

LEAF ANATOMY OF ORCUTTIEAE (POACEAE: CHLORIDOIDEAE): MORE EVIDENCE OF C₄ PHOTOSYNTHESIS WITHOUT KRANZ ANATOMY

LAURA M. BOYKIN^{1,2,3}, WILLIAM T. POCKMAN¹ AND TIMOTHY K. LOWREY¹

¹Department of Biology, University of New Mexico, Albuquerque, NM 87131

ABSTRACT

C₄ photosynthesis without Kranz anatomy (single-cell C₄ photosynthesis) occurs in only 0.003% of known species of C₄ flowering plants. To add insight into the evolution of C₄ photosynthesis, we studied the tribe Orcuttieae (Poaceae: Chloridoideae), which has species that can grow under both aquatic and terrestrial conditions, and utilize single-cell C₄ photosynthesis when growing submerged. Carbon isotope ratios from aquatic, floating, and terrestrial leaves were in the range -12.25 to -14.31, suggesting that all species carry out C₄ photosynthesis. Using light microscopy, we examined the anatomy of aquatic, floating and terrestrial leaves from eight of the nine species in the tribe to assess the pattern of evolution of C₄ photosynthesis and Kranz anatomy among these vernal pool grasses. Kranz anatomy was present in all floating and terrestrial leaves of *Orcuttia californica*, *O. inaequalis*, *O. pilosa*, *O. tenuis*, *O. viscida*, *Tuctoria greenei*, *T. mucronata*, and *Neostapfia colusana*. Although carbon isotope data indicated C₄ photosynthesis, aquatic leaves of all members of *Orcuttia* lacked Kranz anatomy, while aquatic leaves of *Tuctoria* and *Neostapfia* possessed Kranz anatomy. When considered in a phylogenetic context, these findings support previously proposed hypotheses suggesting that Orcuttieae are derived from a terrestrial ancestor and are now becoming more specialized to an aquatic environment.

Key Words: C₄ photosynthesis, Chloridoideae, Kranz anatomy, *Neostapfia*, *Orcuttia*, Orcuttieae, Poaceae, *Tuctoria*, single-cell C₄ photosynthesis, vernal pool.

Since the discovery of C₄ photosynthesis in the 1960's, there has been great interest in documenting the biochemical and anatomical features of the process since it has a much greater photosynthetic capacity as compared to C₃ photosynthesis from which it has been derived (Leegood 2002). In the chloroplast, the C₃ pathway assimilates CO₂ to form phosphoglycerate (PGA), catalyzed by the enzyme ribulose biphosphate carboxylase oxygenase (RUBISCO). The C₄ pathway, observed in 19 plant families (Kellogg 1999; Sage 2004), couples the C₃ pathway with a prior carboxylation step catalyzed by phosphoenolpyruvate carboxylase (PEPcase), producing four-carbon organic acids such as malate and aspartate. Initial carboxylation by PEPcase and subsequent decarboxylation of the C₄ products, acts as a carbon concentrating mechanism that effectively minimizes the oxygenase activity of RUBISCO and minimizes photorespiration, increasing quantum yield at the low CO₂ concentrations (Ehleringer et al. 1991; Sharkey 1988). In most C₄ species, the carbon concentrating activity depends upon the presence of Kranz anatomy in leaves. Kranz anatomy is the wreath of radially arranged mesophyll cells surrounding the bundle sheath Haberlandt (1882, 1914). In these species, PEP-

case activity is restricted to mesophyll cells while RUBISCO activity occurs in the bundle sheath cells, in the high CO₂ microenvironment created by the decarboxylation of C₄ products.

It has been generally accepted that Kranz anatomy is essential for C₄ photosynthesis (Kellogg 1999), but several studies have shown otherwise for submerged monocots (Keeley 1998), submerged dicots (Casati et al. 2000; de Groote and Kennedy 1977; Holaday and Bowes 1980; Lara et al. 2002; Salvucci and Bowes 1983; Spencer et al. 1996), and terrestrial dicots (Freitag and Stichler 2002; Sage 2002; Voznesenskaya et al. 2002; Voznesenskaya et al. 2001). With the exception of a few terrestrial dicot taxa, the majority of species that lack Kranz anatomy are aquatic (Bowes et al. 2002). The aquatic plants that have C₄ photosynthesis without Kranz anatomy are: *Hydrilla verticillata* (Holaday and Bowes 1980; Magnin et al. 1997; Salvucci and Bowes 1983; Spencer et al. 1996), *Egeria densa* (Casati et al. 2000; Lara et al. 2002), *Elodea canadensis* (de Groote and Kennedy 1977), and the tribe Orcuttieae (Keeley 1998).

Orcuttieae is a small tribe of semi-aquatic grasses (three genera and nine species) restricted to vernal pools in California and Baja California. Vernal pools result from an unusual combination of soil conditions, summer-dry Mediterranean climate, topography, and hydrology. These pools form in small depressions that are filled by winter precipitation and retain moisture longer than surrounding grasslands before evaporation dur-

²Current Address: USDA, ARS, USHRL, 2001 South Rock Road, Fort Pierce, FL 34945.

³Author for correspondence (E-mail lboykin@macworld.com)

TABLE 1. LOCALITY INFORMATION PROVIDED FOR ORCUTTIEAE SAMPLES INCLUDED IN THE LEAF ANATOMY AND CARBON ISOTOPE ANALYSIS. Voucher specimens are deposited at University of New Mexico Herbarium (UNM) (except for *Tuctoria fragilis* which is located at University of Arizona [ARIZ]). Collectors of plant material were Laura M. Boykin (LMB), Paula M. Hall (PMH), and John Reeder.

Species	Location	Voucher	Latitude	Longitude
<i>Orcuttia californica</i> Vasey	Riverside, Co.	LMB & PMH 44	33°43'356"	117°03'3045"
<i>Orcuttia inaequalis</i> Hoover	Merced, Co.	LMB & PMH 32	37°14.818"	120°13.521"
<i>Orcuttia viscida</i> (Hoover) J. Reeder	Sacramento, Co.	LMB & PMH 42	39°31'291"	121°11'345"
<i>Orcuttia pilosa</i> Hoover	Tehama, Co.	LMB & PMH 16	39°54.407"	122°58.942"
<i>Orcuttia tenuis</i> A.S.Hitchc.	Shasta, Co.	LMB & PMH 62	40°17'115"	122°07'185"
<i>Tuctoria greenei</i> (Vasey) J. Reeder	Tehama, Co.	LMB & PMH 28	39°54.124"	121°58.963"
<i>Tuctoria fragilis</i> (Swallen) J. Reeder	B.C.S., Mexico	J. Reeder 7141	Baja California Sur	Llanos de Hiray
<i>Tuctoria mucronata</i> (Crampton) J. Reeder	Yolo, Co.	LMB & PMH 19	38°29.509"	121°41.157"
<i>Neostapfia colusana</i> Davy	Yolo, Co.	LMB & PMH 21	37°14.852"	120°37.130"

ing the hot, dry summer causes the pools to shrink and eventually disappear. As a result, vernal pool habitat represents a continuously changing environment, which contains a specialized biota and relatively large numbers of threatened and endangered species (Holland and Jain 1977) including the Orcuttieae. Orcuttieae are unusual among C_4 plants because they spend a large portion of their life cycle as submerged aquatics (Ehleringer and Monson 1993). The presence of C_4 photosynthesis in this group may have been favored because it reduces water loss at anthesis when the pools are dry in late summer and plants are subjected to drought and high temperatures (Keeley 1998).

C_4 plants are divided into three biochemical subtypes that differ mainly in the C_4 acid transported into the bundle sheath cells (malate and aspartate) and in the way in which it is decarboxylated; they are named, based on the enzymes that catalyze their decarboxylation, NADP-dependent malic enzyme (NADP-ME) found in the chloroplasts, NAD-dependent malic enzyme (NAD-ME) found in the mitochondria, and phosphoenolpyruvate (PEP) carboxykinase (PCK), found in the cytosol of the bundle sheath cells (Edwards and Walker 1983; Ghannoum et al. 2001; Hatch 1987; Hatch et al. 1975; Jenkins et al. 1989). Anatomically, the three subtypes of C_4 photosynthesis differ in chloroplast position and cell outline of the bundle sheath cells (Hattersley and Browning 1981; Hattersley and Watson 1976; Hattersley and Watson 1992; Prendergast and Hattersley 1987). Both NADP-ME and PCK have an uneven outline of the bundle sheath cells, while NAD-ME has an even outline. The chloroplasts in the bundle sheath cells of NADP-ME and PCK have a centrifugal position, while NAD-ME has a centripetal position. Keeley (1998) provided photosynthetic and anatomical data for four of the nine species of Orcuttieae: *Orcuttia californica*, *O. viscida*, *Tuctoria greenei*, and *Neostapfia colusana*. In addition, he found that all species examined in his

study had the NADP-ME C_4 photosynthetic subtype based on chloroplast position and bundle sheath cell outline. The exception, *N. colusana*, had high activities of both NADP-ME and NAD-ME in pulse-chase experiments indicating a mixed subtype (Keeley 1998).

The most unexpected finding of Keeley (1998) was that aquatic leaves of *Orcuttia californica* and *O. viscida* carry out C_4 photosynthesis but lack Kranz anatomy while terrestrial and floating leaves of these species carry out C_4 photosynthesis with Kranz anatomy. The other members of the tribe included in his study, *Tuctoria greenei* and *Neostapfia colusana*, carry out C_4 photosynthesis but possess Kranz anatomy in both aquatic and terrestrial leaves. The photosynthetic and anatomical diversity that exists among such closely related taxa provides a unique opportunity to study the evolution of single-cell C_4 photosynthesis. Single-cell C_4 photosynthesis in aquatic plants has generated great interest as the system that may hold promise for genetic engineering in C_3 plants (Leegood 2002).

To assess the anatomical and photosynthetic pathway variation of all species in the Orcuttieae (excluding *Tuctoria fragilis*), we surveyed leaf anatomy at the aquatic, floating and terrestrial stages using light microscopy and carbon isotope ratio of leaf tissue. Leaf anatomy and carbon isotope data for four species of Orcuttieae, *O. inaequalis*, *O. tenuis*, *O. pilosa*, and *T. mucronata* are presented for the first time.

METHODS

Seed and Soil Collection

Seeds, and associated soil, of eight species in Orcuttieae were collected from vernal pools throughout the geographic distribution of *Orcuttia*, *Tuctoria*, and *Neostapfia* (Table 1) (federal permit # TE-029387-0 and California state permit # 00-04). Collections were made from different populations of the species (*Orcuttia*

californica, *O. viscida*, *Tuctoria greenei*, and *Neostapfia colusana*) than those previously sampled by Keeley (1998). Exact locality data for all collections are provided in Stone et al. (1988) and the California Natural Diversity Database (CNDDDB [CDFG 2007]).

The ninth species in Orcuttieae, *Tuctoria fragilis*, could not be sampled due to the lack of living plants in the only known population in Baja California Sur. Cold stratification of the soil collected from the Llanos de Hiray, the only known location for *T. fragilis*, produced no seed germination. Terrestrial leaves for carbon isotope analysis were obtained from herbarium specimens provided by J. Reeder.

Seed Germination

Leaves for anatomy and carbon isotope measurements were obtained for eight species by growing plants from seed modifying the seed germination procedures of Griggs (1974, 1980). *Orcuttia tenuis*, *O. pilosa*, and *Tuctoria greenei* germination requires an initial period of cold stratification in cool, wet conditions followed by a gradual increase in daily temperature to a maximum of 32°C and a minimum of 15°C (Griggs 1974). Experiments with all *Orcuttia* species and *Tuctoria greenei* showed that naked seeds cannot be germinated in the laboratory, regardless of treatment (Griggs 1980). A germination rate of 90–100% is possible when submerged inflorescences containing ripe seeds are allowed to become completely engulfed by an aquatic fungus (Griggs 1980). After unsuccessful attempts using calcium carbonate-rich local clay soil in New Mexico, germination of seeds of all species except *Tuctoria fragilis* was achieved using soil collected from natural vernal pools at the time of seed collection.

Whole inflorescences from field-collected material were placed in petri dishes containing 50 mL of deionized (DI) water and placed at 20°C for three weeks. Soil was placed in 4-inch pots, saturated with DI water, and placed at 20°C for three weeks. Whole inflorescences were buried in the cold-stratified soil after the three-week cold stratification. The pots were submerged in tubs filled with DI water and placed in a greenhouse at the Department of Biology, University of New Mexico. DI water was added to the tubs daily. Approximately four months after the soil and seeds were submerged, the DI water was allowed to evaporate completely and the plants were exposed to the air.

Collection of Leaf Material

Once plants were established in the greenhouse, leaves were sampled as the different stages became available (aquatic, floating and terrestri-

al). Aquatic leaves were collected three months after the seeds and soil were submerged. Floating leaves were collected the first week after they reached the surface of the water. Terrestrial leaves, which developed entirely in air, were collected approximately two weeks after the tubs had completely dried down. All leaf material was cut into small pieces and fixed for one week in formalin acetic acid (FAA) (Berlyn and Miksche 1976).

Dehydration, Infiltration, and Sectioning of Leaf Tissue

Fixed leaf material was removed from the FAA and placed in 70% ethanol overnight and changed five times in the 24 hr period. The leaf material was put through an ethanol dehydration series: 95% ethanol for 20 min, twice; and 100% ethanol for 20 min, three times. Ethanol dehydration was followed by three washes of pure propylene oxide for 20 min each. Leaf samples were then infiltrated with Epon LX112 resin (Ladds Scientific, Williston). An epon mixture of 4:6 (epon A:epon B) was used in all the infiltration steps after mixtures of 3:7 and 2:8 produced unsatisfactory hardness for cross-sectioning. The epon mixture of 4:6 was diluted for the first infiltration step in a 1:1 ratio with propylene oxide for three hours. The tissue was then transferred to a 3:1 mixture of epon (4:6) to propylene oxide and infiltrated overnight. The samples were then placed in molds (Pelco, 105) filled with only epon 4:6 and left for ten hours. The final step was to transfer the leaf material into a new mold filled with epon 4:6 and incubate at 60°C for 18 hr. Sections 7 µm thick were cut with an ultra-microtome (Sorvall MT2-B) and stained with a 1:1 mixture of 1% azure II and 1% methylene blue. Light microscopy was used to determine presence of Kranz anatomy. All leaf sections were photographed at final magnifications of 20× and 40× with a Nikon Coolpix 990 attached to a Nikon Eclipse E400 microscope.

¹³C/¹²C Isotope Values

¹³C/¹²C isotope ratios were determined in leaf tissue to assess the photosynthetic pathway. ¹³C/¹²C isotope ratios of Orcuttieae aquatic and terrestrial leaves were obtained using a Delta E mass spectrometer in the Earth and Planetary Sciences Department at the University of New Mexico. δ¹³C values, a measure of the carbon composition, were determined on plant samples using standard procedure relative to PDB (Pee Dee Belemnite) limestone as the carbon isotope standard (Bender et al. 1973). Isotope studies have demonstrated that C₄ has less negative δ¹³C values than those found in C₃ plants (Bender 1968; Bender 1971; Smith and Epstein 1971). This

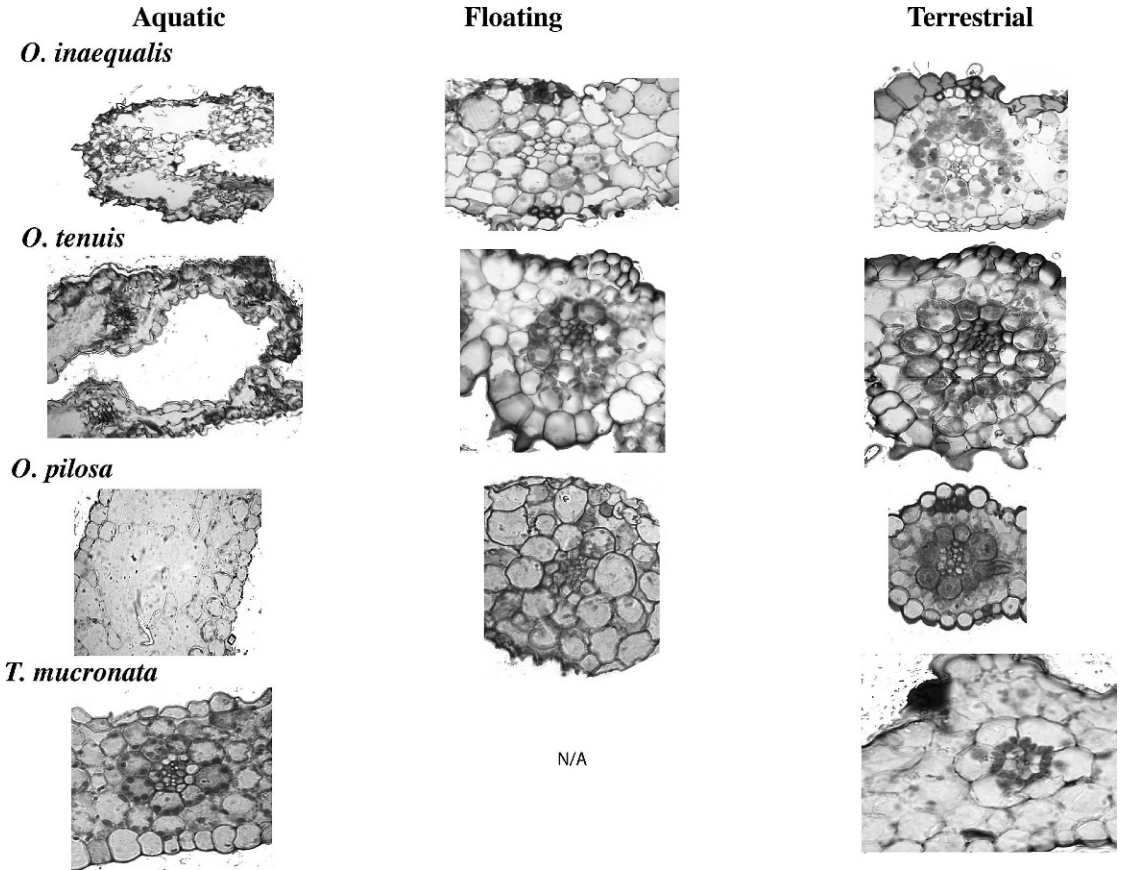


FIG. 1. Transverse sections of aquatic, floating and terrestrial leaves of Orcuttieae. Aquatic leaves were taken at a 20× magnification while floating and terrestrial leaves are a 40× magnification.

difference in isotopic composition has become a standard mechanism for distinguishing plant tissues from these two groups, with C₃ plants having a ratio of -20 to -35‰ and C₄ plants having values of -9 to -14‰ (Bender 1971).

RESULTS

Leaf Anatomy

Among the eight species of Orcuttieae sampled, Kranz anatomy was absent in aquatic leaves of *O. pilosa*, *O. tenuis*, and *O. inaequalis* but present in floating and terrestrial leaves of these species (Fig. 1). In addition, we confirmed this pattern, previously observed in aquatic and terrestrial leaves by Keeley (1998), for the remaining two species in the genus, *O. viscida* and *O. californica* (data not shown, see Keeley 1998 for photos). Kranz anatomy was present in aquatic and terrestrial leaves of *T. mucronata* (Fig. 1), and we also confirmed this pattern in *Tuctoria greenei* and *Neostapfia colusana* (data not shown, see Keeley 1998 for photos).

Considerable morphological and anatomical variation was present in the aquatic leaves of *Orcuttia*. *Orcuttia viscida*, *O. californica*, and *O. pilosa* had linear leaves, while *O. inaequalis* and *O. tenuis* had terete leaves, due to folding of the leaves so the margins nearly touch (Fig. 1). Extensive lacunae were evident in the *Orcuttia* aquatic leaves while they were lacking in aquatic leaves of *Tuctoria* and *Neostapfia* species.

Chloroplast arrangement was variable in the terrestrial leaves of *Orcuttia* (Fig. 1). *Orcuttia inaequalis*, *O. viscida* (not shown), and *O. tenuis* exhibited centrifugal arrangement of chloroplasts in terrestrial leaves, while *O. californica* (not shown) and *O. pilosa* had chloroplasts arranged around the outer edge of the cell. Floating leaves of *Orcuttia* all have centrifugal chloroplast distribution in the bundle sheath cells (Fig. 1 and Keeley 1998). *Neostapfia colusana* and *T. greenei* have centrifugal distribution of chloroplasts in the bundle sheath cells (Keeley 1998). *Tuctoria mucronata* aquatic leaves have the chloroplasts in the bundle sheath cells distributed centripetally (Fig. 1).

TABLE 2. STABLE CARBON ISOTOPE RATIOS IN AQUATIC, FLOATING AND TERRESTRIAL LEAVES OF ORCUTTIEAE. Values shown are the mean of five replicates \pm 0.3 standard error. All dried material excluding *T. fragilis* was obtained from greenhouse material. *Tuctoria fragilis* material was obtained from voucher specimens supplied by Dr. John Reeder. Values represent $\delta^{13}\text{C}$ values (‰).

Species	Aquatic	Floating	Terrestrial
<i>Orcuttia californica</i>	-12.7	-12.7	-12.7
<i>Orcuttia inaequalis</i>	-13.0	-12.8	-13.0
<i>Orcuttia pilosa</i>	-12.7	-12.6	-12.8
<i>Orcuttia tenuis</i>	-12.6	-12.4	-12.3
<i>Orcuttia viscida</i>	-13.6	-13.7	-13.7
<i>Tuctoria greenei</i>	-13.1	n/a	-13.4
<i>Tuctoria fragilis</i>	-13.0	n/a	-13.2
<i>Tuctoria mucronata</i>	-13.6	n/a	-13.6
<i>Neostapfia colusana</i>	-14.3	n/a	-14.2

Carbon Isotope Ratio

The carbon isotope values for all Orcuttieae from aquatic, floating (only present in *Orcuttia*) and terrestrial leaves (Table 2) are within the range exhibited by C_4 plants (-9 to -14‰). Terrestrial, floating and aquatic leaves of all species of *Orcuttia* had $\delta^{13}\text{C}$ values between -12.3 and -13.7‰. *Tuctoria* aquatic and terrestrial leaves had $\delta^{13}\text{C}$ values between -13.0 and -13.6‰. *Neostapfia colusana* aquatic and terrestrial leaves had the most negative values (-14.3 and -14.2‰ respectively).

DISCUSSION

Our findings revealed that C_4 photosynthesis occurs throughout Orcuttieae (Table 2) but with considerable leaf anatomical variation (Fig. 1). Aquatic leaves in all species of *Orcuttia* carry out C_4 photosynthesis (Table 2) in the absence of Kranz anatomy (Fig. 1 and Keeley 1998). The aquatic environment might result in less negative isotope ratios because of diffusion limitations (Osmond et al. 1981; Raven et al. 1982). However, the consistency of the isotopic ratios between aquatic and terrestrial leaves provide strong circumstantial evidence that the underlying biochemical pathway is C_4 . Floating and terrestrial leaves of these same species utilize the C_4 pathway with Kranz anatomy. On the other hand, both aquatic and terrestrial leaves of *Tuctoria mucronata*, *T. greenei* and *Neostapfia* carry out C_4 with Kranz anatomy (Fig. 1).

Orcuttieae is unique among plants exhibiting single-cell C_4 photosynthesis because it produces both an aquatic and a terrestrial phase of growth. A single plant can be submerged for three months with the aquatic leaves lacking Kranz anatomy while three months later (after the water in the pool has evaporated), the same plant produces leaves with Kranz anatomy after exposure to the

terrestrial environment. Aquatic single-cell C_4 photosynthesis has previously been documented from several aquatic species in addition to Orcuttieae: *Hydrilla verticillata* (Holaday and Bowes 1980; Salvucci and Bowes 1983; Spencer et al. 1996; Magnin et al. 1997), *Egeria densa* (Casati et al. 2000; Lara et al. 2002), and *Elodea canadensis* (de Groote and Kennedy 1977). Single-cell C_4 photosynthesis also occurs in several terrestrial species most notably *Bienertia cycloptera* and *Borszczowia aralocaspica* (Chenopodiaceae) (Voznesenskaya et al. 2001; Voznesenskaya et al. 2002; Edwards et al. 2004). Orcuttieae is the only documented group of species that is both aquatic and terrestrial in a given year with attendant photosynthetic and anatomical diversity.

The variation observed in chloroplast arrangement (Fig. 1) suggests that the biochemical C_4 subtype according to C_4 acid decarboxylases (NADP-malic enzyme, NAD-malic enzyme, phosphoenolpyruvate carboxykinase) may differ within Orcuttieae. Such variation, which is common in plant families and genera (Kellogg 1999), has been documented previously in terrestrial leaves of *Orcuttia viscida* (Keeley and Rundel 2003) and *O. tenuis* (Sage et al. 1999) which have a centrifugal (NADP-ME) and centripetal (NAD-ME) chloroplast arrangement, respectively. Additionally, our data showed centrifugal (NADP-ME) and centripetal (NAD-ME) chloroplast arrangement in terrestrial leaves of *Tuctoria greenei* and *T. mucronata*, respectively. Further research on Orcuttieae should include analysis for type of C_4 acid decarboxylases (enzyme assays and western blots) to determine the C_4 photosynthetic subtypes (Edwards et al. 2004).

Although there are no apparent performance advantages between NAD-ME and NADP-ME (Voznesenskaya et al. 1999), the chloroplast variation and inferred C_4 subtype variation in Orcuttieae (Fig. 1) indicates that there may have been multiple independent origins of the NAD-ME and NADP-ME sub-types in the tribe. A switch in subtype could occur if the C_4 pathway were controlled by few regulatory genes, which would make evolutionary changes in the pathway expression relatively easy (Ehleringer and Monson 1993; Ku et al. 1996; Monson 1996; Monson 1999). Variation in environmental conditions such as, depth and length of inundation of the vernal pools might also have led to variation in chloroplast position. However, this is not a likely explanation for our results because all samples in this study were exposed to the same conditions during growth and development.

The vernal pool environment in which *Orcuttia* thrives, has apparently favored different patterns of leaf development across leaf types (aquatic, terrestrial and floating) while preserving the

carbon-concentrating effects of C_4 photosynthesis (Keeley 1998). Aquatic plants that utilize the C_4 pathway are adapted to reduced levels of CO_2 (Keeley 1998; Keeley and Rundel 2003) that are largely unrelated to long-term patterns of atmospheric CO_2 (Keeley and Rundel 2003). For example, *Hydrilla verticillata* switches between C_3 and C_4 photosynthesis in response to changes in CO_2 in the surrounding water driven by biogenic depletion of carbon in dense mats of vegetation (Salvucci and Bowes 1983). Similarly, the occurrence of Kranz anatomy in floating and terrestrial leaves but not in aquatic leaves suggests that leaf anatomical development responds to the relative abundance of CO_2 and O_2 during growth of individual leaves. Besides the relative amount of CO_2 and O_2 around aquatic versus terrestrial leaves, the high diffusive resistance to gases in the aquatic environment may affect leaf anatomical development. Manipulating the CO_2 and O_2 concentrations in water or air during development of aquatic and terrestrial leaves, respectively, could test this hypothesis.

Although the advantages of C_4 in aquatic leaves are known, the relative benefits associated with the shift in leaf anatomy from aquatic to floating and terrestrial leaves of *Orcuttia* remain unclear. The dense vegetation, high light and high temperatures in the shallow seasonal pools inhabited by *Orcuttieae* lead to CO_2 -depletion and O_2 supersaturation (Keeley and Busch 1984). C_4 photosynthesis, with or without Kranz anatomy, is favored because its CO_2 pumping mechanism concentrates CO_2 around the active site of the enzyme Rubisco, maintaining a high $CO_2:O_2$ ratio and eliminating photorespiration (Ehleringer and Monson 1993) relative to C_3 plants under the low $CO_2:O_2$ conditions in the pools. Although the reduction in photorespiration with C_4 photosynthesis represents a clear advantage over C_3 plants in the same environment, any differences in performance between C_4 leaves with and without Kranz anatomy await additional studies. One possibility is that C_4 without Kranz achieves similar rates of CO_2 fixation with a reduced construction cost compared to Kranz anatomy (Sage 2002).

The origin of aquatic C_4 species varies among taxonomic groups. The presence of C_4 photosynthesis in *Hydrilla* and *Egeria*, which comprise separate lineages of Hydrocharitaceae, indicates more than one origin of C_4 within this family. All taxa in the family are aquatic, and C_4 is absent from the nearest sister group (Kellogg 1999), supporting an independent aquatic origin for C_4 . However, the presence of C_4 photosynthesis in aquatic leaves of *Orcuttieae* show a very different origin, one from a terrestrial C_4 ancestor (Keeley 1998). *Orcuttieae* are one of four tribes in the Chloridoideae, a subfamily of 1400 species, all possess C_4 photosynthesis; thus, *Orcuttieae* likely

were derived from upland C_4 ancestors, and the monophyly of the *Orcuttieae* are well supported (Boykin 2003; Boykin et al. in revision; Hilu and Alice 2001). Since *Orcuttia* has many aquatic specializations, including the loss of Kranz anatomy, Reeder (1982) and Keeley (1998) suggest that it is the most derived genus in the tribe. In a study of phylogenetic relationships in the tribe, our data support the derived status of *Orcuttia* (Boykin 2003). With the data presented here together with that from previous studies (Boykin 2003; Keeley 1998; Reeder 1982) we believe *Orcuttieae* originated on land and has subsequently become more specialized to an aquatic environment.

ACKNOWLEDGMENTS

The authors thank Paula Hall for valuable laboratory and field assistance, Jon Keeley and John Reeder for plant material, Tom Turner and Gerry Edwards for valuable comments on the manuscript, Joy Avritt and Jeffrey Lucero for greenhouse assistance, Sergio Flores for field assistance in Baja California. Steve Stricker, Toni Smythe, and Anna Tyler were instrumental in helping process leaf material for cross-sectioning. Zach Sharp and Viorel Autoderi analyzed leaf samples for carbon isotope data. This study was funded by a P.E.O. fellowship, California Native Plant Society, American Society of Plant Taxonomists, Botanical Society of America and various sources at University of New Mexico (Springfield and Grove fellowships, GRAC, SRAC, and RPT). This research represents a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at University of New Mexico.

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