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Original Article

Multivariate statistical analysis of morpho-anatomical data of nine sect. *Caulopterae* species (*Baccharis* – Asteraceae) used in folk medicine

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ABSTRACT

Baccharis species belonging to sect. *Caulopterae* are difficult to identify. Most countries are controlling the quality of herbal medicines destined for the internal market or export. "Carquejas" are used arbitrarily for the same medicinal purposes and only three species of sect. *Caulopterae* are official herbal medicines. In the present study, a morpho-anatomical and statistical analysis was performed with nine species of sect. *Caulopterae*: *Baccharis articulata*, *B. crispa*, *B. gaudichaudiana*, *B. microcephala*, *B. penningtonii*, *B. phyteumoides*, *B. sagittalis*, *B. triangularis* and *B. trimera*, emphasizing the importance of anatomy as a taxonomic tool. A total of 114 populations of these nine species were examined. The first three principal components of morphoanatomical data provided relevant information to classify the species (75.04% of the total variability). The most discriminatory variable in this issue was the stomatal index (1.0530). We determined the qualitative and quantitative variables in order to differentiate the species by using principal components analysis and ANOVA tests. Stomata type, uniseriate trichome type and presence/absence of collenchyma in the wing margin are the qualitative variables that should be analyzed. Regarding quantitative variables, the epidermal ones in superficial view are more important and discriminatory than those of alate stem cross section and they must be considered for proper quality control of the species of this work.

Introduction

The genus *Baccharis* L. consists of over 500 species, with a geographical distribution extending from Canada to Southern Argentina and Chile (Fielding, 2001). In this vast area, the genus occupies a large variety of habitats and is an important element in many vegetation communities (Giuliano, 2001). Some species of this genus are popularly known as "carquejas" and they are morphoanatomically very similar to each other. In popular medicine they are used for their digestive, hepatoprotective and anti-inflammatory properties. The beneficial effects of these species can be attributed at least in part to their antioxidant properties and free radical scavengers (Hieronymus, 1882; Sorarú and Bandoni, 1978; Toursarkissian, 1980; Martínez Crovetto, 1981; Correa, 1985). Giuliano (2001) subdivided the 96 Argentine *Baccharis* species into 15 sect., sect. *Caulopterae* DC. being characterized by the presence of species with alate stems. The species with alate stems are collected and used arbitrarily for the same therapeutic purposes, because they can be easily confused (Ariza Espinar, 1973; Lonni et al., 2005; Simões-Pires et al., 2005; Müller, 2006). Only three of these nine species are official herbal medicines, *Baccharis articulata* (Lam.) Pers. and *Baccharis crispa* Spreng. are included in the National Argentine Pharmacopeia Ed. VI (1978) and *Baccharis trimera* (Less.) DC. in the Brazilian Pharmacopeia Ed. V (2010). There is literature supporting the medicinal use of seven of these species (Stoicke and Leng-Peschlow, 1987; Gamberini et al., 1991; Gené et al., 1992, 1996; Lapa et al., 1992; Fullas et al., 1994; Brandão Torres et al., 2000; De Oliveira et al., 2003, 2012; Oliveira et al., 2005; Guo et al., 2006; Petenatti et al., 2007; Paul et al., 2009; Cifuente et al., 2010; Biondo et al., 2011). The identification of herbal medicines as part of the quality control is not obvious. Minimal morphological differences are described, which are often difficult to determine within the limits of species variability. These problems highlight the need for unequivocal parameters of identification and tests for the verification of its quality.

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The anatomy of *Baccharis* L. genus belonging to Argentina and Bolivia has been studied by Ariza Espinar (1973), Hadad et al. (2013) and Müller (2006) among others. The species with the highest number of anatomical studies are *B. articulata* and *B. crispa* (Ariza Espinar, 1973; Cortadi et al., 1999; Barboza et al., 2001; Budel et al., 2003a; Müller, 2006). The anatomical structure of *B. trimera* has also been studied exhaustively (Cortadi et al., 1999; Budel et al., 2003a; Müller, 2006; Budel and Duarte, 2009). Regarding these three species, Gianello et al. (2000) contributed with quantitative micrographic data to differentiate the raw drug. Rodriguez et al. (2008) provided new micrographic characters such as the number and size of the schizogenous secreting structures in the wing and stem, finding differences between *B. crispa* and *B. trimera*. Petenatti et al. (2007) determined quantitative micrographic characters of *Baccharis sagittalis* (Less.) DC. and *Baccharis triangularis* Hauman species. The species *B. sagittalis* was also studied anatomically by Müller (2006). Freire et al. (2007) studied the epidermis of 38 medicinal species of *Baccharis*, including *B. articulata*, *B. crispa*, *Baccharis gaudichaudiana* DC., *Baccharis microcephala* (Less.) DC. and *B. trimera*, also analyzed in the present work (qualitative and quantitative anatomical variables revision of *Baccharis* species with alate stems are listed in Box 1S and Table 1S (Supplementary Material). However, the information from these studies is inconclusive about the proper differentiation of the nine species of the *Caulopterae* sect.

Numerical methods consist of a number of statistical, mathematical and graphic techniques that analyze many variables simultaneously and are useful for taxonomical purposes (Lonni et al., 2005; Rodriguez et al., 2010). Therefore, the objective of this research is to combine chemometric methods with morpho-anatomical data to identify *Baccharis* species belonging to sect. *Caulopterae*. This work will therefore provide qualitative and quantitative differential micrographic characters of these species contributing with their effective quality control.

Materials and methods

Plant material

The *Baccharis* species (sect. *Caulopterae*), Asteraceae, with alate stems included in the study were: *B. articulata* (Lam.) Pers., *B. crispa* Spreng., *B. gaudichaudiana* DC., *B. microcephala* (Less.) DC., *Baccharis penningtonii* Heering, *Baccharis phyteumoides* (Less.) DC., *B. sagittalis* (Less.) DC., *B. triangularis* Hauman and *B. trimera* (Less.) DC. and samples of each species from different regions of Argentina were examined (114 populations). Specimens from the following herbaria: UNR, SI, CTES, BAF and LP (abbreviations according to Holmgren et al., 1990), or fresh material collected by the authors and checked by Dr MA Gattuso and Dr SJ Gattuso during collecting campaigns were examined. All materials were collected with flowers and/or fruits to enable identification and stored in the UNR herbarium. The superscript numbers indicate the plant material used to obtain the quantitative micrographic variables (1) and the plant material used for microscopic and macroscopic examination (2) (Voucher specimens and locations are detailed in Box 2S and Fig. 1S, Supplementary Material).

Morphoanatomy

The fresh material was fixed in F.A.A. (70° ethanol, glacial acetic acid, formaldehyde and water 50:5:30:15). The herbarium material was hydrated in boiling water with added drops of detergent. Zeiss MC 80 Axiolab light microscope equipped with a photographic camera and Nikon Alphaphot YS light microscope with polarized light and a Nikon Type 104 stereoscopic drawing tube were used for microscopic examination.

Table 1
Correlation between the original variables and the three first components in the characterization of nine *Baccharis* species.

Quantitative variables	Principal components (*R*)		
	*R*1	*R*2	*R*3
Wing width (mm)	0.5743	0.0930	0.6448
Stomatal density	0.1094	0.9155	−0.1835
Stomatal index	0.1129	0.8675	−0.2734
Stomatal length (μm)	0.1786	−0.9450	0.0767
Stomatal width (μm)	0.2514	−0.9091	−0.0126
Number of SSS in wing	0.9080	0.0106	0.1034
Number of SSS per wing (mm)	0.6259	−0.0925	−0.6017
Length of SSS in wing (μm)	0.8255	0.1731	0.0646
Width of SSS in wing (μm)	0.8569	0.0549	0.0796
Density of trichome tufts	−0.1480	−0.0963	−0.5808
Stem perimeter	−0.0399	0.2227	0.7848
Number of SSS in stem	0.8677	−0.0147	−0.0682
Number of SSS per stem mm	0.8033	−0.0416	−0.3870
Length of SSS in stem (μm)	0.7976	0.2437	0.2684
Width of SSS in stem (μm)	0.6987	−0.2138	−0.0964
$\sum^2$ = eigenvalue	5.6275	3.5261	2.1024

The wings were dehydrated with increasing concentrations of alcohol and coated with gold-palladium. Observations were made using a JEOL scanning electron microscope, model 35-CI.

(1) Surface view of epidermis

The stem wings were diaphanised according to Strittmatter's technique (1973) when KOH 10% was used to remove the resin layer.

(2) Cross-sections of winged stems

The material was dehydrated in increasing ethanol concentrations, then in ethanol/xylene and xylene and it was finally embedded in paraffin (Johansen, 1940). Cuts were performed manually with a Minot microtome, obtaining 12 μm thick sections. Diluted Safranine and Safranine-Fast green were used for staining (Strittmatter, 1979). The material was also dehydrated in increasing acetone concentrations, acetone/propylene oxide and propylene oxide, and embedded in Spurr's epoxy resin (Union Carbide International Co.). The stem segments were cut into 1 μm sections with an ultramicrotome equipped with a diamond knife. Toluidine Blue 1% and Acid Fuchsin 1% were used for staining (D'Ambrogio, 1986).

Crystals were observed using weak diluted acid and polarized light analysis (Johansen, 1940).

Both techniques (diaphanised and cross-sections) were used in order to obtain the quantitative micrographic variables (marked by superscript 1 in each sample tested).

Statistical analysis

Population analysis was performed by means of principal components analysis (PCA) using NTSYS-pc 2.11w (Numerical Taxonomy and Multivariate Analysis System) designed by Rohlf (1998). The aim of PCA is to reduce data dimensionality by transforming the original characteristic variables into others that are linear combinations of the first variables (Lonni et al., 2005).

The basic data matrix was prepared by considering fifteen micrographic quantitative variables (listed in Tables 1 and 2) of the alate stems as seen in the cross-section and diaphinased material (in total: 50 populations of nine species were studied).

Variables were grouped as follows: (1) surface view of epidermis: (a) stomatal length, (b) stomatal width, (c) stomatal index, (d) stomatal density, and (e) density of tufts; (2) cross section of winged stems: (a) wing width, (b) number of secreting schizogenous structures (SSS) in the wing, (c) number of SSS per mm stem, (d) SSS length in the wing, (e) SSS width in the wing, (f) stem perimeter,

Table 2
The proportion of explained variance by each original variable on the first three principal components in the characterization of nine *Baccharis* species.

Quantitative variables	Principal components			
	*R*1	*R*2	*R*3	Proportion of variance
Stomatal index	0.2258	0.7525	0.0747	1.0530
Stomatal length (μm)	0.0318	0.8930	0.0058	0.9306
Stomatal width (μm)	0.0632	0.8264	0.0001	0.8897
Stomatal density	0.0119	0.8381	0.0336	0.8836
Number of SSS in wing	0.8244	0.0001	0.0106	0.8351
Number of SSS per stem mm	0.6452	0.0017	0.1497	0.7966
Length of SSS in stem (μm)	0.6361	0.0593	0.0720	0.7674
Number of SSS per wing mm	0.3917	0.0085	0.3620	0.7622
Number of SSS in stem	0.7529	0.0002	0.0046	0.7577
Wing with (mm)	0.3298	0.0086	0.4157	0.7541
Width of SSS in wing (μm)	0.7342	0.0030	0.0063	0.7435
Length of SSS in wing (μm)	0.6814	0.0299	0.0041	0.7154
Stem perimeter	0.0015	0.0495	0.6159	0.6669
Width of SSS in stem (μm)	0.4881	0.0457	0.0092	0.5430
Density of trichome tufts	0.0219	0.0092	0.3373	0.3684

(g) number of SSS in the stem, (h) number of SSS per mm stem, (i) SSS length in the stem, (j) SSS width in the stem.

Quantitative data of the micrographic variables were obtained from the average of ten cross sections and diaphanised replicates of each population.

The ANOVA test and Scheffe's test for multiple comparisons were used to perform an in-depth analysis of the information obtained from the PCA. This univariate analysis was undertaken in order to establish the variables that should be used to differentiate species in this study, using the same fifteen variables as in the multivariate analysis, which was considered statistically significant at $p < 0.05$.

Results

Alate stem anatomy – qualitative variables

Box 1 summarizes the common and differential anatomical qualitative variables of nine species of *Baccharis* with alate stems. Figs. 1–3 show the stomata type, the uniseriate type of trichome and the presence or absence of collenchyma in the wing margin for each species, respectively.

Statistical analysis – quantitative variables

Multivariate analysis

PCA results (using the standardized variables listed in Tables 1 and 2) showed that the first three components (R1–R3) are responsible for 75.04% of the total variability. The first (R1) and second (R2) principal components provide the most relevant information to classify the species. Fig. 4 shows the projection of individuals in the principal plane, although the populations can be classified in six different groups identified as G_1 for *B. articulata* (Ba), G_2 for *B. gaudichaudiana* (Bg), G_3 for *B. trimera* (Bt), G_4 for *B. sagittalis* (Bs) and *B. penningtonii* (Bp), G_5 for *B. microcephala* (Bm) and G_6 for *B. crispa* (Bc), *B. phyteumoides* (Bphy) and *B. triangularis* (Btr). R2 separates G_6 from G_5, G_4, G_2 and G_1; it also separates G_3 from G_2 and G_1, while R1 separates G_2 and G_3 from the others groups and G_1 from G_5.

Figs. 5 and 6 show the projection of individuals in the principal plane (R1 *vs* R3 and R2 *vs* R3, respectively). R3 separates *B. triangularis* from *B. crispa*, and *B. phyteumoides* and *B. sagittalis* from *B. penningtonii* (Figs. 5–6). Therefore, all the species were separated by R1, R2 and R3, which emphasizes the discriminatory power of quantitative micrographic variables.

Results of the eigenvalues and correlation of the original variables on the first three components selected are shown in Table 1.

PCA allowed the determination of the discrimination degree of the studied variables through the proportion of variance explained. Results are presented in Table 2 with the variables arranged in descending order. It should be noted that the variables explaining a greater proportion of variance are more discriminating and so their importance is greater.

The principal component analysis of the morphoanatomical data emphasizes the fact that the first principal component contributed 37.51% of the total variance explained; whereas the first eigenvector coefficients (eigenvalues) of distribution and correlation (Table 1) point out that the SSS number in the wings is the major positive contributing variable of this component (0.9080). Other variables contribute in the following order: the SSS number in the stem (0.8677); SSS size in the wing (0.8255 and 0.8569) and stem (0.7976 and 0.6987) and the SSS number per mm stem (0.8033). A positive contribution of the SSS number and size in the wing and stem indicates that those populations with larger SSS numbers also show a greater SSS size.

The second principal component represented 23.50% of the total variance explained. According to the second eigenvector coefficients and correlation (Table 1), variables with the greatest coefficient values that contributed positively were the stomatal density (0.9155) and stomatal index (0.8675), whereas stomata size, *i.e.* length and width, were the most negatively contributing variables, *i.e.*, −0.9450 (length) and −0.9091 (width). Therefore, from the results, it can be inferred that the second component enables us to distinguish the populations with the largest number of stomata and stomatal index. Consequently, the smallest stomata size (negative contribution) increases toward the axis; the opposite is true for those populations decreasing toward the axis.

The third principal component contributed 14.01% of the total variance explained. According to the third eigenvector coefficients and correlation (Table 1), variables with a greater value of coefficient that contributed positively, were stem perimeter (0.7848) and wing width (0.6448), whereas the negative ones were SSS number per mm wing (−0.6017) and the density of trichome tufts (−0.5808).

In general, the results in Table 2 show that epidermal variables in the surface view of *Baccharis* populations are more important and discriminatory than the alate stem variables in the cross section and therefore, they are of the utmost importance in separating the nine species in the bidimensional space.

Among the variables in the surface view, the stomatal index, whose behavior depends partly on the genotype of the species studied, is in the first place (1.0530), becoming the most discriminating. These results are in agreement with those previously performed on *B. articulata*, *B. gaudichaudiana* and *B. trimera* (Rodriguez et al., 2010).

Univariate analysis

Univariate analysis was undertaken to establish the variables that should be used to differentiate species in this study and the same fifteen quantitative variables that were used in the multivariate analysis were considered. The following tables show the variables of epidermis surface views (Box 2), wing and stem cross sections (Box 3 and 4) and the statistically significant differences between the species studied.

Discussion and conclusion

Regarding alate stem anatomy, it could be observed that:

Box 1
Common and differential anatomical qualitative variables of nine species of *Baccharis* with alate stems.

	Ba	*Bc*	*Bg*	*Bm*	*Bp*	*Bphy*	*Bs*	*Btr*	*Bt*
CS	With circular contour and 2–4 wings well developed								
E	Unilayered with rectangular cells, presents stomata and glandular and non-glandular trichomes in tufts; two types, uniseriate and biseriate, can be distinguished								
C	Laminar collenchyma type, discontinuous, corresponding to ribs, from the endodermis to the epidermis. Palisade parenchyma is discontinuous, compact alternating with the collenchyma. Endodermis is continuous with Caspary bands. Presence of outer extra-endodermal secreting schizogenous structures								
VB	Siphonostele with phloem and xylem in continuous bands. The medullar parenchyma is abundant and has large polygonal cells that leave few intercellular spaces. In this zone there are polyhedral crystals of calcium oxalate								
S	Isobilateral								
ST	Anomocytic Ciclocytic	Anomocytic Anisocytic	Anomocytic Ciclocytic	Anomocytic	Anomocytic	Anomocytic Ciclocytic	Anomocytic Ciclocytic	Anomocytic	Anomocytic Anisocytic
UT	3–4 celled, acutely curved, terminal cell not very long, subterminal cell larger than other cells and terminal cell narrower than remaining cells of the trichome (flagellate trichomes)	2–3 celled with long, terminal cell acute at the apex with thick cell wall, which gives a smooth appearance to its surface (armed trichomes)	3–4 celled, acutely curved, terminal cell not very long, subterminal cell larger than other cells and terminal cell narrower than remaining cells of the trichome (flagellate trichomes)	3–4 celled, acutely curved, terminal cell not very long, subterminal cell larger than other cells and terminal cell narrower than remaining cells of the trichome (flagellate trichomes)	3–4 celled with thin cell wall, ending in a cylindrical rounded tip cell as long as body hair (basal cells). Terminal cell is slightly narrower than the subterminal cell (spatula-shaped trichomes)	3–4 celled with thin cell wall, ending in a cylindrical rounded tip cell as long as body hair (basal cells). Terminal cell is slightly narrower than the subterminal cell (spatula-shaped trichomes)	3–4 celled with thin cell wall, ending in a cylindrical rounded tip cell as long as body hair (basal cells). Terminal cell is slightly narrower than the subterminal cell (spatula-shaped trichomes)	4–5 basal cells, usually wider than long, with straight side walls. Branched terminal cell with acute apices ramifications and thick cell wall, which gives a smooth appearance to its surface (armed trichomes)	3–4 celled, curve triangular terminal cell, not very long with thin cell walls, giving it a rough appearance on the surface. Subterminal cell as wide as the terminal cell (clavate trichomes)
PP	It is composed of 2–5 rows of narrow cells elongated radially								
IB	With fibers and some accompanied by inner extra-endodermal secreting schizogenous structures								
LCMB	Presence	Absence	Presence	Presence (only 1 or 2 rows)	Presence (only 1 or 2 rows)	Presence	Presence	Absence	Absence

C, cortex; CS, cross-sectional; E, epidermis; IB, intermediate bundles; LCMB, laminar collenchymas in marginal bundles; PP, palisade parenchyma; S, structure; ST, stomata type; UT, uniseriate trichome type; VB, vascular bundles.

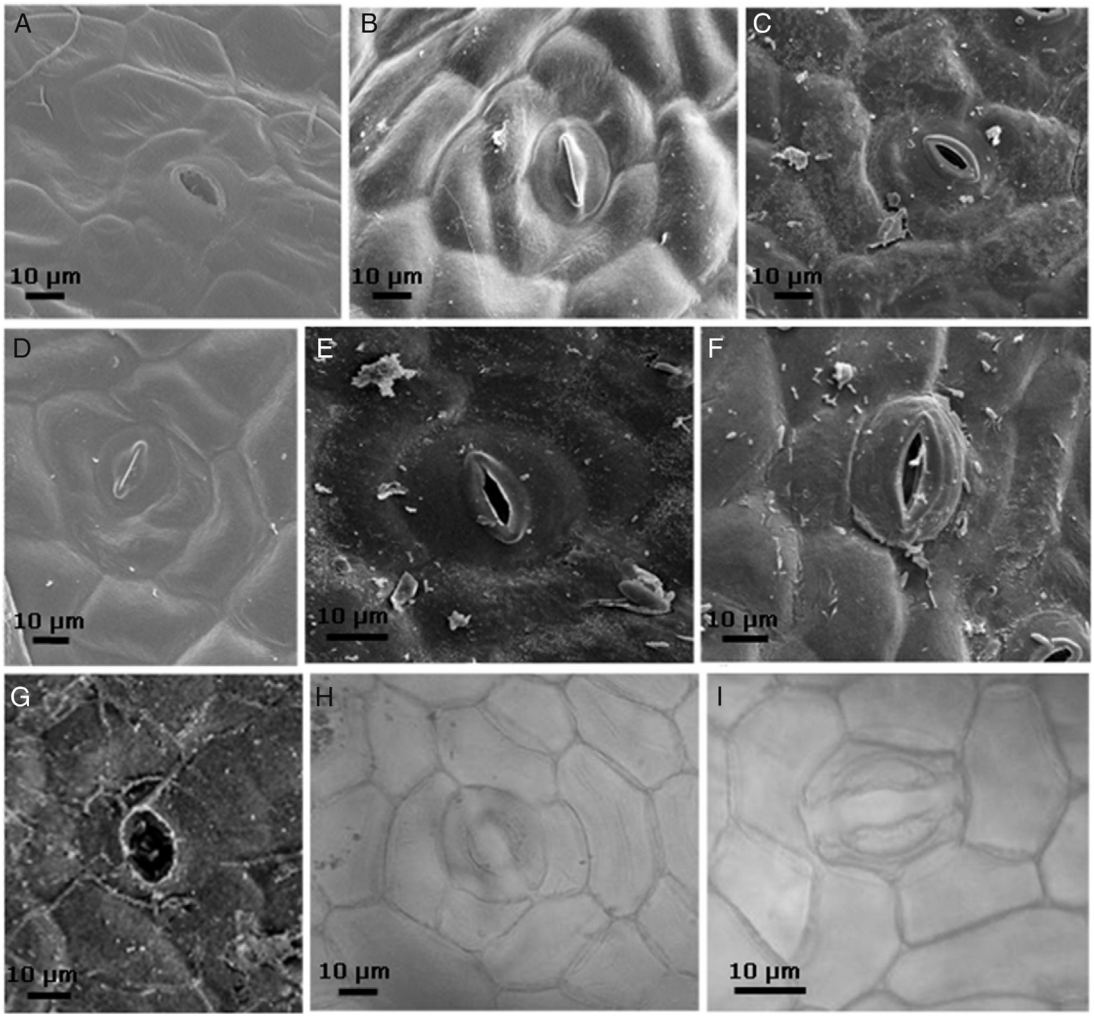

Fig. 1. Stomata type. (A–G) Scanning electron micrograph and (H–I) light micrograph of a surface view of wing epidermis. (A–D) ciclocytic stomata of *Baccharis. gaudiachaudiana*, *B. articulata*, *B. sagittalis* and *B. phyteumoides*, respectively. (E) anisocytic stomata of *B. trimera*. (F–I) anomocytic stomata of *B. crispa*, *B. triangularis*, *B. penningtonii* and *B. microcephala*, respectively.

Box 2
Variables of surface view of epidermis showing statistically significant differences among the species studied ($p < 0.05$).

	Bc	Bg	Bm	Bp	Bphy	Bs	Btr	Bt
Ba	SD, SI, SL, SW		SD, SL, SW	SD, SL, SW	SD, SL	SD, SL, DTT	SD,SI, SL, SW, DTT	SD, SI, SL, SW
Bc		SD, SI, SL, SW	SI	SI		DTT	DTT	
Bg			SD, SL, SW	SD, SL, SW	SD, SI, SL, SW	SD,SI, SL, SW, DTT	SD, SI, SL, SW, DTT	SD, SI, SL, SW
Bm						DTT	SD, SI, SL, DTT	SD, SI, SL
Bp						DTT	SI, DTT	SI
Bphy						DTT	DTT	
Bs							SI, DTT	SI, DTT
Btr								DTT

SD, stomatal density; SI, stomatal index; SL, stomatal length; SW, stomatal width; DTT, density of trichome tufts.

(a) the nine species present unilayered epidermis with rectangular cells of similar size in both epidermis. The epidermis presents stomata and both glandular and non-glandular trichomes. Trichomes are in tufts and two types – uniseriate and biseriate – can be distinguished. Our results are consistent with those published by authors who studied *Baccharis* anatomy (Ariza Espinar, 1973; Cortadi et al., 1999; Barboza et al., 2001; Budel et al., 2003a,b; Müller, 2006; Budel and Duarte, 2009; Petenatti et al., 2007; Rodriguez et al., 2010, 2013). Cortadi et al. (1999) reported only glandular trichomes in *B. articulata* and *B. trimera*, although they found both glandular and non-glandular trichomes in *B. crispa*, which not in tufts but isolated. Budel et al. (2003b) observed only tuft of glandular trichomes in *B. gaudichaudian*. Ariza Espinar (1973) concluded that in the indumentum of *Baccharis* species from central Argentina, trichomes can be found in isolation or forming clusters located in depressions, as in *B. crispa* ("pilose nests"). The indumentum of *Baccharis* was later illustrated in detail by Hellwig (1992), who found that the tuft is uniformly distributed in the indumentum of the leaves and stems in most species of this genus. These tufts usually consist of both uniseriate and biseriate trichomes (Hellwig, 1990). The *Baccharis* indumentum, with trichomes grouped in tufts, is apparently unique within the Asteraceae family (Müller, 2006).

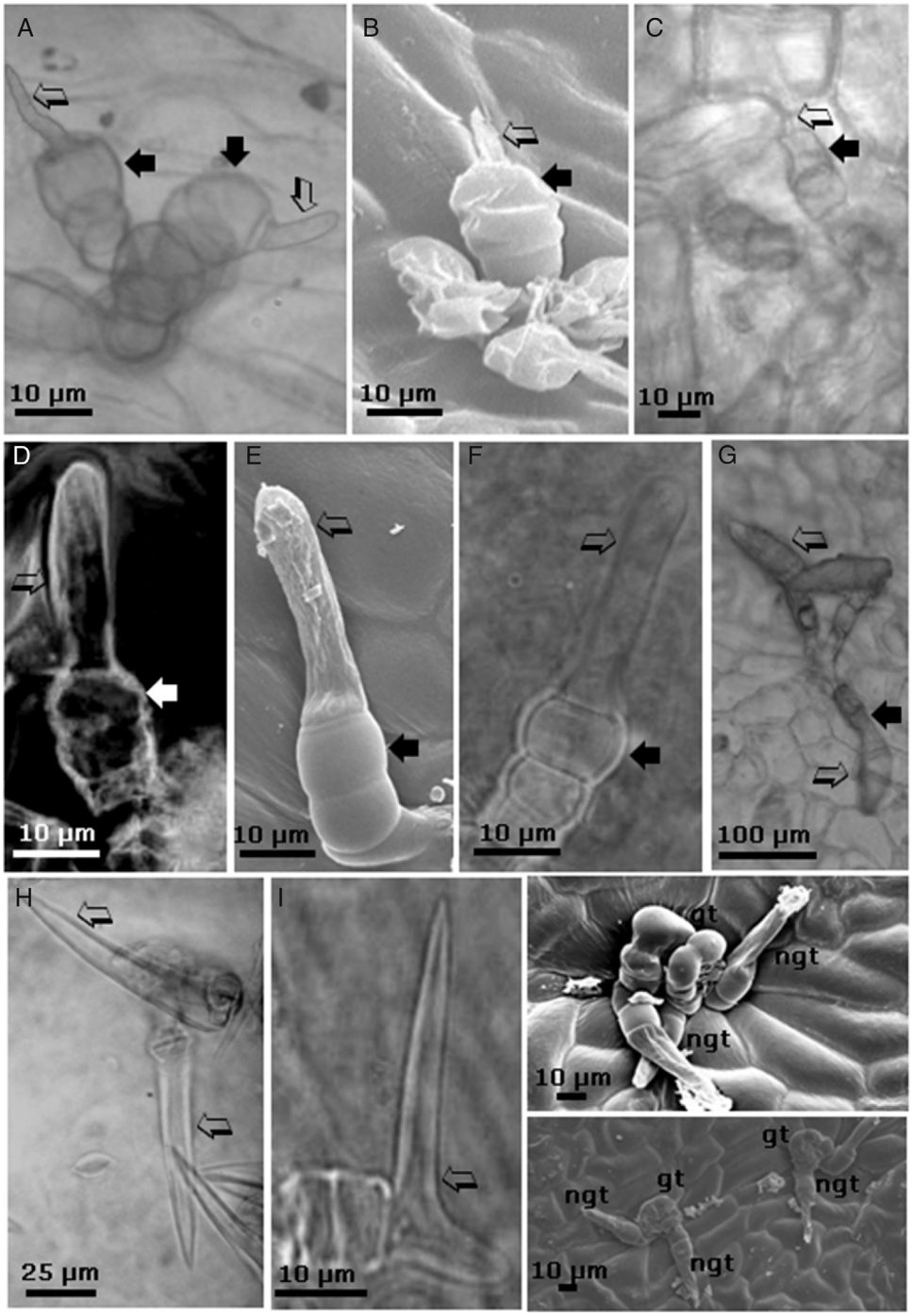

Fig. 2. Uniseriate type of trichome. (A, C, D, F, G–I) Light micrograph and (B, E, J) scanning electron micrograph of a surface view of wing epidermis. (A–C) flagellate trichomes of *Baccharis articulata*, *B. gaudichaudiana* (Rodriguez et al., 2010; Springer Nature license number 4297720745543) and *B. microcephala*, respectively. (D–F) spatula-shaped trichomes of *B. penningtonii*, *B. phyteumoides* and *B. sagittalis*, respectively. (G) clavate trichomes of *B. trimera* (Rodriguez et al., 2010; Springer Nature license number 4297720745543). (H–I) armed trichomes of *B. crispa* and *B. triangularis*, respectively. (J) tufts of uniseriate and biseriate trichomes; gt, glandular trichome; ngt, non-glandular trichome. Arrows indicate terminal cells (⇐) and subterminal cells (⇐).

(b) At least five different types of uniseriate trichomes in the nine species studied were distinguished (Box 1, Fig. 2). Müller (2006) reported tufts of 3–7-celled clavate uniseriate hairs in *Baccharis genistelloides* subsp. *crispa*. This type of trichome was observed in *B. trimera*, but not in *B. crispa* (Budel and Duarte, 2009; Rodriguez et al., 2010, 2013). *B. crispa* presented the trichome type described as a whip by Ariza Espinar (1973), or the 1-armed trichome described by Freire et al. (2007), and Metcalfe and Chalk (1972). Freire et al. (2007) described the bulbiferous flagellate trichome type for *B. articulata*, *B. gaudichaudiana*, *B. microcephala* and *B. trimera*. Rodriguez et al. (2010, 2013) observed this trichome type only in the first three species mentioned but not in *B. trimera*. Budel and Duarte (2009) observed the same non-glandular trichome in *B. microcephala*. Ariza Espinar (1973) reports the whip trichome type in most of *Baccharis* species with different lengths of the terminal cell according to the species. For *B. articulata*, the same author describes the presence of uniseriate glandular and whip

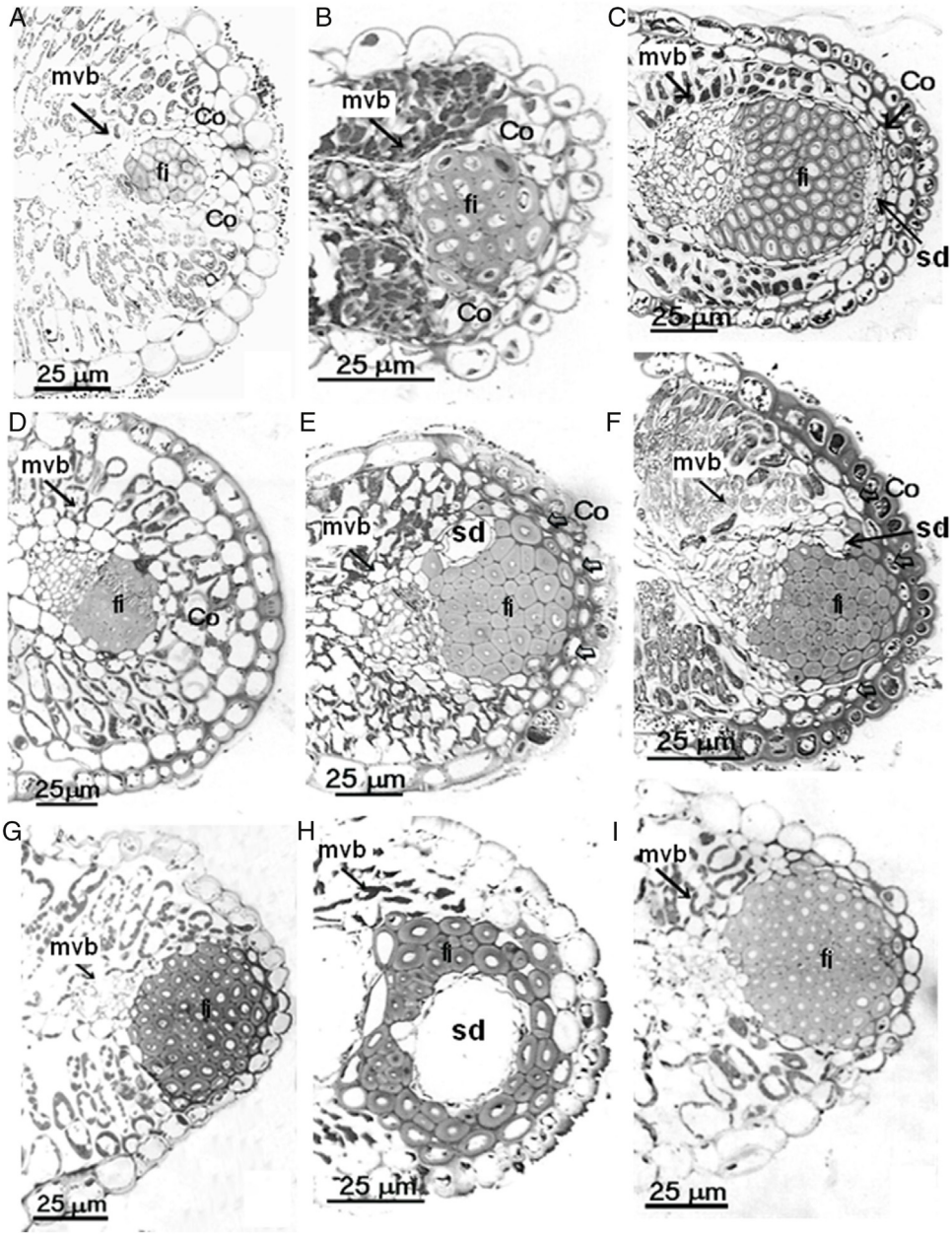

Fig. 3. Light micrograph of wing cross section. (A–D) Presence of collenchyma in the wing margin of *Baccharis articulata*, *B. gaudichaudiana*, *B. phyteumoides* and *B. sagittalis*, respectively. (E–F) Presence of 1–2 rows of collenchyma in the wing margin of *B. microcephala* and *B. penningtonii*, respectively. (G–I) Absence of collenchyma in the wing margin of *B. crispa*, *B. trimera* and *B. triangularis*, respectively. Co, collenchyma; fi, fibers; mvb, marginal vascular bundle; sd, secretory duct/secreting schizogenous structures.

trichomes in tufts (Ariza Espinar, 1973). The same non-glandular trichome type was observed for *B. penningtonii*, *B. phyteumoides* and *B. sagittalis* which resemble the trichome type described by Petenatti et al. (2007) for *B. sagittalis*. Regarding to *B. triangularis* a non-glangular trichome type resembling the 2,4-armed trichome described by Freire et al. (2007) for *B. dracunculifolia* DC was observed.

(c) Anomocytic stomata are present in the nine species studied (Box 1) and this coincides with the reports of Metcalfe and Chalk (1972) for the Asteraceae family; Cortadi et al. (1999) for *B. articulata*, *B. crispa* and *B. trimera*; Budel et al. (2003a,b) for *B. articulata*, *B. gaudichaudiana* and *B. trimera*; Freire et al. (2007) for *B. microcephala*; Petenatti et al. (2007) for *B. sagittalis*; Budel and Duarte (2009) for *B. microcephala* and *B. trimera*; Rodriguez et al. (2010) for *B. articulata*, *B. gaudichaudiana* and *B. trimera* and Rodriguez et al. (2013) for *B. articulata*, *B. crispa*, *B. gaudichaudiana*, *B. microcephala* and *B. trimera*. Cyclocytic stomata in *B. articulata* and anisocytic stomata in *B. trimera* were previously reported by Pertusi (1987). Other authors also reported anisocytic stomata in *B. trimera* (Cortadi et al., 1999; Freire et al., 2007; Budel and Duarte, 2009; Rodriguez et al., 2010) and *B. crispa* (Ariza Espinar, 1973; Cortadi et al., 1999; Barboza et al., 2001; Freire et al., 2007; Rodriguez et al., 2013). Anisocytic stomata were also reported in *B. articulata* by Ariza Espinar (1973), Cortadi et al. (1999) and Barboza et al. (2001) and *B. microcephala* by Budel and Duarte (2009), but no anisocytic stomata were observed for these species by Rodriguez et al. (2010, 2013) in later works. Freire et al. (2007) and Rodriguez et al. (2010, 2013) observed cyclocytic stomata in *B. articulata* and *B. gaudichaudiana*. This type of stoma was also identified for the species *B. phyteumoides* and *B. sagittalis* in the present work (Box 1, Fig. 1).

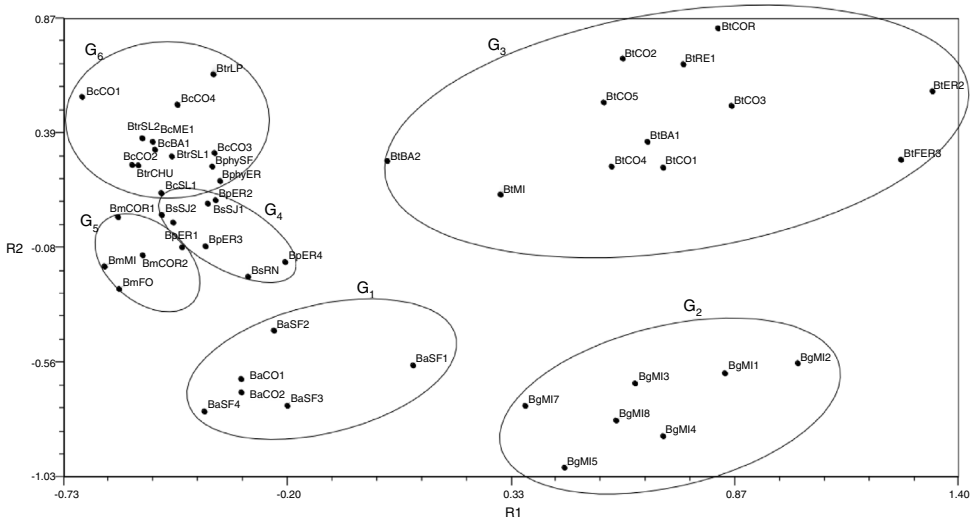

Fig. 4. Two dimensional model (R1 *vs* R2) derived from PCA of 15 quantitative micrographic characters from 50 *Baccharis* populations. Ba, *B. articulata*; Bc, *B. crispa*; Bg, *B. gaudichaudiana*; Bm, *B. microcephala*; Bp, *B. penningtonii*; Bphy, *B. phyteumoides*; Bs, *B. sagittalis*; Btr, *B. triangularis*; Bt, *B. trimera*; BA, Buenos Aires; Cba, Córdoba; Ctes, Corrientes; CHU, Chubut; ER, Entre Ríos; FO, Formosa; LP, La Pampa; M, Misiones; ME, Mendoza; RN, Río Negro; SF, Santa Fe; SJ, San Juan; SL, San Luis. Numbers indicate when there is more than one population of the same species. The first two components represent 61.02% of total variability (37.51 and 23.50%, respectively).

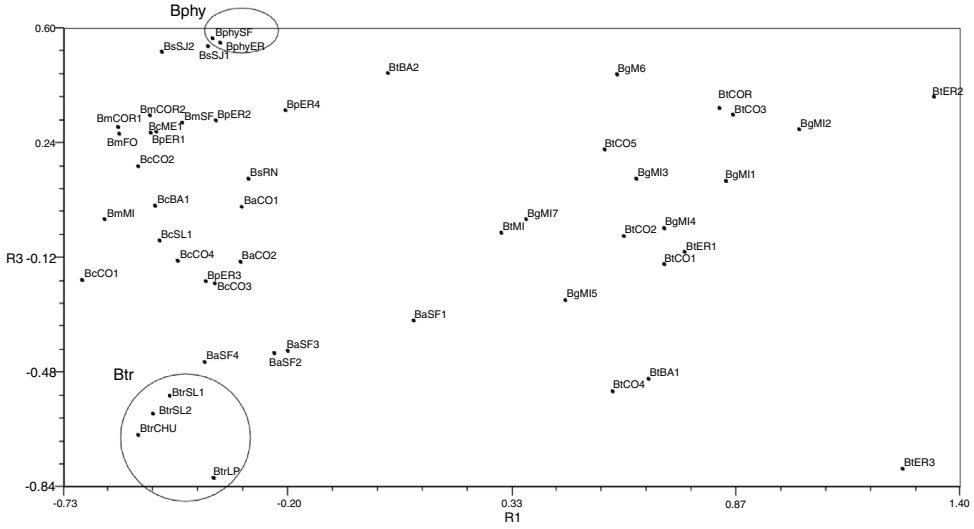

Fig. 5. Two dimensional model (R1 *vs* R3) derived from PCA of fifteen quantitative micrographic characters from fifty *Baccharis* populations. Ba, *B. articulata*; Bc, *B. crispa*; Bg, *B. gaudichaudiana*; Bm, *B. microcephala*; Bp, *B. penningtonii*; Bphy, *B. phyteumoides*; Bs, *B. sagittalis*; Btr, *B. triangularis*; Bt, *B. trimera*; BA, Buenos Aires; Cba, Córdoba; Ctes, Corrientes; CHU, Chubut; ER, Entre Ríos; FO, Formosa; LP, La Pampa; M, Misiones; ME, Mendoza; RN, Río Negro; SF, Santa Fe; SJ, San Juan; SL, San Luis. Numbers indicate when there is more than one population of the same species. R1 represents 37.51% of total variability and R3 represents 14.01% of total variability.

(d) Species in this study show discontinuous, subepidermal collenchyma of the laminar type in their stems, corresponding to the angular ribs. Chlorenchyma, which is also discontinuous and alternates with the collenchyma, is compact and palisade. Stems present endodermis with Caspary bands and outer extra-endodermal secreting schizogenous structures with variable size and number (Box 1). Budel et al. (2003a,b) and Budel and Duarte (2009) observed collenchyma of the angular type for *B. articulata*, *B. gaudichaudiana*, *B. microcephala* and *B. trimera*.

(e) Wing vascular bundles are accompanied by fibers and sometimes by inner extra-endodermal secreting schizogenous structures with variable size and number depending on the species (Rodriguez et al., 2008, 2010, 2013). In *B. trimera* and *B. gaudichaudiana*, the secreting schizogenous structures are larger and more numerous than those in the remaining species. These observations could explain (Simões-Pires et al., 2005) results regarding the large amount of essential oils found in the species *B. trimera*. The absence of fibers in vascular bundles were reported in *B. articulata* by Ariza Espinar (1973), Cortadi et al. (1999) and Müller (2006), as well as the presence of secreting schizogenous structures (Ariza Espinar, 1973; Cortadi et al., 1999). Several authors reported the presence of fibers in *B. crispa* (Ariza Espinar, 1973; Cortadi et al., 1999; Barboza et al., 2001; Müller, 2006). Ariza Espinar (1973) found secreting schizogenous structures in this species, while Cortadi et al. (1999) did not observed any of these structures in *B. crispa*. Müller (2006) observed vascular bundles in *B. sagittalis*, sometimes accompanied by fibers, whereas Petenatti et al. (2007) reported their absence in *B. sagittalis* and *B. triangularis*. This author also reported the presence of secreting schizogenous structures in both species. Cortadi et al. (1999) and Müller (2006) reported vascular bundles for *B. trimera* accompanied by a conspicuous group of fibers and 1–2 secreting schizogenous structures (Cortadi et al., 1999). Budel et al. (2003a,b) and

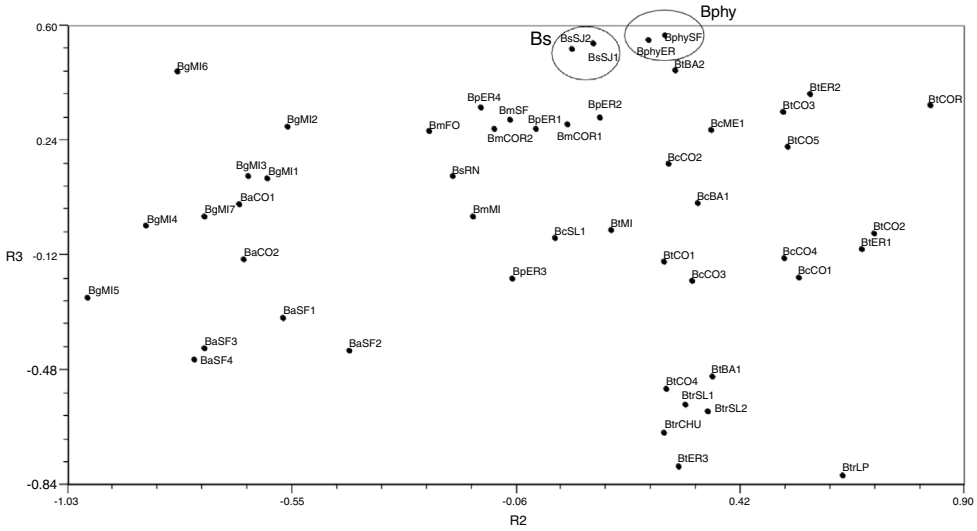

Fig. 6. Two dimensional model (R2 *vs* R3) derived from PCA of 15 quantitative micrographic characters from 50 *Baccharis* populations. Ba, *B. articulata*; Bc, *B. crispa*; Bg, *B. gaudichaudiana*; Bm, *B. microcephala*; Bp, *B. penningtonii*; Bphy, *B. phyteumoides*; Bs, *B. sagittalis*; Btr, *B. triangularis*; Bt, *B. trimera*; BA, Buenos Aires; Cba, Córdoba; Ctes, Corrientes; CHU, Chubut; ER, Entre Ríos; FO, Formosa; LP, La Pampa; M, Misiones; ME, Mendoza; RN, Río Negro; SF, Santa Fe; SJ, San Juan; SL, San Luis. Numbers indicate when there is more than one population of the same species. R2 represents 23.50% of total variability and R3 represents 14.01% of total variability.

Box 3
Variables of cross section of wing showing statistically significant differences between the species studied ($p < 0.05$).

	Bc	Bg	Bm	Bp	Bphy	Bs	Btr	Bt
Ba		WW NSSSW	NSSS/W					WW NSSSW LSSSW
Bc		NSSSW LSSSW						NSSSW NSSS/W LSSSW WSSSW
Bg			NSSSW NSSS/W	NSSSW	NSSSW	NSSSW	WW NSSSW	
Bm								NSSSW NSSS/W LSSSW
Bp								NSSSW
Bphy								NSSSW
Bs								NSSSW LSSSW
Btr								WW NSSSW LSSSW WSSSW

NSSSW, number of secreting schizogenous structures in the wing; NSSS/W, number of secreting schizogenous structures per wing mm; LSSSW, length of secreting schizogenous structures in the wing; WSSSW, width of secreting schizogenous structures in the wing; WW, wing width.

Box 4
Variables of cross section of stem showing statistically significant differences between the species studied ($p < 0.05$).

	Bc	Bg	Bm	Bp	Bphy	Bs	Btr	Bt
Ba		NSSSS LSSSS			SP			NSSSS LSSSS
Bc		NSSSS NSSS/S			SP			NSSSS NSSS/S LSSSS
Bg			NSSSS NSSS/S	NSSSS	SP NSSSS NSSS/S	NSSS/S	NSSSS	
Bm					SP			NSSSS NSSS/S LSSSS
Bp								NSSSS LSSSS
Bphy							SP	SP NSSSS NSSS/S
Bs								NSSS/S LSSSS
Btr								NSSSS LSSSS

NSSSS, number of secreting schizogenous structures in the stem; NSSS/S, number of secreting schizogenous structures per stem mm; SP, stem perimeter; LSSSS, length of secreting schizogenous structures in the stem; WSSSS, width of secreting schizogenous structures in the stem.

Budel and Duarte (2009) reported vascular bundles with fibers and secretory ducts in *B. articulata*, *B. gaudichaudiana*, *B. microcephala* and *B. trimera*.

(f) There is collenchyma in the wing margins of *B. articulata*, *B. gaudichaudiana*, *B. phyteumoides* and *B. sagittalis* which make these species different from *B. crispa*, *B. triangularis* and *B. trimera*, which have a conspicuous cap of schlerenchyma fibers replacing the collenchyma in this position. *B. microcephala* and *B. penningtonii* present only 1–2 rows of collenchyma in the wing margins. The presence or absence of subepidermal collenchyma in the wing margin is a differential character among some species (Box 1, Fig. 3). The presence of subepidermal collenchyma in the wing margin for *B. articulata*, *B. gaudichaudiana*, *B. microcephala* and *B. sagittalis* has previously been reported (Ariza Espinar, 1973; Budel et al., 2003b; Müller, 2006; Rodriguez et al., 2010, 2013).

Regarding the qualitative variables, we could see that while some characters help to establish a difference between two or more species, others showed similarities between them, and therefore it is very difficult to establish unequivocal differences among the nine species studied. As a consequence, it would not be accurate to use qualitative variables alone for differentiating the species of *Baccharis*. Hence we also suggested fifteen quantitative variables to characterize them completely.

The PCA was performed to obtain the probable quantitative variables that would distinguish different species. Firstly, the results show that epidermal variables in surface view are the most important and discriminant in *Baccharis* populations (Table 2).

Regarding the stomatal index, as well as their density and stomata length, it can be observed that ANOVA and Scheffe's test for multiple comparisons showed statistically significant differences among sixteen pairs of species for both quantitative variables ($p < 0.05$, Box 2). Gianello et al. (2000) reported differences in the stomatal index and density between *B. articulata–B. crispa* and *B. articulata–B. trimera* and our results are consistent with those reported by this author (Box 2). Petenatti et al. (2007) also reported differences in these variables between *B. sagittalis* and *B. triangularis*, but our results are only consistent with the difference in the stomatal index (Box 2). *B. articulata* and *B. gaudichaudiana* presented stomata length of 51–58 μm and *B. trimera* of 31 μm. These results agree with Müller (2006), who reported guard cells of 50–80 μm for *B. articulata* and 25–55 μm for *B. trimera*. Freire et al. (2007) reported stomatal lengths between 20 and 60 μm for 38 *Baccharis* species, including *B. trimera*, but with the exception

Box 5
Summary of qualitative and quantitative variables that differ among *Baccharis* species with alate stems in Argentina.

	Bc	*Bg*	*Bm*	*Bp*	*Bphy*	*Bs*	*Btr*	*Bt*
Ba	SD, SI, SL, SW, NSSSS, LSSSS CS in Ba, AS in Bc. LCMB in Ba. Different UT	WW, NSSSW	SD, SL, SW, NSSS/W CS in Ba	SD, SL, SW CS in Ba. Different UT	SD, SL, SP Different UT	SD, SL, DTT Different UT	SD, SI, SL, SW, DTT CS in Ba. LCMB in Ba. Different UT	SD, SI, SL, SW, NSSSS, LSSSS, WW, NSSSW, LSSSW CS in Ba. LCMB in Ba. Different UT
Bc		SD, SI, SL, SW, NSSSS, NSSS/S, NSSSW, LSSSW CS in Bg, AS in Bc. LCMB in Bg. Different UT	SI AS in Bc. LCMB in Bm. Different UT	SI AS in Bc. LCMBin Bp. Different UT	AS in Bc, CS in Bphy. LCMB in Bphy Different UT	DTT AS in Bc, CS in Bs. LCMB in Bs. Different UT	DTT AS in Bc. Different UT	NSSSS, NSSS/S, LSSSS, NSSSW, NSSS/W, LSSSW, WSSSW Different UT
Bg			SD, SL, SW, NSSSS, NSSS/S, NSSSW, NSSS/W CS in Bg	SD, SL, SW, NSSSS, NSSSW CS in Bg. Different UT	SD, SI, SL, SW, SP, NSSSS, NSSS/S, NSSSW Different UT	SD, SI, SL, SW, DTT, NSSS/S, NSSSW Different UT	SD, SI, SL, SW, DTT, NSSSS, WW, NSSSW CS in Bg. LCMB in Bg. Different UT	SD, SI, SL, SW CS in Bg, AS in Bt. LCMB in Bg. Different UT
Bm				Different UT	SP CS in Bphy. Different UT	DTT CS in Bs. Different UT	SD, SI, SL, DTT LCMB in Bm. Different UT	SD, SI, SL, NSSSS, NSSS/S, LSSSS, NSSSW, NSSS/W, LSSSW AS in Bt. LCMB in Bm. Different UT
Bp					CS in Bphy	DTT CS in Bs	SI, DTT LCMB in Bp. Different UT	SI, NSSSS, LSSSS, NSSSW AS in Bt. LCMB in Bp. Different UT
Bphy						DTT	DTT, SP CS in Bphy. LCMB in Bphy. Different UT	SP, NSSSS, NSSS/S, NSSSW CS in Bphy, AS in Bt. LCMB in Bphy. Different UT
Bs							SI, DTT CS in Bs. CLMB in Bs. Different UT	SI, DTT, NSSS/S, LSSSS, NSSSW, LSSSW CS in Bs, AS in Bt. LCMB in Bs. Different UT
Btr								DTT, NSSSS, LSSSS, WW, NSSSW, LSSSW, WSSSW AS in Bt. Different UT

AS, anisocytic stomata; CS, ciclocytic stomata; LCMB, laminar collenchymas in marginal bundle; UT, uniseriate trichome type.

of *B. articulata* and *B. gaudichaudiana*, whose guard cell sizes were larger – about 60 and 75 μm in length.

Although several studies emphasize the stomatal density sensitivity to atmospheric CO_2 concentration and other environmental conditions (Dai et al., 1995; Uprety et al., 2002; Kakani et al., 2003; Christ et al., 2006), progress has been made in understanding the signaling pathway that defines the pattern of stomatal distribution. As a consequence, the first gene in the signaling pathway that affects patterns of stomatal distribution has been found. It is stomatal density and distribution 1 (SDD1), which orientates the asymmetric divisions of the satellite meristemoids (Berger and Altmann, 2000; Von Groll et al., 2002). Therefore, there is a clear genetic component in the stomatal distribution of each species that would justify the use of variables related to them as taxonomic characters within the genus.

Secondly, the PCA results showed that *B. crispa*, *B. phyteumoides* and *B. triangularis* were in the same group when the first two components (R1 and R2) were analyzed. Petenatti et al. (2007) reported close anatomical similarities between *B. crispa* and *B. triangularis*. When analyzing the R3 component and when ANOVA and Scheffe's test for multiple comparisons were performed, density of trichome tufts was the variable used for distinguishing these species, a variable not analyzed by Petenatti et al. (2007). *B. crispa* and *B. triangularis* also presented different non-glandular trichome types. *B. crispa* has anisocytic stomata, which is absent in *B. triangularis*.

The variables stem perimeter, non-glandular trichome type, stomata type and the presence of subepidermal collenchyma in the wing margin of *B. phyteumoides* distinguished *B. crispa* from *B. phyteumoides*. *B. crispa* presented the 1-armed trichome type (Rodriguez et al., 2013) while *B. phyteumoides* presented the spatula-shaped trichomes type. Regarding stoma type, both species presented anomocytic stomata. In addition, *B. crispa* presented anisocytic stomata (Ariza Espinar, 1973; Cortadi et al., 1999; Barboza et al., 2001; Freire et al., 2007; Rodriguez et al., 2013), while *B. phyteumoides* showed the ciclocytic type.

Petenatti et al. (2007) also reported close anatomical similarities between *B. articulata* and *B. sagittalis*. In this case, the variables that allow their differentiation were determined, among them stomatal length and density, the density of trichome tufts and the non-glandular trichome type.

Box 5 displays the differential qualitative and quantitative variables among pairs of species studied. Non-glandular trichome type was the only variable that allowed *B. microcephala* and *B. penningtonii* to be distinguished. The presence of ciclocytic stoma in *B. phyteumoides* allows this species to be distinguished from *B. penningtonii*, which only presents anomocytic stomata type.

As far as we know, this study constitutes the first report on showing morpho-anatomical features of *B. penningtonii* and *B. phyteumoides.* Herein the characteristic qualitative and quantitative variables of these species are provided.

Nowadays there is great interest in controlling quality of raw plant material which will be used as medicine. The first minimum requirement that ensures a correct quality control of the raw material, the intermediates and the finished products, is the botanical identity of the material. In the present study, after an exhaustive morpho-anatomical and population statistical analysis, we include a practical table indicating the qualitative and quantitative variables that should be analyzed to achieve the correct differentiation of the nine *Baccharis* species in the state of raw drug.

Authors' contributions

MVR, MNC, GRB and MF contributed in collecting plant sample and identification and confection of herbarium. MLM, GRB, NA and MVR contributed in running the laboratory work, analysis of the data and drafted the paper. MVR and AAC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.05.002.

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