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THE COMPLEX EVOLUTIONARY DYNAMICS OF ANCIENT AND RECENT POLYPLOIDY IN *LEUCAENA* (LEGUMINOSAE; MIMOSOIDEAE)[1]

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- *Premise of the study:* The evolutionary history of *Leucaena* has been impacted by polyploidy, hybridization, and divergent allopatric species diversification, suggesting that this is an ideal group to investigate the evolutionary tempo of polyploidy and the complexities of reticulation and divergence in plant diversification.
- *Methods:* Parsimony- and ML-based phylogenetic approaches were applied to 105 accessions sequenced for six sequence characterized amplified region-based nuclear encoded loci, nrDNA ITS, and four cpDNA regions. Hypotheses for the origin of tetraploid species were inferred using results derived from a novel species tree and established gene tree methods and from data on genome sizes and geographic distributions.
- *Results:* The combination of comprehensively sampled multilocus DNA sequence data sets and a novel methodology provide strong resolution and support for the origins of all five tetraploid species. A minimum of four allopolyploidization events are required to explain the origins of these species. The origin(s) of one tetraploid pair (*L. involucrata/L. pallida*) can be equally explained by two unique allopolyploidizations or a single event followed by divergent speciation.
- *Conclusions:* Alongside other recent findings, a comprehensive picture of the complex evolutionary dynamics of polyploidy in *Leucaena* is emerging that includes paleotetraploidization, diploidization of the last common ancestor to *Leucaena*, allopatric divergence among diploids, and recent allopolyploid origins for tetraploid species likely associated with human translocation of seed. These results provide insights into the role of divergence and reticulation in a well-characterized angiosperm lineage and into traits of diploid parents and derived tetraploids (particularly self-compatibility and year-round flowering) favoring the formation and establishment of novel tetraploids combinations.

Key words: allopolyploidy; diploidization; human translocation; hybridization; Leguminosae; *Leucaena*; paleopolyploidy; plant; "spene" tree.

Mechanisms of polyploid formation and the overall significance of genome duplication in the evolutionary history of flowering plants have been debated and investigated for nearly 100 years. Recent studies applying new molecular tools have revealed many of the genomic consequences of polyploidy (e.g., Wendel, 2000; Adams and Wendel, 2005; Gaeta et al., 2007) and identified paleopolyploid genomes or genomic segments in most, if not all, angiosperms (e.g., De Bodt et al., 2005; Cui et al., 2006; Soltis et al., 2009; Jiao et al., 2011), demonstrating beyond doubt that genome duplication has played a central role in plant diversification. Whether duplicated genomes are derived through hybridization of divergent genomes (allopolyploidy) or from members of the same population (autopolyploidy), polyploidy can increase allelic diversity, alter genomic complexity, and introduce novel traits (e.g., Osborn et al., 2003; Doyle et al., 2008; Grover and Wendel, 2010). Paleopolyploidization events have also been shown to pre-date and potentially trigger diversification of particular clades (e.g., Cui et al., 2006; Barker et al., 2009; Joly et al., 2009; Jiao et al., 2011), and neopolyploidization, now considered common (Wood et al., 2009), has even generated new species right before our eyes (e.g., Abbott and Lowe, 2004; Soltis et al., 2004). Despite this growing body of knowledge informing our understanding of polyploidy, there are few studies that have managed to track and disentangle potentially complex evolutionary histories involving divergence, reticulation, and polyploidy across all extant species within a lineage. For plant genera with more than a handful of species, there are few for which documentation of taxonomic (species) diversity, chromosome numbers, and genome sizes is complete, for which species boundaries have been tested via dense sampling of intraspecific diversity, for which there is a well-resolved, robustly supported, and completely sampled (all species and multiple

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accessions of all species) diploid phylogeny, and for which there are similarly high quality gene trees that provide evidence about polyploid origins. Detailed studies of a handful of genera such as *Brassica* (Song et al., 1995; Pires et al., 2004), *Gossypium* (Wendel, 2003), *Glycine* (Doyle et al., 2004; Gill et al., 2009; Egan and Doyle, 2010), *Nicotiana* (Lim et al., 2007; Leitch et al., 2008; Clarkson et al., 2010), *Primula* (Guggisberg et al., 2009), *Senecio* (e.g., Abbott and Lowe, 2004), *Spartina* (Ainouche et al., 2004), and *Tragopogon* (Mavrodiev et al., 2008), represent important exemplars. However, even for some of these well-studied groups, incomplete sampling and poorly resolved gene trees remain significant hurdles that limit our understanding of polyploid-diploid evolutionary cycles that characterize the flowering plants. As a result many questions await investigation (e.g., Doyle et al., 2008; Soltis et al., 2010): what are the overall contributions of divergence and reticulation in diversification of the angiosperms? What is the evolutionary tempo of polyploid-diploid cycles and diploidization processes? Do some diploids show greater proclivity to form polyploids than others? What are the consequences of polyploidy and traits that favor the formation and ultimate establishment of polyploids? Our aim in this study has been to assemble as complete a picture of polyploidy for the legume genus *Leucaena* as possible based on thorough investigation of well-resolved and robustly supported plastid and nuclear gene trees and taking into consideration variation in chromosome number, genome size, the geography of species, and the archaeological record.

The genus *Leucaena* comprises 19 diploid (sensu Govindarajulu et al., 2011 in this issue) and five tetraploid species of small to medium-sized trees primarily native to seasonally dry tropical forests and adjacent biomes, from the southern United States, Mexico, Central America, and northern South America (Hughes, 1998a). Seeds of several species have been used as a source of human food in south-central Mexico for at least 6000 years (e.g., MacNeish, 1958; Zárate, 2000). The evolutionary history of *Leucaena* has been impacted by polyploidy, hybridization, and divergent allopatric species diversification (e.g., Hughes, 1998a; Hughes et al., 2002, 2007; Govindarajulu et al., 2011), suggesting that this is an ideal study group for investigating the evolutionary tempo of polyploidy and the complexities of reticulation and divergence. Furthermore, research on *Leucaena* continues to inform our understanding of the impacts of seed translocation and human cultivation on plant lineages (e.g., Hughes et al., 2007).

Repeated patterns of allopatric divergent speciation among diploids across the three main species clades (Govindarajulu et al., 2011), contrast with previous findings suggesting that several tetraploid species of *Leucaena* are allopolyploids whose history likely required sympatry among divergently related parental species (e.g., Hughes et al., 2002, 2007). Previous studies in *Leucaena* using data from allozymes (Harris et al., 1994a), cpDNA RFLPs (Harris et al., 1994b), cpDNA sequence data (Hughes et al., 2007), and nrDNA ITS (Hughes et al., 2002) have met with varying success in the recovery of tetraploid origins. However, in general, limited phylogenetic resolution (Bailey et al., 2004), reliance on single markers, and conflicting signal between some markers in these studies, as well as significant paralogy problems and the frequent occurrence of potentially nonfunctional pseudogene copy types associated with the nrITS data set and gene trees (Hughes et al., 2002; Bailey et al., 2003; see below), have restricted our understanding of polyploid origins in *Leucaena*. Here we used DNA sequences from seven biparentally inherited nuclear-encoded loci and four noncoding maternally inherited cpDNA regions to investigate the origin(s) of tetraploid *Leucaena.* These data are interpreted in the context of a well-resolved hypothesis of divergent diploid species relationships (Govindarajulu et al., 2011). Well-established gene tree and novel "hybrid" species tree/gene tree approaches are used to develop precise hypotheses for polyploid origins and to more fully understand the complex evolutionary history, including paleopolyploidy, neopolyploidy, diploidization, and polyploid establishment, that has resulted in the diverse mix of diploid and polyploid taxa found today.

MATERIALS AND METHODS

***Taxon sampling*—**Multiple accessions of all species were sampled for each DNA sequence locus. This includes a total of 59 diploid (Govindarajulu et al., 2011) and 46 polyploid representatives of *Leucaena* (Appendix 1; Appendix S6, see Supplemental Data with the online version of this article). Outgroups included single accessions of *Desmanthus fruticosus* and *Schleinitzia novoguineensis* (Hughes et al., 2002), in line with what is known about the sister group relationships of *Leucaena* (Hughes et al., 2003; Luckow et al., 2005). Sequences derived from unique clones from one accession are differentiated by a numerical suffix (e.g., 1, 2, 3…).

***Molecular protocols and phylogenetic analyses*—**PCR, cloning, and phylogenetic analyses follow Govindarajulu et al. (2011) with modifications and additions as noted. Accessions that yielded polymorphic reads through direct sequencing were cloned following previously published methods (Hughes et al., 2002); As many as 10 colonies were sequenced for each cloned sample to recover discrete variation consistent with the observed polymorphisms in the directly sequenced PCR product. Sequences were aligned in the program MUSCLE (Edgar, 2004) and manually adjusted in the program WinClada (Nixon, 2002). For parsimony-based gene tree analyses, indels were scored as characters using the simple gap coding method of Simmons and Ochoterena (2000) implemented in the program SeqState ver. 1.4 (Müller, 2005). Parsimony heuristics and bootstraps used WinClada (Nixon, 2002) and the program NONA (Goloboff, 2000) applying a minimum of 500 random additions, tree-bisection-reconnection (TBR) branch swapping, and holding at least 1000 equal trees for the heuristic with bootstraps including at least 500 replicates with 10 random additions/replicate and holding 10 trees/replicate. The best fitting maximum likelihood (ML) tree (model GTR+Γ) and 500 ML bootstrap analyses (model GTR+CAT) were performed using the program RAxML (Stamatakis et al., 2008).

***Recombination*—**Previous work on *Leucaena* has identified potential hybrid origins for several tetraploids and extensive hybridization in artificial sympatry (e.g., Hughes et al., 2002, 2007), highlighting the potential for recombination. Recombinant sequences violate the assumption of divergence in phylogenetics and can reduce support and resolution, potentially leading to an underestimate of our ability to infer polyploid parentage. Each matrix was subject to the phi test (Bruen et al., 2006) for recombination as implemented in the program SplitsTree (Huson, 1997). Irrespective of the result from the phi test, each matrix was also inspected for sequences occupying intermediate splits (indicative of conflicting signal) using SplitsTree. Potentially problematic sequences were subsequently inspected for recombination breakpoints and sequences identified as potential recombinants were checked for potential laboratory-based contig assembly or concatenation assembly errors. Putatively recombinant sequences were omitted from further analyses (see Results).

***Inferred parentage of hybrids/polyploids*—**Sequence characterized amplified region (SCAR)-based anonymous nuclear sequences (23L, 28, A2, A4A5, A9, PA1213) and nrDNA ITS were tested for biparental inheritance through sequencing of an artificially generated triploid hybrid, *L.* ×*mixtec* (accession *Hughes 1715*) (Hughes and Harris, 1998; Bailey et al., 2004). Each locus was considered to be useful in the recovery of divergent allele types (indicative of parentage) if PCR and cloning procedures recovered the expected divergent alleles indicative of biparental inheritance. The maternal inheritance of cpDNA

in *Leucaena* was also confirmed by identifying the maternal alleles recovered from the *L.* ×*mixtec* accession.

Gene tree approach—Standard analyses of each independent genetic locus (i.e., gene trees) were used as one means of inferring parentage of polyploid accessions. For the chloroplast locus, represented by four sequenced regions, sequences for each accession were concatenated into a single terminal and the placements of polyploid accessions in the cpDNA gene tree used to infer the maternal history. Biparentally inherited markers potentially included multiple clones derived from single individuals in each matrix. For these matrices, each unique sequence type represented a separate terminal in the analyses.

The consistent placement of alleles derived from polyploid accessions relative to diploid (and other polyploid accessions) was interpreted as evidence of ancestry. Maternal and paternal parentage of polyploid species was inferred through consistently repeated patterns of ancestry recovered across multiple independent nuclear-encoded gene trees.

"*Spene*" *tree analyses*—A common problem encountered while investigating hybrid origins using gene tree-based approaches is poorly resolved/supported groups derived from analyses of individual genes. This problem is often the result of insufficient variation derived from single loci (e.g., Bailey et al., 2004), which was part of the driving force behind concatenation-based methods of phylogenetic inference (e.g., Nixon and Carpenter, 1996). To infer hybrid parentages, we attempt to resolve sequences from one or more potential hybrid (tetraploid) individuals in the context of the gene trees. When gene tree resolution is lacking, little can be said about potential parentage based on that locus. To overcome this problem, in addition to the standard gene tree approach (noted earlier), we used a novel method, combining the attributes of a species tree approach (using data derived from multiple independent genetic loci) in a manner consistent with Nixon and Carpenter (1996) with aspects of a standard gene tree approach, hereafter referred to as a "spene" tree. This method was developed to identify whether the position of a sequence derived from a tetraploid can be better resolved in the context of a concatenated matrix of divergently related diploids than in the framework of each single gene tree alone.

For this method, we started by generating six replicate copies (hereafter, matrices 1–6) of the diploid-only concatenated matrix (23L, 28, A2, A9, PA1213, and cpDNA respectively) used to investigate divergent relationships among diploid taxa (Govindarajulu et al., 2011). We then added polyploid derived sequences from a single nuclear encoded locus to a concatenated diploid data set (e.g., locus 1 polyploids to matrix 1, locus 2 polyploids to matrix two, and so forth). Each of the "spene" tree matrices therefore contains all multilocus data for divergently related individuals (diploids) together with data for a single locus for accessions with potential reticulate ancestry (polyploids). The logic behind this approach derives from the potential to resolve divergently related accessions based on the combined data (i.e., the species tree component), while the terminal position of the sequences derived from potential hybrid species can only be resolved based on phylogenetic signal from each individual gene segment (i.e., the gene tree component).

We expected two possible outcomes from the approach. First, if a "spene" tree is no better resolved or supported than the original gene tree for a locus, then the problem of resolution resides with lack of signal among polyploid sequences in the "spene" matrix, thus providing no additional information relative to the gene tree alone. Alternatively, if the "spene" tree generates better resolution and support than the corresponding gene tree, the problem of resolution resides with divergently related accessions (i.e., *Leucaena* diploids) that can be resolved by the available multilocus data present in the spene tree matrix. An additional advantage of the "spene" tree approach in this study is the inclusion of outgroups in all "spene" tree matrices, something that was not possible with all individual gene trees (see Results).

***Genome size estimates*—**Prior to this study, genome size estimates were available for one or a few accessions of each species of *Leucaena* (Palomino et al., 1995; Hartman et al., 2000). To extend these estimates to a more comprehensive sample of representatives per species, we employed flow cytometry following the technique of Doležel and Göhde (1995). Approximately 100 mg of greenhouse grown fresh leaves were chopped in Otto I buffer (0.1 mol/L citric acid and 0.5% v/v Tween 20) and stained with propidium iodine in Otto II buffer (0.4 mol/L Na2HPO4, 0.15 µmol/L propidium iodide, and 50 µg/mL RNase). Samples were run on a FACS flow cytometer (Dept. of Biochemistry, University of Oxford) using *Lactuca sativa* (ca. 5.32 pg/2C Michaelson et al., 1991; Koopman, 2002) as a size standard.

RESULTS

For each locus, alignment length, number of indel characters, percentage parsimony informative characters, and basic tree statistics are presented in Table 1. We were highly successful in amplifying and sequencing all loci for each ingroup accession (Govindarajulu et al., 2011); however, the amplification of outgroups continued to be problematic for some of the SCAR loci (Bailey et al., 2004). As a result, only the cpDNA, ITS, and PA1213 gene trees could be rooted with outgroup sequences, while the remaining gene trees were rooted internally with *L. cuspidata* sequences (Hughes et al., 2002). As indicated already, the "spene" tree concatenation approach circumvents this problem of rooting such that "spene" trees are rooted using relevant outgroups.

Gene trees for each locus are presented in Appendix S1 (see Supplemental Data with the online version of this article). With the exception of nrDNA ITS and A4A5 gene trees, sequences from diploids generally form robustly supported monophyletic species clades in each tree, as demonstrated by Govindarajulu et al. (2011), and interlocus comparisons are largely congruent across well-supported nodes. The A4A5 and nrDNA ITS gene trees suggest inclusion of multiple paralogs (online Appendix S1G, H). The latter findings are consistent with and extend the earlier findings of Hughes et al. (2002) and Bailey et al. (2003) for nrDNA ITS in *Leucaena*. Nevertheless, in both cases, the terminal placements of polyploid sequences are largely consistent with findings from the other loci, suggesting that these terminal relationships can provide supporting evidence when viewed in the context of results from all loci.

***Potential recombination*—**The phi test failed to detect evidence for recombination within these complex data sets; however, a laborious network-based and alignment-based evaluations detected potential conflicting signal in loci PA1213 (accession *L. diversifolia* 46-87_3_5), 23L (*L. pallida* 122-92_05, *L. leucocephala* 147-92_3_7), 28 (*L. confertiflora* 127-92_3 and 1730_4, *L. pallida* 2165_9, and *L. involucrata* 146-91_2_4), and A2 (*L. leucocephala* 93-92_2_7 and 80-92_3, *L. pallida* 79-92_3). These were each cloned from an allopolyploid accession (see below), suggesting that recombination is either occurring in vivo or through PCR (e.g., Cronn et al., 2002). Inspection for potential recombination breakpoints further suggested that these are indeed recombinant sequences. As a result, subsequent "spene" tree analyses of polyploid origins excluded these sequences.

TABLE 1. Matrix and tree characteristics. For each locus, the number of aligned base pairs (bp), of indel characters (indel char), percentage parsimony informative characters (% PIC), tree length (L), ensemble consistency (CI), and retention indices (RI) are presented.

Gene region	No. bp	No. indel char	% PIC	L	CI	RI
cpDNA	2600	98	6.7	371	0.53	0.88
23L	881	96	26.9	638	0.48	0.87
28	873	73	17.2	460	0.41	0.86
A9	1260	200	30.3	865	0.61	0.94
PA1213	796	114	24.7	726	0.47	0.87
A2	692	46	14.2	269	0.48	0.88
A4A5 [a]	840	100	37	954	0.51	0.94
ITS [a]	571	64	58	1298	0.41	0.82

[a] Loci with clear gene tree/species tree problems excluded from "spene" tree analyses.

TABLE 2. Hypotheses of polyploid parentage based on the "spene" tree approach (trees presented in online Appendix S2A–F). Support for the maternal and paternal parentage is indicated by maximum likelihood and parsimony bootstraps, respectively (e.g., "100/100" with "<" indicating less than 50% support). When polyploids were resolved with diploids and other polyploids in the same clade of potential parents, diploid progenitors are listed first (see Results). Note that two polyploid-derived sequences were recovered whose positions were inconsistent with the majority of other sequences in the above comparison: a single *L. leucocephala* clone (86-92_1) weakly supported at the base of clade 1 and one accession of *L. confertiflora* (1800) resolved in clade 2 in the 28 and PA1213 results, respectively.

Taxon	Line	cpDNA	23L	28	A9	PA1213	A2
Leucaena confertiflora	Maternal	*L. trichandra* (</<)	*L. trichandra* (91/89) with polyploids *L. diversifolia,* *L. involucrata,* and *L. pallida*	*L. trichandra* (56/<) with polyploids *L. involucrata* and *L. pallida*	no information	*L. trichandra* (</<) with polyploids *L. diversifolia,* *L. involucrata,* and *L. pallida*	*L. trichandra* (94/54) with polyploids *L. diversifolia*, *L. involucrata*, and *L. pallida*
	Paternal	NA	*L. cuspidata* (96/76)	*L. cuspidata* (100/72)	*L. cuspidata* (100/91)	*L. cuspidata* (91/65)	*L. cuspidata* (52/<)
Leucaena diversifolia	Maternal	*L. pulverulenta* (99/71) with polyploid *L. leucocephala*	*L. pulverulenta* (100/81) with polyploid *L. leucocephala*	*L. pulverulenta* (100/93) with polyploid *L. leucocephala*	*L. pulverulenta* (100/99) with polyploid *L. leucocephala*	*L. pulverulenta* (99/99) with polyploid *L. leucocephala*	*L. pulverulenta* (98/90) with polyploid *L. leucocephala*
	Paternal	NA	*L. trichandra* (91/89) with polyploids L. *confertiflora, L. involucrata, and L. pallida*	no information	no information	*L. trichandra* (</<) with polyploids L. *confertiflora, L. diversifolia, and L. pallida*	no information
Leucaena involucrata	Maternal	*L. pueblana* (87/88) with polyploid *L. pallida*	no information	*L. pueblana* (83/80) with polyploid *L. pallida*	*L. pueblana* (100/99) with polyploid *L. pallida*	no information	no information
	Paternal	NA	*L. trichandra* (100/61)	*L. trichandra* (56/<) with polyploids *L. confertiflora* and *L. pallida*	no information	*L. trichandra* (</<) with polyploids L. *confertiflora, L. involucrata, and L. pallida*	*L. trichandra* (94/54) with polyploids *L. diversifolia*, *L. confertiflora,* and *L. pallida*
Leucaena leucocephala	Maternal	*L. pulverulenta* (99/71) with polyploid *L. diversifolia*	*L. pulverulenta* (100/81) with polyploid *L. diversifolia*	*L. pulverulenta* (96/78) with polyploid *L. diversifolia*	*L. pulverulenta* (100/99) with polyploid *L. diversifolia*	*L. pulverulenta* (99/99) with polyploid *L. diversifolia*	*L. pulverulenta* (98/90) with polyploid *L. diversifolia*
	Paternal	NA	*L. cruziana*(99/97) in one clade and *L. cruziana* plus *L. macrophylla* (58/<) in another	*L. cruziana* (</<)	*L. trichandra*(99/95)	*L. cruziana* (96/85)	*L. cruziana* (96/86)
Leucaena pallida	Maternal	*L. pueblana* (87/88) with polyploid *L. involucrata*	*L. pueblana* (63/<) with polyploid *L. involucrata*	*L. pueblana* and *L. matudae* clade (72/<) with polyploid *L. involucrata*	Two sister clades: (1) *L. matudae* (100/99); (2) *L. pueblana* (100/99).	*L. pueblana* and *L. matudae* clade (80/69)	Clade 2 (99/79)
	Paternal	NA	*L. trichandra* (86/64) with polyploids *L. involucrata* and *L. confertiflora*	*L. trichandra* (56/<) with polyploids *L. confertiflora* and *L. involucrata*	*L.trichandra* (100/95)	*L. trichandra* (</<) with polyploids *L. diversifolia, confertiflora,* and *L. involucrata*	*L. trichandra* (54/<) with polyploid *L. involucrata*

Putative polyploid parentage—For the primary inference of polyploid ancestry, detailed next, we focus on results derived from the novel "spene" tree approach (online Appendix S2A–F), applying the same six loci that were used to reconstruct divergent diploid relationships by Govindarajulu et al. (2011). The placements of sequences derived from polyploids that received support within the context of these trees are summarized in Table 2 and Fig. 1. In addition, online Appendices S1 and S3 (phylogenies and summary table, respectively) present comparable results for the individual gene tree analyses.

The following paragraphs summarize the inferred parentage of each polyploid (Figs. 1, 2). In these sections, three conventions are adopted for brevity. First, we refer to three well-supported clades of divergent diploid species recovered by Govindarajulu et al. (2011). These are "clade 1" (12 species; the majority of diploids within *Leucaena*), "clade 2" (three diploid species; *L. esculenta*, *L. matudae*, and *L. pueblana*), and "clade 3" (three diploid species: *L. pulverulenta*, *L. greggii*, and *L. retusa*,) (Fig. 2). Second, although polyploids may be derived from other polyploids rather than from diploid progenitors, when polyploid sequences were resolved with diploids as well as other polyploids, we identify the diploid(s) as the potential progenitor(s). This simplification is adopted because the results for four of the five polyploids are ultimately consistent with polyploids being derived from diploids rather than from other polyploids (see Discussion). Last, whenever an extant diploid species is implicated in the ancestry of a polyploid, we acknowledge the possibility that an extinct or unsampled ancestor could have been involved in the origin of these polyploids but refer to the extant sampled species for simplicity.

There are two polyploid-derived sequences whose gene tree placements were inconsistent with the majority of other sequences within a comparison. These are a single clone of *L. leucocephala* (86-92_1), weakly supported at the base of clade 1 in the 28-gene tree (online Appendix S1C), and one accession of *L. confertiflora* (1800), resolved in clade 2 in the PA1213 gene tree (online Appendix S1E). These two anomalous sequences may indicate evidence of introgression among cultivated tetraploid species of *Leucaena*.

Leucaena confertiflora—The polyploid *L. confertiflora* was first discovered in cultivation in 1980 and initially considered, but not formally described, as a subspecies of *L. cuspidata* by Zárate (1984), and only later described as a distinct species (Zárate, 1994). Clear morphological similarities between *L. confertiflora* and *L. cuspidata* were also noted by Hughes (1998a). However, cpDNA RFLP results identified a well-supported relationship to several species in clade 1 (Harris et al., 1994b; Hughes, 1998a; Hughes et al., 2002) rather than to *L. cuspidata*. In this study, a sister group relationship between sequences derived from *L. trichandra* and *L. confertiflora* was recovered in the best fitting ML tree for cpDNA, 23L, 28, PA1213, and A2 with high to moderate support in 23L (91 ML bootstrap/89 parsimony bootstrap—hereafter abbreviated 91/89) and A2 (94/54) (Table 2). All five of the nuclear-encoded markers also recovered a divergent set of *L. confertiflora* sequences that are strongly supported with alleles from the diploid *L. cuspidata* (Table 2). Thus, the newly available data are consistent with *L. confertiflora* being an allopolyploid derived maternally from *L. trichandra* and paternally from *L. cuspidata,* the multilocus data providing robust evidence for an allopolypoid origin. The variation in the tetraploid chromosome complement of *L. confertiflora* ($2n = 4x = 104/112$, Cardoso et al., 2000) has been suggested as evidence of possible multiple independent origins (Hughes et al., 2002). However, this variation does not appear to correspond to the two morphologically defined varieties *confertiflora* and *adenotheloidea* and remains unexplained. On the basis of current herbarium records, the putative parents of *L. confertiflora* occupy disjunct distributions in dry mid-elevation oak formations and matorrales to the north (*L. cuspidata*) and south (*L. trichandra*) of the central Mexican volcanic axis (figs. 36 and 46 in Hughes, 1998a), with only the latter species occurring sympatrically with *L. confertiflora* itself. This lack of geographic overlap suggests that either one or both parents may have been more widespread in the past, or that one or both species occur more widely but remain to be collected in the intervening mountains of Hidalgo and north Puebla. However, unripe pods and seeds of *L. cuspidata* are harvested and the seeds consumed and marketed locally, and the species is occasionally cultivated in parts of Hidalgo (Hughes, 1998b), suggesting that this species could also have been more widely cultivated in the past (see Discussion).

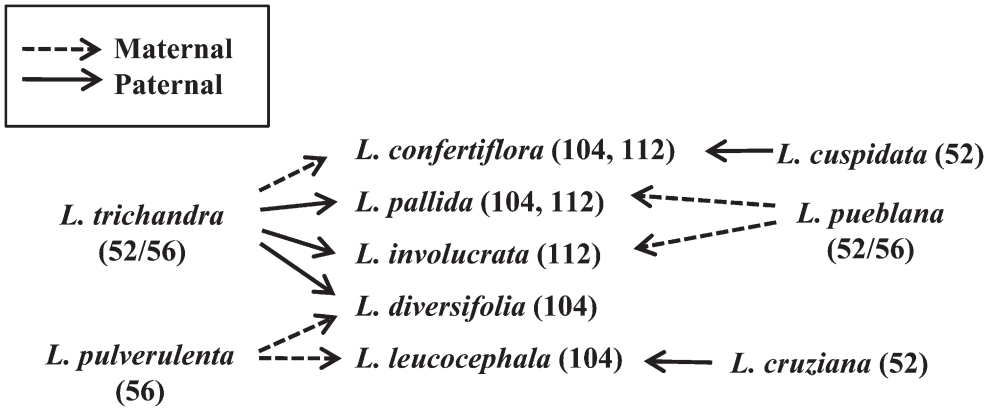

Fig. 1. Inferred pattern of tetraploid origins from diploid progenitors. Maternal origins are based on findings from cpDNA and congruent resolution in nuclear derived gene tree and "spene" tree analyses. Paternal origins derive from the divergent placement of nuclear derived sequences in comparison to the inferred maternal origins.

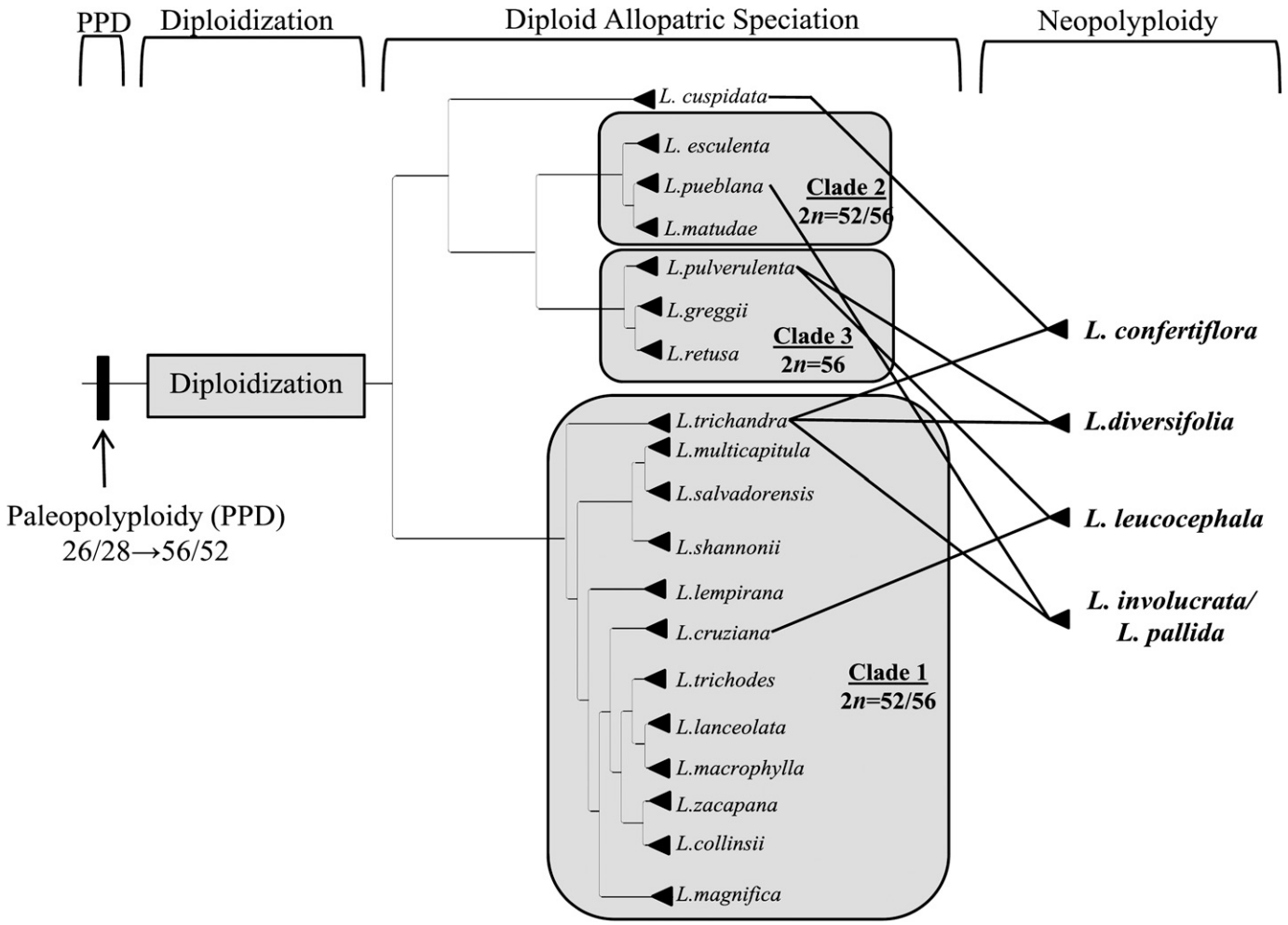

Fig. 2. The evolutionary dynamics of polyploidy in *Leucaena*. The diploid tree summarizes relationships recovered by Govindarajulu et al. (2011). Multiple accessions of each species are collapsed to a single terminal.

Leucaena diversifolia—Historically, the taxon referred to as *L. diversifolia* included both diploid and tetraploid components (Pan and Brewbaker, 1988; Zárate, 1994). The diploid portion of this taxon was segregated by Hughes (1998a) as *L. trichandra.* Previous hypotheses for the origin of the tetraploid *L. diversifolia* ($2n = 4x = 104$) have focused on a possible autopolyploidization event involving *L. trichandra* (Pan and Brewbaker, 1988), with more recent studies suggesting a possible allopolyploid origin (Harris et al., 1994b; Hughes et al., 2007). We recovered strongly supported sister group relationships between sequences of *L. pulverulenta* and *L. diversifolia* in all five nuclear loci and cpDNA (Table 2). In addition, sets of divergent alleles were recovered from 23L and PA1213, supporting *L. diversifolia* as an allopolyploid derived maternally from *L. pulverulenta* and paternally from *L. trichandra.* These findings help to clarify conflict previously noted between morphological and cpDNA affinities in the origin of this tetraploid (Hughes, 1998a). As with *L. confertiflora*, the present day distributions of these two putative diploid progenitors as currently known, do not overlap, with *L. pulverulenta* restricted to the NE flanks of the Sierra Madre Oriental in central-NE Mexico and *L. trichandra* on the inland western flanks. However, in this case, the tetraploid *L. diversifolia* occupies a distribution that spans the gap between these two diploids, occurring sympatrically with *L. pulverulenta* in northern Veracruz, and in areas closely adjacent to *L. trichandra* in northern Oaxaca and Chiapas.

Leucaena involucrata—The most recently discovered and described tetraploid species in *Leucaena*, *L. involucrata* ($2n = 4x = 112$, Cardoso et al., 2000), is also the least understood. Morphological data suggested conflicting affinities to the yellow-flowered clade 3 species *L. greggii* and *L. retusa* on the one hand, and to members of clade 2, and especially *L. pallida*, on the other (Hughes, 1998a). Furthermore, the cpDNA RFLP study of Harris ("sp. nov." in Harris et al., 1994b) recovered conflicting evidence of affinities to both clade 2 and clade 3 species. In the present study, a strongly supported sister relationship was recovered between sequences of *L. pueblana* and *L. involucrata* from cpDNA, 28, and A9 (Table 2), while divergent alleles from this polyploid were resolved with moderate to high support with *L. trichandra* in four of five nuclear-derived trees (Table 2). This pattern is consistent with *L. involucrata* being an allopolyploid derived maternally from *L. pueblana* and paternally from *L. trichandra* with no evidence for parentage from the morphologically similar *L. greggii/L. retusa*. The outlying distribution of

L. involucrata in NW Mexico is isolated from all other species of *Leucaena* and well distant from these putative diploid progenitors adding support to the idea that *L. involucrata* is more likely to be a divergent form of tetraploid *L. pallida* (see Discussion).

Leucaena leucocephala—This species is by far the most widely known in the genus because it has been pantropically spread in cultivation as an important forage and agroforestry tree, has been the focus of tree breeding programs to improve yield, and is now naturalized and frequently invasive in diverse tropical countries (Hughes and Jones, 1999). Previous hypotheses for the origin of *L. leucocephala* ($2n = 4x = 104$) have varied extensively, with most focusing on a possible allopolyploid origin. These included several speculative suggestions about possible diploid parent combinations including *L. collinsii* × *L. trichandra* and *L. shannonii* × *L. trichandra*, as well as some evidence for a maternal origin from *L. pulverulenta* with a paternal contributor from clade 1 (Hughes, 1998a; Hughes et al., 2002, 2007). Here, all loci strongly supported *L. pulverulenta* as the maternal parent and four of five nuclear loci strongly support *L. cruziana* as the paternal parent of *L. leucocephala*. *Leucaena cruziana* was previously treated as conspecific with *L. lanceolata*, but Govindarajulu et al. (2011) recently divided *L. lanceolata* into two distinct species. The 28, PA1213, A2, and 23L gene trees all show *L. leucocephala* sequences strongly supported as derived from the segregate species *L. cruziana* rather than *L. lanceolata* s.s., helping to clarify the precise origin of the species. Unlike the situation for several of the other *Leucaena* polyploids, the present-day geographic distributions of the two putative diploid progenitors of *L. leucocephala* overlap in northern Veracruz. For the fifth nuclear locus, A9, two sequences from *L. leucocephala* were resolved with *L. trichandra*. *Leucaena leucocephala* is known to hybridize extensively in backyard cultivation with *L. diversifolia* (Zárate, 1994; Hughes and Harris, 1998), which provides a possible explanation for the origin of these *L. trichandra*-like A9 alleles in two accessions of *L. leucocephala* (online Appendix S2D).

Leucaena pallida—The tetraploid *L. pallida* ($2n = 4x = 104/112$ see Cardoso et al., 2000) is one of the more widespread species in cultivation across south-central Mexico. Hypotheses for its origin have primarily focused on possible maternal ancestry derived from either *L. esculenta* or *L. pueblana* in Clade 2 (Hughes, 1998a; Hughes et al., 2002) and the paternal side from clade 1 (Hughes et al., 2007). The cpDNA (87/88), A9 (100/99), and 23L (63/<50) topologies support *L. pueblana* as the likely maternal contributor to *L. pallida*, and this result is consistent with weakly resolved/supported components in trees from the nuclear loci 28, PA1213, and A2. However, locus A9 also recovered two accessions of *L. pallida* with *L. matudae* (100/99), a close relative of *L. pueblana*. Although this conflict might be interpreted as evidence of different species combinations giving rise to the morphologically recognizable taxon *L. pallida*, one accession is strongly supported with *L. pueblana* in the cpDNA tree but with *L. matudae* in the alternative 23L topology, suggesting the possibility that lineage sorting could be complicating our understanding of the maternal histories of these closely related taxa. Alongside this evidence for a maternal progenitor from clade 2, all five nuclear-encoded loci support *L. trichandra* as the paternal progenitor of *L pallida* (Table 2).

"*Spene*" *tree approach*—When comparing results from the standard gene tree (Appendix S3) and novel "spene" tree (Table 2) approaches, it is clear that the latter, incorporating assessments of recombination and data for all loci for divergently related diploid accessions alongside data for each of the loci in separate analyses for accessions of hybrid origin (i.e., polyploid *Leucaena*), added resolution and support that aided in the interpretation of polyploid origins. There were five cases for which the "spene" tree data provided greater resolution and no cases with the reverse result. In addition, there were 18 and 19 cases (ML and parsimony, respectively) with a minimum of 5% greater bootstrap support (ML/P respectively) using the "spene" tree approach and just five and eight cases in which a gene tree retained higher support for the same resolution. The resolution recovered through the "spene" tree approach was especially important in pinpointing the maternal origin of *L. confertiflora* as well as the likely origins of *L. involucrata* and *L. pallida*.

***Genome sizes*—**Estimates of genome size via flow cytometry were successfully obtained for most species in clade 1. However, accessions representing species from clades 2 and 3 routinely released excessive mucilage upon homogenization in Otto I buffer, making it impossible to run on the flow cytometer, such that estimates for these taxa are only available for a limited number of accessions from previously published studies. New genome size estimates for diploid *Leucaena* (clade 1 taxa) ranged from 1.30 pg/2C (*L. macrophylla*) to 1.52 pg/2C (*L. salvadorensis*) (Appendix S4). Among polyploid accessions, new genome size estimates ranged from 2.52–2.93 pg/2C in *L. leucocephala* and 2.63–2.86 pg/2C in *L. diversifolia*.

DISCUSSION

This study, alongside the accompanying analyses of diploid species relationships (Govindarajulu et al., 2011), presents the most thorough exploration of relationships in the mimosoid legume genus *Leucaena* undertaken to date. These studies combine dense taxon sampling incorporating multiple accessions of all the known species of *Leucaena*, with use of a set of multiple highly informative nuclear-encoded DNA sequence loci. The comprehensive taxon and character sampling employed, with minimal missing data across data sets, have provided an opportunity to both test species limits (Govindarajulu et al., 2011) and disentangle divergent from reticulate relationships simultaneously across the genus as a whole. One of the challenges to disentangling divergent and reticulate relationships among species has been finding DNA sequence loci that are sufficiently informative to obtain gene trees that are adequately resolved and robustly supported. Lack of sufficient resolution remains a problem in analyses of even some of the more intensively studied polyploid plant genera, including *Brassica*, *Gossypium*, *Nicotiana*, *Primula*, *Senecio*, *Spartina*, and *Tragopogon* that have been prominent in studies of reticulation and polyploidy (e.g., Wendel, 2003; Abbott and Lowe, 2004; Ainouche et al., 2004; Mavrodiev et al., 2008; Guggisberg et al., 2009; Clarkson et al., 2010). The nuclear-encoded SCAR-based DNA sequence loci used in this study are the product of extensive and laborious screening (Bailey et al., 2004), but have proved of sufficient phylogenetic utility to build a set of informative gene trees for *Leucaena*. Next, we use the newly developed genus-wide framework to discuss the scope, pattern, and frequency of polyploid origins, the potential influence of human activities on these plant lineages, as well as the diploid traits that may favor the involvement of one species over another in the formation of polyploids, and polyploid traits favoring establishment and persistence of new minority cytotype lineages.

Hybridization and allopolyploidy—The inferred pattern of polyploid parentages viewed in the context of the most recent estimate of diploid relationships reveals a number of interesting features (Fig. 2). First, all five tetraploids retain components of two divergent nuclear genomes (at multiple loci) representing four different combinations of diploid progenitors. This pattern is consistent with at least four allopolyploidization events giving rise to the formation of *L. diversifolia*, *L. leucocephala*, *L. confertiflora*, and *L. involucrata/L. pallida*. Nuclear-encoded loci identify that the latter pair of polyploids share the same paternal (*L. trichandra*) and maternal (*L. pueblana*) contributors. These two polyploids also share similar genome sizes, estimated at 2.53–2.77 for *L. pallida* and 2.29 for *L. involucrata* (Appendix S4). Two separate allopolyploid origins or a single allopolyploidization event followed by divergent speciation provide equally plausible explanations for the formation of these two species based on evidence from the analyses of DNA sequences and genome size estimates. However, it is notable that the *L. involucrata* sequences form a monophyletic group with, or sister to, the *L. pallida* sequences in all gene trees, except for one anomalous sequence in the 23L gene tree (Appendix S1). If *L. involucrata* and *L. pallida* were independently derived from independent allopolyploidization events from the same diploid parents, we might not expect this consistent resolution, suggesting that it is more plausible that a single allopolyploid ancestor gave rise to these two tetraploids via divergent speciation rather than by two independent allopolyploidizations. This idea is consistent with the geographical distribution of *L. involucrata*, which occupies an isolated outlying range in the mountains of northern Sinaloa and Sonora in northwestern Mexico that is far distant from the distribution of *L. pueblana* in south-central Mexico, but much closer to the northern occurrences of *L. pallida* in Zacatecas (see detailed distribution maps in Hughes, 1998a).

Artificial sympatry facilitates allopolyploid speciation—The identification of a critical role for sympatric allopolyploid speciation in the formation of tetraploid species of *Leucaena* contrasts with patterns of predominantly allopatric distributions and a hypothesis of allopatric divergent speciation for the diploid species (Govindarajulu et al., 2011). This apparent paradox could of course be the result of historical sympatry among wild diploid progenitor taxa that subsequently retracted into a more restricted pattern of allopatric ranges. However, given that species of *Leucaena* have been used as a source of food for at least 6000 years (MacNeish, 1958; Flannery, 1986; Zárate, 2000) and that the three clades of diploid *Leucaena* retain deep allopatric splits (fig. 4 in Govindarajulu et al., 2011 in this issue) dating back 9–11 Myr on average (Lavin et al., 2004; Simon et al., 2009), these interclade derived allopolyploid lineages could also be derived relatively recently from hybridization resulting from artificial sympatry in backyard gardens. Anderson (1949) and Anderson and Stebbins (1954) were the first to posit that human translocation of individuals and informal backyard cultivation were likely to provide important avenues facilitating hybridization among otherwise geographically isolated species. Recently, Hughes et al. (2007) demonstrated that *Leucaena* represents one of the best empirical examples of this phenomenon by uncovering evidence for widespread hybridization in contemporary backyards across south-central Mexico.

A number of lines of indirect evidence provide support for the idea that at least some of these allopolyploidization events could have occurred within the last 10 000 years as a result of translocation of seed by humans. First, populations of the diploid parents of *L. confertiflora* and *L. diversifolia* are geographically isolated by distance and/or the mountains of the central volcanic axis or the Sierra Madre Oriental, making hybridization among wild populations unlikely. Second, the archaeological evidence from *Leucaena* seed remains is consistent with seeds of diploid *Leucaena* being used as minor food source for nearly 4000 years before those of tetraploid species (Zárate, 2000). Seed use of diploids contrasts with the more widespread use of tetraploid species once they appear in the archeological record and that persists into modern usage (Zárate, 2000). Last, the cumulative genome sizes for parental diploids are essentially additive in the allopolyploid genomes of all five tetraploid species (Table 3), suggesting that these allopolyploids have yet to show significant signs of genome downsizing that might be expected in older paleopolyploids (Leitch and Bennett, 2004).

TABLE 3. Genome sizes of tetraploid species and their putative diploid maternal/paternal parents.

Tetraploid	Genome size (pg/4C)	Maternal genome (pg/2C)	Paternal genome (pg/2C)
L. confertiflora	3.31 [c] (1.69[*])	1.56 [a]	1.40 [a]
		1.43–1.51 [b]	
L. diversifolia	2.71 [a]	1.40 [a]	1.40 [a]
	2.63–2.86 [b]		1.43–1.51 [b]
L. involucrata	2.29 [a]	1.00 [a]	1.40 [a]
			1.43–1.51 [b]
L. leucocephala	2.97 [a]	1.40 [a]	1.44 [a]
	2.52–2.93 [b]		1.43–1.51 [b]
L. pallida	2.53–2.77 [c] (1.58[1])	1.00 [a]	1.40 [a]
			1.43–1.51 [b]

Notes: Lettered footnotes designate sizes measured by (a) Hartman et al. (2000), (b) authors of present study, and (c) Palomino et al. (1995). More detailed information on genome sizes derived for the present study is available in online Appendix S4.

[*]The apparent diploid values for tetraploid *L. confertiflora* and *L. pallida* reported by Hartman et al. (2000) do not agree with data of Palomino et al. (1995) and are probably mistaken.

Paleopolyploidy and diploidization Additivity of genome sizes in all five allotetraploid species of *Leucaena* could be attributed either to the recency of their tetraploid origins or older polyploid origins in a system lacking significant diploidization, as found in other plant groups (e.g., some *Nicotiana*, Leitch et al., 2008). If significant diploidization has not occurred, then there would be no reason to expect extensive reduction of genome sizes for these tetraploid *Leucaena* species, regardless of age. To address whether genome reduction is likely to correlate with age of origin in *Leucaena*, we need to bear in mind that "diploid" *Leucaena* have chromosome numbers ($2n$ = 52 and 56) consistent with what would clearly be considered tetraploid genomes in other legumes (Appendix S5; Goldblatt, 1981). Thus, *Leucaena* species referred to here, and elsewhere, as "tetraploids" could equally be considered to be octoploids ($2n$ = 104 and 112). Despite this, *Leucaena* species have consistently been referred to as diploids ($2n$ = 52 and 56) and tetraploids $2n$ = 104 and 112) based on evidence that the diploids show disomic patterns of inheritance (Pan, 1985; Sorensson and Brewbaker, 1989). This usage is maintained here.

In contrast to *Leucaena*, all the closely related mimosoid legume genera (except *Schleinitzia*) for which chromosome counts are available have diploid chromosome complements of $2n$ = 26–28, with just a few sporadic polyploid species. Optimization of chromosome numbers (Appendix S5) onto the most

recent combined ITS and cpDNA phylogeny of genera in the informal *Leucaena* and *Dichrostachys* groups and related mimosoid taxa of Luckow et al. (2005) confirms that the overall pattern of chromosomal evolution across these closely related legume genera appears to be straightforward. This phylogeny suggests a consistent $2n = 26$ or 28 backbone across the tree in line with the known base number $x = 14$ for Mimosoideae (Appendix S5; Goldblatt, 1981), and only one or two paleopolyploidization events derived within the informal *Leucaena* group. Two alternative equally parsimonious optimizations (Appendix S5) identify a paleopolyploidization event predating the diploid *Leucaena* clade or a clade comprising *Schleinitzia* plus all diploid *Leucaena*. Both optimizations clearly indicate that paleopolyploidy preceded the divergence of extant diploid *Leucaena* species (Fig. 2).

Disomic inheritance (discussed earlier) in the 19 "diploid" species derived from the paleopolyploid ancestor(s) could be the result either of diploidization of an autopolyploid ancestor or to an allopolyploid ancestor that may or may not have been subject to diploidization (e.g., reduction to one homeologous locus from a pair). For the five nuclear-encoded loci investigated by Govindarajulu et al. (2011), 93% of 242 gene by accession combinations for diploid species amplified two or fewer sequence types and in 98.7% of cases where multiple alleles were discovered for an accession, these formed monophyletic groups on the respective gene trees (Appendix S7). These findings are consistent with amplification of single loci in diploidized genomes rather than amplification from two homeologous loci in allopolyploids lacking significant diploidization. In contrast, the five allotetraploids investigated here present a nice counter example. In this case, we recovered more than two unique sequence types in 55% of the gene by accession combinations, and sequence types from individual accessions were resolved in divergent clades in 76% of cases. These results are consistent with two homeologous loci being amplified using a single primer pair. Furthermore, the "diploid" species have genome sizes (Appendix S4; Palomino et al., 1995; Hartman et al., 2000) well within the diploid range for other legumes (genome sizes from 0.62–1.60 pg/2C) available in the Kew C-Values database (surveyed October 2010, Bennett and Leitch, 2005). In fact, if the genome size of 0.62 pg/2C for diploid *L. macrophylla* reported by Hartman et al. (2000) is accurate, some individuals of *Leucaena* have the smallest published genome sizes known in the Leguminosae.

Little is known about the tempo or the processes involved in genome reduction whereby polyploid genomes become diploidized over time (Adams and Wendel, 2005; Clarkson et al., 2005; Hawkins et al., 2008; Leitch et al., 2008; Rousseau-Gueutin et al., 2008; Grover and Wendel, 2010). Estimates for the crown age of *Leucaena* range from a mean of 10.1 (SD ± 1.3) Myr (Lavin et al., 2004) to 9.1 Myr (95% CI 3.1–18.5 Myr, unpublished from Simon et al., 2009), while the stem node has been estimated to be 25.3 Myr old (95% CI 13.3–35.7 Myr, unpublished data from Simon et al., 2009). The pattern of genome size variation interpreted in the context of the phylogeny suggest that the last common ancestor of extant diploid *Leucaena* underwent extensive diploidization over ca. 15 Myr (stem to crown node) (Fig. 2). These findings are in line with more extensive shifts in genome size, both increases and decreases, with increasing age of polyploids observed in *Nicotiana* (Hawkins et al., 2008; Leitch et al., 2008).

These data on ancestral shifts in chromosome number and the apparent diploid genetic architecture of the 19 diploid species of *Leucaena* suggest that they are paleotetraploids that have undergone subsequent diploidization. Patterns of chromosomal segregation have not been studied in tetraploid *Leucaena*, but there is little evidence for similar diploidization having impacted the five tetraploids. These genomes retain largely additive sizes with respect to their inferred parents and often harbor three or four alleles per locus, while homeologous sequences are resolved in divergent clades (Appendix S7). We suggest that this lack of evidence for diploidization among these tetraploids provides further support for the idea of recent neopolyploidy for the five tetraploid species of *Leucaena* (see Hughes et al., 2007).

Derived traits influencing the establishment and potential persistence of polyploids—One of the intriguing but as yet largely unanswered questions surrounding polyploid origins relates to whether certain diploid parental traits and variations in the genes controlling meiosis might favor the involvement of a species in the formation of a polyploid (e.g., Doyle et al., 2008; Soltis et al., 2010). The likely involvement of *L. trichandra* in the origins of four of the five *Leucaena* tetraploids (Figs. 1, 2) is striking and supports the idea that certain species could have a proclivity for allopolyploidization. Investigation of pollen formation in *Leucaena* suggested that *L. trichandra* can generate 7% unreduced pollen grains, whereas other diploids produce just 0.0–1.5% (Boff and Schifino-Wittmann, 2003). This tendency for the production of unreduced gametes is consistent with the idea that *L. trichandra* could more likely be involved in allopolyploidization than other species of *Leucaena*. Furthermore, with a distribution from northern Nicaragua to Durango in west-central Mexico, *L. trichandra* is one of the most widespread and morphologically variable diploid species in the genus (Hughes, 1998a), attributes that are likely also to increase the probability that *L. trichandra* will hybridize with other diploids, compared to most other species which occupy more narrowly restricted geographic ranges (Table 10 in Hughes, 1998b). These observations question whether hybridization or polyploidization likely represents the limiting factor in the formation of polyploid lineages in *Leucaena*. Crossing studies on 12 of 19 diploid species of *Leucaena* (including *L. trichandra*) recovered high levels of interfertility (Sorensson and Brewbaker, 1994), and hybrids individuals are common in cultivation (Hughes et al., 2007; Govindarajulu et al., 2011). However, allopatry is the rule between diploid *Leucaena*, and there is little evidence for hybridization among diploids in natural populations (Hughes et al., 2007; Govindarajulu et al., 2011). These patterns suggest that restricted opportunities for hybridization, rather than the process of polyploidization, represents the limiting step in the formation of allopolyploid lineages in *Leucaena* outside of cultivation.

We also considered whether any derived traits in allotetraploids might have favored their establishment and persistence. In natural systems, minority cytotype exclusion has been hypothesized (Levin, 1975; Soltis et al., 2010) and shown (Husband, 2000) to hinder the establishment of a minority cytotype such as a newly formed polyploid. Minority cytotype exclusion occurs through the majority haploid pollen (typically from diploids) fertilizing the minority cytotype (a new tetraploid), leading to the formation of sterile triploid individuals and the ultimate elimination of the minority cytotype. It is striking that all the diploid species of *Leucaena* that have been investigated are self-incompatible, while the tetraploids that have been studied (three of five species) are self-compatible (Sorensson and

Brewbaker, 1994) and tend to have much higher levels of fruit and seed set (Hughes, 1998a). Models examining the ability of polyploids to establish in sympatry with their diploid parents have suggested that self-compatibility, increased fertility, and the frequent production of unreduced gametes (mentioned earlier) are key factors in overcoming minority cytotype exclusion and successful establishment of neopolyploids (Felber, 1991; Rausch and Morgan, 2005). Empirical studies have also found a higher frequency of self-compatibility in polyploid plants compared to diploids, and particularly in allopolyploids rather than autopolyploids (Barringer, 2007; Husband et al., 2008). Another potentially significant derived trait in *L. leucocephala* is year-round flowering and fruiting. A minority self-compatible cytotype with year-round flowering could facilitate establishment if its reproductive cycle is partially (e.g., *L. leucocephala*) or fully isolated from the majority cytotype.

We have previously argued that certain of these derived traits in some *Leucaena* tetraploids, and particularly self-compatibility and year-round flowering in *L. leucocephala*, were desirable for food use, thereby promoting historical cultivation (Hughes et al., 2007), resulting in the possibility that people would have quickly noticed novel polyploids and attempted to maintain them. However, some of these features are not only desirable from a human perspective, but are also likely to have facilitated the indigenous propagation of rare new genotypes. For example, a self-incompatible tetraploid could form through crosses of diploid individuals translocated into backyard gardens; nevertheless, even in this comparative isolation with only a small group of diploids, the polyploid individual is still likely to succumb to minority cytotype exclusion through the production of sterile triploid seed. It could still be difficult or impossible for humans to cultivate and spread seed from single one-off individuals under such a scenario. Thus, minority cytotype exclusion is not only important in natural populations (e.g., Levin, 1975; Buggs and Pannell, 2006; Soltis et al., 2010) but also in plants undergoing incipient domestication.

There is considerable evidence that multiple independent origins may be relatively common for polyploids (e.g., Soltis and Soltis, 1999; Alexander et al., 2010; Cifuentes et al., 2010; Li et al., 2010; Symonds et al., 2010; Zhou et al., 2010). The limited sampling of intraspecific diversity employed in this study, with just a small number of accessions per species, and the general lack of well-supported resolution of clades within species, limit the scope to test for multiple vs. single origins from the same taxa for the five tetraploid species of *Leucaena*. However, there is some preliminary evidence for multiple origins of some tetraploids in some of the individual gene trees. For example, in the A9 gene tree (Appendix S1D), there are two robustly supported clades each containing accessions of diploid *L. pulverulenta* and tetraploid *L. leucocephala* and *L. diversifolia* that suggest the possibility of multiple independent origins of these two tetraploids. The possibility that some allopolyploids arose more than once could also have been an important contribution to their early establishment and success (Soltis et al., 2010); however, multiple origins can also be difficult to differentiate from heterozygous loci and a single origin.

Whatever the factors prompting initial polyploid establishment, several of the *Leucaena* polyploids are now widespread and abundant across large parts of Mexico and, in some cases, beyond. *Leucaena leucocephala* provides perhaps one of the most spectacular examples of geographical expansion of a recent allopolyploid. It is now one of the most common trees both in cultivation throughout tropical Mexico, in extensive secondary stands on ruderal sites, especially in the Yucatan peninsula and adjacent parts of southern Mexico, and in cultivation as a forage and agroforestry tree and an important invasive tree pantropically.

***Conclusions*—**The multilocus phylogenetic approach used here has provided potent insights into the origins of tetraploid species in *Leucaena*. Taken together with findings from Govindarajulu et al. (2011), a more complete picture of the complex evolutionary dynamics of polyploidy in *Leucaena* is emerging. This framework (Fig. 2) includes (1) an ancient paleotetraploidization event followed by extensive diploidization of the last common ancestor to extant *Leucaena* 25–10 Myr; (2) the subsequent largely allopatric divergence of 19 extant diploid species across geographically disjunct seasonally dry tropical habitats in the northern neotropics over the last 10 Myr (Govindarajulu et al., 2011); (3) more recent allopolyploid origins of the five tetraploid species, with a minimum of four unique allopolyploidization events between divergent parental diploids. The available data from archaeology (Zárate, 2000), patterns of artificial sympatry attributable to human translocation of seed and cultivation (Hughes, 1998a; Hughes et al., 2007), and lack of evidence for diploidization of these recent allopolyploids provide evidence that is consistent with some of the tetraploid *Leucaena* being derived via spontaneous hybridization induced by human translocation of seed in south-central México over the last 10 000 yr (Hughes et al., 2007). Our findings also identify potentially important diploid and polyploid traits favoring hybrid speciation both in the wild and under artificial selection in cultivation. This recent polyploidy in *Leucaena* has had important evolutionary and economic impacts and consequences. Several of the tetraploid species are now common, widespread, and abundant elements in secondary vegetation across south-central Mexico; several are among the most commonly cultivated trees in Mexico and are widely used as minor food plants; one tetraploid species, *L. leucocephala*, is a globally important agroforestry tree that is also a well-known invasive weed in many parts of the tropics.

Leucaena represents an interesting study system combining paleopolyploidy, diploidization, diploid divergence, and potentially recent neopolyploidy, each of which has more often been investigated separately in different genera (e.g., Ainouche et al., 2004; Adams and Wendel, 2005). Future studies on *Leucaena* could therefore investigate unique and conserved genomic reactions to allopolyploidy by comparing independently derived lineages within one genus. Last, the potential long-term impacts of cyclical polyploidy on *Leucaena* may well be countered by conversion to self-compatible lineages (in polyploid *Leucaena*), which are known to exhibit high rates of extinction compared to self-incompatible lineages (e.g., Goldberg et al., 2010).

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APPENDIX 1. Voucher information for (A) diploid and (B) polyploid accessions used in this study. (For complete locality information and GenBank accession information, see Appendix S6.) Seedlot number is provided if DNA was extracted from a seedling raised from a seed lot. Herbarium vouchers are all at FHO, with duplicates variously deposited at CAS, EAP, K, MEXU, NY, US, and MO.

Taxon, *Collector, collection number*, seed lot (where applicable—e.g., "51/81"), and locality.

(A) Diploid accessions—

See Govindarajulu et al. (2011, this issue) or online Appendix S6.

(B) Polyploid accessions—

***L. confertiflora* var. *adenotheloidea* (S. Zárate) C.E. Hughes**, *Hughes CE 1800*, -Mexico, Mexico. Hughes CE 1801, -Santa Catalina Oxolotepec, Puebla, Mexico. *Hughes CE 1730*, -Azumbilla, Puebla, Mexico. *Hughes CE 1731*, -Santa Catalina Oxolotepec, Puebla, Mexico. *Hughes CE 2051*, -Francisco I Madero, Puebla, Mexico. *Hughes CE 2063*, -Santa Catalina Oxolotepec, Puebla, Mexico. ***L. confertiflora* var. *confertiflora* S. Zárate**, *Hughes CE 1152*, -Oaxaca, Mexico. *Hughes CE 1653*, 127/92 -El Moral, Oaxaca, Mexico. ***L. diversifolia* (Schlechtendal) Bentham,** *Hughes CE 1613*, 81/92 -San Bartolo Tutotepec, Hidalgo, Mexico. *Hughes CE 1666*, 83/92 -Jalapa de Diaz, Oaxaca, Mexico. *Hughes CE 1693*, 82/92 -Barillas, Huehuetenango, Guatemala. *Hughes CE 921*, 46/87 -Veracruz, Mexico. ***L. involucrata* S. Zárate**, Hughes CE 1522, 146/91 -El Novillo,Sonora, Mexico. *Hughes CE 1572*, 87/92 -El Novillo, Sonora, Mexico. ***L. leucocephala* subsp. *glabrata* (Rose) S. Zárate**, *Hughes CE 1547*, 91/92 -El Pescadero, Baja California Sur, Mexico. *Hughes CE 1568*, 94/92 -Empalme, Sonora, Mexico. *Hughes CE 1578*, 93/92 -Ixmiquilapan, Hidalgo, Mexico. *Hughes CE 1596*, 86/92 -Jalpan de la Sierra, Queretaro, Mexico. *Hughes CE 1638*, 84/92 -Teotitlan del Camino, Oaxaca, Mexico. *Hughes CE 1679*, 136/92 -Cintalapa de Figueroa, Chiapas, Mexico. ***L. leucocephala* subsp. *ixtahuacana* C.E. Hughes**, *Hughes CE 1469*, 24/91 -Ixtahuacan, Huehuetenango, Guatemala. *Hughes CE 1689*, 117/92 -San Miguel, Huehuetenango, Guatemala. ***L. leucocephala* subsp. *leucocephala* (Lamark) de Wit**, *Hughes CE 1671*, 133/92 -Matias Romero, Oaxaca, Mexico. *Hughes CE 1734*, 80/92 -Francisco Escarcega, Campeche, Mexico. *Hughes CE 1735*, 147/92 -Chetumal, Quintana Roo, Mexico. *Hughes CE 1861*, 103/94/04 -Tihuatlan, Veracruz, Mexico. ***L. pallida* Britton & Rose**, *Hughes CE 1620*, 122/92 -Santiago Acatepec, Puebla, Mexico. *Hughes CE 1629*, 79/92 -Tamazulapan,Oaxaca, Mexico. *Hughes CE 1662*, 78/92 -Guelatao de Juarez, Oaxaca, Mexico.