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# Characterisation of chitin in the cuticle of a velvet worm (Onychophora)

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**Abstract:** We characterize the trunk cuticle of velvet worms of the *Peripatoides novaezealandiae*-group (Onychophora) using SEM, TEM, Fourier transform infrared spectroscopy (FT-IR), and thermogravimetric analysis (TGA). TEM and SEM revealed a relatively uniform organization of the delicate cuticle that is covered by numerous bristled and nonbristled papillae with ribbed scales arranged in transverse rows. The cuticle consists of a very thin multilayered epicuticle of varying appearance followed by the largely fibrous procuticle. The irregularly arranged nanofibres of isolated cuticular chitin seen by SEM are considered as bundles of chitin fibres. FT-IR and TGA showed that the chitin is of the α-type. This confirms and broadens the single previous study in which the presence of α-chitin in a velvet worm was demonstrated with a single analysis (X-ray diffraction).

**Key words:** Chitin, Fourier transform infrared spectroscopy, thermogravimetric analysis, *Peripatoides*, cuticle, Ecdysozoa

## 1. Introduction

Onychophora (velvet worms) is an enigmatic taxon with a changing history concerning their systematic position. For a long time, they were considered to link Annelida and Arthropoda (e.g., Lankester, 1904; briefly reviewed in Wright and Luke, 1989). However, at the latest after the final abandonment of the Articulata-concept and the establishment of the Ecdysozoa (e.g., Aguinaldo et al., 1997; Mallatt et al., 2004; Telford et al., 2008; Dunn et al., 2008; Borner et al., 2014), a relationship between the Annelida and Arthropoda is off the table. Currently, velvet worms are united with Tardigrada and Arthropoda in the widely accepted clade Panarthropoda within the monophyletic Ecdysozoa and only the clustering of the three groups is still a matter of debate (see literature above and Hejnol et al., 2009; Meusemann et al., 2010; Rota-Stabelli et al., 2010; Campbell et al., 2011; Mayer et al., 2013). Regarding the cuticle, ultrastructural, (ultra) histochemical, and biochemical studies have noted strong similarities between the onychophoran and the arthropod cuticle in its simplest form (e.g., Robson, 1964; Lavallard, 1977; Wright and Luke, 1989; Krishnan, 1970; Hackmann and Goldberg, 1975).

As far as is known, not only the cuticle of arthropods but also the cuticle of most Ecdysozoa contains chitin (e.g., partly summarized in Greven and Peters, 1986.; Neuhaus et al., 1997a, b; Kristensen and Neuhaus, 1999), and it is widely accepted that the presence of a specific type of chitin, i.e. α-chitin, in cuticle layers near the epidermis is an apomorphic character of this clade. According to data from the literature (summarized in Schmidt-Rhaesa et al., 1998; Nielsen, 2012; Westheide and Rieger, 2013, Greven et al., 2016, 2019), there is no doubt that the vast majority of arthropods has α-chitin in the cuticle (reviewed in Neville, 1975). This should also apply to the cuticle of onychophorans, in which the presence of α-chitin was shown in the last century using X-ray diffraction (Lotmar and Picken, 1950; Rudall, 1955; see also Rudall and Kenchington, 1973). However, today X-ray diffraction alone is not considered to be sufficient to demonstrate and characterize chitin-types (e.g., Kumirska et al., 2010) and its use even may produce erroneous results (see Greven et al., 2019).

In the present article, we therefore study the cuticle of a velvet worm with more modern and stronger methods, such as Fourier transform infrared spectroscopy (FT-IR)

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**Table.** FT-IR bands of commercial α-chitin, *Peripatoides novaezealandiae* chitin, and β-chitin from cuttlebone.

| Functional group and vibration modes | Classification | Commercial α-chitin | *Peripatoides* α-chitin | Cuttlebone β-chitin |
|---|---|---|---|---|
| O–H stretching | - | 3430 | 3434 | - |
| N–H stretching | - | 3104–3261 | 3098–3271 | 3265 |
| $CH_3$ sym. stretch and $CH_2$ asym. stretch | Aliphatic compounds | 2937 | - | 2923 |
| $CH_3$ sym. stretch | Aliphatic compounds | 2874 | 2888 | - |
| C=O secondary amide stretch | Amide I | 1655 | 1654 | 1640 |
| C=O secondary amide stretch | Amide I | 1620 | 1622 | - |
| N–H bend, C–N stretch | Amide II | 1553 | 1551 | 1551 |
| $CH_2$ ending and $CH_3$ deformation | - | 1425 | 1415 | 1427 |
| CH bend, $CH_3$ sym. deformation | - | 1375 | 1377 | 1374 |
| $CH_2$ wagging | Amide III, components of protein | 1307 | 1310 | 1307 |
| Asymmetric bridge oxygen stretching | - | 1154 | 1154 | 1151 |
| Asymmetric in-phase ring stretching mode | - | 1114 | 1113 | 1105 |
| C-O-C asym. stretch in phase ring | Saccharide rings | 1069 | 1069 | 1058 |
| C-O asym. stretch in phase ring | | 1009 | 1022 | 1024 |
| $CH_3$ wagging | A long chain | 952 | 952 | 946 |
| CH ring stretching | Saccharide rings | 894 | 896 | 872 |

and thermogravimetric analysis (TGA), and complete these results with transmission (TEM) and scanning electron microscopy (SEM).

## 2. Materials and methods

### 2.1. Origin of the samples

For the present study, we examined samples of *Peripatoides novazealandiae* (Hutton, 1876) endemic to New Zealand from various sources:

1. Remnants of individuals, which were previously used for studies on muscle proteins (e.g., Prasath et al., 2013) and were obtained from a commercial dealer (www.exotic-pets.co.uk) in 2010. Time and site of collection unknown. These remains were stored in either 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer or 70%–80% ethanol.

2. Two specimens were purchased in 2016 from the Pet Factory (Germany). Time and site of collection unknown. One specimen was also fixed in 2.5% glutaraldehyde (GA) in 0.1 mol cacodylate buffer and processed for TEM (see below).

As *P. novazealandiae* is considered a species complex with several morphospecies (see Trewick, 2000; Pripnow and Ruhberg, 2003), its current status is unclear (Oliveira et al., 2012). Therefore, the second specimen, fixed in 70%, was deposited in the Staatiches Museum für Naturkunde Karlsruhe (Karlsruhe, Germany) (Acc. No. SMNK-ONY0013).

3. Ten specimens were obtained from the collections of the Natural History Museum of Denmark (catalogue number NHMD-274199). Time and site of collection: 15 October 1962, Rotorua, New Zealand. Specimens were fixed with unknown fixative and stored long term in 70% ethanol. The specimens were used for chitin isolation. Two individuals from the same sample are shown in Figures 1A and 1B. Serial photos were taken in different focal planes with a BK+ Imaging System from Visionary Digital (Dun, Inc., http://www.duninc.com) equipped with a Canon EOS 7D camera. The serial photos were stacked and combined with Zerene Stacker (Version 1.04; http://zerenesystems.com).

### 2.2. Scanning electron microscopy (SEM)

Pieces of the previously used GA-fixed material were rinsed in distilled water, dehydrated with hexamethyldisilazane (HMDS), mounted on stubs, sputtered with gold, and viewed in a SEM Leo 1430 (Fa. Zeiss). In addition, pieces of chitin extracted for FT-IR (see below) were processed in the same way and viewed in a SEM Quanta FEG 250.

### 2.3. Transmission electron microscopy (TEM)

Pieces of the trunk were fixed in 2.5% glutaraldehyde in 0.1 mol cacodylate buffer, postfixed in 1% osmiumtetroxide in the same buffer, dehydrated in an acetone series (stained with 1% phototungstic acid plus 0.5% uranylacetate in 70% acetone) and embedded in Spurr's medium. A sample from the previously fixed specimens was also postfixed and embedded. Semithin sections (1 µm, for orientation) were stained with toluidinblue–borax. Ultrathin sections were cut with a Reichert 0MU3 ultramicrotome, mounted

on copper grids, stained with lead and uranylacetate and viewed in a Zeiss Elmiskope EM 109.

**2.4. Isolation of chitin**

Samples stored in ethanol (80%) were dried at room temperature for 4 h. They were then heated in 2 M HCl solution at 50 °C for 4 h. After that, the samples were rinsed with distilled water (up to neutral pH). To remove proteins, the remaining tissue was transferred in 4 M NaOH solution and heated at 100 °C for 12 h, then rinsed with distilled water again until a neutral pH was reached. Subsequently, the sample was treated with 0.2 NaOCl at room temperature for 3 h to remove other organic residues, such as pigments. Finally, the sample was rinsed several times to reach neutral pH again and then dried at room temperature for 24 h. The obtained chitin skeletons from *P. novaezealandiae* are shown in Figures 1C and 1D. Commercial α-chitin was obtained from Sigma-Aldrich (pcode: 1001416772), and β-chitin from the cuttlebone of *Sepia* sp. was supplied from the stock in the Department of Biotechnology and Molecular Biology (Aksaray University).

**2.5. Fourier transform infrared spectroscopy (FT-IR)**

The FT-IR spectrum of chitin from *Peripatoides* exoskeleton was obtained using a Perkin Elmer FT-IR Spectrometer (ATR) (Perkin Elmer 100, Waltham, MA, USA) over the frequency range of 4.000–625 $cm^{-1}$. The FT-IR spectrum was compared with spectra of known α- and β-chitin samples (s. 2.4.).

**2.6. Thermogravimetric analysis (TGA)**

An EXSTAR S11 7300 device was used to examine thermal properties of the isolated chitin. The analysis was conducted at temperatures changing 10 °C per min between 100 and 850 °C under a nitrogen atmosphere (Kaya et al., 2017).

## 3. Results

**3.1. Electron microscopy (SEM and TEM)**

The dorsal body surface of *Peripatoides novaezealandiae* bears transversal rows of scaled large papillae from which sensory bristles extend, and smaller papillae that lack such an extension. Corresponding to the epidermal cells, papillae are formed by small scales that appear to be ribbed (Figure 2A). The cuticle is very thin, ranging from <1 μm in the transversal folds to approximately 2 μm in the sensory organs. It covers the monolayered epidermis, which rests on a thin basal lamella followed by a thick layer of collagen fibrils (Figures 2B and 2C). The general (trunk) cuticle reveals two main layers, the thin multilayered epicuticle and the thick procuticle (Figure 2D).

Epicuticle: The extremely delicate epicuticle needs considerable high magnifications to resolve its layers. Nevertheless, their course and number are not entirely clear. In favourable sections, at least three layers may be visualized, measuring altogether approximately 60 to 80 nm. The innermost layer—inner epicuticle (terminology adopted from Wright and Luke, 1984)—appears electron dense, but is not always continuous (Figures 2E and 2F). In

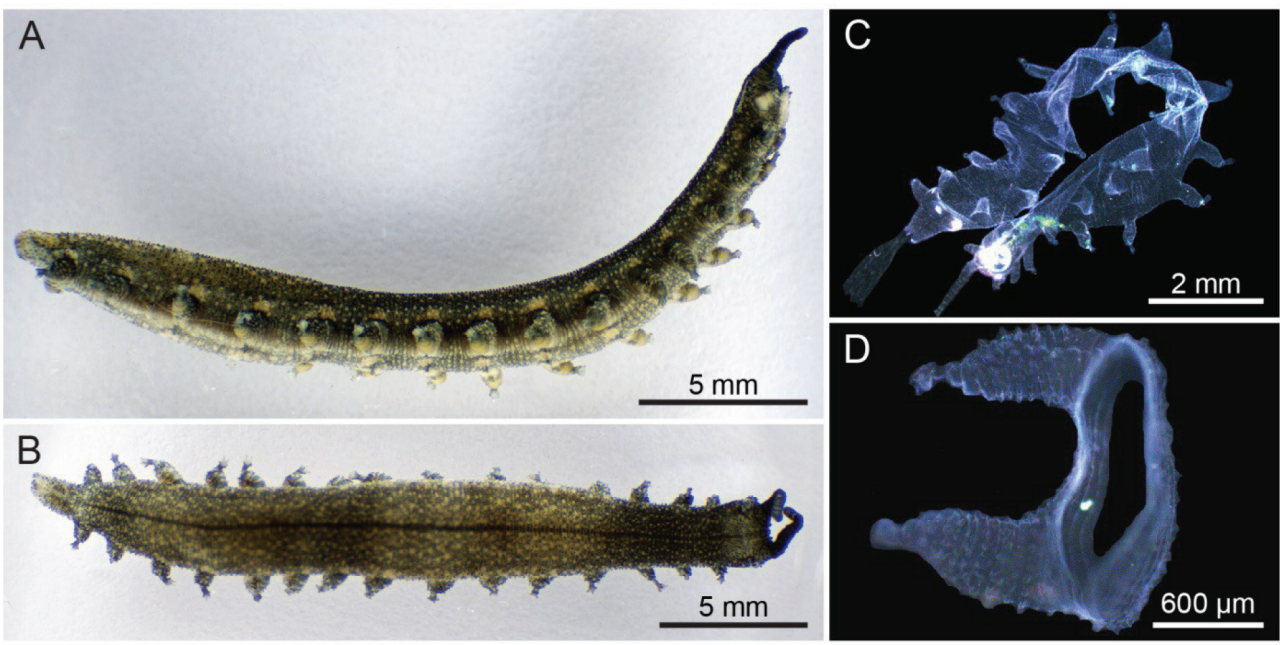


**Figure 1.** Preserved specimens (A–B) and chitin isolates (C–D) of *Peripatoides novaezealandiae*. A: Lateral view. B: Ventral view. C: Chitin skeleton of complete specimen. D: Chitin skeleton of a single segment.

some areas, layer 1 tends to lift away from layer 2, leaving a small space between them that occasionally appears to be bridged by small vertical structures (Figure 2F).

Procuticle: This innermost cuticular layer is relatively uniform and accounts for the main part of the cuticle. Its thickness depends on the body region, measuring approximately 1 to 3 µm. The bulk of the procuticle reveals a fibrous structure, and fibres appear to be randomly oriented. (Figures 2C–2F). In some regions the procuticle appears somewhat denser, obscuring the fibrous pattern (Figures 2D, 2F). Furthermore, we observed discrete structures within the cuticle that sometimes appeared to have a hollow space (Figures 2E and 2F).

Randomly arranged nanofibres are also seen in the samples prepared for FT-IR and viewed by SEM. Here, the width of the nanofibres is between 27 and 36 nm (Figure 2G and inset).

### 3.2. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra of commercial α-chitin, chitin isolated from *Peripatoides novaezealandiae*, and squid pen β-chitin are shown in Figure 3. Commercial α-chitin has a broad, divided, and V-shaped (sharp) amide I band with peaks at around 1655 and 1620 $cm^{-1}$ (Figure 3a). Chitin obtained from *P. novaezealandiae* has the same broad, divided, and V-shaped amide I band with peaks around 1654 and 1622 $cm^{-1}$ (Figure 3b), whereas the spectrum of the β-chitin of cuttlebone has a broad, undivided, U-shaped amide I band around 1640 $cm^{-1}$ (Figure 3c). Data for the amide II band are also given in Figure 3. All other FT-IR absorption bands are listed in the Table.

### 3.2. Thermogravimetric analysis (TGA)

TG/DTG analyses are decisive for determining the purity and type of chitin. The results for chitin extracted from *P. novaezealandiae* are illustrated in Figures 4a and 4b. As is seen here, only a single degradation was recorded between 200 and 400 °C, which can be attributed to the degradation of chitin. The maximum degradation temperature (DTGmax) was 354 °C (Figure 4b). According to the literature, a DTGmax lower than 350 °C is attributed to β-chitin, whereas values higher than 350 °C are characteristic for α-chitin (Kaya et al., 2017). Thus, the DTGmax shown for the chitin of *P. novaezealandiae* clearly indicates the presence of α-chitin.

## 4. Discussion

### 4.1. The structure of the cuticle

The ultrastructure of the trunk cuticle in onychophorans studied thus far appears relatively uniform. The cuticle consists of a compound epicuticle that may be divided into a stratified outer and an inner homogeneous epicuticle, and the procuticle with randomly ordered fibrils, in which a variably sclerotized exocuticle may be identified in some regions (see Robson, 1964; Lavallard, 1965, 1972; Wright and Luke, 1984). The epicuticle is more variable. The appearance of single layers may depend on the fixatives used, and may differ in certain body regions and between species. Furthermore, the number of layers appears not to be standardized. In the epicuticle of the trunk cuticle, four (*Peripatopsis moseleyi*: Robson, 1964), three (*Peripatus* [today *Epiperipatus*] *acacioi*: Lavallard, 1965, 1977), and five layers (*Euperipatoides leukartii*: Wright and Luke, 1984) were distinguished. In *E. leukartii*, the fourth layer situated immediately above the osmiophilic layer (= inner epicuticle)—the counting of layers is not clear in this article (see Figure 13 and text)—is striated, i.e. consists of vertical rods. In addition, Wright and Luke (1984) described 'pore canals' in the epicuticle extending into the procuticle, and helicoidally organized collagen fibres beneath the epidermis not seen so far in the other species. Concerning the epicuticle and the unknown structures in the procuticle, further studies are needed.

The ultrastructure and (ultra)histochemistry (e.g., tanned lipoproteins) of the outer and untanned lipoproteins in the inner epicuticle, α-chitin and proteins in the untanned procuticle, and the occurrence of a discontinuous 'sclerotin' layer (= exocuticle) hardened by quinone tanning, e.g., in sensory setae, claws, and mandibles, as well as cuticle proteins (Robson, 1964; Hackman and Goldberg, 1975; summarized and broadened in Wright and Luke, 1984) prompted the authors to conclude that "the overall scheme of cuticle structure suggests likely homology with the basic arthropod design" (Wright and Luke, 1984; p. 620).

At high magnifications, ultrathin sections of the cuticle, but even clearer SEM images of the chitin samples prepared for FT-IR, show many irregularly arranged fibres, confirming the nonlamellated, nonhelicoidal organisation of the onychophoran cuticle (Robson, 1964; Lavallard, 1965, 1977; Wright and Luke, 1984). When observed with SEM, isolates of insect α-chitin exhibit different surface morphologies ranging from smooth without 'nanopores' (= pore canals) to fibrous with or without nanopores and very distinct nanofibres (e.g. Kaya et al., 2016a). The surface of the α-chitin isolate of *P. novaezealandiae* does not exhibit nanopores (as pore canals appear to be absent, see above), but shows distinct nanofibres.

### 4.2. Type of chitin and arrangement of chitin nanofibres

Chitin, one of the most abundant biopolymers found in nature, is widespread among animals (e.g., Jeuniaux, 1982), where it occurs in two polymorphic crystalline structures, i.e. α- and β-chitin. The less studied γ-chitin (Jang et al., 2004; Lavall et al., 2007; Wang et al., 2013) is probably a combination of the α- and β structures rather than a different allomorph (Kumirska et al., 2010). The cuticle of all Panarthropoda including onychophorans studied so far contains α-chitin exclusively, but studies concerning velvet worms have used either methods generally not specific for chitin (e.g., 'positive Schultze reaction' by Kunike [1925] and Krishnan [1970]) or

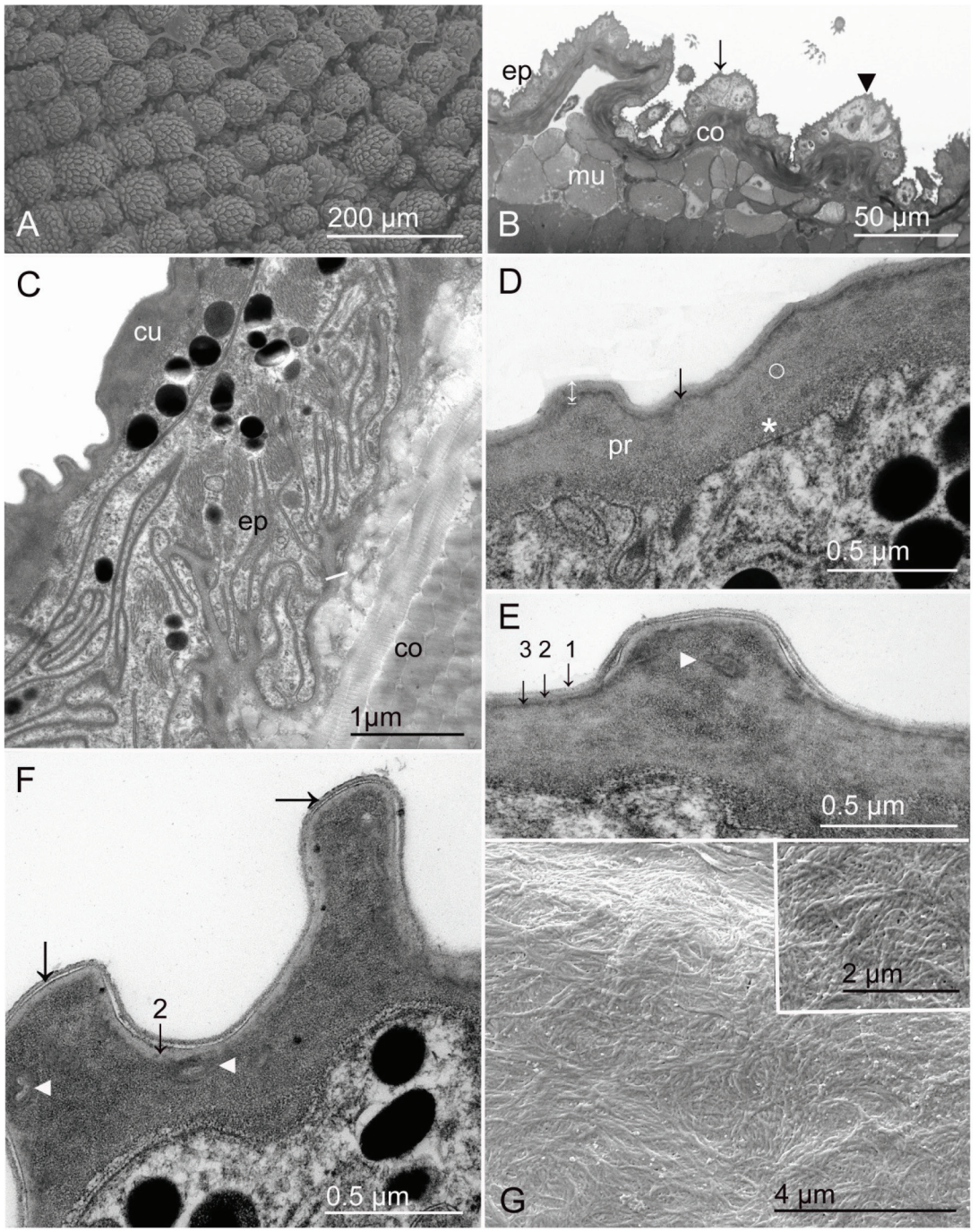


**Figure 2**. Structure of the cuticle of *Peripatoides novaezealandiae*. A SEM image of the dorsal cuticle showing transversely folded ridges with bristled and nonbristled papillae covered by ribbed 'scales'. B: Semithin section; note thinness of the cuticle (arrows). s papilla (arrowhead); co = collagen, ep = epidermis, mu = muscles. C: Low-power TEM image of the epidermis (ep), and cuticle (cu). Note the collagenous layer beneath the epidermis (co). D–F: High power TEM images showing the varying appearance of the epicuticle and the procuticle. D: Epicuticle (double-headed arrow) with several layers, the inner epicuticle (arrow) is osmiophilic; procuticle (pr) with fibrous (asterisk) and more compact parts (circle). E: Largely fibrous procuticle; the osmiophilic inner epicuticle seems to be reduced; note the space between layers 1 and 2 bridged by small vertical structures (arrow); unknown structures in the procuticle (arrowheads): F: Transition from the three-layered epicuticle (1, 2, 3) to the epicuticular parts with the space between layers 1 and 2. G: SEM images of the surface of a chitin isolate showing randomly arranged nanofibres. Inset: high power image of nanofibres.

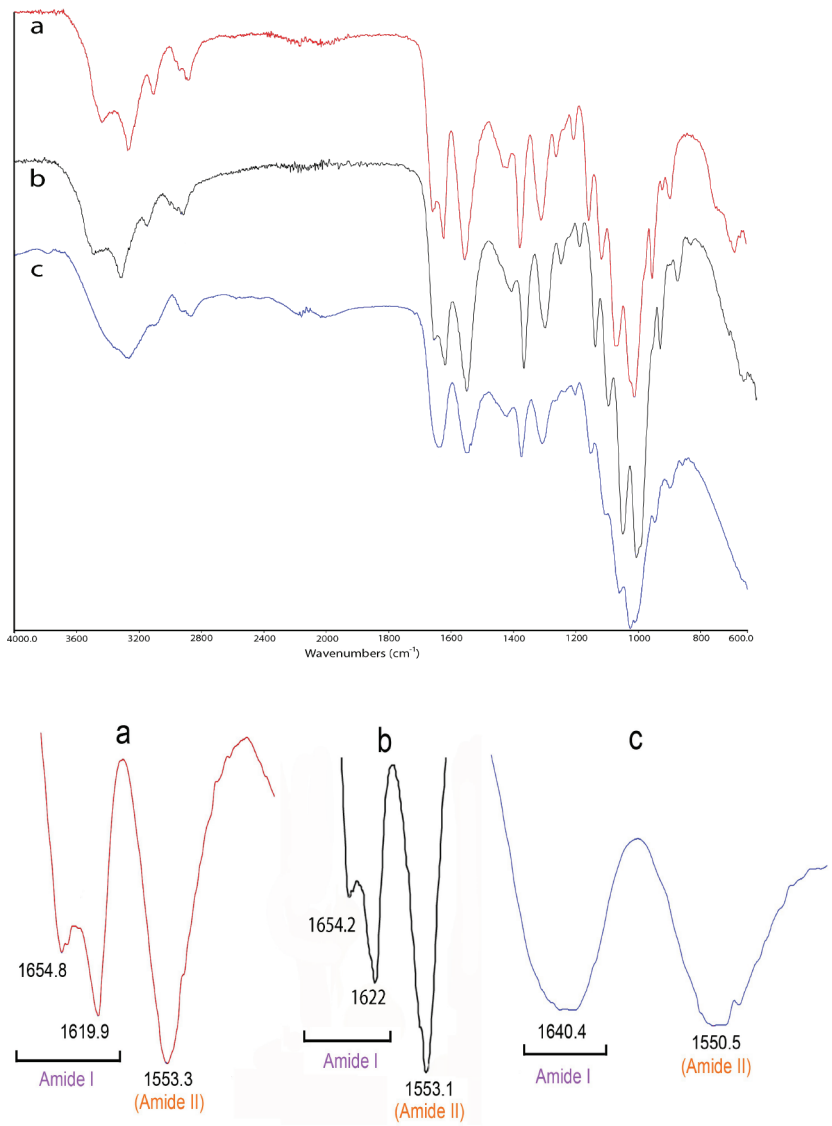


**Figure 3.** FT-IR spectra: overview (top), detail (below). Commercial α-chitin (a), extracted chitin from *Peripatoides novaezealandiae* (b), and β-chitin from cuttlebone (c).

X-ray diffraction to characterize the cuticular chitin more specifically (Lotmar and Picken, 1950; Rudall, 1955; Rudall and Kenchington, 1973). Lotmar and Picken (1950), using the high crystallinity of the chitin as their main criterion (see also Rudall and Kenchington, 1973), mentioned only "The cuticle of *Peripatus* (Onychophora) gave a powder-diagram with rings of remarkable sharpness. It appears to be α-chitin" (p. 59), but did not produce a corresponding diagram of β-chitin for comparison, whereas Rudall (1955; see also Rudall and Kenchington, 1973) studied an isolated but nonpurified (proteins were not removed) cuticle of *Peripatoides* sp., and stated a "resemblance to α-chitin in a similar state of purity" (p. 56).

There are various techniques for demonstrating the presence of chitin and its allomorphic forms, of which X-ray spectroscopy and FT-IR spectra analysis are widely used (for review see Kumirska et al., 2010). Generally, α-chitin has a higher crystallinity than β-chitin (Jang et al., 2004; Kumirska et al., 2010). However, the physical state of the chitin samples (dryness, contamination, particle size) influences the crystallinity of chitin (Cuong et al., 2016). Therefore, X-ray diffraction and FT-IR

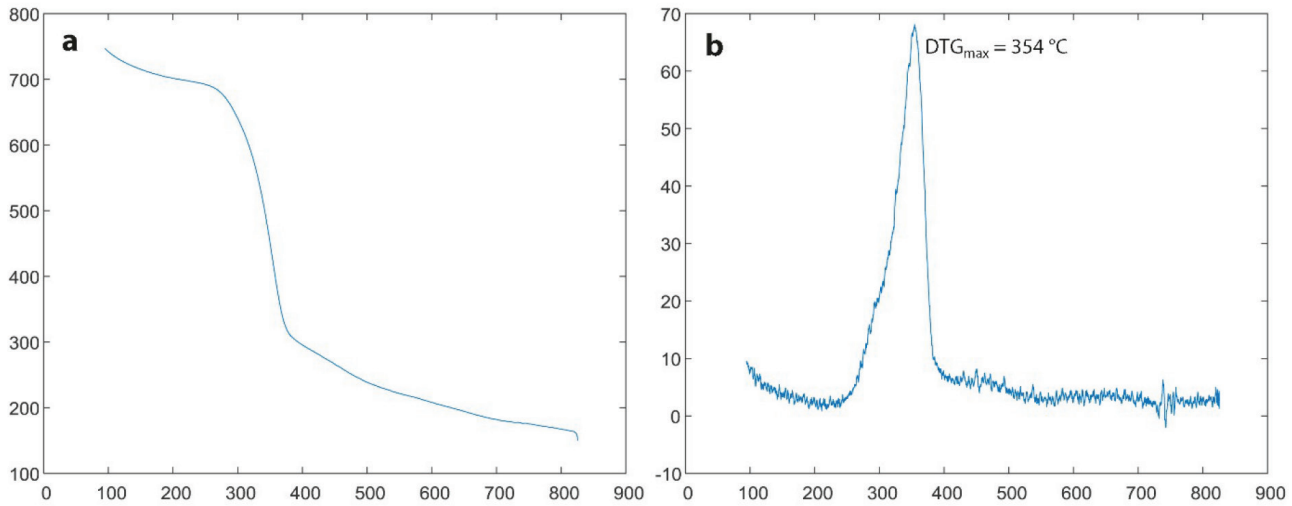


**Figure 4.** (a) Thermogravimetric (TG) and (b) derivative thermogravimetric (DTG) curves of chitin isolated from *Peripatoides novaezealandiae*.

analysis may yield different results that most likely can be attributed to the chitin extraction method. For example, Zhang et al. (2000), using X-ray diffraction, reported 56% crystallinity of α-chitin from the shrimp cuticle, whereas Liu et al. (2012) recorded a much higher crystallinity (89%) from the same source. In the first example, chitin was extracted with mineral acid (1 M HCl at 100 °C for 20 min), alkali solution (1 M NaOH at 80 °C for 24 h), and extra deproteinization (0.4% $Na_2CO_3$). In the second example, chitin was extracted with mineral acid (1 M HCl at 100 °C for 30 min), alkali solution (1 M NaOH at 80 °C for 24 h), and decolorization (1% $KMnO_4$ for 1 h). Thus, the same but differently processed chitin source gave different crystalline chitin samples, whereas FT-IR spectra showed that both chitin isolates had the same allomorphic structure (α-chitin). This means that results obtained by X-ray diffraction should be reexamined with different analytical approaches, such as FT-IR. Recently, we did this with the cuticle pentastomids, which were suggested to contain β-chitin after X-ray diffraction (Karuppaswamy, 1977), but actually contained α-chitin (Greven et al., 2019).

In α-chitin, the piles of polysaccharide chains are disposed in an antiparallel fashion. This leads to strong inter- and intrasheet hydrogen bonding, whereas the sheets of β-chitin adopt a parallel fashion, and a relatively weak intrasheet hydrogen bonding network is formed among the chains. Therefore, some differences can be observed in the infrared spectra of α- and β-chitin. Many characteristic bands resemble one another in the infrared spectra of α- and β-chitin, but some bands, primarily amide I, II, and III bands, allow the distinction between the two polymorphic crystalline structures. These bands are responsible for C=O secondary amide stretch, N–H bend, C–N stretch, and $CH_2$ wagging, respectively (Jang et al., 2004; Kumirska et al., 2010). The divided and sharp amide I band is especially characteristic of α-chitin, while the undivided and broad amide I band indicates β-chitin. The lower degree of crystallinity of β-chitin is indicated by the U-shaped amide band I, whereas the amide band I of α-chitin is V-shaped due to its higher crystallinity (e.g., Jang et al., 2004; Kumirska et al., 2010).

Thermogravimetry represents an additional method to discriminate chitin allomorphs (Jang et al., 2004), with which the maximum degradation temperature (DTGmax) of chitin samples is determined. The relatively high value of more than 350 °C shown for the chitin of *P. novaezealandiae* further supports the presence of α-chitin, which is more robust than other chitin allomorphs due to the above mentioned strong inter- and intrasheet hydrogen bonding (Jang et al., 2004).

### 4.3. Conclusions

Our study demonstrates more clearly and more specifically than before the presence of α-chitin in the cuticle of a velvet worm. As in the arthropod cuticle, its properties, such as stiffness and strength, surely depend on the chitin nanofibres, the type of proteins, the water content, and the interaction of the proteins with the chitin (see Neville, 1973; Vincent and Wegst, 2004). The material properties of the onychophoran cuticle that can be considered as a composite material (as the [pan]arthropod cuticle in general) are to our knowledge not yet explored.

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