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Abstract.—Fern identification usually requires the use of mature sporophytes, since attempts to identify juveniles using morphological traits often provides unsatisfactory results. Here we examined young sporophytes found among boulders in a river basin of a xeric valley in central Peru. Attempts to identify these sporophytes first pointed to four different genera, two in Pteridaceae (*Anogramma* and *Pityrogramma*), and the others in Aspleniaceae (*Asplenium*) and Cystopteridaceae (*Cystopteris*). Here, we resolved this puzzle combining morphology and sequences of DNA (*rbcL* and *trnG-R*) that point to *Pityrogramma trifoliata* of Pteridaceae.

Key Words.—Andes, DNA sequencing, *Pityrogramma*, young sporophytes

Species of the Neotropical fern flora are, in general, morphologically identifiable thanks to floristic treatments (e.g. Tryon, 1989–1994; Mickel and Smith, 2004; Smith et al., 2005) and recent revisionary studies (e.g. Link-Pérez and Hickey, 2011; Vasco, 2011). However, the nature of the fern life cycle, with its two independent phases, offers a challenge for identifiying gametophytes and juveniles sporophytes, since those floristic or taxonomic treatments rely on morphological characters of the mature, sporophytic phase. This study shows an example of how DNA sequencing techniques can expand our capabilities for identification. In this case, they also helped to confirm a surprising range extension.

Fern richness and species distribution in the Neotropics are associated with forested, wet locales. Indeed, in Peru, 70% of fern diversity is found in the eastern Andean slopes that overlook the Amazon. For the Pacific basin (both coastal and western Andes below 7° South), there is a range of xeric to mesic

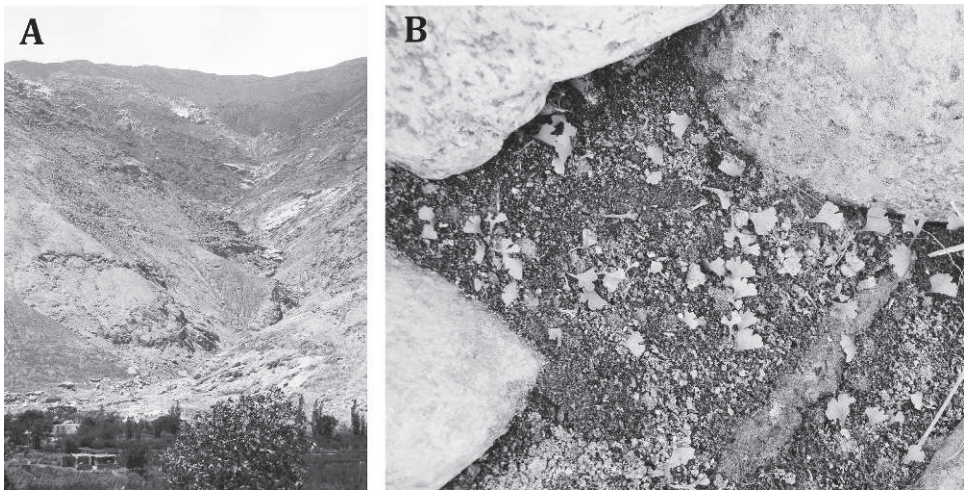

FIG. 1. Locality of the mystery fern in the valley of Mala, in central coastal Peru. A) View of barren hillslopes from the southern side of the valley at 400 m elevation. B) Gametophytes and young sporophytes under boulders near the Mala river.

environments along an altitudinal transect that harbors only 20% of the fern flora, usually occupying patchy habitats. An extreme xeric belt is known between 400 and 1000 m elevation in this basin.

Most members of the Peruvian Pacific basin fern flora belong to three families: Pteridaceae (dominated by cheilanthoids and pteroids *sensu* Schuettpelz et al., 2007; Rothfels, 2008), Aspleniaceae and Dryopteridaceae (León and Valencia, 1988; León and Young, 1999; León et al., 2002). All the coastal species are widely distributed and floristically they represent a subset of the western Andean fern flora (Tryon, 1960; León et al., 2002) comprising over 250 species.

In August 2011, a small population of gametophytes and young sporophytes was found under and among boulders along the river bed of a narrow xeric valley in central Peru, at 400 m elevation in a site with vegetation limited to the river margins (Fig. 1). These plants were initially assumed to belong to the common non-native *Adiantum capillus-veneris* L., which is found in nearby irrigation canals. However, the development of new leaves from material collected for cultivation, showed basal pinnae bearing acroscopic segments, so it was clearly another entity.

Some morphological characters such as indument and venation can aid in the identification of young sporophytes at the family and genus levels. Recently, molecular markers (DNA barcodes) have been used to improve our ability to identify problematic or easily confused ferns (e.g., Schneider and Schuettpelz, 2006; Li et al., 2009, 2011; Pryer et al., 2010; Yansura and Hoshizaki, 2012), and here this combined approach of morphological and molecular data was employed to resolve the identity of this puzzling Peruvian fern.

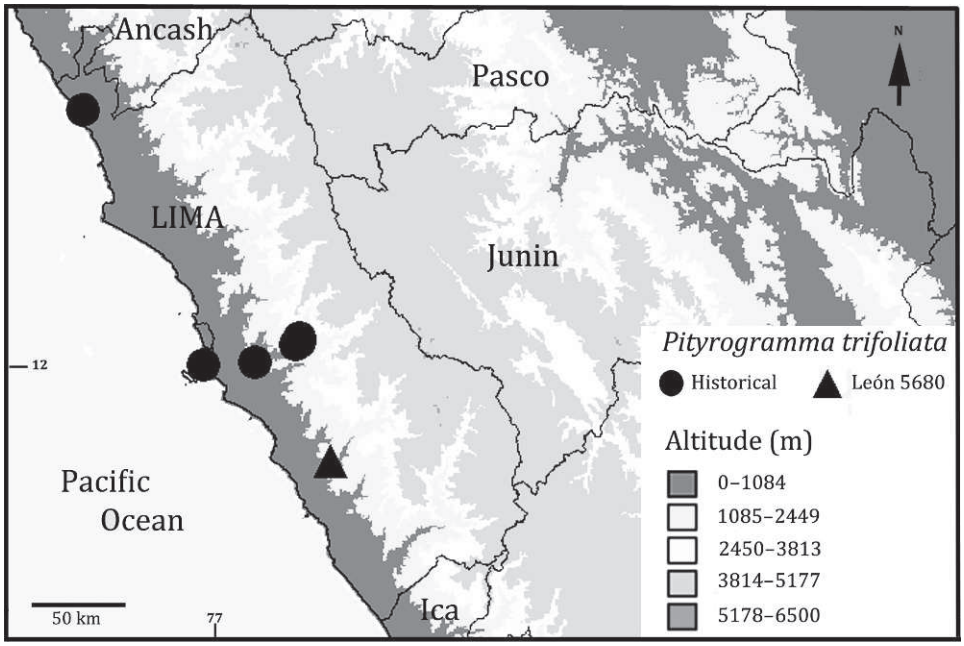

FIG. 2. Localities of all records of *Pityrogramma trifoliata* known from central-western Peru; circles represent historical records; triangle represents recent collection (B. León 5680, USM).

MATERIALS AND METHODS

Plants were obtained in the field, from a site in the Department of Lima, Province Cañete, in the Mala river basin, above the town of Calango (12°31′17.54″S, 76°30′1.11″W), at near 400 m elevation (Fig. 1A–B; Fig. 2), where climate conditions are hyper-arid (Rundel et al., 2007). They were present as small, undeveloped gametophytes and very young sporophytes. Plants and their surrounding soil were collected and maintained in a plastic-covered container for nearly 5 months, from August 2011 until February 2012. Two samples were taken in November 2011 to prepare herbarium vouchers and to extract DNA. Plants did not survive past February 2012.

DNA was isolated from dry tissue using the MP FastDNA® SPIN Kit and FastPrep® instrument (MP Biomedicals LLC, Solon, OH, USA).

Portions of two plastid loci—*rbcL* and the *trnG-trnR* intergenic spacer (henceforth, *trnG-R*)—in 21 μL reactions were amplified following established protocols (Rothfels et al. 2013). PCR was performed with an initial four-minute denaturation step (95°C), followed by 35 cycles of 30 seconds denaturation (95°C), 30 seconds elongation (40°C), and one minute elongation (71°C). The reaction was concluded with a final elongation step at 71°C, for 10 minutes. The *rbcL* amplifications used the primers *ESRBCL1F* and *ESRBCL654R* (Schuettpelz and Pryer, 2007), and the *trnG-R* reactions used *TRNG1F* and *TRNG63R* (Nagalingum et al., 2007). PCR products were purified using Shrimp

Alkaline Phosphatase (USB, Cleveland, Ohio) following established protocols (Rothfels et al., 2012) and sequenced on an ABI Prism 3700 DNA Analyzer (Applied Biosystems) at the Duke University Genome Sequencing and Analysis Core Resource, again using established protocols (Schuettpelz and Pryer, 2007). The forward and reverse chromatograms were assembled and edited in Sequencher 4.5 (Gene Codes Corporation), and the final sequences deposited in GenBank.

Based on the results of the preliminary BLAST comparisons (see below), we aligned the new sequences with the corresponding regions of previously published sequences spanning the Pteridaceae. For the *rbcL* alignment, we used sequences generated by Schuettpelz et al. (2007), with the exception of one sequence (AF336104), which is from Gastony and Johnson (2001). This *rbcL* dataset focuses on pteroids (*sensu* Schuettpelz et al., 2007; Rothfels, 2008) and involved no alignment issues (i.e., there were no indels). Fewer sequences were available for comparison with our *trnG-R* sequence. For this locus, we selected as wide a sample as possible, from Rothfels et al. (2008); it is primarly composed of cheilanthoids (*sensu* Schuettpelz et al., 2007; Rothfels, 2008), but includes one pteroid, and a cryptogrammoid. The sequences were aligned by hand, with ambiguous areas excluded prior to analysis. Heuristic tree searches on both datesets were performed under maximum parsimony in PAUP* v4.0A125 (Swofford, 2002).

Results

Gametophytes were oblong-cordate, nearly symmetrical, and glabrous. Most gametophytes were found in clumps in the field (Fig. 1B, 3A), and in this arrangement the lateral sides of the thalli were oblique to the midrib.

The initial sporophytic leaves were cuneate (Fig. 3A), while later, but still very young leaves had a pair of lateral segments similar in size to the apical one (Fig. 3B). In a month's time, we observed the development of pinnate-pinnatifid leaves (Fig. 3C), and these had a basal pair of pinnae with either an acroscopic segment, or segments along both the acroscopic and basiscopic sides (Fig. 3D). The leaves had an open venation that was 2–3 times furcate, and the apices of veins ended at the toothed margin (Fig. 3E); for young leaves, marginal teeth were not clearly developed. Well developed sporophytes also bore linear-lanceolate rhizome scales that were light brown, sometimes iridescent, and with elongate cells. In leaves with more than one pair of pinnae, indument consisted of multicellular, non-glandular hairs, initially sparse at the base of the rachis.

Attempting an identification based strictly on leaf morphology suggested the possibility of four different genera: *Anogramma*, *Asplenium*, *Cystopteris*, and *Pityrogramma*, all of which include taxa known in the local fern flora. Laminae that were once pinnate, with an acroscopic segment, pointed to a member of the Aspleniaceae (*Asplenium sessilifolium* Desv.) or Cystopteridaceae (*Cystopteris fragilis* (L.) Bernh.). The presence of non-clathrate rhizome scales eliminated *Asplenium*, but the presence of multicellular hairs similar to those

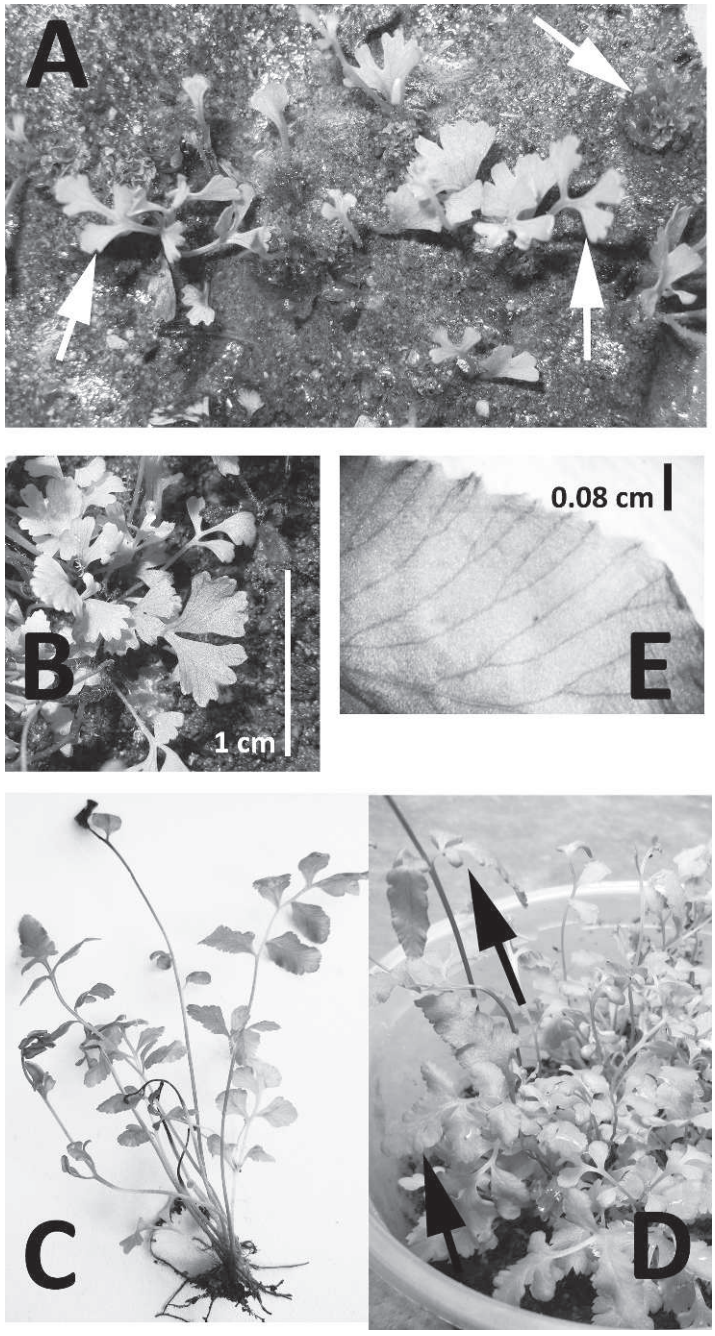

FIG. 3. A) Gametophytes and young sprorophytes showing furcate and pinnatisect laminae. B) Young sporophytes showing open venation. C) Four-month old sporophyte, fronds with elongate apical segments. D) Portion of pinnae, notice veins ending at tip of tooth. E) Young sporophyte, pinnae with acroscopic and basiscopic segments.

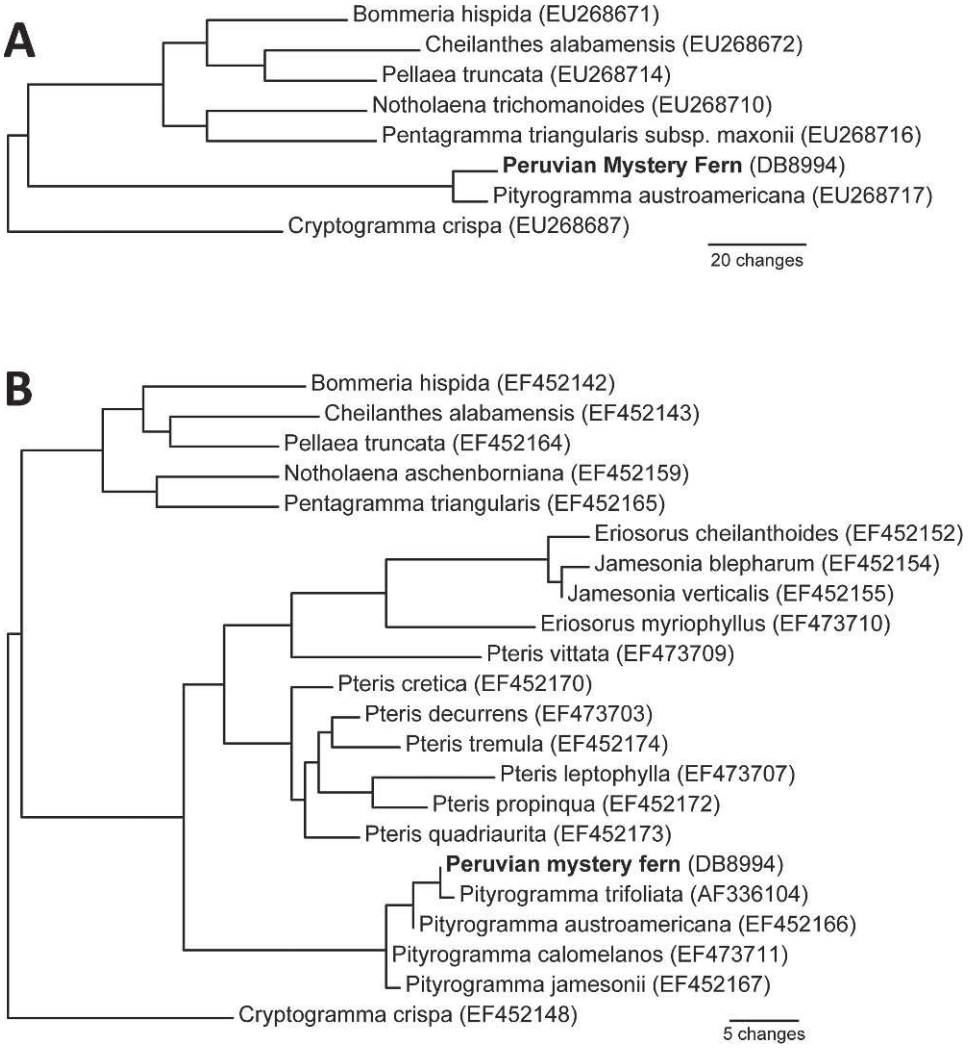

FIG. 4. Molecular phylogenies for the Peruvian mystery fern. A) The single most parsimonious tree for the *trnG-R* alignment, showing the position of the mystery fern among a broad Pteridaceae sample from Rothfels et al. 2008. B) One of 12 most parsimonious trees for the *rbcL* alignment, showing the position of the mystery fern among a broad Pteridaceae sample from Schuettpelz et al. 2007 (AF336104 is from Gastony and Johnson, 2001). Numbers in parentheses are GenBank accession numbers.

of *Cystopteris* supported the latter, as did the presence of toothed margins, although *Cystopteris fragilis* has veins ending in a sinus rather than in a tooth. A third possibility was a member of the Pteridaceae, either *Anogramma leptophylla* (L.) Link or *Pityrogramma chaerophylla* (Desv.) Domin, but in both these species the lamina is gradually reduced, and in the former, the rhizome does not bear scales, but hairs; additionally, the latter has not yet been

recorded in Peru. Another possibility among Pteridaceae, was either *Pityrogramma calomelanos* (L.) Link or *P. trifoliata* (L.) R. M. Tryon; the former has a leaf apex that is gradually reduced and it bears glandular hairs on the laminae, whereas in the latter the terminal segment has a similar shape to the lateral pinnae, and also, for some individuals, laminar glandular hairs can be absent.

BLAST searches supported the identity of the mystery fern as a species of Pteridaceae, apparently closest to *Pityrogramma*. In both datasets (*rbcL* and *trnG-R*), the mystery fern matches closely with accessions of *Pityrogramma* (Fig. 4 A–B). In the more densely sampled *rbcL* dataset, the mystery sequence is particularly closely related to *Pityrogramma trifoliata* (Fig. 4B); these two sequences differ by a single substitution across the 607 aligned sites. *Pityrogramma trifoliata* is sometimes treated as *Trismeria trifoliata* (L.) Diels (e.g. Zuloaga et al., 2008), the only commonly recognized member of that genus, and is morphologically highly distinctive within *Pityrogramma* (at least as mature sporophytes!). The mystery plant then, while not exactly identical in sequence to the published *rbcL* sequence from *P. trifoliata*, is almost certainly that species.

Discussion

The discovery of *Pityrogramma trifoliata* in river beds in the xeric belt of the Andean foothills is a novelty due to the underexplored microhabitat it inhabited and the stark bareness of the surrounding hills (Fig. 1A). Watkins et al. (2007) suggested that for terrestrial ferns, soil disturbance is related to gametophyte establishment success and growth. This also appears to be the case for our findings, as microsites among the riparian cobble provide shade and constantly humid soil essential for gametophyte colonization and development. In turn, these sites are likely highly unstable due to seasonal fluctuation in river discharge.

Pityrogramma trifoliata is known from an altitudinal range of 50 to 2300 m in Peru, but no previous collections are known from the xeric elevational belt itself. Populations of *P. trifoliata* are found in sparse clusters where humidity is constant, such as margins of waterfalls and irrigation channels, and from which a few collections of this species in western Peru have been previously reported. This species and other western Andes ferns are likely characterized by higher dispersability, resilience, and based on this study, the capacity to survive as a gametophyte.

If we had been restricted to using morphological data for identification, we would have required more time and cultivation efforts for the plants to express those morphological characters associated with *P. trifoliata*.

An important note as to the state of our knowledge of the fern flora is the scarcity of collections for ferns that are considered to be common. A more complete set of data on the natural history of ferns is also needed, especially those verifying and updating information on gametophytes, such as recent work within a phylogenetic framework in Pteridaceae (e.g. Gabriel y Galán, 2011; Johnson et al., 2012).

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