



**Pollen morphology of the genus *Omphalodes* Mill.  
(Cynoglosseae, Boraginaceae)**

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3 **Pollen morphology of the genus *Omphalodes* Mill. (Cynoglosseae,**  
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6 **Boraginaceae)**  
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11 **Short running title:** Pollen morphology of *Omphalodes* Mill.  
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30  
31 **Abstract**  
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33 To better understand the taxonomy and phylogeny of the genus *Omphalodes* Mill.  
34 (Boraginaceae), the pollen morphology of 23 species encompassing all major  
35 geographical and morphological groups was studied from acetolysed pollen grains using  
36 light and scanning electron microscopy. Three pollen types were distinguishable through  
37 apertural morphology and sculpture. A dichotomous key to the pollen types and pollen  
38 descriptions are provided. Despite being fairly homogenous, palynological data provided  
39 useful information to understand the relationships of *Omphalodes*, considering the  
40 uselessness of most floral and fruit characters, karyological homogeneity, and striking  
41 disjunct geographical distribution. At the suprageneric level, pollen morphology supports  
42 traditional tribal classification of *Omphalodes* within Cynoglosseae, in contrast to  
43 published molecular phylogenetic analyses. At the infrageneric level, pollen morphology  
44 agrees with phylogenetic analyses suggesting that *Omphalodes* could be a polyphyletic  
45 group. Palynological data would support the segregation of a monophyletic group  
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3 comprising the Japanese species, corroborating recent phylogenetic results, and define  
4 two different pollen types for the *Omphalodes* s.str. group. Biogeographical  
5  
6 considerations are also discussed.  
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12 **Keywords:** *acetolysis, dichotomous key, disjunct biogeographic pattern, ring-like*  
13 *aperture, phylogenetic relationships, pollen types, taxonomy*  
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6 *Omphalodes* Mill. (Boraginaceae, Boraginoideae) comprises 28 species of herbs from  
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8 temperate habitats, including mostly narrow endemics but also some ornamental species  
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10 like *O. verna* Moench. The genus has a striking disjunct distribution in the Northern  
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12 Hemisphere, occurring in three distant areas: Europe and adjacent areas of western Asia,  
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14 northern Mexico and Texas, and Japan, being an unusual variant of the north temperate  
15  
16 disjunct biogeographic pattern (*sensu* Raven, 1972). The genus shows a great variation in  
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18 habit (perennial, biennial and obligate annual species), ecology (ranging from coastal  
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20 dunes to mountain summits or mesic habitats), and morphology (e.g., fruit characters).  
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22 Still, some species seem to form natural subgroups as, e.g., the Japanese species or the  
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24 annual species from south-western Europe. Several sections have been described for the  
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26 western Euroasiatic taxa (de Candolle, 1846; Brand, 1921; Popov, 1953), but their  
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28 evolutionary significance has not been assessed. It has been suggested that the Japanese  
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30 species should be considered a subgenus (Popov, 1953) and that *O. scorpioides* (Haenke)  
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32 Schrank could even deserve an independent genus (Johnston, 1924), albeit no formal  
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34 proposals have been done so far. Despite its biogeographic singularity and the evidence  
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36 for a certain infrageneric structure (Popov, 1953), no attempts have been done to find  
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38 morphological characters that could clarify the internal relationships of the genus.  
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46 The subfamily Boraginoideae has been divided into several tribes that vary from four  
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48 to eight, depending on the author (de Candolle, 1846; Riedl, 1997). *Omphalodes* has been  
49  
50 traditionally ascribed to the tribe Cynoglosseae (Brand, 1921), mainly because of the  
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52 nutlet apical attachment to the gynobase (Johnston, 1924). Indeed, the internal  
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54 systematics of the Boraginoideae, and in particular of the Cynoglosseae (Johnston, 1924;  
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56 Riedl, 1997), heavily relies on fruit traits. However, this classification has been  
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58 considered weak (Al-Shehbaz, 1991) mainly because fruits from this group are suggested  
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3 to be under evolutionary selective pressures, thus, being labile characters (Johnston,  
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5 1924; Selvi et al., 2011) unsuitable for taxonomic purposes. Molecular studies of  
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7 Boraginaceae including all Boraginoideae tribes (Långström & Chase, 2002) and a  
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9 representative of *Omphalodes* (the type species *O. verna*) have been performed to shed  
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11 light on the phylogenetic relationships of the family. Interestingly, the phylogeny  
12  
13 recovered merged the tribes Cynoglosseae, Eritrichieae, Myosotideae and Trigotideae and  
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15 located *Omphalodes* outside of the Cynoglosseae s.l. clade at an unresolved position  
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17 among all tribes of the subfamily Boraginoideae. Recent molecular phylogenetic analyses  
18  
19 of *Omphalodes* species (Serrano et al., unpublished data) recovered five main clades: the  
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21 American species, the annual European species, the western Eurasian perennial species,  
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23 the Japanese species, and *O. scorpioides*. Two clades, the Japanese group and *O.*  
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25 *scorpioides*, are separately nested among other genera of Cynoglosseae, therefore making  
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27 the genus polyphyletic as currently circumscribed. However, morphological and  
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29 karyological support for the inferred relationships is scarce. Floral characters are  
30  
31 relatively homogeneous throughout the genus, and fruit characters, albeit very variable,  
32  
33 hardly show discontinuities that could be clearly correlated with the inferred phylogenetic  
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35 groups. Karyological information is also relatively helpless to clarify the internal  
36  
37 evolutionary relationships of the genus, since the base number of the Boraginaceae ( $x =$   
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39 12; Lewis, 1980) is much conserved within the Cynoglosseae (Luque & Valdés, 1986;  
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41 Coppi et al., 2006; Selvi et al., 2011) and is present as  $2n = 24$  in both western Eurasian  
42  
43 (Grau, 1967) and Japanese (Kadota, 2009) species of *Omphalodes*. The exceptions are the  
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45 European annual taxa, having a derived chromosome number  $2n = 28$ , (Grau, 1967;  
46  
47 Valdés, 1987) and *O. caucasica* Brand with  $2n = 22$  (Grau, 1967).

58 The Boraginaceae show a great diversity in pollen ornamentation, size, shape and  
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60 number of apertures and the family is considered one of the most eurypalynous among

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3 the angiosperms (Díez & Valdés, 1991). Several palynologists have demonstrated the  
4 importance of the palynological characters to understand the taxonomy, ecology and  
5 evolution of Boraginaceae (e.g., Díez et al., 1986; Ning et al., 1992; Scheel et al., 1996;  
6 Retief & van Wyk, 1997; Liu et al., 2010). Pollen morphology has been used as a  
7 valuable tool in delimiting genera within Cynoglosseae (Barbier & Mathez, 1973) and in  
8 suggesting several evolutionary hypotheses (Clarke et al., 1979; Bigazzi et al., 2006). A  
9 few authors published some significant studies of the pollen morphology of *Omphalodes*  
10 using light and, in some cases, scanning electron microscopy (Barbier & Mathez, 1973;  
11 Díez, 1984; Ahn & Lee, 1986; Díez & Valdés, 1991). Still, these are partial studies  
12 including only a few species and, thus, pollen morphology of *Omphalodes* is  
13 insufficiently known.  
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29 The goal of this work was to investigate for the first time the variability of pollen  
30 morphology in *Omphalodes*, studying the majority of the species recognized and  
31 encompassing all major geographical and morphological groups, through light and  
32 scanning electron microscopy of acetolysed material. Since pollen is highly variable in  
33 Boraginaceae, it was hypothesized that the extremely disjunct distribution range of  
34 *Omphalodes* spp. suggests an ancient origin of the genus that may have allowed enough  
35 evolutionary time for accumulation of informative morphological changes. Palynological  
36 information was used to address two main questions in order to shed light to the complex  
37 internal systematics and the striking geographical pattern of *Omphalodes*. First,  
38 considering that molecular phylogenetic analyses questions the position of *Omphalodes*  
39 within the Cynoglosseae (Långström & Chase, 2002), palynological data was studied to  
40 test whether pollen morphology supports the traditional tribal adscription of the genus.  
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3 variation in pollen morphology and the recent phylogenetic results that singularize,  
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5 among others, the Japanese group.  
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## 8 9 10 **Materials and methods**

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12 Pollen samples were collected from a total of 34 herbarium specimens of *Omphalodes*  
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14 genus (see *Specimens investigated*) from the following institutions: COI, IEB, K, MA, P,  
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16 SALAF, SANT, TEX, TI and TNS (abbreviations follow Holmgren et al., 1990). The  
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18 samples included 26 taxa from *Omphalodes* genus with representatives from all the  
19  
20 geographic regions. All pollen samples were subjected to acetolysis according to the  
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22 method proposed by Erdtman (1960). The terminology used for pollen descriptions  
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24 follows Punt et al. (2007) and Hesse et al. (2009).  
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29 For light microscopy (LM), pollen grains were pre-treated with t-butanol, mounted in  
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31 silicone oil (Andersen, 1960) and observed using a Motic BA 310 light microscope with  
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33 an oil immersion objective lens (magnification = 1000×). The exine thickness was  
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35 measured in 15 pollen grains from each taxa using a micrometer.  
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39 For scanning electron microscopy (SEM), the acetolysed pollen grains were air dried  
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41 (Ahn & Lee, 1986), mounted on aluminium stubs, and coated with a 30 nm layer of gold-  
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43 palladium for 8 min at high vacuum in a sputtering chamber (Jeol JFC-1100 Ion Sputter).  
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45 Pollen grains were then observed with a Zeiss FESEM ULTRA plus (operating at 15 kV),  
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47 and micrographs of at least 15 pollen grains were taken using ImageTool (v.3.0 for  
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49 Windows, University of Texas Health Science Center, San Antonio, TX, USA). Due to  
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51 the small pollen size, micrographs were then used for morphometric analysis. The  
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53 following characters were measured in 15 pollen grains from each taxon: polar axis (P),  
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55 equatorial axis (E), maximum width of the grain, pseudocolpi length, colpori ectoaperture  
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57 length, and endoaperture width. The ratio P/E was then calculated. The general pollen  
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3 morphology and particularly the type of sculpture of the margins of the pseudocolpi and  
4 colpori, and the presence/absence of a ring-like aperture were studied also from the SEM  
5 micrographs. The ring-like aperture is an outer circumferential aperture at the equator of  
6 the pollen grain (following Hesse et al., 2009).  
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12 Descriptive statistics (mean and standard deviation of the mean) of the quantitative  
13 variables were calculated for each species (subspecies were not considered due to the low  
14 variability observed within species). Multivariate analyses were performed to investigate  
15 the structural organization of the species studied based on all the quantitative and  
16 qualitative palynological characters combined. Principal component analysis (PCA) was  
17 performed using the specimen mean values for and all the measurements, except exine  
18 thickness.  
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29 Palynological description of pollen types were made based on the quantitative and  
30 qualitative morphologic results.  
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## 36 **Results**

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38 The morphology of the pollen belonging to 23 species of *Omphalodes* (covering 82% of  
39 the genus) was studied under using LM and SEM (Table I, Figures 1-2). This study  
40 constitutes the first survey on the pollen morphology for 19 of the species studied.  
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45 Overall, pollen morphologic features, including polarity, symmetry, shape, size and  
46 exine thickness of the species of *Omphalodes* studied was revealed to be fairly constant  
47 (Figures 1, 2). The most important characters for pollen type delimitation were  
48 qualitative: the sculpture of the margins of colpori and pseudocolpi and the  
49 presence/absence of a ring-like aperture. Despite the continuous gradient of transition  
50 observed among the species for all the quantitative characters (Table I), it was possible to  
51 recognize three pollen types based on the presence/absence of a ring-like aperture and on  
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3 the presence/absence of conspicuous granula in the apertural margins: *O. verna* pollen  
4 type, *O. japonica* pollen type and *O. chiangii* pollen type (see descriptions below).  
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6 Interestingly, the abundance of these sculpture elements seems to be positively correlated  
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8 to annual habits and negatively to perennial American species, and the presence of a ring-  
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10 like aperture is restricted to the Japanese perennial species (Table I, Figures 1, 2).  
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15 The PCA of the specimens studied produced the results presented in Figure 3. The  
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17 first three axes accounted for 86.1% of the total variation (55.1, 17.2 and 13.8% of  
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19 variation explained by the first, second and thirds components, respectively). The first  
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21 component had a high negative loading for all the palynological characters except P/E  
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23 ratio, while the second component had negative loadings for all the characters except E,  
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25 pseudocolpi length and the presence/absence of granula in the ectoapertures margin, for  
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27 which positive loadings were obtained (Figure 3). The multivariate analysis enabled us to  
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29 separate the three pollen types across the two first components (Figure 3). Clearly, a  
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31 group including the Japanese species (*O. japonica* pollen type) separates from the  
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33 remaining species in both axes due to their greater pollen size (reflected in the characters  
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35 measured) and qualitative characters (presence of a ring-like aperture and granula in the  
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37 apertural margins) (Figure 3). The remaining two groups (*O. verna* and *O. chiangii* pollen  
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39 types) show a continuum but can be separated from each other across the second  
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41 component (Figure 3). These observations corroborate the pollen types defined below.  
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#### 50 *General palynological description of Omphalodes*

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52 Pollen grains radially symmetrical; isopolar; rectangular-elliptic and more or less  
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54 constricted at the equator (dumbbell-shaped), more or less rounded at the poles; sub-  
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56 circular to sub-hexagonal in polar view; prolate to perprolate, P/E = 1.3-2.8 ( $2.3 \pm 0.4$ ). P  
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58 = 6.4-10.9 ( $8.6 \pm 0.9$ )  $\mu\text{m}$ , E = 2.2-6.2 ( $3.8 \pm 0.2$ )  $\mu\text{m}$ , maximum width of the grains =  
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2.2-7.5 ( $4.8 \pm 0.6$ )  $\mu\text{m}$ . Apertural system 6-zono-heterocolpate: three pseudocolpi 3.6-9.0 ( $6.3 \pm 0.9$ )  $\mu\text{m}$  long; three colpi elongated-rhomboidal, operculate with ectoaperture 2.7-6.6 ( $4.5 \pm 0.8$ )  $\mu\text{m}$  long and endoaperture lalongate, 0.9-3.8 ( $1.7 \pm 0.5$ )  $\mu\text{m}$  wide; margins of the pseudocolpi and of the colpi without granula or granulate; colpal and pseudocolpal membranes psilate; ring-like aperture absent or present; opercula granulate. Exine 0.5-1.0  $\mu\text{m}$  thick, costae slightly developed. Sculpture finely granulate, perforate.

#### *Dichotomous key to pollen types*

1. Margins of the pseudocolpi and colpi conspicuously granulate.....2  
 Margins of the pseudocolpi and colpi not granulate.....*O. chiangii* pollen type
2. Ring-like aperture present.....*O. japonica* pollen type  
 Ring-like aperture absent.....*O. verna* pollen type

#### *Pollen type descriptions*

*O. japonica* pollen type (Figure 1). – Species included: *O. akiensis*, *O. japonica*, *O. krameri* and *O. laevisperma*. Pollen grains radially symmetrical; isopolar; rectangular-elliptic and more or less constricted at the equator, more or less rounded at the poles; sub-circular to sub-hexagonal in polar view; prolate to perprolate, P/E = 1.8-2.5 ( $2.2 \pm 0.3$ ). P = 8.1-10.7 ( $9.7 \pm 0.5$ )  $\mu\text{m}$ , E = 3.2-5.6 ( $4.4 \pm 0.6$ )  $\mu\text{m}$ , maximum width of the grains = 4.4-6.0 ( $5.4 \pm 0.4$ )  $\mu\text{m}$ . Apertural system 6-zono-heterocolpate: three pseudocolpi 4.7-8.5 ( $7.0 \pm 0.8$ )  $\mu\text{m}$  long; three colpi elongated-rhomboidal with the ectoaperture 2.7-6.5 ( $4.9 \pm 0.9$ )  $\mu\text{m}$  long and the endoaperture lalongate, 0.9-3.3 ( $2.2 \pm 0.7$ )  $\mu\text{m}$  wide; margins of the pseudocolpi and colpi conspicuously granulate; colpal and pseudocolpal membranes psilate; ring-like aperture present. Exine 0.5-1.0  $\mu\text{m}$  thick, costae slightly developed. Sculpture finely granulate, imperforate.

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3 *O. chiangii* pollen type (Figure 2A-D). – Species included: *O. acuminata*, *O. chiangii*, *O.*  
4 *cardiophylla*, *O. erecta*, *O. lojkae* and *O. mexicana*. Pollen grains radially symmetrical;  
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6 isopolar; rectangular-elliptic and more or less constricted at the equator, more or less  
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8 rounded at the poles; sub-circular to sub-hexagonal in polar view; prolate to perprolate,  
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10 P/E = 31.2-3.8 ( $2.4 \pm 0.4$ ). P = 7.0-10.7 ( $8.5 \pm 0.7$ )  $\mu\text{m}$ , E = 2.3-6.2 ( $3.7 \pm 0.8$ )  $\mu\text{m}$ ,  
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12 maximum width of the grains = 3.7-6.3 ( $4.9 \pm 0.6$ )  $\mu\text{m}$ . Apertural system 6-zono-  
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14 heterocolpate: three pseudocolpi 4.6-8.4 ( $6.4 \pm 0.8$ )  $\mu\text{m}$  long; three colpi elongated-  
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16 rhomboidal, operculate, with the ectoaperture 2.7-6.1 ( $4.3 \pm 0.7$ )  $\mu\text{m}$  long and the  
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18 endoaperture lalongate, 1.1-2.7 ( $1.6 \pm 0.3$ )  $\mu\text{m}$  wide; margins of the pseudocolpi and  
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20 colpi not granulate; colp and pseudocolpal membranes psilate; ring-like aperture  
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22 absent; opercula granulate. Exine 0.5-1.0  $\mu\text{m}$  thick, costae slightly developed. Sculpture  
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24 finely granulate, perforate.  
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34 *O. verna* pollen type (Figure 2E-K). – Species included: *O. aliena*, *O. commutata*, *O.*  
35 *kuzinskyanae*, *O. linifolia*, *O. littoralis*, *O. luciliae*, *O. lusitanica*, *O. nitida*, *O.*  
36 *richardsonii*, *O. ripleyana*, *O. rupestris*, *O. scorpioides* and *O. verna*. Pollen grains  
37  
38 radially symmetrical; isopolar; rectangular-elliptic and more or less constricted at the  
39  
40 equator, more or less rounded at the poles; sub-circular to sub-hexagonal in polar view;  
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42 prolate to perprolate, P/E = 1.5-3.5 ( $2.3 \pm 0.4$ ). P = 6.4-10.9 ( $8.5 \pm 0.9$ )  $\mu\text{m}$ , E = 2.6-5.8  
43  
44 ( $3.8 \pm 0.6$ )  $\mu\text{m}$ , maximum width of the grains = 2.2-7.5 ( $4.6 \pm 0.6$ )  $\mu\text{m}$ . Apertural system  
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46 6-zono-heterocolpate: three pseudocolpi 3.6-9.0 ( $6.2 \pm 0.9$ )  $\mu\text{m}$  long; three colpi  
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48 elongated-rhomboidal, operculate, with ectoaperture 2.8-6.6 ( $4.6 \pm 0.8$ )  $\mu\text{m}$  long and  
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50 endoaperture lalongate, 0.9-3.8 ( $1.7 \pm 0.5$ )  $\mu\text{m}$  wide; margins of the pseudocolpi and  
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52 colpi conspicuously granulate; colp and pseudocolpal membranes psilate; ring-like  
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3 aperture absent; opercula granulate. Exine 0.5-1.0  $\mu\text{m}$  thick, costae slightly developed.  
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6 Sculpture finely granulate, perforate.  
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## 10 Discussion

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12 The results of this study revealed that most of the pollen morphology features (including  
13 polarity, symmetry, shape, size and exine thickness) of *Omphalodes* species are quite  
14 constant, although the margins sculpture and presence/absence of a ring-like aperture  
15 varies among the taxa. Overall, the results obtained agreed with previous descriptions  
16 (Liu et al., 2001; Hargrove & Simpson, 2003; Bigazzi et al., 2006), despite some varied  
17 values, most probably due to different methodologies (e.g., Barbier & Mathez, 1973;  
18 Díez & Valdés, 1991; Buchner & Halbritter, 2000-onwards). Regardless of the fairly  
19 continuous gradient observed across the species studied for all the quantitative characters,  
20 it was possible to recognize three pollen types (each one including several species) that  
21 differed mostly in qualitative characters related with the presence/absence of a ring-like  
22 aperture and of conspicuous granula in the apertural margins. The need to include several  
23 con-generic species in one pollen type was already described for the Boraginaceae (e.g.,  
24 Barbier & Mathez, 1973; Clarke, 1977; Díez, 1984, Díez & Valdés, 1991; Sheel et al.,  
25 1996) and some palynologists have even considered the genus, more than the species, as  
26 the basic palynosystematic unit (e.g., Pons, 1970). This view is valid especially when  
27 studying taxonomic groups with low sculptural variability such as Cynoglosseae. In  
28 addition to the three pollen types described, two main patterns arose from the  
29 palynological data obtained in this study. First, the Japanese taxa form a group strongly  
30 individualized by its greater size, presence of a ring-like aperture, and presence of granula  
31 in the apertural margins, supporting recent phylogenetic analyses. Second, although the  
32 perennial species from both the Old and the New world could belong either to the *O.*  
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3 *chiangii* or *O. verna* pollen types, the pollen of all annual species belong exclusively to  
4 the *O. verna* pollen type. It is also interesting to note that within the *O. verna* pollen type,  
5 the pollen grains of *O. scorpioides* are more compact and globose than the pollen grains  
6 of the other species.  
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12 Among the pollen characters studied, the occurrence of a ring-like aperture was of  
13 major importance to discriminate pollen types, namely the Japanese taxa. The existence  
14 of a ring-like aperture has already been described for some genera of Cynoglosseae (e.g.,  
15 *Cryptantha*, *Paracaryum*, *Rindera* and *Solenanthus*; Hargrove & Simpson, 2003; Bigazzi  
16 et al., 2006), but was never reported for *Omphalodes* up to now (e.g., Barbier & Mathez,  
17 1973). The heterocolpate condition was constant to all the species of *Omphalodes*  
18 studied, as well as to other taxa of Cynoglosseae (Valdés, 1987; Díez & Valdés, 1991;  
19 Anh & Lee, 1986; Hargrove & Simpson, 2003; Coutinho et al., unpublished results)  
20 being, most probably, an apomorphic condition for *Omphalodes* and Cynoglosseae  
21 (Hargrove & Simpson, 2003). Three functions have been proposed for the heterocolpate  
22 condition: points for pollen tube growth, points for the exchange of physiologically active  
23 substances, and harmomegathy (Muller, 1979), the latter being solely associated with  
24 pseudocolpi (Hargrove & Simpson, 2003). In *Omphalodes*, harmomegatic reactions are  
25 indeed facilitated by the presence of pseudocolpi that enables the accommodation of  
26 volume changes after (de)hydration, and by a thin exine that increases the flexibility of  
27 the pollen grain (Bolick, 1981; Payne, 1981). The perforations and opercula observed in  
28 *Omphalodes* pollen grains may also constitute important regulatory structures for the  
29 exchange of physiologically active substances (Bolick, 1981; Payne, 1981).  
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55 Phylogenetic analysis based on *atpB* plastid marker places the *Omphalodes* genus in  
56 an isolated position within Boraginoideae and suggests no relation with the Cynoglosseae  
57 (Långström & Chase, 2002). However, the pollen morphological data collected in this  
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3 study does not support this molecular inference; instead, palynological data corroborates  
4 the classical inclusion of the genus within Cynoglosseae. In fact, Cynoglosseae s.l. is the  
5 only tribe of the subfamily Boraginoideae that possess heterocolpate pollen, with the  
6 colporate pollen being restricted to Boragineae and Lithospermeae. Still, independent  
7 evolution of heterocolpate pollen in *Omphalodes* and Cynoglosseae should be tested in a  
8 phylogenetic context using more molecular markers. It is also interesting to notice that  
9 the *O. japonica* pollen type is similar to the *Cynoglossum officinale* pollen type (Clarke,  
10 1977) and the *Cynoglossum creticum* pollen type (Díez, 1984), while *O. verna* pollen  
11 type resembles the *Myosotis arvensis* and *Myosotis stolonifera* pollen types (Clarke,  
12 1977; Díez, 1984).

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27 Based on palynological data it was possible to clearly discriminate the Japanese  
28 species from the remaining species of *Omphalodes*. This corroborates the segregation of  
29 the monophyletic group of Japanese species from *Omphalodes* proposed by recent  
30 molecular phylogenetic analyses (Serrano et al., unpublished data). Our results revealed  
31 that *Omphalodes* is a quite stenopalynous taxon (except for the margin sculpture and  
32 presence of a ring-like aperture), in contrast to other genera of the Cynoglosseae in which  
33 the high variability of pollen morphology significantly assisted taxonomic delimitations  
34 within the tribe (Barbier & Mathez, 1973). The observed low variability within  
35 *Omphalodes* does not support the hypothesis of a relatively ancient diversification of the  
36 genus, as suggested by the disjunct distribution. Alternatively, a possible trend to  
37 palynological stasis in the genus needs to be tested in a dated phylogenetic framework.

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53 Finally, the results obtained have also significant biogeographic implications. Pollen  
54 morphology gives support to a reduced concept of *Omphalodes* that would include only  
55 species with *O. chiangii* or *O. verna* pollen types. These taxa display a disjunct  
56 distribution range between West Eurasia and North America that accommodates in the  
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3 well-known Madrean-Tethyan Flora pattern (Raven & Axelrod, 1974; Wen & Ickert-  
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5 Bond, 2009). However, whether *Omphalodes* disjunction originated in the first half of the  
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7 Tertiary, as proposed by the Madrean-Tethyan hypothesis (Axelrod, 1975), or resulted  
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9 from more recent long distance dispersal events still remains an open question.  
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### 13 14 15 **Conclusions**

16  
17 This study investigated for the first time the variability of pollen morphology in  
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19 *Omphalodes*, encompassing 23 species (82% of the recognized species) and all major  
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21 geographical and morphological groups. Palynological data provided useful information  
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23 to assist the complex systematics of *Omphalodes* genus considering the usefulness of  
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25 most floral and fruit characters, karyological homogeneity, and striking geographical  
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27 distribution. At the suprageneric level, pollen morphology supports traditional tribal  
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29 classification of the genus, in contrast to previously published molecular phylogenetic  
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31 analyses. At the infrageneric level, pollen morphology corroborates the recent  
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33 phylogenetic results for *Omphalodes* and clearly separates, among others, the Japanese  
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35 taxa in a distinct group.  
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52 Department of Life Sciences (University of Coimbra) and to the staff of the Confocal and  
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56 Foundation for Science and Technology financed the work of Sílvia Castro  
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### Specimens investigated

*Omphalodes acuminata* B.L.Rob. Mexico: Nuevo León, Monterrey. Ford M186 (TEX 218154);

Mexico: Nuevo León, Villa Santiago. Johnson & Barkley 1609M (TEX 120476).

*Omphalodes akiensis* Kadota. Japan: Hiroshima pref., Hiramidani gorge. Kubota s.n.

(TNS 737082).

*Omphalodes aliena* A.Gray ex Hemsl. USA: Texas, Presidio. Correll & Rollins 23704. (TEX

35421); Mexico: Nuevo León, Minas Manto Blanco. Johnston, Wendt & Chiang 10248F

(TEX); Mexico: Nuevo León, Rayones. Hinton 20173 (TEX 223071).

*Omphalodes cardiophylla* A.Gray ex Hemsl. México: Coahuila, General Cepeda. Encina 228 &

Závala. (TEX 223076).

*Omphalodes chiangii* Higgins. Mexico: Sierra del Carmen. Carranza s.n. (IEB 15903).

*Omphalodes commutata* G.López. Spain: Málaga, Serranía de Ronda. Fuertes, Ladero, G. López

& Navarro s.n. (SALAF 12451).

*Omphalodes erecta* I.M.Johnst. Mexico: Galeana. Hinton s.n. (IEB 22275); Mexico: Aramberri.

Hinton s.n. (IEB 23145).

*Omphalodes japonica* (Thunb.) Maxim. Japan: Kyoto, Yamashiro. Togashi s.n. (TI); Japan:

Cultivated in Karuizawa Bot. Garden. Arai s.n. (SANT 59953).

*Omphalodes krameri* Franch. & Sav. Japan: Nagano Pref., Kitasaku-gun. Yahara et al. s.n. (TI).

*Omphalodes kuzinskyanae* Willk. Portugal: Cascais, Quinta do Espírito Santo. Torre s.n. COI;

Portugal: Cascais, near Guincho. Carballal & Serrano s.n. (SANT 50016).

*Omphalodes laevisperma* Nakai. Japan: Murakamishi. Togashi s.n. (TI).

*Omphalodes linifolia* (L.) Moench. Spain: Málaga, El Burgo. Perez & Molero s.n. (SANT

12850); Portugal: Serra da Arrábida. Carbajal & Serrano s.n. (SANT 50020).

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3 *Omphalodes littoralis* Lehm. subsp. *gallaecica* M. Laínz. Spain: Ferrol, Covas. Carbajal &  
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5 Serrano s.n. (SANT 51847).  
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7 *Omphalodes littoralis* Lehm. subsp. *littoralis*. France: Charente-Inf., La Rochelle. Giraudias s.n.  
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9 (P 271712).  
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11 *Omphalodes lojkae* Sommier & Levier. Georgia: Svanethi. Schethekauzi s.n. (MA 575919).  
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13 *Omphalodes luciliae* Boiss. Greece: Parnaso massif, Gorge Gouna. Maire & Petitmengin s.n.  
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15 (MA 94701).  
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17 *Omphalodes lusitanica* Schrank in Denksch Spain: Cáceres, Arroyomolinos de la Vera. Ladero &  
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19 Amor s.n. (SALAF 23592).  
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21 *Omphalodes mexicana* S.Watson. Mexico: Nuevo León, Galeana, Cerro el Gallo. Hinton 21036  
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23 (IEB 137190).  
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25 *Omphalodes nitida* Hoffmanns. & Link. Spain: Corunha, Ames. Fagúndez s.n. (SANT 53295).  
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28 *Omphalodes richardsonii* G.L.Nesom. Mexico: Tamaulipas, Gómez Farias. Richardson 1114  
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30 (TEX); Mexico: Tamaulipas, Gómez Farias. Woodruff 160 (TEX).  
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32 *Omphalodes ripleyana* P.H.Davis. Cultivated in Edinburgh. Davis s.n. (K).  
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35 *Omphalodes rupestris* Boiss. Caucasus region. Desoulavy s.n. (B 10 0293510).  
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38 *Omphalodes scorpioides* (Haenke) Schrank Slovakia: Slovensky Kras. Zertova s.n. (MA 219178);  
39  
40 Austria: Niederösterreich, Fuss des Rosaliengebirges. Barta s.n. (MA 732115).  
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42 *Omphalodes verna* Moench. Italy: Udine, Valli del Natisone-Valle dell'Erbezzo. Feoli & Feoli  
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44 s.n. (MA 350089).  
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### Figure captions

Figure 1. SEM micrographs of pollen grains of *Omphalodes*. **A.-G.** *Omphalodes japonica* pollen type. **A. & D.** *O. akiensis*: **A.** equatorial view; **D.** detail of apertural system (ra, ring-like aperture) and granula in the margins of colporus and pseudocolpus (arrow). **B., F. & G.** *O. japonica*: **B.** equatorial view; **F.** detail of apertural system (ra, ring-like aperture) and granula in the margins of colporus and pseudocolpus (arrow); **G.** detail of the exine showing the perforations (arrow). **C. & E.** *O. krameri*: **C.** equatorial view; **E.** detail of apertural system (ra, ring-like aperture) and granula in the margins of colporus and pseudocolpus (arrow). Scale bars – 2  $\mu\text{m}$  (A-C); 1  $\mu\text{m}$  (D-F); 0.5  $\mu\text{m}$  (G).

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Figure 2. SEM micrographs of pollen grains of *Omphalodes*. **A.-D.** *Omphalodes chiangii* pollen type. **A. & C.** *O. cardiophylla*: **A.** equatorial view; **C.** detail of apertural system and margins of colporus and pseudocolpus without granula. **B. & D.** *O. chiangii*: **B.** equatorial view; **D.** detail of apertural system and margins of colporus and pseudocolpus without granula. **E.-K.** *O. verna* pollen type. **E.** *O. linifolia*, equatorial view. **F. & G.** *O. littoralis*: **F.** equatorial view; **G.** detail of apertural system and granula in the margins of colporus and pseudocolpus (arrow). **H.** *O. commutata*, detail of apertural system and granula in the margins of colporus and pseudocolpus (arrow). **I.** *O. luciliae*, equatorial view with visible perforations (arrow). **J.** *O. verna*, equatorial view. **K.** *O. nitida*, detail of apertural system and granula in the margins of colporus and pseudocolpus (arrow). Scale bars – 1.5  $\mu\text{m}$  (A-B, E-F, I-J); 0.5  $\mu\text{m}$  (C-D, G-H, K).

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Figure 3. Principal component analysis performed with the palynological characters and the specimens of *Omphalodes*. Specimens are represented in the first and second PCA components and were marked according with the pollen type: *O. chiangii* pollen type (diamonds), *O. japonica* pollen type (open circles) and *O. verna* pollen type (open squares). Percentage of variance explained by the component factors is also provided in parenthesis in each axis. Inset gives the projection of the variables on the same factor axes with the following variables in a clockwise direction: P/E, colporus,

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P, maximum width of the grain, ring-like aperture, pseudocolpus, E and margin ornamentation.  
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**Table I.** Summary of the quantitative and qualitative pollen morphological characters for the *Omphalodes* species examined.

Taxa	P (µm)	E (µm)	P/E	Maximum grain width (µm)	Pseudocolpus (µm)	Colporus (µm)	Endo-aperture (µm)	Margines ornamentation	Ring-like aperture	Habit and Geographical distribution
<b><i>Omphalodes japonica</i> pollen type</b>										
<i>O. akiensis</i>	9.2-9.6 (9.4 ± 0.2)	3.9-4.8 (4.4 ± 0.4)	1.9-2.4 (2.2 ± 0.3)	4.9-5.2 (5.0 ± 0.1)	7.05-7.06 (7.1 ± 0.0)	5.8-6.1 (6.0 ± 0.1)	2.9-3.1 (3.0 ± 0.1)	Yes	Yes	Perennial, Japan
<i>O. japonica</i>	9.1-10.7 (9.8 ± 0.4)	4.1-5.6 (4.7 ± 0.5)	1.8-2.5 (2.1 ± 0.2)	4.8-6.0 (5.4 ± 0.3)	6.1-7.8 (7.1 ± 0.6)	4.7-5.9 (5.4 ± 0.5)	1.5-3.3 (2.3 ± 0.5)	Yes	Yes	Perennial, Japan
<i>O. krameri</i>	9.1-10.6 (9.9 ± 0.6)	4.2-4.9 (4.6 ± 0.2)	2.0-2.4 (2.1 ± 0.2)	5.2-6.0 (5.6 ± 0.3)	6.9-8.5 (7.6 ± 0.5)	3.6-6.5 (4.7 ± 0.9)	1.1-1.3 (1.2 ± 0.1)	Yes	Yes	Perennial, Japan
<i>O. laevisperma</i>	8.7-10.9 (9.8 ± 0.4)	3.6-5.8 (4.6 ± 0.6)	1.7-2.6 (2.2 ± 0.2)	4.8-5.9 (5.4 ± 0.4)	6.8-9.0 (7.6 ± 0.5)	4.4-7.1 (5.4 ± 0.7)	0.7-2.1 (1.5 ± 0.4)	Yes	Yes	Perennial, Japan
<b><i>Omphalodes chiangü</i> pollen type</b>										
<i>O. acuminata</i>	7.0-8.7 (7.8 ± 0.4)	2.2-3.5 (3.0 ± 0.3)	2.2-3.7 (2.7 ± 0.4)	3.7-4.9 (4.4 ± 0.3)	5.2-7.1 (6.0 ± 0.6)	2.9-5.0 (3.9 ± 0.6)	1.1-2.3 (1.4 ± 0.3)	No	No	Perennial, Mexico
<i>O. cardiophylla</i>	7.6-8.9 (8.1 ± 0.4)	2.3-3.6 (3.0 ± 0.4)	2.1-3.8 (2.8 ± 0.4)	3.9-5.2 (4.4 ± 0.4)	5.1-7.8 (6.2 ± 0.5)	3.6-6.1 (4.4 ± 0.6)	1.1-1.6 (1.3 ± 0.2)	No	No	Perennial, Mexico
<i>O. chiangü</i>	7.3-9.5 (8.6 ± 0.7)	4.9-6.2 (4.7 ± 0.8)	3.6-6.2 (4.7 ± 0.8)	1.3-2.4 (1.9 ± 0.3)	4.1-6.3 (5.2 ± 0.5)	4.7-7.5 (6.0 ± 0.9)	1.4-2.1 (1.8 ± 0.3)	No	No	Perennial, America
<i>O. erecta</i>	8.4-9.9 (9.0 ± 0.4)	3.0-4.4 (3.9 ± 0.4)	2.1-2.9 (2.4 ± 0.2)	4.1-5.4 (4.9 ± 0.4)	5.2-7.8 (6.6 ± 0.7)	4.3-5.6 (4.7 ± 0.4)	1.1-2.1 (1.8 ± 0.5)	No	No	Perennial, Mexico
<i>O. lojkae</i>	8.1-9.8 (8.9 ± 0.5)	3.3-4.4 (3.9 ± 0.3)	2.0-2.7 (2.3 ± 0.2)	4.5-6.1 (5.5 ± 0.4)	5.8-8.0 (6.7 ± 0.6)	4.0-5.1 (4.3 ± 0.4)	1.1-2.1 (1.6 ± 0.4)	No	No	Perennial, Caucasus
<i>O. mexicana</i>	7.9-10.7 (8.5 ± 0.8)	2.8-4.2 (3.6 ± 0.4)	1.9-3.0 (2.4 ± 0.3)	4.9-5.6 (5.1 ± 0.2)	5.7-8.4 (6.9 ± 0.7)	3.8-5.5 (4.6 ± 0.5)	1.3-1.7 (1.5 ± 0.1)	No	No	Perennial, Mexico
<b><i>Omphalodes verna</i> pollen type</b>										
<i>O. aliena</i>	7.8-10.8 (8.8 ± 0.6)	2.9-4.6 (3.4 ± 0.4)	2.2-3.0 (2.6 ± 0.2)	4.2-6.3 (4.8 ± 0.4)	5.2-7.8 (6.2 ± 0.6)	3.7-5.5 (4.4 ± 0.59)	0.9-2.0 (1.4 ± 0.3)	Yes	No	Annual/perennial, Mexico, Texas

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5	<i>O. comuttata</i>	8.0-9.7 (8.1 ± 0.4)	3.1-4.5 (3.7 ± 0.4)	2.0-2.6 (2.4 ± 0.2)	4.1-5.9 (4.6 ± 0.5)	5.9-7.2 (6.5 ± 0.4)	3.6-5.5 (4.6 ± 0.5)	1.4-2.1 (1.7 ± 0.2)	Yes	No	Annual, Iberian Peninsula
6											
7	<i>O. kuzinskyanae</i>	7.4-9.1 (8.4 ± 0.5)	3.0-4.6 (4.0 ± 0.5)	1.8-2.4 (2.1 ± 0.3)	4.0-5.2 (4.6 ± 0.4)	5.5-7.8 (6.2 ± 0.7)	3.1-5.9 (5.0 ± 0.7)	1.1-1.7 (1.4 ± 0.2)	Yes	No	Annual, Iberian Peninsula
8											
9	<i>O. linifolia</i>	8.1-10.7 (9.1 ± 0.7)	2.7-4.8 (3.5 ± 0.5)	1.9-3.5 (2.6 ± 0.3)	2.2-5.0 (4.6 ± 0.6)	5.4-8.1 (6.6 ± 0.6)	2.9-6.0 (4.6 ± 1.0)	1.1-2.3 (1.6 ± 0.3)	Yes	No	Annual, Iberian Peninsula
10											
11	<i>O. littoralis</i> subsp. <i>gallaecica</i>	7.8-10.0 (8.9 ± 0.7)	3.4-4.5 (3.8 ± 0.3)	2.1-2.6 (2.3 ± 0.1)	4.1-5.0 (4.5 ± 0.3)	5.1-7.4 (6.1 ± 0.6)	4.0-6.6 (5.4 ± 0.8)	1.5-1.9 (1.6 ± 0.2)	Yes	No	Annual, Iberian Peninsula
12											
13	<i>O. littoralis</i> subsp. <i>littoralis</i>	8.1-10.2 (9.4 ± 0.5)	3.2-4.3 (3.8 ± 0.3)	2.2-3.0 (2.5 ± 0.2)	4.4-5.4 (5.0 ± 0.3)	4.7-7.4 (6.2 ± 0.7)	2.7-5.1 (3.9 ± 0.7)	0.9-1.6 (1.3 ± 0.2)	Yes	No	Annual, West France
14											
15	<i>O. luciliae</i>	6.4-7.7 (7.1 ± 0.4)	2.8-4.0 (3.5 ± 0.4)	1.8-2.8 (2.1 ± 0.3)	3.4-4.9 (4.0 ± 0.3)	3.6-6.2 (5.0 ± 0.7)	2.9-4.1 (3.4 ± 0.4)	1.3-2.2 (1.6 ± 0.2)	Yes	No	Perennial, Greece, West Asia
16											
17	<i>O. lusitanica</i>	7.4-9.8 (8.3 ± 0.6)	3.3-4.3 (4.0 ± 0.3)	1.7-2.5 (2.1 ± 0.2)	4.3-5.3 (4.7 ± 0.4)	4.8-8.0 (6.2 ± 0.8)	2.8-5.2 (4.2 ± 0.7)	1.1-1.7 (1.4 ± 0.2)	Yes	No	Annual, Iberian Peninsula
18											
19	<i>O. nitida</i>	7.4-9.3 (8.4 ± 0.5)	3.0-4.2 (3.5 ± 0.3)	1.9-2.9 (2.4 ± 0.2)	4.3-5.7 (4.7 ± 0.4)	6.5-8.5 (7.0 ± 0.7)	3.2-4.7 (4.1 ± 0.4)	2.4-3.8 (2.9 ± 0.5)	Yes	No	Perennial, Iberian Peninsula
20											
21	<i>O. richardsonii</i>	7.3-8.7 (7.7 ± 0.4)	2.6-3.2 (2.9 ± 0.2)	2.4-3.1 (2.7 ± 0.2)	3.8-4.4 (4.1 ± 0.2)	4.8-6.3 (5.3 ± 0.5)	3.3-5.4 (4.0 ± 0.6)	1.0-1.7 (1.3 ± 0.2)	Yes	No	Perennial, Mexico
22											
23	<i>O. ripleyana</i>	6.4-8.6 (6.8 ± 0.7)	2.7-4.0 (3.4 ± 0.4)	2.1-2.5 (2.3 ± 0.1)	3.4-4.5 (3.9 ± 0.3)	4.2-5.8 (5.2 ± 0.4)	3.8-6.1 (4.8 ± 0.6)	1.1-1.7 (1.4 ± 0.2)	Yes	No	Perennial, Anatolia
24											
25	<i>O. rupestris</i>	6.5-8.9 (7.6 ± 0.8)	3.7-4.8 (4.7 ± 0.3)	1.5-2.1 (1.8 ± 0.2)	4.2-5.2 (4.6 ± 0.4)	5.5-7.8 (6.4 ± 0.7)	4.1-6.5 (5.0 ± 0.6)	1.5-2.5 (1.9 ± 0.3)	Yes	No	Perennial, Caucasus
26											
27	<i>O. scorpioides</i>	7.4-10.5 (8.4 ± 0.7)	3.7-4.9 (4.4 ± 0.3)	1.7-2.4 (1.9 ± 0.2)	4.4-5.7 (4.8 ± 0.4)	5.4-7.3 (6.2 ± 0.6)	4.0-4.9 (4.4 ± 0.3)	1.1-2.4 (2.6 ± 0.5)	Yes	No	Biennial, Europe
28											
29	<i>O. verna</i>	8.4-9.3 (8.9 ± 0.3)	4.2-4.9 (4.5 ± 0.3)	1.8-2.2 (2.0 ± 0.2)	4.5-7.5 (5.3 ± 0.8)	4.7-7.3 (5.8 ± 0.9)	4.1-6.5 (5.2 ± 0.7)	2.1-3.1 (2.7 ± 0.4)	Yes	No	Perennial, Europe
30											
31											

Notes: P, polar axis; E, equatorial axis, P/E, ratio between polar and equatorial axes; margins ornamentation refers to the presence/absence of conspicuous granula in pseudocolpi and colpi margins. For each *taxon* and character the following information is provided: minimum and maximum, followed by mean ± standard deviation of the mean in parenthesis. Quantitative variables are provided in µm, except P/E. Dichotomous key for the pollen types is provided in *Results* section.

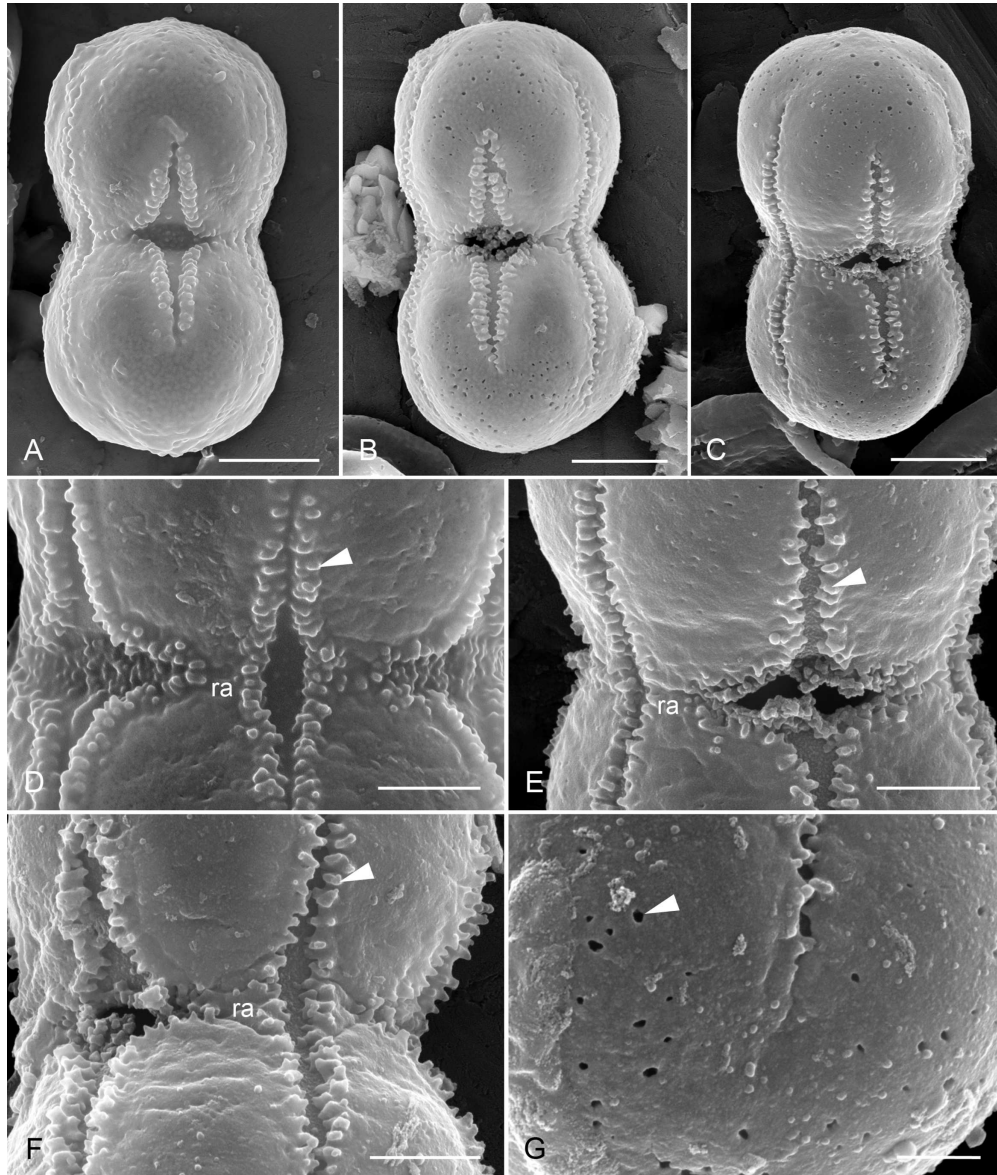


Figure 1. SEM micrographs of pollen grains of *Omphalodes*. A.-G. *Omphalodes japonica* pollen type.  
 A. & D. *O. akiensis*: A. equatorial view; D. detail of apertural system (ra, ring-like aperture) and granula in the margins of colporus and pseudocolpus (arrow). B., F. & G. *O. japonica*: B. equatorial view; F. detail of apertural system (ra, ring-like aperture) and granula in the margins of colporus and pseudocolpus (arrow); G. detail of the exine showing the perforations (arrow). C. & E. *O. krameri*: C. equatorial view; E. detail of apertural system (ra, ring-like aperture) and granula in the margins of colporus and pseudocolpus (arrow). Scale bars – 2  $\mu\text{m}$  (A-C); 1  $\mu\text{m}$  (D-F); 0.5  $\mu\text{m}$  (G).  
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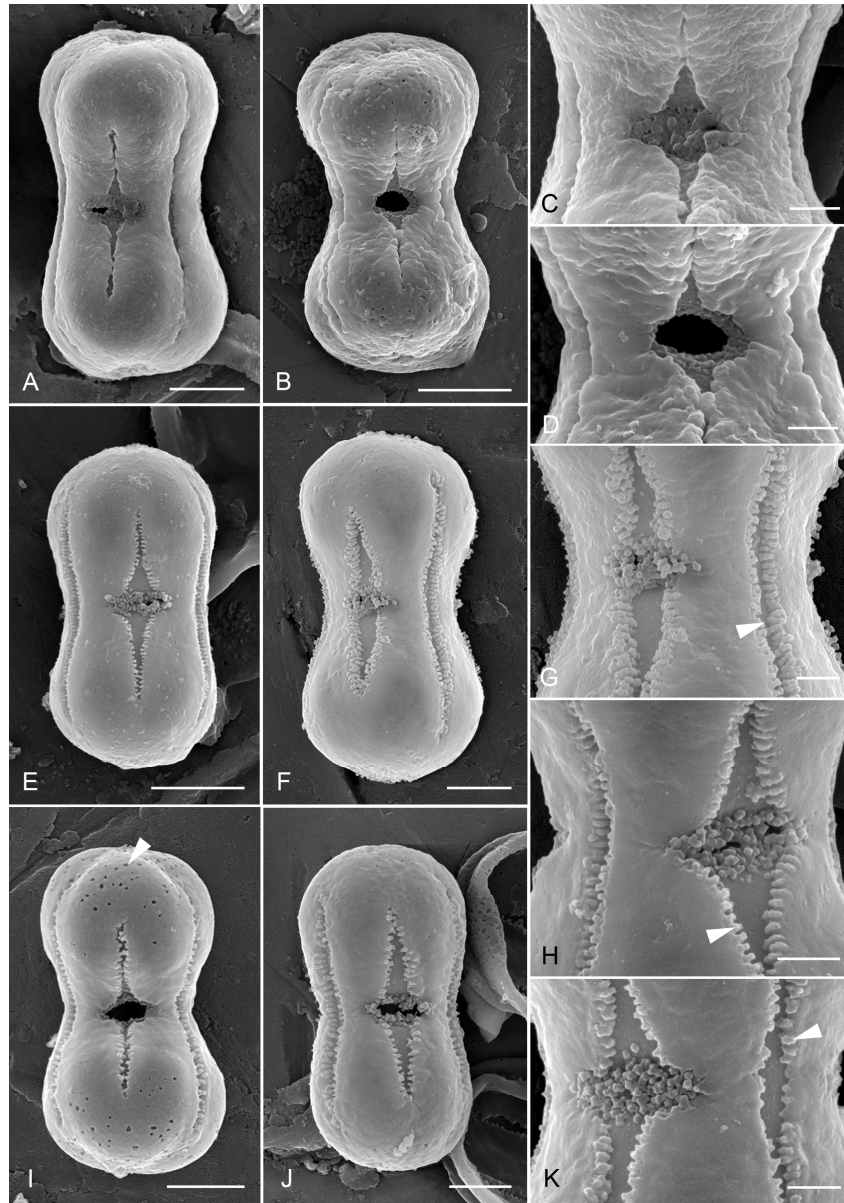


Figure 2. SEM micrographs of pollen grains of *Omphalodes*. A.-D. *Omphalodes chiangii* pollen type. A. & C. *O. cardiophylla*: A. equatorial view; C. detail of apertural system and margins of colporus and pseudocolpus without granula. B. & D. *O. chiangii*: B. equatorial view; D. detail of apertural system and margins of colporus and pseudocolpus without granula. E.-K. *O. verna* pollen type. E. *O. linifolia*, equatorial view. F. & G. *O. littoralis*: F. equatorial view; G. detail of apertural system and granula in the margins of colporus and pseudocolpus (arrow). H. *O. commutata*, detail of apertural system and granula in the margins of colporus and pseudocolpus (arrow). I. *O. luciliae*, equatorial view with visible perforations (arrow). J. *O. verna*, equatorial view. K. *O. nitida*, detail of apertural system and granula in the margins of colporus and pseudocolpus (arrow). Scale bars – 1.5  $\mu\text{m}$  (A-B, E-F, I-J); 0.5  $\mu\text{m}$  (C-D, G-H, K).

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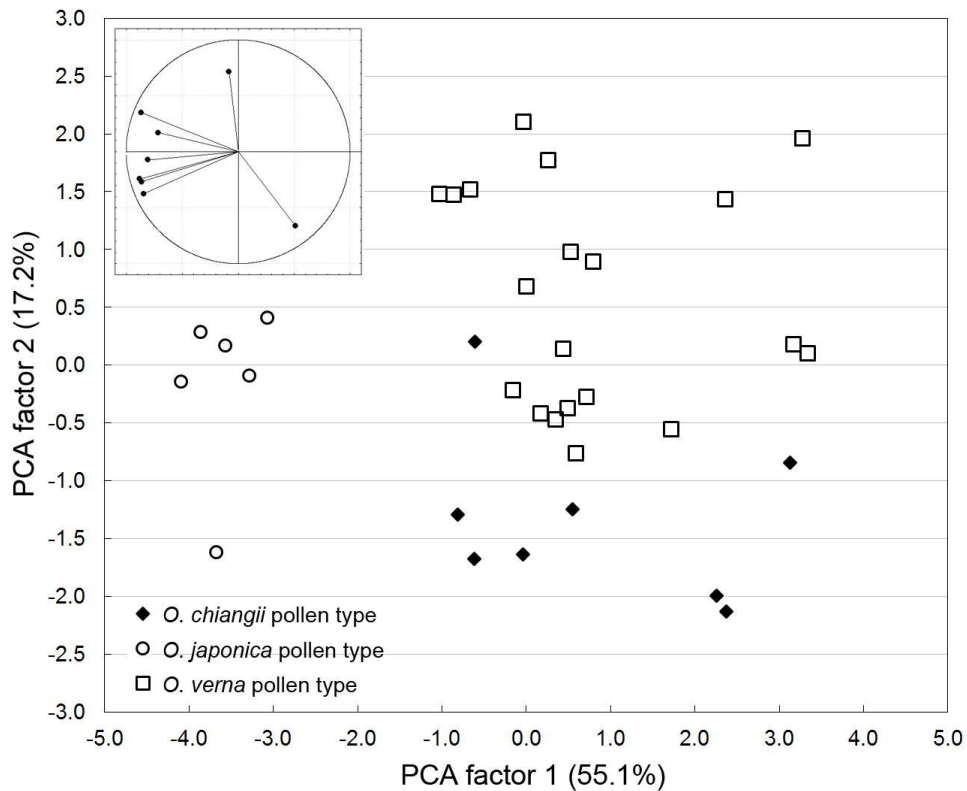


Figure 3. Principal component analysis performed with the palynological characters and the specimens of *Omphalodes*. Specimens are represented in the first and second PCA components and were marked according with the pollen type: *O. chiangii* pollen type (diamonds), *O. japonica* pollen type (open circles) and *O. verna* pollen type (open squares). Percentage of variance explained by the component factors is also provided in parenthesis in each axis. Inset gives the projection of the variables on the same factor axes with the following variables in a clockwise direction: P/E, colporus, P, maximum width of the grain, ring-like aperture, pseudocolpus, E and margin ornamentation.

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