Notes on *Trochiscanthes* Koch (Apiaceae) on the basis of ITS rDNA sequence

ALESSIO PAPINI, STEFANO MOSTI
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ALISSIO PAPINI, STEFANO MOSTI

Dipartimento di Biologia Vegetale dell'Università
Via G. La Pira 4, I-50121 Firenze
E-mail: alessio.papini@unifi.it

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Note su *Trociscauthes* Koch (Apiaceae) sulla base della sequenza ITS rDNA. *Trociscauthes* Koch (Apiaceae) è un genere mono- o multispecifico endemico europeo. Questo contributo si occupa dell'analisi filogenetica di questo genere su base molecolare. Per raggiungere quest'obiettivo sono stati utilizzati i criteri di Massima verosimiglianza, Maximum Likelihood e l'analisi Bayesiana su base della sequenza nucleotidica degli spaziatori ribosomiali interni trascritti (Internal Transcribed Spacers = ITS). Inoltre sono stati aggiunti dati morfologici provenienti dalla sezione classica del frutto osservato al microscopio ottico. Questa sezione presenta una serie di caratteri tradizionali non considerati importanti nelle Apiaceae.


Quest'analisi chiarifica la posizione del genere *Trociscauthes*. Inoltre, poiché *Ligusticum* e *Conoisellum* intesi in senso tradizionale, risultano come non monofiletici in studi precedenti (e in questa analisi) e le specie tipo di questi generi si collocano filogeneticamente piuttosto distanti dal clado "*Conoisellum chimplii*", i pericarpi raramente tassonomici (successivamente ad una più ampia revisione di questo gruppo e dei generi *Ligusticum* e *Conoisellum*) potrebbero rimanere un valida unione nel trasferimento delle specie di *Conoisellum* e *Ligusticum* all'interno del clado "*Conoisellum chimplii*" nel genere *Trociscauthes*.

**Key words**: Bayesian analysis, ITS rDNA sequence, Phylogeny, *Trociscauthes* (Apiaceae)

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**Introduction**

The angiosperms family Apiaceae Lindl. (Umbelliferae Juss.) comprises 300-455 genera and some 3000-3750 species (Pimenov and Levanon, 1993). Many species are of economical interest.

Phylogenetic relationships in the subfamily Apioideae have been particularly difficult to resolve (Katz Downie et al., 1999). The most widely used classification is still that proposed by Drude (1898) in Engler & Prantl's *Die natürlichen Pflanzenfami-

lien*, but many recent contributions using molecular data demonstrated that many of Drude's infrafamil-

ial groups were unnatural.

The genus *Trociscauthes* Koch is monotypic, with only one known species, *Trociscauthes nodiflora* (Vill.) Koch. This species lives in chestnut, beech and larch woods (Käsermann and Moster, 1999), and also in *Quercus coccifera* woods in Italy (Ferrarin, 1987); it is an oxyphtile, living on Alps and Appennines mountains from 600 to 1200 metres (Pignatti, 1982). The area distribution is limited to western and northern Alps.
but also some disjunct stations. All the known stations occur in France (Provence, Dauphiné, Savoy, disjunct stations in Haute Savoie and Aude), Italy (Marianne Alps, Courmayeur, Val di Susa, Aosta, Prato, and disjunct stations in Adige Valley and Piave Valley. Ricciardi, 1987) and Switzerland (Venia). Therefore T. nodiflora is an endemic species. T. nodiflora is not common, at least in the southern limit of the distribution area, in Tuscany (personal observation), and is considered a priority species in Switzerland by the Swiss Commission for Wild Plant Conservation (CPS/SKAVE) (Riemann and Moser, 1999).

After Ferrari (1987) T. nodiflora lives in a habitat typical of a very ancient taxon. These features include being a monotypic genus living in habitats as beech woods, with a predominantly alpine distribution but absent on the central Alps, covered by ice during the glacial age. The disjunct stations in France and eastern Alps are indicative of a past larger distribution area, now restricted.

The systematic position of Trichiscantes has been until now quite neglected. Druce (1898) inserted it in the "Sesiid group" (9b) together with Sesel, Grallina, Mucina, Aithana, Athanasius, Ochthoe, Anetium, Menia, Sonina, and Ligusticum on morphological grounds. No molecular data are available on this genus.

Among the molecular markers used to study phylogeny in Aphiodeae and other Angiosperms the nuclear Internal Transcribed Spacers of ribosomal DNA (Baldwin et al., 1995) are the most widely used and many sequences of Apiaceae provided by several authors are available on GenBank. Even if the general utility of ITS in studying phylogeny has recently raised doubts (Alvarez and Wendel, 2003), a huge amount of ITS data is available for Apiaceae. The utility of the ITS markers has been demonstrated at least comparing them to the plastid markers (Chandler and Plunkett, 2004).

The aim of this work is to assess the phylogenetic position of genus Trichiscantes using the Internal Transcribed Spacers as molecular markers to individualize the closest relatives to this biological entity.

Material and methods

Silica gel preserved samples of leaf tissue of Trichiscantes nodiflora (Vill.) Koch were collected in the field in 24-07-2004 in a beech wood close to Abetone (northern Appennines, near the city of Piscoli), at about 1600 m altitude. The herbarium specimens are conserved at the Herbarium Centrale Italiano (FI) in Florence, Italy.

Genomic DNA was isolated using a modified CTAB extraction protocol (Durell and 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.

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'-CGTAAAGGTTTCCCTAG and on the 25S: 5'-AGTGCGCCCTGATGGGGCCA. The adopted thermal cycling profile consisted of 35 cycles of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 72°C. Clear cut single-banded fragments were observed on 1% agarose gels. The amplification products were purified by running on an 1% agarose gel and cutting and purifying the observed bands with a Machery-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 CIBIACL (Center for Biotechnological Services) of the University of Florence. Asymmetrical PCR cycle Sequencing and the BigDye Terminator Ready Reaction Kit (Applied Biosystems) were used.

Resulting ITS sequences were further checked by eye with the software CHROMAS 1.43 (C. McCrathy, School of Biomolecular and Biomedical Sciences, Brisbane, Australia) while a BLAST (Altschul et al., 1997) search was performed to exclude the sequencing of any contaminant organism.

The new ITS sequences of T. nodiflora produced during our investigation were deposited in GenBank (GenBank accession numbers AY957497 for the ITS1 and AY957498 for the ITS2).

Other sequences available in GenBank were chosen sampling adequately all main clades of Umbelloides observed in previous molecular studies (in particular Katz-Dowd et al., 1999) and in recent analyses by the author on Carum, Bradphenium C. tenuissimum. L. was chosen as outgroup on the basis of its position among Aphiodeae (Chandler and Plunkett, 2004).

Optimal multiple alignment was obtained with CLUSTAL 1.81 (Thompson et al., 1994) and
Table 1 — Accessions of Apiaceae used in these studies (ITS sequences). When a single GenBank (GRAN) accession number is indicated, the whole ITS-5.8S-ITS2 is sequenced; otherwise the first accession corresponds to ITS1 and the second to ITS2. Species sequenced by the authors are underlined. Herbarium samples are available from the authors.

<table>
<thead>
<tr>
<th>GenBank Accession Numbers</th>
<th>Species Name</th>
</tr>
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<tbody>
<tr>
<td>AF204897</td>
<td><em>Angelica tenuissima</em> F. Melis &amp; G. Dalla</td>
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<tr>
<td>AF204898</td>
<td><em>Anethum graveolens</em> L.</td>
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<tr>
<td>AF204899</td>
<td><em>Carum carvi</em> L.</td>
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<tr>
<td>AF204900</td>
<td><em>Cuminum cyminum</em> L.</td>
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<tr>
<td>AF204901</td>
<td><em>Eryngium campestre</em> L.</td>
</tr>
<tr>
<td>AF204902</td>
<td><em>Foeniculum vulgare</em> Mill.</td>
</tr>
<tr>
<td>AF204903</td>
<td><em>Lomatium rotundatum</em> DC.</td>
</tr>
<tr>
<td>AF204904</td>
<td><em>Myrrhis odorata</em> L.</td>
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<tr>
<td>AF204905</td>
<td><em>Murdannia gigantea</em> (Dinter) L.</td>
</tr>
<tr>
<td>AF204906</td>
<td><em>Origanum vulgare</em> L.</td>
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<tr>
<td>AF204907</td>
<td><em>Psoralanum obtusatum</em> L.</td>
</tr>
<tr>
<td>AF204908</td>
<td><em>Satureja montana</em> L.</td>
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<tr>
<td>AF204909</td>
<td><em>Sisymbrium officinale Koch</em></td>
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<tr>
<td>AF204910</td>
<td><em>Sisymbrium campestre</em> L.</td>
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<tr>
<td>AF204911</td>
<td><em>Sisymbrium densiflorum DC.</em></td>
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<tr>
<td>AF204912</td>
<td><em>Sisymbrium angustifolium</em> L.</td>
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<tr>
<td>AF204913</td>
<td><em>Sisymbrium pungens</em> L.</td>
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<tr>
<td>AF204914</td>
<td><em>Sisymbrium altissimum</em> L.</td>
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<tr>
<td>AF204915</td>
<td><em>Sisymbrium arvense</em> (L.) DC.</td>
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<td>AF204916</td>
<td><em>Sisymbrium arvense</em> (L.) DC.</td>
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<tr>
<td>AF204917</td>
<td><em>Sisymbrium arvense</em> (L.) DC.</td>
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Notes on Fruits and Seeds (T. N. Ho)
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 Gapcodex (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 undertaken with 100 replicated heuristic searches, using random-sequence addition of taxa, tree bisection-reconnection (TBR) branch swapping, and MULTREES in effect. Bootstrap (Felsenstein, 1985) resampling was performed using TBR branch swapping with ten random taxon entries per replicate and multireplicate option in effect with 100 replicates.

A maximum likelihood (Felsenstein, 1981) search approach was undertaken with Modeltest 3.66 (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 3.4a4i (Huelsenbeck and Ronquist, 2001).

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

The Bayesian analysis was undertaken 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 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 106 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". Optimality 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 dataset, 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 an exploratory phase (Huelsenbeck et al., 2002) and hence we compared the results with those obtained with maximum parsimony (with bootstrap) and maximum likelihood. Also distances with Kimura's settings (Kimura, 1980) were calculated with PAUP.

Metacarpals were sampled from heterotact specimens obtained from field collection, partially rehydrated in 1% Succiase solution and sectioned with a cryostat Cryocut A/O. The slides were stained with Toluidine Blue and observed at a Leitz light microscope.

Results

The total alignment (ITS1 + ITS2) was 487 bp long, plus 123 characters derived from indels coding (simple gaps coding) in the matrix used for maximum parsimony. ITS1 length of T. nadiflorum was 216 bp while the ITS2 reached 224 bp of length.

For parsimony analysis 59 characters were constant, 119 variable characters were parsimony-uninformative and 400 parsimony-informative.

Maximum parsimony analysis produced 720 maximum parsimony trees 2292 steps long, CI = 0.392 and RI = 0.607. A consensus tree with bootstrap support values is reported in Fig. 1.

The software Modeltest indicated the model TN+I+G after the hierarchical likelihood ratio test. The Maximum likelihood tree with Bayesian support is reported in Fig. 2. The analyses were concordant for the position of T. nadiflorum; the closest relatives were Ligusticum canadense (L.) Britton, L. porteri J. M. Coulte & Rose and C. scoparium (A. Gray) J. M. Coulte & Rose. These last two species clustered together with 82% Bootstrap and 92% Bayesian support. These four taxa together formed a clade with 100% Bootstrap and Bayesian support. The outgroup to this
Fig. 1 — Strict consensus tree of 720 maximum parsimony trees: 2292 steps long, CI = 0.392 and RI = 0.607. Bootstrap support values are reported above branches. The *Conioselinum chinense* clade [with *Tredecassaleria*] is shown by a curly bracket.
clade was Conioselinum chinesense, with 59% Bootstrap and 93% Bayesian support. Mecin aethiopicum was outgroup to this last clade both after the maximum likelihood and maximum parsimony analyses with a low 62% Bootstrap support and 97% Bayesian support. The Kimura's distance among the most important taxa considered in this investigation are reported in Table 2.
Discussion

The analyses were concordant for the position of *T. undiflorum*: the closest relatives were *Ligusticum canadense* (L.) Britton, *L. perrottetii* J. M. Coulter & Rose and *C. scopulorum* (A. Gray) J. M. Coulter & Rose. These taxa together formed a clade with high Bootstrap and Bayesian support, while the outgroup to this clade was *Conioselinum chinense*. These five species correspond to the "Conioselinum chinense clade" after Katz et al. (1999). *Meun athamanticum* was outgroup to this last clade both after the maximum likelihood and maximum parsimony. *Conioselinum* and *Ligusticum* are widely distributed genera, almost cosmopolitan and not monophyletic on the basis of molecular data (Downie et al., 2000), while previous studies on *Ligusticum* (Leute 1969, 1970) and allied genera pointed out the difficulty of delimitation of these genera. Moreover the type species of these two genera, *C. sativum* and *L. scoticum*, clustered quite long away from the "Conioselinum chinense clade". We agree with Downie et al. (2000) about the fact that a more exhaustive revision of the group is necessary before definitive taxonomical rearrangements, but it is necessary to observe that the closely related monotypic genus *Tschicaites* would maintain a valid name and would be a candidate name destined to contain at least some of the species of *Ligusticum* and *Conioselinum*. Kimura's distance of *Tschicaites undiflorus* to *L. canadense* is 0.033, 0.046 to *L. perrottetii* and 0.033 to *C. scopulorum*. These distances are comparable to these between species belonging to the same genus (and relaxed also after the molecular data). For instance in this same matrix: 0.082 between *Conioselinum chinense* and *C. scopulorum*, 0.023 between *L. perrottetii* and *L. canadense*; even 0.117 for *Ligusticum linatum*-*L. scoticum* and 0.012 for *Impatiens balsamina*-*L. athamanticum*. The distance rises quickly to 0.155 between, for instance, *Tschicaites* and *Meun athamanticum* and, in general, almost all distances between the species in Figs. 1 and 2 were higher than 0.1.

Of the genera belonging to Drude's Scelli group 9b (Drude, 1898) *Sceli, Crithmum, Oenanthe, Aethusa, Athamanta, Foeniculum, Anethum* and *Selinum* clustered in clades different from the "Conioselinum chinense clade". *Meun athamanticum* was outgroup to this last clade, while *Ligusticum* was polyphyletic.
The chromosome number in T. nadiflora is 2n=22 (Wanscher, 1934). After the same author 2n=22 was found also in Meunium abstratum, Selinum carafealum, Ligusticum austrocalabrum and L. scoticum. This number is the most common among Apiaceae (Wanscher, 1934) and these data, useful in other situations, do not conflict with the phylogenetic results here reported.

The mericarp section of Trachiscanthus nadiflora (data not shown) showed five prominent ribs and 7-8 sutures (only 4 sutures are described in Flora Europaea). This character is considered to be one of the most relevant morphological markers in Apiaceae. Mericarp sections in Ligusticum and Conioselinum are quite variable (see for instance Pigigian, 1982) and also the observation of the mericarp section of four species of Ligusticum inserted in the phylogenetic analysis didn't provide further morphological support to the molecular data: L. bulbosum had less ridges with respect to the other species of Ligusticum but clustered in a group away from Trachiscanthus, while L. canadense and L. porteri clustered together with Trachiscanthus without showing any evident similarity in mericarp section. A morphological analysis to best redefine these genera following also the molecular results is necessary, but the mericarp section is probably more useful at the species level than to discriminate among different genera in this group.

It is interesting to observe that Ligusticum nadiflorum Vill. is the basionym of Trachiscanthus and hence a relationships between this last and some other species of Ligusticum has already been observed.

In conclusion genus Trachiscanthus resulted strictly linked to the "Conioselinum chineense clade" and hence to representatives of the polyphyletic genera Ligusticum and Conioselinum. Since the type species of these last genera cluster in other clades and the Kimura's distance between the species belonging to the "Conioselinum chineense clade" are compatible with distances among species belonging to the same genus, we propose the insertion of the species of Ligusticum and Conioselinum clustering in the "Conioselinum chineense clade" of Trachiscanthus. Anyway formal recombinations need a revision of these genera because of important morphological and phylogeographical divergence: in particular T. nadiflora and Meunium abstratum are european endemics, while C. scoparium, L. canadense and L. porteri are north american species. M. abstratum was found also in northern stations as Scotland and Southern Norway (Lutece, 1959) and this distribution might be the remnant of a previous circumocean distribution (of Meunium or an extinct ancestor) from which the other species of the "Conioselinum chineense clade" could have dispersed. The position of Meunium, basal to the "Conioselinum chineense clade" would be coherent with this hypothesis.

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Abstract: Trichocissanthus Koch (Apiaceae) is an endemic, monospecific genus. This paper deals with the phylogenetic analysis on molecular basis to assess the relationships of the genus. Parsimony, maximum likelihood and Bayesian support analyses on the basis of the ribosomal internal transcribed spacers were adopted. Moreover data were added to meta-carp section data, one of the most important morphological characters in Apiaceae. Trichocissanthus nodiflorus, Ligustrum porinum, Ligustrum confine and Caricinella spicatum clustered together with 100% Bayesian and Bootstrap (parsimony) support. These clade was sister to Caricinella chinensis (98% Bayesian and 98% Bootstrap support). This clade corresponds to the "Caricinella chinensis" clade, by other authors. All these species clustered together with Meum athamanticum (97% Bayesian and 62% Bootstrap support). This result clarifies the position of genus Trichocissanthus. Moreover, since Ligustrum and Caricinella (in traditional sense) resulted as probably not monophyletic in previous studies and the type species of these genera clustered far away from the "Caricinella chinensis" clade, the necessary taxonomical rearrangements (after a further revision of the group) could find a solution transferring the species currently inserted in Ligustrum and Caricinella but within the "Caricinella chinensis" clade, to genus Trichocissanthus.