

Pollen morphology of *Chamaebuxus* (DC.) Schb., *Chodatia* Paiva and *Rhinotropis* (Blake) Paiva (*Polygala* L., Polygalaceae)

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Abstract

Polygala L. is a large and highly diverse genus with complex taxonomy, but pollen morphological information for this taxon is scarce. In the present study, pollen characters have been used to assess the taxonomic delimitation and phylogenetic relationships of three newly established subgenera of *Polygala*: *Chamaebuxus*, *Chodatia* and *Rhinotropis* (*sensu* Paiva). The pollen morphology of 22 species has been examined using light microscopy and scanning electron microscopy of acetolysed material. The pollen of 15 of the species is examined for the first time. The pollen grains are isopolar, radially symmetrical, tectate and, typically, polyzonocolporate with numerous colpi running parallel to the polar axis, and an endocingulum around the equator. Two pollen types can be distinguished: Type I, which includes species belonging to *Rhinotropis*, and Type II, which includes species from *Chamaebuxus* and *Chodatia*. The two pollen types are described and the pollen of the three studied subgenera is illustrated. Despite the low infrageneric morphological diversity observed within the genus *Polygala*, quantitative characters of pollen grains support the current classification of the subgenera *Chamaebuxus*, *Chodatia* and *Rhinotropis*, and reveal a closer relationship between the first two taxa. Pollen characters are shown to be a useful and informative tool for assessing taxonomic position and phylogenetic relationships within Polygalaceae, especially at higher taxonomic levels.

Keywords: *Acetolysis, endocingulum, polyzonocolporate pollen, taxonomy, infrageneric relationships*

Polygala L. is the most representative genus within Polygalaceae, with more than 700 species widely distributed all over the world (except in the Arctic and New Zealand). It is a highly diverse genus of herbs, shrubs, trees and climbers with specialised mechanisms of pollination and seed dispersal (e.g. Brantjes, 1982; Westerkamp & Weber, 1997; Paiva, 1998; Forest et al., 2007a; Castro et al., 2008a, b). As a result of its diversity, high number of species, and wide distribution range, the taxonomy of the genus *Polygala* is highly fragmented. In the most recent and extensive work on the genus, Paiva (1998) observed many species of *Polygala* from all areas of distribution to gain a clearer understanding

of its taxonomy. This author organised *Polygala* into 12 subgenera and divided the old *Chamaebuxus* DC. into three distinct subgenera: *Chamaebuxus* (DC.) Schb., comprising the species from North Africa and Europe; *Chodatia* Paiva, including the species from Asia; and *Rhinotropis* Paiva, comprising the species from south-west North America and Mexico. Nonetheless, despite the disjunction in distribution of these three subgenera, several morphological characters (e.g. nectar gland and keel appendage morphology) appear to reveal a close relationship between them (Chodat, 1887, 1891; Paiva, 1998).

Due to their structural and morphological diversity, and highly conserved characteristics, pollen grains are

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frequently used in plant taxonomy and in the assessment of evolutionary relationships (e.g. Ortiz & Coutinho, 2001; Wang et al., 2003; Claxton et al., 2005; Sagun et al., 2006). Recently, pollen morphology has been used successfully to assess phylogenetic relationships between early divergent lineages of the Fabales clade (e.g. Claxton et al., 2005; Banks et al., 2008). In Polygalaceae, the distinctive pollen characters have been especially useful for the determination of generic boundaries (Chodat, 1896; Erdtman, 1944; Simpson & Skvarla, 1981; Paiva, 1998). Within the family, pollen grains are characterised by a large number of colpi running parallel to the polar axis; each colpus has an endoaperture and these endoapertures are more or less fused into an endocingulum in the equatorial region (i.e. the polyzonocolporate type: Paiva, 1998). Phylogenetic and pollen morphological studies suggest that the polycolporate condition is a characteristic of this family (Claxton et al., 2005; Forest et al., 2007a; Banks et al., 2008). Within *Polygala*, pollen features are described as highly variable, supporting the polyphyletic origin of the genus suggested by molecular phylogenetic analyses (Persson, 2001; Forest et al., 2007a; Banks et al., 2008). Several studies have revealed the value of pollen morphological characters in the delimitation of subgenera within this genus (Villanueva & Ramos, 1986; Furness & Stafford, 1995; Paiva, 1998). Although there are a number of studies which have analysed the pollen morphology of some *Polygala* species, the genus remains largely unexplored. For example, the pollen of species from subgenus *Chamaebuxus* (*sensu* Paiva, 1998) has been investigated by Merxmüller and Heubl (1983) and occasionally described in other studies (Villanueva & Ramos, 1986; Furness & Stafford, 1995; Paiva, 1998; Banks et al., 2008), but there have been no complete studies or descriptions for pollen of the subgenus based on acetolysed pollen. In addition, pollen morphology of *Chodatia* and *Rhinotropis* is almost completely unknown (only *P. desertorum* and *P. arizonae* from subgenus *Rhinotropis* (Paiva, 1998) and *P. arillata* from subgenus *Chodatia* (Banks et al., 2008), have been previously described).

This investigation focuses on the pollen morphology of the newly established subgenera *Chamaebuxus*, *Chodatia* and *Rhinotropis* (*sensu* Paiva, 1998), which were previously included in the same taxonomic group. The main objectives of the present study were: 1) to assess the taxonomic delimitation and phylogenetic relationships of the three subgenera based on pollen morphological characters, and 2) to describe the pollen morphology of the studied taxa in order to better understand species relationships within the genus *Polygala*. To do this, the pollen has been studied

with light microscopy (LM) to obtain data from quantitative characters, and with scanning electron microscopy (SEM) to observe qualitative characteristics of exine ornamentation.

Material and methods

Pollen samples were collected from a total of 52 herbarium specimens of the subgenera *Chamaebuxus*, *Chodatia* and *Rhinotropis* (see 'Specimens investigated') representing 22 species; the pollen of 15 of these species has not been studied previously. Pollen samples were taken from herbarium specimens in the following institutions: AVE, BM, GB, L, MO and RNG (abbreviations follow Holmgren et al., 1990). Light microscope slide preparations and scanning electron microscope stub preparations are held in the pollen reference collection of the University of Aveiro.

All of the species belonging to *Chamaebuxus* were sampled, 3–6 individuals per species. *Chodatia* and *Rhinotropis* were partially sampled, 1–3 individuals per species, due to unavailable material (up to 77% of the species according to Chodat, 1893). All pollen samples were subjected to acetolysis (Erdtman, 1960). The terminology used follows Punt et al. (2007).

For morphometric analysis, using light microscopy (LM), the pollen samples were pre-treated with t-butanol, mounted in silicon oil (Andersen, 1960), and observed using a Leitz Laborlux S light microscope fitted with a $\times 100$ oil immersion objective lens. Micrometer measurements of 36 pollen grains were taken for the following characteristics in all samples: polar axis (P), equatorial diameter (E), colpus width and length, diameter of the apocolpium, width of the endocingulum, and thickness of the costae in meridional optical section (m.o.s.). Colpi number in equatorial optical section (e.o.s.) and presence vs. absence of colpus ramifications were also recorded. Aborted grains were not measured.

To understand the surface morphology, in particular exine ornamentation, the pollen grains were also studied using scanning electron microscopy (SEM). Pollen grains were dehydrated in ethanol, mounted on metallic stubs and sputter coated with gold/palladium at high vacuum in a Jeol JFC-1100 Ion Sputter. Pollen samples were then observed with a Jeol JSM 5400 SEM, operating at 10 kV.

In order to investigate the differences and relationships among the subgenera, univariate and multivariate analyses were performed. For univariate analysis, descriptive statistics of quantitative variables were calculated for each subgenus, and differences among subgenera in the means of each variable were tested

using a Kruskal–Wallis one-way ANOVA followed by Dunn's method for all pairwise multiple comparisons (Zar, 1996). Descriptive statistics of quantitative variables were also calculated for each species. Multivariate analyses were carried out to determine the structural organisation of individuals based on all pollen morphological characters. Principal component analysis and a cluster analysis (UPGMA, Euclidean distance) were performed using all species and all measurements, except colpus length and width, which were included as a ratio (Sneath & Sokal, 1973).

The pollen type descriptions are based on both quantitative and qualitative data.

Results

Differences and relationships among the subgenera

Pollen measurements taken for all species examined within each subgenus allowed a detailed quantitative study of pollen characters (Tables I and II, respectively). Subsequently, statistical analysis revealed significant differences among subgenera for almost all the evaluated characters ($p < 0.05$; Table I). Pollen grains from subgenus *Rhinotropis* were typically smaller, with a lower number of colpi and smaller apertures (Table I). Despite the statistical differences obtained, it is difficult to distinguish the pollen from subgenus *Chamaebuxus* from that of subgenus *Chodatia* because several characters overlap in their ranges of variation (Tables I and II).

Relationships among subgenera based on pollen morphological data were explored using principal component analysis (PCA) and cluster analysis (CA). The PCA of the individuals studied is presented in Figure 1 and Table III (factor coordinates along the first three axes are provided in Appendix 1). The first three axes ('components') accounted for 84.5% of the total variation (Table III). The first component explained 54.9% of variation and had a high negative loading for all pollen morphological characters. The second component explained 17.4% of variation and had high positive loadings for P/E and percentage of colpus ramifications, and high negative loadings for equatorial diameter (E) and apocolpium diameter. The third component explained 12.1% of variation and had high positive loading for number of colpi and ratio between colpus length and width, and high negative loadings for thickness of the costae, P/E and endocingulum width. The first component clearly separates the species in subgenus *Rhinotropis* from the remaining subgenera (Figure 1A, B). Species from the subgenera *Chamaebuxus* and *Chodatia* overlapped along the first and second components (Figure 1A). However, despite the low percentage of variability explained by the third component,

Table I. Pollen characters and measurements for *Polygala* subgenera.

Values are given as means and standard deviations of the mean, followed in parentheses by minimum and maximum observed for the species. All values are given in μm , except Colpus ramification, which is given as a percentage for pollen grains with ramifications.

Subgenera	P	E	P/E	Colpi			Colpus ramification	Apocolpium	Endocingulum width	Thickness of costae
				n	Width	Length				
<i>Chamaebuxus</i> (DC.) Schb. (5 species)	47.1 \pm 3.7 ^a (44.0–50.0)	40.2 \pm 4.2 ^a (36.8–44.2)	1.18 \pm 0.09 ^a (1.13–1.23)	18 \pm 2 (17–21) ^a	3.3 \pm 0.7 ^a (2.4–3.8)	33.6 \pm 3.5 ^a (30.1–35.9)	16.4% ^a	25.6 \pm 3.3 ^a (22.9–28.9)	7.3 \pm 1.3 ^a (6.7–7.8)	3.7 \pm 0.7 ^a (3.1–4.1)
<i>Chodatia</i> Paiva (7 species)	53.1 \pm 5.6 ^b (46.3–57.5)	42.9 \pm 6.7 ^b (37.2–55.6)	1.26 \pm 0.17 ^b (1.02–1.55)	16 \pm 1 (14–18) ^b	3.7 \pm 0.5 ^b (3.2–4.0)	36.3 \pm 6.5 ^a (30.1–43.7)	27.0% ^b	27.6 \pm 4.6 ^b (23.3–32.3)	9.7 \pm 2.5 ^b (7.7–12.3)	5.3 \pm 0.9 ^b (4.1–6.3)
<i>Rhinotropis</i> (Blake) Paiva (10 species)	34.0 \pm 3.5 ^c (26.4–37.4)	30.7 \pm 4.1 ^c (23.5–34.9)	1.11 \pm 0.09 ^c (0.98–1.22)	12 \pm 2 (11–16) ^c	4.4 \pm 0.9 ^c (3.7–6.0)	23.5 \pm 3.1 ^b (16.8–26.9)	5.8% ^c	19.5 \pm 2.7 ^c (16.1–23.7)	4.3 \pm 1.1 ^c (2.9–5.8)	3.4 \pm 0.5 ^c (3.0–4.0)

Abbreviations, column headers: P – polar length; E – equatorial width; P/E – shape ratio (polar length divided by equatorial width); n – number of colpi. Within columns superscript letters to right of values indicate significant differences at $p < 0.05$.

Table II. Pollen characters and measurements for *Polygala* species.

Values are given as means and standard deviations of the mean, followed in parentheses by minimum and maximum observed for the species. All values are given in μm , except Colpus ramification, which is given as presence (+) or absence (-).

Species	P	E	P/E	n	Colpi width	Colpi length	Colpus ramification	Apocolpium	Endocingulum width	Thickness of costae
Subg. <i>Chamaebuxus</i>										
<i>P. balansae</i>	44.0 \pm 3.0 (39.5–49.6)	36.8 \pm 2.1 (33.0–41.5)	1.20 \pm 0.11 (1.01–1.48)	17 \pm 2 (14–20)	3.4 \pm 0.7 (2.0–4.6)	30.1 \pm 3.0 (23.8–37.0)	–	22.9 \pm 1.5 (20.3–25.4)	7.0 \pm 1.1 (4.6–9.1)	3.7 \pm 0.7 (2.0–4.6)
<i>P. chamaebuxus</i>	50.0 \pm 2.8 (43.6–58.9)	44.2 \pm 2.8 (37.5–48.2)	1.13 \pm 0.06 (1.06–1.26)	17 \pm 1 (16–19)	3.8 \pm 0.6 (2.5–4.6)	35.9 \pm 2.0 (31.4–41.0)	–	28.9 \pm 2.3 (24.3–33.0)	7.5 \pm 1.7 (4.1–11.7)	4.0 \pm 0.1 (2.1–5.9)
<i>P. munbyana</i>	46.9 \pm 3.6 (40.6–53.3)	38.3 \pm 3.0 (33.0–43.6)	1.23 \pm 0.05 (1.08–1.31)	20 \pm 1 (19–23)	2.4 \pm 0.4 (2.0–3.6)	35.5 \pm 2.7 (30.4–41.0)	+	23.9 \pm 2.9 (19.8–29.7)	6.7 \pm 1.1 (5.1–9.1)	3.2 \pm 0.5 (2.5–4.6)
<i>P. vayredae</i>	45.4 \pm 1.6 (42.6–48.7)	38.4 \pm 3.0 (32.5–43.1)	1.19 \pm 0.08 (1.05–1.41)	17 \pm 1 (14–19)	3.8 \pm 0.5 (3.0–5.1)	31.2 \pm 2.4 (24.3–34.5)	–	25.5 \pm 3.2 (19.8–31.4)	7.8 \pm 1.4 (4.6–10.7)	4.0 \pm 0.5 (3.0–5.1)
<i>P. webbiana</i>	48.8 \pm 3.6 (40.7–54.7)	43.1 \pm 3.7 (36.5–50.6)	1.14 \pm 0.09 (0.98–1.33)	18 \pm 1 (16–20)	3.1 \pm 0.5 (2.0–4.1)	35.0 \pm 3.0 (28.4–41.6)	+	26.9 \pm 2.8 (21.8–32.4)	7.5 \pm 1.1 (5.1–9.6)	3.5 \pm 0.7 (2.5–5.1)
Subg. <i>Chodatia</i>										
<i>P. arillata</i>	50.9 \pm 4.6 (42.1–58.8)	44.4 \pm 4.0 (35.5–51.2)	1.15 \pm 0.05 (1.07–1.33)	17 \pm 1 (15–19)	3.2 \pm 0.4 (2.5–4.1)	32.0 \pm 4.2 (24.9–39.6)	–	32.3 \pm 3.2 (24.3–38.6)	7.7 \pm 1.5 (4.1–10.1)	4.4 \pm 0.6 (3.0–5.6)
<i>P. karenium*</i>	52.9 \pm 2.6 (46.2–58.3)	41.5 \pm 1.5 (34.0–44.1)	1.28 \pm 0.04 (1.20–1.35)	16 \pm 1 (14–18)	3.7 \pm 0.4 (3.0–4.6)	37.7 \pm 2.7 (30.9–42.1)	+	24.2 \pm 1.8 (20.3–28.4)	11.3 \pm 1.2 (8.1–13.2)	6.4 \pm 0.6 (5.6–7.1)
<i>P. reinitii*</i>	50.2 \pm 2.1 (45.1–54.8)	37.2 \pm 1.4 (34.0–40.6)	1.35 \pm 0.05 (1.25–1.46)	18 \pm 1 (15–19)	3.3 \pm 0.3 (3.0–4.1)	30.1 \pm 1.5 (26.4–32.5)	+	30.4 \pm 1.4 (26.4–33.0)	7.7 \pm 0.5 (6.6–8.6)	5.6 \pm 0.5 (4.6–6.1)
<i>P. tonkinensis*</i>	56.5 \pm 2.7 (51.7–62.4)	55.6 \pm 3.4 (49.2–61.4)	1.02 \pm 0.07 (0.90–1.16)	14 \pm 1 (13–16)	3.6 \pm 0.4 (3.0–4.1)	36.5 \pm 2.3 (40.0–42.6)	–	33.7 \pm 2.2 (28.4–37.0)	9.8 \pm 1.5 (7.1–12.2)	5.6 \pm 0.5 (4.1–6.6)
<i>P. tricholopha*</i>	46.3 \pm 3.1 (41.6–51.7)	40.1 \pm 2.9 (35.5–44.6)	1.16 \pm 0.04 (1.07–1.21)	16 \pm 1 (15–18)	3.8 \pm 0.3 (3.0–4.1)	31.5 \pm 2.2 (22.3–35.0)	–	23.3 \pm 2.3 (20.3–32.5)	10.4 \pm 1.5 (8.1–13.7)	4.2 \pm 0.4 (3.6–5.6)
<i>P. venenosa*</i>	57.5 \pm 5.4 (49.7–70.0)	37.0 \pm 3.0 (33.0–43.6)	1.55 \pm 0.06 (1.44–1.68)	16 \pm 1 (14–17)	4.0 \pm 0.3 (3.0–4.6)	42.5 \pm 5.5 (35.5–51.7)	+	25.5 \pm 1.6 (21.8–27.9)	8.9 \pm 1.2 (6.6–11.1)	5.0 \pm 0.5 (4.1–6.1)
<i>P. sumatrana*</i>	57.3 \pm 5.9 (47.2–67.0)	44.6 \pm 4.2 (36.5–54.3)	1.29 \pm 0.07 (1.17–1.51)	16 \pm 1 (14–18)	4.0 \pm 0.4 (3.6–4.6)	43.7 \pm 7.6 (31.4–55.8)	+	23.6 \pm 2.0 (20.3–26.9)	12.3 \pm 3.8 (6.6–18.3)	6.1 \pm 5 (46–7.1)
Subg. <i>Rhinotropis</i>										
<i>P. acanthoclada*</i>	26.4 \pm 1.1 (24.9–29.9)	23.5 \pm 1.4 (21.8–26.9)	1.13 \pm 0.04 (1.06–1.21)	13 \pm 1 (12–14)	3.7 \pm 0.3 (3.0–4.1)	16.8 \pm 1.0 (15.2–18.8)	–	16.1 \pm 1.1 (14.2–18.8)	2.9 \pm 0.3 (2.5–3.6)	3.2 \pm 0.3 (2.5–4.1)
<i>P. californica*</i>	36.3 \pm 1.7 (32.4–39.5)	34.1 \pm 2.3 (28.9–38.5)	1.07 \pm 0.05 (0.99–1.30)	12 \pm 1 (11–14)	5.5 \pm 0.5 (4.6–6.6)	25.4 \pm 0.9 (23.3–27.3)	+	22.2 \pm 2.2 (18.2–25.8)	4.2 \pm 0.7 (3.0–6.1)	3.6 \pm 0.5 (3.0–4.6)

<i>P. cornuta</i> ssp.	37.4 ± 2.7 (32.5–43.6)	33.9 ± 2.6 (28.4–39.6)	1.11 ± 0.08 (0.96–1.27)	12 ± 1 (11–13)	6.0 ± 0.5 (5.1–7.6)	26.9 ± 2.5 (21.3–31.4)	–	19.3 ± 2.0 (16.2–25.4)	5.6 ± 0.8 (3.6–6.6)	3.2 ± 0.5 (2.5–4.6)
<i>fishiae</i> *	37.3 ± 1.8 (34.5–41.6)	34.9 ± 1.8 (31.4–38.6)	1.07 ± 0.03 (1.00–1.13)	16 ± 1 (14–17)	4.2 ± 0.4 (3.6–5.1)	24.8 ± 1.3 (22.3–27.4)	–	23.7 ± 1.2 (21.3–26.9)	5.8 ± 0.7 (4.6–7.1)	4.0 ± 0.4 (3.0–4.6)
<i>P. heterorhyncha</i> *	33.9 ± 1.5 (29.9–36.5)	29.1 ± 1.8 (26.4–32.5)	1.17 ± 0.06 (1.04–1.30)	14 ± 1 (13–16)	4.0 ± 0.3 (3.6–4.6)	22.5 ± 1.3 (20.3–25.4)	+	19.6 ± 1.3 (17.2–21.8)	4.3 ± 0.6 (3.0–5.1)	3.6 ± 0.4 (3.0–4.1)
<i>P. lindheimeri</i> *	34.1 ± 2.6 (30.4–39.6)	32.0 ± 4.8 (23.3–39.6)	1.08 ± 0.11 (0.84–1.30)	11 ± 1 (10–12)	4.3 ± 0.5 (3.6–5.6)	25.7 ± 2.3 (22.3–30.9)	+	17.0 ± 1.5 (13.7–20.3)	3.2 ± 0.7 (2.0–4.6)	3.3 ± 0.5 (2.0–4.1)
<i>P. nitida</i> *	33.2 ± 1.8 (30.4–36.5)	29.7 ± 1.9 (27.4–35.5)	1.12 ± 0.09 (0.98–1.30)	11 ± 1 (10–12)	3.8 ± 0.5 (3.0–5.1)	23.2 ± 1.8 (20.3–26.4)	+	18.2 ± 1.1 (16.2–21.3)	4.1 ± 0.7 (2.5–5.1)	3.4 ± 0.4 (2.5–4.1)
<i>P. rusbyi</i> *	32.7 ± 1.6 (30.4–36.5)	26.8 ± 1.6 (23.8–30.4)	1.22 ± 0.06 (1.11–1.36)	15 ± 1 (13–16)	3.8 ± 0.4 (3.0–4.6)	23.4 ± 1.3 (20.8–25.4)	–	17.6 ± 1.1 (14.2–19.3)	4.1 ± 0.4 (3.0–5.1)	3.0 ± 0.4 (2.0–4.1)
<i>P. subspinosa</i> *	35.9 ± 1.4 (30.9–38.0)	30.3 ± 1.5 (27.4–33.0)	1.19 ± 0.06 (1.07–1.33)	13 ± 1 (12–14)	4.3 ± 0.6 (3.0–5.1)	23.5 ± 1.3 (21.3–25.9)	+	20.6 ± 1.2 (18.3–22.8)	4.5 ± 0.7 (3.0–6.1)	3.4 ± 0.5 (2.0–4.6)
<i>P. tweedyi</i> *	32.6 ± 1.3 (30.4–35.5)	33.2 ± 1.3 (30.4–36.5)	0.98 ± 0.05 (0.90–1.06)	11 ± 1 (11–12)	4.8 ± 0.5 (4.1–5.6)	23.1 ± 1.7 (18.3–26.4)	–	20.9 ± 1.4 (18.8–25.4)	3.7 ± 0.4 (3.0–4.6)	3.3 ± 0.3 (3.0–4.1)

Abbreviations, column headers: P – polar length; E – equatorial width; P/E – shape ratio (polar length divided by equatorial width); n – number of colpi. An asterisk (*) indicates first study of pollen morphology for this species.

Chamaebuxus and *Chodatia* species tend to separate along this axis (Figure 1B).

The phenogram illustrated in Figure 2 was produced using cluster analysis. The clusters obtained are in agreement with the spatial arrangement of the individuals produced by PCA. Two clusters can be recognised: a cluster containing species from the subgenus *Rhinotropis* and a cluster containing species from the subgenera *Chamaebuxus* and *Chodatia*. Once again, based on all pollen morphological characters, *Rhinotropis* clearly separates from the remaining subgenera, while species from *Chamaebuxus* and *Chodatia* do not.

Despite low pollen morphological diversity, it is possible to distinguish two pollen types based on differences between a particular set of quantitative characters: pollen grain length and diameter, colpus number and length, and endocingulum width.

Pollen morphological descriptions

Pollen grains of *Polygala* are isopolar, tectate, suboblate to prolate, radially symmetric, outline lobate-circular in equatorial optical section (e.o.s.), and elliptic, elliptic-rectangular, elliptic-rhomboidal or sub-circular in meridional optical section (m.o.s.), apocolpia contracted (cap-like, e.g. Figure 4D, E) or attenuated. The aperture system is polyzonocolporate and endocingulate. The ectoapertures are long emarginate colpi with sub-polar rounded (e.g. Figure 4O) or obtuse apices (e.g. Figure 5L, P). Colpus membrane psilate, scabrate or granulate. The mesocolpia are sometimes ramified, and may be with or without microperforations. The apocolpia may be with or without depressions and microperforations. The nexine is 1–1.5 times thicker than the sexine.

Dichotomous key to pollen types

Average P < 38 μ m, average E < 35.5 μ m, average length of colpi < 28 μ m, average width of the endocingulum < 6.5 μ m, colpus number usually 11–14 (17)

Type I - *Rhinotropis*

Average P $\geq 40 \mu\text{m}$, average E $\geq 36.5 \mu\text{m}$, average length of the colpi $\geq 28 \mu\text{m}$, width of the endocingulum $\geq 6.5 \mu\text{m}$, colpus number (14)15–20 (21)

Type II - *Chamaebuxus* and *Chodatia*

Pollen type descriptions

Type I (Figure 3). – Pollen oblate-spheroidal to subprolate, P/E = 1.11 ± 0.09 (0.98–1.22); lobate-circular in e.o.s.; elliptic, elliptic-rectangular, elliptic-rhomboidal or sub-circular in m.o.s., not

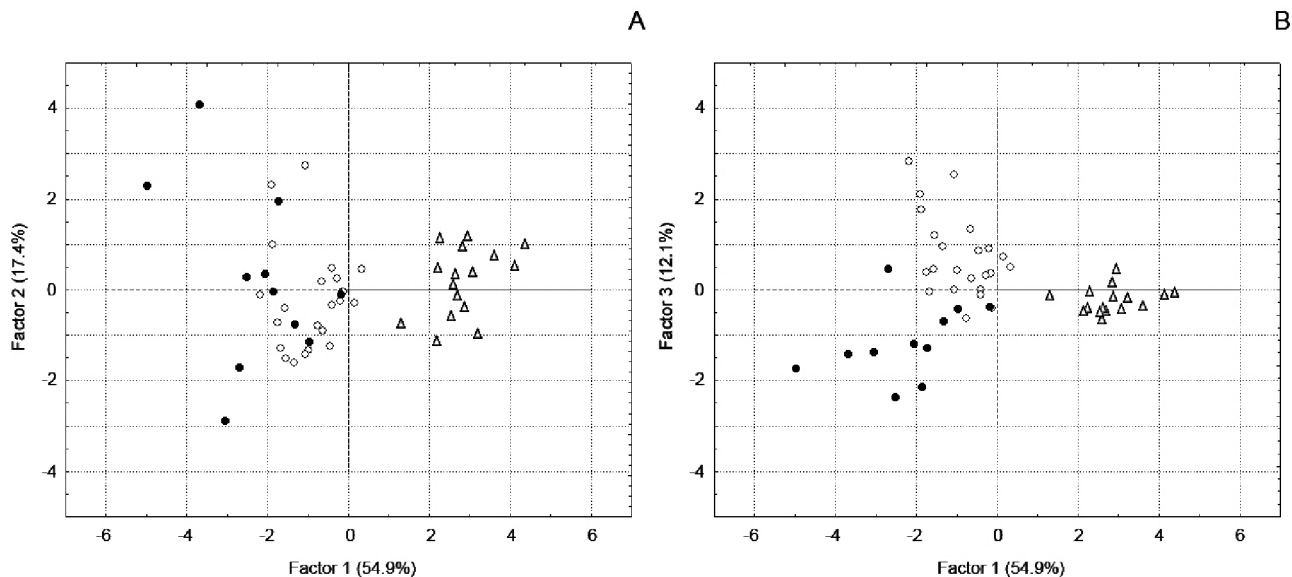


Figure 1. Principal component analysis performed with the pollen morphological characters and the species from the subgenera *Chamaebuxus* (open circles), *Chodatia* (closed circles) and *Rhinotropis* (open triangles). **A.** Scatterplot of the first and second components. **B.** Scatterplot of the first and third components.

Table III. Principal component analysis on nine variables of the 52 individuals from subgenera: *Chamaebuxus*, *Chodatia* and *Rhinotropis*.

Axis	Eigen values	Variance explained (%)	Cumulative variance explained (%)
1	4.9429	54.92	54.92
2	1.5657	17.40	72.32
3	1.0914	12.13	84.45

contracted in the apocolpia. $P = 34.0 \pm 3.5$ (26.0–37.5) μm , $E = 30.7 \pm 4.1$ (23.5–35.0) μm . Number of colpi 12 ± 2 (11–16), with 23.50 ± 3.1 (16.5–27.0) μm long and 4.4 ± 0.9 (3.5–6.0) μm wide; endocingulum 4.3 ± 1.1 (2.5–6.0) μm wide; costae 3.4 ± 0.5 (3.0–4.0) μm . Apocolpium diameter = 19.5 ± 2.7 (16.0–27.0) μm . Sculpture psilate and micro-perforate, rarely micro-rugulate and non-perforate.

Subgenus included: *Rhinotropis*

Type II (Figures 4, 5). – Pollen prolate-spheroidal to prolate, $P/E = 1.22 \pm 0.15$ (1.02–1.55); lobate-circular in e.o.s.; elliptic, sub-rectangular or sometimes sub-circular in m.o.s., frequently contracted in the

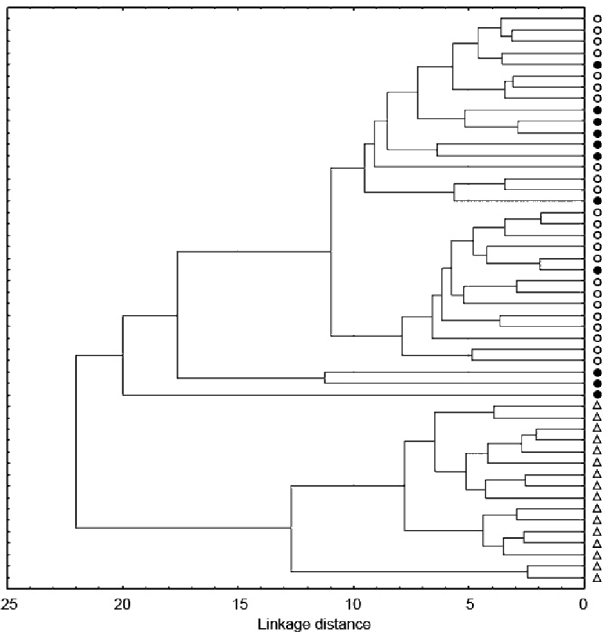
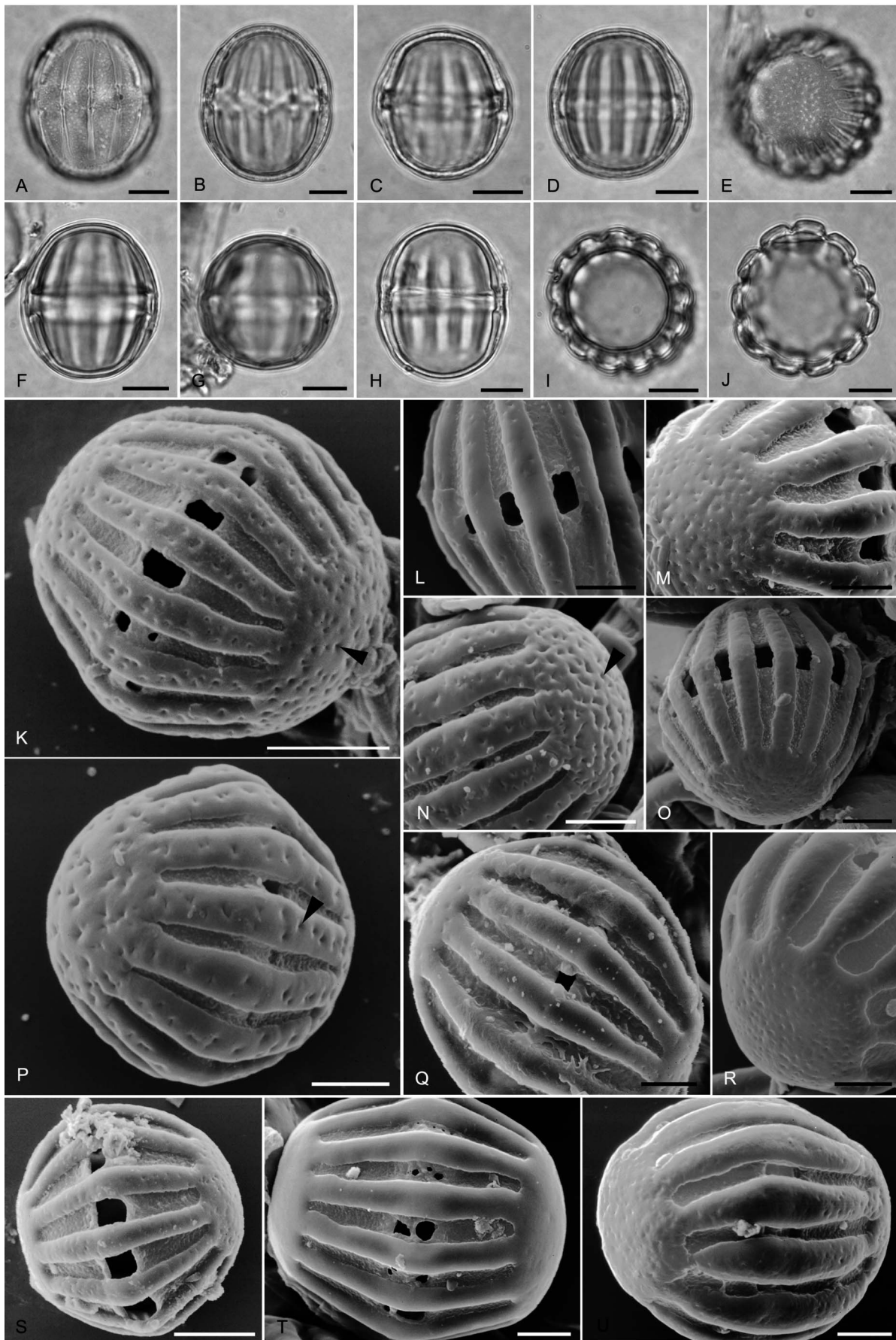
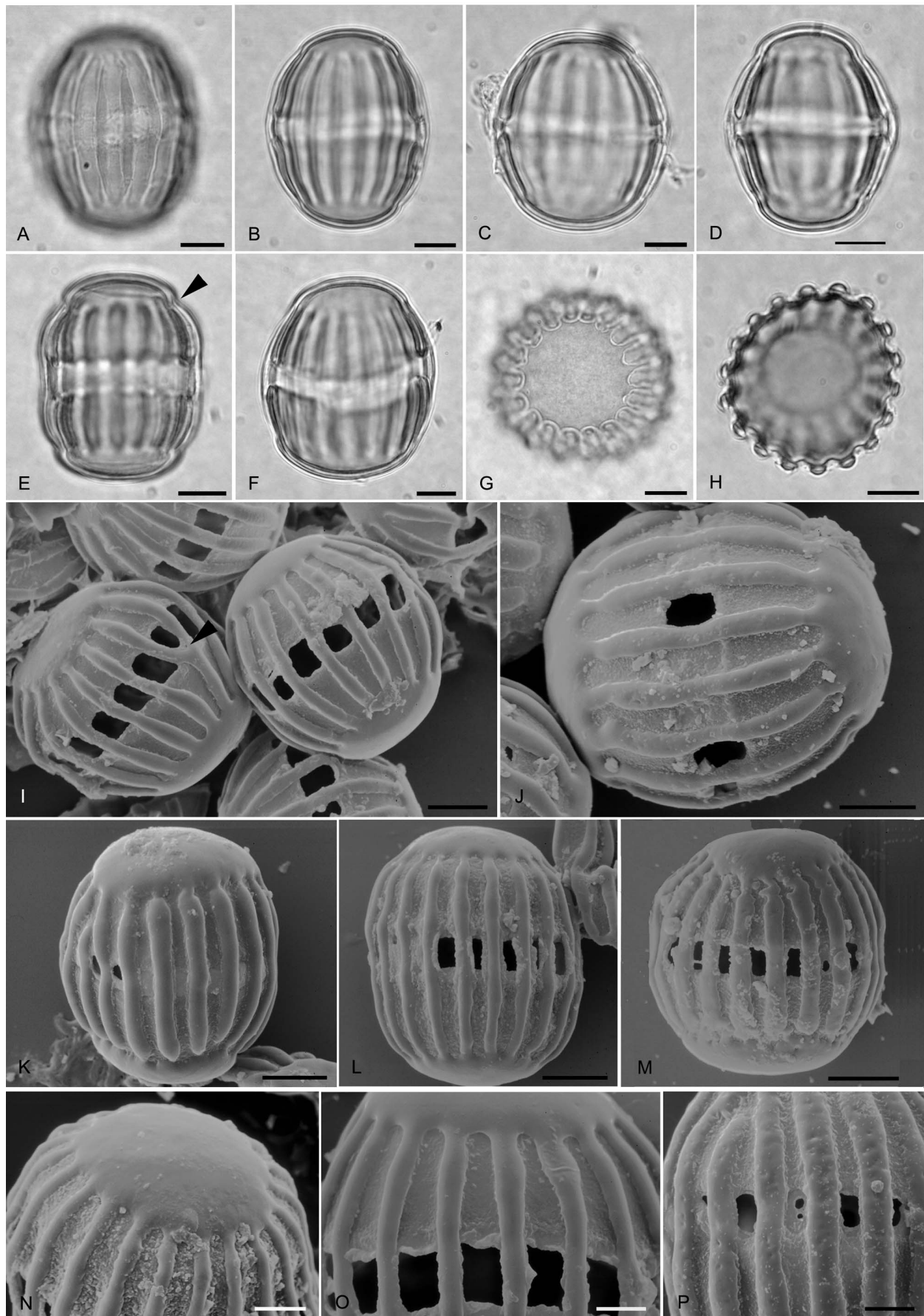


Figure 2. Cluster analysis (UPGMA, Euclidean distance) performed with the pollen morphological characters and the species from the subgenera *Chamaebuxus* (open circles), *Chodatia* (closed circles) and *Rhinotropis* (open triangles).

Figure 3. **A, B.** *Polygala* subg. *Rhinotropis* (**A–J.** LM; **K–U.** SEM). **A, B.** *Polygala fishiae*: **A.** equatorial view; **B.** meridional optical section (m.o.s.). **C & N, P.** *P. acanthoclada*: **C.** m.o.s.; **N.** detail of microperforate apocolpium and mesocolpia (arrow); **P.** equatorial view note microperforations, arrow). **D, E & K.** *P. desertorum*: **D.** m.o.s.; **E.** polar view; equatorial view (note microperforations, arrow). **F & O.** *P. rusbyi*: **F.** m.o.s.; **O.** polar view. **G.** *P. tweedi*, meridional optical section (m.o.s.); **H, I & L, M.** *P. subspinoso*: **H.** m.o.s.; **I.** polar view where the number of colpi can be observed; **L.** detail of an equatorial area; **M.** detail of the apocolpium. **J, R, U.** *P. californica*: **J.** equatorial optical section (e.o.s.) where the number of colpi can be observed; **R.** detail of an apocolpium; **U.** equatorial view. **Q.** *P. lindheimeri*, equatorial view. **S.** *P. nitida*, equatorial view. **T.** *P. heterorhyncha*, equatorial view. Scale bars – 10 μm (**A–K** & **S**); 5 μm (**L–R** & **T–U**).





apocolpia. $P = 50.6 \pm 5.7$ (44.0–57.5) μm , $E = 41.8 \pm 5.9$ (36.5–56.0) μm . Number of colpi 17 ± 2 (14–21), with 35.2 ± 5.6 (30.0–44.0) μm long and 3.5 ± 0.6 (2.0–4.0) μm wide; endocingulum 8.7 ± 2.4 (6.5–12.5) μm wide; costae 4.6 ± 1.2 (3.0–6.5) μm . Apocolpium diameter = 26.8 ± 4.2 (22.5–32.5) μm , infrequently with depressions (Figure 5J, Q). Sculpture generally psilate, less frequently scabrate or granulate.

Subgenera included: *Chamaebuxus* and *Chodatia*

Discussion

Within the order Fabales the family Polygalaceae has pollen grains with remarkably distinct and characteristic morphology (Claxton et al., 2005; Banks et al., 2008). In previous studies, pollen characters were shown to be useful not only for assessing taxonomic positions at lower levels within the family (e.g. Chodat, 1896; Erdtman, 1944; Simpson & Skvarla, 1981; Paiva, 1998), but also for assessing phylogenetic relationships at higher levels (e.g. Claxton et al., 2005; Banks et al., 2008). The present study provides valuable information on the pollen morphology of the genus *Polygala* and offers new insights regarding the relationship between three closely-related subgenera.

Pollen morphology in relation to taxonomy

The pollen of the studied species shares the morphology which is typical for the genus *Polygala*, i.e., isopolar polyzonocolporate pollen grains. However, detailed studies of the pollen have revealed quantitative differences among the studied taxa. The most notable differences include pollen grain size, number of ectoapertures (colpi), and dimensions of the endo- and ectoapertures. Species belonging to subgenus *Rhinotropis* have pollen which is distinctly smaller in size, has smaller endo- and ectoapertures and a lower number of colpi than the pollen of the species in the other two subgenera. The pollen grains of *Chodatia* have larger endoapertures, thicker costae, and a lower mean number of colpi than pollen from *Chamaebuxus*. However, despite these quantitative differences, overall distinction between the pollen from *Chodatia* and *Chamaebuxus* under the microscope is difficult because of a significant

overlap in the range of variation for most characters. Therefore, based on our quantitative data, two pollen types are recognised. Notably, the two pollen types correlate with the recent taxonomic delimitation, thus supporting the current classification of the subgenera *Rhinotropis*, *Chamaebuxus*, and *Chodatia* (Paiva, 1998) and confirming the close affinity between *Chamaebuxus*, and *Chodatia*. Further distinctions at lower taxonomic levels were not possible due to the high pollen variability between species, confirming the observations of other authors (Villanueva & Ramos, 1986; Furness & Stafford, 1995; Banks et al., 2008). The present results, together with previous studies, indicate that within Polygalaceae pollen characteristics are more useful and informative for higher level, than for lower level taxonomic discrimination.

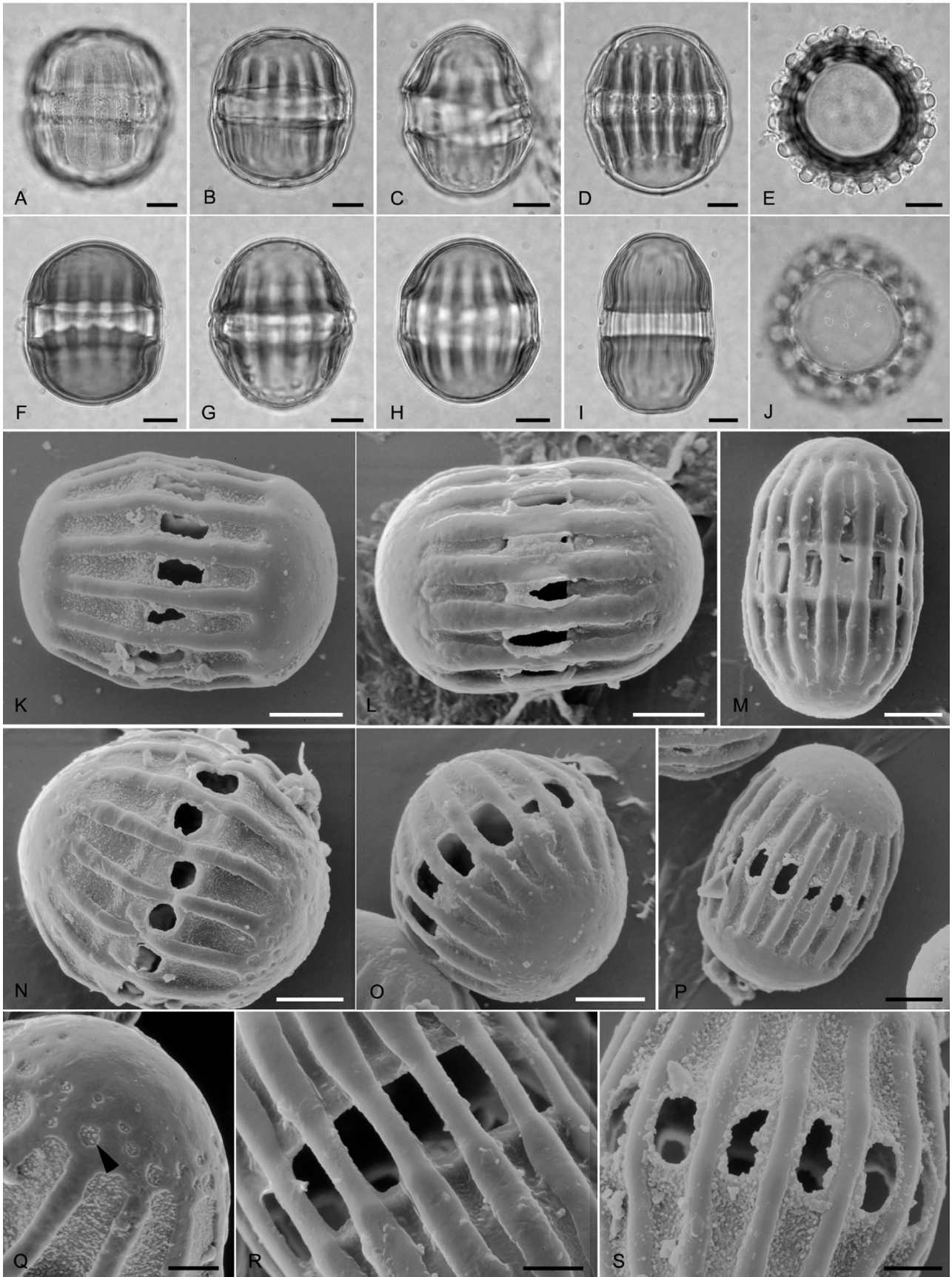
Differences and relationship among subgenera

Multivariate analyses of pollen morphological characters have been successfully performed to assess relationships in several taxonomic groups (e.g. Panajiotidis et al., 2000; Pardo et al., 2000). In the present study the spatial structure obtained from principal components and cluster analyses, using all the data from pollen characters, has revealed different relationships among the studied groups. The pollen characters show the closest relationship between individuals to be in subgenera *Chamaebuxus* and *Chodatia*. Molecular phylogenetic analyses reveal similar results. Despite the limited number of species studied, these data supported findings that subgenera *Chamaebuxus* and *Chodatia* are likely sister groups (Persson, 2001; Forest et al., 2007a), while *Rhinotropis* appears to be in a distinct clade (Forest et al., 2007a).

The close relationship between *Chamaebuxus* and *Chodatia* suggests migration of a common ancestral form from tropical Africa to North Africa and Europe (*Chamaebuxus*) and to Asia (*Chodatia*), while the ancestral taxon of *Rhinotropis* separated at an earlier time (Paiva, 1998). Fossil evidence and molecular estimates show that Polygalaceae originated, at the earliest, in the late Cretaceous (e.g. Lavin et al., 2005; Forest et al., 2007b). According to the theory elaborated by Paiva (1998), the ancestral

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Figure 4. *Polygala*, subg. *Chamaebuxus*. (A–H. LM; I–P. SEM). A, B, L & N. *Polygala munbyana*: A. & L. equatorial view; B. meridional optical section (m.o.s.); N. detail of one apocolpium. C. & J. *P. chamaebuxus*: C. m.o.s.; J. equatorial view. D, H, M. *P. balansae*: D. m.o.s.; H. equatorial optical section (e.o.s) where the number of colpi can be observed; M. equatorial view. E, K, P. *P. vayredae*: E. m.o.s. with conspicuous contractions in apocolpium (arrow); K. equatorial view; P. detail of apertures and microperforations in mesocolpium. F, G, I & O. *P. webbiana*: F. m.o.s.; G. polar view; I. several grains where a ramification can be observed (arrow); O. detail of apertures and smooth mesocolpium. Scale bars – 10 μm (A–M); 5 μm (N–P).



form of Polygalaceae, or even *Polygala*, was already present at the moment of separation of the Afro-Brasilian plate, generating 2–3 tropical centres of diversification of the genus: one in South America, another in Africa, and another in Madagascar. From the tropical African centre, the genus spread via north-west Africa and Gibraltar to Europe, where *Chamaebuxus* most probably originated. This subgenus is currently represented by five species, three in northern Africa (*P. balansae*, *P. munbyana* and *P. webbiana*), one in Spain (*P. vayredae*) and another in the Alps and Central Europe (*P. chamaebuxus*), and their distribution could be interpreted as evidence for the proposed migration line. Another likely migration route follows a line from north-east Africa to tropical Asia, from where *Chodatia* appears to have originated. In South America the ancestral members of Polygalaceae underwent high diversification rates, resulting in eight distinct subgenera, including *Rhinotropis*. However, further studies are needed to confirm this theory.

A range of cladistic analyses of the 12 subgenera of *Polygala* produced differing results (Paiva, 1998). One cladogram places *Chamaebuxus*, *Chodatia* and *Rhinotropis* in the same cladistic line, while in another they appear in separate cladistic lines, with *Rhinotropis* being the most divergent group. However, recent molecular studies suggested that the characters used for defining *Polygala* are plesiomorphic and thus not useful for inferring phylogenetic relationships (Persson, 2001). As a result, the cladistic analyses of Paiva (1998) should be viewed with caution, because some of these plesiomorphic characters were used in the analyses (e.g. fertile stamens and capsule). Despite the insights provided by pollen morphology in the present study, further pollen morphological studies on the remaining subgenera, as well as more extensive molecular analyses, will be necessary to elucidate the evolutionary relationships within *Polygala*.

A particularly interesting pollen characteristic of *Polygala* is the number of ectoapertures. In the present study, polymorphism in aperture number was observed within all studied species. Additionally, in the present study, an increase in the number (minimum – maximum) of ectoapertures was observed between the subgenera: *Rhinotropis* with the lowest number range, *Chodatia* with a higher

number range and *Chamaebuxus*, with the highest number range. This variability within species and differences among taxa are interesting, not only from a taxonomic and evolutionary point of view, but also from a functional perspective. Pollen grain apertures are important characteristics in systematic studies. In life they have key functional roles in the plant life cycle. Their primary role is as the specialised region(s) of the pollen wall for pollen tube germination (Walker & Doyle, 1975). Furthermore, they act as sites for water uptake and accommodation of volume changes (harmomegathy; Wodehouse, 1935), thus playing a major role in the protection of the male gametophyte from dessication, fungal attack and mechanical stress (Edlund et al., 2004), and also allowing the transfer of recognition substances (Blackmore & Crane, 1998; Edlund et al., 2004 and references cited therein). Variability in characters such as colpus number could lead to different functional outputs and different reproductive success, and a number of studies have explored these possibilities, including, for example, Dajoz et al. (1991, 1993). The morphology of angiosperm pollen, most notably in the eudicotyledons, has evolved towards an increasing number of apertures over evolutionary time (e.g. Walker & Doyle, 1975), suggesting that they are subjected to strong selective pressures (e.g. Furness & Rudall, 2004). A higher number of apertures have been correlated with higher germination rates, possibly an adaptive response to enhance pollen competitive ability in the style, and early fertilisation, a trait subsequently correlated with lower survival rates, viewed as a result of increasing efficiency of pollination in animal-pollinated species (Dajoz et al. 1991, and references therein). The variability of aperture number observed in the present study could make *Polygala* an interesting genus in which to study the significance and evolution of increased pollen grain aperture number.

Comparison of the results obtained with previous studies

Despite being slightly smaller, in general, the measurements obtained in the present study were consistent with the ranges of variation obtained in previous studies on this family (Merxmüller & Heubl, 1983; Villanueva & Ramos, 1986; Furness & Stafford, 1995; Paiva, 1998; Banks et al., 2008). Slight fluctuations

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Figure 5. *Polygala*, subg. *Chodatia*. (A–J. LM; K–S. SEM). A, B, E, J, K & S. *Polygala arillata*: A. & K. equatorial view; B. m.o.s.; E. e.o.s.; J. polar view where the number of colpi can be observed; S. detail of the apertures. C. *P. karensium*, equatorial view. D. & P. *P. reinii*: D. m.o.s.; P. equatorial view. F. & L. *P. sumatrana*: F. m.o.s.; L. equatorial view. G, N, Q. *P. tonkinensis*: G. m.o.s.; N. equatorial view; Q. detail of the apocolpium with several depressions (arrow). H. & O. *P. tricholopha*: H. m.o.s.; O. equatorial view. I, M, R. *P. venenosa*: I. m.o.s.; M. equatorial view; R. detail of the apertures. Scale bars – 10 µm (A–P); 5 µm (Q–S).

in the size of pollen grains could be due to different methodological techniques, such as hydration of the samples (e.g. Furness & Stafford, 1995) or the use of different mounting mediums (Andersen, 1960).

Contrary to the report by Merxmüller and Heubl (1983), no depressions were observed in the apocolpium of the pollen grains from *Chamaebuxus*. These authors described simple depressions in the apocolpium of *P. balansae*, *P. munbyana* and *P. webbiana* (one individual per species was observed), but in the present study none of the 12 individuals belonging to these species showed these depressions. The frequency of depressions in the pollen grain population from each individual studied by Merxmüller and Heubl (1983) is also unknown. Thus, it seems that this character could be generally absent, appearing only occasionally in some individuals. However, depressions in the apocolpium of varying shape, distribution, and number have been described for several species of *Polygala* (reviewed in Paiva, 1998), indicating that this character is variable and must be assessed with caution if used in morphological analyses.

In previous studies the shape of the pollen grains was found to be very useful in the characterisation of pollen types (e.g. Villanueva & Ramos, 1986), although in the present study it has not been an especially useful character because of its variability within the subgenera. To some extent this could be a result of the large number of species (and samples) studied, which has amplified the range of pollen shape within the subgenera, and consequently reduced its value as a discriminative character.

Conclusions

Pollen characters are a useful tool to assist in taxonomic delimitation and the investigation of relationships among the subgenera of *Polygala*. Pollen morphology supports the current classification of the subgenera *Chamaebuxus*, *Chodatia* and *Rhinotropis* (Paiva, 1998) and indicates a closer relationship between *Chamaebuxus* and *Chodatia*. Further pollen studies and more extensive molecular analyses, involving the other subgenera, are needed to allow a fuller understanding of the evolution of the genus *Polygala*.

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Specimens investigated

Subg. *Chamaebuxus* (DC.) Lchb.

- Polygala balansae* Coss. Morocco: Ifoane, 1320m. Lynes 189 (BM); Morocco: Ighrem d'Ougdal, 1700m. Davis 53988 (BM); Morocco: NNE Asni, Gorge de Moulay Brahim, 1050m. Jury 14159 (BM); Morocco: High Atlas, S Marrakech, S Tizi-n-Test Pass, 1470m. Jury 14223 (BM); Morocco: Immouzer, 200m. Miller, Russell & Sutton 434 (RNG).
P. chamaebuxus L. Austria: Salzkammergut, SW Bad Goisern. Watson s.n. (RNG); France: Alps, SE Briançon, S Col d'Izoard, 2300m. Jury 6343 (BM); Italy: Frosinone, Cassino, St. Elia. Lupton s.n. (BM); Italy: Stresa, Maggior. Hb. Heard s.n. (BM); Italy: Treviso, Monte Pallone, Possagno, 600–700m. Davis 34074 (RNG); Switzerland: Ticino, Lugano, San Salvatore Mountains. Hb. Lacaita 5925 (BM).
P. munbyana Boiss. & Reuter. Algeria: Oran. Wariay s.n. (MO); Morocco: Djebel Hamman Mt., 400m. Font Quer 291 (BM); Morocco: Imzouene, W Al Hoceima, 420m. Jury 13548 (RNG).
P. vayredae Costa. Spain: Girona, Alta Garrotxa, Montmajor, 1070m. Castro 1 (AVE) (5 distinct individuals).
P. webbiana Coss. Morocco: Jebela. Lynes s.n. (BM, MO); Morocco: Kalaa Mt. near Xauen, 1000m. Font Quer 252 (BM); Morocco: Tétouan. Guindal E7445 (RNG).

Subg. *Chodatia* Paiva

- Polygala arillata* Buch.-Ham. ex D. Don. Burma: Mindat, 2300m. Ward 22238 (GB).
P. arillata Buch.-Ham. ex D. Don. China: Sichuan, Hen Ya, 900m. Cehong 334 (MO); China: Yunnan, Diqing, Weixi, 3050m. Aldén et al. 1600 (GB).
P. karenium Kurz. Thailand: Chiang Mai, Chiang Dao Mt., 1650m. Maxwell 90–767 (MO).
P. reinmii Franch. & Sav. Japan: Kobe, Mount Maya. Yatabe s.n. (BM).
P. sumatrana Chodat. Indonesia: Sumatra, Habinsaran. Bartlett 7936 (L); Indonesia: Sumatra, Jambi, Kerinci, Danau Gunung Tujuh, 2000m. Morley 437 (L).
P. tonkinensis Chodat. Vietnam: Ninh Binh, Cuc Phuong National Park, 100m. Cuong 324 (MO).
P. tricholopha Chodat. Burma: Kachin, Mahtum, 1370m. Kaulback 345; Kaulback 373 (BM).
P. venenosa Juss. ex Poir. Brunei: Temburong, Sungai Temburong, Kuala Belalong. Dransfield 6704 (MO); Malaysia: Sarawak, Padawan, Braang, 360m. Chai S.37366 (MO).

Subg. *Rhinotropis* (Blake) Paiva

- Polygala acanthoclada* A. Gray. USA: California, E Mojave Desert, Sagamore mine, 1580m, Robert, Thorne & Tilforth 44132 (BM).
P. acanthoclada A. Gray. USA: California. Brandegee s.n. (MO).
P. californica Nutt ex Torr. & Gray. USA: California, Santa Cruz Co. Kearney s.n. (GB).
P. californica Nutt ex Torr. & Gray. USA: California, Sonoma Co., 150m. Rose 51004 (GB).
P. cornuta ssp. *fishiae* (Parry) Munz. USA: California, Ventura Co., Black Mountains. Pollard s.n. (GB); USA: California, Ventura Co., W Ojai. Pollard s.n. (GB).

- P. desertorum* Brandege. Mexico: Baja California, Cerro Blanco, 500m. Wiggins & Thomas 138 (BM).
- P. heterorhyncha* (Barneby) T. Wendt. USA: Nevada, Nye Co., Death Valley, 1300m. Wendt 1509 (MO).
- P. lindheimeri* A. Gray. Mexico: Coahuila, Nueva Rosita. Powell & Turner 2716 (MO); USA: Texas, Kinney Co. Correll & Johnshon 19462 (MO).
- P. nitida* Brandege. Mexico: Zacatecas, NE Juchipila, 1500m. Johnston, Chiang & Wendt 12238 (MO).
- P. rusbyi* Greene. USA: Arizona, Montezuma Castle. Nelson & Nelson 2052 (MO); USA: Arizona, Peach Springs. Lemmon & Lemmon s.n. (BM).
- P. subspinoso* S. Watson. USA: Nevada, Nye Co., White River Valley, 1600m. Windham 93–46 (MO); USA: Utah, Mercur, 1670m. Jones s.n. (MO).
- P. tweedyi* Britton. USA: Texas, Coleman Co., Santa Anna. Correll & Johnston 19010 (MO).

Appendix 1

Results of principal component analysis on 9 variables of 52 specimens of *Polygala*: factor coordinates of the variables along the three first axes and percentage of total variance explained by each axis.

Variable	Axis 1	Axis 2	Axis 3
P	-0.967199	-0.037206	-0.092219
E	-0.843296	-0.460451	0.019287
P/E	-0.424637	0.717247	-0.210243
Colpi number	-0.734075	0.057407	0.543974
Colpi length/ width ratio	-0.837471	0.202106	0.421224
Ramifications	-0.384351	0.739734	0.094411
Apocolpium	-0.765199	-0.496422	0.060470
Endocingulum	-0.861417	0.006404	-0.272718
Exine in costae	-0.632766	-0.002621	-0.691423
Variance explained by each axis	54.9%	17.4%	12.1%

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