Plant, Cell & Environment

Plant, Cell and Environment (2012) **35**, 1533–1542 doi: 10.1111/j.1365-3040.2012.02503.x

Quantifying succulence: a rapid, physiologically meaningful metric of plant water storage

R. MATTHEW OGBURN & ERIKA J. EDWARDS

Department of Ecology and Evolutionary Biology, Brown University, Providence, RI, USA

ABSTRACT

Quantification of succulence should ideally convey information about physiological function and yet also be straightforward to measure. While important aspects of succulence and its physiological consequences may be quantified using parameters derived from pressure–volume (P-V) curves, this technique applied to succulent tissues is difficult, time consuming and generally not suitable for large comparative datasets. We performed P-V curves on leaves of 25 taxa from across Caryophyllales and compared the results with direct measures of saturated water content (SWC_{meas}), the ratio of water mass at full saturation to tissue dry mass, for the same taxa. SWC_{meas} was significantly related to relative capacitance, the most physiologically relevant parameter describing tissue succulence. We developed a linear model describing SWC_{meas} as a function of relative capacitance and leaf volume, which is also supported when accounting for the phylogenetic relationships among taxa. These results indicate that SWC_{meas} is a suitable proxy for tissue succulence, and that both cellular properties and variation in gross morphology contribute towards a plant's relative water storage capacity. Quantifying SWC_{meas} across many taxa showing variation in tissue succulence will provide a new avenue for exploring the evolutionary dynamics of this important ecological adaptation.

Key-words: Caryophyllales; capacitance; pressure-volume curve; saturated water content; water relations.

INTRODUCTION

Highly succulent plants, with their pronounced water storage tissues, are a striking example of the synergy between form and function in adaptation to stressful environments (Eggli & Nyffeler 2009). Succulence affords plants a degree of independence from the vagaries of a limited or unpredictable water supply (Calkin & Nobel 1986; Hunt & Nobel 1987; Schulte. Smith & Nobel 1989; Von Willert *et al*. 1990; Eggli & Nyffeler 2009). Common features of these plants include traits that limit water loss to the environment, such as a thick cuticle (Gibson 1982; Ogburn & Edwards 2009), low stomatal density (Gibson & Nobel 1986) and crassulacean acid metabolism (CAM) photosynthesis (Winter *et al*. 1983; Lüttge 2004; Borland *et al*. 2009), or traits that maximize uptake of transiently available sources of water, such as a shallow, spreading root system with rectifier-like properties (Nobel & Sanderson 1984; Preston 1900; North & Nobel 1991; von Willert *et al*. 1992).

The core feature of the succulence syndrome, however, is the ability of water storage tissues to support physiological function in the absence of an external water source. This ability is governed primarily by two traits of water storage tissues: relative capacitance (C) and absolute volume. C is defined as the change in tissue or cell volume for a given change in water potential [$(\Delta V/V)/\Delta\Psi$] (Koide *et al*. 1989; Nobel 2005). Succulent water storage tissues, with high values of C, gain or lose relatively large volumes of water across small gradients in water potential. This property of high C tissues has at least two ecologically relevant corollaries: first, for a given amount of water volume lost, succulent tissues maintain relatively high water potentials, allowing them to extend photosynthetic activity longer into periods of drought. Second, differences in capacitance between neighbouring cell types, for example between high C water storage and lower C chlorenchyma tissues within a leaf, provide a passive mechanism ensuring supply of water from storage tissues to actively photosynthesizing cells (Schmidt & Kaiser 1987; Martin *et al*. 2004; Nobel 2006). Absolute volume of water storage tissues contributes to succulence as well, such that some obviously 'succulent' taxa may not even have particularly high relative capacitance values, instead relying on high absolute capacitance from the total volume of storage tissues (e.g. *Agave deserti*, Calkin & Nobel 1986; *Ferocactus acanthodes*, Hunt & Nobel 1987).

Quantifying succulence should therefore ideally incorporate information about C and volume. While C and other useful parameters may be derived from pressure–volume (P-V) curves of the relationship between water potential and relative water content (Supporting Information Fig. S1), this technique is time consuming and not easily applicable to large numbers of samples. This is more so the case for succulent tissues because they are not amenable to the standard pressure chamber technique for measuring water potential, instead requiring more difficult approaches such as thermocouple psychrometry (Koide *et al*. 1989; Holbrook & Sinclair 1992; Kramer & Boyer 1995). A number of alternative metrics of succulence have been proposed through the years, many of which have never been widely

Correspondence: R. M. Ogburn. E-mail: mogburn@brown.edu

adopted. These include the ratio of water mass at saturation to surface area (degree of succulence; Delf 1912) and the ratio of water mass to chlorophyll mass (mesophyll succulence; Kluge & Ting 1978). von Willert *et al*. (1990) defined succulence as total water mass divided by the dry mass of organic material (total dry mass minus ash content), which they interpreted as providing a measure of water stored per gram carbon expenditure on the part of the plant. Among researchers studying CAM photosynthesis, succulence has commonly been measured as a function of leaf thickness (Teeri, Tonsor & Turner 1981; Winter *et al*. 1983), total fresh mass per leaf area (Borland *et al*. 1998; Maxwell 2002) or both (Nobel & Hartsock 1990; Griffiths *et al*. 2008). While these latter studies provide the best example of a widely used metric of succulence, it has generally been measured as a covariate of CAM expression; the physiological aspects of succulence per se in these studies were not the focus. Anatomical measurements, including cell size and cell packing (Smith & Nobel 1986; Nelson, Sage & Sage 2005; Nelson & Sage 2008) or proportion of parenchyma in stem cross section (Hearn 2009), have also been used to quantify succulence. Among larger-scale comparative studies, succulence is frequently treated as a discrete, 'either-or' characteristic (Jones & Price 1996; Jones, Cardon & Czaja 2003; Klak, Reeves & Hedderson 2004; Hearn 2006; Olson & Rosell 2006; Ogburn & Edwards 2009). Furthermore, most of these larger-scale comparative studies have focused on growth form and developmental aspects in the evolution of these lineages; still largely lacking is the connection between ecophysiology and the evolution of succulence (but see Edwards & Donoghue 2006).

Our goal in this study was to explore an easily measured metric of succulence suitable for use in field work, and to understand its relationship to physiologically important traits, including capacitance and leaf volume, as well as anatomical characters such as water storage cell size. We measured the saturated water content (SWC), defined as total water mass normalized by dry tissue mass, for whole leaves from a range of taxa from the clade Caryophyllales. SWC is thus a simplified version of the succulence quotient of von Willert *et al*. (1990). Our sampling included four halophytic taxa [*Arthrocnemum subterminale* (Parish) Standl., *Limonium carolinianum* (Walter) Britton, *Salicornia maritima* S.L. Wolff & Jefferies, and *Suaeda taxifolia* Standl.], which display a more or less succulent morphology but are thought to use water very differently from drought-adapted succulents (Waisel 1972; Ogburn & Edwards 2010). To infer the underlying factors that determine SWC, we explored phylogenetically informed models relating SWC to relative capacitance and other water use parameters, leaf size and shape, and leaf anatomical traits.

METHODS

P-V curves

We obtained P-V curves for leaf material from 25 taxa from across the plant clade Caryophyllales, representing a broad range in tissue succulence and alternative water use strategies (Appendix; Supporting Information Fig. S2). The majority of leaves were collected in the field, although several taxa were grown in the greenhouse at the Plant Environmental Center at Brown University, Providence, RI. P-V curves were measured using a Wescor Psypro thermocouple psychrometer with Wescor C-52 sample chambers (Wescor, Inc., Logan, UT, USA). Leaves were soaked in distilled water overnight, long enough to allow water potential to reach the range of 0 to –0.1 MPa. Once hydrated, entire leaves or most usually excised segments of leaves, depending on total size, were placed inside sample holders, which were then weighed and sealed inside sample chambers for water potential readings. In the case of excised leaf pieces, all attempts were made to minimize the area of cut surface, as this has been shown to potentially affect water potential readings (Walker, Oosterhuis & Wiebe 1984). Samples were allowed to equilibrate until roughly five water potential readings within a range of 0.1 MPa were obtained in a row. Between readings, samples were removed from the sample chambers and allowed to dry for between 1 and 15 min, depending on the hydration state of the tissue. After drying, sample holders were tightly sealed in parafilm for at least 30 min to allow equilibration of water status throughout the sample. Samples were then weighed again and transferred to sample chambers for the next water potential reading. This process was repeated 10–12 times per leaf sample, enough to generate a full P-V curve (Supporting Information Figs S1 & S2), with subsequently longer pauses for drying as the sample reached lower water potentials. The C-52 sample sensor heads were cleaned and calibrated between each full curve that was generated. P-V curves were graphed as the negative inverse of water potential against 100 – relative water content (RWC). A line was fitted to the linear part of the curve, and the turgor loss point (TLP) assigned as the inflection point of the curve (Supporting Information Fig. S1) (Tyree & Hammel 1972). Water relation parameters derived from P-V curves include saturated water content (SWC_{PV}), osmotic potential at full turgor (Π_o), water potential at turgor loss point (Ψ_{TLP}), relative water content at turgor loss point (RWC_{TLP}), volumetric elastic modulus (ε), capacitance before turgor loss (C_{FT}) and capacitance after turgor loss (C_{TLP}).

Direct measure of SWC

SWC is a dimensionless measure of water mass held by a given tissue or organ at full hydration normalized by the tissue dry mass:

$$[\text{leaf mass (g) at full hydration} - \text{dried leaf mass (g)}]/\text{dried leaf mass (g)}$$

Because excess water may be absorbed and held in the apoplast, it is likely that the maximum water content of an artificially hydrated leaf is well outside of the natural range for plants in the field and is therefore not biologically realistic. This has been held up as a rationale for simply

measuring water content as plants are collected, without further rehydration (von Willert *et al*. 1990). This approach, however, will greatly increase variance in SWC_{meas} because what is being measured is essentially the water status of the plant. While water content at maximum hydration may be outside of the values commonly experienced by plants in the field, we expect that this value relates directly to the water storage capacity (i.e. succulence) of a given tissue and is therefore biologically relevant.

We obtained SWC_{meas} for multiple leaves ($n = 4$ to 10) of the same set of 25 taxa for which we derived P-V curves. Each leaf was taken from a different individual except in the case of the greenhouse taxa for which only one to three individuals were available. In the case of very small leaves, such as those of *Pharnaceum incanum* or *Montiopsis ramosissima*, multiple leaves ($n = 5$ to 10) from a single plant were measured together for each replicate. To obtain SWC_{meas}, leaves were excised from the plant, soaked in distilled or deionized water, then dried on their surfaces and weighed at approximately 12 h intervals. When leaves gained less than 5% in mass between subsequent weighings, they were considered saturated and drying was commenced. For drying in the laboratory, leaves were transferred to an oven at 60 °C for a minimum of 48 h before measuring dry mass. For drying in the field, leaves were placed in small seed envelopes or coffee filters and sealed in small ziplock bags with desiccant, which was refreshed as necessary as it absorbed water. After collecting trips, field-collected samples were transferred to a drying oven for at least 48 h before measuring.

In addition to direct measurements of SWC, we were able to estimate SWC from P-V curves. The approximately linear relationship between sample water mass (total sample mass – dried mass) and water potential is used to extrapolate water mass at 100% RWC (i.e. at zero MPa water potential; Supporting Information Fig. S3). This quantity divided by sample dried mass provides SWC. The two independent methods of estimating SWC provide a useful cross-check of both. We hereafter refer to directly measured SWC as SWC_{meas} and SWC estimated from P-V curves as SWC_{PV}.

Leaf anatomy

We prepared anatomical cross-sections of leaves from a subset of 14 taxa (Appendix). Fresh leaves were initially fixed in FAA (10 parts 95% ethanol: 7 parts distilled water: 2 parts 37% formalin: 1 part glacial acetic acid) or 70% ethanol for a minimum of 48 h. Portions of fixed leaves were excised and completely dehydrated in 100% ethanol for another 48 h, then infiltrated and embedded using the JB-4 resin embedding kit (Polysciences, Inc., Warrington, PA, USA). Samples were sectioned at 5 μm on a rotary microtome and stained with cresyl violet acetate or toluidine blue. Images were taken on a Nikon Eclipse E600 light microscope with a Nikon DXM 1200C digital camera (Nikon Inc., Melville, NY, USA). Large images comprising the total leaf cross-sectional area were stitched together using Nikon NIS-Elements D2.30 imaging software. Two-dimensional area of mesophyll water storage cells and leaf thickness were measured using ImageJ 1.44o (NIH, Bethesda, MD, USA). Water storage cell area was taken as the average of 10 cells per image, and leaf thickness was averaged from three lengths of adaxial to abaxial surface of the leaf (Supporting Information Fig. S4).

Statistics and model selection

We evaluated the relationship between SWC_{meas} and various physiological parameters including C_{FT}, Π_o and SWC_{PV}, and with gross leaf traits including leaf volume and leaf shape. We also considered influences of water storage cell area (i.e. cross-sectional area of non-palisade mesophyll cells) and leaf thickness, as well as whether the plant was considered a halophyte. Leaf shape was treated as a discrete character with three states: bifacial, linear and terete (Appendix). We measured leaf volume using the water displacement method on a subset of leaves from a larger SWC_{meas} dataset (data not shown, $n = 15$ taxa, 4–5 leaves per taxon), and demonstrated a tight and nearly 1:1 relationship between leaf volume and leaf mass at full hydration ($r > 0.99$, slope = 1.07). Because we have leaf mass at full hydration for all 25 taxa as part of the calculation of SWC_{meas}, we used this value as a proxy for leaf volume.

We evaluated single pairwise trait correlations between SWC_{meas} and P-V curve parameters (Table 2), and compared various linear multiple regression models in which SWC_{meas} was described as a function of different combinations of predictor variables. Because not all of the models were nested, we used corrected Akaike information criterion (AICc) scores to compare model fit (Burnham & Anderson 2002). As many of these leaf traits and water relations parameters are non-normally distributed and show correlated errors, we log transformed the data.

Phylogenetic regressions

Of the 25 taxa for which we obtained leaf P-V curves, 12 had previously been included in a recent large-scale phylogeny of the clade Portulacineae (a subclade of Caryophyllales) based on the nuclear marker *phyC* and the chloroplast *trnK-matK* region (Arakaki *et al*. 2011). To this dataset we added new sequences of either *trnK-matK* alone or *trnK-matK* + *phyC* for seven additional taxa from our dataset (Appendix; methods following Arakaki *et al*. 2011) and performed a new phylogenetic analysis using raxML v.7.2.6 (302 taxa total) under a GTRCAT model of evolution with 1000 bootstrap replicates (Stamatakis 2006). We pruned the maximum likelihood tree to the 19 species included in our dataset. We then retested the relationship between SWC_{meas} and various predictor variables taking the tree covariance structure into account using a phylogenetic generalized linear model with the R function pglm 3.3 (Freckleton, personal communication; R Development Core Team 2011), comparing both untransformed molecular branch lengths, branch lengths with a maximum likelihood optimized Pagel's lambda transformation (Pagel 1999) and with

all branch lengths set to length 1. Halophytes were not treated as a factor in the phylogenetic glm analysis because sequence data were not available for any of those taxa.

RESULTS

Relationships between SWC_{meas} and other variables

Physiological parameters measured with P-V curves are listed in Table 1. SWC_{meas} correlated most strongly and showed a nearly 1:1 relationship with SWC_{PV} (Fig. 1a; slope = 0.85, $r = 0.88$, $P < 10^{-8}$). SWC_{meas} showed strong relationships with other parameters as well, in particular C_{FT} (Fig. 1b; $r = 0.63$, $P < 0.001$), ε, Π_o and Ψ_{TLP}, as well as with leaf volume (Table 2) and leaf shape (Supporting Information Fig. S5). Correlations between SWC_{meas} and both leaf anatomical traits were also very high (Table 2; Fig. 1c,d). We also evaluated pairwise trait correlations incorporating phylogenetic information. The phylogenetic topology recovered was completely congruent with current hypotheses of relationships within Caryophyllales (Fig. 2) (Brockington *et al*. 2009; Arakaki *et al*. 2011). Phylogenetic glm with uncorrected branch lengths recovered similar, and generally stronger, pairwise relationships between log SWC_{meas} and the predictor variables surveyed, for example, log SWC_{PV} ($r = 0.96$, $P < 10^{-10}$), log C_{FT} ($r = 0.71$, $P < 10^{-3}$) and log volume ($r = 0.87$, $P < 10^{-5}$).

We tested linear multiple regression models treating combinations of predictor variables to further investigate the components of SWC_{meas}. Because many of the variables examined appear to be highly collinear (Table 2), we performed an exploratory principal components analysis (PCA) on the dataset. PC axes 1 and 2 explained 70.7 and 21.27% of the variation in the dataset, respectively, with volume loading most strongly on PC1 and C_{FT} and ε loading most strongly on PC2. They are thus orthogonal for these axes, with the vectors for SWC_{meas}, SWC_{PV}, water storage cell area, and leaf thickness largely overlapping and falling directly between them (Supporting Information Fig. S6; vectors for water storage cell area, leaf thickness and for variables loading less strongly on PC axes are omitted for clarity). Similar results were obtained using both the full dataset and the subset of taxa on which anatomical measurements were performed. We therefore focused on C_{FT} and leaf volume as primary predictor variables in the linear model, in which SWC_{meas} is the dependent variable. We also included whether the plant is considered a halophyte as a factor in initial analyses; however, the small sample size ($n = 4$) of halophytic taxa resulted in excessive leverage from a few individual data points. We therefore did not include halophyte status as a factor in the final analysis.

Table 1. Water relation parameters derived from pressure–volume curves

Taxon	SWC	Π_o (MPa)	Ψ_{TLP} (MPa)	RWC_{TLP}	ε (MPa)	C_{FT} (MPa^{-1})	C_{TLP} (MPa^{-1})
Alluaudia procera	18.49 (1.48)	–0.90 (0.05)	–0.98 (0.06)	90.78 (0.68)	9.71 (0.32)	0.09 (0.00)	0.49 (0.08)
Anacampseros lanceolata	51.99 (14.97)	–0.29 (0.00)	–0.47 (0.00)	63.90 (6.29)	0.85 (0.19)	0.85 (0.11)	0.55 (0.19)
Anredera baselloides	18.70 (2.04)	–0.42 (0.04)	–0.60 (0.07)	70.66 (2.89)	1.57 (0.24)	0.47 (0.04)	0.40 (0.13)
Arthrocnemum subterminalis	21.34 (3.83)	–0.96 (0.10)	–1.28 (0.13)	72.80 (0.82)	3.56 (0.41)	0.22 (0.03)	0.44 (0.03)
Calandrinia axilliflora	22.76 (6.18)	–0.93 (0.10)	–1.09 (0.10)	88.08 (0.53)	8.69 (0.57)	0.10 (0.01)	0.53 (0.13)
Calandrinia colchaguensis	7.96 (0.77)	–1.20 (0.04)	–1.34 (0.01)	90.98 (2.83)	15.34 (4.57)	0.06 (0.02)	0.41 (0.09)
Cistanthe picta	13.77 (1.58)	–0.67 (0.07)	–0.78 (0.08)	86.05 (3.00)	5.90 (1.41)	0.17 (0.03)	0.69 (0.04)
Cistanthe tweedyi	19.42 (2.21)	–0.71 (0.06)	–0.90 (0.07)	76.38 (0.33)	3.78 (0.52)	0.23 (0.03)	0.45 (0.06)
Claytonia exigua	30.24 (3.90)	–0.74 (0.06)	–0.91 (0.03)	80.82 (1.94)	4.45 (0.50)	0.19 (0.02)	0.54 (0.22)
Claytonia lanceolata	31.09 (3.17)	–0.77 (0.05)	–1.03 (0.10)	77.91 (4.17)	4.21 (0.81)	0.20 (0.03)	0.35 (0.06)
Grahamia bracteata	31.01 (4.68)	–0.27 (0.00)	–0.51 (0.04)	50.80 (5.24)	0.49 (0.07)	1.05 (0.07)	0.25 (0.06)
Limeum africanum	8.09 (0.54)	–1.21 (0.15)	–1.35 (0.15)	89.39 (0.88)	12.17 (3.07)	0.08 (0.02)	0.24 (0.02)
Limonium carolinianum	15.82 (0.05)	–1.82 (0.05)	–2.48 (0.19)	79.18 (3.12)	9.34 (2.11)	0.08 (0.01)	0.11 (0.01)
Mirabilis nyctaginea	8.51 (1.32)	–0.44 (0.02)	–0.52 (0.04)	81.78 (4.63)	3.20 (0.03)	0.27 (0.00)	0.50 (0.24)
Mollugo verticillata	15.29 (1.69)	–0.50 (0.07)	–0.63 (0.07)	82.89 (0.45)	2.97 (0.92)	0.29 (0.07)	0.30 (0.05)
Montiopsis andicola	3.84 (0.04)	–1.11 (0.03)	–1.41 (0.01)	78.92 (1.71)	5.69 (0.17)	0.14 (0.01)	0.39 (0.01)
Montiopsis capitata	11.96 (3.07)	–1.01 (0.11)	–1.39 (0.17)	83.62 (2.05)	7.10 (0.41)	0.11 (0.01)	0.16 (0.04)
Montiopsis gayana	4.45 (0.14)	–1.06 (0.08)	–1.48 (0.09)	76.59 (4.91)	6.37 (0.74)	0.13 (0.01)	0.28 (0.05)
Montiopsis ramosissima	4.90 (0.28)	–0.79 (0.17)	–1.00 (0.15)	78.72 (4.42)	4.49 (2.18)	0.24 (0.07)	0.53 (0.14)
Montiopsis umbellata	2.49 (0.02)	–1.39 (0.10)	–1.79 (0.17)	80.80 (2.29)	7.43 (0.71)	0.11 (0.01)	0.16 (0.01)
Pharnaceum incanum	2.21 (0.34)	–1.05 (0.08)	–1.15 (0.08)	89.72 (1.52)	11.08 (2.64)	0.09 (0.02)	0.37 (0.04)
Pharnaceum microphyllum	9.98 (0.81)	–0.65 (0.05)	–0.94 (0.05)	72.85 (3.13)	2.43 (0.50)	0.30 (0.04)	0.25 (0.03)
Portulaca oleracea	14.24 (2.44)	–0.64 (0.07)	–0.83 (0.05)	79.35 (2.20)	3.84 (1.00)	0.24 (0.05)	0.40 (0.02)
Salicornia maritima	34.06 (6.64)	–0.77 (0.05)	–1.14 (0.20)	71.58 (7.19)	2.82 (0.84)	0.23 (0.02)	0.19 (0.02)
Suaeda taxifolia	15.80 (0.62)	–0.70 (0.06)	–0.97 (0.07)	70.45 (3.22)	2.56 (0.36)	0.30 (0.04)	0.31 (0.02)

Standard errors in parentheses.

SWC, saturated water content; Π_o, osmotic potential at full turgor; Ψ_{TLP}, water potential at turgor loss point; RWC_{TLP}, relative water content at turgor loss point; ε, volumetric elastic modulus; C_{FT}, relative capacitance before turgor loss; C_{TLP}, relative capacitance after turgor loss; MPa, megapascals.

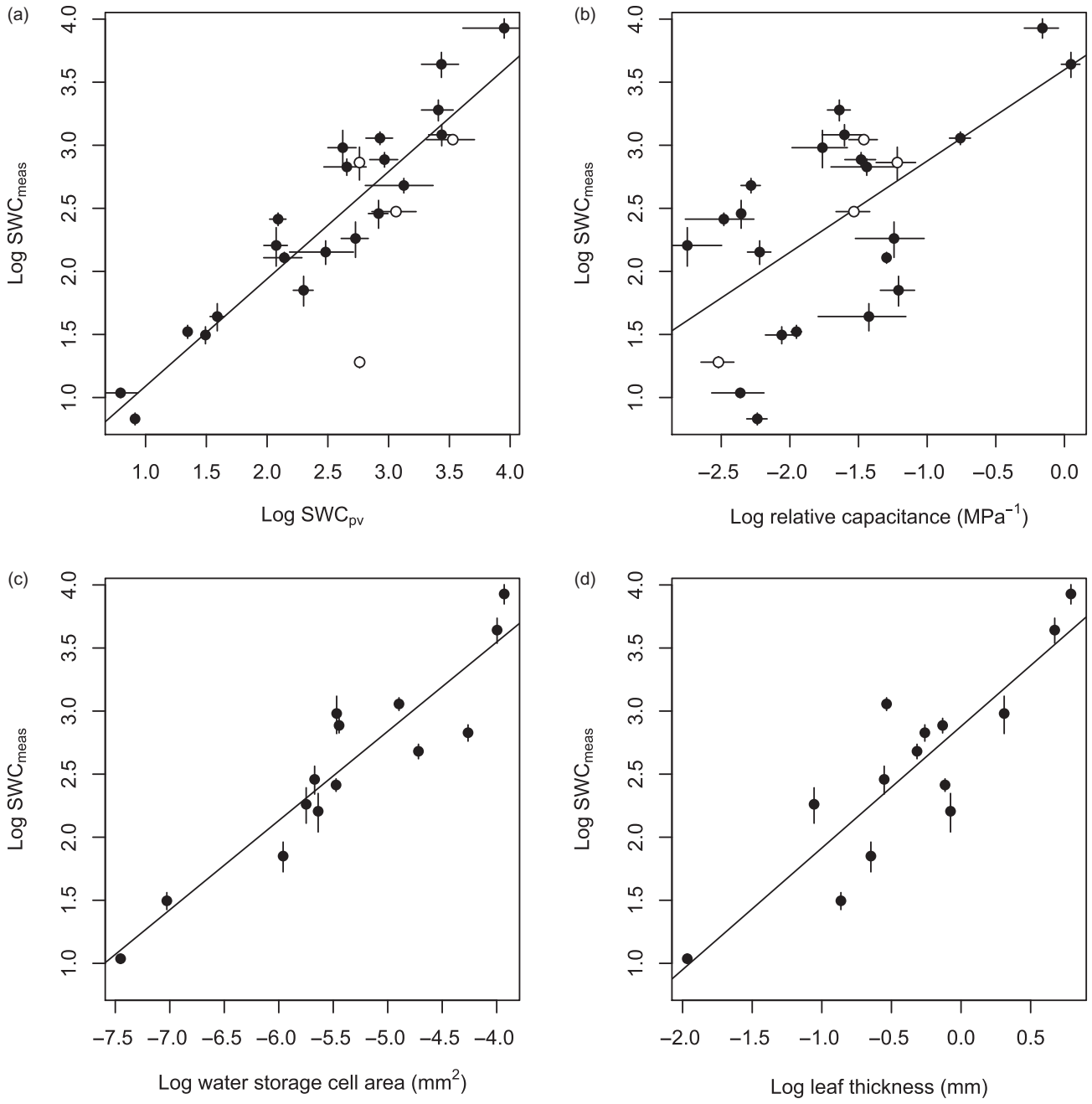

Figure 1. Relationships between SWC_{meas} and other leaf traits. (a) SWC_{PV}. (b) C_{FT}. (c) Cross-sectional area of leaf water storage cells. (d) Leaf thickness in cross section. Black circles, non-halophytes; white circles, halophytes.

Table 3 summarizes the best-fitting linear models with and without phylogenetic information. The aphylogenetic regressions include all 25 taxa, while those incorporating phylogenetic topology include the subset of 19 taxa for which sequence data were available. For this restricted set of taxa, we obtained similar results with lambda-transformed branch lengths, untransformed molecular branch lengths, with all branch lengths set to 1, and with an aphylogenetic regression on this subset of taxa. For the full aphylogenetic dataset and the restricted dataset incorporating lambda-transformed branch lengths, all models within 2 AICc units of the best-fitting model for each condition are shown, as these are not considered to differ substantially in support (Burnham & Anderson 2002). In general, the best-fitting models explained log SWC_{meas} as an additive function of log C_{FT} and log volume (Table 3; Figs 2 & 3).

DISCUSSION

In this study we build on previous efforts to produce a quantitative metric of succulence in plants, explicitly linking SWC_{meas} with physiological parameters, aspects of gross

Table 2. Pairwise Pearson correlation coefficients between SWC_{meas}, P-V curve water relations parameters, leaf volume and leaf anatomical characters

	SWC_{meas}	SWC_{PV}	Π_o	Ψ_{TLP}	RWC_{TLP}	ε	C_{FT}	C_{TLP}	volume	ws cell size	Leaf thickness
SWC_{meas}	1.00	0.88	0.68	0.63	–0.49	–0.62	0.63	0.41	0.56	0.93	0.88
SWC_{PV}		1.00	0.43	0.33	–0.45	–0.54	0.52	0.17	0.68	0.89	0.77
Π_o			1.00	0.97	–0.44	–0.72	0.78	0.57	0.08	0.62	0.41
Ψ_{TLP}				1.00	–0.27	–0.56	0.64	0.67	0.03	0.65	0.40
RWC_{TLP}					1.00	0.91	–0.85	0.14	–0.17	–0.54	–0.51
ε						1.00	–0.99	–0.09	–0.22	–0.61	–0.48
C_{FT}							1.00	0.22	0.21	0.62	0.48
C_{TLP}								1.00	0.15	0.23	0.25
volume									1.00	0.67	0.68
ws cell size										1.00	0.81
leaf thickness											1.00

All data log transformed. For most symbols and abbreviations, see Table 1.
ws, water storage.

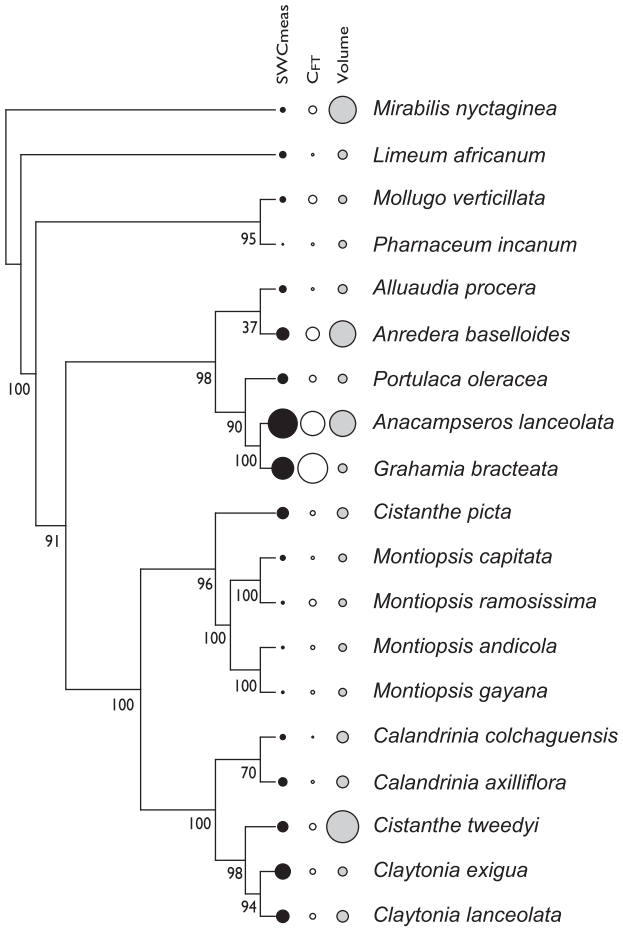

Figure 2. Cladogram of relationships among taxa based on 1000 maximum likelihood bootstrap replicates with the markers *phyC* and *trnK-matK*. Numbers below nodes indicate bootstrap values. Circles indicate relative values of SWC_{meas}, C_{FT} and leaf volume for each taxon.

morphology and leaf anatomy. The high correlation between SWC_{meas} and C_{FT}, and the inclusion of C_{FT} in most of the best-fitting linear models of SWC, supports the conclusion that the relatively rapid and easy-to-measure SWC is a suitable metric for estimating this core physiological aspect of the succulence syndrome. Despite the fact that SWC is a relative measure of water content, the regression models were also significantly improved by inclusion of absolute leaf volume (Figs 2 & 3). This likely reflects surface area to volume relationships; for a given leaf shape, an increase in volume results in a lower surface area to volume ratio (Mauseth 2000). SWC thus captures elements of both tissue-level properties (C_{FT}) and organ-level properties (leaf volume), and furthermore these variables are orthogonal along the main axes of variation in the dataset (Supporting Information Fig. S6). SWC_{meas} is also very strongly correlated with the size of water storage cells in the leaf mesophyll and with leaf thickness (Table 2), linking SWC with previous studies that have used these metrics to determine succulence (Teeri *et al*. 1981; Winter *et al*. 1983; Smith & Nobel 1986; Nobel & Hartsock 1990; Nelson *et al*. 2005; Griffiths *et al*. 2008; Nelson & Sage 2008) and supporting their utility, especially in cases where no fresh material is available to calculate SWC_{meas} using the method described here.

In our dataset, succulence is also significantly associated with leaf shape, with linear leaves having significantly lower values of SWC than terete or bifacial leaves (Supporting Information Fig. S5). This contradicts predicted surface area to volume relationships; for a given volume, a flat, bifacial leaf is expected to have the highest surface area to volume ratio, while a terete leaf will have the lowest. The lower values of SWC in linear leaves here likely reflect the fact that they are also the smallest, and thus this pattern is more strongly driven by the relationship between SWC and volume (Supporting Information Fig. S5).

We had expected that the four halophytic taxa included in our study would potentially show a different relationship between SWC and other parameters such as C_{FT} because of

Table 3. Summary of multiple linear regression models describing SWC_{meas}

#taxa	Phylogenetic information?	Branch lengths	λ	Model	AICc	r^2	Adjusted r^2	*P* value
25	No	NA	NA	log C_{FT} + log volume	46.23	0.59	0.55	5.27E-05
19	Yes	Transformed with Pagel's λ	0.71	log C_{FT} + log volume	29.86	0.54	0.48	0.002
				log volume	30.39	0.37	0.33	0.006

The best-fitting model and any models within 2 AICc units for each condition are shown.
AICc, Akaike information criterion; NA, not applicable.

the combination in these taxa of high stored water content (i.e. a 'succulent' morphology, exhibited by three of the four taxa included here) with a distinct water-use strategy that relies on low capacitance. Because halophytes generally function in conditions of low soil water potentials, their water use strategies centre around tolerance of low xylem and cell water potentials, rather than maintenance of high water potentials as in other highly succulent taxa (Waisel 1972; Trent, Blank & Young 1997). We hypothesized that this distinct water use strategy would pair high values of SWC with low values of C_{FT}, Π_o and Ψ_{TLP}, in contrast to the relationship in non-halophytic taxa. This did not appear to be the case, however; the correlations for SWC and other parameters in halophytes were similar to the values in non-halophytic taxa (Table 1, Fig. 1b). This pattern may potentially be explained by the higher relative contribution of salts to the dried mass in halophyte leaves, which could cause us to overestimate dried mass and underestimate SWC_{meas}. A better measure of SWC in halophytes may require calculation of ash content as recommended by von Willert *et al*. (1990).

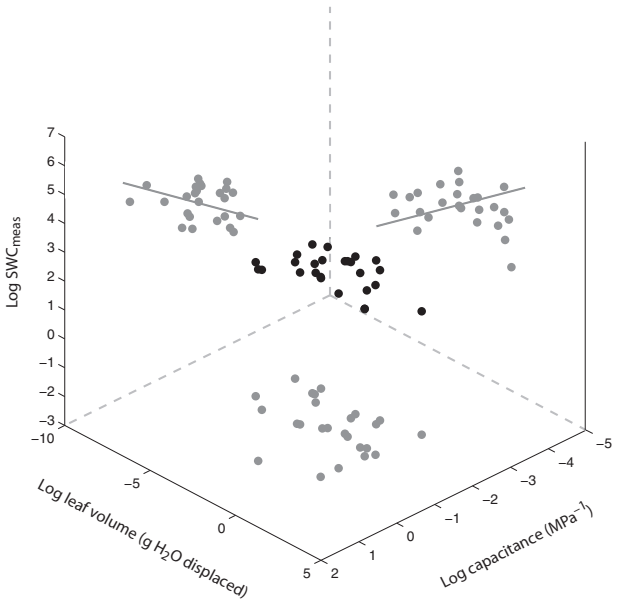

Figure 3. Relationships between SWC_{meas}, C_{FT} and leaf volume (black points), with shadow projections for pairwise relationships between SWC_{meas} and the predictor variables (grey points).

While highly succulent plants are often considered 'drought-tolerant' based on their prominence in arid zones, the positive relationship between SWC_{meas} and Ψ_{TLP} (Table 2), which has been interpreted as an indicator of drought tolerance (Bartlett, Scoffoni & Sack 2012), demonstrates that, at the cellular and tissue level, succulence is a strategy of drought avoidance rather than tolerance (Borland *et al*. 2009; Ogburn & Edwards 2010). This fits well with our understanding of high capacitance as a mechanism for maintaining relatively high water potentials in the face of water loss.

One caveat on the use of SWC is that values may not be directly comparable between different organ types, for example between leaves and stems, because these organs generally contain different ratios of water storage parenchyma to lignified vascular and support tissues. Thus, for a given absolute water storage capacity, stems will tend to have lower values of SWC because of the higher dried mass of extensive lignified cells. Similarly, in comparisons between herbaceous and woody stems, the latter will be biased to lower values of SWC, even in highly succulent woody taxa such as many cacti. In general, excluding the vascular tissues may be the most appropriate method for assessing SWC in stems. Along the same lines, because we are taking SWC as a bulk trait measured on entire leaves, it will not capture functional tissue differentiation within leaves, for example into separate water storage and chlorenchyma compartments (Smith & Lüttge 1985). Taxa with highly differentiated leaf water storage and photosynthetic tissues ('storage succulence', Eggli & Nyffeler 2009) may have lower values of SWC when compared with taxa in which there is little functional differentiation among mesophyll cells ('all-cell succulence', von Willert *et al*. 1990). In this dataset, we included taxa with both strong and weak within-leaf tissue differentiation but saw no clear signal of this in the relationship of SWC to other variables.

These caveats underscore the complex nature of succulence, which involves many dynamic aspects of organismal function (Smith & Lüttge 1985; Calkin & Nobel 1986; Smith & Nobel 1986; Schulte *et al*. 1989; Griffiths *et al*. 2008; Borland *et al*. 2009). We view a metric such as SWC as a useful complement to existing techniques. It will be especially valuable in larger-scale comparative evolutionary studies in which in-depth physiological investigation of each taxon is not feasible, and which to date have tended to suffer for lack of a quantitative estimate of succulence.

CONCLUSION

Tissue succulence in plants is a prominent aspect of plant persistence in water-limited environments. The difficulty in accurately quantifying this complex trait has presented a barrier to placing tissue succulence in a comparative phylogenetic framework. We have demonstrated that a simple metric, SWC, is a useful and practical measure of succulence, capturing important variation at both cellular and whole organ levels. It is our hope that SWC will be incorporated in future comparative studies of the evolutionary and developmental forces shaping the evolution of this important adaptive syndrome.

ACKNOWLEDGMENTS

We thank J.T. Columbus for supplying plant material, and P. Giraldo Ramírez, M. Kalin Arroyo and the herbarium staff at Universidad de Concepción, Chile (CONC) and the Compton Herbarium (NBG) at Cape Town, South Africa for support of field work. Thanks to P.-A. Christin, R. Bhaskar, M. Arakaki, L. Garrison, A. Rahlin and two anonymous reviewers for helpful comments on the manuscript. Thanks to J. Cheney for help with figure design in Matlab. This work was funded in part by the National Science Foundation grant DEB-1026611 to E.J.E.

REFERENCES

Arakaki M., Christin P.-A., Nyffeler R., Lendel A., Eggli U., Ogburn R.M., Spriggs E., Moore M.J. & Edwards E.J. (2011) Contemporaneous and recent radiations of the world's major succulent plant lineages. *Proceedings of the National Academy of Sciences of the United States of America* **108,** 8379–8384.

Bartlett M.K., Scoffoni C. & Sack L. (2012) In press. The determinants of leaf turgor loss point and prediction of drought tolerance of species and biomes: a global meta-analysis. *Ecology Letters*.

Borland A.M., Técsi L.I., Leegood R.C. & Walker R.P. (1998) Inducibility of crassulacean acid metabolism (CAM) in *Clusia* species; physiological/biochemical characterisation and intercellular localization of carboxylation and decarboxylation processes in three species which exhibit different degrees of CAM. *Planta* **205,** 342–351.

Borland A.M., Griffiths H., Hartwell J. & Smith J.A.C. (2009) Exploiting the potential of plants with crassulacean acid metabolism for bioenergy production on marginal lands. *Journal of Experimental Botany* **60,** 2879–2896.

Brockington S.F., Alexandre R., Ramdial J., Moore M.J., Crawley S., Dhingra A., Hilu K., Soltis D.E. & Soltis P.S. (2009) Phylogeny of the Caryophyllales sensu lato: revisiting hypotheses on pollination biology and perianth differentiation in the core Caryophyllales. *International Journal of Plant Sciences* **170,** 627–643.

Burnham K.P. & Anderson D.R. (2002) *Model Selection and Multi-Model Inference: A Practical Information-Theoretic Approach* 2nd edn. Springer-Verlag, New York, NY, USA.

Calkin H.W. & Nobel P.S. (1986) Nonsteady-state analysis of water flow and capacitance for *Agave deserti*. *Canadian Journal of Botany* **64,** 2556–2560.

Delf E.M. (1912) Transpiration in succulent plants. *Annals of Botany* **26,** 409–442.

Edwards E.J. & Donoghue M.J. (2006) *Pereskia* and the origin of the cactus life-form. *The American Naturalist* **167,** 777–793.

Eggli U. & Nyffeler R. (2009) Living under temporarily arid conditions – succulence as an adaptive strategy. *Bradleya* **27,** 15–38.

Gibson A.C. (1982) The anatomy of succulence. In *Crassulacean acid metabolism* (eds I.P. Ting & M. Gibbs), pp. 1–17. Proceedings of the Fifth Annual Symposium in Botany, University of California, Riverside.

Gibson A.C. & Nobel P.S. (1986) *The Cactus Primer*. Harvard University Press, Cambridge, MA, USA.

Griffiths H., Robe W.E., Girnus J. & Maxwell K. (2008) Leaf succulence determines the interplay between carboxylase systems and light use during Crassulacean acid metabolism in *Kalanchoë* species. *Journal of Experimental Botany* **59,** 1851–1861.

Hearn D.J. (2006) *Adenia* (Passifloraceae) and its adaptive radiation: phylogeny and growth form diversification. *Systematic Botany* **31,** 805–821.

Hearn D.J. (2009) Developmental patterns in anatomy are shared among separate evolutionary origins of stem succulent and storage root-bearing growth habits in *Adenia* (Passifloraceae). *American Journal of Botany* **96,** 1941–1956.

Holbrook N.M. & Sinclair T.R. (1992) Water balance in the arborescent palm, *Sabal palmetto*. I. Stem structure, tissue water release properties and leaf epidermal conductance. *Plant, Cell & Environment* **15,** 393–399.

Hunt E.R. & Nobel P.S. (1987) Non-steady-state water flow for three desert perennials with different capacitances. *Australian Journal of Plant Physiology* **14,** 363–375.

Jones C.S. & Price R.A. (1996) Diversity and evolution of seedling Baupläne in *Pelargonium* (Geraniaceae). *Aliso* **14,** 281–295.

Jones C.S., Cardon Z.G. & Czaja A.D. (2003) A phylogenetic view of low-level CAM in *Pelargonium* (Geraniaceae). *American Journal of Botany* **90,** 135–142.

Klak C., Reeves G. & Hedderson T. (2004) Unmatched tempo of evolution in Southern African semi-desert ice plants. *Nature* **427,** 63–65.

Kluge M. & Ting I.P. (1978) *Crassulacean Acid Metabolism: Analysis of An Ecological Adaptation*. Springer-Verlag, Berlin, Germany.

Koide R.T., Robichaux R.H., Morse S.R. & Smith C.M. (1989) Plant water status, hydraulic resistance and capacitance. In *Plant Physiological Ecology: Field Methods and Instrumentation* (eds R.W. Pearcy, J.R. Ehleringer, H.A. Mooney & P.W. Rundel), pp. 161–183. Chapman and Hall, New York, NY, USA.

Kramer P.J. & Boyer J.S. (1995) *Water Relations of Plants and Soils*. Academic Press, San Diego, CA, USA.

Lüttge U. (2004) Ecophysiology of Crassulacean acid metabolism (CAM). *Annals of Botany* **93,** 629–652.

Martin C.E., Lin T.-C., Lin K.-C., Hsu C.-C. & Chiou W.-L. (2004) Causes and consequences of high osmotic potentials in epiphytic higher plants. *Journal of Plant Physiology* **161,** 1119–1124.

Mauseth J.D. (2000) Theoretical aspects of surface-to-volume ratios and water-storage capacities of succulent shoots. *American Journal of Botany* **87,** 1107–1115.

Maxwell K. (2002) Resistance is useful: diurnal patterns of photosynthesis in C_3 and crassulacean acid metabolism epiphytic bromeliads. *Functional Plant Biology* **29,** 679–687.

Nelson E.A. & Sage R.F. (2008) Functional constraints of CAM leaf anatomy: tight cell packing is associated with increased CAM function across a gradient of CAM expression. *Journal of Experimental Botany* **59,** 1841–1850.

Nelson E.A., Sage T.L. & Sage R.F. (2005) Functional leaf anatomy of plants with crassulacean acid metabolism. *Functional Plant Biology* **32,** 409–419.

Nobel P.S. (2005) *Physicochemical and Environmental Plant Physiology,* 3rd edn. Elsevier Academic Press, Burlington, MA, USA.
Nobel P.S. (2006) Parenchyma-chlorenchyma water movement during drought for the hemiepiphytic cactus *Hylocereus undatus*. *Annals of Botany* **97,** 469–474.
Nobel P.S. & Hartsock T.L. (1990) Diel patterns of CO_2 exchange for epiphytic cacti differing in succulence. *Physiologia Plantarum* **78,** 628–634.
Nobel P.S. & Sanderson J. (1984) Rectifier-like activities of roots of two desert succulents. *Journal of Experimental Botany* **35,** 727–737.
North G.B. & Nobel P.S. (1991) Changes in hydraulic conductivity and anatomy caused by drying and rewetting roots of *Agave deserti* (Agavaceae). *American Journal of Botany* **78,** 906–915.
Ogburn R.M. & Edwards E.J. (2009) Anatomical variation in Cactaceae and relatives: trait lability and evolutionary innovation. *American Journal of Botany* **96,** 391–408.
Ogburn R.M. & Edwards E.J. (2010) The ecological water-use strategies of succulent plants. *Advances in Botanical Research* **55,** 179–225.
Olson M.E. & Rosell J.A. (2006)) Using heterochrony to detect modularity in the evolution of stem diversity in the plant family Moringaceae. *Evolution* **60,** 724–734.
Pagel M. (1999) Inferring the historical patterns of biological evolution. *Nature* **401,** 877–884.
Preston C.E. (1900) Observations on the root system of certain Cactaceae. *Botanical Gazette* **30,** 348–351.
R Development Core Team (2011) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.
Schmidt J.E. & Kaiser W.M. (1987) Response of the succulent leaves of *Peperomia magnoliaefolia* to dehydration. *Plant Physiology* **83,** 190–194.
Schulte P.J., Smith J.A.C. & Nobel P.S. (1989) Water storage and osmotic pressure influences on the water relations of a dicotyledonous desert succulent. *Plant, Cell & Environment* **12,** 831–842.
Smith J.A.C. & Lüttge U. (1985) Day-night changes in leaf water relations associated with the rhythm of crassulacean acid metabolism in *Kalanchoë daigremontana*. *Planta* **163,** 272–282.
Smith J.A.C. & Nobel P.S. (1986) Water movement and storage in a desert succulent: anatomy and rehydration kinetics for leaves of *Agave deserti*. *Journal of Experimental Botany* **180,** 1044–1053.
Stamatakis A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22,** 2688–2690.
Teeri J.A., Tonsor S.J. & Turner M. (1981) Leaf thickness and carbon isotope composition in the Crassulaceae. *Oecologia* **50,** 367–369.
Trent J.D., Blank R.R. & Young J.A. (1997) Ecophysiology of the temperate desert halophytes: *Allenrolfia occidentalis* and *Sarcobatus vermiculatus*. *Great Basin Naturalist* **57,** 57–65.
Tyree M.T. & Hammel H.T. (1972) The measurement of the turgor pressure and the water relations by the pressure-bomb technique. *Journal of Experimental Botany* **23,** 267–282.
Von Willert D.J., Eller B.M., Werger M.J.A. & Brinckmann E. (1990) Desert succulents and their life strategies. *Vegetatio* **90,** 133–143.
Von Willert D.J., Eller B.M., Werger M.J.A., Brinckmann E. & Ihlenfeldt H.-D. (1992) *Life Strategies of Succulents in Deserts: With Special Reference to the Namib Desert*. Cambridge University Press, Cambridge, UK.
Waisel Y. (1972) *Biology of Halophytes*. Academic Press, New York, NY, USA.
Walker S., Oosterhuis D.M. & Wiebe H.H. (1984) Ratio of cut surface area to leaf sample volume for water potential measurements by thermocouple psychrometers. *Plant Physiology* **75,** 228–230.
Winter K., Wallace B.J., Stocker G.C. & Roksandic Z. (1983) Crassulacean acid metabolism in Australian vascular epiphytes and some related species. *Oecologia* **57,** 129–141.

Received 6 December 2011; received in revised form 14 February 2012; accepted for publication 15 February 2012

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pressure-volume curves from two representative taxa. The inflection point of the curve indicates the turgor loss point (arrows), after which leaf water potential is determined solely by osmotic potential. The shallower slope for the initial (high water content) portion of the curves in *Anredera baselloides* indicates higher relative capacitance compared with *Montiopsis gayana*. Π_0 is calculated as the negative inverse of the y-intercept for the post-turgor loss portion of the curve; Ψ_{TLP} is the negative inverse of the *y* axis value at the turgor loss point; RWC_{TLP} is 100 min the *x* axis value at the turgor loss point; C_{FT} is the relationship $(\Delta V/V)/\Delta\Psi$, calculated from the pre-turgor loss portion of the curve; C_{TLP} is the same relationship from the post-turgor loss portion; ε is the relationship $\Delta\Psi_P/(\Delta V/V)$ from the pre-turgor loss portion of the curve.
Figure S2. Primary pressure-volume curve data from 12 taxa. Note truncated *y*-axis scale for *Arthrocnemum subterminale*, *Grahamia bracteata* and *Salicornia maritima*.
Figure S3. Estimation of SWC_{pv} from the relationship between sample water mass and water potential for *Montiopsis ramosissima*. Extrapolation of the regression line from the pre-turgor loss portion of the curve (black points) is used to estimate sample water mass at full saturation, taken as the point of zero MPa water potential. This quantity is divided by sample dried mass to yield SWC_{pv}.
Figure S4. Cross-sections from representative leaves, with measurements of water storage cell area and leaf thickness depicted in black. (a) *Mollugo verticillata*, with slightly succulent, bifacial leaves. (b) *Pharnaceum incanum*, with non-succulent, linear leaves. (c) *Grahamia bracteata*, with terete, highly succulent leaves.
Figure S5. Relationships between log SWC_{meas}, leaf shape and volume. Linear leaves have significantly lower values of SWC_{meas}, but this is likely being driven by their smaller volume rather than by a large contribution of leaf shape.
Figure S6. Principal components analysis on all continuous variables. PC axis 1 explains 72.6% of the variation and PC axis 2 explains a further 18.9% of the variation in the dataset. Leaf volume loads most strongly onto PC1, while C_{FT} and ε load most strongly onto PC2. The eigenvectors for

SWC_{meas}, SWC_{PV}, and water storage cell size and leaf thickness (not shown) are all highly overlapping for PC axes 1 and 2 and are located midway between those for leaf volume and C_{FT}. Other P-V curve variables loading weakly onto PCs 1 and 2 are omitted for clarity.

APPENDIX

Taxon	Source	Voucher	Sequence data available	Leaf shape	Leaf anatomy
Alluaudia procera Drake	Greenhouse	Ogburn 265	trnK-matK + phyC	Bifacial	X
Anacampseros lanceolata Sweet	Greenhouse	Ogburn 38	phyC	Terete	X
Anredera baselloides (Kunth) Baill.	Greenhouse	Ogburn 256	trnK-matK + phyC	Bifacial	X
Arthrocnemum subterminale (Parish) Standl.	Field		None	Terete	
Calandrinia axilliflora Barnéoud	Field	Ogburn 249	trnK-matK* + phyC*	Bifacial	X
Calandrinia colchaguensis Barnéoud	Field	Ogburn 273	trnK-matK*	Linear	X
Cistanthe picta (Gillies ex Arn.) Carolin ex Hershk.	Field	Ogburn 286	trnK-matK	Bifacial	X
Cistanthe tweedyi (Gray) Hershkovitz	Field	Ogburn 182	trnK-matK*	Bifacial	X
Claytonia exigua Torr. & Gray	Field	Ogburn 177	trnK-matK + phyC	Terete	
Claytonia lanceolata Pall. ex Pursh	Field	Ogburn 186	trnK-matK	Bifacial	
Grahamia bracteata Gill.	Greenhouse	Ogburn 25	trnK-matK + phyC	Terete	X
Limeum africanum Sieber ex Moq.	Field	Ogburn 140	trnK-matK	Bifacial	X
Limonium carolinianum (Walter) Britton	Field	Ogburn 292	none	Bifacial	
Mirabilis nyctaginea (Michx.) MacM.	Field		trnK-matK	Bifacial	
Mollugo verticillata L.	Field		trnK-matK + phyC	Bifacial	X
Montiopsis capitata (Hook. & Arn.) D.I. Ford	Field	Ogburn 248	trnK-matK*	Linear	
Montiopsis gayana (Barnéoud) D.I. Ford	Field	Ogburn 268	trnK-matK*	Linear	X
Montiopsis ramosissima (Hook. & Arn.) D.I. Ford	Field	Ogburn 277	trnK-matK*	Linear	
Montiopsis umbellata (Ruiz & Pav.) D.I. Ford	Field	Ogburn 253	none	Linear	
Montiopsis andicola (Gillies) D.I. Ford	Field	Ogburn 271	trnK-matK*	Linear	
Pharnaceum incanum L.	Field	Ogburn 148	trnK-matK + phyC	Linear	X
Pharnaceum microphyllum L.f.	Field	Ogburn 151	none	Terete	X
Portulaca oleracea L.	Field	Ogburn 18	trnK-matK + phyC	Bifacial	X
Salicornia maritima S.L. Wolff & Jefferies	Field	Ogburn 291	none	Terete	
Suaeda taxifolia Standl.	Field		none	Linear	

*Sequenced for this study. Sequences deposited in GenBank, GB accession numbers JQ780477–JQ780484. All other sequences downloaded from GenBank.