STOMATA LENGTH IS A RELIABLE CHARACTERISTIC FOR DISTINGUISHING INFRASPECIES AND PLOIDY LEVELS OF *OPUNTIA MESACANTHA* (CACTACEAE)

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ABSTRACT

Opuntia mesacantha includes two subspecies, *O. mesacantha* subsp. *mesacantha* (tetraploid) and *O. mesacantha* subsp. *lata* (diploid), that are difficult to distinguish in the field. We show that (1) stomata length is effective for distinguishing the two subspecies, and (2) this can be visually assessed, reliably and non-destructively, using a microscope with a reticle (ocular data). We compare our ocular results with digital imaging and chromosome counts from mitosis in root tips to confirm that our approach is effective for these taxa.

Key Words: cactus, field techniques, polyploidy, prickly pear

RESUMEN

Opuntia mesacantha incluye dos subespecies, *O. mesacantha* subsp. *mesacantha* (tetraploide) y *O. mesacantha* subsp. *lata* (diploide), que son difíciles de distinguir en el campo. Nosotros señalamos (1) la longitud de los estomas es efectiva para distinguir las dos subespecies, y (2) esto puede asegurarse visualmente, es efectivo y no-destructivo, usando un microscopio con un retículo (ocular). Comparamos nuestros resultados con los imagen digital y recuentos cromosomáticos en mitosis ápices radiculares para confirmar que nuestro sistema es efectivo para estos taxa.

INTRODUCTION

Opuntia mesacantha Raf. (Cactaceae, prickly pear cactus) includes two subspecies: the tetraploid (*n*=22) *O. mesacantha* subsp. *mesacantha* and the diploid (*n*=11) *O. mesacantha* subsp. *lata* (Small) Majure (Majure et al. 2017). While the two subspecies are widespread throughout the Fall Line Sandhills, Piedmont, and Coastal Plains, both are found in small, widely dispersed populations. These taxa are part of the *Humifusa* clade and are becoming increasingly vulnerable to human encroachment, resulting in habitat loss and shrinking populations (Majure et al. 2017). Reliable identifiers are crucial to distinguish members of this group, as few morphological characteristics are consistently different across these taxa. The two subspecies of *O. mesacantha* are difficult to distinguish in the field, although they have two different ploidy levels. Stomata length is known to vary with different ploidy levels and has been used to determine those differences in many species such as *Capsicum annuum*, *Coffea*, *Phleum* spp., *Actinidia deliciosa*, *Acacia mearnsii* (Przywara et al. 1988; Mishra 1997; Beck et al. 2003; Joachimiak & Grabowska-Joachimiak 2000; Shrestha & Kang 2016). In *Opuntia*, stomata length is among the characteristics with the least variability (Conde 1975). All *Opuntia* primarily photosynthesize on their stems (cladodes) (Nobel 1982).

The goal of this study was to determine if stomata length can be used to reliably distinguish between diploid and tetraploid subspecies of *Opuntia mesacantha*. We measured stomata length of *O. mesacantha* subsp. *lata* and subsp. *mesacantha* to determine whether there are consistent and observable differences between these two subspecies. We also performed chromosome counts to confirm ploidy levels of each of our samples. Reliable, field-based approaches are needed for faster and more accessible ways of identifying plants with different ploidy levels, which may also represent different taxa. While chromosome numbers are extremely

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useful, data for morphological variation is crucial for field identification of species and for developing taxonomic descriptions and keys (Baker 2016).

STUDY SITES

Opuntia mesacantha subsp. *mesacantha* (tetraploid) were sampled in Rockdale and DeKalb counties east of Atlanta, Georgia, and in Randolph County, Alabama, on the eastern side of the state. *Opuntia mesacantha* subsp. *lata* (diploid) were sampled south of Macon, Georgia, in Houston and Irwin counties, and west of Macon in Taylor County, Georgia. Habitat ranged from granitic outcrops in the Piedmont, to poorly managed land in the Fall Line Sandhills, to the Tifton Uplands in the Coastal Plains. Sites were selected utilizing known populations from previous work by Majure (2014, 2017) or other sites located by the author with populations that more closely matched the morphologic descriptors given in those same works. All of the study sites and overall range of the species is within the Köppen Climate Classification Cfa, which is a mild temperate climate with hot, humid summers.

METHODS

Three populations of each subspecies were sampled. At each site, one pad was sampled, on each of two occasions. Each sample was tested for ploidy level by measuring stomata length, and ploidy was then verified by chromosome counts of the root-tip meristematic cells.

For the stomata data, impressions or casts were made utilizing a modified version of the technique described by Hilu and Randall (1984) using a 50–50 mixture of clear fingernail polish and acetone, which was found to reduce fracturing of the cast. The pad surface was cleaned, and a uniform, medium-thick layer of polish was carefully applied over a 12 mm^2 area. The polish was allowed to dry for 10 minutes. A piece of 19 mm wide clear film tape (Scotch® Transparent or Shurtape® JLAR) was folded over on itself to provide a 20 mm handle and then cut, leaving another 20 mm length of the sticky surface exposed. At the end of the drying period, the tape was placed firmly over the nail polish cast. The cast was then slowly peeled off of the pad and affixed to a clean microscope slide. The folded handle of the tape was removed with a scalpel. Shrestha and Kang (2016) used a cover slip instead of the tape completely covering the cast. We found that the cover slip had no optical superiority over the tape and experienced less stretching and deformation of the casts if removed when completely covered by the tape. Four casts were made per pad: one distal and one proximal on the front and rear sides of the pad. Each slide was then placed on a compound microscope (Olympus Corp.) and observed under 400× magnification, and images for eight stomata per slide were captured using a CMOS microscope camera (Tucsen Photonics) and measurements were then made using Image J software (NIH, Bethesda, MD). For the eyepiece with reticle measurements (ocular data), one random slide from each specimen was used. Stomata length, as defined in this study, is the linear distance between the junctions of the guard cells at each end of the stomata (Zheng et al. 2013).

Sixteen variables were examined. The digital data included stomata measurements for the six populations (n = 64 for each population: two pads per population x four casts per pad x eight stomata measured per cast) and for the ocular data (n = 16 for each population: two pads per population x one cast per pad x eight stomata measured by reticle per cast). Because pads are typically tilted rather than having their long axis perpendicular to the ground, the digital data were split into top of pad (the adaxial surface) and bottom of pad (the abaxial surface), merging the data for the three locations with the same subspecies. Thus, the last four variables (n = 96 for each) represented all the top readings for *O. mesacantha* subsp. *mesacantha*, the bottom readings for this taxon, and the same two variables for *O. mesacantha* subsp. *lata*. These variables were tested for normality using the Kolmogorov-Smirnov normality test. Because most of the variables deviated significantly from a normal distribution, non-parametric statistics (independent-samples Kruskal-Wallis and Mann-Whitney U tests) were applied. For *O. mesacantha* subsp. *mesacantha* digital data, two of the sites did not deviate significantly from a normal distribution ($P > 0.05$) and the third was marginal ($P = 0.0497$), thus we used one-way

ANOVA for this test comparing the three tetraploid sites. Finally, a Kruskal-Wallis test was employed to test whether the two sides of the pads have significantly different stomata lengths.

For chromosome counts, rapidly growing emergent root tips from collected cladodes were treated for 8–10 hours at room temperature in a 1:1 mixture of 8-hydroxyquinoline (0.002 M) and 0.05% colchicine, then fixed in a cold 3:1 solution of 95% ethanol and glacial acetic acid for a minimum of 24 hours (modified from Tanaka & Kamemoto 1984). Samples not processed immediately were refrigerated until use. Root tips were hydrolyzed in a 1:1 mixture of concentrated hydrochloric acid and 95% ethanol for 5 minutes at room temperature, transferred directly into cold 45% aqueous acetic acid for 15–30 minutes, and then placed on a slide under a dissecting microscope, where the meristematic tissue was teased out in 60% acetic acid (Stock et al. 1972). A cover slip was positioned, the tissue then squashed by thumb pressure and the slide was placed on dry ice for 10 minutes. The cover slip was then removed by quickly prying up with a scalpel (Conger & Fairchield 1953) and the slide was gently heated over an alcohol flame to dry, allowed to cool, stained in 2% Giemsa (Gurr's R66) in distilled water, rinsed briefly in distilled water, and placed vertically to dry. Slides were observed directly, without coverslips, under 400× and 1000× (oil) magnification on a Zeiss Axioscope. A minimum of 5 cells were analyzed per specimen, and chromosome images were digitally captured.

RESULTS

The Taylor County ocular data (n = 16) and the digital data for Randolph, Rockdale, Houston, and Taylor counties (n = 64 each) did not deviate significantly from a normal distribution. The remaining ocular data for DeKalb, Randolph, Rockdale, Houston and Irwin counties did deviate significantly ($P < 0.05$) from a normal distribution, as did the digital data for Irwin county ($P < 0.05$) and DeKalb county marginally ($P = 0.0497$). The adaxial and abaxial data for both subspecies also deviated significantly from a normal distribution.

Results were consistent for all parametric and nonparametric tests of stomata length across all populations, for both the digital and ocular data. The independent-samples Kruskal-Wallis test confirmed no difference ($P = 0.97$, n = 64 at each site) in digital measurements of stomata length in specimens across the three diploid sites sampled (*O. mesacantha* subsp. *lata*). A one-way ANOVA similarly confirmed no difference ($P = 0.44$, n = 64 at each site) in digital measurements across the three tetraploid sites (Table 1). For the ocular data, the Kruskal-Wallis test confirmed there was no significant difference across the three tetraploid sites ($P = 0.52$, n = 16 at each site), or across the three diploid sites ($P = 0.36$, n = 16 at each site) (Table 2). Kruskal-Wallis results show that stomata lengths did not vary between the top and bottom of the pads for either the diploid ($P = 0.43$, n = 96 for each pad face) or tetraploid ($P = 0.78$, n = 96 for each pad face) populations.

The chromosome counts confirmed *Opuntia mesacantha* subsp. *lata* samples were diploid, and those of *O. mesacantha* subsp. *mesacantha* were tetraploid (Fig. 1). Thus, the samples from populations with the same subspecies and ploidy are consistent. We compared stomata length between the subspecies. The independent-samples Mann-Whitney U test confirms that the diploid and tetraploid stomatal lengths are significantly different for both digital data (n = 192 for each taxon, $P < 0.001$, Table 1), and ocular data (n = 48 for each taxon, $P < 0.001$, Table 2). Both the ocular and digital methods confirm that stomata length can be used to distinguish the different subspecies (Fig. 2), and the digital results confirm that the ocular technique is reliable for fast, efficient assessment in the field.

DISCUSSION

This group represents a mature polyploid species complex (Majure et al. 2012; Majure & Ribbens 2012). Its members commonly hybridize (Parfitt 1980; Majure et al. 2017), and they also express highly variable phenotypes in response to the environment (e.g., Drezner 2017a, b). Consequently, identification is complicated and sometimes compromised because of the great variability across and within taxa.

In his study of two *Opuntia* species and three *Cylindropuntia* species, Conde (1975) observes that stomata length shows low variability compared to other anatomical characteristics. Our data confirm that stomata

Table 1. Stomata length as measured by digital imaging and statistical comparisons. Ploidy levels include tetraploid (*2n*=44) and diploid (*2n*=22). States are abbreviated GA (Georgia) and AL (Alabama). Means and standard deviations (SD) are given along with sample size (n = number of stomata measured) and the minimum and maximum values. An independent-samples Mann-Whitney U test comparing the subspecies (n = 192 for each) gives a significance of $P < 0.001$. Voucher specimens were deposited at Valdosta State University Herbarium (VSC) and living plant material is maintained by the author (PA).

Site (County, State)	Ploidy	Stomata Length (µm) Mean	SD	Min	Max	n	Significance
Opuntia mesacantha* subsp. *mesacantha							
DeKalb, GA	44	28.68	0.97	27.07	31.03	64	
Randolph, AL	44	28.88	0.95	27.24	30.83	64	One-Way
Rockdale, GA	44	28.88	1.04	27.00	31.03	64	ANOVA
Combined	44	28.81	0.99	27.00	31.03	192	P = 0.44
Opuntia mesacantha* subsp. *lata							
Houston, GA	22	21.98	0.85	20.13	23.75	64	
Taylor, GA	22	21.97	0.88	20.14	23.44	64	
Irwin, GA	22	21.92	0.89	20.23	23.43	64	Kruskal-Wallis
Combined	22	21.96	0.88	20.13	23.75	192	P = 0.97

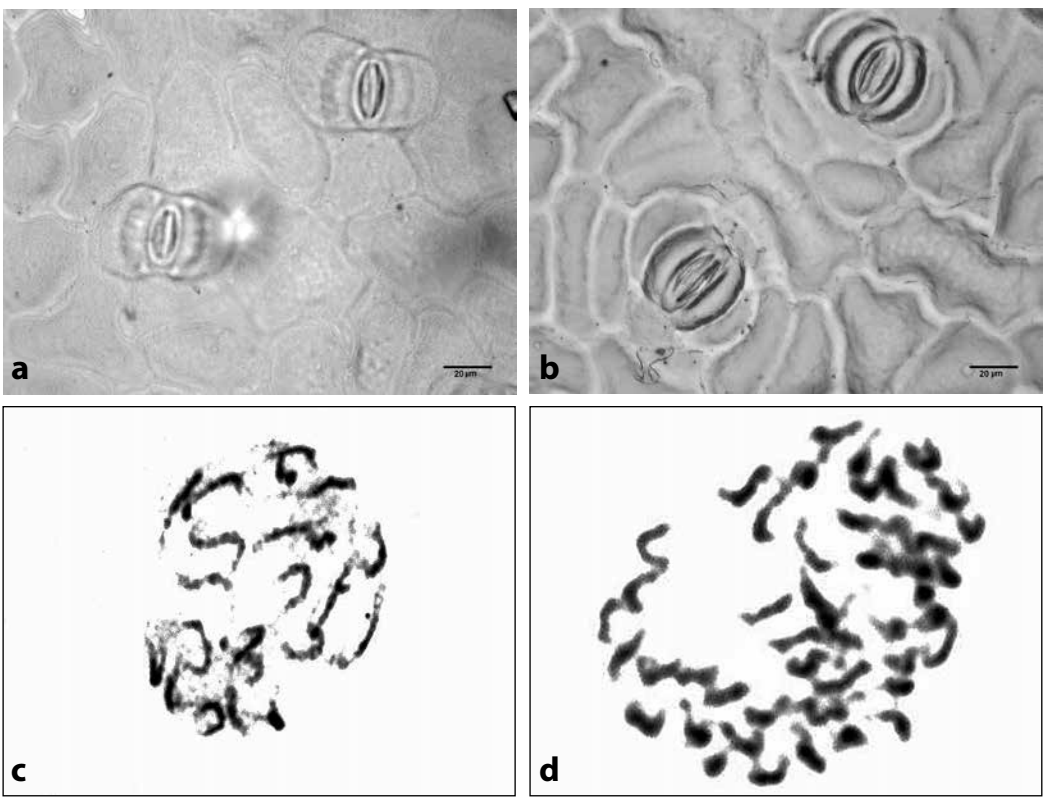

Fig. 1. Stomata in *Opuntia mesacantha* subsp. *lata* (diploid: a, c) and *O. mesacantha* subsp. *mesacantha* (tetraploid: b, d). Top (a, b) differences in stomata length between the two subspecies, with scale bars (20 µm). These differences are associated with different chromosome counts as confirmed by images c (diploid, *2n*=22) and d (tetraploid, *2n*=44).

Table 2. Stomata length as measured by microscope with reticle (ocular data) and statistical comparisons. Ploidy levels include tetraploid (*2n*=44) and diploid (*2n*=22). States are abbreviated GA (Georgia) and AL (Alabama). Mean and standard deviations (SD) are given along with sample size (n, number of stomata measured) and the minimum and maximum values. Independent-samples Kruskal-Wallis tests were performed for each subspecies, and the P-value (Significance) is given for each. An independent-samples Mann-Whitney U test comparing the subspecies (n = 48 for each) gives a significance of $P < 0.001$.

Site (County, State)	Ploidy	Stomata Length (µm) Mean	SD	Min	Max	n	Significance
Opuntia mesacantha* subsp. *mesacantha							
DeKalb, GA	44	29.03	0.74	27.50	30.00	16	
Randolph, AL	44	28.80	0.78	27.50	30.00	16	
Rockdale, GA	44	29.08	0.47	28.00	29.50	16	
Combined	44	28.96	0.67	27.50	30.00	48	P = 0.52
Opuntia mesacantha* subsp. *lata							
Houston, GA	22	23.03	0.77	21.25	23.75	16	
Taylor, GA	22	23.00	0.93	21.25	25.00	16	
Irwin, GA	22	23.45	0.94	22.00	25.00	16	
Combined	22	23.13	0.89	21.25	25.00	48	P = 0.36

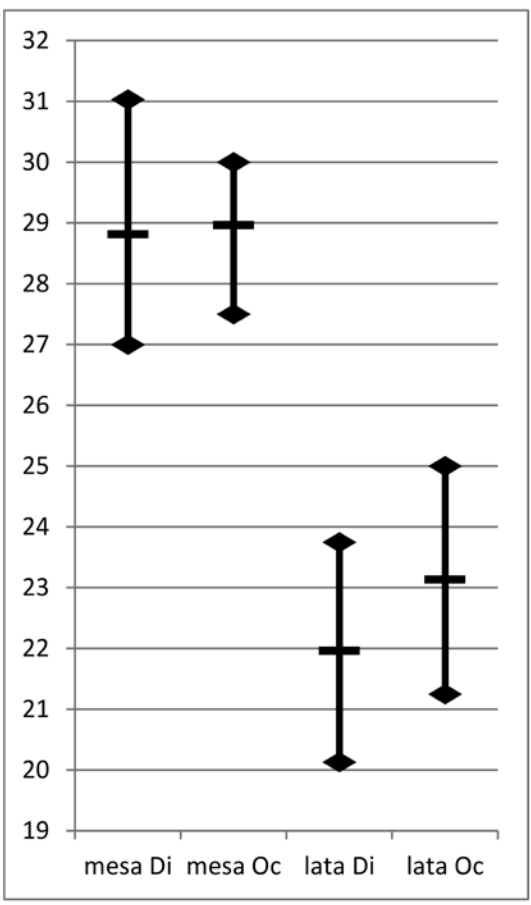

Fig. 2. Comparison of stomata length in the two *Opuntia mesacantha* subspecies using digital (Di) measurements (n = 192 for each subspecies) and ocular (Oc) eyepiece measurements (n = 48 for each subspecies). *Opuntia mesacantha* subsp. *mesacantha* (mesa) is tetraploid, and *O. mesacantha* subsp. *lata* (lata) is diploid. The central horizontal line is the mean and the diamonds the maxima and minima. Vertical axis units are in microns (µm).

lengths are statistically and predictably different in the diploid and tetraploid subspecies of *Opuntia mesacantha*. A morphological approach that uses stomata length to distinguish diploid and tetraploid subspecies of *Opuntia mesacantha* is a feasible and much more field-friendly alternative to chromosome counts, and both ocular and digital stomata length measurements are reliable for distinguishing between the diploid and tetraploid subspecies of *Opuntia mesacantha* and may prove to be useful in distinguishing other members of the recently revised *Humifusa* clade, such as *O. drummondii* (Majure et al. 2017).

Stomata density also has been investigated as a potential indicator of ploidy (e.g., *Coffea*, Mishra 1997). We have observed wide variation in stomata densities within the species and across populations, consistent with other studies that have found large variations in *Opuntia* stomata density (Herrera-Martinez et al. 2015). For example, a population of *O. mesacantha* subsp. *lata* growing in full sun adjacent to a pine tree farm showed extremely low stomata density, perhaps as a result of high CO_2 levels as referenced in Casson and Gray (2007). Because of the high degree of variation within a single subspecies, we did not test stomata density as a potential variable to distinguish the two subspecies in this study.

Our results show that the ocular measurements are successful for determining stomata length in the field, making it valuable for identification and taxonomic work. Our ocular technique is simple and non-destructive, as the pad does not need to be removed from the plant. Our approach offers a time-saving and inexpensive method for determining ploidy level.

APPENDIX A: SPECIMENS EXAMINED

Opuntia mesacantha* subsp. *mesacantha

U.S.A. ALABAMA: Randolph Co.: granite outcrop, ca. 0.1 mi E of intersection Co. Rd 15 and Co. Rd 15, 9 Jul 2016, *Adanick 074* (VSC); 16 May 2017, *Adanick 109* (VSC). **GEORGIA: DeKalb Co.:** disused quarry, Hwy. 124, ca. 1.1 mi N of I-20, 17 Jun 2016, *Adanick 053* (VSC); 16 May 2017, *Adanick 103* (VSC). **Rockdale Co.:** granite outcrop, Harralson Mill Rd., N of Booth Rd, 16 May 2017, *Adanick 114* (VSC); 17 May 2017, *Adanick 115* (VSC).

Opuntia mesacantha* subsp. *lata

U.S.A. GEORGIA: Houston Co.: rough pasture, Marshallville Rd., ca. 0.1 mi W of I-75, 17 Jun 2016, *Adanick 057* (VSC); 11 Feb 2017, *Adanick 100* (VSC). **Irwin Co.:** roadside, Hwy. 32, ca. 1.8 mi W of Irwinville, 31 Dec 2015, *Adanick 011* (VSC); 11 Feb 2017, *Adanick 102* (VSC). **Taylor Co.:** sandy field, Hwy. 96 and Industrial Rd, 18 Jun 2016, *Adanick 066* (VSC); 10 Feb 2017, *Adanick 099* (VSC).

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