Application of confocal laser scanning microscopy in the taxonomy of free-living marine nematodes

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Summary
Confocal laser scanning microscopy (CLSM) can provide high-resolution images of thick nematode specimens and highly detailed three-dimensional reconstructions. Several permanent slides of free-living marine nematode species including a species collected in the 1951 were analyzed with the aid of CLSM. The specimens were excited using an argon laser at 488 nm under the conditions used for Fluorescein Isothiocyanate (FITC) and 543 nm for Tetramethyl Rhodamine Isothiocyanate (TRITC). New details on the morphology of various diagnostic features (the cephalic region and male reproductive system) have been captured for several species allowing a re-description for some of them. Spicules and *gubernaculum*, followed by cuticularised parts of the buccal cavity and precloacal supplements were the most fluorescent parts of the nematode body. The morphological approach here adopted highlights new chances for the study of Museum type material for which CLSM may be decisive in capturing additional, important taxonomical details. Material collected in the 1951 and 1973 still resulted fluorescent making possible the detection of crucial taxonomical data.

Key words: taxonomy, free-living marine nematodes, confocal laser scanning microscopy, autofluorescence.

Introduction

The *phylum* Nematoda includes arguably the most successful free-living metazoans on the Earth (Da Rocha *et al.* 2006; Balsamo *et al.*, 2010). Nematodes have a central role in the energy flows, mineralization rates and nutrients recirculation of the marine ecosystems (Zeppilli *et al.*, 2015). Accordingly, they can give an important and direct contribution in functioning of marine environments (Danovaro *et al.*, 2008; Semprucci and Balsamo, 2012) and are effective tools in the assessment of environmental health and in biomonitoring (Semprucci *et al.*, 2015).

So far ~ 7000 marine species have been described that represent only 19% of the total number of the predicted nematode biodiversity (Appeltans *et al.*, 2012). Biodiversity estimates are crucial for developing conservation programs focused on marine ecosystems. Nematodes are fine and slender worms, mostly one to few millimetres long, with a body diameter 20-40 times less than total length. The body wall is made of a thick, layered cuticle mainly constituted by collagen. The cuticle may appear smooth, transversely annulated, or dotted, and bears a number of sensory structures called *sensilla*, the length and pattern of distribution of which as well as the size, shape and position of the lateral organs (i.e. amphids) are important diagnostic characters for taxonomy. The morphology of the buccal cavity is quite various reflecting the wide range of feeding habits: it may be from absent or minute to armed with mandibles. Cuticularized structures of the male reproductive system such as spicules and *gubernaculum* may considerably vary in shape and size and are normally of great value for the taxonomical identification as are the presence and features of precloacal supplements.

Among the difficulties which hamper the global biodiversity estimate of the *phylum* are the small body size, and the low number of the diagnostic characters that are often minute (Semprucci and Balsamo, 2012). Therefore, new techniques to make their identification easier and capture additional morphological features need to be explored. Furthermore, museum taxonomical collections of nematodes include significant numbers of type materials. These are fundamental not only as reference points for specialists but also as potential sources for obtaining new taxonomical data. However, most types or paratypes of many species have been deposited in the last 10 decades as permanent slides and their conservation status is damaged in some cases. In this regard, advanced microscopic techniques such as Confocal Laser Scanning Microscopy (CLMS) may provide integrative information and even highly detailed tri-dimensional reconstructions of morphological structures thanks to the autofluorescence of specimens.

Autofluorescence may be fixative-induced (e.g. using aldehyde fixatives) or natural and the emission spectra are generally broad compared to the spectra of the dyes or probes (Monici, 2005; Leischner *et al.* 2010). In particular, natural fluorescence has been documented from morphological structures of many animals, plants, fungi and microorganisms when they absorb light (Wu and Warren, 1984; Monici, 2005). The most commonly observed autofluorescing molecules in biological samples are structural molecules such as collagen and elastin as well as molecules involved in metabolic and functional processes (i.e. NADPH and flavins) (Monici, 2005). Forge and MacGuidwin (1989) documented that several nematode genera accumulate fluorescent lipofuscin compounds (lipids) in intestinal cell globules. Instead, Daugschies *et al.* (2001) documented that the eggs of some parasite nematodes emit light and can be easily distinguished from debris. No hypotheses have been advanced about the origin of the eggshell autofluorescence, but nematode eggs consist of vitelline, chitin and lipid-rich layers (Brownell and Nelson, 2006; Altun and Hall, 2009). Zullini and Villa (2006) re-described three species of freshwater nematodes belonging to the Altherr's collection just thanks to their autofluorescence under confocal microscopy. This approach was also used by Semprucci and Burattini (2015) on recent and ancient collection slides of marine nematodes.

Thus, the aid of confocal microscopy may be crucial both to describe new species and possibly also to re-describe some already known species for which morphological and morphometric details are very poor. Accordingly, this study explores possible explanations of nematode autofluorescence and highlights the advantages of CLMS technique in the taxonomical study of free-living marine nematodes.

Materials and Methods

Dorylaimopsis pellucidum (1 specimen), *D. variabilis* (Comesomatidae) (3), *Laxus gerlachi* (Desmodoridae) (1), *Rhinema retrosum* (Monoposthidae) (1), *Craspodema reflectans* (1), *C. octogoniata* (Cyatholaimidae) (2, 1) were the species analyzed in this study. Specimens were fixed with 4% buffered formaldehyde in sea water solution and mounted as permanent slides (Seinhorst, 1959). *C. octogoniata* belongs to the collection of the Zoologisches Institut und Zoologisches Museum of the Hamburg University and its specimens are conserved as permanent slides for which the preparation was not reported in the original description. *C. octogoniata* specimens were collected in French Coasts (St. Honorat and Pierres Noires) in the 1951 and 1973. *D. pellucidum*, *L. gerlachi*, *R. retrosum* and *C. reflectans* were collected in Maldives (Suvadiva atoll, 2009), while *D. variabilis* was collected in Korea (East Sea, 2012).

All specimens were studied with a CLSM, and excited using an argon laser either at 488 nm under the conditions used for Fluorescein Isothiocyanate (FITC, 495 nm excitation and 520 nm emission spectrum peak wavelengths) or at 543 nm for Tetramethyl Rhodamine Isothiocyanate (TRITC, 557 nm excitation and 576 nm emission spectrum peak wavelengths). As reported in Semprucci and Burattini (2015), images were taken with a Leica TCS-SP5 Confocal connected to a DMI 6000 CS Inverted Microscope (Leica Microsystems CMS GmbH), and analysed using the Leica Application Suite Advanced Fluorescence (LAS AF) software. Samples were examined using oil immersion objective lenses (63x N.A. 1.40). CLSM images are presented as single-plane images or Z-stack projections (3D-recostructions) obtained by ImageJ software.

Results

Generally the most fluorescent morphological structures were the buccal cavity (i.e. walls and teeth) and the male spicules and *gubernaculum*. The cuticle, even if visible with both wavelengths, did not present a high fluorescent emission. An exception was represented by some more cuticularized parts such as the helmet (cephalic capsule), the precloacal supplements and the *setae* insertions. Morphological structures were generally visible under both green and red emission with some exceptions reported below.

Dorylaimopsis pellucidum (Cobb, 1920) is a representative species of the family Comesomatidae. As belonging to this genus it is characterized by 3 teeth, cylindrical buccal cavity, 6 + 4 cephalic setae, cuticle with lateral longitudinal rows of dots; spicules elongated and *gubernaculum* apophysis directed caudally. Furthermore, it has a multispiral amphideal fovea with 2.5 turns. Cuticle with 2 lateral longitudinal rows of coarse dots in the middle body region, and 4 in the anterior (i.e. from amphideal fovea to pharyngeal end) and posterior (from spicule to tail) body regions of both sexes. Spicule 110 µm long, with a small distal hook and proximally cephalate. *Gubernaculum* with lateral projections and long apophysis. Pre-cloacal supplements are present, but fine and tubular.

In the male specimen analysed using CLMS, cuticle ornamentation showed a moderate fluorescence that resulted of the same intensity in both green and red emissions (Figure 1A). This was roughly also applicable to the teeth (Figure 1B,C). Instead, spicule and *gubernaculum* showed a relevant emission, especially in green (Figure 1D,E).

Dorylaimopsis variabilis Muthumbi, Soetaert and Vincx 1997 reflects the general features of the genus. In particular, it is characterized by the multispiral amphideal fovea with 2.5–3.0 turns, the cuticle with 2 lateral longitudinal rows of coarse dots in the middle body region, and 3 in the anterior and posterior body regions of both sexes. Spicules 73-127 µm long, arcuate and with a well-developed *capitulum*. *Gubernaculum* with long caudal apophysis. Precloacal supplements with very fine ducts.

The buccal cavity and cuticle ornamentation appeared poorly fluorescent and equivalent in both emissions as in the previous *Dorylaimopsis* species. Spicules and *gubernaculum* showed a higher emission signal and comparable in both the wavelengths (Figure 2A). The 3D-recostructions by CLMS revealed the presence of a greater morphological complexity of the spicule tip than that reported in the original description (Figure 2B,E). These details were also visible to a further analysis by scanning electron (SEM) and differential interference contrast (DIC) microscopies (Figure 2C,D,F).

Laxus gerlachi (Hopper and Cefalu, 1973) belongs to Stilbonematinae (family Desmodoridae). The genus is characterized by the cuticle with fine transverse *striae*. Cephalic cuticle thick with a surface irregularly annulated, reticulated, or sculptured in a fingerprint pattern. Amphideal fovea small, coiled with ~1.5 turns, situated close to the apex. Anterior pharynx slightly swollen and not sharply separated from the narrow middle region. *Gubernaculum* directed dorsally, no dorso-caudal apophysis. Tail short, conical, mostly 1.4 – 2 anal diameters long. Symbiotic bacteria coccoid. In particular, *L. gerlachi* showed cuticle ornamentation starting posteriorly to the amphideal fovea and made of fine transverse *striae* covered by a coat of symbiotic coccoid bacteria. Amphideal fovea multispiral with ~ 3 turns close to the apex. Buccal cavity small without teeth. Spicule 44 µm long and proximally cephalate. *Gubernaculum* present and directed dorsally. Tail short and conical.

The cephalic cuticle emitted a fluorescence in both red and green fluorescence (Figure 3A) and highlighted globular structures that likely were coccoid bacteria. Red fluorescence seemed to better discriminate morphological details than green fluorescence because in the latter the signal emission was too high, so making a clear visualization difficult. The only cuticular parts really fluorescent were the inserting points of the somatic setae that appeared more clearly in green fluorescence (Figure 3A). Single morphological details of the male reproductive system were more fluorescent under FITC than TRITC condition. The tri-dimensional reconstruction of the *gubernaculum* structure was highly informative (Figure 3B). Also the subventral *setae* on the male tail were well-visible (Figure 3C).

Rhinema retrosum Cobb, 1920 belongs to the family Monoposthidae. The head cuticular annules fuse forming a helmet. Buccal cavity is characterized by three teeth (one dorsal and two subventral teeth). Amphideal *fovea* is circular without interruption of its edge. Cuticle exhibits 12 longitudinal rows of *alae* throughout the body. Paired ovaries. Spicules (45 µm long) and *gubernaculum* present.

The specimen analyzed was a female that showed a signal emission in both the wavelengths consid-

ered. In detail, the whole cephalic region (e.g. amphid and helmet) showed a high emission: labial region, buccal cavity and the surrounding pharynx resulted slightly more fluorescent under FITC conditions, while the cuticle of the helmet was comparable with both emissions (Figure 3D,E).

Craspodema reflectans Gerlach, 1964 belongs to the family Cyatholaimidae. Amphideal fovea spiral (3.5 turns), hand-mirror shaped and based on a plaque. The buccal cavity deep with three teeth (one well-developed dorsal tooth and two smaller and equal subventral ones). Lateral differentiation of body cuticle is very prominent as longitudinal rows of enlarged punctations with broad lateral fields between them. Spicules are arcuate (41 µm long) and *gubernaculum* present. Well-developed ventral preanal supplementary organs heavily cuticularized is observed.

Both FITC and TRITC conditions excited the cephalic region of *C. reflectans* (e.g. *rugae* and buccal cavity) (Figure 4A,D). However, *rugae* were slightly more evident using the FITC conditions (Figure 4B), while the buccal cavity walls was slightly more fluorescent under TRITC conditions (Figure 4C). Cuticle and amphideal fovea showed a moderate fluorescence with both wavelengths, while

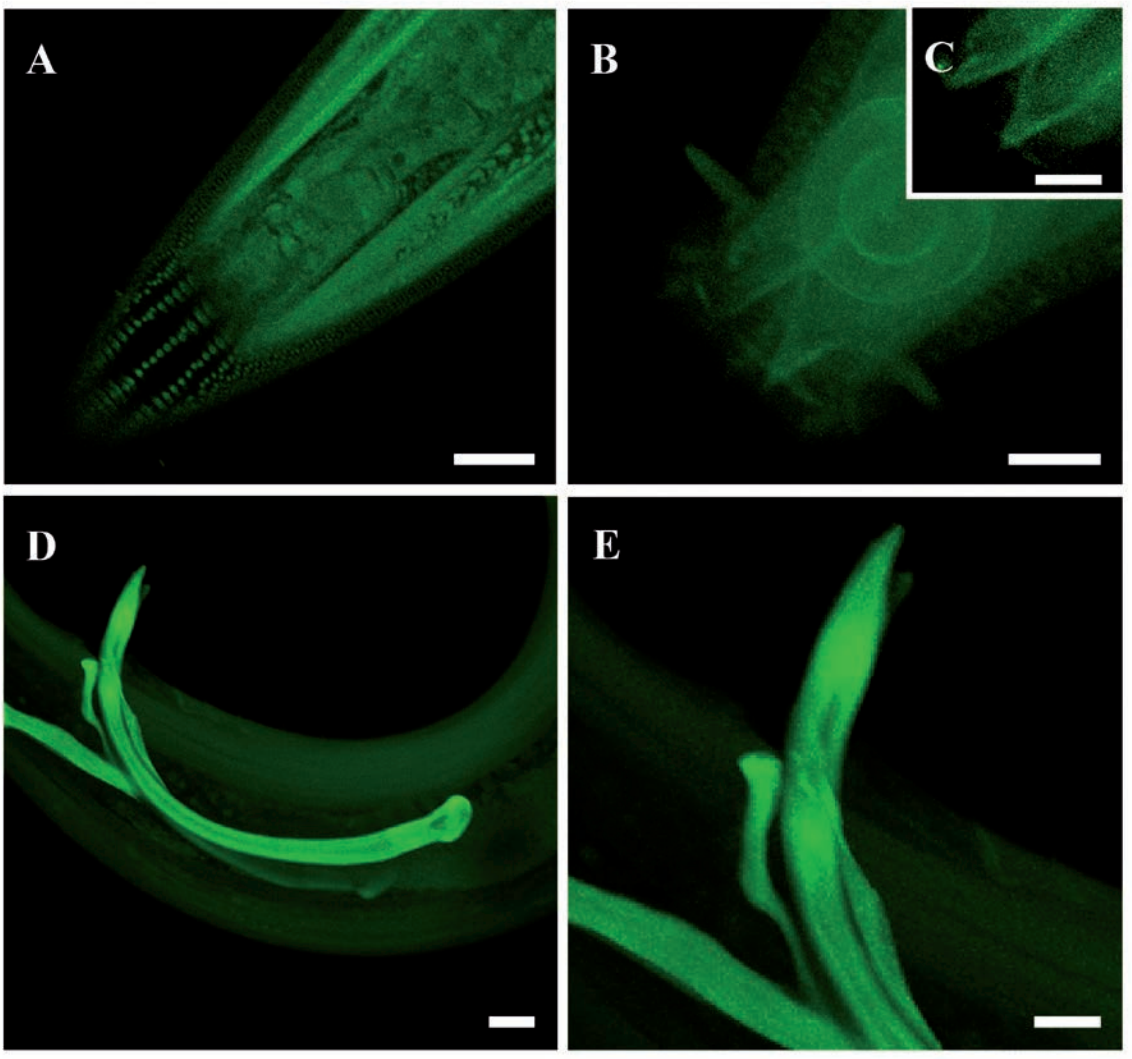

Figure 1. *Dorylaimopsis pellucidum*. A) Detail of cuticular ornamentation by Confocal Laser Scanning Microscopy (CLSM); B) Reconstruction of the buccal cavity by CLSM; inset C. Detail of the teeth; D) Reconstruction of the male copulatory apparatus by CLSM; E) Detail of the spicule tip by CLSM. All images are excited in FITC emission spectra. Scale bars: A, D = 10µm; B,C, E = 5µm.

spicule, *gubernaculum* and precloacal supplements resulted slightly more fluorescent under TRITC conditions. CLMS observations highlighted new details on the *gubernaculum* morphology that have been used to redescribe the species (see below).

Craspodema octogoniata (Gerlach, 1954) shares with *C. reflectans* the cuticle ornamentation typical of the genus, but shows a shallower buccal cavity and a multispiral amphideal *fovea* with ~ 3 turns not based in a plaque. Spicules are arcuate and 42 µm long, the *gubernaculum* is present as well as precloacal supplements. The specimens analyzed belong to the Zoological Museum of the Hamburg University (Germany). In particular, the holotype (collected in the 1951) and the paratype material (1973) showed both fluorescence emissions. However, the conservation state of the holotype is not perfect, especially in the cephalic region. This region, the cuticle as well as the male reproductive structures of *C. octogoniata* appeared fluorescent with both green and red emissions (Figure 5A-D). Also the precloacal supplements were well visible (Figure 5C).

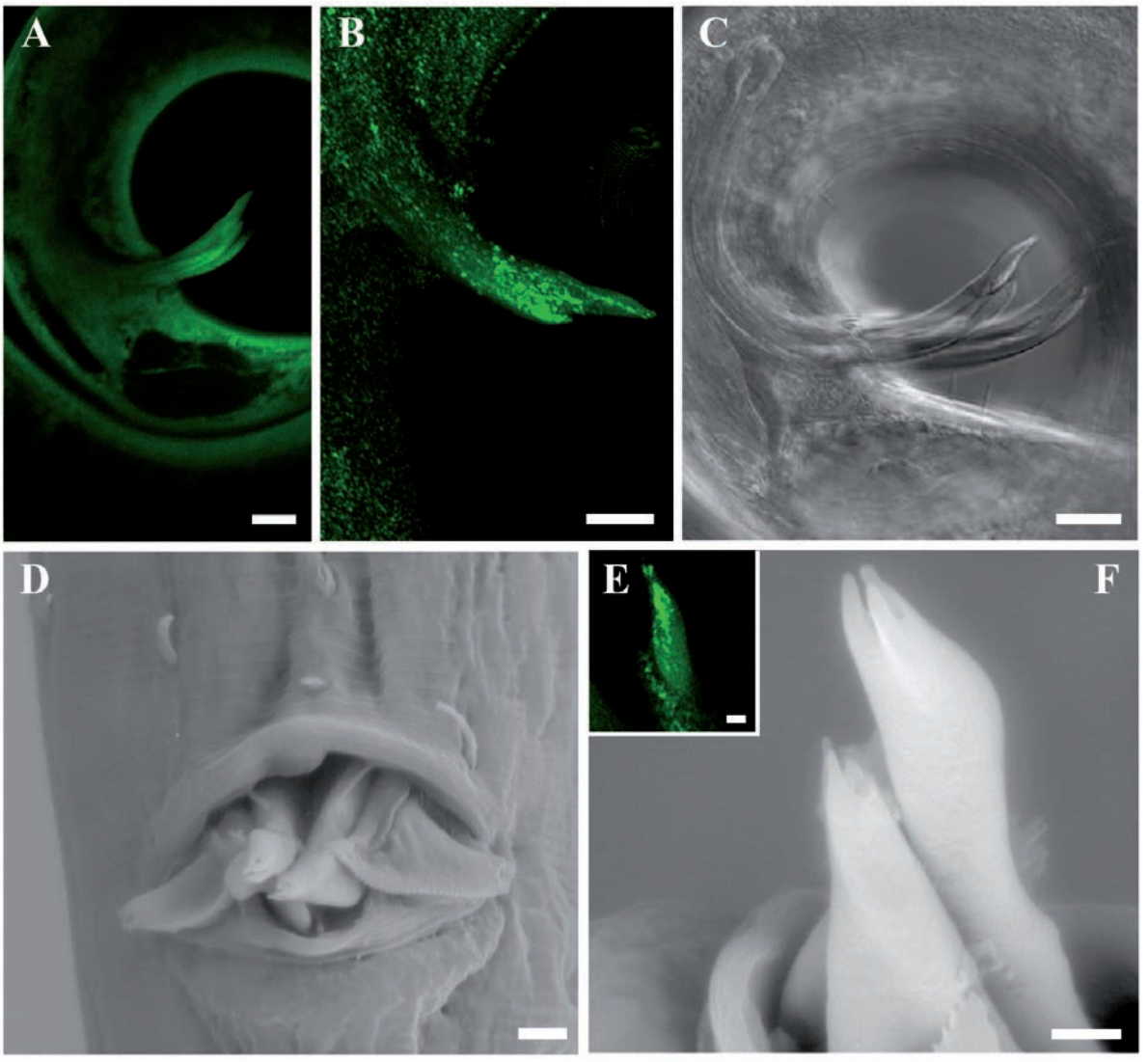

Figure 2. *Dorylaimopsis variabilis*. A) Male spicules by Confocal Laser Scanning Microscopy (CLSM); B) Spicule tip by CLSM; C) Male copulatory apparatus by differential interference contrast microscopy (DIC); D) Male cloaca and distal part of the spicules by scanning electron (SEM); E) Detail of spicule tip region by CLSM; F) Detail of the spicule tip by SEM. Images A,B,E are excited in FITC emission spectra. Scale bars: A, B, C = 10µm; D, E = 2µm; F = 1µm.

Discussion

The autofluorescence of free-living nematodes was documented for the first time by Zullini and Villa (2006), but no possible explanation was given by authors. In all the species observed in the present study, the emission was more marked in spicules and *gubernaculum*, two diagnostic characters that have a fundamental role for the taxonomical identification of nematodes. Depending on the level of the cuticularization of the buccal cavity, the teeth and the buccal cavity walls can be detected, while denticles (very small in size) generally not (see *R. retrosum*). In Cyatholaimidae, Comesomatidae as well as Monoposthidae species, in which the labial region may be rather complex, the 3D-reconstruction may be possible and give spectacular results that may be

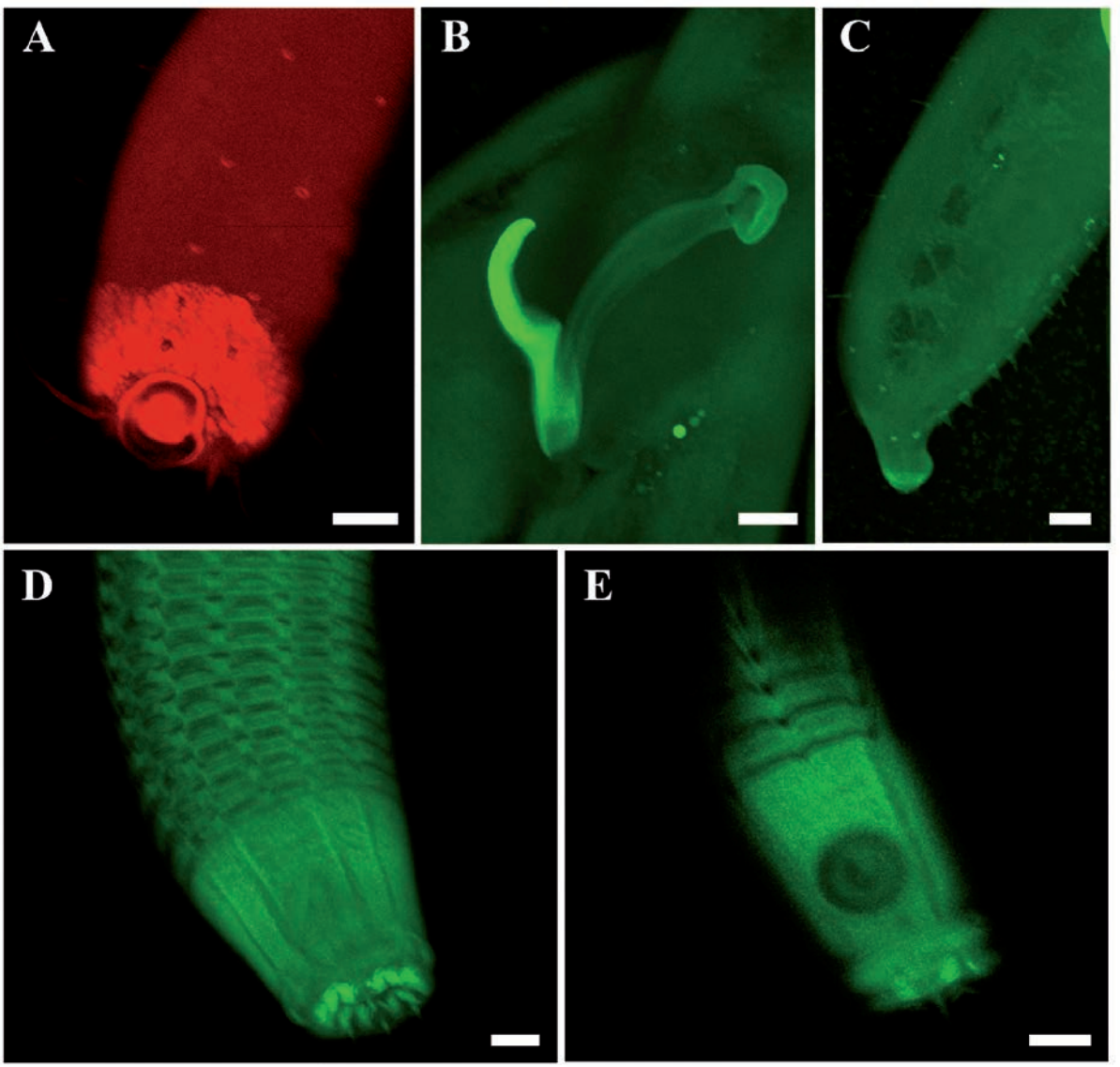

Figure 3. *Laxus gerlachi and Rhinema retrosum* species. A) Detail of helmet region of *L. gerlachi* by Confocal Laser Scanning Microscopy (CLSM); B) Reconstruction of the male spicule and *gubernaculum* of *L. gerlachi* by CLSM; C) Reconstruction of the caudal region of *L. gerlachi* by CLSM; D-E) Detail of helmet region of *R. retrosum* by CLSM. Image A is excited in TRITC emission spectra; images B-E are excited in FITC emission spectra. Scale bars: A, B, C = 10µm; D, E = 5µm.

used for taxonomical purpose (see e.g. Figure 4). The emission from the cuticle ornamentation is often not high, and depends on the cuticle thickness. In general, a greater emission of fluorescence was mainly detected in the cephalic region, the heavy cuticular punctuation or precloacal supplements (e.g. Figures 1A; 3A,D,E; 4A,B,D). The structures of the nematode species that emitted a higher fluorescence were mainly composed by of scleroproteins (e.g. collagen and elastin) (Chitwood and Timm, 1954; Page and Johnstone, 2007) that are known to be autofluorescent because they contain several fluorophores (Georgakoudi *et al.*, 2002; Gerson *et al.*, 2009). In particular, Roshchina (2012) reported their fluorescence spectra over a range of excitation wavelengths: max. 400–430, 465, 495 and 520 nm. This could explain the emission of spicule, *gubernaculum*, cuticularized parts of the buccal cavity, precloacal supplements and cuticle ornamentation both in FITC and (in to a lesser extent) in TRITC conditions. However, it was possible to observe that also internal parts of the nematode bodies, without collagen and elastin, appeared slightly fluorescent. This could be related to the action of formalin that may react with amines and proteins and generate fluorescent products (Leischner *et al.* 2010). Despite this possible formalin influence, the various microscopic techniques that we used (for instance with *D. variabilis*) gave consistent results on the structure of the spicule tip. Thus, formalin seems to amplify the signal emission already released by scleroproteins, and also to induce fluorescence in molecules that are not autofluorescent, as e.g. biogenic amines (Falck *et al.*, 1962; Corrodi and Jonsson, 1967; Rost, 1995). The

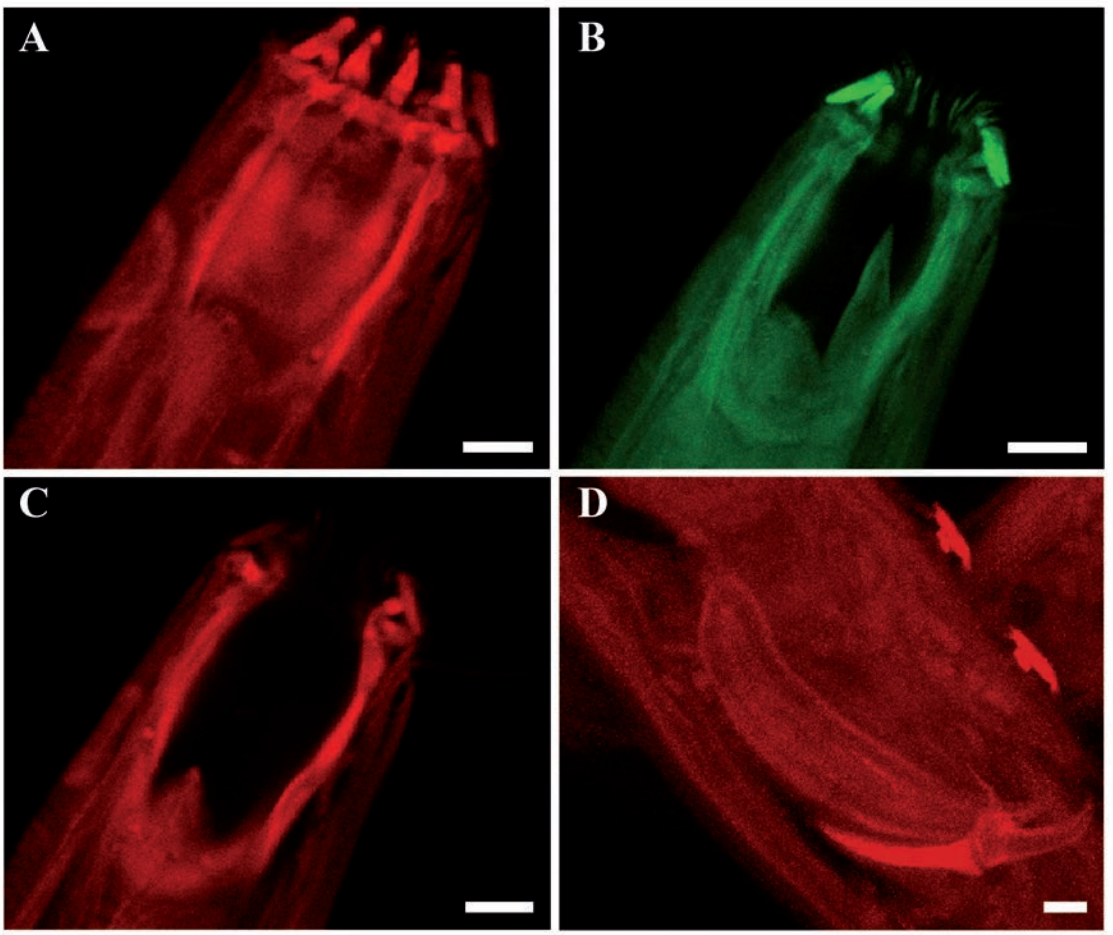

Figure 4. *Craspodema reflectans*. A) Reconstruction of rugae by Confocal Laser Scanning Microscopy (CLSM); B) Detail of teeth by CLSM; C) Buccal cavity walls by CLSM; D) Reconstruction of the male reproductive system. Image B is excited in FITC emission spectra; Images A, C, D are excited in TRITC emission spectra. Scale bars: A, B, C = 5µm; D = 10µm.

details captured by CLMS both in the *gubernaculum* and cephalic region of *C. reflectans* and in the spicule of *D. variabilis* were crucial for the re-description of these species (Figure 2) showing that CLMS is a very powerful tool for nematode study and opens new perspective in the taxonomy of this *phylum*. Indeed, the exploitation of autofluorescence involves low labour costs and make possible excellent 3D-reconstructions of morphological structures without the application of fluorescent tags for target cells or tissues. Furthermore, the approach here adopted highlights new chances for the study of Museum type material. Indeed, old descriptions and drawings of nematodes do not always fit the current taxonomical standards and require further analysis. Type material preserved as permanent slides can

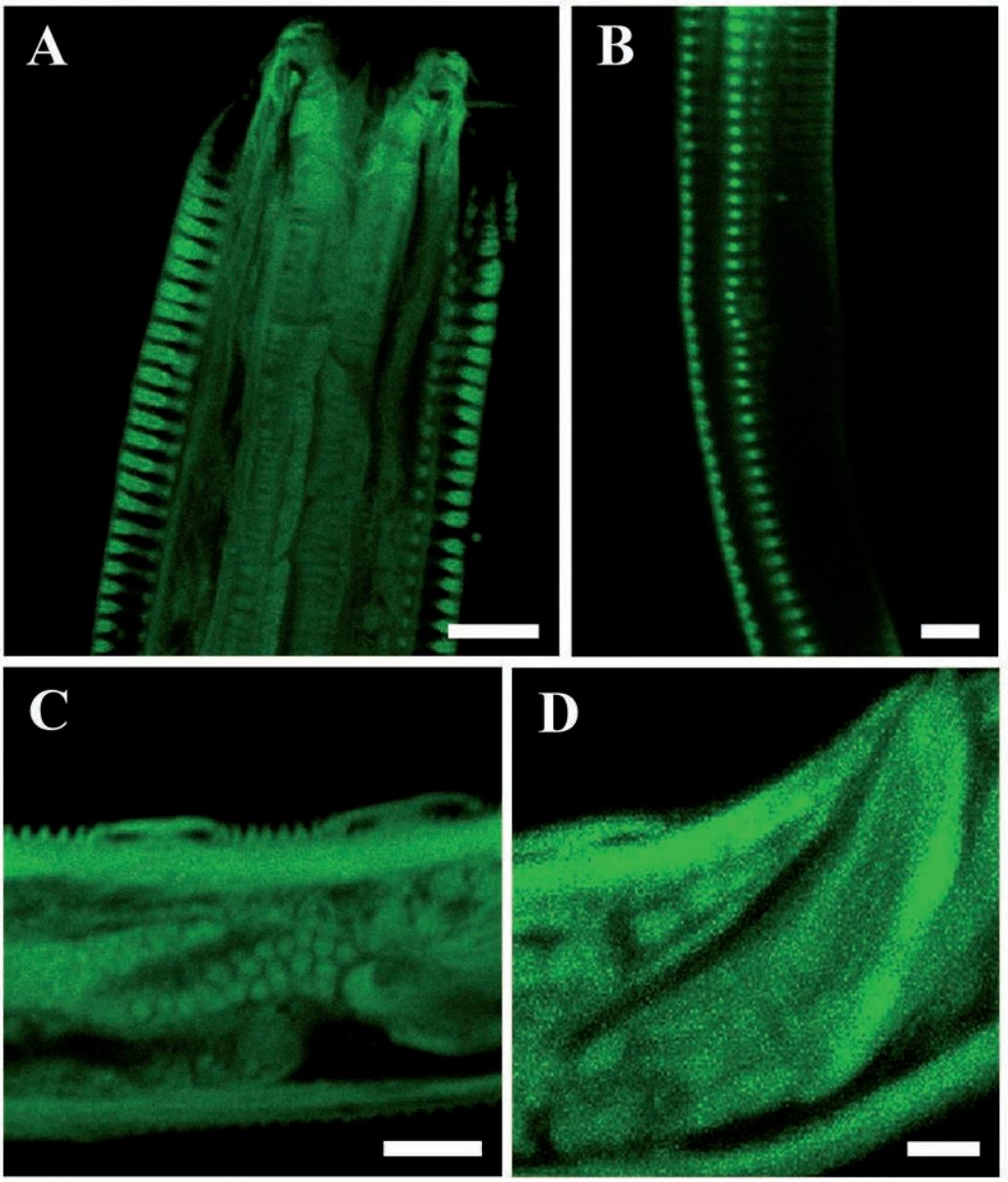

Figure 5. *Craspodema octogoniata*.A) Reconstruction of teeth by Confocal Laser Scanning Microscopy (CLSM); B) cuticle ornamentation in the middle body by CLSM; C) Detail of the precloacal supplements by CLSM; D) Detail of the spicule by CLSM. All images are excited in FITC emission spectra. Scale bars: A, B, C, D = 5µm.

undergo a deterioration or simply cannot be utilized for additional observations such SEM analysis because a part of the necessary diagnostic elements are inside the specimen body and so not directly accessible. In this regard, confocal microscopy can provide high-resolution images of buccal cavity, spicule and *gubernaculum* allowing a 3D-reconstruction of these structures without any damage like the degradation of the cuticle that would be necessary for SEM analysis. The detection of fluorescence in ancient samples of Altherr's collection (deposited in the Museo Cantonale di Storia Naturale of Lugano, Switzerland) (Zullini and Villa, 2006) as well as Gerlach's collections (deposited in the Zoological Museum of the Hamburg University, Germany) (Semprucci and Burattini, 2015) is promising because it really shows that museum specimens may still provide new, precious information. Accordingly, CLSM technique seems to be a significant tool in the effort of making the taxonomical material deposited at the Natural History Museums more accessible and available for scientific purposes.

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