Linear and geometric morphometrics as tools to resolve species circumscription in the *Pseudolaelia vellozicola* complex (Orchidaceae, Laeliinae)

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Background and aims – *Pseudolaelia* is a genus endemic to eastern Brazil, with 12 accepted species predominantly distributed across granitic inselbergs of the Brazilian Atlantic Forest. The aim of the present study was to distinguish between the very similar taxa *P. aguadocensis*, *P. oliveirana*, *P. regentii* and *P. vellozicola*, using morphometric data acquired as linear measurements and outlines capture with Elliptic Fourier Analysis (EFA) of the floral parts.
Material and methods – We sampled 208 specimens from 11 natural populations of the above taxa. We measured 20 floral variables and for the EFA, and we extracted 24 shape variables from the Fourier coefficient matrices, which describe the outlines of the floral parts. In both cases the data were analyzed with multivariate methods (both ordination and clustering).
Key results – We could not find morphological discontinuities with sufficient magnitude to consider *P. aguadocensis*, *P. oliveirana* and *P. regentii* as distinct species from *P. vellozicola*.
Conclusions – We propose that *P. vellozicola* should be considered a polymorphic and widely distributed species, generally supported by both methods.

Key words – Elliptic Fourier Analyis, geometric morphometrics, inselbergs, linear morphometrics, *Pseudolaelia aguadocensis*, *Pseudolaelia oliveirana*, *Pseudolaelia regentii*, *Pseudolaelia vellozicola*, taxonomy.

INTRODUCTION

Morphological analyses through numerical methods are important for taxonomy as they may assist greatly in the evaluation of morphological variation and to circumscription of taxa (Rohlf 1990, Henderson 2006, Pinheiro & Barros 2007). Their objectivity when dealing with the data is a great advantage, especially if many variables are available (Manly 1994).

However, relatively few studies have been carried out with Orchidaceae in relation to the size of the family. Studies about temperate or paleotropical species are more common (e.g. Bateman & Farrington 1989, Tyteca & Gathoye 1993, Peakall et al. 1997, Shaw 1998, Goldman et al. 2004, Bernardos et al. 2005, Gardiner et al. 2005, Sosa & De Luna 2005, Shipunov et al. 2004, 2005, Shipunov & Bateman 2005, Ponsie et al. 2009, Simo-Droissart et al. 2013, 2016, Tsiftsis 2016). Such work is seldom conducted with Neo-

tropical species (e.g. Cardim et al. 2001, Carlini-Garcia et al. 2002, Sosa & De Luna 2005, Pinheiro & Barros 2007, 2009, Ribeiro et al. 2008, Borba et al. 2002, 2007). Even fewer studies have been conducted using geometric morphometrics (Kores et al. 1993, Shipunov & Bateman 2005), even though this tool has been successfully applied to study other plant families (e.g. Premoli 1996, McLellan 2000, Volkova & Shipunov 2007, Andrade et al. 2008, 2010, Fritsch et al. 2009, Magrini & Scoppola 2010), allowing a separate assessment of organ size and organ shape.

Pseudolaelia Porto & Brade (Orchidaceae, Laeliinae) is a genus endemic to eastern Brazil, which includes 12 accepted species (Menini Neto et al. 2013). The species occur predominantly as epiphytes on Velloziaceae distributed along granitic inselbergs of the Brazilian Atlantic Forest and quartzite outcrops of the Cerrado and Caatinga *campos rupestres* in the states of Bahia, Espírito Santo, Minas Gerais

and Rio de Janeiro. The majority of species have the vegetative morphology quite homogeneous, usually varying only the size of the plants, but some species present strong intra- and interpopulation floral polymorphism (Menini Neto 2011, Menini Neto et al. 2013).

This polymorphism is found in *P. vellozicola* (Hoehne) Porto & Brade populations, which is also the most widely distributed species (see Menini Neto et al. 2013), raising uncertainty regarding its delimitation and differentiation from some very similar taxa recently described, viz. *P. regentii* V.P.Castro & Marçal (Castro Neto & Marçal 2007), *P. oliveirana* V.P.Castro & Chiron (Castro Neto & Chiron 2009), and *P. aguadocensis* Campacci (Campacci 2016). The first two names have been previously synonymized as part of a systematic study of the genus (Menini Neto 2011, Menini Neto et al. 2013). They are mostly based on strongly variable flower characteristics as: flower size, lip isthmus shape, apex
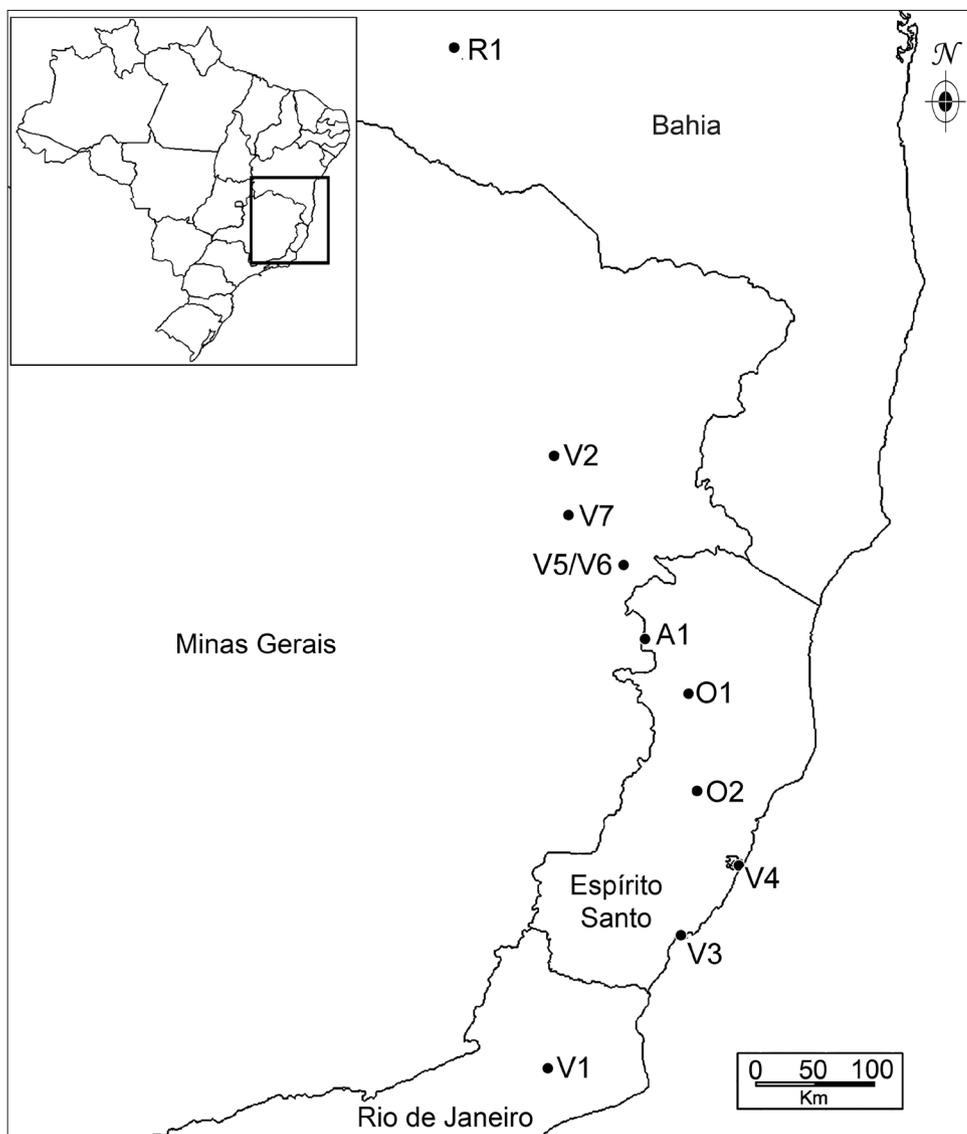
Figure 1 – Location of populations sampled in this study. Populations: R1, Caetité; V2, Caraí; V7, Itaipé; V5/V6, Ataléia (1 and 2); A1, Água Doce do Norte; O1, Águia Branca; O2, Marilândia; V4, Vitória; V3, Piúma; V1, Campos dos Goytacazes. The codes correspond to the populations of original delimitation of *P. aguadocensis* (A), *P. oliveirana* (O), *P. regentii* (R), and *P. vellozicola* (V).

Table 1 – **Populations of *P. vellozicola* complex used in this study.**
State acronyms: BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro. Herbaria acronyms according to Thiers (continuously updated). Populations corresponding to the original delimitation of *P. aguadocensis* (A), *P. oliveirana* (O), *P. regentii* (R), *P. vellozicola* (V).

Code	Populations	Sample size	Altitude (m.s.m.)	Voucher
A1	ES – Água Doce do Norte	22	610	*Fontana* 5053 (MBML)
O1	ES – Águia Branca	20	360	*Fontana* 5040 (MBML)
O2	ES – Marilândia	9	770	*Magnago* 804 (MBML)
R1	BA – Caetité	16	970	*van den Berg* 1915 (HUEFS)
V1	RJ – Campos dos Goytacazes	33	50	*Menini Neto* 520 (CESJ, RB)
V2	MG – Caraí	7	740	*Kollmann* 11746 (MBML)
V3	ES – Piúma	25	100	*Fernandes* s.n. (CESJ 57157)
V4	ES – Vitória	30	50	*Costa* s.n. (CESJ 57158)
V5	MG – Ataléia	20	330	*Menini Neto* 754 (CESJ, RB)
V6	MG – Ataléia	20	280	*Menini Neto* 755 (CESJ, RB)
V7	MG – Itaipé	7	770	*Kollmann* 11734 (MBML)
Total		208		

of median lobe of the lip, width and length of sepals and petals, among others (see electronic appendix 1).

Hence, the present study aimed to test the hypothesis of wide morphological variability of *P. vellozicola* (of which *P. aguadocensis* would also be considered conspecific, along with *P. oliveirana* and *P. regentii*) due to its relative isolation. We also aimed to contribute to understanding the morphological evolution of the plant populations of inselberg, which present an insular distribution. For this, we used an approach often applied in animals and rarely in plants, by integrating morphometrics through linear measurements and Elliptical Fourier Analysis of perianth parts, believing that both methods can contribute in different ways in the answer of the arisen question about the polymorphism of *P. vellozicola*.

MATERIALS AND METHODS

Sampling and specimen preparation

We sampled 208 specimens belonging to eleven populations of the *P. vellozicola* complex (table 1, fig. 1). Sampling encompassed the entire area of distribution of these taxa and is formed by a population from Bahia (related to *P. regentii* and considered endemic to this state); three populations from northwestern Espírito Santo (of which one is related to *P. aguadocensis*, and two are related to *P. oliveirana*), plus seven other populations from Minas Gerais, the coastal region of Espírito Santo and Rio de Janeiro. Figure 2 shows five selected lips of each population, representing part of the observed variation.

We used only flowers in the analyses because the taxa proposed (Castro Neto & Marçal 2007, Castro Neto & Chiron 2009, Campacci 2016) were mainly separated from *P. vellozicola* based on floral features, and the vegetative features in this complex are quite homogeneous (Menini Neto et al. 2013). Materials were collected randomly and sample sizes varied according to the availability of flowers and size of the

populations in the different localities. The flowers were collected from different individuals in the populations and preserved in 70% alcohol for further analysis. In the laboratory, they were dissected, and sepals, petals, and lip were mounted on paper sheets (named floral cards) and scanned at 600 dots per inch on a flatbed scanner.

Linear measurements

Measurements of perianth parts were made from the digitized images with the help of Image J open access software (Image Processing Analysis in Java, available at http://rsbweb.nih.gov/ij/). Twenty quantitative floral variables were measured (table 2, fig. 3) and were accurate to three decimal places.

Two ordination methods were applied to investigate possible distortions between them (Everitt 1978): a Principal Components Analysis (PCA), as an exploratory method for analyzing the general structure of the data and detect outliers (without distinguishing between groups) and a Canonical Variate Analysis (CVA), with the populations used as categorical variables. In the PCA, the number of informative axes was defined by comparing the eigenvalues to chance in a broken-stick distribution (Frontier 1976). We also conducted a cluster analysis, using the Mahalanobis Generalized Distance obtained from the CVA, and using Neighbor-Joining as clustering algorithm. Multivariate analyses were performed using the PAST version 2.04 software (Hammer 2010), Neighbor-Joining (NJ) was performed in PAUP version 4.0 (Swofford 2003), and trees figures generated with FigTree version1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Elliptical Fourier analysis (EFA)

The parts of perianth on the digitized floral cards were separated and the resulting images were edited to enhance contrast and sharpness, suppressing the background to attain a

well-defined outline of each piece. For symmetrical parts (petals and lateral sepals), only the left side of the flower was used. Two reference points (landmarks) were positioned, one at the apex and one at the base of each piece. Next, the outline of each was scanned to extract the set of *xy* coordinates with the tpsDIG software, version 2.12 (Rohlf 2008) and recorded in a ".tps" file for subsequent processing.

These coordinates were imported into the Morpheus et al. software (Slice 2008), which was used to conduct the EFA. Sepals and petals were converted into 20 harmonics and the lip was converted into 30 harmonics, sufficient for reconstruction of their outlines, resulting in arrays of 84 and 124 Fourier coefficients, respectively. The number of harmonics was chosen based on a previously-conducted test through sequential addition until digital reconstruction of the perianth parts outlines were judged by eye as completely reconstructing the shape of each one. The harmonics define the ellipses that estimate the shape's contour and each of them consist

of four values, accounting for the size, angulation and displacement of starting point of an ellipse (Lestrel 1997, Elewa 2010). The first four coefficients of each array were discarded, as they correspond to the zero-th harmonic. The contours were standardized in the Morpheus et al. software (Slice 2008) using the *bookstein superimposition* command, which uses the two previously defined landmarks. This command requires that the orientation, scale, location and starting point settings for the scanning of the floral parts be turned off.

A PCA was conducted for the matrix of Fourier coefficients of each floral part using the Fitopac software, version 1.64 (Shepherd 2006). The first six components obtained represented the variation in outline shape of each of the floral parts, which were reconstructed (Yoshioka et al. 2004), using the "Fourplot" module in the NTSYSpc software, version 2.1 (Rohlf 2000), which redraws the outlines through the set of Fourier coefficients. These contours show the changes expressed independently by each of the major components
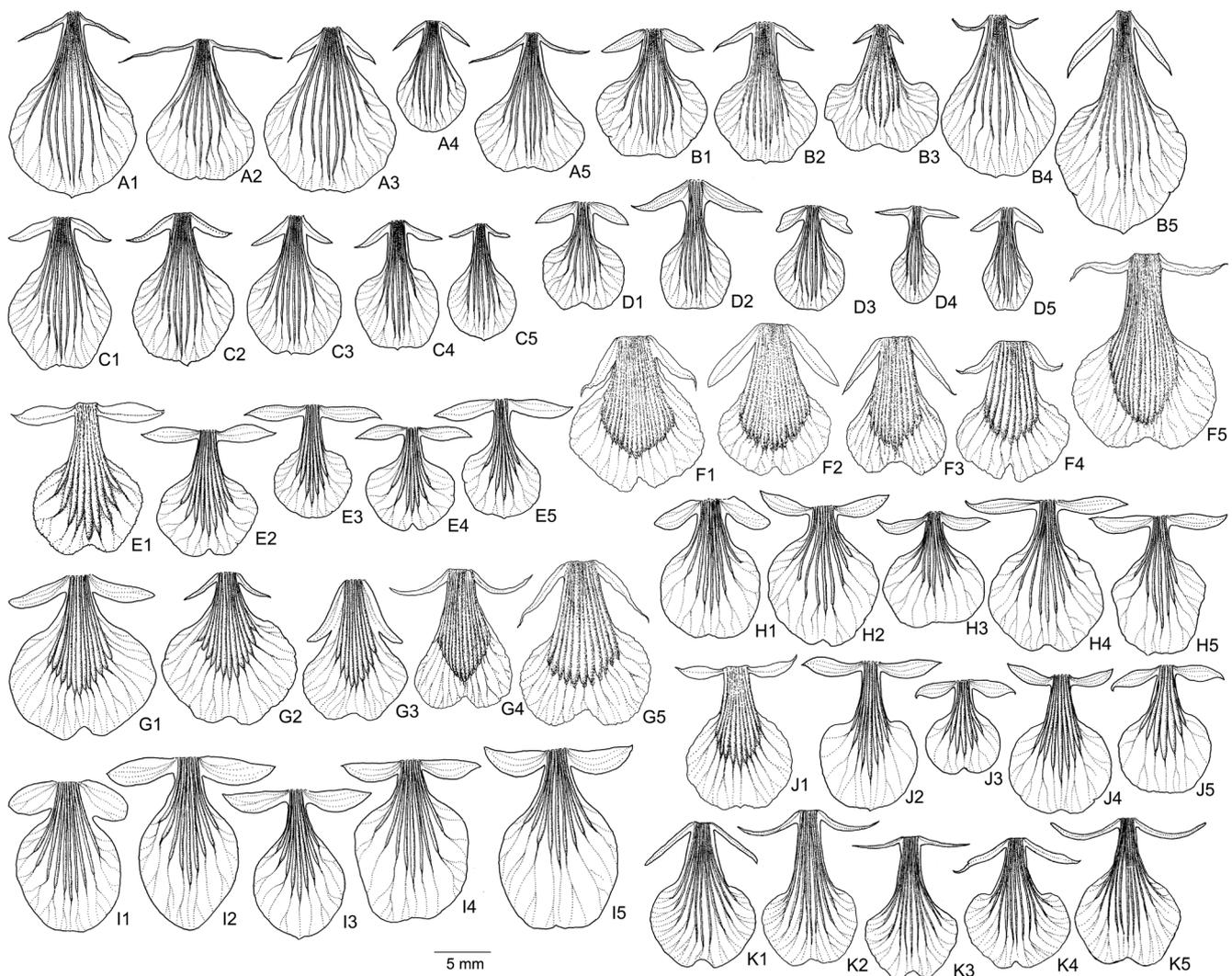
Figure 2 – Selected lips of the flowers showing inter and intrapopulational variation: A1–A5, Águia Branca (O1); B1–B5, Água Doce do Norte (A1); C1–C5, Marilândia (O2); D1–D5, Caetité (R1); E1–E5, Caraí (V2); F1–F5, Ataléia 1 (V5); G1–G5, Ataléia 2 (V6); H1–H5, Vitória (V4); I1–I5, Piúma (V3); J1–J5, Campos dos Goytacazes (V1); K1–K5, Itaipé (V7). The codes in parentheses correspond to the populations of original delimitation of *P. oliveirana* (O), *P. regentii* (R), and *P. vellozicola* (V).

Table 2 – Floral measurements used as variables in the linear morphometric analysis and results of PCA (PC1 and PC2) and CVA (axis 1 and axis 2).
The five most important (largest) values for each component/axis are in bold.

Code	Variable	PC1	PC2	Axis1	Axis2
1. ll/ds	longest length of dorsal sepal	**0.504**	-0.168	-0.017	**-0.044**
2. wid25/ds	width of dorsal sepal taken at 25% of its length	0.042	**0.279**	0.028	-0.009
3. wid50/ds	width of dorsal sepal taken at 50% of its length	0.057	0.234	0.024	-0.012
4. wid75/ds	width of dorsal sepal taken at 75% of its length	0.036	0.145	0.021	-0.006
5. ll/pet	longest length of petal	**0.546**	-0.158	-0.014	**-0.052**
6. wid25/pet	width of petal taken at 25% of its length	0.029	0.221	0.023	-0.014
7. wid50/pet	width of petal taken at 50% of its length	0.060	**0.291**	**0.031**	-0.022
8. wid75/pet	width of petal taken at 75% of its length	0.069	0.219	0.029	-0.023
9. ll/ls	longest length of lateral sepal	**0.521**	-0.233	-0.029	**-0.057**
10. wid25/ls	width of lateral sepal taken at 25% of its length	0.044	**0.317**	**0.036**	-0.003
11. wid50/ls	width of lateral sepal taken at 50% of its length	0.057	0.252	**0.032**	-0.008
12. wid75/ls	width of lateral sepal taken at 75% of its length	0.033	0.151	0.027	0.002
13. lm/lip	lip median length	**0.252**	**0.269**	0.010	-0.021
14. llw/lip	longest width in the lateral lobes of lip	0.129	**0.436**	**0.055**	**0.037**
15. lmw/lip	longest width of median lobe of lip	0.170	0.121	**0.039**	**-0.047**
16. lem/lip	length of median lobe of lip	**0.191**	0.228	0.017	-0.034
17. lel/lip	length of lateral lobe of lip	0.054	0.087	0.013	0.027
18. lwl/lip	longest width of lateral lobe of lip	0.007	0.134	0.018	0.011
19. iw/Iip	width of lip isthmus	0.048	-0.111	-0.016	0.009
20. il/lip	length of lip isthmus	0.036	0.008	0.010	-0.016

shown in figs 4 & 5 (which show the changes of -2, -1, +1 and +2 standard deviations), and remove the effect of the size of flower parts (Andrade et al. 2008, 2010).

The first six principal components then were obtained from the lip, petal, sepal and dorsal side were used to assemble a matrix of 24 columns and 208 rows. A CVA was performed with the data from this matrix, using the population as a categorical variable. We also conducted a cluster analysis with the same methods used for the linear measurements (Mahalanobis Distance and NJ).

Combined analysis

For a combined analysis of linear measurement and shape data, we centered and standardized the linear measurements, and prepared a concatenated matrix by adding the first five PCA scores from each organ. Then we used this matrix for CVA and cluster analyses, in an analogous manner to the separate analyses. Additionally, we performed a Discriminant Analysis (DA) with specimens grouped according to the population they belong. Wilks' Lambda and jackknife classifications were calculated in order to assign unclassified individual to groups.

RESULTS

Linear measurements

For PCA, only the first two axes were considered informative, as these were the only ones displaying a non-random pattern, obtained by the broken-stick null model for the distribution of eigenvalues (values not shown). These first two axes explain 69.58% and 13.53% of the total variation, respectively (electronic appendix 2). In this analysis individuals from the sampled populations tended to cluster, with some exceptions (e.g. Água Doce do Norte and Águia Branca), whose individuals were widely scattered in the multivariate space, showing great variability. However, all populations overlapped on both axes, with no clear separation between them. The five most important values for component 1 are: longest length of dorsal sepal, longest length of petal, longest length of lateral sepal, lip median length, length of median lobe of lip. The five most important values for component 2 are: width of dorsal sepal taken at 25% of its length, width of lateral sepal taken at 25% of its length, width of lateral sepal taken at 50% of its length, lip median length, longest width in the lateral lobes of lip (table 2).

The first two axes of the CVA (fig. 6) represent 48.62% and 14.45% of the total variance, respectively. There was some tendency towards grouping, but an overlap of individuals in almost all populations can also be observed. Axis 1 reveals a tendency for populations closest to the coast and populations in lower elevations (represented by Piúma, Vitória and Campos dos Goytacazes) to separate from other populations further inland which are generally at higher locations. On axis 2, only the population of Águia Branca stands out, with strong overlap between individuals from the other sampled populations. The five most important values in axis 1 are width of petal taken at 50% of its length, width of lateral sepal taken at 25% of its length, width of lateral sepal taken at 50% of its length, longest width in the lateral lobe of lip, longest width of median lobe of lip. The five most important values in axis 2 are longest length of dorsal sepal, longest length of petal, longest length of lateral sepal, longest width in the lateral lobes of lip, longest width of median lobe of lip (table 2).

The cluster analysis (fig. 7A) indicated three general groups. In the first, O1, O2, and A1 are related to V5, V6 and

V7. The coastal populations of *P. vellozicola* (V1, V3 and V4) are related and quite divergent among themselves, and V2 is related to R1.

Elliptical Fourier analysis

The first six principal components (PCs) obtained with PCA represent between 89% and 98% of the total variance. For the sepals and petals, PCs 1, 4 and 6 represent asymmetric modifications and PCs 2, 3 and 5 are symmetrical changes, while for the lip, asymmetric modifications are represented by PCs 1, 2 and 3 and symmetrical modifications are represented by PCs 4, 5 and 6.

For CVA, the first two axes explain 47.4% and 21.6% of the total variance, respectively (fig. 8), and a strong morphological overlap between the populations sampled in both axes is observed, showing a tendency of the Campos de Goytacazes population to separate from the rest only in axis 1. The vectors in figure 8 represent the variables that account for the differences between populations, the most important of these being PC4sd, PC4sl, PC4pet, PC3lab, and PC4lab.

The cluster analysis (fig. 7B) grouped the populations in a similar manner as the linear measurements. Based on shape data, V1, V3, and V4 are still related and divergent, as well as V2 and R1, while A1 grouped together with V5, V6, and V7, being O1 and O2 in another branch.

Combined analysis

The combination of linear measurements and shape data from EFA provided a better discrimination of the groups. Axis 1 of the CVA (fig. 9) indicated a clear separation of the coastal populations of *P. vellozicola* (V1, V3 and V4) from the other populations. Axis 2, in turn, separated another two populations (V5 and R1). However, the main morphological patterns of separation of these populations do not correspond to what was proposed for the circumscription of the other taxa, and in fact, there is broad overlap of the populations of *P. oliveirana* (O1, O2, and O3) with populations of *P. vellozicola* (V5, V6 and V7), and *P. regentii* (V2, R1).

In the cluster analysis of the combined data (fig. 7C), the results have some differences in comparison with the linear measurements and EFA, especially in the position of V2, V3, and R1, also differing in the distance values.

Table 3 presents the classification matrix produced by DA, using populations as categorical variable, and the average percentage of correct classification was 69% (Wilks' Lambda = 0.001001, p < 0.001). The coastal populations show the highest percentage of correct classification (94% for Campos dos Goytacazes, 92% for Piúma and 90% for Vitória), but all populations present some divergent placement of specimens. Specimens of Água Doce do Norte (*P. aguadocensis*), Caetité (*P. regentii*) and Itaipé (*P. vellozicola*) showed deviating position, and were mixed among four populations of different species. The same happened with specimens of Águia Branca (*P. oliveirana*) and Ataléia B (*P. vellozicola*), showing divergent placement of specimens in three populations of different species. It is possible to observe that specimens of all species present some degree of divergent placement among the individuals of different populations/species.
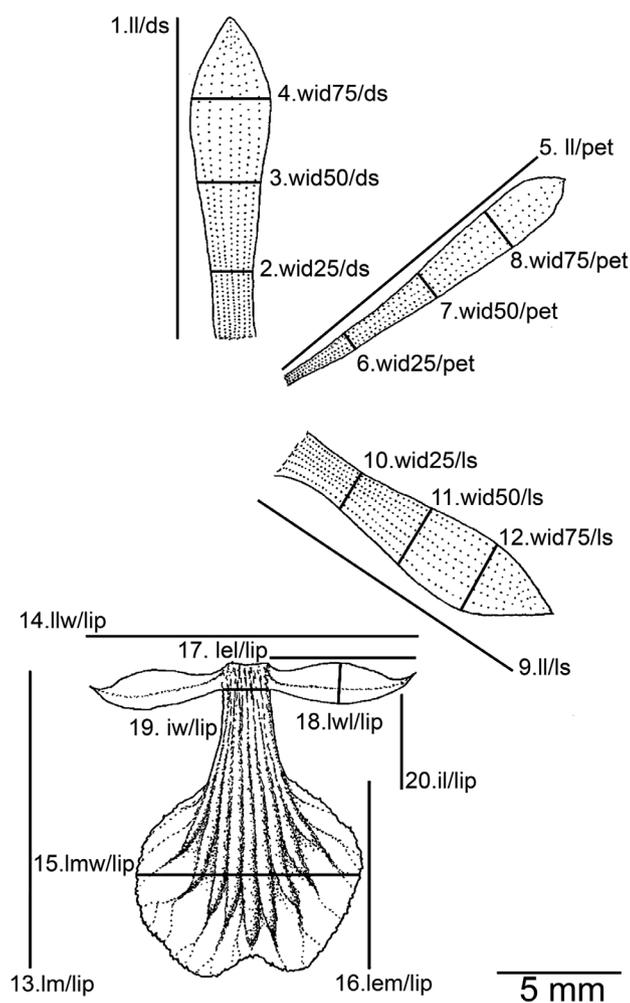

Figure 3 – Floral parts with variables measured in the linear morphometric analysis. (see table 2 for variable codes).

DISCUSSION

The integrated linear and EFA morphometric analyses, to evaluate the hypothesis if *P. vellozicola* complex must be considered as a highly polymorphic species, presented some lack of agreement, but, in general, showed overlap between the populations of the four taxa (*P. aguadocensis*, *P. oliveirana*, *P. regentii* and *P. vellozicola*).

The PCA and CVA analyses yielded very similar results in linear measurements, with strong overlap between individuals from different populations, even though populations of the recognized species mostly remained grouped together. The strong overlap between different populations, regardless of geographical location, stands out in the PCA analysis. Although in this type of analysis the first axis often reflects
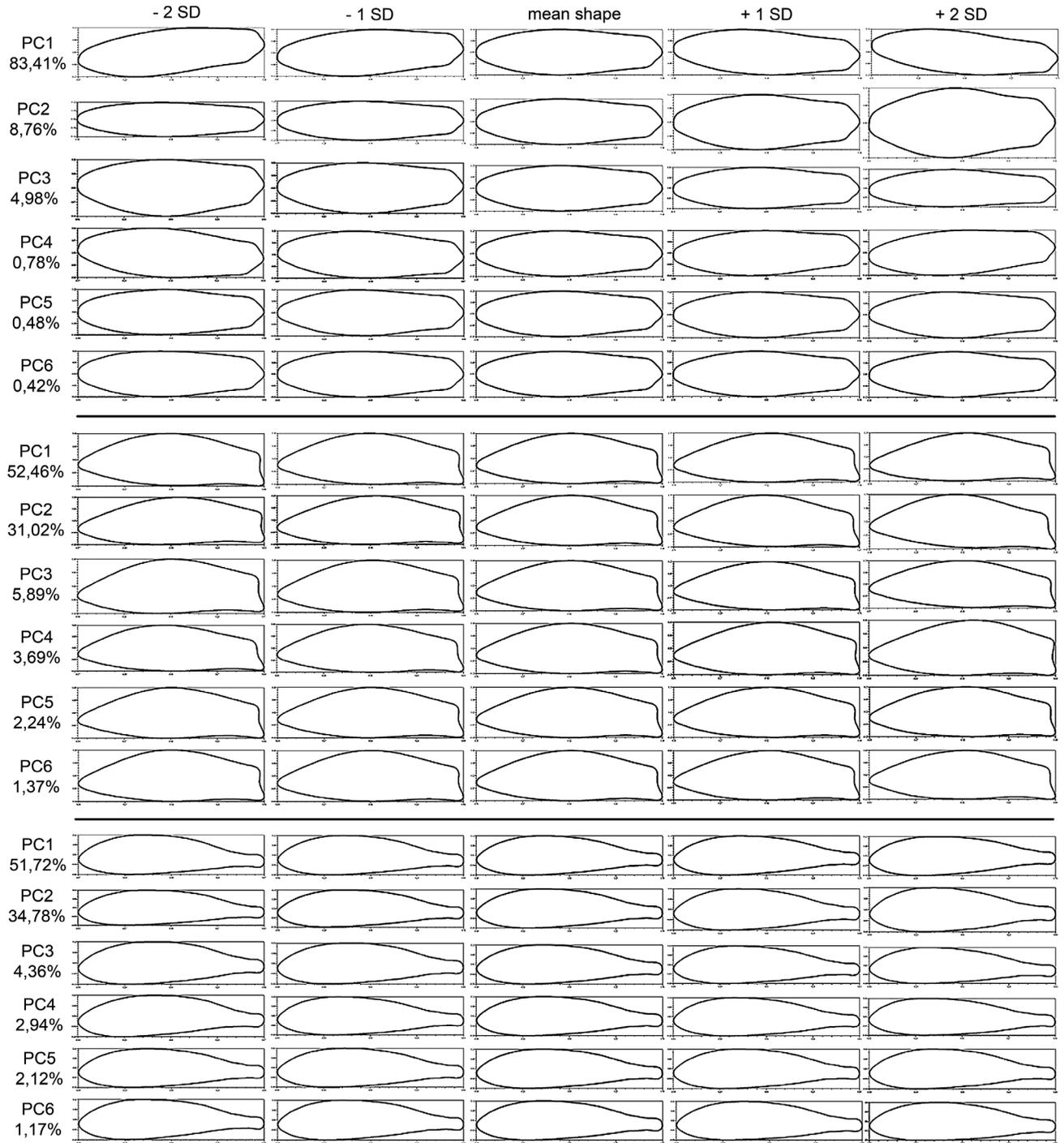
Figure 4 – Reconstructions of six first principal components as shape variables of dorsal sepal, lateral sepal and petal, based on PCA of the floral parts outline.
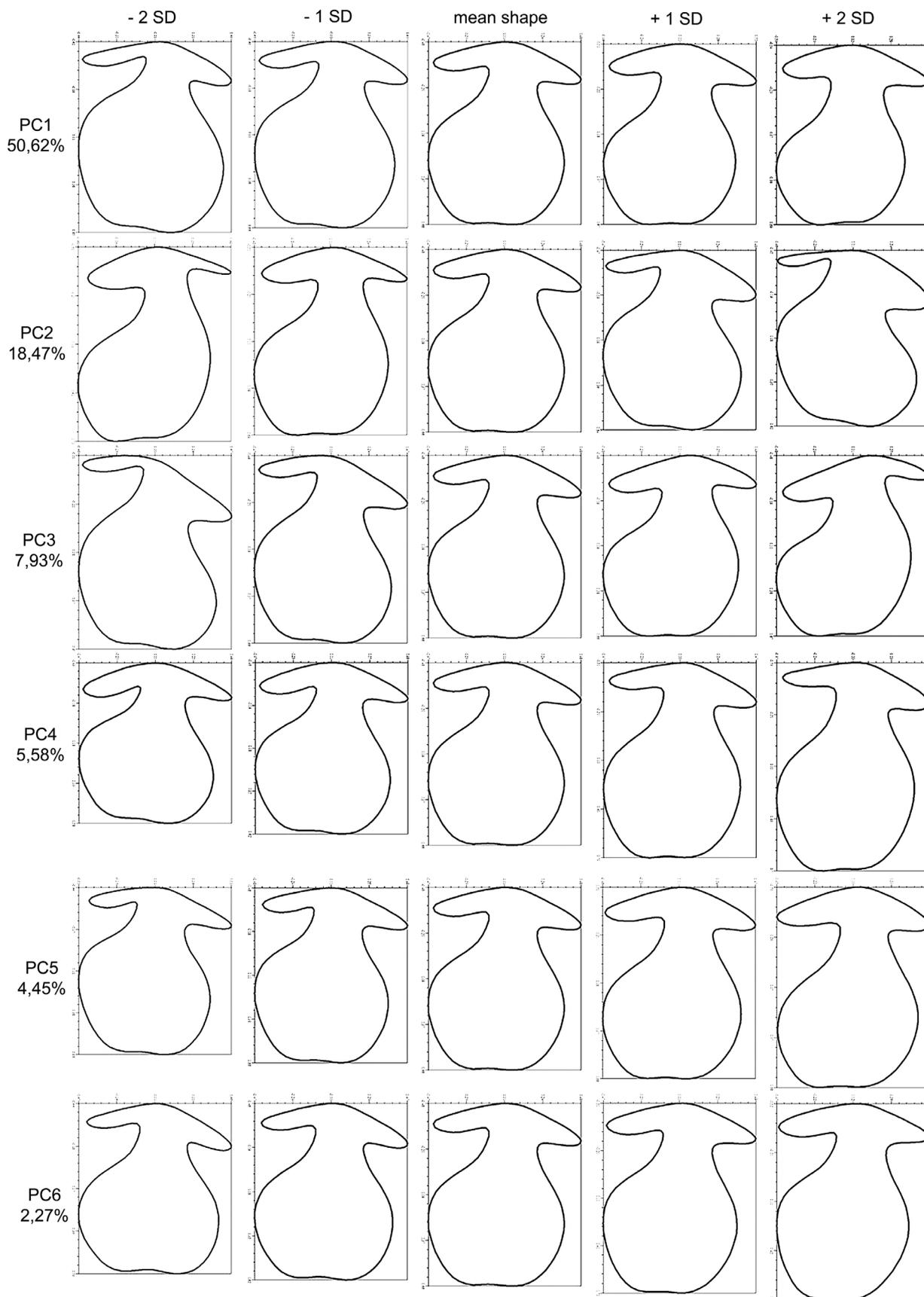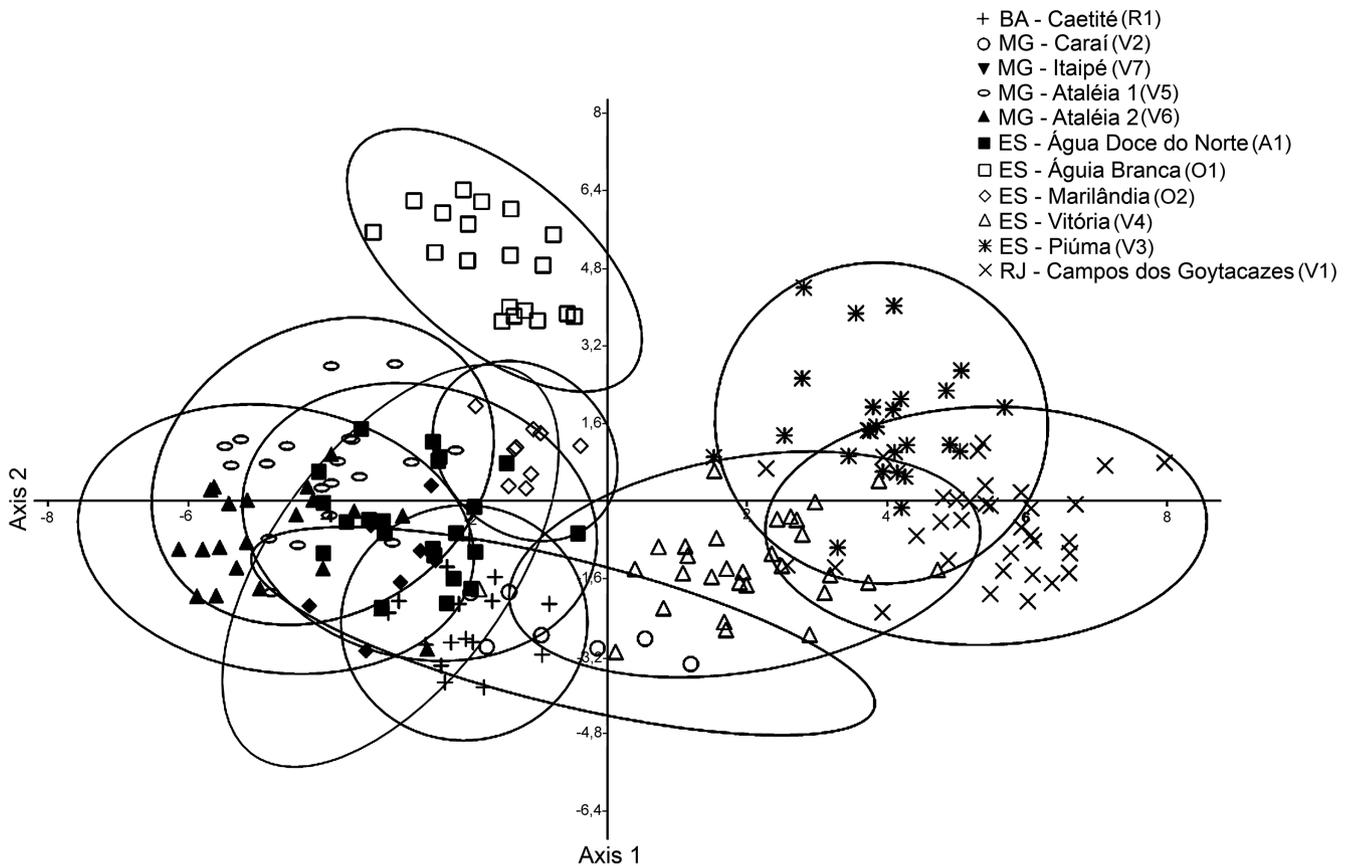
Figure 5 – Reconstructions of six first principal components as shape variables of lip, based on PCA of the floral parts outline.

Table 3 – Classification of the specimens in the discriminant analysis of combined characters (linear and geometric) in 11 populations of the *Pseudolaelia vellozicola* complex. Wilks' lambda = 0.001001, *p* < 0.001.
Populations (Pops.): R1, Caetité (BA); O1, Águia Branca (ES); O2, Marilândia (ES); A1, Água Doce do Norte (ES); V1, Campos dos Goytacazes (RJ); V2, Caraí (MG); V3, Piúma (ES); V4, Vitória (ES); V5, Ataléia 1 (MG); V6, Ataléia 2 (MG); V7, Itaipé (MG). See caption of table 1 for State acronyms.

Pops.	% correct	*P. regentii*	*P. oliveirana*		*P. aguadocensis*	*P. vellozicola*						
		R1	O1	O2	A1	V1	V2	V3	V4	V5	V6	V7
A1	94	–	–	–	–	30	–	–	2	–	–	–
O1	50	–	10	3	6	–	–	1	–	–	–	–
O2	68	–	4	1	15	1	–	–	1	–	–	–
R1	75	12	–	–	1	1	–	–	1	–	1	–
V1	71	–	–	–	–	-	5	–	–	1	–	1
V2	56	–	–	5	3	1	–	–	–	–	–	–
V3	92	–	1	–	1	–	–	23	–	–	–	–
V4	90	–	–	–	–	1	–	–	27	2	–	–
V5	65	–	–	–	1	–	–	–	–	13	6	–
V6	50	–	–	–	–	–	1	–	2	7	10	–
V7	43	1	1	–	–	–	–	1	–	1	–	3
Average	69	–	–	–	–	–	–	–	–	–	–	–
Total	–	13	16	9	27	34	6	25	33	24	17	4

+ BA - Caetité (R1)
○ MG - Caraí (V2)
▼ MG - Itaipé (V7)
◦ MG - Ataléia 1 (V5)
▲ MG - Ataléia 2 (V6)
■ ES - Água Doce do Norte (A1)
□ ES - Águia Branca (O1)
◇ ES - Marilândia (O2)
△ ES - Vitória (V4)
✳ ES - Piúma (V3)
✕ RJ - Campos dos Goytacazes (V1)

Figure 6 – CVA of linear measurements of 208 specimens of the *P. vellozicola* complex, based on 20 floral variables (fig. 3, table 2). Axes 1 and 2 represent 51.62% and 14.65% of the total variation, respectively. The ellipses are centered in the sample average and comprise 95% of individuals of each population. The codes between parentheses correspond to the populations of original delimitation of *P. aguadocensis* (A), *P. oliveirana* (O), *P. regentii* (R), and *P. vellozicola* (V).
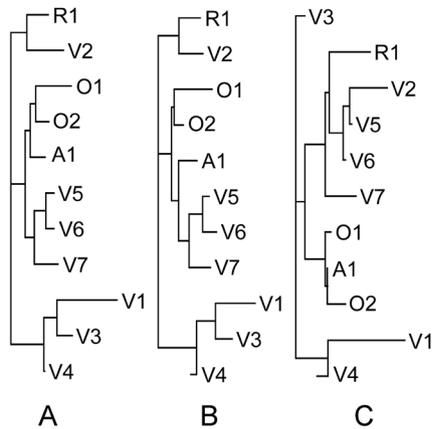
Figure 7 – Dendrogram of phenetic relationships between specimens of populations of the *P. vellozicola* complex, based on 20 floral variables (fig. 3, table 2). A, linear measurements; B, Elliptical Fourier Analysis; C, combined analysis.

the size of the individuals (Peres-Neto 1995), there was an overlap of individuals with flowers of very different sizes. The trend of clustering observed among coastal populations, separating themselves from the inland populations is also observed in the NJ. This tendency towards separation between montane/inland populations and coastal ones was also found for *Actinocephalus polyanthus* (Bong.) Sano (Eriocaulaceae) (Trovó et al. 2008). Given that the environments in which these populations live are similar (inselbergs), it is possible that the proximity of the coast and the altitude explain the observed morphological differences. Similar results regarding elevation gradient and/or inland/coastal distribution were also found, for instance, in *Silene maritima* With. (Caryophyllaceae) (Baker & Dalby 1980), *Mimulus guttatus* Fisch. ex DC. (Phrymaceae) (Lowry et al. 2008), and *Solidago virgaurea* L. (Asteraceae) (Nishizawa 2001, Kiełtyk & Mirek 2014) reinforcing the obtained results.

Castro Neto & Chiron (2009: 22), when describing *P. oliveirana* from material obtained from Pancas (north-
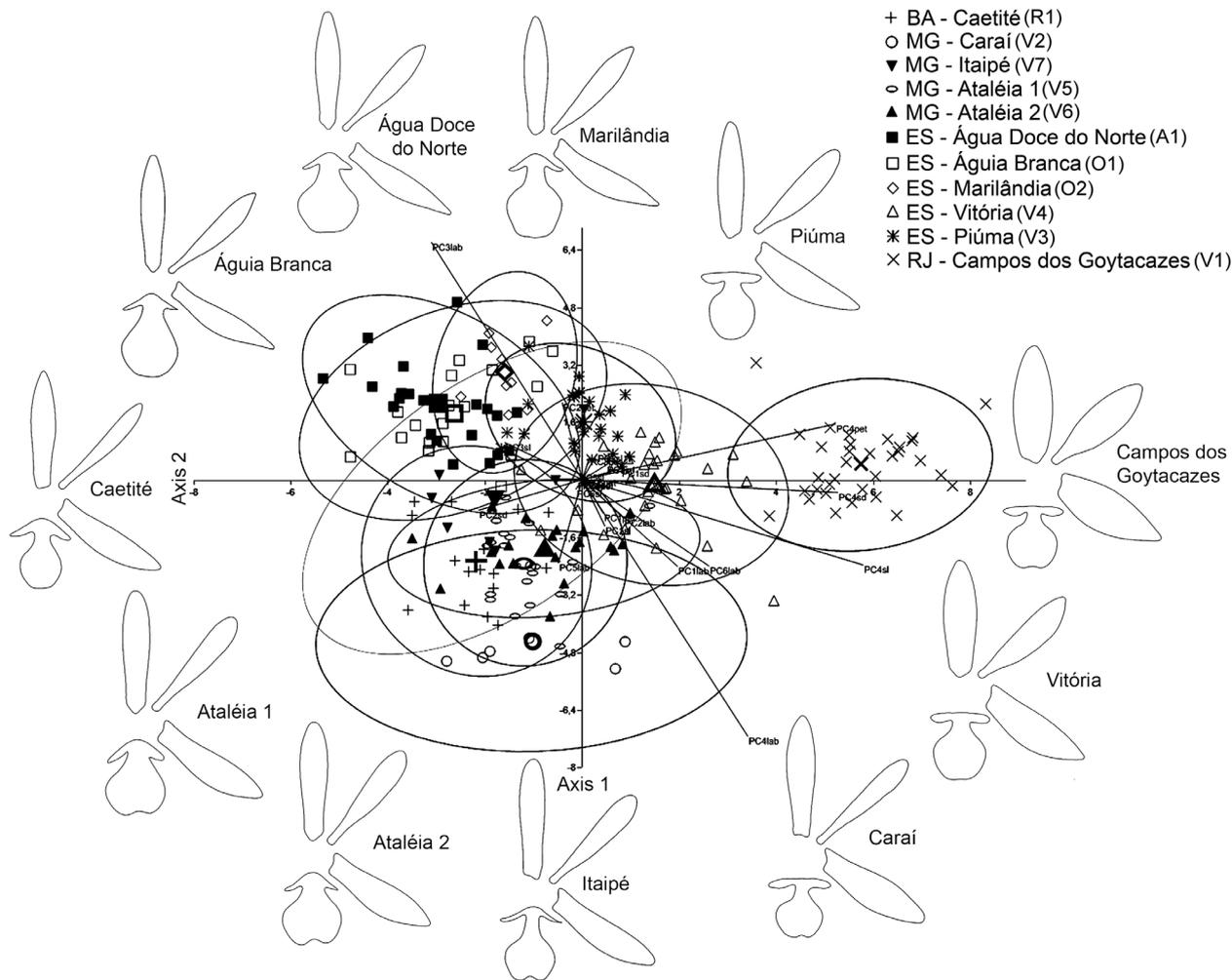

Figure 8 – CVA of Elliptical Fourier Analysis of 208 specimens of the *P. vellozicola* complex. Axes 1 and 2 represent 47.4% and 2.64% of the total variation, respectively. The ellipses are centered in the sample average (represented as larger symbols) and comprise 95% of individuals of each population. Outlines represent the mean shape of floral parts of each population. The codes between parentheses correspond to the populations of original delimitation of *P. aguadocensis* (A), *P. oliveirana* (O), *P. regentii* (R), and *P. vellozicola* (V).

western Espírito Santo, the same region as Águia Branca and Marilândia), mainly presented metric values for their definition of *P. vellozicola* (slightly larger flowers with longer and narrower sepals and petals, lip isthmus shape that makes the median lobe oval and the apex of the lip rounded). Similarly, in the original description of *P. regentii* from material from Bahia, Castro Neto & Marçal (2007: 7) highlighted that flower size is one of the main characteristics distinguishing them from the closest species, as well as petal width, length of the lip isthmus and obtuse apex of the median lobe of the lip. Although they compared it with *P. freyi*, this is clearly a wrong viewpoint, since agreement is certainly greater with *P. vellozicola*. Also, Campacci (2016: 498) presented the smaller size of the plant, lip without lamellae, and acute apex as diagnostic characteristics for *P. aguadocensis* (from Água do Doce do Norte, northwestern Espírito Santo). However, all these features proved to be strongly variable, both between and within populations, which was observed in the analysis of herbarium specimens along the distribution of *P. vellozicola* (Menini Neto et al. 2013) and in the analysis of the individuals of each of the populations in this study.

The smaller flowers observed for the population from Caetité (also observed in other analyzed herbarium specimens from Bahia and northern Minas Gerais), is probably due to the fact that these are among the northernmost populations of *Pseudolaelia*, from the Caatinga, which has a drier environment and the highest altitude (970 m). Similarly, plants from this region are generally smaller than plants from other populations (Menini Neto et al. 2013). Often plants from more arid and/or high environments present smaller vegetative and floral organs (Sapir et al. 2002, Kofidis et al. 2007). Morphological variations related to environmental and latitudinal gradients have already been observed in morphometric analyses of species from different families and different locations, such as Styracaceae (Fritsch & Lucas 2000), Iridaceae (Sapir et al. 2002), Asteraceae (Fici & Lo Presti 2003), Orchidaceae (Goldman et al. 2004, Haraštová-Sobotková et al. 2005), Sarraceniaceae (Ellison et al. 2004), Malvaceae (Assobagdjo et al. 2006), Lamiaceae (Kofidis et al. 2007), Eriocaulaceae (Trovó et al. 2008), and Rosaceae (Shimono et al. 2009).

Some studies of Orchidaceae using morphometric methods have shown that widely distributed plants can display strong morphological variation. This trend was found for example in *Calopogon tuberosus* (L.) Britton, Sterns & Poggenberg (Goldman et al. 2004); *Epidendrum secundum* Jacq. (Pinheiro & Barros 2007); *Bulbophyllum exaltatum* Lindl. (Ribeiro et al. 2008); *Brasiliorchis picta* (Hook.) R.B.Singer, S.Koehler & Carnevali and *B. crysantha* (Barb. Rodr.) R.B.Singer, S.Koehler & Carnevali (Pinheiro & Barros 2009). The results obtained in this study corroborate this trend, pointing to the presence of inter-population morphological variability, possibly due to isolation, since the inselbergs in which they live act as islands, hampering gene flow between populations (Barbará et al. 2007, Menini Neto 2011).
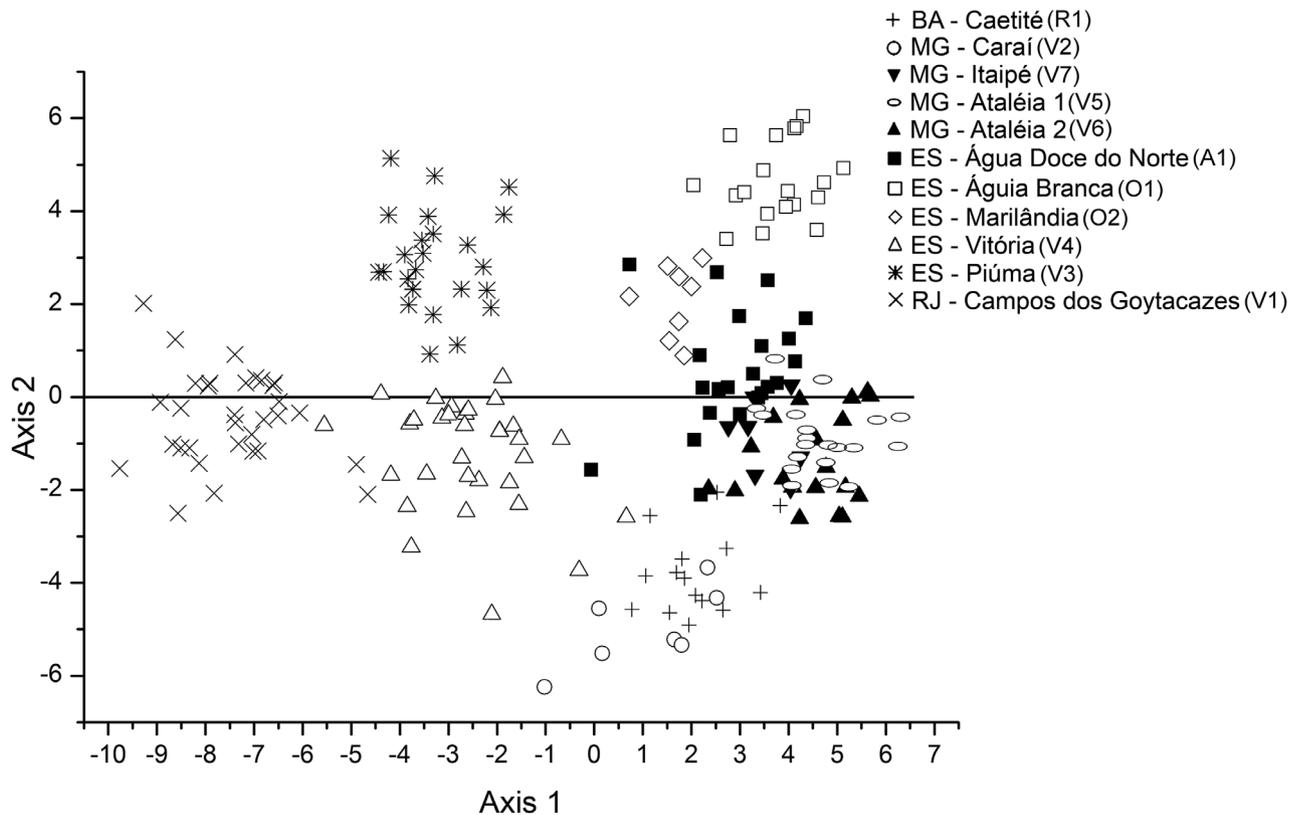
Figure 9 – CVA of combined analysis of 208 specimens of the *P. vellozicola* complex. The codes between parentheses correspond to the populations of original delimitation of *P. aguadocensis* (A), *P. oliveirana* (O), *P. regentii* (R), and *P. vellozicola* (V).

In the present study, asymmetric changes revealed by EFA played an important role in the total variation of flower parts, especially when primarily symmetrical, such as the dorsal sepal (with 83.41% in the PC1) and the lip (with a total of 77.02% in the first three PCs). The effect of symmetric and/or asymmetric changes in the variations in shape of symmetrical flower parts was evaluated in three studies of ornamental species (Yoshioka et al. 2004, 2006, Kawabata et al. 2009). These studies showed that these modifications may have different contributions, even in flower parts with predominantly symmetrical shapes, similar to what was observed for dorsal sepal and lip in this study. Different trends in the importance of the variables responsible for the symmetric and asymmetric changes were also observed in studies employing EFA in leaves (Andrade et al. 2008, 2010, Fritsch et al. 2009, Magrini & Scoppola 2010).

The canonical variables analysis (CVA) showed a strong overlap between populations (with the exception of Campos dos Goytacazes). This result is somewhat similar to that obtained for interpopulation relationships in the linear measurement analysis: in this case, the population of Campos dos Goytacazes is isolated in CVA, but is grouped together in NJ in a branch with Piúma and Vitória. Thus, the trend observed in the traditional morphometrics study for the separation of populations in lowland areas closest to the coast (Campos dos Goytacazes, Piúma and Vitória) from the remaining inland populations in higher altitude (e.g. Baker & Dalby 1980, Trovó et al. 2008) is attenuated in the geometric morphometric study. This is probably due to the smaller effect of the size of the floral parts in this analysis. In addition, this result is in an inverse way related to the one aforementioned regarding the population of Caetité which is the northernmost and whose flowers are the smallest of those analyzed, reflecting the latitudinal/altitudinal gradient of distribution.

Perhaps the isolated placement of the Campos dos Goytacazes population is due in large part to the greater width of sepals and petals compared with the other populations, as well as to the importance of the variables PC4sd, PC4sl and PC4pet. On the other hand, the lip in Campos dos Goytacazes is similar to that of other populations, such as Caraí, Piúma and Vitória. However, evidence for the separation of Campos dos Goytacazes from the rest of the populations is small, since the variables that most stood out in the differentiation of this group explain a small percentage of the total variance: PC3lab (7.93%), PC4lab (5.58%), PC4sd (0.78%), PC4sl (3.69%), and PC4pet (2.94%). The partial genetic isolation of this population (Menini Neto 2011) probably is one responsible to this pattern, because Campos dos Goytacazes is the known southernmost population of *P. vellozicola*, although a subpopulation is genetically related with a population of Bahia, the northernmost of the genus.

On the other hand, the EFA corroborates the results obtained with linear measurements, showing little evidence for the recognition of the Caetité, Água Doce do Norte, Águia Branca and Marilândia populations as separate from *P. vellozicola*, as proposed by Castro Neto & Marçal (2007: 7), Castro Neto & Chiron (2009: 22), and Campacci (2016: 498).

The combined linear and geometric morphometric results show that the wide distribution of *P. vellozicola*, coupled with the relative genetic and geographic isolation of populations in inselbergs and *campos rupestres* (Menini Neto 2011), are likely responsible for the large interpopulation variation, suggesting clinal variation due to environmental gradient (Sapir et al. 2002, Fici & Lo Presti 2003, Etterson et al. 2008). Although jackknife classification presented significant differences between the populations, there is not a single pattern regarding the taxa previously described, with several individuals showing a deviating position in populations belonging to different species.

The observed variability reinforces the need for greater discretion and care in the description of new species and the definition of widely distributed taxa, especially those occurring in some kind of environmental gradient. The misinterpretation of species with broad variation can lead therefore to the description of several "new and microendemic species" causing taxonomic confusion (Dayrat 2005), especially by sampling populations in the extremes of distribution without knowledge of the overall pattern. Such a proliferation of names (often seen today in Orchidaceae) is difficult, for instance, when evaluating threatened species.

The overlap observed in the multivariate analyses indicates the co-specificity of *P. vellozicola*, *P. aguadocensis*, *P. oliveirana* and *P. regentii*. Despite the large inter-population polymorphism (probably due to relative isolation provided by the insular environment of the outcrops), there are no strong morphological discontinuities among the populations from the various localities studied and gradations may be observed in intermediate locations of geographical distribution (clinal variation). Additionally, the patterns obtained showed, in some cases, the lack of agreement between the multivariate analyses and do not provide clear geographical clusters, because many populations at close locations are different, and the reverse can also occur.

Except for *P. aguadocensis*, proper type specimens are not available to corroborate this conclusion (see below, Taxonomy). However, the analysis of lectotypied figures of *P. oliveirana*, *P. regentii* and *P. vellozicola* suggests a similarity (both in shape and dimensions of the floral parts) with some individuals from the populations of Marilândia, Caetités and Vitória, respectively, which also are mixed among other populations. The type specimen of *P. aguadocensis* (*Leitão* 47) is from the same locality of the population sampled in Água Doce do Norte for the present study, and several of the specimens also are morphologically very similar, justifying the treatment adopted here.

Thus, based on our findings, we propose that *P. vellozicola* should be recognized as a widely distributed species with large morphological polymorphism regarding its flowers; *P. aguadocensis* also must be considered as synonym, along *P. oliveirana*, and *P. regentii* (which were synonymized by Menini Neto et al. 2013).

TAXONOMY

Pseudolaelia vellozicola (Hoehne) Porto & Brade (Porto & Brade 1935: 211). – *Schomburgkia vellozicola* Hoehne (Hoehne 1934: 622). – Type: lectotype (designated by

Menini Neto et al. 2011): plate number 11 of the original publication of *Schomburgkia vellozicola*.

P. oliveirana V.P.Castro & Chiron (Castro Neto & Chiron 2009: 22). – Type: Brazil, Espírito Santo, Pancas, s.d., Pinto s.n. (holo-: SP, not deposited). Lectotype: Figure 1 of original work, designated by Menini Neto et al. (2013).

P. regentii V.P.Castro & Marçal (Castro Neto & Marçal 2007: 7). – Type: Brazil, Bahia, Rio de Contas, Paramirim, s.d., Marçal s.n. (holo-: SP, not deposited). Lectotype: Figure 2 of original work designated by Menini Neto et al. (2013).

P. aguadocensis Campacci (Campacci 2016), **synon. nov.** – Type: Brazil, Espírito Santo, Água Doce do Norte, Santa Luzia do Azul, 800m (2,620 feet) elevation, May 2009 (flowered in cultivation in April 2015), *R.V. Leitão* 047 (holo-: ESA).

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (https://www.ingentaconnect.com/content/botbel/plecevo/supp-data) and consist of: (1) photographs of flowers from different populations of the *Pseudolaelia vellozicola* complex; and (2) PCA of linear measurements of 208 specimens of the *Pseudolaelia vellozicola* complex.

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