A taxonomic study of the genus Hellenocarum H. Wolff (Umbelliferae-Apioideae) based on morphology, fruit anatomy, and molecular data

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Abstract: Phylogenetic relationships among the species of Hellenocarum and its close allies (Umbelliferae-Apioideae) were investigated using nuclear (ITS, ETS) and plastid (psbA-trnH intergenic spacer) DNA sequences. The results obtained were supplemented with an examination of morphology from herbarium and field-collected materials, as well as details of fruit anatomy obtained from light and scanning electron microscopy. According to the molecular data, Hellenocarum is not monophyletic. In most molecular analyses, the genus comprises 2 disparate lineages, with each lineage supported by distinct morphological characters (e.g., structure of underground organs, shape of the umbel, petal color). The first line includes Hellenocarum multiflorum (the type species) and H. strictum and corresponds to Hellenocarum sensu stricto. The second lineage includes Hellenocarum amplifolium and H. pisidicum and is recognized herein as the new genus Neomuretia Kljuykov, Degtjareva & Zakharova. Carum depressum is united with Hellenocarum sensu stricto. Three new nomenclatural combinations are proposed: Hellenocarum depressum (Hartvig & Kit Tan) Kljuykov & Zakharova; Neomuretia amplifolia (Boiss. & Hausskn.) Kljuykov, Degtjareva & Zakharova; and Neomuretia pisidica (Kit Tan) Kljuykov, Degtjareva & Zakharova.

Key words: Umbelliferae, Hellenocarum, Neomuretia, ITS, ETS, psbA-trnH, molecular phylogeny, morphology, fruit anatomy, taxonomy

1. Introduction
Hellenocarum H. Wolff (Umbelliferae) is a small genus of geophytic herbs that currently includes the following 4 species distributed in the eastern part of southern Europe, western Turkey, western Iran, and northeastern Iraq: H. amplifolium (Boiss. & Hausskn.) Kljuykov, H. multiflorum (Sm.) H.Wolff, H. pisidicum Kit Tan, and H. strictum (Griseb.) Kljuykov. The relationships among its members, as well as the precise circumscription of the genus and its phylogenetic placement relative to putatively allied genera Bunium L. and Carum L. (especially, C. depressum Hartvig & Kit Tan), have been heretofore unclear.

Hellenocarum was established by Wolff (1927) based on 2 species transferred from Carum [C. multiflorum (Sm.) Boiss. and C. lumpeanum Dörfl. & Hayek]. In subsequent floristic treatments, however, these taxa continued to be treated as species of Carum (Rechinger, 1943; Tutin, 1968; Hedge and Lamond, 1972; Osorio-Tafall and Seraphim, 1973; Meikle, 1977). Carum lumpeanum was placed into synonymy under Bunium strictum Griseb. and later transferred into Carum as a subspecies of C. multiflorum (Tutin, 1967). Engstrand (1973) argued that Hellenocarum is well differentiated from Bunium and Carum and should be maintained as a distinct genus. Kljuykov (1985) also considered Hellenocarum to be a separate genus, and expanded it to include one species from Muretia (M. amplifolia Boiss. & Hausskn.); he also raised Carum multiflorum subsp. strictum to the species rank within Hellenocarum. In contrast, Hartvig (1986) submerged Hellenocarum into Carum, and by so doing increased substantially the morphological heterogeneity of the latter. Tan and Sorger (1986) described a new endemic species from western Turkey as Hellenocarum pisidicum Kit Tan, but also reported that Hellenocarum is only weakly delimited from Carum and might be better recognized at the subgeneric rank. The changing generic concept of Hellenocarum has been influenced by the taxonomic value assigned to a variety of morphological characters; thus, molecular data are required to elucidate the proper circumscription of the genus and its relationship to Bunium and Carum.

To date, molecular systematic investigations including Hellenocarum have only considered the type species, H.
multiflorum. Using sequences from the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) region, Papini et al. (2007) showed that H. multiflorum (treated in their study as Carum multiflorum) formed a strongly supported clade with Carum heldreichii Boiss. and Bunium elegans (Fenzl) Freyn in tribe Pyramidoptereae Boiss. rather than with Carum carvi L., the nomenclatural type of the genus, in tribe Careae Baill. Based on this evidence, Papini et al. (2007) confirmed “the autonomy of Hellenocarum from Carum”. They also reported that further sampling in Bunium and allied genera is necessary to ascertain if C. heldreichii is to be assigned to Hellenocarum, or if both H. multiflorum and C. heldreichii should be transferred into Bunium. Extended taxonomic sampling of Bunium and allied genera of tribe Pyramidoptereae using ITS and plastid psbA-trnH intergenic spacer sequences showed that Hellenocarum multiflorum does not ally closely with Carum heldreichii (Degtjareva et al., 2009; Zakharova et al., 2012). Instead, H. multiflorum comprises a single lineage sister group to a clade containing Bunium section Bunium, which includes the type species, Bunium bulbocastanum L. Based on these molecular systematic investigations, Hellenocarum is now generally accepted as a distinct genus of Umbelliferae (Hand, 2011).

Carum depressum Hartvig & Kit Tan was described in 2001 based on plants from Peloponnese, Greece (Tan and Iatrou, 2001). These plants possess tuberiform roots, a morphological feature more characteristic of Hellenocarum than of Carum, if Hellenocarum is accepted as a separate genus. The phylogenetic relationships among the species of Hellenocarum and Carum depressum are unclear.

Herein, we carry out a taxonomic study of the genus Hellenocarum. Our objectives are: 1) to infer phylogenetic relationships among the species of Hellenocarum and its putative allies in tribe Pyramidoptereae, using 3 molecular markers; 2) to provide detailed descriptions of the morphology and fruit anatomy of these species; and 3) to assess the taxonomic status of these species, based on the results obtained from the aforementioned analyses. In addition to sampling all 4 species currently comprising the genus Hellenocarum, we pay special attention to the putatively allied and rare species Carum depressum.

2. Materials and methods
2.1. Molecular study
We examined sequence data from the nuclear ribosomal DNA (nrDNA) internal and external transcribed spacer regions (ITS and ETS), as these markers have been shown as suitable for phylogenetic analyses of Umbelliferae at low taxonomic levels (Downie et al., 2010; Logacheva et al., 2010). The ETS region has not yet been used to resolve relationships in tribe Pyramidoptereae. In addition, we analyzed variation in the plastid psbA-trnH intergenic spacer region. Previously, it was determined that psbA-trnH sequences may not be informative enough to resolve relationships among closely related species in the Umbelliferae (Degtjareva et al., 2009, 2012). Nevertheless, this spacer does include insertions and deletions that can be used for testing hypotheses inferred by other loci, and preliminary analysis indicated that this was indeed the case for Hellenocarum, Bunium, and Carum.

ITS sequences from single accessions of Carum depressum, Hellenocarum amplifolium, H. multiflorum, H. pisidicum, and H. strictum were obtained and used to modify the alignment of Zakharova et al. (2012). This alignment included 46 ITS sequences from 19 genera, representing not only Hellenocarum, Bunium, and Carum, but also other representatives from Apiaceae tribes Pyramidoptereae and Careae that were sampled in earlier studies (Papini et al., 2007; Degtjareva et al., 2009; Downie et al., 2010; Zakharova et al., 2012). Data for the 5.8S region were unavailable for many previously published sequences; thus, they were not included in the analysis. ETS sequences were generated for 45 of these same accessions (data for Postiella capillifolia could not be obtained despite repeated attempts). PsbA-trnH sequences for 10 species were also newly generated for this study and added to a matrix containing 36 previously published sequences (Degtjareva et al., 2009). Physospermum cornubiense (L.) DC. was used to root all trees. GenBank accession numbers and voucher information for all investigated taxa are presented in the Appendix.

Total genomic DNA was isolated from fruit and leaf tissues using a NucleoSpin Plant DNA isolation kit (Macherey-Nagel, Düren, Germany) following the manufacturer’s instructions. The strategies used to obtain these ITS and ETS data, including primer locations and characteristics, have previously been described (ITS, Valiejo-Roman et al., 2002; ETS, Logacheva et al., 2010). Strategies for amplification and sequencing of the psbA-trnH spacer were the same as described previously for ITS, except that the region was amplified using primers trnH2 (Tate and Simpson, 2003) and psbAF (Sang et al., 1997). PCR products were purified using a DNA cleaning kit (Evrogen, Moscow, Russia). Direct sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and a BigDye Terminator Cycle Sequencing Ready Reaction kit. Both forward and reverse DNA strands were sequenced in their entirety for all samples.

The resulting DNA sequences were edited by eye using the software CHROMAS 1.45 (http://www.techne lysium.com.au/chromas.html). The ETS, ITS, and psbA-trnH sequences were each aligned and then manually adjusted using BioEdit (Hall, 1999). The 3 data matrices were deposited in TreeBASE (study number S15474). Phylogenetic analyses were performed on separate ITS
(i.e. ITS1 + ITS2), ETS, and psbA-trnH data sets, as well as on concatenated data sets representing both nuclear markers only (ITS + ETS) and combined nuclear and plastid sequences (ITS + ETS + psbA-trnH).

Indels in the psbA-trnH matrix were coded as binary characters according to a simple gap-coding algorithm (Simmons and Ochoterena, 2000) using the program SeqState (Müller, 2005). Indels in the nuclear (ITS, ETS) data matrices were not coded because their boundaries could not be unambiguously aligned. In the psbA-trnH region, inversions were also identified for some taxa; these inverted regions were reverse-complemented prior to analysis to avoid distortion of phylogenetic signal (Kelchner and Wendel, 1996).

For each data set, heuristic maximum parsimony (MP) searches using TBR branch swapping were conducted using PAUP* version 4.0b8 (Swofford, 2003), with character states specified as equally weighted. Five hundred random-addition replications were carried out and all shortest trees were saved. Gaps were treated as missing data. Bootstrap (BS) analysis was performed to assess the degree of support for particular branches on the tree (Felsenstein, 1985); values were calculated from 1000 replicate analyses, using TBR branch swapping and random addition sequence of taxa. One thousand most parsimonious trees from each replicate were saved. Both consistency (CI; Kluge and Farris, 1969) and retention (RI; Farris, 1989) indices were calculated.

The incongruence length difference (ILD; Farris et al., 1994) test was carried out using PAUP* to evaluate the congruence between molecular data sets. For the ILD test, 1000 homogeneity replicates of heuristic searches were performed with random taxon addition. The number of additional steps required to force particular taxa into a monophyletic group was examined using the constraint option of PAUP*. The Shimodaira–Hasegawa (SH; Shimodaira and Hasegawa, 1999) test was used to confirm if the differences between optimal tree topologies and those trees with the constraints evoked are statistically significant. The SH test was executed using resampling estimated log-likelihood (RELL) optimization and 1000 BS replicates.

Bayesian analysis was conducted using MrBayes version 3.2.1 (Ronquist et al., 2012) using the GTR + G model for all partitioned and combined data sets. This model was selected by the Akaike information criterion estimator using Modeltest version 3.7 (Posada and Crandall, 1998). All analyses were performed with 2 parallel runs, with 4 Markov chains used for each run. A total of 25,000,000 generations were performed, with trees sampled every 1000 generations. The number of generations discarded was determined by a cold chain log likelihood examination using Tracer version 1.6 (http://beast.bio.ed.ac.uk/Tracer).

2.2. Morphology and fruit anatomy
Specimens of Hellenocarum and Carum were obtained from herbaria C, E, GB, JE, LD, LE, MA, MPU, MW, OXF, and TARI and were supplemented with field-collected specimens of Hellenocarum from Greece. Hellenocarum pisidicum and H. amplifolium are known only from limited localities and are poorly represented in herbaria. We emphasized comparisons among the 4 Hellenocarum species and Carum depressum, as the latter possesses morphological features typical of Hellenocarum. The morphological characters examined were those deemed important by Kljuykov (1985), Hartvig (1986), Tan and Sorger (1986), Rechinger (1987), and Tan and Iatrou (2001) in Hellenocarum species recognition. Standard umbellifer terminology was applied (Kljuykov et al., 2004).

Fruit anatomy was examined under a light microscope, with hand sections made through the middle of the mericarps. Prior to sectioning, the fruit had been kept for 3 days in equal parts glycerin, ethyl alcohol, and water. The sections were treated with phloroglycine and hydrochloric acid and then mounted in glycerin. The number of cotyledons was determined on embryos extracted from mature fruits. Microstructure of fruit surfaces was studied using a JSM-6380LA scanning electron microscope (SEM; JEOL, Tokyo, Japan) at Moscow State University.

2.3. Distribution

3. Results
3.1. Molecular study
Sequence and tree characteristics of the partitioned and combined data sets are summarized in Table 1. The length of concatenated ITS1 and ITS2 sequences ranged from 428 to 440 bp, and that of ETS ranged from 380 bp to 401 bp. Among Hellenocarum species, the length of the ITS
Table 1. Sequence and tree characteristics of the partitioned and combined data sets used in the study.

<table>
<thead>
<tr>
<th></th>
<th>ITS (ITS1 + ITS2)</th>
<th>ETS</th>
<th>ITS + ETS</th>
<th>psbA-trnH</th>
<th>Combined (ITS + ETS + psbA-trnH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of accessions</td>
<td>46</td>
<td>45</td>
<td>46</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Length variation (bp)</td>
<td>428–440</td>
<td>380–401</td>
<td>-</td>
<td>110–357</td>
<td>-</td>
</tr>
<tr>
<td>No. of aligned positions</td>
<td>458</td>
<td>423</td>
<td>881</td>
<td>457 (incl. 32 coded gaps)</td>
<td>1338</td>
</tr>
<tr>
<td>No. of ambiguous aligned positions</td>
<td>27</td>
<td>23</td>
<td>50</td>
<td>275</td>
<td>325</td>
</tr>
<tr>
<td>No. of variable aligned positions</td>
<td>263</td>
<td>277</td>
<td>540</td>
<td>96</td>
<td>636</td>
</tr>
<tr>
<td>No. of parsimony informative aligned positions</td>
<td>176</td>
<td>198</td>
<td>374</td>
<td>64 (incl. 24 coded gaps)</td>
<td>438</td>
</tr>
<tr>
<td>No. of steps in shortest trees</td>
<td>720</td>
<td>717</td>
<td>1451</td>
<td>152</td>
<td>1636</td>
</tr>
<tr>
<td>No. of shortest trees</td>
<td>288</td>
<td>2</td>
<td>24</td>
<td>567</td>
<td>48</td>
</tr>
<tr>
<td>CI/RI of shortest trees</td>
<td>0.542/0.698</td>
<td>0.596/0.792</td>
<td>0.563/0.745</td>
<td>0.789/0.894</td>
<td>0.573/0.748</td>
</tr>
<tr>
<td>CI excluding uninformative characters</td>
<td>0.4695</td>
<td>0.5345</td>
<td>0.4964</td>
<td>0.7895</td>
<td>0.5043</td>
</tr>
</tbody>
</table>
Hellenocarum to monophyly resulted in trees 6 steps longer than those without the constraint invoked, which were rejected in the SH test (P = 0.021). Once more, neither Bunium nor Carum is resolved as monophyletic.

Trees resulting from analyses of the psbA-trnH spacer region (Figure 3) showed poor resolution in comparison with the nuclear markers due to fewer parsimony-informative characters (64 vs. 374 in the nuclear data set; Table 1), and the results showed incongruities in topology relative to the ITS and ETS trees. The Hellenocarum-I clade is allied strongly with Carum depressum (98 BS, 1.00 PP), and their monophyly is also supported by a shared 1-bp
deletion. In turn, this clade is a sister group (77 BS, 0.99 PP) to a clade consisting of 3 Carum species. Two unique insertions, of 10 bp and 20 bp, support the monophyly of the Hellenocarum-I clade plus these 4 species of Carum. Hellenocarum amplifolium and H. pisidicum, members of the Hellenocarum-II clade, did not form a monophyletic
group. Rather, these species were part of a large polytomy containing members of both the Pyramidoptereae and Careae tribes. Constraining Hellenocarum to monophyly resulted in trees 13 steps longer than those without the constraint invoked and this was rejected in the SH test ($P < 0.001$). No indel supports the monophyly of Hellenocarum.

A visual comparison of the ITS, ETS, and psbA-trnH trees indicated that the discrepancies observed among them were related to strongly supported clades. Pairwise ILD tests for ITS or ETS vs. psbA-trnH both resulted in $P = 0.001$, indicating that the plastid marker is significantly incongruent from the nuclear markers. The ILD test between ITS and ETS resulted in $P = 0.049$, which should also be interpreted as evidence of incongruence (Cunningham, 1997). As previously demonstrated, $P$-values should not be taken as evidence that data partitions are not combinable

Figure 3. Bayesian tree obtained from analysis of cpDNA psbA-trnH sequences. Only relationships with greater than 50% posterior probabilities are shown. Branch lengths are proportional to the number of character changes. Bayesian posterior probabilities and maximum parsimony bootstrap percentage values are indicated above nodes. Hellenocarum species are boldfaced. Names of newly accepted genera in this study are in parentheses.
(Hipp et al., 2004); therefore, with the hope of increasing resolution, all nuclear and plastid data (ITS + ETS + psbA-trnH) were combined and analyzed simultaneously. Bayesian and MP analyses of these combined data resulted in trees essentially identical to those obtained from ETS data alone, with comparable or slightly lower measures of branch support (trees not shown).

The results of the molecular study do not support the monophyly of *Hellenocarum*. In all analyses where resolution is achieved, the genus comprises 2 well-supported, disparate clades. The *Hellenocarum*-I clade includes the type species of *Hellenocarum* (*H. multiflorum*) and allies strongly with *Carum depressum* in the psbA-trnH trees. In the ITS and ETS trees, as well as in the trees resolved from analyses of combined data, the *Hellenocarum*-I clade, *C. depressum*, and 5 species of *Bunium* form a monophyletic group, although the group is variably supported. The *Hellenocarum*-II clade also associates with *Bunium* species, but their precise relationship is unclear because of low resolution in this portion of the trees. While these results corroborate the close relationship between *Hellenocarum* and many species of *Bunium* and *Carum*, none of these genera are monophyletic; furthermore, the type species of *Carum* (*C. carvi*) and *Bunium* (*B. bulbocastanum*) are not included within any clade of *Hellenocarum*.

### 3.2. Morphology and fruit anatomy

A comparison of morphological and fruit anatomical features of *Hellenocarum* and *Carum depressum* is presented in Table 2. Mericarp morphology and anatomy of the 4 *Hellenocarum* species are illustrated in Figures 4a–4j and 5a–5j. Detailed descriptions of fruit morphology and anatomy are provided in Section 4.

All species share a similar habit. Each has a tuberiform storage root, 2–4 pinnate leaves with petiolulate primary segments, and entire bracts and bracteoles. Differences are apparent in the structure of their underground organs, type of stem branching, shape of leaves and leaflets, petal color, number of petal vittae, and shape of the umbel, especially during fructification. In the original description of *C. depressum* (Tan and Iatrou, 2001), Hartvig and Tan indicated the shape of its lamina outline as being lanceolate. However, from the photos of the specimen kindly provided by Dr K Tan, it should have been characterized as triangular.

*Hellenocarum* species share many fruit characters. All have an elliptic mericarp shape, obsolete calyx teeth, small exocarp cells, indistinct cell borders on the mericarp surface (Figures 4b and 4g and 5b and 5g), a narrow mericarp commissure, compact vascular bundles situated at the primary rib bases, cyclic vittae (Figures 4e and 4j and 5e and 5j), an endocarp of slightly lignified

### Table 2. Comparison of morphological and fruit anatomical features of *Hellenocarum* and *Carum depressum*.

<table>
<thead>
<tr>
<th>Character</th>
<th>Carum (≡ <em>Hellenocarum</em>) depressum</th>
<th><em>Hellenocarum</em> multiflorum</th>
<th><em>Hellenocarum</em> strictum</th>
<th><em>Hellenocarum</em> (= <em>Neomuretia</em>) amplifolium</th>
<th><em>Hellenocarum</em> (= <em>Neomuretia</em>) pisidicum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taproot</td>
<td>Thick, elongate, not woody</td>
<td>Thick, elongate, not woody</td>
<td>Thick, fusiform, not woody</td>
<td>Thick, napiform, woody</td>
<td>Thick, napiform, woody</td>
</tr>
<tr>
<td>Stem</td>
<td>Some branches adpressed to the ground</td>
<td>Erect</td>
<td>Erect</td>
<td>Erect</td>
<td>Erect</td>
</tr>
<tr>
<td>Upper stem branching</td>
<td>Alternate or opposite</td>
<td>Alternate or verticillate</td>
<td>Alternate or opposite</td>
<td>Alternate or verticillate</td>
<td>Alternate or verticillate</td>
</tr>
<tr>
<td>Basal leaf dissection</td>
<td>2–3 pinnate</td>
<td>2–4 pinnate</td>
<td>2–3 pinnate</td>
<td>3–4 pinnate</td>
<td>2–3 pinnate</td>
</tr>
<tr>
<td>Shape of lamina in outline</td>
<td>Triangular</td>
<td>Triangular</td>
<td>Triangular</td>
<td>Osate</td>
<td>Subtaperiform</td>
</tr>
<tr>
<td>Shape of terminal leaflets</td>
<td>Ovate to lanceolate</td>
<td>Ovate to lanceolate</td>
<td>Ovate</td>
<td>Ovate</td>
<td>Linear-lanceolate</td>
</tr>
<tr>
<td>Upper stem leaves</td>
<td>With pinnate blade</td>
<td>With pinnate blade</td>
<td>Reduced, entire</td>
<td>Reduced, entire</td>
<td>Reduced, entire</td>
</tr>
<tr>
<td>Number of rays in umbel</td>
<td>8–20</td>
<td>12–35</td>
<td>8–15</td>
<td>8–15</td>
<td>16–20</td>
</tr>
<tr>
<td>Petal color</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>Number of vittae in petal</td>
<td>?</td>
<td>1</td>
<td>1–several</td>
<td>Several</td>
<td>?</td>
</tr>
<tr>
<td>Mericarp length, mm</td>
<td>2–2.5</td>
<td>2.7–4</td>
<td>2.5–3.5</td>
<td>2.5–3.5</td>
<td>4–4.5</td>
</tr>
<tr>
<td>Mericarp width, mm</td>
<td>?</td>
<td>0.75–1</td>
<td>0.5–0.75</td>
<td>0.6–0.8</td>
<td>1–1.5</td>
</tr>
<tr>
<td>Stylodium shape</td>
<td>Low conic</td>
<td>Low conic</td>
<td>Low conic</td>
<td>Low conic</td>
<td>Conic</td>
</tr>
<tr>
<td>Style length, mm</td>
<td>0.5–1</td>
<td>0.7–1.5</td>
<td>0.5–0.7</td>
<td>0.75–1</td>
<td>0.5–0.75</td>
</tr>
<tr>
<td>Mericarp ridge in valleculae</td>
<td>Absent</td>
<td>Present or absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Rib secretory ducts in mesocarp</td>
<td>Solitary and small</td>
<td>Solitary and small</td>
<td>Solitary and small</td>
<td>Solitary and small</td>
<td>Obsolete</td>
</tr>
<tr>
<td>Cotyledon number</td>
<td>?</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 4. Comparison of fruit morphology and anatomy. *Hellenocarum multiflorum* [delectus seminum 2000-281 of Museum National d’Histoire Naturelle Paris, Département des Jardins Botaniques et Zoologiques: Grèce, Massif du Timfi, 1015 m]: a - view of mature mericarp, scale = 1 mm; b - details of surface in the middle part of the fruit (SEM) showing indistinct cell borders, smooth or longitudinally sulcate mericarp surface on ribs, and foveolate-tuberculate or longitudinally sulcate mericarp surface on valleculae, scale = 30 µm; c - sparse striate, striate with straight striae or rugulate cuticle on valleculae, scale = 10 µm; d - rugate cuticle on rib, scale = 10 µm; e - schematic transect of mericarp, scale = 1 mm. *Hellenocarum strictum* [NW Macedonia/Kosovo, Šar Planina, 10 km WSW Tetovo, 27.08.1981, Andersson & Franzén 901 (C)]: f - view of mature mericarp, scale = 1 mm; g - details of surface in the middle part of the fruit (SEM) showing indistinct cell borders, rugate mericarp surface on ribs, and undulate, small tubercles mericarp surface on valleculae, scale = 50 µm; h - striato-rugulate cuticle on valleculae, scale = 10 µm; i - striato-rugulate cuticle on rib, scale = 10 µm; j - schematic transect of mericarp, scale = 1 mm. Abbreviations: co - mericarp commissure, df - secretory vittae, en - endocarp, es - endosperm, ex - exocarp, mc - mesocarp, sc - seed coat, vb - vascular bundles.
cells, and a flat endosperm groove on the commissural side. A characteristic trait for *Hellenocarum* is a clearly visible constriction of the mericarps under the stylopodia (Figures 4a and 4f and 5a and 5f). This character is not usually diagnostic in the Umbelliferae (Kljuykov et al., 2004), as it is difficult to interpret in many taxa, but in *Hellenocarum* it is obvious.

Fruit differences include those of mericarp length and width, shape of stylopodia, length of styles, the presence/absence of a filiform ridge in the valleculae, structure and ultrasculpture of exocarp cells as revealed on surface view (SEM), shape of mericarp in transverse section, the presence/absence of rib secretory ducts in the mesocarp, and cotyledon number. In the original description of *H. pisidicum* (Tan and Sorger, 1986), the vittae were indicated as being solitary in valleculae. However, they were observed as being cyclic in a transverse section of the mericarp of the type specimen. *Hellenocarum multiflorum*, *H. strictum*, and *H. amplifolium* each have an embryo with 1 cotyledon, whereas *H. pisidicum* has an embryo with 2 cotyledons.

Some of these fruit differences correlate with *Hellenocarum* clades I and II, as revealed in the molecular study. The *Hellenocarum*-I clade is characterized by underground organs represented by thick, elongate or fusiform, nonwoody roots; corymbose umbels; white petals; and slightly laterally compressed mericarps. The *Hellenocarum*-II clade is characterized by underground organs represented by thick, napiform, woody roots; globular umbels (especially during fructification); yellow petals; and slightly dorsally compressed mericarps.

*Carum depressum* is very similar to *H. multiflorum* of the *Hellenocarum*-I clade in many essential characters. These include life form (monocarpic), structure of underground organs (thick, elongate, nonwoody roots), leaf structure (petiolulate primary segments), shape of upper stem leaves (dissected), obsolete calyx teeth, color of petals (white), shape of the mericarp (constricted under stylopodium), and form of the mericarp ribs (short-winged). The other species of *Carum* are monocarpic or polycarpic, with underground parts represented by a taproot, sessile (rarely petiolulate) primary segments of leaf, and keeled ribs on the fruit. The lateral branches of the stem being adpressed to the ground is a main distinguishing feature of *Carum depressum* and clearly separates it from all other examined species of *Hellenocarum*.

### 3.3. Distribution

The distribution of all *Hellenocarum* species and *Carum depressum* is shown in Figure 6. The species of the *Hellenocarum*-I clade are distributed from southern Italy to Turkey (western Anatolia), with *H. multiflorum* widespread in this region. According to Meikle (1977), the occurrence of *H. multiflorum* in Cyprus should be regarded as questionable, since many of the records from Cyprus in *Flora Graeca* are known to be erroneous. *Hellenocarum strictum* occurs in the northern part of the Balkans and the northern islands of the Aegean Sea. The species of the *Hellenocarum*-II clade are distributed further east than those of the *Hellenocarum*-I clade. *Hellenocarum pisidicum* is a local endemic of limestone cliffs in Antalya (Turkey); *H. amplifolium* is known from only a few locations in western Iran and northern Iraq.

### 4. Discussion

The genus *Hellenocarum*, as traditionally circumscribed, is not monophyletic. Instead, it comprises 2 disparate clades nested within a large, complicated clade that also includes *Tamaneschjanella* Pimenov & Kljuykov, *Postiella* Kljuykov, and several species of *Bunium* and *Carum*. The molecular data reveal a complex picture of relationships, as *Bunium* and *Carum* are not monophyletic either. These 3 genera are taxonomically connected, as, on the one hand, they all possess a rather simple fruit structure, mainly characterized by glabrous mericarps bearing almost equal ribs. On the other hand, and in contrast to many other genera of Umbelliferae and even eudicots, they exhibit variability in cotyledon number. Cotyledon number has been used previously to separate *Bunium* from *Carum* (Drude, 1889; Calestani, 1905; Wolff, 1927). Engstrand (1973) showed that *H. multiflorum* has a pseudomonocotyledonous embryo and used this feature to separate *Hellenocarum* from *Carum*. The morphological study herein revealed that species from the *Hellenocarum*-I clade possess an embryo with 1 cotyledon, whereas species from the *Hellenocarum*-II clade possess an embryo with either 1 (*H. amplifolium*) or 2 (*H. pisidicum*) cotyledons. This variability in cotyledon number also occurs in *Elaeosticta*, another member of tribe Pyramidoptereae (Degtjareva, 2013). In contrast, the dicotyledonous *Bunium* species now treated in the separate genus *Elwendia* Boiss. are closer to other dicotyledonous geophytic genera than they are to the pseudomonocotyledonous species of *Bunium* (Degtjareva et al., 2013). The presence of both pseudomonocotyledonous and dicotyledonous species in both *Elaeosticta* and members of the *Hellenocarum*-II clade diminishes the taxonomic value of this character. It is interesting to note that both *Hellenocarum* clades are nested within a larger clade comprising *Bunium* species having a single cotyledon.

The *Hellenocarum*-I clade includes *H. multiflorum* (type species) and *H. strictum*. These species were segregated from *Carum* by Wolff (1927) solely on the basis of “umbellis involucratis”. In *Carum*, the bracts are absent or are represented only by a few, although this character is actually much more variable in the genus than considered by Wolff (1927). Many authors, however, do not consider
Figure 5. Comparison of fruit morphology and anatomy. *Hellenocarum* (= *Neomuretia*) *amplifolium* [Iran, Chaharmahal-e Bakhtiari: Lordegan, 13.06.1987, Mozaffarian 62101 (TARI)]: a - view of mature mericarp, scale = 1 mm; b - details of surface in the middle part of the fruit (SEM) showing indistinct cell borders, longitudinally sulcate mericarp surface both on ribs and valleculae, scale = 30 µm; c - striate with straight striae cuticle on valleculae, scale = 10 µm; d - striate with straight striae cuticle on ribs, scale = 10 µm; e - schematic transect of mericarp, scale = 1 mm. *Hellenocarum* (= *Neomuretia*) *pisidicum* [S Anatolia, NW Antalya: subdistr. Beskonak, above Dizağaç, 19.07.1982, Ayaşligil 1378B (E)]: f - view of mature mericarp, scale = 1 mm; g - details of surface in the middle part of the fruit (SEM) showing indistinct cell borders, irregularly sulcate mericarp surface on ribs, and longitudinally rugate mericarp surface on valleculae, scale = 30 µm; h - striato-rugulate cuticle on valleculae, scale = 10 µm; i - striato-rugulate cuticle on ribs, scale = 10 µm; j - schematic transect of mericarp, scale = 1 mm. Abbreviation: dr - rib secretory ducts.
this character decisive and treat H. multiflorum and H. strictum in the genus Carum. Since this work by Wolff (1927), additional morphological characters have been used to separate Hellenocarum from Carum. Engstrand (1973) pointed out the pseudomonocotyledonous embryo and fusiform tuber of Hellenocarum; while these features clearly separate the genus from Carum, they also place Hellenocarum closer to Bunium, as Bunium also possesses a pseudomonocotyledonous embryo. Kljuykov (1985) emphasized the petiolulate primary segments of the leaf (vs. mainly sessile in Carum) and the cyclic vittae in the fruits (vs. 1 to several vallecular vittae in Bunium). The molecular results presented herein support earlier conclusions by Wolff (1927), Engstrand (1973), and Kljuykov (1985) in segregating Hellenocarum from Carum. The separation of Hellenocarum from Carum was also revealed in the molecular phylogenetic studies of Papini et al. (2007), Degtjareva et al. (2009), and Zakharova et al. (2012), although only H. multiflorum was considered in each of these studies.

Although this study has clarified relationships among the species of Hellenocarum, the relationship of H. multiflorum and H. strictum to Bunium and Carum is still ambiguous. This ambiguity is manifest in the incongruent relationships recovered for these genera in both nuclear- and plastid-derived trees. Possible evolutionary processes that may help explain this incongruence include hybridization, horizontal gene transfer, incomplete lineage sorting, and gene duplication (e.g., Wendel and Doyle, 1998). In our case, additional study is required (including ascertaining chromosome numbers) before we can speculate on what may have caused this discordance. Moreover, additional plastid data are needed to yield a better-resolved topology for comparison to the nuclear-derived trees.

The close relationship of H. multiflorum and H. strictum is consistent with morphology, as both share many characters; it is also consistent with their distributions, as both occur in the eastern region of southern Europe. Based on overall morphological similarities, Tutin (1967) considered H. strictum (= B. strictum or Carum lumpeanum) as a subspecies of Carum multiflorum. Subsequent studies, emphasizing life form (biennial vs. perennial), number of umbel rays (8–15 vs. more than 15), length of style (0.5–1 mm vs. 1–2 mm), and shape of the upper stem leaves (reduced and entire vs. dissected), distinguished H. strictum from H. multiflorum (Kljuykov, 1985; Hartvig, 1986). In H. multiflorum, the mericarp surface on the ribs is smooth or longitudinally sulcate and on the valleculae it is foveolate-tuberculate or longitudinally sulcate. In H. strictum, the mericarp surface is undulate, with small tubercles. These species also have slightly different ITS and ETS sequences, which serve to delimit them molecularly. However, additional material of each species must be examined to test the significance of these differences for bar coding.

The Hellenocarum-I clade allies with Carum depressum, with this grouping strongest in only the psbA-trnH trees. Carum depressum is very similar to H. multiflorum in its life form (monocarpic), leaf structure (petiolulate primary segments), obsolete calyx teeth, and petal color. Carum depressum also shares a similar distribution in the eastern portion of southern Europe, although it is restricted to only 2 localities in Peloponnisos (Greece). Our results confirm that plants of Carum depressum should be maintained as a distinct species (and are not artifacts caused by trampling or grazing; Tan and Iatrou, 2001), distantly placed from Carum strictum or Carum lumpeanum (-II clade allies with, H. amplifolium and H. pisidicum. Hellenocarum amplifolium was originally described as Muretia amplifolia Boiss. & Hausskn. (Boissier, 1872), and while a close similarity of this species with Carum multiflorum was noted, no transfer of M. amplifolia to Carum or Hellenocarum was done prior to Kljuykov’s (1985) taxonomic study. Muretia amplifolia and H. multiflorum share thick roots, petiolulate primary segments of the leaf, an elliptic mericarp with equally short-winged primary ribs, a narrow commissure, and cyclic...
vittae. The most important difference between *Muretia amplifolia* and *H. multiflorum* is the number of petal vittae (several vs. solitary). This character, however, was treated by Kljuykov (1985) as being insufficient for generic separation; therefore, *Muretia amplifolia* was transferred to *Hellenocarum*. *Hellenocarum amplifolium* and *H. pisidicum* are separated from each other geographically. *Hellenocarum pisidicum* was postulated as being closely related to *H. multiflorum* (Tan and Sorger, 1986). Although all *Hellenocarum* species are morphologically very similar, the present study revealed a set of characters that separate *H. amplifolium* and *H. pisidicum* from *H. multiflorum*. These include structure of underground organs (woody vs. nonwoody), petal color (yellow vs. white), and shape of the umbel (globular vs. corymbose).

The polyphyletic nature of *Hellenocarum* necessitates a taxonomic revision. Such a revision would require 1 of the 5 *Bunium* species most closely allied to *Hellenocarum*. Bunium strictum into a greatly expanded *Bunium. Hellenocarum amplifolium* and *H. pisidicum* from *H. multiflorum*. In the ITS phylogeny, the relationships between *Bunium* species and nongeophytic members are unresolved. These results indicate the necessity of reviewing the taxonomy of *Bunium*.

**Nomenclatural implications**

Based on the molecular phylogenetic hypothesis presented herein and our observations of morphology and fruit anatomy, nomenclatural changes are in order. Because *H. multiflorum* is the nomenclatural type of *Hellenocarum* and is contained within the *Hellenocarum*-I clade, we designate this group as *Hellenocarum sensu stricto*. Carum depressum is to be transferred from *Carum* to *Hellenocarum* sensu stricto and, as a result, *Hellenocarum* now contains 3 species. The species from the *Hellenocarum*-II clade, *H. pisidicum* and *H. amplifolium*, are to be recognized in a separate genus, and a new name must be proposed. We name the new genus *Neomuretia* to honor the Swiss botanist J Muret. The genus *Muretia* was described by Boissier (1844), but it is now abolished as all of its species have been transferred to other genera. *Hellenocarum amplifolium* was initially described as *Muretia amplifolia*. We cannot use the name *Muretia* for the members of the *Hellenocarum*-II clade because the lectotype of *Muretia* (*Muretia tanacensis*) is contained within the *Hellenocarum*-I clade, we designate this group as *Hellenocarum sensu stricto*. Carum depressum is to be transferred from *Carum* to *Hellenocarum* sensu stricto and, as a result, *Hellenocarum* now contains 3 species. The species from the *Hellenocarum*-II clade, *H. pisidicum* and *H. amplifolium*, are to be recognized in a separate genus, and a new name must be proposed. We name the new genus *Neomuretia* to honor the Swiss botanist J Muret.

Below we present a key to the genera of *Bunium, Carum, Hellenocarum,* and *Neomuretia*, followed by descriptions of the new combination *Hellenocarum depressum* and the new genus *Neomuretia*.

**Key to the genera of *Bunium, Carum, Hellenocarum,* and *Neomuretia***

1 Plants without thickened taproot; primary segments of leaf sessile .......................................................... *Carum*

1* Plants with thickened taproot; primary segments of leaf petiolulate ..........................................................

2 Plants polycarpic with spherical tuber, deeply submerged in soil; mericarp ribs filiform or keeled, rarely short-winged; vallecular vittae 1–several.................................................. *Bunium*

Typus: H. multiflorum (Sm.) H.Wolff (Carum multiflorum Sm.) (Kljuykov, 1985)  
Apioidae.  

(3 spp., Asia (SW), Europe (S, SE).

Wolff (1927), in the protologue, did not explicitly address the affinity of his new genus, although he placed it between Carum and the North American genus Ataenia Endl. (= Perideridia Rchb.). Hellenocarum is distinguished from Carum by its life form (geophyte with tuberiform storage roots), 2–4 pinnate leaves with petiolulate basal segments, and numerous vittae in the fruit pericarp. Hellenocarum is similar to Bunium in its life form, but differs from it by possession of an elongate, often branched tuber (vs. spherical), short-winged ribs (vs. keeled or filiform), and numerous cyclic vittae.

Hellenocarum depressum (Hartvig & Kit Tan) Kljuykov & Zakharova comb. nov.


Typus: Greece, the Peloponnese “Nomos Messinias, Eparchia Kalamon, in faucibus Langada, in fissures rupium calcareaum” 770–800 m, 24 May 1998, Kit Tan & Strid 20416 (holo - C!).

Fruit glabrous. Mericarps homomorphic, elliptic, 2.7–4 mm long, 0.75–1 mm broad, slightly compressed laterally, constricted under stylopodium (Figure 4a); with primary ribs and additional ridges in valleculae; primary ribs equally short-winged, straight, with entire margin; ridges in valleculae filiform or invisible. Calyx teeth obsolete. Stylodia low conic, styles 0.7–1.5 mm long, recurved. Cell borders of mericarp surface indistinct, hairs absent (Figure 4b). On ribs, mericarp surface smooth or longitudinally sulcate, cuticle rugate (Figure 4d). On valleculae, mericarp surface foveolate-tuberculate or longitudinally sulcate, cuticle sparse striate, striate with straight striiae, or rugulate (Figure 4e). Stomata rare. Exocarp composed of small cells, interrupted near the carpophore, commissure narrow (Figure 4e). Mesocarp composed of nonlignified parenchyma cells. Vascular bundles compact, situated in the primary rib bases (Figure 4e). Vittae cyclic, situated in valleculae, under the vascular bundles and on the commissure (Figure 4e); rib secretory ducts small, solitary, sometimes invisible, or visible in some ribs only. Endocarp of long, slightly lignified prosenchyma cells. Endosperm flat at commissural side (Figure 4e). Embryo with one cotyledon.

Distribution: SE Italy, S Albania, Greece, Crete, Turkey (W Anatolia: İzmir), E Aegean Islands (Figure 6).


* H. strictum (Griseb.) Griseb. 1843, Spicil. Fl. Rumel. et Bith. 1: 344.

* Carum multiflorum (Sm.) H. Wolff subsp. strictum (Griseb.) Kit Tan, 1986, in Tan and Sorger, Pl. Syst. Evol. 154: 122.


* Carum multiflorum (Sm.) H. Wolff subsp. strictum (Griseb.) Tutin, 1967, Fedd. Repert. 74, 1–2: 31.
Typus: In Macedonia, Frivaldszky. (G-BOISS, GOET).

Typus: Insula Thasos, in rupestris umbrosis marmoreis montis Elias et montis Theologos (23 and 31 05 1891). P. Sintenis & J. Bornmuller, 606, 609 (syn – LD!, B!); Macedonia centralis: Drenovo, in faubicus “Klisura”, 2–300 m s.m. 11 05 1918. J. Bornmuller, 4175 (B!).

Fruits glabrous, carpophore bifid to the middle or the base. Mericarps homomorph, elliptic, 2.5–3.5 mm long, 0.5–0.75 mm broad, slightly compressed laterally, constricted under stylodium (Figure 4f); only primary ribs are present; primary ribs equally short-winged, straight, with entire margin. Calyx teeth obsolete. Stylodinia low conic, styles 0.5–0.7 mm long, recurved. Cell borders of mericarp surface indistinct, hairs absent (Figure 4g). On ribs, mericarp surface rugate, cuticle striato-rugulate (Figure 4i). On valleculae, mericarp surface undulate, with small tubercles, cuticle striato-rugulate (Figure 4h). Stomata rare. Exocarp composed of small cells, interrupted near the carpophore, commissure narrow (Figure 4j). Mesocarp composed of nonlignified parenchyma cells. Vascular bundles compact, situated in the primary rib bases (Figure 4j). Vittae cyclic, situated in valleculae, under the vascular bundles and on the commissural side (Figure 4j); rib secretory ducts small, solitary, sometimes invisible, or visible in some ribs only. Endocarp of long, slightly lignified prosenchyma cells. Endosperm flat at commissural side (Figure 4j). Embryo with one cotyledon.

Distribution: Balkans (Albania, Macedonia, N Greece), N Aegean Islands (Figure 6).

**Neomuretia** Kljuykov, Degtjareva & Zakharova gen. nova (Umbelliferae-Apioideae).

Affinitas: A genero *Hellenocarum* H. Wolff radicibus primariis naphiformibus lateralis umbelliforum (nec carnosis ovatis vel fusiformibus digitatis), petalis flavis (nec albis), umbellis sphaericis (nec corymbose) differt.


Monocarpic perennial herbs, 50–100 cm tall, somewhat glabrous. Root thick, woody, naphiform, with threadlike lateral roots. Stem solitary, thick, to 1–2 cm in diameter at the base, covered with fibrous remains of petioles, branched from the base with alternate branches in lower part and verticillate branches in upper part. Basal leaves petiolate, their blades ovate in outline, 2–3 pinnate. Primary segments of leaves petiolate. Terminal leaflets oblong or linear-lanceolate. Upper stem leaves reduced, entire, triangular. Umbels globular; rays 8–20, subequal, sparsely hairy, thin. Bracts up to 8, linear-lanceolate or lanceolate. Bracteoles several, lanceolate-linear. Calyx teeth obsolete. Petals yellow, glabrous, obcordate, with inflexed apex and several secretory ducts. Stylodinia low conic or conic, styles recurved. Fruits glabrous, carpophore bifid to the middle or the base. Mericarps homomorph, elliptic, 2.5–4.5 mm long, slightly compressed laterally, constricted under stylodium; primary ribs short-winged. Exocarp composed of small cells. Mesocarp composed of parenchyma cells. Vittae cyclic; rib secretory ducts solitary, small or obsolete. Endocarp of 1 cell layer with elongate cells having slightly lignified walls. Endosperm flat at commissural side. Embryo with 1 or 2 cotyledons.

Typus: *N. amplifolia* (Boiss. & Haussk.) Kljuykov, Degtjareva & Zakharova.

The genus includes 2 species, *Neomuretia amplifolia* and *N. pisidica*.

**Neomuretia amplifolia** (Boiss. & Hausskn.) Kljuykov, Degtjareva & Zakharova, comb. nov.


Typus: IRAN: In fissuris rupium calcarae montis Avroman Persiae austro-occidentalis, alt. 6000–7000′, 06.1867, Haussknecht (holo – G-BOIS!; iso –: JE! W!).

Fruit glabrous, carpophore bifid to the middle or the base. Mericarps homomorphic, elliptic, 4–4.5 mm long, 1–1.5 mm broad, slightly compressed dorsally, constricted under stylododium (Figure 5f); with primary ribs and additional ridges in valleculae; primary ribs equally short-winged, straight, with entire margin. Calyx teeth obsolete. Stylododia conic, styles 0.5–0.75 mm long, recurved. Cell borders of mericarp surface indistinct, hairs absent (Figure 5g). On ribs, mericarp surface irregularly sulcate, cuticle striato-rugulate (Figure 5i). On valleculae, mericarp surface longitudinally rugate, cuticle striato-rugulate (Figure 5h). Stomata rare. Exocarp composed of small cells, interrupted near the carpophore, commissure narrow (Figure 5j). Mesocarp composed of nonlignified parenchyma cells. Vascular bundles compact, situated in the primary rib bases (Figure 5e). Vittae cyclic, situated in valleculae, under vascular bundles and on commissural side; rib secretory ducts small, solitary, sometimes invisible, or visible in some ribs only (Figure 5e). Endocarp of long, slightly lignified prosenchnyma cells. Endosperm flat at commissural side (Figure 5e). Embryo with one cotyledon.

Distribution: Iran (W: Kordestan, Kermanshah; C: Chaharmahal va Bakhteyari; S: Khuzestan), Iraq (Figure 6).

Neomuretia pisidica (Kit Tan) Kljuykov, Degtjareva & Zakharova, comb. nov.


Typus: TURKEY: Turkey, C3, prov. of Antalya, subdistrict Beskonak, NE of Duezagac, limestone cliffs, 1250 m s.m., 19.08.1983, Ayaşligil 1638 (holo – Hb. Ayaşligil; iso –: E!).

Fruit glabrous, carpophore bifid to the middle or the base. Mericarps homomorphic, elliptic, 4–4.5 mm long, 1–1.5 mm broad, slightly compressed dorsally, constricted under stylododium (Figure 5f); with primary ribs and additional ridges in valleculae; primary ribs equally short-winged, straight, with entire margin; ridges in valleculae filiform. Calyx teeth obsolete. Stylododia conic, styles 0.5–0.75 mm long, recurved. Cell borders of mericarp surface indistinct, hairs absent (Figure 5g). On ribs, mericarp surface irregularly sulcate, cuticle striato-rugulate (Figure 5i). On valleculae, mericarp surface longitudinally rugate, cuticle striato-rugulate (Figure 5h). Stomata rare. Exocarp composed of small cells, interrupted near the carpophore, commissure narrow (Figure 5j). Mesocarp composed of not lignified parenchyma cells. Vascular bundles compact, situated in the primary rib bases (Figure 5e). Vittae cyclic, situated in valleculae, under vascular bundles and on commissural side (Figure 5j); rib secretory ducts obsolete. Endocarp of long, slightly lignified prosenchnyma cells. Endosperm flat at commissural side (Figure 5j). Embryo with 2 cotyledons.

Distribution: Turkey (S Anatolia: Antalya) (Figure 6).

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References


Appendix. Voucher information and GenBank accession numbers for plants used in the present study. * = sequences newly generated for this study.