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The infrageneric taxonomy of *Chaerophyllum* (Apiaceae) revisited: new evidence from nuclear ribosomal DNA ITS sequences and fruit anatomy

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Evolutionary relationships among 65 *Chaerophyllum* spp. were inferred from nuclear ribosomal DNA internal transcribed spacer (ITS) sequence variation. Thirty-one species, represented by 158 mericarp samples, were analysed for fruit anatomical character variation, employing phylogenetic and phenetic methods to explore their congruence with infrageneric divisions based on molecular data. Phylogenetic trees inferred from molecular data using maximum likelihood (ML) and Bayesian inference (BI) methods corroborated the division of the genus into four sections: *Chaerophyllum*, *Dasypetalon*, *Physocaulis* and *Chrysocarpum*. From among the newly sequenced species, the Greek endemic *C. heldreichii* was grouped with section *Chaerophyllum*, whereas the highly variable Asian *C. reflexum–C. villosum* complex formed an early-branching paraphyletic assemblage in section *Chrysocarpum*. The recently described *C. karsianum* has an identical ITS sequence to *C. bulbosum*, whereas *C. aksekiense* was clearly separated from the morphologically similar *C. macrospermum*. Our study confirmed the postulated synonymy of several species on the basis of morphology, but also demonstrated distant relationships between some morphologically similar species. With the exception of the monotypic section *Physocaulis*, we were unable to find carpological traits matching sectional divisions. We hypothesize that fruit characters evolved rapidly as a result of diversification of members of the genus in different habitats. *Botanical Journal of the Linnean Society*, 2015, **178**, 298–313.

ADDITIONAL KEYWORDS: anatomical cross-sections – ancestral reconstruction – carpology – chervil – molecular taxonomy – principal component analysis (PCA).

INTRODUCTION

Chaerophyllum L. is placed in tribe Scandiceae in the taxonomically complex family Apiaceae. Traditionally, it was recognized as having *c*. 34 species distributed mostly in Eurasia, with two species occurring in North America (Spalik & Downie, 2001). Subsequent molecular studies revealed that the predominantly Southern Hemisphere genus *Oreomyrrhis* Endl. with *c*. 26 species is nested in *Chaerophyllum* (Chung *et al*., 2005). Subsequently, the former was subsumed into the latter (Chung, 2007). The phylogenetic position of the *Oreomyrrhis* clade in *Chaerophyllum* was established using nuclear ribosomal DNA internal transcribed spacer (ITS) sequences and plastid DNA markers (Chung, 2007), and the relationships among the remaining species of the genus (*Chaerophyllum s.s.*) were examined with ITS sequences with limited taxonomic sampling (Spalik & Downie, 2001); they therefore require a detailed study.

Spalik & Downie (2001) provided a taxonomic framework for *Chaerophyllum s.s.*, dividing the genus into

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four sections on the basis of morphological and molecular data: (1) *Chaerophyllum*, with three species distributed in Europe (one) and North America (two); (2) *Dasypetalon* Neilr., with four species occurring in the mountains of Europe; (3) the monotypic *Physocaulis* DC., with one species, *C. nodosum* (L.) Crantz, distributed in southern Europe and the Middle East; and (4) *Chrysocarpum* Spalik & S.R.Downie, encompassing the majority of European and Asian members of the genus. Species of the former *Oreomyrrhis* constitute a clade which may be sister to the North American members of section *Chaerophyllum*, i.e. *C. procumbens* (L.) Crantz and *C. tainturierii* Hook. & Arn. (Chung, 2007). However, because these studies did not include all species of *Chaerophyllum s.s.*, several questions about the relationships in the genus remain unanswered. Two groups are particularly problematic: the *C. reflexum* Lindl.–*C. villosum* DC. complex and the *C. bulbosum* L.–*C. prescottii* DC. group.

The *C. reflexum*–*C. villosum* complex includes several montane taxa distributed in the Himalayas, extending westward to Pakistan and Afghanistan. Hedge & Lamond (1980, 1987) distinguished two species in this complex, namely *C. villosum* and *C. reflexum*, with several varieties (*C. reflexum* var. *acuminatum* (Lindl.) Hedge & Lamond, *C. reflexum* var. *dissectum* (C.B.Clarke) Hedge & Lamond, *C. reflexum* var. *reflexum*, *C. reflexum* var. *occidentale* Hedge & Lamond and *C. reflexum* var. *tuberosum* Hedge & Lamond). In contrast, Mukherjee & Constance (1993), in their monographic study of Indian Apiaceae, treated some of these varieties at the specific level, recognizing five species [*C. acuminatum* Lindl., *C. cachemiricum* C.B.Clarke, *C. orientale* (C.B.Clarke) P.K.Mukherjee, *C. reflexum* and *C. villosum*]. Across the different treatments of the *C. reflexum*–*C. villosum* complex (Hedge & Lamond, 1980, 1987; Mukherjee, 1982; Mukherjee & Constance, 1993), only *C. villosum* has consistently retained its identity.

Similar taxonomic problems are evident for *C. bulbosum* and *C. prescottii*. Some authors (de Candolle, 1830; Schischkin, 1950; Pimenov & Ostroumova, 2012) have treated them as separate species, and others (Hämet-Ahti, 1967; Tutin *et al*., 1968) as two subspecies of the bulbous chervil: *C. bulbosum* ssp. *bulbosum* and *C. bulbosum* ssp. *prescottii* (DC.) Nyman. Regardless of the treatment, all authors considered these taxa to be closely related. The recently described *C. karsianum* Kit Tan & H.Ocakverdi from Turkey (Tan & Ocakverdi, 1986) and a Caucasian endemic *C. caucasicum* (Fisch.) Schischk., the latter usually synonymized with *C. bulbosum*, are also morphologically similar with characteristic underground tubers.

Several species that were not included in previous analyses have restricted distributions. These include *C. heldreichii* Orph. ex Boiss. from Greece, *C. coloratum* L. restricted to the Adriatic coast of the Balkan Peninsula, three endemics of the Caucasus (*C. borodinii* Albov, *C. roseum* M.Bieb. and *C. rubellum* Albov), *C. aurantiacum* Post restricted to Lebanon and *C. syriacum* Hemprich & Ehrenb. ex Boiss. from Syria. Three species that were recently described from a few localities in Turkey are also microendemics: *C. karsianum* (Tan & Ocakverdi, 1986), *C. posofianum* S.Erik & N.Demirkuş (Erik & Demirkuş, 1998) and *C. aksekiense* A.Duran & H.Duman (Duran & Duman, 1999).

Morphological characters are commonly used to infer species relationships and for their identification. However, such traits can be misleading in inferring relationships as a result of homoplasy. A good example of convergent evolution in *Chaerophyllum* is leaf division: a group of species with bipinnate leaves having elliptical or ovate segments (*C. aromaticum* L., *C. atlanticum* Coss., *C. azoricum* Trel., *C. byzantinum* Boiss. and *C. libanoticum* Boiss. & Kotschy) has been demonstrated to constitute several clades in the genus (Spalik & Downie, 2001). Such leaves are also characteristic of *C. angelicifolium* M.Bieb. and *C. heldreichii* which have not been examined at the molecular level.

In their analysis of subtribe Scandicinae, Spalik & Downie (2001) traced 44 discrete vegetative and reproductive characters on the molecular phylogenetic tree. Two sections of *Chaerophyllum*, *Physocaulis* and *Dasypetalon*, were characterized by having heavily setose fruits and ciliate petals, respectively. In spite of the number of traits analysed, they did not find unequivocal diagnostic characters useful for the descriptions of sections *Chrysocarpum* and *Chaerophyllum*. However, Spalik & Downie (2001) did not consider fruit anatomy, despite past reliance on these features in systematic treatments of Apiaceae dating to the early studies of Drude (1898) and his contemporaries. Although taxonomic systems based on fruit anatomy have appeared to be incongruent with the molecular phylogenetic tree of the family, some taxonomic groups delineated by molecular data have well-defined fruit characteristics (Spalik & Downie, 2001; Spalik, Wojewódzka & Downie, 2001a; Liu *et al*., 2006; Calviño, Martínez & Downie, 2008; Feist *et al*., 2012). Moreover, carpological characters are still considered to be important for infrageneric classifications of many Apiaceae, e.g. *Eryngium* L. (Wörz & Diekmann, 2010), *Ferulago* W.D.J.Koch (Akalın Uruşak & Kızılarslan, 2013), *Pimpinella* L. (Khajepiri, Ghahremaninejad & Mozaffarian, 2010), *Marlothiella* H.Wolff (Liu, van Wyk & Tilney, 2007), *Capnophyllum* Gaertn., *Dasispermum* Raf. and *Sonderina* H.Wolff (Magee *et al*., 2009a, b, 2010).

In this study, we test the coherence of the recently proposed taxonomic system of the genus using ITS sequences from a comprehensive sample of almost all

species of *Chaerophyllum*. Despite problems with the use of ITS sequences for phylogenetic inference in some plant groups (Álvarez & Wendel, 2003), this region constitutes the most useful source of phylogenetic information in Apiaceae, especially at lower taxonomic levels (Downie *et al*., 2010; Liu *et al*., 2014). Furthermore, extending the work of Spalik & Downie (2001), we evaluate the utility of anatomical characters of fruits for the delimitation of the main lineages in the genus.

MATERIAL AND METHODS

Taxon sampling

In their revision of subtribe Scandicinae, Spalik & Downie (2001) analysed 21 species of *Chaerophyllum s.s.* We analysed all of these anew and sampled an additional 22 species (Supporting Information Table S1). Among them were five species that had been placed in synonymy under other species on the basis of morphology. Our aim was to verify their status using molecular data. These include *C. calabricum* Guss. ex DC. (= *C. hirsutum* L.), *C. confusum* Woronow (= *C. angelicifolium*), *C. kiapazi* Woronow ex Schischk. [= *C. humile* Steven ex M.Bieb. var. *kiapazi* (Woronow ex Schischk.) Karjagin], *C. caucasicum* (Fisch. ex Hoffm.) Schischk. (= *C. bulbosum*) and *C. millefolium* DC. (= *C. roseum*). We also considered four varieties of *C. reflexum*: *C. reflexum* var. *reflexum*, *C. reflexum* var. *occidentale*, *C. reflexum* var. *tuberosum* and *C. reflexum* var. *acuminatum*. We used several individuals per species for the majority of taxa to account for intraspecific variability (Table S1). To make our sampling as comprehensive as possible, we added 20 species of the '*Oreomyrrhis*' clade from the work of Chung (2007; TreeBase S1622), whereas two species from this group, *C. involucratum* (Hataya) K.F.Chung and *C. eriopodum* (DC.) K.F.Chung, were analysed *de novo*. To root trees, we used four species of Scandicinae (*Myrrhis odorata* Scop., *Scandix stellata* Soland., *Kozlovia paleacea* Lipsky and *Sphallerocarpus gracilis* Koso-Pol.), which formed sister groups to *Chaerophyllum* in former phylogenetic analyses (Downie, Katz-Downie & Spalik, 2000; Spalik & Downie, 2001; Spalik, Wojewódzka & Downie, 2001b; Downie *et al*., 2010). In summary, we sequenced 116 specimens for the entire ITS region (Table S1) and considered 136 in phylogenetic analyses (65 species). Our study did not encompass four taxa (*C. euboeum* Halácsy, *C. posofianum, C. leucolaenum* Boiss. and *C. reflexum* var. *dissectum*), all of which are rare endemics with unavailable herbarium material.

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from *c.* 20 mg of dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Venlo, the Netherlands). The ITS region was amplified using primers N-nc18S10 and C26A (Wen & Zimmer, 1996). For some accessions, the ITS1 and ITS2 regions were amplified separately using the following pairs of primers: 18S-ITS1-F and 5.8S-ITS1-R for ITS1, and ITS-3N and C26A for ITS2 (Spalik & Downie, 2006). Each 20-μL polymerase chain reaction (PCR) was prepared using 1 × PCR buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.2 μM each primer, 2–4 mM $MgCl_2$, 1–2 U Taq DNA polymerase (Qiagen), 5 μL Q solution (Qiagen) and 1–4 μL DNA template. The PCR included an initial denaturation at 94 °C for 5 min, followed by 35 cycles comprising 1 min of denaturation at 94 °C, 1 min of annealing at 50–54 °C and 1 min of extension at 72 °C. A final extension at 72 °C for 10 min was also added. Each PCR product was electrophoresed in a 1% agarose gel, stained with ethidium bromide, and then excised and eluted using the QIAquick Gel Extraction Kit (Qiagen). No obvious polymorphisms (multiple bands from a single PCR product) were observed. Cycle sequencing reactions were performed using the purified PCR product and fluorescent Big Dye terminators (Applied Biosystems, Foster City, CA, USA). The final products were resolved using an automated DNA sequencer at IBB PAN (Warsaw, Poland). We examined both DNA strands to unambiguously determine base identity. The sequences were assembled and edited using SeqMan II ver. 4.0 (DNASTAR, Madison, WI, USA). All newly obtained ITS sequences have been deposited in GenBank (KJ956474–KJ956589, Table S1).

Phylogenetic inference

The DNA sequences were aligned using MUSCLE (Edgar, 2004) through the graphical interface in Seaview 4.4.0 (Gouy, Guindon & Gascuel, 2010), with default parameters for gap penalty and extension. The alignment was then edited manually where necessary. Phylogenetic analyses included Bayesian inference (BI) using MrBayes v. 3.2.1 (Ronquist *et al*., 2012) and maximum likelihood (ML) using RAxML v.7.3.1 (Stamatakis, 2006). Substitution models for BI were selected using the program jModelTest 2.1.4 and the Akaike information criterion (Darriba *et al*., 2012), whereas, for ML, we employed GTR + I + G because it is the only model implemented in RAxML. Two simultaneous BI analyses were carried out using a random starting tree, one cold and three heated chains (at default temperature of 0.1) for 40 million generations, sampling every 1000th generation, and default priors for estimated parameters. As the tree topologies were our main interest, we checked chain convergence using tree diagnostics (compare and slide options) in AWTY (Nylander *et al*., 2008). The initial 25% (10 000) of saved trees were discarded as burn-in and the

consensus and posterior probabilities (PPs) of the clades were calculated on the basis of the remaining trees. ML analysis was repeated 1000 times with distinct randomized maximum parsimony starting trees to obtain a set of 1000 trees. The tree with the highest likelihood score was then used in subsequent analyses. Node support values were assessed using bootstrap analysis with 1000 replicates and summarized on the best ML tree.

For four major lineages of *Chaerophyllum*, there are 15 possible topological hypotheses for their branching order (Fig. 1). The posterior support for these hypotheses is the frequency of Bayesian trees congruent with the respective topologies inferred by filtering the posterior tree sample in PAUP* v. 4.0a125 (Swofford, 1998) with a given constraint (Bergsten, Nilsson & Ronquist, 2013).

Fruit sample preparation and scored morphological characters

Fruit anatomical characters were assessed for 31 *Chaerophyllum* spp. represented by 158 mericarp samples (Supporting Information Table S2). Material for fruit analyses was collected from natural localities or obtained from herbarium specimens from the following institutions: ATH, B, BCN, BM, E, FUMH, G, H, K, L, LE, LISU, MA, MW, S, SOM, WU, Mashhad Botanical Garden (Iran) and the Seed and Plant Improvement Institute in Karaj (Iran). Mature mericarps were soaked in water for 6 days, dehydrated in an increasing ethanol series (50%, 70%, 80%, 96%, 100%) and embedded in paraffin according to standard histological methods (Johansen, 1940; Ruzin, 1999). Paraffin blocks were cut into cross-sections, 20–30 μm thick, with a Carl Zeiss Hyrax S30 microtome and then purified with Bio-Clear (Bio Optica). Permanent microscopic slides were prepared by mounting cross-sections in Histokitt (Assistant-Histokitt, Germany). All slides were documented using a Nikon 8400 digital camera mounted on a Nikon Eclipse E200 microscope (Nikon Corporation, Tokyo, Japan) and deposited in the TRN herbarium.

Twenty quantitative anatomical fruit characters (Fig. 2) and one morphological character (fruit length) were measured. Based on these traits, we constructed 13 variables that were subsequently used in phylogenetic and phenetic analyses (Table 1). The carpological terminology followed Kljuykov *et al*. (2004). All anatomical measurements were performed on digital images using a script written in R (R Core Development Team, 2013), which is available from the corresponding author on request.

Analyses of character evolution

We performed non-phylogenetic and phylogenetic analyses to delimit sections in *Chaerophyllum* on the basis of the phenotypic characters of fruits. Non-phylogenetic methods included principal component analysis (PCA) using 13 fruit characters. Using three axes combinations (1 vs. 2, 2 vs. 3 and 1 vs. 3), we delimited each section by calculating the convex hull as implemented in the R function *chull* (Piwczyński *et al*., 2013; R Core Development Team, 2013).

We used the ML method to estimate the ancestral characters for each node of the tree (Schluter *et al*., 1997). Prior to reconstruction, the tree was ultrametricized by applying the penalized likelihood method implemented in the *chronopl* function in the R package ape (Popescu, Huber & Paradis, 2012) with the root node age set to 18.5 Ma (Spalik *et al*., 2010). To visualize our results, we applied the method of Revell (2013). This method interpolates the internal states along branches assuming Brownian motion (Felsenstein, 1985; Revell, 2013). As a result, we can observe the character evolution along branches of the tree as a colour gradient. Moreover, we added a phenogram, i.e. a projection of the phylogenetic tree in the space defined by phenotype and time (Revell, 2012). This helps to visualize the trait distributions and examine their degree of overlap between different clades.

RESULTS

Phylogenetic analyses

Forty-eight sequences were identical to other sequences in the alignment. Among the new sequences, the identical ones belonged to either the same or morphologically similar species (Fig. 3). These duplicate sequences were removed from the phylogenetic analyses. As a consequence, the final data matrix consisted of 88 sequences. *Chaerophyllum aurantiacum* was represented only by ITS1 because of poor DNA quality and problems with the amplification of ITS2. JModeltest with the Akaike information criterion selected two models, SYM + I + G and GTR + I + G with $\Delta = 0.59$, which differ from each other only by the assumption of equal or unequal base frequencies, respectively. We chose the GTR + I + G model for both ML and BI analyses. Trees from these analyses were topologically similar, although many nodes in the BI tree were unresolved and formed polytomies. A Bayesian 50% majority-rule consensus tree with PPs and corresponding bootstrap values (BSs) from ML analysis is presented in Figure 3. The reduced matrix, ML tree and consensus Bayesian tree with clade support were deposited in TreeBASE, study number 16730.

The division of the genus into four monophyletic sections was corroborated by high support values (Fig. 3). However, the relationships between them

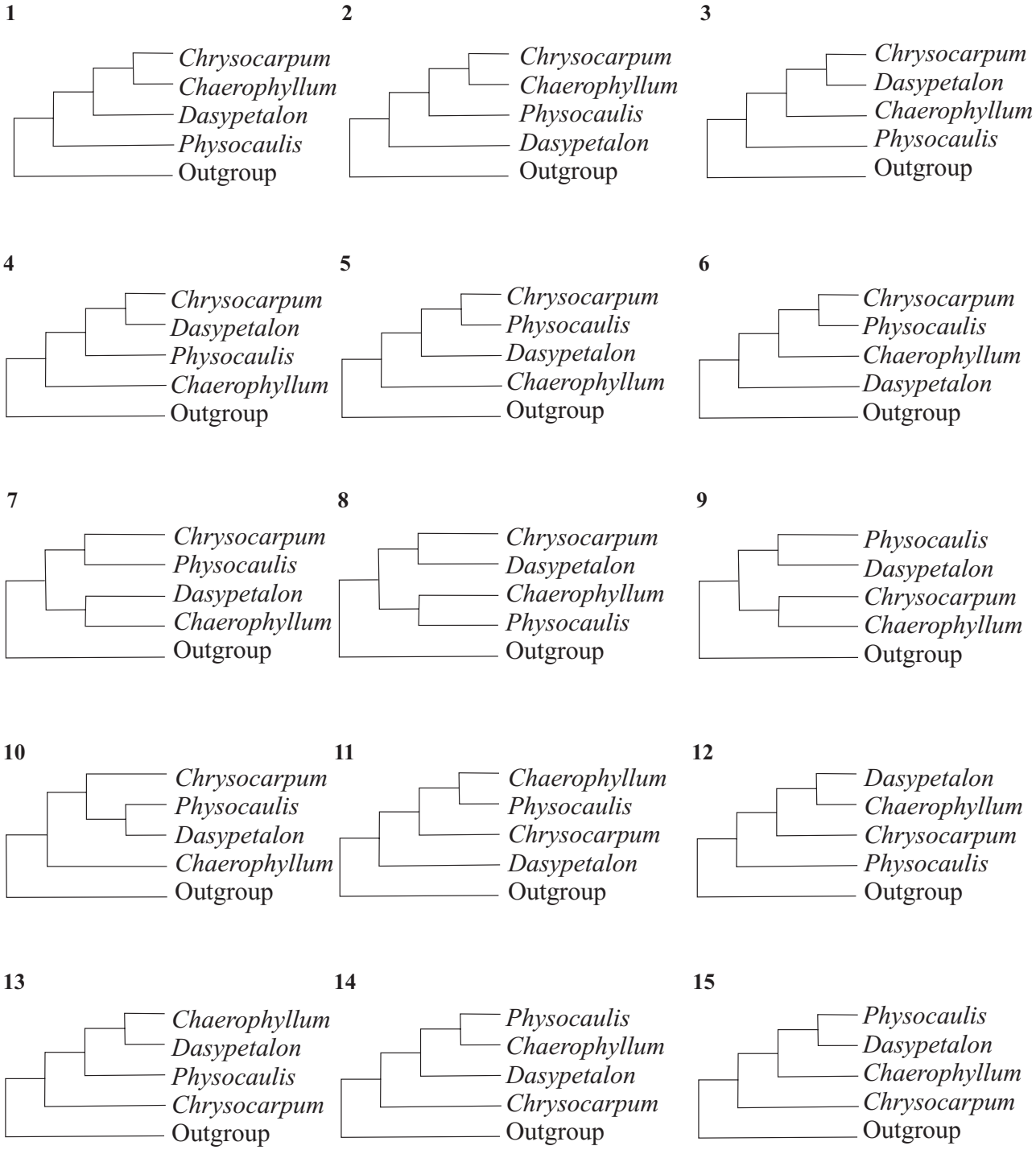

Figure 1. Topological constraints on relationships among major clades of *Chaerophyllum* used in the estimation of their frequency in the posterior set of trees from the Bayesian analysis.

were weakly supported (ML tree) or reduced to a polytomy (BI consensus tree; Fig. 3). This was confirmed by searching the Bayesian posterior tree set for 15 topological hypotheses (Fig. 1), as all were represented in the tree set (Table 2). The fifth scenario with section *Chaerophyllum* branching first and then consecutively *Dasypetalon*, *Physocaulis* and *Chrysocarpum* constituted the highest number of trees in the set (9696/60 000; 16.2%). Similarly, the best ML tree was concordant with this hypothesis

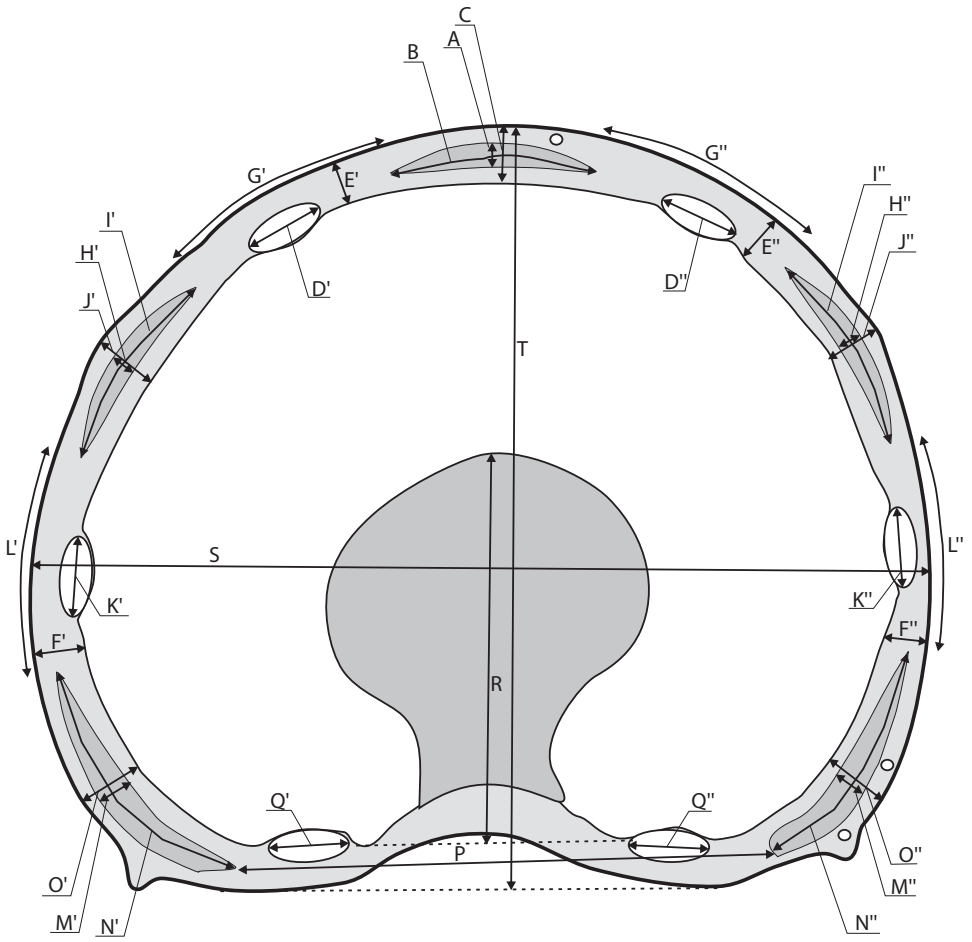

Figure 2. Outline of fruit cross-section of *Chaerophyllum* spp. The characters used for constructing variables in Table 1 are marked. Duplicated letters with prime and double prime symbols refer to symmetric characters. A, Thickness of the vascular bundle in the median rib. B, Width of the vascular bundle in the median rib. C, Thickness of the median rib. D′, D″, Width of vittae (canals) between median and lateral ribs. E′, E″, F′, F″, Pericarp thickness in vallecula (space between ribs). G′, G″, Length of space between median and lateral vascular bundles. H′, H″, Thickness of vascular bundles in lateral ribs. I′, I″, Width of vascular bundles in lateral ribs. J′, J″, Thickness of lateral ribs. K′, K″, Width of vittae between lateral and marginal ribs. L′, L″, Length of space between lateral and marginal vascular bundles. M′, M″, Thickness of vascular bundles in marginal ribs. N′, N″, Width of vascular bundle in marginal ribs. O′, O″, Thickness of marginal ribs. P, Distance between marginal ribs on commissure. Q′, Q″, Commissural vittae width. R, Depth of endosperm furrow measured from line connecting two commissural vittae. S, Mericarp width. T, Mericarp thickness.

(TreeBASE 16730). The lowest number of trees had topologies with section *Chrysocarpum* and *Chaerophyllum* as sister groups (scenarios 1, 2 and 9) and with *Chrysocarpum* as one of the first branches in the genus (scenarios 13 and 15; Table 2, Fig. 1).

The *Chaerophyllum* clade comprised all taxa of that section and *C. heldreichii*. This species was sister to the remaining members of this section (PP = 1.0, BS = 92%). The widely distributed European *C. temulum* L. showed variability in ITS sequences and was divided into two lineages: a western lineage encompassing accessions from France and Algeria and an eastern clade comprising accessions from Russia and Greece, with moderate support in Bayesian and ML analyses. The analyses confirmed the paraphyletic status of *Chaerophyllum s.s.*, as two North American species, *C. tainturieri* and *C. procumbens*, were nested inside the '*Oreomyrrhis*' clade. In the section *Dasypetalon*, only three distinct taxa were recognized (*C. villarsii* W.D.J.Koch, *C. magellense* Ten. and *C. hirsutum*), the latter encompassing *C. elegans* Gaudin and *C. calabricum* which have identical ITS sequences (Fig. 3). The accessions of *C. nodosum* clustered together, forming an isolated lineage that supported its placement in the monotypic section *Physocaulis*. Most members of the genus were placed in section *Chrysocarpum*. Taxa from

Table 1. Anatomical characters of fruits used in the analyses. The letters in character formulae refer to the traits illustrated in Figure 2

No.	Formula	Description	Comments
1	–	Fruit length	There is considerable variation in fruit length among members of the genus. Fruit length is also strongly correlated with mass
2	S/T	Ratio of mericarp width to its thickness	This variable quantifies the shape of the mericarp cross-section. A value above unity means that the mericarp is compressed dorsally
3	A/B	Ratio of thickness of median vascular bundle to its width	All members of the genus have compressed bundles, although to different degrees – from highly flattened to more or less rounded
4	(H′/I′ + H″/I″)/2	Ratio of thickness of lateral vascular bundles to their width	See above
5	(M′/N′ + M″/N″)/2	Ratio of thickness of marginal vascular bundles to their width	See above
6	R/T	Ratio of furrow depth to mericarp thickness	Furrow depth can change considerably from almost flat endosperm to deep endosperm incision on commissure
7	(Q′ + Q″)/(P × 2)	Ratio of width of commissural canals to commissure width	This variable describes to what extent the commissure is composed of canals
8	(D′/G′ + D″/G″)/2	Ratio of width of canals in space between median and lateral vascular bundles to length of this space	The space between bundles can be filled to various degrees with canals. This variable depends not only on the size of canals, but also on the size of vascular bundles. For example, *C. nodosum* has very wide bundles with small spaces between. In consequence, the ratio in this case is above unity as canals overlap with bundles
9	(K′/L′ + K″/L″)/2	Ratio of width of canals in space between lateral and marginal vascular bundles to length of this space	See above
10	(E′ + E″ + F′ + F″)/(4 × C)	Ratio of mean thickness of pericarp to thickness of median rib	The ribs can be variously protuberant relative to the thickness of the pericarp
11	(E′ + E″ + F′ + F″)/(2 × (J′ + J″))	Ratio of mean thickness of pericarp to thickness of lateral ribs	See above
12	(E′ + E″ + F′ + F″)/(2 × (O′ + O″))	Ratio of mean thickness of pericarp to thickness of marginal ribs	See above
13	(E′ + E″ + F′ + F″)/(4 × fruit length)	Ratio of mean thickness of pericarp to fruit length	Fruit length changes dramatically in the genus and this variable quantifies to what degree the thickness of the pericarp changes accordingly

the *C. reflexum–C. villosum* complex were paraphyletic in relation to other species from this clade. A single specimen of *C. reflexum* var. *reflexum* was sister to the remainder of the section, whereas other members of this group constituted three clades with various support: (1) *C. villosum* (PP = 0.93, BS = 95%); (2) *C. reflexum* var. *tuberosum* and var. *acuminatum* (PP = 0.97, BS = 69%); and (3) *C. reflexum* var. *occidentale* (PP = 1.0, BS = 84%). The accessions identified as *C. reflexum* var. *reflexum* were placed in clades 2 and 3.

Apart from the *C. reflexum–C. villosum* complex, the other members of section *Chrysocarpum* formed a clade with high PP (0.99) and moderate BS (85%) support. However, the relationships in this clade were poorly resolved, with many subclades having relatively low internal support. Three species that had been previously synonymized on the basis of morphology clustered with their presumed conspecifics, *C. confusum* with *C. angelicifolium* and *C. kiapazi* (= *C. humile* var. *kiapazi*) with *C. humile*, or had

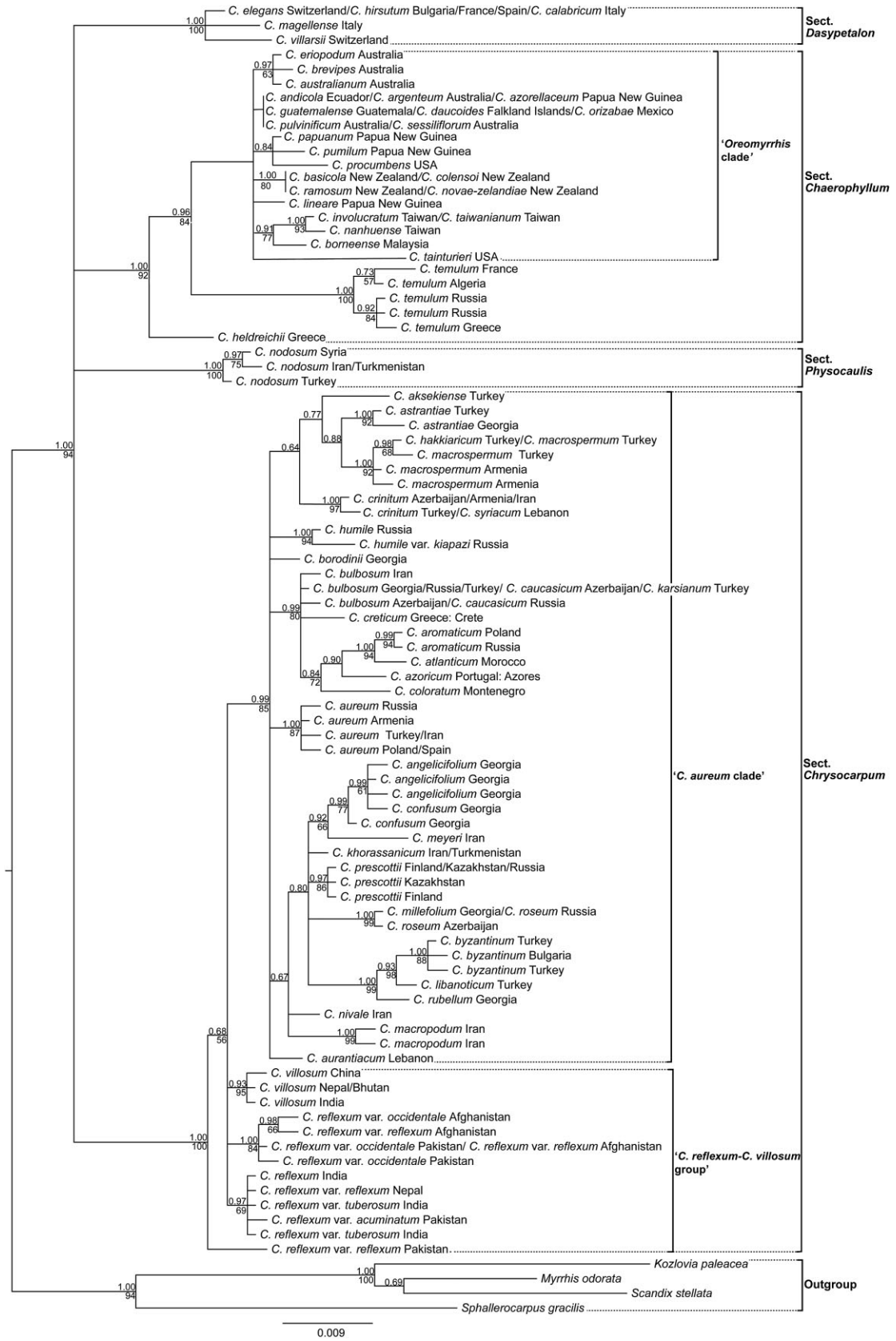

Figure 3. See caption on next page.

Figure 3. 50% majority-rule consensus tree obtained from Bayesian analysis of 92 terminals representing *Chaerophyllum* and outgroups using the GTR + G + I nucleotide substitution model. Posterior probabilities (PP > 0.5) and bootstrap values (BS > 50%) from maximum likelihood analyses are given above and below the branches, respectively. Clades and corresponding section names are given.

Table 2. The number and percentage of trees congruent to 15 topological hypotheses on the relationships among sections of *Chaerophyllum* in a Bayesian posterior set consisting of 60 000 trees. Hypothesis numbers are the same as in Figure 1

Hypothesis no.	Number of trees	Percentage
1	2231	3.72
2	1926	3.21
3	5290	8.82
4	7417	12.36
5	9696	16.16
6	5439	9.07
7	5051	8.42
8	4801	8.00
9	1303	2.17
10	2656	4.43
11	3824	6.37
12	2504	4.17
13	2250	3.75
14	4075	6.79
15	1534	2.56
	Σ59 997	Σ99.99

identical ITS sequences, as in the case of *C. millefolium* and *C. roseum*. Two morphologically similar species, *C. bulbosum* and *C. prescottii*, were distantly related in the section, and *C. caucasicum* (treated as a synonym of *C. bulbosum*) and a recently described taxon from Turkey, *C. karsianum*, had identical ITS sequences to Caucasian and Turkish accessions of *C. bulbosum*. Similarly, *C. syriacum* was indistinguishable from *C. crinitum* Boiss. *Chaerophyllum angelicifolium*, characterized by doubly compound leaves, belonged to the same clade as two species with the same feature, *C. byzantinum* and *C. libanoticum*, although they were not sister taxa. Other species with bipinnate leaves, *C. aromaticum*, *C. atlanticum* and *C. azoricum*, formed another clade in the section. Two morphologically similar Caucasian endemics, *C. roseum* and *C. rubellum*, were not sister taxa. The Balkan endemic *C. coloratum* grouped with a clade comprising *C. aromaticum*, *C. atlanticum*, *C. azoricum*, *C. creticum* Boiss. & Heldr. and *C. bulbosum* with high PP (0.99) and moderate BS (80%) support. The Levantine endemic *C. aurantiacum* was placed with the Turkish endemic *C. aksekiense* in ML analysis, whereas, in BI, it was part of a large polytomy at the base of the clade.

Phenotypic analyses

The morphology and anatomy of the studied species are presented in Supporting Information Figure S1. The first three principal components (PC1, PC2 and PC3) accounted for 70.31% (33.05%, 21.80% and 15.47%, respectively) of the phenotypic variance of anatomical traits. However, there was no trait that tightly correlated with any of the three axes: all loadings were < 0.5. The phenotypic variation of sections outlined by convex hulls overlapped considerably, with the exception of a point representing the monotypic section *Physocaulis*, which, for two projections, PC1 vs. PC2 and PC1 vs. PC3, was placed outside of any convex hull (Fig. 4). The members of the *C. villosum–C. reflexum* group, forming early-branching clades in section *Chrysocarpum* (Fig. 3), were placed inside the convex hull of this section (Fig. 4).

The ancestral reconstructions for 13 phenotypic traits, performed on the best ML tree, revealed a lack of a single continuous character diagnostic for sections *Chaerophyllum*, *Dasypetalon* and *Chrysocarpum*. Two traits, the ratio of the commissural canal width to the commissure width (character 7; Table 1) and the ratio of the width of the canals in the space between the lateral and marginal vascular bundles to the length of this space (character 8), delimited section *Physocaulis* (Fig. 5). These characters were also relatively tightly correlated with PC1 (loadings > 0.3) and were probably responsible for the extreme value for *C. nodosum* on this axis (Fig. 4). However, all other traits were highly variable, with their values overlapping between sections (Supporting Information Fig. S2). Similarly, phenograms and ancestral reconstructions showed that, for each trait, there were always several outliers belonging to different sections and their extreme values evolved rapidly relative to the time of origin of the genus (Figs 5, S2).

DISCUSSION

Molecular taxonomy of the genus *Chaerophyllum*

The affinities in the genus *Chaerophyllum* were inferred in previous phylogenetic studies (Spalik &

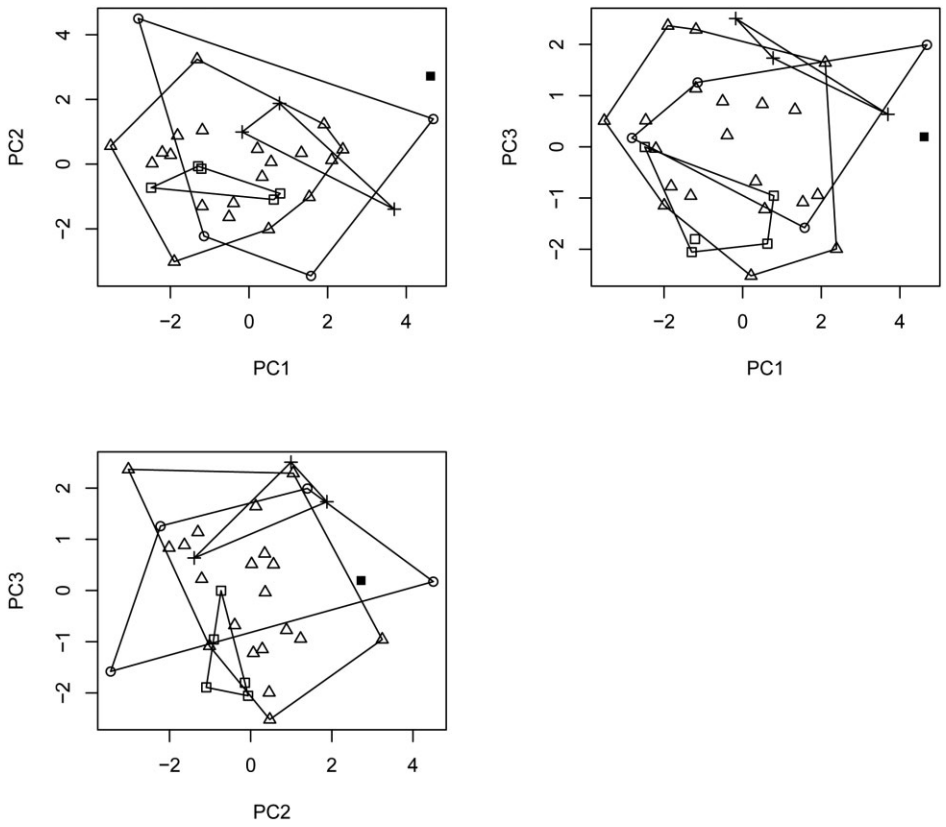

Figure 4. Ordination plots for PC1 vs. PC2, PC1 vs. PC3 and PC2 vs. PC3 for 13 fruit traits of 33 species of *Chaerophyllum*. Convex hulls delimiting sections and the *C. reflexum–C. villosum* complex are illustrated (circles, section *Chaerophyllum*; triangles, section *Chrysocarpum*; open squares, members of *C. reflexum–C. villosum* complex; crosses, section *Dasypetalon*; filled square, section *Physocaulis*).

Downie, 2001; Chung *et al*., 2005; Chung, 2007). However, these studies focused on a specific clade in the genus ('*Oreomyrrhis* clade'; Chung *et al*., 2005; Chung, 2007) or investigated the relationships in the entire subtribe Scandicinae with limited sampling of *Chaerophyllum* (Spalik & Downie, 2001). In our analysis, comprising almost all species of *Chaerophyllum*, we corroborated the results of Spalik & Downie (2001) and showed that, after the addition of species, their taxonomic division is still supported (Fig. 3). Moreover, these authors speculated that most species not included in their study should fall in section *Chrysocarpum*. This was also confirmed by our analysis.

A detailed investigation of intersectional division found that our best ML tree is congruent with the hypothesis (scenario 5; Fig. 1) supported by the highest number of trees in the Bayesian posterior set (Table 2). According to this scenario, section *Physocaulis* does not form the first branch of the genus, despite its morphological and anatomical peculiarities. Moreover, >83% of trees from the posterior set (except scenarios 1, 3 and 12) did not support the first branching position of this section. This suggests a different scenario for trait evolution than the assumption of plesiomorphic fruit characters for *C. nodosum* (Spalik *et al*., 2001a). Two sections, *Chaerophyllum* and *Chrysocarpum*, for which there is a lack of unique characters, do not form sister clades as <10% of all trees support this placement (scenarios 1, 2 and 9). Thus, their reduction to one section is not supported. However, all tested hypotheses had quite even frequencies in the posterior tree set (Table 2), precluding the choice of a convincing best scenario for intersectional relationships.

The new addition to section *Chaerophyllum* is *C. heldreichii*. This Greek endemic does not share a similar morphology with the common *C. temulum*, having doubly compound leaves that are heavily pubescent underneath and long mericarps. However, both species occur in similar habitats: shady and damp places, mainly at the edges of deciduous or coniferous forests (Pimenov & Ostroumova, 2012; see also table 10 in Ellenberg, 2009 and p. 261 in Vladimirov & Tan, 2011). The addition of *C. heldreichii* to section *Chaerophyllum* confirms the European origin of this clade. In summary, this section now includes two European species, two North American

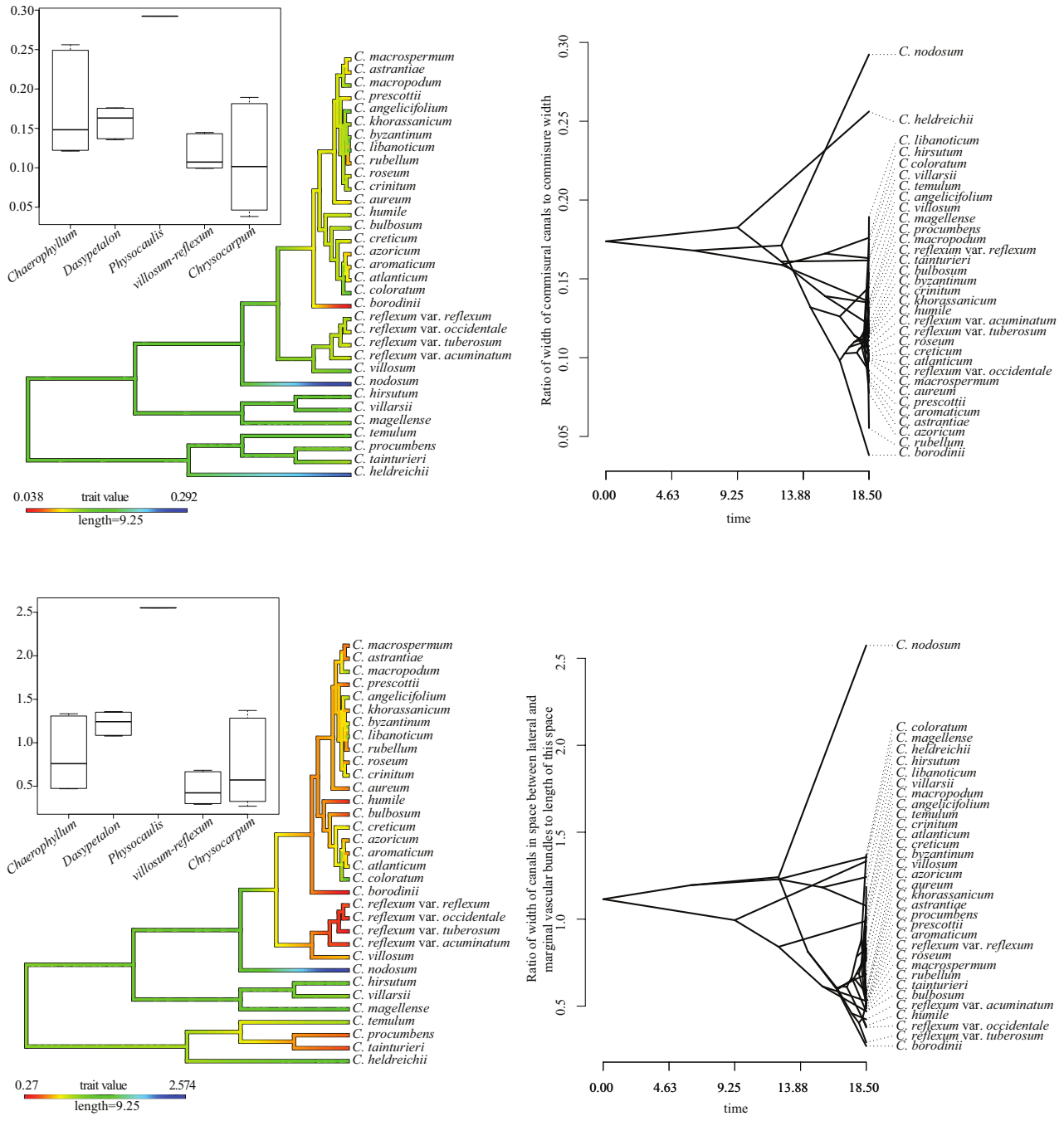

Figure 5. Maximum likelihood ancestral state reconstructions for two traits: ratio of width of commissural canals to commissure width and ratio of width of canals in space between lateral and marginal vascular bundles to length of this space. A phenogram displaying the evolution of traits in phenotypic space and a boxplot showing the distribution of traits in each section and *C. reflexum–C. villosum* complex are also given. The horizontal lines in the middle of the boxes in the boxplot are medians, the boxes delimit 95% of the data, and the horizontal lines marking the box ends are minimum and maximum values.

congeners and >20 members of the mainly Southern Hemisphere '*Oreomyrrhis*' group.

Section *Dasypetalon* comprises species occurring in European mountains. In the *Flora Europaea* account (Tutin *et al*., 1968), three sympatric species (*C. hirsutum*, *C. elegans* and *C. villarsii*) were recognized as the *C. hirsutum* group. These taxa are indeed morphologically similar and differ mainly in the shape of leaf segments. The ITS sequences of *C. hirsutum*, *C. elegans* and *C. calabricum* are identical, suggesting conspecific status. However, more data are needed to reach a firm conclusion. The fruit morphology of *C. elegans* differs from that of *C. hirsutum* to a similar extent as between *C. hirsutum* and genetically different *C. villarsii* and *C. magellense* (Fig. S1). Similar diversity among European montane taxa was found in *Anthriscus* Pers. section *Cacosciadium* (Rchb.) Neilr., also belonging to Scandicinae (Spalik, 1996; Kurzyna-Młynik, 2010). Similarly, as in the *C. hirsutum* group, the differences among taxa were minor and comprised quantitative rather than qualitative characters from leaf and fruit morphology. Allopatric speciation in these complexes was probably triggered by climate change in Europe during the Pleistocene, whereas recent sympatric contact is probably secondary and could lead to admixture at loci that are not under strong disruptive selection (Seehausen *et al*., 2014). The fruit morphology of Italian *C. calabricum* is identical to that of *C. hirsutum*. Its distribution in Calabria constitutes the most southern and isolated occurrence of the *C. hirsutum* group. In conclusion, the determination of the specific status of these taxa requires more data, particularly from population studies and documenting local differentiation and gene flow.

All but one species sampled for the first time for molecular analyses fell in section *Chrysocarpum*. The most interesting result is the early-branching position of the *C. reflexum–C. villosum* complex and its apparent paraphyly with respect to the '*C. aureum* L.' clade comprising the remaining taxa of the section (Fig. 3). The *C. reflexum–C. villosum* clade includes several taxa that are morphologically similar and, as remarked by Hedge & Lamond (1980), 'many intermediates exist and it is not possible to assign every specimen to a particular variety with confidence'. However, our results provided support for the delineation of some taxa. *Chaerophyllum reflexum* var. *tuberosum* and *C. reflexum* var. *acuminatum* (plus an accession of *C. reflexum* var. *reflexum*) were clustered in one clade in agreement with the treatment of Mukherjee & Constance (1993), who united these taxa as one species, *C. acuminatum*. Another clade in the group included specimens of *C. reflexum* var. *occidentale* (plus an accession of *C. reflexum* var. *reflexum*) (Fig. 3). According to Hedge & Lamond (1980), this variety differs from var. *tuberosum* and var. *acuminatum* by having a perennial habit, less divided leaves and non-swollen roots. Mukherjee & Constance (1993) did not recognize this taxon as distinct.

In the *C. reflexum–C. villosum* complex, Mukherjee & Constance (1993) listed *C. orientale* (C.B.Clarke) P.K.Mukherjee non Willd. ex Boiss. This taxon was described by C.B. Clarke from the Naga Hills in India as a variety of *C. reflexum* and later raised to the rank of species by Mukherjee (1982). However, Hedge & Lamond (1980) noted that the specimens from this locality collected by Clarke belong to a distantly related species, *Pimpinella sikkimensis* C.B.Clarke (cf. *Clarke 41861A* and *41861B* in online Kew Herbarium Catalogue; http://apps.kew.org/herbcat/navigator.do). We examined the fruit anatomy of another representative specimen from the Naga Hills, *Koelz 26 220* (L), cited by Mukherjee & Constance (1993; Fig. S1). The shape and size of the vascular bundles and the presence of a layer of weakly lignified parenchymatous cells in the mesocarp indicate that the specimen represents *Pimpinella* L. (Khajepiri *et al*., 2010), therefore confirming the treatment of Hedge & Lamond (1980).

All accessions of annual *C. villosum* formed a clade. Hedge & Lamond (1980) suggested its close relationship with *C. temulum*, but this was not corroborated by molecular data. In contrast, the typical variety of *C. reflexum*, *C. reflexum* var. *reflexum*, was not monophyletic with individual accessions scattered across several lineages (Fig. 3). Apparently, the current taxonomic treatment of the *C. reflexum* group does not mirror the complex speciation pattern in the heterogeneous environment of the highest mountain ranges of Asia.

The '*C. aureum*' clade probably radiated recently, as corroborated by the dated phylogenetic tree of the genus, which shows that the common ancestor of the section appeared at *c.* 5 Myr, whereas the main speciation events leading to recent species took place at *c.* 3–0.5 Myr (Spalik *et al*., 2010). This section is ecologically and phenotypically diversified and it is difficult to pinpoint any morphological character separating it from other sections. Some traits have evidently evolved convergently. For example, the species having bipinnate leaves with broad lobes that were considered as close relatives based on morphology did not cluster together, but formed several clades. Two Caucasian species, *C. roseum* and *C. rubellum*, have been treated as closely related and even subsumed to varietal rank (Pimenov & Ostroumova, 2012) because they differ mainly in the shape and size of the bracteoles and leaf segments. However, molecular data suggest that these taxa are not closely related. Similarly, *C. prescottii* and *C. bulbosum* appear to be distantly related. Conversely, some morphologically close species formed sister taxa or had identical ITS sequences. The

recently described *C. karsianum* has an identical ITS sequence to that of *C. bulbosum*. According to the protologue, it closely resembles *C. bulbosum*, differing only in having sparsely ciliate bracteoles and fading pink petals (Tan & Ocakverdi, 1986). However, sparsely bristled bracteoles are also characteristic for *C. bulbosum* and its inclusive taxon *C. caucasicum* (Schischkin, 1950). Pink petals often occur in montane umbellifers that usually have white petals (e.g. *C. hirsutum* or *Pimpinella major* (L.) Huds., M. Piwczyński, personal observation) and low temperature was found to induce the accumulation of anthocyanins in another member of the family (*Oenanthe stolonifera* DC.: Hasegawa *et al*., 2001). We suspect that a similar problem arises for *C. posofianum* for which DNA sequences are unavailable; all traits that have been used to differentiate this species from widely distributed *C. bulbosum* (see table 1 in Erik & Demirkuş, 1998) fall within its variability (Schischkin, 1950; M. Piwczyński, unpubl. data). However, as in the case of the *C. hirsutum* group, the final decision on the specific status of *C. karsianum* and *C. posofianum* must be postponed until further molecular data are available.

Taxonomic value and evolution of anatomical fruit characters

The majority of fruit anatomical characters do not support the monophyly of sections of *Chaerophyllum* delineated using molecular data. Most of these traits exhibit considerable variation in the sections, resulting in a wide phenotypic space (Fig. 4). An illustrative example is section *Chaerophyllum* which, despite being represented in our analyses by fewer species than section *Chrysocarpum*, has a similar or even larger area inside the convex hull (Fig. 4). Traditionally, classification in Apiaceae has relied heavily on fruit characters. However, molecular evidence has put into question many of the relationships suggested by fruit morphology. The high levels of homoplasy among fruit characters demonstrated by mapping these features on molecular trees could partially be explained by selection, which may support similar solutions in similar environments. For example, wings of dorsally compressed fruits can be interpreted as an adaptation to dispersal in open habitats. This trait evolved many times in the evolutionary history of the family (Liu *et al*., 2006). Despite this homoplasy, fruit morphology can provide valuable structural characters to supplement the results based on molecular data (Liu *et al*., 2006, 2012). In tribe Oenantheae, for instance, the clades formed from traditionally recognized genera *Ptilimnium* Raf. and *Oxypolis* Raf. were delimited by the numbers and size of vittae, the presence and size of lateral wings and the presence or absence of lignified cell layers in the mesocarp (Feist *et al*., 2012). In our study, however, only *C. nodosum* from the monotypic section *Physocaulis* was anatomically different (Fig. 5). This species is also morphologically distinct from other members of the genus and was, until recently, placed in the monotypic genus *Myrrhoides* Heist. ex Fabr. (Pimenov & Ostroumova, 2012). The substantial variation of fruit characters in other sections in *Chaerophyllum* (Figs 4, 5) may be attributed to different life history strategies and habitats occupied by species, which occur in deserts, forests and meadows at low and high elevations, and have a monocarpic or polycarpic strategy. For example, variation in fruit size is substantial within the genus (Figs S1, S2). In the literature, many significant correlations have been identified between seed mass and ecological factors or plant life history strategies. The most important correlates are light regime, water availability, dispersal mode and plant characteristics, such as height (Leishman *et al*., 2000). All may contribute to the evolution of seed size in *Chaerophyllum*. Traits contributing to a given function, however, do not evolve independently, but instead co-vary (Berg, 1960). Our PCA, based on a correlation matrix, showed that size is correlated with several anatomical traits: positively with the ratio of the width of the canals in the space between the vascular bundles to the length of this space ($r = 0.51$; character 8 in Table 1) and negatively with the ratio of the mean thickness of the pericarp to the fruit length ($r = -0.69$, both correlations were significant after Bonferroni correction; character 13). These correlations suggest that, when the size of a fruit increases, the relative thickness of the pericarp decreases and, simultaneously, the plant invests in other protective structures, such as wide bundles and canals. The broadening of vascular bundles by the development of sclerenchymatic tissue may provide a strong barrier against intruders, because larger seeds may be preferentially eaten by insects or vertebrates (e.g. Hare, 1980; Alexander *et al*., 2001). Similar relations were observed in Scandicinae, in which different forms of fruit and endosperm protection rarely occur together; species characterized by a thickened cuticle usually have reduced vittae and bundles (Spalik *et al*., 2001a).

Recently, several taxonomic surveys of genera of Apiaceae have been performed exclusively on the basis of fruit anatomy (e.g. Khajepiri *et al*., 2010; Akalın Uruşak & Kızılarslan, 2013). According to our results, this approach must be supplemented by molecular data. For example, the introduction of new genera based exclusively on fruit characters and morphology (see *Pseudopimpinella* F.Ghahrem, Khajepiri & Mozaff. in Khajepiri *et al*., 2010) may be erroneous, even in the case of high phenotypic distinction of the species, as in *Chaerophyllum nodosum*. The fruit

anatomist should also take into account the intraspecific variation of fruit phenotypes. In both studies cited above, some of the traits are given without standard deviations (e.g. ratio of mericarp width to its thickness); mericarp width is influenced by both measurement method and natural variation. Another problem is the occurrence of aberrations during fruit development. In our study, several mericarps from different species had additional vascular bundles and canals, causing asymmetrical development of fruit (Supporting Information Fig. S1). *Pimpinella tragioides* (Boiss.) Benth. & Hook.f. with seven vascular bundles, in Khajepiri *et al*. (2010), is apparently an example of such aberrant development. Similarly, an illustration of the fruit cross-section of *C. aksekiense* from the species description shows only four vascular bundles with some canals arranged asymmetrically over the bundles (see fig. 1 in Duran & Duman, 1999). Such anomalous structures should not be considered in taxonomic descriptions.

CONCLUSIONS

Our study corroborated the division of the genus *Chaerophyllum* into four sections, *Chaerophyllum*, *Dasypetalon*, *Physocaulis* and *Chrysocarpum*, postulated by Spalik & Downie (2001). The most interesting findings are the addition of Greek endemic *C. heldreichii* to section *Chaerophyllum* and the early-branching position of the highly variable Asiatic *C. reflexum–C. villosum* complex in section *Chrysocarpum*. However, with the exception of *C. nodosum*, we were unable to find carpological traits useful in confirming this division. We hypothesized that anatomical traits may change as a result of natural selection or co-vary with other highly variable characters during the diversification of species in different habitats.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Morphological and anatomical fruit variation in *Chaerophyllum*, anomalous anatomical phenotypes and the cross-section of fruit of a *C. orientale* accession from the Naga Hills.

Figure S2. Maximum likelihood ancestral state reconstruction for fruit traits. A phenogram displaying the evolution of traits in phenotypic space and a boxplot showing the distribution of traits are also given. Boxes represent 95% of the data; horizontal lines denote minimum, median and maximum values.

Table S1. Species names, GenBank accession numbers and voucher information for specimens for which the nuclear rDNA ITS region was sequenced in this study.

Table S2. Accessions of *Chaerophyllum* examined for fruit anatomy.