



## Morphometric studies suggest taxonomic changes in a species complex in *Chromolaena* (Asteraceae, Eupatorieae, Praxelinae)

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### ABSTRACT

The *Chromolaena congesta* complex (Asteraceae, Eupatorieae) presents a difficult challenge for biodiversity researchers due to the intertwined connections of its species and the difficulty of establishing morphological boundaries. In this study, we aimed to use morphometric analyses to evaluate the delimitation of the taxa belonging to the *C. congesta* complex, identify informative morphological traits and to understand the identity of several “atypical” specimens morphologically related to the complex. To achieve this, we used cluster and principal component analysis to evaluate 50 morphological traits from a total of 210 specimens throughout the geographic distribution of the species complex. We found support for the recognition of at least six species - *Chromolaena ascendens*, *C. gentianoides*, *C. hirsuta*, *C. latisquamulosa*, *C. rhinanthacea* and *C. squarrolosa* - according to a phenetic species concept and to corroborate the restoration of three species from synonymy. Furthermore, our results provide a morphological recircumscription of *C. congesta* and *C. elliptica* and indicate two taxonomic novelties, including a new combination and a new species. Despite the answers provided by morphology, many taxonomic issues remain to be solved, and further studies with other types of evidence should be carried to contribute towards a more stable classification.

**Keywords:** *Chromolaena congesta* complex, integrative taxonomy, morphology, morphometrics, Praxelinae, species complex, species delimitation.

## Introduction

The term “species complex” has been widely used in the last decades to describe taxonomically challenging groups of species, which can be defined as groups of actively diverging lineages of organisms (Stoughton *et al.* 2018) or simply as a group of species characterized by unclear boundaries

(Sigovini *et al.* 2016). Much of the difficulty involving disentangling species complexes arises from the primal issue with defining what species are and how they should be circumscribed (Mayr 1996; Mayden 1997; Saikia *et al.* 2008). This scenario has led to an increased interest in discussing species concepts and better approaches for delimiting them (*e.g.* de Queiroz 2005; 2007; Shanker *et al.* 2017).

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In this way, species complexes offer great opportunities for the development of broader, in-depth studies regarding the nature and evolution of species.

*Chromolaena* DC. (Asteraceae, Eupatorieae) has been considered as a challenging genus in terms of species delimitation and identification even before it was segregated from *Eupatorium* L. (Barroso 1950). The apparent difficulty in delimiting species in *Chromolaena* is most likely influenced by the overall morphological uniformity of its species, as well as by the possible widespread occurrence of hybridization, polyploidy, apomixis and irregular meiosis (King & Robinson 1987). The genus is known to include several species complexes and taxa of challenging circumscription (see Christ & Rebouças 2020). The *C. congesta* complex was defined for the first time as a group of ten species - *C. ascendens* (Sch.Bip. ex Baker) R.M.King & H.Rob., *C. caaguazuensis* (Hieron.) R.M.King & H.Rob., *C. congesta* (Hook. & Arn.) R.M.King & H.Rob., *C. elliptica* (Hook. & Arn.) R.M.King & H.Rob., *C. gentianoides* (B.L.Rob.) R.M.King & H.Rob., *C. hirsuta* (Hook. & Arn.) R.M.King & H.Rob., *C. latisquamulosa* (Hieron.) R.M.King & H.Rob., *C. rhinanthacea* (DC.) R.M.King & H.Rob., *C. squarrulosa* (Hook. & Arn.) R.M.King & H.Rob. and *C. xylorhiza* (Baker) R.M.King & H.Rob. - circumscribed by Christ & Ritter (2019) due to their overall morphological similarities, common geographic distribution and intricate taxonomic history. These species are native to grasslands from southeastern South America, occurring in Argentina, Brazil, Paraguay, and Uruguay. Morphologically, the complex is generally characterized by the suffruticose habit, presence of xylopodium, small capitula with few florets (usually between 6–15), corymbose to antellate secondary capitulescences, involucre bracts usually with more or less recurved apices and by the presence of glandular hairs at least on the abaxial surface of the leaves. However, there are deviations of this pattern, and many populations include specimens with great morphological variation. This way, the *C. congesta* complex could be described as a group of populations connected by strong general similarity and plagued by inconsistent morphological boundaries, which hampers even the identification of the most characteristic species.

Due to these morphological uncertainties, past taxonomic treatments circumscribe these species in varying, sometimes even conflicting manners (e.g. Baker 1876; Barroso 1950; Matzenbacher 1979; Cabrera & Klein 1989; Cabrera *et al.* 1996; Perez 2009; Freire & Ariza Espinar 2014; Christ & Ritter 2019). The most recent taxonomic study to include all species of the complex was the treatment of *Chromolaena* produced by Christ & Rebouças (2020) as part of the Flora do Brasil 2020 project. This study was based solely on specimens from Brazil and may have overlooked the particularities of each taxon due to being focused on a country-wise treatment of the entire genus. Thus, it seems reasonable that in-depth analyses through the entire geographic range of the complex are necessary

to better understand the boundaries and relationships among these species.

Even though morphology is of fundamental importance to taxonomy and systematics (Sattler & Rutishauser 1997; Stuessy 2009), and has been traditionally used both by researchers and the public in general for delimitation and recognition of taxa (Mayden 1997; Dayrat 2005; Duminil & Di Michele 2009), its usefulness is greatly diminished when dealing with taxonomic challenging species complexes such as the *C. congesta* complex (e.g. Li *et al.* 2019; Cruz-Lustre *et al.* 2020; Philips & Bytebier 2020). Traditional morphometrics may overcome this issue, given it is a way of quantifying morphological variation (Henderson 2006), avoiding subjectivity, validating species boundaries (Nery *et al.* 2020) and assessing patterns of variation even at intraspecific level (Marhold 2011). Recent examples of applications of traditional morphometrics in plant taxonomy include (but are not limited to) Finot *et al.* (2018), Pastori *et al.* (2018), Viera Barreto *et al.* (2018), Ely *et al.* (2018), Guerra *et al.* (2019), Li *et al.* (2019), Cruz-Lustre *et al.* (2020), Nery *et al.* (2020), and Philips & Bytebier (2020).

Therefore, we use morphometrics as a tool to explore and better understand the morphological variations observed in the *C. congesta* complex. Our specific goals are: 1) to test the boundaries of the species included in the *C. congesta* complex; 2) to detect patterns and morphological relationships in undetermined specimens; 3) to identify useful traits for both delimiting and identifying species of the complex; and 4) to discuss how useful traditional morphometrics and morphology are for the taxonomy of the *C. congesta* complex. This study is part of an on-going research project aiming to disentangle this species complex through the application of integrative taxonomy and systematics, including molecular and cytogenetic analyses, to support taxonomic changes and a more stable classification.

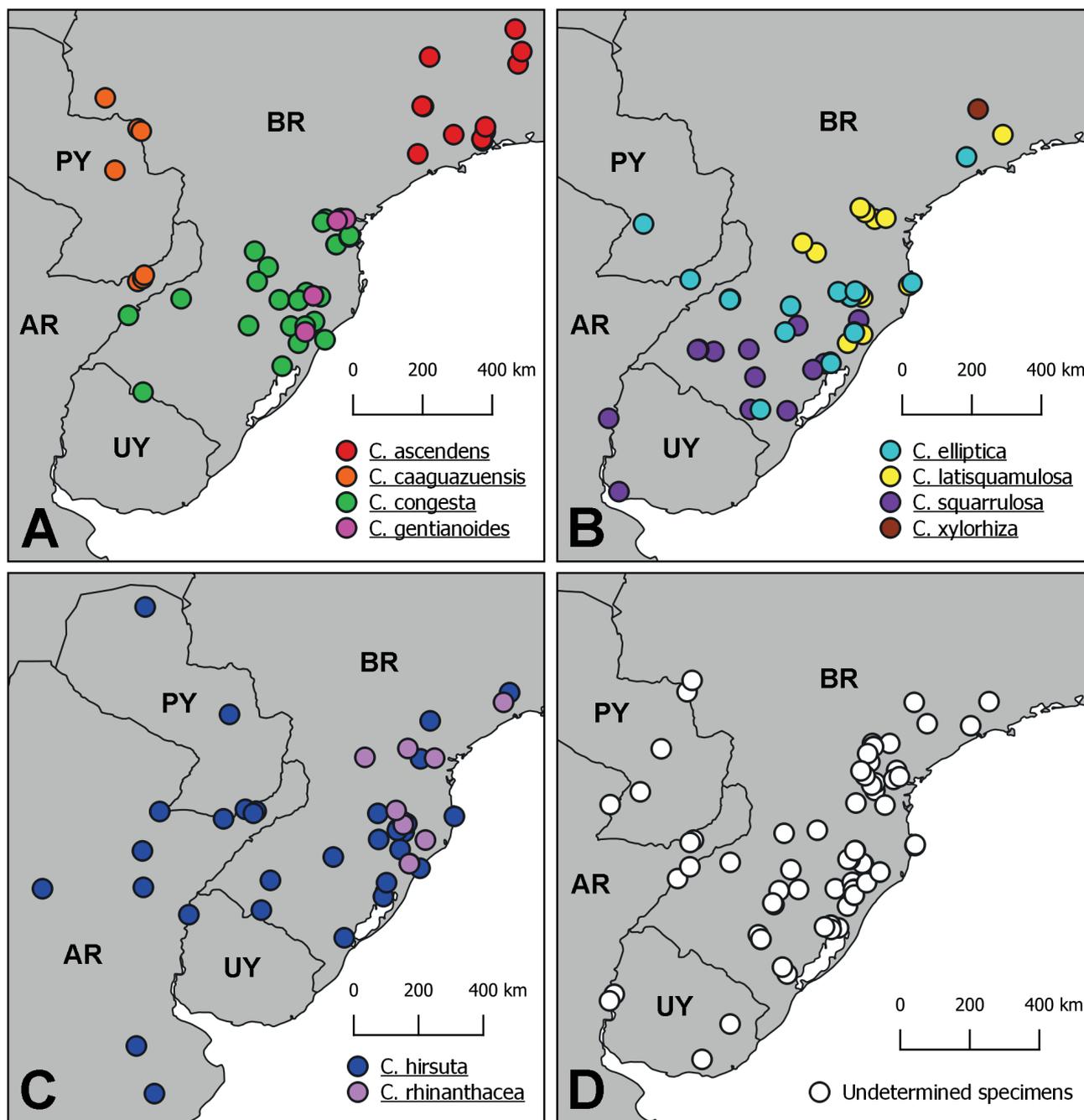
## Material and Methods

### *Specimens and traits examined*

This study was based on specimens deposited in herbaria and new collections made during fieldwork expeditions. Overall, 210 specimens were analyzed, spanning the entire geographical range of the *Chromolaena congesta* complex (Fig. 1), as well as known inter- and intraspecific morphological variation. Representatives of some species of the *C. congesta* complex are exhibited in Fig. 2. Species were delimited according to a morphological species concept based on their original descriptions and type specimens. Specimens that could not be accurately ascribed to specific taxa of the complex were collectively treated in our analyses as “undetermined specimens”. This approach was used due to the difficulty of delimiting and designating names to the different phenotypes observed among them, which



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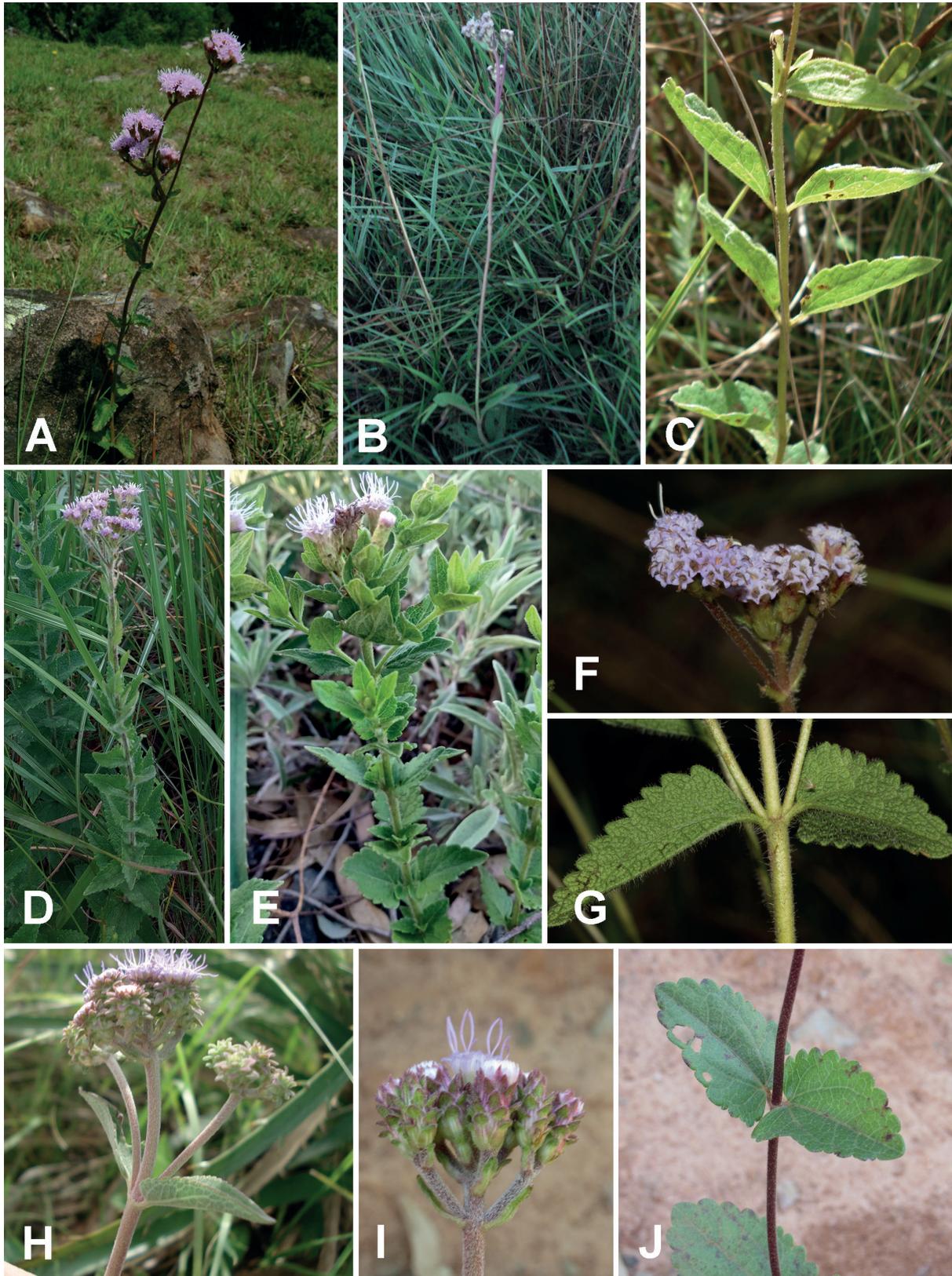


**Figure 1. A-D.** Distribution of specimens used in the morphometric analyses. Species are divided among the presented maps to avoid overlapping occurrence points of different taxa. **A.** *Chromolaena ascendens* (red), *C. caaguazuensis* (orange), *C. congesta* (green) and *C. gentianoides* (magenta). **B.** *Chromolaena elliptica* (cyan), *C. latisquamulosa* (yellow), *C. squarrulosa* (purple) and *C. xylorhiza* (brown). **C.** *Chromolaena hirsuta* (blue) and *C. rhinanthacea* (lilac). **D.** Undetermined specimens (white). Countries were abbreviated as following: Argentina (AR), Brazil (BR), Paraguay (PY) and Uruguay (UY).

would ultimately lead to a matrix with numerous subjective, overlapping potential taxa. This approach is also useful to investigate the boundaries of each species according to their most recent taxonomic treatment (*i.e.* Christ & Rebouças 2020), while also exploring the rest of the dataset in a less biased manner.

Due to the geographic range of each species and the availability of populations on the field and specimens in herbaria, the numbers of samples fluctuated between species. The number of samples per species was *Chromolaena ascendens* (12), *C. caaguazuensis* (7), *C. congesta* (28), *C. elliptica* (17), *C. gentianoides* (5), *C. hirsuta* (31),





**Figure 2.** Representatives of the *Chromolaena congesta* complex. **A.** *C. congesta*. **B.** *C. elliptica*. **C.** Unidentified specimen, possibly *C. congesta* or *C. elliptica*. **D-E.** *C. hirsuta* with alternate (**D**) and opposite (**E**) leaves. **F-G.** *Eupatorium caaguazuense* var. *nervosum*: **F**, capitula; **G**, leaves. **H-J.** *C. squarrulosa*: **H**, capitula and leaves of a typical specimen; **I**, capitula of an atypical specimen; **J**, leaves of an atypical specimen. Photographs by Anderson Christ (A-C, E, H); Rodrigo Trompczynski Dall'Agnol (D); Martin Grings (F-G); and Cleusa Vogel Ely (I-J).



*C. latisquamulosa* (13), *C. rhinanthacea* (10), *C. squarrulosa* (15) and *C. xylorhiza* (1) as well as undetermined specimens (71).

We analyzed specimens included in BHCB, FLOR, FURB, HAS, HUCS, HUFU, ICN, LP, MBM, OUPR, PACA, R, RB, SI, SP, SPF, UEC and UPCB, acronyms according to Thiers (2022, continuously updated). Fieldwork expeditions were also conducted in southeastern and southern Brazil from November 2018 to April 2019 and in February 2020. Complete voucher information on the specimens used in this study is available as supplementary material (Tables S1 and S2). Protologues and high-definition online images of the type specimens of the accepted names and synonyms listed by Christ & Rebouças (2020) were also consulted in order to aid with the identification of the specimens. Since type specimens could only be accessed through digital images, their traits were not included in the final analyses, as many of them (e.g. quantitative traits of phyllaries and cypselae) could not be accurately measured, which would result in a matrix with too many missing data. Images of the type specimens were consulted through the JSTOR database (GPI 2003) and from the online databases of herbaria BM, E, G, GH, K, P and S.

A total of 50 quantitative and qualitative traits were recorded (Table 1). Some of the evaluated traits are present in Fig. 3. Continuous variables were measured using a digital caliper. Whenever possible, we recorded each variable five times per specimen to contemplate morphological variation and then we used the median value of each variable on all analyses. Internodes were measured from base to apex. Leaf measurements were undertaken on leaves from the vegetative part of the plant and not from bracts of the capitulescences; length was measured from the insertion of the petiole in the stem to the tip of the blade and width from one margin to the other at their widest part. The petiole was included in the leaf length due to *C. gentianoides*, *C. elliptica* and *C. rhinanthacea* possessing leaf blades extending into the petiole, thus making it impossible to separate both structures in a practical manner. Conversely, phyllaries and cypselae were measured from base to tip and from margin to margin across their widest part. Lastly, involucre's length were measured from base to the tip of the longest inner phyllary and width was measured across the medium of the involucre, since these structures are cylindrical in shape. Qualitative traits include both vegetative, inflorescence and cypselae traits, and were mostly categorized according to Beentje (2010) in order to avoid subjectivity. The secondary capitulescences refer to the overall shape of the fertile part of the plant, as used by Christ & Ritter (2019), and they were categorized as "corymbose" or "anthelate". The latter was used by Matzenbacher (1979) to describe a capitulescence in which two secondary axes grow longer than the one in between, similar to the inflorescences of some Cyperaceae. All traits (including measurements) were analyzed in dried specimens. Traits that could not be taken in some specimens were reported as missing data (NA).

## Morphometric analyses

In order to explore the morphological relations between the species of the *C. congesta* complex, to attempt to detect patterns in the morphology of undetermined specimens and to test their morphological circumscription, we conducted Cluster Analyses (CA) with the Unweighted Pair Group Method (UPGMA) using the function "hclust" of package "cluster" (Maechler *et al.* 2021) in software RStudio 3.5.1. (RStudio Team 2021). Because our dataset includes both quantitative and qualitative data, we used Gower's coefficient (Gower 1971) to generate the dissimilarity matrix. To select the most suited algorithm for these analyses, we initially calculated single, average and complete linkage algorithms and then a cophenetic correlation between each algorithm and the original dissimilarity matrix. The algorithm with the higher cophenetic correlation, which was selected for the final analyses, was the average linkage. We conducted CA with a complete dataset including all 210 specimens and also with a reduced dataset, excluding the undetermined specimens, and thus contemplating only specimens attributed to the previously recognized taxa. This second dataset included 139 specimens. Analyses with this reduced dataset were conducted in order to evaluate whether the species of the complex would be circumscribed based only on typical specimens, that is, how much undetermined specimens interfere with the classification of these species. These datasets will be simply referred respectively as "complete" and "reduced" throughout this paper. Finally, we calculated a cophenetic correlation coefficient ( $r$ ) for each analysis to evaluate how well they fit the original data.

To compare the morphological traits of the species, we used Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson 2001), which aims to test for differences between the groups' centroids ("location effects"); and Permutational Analysis of Multivariate Dispersions (PERMDISP; Anderson 2006), which is used to test differences between the groups' variances ("dispersion effects") in multivariate space (Anderson & Walsh 2013). For both tests, we assessed the significance of the results using pseudo- $F$  statistics based on 9999 Monte Carlo permutations. We calculated PERMANOVA and PERMDISP respectively using the "adonis" and "betadisper" functions of the package "vegan", also in RStudio 3.5.1. (Oksanen *et al.* 2020). In addition to the PERMDISP test, we used Principal Coordinate Analysis (PCoA) to explore and visualize the location and dispersion effects among the species. We used the square root of Gower dissimilarities to avoid negative eigenvalues in these analyses. To avoid issues when comparing groups with too different sample sizes, we excluded from PERMANOVA and PERMDISP all species with less than ten samples (i.e. *C. caaguazuensis*, *C. gentianoides* and *C. xylorhiza*), while *C. xylorhiza* was also excluded from the PCoA due to being composed of a single specimen. This way, the datasets used for the PCoA included

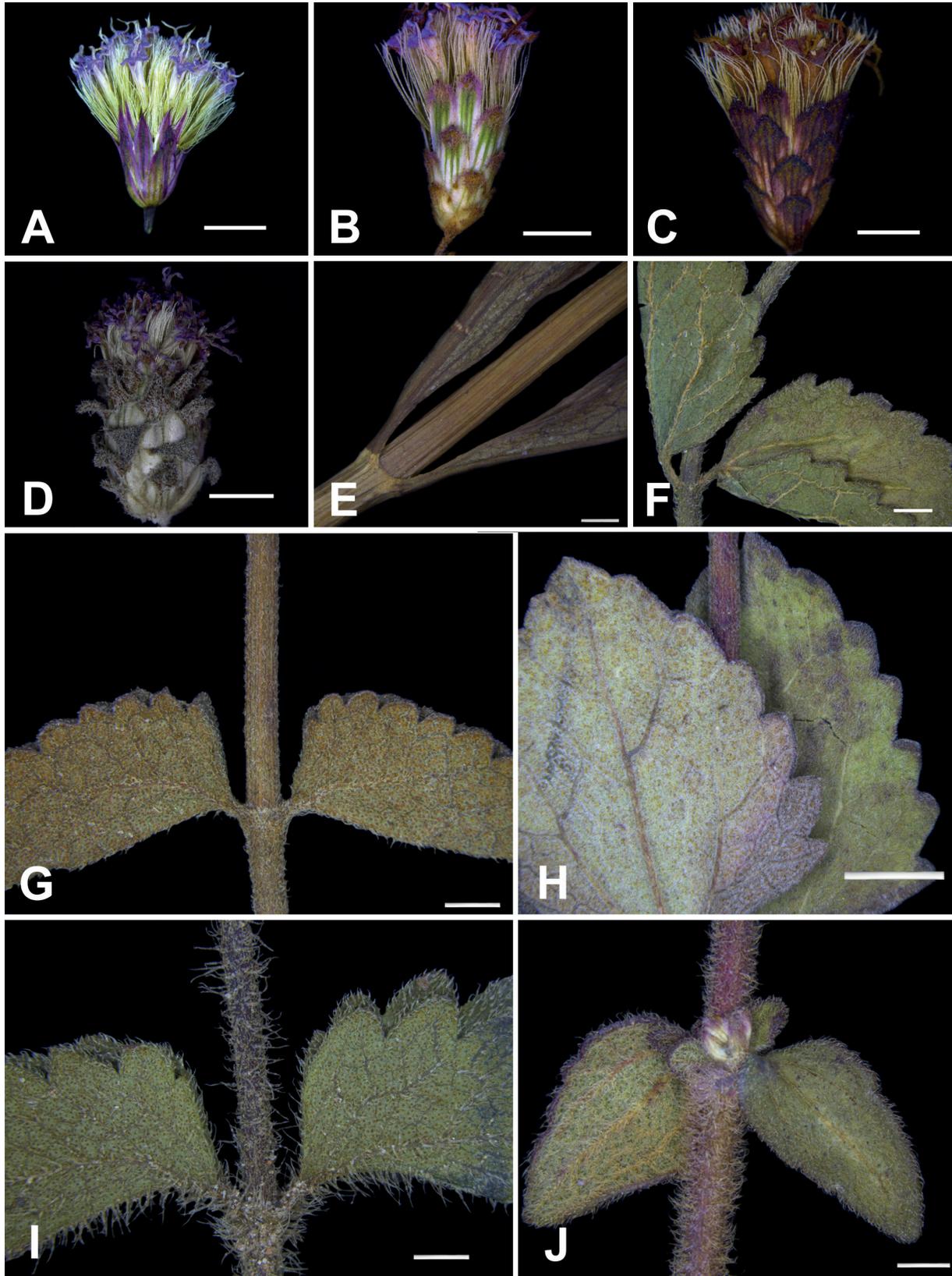


**Table 1.** List of morphological traits selected for the analyses. Traits marked with an asterisk (\*) are the ones used in the dataset used for Linear Discriminant Analysis (LDA).

1. Stem, indumentum (0 glabrous, 1 glabrescent, 2 strigose, 3 puberulous, 4 sericeous, 5 tomentose, 6 villose, 7 hirsute, 8 lanate).
2. Stem, presence of glandular hairs (0 absent, 1 present).
3. Stem, first internode length (mm)*.
4. Stem, last internode length (mm)*.
5. Leaf, phyllotaxy (1 opposite, 2 alternate).
6. Leaf, length (mm)*.
7. Leaf, width (mm)*.
8. Leaf, blade shape (1 ovate, 2 lanceolate, 3 deltate, 4 reniform, 5 oblong, 6 elliptic, 7 orbicular, 8 oblanceolate).
9. Leaf, apex shape (1 cuneate, 2 acute, 3 obtuse, 4 attenuate, 5 acuminate, 6 apiculate, 7 rounded).
10. Leaf, base shape (1 rounded, 2 cordate, 3 subcordate, 4 truncate, 5 cuneate, 6 attenuate, 7 obtuse, 8 cuspidate, 9 acute).
11. Leaf, margins (1 entire, 2 crenate, 3 serrate, 4 dentate, 5 irregular).
12. Leaf, adaxial surface indumentum (0 glabrous, 1 glabrescent, 2 strigose, 3 sericeous, 4 hirsute, 5 villose).
13. Leaf, abaxial surface indumentum (0 glabrous, 1 glabrescent, 2 strigose, 3 sericeous, 4 hirsute, 5 villose, 6 tomentose, 7 lanate).
14. Leaf, glandular hairs on adaxial surface (0 absent, 1 present).
15. Leaf, glandular hairs on abaxial surface (0 absent, 1 present).
16. Leaf, venation emergence (1 emergent, 2 immersed).
17. Primary capitulescence, shape (1 corymbose, 2 glomerate).
18. Secondary capitulescence, shape (0 absent, 1 corymbose, 2 antellate).
19. Axes of capitulescences, indumentum (0 glabrous, 1 strigose, 2 puberulous, 3 sericeous, 4 tomentose, 5 villose, 6 hirsute).
20. Peduncle of the head, length (mm)*.
21. Peduncle of the head, indumentum (0 glabrous, 1 strigose, 2 puberulous, 3 sericeous, 4 tomentose, 5 villose, 6 hirsute).
22. Peduncle of the head, glandular hairs (0 absent, 1 present).
23. Involucre, height (mm)*.
24. Involucre, width (mm)*.
25. Phyllaries, number*
26. Outer phyllaries, length (mm)*.
27. Outer phyllaries, width (mm)*.
28. Outer phyllaries, blade shape (1 oblong, 2 ovate, 3 deltate, 4 elliptic, 5 oblanceolate, 6 orbicular).
29. Outer phyllaries, apex shape (1 acute, 2 cuneate, 3 apiculate, 4 acuminate, 5 rounded, 6 obtuse, 7 attenuate).
30. Outer phyllaries, indumentum (0 glabrous, 1 glabrescent, 2 puberulous, 3 tomentose).
31. Outer phyllaries, glandular hairs (0 absent, 1 present).
32. Outer phyllaries, apex curvature (1 erect, 2 recurved, 3 squarrose).
33. Outer phyllaries, apex color (1 vinaceous, 2 citrine, 3 cinereous).
34. Outer phyllaries, blade color (1 vinaceous, 2 citrine).
35. Inner phyllaries, length (mm)*.
36. Inner phyllaries, width (mm)*.
37. Inner phyllaries, apex shape (1 acute, 2 cuneate, 3 apiculate, 4 acuminate, 5 rounded, 6 obtuse, 7 attenuate).
38. Inner phyllaries, indumentum (0 glabrous, 1 glabrescent, 2 puberulous, 3 tomentose).
39. Inner phyllaries, glandular hairs (0 absent, 1 present).
40. Inner phyllaries, apex curvature (1 erect, 2 recurved, 3 squarrose).
41. Inner phyllaries, apex color (1 vinaceous, 2 citrine, 3 cinereous).
42. Inner phyllaries, blade color (1 vinaceous, 2 citrine).
43. Florets, number*
44. Florets, glandular hairs on the corollas (0 absent, 1 present).
45. Cypselae, length (mm)*.
46. Cypselae, width (mm)*.
47. Cypselae, glandular hairs (0 absent, 1 present).
48. Cypselae, indumentum (0 glabrous, 1 glabrescent, 2 setuliferous).
59. Pappus, length (mm)*.
50. Pappus, number of bristles*.



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**Figure 3.** Capitula, stems and leaves of different species of the *Chromolaena congesta* complex, showcasing some of the morphological traits evaluated in this study. **A.** Capitulum of *C. gentianoides*. **B.** Capitulum of *C. congesta*. **C.** Capitulum of *C. elliptica*. **D.** Capitulum of *C. squarrosa*. **E.** Glabrous stem and leaves of *C. rhinanthacea*. **F.** Strigose stem and glabrescent leaves of *C. latisquamulosa*. **G.** Strigose stem and leaves of *C. congesta*. **H.** Sericeous stem and lanate leaves of an undetermined specimen. **I.** Hirsute stem and leaves of an undetermined specimen. **J.** Villose stem and leaves of *C. ascendens*. (All scale bars = 2 mm).



209 (complete analysis) and 138 (reduced analysis) samples. Finally, since the group collectively treated as undetermined does not correspond to any particular taxa and is actually an artificial collection of specimens, as commented above, we will only present the results of the PERMANOVA and PERMDISP based on the reduced dataset, *i.e.* the one that excludes these specimens.

Lastly, we used Linear Discriminant Analysis (LDA) to evaluate whether the species of the complex (*i.e.* the same taxa used in comparison analyses) could be predicted from quantitative morphological traits (Table 1). Specifically, our interest was to detect which of the quantitative traits better discriminate the species. Here, we defined that the prior probabilities (*i.e.* the probability that an observation or replicate will belong to a certain group) are proportional to the number of samples in each species. All morphological traits were log-transformed before the analysis. We used Bootstrap resampling (9999 resamples) to estimate LDA in Systat (Systat Software, San Jose, CA). All variables with F-values higher than 4 were considered as potentially informative to discriminate among the species of the complex, while the values of tolerance presented indicate how independent each variable is from the others, with the maximum value expected being 1 (completely independent).

### Figures and maps

All figures presented were edited on Adobe Photoshop CS. Photographs of specimens found in fieldwork expeditions were either taken from the personal archives of the authors or acquired with express permission of third-party researchers, with all credits being indicated in their respective captions. Maps were produced with software QGIS 3.10 (QGIS Development Team, 2009), while location coordinates of each specimen analyzed were acquired through Google Earth Pro. In the presented dendrograms, each specimen is assigned to a specific code (combination of letters and numbers) which can be used to assess its specific data in the supplementary material (Tables S1 and S2).

## Results

### Cluster Analyses

The dendrogram using the complete dataset is shown in Figs. 4 and 5. Nine major clusters were identified and are presented below. The cophenetic correlation coefficient ( $r$ ) for this dendrogram is  $r = 0.75$ , which is considered a good fit of the observed data.

Clusters 1 to 7 include all specimens assigned to *C. ascendens*, *C. gentianoides*, *C. latisquamulosa*, *C. rhinanthacea*, *C. squarrulosa* and *C. xylorhiza*, as well as most specimens assigned to *C. hirsuta* and many unidentified specimens (Fig. 4) and are morphologically characterized

mostly according to traits presented in Table 2. Clusters 8 and 9 were considerably larger and more heterogeneous than the other seven (Fig. 5). Regarding Cluster 8, we found out it could be further divided in three (clusters 8A, 8B and 8C) and a further specimen of *C. congesta* (CCO15) which is treated as *incertae sedis* (Fig. 5). Cluster 8B could be morphologically defined by stems mostly strigose to puberulous, leaves usually elliptic, bases usually cuneate, commonly strigose on both surfaces, and cypselae with glandular hairs; and Cluster 8C is characterized mainly by the stems and leaves commonly hirsute to villose (even though other types of indumentum are also observed), venation emergent on the abaxial surface of the leaves, and cypselas without glandular hairs. Cluster 8A does not seem to possess clear morphological boundaries. These three clusters have in common the almost constant presence of cuneate outer phyllaries. On the other hand, Cluster 9 is characterized by outer phyllaries usually acuminate to apiculate, and could be further divided in smaller groups, two of which possess unique and constant sets of morphological traits and are named as distinct clusters. Cluster 9A is majorly composed of specimens attributed to *C. congesta* and could be characterized mostly by stems usually strigose, leaves deltate, apices usually acute, bases usually truncate to rounded, strigose on the adaxial surface and usually strigose to tomentose on the abaxial surface; Cluster 9B included exclusively undetermined specimens, which are mostly overall very similar to specimens from cluster 9A, but differ from it mainly by the stems usually sericeous and leaves sericeous on the adaxial surface and lanate on the abaxial surface.

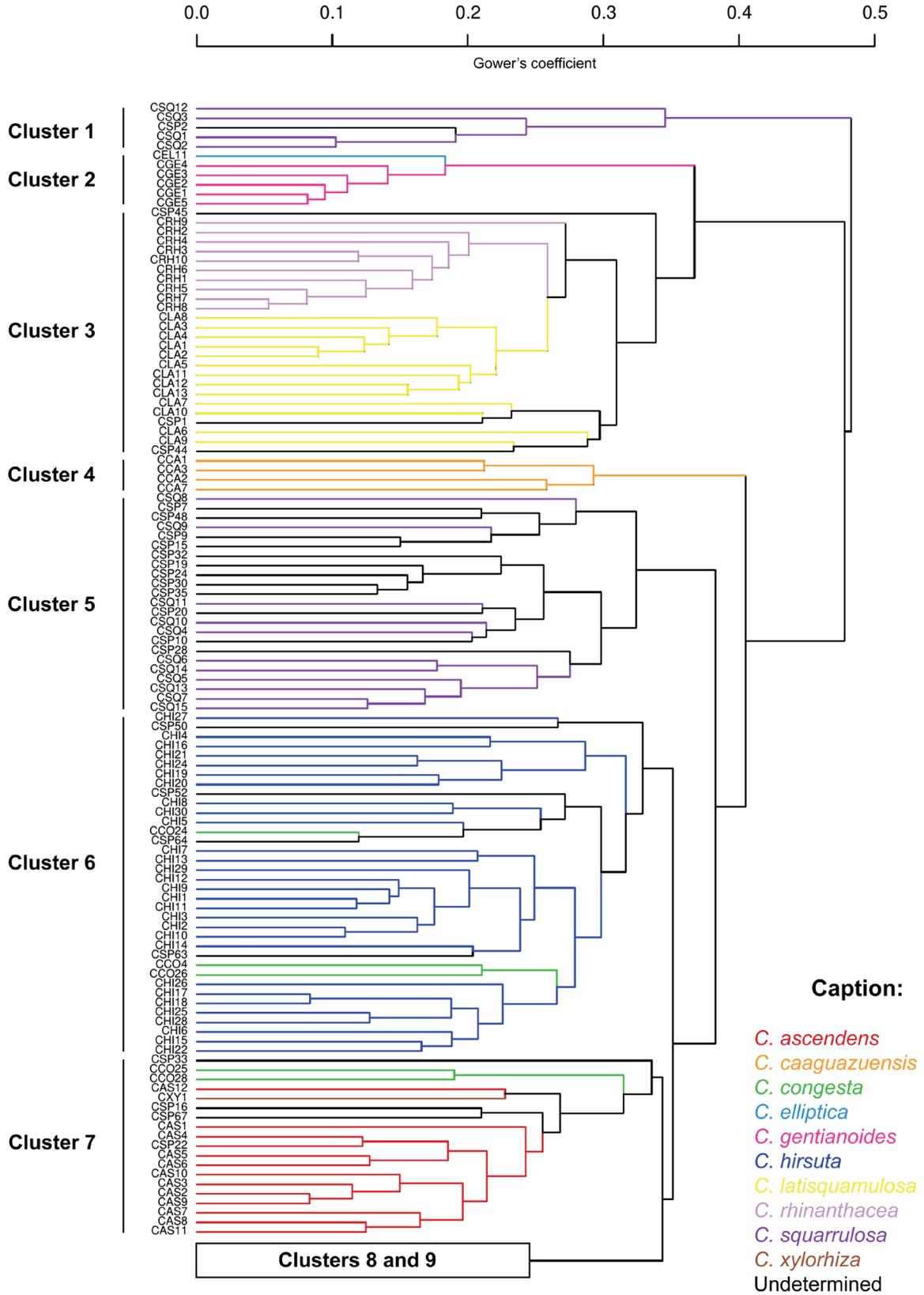
Finally, the cluster analysis based on the reduced dataset produced a much more succinct and comprehensible dendrogram (Fig. 6). Overall, seven major groupings were observed, of which four (Clusters A, C, D and G) correspond to *C. gentianoides*, *C. caaguazuensis*, *C. hirsuta* and *C. squarrulosa* respectively. The remaining groups are cluster B, which includes both *C. latisquamulosa* and *C. rhinanthacea*; cluster E, which includes both *C. congesta* and *C. elliptica*; and cluster F, which includes *C. ascendens* and the single specimen assigned to *C. xylorhiza*. While all specimens assigned to *C. ascendens*, *C. gentianoides*, *C. latisquamulosa*, *C. rhinanthacea* and *C. squarrulosa* were grouped exclusively on single clusters, some specimens of *C. congesta*, *C. elliptica* and *C. hirsuta* were placed in different groups through the dendrogram, and almost half of the specimens belonging to *C. caaguazuensis* did not group in cluster C, where the other half of them is located. The cophenetic correlation coefficient ( $r$ ) of this dendrogram was also  $r = 0.75$ .

### PERMANOVA, PERMDISP and Principal Coordinate Analyses

The results of the PERMANOVA test conducted with the reduced dataset indicate that the species differ based on the evaluated morphological traits (d.f. = 6, 119,

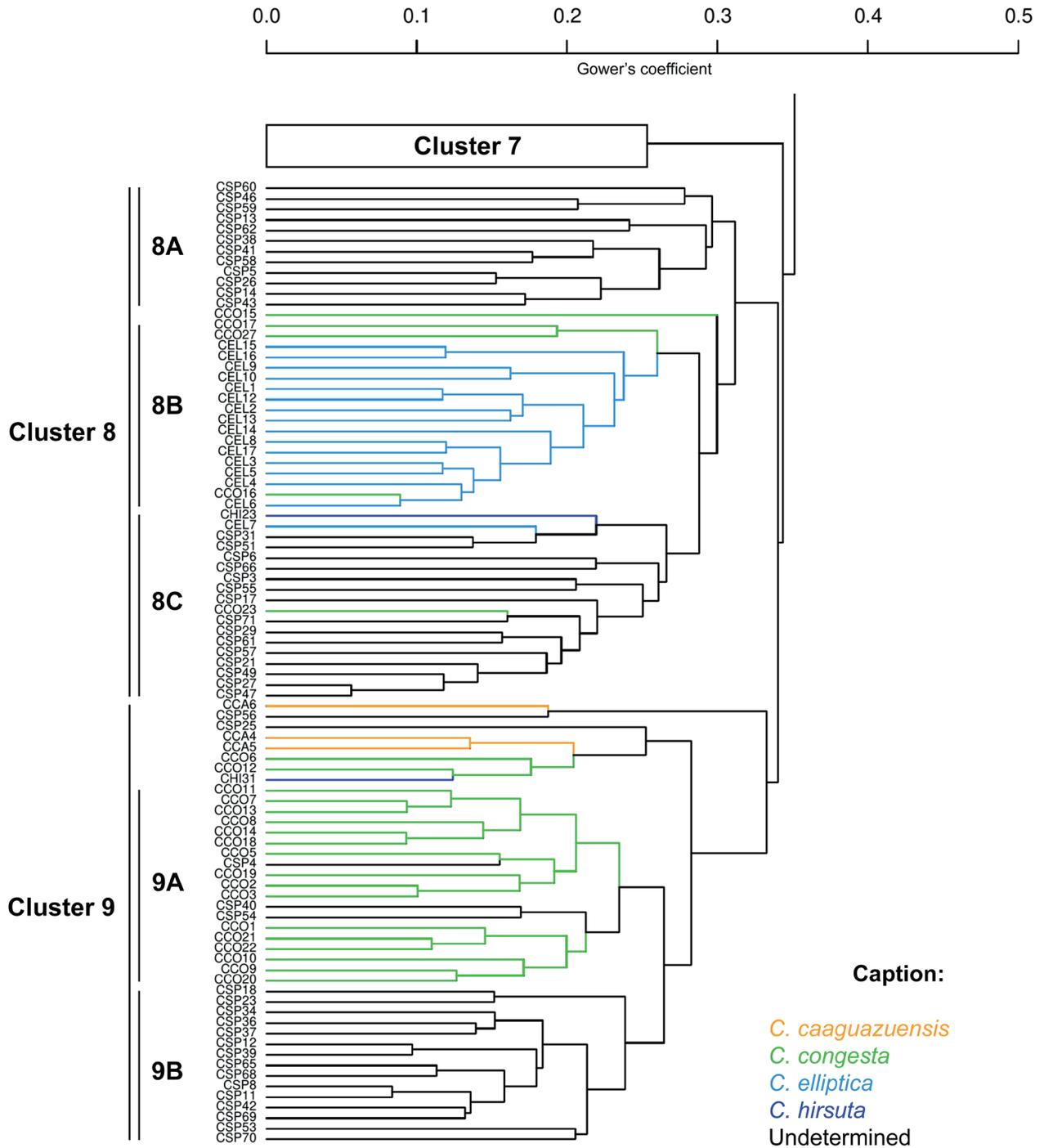


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**Figure 4.** Dendrogram produced through Cluster Analysis of the complete dataset, with clusters 1–7 indicated by black bars to the left of the image. Species are identified through colors indicated on the image itself, while individual specimens are identified by a specific code.





**Figure 5.** Clusters 8 and 9 of the dendrogram presented in Figure 4, with major and minor clusters discussed in the text indicated by black bars to the left (major clusters) and to the right (minor clusters), both positioned on the far left of the image. Species are identified through colors indicated on the image itself, while individual specimens are identified by a specific code.

$F = 29.22$ ,  $P = 0.0001$ ). Similarly, the variance in morphological traits also differed among the species (PERMDISP, d.f. = 6, 119,  $F = 4.12$ ,  $P = 0.0011$ ). Specifically, *C. squarrolosa* (average distance to spatial median = 0.40)

and *C. hirsuta* (average distance to spatial median = 0.37) were the species with the highest morphological variation in the evaluated traits. The other species had similar values and therefore do not differ significantly in the amount of

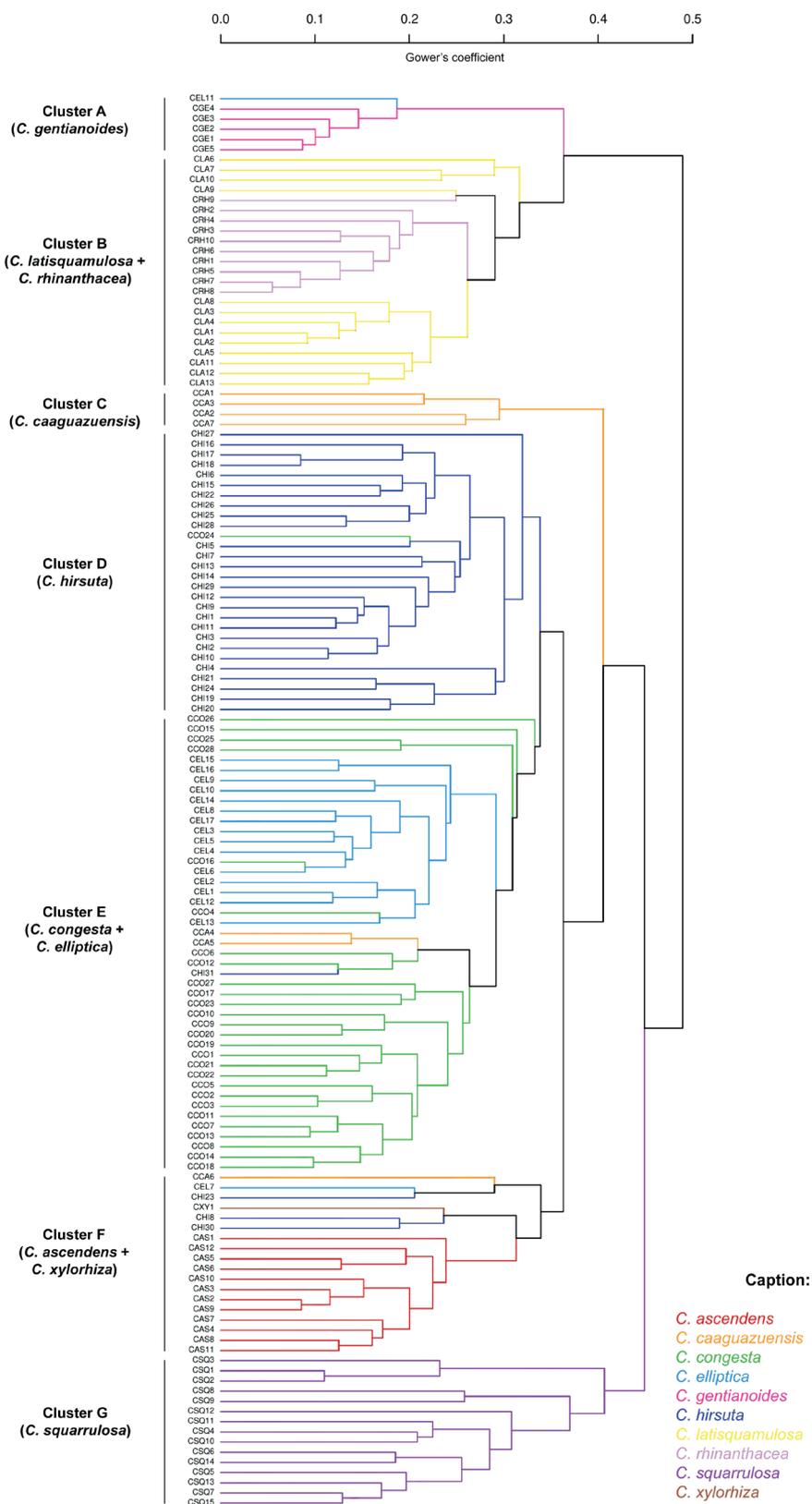


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**Table 2.** Main morphological traits of each taxon of the *Chromolaena congesta* complex according to the clusters observed in both Cluster Analysis. Each taxon is indicated alongside its corresponding clusters according to the complete (clusters 1-9) and reduced (clusters A-G) datasets.

Taxon	Main vegetative traits	Main reproductive traits
<i>Chromolaena ascendens</i> (Clusters 7 and F)	Stems usually villose, glandular or not; leaves opposite, usually orbicular to ovate, apices mostly acute to rounded, bases cuneate to rounded, mostly strigose on the adaxial surface, usually villose on the abaxial surface, glandular only in the abaxial surface.	Axes usually villose; capitula commonly sessile; outer phyllaries commonly cuneate, puberulous, glandular, erect, vinaceous, 1.8-3.4 mm long; florets usually 13-24; cypselae usually shorter than 2 mm long, glabrescent to setuliferous and eglandular; pappus commonly shorter than 4 mm long, usually with less than 30 bristles.
<i>C. caaguazuensis</i> (Clusters 4 and C)	Stems strigose, puberulous, tomentose or villose, mostly eglandular; leaves opposite, ovate, apices mostly acute, bases cordate, strigose to glabrescent on adaxial surface and strigose to tomentose or villose on abaxial surface, glandular on both surfaces.	Axes mostly tomentose; peduncles 1.4-3.2 mm long; outer phyllaries usually acute, puberulous, glandular, erect, usually vinaceous, usually ca. 1.8 mm long; florets 4; cypselae usually glabrous or glabrescent and eglandular; pappus 4.3-5.3 mm long, bristles ca. 37-50.
<i>C. congesta</i> (Cluster 9A and part of cluster E)	Stems usually strigose, rarely villose, glandular or not; leaves opposite, deltate, apices usually acute, bases usually truncate to rounded, strigose on adaxial surface and strigose to tomentose on abaxial surface, glandular only on abaxial surface.	Axes mostly strigose to tomentose; peduncles usually 1-3.8 mm long; outer phyllaries usually apiculate to acuminate, puberulous, glandular, erect to recurved, vinaceous, 1.4-2 mm long; florets 7-13; cypselae usually setuliferous, glandular; pappus usually 3.8-4.2 mm long, bristles ca. 30-40.
<i>C. elliptica</i> (Cluster 8B and part of cluster E)	Stems usually strigose to puberulous, glandular or not; leaves opposite, usually elliptic, apices mostly acute, bases usually cuneate, usually strigose on both surfaces, glandular only on the abaxial surface.	Axes mostly tomentose; capitula mostly 0.8-2.6 mm long; outer phyllaries usually cuneate, puberulous, glandular, usually recurved, vinaceous, usually 2.2-3.3 mm long; florets 6-15; cypselae usually setuliferous, glandular; pappus usually 4-5.3 mm long, bristles ca. 36-41.
<i>C. gentianoides</i> (Clusters 2 and A)	Stems glabrous, eglandular; leaves opposite, elliptic or oblanceolate, apices usually acute to obtuse, bases attenuate, glabrous and eglandular on both surfaces.	Axes usually glabrous; peduncles 3.5-8 mm long; outer phyllaries attenuate, glabrous, eglandular, erect, vinaceous, 2.9-4 mm long; florets 17-21; cypselae glabrous or glabrescent and eglandular; pappus 4.7-5.6 mm long, bristles ca. 33-40.
<i>C. hirsuta</i> (Clusters 6 and D)	Stems usually hirsute to villose, glandular or not; leaves usually alternate (rare opposite), usually deltate to lanceolate, apices usually acute to attenuate, bases usually truncated, mostly hirsute to villose on both surfaces, glandular only on the abaxial surface.	Axes mostly puberulous to hirsute; peduncles usually longer than ca. 2 mm; outer phyllaries commonly acute, apiculate or cuneate, puberulous to tomentose, mostly recurved, vinaceous, 1.8-2.8 mm long; florets 6-14; cypselae usually setuliferous and glandular; pappus 3.8-5.7 mm long, bristles ca. 30-44.
<i>C. latisquamulosa</i> (part of clusters 3 and B)	Stems mostly strigose to glabrescent, eglandular; leaves opposite, orbicular to ovate, apices and bases usually cuneate to rounded, mostly glabrous to glabrescent on both surfaces, eglandular on both surfaces.	Axes usually strigose; peduncles 1.8-2.6 mm long; outer phyllaries acuminate to cuneate, glabrescent or puberulous, eglandular, mostly erect, vinaceous, 1.9-2.6 mm long; florets 10-13; cypselae usually setuliferous, eglandular; pappus 3.6-4.9 mm long, bristles ca. 27-36.
<i>C. rhinanthacea</i> (part of clusters 3 and B)	Stems mostly glabrous, eglandular; leaves opposite, usually elliptic to oblanceolate, apices acute to obtuse, bases acute to attenuate, glabrous and eglandular on both surfaces.	Axes mostly strigose; peduncles usually 1.7-3.2 mm long; outer phyllaries acuminate to cuneate, glabrous or glabrescent, eglandular, mostly erect, vinaceous, 2-3.2 mm long; florets 7-11; cypselae setuliferous, eglandular; pappus usually 4-4.9 mm long, bristles ca. 28-37.
<i>C. squarrolosa</i> (Clusters 1, 5 and G)	Stems mostly sericeous, glandular or not; leaves opposite, usually ovate, apices usually acute, bases usually truncate to rounded, commonly sericeous and glandular on both surfaces.	Axes sericeous to tomentose; capitula sessile or peduncles up to 2 cm long; outer phyllaries usually cuneate, puberulous to tomentose, glandular, recurved to squarrose, vinaceous to cinereous, 2.2-3.3 mm long; florets 9-30; cypselae mostly setuliferous and eglandular; pappus 4.3-5.3 mm long, bristles ca. 32-53.





**Figure 6.** Dendrogram produced through Cluster Analysis of the reduced dataset. Clusters are indicated through black bars positioned to the left of the image and named according to the most prominent species included on it. Species are identified through colors indicated on the image itself, while individual specimens are identified by a specific code.

morphological variation between them: *C. ascendens* (0.31), *C. congesta* (0.35), *C. elliptica* (0.34), *C. latisquamulosa* (0.34), and *C. rhinanthacea* (0.30).

The scatterplot produced through PCoA of the complete dataset (Fig. 7A) presents the specimens in two distinct major groups: one to the left including all specimens assigned to *C. ascendens*, *C. caaguazuensis*, *C. congesta*, *C. hirsuta* and *C. squarrolosa*, as well as most specimens included in *C. elliptica* and undetermined specimens; and one to the right, including all specimens identified as *C. gentianoides*, *C. latisquamulosa* and *C. rhinanthacea*, as well as few undetermined specimens. and one specimen of *C. elliptica*.

In the scatterplot of the reduced dataset (Fig. 7B), four major groups can be defined: one consisting of *C. squarrolosa* (top), one composed of *C. hirsuta* (bottom), one including *C. ascendens*, *C. caaguazuensis*, *C. congesta* and *C. elliptica* (middle) and another one consisting of *C. gentianoides*, *C. latisquamulosa* and *C. rhinanthacea* (right).

### Linear Discriminant Analysis

The complete results of the LDA conducted with all 17 quantitative morphological traits are shown in Table 3. The traits with the highest values - and thus considered as the most important numerical variables evaluated - were last internode length (17.4), leaf length (10.8) and leaf width (9.5). Conversely, cypselas length (1.7), cypselas width (1.5) and inner phyllary width (1.1) were considered as the less informative variables due to their low F-values. The variables with the highest tolerance value were peduncle length (0.7), cypselas length (0.6) and first internode length (0.6).

## Discussion

### Species delimitation and recognition

The morphological circumscriptions of each species of the *C. congesta* complex according to our analyses are presented in Table 2. Specific discussions on the morphological boundaries of each species are presented below. Commentaries on the identity and possible treatments of undetermined specimens are made immediately afterwards.

#### *Chromolaena ascendens* and *C. xylorhiza*

*Chromolaena ascendens* was long treated as a synonym of *C. squarrolosa* (Cabrera *et al.* 1996; Freire & Ariza Espinar 2014; Perez 2009). According to the groups observed in our analyses, *C. ascendens* could be recognized by the morphological traits indicated in Table 2, particularly stems and abaxial surfaces of the leaves villose (Fig. 3J), as well as the tendency of having more than 13 florets in each capitulum, smaller cypselas and fewer than 30 pappus' bristles (Fig. 8E-H). These traits, particularly the

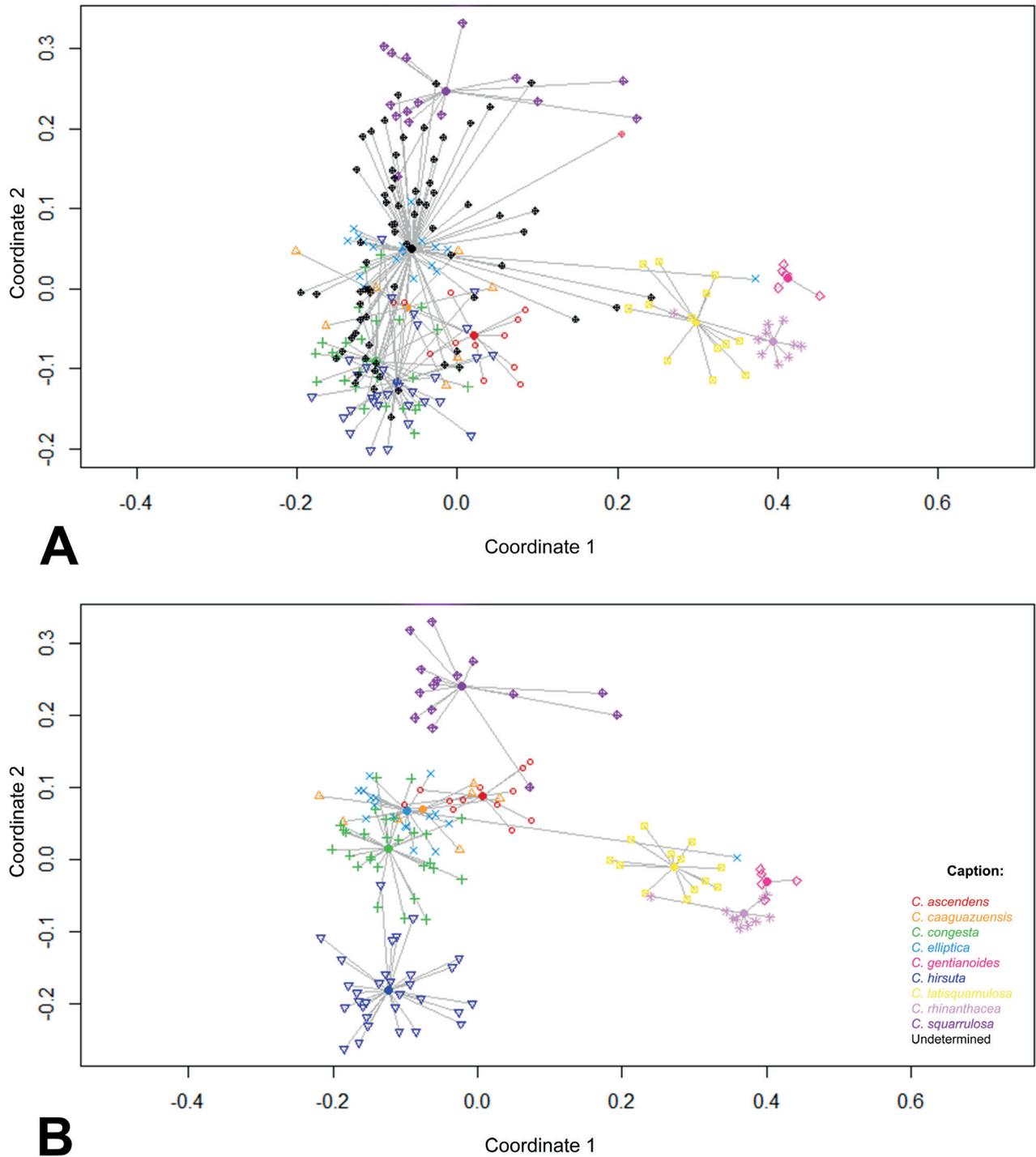
villose indumentum of the stems and leaves, have not been observed in populations of *C. congesta* (which has also been treated as a synonym of *C. squarrolosa*) and *C. squarrolosa*. It is also particularly interesting that all populations with this set of traits occur exclusively in the Brazilian states of São Paulo, Minas Gerais and Rio de Janeiro (Fig. 1), in localities where neither *C. congesta* and *C. squarrolosa* occur. These particularities support the decision of Christ & Rebouças (2020) to recognize *C. ascendens* as distinct from *C. congesta* and *C. squarrolosa* and explain why authors from Argentina, Paraguay and Uruguay (*e.g.* Cabrera *et al.* 1996; Freire & Ariza Espinar 2014; Perez 2009) tend to overlook the morphological distinctions of these species and place *C. ascendens* in synonymy.

The biggest issue with the delimitation of *C. ascendens* is *C. xylorhiza*: both species are practically identical and would be differentiated from each other by the indumentum of the cypselas (Christ & Rebouças 2020) or the presence or absence of peduncles (Baker 1876). Both traits, however, are not constant: most specimens analyzed present sessile capitula, a trait associated with *C. xylorhiza* according to Baker (1876), while the indumentum of the cypselas varies between glabrous (characteristic of *C. ascendens*) and setuliferous (characteristic of *C. xylorhiza*). In addition, most specimens with overall similarity to either *C. ascendens* or *C. xylorhiza* possess glabrescent cypselas, which further complicated the issue of assigning one of these names to them. Thus, the only specimen included in our analyses that could be undoubtedly identified as *C. xylorhiza* is CXY1, which consists of one of the syntypes of its basionym. Since there are no stable morphological traits that differentiate these taxa and since CXY1 did group close to the specimens assigned to *C. ascendens* in both dendrograms, it is possible that *C. xylorhiza* would be more accurately treated as a synonym of *C. ascendens* than as a separate species.

#### *Chromolaena caaguazuensis*

Half of the specimens attributed to *C. caaguazuensis* did not cluster together in the dendrograms produced, while the other half composes clusters 4 (Fig. 4) and C (Fig. 6). The dispersion of the species in the scatterplots connects it to *C. ascendens*, *C. congesta* and *C. elliptica*, and even in a subjective manner it is difficult to identify useful traits to delimitate it, even though the analyzed specimens tended to possess longer pappus and with more bristles than the remaining species (Fig. 8G-H). *Chromolaena caaguazuensis* has been usually identified through the combination of pendant leaves with cordate bases and capitula with usually less than five florets (Hieronymus 1897; Cabrera *et al.* 1996; Freire & Ariza Espinar 2014; Perez 2009), but these traits were not sufficient to delimitate this species in our analyses. This result could be a product of undersampling, as few specimens of *C. caaguazuensis* were available to be included, and further research has to be conducted in order to investigate its identity and circumscription.





**Figure 7.** Scatterplots of the Principal Coordinate Analyses of the complete (**A**) and the reduced dataset (**B**). The full-colored circles in the middle of each network indicate the position of the average value of variation of each species (centroids), with each specimen being positioned relative to it throughout the scatterplot. Dots positioned nearby to each other are thus more similar to each other than to dots positioned further away. Furthermore, dots placed near the centroid of their respective group present similar values of variation to it. Species are identified through colors indicated on the image itself. In the complete scatterplot, the first two axes accounted for 12% and 7% of the variation, respectively; in the reduced scatterplot, the first two axes accounted for 14% and 7% of the variation, respectively.

**Morphometric studies suggest taxonomic changes in  
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**Table 3.** Results of the Linear Discriminant Analysis of 17 quantitative traits. The F-values presented indicate how well each variable contribute to the discrimination between the species, while the value of tolerance indicates how independent each variable is from one another. Characters with high F-value (higher than 4) are presented in bold.

Variable	F-statistics	Tolerance
Stem, first internode length	3.390	0.617
<b>Stem, last internode length</b>	<b>17.442</b>	<b>0.569</b>
<b>Leaf, length</b>	<b>10.812</b>	<b>0.608</b>
<b>Leaf, width</b>	<b>9.575</b>	<b>0.235</b>
<b>Peduncle, length</b>	<b>6.422</b>	<b>0.723</b>
Involucre, length	3.000	0.245
Involucre, width	2.973	0.390
Phyllaries, number	2.869	0.392
<b>Outer phyllaries, length</b>	<b>6.394</b>	<b>0.409</b>
<b>Outer phyllaries, width</b>	<b>5.146</b>	<b>0.539</b>
Inner phyllaries, length	2.033	0.354
Inner phyllaries, width	1.119	0.431
<b>Florets, number</b>	<b>4.621</b>	<b>0.275</b>
Cypselas, length	1.696	0.612
Cypselas, width	1.505	0.447
Pappus, length	2.498	0.380
Pappus, number of bristles	3.181	0.523

#### *Chromolaena congesta*

This species appears intertwined or at least closely connected to *C. elliptica* in all conducted analyses. This is supported by many morphological traits common to both taxa, including the overall indumentum of the stems and leaves (which mostly vary from puberulous to strigose), the number of florets and the presence of glandular hairs in phyllaries and cypselae. These species have been mostly told apart by the shape of leaves (deltate with truncate bases in *C. congesta* and elliptic with cuneate bases in *C. elliptica*) and the habit of the plant, which is usually erect in *C. congesta* (Fig. 2A) and usually procumbent in *C. elliptica* (Fig. 2B) (Barroso 1950; Matzenbacher 1979; Christ & Ritter 2019; Christ & Rebouças 2020).

The close connections of *C. congesta* and *C. elliptica* indicate a need for further in-depth studies focusing on their relationship, such as phylogeographic studies and population genetics, since both commonly co-occur at least in the Brazilian states of Santa Catarina and Rio Grande do Sul and could be producing natural hybrids. Our results, however, suggest there are morphological differences between these species and indicate further morphological traits that could be informative to circumscribe them.

Regarding *C. congesta*, we found out that this species should be re-circumscribed to include only the specimens with morphological traits consistent with the ones included in cluster 9A. These specimens tend to possess deltate leaves with emergent veins on the abaxial surface, usually strigose

stems and leaves (Fig. 3G) and apiculate outer phyllaries (Fig. 3B) mostly shorter than 2 mm long. The shape and length of the outer phyllaries have never before been identified as a useful trait to differentiate *C. congesta* and *C. elliptica*, and is a novelty uncovered by our analyses.

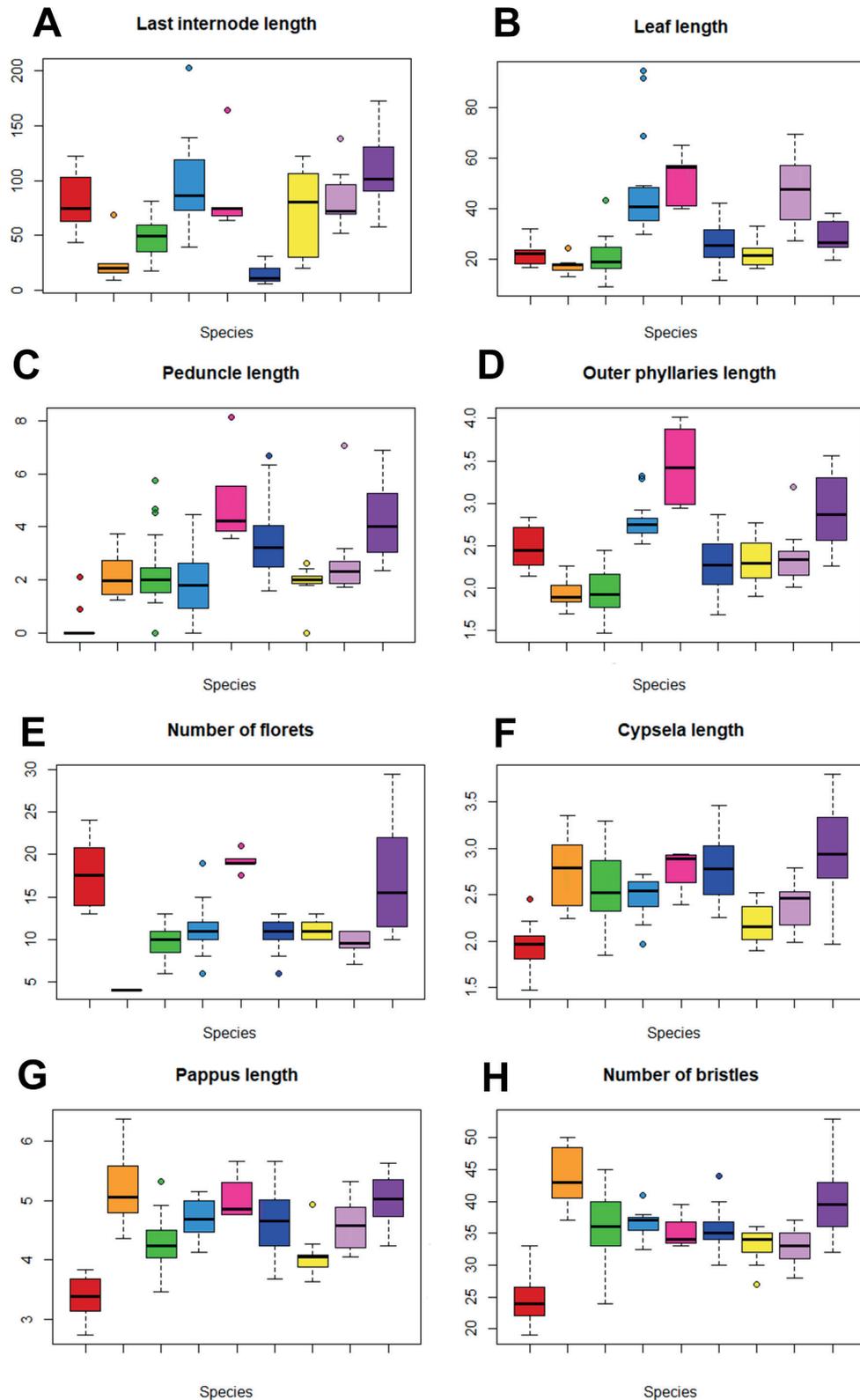
#### *Chromolaena elliptica*

As mentioned above, this species shares morphological traits with *C. congesta* and appears connected to it in all analyses conducted. Despite their intertwined relationship indicated by cluster E (Fig. 6), *C. elliptica* and *C. congesta* cluster separately in Fig. 5 - respectively in cluster 8 and cluster 9 - and possess particular morphological tendencies. While further research should be conducted to clarify the relationship between them, we found out that *C. elliptica* should be treated according to the morphological traits of cluster 8B. This cluster is characterized mostly by leaves usually elliptic and cuneate (or obtuse) outer phyllaries (Fig. 3C), usually longer than 2.5 mm long.

#### *Chromolaena gentianoides*

All specimens of *C. gentianoides* consistently group together in all analyses (cluster 2 - Fig. 4; cluster A - Fig. 6). This species is similar to *C. rhinanthacea* in having glabrous stems and leaves, while also typically lacking glandular hairs much like *C. latisquamulosa* and *C. rhinanthacea*. *Chromolaena gentianoides* is unique among the species of the complex due to its characteristic involucre and





**Figure 8.** Boxplot representations of some of the numerical traits discussed in the text. **A.** Last internode length. **B.** Leaf length. **C.** Peduncle length. **D.** Outer phyllaries length. **E.** Number of florets. **F.** Cypsela length. **G.** Pappus length. **H.** Number of bristles. Species are presented alphabetically from left to right and also identified through colors: red = *C. ascendens*; orange = *C. caaguazuensis*; green = *C. congesta*; cyan = *C. elliptica*; magenta = *C. gentianoides*; blue = *C. hirsuta*; yellow = *C. latisquamulosa*; pink = *C. rhinanthacea*; purple = *C. squarrosula*. Undetermined specimens and *C. xylorhiza* weren't included in these representations due to not corresponding to a single taxon and due to being represented solely by one specimen, respectively.

## Morphometric studies suggest taxonomic changes in a species complex in *Chromolaena* (Asteraceae, Eupatorieae, Praxelinae)

tendency of having longer peduncles (3.5–8 mm long) and outer phyllaries (2.9–4 mm long), and is also notable for possessing overall more florets in its capitula (17–21) (Fig. 8) than related species.

### *Chromolaena hirsuta*

This species has been traditionally identified based on its distinctive alternate leaves at least on the upper half of the stems (Barroso 1950; Matzenbacher 1979; Cabrera *et al.* 1996; Perez 2009; Christ & Rebouças 2020) (Fig. 2D), a trait exclusive to it in the complex and not very common in the species of *Chromolaena* found in southeastern South America (Christ & Rebouças 2020). This trait, however, is subject to variations, as specimens or populations of *C. hirsuta* with opposite leaves have been spotted and collected throughout its distribution (Fig. 2E), and this variation has been accepted by some authors (*e.g.* Christ & Ritter 2019; Perez 2009; Christ & Rebouças 2020). Historically, specimens of *C. hirsuta* with opposite leaves have been more than often misidentified as *C. congesta*, to the point where Malme (1933) have suggested that both species could be producing hybrid offsprings with intermediate traits between them. The difficulty of dealing with these specimens and the apparent close morphological relationship between both species has led some authors to attribute a varietal level to *C. hirsuta* in relation to *C. congesta* (*e.g.* Matzenbacher 1979; Cabrera & Klein 1989). However, our results do not support the interpretation that these species would be particularly morphologically close: despite the existence of misplaced specimens between them in the complete dendrogram (Figs. 4 and 5) and the proximity of a few specimens in the reduced scatterplot (Fig. 7B), both species tend to form distinct groups from one another. Though it is reasonable to think both species could be producing natural hybrids that would hamper their delimitations, our results suggest that *C. hirsuta* could be accurately delimited and distinguished from *C. congesta* through the morphological traits mentioned in Table 2.

### *Chromolaena latisquamulosa*

This species is morphologically closer to *C. rhinanthacea*, with which it shares cluster 3 (Fig. 4) and cluster B (Fig. 6). Despite this scenario, both species form different groups in the scatterplots (Fig. 7) and can be differentiated through specific morphological traits: *Chromolaena latisquamulosa* differs from *C. rhinanthacea* based on the indument of stems, which is usually strigose to glabrescent or puberulous; shape of the leaves, which is mostly orbicular to ovate, with bases mostly rounded on the latter; length of the leaves (shorter than 2.8 cm) and length of the pappus (shorter than 4 mm). We have observed variations in the indumentum of the stems and axes, however, with some specimens identified as *C. latisquamulosa* (*e.g.* CLA6, CLA7, CLA9 and CLA10) being characterized by tomentose to villose stems and axes. These distinctions may explain why these specimens

did not group together with the remaining specimens of *C. latisquamulosa*. Until further studies are conducted to better clarify the circumscription of this species, we suggest these variations in indument should be treated as exceptions of the typical indument of *C. latisquamulosa*, which would be strigose stems and axes.

### *Chromolaena rhinanthacea*

As noted above, this species is close to *C. latisquamulosa*, with which it shares clusters 3 (Fig. 4) and B (Fig. 6), while also possessing morphological similarities with *C. gentianoides* (*i.e.* glabrous stems and leaves). *Chromolaena rhinanthacea* can be differentiated from *C. latisquamulosa* mostly based on the glabrous indument of the stems; shape of the leaves, which are elliptic to oblanceolate, with bases acute to attenuate; length of the leaves (mostly longer than 3.5 cm long) and length of the pappus (usually longer than 4 mm long).

### *Chromolaena squarrolosa*

While both scatterplots (Fig. 7) and the reduced dendrogram (Fig. 6) clearly portray this species as a distinct entity from the remaining complex, the complete dendrogram fragments the specimens in two clusters with no immediate relationship, with the principal morphological discontinuity between them being the indumentum of the cypselae, which tends to be glabrous on cluster 1 and setuliferous on cluster 5. One hypothesis to explain the scenario recovered in the complete dendrogram is the possible interference of noise in the analysis (Kaur *et al.* 2010). In any case, the odd partition of *C. squarrolosa* in this dendrogram echoes its position as the species with the highest variation observed in the PERMDISP test.

This species has been often identified by its characteristic cinereous, squarrose outer phyllaries (Figs. 2H and 3D) and tendency of having longer peduncles (usually longer than 4–5 mm long) and more florets (usually more than 20) in its capitula (Fig. 8). Most specimens identified as *C. squarrolosa* also tend to possess sericeous stems and leaves and more than 15 florets, traits uncommon in other species of the complex. However, specimens and populations with sparser indumentum (Fig. 2J) and fewer florets are common, as well as specimens with capitula with increasingly less-squarrose outer phyllaries (Fig. 2I), which tend to eventually connect the “typical” interpretation of *C. squarrolosa* to the concept of *Eupatorium ascendens* var. *parcisetosum* B.L.Rob.. This variety is usually treated as a synonym of *C. squarrolosa* (Cabrera *et al.* 1996; Freire & Ariza Espinar 2014; Christ & Ritter 2019), despite presenting discontinuities in many morphological traits, including shape and indument of the outer phyllaries.

In the complete cluster dendrogram, almost all undetermined specimens which have been tentatively assigned to *E. ascendens* var. *parcisetosum* prior to the final analyses grouped together with *C. squarrolosa* in cluster 5,



as well as most undetermined specimens interpreted as possessing intermediary morphological traits between *C. squarrulosa* and *C. congesta*, which tend to possess recurved and vinaceous outer phyllaries. While these results support the inclusion of *E. ascendens* var. *parcisetosum* in the synonymy of *C. squarrulosa*, they also seem to indicate that this species should have an expanded morphological concept to include specimens and populations without the characteristic squarrose outer phyllaries. In this way, *C. squarrulosa* should be treated as a highly polymorphic species and circumscribed based on the morphological traits of clusters 1 and 5 (Table 2).

#### Undetermined specimens

As expected, undetermined specimens spread through the complete dendrogram and scatterplot, usually grouping with species with which they show some degree of morphological affinity. Undetermined specimens consist primarily of “atypical” representatives of the species of the complex, that is, specimens with overall morphological similarity to a particular species, but with important differences from the type specimen. In this case, it would be reasonable to modify the taxa's concepts to better allocate these specimens, which would in consequence demand a reinterpretation of their morphological boundaries. This scenario was discussed in the specific concepts of each species above, and is, for example, the case of the undetermined specimens grouped with *C. hirsuta* in cluster 6 (Fig. 4), like CSP50 and CSP63, which differ from “typical” *C. hirsuta* based only on the opposite phyllotaxy. In this case, the concept of the species should be expanded to also accommodate specimens with opposite leaves along the entire stem. Thus, “atypical” specimens should be regarded as morphological variations of already existing taxa and identified as the most similar species possible.

In other scenarios, undetermined specimens bear morphological traits associated with more than a single species, which leads us to informally label them as “intermediary”. Examples of this scenario include many specimens in cluster 5 (Fig. 4), which are interpreted as intermediaries between *C. squarrulosa* and *C. congesta*; and the undetermined specimens included among *C. latisquamulosa* in cluster 3, which are seen as intermediaries between this species and *C. congesta*. While tempting, an assertive interpretation of these specimens as hybrids, however, should not be based solely on morphology (Rieseberg 1995) and would depend on more concrete evidence from cytology, molecular data or reproductive biology. We could also hypothesize that these specimens, rather than hybrids, are evidence of two (or more) different lineages of currently differentiating species that are not yet completely morphologically independent or simply specimens retaining morphological traits from an ancestral lineage (Schilling 2011). As such, intermediary specimens

need to be investigated with more depth before taxonomic decisions can be made regarding their identity, and we suggest these specimens should be treated as undetermined until then.

Apart from these specimens, two distinct clusters composed mostly or exclusively of undetermined specimens with similar morphological traits were recovered in the Cluster Analysis: clusters 8C and 9B. They will be discussed based on their denominations according to this dendrogram (Fig. 5).

Cluster 8C includes mostly specimens similar to the type specimens and original protologue of *Eupatorium caaguazuense* var. *nervosum* Chodat (Figs. 2F-G). This taxon was described by Chodat (1902) and is usually treated as a synonym of *C. squarrulosa* (Freire & Ariza Espinar 2014; Christ & Ritter 2019). However, this treatment seems to be erroneous, considering both species show different morphological traits: *E. caaguazuense* var. *nervosum* differs from *C. squarrulosa* due to the hirsute to villose indumentum of the stem and leaves (Fig. 3I), the prominent venation of the abaxial surface of the leaves and the absence of cinereous, squarrose outer involucral bracts (Fig. 2F). It is interesting that most specimens bearing morphological traits fitting with this variety grouped in a distinct cluster and were found to not be particularly morphologically similar to neither *C. caaguazuensis* nor *C. squarrulosa*. Considering this taxon was never treated as a separate species before and that the name was never combined to *Chromolaena*, our results point towards a new recombination of this name and its recognition as a distinct species.

Cluster 9B (with the exceptions of CSP18 and CSP23) contains a remarkable collection of specimens superficially similar to specimens grouped in cluster 9A (which is understood as being composed of “typical” representatives of *C. congesta*, as discussed above). The most noticeable morphological traits that distinguish these clusters are the sericeous indument of the stems and adaxial surfaces of the leaves and the lanate abaxial surfaces of the leaves (Fig. 3H), traits not observed in specimens of *C. congesta*. The indumentum of the abaxial surface of the leaves is particularly interesting because it is unique among the populations of the *C. congesta* complex. All specimens included in cluster 9B were collected in highland grasslands areas from Southern Brazil (specially in Paraná) and São Paulo, and thus possess a common preference of habitats, while “typical” specimens of *C. congesta* (cluster 9A) tend to occur further south, mostly in the Brazilian states of Rio Grande do Sul and Santa Catarina. The Cluster Analysis indicates that, despite being somewhat related to *C. congesta*, these specimens are morphologically closer to one another than to the remaining specimens assigned to this species. Considering that there is a diagnostic set of traits that can be easily and objectively used to differentiate between these clusters, it seems reasonable that cluster 9B could subsidize a newly described taxon.



### Morphological traits

The results of the LDA indicate that the last internode length, leaf length and leaf width are particularly informative in this group of species and could be used to both delimitate and identify some of them. Species with longer last internodes tend to be procumbent subshrubs, while the ones with shorter last internodes are usually erect subshrubs. The finding that this trait is the most informative numerical variable reinforces the notion that species like *C. congesta* (which is usually erect) (Fig. 2A) and *C. elliptica* (Fig. 2B) (which is usually procumbent) could be differentiated based on their growing habits (Fig. 8A). While aspects of the leaves such as shape and size are influenced by environmental factors such as the availability of light or water (e.g. Xu *et al.* 2008; 2009), our findings suggest these traits, especially the leaf length (Fig. 8B), which has a higher tolerance value than leaf width (Table 3) could still be useful in the taxonomy of this group, particularly to aid the disentangling of *C. elliptica* and *C. congesta*.

Other potentially informative numerical traits include peduncle length, number of florets and length of the outer phyllaries, which also scored significant values in the LDA and show different tendencies among the species (Fig. 8C-E). The peduncle length is especially notorious for scoring the highest tolerance value of the evaluated traits (Table 3), meaning it is less correlated to other variables and thus more independent. This trait is useful not only to support the identification of *C. ascendens*, which tends to present sessile capitula (Fig. 8C), but also to further reinforce the differentiation of *C. gentianoides* and *C. squarrolosa*, which tend to present longer peduncles. Despite not scoring significant values in the LDA, cypsel length, pappus length and the number of pappus bristles also seem to be useful numerical traits to at least recognize *C. ascendens* and differentiate it from similar species, since it tends to possess smaller cypselae and pappus bristles, as well as fewer bristles than species usually confused with it, such as *C. congesta* and *C. latisquamulosa* (Fig. 8F-H). All these traits, however, show variations among the analyzed specimens, and we were only able to identify the different tendencies through graphical visualizations of traits scored through multiple specimens, which means that they probably would not be useful in dichotomous keys or to identify single specimens.

The significance of the categorical variables, on the other hand, is more difficult to assert, considering this type of trait tends to be more subjective and that we could not carry on a similar analysis to the LDA conducted with the numerical variables. Despite many of these traits, particularly vegetative ones, being subject to environmental influences and variations among different individuals (e.g. Xu *et al.* 2008; Dumini & Di Michele 2009; Stuessy 2009), we observed that patterns of variation and morphological tendencies do exist among the species.

Finally, despite our attempt of using an extensive set of traits, there are still a few potentially informative traits that were not included in our analyses and that could be further analyzed in the future. These include the habit of the plant, the position of the leaf blade in relation to the petioles (*i.e.* pendant, patent or ascendant) and the folding of the leaf blade in relation to its median vein (*i.e.* folded or not). Floral “microcharacters” (as labeled by King & Robinson 1987) relative to corollas, stamens and styles were also not evaluated in our study, but according to Grossi *et al.* (2020), these traits could prove informative at least at the generic level, possibly also at specific level, especially in genera known to possess high interspecific variations on these traits, such as *Chromolaena*. Thereby, the evaluation of these floral traits could also be useful in future studies, especially if confronted with other lines of evidence.

### Applicability of morphological species concepts and usefulness of morphology

The variation of many of the traits used to study this species complex indicates that combinations of morphological traits are more useful than simply using few diagnostic traits alone. A phenetic species concept (Sokal & Crovello 1970) could be applied to at least some of the species of the complex, as they comprehend different clusters of individuals with different combinations of traits, which were evaluated through multivariate analyses. This scenario, allied to the overall visualization of the data provided by the scatterplots and the major clusters recovered in CA, support the maintenance of at least six species: *C. ascendens*, *C. gentianoides*, *C. hirsuta* and *C. squarrolosa* are all supported by the cluster analysis, while *C. latisquamulosa* and *C. rhinanthacea*, despite clustering together in the dendrograms, are shown to be separate taxa in the scatterplots. These species can all be circumscribed based on traits indicated in Table 2. Our results also indicate that there are morphological tendencies particular to both *C. congesta* and *C. elliptica*, despite their close connections, and that these species should be re-circumscribed according to the morphological traits of clusters 9A and 8B (Table 2), respectively. However, as previously suspected, the species of the *C. congesta* complex cannot be delimited based exclusively on “typical” specimens and without tolerance on the variation of some morphological traits, including some that have previously been treated as diagnostic by different authors.

One important factor behind the difficult taxonomy of species in the *C. congesta* complex is the prevalence of “atypical” and “intermediary” specimens and populations, which limits the usefulness of morphology in circumscribing and identifying species. This is evidenced by the much clearer separation of the species in the reduced Cluster Analysis (Fig. 6). Thus, we reinforce the view of de Queiroz (2005) that species should be circumscribed based on a unified concept produced through the convergence of different



lines of evidence. We expect that the results of other studies included in this project will contribute to the delimitation and understanding of these species, and will also help us to better understand who these “atypical” and “intermediary” specimens are and how we should deal with them from a taxonomic point of view.

Despite the challenges imposed by the adoption of morphology as a method for delimiting species, our results reinforce the exclusion of *C. latisquamulosa* from the synonymy of *C. elliptica*, which was proposed by Freire & Ariza Espinar (2014); and of *C. ascendens* and *C. congesta* from the synonymy of *C. squarrulosa*, a treatment used by several authors, mostly from outside Brazil (e.g. Cabrera *et al.* 1996; Freire & Ariza Espinar 2014; Perez 2009). These synonymies were previously refuted by Christ & Ritter (2019) based on classic taxonomy and are now reinforced with statistical support. Lastly, our results add up to studies like Grossi & Katinas (2013), Viera Barreto *et al.* (2018), Rodríguez-Craverro *et al.* (2019) and Grossi *et al.* (2020) in the reinforcement of the perception that a morphological interpretation of species and genera within Eupatorieae is challenging and that further studies should be conducted on this topic to elucidate the significance of certain traits and their overall usefulness in the taxonomy and systematics of the tribe itself.

## Conclusion

Despite the presence of different morphological tendencies and the applicability of a phenetic species concept to some species included in our study, we found out that morphological traits have only a limited use in the resolution of taxonomic problems in the *C. congesta* complex. This is mainly due to noticeable variations in practically all traits included in our study, including many previously considered to be diagnostic of some species. Morphological data, however, should not be overlooked nor discarded when attempting to circumscribe or identify species. A unified species concept should be pursued and morphology should be understood only as one of many tools to be used in order to access the diversity of this species complex and to produce a more stable and clear classification. Therefore, we expect that the results of molecular and cytogenetic studies that are currently being conducted with this species complex will help us to better understand the relations among these species and will also aid in identifying useful morphological traits to differentiate them, which will in turn contribute to the development of a more robust taxonomic treatment of this species complex.

Concerning the specific objectives of our study, we found out that: 1) six of the ten species of the *C. congesta* complex could be recognized based on a phenetic species concept: *Chromolaena ascendens*, *C. gentianoides*, *C. hirsuta*, *C. latisquamulosa*, *C. rhinanthacea* and *C. squarrulosa*.

On the other hand, *C. xylorhiza* seems to be identical to *C. ascendens*, while *C. congesta* and *C. elliptica* should be re-circumscribed based on clusters 9A and 8B, respectively; 2) many undetermined specimens could be included in modified morphological concepts of some species, but two taxonomic novelties were suggested and are being described, including a new combination and a new species; 3) while most traits evaluated present some degree of variation among the analyzed species, some of them portray patterns of variation and could be used to support the circumscription and identification of some taxa; these include indumentum of stems and leaves, phyllotaxy, shape of the leaves, presence of glandular hairs, shape of the outer phyllaries' apices, last internode length, leaf length, peduncle length, outer phyllaries length and number of florets; and 4) morphometrics are useful for detecting patterns of variation and testing existing classifications, but morphological data should be approached carefully and in conjunction with other lines of evidence, which will be available in the near future.

## Supplementary material

The following online material is available for this article:

**Table S1** - Relations of traits and states of traits computed for each specimen analyzed. Traits and states of traits are identified by their numbers as assigned in Table 1. Specimens are assigned to codes (combinations of letters as numbers), which in turn are used to identify them in the dendrograms presented. Full information on each specimen is available in Table S2.

**Table S2** - Detailed voucher information for each specimen analyzed. Specimens are assigned to codes (combinations of letters as numbers), which in turn are used to identify them in the dendrograms presented.

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