Research paper

New family and genus of a *Dendrilla*-like sponge with characters of Verongiida. Part II. Discovery of chitin in the skeleton of *Ernstilla lacunosa*

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**Abstract**

Evolution and multi-functionality of the chitin system in sponges (Porifera) is currently a prime focus in modern experimental zoology including in phylogenetic studies of diverse poriferan taxa. Here, we present the first evidence of chitin in the skeleton of the demosponge originally defined as *Dendrilla lacunosa* (Porifera: Dendroceratida: Keratosa). The presence of chitin has been clearly confirmed using special Calcofluor white staining, Fourier-transform Infrared (FTIR) and Raman Spectroscopy, electrospray ionization mass spectrometry (ESI-MS), as well as a chitinase digestion assay. This feature is, however, unusual for Keratosa sponges with skeletons usually made of structural protein — spongion, and at the same time is typical for sponges of the Verongiida (subclass Verongimorpha). Consequently, obtained bioanalytical results, together with our findings about distinctive morphological and molecular characters of this Dendrilla-like sponge confirmed the necessity of renaming this organism as a novel species *Ernstilla laculosa* in the new family Ernstillidae. This study is the first example of the impact of the reporting of chitin on the systematics of sponges.

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**1. Introduction**

Nearly 8600 species of sponges (Porifera) exist today in different oceans and seas worldwide and belonging to four classes: Hexactinellida, Calcarea, Homoscleromorpha and Demospongiae (Van Soest et al. 2012). The Demospongiae includes about 83% of recent described sponges and is represented by three subclasses: Verongimorpha, Keratosa and Heteroscleromorpha (Moron & Cardenas 2015). Prior to 2007, it was recognized that organic skeletons of all demosponges contain a keratin-like structural protein called spongion (see for review Jesionowski et al. 2018). However, in 2007 this belief changed with the discovery of an aminopolysaccharide scaffold, chitin, as the main organic skeletal component within the skeletons of the Verongiida (Ehrlich et al. 2007a) and selected other sponges (Ehrlich et al. 2007b), all within the Demospongiae. In the last 10 years,
chitin has been identified in skeletal structures of diverse representatives of marine (see for review Ziółowska-Aksamitowska et al. 2018a,b) and fresh water (Ehrlich et al. 2013a,b) demosponges. However, only members of the Verongiida were shown to develop three dimensional chitinous scaffolds that closely resemble the shape of their sponge skeletons (Brunner et al. 2009; Ehrlich et al. 2010a; Cruz-Barraza et al. 2012; Wysokowski et al. 2013a). This feature has been effectively used for practical applications such as scaffolding material in tissue engineering of human mesenchymal stromal cells (Mutsenko et al. 2017a,b), and for development of novel composite materials using the Extreme Biomimetics route (Wysokowski et al. 2013b,c, 2015; Petrenko et al. 2017). Intriguingly, this structural feature has been seen for the first time in a marine demosponge previously known as Dendrilla lacunosa Hentschel, 1912 (for details see Part I) (Vacelet et al. 2019) which originally was classified in the order Dendroceratida of the subclass Keratosa. All representatives of Dendroceratida studied to date contain spongin-based skeletons which are not resistant to alkali treatment (Jesionowski et al. 2018), in contrast to the chitin-based skeletons of verongiids. The classical method of isolation of chitin from sponges is based on the treatment of their cell-free skeletons with a solution of 2.5 M NaOH at 37°C (Ehrlich et al. 2007a). Thus, preliminary studies carried out on dried fragments of this sponge (Fig. 1a) showed that cell-free skeletons (Fig. 1b) remain, and are stable, after alkali treatment and the resulting skeletons (Fig. 1c) resemble in shape and consistency the skeletons found in the order Verongiida. Alkali-based depigmentation of the skeleton results in a reddish coloured bromine-containing extract (Fig. 1d), a phenomenon observed mainly in members of the Verongiida (Ehrlich et al. 2007a).

These results, together with our findings in Part I (Vacelet et al. 2019) about distinctive morphological and molecular characters of this sponge, inspired us to investigate the chemical nature of the organic phase of the skeleton of the Dendrilla-like sponge renamed as Ernstilla lacunosa in the new family Ernstillidae (Part I).

To date, there are no reports or even attempts to isolate and identify chitin from any member of Dendrilla-like sponges, which could in some cases be confused with verongiid sponges. Consequently, here we represent the first bioanalytical study on the sponge Ernstilla lacunosa.

2. Materials and methods

2.1. Location and collection

The specimen of the sponge Ernstilla lacunosa, WAM Z95153, was collected by benthic sled in the Kimberley region of Western Australia in March 2016 at a depth of 55.6 m and wet frozen.

2.2. Isolation of chitinous skeleton from the sponge

The isolation of a chitin-based skeleton from E. lacunosa was carried out using techniques previously described in Ehrlich et al.
The protocol includes sequential steps (Fig. 2) starting with the storage of selected fragments of the sponge skeleton in deionized water at room temperature for 24 h in order to remove salts. Afterwards, the samples were treated with 3 M HCl at room temperature twice in 6 h in order to remove calcium carbonates from the skeleton. The decalcified samples were washed several times with deionized water until achieving a pH of 6.5. At the next step, these samples were then treated with 2.5 M NaOH at 37 °C for 24 h to remove pigments and proteins. The residual siliceous phase has been removed after alkali treatment for an additional 72 h.

The influence of alkaline and acidic treatments on the structure of the skeleton of *E. lacunosa* was investigated using stereo, white light and fluorescence microscopy as well as scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX). Finally, the isolated material was washed several times with deionized water to a pH of 6.5. The resulting translucent fibrous material (Fig. 3) was placed into a 500 ml large GLS 80 Duran glass bottle containing deionized water and stored at 4 °C for further analyses.

2.3. Light and fluorescent microscopy imaging

Collected sponge samples and isolated chitinous scaffolds were observed using a BZ-9000 microscope (Keyence) in light as well as fluorescent microscopy.

2.4. Scanning electron microscopy

The surface morphology and microstructure of isolated chitinous scaffolds as well as untreated samples of *E. lacunosa* have been investigated from SEM images obtained using an ESEM XL 30 Philips scanning electron microscope. Prior to analysis, samples were covered with a carbon layer for one minute using an Edwards S150B sputter coater.

2.5. Fourier-transformation infrared spectroscopy

Transmission spectra of chitinous scaffolds were made using a Nicolet 210c FTIR spectrometer. The samples were analysed using an attenuated total reflectance (ATR) system.

**Fig. 2.** Schematic view: step-by-step isolation of chitinous fibers from *E. lacunosa*.

**Fig. 3.** Photograph of the purified skeletal fibres of *E. lacunosa*, which have been identified as α-chitin.
2.6. Raman spectroscopy

Raman spectroscopy was performed using a Raman spectrometer (RamanRxn17™, Kaiser Optical Systems Inc., Ann Arbor, USA) coupled to a light microscope (DM2500 P, Leica Microsystems GmbH, Wetzlar, Germany).

The sample displayed intense fluorescence, which made the acquisition of a high quality Raman spectrum impossible. Consequently, the samples were bleached in a 10% solution of hydrogen peroxide for 3 h followed by washing in distilled water and final drying at room temperature. The Raman spectra were then acquired using an accumulation time of 3 s and summing up to 50 accumulations. Finally, a baseline correction was applied in Matlab to remove the residual fluorescence signal from the spectra and display the Raman scattering.

2.7. Chitinase digestion test

In order to carry out a chitinase digestion test, the Yatalase® enzyme from culture supernatants of Corynebacterium sp. OZ-21 (Cosmo Bio, Japan) was used. One unit of this enzyme released 1 μmol of p-nitrophenyl-N-acetylglucosamine from 0.5% chitin solution and 1 μmol of p-nitrophenol from p-nitrophenyl-N-acetyl-β-D-glucosaminide solution for 1 min at 37 °C and pH 6.0. The completely demineralized fibers of E. lacunosa (Fig. 3) were incubated in an enzyme solution containing 10 mg Yatalase dissolved in 1 mL of citrate phosphate buffer at pH 5.0 for 2 h. The effectiveness of the enzymatic digestion was monitored using an optical microscope (Keyence).

2.8. Estimation of N-acetyl-D-glucosamine (NAG) contents (electrospray ionization mass spectrometry ESI-MS)

The Morgan–Elson assay was used to estimate the N-acetyl-D-glucosamine content released after chitinase treatment, previously described in detail in Ehrlich et al., 2007a,b, 2010a.

Preparation of the samples for ESI-MS was as follows: the demineralized organic matrix of E. lacunosa (Fig. 3) was hydrolysed in 6 M HCl at 50 °C for 24 h. After hydrolysis, the samples were filtered on a 0.4 μm filter and freeze-dried to remove any excess of HCl. The solid samples were dissolved in deionized water for analysis. All ESI-MS measurements were performed on Waters TQ Detector ACQUITY® Uplc mass spectrometer (Waters, USA) equipped with ACQUITY® Uplc pump (Waters, USA) and BEH18 1.7 mm 2.1 × 50 mm UPLC column. Nitrogen was used as the nebulizing and desolvation gas. Graphs were generated using Origin 8.5 for PC.

2.9. Calcofluor White (CFW) staining

In order to evaluate the localization of chitin in the demineralized skeletal fibres of E. lacunosa, Calcofluor White (Fluorescent Brightener M2R, Sigma) was used because it exhibits enhanced fluorescence when linked to chitin (Ehrlich et al. 2007 a, b; Brunner et al. 2009; Wysokowski et al. 2013a). Selected fragments of demineralized fibres of E. lacunosa were placed in 0.1 M KOH-glycerine-water solution, then a few drops of 0.1% solutions of CFW were added and the mixture was placed in darkness for 60 min. Subsequently, the stained fibres were rinsed 5 times with deionized water and dried at room temperature. The resulting materials were investigated using fluorescence microscopy.

3. Results

Figs. 1c and 3 clearly indicate that the chemical treatments in this study (detailed in Fig. 2) lead to isolation of fibrous material from the skeleton of E. lacunosa that has a well-organized anastomosing morphology.

SEM microphotographs of isolated skeletal fibers of E. lacunosa prior to any treatments confirmed the complex chemical character of its skeleton, where inorganic (Ca, Mg, Si) and organic (Br, S, C) elements can be well identified using EDS (Fig. 4). The resulting data were found to be similar to that reported previously for verongiids species such as Verongula gianteana (Hyatt, 1875), Aplysina cavernicola (Vacelet, 1959) and A. caullimaris (Carter, 1882) (Ehrlich et al., 2010b). The verongiid sponge Suberea clavata (Pulitzer-Finali 1982) has been found to be heavily mineralized with respect to both calcium carbonates and silica in the form of mineral containing structural layers (Ehrlich et al. 2017).

The Calcofluor white staining is generally used as a fluorescent dye for staining of β-(1→3) and β-(1→4) linked polysaccharides including chitin, in cases where cellulose and β-glycans can be excluded. As a result, on binding to polysaccharides such as chitin, this fluorochrome emits a bright blue light under UV excitation. Fluorescence microscopy analysis of the skeletal fibers isolated from E. lacunosa after CFW staining display very strong fluorescence even under light exposure times as short as 1/4000 s (Fig. 5). Similar results have been previously reported for chitin of poriferan origin (Ehrlich et al. 2007a, 2010a, b, 2017).

To confirm the presence of chitin in the isolated scaffolds (Figs. 1c, 3 and 5a, b), more powerful and sensitive analytical staining techniques such as FTIR and Raman spectroscopy were applied. Both methods also allowed for the determination of the chitin isoforms (alpha-, beta-, or gamma) (Kaya et al. 2017). FTIR spectra of the isolated fibrous material along with an α-chitin reference are presented in Fig. 6. The peak of the amide band I as well as the characteristic intense band νmax 949 cm⁻¹ are assigned to γCH₃ which were observed in both the α-chitin standard and the isolated sponge chitin samples. Additionally, the α-chitin indicative band assigned to a β-glycosidic bond is observed at νmax 898 cm⁻¹ in FTIR spectra from chitin from E. lacunosa (Fig. 6). Examination results of Raman spectroscopy showed that spectra of chitin from the sponge match the acquired spectrum for the α-chitin reference (see Fig. 7). Similar observations have been reported previously for chitin of verongiid origin (Ehrlich et al. 2007a, 2010a; Brunner et al. 2009; Wysokowski et al. 2013a).

Chitinases are able to hydrolyse chitin directly to low molecular weight chitin oligomers including N-acetylglucosamine (GlcNAc). Consequently, such enzymatic treatment results in the loss of chitin integrity and in release of residual chitin microfibers of steadily decreasing size, these are observable with an optical microscope (Fig. 8). The chitinase digestion test, used to detect chitin in sponges in other studies (Ehrlich et al. 2007a, 2010a, b; Brunner et al. 2009; Wysokowski et al. 2013a; Ziółowska-Aksamitowska et al. 2018a,b; Klinger et al. 2019), confirmed the chitinous nature of the demineralized skeletal fibers isolated from E. lacunosa.

The Morgan–Elson assay has been widely used and is considered as the most accurate method to estimate the amount of GlcNAc released after chitinase treatment (Ehrlich et al. 2013a,b). Determination of GlcNAc in chitin-based fibers of E. lacunosa showed 950 ± 1.5 μg of N-acetyl-glucosamine per mg of chitinous material for this sponge (95% yield). ESI-MS of N-glucosamine (GlcN) standard shows three ion peaks at m/z = 162.08, 180.09 and 359.16. The ion peak at m/z = 180.09 corresponds to the molecular ion peak [M+H]⁺ of a species with a molecular weight 179.09 which is GlcN (calculated: 179.1). The ion peak at m/z = 162.08 corresponds to a fragment [M-H₂O+H]⁺ where GlcN has lost one water molecule (calculated: 162.1), which is very common for this type of molecule. The ion peak at m/z = 359.16 is equivalent to [2M + H]⁺ species, which is a proton-bound GlcN non covalent dimmer.
ESI-MS of the sample hydrolysis revealed many signals. Among these the major signals corresponded to GlcN and its derivatives (m/z = 162.08, 180.09 and 359.12). Other strong signals indicated species with m/z = 341.16 and 502.22, which are common for MS analyses of chitosan hydrolysis products (Ehrlich et al. 2013c). These latter products correspond to the unhydrolyzed oligosaccharides in which the species at m/z = 341.16, 502.22 are equivalent to the disaccharide [(GlcN)\textsuperscript{2} + H\textsuperscript{+}] and trisaccharide [(GlcN)\textsuperscript{3} + H\textsuperscript{+}] (Fig. 9) respectively.

4. Discussion

Demosponges of the genus *Dendrilla* are known as producers of secondary metabolites with antiviral, antibiotic, cytotoxic, anti-inflammatory and antitumor activities. Examples of such biologically active compounds include the furanosesquiterpenoid pallecensone (Cambie et al. 1987), diverse dendrillolide (Bobzin & Faulkner 1989; Fontana et al. 1997), membranolide diterpenes (Ankisetty et al. 2004), terpene dihydrogracilin A (Ciaglia et al. 2017), lamellarins (Hooper & Hobbs 1995; Liu et al. 2007; Reeder et al. 2008; Imbri et al. 2014) and darwinolide (Von Salm et al. 2016) which were isolated from different members of *Dendrilla* collected from diverse geographic localities including tropical and Antarctic waters. Interestingly, only in one instance has bromotyrosine-related bastadin alkaloids been reported in this genus, from *Dendrilla cactos* (Reddy et al. 2006). It is possible that presence of bromotyrosine compounds could be an indication that *D. cactos* or at least the species identified as such by Reddy et al. 2006, is in fact a verongiid.

Evolution and multi-functionality of the chitin system (Wagner 1994) in nature remain an intriguing question also in the case of sponges. The identification of potential chitin synthesis among representatives of the genus *Dendrilla* should gain importance as a result of our findings. The evolution, localization and functions of chitin in other *Dendrilla*-like demosponges as well as in other representatives of the genus *Ernstila* should be examined in future. Additionally, separate studies should be carried out on the identification of chitin synthase genes within genomes of diverse representatives of these genera. Further investigations are essential to obtain a better understanding of the nature and origin of skeletons of these demosponges with respect to the spongine-chitin relationship. It is still unclear how much spongine is present in the chitin-based skeletons of sponges studied to date. Novel approaches should be sought which will bring together molecular
Fig. 5. Dried chitinous fibers of *E. lacunosa* showed spicules-free morphology well visible under SEM (a) and light (b) microscopy. It is additionally confirmed using fluorescence microscopy after specific Calcofluor white staining for chitin (c). (Light exposure time 1/4800 s).

Fig. 6. FTIR analysis of purified chitinous fibers isolated from *E. lacunosa*. 
Fig. 7. Identification of alpha-chitin within chitinous fibers of *E. lacunosa* using Raman spectroscopy.
biology, experimental zoology and modern bioanalytical methods for a better understanding of poriferan chitin synthesis in diverse taxa at a molecular level.

Authors contribution

H.E., J.V., J.F., C.D., D.E. and S.Z.A. designed the study protocol and wrote the manuscript; J.F. collected the sponge materials; D.Y., R.G., and I.P. prepared samples and performed chemical characterization of chitin from *Ernstilla laculosa*; S.Z.A., H.M and I.P. conducted SEM and other microscopy investigations and analysed the data. All the authors critically reviewed and approved the final version of the manuscript.

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