Received: 5 January 2022 | Accepted: 10 March 2022

DOI: 10.1002/ajb2.1850

RESEARCH ARTICLE

American Journal of Botany

Once more unto the breach, dear friends: Resolving the origins and relationships of the *Pellaea wrightiana* hybrid complex

Michael D. Windham[1] | **Layne Huiet**[1] | **Jordan S. Metzgar**[2] | **Tom A. Ranker**[3] | **George Yatskievych**[4] | **Christopher H. Haufler**[5] | **Kathleen M. Pryer**[1]

[1]Department of Biology, Duke University, Durham, 27708NC, USA

[2]Department of Biological Sciences, Virginia Tech, Blacksburg, 24061VA, USA

[3]School of Life Sciences, University of Hawai'i at Mānoa, Honolulu, 96822HI, USA

[4]Billie L. Turner Plant Resources Center, University of Texas, Austin, 78712TX, USA

[5]Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, 66045KS, USA

Correspondence

Michael D. Windham, Department of Biology, Duke University, Durham, NC 27708, USA.
Email: mdw26@duke.edu

Abstract

Premise: The taxonomic status of Wright's cliff brake fern, *Pellaea wrightiana*, has been in dispute ever since it was first described by Hooker in 1858. Previously published evidence suggested that this "taxon" may represent a polyploid complex rather than a single discrete species, a hypothesis tested here using a multifaceted analytical approach.

Methods: Data derived from cytogenetics, spore analyses, leaf morphometrics, enzyme electrophoresis, and phylogenetic analyses of plastid and nuclear DNA sequences are used to elucidate the origin, relationships, and taxonomic circumscription of *P. wrightiana*.

Results: Plants traditionally assigned to this taxon represent three distinct polyploids. The most widespread, *P. wrightiana*, is a fertile allotetraploid that arose through hybridization between two divergent diploid species, *P. truncata* and *P. ternifolia*. Sterile triploids commonly identified as *P. wrightiana*, were found to be backcross hybrids between this fertile tetraploid and diploid *P. truncata*. Relatively common across Arizona and New Mexico, they are here assigned to *P.* ×*wagneri* hyb. nov. In addition, occasional sterile tetraploid plants assigned to *P. wrightiana* are shown here to be hybrids between the fertile allotetraploid and the tetraploid *P. ternifolia* subsp. *arizonica.* These tetraploid hybrids originated independently in two regions of parental sympatry (southern Arizona and west Texas) and are here assigned to *P.* ×*gooddingii* hyb. nov.

Conclusions: Weaving together data from a diversity of taxonomic approaches, we show that plants identified as *P. wrightiana* represent three morphologically distinguishable polyploids that have arisen through repeated hybridization events involving the divergent sexual taxa *P. ternifolia* and *P. truncata*.

KEYWORDS

cytogenetics, enzyme electrophoresis, ferns, morphometrics, plastid and nuclear phylogeny, Pteridaceae, spores

In a world experiencing rampant loss of biodiversity, the ability to accurately draw species boundaries is one of biology's most significant and urgent goals (Soltis and Gitzendanner, 1998; Bickford et al., 2007; Pace and Cameron, 2017). Genetically divergent and reproductively isolated species that are morphologically difficult to distinguish from related taxa present a major challenge to taxonomists (Löve, 1964; Grant, 1981; Soltis et al., 2007). Such cryptic taxa are especially common in seed-free plant lineages, such as ferns and bryophytes, which lack the diversity of morphological features provided by seeds and flowers (Paris et al., 1989; Shaw, 2001; Dauphin et al., 2017, 2018). The key to deciphering these species complexes involves coupling modern molecular data with a variety of traditional techniques such as cytogenetics and morphometrics to achieve as comprehensive a picture as possible. Such an integrative approach can provide unprecedented insights into evolutionary processes such as genetic diversification, ecological adaptation, speciation, gene flow, hybridization, and the origin and establishment of polyploids (e.g., Haufler et al., 1995b; Takamiya et al., 2001; Grusz et al., 2009; Suissa et al., 2022). Here we employed an

unusually broad data set accumulated over 40 years to investigate the xeric-adapted *Pellaea wrightiana* Hook. hybrid complex, a lineage of cheilanthoid ferns that has been described as "intractable" to standard taxonomic analysis (Wagner, 1965) due, in part, to the presence of undescribed, morphologically cryptic taxa.

Although *Pellaea* is nonmonophyletic as currently circumscribed (PPG I, 2016), *P. wrightiana* is sufficiently closely related to the type species (see Grusz et al., 2021) that it will certainly remain in the genus when generic boundaries are redrawn. This taxon has long been included in section *Pellaea*, a group that apparently diversified in Mexico and subsequently colonized much of North and South America (Tryon and Tryon, 1973). Members of this section have been central to a series of investigations focusing on morphology (Tryon, 1957), drought physiology (Pickett, 1931; Hevly, 1963), gametophyte ontogeny (Pray, 1968, 1970, 1971), cytotaxonomy (Tryon and Britton, 1958; Tryon, 1972; Rigby, 1973), apomixis (Tryon, 1968; Whittier, 1968; Gastony and Windham, 1989; Grusz et al., 2021), evolutionary genetics (Gastony and Gottlieb, 1985; Gastony, 1988), and molecular phylogeny (Kirkpatrick, 2007; Grusz et al., 2021). Although these studies have yielded a wealth of systematic information, several of the taxonomic questions raised by Tryon's (1957) sectional monograph remain to be addressed.

One of these questions relates to the appropriate taxonomic status of *Pellaea wrightiana*, which has been a point of contention ever since the taxon was first described by Hooker (1858). In Figure 1, we summarize the various hypotheses put forward over the last 65 years to explain the origin and relationships of this taxon. In her monograph of *Pellaea* section *Pellaea*, Tryon (1957, p. 154) stated that *P. wrightiana* was "essentially similar to *P. ternifolia* var. *ternifolia* in the architecture of the blade and is considered a northern extension of the species having quantitative differences not sufficient for species separation." Hypothesizing that it had arisen through gradual divergence due to local adaptation and partial geographic isolation, Tryon (1957) proposed *P. wrightiana* be treated as a variety of *P. ternifolia*, noting that her circumscription of var. *ternifolia* was cytogenetically diverse, encompassing both diploid and tetraploid populations (Figure 1A). The ploidy of var. *wrightiana* was unknown at the time, thus there was no basis for determining which of the two cytotypes of var. *ternifolia* had been involved in its origin.

The first chromosome count attributed to *Pellaea wrightiana* was published by Knobloch and Britton (1963). A plant collected in the Santa Catalina Mts. near Tucson, Arizona was triploid. The cells exhibited 29 bivalents and 29 univalents during meiosis I, providing strong evidence that the plant was some sort of hybrid. Arguing that hybridization between the known diploid and tetraploid cytotypes of *P. ternifolia* var. *ternifolia* could not explain the morphological divergence between vars. *ternifolia* and *wrightiana*, Knobloch and Britton (1963) inferred that a second species of *Pellaea* must have been involved in the origin of the latter. They hypothesized the other parent to be *P. longimucronata* Hook., which they documented was diploid and showed broad geographic overlap with *P. wrightiana*. Based on these data, Knobloch and Britton (1963) proposed that *P. wrightiana* was a triploid hybrid between tetraploid *P. ternifolia* and diploid *P. longimucronata* (Figure 1B). As noted by the authors, this scenario would be congruent with the chromosome pairing observed in meiosis I if the 29 bivalents were attributed to autosyndetic pairing of the two genomes derived from the *P. ternifolia* parent. The main weakness of the Knobloch and Britton (1963) hypothesis was that their triploid produced only malformed, nonviable spores, an observation at odds with the broad geographic distribution reported for *P. wrightiana*.

The question of how *P. wrightiana*, an apparently sterile triploid according to Knobloch and Britton (1963), could establish a geographic range spanning most of the southwestern United States, was explored in more detail by Wagner (1965). To exclude the possibility of hybridization with other species of *Pellaea,* Wagner (1965) focused on a disjunct occurrence of *P. wrightiana* in North Carolina. Chromosome counts from this population revealed that the plants were tetraploid, forming 58 bivalents during meiosis I. These cytogenetic observations, combined with evidence of morphological intermediacy, led Wagner (1965) to infer that *Pellaea wrightiana* was a fertile amphidiploid (=allotetraploid) hybrid between *P. longimucronata* and diploid *P. ternifolia* (Figure 1C). Under this hypothesis, the triploid plant studied by Knobloch and Britton (1963) was inferred to be a backcross between tetraploid *P. wrightiana* and diploid *P. longimucronata*. This scenario accounted for all relevant data available at the time: (1) *Pellaea wrightiana* was abundant and widespread because it was a fertile tetraploid (not a sterile triploid), (2) the sterile triploid occurred in the Santa Catalina Mountains where both putative parents grew in close proximity, and (3) the 29 bivalents observed during meiosis I in the triploid represented the two genomes derived from *P. longimucronata*, with the genome from *P. ternifolia* remaining unpaired as 29 univalents.

Over the years, most botanists have come to accept Wagner's (1965) interpretation of *Pellaea wrightiana* as an allopolyploid that should be treated as specifically distinct from *P. ternifolia* (Figure 1C). The nomenclature of the other hypothesized parent has since been updated to *P. truncata* Goodd. because *P. longimucronata* is now considered an illegitimate name (see Cronquist et al., 1972). The most likely point of origin for such a hybrid is the southwestern United States where both putative parents occur. However, the taxonomic situation in this region is more complex than previously realized. Windham (1993a) recognized three subspecies among North American populations of *P. ternifolia* (Figure 1D). Subspecies *ternifolia* remained cytogenetically diverse, but the newly recognized subsp. *arizonica* Windham and subsp. *villosa* Windham were described as morphologically distinct tetraploids of uncertain relationship to the nominate subspecies. Subspecies *villosa* was subsequently elevated to species rank (Windham and

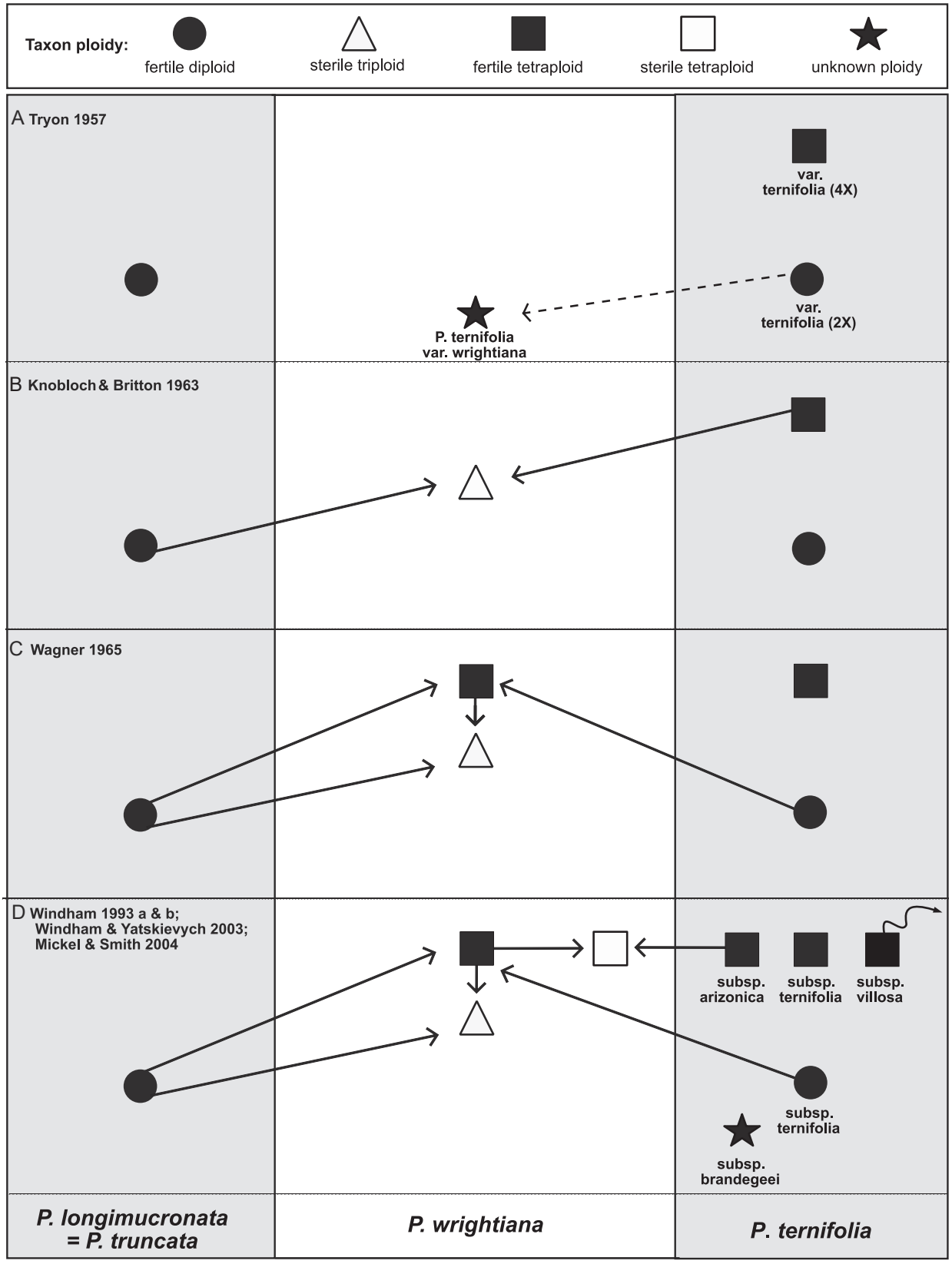

FIGURE 1 (See caption on next page)

Yatskievych, 2003) and will not be discussed further here. But even with this transfer, the number of proposed taxa in the species complex has continued to grow. Additional analysis of southwestern collections attributed to *P. wrightiana* revealed a cryptic tetraploid with malformed spores (Figure 1D), which may represent a hybrid between fertile *P. wrightiana* and *P. ternifolia* subsp. *arizonica* (Windham, 1993b). More recently, Mickel and Smith (2004) recognized yet another subspecies of *P. ternifolia* (subsp. *brandegeei*) whose ploidy and relationship to the other taxa has remained a mystery.

The observations presented above drive home the fact that plants identified as *P. wrightiana* represent a polyploid complex, not a single discrete taxon (see Figure 1). Thus, although Wagner's (1965) hypothesis regarding the allopolyploid origin of *P. wrightiana* is compelling, the systematics of the group cannot be fully resolved until all members of the hybrid complex have been adequately investigated and critically compared to the type collections. Here we employ an eclectic data set derived from cytogenetics, spore analyses, leaf morphometrics, enzyme electrophoresis, and phylogenetic analyses of plastid and nuclear DNA sequences to (1) clarify relationships among the polyploid members of the *Pellaea wrightiana* complex and their putative diploid progenitors and (2) provide an appropriate taxonomic treatment for the group.

MATERIALS AND METHODS

Taxon sampling

Much of the data presented here came from 575 live sporophytes (representing 38 populations) collected by the lead author and associates between 1983 and 1987. Additional morphological and geographical data were obtained from herbarium collections curated at ARIZ, ASC, ASU, COLO, GH, MICH, MNA, MO, MSC, NMC, NY, UC, UNLV, UNM, US, UTC, and UTEP. In all, nearly 2000 plants were examined for this study. Spore measurements were obtained from 62 collections listed in Table 1 and Appendices 1 and 2. These included type specimens of *Pellaea truncata* and *P. wrightiana* housed at the U.S. National Herbarium (US). The value of herbarium specimens was further enhanced by the discovery of long-term spore viability in this species complex (up to 100 years; Windham et al., 1986). Viable spores were obtained (with permission) from specimens at ARIZ, ASC, ASU, COLO, GH, MICH, MSC, NMC, NY, UNLV, UNM, and UTEP. Spores were sown and gametophytes maintained using the methods described by Windham et al. (1986). Gametophytes derived from this source furnished many of the chromosome counts presented here and greatly increased the geographic coverage of our sampling. A complete list of populations and herbarium specimens included in the electrophoretic and phylogenetic analyses is provided in Appendix 2.

Cytogenetic and spore studies

Meiotic materials were obtained from population samples that had been successfully transferred from the field to greenhouses at the University of Kansas. Leaves at the proper stage of development were fixed in a mixture of absolute ethanol and glacial acetic acid (3:1). After 24 h, they were removed from the fixative and stored (up to 5 years) in 70% ethanol at –20°C. Slides were prepared by macerating 50–100 sporangia in a drop of 1% iron acetocarmine; the cells were then squashed in a 1:1 v/v mixture of acetocarmine and Hoyer's medium (Anderson, 1954). All meiotic chromosome counts were derived from sporocytes at diakinesis or metaphase I. In polyploid accessions, earlier meiotic stages (especially pachytene) were examined for evidence of multivalent associations. Mitotic counts were derived from the root tips of greenhouse-grown sporophytes or from gametophytes using the colchicine/Glusulase protocol of Windham and Haufler (1986). Representative cells were photographed using a Zeiss standard microscope equipped with phase contrast optics, a Nikon AFM camera, and Kodak Technical Pan 2415 film.

To obtain spores for ploidy correlations, mature, undehisced sporangia were individually transferred to drops of glycerol and ruptured with the tip of a dissecting needle. Spore number was estimated for each sporangium, and only those containing approximately 64 spores were included in subsequent analyses. Occasional sporangia containing 32 unusually large spores have been detected in several sexually reproducing fern species, including both *Pellaea wrightiana* and *P. truncata* (Windham, 1983; Grusz et al., 2021). These likely represent unreduced products of meiosis and were therefore excluded when attempting to correlate spore size with ploidy level. Twenty-five normally developed spores from each sporangium were selected for measurement by means of a random transect across the slide, and the spores were measured using an ocular micrometer mounted on a phase contrast microscope (either the Zeiss scope used for

FIGURE 1 Overview of taxonomic treatments for *Pellaea wrightiana* and related taxa published between 1957 and 2004 (A–D). Solid circles represent taxa known to be fertile diploids when each taxonomic treatment (cited at upper left of each panel) was published; solid stars represent fertile taxa whose ploidy was unknown at time of publication. Solid squares represent documented fertile tetraploids; open squares are largely sterile tetraploids; open triangles are mostly sterile triploids. For each taxonomic scheme, arrows point from proposed parents to putative hybrids. Dashed arrow in panel A reflects Tryon's (1957) view that *P. wrightiana* was derived from *P. ternifolia* by divergence, rather than hybridization. Wavy arrow in Panel D indicates taxon removed from *P. ternifolia* by Windham and Yatskievych (2003).

chromosome analyses at the University of Kansas or a Meiji MT5310L scope at Duke University). Approximate diameter of the trilete spores was determined by spanning the exospore, visible as a dark ring just below the rugulate spore surface. This procedure eliminates species-dependent variation in the height of perisporal ornamentation (see Kott and Britton, 1982). The malformed spores of sterile triploids and tetraploids were not measured due to our inability to determine the ploidy of spores produced by irregular meiotic events. Spore contents of representative sporangia were documented using a Canon EOS Rebel T3i digital camera mounted on a Meiji MT5310L microscope.

Geographic distributions

Ranges of the three fertile species (*Pellaea ternifolia*, *P. truncata*, and *P. wrightiana*) were approximated by drawing a perimeter closely encircling peripheral populations of each taxon shown on dot maps published by Tryon (1957). This perimeter was then expanded as needed to include new records confirmed through fieldwork or examination of nearly 1400 herbarium specimens obtained on loan from the herbaria listed in the "Taxon sampling" section. Sterile triploids and sterile tetraploids confirmed via cytogenetic and/or spore analyses were georeferenced using U.S. Geological Survey topographic maps and individually plotted on the appropriate base maps.

Leaf morphometric analyses

Wagner (1965) suggested that the southwestern triploid identified as *Pellaea wrightiana* was most likely a backcross between a western version of his allotetraploid *P. wrightiana* and diploid *P. truncata* (as *P. longimucronata*) (Figure 1C). Subsequent studies of the sterile tetraploid "form" of *P. wrightiana* suggested that it might be a cross between Wagner's allotetraploid *P. wrightiana* and a tetraploid form of *P. ternifolia* (Yatskievych and Windham, 1986; Figure 1D). Based on these observations, we subdivided our morphological analysis into a univariate study of triploid individuals and their putative parents, and a multivariate analysis of the tetraploid "cytotypes" of *P. wrightiana* and *P. ternifolia*. The more complex multivariate approach was utilized in the latter case because the morphological differences between these taxa are more subtle (Tryon, 1957). Sporulating leaves from both herbarium specimens and greenhouse-grown plants were included in these analyses, but all fresh materials were dried in a plant press at ambient temperatures for several weeks to ensure that measurements would be comparable. The identity of each individual sampled was verified using chromosome counts, spore measurements, electrophoretic markers, or qualitative morphological characters.

Our univariate analysis of triploid plants was based on five quantitative leaf characters commonly used to distinguish *P. wrightiana* from *P. truncata*: (1) blade length (measured from the lowest basiscopic pinnule to the tip of the apical leaf segment) and maximal values for (2) blade width, (3) rachilla length, (4) pinnule length, and (5) number of pinnules per pinna. The sampling used for this analysis included 100 triploids and 200 plants each of *P. truncata* and fertile *P. wrightiana*. Multivariate analyses of the three tetraploid taxa (fertile *P. wrightiana*, *P. ternifolia* subsp. *arizonica*, and the putative hybrid between them; Figure 1D) incorporated a total of nine quantitative leaf characters. In addition to the five mentioned above, measurements were recorded for petiole length, distance from the base of the blade to the widest point, number of pinna pairs per leaf, and maximum distance between pinnae. The sample consisted of 22 individuals of *P. ternifolia* subsp. *arizonica*, 31 sterile tetraploids, and 47 plants of fertile *P. wrightiana* collected from the region of sympatry. Data from the three tetraploids were analyzed using the BMDP7M stepwise discriminant analysis program (Dixon, 1983). The criterion for entry of each variable in the discriminant model was the approximate *F*-ratio at each step, which was estimated using the principle of Bonferroni's inequality (Morrison, 1976; Ranker and Schnabel, 1986).

Enzyme electrophoresis

Active enzymes were extracted by crushing small quantities of living (photosynthetic) tissue in porcelain spot plates using the phosphate-PVP grinding buffer of Soltis et al. (1983) modified by the addition of DMSO to a final concentration of 10% (v/v). Sporophytic samples were prepared by grinding small pieces (ca. 50 mm^2) of immature leaf tissue in 10 drops of grinding buffer. Gametophytes were crushed individually (in 1–2 drops of buffer) for progeny analyses or in small groups to determine the enzyme phenotype of the parental sporophyte. The slurry produced by grinding was absorbed into Whatman 3MM chromatography paper wicks that were inserted into 12.5% starch gels for electrophoresis. Once we determined that frozen extracts gave the same results as fresh material, samples were prepared 1–2 days in advance and stored in an ultracold freezer at −80°C. Four buffer systems were used to survey enzyme variability: System 6 (from Soltis et al., 1983), Systems 8 and 11 (as modified by Haufler, 1985), and System M, a 7.5 pH version of the morpholine–citrate system used by Odrzykoski and Gottlieb (1984). Whenever possible, enzymes were run on two or more buffer systems to ensure that co-migration of bands in different individuals was a true reflection of genetic similarity. The following 12 enzyme systems were assayed (enzyme abbreviation and buffer systems appear in parentheses): aconitase (ACO; 11,6), aldolase (ADL; 11,8), aspartate aminotransferase (AAT; 8,6), hexokinase (HK; 8), isocitrate dehydrogenase (IDH; 11,M), leucine aminopeptidase (LAP; 8,6), malate dehydrogenase (MDH; M,11), phosphoglucomutase (PGM; 6,11), 6-phosphogluconate dehydrogenase (6PGDH; 11,M), phosphoglucose isomerase (PGI; 6,8), shikimate

dehydrogenase (SkDH; M,11), and triosephosphate isomerase (TPI; 6,8). Assays for MDH and PGM were described by Werth (1985); all other staining schedules were adapted from Soltis et al. (1983). Stained gels were photographed using a red filter and Kodak Technical Pan 2415 film.

Because attempts to hybridize gametophytes with known enzyme phenotypes were unsuccessful, most genotypes were inferred directly from electromorphs observed on the stained gels. For multilocus enzymes, allozymes (allelic variants at a single genetic locus) were distinguished from isozymes (electromorphs produced by different loci) through (1) analyses of meiotic segregation in gametophyte progeny, (2) studies of electrophoretic variability in natural sporophyte populations, and (3) assumptions that enzyme substructure and compartmentalization in *Pellaea* parallel those observed in other ferns (Gastony and Darrow, 1983; Wolf et al., 1987) and in flowering plants (Gottlieb, 1981). For each enzyme, the loci coding for different isozymes were numbered sequentially from anode to cathode. Within a single locus, the most anodal allozyme was designated "a", and slower-migrating allelic products were labelled "b", "c," etc. The identity of allozymes shared between populations and species were verified by running samples side-by-side on the same gels.

DNA isolation, amplification, cloning, and sequencing

Protocols for isolating genomic DNA and for the amplification and sequencing of the plastid *rbcL* and *atpA* regions followed Schuettpelz and Pryer (2007). The plastid region *trnG-R* was amplified and sequenced following Nagalingum et al. (2007) and Beck et al. (2010). DNA isolation, amplification, cloning, and sequencing of the nuclear *gapCp* region followed Schuettpelz et al. (2008). Protocols for identifying *gapCp* alleles followed Schuettpelz et al. (2015). All plastid and nuclear sequence data were deposited in GenBank (see Appendix 2).

Sequence alignment and phylogenetic analysis

Sequences for each locus were aligned manually using Mesquite v.3.70 (Maddison and Maddison, 2021). The *atpA*, *rbcL,* and *trnGR* alignments were 1831, 1309, and 1162 bp long, respectively. Portions of the alignments with indels or ambiguous alignments were excluded, and no gap-coding method was employed. No characters were excluded from the *atpA* or *rbcL* alignments, while the *trnGR* alignment had 63 bp excluded due to ambiguous alignment. The *gapCp* sequence reads for each accession were compared manually and used to construct a phylogeny that was also inspected for evidence of PCR artifacts including chimeric sequences. The resulting 35 unique sequences were then combined into an alignment that was 657 bp long with 46 ambiguously aligned characters excluded.

We checked for topological conflict among the single-gene plastid data sets by calculating a majority-rule consensus topology for each locus. The resulting phylogenies were manually inspected following Mason-Gamer and Kellogg (1996) using a 0.95 posterior probability (PP) value as the minimum threshold for conflict. We did not observe any topological conflict among the plastid data sets, so we combined all three into a single data set with a length of 4302 bp. The nuclear *gapCp* alignment and the combined plastid alignment were analyzed separately with *gapCp* and each of the plastid loci assigned its own model of sequence evolution based on Akaike information criterion scores calculated in ModelTest-NG v0.1.5 (Darriba et al., 2020).

All phylogenetic analyses were performed using Bayesian Markov chain Monte Carlo (B/MCMC) analyses in MrBayes v.3.2.6 (Ronquist et al., 2012) and implemented on the CIPRES portal (Miller et al., 2010). All analyses comprised four runs of four chains each, with each chain run for 50 million generations. We used default prior values and chains were sampled every 5000 generations. We assessed stationarity by plotting likelihood and generation scores using Tracer v1.6 (Rambaut and Drummond, 2007). The first 12.5 million generations were conservatively discarded as the burn-in period. The majority-rule consensus trees were calculated from the remaining 30,000 trees.

RESULTS

Previous studies have indicated that plants classified as *Pellaea wrightiana* represent three cytogenetically distinguishable polyploids: (1) a fertile tetraploid (Wagner, 1965), (2) a mostly sterile triploid (Knobloch and Britton, 1963), and (3) a largely sterile tetraploid (Windham, 1993b). Because the data presented below indicate that these entities contain different combinations of parental genomes and are morphologically recognizable as well, we assigned each a binomial name (validated in the Taxonomic Treatment). To simplify presentation of our results and link these data to a formal nomenclatural construct, we use these new names from this point forward. Given that the type specimens of *P. wrightiana* at K (holotype) and US (isotype) have well-formed spores (Figure 3A) with an average length close to the grand mean of the fertile tetraploid (Figure 4; Appendix 1), we associate Hooker's original name with the widespread fertile tetraploid. The mostly sterile triploid will hereafter be called *P.* ×*wagneri* Windham, while the largely sterile tetraploid is designated *P.* ×*gooddingii* Windham.

Cytogenetic and spore studies

Chromosome counts were obtained from 63 populations representing six taxa (Table 1). Nineteen populations of *P. wrightiana* s.s. scattered throughout the southwestern United States (one from Mexico) were included. All collections were tetraploid and formed 58 bivalents at diakinesis (Figure 2A); the spores were well formed and more or less uniform in size (Figure 3A), with mean spore

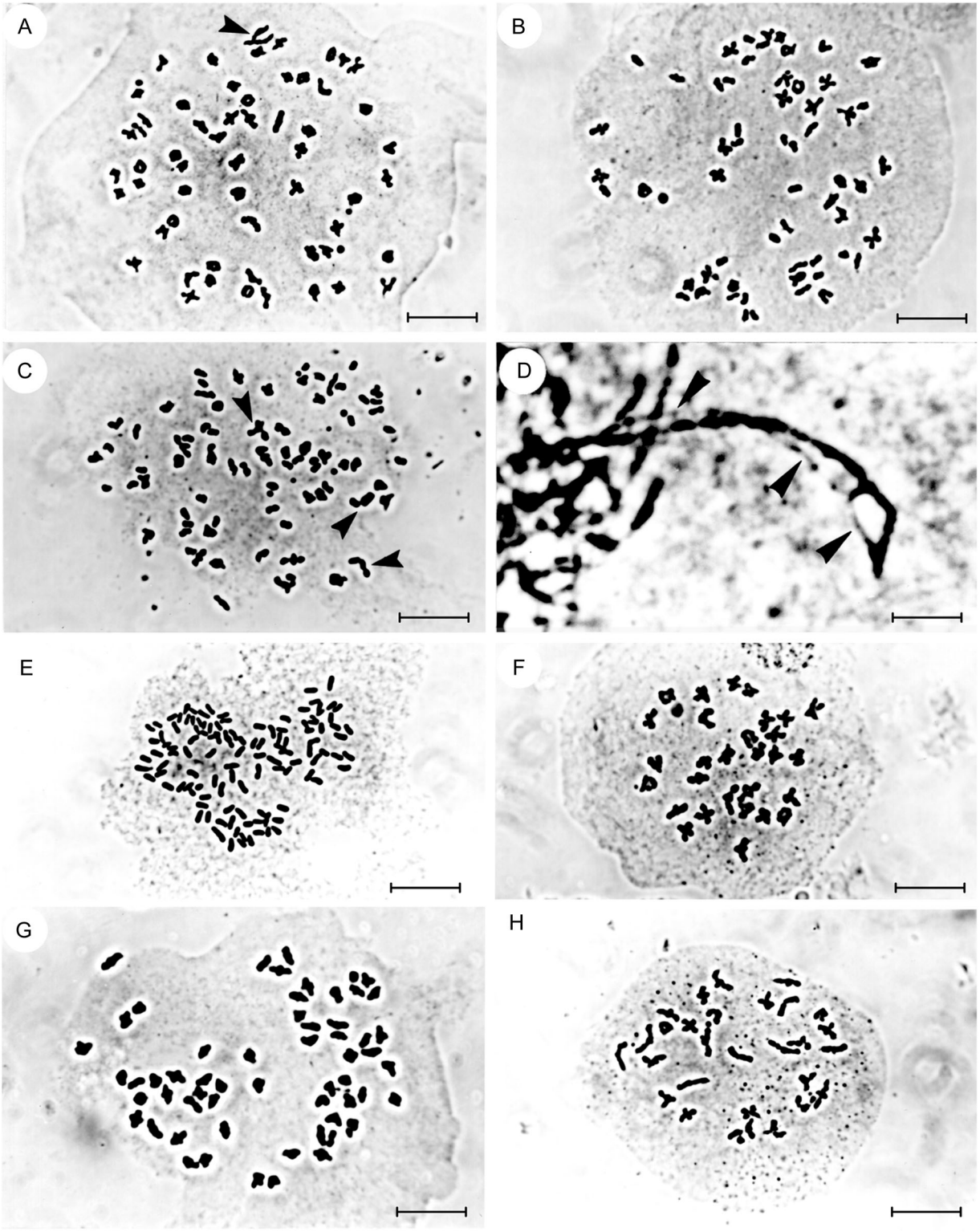

FIGURE 2 Chromosome squashes of *Pellaea wrightiana* and related taxa. Scale bars: A–C, E–H = 20 μm, D = 5 μm. (A) Fertile allotetraploid *P. wrightiana* with 58 bivalents (overlapping bivalents indicated by single arrowhead). (B) Triploid *P.* ×*wagneri* showing 29 bivalents and 29 univalents. (C–E) Tetraploid *P.* ×*gooddingii*. (C) Sporocyte at diakinesis exhibiting univalents, bivalents, and trivalents; three arrowheads identify the most obvious trivalents. (D) Trivalent association at pachynema; three arrowheads identify partially synapsed chromosome. (E) Mitotic preparation of root tip cell exhibiting $2n = 116$. (F) Diploid *P. ternifolia* showing 29 bivalents. (G) Tetraploid *P. ternifolia* subsp. *arizonica* with 58 bivalents. (H) Sporocyte of diploid *P. truncata* exhibiting 29 bivalents.

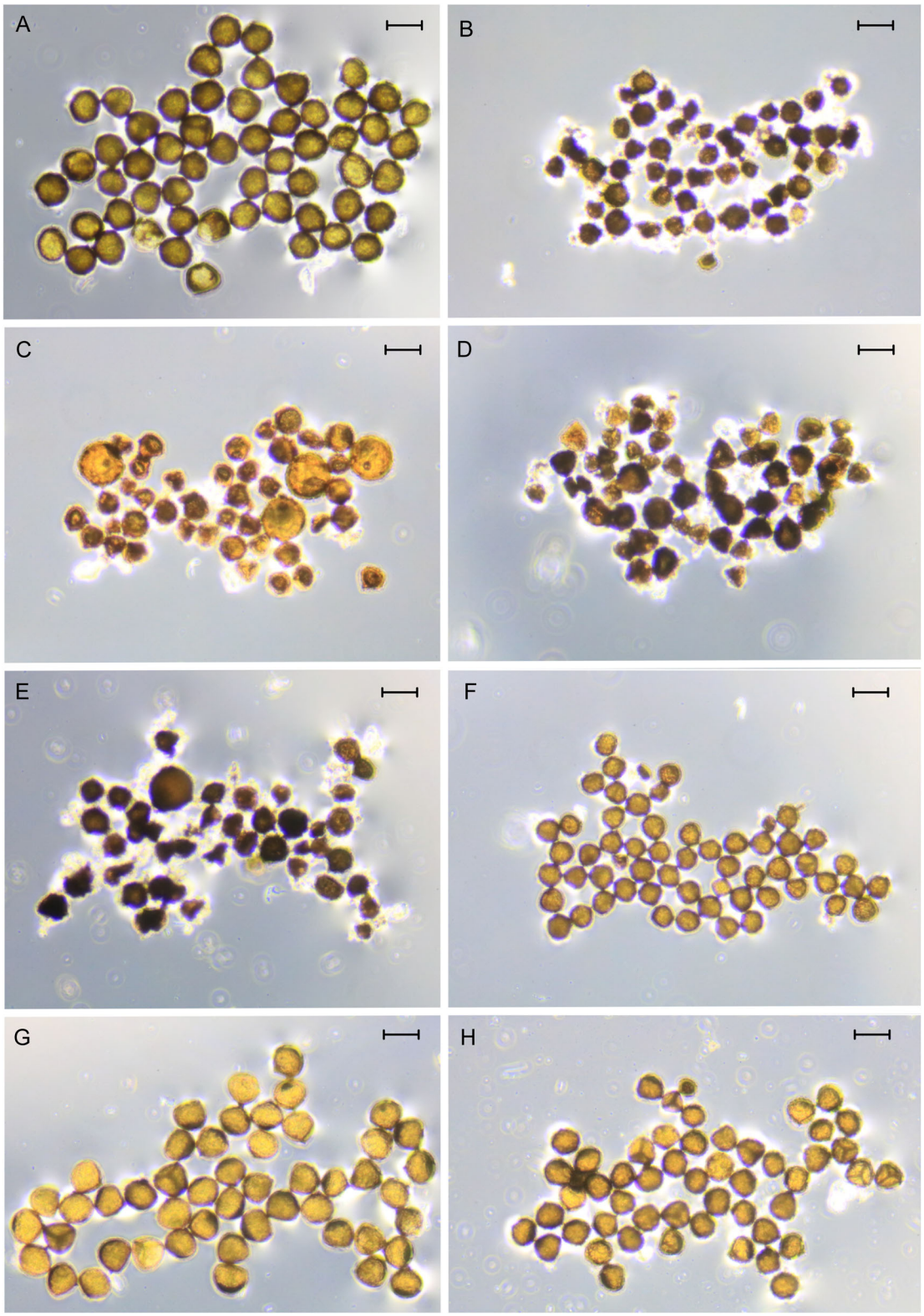

FIGURE 3 Spore contents of individual sporangia from *Pellaea wrightiana* and related taxa. Scale bars = 50 μm. (A) Fertile tetraploid *P. wrightiana* (mean spore length = 46.6 μm). (B, C) Sterile triploid *P. wrightiana* (=*P.* ×*wagneri*). (D, E) Sterile tetraploid *P. wrightiana* (=*P.* ×*gooddingii*). (F) Fertile diploid *P. ternifolia* subsp. *ternifolia* (mean spore length = 33.9 μm). (G) Fertile tetraploid *P. ternifolia* subsp. *arizonica* (mean spore length = 47.6 μm). (H) Fertile diploid *P. truncata* (mean spore length = 38.3 μm).

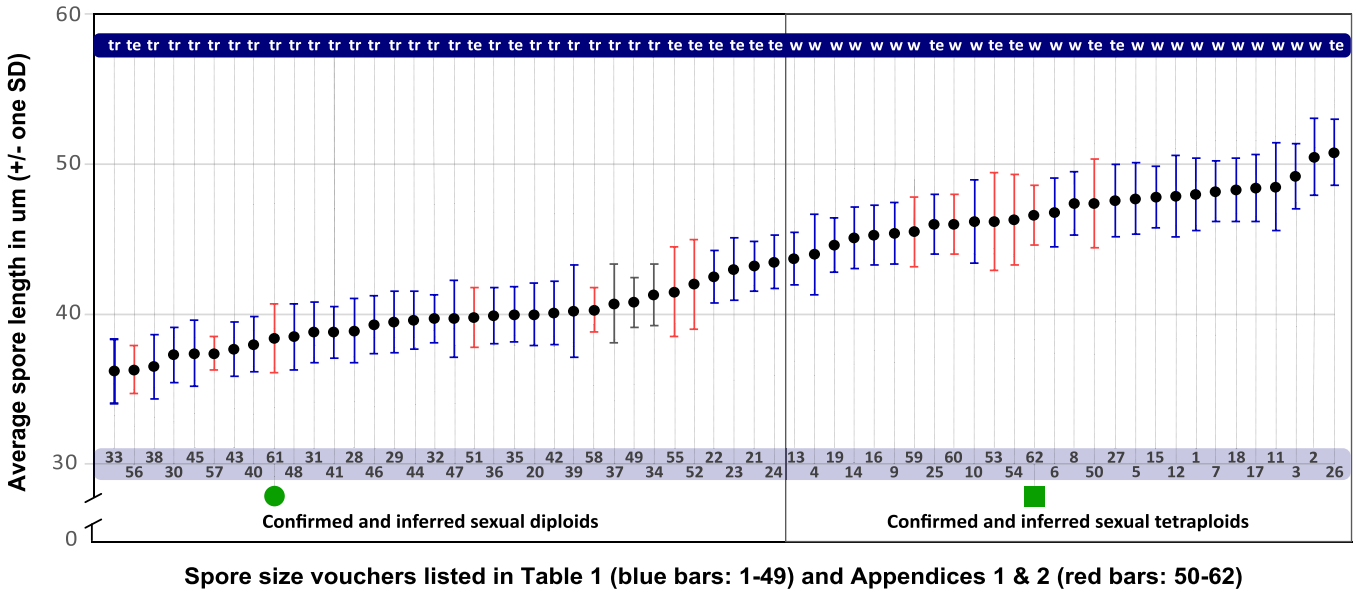

FIGURE 4 Average spore lengths (μm) for specimens of *Pellaea wrightiana* and related taxa; error bars indicate one standard deviation. Number below each sample is a unique identifier linked to voucher data in Table 1 and Appendices 1 and 2. Ploidy of samples 1–49 (blue bars) directly determined by chromosomes counts (see Table 1). Ploidy of samples 50–62 (red bars) inferred from spore length data (Appendices 1 and 2). Abbreviations in blue bar across top of figure identify species sampled: te = *P. ternifolia*, tr = *P. truncata*, w = *P. wrightiana.* Green circle = isotype (US 692689) of *P. truncata*; green square = isotype (US 62535) of *P. wrightiana.*

lengths ranging from 43.7–50.5 μm ($\bar{X}$ = 47.0) (Table 1, Figure 4). Plants representing *P.* ×*wagneri* were obtained from 11 localities in Arizona and New Mexico (Table 1); sporocytes from these collections showed 29 bivalents and 29 univalents at diakinesis (Figure 2B). Although most sporangia contained only shrunken and/or malformed spores (Figure 3B), a few also produced a small number of unusually large spores (Figure 3C) that germinated to form unreduced, triploid gametophytes. These provided an additional chromosome count of $n = 87$ from the Organ Mountains of New Mexico (*Dunn 8527*, UNM).

Plants representing *P.* ×*gooddingii* were obtained from two localities in Arizona and one in Texas (Table 1). All showed a mixture of univalents, bivalents, and trivalents at diakinesis (Figure 2C). The presence of trivalent associations was confirmed by observing partial synapsis between thin (unpaired) and thick (paired) chromosome strands at pachytene (Figure 2D). The number of trivalents per cell varied from 6–15, making it difficult to obtain accurate meiotic chromosome counts. However, root tip preparations consistently yielded counts of $2n = 116$ for this taxon (Figure 2E). Most sporangia contained only shrunken and/or malformed spores (Figure 3D), and the production of unusually large, potentially viable spores (Figure 3E) occurred at much lower frequency than that observed among plants of *P.* ×*wagneri* (Figure 3C).

Our cytogenetic sampling also included 30 populations of the two species proposed as parents of *P. wrightiana*. *Pellaea ternifolia* subsp. *ternifolia* was represented by six collections (Table 1) encompassing two cytotypes: five diploid individuals showing 29 paired chromosomes at diakinesis (Figure 2F) and a single tetraploid with 58 pairs (Figure 2G). Though generally considered to be an autopolyploid because of its close resemblance to the diploid, the tetraploid cytotype of subsp. *ternifolia* showed no evidence of multivalent formation during meiosis. Two populations of *P. ternifolia* subsp. *arizonica* were analyzed as well, both of which were shown to be tetraploid (Table 1). Mean spore length ranged from 40.0 to 43.5 μm ($\bar{X}$ = 42.3) in confirmed diploid plants of *P. ternifolia* s.l. and from 46.0 to 50.8 μm ($\bar{X}$ = 48.1) in confirmed tetraploids (Figure 4, Table 1). Finally, 22 populations of *P. truncata* were also included in the cytogenetic analyses. These spanned the geographic range of the taxon, including plants collected near the type locality in the Mule Mountains of Arizona (Table 1, ref. no. 28). All were diploid with 29 paired chromosomes at diakinesis (Figure 2H) or $n = 29$ in gametophytic preparations; the spores were well formed and more or less uniform in size (Figure 3H), with mean spore lengths ranging from 36.2 to 41.3 μm ($\bar{X}$ = 39.0) (Table 1, Figure 4).

Geographic distributions

Ranges of the three polyploid taxa included in the *P. wrightiana* complex were plotted based on herbarium specimens and extensive fieldwork. The prevalence of well-formed spores was used to separate *P. wrightiana* s.s. from *P.* ×*wagneri* and *P.* ×*gooddingii*, and the two hybrids were distinguished using a combination of morphological, cytogenetic, and electrophoretic data. As might be expected, the fertile tetraploid showed a more extensive distribution than either of the largely sterile taxa. It had been collected in the

TABLE 1 Confirmed chromosome counts, collection localities, and spore length data for taxa related to *Pellaea wrightiana* s.l. Locations of voucher specimens indicated by standard herbarium acronyms (Thiers, 2016)

Ref no.	Country	State	Collection locality and voucher information		Mean spore length, µm (SD)
***Pellaea wrightiana s.s.*, fertile tetraploid, *n* = 58**					
1	MEXICO	Sonora	NE of Nacozari on rd. to Angustura. *Reeves* et al. *6369* (ASU)[a]		48.0 (2.44)
2	USA	Arizona	Cochise Co.	Dragoon Mts. Sheepshead Pass. *Windham 247* (ASC)	50.5 (2.55)
3				Huachuca Mts. Huachuca Canyon. *Windham & Haufler 639* (DUKE)	49.2 (2.16)
4			Coconino Co.	Elden Mtn. NE of Flagstaff. *Windham 396B* (ASC)	44.0 (2.69)
5			Gila Co.	Mazatzal Mts., Barnhardt Canyon. *Windham & Windham 441* (DUKE)	47.7 (2.38)
6			Graham Co.	near Arcadia Campground. *Keil* et al. *10098* (ASU)[a]	46.8 (2.31)
7			Pima Co.	Santa Catalina Mts., Molino Basin. *Windham & Windham 439* (DUKE)	48.2 (2.00)
8				Windy Point. *Windham & Yatskievych 778* (DUKE)	47.4 (2.11)
9				Bear Canyon. *Windham & Yatskievych 780* (DUKE)	45.4 (2.06)
10			Yavapai Co.	Dry Creek Canyon N of Sedona. *Windham & Czech 597* (DUKE)	46.2 (2.79)
11				Long Canyon. *Morefield & Windham 1371* (ASC)	48.5 (2.92)
12				Forests S of Prescott. *Clark s.n.* (UNM)[a]	47.9 (2.71)
13		Colorado	Baca Co.	Picture Canyon SW of Campo. *Howard s.n.* (COLO)[a]	43.7 (1.76)
14		Oklahoma	Cimarron Co.	E of Kenton, *Windham & Yatskievych 802* (DUKE)	45.1 (2.08)
15		New Mexico	Hidalgo Co.	Peloncillo Mts., Clanton Canyon, *Castetter 9946* (UNM)[a]	47.8 (2.05)
16			Lincoln Co.	Three Rivers Canyon, *Hutchins 2754* (UNM)[a]	45.3 (1.99)
17		Texas	Brewster Co.	Chisos Mts., *Windham & Yatskievych 754* (DUKE)	48.4 (2.24)
18			Burnet Co.	Inks Lake, *Windham & Yatskievych 733* (UT 114360)	48.3 (2.10)
19			Jeff Davis Co.	Davis Mts. Resort, *Worthington 10* (COLO)[a]	44.6 (1.80)
***Pellaea* × *wagneri*, sterile triploid, *n* = 29II + 29I**					
—	USA	Arizona	Cochise Co.	Dragoon Mts., *Windham & Haufler 631* (DUKE)	*
—			Coconino Co.	Elden Mtn. NE of Flagstaff. *Windham 464* (DUKE)	*

TABLE 1 (Continued)

Ref no.	Country	State	Collection locality and voucher information		Mean spore length, µm (SD)
—			Gila Co.	Mazatzal Mts. Barnhardt Canyon. *Windham & Windham 442* (DUKE)	*
—			Pima Co.	Santa Catalina Mts., Molino Basin. *Windham & Windham 440* (DUKE)	*
—				Santa Catalina Mts., Bear Canyon. *Windham & Yatskievych 779* (DUKE)	*
—			Yavapai Co.	Black Canyon S of Bumble Bee. *Windham & Windham 859* (DUKE)	*
—				Dry Creek Canyon N of Sedona. *Windham & Windham 434, 436* (DUKE, both)	*
—				Long Canyon NW of Sedona. *Morefield & Windham 1374* (ASC)	*
—				Secret Canyon NNW of Sedona. *Windham & Lyngholm 425* (DUKE)	*
—		New Mexico	Dona Ana Co.	W slope of the Organ Mts. *Dunn 8527* (UNM)[b]	*
—			Sierra Co.	along road W of Hillsboro. *Windham & Haufler 609* (DUKE)	*
***Pellaea* × *gooddingii*, sterile tetraploid, 2*n* = 116**					
--	USA	Arizona	Cochise Co.	Dragoon Mts., Sheepshead Pass. *Windham & Haufler 630* (DUKE)	*
--				Huachuca Mts., Huachuca Canyon. *Windham & Haufler 640* (DUKE)	*
--		Texas	Brewster Co.	Chisos Mts., *Windham & Yatskievych 753* (DUKE)	*
***Pellaea ternifolia* subsp.*"ternifolia 1"* (2*x*), *n* = 29**					
20	MEXICO	Chihuahua	Mpio. de Guachochic, Cascada de Cusarare. *Bye & Weber 8176* (COLO)[a]		40.0 (1.82)
21		Durango	Along Mex. Rte. 40 W of Cd. Durango. *Ranker & Yatskievych 829a* (UT)		43.2 (1.68)
22		Michoacan	Along Mex. Rte. 15 E of Morelia. *Benham & Hevly 1161* (ASC)		42.5 (1.77)
23	USA	Hawaii	Hawaii Volcanoes National Park. *Weber & Bujakiewicz 16507* (COLO)[a]		43.0 (2.08)

(Continues)

TABLE 1 (Continued)

Ref no.	Country	State	Collection locality and voucher information		Mean spore length, μm (SD)
24		Texas	Brewster Co. Chisos Mts. (29.2501 N 103.2947 W), *Windham & Yatskievych 749* (UT 115153)		43.5 (1.79)
***Pellaea ternifolia* subsp. *"ternifolia 2"* (4*x*), *n* = 58**					
25	MEXICO Dist. Fed.		El Pedregal, S of Mexico City. *Windham* et al. *573* (DUKE) 46.0 (1.99)		
***Pellaea ternifolia* subsp. *arizonica* (4*x*), *n* = 58**					
26	USA	Arizona	Cochise Co.	Dragoon Mts. (31.8719 N 109.9825 W), *Windham 246* (ASC)	50.8 (2.23)
27				Huachuca Mts., Huachuca Canyon. Windham 303 (ASC)	47.6 (2.44)
***Pellaea truncata* (2*x*), *n* = 29**					
28	USA	Arizona	Cochise Co.	Mule Mts., Box Canyon. *Yatskievych 84-196* (DUKE)	38.9 (2.13)
29				Baldy region NE of Pomerene. *Goodding 114-54* (ARIZ)[a]	39.5 (2.04)
30			Coconino Co.	Oak Creek Canyon N of Sedona. *Windham & Windham 435* (DUKE)	37.3 (1.84)
31			Gila Co.	Mazatzal Mts., Barnhardt Canyon. *Windham & Lyngholm 722* (DUKE)	38.8 (2.02)
32			Graham Co.	Swift Trail W of US 666. *Lehto* et al. *10253* (ASU)[a]	39.7 (1.58)
33			Maricopa Co.	Apache Trail SW of Canyon Lake. *Knobloch 1796* (MSC)[a]	36.2 (2.14)
34			Mohave Co.	Along Interstate 40 near Willow Creek. *Windham 720* (DUKE)	41.3 (2.06)
35			Pima Co.	Santa Catalina Mts., Molino Basin. *Windham 438* (DUKE)	40.0 (2.07)
36			Pinal Co.	Along US 60 E of Superior. *Lehto 18109* (ASU)[a]	39.9 (1.89)
37			Santa Cruz Co.	Pajarito Mts. *Windham & Lyngholm 437* (DUKE)	40.7 (2.63)
38			Yavapai Co.	Black Canyon S of Bumble Bee. *Windham 203* (ASC)	36.5 (2.13)
39				Dry Creek Canyon N of Sedona. *Windham & Czech 598* (DUKE)	40.2 (3.08)
40		Colorado	Fremont Co.	Phantom Canyon. *Howard 1982* (COLO)[a]	38.0 (1.83)
41		Nevada	Clark Co.	W slope of Newberry Mts. *Nickell 529* (UNLV)[a]	38.8 (1.73)
42		New Mexico	Bernalillo Co.	Sandia Mts. *Windham & Windham 22* (ASC)	40.1 (2.11)
43			Grant Co.	W of Mule Creek. *Martin 5146* (UNM)[a]	37.7 (1.81)

TABLE 1 (Continued)

Ref no.	Country	State	Collection locality and voucher information	Mean spore length, µm (SD)
44			Hidalgo Co. Guadalupe Canyon E of Douglas. *Hess 1796* (NMC)[a]	39.6 (1.93)
45			along US 80 in Peloncillo Mts. *Castetter 4300* (UNM)[a]	37.4 (2.20)
46			Luna Co. S peak of Tres Hermanas Mts. *Clark 10711* (UNM)[a]	39.3 (1.95)
47		Texas	El Paso Co. Franklin Mts. *Johnston s.n.* (MSC)[a]	39.7 (2.59)
48			Hudspeth Co. N end of Quitman Mts. *Windham & Yatskievych 763* (DUKE)	38.5 (2.19)
49		Utah	Washington Co. W of Santa Clara. *Cottam 8774* (COLO)[a]	40.8 (1.65)

*Spores predominantly malformed.
[a]Gametophytic counts obtained by germinating spores from herbarium specimen.
[b]Count of $n = 87$ from unreduced gametophyte.

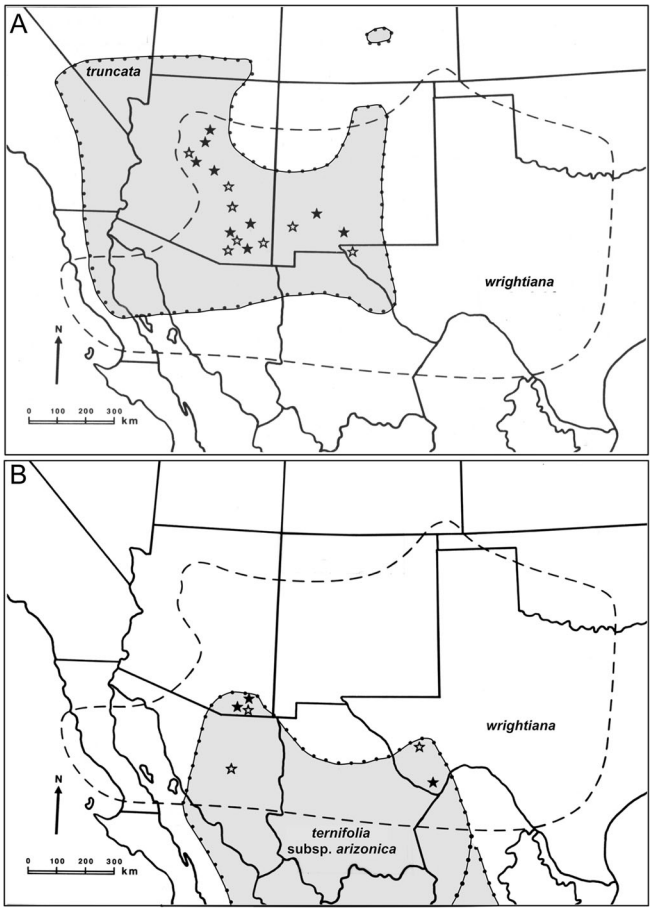

FIGURE 5 (A) Geographic distribution of triploid *Pellaea ×wagneri* samples (stars) relative to the known ranges of allotetraploid *P. wrightiana* (dashed line) and diploid *P. truncata* (connected dots and gray fill). (B) Geographic distribution of tetraploid *Pellaea ×gooddingii* samples (stars) compared to the known ranges of allotetraploid *P. wrightiana* (dashed line) and *P. ternifolia* subsp. *arizonica* (connected dots and gray fill). Solid stars represent chromosomal and electrophoretic samples; open stars are collections identified by morphology and malformed spores.

four northernmost states of Mexico, plus Arizona, New Mexico, Colorado, Texas, and Oklahoma (Figure 5). In addition, disjunct populations have been found on granite outcrops in North Carolina (Wagner, 1965; Heafner, 2001) and South Carolina (McMillan et al., 2018). *Pellaea ×wagneri* was represented by specimens from 17 localities in Arizona, New Mexico, and Texas (Figure 5A; stars). All were found within the region where *P. wrightiana* and *P. truncata* were sympatric and, in most cases, both putative parents were present at the collection sites. *Pellaea ×gooddingii* was identified from six localities in Arizona, Texas, and Sonora; it grew in close proximity to both putative parents at all three localities visited by the lead author (Figure 5B; solid stars).

Morphometric analyses

Univariate analyses of plants assigned to *P. ×wagneri* revealed that they were intermediate between *P. wrightiana*

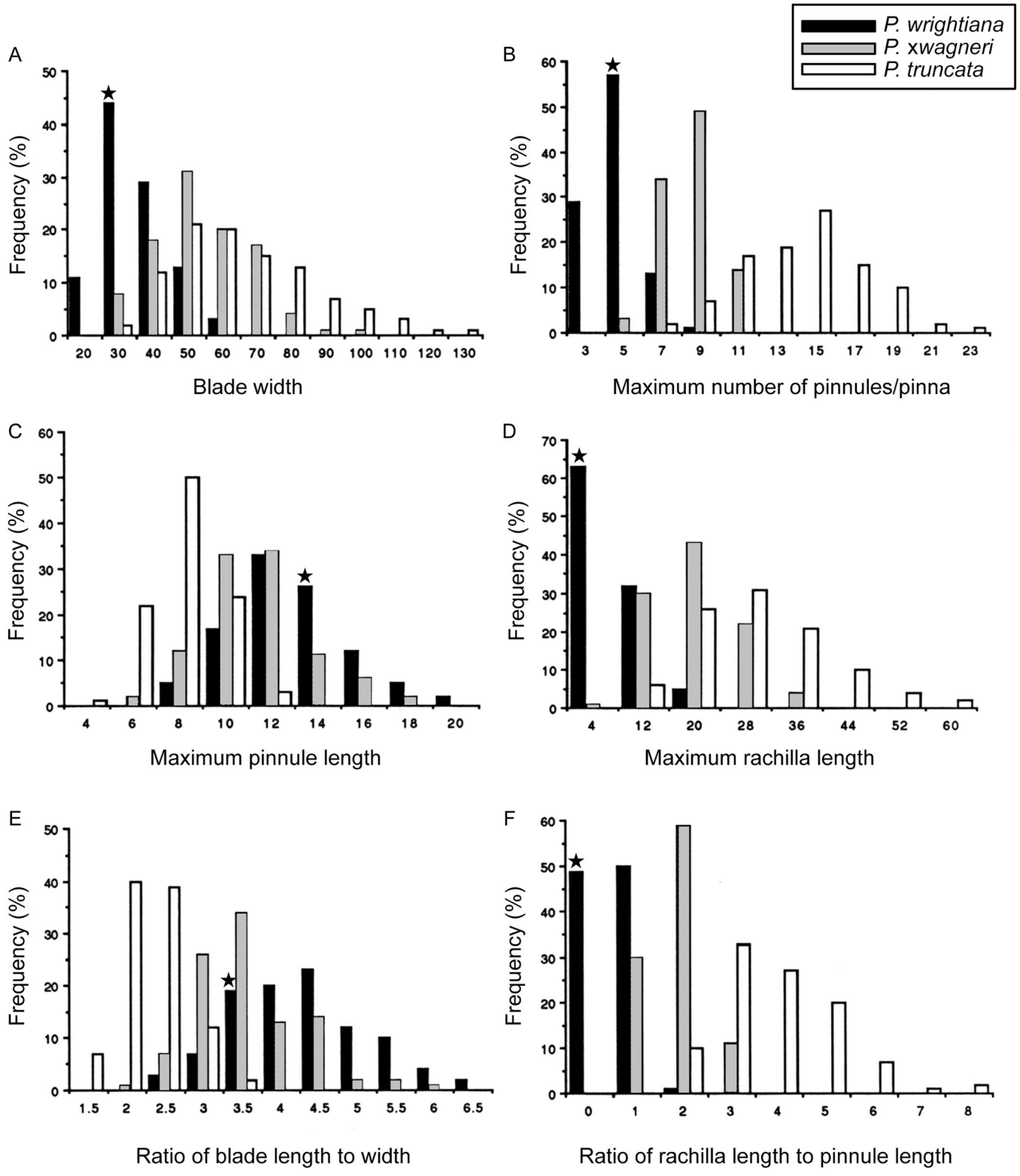

FIGURE 6 Histogram summaries of morphological characters and character ratios investigated in triploid *Pellaea* ×*wagneri* and its inferred parents. Open bars = *P. truncata*; black bars = *P. wrightiana*; gray bars = *P.* ×*wagneri*. Stars indicate values recorded for isotype (US 62535) of *P. wrightiana*.

and *P. truncata* in four of the five characters examined (Figure 6A–D). The only exception was blade length; the ranges and means of the putative parents were nearly identical, but values recorded for the triploid were somewhat higher (data not shown). The character most useful in distinguishing the three taxa was the maximum number of pinnules per pinna (Figure 6B). Seventy-four percent of *P. truncata* specimens and 29% of *P. wrightiana*

plants were identifiable solely on the basis of this character. When maximum pinnule number per pinna was combined with gross spore morphology (i.e., well-formed vs. malformed spores; Figure 4), it was possible to accurately classify more than 90% of the 500 individuals sampled.

Because blade and pinna shape are often employed to differentiate *P. wrightiana* from *P. truncata* (e.g., Tryon, 1957), an attempt was made to quantify these characters using two ratios: blade length/blade width and rachilla length/pinnule length. When displayed in the form of histograms (Figure 6E, F), these ratios showed the same pattern of triploid intermediacy observed in the univariate plots. In both cases, the ratios provided better resolution of the three taxa than did the original characters. In fact, the rachilla/pinnule ratio (Figure 6F) showed even greater discriminatory power than maximum pinnule number (Figure 6B). Fifty-seven percent of *P. truncata* specimens and 49% of *P. wrightiana* plants could be identified using only this ratio. When the rachilla/pinnule ratio was combined with maximum pinnule number and gross spore morphology, *P. truncata*, *P. wrightiana,* and *P. ×wagneri* could be distinguished with 100% accuracy.

Plants identified as *P. ×gooddingii* were intermediate between *P. wrightiana* and *P. ternifolia* subsp. *arizonica* in five of the nine characters included in the multivariate analysis (Table 2, nos. 3, 4, 7–9). *Pellaea ×gooddingii* showed mean values somewhat higher than either putative parent for petiole length, blade length, number of pinna pairs per leaf, and maximum distance between pinnae (Table 2, nos. 1, 2, 5, 6). Judging from the covariance matrix of the discriminant analysis (see below), these four characters were strongly correlated and associated with increased leaf size in *P. ×gooddingii.* An examination of the percentage overlap data indicated that maximum pinnule length provided the best single character for distinguishing the three taxa (Table 2, no. 8). Twenty-three percent of *P. ternifolia* subsp. *arizonica* specimens and 45% of *P. wrightiana* individuals could be identified using only this character. When maximum pinnule length was combined with gross spore morphology, it was possible to accurately classify 72% of the 100 individuals sampled.

To determine whether the three tetraploid taxa could be more clearly resolved through a combination of variables, the data for nine quantitative characters were included in a discriminant function analysis (Table 3, Figure 7). Four variables satisfied Bonferroni's criterion of significance and were entered into the discriminant model: maximum pinnule length, blade length, maximum pinnule number, and distance from the base of the blade to the widest point (Table 2, nos. 2, 4, 7, 8). The first canonical axis (CA 1) accounted for 84.1% (Table 3) of the total variance and separated most individuals of *P. ternifolia* subsp. *arizonica* from *P. wrightiana* (Figure 7). Character loadings on CA 1 indicated that the most important characters distinguishing these groups were maximum pinnule length, blade length, and position of the widest point on the blade (Table 3). *Pellaea wrightiana* and *P. ×gooddingii* were distinguished on the second canonical axis, which explained all remaining variance (15.9%; Table 3). Character loadings on CA 2 (Table 3) indicated that blade length and maximum pinnule number were the most useful macromorphological characters for separating *P. wrightiana* and *P. ×gooddingii*. The minimal overlap between these groups (Figure 7) may have resulted from the inclusion of several greenhouse-grown leaves of *P. ×gooddingii* that were much smaller than those collected in nature. Although the discriminant analysis indicated that *P. ×gooddingii* bears a stronger resemblance to *P. wrightiana* than to *P. ternifolia* subsp. *arizonica,* it was intermediate between these taxa with respect to both canonical axes (Figure 7). The discriminant model produced a jackknifed classification matrix that correctly classified 88% of the individuals sampled. All misclassifications involved a transposition of fertile and sterile collections; specimens of *P. wrightiana* were never misidentified as *P. ternifolia* subsp. *arizonica* and vice versa. Thus, when the discriminant model was combined with gross spore morphology (facilitating the recognition of sterile individuals), *P. ternifolia* subsp. *arizonica*, *P. wrightiana*, and *P. ×gooddingii* could be distinguished with 100% accuracy.

Enzyme electrophoresis

Of the 20 putative gene loci examined during this study, 12 showed consistent activity and interpretable banding patterns for all taxa and individuals. These were SkDH, IDH, 6PGDH-1, MDH-1, MDH-3, HK, AAT, PGI-2, TPI-2, LAP, PGM-1, and PGM-2. At most of these loci, plants identified as *P. wrightiana* showed evidence of multiple banded patterns and fixed heterozygosity characteristic of allopolyploid plants. Band patterns were largely symmetrical (balanced) in fertile *P. wrightiana* s.s. but distinctly asymmetrical in *P. ×wagneri* and *P. ×gooddingii* due to the unequal representation of alleles (gene dosage effects).

To evaluate Wagner's (1965) hypothesis on the origin of *P. wrightiana* s.s., band patterns in this taxon were compared to those observed in *P. truncata* and diploid *P. ternifolia* subsp. *ternifolia* from the southwestern United States and northern Mexico. This survey revealed additive isozyme expression at all 12 clearly resolved loci. For example, in the monomeric enzyme HK (Figure 8A), a slow band from *P. truncata* was combined with a fast band from diploid *P. ternifolia* to form the two-banded phenotype ubiquitous in *P. wrightiana.* The fixed three-banded AAT electromorph observed in plants of *P. wrightiana* (Figure 8B, lanes 3, 4, 9, 10) included a slow band derived from *P. truncata*, a fast band derived from *P. ternifolia,* and a novel heterodimer produced by combining parental subunits to form a functional molecule of intermediate mobility. The pattern observed at MDH-3 (Figure 8C) was similar except that the relative mobility of the parental allozymes was reversed. Our broad survey of *P. wrightiana* populations detected two widespread electromorphs at the monomeric SkDH locus (Figure 8D, lanes 3 and 4 vs. 9 and

TABLE 2 Descriptive statistics for quantitative characters used in the multivariate analysis of the three tetraploid taxa. Sample sizes: $N = 22$ for *P. ternifolia* subsp. *arizonica*; $N = 31$ for *P.* ×*gooddingii*; $N = 47$ for fertile *P. wrightiana*. Measurements are in mm. Percent overlap based on number of individuals falling within observed limits of other taxa

Character	Taxon	Mean, mm (SD)	Observed limits	% Overlap
1. Petiole length	*P. ternifolia* subsp. *arizonica*	78.7 (35.82)	35–149	100
	P. ×*gooddingii*	92.6 (32.17)	48–184	90
	P. wrightiana	71.0 (27.76)	34–145	98
2. Blade length	*P. ternifolia* subsp. *arizonica*	150.5 (58.52)	86–357	95
	P. ×*gooddingii*	219.6 (51.74)	121–301	100
	P. wrightiana	135.2 (40.06)	65–251	91
3. Maximum blade width	*P. ternifolia* subsp. *arizonica*	54.9 (12.10)	29–82	91
	P. ×*gooddingii*	47.6 (9.16)	29–63	100
	P. wrightiana	35.1 (8.88)	18–66	72
4. Distance from base of blade to widest point	*P. ternifolia* subsp. *arizonica*	78.0 (23.08)	29–123	100
	P. ×*gooddingii*	70.4 (28.41)	16–127	97
	P. wrightiana	27.9 (22.72)	6–87	53
5. Number of pinna pairs per leaf	*P. ternifolia* subsp. *arizonica*	7.6 (2.99)	4–17	73
	P. ×*gooddingii*	13.3 (3.29)	8–21	94
	P. wrightiana	10.3 (2.55)	6–16	100
6. Maximum distance between pinnae	*P. ternifolia* subsp. *arizonica*	23.5 (6.01)	14–33	100
	P. × *gooddingii*	28.5 (6.36)	18–42	77
	P. wrightiana	20.6 (4.98)	11–31	91
7. Maximum number of pinnules per pinna	*P. ternifolia* subsp. *arizonica*	3.0 (0.00)	3	100
	P. ×*gooddingii*	3.4 (0.71)	3-5	100
	P. wrightiana	4.6 (1.17)	3–7	89
8. Maximum pinnule length	*P. ternifolia* subsp. *arizonica*	25.5 (6.22)	13–42	77
	P. ×*gooddingii*	21.8 (4.15)	13–29	100
	P. wrightiana	12.9 (2.48)	8–18	55
9. Maximum rachilla length	*P. ternifolia* subsp. *arizonica*	1.0 (0.21)	1–2	100
	P. ×*gooddingii*	2.5 (1.41)	1–7	100
	P. wrightiana	5.2 (3.78)	1–19	83

10). Both exhibited two bands of equal intensity and shared a slow-migrating allozyme derived from *P. truncata*. The faster bands comigrated with different allozymes observed in diploid *P. ternifolia* and were likely incorporated into *P. wrightiana* through recurrent hybrid origins.

Isozyme band patterns observed at all 12 loci in triploid *P.* ×*wagneri* were additive expressions of alleles derived from *P. wrightiana* and *P. truncata*. In the dimeric enzyme IDH (Figure 8E), *P. wrightiana* showed a balanced three-banded electromorph in which the heterodimer (middle band) was twice as intense as either homodimer. Plants of *P. truncata* were homozygous for the allele coding the slower-migrating band. Collections of *P.* ×*wagneri* were characterized by an unbalanced three-banded pattern in which the heterodimer and slow homodimer were equally intense, but the fast homodimer was faintly expressed. This pattern approximated the 4:4:1 staining ratio expected in plants carrying two alleles (one from *P. truncata* and one from *P. wrightiana*) coding for the slower allozyme and one (from *P. wrightiana* but ultimately traceable to *P. ternifolia*) coding for the faster allozyme. In the monomeric enzyme SkDH (Figure 8F), *P. wrightiana* consistently showed a balanced, two-banded electromorph. Most sampled plants of *P. truncata* were homozygous for an allele coding the slower-migrating band (Figure 8F, lanes 7, 8), but several were heterozygous for this and a second allele producing a

slightly faster band (Figure 8F, lanes 5, 6). Through recurrent hybridization (i.e., multiple origins), *P.* ×*wagneri* has acquired both of these *P. truncata* alleles, with some individuals expressing a balanced three-banded pattern (Figure 8F, lanes 3, 4), and others a two-banded pattern (Figure 8F, lanes 9, 10) unbalanced in favor of the slow band shared by its inferred parents.

Isozyme band patterns observed at all 12 loci in *P.* ×*gooddingii* also combined allozymes derived from its inferred parents, *P. wrightiana* and *P. ternifolia* subsp. *arizonica*. For example, in the dimeric enzyme AAT (Figure 8G), *P. wrightiana* exhibited a balanced, three-banded electromorph and *P. ternifolia* subsp. *arizonica* was homozygous for the allele coding the faster migrating band. Collections of *P.* ×*gooddingii* were characterized by an asymmetrical, three-banded pattern in which the fast homodimer was very dark, the heterodimer somewhat lighter, and the slow homodimer very faint (Figure 8G, lanes 3, 4, 7, 8). This pattern approximated the 9:6:1 staining ratio expected in a plant carrying three alleles coding for the faster allozyme and one coding for the slower allozyme. In the monomeric enzyme SkDH (Figure 8H), *P.* ×*gooddingii* produced a four-banded pattern, the maximum number of bands a tetraploid can express at a single locus. The fastest and slowest migrating alleles were contributed by *P. wrightiana* (Figure 8H, lanes 1, 2, 9, 10), while those of intermediate mobility were derived from *P. ternifolia* subsp. *arizonica* (Figure 8H, lanes 5, 6).

TABLE 3 Description of canonical axes (CA) from the discriminant function analysis of tetraploid *Pellaea ternifolia* and the fertile and sterile tetraploid "forms" of *P. wrightiana*.

Descriptor	CA 1	CA 2
Eigenvalue	4.945	0.937
Proportion of variance explained (%)	84.1	15.9
Character loadings of variables		
Maximum pinnule length	1.514	0.363
Leaf blade length	−1.184	−1.219
Position of widest point	0.823	0.237
Maximum pinnule number	0.163	0.688

Combined plastid data set phylogenetic analysis

The majority rule consensus topology (Figure 9) generated by the B/MCMC analysis possessed a mean likelihood of −6457.34, and all effective sample sizes (ESSs) were well over 500. Combined analysis of the three plastid markers (*rbcL*, *atpA*, and *trnG-R*) produced a strongly supported tree, with all deep branches exhibiting PP ≥ 0.99 (Figure 9). Designating two of Tryon's (1957) "light-stiped" species (*Pellaea intermedia* and *P. ovata*) as outgroups, the seven "dark-stiped" taxa sampled here form a well-supported monophyletic group, with *P. bridgesii* sister to a clade encompassing the other six. The next branching event separates the *P. ternifolia* lineage (plus one sample of *P.* ×*gooddingii*) from a clade of taxa related to *P. truncata*. In the latter group, *P. brachyptera* is sister to a polytomy comprising (1) all five samples of *P. truncata* (including three confirmed diploids), (2) five documented tetraploid collections of *P. wrightiana*, (3) one triploid specimen of *P.* ×*wagneri*, and (4) one tetraploid sample of *P.* ×*gooddingii* from Texas. The minimal divergence observed among the latter four taxa suggests that diploid *P. truncata* was the original source of the plastomes found in all three polyploid hybrids in this clade.

Pellaea ternifolia, the other hypothesized parent of allotetraploid *P. wrightiana*, is unexpectedly diverse (Figure 9). The first branching event separated *P. ternifolia* subsp. *ternifolia* from a polytomy comprising samples of subsp. *brandegeei*, subsp. *arizonica*, and Arizona *P.* ×*gooddingii*. This latter individual appears to have obtained its plastome from *P. ternifolia* subsp. *arizonica*, supporting a hybrid origin separate from that of the *P.* ×*gooddingii* sample

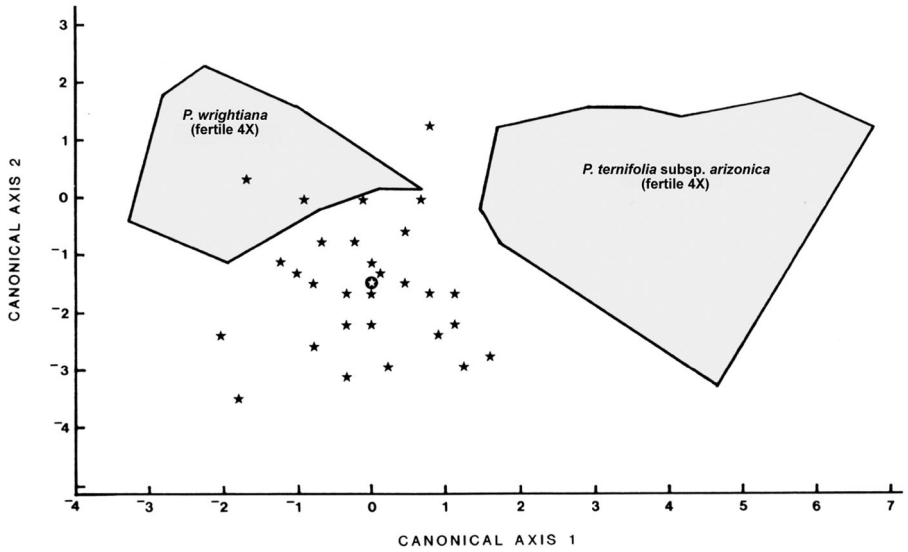

FIGURE 7 Plot of first two canonical axes from a discriminant analysis of *Pellaea* ×*gooddingii* and its inferred parents (allotetraploid *P. wrightiana* and tetraploid *P. ternifolia* subsp. *arizonica*). Outlines circumscribe limits of the two fertile tetraploids. Solid stars identify the locations of discriminant scores for individual plants of *P.* ×*gooddingii* and open circle represents the centroid for this taxon.

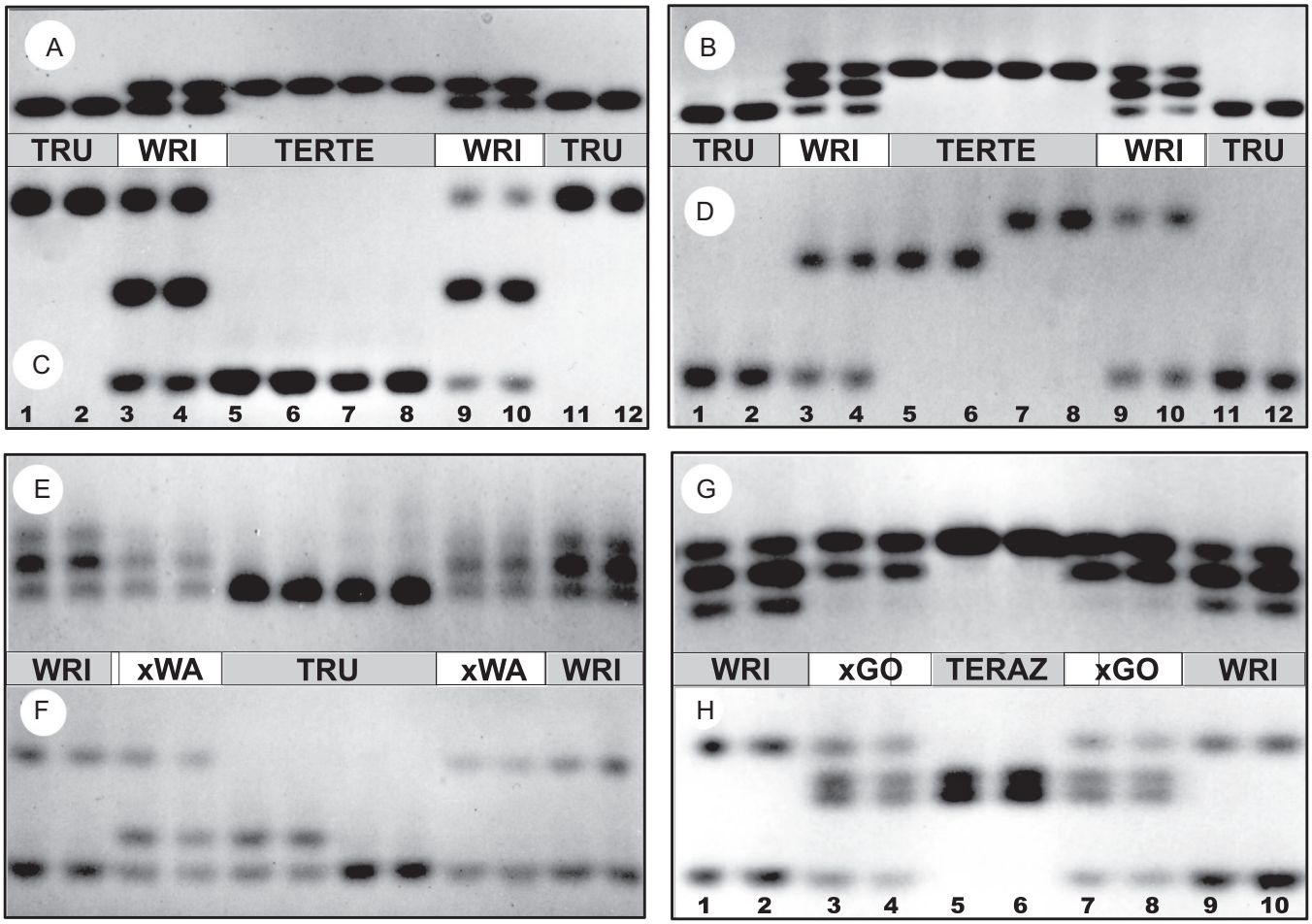

FIGURE 8 Enzyme electrophoresis gel photos showing allozyme additivity and variability in the *Pellaea wrightiana* complex (A–H). Anode is toward the top of each figure. Enzymes are identified by panel letters (see below). In photos documenting the hybrid origin of tetraploid *P. wrightiana* s.s. (A–D), lanes 1, 2, 11, and 12 are diploid *P. truncata*; lanes 3, 4, 9, and 10 are *P. wrightiana*; lanes 5–8 are diploid *P. ternifolia* subsp. *ternifolia*. (A) HK. (B) AAT. (C) MDH-3. (D) SkDH. In photos documenting the hybrid origin of triploid *P. ×wagneri* (E, F), lanes 1, 2, 11, and 12 are *P. wrightiana* s.s.; lanes 3, 4, 9, and 10 represent *P. ×wagneri*; lanes 5–8 are diploid *P. truncata*. (E) IDH. (F) SkDH. In photos documenting the hybrid origin of tetraploid *P. ×gooddingii* (G, H), lanes 1, 2, 9, and 10 are *P. wrightiana* s.s.; lanes 3, 4, 7, and 8 represent *P. ×gooddingii*; lanes 5 and 6 are tetraploid *P. ternifolia* subsp. *arizonica*. (G) AAT. (H) SkDH.

from Texas. The six samples of *P. ternifolia* subsp. *ternifolia* split evenly between two well-supported clades, one of which includes only known or inferred diploids, while the other consists solely of known or inferred tetraploids (Figure 9).

Nuclear gapCp data set phylogenetic analysis

The *gapCp* "short" analysis generated a well-supported phylogeny (Figure 10) with a mean likelihood value of −1684.87 and all ESSs > 500. Using alleles from Tryon's (1957) "light-stiped" species *Pellaea intermedia* as the outgroup, the "dark-stiped" taxa sampled here form a well-supported monophyletic group. The substantial phylogenetic divergence between *P. truncata* and *P. ternifolia* (the proposed parents of the *P. wrightiana* polyploid complex) observed in the plastid tree (Figure 9) is apparent in the nuclear data as well. Samples of all proposed hybrids (*P. wrightiana* s.s., *P. ×wagneri,* and *P. ×gooddingii*) contain alleles from both parental clades (Figure 10). The only sample of *P. ×wagneri* included in the nuclear DNA analysis was indistinguishable from *P. wrightiana* s.s. In this case, we were unable to detect a predicted second allele derived from *P. truncata* due to poor quality of the triploid sample that yielded just two clones.

Cloning was more successful on the only sample of *P. ×gooddingii*, which exhibited four alleles (Figure 10). These are (1) a *truncata* allele nearly identical to that found in a geographically proximate sample (4910) of *P. wrightiana* (i.e., the *truncata* allele derived from the *P. wrightiana* parent of *P. ×gooddingii*), (2) a *ternifolia* allele representing a clade shared by all samples of *P. wrightiana* (i.e., the *ternifolia* allele contributed by the *P. wrightiana* parent of *P. ×gooddingii*, and (3) two additional *ternifolia* alleles nearly identical to those detected in our only sample of *P. ternifolia* subsp. *arizonica* (the other hypothesized parent of *P. ×gooddingii*).

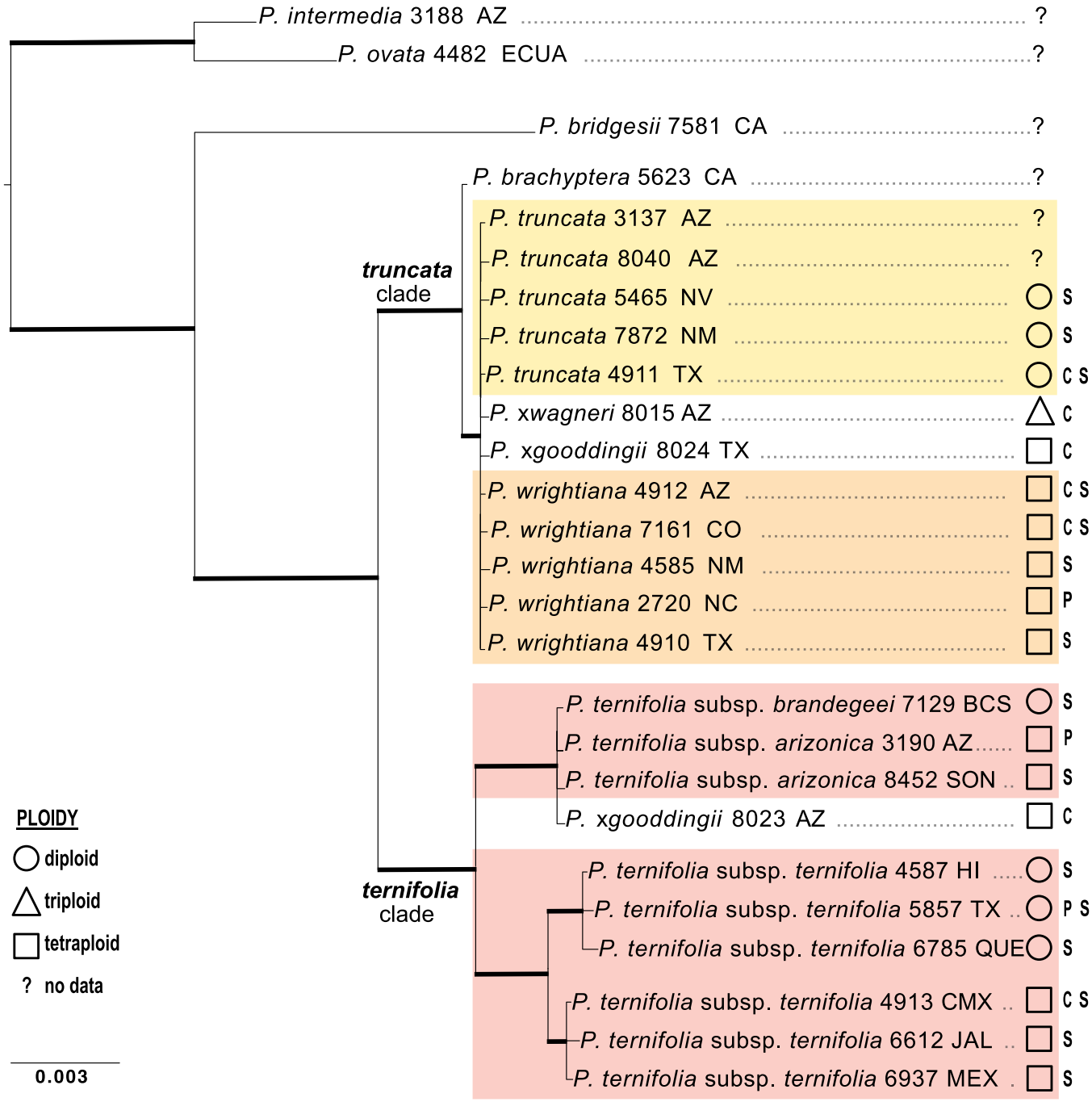

FIGURE 9 Plastid topology of the *Pellaea wrightiana* hybrid complex and close relatives. A majority rule consensus phylogeny was generated using a combined, three-locus (*rbcL*, *atpA*, *trnGR*) plastid data set analyzed by Bayesian Markov chain Monte Carlo (B/MCMC). Deep divergences were well resolved, and all nodes in the phylogeny had a PP ≥ 0.99 (thickened branches). Where available, ploidy level is indicated for each taxon with supporting data from chromosome counts (C = individual, P = population), spore measurements (S), or both. The plastid data support that tetraploid *P. wrightiana* (outlined by orange box) is an allotetraploid hybrid resulting from a cross between diploid *P. truncata* (yellow box) as female progenitor and diploid *P. ternifolia* (pink box) as male progenitor. *Pellaea intermedia* and *P. ovata* were used as outgroups based on Grusz et al. (2021).

DISCUSSION

The absence of hyperdiverse structures comparable to flowers and seeds means that ferns have far fewer morphological features suitable for distinguishing close relatives (Paris et al., 1989). Because of this, ferns usually require evidence from a broad array of techniques before their species boundaries and relationships are fully apparent (Dauphin et al., 2017, 2018). Thus, obtaining crucial biodiversity data for ferns usually requires the integration of next-generation molecular data with more traditional techniques. Among the latter, chromosome studies are

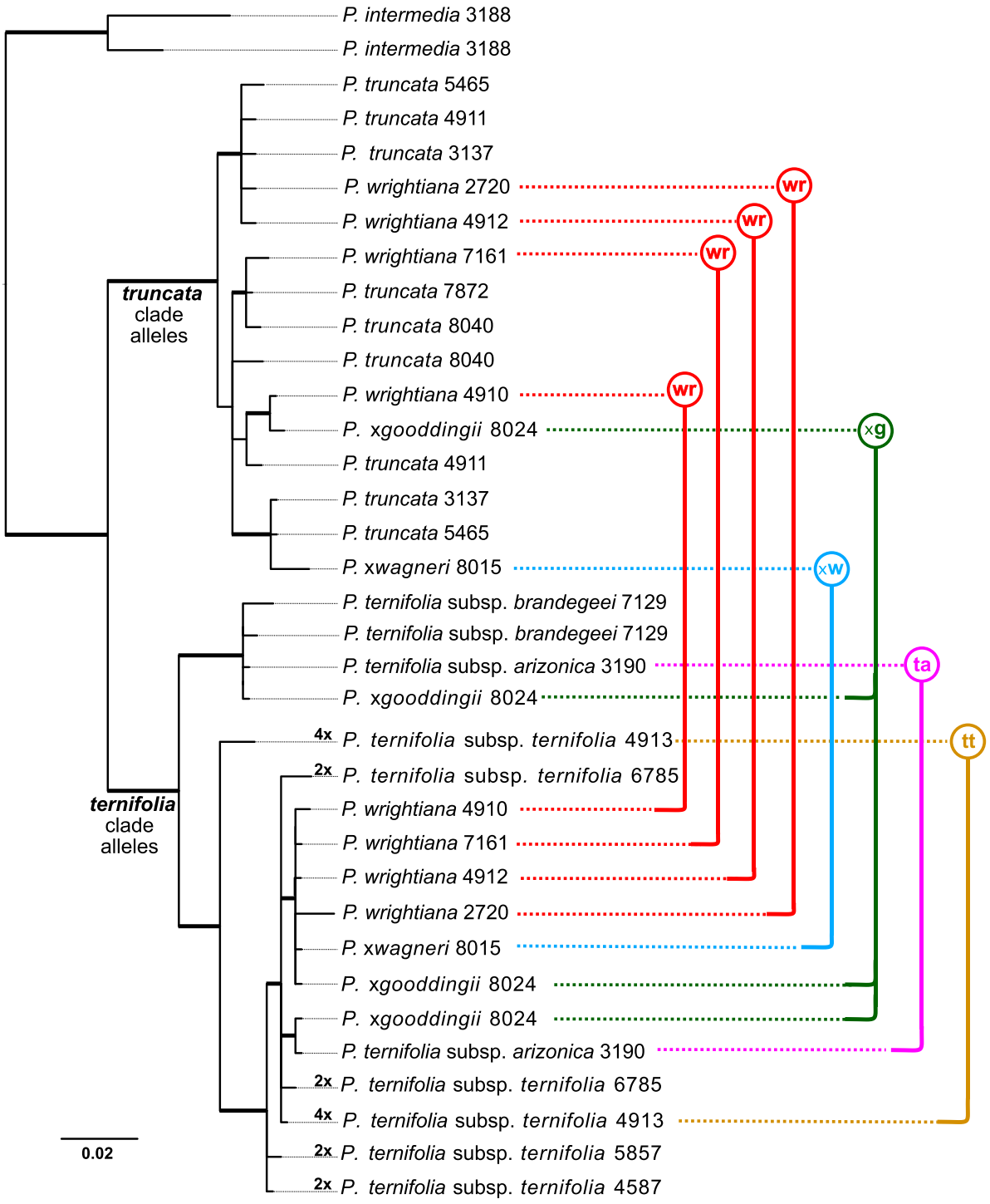

FIGURE 10 Nuclear *gapCp* "short" topology of the *Pellaea wrightiana* hybrid complex and close relatives recovered from a Bayesian Markov chain Monte Carlo (B/MCMC) analysis. Most nodes were well supported (PP ≥ 0.95; thickened branches) with the exception of two nodes in the *truncata* clade. Four individual samples of *P. wrightiana* each possess one allele associated with the *P. truncata* clade and another from the *P. ternifolia* lineage (tracked on the right-hand side of the phylogeny by red lines). The same is true for the only sample of *P.* ×*wagneri* (blue lines). The tetraploid hybrid *P.* ×*gooddingii* exhibited four distinct alleles, as expected (green lines). Tetraploid *P. ternifolia* subsp. *arizonica* carries alleles from both diploid subsp. *brandegeei* and *ternifolia* (pink lines). Tetraploid subsp. *ternifolia* contains two divergent alleles (tan lines) placing diploid (2*x*) and tetraploid (4*x*) collections of subsp. *ternifolia* in distinct clades. *Pellaea intermedia* alleles were used as outgroups.

especially important (Windham et al., 2020) because they provide the means to calibrate flow cytometry and spore measurement data commonly used to sort taxa in heteroploid species groups. The *Pellaea wrightiana* hybrid complex provides a textbook example of this. In the last 60 years, the number of recognized or hypothesized taxa in the group has more than doubled, increasing from four (Figure 1A, B) to nine in the current study (Figure 11). This

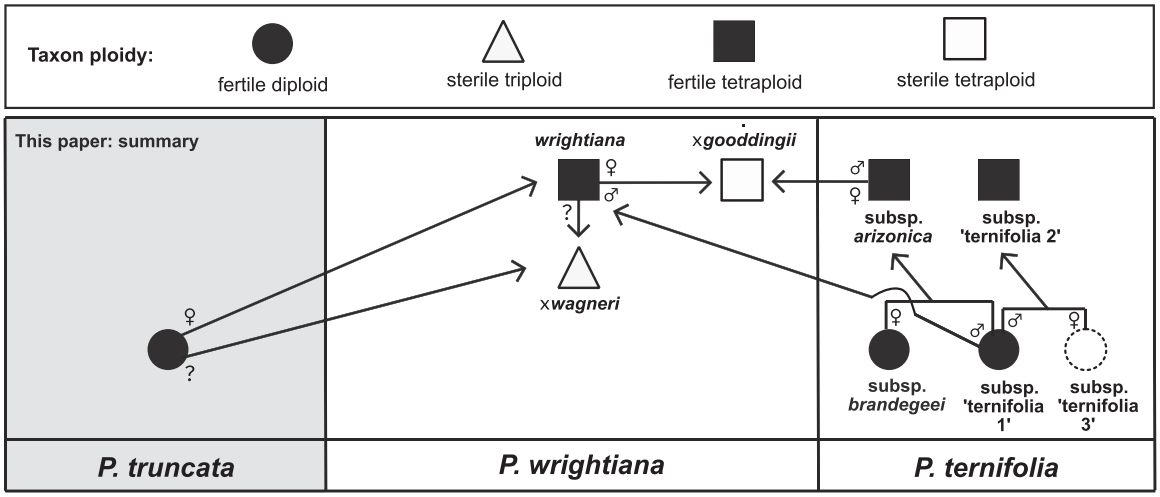

FIGURE 11 Summary of resolved relationships for the *Pellaea wrightiana* hybrid complex based on data from this study. Arrows point from parents to offspring. Circles, triangles, and squares depict ploidy level of taxon (diploid, triploid, and tetraploid, respectively). Largely sterile triploid and tetraploid hybrid taxa are drawn as an open triangle and open square, respectively. Open dotted circle depicts unnamed inferred diploid subspecies of *P. ternifolia*. Where known, the sex of the contributing parent is shown as either ♀, ♂, or ♂/♀; ? = gender of gamete unknown.

complex now encompasses (1) three known fertile diploids, (2) three known fertile tetraploids, (3) two largely sterile hybrids (one triploid, one tetraploid), and (4) one hypothesized fertile diploid. Based on current information, all of the fertile diploids are phylogenetically divergent (Figures 9 and 10), and all fertile tetraploids (including supposed autopolyploids) show strict bivalent formation in meiosis and appear to be genetically isolated from their progenitor diploids and each other. A summary of what we now know about the *P. wrightiana* hybrid complex and its progenitors is provided below.

Pellaea wrightiana s.s.

Our analyses confirm the existence of three distinct polyploid taxa (one fertile, two largely sterile) among plants traditionally referred to *Pellaea wrightiana*. Spore analyses of the type specimens of *P. wrightiana* at K (holotype) and US (isotype) indicate that Hooker's name is unequivocally associated with the fertile tetraploid (Figures 3 and 4). Leaf morphology strongly supports this conclusion as well. In all three measures providing the greatest discriminatory power (i.e., maximum number of pinnules per pinna, maximum rachilla length, and rachilla/pinnule ratio), the US isotype exhibits values that are exclusive or nearly exclusive to the fertile tetraploid (Figure 6). Thus, Knobloch and Britton's (1963) hypothesis that *P. wrightiana* is equivalent to the mostly sterile triploid, herein called *P.* ×*wagneri* (Figure 1), can be confidently rejected. Our chromosomal analyses confirm Wagner's (1965) hypothesis that *P. wrightiana* s.s. is a fertile tetraploid. Nineteen geographically representative samples of *P. wrightiana* s.s. were analyzed cytogenetically (Table 1), and all were established to be tetraploid. The strong correlation between ploidy and spore size in this group (Figures 2–4) allowed us to infer the ploidy of additional collections (e.g., the type specimens and DNA vouchers) that could not be analyzed chromosomally. As a result, we can say with confidence that *P. wrightiana* s.s. is uniformly tetraploid and very likely to be an allopolyploid (i.e., there was no evidence of a cryptic diploid progenitor within *P. wrightiana*).

Resolving the evolutionary origin of tetraploid *P. wrightiana* s.s. is crucial to understanding relationships within the complex because it was evidently involved in the formation of both sterile taxa. Whereas Tryon (1957) stated that the taxon she treated as *P. ternifolia* var. *wrightiana* descended from *P. ternifolia* s.s. through gradual divergence, Wagner (1965) hypothesized it was an amphidiploid hybrid between *P. truncata* (as *P. longimucronata*) and *P. ternifolia* (compare Figure 1A, C). Wagner supported his argument by demonstrating that *P. wrightiana* was morphologically intermediate between the two aforementioned diploid species, and plants of *P. wrightiana* formed 58 bivalents during meiosis, as would be expected in an allotetraploid hybrid between two divergent *Pellaea* species. However, it should be noted that the chromosome pairing data do not provide unequivocal support for Wagner's hypothesis. Some ferns widely accepted as autopolyploids show normal bivalent formation (Bouharmont, 1972a, 1972b; Lovis, 1977), including tetraploid *P. ternifolia* subsp. *ternifolia* (Figure 2G). Chromosome pairing is governed both by homology and the action of regulatory genes (such as the well-known *Ph1* locus in wheat), and genic control of pairing is common in plants (see Jackson and Casey, 1980; Jenczewski and Alix, 2004; Soares et al., 2021 and references therein). These mechanisms of pairing control often go undetected until they fail to function properly, as they often do in hybrids between genetically incompatible systems. Plants of *Pellaea* ×*gooddingii* (=*P. wrightiana* × *P. ternifolia* subsp. *arizonica*) provide an intriguing example of this. Although both putative tetraploid

parents of this hybrid produced only bivalents, the sterile tetraploid showed a significant number of trivalent associations (Figure 2C and D). This observation suggests that intergenomic homology in one (or both) is suppressed through some form of genic pairing control.

By contrast, the enzyme electrophoretic data are unequivocal regarding the origin of *P. wrightiana* s.s. All 12 enzyme loci showed band patterns combining allozymes from *P. truncata* and *P. ternifolia* (Figure 8A–D), and progeny analyses indicated that these heterozygous electromorphs did not segregate during meiosis. Such high levels of fixed heterozygosity in a polyploid are maintained through strict disomic inheritance (see Soltis and Rieseberg, 1986) and are often considered an indicator of amphidiploidy (Crawford, 1985; Bryan and Soltis, 1987). Thus, the enzyme electrophoretic study strongly supports the hypothesis (previously based primarily on morphological data) that *P. wrightiana* s.s. originated through hybridization between diploid populations of *P. truncata* and *P. ternifolia* subsp. *ternifolia*. Enzyme analyses further indicate that existing populations of *P. wrightiana* s.s. are the products of recurrent hybridization rather than a single origin event. More than half the loci studied exhibited two or more alleles traceable to a single parental taxon. For example, different populations of *P. wrightiana* s.s. contain different SkDH alleles derived from *P. ternifolia* (Figure 8D). The most likely explanation for this is multiple hybrid origins. However, if only two alleles are involved (as is the case with SkDH in *P. wrightiana* s.s.), it is possible (though improbable) that such allelic variation could have arisen from a single hybridization event in which both parental gametophytes were unreduced (Grusz et al., 2021). In the case of *P. wrightiana* s.s., the occurrence of three or more alleles traceable to *P. truncata* (as seen in the enzyme loci IDH, PGI-2, and PGM-1; Windham, 1988) is strong evidence of multiple hybrid origins.

DNA sequence data bring a new level of resolution to our understanding of *P. wrightiana* s.s. The plastid tree reveals that the parental species of this allotetraploid (*P. truncata* and *P. ternifolia*) are relatively divergent, nonsister taxa (Figure 9). The five samples of *P. truncata* have nearly identical sequences at the three chloroplast loci, and they are part of a polytomy that encompasses all samples of *P. wrightiana*, *P. ×wagneri*, and *P. ×gooddingii* as well. Because plastids have been shown to be maternally inherited in *Pellaea* (Gastony and Yatskievych, 1992), we infer that all samples of *P. wrightiana* s.s. included in the chloroplast tree descend from hybridization events in which the *P. truncata* gametophyte was female and the *P. ternifolia* gametophyte was male (Figure 11).

Results from nuclear DNA analyses provide additional support for Wagner's (1965) hypothesis regarding the origin of *P. wrightiana* s.s. Each of the four samples of this taxon possess one allele associated with the *P. truncata* clade and another from the *P. ternifolia* lineage (Figure 10). All *ternifolia* alleles recovered from *P. wrightiana* s.s. form a well-supported clade with alleles from a west Texas plant of *P. ternifolia* subsp. *ternifolia* (6785), which coincidentally represents an isolated diploid population closest to the range of *P. truncata*. A geographically isolated population of *P. wrightiana* s.s. from North Carolina exhibits the most divergent *ternifolia* allele, but it remains unclear whether this signals a distinct hybridization event or post-origin divergence. The *truncata* alleles recovered from *P. wrightiana* s.s. are more informative in this regard, representing three well-supported clades (Figure 10). As in the case of the enzyme data, it is *P. truncata* (the highly outcrossed diploid with greater allelic variability in the probable region of origin; Windham, 1988) that provides the best evidence of recurrent hybrid origins.

Pellaea ×wagneri

The mostly sterile triploid plants included in our study were morphologically intermediate between *P. wrightiana* s.s. and *P. truncata* in all quantitative characters shown in Figure 6. The only non-intermediate character was blade length, reflecting the fact that *P. ×wagneri* produced larger leaves than either inferred parent. Increases in leaf size are among the most common expressions of heterosis, and they may result from complementary enhancement of mitochondrial respiration in hybrid individuals (Grant, 1975). Characters showing strong heterotic effects are unusually sensitive to changes in the internal genetic environment and thus do not present a serious challenge to hypotheses of relationship based on more stable morphological traits.

The results of cytogenetic and electrophoretic analyses of *P. ×wagneri* are fully concordant with the evidence from morphology. Sporocytes of the triploid invariability show *Drosera*-type chromosome pairing (Grant, 1981) with 29 bivalents and 29 univalents, suggesting the presence of two homologous genomes and one nonhomologous genome. Given the ploidy and parentage of *P. wrightiana* s.s., a hybrid with *P. truncata* would be triploid with two sets of chromosomes from *P. truncata* and one from *P. ternifolia*. However, this pattern of chromosome association would also be expected in a triploid containing two genomes from *P. ternifolia* and one from *P. truncata*. This genetic interpretation was, in fact, advanced by Knobloch and Britton (1963) when the triploid was first discovered. Morphological and geographical data accumulated since that time indicate that the widespread triploid is unlikely to contain two genomes of *P. ternifolia*, but enzyme electrophoresis provides a conclusive test. The *truncata* and *ternifolia* genomes are characterized by distinct marker alleles at most enzyme loci (Figure 8A–D), and gene dosages in the triploid can be used to determine which diploid genome is more strongly represented. In nearly every individual displaying alleles of known origin, gene dosage was 2:1 in favor of the *P. truncata* contribution (Figure 8E, F).

Although congruent with the more extensive isozyme data, our DNA analyses of *P. ×wagneri* provide no

additional resolution. Whereas the allozymes yield clear evidence of multiple hybrid origins (Figure 8F), the single sample of *P.* ×*wagneri* included in the DNA study is insufficient to address this topic. And though the nuclear DNA data indicate that this plant (like *P. wrightiana* s.s.) contains alleles from both *P. truncata* and *P. ternifolia* (Figure 10), the recovery of just two clones (Appendix 2) does not allow us to confirm the 2:1 gene dosage clearly evident in the isozyme data (Figure 8E, F). We are also unable to determine which taxon functioned as the female parent and which was the male (Figure 11) given that all analyzed samples of the two inferred parents (*P. truncata* and *P. wrightiana* s.s.) exhibited a plastome derived from *P. truncata* (Figure 9). Further study of *P.* ×*wagneri*, which is relatively common in Arizona and New Mexico (Figure 5A), is clearly warranted.

Pellaea ×*gooddingii*

These largely sterile tetraploids were intermediate between *P. wrightiana* s.s. and *P. ternifolia* subsp. *arizonica* for five of the nine characters included in the multivariate analysis (Figure 7). Chromosome squashes provide little additional information due to meiotic irregularities (Figure 2C, D) that complicate a thorough cytogenetic analysis. While the available chromosome data are congruent with the hypothesis that *P.* ×*gooddingii* is a hybrid between *P. wrightiana* s.s. and *P. ternifolia* ssp. *arizonica*, they are not sufficient to rule out the alternative that *P.* ×*gooddingii* is simply a meiotically unstable tetraploid form of *P. ternifolia*. Once again, however, the genomic markers provided by electrophoresis resolve the situation. If the proposed parentage of *P.* ×*gooddingii* is correct (Figure 1D), each enzyme locus should show three alleles derived from the *P. ternifolia* clade and one attributable to *P. truncata*. The electromorphs observed in plants of *P.* ×*gooddingii* exactly fit the predicted pattern (Figure 8G, H) at all but one locus. The only exception involves the AAT locus, which occasionally exhibits four *ternifolia* alleles in *P.* ×*gooddingii*. However, these exceptions actually strengthen the hybrid hypothesis while simultaneously providing evidence of multiple origins. The *P.* ×*gooddingii* plants with four *ternifolia* alleles co-occur with a lineage of *P. wrightiana* s.s. that has lost its *P. truncata* allele through a homoeologous chromosome pairing event (Windham, 1988). These genotypes grow intermixed with "normal" *P. wrightiana* s.s, and "normal" *P.* ×*gooddingii* in the Dragoon Mountains of Arizona. Thus, the diverse but consistently additive enzyme electromorphs of *P.* ×*gooddingii* support the hypothesis that this taxon arose through repeated hybridization between *P. wrightiana* and *P. ternifolia* subsp. *arizonica*.

Our plastid DNA tree (Figure 9) provides additional evidence that *P.* ×*gooddingii* has arisen multiple times. The two samples included in this analysis obtained their plastids from different sources; the Texas plant (8024) exhibits a *truncata* clade plastid attributable to its *P. wrightiana* s.s. parent, whereas the Arizona plant (8023) contains a *ternifolia* clade plastid derived from its *P. ternifolia* subsp. *arizonica* parent (Figure 9). Thus, based on what we know about plastid inheritance in *Pellaea* (Gastony and Yatskievych, 1992), *P.* ×*gooddingii* joins the growing list of polyploid ferns (Stein and Barrington, 1990; Haufler et al., 1995a; Xiang et al., 2000; Sigel et al., 2014; Testo et al., 2015; Jorgensen and Barrington, 2017; Sessa et al., 2018; Kao et al., 2019; Suissa et al., 2022) showing clear evidence of reciprocal hybrid origins (Figure 11).

Pellaea ternifolia

This species was included in our analyses chiefly to assess its role in the origins of the three polyploids included within *P. wrightiana* s.l. We did not investigate the substantial morphological variability present within *P. ternifolia*, and we were fortunate that our limited cytogenetic, electrophoretic, and DNA sampling of this taxon was sufficient to address our original research questions. Despite the small sample sizes, our studies reveal considerable genetic diversity within *P. ternifolia* s.l. (Figures 9, 10) and support the recognition of all previously named North American taxa (Figure 11). Our only sample of subsp. *brandegeei* is diploid based on spore measurements (Figure 4, ref. no. 51) and is phylogenetically divergent in both the plastid (Figure 9) and nuclear (Figure 10) trees from the other documented diploids assigned to subsp. *ternifolia*. The only sample of tetraploid subsp. *arizonica* included in the *gapCp* analysis contains alleles derived from both recognized diploids. On the basis of previously documented patterns of plastid inheritance in *Pellaea* (Gastony and Yatskievych, 1992), we infer that both samples of subsp. *arizonica* in the plastid tree descend from hybridization events in which the subsp. *brandegeei* gametophyte was female and the subsp. *ternifolia* gametophyte was male (Figure 11).

Although Tryon (1957, 1968) and Tryon and Britton (1958) hypothesized that tetraploid plants of *P. ternifolia* were autopolyploids, we found no evidence of strict autopolyploidy within our limited sampling of this species. The tetraploid attributed to subsp. *ternifolia* (4913) in the cytogenetic and nuclear DNA analyses displays only bivalents at diakinesis (Figure 2G) and contains two divergent *gapCp* alleles (Figure 10). These observations help explain the plastid data (Figure 9), which separate diploid and tetraploid collections of subsp. *ternifolia* into two distinct clades. Our tetraploid sample from central Mexico (designated subsp. "*ternifolia 2*" in Figure 11) apparently arose through hybridization between a male gametophyte of diploid subsp. "*ternifolia 1*" and a female gametophyte from a morphologically cryptic, as yet undiscovered diploid (subsp. "*ternifolia 3*"). Future resolution of the taxonomy of *P. ternifolia* will require extensive sampling from South American populations, the source of the type collection (whose ploidy is unknown) and the region with the greatest morphological diversity (Tryon, 1957).

Assessing the potential for gene flow and introgression

All three polyploid taxa in the *Pellaea wrightiana* hybrid complex are identifiable using a combination of morphological characters (Table 4). Although this observation suggests that gene flow among the taxa is limited, other data must be considered. Rieseberg et al. (1988) found that studies of introgression relying solely on morphology were misleading, and they recommended the use of precise genetic markers to examine the movement of genes from one taxon to another. Our electrophoretic analyses of *Pellaea* indicate that genetic identities are low, and species are distinguished by a large number of marker alleles. Thus, enzyme loci visualized through electrophoresis can provide the resolution necessary to track gene migration.

Most studies of introgression have examined gene movement among members of homoploid complexes (e.g., Bloom, 1976; Davis, 1985; Rieseberg, 1991; Rieseberg and Ellstrand, 1993; Linder and Rieseberg, 2004; Nieto Feliner et al., 2017). Problems arise when two or more ploidy levels are involved, and it becomes necessary to recognize two types of intertaxon gene flow: (1) introgression, involving the permanent movement of genes into parental taxa via hybrid intermediates, and (2) genetic enrichment of hybrid derivatives through recurrent hybridization (Soltis and Soltis, 1999). Our electrophoretic data indicate that the gene pools of all three polyploids in the *P. wrightiana* complex have been enriched by recurrent hybridization. However, the genetic markers also indicate that gene movement has been entirely unidirectional —from parents to hybrids. *Pellaea truncata* and *P. ternifolia* have contributed a substantial proportion of their genetic variability to fertile *P. wrightiana*. Despite the existence of backcross individuals (the triploids and sterile tetraploids of this study), there is no evidence that a single gene has moved from one parental diploid to the other. Gene migration from *P. ternifolia* and *P. truncata* to fertile *P. wrightiana* via hybrid intermediates also appears to be nonexistent. Rates of interpopulational gene flow are very low in *P. wrightiana s.s.* (Windham, 1988), so the appearance of new genes in hybrid populations would be easily detected. However, populations located hundreds of kilometers from hybrid zones are genetically indistinguishable from those in direct contact with other species. These data suggest that introgression has not played a significant role in the recent evolution of this polyploid complex. Gene flow barriers imposed by ploidy level and hybrid sterility appear to be very robust, facilitating the recognition of three distinct taxa.

Taxonomic treatment

As previously defined, *Pellaea wrightiana* comprised a polyploid complex including three genetically distinct taxa. The holotype at Kew and an isotype at the U.S. National Herbarium (US 62535) were morphologically most similar to plants identified here as fertile tetraploids (Figure 6). More importantly, both produced normally developed spores averaging 47.6 µm in length, which is very close to the grand mean of the fertile tetraploid (47.0 µm). Thus, the epithet *wrightiana* is securely associated with the fertile allotetraploid hybrid between *P. truncata* and *P. ternifolia.* As pointed out by Wagner (1965), it is inappropriate to treat such a hybrid as a variety of either parent, and *Pellaea wrightiana* should be accorded species rank. A survey of the literature and selected type specimens reveals that neither of the largely sterile hybrids has received formal nomenclatural recognition. Because these taxa (especially the triploid) are

TABLE 4 Summary of morphological characters most useful for distinguishing the three taxa formerly included in *Pellaea wrightiana*. (Mean values are in parentheses.)

Characters	*P. ×gooddingii*	*P. wrightiana*	*P. ×wagneri*	Illustrations
Maximum number of pinnules per pinna (mm)	2-(3)-5	3-(5)-9	6-(9)-11	Figure 13A–C
Maximum rachilla length (mm)	1-(3)-7	1-(5)-19	7-(18)-37	Figure 13A–C
Maximum pinnule length (mm)	13-(22)-29	8-(13)-19	7-(11)-17	Figure 13A–C
Ratio of rachilla length to pinnule length	0.04-(0.14)-0.30	0.1-(0.6)-1.5	0.9-(1.8)-3.2	Figure 13A–C
Shape of rachis/petiole in cross section	Adaxially slightly flattened or terete	Adaxially flattened or grooved	Adaxially flattened or grooved	Figure 13D–F
Rachis/petiole color	Purplish-brown	Dark brown	Brown to dark brown	Figure 13D–F
Farina-producing glands in sorus	Absent	Sparse	Common	Figure 13G–I
Average width of recurved pinnule margin (mm)	1.1	0.8	0.5	Figure 13G–I
Pseudoindusium margin	Denticulate	Entire to crenulate	Entire to crenulate	Figure 13J–L
Spores	Mostly malformed and variable in size	Well-formed and ± uniform in size	Mostly malformed and variable in size	Figure 3A–E, 13M–O

commonly encountered in the southwestern United States, we propose the following hybrid binomials.

(1) *Pellaea* ×*gooddingii* Windham, hyb. nov. TYPE: United States, Arizona, Cochise Co., WSW of Sierra Vista in the Huachuca Mts. on slope above Huachuca Canyon Road ca. 2.7 road miles SW of military housing area at Fort Huachuca. Lat./Long.: 31.5106 N 110.3902 W (WGS84 Datum); on moss-covered quartzite ledges near base of N-facing cliff in evergreen oak-pine woodland, elev. 6000 ft, 04 Aug 1984, *M. D. Windham and C. H. Haufler 640* (DUKE; isotypes MO, NY, US) (Figure 12A).

A tetraploid hybrid morphologically intermediate between its parents, *Pellaea wrightiana* and *P. ternifolia* subsp. *arizonica*, and distinguished from both by the production of mostly malformed spores; further differentiated from *P. wrightiana* by having rachises and petioles purplish brown and slightly flattened adaxially or terete (vs. dark brown and grooved or flattened adaxially, margins of the pseudoindusia denticulate (vs. entire to crenulate), longer pinnules (the largest averaging 22 mm vs. 13 mm), and mostly entire pinnae on the distal third of the blade; further distinguished from *P. ternifolia* s.l. by having the terminal lobe of the largest pinnae separated from adjacent lobes by a short, brownish stalk that renders the pinna fully pinnate (vs. deeply ternately lobed in *P. ternifolia*).

The epithet honors Mr. Leslie N. Goodding (1880–1967), an avid student of southwestern ferns who was apparently the first to collect this hybrid and suggest that it might be an undescribed taxon.

(2) *Pellaea* ×*wagneri* Windham, hyb. nov. TYPE: United States, Arizona, Yavapai Co., NNW of Sedona along Secret Canyon just N of confluence with Dry Creek. Lat./Long.: 34.9306 N 111.8074 W (WGS84 Datum); In sandy alluvial soil among sedimentary and basalt rocks on gentle WSW-facing slope in mixed chaparral/oak-juniper woodland, elev. 4700 ft, 6 Nov 1982, *M. D. Windham and D. Lyngholm 425* (DUKE; isotypes MO, NY, US) (Figure 12B).

A triploid hybrid morphologically intermediate between its parents, *Pellaea wrightiana* and *P. truncata,* and distinguished from both by the production of mostly malformed spores;

FIGURE 12 Holotypes of new hybrid taxa described here. (A) Tetraploid *Pellaea* ×*gooddingii*. (B) Triploid *P.* ×*wagneri.*

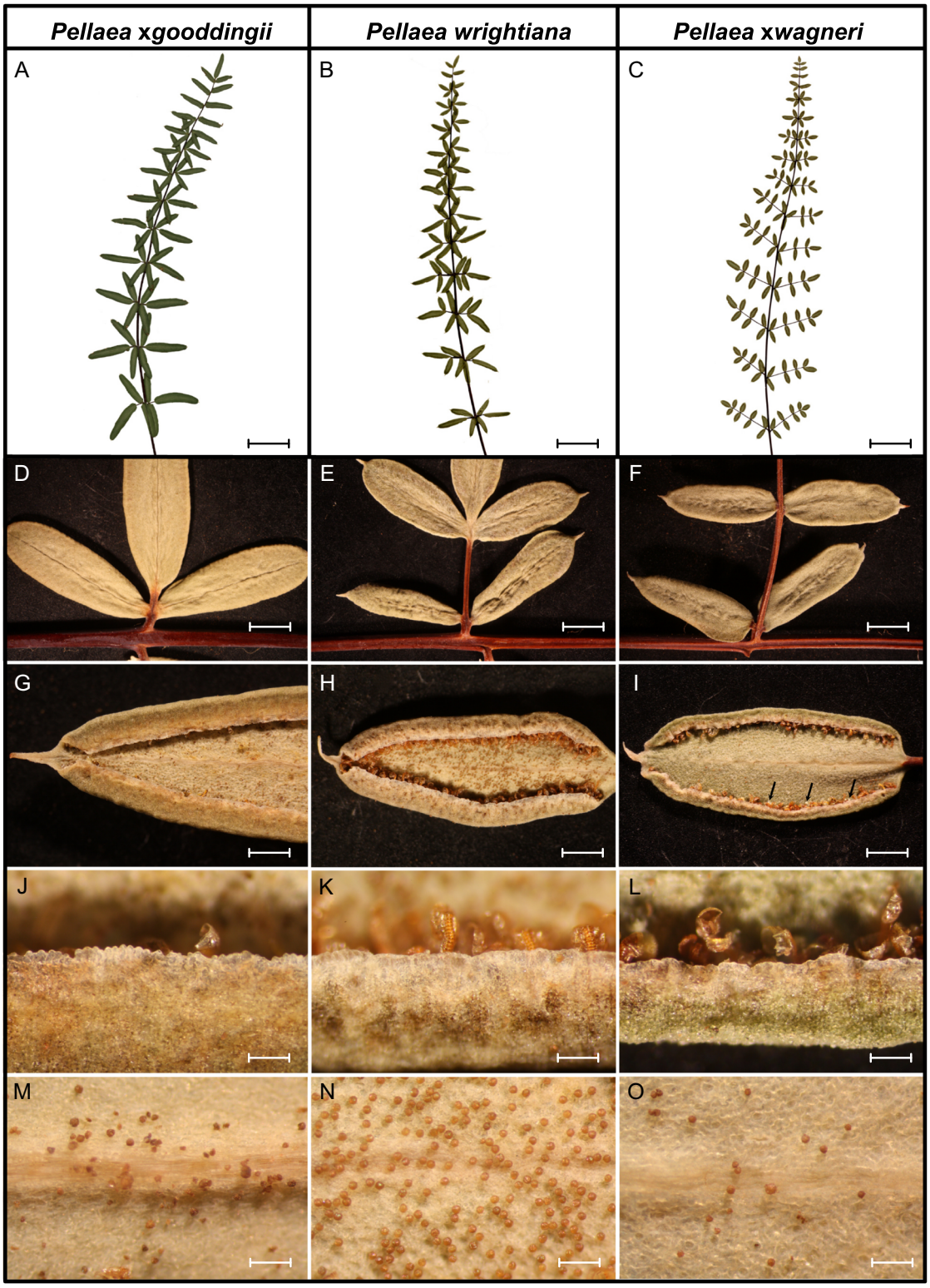

FIGURE 13 Morphological features distinguishing *Pellaea ×gooddingii, P. wrightiana*, and *P. ×wagneri* at low to medium (25×) magnification. (A–C) Representative leaf blades (those of *P. ×gooddingii* and *P. ×wagneri* from the respective holotypes); scale bars = 20 mm. (D–F) Adaxial view of lower rachis and basal pinna; scale bars = 3 mm. (G–I) Abaxial surface of distal portion of terminal pinnule of basal pinna; scale bars = 1.5 mm; arrows on I point to farina-producing paraphyses. (J–L) Pseudoindusium formed by recurved pinnule margin; scale bars = 0.25 mm. (M–O) Dispersed spores on abaxial pinnule surface; scale bars = 0.25 mm.

further differentiated from *P. wrightiana* in having farina-producing glands common among the sporangia, more pinnules on the largest pinnae (averaging 9 vs. 5), and longer rachillae (the largest averaging 18 mm vs. 5 mm); further distinguished from *P. truncata* by having fewer pinnules on the largest pinnae (averaging 9 vs. 15), and lower rachilla length/pinnule length ratios (<2.5 vs. >2.5).

The epithet honors Dr. Warren H. Wagner, Jr. (1920–2000), who correctly surmised the parentage of this hybrid and whose careful analysis of *P. wrightiana* laid the groundwork for this study.

The morphological characters most useful for separating *P. wrightiana* s.s., *P.* ×*gooddingii*, and *P.* ×*wagneri* are listed in Table 4 and illustrated in Figure 13. Though most of these are self-explanatory, two require additional commentary. With regard to the presence of farina-producing glands in the sorus, these are visible as tufts of cream-colored powder scattered among the sporangia (Figure 13I). This feature is shared by all diploid members of the *P. truncata* clade (Figure 9; see Grusz et al., 2021 for broader sampling). Among the polyploids of the *P. wrightiana* hybrid complex, these tufts are common only in triploid *P.* ×*wagneri*, which contains two genomes from a farinose progenitor (*P. truncata*) and one from a nonfarinose species (*P. ternifolia*). Such tufts of farina are sparse in *P. wrightiana* s.s. (with a 2:2 ratio of these parental genomes) and apparently absent in *P.* ×*gooddingii* (3:1 ratio in favor of the nonfarinose parent). For identifying polyploids in the *P. wrightiana* complex, the observation of multiple farina tufts is diagnostic for *P.* ×*wagneri*, though their absence is not definitive because the farina can be melted by excessive heat in drying (M. Windham, personal observations). Another character (average width of recurved pinnule margin; Table 4, Figure 13G–I) can be useful if the pinnules are symmetrically flattened with the edges of the pseudoindusia clearly visible. However, this character can be misleading if either of these conditions is not met.

AUTHOR CONTRIBUTIONS

This study was conceived of and organized by M.D.W., with project development by C.H.H and K.M.P. Samples were gathered by M.D.W., G.Y., and T.A.R. Data collection and analyses were conducted by M.D.W., L.H., J.S.M., and T.A.R. All authors discussed the results and contributed to the final manuscript.

ACKNOWLEDGMENTS

Much of this work was completed in partial fulfillment of a doctoral dissertation in Botany in 1988 at the University of Kansas by M.D.W. under the direction of C.H.H. Research support to M.D.W. from various sources at the University of Kansas and to C.H.H. from NSF (grants #8717499 and #91067634) is gratefully acknowledged. Funding for the molecular sequencing was provided by NSF-DEB 0717398 to M.D.W. and K.M.P and NSF-DEB 0717430 to G.Y. We thank the curators of the following herbaria for providing specimen loans and permission to remove spores and small fragments of leaf tissue for microscopic examination, germination studies, and/or for DNA analysis: ARIZ, ASC, ASU, COLO, DUKE, GH, JEPS, MICH, MNA, MO, MSC, NMC, NY, PTBG, UC, UNLV, UNM, US, UTC and UTEP. Thanks also to the staff of the University of Kansas greenhouses, who nurtured the many live plants included in this study. We are grateful to Dr. Derick Poindexter and Eric Rabe for help with various aspects of microphotography and to the reviewers and Associate Editor for their useful comments on the manuscript. This work is dedicated to Donavon Lyngholm, a treasured friend who financially supported M.D.W.'s early career change from archaeology to botany.

DATA AVAILABILITY STATEMENT

DNA sequences used in this study are deposited in GenBank (Appendix 2).

ORCID

Michael D. Windham http://orcid.org/0000-0002-1216-3101
Jordan S. Metzgar https://orcid.org/0000-0002-4641-9929
George Yatskievych https://orcid.org/0000-0002-2314-7852
Kathleen M. Pryer https://orcid.org/0000-0002-9776-6736

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How to cite this article: Windham, M. D., L. Huiet, J. S. Metzgar, T. A. Ranker, G. Yatskievych, C. H. Haufler, and K. M. Pryer. 2022. Once more unto the breach, dear friends: resolving the origins and relationships of the *Pellaea wrightiana* hybrid complex. *American Journal of Botany* 109(5): 1–30. https://doi.org/10.1002/ajb2.1850

APPENDIX 1: Spore measurement data for type specimens of *Pellaea truncata* and *P. wrightiana*. Taxon, collection data, collector (voucher location), mean spore diameter (sd; sample no. in Figure 4)

***Pellaea truncata*:** USA; Arizona, Cochise Co., Mule Mts., in dry rocky place, August 1911, *Goodding 977* (isotype: US 692689), 38.4 µm (2.30; 61). ***Pellaea wrightiana*:** USA; New Mexico,1851-1852, *Wright 2130* (isotype: US 62535), 46.6 µm (1.98; 62).

APPENDIX 2: Sample information for specimens included in molecular phylogenetic analyses. Taxon, collection data, *collector* (voucher location), pryer lab fern database # (see http://www.pryerlab.net/DNA_database.shtml), mean spore diameter in µm (s.d.; sample # in Figure 4), *rbcl* GenBank accession #, *atpa* GenBank accession #, *trngr* GenBank accession #; *gapcp* GenBank accession #(s) (number of *gapcp* clones analyzed), in that order

***Pellaea brachyptera*:** USA; California, Trinity Co., W Fork of Smoky Creek, *Oswald 8751* (JEPS), DB5623, —(—), MW057278, MW057302, MW057326; —(—). ***Pellaea bridgesii*:** USA; California, Fresno Co., E side of Dinkey Creek *Alexander 1025* (DUKE), DB7581, —(—), MW057291, MW057315, MW057339; —(—). ***Pellaea intermedia*:** USA; Arizona, Cochise Co., Dragoon Mts., *Schuettpelz* et al. *481* (DUKE), DB3188, —(—), EF452163, EU268765, EU268713; OM942800/OM942801 (2). ***Pellaea ovata*:** Ecuador; Reserva Geobotanica Pululahua, *Schuettpelz & Sundue 996* (DUKE), DB4482, —(—), OM914498, OM938538, OM914505; —(—). ***Pellaea ternifolia*subsp.*arizonica*:** MEXICO; Sonora, Sierra de Mazatan, *Reina G.* et al. *2005-218* (MO), DB8452, 47.4 µm (2.96; 50), —, —, OM914519; —(—). USA; Arizona, Cochise Co., Dragoon Mts., *Schuettpelz* et al. *483* (DUKE), DB3190, —, (same population as 26), OM914491, OM938539, OM914512; OM942818/OM942826 (2). ***Pellaea ternifolia*subsp.*brandegeei*:** MEXICO; Baja California Sur, Sierra la Laguna, *O'Kane & Anderson 3564* (MO), DB7129, 39.8 µm (1.99; 51), OM914490, —, OM914507; OM942817/OM942820 (2). ***Pellaea ternifolia*subsp.*ternifolia*:** MEXICO; Distrito Federal, El Pedregal, *Windham* et al. *573* (DUKE), DB4913, 46.0 µm (1.99; 25), OM914492, OM938541, OM914514; OM942821/OM942829 (2). Queretaro, Cerro El Zamorano, *Rothfels* et al. *3449* (DUKE), DB6785, 42.0 µm (3.00; 52), OM914489, —, OM914516; OM942822/OM942828 (2). Jalisco, 3.7 km W of Sayulapa, *Rothfels* et al. *3449* (DUKE), DB6612, 46.2 µm (3.25; 53), OM914497, —, —; —(—). Estado de Mexico, near Lagunas de Zempoala, *Beck* et al. *1227* (DUKE), DB6937, 46.3 µm (3.01; 54), OM914496, —, OM914518; —(—). USA; Hawaii, Kauai, *Wood 10960* (PTBG), DB4587, 41.5 µm (3.00; 55), OM914486, OM938540, OM914513; OM942825 (1). Texas, Brewster Co., Chisos Mts., *Windham* et al. *3548* (DUKE), DB5857, 36.3 µm (1.60; 56), OM914485, OM938542, OM914515; OM942824 (1). ***Pellaea truncata*:** USA; Arizona, Cochise Co., Dragoon Mts., *Windham & Yatskievych 777* (DUKE), DB8040, —, (—), —, —, OM914522; OM942810/OM942816 (2). Yavapai Co., Fay Canyon, *Schuettpelz* et al. *430* (DUKE), DB3137, —(—), EF452164, EU268766, EU268714; OM942806/OM942807 (2). Nevada, Clark Co., Christmas Tree Pass area, *Rothfels* et al. *2657* (DUKE), DB5465, 37.4 µm (1.11; 57), —, —, OM914520; OM942802/OM942808 (2). New Mexico, Otero Co., Jarilla Mts., *Worthington 35524* (DUKE), DB7872, 40.3 µm (1.47; 58), —, —, OM914521; OM942815 (1). Texas, Hudspeth Co., Quitman Mts., *Windham & Yatskievych 763* (DUKE), DB4911, 38.5 µm (2.19; 48), OM914500, OM938543, OM938537; OM942803/OM942813 (2). ***Pellaea wrightiana*:** USA; Arizona, Pima Co., Santa Catalina Mts., *Windham & Yatskievych 780* (DUKE), DB4912, 44.1 µm (2.69; 9), MW057290, MW057314, MW057338; OM942805/OM942831 (2). Colorado, Baca Co., SW of Campo, *Howard s.n.* (COLO), DB7161, 43.7 µm (1.76; 13), OM914504, —, OM914531; OM942814/OM942832 (2). New Mexico, Otero Co., Hueco Mts., *Worthington* 34632 (DUKE), DB4585, 45.5 µm (2.34; 59), —, —, OM914528; — (—). North Carolina, Alexander Co., Little Joe Mtn., *Christenhusz 3822* (DUKE), DB2720, — (—), OM914501, —, OM914526; OM942804/OM942823 (2). Texas, Brewster Co., Chisos Mts., *Windham & Yatskievych 746* (DUKE), DB4910, 46.0 µm (2.00; 60), OM914502, OM938544, OM914529; OM942811/OM942830 (2). ***Pellaea* ×*gooddingii*:** USA; Arizona, Cochise Co., Dragoon Mts., *Windham & Haufler 630* (DUKE), DB8023, —(—), —, —, OM914510; —(—). Texas, Brewster Co., Chisos Mts., *Windham & Yatskievych 753* (DUKE), DB8024, (—), —, —, OM914511; OM942812/OM942819/OM942827/OM942833 (4). ***Pellaea* ×*wagneri*:** USA; Arizona, Cochise Co., Dragoon Mts., *Windham & Haufler 631* (DUKE), DB8015, —(—), —, —, OM914525; OM942809/OM942834 (2).