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COMPLEX TAXONOMY IN OPUNTIOIDEAE: IS FLORAL MORPHOMETRY ESSENTIAL TO IDENTIFY *OPUNTIA* SPECIES?

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Abstract

Correct species identification is critical for studies on biodiversity, ecology, and conservation. Determining *Opuntia s.s.* species is difficult because they have similar traits and are phenotypically plastic. Taxonomic keys are based on vegetative traits, rather than reproductive ones such as flowers, because they are assumed to be too similar. We analyzed morphometric characteristics of flowers and cladodes over 6 years to determine which of these is most useful for differentiating *Opuntia* species from the Chihuahuan Desert. For each species (*Opuntia robusta*, *O. cantabrigiensis*, *O. tomentosa*, and *O. streptacantha*), we tagged 20 hermaphroditic and 40 dioecious plants (totaling 100) from 2014-2020, to complete the sample size of flowers and cladodes. Seventeen morphometric characters were measured for new cladodes and 15 for flowers, and discriminant analysis was applied to determine which traits enabled species delimitation. Six of the 17 cladode characteristics combined explained 89% of the variation, while nine floral characteristics combined explained 94% of the variation. Floral morphometrics proved to be very useful to accurately differentiate species and should be included, in addition to cladodes, in future taxonomic studies. Here, we provide the first taxonomic key that includes floral traits to identify *Opuntia* and a new description of each studied species.

Key words. Cladodes, floral morphometry, *Opuntia,* taxonomic key.

1. Introduction

Opuntia s.s. (Cactaceae) are an important source of food and shelter for a variety of vertebrate (Illoldi-Rangel et al. 2012) and invertebrate (Mandujano et al. 1996; Reyes-Agüero et al. 2006) species. Humans in many cultures have historically gathered *Opuntia* products, a practice that has expanded worldwide to more than 30 countries after their introduction to the Old World (Inglese et al. 2002; Novoa et al. 2015; Adli et al. 2017). Prickly pears (cactus pear cacti, *Opuntia s.s.*) are grown worldwide in small patios, gathered as nontimber products, or cultivated extensively in plantations for their edible fruits and cladodes, and as a natural dye (Bravo-Hollis 1978; Pinkava et al. 1985; Russell and Felker 1987; Novoa et al. 2015). The capacity for clonal regeneration and resistance to harsh conditions have made them particularly successful species after their introduction (Mandujano et al. 2001, 2007; Gallegos-Vázquez et al. 2012), to the point of being invasive in many countries (Novoa et al. 2015).

Interestingly, *Opuntia s.s.* is easily distinguished from other 174 cacti genera. It is characterized by flattened stem segments, racket-shaped paddles (cladodes), without spine sheaths around permanent spines, often has transient leaves in areoles, and pollen grains are reticulated and spherical with a polyporate surface (Bravo-Hollis 1978; Kiesling 1984; Pinkava et al. 1985; Rebman and Pinkava 2001). The flowers are typical for the entire group—large, solitary, actinomorphic, hermaphroditic, with numerous stamens, a single pistil and lobulate stigma, an inferior ovary, within a green pericarpel containing areoles, which bear small retrorse spines (glochids). The ovary consists of several fused carpels with numerous ovules with parietal placentation. The perianth has segments arranged in a spiral, which can be orange, yellow, red, or pink. The styles have lobulate stigmas, and the numerous stamens have yellow anthers (Bravo-Hollis 1978; Grant and Hurd 1979; Kiesling 1984; Pinkava et al. 1985; Anderson 2001). The fruit is a berry (Bravo-Hollis 1978; Grant and Hurd 1979; Kiesling 1984; Pinkava et al. 1985).

The classification of *Opuntia s.s.* is known to be difficult (Bravo-Hollis 1978; Kiesling 1984; Anderson 2001; Pinkava 2002; Hunt et al. 2006). Not surprisingly, the number of described species of *Opuntia* varies, not only because of their natural diversity and ability to hybridize, but also because the distinctive characters that were previously used to define species in this genus (e.g., shape of the stems) have changed over time. Currently, the habit of the plant and the shape of the stems have been used to split *Opuntia* into different genera: cylindrical stems for *Cylinropuntia*, columnar stems for *Grusonia*, and flattened stems for *Opuntia s.s*. The flattened shape was previously used to describe both *Opuntia* and *Nopalea*, but the two genera are now merged as *Opuntia s.s*. *Opuntia* currently includes *ca.* 200 species (Bravo-Hollis 1978; Anderson 2001; Hunt et al. 2006) and several hybrids and polyploids (Pinkava et al. 1985; Moran and Zimmermann 1991; Mayer et al. 2000). For this reason, *Opuntia* is as a complex genus (Kiesling 1984; Rebman and Pinkava 2001; Pinkava 2002) with reticulated evolution (Pinkava 2002; Majure et al. 2012). *Opuntia s.s*. species are distributed naturally throughout the Americas, mainly in dry environments (including arid, semiarid, and dry tropical areas) with some cosmopolitan species. Mexico is a center of diversification for the Cactaceae as well as for the genus, where about half of the species can be found, and ca. 85% of these species are endemic (Bravo-Hollis 1978; Rebman and Pinkava 2001; Guzmán et al. 2003; Golubov et al. 2005). The wide geographical distribution of *Opuntia s.s.* contrasts with all other cacti genera, which usually have limited distributions. For example, *Mammillaria*, which is the most speciose genus of the family, is distributed exclusively in arid areas of North America and the Caribbean (Anderson 2001).

Despite *Opuntia s.s.* being a highly distinctive genus, its species-level taxonomy is confusing. Morphological characters have not yet been determined to clearly differentiate taxonomic units, and molecular phylogenies generally result in unresolved trees with low branch support and multiple possible relationships, yielding a reticulate phylogeny, probably due to hybridization (Pinkava et al. 1985; Majure et al. 2012). This morphological diversity has resulted into descriptions of many varieties or subspecies. For example, *Opuntia albicarpa* in central Mexico covers a complex of 22 varieties, each one with unique fruit characteristics, and some with sterile seeds (e.g., *O. albicarpa* cultivar Amarilla Olorosa [meaning fragrant yellow]; (Reyes-

Agüero et al. 2009). *Opuntia albicarpa* cultivars have different common or vernacular names, and three varieties that are cultivated at large scales (i.e., chapeada, which means rosy cheeks; Copena, which is an a proper name and blanca San José, which means San Jose white; (Gallegos-Vázquez and Mondragón 2011). In addition, *Opuntia* s.s. is a genus with persistent clonality (Mandujano et al. 2001, 2007), and there are clonal microspecies in which no sexual reproduction or fruits are observed (Grant and Grant 1980; Reyes-Agüero et al. 2009; Elbehi et al. 2015).

Determining which quantitative characters are useful for distinguishing taxa at the specific and infraspecific levels are especially relevant for estimating diversity in complicated taxa with subspecies or varieties such as *Opuntia s.s.* (Anderson 2001; Reyes-Agüero et al. 2009). Morphometric analyses make quantitative characters important for distinguishing taxa (Lihová et al. 2004) and can help to establish differences between related species that are easily observable and measurable. Morphological characteristics can also be used to generate accurate taxonomic descriptions, define discriminating traits, establish kinship relationships, and compare related taxa (Anderson 2001; Benítez et al. 2006).

Floral characters are preferred over vegetative characters in the taxonomic descriptions of most angiosperms because many vegetative characters can vary widely within a genus or even within a species. In addition, in most species, floral characters are produced for a short time and are subject to lower selective pressure than vegetative parts (Anderson 2001; Benítez et al. 2006). Contrary to the convention of using floral morphology for taxonomic descriptions in most angiosperms, taxonomic descriptions of *Opuntia s.s.* are based on vegetative characters, such as growth form, plant size and habit, cladode traits and spine number/position, and only rarely include qualitative characteristics of the flowers (Britton and Rose 1919; Bravo-Hollis 1978; Guillot-Ortiz and Der Meer 2006). A review of taxonomic keys to determine *Opuntia* species indicates that only one of 80 keys includes a floral trait, and some species descriptions do not include any floral attributes at all (Mandujano personal obs., e.g., *Opuntia pycnantha* Engelm., *O. strigil* Engelm.; Britton and Rose 1919; Bravo-Hollis 1978).

Our goal was to identify the morphometric characteristics of cladodes and flowers of four sympatric *Opuntia* species (*Opuntia robusta, O. cantabrigiensis, O. tomentosa* and *O. streptacantha*; (Bravo-Hollis 1978; Guzmán et al. 2003; "The Plant List" 2019) to determine which characters were informative for differentiating species and their hybrids. The four species are widely distributed but co-occur in a 7-ha area and can be differentiated from each other. The first two species are superficially similar, since both have a shrubby growth form, rounded cladodes, large spines, and yellow flowers. *Opuntia tomentosa* and *O. streptacantha* also have similarities. The color and shape of their cladodes are often confusing, especially in young individuals. In addition, the flowers of both species are orange at the study site, although some individuals of *O. streptacantha* have yellow flowers.

2. Materials and Methods

2.1 Study site

The studied populations are in a xerophytic crassicuale scrubland at Caderetya de Montes, Queretaro, Mexico (20º41'15.8"N, 99º48'17.7"W). The climate is semi-dry with summer rains [Köppen climate group $BS_1kw(w)$, modified by García (2004)], at 2,046 masl. Mean annual temperature varies from 12º to 19ºC, and annual precipitation averages 550 mm ("Jardín Botánico Regional de Cadereyta" 2008).

2.2 Study species

Opuntia robusta H.L. Wendl. ex Pfeiff., 1837 (Figure 1a, Table 1). The plants are shrubby to treelike, up to 3 m tall. Trunks are more or less distinct, and racquet-shaped stems are glaucous green-blue or silvery-blue, 1.5–2.5 cm thick, round to oblong, from 14 to 40 cm long. Areoles are 4–5.5 cm wide, with spines 2–5 cm long. Glochids are numerous, yellowish to brownish (Bravo-Hollis 1978; Anderson 2001; Reyes-Agüero et al. 2006). Flowers are yellow, 5–7 cm long, up to 5 cm in diameter. Female flowers (pistillate) have a fully developed gynoecium, yellow stigma with yellow lobes and collapsed anthers. Male flowers (staminate) have well-developed, pollen-filled anthers, and a gynoecium with a yellow stigma with atrophied lobes (Hernández-Cruz et al. 2019). Fruits are globose to ellipsoid, deep red to purple when ripe, somewhat tuberculate with an elongated podarium, 7–9 cm long. The studied population is dioecious, so only female plants bear fruit. Bravo-Hollis (1978) assumed that this species hybridizes with *O. streptacantha* and *O. cantabrigiensis.*

Opuntia cantabrigiensis Lynch (1903) (Figure 1b). Plants grow as rounded shrubs. Cladodes are 12–20 cm long and light green. The areoles are far apart, large, covered with brown felt and generally have 3–6 spines per areole, but sometimes more; spines are yellow with reddish base, 1.5–5 cm long; numerous glochids, large, yellowish to dark yellow. Flowers are 5–6 cm long, yellowish with reddish center; stigma lobes are green. Fruits are globose, approximately 4 cm in diameter, purple with crimson pulp, with numerous seeds 4 mm in diameter (Bravo-Hollis 1978; Guzmán et al. 2003). This species is considered synonymous with *Opuntia engelmannii* Salm-Dyck ex Engelm. (valid name) (Roskov et al. 2017).

Opuntia tomentosa Salm-Dyck (Figure 1c). Arborescent plants 3–5 m tall, with a smooth trunk 10–30 cm wide. The stem is divided into cladodes that are matte green and velvety due to a dense covering of short, fine hairs. The cladodes are 15–35 cm long, 8–12 cm wide and 1.5–2 cm thick. Young plants have two to four white or pale-yellow spines 2.5 cm long at the areoles. The flowers are deep orange, with a pale pink style. The red fruits are ovoid, about 5 cm long and 3 cm wide. Seeds are in a reddish pulp (Bravo-Hollis 1978).

Opuntia streptacantha Lem., 1839 (Figure 1d). Arborescent with many branches, up to approximately 5 m in height. The trunk is well defined, sometimes up to 45 cm in diameter. Stem segments obovate to orbicular, 25–30 cm long, dark green. Areoles are small and close together. Spines are numerous, extended, white; glochids are reddish brown and very short. Flowers are 7–9 cm wide, with yellow to orange or pink petals and reddish sepals, 8–12 usually green stigma lobes, and greenish or reddish filaments. Fruits are globose, 5 cm in diameter, dark red or sometimes yellowish inside (pulp) and outside (pericarp). There are numerous hybrids and varieties (Bravo-Hollis 1978; Reyes-Agüero et al. 2009).

2.3 Cladode morphometry

Ten young lateral cladodes (with no-hardened spines) of 20 individuals per species were measured with a digital caliper. We measured the same cladode morphometric variables used by Reyes-Agüero et al. (2009) and Muñoz-Urias et al. (2008; Figure 2). Additionally, the number and type of spines in areoles were counted, measured, and classified as erect (spines at 80–90° with respect to the stem), porrect (deflexed or perpendicular to the stem with an angle of 46–75°), reflexed (angle of 16–40°), or adpressed (angle of $<10°$; Reyes-Agüero et al., 2009).

2.4 Floral morphometry

Flowers of *Opuntia robusta* ($n = 20$ of each sex), *O. cantabrigiensis* ($n = 20$), *O. tomentosa* ($n = 20$) and *O. streptacantha* ($n = 20$) from different plants were collected from the population. We measured the maximum opening of the perianth before collection and preserved flowers in FAA (10:50:5:35 v/v, formalin–95% ethanol–acetic acid–distilled water) for morphometric measurements in the laboratory. For each flower, we measured different morphological attributes of the corolla or perianth, pistil and style, and pericarpel (Figure 3; Mandujano et al., 2010). Flower color was not included in the discriminant analysis because there are two color groups in the study population—yellow flowers (*Opuntia robusta* and *O. cantabrigiensis*) and orange flowers (*O. tomentosa* and *O. streptacantha*). However, it was included in the species description.

Morphometric data for cladodes and flowers were tested for differences among the four species using either an ANOVA with Tukey's post-hoc test (significance level of 0.05) for continuous variables, or GLM log-linear models with Poisson error distribution for discrete counts with a *t*-test for contrasts (Crawley 1993). A principal component analysis (PCA) was used to transform data from floral and cladode morphological variables. In addition, we did a linear discriminant analysis to compare between the groups and determine their possible separation (López-Borja et al. 2017). We tested the data to ensure that assumptions of ANOVA and PCA models were met. For all analyses, built-in functions of R version 3.5.3 (R Core Team 2021) and R packages MASS (Venables and Ripley 2002) and emmeans (Russell 2021) were used.

3. Results

3.1 Cladode morphometry

Some of the morphometric variables (mean ± SE) for the cladodes differed among the four species. *Opuntia streptacantha* had the longest cladodes (30.77 ± 6.75 cm). All four species differed significantly in cladode width and thickness; *O. tomentosa* had the thinnest (11.11 ± 2.98 cm) and *O. robusta* the thickest cladodes (2.88 ± 0.75 cm). *Opuntia tomentosa* and *O. cantabrigiensis* were similar in distance from the widest part of the cladode to the apex (12.47 ± 3.28 cm and 12.08 ± 2.60 cm, respectively). Consistent with their length and widths, the cladodes of *O. robusta* and *O. cantabrigiensis* were the most rounded. For a particular species, the distance between the areoles and distance between lines of areoles was nearly the same, but when compared to each other, they differed significantly among the four species. *Opuntia streptacantha* was the only species that consistently presented adpressed spines (Table 1).

Four main components in the PCA of cladode morphometry together explained 80.89% of the variation among species. The first component explained 43.32% and consisted of cladode width, distance from apex to widest point and from base to widest point. The second component explained 19.03% with only one character (areole size). The third component explained 12.25% with only one character (number of spines per areole), and the last component explained 6.29% of the variation with only one character (thickness).

The linear discriminant analysis of cladode morphometry explained 87.56% of the variation with the first two discriminant functions. *Opuntia robusta* and *O. cantabrigiensis* were clearly separated from the other species, and *O. tomentosa* and *O. streptacantha* had intermediate characteristics that did separate groups (Figure 4a). The analysis was able to correctly classify 88% of the individuals within species. *Opuntia cantabrigiensis* had the most correctly classified individuals, and *O. streptacantha* had the fewest correctly classified individuals (Table 2).

3.2 Floral morphometry

The four species had some similarities in their floral morphometric characteristics. For example, the length of the stigma, the minimum distance between the anthers and the stigma, and the polar diameter of the ovary did not differ among the species. There were also traits that distinguished one species from the rest, as in *O. tomentosa*, which had the narrowest pericarpel (mean ± SE, 19.21 ± 2.17 mm), and female *O. robusta* which had the fewest and small stamens and the largest equatorial diameter of the ovary (Table 3).

The PCA revealed five principal components for floral morphometry, explaining 80.44% of the floral variation. The first component explained 36.77% (number of ovules, equatorial diameter of the ovarian chamber, and polar diameter of the ovarian chamber), the second component

15.67% (perianth height, style size, and longest stamen), the third component 12.52% (aperture of the perianth, height of the pericarpel), the fourth component 8.51% (height of the pericarpel) and the last component 6.96% of the variation (minimum distance between the anthers and the stigma).

According to the linear discriminant analysis, the floral morphometric variables clearly separated the four species. Two discriminant functions of the floral morphometry explained 93.65% of the variation (Figure 4b). The analysis was able to correctly classify 99% of the individuals in the species to which they belong. *Opuntia streptacantha* was the only species with a misclassification error based on floral morphometrics (Table 4).

3.3 Identification key for the *Opuntia* species included in the study

1. Aperture of the perianth up to 4 cm ……………. 2

2. Style length smaller than 15 mm ……………. 4

4. Flower 4.51–6.68 cm long, 106–312 stamens, 183–415 ovules, yellow with reddish tones, unisexual, stigma lobes open and yellow. Cladodes 6.5–39.4 cm width, 0.9–5.10 cm thickness, 0.30–1 cm areole size ……………. *Opuntia robusta* ♀

2. Style length greater than 15 mm ……………. 5

5. Height of the pericarpel lower than 30 mm ……………. 6

6. Polar diameter of the ovary lower than 5 mm ……………… 8

8. Flower 2.04–6.09 cm long, 154–498 stamens, no ovules, yellow with reddish tones, unisexual, stigma lobes open and yellow. Cladodes 6.5–39.4 cm width, 0.9–5.10 cm thickness, 0.30–1 cm areole size ……………. *Opuntia robusta* ♂

6. Polar diameter of the ovary greater than 5 mm ……………. 9

9. Flower 3.40–5.27 cm long, 228–508 stamens, 36–136 ovules, deep orange, hermaphrodite, style closed and pink. Cladodes 5–29 cm width, 0.9–4.3 cm thickness, 0.10–0.9 cm areole size ……………. *Opuntia tomentosa*

5. Height of the pericarpel greater than 30 mm ……………. 7

7. Flower 4.90–6.80 cm long, 258–490 stamens, 42–272 ovules, yellow to orange, hermaphrodite, style closed and cream to yellow. Cladodes 8–35.8 cm width, 1.1–5.5 cm thickness, 0.09–2.2 cm areole size ……………. *Opuntia streptacantha*

1. Aperture of the perianth more than 4 cm ……………. 3

3. Flower 4.27–5.68 cm long, 240–422 stamens, 112–267 ovules, yellow with reddish tones, hermaphrodite, stigma lobes closed and green. Cladodes 5–25 cm width, 0.3–3.9 cm thickness, 0.05–1.3 cm areole size ……………. *Opuntia cantabrigiensis*

3.4 Species local descriptions

Opuntia robusta H.L. Wendl. ex Pfeiff., 1837 (Figure 1a, Table 1)

Shrubby, 1.70–2 m in height, highly branched, trunk more or less defined. **Stem segments or cladodes**, 12–53 cm long or longer, orbicular, oblong to obovate, very robust, 0.90–5.10 cm thick, light bluish-green; **areoles** measuring 0.3–1 cm, separated by a distance of 2.30–8.30 cm;

spines 1–9 per areole, erect, porrect, or reflexed, 5 cm long. **Flowers**, yellow [During flowering, the study population was identified as dioecious.], female 4.51–6.68 cm long, males 2.04–6.09 long; flowers of both sexes have **stigmas**, males have stigmas with different degrees of atrophy, females have stigmas with large open lobes and yellow; **stamens** 106–312, stamens atrophied and pollen absent in female flowers; male flowers have 154–498 stamens with abundant pollen; **ovules** 183–415 in female flowers, absent in male flowers. **Fruits** broadly subglobose, purplish. **Seeds** numerous, 273.89 ± 34.57, small, 4 mm in diameter

Distribution: Chihuahua, Mexico City, Durango, Guanajuato, Hidalgo, Jalisco, Mexico, Michoacan, Queretaro, San Luis Potosi, Sonora, Zacatecas (Bravo-Hollis 1978; Guzmán et al. 2003).

Bravo-Hollis (1978) suggests that the species frequently hybridizes with *O. streptacantha* and *O. cantabrigiensis.*

Opuntia cantabrigiensis Lynch, 1903 (Figure 1b)

Shrubby, 0.7–1 m in height. **Stem segments or cladodes** 6–34 cm long, orbicular, 0.30–3.90 cm thick, pale green; **areoles** 0.05–1.30 cm wide, 1.70–7.60 cm apart, with brown felt; **spines** 1–10 per areole, erect, porrect, 1.5–5 cm long, yellow with reddish base, glochids numerous, large, yellowish to dark yellow. **Flowers** 4.27–5.68 cm long, yellow with reddish center, **hermaphroditic**; **stigma** lobes green, closed; **style** white; **stamens** 240–422; **ovules** 112–267. **Fruits** globose, 4 cm in diameter, magenta, pulp crimson. **Seeds** numerous 75.71 ± 13.34 (Martínez-Ramos, L.M. personal obs.), small, 4 mm in diameter.

Distribution: Guanajuato, Hidalgo, Jalisco, Querétaro, San Luis Potosi (Bravo-Hollis 1978; Guzmán et al. 2003)

This species is considered by some authors to be synonymous with *Opuntia engelmannii* Salm-Dyck ex Engelm. (valid name, Roskov et al. 2017; but see discussion.

Opuntia tomentosa Salm-Dyck, 1822 (Figure 1c)

Shrubby or arborescent 1.30–1.50 m tall, trunk smooth. **Stem segments or cladodes** 11–45 cm long, oval, 5–4.30 cm thick, matte green, velvety to the touch; **areoles** 0.10–0.90 cm wide, 0.70–6.30 cm apart; **spines** 1–6 per areole, erect, porrect, rarely reflexed or adpressed, 2.5 cm long, yellow. **Flowers** 3.40–5.27 cm long, deep orange, **hermaphroditic**; **stigma** white with lobes closed; **style** pink; **stamens** 228–508; **ovules** 36–136. **Fruits** ovoid, 5 cm long, 3 cm wide, dark red, pulp reddish. **Seeds** numerous 54.21 ± 3.30 (Martínez-Ramos, personal obs.), small, 4 mm in diameter.

Distribution: Mexico City, Guanajuato, Guerrero, Hidago, Jalisco, Mexico, Michoacan, Morelos, Oaxaca, Puebla, Querétaro and San Luis Potosi (Guzmán et al. 2003).

Opuntia streptacantha Lem., 1839 (Figure 1d)

Shrubby or arborescent 3.50–4 m tall, highly branched, trunk well defined. **Stem segments or cladodes** 15.20–48 cm long, obovate to orbicular, 1.10–5.55 cm thick, dark green; **areoles** 0.09–2.22 cm wide, 1.20–7.70 cm apart; **spines** 1–9 per areole, erect, porrect, frequently reflexed or adpressed, white or yellow; **glochids** reddish brown, very short. **Flowers** 4.90–6.80 cm long, yellow, orange, or pink after more than one day, reddish **sepals, hermaphroditic**; **stigma** light yellow with closed or rarely open lobes; **style** white to light yellow; **stamens** 258–490, **filaments** greenish or reddish; **ovules** 42–272. **Fruits** globose, 3.73 ± 0.62 cm in diameter, 4.76 ± 1.06 cm long, dark red or sometimes yellowish, same color internally and externally. **Seeds** numerous 97.35 ± 4.65 (Manzanarez-Villasana, personal obs.), small, 4 mm in diameter.

Distribution: Hidalgo, Querétaro, Guanajuato, San Luis Potosí, Aguascalientes, Zacatecas, Durango, Jalisco, Oaxaca, Puebla, México Valley (Bravo-Hollis 1978; Guzmán et al 2003).

4. Discussion

Species descriptions of *Opuntia s.s.* have been based on stem shape, presence of glochids, or sometimes, a general description of the flowers is provided (Buxbaum, 1953; Bravo-Hollis, 1978). Several authors have claimed that the seeds, pollen, and flowers are similar among the species within this genus (e.g., Bravo-Hollis 1978; Anderson 2001; Guzmán et al. 2003). In particular, flowers were traditionally ruled out for species discrimination because floral characters in *Opuntia s.s.* have been considered relatively uniform and therefore not useful for the taxonomic diagnosis of sections or species (Grant and Hurd 1979). As such, identification keys at the species level had been based on vegetative characters of adult plants: habit (shrub, tree, creeping), trunk (absent or present), areoles (size, protuberance, color), distance between areoles, spines (color, position, texture, size), persistence of the stem, epidermis (glabrous or tomentose), cladode color (green, olive, bluish), and cladode size (quantitative and qualitative). Fewer than 10 species can be distinguished by fruit characteristics (juicy or dry when mature, large or small) or spines (present or absent), or flower color. For example, members of the series *Basilaris* (*Opuntia microdasys* and *Opuntia basilaris*) share dry fruits and absent spines (Bravo-Hollis 1978). For each species, five varieties are recognized, by the glochids color (from white to yellow shades) and cladodes size and shape in the case of *O. microdasys* and by the size of the cladodes in the case of *O. basilaris* (Bravo-Hollis 1978). Both species can be distinguished from each other by a reproductive character such as flower color.

There are, however, species that have been described without using any reproductive structures or without a place of origin even though Van Wyk and Smith (2001) proposed that geographic range is one of the key taxon characteristics. Our results indicate that nine floral characters can differentiate the four species, and can be included in descriptions of *Opuntia* species for unambiguous taxonomic classification. We consider that geographic information for wild *Opuntia* species, in addition to the reproductive structures that we have proposed are important elements to include in taxa descriptions. However, caution should be exercised because geographic range may have little value as a diagnostic character for plants that have been widely distributed over time and space by humans.

The discriminant analysis of the cladodes separated two species: *Opuntia robusta* and *O. cantabrigiensis* but showed intermediate values for some individuals of *Opuntia streptacantha* and *O. tomentosa*, which indicates that the morphometric characteristics of the cladodes were not sufficient for differentiating these two species. However, as noted earlier, the species of *Opuntia s.s.* are very similar because of their highly variable vegetative characteristics and flower color. For example, *O. streptacantha* can have yellow or orange flowers (Bravo-Hollis 1978), and species can share characteristics such as plant growth form (arborescent or shrubby) that confound identification. Morphological characteristics such as growth habit, stem pubescence, spine length, number of spines per areole, color of cladodes (e.g., betacyanins in stems) and flowers, flower shape, reproductive season, fruit type, seed size, glochid presence, and leaves on the flower and vegetative buds, among others are all phenotypically plastic (Muñoz-Urias et al. 2008; Mosco 2012). For example, *Opuntia excelsa* is a tree with a well-defined trunk in the tropical deciduous forest of the Pacific coast of Mexico, but grows as a shrub in marshes, and as a creeping plant on coastal dunes (Bravo-Hollis, 1978).

The morphological similarities between species as those found between *O. streptacantha* and *O. tomentosa* are frequent in several taxa. The classic species concept of a group of similar organisms evolving together and presenting etiological, morphological, and genetic differences that facilitate recognition among members of the same species and to ensure reproductive isolation from other groups (Mayr 1991; De Haro 1999; De Queiroz 2007) does not apply to

Opuntia s.s. The lack of reproductive isolation is the reason why *Opuntia s.s.* have been referred to as a species complex (Grant and Hurd 1979; Pinkava et al. 1985; Mayer et al. 2000; Pinkava 2002), or a taxonomic series (Britton and Rose 1919; Bravo-Hollis 1978), or as adhering to the cohesive species concept (Templeton 2008). In any case, molecular evidence is needed to distinguish taxonomic entities within species complexes is needed to allow the separation of taxonomic entities within species complexes even if there is a proportion of overlap or hybridization among them (e.g. Pinus; Majure et al. 2012; Willyard et al. 2017).

With respect to *Opuntia* species identities, there is a lack of consensus among authors and of monographs that formally review individual species beyond just their names. Regarding the taxonomic confusion among the four study species, *Opuntia cantabrigensis* Lynch is proposed to be synonymous with *Opuntia engelmannii* Salm-Dyck ex Engelm. (valid name, Integrated Taxonomic Information System-ITIS). In addition, *O. rastrera* Weber, *Opuntia bensonii* Sánchez-Mej., *Opuntia neochrysacantha* Bravo and *Opuntia subarmata* Griffiths, with six varieties or subspecies have been also reclassified as *O. engelmannii* (Hunt et al. 2006; Roskov et al. 2017). The reclassification, validation of names or removal of *Opuntia* species in checklists has often lacked solid evidence based on monographs, taxonomic revisions, or other publications to justify the changes. That is, without a solid taxonomic or molecular study, we consider Hunt (2006) *Opuntia s.s.* classifications unsupported. To clarify the taxonomic classification of *Opuntia s.s*. we propose to consider local floristic listings, including the locality of the study taxon, preparing herbarium specimens, and reporting measurements for cladodes, flowers, fruits, seeds, and ideally pollen. In addition, molecular phylogenies should be reconstructed to clarify relationships and support a more stable taxonomic classification (Majure et al. 2012; Guerrero et al. 2019).

Several authors support the existence of intermediate variants that are likely the result of hybridization events between species (Moran and Zimmermann 1991; Fritz et al. 1994; Primot et al. 2005). Plants that most often hybridize are generally perennial, obligate allogamous, or reproduce vegetatively (Vaco 2011), as is the case for most species of *Opuntia*. Furthermore, Muñoz-Urias et al. (2008), who studied three of the species in the present study from populations in the Chihuahuan Desert, stated that hybridization was not common among *Opuntia* species in the wild (Muñoz-Urias et al. 2008). However, according to Pinkava et al. (1985), hybridization is very common in *Opuntia*, and *O. streptacantha* has been proposed to commonly hybridize with other species such as *O. robusta* (Bravo-Hollis 1978; Gallegos-Vázquez et al. 2012). Hybridization events are often supported by the intermediate morphology between *Opuntia* species (e.g., *O. streptacantha* × *O. tomentosa*, Baker et al. 2009; *Opuntia ×andersonii*, Hernández et al. 2002; *Opuntia ×carstenii*, Puente and Hamann 2005). It is also worth mentioning that *O. streptacantha* and *O. tomentosa* are phylogenetically closely related (Valadez-Moctezuma et al. 2015), which could favor interspecific hybridization. This interspecific hybridization generates new morphologies that increase the difficulty of taxonomic study in this group of plants (Mercado 2014). Notwithstanding, the morphological affinity of the parent plants could be incorrect. For example, *O. aurantiaca* is proposed to be a hybrid between *O. discolor* Britton and Rose and *O. salmiana* Parm., but later studies showed that *O. salmiana* is sterile, ruling it out as the parent plant (Moran and Zimmermann 1991).

Recognizing morphological characters that allow the separation of *Opuntia s.s.* species is highly important for other disciplines, for example, for the biological control of species that are cultivated outside their natural distributions and become invasive (e.g., *O. aurantiaca*). Biological control depends on a correct taxonomic determination because plant-insect interactions are usually specific (e.g. a specific biotype of the cochineal insect *Dactylopius coccus* feeds on *Opuntia* (Moran and Zimmermann 1991; Githure et al. 1999).

Floral traits and other reproductive characters such as fruit and seed traits can be a source of taxonomic information for taxa that possess relatively similar vegetative characters as found in *Opuntia* and members of the Umbelliferae (Ornduff 1978), Rapateaceae (Oriani and Scatena 2013), *Thottea* (Aristolochiaceae) and *Malus* (Rosacea; Shaiju and Omanakumari 2010; Zhou et al. 2021). Using these traits is better than relying solely on the sporophyte phase, as traditional taxonomy often does in such cases, which in isolation restricts our understanding of plant diversity (Ornduff 1969, 1978). The use of selected vegetative and reproductive characters can help resolve well-supported relationships among taxa where it is difficult to differentiate species (e.g., Ajao and Moteetee 2020). Shaiju and Omanakumari (2010) reported high levels of intra- and interspecific variations in floral morphology in the genus *Thottea*; they found that traits related to the perianth, stamens disposition and gynoecium color are important to differentiate eight species for the genus. They were also able to devise an identification key that included floral.

Floral morphology comprises a set of traits that can facilitate separation from subfamilies to species and might be better for elucidating relationships between taxa (Bredenkamp and Van Wyk 2001; Oriani and Scatena 2013; Zhou et al. 2021). Our PCA for flowers also indicates that *Opuntia* species can be separated with 99% certainty based almost entirely on floral traits. This information should be considered in future studies of the genus *Opuntia s.s*. Based on our results, we conclude that floral traits must be used for species differentiation, in contrast to past *Opuntia* treatments that were usually based on vegetative morphology alone (Tapia et al. 2016). Together, morphometric variables with genetic, ecological, environmental characteristics and geographic distribution of the species will help disentangle this complex.

Data statement

All the data supporting the findings of this study will be available as Supplementary materials published online. Morphology data will be available at GitHub in the repository: Gerardo-Manzanarez-Villasana/Galicia-Perez_etal

Author statement

Alda Galicia-Pérez: Original research project, data collection, writing—original draft, review and editing; figure design. **Jordan Golubov**: Funding acquisition; investigation, data collection, data analysis, writing—review and editing. **Gerardo Manzanarez-Villasana:** Data collection, herbarium vouchers, data analysis, writing—review and editing, new species descriptions, identification key. **Linda Mariana Martínez-Ramos:** Data collection, writing—review and editing. **Salvador Arias:** Species identification, writing—review and editing, new species descriptions, identification key. **Judith Márquez-Guzmán:** writing—review and editing. **María C. Mandujano:** Original research project, conceptualization, funding acquisition, data collection, data analysis, writing—original draft, review and editing, new species descriptions and identification key.

Declaration of competing interest

The authors declare that they have no know competing financial interest or personal relationships that could have influenced the work reported in this paper.

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Figure 1. Habit (left images), cladode (middle), and flower (right) of Opuntia species in Cadereyta de Montes, Queretaro, Mexico. (a) <i>Opuntia robusta</i>. Photo: Mariana Paola y Pedro Nájera. (b) <i>O. cantabrigiensis<i/>. Photos: Diana Cárdenas. (c) <i>O. tomentosa</i>. Photos: Linda Mariana, Ruth Julieta and Fernando Cecor. (d) <i>O. streptacantha</i>. Photos: Gerardo Manzanarez. Scale bar = 5 cm.

Figure 2. Morphometric variables of Opuntia cladodes. 1: length (cm), 2: width (cm), 3: distance from the widest point to the apex (cm), 4: distance from the widest point to the base (cm), 5: number of lines of areoles, 6: areole size (cm), 7: distance between areoles (cm), 8: distance between lines of areoles (cm), 9: thickness (cm), 10: spine length (cm) and 11: number of spines.

Figure 3. Longitudinal section showing the morphometric variables of <i>Opuntia</i> flowers. Flower drawings adapted from Comentuna, Red nopal and Conabio (2009).

Figure 4. Linear discriminant analysis using (a) cladode morphometry (length, width, thickness, distance between lines of areoles, distance between areoles, and number of adpressed spines) and (b) floral morphometry (aperture diameter of the perianth, total height, pericarpel width, stigma length, ovary polar and equatorial diameter, nectar chamber, pericarpel width, anther–stigma distance, and perianth height).

Table 1. Mean and standard error (±) of the cladode morphological characteristics that were measured and counted in the four species of *Opuntia* in Cadereyta de Montes, Querétaro, México (*n* = 200 cladodes per species). Levels with different letters indicate groups that are significantly different (a) ANOVA and Tukey, for continuous variables, and (b) GLM with Poisson distribution and contrast for +discrete variables, *P*<0.05*, non-significant n.s.). + Sample size for spines varies among species because some of them were absent. ++*Opuntia robusta* was excluded as it did not bare adpressed spines.

(a) Species/Cladode characteristics	*Opuntia cantabrigiensis*	*Opuntia robusta*	*Opuntia streptacantha*	*Opuntia tomentosa*	$F_{3,796}$
Length (cm)	$19.82±0.30^{B}$	$29.96±0.426^{A}$	$30.77±0.48^{A}$	$24.8±0.41^{C}$	156.30^{*}
Width (cm)	$16.08±0.23^{A}$	$24.92±0.38^{B}$	$17.54±0.30^{C}$	$11.11±0.21^{D}$	395.10^{*}
Thickness (cm)	$1.36±0.03^{A}$	$2.88±0.05^{B}$	$2.55±0.05^{C}$	$2.36±0.04^{D}$	185.56^{*}
Distance from the widest part to the apex (cm)	$12.08±0.18^{A}$	$18.73±0.33^{B}$	$15.90±0.28^{C}$	$12.47±0.23^{C}$	142.90^{*}
Distance from the widest part to the base (cm)	$15.59±0.26^{A}$	$21.97±0.35^{A}$	$20.97±0.36^{B}$	$15.24±0.27^{B}$	126.90^{*}
Number of lines of areoles	$5.94±0.07^{A}$	$5.6±0.07^{B}$	$8.65±0.13^{BC}$	$6.19±0.10^{C}$	205.820^{*}
Areole size (cm)	$0.58±0.01^{A}$	$0.53±0.008^{B}$	$0.38±0.01^{C}$	$0.29±0.007^{D}$	153.30^{*}

Distance between areoles (cm)	3.7±0.06^{A}	4.72±0.07^{B}	3.08±0.06^{C}	2.59±0.06^{D}	209.10*
Distance between lines of areoles	3.78±0.06^{A}	4.08±0.07^{B}	3.28±0.06^{C}	2.61±0.05^{D}	216.20*
Total height of the individual (cm)	85.4±13.88^{A}	180.75±8.33^{B}	371.84±12.80^{B}	142.68±8.93^{C}	119.70*

(b) Species/Cladode characteristics	***Opuntia cantabrigiensis***	***Opuntia robusta***	***Opuntia streptacantha***	***Opuntia tomentosa***	**Chi-square df = 3**
+Number of spines per areole	4.18±0.10^{A}	2.95±0.09^{B}	3.69±0.10^{C}	2.35±0.07^{D}	126.12*
+Number of erect spines	1.16±0.09^{A}	1.05±0.08^{B}	0.75±0.06^{A}	0.72±0.04^{C}	29.30**
+Number of porrect spines	1.98±0.12^{A}	1.3±0.08^{B}	0.75±0.07^{C}	1.2±0.07^{B}	104.90*
+Number of reflexed spines	0.44±0.07	0.46±0.07	0.53±0.06	0.37±0.04	5.90$^{n.s}$.
++Number of adpressed spines	0.06±0.03^{A}	0	1.48±0.11^{B}	0.01±0.01^{A}	535.49

Table 2. Classification of four selected *Opuntia* species based on cladode measurements from the linear discriminant analysis. Cladodes were similar in both sexual morphs in *Opuntia robusta*.

Species	***Opuntia cantabrigiensis***	***Opuntia robusta***	***Opuntia streptacantha***	***Opuntia tomentosa***	**Correctly classified individuals**
O. cantabrigiensis	189	13	8	2	189
O. robusta	6	179	8	2	179
O. streptacantha	3	3	154	17	154
O. tomentosa	2	5	30	179	179
n	200	200	200	200	800 (100%) / 701 (88%)

Table 3. Mean and standard error (±) of the morphological characteristics of the flowers from the four species of *Opuntia* in Cadereyta de Montes, Querétaro, Mexico. Levels with different letters indicate groups that are significantly different (a) ANOVA and Tukey, for continuous variables, and (b) GLM with Poisson distribution and contrast for discrete variables, *P*<0.05*, non-significant n.s.).

a) Species/Floral characteristic	*Opuntia cantabrigiensis*	*Opuntia robusta*		*Opuntia streptacantha*	*Opuntia tomentosa*	$F_{4,51}$
		♀	♂			
Aperture of the perianth (mm)	56.03±2.60^{A}	38.08±2.46^{B}	36.69±3.74^{B}	30.41±2.66^{BC}	23.91±1.24^{C}	34.58*
Flower height (mm)	51.38±1.30^{AB}	56.76±3.38^{A}	45.01±4.15^{BC}	57.82±2.45^{A}	43.70±1.26^{C}	8.88*
Height of the pericarpel (mm)	22.86±1.52^{C}	31.63±1.91^{AB}	24.20±2.32^{BC}	34.34±1.97^{A}	24.80±0.95^{BC}	8.96*
Width of the pericarpel (mm)	22.83±0.91^{A}	24.53±0.72^{A}	24.86±1.65^{A}	23.13±1.08^{A}	19.21±0.48^{B}	7.55*
Height of the perianth (mm)	32.02±1.75^{A}	26.34±1.76^{B}	25.57±1.34^{B}	25.18±0.95^{B}	18.90±0.41^{C}	22.07*
Style length (mm)	21.37±0.49^{A}	13.87±1.09^{C}	19.82±1.14^{AB}	18.25±0.66^{B}	20.69±0.35^{A}	15.91*
Stigma length (mm)	4.96±0.14^{B}	7.36±0.61^{A}	3.81±0.43^{C}	5.57±0.26^{B}	4.78±0.09^{B}	18.67*
Length of the nectar chamber (mm)	1.68±0.22^{B}	2.19±0.55^{AB}	0.61±0.32^{C}	2.93±0.24^{A}	2.40±0.08^{AB}	12.29*
Equatorial diameter of the	4.15±0.42^{C}	8.04±0.45^{A}	1.22±0.49^{D}	6.14±0.57^{AB}	4.68±0.20^{BC}	27.13*

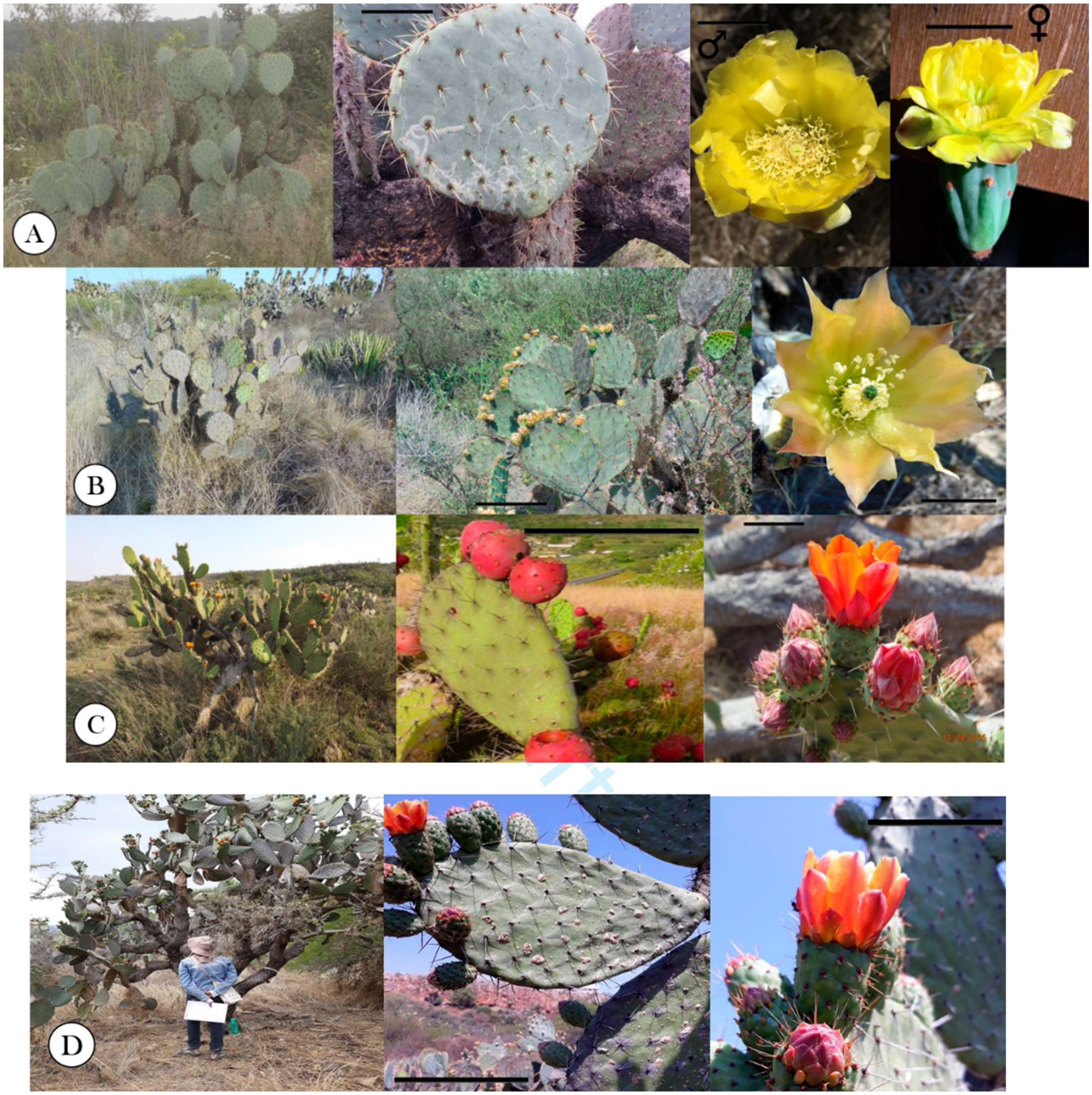
ovary (mm)						
Polar diameter of the ovary (mm)	4.46 ± 0.18^{C}	14.13 ± 1.26^{A}	1.59 ± 0.67^{D}	7.77 ± 1.27^{B}	6.11 ± 0.31^{BC}	33.46^{*}
Stamen length (mm)	16.11 ± 0.87^{A}	10.93 ± 0.91^{C}	13.19 ± 0.59^{BC}	11.78 ± 0.52^{C}	14.50 ± 0.41^{AB}	8.7^{*}
Minimum distance between the anthers and the stigma (mm)	0.90 ± 0.31	0.48 ± 0.34	2.21 ± 0.82	0.66 ± 0.35	1.36 ± 0.09	$2.84^{n.s.}$

b) Species/Floral characteristic	*Opuntia cantabrigiensis*	*Opuntia robusta*		*Opuntia streptacantha*	*Opuntia tomentosa*	**Chi-square df = 4**
		♀	♂			
Number of ovules	197 ± 11.98^{B}	273.50 ± 33.22^{A}	0	144.77 ± 29.19^{C}	85.75 ± 6.37^{D}	35.43^{*}
Number of stigma lobes	7.15 ± 0.29^{C}	10.00 ± 0.89^{A}	$2.75\pm0.77D^{D}$	8.44 ± 0.44^{B}	6.85 ± 0.16^{C}	36.27^{*}
Number of stamens	346.92 ± 16.92^{B}	223.00 ± 35.54^{D}	329.25 ± 4.69^{C}	325.55 ± 24.65^{C}	396.80 ± 13.66^{A}	460.56^{*}
Stigma color	Green	Yellow	Yellow	Yellow	Yellow	-
Stigma lobes	Closed	Open in female flower	Closed-atrophied in male flower	Closed	Closed	-

Pollen	Present	Absent in female flowers	Present in male flower	Present	Present	-
Color of the style	White	White	White	White	Pink	-

Table 4. Classification of the individuals based on flower measurements using the linear discriminant analysis.

Species	***Opuntia cantabrigiensis***	***Opuntia robusta*** ♂	♀	***Opuntia streptacantha***	***Opuntia tomentosa***	**Correctly classified individuals**
O. cantabrigiensis	20	0	0	0	0	20
O. robusta ♂	0	20	0	0	0	20
O. robusta ♀	0	0	20	0	0	20
O. streptacantha	0	0	0	19	0	19
O. tomentosa	0	0	0	1	20	20
n	20	20	20	20	20	100 (100%) / 99 (99%)


A
B
C
D

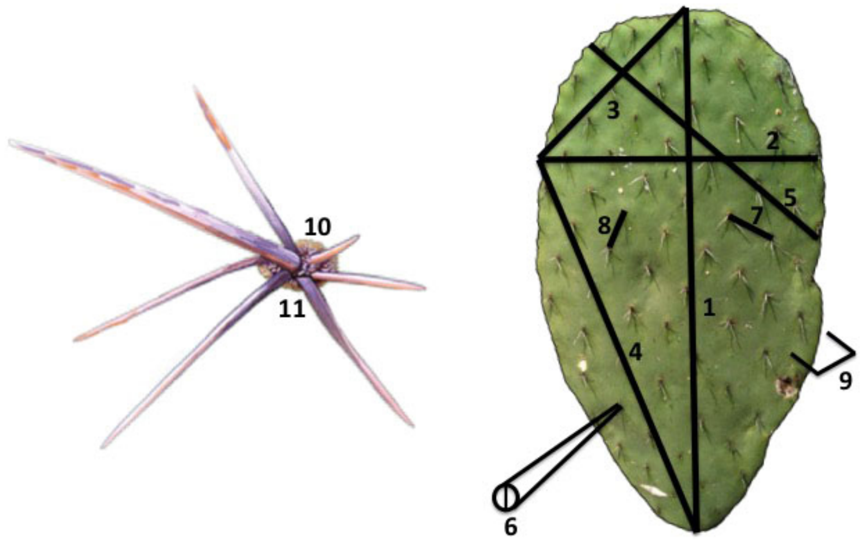
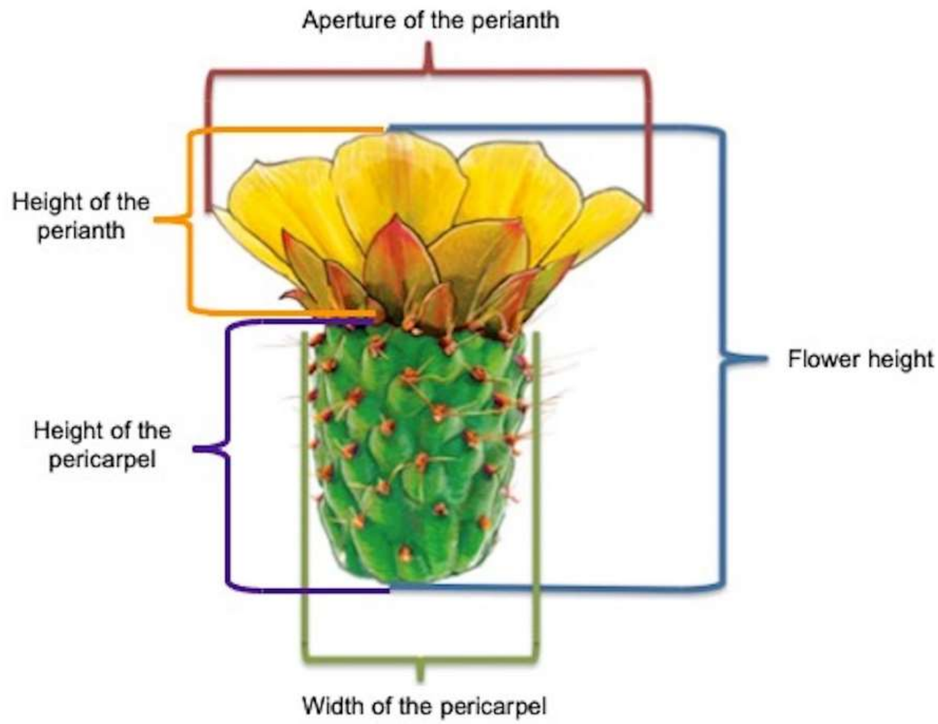
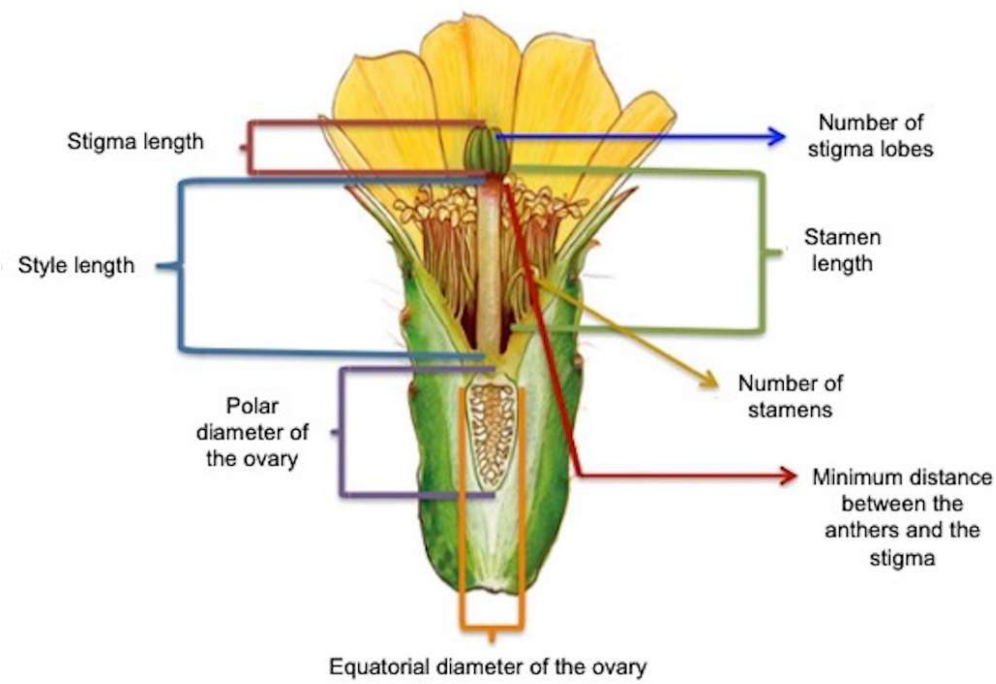
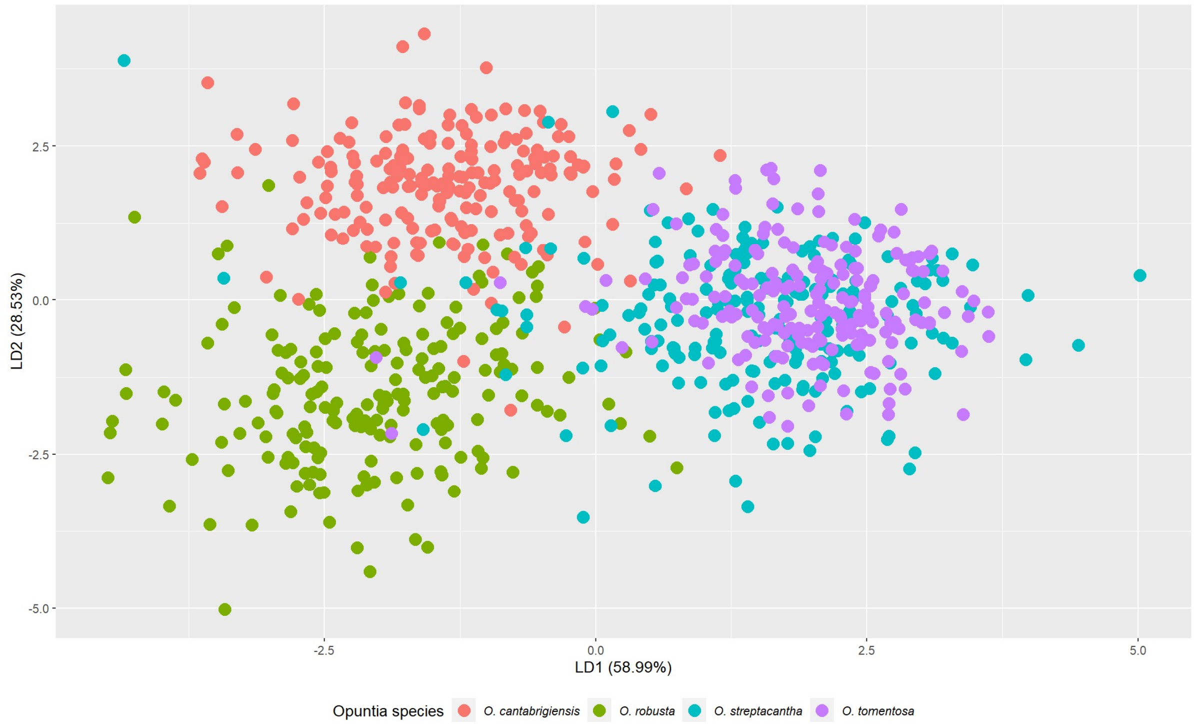


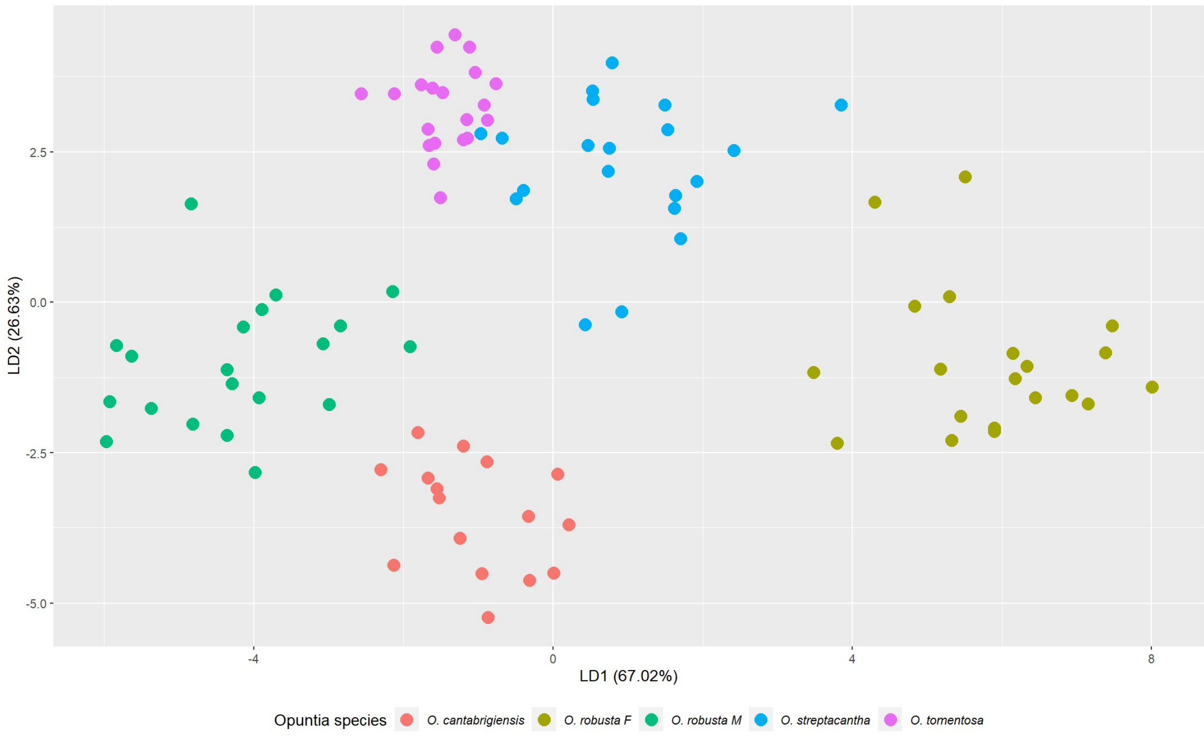
Figure 2. Morphometric variables of Opuntia cladodes. 1: length (cm), 2: width (cm), 3: distance from the widest point to the apex (cm), 4: distance from the widest point to the base (cm), 5: number of lines of areoles, 6: areole size (cm), 7: distance between areoles (cm), 8: distance between lines of areoles (cm), 9: thickness (cm), 10: spine length (cm) and 11: number of spines.

254x190mm (72 x 72 DPI)


Aperture of the perianth
Height of the perianth
Flower height
Height of the pericarpel
Width of the pericarpel


Stigma length
Number of stigma lobes
Style length
Stamen length
Number of stamens
Polar diameter of the ovary
Minimum distance between the anthers and the stigma
Equatorial diameter of the ovary


LD2 (28.53%)
LD1 (58.99%)
2.5
0.0
-2.5
-5.0
-2.5
0.0
2.5
5.0
Opuntia species
O. cantabrigiensis
O. robusta
O. streptacantha
O. tomentosa


LD2 (26.63%)
LD1 (67.02%)
2.5
0.0
-2.5
-5.0
-4
0
4
8
Opuntia species
O. cantabrigiensis
O. robusta F
O. robusta M
O. streptacantha
O. tomentosa