American Journal of Botany 92(11): 1911–1928. 2005.

PHYLOGENY OF *FLAVERIA* (ASTERACEAE) AND INFERENCE OF C_4 PHOTOSYNTHESIS EVOLUTION[1]

ATHENA D. MCKOWN,[2] JEAN-MARC MONCALVO,[2,3] AND NANCY G. DENGLER[2,4]

[2]Department of Botany, University of Toronto, Toronto, Ontario, M5S1A1, Canada; and
[3]Department of Natural History, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario, M5S 2C6, Canada

A well-resolved phylogeny of *Flaveria* is used to infer evolutionary relationships among species, biogeographical distributions, and C_4 photosynthetic evolution. Data on morphology, life history, and DNA sequences (chloroplastic *trnL-F*, nuclear ITS and ETS) for 21 of 23 known species were collected. Each data set was analyzed separately and in combination using maximum parsimony and Bayesian analyses. The phylogeny of *Flaveria* is based on the combined analysis of all data. Our phylogenetic evidence indicates that C_3 *Flaveria* are all basal to intermediate (C_3-C_4 and C_4-like) and fully expressed C_4 *Flaveria* species. Two strongly supported clades (A and B) are present. Using this phylogeny, we evaluate the current systematics of the genus and suggest the removal and reevaluation of certain taxa. We also infer the center of origin and dispersal of *Flaveria* species. Multiple origins of photosynthetic pathway intermediacy in *Flaveria* are recognized. C_3-C_4 intermediacy has evolved twice in the genus and is found to be evolutionarily intermediate in clade A, but not necessarily in clade B. C_4-like photosynthesis is also derived once in each clade. In addition, fully expressed C_4 photosynthesis may have evolved up to three times within clade A.

Key words: Asteraceae; C_3-C_4 intermediates; C_4 photosynthesis; ETS; *Flaveria*; ITS; phylogeny; *trnL-F*.

C_4 photosynthesis is a complex adaptation that has generated interest for decades and produced a wealth of research, yet details of the origins and modes of C_4 evolution have remained somewhat enigmatic. Numerous lines of evidence suggest that C_4 photosynthesis evolved from C_3 photosynthesis in response to low levels of atmospheric CO_2 and environmental conditions promoting the energetically wasteful oxygenase reaction (photorespiration) of the photosynthetic enzyme ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) (reviewed in Sage, 2001, 2004). In C_4 plants, CO_2 enters the C_4 cycle first and is incorporated into a 4-carbon organic acid by phosphoenolpyruvate carboxylase (PEPCase). The C_4 acid diffuses to the site of Rubisco and is decarboxylated to release and concentrate CO_2 near Rubisco, thereby suppressing the enzyme's oxygenase activity and essentially eliminating photorespiration. A significant advantage for plants with this complex adaptation is surviving and thriving in environmental conditions that favor photorespiration, such as warm, saline, or arid habitats.

The C_4 syndrome is extremely successful in the monocotyledonous families Poaceae and Cyperaceae, reflected in the global diversity and widespread distribution of C_4 grasses and sedges. C_4 photosynthesis is documented in one additional monocotyledonous family and 16 dicotyledonous families and is currently known to have at least 45 origins in the angiosperms as a whole, based on molecular phylogenetic data (Sage, 2004). Studies show that C_4 photosynthesis has arisen more than once in some orders or families (Sinha and Kellogg, 1996; Soros and Bruhl, 2000; Giussani et al., 2001; Kadereit et al., 2003) and even within a single genus (Kopriva et al., 1996; Pyankov et al., 2001; Kadereit et al., 2003; Sage, 2004). In addition, 10 genera (four monocot and six dicot) include species that have features that are between C_3 and C_4 values (e.g., degree of inhibition by photorespiration) and may include a combination of characteristics that reflect a partially to nearly complete C_4 photosynthetic cycle (Bruhl et al., 1987; Edwards and Ku, 1987; Monson and Moore, 1989; Bruhl and Perry, 1995; Sage et al., 1999; Monson and Rawsthorne, 2000). These species are classified as photosynthetic ''intermediates'' and have been used in numerous comparative studies of physiology and molecular biology that have formed the basis of evolutionary models hypothesizing the stepwise acquisition of C_4 traits (Edwards and Ku, 1987; Monson and Moore, 1989; Monson, 1999, 2003; Monson and Rawsthorne, 2000; Svensson et al., 2003; Westhoff and Gowik, 2004; Sage, 2004).

The genus *Flaveria* Juss. (Asteraceae) in particular has been used extensively in research of C_4 photosynthesis evolution. This small genus of 23 known species includes both strictly C_3 and C_4 species (NADP-malic enzyme type [Ku et al., 1983; Bauwe, 1984]), in addition to a large number of intermediate species (C_3-C_4 and C_4-like) (Table 1), and is believed to have evolved C_4 photosynthesis at least twice (Powell, 1978; Kopriva et al., 1996; Monson, 1996; Kellogg, 1999). Compared to C_3 *Flaveria* species, intermediate *Flaveria* species have reduced photorespiration, lower CO_2 compensation concentrations, partial to complete Kranz anatomy, and certain intermediates also have increased PEPCase and NADP-malic enzyme activities, and a partially functional C_4 cycle (Apel and Maass, 1981; Ku et al., 1983; Bassüner et al., 1984; Bauwe, 1984; Holaday et al., 1984; Rumpho et al., 1984; Reed and Chollet, 1985; Bauwe and Chollet, 1986; Brown et al., 1986; Monson et al., 1986; Edwards and Ku, 1987; Moore et al., 1987, 1988; Cameron et al., 1989; Cheng et al., 1988; Hylton et al., 1988; Brown and Hattersley, 1989; Ku et al., 1991, Dai et al., 1996; Drincovich et al., 1998). While the physiological properties of the intermediate *Flaveria* species fall between

[1] Manuscript received 2 April 2005; revision accepted 16 August 2005.

This work was supported by NSERC (NGD) and the Canada Foundation for Innovation/Ontario Innovation Trust (grant no. 7475 to JMM). The authors thank Dr. M. Powell for advice and plant material, Dr. T. Dickinson for curatorial assistance and helpful discussion, Drs. J. Bruhl and R. Sage for manuscript review, S. Margaritescu and O. Haddrath for technical assistance, E. Sudderth for plant material, Dr. S. Malcomber for donation of *trnL-F* primers, and Drs. J. E. Eckenwalder and J. L. Villaseñor for helpful discussion.

[4] Author for correspondence (e-mail: dengler@botany.utoronto.ca)

TABLE 1. Photosynthetic type and geographical distribution of *Flaveria* species and outgroup taxa *Haploësthes* and *Sartwellia*.

Species	Photosynthetic type	Geographical distribution[b,k]
Flaveria angustifolia (Cav.) Persoon	C_3-C_4[h,i]	South central Mexico (Distrito Federal, Guerrero, Oaxaca, Puebla)
Flaveria anomala B.L. Robinson	C_3-C_4[c]	North central Mexico (Coahuila, Nuevo León, Querétaro, San Luis Potosí, Tamaulipas, Zacatecas)
Flaveria australasica Hooker	C_4[a,b]	Australia (recorded in all states and territories)
Flaveria bidentis (L.) Kuntze	C_4[a,b]	Africa (Botswana, Egypt, Ethiopia, Lesotho, Namibia, South Africa, Swaziland, Zimbabwe), Caribbean (Antigua, Cuba, Dominican Republic, Puerto Rico), Great Britain (England), South America (Argentina, Bolivia, Brazil, Chile, Ecuador, Paraguay, Peru), USA (Alabama, Florida, Georgia, Massachusetts)
Flaveria brownii A.M. Powell	C_4-like[g]	Northeastern Mexico (Tamaulipas), southern USA (coastal Texas)
Flaveria campestris J.R. Johnston	C_4[b,l]	Mexico (state not recorded), southern and central USA (Arizona, Colorado, Kansas, Missouri, New Mexico, Oklahoma, Texas, Utah)
Flaveria chloraefolia A. Gray	C_3-C_4[e]	Northern Mexico (Chihuahua, Coahuila, Nuevo León), southern USA (New Mexico, Texas)
Flaveria cronquistii A.M. Powell	C_3[b]	South central Mexico (Oaxaca, Puebla)
Flaveria floridana J.R. Johnston	C_3-C_4[b,e]	South eastern USA (western Florida)
Flaveria haumanii Dimitri & Orfila	C_4-like or C_4[j]	Northern Argentina[f]
Flaveria kochiana B.L. Turner	C_4-like or C_4[l]	South central Mexico (Oaxaca, Puebla)[m]
Flaveria linearis Lagasca	C_3-C_4[b,d]	Caribbean (Bahamas Islands, Cuba), Central America (Belize, Honduras), southeastern Mexico (Campeche, Quintana Roo, Yucatán), southeastern USA (Keys, mainland Florida)
Flaveria mcdougallii Theroux, Pinkava & Keil	C_3[b]	Southern USA (Arizona)
Flaveria oppositifolia (DC.) Rydberg	C_3-C_4[b,h,i]	Central Mexico (Aguascalientes, Coahuila, Durango, Hidalgo, Nuevo León, San Luis Potosí, Tamaulipas, Zacatecas), southern USA (Texas)
Flaveria palmeri J.R. Johnston	C_4-like[e]	North central Mexico (Coahuila, Nuevo León, San Luis Potosí)
Flaveria pringlei Gandoger	C_3[a,b]	South central Mexico (Guerrero, Oaxaca, Puebla)
Flaveria pubescens Rydberg	C_3-C_4[c]	East central Mexico (Querétaro, San Luis Potosí, Tamaulipas)
Flaveria ramosissima Klatt	C_3-C_4[a,d]	South central Mexico (Oaxaca, Puebla)
Flaveria robusta Rose	C_3[a,i]	West central Mexico (Colima, Jalisco, Michoacán)
Flaveria sonorensis A.M. Powell	C_3-C_4[h,i]	Northwestern Mexico (Chihuahua, Sonora)
Flaveria trinervia (Spreng.) C. Mohr	C_4[a,b]	Africa (Ethiopia, Eritrea, Kenya, Somalia, Tanzania, Zambia, Zimbabwe), Caribbean (Bahamas Islands, Cuba, Dominican Republic, Jamaica, Haiti, Puerto Rico, Turks and Caicos), Central America (Belize), Mexico (recorded in nearly every state), Indian ocean (India, Mascarene Islands), Middle East (Iraq, Saudi Arabia, Yemen), South America (Brazil, Ecuador, Peru, Venezuela), USA (Arizona, Florida, Hawaii, Oklahoma, Massachusetts, Missouri, New Mexico, Texas)
Flaveria vaginata B.L. Robinson & Greenman	C_4-like[e]	South central Mexico (Oaxaca, Puebla)
Haploësthes greggii A. Gray	C_3[b]	Northern Mexico (Coahuila, Nuevo León), southern USA (New Mexico, Oklahoma, Texas)
Sartwellia flaveriae A. Gray	C_3[b]	Southern USA (New Mexico, western Texas)
Sartwellia mexicana A. Gray	C_3[b]	North central Mexico (Coahuila, Nuevo León, San Luis Potosí, Zacatecas)

[a] Smith and Turner, 1975.
[b] Powell, 1978.
[c] Apel and Maass, 1981.
[d] Ku et al., 1983.
[e] Holaday et al., 1984.
[f] Dimitri and Orfila, 1986.
[g] Monson et al., 1987.
[h] Moore et al., 1987.
[i] Ku et al., 1991.
[j] Petenatti and Del Vitto, 2000.
[k] A.D. McKown, collection notes.
[l] A.D. McKown, unpublished data.
[m] E. Sudderth, unpublished collection data.

those of C_3 and fully expressed C_4 *Flaveria* species, the evolutionary position of the intermediates still remains unclear (Kopriva et al., 1996). It is possible the intermediates represent stabilized, surviving taxa at various stages of C_4 photosynthetic evolution; however, others have also suggested that these species represent stable endpoints in themselves and are not evolving towards full C_4 photosynthesis (Edwards and Ku, 1987; Monson, 1989; Monson and Moore, 1989).

Taxonomically, *Flaveria* is circumscribed in the Asteraceae tribe Tageteae (Baldwin et al., 2002), subtribe Flaveriinae (Turner and Powell, 1977; Baldwin et al., 2002), with two sister genera *Sartwellia* and *Haploësthes* (Turner, 1971, 1975; Turner and Powell, 1977). *Flaveria* remains the sole C_4 genus in the Flaveriinae, although another occurrence of C_4 photosynthesis exists in the Tageteae (*Pectis*; Kellogg, 1999). Two C_4 *Flaveria* species are cosmopolitan, especially as weeds in tropical and subtropical areas, and one C_4 species occurs in Australia; however, the majority of *Flaveria* species are located in the southern USA, Mexico, and the West Indies (Table 1). Powell (1978) hypothesized evolutionary relationships within the genus based on morphological and hybridization data. The 21 then-known *Flaveria* species were segregated into two subgeneric groups based on the number of large floral bracts (involucral phyllaries) surrounding each head-type inflorescence (capitulescence), and species were placed either into the 3–4 phyllary or the 5–6 phyllary lineage. Intermediate and C_4 (or C_4-like) photosynthetic *Flaveria* species are found within both lineages; therefore, Powell (1978) suggested the possibility of two separate origins of C_4 photosynthesis within the genus. A morphological phylogeny produced by Monson (1996) with 15 characters obtained from Powell's monograph (1978) suggested three origins of C_4 photosynthesis in *Flaveria*; however, the phylogenetic tree was only moderately resolved and homoplasy was high. The first molecular-based phylogeny of *Flaveria* used the H-protein of the glycine cleavage system (338 bp fragments) from cDNA clones of 12 *Flaveria* species (Kopriva et al., 1996). The resulting tree topology supported Powell's phyllary-based groupings of *Flaveria*, although the 5–6 phyllary lineage was nested within the 3–4 phyllary group. Furthermore, the tree supported the hypothesis of C_4 or C_4-like photosynthesis evolving twice (once in each *Flaveria* ''clade''), but only one origin of photosynthetic intermediacy was suggested based on H-protein marker evidence. High bootstrap support (>75%) was only achieved at a few nodes of the tree, and the authors stated that the addition of *Flaveria* species and molecular markers was necessary to fully understand the evolutionary history of *Flaveria*. Another molecular phylogeny by Westhoff and Gowik (2004) using PEPCase (500 bp *ppc*A1 promoter sequences) produced a phylogenetic tree similar to that of Kopriva et al. (1996); however, this study also included only 12 *Flaveria* species, did not achieve high bootstrap support (>75%) at most nodes on the tree, and did not resolve the phylogeny of *Flaveria* better than the previous molecular study. In both molecular phylogenetic studies, the gene markers chosen would not be considered neutral, but under selection pressure for C_4 photosynthesis.

The presence of intermediate species within the genus *Flaveria* has provided the basis for several models involving C_4 genetic, enzyme, and physiological evolution (Rosche and Westhoff, 1995; Drincovich et al., 1998; Monson, 1999, 2003; Engelmann et al., 2003; Sage, 2004; Westhoff and Gowik, 2004). It is evident, however, that a robust phylogeny is necessary to provide an evolutionary framework for previous and future research on *Flaveria* thus enabling a further understanding of the evolution of C_4 photosynthesis in this genus. We infer phylogenetic relationships among 21 of the 23 known *Flaveria* species using morphological, life history, chloroplastic (*trnL-F*), and nuclear (ITS, ETS) DNA sequence data. This study has three objectives: (1) to provide a phylogeny of *Flaveria*, (2) to reassess *Flaveria* systematics and biogeography, and (3) to evaluate previous hypotheses concerning the phylogenetic placement of photosynthetically intermediate *Flaveria* species and diversification of photosynthetic pathways in the genus in an evolutionary framework.

MATERIALS AND METHODS

Sampling strategy and material—A large collection of Flaveriinae specimens (*Flaveria*, *Sartwellia*, *Haploësthes*) was studied from various herbaria and sampled for genetic, morphological, and biogeographical data. Specimens were from the University of Toronto *Flaveria* research collection (grown by F. Kocacinar/voucher specimens housed at the Royal Ontario Museum [TRT]); Arnold Arboretum, Harvard University (AA); Gray Herbarium, Harvard University (GH); Royal Botanical Gardens, Kew (K); New York Botanical Gardens (NY); Sul Ross State University (SRSC); University of Texas (TEX/LL); Texas A & M University (TAES); and Museum of Natural History, Vienna (W) (Table 2; DNA GenBank codes listed in Appendix). Additional plant materials of *Flaveria* for DNA extraction were donated by A. M. Powell (Sul Ross State University, Alpine, Texas, USA) and E. Sudderth (Harvard University, Cambridge, Massachusetts, USA). Biogeographical data were recorded for specimens from these institutions and compared to the web-based Missouri Botanical Garden Vascular Tropicos Nomenclatural database (W^3TROPICOS) and Mexican Biodiversity Information Network database (Red Mundial de Información Sobre Biodiversidad, REMIB). Sequence and morphological data were obtained for specimens of 21 of the 23 described *Flaveria* species (Powell, 1978; Dimitri and Orfila, 1986; Turner, 1995). Of the two *Flaveria* species missing from this study, *F. intermedia* J. R. Johnst. (type specimen collected in 1896, located at GH) was studied for morphology, but is very similar to *F. palmeri* and remains questionable as a distinct species (Powell, 1978; A. D. McKown, personal observation). The other species, *F. haumanii* Dimitri and Orfila, is located in northern Argentina and considered to be closely related to *F. bidentis* (Dimitri and Orfila, 1986), but was unavailable for this study. *Haploësthes greggii*, *Sartwellia flaveriae*, and *S. mexicana* were used as outgroup taxa to root the *Flaveria* phylogeny, because *Haploësthes* and *Sartwellia* are both considered to be closely related to *Flaveria* (Turner and Powell, 1977; Baldwin et al., 2002).

Data for phylogenetic inference were obtained from three gene markers, a morphological data matrix assembled from herbarium specimens, and published life history characters (Powell, 1978). Gene sequences were amplified from the chloroplast *trnL-F* spacer region, and the nuclear ribosomal internal (ITS) and external (ETS) transcribed regions. Morphological data from over 600 Flaveriinae samples (most are independent acquisitions), including type specimens, and life history characters were coded with symbols (0, 1, 2, etc.). Only discrete characters were selected and used for analysis, eliminating bias from characters occurring on a gradient, such as length or height measurements.

DNA isolation—Total genomic DNA for all taxa was obtained from dried herbarium leaf material, dried fruit material, or alcohol-preserved leaf tissue (Table 2). Herbarium specimens were sampled with as little destruction as possible using 0.5 cm^2 of tissue or less. Recent collections were chosen (the oldest sample was 50 yr old), and green portions of the leaf were sampled. Samples were ground in 1 mL of 2.5% SDS with 1.0 mm zirconia/silica beads and a single ceramic bead (Bio Spec Products, Bartlesville, Oklahoma, USA) in a Fast Prep FP120 mixer mill (Thermo Electron Corp., Milford, Massachusetts, USA). Following complete pulverization of tissues, samples were centrifuged for 4–5 min at 13 000 rpm. Supernatants were collected and washed twice with an equivalent volume of 24 : 1 chloroform : isoamyl alcohol for removal of proteins, lipids, pigments, and debris. Approximately

TABLE 2. Voucher, herbarium and collection locality information of *Flaveria* (*F*), *Haploësthes* (*H*) and *Sartwellia* (*S*) specimens used for amplification of gene markers *trnL-F*, ITS and ETS. Specimen replicates are marked as letters (A, B, C, etc.).

Taxon	Collector, voucher number, herbarium	Collection locality
F. angustifolia A	McKown, 10906, TRT	University of Toronto greenhouse collection
F. angustifolia B	Calzada, 19400, NY	Santiago Juxtlahuaca, Oaxaca, Mexico
F. anomala A	McKown, 10907, TRT	University of Toronto greenhouse collection
F. anomala B	Powell, 2579, fruit material	Mexico
F. anomala C	Nesom and Wells, 6630, TEX	Villa Juárez, San Luis Potosí, Mexico
F. anomala D	Powell, 2599, SRSC	Conceptión del Oro, Coahuila, Mexico
F. anomala E	Hinton et al., 22360, TEX	Aramberri, Nuevo León, Mexico
F. anomala F	Hinton et al., 19564, TEX	Galeana, Nuevo León, Mexico
F. australasica A	McKown, 10911, TRT	University of Toronto greenhouse collection
F. australasica B	Powell, 5843, fruit material	Millstream, Western Australia, Australia
F. australasica C	Pedley, 763, NY	Darling Downs district, Queensland, Australia
F. australasica D	Craven, 5278, AA	Wiluna, Western Australia, Australia
F. bidentis A	McKown, 10909, TRT	University of Toronto greenhouse collection
F. bidentis B	Powell, 361, fruit material	Argentina
F. bidentis C	Nee, 51694, TEX	Santiesteban, Bolivia
F. brownii A	McKown, 10912, TRT	University of Toronto greenhouse collection
F. brownii B	Loring, s.n., 1993, SRSC, s.n.	Nueces, Texas, USA
F. brownii C	Richardson et al., 2605, TEX	Cameron, Texas, USA
F. brownii D	Powell, 2802, SRSC	San Patricio, Texas, USA
F. campestris A	Powell and Powell, 3011, SRSC	Socorro, New Mexico, USA
F. campestris B	Loring, 2000-94, SRSC	Woods, Oklahoma, USA
F. campestris C	Wagenknecht, 3144, GH	Stafford, Kansas, USA
F. chloraefolia A	McKown, 10904, TRT	University of Toronto greenhouse collection
F. chloraefolia B	Powell and Powell, 3036, NY	Culberson, Texas, USA
F. chloraefolia C	Hinton et al., 21951, TEX	Aramberri, Nuevo León, Mexico
F. chloraefolia D	Villarreal and Carranza, 2307, TAES	Presa El Tulillo, Coahuila, Mexico
F. cronquistii A	Anderson and Anderson, 5341, NY	Coxcatlán, Puebla, Mexico
F. cronquistii B	Turner, 0-31, SRSC	Tehuacán, Oaxaca, Mexico
F. cronquistii C	Rzedowski, 37186, NY	Teotitlán del Camino, Oaxaca, Mexico
F. floridana A	McKown, 10903, TRT	University of Toronto greenhouse collection
F. floridana B	Powell, 342, fruit material	Florida, USA
F. floridana C	Moldenke and Moldenke, 29697, LL	Fort Myers, Florida, USA
F. floridana D	Brumbach, 8868, NY	Sanibel, Florida, USA
F. kochiana A	Zamudio and Ocampo, 10973, TEX	Laguna Encantada, Oaxaca, Mexico
F. kochiana B	Sudderth, 8, leaf material	Puebla, Mexico
F. kochiana C	Sudderth, 10, leaf material	Puebla, Mexico
F. kochiana D	Sudderth, 11, leaf material	Puebla, Mexico
F. linearis A	McKown, 10908, TRT	University of Toronto greenhouse collection
F. linearis B	Brown and Evans, F-22, SRSC	Key West, Florida, USA
F. linearis C	Nee and Atha, 46867, LL	San Pedro, Belize
F. linearis D	Correll, 43462, NY	South Andros Isle, Bahamas
F. linearis E	Hill, 13417, NY	Park Key, Florida, USA
F. linearis F	Brown, 14463, TEX	Big Cypress, Florida, USA
F. mcdougallii	Scott et al., 884, TEX	Coconino, Arizona, USA
F. oppositifolia A	Powell and Turner, 2710, SRSC	Nadadores, Coahuila, Mexico
F. oppositifolia B	Powell and Tomb, 2551, SRSC	Saltillo, Nuevo León, Mexico
F. oppositifolia C	Hinton et al., 20471, TEX	Galeana, Nuevo León, Mexico
F. palmeri A	Powell, 851, fruit material	Mexico
F. palmeri B	Powell and Tomb, 2621, SRSC	Cuatro Ciénegas, Coahuila, Mexico
F. palmeri C	Powell and Tomb, 2611, SRSC	San Pedro de las Colonias, Coahuila, Mexico
F. pringlei A	McKown, 10902, TRT	University of Toronto greenhouse collection
F. pringlei B	Panero and Salinas, 1146, TEX	Tamazulapan, Oaxaca, Mexico
F. pringlei C	Salinas and Ramos, F-3798, TEX	Tehuacán, Puebla, Mexico
F. pringlei D	Boege, 2101, GH	Tehuacán, Puebla, Mexico
F. pringlei E	Rzedowski, 30525, GH	Chilpancingo, Guerrero, Mexico
F. pringlei F	Sudderth, 7, leaf material	Puebla, Mexico
F. pringlei G	Sudderth, 3, leaf material	Puebla, Mexico
F. pubescens A	Hartman et al., 3823, LL	Río Verde, San Luis Potosí, Mexico
F. pubescens B	Ward, 8106, NY	Río Verde, San Luis Potosí, Mexico
F. ramosissima A	Cowan et al., 5773, NY	Altepexi, Puebla, Oaxaca
F. ramosissima B	Steinmann and Cervantes-Maldonado, 1396, NY	Tehuacán, Puebla, Mexico
F. ramosissima C	Cronquist, 11235, NY	Teotitlán del Camino, Oaxaca, Mexico
F. robusta A	McKown, 10901, TRT	University of Toronto greenhouse collection
F. robusta B	Powell, 168, fruit material	Manzanillo, Colima, Mexico
F. robusta C	Sanders et al., 11760, TEX	Colima, Colima, Mexico
F. robusta D	Ayers et al., 94, TEX	Zapotiltlic, Jalisco, Mexico
F. sonorensis A	McKown, 10905, TRT	University of Toronto greenhouse collection
F. sonorensis B	Arguelles, 82, GH and TEX	San Bernardo, Sonora, Mexico

Table 2. Continued.

Taxon	Collector, voucher number, herbarium	Collection locality
F. trinervia A	McKown, 10910, TRT	University of Toronto greenhouse collection
F. trinervia B	Hinton et al., 19547, TEX	Galeana, Nuevo León, Mexico
F. trinervia C	Annable and Sickle, 3138, NY	Oahu, Hawaii, USA
F. trinervia D	Correll, 46028, NY	Great Inagua, Bahamas
F. trinervia E	Meleady et al., 216, NY	Corozal, Belize
F. vaginata A	McKown, 10914, TRT	University of Toronto greenhouse collection
F. vaginata B	King, 2922, TEX	Izúcar de Matamoros, Puebla, Mexico
H. greggii	Turner, 23-109, SRSC	Brewster, Texas, USA
S. flaveriae	Powell and Powell, 6389, SRSC	Ward, Texas, USA
S. mexicana	Hinton et al., 19446, TEX	Galeana, Nuevo León, Mexico

800 μL of supernatant was recovered for each sample, and DNA was precipitated from this solution by adding 500 μL of isopropanol. DNA precipitates were rinsed with 1 mL 80% ethanol at −20°C, resuspended in 100 μL distilled, deionized water and stored at −20°C. Extractions with poor amplifications were further cleaned by ''re-extracting'' the DNA using a DNeasy Plant mini kit (QIAGEN, Mississauga, Ontario, Canada) following the manufacturer's instruction.

Gene markers and amplification—Gene markers selected for this study included chloroplastic *trnL-F* (primers ''c'' and ''f'' from Taberlet et al., 1991 donated by S. Malcomber, University of Missouri, St. Louis, Missouri, USA) (Bruneau et al., 2001), and nuclear ITS (primers ITS1 and ITS4 from White et al., 1990) and ETS (primers Hel-1 and 18S from Baldwin and Markos, 1998). All gene markers were amplified with a polymerase chain reaction (PCR) using the same PCR ''cocktail'': 4 μL 10 mM dNTPs, 3.5 μL ddH_2O, 2.5 μL 10× PCR buffer (QIAGEN), 2.5 μL bovine albumin serum, 0.5 μL *Taq* DNA polymerase (QIAGEN), and 1 μL each of forward and reverse 10 μM primers. Some samples with low PCR product yield required lowering the water content by 1 μL and adding 1 μL 25 mM $MgCl_2$ solution (QIAGEN). All PCR amplifications were performed with the same thermal cycler settings (10 s at 95°C, 36 cycles of 1 min at 95°C, 30 s at 50°C, 4 min at 72°C, followed by a 10-min final extension at 72°C). PCR products were purified with QIAquick PCR purification kit (QIAGEN) and concentrated to approximately 10 ng DNA/μL for direct sequencing. Samples from *Flaveria brownii* showed allelic variation of ITS, and samples from *F. pringlei* and *F. anomala* had multiple alleles of both ITS and ETS, all of which required cloning with a QIAGEN PCR cloning kit using QIAGEN competent cells prior to sequencing.

DNA sequencing—Cleaned PCR products were sequenced in both directions using Big Dye Terminator ver. 3.5 (Applied Biosystems, Foster City, California, USA). Sequencing products were cleaned with Sephadex G-50 Fine DNA grade (Amersham Biosciences, Uppsala, Sweden), heat and vacuum-dried, and resuspended in Hi-Di formamide (Applied Biosystems). Sequences were read with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, California, USA), and the resulting gene sequences were edited with Sequencher ver. 2.2 (Gene Codes, Ann Arbor, Michigan, USA). Sequences containing single nucleotide polymorphisms were marked for the polymorphism. All sequences were submitted to GenBank (samples listed in Appendix).

Phylogenetic analysis—Nucleotide sequences were aligned using ClustalW (Chenna et al., 2003). Sequence alignments were optimized by eye in Se-Al manual sequence alignment editor (Rambaut, 1996), and ambiguously aligned regions were removed from the phylogenetic analyses. Aligned files were analyzed with maximum parsimony using PAUP* version 4.0 (Swofford, 2003) and Bayesian inference using MrBayes version 3.0 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Parsimony searches were conducted for each data set separately and in combination assuming gaps as missing data, multiple states as uncertainty, and with a minimum *F* value for character state optimization. Full heuristic searches of alignment files were run with 1000 repetitions of random addition sequences and using tree-bisection-reconnection (TBR) branch swapping. To estimate support and robustness of the parsimony tree, 1000 bootstrap replicates were run with 100 full heuristic searches per replicate. All data sets were compared in PAUP* 4.0 for congruency with the partition homogeneity test (PHT) using 1000 replicates with 10 full heuristic searches per replicate. Bayesian analyses were run for separate and combined data sets with one million generations using four Markov chains and assuming a general model of DNA substitution/character change with gamma-distributed (DNA) or beta-distributed (morphological) rate variation across sites. Trees were sampled and saved at every 100th generation, and the initial 500 trees were discarded (as burn-ins). Bayesian consensus trees were constructed from 9500 trees and compared to parsimony consensus trees for congruence by eye. A single tree from parsimony analysis is presented for each data set indicating branch lengths (where applicable), and bootstrap/posterior probability values.

RESULTS

Alignment and tree statistics (alignment lengths, number of variables and phylogenetically informative sites, number of most parsimonious trees found, tree length, consistency, and retention indices) are given in Table 3.

trnL-F—The chloroplast spacer *trnL-F* is the most easily amplified of the three gene markers and worked for all the investigated herbarium specimens, regardless of condition, age, or treatment (such as chemical spraying). Sequences of *trnL-F* from *Flaveria* show relatively little variation, the consistency index of these data is very high (Table 3), and the consensus parsimony and Bayesian tree topologies are congruent (Fig. 1). Most branches receive high posterior probability support (>95%) in the Bayesian analysis; however, there is a lack of high bootstrap values (>75%) for some branches of the parsimony tree, likely due to the limited number of variable sites in the *trnL-F* data (Table 3). This marker shows that certain species of *Flaveria*, believed to be closely related (Powell, 1978), have identical *trnL-F* sequences, such as *F. australasica* and *F. trinervia*, or *F. campestris* and *F. palmeri*. Multiple samples from different species of *Flaveria* cluster together, except samples of *F. chloraefolia* and *F. linearis*, which do not demonstrate strict monophyletic species groupings. All C_3 *Flaveria* (*F. cronquistii*, *F. mcdougallii*, *F. pringlei*, and *F. robusta*) are located at the base of the tree and C_4 *Flaveria* (*F. australasica*, *F. bidentis*, and *F. trinervia*) are found at the tips of the tree. *Flaveria campestris* (C_4) and *F. palmeri* (C_4-like) are also at the tips of the tree, but are not clustered with the other true C_4 species where they have traditionally been grouped (Powell, 1978).

ITS—Nucleotide sequences from the nuclear spacer ITS show greater variation than the chloroplast marker and dem-

TABLE 3. Summary of characteristics from separate and combined analyses of *trnL-F*, ITS, ETS, and morphological data for *Flaveria*, *Haploësthes*, and *Sartwellia*.

Characteristic	*trnL-F*	ITS	ETS	Morphology	Combined genes[a]	Combined genes and morphology[b]
Aligned sequence length/number of characters	871	708	452	30	2031	2061
Variable positions	27	232	135	30	367	398
Phylogenetically informative positions	19	179	79	27	174	200
Most parsimonious trees	2	654	16	3	1	2
Tree length (including uninformative data)	30	413	204	119	575	727
Polytomies in consensus tree	5	4	2	1	2	1
Consistency index	0.90	0.69	0.78	0.42	0.76	0.68
Retention index	0.98	0.94	0.91	0.65	0.82	0.76

[a] *Flaveria linearis* samples labeled A and B not included in morphological analysis.
[b] *Flaveria chloraefolia* sample labeled C not included in combined analysis using gene and morphological data.

onstrate better resolution of *Flaveria* species' relationships, despite a slightly lower consistency index than *trnL-F* (Table 3). Consensus trees from parsimony and Bayesian analyses are congruent with each other, and posterior probability support is stronger than bootstrap support for most branches of the tree (Fig. 2). At the base of the tree, both analyses split *F. mcdougallii* from the remainder of the *Flaveria* species with 100% bootstrap and posterior probability support. C_3 *Flaveria* species (*F. cronquistii* and *F. pringlei*) are located at the base of the tree with 100% posterior probability support. The following deep node is a polytomy in both parsimony and Bayesian analyses, which consists of two strongly supported clades (A and B) and three species that do not fall into either clade with any significant statistical support. Of note, ITS copies from three *F. pringlei* specimens are grouped with *F. angustifolia*, while other ITS copies from the same specimens cluster with the remaining *F. pringlei* specimens. Species in clade A have well-resolved relationships amongst each other based on the high bootstrap values and posterior probabilities (100% for most branches). In contrast, the relationships among species of *Flaveria* in clade B are not as clear. While *F. anomala* is well-supported as the basal species of this clade, the following node is a polytomy, and thus it is difficult to determine the phylogenetic history of the other species. Multiple samples of the different species in clade B form monophyletic species groups with the exception of samples labeled *F. chloraefolia* and *F. linearis*.

ETS—Analyses of ETS sequence data produce similar results to ITS analyses with a higher consistency index (Table 3). While parsimony and Bayesian analyses of ETS yield congruent tree topologies, the Bayesian analysis resolves the topology better than parsimony with high posterior probability supports (Fig. 3). Both analyses separate *Flaveria mcdougallii* from the other species of *Flaveria* with very high bootstrap (98%) and posterior probability (100%) supports. Analysis of this gene marker also places C_3 species at the base of the tree; however, ETS results suggest *F. cronquistii* to be more basal than *F. pringlei*. Similar to the ITS analysis, there is a lack of resolution at the subsequent node. Also comparable is the grouping of ETS copies from two *F. pringlei* samples with *F. angustifolia* rather than with other members of *F. pringlei*. In contrast to the ITS analysis is the sister-relationship of *F. robusta* and *F. sonorensis*, although this is only supported by parsimony analysis. The well-supported clade A from the ITS results is also present in the ETS analyses with 100% posterior probability and 97% bootstrap supports. The clade B from ITS is also present with 96% posterior probability and 71% bootstrap supports, although the ETS data lack support for inclusion of *F. anomala* in this clade. Similar to results from *trnL-F* and ITS, members labeled *F. chloraefolia* and *F. linearis* do not form monophyletic groups.

Morphology and life history characters—A large number of specimens were studied for most species of *Flaveria*, and the resulting morphological data are coded to reflect the range of each character observed. Sixty-five morphological and life history characters were gathered; however, only 30 were eventually utilized for parsimony analysis (data matrix in Appendix). While it is unknown whether variation in certain characters could represent environmentally induced responses, the large number of *Flaveria* specimens studied was deemed sufficient to assess the utility of each character for systematics.

The consensus tree topology of the morphological characters from both parsimony and Bayesian analyses does not retrieve the exact tree topology of any of the gene analyses (Fig. 4). The consistency index of this data set is very low (Table 3), indicating extensive homoplasy in morphological features; however, the consensus tree topology does retrieve Powell's subgeneric phyllary groups (excepting the position of *Flaveria cronquistii*). Portions of the morphological tree correspond to the gene tree topologies, such as the placement of *F. mcdougallii* at the base of the morphological tree, along with the glabrous 3–4 phyllary *F. cronquistii*. A clade comprising species from *F. ramossisima* upwards is similar to clade A from the gene analyses (excepting the placement of *F. angustifolia*). Species designated as 5–6 phyllary by Powell (1978) are found in more basal positions along the tree, but the group is paraphyletic. In contrast with the ITS analysis, the morphological tree suggests that species in clade A (ITS) are more derived compared to species placed in clade B (ITS). In addition, while the gene markers differentiate among *F. chloraefolia* samples collected in northern areas of Mexico/southern USA and the *F. chloraefolia* specimen from Nuevo León, Mexico (labeled C in the gene analyses) (Figs. 1–3), no single character used in the morphological analysis distinguishes between these specimens. In contrast, morphological differences among collections of *F. linearis* were observed, suggesting agreement between the morphological data and the non-monophyletic grouping of samples labeled *F. linearis* in the gene analyses (Figs. 1–3). The *F. linearis* sample from Belize (labeled ''Yucatan'' in Fig. 4) is more robust in appearance with broader leaves and loosely aggregated inflorescences, compared to samples from Florida and the Bahamas (Appendix). Among the West Indies samples, the most striking difference is the presence or absence of ray florets in the inflorescence.

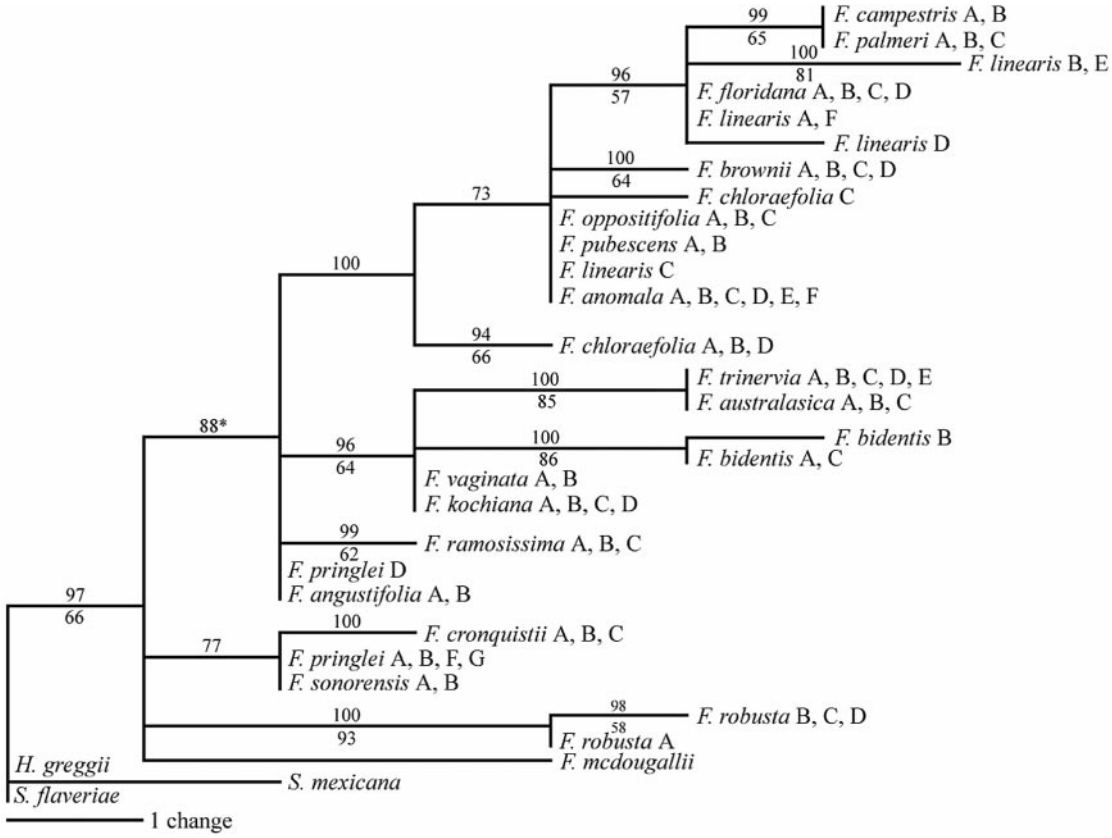

Fig. 1. Consensus tree of chloroplast *trnL-F* sequences from *Flaveria* (*F*), *Haploësthes* (*H*), and *Sartwellia* (*S*) species indicating branch lengths. Bayesian posterior probabilities are placed above branches and bootstrap values are placed below. Capital letters represent species samples corresponding with voucher information provided in Table 2. * indicates a supported node when *F. pringlei* sample D (hybrid) is excluded from the analysis.

While this character varies among species of *Flaveria*, it does not vary within any other *Flaveria* species, suggesting at least two entities in the Caribbean are included under the taxonomic label ''*F. linearis.*''

In a second parsimony analysis, morphological characters previously distinguished as important features for determining *Flaveria* species and their phylogenetic placement (e.g., life history, phyllary number, capitulescence [= capitula] shape, and inflorescence types; Powell, 1978) were given twice the weight assigned to other morphological characters. The resulting parsimony tree topology is identical to the tree from analysis of equally weighted characters except that it resolves the basal polytomy shown in Fig. 4, and bootstrap supports are lower (tree not shown). Excluding the outgroup species and re-rooting the data set with either *F. cronquistii* or *F. pringlei* does not yield any tree topology similar to those observed in the gene analyses and lowers the consistency index further (trees not shown).

Combined data—The gene marker data sets were not found to be statistically different from each other using PHT (*trnL-F* vs. ITS: $P = 0.05$, *trnL-F* vs. ETS: $P = 0.06$, ITS vs. ETS: $P = 0.06$). The different data partitions were therefore combined and analyzed under the same conditions used for the separate gene marker analyses. The combined data set was constructed with each species represented by one specimen with all three markers sequenced (chosen randomly if multiple specimens were available). Where cloned sequences exist for a specimen (e.g., *Flaveria pringlei*), the representative sequence was chosen randomly (the resulting tree topology was not affected by changing clone sequences). The consensus tree topologies obtained from either parsimony or Bayesian analyses of the combined gene data are congruent, and most nodes are supported with high posterior probabilities and bootstrap values (Fig. 5, Table 3). In the consensus tree, *F. mcdougallii* is separated from the other species of *Flaveria* with 100% bootstrap and posterior probability supports. *Flaveria cronquistii* is located at the base of the tree along with *F. pringlei*, and a node with good posterior probability support (85%) separating *F. robusta* and *F. sonorensis* is present that was not supported in the separate gene analyses. Following this node are two well-supported clades (previously ascribed as clades A and B; Figs. 2, 3). Although *F. angustifolia* is placed at the base of clade B, this placement is not strongly supported statistically (65%). A polytomy exists in clade B, indicating insufficient genetic signal to resolve species' relationships.

The morphological data set was not statistically different from the combined gene data set using PHT (morphology vs. gene: $P = 0.05$) and therefore, all data were combined. Addition of the morphological data set lowers the consistency index of the parsimony analysis, due to inclusion of many homoplasic morphological characters (Table 3); however, the resulting consensus tree topology from parsimony and Bayesian analyses, inclusive of the morphological data, is nearly identical to that of the combined gene analysis. Furthermore, addition of the morphological data set contributes significant

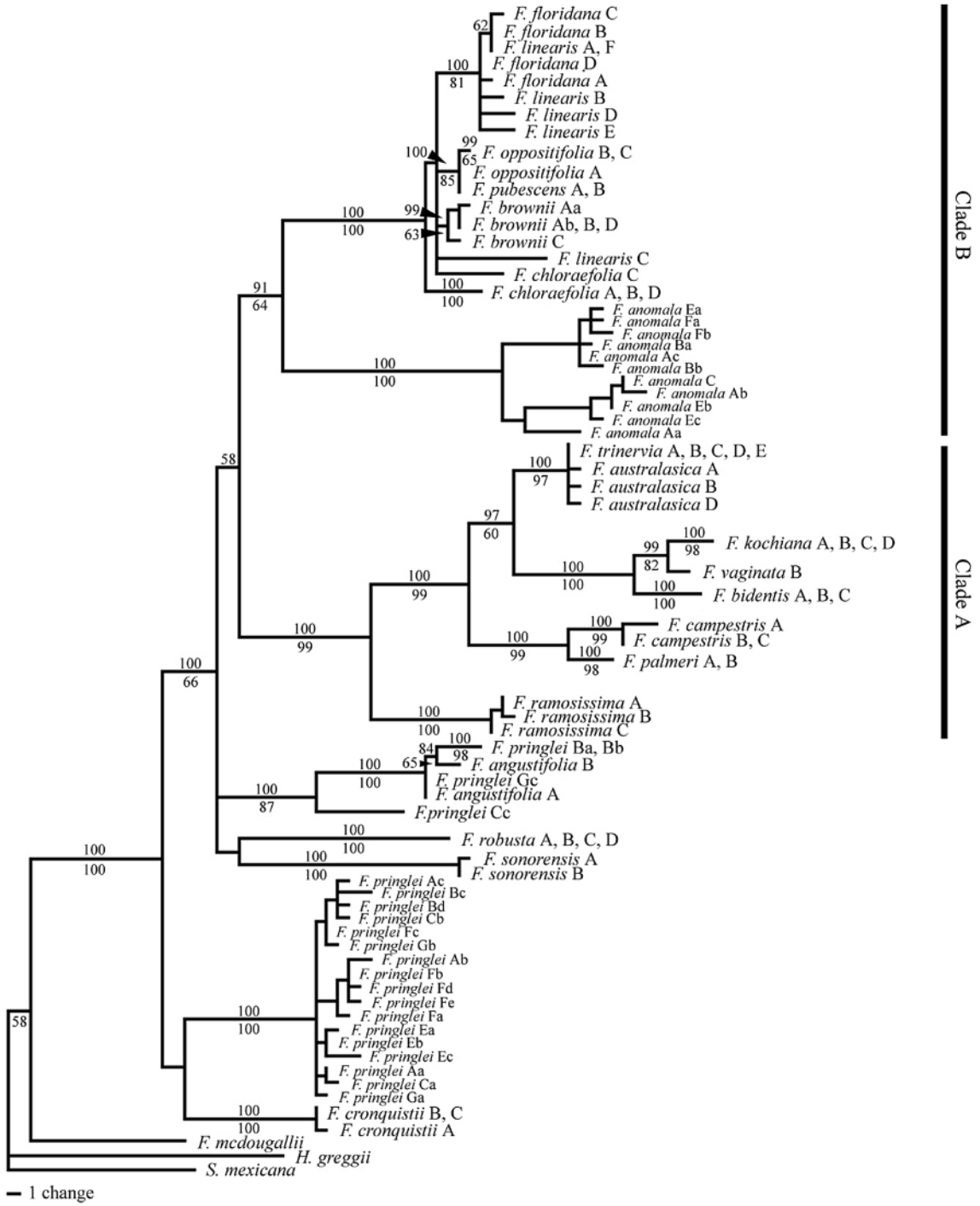

Fig. 2. Consensus tree of nuclear ribosomal ITS sequences from *Flaveria* (*F*), *Haploësthes* (*H*), and *Sartwellia* (*S*) species indicating branch lengths. Bayesian posterior probabilities are placed above branches and bootstrap values are placed below. Capital letters represent species samples corresponding with voucher information provided in Table 2. Small letters are cloned replicates from species with variable ITS sequences. Solid vertical lines represent well-supported clades A and B.

support to some nodes within the tree and recovers additional nodes, which are not present in the combined gene parsimony analysis alone (values marked with parentheses on Fig. 5). Inclusion of the morphological evidence strengthens the position of *F. angustifolia* at the base of clade B with a 100% posterior probability support. Considering all lines of evidence, the C_3 species (*F. cronquistii*, *F. mcdougallii*, and *F. pringlei*) are located at the base of the tree, while C_4 species (*F. australasica*, *F. bidentis*, *F. campestris*, and *F. trinervia*), and C_4-like species (*F. brownii*, *F. palmeri*, and *F. vaginata*) occur at the tips. Only intermediate species *F. ramosissima* (C_3-C_4) in clade A is in an unequivocal intermediate phylogenetic placement between C_3 ancestral species and derived C_4 species.

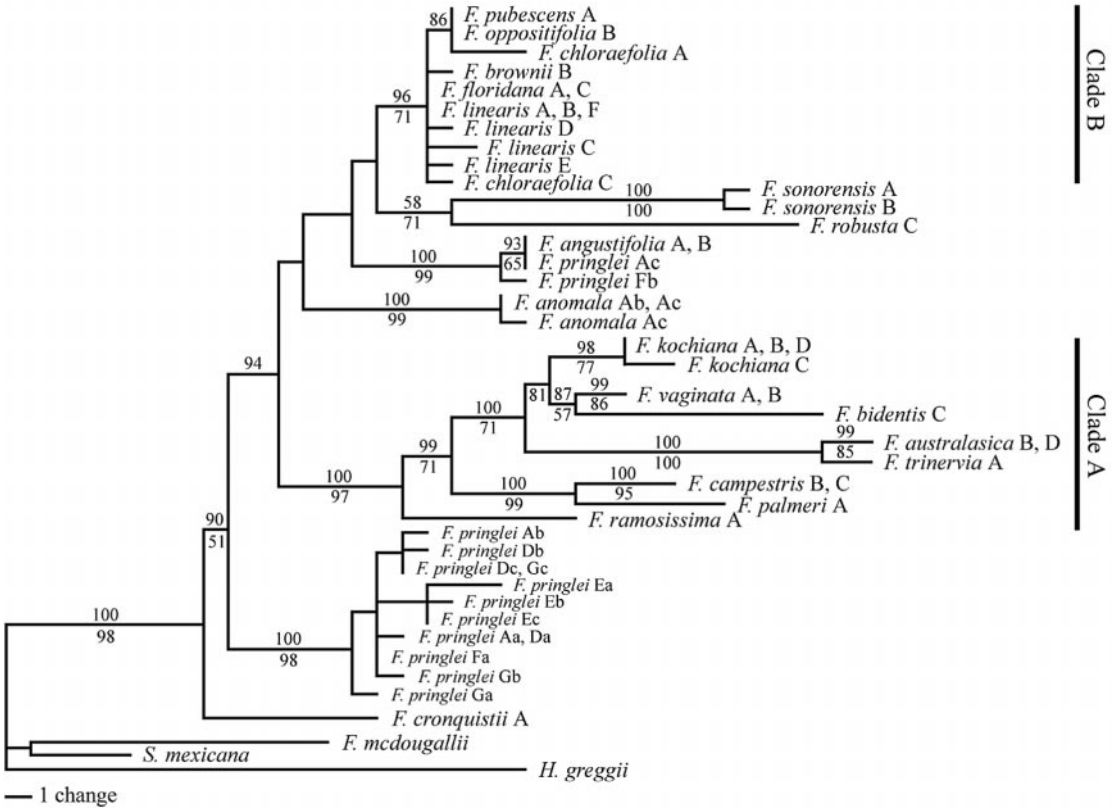

Fig. 3. Consensus tree of nuclear ribosomal ETS sequences from *Flaveria* (*F*), *Haploësthes* (*H*), and *Sartwellia* (*S*) species indicating branch lengths. Bayesian posterior probabilities are placed above branches and bootstrap values are placed below. Capital letters represent species samples corresponding with voucher information provided in Table 2. Small letters are cloned replicates from species with variable ETS sequences. Solid vertical lines represent well-supported clades A and B.

DISCUSSION

This study uses nucleotide sequence data from one chloroplastic and two nuclear genes in combination with morphological and life history data (Fig. 6). It is the first to provide a nearly fully resolved phylogeny for virtually all known *Flaveria* species, and the resulting phylogeny enables us to infer the evolution of C_4 photosynthesis in *Flaveria* in an evolutionary context.

***Flaveria* systematics and biogeography**—*Basal species of Flaveria: F. cronquistii, F. mcdougallii, and F. pringlei*—In each analysis (Figs. 1–5), *F. mcdougallii* is located at the base of the tree and is separated from the other species of *Flaveria* with very high bootstrap and posterior probability supports in all analyses (except the *trnL-F* analysis). In addition, morphological characters, such as strongly exserted floral corollas, pappus scales, and pubescent, flattened, oblanceolate cypselas, suggest more similarity between *F. mcdougallii* and *Haploësthes*/*Sartwellia* than to other *Flaveria* species. These results correspond with hybridization experiments in which *F. mcdougallii* did not hybridize with any *Flaveria* species, but demonstrated a low crossability with *Haploësthes greggii* var. *texana* and *Sartwellia puberula* (Powell, 1978). Based on the hybridization and morphological studies, Powell suggested that this species might represent a distinct, monotypic genus. The results of our phylogenetic analyses and the previous study by Powell (1978) concur in the exclusion of *F. mcdougallii* from the genus *Flaveria* (Fig. 6).

The removal of *Flaveria mcdougallii* from *Flaveria* indicates that the true basal species are the large shrub/short tree species, *F. cronquistii* and *F. pringlei* (Fig. 6). This suggests that the ancestral condition of *Flaveria* was a large, shrubby, self-incompatible perennial with glabrous stems and leaves, loosely aggregated, paniculate corymbs, and inflorescences of solely disc-type flowers with 3–4 phyllaries. *Flaveria cronquistii* is found in the Tehuacán valley of southern Mexico (southeast Puebla and northwest Oaxaca) (Powell, 1978; A. D. McKown, collection notes). *Flaveria pringlei* occurs sympatrically with *F. cronquistii* in the Tehuacán valley region, but is also located across the southern half of Puebla, the northern part of Oaxaca and west into central Guerrero (Powell, 1978; A. D. McKown, collection notes). The proximity of these two basal *Flaveria* species suggests that the origin and distributional center for the genus is the south-central region of Mexico (Puebla-Oaxaca) (Fig. 7). There is no evidence of hybridization between *F. cronquistii* and *F. pringlei*; however, *F. pringlei* forms hybrids with *F. angustifolia*, as previously suggested by Kopriva et al. (1996). Four of our seven *F. pringlei* samples (labeled A, B, D, and G in Figs. 1–3) show direct genetic evidence of hybridization by possessing chloroplast or nuclear gene sequences that are identical to those from *F. angustifolia* samples. Further morphological evidence of hybridization is observed in the *F. pringlei* sample labeled B, because ray florets are present in the inflorescences, stem and leaf surfaces are moderately pubescent, and leaf margins are toothed—characters that are observed in *F. angustifolia* but

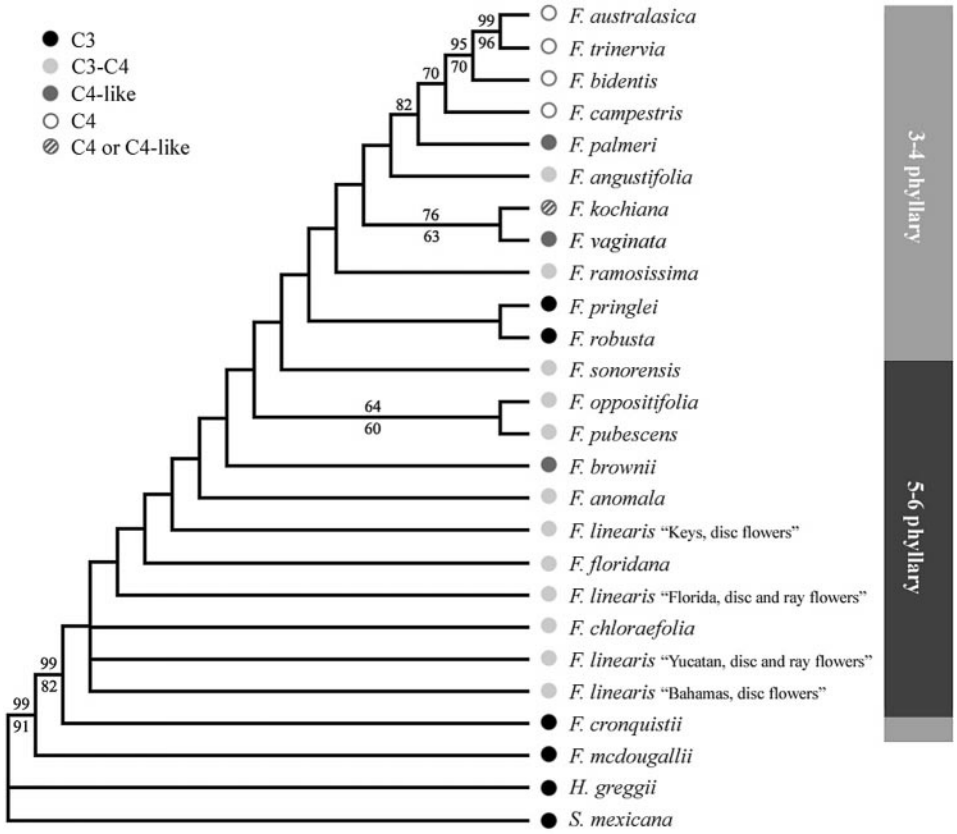

Fig. 4. Consensus tree of morphological data from *Flaveria* (*F*), *Haploësthes* (*H*), and *Sartwellia* (*S*) species using parsimony analysis. Bayesian posterior probabilities are placed above branches and bootstrap values are placed below. Light and dark gray bars correspond to Powell's (1978) 3–4 and 5–6 phyllary groups. Coloured dots indicate photosynthetic type: black = C_3 photosynthesis, light gray = C_3-C_4 photosynthesis, dark gray = C_4-like photosynthesis, white = C_4 photosynthesis, white with gray hatches = C_4-like or C_4 photosynthesis.

are absent in typical *F. pringlei*. In addition to evidence of hybridization, most of our *F. pringlei* samples have copies of ITS and/or ETS that are more similar to sequences from *F. angustifolia* than to the remaining *F. pringlei* sequences. For instance, one allele of ITS from sample C and one allele of ETS from sample F cluster with *F. angustifolia* sequences (Figs. 2, 3); however, the clustering of these alleles from *F. pringlei* samples C and F with *F. angustifolia* sequences occurs in a basal position, suggesting retention of a more ancestral copy of the gene in *F. pringlei*. Powell (1978) considered *F. pringlei* to have evolved from *F. angustifolia*; however, according to our phylogeny, *F. angustifolia* is more derived. Overall, we speculate that hybridization between these two species still occurs, because this genus is relatively young and the geographical ranges of the two species are extremely similar (Powell, 1978; A. D. McKown, collection notes). Hybridization likely accounts for reports of ''*F. angustifolia*-like'' morphological characters and gene copies in some *F. pringlei* specimens.

Derived species of Flaveria: F. robusta and F. sonorensis—An early branch of the basal *Flaveria* species includes two species, *F. robusta* and *F. sonorensis* (Fig. 6). These species are both small, shrubby perennials and are similar enough in morphology that early collections of *F. sonorensis* were labeled ''*F. robusta*'' (Powell, 1978; A. D. McKown, collection notes). *Flaveria robusta* is found northwest of the Puebla-Oaxaca region (in Colima, northern Michoacán, and southern Jalisco) whereas *F. sonorensis* is substantially further northwest in Sonora and Chihuahua, Mexico (Fig. 7; Powell, 1978; A. D. McKown, collection notes). Based on the results of the combined analysis, it is feasible that *F. robusta* is the sister species to *F. sonorensis* and that long reproductive isolation has resulted in significant genetic variation, as indicated by the long branch lengths observed in our gene marker analyses. The placement of these two species in a separate branch of *Flaveria* indicates that both Powell's subgeneric phyllary lineages are paraphyletic (Fig. 6).

Clade A—Clade A is the most unambiguous and well-supported group in all analyses, and most species within this clade are well-defined genetically (Figs. 1–3, 5). Clade A includes *Flaveria ramosissima*, *F. palmeri*, *F. campestris*, *F. australasica*, *F. trinervia*, *F. bidentis* (*F. haumanii*), *F. kochiana*, and *F. vaginata* (Fig. 6). *Flaveria vaginata* and *F. kochiana* have been suggested as representing a single species as these plants are sympatric and morphologically similar (J. L. Villaseñor, Instituto de Biología, Universidad Nacional Autonóma de México, México D. F., México, personal communication); however, hybridization was not detected from our gene data and the phylogenetic analyses strongly support two independent species. Identical *trnL-F* sequences and low variability in the nuclear sequences confirm a recent divergence between *F. australasica* and *F. trinervia* (Powell, 1978; Kopriva et al.,

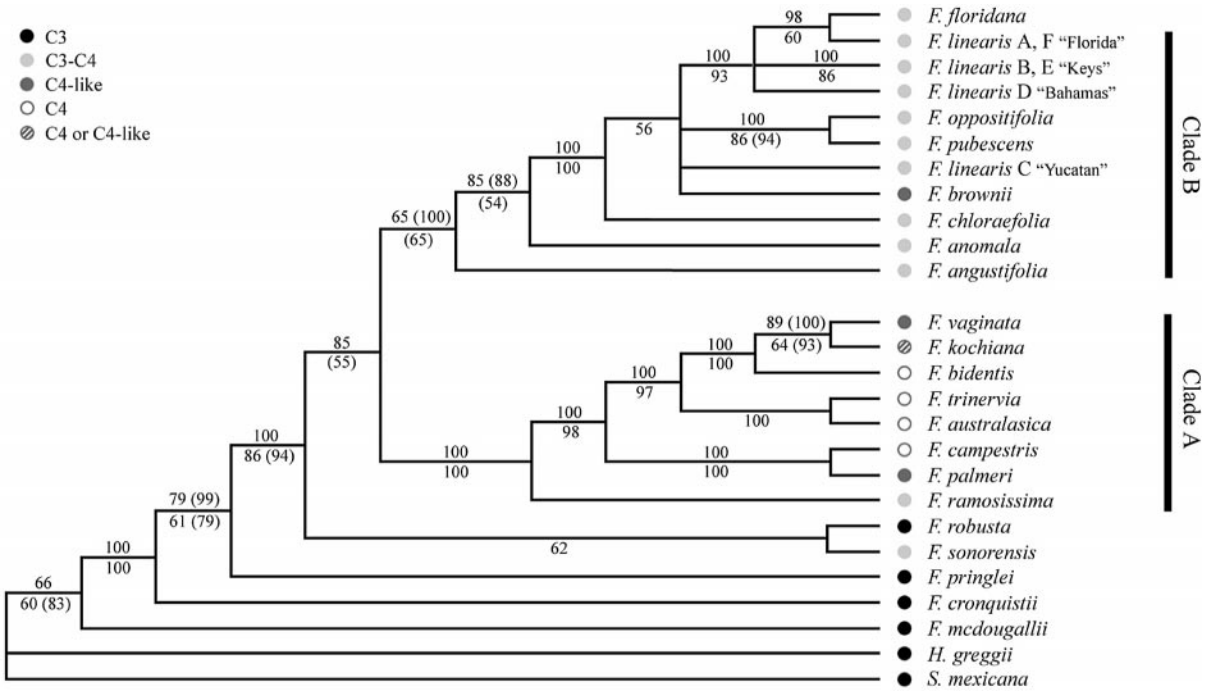

Fig. 5. Consensus tree of the combined gene data analysis from *Flaveria* (*F*), *Haploësthes* (*H*), and *Sartwellia* (*S*) species. Bayesian posterior probabilities are placed above branches and bootstrap values are placed below. Values in brackets indicate nodes that gain support with the inclusion of morphological data in the combined analysis. Capital letters mark species with genetic variability resulting in non-monophyletic species groupings and correspond with voucher information provided in Table 2. Solid vertical lines represent well-supported clades A and B. Colored dots indicate photosynthetic type: black = C_3 photosynthesis, light gray = C_3-C_4 photosynthesis, dark gray = C_4-like photosynthesis, white = C_4 photosynthesis, white with gray hatches = C_4-like or C_4 photosynthesis.

1996). The latter is most likely the result of successful establishment of *F. trinervia* in Australia and, given the identical morphology and high genetic similarity, could be considered a subspecies of *F. trinervia*, as suggested by Powell (1978).

The species of clade A fall within Powell's 3–4 phyllary lineage and all are annuals, with the exception of *F. vaginata* (Powell, 1978) and possibly *F. kochiana* (E. Sudderth, Harvard University, Cambridge, Massachusetts, USA, personal communication). The only self-compatible *Flaveria* species are also fully C_4, and these species are all included within this clade: *F. trinervia*, *F. australasica*, *F. campestris*, and *F. bidentis* (Fig. 6; Powell, 1978). Species in clade A are small to moderately sized herbaceous plants with moderate pubescence, strongly serrated leaves (excepting *F. vaginata* and *F. kochiana*), and inflorescences with both ray and disc flowers. In general, floral features are reduced within this clade. For instance, capitulescence shape is contracted to a scorpioid corymb (*F. ramosissima*, *F. campestris*, *F. palmeri*, and *F. bidentis*) or further reduced to a globose or glomerule shape (*F. trinervia*, *F. australasica*, *F. vaginata*, and *F. kochiana*). In addition, this clade shows a reduction in phyllary number and shape, from 4–5 broadly elliptic phyllaries in *F. ramosissima* to 3–4 narrow phyllaries in other species of clade A, and further reduced to two oblong and 1–2 linear phyllaries in *F. trinervia* and *F. australasica*. The outermost phyllaries in *F. trinervia* and *F. australasica* are narrow and not laterally expanded. These were previously described as ''chaff-like setae'' (Powell, 1978); however, these structures are vascularized and are not always completely reduced to narrow, linear structures, but can appear similar to the larger phyllaries.

The basal species of clade A, *F. ramosissima*, occurs in southern Puebla and northern Oaxaca sympatrically with the basal species of *Flaveria* and more derived members of clade A (*F. vaginata*, *F. kochiana*) (Fig. 7; Powell, 1978; A. D. McKown, collection notes; E. Sudderth, Harvard University, personal communication). One branch of clade A suggests a northward radiation, as *F. palmeri* is found in northcentral Mexico and *F. campestris* occurs across southern and central USA (Fig. 7; Powell, 1978; A. D. McKown, collection notes). Another branch of clade A represents a possible southern dispersal, as *F. bidentis* occurs in Central America, the West Indies, and across South America, in addition to being established in other continents as a cosmopolitan weed (Fig. 7; Powell, 1978; A. D. McKown, collection notes). *Flaveria haumanii*, the relative of *F. bidentis*, is located in northern Argentina (Dimitri and Orfila, 1986) and should also be considered as part of this southern lineage. The other derived species, *F. trinervia*, is widespread throughout Mexico and the southern USA and is a very successful cosmopolitan weed established on nearly every continent (Powell, 1978; A. D. McKown, collection notes).

Clade B—Clade B in *Flaveria* is as diverse in species as clade A, and includes *F. angustifolia*, *F. anomala*, *F. brownii*, *F. chloraefolia*, *F. floridana*, *F. linearis*, *F. oppositifolia*, and *F. pubescens* (Fig. 6). In the gene analyses, branch lengths of species in this clade, excepting *F. angustifolia* and *F. anomala*, are very short and the lack of genetic variation makes the phylogeny of this clade difficult to resolve without the inclusion of morphological data (Figs. 1–3). The radiation of more derived members in this clade appears to have occurred rapidly, as indicated by lower genetic divergence, and this difficulty in resolution is also evident in the analyses of Kopriva et al. (1996) and Westhoff and Gowik (2004). The basal spe-

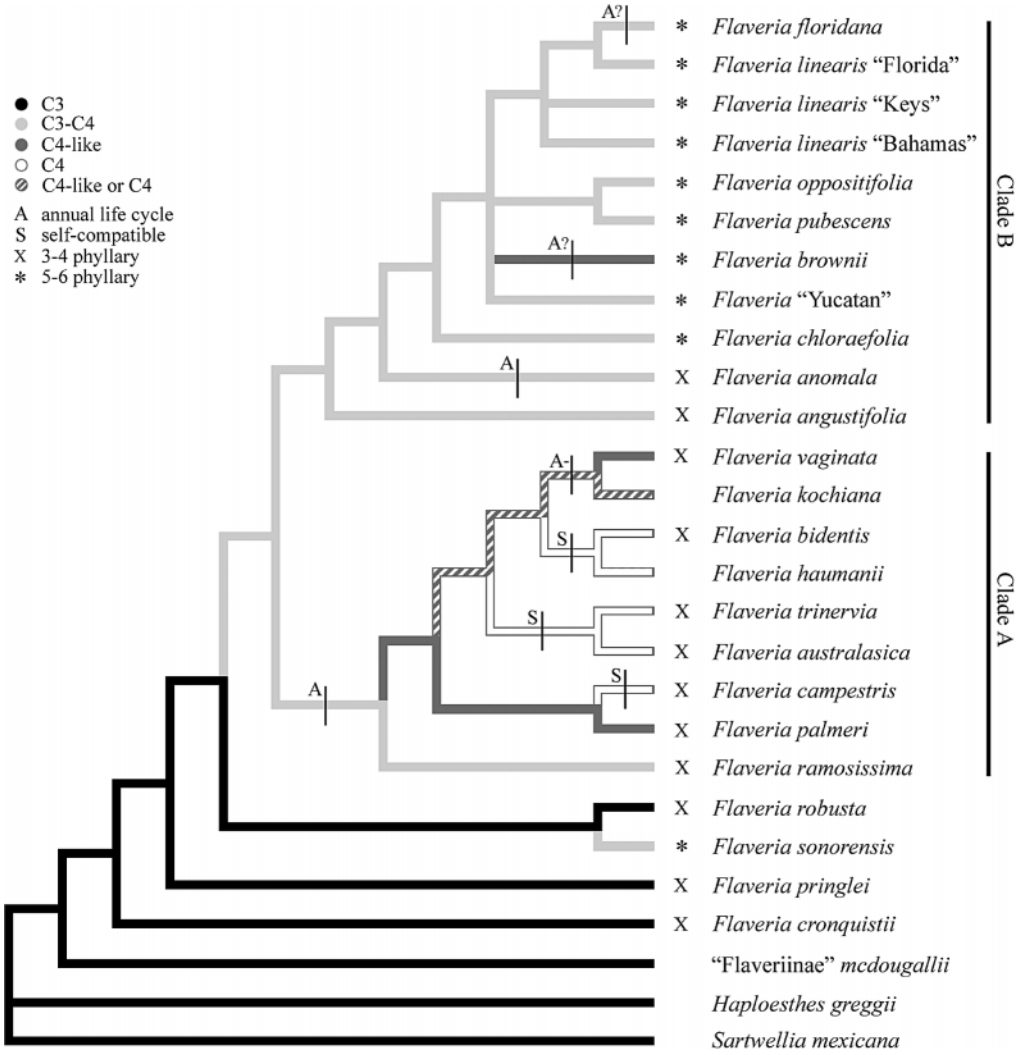

Fig. 6. Phylogeny of *Flaveria* based on combined gene and morphological data analysis from *Flaveria*, *Haploësthes* and *Sartwellia* species. Solid vertical lines represent well-supported clades A and B. Branch shading indicates photosynthetic types (listed in Table 1): black = C_3 photosynthesis, light gray = C_3-C_4 photosynthesis, dark gray = C_4-like photosynthesis, white = C_4 photosynthesis, white with gray hatches = C_4-like or C_4 photosynthesis. Symbols correspond to Powell's (1978) 3–4 (X) and 5–6 (*) phyllary groups. Derived life history features are indicated: annualism (A) or self-compatibility (S).

cies, *F. angustifolia*, is included in clade B by both Bayesian and parsimony analyses of the combined data; however, the posterior probability is low (65%) without the inclusion of morphological and life history data (Fig. 5). Morphological characters observed in *F. angustifolia*, such as stem pubescence and scorpioid corymb capitulescence, are features that are similar to the basal species of clade A (*F. ramosissima*); however, other characters, such as shallowly toothed leaf margins, corky phyllaries, campanulate corollas, and pubescent corolla tubes, are shared with the other basal species of clade B (*F. anomala*). In addition, growth habit and life history of *F. angustifolia* is more similar to other clade B species than it is to clade A species. The phyllary number of *F. angustifolia* (3–4) does not correspond with *F. ramosissima* (4–5) or *F. anomala* (2–4), and is consistent with species in clade A (3–4) rather than clade B (5–6), indicating homoplasy in the focal character previously used to designate subgeneric groups (Powell, 1978).

The species in clade B are circumscribed in Powell's 5–6 phyllary line, with the exception of *F. angustifolia* and *F. anomala* (Fig. 6; Powell, 1978). Species in clade B species are all self-incompatible and most are perennial, excepting *F. anomala*, which is annual, although it is suggested that *F. brownii* and *F. floridana* might be long-lived annuals (Fig. 6; Powell, 1978). Unlike clade A, there is not a strong trend in reducing floral features, and extensive homoplasic variation in vegetative and floral characters is present among these species. Plants of clade B species are moderately sized herbaceous or shrubby plants with sessile, linear to narrowly lanceolate leaves (excepting *F. chloraefolia*). The capitulescence shape tends to be a paniculate corymb, and corolla tubes are sparsely to moderately pubescent in these species. All samples from the different *Flaveria* species within clade B form respective monophyletic groups except samples labeled *F. chloraefolia* and *F. linearis* (Figs. 1–3). The genetically distinct, but morphologically similar *F. chloraefolia* sample labeled C (Figs. 1–3) is distinguished only by leaf length from other *F. chloraefolia* specimens and by its disjunct geographical location (Nuevo León). Further study is required to determine the extent of the range of this plant and whether any other morpho-

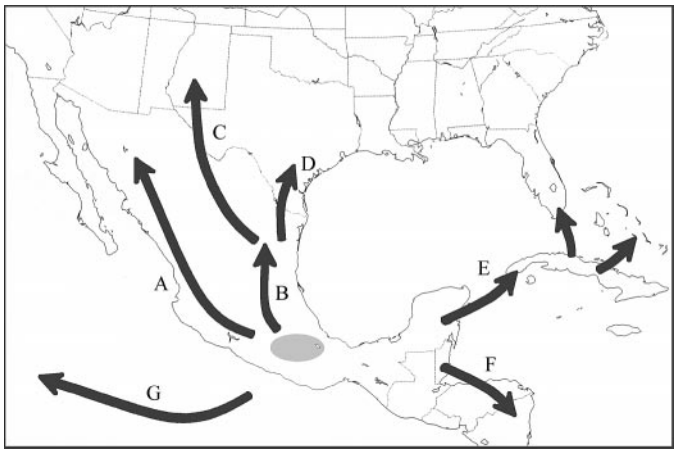

Fig. 7. Hypothesized radiations of species of *Flaveria* based on current distributions are indicated by dark gray arrows. Light gray shading indicates the Puebla-Oaxaca region of Mexico where basal species of *Flaveria*, in addition to clade A and clade B species of *Flaveria*, are located. A: *F. robusta* and *F. sonorensis*; B: clade A species (*F. palmeri*) and clade B species (*F. anomala*, *F. oppositifolia*, *F. pubescens*; C: clade A species (*F. campestris*) and clade B species (*F. chloraefolia*); D: clade B species (*F. brownii*); E: clade B species (*F. linearis* and *F. floridana*); F: clade A species (*F. bidentis* and *F. haumanii*); G: cosmopolitan species (*F. trinervia* and *F. bidentis*). Map generated using MapPad 2.0 (Keltner and Maher, 1996).

logical differences distinguish it from *F. chloraefolia* sensu stricto. The other paraphyletic taxon, *F. linearis*, likely represents more than one distinct entity, evident from both the genetic and morphological data. The more basal specimen according to our phylogeny (sample labeled C; Figs. 1–3, 5) is found in the Yucatán Peninsula area (A. D. McKown, collection notes) and is separated from other *F. linearis* samples with 100% posterior probability and 93% bootstrap supports (Fig. 5). Powell (1978) observed that *F. linearis* plants from this region are similar in morphology to *F. brownii*, which is consistent with our molecular phylogenetic results and morphological observations (Appendix). Features of these robust plants from the Yucatán Peninsula that are comparable to *F. brownii* include glabrous stems, glabrous, entire to shallowly toothed, narrowly lanceolate leaves, and loose, paniculate corymbs with ray and disc-type flowers. The other *F. linearis* specimens (samples labeled A, B, D–F; Figs. 1–3, 5) from the Bahamas and Florida form a clade with *F. floridana* but do not demonstrate cohesion either genetically or morphologically. Variability in morphological characters of *F. linearis* has also been noted by Long and Rhamstine (1968) and Powell (1978), in addition to physiological variability (Edwards and Ku, 1987). In this study, Floridean samples A and F and *F. floridana* cluster together with 98% posterior probability support and exclude samples labeled B and E (Keys) and D (Bahamas) (Fig. 5). The samples from the Keys and Bahamas have only disc flowers, whereas all other samples demonstrate ray and disc flowers. While samples from the Keys cluster together with 100% posterior probability (Fig. 5), there is little morphological similarity beyond inflorescence characters (Appendix). Therefore, it is clear that further detailed study of these entities, including *F. floridana*, is required to determine the history of this group and the potential number of taxa that may be included under ''*F. linearis*.''

The radiation of clade B has been restricted to North America and the West Indies (Fig. 7; Powell, 1978; A. D. McKown, collection notes). No species in this clade demonstrates the wide geographic range of the C_4 cosmopolitan weeds in clade A. The basal species *F. angustifolia* is found in the Tehuacán valley, and across the southern half of Puebla, northern part of Oaxaca, and west into central Guerrero (Powell, 1978; A. D. McKown, collection notes) sympatrically with *F. pringlei*. The other basal species, *F. anomala*, is found in the northeastern states of Mexico (in southern Coahuila, Durango, Nuevo León, San Luís Potosí, Queretaro, Tamaulipas, and eastern Zacatecas) (Fig. 7; Powell, 1978; A. D. McKown, collection notes). *Flaveria chloraefolia* sensu stricto, *F. oppositifolia*, and *F. pubescens* are also found in this area of northeast Mexico, and the range of *F. chloraefolia* sensu stricto also extends into Chihuahua, Texas, and New Mexico (Fig. 7; Powell, 1978; A. D. McKown, collection notes). *Flaveria brownii* occurs northwards from this northeastern area in Mexico in Tamaulipas and along the Texan Gulf of Mexico coast (Fig. 7; Powell, 1978; A. D. McKown, collection notes). A southeastern branch of clade B *Flaveria*, including *F. floridana* and the variable entities of *F. linearis*, extends from the Yucatán Peninsula to the West Indies and Florida (Fig. 7). The more basal *F. linearis* (represented by sample C) is found in the Yucatán and Quintana Roo (Mexico), Belize, and Honduras (Powell, 1978; A. D. McKown, collection notes) and may extend into Cuba based on morphological similarities of the specimens studied. If this entity is indeed the most basal of the *F. linearis* complex, it supports Powell's (1978) hypothesis that *F. linearis* originated in Mexico and spread eastward to the West Indies and into Florida. Consistent with this hypothesis is the recent divergence between *F. floridana* and *F. linearis* specimens from Florida.

Evolution of C_4 photosynthesis—The results of this study demonstrate two independent origins of C_3-C_4 intermediacy and C_4-like photosynthesis from C_3 ancestry. They also provide a phylogenetic context to examine physiological, genetic and ecological factors promoting the evolution of C_4 photosynthesis in dicots.

Multiple origins of C_3-C_4, C_4-like, and C_4 photosynthesis—All *Flaveria* species with C_3 photosynthesis are restricted to the basal portions of the phylogeny (Figs. 5, 6). This result is also supported by the H-protein phylogeny of Kopriva et al. (1996), but is not apparent in the Westhoff and Gowik (2004) PEPCase phylogeny. The inclusion of all known C_3 *Flaveria* species in our study confirms that the ancestral condition in *Flaveria* is unambiguously C_3 photosynthesis. Photosynthetically intermediate *Flaveria* species are all placed within clades A and B, excepting C_3-C_4 *F. sonorensis*. The phylogenetically disjunct *F. sonorensis* is also geographically distant from the other intermediate *Flaveria* species and represents an independent evolution of C_3-C_4 intermediacy. Physiological characteristics of *F. sonorensis* indicate that photosynthesis in this species is more C_3-like (Moore et al., 1987; Ku et al., 1991). The CO_2 compensation point of *F. sonorensis* is lower than that of its sister species *F. robusta*, but there is relatively little difference in O_2 inhibition between the two species. In addition, *F. sonorensis* does not operate a partial C_4 cycle (Ku et al., 1991) and should be considered a type I intermediate (no C_4 cycle [Edwards and Ku, 1987]). Within clade A and B, the absence of C_3 species at the base of both clades suggests a second origin of C_3-C_4 intermediacy in the shared common ancestor of these clades. This shared origin of intermediacy

giving rise to both lineages was also suggested by Kopriva et al. (1996). While the basal species of clades A and B (*F. angustifolia* and *F. ramosissima*) are C_3-C_4 photosynthetic intermediates, the physiological characteristics of both species differ. *Flaveria angustifolia* is similar to *F. sonorensis*, classifying it as a type I intermediate; however, *F. ramosissima* has a substantially lower CO_2 compensation point, lower O_2 inhibition, and a limited C_4 cycle compared to C_3 species and type I C_3-C_4 intermediates (Ku et al., 1991), and is a type II intermediate (with C_4 cycle [Edwards and Ku, 1987]).

Within clade A, type II C_3-C_4 photosynthesis coincides with the evolution of an annual life cycle in *Flaveria ramosissima* (Fig. 6). The phylogenetic position of *F. ramosissima* suggests that its common ancestor with other clade A species (C_4-like and C_4) was C_3-C_4, thereby supporting an evolutionary intermediate position for C_3-C_4 photosynthesis. The common ancestor to the other derived clade A species (C_4-like and C_4) may have been an advanced intermediate similar to the C_4-like species *F. palmeri*, suggesting that fully expressed C_4 photosynthesis may have evolved up to three times in clade A (once each in *F. australasica*/*trinervia*, *F. bidentis*/*haumanii*, and *F. campestris*) along with the evolution of self-compatibility (Fig. 6). An alternative hypothesis, although unlikely, is that the common ancestor to C_4-like and C_4 clade A species was fully expressed C_4 photosynthesis and that both C_4-like *F. palmeri* and *F. vaginata* are reversals from C_4 photosynthesis (Monson and Moore, 1989). Further study to determine the photosynthetic type of *F. kochiana* (currently known to be either C_4-like or C_4 [A. D. McKown, unpublished data]) may assist in interpretation of the photosynthetic evolutionary history in this clade.

All species in clade B are C_3-C_4 intermediate, excepting *Flaveria brownii*, which is classified as having C_4-like photosynthesis, and fully expressed C_4 photosynthesis is absent in clade B. The short branch lengths of the more derived portion of clade B in the genetic analyses (Figs. 2, 3) may indicate a rapid evolution and radiation of species and prevent the full reconstruction of the evolutionary history of this clade from our data. As a result, only the basal C_3-C_4 species *F. angustifolia* and *F. anomala* are placed with certainty in a phylogenetically intermediate position between C_3 photosynthetic species and C_4-like *F. brownii*. The other C_3-C_4 species do not demonstrate unequivocal intermediate phylogenetic placements, although the *trnL-F* and ITS analyses suggest that *F. chloraefolia* should be considered phylogenetically basal to C_4-like *F. brownii*. The basal species *F. angustifolia* is designated as a type I intermediate; however, the other basal species, *F. anomala*, has physiological characteristics supporting its classification as a type II intermediate species (Edwards and Ku, 1987; Moore et al., 1987; Ku et al., 1991). Similar to *F. ramosissima* of clade A, the appearance of type II intermediacy in *F. anomala* coincides with annualism (Fig. 6). Of the remaining C_4-C_4 intermediate species, most demonstrate little C_4 cycle activity, except *F. floridana* (Monson et al., 1986; Moore et al., 1987; Chastain and Chollet, 1989; Ku et al., 1991). The derived species *F. brownii* with C_4-like photosynthesis is clearly not directly related to the C_4-like and C_4 species of clade A, supporting Powell's (1978) hypothesis that C_4 (or C_4-like) photosynthesis in *Flaveria* has evolved independently at least twice (Fig. 6). The presence of one ''advanced'' C_3-C_4 species (*F. floridana*) and one C_4-like species (*F. brownii*) at the tips of clade B does not signify that all C_3-C_4 species in this clade are evolving towards full C_4 photosynthesis. It is possible that *F. linearis* (all entities) and *F. floridana* are both undergoing photosynthetic-type reversal (Monson and Moore, 1989), but the recent origin of these species, as indicated by the phylogenetic analyses, does not suggest sufficient evolutionary time for this scenario. The evolutionary intermediate positions of C_3-C_4 photosynthesis (in *F. angustifolia* and *F. anomala*) and C_4-like photosynthesis (in *F. brownii*) is clear; however, caution should be exercised in the interpretation of other clade B *Flaveria* species.

Photosynthetic intermediate species in comparative studies are generally interpreted to represent evolutionary steps progressing towards the development of fully expressed C_4 photosynthesis (Edwards and Ku, 1987; Monson, 1989; Monson and Moore, 1989). Evolution patterns in *Flaveria* demonstrate this to be the case with intermediacy in clade A, although this is not as clear among intermediates in clade B. Monson and Moore (1989) discussed three alternatives for the origin of C_3-C_4 intermediate species. The first considers C_3-C_4 intermediates as a product of reverse evolution from C_4 photosynthesis; however, the ecology of the intermediates and the unapparent adaptive advantage this strategy would confer do not support this hypothesis (Monson and Moore, 1989). The recent divergence of *Flaveria* species, especially derived clade B species, suggested by our phylogeny also rejects this hypothesis. A second hypothesis suggests that C_3-C_4 intermediates are the result of hybridization between C_3 and C_4 plants. Within *Flaveria*, reproductive isolation exists among some species, and the only C_3 species observed to form hybrids does not do so with C_4 plants (Powell, 1978). In addition to the naturally occurring *F. pringlei* × *F. angustifolia* hybrids, only two other hybrids were found. These were between research collection plants but were not hybrids of C_3 and C_4 species. Therefore, it is highly unlikely that the C_3-C_4 *Flaveria* intermediates arose from hybridization between C_3 and C_4 *Flaveria*. Despite the recent and rapid radiation inferred by the phylogenetic results of clade B and the high crossability of species in this clade (Powell, 1978), lack of hybrid evidence suggests that intermediacy was not spread through this clade by means of hybridization. The third hypothesis is that C_3-C_4 intermediates are evolutionary ''dead-ends'' and will not evolve fully expressed C_4 metabolism (Monson and Moore, 1989). Phylogenetic evidence suggests that this is not the case in clade A (Figs. 5, 6). The diversity of physiological characteristics in clade B photosynthetic intermediates (type I and type II) demonstrates that C_3-C_4 intermediacy is not identical in these species (Edwards and Ku, 1987; Ku et al., 1991, Dai et al., 1996), which is also suggestive that these intermediates are not ''dead-ends.'' As mentioned by Monson and Moore (1989), niches can exist for intermediate plants, and therefore the potential to evolve fully expressed C_4 photosynthesis exists, but is not realized without C_4-selecting conditions.

Ecological conditions and life history traits—Sage (2004) outlines that under current atmospheric conditions and at warm temperatures (>30°C), photosynthesis can be inhibited by 30% due to photorespiration. Some intermediate species of *Flaveria* (type II with a limited C_4 cycle [Edwards and Ku, 1987]) demonstrate an increase in initial assimilation of CO_2 into C_4 acids under low CO_2 levels, which promotes photorespiration (Chastain and Chollet, 1989). This supports the hypothesis that CO_2 levels play a selective role in C_4 photosynthesis in *Flaveria*. In addition to the lower CO_2 levels recorded in recent geological time, other environmental conditions pro-

moting photorespiration could act as selective forces, such as heat, drought, low humidity, or salinity (Sage, 2001, 2004). The Puebla-Oaxaca region of Mexico is warm and arid, and two C_3-C_4 intermediate species in this region (*F. angustifolia* of clade B and *F. ramosissima* of clade A) are known to occur in sandy and possibly gypseous soils (Powell, 1978). The aridity of this environment and the potential nutrient-deficiency of these soils (Jafarzadeh and Zinck, 2000) could act to select for adaptations compensating for a higher rate of photorespiration and water use efficiency (Sage, 2004). The two basal C_3 species (*F. cronquistii* and *F. pringlei*) occur, however, in the same region and in similar habitats as these intermediates (Powell, 1978). C_3-C_4 intermediate *F. sonorensis* and species of clade B are all described as occurring in saline or gyseous soils, but are also generally found in more mesic habitats (Long and Rhamstine, 1968; Powell, 1978). In particular, C_4-like *F. brownii* is found along saline, sandy coastal flats and brackish marshes of the Gulf of Mexico. This highly saline environment may have represented a more stringent selective force (e.g., for higher water use efficiency) and propelled *F. brownii* towards developing C_4-like photosynthesis. Supporting this hypothesis are the ecological and physiological data from *F. floridana*, as this species also occurs in coastal saline, sandy soils near brackish marshy areas in Florida (Long and Rhamstine, 1968) and is a physiologically advanced C_3-C_4 intermediate in clade B (Monson et al., 1986, 1988; Moore et al., 1987; Brown and Hattersley, 1989; Chastain and Chollet, 1989; Ku et al., 1991; Dai et al., 1996). Monson and Jaeger (1991) demonstrated that C_3-C_4 intermediacy in *F. floridana* conveys ecological advantages (e.g., higher rates of photosynthesis) compared to C_3 plants in the same natural habitat at high temperatures. Thus, despite variability in habitats, the ecological conditions *Flaveria* species currently experience (high light intensity, heat, aridity, and saline or gypseous soil) suggest environments that promote photorespiration and might exert adaptive pressure towards evolving the development of full C_4 photosynthesis.

Flaveriinae species (*Flaveria*, *Haploësthes*, and *Sartwellia*) are found in gypseous soils, indicating a common ecological niche; however, photosynthetic intermediacy and C_4 photosynthesis are only known in *Flaveria*. Gene duplication and neofunctionalization are outlined in *Flaveria* as preceding the evolution of many C_4 biochemical attributes (reviewed in Monson, 2003). It is likely that this genetic ''preconditioning'' exists in *Flaveria* species of clades A and B, but is lacking in the basal C_3 *Flaveria*, *Haploësthes* and *Sartwellia* species. Assuming similar genetic preconditioning for *Flaveria* species, the rate at which the C_4 syndrome can evolve in each species may be determined by the species' life history (Monson, 2003). This hypothesis is supported by the co-existence of annualism and C_4, C_4-like or physiologically advanced C_3-C_4 intermediate photosynthesis in both derived clades of *Flaveria* species (Fig. 6). In annual plants, generation time is shorter and the rate of gene recruitment and modification for C_4 photosynthesis is potentially higher (Monson, 2003). Gene recruitment processes may be enhanced by inbreeding in *Flaveria*, as all known C_4 species of *Flaveria* are self-compatible (Powell, 1978). The recent evolution of C_4-like photosynthesis in *F. brownii* indicates that the intensity of environmental selection pressures, such as heat and salinity, also plays a major selective role in the evolution of C_4 photosynthesis in the genus *Flaveria*. Together, the number of fully developed C_4 species in this genus and the multiple evolutions of intermediacy all suggest a latent ability to evolve C_4 photosynthesis. Within *Flaveria*, however, we hypothesize that the evolution of this complex adaptation is realized through the intensity of environmental selection pressure and through modification of a species' life history to exploit the presence of genetic preconditioning outlined in *Flaveria*.

Concluding remarks—The well-resolved phylogeny of nearly all *Flaveria* species enables a better understanding of the evolutionary relationships among *Flaveria* species and the biogeographical patterns of dispersal in this genus. The presence of intermediate (C_3-C_4 and C_4-like) species in separate clades of *Flaveria* is in agreement with earlier studies that have suggested multiple origins of photosynthetic intermediacy in this genus (Powell, 1978; Kopriva et al., 1996; Monson, 1996; Westhoff and Gowik, 2004). Our phylogenetic evidence indicates more precisely where, and how many times, multiple and parallel evolutions of C_3-C_4 or C_4-like photosynthesis have occurred. This information will be useful in evaluating the appropriateness of species choice in future comparative studies and supports review of the large body of existing *Flaveria* research in a phylogenetic context. *Flaveria* characteristics, especially of the intermediate species, underpin many models of C_4 evolution and phylogeny (Rosche and Westhoff, 1995; Drincovich et al., 1998; Monson, 1999, 2003; Engelmann et al., 2003; Sage, 2004; Westhoff and Gowik, 2004). Within the phylogenetic context proposed, characteristics in the progression of C_3-C_4 to C_4-like/C_4 photosynthesis in clade A, and traits that have evolved in parallel among species of clade A and B can be identified. This will greatly facilitate testing stepwise evolutionary models for C_4 photosynthesis in dicots in general, thereby advancing our understanding of C_4 photosynthetic evolution in plants.

LITERATURE CITED

Apel, P., and I. Maass. 1981. Photosynthesis in species of *Flaveria*. CO_2 compensation concentration, O_2 influence on photosynthetic gas exchange and $\delta^{13}C$ values in species of *Flaveria* (Asteraceae). *Biochemie und Physiologie der Pflanzen* 176: 396–399.

Baldwin, B. G., and S. Markos. 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S–26S rDNA: congruence of ETS and ITS trees of Calycadenia (Compositae). *Molecular Phylogenetics and Evolution* 10: 449–463.

Baldwin, B. G., B. L. Wessa, and J. Panero. 2002. Nuclear rDNA evidence for major lineages of helenioid Heliantheae (Compositae). *Systematic Botany* 27: 161–198.

Bassüner, B., O. Keerberg, H. Bauwe, T. Pärnik, and H. Keerberg. 1984. Photosynthetic CO_2 metabolism in C_3-C_4 intermediate and C_4 species of *Flaveria* (Asteraceae). *Biochemie und Physiologie der Pflanzen* 179: 631–634.

Bauwe, H. 1984. Photosynthetic enzyme activities and immunofluorescence studies on the localization of ribulose-1,5-bisphosphate carboxylase/oxygenase in leaves of C_3, C_4, and C_3-C_4 intermediate species of *Flaveria* (Asteraceae). *Biochemie und Physiologie der Pflanzen* 179: 253–268.

Bauwe, H., and R. Chollet. 1986. Kinetic properties of phosphoenolpyruvate carboxylase from C_3, C_4 and C_3-C_4 intermediate species of *Flaveria* (Asteraceae). *Plant Physiology* 82: 695–699.

Brown, R. H., C. L. Bassett, R. G. Cameron, P. T. Evans, J. H. Bouton, C. C. Black, L. O. Sternberg, and M. J. DeNiro. 1986. Photosynthesis of F1 hybrids between C_4 and C_3-C_4 species of *Flaveria*. *Plant Physiology* 82: 211–217.

Brown, R. H., and P. W. Hattersley. 1989. Leaf anatomy of C_3-C_4 species as related to evolution of C_4 photosynthesis. *Plant Physiology* 91: 1543–1550.

Bruhl, J. J., and S. Perry. 1995. Photosynthetic pathway-related ultrastructure of C_3, C_4 and C_3-like C_3-C_4 intermediate sedges (Cyperaceae) with

special reference to *Eleocharis*. *Australian Journal of Plant Physiology* 22: 521–530.

BRUHL, J. J., N. E. STONE, AND P. W. HATTERSLEY. 1987. C_4 acid decarboxylation enzymes and anatomy in sedges (Cyperaceae): first record of NAD-malic enzyme species. *Australian Journal of Plant Physiology* 14: 719–728.

BRUNEAU, A., F. FOREST, P. S. HERENDEEN, B. B. KLITGAARD, AND G. P. LEWIS. 2001. Phylogenetic relationships in the Caesalpinioideae (Leguminosae) as inferred from chloroplast *trn*L intron sequences. *Systematic Botany* 26: 487–514.

CAMERON, R. G., C. L. BASSETT, J. H. BOUTON, AND R. H. BROWN. 1989. Transfer of C_4 photosynthetic characters through hybridization of *Flaveria* species. *Plant Physiology* 90: 1538–1545.

CHASTAIN, C. J., AND R. CHOLLET. 1989. Interspecific variation in assimilation of ^{14}C into C_4 acids by leaves of C_3, C_4, and C_3-C_4 intermediate *Flaveria* species near CO_2 compensation point (concentration). *Planta* 179: 81–88.

CHENG, S. H., B. D. MOORE, AND G. E. EDWARDS. 1988. Photosynthesis in *Flaveria brownii*, a C_4-like species. Leaf anatomy, characteristics of CO_2 exchange, compartmentation of photosynthetic enzymes, and metabolism of $^{14}CO_2$. *Plant Physiology* 87: 867–873.

CHENNA, R., H. SUGAWARA, T. KOIKE, R. LOPEZ, T. J. GIBSON, D. G. HIGGINS, AND J. D. THOMPSON. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research* 31: 3497–3500.

DAI, Z., M. S. B. KU, AND G. E. EDWARDS. 1996. Oxygen sensitivity of photosynthesis and photorespiration in different photosynthetic types in the genus *Flaveria*. *Planta* 198: 563–571.

DIMITRI, M. J., AND E. N. ORFILA. 1986. Acerca del nuevo taxón *Flaveria haumanii* Dim. & Orf. (Compositae) de la Flora Argentina. *Sociedad Científica Argentina, Buenos Aires*: 1–13.

DRINCOVICH, M. F., P. CASATI, C. S. ANDREO, S. J. CHESSIN, V. R. FRANCESCHI, G. E. EDWARDS, AND M. S. KU. 1998. Evolution of C_4 photosynthesis in *Flaveria* species: isoforms of NADP-malic enzyme. *Plant Physiology* 117: 733–744.

EDWARDS, G. E., AND M. S. B. KU. 1987. Biochemistry of C_3-C_4 intermediates. *In* P. K. Stumpf and E. E. Conn [eds.], The biochemistry of plants, vol. 10, Photosynthesis, 275–325. Academic Press, London, UK.

ENGELMANN, S., O. E. BLASING, U. GOWIK, P. SVENSSON, AND P. WESTHOFF. 2003. Molecular evolution of C_4 phosphoenolpyruvate carboxylase in the genus *Flaveria*—a gradual increase from C_3 to C_4 characteristics. *Planta* 217: 717–725.

GUISSANI, L. M., J. H. COTA-SÁNCHEZ, F. O. ZULOAGA, AND E. A. KELLOGG. 2001. A molecular phylogeny of the grass subfamily Panicoideae (Poaceae) shows multiple origins of C_4 photosynthesis. *American Journal of Botany* 88: 1993–2012.

HOLADAY, A. S., K. W. LEE, AND R. CHOLLET. 1984. C_3-C_4 intermediate species in the genus *Flaveria*: leaf anatomy, ultrastructure, and the effect of O_2 on the CO_2 compensation concentration. *Planta* 160: 25–32.

HUELSENBECK, J. P., AND F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* 17: 754–755.

HYLTON, C. M., S. RAWSTHORNE, A. M. SMITH, D. A. JONES, AND H. W. WOOLHOUSE. 1988. Glycine decarboxylase is confined to the bundle sheath cells of leaves of C_3-C_4 intermediate species. *Planta* 175: 452–459.

JAFARZADEH, A. A., AND J. A. ZINCK. 2000. Worldwide distribution and sustainable management of soils with gypsum. *In* Proceedings of the International Symposium on Desertification, June 2000, Konya, Turkey, 362–368. Soil Science Society of Turkey, Turkey.

KADEREIT, G., T. BORSCH, K. WEISING, AND H. FREITAG. 2003. Phylogeny of Amaranthaceae and Chenopodiaceae and the evolution of C_4 photosynthesis. *International Journal of Plant Sciences* 164: 959–986.

KELLOGG, E. A. 1999. Phylogenetic aspects of the evolution of C_4 photosynthesis. *In* R. F. Sage and R. K. Monson [eds.], C_4 plant biology, 411–444. Academic Press, San Diego, California, USA.

KELTNER, J., AND L. MAHER. 1996. MapPad, version 2.0. Computer program distributed by the author; available at website, http://www.ncdc.noaa.gov/paleo/softlib/mappad.html.

KOPRIVA, S., C. C. CHU, AND H. BAUWE. 1996. Molecular phylogeny of *Flaveria* as deduced from the analysis of nucleotide sequences encoding the H-protein of the glycine cleavage system. *Plant, Cell & Environment* 19: 1028–1036.

KU, M. S. B., R. K. MONSON, AND R. O. LITTLEJOHN JR. 1983. Photosynthetic characteristics of C_3-C_4 intermediate *Flaveria* species: leaf anatomy, photosynthetic responses to O_2 and CO_2, and activities of key enzymes in the C_3 and C_4 pathways. *Plant Physiology* 71: 944–948.

KU, M. S. B., J. WU, AND Z. DAI. 1991. Photosynthetic and photorespiratory characteristics of *Flaveria* species. *Plant Physiology* 96: 518–528.

LONG, R. W., AND E. L. RHAMSTINE. 1968. Evidence for the hybrid origin of *Flaveria latifolia* (Compositae). *Brittonia* 3: 238–250.

MONSON, R. K. 1989. The relative contributions of reduced photorespiration, and improved water- and nitrogen-use to the advantages of C_3-C_4 intermediate photosynthesis in *Flaveria*. *Oecologia* 80: 215–221.

MONSON, R. K. 1996. The use of phylogenetic perspective in comparative plant physiology and developmental biology. *Annals of the Missouri Botanical Garden* 83: 3–16.

MONSON, R. K. 1999. The origins of C_4 genes and evolutionary pattern in the C_4 metabolic phenotype. *In* R. F. Sage and R. K. Monson [eds.], C_4 plant biology, 49–87. Academic Press, San Diego, California, USA.

MONSON, R. K. 2003. Gene duplication, neofunctionalization, and the evolution of C_4 photosynthesis. *International Journal of Plant Sciences* 164 (Supplement): S43–S54.

MONSON, R. K., AND C. H. JAEGER. 1991. Photosynthetic characteristics of C_3-C_4 intermediate *Flaveria floridana* (Asteraceae) in natural habitats: evidence of advantages to C_3-C_4 photosynthesis at high leaf temperatures. *American Journal of Botany* 78: 795–800.

MONSON, R. K., AND B. D. MOORE. 1989. On the significance of C_3-C_4 intermediate photosynthesis to the evolution of C_4 photosynthesis. *Plant, Cell and Environment* 12: 689–699.

MONSON, R. K., B. D. MOORE, M. S. B. KU, AND G. E. EDWARDS. 1986. Co-function of C_3- and C_4-photosynthetic pathways in C_3, C_4 and C_3-C_4 intermediate *Flaveria* species. *Planta* 168: 493–502.

MONSON, R. K., AND S. RAWSTHORNE. 2000. CO_2 assimilation in C_3-C_4 intermediate plants. *In* R. C. Leegood, T. D. Sharkey, and S. C. von Caemmerer [eds.], Photosynthesis: physiology and metabolism, 533–550. Kluwer, Dordrecht, Netherlands.

MONSON, R. K., W. S. SCHUSTER, AND M. S. B. KU. 1987. Photosynthesis in *Flaveria brownii* A. M. Powell. A C_4-like C_3-C_4 intermediate. *Plant Physiology* 85: 1063–1067.

MONSON, R. K., J. TEERI, M. S. B. KU, J. GUREVITCH, L. METS, AND S. DUDLEY. 1988. Carbon-isotope discrimination by leaves of *Flaveria* species exhibiting different amounts of C_3- and C_4- cycle co-function. *Planta* 174: 145–151.

MOORE, B. D., M. S. B. KU, AND G. E. EDWARDS. 1987. C_4 photosynthesis and light-dependent accumulation of inorganic carbon in leaves of C_3-C_4 and C_4 *Flaveria* species. *Australian Journal of Plant Physiology* 14: 657–668.

MOORE, B. D., R. K. MONSON, M. S. B. KU, AND G. E. EDWARDS. 1988. Activities of principal photosynthetic and photorespiratory enzymes in leaf mesophyll and bundle sheath protoplasts from the C_3-C_4 interemediate *Flaveria ramosissima*. *Plant Cell Physiology* 29: 999–1006.

PETENATTI, E. M., AND L. A. DEL VITTO. 2000. Estructura Kranz en las especies argentinas de *Flaveria* (Asteraceae-Helenieae). [Kranz structure in Argentinian species of *Flaveria* (Asteraceae-Helenieae).] *Kurtziana* 28: 251–257.

POWELL, A. M. 1978. Systematics of *Flaveria* (Flaveriinae-Asteraceae). *Annals of the Missouri Botanical Garden* 65: 590–636.

PYANKOV, V. I., E. G. ARTYUSHEVA, G. E. EDWARDS, C. C. BLACK JR. AND P. S. SOLTIS. 2001. Phylogenetic analysis of tribe Salsoleae (Chenopodiaceae) based on ribosomal ITS sequences: implications for the evolution of photosynthesis types. *American Journal of Botany* 88: 1189–1198.

RAMBAUT, A. 1996. Se-Al: Sequence alignment editor. Computer program distributed by the author, available at website, http://evolve.zoo.ox.ac.uk/.

REED, J. E., AND R. CHOLLET. 1985. Immunofluorescent localization of phosphoenolpyruvate carboxylase and ribulose 1,5-bisphosphate carboxylase/oxygenase proteins in leaves of C_3, C_4, and C_3-C_4 intermediate *Flaveria* species. *Planta* 165: 439–445.

ROSCHE, E., AND P. WESTHOFF. 1995. Genomic structure and expression of the pyruvate, orthophosphate dikinase gene of the dicotyledonous C_4 plant *Flaveria trinervia* (Asteraceae). *Plant Molecular Biology* 29: 663–678.

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

RUMPHO, M. E., M. S. B. KU, AND S. H. CHENG. 1984. Photosynthetic characteristics of C_3-C_4 intermediate *Flaveria* species: reduction of photores-

piration by a limited C_4 pathway of photosynthesis in *Flaveria ramosissima*. *Plant Physiology* 75: 993–996.

SAGE, R. F. 2001. Environmental and evolutionary preconditions for the origin and diversification of the C_4 photosynthetic syndrome. *Plant Biology* 3: 202–213.

SAGE, R. F. 2004. The evolution of C_4 photosynthesis. *New Phytologist* 161: 341–370.

SAGE, R. F., M. R. LI, AND R. K. MONSON. 1999. The taxonomic distribution of C_4 photosynthesis. *In* R. F. Sage and R. K. Monson [eds.], C_4 plant biology, 551–584. Academic Press, San Diego, California, USA.

SINHA, N. R., AND E. A. KELLOGG. 1996. Parallelism and diversity in multiple origins of C_4 photosynthesis in the grass family. *American Journal of Botany* 83: 1458–1470.

SMITH, B. N., AND B. L. TURNER. 1975. Distribution of Kranz syndrome among Asteraceae. *American Journal of Botany* 62: 541–545.

SOROS, C. L., AND J. J. BRUHL. 2000. Multiple evolutionary origins of C_4 photosynthesis in the Cyperaceae. *In* K. L. Wilson and D. A. Morrison [eds.] Monocots, vol. 1, Systematics and evolution, 629–636. CSIRO, Collingwood, Australia.

SVENSSON, P., O. E. BLÄSING, AND P. WESTHOFF. 2003. Evolution of C_4 phosphoenolpyruvate carboxylase. *Archives of Biochemistry and Bioscience* 414: 180–188.

SWOFFORD, D. L. 2003. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4. Sinauer, Sunderland, Massachusetts, USA.

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

TURNER, B. L. 1971. Taxonomy of *Sartwellia* (Compositae: Helenieae). *Sida* 4: 265–273.

TURNER, B. L. 1975. Taxonomy of *Haploësthes* (Asteraceae-Senecioneae). *Wrightia* 5: 108–119.

TURNER, B. L. 1995. A new species of *Flaveria* (Asteraceae, Helenieae) from Oaxaca, Mexico. *Phytologia* 78: 400–401.

TURNER, B. L., AND A. M. POWELL. 1977. Helenieae—systematic review. *In* V. H. Heywood, J. B. Harborne, and B. L. Turner [eds.], The biology and chemistry of the Compositae, vol. 2, 699–737. Academic Press, London, UK.

WESTHOFF, P., AND U. GOWIK. 2004. Evolution of C_4 phosphoenolpyruvate carboxylase. Genes and proteins: a case study with the genus *Flaveria*. *Annals of Botany* 93: 13–23.

WHITE, T. J., T. BRUNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* M. A. Innis, D. H. Gelfand, J. Sninsky, and T. J. White [eds.], PCR protocols: a guide to methods and applications, 315–322. Academic Press, New York, New York, USA.

APPENDIX. Taxa used in this study, specimen replicate, GenBank accession numbers for the three sequences studied, collector, collector's number, and herbarium. GenBank accession numbers are listed in the following order: trnL-F, ITS, ETS. A dash indicates the region was not sampled. Voucher specimens are located in the following herbaria: AA = Arnold Arboretum, Harvard University, Cambridge, Massachusetts, USA; GH = Gray Herbarium, Harvard University; LL = Lundell Herbarium, University of Texas, Austin, Texas, USA; NY = New York Botanical Gardens, Bronx, New York, USA; SRSC = Sul Ross State University, Alpine, Texas, USA; TAES = Tracy Herbarium, Texas A & M University, College Station, Texas, USA; TEX = University of Texas, Austin, Texas, USA; TRT = Royal Ontario Museum, Toronto, Ontario, Canada. Additional plant materials of *Flaveria* were donated by A. M. Powell (Sul Ross State University, Alpine, Texas, USA) and E. Sudderth (Harvard University).

Taxon; replicate; *trnL-F*; ITS; ETS; Voucher information.

Flaveria angustifolia (Cav.) Persoon; A; DQ122561; DQ122483; DQ122426; *McKown, 10906,* TRT. ***F. angustifolia*** (Cav.) Persoon B; DQ122560; DQ122482; DQ122427; *Calzada, 19400,* NY. ***F. anomala*** B. L. Robinson; A; DQ122586; DQ122509, DQ122516, DQ122518; DQ122448, DQ122449, DQ122450; *McKown, 10907,* TRT. ***F. anomala*** B. L. Robinson; B; DQ122585; DQ122508, DQ122513; —; *Powell, 2579,* fruit material. ***F. anomala*** B.L. Robinson; C; DQ122589; DQ122514; —; *Nesom and Wells, 6630,* TEX. ***F. anomala*** B.L. Robinson; D; DQ122590; —; —; *Powell, 2599,* SRSC. ***F. anomala*** B.L. Robinson; E; DQ122587; DQ122510, DQ122515, DQ122517; —; *Hinton et al., 22360,* TEX. ***F. anomala*** B.L. Robinson; F; DQ122588; DQ122511, DQ122512; —; *Hinton et al., 19564,* TEX. ***F. australasica*** Hooker; A; DQ122602; DQ122529; —; *McKown, 10911,* TRT. ***F. australasica*** Hooker; B; DQ122603; DQ122530; DQ122402; *Powell, 5843,* fruit material. ***F. australasica*** Hooker; C; DQ122601; —; —; *Pedley, 763,* NY.***F. australasica*** Hooker; D; —; DQ122531; DQ122403; *Craven, 5278,* AA.***F. bidentis*** (L.) Kuntze; A; DQ122617; DQ122544; —; *McKown, 10909,* TRT. ***F. bidentis*** (L.) Kuntze; B; DQ122615; DQ122542; —; *Powell, 361,* fruit material. ***F. bidentis*** (L.) Kuntze; C; DQ122616; DQ122543; DQ122410; *Nee, 51694,* TEX. ***F. brownii*** A. M. Powell; A; DQ122578; DQ122500, DQ122501; —; *McKown, 10912,* TRT. ***F. brownii*** A.M. Powell; B; DQ122577; DQ122499; DQ122419; *Loring, s.n., 1993,* SRSC. ***F. brownii*** A.M. Powell; C; DQ122579; DQ122502; —; *Richardson et al., 2605,* TEX. ***F. brownii*** A. M. Powell; D; DQ122580; DQ122503; —; *Powell, 2802,* SRSC. ***F. campestris*** J.R. Johnston; A; DQ122605; DQ122533; —; *Powell and Powell, 3011,* SRSC. ***F. campestris*** J.R. Johnston; B; DQ122604; DQ122532; DQ122412; *Loring, 2000–94,* SRSC. ***F. campestris*** J.R. Johnston; C; —; DQ122534; DQ122413; *Wagenknecht, 3144,* GH. ***F. chloraefolia*** A. Gray; A; DQ122583; DQ122506; DQ122417; *McKown, 10904,* TRT. ***F. chloraefolia*** A. Gray; B; DQ122582; DQ122505; —; *Powell and Powell, 3036,* NY. ***F. chloraefolia*** A. Gray; C; DQ122571; DQ122493; DQ122425; *Hinton et al., 21951,* TEX. ***F. chloraefolia*** A. Gray; D; DQ122584; DQ122507; —; *Villarreal and Carranza, 2307,* TAES. ***F. cronquistii*** A.M. Powell; A; DQ122555; DQ122475; DQ122431; *Anderson and Anderson, 5341,* NY. ***F. cronquistii*** A.M. Powell; B; DQ122553; DQ122473; —; *Turner, 0–31,* SRSC. ***F. cronquistii*** A.M. Powell; C; DQ122554; DQ122474;—; *Rzedowski, 37186,* NY. ***F. floridana*** J.R. Johnston; A; DQ122566; DQ122488; DQ122422; *McKown, 10903,* TRT. ***F. floridana*** J.R. Johnston; B; DQ122563; DQ122485; —; *Powell, 342,* fruit material. ***F. floridana*** J.R. Johnston; C; DQ122562; DQ122484; DQ122421; *Moldenke and Moldenke, 29697,* LL. ***F. floridana*** J.R. Johnston; D; DQ122565; DQ122487; —; *Brumbach, 8868,* NY. ***F. kochiana*** B.L. Turner; A; DQ122611; DQ122538; DQ122404; *Zamudio and Ocampo, 10973,* TEX. ***F. kochiana*** B.L. Turner; B; DQ122612; DQ122539; DQ122405; *Sudderth, 8,* leaf material. ***F. kochiana*** B. L. Turner; C; DQ122613; DQ122540; DQ122406; *Sudderth, 10,* leaf material. ***F. kochiana*** B.L. Turner; D; DQ122614; DQ122541; DQ122407; *Sudderth, 11,* leaf material. ***F. linearis*** Lagasca; A; DQ122564; DQ122486; DQ122418; *McKown, 10908,* TRT. ***F. linearis*** Lagasca; B; DQ122567; DQ122489; DQ122420; *Brown and Evans, F-22,* SRSC. ***F. linearis*** Lagasca; C; DQ122581; DQ122504; DQ122424; *Nee and Atha, 46867,* LL. ***F. linearis*** Lagasca; D; DQ122568; DQ122490; DQ122423; *Correll, 43462,* NY. ***F. linearis*** Lagasca; E; DQ122569; DQ122491; DQ122446; *Hill, 13417,* NY. ***F. linearis*** Lagasca; F; DQ122570; DQ122492; DQ122447; *Brown, 14463,* TEX. ***F. mcdougallii*** Theroux, Pinkava & Keil; A; DQ122619; DQ122546; DQ122451; *Scott et al., 884,* TEX. ***F. oppositifolia*** (DC.) Rydberg; A; DQ122574; DQ122496; —; *Powell and Turner, 2710,* SRSC. ***F. oppositifolia*** (DC.) Rydberg; B; DQ122573; DQ122495; DQ122416; *Powell and Tomb, 2551,* SRSC. ***F. oppositifolia*** (DC.) Rydberg; C; DQ122572; DQ122494; —; *Hinton et al., 20471,* TEX. ***F. palmeri*** J.R. Johnston; A; DQ122606; DQ122535; DQ122411; *Powell, 851,* fruit material. ***F. palmeri*** J.R. Johnston; B; DQ122607; DQ122536; —; *Powell and Tomb, 2621,* SRSC. ***F. palmeri*** J.R. Johnston; C; DQ122608; —; —; *Powell and Tomb, 2611,* SRSC. ***F. pringlei*** Gandoger; A; DQ122548; DQ122456, DQ122457, DQ122458; DQ122432, DQ122433, DQ122434; *McKown, 10902,* TRT. ***F. pringlei*** Gandoger; B; DQ122549; DQ122454, DQ122455, DQ122480, DQ122481; —; *Panero and Salinas, 1146,* TEX. ***F. pringlei*** Gandoger; C; —; DQ122459, DQ122460, DQ122461; —; *Salinas and Ramos, F-3798,* TEX. ***F. pringlei*** Gandoger; D; DQ122550; —; DQ122435, DQ122436, DQ122437; *Boege, 2101,* GH. ***F. pringlei*** Gandoger; E; —; DQ122462, DQ122463, DQ122464; DQ122438, DQ122439, DQ122440; *Rzedowski, 30525,* GH. ***F. pringlei*** Gandoger; F; DQ122551; DQ122465, DQ122466,

DQ122467, DQ122468, DQ122469; DQ122441, DQ122442; *Sudderth, 7,* leaf material. ***F. pringlei*** Gandoger; G; DQ122552; DQ122470, DQ122471, DQ122472; DQ122443, DQ122444, DQ122445; *Sudderth, 3,* leaf material. ***F. pubescens*** Rydberg; A; DQ122575; DQ122497; DQ122415; *Hartman et al., 3823,* LL. ***F. pubescens*** Rydberg; B; DQ122576; DQ122498; —; *Ward, 8106,* NY. ***F. ramosissima*** Klatt; A; DQ122593; DQ122521; DQ122414; *Cowan et al., 5773,* NY. ***F. ramosissima*** Klatt; B; DQ122594; DQ122522;—; *Steinmann and Cervantes-Maldonado, 1396,* NY. ***F. ramosissima*** Klatt; C; DQ122595; DQ122523; —; *Cronquist, 11235,* NY. ***F. robusta*** Rose; A; DQ122559; DQ122479; —; *McKown, 10901,* TRT. ***F. robusta*** Rose; B; DQ122558; DQ122478; —; *Powell, 168,* fruit material. ***F. robusta*** Rose; C; DQ122556; DQ122476; DQ122430; *Sanders et al., 11760,* TEX. ***F. robusta*** Rose; D; DQ122557; DQ122477; —; *Ayers et al., 94,* TEX. ***F. sonorensis*** A.M. Powell; A; DQ122591; DQ122519; DQ122428; *McKown, 10905,* TRT. ***F. sonorensis*** A.M. Powell; B; DQ122592; DQ122520; DQ122429; *Arguelles, 82,* GH and TEX. ***F. trinervia*** (Spreng.) C. Mohr; A; DQ122599; DQ122527; DQ122401; *McKown, 10910,* TRT. ***F. trinervia*** (Spreng.) C. Mohr; B; DQ122596; DQ122524; —; *Hinton et al., 19547,* TEX. ***F. trinervia*** (Spreng.) C. Mohr; C; DQ122600; DQ122528; —; *Annable and Sickle, 3138,* NY. ***F. trinervia*** (Spreng.) C. Mohr; D; DQ122597; DQ122525; —; *Correll, 46028,* NY. ***F. trinervia*** (Spreng.) C. Mohr; E; DQ122598; DQ122526; —; *Meleady et al., 216,* NY. ***F. vaginata*** B.L. Robinson & Greenman; A; DQ122610; —; DQ122408; *McKown, 10914,* TRT. ***F. vaginata*** B.L. Robinson & Greenman; B; DQ122609; DQ122537; DQ122409; *King, 2922,* TEX.

Haploësthes greggii A. Gray; A; DQ122618; DQ122545; DQ122452; *Turner, 23–109,* SRSC.

Sartwellia flaveriae A. Gray; A; DQ122621; —; —; *Powell and Powell, 6389,* SRSC. ***S. mexicana*** A. Gray; A; DQ122620; DQ122547; DQ122453; *Hinton et al., 19446,* TEX.