Predation drives convergent evolution of the thick and baggy cuticle in nematodes

Kanata ICHIISHI 1, Taisuke EKINO 1, Natsumi KANZAKI 2 and Ryoji SHINYA 1,*

1 School of Agriculture, Meiji University, Kawasaki, Kanagawa 214-8571, Japan
2 Kansai Research Center, Forestry and Forest Products Research Institute, Fushimi, Kyoto 612-0855, Japan

ORCID iD: Shinya: 0000-0002-2450-3054

Received: 14 July 2022; revised: 22 August 2022
Accepted for publication: 24 August 2022; published online: 28 September 2022

Summary – The nematode cuticle is an important structure that provides protection from abiotic environmental stresses and natural enemies. The cuticle ultrastructure of a Myolaimus species (culture code NKZ384) isolated from Kyoto, Japan, was examined in relation to its avoidance of predation by an aphelenchoidid predator, Seinura caverna. The survivability of Myolaimus sp. co-cultured with the predator was examined and compared with those of four Poikilolaimus spp. previously reported by the present authors. Myolaimus and two of the four Poikilolaimus spp. share a ‘baggy’ cuticle and resisted predation effectively. However, the ultrastructure differed between these two genera: i.e., the cuticle of Myolaimus sp. is seven-zoned, while that of P. regenfussi and P. oxycercus is five-zoned. In addition, Myolaimus sp. does not possess the characteristic osmophilic zone reported in Poikilolaimus spp. Therefore, although the ultrastructure differs, the thick and baggy cuticle found in these two phylogenetically distant genera exhibits functional convergence to resist predation.

Keywords – anti-predatory mechanism, convergent evolution, cuticle ultrastructure, Myolaimus, Seinura caverna, transmission electron microscopy.

The nematode cuticle, a body surface consisting of collagenous protein material, has received much attention due to its multi-functionality and structural diversity (Decraemer et al., 2003; Page & Johnstone, 2007). Protection of the nematode body from environmental stresses is an essential function of the cuticle. The cuticle is the first organ to encounter a variety of fatal stressors, such as toxic material, desiccation, osmotic stress and predator attacks (Manton & Ramsay, 1937; Hadley, 1984; Wright, 1989). Each nematode species appears to have evolved a unique cuticle structure for dealing with the stresses existing in its surrounding environment.

We previously reported that Poikilolaimus oxycercus and P. regenfussi possess a thick and unique cuticle structure: i.e., the cuticles contain an ‘osmophilic median zone’ that is not observed in their congener, P. floriden-sis and P. carsiops (Ichiishi et al., 2021). The thickness of the osmophilic median zone and total cuticle thickness are both positively correlated with survival in the presence of an aphelenchoidid predator, Seinura caverna, which preys upon nematodes using a stylet. To date, this relationship between a thick cuticle and an anti-predatory function has been reported only in the genus Poikilolaimus. To confirm that the characteristic cuticles were independently acquired as an anti-predatory mechanism in an evolutionary context, i.e., convergent evolution, we must explore whether other nematode species, e.g., Myolaimus, Delaia, Diphtherophora and Trichodoridae, which have a baggy cuticle, with a similar cuticle structure also experience high survival rates following exposure to predator nematode species (Nedelchev & Choleva, 1989; Deacrae-mer & Robertson, 1998; Holovachov & Boström, 2006; Slos et al., 2018). Here, we focus on the genus Myolaimus (suborder Myolaimina), which is phylogenetically distinct from the genus Poikilolaimus (family Rhabditidae) (Meldal et al., 2007). Microscopic observations revealed that Myolaimus possesses a baggy cuticle (Slos et al., 2018). Moreover, Giblin-Davis et al. (2010) observed a rough cuticle ultrastructure in M. byersi using transmission electron microscopy (TEM). These studies revealed that M. byersi exhibits a dissociation or dissolution of the median zone of the cuticle. In terms of anti-predator
mechanisms, Fürst von Lieven (2008) reported that a diplogastrid predatory nematode, which preys upon nematodes using a tooth, was unable successfully to depredate first- and second-stage juveniles of *Myolaimus* sp. Therefore, *Myolaimus* can serve as an appropriate comparative group to confirm the relationship between cuticle ultrastructure and anti-predator ability in an evolutionary context. However, no information currently exists regarding the detailed cuticle ultrastructure of *Myolaimus* spp. or their survival rates in the presence of aphelenchoidid predatory species. In the present study, we examined the cuticle ultrastructure of *Myolaimus* sp. NKZ384 and determined the thickness of each cuticle zone using TEM. We then assessed the survival of *Myolaimus* sp. NKZ384 following exposure to a predatory nematode, *S. caverna*.

**Materials and methods**

**Nematode strains**

We used *Myolaimus* sp. NKZ384 that had been isolated from dead wood of an unidentified species of broad-leaved tree in Kyoto, Japan, and subsequently maintained in the laboratory. *Myolaimus* sp. NKZ384 was cultured on *Escherichia coli* OP50 in nematode growth medium with 4% agar at 25°C. We used adult females of *Myolaimus* sp. NKZ384 in all experiments.

The predatory nematode *S. caverna* was cultivated using the method described by Kanzaki et al. (2019). Briefly, *Acrobeloides* sp. NKZ393 was first propagated on a nematode growth medium agar plate as a food source for *S. caverna*. Then, *S. caverna* was inoculated onto the same plate. After incubation for several days at 20°C, adult hermaphrodites of *S. caverna* were retrieved using platinum wire and used for each experiment.

**Cuticle ultrastructure observations**

We prepared samples for TEM following the method of Ekino et al. (2017), with minor modifications. *Myolaimus* sp. NKZ384 was first fixed in a solution of 4% paraformaldehyde and 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for at least 24 h. Following Saikawa (1999), we then embedded the fixed nematodes in a 2% agarose pad and arranged them in a parallel array on a 2% agarose pad placed on a microscope slide (Bargmann & Avery, 1995). We then dripped 2% agarose liquid onto the pad. After the agarose had solidified, we trimmed it to a cube shape containing five nematodes. After rinsing in phosphate buffer (six times, 10 min each), the cubes were post-fixed in 1% osmium tetroxide for 90 min in phosphate buffer. The fixed nematodes were then dehydrated in a graded ethanol series (50, 70, 80, 90% and three times with 99.5%). Next, they were cleaned with propylene oxide (three times, 10 min each) and left to infiltrate overnight in a mixture of 50% Eponate resin and 50% propylene oxide, followed by undiluted resin. Finally, the nematodes were embedded in Eponate resin. Their midbody regions were sectioned using a diamond knife in an ultramicrotome. Sections were collected on copper grids for electron microscopy. The grids were stained with EM Stainer (Niisshin EM) for 30 min, followed by lead citrate for 3 min. Grid-mounted sections were examined and photographed at 100 kV using the JEM-2010EX and JEM-1400Plus (JEOL) transmission electron microscope. We took measurements of all cuticle zones that were clearly visible using the photographs obtained from the microscope. The thickness of each zone and the total thickness of the cuticle were measured using ImageJ software (Rasband, 2014) (https://imagej.nih.gov/ij/). To compare the cuticular measurements with the four *Poikilolaimus* spp., we used the data from Ichishi et al. (2021). Cuticle zones were determined following Bird (1980, 1984) and Jones et al. (1993).

**Survival assay of *Myolaimus* sp. following exposure to *S. caverna***

We conducted this assay following the methods of Ichishi et al. (2021), with minor modifications. We assessed the survival rates of *Myolaimus* sp. NKZ384 following exposure to *S. caverna*. *Myolaimus* sp. NKZ384 and *S. caverna* were rinsed in M9 buffer and ion-exchanged water, respectively. We placed equidistantly six drops of sterile distilled water (50 μl) on a 60 mm diam. Petri dish with 4% water agar. We then selected 30 individuals of *Myolaimus* sp. NKZ384 adult females and 30 starved hermaphrodites of *S. caverna*. We transferred ten individuals of *Myolaimus* sp. NKZ384 to each of three sterile distilled water drops and ten individuals each of *S. caverna* to the other three water drops. As a control, we assessed the survival rates of the prey nematodes in the absence of the predator. We also compared the survival rates of *Myolaimus* sp. NKZ384 with those of *P. floridensis* and *P. carpio* that possess thin cuticles, and *P. regenfussi* and *P. oxy cercus* that possess thick cuticles, published in Ichishi et al. (2021). Following incubation at 25°C for 24 h, the numbers of living and dead nematodes were determined using a stereomicroscope (SZX, Olympus). We conducted the assay in triplicate.
Predation drives convergent evolution of cuticle form

Statistical differences in survival rates among species after exposure to predators were examined using Welch’s t-test with Bonferroni-corrected P values in Microsoft Excel (Microsoft Office 2019).

Results

CUTICLE ULTRASTRUCTURE

The cuticle ultrastructure of Myolaimus sp. NKZ384 consisted of seven zones that could be grouped into the epicuticle, cortical zone, median zone and basal zone (Fig. 1). The epicuticle (L1) consisted of an electron-dense outermost layer (surface coat) and double inner layers. The cortical zone (L2) was an electron-dense zone under the epicuticle. The median zone was divided into three zones: the elastic zone (EZ), an electron-lucent zone; L4, an electron-dense zone; and L5, an electron-lucent zone compared with L4. The basal zone was divided into two zones: L6, a radially striated zone, and L7, an electron-lucent zone similar to L5. The unspecified position was thick in the EL, and the overall cuticle was baggy (Fig. 2). Measurements of cuticle thickness and of each zone are shown in Table 1.

SURVIVABILITY OF MYOLAIMUS SP.

Myolaimus sp. NKZ384 exhibited an average survival of > 78% in the predator treatment. In the absence of predators, 97% of Myolaimus sp. NKZ384 survived. The survival of Myolaimus sp. NKZ384 did not differ significantly (P > 0.05) in the presence vs absence of S. caverna (Fig. 3).

Discussion

In a previous study, we demonstrated that nematode cuticle structure differs among four Poikilolaimus spp. Of these four species, two possessed a thick cuticle with an osmophilic zone, while this zone was absent in the other two species. The former two species resisted S. caverna predation more effectively compared with the latter two species (Ichiishi et al., 2021). To determine whether these traits are unique features of Poikilolaimus spp. or whether they are more common among nematodes with thick and baggy cuticles, we investigated the cuticle ultrastructure and thickness of Myolaimus sp. NKZ384, which is phylogenetically distant from Poikilolaimus spp.

Our findings revealed substantial differences in the cuticle zone ultrastructure between the two genera Myolaimus and Poikilolaimus. Myolaimus did not possess an osmophilic median zone, which has been suggested to function in predator avoidance in Poikilolaimus spp. Our observations indicate that the osmophilic median zone is not essential for resisting predation in nematodes, although it may serve an important anti-predatory role in Poikilolaimus spp. The osmophilic median zone is electron-dense and combines readily with osmium oxide, which oxidises unsaturated fatty acids with a double bond (Ioannou et al., 2017). This particular zone was found only in Poikilolaimus, but a similar electron-dense zone was found in unhatched J2 of Globodera rostochiensis, which is a plant-parasitic nematode and phylogenetically distinct from the genus Poikilolaimus (Jones et al., 1993).

The thickness of the cuticle of Myolaimus sp. NKZ384 adult females (0.803 ± 0.028 μm) was comparable with
Fig. 2. A: Swollen elastic zone (EZ). Scale bar = 500 nm; B: Baggy cuticle of *Myolaimus* sp. NKZ384. Arrows indicate the baggy portion. EZ = elastic zone. Scale bar = 5 μm.
Table 1. Measurements of total cuticle thickness and the thickness of each zone in *Myolaimus* sp. NKZ384 and four species of *Poikilolaimus*.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Cuticle thickness (μm)</th>
<th>EPI + CZ (μm)</th>
<th>MZ (μm)</th>
<th>BZ (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Myolaimus</em> sp. adult female</td>
<td>5</td>
<td>0.803 ± 0.0280&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.219 ± 0.0064</td>
<td>0.435 ± 0.0135</td>
<td>0.138 ± 0.0031</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.635–1.05)</td>
<td>(0.098–0.327)</td>
<td>(0.297–0.663)</td>
<td>(0.108–0.187)</td>
</tr>
<tr>
<td><em>P. regenfussi</em> adult hermaphrodite</td>
<td>5</td>
<td>0.762 ± 0.0280&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.228 ± 0.0038</td>
<td>0.405 ± 0.0073</td>
<td>0.118 ± 0.0028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.687–0.846)</td>
<td>(0.183–0.292)</td>
<td>(0.272–0.489)</td>
<td>(0.105–0.133)</td>
</tr>
<tr>
<td><em>P. oxy cercus</em> adult female</td>
<td>5</td>
<td>1.232 ± 0.0337&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.443 ± 0.0114</td>
<td>0.635 ± 0.0168</td>
<td>0.155 ± 0.0028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.118–1.323)</td>
<td>(0.297–0.655)</td>
<td>(0.470–0.965)</td>
<td>(0.140–0.169)</td>
</tr>
<tr>
<td><em>P. floridensis</em> adult female</td>
<td>5</td>
<td>0.242 ± 0.0108</td>
<td>0.147 ± 0.0055</td>
<td>Absent</td>
<td>0.080 ± 0.0042</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.204–0.265)</td>
<td>(0.116–0.170)</td>
<td></td>
<td>(0.056–0.099)</td>
</tr>
<tr>
<td><em>P. carsiops</em> adult female</td>
<td>5</td>
<td>0.483 ± 0.0326</td>
<td>0.260 ± 0.0081</td>
<td>Absent</td>
<td>0.105 ± 0.0025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.376–0.567)</td>
<td>(0.252–0.356)</td>
<td></td>
<td>(0.097–0.323)</td>
</tr>
</tbody>
</table>

The data of four species of *Poikilolaimus* presented here are reproduced from our previous study (Ichiishi *et al.*, 2021) and used for comparisons. All numerical values are reported as the mean ± standard error (range). In *Myolaimus*, EPI (L1) = epicuticle; CZ (L2) = cortical zone; MZ (EZ–L5) = median zone; BZ (L6–L7) = basal zone. In *Poikilolaimus*, EPI = epicuticle; CZ = cortical zone; MZ (osmophilic zone–electron-lucent median zone) = median zone; BZ = basal zone. Different letters (a, b) in each column indicate significant differences as $P < 0.0000001$ according to the Tukey-Kramer test.
that of adult females of *P. regenfussi* (0.762 ± 0.0280 μm) and *P. oxy cercus* (1.232 ± 0.0337 μm) (Ichiishi et al., 2021). Although cuticle thickness did not differ significantly (*P* > 0.05) between *Myolaimus* and *P. regenfussi* females, *Myolaimus* and *P. oxy cercus* females did exhibit significant differences (*P* < 0.0000001) in cuticle thickness (Tukey-Kramer test). In a previous study, we reported a strong positive correlation between cuticle thickness and survival; thus, cuticle thickness may play an important anti-predator role in both *Myolaimus* and *Poikilolaimus*. Given that these two genera are phylogenetically distinct, and that many nematode species of both genera lack a thick cuticle, the two species must have evolved thick cuticles independently. To reveal the actual function of these thick cuticles, additional studies are necessary using *Caenorhabditis elegans* mutants that have thick cuticles, such as a Bli-2 mutant (Moribe et al., 2004).

In addition to the thickness of the cuticle, the number of cuticle zones may also be important in resisting predation. This study demonstrated that the cuticle of *Myolaimus* sp. NKZ384 is composed of seven zones, which is the largest number of zones reported among rhabditid nematodes to date (Decraemer et al., 2003). For example, the cuticle of the free-living nematode *C. elegans* is composed of four zones (Cox et al., 1981). Among *Poikilolaimus* nematodes, *P. regenfussi* and *P. oxy cercus* have five-zoned cuticles, while the cuticles of *P. floridensis* and *P. carsiops* are three-zoned (Ichiishi et al., 2021). We propose two possible interpretations of the role of a multi-layered cuticle. First, a multi-layered cuticle is widely useful for resisting predation in nematodes. In *Poikilolaimus* spp., the number of cuticle zones was higher in the species more resistant to predation (*P. regenfussi* and *P. oxy cercus*) than in those more susceptible to predation. However, this cannot fully explain the role of a multi-layered cuticle because some nematode species with a four-zoned cuticle, such as *Aphe lenchus avenae* and *C. elegans*, were preyed upon by *Seinura caverna* at high frequencies (Johnson et al., 1970; Cox et al., 1981; Kanzaki et al., 2019). Moreover, though Trichoridae nematodes also possess the baggy and six-zoned cuticle, Trichoridae nematodes were preyed on by Dorylaimida predatory nematodes, which use odontology to catch the prey nematodes like *Seinura* (Khan et al., 1995; Decraemer & Robertson, 1998; Karanastasi et al., 2001). The second interpretation is that a multi-layered cuticle is useful only for resisting predation in *Myolaimus*; *Poikilolaimus* may primarily resist predation by other mechanisms, e.g., via the osmophilic zone. In this case, although the phenotype of predation resistance is similar between *Myolaimus* and *Poikilolaimus*, the underlying mechanisms would differ. In general, multi-zone structures, which include chemically different characteristic materials, are highly elastic and stiff (Hampson & Moatamedi, 2007; Ali et al., 2016). In a previous study, we suggested that the *Poikilolaimus* cuticle exhibits a sandwich structure, which is fabricated by attaching a stiff layer to a soft layer such as the osmophilic zone (Kim et al., 1999; Ichiishi et al., 2021). Although the osmophilic zone was absent in *Myolaimus*, and the physicochemical characteristics of the *Myolaimus* cuticle remain unclear, Giblin-Davis et al. (2010) reported that *Myolaimus* has a uniquely baggy cuticle that drapes loosely around moving animals. The present TEM observations demonstrated that *Myolaimus* sp. NKZ384 has an extremely thick and swollen EZ (Figs 1A; 2A). The high elasticity in the cuticle of *Myolaimus* is probably due to the unique EZ and could function to resist predation. When *Seinura* spp. prey upon other nematodes, they sting the prey to inject toxin(s) and digestive enzyme(s) into the pseudocoelom, an internal body structure (Hechler, 1963). During this feeding behaviour, the baggy cuticle may mechanically hinder the predator sting by its flexibility; i.e., when the
predator stylet penetrates the cuticle the prey can escape before the stylet reaches an internal structure. Therefore, although the cuticle structure differs between *Myolaimus* and *Poikilolaimus*, the structure may serve the same function to resist predation; i.e., functional convergence has occurred in these two phylogenetically distant genera.

Although remarkable differences were observed in the ultrastructure of the cuticles of *Myolaimus* sp. NKZ384 and *Poikilolaimus* spp., the two genera appear to have independently evolved a thick cuticle, which likely contributes to resisting predation by *S. caverna*. Because *Myolaimus* sp. NKZ384, *P. regenfussi* and *P. oxy cercus* have frequently been isolated from similar environments, *i.e.*, rotting wood (Holovachov & Boström, 2006), predation by predators existing within the same habitat (*e.g.*, predatory nematodes, predatory arthropods, and fungi) may have served as an evolutionary driving force leading to the convergent evolution of a thick cuticle and predator avoidance ability. In addition, other type of predators, *e.g.*, *Mononchoides*, which use large teeth to kill and feed on prey species, are also found in similar habitats (*e.g.*, Sudhaus & Fürst von Lievern, 2003), and *P. oxy cercus* exhibits relatively high survival when co-cultured with *Mononchoides* sp. (Kanzaki, unpubl.). Studies of the function of these cuticle structures will provide further insight into the survival strategy of nematodes with a thick and baggy cuticle.

**Acknowledgements**

The authors thank Drs Ryota Kose and Ryo Funata, Tokyo University of Agriculture and Technology, for use of TEM facilities. This study was funded by grants from JSPS Grant-in-Aid for Early-Career Scientists JP19K15853 (to RS), and JST FOREST Grant no. JPMJFR210A (to RS).

**References**


