Triparticalcar equi is a new coprophilous species within Spizellomycetales, Chytridiomycota

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ABSTRACT

Herbivore dung is a well-known habitat for filamentous fungi; however, we are only beginning to examine this substrate for chytridiomycete diversity. During a recent survey of chytrids of Tuscaloosa County, Alabama, we isolated a novel species in the genus *Triparticalcar* from herbivore dung. We examined two strains (WJD101, WJD156) with light and transmission electron microscopy and compared them to the type species, *T. articum*. We also analyzed partial nuc 28S rDNA D1-D2 domains (28S) and nuc rDNA regions encompassing the internal transcribed spacers 1 and 2 and 5.8S (ITS) to determine the phylogenetic placement of the strains within a broader sampling of Spizellomycetales. Our molecular phylogeny confirmed that the two strains belong to a new phylogenetic species within *Triparticalcar*. The two isolates are distinguishable from the type by the development and morphology of their rhizoids, and we describe the new species as *T. equi*, the first formal description of a new species in this genus since it was erected. Published on-line www.phytologia.org *Phytologia 98(4): 241-249 (Oct 6, 2016)*. ISSN 030319430.

KEY WORDS: *Triparticalcar equi* **sp. nov.**, chytrid, dung, molecular phylogeny, Spizellomycetales, taxonomy, zoospore ultrastructure.

Herbivore dung is a well-known habitat for ascomycetes, basidiomycetes, and fungi formerly classified as zygomycetes (Bell, 1983). However, the diversity of chytrid fungi (Chytridiomycota) inhabiting dung is under explored. Wakefield et al. (2010) first reported strains representing the Spizellomycetales genera *Gaertneriomyces* D. J. S. Barr and *Triparticalcar* D. J. S. Barr from dung, and some of these dung strains were molecularly divergent from existing species of the genera and probably are novel taxa. Expanding the list of dung chytrids within the Spizellomycetales, Simmons (2011) reported the genus *Geranomyces* D. R. Simmons from dung, and Simmons and Longcore (2012) described a new genus, *Fimicolochytrium* D. R. Simmons & Longcore, based on a species isolated from dung. A new member of Lobulomycetales, *Alogomyces tanneri* D. R. Simmons and Letcher, was also isolated from dung (Simmons et al., 2012). Simmons (2012) examined biodiversity within herbivore dung with next-generation sequencing and found it rich in sequences that form a well-supported clade with *Triparticalcar arcticum* (D. J. S. Barr) D. J. S. Barr.

Barr (1970) isolated *Triparticalcar arcticum*, the type and currently only species of *Triparticalcar* (Barr, 1980), from Canadian high arctic saline clay soil and originally placed it in the genus *Phlyctochytrium* J. Schröt based on thallus morphology and development. Isolated from pollen, it had one to many papillae, a globose or peg-like apophysis, and spherical zoospores with the ability to become amoeboid. Based on its distinct zoospore ultrastructure (Barr and Allan, 1981; Chong and Barr, 1973), including a tripartite spur, anterior position of the nucleus and lipid globules, and posterior placement of mitochondria, Barr (1980) placed the species into the new genus *Triparticalcar*. The molecular phylogeny of Wakefield et al. (2010) validated the monophyly of *Triparticalcar* and its divergence from other spizellomycetalean genera.

During an investigation of chytrid diversity in Tuscaloosa County, Alabama, we baited horse and cow dung samples for chytrids. The resulting strains, designated WJD 101 and WJD 156, were morphologically similar to *T. arcticum*, and in a molecular phylogeny based on partial nuc 28S rDNA D1-D2 domains (28S), the two strains grouped sister to *T. arcticum* (Davis et al., 2013). Davis et al.

(2013) suggested that the strains represented a new species within the genus; however, their analysis did not include other members of the *Triparticalcar* clade from Wakefield et al. (2010) nor was a morphological description included. Herein, we infer a new molecular phylogeny that includes both the new isolates and members of the *Triparticalcar* clade from Wakefield et al (2010) and compare morphology of cultures WJD 101 and WJD 156 with that of the extype culture of *Triparticalcar arcticum* (strain BR 59). Based on the results, we describe the new species *Triparticalcar equi.*

MATERIALS AND METHODS

Isolation and culture: Dr. Pete Letcher collected cow dung from a farm in Duncanville, Alabama, and Dr. Carol Duffy collected horse dung from Timber Acres Ranch in Buhl, Tuscaloosa County, Alabama. A subsample of each dung collection was placed in a sterile Petri dish, flooded with sterile water, and baited with pine pollen (Davis et al., 2013). Strain WJD 101 was cultured from cow dung and strain WJD 156 from horse dung. Strain BR 059 was obtained from the American Type Culture Collection (ATCC #18785). All cultures were maintained on PmTG (Barr, 1987; 1 g peptonized milk, 1 g tryptone, 5 g glucose, 10 g agar, 1 L double-distilled water) nutrient agar in Parafilm-sealed Petri dishes at room temperature (20–25°C) in the dark.

DNA extraction and amplification: We extracted genomic DNA as described in Davis et al. (2013) and amplified the nuc rDNA region encompassing the internal transcribed spacers 1 and 2 and 5.8S (ITS) with the ITS4/ITS5 primer pair (White et al., 1990). PCR conditions and cycles were those used in Davis et al. (2013). Amplicons were sequenced by Macrogen Corp USA (Rockville MD), and we assembled sequenced amplicons into contiguous sequences using default settings in Sequencher 4.5 (Genecodes).

Phylogenetic analysis: We downloaded the 28S sequences of WJD 101 and WJD 156 (Davis et al., 2013) and 17 additional members of Spizellomycetales (Simmons, 2011; Wakefield et al., 2010) from GenBank and aligned them with ClustalX (Thompson et al., 1997) followed by manual adjustment in BioEdit (Hall, 1999). The alignment was deposited in TreeBase http://purl.org/phylo/treebase/phylows/study/TB2:S190 96. We used PAUPRat (Sike and Lewis, 2001) to infer maximum parsimony (MP) trees and generated support values as heuristic searches with 500 replicates, each with 10 random-addition replicates. We determined the best-fit model of nucleotide substitution with MrModeltest 2.3 (http://www.abc.se/nylander) and inferred Maximum Likelihood (ML) trees with GARLI 0.951 (Zwikl, 2006). Branch support was assessed with 500 bootstrapping replicates. The inferred trees included 19 spizellomycetalean members and were rooted with two members of the sister order Rhizophlyctidales. Molecular divergence among strains WJD 101, WJD 156, and BR 059 in the 28S and ITS rDNA regions were determined by pair-wise similarity comparisons in BioEdit (Hall, 1999).

Morphology: We inoculated nutrient agar plates (PmTG) with zoospore suspensions of the three strains and recorded morphology of different developmental stages with brightfield and phase contrast light microscopy (Zeiss Axioskop with a Zeiss AxioCam MRc3 camera). Mature sporangia were stained with 0.1% toluidine blue to observe morphology of discharge papillae (Letcher et al., 2015; Parker et al., 1982).

Zoospore ultrastructure: Zoospores were collected and fixed for examination with a Hitachi 7650 transmission electron microscope (TEM). Three- to four-day old plates of WJD 156 and WJD 101 were flooded with sterile water to initiate zoospore discharge. Zoospores were collected in 15 min intervals and fixed in 2.5% glutaraldehyde in 0.1 M *s*-collidine buffer overnight at 20 C. Fixation was continued in1% osmium tetroxide in 0.1M *s*-collidine buffer overnight in the dark at 20 C. Centrifugation at 3 *g* was used to produce a pellet of fixed zoospores, which was then embedded in molten agar. The pellet was trimmed into 1 mm × 1mm blocks and suspended in saturated aqueous uranyl acetate overnight in the dark on a

shaker. The blocks were dehydrated in a graded acetone series (10%, 30%, 50%, 70%, 85%, 95%, 100%, 100%) held for 15 min at each step. A graded series was used to infiltrate the blocks with EPON resin: 12% for 1 h, 25% for 4 h, 50% for 4 h, 75% for 12 h, 100% for 24 h, and 100% for 24 h. The EPON was polymerized for 3 days at 72 C. Sections of 100 nm thickness were obtained using a diamond knife on a Leica Ultracut microtome and were collected on 300-mesh hexagonal nickel grids. Grids with sections were treated with 1% periodic acid (4 min) to enhance staining and post-stained with uranyl acetate in 70% ethanol (10 min) and lead citrate in the presence of sodium hydroxide (6 min). Sections were observed at 60 kV with a Hitachi 7650 transmission electron microscope (TEM).

RESULTS

Phylogenetic analysis: The 28S sequences of WJD 156 and WJD 101 were 99% similar to each other and 90% similar to *Triparticalcar arcticum* BR 059. Internal transcribed spacer sequences of WJD 156 and WJD 101 were 89% similar to each other and 57% similar to *T. arcticum* BR 059, which made them unalignable even within the genus; thus, ITS was excluded from tree inference. The alignment of 28S sequences contained 861 characters of which 315 were parsimony informative. From 1005 PAUPRat inferred trees, 990 were equally parsimonious (L= 1370 steps, CI= 0.393, RI= 0.711). The best model of base pair substitution was GTR + G. MP and ML (-lnL = 4212.74) trees were congruent and only the ML tree is depicted here (FIG.1). Figure 1 shows that isolates WJD 156 and WJD 101 form a well-supported (100% bootstrap support) sister clade to *T. arcticum* (BR 059, JEL 554, JEL 555, JEL 560). The relationships between the *Triparticalcar* clade and other members of Spizellomycetales are not fully resolved in this analysis (FIG. 1).

Morphology: The development and morphology of *T. arcticum* are treated extensively elsewhere (Barr 1970, 1984) and will be treated briefly here. Descriptions will follow the terminology of Barr (1984). On agar, BR 059 germlings have a broad, peg-like rhizoidal axis (FIG. 2A) with rhizoids typically developing at the end of the rhizoidal axis and occasionally developing laterally (FIG. 2A, B). Rhizoids are generally stout, branch terminally, and taper evenly into blunt ends (FIG. 2B). At maturity, the main rhizoidal axis becomes bulbous or a multibranched process (FIG. 2C). Rhizoids form a short, dense network (FIG. 2C), and zoosporangia produce two to three discharge papillae (FIG. 2D).

WJD 156 and WJD 101 followed similar developmental pathways and share the same morphological features. Hence, observations made on both are reported together, and only images of WJD156 are shown. On nutrient agar, zoospore cysts form narrow, long rhizoidal axes (FIG. 2E), and rhizoids develop laterally and terminally (FIG. 2F). Through development, rhizoids are long, isodiametric, and blunt-ended with lateral and terminal branches, and the rhizoidal axis is isodiametric initially and becomes bulbous or inflated in shape (FIG. 2F). At maturity, the rhizoidal system is dense with long, extensively branched rhizoids that obscure the rhizoidal axis (FIG. 2G). Mature zoosporangia are covered with numerous cylindrical, protruding discharge papillae approximately 2-5μm high (FIG. 2H).

Zoospore ultrastructure: The nucleus and several lipid globules are anteriorly positioned (FIG. 3A). In longitudinal section, mitochondria are predominantly posteriorly positioned (FIG. 3A), and in transverse section, mitochondria are also observed anteriorly positioned (FIG. 3B). Microbodies are associated with the lipid globules with mitochondria located close by (FIG. 3B). Ribosomes are dispersed (FIG. 3A, B). A tripartite spur and microtubule complex extend from the kinetosome to the nucleus (FIG. 3A, D, E). The posterior of the non-flagellated centriole is angled approximately 60° away from the kinetosome, and the cell membrane is invaginated near the flagellum (FIG. 3A, C).

TAXONOMY

Triparticalcar equi W. J. Davis, Letcher, & M. J. Powell **sp. nov.** FIGS. 2–3
MycoBank MB 816514

Typification: USA. ALABAMA: Tuscaloosa County, Buhl, Timber Acres Ranch, 33.227526, -87.739037. Pine pollen, horse dung, October 2010, *W. J. Davis 156* (**holotype** FIG. 2G, in Davis et al. 2016. Phytologia 98: 248).

Ex-type strain WJD 156 (UACCC); GenBank KC691398 (28S) and KX019807 (ITS).

Etymology: The specific epithet is the genitive noun of the Latin word for horse, *equus*, and the source of the dung that yielded the holotype culture.

Description: On nutrient agar, germlings have slender, long germ tubes; rhizoids develop laterally and terminally. Rhizoids are long with lateral and terminal branches, isodiametric with blunt ends, and dense at maturity. Rhizoidal axis is bulbous or inflated in shape and obscured at maturity. Mature zoosporangia have numerous discharge papillae 2-5μm high. Zoospores have a *Triparticalcar*-type ultrastructure.

Additional specimens examined: USA. ALABAMA: Tuscaloosa County, Duncanville, a farm. Pine pollen, cow dung, October 2010, *W. J. Davis 101*(UACCC WJD101); GenBank KC788571 (28S) and KX019806 (ITS).

DISCUSSION

Triparticalcar was first erected to accommodate a species isolated on pine pollen from high arctic soils (Barr, 1970, 1980). Subsequently, a number of phylogenetically divergent strains have been observed in this genus but not yet formally described (Davis et al., 2013; Simmons, 2012; Wakefield et al., 2010). Thus, we formally describe and name a new member of this genus, *T. equi*, a species firmly established as a member of this genus molecularly (FIG. 1) and ultrastructurally by the presence of the tripartite spur extending from the kinetosome to the nucleus (FIG. 3A, D, E).

Triparticalcar equi can be distinguished from the type *T. arcticum*. Developmentally, *T. equi* germlings have a narrower and longer germ tube than *T. arcticum* and rarely appear carrot or turnip shaped. In *T. equi* rhizoids develop laterally and terminally whereas in *T. arcticum* the rhizoids develop from the base of the germ tube. The rhizoids of *T. equi* are longer than those of *T. arcticum*, both during development and at maturity. Branching of rhizoids tends to occur laterally and terminally in *T. equi* rather than primarily terminally as in *T. arcticum*.

Molecularly, *T. arcticum* and *T. equi* are 10% divergent in the 28S rDNA region, which is comparable to other species in Chytridiomycetes. For example, in the Lobulomycetales, *Lobulomyces poculatus* (Willoughby & Townley) D. R. Simmons and *L. angularis* (Longcore) D. R. Simmons are 11% divergent in the 28S rDNA region (Simmons et al., 2009). In the Polychytriales, *Arkaya lepida* Longcore & D.R. Simmons and *A. serpentine* (Dogma) Longcore & D. R. Simmons are 7% divergent (Longcore and Simmons, 2012). In Chytridiales, the described species *Pseudorhizidium endosporangiatum* M. J. Powell, Letcher & Longcore is 8% divergent from an undescribed species in the genus (Letcher and Powell, 2014; Powell et al., 2013). In the Rhizophydiales, *Rhizophydium globosum* (A. Braun) Rabenh. and *R. brooksianum* Longcore are 7% divergent (Letcher et al., 2006).

Since this is the first species of *Triparticalcar* described since the genus was established with the single species, *T. arcticum*, our results highlight the undescribed diversity in this group (Barr, 1980). It is interesting that strains JEL554, JEL 555, and JEL 560 that are in the *T. arcticum* clade were also isolated on horse and cow manure (Wakefield et al., 2010). Strains JEL 250, JEL 355, PL 162 formed a clade sister of the *Triparticalcar* clade in our study (labeled unidentified sp.) but were included in the

Triparticalcar clade in Wakefield et al. (2010). Preliminary ultrastructural investigations indicate this sister clade is a new genus. Thus, our study demonstrates that as *Triparticalcar* is currently circumscribed with two species, *T. articum* and *T. equi*, it is a monophyletic genus.

Dung is a well-established source of basidiomycetes, ascomycetes, and fungi formerly classified as zygomycetes (Bell, 1983). However, we are in the early stages of observing the diversity of chytrid fungi that inhabit dung (Wakefield et al., 2010). Much of the diversity observed thus far can be placed in the Spizellomycetales; however, as exemplified by *Alogomyces tanneri*, additional orders may be present as well. More diversity awaits discovery and classification.

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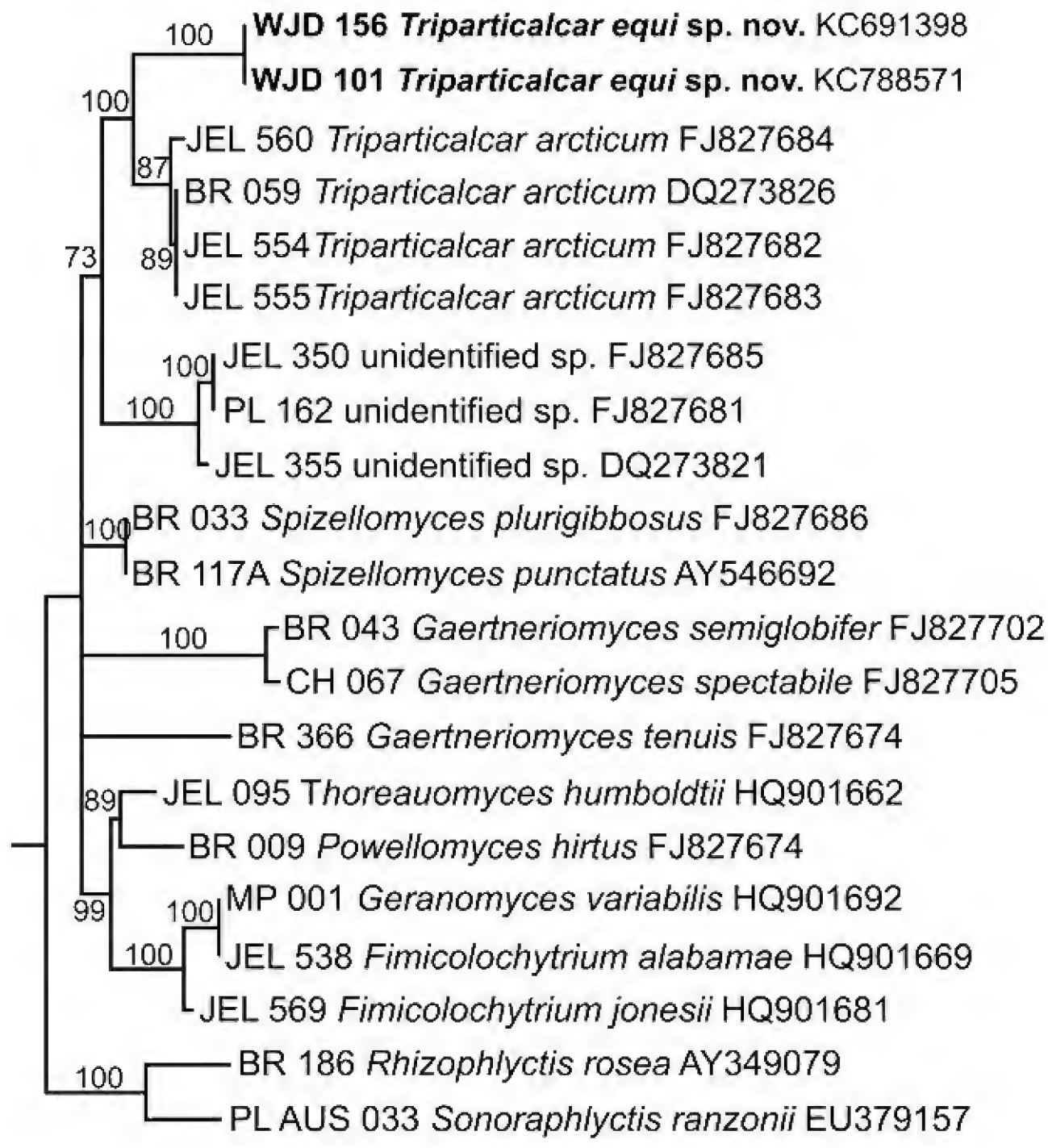

FIG. 1. Molecular phylogeny of 19 taxa in Spizellomycetales inferred from analyses of partial 28S rRNA gene sequences, with two members of Rhizophlyctidales as outgroup. Numbers above branches are ML bootstrap support values; –lnL = 4212.74

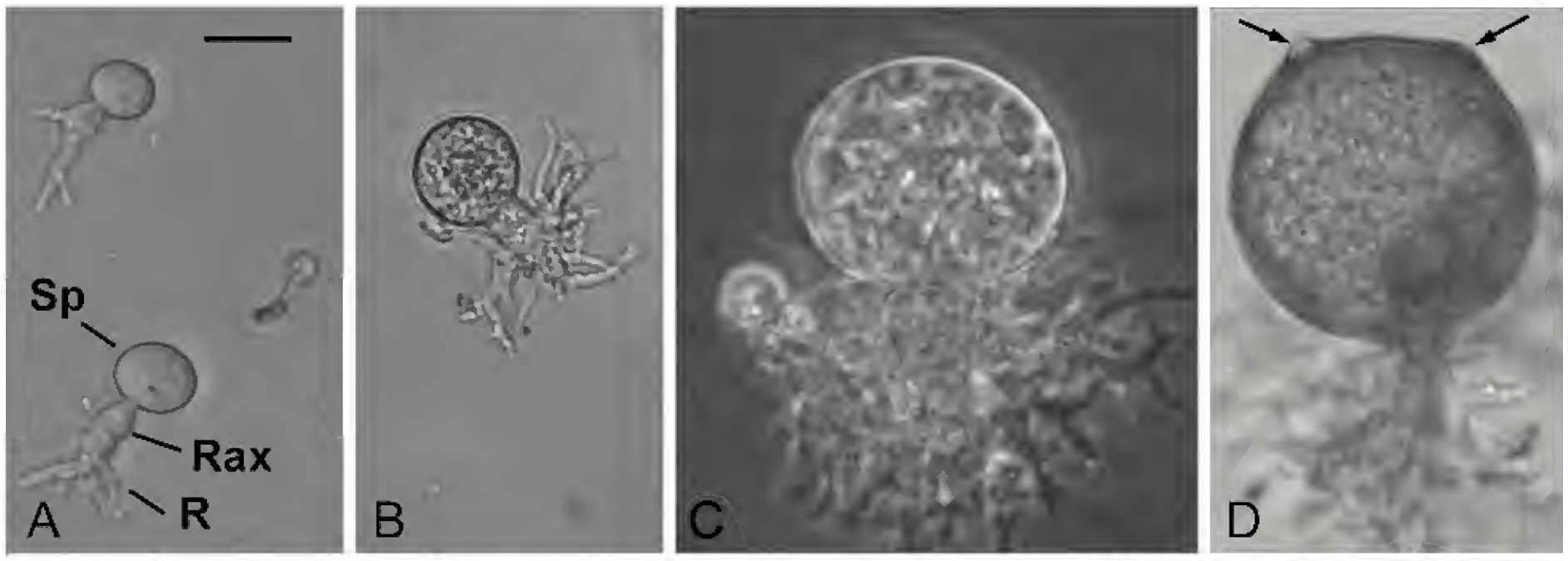

FIG. 2. Light micrographs of *Triparticalcar arcticum* BR 059(A-D) and *T. equi* WJD 156 (E-G) on nutrient agar. A-D. *T. arcticum*, BR 059, type. A. Germlings with stout rhizoidal axis and stubby rhizoids. B. Immature thallus with terminal branching of rhizoids. C. Maturing thallus with compact mass of stout, short rhizoids. D. Mature sporangium with two discharge papillae (arrows) stained with toluidine blue. E. Germinating zoospore with long, narrow rhizoidal axis. F. Immature thallus with stout rhizoidal axis and terminal and laterally branching rhizoids. G. Maturing thallus with compact mass of long, slender rhizoids. H. Mature sporangium with multiple discharge papillae (arrows) stained with toluidine blue. Abbreviations: Sp, sporangium; R, rhizoids; Rax, rhizoidal axis. Scale bar = 10 µm (A, B, E), 20 µm (C, D, F, G).

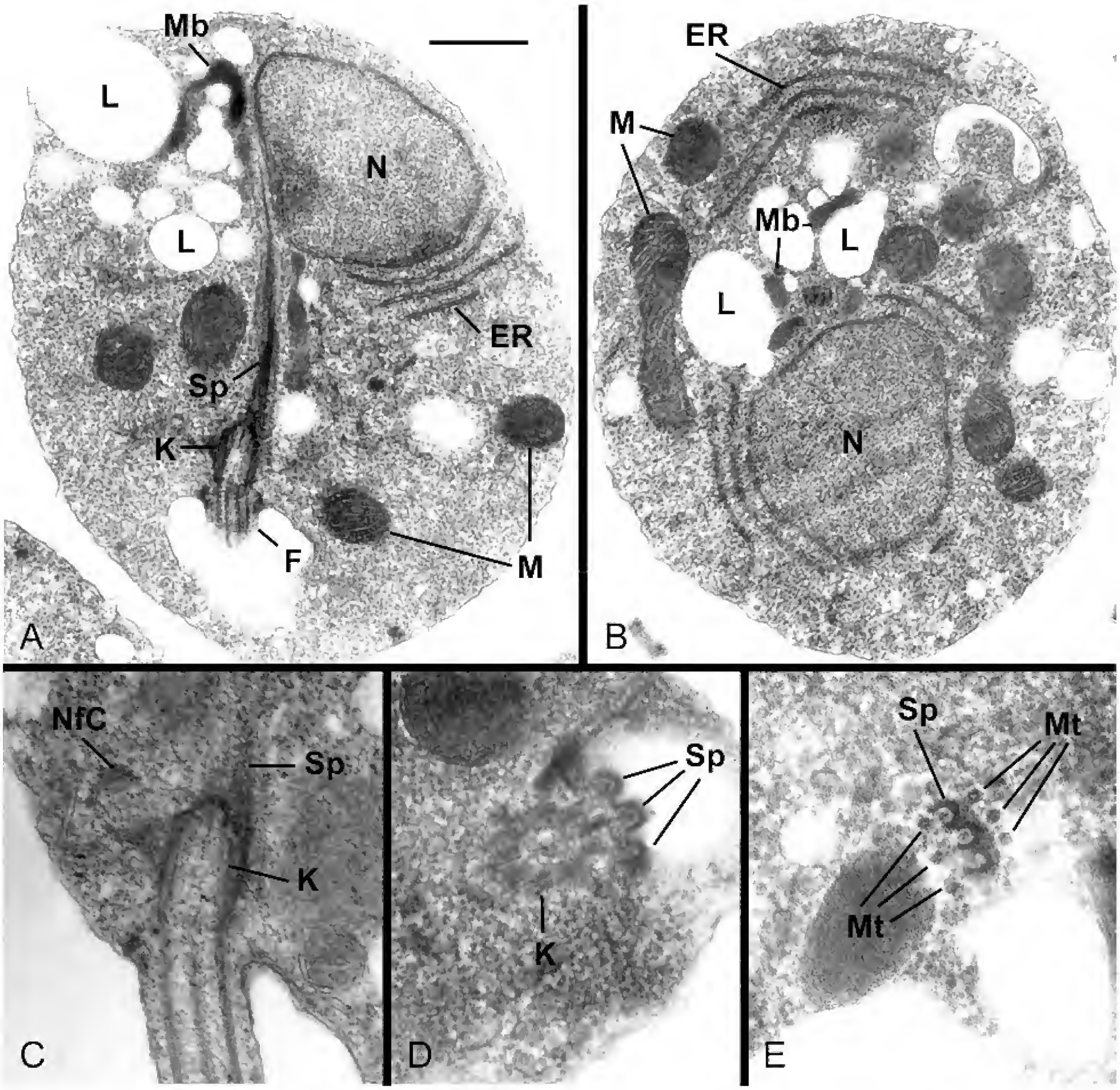

FIG. 3. Ultrastructural features of zoospore of *Triparticalcar equi.* A. Longitudinal section (LS), with an elongate spur originating at the kinetosome and extending anteriorly, microbodies interspersed among multiple lipid-globules, nucleus backed by multiple layers of endoplasmic reticulum, and multiple mitochondria. Ribosomes dispersed in the cytoplasm. B. Transverse section (TS). C. LS illustrating angle (~60°) between kinetosome and non-flagellated centriole. D. TS of kinetosome and spur. E. TS through spur and microtubules, approximately midway through zoospore body. Abbreviations: ER, endoplasmic reticulum; F, flagellum; K, kinetosome; L, lipid globules; M, mitochondria; Mb, microbody; Mt, microtubules; N, nucleus; NfC, non-flagellated centriole; Sp, spur. Scale bar in A = 0.5 µm (A, B), 0.25 µm (C, D), 0.2 µm (E).

Variation in Vegetative and Floral Characteristics of Potential Commercial Significance in Four Native Texas Coastal Species

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ABSTRACT

With increasing demand for high quality irrigation water and active regional coastal development, new plants need to be developed that thrive with the use of saline irrigation and provide an alternative to invasive exotic landscape plants. Regionally native coastal species offer a potential solution. Accessions of *Erigeron procumbens* (Houst. *ex* Mill.) G.L. Nesom, *Borrichia frutescens* (L.) DC., *Sesuvium portulacastrum* (L.) L., and *Oenothera drummondii* Hook. were collected along the Texas coast from Port Isabel to Port Arthur. Then taxa were screened for phenotypic variability in morphological traits that may benefit the landscape industry. There were differences among accessions for all four species and there were regional differences in flowering and height for *B. frutescens* and *O. drummondii*. Mean height for *O. drummondii* accessions ranged from 8 to 68 cm. Flower count varied among *O. drummundii* accessions, with those collected from the southern region tending to only flower in the fall. Flower size and color were not variable for *O. drummondii*, however foliage color was variable among accessions. Plant height for *B. frutescens* accessions ranged from 17 to 78 cm. Phenotypic variability appeared most promising in *O. drummondii* and *B. frutescens* for future breeding efforts. Regional trends were identified in many traits within each species. Published on-line www.phytologia.org *Phytologia 98(4): 250-276 (Oct 6, 2016).* ISSN 030319430.

KEY WORDS: Native plants, phenotypic variability, *Oenothera drummondii*, *Borrichia frutescens*, *Erigeron procumbens*, *Sesuvium portulacastrum*

With the decreasing availability of high quality irrigation water in urban areas, new ornamental crops need to be developed for landscapes that will thrive with lower quality saline irrigation water. One source of irrigation water in arid climates is recycled treated effluent water. One concern with this source of irrigation is elevated salinity that can be as much two to three times the content of potable water (Khurram and Miyamoto, 2005; Wu et al., 2001). When introducing plants to the landscape or nursery trade it is important to determine the extent of variation present in native populations for ornamental traits. *Oenothera drummondii* Hook., *Sesuvium portulacastrum* (L.) L., *Borrichia frutescens* (L.) DC., and *Erigeron procumbens* (Houst. *ex* Mill.) G.L. Nesom were selected from Texas coastal regions based on their close proximity to the coast (Correll and Johnston, 1970; USDA Plants Database, 2009). This proximity to the coast would likely provide natural tolerance to salt exposure, especially in the form of sodium and chlorine ions because in these habitats plants are exposed to saline conditions (Taiz and Zeiger, 2006). Regional native plants were also selected because of growing trends toward use of natives in built landscapes for their adaptability to their endemic region and low potential to become invasive. Sensitive coastal ecosystems can be threatened by invasive exotics such as Brazilian pepper tree (*Schinus terebinthifolius* Raddi), melaleuca (*Melaleuca quinquenervia* (Cav.) S.F. Blake), and water hyacinth (*Eichhornia crassipes* (Mart.) Solms) (Ewe and Sternberg, 2002; Turner et al., 1998; Villamagna and Murphy, 2010). Use of native species could avoid this problem.

Not all native plants may be suitable for general use in built environments, particularly in coastal locations. Plants selected must be able to adapt to commercial container nursery production techniques, tolerate low quality irrigation water, tolerate salt exposure, and have some form of regional and/or genetic variation to provide a basis for the future improvement of cultivated selections.

Documenting the amount and kind of variation in desirable traits within a species is important for the success of a plant improvement program (Zobel and Talbert, 1984). Variation that is present due to geographic differences should be documented first, followed by variation that occurs from other sources (Zobel and Talbert, 1984). "Ecotypic variation is a distinct morphological or physiological form, or population, resulting from selection by a distinct ecological condition" and "is the whole basis of provenance studies" (Arnold, 2008). Provenance studies should provide the foundation for genetic improvement of plant species (Morganstern, 1996). It appears that most adaptability traits are additive in nature and gains in improvement programs can be made by selecting individuals that already possess traits permitting grow in suboptimal conditions (Zobel and Talbert, 1984).

Ecoptypic variation in leaf morphology and plant height has been documented in several species including *Helianthus annuus* L. (sunflower), *Carya illinoinensis* (Wangenh.) K. Koch (pecan), *Spartina patens* (Aiton) Muhl. (saltgrass) and *S. portulacastrum* (Hester et al., 1996; Lokhande et al., 2009; Nooryazdan et al., 2010; Wood et al. 1998).

The objectives of experiments described herein were to begin to characterize the variation in traits of ornamental interest in Texas' coastal populations of *O. drummondii*, *B. frutescens*, *E. procumbens*, and *S. portulacastrum* in a common field location and under container nursery conditions.

MATERIALS AND METHODS

Clonal material of *B. frutescens*, *E. procumbens*, *S. portulacastrum*, and *O. drummondii* was collected from locations along the Texas coast from South Padre Island, Texas to Port Arthur, Texas. Global positioning system (GPS) data and physical location data were recorded (see appendix). Stock plants were generated from the collected material and used to conduct this provenance study in College Station, Texas.

Tip cuttings, 4-6 cm long, were taken on 17 April 2010, from containerized stock plants maintained in a gravel bottom nursery in College Station, TX (30° 37' 24.24", -97° 22' 0.17"). Basal ends of cuttings were dipped in talc based indolebutyric acid at the concentration of 1 $g \cdot kg^{-1}$ (Hormodin® 1, OHP, Inc., Mainland, PA). Cuttings were placed in 36 cm x 51 cm x 10 cm deep flats (Kadon Corp., Dayton, OH) filled with coarse perlite (Sun Gro Horticulture Canada Ltd., Seba Beach, AB). Intermittent mist was applied at 16 min intervals for a 15 sec duration using reverse osmosis water from 1 h before sunrise to 1 h after sunset. On 13 May 2010, rooted cuttings were potted in 0.47 L black plastic pots (Dillen Products, Middlefield, OH) containing Metro-Mix 700 media (Sun Gro Horticulture Canada Ltd., Vancouver, BC.

Container responses: rooted cuttings generated as described above, from each accession collected, were potted into 2.3-L black plastic containers (C400, Nursery Supplies Inc., Kissimmee, FL) containing Metro-Mix 700 media (Sun Gro Horticulture Canada Ltd, Vancouver, BC with 6.53 $kg \cdot m^{-3}$ 15N-3.9P-9.9K controlled release fertilizer (3-4 month Osmocote® Plus, Scotts Co., Marysville, OH) on 3 June 2010. Plants were placed in an outdoor gravel bottom nursery with full sun exposure in a completely randomized design with three replicates of each genotype collected (n=3). Plants were irrigated as needed by hand using tap water with constant fertilizer injection (300 $mg \cdot L^{-1}$ of N, Peters Professional 20N-8.74P-16.6K, Scotts Co., Marysville, OH). On 10 July 2010 plant height, leaf lamina length, leaf width, internode length, stem diameter, and flower diameter were recorded for each as was done with

other species in prior studies (Hester et al., 1996; Nooryazdan et al., 2010; Wood et al., 1998). Leaf and internode measurements were taken from three fully expanded leaves per plant. Flower data were taken from three open flowers per plant.

Landscape responses: rooted cuttings generated as described in the container nursery experiment were planted in field conditions at the Texas A&M University Horticulture Farm (30° 37' 34.0608", -96° 22' 14.2104") with five replicates of each genotype (n=5) on 1 m in row spacings and 4 m between row spacings on 2 June 2010. The soil was a sandy clay loam (66% sand, 8% silt, 26% clay) with a pH of 6.0. Plants were drip irrigated (T-Tape Model 505, Deere and Company, Moline, IL) as needed to maintain turgidity. Flower counts, growth index (height x width in the widest direction x width perpendicular to the widest direction), and an ornamental rating were taken at the end of the growing season (1 November 2010). End of the season plant height, leaf lamina length, leaf width, internode length, and flower diameter were recorded for each genotype (Hester et al., 1996; Nooryazdan et al., 2010; Wood et al., 1998). Leaf and internode measurements were taken on three fully expanded leaves on each plant. Flower width at the widest point was collected from three open flowers on each plant.

An ornamental rating of 1 to 5 was recorded by the same observer at harvest, with 1) representing a dead plant or plant near death (unacceptable for ornamental use), 2) plant with severe damage to the canopy but surviving, 3) plant with open holes in the canopy, erratic growth, and general lack of flowers, 4) canopy was full with uniform growth throughout, with or without flowers (acceptable ornamental landscape plant), and 5) canopy is full with uniform growth throughout with flowers covering at least 10 % of the canopy (acceptable ornamental landscape plant).

The accessions were separated into large regional groupings based on collection site along the Texas coast (Fig. 1.), then statistically analyzed using ANOVA in JMP (SAS Institute Inc., Cary, NC). Effects were considered significant at $P \leq 0.05$. Hierarchical cluster analysis with Wards distance was performed. All non-normal data were analyzed using permutations in the lmPerm package (Wheeler, 2010) in R (R Core Team, 2013), set to defaults.

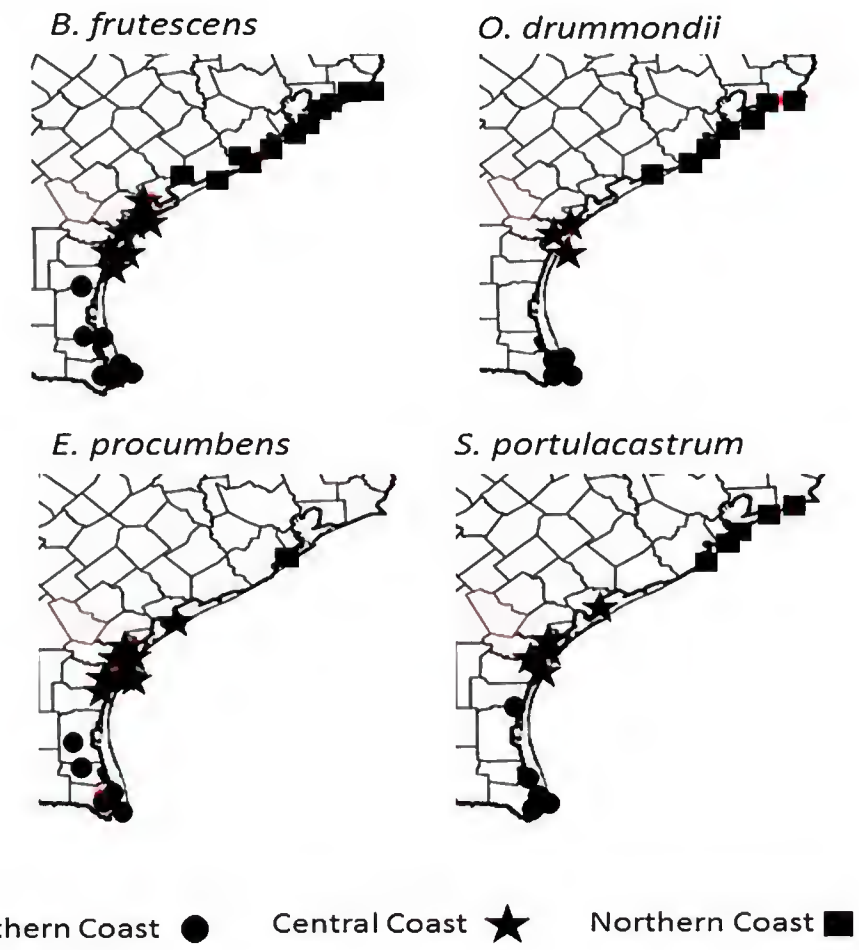

Fig. 1. Collection regions for accessions of *B. frutescens, O. drummondii, E. procumbens*, and *S. portulacastrum*.

RESULTS AND DISCUSSION

Oenothera drummondii

Differences occurred ($P \leq 0.05$) amongst accessions of *O. drummondii* (beach evening primrose) for height, height/width ratio, flower count at harvest, flower diameter, leaf length, leaf width, petiole length and the number of serrations present on each leaf (Tables 1 and 2a). When accessions were allocated to regional groups along the Texas Coast (South, Central, and Northern) based on original

collection location there were differences among regional groups and accessions for height, height/width ratio, flower count, leaf length, leaf width, and petiole length. Internode length was only significant for environment (nursery versus field locations) but not for accession or collection region.

Height varied from 68 cm to 8 cm with a mean across all accessions of 25.2 cm and height:width ratio ranged from 0.67 to 0.05 with a mean of 0.30 (Table 3). Larger height:width ratios are characteristic of upright plants and lower ratios are indicative of a spreading habit. In general, accessions from the southern coast were taller in field conditions than plants from either the central or northern Texas coast (Table 2a). This would explain the negative correlation between height and latitude of original collection site ($r = 0.56$) and the negative correlation between height:width ratio and latitude of original collection site ($r = 0.49$) (Table 3). All accessions, except O10, were not as tall in the nursery environment as they were in the field environment. There was an interaction for environment by accession for height (Table 1). In the field environment, O10 had a mean height of 12.8 cm and in the nursery environment O10 had a mean height of 13.7 cm. All other accessions had reduced height in the nursery compared to the field environment.

Flower count was different ($P \leq 0.05$) among the individual accessions, dependent on the environment in which they were tested, yielding a significant accession by environment interaction (Table 1). The accessions from central and northern collection sites tended to have more flowers in both the nursery and field environments (Table 1). All groups did not flower as freely in the nursery environment as they did in the field conditions. Some accessions came into flower sooner such as O13 in the nursery environments and O16 in the field environment (Fig. 2). Early flowering accessions were not consistent between the two environments and some accessions came into heavier flower later during the experiment (Fig 2). This could be due to the longer natural photoperiod at time of harvest and a more constricted root zone when the plants were grown in containers and the smaller size of the container-grown plants. Plants were smaller across genotypes in containers. The mean growth indices (height x width at widest point x width perpendicular to widest point, a pseudo-volumetric estimate of canopy size) was 63,958 cm^3 in the nursery compared to 551,452 cm^3 in the field, nearly a nine fold difference in size. Several accessions from the southern collection region might be photoperiod sensitive (Tables 2a, b, and 5). Nursery grown plants were harvested in late summer (7 July 2010) and field grown plants were harvested at the end of the season (1 Nov 2010); if the accessions were sensitive to day length, then field grown plant were exposed to shorter days. There are many reports of members of the genus *Oenothera* L. being sensitive to day length, so the presence of day length sensitivity in some accessions would not be surprising (Clough et al., 2001; Gimenez et al., 2013; Kachi and Hirose, 1983).

Further studies need to be performed to determine whether it is indeed photoperiodicity or other factors such as plant size, temperature, or general reluctance to flower that dictate differences among accessions. Further testing is needed due to a lack of sampling dates (Fig. 2). Collections from the southern region had lower flower counts but, as far as ornamental value is concerned, better growth habits with fewer defoliated sections in the canopy as shown in Figure 3.

This is also analogous to results reported by Gratani et al. (2003), who found that *Quercus ilex* L. (holly oak) leaf morphology was related to provenance in varying mesic and xeric climates. This could explain the smaller leaves in the field on accessions from the southern coast. Average rainfall along the Texas coasts varies from 61 – 71 cm in the southern region, 91 – 101 cm in the central coast to 132 – 142 cm in the northern coast (Texas Water Development Board, 2014).

In general, all leaf measures increased in the nursery environment, most likely from more favorable cultural conditions in the form of ample water and nitrogen fertilizer. When accessions were grouped by collection region, accessions from the south had shorter leaves than plants from either the central or northern collection zones in the field. However, when grown in nursery conditions, plants from

the southern region had larger leaves than plants from either the northern or central regions (Table 2a). This suggests leaf morphology is more plastic in accessions from the southern Texas coast and may provide some form of adaptability to harsher environments as has been reported for other taxa (Sultan, 1987; Wood et al., 1998; Gratani et al., 2003).

The number of leaf serrations is also reduced in the southern region accessions, with *O. drummondii* from the northern regions having more leaf serrations on average (Table 2a). In addition to reduced leaf serrations, accessions from the southern collection region tended to have blue foliage, (Chi Square $P = 0.0001$) whereas the other collection locations tended to have green foliage. Sixty-six percent of blue observations were collected from the southern location. The blue foliage color is brought on by the increased presence of pubescence on the leaves, another drought adaptation strategy employed by many plants (Sandquist and Ehleringer, 1998; Ehleringer and Mooney, 1978), and likely reflecting the reduced rainfall in the southern collection region.

Leaf length and plant height, and number of leaf serrations and plant height were both negatively correlated -0.45 and -0.54, respectively (Table 3). Wood et al. (1998) also found correlations among height and latitude and other leaf characteristics such as leaflet droop angle and leaflet tilt angle and latitude in pecan [*Carya illinoinensis* (Wangenh.) K. Koch]. Pecan tree height and latitude were negatively correlated with increasing height and decreasing latitude (Wood et al., 1998), very similar to what was found in *O. drummondii* in this study. Number of leaf serrations and height were also correlated to the longitude of the original collection site (Table 3). Flower count was weakly correlated to leaf width and length, but not to latitude of collection site (Table 3). This suggests that in each group there might be free-flowering and not free-flowering accessions.

Based on hierarchical cluster analysis using only morphologic measures, accessions clustered into two large groups (Fig. 4). This is different than the expected three clusters based on location of collection. Accessions collected from central and northern locations formed one large cluster and accessions from the southern collection locations formed a separate cluster. This is in line with Nooryazdan et al. (2010) who also found that sunflowers (*Helianthus annuus* L.) from similar climatic zones clustered together. One accession of *O. drummondii* collected from the central coast (O2) clustered in the southern group as did one accession (O1) from the southern region which clustered with the northern accessions. Neither accession O1 or O2 were from transition zones. These clustering patterns were also supported by least significant difference means separation performed on the means of the three regional groups for height, flower count and height:width ratio (Table 4). For these measures only plants from the southern region were significantly different from the other collection locations.

There is variation among accessions of *O. drummondii* when sampled from the southern, central, and northern coasts of Texas for height, propensity to flower, growth form, leaf length and width, as well as the number of serrations on the margin of the leaf. We did not find variation in flower diameter based on the region of collection but it was present amongst the accessions as a whole. There was no significant variation in internode length associated with region of collection or accession. There also was no significant variation found in flower color based on visual observation (data not presented) all were of a similar shade of yellow. Plants from the southern collection region tended to have blue foliage.

Table 1. Means of growth measures by accession and regional grouping along the Texas coast of *Oenothera drummondii* grown in field and nursery conditions.

	Height (cm)		Height/width ratio ($cm \cdot cm^{-1}$)		Internode length (mm)		Flower Count (No./plant)		Flower Diameter (mm)	
Accession	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
1	31.0 ± 5.8[T]	21.7 ± 5.2	0.27 ± 0.04	0.31 ± 0.07	8.7 ± 1.0[V]	34.3 ± 7.0	0.6 ± 0.4	0.0 ± 0.0	51.4 ± 1.4	-
2	42.8 ± 3.0	22.0 ± 2.3	0.31 ± 0.03	0.31 ± 0.03	13.7 ± 1.8	18.1 ± 3.0	17.6 ± 4.8	1.0 ± 1.0	62.3 ± 1.4	60.6 ± 2.8
3	47.6 ± 4.6	22.7 ± 3.5	0.30 ± 0.03	0.33 ± 0.10	11.6 ± 1.4	17.6 ± 2.7	4.8 ± 1.0	0.0 ± 0.0	55.1 ± 1.7	59.7 ± 2.7
4	52.75 ± 4.4	23.0 ± 2.6	0.33 ± 0.04	0.39 ± 0.06	14.3 ± 2.4	21.9 ± 2.9	11.8 ± 3.6	0.3 ± 0.3	59.4 ± 1.4	-
5	51.0 ± 17.0	24.7 ± 2.0	0.39 ± 0.09	0.40 ± 0.06	11.0 ± 1.0	17.2 ± 2.6	17.0 ± 16.0	0.0 ± 0.0	54.6 ± 1.5	-
6	49.6 ± 5.0	23.0 ± 2.6	0.36 ± 0.08	0.37 ± 0.02	11.6 ± 1.1	24.4 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	47.9 ± 1.2	-
7	23.6 ± 4.0	14.0 ± 1.5	0.35 ± 0.08	0.24 ± 0.03	11.2 ± 0.9	19.6 ± 2.5	4.2 ± 0.8	0.7 ± 0.7	55.0 ± 2.4	59.8 ± 3.3
8	14.2 ± 2.3	13.3 ± 1.8	0.13 ± 0.03	0.20 ± 0.03	11.4 ± 1.3	24.2 ± 3.5	7.2 ± 2.9	1.7 ± 0.3	62.5 ± 1.7	58.3 ± 5.1
9	23.0 ± 4.1	14.7 ± 3.3	0.20 ± 0.04	0.18 ± 0.04	13.7 ± 1.4	24.1 ± 1.9	11.4 ± 3.9	3.3 ± 1.3	65.0 ± 2.4	51.4 ± 6.8
10	12.8 ± 1.5	13.7 ± 2.4	0.12 ± 0.02	0.18 ± 0.03	10.5 ± 0.6	22.7 ± 2.9	9.4 ± 2.7	3.7 ± 0.6	51.8 ± 1.8	56.4 ± 2.3
11	20.0 ± 4.5	15.0 ± 2.0	0.15 ± 0.04	0.20 ± 0.03	10.5 ± 0.7	19.8 ± 1.8	34.0 ± 11.7	3.3 ± 1.3	53.5 ± 1.2	57.1 ± 0.8
12	25.6 ± 3.7	22.0 ± 2.5	0.19 ± 0.02	0.35 ± 0.04	12.7 ± 1.1	34.0 ± 4.8	33.4 ± 9.1	0.3 ± 0.3	60.3 ± 2.0	63.6 ± 3.6
13	17.8 ± 2.8	15.0 ± 1.5	0.29 ± 0.03	0.27 ± 0.01	8.0 ± 0.6	14.6 ± 1.1	4.0 ± 1.4	2.0 ± 1.0	55.0 ± 1.8	53.7 ± 3.3
14	28.2 ± 2.6	13.7 ± 1.2	0.19 ± 0.02	0.18 ± 0.01	10.6 ± 1.3	24.9 ± 3.7	20.2 ± 3.3	0.3 ± 0.3	57.8 ± 2.9	60.6 ± 1.4
15	24.0 ± 3.3	20.3 ± 5.4	0.23 ± 0.03	0.29 ± 0.04	13.4 ± 1.5	28.11 ± 3.8	9.0 ± 2.6	2.7 ± 0.9	50.0 ± 1.4	55.1 ± 0.9
16	24.4 ± 2.2	16.0 ± 1.2	0.13 ± 0.02	0.22 ± 0.02	11.3 ± 1.1	27.11 ± 4.7	52.2 ± 8.4	1.0 ± 0.6	49.3 ± 0.7	48.4 ± 3.6
ANOVA										
Environment	***[W]		NS		***		***		*	
Accession	***		***		NS		***		*	
Environment x Accession	***		***		NS		***		NS	
Accessions grouped by region										
Location	Field	Nursery	Combined		Field	Nursery	Field	Nursery	Combined	
South	45.4 ± 3.0[X]	23.0 ± 1.2	0.34 ± 0.02		11.3 ± 0.7[Y]	23.1 ± 1.9	5.1 ± 1.8	0.06 ± 0.06	53.6 ± 0.8[Z]	
Central	25.8 ± 2.3	17.1 ± 1.4	0.29 ± 0.02		12.2 ± 0.6	23.4 ± 1.6	18.0 ± 4.1	1.4 ± 0.3	56.2 ± 0.8	
North	21.2 ± 1.6	15.7 ± 1.0	0.20 ± 0.01		11.0 ± 0.7	23.3 ± 1.4	18.7 ± 3.2	2.2 ± 0.5	57.1 ± 0.9	
ANOVA										
Environment	***[Z]		NS		***		***		NS	
Location	***		***		NS		*		NS	
Envir. x Loc.	***		NS		NS		NS		NS	

[T]Values represent mean (± standard errors) of 5 observations for the field environment and 3 observations for the nursery environment. [U]Environments combined when not significant to $P \leq 0.05$. [V]Values represent mean (± standard errors) internode extension of 15 observations for field environment and 9 observations for nursery environment. [W]NS,*,**,***Non-significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively. [X]Values represent means (± standard errors) of 21, 25, and 30 observations for south, central, and northern coast, respectively for field environment and observation of 15, 15, and 18 observations for south, central, and northern coast, respectively for nursery environment. [Y]Values represent means (± standard errors) internode extension of 108, 120, and 144 observations for south, central, and northern coast, respectively. [Z]Values represent means (± standard errors) flower diameter of 59, 102, and 107 observations for south, central, and northern coast, respectively.

Table 2a. Means of leaf measures by accession of *Oenothera drummondii* grown in both field and nursery conditions.

	Lamina length (mm)		Lamina width (mm)		Petiole length (mm)		Serrations (No./leaf)	
Accession	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
1	25.8 ± 0.8[x]	46.3 ± 1.3	10.7 ± 0.3[y]	18.2 ± 0.6	4 ± 0.5	5.7 ± 0.2	1.9 ± 0.3	7.2 ± 0.5
2	23.7 ± 1.4	28.9 ± 0.7	10.7 ± 0.2	13.0 ± 0.3	2.8 ± 0.3	3.9 ± 0.4	2.4 ± 0.4	5.1 ± 0.5
3	29.8 ± 1.0	45.9 ± 1.6	11.2 ± 0.3	16.7 ± 0.7	4.5 ± 0.2	7.3 ± 0.4	4.9 ± 0.3	6.3 ± 0.5
4	27.3 ± 1.0	50.2 ± 1.2	10.1 ± 0.4	15.9 ± 0.4	3.6 ± 0.3	4.6 ± 0.4	2.6 ± 0.5	4.3 ± 0.3
5	24.9 ± 1.3	41.7 ± 0.9	7.9 ± 0.7	10.7 ± 0.4	2.3 ± 0.3	3.6 ± 0.3	2.3 ± 0.6	4.6 ± 0.5
6	24.3 ± 1.3	34.2 ± 0.5	10.7 ± 0.3	13.2 ± 0.5	2.3 ± 0.2	2.1 ± 0.3	1.2 ± 0.1	2.4 ± 0.3
7	32.6 ± 1.5	34.6 ± 0.6	13.4 ± 0.5	15.2 ± 0.7	3.5 ± 0.4	3.1 ± 0.3	5.9 ± 0.5	6.3 ± 0.5
8	39.7 ± 2.1	46.1 ± 0.5	12.4 ± 0.6	16.8 ± 0.4	3.8 ± 0.4	4.7 ± 0.3	8.7 ± 0.8	8.2 ± 0.4
9	36.3 ± 1.2	38.7 ± 1.8	11.3 ± 0.8	14.8 ± 0.4	2.5 ± 0.2	2.9 ± 0.2	8.6 ± 0.5	9.0 ± 0.6
10	31.0 ± 1.0	37.2 ± 1.6	10.7 ± 0.3	12.7 ± 0.8	2.6 ± 0.3	2.7 ± 0.4	8.7 ± 0.6	9.1 ± 0.6
11	24.8 ± 1.0	29.9 ± 0.7	10.0 ± 0.4	11.2 ± 0.3	2.3 ± 0.3	2.4 ± 0.2	10.9 ± 0.3	10.1 ± 0.5
12	31.7 ± 0.8	34.4 ± 1.0	10.8 ± 0.2	12.1 ± 0.4	3.1 ± 0.3	3.3 ± 0.2	10.0 ± 0.6	11.0 ± 0.9
13	37.9 ± 1.1	35.2 ± 1.6	11.1 ± 0.4	11.1 ± 0.8	3.7 ± 0.3	2.1 ± 0.4	5.7 ± 0.5	4.0 ± 0.7
14	25.7 ± 0.9	35.9 ± 1.7	10.4 ± 0.3	13.7 ± 0.4	2.6 ± 0.2	2.1 ± 0.2	4.9 ± 0.6	8.3 ± 0.3
15	29.2 ± 1.2	33.4 ± 1.2	9.5 ± 0.4	15.6 ± 0.5	2.1 ± 0.2	2.8 ± 0.2	4.4 ± 0.6	6.4 ± 0.5
16	26.3 ± 1.0	36.8 ± 1.7	10.2 ± 0.5	15.8 ± 0.3	2.3 ± 0.2	3.2 ± 0.4	6.3 ± 0.5	7.8 ± 0.4
ANOVA								
Environment	***		***		***		***	
Accession	***		***		***		***	
Environment x Accession	***		***		***		***	
Location	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
South	26.6 ± 0.5[y]	43.7 ± 1	10.4 ± 0.2	14.9 ± 0.5	3.5 ± 0.2	4.6 ± 0.3	2.6 ± 0.2	5.0 ± 0.3
Central	30.3 ± 0.9	36.0 ± 1	11.2 ± 0.3	15.3 ± 0.3	2.9 ± 0.1	3.5 ± 0.2	5.5 ± 0.3	6.8 ± 0.3
North	31.2 ± 0.7	35.2 ± 0.7	10.7 ± 0.2	12.6 ± 0.3	2.8 ± 0.1	2.6 ± 0.1	8.2 ± 0.3	8.6 ± 0.4
ANOVA								
Environment	***[z]		***		***		***	
Location	*		***		***		***	
Environment x Location	***		***		***		***	

[x]Values represent mean (± standard errors) of 15 observations for the field environment and 9 observations for the nursery environment. [y]Values represent means (± standard errors) of 63, 75, and 90 observations for south, central, and northern coast, respectively for field environment and observations of 45, 45, and 54 for south, central, and northern coast, respectively for the nursery environment. [z] NS,*,**,***Non-significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 2b. Maximum, minimum, mean, standard deviation, and coefficient of variation of growth measures combined for all accessions of *Oenothera drummondii* across environments of field and nursery.

Growth characteristic	Maximum	Minimum	Mean	Standard deviation	Coefficient of variation
Plant height (cm)	68	8	25.2[y]	13.3	53.0
Flower count	85	0	9.5	15.3	160.1
Height/width ratio	0.67	0.05	0.3	0.1	44.3
Ornamental rating	5	2	3.1	0.7	22.4
Internode length (mm)	52	1	16.1[z]	9.1	57.3
Flower diameter (mm)	79	35	56.0	8.2	14.7
Lamina length (mm)	56	17	32.9	7.9	24.1
Lamina width (mm)	21	2	12.1	2.8	22.9
Petiole length (mm)	11	1	3.2	1.4	45.0
Number of Teeth	15	1	6.2	3.3	52.9

[y]Means combined across all accessions and environments, n=124.
[z]Means combined across all accession and environments; n=372 for internode mean and n=268 for floral data.

Table 3. Correlation coefficients between morphological characteristics and collection location coordinates of *Oenothera drummondii* accessions from the Texas coast.

	Height	Flower count	Ht/W	Leaf length	Leaf width	Petiole length	# of leaf serrations	Orn. rating	Lat.	Long.
Height	1	0.05	0.56	-0.44	-0.33	-0.02	-0.55	0.28	-0.56	0.45
Flower count	0.05	1	-0.38	-0.39	-0.41	-0.23	0.17	0.3	0.25	-0.13
Height/width	0.56	-0.38	1	0.07	0.12	0.14	-0.44	-0.05	-0.5	0.38
Leaf length	-0.44	-0.39	0.07	1	0.74	0.53	0.29	-0.18	-0.03	-0.03
Leaf width	-0.33	-0.41	0.12	0.74	1	0.51	0.19	-0.01	-0.11	0.14
Petiole length	-0.02	-0.23	0.14	0.53	0.51	1	-0.01	0.06	-0.39	0.27
Number of leaf serrations	-0.55	0.17	-0.44	0.29	0.19	-0.01	1	-0.04	0.67	-0.62
Ornamental rating	0.28	0.3	-0.05	-0.18	-0.01	0.06	-0.04	1	-0.23	0.14
Latitude	-0.56	0.25	-0.5	-0.03	-0.11	-0.39	0.67	-0.23	1	-0.87
Longitude	0.45	-0.13	0.38	-0.03	0.14	0.27	-0.62	0.14	-0.87	1

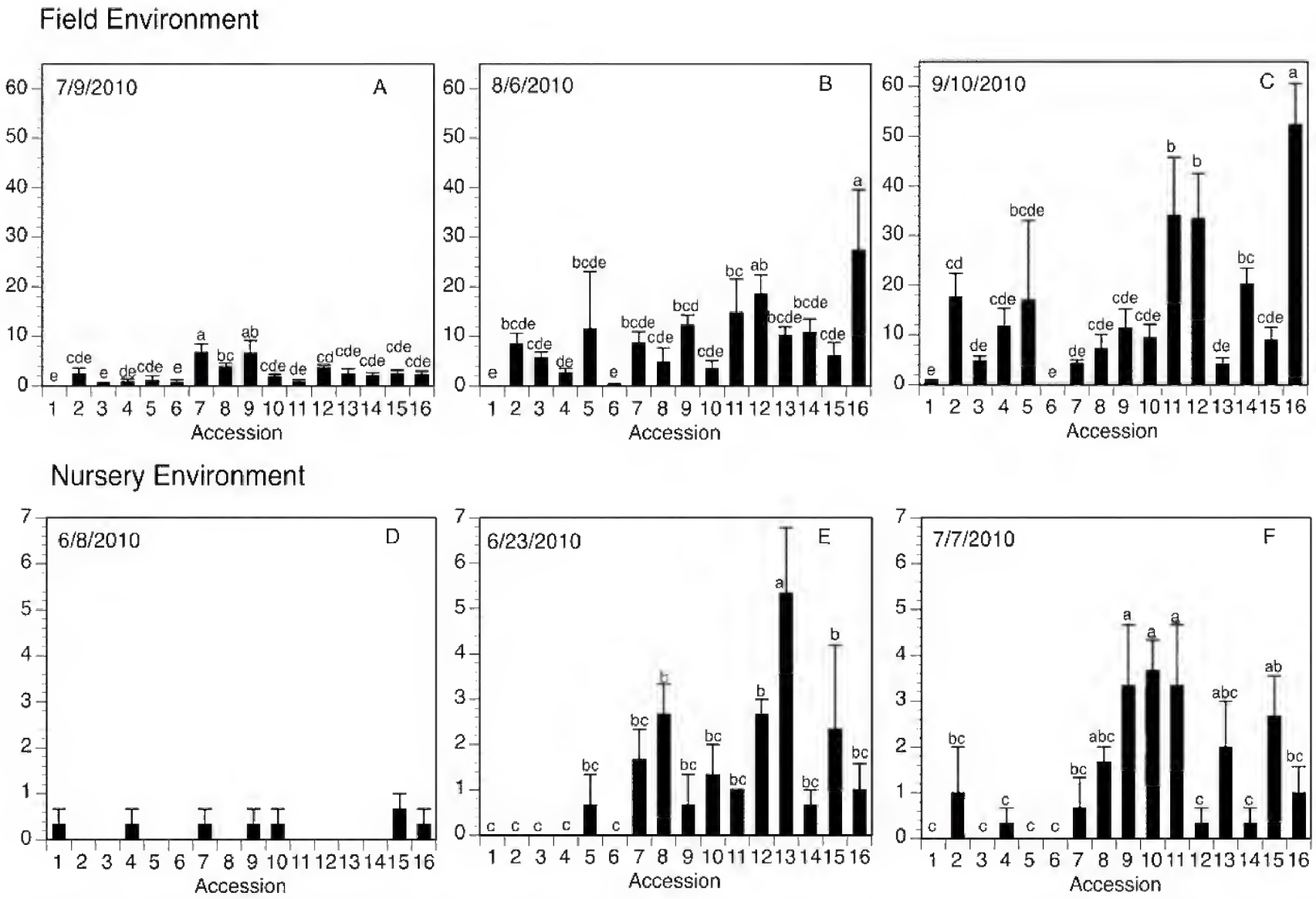

Fig. 2. Mean flower count of *Oenothera drummondii* on three sampling dates planted in field conditions or grown in 2.3 L containers in a nursery. Values represent mean (± standard errors) of 5 observations for the field environment and 3 observations for the nursery environment. There were no significant differences among accessions ($P \leq 0.05$) for sampling date 6 Jun 2010 (A). Any two means within a sampling date not followed by the same letter are significantly different at $P \leq 0.05$ using LSD mean separation.

Fig. 3. Example of *Oenothera drummondii* accessions exhibiting green foliage, blue foliage intact canopies and defoliated holes in canopy. Example of an *O. drummondii* exhibiting green foliage and defoliated holes in the canopy (A) and an example of an *O. drummondii* accession exhibiting blue foliage and an intact canopy (B).

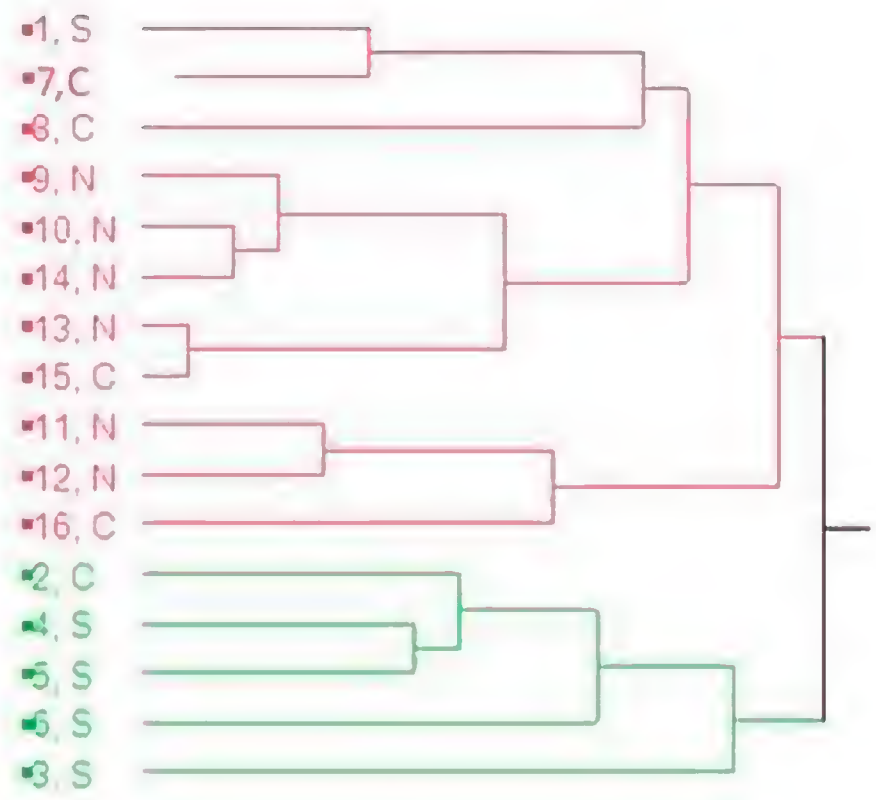

Fig. 4. Hierarchical Cluster analysis using Wards distance of *Oenothera drummondii* accessions based on morphological traits. Digits represent accession numbers of *O. drummondii* and letters the represent accessions' collection region along the coast S=Southern coast, C= Central coast, and N= Northern coast. Clusters separated by color.

Table 4. Means of growth measures separated by origin of *Oenothera drummondii* accession along Texas coast combined across both field and nursery environments.

Location	Height (cm)	Flower (No./plant)	Height:width ratio ($cm \cdot cm^{-1}$)
South	36.08a[v]	3.03b	0.34a
Central	22.56b	11.80a	0.24b
North	19.15b	12.52a	0.20b
ANOVA			
Location	***	***	***

[v] Values represent means of 21, 25, and 30 observations for south, central, and northern coast, respectively for field environment and observations of 15, 15, and 18 for south, central, and northern coast, respectively for nursery environment. Any two means within a column not followed by the same letter are significantly different at $P \leq 0.05$ using LSD mean separation.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

In general the southern forms of *O. drummondii* had a more upright and less spreading subshrub habit, whereas the plants from the central and northern areas had a shorter, more spreading groundcover growth form and a greater tendency to be free flowering. This will allow targeting collection efforts to regions based on characteristics and to potentially combine desirable traits via controlled crosses.

Borrichia frutescens

Significant ($P \leq 0.05$) differences existed among the accessions of *B. frutescens* (sea marigold) for height, height:width ratio, internode extension, flower count, flower diameter, leaf length leaf width, petiole length and number of serrations along the margin of the leaf (Tables 5 and 6). When the accessions were grouped by their region of collection along the Texas coasts, there were differences in height, flower count, flower diameter, internode extension, leaf length, leaf width, petiole length and the number serrations along the leaf margin (Tables 7 and 8).

Plant height ranged from a maximum of 78 cm to a minimum of 17 cm and had a coefficient of variation of 24.1%. Plants collected from the southern and central regions were on average shorter than *B. frutescens* collected from the northern coast. Environment affected mean plant height when accessions were grouped; based on collection location, mean height for plants grown in the field was 42 ± 0.9 cm and mean height for plants grown in the nursery was 45 ± 1.1 cm. Environment was a significant factor when analyzed as individual accessions instead of as part of the northern, central or southern collection zones. Plants could have been taller in the nursery due to ample water and nitrogen fertilizer. *Borrichia frutescens* has been reported to respond vigorously to increased fertility in container nursery production (King, 2015). Flower (inflorescence) count was variable among accessions, and highly significant for accession but not for environment (Table 5). Stability of flower production across growing environments could be an important attribute for acceptance of *B. frutescens* by the green industry and by consumers as a substitute for invasive exotic species. When grouped in collection areas, plants from the southern sites had a larger mean flower count of 5.2 flowers per plant compared to northern sites with 3.0 flowers per plant. Southern collection sites had larger flowers with a mean of 31.1 mm compared to 28.1 mm for plants collected from northern locations when planted in the field, but plants collected from northern locations had larger flowers than southern accessions when grown in the nursery (Table 5). Flower count was much more variable with a CV (coefficient of variation) of 106.4 than flower diameter with a CV of 14.2 (Table 9).

Leaf width, length, petiole and leaf margin serration were significantly different ($P \leq 0.05$) among regional groups and among accessions for *B. frutescens* (Table 6). Leaves tended to be larger in accessions for the central collection sites, with longer and wider leaf laminae (Table 8). The northern plants had longer petioles compared to plants collected from either the central or southern locations. The size of the leaves was different among field and nursery grown plants, with plants generally producing larger leaves when grown in the nursery (Table 6 and 8). The larger leaves were most likely the result of more favorable cultural conditions found in the nursery. Plants from the northern Texas coast had more entire margins on their leaves compared to plants collected from either the central or southern locations (Table 8).

Latitude was only significantly correlated with number of leaf serrations (r=-0.59); all other variables measured had correlation coefficients between 0.25 and -0.18. Longitude was positively correlated with the number of leaf serrations (r = 0.46) and petiole length (r = -0.32). Leaf lamina length was strongly correlated with leaf width (r = 0.70), petiole length (r = 0.65), and internode length (r = 0.46). Flower diameter was correlated with both leaf lamina length (r = 0.40) and leaf lamina width (r = 0.52).

Cluster analysis based on Wards method using all collected growth measures was not aligned ($P > 0.05$) with region of collection. Three clusters were developed and accessions from all three collection zones were randomly dispersed throughout.

Flower count was variable and significantly different ($P \leq 0.05$) among accessions. Flower diameter was also correlated with leaf width (r = 0.52). Southern accessions had more flowers and larger diameter flowers. Therefore, collections can be targeted for certain traits of interest and there is most likely a source of variation which exists for the creation of improved populations in the wild, though not all morphological measures may be correlated with the region of the Texas coast where plants are collected.

Table 5. Means *Borrichia frutescens* growth measures by accession when grown in 2.3 L containers in the nursery or planted to the field.

	Height (cm)		Height:width ratio ($cm \cdot cm^{-1}$)		Internode length (mm)		Flower count (No./plant)	Flower diameter (mm)	
Accession	Field	Nursery	Field	Nursery	Field	Nursery	Combined[x]	Field	Nursery
1	43.0 ± 4.0[w]	50.0 ± 10.5	1.0 ± 0.2	1.1 ± 0.3	16.9 ± 1.0[y]	50.3 ± 3.2	2.0 ± 0.5	34.4 ± 0.0	33.2 ± 1.7
2	37.6 ± 2.8	37.0 ± 7.0	0.6 ± 0.1	0.9 ± 0.2	24.1 ± 1.6	52.4 ± 1.9	4.4 ± 1.0	30.7 ± 1.2	33.3 ± 2.3
3	56.6 ± 3.6	46.0 ± 6.8	1.4 ± 0.1	1.3 ± 0.3	21.4 ± 1.4	59.4 ± 3.4	3.4 ± 1.4	37.2 ± 1.0	35.8 ± 0.9
4	40.6 ± 1.3	47.7 ± 3.7	0.7 ± 0.0	1.1 ± 0.1	26.4 ± 1.4	46.9 ± 3.0	14.9 ± 3.4	28.9 ± 0.5	28.3 ± 0.6
5	36.6 ± 1.8	49.0 ± 1.2	1.0 ± 0.1	1.4 ± 0.1	20.4 ± 1.7	40.3 ± 1.3	2.9 ± 0.7	26.6 ± 0.7	26.5 ± 0.5
6	46.6 ± 1.7	42.0 ± 2.1	1.0 ± 0.1	1.4 ± 0.1	17.5 ± 0.7	44.6 ± 2.5	3.0 ± 0.7	29.3 ± 0.4	35.6 ± 0.5
7	37.8 ± 2.1	45.7 ± 4.3	1.1 ± 0.1	1.3 ± 0.2	20.7 ± 1.3	43.8 ± 1.3	3.8 ± 1.4	30.6 ± 0.0	29.3 ± 0.7
8	36.4 ± 2.4	45.3 ± 3.0	0.7 ± 0.1	1.2 ± 0.2	15.2 ± 2.0	41.3 ± 2.6	6.3 ± 2.4	0.0 ± 0.0	27.6 ± 0.7
9	44.4 ± 4.0	42.3 ± 5.4	0.8 ± 0.1	1.0 ± 0.2	17.8 ± 0.9	45.4 ± 2.6	4.0 ± 1.0	28.6 ± 1.2	29.7 ± 1.2
10	50.2 ± 2.9	46.7 ± 0.9	1.0 ± 0.1	1.5 ± 0.2	20.3 ± 1.7	43.3 ± 3.1	5.5 ± 2.1	34.0 ± 1.7	28.5 ± 0.6
11	43.2 ± 3.5	39.7 ± 5.9	0.8 ± 0.1	1.1 ± 0.2	20.3 ± 1.6	40.8 ± 2.5	8.0 ± 1.7	30.0 ± 0.7	34.0 ± 0.8
12	26.2 ± 3.1	31.3 ± 2.7	0.6 ± 0.1	0.7 ± 0.1	20.7 ± 1.5	49.7 ± 3.1	2.8 ± 0.6	31.3 ± 1.2	29.4 ± 0.5
13	36.2 ± 2.2	33.0 ± 2.1	0.6 ± 0.0	0.6 ± 0.1	18.3 ± 1.7	50.4 ± 3.0	3.1 ± 1.0	27.0 ± 0.6	0.0 ± 0.0
14	40.2 ± 2.1	45.0 ± 1.2	0.9 ± 0.1	1.1 ± 0.1	17.3 ± 1.4	39.9 ± 2.0	1.5 ± 0.3	27.6 ± 2.1	32.3 ± 1.8
15	44.4 ± 2.3	56.7 ± 0.9	0.8 ± 0.1	1.4 ± 0.1	17.9 ± 1.4	40.3 ± 1.5	3.4 ± 0.5	21.8 ± 0.6	30.0 ± 1.1
16	32.0 ± 2.3	38.7 ± 2.9	0.7 ± 0.1	1.1 ± 0.0	15.2 ± 1.5	46.7 ± 2.8	5.8 ± 1.2	26.0 ± 1.0	27.2 ± 0.9
17	40.2 ± 3.8	52.0 ± 8.6	0.9 ± 0.1	1.3 ± 0.3	15.3 ± 1.7	47.2 ± 3.3	2.4 ± 0.3	27.3 ± 1.5	26.7 ± 0.9
18	49.4 ± 2.1	48.3 ± 0.7	0.9 ± 0.0	1.6 ± 0.2	18.6 ± 1.0	39.9 ± 1.5	6.8 ± 1.4	27.5 ± 0.7	27.1 ± 0.7
19	48.6 ± 1.1	51.7 ± 4.9	0.9 ± 0.1	1.3 ± 0.1	16.3 ± 0.9	44.1 ± 1.2	3.9 ± 1.0	27.4 ± 0.6	34.2 ± 1.5
20	69.8 ± 2.9	63.3 ± 0.3	0.9 ± 0.0	1.7 ± 0.1	18.0 ± 1.2	39.2 ± 2.0	2.1 ± 1.0	28.6 ± 1.5	0.0 ± 0.0
21	37.0 ± 3.4	43.7 ± 4.7	0.6 ± 0.1	1.0 ± 0.1	11.8 ± 1.3	48.2 ± 2.2	0.8 ± 0.3	0.0 ± 0.0	33.3 ± 2.7
22	41.6 ± 3.4	45.0 ± 5.3	0.8 ± 0.1	0.9 ± 0.2	18.6 ± 2.3	52.2 ± 2.7	4.1 ± 1.0	27 ± 1.3	32.0 ± 1.1
23	58.2 ± 1.9	63.3 ± 1.7	1.3 ± 0.1	1.7 ± 0.2	16.8 ± 1.6	48.7 ± 3.0	0.9 ± 0.4	28.4 ± 3.2	0.0 ± 0.0
24	34.6 ± 5.0	29.7 ± 4.2	1.0 ± 0.1	1.0 ± 0.1	14.3 ± 1.4	37.4 ± 2.7	6.6 ± 2.0	25.4 ± 0.8	30.3 ± 1.2
26	43.4 ± 3.7	49.3 ± 2.3	0.8 ± 0.1	1.2 ± 0.1	18.6 ± 0.9	57.0 ± 3.3	2.0 ± 0.8	32.6 ± 1.6	37.0 ± 0.6
27	39.0 ± 2.2	42.3 ± 1.2	1.0 ± 0.1	1.2 ± 0.0	13.7 ± 1.0	43.0 ± 2.9	4.3 ± 0.8	29.2 ± 0.7	30.6 ± 1.3
28	33.2 ± 2.9	40.7 ± 2.8	0.7 ± 0.0	1.1 ± 0.1	12.6 ± 1.8	44.9 ± 1.6	6.8 ± 1.9	29.1 ± 0.9	28.5 ± 0.5
Environment	**[z]		***		***		NS	***	
Accession	***		***		***		***	***	
Environment x Accession	NS		**		***		NS	***	

[w]Values represent mean (± standard errors) of 5 observations for field environment and 3 observations for nursery environment. [x]Environments combined when not significant to $P \leq 0.05$. [y]Values represent mean (± standard errors) internode extension of 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 6. Mean of leaf measures by accession for *Borrichia frutescens* when grown in 2.3 L containers in the nursery or planted to the field.

	Lamina length (mm)		Lamina width (mm)		Petiole length (mm)		Teeth/serrations (No./leaf)	
Accession	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
1	36.8 ± 0.9[y]	41.3 ± 1.8	13.5 ± 0.7	23.6 ± 1.7	4 ± 0.2	6.1 ± 0.4	12.1 ± 1	19.7 ± 1.8
2	38.8 ± 1.6	39.4 ± 1.6	18.6 ± 1.3	24 ± 2.3	6.3 ± 0.3	6.6 ± 0.4	6 ± 1.4	15.1 ± 1.8
3	36.6 ± 0.7	39.6 ± 1.1	16.9 ± 0.6	29 ± 1.4	6.1 ± 0.3	6.2 ± 0.3	6.1 ± 0.9	17 ± 2.1
4	31.2 ± 1.1	41.1 ± 1.1	9.8 ± 0.4	19.2 ± 1.3	4.4 ± 0.2	5.9 ± 0.4	1.7 ± 0.5	10.9 ± 2.2
5	27 ± 0.7	29.8 ± 0.7	11.6 ± 0.5	16.2 ± 1.1	3.9 ± 0.2	5.1 ± 0.3	13.5 ± 1.1	18 ± 0.9
6	37.9 ± 1.5	40.4 ± 0.9	15.6 ± 0.9	28 ± 1.3	5.6 ± 0.2	6.8 ± 0.3	5.4 ± 0.9	15.9 ± 1.6
7	31 ± 1.3	36.3 ± 2.3	8.9 ± 0.5	16.9 ± 1.3	4.3 ± 0.2	6 ± 0.3	9.5 ± 1.2	19.3 ± 0.7
8	23.7 ± 0.8	26.8 ± 0.7	10.1 ± 0.5	15.1 ± 1	3.9 ± 0.2	5.7 ± 0.4	11.7 ± 1.2	16.9 ± 1.3
9	35.4 ± 1.2	36.2 ± 2	14.8 ± 0.6	19.1 ± 1.7	4.5 ± 0.2	6.4 ± 0.5	14.5 ± 0.8	14.2 ± 1
10	43.7 ± 1.2	35.1 ± 1.4	26.0 ± 1.0	25.4 ± 1.7	7.2 ± 0.5	6.2 ± 0.5	26.7 ± 1.3	23.9 ± 2.5
11	31.4 ± 1.4	38.2 ± 1.3	11.8 ± 0.7	24.4 ± 1.8	5 ± 0.3	6.9 ± 0.3	2.5 ± 0.6	15.6 ± 1.8
12	32.3 ± 1.3	39.3 ± 1.5	10.9 ± 0.7	21.7 ± 1.3	4.5 ± 0.3	7.3 ± 0.5	2.7 ± 0.3	12.8 ± 1.5
13	35.9 ± 0.9	39.3 ± 1.6	10.2 ± 0.3	16.8 ± 1.4	5.5 ± 0.2	8.2 ± 0.5	1.1 ± 0.1	6.8 ± 1.2
14	37.8 ± 1.4	39.6 ± 1.4	16 ± 1.3	24.2 ± 2.3	6 ± 0.4	8 ± 0.4	1.5 ± 0.4	15.1 ± 2.5
15	35.9 ± 1.1	38.4 ± 1.3	11.9 ± 0.8	19.2 ± 1.3	4.3 ± 0.3	5.9 ± 0.3	1.6 ± 0.4	4.2 ± 0.5
16	32 ± 1.4	42.2 ± 2.3	7.6 ± 0.5	20.1 ± 1.6	4.8 ± 0.3	8.4 ± 0.5	2.0 ± 0.6	11.2 ± 1.1
17	29.1 ± 1.9	37.4 ± 1.5	11.7 ± 0.8	19.7 ± 1.4	4.6 ± 0.2	6.8 ± 0.3	2.1 ± 0.7	6.3 ± 1.2
18	37.8 ± 1.2	41.8 ± 1.6	13.5 ± 0.7	23.8 ± 0.6	5.5 ± 0.2	6.8 ± 0.4	5.2 ± 1.3	14.2 ± 1
19	34.4 ± 1.2	39.3 ± 1.7	13.6 ± 0.7	22.6 ± 1.8	4.9 ± 0.3	6.7 ± 0.3	2.3 ± 0.5	14.4 ± 1.4
20	32 ± 1.1	36.3 ± 1.4	15.8 ± 0.7	23.7 ± 0.9	6 ± 0.3	8.1 ± 0.5	1.0 ± 0.0	2 ± 0.3
21	31.6 ± 1.3	42.4 ± 1.2	9.7 ± 0.7	22 ± 1.1	4.6 ± 0.2	7.8 ± 0.3	1.0 ± 0.0	2.3 ± 0.3
22	30.2 ± 1.1	35.3 ± 1.4	12.3 ± 0.6	18.1 ± 1.4	6.6 ± 0.3	8.4 ± 0.5	1.3 ± 0.3	8 ± 1.9
23	36 ± 1.1	43.4 ± 1.9	13.9 ± 0.6	20.1 ± 1.1	5.6 ± 0.3	8.7 ± 0.5	1.0 ± 0.0	5.9 ± 1.2
24	26.7 ± 1.0	32.7 ± 1	11.1 ± 0.6	20.6 ± 1.6	4.2 ± 0.2	6.9 ± 0.4	1.0 ± 0.0	3.1 ± 0.5
26	42.1 ± 1.7	40.1 ± 0.9	15.9 ± 0.9	20.8 ± 1.3	7 ± 0.4	8.1 ± 0.4	1.1 ± 0.1	1.6 ± 0.2
27	29.2 ± 1	35.9 ± 1.4	11.1 ± 0.6	21.8 ± 1.6	4.8 ± 0.1	6.2 ± 0.4	4.6 ± 0.7	14.9 ± 1.8
28	33.1 ± 1.7	41 ± 1.1	12.2 ± 0.8	21.3 ± 1.3	4.2 ± 0.2	5.9 ± 0.3	1.2 ± 0.2	5.8 ± 1.2
ANOVA								
Environment	***[z]		***		***		***	
Accession	***		***		***		***	
Environment x Accession	***		***		***		***	

[y]Values represent mean (± standard errors) of 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 7. Mean of growth measures separated by origin of accession along Texas coast for *Borrichia frutescens* when grown in 2.3 L containers in the nursery or planted to the field.

	Height (cm)	Flower count (No./plant)	Height:width ratio ($cm \cdot cm^{-1}$)		Internode length (mm)		Flower diameter (mm)	
Location	Combined[u]	Combined	Field	Nursery	Field	Nursery	Field	Nursery
South	42.6 ± 1.2b	5.2 ± 0.5a	0.9 ± 0.0[v]	1.2 ± 0.1	20.0 ± 0.5	46.1 ± 1.0	31.1 ± 0.5[x]	29.8 ± 0.5
Central	42.5 ± 1.0b	4.4 ± 0.4ab	0.8 ± 0.0	1.1 ± 0.1	17.5 ± 0.5	45.2 ± 0.8	27.6 ± 0.5	30.5 ± 0.6
North	46.9 ± 1.3a	3.0 ± 0.6b	0.9 ± 0.0	1.2 ± 0.1	16.0 ± 0.6[x]	46.5 ± 1.1	28.1 ± 0.7	31.8 ± 0.7
ANOVA								
Environment	NS[z]	NS	***		***		***	
Location	*	*	NS		*		**	
Environment x Location	NS	NS	NS		NS		***	

[u]Environments combined when not significant to $P \leq 0.05$. Values represent means (± standard errors) of 72, 88, and 56 observations for south, central, and northern coasts, respectively.
[v]Values represent means (± standard errors) of height: width ratio of 45, 55, and 35 observations for south, central, and northern coasts, respectively for field environment and observations of 27, 33, and 21 for south, central, and northern coasts, respectively for nursery environment.
[x]Values represent means (± standard errors) of internode extension for 135, 164, and 104 observations for south, central, and northern coasts, respectively for field environment and of 81, 99, and 63 observations for south, central, and northern coasts, respectively for nursery environment.
[y]Values represent means (± standard errors) of flower diameter for 57, 73, and 37 observations for south, central, and northern coasts, respectively for field environment and 60, 53, and 32 observations for south, central, and northern coasts, respectively for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 8. Mean of leaf measures separated by origin of accession along Texas coast for *Borrichia frutescens* when grown in 2.3 L containers in the nursery or planted to the field.

	Lamina length (mm)		Lamina width (mm)		Petiole length (mm)		Teeth/serrations (No./leaf)	
Location	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
South	33.2 ± 0.6[y]	36.1 ± 0.7	13.8 ± 0.5	21.2 ± 0.7	5.0 ± 0.1	6.2 ± 0.1	10.2 ± 0.7	16.5 ± 0.7
Central	34.9 ± 0.5	39.8 ± 0.5	12.8 ± 0.3	21.8 ± 0.5	5.0 ± 0.1	6.9 ± 0.1	3.4 ± 0.3	11.7 ± 0.6
North	32.5 ± 0.6	38.0 ± 0.7	12.8 ± 0.3	21.0 ± 0.5	5.5 ± 0.1	7.7 ± 0.2	1.6 ± 0.2	5.4 ± 0.7
ANOVA								
Environment	***[z]		***		***		***	
Location	*		***		***		***	
Environment x Location	***		***		***		***	

[y]Values represent mean (± standard errors) of 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 9. Maximum, minimum, mean, standard deviation, coefficient of variation of growth measures combined for all accessions across all environments for accessions of *Borrichia frutescens*.

	Maximum	Minimum	Mean	Standard Deviation	CV[x]
Plant height (cm)	78	17	43.6[y]	10.5	24.1
Flower count	34	0	4.3	4.5	106.4
Height:width ratio	2.0	0.3	1.0	0.3	33.8
Ornamental rating	5	2	3.1	0.8	24.2
Growth index (cm^3)	507744.0	9500	81276.5	64607.0	79.5
Internode length (mm)	73.0	2.3	28.5[z]	15.4	54.2
Flower diameter (mm)	42.53	18.1	29.7	4.2	14.2
Pedicle length (mm)	76.0	14.0	38.3	11.7	30.5
Lamina length (mm)	57.0	18.4	35.4	6.6	18.6
Lamina width (mm)	37.0	3.9	16.2	6.3	38.8
Petiole length (mm)	12.0	2.6	5.8	1.7	28.6
Teeth/serrations (no./plant)	37	1	7.6	7.5	98.6

[x]Coefficient of Variation.
[y]Means combined across all accessions and environments, N=216.
[z]Means combined across all accession and environments N=646 for internode mean and N=312 for floral data.

Erigeron procumbens

Significant differences ($P \leq 0.05$) in height, height:width ratio, internode extension, lamina length, lamina width and petiole length were found among accessions and regional collection of *E. procumbens* (Corpus Christi fleabane) (Table 10 and 13). This species primarily blooms in cooler seasons in warm climates such as Texas (Arnold, 2011). There were no differences among collections ($P > 0.05$) in flower (inflorescence) count, though floral analyses were constrained by small sample size on the date of data collection. There was only one accession collected from the northern region of the Texas coast. This is to be expected, encountering *E. procumbens* in this region would be rarer due to this being the extreme northern end of its natural range. In statistical analysis with a regional effect, only the southern and central regions were considered because of the small sample size from the northern region.

Accessions from the southern collection region in the vicinity of Brownsville were taller in both the nursery and field environment (Table 11). Plants from the southern collection region also had a larger height:width ratio when grown in containers (Table 11) than *E. procumbens* from the other collection area. This greater height:width ratio indicated that plants were not only taller but also had less of a prostrate habit than the wild accessions collected from the central coast of the Texas.

All leaf growth measures of *E. procumbens* were different ($P \leq 0.05$) among the accessions and between the collection groups (Tables 13 and 14). Plants collected from the southern collection region had larger leaves in both length and width of the leaf laminae. There was only an interaction among environments and regions of collection for leaf width; plants collected from the central Texas coast had a much larger increase in leaf width when grown in containers than in the field. On the accession level, most plants had larger leaves in terms of width, length, and petiole length when grown in the nursery environment (Table 13). This could be explained by the more favorable cultural conditions provided by the nursery compared to the field. The interaction among accessions and environments for *E. procumbens* could be explained by not all accessions being equally plastic in phenotype. Differences in plasticity are shown by not all accessions having similar increases in leaf size (Table 13). Some accessions of *E. procumbens* increased leaf size by 53% when grown in the nursery and other accessions (e.g. 18) only increased leaf size by 4 % when grown in nursery conditions compared to the field.

Table 10. Means of *Erigeron procumbens* growth measures by accession when grown in 2.3 L containers in the nursery or planted to the field.

	Height (cm)		Height:width ratio ($cm \cdot cm^{-1}$)		Internode length (mm)	
Accession	Field	Nursery	Field	Nursery	Field	Nursery
1	5.8 ± 0.4	11.0 ± 3.2	0.1 ± 0.0	0.2 ± 0.1	16.2 ± 1.5	28.8 ± 3.7
2	7.0 ± 1.4	8.7 ± 1.2	0.1 ± 0.0	0.2 ± 0.0	17.9 ± 1.2	22.6 ± 1.0
3	5.4 ± 0.5	6.3 ± 0.7	0.1 ± 0.0	0.1 ± 0.0	13.6 ± 0.9	19.4 ± 2.0
4	6.5 ± 1.3	8.7 ± 2.3	0.1 ± 0.0	0.2 ± 0.0	11.4 ± 1.5	18.4 ± 2.6
5	10.8 ± 2.1	11.7 ± 1.9	0.1 ± 0.0	0.2 ± 0.0	15.0 ± 1.3	17.0 ± 1.0
6	4.2 ± 0.9	6.3 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	16.6 ± 1.6	24.2 ± 2.4
7	5.6 ± 0.7	7.3 ± 1.5	0.1 ± 0.0	0.1 ± 0.0	18.0 ± 1.0	24.9 ± 3.1
8	6.8 ± 0.7	9.3 ± 1.5	0.1 ± 0.0	0.2 ± 0.0	15.1 ± 0.9	22.9 ± 2.4
9	6.2 ± 0.4	10.3 ± 1.9	0.1 ± 0.0	0.2 ± 0.0	13.5 ± 0.8	20.1 ± 1.7
10	7.8 ± 1.4	7.7 ± 1.2	0.1 ± 0.0	0.1 ± 0.0	15.5 ± 1.1	23.8 ± 2.8
11	9.0 ± 1.3	14.0 ± 1.5	0.1 ± 0.0	0.3 ± 0.0	14.9 ± 1.5	17.3 ± 1.2
12	4.8 ± 0.7	6.7 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	15.1 ± 1.4	22.8 ± 1.4
13	5.0 ± 0.8	7.0 ± 0.6	0.0 ± 0.0	0.1 ± 0.0	17.7 ± 0.7	23.8 ± 2.1
15	7.4 ± 1.6	5.3 ± 0.9	0.1 ± 0.0	0.1 ± 0.0	21.9 ± 2.2	25.9 ± 2.0
16	4.6 ± 0.5	5.0 ± 0.6	0.1 ± 0.0	0.1 ± 0.0	13.8 ± 1.2	21.7 ± 1.5
17	5.2 ± 0.8	8.7 ± 0.9	0.1 ± 0.0	0.2 ± 0.0	18.7 ± 2.2	25.6 ± 1.8
18	6.8 ± 0.6	7.0 ± 0.6	0.1 ± 0.0	0.1 ± 0.0	11.9 ± 1.0	18.8 ± 1.0
ANOVA						
Environment	***[z]		***		***	
Accession	***		***		***	
Environment x Accession	NS		*		NS	

Values represent mean (± standard errors) of 5 observations for field environment and 3 observations for nursery environment.
[x]Environments combined when not significant to $P \leq 0.05$.
[y]Values represent mean (± standard errors) internode extension of 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Ornamental ratings of *E. procumbens* were different among the accessions (Chi square P=0.02) and the two regional collection (Chi square P=0.01) groups. The accessions and plants collected from the southern region tended to have a greater ornamental rating than *E. procumbens* from the central Texas coast in both the field and nursery environments.

There were differences in height, height:width ratio, ornamental rating, internode extension, lamina length, lamina width and petiole length among accessions and regional collections of *E. procumbens*. The relatively low CV for ornamental characteristics of interest such as height, height:width ratio, and ornamental rating (Table 12) indicate there is little variability for selection. Flower (inflorescence) count was not significant among accessions or regional collection groups in this study. The lack of differences in flower count could be due to the tendency of *E. procumbens* to flower in flushes, peaking during the cooler spring temperatures (Arnold, 2011).

Sesuvium portulacastrum

Height, height:width ratio, flower count, flower diameter, internode, leaf length, leaf width, petiole length, and stem diameter were different ($P \leq 0.05$) among *S. portulacastrum* (sea purslane) accessions (Tables 15 and 16). When grouped based on region of collection along the Texas coast, there

were differences ($P \leq 0.05$) among the regions for height, flower diameter, leaf length, leaf width, petiole length and stem diameter. Only flower count, height:width ratio, growth index, and pedicle length had highly variable traits with CVs near 100 (Table 17).

Table 11. Means of growth measures separated by origin of accession along Texas coast for *E. procumbens* when grown in 2.3 L containers in the nursery or planted to the field.

	Height (cm)		Height:width ratio ($cm \cdot cm^{-1}$)		Internode length (mm)	
Location	Field	Nursery	Field	Nursery	Field	Nursery
South[x]	7.7 ± 0.6[y]	10.1 ± 0.8	0.1 ± 0[y]	0.2 ± 0	15.33 ± 0.47	21 ± 0.95
Central	5.6 ± 0.3	7.4 ± 0.5	0.1 ± 0	0.1 ± 0	16.4 ± 0.51	23.31 ± 0.72
North	6.8 ± 0.6	7 ± 0.6	0.1 ± 0	0.1 ± 0	11.9 ± 0.95	18.78 ± 1
ANOVA						
Environment	***[z]		***		***	
Location	***		***		***	
Environment x Location	NS		*		NS	

[x]Location is for Central and Southern Region only, due to the lack of samples from Northern Region.
[y]Values represent mean (± standard errors) of 5 observations for field environment and 3 observations for nursery environment for height, height /width ratio. Means (± standard errors) for internode extension represent 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Although all *S. portulacastrum* had low spreading groundcover type habits, plants collected from the southern region, like southern accessions of *O. drummondii*, were on average 69 % taller than plants collected from the northern range (Table 18). Most of this increase in the average height could be explained by accession 1 and accession 7, with mean heights when grown in the field of 14.4 ± 0.9 cm and 24.2 ± 1.6 cm, respectively (Table 15). There was not a significant environmental effect ($P > 0.05$), but there was an interaction ($P \leq 0.05$) between environment and accession. All but one accession of *S. portulacastrum* collected from the southern coast decreased in mean height when grown in the nursery environment. This decrease in height ranged from 13 % for accession 4 to 40 % for accession 5 (Table 15). In contrast, all *S. portulacastrum* accessions collected from the northern region range had taller mean heights when grown in the nursery. One accession, accession 10, increased nursery height 60 % compared to field conditions (Table 15).

Internode extension of *S. portulacastrum* was generally greater in the nursery environment, most likely from favorable cultural conditions. Not all accessions were equally plastic. For example, accession 1 only increased internode extension by 66 %, whereas accession 6 increased internode extension by 220 % when grown in the nursery compared to the field (Table 15). This difference in plasticity of internode extension would explain the accession x environment interaction. The region of collection had no effect on the internode extension in this study.

Table 12. Maximum, minimum, mean, standard deviation, coefficient of variation of growth measures combined for all accessions across all environments for accession of *Erigeron procumbens*.

	Maximum	Minimum	Mean	Standard Deviation	CV[x]
Plant height (cm)	19.0	2.0	7.1[y]	3.0	41.9
Flower count	34	0	8.9	8.7	97.3
Height/width ratio	0.3	0.02	0.1	0.1	62.5
Ornamental rating	5	1	3.13	0.7	21.3
Growth index (cm^3)	327750.0	580.0	50548.7	47062.4	93.1
Internode length (mm)	53.0	2.1	18.3	6.8	36.9
Flower diameter (mm)	25.0	15.0	19.5	2.2	11.1
Pedicle length (mm)	184	81	121.0	21.6	17.8
Lamina length (mm)	31.0	8.7	19.2	4.3	22.3
Lamina width (mm)	24.0	5.3	12.1	3.0	25.0
Petiole length (mm)	13.4	2.7	6.8	2.0	29.2

[x]Coefficient of variation.
[y]Means combined across all accessions and environments, N=135.
[z]Means combined across all accession and environments N=387 for internode mean and N=59 for floral data.

There were differences ($P \leq 0.05$) in leaf lamina length, leaf lamina width, petiole length, and stem diameter of *S. portulacastrum* on the regional and accession levels. Accessions of *S. portulacastrum* from the southern region had longer leaves, wider leaves, longer petioles and thicker stems than accessions from either the central or northern collection areas (Table 19). Like internode extension, leaf measures generally increased when *S. portulacastrum* were grown in the nursery environment, with some genotypes like accession 6 increasing leaf length 51 % and leaf width 24 % in the nursery environment (Table 16).

Accessions 11, 12, and 15 exhibited decreases in leaf measures in the nursery environment compared to the field. Leaf length was correlated with leaf width (r=0.59) and internode extension (r=0.53). Leaf width was strongly correlated with latitude of collection site (r=-0.59) and stem diameter (r=0.83). These changes in leaf size indicate that *S. portulacastrum* leaves are plastic in response to environmental conditions.

Flower count of *S. portulacastrum* was not affected ($P > 0.05$) by region of collection, but there were differences ($P \leq 0.05$) among accessions, with a strong environmental effect. Accessions flowered more in the field than in the nursery environment. This is most likely because *S. portulacastrum* were larger in the field than in the nursery due to a longer growing season. Even though fewer flowers were produced in the nursery, the nursery flowers were larger for most accessions of *S. portulacastrum* compared to flowers of plants grown in the field. Flower diameter was correlated to stem diameter (r=0.52). Some accessions, such as 4 and 15, produced smaller flowers in the nursery than in the field (Table 15).

Table 13. Means of leaf measures by accession of *Erigeron procumbens* when grown in 2.3 L containers in the nursery or planted to the field.

	Lamina length (mm)		Lamina width (mm)		Petiole length (mm)	
Accession	Field	Nursery	Field	Nursery	Field	Nursery
1	18.9 ± 1.8	21.4 ± 1.6	11.1 ± 0.9	15.2 ± 1.0	8.4 ± 0.8	9.3 ± 0.4
2	15.6 ± 0.7	20.8 ± 1.0	10.6 ± 0.5	14.7 ± 0.6	4.5 ± 0.3	7.2 ± 0.1
3	16.7 ± 0.8	21.6 ± 1.3	10.8 ± 0.5	14.2 ± 0.8	5.8 ± 0.4	7.6 ± 0.4
4	16.6 ± 0.6	21.8 ± 0.9	12.0 ± 0.4	15.4 ± 0.6	6.2 ± 0.5	7.2 ± 0.5
5	20.3 ± 0.7	22.3 ± 0.9	12.6 ± 0.5	14.3 ± 0.9	8.0 ± 0.5	8.2 ± 0.5
6	18.9 ± 1.4	23.4 ± 1.3	11.4 ± 0.9	14.6 ± 0.6	6.0 ± 0.6	6.6 ± 0.3
7	18.5 ± 0.9	21.1 ± 0.8	12.0 ± 0.4	14.7 ± 0.5	7.2 ± 0.4	8.0 ± 0.4
8	15.5 ± 0.7	21.7 ± 0.4	11.5 ± 0.5	16.0 ± 0.7	5.0 ± 0.4	7.2 ± 0.3
9	17.6 ± 0.9	23.8 ± 1.1	10.0 ± 0.7	13.1 ± 0.9	6.4 ± 0.4	9.0 ± 0.6
10	17.4 ± 1.1	22.3 ± 1.2	9.7 ± 0.4	11.8 ± 1.0	5.5 ± 0.5	7.6 ± 0.9
11	20.9 ± 1.1	22.4 ± 0.9	11.7 ± 0.7	12.1 ± 0.8	8.0 ± 0.4	9.2 ± 0.7
12	12.6 ± 1.0	19.3 ± 1.4	7.2 ± 0.3	10.1 ± 0.7	4.1 ± 0.3	5.4 ± 0.3
13	17.7 ± 0.8	25.4 ± 1.1	10.5 ± 0.6	16.6 ± 1.3	5.4 ± 0.3	9.3 ± 0.5
15	18.3 ± 0.8	22.4 ± 0.6	11.0 ± 0.6	15.4 ± 0.8	6.9 ± 0.3	7.2 ± 0.1
16	17.0 ± 1.1	18.9 ± 1.0	10.2 ± 0.6	12.9 ± 0.7	6.2 ± 0.4	6.7 ± 0.3
17	17.4 ± 1.1	19.3 ± 0.8	12.2 ± 0.6	14.3 ± 0.7	5.6 ± 0.3	6.8 ± 0.3
18	18.8 ± 1.2	19.6 ± 0.7	10.0 ± 0.6	11.0 ± 0.7	6.7 ± 0.5	6.8 ± 0.5
ANOVA						
Environment	***[z]		***		***	
Accession	***		***		***	
Environment x Accession	*		*		***	

[y]Values represent mean (± standard errors) of 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 14. Means of leaf measures separated by origin of *Erigeron procumbens* accession along Texas coast when grown in 2.3 L containers in the nursery or planted to the field.

	Lamina length (mm)		Lamina width (mm)		Petiole length (mm)	
Location	Field	Nursery	Field	Nursery	Field	Nursery
South	18.37 ± 0.41	22.28 ± 0.38	11.23 ± 0.25	13.67 ± 0.38	6.68 ± 0.21	8.2 ± 0.25
Central	16.96 ± 0.35	21.44 ± 0.39	10.69 ± 0.22	14.34 ± 0.30	5.86 ± 0.16	7.33 ± 0.16
North	18.75 ± 1.17	19.56 ± 0.73	10.00 ± 0.58	11.00 ± 0.69	6.71 ± 0.47	6.78 ± 0.46
ANOVA						
Environment	***[z]		***		***	
Location	***		NS		***	
Environment x Location	NS		***		NS	

[y]Values represent mean (± standard errors) of 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 15. Means of *Sesuvium portulacastrum* growth measures by accession when grown in 2.3 L containers in the nursery or planted to the field.

	Height (cm)		Height:width ratio ($cm \cdot cm^{-1}$)		Internode length (mm)		Flower count (No./plant)		Flower diameter (mm)	
Accession	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
1	14.4 ± 0.9[x]	11.7 ± 1.5	0.09 ± 0.01	0.26 ± 0.06	44.2 ± 3.9[y]	73.5 ± 9.6	20.8 ± 7.7	3.3 ± 0.9	14.6 ± 0.9	19.3 ± 0.3
2	10.8 ± 2.1	8.0 ± 3.0	0.08 ± 0.02	0.10 ± 0.04	40.8 ± 3.6	79.9 ± 6.1	16.8 ± 4.6	3.3 ± 0.7	15.6 ± 0.3	17.5 ± 0.5
3	14.2 ± 1.2	8.0 ± 0.6	0.10 ± 0.01	0.10 ± 0.01	25.3 ± 3.1	61.4 ± 5.1	21.8 ± 3.2	18.0 ± 7.5	13.2 ± 1.1	15.5 ± 0.6
4	8.4 ± 0.4	7.3 ± 0.7	0.04 ± 0.00	0.13 ± 0.01	26.3 ± 1.5	54.6 ± 1.7	40.2 ± 19.9	13.0 ± 1.0	16.4 ± 0.5	15.3 ± 0.4
5	12.0 ± 1.7	7.3 ± 1.2	0.06 ± 0.01	0.14 ± 0.03	20 ± 1.2	41 ± 4.2	185.0 ± 42.7	0.7 ± 0.3	15 ± 0.5	16.4 ± 0.2
6	12.7 ± 1.2	13.3 ± 2.0	0.13 ± 0.05	0.15 ± 0.02	30.2 ± 3.7	97.6 ± 5.8	14.7 ± 7.7	0.3 ± 0.3	18 ± 1.1	18.5 ± 0.6
7	24.2 ± 1.6	20.3 ± 1.9	0.18 ± 0.02	0.26 ± 0.02	45.7 ± 2.3	107.2 ± 4.7	15.4 ± 1.8	10.7 ± 0.7	20.4 ± 0.6	19.2 ± 0.5
8	10.4 ± 0.9	12.3 ± 2.3	0.07 ± 0.01	0.13 ± 0.02	27.4 ± 2.0	76.1 ± 2.8	55.6 ± 12.3	11.0 ± 3.5	17.7 ± 0.5	19.6 ± 0.7
9	8.0 ± 1.2	10.0 ± 2.0	0.06 ± 0.00	0.13 ± 0.04	26.9 ± 3.2	54 ± 4.1	67.7 ± 30.6	9.0 ± 2.9	16 ± 0.9	15 ± 0.4
10	5.8 ± 1.1	9.3 ± 1.3	0.03 ± 0.00	0.10 ± 0.02	37.6 ± 4.0	71.8 ± 4.2	70.3 ± 11.9	10.0 ± 2.1	12.8 ± 0.3	15.9 ± 0.6
11	7.8 ± 0.5	10.3 ± 2.9	0.04 ± 0.01	0.13 ± 0.03	41.6 ± 2.2	72.9 ± 3.8	79.0 ± 17.2	8.0 ± 2.7	13.5 ± 0.5	15.8 ± 0.7
12	7.0 ± 1.0	8.0 ± 2.1	0.07 ± 0.01	0.08 ± 0.02	45.7 ± 6.8	83.3 ± 2.6	46.3 ± 20.2	14.7 ± 1.9	15.9 ± 1.4	17.4 ± 0.2
13	7.5 ± 0.9	7.7 ± 0.7	0.26 ± 0.21	0.08 ± 0.01	24.3 ± 1.8	71.1 ± 4.8	25.0 ± 10.2	3.3 ± 0.3	14.7 ± 0.7	18 ± 0.4
14	16.0 ± 0.8	21.0 ± 6.0	0.09 ± 0.01	0.25 ± 0.04	29.2 ± 1.9	49.4 ± 2.0	36.4 ± 3.1	11.0 ± 0.6	15.3 ± 0.3	16 ± 0.4
15	12.8 ± 1.1	12.0 ± 0.6	0.07 ± 0.01	0.13 ± 0.02	37.1 ± 5.7	63.1 ± 4.1	56.5 ± 20.7	3.3 ± 0.8	16.7 ± 0.4	15.2 ± 0.3
ANOVA										
Environment	NS[z]		***		***		***		***	
Accession	***		*		***		***		***	
Environment x Accession	*		NS		***		***		***	

[w]Values represent mean (± standard errors) of 5 observations for field environment and 3 observations for nursery environment.
[y]Values represent mean (± standard errors) internode extension of 15 observations for field environment and 9 observations for nursery environment.

Table 16. Means of leaf measures by accession for *Sesuvium portulacastrum* when grown in 2.3 L containers in the nursery or planted to the field.

	Lamina length (mm)		Lamina width (mm)		Petiole length (mm)		Stem Diameter (mm)	
Accession	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
1	29.9 ± 0.8[y]	38.3 ± 1.6	8.6 ± 0.2	8.6 ± 0.3	11.7 ± 0.3	8.0 ± 0.5	4.5 ± 0.1	3.9 ± 0.4
2	20.6 ± 1.0	28.2 ± 0.8	7.5 ± 0.4	8.3 ± 0.3	9.6 ± 0.4	7.0 ± 0.5	3.5 ± 0.1	3.6 ± 0.1
3	23.7 ± 0.4	33.8 ± 1.1	8.0 ± 0.4	10.1 ± 0.3	7.6 ± 0.3	6.4 ± 0.5	3.5 ± 0.1	3.7 ± 0.1
4	24.1 ± 0.8	32.9 ± 1.3	7.1 ± 0.2	7.7 ± 0.2	7.9 ± 0.4	4.4 ± 0.2	2.8 ± 0.1	3.6 ± 0.1
5	24.7 ± 0.6	31.3 ± 1.1	6.4 ± 0.4	7.9 ± 0.3	6.2 ± 0.2	4.3 ± 0.3	3.3 ± 0.1	3.6 ± 0.2
6	28.4 ± 1.4	42.9 ± 1.6	11.9 ± 0.5	14.8 ± 0.3	10.4 ± 0.3	10.5 ± 0.4	4.2 ± 0.2	5.3 ± 0.2
7	32.8 ± 0.9	48.9 ± 1.0	15.2 ± 0.4	17.7 ± 0.6	12.8 ± 0.4	10.8 ± 0.3	5.3 ± 0.2	6.0 ± 0.2
8	25.5 ± 0.8	33.1 ± 1.3	6.1 ± 0.2	6.9 ± 0.3	6.7 ± 0.2	4.3 ± 0.2	3.2 ± 0.1	3.9 ± 0.1
9	21.5 ± 0.8	25.0 ± 0.9	6.2 ± 0.2	5.9 ± 0.3	7.9 ± 0.3	5.3 ± 0.2	2.9 ± 0.1	2.9 ± 0.1
10	26.0 ± 0.6	26.3 ± 0.3	5.6 ± 0.5	5.9 ± 0.3	6.4 ± 0.4	3.9 ± 0.2	2.5 ± 0.1	2.6 ± 0.1
11	27.2 ± 0.8	26.6 ± 0.6	6.7 ± 0.3	5.4 ± 0.2	6.1 ± 0.4	3.9 ± 0.4	2.6 ± 0.1	2.7 ± 0.1
12	29.2 ± 1.0	28.2 ± 1.0	6.9 ± 0.3	7.0 ± 0.3	8.1 ± 0.4	5.1 ± 0.4	3.2 ± 0.2	3.3 ± 0.1
13	20.7 ± 1.2	29.2 ± 0.6	5.3 ± 0.3	5.7 ± 0.2	7.3 ± 0.5	5.8 ± 0.2	2.5 ± 0.1	2.8 ± 0.1
14	26.0 ± 1.0	31.9 ± 0.9	7.4 ± 0.3	6.8 ± 0.3	8.5 ± 0.2	4.4 ± 0.3	3.7 ± 0.1	3.2 ± 0.1
15	33.7 ± 7.4	29.8 ± 0.6	6.5 ± 0.4	5.2 ± 0.2	8.8 ± 0.6	5.1 ± 0.3	3.0 ± 0.1	3.0 ± 0.1
ANOVA								
Environment	***[z]		***		***		***	
Accession	***		***		***		***	
Environment x Accession	***		***		***		***	

[y]Values represent mean (± standard errors) of 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 17. Maximum, minimum, mean, standard deviation, coefficient of variation of growth measures combined for all accessions of *Sesuvium portulacastrum* across all environments.

	Max	Min	Mean	Standard Deviation	CV[x]
Plant height (cm)	29.0	3.0	11.5[y]	5.3	45.8
Flower count	303.0	0.0	33.3	47.5	142.6
Height/width ratio ($cm \cdot cm^{-1}$)	0.89	0.2	0.11	0.1	88.6
Ornamental rating	5	1	3.0	0.7	24.7
Growth index (cm^3)	785672.0	864.0	199752.0	180270.1	90.2
Internode length (mm)	126.0	8.6	48.7[z]	25.1	51.6
Flower diameter (mm)	23.0	10.9	16.4	2.4	13.7
Pedicle length (mm)	113.0	1.0	8.6	8.3	96.8
Lamina length (mm)	92.1	15.3	28.7	7.4	25.7
Lamina width (mm)	20.0	2.8	8.0	3.1	39.4
Petiole length (mm)	16.2	2.0	7.4	2.7	36.2

[x]Coefficient of variation.
[y]Means combined across all accessions and environments, N=111.
[z]Means combined across all accession and environments N=322 for internode mean and N=230 for floral data.

Table 18. Means of growth measures separated by origin of accession along Texas coast for *Sesuvium portulacastrum* when grown in 2.3 L containers in the nursery or planted to the field.

	Height (cm)	Flower count (No./plant)		Height:width ratio ($cm \cdot cm^{-1}$)		Internode length (mm)		Flower diameter (mm)	
Location	Combined[u]	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
South	13.5 ± 1.0	58.74 ± 17.21	6.6 ± 2.37	0.1 ± 0.01[v]	0.19 ± 0.02	33.57 ± 1.7[x]	74.8 ± 4.53	17.15 ± 0.46[y]	17.17 ± 0.35
Central	12.6 ± 0.8	36.63 ± 5.28	6.33 ± 1.01	0.08 ± 0.01	0.14 ± 0.02	31.5 ± 1.54	66 ± 2.46	16.19 ± 0.26	16.79 ± 0.34
North	8.0 ± 0.4	58.84 ± 8.51	10.53 ± 1.17	0.09 ± 0.04	0.1 ± 0.01	35.48 ± 1.83	70.62 ± 2.22	14.17 ± 0.36	16.42 ± 0.27
ANOVA									
Environment	NS[z]	***		***		***		***	
Location	***	NS		NS		NS		***	
Environment x Location	NS	NS		NS		NS		***	

[u]Environments combined when not significant to $P \leq 0.05$. Values represent means (± standard errors) of 38, 39, and 34 observation for south, central, and northern coasts, respectively.
[v]Values represent means (± standard errors) of height: width ratio of 23, 24, and 19 observations for south, central, and northern coasts, respectively for field environment and of 15, 15, and 15 observations for south, central, and northern coasts, respectively for nursery environment.
[x]Values represent means (± standard errors) of internode extension for 69, 69, and 51 observations for south, central, and northern coasts, respectively for field environment and of 44, 45, and 45 observations for south, central, and northern coasts, respectively for nursery environment.
[y]Values represent means (± standard errors) of flower diameter for 34, 51, and 29 observations for south, central, and northern coasts, respectively for field environment and of 29, 42, and 45 observations for south, central, and northern coasts, respectively for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively

Table 19. Means of leaf measures separated by origin of *S. portulacastrum* accession along Texas coast when grown in 2.3 L containers in the nursery or planted to the field.

Location	Lamina length (mm)		Lamina width (mm)		Petiole length (mm)		Stem diameter (mm)	
	Field	Nursery	Field	Nursery	Field	Nursery	Field	Nursery
South	27.93 ± 0.55[y]	38.86 ± 1.15	9.65 ± 0.43	11.39 ± 0.65	9.72 ± 0.34	7.53 ± 0.46	4.00 ± 0.13	4.52 ± 0.18
Central	25.23 ± 1.08	31.36 ± 0.52	7.17 ± 0.16	7.47 ± 0.27	8.19 ± 0.2	5.47 ± 0.23	3.43 ± 0.06	3.48 ± 0.06
North	25.09 ± 0.56	27.07 ± 0.39	6.14 ± 0.18	5.98 ± 0.14	6.91 ± 0.21	4.80 ± 0.16	2.70 ± 0.06	2.85 ± 0.05
ANOVA								
Environment	***[z]		*		***		***	
Location	***		***		***		***	
Environment x Location	***		*		NS		NS	

[y]Values represent mean (± standard errors) of 15 observations for field environment and 9 observations for nursery environment.
[z] NS,*,**,***Non significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Cluster analysis identified one group that was formed by three of the five southern accessions, and a second group with all other accessions (Fig. 5). The three accessions forming their own group had larger leaves, stems, and were taller than the other accessions.

The accessions from the southern collection had larger leaves, thicker stems, and a more upright habit than collections from either the central or northern coast. Leaf morphology was plastic in response to environment for most accessions of *S. portulacastrum*. Flowering seemed more dependent on the environment than region of collection, so a region cannot be targeted for future collection areas.

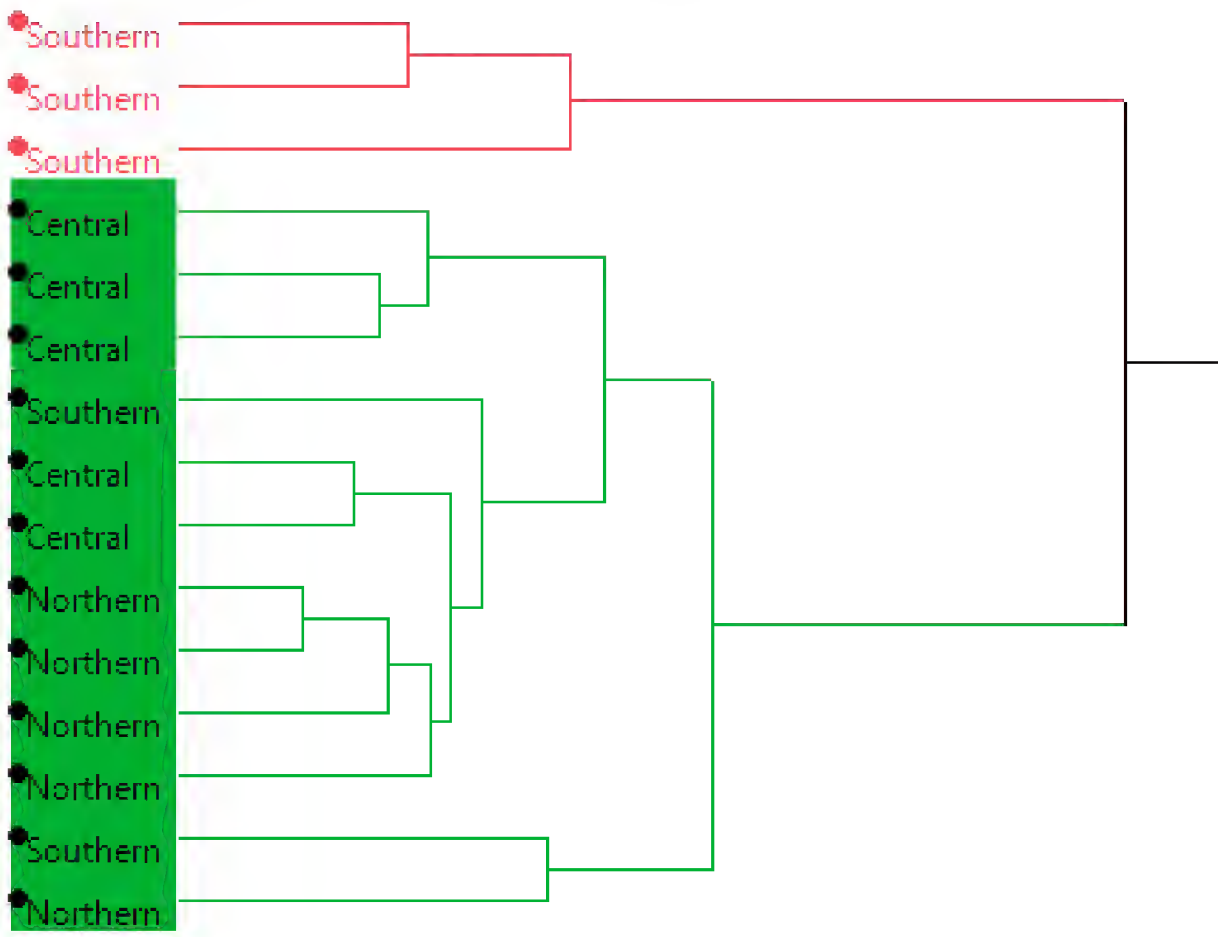

Fig. 5. Hierarchical Cluster analysis using Wards distance of *S. portulacastrum* accessions based on morphological traits. Different colors indicate different cluster groups and labels indicate accessions collection region.

CONCLUSIONS

We found differences in potential commercially important vegetative and floral traits among accessions for all four of the species tested and regional differences in traits of interests in *B. frutescens* and *O. drummondii*. This information could be used to guide the collection of future genotypes of *B. frutescens* and *O. drummondii*. This will assist future collectors of germplasm to target their collection efforts to regions based on the characteristics of material in which they are interested. Further collection of *E. procumbens* needs to be performed to test for differences in northern regional populations.

In the future, studies need to be performed to calculate heritability and stability of these characteristics in more environments to determine if these traits can be used for selection to make gains in ornamental performance over a broader range of environments.

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APPENDICES

Appendix 1. GPS coordinates and location description of collection site for each accession of *Oenothera drummondii*.

Accession	Latitude	Longitude	Description of location	Regional location along Texas coast
1	26° 06.879	97° 09.965	Gulf and Gardenia on Gulf Beach South Padre Island, TX	South
2	27° 38.623	97° 11.288	Hwy 361 and Gulf Access Rd. 3 Top of Dune	Central
3	26° 06.070	97° 09.864	Gulf and E. Martin Gulf Beach South Padre Island, TX	South
4	26° 07.472	97° 10.039	Gulf and Georgia Ruth Gulf Beach South Padre Island, TX	South
5	26° 14.445	97° 11.120	Where Park Rd 100 ends north of South Padre Island, TX	South
6	26° 11.814	97° 10.643	Park Rd 100 North of South Padre Island, Tx	South
7	28° 35.886	95° 58.718	Beach in Matagorda Beach TX	Central
8	28° 36.291	95° 57.588	Beach in Matagorda Beach TX	Central
9	28° 57.043	95° 17.588	Surfside Beach	North
10	29° 06.698	95° 04.956	Beach Access 2 Jamaica Beach	North
11	29° 40.203	94° 03.950	Side of Rd Near end of Hwy 87 Mcfaddin NWR	North
12	29° 12.519	94° 55.596	Galveston 3005 Rd Beach Access 14 in Dunes	North
13	29° 33.076	94° 23.333	Hwy 87 and 124	North
14	29° 26.297	94° 39.666	Off of HWY 87 on Gulf View on Crystal Beach	North
15	27° 51.816	97° 20.057	Sunset Park Portland Texas growing in oyster shell	Central
16	28° 5.1027	97° 20.057	Fulton Beach Rd in front of Royal Oaks Subdivision	?Central

[z]Latitude and Longitude presented in degrees and decimal minutes format

Appendix 2. GPS coordinates and location description of collection site for each accession of *Borrichia frutescens*.

Accession	Latitude	Longitude	Description of location	Regional location along Texas coast
1	27° 42.341	97° 09.224	Hwy 361 and Gulf Access Rd. 2	central
2	27° 38.867	97° 11.587	Hwy 361 and Gulf Access Rd. 3	central
3	26° 06.742	97° 10.212	Laguna St. and Campeche	southern
4	27° 17.363	97° 39.710	End of Rd. 771 in Rivera Beach	southern
5	26° 06.068	97° 09.864	Gulf and E. Martin South Padre Island, TX	southern
6	26° 08.435	97° 10.492	Convention Center in South Padre Island, TX	southern
7	26° 04.353	97° 22.510	Port Isabel Texas next to Whataburger	southern
8	26° 04.715	97° 12.712	Shore Dr. Port Isabel, TX	southern
9	26° 33.535	97° 25.568	Mansfield and North Shore Port Mansfield, TX	southern
10	26° 07.175	97° 09.945	Gulf and E. Mars South Padre Island, TX	southern
11	27° 38.647	97° 17.057	Laguna Shores Rd. Flour Bluff	central
12	26° 34.163	97° 25.774	Fred Stone Park Port Mansfield, TX	southern
13	28° 41.805	95° 57.570	Matagorda Beach along main road	central
14	28° 39.614	96° 24.754	End of 172 Rd in Port Alto, TX	central
15	28° 23.470	96° 50.245	Town Park in Austwell, TX	central
16	28° 33.601	96° 32.247	Public Beach in Magnolia Beach, TX	central
17	28° 27.159	96° 24.326	Park in Port O'Connor, TX	central
18	28° 24.581	96° 43.542	Park living in effluent stream, Sea Drift, TX	central
19	28° 02.160	97° 02.520	Beginning of Fulton Beach Rd. Rockport TX	central
20	28° 57.017	95° 17.142	End of RD332 Surfside	northern
21	29° 22.040	94° 45.607	Hwy 87 Side of RD Bolivar	northern
22	29° 40.091	94° 04.279	McFaddin NWR on Beach	northern
23	29° 42.612	93° 51.539	1st St. in Sabine TX	northern
24	29° 22.042	94° 45.606	Hwy 87 Side on side of RD Bolivar	northern
26	29° 33.079	95° 22.336	On Bay Beach Park View and Port Velasco	northern
27	29° 12.522	94° 55.598	124 @ Hwy 87 High Island in Ditch	northern
28	29° 08.671	97° 03.506	3005 Rd Beach Access 14 Beach in Galveston	northern

[z]Latitude and Longitude presented in degrees and decimal minutes format

Appendix 2. GPS coordinates and location description of collection site for each accession of *Erigeron procumbens*.

Accession	Latitude	Longitude	Description of location	Regional location along Texas coast
1	27° 48.886[z]	97° 04.355	2016 11TH St. Port Aransas, TX	Central
2	27° 42.343	97° 09.240	Hwy 361 and Gulf Access Rd. 2	Central
3	27° 53.658	97° 18.440	Walmart Parking lot Portland, TX	Central
4	27° 40.141	97° 17.239	Wells Fargo Parking lot Flour Bluff, TX	Central
5	26° 07.093	97° 10.165	Park Rd 100 and Mars St. South Padre Island, TX	Southern
6	27° 54.524	97° 08.947	Central Park Aransas Pass, TX	Central
7	27° 08.072	97° 47.561	Hwy 77 Kennedy County Rest Stop	Southern
8	26° 07.185	97° 10.256	Laguna and Constellation South Padre Island, TX	Southern
9	26° 07.598	97° 10.069	Gulf and Cora Lee South Padre Island, TX	Southern
10	26° 30.713	97° 27.841	Hwy 186 in Ditch with Sand	Southern
11	26° 06.738	97° 10.210	Laguna and Mars South Padre Island, Texas	Southern
12	27° 37.411	97° 13.468	14175 Jack Fish Ave. The Island Corpus Christi, TX	Central
13	27° 48.341	97° 04.823	11th St. and Gulf Access Rd 1A Port A, TX	Central
14	27° 38.808	97° 16.958	Laguna Shores Rd. Flour Bluff, TX	Central
15	27° 38.877	97° 11.582	Hwy 361 and Gulf Access Rd. 3	Central
16	28° 27.154	96° 24.327	Water front park in Port O'Connor, TX	Central
17	28° 08.317	96° 58.153	4th St growing in ditch, Lamar, TX	Central
18	29° 05.631	95° 06.601	Just north of Toll Bridge on County RD 3005	Northern

[z]Latitude and Longitude presented in degrees and decimal minutes format

Appendix 3. GPS coordinates and location description of collection site for each accession of *Sesuvium portulacastrum*.

Accession	Latitude	Longitude	Description of location	Regional location along Texas coast
1	26° 34.164[z]	97° 25.745	Fred Stone Park Port Mansfield	Southern
2	27° 48.201	97° 4.654	Hwy 361 and Gulf Access Rd 1A	Central
3	27° 17.363	97° 39.71	End of Rd 771 in Rivera Beach	Central
4	26° 4.715	97° 12.714	Shore Dr. Port Isabel, TX	Southern
5	26° 4.354	97° 12.718	Port Isabel, TX	Southern
6	26° 4.716	97° 12.715	Port Isabel, TX	Southern
7	26° 7.47	97° 10.042	Gulf Beach South Padre Island, TX	Southern
8	28° 27.163	96° 24.325	Park in Port O'Connor, TX	Central
9	29° 6.698	95° 4.956	Beach Access Rd 2 Jamaica Beach	Northern
10	29° 40.09	94° 4.279	McFaddin NWR	Northern
11	29° 33.076	94° 23.338	124 @ HWY87 High Island	Northern
12	28° 57.02	95° 17.148	End of Rd 332 Surfside Beach	Northern
13	29° 12.523	95° 55.598	Beach Access 14 in dunes Galveston	Northern
14	27° 51.719	97° 20.446	Sunset Park Portland Texas	Central
15	28° 8.671	97° 3.506	Fulton Beach Rd	Central

[z]Latitude and Longitude presented in degrees and decimal minutes format

Inheritance of nrDNA in artificial hybrids of *Hesperocyparis arizonica* x *H. macrocarpa*

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ABSTRACT

Sequencing nrDNA of parents (*Hesperocyparis arizonica, H. macrocarpa)* found their nrDNA differ at 8 sites. Analysis of 18 artificial hybrids, revealed each of the hybrids had nrDNA that was heterozygous at each of the 8 sites. However, the peak ratios in the chromatograms were not 1:1 as expected, but varied from 1:1 to 3:1, being more like *H. arizonica*. PCO of the variation in the peak heights revealed four groups of hybrids that might be associated with chromosome inheritance. However, PCO clearly distinguished the parents and the hybrids. nrDNA, if appropriately coded, can be utilized in multivariate methods for the detection of hybrids. Due to concerted evolution, nrDNA may underestimate the degree of introgression. Analysis of petN-psbM (cp DNA) confirmed that all the hybrids inherited the cp genome from their pollen-parent (*H. arizonica*) confirming cp genome inheritance via pollen in *Hesperocyparis* (Cupressaceae). Published on-line **www.phytologia.org** *Phytologia 98(4): 277-283 (Oct. 6, 2016).* ISSN 030319430.

KEY WORDS: *Hesperocyparis arizonica, H. macrocarpa,* Cupressaceae, hybrids, nrDNA, petN-psbM, inheritance of cp genome via pollen.

Sequencing of nrDNA spacer regions has been an important source of phylogenetic information in plant systematics for several years. The conserved nature of the multi-copy nrDNA (thousands of copies per cell) might be due to concerted evolution (Liao, 1999). Liao (1999) argues that because rRNAs are structural molecules, multiple gene copies are necessary to supply the demand for ribosomal subunits in the cell. Because these sub-units function only when assembled into a large complex, homogeneity of rRNAs is critical for regular, functional ribosome assembly and translation to function normally. Liao (1999) concludes that "a possible biological function of concerted evolution is to maintain homogeneous gene copies in a family so that homogeneous transcripts can be produced." However, concerted evolution is thought to be a slow process over numerous generations. Hybrids would seem likely to be heterozygous for both parents nrDNA. Thus, nrDNA (ITS) is often used for the analysis of hybridization. Recently, Adams (2015a,b) found that nrDNA detected 15 hybrids, whereas, maldehy, a single copy nuclear gene (SCN), detected 25 hybrids. nrDNA appeared more often to be the same as one of the parents, whereas the SCN gene (maldehy) was heterozygous, indicating the plant(s) were of hybrid origin.

Chaing et al. (2001) found that in the artificial hybrids between *Begonia aptera* (pollen) and *B. formosana* (maternal), nrDNA was predominantly that of the maternal parent, *B. formosana* (diamonds, Fig. 1). Volkov et al. (1999) reported that one of the parental nrDNAs was eliminated in the allopolyploid genome of cultivated tobacco. Fukuoka et al. (1994) found that the nrDNA in γ-ray irradiated tetraploid rice was homogenized in a short time.

Aguilar et al. (1999) made artificial hybrids between *Armeria villosa* ssp. *longiaristata* and *A. colorata*, then examined the inheritance of nrDNA in F_1 and F_2 generations. They found the expected

additive pattern in polymorphisms for five of the six variable sites in F_1 plants. However, in the F_2 generation, there was a bias towards one parent (*A. colorata*). Backcrosses showed homogenization of five of the polymorphic sites to the recurrent parent.

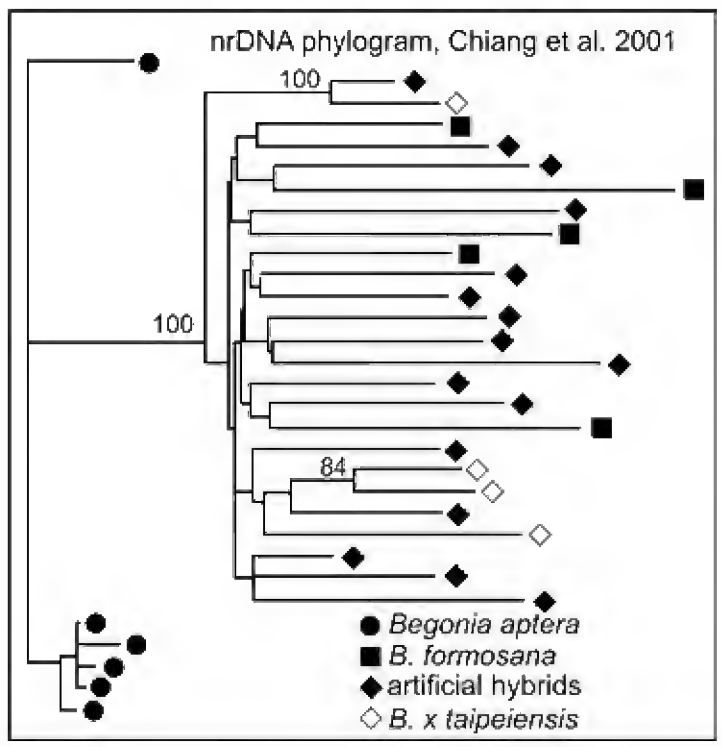

Figure 1. Phylogram based on nrDNA for *Begonia* and hybrids (adapted from Chiang et al. 2001). Notice the grouping of the hybrids (triangles, nrDNA) with the maternal parent, *B. formosana* (shaded squares), rather than with the pollen (paternal) parent *(B. aptera*, shaded circles).

Okuyama et al. (2005) examined introgression in *Mitella* using nrDNA ITS and ETS, and cpDNA and found that cpDNA revealed the most introgression, ITS regions showed a moderate amount of introgression and the ETS region gave no evidence of introgression. They concluded that non-uniform concerted evolution between the ETS region and ITS regions may explain these different patterns of introgression.

Adams and Matsumoto (2016) sequenced nrDNA of *Cryptomeria japonica* D. Don (Sugi) cv. *Haara* and cv. *Kumotooshi* (Kumo) and found 3 variable sites at positions 154, 468 and 505. In Kumo, each of these three positions was heterozygous, indicating that Kumo is a hybrid between Haara x *C. japonica*. If so, then the progeny of this cross would be backcrosses [(Haara x *C. japonica*) x Haara].

Table 1. Variable sites in the nrDNA sequence for *Cryptomeria japonica* cv. *Haara, C. japonica* cv. *Kumotooshi* and their hybrids. The ratios of bases in parenthesis ( ) were obtained by measurements of the peak sizes on the chromatogram. NA = not available.

					petN-psbM (from pollen)		
	site 154	site 468	site 505	nrDNA type	site 145	site 146	cp type
Haara	C	T	G	Haara	A	T	Haara
Kumo	C/G (1:0.7)	T/C (0.64:1.0)	G/A (0.76:1)	Haara x Kumo	C	G	Kumo
progeny (backcrosses to Haara?):					Pollen parent of progeny		
14519	C/G (1:0.7)	T/C (1:1)	G/A (0.7:1)	Haara x Kumo	C	G	Kumo
14520	C/G (1:0.7)	T/C (0.46:1)	G/A (0.58:1)	Haara x Kumo	C	G	Kumo
14521	C/G (1:0.2)	T/C (1:0.5)	NA	Haara x Kumo	C	G	Kumo
14522	C	T	NA	Haara	C	G	Kumo
14523	C	T	NA	Haara	C	G	Kumo
14524	C	T	NA	Haara	C	G	Kumo
14525	C	T	NA	Haara	C	G	Kumo

Three of the seven progeny (BC) had nrDNA very similar to the (Haara x Kumo) parent, with some variation in the ratio of bases (Table 1). 14519 showed a small shift in frequency toward the recurrent parent (Haara) at position 468 (1:1 vs. 0.64:1 in Kumo). 14620 showed a shift in frequency toward Haara at position 468. 14621 shifts toward Haara at positions 154 and 468. Overall, all 3 of these progeny showed some shift in frequencies toward the recurrent parent (Haara), as one might expect from backcrossing.

The nrDNAs of four progeny (BC) (Table 1) were the same as the recurrent parent Haara. Positions 154 and 468 showed a complete shift in frequencies to the recurrent parent Haara by this single backcrossing event. This suggests that perhaps, nrDNA can rather quickly revert to the pattern of one of the parents in a backcrossing event by concerted evolution.

In the Cupressaceae, breeding programs are rare, so the existence of parents and artificial (verified) hybrids is an important resource for studies on inheritance. Scion Research Institute, Rotorua, New Zealand has a breeding program that involves crossing *Cupressus* and *Hesperocyparis* species. Sequencing nrDNA of *H. arizonica* (2003.017) and *H. macrocarpa* (896.752) found they differ at 8 sites and were each monomorphic in each taxon for each of the 8 sites. The breeding program afforded an unusual opportunity to examine the inheritance of nrDNA in hybrids in the Cupressaceae. As far as known, this report will be the second on the inheritance of nrDNA in the Cupressaceae (or in conifers). The purpose of this paper is to report on the inheritance of nrDNA in artificial hybrids of *H. arizonica* x *H. macrocarpa*.

MATERIALS AND METHODS

Plant material: Crosses were made at the Scion Research Institute, Rotorua, New Zealand using pollen of *H. arizonica* (2003.017) onto receptive seed cones of *H. macrocarpa* (896.752). Seedlings were obtained and greenhouse grown to 50-80 cm, then field planted. Leaf samples were taken after approximately one year in the field (plants about 1 m tall). Parents: *Adams 14854 H. arizonica* (2003.017), *Adams 14858 H. macrocarpa* (896.752), (leaves in silica gel)
Eighteen (18) Hybrids (leaves in silica gel) (lab accession #): *Adams 14914 - Adams 14931.*

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.
Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.).

RESULTS AND DISCUSSION

As mentioned in the introduction, sequencing nrDNA of *H. arizonica* (2003.017) and *H. macrocarpa* (896.752) resulted in 1249 bp with differences at 8 sites. Each of these 8 sites were monomorphic in each parent (Table 2). Sequencing nrDNA of the 18 hybrids revealed every hybrid was polymorphic at each of the 8 sites (Table 2). In contrast to the theoretical ratio of 1:1, none of the hybrids had exactly that ratio for the 8 sites (Table 2). However, 3 of the hybrids (14915, 14920, 14931, in bold,

Table 2. Variable sites in hybrids between *H. arizonica* (14854, 2003.017) x *H. macrocarpa* (14858, 896.752) cross which differ at 8 sites. nrDNA (ITS) numbering is from the 5' end. Chromatogram peak heights are ratio of peaks expresses as arizonica bp/ macrocarpa bp. (i.e. for 124, C:T 2:1 = 2.0; C:T 60:40 = 1.5, etc.). na = not available. petN sequence pattern (right-most column) shows the paternal (pollen) parent of the hybrid. Hybrids are grouped by similarities in boldface, italics, normal, and the second boldface group.

	124	177	295	313	370	516	775	1107	petN seq.
arizonica	C	C	T	C	G	C	G	A	*arizonica*
macrocarpa	T	G	C	T	T	T	A	G	*macrocarpa*
	C/T	C/G	T/C	C/T	G/T	C/T	G/A	A/G	ratios
theoretical F1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
14915 group A	**1.0**	**1.5**	**2.0**	**1.0**	**3.0**	**1.0**	**1.0**	**1.0**	*arizonica*
14920	**1.0**	**2.0**	**1.0**	**1.0**	**2.0**	**0.5**	**1.0**	**1.0**	*arizonica*
14931	**1.0**	**1.5**	**1.2**	**1.0**	**2.0**	**1.0**	**1.0**	**1.5**	*arizonica*
14916 group B	*1.5*	*1.0*	*1.2*	*1.5*	*1.5*	*0.5*	*1.0*	*1.0*	*arizonica*
14923	*1.5*	*1.0*	*1.0*	*1.0*	*1.5*	*1.5*	*1.5*	*na*	*arizonica*
14924	*1.5*	*1.0*	*1.0*	*1.5*	*1.5*	*2.0*	*1.0*	*1.0*	*arizonica*
14925	*1.5*	*1.0*	*1.0*	*1.5*	*1.5*	*1.5*	*1.0*	*1.0*	*arizonica*
14917 group C	***1.0***	***1.5***	***2.0***	***1.0***	***2.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14921	***1.0***	***1.5***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14918	***1.0***	***2.0***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14919	***1.0***	***2.0***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14922	***1.0***	***2.0***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14926	***1.0***	***2.0***	***2.0***	***1.0***	***3.0***	***1.0***	***1.0***	***2.0***	***arizonica***
14914 group D	1.0	2.0	2.0	1.0	3.0	1.0	1.5	2.0	*arizonica*
14927	1.0	2.0	1.0	1.0	2.0	1.0	1.5	3.0	*arizonica*
14929	1.0	3.0	1.0	1.0	3.0	1.0	1.5	3.0	*arizonica*
14928	1.0	2.0	2.0	1.0	3.0	1.0	1.5	3.0	*arizonica*
14930	1.0	2.0	2.0	1.5	3.0	1.0	1.5	3.0	*arizonica*
Average	1.11	1.72	1.58	1.11	2.44	1.06	1.17	1.91	
Sd	0.64	1.56	1.47	0.64	1.99	1.01	0.73	2.26	

Table 2) were closest to 1:1 ratios. Many of the hybrids had sites 177, 295, 370, and 1107 with 2:1 or 3:1 ratios. Of course, the parents were judged to be monomorphic based on visual inspection of the chromatograms at the 8 sites. There may have been some (~10% or less) polymorphism that was not evident on the chromatograms.

In contrast, sites 124, 313, 516, and 775 were generally inherited near the expected 1:1 ratio with average ratios of 1.11, 1.11, 1.06, and 1.17 (Table 2).

To further examine the variation among the hybrids, the nucleotides at each of the 8 sites were coded as the proportion of each base present (Table 3). Then, these data were subjected to Principal Coordinates Ordination (PCO). Factoring the similarity matrix resulted in three eigenroots that were larger than the average diagonal value. In addition, the eigenroots appeared to asymptote after the third eigenroot. These three eigenroots accounted for 79.45% of the variance among the hybrids and the theoretical hybrid.

Table 3. Variable sites in hybrids between *H. arizonica* (14854, 2000.75,0.2517) x *H. macrocarpa* (14858, 896.752) cross which differ at 8 sites. nrDNA (ITS) numbering is from the 5' end. Chromatogram peak heights are ratio of peaks expressed as arizonica bp + macrocarpa bp. (i.e. for 124, C:T 2:1 = 0.67,0.33; C:T 60:40 = 0.6,0.4, etc.). na = not available. petN sequence pattern (right-most column) shows the paternal (pollen) parent of the hybrid.

	124	177	295	313	370	516	775	1107	petN seq.
arizonica	C 1.0	C 1.0	T 1.0	C 1.0	G 1.0	C 1.0	G 1.0	A 1.0	*arizonica*
macrocarpa	T 1.0	G 1.0	C 1.0	T 1.0	T 1.0	T 1.0	A 1.0	G 1.0	*macrocarpa*
	C,T	C,G	T,C	C,T	G,T	C,T	G,A	A,G	
theoretical hybrid	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.5, 0.5	
14914	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.6, 0.4	0.67,0.33	*arizonica*
14915	0.5, 0.5	0.6, 0.4	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.5, 0.5	*arizonica*
14916	0.6, 0.4	0.5, 0.5	0.55, 0.45	0.6, 0.4	0.6, 0.4	0.5, 0.5	0.5, 0.5	0.5, 0.5	*arizonica*
14917	0.5, 0.5	0.6, 0.4	0.67, 0.33	0.5, 0.5	0.67, 0.33	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14918	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14919	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14920	0.5, 0.5	0.67,0.33	0.5, 0.5	0.5, 0.5	0.67, 0.33	0.5, 0.5	0.5, 0.5	0.5, 0.5	*arizonica*
14921	0.5, 0.5	0.6, 0.4	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14922	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14923	0.6, 0.4	0.5, 0.5	0.5, 0.5	0.5, 0.5	0.6, 0.4	0.6, 0.4	0.6, 0.4	na	*arizonica*
14924	0.6, 0.4	0.5, 0.5	0.5, 0.5	0.6, 0.4	0.6, 0.4	0.67,0.33	0.5, 0.5	0.5, 0.5	*arizonica*
14925	0.6, 0.4	0.5, 0.5	0.5, 0.5	0.6, 0.4	0.6, 0.4	0.6, 0.4	0.5, 0.5	0.5, 0.5	*arizonica*
14926	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.5, 0.5	0.67,0.33	*arizonica*
14927	0.5, 0.5	0.67,0.33	0.5, 0.5	0.5, 0.5	0.67, 0.33	0.5, 0.5	0.6, 0.4	0.75,0.25	*arizonica*
14928	0.5, 0.5	0.67,0.33	0.67, 0.33	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.6, 0.4	0.75,0.25	*arizonica*
14929	0.5, 0.5	0.75,0.25	0.5, 0.5	0.5, 0.5	0.75, 0.25	0.5, 0.5	0.6, 0.4	0.75,0.25	*arizonica*
14930	0.5, 0.5	0.67,0.33	0.67, 0.33	0.6, 0.4	0.75, 0.25	0.5, 0.5	0.6, 0.4	0.75,0.25	*arizonica*
14931	0.5, 0.5	0.6, 0.4	0.55, 0.45	0.5, 0.5	0.67, 0.33	0.5, 0.5	0.5, 0.5	0.6, 0.4	*arizonica*

PCO ordination revealed four groups (Fig. 2). Group A (15, 20, 31) appears most like the theoretical hybrid. Group B is the most distinct group. Group C is the most uniform and as hybrids 18, 19, 22, 16 had identical compositions. Group D is the least similar to the theoretical hybrid at the 8 polymorphic nrDNA sites.

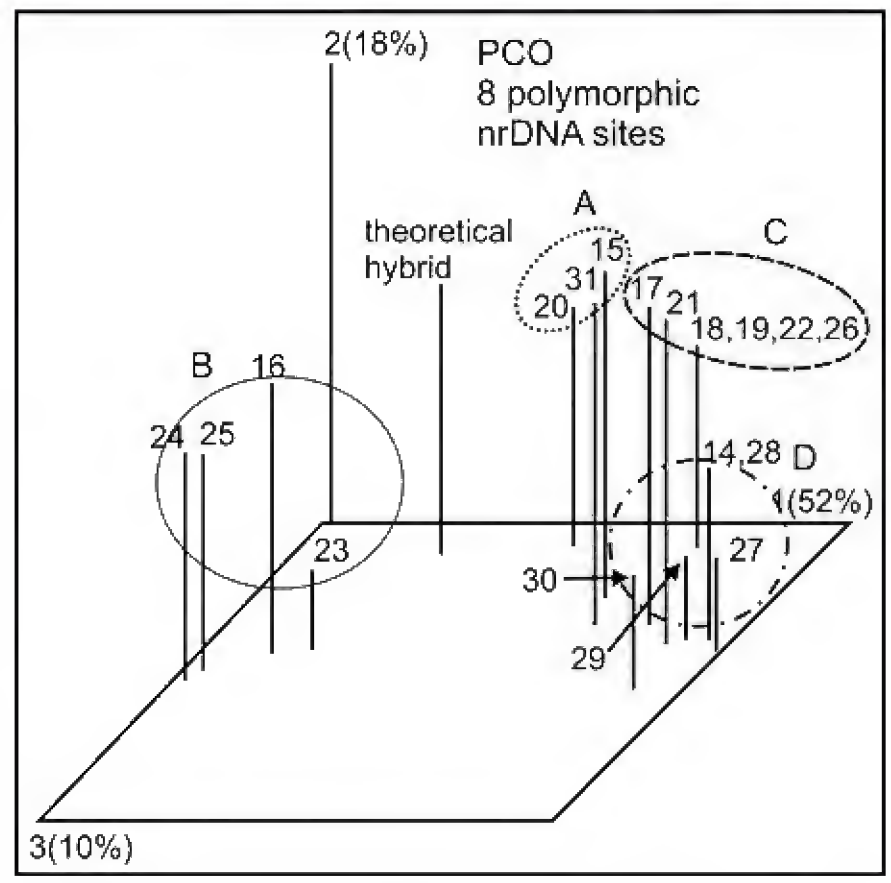

Fig. 2. PCO of hybrids based on relative peak heights. Numbers are the last two digits of the 5 digit plant number in Table 2.

It is interesting to note that if one denoted the *H. arizonica* parent's nrDNA as a1 (from one parent and a2 from the other parent), and likewise *H. macrocarpa* parent's nrDNA as m1, m2, then the nrDNA of the parents would be inherited in the progeny as: a1, m1 (25%), a1, m2 (25%), a2, m1 (25%), a2, m2 (25%). The four groups of nrDNA (Table 2) are: A: 3/18, 16.7%; B: 4/18, 22.2%; C: 6/18, 33.3%; D: 5/18, 27.7%). These data suggests the parents may have not been pure for their nrDNA.

To investigate the potential to utilize this nrDNA for the detection of hybrids, a PCO was performed including both parents and hybrids in
which data were coded as in Table 3. Factoring the similarity matrix resulted in four eigenroots that were larger than the average diagonal value. In addition, the eigenroots appeared to asymptote after the fourth eigenroot. The first three eigenroots accounted for 81.91% of the variance among the parents, hybrids and the theoretical hybrid. Ordination reveals (Fig. 3) the parents are well resolved with the hybrids in an intermediate position, but nearer to *H. arizonica*. This is quite similar to the U (or V) shaped pattern obtained by PCO using morphological data for artificial crosses in *Lepomis* (sunfish) (Adams, 1982, Fig. 4). Adams (1982, Fig. 9) also obtained a U (or V) shaped pattern in PCO based on 30 leaf terpenoids for *J. horizontalis*, *J. scopulorum* and putative hybrids. Thus, it appears, that nrDNA if appropriately coded can be utilized in multivariate methods for the detection of hybrids in Cupressaceae.

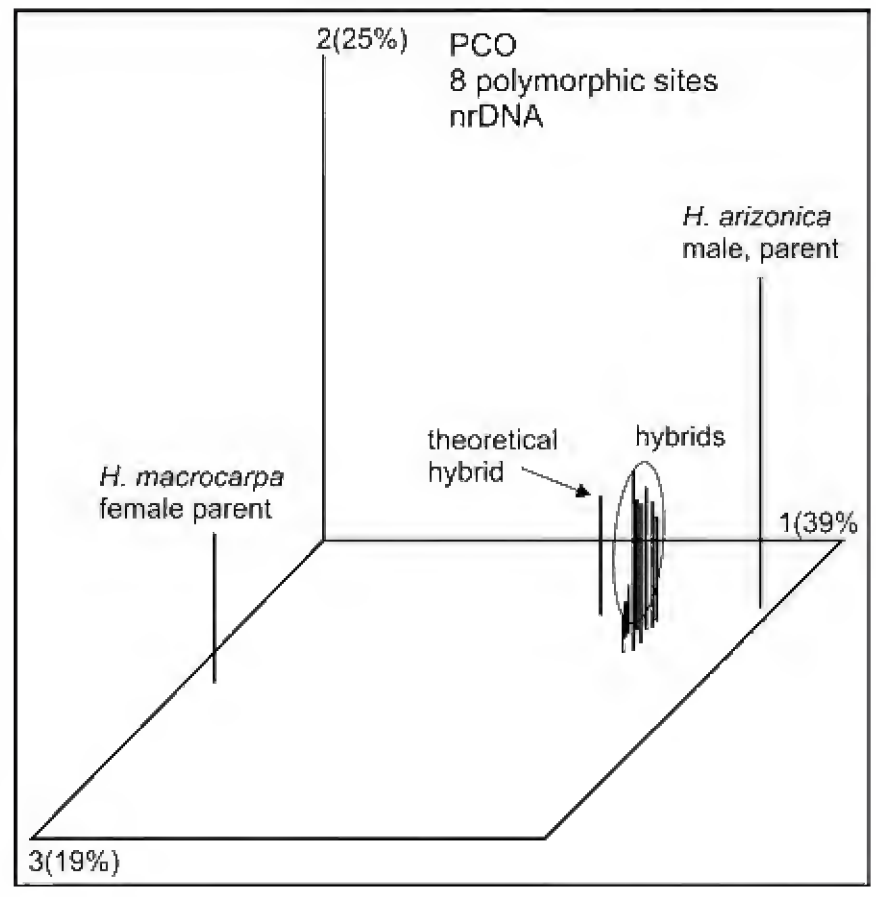

Fig. 3. PCO of parents, artificial hybrids, and the theoretical hybrid.

Analysis of petN-psbM (cp DNA) confirmed that all the hybrids inherited the cp genome from their pollen parent (*H. arizonica*).

The present study, shows a rather uniform inheritance of nrDNA in hybrids. The previous study on 7 putative backcross individuals of *Cryptomeria japonica* cultivars (Adams and Matsumoto, 2016) reported 3 of the 7 progeny had nrDNA very similar to parent (Haara x Kumo), and 4 progeny had nrDNA the same as recurrent parent Haara at positions 154 and 468 (Table 1). It appears that

backcrossing, some (4) of the backcrossed progeny reverted to the nrDNA of the recurrent parent, suggesting that nrDNA may be of somewhat limited value for the analysis of introgression.

The results of the *Cryptomeria japonica* backcrosses-study appear to parallel the Aguilar et al. (1999) study that found backcrosses showed homogenization of five of the polymorphic sites to the recurrent parent.

The present study on *Hesperocyparis* hybrids supports the use of nrDNA for the detection hybrids. But, due to concerted evolution (lineage sorting) in backcrosses (cf. *Cryptomeria japonica* and *Armeria* studies cited above), perhaps nrDNA analysis may underestimate the degree of introgression.

ACKNOWLEDGEMENTS

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Taxonomy and distribution of *Euphorbia stictospora* (Euphorbiaceae)

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ABSTRACT

The taxonomy of *Euphorbia stictospora* is reviewed. It is treated as a widespread highly variable species occurring from south central Mexico to north central USA. No meaningful infra-specific categories are recognized, although several named varieties have been proposed. It belongs to the sect. Anisophyllum of *Euphorbia*, this largely circumscribed by its 3 linear style branches. Published on-line www.phytologia.org *Phytologia 98(4): 284-287 (Oct 6, 2016).* ISSN 030319430.

KEY WORDS: Euphorbiaceae, *Chamaesyce*, *Euphorbia*, *E. stictospora*, USA, Mexico

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A taxonomic interest in Mexican gypseous Euphorbias has occasioned the present paper, following that of Turner (2016). Acceptance of the genus **Euphorbia** so as to include *Chamaesyce* is based upon the DNA work of Yang et al. (2012).

Euphorbia stictospora Engelm., U.S. Mex. Bound. Surv. Bot. 187. 1858.
Anisophyllum senile Klotzsch & Garcke
Chamaesyce interaxillaris (Fernald) Millsp.
Chamaesyce stictospora (Engelm.) Small
Chamaesyce stictospora var. *guadalupensis* Small
Chamaesyce stictospora var. *sublaevis* (M.C. Johnst.) Raju & Rao
Chamaesyce stictospora var. *texensis* Millsp.
Euphorbia interaxillaris Fernald
Euphorbia stictospora var. *sublaevis* M.C. Johnst.
Euphorbia stictospora var. *texensis* (Millsp.) Fedde

Annual or rarely perennial, prostate to weakly ascending, herbs; stems nearly glabrous to most often densely pubescent; leaves opposite, glabrous to markedly pubescent; stipules triangular to deltoid; blades variously broadly ovate to rotund, 3-10 mm long, 3-8 mm wide, asymmetric and rounded apically, the margins to some degree serrate; petioles ca 1 mm long; involucres ca 1 mm high; glands 4, ca equal in size, 0.5-1.0 mm wide, usually with short, white to wine-colored, appendages; stamens 3-10; capsules 1.0-1.5 mm long, glabrous to variously pubescent; style branches usually 3, ca 0.3 mm long, these rarely bifid; seeds 3-4 sided, weakly rugose at most, 0.9-1.2 mm long; chromosome number, n = 6 pairs (Powell & Turner (2005).

The species is known to occur in nearly all soil types throughout its distribution, igneous, sandy, limestone or pure gypsum (especially in north central Mexico). The Type is reportedly from southwestern Kansas. It is a widespread, highly variable, species from which several varieties have been segregated, the most recent being that of var. *sublaevis* from north central Mexico, largely recognized by its sparsely pubescent stems and foliage, this proposed by M.C. Johnston (1975), who nonetheless noted that the variety was growing near the typical variety w/o signs of intergradation. Examination of the several sheets cited by Johnston as var. *sublaevis*, including some of my own collections, show sporadic gradation into the typical variety over a large area. Indeed, Small (1913) in his treatment of *Chamaesyce stictospora* notes that it contains "A Texan variety [Type from Cherokee Co., eastern Texas] with less pubescent foliage, broader and more strongly nerved leaf-blades smaller and broader seeds and narrower glandular appendages, is *C. stictospora Guadalupensis* Small [= *E. s.* var. t*exensis* Millsp., not *E. texana* Boiss.]."

In short, numerous localized forms from throughout the range of the species might be treated as this or that infra-specific category.

Euphorbia stictospora is seemingly close to the recently described **E. rayturneri** (Steinmann & Jercinovic, 2013), so far as known, a novelty restricted to southwestern New Mexico, readily separated from the former by its distinct seeds, uppermost stem pubescence, and yet other characters.

Maps 1 and 2, showing distribution of **E. stictospora** in the USA and Mexico, are based upon specimens in the LL-TEX Herbarium and additional sites provided from the USDA plants database (plants.usda.gov).

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My editorial assistant, Jana Kos, provided meaningful input, as did the Curator at LL-TEX, George Yatskievych, for which I am grateful.

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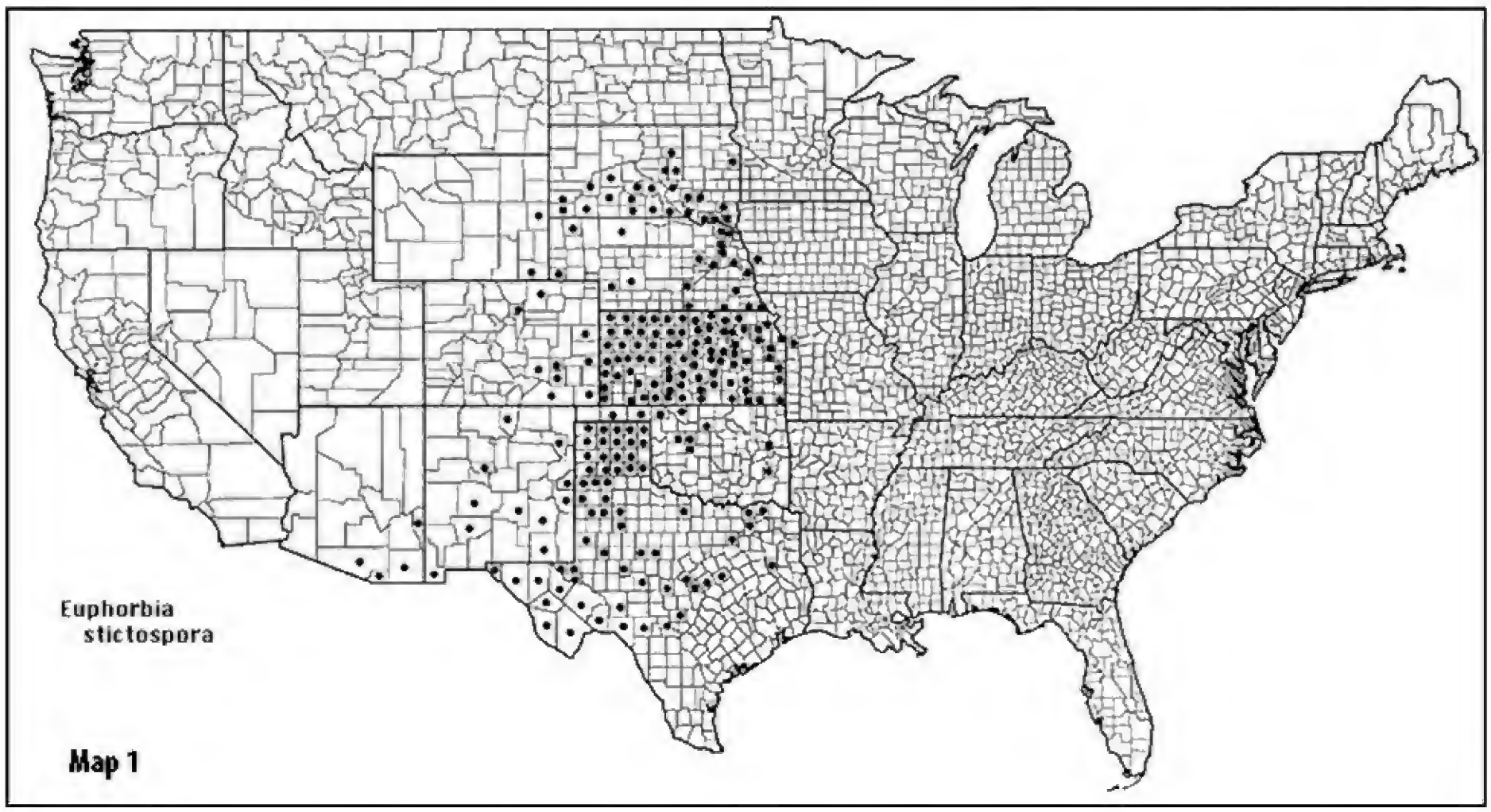
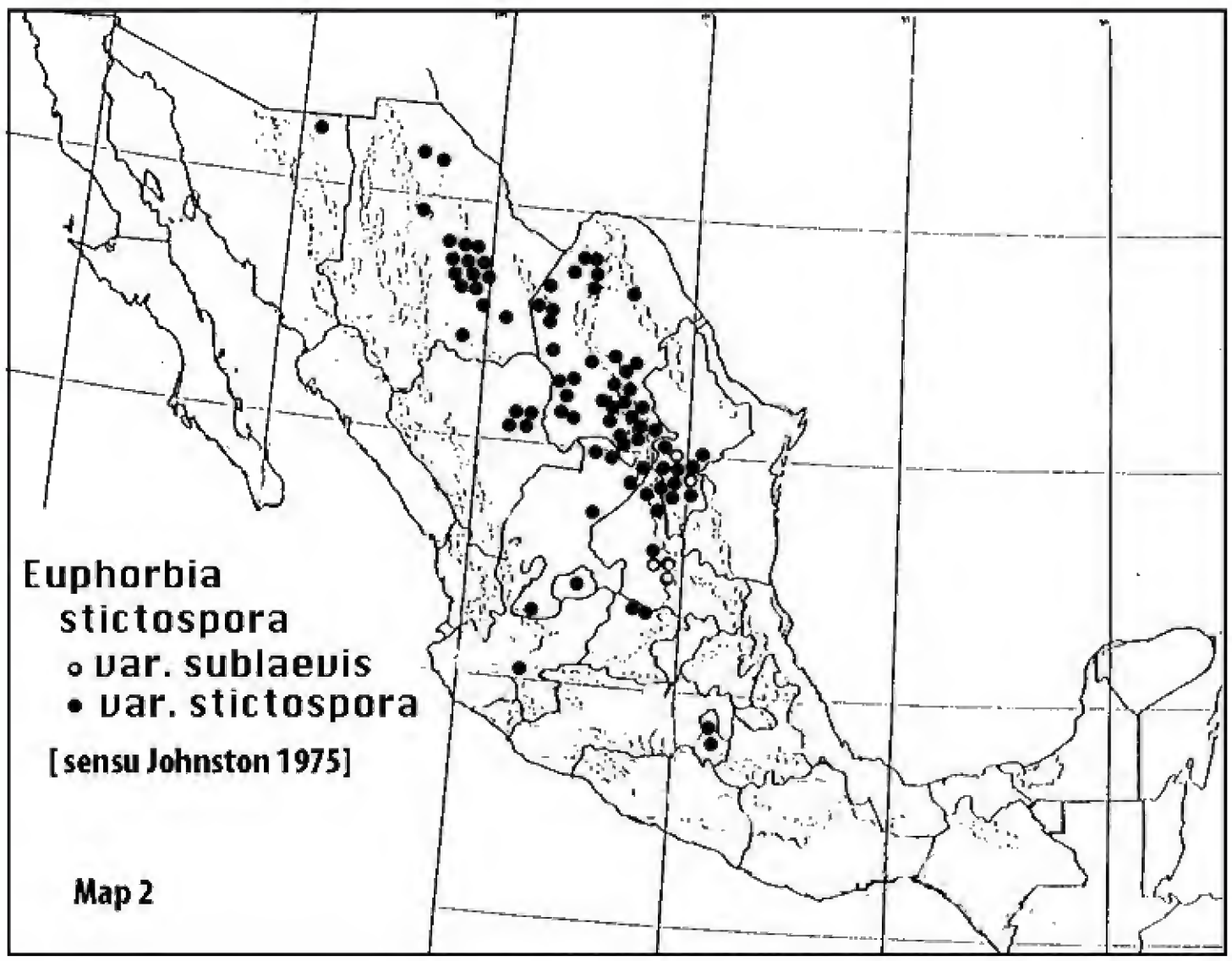


Map 1


Euphorbia
stictospora
var. sublaevis
var. stictospora
[sensu Johnston 1975]
Map 2

Addendum to Comps of Mexico, Vol 22, The genus *Wyethia*

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KEY WORDS: *Wyethia ovata, Agnorhiza ovata,* taxnomy.

In my treatment of The Comps of Mexico (Vol. 22: 2015) I neglected to account for the occurrence of the genus **Wyethia** in Baja California, as treated by Panero (2006) and Moore and Bohs (2003); both workers recognized the genus in its broad sense, preferring not to recognize **Agnorhiza** (sensu Weber 2006). I accept here the taxonomy of Weber, who treated the section Agnorhiza as a distinct genus in his treatment for the Flora of North America. The only species of the complex in Mexico is **A. ovata** (Torr. & Gray) W.A. Weber, presumably confined to the lower elevations of Sierra San Pedro Martir of northern Baja California. Distribution of the taxon concerned for the USA and Mexico is presented (Maps 1 and 2).

The species will key to the genus **Vigethia** in my treatment of the subtribe Engelmanniinae (Turner 2015), the genus accepted by Panero (2007) and yet other workers.

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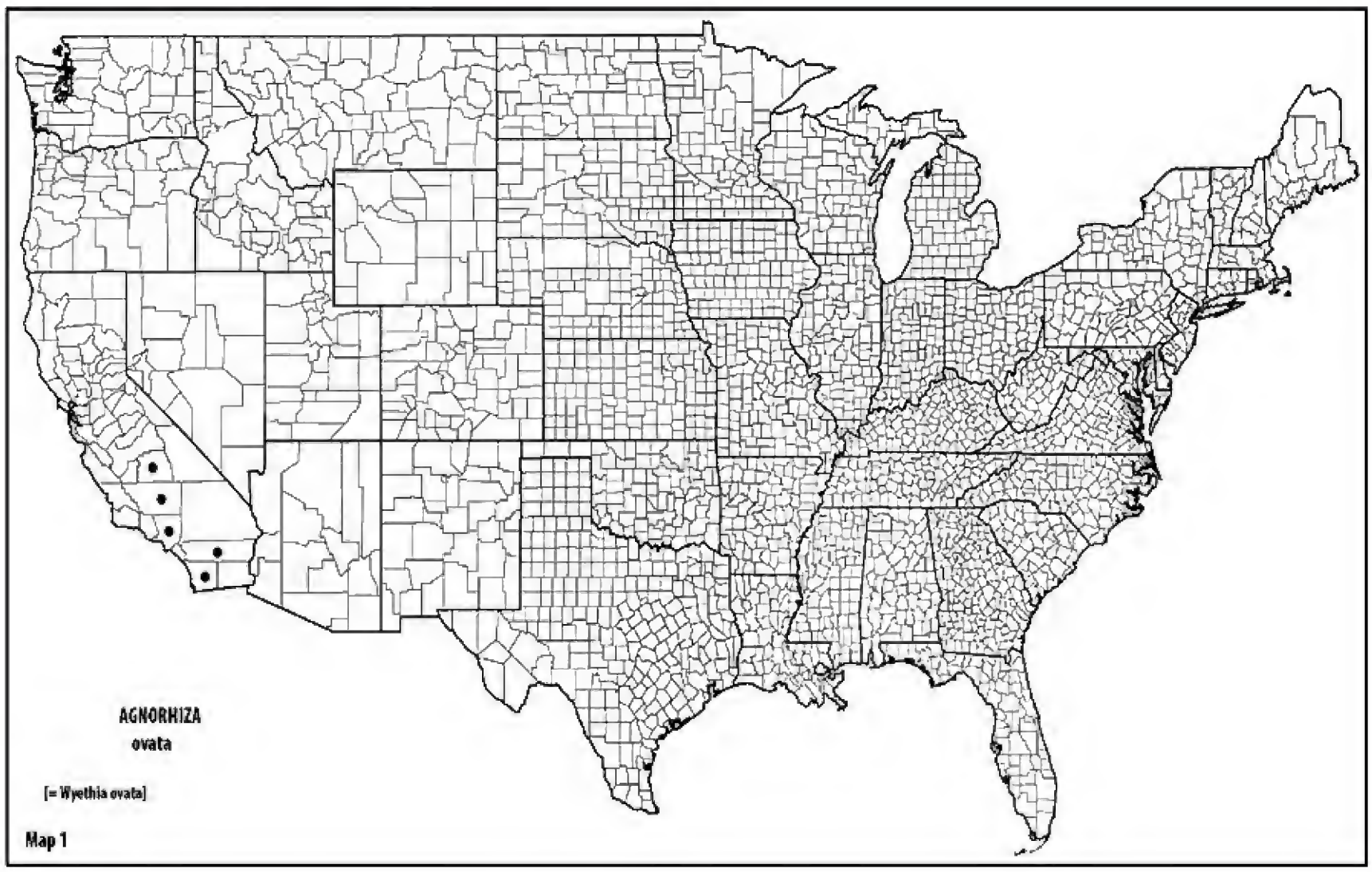
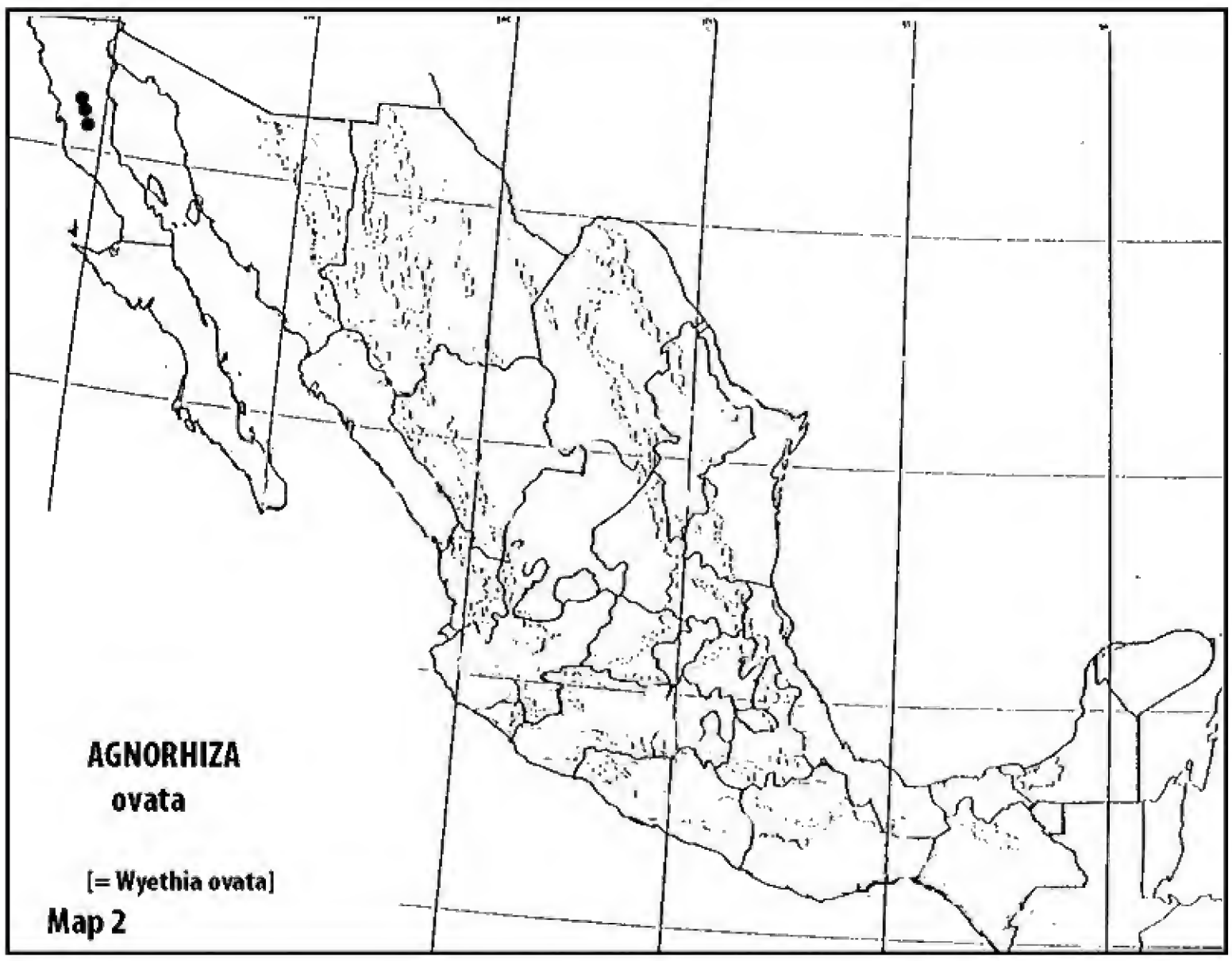


AGNORHIZA
ovata

[= Wyethia ovata]

Map 1



AGNORHIZA
ovata

[= Wyethia ovata]

Map 2

Ontogenetic variation in pentane extractable hydrocarbons from *Helianthus annuus*

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ABSTRACT

Yields of total hydrocarbons (HC) from leaves of *Helianthus annuus* cv. Little Becka and cv. Firecracker reached their maximum for both % yields and g HC/ plant at first flowering (stage R 5.1). Hydrocarbon yields, as % yields and as g/ plant, showed nearly identical patterns with ontogeny. Yields of HC (g/ plant) from leaves plus seed heads reached a maximum with seed filling at R 6 stage (rays wilted). Biomass of leaves, stems and seed heads were examined and total biomass was found to reach a maximum at stage R 6 (rays wilted), with a slight decline in biomass at stage R 8 (seeds filled, head nodding). The optimum time to sample leaves for hydrocarbons is at first flowering (R 5.1), when leaf hydrocarbons are at a maximum. Published on-line www.phytologia.org *Phytologia 98(4): 290-297 (Oct 6, 2016)*. ISSN 030319430.

KEY WORDS: *Helianthus annuus,* Sunflower, ontogenetic variation in leaf hydrocarbon storage.

Due to the uncertain crude oil production in the United States, there is a renewed interest in sustainable, renewable resources fuel and petrochemicals. Sunflowers have been developed as an important crop, primarily for their oil and edible seeds (Heiser et al. 1969). The triglycerides have been used as bio-diesel (Hoffman et al 1980; Morgan and Shultz, 1981).

Adams and Seiler (1984) surveyed 39 taxa of sunflowers for their cyclohexane (hydrocarbon) and methanol (resins) concentrations. The highest cyclohexane (bio-crude) yielding taxa were *H. agrestis*, an annual, Bradenton, FL (7.38%) and *H. annuus*, Winton, OK (7.09%). Adams et al. (1986) screened 614 taxa from the western US for their hydrocarbon (hexane soluble) and resin (methanol soluble) yields. They reported 2 plants of *H. annuus* from Idaho with 8.71% and 9.39% hydrocarbon yields.

Seiler, Carr and Bagby (1991) reported on 28 *Helianthus* taxa for their yields of oil, polyphenols, protein and rubber. The rubber was found to be of lower molecular weight than *Hevea* rubber, but still appeared to be useful as a plasticizing additive and for coatings inside pipes and containers.

Pearson et al. (2010a) demonstrated that Accelerated Solvent Extraction (ASE) could be utilized for the quantification of natural rubber in sunflower. Agronomic and rubber characteristics were reported for *H. annuus* by Pearson et al. (2010b). They reported from 0.9% to 1.7% rubber in sunflower cultivars (Fig. 4, Pearson et al. 2010b).

There does not appear to be any information on the production of hydrocarbons during ontogenetic development of *H. annuus*. The purpose of this report is to present new information on ontogenetic variation of the yields of pentane extractable hydrocarbons in four sunflower cultivars. The major focus of this research was to determine a stage of development when yields of hydrocarbons are at or near their maximum so plant collections could be taken at comparable growth stages across geographic regions.

MATERIALS AND METHODS

Seed was obtained from nursery seed dealers for 4 cultivars: Firecracker, Hopi, Little Becka and Sunrich Orange. Seeds were germinated in 1" tubes, then seedlings were transplanted (at 2" tall) into 6" plastic pots using a commercial potting soil (Berger BM 7). Plants were watered as needed to avoid wilting. No fertilizer or pesticide spray was added. Firecracker and Little Becka were grown in the greenhouse at Oklahoma Panhandle State University (OPSU) under ambient sunlight with 50% shade screen (spring, 2016). Hopi and Sunrich Orange were grown in the Baylor greenhouse at Oslo, TX under ambient light with 50% shade screen. Six plants were randomly selected for analysis at 5 growth stages (see Schnetter and Miller, 1981 for a description of stages): 1. 1st flower bud mature but not opened (R 3, see Fig. 1); 2. 1st flower opened with mature rays (R 5.1); 3. head nearly (90%) filled with disk flowers (R 5.9); 4. rays wilted on 1st flower (R 6); 5: terminal head yellow and nodding (R 8) (seeds filled).

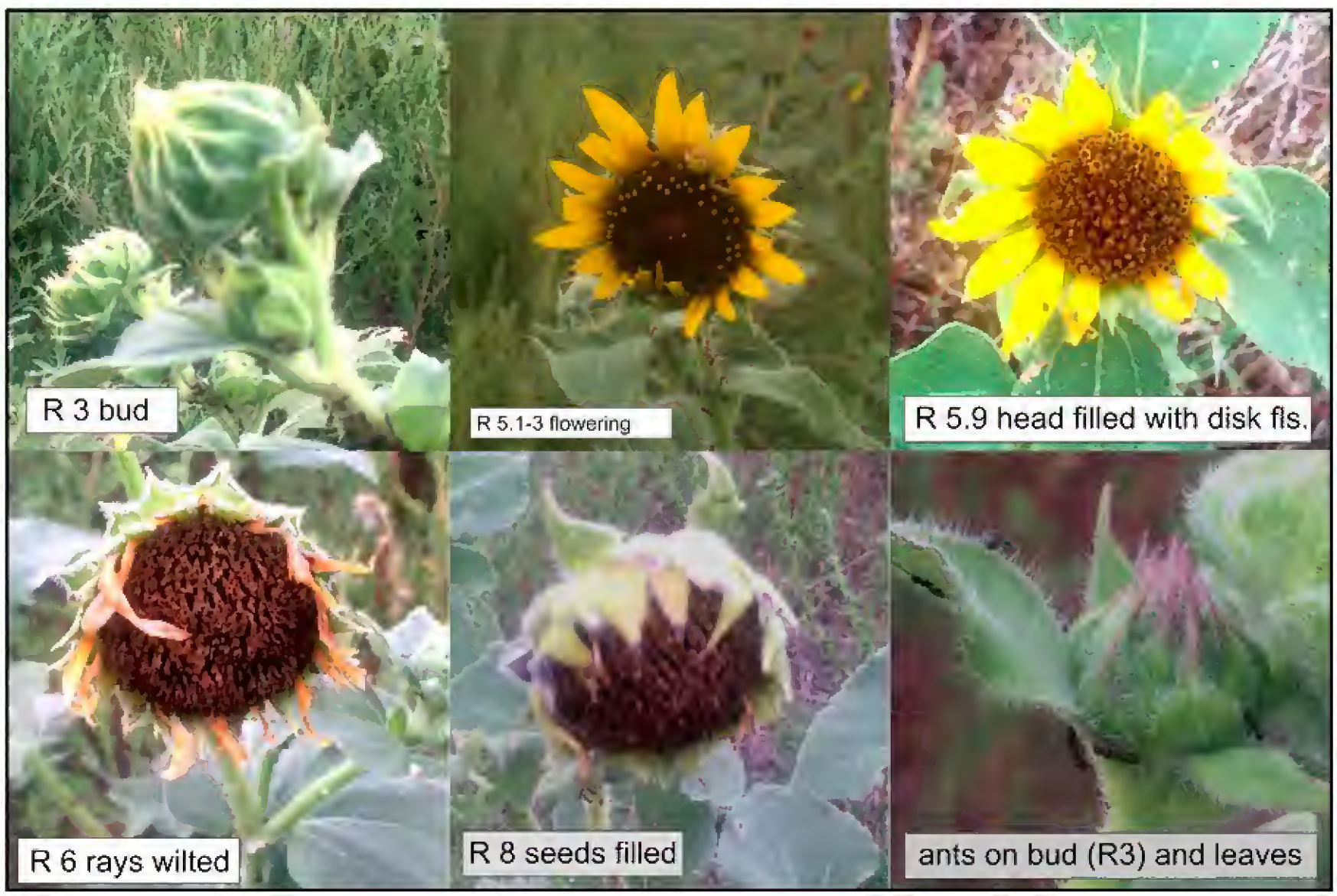

Figure 1. Sampling times at growth stages of wild (*H. annuus*) sunflowers, Gruver, TX. Note black ants on the bud and leaves in lower right photo.

The six plants were divided into leaves, stems, and heads and each part dried (24 h, 45-50° C) then weighed. Leaves and heads were ground in a coffee mill (1mm). 3 g of air dried

material (7% moisture) was placed in a 125 ml, screw cap jar with 20 ml pentane, the jar sealed, then placed on an orbital shaker for 18 hr. The pentane soluble extract was decanted through a Whatman paper filter into a pre-weighed aluminum pan and the pentane evaporated on a hot plate (50°C) in a hood. The pan with hydrocarbon extract was weighed and tared.

RESULTS

Tables 1 and 2 contain growth and yields for Little Becka and Firecracker cultivars. Notice (Fig. 2, upper graph) that both cultivars show a maximum % yields of HC from leaves at the first flower stage (R 5.1) with a decline in % yield as the disk flowers develop (R 5.9). The yield increased (Fig. 2) in Little Becka at R 6 (ray flowers wilted), then declined with the filling of seeds (R 8).

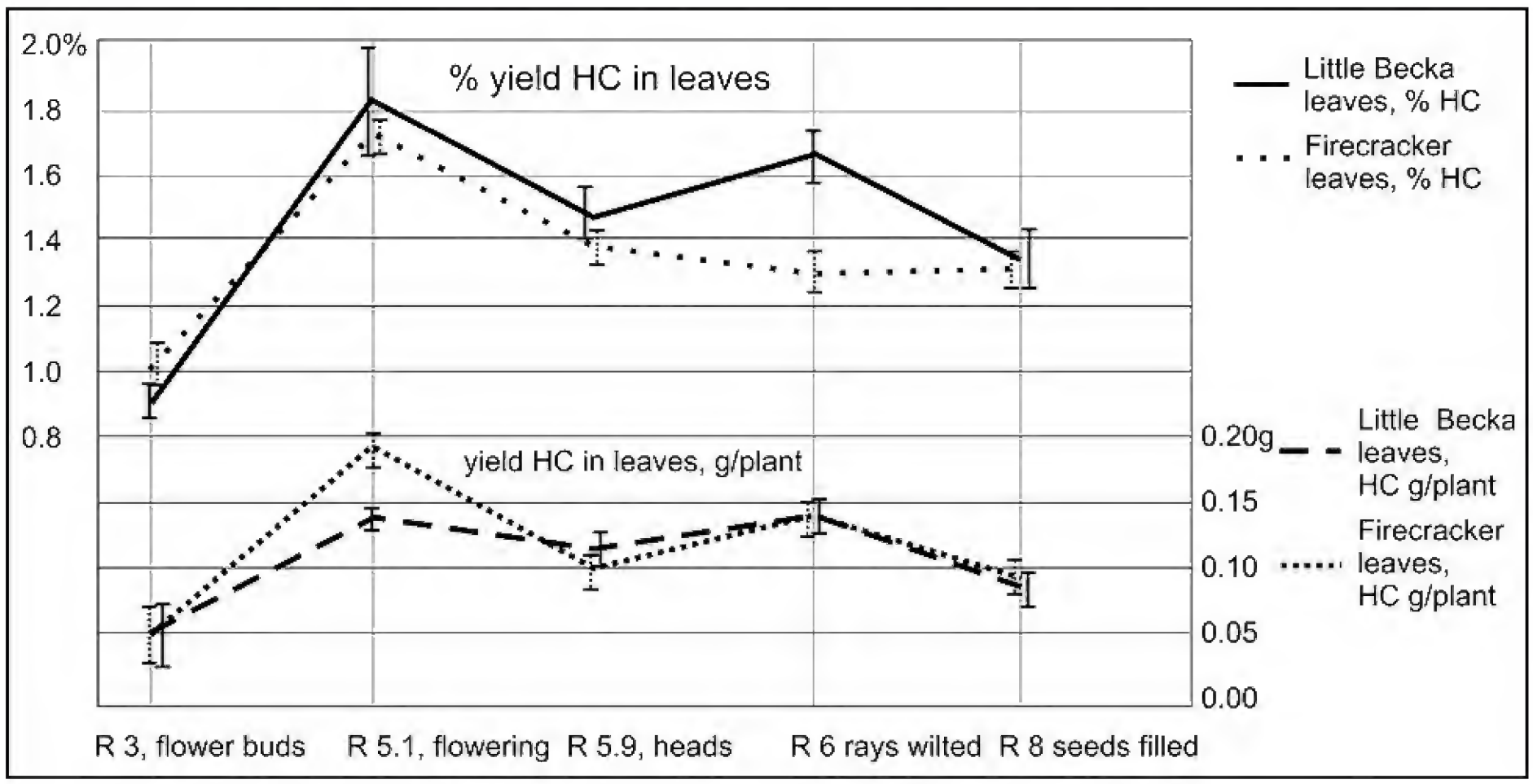

Figure 2. Comparison of hydrocarbon (HC) % yields (upper) between Little Becka and Firecracker cv. Bars are standard error the mean.

The pattern for total HC yields (g/plant) from leaves mirrored the % yield pattern with the g/ plant yields maximized at stage R 5.1 (Fig. 1, Tables 1, 2). A slight, non-significant, rise in HC is suggested at stage R 6 (Fig. 1).

Examination of total g of HC from stems was not a focus of this study. However, we did find that for Firecracker, at stage R 3, the % HC yield from leaves was 1.11% vs. 0.51% from stems. Of course in a farming operation, stems and leaves would be swathed and baled together for processing. As it is far simpler to collect, dry and grind only leaves, that will be the focus in future studies in screening wild sunflower plants.

Yields of HC (g/ plant) from leaves plus seed heads reached a maximum (Fig. 2) with seed filling at R 6 stage (rays wilted). The first sampling of seed heads (R 5.1, first flower opening) is before any seeds are formed. The rather large amounts of HC (Fig. 3, Table 1) of 2.92% in heads vs. 1.81% from leaves seems to be due to large amounts of resin in the flower head bracts. In fact, resin is often excreted and appears to attract small black (sugar) ants (Fig. 1, lower right). The resin is also on the stem and at the base of the leaf blades where black ants

congregate. The increase in seed head HC at stage R 6 may be largely due to the synthesis of triglycerides (fats) in the maturing sunflower seeds (stage R 6, Fig. 3, Table 1).

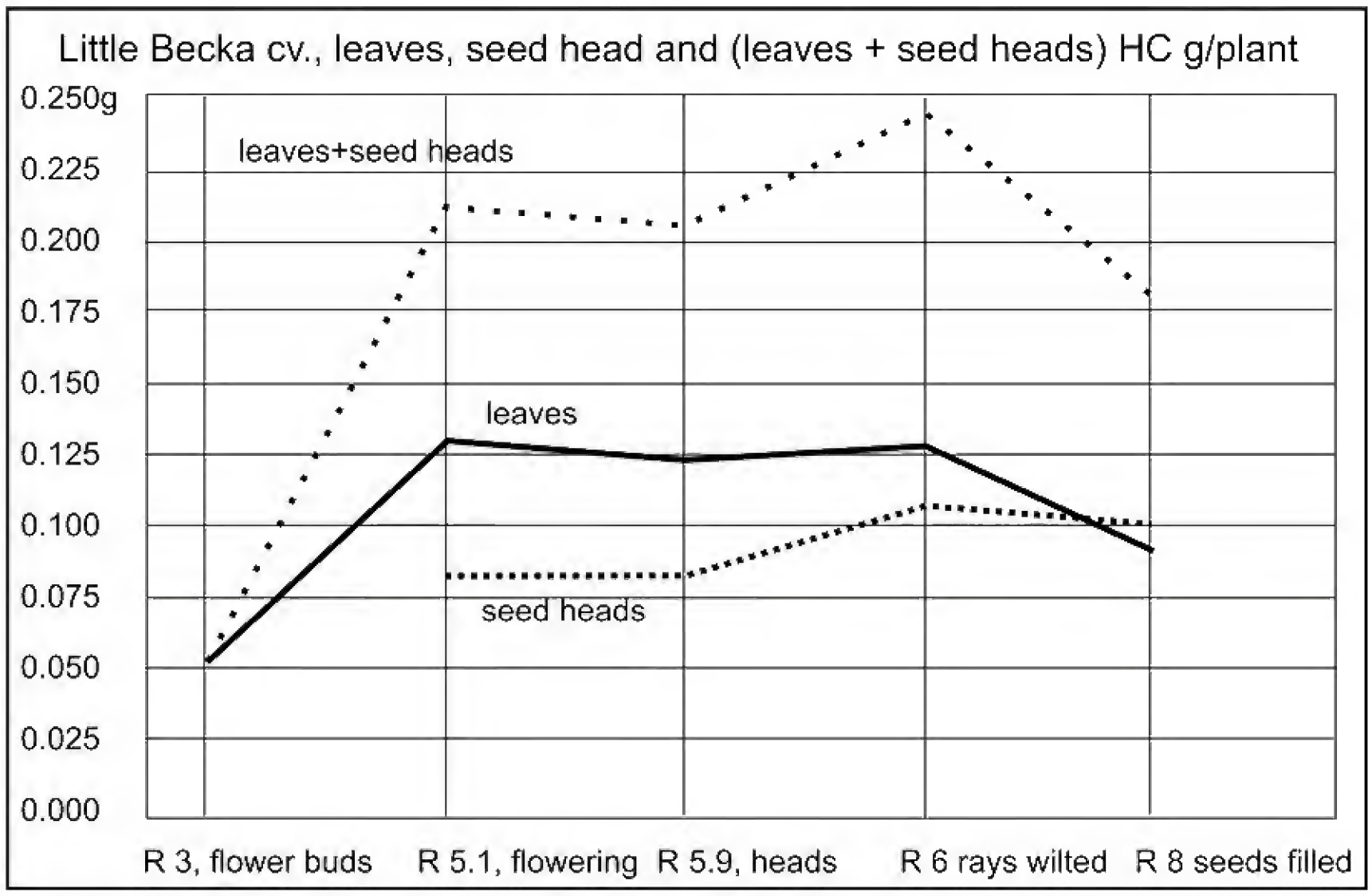

Figure 3. Distribution of HC (g/plant) in leaves, seed heads and leaves + seed heads in Little Becka during the growing season.

The growth and HC yields for Sunrich Orange and Hopi cv. are given in Tables 3, 4. Both of experiments were flawed due to lower ambient light levels that caused the plants to elongated their leaf internodes. This may have affected the levels of HC in the leaves and heads.

Partitioning of biomass between leaves, stems and seed heads for Little Becka and Firecracker is shown in Figs. 4 and 5. Both cultivars show similar patterns, in that leaf biomass declines when flowering commences, stem biomass remains relative stable, and seed head biomass increases as seeds are being filled. For Little Becka, leaf, stem and seed head biomass are about equal at stage 8 (1/3 each, table 1, Fig. 4.). However, for Firecracker at stage 8, seed head biomass is 46.2%, compared to just 20.2% for leaves (Table 2, Fig. 5). It is interesting that the mass (g wt./plant) of stems and leaves both actually decline as the seeds fill (Tables 1, 2). This seems likely due to the transport of sugars and other metabolites from leaves and the stem to the seed heads.

Because Sunrich Orange and Hopi produced elongated stems under sub-optimum light, the biomass of the stem is often greater than the leaf biomass (Tables 3, 4). Notice (Table 3) at stage 8, Sunrich Orange has allocated biomass as follows: leaves, 22.2%; stems, 31.2%; and seed heads, 46.4%. In contrast, Hopi (Table 4) allocated: leaves, 13.3%; stems, 59.8%; and seed heads, 26.9%.

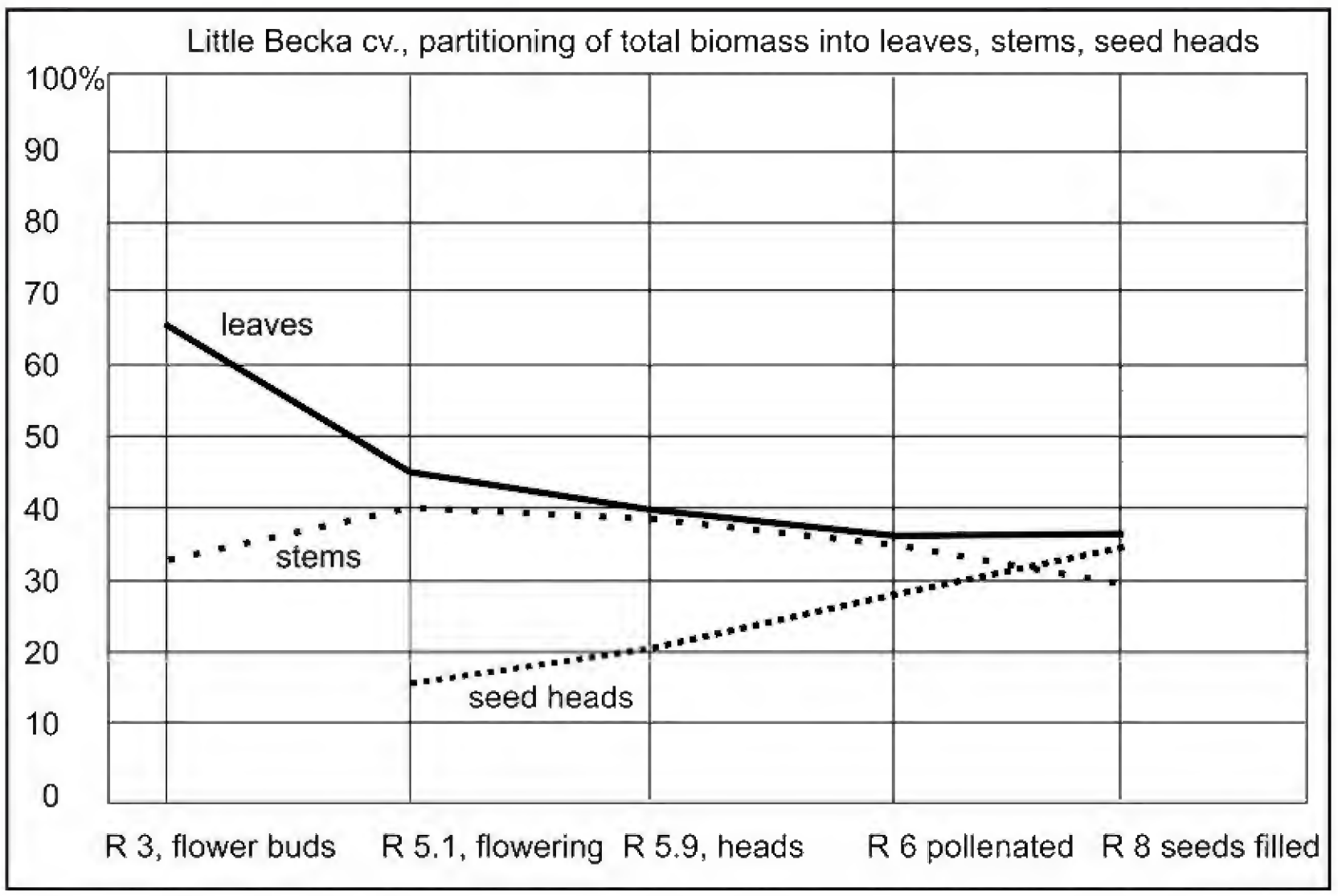

Figure 4. Partitioning biomass among leaves, stems and seed heads during the growing season for Little Becka.

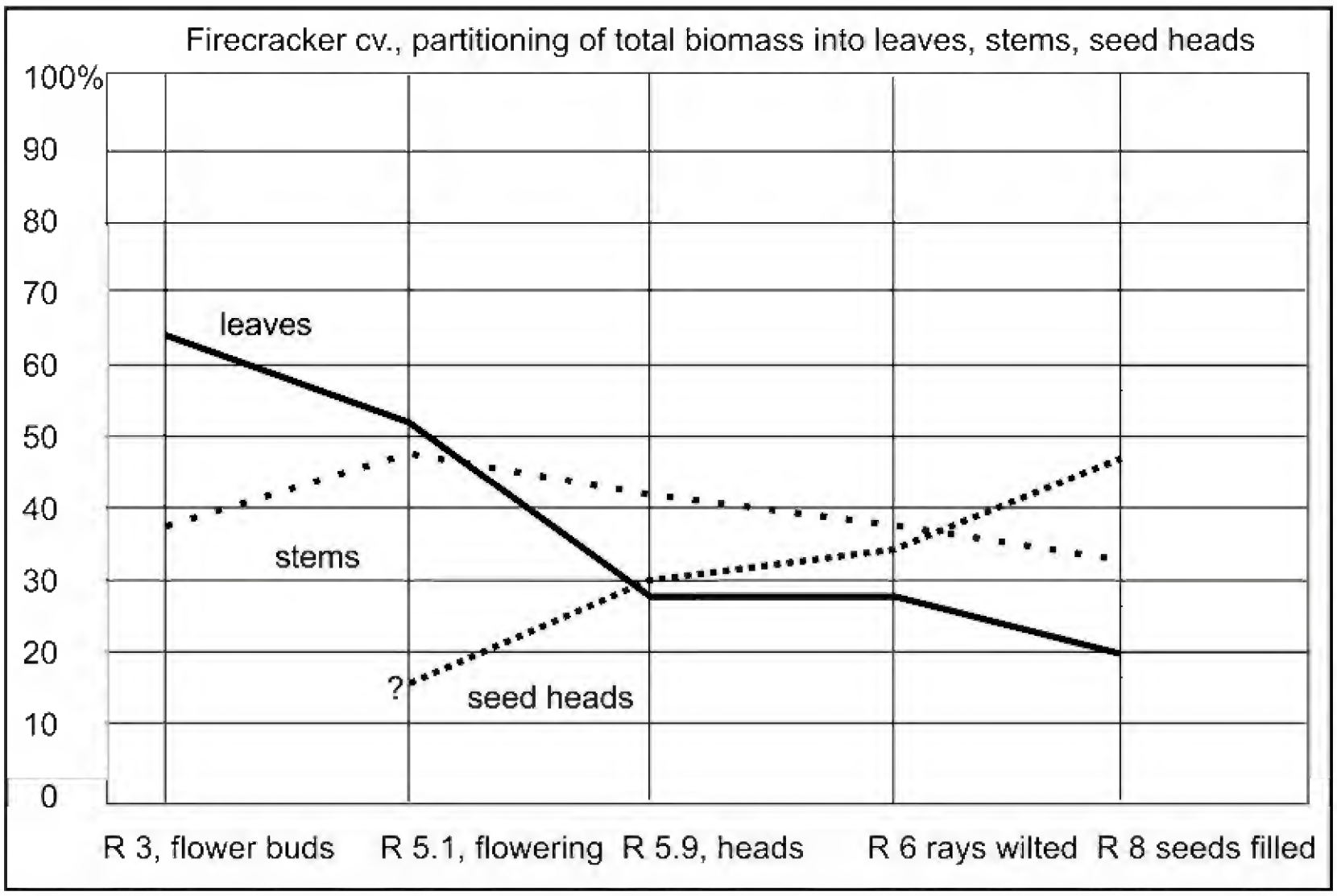

Figure 5. Partitioning biomass among leaves, stems and seed heads during the growing season for Little Becka.

The total biomass varied by stage and among cultivars (Tables 1-4, Fig. 6). Firecracker had the largest changes in biomass, reaching a maximum at R 6, then declining at R 8. This decline in biomass appears mostly due to the decrease in biomass for both leaves and stems. In sunflowers, lower leaves turn yellow as the seeds are filled in the heads. Little Becka and Sunrich Orange show similar patterns to Firecracker by having their peak biomass at R 6, then decline in total biomass at R 8 (Fig. 6). Hopi displayed a slightly different pattern (Fig. 6), increasing from R 6 to R 8 (Fig. 6).

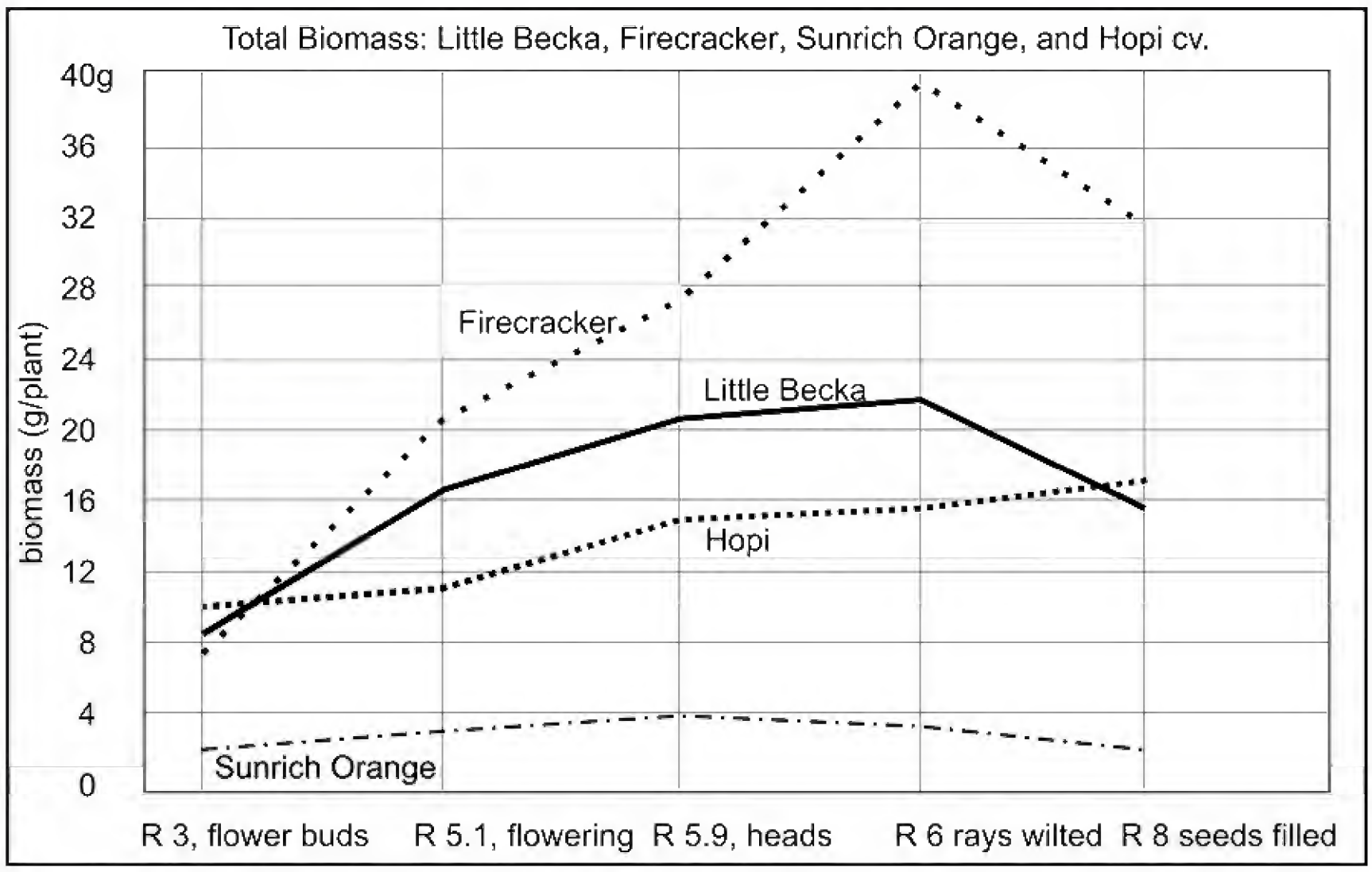

Figure 6. Variation in total biomass with plant maturity and among cultivars.

CONCLUSION

The primary focus of this study was to determine the optimum time to collect leaves to maximize HC yields. Yields of total hydrocarbons (HC) from leaves of *Helianthus annuus* cv. Little Becka and cv. Firecracker reached their maximum for both % yields and g HC / plant at first flowering (stage R 5.1). Hydrocarbon yields from leaves (as g/ plant) showed a very similar trend. Yields of HC (g/ plant) from leaves plus seed heads reached a maximum seed filling at R 6 stage (rays wilted). Total biomass of leaves, stems and seed heads was examined and biomass was found to reach a maximum at stage R 6 (rays wilted), with a slight decline in biomass at stage R 8 (seeds filled, head nodding). The optimum time to sample leaves is at the first flowering on a plant (R 5.1), when leaf hydrocarbons are at a maximum.

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Table 1. Growth, biomass distribution and HC yields for Little Becka cv. Biomass is based on all leaves or seed heads from each of six plants.

Little Becka growth stage	biomass leaves wt. (% total)	biomass stem(s) (% total)	biomass heads (% total)	total biomass g/plant	% yield, pentane shaker leaves	% yield pentane shaker flowers/ heads	HC yield ex total leaves/ plant	HC yield ex total heads/ plant	HC yield lvs + heads/ plant
R 3 bud	5.94g (66.2%)	3.02g (33.8%)	na	8.96	0.88	na	0.052g	na	0.052g
R 5.1-5.3	7.46g (43.6%)	6.90g (40.3%)	2.76 (16.1%)	17.12	1.81	2.92	0.135g	0.081g	0.216g
R 5.9	8.66 (40.2%)	8.43 (39.1%)	4.46 (20.7%)	21.55	1.43	1.89	0.123g	0.084g	0.207g
R 6	8.16 (36.9%)	7.96 (36.1%)	5.98 (27.01%)	22.10	1.64	1.86	0.134g	0.111g	0.245g
R 8	5.83 (37.1%)	4.65 (29.5%)	5.26 (33.4%)	15.74	1.35	1.94	0.079g	0.102g	0.181g

Table 2. Growth, biomass distribution and HC yields for Firecracker cv. Based on 6 plants. HC yield per plant from leaves vs. stems was 0.051g vs. 0.014g (3.6:1).

Fire-cracker growth stage	biomass leaves wt. (% total)	biomass stem(s) (% total)	biomass heads (% total)	total biomass g/plant	% yield, pentane shaker leaves	% yield pentane shaker heads	HC yield ex total leaves/ plant	HC yield ex total heads/ plant	HC yield lvs + heads/ plant
R 3 bud	4.63g (62.4%)	2.78g (37.6%)	na	7.41	1.11	na	0.051g	na	0.051g
R 5.1-5.3	11.05* (52.1%)	10.18 (47.9%)	na	21.23	1.73*	na	0.191g	na	0.191g
R 5.9	7.49 (27.0%)	11.86 (42.8%)	8.38 (30.2%)	27.73	1.38	2.01	0.103g	0.168g	0.271g
R 6	10.66 (27.2%)	15.15 (38.6%)	13.43 (34.2%)	39.24	1.27	1.47	0.135g	0.197g	0.232g
R 8	6.37 (20.2%)	10.58 (33.6%	14.57 (46.2%)	31.52	1.32	1.78	0.084g	0.260g	0.344g

*with flowering heads included.

Table 3. Growth, biomass distribution and HC yields for Sunrich Orange cv. Based on 6 plants. Plants with elongated stems due to low light in greenhouse.

Sunrich Orange growth stage	biomass leaves wt. (% total)	biomass stem(s) (% total)	biomass heads (% total)	total biomass g/plant	% yield, pentane shaker leaves	% yield pentane shaker heads	HC yield ex total leaves/ plant	HC yield ex total heads/ plant	HC yield lvs + heads/ plant
R 3 bud	1.20g (45.1%)	1.46g (54.9%)	na	2.66	1.77	na	0.021g	na	0.021g
R 5.1-5.3	1.66* (51.7%)	1.55 (48.3%)	na	3.21	1.97*	na	0.033g*	na	0.033g
R 5.9	2.51* (60.3%)	1.65 (39.7%)	na	4.16	1.56*	na	0.039g*	na	0.039g
R 6	0.74 (19.5%)	1.45 (38.2%)	1.61 (42.3%)	3.80	1.77	1.80	0.013g	0.029g	0.042g
R 8	0.59 (22.4%)	0.82 (31.2%)	1.22 (46.4%)	2.63	2.01	2.14	0.012g	0.026g	0.038g

*with flowering heads included.

Table 4. Growth, biomass distribution and HC yields for Hopi cv. Based on 6 plants. Plants with very elongated stems due to low light in greenhouse.

Hopi growth stage	biomass leaves wt. (% total)	biomass stem(s) (% total)	biomass heads (% total)	total biomass g/plant	% yield, pentane shaker leaves*	% yield pentane shaker heads	HC yield ex total leaves*/ plant	HC yield ex total heads/ plant	HC yield lvs + heads/ plant
R 3 bud	2.80 (27.7%)	7.36 (73.3%)	na	10.16	1.73*	na	0.048g*	na	0.048g
R 5.1-5.3	1.91 (17.8%)	8.14 (75.9%)	0.68 (6.3%)	10.73	1.67*	na	0.043g*	na	0.043g
R 5.9	2.07 (14.1%)	11.37 (68.2%)	1.31 (8.8%)	14.75	1.56*	na	0.053g*	na	0.053g
R 6	2.58 (17.0%)	10.58 (69.5%)	2.07 (13.5%)	15.23	1.42*	na	0.066g*	na	0.066g
R 8	2.33 (13.3%)	10.49 (59.8%)	4.73 (26.9%)	17.55	3.51*	na	0.248g*	na	0.028g

*with flowering heads included.

Molecular and ultrastructural detection of plastids in *Juniperus* (Cupressaceae) pollen

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ABSTRACT

Transmission electron microscopy (TEM), PCR and sequencing studies can provide important evidence about the presence of plastids in pollen grains. Their presence is critical if one proposes to develop plastid primers for the amplification of DNA from pollen for species identification. Differential interference contrast microscopy (DIC) and TEM were used to investigate the presence of plastids in pollen from *Juniperus* species, a major cause of airborne allergies in North America. Standard PCR techniques were used to amplify DNA from the pollen using universal primers targeting known plastid DNA genes including the *rbc*L and species-specific primers targeting the *mat*K coding regions. Studies using TEM confirmed the presence of plastids in *Juniperus* pollen at three increasing stages of hydration. The *rbc*L and *mat*K genes were successfully amplified using DNA extracted from pollen of *J. ashei, J. pinchotii* and *J. virginiana*. The results open a wide range of possibilities for using plastid primers in general pollen research especially when the interspecific variation among the standard set of nuclear genes (for e.g., ITS-1, ITS-2) is minimal. TEM results coupled with PCR results confirmed that plastid primers can be used to amplify DNA from pollen of *Juniperus*. This can be used to study the role of plastid DNA in the quantification of airborne, allergenic pollen from various *Juniperus* sources.
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KEY WORDS: DNA, *Juniperus*, *mat*K, PCR, plastids, pollen, *rbc*L, TEM.

Juniperus (Cupressaceae) is regarded as a major source of airborne allergens due to its wide distribution and heavy pollen production (Pettyjohn and Levetin, 1997; Bunderson et al., 2012). The allergenicity of pollen from *J. ashei, J. pinchotii* and *J. occidentalis* are the most significant, while other species such as *J. virginiana, J. communis,* and *J. horizontalis* are only occasionally reported as allergenic (Lewis et al., 1983).

Light microscopy (LM) has shown that *Juniperus* pollen grains are spherical when hydrated (Nepi et al., 2005). The exine is thin with granules on the surface; the intine is thick; and the protoplast appears pentagonal or star-like (Kurmann, 1994). The grains appear inaperturate in the light microscope; however, a small pore is visible when the pollen grains are viewed with a scanning electron microscope (SEM) (Kurmann, 1994). Although there are some differences in size, pollen grains from various *Juniperus* species cannot be distinguished by LM. In fact, pollen grains produced by members of the Cupressaceae are considered morphologically uniform (Lewis et al., 1983; Kurmann, 1994).

Cupressaceae pollen structure

Although there have been no specific transmission electron microscope (TEM) studies of *Juniperus* pollen, the ultrastructure of two members of the Cupressaceae, *Cupressus sempervirens* and *Cryptomeria japonica*, has been studied (Kurmann, 1994; Suárez-Cervera et al., 2003). These studies have primarily focused on the pollen wall layers. The sporoderm of cupressacean pollen consists of a thin exine and thick intine that surrounds the protoplast (Suárez-Cervera et al., 2003; Chichiriccò and Pacini, 2008). The exine ranges from 0.3-0.9 µm in thickness (Kurmann, 1994). The outer layer of the exine, the ectexine, is made up of granules while the inner layer, the endexine, is more electron dense and lamellate, ranging up to 0.5 µm in thickness (Kurmann ,1994; Suárez-Cervera et al., 2003). Chichiricco and Pacini

(2008) described the detailed structure of the intine of *Cupressus arizonica*. The intine consists of three layers; the outermost layer is thin, homogeneous and rich in pectins. This outer intine layer is highly plastic and triples its diameter when hydrated. The middle layer is large, spongy and non-homogeneous. This layer is bordered by a mesh of large, branched fibrils and is rich in pectin. The innermost intine layer is considered to be the persistent wall of the sporoderm and consists of both cellulose and callose.

Pollen grains have storage reserves for use after pollen release and during pollen germination. These storage reserves can be oils, proteins and/or carbohydrates. Lipids are generally the main reserve for entomophilous pollen grains, while anemophilous pollen grains typically have starch as their main reserve (Stanley, 1971; Baker and Baker, 1979). Nonetheless, research has shown that the anemophilous pollen of the Cupressaceae contain large amounts of lipids and few starch grains (Owens, 1993).

Cupressaceae pollen has both aerodynamic and hydrodynamic properties. The aerodynamic properties are related to the small size of the pollen grains, which are readily dispersed by wind over long distances. The hydrodynamic properties are related to changes in the sporoderm that occur when pollen lands on the pollination drop produced by ovules (Tomlinson and Takaso, 1998; Fernando et al., 2005). Cupressaceae pollen swells at first contact with the pollination drop (Tomlinson and Takaso, 1998; Fernando et al., 2005). Experiments with *J. communis* indicated that there are various stages of hydration. At stage zero, the pollen was dehydrated, wrinkled and consisted of a star-like protoplast. In the next stage, hydration started in the intine, which increased its volume, and then continued to the protoplast. In the subsequent stage, the protoplast underwent complete hydration and became spherical. At this stage, the splitting and shedding of the exine was observed. During the final stage, the intine increased in volume, the protoplast increased in volume and the outer two layers of intine ruptured and were shed (Nepi et al., 2005). The mechanism of pollen rupturing—shedding the exine and parts of the intine when it comes in contact with the water, is thought to guarantee an easy entry of the pollen protoplast into the ovule (Ottley ,1909; Tomlinson and Takaso, 1998).

Airborne Cupressaceae pollen

Airborne pollen of the Cupressaceae is widely reported (qualitatively and quantitatively) by air sampling networks (Caiaffa et al., 1993; Pendell et al., 2008; Sabariego et al., 2012) and has been well documented in Oklahoma (Rogers and Levetin, 1998; Levetin and Van de Water, 2003). Several *Juniperus* species that occur in Oklahoma, Texas, and New Mexico have overlapping pollination periods. Pollen season start times show considerable year-to-year variability based on local meteorological conditions. The pollination season of *J. pinchotii* starts in late September and lasts through November (Levetin et al., 2012; Adams, 2014). *Juniperus ashei* can start releasing pollen as early as late November and continue through early February (Adams, 2014). *Juniperus virginiana* starts pollinating in early February in Tulsa and continues through April (Levetin ,1998; Adams, 2014). Even though Cupressaceae pollen is detected by air sampling during these overlapping periods, pollen from different *Juniperus* species cannot be distinguished microscopically (Lewis et al., 1983). In contrast to microscopic analysis, some molecular approaches offer opportunities to identify pollen at the species-level.

Molecular markers

Nuclear markers have been previously used in molecular studies of pollen grains (Zhou et al., 2007; Longhi et al., 2009). In contrast, the use of chloroplast markers for pollen studies has been limited. In the case of *Juniperus* species, numerous nuclear genes are available in the NCBI database, but interspecific variation is limited (unpublished observations). The low discrimination at the nucleotide level among the nuclear genes precludes them as potential sources for species-specific primers. Although the number of published chloroplast genes is limited, some variation at the interspecific level is present (unpublished observation).

Phylogenetic investigations of plastid genes have identified seven commonly used markers. Among these seven markers, four are parts of coding genes (*mat*K, *rbc*L, *rpo*B, and *rpo*C1) and three are noncoding spacers (*atp*F-*atp*H, *trn*H-*psb*A, and *psb*K-*psb*I) (CBOLPlantWorkingGroup et al., 2009). The *mat*K gene is approximately 1500 base pairs (bp) long and is situated within the intron of the chloroplast gene *trn*K (Hilu and Liang, 1997). The *mat*K gene is the most rapidly evolving of all plastid coding regions (CBOLPlantWorkingGroup et al., 2009). The *mat*K gene is the only chloroplast-encoded group II intron maturase whose function is to regulate plant development. Expression analysis of the *mat*K gene reveals that "genetic buffers" are in operation, which compel its evolution and thus, low intraspecific variation is coupled with high interspecific differences (Lahaye et al., 2008). The *mat*K gene has high discriminatory power when used in phylogenetic analyses (CBOLPlantWorkingGroup et al., 2009; Hollingsworth et al., 2011). The best characterized gene among the plastid regions is the *rbc*L gene (CBOLPlantWorkingGroup et al., 2009). The *rbc*L gene codes for the large subunit of ribulose-1, 5-biphosphate carboxylase (Chase et al., 1993). While *rbc*L has only modest discriminatory power in phylogenetic analyses when compared to *mat*K (CBOLPlantWorkingGroup et al., 2009), it is convenient to amplify, sequence, and align in most of the land plants. The primers for *rbc*L are universal for virtually all land plants (CBOLPlantWorkingGroup et al., 2009; Hollingsworth et al., 2011).

The goals of the current investigation are to confirm the existence of plastids in *Juniperus* pollen through the use of TEM and to determine if plastid-specific primers can be used to amplify pollen DNA. A new toolbox for the differentiation of *Juniperus* plastid genes has the potential to provide a more effective means of identifying and quantifying pollen that causes widespread hay fever.

MATERIALS AND METHODS

LM and TEM of Juniperus pollen

Samples of *J. ashei* pollen were collected from Lampasas, Texas in January 2011, *J. pinchotii* pollen was collected from Sonora, Texas in October 2013 and *J. virginiana* pollen was collected in Tulsa, Oklahoma in March 2014.

Four samples for each pollen species were prepared for LM with differential interference contrast optics (DIC). The pollen from the first sample was not hydrated; whereas, the second and third samples were hydrated in water for one hour and 24 hours, respectively. Following hydration, the pollen was observed using DIC. The fourth pollen sample from each *Juniperus* species was stained with IKI (1%) solution and then observed using DIC for detection of starch grains in pollen.

For TEM studies, three small aliquots of pollen were prepared for each of the species. The first aliquot contained pollen without rehydration, the second aliquot was hydrated in deionized (DI) water for one hour to stimulate exine rupture, and the third aliquot was hydrated in DI water for 24 to 48 hours. The second and third aliquots were centrifuged at 16,004 g for 10 minutes and the supernatant was discarded. The pellet, containing pollen with a small amount of water, was pipetted and suspended in mini-petri dishes, containing 2% water agar (2g agar per 100 ml water) in the molten state. Similarly, the first aliquot of dry pollen was directly added to the liquid agar. After the agar solidified, a small block of agar containing pollen was cut from each petri dish. These blocks were fixed overnight in 5% glutaraldehyde. The following day, the blocks were washed with 0.1 M sodium phosphate buffer (pH 6.8) (a mixture of sodium phosphate monobasic (0.7 g) and sodium phosphate dibasic (1.31 g) added to 100 ml of water) three times at ten minutes per wash. The pollen blocks were post-fixed for one hour at room temperature with 2% osmium tetroxide dissolved in 0.1 M sodium phosphate buffer. The post-fixation was followed by the wash and dehydration steps with ascending concentrations of ethanol, starting from 25% to 100% for 10 min for each, followed by three steps with 100% acetone. The infiltration step was performed with Spurr's epoxy resin (Spurr, 1969). The following day, the blocks were embedded in 100% Spurr's epoxy resin in flexible silicone rubber molds. To cure the resin, polymerization was performed for 3 hours at 70° C. After curing, the blocks were trimmed with a razor blade and thin sections were cut

using an ultramicrotome and a diamond knife. Copper grids were used to collect the ultrathin sections, which were stained using lead citrate and uranyl acetate and observed using a Hitachi H7000 TEM.

Extraction and amplification of Juniperus pollen DNA

Samples of *J. ashei, J. pinchotii* and *J. virginiana* pollen weighing 0.1 mg were placed in 2 ml screw-capped tubes. One millimeter glass beads corresponding to approximately 700 µl of volume were placed in each tube containing the pollen grains and 500 µl of Fawley's extraction buffer (Fawley et al., 2004) (1 M NaCl, 70 mM Tris, 30 mM Na_2EDTA, pH 8.6), 15 µl of 10 % CTAB extraction buffer and 10 µl of β-mercaptoethanol was added to each tube. This was followed by bead-beating of the samples in a mini bead-beater (Biospec Products, Bartlesville, OK USA) for 3 min and incubation at 75° C for one hour. Incubation was followed by addition of 500 µl of chloroform and isoamyl alcohol (49:1) and centrifugation at 16,004 g for 20 minutes. The aqueous phase was removed and placed in a separate microfuge tube. An additional 500 µl of chloroform and isoamyl alcohol (49:1) was added to the tube with beads and centrifuged. The aqueous phase was added to the previous supernatant and an additional 500 µl of chloroform and isoamyl alcohol (49:1) was added and centrifuged at full speed for 20 minutes. Forty five microliters of 3 M sodium acetate and 900 µl of ice-cold, 100% ethanol were added to the aqueous phase. Samples were inverted and kept at -20° C for one hour. This was followed by centrifugation at 16,004 g for 15 min to pellet the nucleic acid which were washed with 70 % ethanol and dried by placing them in a 40° C water bath. Around 20-120 µl of RNAse free water was added to the dried pellet depending on the size of the DNA pellet.

The *rbcL* region of *Juniperus* pollen DNA was amplified by the PCR using two universal primers: *rbcL*F*Juniperus* and *rbcL*R*Juniperus* (Wolf et al., 1994; Pryer et al., 2001). Similarly, the primers, *asheimat*KF1 and *asheimat*KR1 (Table 1.) were designed to amplify the *mat*K region of *J. ashei* pollen DNA. Pollen DNA from *J. virginiana* was amplified using JV*mat*KF1 and JV*mat*KR1 (Table 1.). A total of 16 µl of PCR mix (final concentration: 10 mM of forward and reverse primer, 5 units of *Taq* polymerase, 10 mM dNTP, 25 mM $MgCl_2$, PCR buffer and water; Promega, Madison, WI) was added to each PCR tube. For both *rbc*L and *mat*K the following amplification protocols were implemented: an initial heating step of 5 min at 94° C, 36 repetitions of each of (1) a denaturation step of 1 min at 94° C, (2) an annealing step of 45s at 54° C and (3) an extension step of 1 min 10s at 72° C. All reactions were terminated with a final extension of 7 min at 72° C. Agarose gel electrophoresis (0.8% in TBE) was performed to verify the presence of suitable amplified products.

Sequence analysis

In order to verify the *mat*K sequence for *J. ashei, J. virginiana and J. pinchotii*, *mat*KJF3, *mat*KJR3, *mat*KJF4, *mat*KJR4 primers were designed (see Table 1.). Cycle-sequencing was performed with each of the primers and the amplicons were prepared for capillary sequencing using an ABI 3130xl Genetic Analyzer (Life Technologies, Grand Island, NY, USA). All sequence fragments were assembled by using Sequencher v 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA) followed by a nucleotide BLAST search with the query sequences.

RESULTS AND DISCUSSION

DIC and TEM of Pollen Grains

Three different stages of *Juniperus ashei* pollen were observed using DIC. Stage 1 characterized an intact pollen grain with exine, intine and a star-shaped protoplast (Figure 1A). In stage two, the exine was released leaving behind the protoplast with the intine. This transformation took place within an hour of hydration of the pollen. In the third stage, which occurred after 24 hours of hydration, the intine was also released. Stages two and three are evident in Figure 1B. Figure 1C depicts the pollen grain loaded with starch grains stained with IKI.

The DIC studies of *Juniperus* pollen were supplemented with the TEM studies, which provided more detailed images of the pollen. The wall of the intact pollen grain consisted of an outer exine, which is two layered including the ectexine and endexine, and a triple layer intine. The pollen grains contained amyloplasts bearing starch grains. Sections of intact pollen reveal the two-layered exine and three-layered intine (Figures 2A, B, C, D). A large nucleus, many lipid bodies without any membrane and a developing vacuole were also detected. The ectexine surface contains some orbicules (Figures 2A, B, C, D). The exine was shed from pollen that was hydrated for one or more hours. The middle layer of intine was swollen and the cytoplasm contained amyloplasts (Figures 3A, B, C, D). Cytoplasm from pollen which had lost both its exine and outer layers of intine after hydration for 24 hours showed several amyloplasts, a large nucleus and many lipid bodies (Figures 4A, B, C, D). Fig 5A and B reveals both amyloplasts and mitochondria in the *J. ashei* pollen after hydration for 24 hours.

PCR and DNA Sequencing

The *rbc*L gene was successfully amplified using DNA extracted from pollen of *J. ashei, J. pinchotii and J. virginiana* pollen DNA by using the *rbc*L primers (Figure 6A). Similarly, *mat*K species-specific primers were used to successfully amplify pollen DNA from *J. ashei* and *J. virginiana* (Figures 6B and 6C). The *mat*K primers for *J. pinchotii* were not species-specific and thus they amplified pollen DNA from all four species of *Juniperus*. Sequence analysis confirmed that the products of amplification for each set of primers corresponded to the targeted gene (KT698211, KT698212, KT698213, KT698214).

Though there are several reports in which LM and SEM were used to study Cupressaceae pollen (Duhoux ,1982; Kurmann, 1994; Chichiriccò and Pacini 2008; Danti et al., 2011), few of them used TEM. One previous TEM study focused on the morphology of *Cupressus sempervirens* pollen grains with emphasis on exine and intine changes during hydration (Kurmann 1994). Although no mention is made of plastids, one image appears to show starch grains (Figure 4, Kurmann, 1994). Similarly, Uehara and Sahashi (2000) present a TEM study of wall development in *Cryptomeria japonica* pollen. Again, starch grains are not mentioned but can be noted in several figures (Uehara and Sahashi, 2000). Suárez-Cervera et al (2003) used TEM of *Cupressus sempervirens* pollen grains for the localization of the pollen allergen. Although various organelles were labeled in the micrographs, no plastids or proplastids were either noted or visible in the published micrographs.

Here we report the presence of plastids and proplastids in all three stages of pollen hydration; these were visible in intact pollen with both exine and intine, pollen without the exine, and pollen without the exine and outer layers of the intine. Previous microscopical studies of *C. macrocarpa* pollen (Hidalgo et al., 2003) made no mention of starch grains. However, starch grains can be observed in the LM micrographs that recorded the early stages of microsporogenesis (see Figure 1f. in Hidalgo et al., 2003). Starch grains are no longer visible in mature *Cupressus* pollen (see Figure 1h in Hidalgo et al., 2003).

In most angiosperms chloroplasts are maternally inherited and plastid exclusion occurs at various stages including during first haploid mitosis, during sperm cell formation or development and during fertilization (Hagemann and Schröder, 1989). In gymnosperms like *Sequoia sempervirens*, chloroplast DNA was paternally inherited whereas, in *Cunninghamia konishii* (Cupressaceae), maternal inheritance of chloroplasts was seen and no paternal linkage was evident (Neale et al., 1989; Lu et al., 2001). Also, the F1 individuals from crosses between *Cunninghamia lanceolata* and *Cryptomeria fortunei* showed maternal inheritance of chloroplast DNA (Qi et al., 1998). Although it has been suggested that paternal inheritance of chloroplasts occurs in all gymnosperms (Neale and Sederoff ,1988; Neale and Sederoff, 1989; Reboud and Zeyl 1994), these studies indicate that maternal inheritance occurs in some members of the Cupressaceae. This emphasizes the importance of checking for the presence or absence of plastids in the pollen grains of other members of the Cupressaceae.

In our studies, we observed the contents of mature *J. ashei*, *J. pinchotii* and *J. virginiana* pollen grains when they came in contact with water. Expansion occurs mainly because of the intine swelling. The same expansion and exine rupture occurs when the mature pollen grain reaches a pollination drop and may facilitates rapid entry of pollen into the micropyle (Tomlinson and Takaso, 1998; Nepi et al., 2005). Our goal was to determine if hydration of the pollen grains was accompanied by changes to the microanatomy of the cell. About 10-16% of the pollen grains examined in this study showed at least one amyloplast as observed under TEM. We also determined that virtually all pollen grains had proplastids even when amyloplasts were not evident. This observation indicates that plastids are retained at least through the pollen hydration stage of gametophyte development. Further work is needed to determine subsequent stages of plastid inheritance in *Juniperus* species.

Primers from nuclear genes such as *needly*, *waxy*, *phantastica* and the internal transcribed spacer have been used as pollen markers (Zhou et al., 2007; Longhi et al., 2009) but the use of chloroplast primers is limited (Fumio et al., 2013). The preference for nuclear markers in pollen characterization may simply be a consequence of a large selection of nuclear genes that are available to the researcher. An alternative explanation for this limited use of plastid genes may be the uncertainty regarding the status of plastids in pollen grains. Regardless of why there is a dearth of pollen studies that use plastid genes, our initial results indicate that the use of chloroplast data from pollen for the study of plant dispersal, pollen dispersal (Mohanty et al., 2015) and diversity needs to be explored. Moreover, our results from preliminary experiments reveal that the plastid primers can be used in rapid quantification of *Juniperus* pollen as an alternative to the time-consuming and labor-intensive microscopy method (Mohanty et al., 2016).

Anemophilous pollen like that of *Juniperus* are light- weight and can travel long distances in large numbers if carried by wind (Levetin and Buck, 1986; Rogers and Levetin, 1998; Levetin and Van de Water, 2003; Bunderson et al., 2014). Population geneticists have exploited genetic evidence from pollen and seeds in the study of plant dispersal (Ennos, 1994; Jordano, 2010; Hampe et al., 2013). Confirmation of plastid gene diversity (Mohanty et al., 2015, 2016) in *Juniperus* pollen could be used to test hypotheses regarding introgression (Hall et al., 1961).

CONCLUSION

Our PCR results using chloroplast primers coupled with the TEM images of plastids in *Juniperus* pollen grains from three different species confirmed that chloroplast primers can be used for the amplification of DNA from pollen of *Juniperus*. The evidence of chloroplast DNA in pollen of *Juniperus* opens up new avenues for the study of *Juniperus* population dynamics (e.g., the invasive *J. virginiana*). Of more immediate impact is the role that plastid DNA will play in the quantification of airborne, allergenic pollen from various *Juniperus* sources.

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Table 1. List of *rbc*L and *mat*K primers used in PCR and sequencing.

Primer name	Primer sequences (5'-3')
*asheimat*KF1	ATCCAACAGGTTATTCTTG
*asheimat*KR1	TGGATTCTAATGATTTTGT
JV*mat*KF1	CGTAAACAGAATCAGAAT
JV*mat*KR1	GATTCTCTTTCTTTTGAAA
*mat*KJF3	GTTCTCCCTGTTCCTTT
*mat*KJR3	TCAAGACTGCATATCCT
*mat*KJF4	TCATCTGTTTCATTTTTGGC
*mat*KJR4	CTCTGTGAACGAGTTTTT
*rbc*LFjuniper	ATGTCACCACAAACAGAAACTAAAGCAAGT
*rbc*LRjuniper	TCACAAGCAGCAGCTAGTTCAGGACTC
*rbc*LBFjuniper	GCAAATACTTCGTTGGCTCA
*rbc*LARjuniper	TGAGCCAACGAAGTATTTGC

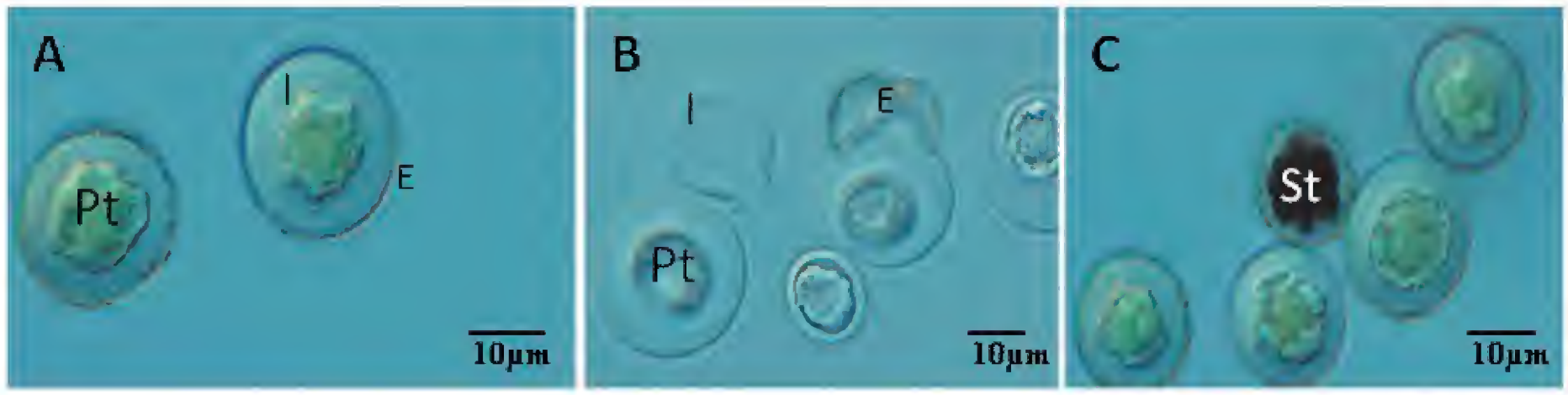

Figure 1. Differential interference contrast micrographs of *Juniperus* pollen (A-C); (A) Intact pollen grain of *Juniperus ashei* with exine, intine and the star-shaped protoplast. (B) Pollen grains of *Juniperus ashei* in which the exine was released leaving behind the intine with protoplast and one pollen grain in which exine and intine both were released leaving behind only protoplast. (C) Pollen grain of *Juniperus ashei* with numerous starch grains visible when stained with IKI solution.

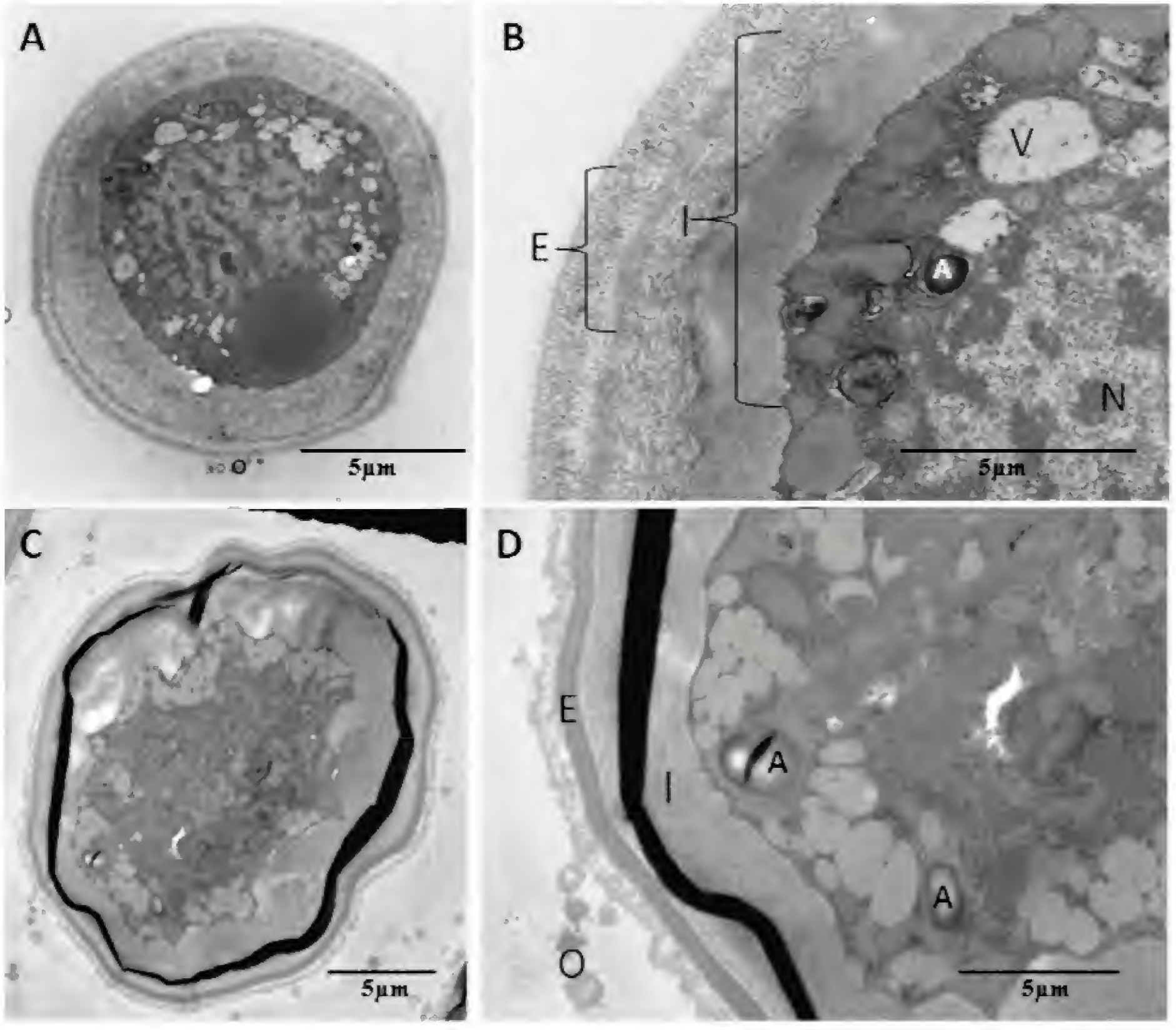

Figure 2. Transmission electron micrographs of intact pollen grains. (A-B) *Juniperus ashei* intact pollen grain and (C-D) *Juniperus pinchotii* pollen with nucleus, lipid bodies, amyloplasts with starch grain, and vacuole. Images show a double-layered exine, a triple-layered intine and orbicules on the exine surface.

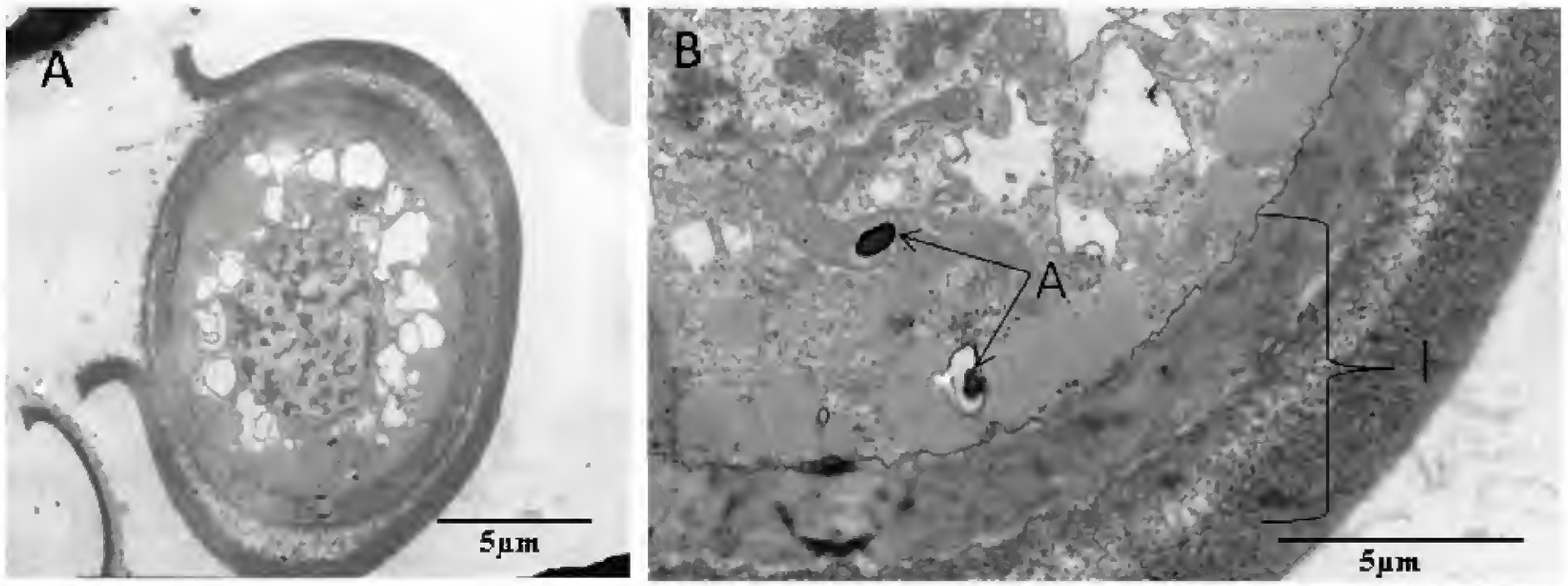

Figure 3. Transmission electron micrographs of hydrated pollen grains lacking exine. (A-B) *Juniperus virginiana* pollen grain and (C-D) *Juniperus pinchotii* pollen grain lacking exine. Amyloplasts with starch grain and triple-layered intine are evident.

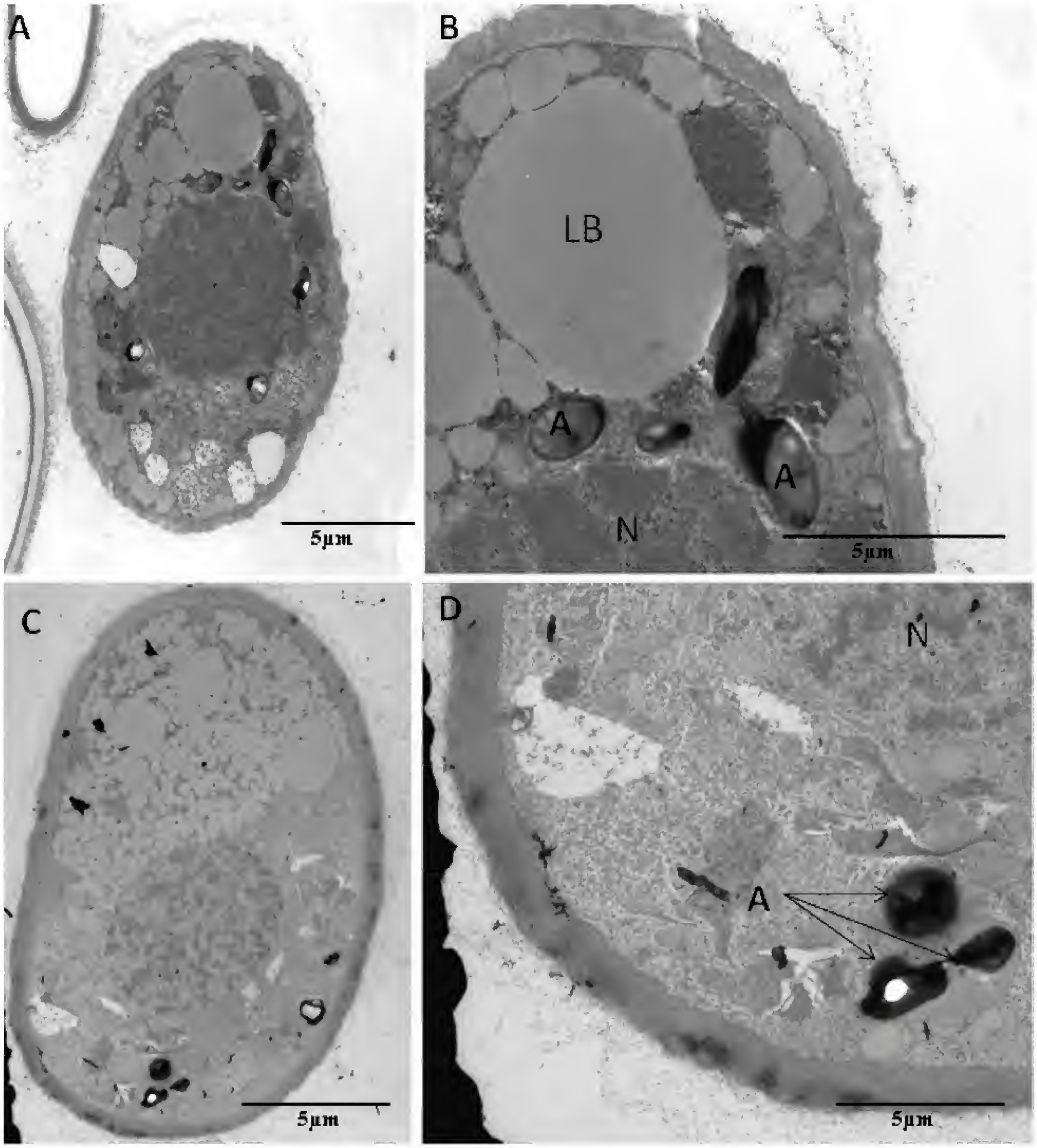

Figure 4. Transmission electron micrographs of pollen grains lacking exine and intine. (A-B) *Juniperus ashei* pollen grain and (C-D) *Juniperus pinchotii* pollen grain lacking exine and most of the intine. It represents nucleus, lipid bodies and amyloplasts with starch grains. A: amyloplasts, E: exine, GB: golgi bodies, I: intine, LB: lipid bodies, N: nucleus, O: orbicules, P: protoplast, V: vacuoles.

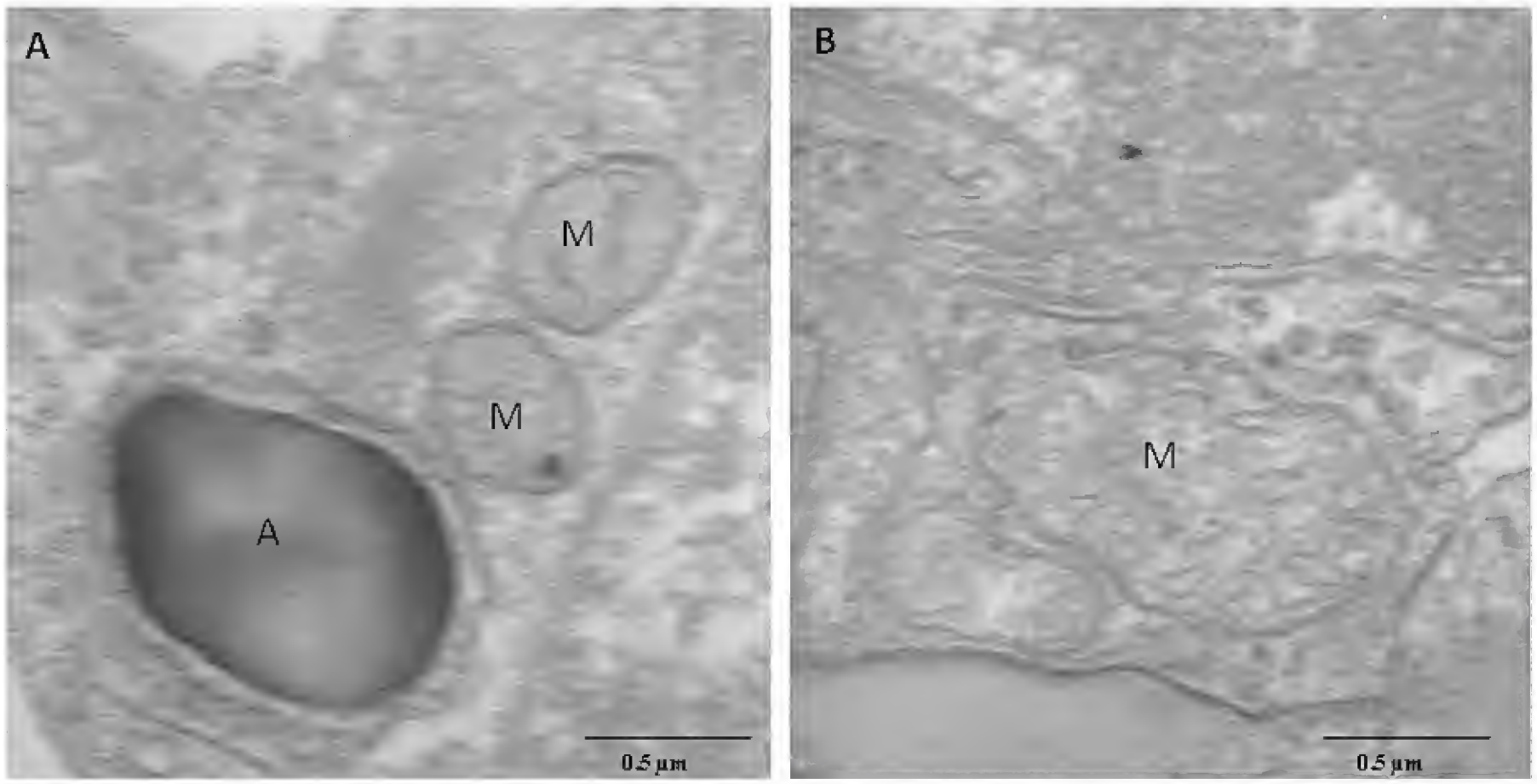

Figure 5. Transmission electron micrographs of *Juniperus ashei* pollen grains. (A-B) *Juniperus ashei* pollen grains with both amyloplasts and mitochondria. A: amyloplasts, M: mitochondria.

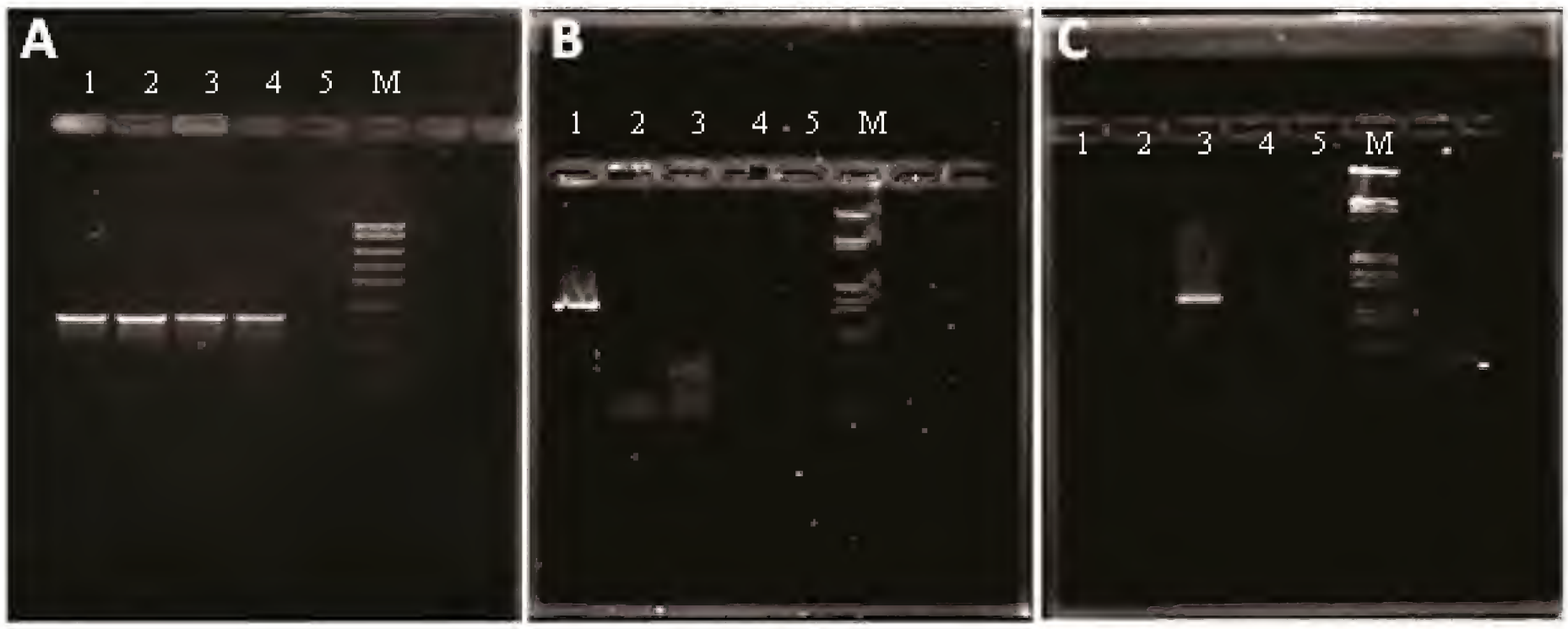

Figure 6. Agarose gel electrophoresis of amplified plastid genes. (A) *Juniperus ashei, J. pinchotii, J. virginiana* and a positive control pollen DNA amplified by *rbc*L primers (B) *Juniperus ashei* pollen DNA amplified by species- specific *mat*K primers. (C) *Juniperus virginiana* pollen DNA amplified by species-specific *mat*K primers. NOTE: Lane 1: *Juniperus ashei* pollen DNA, Lane 2: *Juniperus pinchotii* pollen DNA, Lane 3: *Juniperus virginiana* pollen DNA, Lane 4: Positive control in (A) and blank in (B) and (C), Lane 5: Negative control, Lane 6: λ-DNA ladder as marker (M).

Distribution of *Euploca confertifolia* (Boraginaceae), including it two varietal components

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ABSTRACT

Euploca confertifolia is treated as having two intergrading infraspecific taxa, var, *confertifolia* and var. *coldenioides*, the latter restricted to northeastern Mexico. Distribution maps are provided for both. Published on-line www.phytologia.org *Phytologia 98(4): 311-312 (Oct 6, 2016).* ISSN 030319430.

KEY WORDS: Boraginaceae, *Heliotropium, Euploca confertfolia*

Feuillet and Halse (2016), following the molecular studies of Hilger and Diane (2003), treated *Heliotropium* sect. Orthostachys as constituting the genus **Euploca**. I have accepted their treatment changing the names in My Atlas of Texas Plants (Turner et al. 2003) accordingly. The former workers did not account for infraspecific taxa of the species concerned, mainly because their studies were confined to taxa occurring in the USA.

While **Euploca confertiflora** is represented in Texas by the typical variety **confertifolia** (Map 1), in north-central Mexico it is largely allopatric with a populational system given the name *Heliotropium confertifolium* var. *coldenioides* by I.M. Johnston. This variety does not occur in the USA, but it is quite common in Mexico where it is closely allopatric with the typical var. **confertifolia**, as indicated in Map 2. The following combination will legitimize its existence:

Euploca confertifolia var. **coldenioides** (I.M. Johnston) B.L. Turner, **comb. nov.**
Based upon *Heliotropium confertifolium* var. *coldenioides* I.M. Johnston, J. Arnold Arb. 29: 229. 1948. TYPE: **MEXICO. TAMAULIPAS:** 4 km W of Miquihuana, *Stanford et al. 744* (GH).

According to Johnston, var. **coldenioide**s "is a prostrate or distinctly caespitose plant and does not develop the erect or ascending leafy stems of the typical form of the species." Which seems to be the case. Additionally, it has mostly larger corollas borne upon shorter lateral stems, giving the entire plant a "heady-flowering" mat-like look, readily distinguished from the more erect typical variety.

Johnston also noted "Forms transitional to the variety *coldenioides* are found in Coahuila only at low altitudes in the eastern parts of the state." I myself have examined 3 hypothetical intermediates from southeastern Coahuila (LL, TEX): 45 km SW of Monterrey, *Barkley & Johnson 16254M*; 30 mi SW of Monterrey, *Barkley & Warnock 14725M*; 7 mi W of Saltillo, *Rollins & Tryon 58309.*

ACKNOWLEDGEMENTS

Distribution maps are mostly based upon specimens on file at LL-TEX. Jana Kos provided editorial input.

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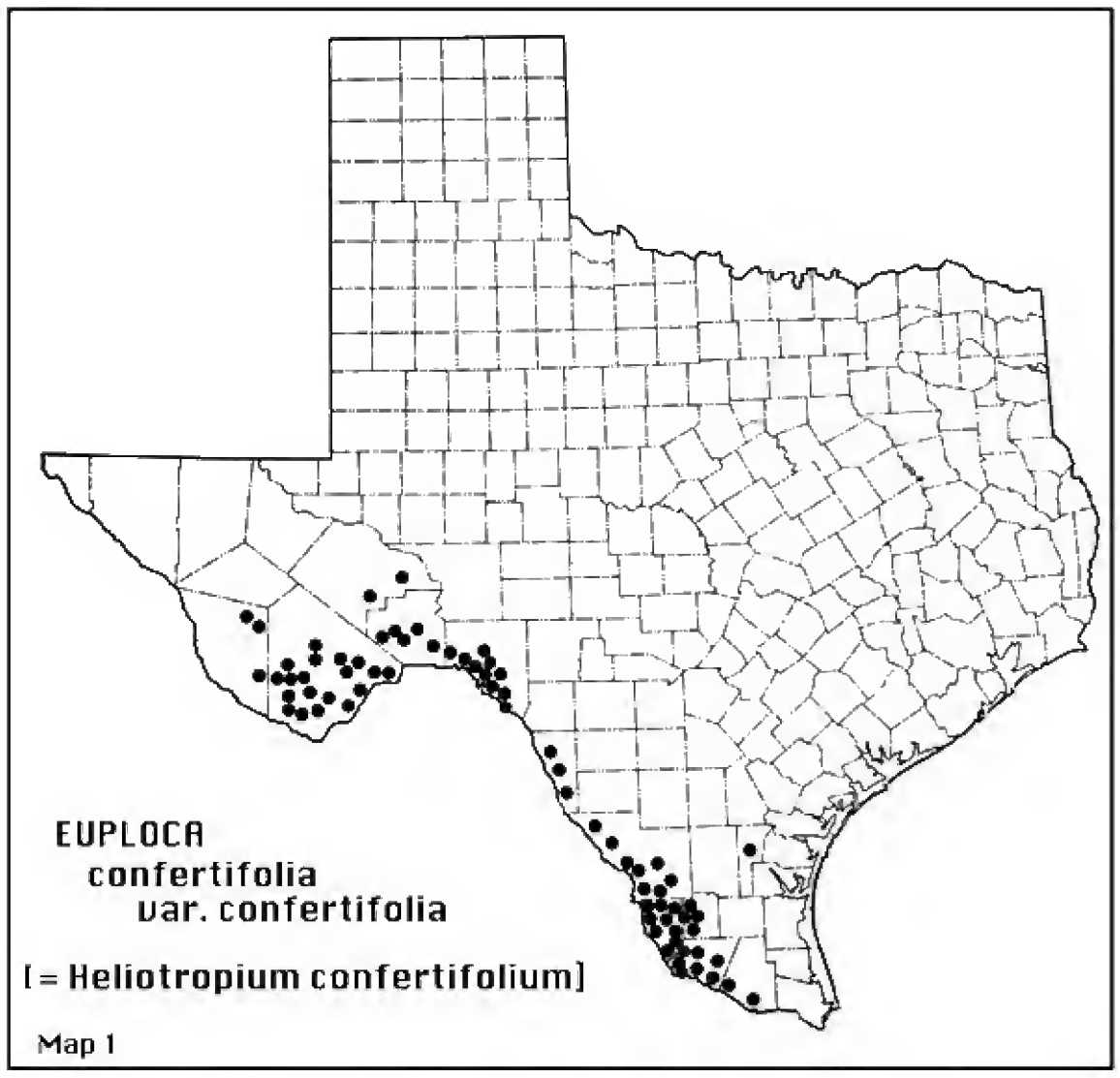
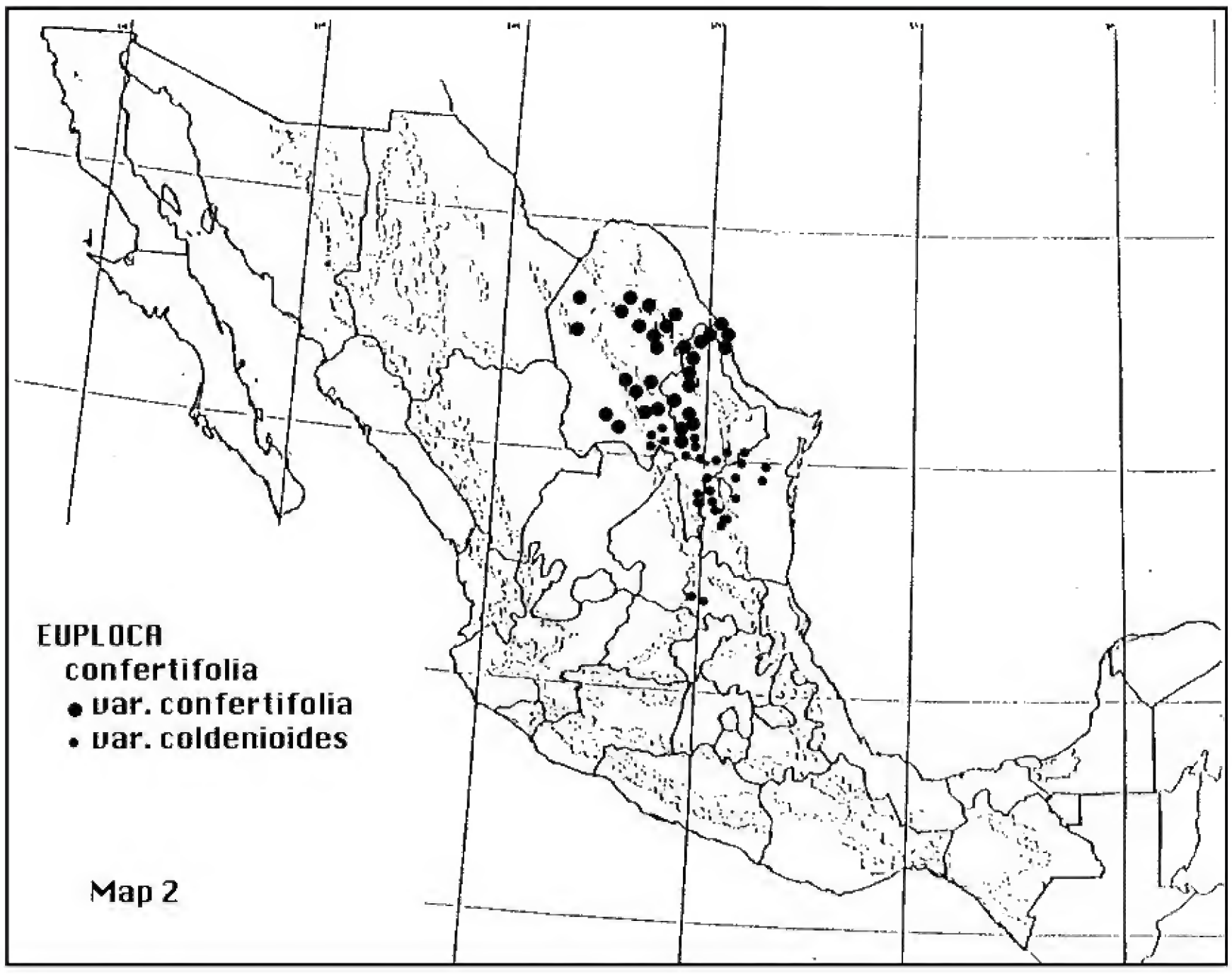

EUPLOCA
confertifolia
var. confertifolia
[= Heliotropium confertifolium]

Map 1


EUPLOCA
confertifolia
• var. confertifolia
• var. coldenioides

Map 2

Pollination does not affect floral nectar production, and is required for fruit-set by a hummingbird-visited Andean plant species

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ABSTRACT

Copious red nectar is produced in the flowers of *Jaltomata calliantha* (Solanaceae), a rare, herbaceous perennial native to Andean Peru. In greenhouses we determined that although self-compatible, flowers are not autogamous: pollination by an external vector is required for fruits to be set. Nectar is produced in all flowers, up to 94 µl per flower per day, first as nearly clear liquid, and then turning bright orange to red. Total nectar production over the life of a flower (with daily removal) ranged from 20–133 µl. Hand-pollination does not affect the rate or total volume of nectar produced. Published on-line www.phytologia.org *Phytologia 98(4): 313-317 (Oct 6, 2016).* ISSN 030319430.

KEY WORDS: floral corona, fruit-set, *Jaltomata calliantha*, nectar volume, pollination, red nectar, self-compatible

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The genus *Jaltomata* (Solanaceae) includes 70 species distributed from Arizona, USA, through Central America and Andean South America (Mione et al., 2015a, 2015b, 2016). The focus of this study was on *J. calliantha* S. Leiva & Mione, a wild, herbaceous perennial restricted to only two known locations in Peru. Flowers of *J. calliantha* are visited by hummingbirds in the more northern of the two populations (Department La Libertad). Only fruits were present (no flowers) when this species was collected in Department Ancash, and thus the floral visitors of this population are unknown. Given that colored nectar is correlated with vertebrate pollinators, mainly birds (Hansen et al., 2007), and that the flowers of the two populations are indistinguishable, it is likely that both populations are pollinated by hummingbirds. Flowers are solitary, and pistillate for about a day before the anthers dehisce. The corolla is green and 3–4 cm across. The floral corona (Figure 1A, left arrow) creates nectar troughs between the stamens. Floral nectar is amber in color when the flowers open (Figure 1A), but darkens with age becoming conspicuously orange to blood-red (Figure 1B), apparently serving as an attractant and reward to pollinators.

Animal pollinators are a requirement for sexual reproduction by 60–90% of plant species (Kremen et al., 2007). If pollinators are lost (regionally extirpated or extinct) due to habitat loss or climate change, how will biotically pollinated species fare? With this question in mind, we explored the question of whether pollination is required for fruits to be set by this Andean species, or if flowers automatically self-pollinate (autogamy) and set fruit in the absence of an external pollen vector. Given that nectar production is energetically expensive (Pyke, 1991), we also offered the hypothesis that a physiological mechanism might have evolved to decrease or cease nectar production following pollination.

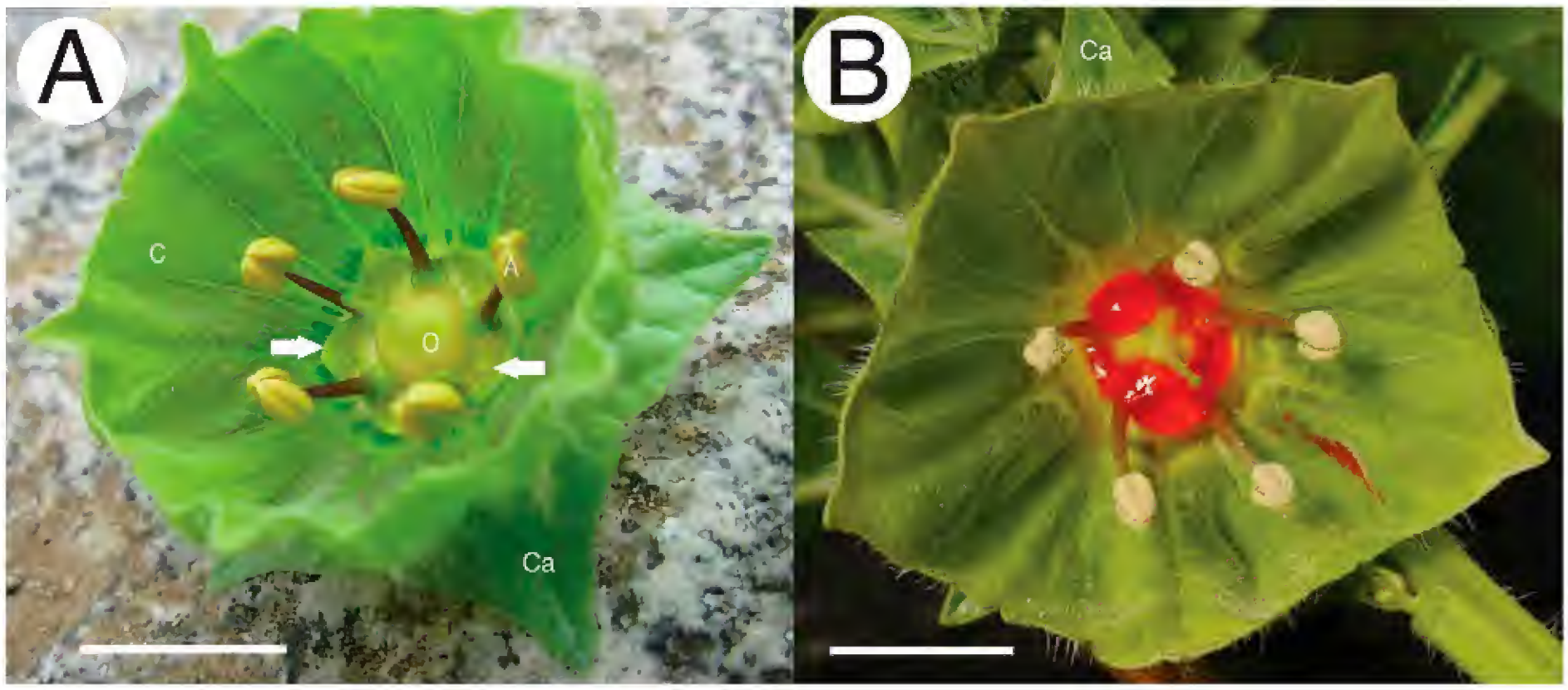

Figure 1. *Jaltomata calliantha.* **A**—Pistillate-phase flower: left arrow - corona, right arrow - the first nectar produced is amber in color, Ca - calyx, C - corolla, A - anther, O - ovary. Bar length = 1 cm, *Mione et al. 758*, photographed by T. M. in Peru. **B**—Hermaphroditic-phase flower: Ca - calyx, nectar is bright red at center of flower. Bar length = 1 cm. *Mione et al. 855/856*, photographed by T. M. at Central Connecticut State University.

MATERIALS AND METHODS

Seeds were collected in the wild in Peru, from 1379 to 2100 m elevation in Department Ancash (*Mione et al. 855 & 856,* CCSU) and treated with gibberellic acid (300 ppm) for 20 hours to induce germination. Plants were grown in greenhouses at Central Connecticut State University. Prior to anthesis pairs of flower buds from the same plant were tagged on the same day, 21 pairs divided nearly equally among five plants. The first day that the flowers opened, while still in their pistillate phase, one randomly chosen flower of each pair was hand-pollinated and the other flower remained unpollinated (the control). Given that anthers of a given flower have not yet dehisced during this pistillate phase, pollen for hand-pollinations was taken from other hermaphroditic-phase flowers on the same plant (i.e., geitonogamous pollination). Nectar was extracted and measured with volumetric microcapillary tubes from all flowers at nearly the same time each day, until flowers ceased producing nectar. This procedure continued until the corolla senesced, which was usually after five days. Flowers were revisited after three weeks to assay for fruit-set, but one branch had broken, reducing the number of flower pairs to 20.

RESULTS AND DISCUSSION

Ninety percent (18/20) of the flowers that were manually self-pollinated set fruit (Figure 2) containing the usual number of full-sized seeds, but none of the unpollinated (control) flowers set fruit. Obviously, the null hypothesis of equal fruit-set from pollinated vs. unpollinated flowers was rejected (Wilcoxon matched-pairs signed rank test, two-tailed, $P < 0.0001$). These tests demonstrate self-compatibility given that the source of pollen was always the same plant. Hand-pollinated flowers received pollen prior to anther dehiscence, and thus this fruit-set certifies stigma receptivity during day one (prior to anther dehiscence). Consequently, we refer to the phase prior to anther dehiscence as the pistillate phase. We refer to the following phase as the hermaphroditic phase because the stigma is also receptive after anthers dehisce (Mione, unpubl. data).

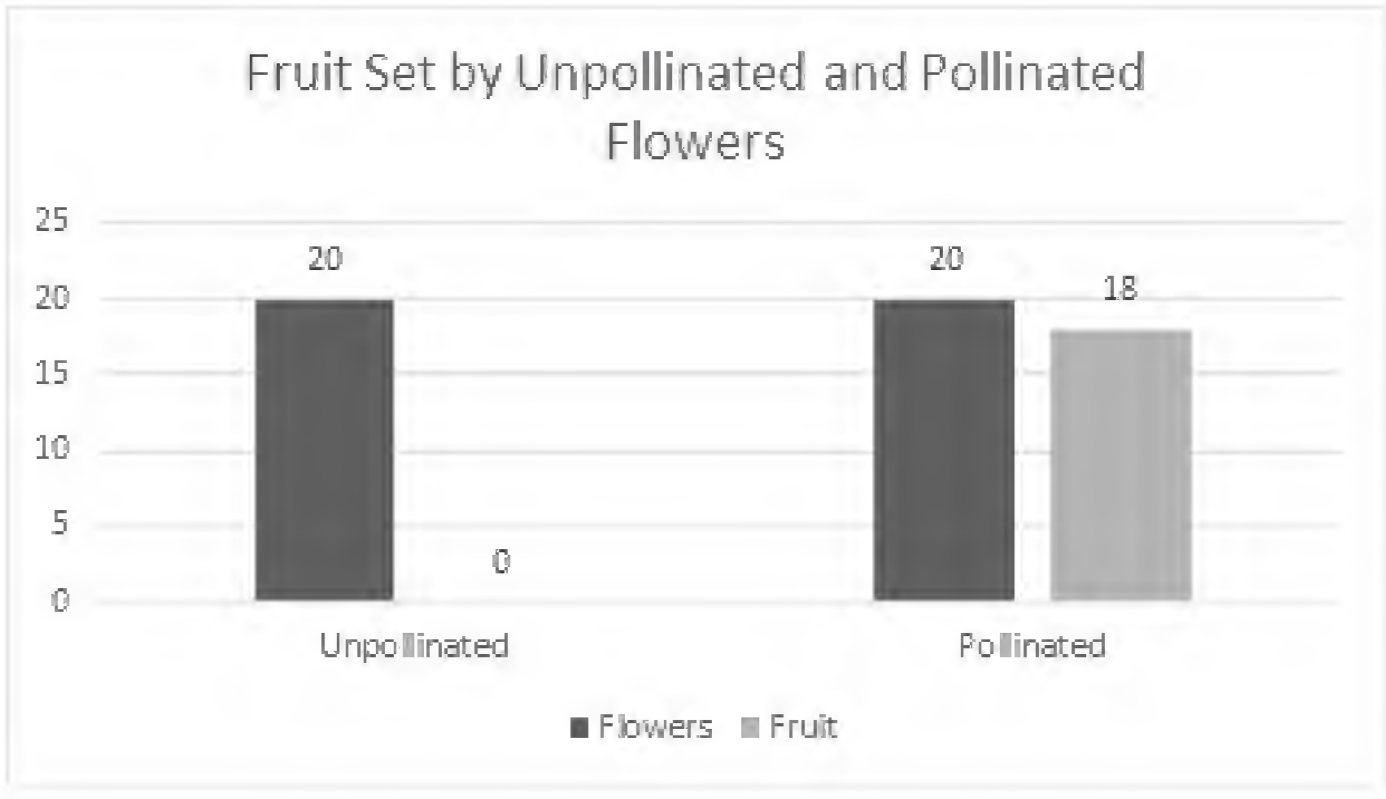

Figure 2. Fruit set by unpollinated (control) versus hand-pollinated flowers.

Considering both 21 pollinated and 21 unpollinated flowers, daily nectar production ranged from 0–94 µl. The average total nectar volume (over the life of the flower, with daily removal) was 52 µl, ranging from 20–133 µl (Figure 3). Variation in nectar production among flowers of the same plant is expected (Pleasants and Chaplin, 1983). The nectar volumes we measured suggest that *Jaltomata calliantha* provides a considerable supply of water and calories to floral visitors, and are similar to those measured for other hummingbird-pollinated flowers (Stiles and Freeman, 1993; Fonseca et al., 2015). Hummingbirds have been observed visiting the flowers and feeding on the floral nectar of *J. calliantha* in Peru (Mione & Leiva González, field observation).

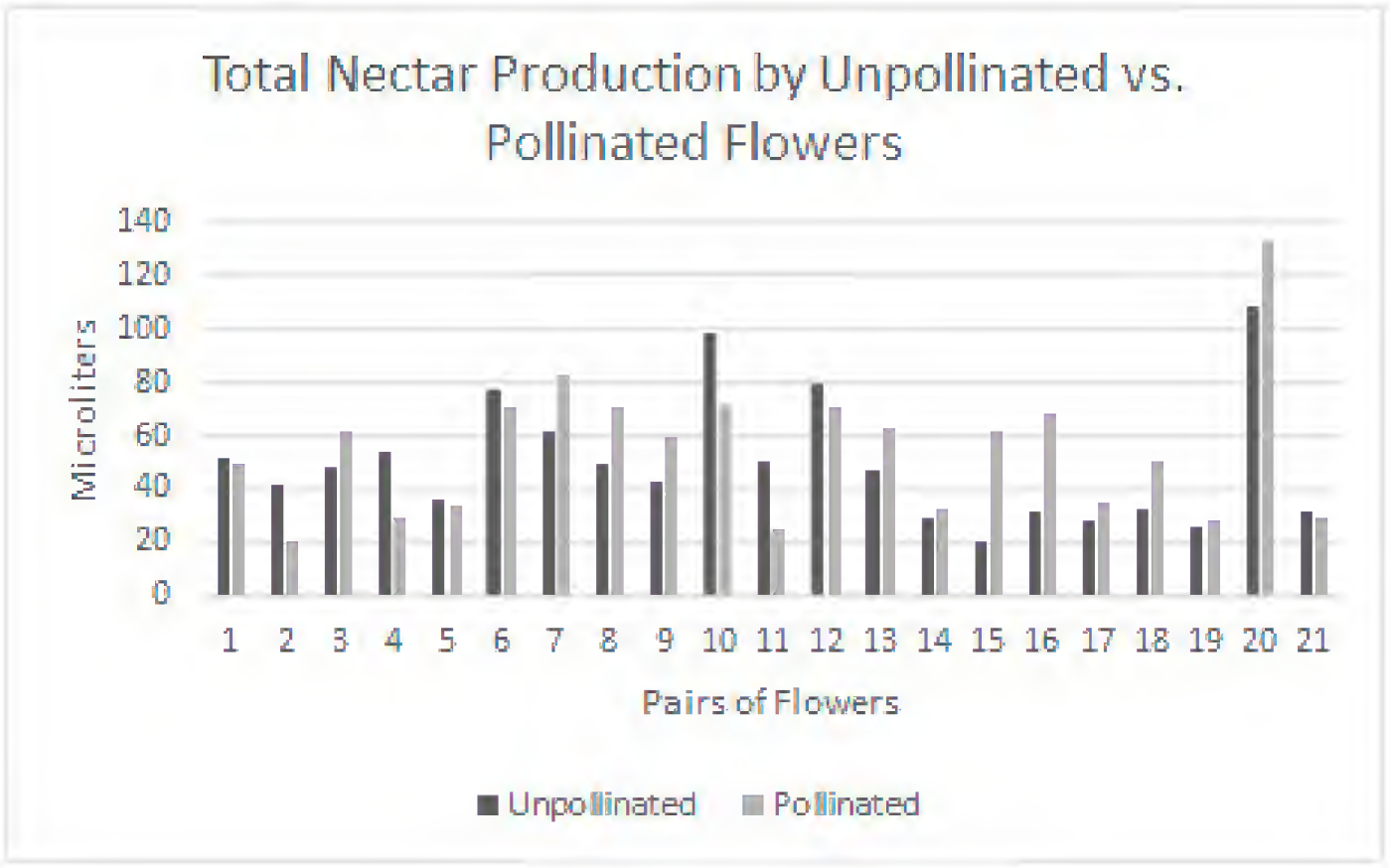

Figure 3. Total nectar production in unpollinated (control) versus hand-pollinated flowers.

Pollination has no effect on nectar production. Nectar volume in pollinated vs. unpollinated flowers is not significantly different (each pair of observations was on the same plant, with one flower pollinated and the other untreated, paired two-tailed T-test, P=0.2744, df = 20). However, during our study water was never limiting, and results may be different in the field where resources are likely scarce

at times. Aizen and Basilio (1998), studying a bumblebee-pollinated Andean, perennial herb (*Alstroemeria aurea*), and Delph and Lively (1989) studying a bird-pollinated tree (*Fuchsia excorticata)* of New Zealand, both found that hand-pollination had no effect on nectar production. In contrast, Stpiczyńska (2003) found that pollination actually increased nectar production in an orchid species.

As noted above, while 90 % of hand-pollinated flowers produced fruit, none of the 20 unpollinated flowers set fruit. Some 180,000 flowering plant species completely rely on biotic pollinators for their survival (Bodbyl Roels and Kelly, 2011). This rare plant may be vulnerable to extinction if it loses its pollinator or pollinators to habitat loss or to global warming (Buermann et al., 2011). At least 1,000 of the nearly 200,000 angiosperms that rely on biotic pollinators are used for agriculture (Bodbyl Roels and Kelly, 2011); thus, like the *Jaltomata* we studied, understanding the biology of pollination and protecting both the plants and their pollinators is not only important, but, for our crops, critical.

CONCLUSIONS

Jaltomata calliantha produces copious bright-red floral nectar (Figure 1B) that may serve as both an attractant and a reward to the hummingbirds that visit its flowers in the Andes. Continuous nectar production likely results in multiple hummingbird visits enhancing both female and male reproductive success (Fenster et al., 2006). Although self-compatible, flowers are not autogamous: pollination by an external vector is required for fruits to be set. In greenhouses hand-pollination does not affect nectar volume.

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