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Short Communication

A comparison of nrDNA ITS and ETS loci for phylogenetic inference in the Umbelliferae: An example from tribe Tordylieae

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ABSTRACT

The Umbelliferae is a large and taxonomically complex family of flowering plants whose phylogenetic relationships, particularly at low taxonomic levels, are generally obscure based on current and widely used molecular markers. Thus, information on the phylogenetic utility of additional molecular markers at these levels is highly favorable. We investigate the utility of nuclear ribosomal DNA (nrDNA) external transcribed spacer (ETS) sequences for phylogenetic inference in Umbelliferae tribe Tordylieae, a group whose relationships have been previously difficult to resolve owing to low sequence variability, and compare the results to those obtained from the nrDNA internal transcribed spacer (ITS) region. We report that the ETS region evolves at a slightly faster rate and has a higher percentage of parsimony informative characters than that of ITS and all chloroplast DNA loci examined to date. The ETS region is a valuable phylogenetic marker in Umbelliferae for low level analysis, especially when used in combination with ITS.

1. Introduction

The search for a molecular marker suitable for low-level phylogenetic analysis remains a challenging problem. In plants, the most widely used marker at this level is the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA). In addition, several chloroplast genes and intergenic regions are employed however they are often not variable enough to yield well resolved and reliable phylogenies. To overcome this problem, the use of non-coding regions of single or low-copy nuclear genes has been promoted (e.g., Mort and Crawford, 2004). The latter regions, however, have severe limitations, such as the absence of universal primers, the difficulties related to gene duplication and resultant paralogy, and the frequent failure of PCR when dealing with herbarium samples. Thus, information on the phylogenetic utility of additional molecular markers that might be useful for low-level phylogenetic reconstruction is highly desirable. One of the most prospective candidates is the nrDNA external transcribed spacer (ETS) region – it belongs to the same locus as ITS and is also present in high-copy numbers and subject to homogenization. Previous studies have demonstrated that the ETS region is often more variable and phylogenetically informative than ITS for low-level phylogenetic reconstruction (Poczai and Hyvönen, 2010). However, because the region is flanked by a highly variable non-transcribed spacer there are no universal primers available for PCR and sequencing of this region, thus it is not used as widely as ITS.

In this study, we assess the ETS region as a source of phylogenetic information in the plant family Umbelliferae (or Apiaceae). This family is large, widely distributed, taxonomically complex, and has heretofore yet to be analyzed using ETS data. We examine the phylogenetic relationships of the genera *Heracleum, Semenovia, Tetrataenium* and their closest allies in Umbelliferae tribe Tordylieae. All of these taxa are characterized by great morphological diversity and a complicated taxonomic history. Moreover, our previous studies of the tribe, based exclusively on ITS and chloroplast DNA (cpDNA) markers, revealed that sequence variation of these loci is quite low, resulting in poorly resolved and weakly supported trees (Logacheva et al., 2008; Ajani et al., 2008). Therefore Umbelliferae tribe Tordylieae provides an excellent group for examining the utility of the ETS region for inferring phylogeny among closely related taxa.

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2. Materials and methods

Seventy-five accessions were sampled (see Supplement for voucher information and GenBank accession numbers). These accessions represent all major subdivisions within the genera *Heracleum*, *Tetrataenium,* and *Semenovia*, as well as several other genera for which relationships to *Heracleum* and/or *Semenovia* and *Tetrataenium* have been proposed. Two rare and little-known species of *Peucedanum* were also included because of their morphological similarity to South Indian *Tetrataenium* species. As outgroups, we considered members of the closely related *Cymbocarpum* clade, *Conium* spp., *Azilia eryngioides*, and *Smyrniopsis aucheri*, based on results of previous, higher-level phylogenetic studies of subfamily Apioideae (Ajani et al., 2008; Winter et al., 2008); all phylogenetic trees were rooted with *Smyrniopsis aucheri*. Multiple accessions of *Conium maculatum* were included to compare intraspecific ITS and ETS sequence diversity.

Total genomic DNA was extracted using a NucleoSpin Plant II genomic DNA extraction kit (Macherey–Nagel, Germany), following the manufacturer's protocol. Details of the protocols for PCR and sequencing of the ITS region are provided elsewhere (Valiejo-Roman et al., 2002). Sequences of the 5.8S rRNA gene were excluded from the analysis because of their absence from several previously published ITS sequences.

The portion of the ETS locus sequenced in this study corresponds to the ETSf region of Bena et al. (1998) or the ETS 1f region of Starr et al. (2003); hereafter, we refer to this region simply as "ETS." To PCR amplify and sequence this region, we used a primer annealing in the highly conserved 18S gene (18S-ETS; 5′-ACTTAC ACATGCATGGCTTAATCT-3′; Baldwin and Markos, 1998) in combination with a reverse primer generated specifically for the Umbelliferae (Umb-ETS; 5′-GCGCATGAGTGGTGAWTKGTA-3′). The last nine nucleotides of this primer correspond to the conserved motif identified by Bena et al. (1998). Design of the Umb-ETS primer required amplification of the entire intergenic spacer (IGS) region using long PCR (using Long PCR Enzyme Mix; Fermentas, Lithuania), with universal primers annealing at the 5′end of 18S (18S-ETS) and at the 3′ end of 26S (26S-IGS; 5′-GGATTGTTCACCCACCAA TAGGGAACGTGAGCTG-3′). The 10-μl PCR reactions contained 5.5 μl of sterile water, 1.0 μl of 10× Long PCR Buffer with 15 mM $MgCl_2$, 0.2 mM of each dNTP, 4 pmol of each primer, 0.2 unit Long PCR Enzyme Mix (0.1 μl), 0.4 μl of DMSO, and 1.0 μl of DNA template. The PCR protocol included an initial denaturation (3 min at 94 °C) followed by 35 cycles of 30 s denaturation (94°), 30 s annealing (58–64 °C), and 6 min extension at 68 °C, followed by a final extension of 10 min at 68 °C. Such a protocol was applied to five species of Umbelliferae (*Aegopodium alpestre*, *Heracleum dissectum*, *Ducrosia anethifolia*, *Prangos pabularia*, *Anthriscus sylvestris*). The PCR products were run on a 1% agarose gel, then bands were cut out and purified using the JETSorb Gel Extraction Kit (Genomed, Germany) and then sequenced from the 3' end using the 18S-ETS primer. These sequences, along with a *Daucus carota* IGS sequence obtained from GenBank (accession number D16103), were aligned and used to develop the Umb-ETS primer. This primer anneals approximately 450 bp upstream from the annealing site of the 18S primer. Conventional PCR for amplification of ETS was performed using the same program and reagents as for ITS. All PCR products from conventional PCR were purified with a Gel Extraction and PCR Cleanup Kit (Cytokine, Russia). For most PCR products, direct sequencing was applied, except for the ETS region of *Peucedanum siamicum*. The latter was gel-purified and cloned using the InsT/A Cloning System (Fermentas, Lithuania), following the manufacturer's protocol. Five clones that contained the insert were sequenced. All sequences were obtained using an ABI PRISM 3100 Genetic Analyzer.

The ETS and ITS sequences were aligned separately using MUSCLE (Edgar, 2004) and then concatenated and manually adjusted using BioEdit (Hall, 1999). Uncorrected *p*-distances were calculated using PAUP* version 4.0b8 (Swofford, 2003). Maximum parsimony analysis involved a heuristic search conducted with PAUP* using tree bisection-reconnection (TBR) branch swapping with character states specified as equally weighted. One thousand replicates with random addition of sequences were performed. Bootstrap (BS) values (Felsenstein, 1985) were calculated from 1000 replicate analyses with TBR branch swapping and random addition sequence of taxa, saving 1000 most-parsimonious trees from each replicate. The incongruence length difference test (ILD; Farris et al., 1995) was carried out as implemented in PAUP* using the parsimony optimality criterion and 1000 addition replicates. Bayesian inference of phylogeny was explored using the program MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The GTR + I + Γ model of substitution for ITS data and the GTR + Γ model for ETS data were selected by the Akaike information criterion (AIC) in Modeltest (Posada and Crandall, 1998). The concatenated data matrix was analysed both as partitioned data using the aforementioned models and as unpartitioned data using the GTR + Γ model of substitution only, which was also selected by the AIC. Bayesian analysis was performed with three chains in each of the two runs, each chain starting with a random tree, with 15,000,000 replicates generated. The trees obtained were sampled every 1000 generations. The number of generations to be discarded was determined using a convergence diagnostic (i.e., by the examination of the average standard deviation of split frequencies).

3. Results

Characteristics of the ITS and ETS sequences are summarized in Table 1. Values for the former are similar to those reported in a previous study of *Heracleum* and related genera (Logacheva et al., 2008); they are also congruent with the general patterns of ITS sequence polymorphism reported for other Umbelliferae (e.g., Downie and Katz-Downie, 1996). For the ETS region, uncorrected pairwise sequence divergence values ranged from identity to 0.277. An intragenomic polymorphism was found in *Peucedanum siamicum,* where the two versions of ETS obtained differed by one nucleotide indel.

The results of maximum parsimony analyses of these two data sets are summarized in Table 2. The strict consensus trees derived from these analyses (not shown) are consistent, although the ITS strict consensus tree is less resolved than that derived from ETS. Greater homoplasy was also evident in the ITS data. The results

Table 1
Characteristics of the nrDNA ITS and ETS regions from 75 accessions of Umbelliferae tribe Tordylieae and outgroups examined in this study.

Characteristic	ITS	ETS
Total size range (bp)	430–447	379–401
GC content,%	54.6	47.6
Pairwise sequence divergence (minimal–maximal/mean)		
Overall	0–0.212/0.089	0–0.277/0.11
Heracleum sensu stricto (Clade 1) + Pastinaca	0–0.087/0.048	0–0.19/0.076
Tetrataenium I (Clade 2)	0–0.094/0.062	0–0.079/0.058
Tetrataenium II (Clade 3)	0.005–0.088/0.05	0.03–0.107/0.058
Semenovia (Clade 4)	0.005–0.078/0.041	0–0.086/0.044
Conium maculatum accessions (in parentheses between *Conium maculatum* and *C. sphaerocarpum*)	0.007–0.016/0.012 (0.016–0.02/0.018)	0.003–0.015/0.008 (0.005–0.015/0.009)

Table 2
Results of maximum parsimony analyses of ITS and ETS sequences, separately and combined, for 75 accessions of Umbelliferae tribe Tordylieae and outgroups.

Characteristic	ITS	ETS	Combined
Number of variable characters	274	278	552
Number of parsimony informative characters (and %)	198 (46.7%)	199 (50%)	397 (48.2%)
Number (upper line) and length (lower line) of most-parsimonious trees	>94,000 761	112 737	512 1516
Consistency index (uninformative characters included)	0.535	0.560	0.541
Retention index	0.732	0.804	0.766

of the ILD test revealed that these loci yield significantly different phylogenetic estimates ($P = 0.026$). However, when outgroups are excluded and only members of tribe Tordylieae are considered, the datasets yield congruent results ($P = 0.09$). Thus, data from all accessions were combined for simultaneous analysis by taking into account the numerous reports indicating that the results of an ILD test do not adequately assess data combinability (e.g., Hipp et al., 2004). Maximum parsimony analysis of these combined data resulted in 512 minimum length trees, each with a length of 1516 steps, a CI of 0.541, and a RI of 0.766. These trees showed much resolution of relationships among all taxa. Bayesian analyses of partitioned data matrices resulted in trees (Fig. 1) very similar to those inferred using maximum parsimony, both in topology and their degree of resolution, but once more the ITS tree was less resolved than that of ETS. However, neither ITS nor ETS alone provided high support for nodes; in the ITS-derived trees, only 32 nodes had posterior probability (PP) values greater than 0.9, whereas in the ETS trees, 44 nodes had PP values greater than 0.9. In contrast, the Bayesian inference tree derived from combined data had 51 nodes with high support (Fig. 2); this tree also provided the greatest resolution of relationships.

The trees resulting from both maximum parsimony and Bayesian analyses of combined data were congruent in topology (except for the placement of *Ormosciadium*, discussed below). Within the ingroup, four major clades are resolved and include: Clade 1 – the *Heracleum* sensu stricto clade, comprising 13 species of *Heracleum*, *Symphyoloma graveolens*, and *Mandenovia komarovii*. The nomenclatural type of *Heracleum*, *H. sphondylium* L., was not included in this analysis; since *H. sibiricum*, a species that is closely related and sometimes treated as a subspecies of *H. sphondylium* (e.g. Zych 2007) is included. This clade is a strongly supported sister group (99% BS, 1.0 PP) to a clade comprising two species of *Pastinaca*, the latter comprising the *Pastinaca* clade of earlier study (Logacheva et al., 2008); Clade 2 – the *Tetrataenium* I clade, comprising six species of *Tetrataenium, Lalldhwojia pastinacifolia*, and *Pinda concanensis*; Clade 3 – the *Tetrataenium* II clade, or *Tetrataenium* s. str., comprising five species of *Tetrataenium* from Peninsular India, including the nomenclatural type, *T. rigens*. Also included within this clade are two species of *Peucedanum*, *Lalldhwojia acro-*

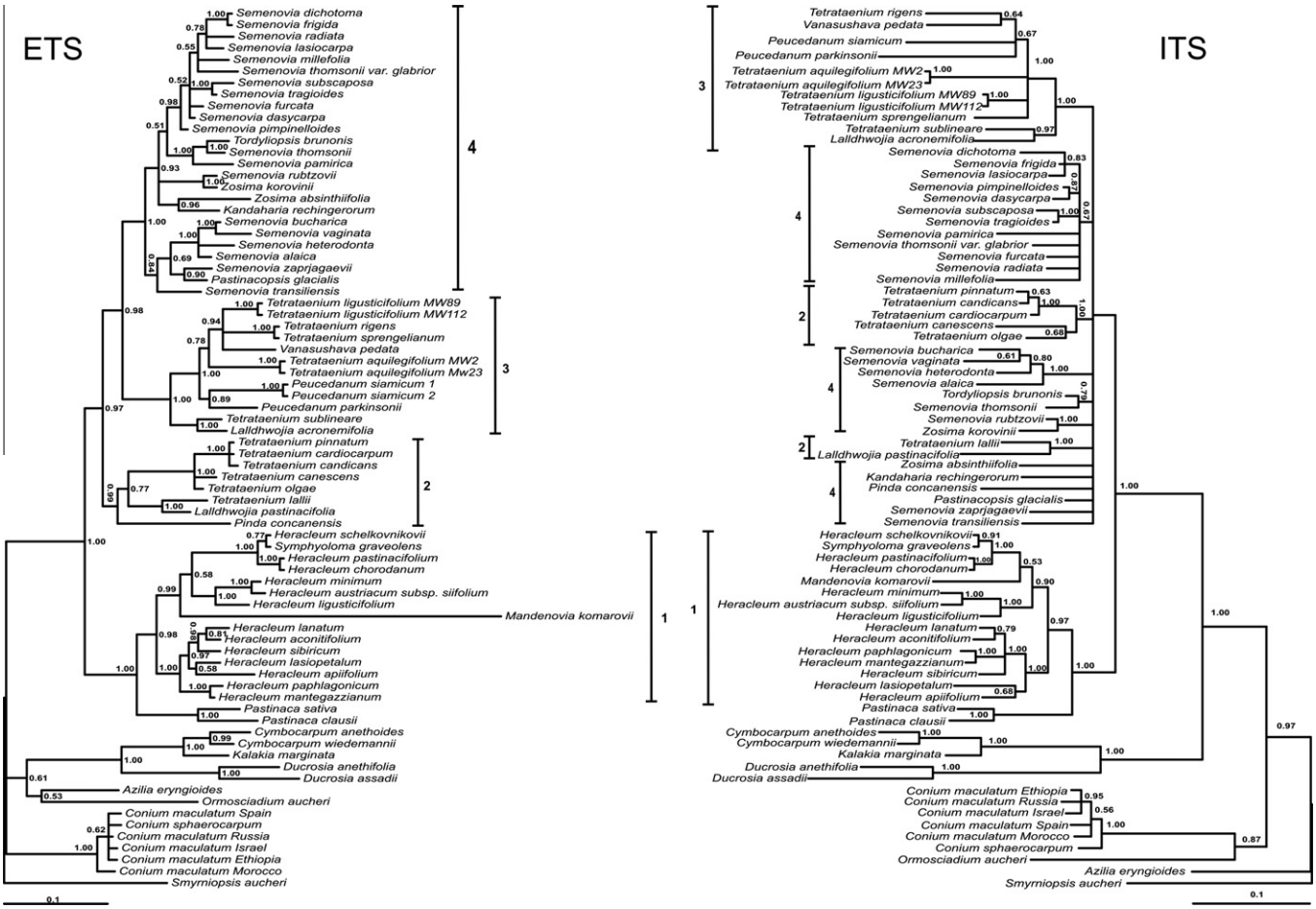

Fig. 1. Phylogenetic trees inferred from Bayesian analysis of ETS (left) and ITS (right) sequences from Umbelliferae tribe Tordylieae and outgroups. Data were analysed under GTR + Γ (ETS) and GTR + I + Γ (ITS) substitution models. Numbers at nodes represent posterior probabilities (only those greater than 0.5 are shown). Branch lengths are proportional to the number of the expected nucleotide substitutions (scale bar corresponds to 1 substitution per 10 sites). Numbered bars represent the four major clades discussed in the text.

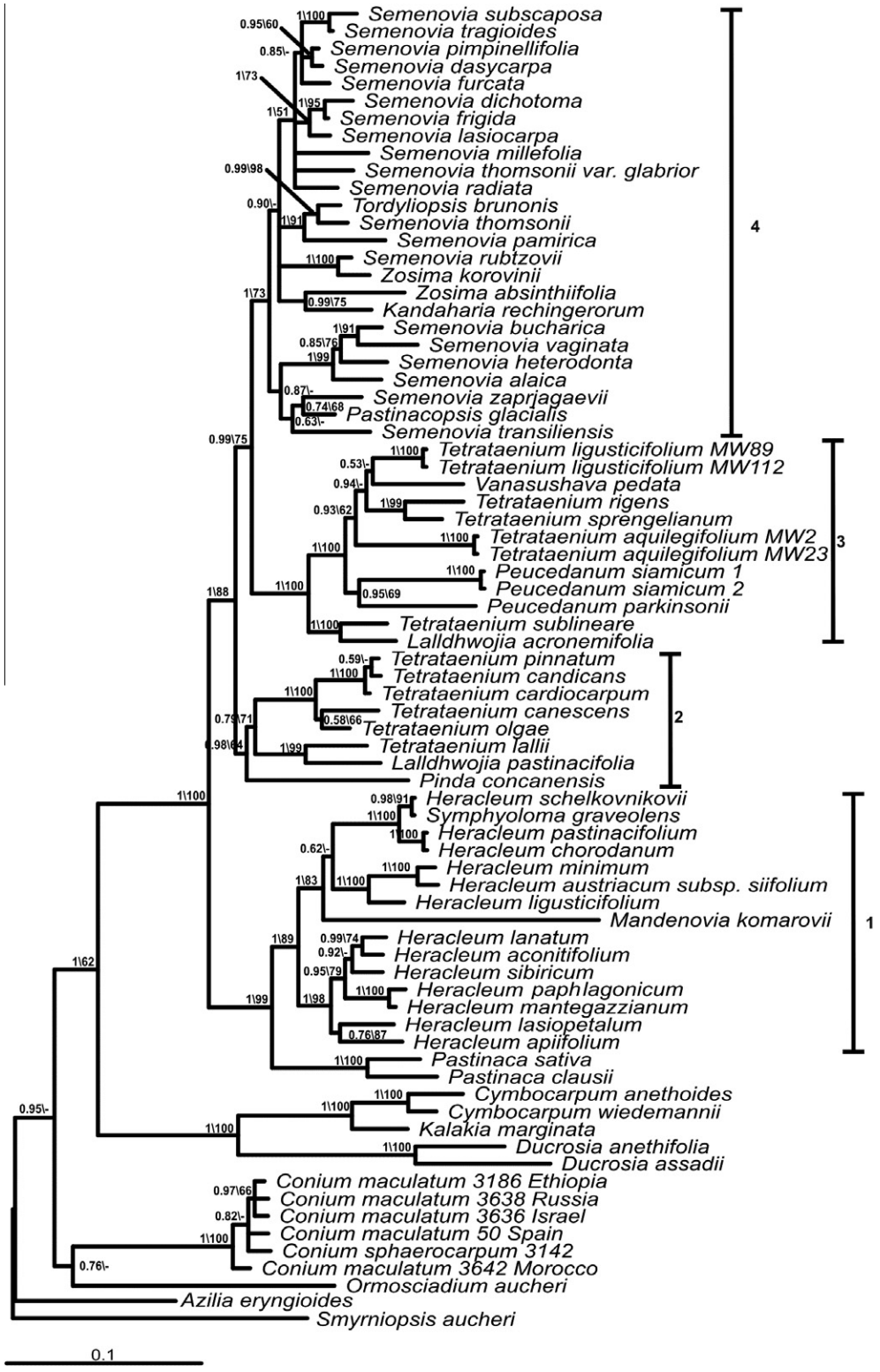

Fig. 2. Phylogenetic tree inferred from Bayesian analysis of combined ITS and ETS sequences (unpartitioned dataset was analysed under the GTR + Γ substitution model; partitioned data analysis yields the same topology) from Umbelliferae tribe Tordylieae and outgroups. Numbers at nodes represent maximum parsimony bootstrap support and posterior probability values (only those greater than 50% and 0.5 are shown). Branch lengths are proportional to the number of the expected nucleotide substitutions (scale bar corresponds to 1 substitution per 10 sites). Numbered bars represent the four major clades discussed in the text.

nemifolia, and *Vanasushava pedata*; and Clade 4 – the *Semenovia* clade, comprising 20 accessions of *Semenovia* including the nomenclatural type, *S. transiliensis*, two species of *Zosima*, and the monotypic genera *Tordyliopsis*, *Pastinacopsis,* and *Kandaharia*. Each of these four major clades is moderately to strongly supported (64–100% BS, 0.98–1.0 PP). In all trees, the *Heracleum* s. str. plus *Pastinaca* clade is basal to the three other major clades, and in no trees do the two clades of *Tetrataenium* ally as monophyletic. In the less-resolved ITS trees (Fig. 1), clades 2 and 4 are each not monophyletic and collectively comprise a paraphyletic group relative to

clade 3. In the ETS trees, clades 1–4 are distinct. Successive sister groups to the clade of all aforementioned taxa in the combined analyses include the *Cymbocarpum* clade (*Cymbocarpum, Ducrosia, Kalakia*), a weakly supported clade of *Conium* plus *Ormosciadium*, and *Azilia eryngioides*. Such basal relationships are the same as reported in previous, higher-level phylogenetic studies of the family (Ajani et al., 2008; Winter et al., 2008), save for *Ormosciadium* which is included now in a phylogenetic study for the first time.

4. Discussion

Phylogenetic analyses of nrDNA ETS and ITS sequence data reveal novel relationships among *Heracleum, Semenovia, Tetrataenium*, and other genera of tribe Tordylieae. In many modern classification systems, the genera *Semenovia* and *Tetrataenium* are not widely accepted, with their species treated within *Heracleum* (e.g., Mukherjee and Constance, 1993; Pu and Watson, 2005). In contrast, these sequence data corroborate morphology-based hypotheses in suggesting that these genera are indeed distinct from *Heracleum*. However, these same molecular data also imply that the taxonomy of this group needs extensive revision. Such a revision would entail the splitting of the genus *Tetrataenium* into two disparate genera, as it was demonstrated to be paraphyletic, and the integration of *Semenovia* with *Zosima*. Furthermore, the generic status of each of *Pinda, Vanasushava, Tordyliopsis, Pastinacopsis, Lalldhwojia, Symphyoloma, Mandenovia*, and *Kandaharia* is not justified by our data, as they fall within larger clades consisting of *Semenovia*, *Tetrataenium* or *Heracleum* species. The placement of *Symphyoloma* and *Mandenovia* within *Heracleum* was reported in our previous ITS study (Logacheva et al. 2008) and now has gained additional support using ETS data. *Ormosciadium* does not ally with any currently included member of tribe Tordylieae; its phylogenetic placement, however, is contradictory in the trees inferred herein. This discrepancy might reflect the effects of poor sampling of taxa basal to tribe Tordylieae in our study, and will require additional exploration using a broader range of species.

This is the first study to use ETS sequences to infer phylogeny of the Umbelliferae. In terms of their phylogenetic utility, these sequences are quite useful, having proportionally a slightly higher percentage of parsimony informative characters than ITS, although both regions contributed approximately the same numbers of variable and parsimony informative characters to the analysis (Table 2). A comparison of uncorrected *p*-distances revealed that, depending upon the taxon or clade, either ITS or ETS were more divergent, with both loci demonstrating variation at the intraspecific level. In *Conium maculatum* (five accessions), the ITS region was about 1.6 times more variable than ETS; in the *Semenovia* clade (25 accessions) and both *Tetrataenium* I and *Tetrataenium* II clades (8 and 11 accessions, respectively) the average amount of variation within each locus was about the same; and in the *Heracleum* s. str. plus *Pastinaca* clade (17 accessions), ETS had 1.6 times greater nucleotide divergence than ITS. For all accessions comprising each data matrix, there was greater sequence divergence in ETS than in ITS (0.277 vs. 0.215, respectively). Phylogenetic studies using ETS sequences in other angiosperm families have reached similar results — in general, the ETS region evolves at a slightly faster rate and has a higher percentage of phylogenetically informative characters than ITS (Poczai and Hyvönen, 2010). The ETS region of *Peucedanum siamicum* revealed intra-individual polymorphism, with the two versions of this region differing by one nucleotide indel. Such a difference may be attributed to the lack of homogenization of this locus by concerted evolution (e.g., Chung et al., 2005). Both sequences, however, ally as a well-supported clade in the phylogeny, thus this polymorphism does not represent a problem for phylogeny estimation. Its only evident shortcoming is that it precludes the use of direct sequencing for some taxa.

Phylogenetic analyses of the two data sets resulted in a greater number of minimal length trees for ITS and a slightly lower CI, indicating greater homoplasy than ETS. Moreover, the ITS-derived trees were, in general, less resolved than those of ETS. The ETS data resolved nodes among closely related taxa of *Semenovia* and *Tetrataenium*, as well as resolved the same four major clades as inferred through analyses of combined data. However, ITS resolved the basalmost branches of the trees and, for these clades, provided greater branch support than that of ETS. The incongruence between ITS and ETS datasets is also confined to the outgroup lineages, presumably reflecting the differences in their substitution rates and the overall higher variability of ETS. The range of applicability of ETS is not completely overlapping with that of ITS, making the former a region of choice for phylogenetic analyses of taxa in which ITS variability is too low. Though the data partitions were not found to be congruent when all accessions were considered, the analysis of combined nrDNA data resulted in trees that are better resolved with branches more greatly supported than trees based on partitioned data. We conclude that despite the smaller size of ETS over ITS, in tribe Tordylieae and allies it provides more informative variation for phylogenetic reconstruction and allows for better resolution of relationships than ITS. It is therefore a valuable marker for phylogenetic studies in the Umbelliferae, especially when used in conjunction with ITS. Similar conclusions were revealed through analyses of members of the Araliaceae, a closely related family to the Umbelliferae (Tronchet et al., 2005). Though we present here results for just one major tribe of Umbelliferae, the ETS primers reported in this study are suited for amplification and sequencing of this region across the entire family, including basal representatives of subfamily Apioideae and several genera of subfamily Saniculoideae (E.I. Terentieva and C.M. Valiejo-Roman, unpublished data). This is consistent with the fact that the Umb-ETS primer anneals at the most conserved region within ETS.

To date, very few molecular markers have been used in high-level phylogenetic studies of Umbelliferae. These include the ITS region plus several cpDNA loci, with ITS evolving most rapidly as evidenced by a greater percentage of sites that are parsimony informative and its higher rate of sequence change (Downie et al., 2001). Recently, the cpDNA *psbA-trnH* intergenic spacer region has been proposed for DNA barcoding in flowering plants (Kress et al., 2005), yet a comparison of ITS vs. *psbA-trnH* intergenic spacer variation across 33 species of *Heracleum* and its close allies revealed that this cpDNA spacer region is actually quite conserved, with no to very little variation among species, even in some intergeneric comparisons (Logacheva et al., 2008). The higher variability of ETS over ITS and all chloroplast loci examined to date suggests that the ETS region has great potential for species identification through barcoding. The obvious limitation of using ETS, however, is that there are no universal primers for amplification and sequencing of the region, because DNA barcoding requires a universal marker for species identification (Lahaye et al., 2008; Kress and Erickson, 2008). There is increasing evidence that for some plant groups a universal marker (or even several markers suited for species identification) may be impossible to find (for discussion see Spooner, 2009). Within some genera of Umbelliferae, for example, both ITS and *psbA-trnH* sequence data may be very similar, even between morphologically distinct species (Logacheva et al., 2008; Degtjareva et al., 2009). Therefore, the use of additional "taxon-specific barcodes," such as ETS, might be useful for species identification in the family.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.06.001.

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