American Journal of Botany

American Journal of Botany 98(9): 1486–1503. 2011.

Phylogenetics of the Chamaesyce clade (*Euphorbia*, Euphorbiaceae): Reticulate evolution and long-distance dispersal in a prominent C_4 lineage[1]

Ya Yang[2,4] and Paul E. Berry[2,3]

[2]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, 830 North University Avenue, Ann Arbor, Michigan 48109-1048 USA; and [3]University of Michigan Herbarium, 3600 Varsity Drive, Ann Arbor, Michigan 48108-2287 USA

- *Premise of the study:* The Chamaesyce clade of *Euphorbia* is the largest lineage of C_4 plants among the eudicots, with 350 species including both narrow endemics and cosmopolitan weeds. We sampled this group worldwide to address questions about subclade relationships, the origin of C_4 photosynthesis, the evolution of weeds, and the role of hybridization and long-distance dispersal in the diversification of the group.
- *Methods:* Two nuclear (ITS and exon 9 of *EMB2765*) and three chloroplast markers (*matK*, *rpl16*, and *trnL-F*) were sequenced for 138 ingroup and six outgroup species. Exon 9 of *EMB2765* was cloned in accessions with >1% superimposed peaks.
- *Key results:* The Chamaesyce clade is monophyletic and consists of three major subclades [1(2,3)]: (1) the Acuta clade, containing three North American species with C_3 photosynthesis and C_3-C_4 intermediates; (2) the Peplis clade, mostly North American and entirely C_4; and (3) the Hypericifolia clade, all C_4, with both New World and Old World groups. Incongruence between chloroplast and ITS phylogenies and divergent cloned copies of *EMB2765* exon 9 suggest extensive hybridization, especially in the Hawaiian Islands radiation.
- *Conclusions:* The Chamaesyce clade originated in warm, arid areas of North America, where it evolved C_4 photosynthesis. From there, it diversified globally with extensive reticulate evolution and frequent long-distance dispersals. Although many species are weedy, there are numerous local adaptations to specific substrates and regional or island radiations, which have contributed to the great diversity of this group.

Key words: C_4 photosynthesis; *Chamaesyce*; *Euphorbia*; Hawaiian Islands; long-distance dispersal; low-copy nuclear marker; reticulate evolution; short chloroplast genome inversion; weeds.

Within the large genus *Euphorbia* L., with some 2000 species, the Chamaesyce clade (*Euphorbia* subgenus *Chamaesyce* section *Anisophyllum* Roeper) comprises a group of ca. 350 species that are remarkably distinct within the genus. This group is cosmopolitan in distribution, but with a majority of species native to the New World (210 vs. 140 native to the Old World), running counter to the prevailing pattern in most other large clades of *Euphorbia* that are more diverse in the Old World (Steinmann and Porter, 2002). The Chamaesyce clade is probably best known for its globally pervasive weedy species, such as *E. maculata* L. (spotted spurge), a mainly temperate species with an affinity for sidewalk cracks, and *E. hirta* L., a species widespread in warm temperate and tropical regions. The weediness of these and other species is facilitated by precocious flowering, quick generation turnover (up to several generations per growing season), high seed set, and a specialized seed coat that becomes sticky when wet (Jordan and Hayden, 1992; Suzuki and Teranishi, 2005). On the other hand, many species in the Chamaesyce clade are quite restricted geographically, such as the eight species endemic to the Galapagos Islands (Burch, 1969) and the 29 taxa in 16 species limited to the Hawaiian Islands (Wagner et al., 1999). Although all members of the Chamaesyce clade share the pseudanthial cyathium that is a synapomorphy for *Euphorbia* (Prenner and Rudall, 2007), the clade differs markedly from the rest of the genus in having opposite, mostly asymmetrical leaves with interpetiolar stipules (Fig. 1). Most species are small, prostrate to ascending herbs, with a dichotomous branching pattern that is associated with the early abortion of the apical meristem (Fig. 1A; Degener and Croizat, 1938; Hayden, 1988). Another unique feature of the Chamaesyce clade within *Euphorbia* is the predominance of C_4 photosynthesis, which is both a physiological and anatomical system generally associated with plants adapted to warm, arid conditions (Fig. 1B) (Sage et al., 2011a). All these factors lead to interesting questions concerning the geographical origin of the Chamaesyce clade, the evolution of C_4 photosynthesis in

[1]Manuscript received 3 December 2010; revision accepted 6 June 2011.

The authors thank the authorities of Miami-Dade County Department of Parks and Recreation, the Institute for Regional Conservation (Miami, FL), Everglades National Park, Big Bend National Park, and the Nature Conservancy for collection permits; V. W. Steinmann, Y. Ramírez-Amezcua, R. Povilus, A. M. Powell, W. Weckesser, C. W. Morden, K. A. Bradley, J. Possley, A. Warren, and T. A. Glancy for help during fieldwork; S. Pereira, H. Draheim, and R. Povilus for help with laboratory work; B. van Ee, J. A. Peirson, R. Riina, J. J. Morawetz, Y.-W. Yuan, and B. Dorsey for valuable discussion and help revising the manuscript; J. W. Brown for assistance with data analysis; and two anonymous reviewers for help improving the manuscript. Funding was provided by the National Science Foundation through a Planetary Biodiversity Inventory Grant (DEB-0616533) to P.E.B., the Department of Ecology and Evolutionary Biology of the University of Michigan through a Block Grant to Y.Y., and the University of Michigan Matthaei Botanical Garden through a Winifred B. Chase Fellowship to Y.Y.

[4]Author for correspondence (e-mail: yangya@umich.edu)

doi:10.3732/ajb.1000496

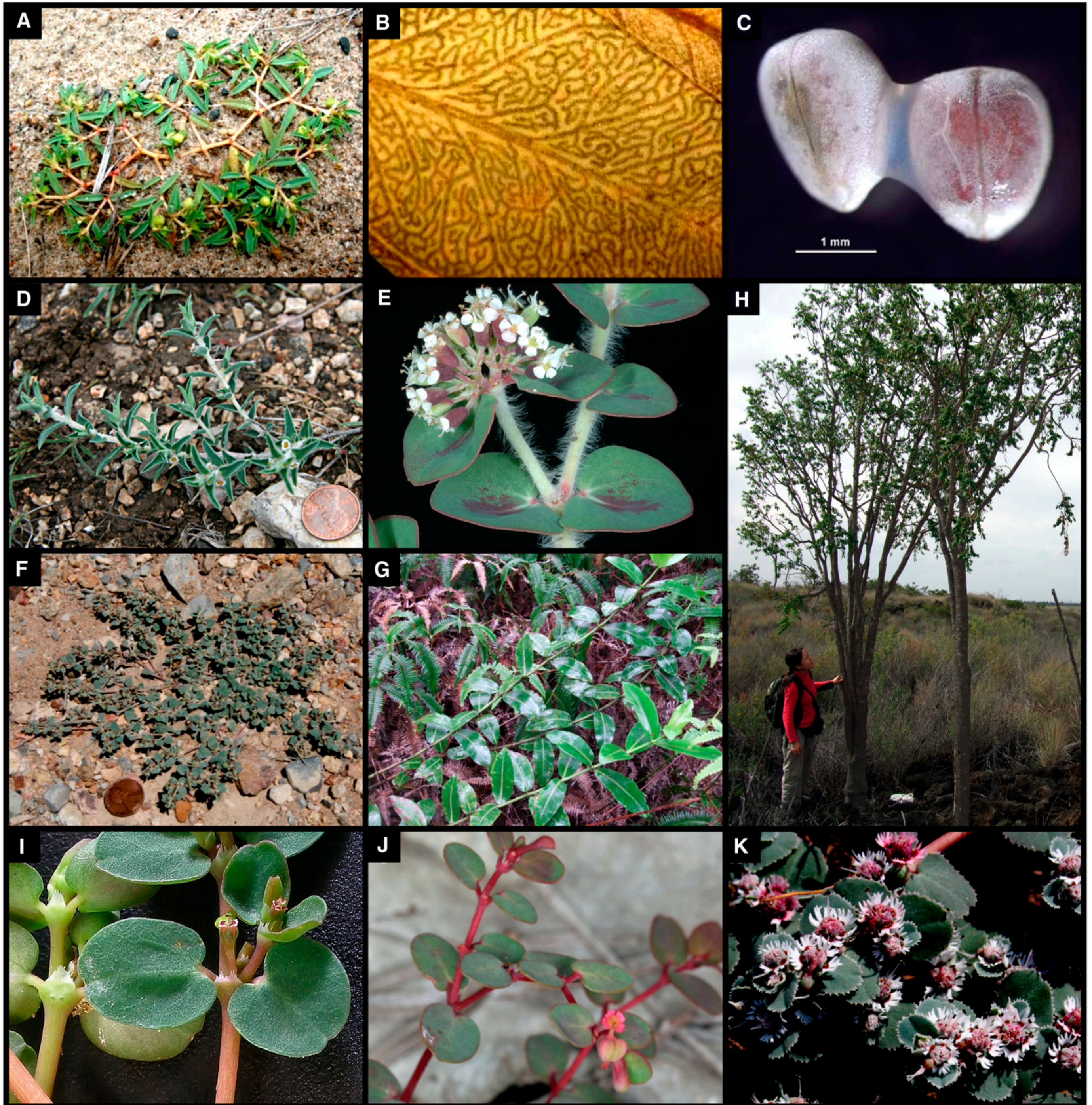


Fig. 1. Diversity of morphology and habitats in the Chamaesyce clade of *Euphorbia*. (A) *Euphorbia polygonifolia*, showing the typical prostrate, dichotomously branching growth form of the Chamaesyce clade (*Berry 7916*, MICH). (B) Dark-green veins associated with Kranz anatomy that are often visible on C_4 Chamaesyce leaves (*E. deppeana* Boiss., *Lau 2817*, BISH). (C) Two seeds of *E. polygonifolia* (*Berry 8023*, MICH), showing mucilaginous seed coats after a drop of water was added. (D) *E. acuta*, a C_3-C_4 intermediate species in the Acuta clade (*Yang 23*, MICH). (E) *E. umbellulata,* showing dichotomous branching and well-developed cyathial gland appendages (*Yang 91*, MICH). (F) *E. cinerascens*, a North America species closely related to the woody Hawaiian Chamaesyces (*Yang 6*, MICH). (G) *E. remyi* var. *remyi*, a C_4 wet-forest understory shrub endemic to the island of Kauai. (H) *E. olowaluana*, a C_4 tree and pioneer species on recently formed lava fields, Hawaii. (I–K) Members of the *E. serpens* species complex. (I) *E. serpens,* a prostrate herb widespread in the New World and introduced to the Old World (*Aedo 18005*, MA). (J) *E. porteriana*, an ascending herb restricted to limestone outcrops in southern Florida (*Yang 131*, MICH). (K) *E. hooveri*, an annual species endemic to vernal pools in the Central Valley of California. *Photo credits:* (D&F), R. Povilus; (H), C. W. Torres-Santana; (E), V. W. Steinmann; (I), C. Aedo.

Euphorbia, adaptations for weediness and long-distance dispersal, and mechanisms that might explain the multiple radiations of species in different regions of the globe.

Taxonomic issues in the Chamaesyce clade have revolved mainly around the appropriate rank at which to recognize the group. Some botanists (for example, Wheeler, 1941; Burch, 1965; Hassall, 1977; Koutnik, 1987) recognized the group as a separate genus, *Chamaesyce* Gray, because it is very easy to distinguish from other *Euphorbia* species. Others, from Boissier (1862) to Bruyns et al. (2006), treated the group as part of *Euphorbia*, usually as a section, and Boissier was correct in treating it as section *Anisophyllum* Roeper. To date, Boissier (1862) was the only botanist to propose subclades within the Chamaesyce clade, in which he recognized eight subsections. Six of these represented small, relatively well-circumscribed groups of species. The other two subsections, however, were large and diverse, including both Old World and New World species; subsect. *Chamaesyce* included a group of 99 mostly prostrate species with solitary cyathia, and subsect. *Hypericifoliae* Boiss. comprised a group of 30 mostly erect species with clustered cyathia. All genus-wide molecular studies to date have unequivocally placed the Chamaesyce clade within *Euphorbia*, in the same subgeneric clade as the New World "Agaloma alliance", which includes *E. pulcherrima* Willd., the familiar Christmas poinsettia (Steinmann and Porter, 2002; Bruyns et al., 2006; Park and Jansen, 2007; Zimmermann et al., 2010). The problem with inferring deeper relationships within the Chamaesyce clade is that until now only 11 species have been molecularly sampled, so there has been insufficient coverage to assess any of the main questions raised above or to assess Bossier's subsectional classification.

Euphorbia is the only plant genus known to exhibit C_3, C_4, and CAM photosynthetic systems. Webster et al.'s (1975) carbon isotope ratio determinations in *Euphorbia* found that C_4 species were restricted to the Chamaesyce clade, although two species in this group endemic to the southwestern United States and northern Mexico, *E. acuta* Engelm. and *E. angusta* Engelm., had isotope ratios consistent with C_3 photosynthesis. These findings led Webster to hypothesize that the Chamaesyce clade had originated in subtropical and warm temperate areas in North America from C_3 ancestors, with *E. acuta* and *E. angusta* representing a transitional stage. Sage et al. (2011b) subsequently used more refined techniques to confirm that *E. angusta* has a C_3 system, whereas the closely related *E. acuta* and *E. johnstonii* Mayfield are actually intermediate C_3-C_4 species. There are no clear reversals to C_3 photosynthesis in the Chamaesyce clade, although there is a radiation of 16 woody species in the Hawaiian Islands that includes several species restricted to wet montane forest understories or bogs and one species that forms trees up to 10 m tall (Fig. 1G, H; Koutnik, 1987; Lorence and Wagner, 1996). C_4 species that grow in such mesic habitats or as trees are highly unusual, and there is evidence that some of these species have experienced modifications of the specialized Kranz leaf anatomy (Herbst, 1971, 1972; Pearcy and Troughton, 1975; Sporck and Sack, 2010). By including more samples among these species in particular, we aim to better understand the dynamics of C_4 photosynthesis in the Chamaesyce clade.

In this study, we used comprehensive taxon sampling and sequencing of the nuclear ribosomal ITS region, the nuclear low-copy coding region exon 9 of *EMBRYO DEFECTIVE 2765*, and three chloroplast loci (*matK*, the *rpl16* intron, and the *trnL-F* spacer) to reconstruct the phylogenetic relationships within the Chamaesyce clade of *Euphorbia*. We first wanted to test the monophyly of the entire clade and then determine the precise placement of the C_3 and C_3-C_4 intermediate species in relation to the more numerous C_4 species. We then used the resulting phylogeny to determine the distribution of New World vs. Old World taxa in the clade, looking for evidence of long-distance dispersal events and correlations with particular habitat types such as deep sand substrates or beach strand vegetation, as well as inferring the position of weedy taxa and their role in the diversification of the clade. Finally, after detecting evidence of reticulate evolution through contrasting nuclear and chloroplast phylogenies, we cloned the nuclear low-copy coding region exon 9 of *EMB2765* in a subset of species to detect the presence of multiple alleles and further evidence of hybridization.

MATERIALS AND METHODS

Taxon sampling—A total of 450 accessions from 138 species within the Chamaesyce clade were sequenced for this study. Of these, 149 ingroup accessions were used in the analyses presented here, and duplicate accessions of a given taxon with identical or nearly identical sequences were excluded. In addition, six outgroup taxa were selected following previous molecular phylogenetic studies in *Euphorbia* (Steinmann and Porter, 2002; Bruyns et al., 2006; Park and Jansen, 2007; Zimmermann et al., 2010). Silica-dried material was obtained from collecting trips from 2004 to 2009 covering the major biogeographical regions where natural populations of Chamaesyce species occur: (1) North America: southern United States and northern Mexico; (2) the Caribbean: Dominican Republic, Puerto Rico, and Cuba; (2) South America: Argentina, Brazil, Colombia, and Venezuela; (3) Africa: Morocco, Kenya, Tanzania, South Africa, and Madagascar; and (4) Eurasia: Portugal, Spain, Italy, Greece, Oman, and Russia. Additional silica-dried materials were obtained from collaborators from Thailand and northern Mexico. DNA of eight Hawaiian species was contributed by the Hawaiian Plant DNA Library (Morden et al., 1996; Randell and Morden, 1999). Leaf fragments were sampled from herbarium material to fill in sampling gaps, especially native species from Australia, Pacific and Atlantic islands, eastern Africa, and South America. Voucher information is presented in Appendix 1.

DNA extraction, PCR, and sequencing—Total genomic DNA was extracted from silica-dried leaf fragments using DNeasy Plant Mini Kits (QIAGEN, Valencia, California, USA) following the manufacturer's instructions, with modified protocols for herbarium material. Genomic DNA was diluted 10–50 times to reduce inhibition of PCR enzymes by secondary compounds.

More than 30 previously published primer pairs were tested for polymerase chain reaction (PCR) amplification specificity, numbers of phylogenetically informative sites, indel richness, and the presence of long poly A/T regions that interrupt sequencing reactions. We also screened nuclear low-copy markers to verify that only one orthologous copy is amplified in the majority of ingroup taxa. Of these, five regions were selected for this study: the nuclear ribosomal internal transcribed spacer (ITS) region; a nuclear low-copy coding region, exon 9 of *EMBRYO DEFECTIVE 2765* (*EMB2765*); the chloroplast (cpDNA) coding region *matK* with adjacent partial *trnK* intron, and noncoding regions *rpl16* intron and *trnL-F* spacer.

All PCR reactions from genomic DNA were carried out using *Ex Taq* (Takara Bio, Otsu, Shiga, Japan). A negative control using nuclease-free water instead of template DNA was included in each PCR reaction to test for contamination. The PCR mixture contained 0.1 μL of 5 units/μL *Ex Taq* (increased to 0.15 μL with difficult samples), 1.5 μL 10× Ex Taq Buffer, 1.2 μL dNTP (2.5 mmol/L), 0.5 μL of each primer (10 μmol/L), 0.5 μL betaine solution (5 mol/L, Sigma-Aldrich, St. Louis, Missouri, USA), 2 μL of diluted template DNA, and ddH_20 to bring the final volume to 15 μL.

The ITS region was amplified using primer pair ITS-I (Urbatsch et al., 2000) and ITS4 (White et al., 1990). When amplification failed, generally in herbarium samples, internal primers ITS2 and ITS3 (White et al., 1990) were used to amplify the ITS region in two pieces with ITS-I-ITS2 and ITS3-ITS4, respectively. The PCR profile consisted of an initial 2 min denaturing step at 95°C; 40 cycles of 45 s denaturation at 95°C, 45 s annealing at 48°C, and 45 s extension at 72°C; and a 4 min final extension at 72°C. The primer pair trnK570f and matK1710r (Samuel et al., 2005) was used to amplify the *matK* coding region

and the adjacent partial *trnK* intron. When those primers were unsuccessful, two additional internal primers newly designed for this study were used to amplify the region in two pieces with trnK570f-matK1100r (5′-TTC TGG TTG AAA CCA CAC-3′) and matK880f (5′-GCG TCT TTC TTG AAC GAA T-3′)-matK1710r, respectively. Similarly, the *rpl16* intron was amplified using primer pair rpl16-71f (Jordan et al., 1996) and rpl16-1516r (Kelchner and Clark, 1997), and internal primers were designed to amplify this region in two pieces in difficult materials, with rpl16-71f-rpl16-770r (5′-GAG AGG TAA CCC ATG ATC TC-3′) and rpl16-431f (5′-AGA AGT GAT GGG AAC GAT GG-3′)-rpl16-1516r, respectively. The *trnL-F* spacer was amplified using primer pair trnL-e and trnL-f (Taberlet et al., 1991). The PCR profile for all three cpDNA regions consisted of an initial 2 min denaturing step at 95°C, 40 cycles of 45 s denaturing at 95°C, 45 s annealing at 54°C, and 1.5–2 min/kb "slow and cold" extension at 65°C (Shaw et al., 2007); with a final extension of 8 min at 65°C. *EMB2765* was PCR-amplified using the primer pair EMB2765ex9F2 and EMB2765ex9R (Wurdack and Davis, 2009). The PCR profile consisted of an initial 2 min denaturing step at 95°C; 40 total cycles of 50 s denaturing at 95°C; a touchdown program of 1 min annealing at 60°C for 1 cycle, 59°C for 2 cycles, 58°C for 3 cycles, 57°C for 4 cycles, 55°C for 5 cycles, 52°C for 6 cycles and 50°C for 19 cycles, and a 1.5 min extension at 72°C for all 40 cycles to minimize PCR-induced recombination (Cronn et al., 2002); then a 10 min final extension at 72°C.

EMB2765 PCR products with greater than 1% superimposed peaks were purified with QIAquick PCR Purification Kit (QIAGEN) and cloned using TOPO TA Cloning Kit (Invitrogen, Carlsbad, California, USA) following the manufacturer's instruction. Transformed clones were incubated for 20 h at 37°C. Positive clones were picked and PCR-amplified directly. Each PCR reaction contained 0.05 μL Taq (5 units/μL, Qiagen, Valencia, California, USA), 1.5 μL 10× buffer, 0.5 μL $MgCl_2$ (2 mmol/L), 1.2 μL dNTP mix (2.5 mmol/L), 0.5 μL each of M13 primers (10 μmol/L, supplied with the TOPO TA kit) and 10.95 μL of ddH_2O. Cycling conditions were: 94°C for 4 min for cell lysis; 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1 min; and a final extension step of 72°C for 4 min.

All PCR products were examined by gel electrophoresis on 1% agarose gels. When positive, products were purified with ExoSap-IT (USB Corp., Cleveland, Ohio, USA). For weak PCR products, or products with primer dimers, the QIAquick PCR Purification Kit was used instead of ExoSap-IT. Cleaned PCR products were sequenced at the University of Michigan DNA Sequencing Core using the same PCR primers. For PCR products longer than 1 kb (*matK* and *rpl16*), internal PCR primers were also used for sequencing to ensure double coverage. For PCR-amplified positive clones, typically eight clones with the correct insertion size were sequenced once using the EMB2765ex9R primer only.

Phylogenetic analyses—Sequences were assembled and edited in the program Sequencher v. 4.10.1 (Gene Codes, Ann Arbor, Michigan, USA). Sequences were aligned in the program MUSCLE v. 4 (Edgar, 2004) using the default parameters and manually adjusted in the program MacClade v. 4.08 (Maddison and Maddison, 2005). The full-length data matrices are archived in TreeBASE (http://www.treebase.org, study number 11056), and sequences are deposited in GenBank (Appendix 1).

Segments of chloroplast gene regions (*matK*, *rpl16*, and *trnL-F*) with poly A/T length variation or variable short repeats of uncertain homology were excluded from the analyses. Two short chromosomal inversions were detected in the *rpl16* intron region (Fig. 2). Both regions were inverted and complemented for phylogenetic analysis without scoring them as binary data (Kim and Donoghue, 2008). A separate analysis was done excluding regions with the inversion.

Indels were not coded for ITS and *EMB2765*. For *matK*, *rpl16*, and *trnL-F*, indels that could be unambiguously aligned were coded as binary characters following the simple gap coding criteria of Simmons and Ochoterena (2000), as implemented in the IndelCoder module of the program SeqState v. 1.4.1 (Müller, 2006).

Each of the three cpDNA gene regions was initially analyzed separately using maximum likelihood (ML) and Bayesian inference (BI). Congruence between the individual chloroplast gene trees was visually inspected before concatenating the three regions into the first three character sets of the cpDNA matrix. Binary indels from all three cpDNA markers were concatenated and became the fourth character set of the cpDNA matrix.

EMB2765 sequences with less than 1% superimposed peaks were coded as ambiguous at those sites, but sequences with more than 1% superimposed peaks were excluded and replaced by sequences from clones. When multiple sequenced clones had identical sequences, they were represented by a single sequence. ITS, *EMB2765*, and combined cpDNA matrices were each subjected to the analyses described next.

Models of sequence evolution that best fit each gene region were determined by the Akaike information criterion (AIC) implemented in the program MODELTEST v. 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). Maximum likelihood (ML) analyses were performed in the program GARLI v. 1.0 (Zwickl, 2006) for ITS and *EMB2765* using the best-fit model. Grouping credibility was assessed with 1000 bootstrap replications. Since GARLI was unable to conduct partitioned analyses, the combined cpDNA data set was analyzed using the program RAxML v. 7.0.3 (Stamatakis, 2006), partitioning each marker. Indels were excluded since neither GARLI nor RAxML is able to analyze binary data in their present versions. The nucleotide substitution model was set to GTR + γ as recommended by the RAxML manual, and 1000 ML bootstrap replicates were performed, followed by a thorough search for the best tree.

Bayesian inference was conducted in the program MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Four independent runs of four chains each (three heated, one cold), starting from random trees, using the default temperature of 0.2, were run for 25 million generations (10 million for cpDNA). Trees were sampled every 5000 generations (1000 for cpDNA). Each analysis was conducted using the nucleotide substitution model GTR + I + γ as selected by AIC in MrModeltest v. 2.3 (Nylander, 2004). For the cpDNA data, the three concatenated gene regions, plus a binary indel data set, were partitioned into four character sets, allowing all parameters to be unlinked except branch length and topology. The binary indels were subject to "rates=gamma" since there is no invariable character in this data set. A branch length prior "brlenspr=unconstrained:exponential(100.0)" was applied to both the cpDNA and *EMB2765* analyses to prevent unrealistically long branches (Brown et al., 2010; Marshall, 2010). All parameters were visually examined in the program Tracer v. 1.5 (Rambaut and Drummond, 2007) to verify stationary status. Trees from the first 2.5 million generations were discarded as the burn-in period, and the remaining trees were used to compute the majority rule consensus.

Before combining ITS, *EMB2765*, *matK*, *rpl16*, and *trnL-F* into a 5-locus data set, suspected hybrid accessions were excluded if they had divergent copies of *EMB2765* in the 50% majority-rule consensus tree recovered from the Bayesian analysis. When *EMB2765* data were not available, accessions were also excluded if they had incongruent ITS and cpDNA placement with Bayesian posterior probability (PP) ≥0.95 or ML bootstrap ≥70%. The 114 remaining accessions were combined into the 5-locus data set. No binary indel data were coded in this data set. The ML and Bayesian analyses were carried out following the same methods as for the cpDNA data set, except the 5-locus data set was partitioned into three character sets by ITS, *EMB2765*, and cpDNA regions.

RESULTS

Monophyly of the Chamaesyce clade is highly supported by both ML and BI analyses of all data sets. Results also support a clade consisting of *E. angusta*, *E. acuta*, and *E. johnstonii* as sister to the rest of the Chamaesyce clade (Figs. 3–5). These three species correspond to *Euphorbia* subsect. *Acutae* Boiss., as modified by Mayfield (1991), hereafter referred to as the Acuta clade. Overall statistics of all gene regions sequenced for this study are summarized in Table 1, and results of the phylogenetic analyses are shown in Figs. 3–5.

Chloroplast* matK, rpl16, trnL-F, *and the combined cpDNA data set—We were able to obtain sequences of all three cpDNA gene regions from 150 of the 155 total accessions. The remaining five had either one or two regions that did not amplify due to degraded template DNA. The cpDNA alignments were rich in highly variable poly A/T and microsatellite repeats, especially in *trnL-F*, in which 227 of the 767 characters were excluded in three poly A/T sections and a 102-bp microsatellite repeat region. After excluding poly A/T and microsatellite regions, the remaining alignments were well aligned yet indel-rich, especially in *rpl16*, in which 184 indels were coded from the 1752-bp alignment. In the *matK* marker, the majority of indels came from the noncoding partial *trnK* intron region (Table 1).

Two short chromosomal inversions were detected in the *rpl16* intron region. The first, a 33-bp inversion starting from base pair 1280 in the initial alignment (Fig. 2A), was observed in all

TABLE 1. Summary of chloroplast and nuclear gene regions used in this study. The cpDNA matrix comes from concatenated *matK*, *rpl16*, *trnL-F*, and the binary indel data set. The 5-locus data set comes from concatenated exon 9 of *EMB2765*, ITS, *matK*, *rpl16*, and *trnL-F*.

	Chloroplast gene regions					Nuclear gene regions		
Matrix information	*matK*	*rpl16*	*trnL-F*	Combined cp indels	Combined cpDNA	ITS	*EMB2765* exon 9	5-Locus data set
Number of accessions	153	154	150	154	154	153	124	114
Range of raw length [a] (bp)	839–1944	446–1366	258–481	334	802–3572	346–1068	621–767	1976–5136
Excluded characters	32	117	227	–	376	–	–	–
Aligned length (after exclusion)	2128	1752	540	334	4754	1213	767	6286
Variable sites (proportion)	691 (32.5%)	613 (35.0%)	203 (37.6%)	334 (100%)	1844 (38.8%)	491 (40.5%)	321 (41.9%)	2090 (33.2%)
No. of indels coded	88	184	62	–	334	–	–	–
Nucleotide substitution model selected by AIC	TVM+γ	GTR+I+γ	TVM+γ	–	TVM+I+γ	GTR+I+γ	TrN+I+γ	–

[a] Lower ends of raw lengths are from partial sequences that the full-length sequences failed to amplify or sequence.

sequenced accessions of *E. hirta*, *E. jejuna* M.C.Johnst. & Warnock, *E. riebeckii* Pax, *E. schizolepis* F.Muell. ex Boiss., and *E. potentilloides* Boiss., and in one of the four sequenced accessions of *E. cinerascens* Engelm. Monophyly of these six accessions is strongly rejected by all other cpDNA and nrDNA markers, as well as the rest of *rpl16*. The second inversion in *rpl16* is 38 bp long (Fig. 2B) and is found in *E. stictospora* Engelm., *E. velleriflora* (Klotzsch & Garcke) Boiss., and in one of the two sequenced accessions of *E. mendezii* Boiss., starting from base pair 1438 in the initial alignment. Monophyly of these three accessions is strongly supported by ITS and cpDNA, but not by *EMB2765*. Since only two base-pair substitutions occurred in the 33-bp inversion, and all three accessions in the 38-bp inversion had identical sequences, we reversed and complemented both inversions and included them in the alignment rather than coding them as binary data (Kim and Donoghue, 2008). In this manner, the inversion events were not scored for analysis, but the phylogenetic signal in corresponding aligned segments was retained. Analyses excluding both aligned segments containing inversions gave the same tree topology and highly similar branch support values (results not shown).

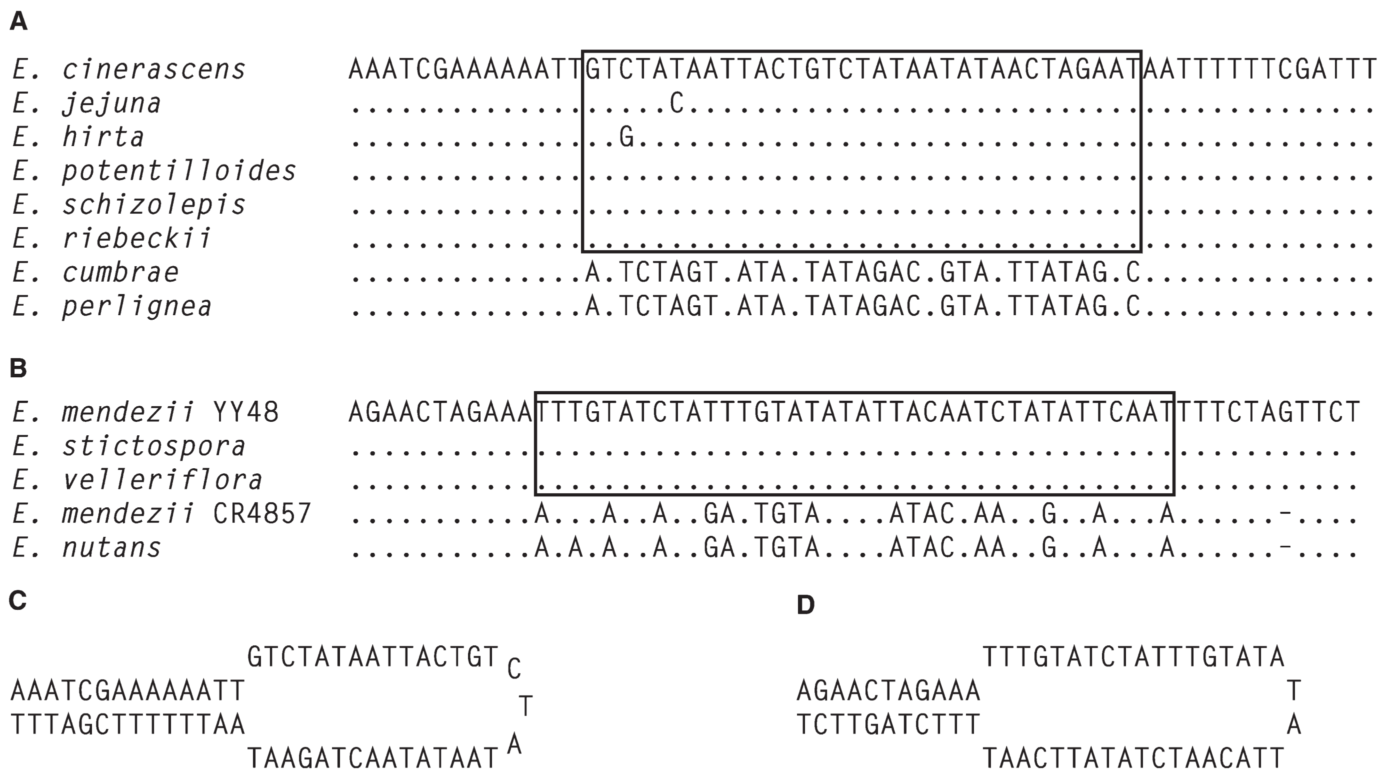


Fig. 2. Short inversions and flanking inverted repeats found in the *rpl16* intron region. (A) The box identifies the 33-bp inversion shared by *E. cinerascens*, *E. jejuna*, *E. hirta*, *E. potentilloides*, *E. schizolepis*, and *E. riebeckii*, flanked by 14-bp inverted repeats on both sides. (B) The box identifies the 38-bp inversion shared by *E. stictospora*, *E. velleriflora*, and one of the two sequenced accessions of *E. mendezii*, flanked on both sides by 11-bp inverted repeats. (C) Secondary stem-loop structure of the DNA region shown in (A) inferred for *E. cinerascens*. (D) Secondary stem-loop structure of the DNA region shown in (B) inferred for *E. stictospora*. Dots represent bases that are identical to the first row in the alignment; dashes indicate gaps created by a single base-pair insertion.

Analysis of the cpDNA sequences produced a well-resolved backbone within the Chamaesyce clade, with three highly supported subclades, which we call the Acuta clade, the Peplis clade, and the Hypericifolia clade (Fig. 3). The Peplis clade and the Hypericifolia clade are strongly supported as sister to each other, and together we call them core Chamaesyce. The ML and BI analyses produced congruent tree topologies. However, a few clades that are supported by ML with bootstrap values ≥70% also received Bayesian PP <0.50. Similarly, a few clades with ≥0.95 Bayesian PP received ML bootstrap values of 60% or less. None of these discrepancies are located along the backbone, and they only affect the interpretation of relationships among closely related species. These local discrepancies could be explained by the fact that BI incorporates the binary indel data, whereas ML implemented in RAxML does not.

Nuclear ribosomal ITS—The nuclear ribosomal ITS region was successfully PCR-amplified in all but two of the 155 accessions. Occasional superimposed peaks (<1% in each sequence) were observed in a number of taxa. Higher rates of superimposed peaks (>2% in each sequence) were found in all native Hawaiian taxa. When we cloned the ITS sequences of the Hawaiian taxa, we recovered more than eight different alleles, including a possible pseudogene copy as evidenced by an unusually variable 5.8S-coding region (data not shown). Two other species, *E. leucantha* (Klotzsch & Garcke) Boiss. and *E. tamanduana* Boiss., had continuously superimposed ITS sequences, and their sequences were excluded from the ITS analyses.

The ITS region has a relatively high proportion of variable (40.5%) sites compared to cpDNA loci (Table 1). The BI and ML results are congruent with the same taxon grouping when ML bootstrap support is ≥70% and Bayesian PP is ≥0.95 (Fig. 3). Monophyly of the entire Chamaesyce clade, the Acuta clade, and core Chamaesyce are each well supported. Relationships among the major lineages within core Chamaesyce are less resolved compared to the cpDNA results, although in general the fine-scale relationships are better resolved by ITS.

Well-supported clades (Bayesian PP ≥ 0.95 and ML bootstrap ≥ 70%) are generally congruent between ITS and cpDNA, but there are 16 species with conflicting placement between ITS and cpDNA in well-supported clades (Fig. 3, species joined by lines between the trees). Also, the 17 taxa on the ITS phylogeny from *E. deltoidea* down to *E. jejuna* are grouped within the Peplis clade with moderate to weak support (Bayesian PP = 0.92, ML bootstrap < 50%), whereas cpDNA data strongly support placement of these taxa within the Hypericifolia clade (Bayesian PP = 1.00, ML bootstrap ≥ 85%). Given the low support levels of the branches leading to this group in the ITS phylogeny compared to the robust support values in the cpDNA tree, we included these taxa in the Hypericifolia clade in Fig. 3.

***Nuclear low-copy coding region* EMB2765**—PCR amplification and direct sequencing of PCR products were successfully carried out in 124 of the 154 accessions for *EMB2765*. Among them, 94 had less than 1% superimposed peaks, and the remaining 30 accessions with higher levels of superimposed peaks were cloned. Monophyly of the Chamaesyce clade, the Acuta clade, and core Chamaesyce are each highly supported, but relationships among major lineages within core Chamaesyce are poorly resolved (Fig. 4). Branches that are well-supported are generally congruent among *EMB2765*, ITS, and cpDNA (Figs. 3, 4). When placement of species in the ITS and cpDNA phylogenies conflict with each other (Fig. 3), they often correspond to divergent placements of *EMB2765* copies, as seen in *E. eichleri* Müll.Arg., *E. carissoides* F.M.Bailey, and *E. porteriana* (Small) Oudejans (Fig. 4). *EMB2765* also reveals a number of cases in which multiple divergent alleles were recovered even when there is no significant conflicting placement between ITS and cpDNA, such as in *E. maculata* (Fig. 4, in pink) and all native Hawaiian species in our sampling (Fig. 4, in red). The majority of cloned accessions in the Hypericifolia clade have alleles that are resolved in different positions within that clade, and these separations are at least moderately supported by Bayesian PP ≥ 0.80 or ML bootstrap ≥ 50%. In contrast, the majority of accessions cloned in the Acuta clade and the Peplis clade have alleles that are clustered together with their conspecific copies (Fig. 4, in green), except in the case of *E. hooveri* Wheeler, which has two divergent alleles, one of them nested within accessions of *E. albomarginata* Torr. & A.Gray and the other nested within *E. serpens* Kunth (Fig. 4, in orange). In addition, there are four species that have divergent copies resolving in both the Peplis clade and the Hypericifolia clade, namely *E. blodgettii* Engelm. ex Hitchc., *E. garberi* Engelm. ex Chapm, *E. porteriana*, and *E. klotzschii* Oudejans (Fig. 4, in brown).

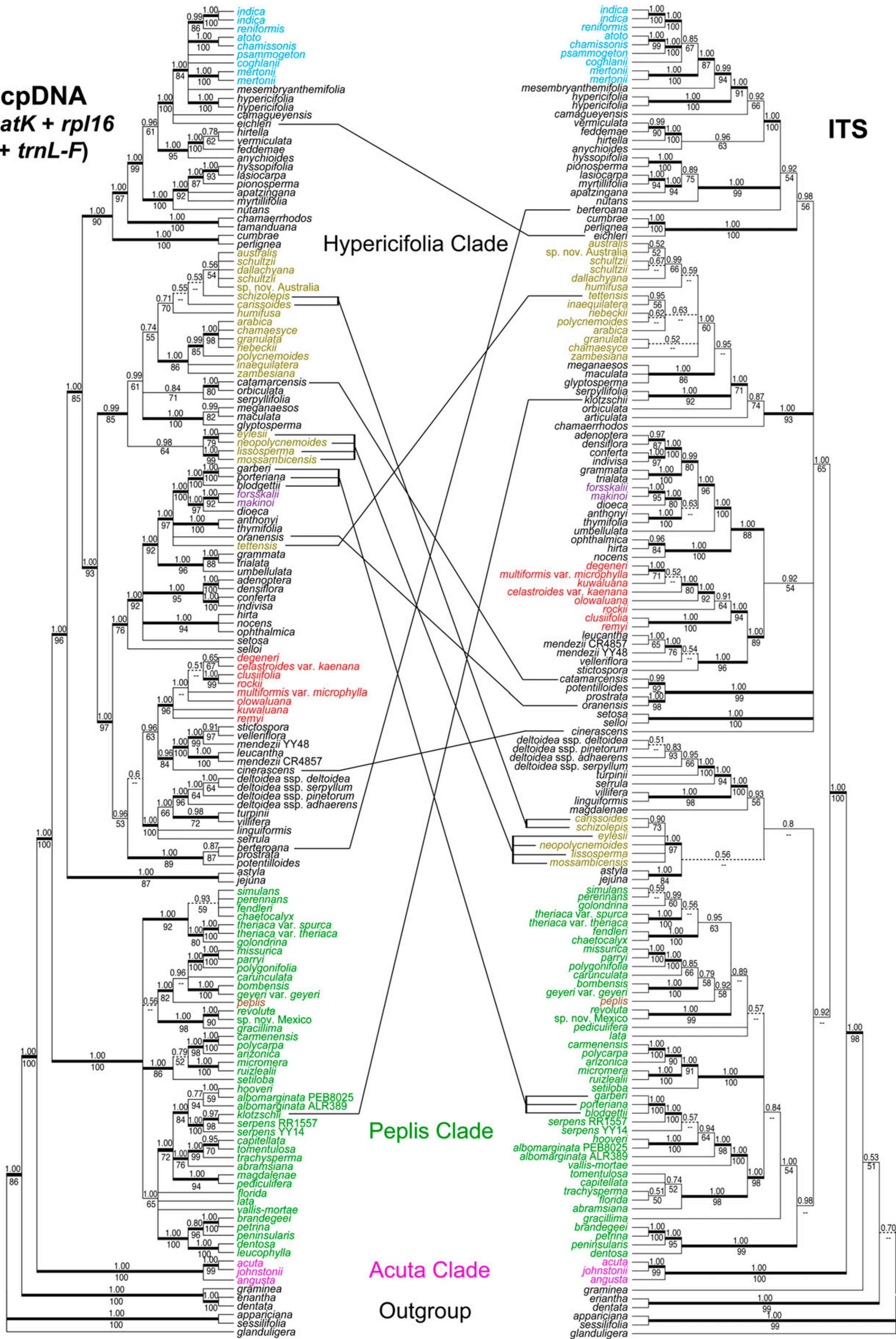
Five-locus data set—Data from all five loci were combined after removal of 35 accessions that were identified as possible hybrids (Appendix 2). The 114 taxa remaining in the 5-locus data set produced a well-supported phylogeny, with most branches having Bayesian PP ≥0.95 and ML bootstrap ≥70% (Fig. 5).

DISCUSSION

Three major subclades within the Chamaesyce clade—In agreement with previous molecular studies (Steinmann and Porter, 2002; Bruyns et al., 2006; Park and Jansen, 2007; Zimmermann et al., 2010), all our data sets (ITS, cpDNA, *EMB2765*, and 5-locus) strongly support the monophyly of the Chamaesyce clade and its nested position within *Euphorbia.* Three major lineages within the Chamaesyce clade are strongly supported by both the cpDNA and the 5-locus data sets, namely, the Acuta clade, the Peplis clade, and the Hypericifolia clade (Figs. 3, 5, 6). Morphologically, species in the Acuta clade can be distinguished from the core Chamaesyce by their reduced stipules, lack of the typical C_4 Kranz anatomy (Webster et al., 1975), and decussate rather than distichous leaves. In the core Chamaesyce clade, however, there is no single character that can readily distinguish a species in the Peplis clade from those in the Hypericifolia clade. Nonetheless, it is notable that the majority of species in the Peplis clade are glabrous, perennial herbs with entire leaf margins. Species in the Hypericifolia clade are considerably more diverse, varying from annual and perennial herbs to woody perennials, often with toothed leaf margins and usually with some kind of pubescence.

There is very little correspondence between Boissier's (1862) large subsections *Hypericifoliae* and *Chamaesyce* and either the Peplis or Hypericifolia clades identified from our molecular data. Therefore, Boissier's previous classification appears to be of little value in designating major monophyletic groups. His other small subsections are nested within the Hypericifolia clade (two were not represented in our sampling), except for subsect. *Acutae*, which corresponds to our Acuta clade.

Evolution of C_4 photosynthesis—The C_4 photosynthetic system evolved at least 62 times in the angiosperms, with 36


cpDNA
(matK + rpl16
+ trnL-F)
ITS
Hypericifolia Clade
Peplis Clade
Acuta Clade
Outgroup

independent occurrences in the eudicots (Sage et al., 2011a). Within Euphorbiaceae, the only known C_4 species are members of the Chamaesyce clade. This is supported by genus-wide surveys of Kranz anatomy, CO_2 compensation points and $^{13}C/^{12}C$ isotope ratios (Webster et al., 1975; Batanouny et al., 1991). With 350 species, the Chamaesyce clade is the most species-rich C_4 eudicot lineage, containing around one fifth of all C_4 eudicot species. The question of where the C_3 to C_4 transition occurred, however, has been unclear because of the uncertainty of photosynthetic states and lack of knowledge about the phylogenetic relationships of the species.

Due to their general morphological resemblance and their largely overlapping distribution, Boissier (1862) grouped *E. acuta*, *E. angusta*, and *E. lata* Engelm. into subsect. *Acutae.* This classification was followed by Webster et al. (1975), who included all three species in the transitional group between C_3 outgroups and the remaining C_4 species. Mayfield (1991) subsequently modified this circumscription by removing the C_4 *E. lata* from subsect. *Acutae* and adding to the group a newly described species, *E. johnstonii*, a northern Mexican endemic that also possesses reduced stipules and lacks Kranz anatomy. Mayfield's taxonomy is confirmed by the molecular data presented here, with all four data sets grouping *E. acuta*, *E. angusta*, and *E. johnstonii* in the Acuta clade (= subsect. *Acutae*), whereas *E. lata* was recovered nested within the Peplis clade (Figs. 3, 5).

Webster's scenario of a transitional C_3 Acuta clade was complicated by the findings of Sage et al. (2011a), who provided detailed data indicating that both *E. acuta* and *E. johnstonii* are in fact C_3-C_4 intermediates. In *E. acuta*, which was examined in more detail, there is low activity of key C_4 enzymes, and therefore it has a C_3-like carbon isotope ratio in its leaves. However, Kranz-like anatomy and localization of glycine decarboxylase in the bundle sheath cells of *E. acuta* indicate that it is able to scavenge CO_2 produced by photorespiration in enlarged bundle sheath cells. This trait is considered to be an early and key step in the evolution from C_3 to C_4 photosynthesis. On the other hand, *E. angusta* was confirmed to be a true C_3 species, and the species now excluded from subsect. *Acutae*, *E. lata*, has typical C_4 features in anatomy, gas exchange, and activities of key photosynthetic enzymes. Thus, the results of Sage et al. (2011a) confirm the recircumscription of subsect. *Acutae* by Mayfield (1991), and there is likely a partial transition from C_3 to C_4 in the Acuta clade.

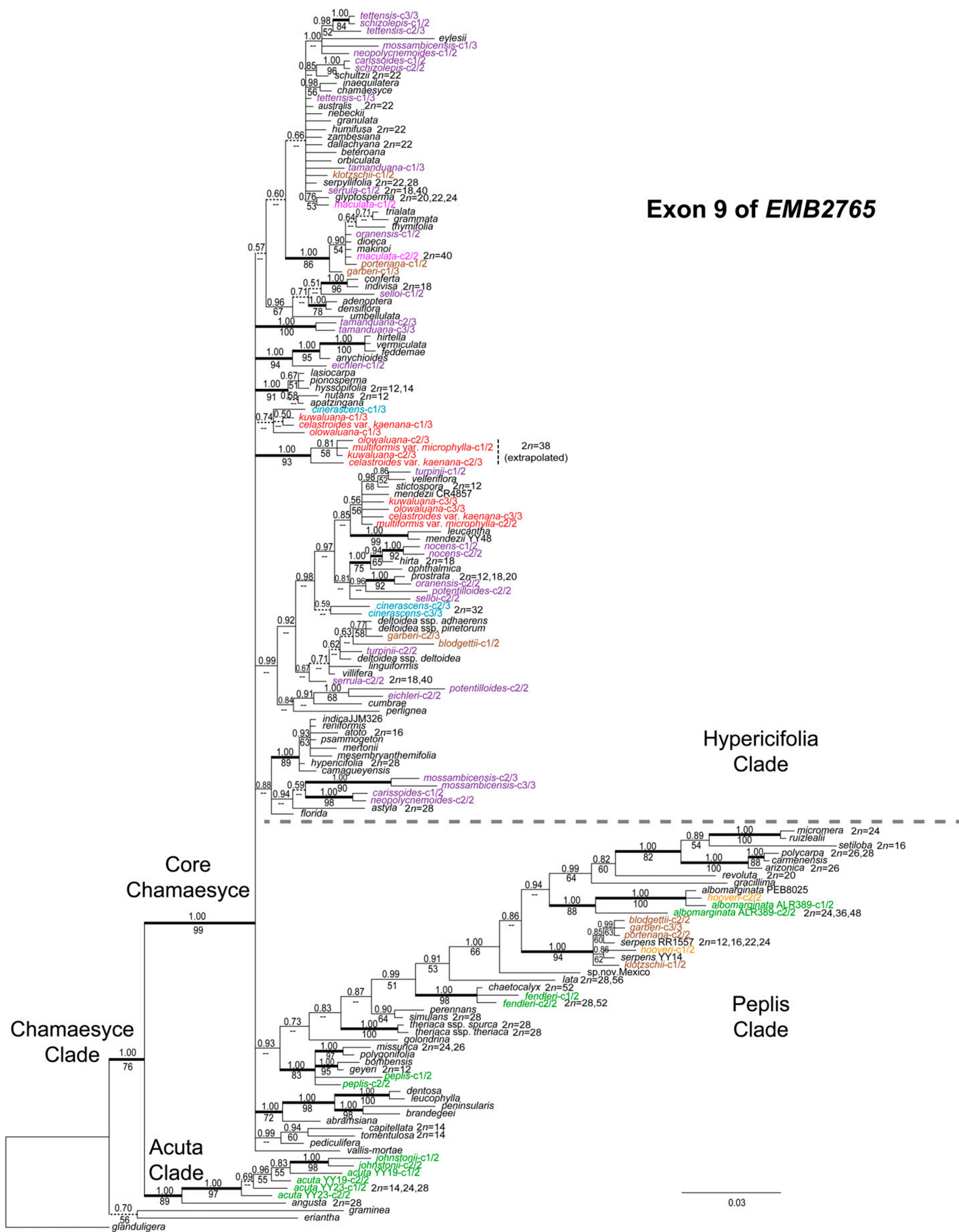
According to our present knowledge on phylogenetic relationships and photosynthetic states (Fig. 6), there are three possible scenarios for the evolution of C_4 photosynthesis in the Chamesyce clade: (1) C_3 to C_4 transitions could have originated at least twice, once within the Acuta clade and another time on the stem leading to core Chamaesyce; (2) alternatively, C_4 photosynthesis could have evolved once in the common ancestor of the entire Chamaesyce clade and then have gone through various degrees of reversal to C_3 in the Acuta clade; or (3) an ancestral C_3-C_4 intermediate type in the common ancestor of the Chamaesyce clade could have given rise to all the C_3, C_4, and intermediate types in the extant species of the clade (Christin et al., 2010). Given the complexities of the intermediate photosynthetic types in *E. acuta* and *E. johnstonii*, and the small size and sister relationship of subsect. *Acutae* to the rest of the clade, we cannot yet be certain which of these scenarios might explain the evolution of C_3, C_4, and intermediate systems within the Chamaesyce clade.

North American origin of the Chamaesyce clade**—**According to evidence from morphology, geographic centers of diversity, chromosome counts, and photosynthetic types, Webster et al. (1975) hypothesized that the Chamaesyce clade originated in subtropical and warm temperate areas of North America. This scenario is strongly supported by our molecular phylogenetic analysis (Figs. 3–5). The outgroup lineage sister to the Chamaesyce clade is mainly North American and corresponds to the former *Euphorbia* subgenus *Agaloma* (Raf.) House (Steinmann and Porter, 2002). Within the Chamaesyce clade, the entire Acuta clade and all but two species that are deeply nested in the Peplis clade, *E. peplis* L. and *E. serpens*, are endemic to the southern United States and Mexico (Fig. 5). Species in the Hypericifolia clade, in contrast, have many different distribution patterns, including both cosmopolitan weeds as well as narrow endemics in both the New World and the Old World. Even so, a small clade consisting of *E. astyla* Engelm. ex Boiss. and *E. jejuna*, two Chihuahuan Desert endemics, is sister to the rest of the Hypericifolia clade, and all of the Old World groups are deeply nested in predominantly New World groups. Consequently, our data are consistent with a North American origin for the Chamaesyce clade as well as for each of the three major subclades. This makes it very likely that C_4 photosynthesis originated in this area as well.

Long-distance dispersal events from the New World to the Old World**—**There is a group of species in the Hypericifolia clade that occurs either on oceanic islands or in coastal areas of Old World continents, represented in our sampling by seven species beginning with *E. atoto* G.Forst. (Fig. 3, in blue; Fig. 5, shaded box). While cpDNA and *EMB2765* do not fully resolve this group, both ITS and the 5-locus data sets support the monophyly of the seven species. Both data sets recover *E. mesembryanthemifolia* Jacq., a shrub native to the Caribbean, as the group's closest New World relative, followed by *E. hypericifolia* L., a weedy species native to the neotropics. Both of these closely related New World species are characterized by relatively large leaves and a more or less woody, ascending habit. Therefore, this oceanic Old World group most likely originated once from an ascending and shrubby ancestor in the neotropics. Subsequent dispersals have occurred throughout the Pacific coastlines, with widespread species such as *E. atoto* and *E.*

←

Fig. 3. Majority rule consensus trees recovered from Bayesian analyses of the chloroplast DNA data (*matK* + *rpl16* + *trnL-F* + indels, cpDNA) and the nuclear ITS data. Numbers above the branches are Bayesian posterior probabilities (PP) and numbers below the branches are maximum likelihood bootstrap percentages (MLB). Thick branches indicate PP ≥0.95 and MLB ≥70%, and branches in dashed lines have Bayesian PP <0.80 and MLB <50%. Presumed hybrid accessions with different placement between the ITS and cpDNA phylogeny that are well-supported by MLB and PP are connected by lines. Taxa belonging to the Acuta clade near the bottom are colored pink; taxa in the Peplis clade are colored green, except *E. peplis*, the only taxa in the Peplis clade that is native to the Old World and therefore colored darker brown. Taxa in the Hypericifolia clade are colored black if they are native to the New World. The four groups native to the Old World are colored as follows: the oceanic group in blue, the continental group in tan, *E. forsskalii* and *E. makinoi* in purple, and the Hawaiian group in red.


Exon 9 of EMB2765
tettensis-c3/3
schizolepis-c1/2
tettensis-c2/3
eylesii
mossambicensis-c1/3
neopolycnemoides-c1/2
carissoides-c1/2
schizolepis-c2/2
schultzii 2n=22
inaequilatera
chamaesyce
tettensis-c1/3
australis 2n=22
riebeckii
granulata
humifusa 2n=22
zambesiana
dallachyana 2n=22
beteroana
orbiculata
tamanduana-c1/3
klotzschii-c1/2
serpyllifolia 2n=22,28
serrula-c1/2 2n=18,40
glyptosperma 2n=20,22,24
maculata-c1/2
trialata
grammata
thymifolia
oranensis-c1/2
dioeca
makinoi
maculata-c2/2 2n=40
porteriana-c1/2
garberi-c1/3
conferta
indivisa 2n=18
selloi-c1/2
adenoptera
densiflora
umbellulata
tamanduana-c2/3
tamanduana-c3/3
hirtella
vermiculata
feddemae
anychioides
eichleri-c1/2
lasiocarpa
pionosperma
hyssopifolia 2n=12,14
nutans 2n=12
apatzingana
cinerascens-c1/3
kuwaluana-c1/3
celastroides var. kaenana-c1/3
olowaluana-c1/3
olowaluana-c2/3
multiformis var. microphylla-c1/2
kuwaluana-c2/3
celastroides var. kaenana-c2/3
2n=38 (extrapolated)
turpinii-c1/2
velleriflora
stictospora 2n=12
mendezii CR4857
kuwaluana-c3/3
olowaluana-c3/3
celastroides var. kaenana-c3/3
multiformis var. microphylla-c2/2
leucantha
mendezii YY48
nocens-c1/2
nocens-c2/2
hirta 2n=18
ophthalmica
prostrata 2n=12,18,20
oranensis-c2/2
potentilloides-c2/2
selloi-c2/2
cinerascens-c2/3
cinerascens-c3/3 2n=32
deltoidea ssp. adhaerens
deltoidea ssp. pinetorum
garberi-c2/3
blodgettii-c1/2
turpinii-c2/2
deltoidea ssp. deltoidea
linguiformis
villifera
serrula-c2/2 2n=18,40
potentilloides-c2/2
eichleri-c2/2
cumbrae
perlignea
indica JJM326
reniformis
atoto 2n=16
psammogeton
mertonii
mesembryanthemifolia
hypericifolia 2n=28
camagueyensis
mossambicensis-c2/3
mossambicensis-c3/3
carissoides-c1/2
neopolycnemoides-c2/2
astyla 2n=28
florida
Hypericifolia Clade
micromera 2n=24
ruizlealii
setiloba 2n=16
polycarpa 2n=26,28
carmenensis
arizonica 2n=26
revoluta 2n=20
gracillima
albomarginata PEB8025
hooveri-c2/2
albomarginata ALR389-c1/2
albomarginata ALR389-c2/2 2n=24,36,48
blodgettii-c2/2
garberi-c3/3
porteriana-c2/2
serpens RR1557 2n=12,16,22,24
hooveri-c1/2
serpens YY14
klotzschii-c1/2
sp.nov.Mexico
lata 2n=28,56
chaetocalyx 2n=52
fendleri-c1/2
fendleri-c2/2 2n=28,52
perennans
simulans 2n=28
theriaca ssp. spurca 2n=28
theriaca ssp. theriaca 2n=28
golondrina
missurica 2n=24,26
polygonifolia
bombensis
geyeri 2n=12
peplis-c1/2
peplis-c2/2
dentosa
leucophylla
peninsularis
brandegeei
abramsiana
capitellata 2n=14
tomentulosa 2n=14
pediculifera
vallis-mortae
Peplis Clade
Core Chamaesyce
johnstonii-c1/2
johnstonii-c2/2
acuta YY19-c1/2
acuta YY19-c2/2
acuta YY23-c1/2 2n=14,24,28
acuta YY23-c2/2
angusta 2n=28
graminea
eriantha
glanduligera
Acuta Clade
Chamaesyce Clade
0.03

chamissonis (Klotzsch & Garcke) Boiss., as well as others that colonized coastal Australia such as *E. coghlanii* F.M.Bailey and *E. psammogeton* P. S. Green, southeast Asia (e.g., *E. reniformis* Blume) and the Indian Ocean (e.g., *E. mertonii* Fosberg and *E. indica* Lam.). None of the native Hawaiian species is recovered in this oceanic group, however, despite Hawaii's intermediate geographic position between the New World and the members of the *E. atoto* group. Therefore, a close relationship between Hawaiian Chamaesyce and *E. atoto,* as proposed by Degener and Croizat (1938) and Koutnik (1982), is not supported by our molecular data.

Many species in the Chamaesyce clade possess a seed coat that becomes mucilaginous and sticky when wet (Fig. 1C; Jordan and Hayden, 1992), and this type of seed coat is otherwise rare in *Euphorbia*. Mucilaginous seed coats have been shown to facilitate seed germination in other plant groups, particularly in weedy species or in desert habitats (Gutterman and Shem-Tov, 1997; Ebrahimzadeh et al., 2000; Penfield et al., 2001). The small, sticky seeds of the Chamaesyce clade can easily adhere to birds and thus enhance the likelihood of long-distance dispersal (Jordan and Hayden, 1992; Steinmann and Porter, 2002). A sticky seed coat is notably absent in the C_3 *E. angusta,* which could be interpreted as retaining the ancestral state of the clade, although it is present in the closely related *E. acuta* and *E. johnstonii*. A mucilaginous seed coat is also missing in inland Hawaiian species, but in this case it has been attributed to a secondary loss in insular habitats (Jordan and Hayden, 1992). Certain species such as *E. mesembryanthemifolia* and *E. atoto* also lack a mucilaginous seed coat. Instead, their seeds are able to float in seawater, which could explain their distribution on islands and in coastal areas (Carlquist, 1966; Jordan and Hayden, 1992).

In contrast to the oceanic dispersal pattern exemplified by the shrubby *E. atoto* and its allies, there is another Old World group in the Hypericifolia clade that shows evidence of long-distance dispersal between inland continental habitats. This clade (Fig. 3, in tan; Fig. 5, shaded) consists of prostrate to ascending herbs and subshrubs. It includes a number of African and Eurasian species such as *E. humifusa* Willd. and the seven species from *E. arabica* Hochst. & Steud. ex Anderson to *E. zambesiana* Benth. It also includes inland Australian species such as *E. australis* Boiss., *E. dallachyana* Baill., *E. schultzii* Benth., and *E.* sp. nov. Australia. Some species in this inland group show incongruent relationships between ITS and cpDNA, and some of them also have divergent *EMB2765* copies. These include inland African species such as *E. tettensis* Klotzsch, *E. neopolycnemoides* Pax & K.Hoffm., *E. mossambicensis* (Klotzsch & Garcke) Boiss., *E. lissosperma* S.Carter, and *E. eylesii* Rendle, as well as inland Australian species like *E. carissoides* and *E. schizolepis* (Fig. 3, in tan and with lines connecting incongruent placements; Fig. 4). However, accessions of many of the Old World species we were able to sample in this group came from herbarium specimens and were difficult to amplify or clone, so we may have failed to detect additional copies of *EMB2765*. Better sampling with fresh leaf material among the inland Old World species is needed to better understand the relationships among species in this region.

Another Old World group in the Hypericifolia clade consists of only two species sampled here (Fig. 3, in purple): *E. forsskalii* J.Gay is native to Africa, the Mediterranean region, and the Arabian Peninsula, whereas *E. makinoi* Hayata is native to eastern and southeastern Asia. Both ITS and cpDNA support the two as sister to each other, and together they are sister to *E. dioeca* Kunth, a widespread New World species. Like the previous group, this group would benefit from freshly collected material to verify the relationship suggested here, as well as to check for multiple copies of *EMB2765.*

Contrary to the different kinds of long-distance dispersals evoked within the Hypericifolia clade, the Peplis clade is entirely New World except for *E. peplis*, which has a Macaronesian, Mediterranean, and European distribution. This species is nested in a clade of six other species, and all seven of them are specialized on either inland deep sand deposits or sandy beach habitats (Figs. 3–5). *Euphorbia peplis* is thus the only species in the Peplis clade that appears to represent a dispersal event from the New World to the Old World.

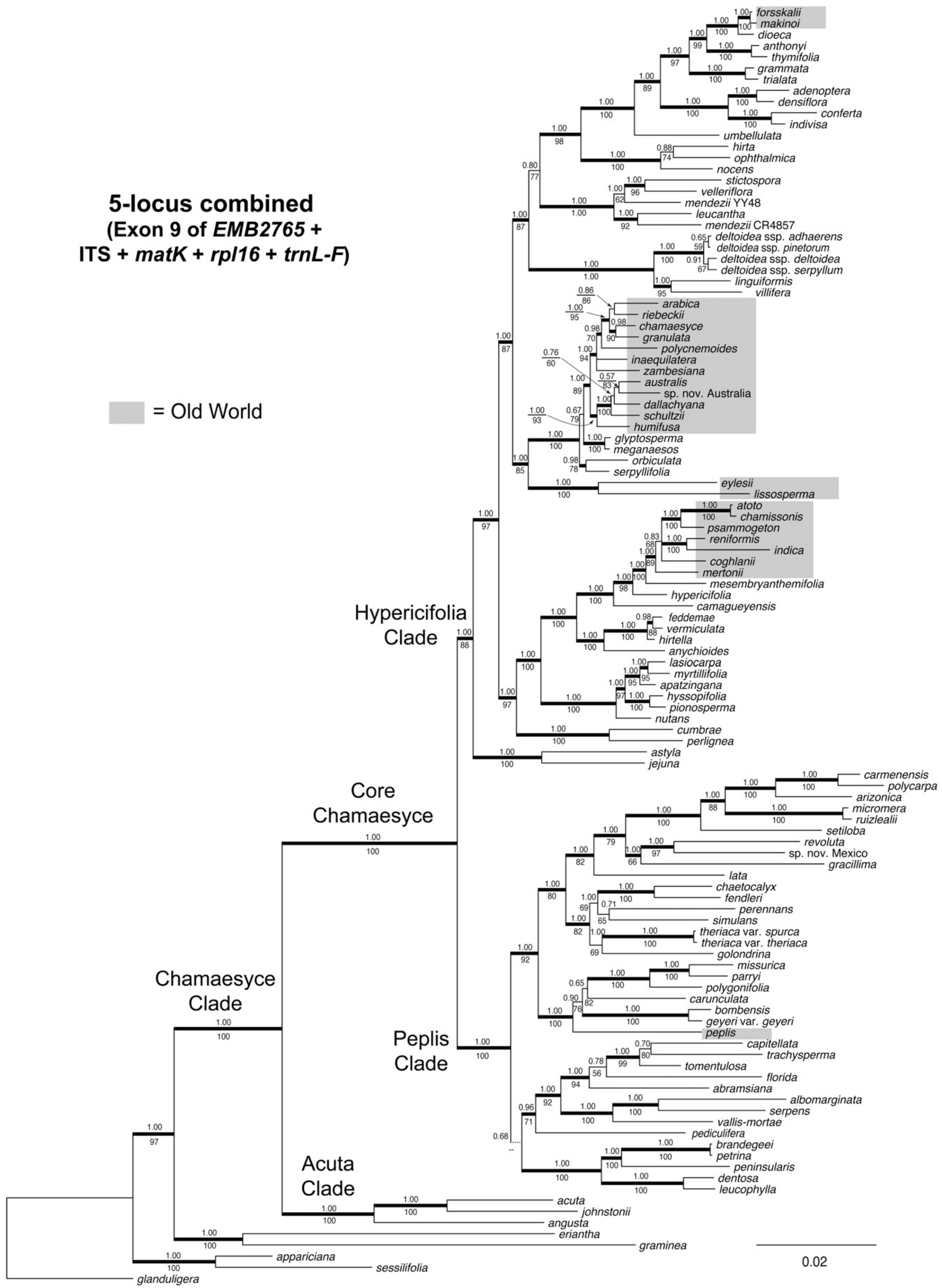
Finally, more recent human-assisted dispersal has probably contributed to the cosmopolitan distribution of weeds such as *E. maculata*, *E. hirta*, *E. prostrata* Aiton, and *E. thymifolia* L., although we cannot rule out the possibility of prehuman dispersal events.

Widespread reticulate evolution—Divergent copies of *EMB2765* and incongruence between chloroplast and nuclear data sets allow us to hypothesize 35 taxa of possible hybrid origin among the species of the Chamaesyce clade sampled here (Appendix 2). To untangle their relationships, we made four assumptions: (1) The chloroplast genome is contributed by the maternal parent, and thus a species of hybrid origin would group with the species most closely related to the maternal parent in the cpDNA tree. (2) Due to concerted evolution, ITS can be homogenized either toward the paternal or maternal sequence (Alvarez and Wendel, 2003). Therefore, ITS and cpDNA could be incongruent if our ITS sequence had retained the paternal copy in a taxon of hybrid origin. (3) If we found two copies of *EMB2765*, we expected divergent copies to cluster with both the maternal and paternal parents, due to the biparental nature of nuclear low-copy genes. (4) When a third copy of *EMB2765* was found that resolved in a different phylogenetic position from the other two copies, it could be indicative of further hybridization events in the history of that taxon.

We should note that due to the broad scale of this study, in which we sequenced between two and four accessions for most

←

Fig. 4. Majority rule consensus tree recovered from Bayesian analyses of the nuclear coding region exon 9 of *EMB2765*. Numbers above the branches are Bayesian posterior probabilities (PP) and numbers below the branches are maximum likelihood bootstrap percentages (MLB). Thick branches indicate PP ≥0.95 and MLB ≥0.70%, and branches in dashed lines have Bayesian PP <0.80 and MLB <50%. Branch length scale on lower right. Previously published chromosome numbers are listed next to their respective taxa (Perry, 1943; Hans, 1973; Urbatsch et al., 1975; Hassall, 1976; Carr, 1985; Xue et al., 2007; A. M. Powell, unpublished manuscript). Cloned accessions in the Acuta and Peplis clades with their conspecific copies clustered together are colored green; divergent cloned copies of *E. hooveri* are colored orange. Divergent cloned copies of the *E. serpens* complex that span both the Peplis clade and the Hypericifolia clade are colored brown. Within the Hypericifolia clade, accessions of *E. maculata* are in pink, Hawaiian endemics in red, and the closely related *E. cinerascens* in blue. Remaining accessions with divergent cloned copies are purple. The separation of the Hypericifolia clade from the Peplis clade in this tree is inferred from the more robust cpDNA and 5-locus phylogenies and is indicated by a dashed line.


5-locus combined
(Exon 9 of EMB2765 +
ITS + matK + rpl16 + trnL-F)
= Old World
forsskalii
makinoi
dioeca
anthonyi
thymifolia
grammata
trialata
adenoptera
densiflora
conferta
indivisa
umbellulata
hirta
ophthalmica
nocens
stictospora
velleriflora
mendezii YY48
leucantha
mendezii CR4857
deltoidea ssp. adhaerens
deltoidea ssp. pinetorum
deltoidea ssp. deltoidea
deltoidea ssp. serpyllum
linguiformis
villifera
arabica
riebeckii
chamaesyce
granulata
polycnemoides
inaequilatera
zambesiana
australis
sp. nov. Australia
dallachyana
schultzii
humifusa
glyptosperma
meganaesos
orbiculata
serpyllifolia
eylesii
lissosperma
atoto
chamissonis
psammogeton
reniformis
indica
coghlanii
mertonii
mesembryanthemifolia
hypericifolia
camagueyensis
feddemae
vermiculata
hirtella
anychioides
lasiocarpa
myrtillifolia
apatzingana
hyssopifolia
pionosperma
nutans
cumbrae
perlignea
astyla
jejuna
Hypericifolia Clade
Core Chamaesyce
carmenensis
polycarpa
arizonica
micromera
ruizlealii
setiloba
revoluta
sp. nov. Mexico
gracillima
lata
chaetocalyx
fendleri
perennans
simulans
theriaca var. spurca
theriaca var. theriaca
golondrina
missurica
parryi
polygonifolia
carunculata
bombensis
geyeri var. geyeri
peplis
capitellata
trachysperma
tomentulosa
florida
abramsiana
albomarginata
serpens
vallis-mortae
pediculifera
brandegeei
petrina
peninsularis
dentosa
leucophylla
Chamaesyce Clade
Peplis Clade
Acuta Clade
acuta
johnstonii
angusta
eriantha
graminea
appariciana
sessilifolia
glanduligera
0.02

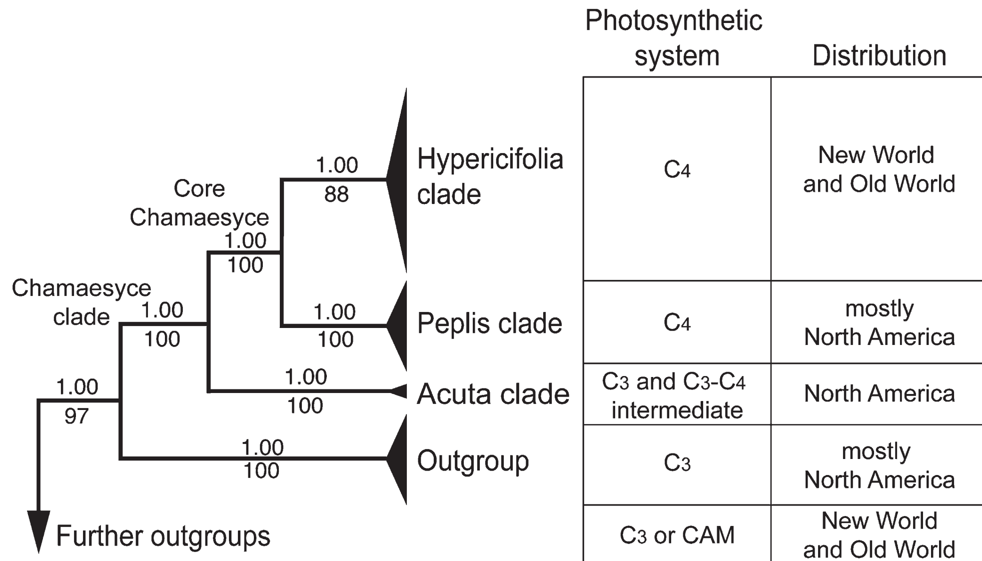


Fig. 6. Summary of the three major subclades within the Chamaesyce clade recovered from results of the 5-locus data set (exon 9 of *EMB2765* + ITS + *matK* + *rpl16* + *trnL-F*), with corresponding photosynthetic systems and geographical distributions indicated. Numbers above the branches indicate Bayesian posterior probabilities and numbers below the branches are maximum likelihood bootstrap percentages. Photosynthetic systems are from Webster (1975) and Sage et al. (2011a); closely related outgroups follow Steinmann and Porter (2002).

taxa in our unreduced 450-accession data set, the hybrid relationships proposed here (taxa joined by lines in Fig. 3 and taxa with divergent copies of *EMB2765* in Fig. 4; summarized in Appendix 2) are meant to be taken as working hypotheses for more detailed, population-level sampling involving both cytological and molecular studies. Because of the high number of taxa of possible hybrid origin that emerged from this study, we cannot examine each one in detail here. Instead, three of the most notable species or species complexes of hypothesized hybrid origin are presented below as examples.

Allopolyploid origin of the woody Hawaiian Chamaesyce—With 16 recognized species, the Hawaiian Chamaesyce clade represents one of the most notable radiations of woody taxa in the Hawaiian Archipelago (Fig. 1, G and H) (Ziegler, 2002). Monophyly of the Hawaiian Chamaesyce clade was reported by Motley and Raz (2004) based on ITS sequence data, with extensive taxon sampling among Pacific Island species, but relatively little sampling from North America. Their study suggested that the closest relatives of the Hawaiian Chamaesyce clade were from the New World instead of other Pacific Islands. Our expanded sampling also supports the monophyly of the Hawaiian Chamaesyce clade (Fig. 3, in red) and recovers four small annual species commonly found in disturbed habitats in the southern United States, northern Mexico, and the Caribbean as the closest relatives of the group. These include *E. stictospora*, *E. velleriflora*, *E. mendezii*, and *E. leucantha* (Fig. 3, ITS), which are all morphologically quite similar to each other. A fifth species, *E. cinerascens* (Fig. 1F), is a perennial species endemic to the Chihuahuan Desert, and it was identified by cpDNA as an additional member of the sister clade to Hawaiian Chamaesyce (Fig. 3, cpDNA). Cloning of *EMB2765* PCR products detected three copies in taxa of the Hawaiian Chamaesyce clade (Fig. 4, in red). Each species surveyed had all three copies, except for *E. multiformis* Gaudich. ex Hook. & Arn., which had only two copies. One of these copies supports the Hawaiian species as being closely related to *E. stictospora*, *E. velleriflora*, *E. mendezii*, *E. leucantha*. There are also two copies of *E. cinerascens* that are placed close to this clade, but with low support (Fig. 4, blue), which is consistent with the cpDNA pattern observed in Fig. 3. A second *EMB2765* copy in the Hawaiian species gives weak support for them being sister to the third, divergent copy of *EMB2765* in *E. cinerascens*. The third copy of *EMB2765* in the Hawaiian species, however, does not reveal a highly supported sister group for this clade. Given the high chromosome numbers in counts of the four Hawaiian species surveyed thus far compared to other closely related Chamaesyce species (Fig. 4) ($2n$ = 38, Carr, 1985), allopolyploidy appears to have evolved early within the native Hawaiian species of the Chamaesyce clade. Also, since *E. cinerascens* has multiple copies of *EMB2765,* and its placement in the ITS phylogeny is different from the relationships inferred by the cpDNA tree, it may also have originated by interspecific hybridization.

According to our earlier assumptions, both *E. cinerascens* and the Hawaiian Chamaesyce clade appear to share the same or a closely related maternal genome, related to the clade of *E. stictospora*, *E. velleriflora*, *E. mendezii*, and *E. leucantha*. A different shared paternal parent for both *E. cinerascens* and the Hawaiian Chamaesyce clade is suggested by the second copy of

←
Fig. 5. Majority rule consensus tree recovered from Bayesian analyses of the 5-locus data set (exon 9 of *EMB2765* + ITS + *matK* + *rpl16* + *trnL-F*), with putative hybrid taxa removed (see Appendix 2). Numbers above the branches indicate Bayesian posterior probabilities (PP); numbers below the branches are maximum likelihood bootstrap percentages (MLB). Thick branches indicate PP ≥0.95 and MLB ≥0.70%, and branches in dashed lines have Bayesian PP <0.80 and MLB <50%. Branch length scale is on lower right.

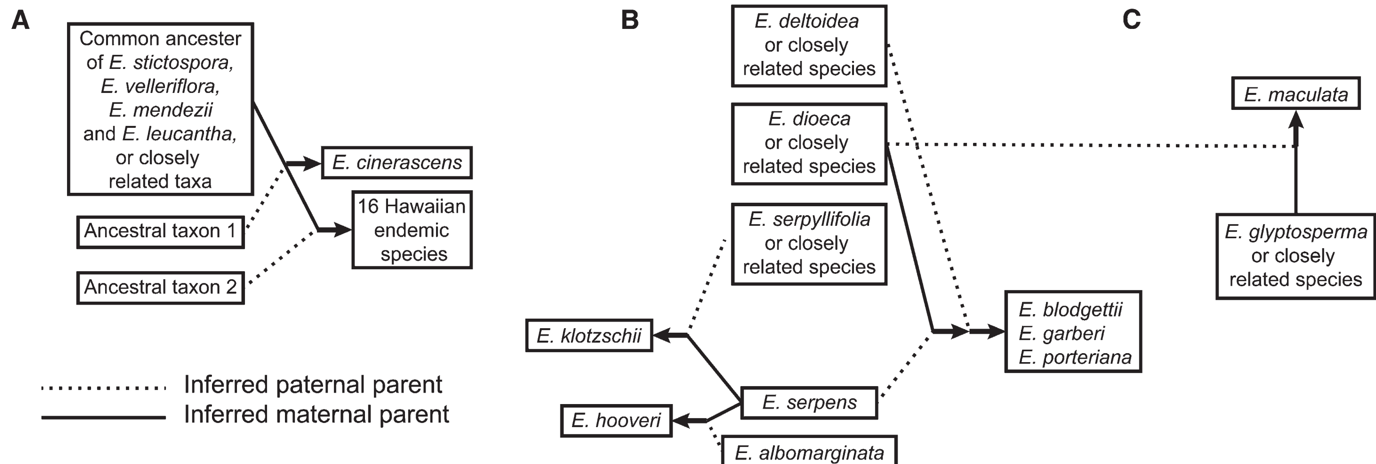


Fig. 7. Hypothetical hybrid relationships inferred from the ITS, cpDNA, and *EMB2765* data sets. Arrows go from putative parents toward derived hybrid taxa. The inferred paternal parent is indicated by dotted lines, and maternal parent by solid lines. (A) Endemic Hawaiian Chamaesyce and proposed New World progenitors. (B) *E. serpens* species complex. (C) *E. maculata.*

EMB2765, albeit with weak support ("ancestral taxon 1" in Fig. 7A). This initial hybrid may have served as the maternal parent in a subsequent hybridization event ("ancestral taxon 2" in Fig. 7A), as evidenced by the third divergent copy of *EMB2765* in the Hawaiian Chamaesyce. It would presumably have been this secondary hybrid that eventually reached the Hawaiian Islands through long-distance dispersal and subsequently radiated into the 16 species present there now.

Our results are consistent with the finding that a number of other Hawaiian angiosperm radiations are of North American origin involving hybrids and/or polyploids as well (Carr, 1998; Baldwin and Wagner, 2010), including the Hawaiian silversword alliance (Barrier et al., 1999), the Hawaiian mints (Lindqvist and Albert, 2002), *Viola* (Ballard and Sytsma, 2000), and *Cuscuta* (Stefanovic and Costea, 2008). These radiations appear to be associated with dispersal by birds and with hybridization before long-distance dispersal (Baldwin and Wagner, 2010). Allopolyploid taxa can exhibit great adaptive plasticity through increased heterozygosity, better masking of recessive deleterious alleles, and lower susceptibility to inbreeding depression (Comai, 2005). Such adaptive plasticity may facilitate colonization of new niches, such as in the Hawaiian Islands.

Apart from the Hawaiian radiation, there are also eight species in the Chamaesyce clade that are endemic to the Galapagos Islands (Burch, 1969). Although we were not able to sample any taxa from this area, it would be an excellent group to study to determine whether polyploidy and hybridization were involved in their radiation as well.

Euphorbia serpens *species complex*—*Euphorbia serpens*, its sister species *E. albomarginata*, and five other species that appear to involve *E. serpens* as one of their parents, together form a complex that we infer to have highly reticulate relationships (Fig. 7B). Three of the species in this complex are very similar morphologically and are monophyletic in both the ITS and cpDNA phylogenies. These include *E. blodgettii*, which is widespread from the southeastern United States to Central America in somewhat disturbed habitats, and *E. garberi* and *E. porteriana* (Fig. 1J), which are both narrow endemics restricted to limestone outcrops in southern Florida. The cpDNA data places these three species together and sister to a small clade consisting of *E. dioeca* (Mexico to South America) and the Old World *E. forsskalii* and *E. makinoi* in the Hypericifolia clade, whereas ITS data places them nested among accessions of *E. serpens* in the Peplis clade (Fig. 3). In the *EMB2765* phylogeny, each of these species has a copy of *EMB2765* nested among multiple *E. serpens* accessions (Fig. 4, in brown, with only 2 of 20 accessions of *E. serpens* shown), in agreement with the ITS placement. Both *E. garberi* and *E. porteriana* also have a second copy of *EMB2765* that is closely related to *E. dioeca*, in the Hypericifolia clade. This topology is consistent with the relationships shown in the cpDNA tree (Fig. 3), except that an *EMB2765* copy of *E. blodgettii* is presumably missing. A third *EMB2765* copy of *E. garberi* and a second copy of *E. blodgettii* are both clustered with other tropical New World species of the Hypericifolia clade that are also specialized on limestone substrates, such as *E. deltoidea* Engelm. ex Chapm. and *E. turpinii* Boiss. We hypothesize that a hybridization event occurred between *E. dioeca* or a closely related extant or ancestral species as the maternal donor and *E. serpens* as the paternal donor. This initial hybrid plant subsequently hybridized with *E. deltoidea* or a closely related species (Fig. 7B), followed by differentiation of *E. garberi*, *E. blodgettii*, and probably *E. porteriana*. Both *E. garberi* and *E. porteriana* are sympatric with *E. deltoidea*, and all three species are restricted to pine rocklands on limestone outcrops in southern Florida; *E. blodgettii* also occurs in southern Florida, but extends into the southeastern United States and Central America.

Another suggested hybridization event between the Peplis clade and the Hypericifolia clade also involves *E. serpens*. *Euphorbia klotzschii* from southern South America is nested among accessions of *E. serpens* in the Peplis clade according to cpDNA data, whereas ITS sequence data place it sister to *E. serpyllifolia* Pers. in the Hypericifolia clade (Fig. 3). *EMB2765* recovered two copies of *E. klotzschii* that correspond to the different ITS and cpDNA placements (Fig. 4). This implies that *E. serpens* could have been the maternal donor, and *E. serpyllifolia*, or a closely related species, was the paternal donor that led to *E. klotzschii*.

A third proposed hybrid species origin involves *E. serpens* and *E. albomarginata,* both of which are small, prostrate, glabrous herbs with white, membranous stipules and entire leaf

margins. *Euphorbia hooveri*, a species that is morphologically quite distinct from *E. serpens* and *E. albomarginata* (see Fig. 1, I and K), was recovered in both the ITS and cpDNA phylogenies nested among *E. albomarginata* accessions in the Peplis clade (Fig. 3). However, *E. hooveri* has two *EMB2765* copies, one nested among *E. albomarginata* accessions and the other nested with *E. serpens* accessions (Fig. 4, in orange). Placement of these two divergent alleles suggests that *E. hooveri* may be of hybrid origin from ancestors allied to *E. albomarginata* and *E. serpens*. *Euphorbia hooveri* is a rare summer annual restricted to mud flats in ephemeral vernal pools in the Central Valley of California, whereas both putative parental species occur on a variety of soil types nearby in more upland habitats (Hickman, 1993).

One of the caveats of inferring parentage and reticulate relationships within a species complex is that population level sampling is required to account for complications such as lineage sorting, introgression, and other confounding factors. In our unreduced 450-accession data set, we analyzed ITS and cpDNA data in 20 accessions of *E. serpens* throughout its full range of distribution, as well as three accessions of *E. blodgettii*, two accessions of *E. porteriana*, and four accessions of *E. albomarginata*. When the multiple accessions are analyzed together, all five putative hybrid species discussed earlier continue to have either ITS or cpDNA sequences deeply nested in *E. serpens* or *E. albomarginata* with strong support (data not shown), showing the same pattern as seen from the reduced data set in Fig. 3. Although no conclusive evidence can be drawn, a consistent pattern within the more densely sampled species further supports the hypothesized reticulate evolution in *E. serpens* complex.

Euphorbia maculata—*Euphorbia maculata* is a small, prostrate summer annual, able to go through multiple overlapping generations within a single growing season (Suzuki and Teranishi, 2005), and it is one of the most common weeds across temperate North America and is naturalized worldwide. While both ITS and cpDNA analyses place it sister to the North American species *E. meganaesos* Featherman and *E. glyptosperma* Engelm. in the Hypericifolia clade (Fig. 3), two distinct *EMB2765* alleles were recovered (Fig. 4). The first allele corresponds to the ITS and cpDNA placement, grouped together with species that have chromosome numbers of $2n = 22$, while the other allele is closely related to *E. dioeca*, a species that may also be involved in the *E. serpens* species complex as well as other hybrid relationships. With a relatively high chromosome count ($2n = 40$, Xue et al., 2007; Fig. 4), *E. maculata* likely has an allopolyploid origin from species closely related to *E. dioeca* and *E. glyptosperma* (Fig. 7C).

Conclusion—Through a complex suite of character switches, including physiology and anatomy (C_4 photosynthesis), seed morphology (sticky surface and small size), and life-history (reduced vegetative growth and prolonged reproductive stages), the Chamaesyce clade of *Euphorbia* has successfully adapted to warm and dry areas in subtropical North America, diversified locally into three major clades, and subsequently achieved worldwide distribution through multiple long-distance dispersal events. During this process, genetic mixing through reticulate evolution and changes in ploidy level have produced new species with novel adaptations. This study provides a phylogenetic framework for further study into the physiology, biogeography, character evolution, and conservation status of the most diverse C_4 lineage among the eudicots. It also demonstrates the ongoing evolutionary potential of weedy plant lineages through dispersal followed by local adaptation, producing diverse derivative endemic lineages such as the Hawaiian radiation.

LITERATURE CITED

Alvarez, I., and J. F. Wendel. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution* 29: 417–434.

Baldwin, B. G., and W. L. Wagner. 2010. Hawaiian angiosperm radiations of North American origin. *Annals of Botany* 105: 849–879.

Ballard, H. E., and K. J. Sytsma. 2000. Evolution and biogeography of the woody Hawaiian violets (*Viola*, Violaceae): Arctic origins, herbaceous ancestry and bird dispersal. *Evolution* 54: 1521–1532.

Barrier, M., B. G. Baldwin, R. H. Robichaux, and M. D. Purugganan. 1999. Interspecific hybrid ancestry of a plant adaptive radiation: Allopolyploidy of the Hawaiian silversword alliance (Asteraceae) inferred from floral homeotic gene duplications. *Molecular Biology and Evolution* 16: 1105–1113.

Batanouny, K., W. Stichler, and H. Ziegler. 1991. Photosynthetic pathways and ecological distribution of *Euphorbia* species in Egypt. *Oecologia* 87: 565–569.

Boissier, E. 1862. *Euphorbia* sect. *Anisophyllum. In* A. P. de Candolle [ed.], Prodromus systematis naturalis regni vegetabilis, vol. 15, part 2, 11–52. Victor Masson & Fils, Paris, France.

Brown, J. M., S. M. Hedtke, A. R. Lemmon, and E. M. Lemmon. 2010. When trees grow too long: Investigating the causes of highly inaccurate Bayesian branch-length estimates. *Systematic Biology* 59: 145–161.

Bruyns, P. V., R. J. Mapaya, and T. Hedderson. 2006. A new subgeneric classification for *Euphorbia* (Euphorbiaceae) in southern Africa based on ITS and *psbA-trnH* sequence data. *Taxon* 55: 397–420.

Burch, D. 1965. A taxonomic revision of the genus *Chamaesyce* (Euphorbiaceae) in the Caribbean. Ph.D. dissertation, University of Florida, Gainesville, Florida, USA.

Burch, D. 1969. Notes on the Galapagos Euphorbieae (Euphorbiaceae). *Annals of the Missouri Botanical Garden* 56: 173–178.

Carlquist, S. 1966. The biota of long-distance dispersal. III. Loss of dispersibility in the Hawaiian flora. *Brittonia* 18: 310–335.

Carr, G. D. 1985. Additional chromosome numbers of Hawaiian flowering plants. *Pacific Science* 39: 302–306.

Carr, G. D. 1998. Chromosome evolution and speciation in Hawaiian flowering plants. *In* T. F. Stuessy and M. Ono [eds.], Evolution and speciation of island plants, 5–47. Cambridge University Press, Cambridge, UK.

Christin, P.-A., R. P. Freckleton, and C. P. Osborne. 2010. Can phylogenetics identify C_4 origins and reversals? *Trends in Ecology & Evolution* 25: 403–409.

Comai, L. 2005. The advantages and disadvantages of being polyploid. *Nature Reviews Genetics* 6: 836–846.

Cronn, R., M. Cedroni, T. Haselkorn, C. Grover, and J. F. Wendel. 2002. PCR-mediated recombination in amplification products derived from polyploid cotton. *Theoretical and Applied Genetics* 104: 482–489.

Degener, O., and L. Croizat. 1938. *Chamaesyce*, part 1, 2 pp., reprint with changes. *In* O. Degener [ed.], Flora hawaiiensis. Published privately.

Ebrahimzadeh, H., V. Niknam, and A. A. Maassoumi. 2000. Mucilage content and its sugar composition in *Astragalus* species from Iran. *Pakistan Journal of Botany* 32: 131–140.

Edgar, R. C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792–1797.

Gutterman, Y., and S. Shem-Tov. 1997. Mucilaginous seed coat structure of *Carrichtera annua* and *Anastatica hierochuntica* from the Negev Desert highlands of Israel, and its adhesion to the soil crust. *Journal of Arid Environments* 35: 695–705.

Hans, A. S. 1973. Chromosomal conspectus of the Euphorbiaceae. *Taxon* 22: 591–636.

HASSALL, D. C. 1976. Numerical and cytotaxonomic evidence for generic delimitation in Australian Euphorbiaceae. *Australian Journal of Botany* 24: 633–640.

HASSALL, D. C. 1977. Systematic studies in Australian Euphorbieae (Euphorbiaceae). Ph.D. dissertation, University of Queensland, St. Lucia, Queensland, Australia.

HAYDEN, W. J. 1988. Ontogeny of the cotyledonary region of *Chamaesyce maculata* (Euphorbiaceae). *American Journal of Botany* 75: 1701–1713.

HERBST, D. 1971. Disjunct foliar veins in Hawaiian euphorbias. *Science* 171: 1247–1248.

HERBST, D. 1972. Ontogeny of foliar venation in *Euphorbia forbesii. American Journal of Botany* 59: 843–850.

HICKMAN. J. C. 1993. The Jepson manual: Higher plants of California. University of California Press, Berkeley, California, USA.

HUELSENBECK, J. P., AND F. RONQUIST. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.

JORDAN, M. S., AND W. J. HAYDEN. 1992. A survey of mucilaginous testa in *Chamaesyce. Collectanea Botanica* 21: 79–89.

JORDAN, W. C., M. W. COURTNEY, AND J. E. NEIGEL. 1996. Low levels of intraspecific genetic variation at a rapidly evolving chloroplast DNA locus in North American duckweeds (Lemnaceae). *American Journal of Botany* 83: 430–439.

KELCHNER, S. A., AND L. G. CLARK. 1997. Molecular evolution and phylogenetic utility of the chloroplast *rpl16* intron in *Chusquea* and the Bambusoideae (Poaceae). *Molecular Phylogenetics and Evolution* 8: 385–397.

KIM, S. T., AND M. J. DONOGHUE. 2008. Molecular phylogeny of *Persicaria* (Persicarieae, Polygonaceae). *Systematic Botany* 33: 77–86.

KOUTNIK, D. L. 1982. A taxonomic revision of the Hawaiian species of the genus *Chamaesyce* (Euphorbiaceae). Ph.D. dissertation, University of California, Davis, California, USA.

KOUTNIK, D. L. 1987. A taxonomic revision of the Hawaiian species of the genus *Chamaesyce* (Euphorbiaceae). *Allertonia* 4: 331–388.

LINDQVIST, C., AND V. A. ALBERT. 2002. Origin of the Hawaiian endemic mints within North American *Stachys* (Lamiaceae). *American Journal of Botany* 89: 1709–1724.

LORENCE, D. H., AND W. L. WAGNER. 1996. A new species of *Chamaesyce* section *Sclerophyllae* (Euphorbiaceae) from Kaua'i. *Novon* 6: 68–71.

MADDISON, D. R., AND W. P. MADDISON. 2005. MacClade 4.08: An analysis of phylogeny and character evolution. Sinauer, Sunderland, Massachusetts, USA.

MARSHALL, D. C. 2010. Cryptic failure of partitioned Bayesian phylogenetic analyses: Lost in the land of long trees. *Systematic Biology* 59: 108–117.

MAYFIELD, M. H. 1991. *Euphorbia johnstonii* (Euphorbiaceae), a new species from Tamaulipas, Mexico, with notes on *Euphorbia* subsection *Acutae. SIDA, Contributions to Botany* 14: 573–579.

MORDEN, C. W., V. C. CARAWAY, AND T. J. MOTLEY. 1996. Development of a DNA library for native Hawaiian plants. *Pacific Science* 50: 324–335.

MOTLEY, T. J., AND L. RAZ. 2004. Origins, evolution, and systematics of Hawaiian and Pacific *Chamaesyce* (Euphorbiaceae). Botany 2004: Annual Meeting of the Botanical Society of America, Salt Lake City, Utah, USA [abstract, http://www.2004.botanyconference.org/engine/search/index.php?func=detail&aid=360].

MÜLLER, K. 2006. Incorporating information from length-mutational events into phylogenetic analysis. *Molecular Phylogenetics and Evolution* 38: 667–676.

NYLANDER, J. A. A. 2004. MrModeltest, version 2.3 for Mac. Program distributed by the author, website http://www.abc.se/~nylander/ [accessed 4 January 2010].

PARK, K. R., AND R. K. JANSEN. 2007. A phylogeny of Euphorbieae subtribe Euphorbiinae (Euphorbiaceae) based on molecular data. *Journal of Plant Biology* 50: 644–649.

PEARCY, R. W., AND J. TROUGHTON. 1975. C_4 photosynthesis in tree form *Euphorbia* species from Hawaiian rainforest sites. *Plant Physiology* 55: 1054–1056.

PENFIELD, S., R. C. MEISSNER, D. A. SHOUE, N. C. CARPITA, AND M. W. BEVAN. 2001. *MYB61* is required for mucilage deposition and extrusion in the *Arabidopsis* seed coat. *Plant Cell* 13: 2777–2791.

PERRY, B. A. 1943. Chromosome number and phylogenetic relationships in the Euphorbiaceae. *American Journal of Botany* 30: 527–543.

POSADA, D., AND T. R. BUCKLEY. 2004. Model selection and model averaging in phylogenetics: Advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology* 53: 793–808.

POSADA, D., AND K. A. CRANDALL. 1998. MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

PRENNER, G., AND P. J. RUDALL. 2007. Comparative ontogeny of the cyathium in *Euphorbia* (Euphorbiaceae) and its allies: Exploring the organ flower inflorescence boundary. *American Journal of Botany* 94: 1612–1629.

RAMBAUT, A., AND A. DRUMMOND. 2007. Tracer v1.4. Computer program and documentation distributed by the author, website: http://beast.bio.ed.ac.uk/Tracer [accessed 14 Jul 2010].

RANDELL, R. A., AND C. W. MORDEN. 1999. Hawaiian Plant DNA library II: Endemic, indigenous, and introduced species. *Pacific Science* 53: 401–417.

RONQUIST, F., AND J. P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

SAGE, R. F., P.-A. CHRISTIN, AND E. J. EDWARDS. 2011a. The C_4 plant lineages of planet Earth. *Journal of Experimental Botany* 62: 3155–3169.

SAGE, T. L., R. F. SAGE, P. J. VOGAN, B. RAHMAN, D. C. JOHNSON, J. C. OAKLEY, AND M. A. HECKEL. 2011b. The occurrence of C_2 photosynthesis in *Euphorbia* subgenus *Chamaesyce* (Euphorbiaceae). *Journal of Experimental Botany* 62: 3183–3195.

SAMUEL, R., H. KATHRIARACHCHI, P. HOFFMANN, M. H. J. BARFUSS, K. J. WURDACK, C. C. DAVIS, AND M. W. CHASE. 2005. Molecular phylogenetics of Phyllanthaceae: Evidence from plastid *matK* and nuclear *PHYC* sequences. *American Journal of Botany* 92: 132–141.

SHAW, J., E. B. LICKEY, E. E. SCHILLING, AND R. L. SMALL. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *American Journal of Botany* 94: 275–288.

SIMMONS, M. P., AND H. OCHOTERENA. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49: 369–381.

SPORCK, M. J., AND L. SACK. 2010. Adaptive radiation of leaf venation and the meaning of disjunct veins in the C_4 Hawaiian *Euphorbia*. Botany 2010: Annual Meeting of the Botanical Society of America, Providence, Rhode Island, USA [online abstract, website http://2010.botanyconference.org/engine/search/index.php?func=detail&aid=475].

STAMATAKIS, A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688–2690.

STEFANOVIC, S., AND M. COSTEA. 2008. Reticulate evolution in the parasitic genus *Cuscuta* (Convolvulaceae): Over and over again. *Botany* 86: 791–808.

STEINMANN, V. W., AND J. M. PORTER. 2002. Phylogenetic relationships in Euphorbieae (Euphorbiaceae) based on ITS and *ndhF* sequence data. *Annals of the Missouri Botanical Garden* 89: 453–490.

SUZUKI, N., AND S. TERANISHI. 2005. Phenology and life cycle of the annual, *Chamaesyce maculata* (L.) Small (Euphorbiaceae), with multiple overlapping generations in Japan. *Ecological Research* 20: 425–432.

TABERLET, P., L. GIELLY, G. PAUTOU, AND J. BOUVET. 1991. Universal primers for amplification of three noncoding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

URBATSCH, L. E., J. D. BACON, R. L. HARTMAN, M. C. JOHNSTON, T. J. WATSON JR., AND G. L. WEBSTER. 1975. Chromosome numbers for North American Euphorbiaceae. *American Journal of Botany* 62: 494–500.

URBATSCH, L. E., B. G. BALDWIN, AND M. J. DONOGHUE. 2000. Phylogeny of the coneflowers and relatives (Heliantheae: Asteraceae) based on nuclear rDNA internal transcribed spacer (ITS) sequences and chlorplast DNA restriction site data. *Systematic Botany* 25: 539–565.

WAGNER, W. L., D. R. HERBST, AND S. H. SOHMER. 1999. Manual of the flowering plants of Hawai'i, revised edition with supplement by W. L. Wagner and D. R. Herbst, 1855–1918. Bishop Museum Special Publication, vol. 97. University of Hawai'i Press, Honolulu, Hawai'i, USA.

Webster, G. L., W. V. Brown, and B. N. Smith. 1975. Systematics of photosynthetic carbon fixation pathways in *Euphorbia. Taxon* 24: 27–33.

Wheeler, L. C. 1941. *Euphorbia* subgenus *Chamaesyce* in Canada and the United States exclusive of southern Florida. *Rhodora* 43: 97–154, 168–205, 223–286, pls. 654–668.

White, T. J., T. Bruns, S. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White [eds.], PCR protocols: A guide to methods and applications, 315–324. Academic Press, San Diego, California, USA.

Wurdack, K. J., and C. C. Davis. 2009. Malpighiales phylogenetics: Gaining ground on one of the most recalcitrant clades in the angiosperm tree of life. *American Journal of Botany* 96: 1551–1570.

Xue, H. G., S. D. Zhou, X. J. He, and Y. Yu. 2007. Karyotype in fifteen populations belonging to thirteen species of *Euphorbia* (Euphorbiaceae) in China. *Acta Phytotaxonomica Sinica* 45: 619–626.

Ziegler, A. C. 2002. Hawaiian natural history, ecology, and evolution. University of Hawai'i Press, Honolulu, Hawai'i, USA.

Zimmermann, N., C. Ritz, and F. Hellwig. 2010. Further support for the phylogenetic relationships within *Euphorbia* L. (Euphorbiaceae) from nrITS and *trnL-trnF* IGS sequence data. *Plant Systematics and Evolution* 286: 39–58.

Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence data sets under the maximum likelihood criterion. Ph.D. dissertation, University of Texas at Austin, Austin, Texas, USA.

Appendix 1. Voucher and GenBank accession numbers for plant materials used in this study. Abbreviations: BISH = Bishop Museum; BRI = Queensland Herbarium, Australia; CORD = Universidad Nacional de Córdoba Herbarium, Argentina; DAV = University of California, Davis Herbarium; HAW = University of Hawai'i Herbarium; IEB = Instituto de Ecología, Pátzcuaro, Mexico; LSU = Louisiana State University Herbarium; MICH = University of Michigan Herbarium; MO = Missouri Botanical Garden Herbarium; PRE = South African National Biodiversity Institute Herbarium, South Africa; SD = San Diego Natural History Museum; SP = Instituto de Botânica, Brazil; SRSC = Sul Ross State University Herbarium.

Taxon, Collection locality, Collection number (herbarium), GenBank accession: ITS, exon 9 of *EMB2765*, *rpl16* intron, *trnL-F* spacer, *matK*.

Ingroup—***Euphorbia abramsiana*** L.C.Wheeler, Mexico: Sonora, *T. Van Devender2006-644* (MICH), HQ645217, HQ650889, HQ645369, HQ645523, HQ645673; ***Euphorbia acuta*** Engelm., USA: Texas, *Y. Yang 19* (MICH), –, [HQ650891 (clone 1), HQ650890 (clone 2)], –, –, –; ***Euphorbia acuta*** Engelm., USA: Texas, *Y. Yang 23* (MICH), HQ645218, [HQ650893 (clone 1), HQ650892 (clone 2)], HQ645370, HQ645524, HQ645674; ***Euphorbia adenoptera*** Bertol., Dominican Republic, *B. van Ee 636* (MICH), HQ645219, HQ650894, HQ645371, HQ645525, HQ645675; ***Euphorbia albomarginata*** Torr. & A.Gray, USA: California, *P.E. Berry 8025* (MICH), HQ645220, HQ650895, HQ645372, HQ645526, HQ645676; ***Euphorbia albomarginata*** Torr. & A.Gray, Mexico: Sonora, *A.L. Reina-G. 2006-389* (MICH), HQ645221, [HQ650897 (clone 1), HQ650896 (clone 2)], HQ645373, HQ645527, HQ645677; ***Euphorbia angusta*** Engelm., USA: Texas, *Y. Yang 41* (MICH), HQ645222, HQ650898, HQ645374, HQ645528, HQ645678; ***Euphorbia anthonyi*** Brandegee, Mexico, *R. Moran 5917* (SD), HQ645223, –, HQ645375, HQ645529, HQ645679; ***Euphorbia anychioides*** Boiss., Mexico, *Y. Yang 107* (MICH), HQ645224, HQ650899, HQ645376, HQ645530, HQ645680; ***Euphorbia apatzingana*** McVaugh, Mexico, *Y. Yang 89* (MICH), HQ645225, HQ650900, HQ645377, HQ645531, HQ645681; ***Euphorbia arabica*** Hochst. & Steud. ex Anderson, Ethiopia, *M. Gilbert 168* (MO), HQ645227, –, HQ645379, HQ645533, HQ645683; ***Euphorbia arizonica*** Engelm., USA: Texas, *Y. Yang 31* (MICH), HQ645228, HQ650901, HQ645380, HQ645534, HQ645684; ***Euphorbia articulata*** Burm., N/A, AF537446 (downloaded from GenBank), –, –, –, –; ***Euphorbia astyla*** Engelm. ex Boiss., USA: Texas, *B.H. Warnock 20328* (SRSC), HQ645229, HQ650902, HQ645381, HQ645535, HQ645685; ***Euphorbia atoto*** G. Forst., New Hebrides, *G.L. Webster 19361* (DAV), HQ645230, HQ650903, HQ645382, HQ645536, HQ645686; ***Euphorbia australis*** Boiss., Australia, *D. Halford Q9233a* (BRI), HQ645231, HQ650904, HQ645383, HQ645537, HQ645687; ***Euphorbia berteroana*** Balb. ex Spreng., Argentina, *B. van Ee 647* (MICH), HQ645232, JN542512, HQ645384, HQ645538, HQ645688; ***Euphorbia blodgettii*** Engelm. ex Hitchc., USA: Florida, *Y. Yang* 138 (MICH), HQ645233, [HQ650905 (clone 1), HQ650906 (clone 2)], HQ645385, HQ645539, HQ645689; ***Euphorbia bombensis*** Jacq., USA: Florida, *Y. Yang 177* (MICH), HQ645234, HQ650907, HQ645386, HQ645540, HQ645690; ***Euphorbia brandegeei*** Millsp., Mexico, *B. van Ee 706* (MICH), HQ645235, HQ650908, HQ645387, HQ645541, HQ645691; ***Euphorbia camagueyensis*** (Millsp.) Urb., Cuba, *J. Gutierrez. HAJB 81994* (MICH), HQ645236, HQ650909, HQ645388, HQ645542, HQ645692; ***Euphorbia capitellata*** Engelm., Mexico: Sonora, *A.L. Reina-G. 2006-916* (MICH), HQ645237, HQ650910, HQ645389, HQ645543, HQ645693; ***Euphorbia carissoides*** F.M. Bailey, Australia, *K.R. McDonald 5073* (BRI), HQ645239, [HQ650911 (clone 1), HQ650912 (clone 2)], HQ645391, HQ645545, –; ***Euphorbia carmenensis*** N.E.Rose, Mexico: Baja California Sur, *V.W. Steinmann 6450* (MICH), HQ645240, HQ650913, HQ645392, HQ645546, HQ645695; ***Euphorbia carunculata*** Waterf., USA: Texas, *B.H. Warnock 20916* (SRSC), HQ645241, –, HQ645393, HQ645547, HQ645696; ***Euphorbia catamarcensis*** (Croizat) Subils, Argentina, *F.N. Biurrun 4748* (CORD), HQ645242, –, HQ645394, HQ645548, HQ645697; ***Euphorbia celastroides* var. *kaenana*** Sherff, USA: Hawaii, MMR C-2-60, HQ645243, [HQ650916 (clone 1), HQ650915 (clone 2), HQ650914 (clone 3)], HQ645395, HQ645549, HQ645698; ***Euphorbia chaetocalyx*** (Boiss.) Tidestr., USA: Texas, *Y. Yang 30* (MICH), HQ645244, HQ650917, HQ645396, HQ645550, HQ645699; ***Euphorbia chamaerrhodos*** Boiss., Brazil, da Silva 2945 (SP), HQ645245, –, HQ645397, HQ645551, HQ645700; ***Euphorbia chamaesyce*** L., Greece, Riina, R. 1558 (MICH), HQ645246, HQ650918, HQ645398, HQ645552, HQ645701; ***Euphorbia chamissonis*** (Klotzsch & Garcke) Boiss., Malaysia, *J. Beaman 9736* (DAV), HQ645247, –, HQ645399, HQ645553, HQ645702; ***Euphorbia cinerascens*** Engelm., USA: Texas, *Y. Yang 6* (MICH), HQ645248, [HQ650919 (clone 1), HQ650921 (clone 2), HQ650920 (clone 3)], HQ645400, HQ645554, HQ645703; ***Euphorbia clusiifolia*** Hook. & Arn., USA: Hawaii, *T.J. Motley 1576* (BISH), HQ645249, –, HQ645401, HQ645555, HQ645704; ***Euphorbia coghlanii*** F.M.Bailey, Australia, *D. Halford Q8601* (BRI), HQ645250, –, HQ645402, HQ645556, HQ645705; ***Euphorbia conferta*** (Small) B.E.Sm., USA: Florida, *Y. Yang 162* (MICH), HQ645251, HQ650922, HQ645403, HQ645557, HQ645706; ***Euphorbia cumbrae*** Boiss., Mexico, *Y. Yang 49* (MICH), HQ645252, HQ650923, HQ645404, HQ645558, HQ645707; ***Euphorbia dallachyana*** Baill., Australia, *D. Halford Q8109* (BRI), HQ645261, HQ650930, HQ645413, HQ645567, HQ645716; ***Euphorbia degeneri*** Sherff, USA: Hawaii, *C.W. Morden 1274* (HAW), HQ645253, –, HQ645405, HQ645559, HQ645708; ***Euphorbia deltoidea* subsp. *adhaerens*** (Small) Oudejans, USA: Florida, *Y. Yang 147* (MICH), HQ645254, HQ650924, HQ645406, HQ645560, HQ645709; ***Euphorbia deltoidea* subsp. *deltoidea*** Engelm. ex Chapm., USA: Florida, *Y. Yang 159* (MICH), HQ645255, HQ650925, HQ645407, HQ645561, HQ645710; ***Euphorbia deltoidea* subsp. *pinetorum*** (Small) Oudejans, USA: Florida, *Y. Yang 145* (MICH), HQ645256, HQ650926, HQ645408, HQ645562, HQ645711; ***Euphorbia deltoidea* subsp. *serpyllum*** (Small) Oudejans, USA: Florida, *Y. Yang 132* (MICH), HQ645257, –, HQ645409, HQ645563, HQ645712; ***Euphorbia densiflora*** (Klotzsch and Garcke) Klotzsch, Mexico: Sonora, *A.L. Reina-G. 2006-149* (MICH), HQ645258, HQ650927, HQ645410, HQ645564, HQ645713; ***Euphorbia dentosa*** I.M.Johnst., Mexico: Baja California Sur, *Y. Yang 204* (MICH), HQ645259, HQ650928, HQ645411, HQ645565, HQ645714; ***Euphorbia dioeca*** Kunth, Mexico, *Y. Yang 102* (MICH), HQ645260, HQ650929, HQ645412, HQ645566, HQ645715; ***Euphorbia eichleri*** Müll.Arg., Argentina, *B. van Ee 671* (MICH), HQ645264, [HQ650933 (clone 1), HQ650934 (clone 2)], HQ645416, HQ645570, HQ645719; ***Euphorbia eylesii*** Rendle, Namibia, *Giess 10005* (PRE), HQ645265, HQ650935, HQ645417, HQ645571, HQ645720; ***Euphorbia feddemae***

McVaugh, Mexico, *Y. Yang 112* (MICH), HQ645266, HQ650936, HQ645418, HQ645572, HQ645721; ***Euphorbia fendleri*** Torr. & A.Gray, USA: Texas, *Y. Yang 7* (MICH), HQ645267, [HQ650938 (clone 1), HQ650937 (clone 2)], HQ645419, HQ645573, HQ645722; ***Euphorbia florida*** Engelm., Mexico: Sonora, *A.L. Reina-G. 2006-476* (MICH), HQ645268, HQ650939, HQ645420, HQ645574, HQ645723; ***Euphorbia forsskalii*** J.Gay, French Guinea, *J.G. Adam 25916* (MO), HQ645269, –, HQ645421, HQ645575, HQ645724; ***Euphorbia garberi*** Engelm. ex Chapm., USA: Florida, *Y. Yang 164* (MICH), HQ645270, [HQ650940 (clone 1), HQ650941 (clone 2), HQ650942 (clone 3)], HQ645422, HQ645576, HQ645725; ***Euphorbia geyeri* var. *geyeri*** Engelm. & A.Gray, USA: Texas, *B.H. Warnock 20915* (SRSC), HQ645271, HQ650943, HQ645423, HQ645577, HQ645726; ***Euphorbia glyptosperma*** Engelm., USA: Texas, *Y. Yang 35* (MICH), HQ645273, HQ650945, HQ645425, HQ645579, HQ645728; ***Euphorbia golondrina*** L.C. Wheeler, USA: Texas, *Y. Yang 27* (MICH), HQ645274, HQ650946, HQ645426, HQ645580, HQ645729; ***Euphorbia gracillima*** S.Watson, Mexico: Sonora, *A.L.Reina-G. 2006-579* (MICH), HQ645275, HQ650947, HQ645427, HQ645581, HQ645730; ***Euphorbia grammata*** (McVaugh) Oudejans, Mexico, *Y. Ramirez-Amezcua 697* (MICH), HQ645276, HQ650948, HQ645428, HQ645582, HQ645731; ***Euphorbia granulata*** Forssk., Morocco, *R. Riina 1800* (MICH), HQ645277, HQ650949, HQ645429, HQ645583, HQ645732; ***Euphorbia hirta*** L., Mexico: Sonora, *A.L. Reina-G. 2006-470* (MICH), HQ645278, HQ650950, HQ645430, HQ645584, HQ645733; ***Euphorbia hirtella*** Boiss., Argentina, *B. van Ee 621* (MICH), HQ645279, HQ650951, HQ645431, HQ645585, HQ645734; ***Euphorbia hooveri*** Wheeler, USA: California, *P.E. Berry 7761* (MICH), HQ645280, [HQ650952 (clone 1), HQ650953 (clone 2)], HQ645432, HQ645586, HQ645735; ***Euphorbia humifusa*** Willd., Russia, *W. Jin 16* (MICH), HQ645281, HQ650954, HQ645433, HQ645587, HQ645736; ***Euphorbia hypericifolia*** L., USA: Florida, *Y. Yang 128* (MICH), HQ645282, HQ650955, HQ645434, HQ645588, HQ645737; ***Euphorbia hypericifolia*** L., Puerto Rico, *W. Jin 36* (MICH), HQ645353, –, HQ645506, HQ645656, HQ645809; ***Euphorbia hyssopifolia*** L., Mexico: Sonora, *T.R. Van Devender 2006-463* (MICH), HQ645283, HQ650956, HQ645435, HQ645589, HQ645738; ***Euphorbia inaequilatera*** Sond., Tanzania, *J.J. Morawetz 452* (MICH), HQ645284, HQ650957, HQ645436, HQ645590, HQ645739; ***Euphorbia indica*** Lam., Madagascar, *B. van Ee 1025* (MICH), HQ645350, –, HQ645503, HQ645653, HQ645806; ***Euphorbia indica*** Lam., Oman, *J.J. Morawetz 326* (MICH), HQ645352, HQ651029, HQ645505, HQ645655, HQ645808; ***Euphorbia indivisa*** (Engelm.) Tidestr., Mexico: Sonora, *T.R. Van Devender 2006-723* (MICH), HQ645285, HQ650958, HQ645437, HQ645591, HQ645740; ***Euphorbia jejuna*** M.C.Johnst. & Warnock, USA: Texas, *B.L. Turner 24-416* (SRSC), HQ645286, –, HQ645438, –, HQ645741; ***Euphorbia johnstonii*** Mayfield, Mexico, *R.F. Sage s.n.* (MICH), HQ645287, [HQ650959 (clone 1), HQ650960 (clone 2)], HQ645439, HQ645592, HQ645742; ***Euphorbia klotzschii*** Oudejans, Argentina, *B. van Ee 619* (MICH), HQ645314, [HQ650996 (clone 1), HQ650995 (clone 2)], HQ645467, HQ645620, HQ645770; ***Euphorbia kuwaluana*** O.Deg. & Sherff, USA: Hawaii, *C.W. Morden 2222* (HAW), HQ645288, [HQ650962 (clone 1), HQ650961 (clone 2), HQ650963 (clone 3)], HQ645440, HQ645593, HQ645743; ***Euphorbia lasiocarpa*** Klotzsch, Jamaica, *B. van Ee 764* (MICH), HQ645289, HQ650964, HQ645441, HQ645594, HQ645744; ***Euphorbia lata*** Engelm., USA: Texas, *Y. Yang 13* (MICH), HQ645290, HQ650965, HQ645442, HQ645595, HQ645745; ***Euphorbia leucantha*** (Klotzsch & Garcke) Boiss., Mexico, *Y. Yang 98* (MICH), HQ645291, HQ650966, HQ645443, HQ645596, HQ645746; ***Euphorbia leucophylla*** Benth., Mexico: Baja California Sur, *V.W. Steinmann 6437* (MICH), –, HQ650967, HQ645444, HQ645597, HQ645747; ***Euphorbia linguiformis*** McVaugh, Mexico, *Y. Yang 97* (MICH), HQ645292, HQ650968, HQ645445, HQ645598, HQ645748; ***Euphorbia lissosperma*** S.Carter, Kenya, *R.B. Faden 74/778* (MO), HQ645293, –, HQ645446, HQ645599, HQ645749; ***Euphorbia maculata*** L., USA: Michigan, *P.E. Berry 7762* (MICH), HQ645294, [HQ650970 (clone 1), HQ650969 (clone 2)], HQ645447, HQ645600, HQ645750; ***Euphorbia magdalenae*** Benth., Mexico, Dominguez L., M. 1476 (IEB), HQ645295, –, HQ645448, HQ645601, HQ645751; ***Euphorbia makinoi*** Hayata, Taiwan, *C. Lin 690* (MO), HQ645296, HQ650971, HQ645449, HQ645602, HQ645752; ***Euphorbia meganaesos*** Featherman, USA: Louisiana, *R. Neyland 1092* (LSU), HQ645297, –, HQ645450, HQ645603, HQ645753; ***Euphorbia mendezii*** Boiss., Mexico, *Y. Yang 48* (MICH), HQ645298, HQ650972, HQ645451, HQ645604, HQ645754; ***Euphorbia mendezii*** Boiss., Mexico, *P. Carrillo-Reyes 4857* (IEB), HQ645299, HQ650973, HQ645452, HQ645605, HQ645755; ***Euphorbia mertonii*** Fosberg, Seychelles, *D. Potter 920501-04* (DAV), HQ645300, HQ650974, HQ645453, HQ645606, HQ645756; ***Euphorbia mertonii*** Fosberg, Madagascar, *B. van Ee 1086* (MICH), HQ645351, –, HQ645504, HQ645654, HQ645807; ***Euphorbia mesembryanthemifolia*** Jacq., USA: Florida, *Y. Yang 136* (MICH), HQ645301, HQ650975, HQ645454, HQ645607, HQ645757; ***Euphorbia micromera*** Boiss., USA: Texas, *Y. Yang 36* (MICH), HQ645302, HQ650976, HQ645455, HQ645608, HQ645758; ***Euphorbia missurica*** Raf., USA: Texas, *Y. Yang 29* (MICH), HQ645303, HQ650977, HQ645456, HQ645609, HQ645759; ***Euphorbia mossambicensis*** (Klotzsch & Garcke) Boiss., South Africa, *R. Becker 1338* (MICH), HQ645304, [HQ650980 (clone 1), HQ650978 (clone 2), HQ650979 (clone 3)], HQ645457, HQ645610, HQ645760; ***Euphorbia multiformis* var. *microphylla*** Boiss., USA: Hawaii, *M.J. Spork s.n.*, HQ645305, [HQ650981 (clone 1), HQ650982 (clone 2)], HQ645458, HQ645611, HQ645761; ***Euphorbia myrtillifolia*** L., Jamaica, *B. van Ee 754* (MICH), HQ645306, JN542511, HQ645459, HQ645612, HQ645762; ***Euphorbia neopolycnemoides*** Pax & K.Hoffm., South Africa, *R. Becker 1339* (MICH), HQ645307, [HQ650984 (clone 1), HQ650983 (clone 2)], HQ645460, HQ645613, HQ645763; ***Euphorbia nocens*** (L.C.Wheeler) V.W.Steinm., Mexico, *Y. Yang 43* (MICH), HQ645308, [HQ650986 (clone 1), HQ650985 (clone 2)], HQ645461, HQ645614, HQ645764; ***Euphorbia nutans*** Lag., USA: Michigan, *P.E. Berry 7763* (MICH), HQ645309, HQ650987, HQ645462, HQ645615, HQ645765; ***Euphorbia olowaluana*** Sherff, USA: Hawaii, *M.J. Spork s.n.*, HQ645310, [HQ650988 (clone 1), HQ650990 (clone 2), HQ650989 (clone 3)], HQ645463, HQ645616, HQ645766; ***Euphorbia ophthalmica*** Pers., Mexico, *Y. Yang 101* (MICH), HQ645311, HQ650991, HQ645464, HQ645617, HQ645767; ***Euphorbia oranensis*** (Croizat) Subils, Argentina, *B. van Ee 685* (MICH), HQ645312, [HQ650993 (clone 1), HQ650992 (clone 2)], HQ645465, HQ645618, HQ645768; ***Euphorbia orbiculata*** Kunth, Colombia, *R. Riina 1589* (MICH), HQ645313, HQ650994, HQ645466, HQ645619, HQ645769; ***Euphorbia parryi*** Engelm., USA: Texas, *B.H. Warnock 18715* (SRSC), HQ645315, –, HQ645468, HQ645621, HQ645771; ***Euphorbia pediculifera*** Engelm., Mexico: Sonora, *T.R. Van Devender 2006-938* (MICH), HQ645317, HQ650997, HQ645470, HQ645623, HQ645773; ***Euphorbia peninsularis*** I.M.Johnst., Mexico: Baja California Sur, *Y. Yang 201* (MICH), HQ645318, HQ650998, HQ645471, HQ645624, HQ645774; ***Euphorbia peplis*** L., Greece, *R. Riina 1566* (MICH), HQ645319, [HQ650999 (clone 1), HQ651000 (clone 2)], HQ645472, HQ645625, HQ645775; ***Euphorbia perennans*** (Shinners) Warnock & M.C.Johnst., USA: Texas, *Y. Yang 3* (MICH), HQ645320, HQ651001, HQ645473, HQ645626, HQ645776; ***Euphorbia perlignea*** McVaugh, Mexico, *V.W. Steinmann 3045* (MICH), HQ645321, HQ651002, HQ645474, HQ645627, HQ645777; ***Euphorbia petrina*** S.Watson, Mexico: Sonora, *A.L. Reina-G. 2006-1403* (MICH), HQ645322, –, HQ645475, HQ645628, HQ645778; ***Euphorbia pionosperma*** V.W.Steinm. & Felger, Mexico, *V.W. Steinmann 1006* (IEB), HQ645323, HQ651003, HQ645476, HQ645629, HQ645779; ***Euphorbia polycarpa*** Benth., Mexico: Sonora, *T.R. Van Devender 2006-551* (MICH), HQ645325, HQ651004, HQ645478, HQ645630, HQ645781; ***Euphorbia polycnemoides*** Hochst. ex Boiss., Malawi, *J. Pawek 12716* (MO), HQ645324, –, HQ645477, –, HQ645780; ***Euphorbia polygonifolia*** L., Canada, *P.E. Berry 7765* (MICH), HQ645326, HQ651005, HQ645479, HQ645631, HQ645782; ***Euphorbia porteriana*** (Small) Oudejans, USA: Florida, *Y. Yang 131* (MICH), HQ645327, [HQ651006 (clone 1), HQ651007 (clone 2)], HQ645480, HQ645632, HQ645783; ***Euphorbia potentilloides*** Boiss., Argentina, *G. Ocampo 1557* (IEB), HQ645328, [HQ651008 (clone 1), HQ651009 (clone 2)], HQ645481, HQ645633, HQ645784; ***Euphorbia prostrata*** Aiton, Mexico: Sonora, *A.L. Reina-G. 2006-473* (MICH), HQ645329, HQ651010, HQ645482, HQ645634, HQ645785; ***Euphorbia psammogeton*** P.S.Green, Australia, *D. Halford Q8340a* (BRI), HQ645330, HQ651011, HQ645483, HQ645635, HQ645786; ***Euphorbia remyi*** A.Gray ex Boiss, USA: Hawaii, *C.W. Morden 1365* (HAW), HQ645331, –, HQ645484, HQ645636, HQ645787; ***Euphorbia reniformis*** Blume, Thailand, *H-J. Esser 08-03* (MICH), HQ645332, HQ651012, HQ645485, HQ645637, HQ645788; ***Euphorbia revoluta*** Engelm., Mexico: Sonora, *A.L. Reina-G. 2006-661* (MICH), HQ645333, HQ651013, HQ645486, HQ645638, HQ645789; ***Euphorbia riebeckii*** Pax, Oman, *J.J. Morawetz 361a* (MICH), HQ645334, HQ651014, HQ645487, HQ645639, HQ645790; ***Euphorbia rockii*** C.N.Forbes, USA: Hawaii, *T.J. Motley 1699* (BISH), HQ645335, –, HQ645488, HQ645640, HQ645791; ***Euphorbia ruizlealii*** Subils, Argentina, *B. van Ee 675*

(MICH), HQ645336, HQ651015, HQ645489, HQ645641, HQ645792; ***Euphorbia schizolepis*** F.Muell. ex Boiss., Australia, *B. Wannan 2640* (BRI), HQ645337, [HQ651017 (clone 1), HQ651016 (clone 2)], HQ645490, HQ645642, HQ645793; ***Euphorbia schultzii*** Benth., Australia, *D. Halford Q9220a* (BRI), HQ645238, –, HQ645390, HQ645544, HQ645694; ***Euphorbia schultzii*** Benth., Australia, *I.D. Cowie 5234* (BRI), HQ645338, HQ651018, HQ645491, HQ645643, HQ645794; ***Euphorbia selloi*** (Klotzsch & Garcke) Boiss., Argentina, *G. Ocampo 1558* (IEB), HQ645339, [HQ651020 (clone 1), HQ651019 (clone 2)], HQ645492, HQ645644, HQ645795; ***Euphorbia serpens*** Kunth, Greece, *R. Riina 1557* (MICH), HQ645340, HQ651021, HQ645493, HQ645645, HQ645796; ***Euphorbia serpens*** Kunth, USA: Texas, *Y. Yang 14* (MICH), HQ645341, HQ651022, HQ645494, HQ645646, HQ645797; ***Euphorbia serpyllifolia*** Pers., Mexico, *Y. Yang 46* (MICH), HQ645342, HQ651023, HQ645495, HQ645647, HQ645798; ***Euphorbia serrula*** Engelm., Mexico: Sonora, *T.R. Van Devender 2006-406* (MICH), HQ645343, [HQ651025 (clone 1), HQ651024 (clone 2)], HQ645496, HQ645648, HQ645799; ***Euphorbia setiloba*** Engelm. ex Torr., Mexico: Sonora, *A.L. Reina-G. 2006-478* (MICH), HQ645345, HQ651026, HQ645498, HQ645650, HQ645801; ***Euphorbia setosa*** (Boiss.) Müll.Arg., Brazil, *I. Cordeiro 3025* (MICH), HQ645346, –, HQ645499, HQ645651, HQ645802; ***Euphorbia simulans*** (L.C.Wheeler) Warnock & M.C.Johnst., USA: Texas, *Y. Yang 2* (MICH), HQ645347, HQ651027, HQ645500, HQ645652, HQ645803; ***Euphorbia* sp. nov.** Australia, Australia, *R. Booth 3536* (BRI), HQ645348, –, HQ645501, –, HQ645804; ***Euphorbia* sp. nov.** Mexico, Mexico, *V.W. Steinmann 1007* (IEB), HQ645349, HQ651028, HQ645502, –, HQ645805; ***Euphorbia stictospora*** Engelm., USA: Texas, *Y. Yang 24* (MICH), HQ645355, HQ651031, HQ645508, HQ645658, HQ645811; ***Euphorbia tamanduana*** Boiss., Brazil, *M. Caruzo 136* (MICH), –, [HQ651032 (clone 1), HQ61033 (clone 2), HQ651034 (clone 3)], HQ645509, HQ645659, HQ645812; ***Euphorbia tettensis*** Klotzsch, South Africa, *N. Zambatis 2024* (PRE), HQ645356, [HQ651035 (clone 1), HQ61036 (clone 2), HQ651037 (clone 3)], HQ645510, HQ645660, HQ645813; ***Euphorbia theriaca*** L.C. Wheeler, USA: Texas, *A.M. Powell 6349* (SRSC), HQ645357, HQ651038, HQ645511, HQ645661, HQ645814; ***Euphorbia theriaca* var. *spurca*** M.C.Johnst., USA: Texas, *Y. Yang 37* (MICH), HQ645354, HQ651030, HQ645507, HQ645657, HQ645810; ***Euphorbia thymifolia*** L., Mexico: Sonora, *T.R. Van Devender 2006-628* (MICH), HQ645358, HQ651039, HQ645512, HQ645662, HQ645815; ***Euphorbia tomentulosa*** S.Watson, Mexico: Baja California Sur, *Y. Yang 196* (MICH), HQ645359, HQ651040, HQ645513, HQ645663, HQ645816; ***Euphorbia trachysperma*** Engelm., Mexico: Sonora, *T.R. Van Devender 2007-688* (MICH), HQ645360, –, HQ645514, HQ645664, HQ645817; ***Euphorbia trialata*** (Huft) V.W.Steinm., Mexico, *Y. Yang 88* (MICH), HQ645361, HQ651041, HQ645515, HQ645665, HQ645818; ***Euphorbia turpinii*** Boiss., Dominican Republic, *B. van Ee 643* (MICH), HQ645362, [HQ651042 (clone 1), HQ651043 (clone 2)], HQ645516, HQ645666, HQ645819; ***Euphorbia umbellulata*** Engelm. ex Boiss., Mexico, *Y. Yang 99* (MICH), HQ645363, HQ651044, HQ645517, HQ645667, HQ645820; ***Euphorbia vallis-mortae*** (Millsp.) J.T.Howell, USA: California, *P.E. Berry 8027* (MICH), HQ645364, HQ651045, HQ645518, HQ645668, HQ645821; ***Euphorbia velleriflora*** (Klotzsch & Garcke) Boiss., Mexico: Sonora, *T.R. Van Devender 2006-513* (MICH), HQ645365, HQ651046, HQ645519, HQ645669, HQ645822; ***Euphorbia vermiculata*** Raf., Canada, *M.J. Oldham 20515* (MICH), HQ645366, HQ651047, HQ645520, HQ645670, HQ645823; ***Euphorbia villifera*** Scheele, USA: Texas, *Y. Yang 26* (MICH), HQ645367, HQ651048, HQ645521, HQ645671, HQ645824; ***Euphorbia zambesiana*** Benth., Tanzania, *J.C. Lovett 4703* (MO), HQ645368, HQ651049, HQ645522, HQ645672, HQ645825.

Outgroup—***Euphorbia appariciana*** Rizzini, Brazil, *M. Caruzo 138* (MICH), HQ645226, –, HQ645378, HQ645532, HQ645682; ***Euphorbia dentata*** Michx., USA: Texas, *Y. Yang 40* (MICH), HQ645316, –, HQ645469, HQ645622, HQ645772; ***Euphorbia eriantha*** Benth., USA: Texas, *Y. Yang 1* (MICH), HQ645262, HQ650931, HQ645414, HQ645568, HQ645717; ***Euphorbia glanduligera*** Pax, Angola, *P.V. Bruyns 10692* (BOL), HQ645272, HQ650944, HQ645424, HQ645578, HQ645727; ***Euphorbia graminea*** Jacq., Mexico, *V.W. Steinmann 5818* (MICH), HQ645263, HQ650932, HQ645415, HQ645569, HQ645718; ***Euphorbia sessilifolia*** Klotzsch ex Boiss., Brazil, *M. Caruzo 133* (MICH), HQ645344, –, HQ645497, HQ645649, HQ645800.

Appendix 2. Presumably hybrid taxa that were excluded from the 5-locus data set, inferred from divergent copies of *EMB2765* exon 9 or divergent placement between ITS and cpDNA phylogenies.

Euphorbia berteroana, E. blodgettii, E. carissoides, E. catamarcensis, E. celastroides var. kaenana, E. chamaerrhodos, E. cinerascens, E. clusiifolia, E. degeneri, E. dentata, E. eichleri, E. garberi, E. hooveri, E. klotzschii, E. kuwaluana, E. maculata, E. magdalenae, E. mossambicensis, E. multiformis var. microphylla, E. neopolycnemoides, E. olowaluana, E. oranensis, E. porteriana, E. potentilloides, E. prostrata, E. remyi, E. rockii, E. schizolepis, E. schultzii, E. selloi, E. serrula, E. setosa, E. tamanduana, E. tettensis, E. turpinii.