The *Polystichum munitum--Polystichum imbricans* alliance in Western North America: evolutionary origins and contributions to the allopolyploid species *P. californicum*

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ABSTRACT

***Premise of the study*—**Allopolyploid species, the products of hybridization of genetically isolated but allied species followed by genome duplication, are important to the diversity and evolution of many fern clades, including the genus *Polystichum.* In western North America, the closely related diploid species *P. munitum* and *P. imbricans* have been identified as progenitors of four allopolyploid species, including *P. californicum,* whose second progenitor is known to be *P. dudleyi*. However, the possibility of multiple origins of the allopolyploids, the lack of clarity in regards to the evolutionary relationships of the members of *P. munitum-P. imbricans* group, and the variable morphology of *P. californicum* raise questions about this species' origin. I address this complexity by using genetic and morphological characters to assess the phylogeny of the *P. munitum-P. imbricans* group and *P. californicum,* allowing me to test hypotheses about the identities *P. californicum*'s progenitors.

***Methods*—**Sequence variation in chloroplast and nuclear DNA markers as well as ploidy assessed with flow cytometry were used to clarify the history of speciation within the *P. munitum-P. imbricans* group and to test hypotheses about the origins of *P. californicum* in the light of morphological variation found among the species in this group.

***Key results*—**The current taxonomy of the *P. munitum-P. imbricans* clade was supported, and *P. imbricans* ssp. *imbricans* was revealed to be the maternal progenitor of the *P. californicum* and *P. californicum* hybrid accessions included in this study. However, the identification of hybrids between *P. dudleyi* and both *P. munitum* and *P. imbricans* ssp. *curtum* indicate that multiple origins for *P. californicum* remain a possibility.

Key words: allopolyploidy; Dryopteridaceae*;* hybridization; polyploidy; *Polystichum*

INTRODUCTION

Speciation among temperate ferns is often described using an allopatric model. Haufler et al. (2000) argue that while tropical ferns may often speciate via ecological specialization within shared geographic ranges, temperate fern species do not appear to form frequently in this manner, perhaps because the areas they inhabit have less complex ecological niches. As an example, Haufler et al. concluded that ferns in the North Temperate and Boreal *Polypodium sibericum* Siplivinskij group, whose members are similar in leaf morphology but have a low interspecific genetic identity, diverged into species following changes in geographic range linked to glaciation and climate change. As populations were isolated from each other, genetic differences accumulated and post-zygotic genetic barriers developed, so that interbreeding between previously conspecific groups resulted in inviable zygotes or healthy but sterile hybrid individuals. According to Haufler et al., the near-ubiquity of postzygotic genetic barriers in temperate species supports the general applicability of their model. Nevertheless, ecological rather than geographic isolation may underlie the speciation that yielded some of the species diversity in temperate regions. Regardless of the means of speciation, once established, fern species can remain autonomous, isolated in a variety of ways—by geographic barriers, reproductive barriers, or barriers related to specialized habitat—while forces such as gene flow and developmental constraints can maintain each species' unity.

However, hybridization occurs frequently between fern species, and although the hybrids are almost always sterile, fertility can be restored through genome duplication (Manton, 1950; Kentner and Mesler, 2000), resulting in fertile, allopolyploid individuals. These allopolyploids, like their originating hybrids, tend be intermediate between their progenitors in both morphology and habitat preference (Mayer and Mesler, 1993; Kentner and Mesler, 2000), and can be important in the evolution of many fern lineages. Manton (1950) argued that while allopolyploidy could occur occasionally under stable conditions, it is most probable when species ranges are disrupted, bringing fern species into contact with relatives from which they were previously separated geographically.

Individuals grouped into a single polyploid species may in fact be the result of multiple instances of hybridization between their progenitor species. In some cases, lineages within a species may differ in their plastid genomes if reciprocal crosses occurred between the progenitor species, resulting in hybrids with different maternal parents. Sigel et al. (2014) documented one example of this pattern, in the allotetraploid fern *Polypodium hesperium* Maxon. Using both plastid (uniparentally inherited) and nuclear (biparentally inherited) markers, Sigel et al. supported the hypothesis that *P. hesperium* comprised more than one lineage, as they documented two different plastid-donating progenitors. The lineages were geographically patterned: allotetraploids with the plastid genome of one progenitor were limited to the northwestern United States, while those with the plastid genome from the other progenitor were found in the southwestern states and northern Mexico. Sigel et al. hypothesized that each of the two plastid genomes may be more beneficial to *P. hesperium* in different parts of its range, leading to differential selection on the two hybrid lineages. However, this geographic differentiation may instead be due to which species, by chance, happened to be the maternal progenitor in the different parts of *P. hesperium*'s range. A similar example of multiple origins documented with different maternal progenitors can be seen in *Polystichum braunii* (Spenn.) Fée, a circumboreal allotetraploid (Jorgensen and Barrington, 2016).

However, *Polystichum braunii* is hardly the only example of allopolyploidy in *Polystichum* Roth (Dryopteridaceae Herter), a genus in which this phenomenon is common

(Kentner and Mesler, 2000). One of the earliest demonstrations of allopolyploidy in ferns was Manton's (1950) study of the origins of the European *Polystichum aculeatum* (L.) Roth. Manton observed that *P. aculeatum*'s chromosome number was twice that of *P. lonchitis* (L.) Roth. and that the suspected hybrid between them, P. ×*illyricum* Hahne., had a chromosome number appropriate for a triploid hybrid between the two. *Polystichum* ×*illyricum* is morphologically intermediate between *P. lonchitis*, a once-pinnate species, and *P. aculeatum*, whose pinnae are deeply divided although the fronds are not fully bipinnate. Based on her study of chromosome pairing during meiosis in these plants, Manton concluded that *P. aculeatum*, which is an allotetraploid, had the diploid *P. lonchitis* as one of its progenitors. Reticulate patterns of evolution have also been documented in the montane tropics, for two Costa Rican *Polystichum* allotetraploids and their hybrids, by Barrington (1990). The study group in question contained both diploids and related allotetraploids, and provides another an example of hybridization occurring between morphologically disparate species of *Polystichum.*

Homoeologous chromosome pairing (i.e. between genomes of hybridizing species) in *Polystichum* was investigated in greater detail in a hybridization program at the University of Leeds, designed to further investigate the origins and relationships of *Polystichum aculeatum* and *P. braunii* (Sleep, 2014). In this program, a wide variety of *Polystichum* species presumed to be remotely related to the two tetraploids based on morphology were artificially hybridized with the two species in question. The artificial hybrids were unexpectedly easy to create between progenitors that were, because of differences in their morphology and geographic distribution, presumed to be only distantly related. Additionally, at least some degree of chromosome pairing was observed in all the hybrids assessed regardless of ploidy levels. Unusually high levels of homoeologous chromosome pairing can also be seen in *Polystichum* hybrids from northern temperate regions of North America (W. H. Wagner, 1973; Barrington, 1986). Overall, in the cases studied, it appears that *Polystichum* species readily hybridize in nature as well as in experimental settings. Although the diploid species have differentiated noticeably in terms of morphology, they apparently remain similar enough on the chromosome level to maintain enough homology between chromosomes to allow the formation of some bivalents in the *Polystichum* hybrids studied. This frequency of chromosome pairing in hybrids has not been found elsewhere within the pteridophytes (Sleep, 2014), suggesting that *Polystichum* may have different barriers to interspecific reproduction than other ferns. According to Sleep (2014), *Asplenium* is the only other fern genus in which hybridization and chromosome pairing had been studied in a manner similar to *Polystichum*, and when the diploid species were experimentally crossed, the hybrids did not show chromosome pairing.

W. H. Wagner's (1973) study of reticulate evolution using morphology and chromosome pairing in western North American *Polystichum* set the stage for more modern research into *Polystichum* in that region. Wagner noted that a surprisingly large number of sterile diploid plants resembling the fertile allotetraploid *P. californicum* (D.C. Eaton) Diels were found growing together with *P. californicum* and its presumed progenitors, the diploids *P. dudleyi* Maxon and *P. munitum* (Kaulf.) C. Presl. Both triploid backcrosses were also noted at the research sites. *Polystichum californicum* is morphologically intermediate between bipinnate *P. dudleyi* and pinnate *P. munitum*, and the sterile diploid hybrids were nearly indistinguishable from *P. californicum*, while the morphology of the triploids was intermediate between *P. californicum* and either one or the other of the two diploid species. Wagner noted that triploid backcrosses between *P. californicum* and *P. munitum* had equal numbers of univalents and bivalents, which supported the hypothesis that *P. munitum* was a progenitor of *P. californicum*.

Wagner's observations of *de novo* hybrids, allotetraploids, diploid progenitors, and backcrossed hybrids growing together makes it likely that the allotetraploids have originated multiple times, possibly with different maternal parents in different origins. Overall, in *Polystichum*'s western North American range, interspecific hybridization between *Polystichum* species is frequent. Consequently, at least some of the other allopolyploid species in western North America besides *P. braunii* probably originated multiple times, complicating the evolutionary history of this genus (Soltis et al., 1989).

Several years after W. H. Wagner's research, D. H. Wagner (1979) recognized a second species, *Polystichum imbricans* (D.C. Eaton) D. H. Wagner, as distinct from *P. munitum* on the basis of morphology and cytology. He further divided *P. imbricans* into two subspecies: subsp. *imbricans* and subsp. *curtum* (Ewan) D. H. Wagner. According to Wagner (1979), *Polystichum imbricans* subsp. *curtum* is morphologically intermediate between *P. munitum* and *P. imbricans* subsp. *imbricans*. *Polystichum munitum,* which is found from Alaska to California as well as in Idaho and Montana, favors mesic forests. *Polystichum imbricans* favors exposed, dry, rocky slopes (D. Wagner, 1979; Soltis et al., 1990). *Polystichum imbricans* ssp. *imbricans* is found from southern British Columbia to southern California, while *P. imbricans* ssp. *curtum* is found in central to southern California (D. Wagner, 1979).

There is an array of possibilities for the divergence of the three once-pinnate western North American taxa allied to *P. munitum* (Figure 1). Wagner (1979) suggested that *P. imbricans* subsp. *curtum* might be closer to the common ancestor of the group than either *P. munitum* or *P. imbricans* subsp. *imbricans*. An alternate hypothesis would be that *P. imbricans* subsp. *curtum* shares a common ancestor with *P. munitum* that *P. imbricans* subsp. *imbricans* does not share.

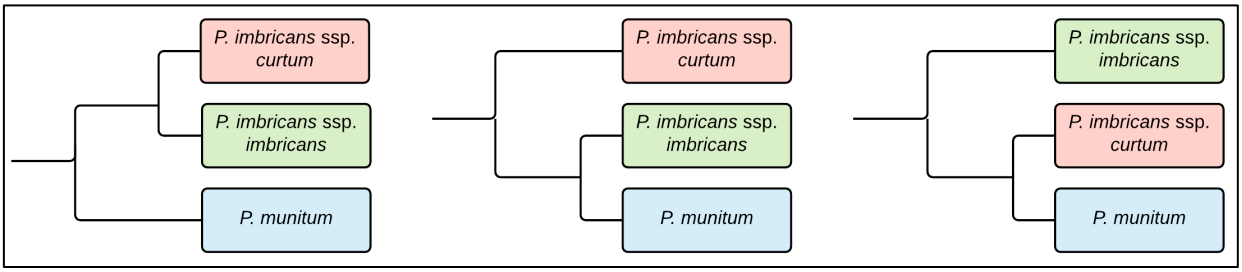

Figure 1: The currently accepted phylogeny of the *P. munitum- P. imbricans* group (left), David Wagner's hypothesized phylogeny (1979) (center), and a third alternative hypothesized phylogeny (right).

Overall, the trio is presumed to be monophyletic based on shared geography and morphology. The close alliance of these taxa is indicated by evidence that reproductive isolation between *P. munitum* and *P. imbricans* is incomplete: fertile diploid hybrids and introgression have both been documented (Mayer and Mesler, 1993; Mullenniex et al., 1998; Kentner and Mesler, 2000). However, the two species continue to be recognized as separate due to their habitat differences: Kentner & Mesler (2000) hypothesize that *P. munitum* and *P. imbricans* ssp. *imbricans* remain generally distinct in nature because of their distinct habitat requirements, with habitats intermediate between those favored by the two species being sometimes populated by their hybrids. They did not, however, consider *P. imbricans* ssp. *curtum* in their work.

In total, *Polystichum munitum* and *Polystichum imbricans* are believed to be progenitors of four of the six allopolyploid species found in Western North America (W. H. Wagner, 1973; D. Wagner, 1979; Soltis et al., 1991). They and their hybrid offspring appear to present examples of speciation closely connected to habitat preferences (Soltis et al., 1990; Mayer and Mesler,

1993; Haufler et al., 2000; Kentner and Mesler, 2000), a situation that is noteworthy in light of the prevalence in temperate ferns of speciation connected to geographic rather than ecological isolation. However, current understanding of the allopolyploids' origins is complicated by a lack of clarity in regards to the evolutionary relationships of *Polystichum munitum* and the two *P. imbricans* subspecies.

Polystichum californicum is one of the allotetraploids whose origin remains unclear due to the current ambiguity in regards to the evolution of the *P. munitum-P. imbricans* groups. W.H. Wagner (1973) named *P. dudleyi* and *P. munitum* as the progenitors of *P. californicum* prior to D. H. Wagner's splitting of *P. munitum* into two species, making it unclear which member of the *P. munitum-P. imbricans* lineage was the progenitor. As the morphology of *P. californicum* varies across its range, D. H. Wagner (1979) argued that *P. californicum* was actually comprised of two allotetraploid lineages that were similar in appearance: one formed through hybridization between *P. munitum* and *P. dudleyi* and the other formed through hybridization between *P. imbricans* and *P. dudleyi*. Later, Soltis et al. (1991) argued that *P. imbricans* was the sole second progenitor based on their allozyme and cpDNA restriction-fragment analyses. However, they did not differentiate between the two subspecies of *P. imbricans,* and did not sample *P. imbricans* in the range where subsp. *curtum* is found, thus leaving it out of their analysis. In sum, P. *californicum's* variable morphology, the lack of specificity as to which subspecies of *P. imbricans* was involved, and the remaining possibility of multiple origins leave the identity or identities of its second progenitor in doubt.

In this study, I focused on two problems in western North American *Polystichum.* First, I addressed the relationships of the three diploid lineages in the *Polystichum munitum-Polystichum imbricans* group based on analysis of genetic and morphological features. Second, I sought to discover the role of the members of the trio in the origin of the allotetraploid *Polystichum californicum* (D. Wagner, 1979). I pursued the latter inquiry with the following possibilities as alternative—but not exclusive—hypotheses:

1. *Polystichum californicum* originated before *P. munitum* and *P. imbricans* diverged from one another.
2. Either *Polystichum imbricans* subsp. *imbricans* or *P. imbricans* subsp. *curtum* was involved in the origin of *P. californicum* (Soltis *et. al.* 1991).
3. *Polystichum californicum* is the product of repeated origins, the result of *P. dudleyi* hybridizing with both *P. munitum* and the *P. imbricans* subspecies separately, with genome duplication following these hybridization events (D.H. Wagner 1979).

I considered these hypotheses first using molecular-genetic data. Then I interpreted data from a morphometric analysis in the light of the molecular data set in order to improve understanding of the structural differences between the members of the trio and their tetraploid derivative *P. californicum.* Finally, I used flow-cytometry data to further constrain my inferences about the evolutionary history of the group.

MATERIALS AND METHODS

Study Set and Sample Storage— A total of 39 accessions representing *Polystichum munitum*, *P. imbricans* ssp. *imbricans*, *P. imbricans* ssp. *curtum*, *P. californicum*, *P. dudleyi*, and hybrid individuals were included in this study (Appendix I). All but two of these samples were collected within the last year and sent to the Barrington lab in the form of herbarium sheets for morphological analysis, pinnae dried in silica for DNA extraction, and fresh pinnae for flow cytometry and DNA extraction. Both fresh and dried pinnae were stored at 4°C. The two older

specimens (*Stensvold 8418* and *Jorgensen 75*) had been collected for previous research in the Barrington Lab, dried in silica, and stored at -80°C until time of use.

DNA Extraction, Amplification, and Sequencing—Total genomic DNA was extracted from fresh or silica-dried pinnae following a modified CTAB protocol (Doyle and Doyle, 1987). The chloroplast markers amplified in this study were the *trnL-trnF* intergenic spacer (hereafter *trnL-F*) and the *rbcL* gene. The nuclear markers amplified were *pgiC* exons 6–8 and 14–16. Primers (Table 1) were drawn from previous literature for *trnL-F* (Taberlet et al., 1991, primers e and f), *rbcL* (Little and Barrington, 2003, primers 1F and 1379R) and *pgiC* 14-16 (Ishikawa et al., 2002, primers 14F and 16R). For *pgiC6–8*, the primers 6F1999 and 8R2159 were designed by Brendan Lyons in the Barrington lab. Amplification by the polymerase chain reaction was performed in a TC-312 or TC-3000 thermal cycler (Techne, Burlington, New Jersey, USA) in 23µL reactions with the following components: 1 µL DNA (>50ng/µL), 1 µL of each 10 µM primer, Taq polymerase (TaKaRa, Gene Clone, Madison, Wisconsin, USA), and water to volume. Success of PCR was determined using gel electrophoresis. PCR products were cleaned using ExoSap-IT (USB Corporation, Cleveland, Ohio, USA) and sequenced at the Vermont Cancer Center DNA Analysis Facility or Beckman Coulter Genomics (Danvers, Massachusetts).

Table 1: Primers used in this study

Marker	Primer Name	Primer Sequence
trnL-F	tab-e	GGT TCA AGT CCC TCT ATC CC
	tab-f	ATT TGA ACT GGT GAC ACG AG
rbcL	1F	ATG TCA CCA CAA ACA GAA ACT AAA GCA AGT
	1379R	TCA CAA GCA GCA GCT AGT TCA GGA CTC
pgic 6-8	6F1999	AAC TGG GAA ACC ATT GAC TGA TG
	8R2159	TCA ATA GGA TCC ACA TTT GCC A
pgic 14-16	14F	GTG CTT CTG GGT CTT TTG AGT G
	16R	GTT GTC CAT TAG TTC CAG GTT CCC C

***Sequence alignment and phylogenetic analysis*—**Sequences for cp and nuclear markers were aligned with MAAFT as implemented by Geneious Pro version 9.1.2 (http://www.geneious.com, Kearse et al., 2012). After inspecting the alignments, it was determined that the results for *pgiC6–8* were not of sufficient quality to include in further analysis. The chloroplast markers were concatenated in Geneious. A *Polystichum lemmonii* sequence was included as the outgroup. Bayesian Inference (BI) was implemented in MrBayes v3.2.2, for the concatenated sequences. BI using MrBayes was run using four chains sampling every 1000 generations. Stationarity was set at a threshold of 0.01. After this threshold was reached, MrBayes was rerun four times as long as the generation time seen at this point, using the default burn-in of 25%. After discarding the first 25% of trees, a 50% majority rule consensus tree was calculated for the remaining trees. Posterior probabilities were obtained using MrBayes v.3.2.2. The program FigTree 1.4.2 was used to view a 50% majority rule consensus tree with posterior probabilities.

The aligned forward sequences for *pgiC1416* were used for summation analysis, in which the chromatograms were inspected for instances where the *P. californicum* accessions showed double peaks that summed the peaks shown by the other species. The span from bp 103 to bp 450 of *pgiC14-16* was assessed, as the chromatograms from the forward sequences were well

resolved within this region. However, there were some regions within this span where the chromatograms were unclear and could not be interpreted for all accessions. Scoreable sites were required to have unambiguous bands and an unambiguous neighbor on either side (i.e. clear single peaks or, rarely, double peaks).

Flow cytometry— To determine ploidy, flow cytometry was performed on selected representatives of each taxon in the dataset. At least 1 cm^2 of fresh or silica-dried unfrozen pinna tissue was placed in a plastic Petri dish with 1.2mL ice-cold LB01 buffer with 1%PVP-40 added, 100µL RNA-ase stock, and 100µL of Sybr green (1mg mL^{-1}) stock. The pinna tissue was then chopped with a razor blade for about 3 minutes at about 5 chops per second, keeping the tissue mixed with the liquid so it would not dry out. The homogenate was then pipetted through a 30µm-diameter cell filter into a microcentrifuge tube and analyzed as soon as possible (usually within approximately 20 minutes). Samples were analyzed on a Coulter Analytical Flow Cytometer equipped with a solid state laser using a gating protocol designed for Sybr Green (λ=497nm) housed in the Flow Cytometry Facility at the Vermont Cancer Center, Burlington, Vermont, USA. The instrument was calibrated before each run using a biological standard, *Hordeum vulgare* variety "Morex" (acquired from U.S. National Plant Germplasm System). Standard plants were cultivated in the laboratory from seed; leaf tissue from these seedlings was used as a standard. The parameters recorded for each sample included blue fluorescence (FL1), green fluorescence (FL2), forward scatter (FSC), and side scatter (SSC). These parameters were observed alone and in combined histograms including: FL2 vs. FSC, FL2 vs. SSC, FL2 vs. FL3, and FSC vs. SSC. Genome size was calculated using the following formula: Sample channel / standard channel * *Hordeum* genome size = Sample genome size. The yields were 2C values for the cells in each sample.

Morphological analysis—Characters to analyze were selected based on those described as important by Wagner (1979), and based on my own observation of the plants. The following characters were included:

1. Indusium margin
2. Presence of spinules on pinna margin
3. Degree of leaf dissection
4. Scales on abaxial side of rachis
5. Middle pinna length, width, and width:length ratio
6. Sorus position
7. Pinna base shape
8. Pinna apex shape

In all cases, a pinna from the middle of the frond was measured rather than one from the base or tip. Morphology was assessed by viewing the herbarium sheets under a dissecting scope (characters 1-4), or by scanning the herbarium sheets and measuring the resulting images in ImageJ 1.47v (characters 4-8). Indusium margins and rachis scales proved to be too variable to code in a reliable manner by looking at them under a dissecting microscope, and image resolution was not high enough to quantify these characters in ImageJ. Although it is possible that these features could be assessed reliably by mounting them on slides and viewing them under a microscope, this was not a feasible option given the timeframe of the project, and so these characters were left out of subsequent analysis. Spinule presence was also not included in subsequent analyses because all specimens assessed had spinules. Methods for quantifying the remaining characters are illustrated in Figure 2. Principal component analysis (PCA) was then performed in R and RStudio Version 0.98.1028 using data from representatives of each species.

Histograms of the traits seen in the *Polystichum munitum-Polystichum imbricans* group were created in GraphPad Prism 6.

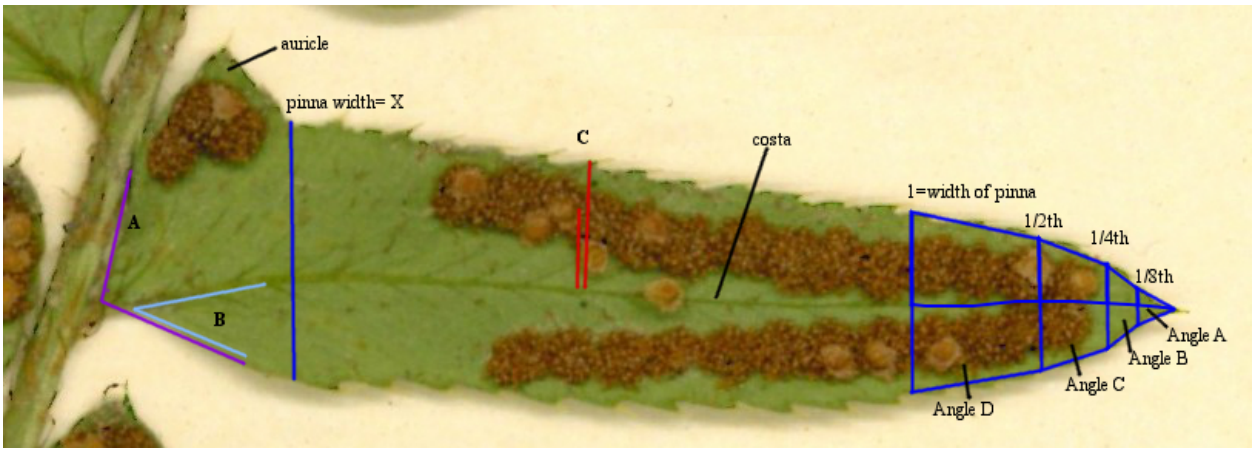

Figure 2: Pinna length was measured along the costa to account for the curvature of some of the pinnae. Pinna width was measured immediately past the auricle, at a right angle to the costa. Pinna base shape was described by two angles, one formed by the basioscopic margin and the acroscopic margin (up to the point where the pinna begins to curve away towards the auricle)(A), and the other between the costa and the basioscopic margin of the pinna (B). Sorus position (C), measured in the middle of the pinna, was scored as the ratio of distance from the costa to the middle of the sorus line to the distance from costa to margin at the same point. The pinna-apex shape was measured relative to the width of the pinna (hereafter *x*). The pinna was marked at five points: the apex, x/8 cm from the apex, x/4 cm from the apex, x/2 cm from the apex, and x cm from the apex. Four angles were then measured between the margin of the pinna and the costa, with the outer arm of the angle along the pinna margin at the points as indicated. For example, Angle A's vertex is at the apex of the pinna and its two arms end at the x/8 mark, while Angle C's two arms pass through the x/2 and x/4 lines where they intersect the pinna margin on either side (with the vertex placed wherever necessary to form this angle). Because the change in angle is what is important for describing the shape of the pinna apex, ratios of adjacent angles were then calculated, making the final measurements of pinna apex shape be Angle A:Angle B, Angle B:Angle C, and Angle C:Angle D.

RESULTS

Phylogenetic analysis—The unrooted tree for the *Polystichum munitum-Polystichum imbricans* group based on the concatenated chloroplast data matrix (Figure 3) resolved three distinct, well-supported clades (posterior probability [PP] = 1 for all three clades). One clade included all *Polystichum munitum* accessions. The second clade contained all *P. imbricans* ssp. *curtum* accessions, as well as two accessions initially identified as *P. imbricans* ssp. *imbricans* (imb04, unc08). The third clade contained all other specimens initially identified as *P. imbricans* ssp. *imbricans*, as well as all specimens initially identified as *P. imbricans* but not identified to the subspecies level (unc01-06).

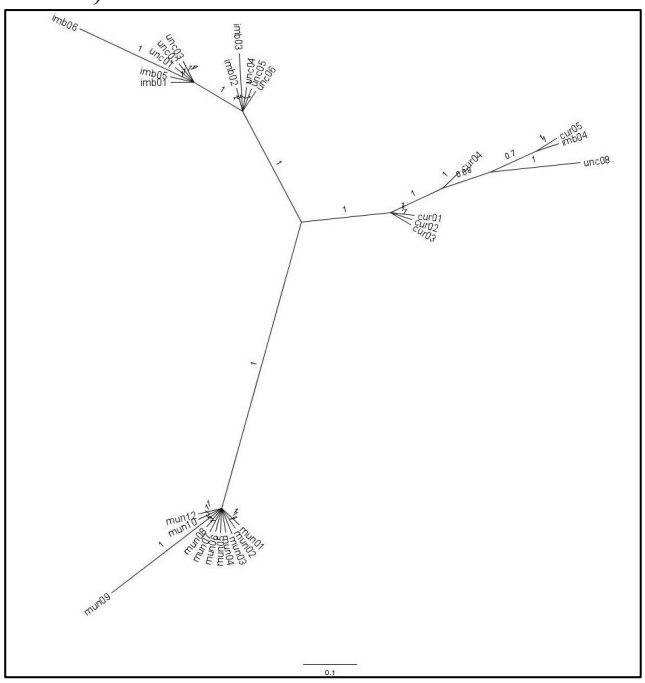

Figure 3: Unrooted tree for the *P. munitum-P. imbricans* group, based on the *trnL-F* and rbcL datasets. Collectors, collection numbers, and species associated with each specimen ID code can be found in Appendix I.

Resolved within the rooted cpDNA tree for all accessions (Figure 4) were two well-supported clades, one of which contained two equally well-supported sister subclades (clades 1A (PP=1), 1B (PP=1), and 2 (PP=1); Figure 4). A single specimen identified as *Polystichum dudleyi* based on its morphology (dud05) fell outside these clades alongside the *Polystichum lemmonii* accession chosen to serve as the outgroup for the analysis.

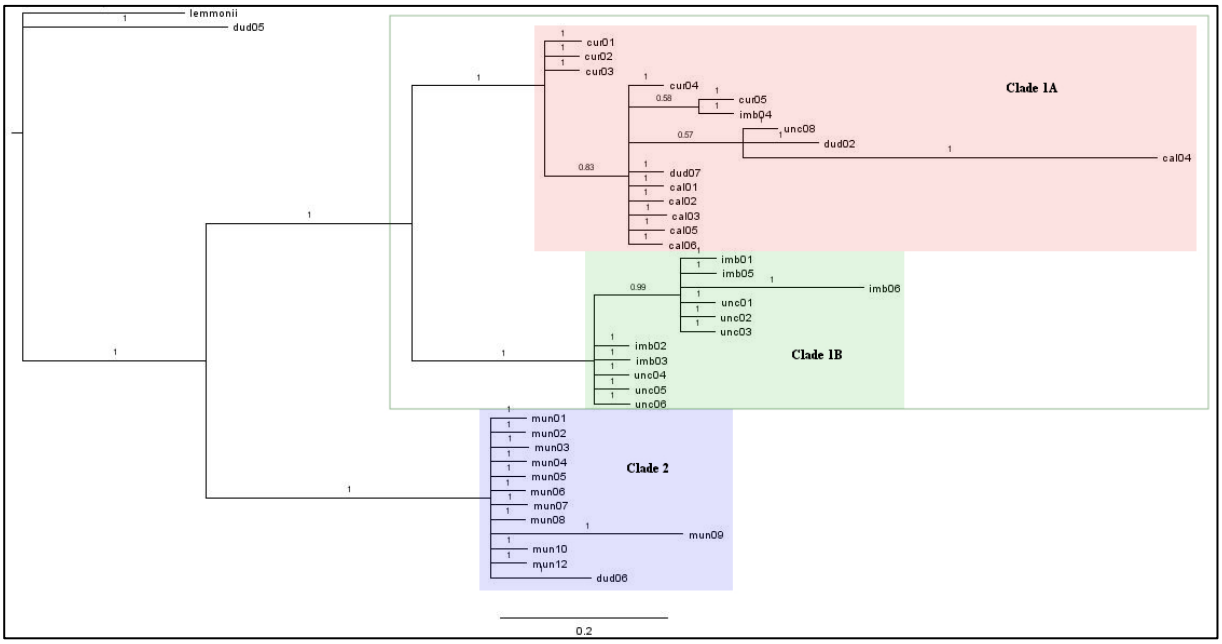

Figure 4: Phylogeny for all accessions, based on the *trnL-F* and rbcL datasets. Clade1A represents *P. imbricans* ssp. *curtum* (diploid) and individuals with this subspecies as a maternal progenitor (a set including diploid and triploid hybrids, and the tetraploid *P. californicum*), Clade 1B represents *P. imbricans* ssp. *imbricans* (diploid), and Clade 2 represents *P. munitum* (diploid) and a hybrid individual (diploid) for whom *P. munitum* is the maternal progenitor.

Clade 1 comprises the sister subclades 1A and 1B. Subclade 1A (the *curtum* clade) contains the five specimens identified based on morphology to be *Polystichum imbricans* ssp. *curtum* (cur01-05), two specimens initially identified as *P. imbricans* ssp. *imbricans* (imb04, unc08), and eight specimens initially identified as *P. californicum*, hybrids with *P. californicum* as one progenitor, or members of the *P. californicum-dudleyi* complex (cal01-06, dud02, dud07). Subclade1B (the *imbricans* clade) contained five specimens initially identified based on morphology as *P. imbricans* ssp. *imbricans* (imb01-03, imb05-06) and six specimens initially identified as *P. imbricans* but not identified to subspecies level (unc01-06). A group of six accessions (imb 01,05 and 06, unc01-03) are united in a derived clade within subclade B with high support (PP=0.99). Clade 2 (the *munitum* clade) contained all samples initially identified based on morphology as *Polystichum munitum*, as well as dud06, a specimen initially identified as belonging to the californicum-dudleyi complex.

Flow Cytometry-- Flow cytometric analyses of nine *Polystichum* plants resulted in histograms with sharp peaks for the samples and the biological standard (*Hordeum vulgare* "Morex"). There was congruence between actual (= measured by flow cytometry) and expected (= inferred from the identification of the accession) relative genome size values, but with some exceptions (Table 2). Representative *P. munitum* accessions (mun06, 09) were determined to be

Table 2: Flow Cytometry Results

Specimen ID Code	Identification by collector	Clade in chloroplast phylogeny	Ploidy Expected	Ploidy Retrieved	Final Identification
cal01	*P. californicum*	1A: curtum	**2n or 4n**	**3n or 4n**	*P. californicum* or *P. californicum* hybrid
cal03	*P. californicum*	1A: curtum	2n or 4n	2n	*P. dudleyi X imbricans* ssp. *curtum*
cal04	*P. californicum*	1A: curtum	**2n or 4n**	**3n**	*P. californicum* hybrid
cal05	*P. californicum*	1A: curtum	2n or 4n	4n	*P. californicum*
dud06	*P. californicum-dudleyi* complex	2: munitum	2n	2n	*P. dudleyi X munitum*
dud07	*P. californicum-dudleyi complex*	1A: curtum	2n	3n	*P. dudleyi X californicum*
imb06	*P. imbricans* ssp. *imbricans*	1B: imbricans	2n	2n	*P. imbricans* ssp. *imbricans*
mun06	*P. munitum*	2: munitum	2n	2n	*P. munitum*
mun09	*P. munitum*	2: munitum	2n	2n	*P. munitum*
unc08	*P. imbricans* ssp. *imbricans*	1A: curtum	2n	2n	*P. imbricans* ssp. *imbricans X* ssp. *curtum?*

diploid, as expected for this species. dud06, which fell in the munitum clade along with the *P. munitum* specimens, was determined to be diploid, as expected for *P. dudleyi*, or *P. munitum*, or their hybrid. Flow cytometry also confirmed unc08, which falls in the *curtum* subclade (1A) in the phylogenetic analysis, and imb06, which falls in the *imbricans* subclade (1B), to be diploid, as expected. Representatives of the accessions determined as *P. californicum* included diploids (cal03) and tetraploids (cal05) as expected. However, three accessions did not yield the expected ploidies. dud07 was retrieved as triploid. Among the californicums, cal04 was retrieved as triploid, and an ambiguous result suggesting either triploidy or tetraploidy was retrieved for cal01.

Analysis of nucleotide additivity—At nine positions on the *pgiC14-16* alignment (bold, Table 3), the accessions initially identified as *P. californicum* or members of the *Polystichum californicum- dudleyi* complex that fell in the ssp. *curtum* clade (1A) of the chloroplast phylogeny include both a nucleotide fixed in *Polystichum dudleyi* and a nucleotide that was fixed in *Polystichum imbricans* but not in *P. munitum*. Two sites (bp108, bp320) combined two

nucleotides, one of which was not encountered in my sample of the once-pinnate progenitors. Differentiating between the two *P. imbricans* subspecies was not possible with these data.

Table 3: Additivity Analysis.
Nucleotide codes: K=G or T; M=A or C; Y=C or T; R=A or G; S=G or C

		Position in *pgiC14-16* Alignment											
Specimen ID	Clade in chloroplast phylogeny	108	**165**	**170**	**233**	**248**	**263**	286	**301**	*320*	**336**	**357**	**405**
cal03	1a: curtum	K	M	Y	K	K	Y	Y	Y	Y	R	S	M
cal05	1a: curtum	K	M	Y	K	K	Y	Y	Y	Y	R	S	M
cal06	1a: curtum	K	M	Y	K	K	Y	Y	Y	Y	R	S	M
cal02	1a: curtum	K	M	Y	K	K	Y	Y	Y	Y	R	S	M
dud02	1a: curtum	K	M	Y	K	K	Y	Y	Y	Y	R	S	M
cal04	1a: curtum	K	M	Y	K	K	Y	Y	Y	Y	R	S	M
cal01	1a: curtum	K	M	Y	K	T	Y	Y	Y	Y	R	S	M
dud05	dudleyi	G	C	T	T	G	C	T	C	C	G	G	A
dud06	2: munitum	G	?	?	T	?	?	T	?	C	?	G	?
mun06	2: munitum	G	C	T	T	G	C	Y	C	C	G	G	A
mun07	2: munitum	G	Y	?	T	?	?	?	?	?	?	?	A
mun08	2: munitum	G	C	T	T	G	C	C	C	C	G	G	A
mun09	2: munitum	G	C	T	T	G	C	C	C	C	G	G	A
mun10	2: munitum	G	C	T	T	G	C	C	C	C	G	G	A
mun12	2: munitum	G	C	T	T	G	C	C	C	C	G	G	A
cur03	1a: curtum	G	A	C	?	T	?	C	?	?	?	?	?
unc08	1a: curtum	G	A	T	G	T	T	C	T	C	A	C	C
imb01	1b: imbricans	G	A	Y	?	?	T	?	T	?	?	?	C
imb05	1b: imbricans	G	A	C	G	T	T	C	T	C	A	C	?
imb06	1b: imbricans	G	A	C	G	T	T	C	T	C	A	C	?
unc01	1b: imbricans	G	A	C	G	T	T	C	T	C?	A	C	C
unc02	1b: imbricans	?	?	?	?	?	?	Y	T	?	A	?	?
unc05	1b: imbricans	G	A	Y	?	T	?	C	?	C	?	?	?

Morphological analysis— In the histograms for single characters, the two species could be differentiated by pinna length (Figure 5); *P. munitum* specimens have pinnae that are 2-7cm longer than the longest pinna measured for the *P. imbricans* specimens. The pinnae of *P. munitum* were also generally wider than those of *P. imbricans* (Figure 5); the two species could be differentiated by the ratio of pinna width to pinna length. There was a small degree of overlap

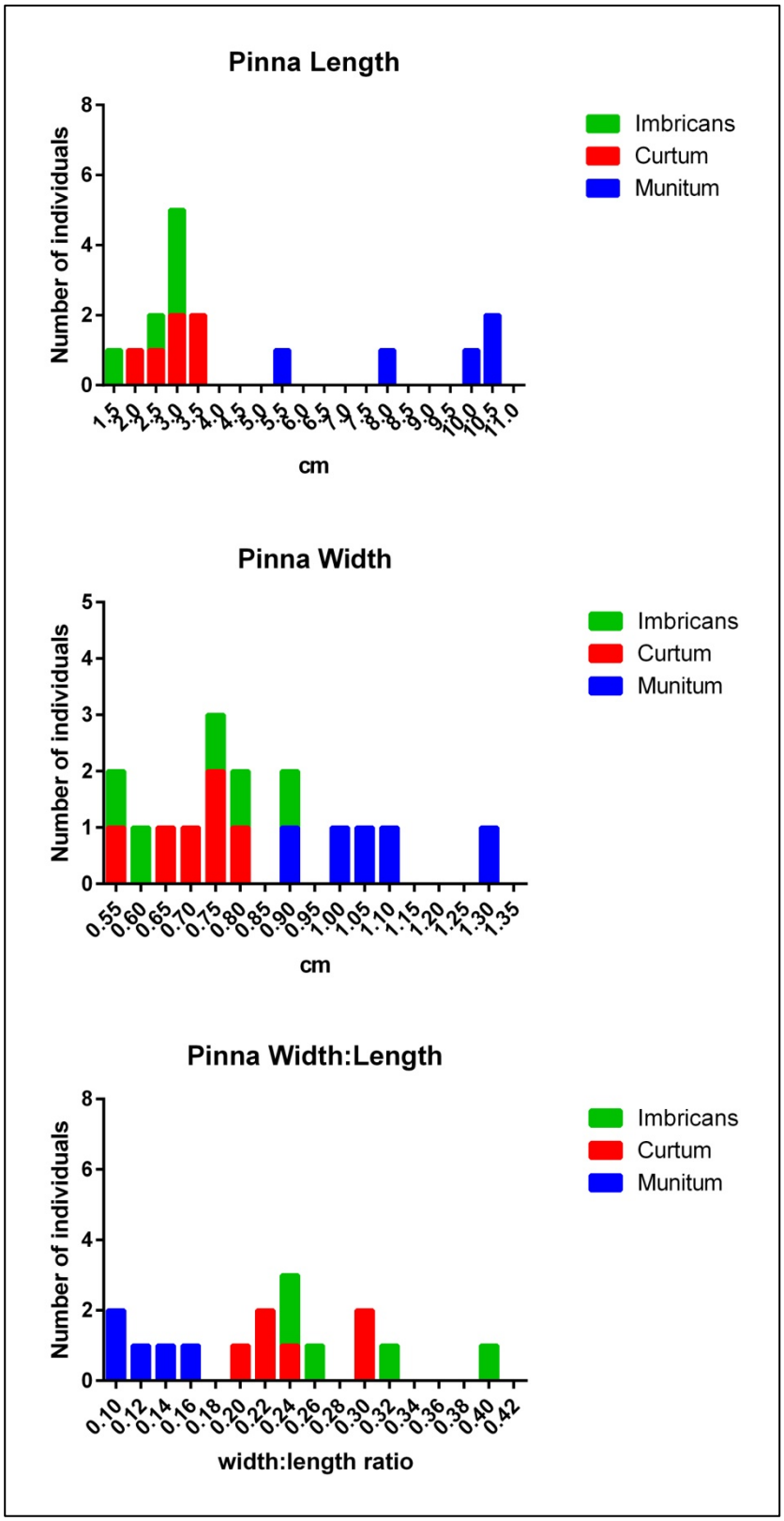

Figure 5: Pinna length and width data for members of the *P. munitum-P. imbricans* group, categorized based on the clades seen in the cpDNA phylogeny.

in trait values exhibited by *P. munitum* and *P. imbricans* for the angle described by the acroscopic and basioscopic margins of the pinna base (Figure 6) and the ratio of apex angles A:B (top, Figure 7). The range of variation in the *P. munitum* specimens overlapped substantially with that of the *P. imbricans* specimens in sorus position (Figure 6), ratio of apex angles B:C (middle, Figure 7) shape, and the ratio of apex angles C:D (lower, Figure 7). For all traits, the range of values seen in the accessions resolving in the *imbricans* subclade of the cpDNA tree overlapped with the range of traits seen in the accessions resolving in the *curtum* subclade.

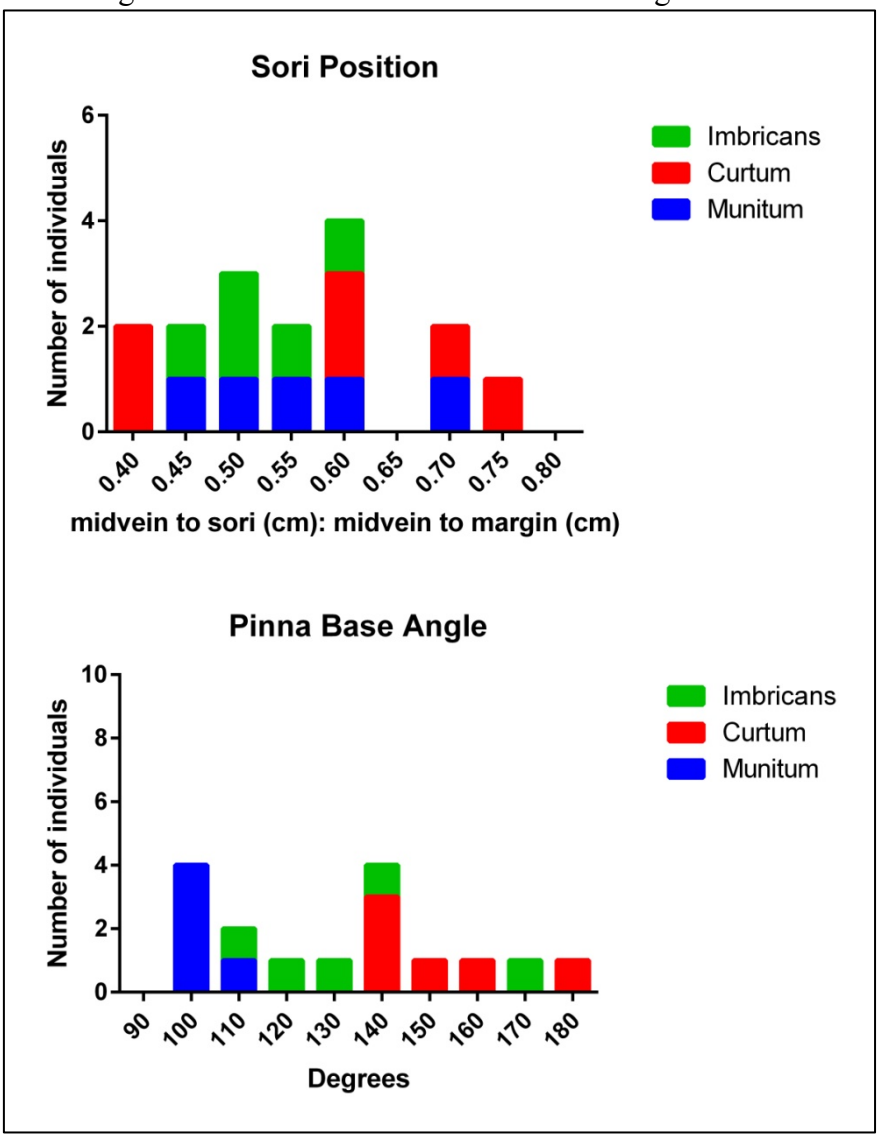

Figure 6: The position of the line of sori relative to the pinna margin and the angle described by the acroscopic and basioscopic margins of the pinna base for members of the *P. munitum-P. imbricans* group, categorized based on the clades seen in the cpDNA phylogeny.

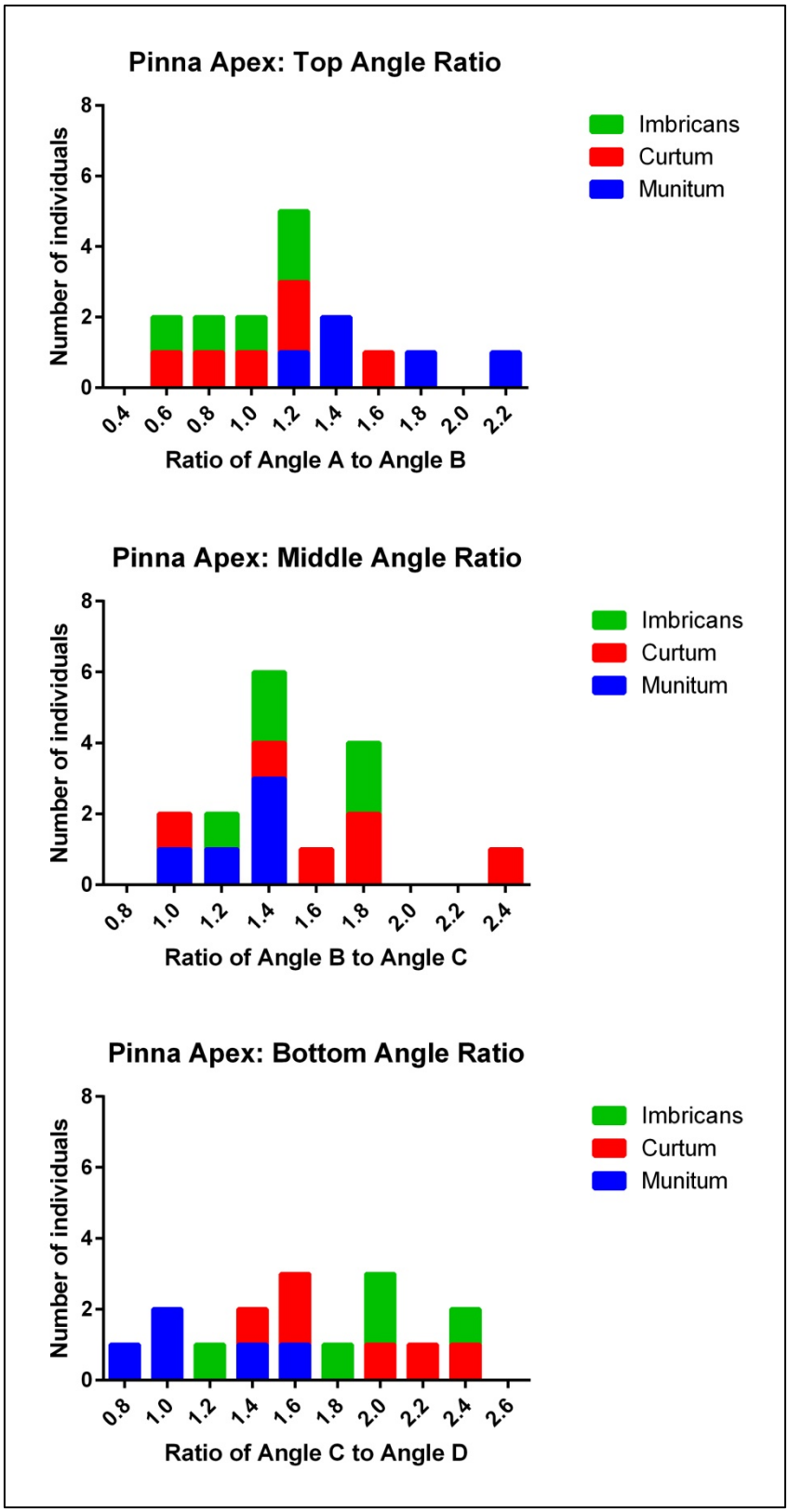

Figure 7: Ratios of angles describing pinna apex shape of members of the *P. munitum-P. imbricans* group, categorized based on the clades seen in the cpDNA phylogeny.

For the Principal Component Analysis, the once-pinnate specimens were grouped in the same manner used to make the histograms, and the twice-pinnate specimens were grouped separately, but also based on the clades in which they fell. Principal component 1 (PC1) explained 41.2% of the variance, principal component 2 (PC2) explained 23.8% of the variance, and principal component 3 (PC3) explained 13.7% (details, Appendix II) for a total of 78.7%. PC1 weighted the ratio of apex angles B:C, the pinna width:length ratio, and the ratio of apex angles C:D most positively, and pinna length, pinna width, and the ratio of apex angles A:B most negatively. PC2 also weighted the ratio of apex angles B:C most positively, and had the most negative values for the angle between the costa and the basioscopic margin of the pinna, the degree of leaf dissection, the angle described by the acroscopic and basioscopic margins of the pinna base, and the pinna width:length ratio. The most important characters for PC3 were sorus position, the ratio of apex angle C:D, and the angle of the acroscopic and basioscopic margins of the pinna base.

When PC1 is graphed against PC2 (Figure 8), the *P. munitum* accessions fall in a cluster distinct from the *P. imbricans* accessions, but the two subspecies are not resolved. The single *P. dudleyi* accession was clearly separate from *P. imbricans* and *P. munitum*, and dud06, which resolved with *P. munitum* in the cpDNA phylogeny, fell between the *P. dudleyi* and *P. munitum* clusters. dud02, which resolved with *P. imbricans* ssp. *curtum* in the phylogeny and is presumed to be *P. californicum* or a *P. californicum* hybrid, fell close to other *P. californicum* specimens. dud07, which also resolved with *P. imbricans* ssp. *curtum* and is presumed to be *P. dudleyi* × *californicum*, fell between the *P. dudleyi* specimen and the cluster containing the *P. imbricans* ssp. *imbricans, P. imbricans* ssp. *curtum*, and *P. californicum.*

Although the two subspecies of *P. imbricans* overlapped on the PC1:PC2 graph, when PC3 was graphed against PC1 or PC2 (Figures 9-10), there was less overlap between ssp. *imbricans* and ssp. *curtum*. Notably, PC3 made it possible to separate the accessions from the *curtum* (1A) clade into two clusters: one that comprised the specimens originally classified as *P. imbricans* ssp. *curtum,* and the other comprising imb04 and unc08. The latter had been identified morphologically as ssp. *imbricans* but resolved with the *curtum* specimens in the cpDNA network and phylogeny. It fell closer to the ssp. *imbricans* cluster than the rest of the ssp. *curtum* accessions did. The PC3:PC2 graph (Figure 10) also indicated that the *P. munitum* accessions were similar to *P. imbricans* ssp. *imbricans* on PC2.

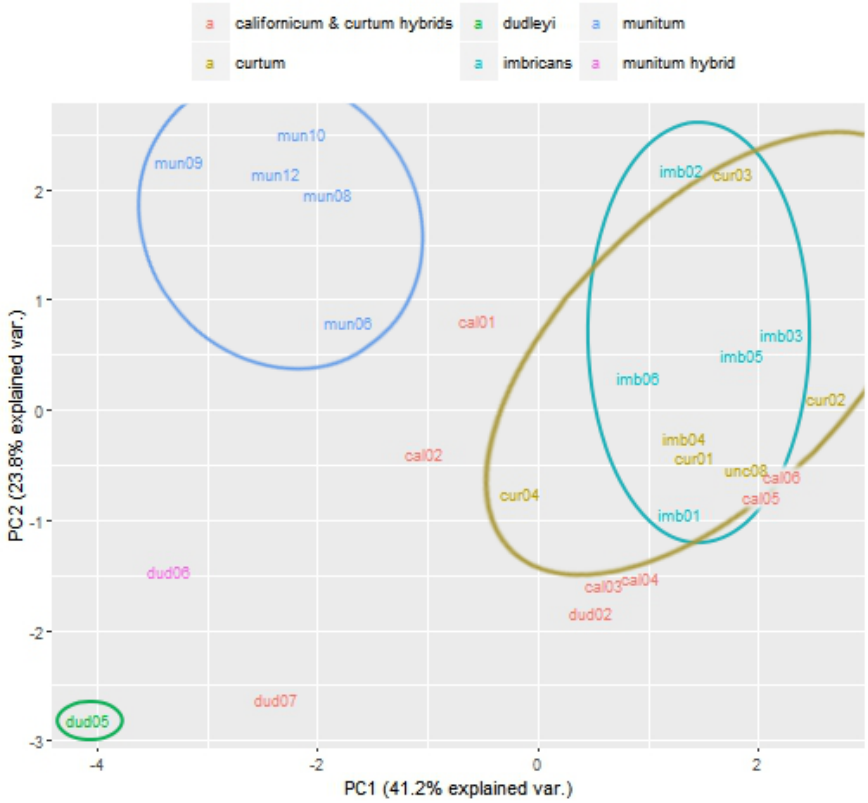

Figure 8: Principal Component Analysis: PC1 vs. PC2.

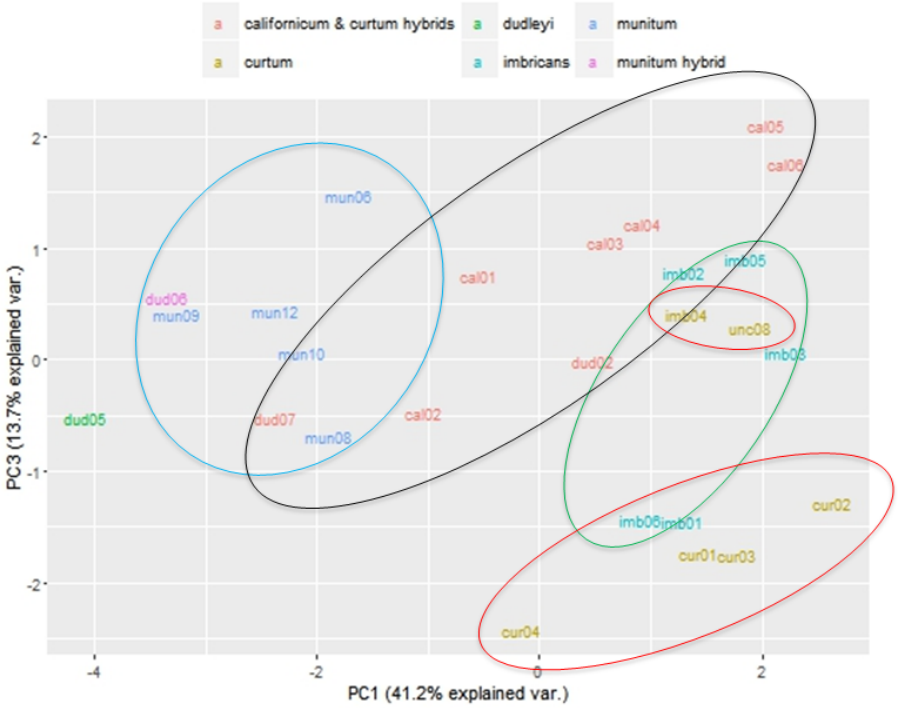

Figure 9: Principal Component Analysis: PC1 vs. PC3.

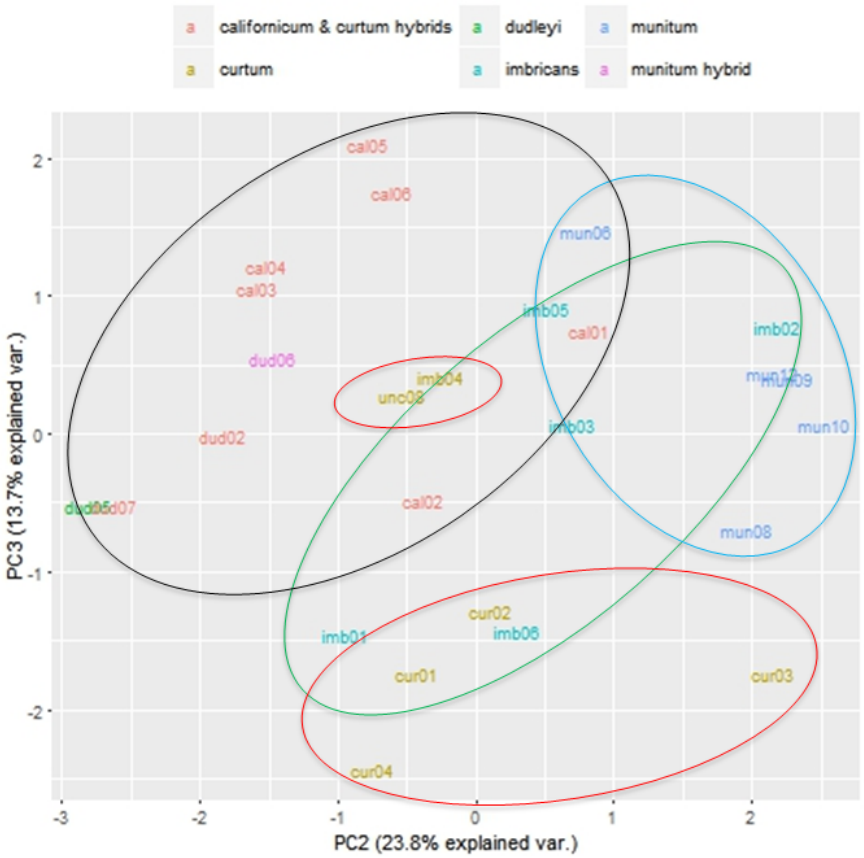

Figure 10: Principal Component Analysis: PC2 vs. PC3.

DISCUSSION

The four datasets gathered in this study provided the basis for a final assignment of accessions to species and hybrids. These decisions made it possible to resolve the relationships of the three once-pinnate diploids and test the hypotheses for the origin of *P. californicum* (Figure 11). Here, I provide details of my approach to the accessions whose assignment was changed.

***P. imbricans* subspecies—** imb04 and unc08, the two accessions initially identified as *P. imbricans* ssp. *imbricans* that fell within the ssp. *curtum* clade, appear based on the principal component analysis to be morphologically similar to *P. imbricans* ssp. *imbricans* in some respects despite being different in their chloroplast DNA. It is plausible that these two specimens are *P. imbricans* ssp. *imbricans* × ssp. *curtum*, with ssp. *curtum* as the maternal progenitor, explaining their positioning in the tree.

***P. californicum* and related hybrids—**I was able to distinguish the two cytotypes of *P. californicum* as well as hybrids with its progenitor species *P. dudleyi* and *P. imbricans* ssp. *curtum*. The eight specimens initially identified as *P. californicum* or *P. californicum* hybrids (cal01-06, dud02, dud07) were all resolved in the ssp. *curtum* clade in the cpDNA phylogeny. They were shown by flow cytometry to be variously diploid (cal03), triploid (cal04, dud07), tetraploid (cal05), or ambiguous in terms of ploidy based on the data gathered (cal01; 3n or 4n). Based on morphology and the cpDNA phylogeny, cal01, which was determined to be either triploid or tetraploid, and cal06, whose ploidy was not tested, appeared to be *P. californicum* or *P. californicum* backcrossed with a progenitor species, with *P. imbricans* ssp. *curtum* being the maternal progenitor. cal04, determined to be triploid, also appears to be the result of *P. californicum* backcrossing with a progenitor. cal05 (a tetraploid) and cal02, however, appear to be true representatives of *P. californicum* based on their morphology, though for cal02 the placement has not been confirmed with flow cytometry data. While all *P. californicum* and *P. californicum* hybrids identified in this study had ssp. *curtum* as a maternal progenitor, the possibility that ssp. *imbricans* has served as the progenitor in other cases has not been ruled out since it could be serving as the progenitor at locations not sampled in this study.

Diploid hybrids involving *P. dudleyi*—Hybrids were also identified between *P. dudleyi* and both *P. imbricans* ssp. *curtum* and *P. munitum*. cal03—which exhibits morphology intermediate between *P. imbricans* and *P. dudleyi,* has some malformed spores, and is diploid—appears to be *P. dudleyi* × *imbricans* ssp. *curtum*, with ssp. *curtum* again as the maternal progenitor. Accession dud06, tentatively identified at the start of this project as a member of the *P. californicum-dudleyi* complex, resolved in the *P. munitum* clade and was determined to be diploid by flow cytometry. The principal component analysis showed its morphology to be intermediate between *P. munitum* and *P. dudleyi*, and visually it appears to be a hybrid since its pinnae are pinnatifid rather than fully divided as in *P. dudleyi*. Together, these features suggest that this accession is P. *dudleyi* ×*munitum*, with *P. munitum* being the maternal progenitor.

Taxonomy of the *P. munitum- P. imbricans* clade— As anticipated, the *P. dudleyi* accession, dud05, falls outside the *P. munitum-P. imbricans* clade. While the chloroplast tree places *P. californicum, P. californicum* hybrids and *P. dudleyi* hybrids within the *munitum* and *curtum* clades, the retrieved pattern is consistent with *P. munitum* or *P. imbricans* ssp. *curtum* serving as the maternal progenitor to these individuals. The data gathered in this study supports D. Wagner's (1979) taxonomy of the *P. munitum-P. imbricans* clade, as they show that the common ancestor of subspecies *imbricans* and subspecies *curtum* diverged from *P. munitum*. Because *P. imbricans* ssp. *curtum* is shown to be the sister clade to *P. imbricans* ssp. *imbricans,*

the phylogeny refutes D. Wagner's (1979) alternative hypothesis (Figure 1) that ssp. *curtum*, which he described as being morphologically intermediate between *P. munitum* and *P. imbricans* ssp. *imbricans*, could be closer to the common ancestor of the *P. munitum*-*P. imbricans* clade than either *P. munitum* or *P. imbricans* ssp. *imbricans.* Although the principal component analysis does not show ssp. *curtum* to be morphologically intermediate between *P. munitum* and *P. imbricans* ssp. *imbricans*, I assessed only some of the characters that Wagner (1979) considered important. The possibility that *P. imbricans* ssp. *curtum* shares a common ancestor with *P. munitum* that neither share with *P. imbricans* subsp. *imbricans* (Figure 1) is not supported by this phylogeny

Origins of P. californicum—The genetic, morphological, and cytometry data together yielded new insights into the evolutionary history of *P. californicum.* Since all *P. californicum* and *P. californicum* hybrids fell within the ssp. *curtum* clade on the chloroplast phylogeny (indicating that *P. imbricans* ssp. *curtum* served as the maternal progenitor of all of these individuals), the hypothesis that *P. californicum* originated before *P. munitum* and *P. imbricans* diverged from one another was rejected. The summation analysis also supports the conclusion that *Polystichum imbricans* and not *P. munitum* hybridized with *P. dudleyi* to form allotetraploid *P. californicum.* However, the possibility of repeated origins of *P. californicum* involving both *P. munitum* and *P. imbricans* continues to stand. My data suggest that both *P. dudleyi* × *munitum* and *P. dudleyi* × *imbricans* ssp. *curtum* are present in this dataset, representing the diploid hybrids that Wagner (1979) hypothesized would (after genome duplication) form *P. californicum* in different parts of its range, thus accounting for its variable morphology. Additionally, the *P. dudleyi* × *munitum* accession (dud06) was found at the same locality as *P. munitum, P. dudleyi, P. californicum,* and *P. californicum* hybrids, indicating a site where the formation of allopolyploids from *P. munitum* and *P. dudleyi* might potentially be occurring. It is also noteworthy that *P. californicum, P. californicum* triploid hybrids, and *P. dudleyi* × *imbricans* ssp. *curtum* (cal03-06) were found at the same locality, indicating that regardless of whether or not *P. munitum* has been involved in multiple origins in *P. californicum*'s history, it is likely that *P. californicum* has formed repeatedly through hybridization between *P. dudleyi* and *P. imbricans* ssp. *curtum.*

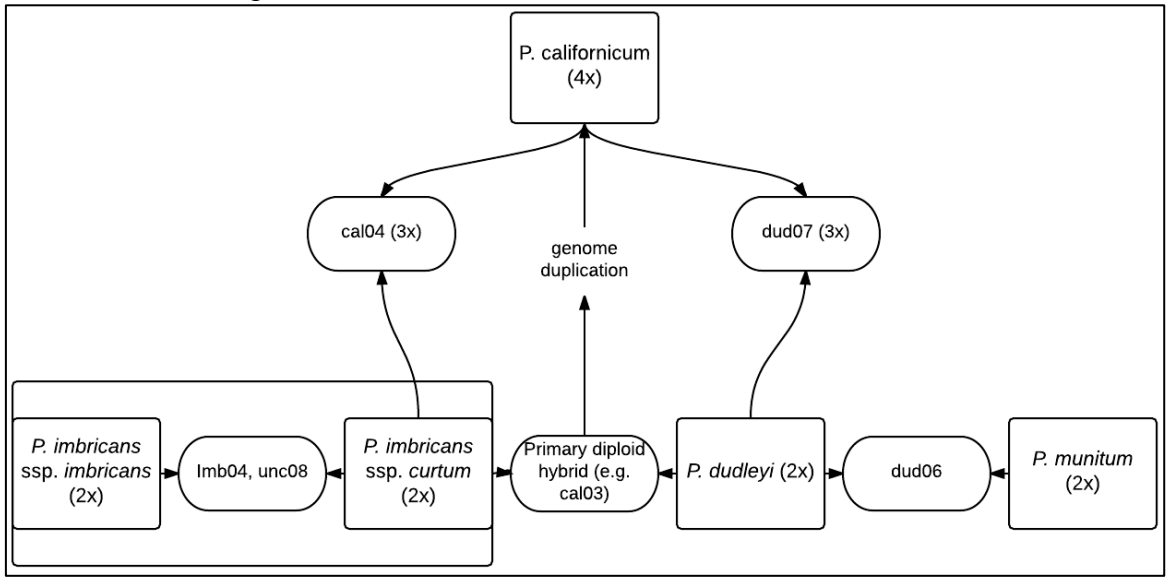

Figure 11: Hypothesized model of relationships between the species studied (rectangles), including the hybrid accessions (ovals) identified in this research.

Conclusions—This research builds on previous understandings of the relationships between the study species, and shows that *P. californicum* remains an intriguing candidate for further study of possible multiple origins in allotetraploid species. In 1973, W. H. Wagner observed that populations of both diploid and tetraploid individuals resembling *P. californicum* could be found together with the presumed progenitor species, a situation that was also seen in the populations of hybrids identified in this research and that suggests the possibility of multiple origins for *P. californicum.* Following D. H. Wagner's (1979) recognition of *P. imbricans* and its two subspecies as separate from *P. munitum*, a classification that is supported by this research, Soltis et al. (1991) concluded that *P. imbricans* rather than *P. munitum* was the progenitor of *P. californicum*. Their research, however, did not address which subspecies of *P. imbricans* might be involved. This project expands on their results by identifying *P. imbricans* ssp. *curtum* as the maternal progenitor for the accessions included in this study, using samples from a wider geographic range than that covered by Soltis et al. Finally, the identification of hybrids between *P. dudleyi* and both *P. munitum* and *P. imbricans* ssp. *curtum* suggests that the possibility that *P. munitum* may be a progenitor of *P. californicum* in some parts of its range continues to stand, meriting further investigation into sites inhabited by potential hybrids to search for examples of multiple origins of *P. californicum*-like individuals with different progenitors. Further research should also include gathering more data to strengthen our identifications of the hybrids: examining spore abortion as a measure of fertility would be useful, as would exploring additional morphological characters and adding more specimens to the PCA.

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