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Seed morphology in the tribe Chloraeeae (Orchidaceae): combining traditional and geometric morphometrics

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Previous work on orchid seeds has shown that characters associated with the seed coat may be useful for classification and phylogeny at a suprageneric level. The seed morphology of several species of the tribe Chloraeeae was analysed using traditional morphometrics, and the seed shape was studied, for the first time, using tools of geometric morphometrics. Seed characters were evaluated by their discriminative power and the information they may provide in a phylogenetic context. By contrast with previous findings, seed shape resulted in a continuum among the taxa studied, and in only a few cases could genera or groups of species be discriminated on the basis of shape. However, seed size, expressed as centroid size, was a variable character and informative at a phylogenetic level. Traditional measures of seed coat, mainly those of seed coat cells, were also helpful for the discrimination of genera and species, agreeing with previous statements about their utility in taxonomy and phylogeny. © 2009 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2009, **160**, 171–183.

ADDITIONAL KEYWORDS: *Bipinnula* – *Chloraea* – *Gavilea* – *Geoblasta* – orchid seeds – seed shape.

INTRODUCTION

The systematic position of the South American orchids *Bipinnula* Comm. ex A.Juss., *Chloraea* Lindl., *Gavilea* Poepp. and *Geoblasta* Barb. Rodr. is unclear. The four genera were grouped in subtribe Chloraeinae Rchb.f. by Dressler (1981, 1993), Szlachetko (1995), Kores *et al.* (2001) and Clements *et al.* (2002). Dressler (1981, 1993) placed Chloraeinae in the tribe Diurideae Endl., but Szlachetko (1995) placed it in Geoblasteae Barb. Rodr. Molecular phylogenetic studies placed the subtribe in tribes Spirantheae Endl. (Kores *et al.*, 2001) and Cranichideae (Kores *et al.*, 2000; Clements *et al.*, 2002; Cameron, 2006). Finally, Pridgeon *et al.* (2003), on the basis of morphological and phylogenetic observations, treated these taxa at the tribal level, including four genera in Chloraeeae (Rchb.f.) Pfeiff.: *Bipinnula*, *Chloraea*, *Gavilea* and *Geoblasta*. The relationships among these four genera are not yet clear: none of the phylogenetic trees published to date has included species of *Bipinnula* or *Geoblasta*, and *Chloraea* and *Gavilea* are underrepresented (for example, Kores *et al.*, 2000, 2001; Clements *et al.*, 2002; Cameron, 2006). In this context, the analysis of the seeds of *Bipinnula*, *Chloraea*, *Gavilea* and *Geoblasta* may help to determine important characters for phylogenetic analyses, given that there are many conservative and phylogenetically informative characters in the seed coat (Molvray & Kores, 1995).

Clifford & Smith (1969) made one of the first analyses of seed morphology, and were the first to propose that seed morphology can be an aid to classification in orchids. Subsequently, several studies on orchid seeds have been published. Most of them are primarily descriptive, and examine differences in morphometry, morphology, size and ultrastructural characters revealed by scanning electron microscopy (SEM) at the generic and suprageneric levels (for example, Barthlott, 1976, 1984; Arditti, Michaud & Healey,

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1979, 1980; Healey, Michaud & Arditti, 1980; Chase & Pippen, 1988, 1990; Swamy *et al.*, 2004; Gamarra *et al.*, 2007). Most analyses of orchid seeds have shown that genera are uniform in seed structure, and most subtribes are also regular in qualitative seed structure (Clifford & Smith, 1969; Barthlott & Ziegler, 1981; Chase & Pippen, 1988; Kurzweil, 1993; Molvray & Kores, 1995; Gamarra *et al.*, 2007).

Seed shape is variable. Clifford & Smith (1969) classified orchid seeds into five basic shapes (presented as a figure in their work), whereas Molvray & Kores (1995) identified four shapes: fusiform, filiform, clavate and ellipsoidal. Other notable characters for orchid seed analyses are the number of cells of the testa and the form and size of the individual, ranging from uniform to noticeably larger or smaller in areas such as the medial region or the chalazal end (Molvray & Kores, 1995). Characters associated with testa cell walls can also be informative. These include the height of the anticlinal wall, the presence of gaps between the walls and the sculpturing of the walls, among others (Molvray & Kores, 1995).

Several authors have attempted to classify orchid seeds and have proposed different classes of seeds (Barthlott & Ziegler, 1981; Kurzweil, 1993; Molvray & Kores, 1995). Molvray & Kores, (1995) included in their analysis three species of the tribe Chloraeeae: *Bipinnula polysyka* Kraenzl., *Chloraea densipapillosa* C.Schweinf. and *Gavilea lutea* (Pers.) M.N.Correa. According to their findings, *Chloraea* and *Gavilea* have the 'goodyeroid' seed type and *Bipinnula* has the 'diuroid' seed type. However, apart from the species included in the work of Molvray & Kores, (1995), no further analyses have been made on the seeds of Chloraeeae. In this context, this contribution is the first to include a large sampling of species of the tribe.

Since the early 1990s, new techniques have been developed to analyse morphological structures. The geometric morphometric revolution has produced more effective approaches for capturing the shape of an organism (Rohlf & Marcus, 1993). These have been widely used in zoology (for example, Marcus, Hingst-Zaher & Zaher, 2000; Swiderski, 2003; Sheets *et al.*, 2006), but plant systematists have found it difficult to recognize landmarks in plant structures and, consequently, did not participate in the morphometric revolution (Jensen, 2003). Indeed, all published studies on orchid seeds have only used a traditional morphometric approach. However, most authors have mentioned the shape of seed in their studies, and it is one of the diagnostic characters utilized in the classifications of Barthlott & Ziegler (1981) and Kurzweil (1993). Therefore, a statistical method for the measurement of seed shape could be useful in orchid seed analyses.

The aims of the present study were to analyse the seed morphology of some representatives of Chloraeeae using light microscopy, SEM and traditional and geometric morphometrics, and to identify diagnostic characters that can contribute to the systematics of the tribe and help resolve the phylogenetics of this group. Six species of *Chloraea*, five of *Gavilea*, one of *Bipinnula* and the only species of *Geoblasta*, were included.

MATERIAL AND METHODS

Seeds of 13 species, representing the four genera (Table 1), were obtained from herbarium specimens from SI and BAB. Some authors have stated that herbarium material is unreliable for seed analyses because of the inaccuracy of the determination of the species identity of fruiting specimens (for example, Chase & Pippen, 1988). However, the species used in this analysis usually have flowers and mature capsules on the same inflorescence, allowing for an appropriate identification of the species. All the seeds analysed were at the same state of maturity, when dehiscence was imminent and the embryo was well developed. Nonviable seeds, which are smaller than viable ones, were discarded from the analyses.

For SEM observations, dry seeds were mounted on SEM stubs, coated with gold–palladium and examined with a Philips XL30 microscope. At least one seed of each species was photographed.

For observations under a light microscope, seeds were rehydrated with a 1% photographic wetting agent solution in water at 60 °C for 4 h. The seeds were then fixed using a mixture of formaldehyde (5%) and acetic acid (5%), and mounted on polyvinyl alcohol–lactic acid medium, containing 16.6 g of polyvinyl alcohol, 100 mL of lactic acid, 5 mL of glycerin and 100 mL of distilled water.

GEOMETRIC MORPHOMETRICS

An average of 20 seeds per species were photographed for the analysis; in total 254 seeds were measured (Table 1). Seeds were oriented with the longitudinal axis parallel to the microscope slide. Seeds with a broken or bent margin were discarded from the analysis.

Landmarks should ideally be homologous, of regular and repeatable position, and provide adequate coverage of the form (Zelditch *et al.*, 2004). However, in the case of curved structures, such as the seeds analysed here, most of these criteria are difficult to accomplish. To deal with forms that lack reliable point-like landmarks, Bookstein (1997) developed the semi-landmark technique. This technique allows a spline relaxation so that the landmarks are free to slide along the lines. There are two sliding semi-landmark methods: the minimum

Table 1. List of species examined, vouchers and numbers of seed analysed

Taxon	Voucher	Seeds for GM	Seeds for TM
Bipinnula fimbriata (Phil.) I.M.Johnst.	*Boelcke 91373* (BAB)	8	–
Chloraea cylindrostachya Poepp.	*Soriano 2564* (SI)	21	–
Chloraea lechleri Lindl. ex Kraenzl.	*Junge 1078* (SI)	17	7
Chloraea magellanica Hook.f.	*Morrone 5396* (SI); *Morrone 5403* (SI)	21	7
Chloraea membranacea Lindl.	*Hubrich 23869* (SI)	22	5
Chloraea philippii Rchb.f.	*Jonson 1046* (SI)	27	7
Chloraea virescens (Willd.) Lindl.	*Belgrano 129* (SI)	21	7
Gavilea araucana (Phil.) M.N.Correa	*Correa 920* (BAB); *Crespo 55* (SI); *Esk & Klein 1622-11* (BAB)	19	6
Gavilea glandulifera (Poepp.) M.N.Correa	*Crespo 2122* (BAB); *Illin 276* (SI); *Krapovickas 3930* (BAB); *Kreibohm 650* (SI); *Morrone 5391* (SI)	26	7
Gavilea kingii (Hook.f.) M.N.Correa	*Boelcke 1967* (SI); *Correa 1657* (SI)	22	6
Gavilea lutea (Pers.) M.N.Correa	*Belgrano 545* (SI); *Morrone 5397* (SI); *Nicora 9652* (SI)	20	–
Gavilea supralabellata M.N.Correa	*Correa 3033* (BAB); *Kreibohm 677* (SI)	6	–
Geoblasta penicillata (Rchb.f.) Hoehne	*Cabrera 10378* (SI); *Hicken 25864* (SI)	24	6

GM, geometric morphometrics; TM, traditional morphometrics.

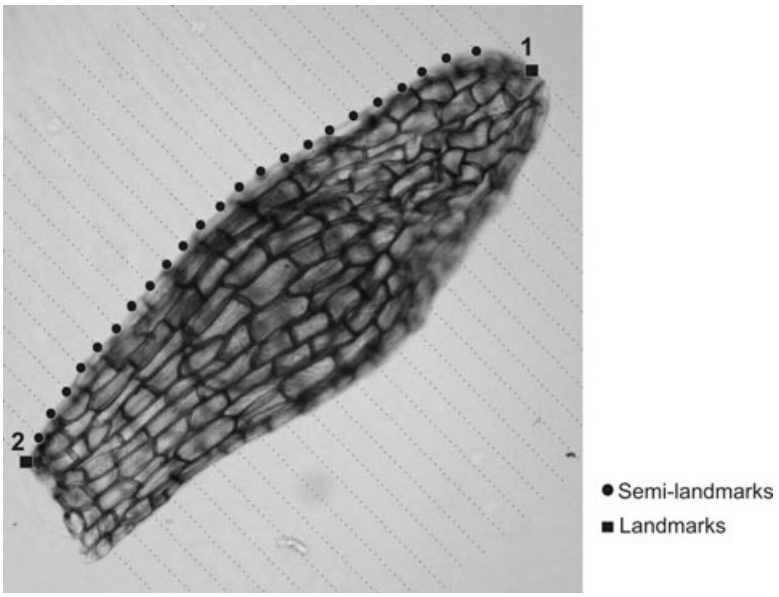

Figure 1. Landmarks and semi-landmarks recorded on seeds.

Procrustes distance, or perpendicular projection (PD; Sampson *et al.*, 1996), and the minimum bending energy (BE; Bookstein, 1997). The BE criterion minimizes part of the total variation resulting from local changes or differences in the area of different points. In addition to the local variation, PD also minimizes the tangential variation along outlines (Perez, Bernal & Gonzalez, 2006). We used both sliding methods in these analyses, but, as they produced similar results, only the BE results are presented. In addition, according to Slice (2007), BE minimization is better if one is going to construct thin-plate spline visualizations.

Two landmarks were obtained, one at the most external point of the chalazal pole (Fig. 1, landmark 1) and the other at the margin of the micropylar pole (Fig. 1, landmark 2), and 23 semi-landmarks were placed on the seeds (Fig. 1). As the seeds analysed here are bilaterally symmetrical, only half a seed was measured, in order to reduce the number of semi-landmarks and, consequently, the number of degrees of freedom. The application MakeFan6 of the IMP package (Sheets, 2003) was used to guarantee consistent placement of the semi-landmarks; a comb of 25 lines was used for this purpose.

Both landmarks and semi-landmarks were digitalized using tpsDIG 2.10 (Rohlf, 2006). The alignment of the landmarks and semi-landmarks, partial warps (PWs; components that explain part of the total deformation that affects some landmarks and not others) and relative warps (RWs; principal components of the PWs) were performed using tpsRelw 1.45 (Rohlf, 2006). For the sliding of the semi-landmarks, 40 iterations were used. All the analyses were performed using $\alpha = 0$, which means that all the principal warps had the same weight when the RWs were calculated. Scores of the RW matrix were used to perform scatterplots, discriminant function analyses and multivariate analysis of variance (MANOVA) using the software STATISTICA 6 (StatSoft Inc., 2001). In the discriminant analyses, all the groups had the same probability, and so a specimen could be assigned to any group independent of the size of the group. The percentage of correct posterior classification was used as an indicator of the performance of the function. In MANOVA, the *post hoc* Tukey test was used to check the significance of pairwise differences. For the analysis of the effect of shape due to size, a regression of shape (i.e. PWs) on centroid size was performed using the program tpsREGR (Rohlf, 2006). Centroid size is the size measure used in geometric morphometrics, and is calculated as the square root of the summed squared distances of each landmark from the centroid of the landmark configuration (Zelditch *et al.*, 2004). Significance in the statistical tests, if not otherwise specified in the text, was checked with $P < 0.05$.

Traditional morphometrics

Seven of the characters used by Molvray & Kores (1995) for the delimitation of seed types were measured here: the number of cells lengthwise multiplied by the number of cells widthwise (cellno), the suddenness of transition between medial and other cells (5celldiff), the average medial cell length in relation to seed length (avmed/se), the average medial cell length (avmedcel), the proportion of gaps at cell vertices in medial section (intercel), the median of obtuse angle measures (medianob) and the parallelism between opposing cell walls (maxminpar). For this, a subsample of the seeds used for geometric morphometric analyses was used (Table 1). Some species were not included because these characters could not be measured accurately (Table 1). Measurements of lengths, widths and angles were performed using the tools available in tpsDIG 2.10 (Rohlf, 2006). As the data did not fulfil the requirements of normality and linear relationship among variables, principal components analysis (PCA) could not be performed (James & McCulloch, 1990). Instead, a non-parametric multidimensional scaling (MDS) was performed, using as input a Euclidean distance matrix. In addition, data were analysed using boxplots and the Mann–Whitney *U*-test (MWU). We tested for homoscedasticity using the Levene test (Levene, 1960). However, according to Bautista & Gómez (2007), the MWU test is robust to heteroscedasticity, and reliable *P* values could be obtained under such circumstances. Therefore, the MWU test was performed even in the absence of homoscedasticity, and in such cases the results were compared with the boxplots to determine whether they were coherent. The MDS analyses were performed using the software PAST ver. 1.89 (Hammer, Harper & Ryan, 2001); the remaining statistical tests and analyses were performed using the software STATISTICA 6.

Evaluation of the phylogenetic information present in the morphometric characters

To test the phylogenetic utility of the variables, a morphological matrix was built using the seven variables from the traditional morphometric analysis, the centroid size obtained in the geometric morphometric analysis and the first ten RWs, which accounted for 95% of the explained variance. Characters were transformed by dividing by the average. In the case of RW, the values were previously transformed by subtracting the highest value.

The characters were analysed as continuous, and their states were represented by a range comprising the first and third quartile. Maximum parsimony analysis was performed using TNT (Goloboff, Farris & Nixon, 2008), which allows the use of continuous characters without transformation (Goloboff, Mattoni & Quinteros, 2006). Implicit enumeration searches were performed under equal weights. Branch support was evaluated using symmetric resampling (Goloboff *et al.*, 2003). As the intention of this analysis was only to check the phylogenetic information of the characters, the selection of the root was trivial, and so the trees were rooted with *Geoblasta penicillata* (Rchb.f.) Hoehne.

RESULTS

Geometric morphometrics

The 25 landmarks and semi-landmarks analysed generated 46 RW measures for each specimen. The first two RWs explained 56.91% and 22.37% of the variation, respectively (79.28% accumulated). A graphical representation of these two RWs showed a clear overlap among the species (data not shown). However, MANOVA indicated that there were significant differences among the species (Wilk's λ, 0.593; $P < 0.000001$). The remaining RWs explained less

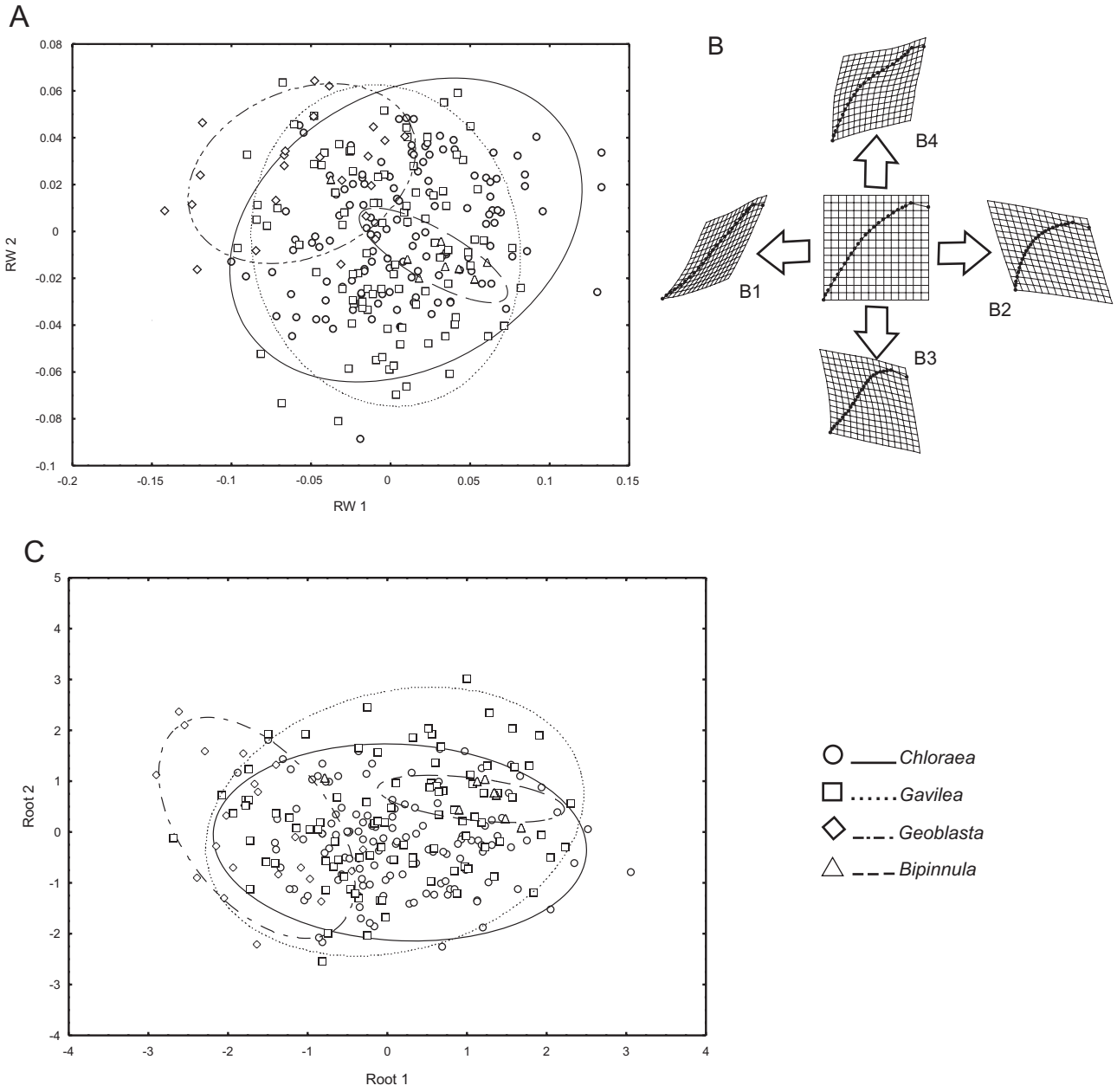

Figure 2. Geometric morphometric results. A, Scatterplots of relative warp (RW) 1 and RW 2 for the four genera. B, Shape changes depicted by RW 1 and RW 2: B1, deformation relative to the mean shape towards the negative direction of RW 1; B2, deformation relative to the mean shape towards the positive direction of RW 1; B3, deformation relative to the mean shape towards the negative direction of RW 2; B4, deformation relative to the mean shape towards the positive direction of RW 2. C, Graphical representation of the discriminant analysis of the four genera of the tribe.

than 7% of the variation each, and their graphs showed a worse superimposition of the specimens analysed.

When analysing the differences between genera, there was a clear overlap between the four genera, and *Bipinnula* was nested within the distribution of *Chloraea* and *Gavilea*. However, seeds of *Chloraea* and *Gavilea* were never as elongated nor as thin as those of *Geoblasta* (Fig. 2A, B). The scatterplot showed that *Geoblasta* had elongated seeds with a fusiform shape, as it was located in the top left portion of the graph (Fig. 2A, B). *Bipinnula* seed shape was similar to the consensus or even shorter and more rounded, and was distributed positively along the first RW and negatively in the second RW (Fig. 2A, B). *Chloraea* and *Gavilea* seeds were mor-

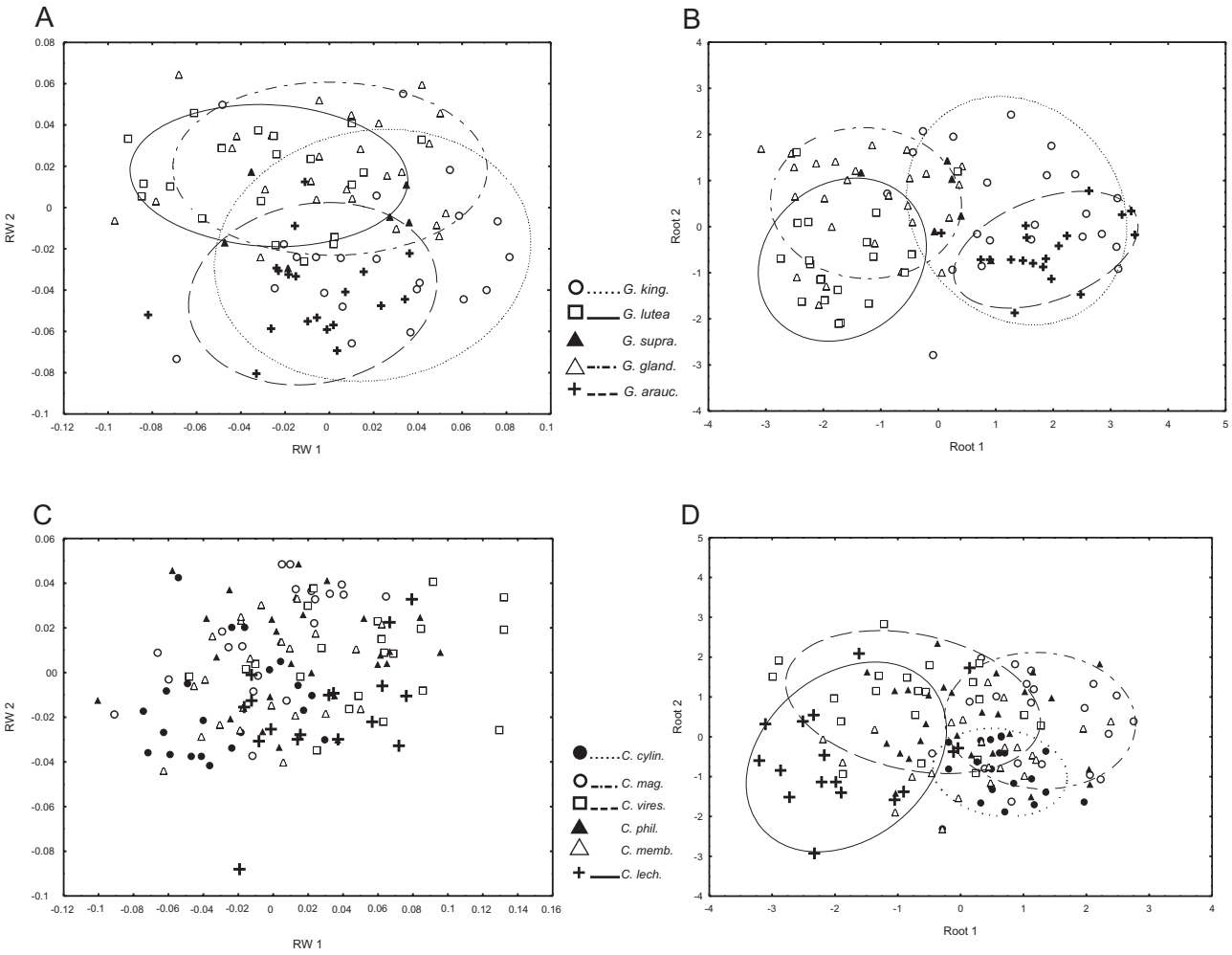

Figure 3. Geometric morphometric results. A, Scatterplots of relative warp (RW) 1 and RW 2 for the five *Gavilea* species. B, Canonical variate axes for canonical variate analyses for *Gavilea* species. C, Scatterplots of RW 1 and RW 2 for the six *Chloraea* species. D, Graphical representation of the discriminant analysis for the six *Chloraea* species. *C. cylin*., *Chloraea cylindrostachya*; *C. lech*., *C. lechleri*; *C. mag*., *C. magellanica*; *C. memb*., *C. membranacea*; *C. phil*., *C. philippii*; *C. vires*., *C. virescens*; *G. arauc*., *Gavilea araucana*; *G. gland*., *G. glandulifera*; *G. king*., *G. kingii*; *G. lutea*, *Gavilea lutea*; *G. supra.*, *G. supralabellata*.

phologically diverse, and this was supported by their wide distribution in the scatterplot. The only differences between these genera were that some *Gavilea* seeds had the lowest values on the second RW, associated with a straighter lateral margin, and some *Chloraea* species had higher values on the first RW, showing a more convex, almost elliptical, shape (Fig. 2A, B). The discriminant analysis (Wilk's λ, 0.593; $F(30,705) = 4.578$; $P < 0.0001$) gave a poor classification, as anticipated by the RW results, performing a percentage of correct posterior classification of only 38.34%. *Bipinnula* and *Geoblasta* had >85% of correct posterior classification, showing that, although there was a continuum among the four genera in the scatterplot, the seeds of *Bipinnula* and *Geoblasta* were distinguishable from those of the other genera (Fig. 2C). The *post hoc* Tukey test also confirmed this distinction, showing that there were significant differences between *Bipinnula* and *Geoblasta*, *Bipinnula* and *Chloraea*, *Bipinnula* and *Gavilea*, *Geoblasta* and *Chloraea* and *Geoblasta* and *Gavilea*, but not between *Chloraea* and *Gavilea*.

After analysing the seed shape in *Gavilea*, it was found that *G. glandulifera* (Poepp.) M.N.Correa and *G. lutea* (Pers.) M.N.Correa had concave and fusiform seeds, according to their distribution on the scatterplot with the highest values for the second RW (Figs 2B, 3A). The seeds of *G. lutea* were more filiform than those of *G. glandulifera*, as evident from their distribution along the first RW. *Gavilea kingii*

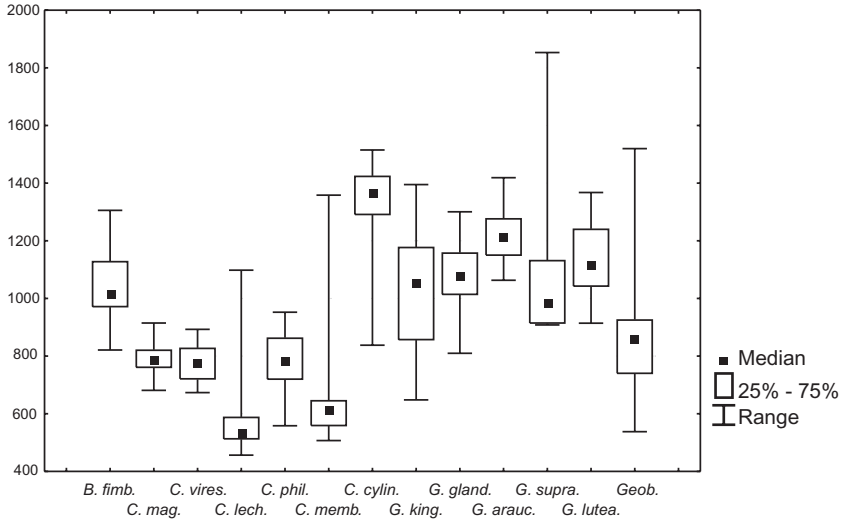

Figure 4. Boxplots of centroid size for the species analysed. For abbreviations, see legend to Fig. 3. *B. fimb*., *Bipinnula fimbriata*; *Geob*., *Geoblasta penicillata*.

(Hook.f.) M.N.Correa and *G. araucana* (Phil.) M.N.Correa had more convex and shorter seeds, as shown by their position with lower values on the second RW. Seeds of *G. kingii* were more elliptical than other *Gavilea* seeds, as shown by their higher values on the first RW. *Gavilea supralabellata* M.N.Correa had elliptical and almost fusiform seeds, and its distribution was similar to that of *G. glandulifera* (Figs 2B, 3A). The discriminant analysis of these five species (Wilk's λ, 0.174; $F(40,301) = 4.411$; $P < 0.0001$) yielded a medium level of correct posterior classification (64.52%) of the species. *Gavilea kingii* was the species with the lowest percentage of correctly classified cases (36.36%; Fig. 3B), whereas *G. araucana* and *G. lutea* had almost 80% correct posterior classification. The canonical analysis showed two overlapping groups, one formed by *G. glandulifera* and *G. lutea* and the other formed by *G. kingii* and *G. araucana*. *Gavilea supralabellata* was placed in the middle of these groups (Fig. 3B). There was a clear separation between *G. araucana* and *G. glandulifera* from *G. lutea* (Fig. 3B), which was also obvious in the posterior classification matrix. MANOVA showed significant differences between the species of both groups *G. glandulifera–G. lutea* and *G. kingii–G. araucana*. *Gavilea supralabellata* only showed significant differences from *G. araucana* (although P was relatively high, $P = 0.02$).

Differences among *Chloraea* spp. were not so evident. The scatterplot of RW did not allow the differentiation of species or groups of species (Fig. 3C). The canonical analysis (Wilk's λ, 0.2744; $F(50,523) = 3.431$; $P < 0.0001$) showed a better separation of the species than did RW, and split *C. lechleri* Lindl. ex Kraenzl. from *C. cylindrostachya* Poepp. and *C. magellanica* Hook.f. The overlap between *C. cylindrostachya* and *C. virescens* (Willd.) Lindl. was minimal (Fig. 3D). The posterior classification matrix agreed with these results (57.36% of correct classification), and showed that *C. magellanica* seeds were never classified as *C. lechleri* seeds, and vice versa, and *C. cylindrostachya* seeds were never classified as *C. magellanica*, *C. virescens* or *C. lechleri* seeds, indicating that seed shape may help to discriminate the seeds of these species. The *post hoc* Tukey test of MANOVA also supported the separation of these species.

Centroid size was analysed as boxplots. *Chloraea cylindrostachya* had the largest seeds and *C. lechleri* the smallest. Other *Chloraea* spp. had seeds smaller than *Geoblasta*, *Bipinnula* and *Gavilea*. After *C. cylindrostachya*, *Gavilea* spp. had the largest seeds (Fig. 4).

Regressing shape onto centroid size showed significant differences (Wilk's λ, 0.698; $F(46,207) = 1.942$; $P = 0.0009$), although size explained little of the PW variation in the sample analysed (approximately 4.5%). The variance explained showed a slight tendency for smaller seeds to be rounded and larger seeds to be more fusiform.

Traditional morphometrics

The MDS analysis was performed using three dimensions, which produced acceptable values of stress (0.1108). The plot of the first two dimensions showed two marked groups: *Geoblasta* on one side, and *Gavilea* overlapping with *Chloraea* on the other

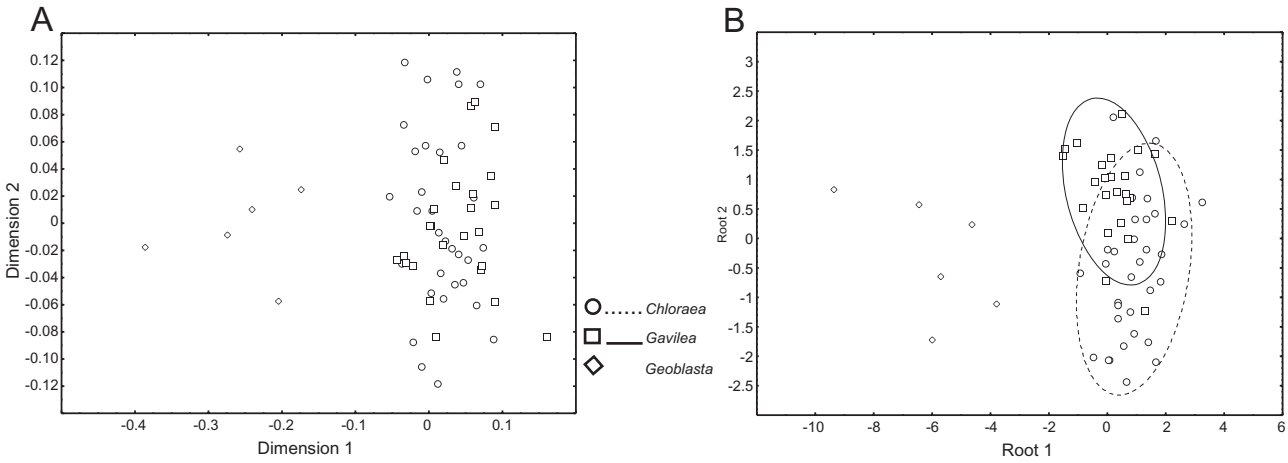

Figure 5. Multidimensional scaling results for variables based on Molvray & Kores (1995). A, Scatterplot of the first two dimensions. B, Graphical representation of the discriminant analysis.

(Fig. 5A). The analysis of dimensions 2 and 3 did not allow the separation of any groups. The discriminant analysis of the scores of the three dimensions showed better results, and allowed partial separation of *Chloraea* and *Gavilea* (although some overlap was observed; Fig. 5B). The posterior classification matrix resulted in 84% correct classification, *Geoblasta* with 100% correct classification and both *Gavilea* and *Chloraea* with almost 80% correct classification. No intrageneric differences were detectable using this method.

When analysing the boxplots, most of the variables measured showed differences among the three genera analysed (*Chloraea*, *Gavilea* and *Geoblasta*) and, in some cases, differences between the species.

The cell number showed clear differences among genera and among species (Fig. 6A). On average. *Gavilea* seeds had more cells than *Chloraea* seeds (MWU test significant: $U = 75.5$; $P < 0.000001$). Only *C. magellanica* had seeds with a cell number comparable with those of *Gavilea*. *Geoblasta* had seeds with the lowest number of cells (MWU test significant: $U = 15$; $P = 0.001$ with *Chloraea*; $U = 0$; $P = 0.0002$ with *Gavilea*). The Levene test was significant, but the results of the MWU test were coherent with the tendency observed in the boxplots.

Variation in size between medial and terminal cells showed no significant differences between seeds of *Chloraea* and *Gavilea* (MWU test: $U = 269$; $P > 0.05$), both having low '5celldiff' values. However, seeds of *Geoblasta* showed a marked transition between medial and terminal cells (Levene test significant; MWU test significant: $U = 0$; $P = 0.0001$ with *Chloraea*; $U = 0$; $P = 0.0002$ with *Gavilea*).

The medial cell length in relation to seed length also showed clear differences among the seeds of the three genera (Fig. 6B). *Geoblasta* had the highest values (MWU test significant: $U = 0$; $P = 0.0001$ with *Gavilea* and *Chloraea*) and *Gavilea* spp. had the lowest (Levene test significant: $U = 59$; $P < 0.000001$ with *Chloraea*). In this attribute, there were also intrageneric differences, mainly between the seeds of *Chloraea* spp. When analysing the raw data for the length of the medial cells, differences were not so obvious (data not shown), and between the seeds of *Chloraea* and *Gavilea* there were no significant differences. Even between species, differences were not so evident; the only tendency observed was for *Geoblasta* to have seeds with the largest medial cells (Levene test significant; MWU test significant: $U = 0$; $P = 0.0001$ with *Chloraea*; $U = 0$; $P = 0.0002$ with *Gavilea*).

The presence of intercellular gaps was a character that, on average, showed no differences between genera (MWU test: $P > 0.5$ for all the comparisons), but within genera there was more variability (Fig. 6C). *Chloraea lechleri* had seeds with the largest number of gaps and *Geoblasta penicillata* the lowest (excluding *C. magellanica* and *C. membranacea*, which lacked intercellular gaps).

Measurements of the obtuse angles revealed no significant differences among the seeds of the three genera. Between species, slight differences were observed, but there was no clear pattern and all the species overlapped, at least for the quartiles (data not shown). Parallelism between opposite cell walls showed that *Gavilea* had seeds with more disordered cells than did *Chloraea* and *Geoblasta* (MWU test significant: $U = 184$; $P = 0.0006$ with *Chloraea*; $U = 14$; $P = 0.002$ with *Geoblasta*). When analysing the species separately, it could be seen that *C. magellanica* had the most regular cell organization in the

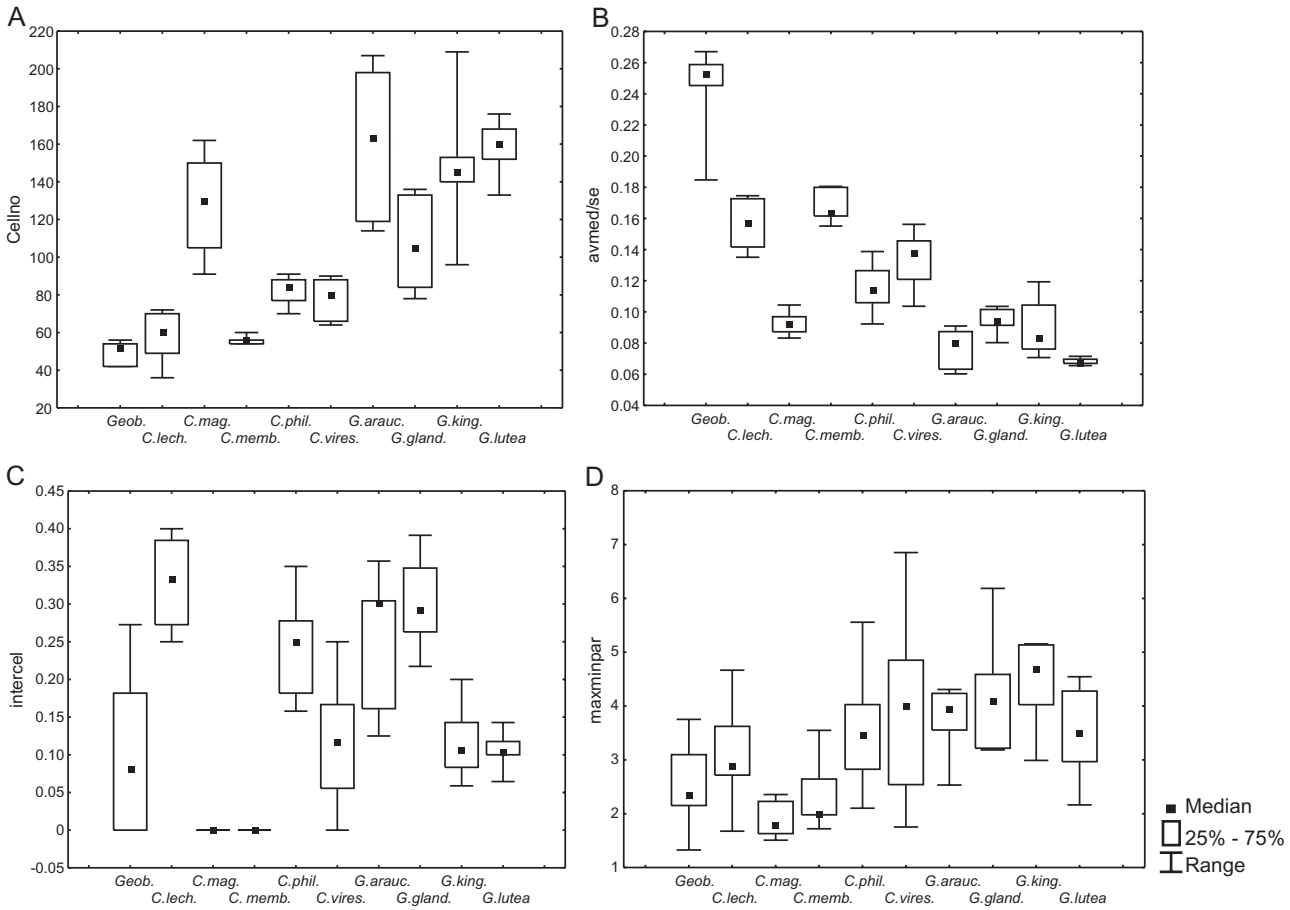

Figure 6. Boxplots for variables based on Molvray & Kores (1995). A, Cell number. B, Medial cell length in relation to seed length. C, Proportion of gaps at cell vertices. D, Parallelism between opposing cell walls. For abbreviations, see legend to Fig. 3. *Geob*., *Geoblasta penicillata*.

seed coat and *G. kingii* had the most unordered pattern of cell organization.

Using SEM, seeds of most of the species analysed had smooth, unsculptured testa cells (Fig. 7A–D, F), but *C. membranacea* had seeds with unordered, thin ridges in the periclinal cell wall (Fig. 7E). Different heights were observed in the anticlinal cell walls. Most of the species had seeds with high anticlinal cell walls (Fig. 7C, D), but those of *G. araucana*, *G. glandulifera*, *G. kingii* and *G. supralabellata* had lower cell walls (Fig. 7G). The anticlinal cell walls did not have any ornamentations or sculpturing.

Evaluation of the phylogenetic information present in the morphometric characters

When performing the analysis on all taxa, 247 trees of 8156 steps were obtained, and the strict consensus tree was fully collapsed. Using the command 'Agreement Subtree' in TNT, that builds a reduced consensus tree, three taxa were identified as 'wild cards' (that is they could be placed in any node, probably because of a lack of information; Nixon & Wheeler, 1992): *B. fimbriata*, *C. cylindrostachya* and *G. supralabellata*. These species were not included in the traditional morphometric analysis, and their exclusion from this analysis resulted in only one most parsimonious tree (length, 8029 steps). With *Geoblasta* as the root, *Gavilea* was nested within *Chloraea*. All nodes were supported by synapomorphies, with branch support of at least 30 (Fig. 8).

The ten RWs were not informative, and only the second RW changed in the tree as an autapomorphy for *G. araucana*. Centroid size was informative, and supported the *Gavilea* node (Fig. 8, node F) and the node that grouped *C. virescens* and *C. lechleri* (Fig. 8, node C). The seven characters taken from Molvray & Kores (1995) were informative and were synapomorphies for several branches. The most significant characters were 'cellno' and 'avmed/se', which supported nodes A, B, D and G. Node H was supported by character 'intercel' and node F was also supported by 'maxminpar'.

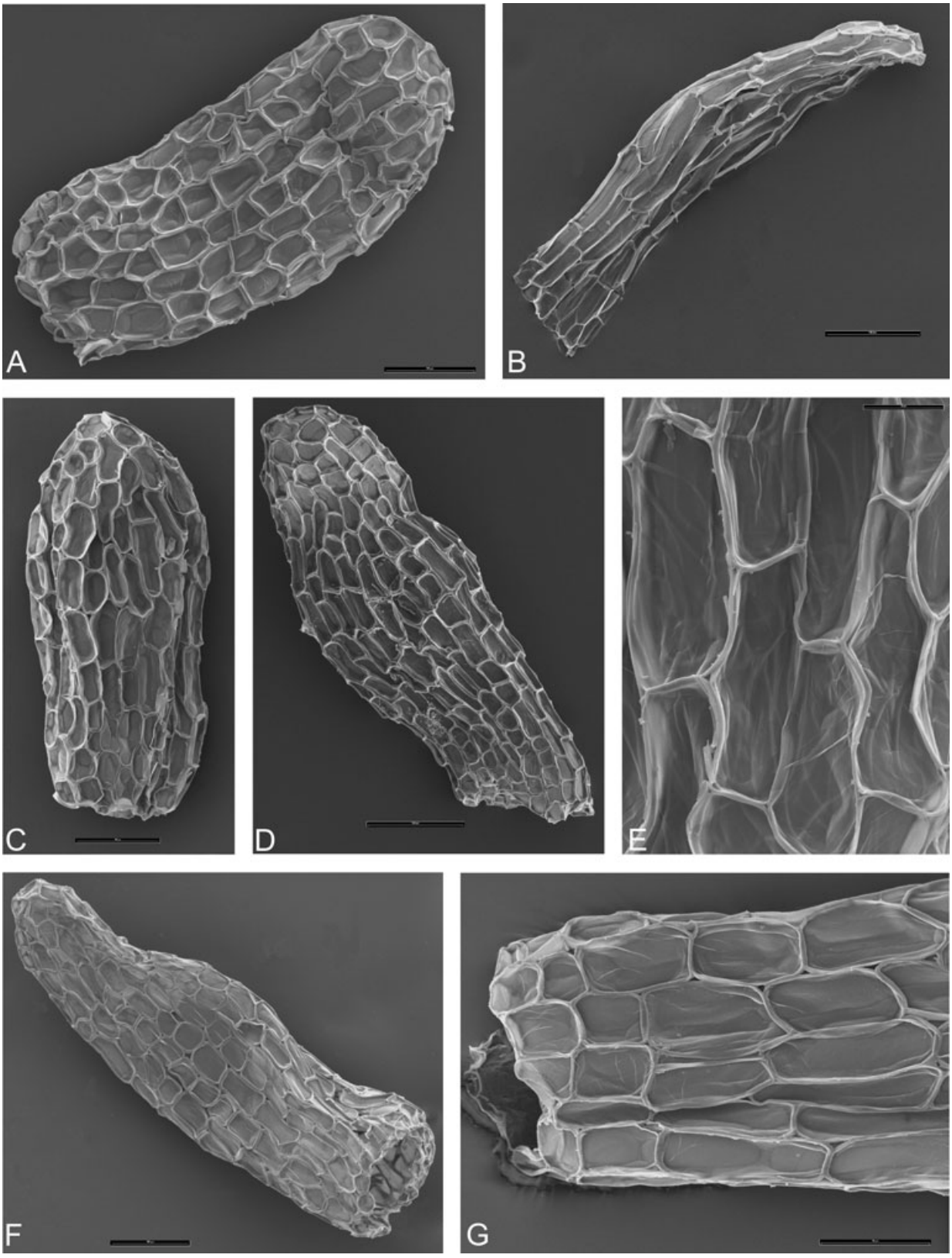

Figure 7. Scanning electron micrographs of seeds of Chloraeeae. A, *Bipinnula fimbriata*. B, *Geoblasta penicillata*. C, *Chloraea virescens*. D, *Chloraea magellanica*. E, Sculpturing of the anticlinal walls of *Chloraea membranacea*. F, *Gavilea glandulifera*. G, Low anticlinal walls of *Gavilea araucana*. Bars: 100 μm in A–D, F; 20 μm in E; 50 μm in G.

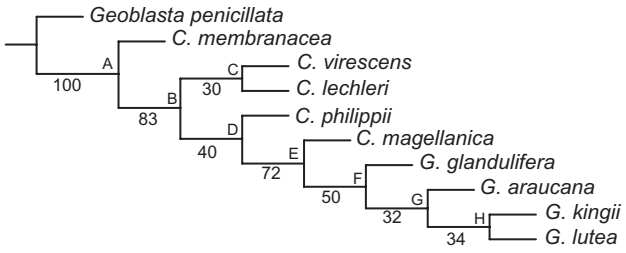

Figure 8. Most parsimonious tree obtained based on the continuous characters discussed in this study. Support values are displayed below the branches. Letters above the branches indicate the nodes.

DISCUSSION

Two approaches were used to analyse orchid seeds in this work. The traditional morphometric approach has been chosen by previous authors and, for the seeds analysed here, showed some differences among genera and species. The RW analysis revealed overlap among the taxa, discriminating only a few species in some plots (see Results).

Several studies of orchid seed classification (Barthlott & Ziegler, 1981; Kurzweil, 1993; Molvray & Kores, 1995) have led to different classifications for different groups of Orchidaceae. Molvray & Kores (1995) focused on the seeds of Diurideae, including the genera treated here, placed under subtribe Chloraeinae. According to their findings, *Chloraea* and *Gavilea* had a 'goodyeroid' seed type, with highly elongate, filiform seeds, with small cells of almost constant size and many intercellular gaps. *Bipinnula* had a 'diuridoid' type, with fewer cells, elongated in the medial region of the seed and occasional intercellular gaps. Most of the characters used by Molvray & Kores (1995) were analysed in this work, although they were analysed under a different statistical approach (i.e. MDS and boxplots instead of PCA). MDS showed that *Chloraea* and *Gavilea* formed a group separated from *Geoblasta*. The overlap between *Chloraea* and *Gavilea* in the scatterplot is consistent with the results of Molvray & Kores (1995), who grouped these genera under the same seed type. However, the analysis of each variable independently showed a different perspective, as the three genera analysed had differences in many characters, revealing a marked variability in the tribe. The seeds of *Geoblasta* had few cells, with larger medial cells and an abrupt difference between medial and terminal cells, whereas the seeds of *Gavilea* presented many cells with smaller medial cells and, on average, a less noticeable transition zone. Nonetheless, it was impossible to classify all the seeds studied here using the descriptions provided by Molvray & Kores (1995). For example, *C. magellanica* seeds had a large number of cells, which is typical of the 'goodyeroid' type, but no intercellular gaps, which does not agree with the 'goodyeroid' type description. Not all *Chloraea* and *Gavilea* spp. included in this analysis had elongated and fusiform seeds (typical of the 'goodyeroid' type). In conclusion, for the group analysed here, the seed classification proposed by Molvray & Kores (1995) could not be applied. At least for Chloraeeae, the seed types should be revisited and modified to accommodate the species and genera of which it is composed. A larger sampling is needed for such a revision, with more species per genus and more seeds per species.

Molvray & Kores (1995) proposed that their classification should be used for character delimitation in phylogenetic analyses. However, their seed classification was based on a combination of characters, and was not a character itself. Most of the variables analysed by Molvray & Kores (1995) can be used individually in phylogenetic analyses, as they are variable and potentially phylogenetically informative, and most are independent of each other. Molvray & Kores (1995) criticized seed coat characters because of their continuous nature, but now phylogenetic programs, such as TNT, allow the treatment of continuous characters without the need to transform them into discrete entities (Goloboff *et al.*, 2006).

Characters associated with the shape of the seed have been widely mentioned in the literature, and include the clavate, ellipsoidal, filiform and fusiform forms, etc. (for example, Clifford & Smith, 1969; Chase & Pippen, 1988; Swamy *et al.*, 2004; Gamarra *et al.*, 2007), but the shape has always been considered as a discrete and qualitative character. Geometric morphometrics is a technique used to study the geometrical character of biological shape in a statistical manner (Monteiro & Reis, 1999), and so it seemed to be a helpful tool to analyse the shape of orchid seeds. The results of the RW analysis showed that seed shape is a continuum rather than a discrete character in this group, and none of the seeds of the four genera could be clearly separated on the basis of the shape in the scatterplot of RW (but see Results). However, MANOVA of RW was significant, possibly because the seeds of *Geoblasta* and *Bipinnula* were moderately separated from the others. Discrimination among taxa was also indicated by the proportion of correct posterior classification of these genera in the discriminant analysis (see above). *Gavilea* seeds formed two distinctive groups, although their distribution on the scatterplot and the canonical analysis showed overlap. These two groups showed no correspondence with the taxonomic classification of the genus or with the species distribution. *Gavilea supralabellata* fell in the middle of both groups, although few seeds were analysed for this species. A larger sampling of *G. supralabellata* and the inclusion of

additional species of *Gavilea* could help to determine whether these two groups are real or just a sampling artefact. Species of *Chloraea* showed clear overlap in the scatterplot and the canonical analysis. The latter, however, revealed that some species were separate from others, but this separation was not as clear as in the case of *Gavilea*, and it was not possible to refer to groups of seeds. In all analyses, RW yielded significant differences in MANOVA; this was clearly an effect of the partial separations mentioned above.

SEM observations showed that cell wall micromorphology was fairly constant among the species included here. Only *C. membranacea* cell walls were sculptured. Future analysis including more species will demonstrate whether this is an autoapomorphy for this species, or whether there are more species in the group with this character. Another character revealed by SEM that could be useful was the height of the anticlinal cell walls, as, in the taxa analysed here, species with high and low anticlinal cell walls were observed.

The cladogram constructed using the characters investigated here showed resolution and support. This tree was not built with the objective of resolving the relationships among the species, and was merely an exploratory phylogenetic analysis to determine the utility of the characters. However, the topology obtained was congruent with the preliminary results of a molecular phylogeny for the group in development (M. A. Chemisquy, unpubl. data). As expected from the observations made above, RWs were not phylogenetically informative because of the superimposition of the species in the analysis. This does not mean that geometric morphometric results should be discarded as useful in a cladistic analysis (see González-José *et al.*, 2008), and perhaps this analysis with a wider taxonomic sample, or with other structures, could be as informative as traditional characters. However, centroid size and the other variables included in the traditional morphometric analysis were useful and led to a congruent phylogenetic hypothesis.

In summary, the results presented here agree with those of several other authors, showing that orchid seeds possess several characters of systematic and phylogenetic value (Clifford & Smith, 1969; Chase & Pippen, 1988; Molvray & Kores, 1995; Swamy *et al.*, 2004). Several of the variables used by Molvray & Kores (1995) to classify orchid seeds were useful, although their idea of using the seed type as a phylogenetic character could be replaced by using the measures separately. At least for this group of species, the classification proposed by these authors should be reviewed. The shape variation, represented by RW, was phylogenetically uninformative, consistent with the continuum observed in the graphical representations of RW. Centroid size was sufficiently informative to be considered as diagnostic or potentially useful in phylogenetic studies.

This is a first attempt at seed analysis in Chloraeeae. More species should be sampled and the number of seeds per species should be increased in order to perform new classifications. In the context of published phylogenetic analyses, new taxa from sister groups should be included to allow comparison of results and exploration of the phylogenetic utility of the characters on a larger scale.

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