

## Molecular phylogeny of *Daucus* (Apiaceae): Evidence from nuclear ribosomal DNA ITS sequences

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The Apiaceae genus *Daucus* consists of approximately 25 species (including carrots) which are characterized by the presence of bracts in pedunculate umbels, dorsally compressed mericarps, hairs on primary ridges, and uniseriately arranged spines on the secondary ridges of the fruit. Taxonomically, *Daucus* has been considered to be one of the most problematic genera in the Apiaceae due to the highly variable fruit morphology. Despite taxonomic controversy and economic importance of the genus, no rigorously constructed estimate of phylogenetic relationships exists. To examine generic limit and relationships among species of *Daucus* and its putatively related taxa, phylogenetic analyses of characters derived from nuclear ribosomal DNA ITS sequences were conducted. Two major clades emerged within *Daucus*, but neither of them have been previously recognized using morphological characters. The phylogeny also provides taxonomic status of recently reported new species of *Daucus*, *D. arcanus* and *D. conchitae*.

Keywords: Apiaceae, *Daucus*, ITS, Umbelliferae

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### INTRODUCTION

The genus *Daucus* L. (Apiaceae Lindl.) is defined morphologically by a suite of easily observed and well-known characters, including dorsally compressed mericarps, hairs on primary ridges, and singly arranged spines on the secondary ridges of the fruit (Okeke, 1978). The genus is mainly distributed in Europe including the Mediterranean regions, southwestern and central Asia, and tropical Africa. A few species are also found in the other continents; *D. glochidiatus* (Labill.) Fischer & C.A. Mey. is restricted to Australia and New Zealand, *D. montanus* Humb. & Bonpl. ex Spreng. to central and South America, and *D. pusillus* Michx. to North and South America (Heywood, 1983). The genus is economically important; it includes the common carrot, *Daucus carota* L. ssp. *sativus* (Hoffm.) Arcang., a root crop which is widely cultivated in most parts of the world. The carrot is medicinally important as stimulant, deobstruent, and excitant and also valuable for other urinary problems, skin affliction, jaundice, dropsy, and uterine ailments (Heywood, 1978; Okeke, 1978).

Taxonomically, *Daucus* and other spiny-fruited genera (e.g., *Caucalis* L., *Torilis* Adans., and *Orlaya* Hoffm.)

were treated into tribe Caucalideae Tausch on the basis of the unique presence of spines, hooks, tubercles, or bristly hairs on the primary and/or secondary ridges of their fruits (Bentham and Hooker, 1867). This is in contrast to Drude (1898), who distributed these spiny-fruited plants into tribe Dauceae and tribe Scandiceae subtribe Caucalidinae. Drude believed that members of Dauceae were allied to plants in his tribe Laserpitieae, whose members have fruits without spines but with primary and prominent secondary ridges. Koso-Poljansky (1916; 1917), following Drude's segregation of the spiny-fruited umbles into Dauceae and Caucalidinae, expanded tribe Dauceae including many representatives of Drude's Laserpitieae. However, all the spiny-fruited taxa (e.g., Drude's Dauceae and Scandiceae subtribe Caucalidinae) were united as a single tribe Caucalideae on the basis of phytochemistry and micro-characters of fruits using scanning electron microscopy (Heywood and Jury in Heywood, 1982) whereas the tribe Laserpitieae was retained as an independent tribe (Heywood, 1978).

Infrageneric classification of *Daucus* L. largely based on anatomical and morphological features of the mature fruit has been also controversial. The genus *Daucus* were variously divided into sections, subgenera, or some species of the genus were even treated into separated genera,

based on anatomical and morphological features of the flower and fruit (Drude, 1898; Calestani, 1905; Thellung, 1926). Thellung (1926), in his worldwide monograph of *Daucus*, recognized six sections (i.e. *Anisactis* DC., *Leptodaucus* Thell., *Carota* [= *Daucus*], *Platyspermum* DC., *Pseudoplatyspermum* Thell., and *Chrysodaucus* Thell.) based on morphological and anatomical features of the flower and fruits including length of styles, stylopodia, petals, and vittae size and shape. However, his treatment was based on the limited materials available at that time and his descriptions lacked detailed information on some important features of fruits. On the other hand, Okeke (1978) divided the genus *Daucus* into four sections; *Meiodes*, *Daucus*, *Platyspermum*, and *Anisactis*. He recognized sect. *Leptodaucus* as a subsect. of sect. *Daucus*, suggesting that species belonging to *Leptodaucus* might be intermediates between sects. *Daucus* and *Anisactis*. In addition, Okeke (1978) treated sects. *Pseudoplatyspermum* and *Chrysodaucus* as subsects. of sect. *Platyspermum*, suggesting that subsect. *Platyspermum* might be an intermediate between subsects. *Pseudoplatyspermum* and *Chrysodaucus*. Latetr, Sáenz (1981) followed Thellung's (1926) system in general, but placed species belonging to sect. *Leptodaucus* and *D. crinitus* of *Meiodes* into sect. *Daucus* on the basis of the triangular shape of vittae. Heywood (1982), however, elevated all the subsections Okeke (1978) treated to sectional status and recognized seven sections of the genus *Daucus*; *Anisactis*, *Chrysodaucus*, *Daucus*, *Leptodaucus*, *Meiodes*, *Platyspermum*, and *Pseudoplatyspermum*. Although twenty-one species were reported to be members of *Daucus* (Heywood, 1982), it is difficult to determine the exact size of the genus because of disagreements about the delimitation of the species. If slight variations in the subspecies of *Daucus carota* L. were considered to be important for recognition of species, number of species within *Daucus* increased up to about sixty species (Cheeseman, 1925; Zohary, 1972). Two more species have been also reported in Europe as new taxa; *D. arcanus* Garcia and Silvestre, and *D. conchitae* Greuters in the Heywood's (1982) checklist of the genus.

Recently, several studies have demonstrated that the utility of molecular data in examining evolutionary relationships of the spiny-fruited umbels using nuclear rDNA ITS sequences (Lee and Downie, 1999) and cpDNA restriction sites (Lee and Downie, 2000). These studies implied that the genus *Daucus* may not be monophyletic: several other genera including *Pseudorlaya* Murb. and *Agrocharis* Hochst. were nested within the genus *Daucus*. Unfortunately, the purpose of each of these studies was not to resolve the infrageneric relationships within the specific genus but rather to infer the higher-level groupings within the family or tribes. Therefore, they did not address generic limits, especially in the larger genera

such as *Daucus* because of the limited number of species investigated.

As part of investigations on the evolutionary relationships of the genus *Daucus*, we have examined nuclear ribosomal DNA ITS sequences of 35 taxa belonging to *Daucus*, *Pseudorlaya*, and *Agrocharis*. Our objectives are as follows: (1) to delimit a generic boundary of the genus *Daucus*; (2) to ascertain phylogenetic relationships of the seven sections within *Daucus*; (3) to determine the taxonomic position and status of the newly reported species of *Daucus*.

## MATERIALS AND METHODS

### Terminal taxa

Twenty-six accessions from 18 species of *Daucus*, all three species of *Pseudorlaya*, all four species of *Agrocharis*, and monotypic *Pachyctenium* Maire & Pamp. ex Pamp. were examined for ITS sequence variation (Table 1). In addition, one accession of *Agrocharis pedunculata* (Baker) Heywood & Jury collected in Tanzania without glochidiate apex of the secondary spines was included. In total, 35 accessions were considered in this study. Three species of *Orlaya* (i.e; *O. daucooides* (L.) Greuter, *O. daucorlaya* Murb., and *O. grandiflora* (L.) Hoffm.) and *Laserpitium hispidum* M. Bieb. were used to root the trees based on previous taxonomic schemes and previously reported nuclear ITS- and cpDNA-derived phylogenies (Lee and Downie, 1999; 2000; Downie *et al.*, 2000).

### Experimental strategy

Leaf materials for DNA extraction were obtained either directly from the field, from plants cultivated from seeds in the greenhouse, or from accessioned plants cultivated at several botanic gardens. For some species, DNAs were extracted from herbarium specimens. All plants were identified using published keys and comparison to herbarium specimens. Details of the DNA extraction procedures have been presented in Lee and Downie (1999). Double-stranded DNAs of the complete ITS region in each genomic DNA were amplified by the PCR (polymerase chain reaction) technique using primers "ITS 5" and "ITS 4" in an equimolar ratio (White *et al.*, 1990). For some DNAs extracted from herbarium materials, optimum amplification was achieved when the template DNA was diluted 1 : 100 or when the concentration of MgCl<sub>2</sub> was increased from 1.5 mmol/L to 3.0 mmol/L. Successful PCR amplifications resulted in a single DNA band corresponding to approximately 700 bp in size. The sequence data were obtained using both Applied Biosystem (Foster City, California, USA) 310 Automatic DNA sequencer with Stretch upgrade and manual sequencing.

**Table 1.** Accessions of the genus Apiaceae genus *Daucus* and putatively allied genera examined for nuclear rDNA ITS variation. GenBank accession numbers for ITS1 and ITS2 sequences are provided. Sections of *Daucus* are based on Heywood and Jury (in Heywood, 1982). Herbarium acronyms were referred according to Holmgren *et al.* (1990). Recently reported two species were indicated by an asterisk (\*).

Taxon	Source and voucher information	Genbank accession no.
<b>Genus <i>Daucus</i> L.</b>		
<b>Section <i>Daucus</i></b>		
<i>D. capitillifolius</i> Gilli	Libya, near Tripoli (E)	AY065318, AY065319
<i>D. carota</i> L.		
subsp. <i>azoricus</i> Franco	Morocco, Marruecos, anonymous (RNG)	AY065312, AY065313
subsp. <i>carota</i>	Kazakhstan, cult. UIUC from seeds obtained from USDA acc. no. 47882	AF077779, AF077094
subsp. <i>drepanensis</i> (Arcang.) Heywood	Portugal, cult. the Reading Univ. U.K.	AY065314, AY065315
subsp. <i>gudecaei</i> (Rony and Camus) Heywood	France, cult. the Reading Univ. U.K.	AY065316, AY065317
subsp. <i>gummifer</i> Hook. f.	cult. UIUC from seeds obtained from Jardin botanique de Caen, France	AF077782, AF077097
subsp. <i>halophilus</i> (Brot.) Okeke	cult. UIUC from seeds obtained from J.-P. Reduron, Mulhouse, France	AF077782, AF077097
subsp. <i>maximus</i> (Desf.) Bail	cult. UIUC from seeds obtained from Institut für Pflanzen-genetik und Kulturpflanzenforschung, Gatersleben, Germany	AF077778, AF077093
subsp. <i>sativus</i> (Hoffm.) Arcang.	cult. UIUC from seeds obtained from Institut für Pflanzen-genetik und Kulturpflanzenforschung, Gatersleben, Germany	AF077780, AF077095
<i>D. gracilis</i> Steinh.	Algeria, Davis 52098 (RNG)	AY065344, AY065345
<i>D. guttatus</i> Sibth. and Sm.	Greece and Aegean Islands, North Cyclades, Andros. Jury and Warren 209 (RNG)	AY065336, AY065337
<i>D. involucreatus</i> Sibth. and Sm.	Crete, Nomos chaniou, Hora Sfakion. Bowen 8896 (E)	AY065334, AY065335
<i>D. sahariensis</i> Murb.	Algeria, JGR and AA 129-108 (RNG)	AY065320, AY065321
<i>D. syrticus</i> Murb.	Libya, Tripoli, near the Univ. of Libya (RNG)	AY065324, AY065325
<i>D. tenuisectus</i> Cosson ex Battand.	Morocco, South of Marrakech. Jury and Springate s.n. (RNG)	AY065326, AY065327
<b>Section <i>Meoides</i> Lange</b>		
<i>D. crinitus</i> Desf.	cult. UIUC from seeds obtained from Jardin Botaniques Lisboa, Portugal	AF077786, AF077101
<b>Section <i>Anisactis</i> DC.</b>		
<i>D. duriena</i> Lange	Israel, Samarian desert, near Sartaba, O. Cohen s.n. (HUJ)	AF077790, AF077105
<i>D. glochidiatus</i> (Labill.) Fischer and C. A. Meyer	Australia, New South Wales, North of Araleun, B.J. Lepschi 449 (CANB)	AY065340, AY065341
<i>D. montanus</i> Humb and Bonpl.	Argentina, cult. Botanical Garden, Univ. California, Berkeley, Constance 94.0563	AF077789, AF077104
<b>Section <i>Leptodaucus</i> Thell.</b>		
<i>D. pusillus</i> Michaux	cult. Botanical Garden, Univ. California, Berkeley Constance 92.0891	AF077788, AF077103
<b>Section <i>Chrysodaucus</i> Thell.</b>		
<i>D. aureus</i> Desf.	cult. UIUC from seeds obtained from Institut für Pflanzen-genetik und Kulturpflanzenforschung, Gatersleben, Germany, Lee 57 (ILL)	AF077784, AF077099

Table 1. Continued.

Taxon	Source and voucher information	Genbank accession no.
Section <i>Platyspermum</i> (Hoffm.) DC.		
<i>D. muricatus</i> L.	cult. UIUC from seeds obtained from Institut für Pflanzenzengenetik und Kulturpflanzenforschung, Gatersleben, Germany, Lee 36 (ILL)	AF077785, AF077100
Section <i>Pseudoplatyspermum</i> Thell.		
<i>D. bicolor</i> Sibth. and Sm.	Israel, Judean Mountains, Har Herzal, near Jerusalem, O. Cohen s.n. (HUJ)	AF077791, AF077106
subsp. <i>bicolor</i>	Lebanon, cult. UIUC, from seeds obtained from USDA accession	AF077783, AF077098
subsp. <i>broteri</i> (Ten.) Okeke	no. 286611, Lee 185 (ILL)	
* <i>Daucus arcanus</i> García and Silvestre	Spain, Huelva, Almonte, Matalascañas. García and Silvestre s.n. (E)	AY065338, AY065339
* <i>Daucus conchitae</i> Greuter	Turkey, Mugla, East of marmaris. Jury and Warren 366 (RNG)	AY065332, AY065333
Genus <i>Agrocharis</i>		
<i>A. incognita</i> (Norman) Heywood and Jury	Kenya, Nairobi, E. Knox 2578	AF077793, AF077108
<i>A. gracilis</i> Hooker	Cameroun, 35 km NW Fouban, R. Letouzey 12123 (K)	AY065344, AY065345
<i>A. melanantha</i> Hochst.	Kenya, Nairobi, E. Knox 2579	AF077794, AF077109
<i>A. pedunculata</i> (Baker) Heywood and Jury	Malawi, Limbe, Mpingwe Hill, Hillard and Burt 4131 (E)	AF077792, AF077107
<i>A. pedunculata</i> (Baker) Heywood and Jury	Tanzania, Ludewa district, Livingstone mountain, R.E. Gereau and C. J. Kayambo 38870 (E)	AY065342, AY065343
Genus <i>Pachyctenium</i> Maire and Pamp.	Libya, E. Shahat, Cyrene, Davis 50249 (E)	AF077787, AF077102
<i>P. mirabile</i> Maire and Pamp.		
Genus <i>Pseudorlaya</i> Murb.	Algerie, Tindouf. Cult. Univ. of California from seeds from Centre National de la Recherche Scientifique, Beni-Abbes. accession no. C-958.	AY065328, AY065329
<i>P. biseriatus</i> (Murb.) Sáenz	Spain, anonymous (RNG)	AY065330, AY065331
<i>P. minuscula</i> (Pau) Lainz	cult. UIUC from seeds obtained from Jardin. Botaniques	U30522, U30523
<i>P. pumila</i> (L.) Grande		
Outgroup		
<i>Laserpitium hispidum</i> Bieb.	cult. UIUC from seeds obtained from Botanical Garden of Research Institute of Ecology and Botany, Hungarian Academy of Sciences, Hungary	U78361, U78421
<i>Orlaya daucooides</i> (L.) Greutter	cult. UIUC from seeds obtained from Botanical Garden of Research Institute of Ecology and Botany, Hungarian Academy of Sciences, Hungary	AF077797, AF077113
<i>Orlaya daucorlaya</i> Murb.	Yugoslavia, Macedonia, Kuceviste, Edmondson 27 (E)	AF077798, AF077113
<i>Orlaya grandiflora</i> (L.) Hoffm.	cult. UIUC from seeds obtained from Botanical Garden	U30524, U30525

Cyclic sequencing reactions were carried out in a PTC-100 thermocycler (M. J. Research, Cambridge, Massachusetts, USA) using the purified PCR products. Ampli Taq DNA polymerase, and fluorescent dye-labeled terminators (Perkin-Elmer, Norwalk, Connecticut, USA). All automated and manual-sequenced outputs were checked visually and edited for correct automated base-calling.

### Phylogenetic analyses

Only ITS 1 and ITS 2 regions were included in the analysis since sequence data for the intervening 5.8S subunit were incomplete for many taxa, and those data available were not sufficiently variable. DNA sequences were aligned using CLUSTAL V (Higgins *et al.*, 1992), adjusted manually where necessary, and imported into PAUP\* version 4.0b4a (Swofford, 2000). Only those positions that were in obvious alignment were used in the distance calculations and phylogenetic analyses. Pairwise nucleotide differences of unambiguously aligned positions were determined using the distance matrix option in PAUP\*. In the phylogenetic analysis, all gaps were treated as missing data. Transition/transversion (Ts/Tv) ratios were calculated using MacClade (version 3.0; Maddison and Maddison, 1992) across all maximally parsimonious trees obtained. The ITS data were analyzed initially using maximum parsimony (MP). All heuristic searches were conducted with 100 random addition replicates and tree bisection-reconnection branch swapping. The options mulpars, steepest descent, collapse, and acctran optimization were selected. To assess the relative support for each clade, bootstrap values (Felsenstein, 1985) were calculated from 100 replicate analyses using the heuristic search strategy and simple addition sequence of taxa. To complete the decay analysis, the computer program AutoDecay (Eriksson, 1997) was used, following the converse-constraint method (Baum *et al.*, 1994). The amount of phylogenetic information in the parsimony analysis was estimated using the consistency (CI; Kluge and Farris, 1969) and retention (RI; Farris, 1989) indices. Distance trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987), implemented using the Neighbor program in Felsenstein's (1993) PHYLIP (version 3.572). Distance matrices were calculated using the DNADIST program of PHYLIP, and the numbers of nucleotide substitutions were estimated using Kimura's (1980) two parameter method. A bootstrap analysis of the data was done using 100 resampled data sets generated using the SEQBOOT program prior to calculating the distance matrices and neighbor-joining trees. PHYLIP's CONSENSE program was then implemented in order to construct a strict consensus tree. The maximum likelihood (ML) method was also applied to these ITS data using the program fastDNAML (version 1.0.6; Olsen *et al.*, 1994), based on

the procedures of Felsenstein (1981). ML tree was inferred using a Ts/Tv rate ratio of 1.6, randomizing the input order of sequences (jumble), and by invoking the global branch swapping search option.

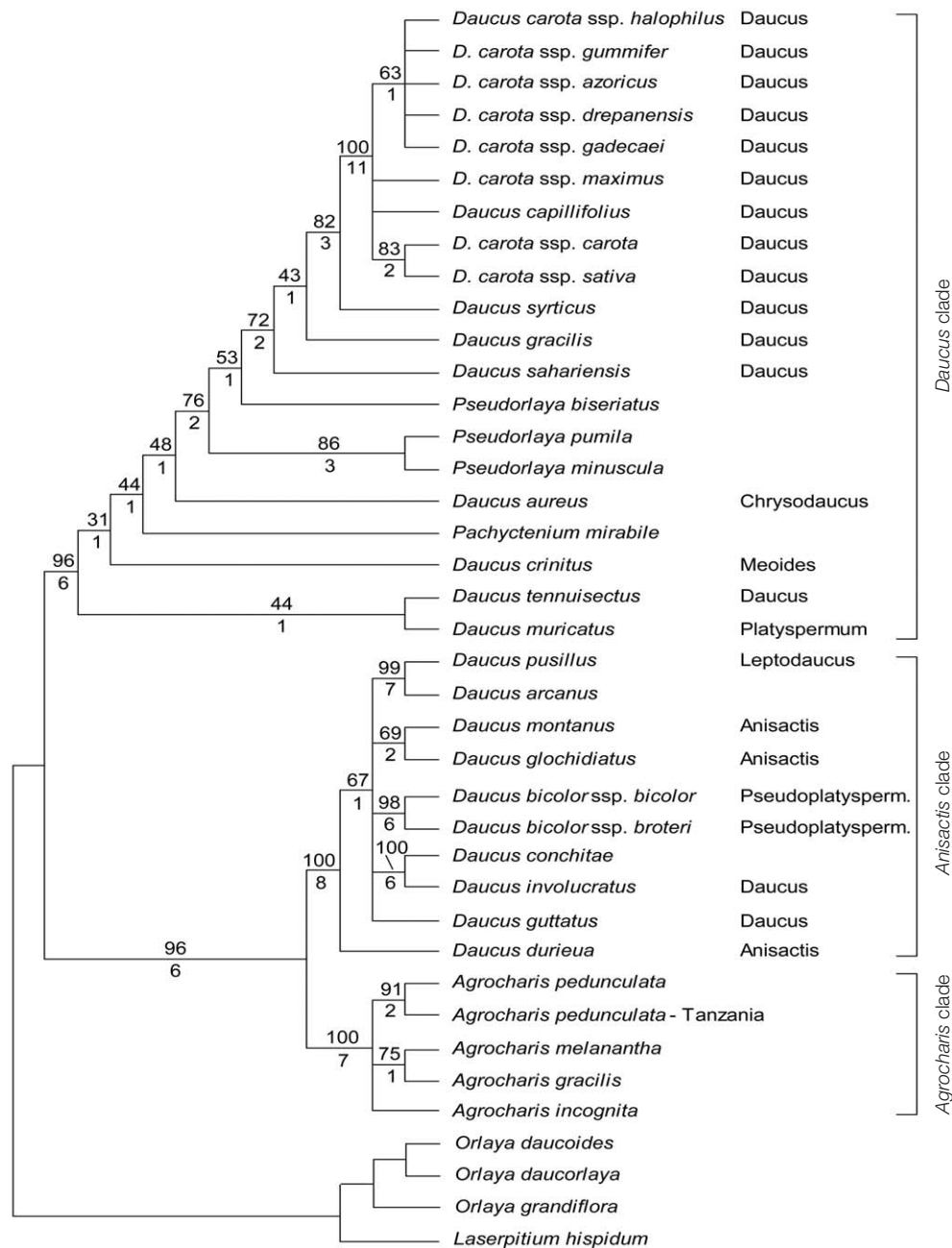
## RESULTS

### Sequence divergence

The ITS region in *Daucus* and putatively related genera ranges from 438 to 442 bp; these sizes are comparable to sizes reported in other Apiaceae genera (Downie and Katz-Downie, 1996; Katz-Downie *et al.*, 1999; Downie *et al.*, 2000). On average, the ITS1 region (216.87 bp in size) is slightly shorter than the ITS2 region (223.26 bp). Alignment of all 39 complete ITS1 and ITS2 sequences resulted in a matrix of 468 nucleotide positions. Of the 468 initial alignment positions, 40 positions (approximately 8.5% of the sequences) were deleted due to alignment ambiguities. Of the remaining 428 unambiguously aligned positions, 145 (33.9%) were potentially parsimony informative, 214 (50.0%) were constant, and 69 (16.1%) were unique to individual taxa. In direct pairwise comparisons of unambiguous positions among all 39 accessions, sequence divergence values ranged from identity to 22.2% of nucleotides in ITS1 and from identity to 24.2% of nucleotides in ITS2. Comparisons of sequence pairs across both spacer regions gave divergence values ranging from identity among four subspecies of *D. carota* (subsp. *halophilus*, subsp. *gummifer*, subsp. *azoricus*, and subsp. *gadecaei*) to 21.5% between *Daucus guttatus* and *Orlaya daucooides*. Within the genus *Daucus*, sequence divergence values ranged from identity to 17.1% between *D. guttatus* and *D. aureus* or *D. muricatus*. Variation in the ITS sequences of recently established new species was not extensive and indicated a close homology with its putatively related taxa; 0.73% between *D. arcanus* and *D. pusillus*, 0.48% between *D. involuocratus* and *D. conchitae*.

### Phylogenetic analysis

Parsimony analysis of 39 combined ITS1 and ITS2 sequences resulted in 11 minimal length trees; the strict consensus of these trees, with accompanying bootstrap and decay values, is presented in Fig. 1. Each of these trees had a length of 431 steps, CI's of 0.6613 and 0.5817 (with and without uninformative characters, respectively), and a RI of 0.8387. The neighbor-joining tree, calculated with a Ts/Tv rate ratio of 1.6 based on the actual inferred frequencies determined over all 11 maximum parsimony trees by MacClade, is presented in Fig. 2. On this tree, bootstrap values < 20% are not indicated. The same topology resulted when Ts/Tv rate ratio of 1.0 or



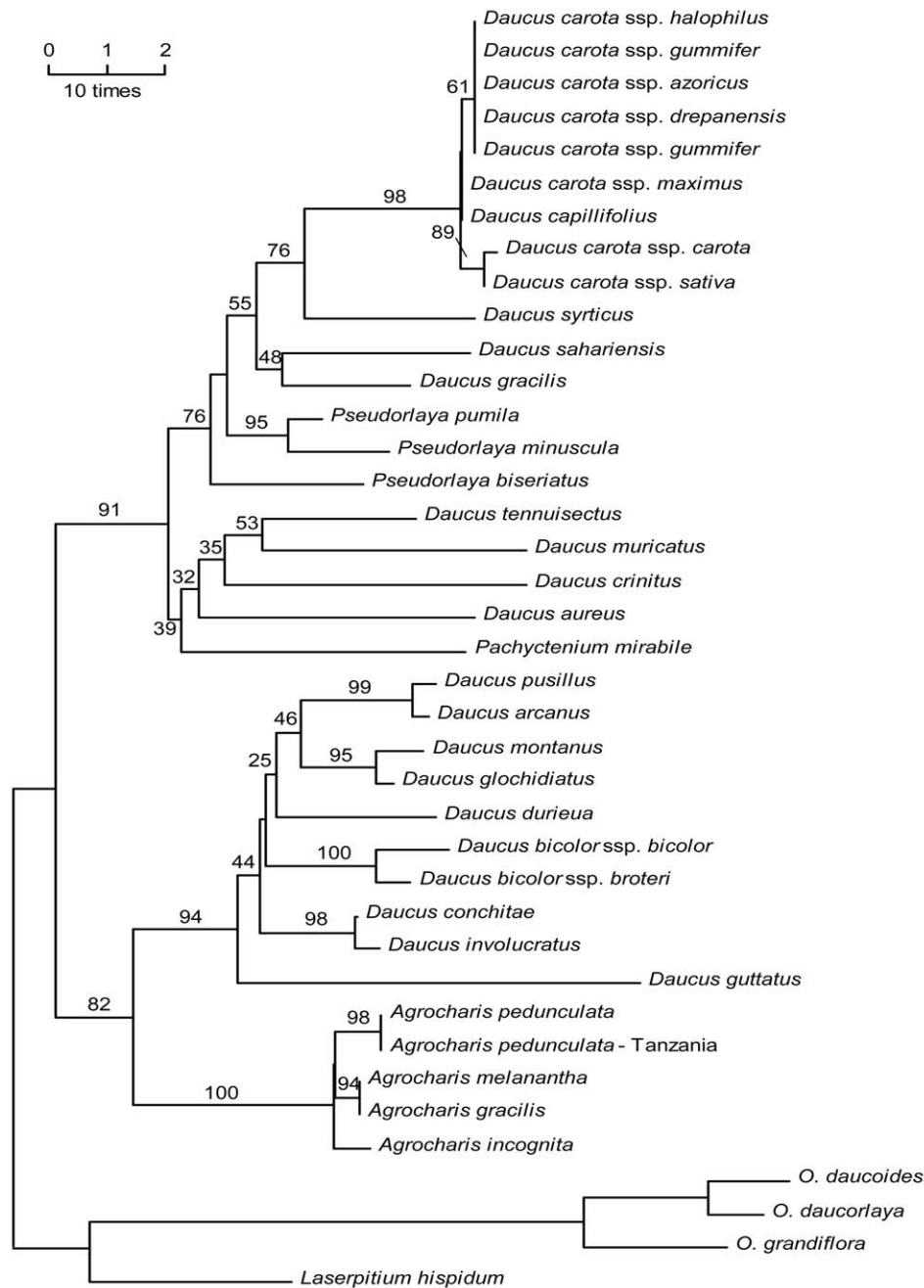
**Fig. 1.** Strict consensus of 11 parsimony trees derived from equally-weighted parsimony analysis of combined nuclear DNA ITS1 and ITS2 sequences from *Daucus* and its relatives using all unambiguously-aligned positions (CIs with and without uninformative characters = 0.6613 and 0.5817; RI=0.8387). From the left to the right, names of taxa, sections, and clades are given. Numbers above the nodes indicate the number of times a monophyletic group occurred in 100 bootstrap replicates; AutoDecay values are given below.

2.0 were used. The best maximum likelihood tree, also calculated with a Ts/Tv rate ratio of 1.6, had a long likelihood value of  $-2938.56$ .

### Phylogenetic resolutions

Phylogenies estimated using maximum parsimony,

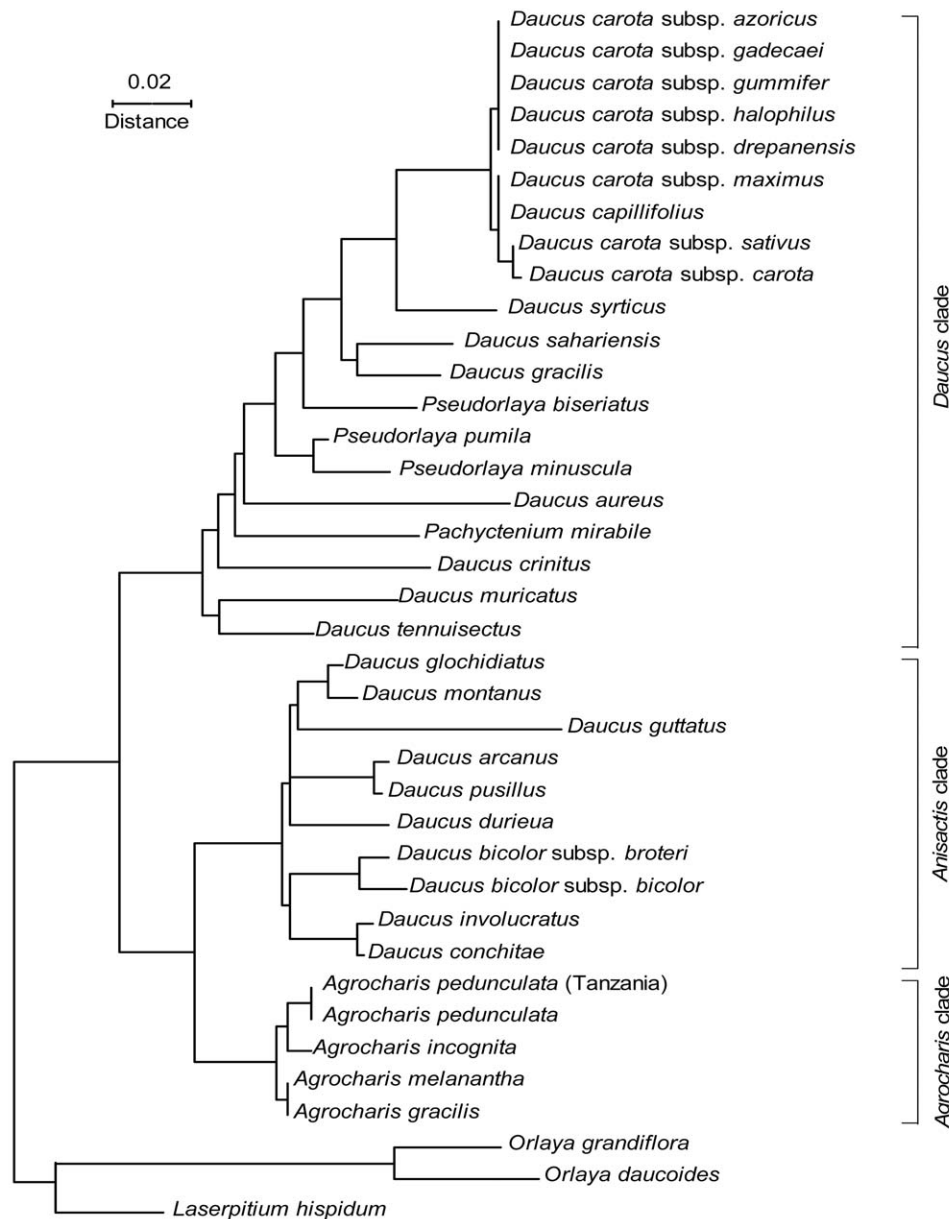
neighbor-joining, and maximum likelihood methods give essentially similar topologies, with those few areas of discord noted below. In each of these trees, three major clades of taxa are clearly discernable with high values of bootstrap and decay. The first group includes the species, *Daucus carota*, *D. capillifolius*, *D. syrticus*, *D. gracilis*, *D. sahariensis*, three species of *Pseudorlaya* (*P. biseria-*



**Fig. 2.** Neighbor-joining tree inferred from the analysis of 39 nuclear rDNA ITS1 and ITS2 sequences from Apiaceae genus *Daucus* and its relatives using a transition/transversion rate ratio of 1.6. Branch lengths are proportional to distance estimated from the two parameter method of Kimura. Numbers at nodes indicate bootstrap values for 100 replicate analyses. On this tree, bootstrap values <20% are not indicated.

*tus*, *P. pumila*, and *P. minuscula*), *D. aureus*, *Pachyctenium*, *D. crinitus*, *D. tenuisectus*, and *D. muricatus*. The second group includes *Daucus pusillus*, *D. arcanus*, *D. montanum*, *D. glochidiatus*, *D. bicolor*, *D. conchitae*, *D. involucratus*, *D. guttatus*, and *D. durieua*. The third group is consisted exclusively of species of *Agrocharis* (*A. pedunculata*, *A. incognita*, *A. melanantha*, and *A. gracilis*).

We have named these three groups the *Daucus*, *Anisactis*, and *Agrocharis* clades, respectively (Figs. 1, 3). The close relationship between our second and third clades is largely congruent as a result of each of the phylogenetic analyses. With regard to the *Daucus* clade, *D. carota*, represented herein by eight subspecies, is not monophyletic due to inclusion of north African *D. capillifolius* within



**Fig. 3.** Maximum likelihood tree constructed from 38 nuclear rDNA ITS1 and ITS2 sequences from Apiaceae genus *Daucus* and relatives using a transition/transversion rate ratio of 1.6. Branch lengths are proportional to the number of expected nucleotide substitutions per site.

the species. This subclade, in turn, is sister to *Daucus syrticus*. Next is *D. gracilis*, followed by *D. sahariensis*. *D. gracilis* and *D. sahariensis* ally weakly in both NJ and ML trees (Figs. 2, 3) but not in the MP tree (Fig. 1). *Pseudorlaya* arises next, but not monophyletic because *P. biseriatus* is placed outside from the genus. *Daucus aureus*, *D. crinitus*, and *Pachyctenium* each arises separately forming unresolved branches in the MP tree whereas *D. muricatus* and *D. tenuisectus* ally in all MP, NJ, and ML trees. Within the *Anisactis* clade, four paired groups are evident. Each of these groups with the exception of a group comprising *D. montanus* and *D. glochidiatus* is

supported by a high bootstrap value. *D. guttatus* is variably positioned depending upon the method of tree construction used. In the MP tree, *D. guttatus* arises as an independent branch of a polychotomy, whereas it is sister to all other taxa within the *Anisactis* clade in the NJ tree (Fig. 2). In the other hand, *D. guttatus* is sister to a small clade comprising *D. glochidiatus* and *D. montanus* in the ML tree (Fig. 3). *Daucus conchitae*, newly established from Turkey, is closely related to *D. involucratus*. Surprisingly, the close relationship between American *D. pusillus* and another new species from Spain, *D. arcanus*, is strongly supported by high bootstrap values (99% in



both MP and NJ trees). However, two species (*D. pusillus* and *D. montanus*) with a closer continental relationship between South and North America fail to form a clade. South American *D. montanus* allies with Australian *D. glochidiatus*, not North American *D. pusillus* in all trees constructed. The monophyly of the tropical African *Agrocharis* is strongly supported by 100% of the bootstrap values in both MP and NJ trees.

## DISCUSSION

Previous molecular and morphological systematic studies (Lee and Downie, 1999; 2000; Lee *et al.*, 2001) of tribe Caucalideae revealed that the monophyly of the genus was not suggested. Expanded sampling of *Daucus* species in this study supports the previous studies showing not monophyly of the genus *Daucus*. Here, we discuss the monophyly of *Pseudorlaya*, sectional relationships within *Daucus*, and phylogenetic relationships of each species investigated.

### Monophyly of the genus *Pseudorlaya*

Morphologically, *Pseudorlaya* is distinguished from *Daucus* in having two rows of fruit secondary spines instead of one (Davis, 1972; Zohary, 1972). This justifies the transfer of *D. biseriatus* to *Pseudorlaya* by Sáenz de Rivas and Heywood (1974). Molecular phylogeny derived from ITS sequences of all the species of *Pseudorlaya* including *P. biseriatus* and *P. minuscula* does not support monophyly of the genus. Failure of monophyly of the genus suggests that the number of spine arrangements is not a good character to delimit the generic boundary of *Pseudorlaya*. Furthermore, Lee *et al.* (2001) reported that these paired spines are alternate with single ones in *Pseudorlaya pumila*. Additional evidence from molecular phylogenies, along with other information, suggests that arrangement rows of secondary spines be not enough to retain *Pseudorlaya* as a distinct genus. Another diagnostic character for *Pseudorlaya* is dome-shaped primary ridges (Lee *et al.*, 2001). However, *P. minuscula* is not obviously widened at the fruit base from recent observation of the type material (Arnold and Jury, 1996). Otherwise, *Pseudorlaya* is very similar to *Daucus*, both morphologically and chemically (Harborne *et al.*, 1969; Heywood and Dakshini, 1971; Williams and Harborne, 1972) and thus might be included within the genus *Daucus*.

### Sectional relationships within the genus *Daucus*

Most classification systems of the family Apiaceae rely on a diverse array of subtle fruit differences to demarcate major taxonomic groups. However, serious doubts have been raised on the phylogenetic utility of these characters

(Theobald, 1971; Davis, 1972; Jury, 1982; Shneyer *et al.*, 1992). Considering the genus *Daucus*, each section possesses a unique set of fruit surface features (Heywood and Dakshini, 1971; Sáenz de Rivas *et al.*, 1982). These characters, while permitting the easy recognition of species, are less useful in providing information on infrageneric relationships. Heywood and Jury's (in Heywood, 1982) division of the genus into seven sections which is largely refinement of Thellung's (1926) treatment is most commonly used. Section *Daucus* has been considered unnatural in a sense that several species were placed in the section primarily based on the superficial similarity of morphological and anatomical characters, as mentioned by Heywood (1971). In observation of microcharacters of fruits, Heywood and Dakshini (1971) found that morphological features of some species overlap with those of other sections of the genus, suggesting that section *Daucus* seems not to be natural. However, on the basis of morphological similarities in relative length of spines to width of the mericarp, degree of connation at the base of spines, length of styles, and anatomical features of the fruits, Okeke (1977) and Heywood (1982) recognized 11 species within the section. Based on our results using nine species of the section, sect. *Daucus* is not monophyletic because *D. tenuisectus*, *D. guttatus*, and *D. involucratus* are closely related to other sections. Section *Anisactis* includes *Daucus durieua*, *D. glochidiatus*, *D. hochstetteri*, and *D. montanus* (Heywood, 1982). The umbel characters of *D. durieua*, *D. glochidiatus*, and *D. hochstetteri* are remarkably distinct from those of other sections. They include axillary umbels, few rays, whorled cauline leaves, and lack of peduncles and normal bracts, suggesting close relationships among these three species (Okeke, 1978). Results of our molecular phylogenies, however, do not support the monophyly of the section because *D. durieua* is not included within. *Daucus* species in the New World (i.e., *D. pusillus* and *D. montanus*) were treated separately into different sections based on the shape of vittae (Thellung, 1926; Sáenz, 1981; Heywood, 1982), which is consistent with phylogenies derived from ITS sequences herein. However, the phylogenies inferred from the morphology and chloroplast DNA restriction variation suggest the close relationship between two taxa based on all perfect flowers and obsolete calyx teeth, and five nonhomoplastic chloroplast DNA restriction site variation (Lee and Downie, 2000; Lee *et al.*, 2001). *D. glochidiatus*, endemic to Australia, showed the close relationships with South American *D. montanus*. These two species share polyploidy of  $x=11$  as well as morphological and anatomical characters of fruits (Okeke, 1978). Section *Meoides* contains *D. crinitus* and *D. setifolius* and is characterized by the presence of unique pseudo-verticillate leaf arrangement, perennial vegetation, and the absence of glochidiate apex on the second

dary spines. However, Sáenz (1981) incorporated *D. crinitus* into section *Daucus* on the basis of similarity of the large and triangular-shaped vittae, leaving *D. setifolius* as the sole member of *Meoides*. Our results do not confirm Sáenz's (1981) transfer of *D. crinitus* into section *Daucus* because the close relationship between them was not supported. From the result, it can be assumed that the large and triangular-shaped vittae seen in many members of section *Daucus* have arisen independently. The results of molecular phylogenies investigated herein agree generally with the recognition and independent separation of each of Heywood's (1982) mono- or di-typic sections (i.e., sections *Chrysodaucus*, *Platyspermum*, *Leptodaucus*, and *Pseudoplatyspermum*) within the genus. The close relative of section *Chrysodaucus* is suggested to be section *Platyspermum* from recent phylogenetic studies using morphological characters (Lee *et al.*, 2001). This result is consistent with Okeke's (1978) classification system which treated *D. aureus* (sect. *Chrysodaucus*) as a member of sect. *Platyspermum*. Although these two sections share closely located vascular bundles on the commissural surface, reduced elliptic vittae, and sclerenchymatous cells between their commissural vittae (Okeke, 1978), our molecular phylogenies do not support the union between these two sections. Homologically, section *Chrysodaucus* including *D. aureus* is distinct from other sections in having overwhelmingly swollen calyx below the stylopodium, and varying degrees of heterocarpic fruits. In addition, the section develops strong secondary spines with glochidiate apex around the outmost margins of the umbel whereas it has very shortened secondary spines without glochidiate apex in inner areas of the same umbel. Section *Platyspermum* is unique in particularly large size of fruits and strong confluence at the base of secondary spines whereas section *Pseudoplatyspermum* can be distinguished from all other *Daucus* species by shapes of bracts. Bracts of the section are trifid rather than pinnatisect or leaf-like, and the unique color of its rays and styles (Okeke, 1982; Lee *et al.*, 2001). Emphasizing the presence of the triangular shape of vittae, Okeke (1978) and Sáenz (1981) treated American endemic *D. pusillus* as a member of European section *Daucus*. However, ITS molecular phylogenies do not support the inclusion of *D. pusillus* into section *Daucus* showing closer relationship among *D. pusillus*, sections *Anisactis* and *Pseudoplatyspermum*.

### Species relationships of the genus *Daucus*

The ITS phylogeny recognizes two major clades within the genus, with some *Daucus* species allied with all the species of *Pseudorlaya* and *Pachyctenium mirabile*, and others with *Agrocharis*. Taxonomic delimitation of each *Daucus* species will be discussed in the following

sections.

***Daucus carota*:** The delimitation of *Daucus carota* L. has been one of the most confused problems in Apiaceae classification because well-defined sterility barriers have not been developed among subspecies of *D. carota*, and even between *D. carota* and its relative species (i.e., *D. capillifolius*), often leading to viable hybrid progenies (McCollum, 1975; 1977; Small, 1978; Debonte *et al.*, 1984). Thellung (1926) and Ono (1936) recognized two groups (*Gummiferi* and *Eucarota*) on the basis of shapes of fruiting umbels, fleshiness and shininess of the leaf, and dissection degree in leaf segments and bracts. In the most recent classification system of *D. carota* complex, Heywood (1983) recognized 13 subspecies which were assigned into two groups. Of Heywood's subspecies of *D. carota*, *Carota* group includes subspecies *carota*, *sativus*, *azoricus*, *maritimus*, and *major* whereas the other *Gingidium* group contains other remaining subspecies (i.e., *gadecaei*, *gummifer*, *drepanensis*, and *hispanicus*). Upon consideration of available ITS sequences, group separation among *Daucus carota* is not supported because *D. carota* ssp. *azoricus* was nested within the group *Gingidium*. Not enough variation of ITS sequences among subspecies of *D. carota* indicates that these sequences are less useful in resolving intraspecific relationships within *D. carota*. No clear pattern of division is consistent with results derived from phytochemical (Williams and Harborne, 1972), karyotype (Owens, 1974; McCollum, 1975), and morphological studies (Small, 1978). As suggested in isozyme analyses (Pierre *et al.*, 1990), subspecies of *D. carota* may be a young taxa in terms of evolutionary history with marginal groups have not yet been separated genetically from each other.

***Daucus capillifolius*:** Morphologically, *D. capillifolius* is distinctive from *D. carota* by having glabrous 2- to 3-pinnatisect leaves with filiform segments. The ITS phylogeny investigated herein does not support separation of *D. capillifolius* from *D. carota*, showing that *D. capillifolius* is nested within the latter. The close relationship between *D. capillifolius* and *D. carota* is also suggested by a hybridization study performed by McCollum (1975; 1977). He and his colleagues were able to produce hybrids between *D. capillifolius* and some subspecies of *D. carota*. Although morphological, anatomical, and phytochemical variation between *D. carota* and *D. capillifolius* is extensive, successful hybridization and close phylogenetic relationships between *D. carota* and *D. capillifolius* raise some doubts on the specific status of *D. capillifolius*, and thus it maybe treated as a subspecies of the *Daucus carota*.

***Daucus syrticus*, *D. gracilis*, and *D. sahariensis*:** Although *Daucus syrticus*, *D. gracilis*, and *D. sahariensis*

did not form a clade in the MP and ML trees, they are probably the most closely related plants to *D. carota*. These three north African and east Mediterranean species are characterized morphologically by the presence of linear to filiform leaf segments, irradiated petals on the scattered umbels, and the same base chromosome number ( $x=9$ ) as those of *D. carota* and *D. capillifolius*. The sister relationship of *D. syrticus* to *D. carota* sensu lato strongly supported from ITS phylogeny is also congruent to the results of a cytological study. In the comparison of karyotype morphology among seven species of *Daucus* (Owens, 1984), the general similarities were found between *D. carota* and *D. syrticus*, reflecting that the two species can be hybridized potentially to improve germplasm of *D. carota* sensu lato.

***D. involucratus* and *D. guttatus*:** The second clade of plants within *Daucus*, allied with *Agrocharis*, is supported strongly with a high bootstrap value of 96% and decay index of six in the MP tree. Preliminary investigation of morphological and anatomical characters on genus *Daucus* reveals that the number of rows of primary hairs (i.e., over three rows) is a probable character to support, in part, the relationships among these plants. Two species of *Daucus* (i.e., *D. involucratus* and *D. guttatus*) were treated as members of section *Daucus* by Heywood (1982), but they did not show close relationships with any species of the section in the ITS phylogeny. Furthermore, careful observation on overall morphology identified several morphological characters to support the separation of these two species from section *Daucus*. One of them is the floral structure. *D. involucratus* is remarkably similar in the floral structure to those of *D. durieua*, *D. glochidiatus*, *D. hochstetteri*, *D. montanus* and *D. pusillus*. Their petals are minute and tend to be unlobed at the apex, and their styles are very short. The fruit of *D. guttatus* shows a remarkable development of tubercules which were not observed in any species of the section *Daucus*. Strongly tuberculated spine is one of the most conspicuous features in delimiting section *Anisactis*. The close relationship between *D. guttatus* and section *Anisactis* is also congruent to results of phytochemical investigations (Okeke, 1978). *D. guttatus* and *D. durieua* contain a flavonol, kaempferol whereas other species of section *Daucus* have either flavones (e.g., luteoline, apigenin) or a flavonol (e.g., quercetin). These morphological and phytochemical peculiarities of *D. involucratus* and *D. guttatus* which are not met elsewhere in the section *Daucus* provide further support for inclusion of these two species within the *Anisactis* clade.

***Daucus conchitae* and *D. arcanus*:** *Daucus conchitae*, established by Greuter (1979), was suggested to fall outside the range of morphological variation of the putatively related *D. guttatus* and *D. involucratus*. Greuter (1979)

proposed that the closely related species of *D. conchitae* might be *D. involucratus* on the basis of similarities in general habit. The results of molecular phylogenies investigated here support the close relationship between these two species, with 100% and 98% bootstrap values in MP and NJ trees, respectively. However, low divergence values (0.48%) of ITS sequences between *D. conchitae* and *D. involucratus* indicate that they were separated relatively recently. *Daucus arcanus*, another new species recently established from southern Spain by Martin and Silvestre (1990), is closely related to north American *D. pusillus*. Despite long distance between habits of these species, the sequence divergence of ITS is extremely low, less than 1%. Morphologically, these two species are very similar to each other except the much smaller size of *D. arcanus*. They also share a base chromosome number of eleven. To date, other molecular information is not available for *D. arcanus*, but we consider that this new species may be infraspecific taxon of *D. pusillus*. The fruits of genus *Daucus* are dispersed by wind and animals (Lacey, 1981). The smallness, dryness and lightness of the fruits render them easily wind-borne. The fruits may also be carried on animal fur, attached by their hooked spines, tubercles and spine barbs (Okeke, 1978). The latter may be responsible for the introduction of *D. pusillus* from America to parts of Europe (i.e., Spain) as *D. carota* subsp. *carota*, a Mediterranean taxon, to most areas of the world, and Australian *Daucus glochidiatus* to most parts of Europe (Okeke, 1978).

Phylogenetic analyses using nuclear ribosomal DNA ITS sequences reveal that the genus *Daucus* is not monophyletic with genera *Pseudorlaya*, *Pachyctenium*, and *Agrocharis* nested within. Two groups within *Daucus* and a group comprising exclusively *Agrocharis* were recognized from the analysis; we have provisionally named these three groups the *Daucus*, *Anisactis*, and *Agrocharis* clades until more formal nomenclature can be applied. We are continuing our investigation of genus *Daucus* phylogeny by examining data from the chloroplast genome. Detailed morphological and anatomical investigations are also underway, and when completed will provide insight into character evolution, including the identification of morphological synapomorphies supporting each of the major clades identified herein on the basis of ITS data. Additional data from both chloroplast DNA and morphology are currently being pursued in order to clarify relationships within the polymorphic *Daucus* and their close relatives, such as *Pseudorlaya*, *Pachyctenium*, and *Agrocharis*.

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