Nutlet micromorphology of the genus Marrubium L. and allies and its systematic implication (Lamiaceae: tribe Marrubieae)

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Abstract. Nutlets of 20 taxa of the tribe Marrubiaceae were examined by scanning electron microscopy (SEM) and detailed descriptions of nutlet micromorphological features for all examined taxa are provided. The nutlets exhibited variation in size, shape, color and surface sculpturing. The nutlets shape of most species studied is ovate, but rounded, broad ovate, elliptic, lanceolate, triangular and oblong can also be found in a few species. Six basic types of the sculpturing pattern of nutlet surface can be distinguished: reticulate, foveolate, scalariform, ruminate, pustulate and colliculate-granulate. The most common type of nutlet sculpturing among the studied species is reticulate, but the variation in size and shape of their composing cells provided useful diagnostic characters. Our investigation revealed that the type of sculpturing was more useful in taxon delimitation among Marrubium species and allies at the species rank.

Keywords. diagnostic characters, Labiatae, nutlet sculpturing, scanning electron microscopy, taxonomy

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INTRODUCTION

Marrubieae Vis., with five genera and ca. 91 species, is one of the 10 Lamioideae tribes distributed mainly in southern Europe and North Africa (Siadati et al., 2018). They are mostly nonaromatic herbs or subshrubs with campanulate to rotate calyx and often with secondary calyx lobes, zygomorphic and 2-lipped corolla and stamens included or shortly exerted from the corolla (Siadati et al., 2018; Harley et al., 2004).

According to one of the most recent phylogenetic studies, the monophyletic Marrubieae contains four major clades: the first one including member of Acanthoprasium (Benth.) Spach and Moluccella L., the second one comprising species of Ballota L., the third clade containing Pseudodictamnus Fabr. and related species and the fourth one including Marrubium L. species (Siadati et al., 2018). Acanthoprasium, with two species, is a European genus with a woody habit, spiny bracteoles and upper lip of corolla totally hairy. According to Bendiksby et al. (2011), the annual or short-lived perennials Moluccella, contains eight species in southwestern Asia and the Mediterranean regions, are characterized by a zygomorphic calyx more or less expanded at the mouth, internally glabrous, and usually lobed with both primary and secondary indentations. The genus Ballota are subshrubs to perennial herbs with herbaceous bracteoles and shortly exerted stamens from the corolla and extend from Macaronesia, Europe to Mediterranean and Western Asia (Siadati et al., 2018). According to Bentham (1832-1836), the genus Ballota had traditionally been divided into three sections based on two main morphological characters, woody against herbaceous habit and the type of bracteoles (spinose against herbaceous): (1) sect. Acanthoprasium Benth., which is raised to generic rank by Bendiksby et al. (2011), (2) sect. Beringeria (Neck.) Benth., which is recently raised to generic rank by Siadati et al. (2018) and (3) sect. Ballota Benth. (Bentham 1832-1836). Based on recent phylogenetic studies, the genus Ballota re-circumscribed to encompass the members of sect. Ballota with about three species. Moreover, members of sect. Beringeria recently raised to generic rank as Pseudodictamnus, which accommodates perennial herbs with herbaceous bracteoles, expanded calyx limb with 6-20 teeth and widely distributed from Eurasia to North Africa (Siadati et al., 2018). The genus Marrubium with about 50 species have usually toothed and petiolate leaves, 5-15 (~30) lobed calyx covered by dense stellate trichomes, corolla tube shorter than calyx and stamens included in the corolla tube (Harley et al., 2004).

Studies on nutlet micromorphology within Lamiaceae showed that nutlets features e.g., shape, morphology of the abscission scar and surface sculpturing, were potentially useful at different taxonomic levels (e.g. Demissew & Harley, 1992; Marin et al., 1994; Ryding, 1995; Oran, 1996; Ryding, 1998; Salmaki et al., 2008; Moon et al., 2009; Satil et al., 2012; Krawczyk & Glowacka, 2015; Eyvaz?zadeh Khosroshahi & Salmaki, 2018). Among different nutlet characters, type of sculpturing has been considered to be taxonomically most important (Oran, 1996; Kahraman et al., 2011), however, color, size and shape of nutlets were considered unimportant, either because they did not vary or the variation was random or too great (Oran, 1996). Abscission scars were invariable, but the variation at higher levels may be significant (Guerin, 2005). There are no reports on the taxonomic significance of the nutlets micromorphology among different genera of Marrubieae, except that by Mosquero et al. (2007), who provided a description of the morphology and anatomy structure in Marrubium vulgare L. Akgül et al. (2008) illustrated the range of variability in seed characters in Marrubium species found in Turkey and Hassan & Al-Thobaity (2015) provided a detailed description of the morphological nutlet characteristics of Marrubium vulgare in Saudi Arabia. Thus, the main goal of this study was to provide a detailed description of nutlet micromorphology of the genus Marrubium and allies.

MATERIALS AND METHODS

Nutlets of 20 species representing all five genera of tribe Marrubieae were selected to investigate the value of seed characters in the classification of genera and species. Nutlets were collected from herbarium specimens, deposited in the herbaria M (Botanische Staatssammmlung München), MSB (Münich Systematic Botany), and TUH (Central Herbarium of Tehran University). A list of voucher specimens is presented in Table 1. A total of two species of Acanthoprasium, two species of Moluccella, four taxa representing three species of Ballota, two species of Pseudodictamnus and 10 species of Marrubium were analyzed. Nutlets were observed in advance, using a stereomicroscope to ensure that they were of normal size and maturity. Nutlets observations were made using scanning electron microscopy.

For SEM observations, dried nutlets were mounted on aluminum stubs using double-sided adhesive and sputter-coated with a thin layer (ca. 30 nm) of gold and examined by means of a Hitachi SU3500 (Japan) scanning electron microscopy at an accelerating voltage of 5-30 kV. This paper follows the terminology of Bojnanský & Fargašová (2007) and Stearn (1983) for the seed shape and surface ornamentation.
Table 1. Taxa, voucher specimens and collection data of selected species of Marrubium and its allied genera deposited in the herbaria M (Botanische Staatssammlung München), MSB (Münich Systematic Botany), and TUH (Central Herbarium of Tehran University).

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthoprasium frutescens</td>
<td>France: Schlucht von Aiglum, 30 km westlich und nördlich Nice, O. Angerer s.n. (M)</td>
</tr>
<tr>
<td>(L.) Spenn.</td>
<td>Cyprus: – 1880 (M)</td>
</tr>
<tr>
<td>Acanthoprasium integrifolium</td>
<td>Turkey: Iggid, near Yazd, Yazili, P. Rasmont 55330 (M)</td>
</tr>
<tr>
<td>(Benth.) Ryding</td>
<td>Yugoslavia: Makedonija, 13 km ONO von Skocivir an der Straße zum Kajmakchalan (Grenzgebirge), D. Podlech &amp; Lippert 26027 (M)</td>
</tr>
<tr>
<td>Ballota nigra subsp. anatolica</td>
<td>Iran: Mazandaran, Zentral- Elburs, Im Einzugsgebiet des oberen Tedschen-Flusses, unterhalb Kom-rud bala, locchere Gebuschvegetation, Felsschutt; 60 km ostlich von Firuzkuh, E. Behboudi &amp; P. Aellen s.n. (M)</td>
</tr>
<tr>
<td>P. H. Davis, Cult.</td>
<td>Spain: Valencia Segundo, inruinis, 508 (M)</td>
</tr>
<tr>
<td>Ballota nigra subsp. ruderalis</td>
<td>Iran: Chahar-Mahal-e Bakhtiari, Kuh-e Rig from Dehno, Zarre 17941 (TUH)</td>
</tr>
<tr>
<td>(SW.) Briq.</td>
<td>Marokko: d’ Oujda, 5 km W El Aioun; Felder, Eucalyptushainen N der Straße nach Taza (P 1), W. Lippert 21821 (M)</td>
</tr>
<tr>
<td>Ballota saxatilis Sieber ex C.Presl</td>
<td>Caucasus: Krasnoselskoie, montes Arenguni, in vicinitate pagi Tokludza, V. Vašák 257082 (M)</td>
</tr>
<tr>
<td>Pseudodictamnus hispanicus J.</td>
<td>Caucasica: M. Senser 257078 (M)</td>
</tr>
<tr>
<td>(L.) Salmaki &amp; Siadati</td>
<td>Iran: Kurdistan, In graminosis siccis jugi prope Salavatabad 25 km E Sanandaj, K.H. Rechinger 42784 (M)</td>
</tr>
<tr>
<td>Pseudodictamnus aucheri (Boiss.</td>
<td>Caucasus: Kislovodsk, in abruptis, I. Akinfiev 7382 (MSB)</td>
</tr>
<tr>
<td>Siadati</td>
<td>Marokko: d’Er-Rachidia, Hoher Atlas, Tizi n’Talent an der Straße von Midelt nach Strassenrand, D. Podlech 47337 (MSB)</td>
</tr>
<tr>
<td>Marrubium alyssoides Pomel,</td>
<td>Austria: Nordburgenland, Pardnoder Platte, trockene, grasige Stellen am E-Fuß des Heidli bei Nickelsdorf, ca. 0.45 km SSE des Bahnübergangs am NE-Fuß des Heidli (Kote 133), T. Barta 2004-351 (M)</td>
</tr>
<tr>
<td>Marrubium anisodon K. Koch</td>
<td>Iran: Ararat, Ararat valley 12 km NNW of Ararat town, 7 km NW of Vedi, Ehrange, Fauvus &amp; al. XI/1040 (M)</td>
</tr>
<tr>
<td>Marrubium astracanicum Jacq.</td>
<td>Caucasus: Krasnoselskoie, montes Arenguni, in vicinitate pagi Tokludza, V. Vašák s.n. (M)</td>
</tr>
<tr>
<td>Marrubium catarifolium Desr.</td>
<td>Ion: Baluchistan, 20 km a Zahedan meridiem versus, K.H. Rechinger 14562 (M)</td>
</tr>
<tr>
<td>Marrubium cuneatum Banks &amp; Sol.</td>
<td>Marrubium litardieri Marrey</td>
</tr>
<tr>
<td>Marrubium leonuroides Desr.</td>
<td>Punstam et Pania, ca 500 m. 11230 (M)</td>
</tr>
<tr>
<td>Marrubium peregrinum L.</td>
<td>Iran: Erbil (Kurdistan). In agris demiss inter Mirza Punstam et Pania, ca 500 m. 11230 (M)</td>
</tr>
<tr>
<td>Marrubium persicum C.A. Mey.</td>
<td>Armenia: Ararat, Ararat valley 12 km NNW of Ararat town, 7 km NW of Vedi, Ehrange, Fauvus &amp; al. XI/1040 (M)</td>
</tr>
<tr>
<td>Marrubium propinquum Fisch. &amp; C.A. Mey.</td>
<td>Caucasus: Krasnoselskoie, montes Arenguni, in vicinitate pagi Tokludza, V. Vašák s.n. (M)</td>
</tr>
<tr>
<td>Moluccella aucheri (Boiss.) Scheen,</td>
<td>Iran: Baluchistan, 20 km a Zahedan meridiem versus, K.H. Rechinger 14562 (M)</td>
</tr>
<tr>
<td>Moluccella laevis (L.)</td>
<td>Morocco: d’Oujda, 5 km W El Aioun; Felder, Eucalyptushainen N der Straße nach Taza (P 1), W. Lippert 21821 (M)</td>
</tr>
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</table>

RESULTS AND DISCUSSION

Several main features of the investigated nutlets i.e. shape, length, width, as well as sculpturing patterns, projections of outer periclinal walls and anticlinal walls are summarized in Table 2. Selected SEM micrographs of nutlets studied were presented in Figures 1-3. In general, the color of nutlets in all studied species was dark brown to black. The nutlets were oblong (e.g., *M. catarifolium* Desr., Fig. 2G), triangular (e.g., *Mo. laevis* L., Fig. 3H), broad ovate (e.g., *B. nigra* subsp. *ruderis* (Sw.) Briq., Fig. 1F) to rounded (e.g., *P. aucheri* (Boiss.) Salmaki & Siadati, Fig. 1L) and elliptic (e.g., *A. frutescens* (L.) Spenn) to lanceolate (e.g., *B. nigra* subsp. *anatolica* P.H. Davis, Fig. 1D) in shape, but the most common type was ovate (e.g., *B. platyloma* Rech. f., Fig. 1H; *M. cuneatum* Banks & Sol., Fig. 2I and *M. persicum* C.A.Mey., Fig. 3B). The size of nutlet ranged from 1.34×1.08 mm (in *M. leonuroides* Desr.) to 4.04×1.45 mm (in *Mo. aucheri* (Boiss.) Scheen). Nutlets of investigated taxa exhibited six types of surface sculpturing patterns. The most common type
of sculpturing pattern was reticulate (e.g., A. frutescens, Fig. 1A), however, other types of sculpturing patterns, like scariosum (A. integrifolium (Benth.) Ryding, Fig. 1C), foveolate (M. persicum, Fig. 3C), ruminate (M. alyssonides Pomel, Fig. 2B), pusticulate (M. astracanicum Jacq., Fig. 2F) and colliculate-granulate (Mo. laevis, Fig. 3I), were observed.

Our study represents the first investigation on nutlet micromorphology on the genus Marrubium and its allied genera. Variation in shape, size, presence or absence of trichomes at the apex of nutlets and particularly surface sculpturing appeared to have taxonomic value in some groups of Lamiaceae (Husain et al., 1990; Oran, 1996; Navarro & El-Qualidi, 2000; Moon & Hong, 2006; Moon et al., 2009; Kahraman et al., 2011). Özkan et al. (2009) found that variation of shape, size, surface sculpturing and color were useful in distinguishing groups, species and subspecies among 12 examined species of Salvia. In Stachys (Salmaki et al., 2008) nutlet micromorphology provided valuable data in separating the related species within sections, although these characters were not useful in

separating large natural groups. Variations in size and the type of sculpturing as well as the shape of its composing cells had been considered to provide the most valuable characters at species level (e.g., Kahraman et al., 2011; Tarimcilar et al., 2013; Salmaki et al., 2008; Eyvazadeh Khosroshahi & Salmaki, 2018). For instance, the polygonal cells in M. peregrinus L. (Fig. 3A) were much smaller than those in M. leonuroides (Fig. 2L). Moreover, M. anisodon K.Koch (Fig. 2D) and M. litardierei (Fig. 2N) were different in the depth of pits on surface. Due to the particular value of microsculpturing, a comprehensive discussion is given below, indicating the importance of sculpturing patterns and its
systematic importance in the genus *Marrubium* and its allied genera.

Fig. 2. Scanning electron micrographs of *Marrubium* and its allied genera. A, B. *Marrubium alyssoides*; C, D. *M. anisodon*; E, F. *M. astracanicum*; G, H. *M. catariifolium*; I, J. *M. cuneatum*; K, L. *M. leonuroides*; M, N. *M. litardierei*, O. *M. peregrinum*.

**Acanthoprasium**—This genus includes two species based on the latest phylogenetic study (Bendiksby et al., 2011), characterized by simple hairs and broadly campanulate calyx with spiny lobes. Both species of *Acanthoprasium* are well distinguished by having bearded nutlets. The present study showed that the presence of trichomes at the apex of nutlets could be used as a taxonomic marker in the delimitation of *Acanthoprasium* nutlets from the rest of Marrubieae. However, these two species were different in shape and sculpturing pattern. While, *A. frutescens* is characterized by elliptic nutlets as well as reticulate pattern. While, *A. integrifolium* is distinguished by triangular nutlets and scalariform sculpturing (Fig. 1B–C).

**Moluccella**—The genus *Moluccella*, characterized by glabrescent stems and leaves as well as an expanded calyx, is distributed from southern Europe to central Asia, Pakistan and Kashmir (Scheen et al., 2010; Bendiksby et al., 2011). In the present study, two out of eight species of this genus were examined. Although both of these species were similar in shape and possession of the largest nutlets (Fig. 3F–H), they were different in the pattern of nutlet sculpturing. *Moluccella aucheri*, distinguished by reticulate pattern of nutlet sculpturing, differs from *Mo. laevis* with colliculate-granulate pattern of nutlet sculpturing. In addition, the type of anticlinal walls was different between these two species. While *Mo. aucheri* possesses raised anticlinal walls, *Mo. laevis* is characterized by anticlinal walls represented by channels. Due to the limited taxon sampling on the genus *Moluccella*, a more comprehensive study needs to be done for indicating the taxonomic importance of nutlet characters.

**Ballota**—Recently, a narrower circumscription of the genus *Ballota* was proposed by Siadati et al. (2018) to encompass the members of sect. *Ballota* (sensu Patzak, 1958) with about three species. These three species are characterized by the following morphological features: five main calyx teeth, rarely with a few additional minute teeth as well as simple and glandular indumentum (Siadati et al., 2018). Although nutlet morphology was not useful in the recognition of the genus *Ballota* from the other
genera of the tribe Marrubiaceae, it was helpful in the delimitation of taxa at species level. Ballota nigra subsp. anatolica with lanceolate nutlets was characterized by foveolate pattern of nutlet sculpturing (Fig. 1D–E). On the other hand, B. nigra subsp. ruderalis (Fig. 1F–G) and B. platyloma (Fig. 1H–I) have broadly ovate and ovate nutlets, respectively, and possess reticulate pattern of nutlet sculpturing. 

Pseudodictamnus— The genus Pseudodictamnus, with about 28 species, comprises perennial herbs with herbaceous bracteoles, expanded calyx limb with 6-20 teeth and corolla tube shorter than or equaling the calyx with branched and simple hairs (Siadati et al., 2018). This genus, which is phylogenetically known to be the closest relative of Ballota and Marrubium, shares similar nutlet features with them. For instance, the pattern of nutlet sculpturing among all three species of the genus Pseudodictamnus was reticulate, which is a common type among the species of Ballota and Marrubium. Moreover, these three species were similar in the following characters: reticulate pattern of nutlet sculpturing with concave projection in outer periclinal wall and raised anticlinal walls. However, they were different in the shape of nutlets (Fig. 1 J, L, N).

Marrubium— Marrubium, with ca. 50 species, is morphologically well characterized by the following features: usually toothed and petiolate leaves, thyrsoid inflorescence; calyx 5-15(-30)-lobed, corolla tube shorter than calyx; stamens included in corolla-tube, posterior corolla lip 2-lobed (Harley et al., 2004). In addition, several characters, such as rounded or subtruncate nutlets at apex and glabrous or with sessile glands at apex, have been mentioned in previous studies (Harley et al., 2004).

It is not surprising that Marrubium, the largest genus of the tribe Marrubiaceae, shows considerable diversity in nutlet characters such as size, shape, and sculpturing pattern. Marrubium leonuroides and M. catarifolium possessed the smallest and biggest nutlets among the examined species, respectively. The common nutlet shapes among the Marrubium species were ovate and oblong. The common sculpturing pattern of nutlet in Marrubium species was reticulate, however, they showed some variation in shape and size of the composing cells.

Based on morphological characters the genus Marrubium has been divided into six sections by Seybold (1978). Marrubium sect. Microdonta, which was represented here by M. astracanicum, M. catarifolium, M. leonuroides and M. propinquum, morphologically characterized by five straight or curved calyx teeth and purple corolla. Although all the species attributed to this section were different in shape, they showed similar type of sculpturing, except in M. astracanicum, which possessed pusticulate sculpturing. While Marrubium alyssoides and M. persicum, belonging to Marrubium sect. Stellata, were similar in shape of nutlets but differed in the type of sculpturing, both studied species of Marrubium sect. Marrubium (M. anisodon and M. cuneatum) differed in the shape of nutlets but possessed a similar reticulate type of sculpturing. Marrubium peregrinum, belonging to sect. Ramosa, possessed the smallest oblong nutlets (1.65×0.96 mm) among the studied species and simple hairs at apex.

This section is morphologically well characterized by widely branched stems and few-flowered verticillasters with white corolla (Davis, 1982). Nutlet sculpturing feature was helpful in the separation of species, however, it appeared uninformative at the level of section.

CONCLUSION

Nutlet micromorphology provided valuable data in the separation of the related species within genera of the tribe. Our findings revealed that the nutlet shape, sculpturing pattern and the absence/presence of simple hairs at apex of nutlet were the most significant features in the separation of taxa at species level. However, these characters were not useful in the separation of sections within genera. Our results, in accordance with previous studies of Lamiaceae (e.g., Oran, 1996), showed that the shape of the nutlets were insignificant in assessing the relationships among Marrubium species and its relatives in the tribe Marrubiaceae. It seemed also that, contrary to other genera of Lamiaceae (e.g., Guerin, 2005), nutlet characters were of low phylogenetic value in Marrubiaceae.

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