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Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology

Official Journal of the Societa Botanica Italiana

ISSN: 1126-3504 (Print) 1724-5575 (Online) Journal homepage: https://www.tandfonline.com/loi/tplb20

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To cite this article: Anna Scoppola & Sara Magrini (2019) Comparative palynology and seed morphology in annual pansies (*Viola* sect. *Melanium,* Violaceae): implications for species delimitation, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 153:6, 883-899, DOI: 10.1080/11263504.2019.1610113

To link to this article: https://doi.org/10.1080/11263504.2019.1610113

PLANT BIOSYSTEMS - AN INTERNATIONAL JOURNAL DEALING WITH ALL ASPECTS OF PLANT BIOLOGY
2019, VOL. 153, NO. 6, 883–899
https://doi.org/10.1080/11263504.2019.1610113

Comparative palynology and seed morphology in annual pansies (*Viola* sect. *Melanium,* Violaceae): implications for species delimitation

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ABSTRACT

This integrated study provides new insights into pollen and seed morphology and pollen heteromorphism of four closely related annual taxa of *Viola* sect. *Melanium*. The plant material, both fresh and dried, was collected in Italy and studied using light and scanning electron microscope. Palynological data for *V. hymettia* together with a detailed comparative analysis of seed morphology and micromorphology of the four species are reported for the first time. Results of this work highlight some pollen and seed features as useful diagnostic characters. The pollen size proves to be of diagnostic value to easily separate *V. kitaibeliana,* having the smallest pollen grains, from the others, especially from *V. arvensis* with the largest ones. Exine ornamentation is microreticulate, showing no relevant differences among species. We can partially confirm the diagnostic value of the prevailing pollen morph as it can be useful only for *V. arvensis* (five-aperturate) versus *V. tricolor* and *V. hymettia* (four-aperturate). The macro- and micromorphology of seeds provide additional useful distinguishing characters. Particularly, seed size was found to be a good delimitating character, especially to distinguish *V. kitaibeliana* (with the smallest seeds) from *V. arvensis,* and easy to be measured with no need of particular equipment.

ARTICLE HISTORY
Received 11 December 2018
Accepted 12 April 2019

KEYWORDS
Diagnostic characteristics; Europe; pollination ecology; pollen morphology; reproductive biology; scanning electron microscopy; seed coat; seed dispersal

Introduction

Viola L. is the largest genus of the Violaceae family with 580–620 taxa distributed throughout the most frost-free regions of the world (Ballard et al. 1998; Yockteng et al. 2003; Wahlert et al. 2014; Marcussen et al. 2015). Most of the species occur in the northern temperate zone (Erben 1996). Evidence from biogeography, karyology, and phylogeny suggest that the genus originated in South America and subsequently spread to the Northern Hemisphere (Clausen 1929; Ballard et al. 1998; Marcussen et al. 2012, 2015). Here, most of the secondary centres of morphological and taxonomic diversity occur, especially in the Alps and Mediterranean region, the Himalayas and mountainous Eastern Asia (Ballard et al. 1998).

The section *Melanium* DC. ex Ging., which includes the so-called pansies, is a derived, monophyletic and morphologically well-defined group comprising 125 species, both perennials and annuals (Marcussen et al. 2015). It is allotetraploid by origin, but further diversification appears to have happened at higher ploidy levels (Marcussen et al. 2015). It shows highly reduced genetic variation (Yockteng et al. 2003) and the interspecific isolation mechanisms are rather weak (Słomka et al. 2017). Its geographical distribution spans Europe and westernmost Asia, with a few species in Northern Africa and one disjunct in North America (Clausen et al. 1964; Yockteng et al. 2003; Randall 2004; Marcussen et al. 2015). The section has its centre of diversity on the hills and mountains of southern Europe, especially in the Balkan Peninsula and Italy (Erben 1996). Pansies are represented in Europe by over 50 taxa (Erben 1985), including about 20 annual or short-lived biennial species closely related to *V. tricolor* L., the common pansy (Valentine et al. 1968; Erben 1985; Espeut 2004; Magrini and Scoppola 2015b). In Italy, 23 species are known, five of which are annuals (Pignatti 2017).

Pansies are highly adapted to entomophilous pollination producing chasmogamous flowers, even if *V. arvensis* Murray and *V. kitaibeliana* Schult. are reported as self-fertile and predominantly autogamous (Beattie 1974; Randall 2004; Marcussen and Karlsson 2010). Cleistogamy occurs in most groups of the genus *Viola* but not in the section *Melanium*, with the exception of the North American *Viola bicolor* Pursh having a seasonal cleistogamy (Clausen et al. 1964).

The genus *Viola* is basically myrmecochorous but different selective pressures, especially seed predation, have produced a clear divergence in dispersal systems. Diplochory is exhibited by most species which have evolved a system combining ballistic and ant seed dispersal: the seeds are explosively ejected from the capsule (typically 1–5 m away) prior to being carried by ants which provides the adaptive advantage of predator avoidance (Beattie and Lyons 1975; Beattie 1985).

Taxonomy and cytology of the annual species of the section Melanium

The taxonomy of this section is considered critical (Pettet 1964b), particularly regarding the annual taxa, due to the

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Published online 04 Jun 2019

reduced number of species-delimitating characters, the strong phenotypical plasticity, the ambiguity of some morphological characters, the seasonal variability, and the conflicting taxonomic treatments (Schmidt 1964; Erben 1996; Scoppola and Lattanzi 2012; Magrini and Scoppola 2015a). Arguably, also the young age of the section *Melanium*, the frequency of polyploidy, dysploidy, and hybridisation events (Marcussen et al. 2015), coupled with the lack of an informative phylogeny (Yockteng et al. 2003) may have contributed to such conflicting treatments.

Particularly, during the last two centuries, many infra-specific taxa described at a local level were attributed on a morphological basis to the three annual species, *V. tricolor* (De Candolle 1824; Grenier and Godron 1848; Boissier 1867; Coutinho 1892), *V. arvensis* (Besnou 1881; Halácsy 1900; Becker 1904) and *V. kitaibeliana* (Rouy and Foucaud 1896; Becker 1910). Numerous taxonomic studies on particular species complexes of section *Melanium* have been published based largely on morphology (Becker 1910; Nauenburg 1991; Colombo et al. 2007; Scoppola and Lattanzi 2012), on chromosome numbers (Clausen 1927, 1929; Erben 1985, 1996; Espeut 2004; Magrini and Scoppola 2015a; Tomović et al. 2016), and on molecular phylogenetics (Ballard et al. 1998; Nadot et al. 2000; Yockteng et al. 2003; Marcussen et al. 2015) that have clarified the composition and relationships of the main groups and re-evaluated the placement of controversial assemblages.

Cytological diversity seems to be a striking feature of section *Melanium*. The base chromosome number of *Viola* is believed to be $x = 7$ (Marcussen et al. 2015), preserved in some South American groups, with a transition to $x = 6$ prior to the colonisation of the northern hemisphere; $x = 6$ is preserved in the diploid sect. *Chamaemelanium* Ging., while the numbers $x = 10$ and $x = 12$ are secondary base numbers in the allotetraploid groups (Marcussen et al. 2015). Owing to dysploidy, no base number is inferable for section *Melanium*, where it ranges from $x = 2$, in *V. modesta* Fenzl (Erben 1996), to $x = 60$, in *Viola corsica* Nyman (Erben 1996; Yockteng et al. 2003; Marcussen et al. 2015). On the other hand, chromosome number may serve as a species-delimiting character in annual pansies, especially when morphological characters are ambiguous as in poorly developed specimens or in hybrids. So, before detailing the observations on pollen and seed morphology of this group of pansies it would be wise to define the cytotype of taxa used in this account: *V. arvensis* has $2n = 34$, *V. tricolor* $2n = 26$, *V. hymettia* Boiss. & Heldr. $2n = 16$, *V. kitaibeliana* $2n = 16$ (Magrini and Scoppola 2015a). It would thus appear that chromosome number offers an important criterion for the separation of *V. arvensis* from *V. tricolor*, and *V. arvensis* from *V. kitaibeliana*, two pairs of taxa quite difficult to delimit satisfactorily on purely morphological criteria (Pettet 1964a), so chromosome counting was used to have a certain identification of the studied populations.

There is, perhaps, little need to stress the value of cytology in understanding the complex variation in the pansies but the potential value of pollen morphology appears relatively unknown (Pettet 1964a; Magrini and Scoppola 2015a).

Pollen aperture heteromorphism

Pollen heteromorphism, defined as the production by the same plant of several pollen morphs with different aperture number (Mignot et al. 1994; Dajoz et al. 1995; Till-Bottraud et al. 1995), occurs in over 30% of angiosperm species and it is particularly common in this section of *Viola* in which over 80% of the species produces pollen morphs with three to six apertures (Dajoz et al. 1993; Till-Bottraud et al. 1999; Nadot et al. 2000; Słomka et al. 2018). In *Viola*, the three-aperturate pollen morph is the ancestral condition and the occurrence of four-, five- or six-aperturate pollen grains are considered as derived (Nadot et al. 2000), with the increasing of the pollen aperture number with ploidy level, probably as a function of genome and cell size (Mignot et al. 1994).

The different morphs have different selective values. An increase in aperture number is linked to faster germination of the pollen tube but to a reduced life expectancy (Dajoz et al. 1991; Słomka et al. 2018): the lower the number of apertures, the longer-lived the pollen (Till-Bottraud et al. 1999; Słomka et al. 2014). Furthermore, the proportion of the different morphs seem to be heritable (Dajoz et al. 1993, 1995; Słomka et al. 2018) and strongly related to the pollination strategy: e.g. predominantly autogamous species like *Viola arvensis* have mainly five-aperturate pollen grains with a few four-aperturate ones, while in *Viola tricolor*, showing entomophilous pollination, four-aperturate grains dominate (Pettet 1964a; Till-Bottraud et al. 1999; Hildebrandt et al. 2006; Marcussen and Karlsson 2010).

Wittrock (1897) was the first to describe the quantitative differences in the heteromorphic pollen assemblages in *V. tricolor* and *V. arvensis* and to use them as an additional character in discriminating between the two taxa (Pettet 1964a). Since then, the diagnostic/taxonomic value of such characteristic has been remarked by many authors (e.g. Mullenders and Mullenders 1957; Clapham et al. 1987; Nadot et al. 2000; Randall 2004; Jäger and Werner 2005; Marcussen and Karlsson 2010; Słomka et al. 2014). Mullenders and Mullenders (1957) used it as a criterion for deciding whether the taxon "*V. maritima*" Schweigg. [= *V. tricolor* subsp. *curtisii* (E. Forst.) Syme] should be considered as distinct from *V. tricolor* subsp. *tricolor*. Other authors included the prevalent pollen morph in the descriptions of the species (Gams 1926; Clapham et al. 1987; Espeut 2004). Jäger and Werner (2005), Marcussen and Karlsson (2010) and Tison and de Foucault (2014) used it as a useful characteristic to distinguish *V. tricolor* from *V. arvensis*. For many authors, the prevalent pollen morph was thought to be important to discriminate close species within the section *Melanium* but can it really be used as a diagnostic character to distinguish among species?

Seed morphology

Several authors highlighted the systematic utility of both macro- and micromorphology of seeds in providing useful characters to discriminate among species (e.g. Clark and Jernstedt 1978; Barthlott 1981; Boesewinkel and Bouman 1995; Gil-ad 1998; Seo 2010; Pahlevani and Akhani 2011).

Figure 1. The studied pansies in their natural habitats in Italy: A) *Viola arvensis* subsp. *arvensis* (VA-7 and VA-2), B) *Viola kitaibeliana* (VK-5), C) *Viola hymettia* (VH-8, photograph by S. Buono), D) *Viola tricolor* subsp. *tricolor* (VT-4, photograph by S. Buono). For the explanation of population codes reported in brackets see Table 1.

Particularly, many surface characters can be of diagnostic significance when characterizing the lowest taxonomic categories or groups of related species, being often stable and little affected by the environmental conditions (Barthlott 1981; Boesewinkel and Bouman 1995). Nevertheless, only a few studies were focused on *Viola* seeds (e.g. Gil-ad 1998; Colombo et al. 2007; Seo 2010; Ballard et al. 2014) and, usually, seed features are not considered as diagnostic characters to distinguish among species.

This paper is part of broader research on the taxonomy, cytology, biology, and ecology of annual taxa of *Viola* Section *Melanium* (Scoppola and Lattanzi 2012; Scoppola et al. 2014; Magrini and Scoppola 2015a, 2015b, 2015c, 2015d). In light of the nomenclatural confusion and the modest number of species-delimitating morphological features within some annual pansies, pollen heteromorphism and pollen and seed morphology have been assessed to find new characteristics useful for the delimitation of these species. In particular, here we focus on an integrated palynological and morphological study of four closely related European pansies, *Viola arvensis* subsp. *arvensis*, *V. hymettia*, *V. kitaibeliana*, and *V. tricolor* subsp. *tricolor*, aimed at 1) a descriptive and quantitative investigation of pollen morphology and heteromorphism, and seed morphology/micromorphology; 2) the assessment of the value as diagnostic characters of pollen size and morphology, of the prevalent pollen morph, of seed size and ornamentation, and 3) the assessment of their ecological/adaptive significance.

Materials and methods

Study species

Viola arvensis subsp. *arvensis* (field pansy), hereafter called *V. arvensis*, is a Mediterranean-Eurasiatic element widespread throughout almost the whole of Europe and SW Asia and linked to fields, other synanthropic habitats, and open shrublands (Gams 1926; Valentine et al. 1968; Marcussen and Karlsson 2010; Magrini and Scoppola 2015c). It is a mostly autogamous species with a pale cream-yellow corolla, shorter, equalling or a little longer than the calyx (Figure 1(A)) and a very small stylar flap (labellum) (Scoppola and Lattanzi 2012). Its chromosome number is $2n = 34$.

Viola kitaibeliana (dwarf pansy) is considered a Mediterranean-Caucasian species which extends to central Europe where it is a component of early stages of grasslands, stony slopes and other open places (Werner 1988; Randall 2004; Magrini and Scoppola 2015a, 2015c). It is a small-flowered and mostly autogamous species, with a cup-shaped corolla, not exceeding or equalling the calyx (Figure 1(B)), and an inconspicuous stylar flap (Erben 1985; Scoppola and Lattanzi 2012). Recently, *V. kitaibeliana* has been split in three species, separating two Atlantic taxa, *V. henriquesii* (Willk. ex Cout.) W. Becker and *V. nana* (DC.) Le Jolis, which have been reaffirmed as independent species (Magrini and Scoppola 2015b). Therefore, in the present study we have considered *V. kitaibeliana* in the strict sense. According to Magrini and Scoppola (2015a), its chromosome number is $2n = 16$ and all the other cytotypes reported for plants named *V. kitaibeliana*

($2n = 14$, 18, 24, 36, 40, and 48; e.g. Valentine et al. 1968; Randall 2004) must be referred to other taxa or to hybrids or to mistakes.

Viola hymettia (Mount Hymettus pansy) is a SE-European species widespread in the South Balkans and in the Aegean Islands, quite rare in Central and South Italy and in Sicily, mostly on sub-acid soils, linked to stony pastures, dry open habitats and shrub fringes (Erben 1985; Raus 1986; Scoppola and Lattanzi 2012; Dimopoulos et al. 2013; Vangjeli 2015). It has a corolla distinctly exceeding the calyx, cream and yellow coloured, often suffused with violet (Figure 1(C)), and a slightly protruding labellum. Its chromosome number is $2n = 16$.

Viola tricolor subsp. *tricolor* (wild pansy), hereafter called *V. tricolor*, is a European element, occurring in most of Europe, in habitats linked to woodlands and semi-natural lands in South Europe. The corolla is large, distinctly exceeding the calyx, usually blue-violet and cream coloured with yellow markings (Figure 1(D)); the labellum is large and protruding (Scoppola and Lattanzi 2012). Its chromosome number is $2n = 26$.

Collecting materials

Flowering plants and seedlings were collected in Italy from February 2011 to May 2015 in about 40 wild populations of the four study species (Table 1). Voucher specimens are preserved in UTV Herbarium (acronym according to Thiers 2018) and Tuscia Germplasm Bank (TGB, Tuscia University) (Table 1). Additional material (seeds and pollen grains) was taken from specimens in UTV, APP, and AO herbaria. New chromosome counts (indicated with * in Table 1) and published ones (Scoppola et al. 2014) were used to have the certain determination of the studied populations, except for some well-developed specimens of *V. arvensis*, *V. hymettia*, and *V. tricolor*. Overall, more than 800 specimens have been gathered to carry out palynological and morphological investigations and to find features to be used as diagnostic characters.

Palynological study

Pollen grains of the four *Viola* species were sampled from 10–20 living plants per population (five to ten different populations for each species in order to assess the constancy of pollen characters within species). They were studied by means of scanning electron microscopy (SEM) and light microscopy. Flowers were harvested before full blooming (large, nearly open buds) to collect pollen from closed anthers, from one to nine different flowers per plant (based on availability). All the flowers were dissected immediately after harvesting to avoid pollen loss.

For SEM observations, the anthers were opened directly over metallic stubs with double-sided tape and sputter-coated with gold (Balzers MED 010). The SEM examination was carried out on a Jeol Scanning Electron Microscope JSM 5200 and the particulars of the exine ornamentation were photographed with a Jeol Scanning Electron Analytic Microscope JSM 6010 LA. The measurements of polar axis (*P*) and equatorial diameter (*E*) were made on 30 grains from each stub under a magnification of 1500×. Pollen terminology used is in accordance with Hesse et al. (2009). Pollen shape has been classified on the basis of the ratio of the length of the polar axis (*P*) to the equatorial diameter (*E*) according to the Erdtman's system of shape classes (Erdtman 1952).

For analysing pollen assemblage, anthers were removed from one to three flower buds for each plant and put directly on a microscope slide, immersing them in a drop of lactic acid to let out all the pollen grains (Magrini and Scoppola 2015c). Observations of the entire pollen assemblage were carried out under a light microscope Leitz HM-LUX3 with a magnification of 100× (resolution: 0.25 μm). This usually entailed the counting of 200–1600 grains and over. The different pollen morphs were counted according to the following scheme:

- triangular shape = three-aperturate grain,
- squared shape = four-aperturate grain,
- pentagonal shape = five-aperturate grain,
- hexagonal shape = six-aperturate grain,
- round, elliptical, irregular shape = immature, empty, aborted, or unidentifiable grains.

For each species, pollen assemblage with the average percentage of the prevalent morph lower than 90% was reported as "heteromorphic" (HM) and when the other morphs represented less than 10% the pollen assemblage was considered "crypto-heteromorphic" (Till-Bottraud et al. 1995).

Seed morphology study

Mature seeds were randomly sampled from both fresh plants and herbarium specimens, from a total of 30 populations of the four species (Table 1).

For the SEM studies, seeds were fixed over metallic stubs with double-sided tape and sputter-coated with gold (Balzers MED 010). The SEM micrographs were taken using a Jeol Scanning Electron Analytic Microscope JSM 6010 LA at 60×, 150×, and 1300× magnification. At least ten seeds from each stub were scanned to ensure the consistency of seed-coat characters. Seed coat terminology used is in accordance with Barthlott (1981) and Corner (1976).

For the morphometric analysis, at least 30 seeds from each population have been photographed under a Nikon SMZ 1000 stereo-microscope with a digital camera Nikon DXM1200. The following seed morphometric features have been measured using the software LUCIA Measurement vers. 4.80 for NIKON Instruments: length (*L*) as the longitudinal axis, width (*W*) as the transverse axis, perimeter (*P*), and area (*A*). Other seed shape parameters were derived from the measured features according to Bacchetta et al. (2008), as follows: Feret ratio, calculated as the width-to-length ratio; shape factor, estimated as $SF = 4\pi\frac{A}{P^2}$; roundness factor, estimated as $RF = 4\frac{A}{\pi L^2}$.

Table 1. The studied material with information about each population: species, locality, habitat, altitude, chromosome number, original (*) and published (Scoppola et al. 2014), and voucher specimen code preserved in UTV, APP, and AO herbaria (acronyms according to Thiers 2018) and in the Tuscia Germplasm Bank (TGB).

Species/code	Locality	Habitat	Altitude (m a.s.l.)	Chromosome number	Voucher specimen
Viola arvensis subsp. *arvensis*					
VA-1	Saint Denis, Del (Aosta)	Edge of stony path between fields	760	$2n = 34$	AO N.SFV-2939. TGB-A-29313
VA-2	Mt. Cetona, Sarteano (Siena)	Edge of stony path in calcareous open grassland	1100	$2n = 34$	UTV 30445. TGB-A-29512
VA-3	Isola del Giglio (Grosseto)	Arid grassland within a garrigue	250	–	UTV 12594
VA-4	San Martino al Cimino, Viterbo (Viterbo)	Synanthropic habitat near pine trees	550	$2n = 34$	UTV 29497. TGB-A-29912
VA-5	Mt. Palanzana, Viterbo (Viterbo)	Roadside edge in a hazel cultivation	480–652	$2n = 34^*$	UTV 30447. UTV 34932
VA-6	Riello, Viterbo (Viterbo)	Resting field	303	$2n = 34$	UTV 30441. TGB-A-14510
VA-7	Bullicame, Viterbo (Viterbo)	Synanthropic habitat	289	$2n = 34^*$	UTV 29494
VA-8	Colonna, Bomarzo (Viterbo)	Fields on calcareous soil	219	$2n = 34$	UTV 30371
VA-9	Mt. Tancia, Monte San Giovanni in Sabina (Rieti)	Stony grassland on calcareous soil	1180	$2n = 34^*$	UTV 32125. TGB-A-28614
VA-10	Mt. Gennaro, Palombara Sabina (Roma)	Grassland among olive groves on calcareous soil	430	–	APP 58219
VA-11	Forca d'Ancarano, Norcia (Perugia)	Field edge	1013	$2n = 34^*$	UTV 32643. TGB-A-29612
Viola kitaibeliana					
VK-1	La Valle, Serravalle di Chienti (Macerata)	Open shrubland with stony grassland on calcareous soil	735	$2n = 16$	UTV 30448
VK-2	Cerreta, Nespolo (Rieti)	Open grassland in rural environment on calcareous soil	1000	$2n = 16$	UTV 29512. TGB-A-14710
VK-3	Mt. Navegna, Varco Sabino (Rieti)	Stony grassland on calcareous soil	1184	$2n = 16^*$	UTV 32126. TGB-A-28714
VK-4	Forca d'Ancarano, Norcia (Perugia)	Stony meadow between fields on calcareous soil	1010	$2n = 16$	UTV 30449. TGB-A-29712
VK-5	Bassano Scalo, Orte (Viterbo)	Arid and stony grassland on calcareous soil	70	$2n = 16$	UTV 30152. TGB-A-17211
VK-6	Le Vigne, Ofena (L'Aquila)	Arid and stony grassland on calcareous soil	481	$2n = 16$	UTV 30537. TGB-A-30712
VK-7	S. Colombo, Barisciano (L'Aquila)	Arid grassland in open woodland on calcareous soil	940	$2n = 16$	UTV 30155
Viola hymettia					
VH-1	Asinello, Viterbo (Viterbo)	Open grassland and uncultivated land on volcanic soil	250	$2n = 16$	UTV 29470
VH-2	Mt. Palanzana, Viterbo (Viterbo)	Dry open habitat and shrub fringes on volcanic soil	534	$2n = 16$	UTV 30151
VH-3	Palanzanella, Viterbo (Viterbo)	Dry open habitat and shrub fringes on volcanic soil	518	$2n = 16^*$	UTV 29514
VH-4	Riello, Viterbo (Viterbo)	Uncultivated escarpment with pine trees and shrubs	323	$2n = 16^*$	UTV 29479. TGB-A-17311
VH-5	Ponte del Diavolo, Viterbo (Viterbo)	Arid and stony escarpment with shrubs	243	$2n = 16^*$	UTV 29508
VH-6	Mt. Pizzo, Viterbo (Viterbo)	Uncultivated escarpment with shrubs	452	–	UTV 30548
VH-7	Montigliano, Viterbo (Viterbo)	Uncultivated land on volcanic soil	366	–	UTV 29511
VH-8	St. Barbara, Vetralla (Viterbo)	Dry open habitat and shrubs fringe on volcanic soil	158	$2n = 16$	UTV 30455. TGB-A-40315
VH-9	Grotta Porcina, Vetralla (Viterbo)	Dry grassland on volcanic soil	199	–	UTV 30154
VH-10	Necropoli di Norchia, Viterbo (Viterbo)	Dry grassland on volcanic soil	150-163	–	UTV 30456. UTV 29471
VH-11	Mt. Trave, Ferentino (Frosinone)	Open grassland and stony slopes on calcareous soil	300	$2n = 16$	UTV 30150
VH-12	Le Vigne, Ofena (L'Aquila)	Uncultivated land in olive grove on calcareous soil	492	$2n = 16$	UTV 30454. TGB-A-30812
VH-13	Gravine, Palagianello (Taranto)	Grassland and uncultivated land on calcareous soil	115	$2n = 16$	UTV 30450. TGB-A-29013
Viola tricolor subsp. *tricolor*					
VT-1	Alviano (Terni)	Uncultivated land	251	–	UTV 25847
VT-2	Mt. Palanzana, Viterbo (Viterbo)	Shrub fringe in semi-natural land	650–655	$2n = 26$	UTV 30443. UTV 30546
VT-3	Le Pantanacce, Caprarola (Viterbo)	Humid grassland with shrubs	512	–	UTV 12650
VT-4	La Mola, Oriolo Romano (Viterbo)	Edge of oak forest	317	$2n = 26$	UTV 30444
VT-5	Acquaforte, Vejano (Viterbo)	Shrub fringe in semi-natural land	336	$2n = 26$	UTV 30440. TGB-A-41517
VT-6	Selva del Lamone, Farnese (Viterbo)	Edge of oak forest	328	–	UTV 30543
VT-7	Mainarde, Vallerotonda (Frosinone)	Shrub fringe in semi-natural land	950	–	UTV 31247

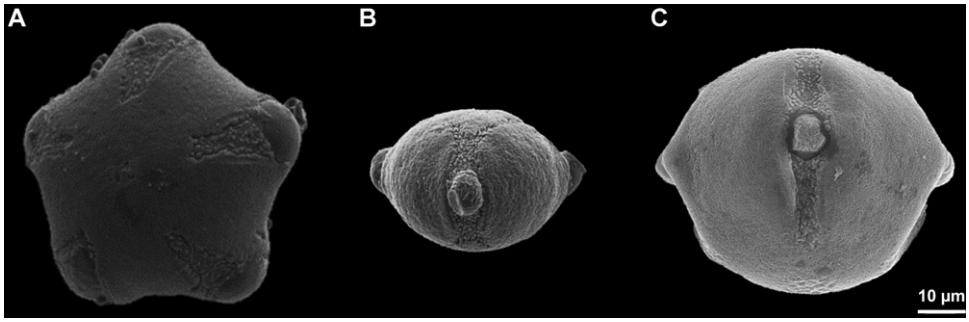

Figure 2. SEM micrographs of pollen grains in hydrated conditions at the magnification of 1500×: A) 5-colporate grain of *Viola arvensis* subsp. *arvensis* in polar view; B) 4-colporate grains of *Viola hymettia* and of C) *Viola tricolor* subsp. *tricolor* in equatorial view.

Table 2. Pollen features of the studied *Viola* species.

Species	*P* (µm) (*M* ± SD)	*E* (µm) (*M* ± SD)	*P/E* ratio (*M* ± SD)	Outline in polar view of the main pollen morph	Shape *P/E*	Shape (dry pollen)
Viola arvensis subsp. *arvensis*	51.37 ± 3.04^{a}	64.34 ± 7.85^{a}	0.81 ± 0.12^{a}	Pentagonal	Subspheroidal-suboblate (oblate to oblate spheroidal)	Prolate, lobate
Viola kitaibeliana	33.04 ± 1.50^{b}	37.94 ± 2.64^{b}	0.87 ± 0.02^{a}	Tetragonal	Subspheroidal-suboblate	Perprolate, lobate
Viola hymettia	39.89 ± 1.86^{c}	44.68 ± 4.36^{bc}	0.90 ± 0.05^{a}	Tetragonal	Subspheroidal-oblate spheroidal	Perprolate, lobate
Viola tricolor subsp. *tricolor*	39.25 ± 3.87^{c}	53.29 ± 2.05^{ac}	0.74 ± 0.11^{a}	Tetragonal	Oblate	Prolate, lobate
***p* value**	**<0.0001**	**<0.0001**	0.1208			
F	**51.62**	**20.94**	2.221			
R^2	**0.8907**	**0.7773**	0.2701			

P: polar axis; *E*: equatorial diameter; shape *P/E*: Shape classes defined as a ratio between the polar axis and the equatorial diameter (Erdtman 1952); *M*: mean value; SD: standard deviation. For each parameter, the values followed by the same letter are not significantly different at the 5% level of probability, as determined by Tukey's test. ANOVA results: *p*: probability; *F*: *F*-test distribution value; R^2: is the fraction of the overall variance attributable to differences among the group means; boldfaced values indicate statistically significant difference among the species.

Statistical analysis

For statistical analysis of palynological data, we have considered only the data-sets with more than 200 pollen grains and with percentages of immature or aborted grains less than 20% (Magrini and Scoppola 2015c). Immature, empty, aborted or unidentifiable grains were not considered to calculate the percentages of the different pollen morphs. Data-sets of both pollen and seed features were processed singularly by means of one-way ANOVA (after a check of Bartlett's statistics) using GraphPad Prism Software, version 5.01 (San Diego, CA, www.graphpad.com), followed by Tukey's multiple comparison test to assess the occurrence of statistically significant differences among species. When Bartlett's statistics revealed a non-homoscedasticity of variance ($p < 0.05$), then the non-parametric Kruskal–Wallis test for independent samples (followed by Dunn's multiple comparison test) was used.

Box and whiskers graphs were plotted for the most significant characteristics, with whiskers indicating the 10–90 percentile and outliers plotted as individual points.

Results

Palynological study

General description of pollen

Pollen grains are radially symmetrical, isopolar and shed in monads. Pollen shape in hydrated condition is subspheroidal in equatorial view in *V. arvensis*, *V. kitaibeliana*, and *V. hymettia*, oblate in *V. tricolor* (Figure 2; Table 2). It is prolate to perprolate in dry condition, lobate in all the species, typically infolded as a consequence of harmomegathy, with apertures sunken and interapertural areas slightly sunken (Figure 3). The outline is polygonal in polar view, mainly quadrangular and pentagonal, with very low percentages of triangular or hexagonal grains in addition (Figure 3).

The apertures are colporate and equatorially elongated, reaching the polar areas of the pollen grain with entire or horned ends (Figure 3). The exine is microreticulate, with lumina smaller than 1 µm, having a different size in the different species. Particularly, in *V. arvensis* and *V. hymettia*, the reticulum is tight and regularly arranged, with lumina much smaller than 1 µm and narrow muri; in *V. kitaibeliana* and *V. tricolor*, the reticulum is irregularly arranged, also with larger lumina, even anastomosing, and with broader muri. The presence of pollen coatings like pollenkitt or granula with different size and shape has been observed. The polar area presents rounded tenuitates (Figure 3).

Morphometric study

Measurements of pollen grains are shown in Table 2. No significant difference has been detected between the size of the different pollen morphs in the same species.

The range of the polar axis (*P*) varies among the species from 31 to 57 µm and the equatorial diameter (*E*) from 35 to 76 µm. *Viola kitaibeliana* has the significantly smallest pollen grains ($P = 31.25$–35.00 µm, $E = 35.20$–41.07 µm; Tukey's test:

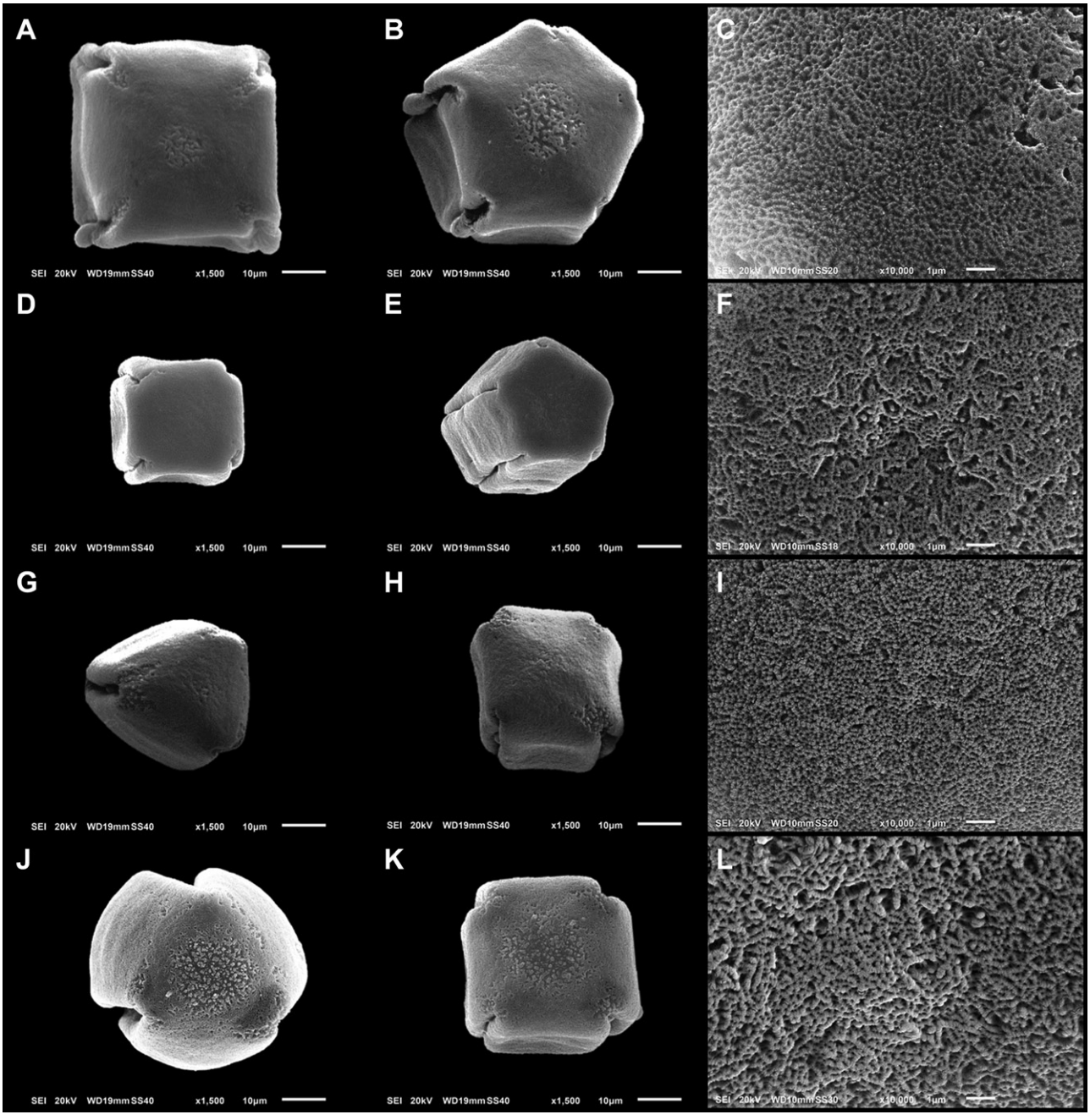

Figure 3. SEM micrographs of dry pollen grains in polar view at a magnification of 1500× with the detail of the exine surface (10,000×): A–C) *Viola arvensis* subsp. *arvensis* (VA-5 and VA-6); D–F) *Viola kitaibeliana* (VK-5, VK-2, and VK-1); G-I) *Viola hymettia* (VH-9, VH-5, and VH-8); J–L) *Viola tricolor* subsp. *tricolor* (VT-5). For the explanation of population codes reported in brackets see Table 1.

$p < 0.05$) and *V. arvensis* the largest ($P = 48.06–57.50\,\mu m$, $E = 53.95–76.28\,\mu m$; Tukey's test: $p < 0.001$). Particularly, significant differences in the pollen size (both *P* and *E*) have been recorded between *V. arvensis* and both *V. kitaibeliana* and *V. hymettia* (Tukey's test: $p < 0.001$), and between *V. kitaibeliana* and *V. tricolor* (Tukey's test: $p < 0.05$) (Table 2, Figure 4). Other significant differences have been recorded in the length of the polar axis between *V. arvensis* and *V. tricolor* (Tukey's test: $p < 0.001$), and between *V. kitaibeliana* and both *V. hymettia* (Tukey's test: $p < 0.01$) and *V. tricolor* (Tukey's test: $p < 0.05$). The *P*/*E* ratio varied between 0.6 and 0.9 showing no significant differences among the species (Table 2).

Pollen heteromorphism

Pollen heteromorphism (*sensu* Mignot et al. 1994) has been observed in all the species with the prevalence of four- or five-aperturate pollen grains and mean percentages of the prevalent morph ranging from 83% to 96% (Figure 3 and Table 3).

Pollen four-aperturate is highly predominant (>90%) in *Viola hymettia*, *V. tricolor*, and *V. kitaibeliana*, with low percentages of five-aperturate grains (<5%) and lower percentages of three-aperturate ones (<0.3%). In *V. arvensis*, five-aperturate grains prevail (>86%), with low percentages of four-aperturate grains (<15.0%) and low percentages of six-aperturate grains (<0.5%). In two out of seven populations

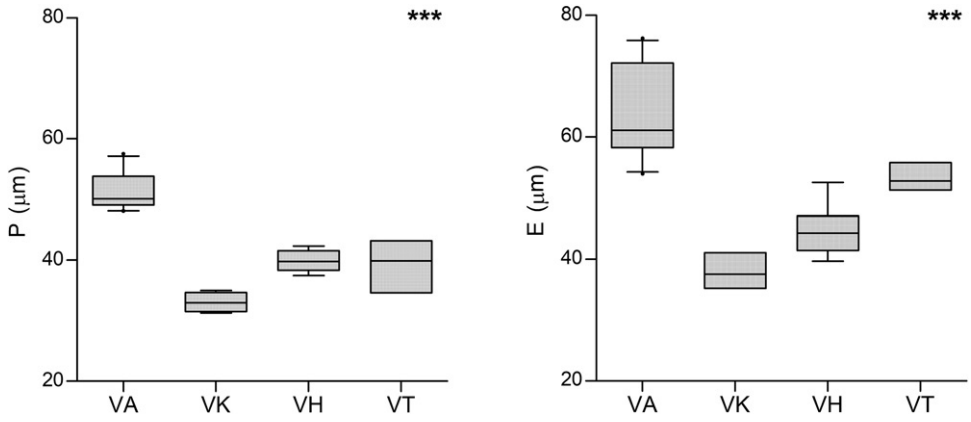

Figure 4. Box-and-whisker plots of pollen features of *Viola arvensis* subsp. *arvensis* (VA), *V. kitaibeliana* (VK), *V. hymettia* (VH), and *V. tricolor* subsp. *tricolor* (VT). *P*: polar axis; *E*: equatorial diameter. Whiskers indicate the 10–90 percentile; outliers are plotted as individual points. ***Significance with $p < 0.0001$, as determined by one-way ANOVA.

Table 3. Percentages of pollen grains for each morph (mean value ± standard deviation, with minimum-maximum values in brackets) and pollen heteromorphism of the four species of *Viola*.

	Pollen grains (%)					
Species	Three-aperturate	Four-aperturate	Five-aperturate	Six-aperturate	HM (<90 %)	Main pollen morph
Viola arvensis subsp. *arvensis*	–	13.50 ± 6.41 (3.33–22.07)	**86.46 ± 6.46 (77.48–96.67)**	0.03 ± 0.12 (0.00–0.45)	HM	5-colporate
Viola kitaibeliana	0.31 ± 0.47 (0.00–1.07)	**95.91 ± 2.05 (94.07–98.41)**	3.78 ± 2.13 (1.12–5.93)	–	–	4-colporate[a]
	–	14.06 ± 5.04 (8.24–17.22)	**85.90 ± 5.08 (82.67–91.76)**	0.04 ± 0.07 (0.00–0.12)	HM	5-colporate[b]
Viola hymettia	0.20 ± 0.37 (0.00–0.94)	**95.54 ± 3.83 (89.07–99.18)**	4.29 ± 3.99 (0.43–10.93)	–	–	4-colporate
Viola tricolor subsp. *tricolor*		**95.50 ± 3.60 (91.55–98.59)**	4.50 ± 3.60 (1.41–8.45)	–	–	4-colporate

The percentages of the main pollen morph for each species are reported in bold. Abbreviations: HM: heteromorphic *sensu* Till-Bottraud et al. (1995).
[a]Found in most of the populations of *V. kitaibeliana* (VK-1, VK-3, VK-5, VK-6, and VK-7).
[b]Only found in two out of seven populations of *V. kitaibeliana* (VK-2 and VK-4).

of *V. kitaibeliana* (VK-2 and VK-4), the prevalence of a different morph (five-aperturate) has been detected. The pollen assemblage of *V. arvensis* and of the populations of *V. kitaibeliana* with the prevalence of five-aperturate pollen morph have mean percentages lower than 90% and can be defined heteromorphic *sensu* Till-Bottraud et al. (1995) (Table 3). Those of *V. hymettia*, *V. tricolor* and of the remaining populations of *V. kitaibeliana*, with a large prevalence of four-aperturate morph, can be defined crypto-heteromorphic *sensu* Till-Bottraud et al. (1995).

Seed morphology study

The analysed seeds are brown to dark brown (only immature ones are pale brown), ovoid to obovoid in shape, all with an exostomal aril, or elaiosome, in the micropylar area (Figure 5), and a conspicuous raphe (Figure 6(A)).

Seed micromorphology

The seed coat (testa) is made up of three layers (Figure 6(C)), with no difference among the species. The first, the outer epidermis, is cuticularised. Stomata have been observed near the chalaza in all the species (Figure 6(A,B)); only in *V. kitaibeliana* and *V. hymettia* a few stomata have been found also higher up along the raphe, and even up to the micropylar area. Epidermal cells are tangentially elongated along the main axis of the seed (Figure 5) and characterized by a cellulose thickening of reticulate type on their radial walls and lumen almost completely obliterated (Figure 6(D)). The cells of the second layer are tangentially elongated with poor cytoplasmatic contents (Figure 6(D)). Each cell of the third layer, the 'crystal layer' ("*Kristallschicht*", Huber 1985), contains a large prismatic crystal of calcium oxalate; their inner walls are extremely thickened (Figure 6(E)).

Different levels of outer seed coat sculpturing were observed in the studied species (Table 4), according to the classification in Barthlott (1981). The cellular pattern showed no variation in cell shape, with the outline polygonal to elongate in the longitudinal direction in all the species, while differences were found in the relief of the cell boundary, another characteristic of the primary sculpture (Table 4), resulting raised instead of channelled only in *V. arvensis*. All the species have the same secondary sculpture, having a smooth surface of the outer cell wall. An evident difference was observed in the tertiary structure with the occurrence of more or less protruding papillae, irregularly distributed on the seed surface, only in *V. kitaibeliana* and *V. hymettia* (Table 4 and Figure 5).

Morphometric study

All the considered seed morphometric features result statistically discriminant among the species (Table 5), especially

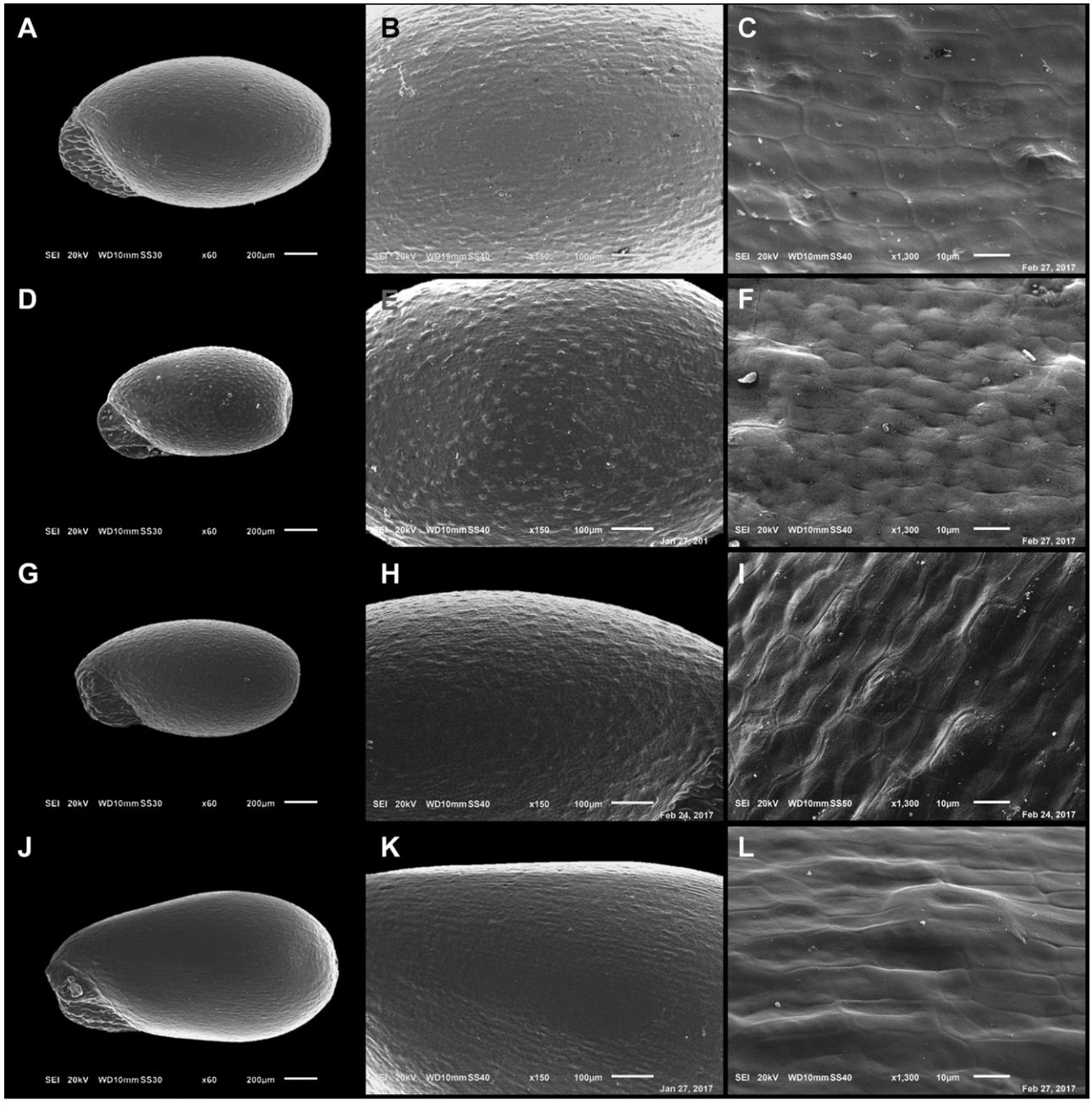

Figure 5. SEM micrographs of seeds in lateral view and seed coats at a magnification of 60×, 150×, and 1300×. A–C) *Viola arvensis* subsp. *arvensis* (VA-6); D–F) *Viola kitaibeliana* (VK-2); G–I) *Viola hymettia* (VH-8); J–L) *Viola tricolor* subsp. *tricolor* (VT-7). For the explanation of population codes reported in brackets see Table 1.

seed length and width (Figure 7). *Viola kitaibeliana* has significantly shorter seeds ($L = 1.19$–1.56 mm), particularly than *V. tricolor* and *V. arvensis* (Tukey's test: $p < 0.001$). Seed length of *V. hymettia* is significantly different from *V. arvensis* (Tukey's test: $p < 0.01$) and both *V. tricolor* and *V. kitaibeliana* (Tukey's test: $p < 0.05$). The seed width is highly significantly different between *V. kitaibeliana* and both *V. tricolor* and *V. arvensis* (Tukey's test: $p < 0.001$), and *V. hymettia* (Tukey's test: $p < 0.01$), and between *V. hymettia* and *V. tricolor* (Tukey's test: $p < 0.001$). Significant differences in seed size were found also within *V. kitaibeliana*, specifically between the populations with the prevalence of four-aperturate and five-aperturate pollen morph when comparing both length (1.36 ± 0.09 mm and 1.45 ± 0.07 mm, respectively; *t*-test, $p = 0.0423$) and width (0.73 ± 0.03 mm and 0.81 ± 0.03 mm, respectively; *t*-test, $p < 0.0001$).

The average Feret ratio ranges between 0.52 and 0.55 showing significant differences only between *V. arvensis* and *V. kitaibeliana* (Tukey's test: $p < 0.05$). Significant differences in all the considered seed morphometric features were recorded only between *V. arvensis* and *V. kitaibeliana* (Tukey's test: $p < 0.001$ and $p < 0.05$ for Shape factor and Feret ratio). No significant differences in Feret ratio, Shape factor and Roundness factor were detected between *V. arvensis* and *V. tricolor*, nor between *V. hymettia* and *V. kitaibeliana*.

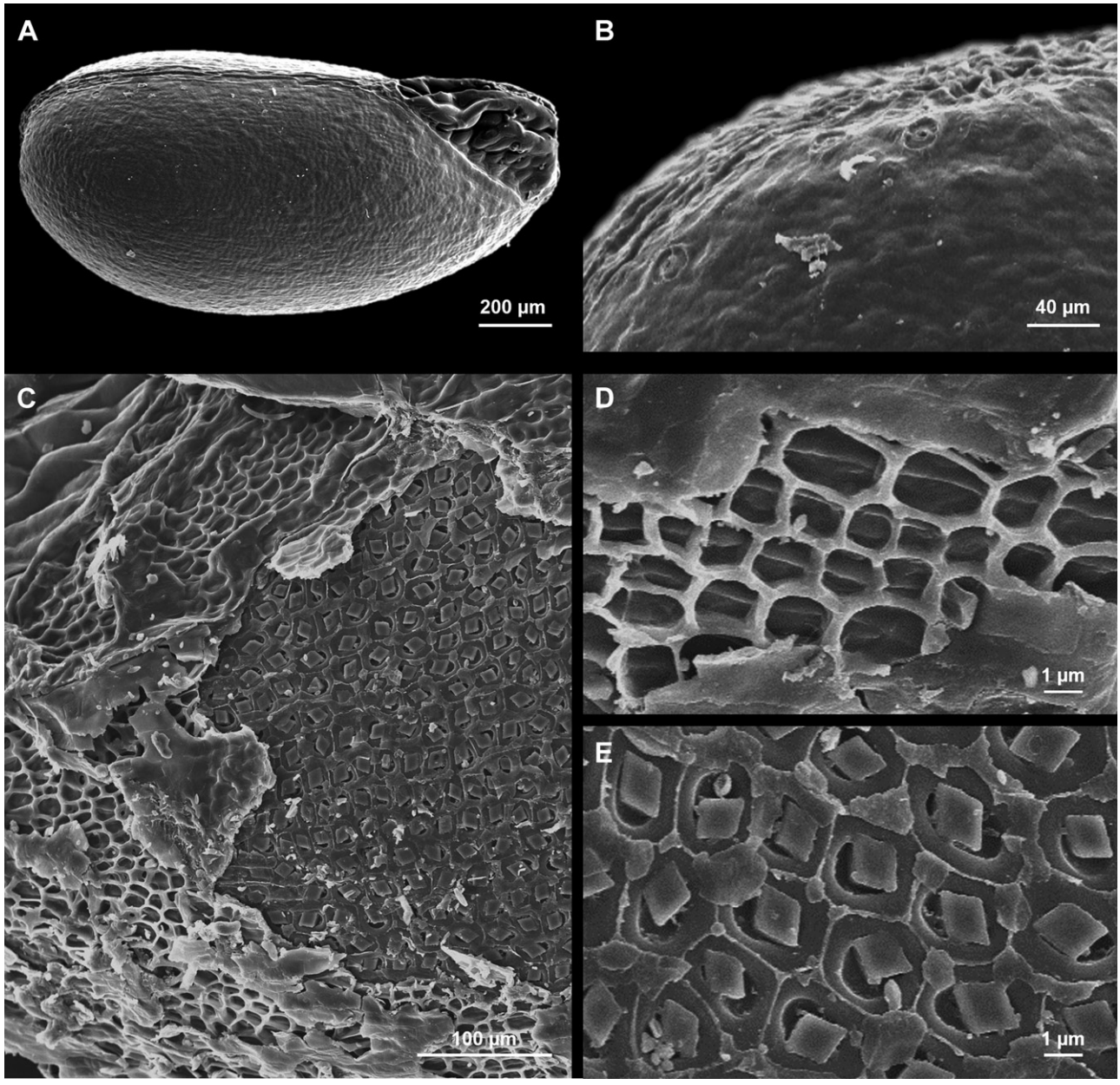

Figure 6. SEM micrographs of a seed of *Viola hymettia* (VH-12): A) evident raphe and aril (60×); B) particular of stomata in the chalazal region (600×); C) surface view of a macerated seed coat showing the three layers of the outer integument (170×); D) particular of the cuticularised epidermis with cells showing a thickening of reticulate type and elongated cells of the second layer; E) particular of the crystal layer with a prismatic crystal of calcium oxalate in each cell. For the explanation of population code reported in brackets see Table 1.

Table 4. Comparison of the primary, secondary and tertiary seed-coat sculpture of the studied species.

Species	Epidermal cell shape	Anticlinal cell wall boundaries	Secondary cell wall sculpture	Tertiary cell wall sculpture	Stomata
Viola arvensis subsp. *arvensis*	Polygonal to elongate in longitudinal direction	Straight and raised	Smooth	None	In the chalazal region
Viola kitaibeliana	Polygonal to elongate in longitudinal direction	Straight and channelled	Smooth	Papillae	Mainly in the chalazal region, also higher up along the raphe
Viola hymettia	Polygonal to elongate in longitudinal direction	Straight and channelled	Smooth	Papillae	Mainly in the chalazal region, also higher up along the raphe
Viola tricolor subsp. *tricolor*	Polygonal to elongate in longitudinal direction	Straight and channelled	Smooth	None	In the chalazal region

Table 5. Features of mature seeds of the four *Viola* species (mean value ± standard deviation).

Species	Length (mm)	Width (mm)	Feret ratio	Shape factor	Roundness factor
Viola arvensis subsp. *arvensis*	1.67 ± 0.07^{a}	0.87 ± 0.08^{a}	0.52 ± 0.04^{a}	0.83 ± 0.03^{a}	0.50 ± 0.04^{a}
Viola kitaibeliana	1.38 ± 0.08^{b}	0.74 ± 0.05^{b}	0.55 ± 0.03^{b}	0.85 ± 0.03^{b}	0.54 ± 0.04^{b}
Viola hymettia	1.52 ± 0.07^{c}	0.85 ± 0.06^{c}	0.55 ± 0.03^{ab}	0.85 ± 0.02^{b}	0.52 ± 0.03^{b}
Viola tricolor subsp. *tricolor*	1.74 ± 0.09^{d}	0.92 ± 0.12^{d}	0.54 ± 0.03^{ab}	0.82 ± 0.03^{ab}	0.51 ± 0.03^{ab}
***p* value**	**< 0.0001**	**< 0.0001**	**0.0163**	**0.0052**	**0.0004**
F	93.72	22.29	3.622	4.564	6.709
R^2	0.7555	0.4432	0.1145	0.1401	0.1933

For each parameter, the values followed by the same letter are not significantly different at the 5% level of probability, as determined by Tukey's test.
ANOVA results: *p*: probability; *F*: *F*-test distribution value; R^2: is the fraction of the overall variance attributable to differences among the group means; boldfaced values indicate statistically significant difference among the species.

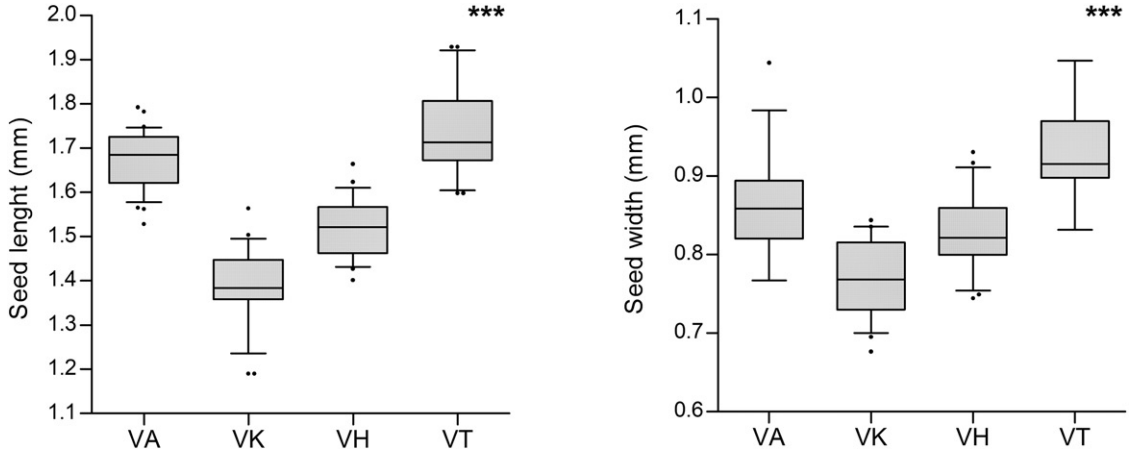

Figure 7. Box-and-whisker plots of length and width of the seeds of *Viola arvensis* subsp. *arvensis* (VA), *V. kitaibeliana* (VK), *V. hymettia* (VH), and *V. tricolor* subsp. *tricolor* (VT). Whiskers indicate the 10–90 percentile. Outliers are plotted as individual points. ***Statistical significance with $p < 0.0001$, as determined by one-way ANOVA.

Discussion

The observations carried out on the Italian populations of the four species confirmed that the taxa more difficult to delimit satisfactorily on purely macromorphological criteria are *V. kitaibeliana* and *V. arvensis*, particularly on specimens growing on poor soil (Yousefi et al. 2012; Scoppola and Lattanzi 2012). In such conditions, size, shape, and colour of the flowers are not worthy of consideration, being influenced by developmental and environmental factors (Kristofferson 1923). However, this study provides further morphological characters useful for species delimitation, especially to separate *V. kitaibeliana* from *V. arvensis* and *V. arvensis* from *V. tricolor*.

Comparison of pollen features and their usefulness as diagnostic characters

SEM has previously been employed in *Viola* to examine differences in pollen features among species of the section *Chamaemelanium* (Fabijan et al. 1987) and *Melanium* (Gorb 1994; Kuta et al. 2012; Saeidi Mehrvarz et al. 2014). To our knowledge, pollen morphology of *Viola hymettia* had never been studied before.

The examined species show no considerable variation in pollen micromorphology. Even if exine ornamentation proved to be a valuable taxonomic character within the section *Melanium* in Iran (Saeidi Mehrvarz et al. 2014), which included additional sublineages of *Melanium* (cf. Yockteng et al. 2003), no relevant difference has been observed among the study species showing the same microreticulate ornamentation, even if more or less thick and irregular. On the other hand, significant differences have been recorded in other features such as shape, size, and aperture number of pollen grains. Size of pollen grains in the section *Melanium* is significantly larger when compared to other sections of *Viola* (Saeidi Mehrvarz et al. 2014) and appears to be a morphological synapomorphy of pansies (Yockteng et al. 2003). Our data on pollen size of *V. arvensis*, *V. kitaibeliana*, and *V. tricolor* are not exactly in accordance with data in Saeidi Mehrvarz et al. (2014) from Iran and in Zvadová et al. (2004) from Slovakia, probably depending on the different method used or on differences in sampling methods that may often lead to biased estimates, but also on geographical differentiation. In accordance with Saeidi Mehrvarz et al. (2014), the pollen size has proved to be of diagnostic value (Table 6). *V. arvensis*, having the largest pollen grains, is easily separated from the other annual species of the section, especially from *V. kitaibeliana* which has the significantly smallest pollen grains. This is in accordance with previous results for the *Viola nuttallii* complex, section *Chamaemelanium* (Fabijan et al. 1987), and for the section *Viola* (s.str.) (McPherson and Packer 1974), suggesting a trend toward the increase in pollen size with increasing chromosome number and with higher ploidy level (Fabijan et al. 1987; Ballard et al. 2014).

Table 6. Synoptic table reporting the characters of pollen and seeds useful for the distinction between pairs of species.

	Viola arvensis subsp. *arvensis*	*Viola kitaibeliana*	*Viola hymettia*
Viola kitaibeliana	• Pollen size • Seed size • Seed coat ornamentation	–	–
Viola hymettia	• Pollen size • Prevalent pollen morph • Seed size • Seed coat ornamentation	• Pollen size • Seed size	–
Viola tricolor subsp. *tricolor*	• Pollen size • Prevalent pollen morph • Seed size	• Pollen size • Seed size • Seed coat ornamentation	• Seed size • Seed coat ornamentation

Prevailing pollen morph as a diagnostic character

Several pollen characters can be heteromorphic in *Viola* (size, shape, pollen wall structure, etc.) but many authors have focused on pollen assemblage in heteromorphic species mainly because the number of apertures in pollen grains is a discrete character (e.g. Till-Bottraud et al. 1995). We cannot confirm the general diagnostic value of the prevailing pollen morph to discriminate closely related species within the annual pansies as suggested by Randall (2004), Jäger and Werner (2005), Marcussen and Karlsson (2010), Słomka et al. (2014). In fact, even if it is widely reported that *V. tricolor* and *V. arvensis* can readily be separated on the basis of the prevalent pollen morph (Table 6) (Pettet 1964a, 1964b; Dajoz et al. 1995; Nadot et al. 2000; Hildebrandt et al. 2006; Saeidi Mehrvarz et al. 2014; Porter and Foley 2017; Słomka et al. 2018), this feature does not work to distinguish *V. kitaibeliana,* having populations with the prevalence of four- or five-aperturate pollen grains, from *V. arvensis* or *V. hymettia*. We do not agree with previous published data about the prevalence of five-aperturate pollen grains in *V. tricolor* (Mullenders and Mullenders 1957) and of four-aperturate ones in *V. arvensis* (Erdtman 1952), which appear to be based on a wrong identification of the two species (Pettet 1964a; Saeidi Mehrvarz et al. 2014). Moreover, in Dajoz et al. (1995) four-aperturate pollen grains prevail in *V. arvensis* based on the analysis of only 200 pollen grains from opened flowers.

Results herein on a larger data-set of *V. arvensis* subsp. *arvensis* do not agree with our preliminary findings concerning a single population of *V. arvensis* s.l. with predominant four-aperturate grains (Magrini and Scoppola 2015c), erroneously attributed in the past to *V. kitaibeliana* only on a morphological basis (Montelucci 1947; Scoppola and Lattanzi 2012). This population, having different morphology and phenology, and growing in a different habitat, is still under taxonomic investigation (unpublished data).

Diagnostic characters in seeds

The macro- and micromorphology of seeds provide additional useful characters (Table 6) to discriminate among species, as reported for *Viola* (Gil-ad 1998) and also for other genera (e.g. Clark and Jernstedt 1978; Seo 2010; Pahlevani and Akhani 2011). Valuable information was provided by seed morphometric characters all resulting in statistical significance. Particularly, the diagnostic value of seed length and width is found to be noteworthy and seed size was found to be a good delimitating character among the study species and easy to be measured with no need of particular equipment. It is especially useful to distinguish *V. kitaibeliana* (having the smallest seeds) from *V. arvensis*, two species often difficult to delimit satisfactorily on purely macro-morphological criteria. The small size of the seeds measured from Italian populations of *V. kitaibeliana* (especially the seeds of the populations having mainly four-aperturate pollen grains) is in accordance with the average seed length measured in BP and W herbaria in the specimens from "Pannonia", the locus classicus (1.39 ± 0.06 mm).

The study of seed coat ornamentation has provided taxonomic, evolutionary, and ecological insights in a number of groups (Clark and Jernstedt 1978; Pahlevani and Akhani 2011) as epidermal characters seem to be little affected by the environmental conditions with evidence suggesting a strong genetic control of these features (Cutler and Brandham 1977; Barthlott 1981). For example, SEM study of seed coat micro-sculpturing was used to delimit species and hybrids in *Viola* subsect. *Boreali-Americanae* (W. Becker) Gilad (Gil-ad 1998) and in supporting the recognition of several phylogenetically distinct lineages currently lumped under the polyphyletic assemblage *Hybanthus* Jacq., also Violaceae (Seo 2010; Ballard et al. 2014). Many surface characters, even if of minor taxonomic importance, are often stable and can be of diagnostic significance when characterizing the lowest taxonomic categories or groups of related species, especially in small seeds (Barthlott 1981; Boesewinkel and Bouman 1995). On the other hand, even if a number of taxa could be identified using the micromorphological characters alone, we suggest avoiding to distinguish pansies exclusively on a micromorphological basis but together with a set of macromorphological characters and habitat preferences, when possible (Gil-ad 1998). In particular, the diversity in the surface sculpturing detected by SEM analyses between *V. kitaibeliana/V. hymettia* (with papillae) and *V. arvensis/V. tricolor* (without papillae), proved to be a useful trait together with other characteristics, especially to distinguish *V. kitaibeliana* from *V. arvensis* (Table 6).

Seeds typically do not have stomata on their coats but they have been reported occasionally on seeds from species scattered among about 30 dicot and monocot families (Boesewinkel and Bouman 1995). They occur mainly in the "lower orders" of dicotyledons with endotestal (e.g. Magnoliaceae and Myristicaceae) or exotegmic seeds, as

Violaceae, suggesting that the presence of stomata may be a primitive or non-specialized feature of the outer epidermis of the testa (Corner 1976). Their presence is not known to be a constant characteristic of any family and in Violaceae seed-coat stomata are reported only for the genera *Viola* and *Rinorea* Aubl. (Corner 1976). Particularly, all of the taxa of subsect. *Boreali-Americanae* possess seed-coat stomata (Gil-ad 1998) but, to our knowledge, they are previously reported in the sect. *Melanium* only in the seed-coat of three taxa: *V. tricolor* (Corner 1976) and two perennials of the *Viola nebrodensis* group, *Viola ucriana* Erben & Raimondo and *V. tineorum* Erben & Raimondo (Colombo et al. 2007). We observed stomata in the seed coat of all the study species, typically in the chalazal region, even if in *V. kitaibeliana* and in *V. hymettia* a few stomata have been found also higher up along the raphe and even up to the micropylar area. However, according to Gil-ad (1998), the presence and the distribution of stomata seem to be variable from seed to seed so they are not helpful for species delimitation. There is limited information about the function of seed-coat stomata even if some hypotheses have been developed (Wang and Hasenstein 2016). Several authors suggested that they are involved in gas exchange for photosynthetic activity in the seeds of *Hymenocallis occidentalis* (Leconte) Kunth. (Amaryllidaceae) (Flint and Moreland 1943), *Eschscholzia californica* Cham. (Papaveraceae) (Jernstedt and Clark 1979), and *Brassica napus* L. (Brassicaceae) (Ruuska et al. 2004). On the other hand, Paiva et al. (2006) found a correlation between imbibition rates and stomata density in *Swietenia macrophylla* King (Meliaceae), suggesting that water uptake may be facilitated through stomata, especially in thick-coated species such as pansies.

Generally, the occurrence of crystals seems to have arisen polyphyletically and does not necessarily have taxonomic implications. Much is not known about the function of crystals in seed-coats. They may serve as storage organs of waste products, defend against animal feeders or assist in mechanical protection, especially in the case of silicate (Boesewinkel and Bouman 1984). In pansies, the seeds have hard and even surfaces that most likely prevent cracking of the seeds by the mandibles of the ants.

Finally, a clear cut-distinction can be made between two pairs of species with similar seed size and micromorphology having as well similar macromorphology. Basically, two seed types can be recognized: short seeds (length $<$ 1.6 cm) with papillae on the seed coat (*V. kitaibeliana/V. hymettia*) and long seeds (length $>$ 1.6 cm) without papillae (*V. arvensis/V. tricolor*). These characters permit the identification of specimens bearing mature seeds when other data are lacking or incomplete.

The interaction between environment and reproductive strategies

Considering the structure of flowers in the genus *Viola* as an evolutionary trait adapted to pollinators and to ecological conditions is widely accepted (e.g. Beattie 1974; Kuta et al. 2012; Słomka et al. 2018). An example is the position of the stigmatic orifice at the top of the gynoecium in pansies, clearly appearing downturned in prevailing inbreeder species and forward turned in outbreeder ones (Kuta et al. 2012; Scoppola and Lattanzi 2012; Słomka et al. 2018). Other features are, for instance, the nectar guides, the spur length and the pollen assemblage (Ballard et al. 2014).

Several authors have highlighted microenvironmental conditions affecting the proportion of the different pollen morphs in species of the sect. *Melanium*, such as soil metal-pollution, microtopography, daily duration of direct sunlight, atmospheric moisture, surrounding vegetation type, as well as elevation and exposure (Dajoz et al. 1993, 1995; Till-Bottraud et al. 1999, 2001; Moroń et al. 2014; Słomka et al. 2014). Recently, Słomka et al. (2018), partially endorsing the suggestions of Nadot et al. (2000), stated that pollen morphs proportions in pansies (not in the other sections of the genus) are not under the general pressure of ecological conditions, as previously hypothesized, and are adaptive but dependent on the breeding system and the pollinator behaviour. Our results and in situ observations essentially agree with their assessments and with main recent literature, particularly on the features of outbreeder large flowered *V. tricolor* and *V. hymettia*, inhabiting fringes and shady semi-natural habitats, where four-aperturate morph prevails (Pettet 1964a, 1964b; Marcussen and Karlsson 2010; Saeidi Mehrvarz et al. 2014; Magrini and Scoppola 2015c). The favourable microenvironmental conditions in shady margins and habitats linked to woods, prolonging the duration of flowering and pollinators activity, seems to promote the performance of four-aperturate long-lived pollen grains than five-aperturate ones, recorded in very low percentages in these populations. Heteromorphic pollen assemblage (*sensu* Till-Bottraud et al. 1995) was not found in *V. tricolor* and *V. hymettia* showing always a crypto-heteromorphic assemblage type (Till-Bottraud et al. 1995).

The ecological requirements of *V. arvensis* are normally different than those of *V. tricolor* in South Europe, being linked to ephemeral habitats mainly occurring in fields and synanthropic places (Erben 1985; Scoppola and Lattanzi 2012; Pignatti 2017; Porter and Foley 2017). On the other hand, neighbouring and even cohabiting populations of the two species can be found (e.g. VA-5 and VT-2, Table 1). In these conditions, a heteromorphic pollen assemblage with predominant five-aperturate morph was recorded in all the populations of *V. arvensis*. According to Till-Bottraud et al. (1995, 1999) and Słomka et al. (2018), the production of both more competitive (five-aperturate) and long-lived (four-aperturate) pollen grains by the same individual is perceived as a bet-hedging strategy favoured in inconstant, anthropogenic environments, where pollination conditions vary in an unpredictable way. Here, the fast-germinating five-aperturate grains could act when pollination takes place very quickly after anthesis and the long-lived four-aperturate could get involved if pollination occurs late (Dajoz et al. 1993). Our findings suggest that *V. arvensis* proves to have a clear role as a weed species, with the more competitive pollen assemblage and the largest pollen size, both positively affecting the germination speed and the pollen tube growth (Dajoz

et al. 1993). This is also supported by the floral structure, the fast life cycle, the great phenotypic plasticity, the production of a high number of large seeds, the efficient dispersal, and the wide distribution within its range (Degenhardt et al. 2005; Euro + Med 2006 onwards; Porter and Foley 2017).

The winter annual small-flowered *V. kitaibeliana* showed variability in the frequencies of the different pollen morphs as well as differences in the prevalent aperture number among populations that do not seem to be affected by the difference in the elevation range. Słomka et al. (2018) suggest that these differences are more frequently observed in predominantly inbreeder species rather than in outbreeder ones. Therefore, species like *V. kitaibeliana* are usually geographically dependent and secondly influenced by pollination ecology. In our study, however, heteromorphic pollen assemblages with predominant five-aperturate morph, as previously reported for Germany (Jäger and Werner 2005), were recorded in only two out of seven populations (VK-2 and VK-4, Table 1), both with cup-shaped corolla and growing in fairly disturbed open areas close to arable fields. Moreover, most of the populations from secondary grasslands and rocky pastures on Central Apennines have shown four-aperturate pollen grains prevailing (VK-1, 3, 5, 6, 7, Table 1) as previously displayed by Magrini and Scoppola (2015c) for Italy and Saeidi Mehrvarz et al. (2014) for Iran. *V. kitaibeliana* is among the earliest species blooming in spring on Apennine grasslands when pollination conditions are erratic due to the unstable atmospheric conditions (Beattie 1971). We could assume that the pollination reliability is rather limited in such circumstances and that this fact may have selected the prevalence of four-aperturate long-lived pollen grains than five-aperturate ones with also the occurrence of three-aperturate long-lived ones (Till-Bottraud et al. 1999). Even if, no noticeable difference in the pollination strategy has been detected among the populations (e.g. in phenology, corolla size and shape, stigma orifice, etc.), the brighter coloured nectar guides observed in plants with a slightly open corolla (particularly, in VK-3 and VK-6) could suggest the presence of a double breeding strategy in *V. kitaibeliana* when four-aperturate pollens prevail.

Seeds of pansies are dispersed in two steps, first by autochory and subsequently by ants (diplochory). The combination of two dispersal modes appears to be an adaptation to multiple selective pressures, because the first and second dispersal modes often have different effects (Beattie and Lyons 1975; Culver and Beattie 1978, 1980), and the relative importance of the two dispersal modes varies in response to different selection complexes in different habitats (Beattie and Lyons 1975; Ohkawara and Higashi 1994). Autochory only permits the seeds to be expelled from the capsule reaching short distances of dispersal, positively related to plant height and negatively to seed weight (Thomson et al. 2011). However, it may be important in reducing the competition propelling the seeds away from the parent plant and in different directions (Roberts and Haynes 1983; Lisci and Pacini 1997). On the other hand, even the opportunities for long-distance dispersal of ant-dispersed seed are very limited, because seeds dispersed by ants are not usually transported further than 10 m (Beattie and Lyons 1975; Culver and Beattie 1978; Higashi et al 1989). The study pansies can be divided into two groups, as already stated, also based on differences in the dispersal strategy. *V. arvensis* and *V. tricolor* can reach a height of even 40–60 cm (Valentine et al. 1968; Erben 1985; Tison and de Foucault 2014), producing many fruits and seeds during a fairly long life cycle. They are both able to disperse larger numbers of seeds at longer distances than smaller pansies (usually less than 20 cm tall) like *V. hymettia* and, particularly, *V. kitaibeliana* (Valentine et al. 1968; Erben 1985; Tison and de Foucault 2014), which produce smaller seeds with papillae during a very short growing season. The presence of a seed surface with more or less developed protuberances, as papillae, can have some ecological and adaptative advantages. For instance, Barthlott (1981) suggested that the surface roughness may cause turbulence in laminar air flow so helping to control the temperature in sunlight; on the other hand, it probably contributes to further limit the low efficiency of seed ballistic dispersal, especially in such small plants (Thomson et al. 2011). However, short dispersal distances can have an adaptative advantage providing suitable sites for seedling establishment, especially in habitats where microsites optimal for seed growth are scarce (Davidson and Morton 1981; Andersen 1988; Hughes et al. 1993), as observed in *V. kitaibeliana* having small populations always restricted to specific niches within the same limited areas. Short distance dispersal together with the autogamous pollination might enhance speciation rates because of the ease with which populations can become geographically isolated and results potentially subjected to inbreeding (Slingsby and Bond 1985).

Conclusions

In this integrated study concerning four closely related European pansies, palynological data for *V. hymettia* together with a detailed comparative analysis of seed morphology and micromorphology of the four species are reported for the first time. Results have highlighted some pollen and seed features as useful diagnostic characters. The pollen size has proved to be of diagnostic value to easily separate *V. kitaibeliana,* having the significantly smallest pollen size, from the others, especially from *V. arvensis* with the largest pollen grains. We can only partially confirm the diagnostic value of the prevailing pollen morph: *V. arvensis* can readily be separated from *V. tricolor* and *V. hymettia* but not *V. kitaibeliana* from *V. arvensis* or *V. hymettia*. The macro- and micromorphology of seeds of these annual pansies provide additional useful distinguishing characters. Seed size could be sufficient to discriminate among all the study species, even if we suggest using a larger set of features, and it results particularly useful in poor specimens bearing capsules with mature seeds when other vegetative/reproductive characters are lacking or incomplete.

This micromorphological approach applied to both pollen and seeds could be useful to clarify the species delimitation in other critical groups and also among other species within

the Section *Melanium*, e.g. *V. calcarata* L. and *V. aetnensis* (DC.) Strobl.

Acknowledgements

We are grateful to A. Gennaro and S. Bitti of the Laboratory of Plant Cytogenetics (Tuscia University, Viterbo) for cytological analyses. We also thank S. Buono, S. Ballelli, F. Bartolucci, M. Bovio, F. Conti, E. Lattanzi, and F. Prosser for materials and useful information. The authors gratefully acknowledge MIUR (Ministry for Education, University and Research) for financial support (Law 232/216, Department of excellence). The authors are grateful to T. Marcussen and the reviewers for their valuable and constructive comments which have helped in improving the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The study visits in the Herbaria of Copenhagen (C), Budapest (BP), Madrid (MA), and Wien (W) received support from the SYNTHESYS Projects DK-TAF-4177, HU-TAF-4506, ES-TAF-7076, and AT-TAF-7074 (www.synthesys.info) which were financed by European Community Research Infrastructure Action under the FP7 "Capacities" Programme.

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