The systematic position of Chamaesciadium C. A. Meyer (Umbelliferae) on the basis of nuclear ITS sequence

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Abstract

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The position of the genus *Chamaesciadium* C. A. Meyer (*Apiaceae*) was investigated with a phylogenetic analysis based on the ribosomal internal transcribed spacers. Parsimony, maximum likelihood and Bayesian support analyses were adopted. *Chamaesciadium* resulted nested within tribe *Careae*. The results indicated that the genera resulting closer to *Chamaesciadium* were *Carum* s. s. (the part of the genus to which *Carum carvi* belongs), *Fuernrohria* and *Grammosciadium*: these genera clustered together in a clade with 100% bayesian and bootstrap support. This group was sister the other genera of tribe *Careae* here considered (*Rhabdosciadium*, *Falcaria*, *Aegokeras*, *Aegopodium*) with 100% Bayesian and 91% bootstrap support. Tribe *Careae* resulted outgroup to tribe *Pyramidoptereae* and other species of genus *Carum* resulted nested in this last tribe rather than in tribe *Careae*.
The possible previously indicated relationship of *Chamaesciadium* to *Pycnocycla* was excluded at least with reference to *Pycnocycla aucherana*, since this species clustered far away from tribes *Careae* and *Pyramidoptereae* resulting more strictly related, among the considered species, to *Trachyspermum aethusifolium*.

Introduction

Phylogenetic relationships in family *Apiaceae* subfam. *Apioideae* have been particularly difficult to resolve (Katz-Downie & al. 1999). Despite in the last years many researchers have worked on this group, often indicating incongruence between molecular data and previous taxonomical treatements, the most widely used classification is still that proposed by Drude (1897-1898) in Engler & Prantl's 'Die natürlichen Pflanzenfamilien' and derivation of it. Despite it, most recent cladistic analysis of molecular data (for instance Downie and Katz-Downie 1996, Downie & al. 1998; Kondo & al. 1996; Valiejo-Roman & al. 1998; Downie & al. 2000) supported the hypothesis that many of Drude's tribal and subtribal taxa were not monophyletic.

Chamaesciadium C. A. Meyer is an interesting old world mountain genus of *Umbelliferae* family ranging from Caucasus to Iran to Himalaya (Heywood 1971; Farille & al. 1985). Genus *Chamaesciadium* is monotypic after Heywood (1971), while Hiroe

(1979) included in genus *Chamaesciadium* also the representatives of genus *Pycnocycla* Lindl., considered by other authors as a member of Subfamily *Apioideae*, Tribe *Echinophoreae* (Heywood 1971; Hedge & Lamond 1973, 1978). Instead *Chamaesciadium* is considered a member of Subfamily *Apioideae*, Tribe *Apieae* by Pimenov and Leonov (1993). More recent general treatment on Umbellifers maintain *Pycnocycla* autonomous from *Chamaesciadium* (Pimenov & Leonov 1993).

One of the most used molecular markers in Apioideae and other Angiospermae have been the nuclear Internal Transcribed Spacers of ribosomal DNA (Baldwin & al. 1995) and a wide sampling of sequences of Apiaceae are available on Genbank from previous studies. Even if the general utility of ITS in studying phylogeny has been recently posed in doubt (Alvarez & Wendel 2003), a huge amount of ITS data is available for *Apiaceae* and the utility of the ITS markers has been demonstrated at least comparing them to the plastidial markers (Chandler & Plunkett 2004).

The aim of this work was to assess the phylogenetic position of genus *Chamaesciadium* using the Internal Transcribed Spacers as molecular markers.

Material and methods

1. Examined material

Silica gel preserved samples of leaf tissue of *Chamaesciadium acaule* C. A. Meyer were collected during the OPTIMA XI ITER TO ARMENIA (June-July 2002) in the following location: Aragatsotn province; Ashtarak distr., Mt. Aragats, c. 20 km N of Ashtarak, c. 1 km SE observatory, vicinity of the Lake Karilich, 3160 m a.s.l.; 44°11'"E/40°28'"N; alpine meadows, on the 30.06.2002. The herbarium exsiccata are conserved by the Herbarium Centrale Italicum in Florence, Italy and by the other herbaria receiving the OPTIMA specimens (for further details: http://www.nhm-wien.ac.at/nhm/Botanik/news.htm). Also *Carum multiflorum* (Sibth. & Sm.) Boiss. = *Hellenocarum multiflorum* (Sibth. & Sm.) Wolff, *Carum heldreichii* Boiss and *Carum appuanum* (Viv.) Grande were used to obtain thei ITS sequences that were inserted in this study (Tab. 1).

2. DNA Extraction

Genomic DNA was isolated using a modified CTAB extraction protocol (Doyle & Doyle 1990; tissue ground in sea-sand, 70% [v/v] isopropanol substituted for the RNase step). Approximately 40 mg of leaf tissue were used for each extraction.DNA concentrations were estimated by gel electrophoresis on 1% agarose.

3. PCR conditions

PCR reactions were carried out with 10 ng of genomic DNA in 50 µl volume with 1,25 U of Taq polymerase (by Takara) for each reaction. The primers were: on the 18S sequence: 5'-CGTAACAAGGTTTCCGTAG and on the 25S: 5'-AGTCCGCCCTGATGGGCGA. The adopted thermal cycling profile consisted in 35 cycles of 1 min at 94°

Table 1. Accession of Apiaceae used in these study (ITS sequences). When a single Genbank (GBAN) accession number is indicated, the whole ITS1-5.8S-ITS2 is intended, otherwise the first accession correspond to the ITS1 and the second accession to the ITS2. Species sequenced by the author are underlined. Herbarium samples are available by the authors.

Aegokeras caespitosa (Sibth. & Sm.) Raf.	Downie & al. 1998	GBAN U78379, GBAN U78439
Aegopodium alpestre Ledeb.	Downie & al. 1998	GBAN U78376, GBAN U78436
Aegopodium podagraria L.	Downie & Katz-Downie, 1996	GBAN U30536, GBAN U30537
Angelica archangelica L.	Downie & Katz-Downie, 1996	GBAN U30576, GBAN U30577
Anthriscus cerefolium (L.) Hoffm.	Downie & al. 1998	GBAN U30532, GBAN U30533
Apium graveolens L.	Downie & al. 1998	GBAN U30552, GBAN U30553
Arracacia brandegei J. M. Coult. & Rose	Downie & Katz-Downie, 1996	GBAN U30570, GBAN U30571
Bunium elegans (Fenzl) Freyn	Downie & al. 2000	GBAN AF073543, GBAN AF073544
Capnophyllum dichotomum Lag.	Downie & al. 1998	GBAN U78390, GBAN U78391
Carum appuanum (Viv.) Grande	Monte Matanna, Alpi Apuane, Tuscany	GBAN AY840984, GBAN AY840985
Carum carvi L. (a)	Valiejo-Roman & al. 1998	GBAN AF077878
Carum carvi L. (b)	Downie & al. 1998	GBAN U78377, GBAN U78437
Carum heldreichii Boiss.	Lago Scaffaiolo, Appennines, Tuscany	GBAN AY840988, GBAN AY840989
Carum multiflorum (Sibth. & Sm.) Boiss. = *Hellenocarum multiflorum* (Sibth. & Sm.) Wolff	Gravina di Laterza (Taranto), South-East Italy	GBAN AY840986, GBAN AY840987
Chaerophyllum aureum L.	Downie & al. 2000	GBAN AF073655, GBAN AF073656
Chamaesciadium acaule C. A. Meyer	Mt. Aragats, Armenia	GBAN AY957495, GBAN AY957496
Ciclospermum leptophyllum (Pers.) Sprague	Downie & al. 2002	GBAN AF358471, GBAN AF358538
Cnidium silaedium Fiori & Paol.	Downie & al. 1998	GBAN U78407, GBAN U78467
Coriandrum sativum L.	Downie & Katz-Downie, 1996	GBAN U30586, GBAN U30587
Crithmum maritimum L.	Downie & Katz-Downie, 1996	GBAN U30540, GBAN U30541
Elaeosticta allioides (Regel & Schmalh.) E.V.Klyuikov , M.G.Pimenov & V.N.Tikhom.	Downie & al. 2000	GBAN AF73547, GBAN AF73548
Falcaria vulgaris Bernh.	Downie & al. 1998	GBAN U78378, GBAN U78438
Ferula assa-foetida L.	Downie & al. 1998	GBAN U78391, GBAN U78451
Foeniculum vulgare Mill.	Downie & al. 1998	GBAN U78385, GBAN U78445
Fuernrohria setifolia K. Koch	Katz-Downie & al. 1999	GBAN AF008633, GBAN AF009112
Grammosciadium daucoides DC.	Downie & al. 2000	GBAN AF073559, GBAN AF073560
Grammosciadium macrodon Boiss.	Downie & al. 2000	GBAN AF073553, GBAN AF073554
Grammosciadium platycarpum Boiss. & Hausskn.	Downie & al. 2000	GBAN AF073551, GBAN AF073552
Grammosciadium pterocarpum Boiss.	Downie & al. 2000	GBAN AF073557, GBAN AF073558
Grammosciadium scabridum Boiss.	Downie & al. 2000	GBAN AF073555, GBAN AF073556
Hacquetia epipactis DC.	Valiejo-Roman & al. 1998	GBAN AF07792
Heracleum sphondylium L.	Downie & Katz-Downie, 1996	GBAN U30544, GBAN U30544
Komarovia anisosperma Korovin	Downie & al. 1998	GBAN U78381, GBAN U78441

Table 1. Continued.

Lagoecia cuminoides L.	Valiejo-Roman & al. 2002	GBAN AF337179, GBAN AF337187
Laserpitium siler L.	Downie & al. 1998	GBAN U30528, GBAN U30529
Levisticum officinale Koch	Downie & al. 1998	GBAN U78389, GBAN U78449
Ligusticum porteri J. M. Coult. & Rose	Downie & al. 1998	GBAN U78375, GBAN U78435
Oedibasis platycarpa (Lipsky) Koso-Pol.	Katz-Downie & al. 1999	GBAN AF008632, GBAN AF009106
Oenanthe pimpinelloides L.	Downie & al. 1998	GBAN U78371, GBAN U78431
Pastinaca sativa L.	Downie & al. 1998	GBAN U30546, GBAN U30547
Peucedanum coriaceum Rchb.	Spalik & al. 2004	GBAN AF495824, GBAN AF495825
Physospermum cornubiense (L.) DC.	Downie & al. 1998	GBAN U78382, GBAN U78442
Pimpinella peregrina L.	Downie & al. 1998	GBAN U30592, GBAN U30593
Prangos pabularia Lindl.	Downie & al. 1998	GBAN U78409, GBAN U78469
Pycnocycla aucherana Boiss.	Downie & al. 2000	GBAN AF073533, GBAN AF073534
Pyramidoptera cabulica Boiss.	Katz-Downie & al. 1999	GBAN AF008631, GBAN AF009110
Rhabdosciadium aucheri Boiss.	Downie & al. 2000	GBAN AF073549, GBAN AF073550
Rhodosciadium argutum (Rose) Mathias & Constance	Downie & Katz-Downie, 1996	GBAN U30566, GBAN U30567
Sanicula europaea L.	Vargas & al. 1998	GBAN AF031964
Scaligeria moreana Engstrand	Downie & al. 2000	GBAN AF73545, GBAN AF73546
Scandix iberica M. Bieb.	Downie & al. 2000	GBAN AF073627, GBAN AF073628
Seseli krylovii (V.Tichom.) Pimenov & Sdobnina	Downie & al. 1998	GBAN U78402, GBAN U78462
Smyrniopsis aucheri Boiss.	Downie & al. 1998	GBAN U78393, GBAN U78453
Smyrnium olusatrum L.	Downie & al. 1998	GBAN U30594, GBAN U30594
Torilis nodosa (L.) Gaertn.	Downie & al. 1998	GBAN U30534, GBAN U30535
Trachyspermum aethusifolium Chiov.	Downie & al. 2000b	GBAN AF164845, GBAN AF164870
Trachyspermum ammi (L.) Sprague	Downie & al. 1998	GBAN U78380, GBAN U78440

C, 1 min at 55° C, 2 min at 72° C. Clear cut single-banded fragments were visualised on 1% agarose gels. The amplification products were purified by run on 1% agarose gel and cutting and purifying the observed bands with a Macherey-Nagel kit. The fragments were directly sequenced in both directions by using the above described primers with an automated sequencer 310 by Perkin Elmer by the CIBIACI (Center for Biotechnological Services) of the University of Florence. Asymmetrical PCR cycle Sequencing and the BigDye Terminator Ready Reaction Kit (Applied Biosystems) were used.

4. Sequence and phylogenetic analysis

Resulting ITS sequences were further checked by eye with the software CHROMAS 1.43 (C. McCarthy, School of Biomolecular and Biomedical Sciences, Brisbane, Australia)

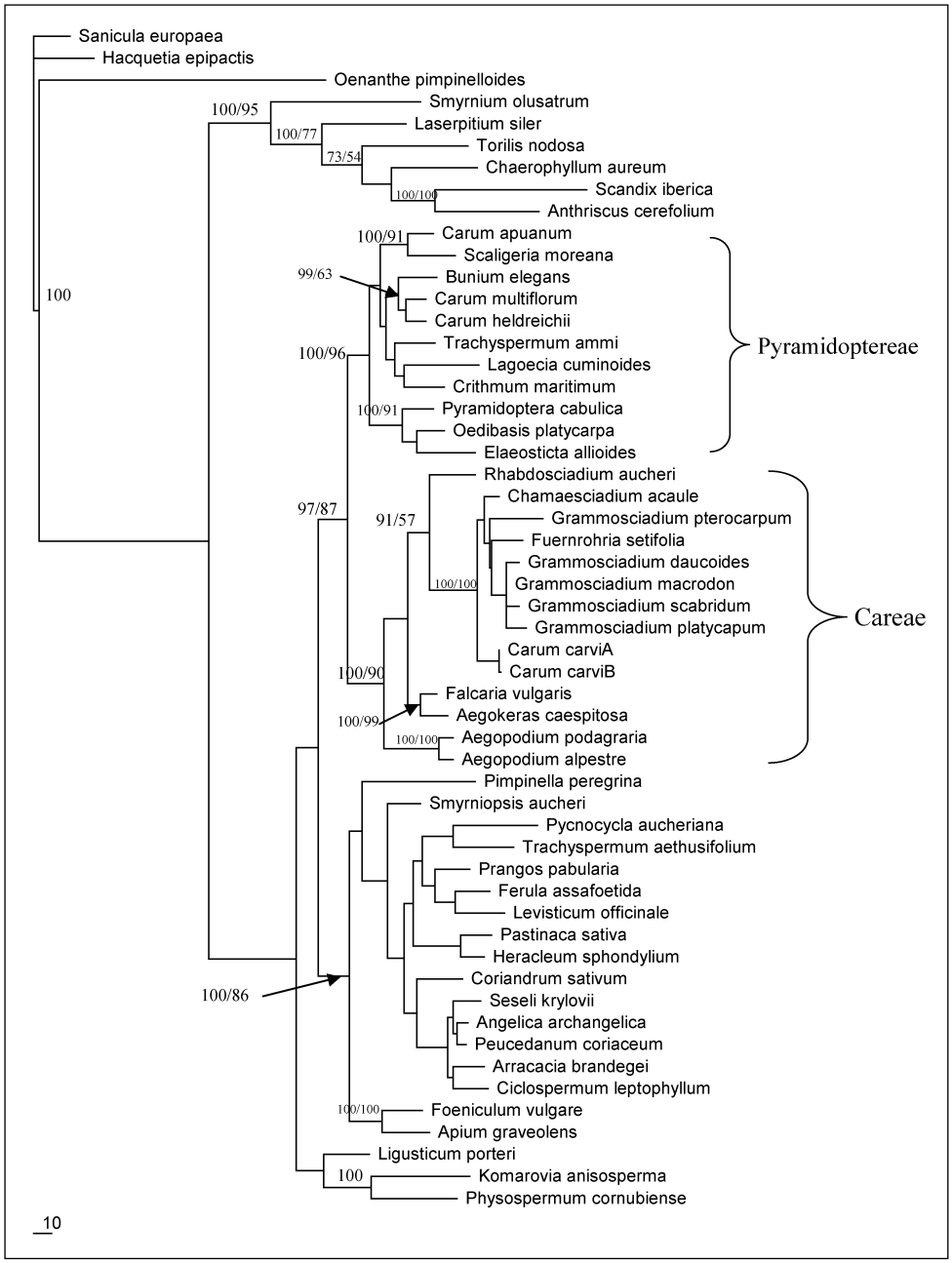

Fig. 1. One of the 100 maximum parsimony trees, 1895 steps long, CI = 0.437 and RI = 0.615. This tree corresponds to the maximum likelihood tree. Parsimony bootstrap (on the left) and Bayesian (on the right) support (separated by a slash) indicated above branches. If only one value is present it is the only Bayesian support value while the bootstrap support value was lower than 50%. Support values on branches less important for the aim of this paper were omitted to enhance readability of the figure.

while a BLAST (Altschul & al. 1997) search was performed to exclude the sequencing of any contaminant organism.

The new ITS sequences of *Chamaesciadium acaule* produced during our investigation were deposited in Genbank (Genbank accession numbers AY957495 for the ITS1 and AY957496 for the ITS2).

Other sequences available in Genbank were chosen sampling adequately all main clades of Umbellifers observed in previous molecular studies (in particular Katz-Downie & al. 1999) and in recent analyses by the author on genus *Carum*. Two members of Apiaceae subfamily Saniculoideae, *Sanicula europaea* L. and *Hacquetia epipactis* (Scop.) DC were chosen as outgroups.

Optimal multiple alignment was obtained with CLUSTALW 1.81 (Thompson & al. 1994) and checked by eye. Parsimony analysis was performed with PAUP 4.0b1 (Swofford 1998) for PC.

All characters were weighted equally, and character state transitions were treated as unordered. Gaps were treated after Simmons & Ochoterena (2000) and coded with Simple Gap Coding using the software Gapcoder (Young & Healy 2003). This process codes indels as separate characters in a data matrix, which is then considered along with the DNA base characters in phylogenetic analysis.

The maximum parsimony analysis was done with with 100 replicated heuristic searches, using random stepwise addition of taxa, tree bisection reconnection (TBR) branch swapping, and MULPARS in effect. Bootstrap (Felsenstein 1985) resampling was performed using TBR branch-swapping with ten random taxon entries per replicate and multrees option in effect with 100 replicates.

A maximum likelihood (Felsenstein 1981) search approach was done as follows: we used Modeltest 3.06 (Posada and Crandall 1998) to evaluate the likelihood of 56 different models of sequence evolution on the basis of our data. The likelihood ratio test option in Modeltest 3.06 was used to compare likelihood scores in a nested design. We used the most likely model of evolution from Modeltest 3.06 as settings in a maximum likelihood (ML) phylogenetic analysis in PAUP. We used also MrMODELTEST 2.0 (Nylander 2004) to evaluate the best likelihood model for comparing with results of Modeltest and because the output of this second software is faster to use with the program for Bayesian Inference MrBayes 3.4b4 (Huelsenbeck 2001).

The maximum likelihood heuristic search was done with 10 random additions and TBR branch swapping, and the command ADDSEQ = ASIS with PAUP.

The Bayesian analysis was done using the model of sequence evolution indicated by MRMODELTEST based on the Akaike Information criterion (Akaike 1974). The Bayesian phylogenetic analysis was used for assessing the robustness of tree topology and the support for clades. The posterior probability of the phylogenetic model was estimated using Markov chain Monte Carlo (MCMC) sampling with the Metropolis-Hastings-Green algorithm. Four chains were run, three heated and one cold, for 10^6 generations and sampled every 100 generations. Following the analysis, the posterior probabilities were checked in the output of Mrbayes to estimate the number of trees that should be discarded as "burn-in". Stationarity was reached at approximately generation 20,000, so the first 200 trees or "burn-in" period of the chain were discarded. Phylogenetic inferences are therefore based on those trees sampled after generation 20,000.

After the "burn-in" trees were removed from the data set, the remaining trees were used to produce a 50% majority-rule consensus tree (with PAUP) in which the percentage support indicated a measure of the Bayesian posterior probabilities.

The use of Bayesian analyses for phylogenetic inference is still in exploratory phase (Huelsenbeck & al. 2002) and hence we compared the results with those obtained with maximum parsimony (with bootstrap) and maximum likelihood.

5. Mericarp sections

Mericarps were taken from herbarium specimens, partially rehydrated in 1% Saccharose solution and sectioned with a cryostat Cryocut A/O. The slides were stained with Toluidine Blue and observed at a Leitz light Microscope.

Results and discussion

The total alignment (ITS1+ITS2) was 484 bp long, plus 104 characters derived from indels coding (simple gaps coding) in the matrix used for maximum parsimony. ITS1 length of *Chamaesciadium* was 215 bp while the ITS2 reached 224 bp of length.

For parsimony analysis 104 characters resulted constant, 129 variable characters were parsimony-uninformative and 355 parsimony-informative.

Maximum parsimony analysis produced 100 maximum parsimony trees 1953 steps long, CI = 0.434 and RI = 0.612.

The software Modeltest indicated the model TrN+I+G after the hierarchical likelihood ratio test. In Fig. 1 one of the maximum parsimony trees corresponding to the maximum likelihood tree is described. Bootstrap (parsimony) and bayesian support are reported above branches. Maximum parsimony with bootstrap support and Maximum likelihood with Bayesian support were concordant for the position of genus *Chamaesciadium*. This genus clustered within tribe *Careae* and resulted strictly related to *Carum carvi*, *Grammosciadium* and *Fuernrohria* (100% Bootstrap and Bayesian support). *Rhabdosciadium aucheri* resulted outgroup to this clade (57% Bootstrap and 91% Bayesian support). *Aegopodium*, *Falcaria* and *Aegokeras* resulted the most basal genera of tribe *Careae*. Tribe *Careae* received a 100% Bayesian support as tribe *Pyramidoptereae* (90% and 96% of Bootstrap, respectively). This two tribes resulted sister groups with 87% Bootstrap and 97% Bayesian support. Other investigated species of *Carum* clustered within tribe *Pyramidoptereae*, rather than in tribe *Careae* as the type species of the genus – *Carum carvi* – did.

The mericarp section of *Chamaesciadium acaule* (Fig. 2), for number and position of vittae resulted quite similar to those indicated in literature for *Carum carvi and other species of tribe Careae* as *Fuernrohria setifolia*. This result agreed with molecular data analysis.

After Wolff (1927) genera *Carum, Falcaria, Olymposciadium, Hellenocarum* (within *Carum* after other authors) belong to subfamily *Apioideae*, subtribus *Ammineae* genuinae Drude, series II Ammiformes Wolff, while *Chamaesciadium* was inserted in the same subtribus but in series IV Pimpinelliformes Wolff and hence considered closer to *Pimpinella*,

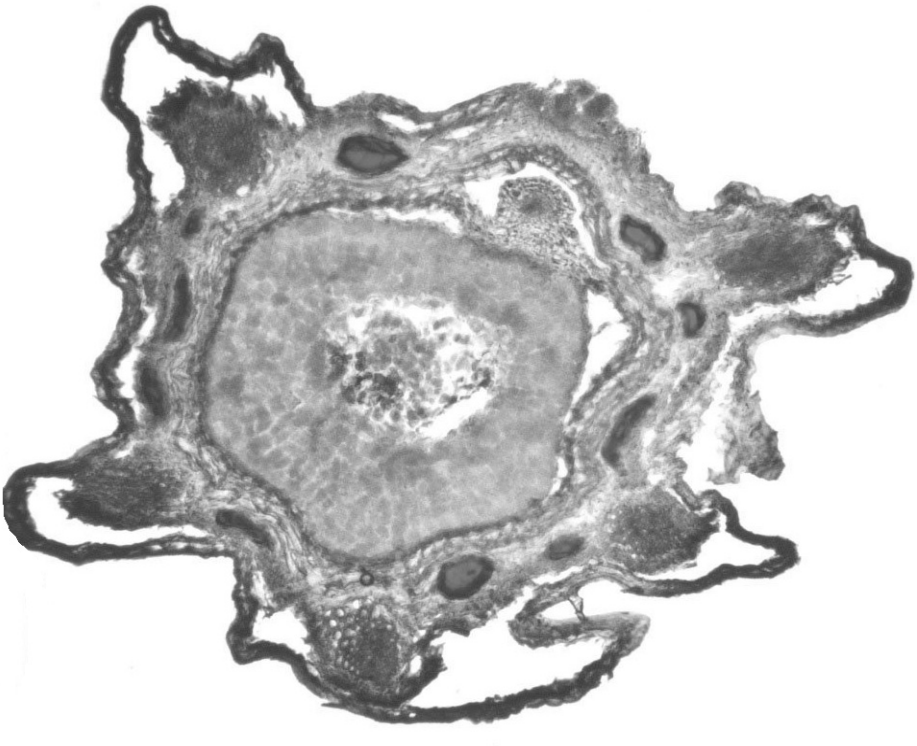

Fig. 2. Mericarp transverse section of *Chamaesciadium acaule*.

Berula and *Sium*. Also *Aegopodium* was inserted in series IV. The molecular data indicated that *Aegopodium* and *Chamaesciadium* are to be inserted in tribe Careae. The mericarp section of *Chamaesciadium* indicated that the position of vittae is similar to that of *Carum carvi and Falcaria vulgaris* (see mericarp sections drawings by Hiroe 1979 and Pignatti 1982) but more numerous (normally 2 vittae among two ridges and not one) while ridges were more prominent in *Chamaesciadium*. The conflict between seed anatomy and molecular evidence would indicate an insufficient reliability of mericarp section as indicator of phylogenetic relationships in this tribe.

After Nazarova and Ghukasyan (2004) *Chamaesciadium acaule* owns $2n = 20$ chromosomes, that confirmed our results since this number is quite common among the genera resulting closer after the molecular data and in general in tribe Careae. In tribe Careae chromosome counts indicated $2n = 20$ for *C. carvi* (Loeve & Loeve 1982) and for the closely related *Grammosciadium daucoides* and *G. platycarpum* (Nazarova & Ghukasyan 2004), $2n = 22$ for *Fuernrohria setifolia* (Daushkevich & al. 1991) and *Falcaria vulgaris* (Kiehn & al. 2000); in genus *Aegopodium* very variable counts are known: from $2n = 21$-22 to 44 in *Aegopodium podagraria* (Stepanov & Muratova 1995) and from $2n = 50$ to $2n = 88$ in *Aegopodium alpestre* (Vasil'eva & al. 1994).

A possibile relationhip between *Chamaesciadium* and *Pycnocycla* was indicated by Hiroe who synonymized this second genus with *Chamaesciadium* (Hiroe, 1979). On the contrary our analysis of molecular data indicated that *Pycnocycla aucherana* is related to *Trachyspermum aethusifolium,* tribe Echinophoreae, far away from tribes Careae and Pyramidoptereae. Since molecular analysis is in agreement with previous taxonomical treatment separating *Pycnocycla* from *Chamaesciadium* on morphological ground (Hedge & Lamond 1973), all species of *Pycnocycla* synomymized by Hiroe (1970) to *Chamaesciadium* should be definitevely reinserted in genus *Pycnocycla* Lindl.

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