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Comparative assessment of pollen micromorphology and meiotic observations in some species from the genus *Salvia* L. (Lamiaceae) in Iran

MASOUMEH SAFAEISHAKIB[1], BEHNAZ ALIJANPOOR[2] & MASOUD SHEIDAI[3]

[1, 3] Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran
[2] Research Center of Agriculture and Natural Resource of Tehran Province, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

ORCID iD. M. SAFAEISHAKIB: https://orcid.org/0000-0002-3557-0287,
B. ALIJANPOOR: https://orcid.org/0000-0002-9698-2592, M. SHEIDAI: https://orcid.org/0000-0003-3983-6852

Author for correspondence: M. Safaeishakib (safaeishakib@gmail.com)

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Abstract

Comparative assessment of pollen micromorphology and meiotic observations in some species from the genus *Salvia* l. (Lamiaceae) in Iran.— This study presents some detailed observations on the meiotic behavior and a comparative palynological study of some selected species of *Salvia* L. sect. *Aethiopis* Benth. ANOVA test was used to compare chiasma frequency, distribution and chromosomal associations, revealing a significant difference in all meiotic characteristics among the *S. hypoleuca, S. limbata, S. reuteriana, S. spinosa*, and *S. xanthocheila* species. Moreover, some meiotic abnormalities such as chromosome stickiness, laggard chromosomes, as well as frequent tripolar, multipolar cell formation and cytomixis occurred in these species. Light Microscopy (LM) and Scanning Electron Microscopy (SEM) were used for analyzing pollen. Some micromorphological characteristics such as pollen shape, size, polar axis length, equatorial axis length, aperture numbers and exine ornamentation, exhibited remarkable differences amongst the studied species.

Key words: Light Microscopy (LM); meiotic behavior; pollen micromorphology; *Salvia*; Scanning Electron Microscopy (SEM).

Resumen

Evaluación comparativa de la micromorfología del polen y las observaciones meióticas en algunas especies del género *Salvia* l. (*Lamiaceae*) en Irán.— Este estudio presenta algunas observaciones detalladas sobre el comportamiento meiótico y un estudio palinológico comparativo de algunas especies seleccionadas de *Salvia* L. sect. *Aethiopis*. Se usa la prueba ANOVA para comparar la frecuencia, la distribución y las asociaciones cromosómicas de los quiasmas, lo que revela una diferencia significativa en todas las características meióticas entre las especies *S. hypoleuca*, *S. limbata*, *S. reuteriana*, *S. spinosa* y *S. xanthocheila*. Además, en estas especies se producen algunas anomalías meióticas, como la adherencia de los cromosomas, los cromosomas rezagados, así como la formación frecuente de células tripolares, multipolares y citomixis. Se usa microscopía de luz (LM) y microscopía electrónica de barrido (SEM) para analizar el polen. Algunas características micromorfológicas como la forma del polen, el tamaño, la longitud del eje polar, la longitud del eje ecuatorial, el número de aperturas y la ornamentación de la exina, exhiben diferencias notables entre las especies estudiadas.

Palabras clave: comportamiento meiótico; micromorfología del polen; microscopía de luz (LM); microscopía electrónica de barrido (SEM); *Salvia*.

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INTRODUCTION

Salvia L. (family Lamiaceae) consists of nearly 1000 species with a notable diversity and cosmopolitan distribution. *Salvia* species are used in traditional medicine throughout the world, possessing antioxidant, antiplasmodial and anti-inflammatory features (Ulubelen, 2003; Kamatou *et al*., 2008; Safaeishakib & Ghaffarzadegan, 2022). Four regions such as central and South America, western and eastern Asia are the major distribution centers of this large genus (Hedge, 1982*a*; Wu & Li, 1982; Walker & Sytsma, 2007). Based on *Flora Iranica* (Hedge, 1982*a*, *b*), the genus *Salvia* consists of 58 species in Iran, among which 17 are endemic (Hedge, 1982*a*, *b*). Cytological studies in *Salvia* species have been conducted by many researchers from Europe, America and Asia; however, most of this research has been carried out only based on chromosome counts (Patudin *et al*., 1975; Afzal-Rafii, 1976; Bhattacharya, 1978; Haque & Ghoshal, 1980; Mercado *et al*., 1989; Harley & Heywood, 1992; Yang *et al*., 2004; Song & Li, 2009; Wang *et al*., 2009).

Detailed chromosomal data and meiotic behavior with palynological research reports on the genus grown in Iran are limited to these studies (Jafari & Nikian, 2008; Sheidai *et al*., 2010; Sheidai & Alijanpoor, 2011; Kharazian, 2011; Ranjbar *et al*., 2015; Alijanpoor & Safaeishakib, 2022). Besides, studies on pollen morphology in *Salvia* have been conducted by many authors worldwide (see e.g. Henderson *et al*., 1968; Trudel & Morton, 1992; Hassan *et al*., 2009; Kahraman *et al*., 2009, 2010; Özler *et al*., 2011, 2020). Yet, research on this genus and related taxa had been mainly based on observations with light microscopy (LM) in the *Aethiopis* Benth. section (Afzal-Rafii, 1980, 1981), the focus of the present work. The main features of this section are false upper crown lips, long-attached stamens, reduced lower theca to usually double-shaped plate, and articular stamens (Hedge, 1982*a*; Walker & Systma, 2007). There are 34 species from sect. *Aethiopis* in Iran, 11 being endemic to the country (Boissier, 1879; Hedge, 1982*b*). From this section, species *S. hypoleuca* Benth. (one of the endemisms growing in the north and central parts of Iran; Hedge, 1982*b*), *S. limbata* C. A. Mey., *S. reuteriana* Boiss., *S. spinosa* L. and *S. xanthocheila* Boiss. have been investigated in this study. The present survey describes the behavior of the chromosomes in meiosis and the occurrence of unreduced pollen grain formation, in addition to the micromorphology of their pollen grain.

MATERIALS AND METHODS

Plant material

The materials for cytological and pollen micromorphology analysis are presented in Table 1. Voucher specimens were deposited at the herbarium of Shahid Beheshti University (HSBU). Samples were collected from five different regions of Iran.

Cytological and pollen grain preparation

To check for stages of meiosis, the presence of abnormalities and the possible failure of meiosis, preparation and counting of pollen were performed according to previous works (Sheidai *et al*., 2002; Safaei *et al*., 2016; Alijanpoor & Safaeishakib, 2022). For the assessment of the behavior of chromosomes in meiosis, young flower buds were selected and fixed in an acetic alcohol mixture (1:2) for 24 hours. For further storage, 70% ethanol at 4°C was adopted. Pollen fertility size frequencies and stain ability tests were done with 2% acetocarmine:50% glycerin (1:1) for about 30 min. Approximately 1000 pollen grains were evaluated. The squashing technique and 2% acetic-orcein (as the

Table 1. *Salvia* species collected for cytological and palynological studies.

Species	Locality	2*n*	Voucher no.
S. hypoleuca Benth.	Qaemshahr-shirgah	22	HSBU 2012179
S. limbata C. A. Mey.	Semnan-sorkheh	22	HSBU 2012177
S. reuteriana Boiss.	Kandovan	20	HSBU 2012167
S. spinosa L.	Hamedan-Bahar	20	HSBU 2012173
S. xanthocheila Boiss.	Dizin	22	HSBU 2012157

stain) were applied for cytological preparations. Round complete pollen grains with stained nuclei were taken as apparently fertile, while shriveled and unstained pollen grains were considered sterile. One hundred pollen mother cells (PMCs) were analysed for chiasma frequency, distribution at diakinesis/metaphase stage and 500 PMCs were analysed for chromosome segregation during the anaphase and telophase stages. All stages of meiosis and morphology of the chromosomes, presence of abnormalities and possible meiosis failure were all considered (Fig.1). The present study of pollen morphology was evaluated by LM (Light Microscopy) and SEM (Scanning Electron Microscopy). Freshly prepared slides were observed by Nikon 80i Eclipse digital imaging system. For SEM the acetolyzed pollen grains were attached and fixed to aluminum stubs with double-sided cellophane tape, air-dried at room temperature and coated with gold (JEOL 6060, JSM 6400). The specimens were tested with a Philips XL 20 SEM at 20kV. The UTHSCSA Image Tool v5 software was applied for pollen measurements. Quantitative and qualitative characters such as diameter of the mesocolpial area, diameter of lumina and polar axis length, equatorial axis length, thickness and number of colpus, and pollen type were considered. The terminology and pollen shape classification used is in accordance with Punt *et al.* (2007) and Özler *et al.* (2011, 2013). Correspondence Analysis (CA) is a multivariate graphical technique based on Hill & Gauch (1980) that was performed with PAST software v4.06b (Hammer *et al*., 2001).

Statistical analyses

One-way Analysis of Variance (ANOVA) and *t*-test were applied ($p < 0.05$) to decide the contrasts in chiasma frequency, chromosomes association and size of unreduced and reduced pollen grains findings. The Pearson coefficient of correlation was applied to address the relationship between pollen fertility, anaphase, metaphase and telophase stickiness.

RESULTS AND DISCUSSION

The highest mean number of ring bivalents occurred in *S. spinosa* (3.33) while the lowest value occurred in *S. xanthocheila* (1.1). The highest mean number of rod bivalents occurred in *S. hypoleuca, S. limbata* (0.64), while the lowest value occurred in *S. reuteriana* (0.52). Subsequently, the highest mean number of quadrivalents occurred in *S. hypoleuca* (2.44) and the lowest value gained in the *S. spinosa* (0.18) (Table 2). Chromosome stickiness was observed in all three phases (anaphase I, metaphase I and telophase). The highest value of stickiness during anaphase and telophase I occurred in *S. xanthocheila.* Significant correlation was observed between pollen fertility and the anaphase I laggard chromosomes. Therefore, this meiotic abnormality produced pollen sterility (Sheidai *et al*., 2010). The occurrence of meiotic abnormalities such as chromosome stickiness, laggard chromosome, formation of micronuclei in tetrad cells and cytomixis are provided in Fig. 1 and Table 3. ANOVA test conducted on chiasma frequency, distribution and chromosomal association revealed a significant difference in all meiotic characteristics among the species studied, indicating genomic differences. Correspondence Analysis (CA) (Fig. 2) was done to elucidate the correspondence between profiles of selected species and meiotic data. The main features of the pollen are illustrated in Figs. 3 and 4 and summarized in Table 4. The *t*-test analysis

Table 2. Meiotic data and chiasma frequency chromosomes association in *Salvia* species. TX: mean number of terminal chiasmata; IX: mean number of intercalary chiasmata; TOX: mean number of total chiasmata; IXN: mean number of intercalary chiasmata/bivalent; TXN: mean number of terminal chiasmata/bivalent; TOXN: mean number of total chiasmata/bivalent; RODN: mean number of rod bivalents; RB: mean number of ring bivalents; U: mean number of univalent; Q: mean number of quadrivalent.

Species	TX	IX	TOX	IXN	TXN	TOXN	RODN	RB	U	Q
S. hypoleuca Benth.	11.11	1.36	12.77	0.02	1.64	1.66	0.64	3.22	0.39	2.44
S. limbata C. A. Mey.	11.66	0.29	11.88	0.02	1.31	1.33	0.64	1.49	0.79	0.58
S. reuteriana Boiss.	12.65	11.66	11	0.06	1.46	1.52	0.52	1.73	1.50	1.15
S. spinosa L.	5.55	2.51	8.14	0.03	1.02	1.24	0.59	3.33	0.96	0.18
S. xanthocheila Boiss.	10.26	0.37	10.66	0.04	1.31	1.36	0.61	1.1	1.24	0.45

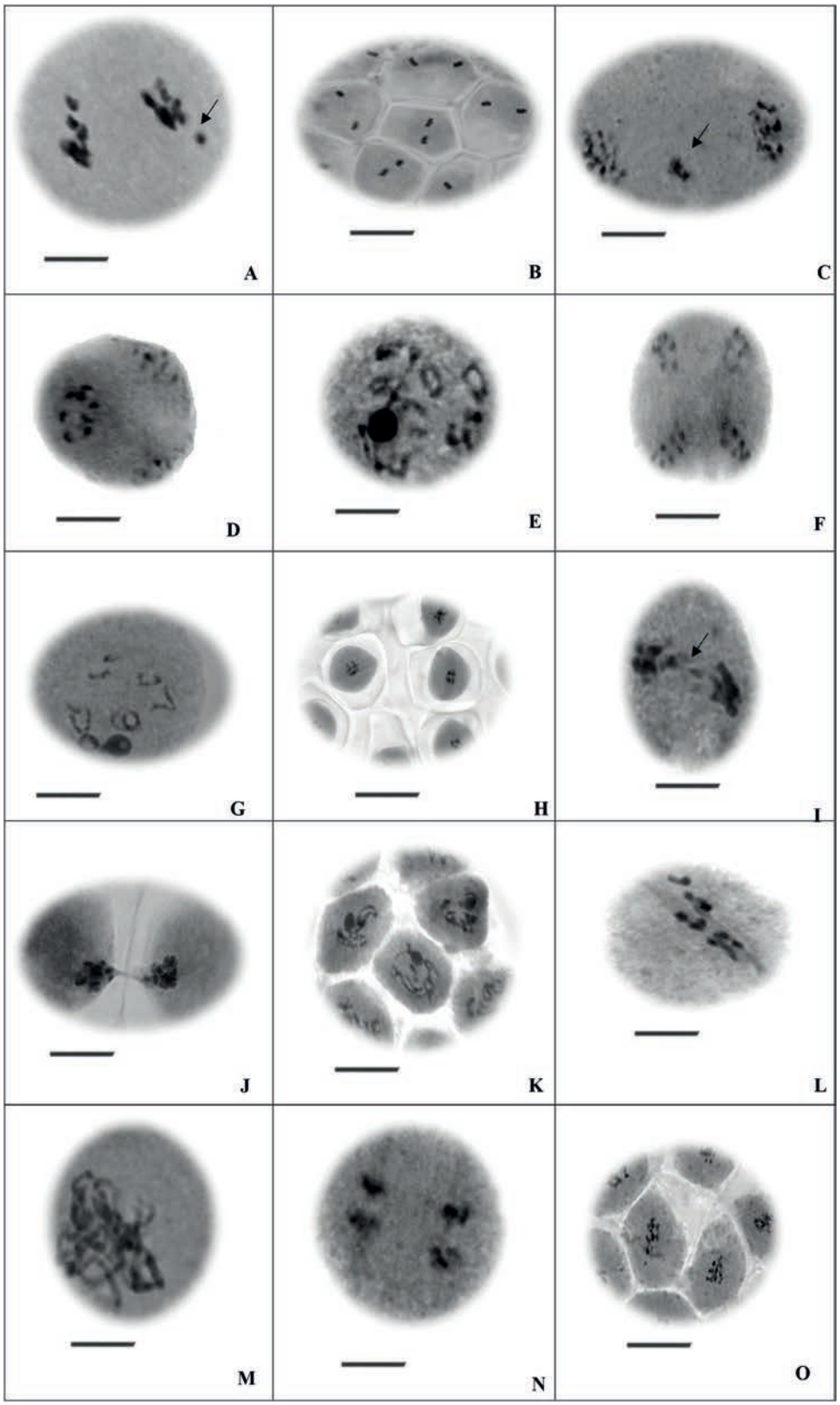

Figure 1. Representative miotic cells in *Salvia* species studied. Scale bar = 10 µm. (A–C), *S. xanthocheila*: (A), micronucleus (arrow) telophase I; (B), metaphase II; (C), laggard (arrow). (D–F), *S. hypoleuca*: (D), triad; (E), diakinesis; (F), tetrad. (G–I), *S. reuteriana*: (G), diakinesis; (H), metaphase I stickiness; (I), laggard (arrow). (J–L), *S. spinosa*: (J), cytomixis; (K), diakinesis; (L), metaphase I stickiness. (M–O), *S. limbata*: (M), pachyten; (N), telophase II; (O), anaphase II stickiness.

Table 3. Different stages of meiosis, number of abnormalities cells and size of pollen grains per species. Dia: diakinesis (%); MI: metaphase I (%); AI: anaphase I (%); TI: telophase I (%); MII: metaphase II (%); AII: anaphase II (%); TII: telophase II (%); Cyt: cytomixis (%); Mic: micronucleus (%); AIS: anaphase I stickiness (%); MIS: metaphase I stickiness (%); TIS: telophase I stickiness (%); Lag: laggard (%); Tet: tetrad (%); PF: pollen fertility (%).

Species	Dia	MI	AI	TI	MII	AII	TII	Cyt	Mic	A1S	M1S	T1S	Lag	Tet	2*n* µm	*n* µm	2*n* Freq.	Infertile pollen	PF
S. hypoleuca Benth.	184	27	14	70	22	4	121	3	-	27	3	4.4	19	105	37.70	25.44	10.36	9.38	80.26
S. limbata C.A. Mey.	150	14	10	77	64	2	151	-	2	25	4	1	23	111	34.50	50.81	8.5	1.5	90.35
S. reuteriana Boiss.	147	21	11	68	48	3	117	2	1	21	14.5	11	15	98	49.55	46.29	14.5	10	75.67
S. spinosa L.	103	33	13	57	55	5	131	1	2	30	7	5	10	133	38.04	28.84	9.5	1	89.30
S. xanthocheila Boiss.	115	18	10	89	21	1	114	4	1	31	10.5	16.67	4	115	36.80	49.60	19.5	5.5	75.61

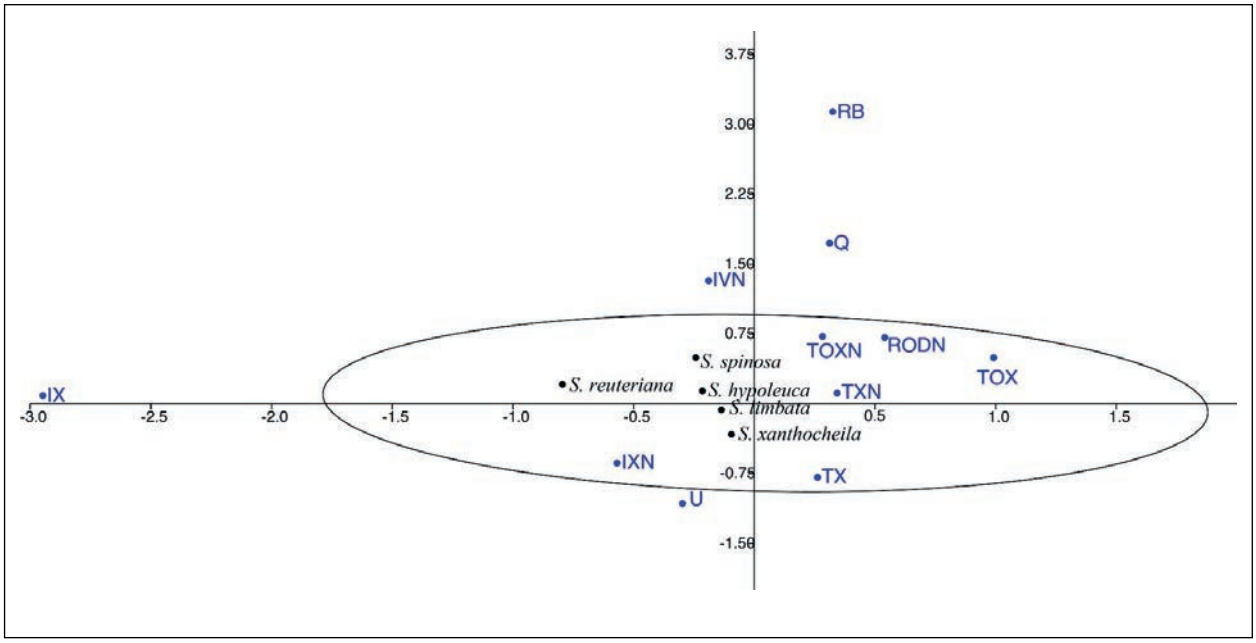

Figure 2. Correspondence analysis (CA) biplots performed in the meiotic data: (TX), terminal chiasma; (IX), intercalary chiasma; (TOX), total chiasma; (IXN), intercalary chiasmata/bivalent; (TXN), terminal chiasmata/bivalent; (TOXN), total chiasmata/bivalent; (RODN), rod bivalents; (RB), ring bivalent; (U), univalent; (Q), quadrivalent.

disclosed a significant difference ($p < 0.05$) in the size of unreduced pollen grains as compared to that of reduced pollen grains. Pollen grains (*n*, 2*n*, and infertile) were observed in all studied species. The diameter of normal pollen grains ranged from 25.44 to 50.81 µm while the diameter of the 2*n* pollen grains ranged from 34.50 to 49.55 µm in the studied species. The frequency of 2*n* pollen grains varied from 8.5 to 19.5 µm and the highest occurrence of large pollen grains (2*n* pollen grains), with a frequency of 19.5%, was observed in *S. xanthocheila*. Pollen grain type ranged from sub-oblate to oblate and subspherodial; also, exine sculpturing (ornamentation) showed bireticulate perforation. Correspondence Analysis (CA) showed that species were mainly influenced by wall thickness and pore diameters than polar and equatorial axis, ratio of polar axis/equatorial diameter, lumen and mesocolpia diameter traits (Fig. 5). The smallest ratio of polar axis/equatorial diameter (P/E) was detected in *S. limbata*, whereas the largest was observed in *S. hypoleuca*. The thickness of exine varied between 372.3 µm in *S. hypoleuca* and 642.9 µm in *S. xanthocheila*. The diameter of the mesocolpial area varied from 3.92 µm in *S. hypoleuca* to 16.41 µm in *S. limbata*. Moreover, the observation of whole pollen grains displayed radial symmetry with isopolar character. Among the investigated species the highest lumen

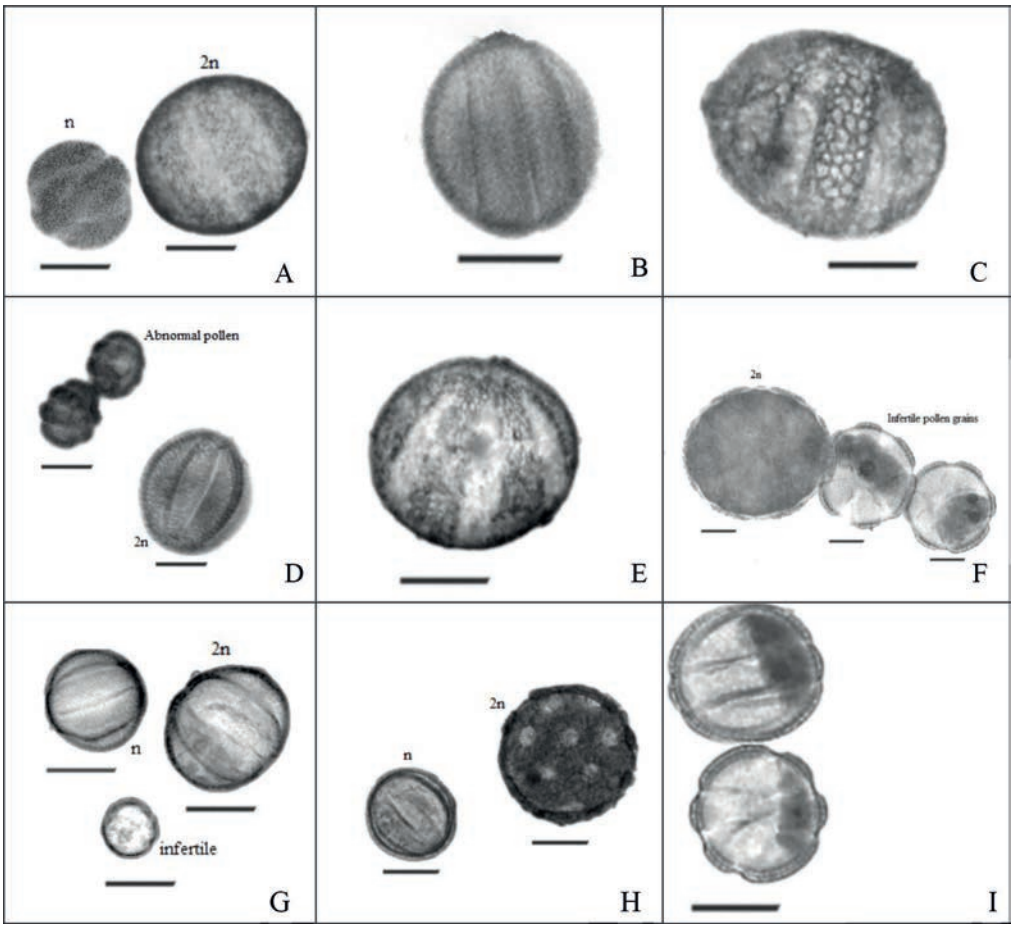

Figure 3. Representative LM photographs of pollen grains in *Salvia* species studied: (A–B), equatorial view of *S. spinosa*; (C–D), equatorial view of *S. xanthocheila*, 2*n*, *n* and abnormal pollen grain; (E–F), polar and equatorial views of different pollen grains in *S. limbata*, 2*n* and infertile; (G–H), equatorial and polar view of *S. hypoleuca* various pollen grains; (I), polar and equatorial views of infertile pollen grain in *S. reuteriana.*

size was obtained in *S. limbata*. Types of pollen apertures were hexacolpate with the highest diameter gained in *S. hypoleuca* (Table 2).

A detailed cytological study of *Salvia* species showed the occurrence of abnormalities (Sheidai *et al*., 2010; Alijanpoor & Safaeishakib, 2022), responsible for the production of unreduced pollen grains (Bretagnolle & Thomson, 1995). The present work demonstrates that the investigated whole pollen grains are hexacolpate and not octacolpate as Erdtman (1945) had reported in Nepetoideae subfamily. In accordance with LM observations, the pollen grains of these species of *Salvia* are oblate in equatorial and elliptic in polar view, with a narrowing at the poles. Lateral mesocolpia were longer and thicker than the four medial mesocolpia which supported the finding from Henderson *et al*. (1968). The existence of big grains is known as an indication of the production of 2*n* pollen. The most straightforward and the easiest method for screening of pollen grains involves the assessment of the range of pollen sizes generated by an individual; as the DNA content increases, the cell volume increases, which in turn affects the pollen diameter (Vorsa & Bingham, 1979). These observations were made on pollen morphological characters, and together with our previous work, they completely support the palynological findings of *Salvia* (Özler *et al.,* 2011, 2020). Micromorphological measurements such as pollen shape, size, polar axis length (P), equatorial axis length (E), aperture numbers, and exine ornamentation, in the pollen grains of these studied species of *Salvia*, can become useful at the systematics/taxonomic level, as they exhibit remarkable differences for the distinction of species.

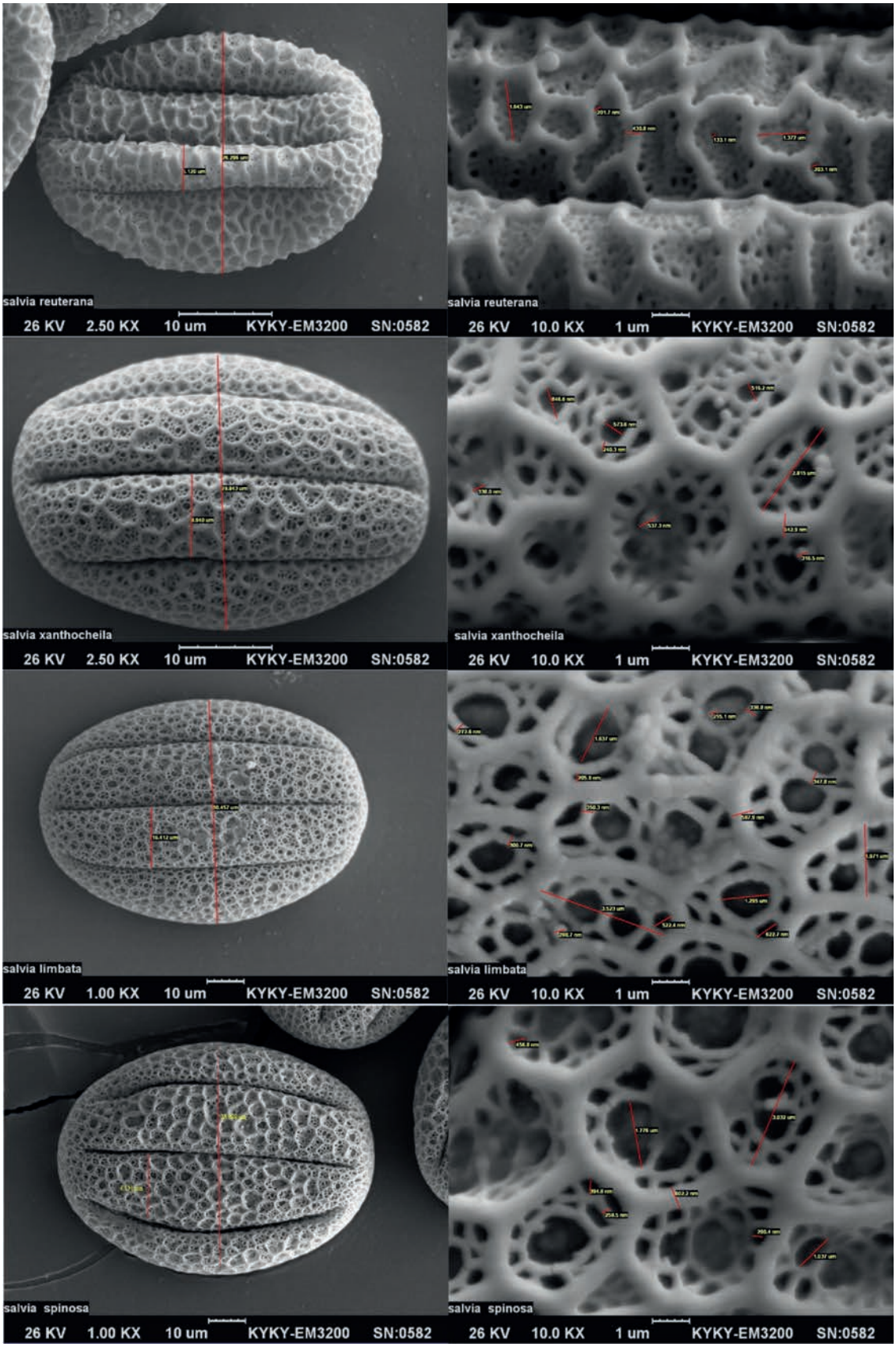

Figure 4. SEM micrographs of pollen grains in *Salvia* species general appearance and exine ornamentation in detail.

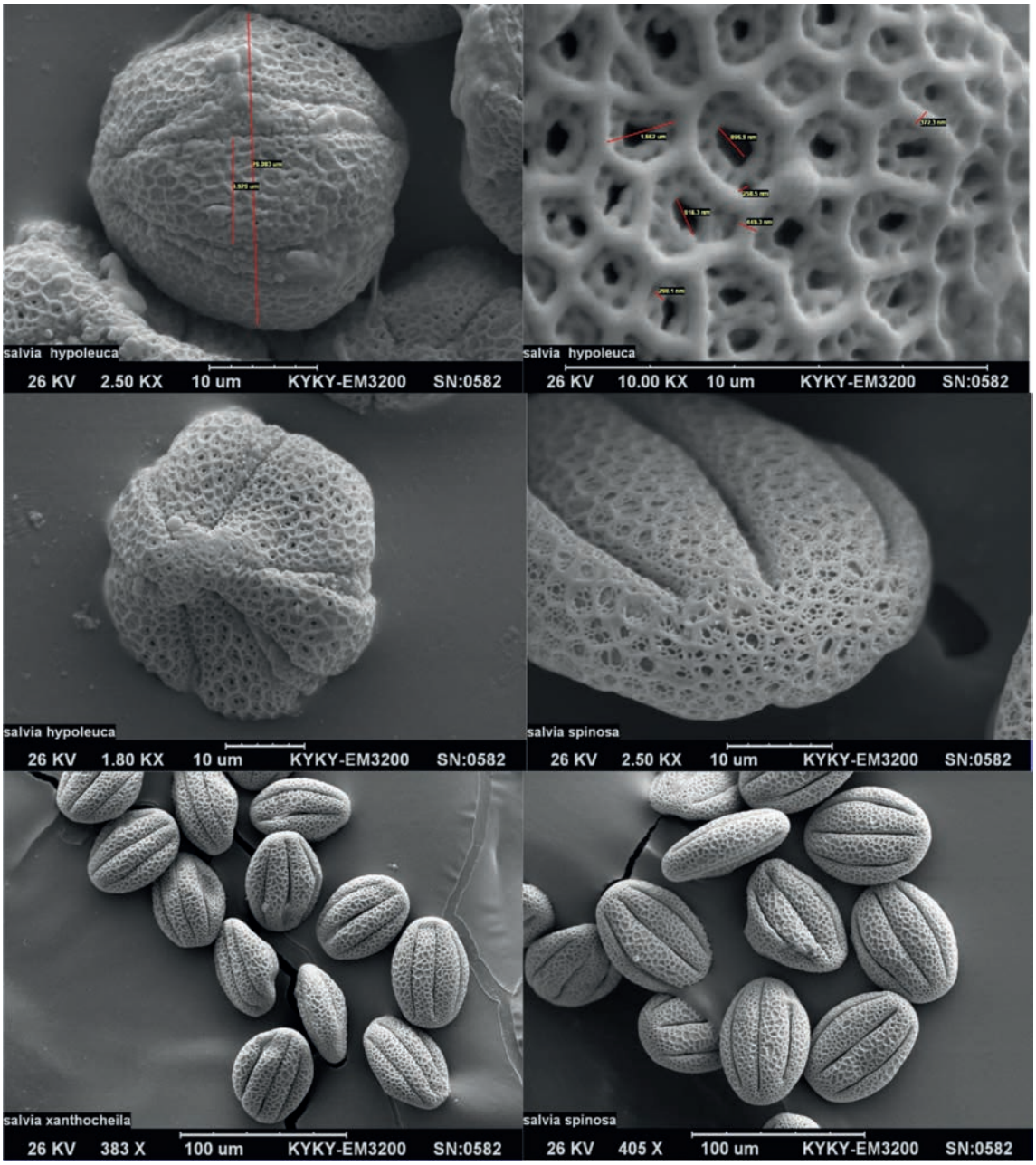

Figure 4 (cont.). SEM micrographs of pollen grains in *Salvia* species general appearance and exine ornamentation in detail.

CONCLUSIONS

This study examined five species of the genus *Salvia* from the sect. *Aethiopis*. The occurrence of meiotic abnormalities such as chromosome stickiness, laggard chromosomes, the formation of micronuclei in tetrad cells and cytomixis are reported in these species. Similarly, the pollen grain type ranged from sub-oblate to oblate and subspherodial; also, exine sculpturing displayed bireticulate perforation. Based on the present analysis, the pollen morphology of these species is more influenced by wall thickness and pore diameters than any other characteristics.

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Table 4. Pollen quantitative and qualitative characters reviewed in this study. P: polar axis (µm); E: equatorial axis (µm); P/E: ratio of polar axis/equatorial diameter (µm).

Quantitative traits								Qualitative traits				
Species	**P (µm)**	**E (µm)**	**P/E (µm)**	**Lumen dia. (µm)**	**Mesocolpia dia. (µm)**	**Pore dia. (µm)**	**Wall thickness (nm)**	**Pollen type**	**Colpus thickness**	**Colpus no.**	**Lumen shape**	**Exine ornamentation**
S. hypoleuca Benth.	29.09	28.51	1.02	1.66	3.92	896.2	372.3	Subspherodial	Narrow	6	Angular	Bireticulate perforate
S. limbata C. A. Mey.	50.45	89.35	0.56	3.52	16.41	522.4	587.9	Oblate	Narrow	6	Angular	Bireticulate perforate
S. reuteriana Boiss.	26.29	36.91	0.71	1.64	5.59	133.1	430.8	Oblate	Narrow	6	Angular	Bireticulate perforate
S. spinosa L.	30.2	34.32	0.87	3.03	8.8	290.4	602.2	Sub-oblate	Narrow	6	Angular	Bireticulate perforate
S. xanthocheila Boiss.	29.94	45.6	0.65	2.81	8.64	573.6	642.9	Oblate	Narrow	6	Angular	Bireticulate perforate

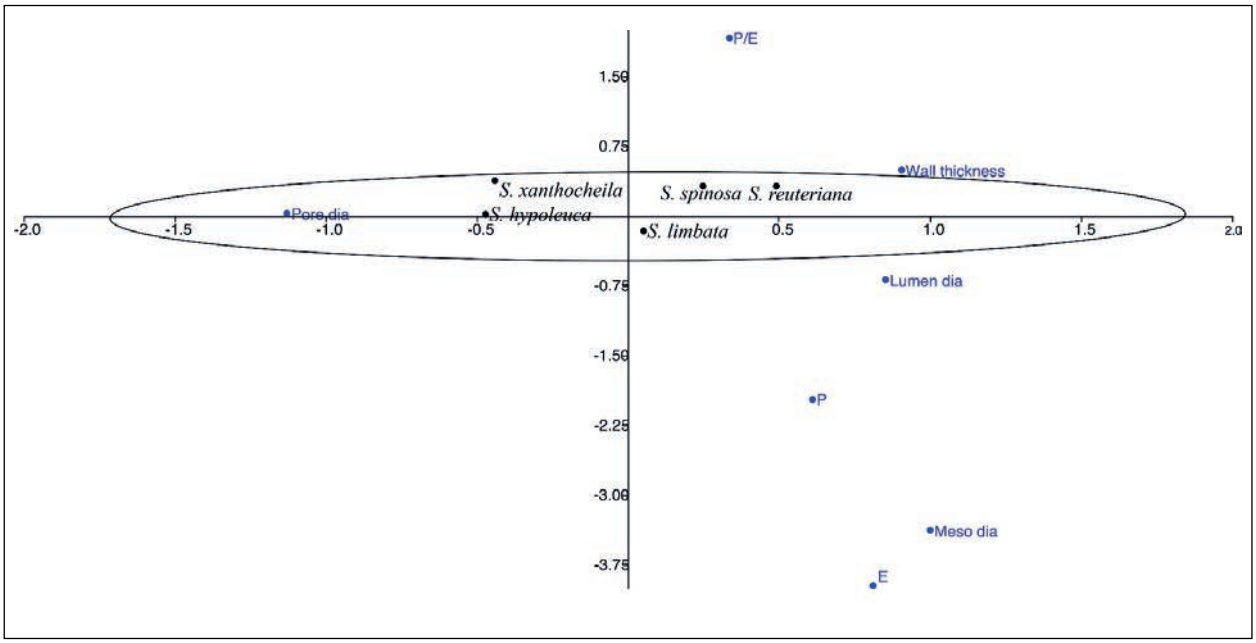

Figure 5. Correspondence analysis (CA) biplots carried out on pollen data: (P), polar axis; (E), equatorial axis; (P/E), ratio of polar axis/equatorial diameter.

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