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Scanning electron microscopy observations of pollen morphology in common vetch (*Vicia sativa*) subspecies

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Abstract

Common vetch (*Vicia sativa*) is an important forage and green manure crop that is widely cultivated around the world. However, the large number of subspecies and accessions of common vetch has made taxonomic research on this species difficult. Pollen morphology data can provide important evidence in the study of plant phylogeny. Therefore, in this research, light microscopy and scanning electron microscopy were used to observe seven morphological traits of pollens from 22 common vetch accessions, and residual maximum likelihood and pattern analysis was conducted. The results showed that the pollen grains of the 22 accessions were all monad pollen and the polar view revealed three-lobed circular and triangular shapes, while the equatorial view mainly revealed an oblate shape; only one accession showed an oblate spherical shape. All accessions were 3-zonocolporate and the colpus length extended close to the poles. The polar axis length was (19.39 ± 0.97)–(42.12 ± 0.76) μm and the equatorial axis length was (35.97 ± 1.27)–(45.25 ± 0.81) μm. We found that qualitative traits of pollen shape, aperture polar view and ornamentation were highly stable. Among them, polar axis length, equatorial axis length and colpus length and width had significant differences among the accessions. The ratios of the equatorial and polar axes had significant differences among the subspecies. This trait information could be used for the classification and identification of common vetch accessions and subspecies.

Introduction

Common vetch (*Vicia sativa*) is an economically important self-pollinating annual legume forage in the genus *Vicia* that has a short growth period, strong adaptability and the ability to fix nitrogen and improve soil structure. Common vetch can be used as feed, green manure, hay and farmland cover (Cakmakci *et al*., 2006; Liu *et al*., 2013, 2014; Dong *et al*., 2016). Due to its excellent traits and wide use, common vetch is widely planted in China, Turkey, New Zealand and other countries, and the area planted in Turkey amounts to 579 684 hectares (Firincioglu *et al*., 2014). This species is commonly planted in the middle and lower reaches of the Yangtze River, North China and Northwest China (Chen and Jia 2002) and is an important pasture and green manure crop in China's high altitude regions.

There are approximately 190 species of *Vicia* in the world, with approximately 40 species in China (Abozeid *et al*., 2017); the genus is mainly distributed in Europe, Asia and North America and extends to temperate South America and tropical Africa (Maxted, 1993; Jaaska, 2005). The highest specific diversity is found in Turkey and Northwest Asia (Maxted and Hawkes, 1997). Ball (1968) divided the genus into four subgenera: *Vicia, Cracca, Ervum* and *Faba*. Afterwards, these species were classified by Kupicha (1976) into two subgenera (subgenus *Vicilla* and subgenus *Vicia*) and 22 sections. Maxted (1993) and others further divided the subgenera into nine series, 38 species and 14 subspecies on the basis of phenotypic classification. Common vetch includes a large number of subspecies and accessions (Maxted, 1993) with high diversity, but the taxonomic information between subspecies is not yet complete. Taxonomic studies of four common vetch subspecies have been reported, but studies of other subspecies are still rare.

Conventional morphotypological taxonomy has been used as the basis for taxonomic treatments in the genus *Vicia* and diagnostic characters chosen on different bases have been used to delimit the subgenera and sections (Jaaska, 2005). Later, new classification methods were also widely used. For example, certain methods such as DNA RAPD and restriction fragment characteristics by Potokina *et al*. (1999) and isozymes by Jaaska (1997) have been utilized in studying the taxonomic relationships prevailing between species in the *Vicia* subgenus. Leht and Jaaska (2002) examined the subgenus based on cladistics and phenetics of both morphological and isozyme characteristics. Chung *et al*. (2013) used next-generation sequencing to develop

Table 1. Name and origin of 22 common vetch accessions

Accession No.	Accession	NPGS numbers	Origin	No.	Accession	NPGS numbers	Origin
1	V. sativa	PI-179113	Turkey	12	*V. sativa* subsp. *nigra*	PI-440763	Russian Federation
2	*V. sativa*	PI-220881	Belgium	13	*V. sativa* subsp. *nigra*	PI-440767	Russian Federation
3	*V. sativa*	PI-220897	Belgium	14	*V. sativa* subsp. *nigra*	PI-203250	Japan
4	*V. sativa*	PI-600818	United States	15	*V. sativa* subsp. *nigra*	PI-535572	Tunisia
5	*V. sativa*	PI-372251	Uruguay	16	*V. sativa* subsp. *sativa*	PI-289519	Hungary
6	*V. sativa*	PI-381066	Iran	17	*V. sativa* subsp. *sativa*	PI-664291	Pakistan
7	*V. sativa*	PI-632677	Bulgaria	18	*V. sativa* subsp. *sativa*	PI-170009	Turkey
8	*V. sativa*	PI-628318	Syria	19	*V. sativa* subsp. *sativa*	PI-251221	Afghanistan
9	*V. sativa*	PI-289510	Hungary	20	*V. sativa* subsp. *cordata*	PI-393867	Czech Republic
10	*V. sativa* subsp. *nigra*	PI-217969	Pakistan	21	*V. sativa* Lanjian No.1		China
11	*V. sativa* subsp. *nigra*	PI-220289	Afghanistan	22	*V. sativa* Lanjian No.3		China

NPGS, National Plant Germplasm System of the USA.

65 novel polymorphic cDNA-SSR markers in *Vicia sativa* subsp. *sativa* to make further contributions in molecular and breeding genetics studies of this species.

The aim of this study was to carry out a comparative study of the pollen morphology of the 22 taxa belonging to *Vicia sativa* L. from 15 countries by using light microscopy (LM) and scanning electron microscopy (SEM) and to assess the systematic significance of the examined pollen characteristics in terms of understanding the variation among the sections and taxa. In addition to accumulating information for systematic taxonomy within common vetch species, this work also lays a foundation for related palynological research.

Materials and methods

Plant materials

A total of 20 wild accessions and two local cultivar checks, Lanjian No. 1 and Lanjian No. 3, of common vetch were used in this study (Table 1). The accessions were obtained from the National Plant Germplasm System of the USA and College of Pastoral Agriculture Science and Technology, Lanzhou University of China.

Field trials

The experimental site was located at Yuzhong Experimental Station (104°09′ E, 35°89′ N, 1720 m a.s.l.), College of Pastoral Agricultural Science and Technology, Lanzhou University, Gansu Province. Yuzhong is located in the western Loess Plateau and belongs to a semi-arid climate zone. The soil type is loess and the area is characterized by an annual average temperature of 6.7 °C, average annual precipitation of 382 mm and average annual evaporation of 1343 mm (Dong *et al*., 2016). The total monthly precipitation and the mean monthly minimum and maximum temperatures during the trial period are shown in Fig. 1.

Experimental method

The experimental layout of the field trial was a randomized complete block design containing three replicates. Each accession of each replicate was planted with 30 individual plants, and the spacing of each individual plant was 50 cm within rows and 50 cm between rows. The experimental materials were sown on 20 April 2016 and pollen samples were collected when the various accessions reached the flowering stage from June to August of the same year. Mature pollen grains were obtained from randomly selected mature flower buds of three individual plants from each accession of each replicate and used as materials for observation by LM and SEM.

Light microscopy

Pollen grains were first treated with 70% alcohol to remove oily substances and then embedded in glycerine jelly stained with basic fuchsin following the method of Wodehouse (1935) (Kahraman *et al*., 2013). The polar axis length (*P*), equatorial axis length (*E*), colpus length (*Clg*) and colpus width (*Clt*) were observed by a LEICA-DM3000 microscope and 30 pollen grains were repeatedly measured for each accession. Then, the *P/E* ratio was determined.

Scanning electron microscopy

A double-sided conductive adhesive was used to fix the dried pollen grains on a metal sample stand and low vacuum sputter was used to coat the samples with gold for 90 s (K650X, this is a Quorum Sputter Coating Systems). Samples were observed with a scanning electron microscope (JSM-5600 LV, JEOL SEM), the acceleration voltage was 20 kV and the working distance was 10 mm. We observed 30 pollen grains of each accession in each replicate to determine pollen ornamentation and pole characteristics.

The size of the pollen grains was represented by the polar axis length and equatorial axis length ($P \times E$). The ratio of polar axis length and equatorial axis length (*P/E*) represented the shape of the pollen grain. A *P/E* of 0.5 ~ 0.8 is oblate, a *P/E* of 0.8 ~ 1.0 is oblate spherical and a *P/E* of 1.0 ~ 2.0 is prolate. The terminology of Punt *et al*. (2007) was used in descriptions of the pollen.

Data processing

Data analysis was based on (1) variance component analysis to assess the significance and magnitude of the genotypic variation

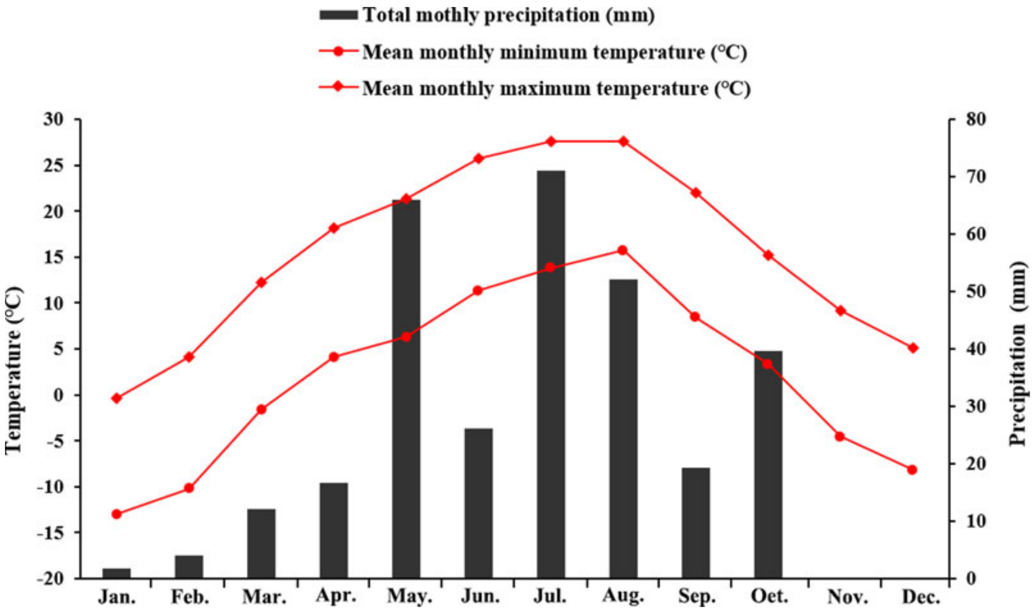

Fig. 1. Mean monthly minimum temperature (°C), mean monthly maximum temperature (°C) and total monthly precipitation (mm) in 2016 in Yuzhong County.

among accessions and (2) pattern analysis, which consisted of a combination of cluster analysis and principal component analysis (PCA), to provide a graphical summary of the accession-by-multi trait data matrices (Gabriel, 1971; Kroonenberg, 1994; Watson *et al.*, 1995).

The residual maximum likelihood (REML) option in GenStat 7.1 (2003) was used for variance component analysis. A random linear model was used to analyse data within subspecies data. The analysis was based on best linear unbiased predictors (BLUP) (White and Hodge, 1989) and generated accession means for pollen traits. The mixed linear model analysis was also performed with accessions as fixed effects to study the differences among accessions for each of the traits measured. SPSS (22) was used to analyse the measures of dispersion indexes.

According to the Fehr (1987) method, the variance components of genotypic (σ_g^2) and experimental error (σ_ε^2) and the number of replications (n_r) in the REML analysis were used to estimate the accession mean repeatability (R) of the five traits of each accession:

$$R = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_\varepsilon^2}{n_r}} \tag{1}$$

Results and analysis

The results of pollen morphological measurements are given in Table 2. SEM images of the representative pollen grains studied are shown in Figs 2–4.

Morphological characteristics

The pollen of 22 common vetch accessions was observed and all accessions were monad pollen (Table 2). Among them, 21 accessions were oblate and one (No. 6) was oblate spherical. The polar views of 19 accessions revealed three-lobed circles and three accessions exhibited triangle shapes (Table 2). The comparison of pollen grain size showed that there were significant differences in pollen grain size among different accessions. Among them, the polar and equatorial axis lengths of accession No. 6 were significantly larger than those of the other 21 accessions ($P > 0.05$). In addition, seven types of pollen ornamentation were recognized: reticulate (seven accessions), rugulate (seven accessions), reticulate-rugulate (three accessions), quarse regulate (two accessions), psilate-reticulate-rugulate (one accession), psilate-perforate (one accession) and reticulate-retipilate (one accession). The pollen colpus size of the various accessions in common vetch was significantly different and the colpus length and width of accession No. 6 were significantly larger than those of the other 21 accessions. The apertures of 22 accessions were 3-zonocolporate and close to the poles, with different widths and the colpus was smooth or showed wart-like protrusions, but there were obvious depressions in the colpus of accession No. 11. The pollen polar axis length of the 22 accessions ranged from 19.39 ± 0.97 to 42.12 ± 0.76 µm and the equatorial axis length ranged from 35.97 ± 1.27 to 45.25 ± 0.81 µm. The colpus length was between 23.95 ± 1.87 and 32.23 ± 2.38 µm and the colpus width was between 2.22 ± 0.32 and 5.05 ± 1.13 µm for the 22 accessions.

In general, there were significant differences between accessions No. 6 and No. 11 and the other accessions in the qualitative pollen traits, such as equatorial view (with nearly all accessions being oblate), aperture (all 3-zonocolporate), ornamentation and polar view. The other 20 accessions were not significantly different and high conservation among the various accessions was observed.

Morphological analysis between subspecies

As shown in Table 3,

Group 1: *V. sativa* subsp. *nigra* (Table 3; Figs 2(*j*—*o*), 3(*j*—*o*), (4*j*—*o*))

Pollen dimensions: $P = 21.03–24.22$ µm, $E = 35.97–40.78$ µm.

Pollen shape: oblate, $P/E = 0.54–0.61$ µm.

Apertures: 3-zonocolporate, apertures long, broader on pori in mesocolpium, $Clg = 26.10–30.97$ µm, $Clt = 2.22–3.30$ µm.

Table 2. Main characteristics of the pollen morphology in 22 accessions of common vetch

No.	Accession	*P* (μm)	*E* (μm)	*P/E*	Ev	Pv	Clg (μm)	Clt (μm)	Aperture	Orn
1	*V. sativa*	24.84 ± 0.83b	38.23 ± 1.99e	0.65	Oblat	Triangle	30.50 ± 1.12b	2.93 ± 0.52de	3-zonocolporate	Psilate-perforate
2	*V. sativa*	21.05 ± 1.08e	38.42 ± 2.17de	0.55	Oblat	Three-lobed circular	28.36 ± 1.92c	2.72 ± 0.44de	3-zonocolporate	Rugulate
3	*V. sativa*	21.17 ± 0.95e	33.91 ± 1.11g	0.62	Oblat	Three-lobed circular	25.20 ± 2.21de	4.25 ± 0.70b	3-zonocolporate	Reticulate-rugulate
4	*V. sativa*	24.35 ± 0.84b	39.82 ± 1.33cd	0.61	Oblat	Three-lobed circular	28.63 ± 2.58c	2.96 ± 0.62de	3-zonocolporate	Reticulate-rugulate
5	*V. sativa*	21.79 ± 0.89de	40.92 ± 1.61cd	0.53	Oblat	Three-lobed circular	27.36 ± 2.40cd	2.64 ± 0.38e	3-zonocolporate	Reticulate
6	*V. sativa*	42.12 ± 0.76a	45.25 ± 0.81a	0.93	Oblate spherical	Three-lobed circular	32.23 ± 2.38a	5.05 ± 1.13a	3-zonocolporate	Reticulate
7	*V. sativa*	24.84 ± 1.14b	40.43 ± 1.99cd	0.61	Oblat	Triangle	30.15 ± 1.74bc	2.34 ± 0.30ef	3-zonocolporate	Psilate-Reticulate-rugulate
8	*V. sativa*	22.48 ± 0.87d	41.14 ± 2.24c	0.55	Oblat	Three-lobed circular	27.10 ± 2.53cd	2.90 ± 0.63de	3-zonocolporate	Rugulate
9	*V. sativa*	19.39 ± 0.97f	36.47 ± 2.16f	0.53	Oblat	Three-lobed circular	23.95 ± 1.87e	2.58 ± 0.31ef	3-zonocolporate	Reticulate
10	*V. sativa* subsp. *nigra*	24.22 ± 2.23bc	39.70 ± 0.79d	0.61	Oblat	Three-lobed circular	30.97 ± 1.82ab	3.30 ± 0.51d	3-zonocolporate	Rugulate
11	*V. sativa* subsp. *nigra*	21.50 ± 0.97e	35.97 ± 1.27f	0.60	Oblat	Three-lobed circular	27.30 ± 1.92cd	2.83 ± 0.63de	3-zonocolporate	Rugulate
12	*V. sativa* subsp. *nigra*	23.00 ± 1.53cd	37.45 ± 1.14ef	0.61	Oblat	Three-lobed circular	29.11 ± 2.85bc	2.92 ± 0.71de	3-zonocolporate	Rugulate
13	*V. sativa* subsp. *nigra*	22.31 ± 1.44de	36.56 ± 2.14f	0.61	Oblat	Three-lobed circular	30.47 ± 0.00b	2.22 ± 0.32f	3-zonocolporate	Rugulate
14	*V. sativa* subsp. *nigra*	21.03 ± 0.89e	36.32 ± 3.07f	0.58	Oblat	Three-lobed circular	28.47 ± 1.68c	2.41 ± 0.30 ef	3-zonocolporate	Reticulate-retipilate
15	*V. sativa* subsp. *nigra*	22.11 ± 1.11de	40.78 ± 1.45cd	0.54	Oblat	Three-lobed circular	26.10 ± 2.96d	2.60 ± 0.31ef	3-zonocolporate	Reticulate
16	*V. sativa* subsp. *sativa*	24.71 ± 1.14b	37.89 ± 1.98ef	0.65	Oblat	Three-lobed circular	27.25 ± 2.43cd	3.49 ± 0.91c	3-zonocolporate	Reticulate-rugulate
17	*V. sativa* subsp. *sativa*	21.18 ± 1.39e	35.70 ± 1.81f	0.59	Oblat	Three-lobed circular	24.99 ± 2.11de	2.81 ± 0.49de	3-zonocolporate	Quarse rugulate
18	*V. sativa* subsp. *sativa*	22.33 ± 1.21de	39.05 ± 2.61de	0.57	Oblat	Three-lobed circular	30.83 ± 3.61ab	2.66 ± 0.35de	3-zonocolporate	rugulate
19	*V. sativa* subsp. *sativa*	20.72 ± 0.96e	40.41 ± 1.59cd	0.51	Oblat	Triangle	31.33 ± 1.79ab	2.41 ± 0.23ef	3-zonocolporate	Reticulate
20	*V. sativa* subsp. *cordata*	23.37 ± 1.65c	42.84 ± 1.55b	0.55	Oblat	Three-lobed circular	28.44 ± 2.66c	2.71 ± 0.45de	3-zonocolporate	Reticulate
21	*V. sativa*	23.50 ± 0.78c	37.95 ± 2.78ef	0.62	Oblat	Three-lobed circular	27.25 ± 2.49cd	2.88 ± 0.45de	3-zonocolporate	Quarse rugulate
22	*V. sativa*	23.21 ± 1.21cd	40.40 ± 1.16cd	0.57	Oblat	Three-lobed circular	30.74 ± 3.19ab	2.56 ± 0.34ef	3-zonocolporate	Reticulate

(*P*) polar axis, (*E*) equatorial axis, (*P/E*) ratio of equatorial and polar axes, (*Ev*) equatorial view, (*Pv*) polar view, (*Clg*) colpus length, (*Clt*) colpus width, (*Orn*) ornamentation.
Note: Different letters after the same data indicate significant differences at $P < 0.050$.

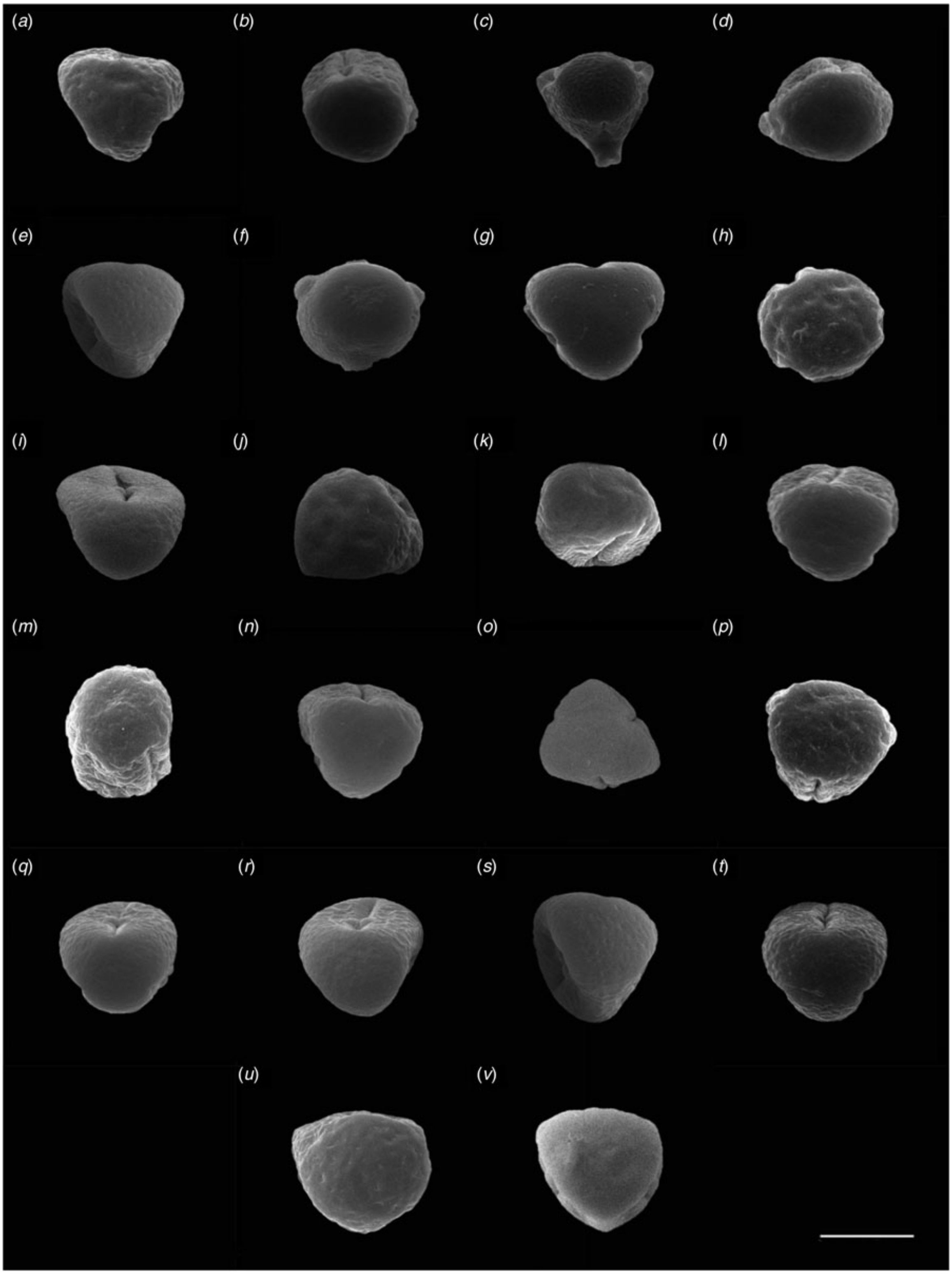

Fig. 2. Scanning electron microscopy (SEM) of pollen grains. Polar view: (*a*) *V. sativa* (No. 1), (*b*) *V. sativa* (No. 2), (*c*) *V. sativa* (No. 3), (*d*) *V. sativa* (No. 4), (*e*) *V. sativa* (No. 5), (*f*) *V. sativa* (No. 6), (*g*) *V. sativa* (No. 7), (*h*) *V. sativa* (No. 8), (*i*) *V. sativa* (No. 9), (*j*) *V. sativa* subsp. *nigra* (No. 10), (*k*) *V. sativa* subsp. *nigra* (No. 11), (*l*) *V. sativa* subsp. *nigra* (No. 12), (*m*) *V. sativa* subsp. *nigra* (No. 13), (*n*) *V. sativa* subsp. *nigra* (No. 14), (*o*) *V. sativa* subsp. *nigra* (No. 15), (*p*) *V. sativa* subsp. *sativa,* (No. 16), (*q*) *V. sativa* subsp. *sativa* (No. 17), (*r*) *V. sativa* subsp. *sativa* (No. 18), (*s*) *V. sativa* subsp. *sativa* (No. 19), (*t*) *V. sativa* subsp. *cordata* (No. 20), (*u*) *V. sativa* (No. 21), (*v*) *V. sativa* (No. 22). Scale bars = 10 μm.

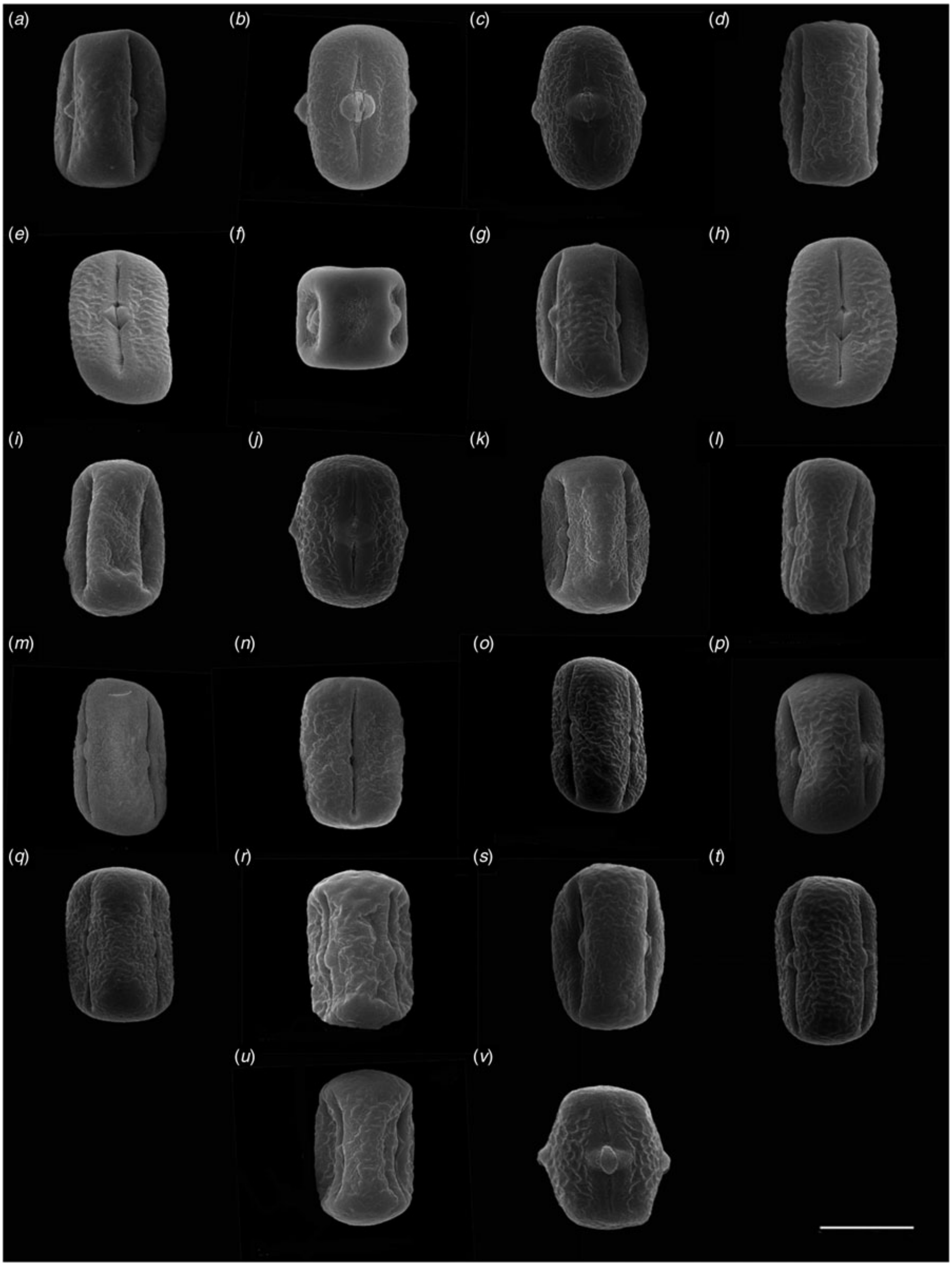

Fig. 3. Scanning electron microscopy (SEM) of pollen grains. Equatorial view: **(***a***)** *V. sativa* (No. 1), **(***b***)** *V. sativa* (No. 2), (*c*) *V. sativa* (No. 3), (*d*) *V. sativa* (No. 4), (*e*) *V. sativa* (No. 5), (*f*) *V. sativa* (No. 6), (*g*) *V. sativa* (No. 7), (*h*) *V. sativa* (No. 8), (*i*) *V. sativa* (No. 9), (*j*) *V. sativa* subsp. *nigra* (No. 10), (*k*) *V. sativa* subsp. *nigra* (No. 11), (*l*) *V. sativa* subsp. *nigra* (No. 12), (*m*) *V. sativa* subsp. *nigra* (No. 13), (*n*) *V. sativa* subsp. *nigra* (No. 14), **(***o***)** *V. sativa* subsp. *nigra* (No. 15), (*p***)** *V. sativa* subsp. *sativa,* (No. 16), **(***q***)** *V. sativa* subsp. *sativa* (No. 17), (*r*) *V. sativa* subsp. *sativa* (No. 18), (*s*) *V. sativa* subsp. *sativa* (No. 19), (*t*) *V. sativa* subsp. *cordata* (No. 20), **(***u***)** *V. sativa* (No. 21), (*v*) *V. sativa* (No. 22). Scale bars = 10 µm.

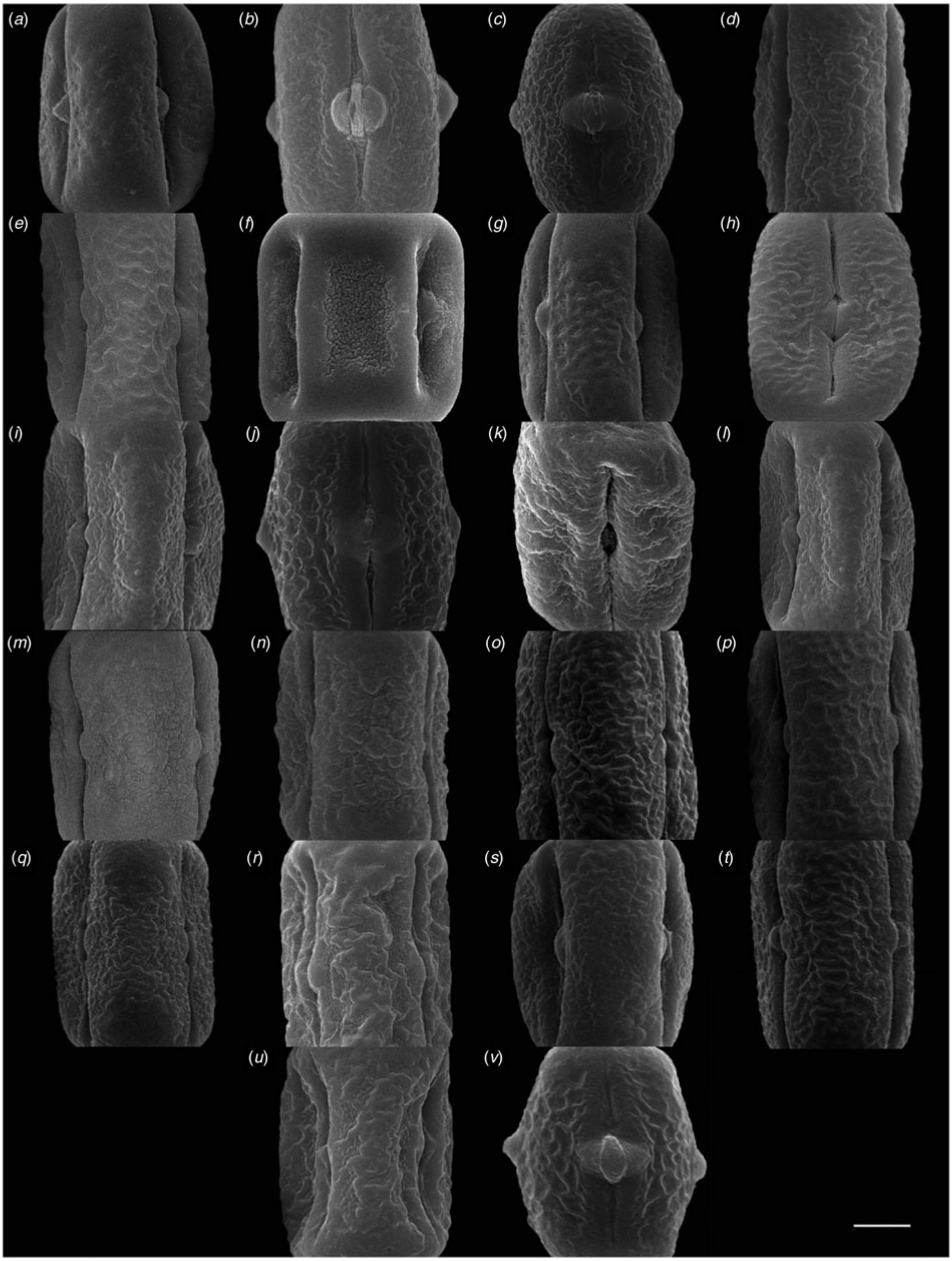

Fig. 4. Scanning electron microscopy (SEM) of pollen grains. Ornamentation: (*a*) *V. sativa* (No. 1), (*b*) *V. sativa* (No. 2), (*c*) *V. sativa* (No. 3), (*d*) *V. sativa* (No. 4), (*e*) *V. sativa* (No. 5), (*f*) *V. sativa* (No. 6), (*g*) *V. sativa* (No. 7), (*h*) *V. sativa* (No. 8), (*i*) *V. sativa* (No. 9), (*j*) *V. sativa* subsp. *nigra* (No. 10), (*k*) *V. sativa* subsp. *nigra* (No. 11), (*l*) *V. sativa* subsp. *nigra* (No. 12), (*m*) *V. sativa* subsp. *nigra* (No. 13), (*n*) *V. sativa* subsp. *nigra* (No. 14), (*o*) *V. sativa* subsp. *nigra* (No. 15), (*p*) *V. sativa* subsp. *sativa* (No. 16), (*q*) *V. sativa* subsp. *sativa* (No. 17), (*r*) *V. sativa* subsp. *sativa* (No. 18), (*s*) *V. sativa* subsp. *sativa* (No. 19), (*t*) *V. sativa* subsp. *cordata* (No. 20), (*u*) *V. sativa* (No. 21), (*v*) *V. sativa* (No. 22). Scale bars = 5 μm.

Table 3. Main characteristics of the pollen morphology of the three subspecies and two cultivars of 22 accessions of common vetch

Group	Taxa	*P* (μm)	*E* (μm)	*P/E*	Clg (μm)	Clt (μm)
1	*V. sativa* subsp. *nigra*	21.03–24.22 (22.36 ± 1.23b)	35.97–40.78 (37.45 ± 2.46d)	0.54–0.61 (0.60 ± 0.05a)	26.10–30.97 (28.08 ± 2.83a)	2.22–3.30 (2.67 ± 0.56a)
2	*V. sativa* subsp. *sativa*	20.72–24.71 (22.24 ± 1.89b)	35.70–40.41 (38.64 ± 2.72c)	0.51–0.65 (0.58 ± 0.06b)	24.99–31.33 (28.60 ± 3.64a)	2.41–3.49 (2.84 ± 0.68a)
3	*V. sativa* subsp. *cordata*	(23.37 ± 0.96a)	(43.67 ± 1.14a)	(0.54 ± 0.03c)	(28.44 ± 2.66a)	(2.71 ± 0.45a)
4	*V. sativa*	23.21–23.50 (23.36 ± 1.23a)	37.95–40.40 (39.84 ± 2.46b)	0.57–0.62 (0.59 ± 0.05ab)	27.25–30.74 (28.00 ± 3.32a)	2.56–2.88 (2.72 ± 0.43a)
Group	Taxa	Ev	Pv	Aperture	Orn	
1	*V. sativa* subsp. *nigra*	Oblat	three-lobed circular	3-zonocolporate	Rugulate/reticulate/reticulate-retipilate	
2	*V. sativa* subsp. *sativa*	Oblat	three-lobed circular/ triangle	3-zonocolporate	Rugulate/reticulate/reticulate-rugulate/ quarse rugulate	
3	*V. sativa* subsp. *cordata*	Oblat	three-lobed circular.	3-zonocolporate	Reticulate	
4	*V. sativa*	Oblat	three-lobed circular	3-zonocolporate	Reticulate/quarse rugulate	

(*P*) polar axis, (*E*) equatorial axis, (*P/E*) ratio of equatorial and polar axes, (*Ev*) equatorial view, (*Pv*) polar view, (*Clg*) colpus length, (*Clt*) colpus width, (*Orn*) ornamentation.
Group one contains six accessions (No. 10–15). Group two contains four accessions (No. 16–19).
Group three contains one accession with No. 20. Group four contains two accessions with No. 21–22.
Note: Different letters after the same data indicate significant differences at $P < 0.050$.

Table 4. The estimated genotypic (σ^2_g) and experimental error (σ^2_ε) variance components and associated standard error (±s.e.) within 22 accessions of common vetch

Traits	*P* (μm)	E (μm)	*P/E*	Clg (μm)	Clt (μm)
Average	23.42	38.89	0.60	28.49	2.92
Max	42.12	45.25	0.93	32.23	5.05
Min	19.39	33.91	0.51	23.95	2.22
$l.s.d._{0.05}$	0.018	0.027	0.001	0.016	0.001
σ^2_g	19.56 ± 6.02	8.31 ± 2.62	0.007 ± 0.002	4.56 ± 1.56	0.39 ± 0.13
σ^2_ε	0.36 ± 0.08	0.50 ± 0.11	0.0004 ± 0.00008	1.48 ± 0.32	0.07 ± 0.02
R	0.99	0.98	0.98	0.90	0.94

(*P*) polar axis, (*E*) equatorial axis, (*P/E*) ratio of equatorial and polar axes, (*Clg*) colpus length, (*Clt*) colpus width.

Outlines: equatorial view oblate; polar view three-lobed circular.

Ornamentation: reticulate-retipilate, reticulate or rugulate, obvious.

Group 2: *V. sativa* subsp. *sativa* (Table 3; Figs 2(*p*—*s*), 3(*p*—*s*), 4(*p*–*s*))).

Pollen dimensions: $P = 20.72–24.71$ μm, $E = 35.70–40.41$ μm.

Pollen shape: oblate, $P/E = 0.51–0.65$ μm.

Apertures: 3-zonocolporate, apertures long and slightly deep, broader on pori in mesocolpium, $Clg = 24.99–31.33$ μm, $Clt = 2.41–3.49$ μm.

Outlines: equatorial view oblate; polar view three-lobed circular or triangular.

Ornamentation: reticulate, regulate, reticulate-rugulate or quarse rugulate, obvious.

Group 3: *V. sativa* subsp. *cordata* (Table 3; Figs 2(*t*), 3(*t*), 4(*t*)).

Pollen dimensions: $P = 23.37$ μm, $E = 43.67$ μm.

Pollen shape: oblate, $P/E = 0.54$ μm.

Apertures: 3-zonocolporate, apertures long and slightly shallow, broader on pori in mesocolpium, $Clg = 28.44$ μm, $Clt = 2.71$ μm.

Outlines: equatorial view oblate; polar view three-lobed circular.

Ornamentation: reticulate, obvious.

Group 4: *V. sativa* (Table 3; Figs 2(*u*) and (*v*), 3(*u*) and (*v*), 4(*u*) and (*v*)).

Pollen dimensions: $P = 23.21–23.50$ μm, $E = 37.95–40.40$ μm.

Pollen shape: oblate, $P/E = 0.57–0.62$ μm.

Apertures: 3-zonocolporate, apertures long and slightly deep, broader on pori in mesocolpium, $Clg = 27.25–30.74$ μm, $Clt = 2.56–2.88$ μm.

Outlines: equatorial view oblate; polar view three-lobed circular.

Ornamentation: reticulate or quarse rugulate, obvious.

It was found that the values of *P*, *E*, *Clg* and *Clt* of *V. sativa* subsp. *cordata* subspecies were higher than those of the other three subspecies. This could be used to classify *V. sativa* subsp. *cordata* subspecies with other subspecies. Among all accessions, the traits *P*, *E* and *P/E* were significantly different, but the traits *Clg* and *Clt* were not significantly different.

Genotypic variation analysis of traits

The genotypic variance components estimated for five pollen traits showed significant differences ($P < 0.05$) among the 22

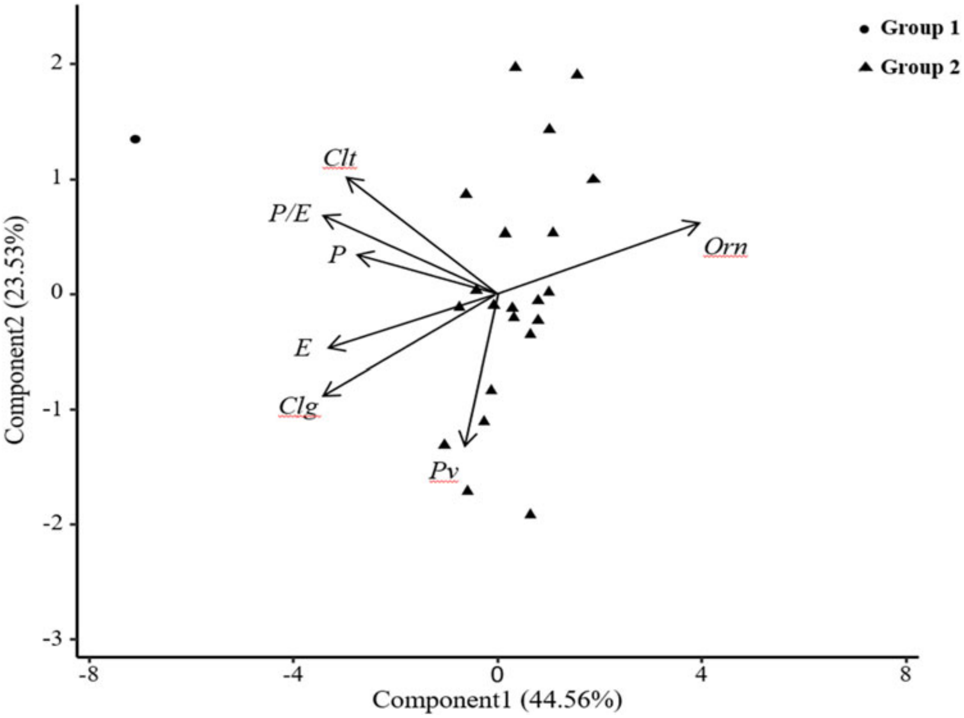

Fig. 5. Pattern analysis of pollen status for 22 common vetch accessions. (*P*) polar axis, (*E*) equatorial axis, (*P/E*) ratio of equatorial and polar axes, (*Pv*) polar view, (*Clg*) colpus length, (*Clt*) colpus width, (*Orn*) ornamentation.

common vetch accessions (Table 4). The traits *P*, *E*, *P/E*, *Clg* and *Clt* all had higher accession mean repeatability (*R*). Among them, the *R* values of traits *P*, *E* and *P/E* were all above 0.98, and the traits *Clg* and *Clt* had relatively lower *R* values, which were 0.90 and 0.94, respectively. Higher *R* values indicate that pollen traits are more affected by genotypes and are more conservative.

Pattern analysis and phenotypic correlation

Pattern analysis is a combination of cluster analysis and PCA, which provides a graphical summary of trial entry-by-multi trait data matrices. We analysed and compared the differences between the pollen traits of 22 common vetch accession pollens and visually displayed the correlation information (positive or negative) between the traits (Jahufer *et al*., 2006). The pattern analysis results of 22 common vetch accessions based on seven traits, including *P*, *E*, *P/E*, *Clg*, *Clt*, *Pv* and *Orn,* are shown in Fig. 5. In the biplot, the first principal component explained 45% of the total trait variation, and the second principal component explained 24%. Among 22 common vetch accessions, the traits *P*, *E*, *P/E*, *Clg* and *Clt* showed a positive correlation (the angle between the direction vectors was <90°) (Fig. 5) and *Pv* also showed a positive correlation with *P*, *E* and *Clg*. *Orn* had a negative correlation with the traits *P*, *E*, *P/E*, *Clg*, *Clt* and *Pv* (the angle between the direction vectors is >90°).

Using pattern analysis, 22 common vetch accessions were divided into two groups by *K*-means clustering (stats package). Among them, the first group included accession No. 6, and the second group included the other 21 accessions and the clustering results were consistent with the differences in pollen traits. The phenotypic correlation coefficient (r_p) shown in Table 5 further verified the positive correlation between the traits *P*, *E*, *P/E*, *Clg* and *Clt* shown in Fig. 5; among them, *P* and *E*, *P/E*, *Clt* were extremely significantly correlated, and *P* and *Clg* were significantly correlated.

Cluster analysis

To further study the common vetch accessions in this study, we selected seven traits of common vetch and used hierarchical clustering in the R Programming Language (NbClust package) for cluster analysis. The results are shown in Fig. 6. When the fusion level value was five, the clustering results were roughly grouped into two groups, which were determined to be the optimal clustering groups. The two groups were as follows:

Accession No. 6 was clustered into a single group, representing the first type. The pollen polar axis length of this accession was 42.12 µm, the equatorial axis length was 45.25 µm, the *P/E* value was 0.93, the colpus length was 32.23 µm and the width was 5.05 µm, as shown in Table 6. Regardless of the size of the pollen or the length and width of the colpus, the pollen was significantly larger than that of other accessions; additionally, these pollen grains alone, among all the common vetch accessions tested, exhibited an oblate spherical shape.

The other 21 common vetch accessions were grouped into the second group. The average length of the polar axis of this accession group was 22.53 µm, the average length of the equatorial axis was 38.59 µm, the average length of the colpus was 28.31 µm and the average width was 2.80 µm. These pollen shapes were oblate, the *P/E* values were less than 0.8, the pollen and colpus were similar in size and there was no significant difference among the various traits.

The above results are completely consistent with the results of the pattern analysis.

In this cluster analysis performed by selecting the relevant traits of common vetch, although the 22 accessions were roughly

Table 5. Phenotypic correlation coefficients (r_p) between traits based on 22 common vetch accessions

	P	*E*	*P/E*	Clg	Clt	Pv	Orn
P	–						
E	0.572**	–					
P/E	0.911**	0.185	–				
Clg	0.522*	0.606**	0.330	–			
Clt	0.741**	0.182	0.796**	0.133	–		
Pv	0.004	0.084	−0.029	0.425*	−0.107	–	
Orn	−0.213	−0.303	−0.152	−0.364	−0.008	−0.428	–

(*P*) polar axis, (*E*) equatorial axis, (*P/E*) ratio of equatorial and polar axes, (*Clg*) colpus length, (*Clt*) colpus width, (*Pv*) polar view, (*Orn*) ornamentation.
**indicates that the difference is significant at $P < 0.010$.
*indicates that the difference is significant at $P < 0.050$.

grouped, the classification results at the subspecies level were not ideal. For example, the four accessions No. 16, No. 17, No. 18 and No. 19 belonged to *V. sativa* subsp. *sativa* subspecies should have been clustered in the same group but were divided into four different groups. Through related research, we found that cluster analysis of common vetch accessions has been focused at levels above the subspecies level, but no related cluster analysis has been performed on accessions within a subspecies (e.g. LPWG, 2017; Banks and Lewis, 2018). The reasons for this finding need to be further explored.

Box-and-whisker plot analysis

As shown in Fig. 7, we conducted data measures of dispersion analysis on the five indexes of *P, E, P/E, Clg* and *Clt* of the 22 common vetch samples tested. Among the two indexes of *P* and *P/E*, the only accession No. 6 showed a relatively large degree of deviation from the other accessions, with significant differences. However, for the three indicators *E, Clg* and *Clt*, the degree of deviation among the 22 accessions was not significant.

Discussion

Previous studies have studied the pollen morphology of *Vicia*. For example, Endo and Ohashi (1996) performed SEM observations of pollen from 32 species of the genus *Vicia* and found that the pollen interstitia of the examined species were regular columella, irregular columella or granular. Liu *et al.* (2015) performed SEM observations of pollen from 16 *Vicia* plants from 11 countries and concluded that the pollen morphology of this genus is highly conserved. However, these studies mainly focused on the observation of pollen of different species of *Vicia* under SEM, and a comparative study of different subspecies and accessions within a single species of common vetch was not performed. This study collected 22 pollen materials from common vetch accessions from 15 countries to accumulate data for systematic taxonomy and palynology-related research within common vetch species.

Kahraman *et al.* (2013) carried out SEM observations of *Vicia* from 11 taxa in Turkey, in which the pollen polar axis length (*P*) of the accessions of *V. sativa* subsp. *sativa* was 30.88–36.67 µm, and the equatorial axis length (*E*) was 23.63–28.55 µm. In the present study, *V. sativa* subsp. *sativa* taxa showed *P* ranging from 20.72 to 24.71 µm, and *E* ranging between 35.70 and 40.41 µm, which is quite different from the experimental results of Kahraman *et al.* (2013). In the study of Liu *et al.* (2015), the pollen equatorial view of accession No. 21 revealed an oblate spherical shape, and the equatorial axis length and polar axis length were relatively short. However, in this study the equatorial view of this accession revealed an oblate shape, and the equatorial axis length and polar axis length were both relatively long, which is inconsistent with the research results of Liu *et al.* (2015) (Fig. 3 (*u*)).

In this study, the similarity between *Clg* and *Clt* in the one subspecies accession of common vetch was higher than those of the other traits and the differences were not significant. At the same time, in the cluster analysis, we found that the accessions of the same subspecies could not be clustered in the same unit. Through other related studies, it was found that cluster analysis of common vetch accessions has only been reported above the subspecies level (e.g., LPWG, 2017; Banks and Lewis, 2018). In the boxplot analysis, we also found that apart from the special accession No. 6, the remaining 21 accessions had no significant degree of deviation in the five indicators of *P*, *E*, *P/E*, *Clg* and *Clt*. Common vetch is a strictly self-pollinated plant (Dong *et al.*, 2016) and its pollen morphology has strong conservation and genetic stability (Lee *et al.*, 2011). In this study, *P, E, P/E, Clg* and *Clt* all had high *R* values, which also indicates that the heredity of the pollen traits of common vetch is mainly affected by genotype and has high genetic stability. These reasons have caused the subspecies of common vetch to be poorly characterized in cluster analysis. However, the pollen morphology analysis among the subspecies of common vetch (Table 3) found that *E* and *P/E* have significant differences among the subspecies. This could provide extremely useful information for future work related to the classification of common vetch subspecies.

Pollen morphology plays an important role in the taxonomy and phylogenetic history of plants. Research in the field of palynology provides valuable information for the identification of closely related and complicated classifications as well as taxa whose taxonomy is contested (Jafar and Karm, 2007; Quamar *et al.*, 2017). In the process of generational transmission, pollen ornamentation, shape, surface, symmetry, colpus length, width and wall structure basically maintained their original morphological characteristics. These morphological and structural characteristics are important bases for identifying plant species (Bahadur *et al.*, 2018). After studying the pollen traits of 22 common vetch accessions, the pollen morphologies of various accessions not only share common characteristics but also have obvious specificities among some accessions. For example, the 22 common vetch accessions all showed pollen apertures that

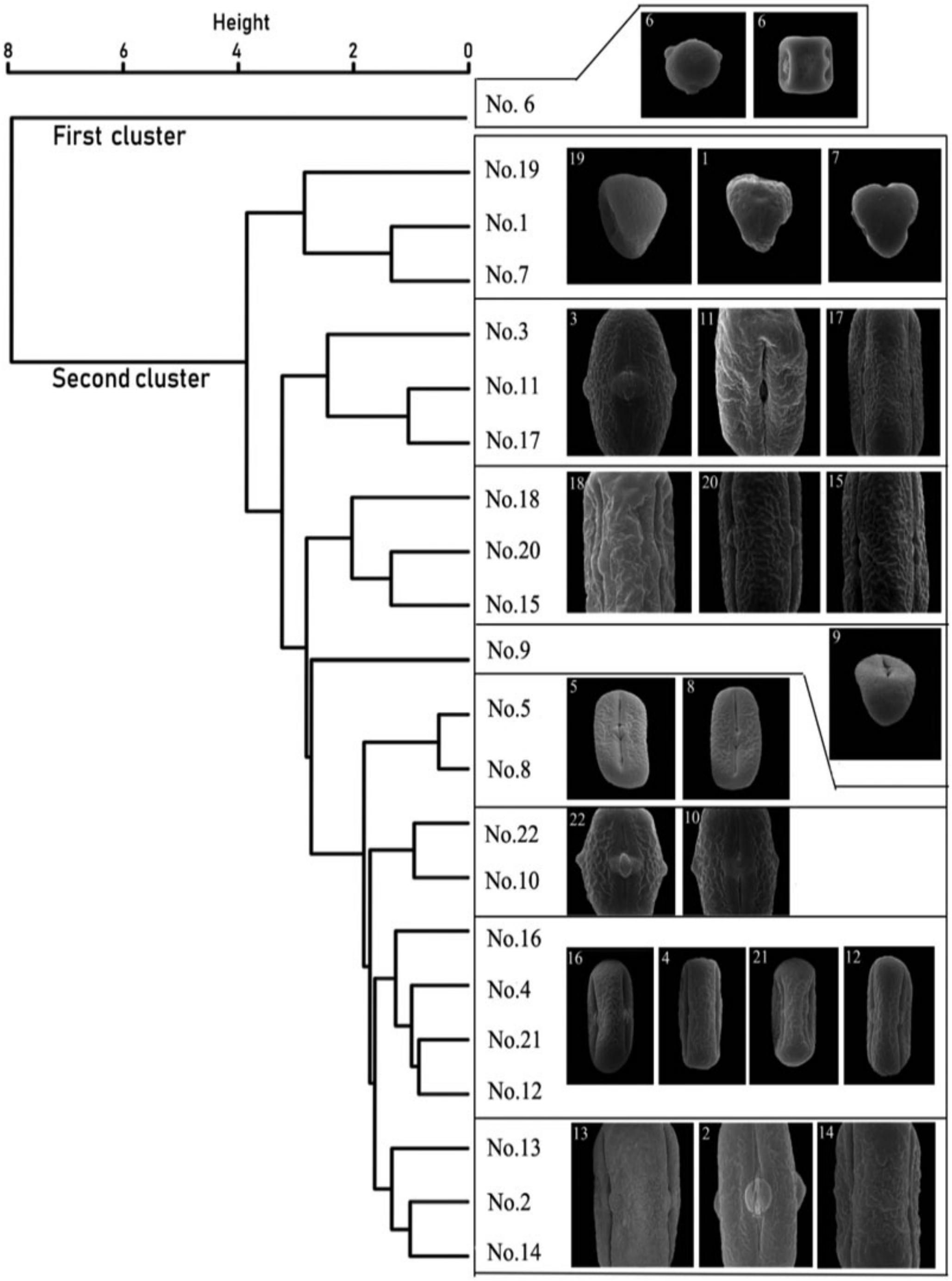

Fig. 6. Cluster analysis of pollen status for 22 common vetch accessions.

Table 6. Quantitative trait means for each of the two groups of the 22 common vetch accessions generated from cluster analysis

Group	Accession members	*P* (μm)	*E* (μm)	*P*/*E*	Clg (μm)	Clt (μm)
1	1	42.12	45.25	0.93	32.23	5.05
2	21	22.53	38.59	0.58	28.31	2.80

(*P*) polar axis, (*E*) equatorial axis, (*P*/*E*) ratio of equatorial and polar axes, (*Clg*) colpus length, (*Clt*) colpus width.

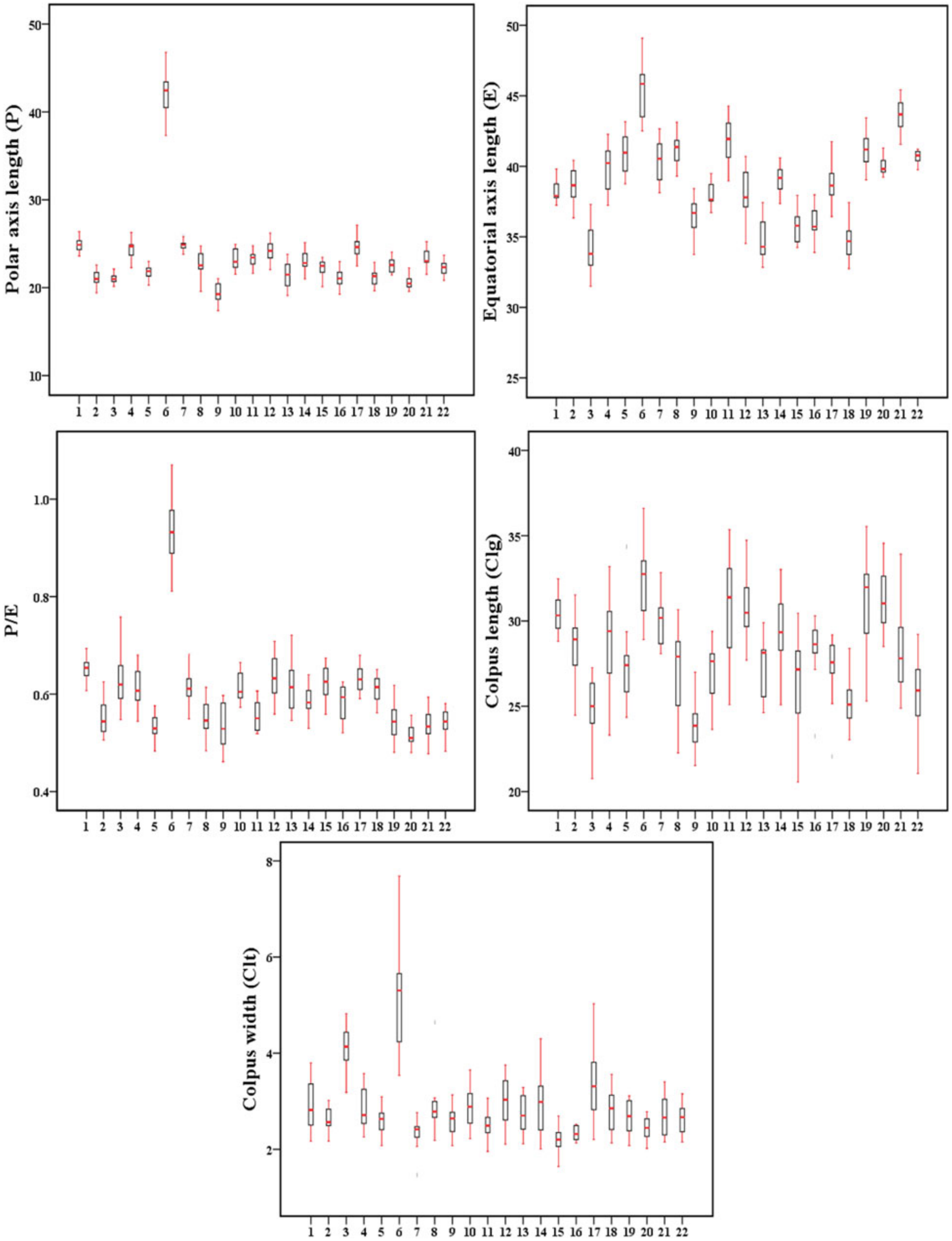

Fig. 7. Box-and-whisker plots for the polar axis (*P*), equatorial diameter (*E*), *P/E* ratio, colpus length (*Clg*) and colpus width (*Clt*) of 22 common vetch accessions.

were 3-zonocolporate, with colpi close the poles and the colpi were smooth or had wart-like protrusions; however, there were obvious depressions in the middle of the colpus of accession No. 11, which provides important information for the identification of this accession. As another example, among the 22 common vetch accessions, 21 accessions had *P/E* values between 0.5

and 0.8 and an oblate pollen morphology, while only the pollen *P/E* value of accession No. 6 was greater than 0.8 and its pollen morphology was oblate spherical. These conclusions indicate that some accessions have their own unique characteristics at present and the identification of plant species can still mainly be based on morphological characteristics. The data from this study provide a basis for the identification of related species (Kahraman *et al*., 2013).

Wodehouse (1935) believed that the more evolved the pollen, the stronger its regulatory function and the longer the pollen. The equatorial axis lengths of 22 common vetch pollens in this study in order from large to small were No. 6, No. 20, No. 8, No. 5, No. 15, No. 7, No. 19, No. 22, No. 4, No. 10, No. 18, No. 2, No. 1, No. 21, No. 16, No. 12, No. 13, No. 9, No. 14, No. 11, No. 17 and No. 3. According to the evolution theory of Wodehouse (1935), the pollens of accessions such as No. 6 and No. 20 were longer and more evolved, while the pollens of accessions No. 3 and No. 17 were shorter and more primitive. At the same time, Beck (1981) reported that pollen ornamentation can also reflect the evolution of pollen and pointed out that the evolutionary trend of ornamentation is surface psilate → surface foveolate, fossulate → surface scabrate, verrucate → surface granular → surface reticulate. The differences in the pollen ornamentation of the 22 common vetch accessions indicated that they have different degrees of evolution. According to the evolution theory of Beck (1981), the pollen ornamentations of accessions No. 3, No. 17 and No. 11 are rugulate and are thus more vague and primitive. The pollen ornamentations of No. 6, No. 20 and other accessions were reticulate and thus more evolved. The pollen morphological characteristics of 22 common vetch accessions observed in this experiment were verified by the research conclusions of Wodehouse (1935) and Beck (1981), and the same results were obtained. This shows that it is feasible to study the evolution of the accessions of common vetch from the perspective of pollen morphology.

Conclusion

In conclusion, there are no previous detailed studies on the pollen morphology of different common vetch subspecies and their various accessions. In this study, the microscopic features of pollen from 22 accessions of common vetch were analysed using LM and SEM. The results showed that there are significant differences in the pollen traits, including polar axis length, equatorial axis length and colpus length and width, among accessions. The traits of the equatorial axis and the ratio of equatorial and polar axes have significant differences among subspecies. This trait information could be used for classification research between common vetch subspecies. The palynological properties of the concerned taxa are described in detail. Therefore, we suggest that the microscopic morphological structures of pollen can be useful to assess whether there are significant differences among accessions of the subspecies of common vetch. The palynological characteristics described for these taxa combined with molecular markers and other methods will help further research on this species.

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Conflict of interest. None.

Ethical standards. Not applicable.

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