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# Single Seed Raman Measurements Allow Taxonomical Discrimination of Apiaceae Accessions Collected in Gene Banks

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**Abstract:** NIR-FT-Raman spectroscopy was applied for a nondestructive analysis of single seeds (fruit mericarps) of 36 accessions belonging to various species of the Apiaceae family. Main seed components such as fatty acids, polysaccharides, proteins, and lignin were identified based on the obtained Raman spectra. Variation at the species and genus level was related to differences observed between spectra. The application of cluster analysis discriminated among most of the species evaluated and grouped them according to their taxonomical classification. The spectroscopically analyzed seeds germinated and developed into normal seedlings to demonstrate the additional advantage that Raman spectroscopy is nondestructive and can be applied to living seed without harm. These results indicate that Raman spectroscopy is a valuable tool for the rational evaluation and management of genetic resources in *ex situ* seed collections by providing useful information for taxonomical validation of the accessions. © 2006 Wiley Periodicals, Inc. *Biopolymers* 81: 497–505, 2006

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**Keywords:** FT-Raman spectroscopy; *in situ*; nondestructive; chemotaxonomy; *Daucus*; seed composition; Umbelliferae

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## INTRODUCTION

Conservation of Apiaceae (Umbelliferae) genetic resources is a challenging task due to the wide diversity existing in this cosmopolitan family, which comprises 455 genera and over 3500 species.<sup>1</sup> This complexity complicates the efforts of gathering, storing, and identifying seeds in ex situ collections. The US (GRIN database <http://www.ars-grin.gov/npgs>) and European (ECP/GR Umbellifer database <http://www.hri.ac.uk/gru>) Apiaceae collections are predominantly comprised of cultivated forms of carrot, celery, parsley, and several condiments, while wild relatives constitute a minority of the germplasm preserved in these collections. The biodiversity represented by these wild species is valuable for research and crop improvement, but they present a challenge. Part of the collected germplasm resources have not been morphologically characterized for several Apiaceae accessions and thus their exact taxonomy has not been validated. Furthermore, new accessions donated to the collections are often misclassified so that rational management of these genetic resources is difficult and the distribution of such material problematical. With these numerous challenges, the need for techniques that could assist in the characterization and particularly in the confirmation of the taxonomical identity of ex situ collections has been announced at the meeting of Umbelliferae Working Group within the European Cooperative Programme for Crop Genetic Resources.<sup>2</sup>

The broad diversity of Apiaceae provokes discussions on its proper delimitation and the criteria used for natural subdivisions.<sup>3,4</sup> Taxonomical classification of Apiaceae relies mainly on morphological characters of fruits and floral parts as proposed by Drude<sup>5</sup> over a century ago. Apiaceae fruit is dry and easily splits apart into two seed-bearing mericarps (commonly called seeds). The pericarp (fruit wall) is hard with ridges and for several species is covered with spines of varying morphology. The use of these morphological characters for classification purpose is however often problematical.<sup>3,6,7</sup> Chemotaxonomical studies have considerably broadened the knowledge about this complex family and have provided additional data on taxonomical relationships. For example, polyacetylenes, umbelliferose, and petroselinic acid are found to be characteristic for Apiaceae family, phenolic compounds, such as flavones and flavonols occurring in leaves, can be related to tribal divisions and were used to distinguish genera in the tribe Caucalideae, and specific terpenes, saponins, seed soluble proteins, and enzymes (esterase, peroxidase) can be used to make distinctions at tribal and generic levels.<sup>8–11</sup> The significance of chemotaxonomy has

been recently underlined by Grayer et al.,<sup>12</sup> who indicated that phylogeny of several families derived from chemical studies, particularly those included in the monumental work of Hegnauer “Chemotaxonomie der Pflanzen,” are congruent with inferences drawn from analyses of DNA sequences.

New instrumentation tools, including spectroscopy, have stimulated a rebirth of chemotaxonomical approaches to plant systematics.<sup>13,14</sup> In higher plants, Kim et al.<sup>15</sup> have applied FT–IR spectroscopy for phylogeny of seven distantly related species (3 monocots and 4 dicots). Chemotaxonomical classification of *Chamomilla*, *Ocimum*, *Origanum*, and *Thymus* has been performed at intrageneric level based on the composition of essential oils by using ATR–IR, NIR, and NIR–FT–Raman spectroscopy.<sup>16–18</sup> Furthermore, surface-enhanced Raman measurements performed directly on glandular trichomes in the stem sections have allowed five mint taxa to be discriminated.<sup>19</sup> Discrimination at generic level using Raman spectroscopy has been shown only in lichens by Jorge Villar et al.<sup>20</sup> who have characterized eight species belonging to four genera.

In this work we apply FT–Raman spectroscopy for analysis of single Apiaceae fruits (seeds) at the species as well as at the genus level. Using primarily validated specimens from the Mediterranean region we show that Raman spectroscopy is a useful tool for rapid evaluation and discrimination between taxa, with potential value for streamlining the management, characterization, and taxonomical validation of seed collections. Additionally, we demonstrate for the first time the nondestructive feature of Raman spectroscopy to living seed, since most seeds evaluated were subsequently able to germinate and produce normal plants.

## EXPERIMENTAL

### Plant Material

Seed samples of 36 accessions of Apiaceae from various geographical locations were obtained from ex situ collections (Table I). Their taxonomical classification had been previously validated at the North Central Regional Plant Introduction Station in Ames, Iowa and included in the Germplasm Resources Information Network (GRIN, USDA–ARS) database ([www.ars-grin.gov/npgs](http://www.ars-grin.gov/npgs)). *Daucus capillifolius* and *D. pusillus* were reproduced at the BAZ experimental station.

### Raman Measurements

Raman spectra were recorded using a NIR–FT–Raman Spectrometer (model RFS 100) from Bruker (Ettlingen,

**Table I** Apiaceae Used for Raman Measurements

Gene Bank No. <sup>1</sup>	Accession, Collecting No.
Ames 25624	<i>Caucalis platycarpus</i> L., G098
Ames 25797	<i>Caucalis platycarpus</i> L., T005
Ames 25804	<i>Caucalis platycarpus</i> L., T013
Ames 25875	<i>Caucalis platycarpus</i> L., T089
Ames 25736	<i>Daucus broteri</i> Ten., S066
Ames 25743	<i>Daucus broteri</i> Ten., S076
Ames 25752	<i>Daucus broteri</i> Ten., S086
HRI 7190	<i>Daucus capillifolius</i> Gilli
Ames 25575	<i>Daucus carota</i> L., G088
Ames 25762	<i>Daucus carota</i> L., S097
Ames 25841	<i>Daucus carota</i> L., T053
Ames 25590	<i>Daucus guttatus</i> Sm., G007
Ames 25593	<i>Daucus guttatus</i> Sm., G015
Ames 25824	<i>Daucus guttatus</i> Sm., T035
Ames 25813	<i>Daucus involucratus</i> Sm., T022
Ames 25846	<i>Daucus involucratus</i> Sm., T058
Ames 25876	<i>Daucus involucratus</i> Sm., T090
IGK DAL340/00	<i>Daucus pusillus</i> Michx.
HRI 8254	<i>Daucus pusillus</i> Michx.
Ames 25614	<i>Orlaya daucorlaya</i> Murb., G003
Ames 25616	<i>Orlaya daucorlaya</i> Murb., G016
Ames 25618	<i>Orlaya daucorlaya</i> Murb., G033
Ames 25726	<i>Pimpinella peregrina</i> L., S051
Ames 25730	<i>Pimpinella peregrina</i> L., S058
Ames 25754	<i>Torilis arvensis</i> (Huds.) Link, S088
Ames 25742	<i>Torilis arvensis</i> subsp. <i>neglecta</i> (Spreng.) Thell., S075
Ames 25757	<i>Torilis arvensis</i> subsp. <i>neglecta</i> (Spreng.) Thell., S091
Ames 25751	<i>Torilis leptophylla</i> L. (Rchb.) f., S085
Ames 25759	<i>Torilis leptophylla</i> L. (Rchb.) f., S093
Ames 25805	<i>Torilis leptophylla</i> L. (Rchb.) f., T014
Ames 25810	<i>Torilis nodosa</i> L. (Gaertn.), T019
Ames 25815	<i>Torilis nodosa</i> L. (Gaertn.), T026
Ames 25844	<i>Torilis nodosa</i> L. (Gaertn.), T056
Cultivated carrot <i>Daucus carota</i> subsp. <i>sativus</i> (Hoffm.) Arcang.	
HRI5779	Nagykallo, landrace
NGB 1853	Nantes Fancy, cultivar
GAT 55504	Senta, cultivar

<sup>1</sup> Ames, North Central Regional Plant Introduction Station, Ames, Iowa, USA.; GAT, Gene Bank, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany; HRI, Genetic Resources Unit, Warwick Horticulture Research International Wellesbourne, United Kingdom; IGK, Institute of Horticultural Crops; BAZ, Quedlinburg, Germany; NGB, Nordic Gene Bank, Alnarp, Sweden.

Germany) equipped with a Nd:YAG laser, emitting at 1064 nm, and a germanium detector cooled with liquid nitrogen. The instrument was equipped with an *xy* stage, a mirror objective, and a prism slide for redirection of the laser beam. Compared with the standard vertical sampling arrangement, the samples were mounted horizontally. Single mericarps (hereafter called seeds) were placed on the stage without any preparation of the sample and exposed to the laser beam at commissure side. At least five seeds were measured per accession. All spectra were measured in the range from 100 to 4000 cm<sup>-1</sup> with an unfocused laser beam

of about 1 mm diameter and with 50 mW power; 64 scans were collected with spectral resolution of 4 cm<sup>-1</sup>.

Spectra obtained were processed by the Bruker Opus/map software package v. 4.3. Before analysis, the spectra were background corrected. Variability of the measurements was evaluated based on the distance between individual spectra and the averaged spectrum, which was derived for each taxon or accession. Hierarchical cluster analysis was used to distinguish the most homogenous groups of seeds that differ in chemical composition and to relate this information with taxonomical classification of the accessions. For

that purpose, either individual or averaged spectra were vector normalized and then the distance between them was calculated using the factorization method.<sup>19</sup> Wavenumber ranges for the analysis were set up individually for the investigated data set. The first few most contributing factors were chosen to produce dendrograms using the Ward's algorithm.

## Seed Germination Test

Sixty seeds of carrot cultivar 'Nantes Fancy' were used for the germination test; one part was measured using NIR-FT-Raman spectroscopy as described above while the other was used as a nonmeasured control. Seeds of both samples were surface sterilized by immersion in 70% ethanol for 1 min and then 12.5% sodium hypochlorite for 30 min and washed three times with sterile water. They were plated on 8% agar in sterile Petri dishes and incubated in light at 25°C. The number of germinating seeds and normal seedlings was counted after 7 and 14 days, respectively. The significance of the effect of Raman measurements was assessed using the *t* test for discrimination between two subsamples.

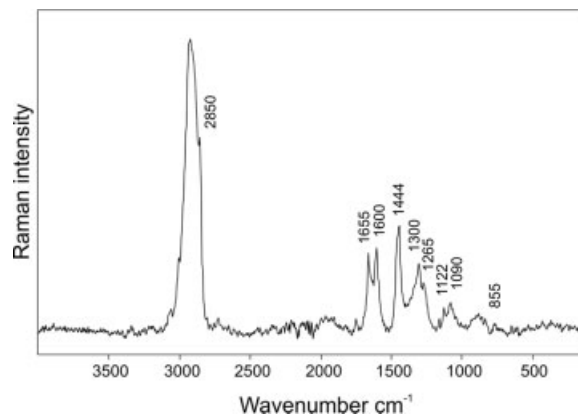
## RESULTS AND DISCUSSION

### Raman Spectra of Apiaceaeous Seeds

Raman spectra from measurements of intact single seeds (mericarps) have several intensive bands indicative of specific fruit constituents. For *D. pusillus* the predominant bands are related to fatty acids (Figure 1).

The signal at 1655 cm<sup>-1</sup> is typical for a  $\nu(\text{C}=\text{C})_{\text{cis}}$  stretching vibration of unsaturated fatty acids. The intensive band at 1444 cm<sup>-1</sup> can be assigned to CH<sub>2</sub> scissoring deformation vibration. The degree of fatty acid unsaturation can be estimated from the peak area of the bands at 1265 and 1300 cm<sup>-1</sup>, which are due to in-phase =CH- symmetric rocking and methylene twisting vibration, respectively. The ratio of the discussed bands indicates a low degree of fatty acids unsaturation in the seed evaluated. Additional confirmation of this fact comes from the narrow band at 3008 cm<sup>-1</sup> of an intensity indicating unsaturation. Apiaceaeous seeds are rich in fatty acids deposited in storage oil bodies.<sup>21,22</sup> The main and characteristic fatty acid of this family is petroselinic acid (C18:1), which occurs in amounts accounting for up to 85% of seed lipids.<sup>23</sup> Thus we conclude that the predominant bands observed in the Raman spectrum of *D. pusillus* seed can be assigned to petroselinic acid.

Another strong signal seen at about 1600 cm<sup>-1</sup> can be attributed to lignin, which is the main component of cell walls present in hard pericarp.<sup>5</sup> In fact, in this range lignin gives a doublet band, with one maximum at about 1600 cm<sup>-1</sup> (aromatic vibrations) and a peak of



**FIGURE 1** FT-Raman spectrum of *D. pusillus* single seed.

lower intensity at higher wavenumbers in the range of 1630–1660 cm<sup>-1</sup>. The latter band also coincides with C=C stretching mode of fatty acids discussed previously and observed at 1655 cm<sup>-1</sup>. Some bands in the spectrum of *D. pusillus* can also be assigned to polysaccharides. Two signals seen at 1090 and 1122 cm<sup>-1</sup> due to -C-O- stretching vibrations of carbohydrates are very characteristic for this chemical group. However, the most intensive band of polysaccharides is observed at 1460 cm<sup>-1</sup> (-O-CH<sub>3</sub> stretch). This mode slightly overlaps with previously described CH<sub>2</sub> scissoring deformation of fatty acids observed at 1444 cm<sup>-1</sup>. Other polysaccharide signals occurring between 1300 and 1400 cm<sup>-1</sup> (CH deformations) contribute to the elevated spectral line in this range. It has been reported that Apiaceaeous fruits contain various polysaccharides in cell walls.<sup>10</sup> However, beyond the report by Hopf and Kandler<sup>24</sup> that  $\beta(1-4)$ -mannan is the prevalent carbohydrate in *Carum carvi* endosperm, very few detailed reports are available concerning the composition of these compounds to our knowledge.

Storage proteins localized in protein bodies are important components in the endosperm of Apiaceae. Raman bands related to proteins are generally observed at about 1450 cm<sup>-1</sup> (CH deformation) and in the range of 1645–1660 cm<sup>-1</sup> (amide I vibration). However, they overlap with signals of other plant components and cannot be uniquely associated with proteins.

Other spectral regions comprise mainly weak signals, although some of them can also be attributed to specific compounds, such as bands detected at about 1474 and 1492 cm<sup>-1</sup>. Some Apiaceaeous seeds contain monohydrate calcium oxalate inclusions in endosperm protein bodies,<sup>25</sup> and their presence has contributed to taxonomical classifications.<sup>5</sup> Monohydrate calcium oxalate gives two characteristic Raman sig-

**Table II Intra- and Interspecific Variation of Raman Spectra as Measured by the Mean Distance between the Spectra of Individual Seeds within Accessions and the Averaged Spectra for the Taxon**

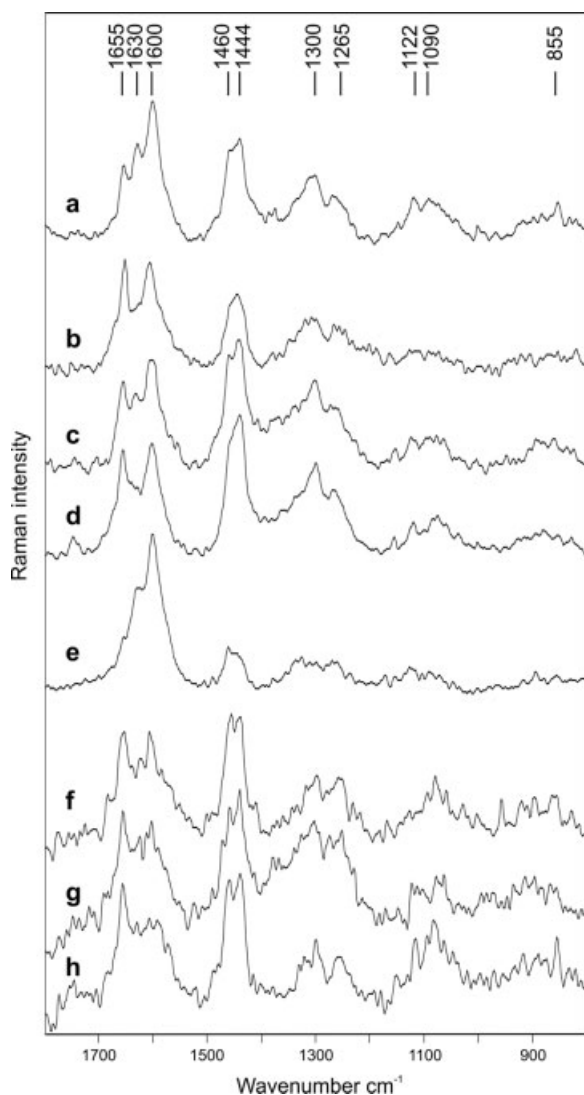
Taxon	Number of Accessions	Number of Seeds	Mean Distance	Standard Deviation
<i>Caucalis platycarpos</i>	4	14	0.29	0.07
<i>Daucus</i>	18	82	0.47	0.18
<i>D. broteri</i>	3	15	0.27	0.06
<i>D. capillifolius</i>	1	5	0.24	0.03
<i>D. carota</i> (wild)	3	13	0.33	0.12
<i>D. carota</i> subsp. <i>sativus</i> (cult.)	3	13	0.29	0.16
<i>D. guttatus</i>	3	13	0.38	0.21
<i>D. involucratius</i>	3	13	0.59	0.13
<i>D. pusillus</i>	2	10	0.15	0.04
<i>Orlaya daucorlaya</i>	3	13	0.24	0.09
<i>Pimpinella peregrina</i>	2	10	0.75	0.37
<i>Torilis</i>	9	38	0.27	0.10
<i>T. arvensis</i>	1	3	0.22	0.13
<i>T. arvensis</i> subsp. <i>neglecta</i>	2	9	0.20	0.07
<i>T. leptophylla</i>	3	13	0.23	0.13
<i>T. nodosa</i>	3	13	0.27	0.10

nals at 1463 and 1496  $\text{cm}^{-1}$  whereas dihydrate calcium oxalate presents a signal at 1475  $\text{cm}^{-1}$ .<sup>26</sup> In the spectra measured from the seeds we evaluated, the band at 1463  $\text{cm}^{-1}$  is probably masked by the  $-\text{O}-\text{CH}_3$  mode of polysaccharides so only a signal at about 1492  $\text{cm}^{-1}$  is evident, and it is likely related to the presence of monohydrate calcium oxalate. A Raman signal observed at 1474  $\text{cm}^{-1}$  in *Torilis* seeds indicates that dihydrate calcium oxalate may also be formed. These bands were more pronounced in the spectra of *Torilis* and *Orlaya* mericarps, which possess such crystals not only in the endosperm tissue but also at the commissure surface.

### Intra- and Intergeneric Variation

The chemical composition of seeds varies upon seed developmental stage as well as environmental and genetic factors. Therefore we estimated the spectral variation within seeds (intraseed variation) and among seeds in an accession (intraaccession variation) and compared them to intraspecific, interspecific, and intergeneric variations. Intraseed variation was estimated by taking measurements from five randomly distributed points for each of 20 seeds of four species. We found that 87% of the spectral distances fit in the range of 1.2 SD from the mean. Intraaccession variation was at a similar level as intraseed variation. In general, variation at seed and accession levels was low while variation at the species and generic level was higher, thus enabling taxonomical compari-

son. The most homogenous species, as reflected by both low mean distance and standard deviation, were *D. pusillus*, *D. capillifolius*, *O. daucorlaya*, and *Torilis* spp. (Table II). In contrast, *P. peregrina* was a very heterogeneous species with mean and SD five- and ninefold higher, respectively, than *D. pusillus*. For this reason *P. peregrina* was excluded from further consideration. Interspecific variation of *Daucus* was higher than intraspecific variation, except for *D. involucratius*. Heterogeneity of the genus *Daucus* resulted from both the high intraspecific variability of *D. involucratius*, *D. guttatus*, and *D. carota* and interspecific variation between the species. Genus *Daucus* is divided into five sections with *D. broteri* belonging to section *Platyspermum* while the other species evaluated here belong to section *Daucus*.<sup>27</sup> That relationship of *Daucus* species based on fruit morphological and anatomical features has recently been supported by analysis of restriction fragments of mitochondrial DNA.<sup>28</sup> The broad diversity of *Daucus* is not surprising since it is one of the largest Apiaceae genera comprising 22 species. *D. carota* is particularly complex as it consists of cultivated carrot plus 12 wild subspecies that can freely hybridize due to the lack of natural crossing barriers.<sup>29</sup> In contrast, *Torilis* is another large, phenotypically diverse genus but it was much more homogenous than *Daucus*. The observed differences in degree of variation coincide with molecular systematic investigations. Sequence analysis of nuclear ribosomal DNA (rDNA) internal transcribed spacers (ITS) and restriction site data of chloroplast



**FIGURE 2** Averaged FT-Raman spectra of (a) *C. platycarpus*, (b) *D. broteri*, (c) *D. capillifolius*, (d) *D. pusillus*, (e) *O. daucorlaya*, (f) *T. arvensis*, (g) *T. leptophylla*, and (h) *T. nodosa* seeds.

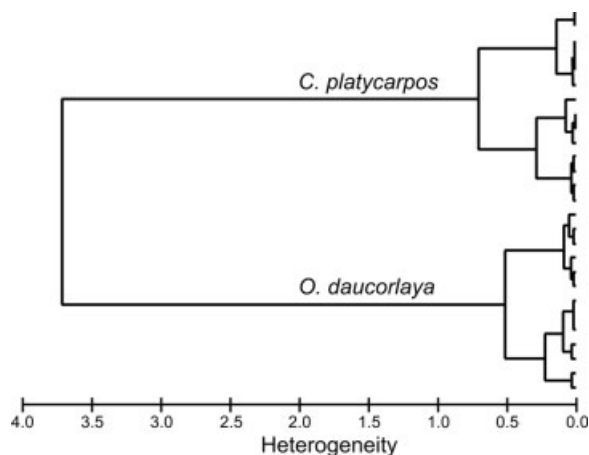
DNA (cpDNA) demonstrate the dichotomy of *Daucus* with *D. pusillus* native to the New World being separated from most Mediterranean species.<sup>30,31</sup> The same studies strongly support monophyly of *Torilis*.

Differences in averaged spectra of *C. platycarpus* (a), *D. broteri* (b), *D. capillifolius* (c), *D. pusillus* (d), *O. daucorlaya* (e), *T. arvensis* (f), *T. leptophylla* (g), and *T. nodosa* (h) of Figure 2 reflect quantitative differences in fatty acid, lignin, and polysaccharide content. More similarity was observed among spectra of *Torilis* species than spectra of *Daucus* species; averaged spectra of *Caucalis* and *Orlaya* were the most distinct. At the generic level, seeds of *Daucus* and *Torilis* have quite similar spectra, but some differences are evident. Slightly lower intensity of the band at

about 1600  $\text{cm}^{-1}$  observed for *Torilis mericarps* suggests that they contain less lignin while a more pronounced band at 1460  $\text{cm}^{-1}$  indicates higher amounts of polysaccharides. This observation corresponds with the presence of an additional band at 855  $\text{cm}^{-1}$  (–C–O–C– skeletal mode of  $\alpha$ -anomer carbohydrate), which is characteristic for pectins. It is worth notice that the spectra of *Daucus* seeds are of significantly higher quality, which could be due to their morphological features. For example, the commissure face of *Daucus mericarps* is usually flat while in *Torilis* species it is hollowed with exposed side ridges and spines, which can additionally disperse the laser beam. Raman spectra of *C. platycarpus* and *O. daucorlaya mericarps* are characterized by a very intensive band at 1600  $\text{cm}^{-1}$ , indicating the presence of a high amount of lignin. That fact corresponds well to the morphology of the fruits as both species have a very hard and thick pericarp. Endosperm of *O. daucorlaya* likely contains a low level of fatty acids based upon a weak band at 1444  $\text{cm}^{-1}$ . In this species, the lower intensity of other bands characteristic for endosperm constituents may be also due to a hard pericarp that can affect laser penetration into seed tissue. Other differences dispersed throughout the spectrum and noticeable as weak signals may be related to essential oils characteristic for these species.<sup>8–11</sup>

## Taxonomical Classification

Hierarchical cluster analysis of Raman spectra for essential oils has successfully demonstrated taxonomical relationships of plants.<sup>18,19</sup> In this study, cluster analysis was used for taxonomical classification of the individual Apiaceae seeds. Using the wavenumber range characteristic for fatty acids and polysaccharides (1200–1700  $\text{cm}^{-1}$ ) and the first two factors of the factor algorithm for the calculation of spectral distance, all *C. platycarpus* seeds were different from *O. daucorlaya*. To allow better discrimination, a wider wavenumber range that comprises additional information coming from the minor bands was taken into consideration. Using four particular wavenumber ranges (320–560, 1100–1500, 1550–1700, and 2420–2960  $\text{cm}^{-1}$ ) and the first two algorithm factors (Figure 3), the expected taxonomical distinction of the two groups was realized. Similarly, seeds of three *Daucus* species (*D. broteri*, *D. capillifolius*, and *D. pusillus*) were separated into three groups using three other spectral regions (500–900, 1550–1750, and 2800–3000  $\text{cm}^{-1}$ ) and the first two algorithm factors (Figure 4). With few exceptions, the application of cluster analysis to spectra of individual seeds allowed species to be distinguished from all other species. Therefore, this



**FIGURE 3** Dendrogram showing classification of *C. platycarpus* and *O. daucorlaya* individual seeds after cluster analysis of the single spectra at the wavenumber ranges of 320–560, 1000–1500, 1550–1700, 2520–2960  $\text{cm}^{-1}$  using the first two factors for calculation of spectral distances and the Ward's algorithm.

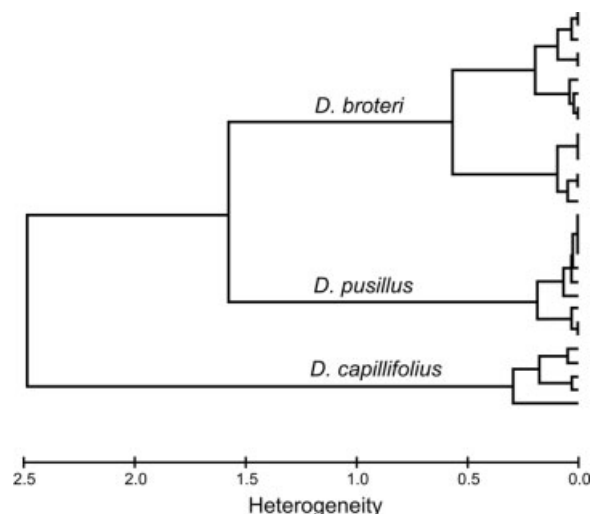
method can be utilized to discriminate and classify species. Only *D. involucratus* and *D. carota* were unable to be distinguished. This can be explained by their high heterogeneity described above. The small intragenetic variation among *Torilis* species also prohibited their unequivocal discrimination. That feature has appeared however very valuable for defining genus *Torilis*, which was clearly set apart from all other genera used in this work.

Using the averaged spectra for accessions, it was possible to classify correctly higher numbers of taxa. Figure 5 presents an exemplary dendrogram consisting of seven species belonging to four genera distributed in distinct clusters. The structure of the first cluster corresponds to two small groups: the first one composed of *C. platycarpus* accessions and a second one of *Daucus* (*D. broteri*, *D. pusillus*). A second cluster consists of only *O. daucorlaya* accessions with another cluster consisting of *Torilis* spp. The hierarchical arrangement of the tree depended on the number of factors used for calculation of spectral distances. For example, *Torilis* and *Orlaya* did not necessarily form a common cluster and thus *Orlaya* could be located closer to *Daucus* and *Caucalis* by evaluating more factors. In contrast, the composition of the groups was more conservative and did not change within main clusters by varying the number of factors evaluated.

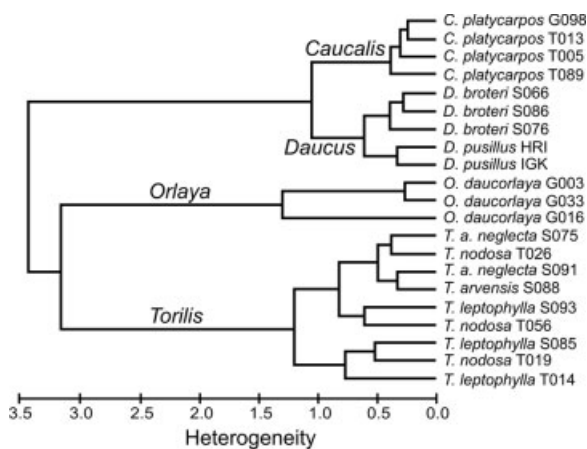
In the recent years, much investigation on Apiaceae phylogeny has been done using molecular approaches. The analyses of rDNA ITS, cpDNA restriction sites, and chloroplast *rps16* intron sequences revealed high similarity between tribe *Dauceae* and

*Scandiceae* subtribe *Caucalidinae* (*sensu* Drude)<sup>5</sup> and supported changes made to taxonomy by their reunion into a new tribe *Caucalideae*, which now include also genera *Caucalis*, *Daucus*, *Orlaya*, and *Torilis*. Also in contrast to Drude's classification based on morphological and anatomical study, molecular approaches indicate that *Orlaya* is more closely related to *Daucus* while *Caucalis* is more closely related to *Torilis* and thus they are classified to *Daucus* and *Torilis* subclades, respectively.<sup>30,32</sup> Although the *Daucus* subclade is adjacent to *Torilis*, the relationship between them is not clear.<sup>32</sup> Our results also demonstrated that these genera can be distinguished spectroscopically, but their relationship is difficult to assess. With both analytical methods *Daucus* differs from *Torilis*, but for some analyses *Caucalis* is clustered with *Daucus* while for other analyses *Torilis* is more closely allied with *Daucus*. That discrepancy may be due to high intragenetic variation of seed constituents. The inclusion of so many *Daucus* species in our cluster analysis may have also contributed to the variability in our results. Nevertheless we conclude that Raman spectroscopy can be used for phylogenetic investigation as a tool for taxonomical classification.

While fruit morphological characters are still the principal criteria for taxonomical classification of Apiaceae, correct identification based on fruit morphology can be difficult, particularly when fruits are collected at maturity from dry plants found in their natural habitat. Furthermore, visual evaluation is la-



**FIGURE 4** Dendrogram showing classification of *D. broteri*, *D. capillifolius*, and *D. pusillus* individual seeds after cluster analysis of the single spectra at the wavenumber ranges of 500–900, 1500–1745, and 2800–3000  $\text{cm}^{-1}$  using the first two factors for calculation of spectral distances and the Ward's algorithm.



**FIGURE 5** Dendrogram showing classification of *C. platycarpus*, *D. broteri*, *D. pusillus*, *O. daucorlaya*, and *Torilis* spp. after cluster analysis of the averaged spectra for each accession at the wavenumber ranges of 500–900, 1500–1745, and 2800–3000  $\text{cm}^{-1}$  using the first two factors for calculation of spectral distances and the Ward's algorithm.

borious, time consuming, and requires cooperation of specialized taxonomists. Therefore, supplementary methods are of great value. In this study we selected Apiaceae collected ex situ from diverse environments, which had presented ambivalent results in subsequent taxonomical evaluation of both genus and species. The results presented here indicate that Raman spectroscopy can be a helpful tool for a rapid discrimination between taxa.

### Nondestructive Effect of Raman Measurement on Seeds

NIR–FT–Raman spectroscopy requires minimal sample preparation to generate useful measurements. Moreover, while this method is considered to be non-destructive to biological material (since it causes neither thermal, chemical, or physical tissue decomposition),<sup>33</sup> there are no reports evaluating Raman spectroscopy effects on living seed material. We therefore investigated seed viability of the cultivated carrot ‘Nantes Fancy’ following Raman measurements. After measurement, seeds were still able to germinate at a similar frequency as the control (0.95 and 0.98, respectively). In a few seeds, primary roots of seedlings evaluated emerged but did not grow further and the shoot apical growth was inhibited. These observations suggest that the penetrating laser may injure the embryo. However, similar abnormal development was also observed in the control seed lot. The proportion of normal seedlings (0.75) developed from

Raman measured seeds was slightly lower than for the control (0.83), but the difference was statistically nonsignificant ( $p = 0.42$ ). The results of the germination test indicate that Raman measurement had no significant effect on seed germination or seedling development, and normal plants were produced.

### CONCLUSION

We demonstrated that NIR–FT–Raman spectroscopy provides spectral information able to discriminate among many Apiaceae species, including those of similar morphology. Therefore, Raman spectroscopy may be a supplementary tool for taxonomical validation of plant germplasm resources gathered in seed collections. This nondestructive technique has potential application for preliminary screening of collections without reducing germination or inducing abnormal growth. Moreover, we have demonstrated that Raman information gathered from the analysis of a few seeds was sufficient to discriminate taxa. That makes this method of considerable importance for evaluation of seed material stored in ex situ collections, even when a very limited number of seeds often is available. This method can contribute to rational management of genetic resources. Seeds that have been evaluated can be sown for plant production, characterization, taxonomical validation, and reproduction. Implementation of the technique, however, should be verified with many more species and genera to create a reference library for further widescale comparisons of taxa in large seed collections.

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