lineata* or *V. appalachiana*. What follows is a brief description of each species, a history of their collection, notes on the ecology of the site in which they were found, and results of a molecular analysis used to identify the two Broxton Rocks specimens.

Vittaria graminifolia.—This species grows mainly in the neotropics of Central and South America, where it readily produces sporophytes and is predominantly epiphytic. In the U.S., *V. graminifolia* has a chequered history of misidentification and collection. Edgar T. Wherry first reported the species as growing in Florida in his book the *Southern Fern Guide* (1964). Unfortunately, the identification proved to be incorrect, as Gerald Gastony later concluded that Wherry's specimen, as well as one Gastony himself had collected in the swamps of Collier County, FL, were both actually *V. lineata* based on morphological characters described by Tryon (1964; Gastony, 1980).

The first correctly identified specimen of *V. graminifolia* in North America was reported some ten years later, found by Donald Farrar and Garry Landry in St. Helena Parish, Louisiana (1987). They observed populations of *D. petersii*, which grew as sporophytes, and *V. graminifolia*, the latter only as independent gametophytes. The populations of both species were found growing on the buttresses of beech trees (*Fagus grandifolia* Ehrh.) and were identified based on allelic banding patterns, as well as distinctive morphology (Farrar and Landry, 1987).

Didymoglossum petersii.—This species has also been reported in the neotropics. Though apparently somewhat less widespread than *V. graminifolia*, the two have been reported in several of the same countries, including Guatemala, Costa Rica, El Salvador, and Mexico (Mickel and Smith, 2004). *Didymoglossum petersii* has several documented populations in the United States as well, in the southern Appalachian Plateau and Boston Mountains of Arkansas (Farrar, 1993b). The gametophytes of this species are filamentous and long-lived, thus enabling gametophytic populations to exist in the absence of sporophytes. In Arkansas, for example, independent gametophytes were observed growing some 50 km from the nearest known population of sporophytes (Bray, 1996).

Unfortunately, the gametophytes for this species are morphologically indistinguishable from many Hymenophyllaceae species with the same growth habit, such as the widespread and sporophyteless *Crepidomanes intricatum* (Farrar) Ebihara & Weakley. Although *D. petersii* is listed as occurring in Broxton Rocks based on the discovery of sporophytes there (Chafin, 2007; Edwards *et al.*, 2013), the presence of independent gametophyte colonies that lack sporophytes necessitates the use of genetic markers to determine whether these populations are *C. intricatum*.

Geography and ecology.—Both *V. graminifolia* and *D. petersii* were found growing together in Broxton Rocks Preserve, located in Coffee County, Georgia, in July of 2015. The preserve is owned by the Nature Conservancy and supports a unique ecosystem in the Atlantic coastal plain. While much of the area is dominated by longleaf pine (*Pinus palustris* Mill.), large sandstone

TABLE 1. Voucher and accession information for specimens sequenced in this study.

Species	Specimen location	Collector(s) & collection date	Tissue source & voucher location	Voucher number
<i>Didymoglossum petersii</i>	GA, Coffee Co.	J. Pinson, S. Chambers 2015	Field collected FLAS	FLAS 261024
<i>Didymoglossum petersii</i>	AL, Winston Co.	J. Pinson, S. Chambers 2017	Field Collected SEL	SEL113690
<i>Didymoglossum petersii</i>	TN, Blount Co.	E. Wofford 1999	Herbarium LSU	LSU00070729
<i>Vittaria graminifolia</i>	GA, Coffee Co.	J. Pinson, S. Chambers 2015	Field collected FLAS	FLAS 261025

outcrops also punctuate the landscape. These outcrops comprise the largest extrusion of what is known as the Altamaha Grit formation, a layer of sandstone covering 15,000 square miles, that ranges in age from the Oligocene epoch (33.9–23mya) to the Pleistocene epoch (2.6mya–11,700ya) (Huddleston, 1988; Edwards *et al.*, 2013). The Broxton Rocks preserve is the only place where the Altamaha Grit sandstone has been exposed through erosion, creating desert-like conditions on its flat-topped surface and exposed rock outcrops below.

Along the face of these exposed outcrops are small crevices in the rock that harbor the populations of ferns examined here, which are often growing in close proximity to one another. Similar outcrops exist throughout the Appalachian Mountain Range and Plateau and provide habitat for other fern species that exhibit a pattern of spatially separated sporophytes and gametophytes (Farrar, 1967; Pinson *et al.*, 2017). These Appalachian outcrops are known to buffer seasonal and daily temperature variation (Chambers and Emery, 2016; Farrar, 1998) and provide extremely shaded conditions. Thus, the outcrops and crevices present in the Broxton Rocks preserve likely serve a similar function as the Appalachian outcrops by protecting the ferns from fluctuations in temperature and exposure to direct sunlight.

MATERIALS AND METHODS

Taxonomic sampling.—Specimens of the two unknown taxa were collected in 2015 with Frankie Snow, who helped guide us to the populations. Samples were dried using silica gel (Chase and Hills, 1991). An additional specimen from a known population of *D. petersii* producing sporophytes in Winston County, AL was collected during the summer of 2017 to serve as a comparison in the phylogenetic analysis, and an additional herbarium specimen of *D. petersii* from Blount County, TN was used for the same purpose. Field-collected specimens were deposited in either the Florida Museum of Natural History (FLAS) or Marie Selby Botanical Gardens (SEL) herbaria (Table 1).

Extraction and amplification.—Total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Germany). Lab techniques used existing

primers and protocols for polymerase chain reaction (PCR) from previous analyses of *rbcL* (Korall *et al.*, 2006) and *rpoA* (Pinson and Schuettpelz, 2016). Each 25 μ L reaction contained 4.5 μ L of purified water, 12.5 μ L of Amplitaq Gold DNA polymerase (Applied Biosystems), 2 μ L of each primer (10 μ M), and 4 μ L of DNA. Thermocycling conditions followed those of Pinson and Schuettpelz (2016). PCR products were then visualized on agarose gels. Sequencing was performed either by the University of Florida Interdisciplinary Center for Biotechnology Research (Gainesville, FL) or Eurofins Genomics (Louisville, KY). Sequences were assembled in Geneious v. 7.1.8 (Kearse *et al.*, 2012).

Phylogenetic analyses.—In order to confirm the identities of the two populations, we conducted two separate analyses. For the population suspected to be *V. graminifolia*, we used a nucleotide BLAST search to identify the sequences, because many accessions of *Vittaria* species (including *V. graminifolia*) have been sequenced for these loci and are available on GenBank. For the population believed to be *D. petersii*, we used a tree-building approach, because there are no sequences available for this taxon and relatively few for *Didymoglossum*. We conducted a phylogenetic analysis that included our newly-generated sequences, plus *rbcL* sequences downloaded from GenBank for: *Didymoglossum krausii* (Hook. & Grev.) C. Presl, *D. reptans* (Sw.) C. Presl, *D. hymenoides* (Hedw.) Copel., *D. tahitense* (Nadeaud) Ebihara & K. Iwats., *D. motleyi* (Bosch) Ebihara & K. Iwats., *D. lorencei* (Tardieu) Ebihara & Dubuisson, *D. ovale* E. Fourn., and *Polyphlebium angustatum* (Carmich.) Ebihara & Dubuisson, which served as an outgroup. Sequences were aligned by hand using Mesquite (v. 3.10; Maddison and Maddison, <http://mesquiteproject.org>). To determine which model best fit our data, we ran PartitionFinder (v 1.1.1 Lanfear *et al.*, 2012). We then performed a maximum likelihood analysis with a GTR+G model of substitution in RAxML (v. 8.0.0 Stamatakis, 2014), completing 1,000 bootstrap replicates and a search for the best tree in a single run (option `-f a`). The outgroup was constrained to be *P. angustatum*. Our alignment and tree files were uploaded to TreeBASE (submission ID 21631).

RESULTS

Based on our GenBank BLAST search, the *Vittaria* specimen collected at Broxton Rocks is *Vittaria graminifolia*. Both the *rbcL* and *rpoA* sequences from our field-collected specimens were matched at 99% identity to other *V. graminifolia* specimens on GenBank. For *D. petersii*, the full *rbcL* locus was amplified for the specimen collected at Broxton Rocks, as well as for an additional specimen collected in northern Alabama (Table 1). We had little success with amplifying sequences from herbarium samples but were able to amplify half of *rbcL* from one such specimen. The Broxton sample is resolved with 100% bootstrap support as being sister to a clade containing the two other *D. petersii* individuals (Fig. 1).

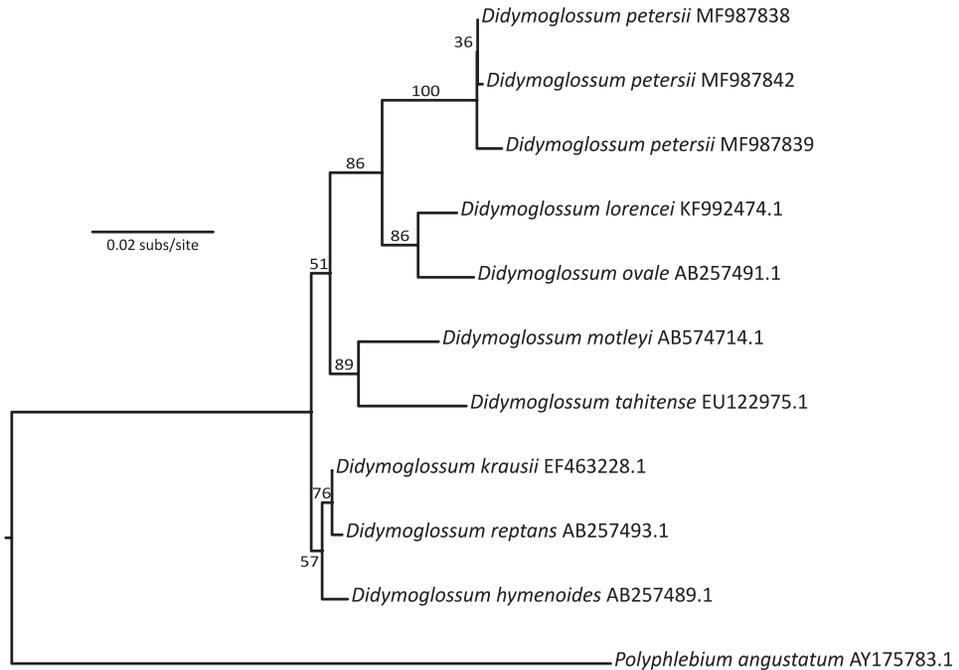


FIG. 1. The best tree topology based on a maximum likelihood analysis performed in RAxML. The *D. petersii* sample collected in Broxton (MF987839) is supported with 100% bootstrap as being sister to a clade containing two additional *D. petersii* taxa.

DISCUSSION

Vittaria graminifolia.—Based on our results, the sporophytic tissue collected from Broxton Rocks, GA belongs to *Vittaria graminifolia*. This is the first time a sporophyte of this species has been confirmed as growing in the United States. According to the Flora of North America, the closest population producing viable sporophytes is likely in Cuba (Farrar, 1993a); we therefore infer that the population at Broxton Rocks is the result of long-distance dispersal from that island or elsewhere in the tropics. However, studies should be conducted to examine the population structure of *V. graminifolia* and should include samples from this location and from the population reported from Louisiana (if it still exists). Such studies will help to support or refute our hypothesis concerning the origin of the Broxton Rocks plants and may help to determine how individuals in this population relate to those in Louisiana, which are solely gametophytes. The habitat differences between the population in Georgia (protected sandstone outcrops) and the one lacking sporophytes in Louisiana (beech tree buttresses) suggest that fine-scale environmental conditions may strongly influence the production of sporophytes in this species. It is also possible that the Louisiana population was founded by the long-distance dispersal of a single spore, and the lack of sporophytes there may represent an inability to self. It has been hypothesized that the more vegetatively prolific

morphology of some epiphytic fern gametophytes, including these species, is an adaptation to prolong their lifespan, and that these gametophytes can therefore ‘wait’ for long periods of time until the arrival of one or more conspecific spores permits outcrossing (Dassler and Farrar, 2001). The population in Louisiana may have been playing just such a waiting game.

Didymoglossum petersii.—The affinities of Hymenophyllaceae species in the eastern United States are poorly understood, but our analysis confirms that *D. petersii* is present in Georgia. This is the first time this species has been included in a phylogenetic analysis. Our maximum likelihood tree resolves *D. petersii* as being most closely related to a clade containing both *D. ovale* and *D. lorencei*. While the former grows in Central and South America, *D. lorencei* grows on the Mascarene Islands of the Paleotropics. Even more surprising is the more distant relationship in our tree of *D. petersii* with *D. krausii*, which grows nearby in southern Florida. These relationships suggest multiple colonization events of North America via long-distance dispersal by species already established in the tropics. *Didymoglossum* is estimated to include about 30 species in total (PPG 1, 2016), and sequencing of additional species than the few currently available and included here will be essential for clarifying the biogeographic history of this group.

Hymenophyllaceae in North America.—There are several other species of Hymenophyllaceae present in North America, but there are few for which molecular data are available. Of the eleven purportedly growing in North America, only three have GenBank accessions from populations in the U.S., including our present submission of *D. petersii*. This makes it difficult to infer the biogeographic history of the family as a whole in this region. Phylogenetic and historical reconstructions are further confounded by differences in distribution patterns of the life cycle stages and life history traits that make it difficult to positively identify gametophyte and sporophyte specimens to species (as is demonstrated by the species discussed here; see introduction above). Some species have limited distributions with sporophytes present, which is the case for most of the species growing in the southeastern United States (e.g. *Didymoglossum krausii*, *Trichomanes holopterum* Kunze). In contrast, *Hymenophyllum tayloriae* Farrar and Raine and *Crepidomanes intricatum*, both endemic to the U.S., are two of three fern species for which there are no known sporophytes anywhere in the world (Pinson *et al.*, 2017), the latter having the largest distribution of any of the North American Hymenophyllaceae (Farrar 1993b). Finally, there is evidence of dispersal to North America from at least three centers of diversity. Both *C. intricatum* and another species found in the northwestern U.S. and Canada, *Hymenophyllum wrightii* Bosch, have either conspecifics or close relatives growing in Asia, whereas *Hymenophyllum tunbridgense* L. Sm. grows in both the Appalachian Mountains and in Europe (Duffy, Stensvold, and Farrar, 2015; Ebihara, Farrar, and Ito, 2008; Farrar, 1967). The remaining species are either endemic to eastern North America (i.e. *H. tayloriae*, *Vandenboschia boschiana* (J.W. Sturm ex Bosch) Ebihara & K. Iwats., and *C. intricatum*) or have additional distributions in Central and/or South America (Farrar, 1993b). These

complications of life history and distribution underscore the need for further work on filmy ferns in North America, to understand both the relationships of these species as well as their biogeographic history.

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APPENDIX 1. INDIVIDUALS USED IN THIS STUDY, WITH INFORMATION PROVIDED IN THE FOLLOWING ORDER: SPECIES NAME, VOUCHER INFORMATION, PLACE OF ORIGIN, AND GENBANK ACCESSION NUMBERS.

Didymoglossum hymenoides (Hedw.) Copel., Dubuisson HG2004-27 (P), Guadeloupe, AB257489.1; *Didymoglossum krausii* (Hook. & Grev.) C. Presl, Schuettpelz 220 (DUKE), Ecuador: Napo Province, EF463228.1; *Didymoglossum lorencei* (Tardieu) Ebihara & Dubuisson, J.-Y. Dubuisson HR 2002-7 (P, REU), Reunion, KF992474.1; *Didymoglossum motleyi* (Bosch) Ebihara & K. Iwats., TNS:759342, Japan, AB574714.1; *Didymoglossum ovale* E. Fourn, T.A.Ohsawa 178-10 (TNS), Bolivia, AB257491.1; *Didymoglossum petersii* (A. Gray) Copel., Eugene B. Wofford s.n. (LSU), United States: Tennessee, MF987838; *Didymoglossum petersii* (A. Gray) Copel., Jerald Pinson 2 with Sally Chambers (FLAS), United States: Georgia, MF987839; *Didymoglossum petersii* (A. Gray) Copel., JP51 (SEL), United States: Alabama, MF987842; *Didymoglossum reptans* (Sw.) C. Presl, Lamieux 2275, Costa Rica, AB257493.1; *Didymoglossum tahitense* (Nadeaud) Ebihara & K. Iwats., J.Nitta005, French Polynesia: Moorea, EU122975.1; *Polyphlebium angustatum* (Carmich.) Ebihara & Dubuisson, M. Kessler 10957, Bolivia, AY175783.1; *Vittaria graminifolia* Kaulf., Jerald Pinson 1 with Sally Chambers (FLAS), United States: Georgia, MF987840, MF987841.