# مطالعه مقایسه‌ای ریخت‌شناسی، گرده‌شناسی و روغن‌های ضروری سه گونه از سرده شنبلیله (تیره باقلائیان) در ایران

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**چکیده.** سرده شنبلیله یکی از سرده‌های مهم تیره حبوبات است که بسیاری از گونه‌های آن ارزش تغذیه‌ای و دارویی دارند. در این پژوهش ویژگی‌های ریخت شناسی سه گونه از سرده شنبلیله (*T. disperma*، *T. subenervis* و *T. aphanoneura*) مورد بررسی قرار گرفت. روغن‌های ضروری حاصل از بخش-های هوایی دو گونه *T. aphanoneura* و *T. subenervis* با استفاده از GC و GC/MS مورد بررسی قرار گرفت و با گونه *T. disperma* مقایسه شد. اسپاتولنول (۱۵/۱٪) فراوان‌ترین جزء موجود در روغن ضروری گونه *T. subenervis* بود. همچنین روغن این گونه دارای مقدار اندکی از ژرماکرن D (۶٪) بود. روغن گونه *T. aphanoneura* نیز غنی از اسپاتولنول (۱۰/۴٪) بود. چهار ترکیب دیگر نیز کمتر از ۱٪ روغن این گونه را به خود اختصاص دادند، لیکن n- اوکتیل استات کمترین مقدار را نشان داد. بر طبق نتایج این تحقیق، داده‌های مورفولوژیکی و فیتوشیمیایی بهتر از داده‌های گرده، روابط گونه‌های مورد مطالعه را روشن می‌کند.

**واژه‌های کلیدی.** بقولات، روغن‌های ضروری، ریخت‌شناسی، گرده

# Comparative study on the morphology, palynology and essential oil composition of three *Trigonella* L. species (Fabaceae) from Iran

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**Abstract.** *Trigonella* L. is one of the important genera of the family Fabaceae, many species of which have nutritional and medicinal properties. In this investigation, morphological characters related to habit and pollen grain of three Trigonella species i.e., *T. disperma*, *T. subenervis* and *T. aphanoneura* were studied. Oils from the aerial parts of two species were investigated by GC and GC/MS methods and compared with *T. disperma*, which had been studied in advance. *T. subenervis* represented oils rich in spathulenol (15.1%). It also contained a small amount of germacrene D (0.6%). *T. aphanoneura* also represented oils rich in spathulenol (10.4%). The amount of four compounds in this species was ≤1%, and n-octyl acetate was the lowest component in quantity. According what we found out, phytochemical and morphological data could clarify the relationships among the *Trigonella* species better than pollen morphological data.

**Keywords.** essential oil composition, Leguminosae, morphology, pollen, *Trigonella*

## INTRODUCTION

*Trigonella* L. is a large genus with approximately 135 species, belonging to the family Fabaceae (Kawashty *et al.*, 1998). In Iranian plateau, the genus is represented by 58 annual and perennial species grouped into 12 sections (Boissier, 1872; Janighorban, 2004; Townsend, 1974). Many of the perennial species are endemic to Iran and belong to sect. *Ellipticae* (Rechinger, 1984). Various species of the genus are important for their medical and culinary properties (Abdel-Barry *et al.*, 2000; Chopra *et al.*, 1956; Giradon *et al*., 1985; Rapior & Bessiere, 2000; Sood, 1975).

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**Table 1.** The collections of three *Trigonella species.*

| Species | Voucher specimen | Locality | Date | Altitude (m) |
|---|---|---|---|---|
| *T. disperma* Vassilcz. | BASU 11089 | Kordestan: Ghorveh, after Hamekasi | 26.06.2010 | 1700 |
| *T. aphanoneura* Rech.f. | BASU 7222 | Bakhtiary: Boroujen protected area | 30.02.2010 | 1100 |
| *T. subenervis* Rech.f. | BASU 14337 | Khorassan: 15 km after Rivash, 80 km to Neyshabour | 04.07.2010 | 1700 |

One of them, *T. foenumgraecum* L. is commonly grown as a vegetable (Grossheim, 1945; Hamzehee, 2000; Hedge, 1970). Many bioactive compounds such as protodioscin, trigoneoside, diosgenin, yamogenin have been isolated from fenugreek seeds (Giradon *et al*., 1986; Kabilan *et al*., 2002; Murakami *et al*., 2000; Pandita *et al*., 1991; Small *et al*., 1990; Yoshikawa *et al*., 1992). The important chemical constituents are saponins, coumarin, fenugreekine, nicotinic acid, phytic acid, scopoletin and trigonelline (Ullah Khan *et al*., 2009).

Studies on the pollen grains of leguminous plants (Brookes & Small, 1988; Clarke & Kupicha, 1976; Diez and Ferguson, 1994; Ferguson, 1990; Ferguson & Skvarla, 1981; Ferguson & St-riton, 1993; Hughes, 1997; Kovach 1985-2002; Small & Bassett, 1981) dealt mainly with the description of the pollen grains of certain genera, e.g. *Trigonella*, or tribes. However, there are only two reports on the oil composition in the genus dealt with *T. foenum-graecum* and *T. disperma* (Ahmadiani *et al*., 2004; Ranjbar *et al*., 2009).

In this investigation three *Trigonella* species, *i.e. T. disperma, T. subenervis* and *T. aphanoneura*, were studied from different aspects of morphology, pollen morphology and essential oil composition, and the relationships between the species were discussed. These species are endemic to Iran and belong to sect. *Ellipticae*. We report here the composition of the essential oils of *T. aphanoneura* and *T. subenervis* for the first time and compare them with those of *T. disperma.* The aim of the present study was to investigate whether or not the secondary compounds could be used as discriminative taxonomic markers in this group, especially between the closely related taxa.

## MATERIAL AND METHODS

### Plant material

Fresh materials of each species were sampled from nature. The locations of the collected species are given in Table 1. The voucher specimens were deposited at the Bu-Ali Sina University Herbarium (BASU), Hamedan, Iran.

### Morphology

In morphological study, 28 qualitative and quantitative characters (Table 2) related to reproductive and vegetative organs were studied in 10 individuals of each species. Data were entered in Microsoft Excel. The spread sheet was later transformed into a file format suitable for phenetic analysis. Then, the principal coordinate analysis (PCO) was carried out using MVSP version 3.1, with a matrix of standardized data (Adams, 2001).

### Essential oil composition

The air-dried aerial parts of the plants were subjected to hydrodistillation using a Clevenger-type apparatus for 3 h. Prior to the analysis, the oils obtained were dried over anhydrous sodium sulfate and stored in sealed glass vials at 4c. The oil was analyzed by GC and GC/MS.

GC analysis was performed on a Thennoquest-Finnigan Trace GC instrument equipped with FID detector and a capillary fused silica column (60 m×0.25 mm ID, coated with DB-l, film thickness, 0.25 μm).

The column temperature was kept at 60°C for 3 min and then programmed to 250°C at a rate of 5°C/min and then kept constant at 250°C for 10 min. Injector and detector temperatures were 250°C and 280°C, respectively. The flow rate of $N_2$ (carrier gas) was 1.1 ml/min; split ratio, 1:50. GC/MS analysis was carried out on a Thennoquest-Finnigan Trace GC-MS system equipped with a fused silica capillary column;
(60 m × 0.25 mm ID, coated with DB-l, film thickness, 0.25 μm) and interfaced with a quadruple detector.

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**Table 2.** Mean value of morphological features and character state matrix of three *Trigonella* species.

| Morphological features | *T. subenervis* | *T. aphanoneura* | *T. disperma* |
|---|---|---|---|
| Stem length (cm) | 26±0.85 | 23.5±0.88 | 45±1.05 |
| Internode length (cm) | 0.36±3 | 2.2±0.61 | 2.7±0.50 |
| Leaf length (mm) | 10.5±0.15 | 6±0.35 | 10.5±0.22 |
| Leaflet length (mm) | 7.5±0.04 | 6±0.11 | 7±0.08 |
| Leaflet width (mm) | 7±0. 07 | 4.5±0.01 | 5±0.02 |
| Petiole length (mm) | 8±0.04 | 4±0.03 | 8.5±0.12 |
| Stipule length (mm) | 3±1.88 | 3±2.31 | 4±2.60 |
| Stipule width (mm) | 0.5±0.32 | 1±0.26 | 0.7±0.18 |
| Peduncle length (cm) | 2.3±0.05 | 3.4±0.21 | 2.6±0.09 |
| Calyx tube length (mm) | 3±0.05 | 2.5±0.06 | 2.5±0.10 |
| Calyx tooth length (mm) | 2±0.05 | 1±0.05 | 1±0.10 |
| Calyx length (mm) | 5±1.10 | 3.5±1.10 | 3.5±0.90 |
| Standard length (mm) | 10±0.08 | 7±0.09 | 10±0.09 |
| Standard width (mm) | 7±1.23 | 7±1.51 | 8±1.08 |
| Standard claw length (mm) | 2±1.38 | 1±1.60 | 2±1.29 |
| Wing length (mm) | 10±0.09 | 7±0.06 | 9±0.04 |
| Wing width (mm) | 2±2.05 | 2±2.14 | 3±1.98 |
| Keel length (mm) | 9±0.70 | 7.5±0.72 | 8±0.55 |
| Number of flowers | 4±1.13 | 8±1.65 | 7±1.41 |
| Pedicel length (mm) | 3±0.11 | 3.7±0.11 | 3.5±0.10 |
| Leaflet beak length (mm) | 0.6±0.46 | 0.6±0.21 | 0.4±0.08 |
| Pod length (mm) | 10±0.76 | 16.5±0.88 | 12.5±0.68 |
| Pod width (mm) | 2.5±1.06 | 3.5±1.11 | 5±1.24 |
| Pod stipa length (mm) | 0.5±0.06 | 2±0.08 | 1.5±0.12 |
| Seed number | 1±1.01 | 3±0.98 | 2±0.86 |
| Hair density on bract (dense = 1, glabrous = 0) | 0 | 1 | 0 |
| Fruit wing (absent = 0, present = 1) | 0 | 0 | 1 |
| Standard color (yellow = 0, violet = 1) | 1 | 0 | 0 |
| Standard form (spheroid = 0, obovate = 1) | 0 | 0 | 1 |

The column temperature was kept at 60°C for 3 min and then programmed to 250°C at a rate of 5°C/min and then kept constant at 250°C for 10 min. Injector and transfer line temperatures were 250°C. The flow rate of Helium (carrier gas) was 1.1 ml/min; split ratio, 1:50.

The MS operating parameters were as follows: ionization energy, 70 eV; scan time, 1 s; mass range, 45-465 amu; ionization current, 150 µA.

The components of the oil were identified by their retention times, retention indices (RI) relative to $C_6$-$C_{24}$ *n*-alkanes (on DB-1 column under the same conditions) and their mass spectra with those reported in literature and by computer matching with the WILEY 7.0 library as well as whenever possible, co-injection with authentic compounds available (Jennings & Shi-bamoto, 1980).

The percentage composition of the identified compounds was computed from the GC peak area without any correction factor and calculated relatively. The data related to oil compositions were analyzed with MVSP ver. 3.1 to understand the relationships between the species (Adams, 2001).

**Pollen morphology**

For light microscopy (LM), pollen samples were obtained from herbarium specimens and prepared using the standard method described by Erdtman (1960). Pollen grains were mounted on unstained glycerin jelly and observed with a Nikon type-2 microscope. Measurements were conducted on the basis of 25 readings from each 10 specimens. Polar axis (P), equatorial diameter (E), colpus length (L), colpi interval in granule site (G) and in non-granule site (NG), and shape index (P/E) were measured.

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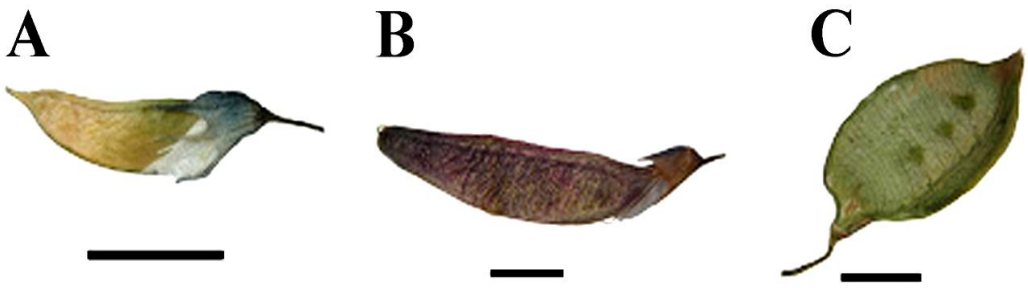



**Fig. 1.** Pods. **A**: *T. subenervis*; **B**: *T. aphanoneura*; **C**: *T. disperma* (with wing). Scale bar = 5 mm.

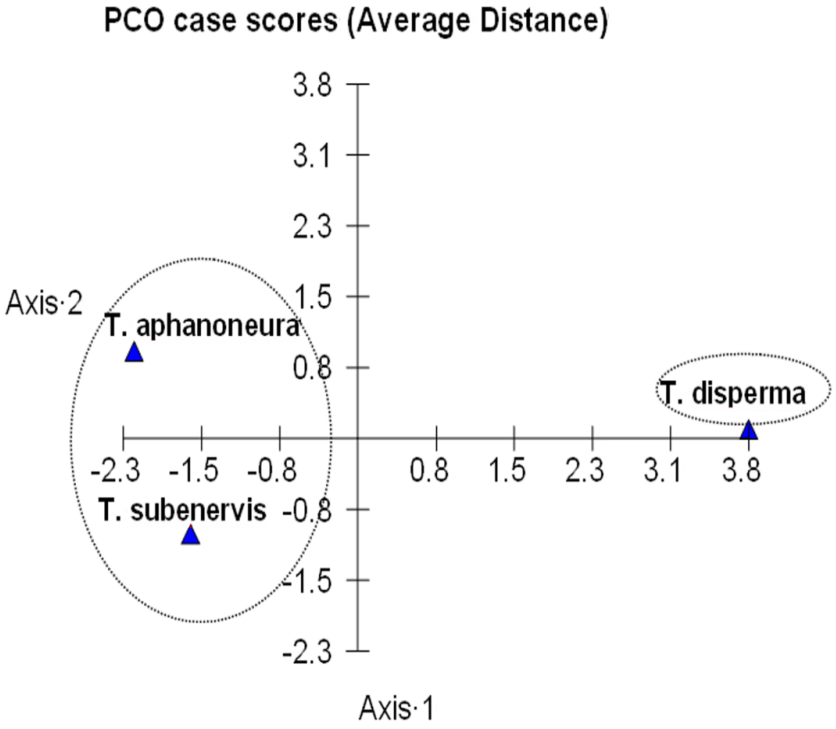



**Fig. 2.** PCO analysis of three *Trigonella* species based on morphological characters.

PCO analysis was performed using average distances in order to show phenetic relationships between the species based on pollen morphological characters.

## RESULTS AND DISCUSSION

### Morphology

This study reports the taxonomic relationnships between three *Trigonella* species armed with the knowledge about morphology, pollen morphology and oil composition. Three species studied were similar in morphology, but some characters related to pods separated them. Pod characters can be useful when the plants are in reproductive stage, but distinguishing among three species in their vegetative stage is so difficult. *T. disperma* is differentiated from other species by its longer pods (± 5 mm) with wings 1-1.5 mm in width (Fig. 1). The results from PCO analysis of the species based on morphological characters are illustrated in Figure 2.

### Chemical composition of essential oils

The chemical composition of the essential oils of *T. subenervis* and *T. aphanoneura* were analyzed by means of GC/MS. The GS-separated compounds were identified by the recorded mass spectra in comparison with the mass spectra from the GS library. Table 3 gives the oil composition of representative samples of the three *Trigonella* species. In total, 23 compounds were identified in *T. subenervis*, *T. aphanoneura* and *T. disperma*, amounting to 41.3%, 40.6% and 87% of the oils, respectively.

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**Table 3.** Compounds identified in the aerial parts oil of three *Trigonella* species.

| Compound[a] | Ri[b] | *T. subenervis* (%) | *T. aphanoneura* (%) | *T. disperma* (%) |
|---|---|---|---|---|
| *p*-Xylene | 800 | - | - | 1.0 |
| Decanal | 1184 | - | - | 0.6 |
| *n*-Octyl acetate | 1190 | 1.3 | 0.4 | - |
| Thymol | 1265 | 1.5 | 0.9 | 1.1 |
| Neryl acetate | 1431 | 0.9 | - | 0.7 |
| *β*-Lonene | 1468 | - | - | 2.9 |
| Germacrene D | 1483 | 0.6 | 0.8 | - |
| Pentacosane | 1498 | - | - | 27.3 |
| Nerolidol | 1574 | 0.8 | - | - |
| Spathulenol | 1572 | 15.1 | 10.7 | 17.8 |
| Caryophyllen oxide | 1580 | - | - | 7.9 |
| Hexadecane | 1597 | - | - | 3.9 |
| NI[c] | 1661 | 6.7 | 11.2 | 5.3 |
| NI[c] | 1667 | 1.5 | 1.9 | 1.4 |
| Heptadecane | 1699 | - | - | 2.4 |
| NI[c] | 1708 | 3.2 | 4.8 | 3.1 |
| NI[c] | 1716 | 2.1 | 5.1 | 1.8 |
| NI[c] | 1728 | 1.2 | 6.0 | 1.3 |
| Myristic acid | 1738 | 1.8 | 3.1 | 2.1 |
| Octadecane | 1799 | - | - | 1.2 |
| Hexahydroxyfarnesyl acetone | 1829 | 5.8 | - | 6.7 |
| Nonadecane | 1898 | - | 0.6 | 0.9 |
| Dibutyl phthalate | 1920 | 2.7 | 1.8 | 3.9 |
| Palmitic acid | 1938 | 0.9 | - | 1.8 |
| Farnesyl acetate | 1990 | 1.3 | 1.0 | - |
| Phytol | 2100 | - | - | 4.0 |
| Tricosane | 2297 | 1.1 | - | 0.9 |
| Tetracosane | 2389 | 1.8 | 1.2 | - |
| Total | | 50.3 | 49.5 | 100 |

[a]Compounds listed in order of their RI.

[b]RI (retention index) measured relative to *n*-alkanes ($C_6$-$C_{24}$) on DB-1 column.

[c]NI, not identified.

The oil composition of *T. aphanoneura* was found to be more similar to *T. subenervis* than *T. disperma*. Most of the compounds were volatile esters. Ranjbar *et al*. (2009) reported that in *T. disperma*, 87.1% of the oil comprised a total of 18 compounds. The major constituents of the oil were pentacosane (27.3%) and spathulenol (17.8 %). Decanal (0.6%) and neryl acetate (0.7%) were observed to have the lowest percentages in the oil. It was found that spathulenol had a high amount in both *T. subenervis* and *T. aphanoneura* (Table 3).

Some of the compounds such as one compound with high amount in *T. aphanoneura* (11.2%), were not identified. In addition, *n*-octyl acetate (0.4%) and germacrene D (0.6%) had the lowest amounts of *T. subenervis* and *T. aphanoneura*., respectively.

*T. disperma* showed major differences in its oil composition in comparison to other species. Its oil includes some compounds such as *p*-xylene, decanal, caryophyllen oxide, hexadecane, heptadecane, octadecane and phytol, which are all absent in other studied species.

In contrast, germacrene D and farnesyl acetate were not observed in *T. disperma*. A detailed list of the oil components of the species is given in Table 3 and PCO analysis based on oil composite-on data is illustrated in Figure 3. These phytochemical data verified morphological results and the separation of the species well. To sum up, these species synthesized many similar compounds in their essential oils.This fact can be justified by their similar morphology (biochemical convergence).

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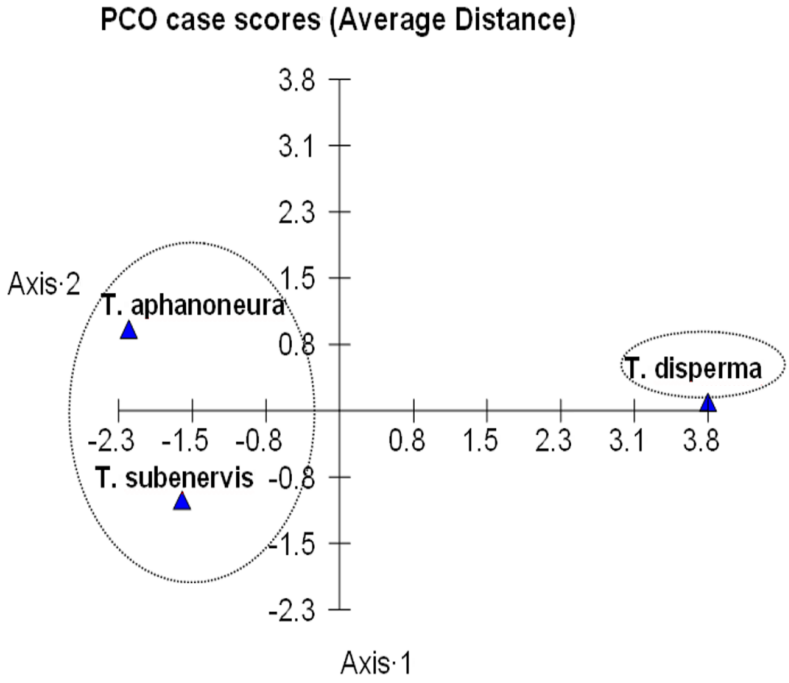



**Fig. 3.** PCO analysis of three *Trigonella* species based on leaf oil composition data.

**Table 4.** Taxa examined with measurements (µm) of the mean and ranges for pollen features of three *Trigonella*.

| Species | E | P | G | NG | L | P/E |
|---|---|---|---|---|---|---|
| *T. disperma* | 22(24±0.28)28.5 | 29(30.1±0.21)31 | 17(17.3±0.0.08)20 | 13(14.3±0.0.20)17 | 24(25.1±0.76)26 | 1.34 |
| *T. aphanoneura* | 21.3(22±0.46)24 | 28(28.5±0.15)31 | 17(18±0.11)20 | 13(13.9±0.18)15 | 24(25.0±0.89)26 | 1.33 |
| *T. subenervis* | 20(21.5±0.53)23 | 27(27.9±0.19)29 | 15(16.4±0.13)17 | 12(12.1±0.26)13 | 22(22.9±0.91)24 | 1.29 |

**E**: Equatorial diameter; **P**: Polar axis; **L**: Colpus length; **G** and **NG**: Colpi interval in granule site and non-granule site, respectively; **P/E**: Shape index. Means in parenthesis were expressed as ± SE.

However, the oil of *T. disperma* differs from the other two species significantly. PCO analysis verified the results of morphological study and showed that the differences referred to some constituents. In addition, the taxonomic distance of the species could be confirmed by chemical data.

**Pollen morphology**

The morphology of pollen grains represents more evidence which can be used to clarify the relationships between the species studied. The pollen grains of the tribe Trifolieae are radially symmetrical, isopolar trizonocolporate, with perforate tectume and varying shape and sculpture. The pollen morphology of this tribe shows that Trifolieae is a eurypalynous tribe (Gamal, 2006; Small & Bassett, 1981). In sect. *Ellipticae*, pollen grains are elliptic to oblong with often perforate ornamentation. Some features are common to several species of the section, while others are unique (Ranjbar *et al.*, 2012). The measurements of six pollen characters of the taxa studied are shown in Table 4, and the light micrographs are presented in Figure 4. The results of PCO analysis, based on pollen morphology, are not in agreement with morphology and phytochemistry (Fig. 5). In contrast to the phytochemistry and morphology-based analyses, *T. subenervis* is separated from other species because of its smaller pollen grains (Table 4).

## CONCLUSION

According to what we found out, phytochemical and morphological data from our study could clarify the relationships among the *Trigonella* species better than pollen morphological data.

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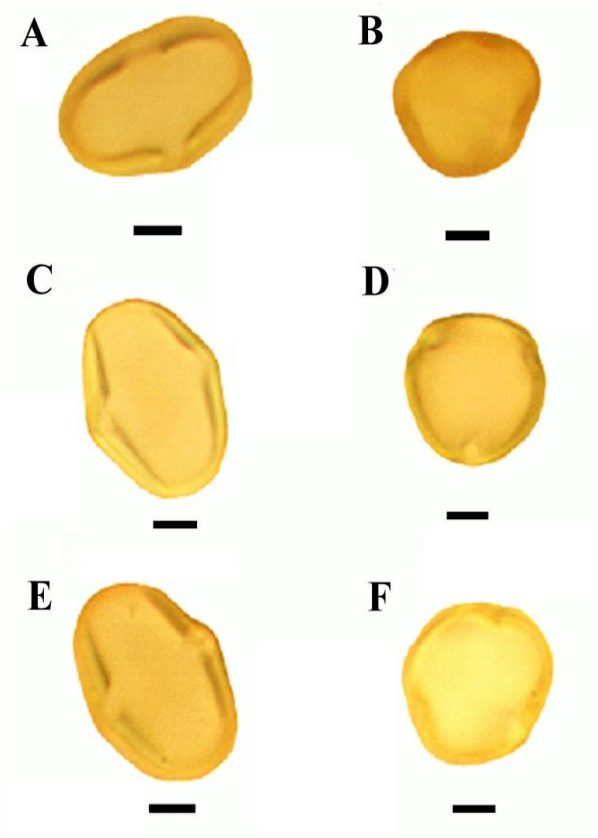



**Fig. 4.** LM polar and equatorial views of the pollen grains in three *Trigonella* species: **A, B**: *T. aphanoneura*; **C, D**: *T. disperma*; **E, F**: *T. subenervis*. Scale bars = 6 µm.

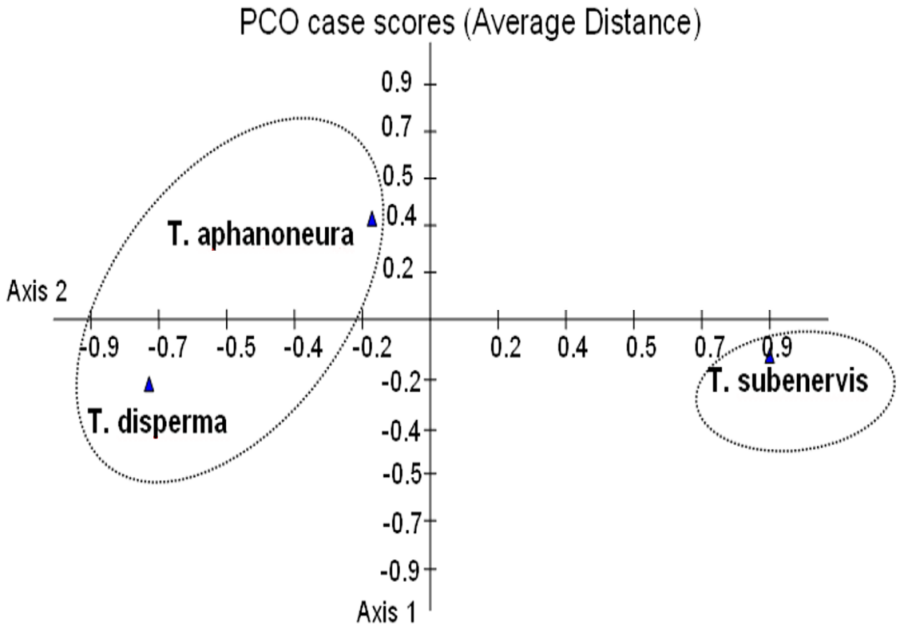



**Fig. 5.** PCO analysis of three *Trigonella* species based on pollen morphological characters.

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