Fourier transform infrared microspectroscopy as a new tool for nematode studies

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Abstract **We report the results of a microspectroscopy study on the Fourier transform infrared (FT-IR) absorption spectra of *Caenorhabditis elegans*, collected from the different parts of a single intact specimen – pharynx, intestine and tail regions. The principal absorption bands were assigned to the molecular species present in *C. elegans*, with an excellent reproducibility for the pharynx spectrum. These results enabled us to explore if FT-IR microspectroscopy could offer a new tool for nematode identification. As an example, the discrimination among four well characterised nematode taxa is reported. The FT-IR results completely match those obtained by Blaxter and colleagues through molecular biology [Nature 392 (1998) 71].**

Keywords: Fourier transform infrared microspectroscopy; Nematode taxonomy; Collagen; *Caenorhabditis elegans*

1. Introduction

Nematodes occupy all biotic habitats on earth with more than 26 000 known species and their identification is an emerging problem in agriculture, in environmental sciences, and in clinics. The study of nematode distribution and biodiversity is an important issue in biogeographical and ecological research, requiring a rapid and reliable method for their recognition [2]. At present, nematode recognition is not an easy task and it is often based on minor morphological characteristics, requiring time and resources consuming methods.

Furthermore, in the last decades important advances were made possible by molecular biology techniques, leading to the determination of the complete genome sequence of *Caenorhabditis elegans* [3] and allowing the study of biodiversity at molecular level. By the polymerase chain reaction (PCR) and sequencing of the small ribosomal subunit from 53 species, Blaxter and collaborators [1] presented a phylogenetic analysis of a large number of nematodes, identifying five major clades within this phylum, all of which include parasitic species.

In this work, we propose the use of Fourier transform infrared (FT-IR) microspectroscopy as a new technique for nematode characterisation and identification. The absorption of electromagnetic radiation in the infrared spectral region, due to its interaction with the vibrational modes of the atoms and chemical bonds of a system, allows to obtain important information on the system molecular composition and interactions [4]. Metabolic fingerprints can be identified in the infrared absorption spectrum of the system under study making it possible to monitor the occurrence of biochemical events at molecular level. Indeed, in the last decades it has been found that infrared spectroscopy, with the support of multivariate statistical analysis, can be a powerful tool for the characterisation and identification of microorganisms [5,6] and complex biological systems [7]. Furthermore, FT-IR microspectroscopy has been recently used to assess preservation methods for natural history specimens such as nematodes, which are often damaged by storage fluids that can induce cuticle structure deterioration [8].

Before exploring the possibility of discriminating among different nematode taxa, we have first studied the infrared absorption spectrum of *C. elegans*. Being the most studied nematode [9,10], *C. elegans* is the ideal model system to asses the potential of this spectroscopic technique. Indeed, after the completion of its genomic sequence [3], *C. elegans* continues to be a powerful model system for the study of gene expression, thanks to the availability of several *C. elegans* clones [11]. In this perspective, we have found that FT-IR microspectroscopy could offer a new approach for the study of these processes in situ, at the level of the different anatomic parts of a single nematode specimen.

We report here a FT-IR microspectroscopy study of a dried but intact *C. elegans* specimen, which is – at our best knowledge – the first FT-IR investigation on a complex whole animal. By the use of an infrared microscope, it was possible to collect the absorption spectrum from the different anatomic parts of a single intact nematode – pharynx, intestine and tail regions. High reproducibility was obtained for the pharynx spectral response, with an excellent statistical significance. These results allowed us to discriminate among different nematode taxa, by studying the absorption spectra of their pharyngeal region. The FT-IR identification was found to be in excellent agreement with that from molecular biology, reported by Blaxter [1].

2. Materials and methods

2.1. Nematode species, growth conditions and sample preparation

Among the examined four soil free-living nematode species three belong to our laboratory collection cultures (*Caenorhabditis elegans*, *Pristionchus lheritieri* and *Panagrolaimus rigidus*) and one was directly

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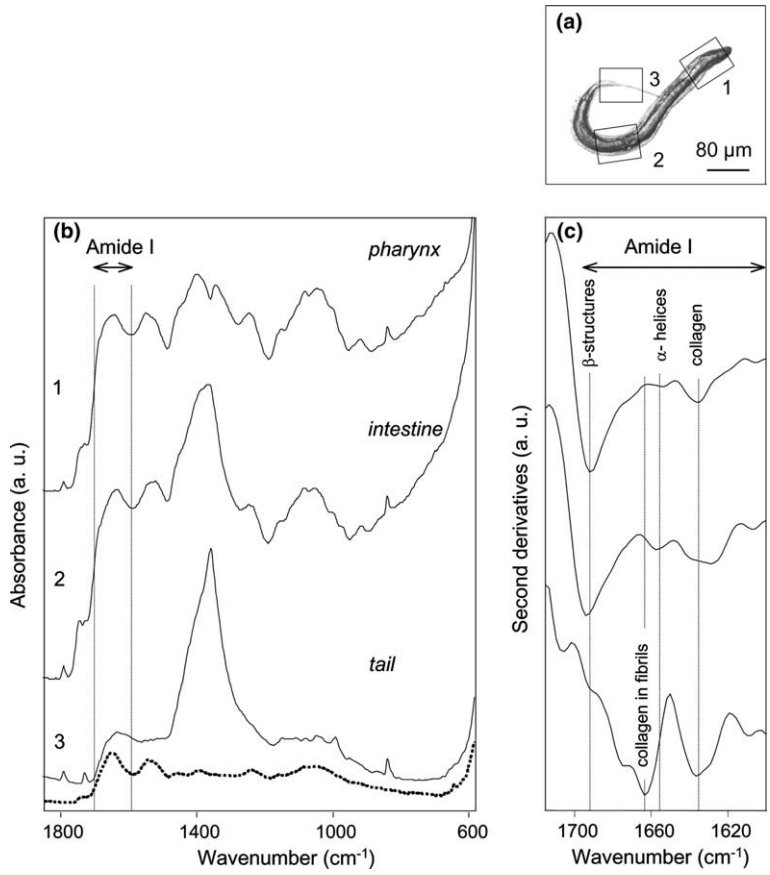


Fig. 1. Infrared microspectroscopy of *Caenorhabditis elegans*. (a) Optical image of a dried hermaphrodite adult specimen. The rectangular frames indicate the regions selected by the infrared microscope. (b) FT-IR absorption spectra of pharynx, intestine and tail regions (tail without surface coat: dotted line). (c) Second derivative absorption spectra of *C. elegans* pharynx, intestine and tail in the Amide I region. The dotted lines indicate the position of the Amide I band components.

collected from moss (*Geomonhystera* sp.). Female adult specimens were examined and, in the case of *C. elegans*, adult hermaphrodite specimens just after the development of the fourth juvenile stadium were examined. In the case of *C. elegans*, specimens with length varying from 400 to 600 μm were examined. All the species were grown on agar plates and fed with *Escherichia coli* OP50, following the Brenner method. For FT-IR absorption measurement, single specimens, taken from a nematode water suspension, were washed twice in distilled water, deposited onto a ZnSe window, and dried at room temperature for about 30 min. Samples prepared in this way led to a spectral response that was found to be constant over several hours. To speed up the drying process, we also tried to reduce the water drop volume by the tip of a filter paper. However, this procedure resulted detrimental, causing the removal of the surface coat – glycocalyx or fuzzy coat – as shown by the infrared absorption spectrum reported in Fig. 1b.

2.2. FT-IR microspectroscopy

FT-IR absorption spectra from 4000 to 600 cm^{-1} were acquired by coupling an UMA 500 infrared microscope to a FTS 40A spectrometer (Digilab-USA) under the following conditions: 4 cm^{-1} spectral resolution, 20 kHz speed, 256 scan coadditions, and triangular apodisation. In the UMA 500 microscope used in the transmission mode, the area of interest in the sample is selected by a variable diaphragm aperture [12] that was adjusted in this study at about 60 μm × 80 μm. In this way it was possible to isolate, within a single nematode specimen, the different anatomical parts – pharynx, intestine, and tail – in order to collect at the detector only the transmitted beam from the region of interest.

2.3. Statistical analysis of the spectral data

A second derivative analysis of the spectra was performed for the assignment of the absorption bands and for the analysis of the Amide I band components (Figs. 1 and 2). A 9–11 points smoothing was applied to the measured spectra before calculating their second derivative by the Savitzky–Golay method, using the GRAMS/32 software (Galactic Industries Corporation, USA).

In order to evaluate the statistical significance of the absorption data and the spectral differences between the responses of the different nematode taxa, we performed a hierarchical cluster analysis of the second derivative spectra in the range 1800–900 cm^{-1} by using the program of the Statistics Toolbox of Matlab 5.3 (The Math Works Inc., USA) and employing Euclidean distance and Ward's algorithm. To assess the similarity among the spectra, the input objects – the second derivative spectra – are represented in a dendrogram, where small distances correspond to similar spectra within each cluster [6]. Spectra from specimens belonging to independent nematode cultures – grown under the same experimental conditions – were examined for each nematode taxon.

3. Results and discussion

The optical image of a dried hermaphrodite *C. elegans* specimen is reported in Fig. 1a, where the frames show the body parts selected by the infrared microscope aperture [12]. The absorption spectra from 1800 to 600 cm^{-1} – collected from pharynx, intestine and tail regions – are different one from the other, as shown in Fig. 1b. They display a number of well resolved absorption bands that can be assigned to the molecular components of the nematode structures. Quite interesting are the features of the Amide I and Amide II protein bands, which arise from the vibrational modes of the backbone amide bonds. As extensively described in the

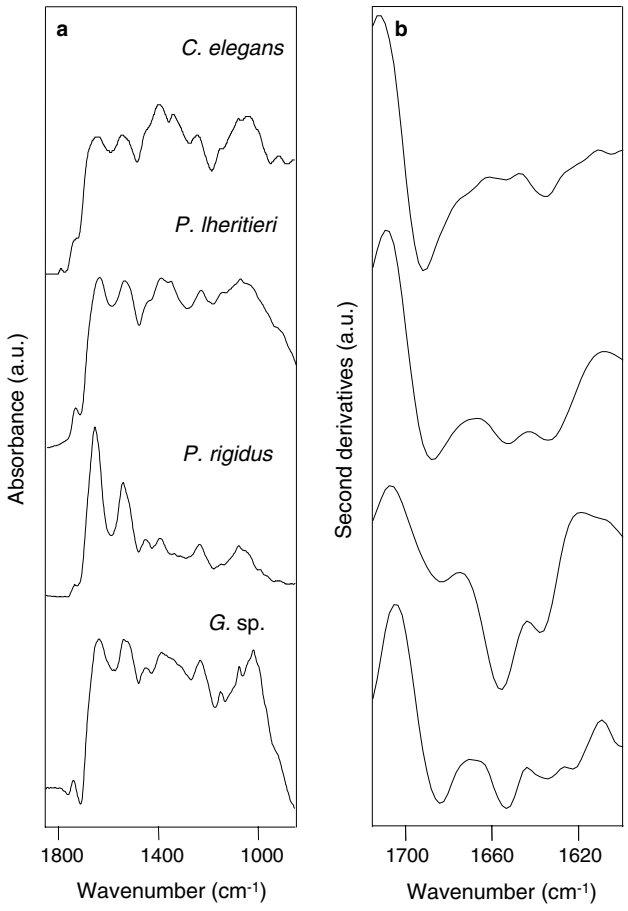
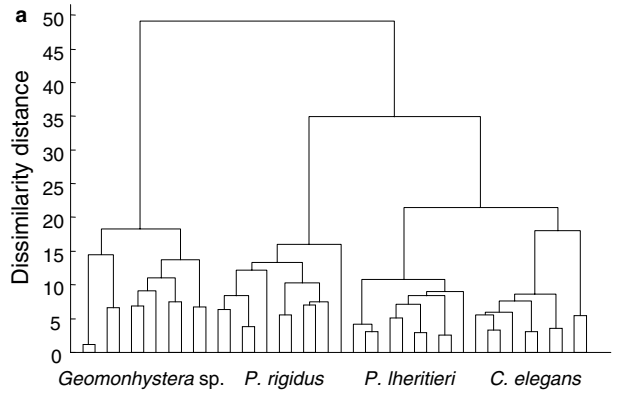


Fig. 2. Infrared microspectroscopy of *Caenorhabditis elegans*, *Pristionchus lheritieri*, *Panagrolaimus rigidus* and *Geomonhystera* sp. (a) FT-IR absorption spectra of the pharyngeal region. (b) Second derivative spectra of the pharyngeal region.



b

Order	Suborder	Family	Genus, species
Monhysterida	Monhysterina	Monhysteridae	*Geomonhystera* sp.
Rhabditida	Tylenchina	Panagrolaimidae	*Panagrolaimus rigidus*
Rhabditida	Rhabditina	Diplogasteridae	*Pristionchus lheritieri*
Rhabditida	Rhabditina	Rhabditidae	*Caenorhabditis elegans*

Fig. 3. Phylogenetic analysis of the examined nematode species. (a) Relationships obtained by the cluster analysis of the second derivative FT-IR spectra from 1700 to 900 cm^{-1}. (b) Relationships obtained by Blaxter and colleagues [1] through molecular biology analysis.

literature [13], the Amide I band due to the stretching mode of the carbonyl group C=O is sensitive to the protein secondary structure. In this study, the second derivative analysis of the absorption spectra (Fig. 1c) enabled us to identify the secondary structures of the proteins present in the different nematode regions, each structure being assigned to a component of the Amide I band detected by the derivative procedure. These components were seen to vary along the nematode body. In the pharynx region (Fig. 1c) three well resolved bands were observed: two at 1691.3 cm^{-1} and at 1653.9 cm^{-1} assigned, respectively, to β-sheets/turns and to α-helix protein structures; a third component at 1635.2 cm^{-1} was assigned to the carbonyl stretching vibration of collagen proline facing the aqueous phase [14,15]. Also in *C. elegans*, collagens, which are mostly found in the cuticle, are made by three polypeptides chains coiled in a left handed helix. Each chain contains a (Gly–X–Y) repeat sequence, where X and Y are prolines; often in position Y hydroxyproline is found instead of proline [16].

Similarly, the Amide I components occurred in the intestine spectrum at approximately the same band position, with the α-helical component more important in this case (Fig. 1c).

In the tail spectrum (Fig. 1c), instead, the features of the Amide I band were different, displaying components at 1691.0, 1662.4 and 1635.5 cm^{-1}. While the first component was almost negligible in this case, the remaining components were both assigned to the carbonyl stretching mode of proline in collagen triple helix, the one at 1662.4 cm^{-1} being characteristic of collagen in fibrils, while that at 1635.5 cm^{-1} is again due to collagen proline facing the aqueous phase [15]. The analysis of the Amide I band indicates therefore – as expected – that different proteins are present in the different nematode regions, and suggests that collagen is the principal protein of the tail. These results – partially confirmed by the behaviour of the Amide II band (data not shown) – offer new insights on the protein content of *C. elegans*.

In addition, the spectra collected from the three body parts of *C. elegans* displayed a prominent absorption between 1500 and 1200 cm^{-1}, which is mainly due to the collagen glycine and proline amino acid sequence [14–16]. Also, the absorption in the region 1200–900 cm^{-1} can be assigned to collagen and to glycogen [14], the most important cuticle components [16]. In addition to these molecular species, nitrogenous compounds were seen to contribute to the absorption band in the region between 1380 and 1280 cm^{-1}. This band can be attributed to nematode waste products – including ammonia – secreted through the excretory pores and through the cuticle. We have found that when the surface coat is removed from the specimen, the intense band at 1354.0 cm^{-1} in the tail spectrum completely disappears, as shown in Fig. 1b.

As regards the reproducibility of the measured spectra, this was found to be excellent for the absorption of pharynx and tail. Quite different was instead the intestine response, where band intensities were seen to vary not only depending on growth conditions, but also among specimens belonging to the same population. This result is actually expected, as several molecular species could contribute to the absorption depending upon specimen gut content.

As a conclusive remark, we can say that all the principal peaks observed in the absorption spectra of *C. elegans* can be assigned to its relevant molecular species. Once more this

"nature's gift" [10] has proved to be an excellent model system [9] to understand the infrared spectral response of nematodes.

To explore the potential of FT-IR microspectroscopy as a new tool for nematode identification, we present here the study of the absorption spectra from the pharyngeal region of four nematode species with clear phylogenetic relationships: *Caenorhabditis elegans*, *Pristionchus lheritieri*, *Panagrolaimus rigidus* and *Geomonhystera* sp. Excluding the intestine spectrum for the reasons discussed above, the spectrum from the tail – even if highly reproducible – was found not to be suitable for identification purposes, since it does not contain any fingerprint of the examined species.

The absorption spectra from the pharyngeal region of the four examined species are reported in Fig. 2a. Even if the spectra display similar patterns, their band positions and intensities change, leading to overall spectra that differ appreciably one from the other. This is particularly evident if one examines the second derivative spectra in the Amide I and Amide II region from 1700 to 1500 cm^{-1}, whose band components vary among the different nematode taxa (see Fig. 2b).

To evaluate the statistical significance of these spectral differences, it was necessary to perform a chemiometric analysis. A cluster analysis of the second derivative spectra led us to the dendrogram reported in Fig. 3a. The input objects of the dendrogram were the spectral data collected from specimens of four independent cultures for each nematode taxon. Four well separated and homogeneous groups were obtained, each corresponding to a given single species. An excellent separation among spectral data of the different species was found, significantly larger than the variance within each species. This dendrogram completely matches with that obtained through the molecular analysis performed by Blaxter and colleagues [1], whose taxonomic similarities (see Fig. 3b) are indicated by the relationship:

3.1. *Geomonhystera (Panagrolaimus (Pristionchus, Caenorhabditis))*

This result therefore suggests that FT-IR microspectroscopy can offer a new useful method for nematode identification in different fields of application. The advantages of this approach, that can be used as a complement to other powerful molecular biology methods, are its rapidity, low cost and reliability. The specimen is examined in a non-invasive way and a minimal amount and manipulation of the sample are required. This study, therefore, highlights the potential of FT-IR microspectroscopy to support the taxonomy of animals, which is a difficult task in the cases of insects and nematodes [2]. The identification approach proposed here will be possible after the construction of a database of infrared spectra from known and well-characterised taxa.

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