# Rediscovery of *Polypodium calirhiza* (Polypodiaceae) in Mexico

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**Abstract**. This study addresses reported discrepancies regarding the occurrence of *Polypodium calirhiza* in Mexico. The original paper describing this taxon cited collections from Mexico, but the species was omitted from the recent *Pteridophytes of Mexico*. Originally treated as a tetraploid cytotype of *P. californicum*, *P. calirhiza* now is hypothesized to have arisen through hybridization between *P. glycyrrhiza* and *P. californicum*. The tetraploid can be difficult to distinguish from either of its putative parents, but especially so from *P. californicum*. Our analyses show that a combination of spore length and abaxial rachis scale morphology consistently distinguishes *P. calirhiza* from *P. californicum*, and we confirm that both species occur in Mexico. Although occasionally found growing together in the United States, the two species are strongly allopatric in Mexico: *P. californicum* is restricted to coastal regions of the Baja California peninsula and neighboring Pacific islands, whereas *P. calirhiza* grows at high elevations in central and southern Mexico. The occurrence of *P. calirhiza* in Oaxaca, Mexico, marks the southernmost extent of the *P. vulgare* complex in the Western Hemisphere.

**Key Words:** Chromosomes, ploidy, rachis scales, spore size.

The *Polypodium vulgare* L. (Polypodiaceae) complex is an iconic group of ferns with a primarily north-temperate distribution. In North America, this complex is represented by six diploid species, four allotetraploids, and various sterile hybrids (Haufler et al., 1993, 1995a, 1995b). One of the more recently described allotetraploids is *P. calirhiza* S. A. Whitmore & A. R. Sm., hypothesized to have arisen through hybridization between the diploid species *P. glycyrrhiza* Maxon and *P. californicum* Kaulf. (Whitmore & Smith, 1991). Although formerly treated as a tetraploid cytotype of *P. californicum* (Manton, 1951; Lloyd, 1963; Lloyd & Lang, 1964), *P. calirhiza* shows additivity of isozyme bands (Haufler et al., 1995a) and a morphology that is subtly intermediate between the hypothesized parental species (Whitmore & Smith, 1991).

*Polypodium calirhiza* can be difficult to distinguish from its putative parents, and the situation is exacerbated by morphological variation in the allotetraploid that may be the genetic legacy of multiple, independent hybridization events (Haufler et al., 1995a, 1995b). Although *P. calirhiza* is not known to form triploid backcrosses with *P. californicum* as it does with *P. glycyrrhiza* (Haufler et al., 1993), it is more difficult to separate morphologically from *P. californicum*. Previous authors (Whitmore & Smith, 1991; Haufler et al., 1993) have proffered a number of characteristics that, taken together, appear to distinguish *P. calirhiza* from *P. californicum.* These include spore length (> 58 μm in *P.*

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*calirhiza* vs. < 58 μm in *P. californicum*); leaf blade shape (widest above the base in *P. calirhiza* vs. widest near the base in *P. californicum*); and degree of reticulation among leaf veins (areoles rare in *P. calirhiza* vs. common in *P. californicum*).

Geography also plays a useful role in distinguishing *P. calirhiza* and its putative parents in the region covered by the *Flora of North America* (Haufler et al., 1993). The three species inhabit the Pacific Rim of North America, usually in close proximity to the coast but with inland populations of *P. calirhiza* occurring in the Sierra Nevada of California (Whitmore & Smith, 1991). The range of *P. glycyrrhiza* extends from central California north to Alaska, with additional localities reported for the Kamchatka Peninsula (Haufler et al., 1993). *Polypodium californicum* ranges from central California south to Baja California, with some authors (i.e., Mickel & Beitel, 1988; Mickel & Smith, 2004) reporting its occurrence in southern Mexico. The allotetraploid *P. calirhiza* is distributed from central Oregon south to the Southern Coastal Ranges and High Sierra Nevada Region of California (Haufler et al., 1993; Baldwin et al., 2012). Notably, Whitmore and Smith (1991) reported disjunct occurrences of *P. calirhiza* in the Mexican states of Mexico and Oaxaca, yet Mickel and Smith (2004) subsequently treated these specimens as *P. californicum*.

A recent survey of *Polypodium* specimens at the DUKE herbarium conducted during a phylogenetic study of the *P. vulgare* complex (Sigel et al., in review) identified a potential new record of *P. californicum* from the western Mexican state of Jalisco (*R. Dyer 51;* Table I). Upon microscopic examination, however, this specimen proved more similar to *P. calirhiza* than to *P. californicum,* and molecular analysis of the nuclear locus *gapCp*-short revealed that the collection was nearly identical to a *P. calirhiza* specimen from Oregon (Sigel et al., in review). This led to the discovery of another specimen of *P. calirhiza* (*J. Mickel 7426*; Table I), identified by Whitmore and Smith (1991) as this species but referred to as *P. californicum* in *The Pteridophytes of Mexico* (Mickel & Smith, 2004). The result of the latter identification was to exclude *P. calirhiza* from the Mexican flora.

This discrepancy provided the impetus to revisit the distinctions between *P. californicum* and *P. calirhiza* and reassess the reported occurrence of the latter species in Mexico. Here, we use spore length measurements derived from chromosome vouchers to calibrate spore-based ploidy estimates for a series of Mexican *Polypodium* specimens previously identified as *P. californicum*.These data are combined with observations of abaxial rachis scale morphology and geographic/ecological distribution to ascertain whether some of the specimens assigned to *P. californicum* in *The Pteridophytes of Mexico* (Mickel & Smith, 2004) actually represented *P. calirhiza*.

## Materials and Methods

Seventeen accessions of *Polypodium calirhiza* and *P. californicum* from Oregon, California, and Mexico were examined for this study (Table I). Special effort was made to obtain specimens examined by Whitmore and Smith (1991) and/or cited in *The Pteridophytes of Mexico* (Mickel & Smith, 2004). Two of these (*Smith 836* and *Lemieux s.n.*) were voucher specimens for published chromosome counts (Whitmore & Smith, 1991), and two others (*Windham 706* and *835a*) provided new cytogenetic data. For the latter, leaves undergoing meiosis were fixed in the field using Farmer's solution (3 parts ethanol: 1 part glacial acetic acid). Protocols for the preparation of chromosome squashes followed Windham and Yatskievych (2003).

Average spore length was determined for all seventeen accessions included in Table I. Because spore size varies with maturity in *Polypodium*, sampling was limited to specimens with sporangia producing fully mature, yellow spores. To obtain spore size data, 10–50 spores (obtained from one to five sporangia) from each specimen were placed in a drop of glycerol and photographed at 100× magnification using a Leica MZ 12.5 dissecting microscope and a Canon EOS Rebel XSi camera. Spore length was measured using ImageJ version 1.38 (Abramoff et al., 2004) calibrated with a slide micrometer. Mean spore length and standard deviation were calculated for each specimen. Spore

TABLE I

Specimens of *Polypodium* used in this study. Taxon names are as confirmed or redetermined by the authors of this study. Numbers under taxon names are unique specimen identifiers, and superscripts indicate chromosome vouchers and specimens included in previous publications. Average spore length and rachis scale width/shape are given for each specimen, with some specimens exhibiting two types of scales. "NA" indicates that no rachis scales were observed.

| Taxon | Voucher Information | Average Spore Length±One Standard Deviation in μm (Number of Spores Measured) | Rachis Scale Width (Cell Number) / Shape |
|---|---|---|---|
| ***Polypodium californicum* Kaulf.** | | | |
| 1[d] | Mexico: Baja California, Guadalupe Island, *J. N. Rose 16015* (NY) | 56.27±2.45 (30) | >6 / lanceolate-caudate |
| 2 | Mexico: Baja California, Guadalupe Island, *G. B. Newcomb 170* (UC) | 56.64±3.67 (10) | NA |
| 3[a] | USA: California, Orange County, *M. D. Windham 706* (DUKE) | 57.08±3.95 (50) | 3–6 / linear-lanceolate<br>≥6 / lanceolate-caudate |
| 4[d] | Mexico: Baja California, Coronado Islands, *A. W. Anthony s.n.* (UC) | 57.25±3.95 (32) | NA |
| 5 | Mexico: Baja California, San Quentin Bay, *E. Palmer 703* (NY) | 58.18±1.54 (20) | 3–6 / linear-lanceolate<br>>6 / lanceolate-caudate |
| 6 | Mexico: Baja California, Guadalupe Island, *A. W. Anthony 256* (UC) | 58.32±1.87 (15) | 3–6 / linear-lanceolate<br>>6 / lanceolate-caudate |
| 7 | USA: California, Orange County, *J. Metzgar 176* (DUKE) | 58.32±2.92 (30) | 3–6 / linear-lanceolate<br>>6 / lanceolate-caudate |
| 8 | Mexico: Baja California Sur, Todos Santos, *T. S. Brandegee s.n.* (UC) | 58.40±2.84 (12) | 3–6 / linear-lanceolate<br>>6 / lanceolate-caudate |
| 9 | USA: California, San Mateo County, *L. Huiet 138* (DUKE) | 59.09±2.19 (30) | 3–6 / linear-lanceolate<br>>6 / lanceolate-caudate |
| ***Polypodium calirhiza* S. A. Whitmore & A .R. Sm.** | | | |
| 10[bd] | Mexico: Mexico, Nevado de Toluca, *J. N. Rose & J. H. Painter 7946* (NY) | 67.55±2.85 (30) | ≤3 / linear<br>3–6 / linear-lanceolate |
| 11[ac] | USA: California, Marin County, *A. R. Smith 836* (UC) | 67.69±2.04 (10) | 3–6 / linear-lanceolate |
| 12[abc] | USA: California, Mendocino County, *T. Lemieux s.n.* (UC) | 68.71±3.31 (36) | ≤3 / linear<br>3–6 / linear-lanceolate |
| 13[b] | Mexico: Veracruz, Xalapa, *Hahn s.n.* (UC) | 68.92±2.88 (20) | ≤3 / linear<br>3–6 / linear-lanceolate |
| 14[bd] | Mexico: Oaxaca, Distrito Ixtlán, *J. T. Mickel 7426* (UC) | 69.09±2.26 (30) | ≤3 / linear<br>3–6 / linear-lanceolate |
| 15[a] | USA: Oregon, Lane County, *M. D. Windham 835a* (DUKE) | 69.26±3.22 (40) | ≤3 / linear<br>3–6 / linear-lanceolate |
| 16[b] | Mexico: Jalisco, Volcán Nevado de Colima, *R. Dyer 51* (DUKE) | 69.86±5.44 (40) | ≤3 / linear<br>3–6 / linear-lanceolate |
| 17 | USA: Oregon, Curry County, *R. Halse 7580* (NY) | 70.29±2.85 (40) | ≤3 / linear<br>3–6 / linear-lanceolate |

[a] Chromosome voucher (chromosome counts indicated in Fig. 1)

[b] Specimen formerly identified as *P. californicum*; [c] Specimen identified as *P. calirhiza* by Whitmore and Smith (1991)

[d] Specimen identified as *P. californicum* in *The Pteridophytes of Mexico* (Mickel & Smith, 2004)

length measurements for the aforementioned chromosome vouchers were used to correlate spore size with ploidal level. A Mann-Whitney *U* test was performed in R (R Development Core Team, 2008) to determine whether mean spore lengths of specimens

representing different ploidal levels were significantly different. Summary statistics were calculated using Excel v. 14.1 (Microsoft, 2011).

All 17 accessions were visually inspected to determine leaf blade shape and degree of reticulation among leaf veins. The leaves of each were assigned to one of two categories as circumscribed in the *Flora of North America* treatment of the genus *Polypodium* (Haufler et al., 1993): 1) widest above the base (proximal 1–3 pinnae shorter than more distal pinnae) or 2) widest near the base (proximal 1–3 pinnae equal to or longer than more distal pinnae). In order to determine the prevalence of vein reticulation, five to ten pinnae per specimen were inspected under 10× magnification to estimate the number of areoles/pinna. In addition, all plants included in the study were examined at 10× magnification for the presence of small scales on the abaxial leaf rachis. These scales, which are nearly ubiquitous in the *Polypodium vulgare* complex but deciduous with age, were observed on fifteen of the seventeen specimens. For each plant with rachis scales, two to four scales were removed, placed on a slide in a drop of soapy water under a cover slip, and photographed at 50× magnification with the same microscope and camera used to document spore sizes. Rachis scales for each specimen were categorized according to their width (≤ 3 cells wide, 3 to 6 cells wide, or>6 cells wide) and shape (linear, linear-lanceolate, or lanceolate-caudate).

## Results

A new meiotic chromosome count of $n=37$ was obtained for *Polypodium californicum* 3 (Table I; Fig. 1; *M. D. Windham 706*), and a new count of $n=74$ was obtained for *P. calirhiza* 15 (Table I; Fig. 1; *M. D. Windham 835a*). Figure 1 illustrates the average spore length and standard deviations for the 17 specimens included in our analyses (see Table I for actual measurements). Spore measurements formed two statistically distinct groups (p-value<0.0001; Mann-Whitney *U* test): specimens 1 – 9 formed one group with an average spore length of 57.72 μm±0.32 μm (standard error of the mean), while specimens 10 – 17 formed a second group with an average spore length of 68.92 μm±0.34 μm (standard error of the mean).

Although the spore differences between tetraploid *P. calirhiza* and diploid *P. californicum* were striking, our study failed to detect clear differences in leaf blade shape and vein reticulation. Leaf blades that were widest above the base and leaf blades widest near the base were encountered in both taxa, with some individual plants exhibiting both leaf shapes. Leaf venation presented a different sort of problem when applied to herbarium specimens. This character could not be scored without clearing leaves in nearly half the accessions included this study due to the thickness of the leaf blades and/or abundance of sporangia. Among the specimens with visible venation, we observed individuals of both small- and large-spored specimens with no or few areoles per pinnae (one to two). Just two of the small-spored accessions (Table I, *P. californicum* 1 and 5) had pinnae with numerous areoles (five to 14).

In contrast to leaf blade shape and venation, there does appear to be a strong correlation between abaxial rachis scale morphology and spore length (Table I). Large-spored specimens exhibited linear to linear-lanceolate scales up to 6 cells wide, usually with at least some scales (often the majority)≤3 cells wide (Fig. 2). In contrast, the small-spored specimens that retained their abaxial rachis scales consistently exhibited some lanceolate-caudate scales>6 cells wide (Fig. 2), as well as linear-lanceolate scales 3 to 6 cells wide, but narrower scales were absent.

## Discussion

A strong correlation between spore size and sporophyte ploidal level has been documented in many fern lineages (Wagner, 1974; Kott & Britton, 1982; Pryer & Britton, 1983; Barrington et al., 1986; Grusz et al., 2009; Beck et al., 2010; Sigel et al., 2011; Li et al., 2012). As a general rule, diploid species produce smaller spores than closely related, congeneric polyploid species. Previous work on the *Polypodium californicum* complex (see Barrington et al., 1986; Whitmore & Smith 1991) suggested that this correlation

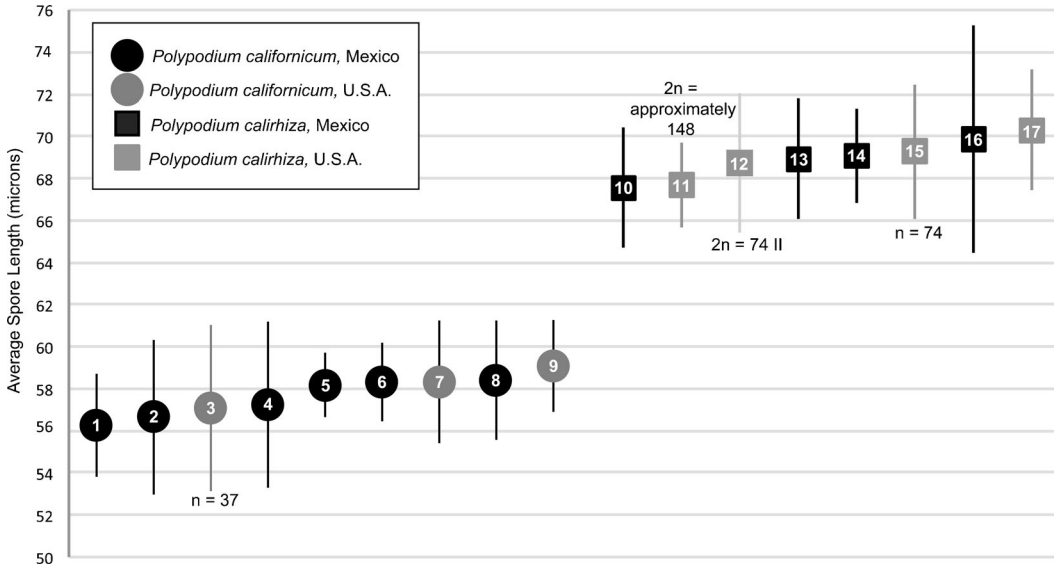


**Fig. 1.** Average spore lengths for specimens listed in Table I; numbers on symbols correspond to specific specimens. Error bars indicate one standard deviation. Specimens originating from Mexico are indicated in black, and those from the United States of America in gray; round symbols are diploids, square symbols are tetraploids. Chromosome counts are provided for the four chromosome voucher specimens listed in Table I.

might provide one of the best ways to separate *P. calirhiza* from *P. californicum*, and spore length was one of three morphological characters used in the *Flora of North America* to distinguish these taxa. In that work, the tetraploid *P. calirhiza* was characterized as having "spores usually more than 58 μm" whereas diploid *P. californicum* had "spores usually less than 58 μm" (Haufler et al., 1993: page 317).

By comparing the mean spore length of specimens of unknown ploidy to similar data derived from chromosome voucher specimens, we confirm that spore size can be used to differentiate *P. californicum* from *P. calirhiza.* Our new mitotic chromosome counts for *P. californicum* 3 (*M. D. Windham 706;* Table I; Fig. 1) and *P. calirhiza* 15 (*M. D. Windham 835a*) are consistent with previously reported counts for each species (Whitmore & Smith, 1991) and demonstrate that these specimens are diploid and tetraploid, respectively. The calibrated spore length data (Fig. 1) reveal two statistically distinct groups for which the group means are separated by approximately 11 μm and with no overlap in their standard deviations. The midpoint between these two distinct groups is approximately 63 μm. In this case, the small-spored (*P. californicum*) and large-spored (*P. calirhiza*) groups are amply distinct from one another, more so than many other diploid-tetraploid pairs of ferns (Whittier & Wagner, 1971; Kott & Britton, 1982; Pryer & Britton, 1983; Barrington et al., 1986).

Spore lengths observed among the specimens sampled for this study differ somewhat from those reported in the *Flora of North America*, which states that the spores of tetraploid *P. calirhiza* are "usually more than 58 μm" whereas those of diploid *P. californicum* are "usually less than 58 μm" (Haufler et al., 1993: page 317). Our data indicate that accessions of *P. calirhiza* from the United States have average spore lengths ranging from approximately 68 μm to 70 μm, with some individual lengths in excess of 73 μm (e.g., *P. calirhiza* 16 & 17; Table I, Fig. 1). Though these are higher than the values reported by Haufler et al. (1993), they do not contribute to specimen misidentification because they are above the stated threshold of 58 μm. In this case the problem is *P. californicum,* which our data indicate can exhibit average spore lengths as high as 59 μm and individual lengths exceeding 61 μm (e.g., *P. californicum* 9; Table I; Fig. 1).

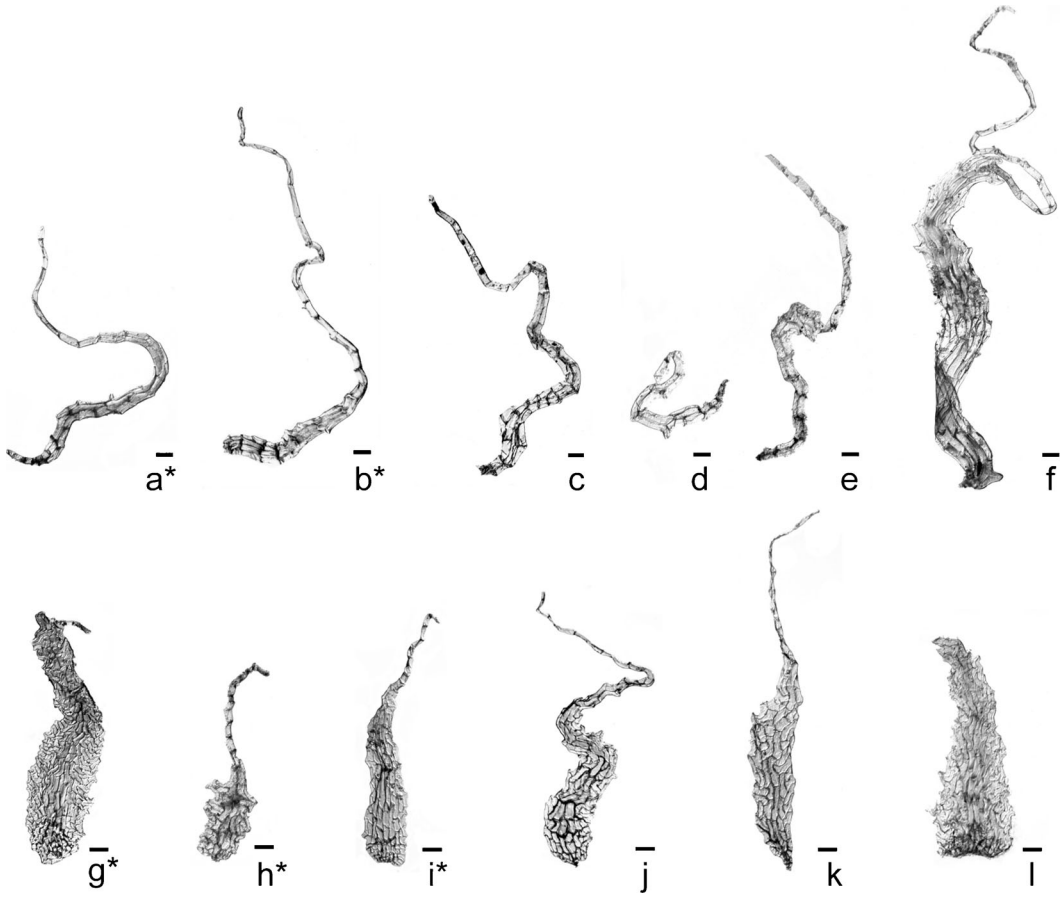


**Fig. 2.** Images of abaxial rachis scales for specimens of *Polypodium calirhiza* (a–f) and *P. californicum* (g–l): a*. *P. calirhiza* 14, *J. T. Mickel 7426*; b*. *P. calirhiza* 10, *J. N. Rose & J. H. Painter 7946*; c. *P. calirhiza* 12, *T. Lemieux* s.n.; d–e. *P. calirhiza* 17, *R. Halse 7580*; f. *P. calirhiza* 11, *A. R. Smith 836*; g*. *P. californicum* 5, *E. Palmer 703*; h*. *P. californicum* 1, *J. N. Rose 16015*; i*. *P. californicum* 8, *T. S. Brandegee s.n.;* j–k. *P. californicum* 9, *L. Huiet 138*; l. *P. californicum* 7, *J. Metzgar 176*. * indicates Mexican specimens. All photographs were taken at 50× magnification, cropped, and converted from RGB to 8-bit grayscale. Scale bars represent 100 μm.

These discrepancies may be due to expanded geographic sampling or slightly different measurement methodologies. However, given that our results closely match those of Whitmore and Smith (1991), it appears that the dividing line between *P. californicum* and *P. calirhiza* in terms of spore length should be 63 μm rather than the lower value of 58 μm suggested by Haufler et al. (1993).

In contrast to the clear separation provided by spore size, the differences in leaf blade shape and venation reported by Whitmore and Smith (1991) and Haufler et al. (1993) are not always effective for clearly distinguishing *P. californicum* from *P. calirhiza*. Leaf blade shape is highly variable and difficult to quantify, and the two supposed character states (leaves widest near the base vs. widest above the base) occasionally occur on leaves from the same plant. Though potentially a more valuable character, the widespread use of leaf venation as a distinguishing feature is inhibited by the thick leaf tissue and abundant sporangia of many specimens. While some specimens of *P. californicum* did exhibit a high degree of reticulation among veins, others exhibited few areoles and could not be differentiated from *P. calirhiza* based on this character alone.

Besides spore length, here we demonstrate the utility of abaxial rachis scale morphology

for differentiating *P. calirhiza* from *P. californicum*. By observing the abaxial rachis scales of previously annotated specimens of these species from California and Oregon, we were able to identify scale types diagnostic for each taxon (Table I; Fig. 2). While both species have linear-lanceolate scales that are 3–6 cells wide, only *P. calirhiza* has hairlike linear scales≤3 cells wide. At the other end of the continuum, *P. californicum* always has at least some lanceolate-caudate scales > 6 cells wide, a scale type that was not found in *P. calirhiza*. These abaxial rachis scale types are consistent with those described in treatments of *P. calirhiza* and *P. californicum* (Whitmore & Smith, 1991; Haufler et al., 1993), but previously have not been emphasized as a valuable character for distinguishing between the two taxa.

The strong correlation between ploidal level and spore size documented in this study allows us to infer that both diploids and tetraploids are present among the Mexican specimens assigned to *P. californicum* by Mickel and Smith (2004). Differences in rachis scales mirror those observed between *P. californicum* and *P. calirhiza* in California, allowing us to reevaluate the species identifications for all included accessions (Table I). We were able to identify four specimens of *Polypodium calirhiza* from Mexico (Table I; *P. calirhiza* 10, 13, 14, & 16), including two specimens (*P. calirhiza* 10 & 14) cited as *P. californicum* in *The Pteridophytes of Mexico* (Mickel & Smith, 2004). These results, combined with preliminary molecular analyses (Sigel et al., in review) justify the inclusion of *P. calirhiza* in the flora of Mexico.

During the course of this study, the highly allopatric geographic distributions of *Polypodium californicum* and *P. calirhiza* in Mexico became apparent. *Polypodium californicum* seems to be restricted to the western coast of the Baja California peninsula and outlying Pacific islands (Fig. 3). It reaches the southern and eastern limits of its

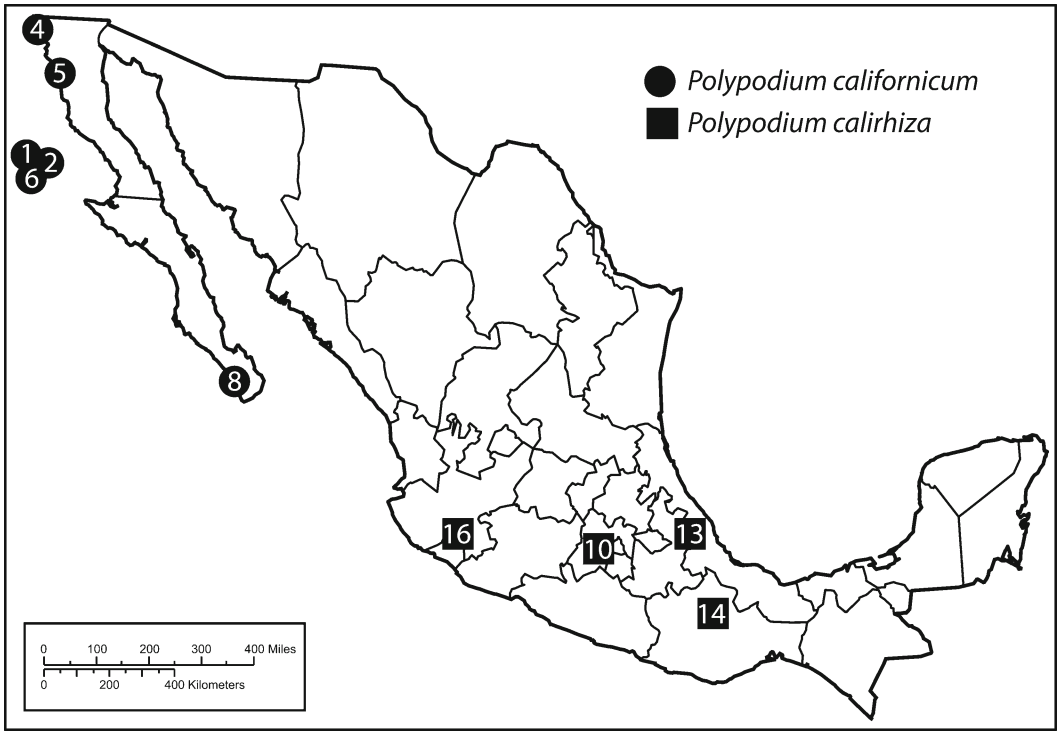


**Fig. 3.** Map of Mexico showing the localities for the Mexican specimens of *Polypodium calirhiza* and *P. californicum* listed in Table I. Symbol shape corresponds to species (see legend on figure), and numbers shown in symbols refer to specific specimens (Table I). Best estimates of collection localities were obtained from herbarium labels, some with limited geographic information.

range in the Sierra de la Laguna Mountains at the tip of Baja California Sur (Table I, *P. californicum* 8). Its restriction to low elevations in the Pacific coastal region mirrors its distribution in California (100-600 m; Mickel & Smith, 2004). This is likely due to ecological similarities, as the California Floristic Province extends to Baja California and Guadalupe Island (Raven & Axelrod, 1978; Hugo & Exequiel, 2007; Baldwin et al., 2012). The one Mexican specimen of *P. californicum* from Baja California Sur was collected in a low-elevation coastal area of the Cape Region (de la Luz et al., 2000).

In sharp contrast, the Mexican collections now identified as *Polypodium calirhiza* appear to be restricted to the central and southern mountains (Fig. 3) in the states of Jalisco, Mexico, Oaxaca, and Veracruz (Table I). Available collection data suggest that the species may be confined to high elevation sites in this region; the specimen from Oaxaca (Table I, *P. calirhiza* 14) was collected at approximately 3100 m, and the specimen from Mexico (Table I, *P. calirhiza* 10) was collected on the Nevado de Toluca volcano, the base of which is about 2500 m elevation. Although *P. calirhiza* often is found at higher elevations than *P. californicum* in the United States, it is not known to occur above 1500 m elevation in its North American range (Haufler et al., 1993). These high elevation sites in Mexico represent a remarkable disjunction of nearly 2500 km from the southernmost populations of *P. calirhiza* in California. To the best of our knowledge, the Oaxacan specimen, cited above and now confidently assigned to *P. calirhiza*, marks the southernmost limit of the *P. vulgare* complex in the Western Hemisphere.

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