A descriptive study of some *Trifolium* L. (Clover) taxa grown in Bolu Province

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Abstract: Micromorphological characteristics of 13 *Trifolium* species, which are grown in the province of Bolu, were examined using a scanning electron microscope (SEM). The morphological characteristics determined from the study were subjected to morphometric analyses and their variation spectrums were defined. Tricolporate pollen grains showed two pollen shapes, prolate and subprolate, according to equatorial view. The smallest size of polar axis length (18.00 μm) was found in *T. micranthum* Viv. from section *Chronosemium* Ser.; the largest (88.95 μm) was found in *T. hirtum* All. from section *Trifolium* L.. Statistically, the smallest seeds were found in *T. resupinatum* L. from the section *Vesicaria* Crantz. with an average size of 1.01 mm. However, the largest seeds were observed in *T. hirtum* species from the section *Trifolium* with an average size of 2.04 mm.

The results gave similar groupings in cluster (CA) and principal coordinate analysis (PCoA). Main taxa were grouped under section *Trifolium*; *T. campestre* Schreb. and *T. micranthum* produced section *Chronosemium*; *T. physodes* Steven & M. Bieb. and *T. resupinatum* formed the section *Vesicaria*; and finally, *T. nigrescens* Viv. presented the section *Lotoidea* Crantz. Besides taxonomical classification, pollen and seed properties of 13 *Trifolium* taxa were also investigated.

Key words: *Trifolium*, macromorphology, micromorphology, Bolu, Turkey

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1. Introduction

The genus *Trifolium* (Fabaceae) includes a large amount of important leguminous forage crops for livestock agriculture. Therefore, these plants in the family Fabaceae are of great importance to animals. They are consumed for their contribution to animal health (Foo et al., 2000); due to their antioxidant and antiinflammatory properties they are useful remedies for cardiovascular disease and cancers (Baytop, 1994; Khaled et al., 2000; Heinonen et al., 2004). Furthermore, many of them are considered ornamental plants (Baytop, 1994). Many of their byproducts are also used in producing paint, gum, and oil. Apart from these usages, legumes are relevant to different applications in their medicinal aspects (isoflavones), biodegradable plastics, oil and furniture production (Stern et al., 2003; Sheokand et al., 2012). *Trifolium* species are also the favorite plants of bees (Koçyiğit et al., 2013).

*Trifolium* L., containing 260 species in the world, is represented by 104 species in Turkey (Keskin, 2012). Among them, 13 taxa are endemic and endemism rate is 10% (Davis et al., 1988). Although this genus is a cosmopolitan, the main distribution area is the countries in the Mediterranean region and its periphery. Zohary and Heller (1984) included about 255 species of *Trifolium* in their study, which are divided into eight sections. According to molecular phylogenetic studies, Ellison et al. (2006) have distinguished them into two valid subgenera of *Trifolium*: subgenus *Chronosemium* and subgenus *Trifolium*.

Research on *Trifolium* also reveals the importance of studies on pollen analyses in beekeeping. Earlier pollen studies of *Trifolium* were done by Erdtman et al. (1963). They have introduced a pollen diagnostic key based on the size, shape, and light microscopic images of pollen grains belonging to 11 *Trifolium* species from Scandinavia. Research on pollens of 45 *Trifolium* species naturally growing in North America is an important study using both scanning electron microscope and light microscope (Gillett, 1970).

On the other hand, seed and pollen characteristics are important in plant systematic studies, especially for the generic and specific levels, as they reflect evolutionary processes. There is quite a high number of research studies on this topic (Brochmann, 1992; Algan and Büyükkartal, 2000; Bernard, 2000; Koul and Ranina, 2000; Seggara and Mateui, 2001; Hassan et al., 2005; Zoric et al., 2010). Seed...
characterizations are very useful in the identification of many species and genera (Marin et al., 1998; Juan et al., 2000; Moro et al., 2001; Seggarra and Mateui, 2001). In many cases, morphological characteristics such as seed shape and testa surface morphology can be used to distinguish species and varieties from each other (Aniszewski et al., 2001). Karam (1997) investigated 16 *Trifolium* species seed surface structures. Similar studies were performed for other Turkish taxa of *Hesperis* L. (Pınar et al., 2009) and of *Velezia* L. (Poyraz and Ataşlar, 2010).

In the studies by George et al. (2013) on the seeds of 61 taxa belonging to the sections *Trifolium*, *Lotoidea* Crantz, *Mistyllus* (C. Presl) Godr, *Vesicaria* Crantz, and *Chronosemium* Ser., the CA results of SDS PAGE profiles, with some restrictions, supported the sectional classification of Zohary and Heller (1984).

Watson et al. (2000) carried out a phylogenetic classification of the *Trifolium* taxa in Asia, Europe, and Africa. In their study, ITS sequences of nuclear DNA and chloroplast DNA (rbcL, trnK, and rpoC1-C2) of *Trifolium* were determined. The results of Watson and colleagues showed that the section *Chronosemium* was monophyletic, although the genus *Trifolium* was a monophyletic group. Despite this finding, in the study of traditional classification (Zohary and Heller, 1984) the two large sections, *Trifolium* and *Lotoidea*, were found not to be monophyletic groups.

In another genetic study carried out by Vizintin et al. (2006) through nuclear DNA content and ITS rDNA region, they also found results not supporting traditional classification of Zohary and Heller (1984), but more or less supporting the phylogenetical results of Watson et al. (2000).

In this study, 13 Turkish *Trifolium* species were viewed based on both their pollen grains and seed characteristics. This study aimed to examine the potential contribution of classifications at the level of the section previously presented by other researchers in traditional (Zohary and Heller, 1984) and phylogenetic (Ellison, et al., 2006) classifications. Additionally, this study aimed to provide important contributions to taxonomic classification of the *Trifolium* species, identification of genetic characteristics, and to the work done in the agriculture and apiculture.

### 2. Materials and methods

Seeds and pollen grains belonging to 13 taxa from 11 stations (Table 1) were collected in Bolu Province during the summer of 2011. Among them, *T. elongatum* is endemic in Turkey (Keskin, 2012). Specimens were identified according to Davis’ Flora of Turkey (Zohary, 1970) and the Monograph of Genus *Trifolium* (Zohary and Heller, 1984). These taxa were included in four sections of Zohary and Heller’s classification, such as *Lotoidea*, *Vesicaria*, *Chronosemium*, and *Trifolium* (Table 1). The plant specimens of the study are stored in the Herbarium of Bolu Abant Izzet Baysal University (AIUB), Turkey.

Pollen grain samples were prepared according to the acetolysis method of Erdtman (1960). For the light microscopic examinations, Olympus BX51 and Olympus

### Table 1. Names of taxa and their location information sampled in Bolu Province.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Location</th>
<th>Altitude</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. nigrescens</em> Viv. (L_NIG)</td>
<td>Kibrıscık</td>
<td>1350 m</td>
</tr>
<tr>
<td><em>T. physodes</em> Steven &amp; M. Bieb. (V_PHY)</td>
<td>CCampus of B. A. I. B. U.*</td>
<td>836 m</td>
</tr>
<tr>
<td><em>T. resupinatum</em> L. (V_RES)</td>
<td>Karacasu</td>
<td>740 m</td>
</tr>
<tr>
<td><em>T. campestre</em> Schreb. (C_CAM)</td>
<td>Kibrıscık</td>
<td>1385 m</td>
</tr>
<tr>
<td><em>T. micranthum</em> Viv. (C_MIC)</td>
<td>Hill of Kartalkaya</td>
<td>2010 m</td>
</tr>
<tr>
<td><em>T. hirtum</em> All. (T_HIR)</td>
<td>Campus of B.A. I. B. U.*</td>
<td>860 m</td>
</tr>
<tr>
<td><em>T. ochroleucum</em> Huds. (T_OCH)</td>
<td>Around lake Abant</td>
<td>1375 m</td>
</tr>
<tr>
<td><em>T. striatum</em> L. (T_STR)</td>
<td>Gerede-Kızılabahamams</td>
<td>1300 m</td>
</tr>
<tr>
<td><em>T. medium</em> L. var. medium(T_MEDm)</td>
<td>Kibrıscık</td>
<td>1385 m</td>
</tr>
<tr>
<td><em>T. pratense</em> L. (T_PRA)</td>
<td>Mudurnu-Gövem</td>
<td>880 m</td>
</tr>
<tr>
<td><em>T. bocconei</em> Savi. (T_BOC)</td>
<td>Sarialan plateau</td>
<td>1527 m</td>
</tr>
<tr>
<td><em>T. elongatum</em> Willd. (T_ELO)</td>
<td>Gerede-Kızılabahamams</td>
<td>1425 m</td>
</tr>
<tr>
<td><em>T. echinatum</em> M. Bieb. (T_ECH)</td>
<td>Yumrukaya village</td>
<td>799 m</td>
</tr>
</tbody>
</table>

*Bolu Abant Izzet Baysal University*
DP71 digital imaging systems were used. For the SEM examinations, pollen grains and dry, mature seeds were fixed with double-sided adhesive tape and coated with gold by keeping them under 4 mA electric current for 200 s on the CoXem Ion Coating (KIC-1A) brand device. Subsequently, the samples were examined on a JEOL 6390-LV scanning electron microscope (SEM) at 20–30 kV with a resolution of 3 nm.

For numerical analysis, a total of 16 characters were measured and coded, 10 of which from seeds and 6 from pollen grains (Table 2). Seven of these are continuous, five are nominal, three are binary, and one is ordinal type of characters. Quantitative character measurements were made by taking 30 pollens and 25 seeds from each plant specimen and their mean values were calculated. Extracted qualitative characters were scored according to their types (Table 2).

Seed surface structures of the study were determined according to Zoric et al. (2010) and pollen grain surface types were determined based on the studies by Zohary and Heller (1984). All data were subjected to statistical analyses such as cluster analysis (CA) and principal coordinate analysis (PCoA). The mixed similarity matrix was used in the clustering analysis of the PAST computer program (Hammer et al., 2001). In the calculation of this matrix, Gower’s distance measures were employed for continuous and ordinal variables (Gower, 1971). Hamming distance (Roth, 2006) and Jaccard coefficient (Sneath and Sokal, 1973) were used for nominal and binary variables, respectively. Since the number of cases is smaller than the number of variables, PCoA was used, as it is more suitable for numerical taxonomic trends (Marhold, 2011). In PCoA, the mixed matrix was also employed for measuring distances between taxa, and scatter diagrams were plotted according to the UPGMA (unweighted pair group method with arithmetic mean) method, in which transformation exponent was used as $c = 2$ (Podani and Miklos, 2002). Moreover, the correlations of variables were controlled by Pearson’s correlation coefficients (Pearson, 1896).

3. Results
3.1. Pollen studies
Although according to equatorial view 13 taxa included two types of pollen shape, prolate and subprolate (Table 2), they are all tricolporate and isopolar pollen grains type. Generally, the prolate pollen shapes were found in eight taxa belonging to all 4 sections (Lotoidea, Vesicaria, Chronosemium, and Trifolium). However, the subprolate pollen shapes were observed in five taxa belonging to two sections (Chronosemium and Trifolium). In the SEM

<table>
<thead>
<tr>
<th>No</th>
<th>Character name and abbreviation</th>
<th>Data types*</th>
<th>Character state and code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Seed shape (SSHP)</td>
<td>Nominal</td>
<td>Round (0) / ovoid (1) / elongated ovoid (3) / heart-shaped (4)</td>
</tr>
<tr>
<td>2</td>
<td>Seed color (SCOL)</td>
<td>Nominal</td>
<td>Light brown (0) / brown (1) / creamy brown (2) / yellowish brown (3)</td>
</tr>
<tr>
<td>3</td>
<td>Radicle lobe length (SRDL)</td>
<td>Ordinal</td>
<td>Radicle lope up to 1/2 (0) / up to 2/3 (1) same as cotyledon length</td>
</tr>
<tr>
<td>4</td>
<td>Hilum positions (HPOS)</td>
<td>Nominal</td>
<td>Lateral (0) / almost apical (1) lateral-end (2)</td>
</tr>
<tr>
<td>5</td>
<td>Hilum shape (HSHP)</td>
<td>Binary</td>
<td>Broad elliptic (0) / elliptic (1)</td>
</tr>
<tr>
<td>6</td>
<td>Hilum color (HCOL)</td>
<td>Binary</td>
<td>White cream (0) / brown (1)</td>
</tr>
<tr>
<td>7</td>
<td>Seed coat ornament (SCOR)</td>
<td>Nominal</td>
<td>Smooth, glabrous (0) / roughed (1) / papillose (2)</td>
</tr>
<tr>
<td>8</td>
<td>Seed length (SLEN)</td>
<td>Continuous</td>
<td>Numerical</td>
</tr>
<tr>
<td>9</td>
<td>Seed width (SWID)</td>
<td>Continuous</td>
<td>Numerical</td>
</tr>
<tr>
<td>10</td>
<td>Seed index (SIND)</td>
<td>Continuous</td>
<td>Numerical</td>
</tr>
<tr>
<td>11</td>
<td>Pollen shape (PSHP)</td>
<td>Binary</td>
<td>Prolate (0) / subprolate (1)</td>
</tr>
<tr>
<td>12</td>
<td>Pollen surface ornaments (PSOR)</td>
<td>Nominal</td>
<td>Irregular reticulate-scrobiculate, aperture edges and polar ends smooth (0) Reticulate-faveolate, aperture edges and polar ends smooth (1) Rough rugulate all surface (2)</td>
</tr>
<tr>
<td>13</td>
<td>Polar axis length (PPOL)</td>
<td>Continuous</td>
<td>Numerical</td>
</tr>
<tr>
<td>14</td>
<td>Equatorial axis length (PEKL)</td>
<td>Continuous</td>
<td>Numerical</td>
</tr>
<tr>
<td>15</td>
<td>Pollen index (PIND)</td>
<td>Continuous</td>
<td>Numerical</td>
</tr>
<tr>
<td>16</td>
<td>Colpus length (PCOL)</td>
<td>Continuous</td>
<td>Numerical</td>
</tr>
</tbody>
</table>

*Seed character continuous data type (mm), pollen character continuous data type (µm)
studies, pollen varieties were revealed based on pollen sculpture types of Zohary and Heller (1984). Thus, a total of three types of pollen sculpture are observed (Table 2; Figures 1 and 2).

**Type I – reticulate-scrobiculate**: Surface ornaments are irregular reticulated-scrobiculate structures. However, edges of furrows and the pole ends are smooth to some extent. This type of pollen sculpture includes *T. campestre*, *T. micranthum*, *T. nigrescens*, *T. physodes*, and *T. resupinatum* in Figures 1A–1C, 1E, and 2K.

**Type II – reticulate-faveolate**: The surface of the pollen lumens is an irregular reticulate-foveolate. Polar ends and along the edges of furrow are areolate-reticulate and sometimes smooth. It contains *T. striatum* and *T. bocconei* in Figures 1D and 1F.

**Type III – coarsely rugulate**: Pollen surfaces, shapes, and intermediate spaces are irregular, with deep lumen roughly rugulate structure. However, it has a structure ranging from areolate to foveolate at the polar ends and along the furrows. This type of structure was found in

Figure 1. A: *T. campestre*; B: *T. micranthum*; C: *T. nigrescens*; D: *T. striatum*; E: *T. resupinatum*; F: *T. bocconei*. A₁–F₁: general SEM view of pollen shape. A₂–F₂: pollen surface sculpture in SEM. There are small pictures representing *Trifolium* species pollen under light microscope (top: polar; bottom: equatorial) views.
T. echinatum, T. hirtum, T. ochroleucum, T. pratense, T. medium var. medium, and T. elongatum in Figures 2H–2J, 2L, and 2M.

The shortest pollen was found in T. micranthum from the section Chronosemium with an average polar axis length of 18.00 μm (min 16.47 μm, max 23.3 μm) (Table 3).

The longest pollen was observed in the T. hirtum species from the section Trifolium with an average polar axis length of 88.95 μm (min 84.21, max 92.11 μm). Of lowest (with an average of 1.04 μm) and highest (with an average of 2.14 μm) pollen indices contained T. micranthum species from the section Chronosemium and T. medium

Table 3. Exploratory data table for the pollen characters. *mean ± standard deviation. **minimum and maximum values of the distribution (see Table 1 for section abbreviation).

<table>
<thead>
<tr>
<th>Sect. Taxon</th>
<th>Polar axis length (µm)</th>
<th>Equatorial axis length (µm)</th>
<th>Pollen index (µm/µm)</th>
<th>Colpus length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>39.67 ± 1.48 (37.78 – 42.22)</td>
<td>18.67 ± 1.15 (17.78 – 20.00)</td>
<td>2.14 ± 0.17 (1.89–2.38)</td>
<td>32.00 ± 2.15 (26.67 – 33.33)</td>
</tr>
<tr>
<td>V. T. physodes</td>
<td>68.13 ± 2.61 (64.58–72.92)</td>
<td>35.00 ± 2.91 (31.25–39.58)</td>
<td>1.96 ± 0.17 (1.72–2.00)</td>
<td>57.71 ± 2.61 (54.17–60.42)</td>
</tr>
<tr>
<td>T. resupinatum</td>
<td>32.09 ± 2.20 (27.91–34.88)</td>
<td>18.60 ± 1.55 (16.28–20.93)</td>
<td>1.73 ± 0.13 (1.50–1.88)</td>
<td>21.86 ± 1.20 (20.93–23.26)</td>
</tr>
<tr>
<td>C. T. campestre</td>
<td>18.00 ± 2.08 (16.47–23.53)</td>
<td>17.41 ± 1.34 (15.29–20.00)</td>
<td>1.04 ± 0.10 (0.88–1.25)</td>
<td>11.29 ± 1.26 (9.41–14.12)</td>
</tr>
<tr>
<td>T. micranthum</td>
<td>88.95 ± 3.24 (84.21–92.11)</td>
<td>51.58 ± 3.33 (47.37–57.89)</td>
<td>1.73 ± 0.10 (1.57–1.89)</td>
<td>58.95 ± 5.98 (52.63–65.79)</td>
</tr>
<tr>
<td>T. hirtum</td>
<td>45.58 ± 2.73 (41.86–51.16)</td>
<td>25.12 ± 1.47 (22.26–27.91)</td>
<td>1.82 ± 0.11 (1.64–2.00)</td>
<td>38.18 ± 3.83 (34.88–44.19)</td>
</tr>
<tr>
<td>T. ochroleucum</td>
<td>37.75 ± 0.79 (37.50–40.00)</td>
<td>23.00 ± 1.05 (22.50–25.00)</td>
<td>1.65 ± 0.08 (1.50–1.78)</td>
<td>28.60 ± 2.58 (25.00–30.00)</td>
</tr>
<tr>
<td>T. striatum</td>
<td>54.59 ± 2.13 (51.35–59.46)</td>
<td>26.76 ± 1.00 (24.32–29.73)</td>
<td>2.05 ± 0.13 (1.82–2.22)</td>
<td>45.95 ± 3.60 (40.54–51.35)</td>
</tr>
<tr>
<td>T. medium var. medium</td>
<td>42.80 ± 3.01 (40.00–48.00)</td>
<td>27.40 ± 2.50 (24.00–30.00)</td>
<td>1.57 ± 0.15 (1.33–1.89)</td>
<td>33.40 ± 2.32 (30.00–38.00)</td>
</tr>
<tr>
<td>T. pratense</td>
<td>46.91 ± 1.15 (45.24–47.62)</td>
<td>25.00 ± 3.42 (19.05–30.95)</td>
<td>1.91 ± 0.26 (1.54–2.38)</td>
<td>40.48 ± 3.18 (35.71–45.24)</td>
</tr>
<tr>
<td>T. bocconei</td>
<td>31.86 ± 1.47 (30.23–34.88)</td>
<td>21.86 ± 1.96 (18.60–25.58)</td>
<td>1.47 ± 0.13 (1.18–1.69)</td>
<td>27.21 ± 1.91 (23.26–30.23)</td>
</tr>
<tr>
<td>T. elongatum</td>
<td>60.25 ± 2.19 (57.50–62.50)</td>
<td>34.00 ± 2.11 (32.50–37.50)</td>
<td>1.78 ± 0.11 (1.53–1.92)</td>
<td>45.75 ± 2.06 (42.50–50.00)</td>
</tr>
<tr>
<td>T. echinatum var. campestre</td>
<td>31.86 ± 1.47 (30.23–34.88)</td>
<td>21.86 ± 1.96 (18.60–25.58)</td>
<td>1.47 ± 0.13 (1.18–1.69)</td>
<td>27.21 ± 1.91 (23.26–30.23)</td>
</tr>
</tbody>
</table>

3.2. Seed studies

The *Trifolium* seeds are compressed from two sides. They usually have two lobes separated by hilum. The larger ones include cotyledons and the smaller ones contain the radicle. The radicular lobe may be more prominent than the cotyledon. Four types of seed shapes were observed according to the lobes: round (0), ovoid (1), elongated ovoid (2), and heart-shaped (3). Four types of seed coat colors were identified: light brown (0), brown (1), creamy brown (2), and yellowish brown (3). The proportions of root and cotyledon lobe lengths, which usually make the seed appear angular, form an important character (Zoric et al., 2010). The length of the radicle lobe is up to half the length of the cotyledon lobe (0); the length of the radicle lobe is up to two-thirds of the length of the cotyledon lobe (1); and the root lobe is the same length as the cotyledon lobe (2). Salimpour et al. (2007) indicated that the two hilum shapes, which distinguish one lobe from the other, were important. In addition to these, the hilum colors are also included in the seed character matrix, considering that they are informative characters. Two hilum characters, the position of the hilum, and the hilum shape (Salimpour et al., 2007) were also included. All these characters and their character states were presented in Table 2.

In the SEM studies, seed types were determined based on seed surface ornaments of Zoric et al. (2010). In this study, only three types of seed surface sculptures were observed from the eight types identified by Zoric et al. (2010) (Table 4; Figures 3 and 4).

**Type 1 – smooth and glabrous (0):** The appearance of epidermal cells on the seed surface does not contain roughness and hair. This type of seed surface includes *T.campestre*, *T. micranthum*, and *T. striatum* in Figures 3A, 3B, and 3D.

**Type 2 – roughed (1):** The walls of the epidermal cells on the seed surface have a convex structure and therefore appear rough. It contains *T. resupinatum*, *T. echinatum*, *T. ochroleucum*, *T. pratense*, and *T. physisodes* in Figures 3E, 3F, 4H, 4L, and 4M).

**Type 3 – papillose (2):** It is a papillous structure and consists of a group of epidermal cells with strongly convex walls. It is found in *T. nigrescens*, *T. bocconei*, *T. hirtum*, *T. medium* var. *medium*, *T. elongatum* (Figures 3C, 3F, 4H, 4L, and 4M).

Statistically, the smallest-sized seeds were found in *T. resupinatum* from the section *Vesicaria* with an average of 1.01 mm (min 0.90 mm, max 1.10 mm). On the other hand, the largest-sized seeds were observed in *T. hirtum* species from the section *Trifolium* with an average of 2.04

var. *medium* taxon from the section *Trifolium*, respectively.
mm (min 1.75 mm, max 2.20 mm). Seeds with the narrowest width were found in the *T. campestre* species from the section *Chronosemium* with a mean of 0.61 mm (min 0.55 mm, max 0.75 mm). Seeds with the largest width were also found in *T. hirtum* from the section *Trifolium* with an average of 1.61 mm (min 1.35 mm, max 1.75 mm). *T. campestre* from the section *Chronosemium* and *T. bocconei* from the section *Trifolium* had the smallest (with 1.16 mm average) and largest (with 1.76 mm average) seed indices, respectively. In general, the smallest and largest seeds were observed in *T. resupinatum* and *T. hirtum* species, belonging to sects. *Vesicaria* and *Trifolium*, respectively.

### 3.3. Numerical analysis

The Pearson product-moment correlation coefficient (r) of the seed and pollen characteristics of the *Trifolium* taxa were calculated. In this study, it was accepted that the coefficients of correlation were 0.65 and greater. The values of correlation coefficients which can be taken into consideration in pollen and seed characters can be divided into 3 groups. These are the characters that correlate with each other at reasonable (*R* < 0.70; PCOL-PIND, SWID-HPOS, HSHP-HPOS, PSOR-SWID), medium (0.70 < *R* < 0.80; SLEN-SCOL, PIND-PSHP, SLEN-HSHP, PSOR-SLEN, PCOL-PEKL), and very strong (*R* > 0.80; PSOR-HSHP, SWID-SLEN, PEKL-PPOL, PCOL-PPOL) values (code explanations can be found in Table 2).

Except for pollen colpus length (PCOL), pollen index (PIND), pollen ornaments (PSOR), and seed width (SWID), the other seed characters have shown strong correlations with each other. However, the pollen index (PIND) and pollen shape (PSHP) characters also showed negative correlation with each other (−0.77). On the other hand, pollen ornaments (PSOR) and seed hilum shape (HSHP) characters, containing a 0.92 correlation coefficient, seed width (SWID) and seed length (SLEN) characters with a 0.93 correlation coefficient, are included in the group of strongly correlated characters. Pollen equatorial length (PEKL) with pollen polar length (PPOL), pollen colpus length (PCOL) and pollen polar length (PPOL) characters, show correlation coefficients between 0.94 and 0.96, respectively.

### 3.4. Cluster analysis

The characters are coded according to numerical taxonomic methods. Thus, a total of 16 qualitative and quantitative characters (Table 2) of 13 taxa (OTUs) were collected from Bolu Province and a 13 × 16 taxonomic matrix was formed. When the dendrogram was examined (Figure 5), two groups were obtained at the 0.400 similarity level.

<table>
<thead>
<tr>
<th>Sect.</th>
<th>Taxon</th>
<th>Seed length (mm)</th>
<th>Seed width (mm)</th>
<th>Seed index (mm/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L_</td>
<td><em>T. nigrescens</em></td>
<td>1.18 ± 0.07* (1.05–1.25)**</td>
<td>0.96 ± 0.11 (0.75–1.10)</td>
<td>1.29 ± 0.13 (1.09–1.60)</td>
</tr>
<tr>
<td>V_</td>
<td><em>T. physodes</em></td>
<td>1.53 ± 0.12 (1.35–1.80)</td>
<td>1.26 ± 0.09 (1.00–1.35)</td>
<td>1.22 ± 0.09 (1.04–1.40)</td>
</tr>
<tr>
<td></td>
<td><em>T. resupinatum</em></td>
<td>1.01 ± 0.06 (0.90–1.10)</td>
<td>0.76 ± 0.08 (0.60–0.90)</td>
<td>1.35 ± 0.17 (1.17–1.83)</td>
</tr>
<tr>
<td>C_</td>
<td><em>T. campestre</em></td>
<td>1.08 ± 0.08 (1.00–1.25)</td>
<td>0.61 ± 0.06 (0.55–0.75)</td>
<td>1.79 ± 0.12 (1.54–1.94)</td>
</tr>
<tr>
<td></td>
<td><em>T. micranthum</em></td>
<td>1.05 ± 0.11 (0.90–1.25)</td>
<td>0.82 ± 0.10 (0.60–0.95)</td>
<td>1.29 ± 0.12 (1.11–1.54)</td>
</tr>
<tr>
<td>T_</td>
<td><em>T. hirtum</em></td>
<td>2.04 ± 0.11 (1.75–2.20)</td>
<td>1.64 ± 0.14 (1.35–1.75)</td>
<td>1.25 ± 0.12 (1.11–1.59)</td>
</tr>
<tr>
<td></td>
<td><em>T. ochroleucum</em></td>
<td>1.70 ± 0.09 (1.55–1.80)</td>
<td>1.18 ± 0.09 (1.00–1.35)</td>
<td>1.44 ± 0.10 (1.30–1.65)</td>
</tr>
<tr>
<td></td>
<td><em>T. striatum</em></td>
<td>1.61 ± 0.10 (1.50–1.75)</td>
<td>1.25 ± 0.12 (1.10–1.45)</td>
<td>1.29 ± 0.09 (1.15–1.45)</td>
</tr>
<tr>
<td></td>
<td><em>T. medium var. medium</em></td>
<td>1.38 ± 0.16 (1.15–1.60)</td>
<td>1.04 ± 0.09 (0.90–1.25)</td>
<td>1.34 ± 0.12 (1.19–1.53)</td>
</tr>
<tr>
<td></td>
<td><em>T. pratense</em></td>
<td>1.83 ± 0.20 (1.50–2.10)</td>
<td>1.34 ± 0.16 (1.00–1.55)</td>
<td>1.37 ± 0.09 (1.23–1.54)</td>
</tr>
<tr>
<td></td>
<td><em>T. bocconei</em></td>
<td>1.45 ± 0.08 (1.25–1.55)</td>
<td>1.25 ± 0.07 (1.25–1.55)</td>
<td>1.16 ±0.06 (1.04–1.33)</td>
</tr>
<tr>
<td></td>
<td><em>T. elongatum</em></td>
<td>1.84 ± 0.10 (1.65–2.00)</td>
<td>1.21 ± 0.11 (0.95–1.35)</td>
<td>1.53 ± 0.11 (1.35–1.95)</td>
</tr>
<tr>
<td></td>
<td><em>T. echinatum</em></td>
<td>1.73 ± 0.11 (1.50–1.90)</td>
<td>1.33 ± 0.12 (1.00–1.50)</td>
<td>1.30 ± 0.10 (1.17–1.50)</td>
</tr>
</tbody>
</table>
The first group is composed of the taxa belonging to the section *Trifolium* (T_) and the second group consists of taxa from the sections of *Lotoidea* (L_), *Vesicaria* (V_), and *Chronosemium* (C_).

When describing the dendrogram results, the taxon names were replaced by their abbreviations (Table 1). A phenon line drawn at the similarity level of 0.525 divides the dendrogram into the five groups. Group I is formed by T_ECH, T_MEDm, T_OCH, T_ELO, and T_PRA taxa belonging to the section *Trifolium*. Group II is composed of T_STR and T_BOC taxa, belonging to the section *Trifolium* as well. The T_HIR taxon belonging to the section *Trifolium* constitutes group III (Figure 5). The C_CAM and C_MIC taxa belonging to the section *Chronosemium* and the L_NIG taxon of the section *Lotoidea* together form group IV, while the two taxa of sect. *Vesicaria*, V_RES and V_PHY form group V (Figure 5). When the dendrogram was examined, the three groups (I, II, and III) of the section *Trifolium* converged to form a single unique *Trifolium* group, while the rest of the sections *Chronosemium*, *Lotoidea*, and *Vesicaria* formed the complex groups (IV and V). In the resulting

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**Figure 3.** A: *T. campestre*; B: *T. micranthum*; C: *T. nigrescens*; D: *T. striatum*; E: *T. resupinatum*; F: *T. bocconei*. A₁–F₁: general SEM view of seeds. A₂–F₂: seed surface sculpture in SEM.
3.5. Principal coordinate analysis (PCoA) results

The PCoA program used in this study calculates the eigenvalues and eigenvectors matrix. Similarity values are amplified to the power of the standard value c (“Transformation base”) before the eigenvector analysis. If this value is taken higher, the effect of the horseshoe on the graph may be reduced (Podani and Miklos, 2002). Standard c value was taken as 2 in the analysis. A mixed similarity matrix was used, and the distribution of taxa was plotted according to the UPGMA technique. The sum of the eigenvalues and percentages of the first three axes is 0.89% and 68.77%, respectively. In the study, only the PCoA graph generated according to 1st and 2nd coordinates was depicted (Figure 6).

In the graph, although C_CAM and C_MIC taxa belonging to the section Chronosemium together with the L_NIG taxon of the section Lotoidea formed the second subgroups, in terms of compliance with the classification of Zohary and Heller (1984), L_NIG taxon alone is acceptable to form the first group, while the other subgroup (section Chronosemium taxa) is accepted as group II. In the lower right corner of the graph, V_PHY and V_RES taxa belonging to the section Vesicaria constitute group III among themselves. The last group, T_ECH, T_STR, T_OCH, and T_MEDm taxa with T_HIT and T_PRA taxa belonging to the section Trifolium in group IV are found to be closer to each other and constitute the 1st and 2nd subgroups, respectively. On the other hand, T_ELO and T_BOC taxa were closer to each other than other subgroups. As a result, the subgroups that formed group IV included only taxa belonging to the section Trifolium (Figure 6).

4. Discussion

Pollen grains of Trifolium (Fabaceae) are tricolporate type, as shown by many different authors (Gillet, 1970; Zohary and Heller, 1984; Lashin, 2006; Koçyiğit et al., 2013; etc.). Tricolporate pollen grains showed two pollen shapes, prolate and subprolate, according to equatorial appearance, same as the other studies (Zohary and Heller, 1984; Lashin, 2006; Koçyiğit et al., 2013). The prolate shape pollen grains were generally observed in the members of all four sections (a total of nine taxa) and the subprolated ones in
two sections (a total of 4 taxa). Therefore, it is a character with little effect on a meaningful grouping. Koçyiğit et al. (2013) grouped the pollen polar axis length of 10–25 µm as small and 25–50 µm as the middle group. However, in this study, <30.00 µm, 30.00–50.00 µm, and >50.00 µm can be divided into three groups as small, medium, and large, respectively (Table 3). According to the equatorial length of pollen, they can be grouped as follows: narrow (<20 µm), medium (20–30 µm), and wide (>30 µm). Only medium-sized pollen grains were observed in six taxa belonging to the section *Trifolium*. The other three groups were formed by the taxa of the remaining sections. According to pollen study, the studied *Trifolium* taxa presented similar results in Koçyiğit et al. (2013). The size and shape of the pollen grains were found in overlapping ranges (Table 3, Figures 1 and 2).

When the seed sizes are grouped it can be simply divided into three-character states such as small (<1.50 mm), medium (1.50–1.80 mm), and large (>1.80 mm). Seed width character can also be divided into three character states: <1.10 mm, narrow; 1.10–1.30 mm, medium; >1.30 mm, wide (Table 4). Despite these codings of seed characters, it did not create a clear-cut grouping based on the section classification. Although the hilum character created some groupings, it did not conform to the section classification of Zohary and Heller (1984). Seed shape contained four types. These are round, ovoid, elongated ovoid, and heart-shaped. Four character states, such as light brown, brown, creamy brown, and yellowish brown were observed in seed color character. Similarly, the ratio of seed radicula and cotyledon protrusions to each other and the positions of the seed hilum were not well matched to the sectional classification of Zohary and Heller (1984). The hilum shape character includes broad elliptic and elliptic character states. In the section *Trifolium*, all taxa, except for *T. bocconei* (T_BOC), include elliptical character state, and the large elliptical character state is contained by all the taxa of the remaining different sections, except *T. physodes* from the section *Vesicaria* (V_PHY). This character can be considered to form good groups to some degree. The seed hilum color character, which contains creamy brown and brown character states did not create any reasonable groups. The seed surface structures, which were defined according to the types defined by Zoric et al. (2010), did not give any conformity with the classification of Zohary and Heller (1984). It should be kept in mind that the seed coat structures defined in several studies (Zohary and Heller, 1984; Zoric et al., 2010; Salimpour et al., 2007, etc.) can be used in seed diagnostics in agriculture. According to seed study, the

Figure 6. Principle coordinate analysis graphic was drawn by Gower similarity coefficient. In this analysis, “Transformation exponent” (c) is accepted as 2 for the similarity values.
The smallest seed was found among the section *Chronosemium* taxa (*T. micranthum* and *T. campestre*). The largest seed was presented by the section *Trifolium* taxa (Table 4; Figures 3 and 4). These findings support the result of Zoric et al. (2010).

The CA and PCoA taxa grouping results of both studies are similar (Figures 5 and 6). Because the similarities between the subgroups of the large *Trifolium* group and the three small sections were relatively low, all groups obtained from the statistical analyses formed clearly and are distinct from each other. All these groups of the study supported the section classification of Zohary and Heller (1984). Results from the groupings of taxa used in this study, except for the traditional classification (sectional classification of Zohary et al., 1984), did not support the phylogenetic classification of Ellison et al. (2006) or Watson et al. (2000). Although Ellison et al. (2006) interpreted that the subg. *Trifolium* was a monophyletic group, there is currently a high incongruency between the traditional (Zohary and Heller, 1984) and the phylogenetic classifications (Ellison et al., 2006). On the other hand, Watson et al. (2000) indicated that only the section *Lotoidae* in the genus *Trifolium* was monophyletic. Their result was not supported by the phylogenetic study of Ellis et al. (2010) either.

It was shown that all characters were affected by variations within the seed and pollen morphology. On the other hand, when the characters that have high correlation coefficients are taken into consideration, it is seen that five of them are among the pollen characters. However, higher correlation values are seen among the seven seed characters. According to these results, it can be said that seed macro- and microcharacters play a more important role in the classification of the taxa.

This study’s results revealed that using only pollen or only seed characters was not useful for creating meaningful infrageneric classifications. Similarly, the groupings of Zoric et al. (2010) according to the seed mantle structures only, did not show any similarities with the classification of Zohary and Heller (1984). Unlike morphological or anatomical studies of species which were made previously, pollen and seeds were used together in this study. At the same time, the results of the study were evaluated taxonomically using statistical analyses. Thus, not only one or a few taxa of the studied species-level comparisons, but comparisons on the infrageneric and interspecific level had also been made.

Genetic characterization of some *Trifolium* species with DNA content and ITS rDNA study by Vizintin et al. (2006) did not support the traditional sectional (Zohary and Heller, 1984) groupings of *Trifolium*. In addition, they also found quite a high (two-fold) variation within the section *Trifolium*. Similar results were found in our study, such as three subgroups of the section *Trifolium* (Figure 5) as being in the study of Vizintin et al. (2006).

Both pollen and seed characteristics were used together in the study, and the results were consistent with the conventional grouping of Zohary and Heller (1984). In the future, it is possible to use as many other taxonomic characters (morphological, anatomical, chemical, DNA, etc.) as possible in similar studies, so that the classifications obtained may conform to conventional and/or phylogenetic classifications. Beside taxonomical contributions, pollen features and seed properties of the 13 studied *Trifolium* taxa from Bolu were also investigated.

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**References**


