RESEARCH ARTICLE

Identification and characterization of epicuticular proteins of nematodes sharing motifs with cuticular proteins of arthropods

Bruno Betschart1,*, Marco Bisoffi2, Ferial Alaeddine1

1 Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland, 2 Chemistry and Biochemistry, Schmid College of Science and Technology, Chapman University, Orange, California, United States of America

* These authors contributed equally to this work.
* bruno.betschart@unine.ch

Abstract

Specific collagens and insoluble proteins called cuticulins are major constituents of the nematode cuticles. The epicuticle, which forms the outermost electron-dense layer of the cuticle, is composed of another category of insoluble proteins called epicuticulins. It is distinct from the insoluble cuticulins localized in the cortical layer and the fibrous ribbon underneath lateral alae. Our objective was to identify and characterize genes and their encoded proteins forming the epicuticle. The combination between previously obtained laboratory results and recently made available data through the whole-genome shotgun contigs (WGS) and the transcriptome Shotgun Assembly (TSA) sequencing projects of *Ascaris suum* allowed us to identify the first epicuticulin gene, *Asu-epic-1*, on the chromosome VI. This gene is formed of exon1 (55 bp) and exon2 (1067 bp), separated by an intron of 1593 bp. Exon 2 is formed of tandem repeats (TR) whose number varies in different cDNA and genomic clones of *Asu-epic-1*. These variations could be due to slippage of the polymerases during DNA replication and RNA transcription leading to insertions and deletions (Indels). The deduced protein, *Asu-EPIC-1*, consists of a signal peptide of 20 amino acids followed by 353 amino acids composed of seven TR of 49 or 51 amino acids each. Three highly conserved tyrosine motifs characterize each repeat. The GYR motif is the Pfam motif PF02756 present in several cuticular proteins of arthropods. *Asu-EPIC-1* is an intrinsically disordered protein (IDP) containing seven predicted molecular recognition features (MoRFs). This type of protein undergoes a disorder-to-order transition upon binding protein partners. Three epicuticular sequences have been identified in *A. suum*, *Ascaris lumbricoides*, and *Toxocara canis*. Homologous epicuticular proteins were identified in over 50 other nematode species. The potential of this new category of proteins in forming the nematode cuticle through covalent interactions with other cuticular components, particularly with collagens, is discussed. Their localization in the outermost layer of the nematode body and their unique structure render them crucial candidates for biochemical and molecular interaction studies and targets for new biotechnological and biomedical applications.
New insoluble epicuticular proteins identified in nematode cuticles

Introduction

The phylum Nematoda, classified with Arthropoda in the clade Ecdysozoa, inhabits a broad range of environments. Nematodes are present in freshwater, marine, and diverse terrestrial environments accounting for about 80% of all animals [1]. Free-living species play an essential role in the decomposition of organic material. Other nematodes parasitize humans, vertebrate animals, or insects. Finally, nematodes can also be parasites of plants, causing significant crop losses [2]. Nematodes undergo up to four molts during their life cycle, thereby shedding the old cuticle and replacing it with a new one [3, 4]. The nematode cuticle is a complex extracellular structure whose morphology varies considerably between species and developmental stages [5]. The cuticle, acting as a protective exoskeleton, can be associated with glycoproteins and lipids [6–10]. Several glycoproteins are associated with a surface coat and are secretory products involved in the interaction of the nematodes with their environment [11, 12]. Other glycoproteins, for example, filariae’s 29 kDa glycoprotein (gp29), a homolog of the enzyme glutathione peroxidase (GSHPx), is considered intrinsic to the cuticle [13, 14]. Intrinsic proteins are synthesized in the hypodermal layer and secreted into the cuticle [9]. Gp29 could be involved in the defense against immune-mediated cytotoxicity or in catalyzing the formation of crosslinking residues, such as dityrosine, trityrosine, and isotrityrosine [15]. Such residues are present in cuticular collagens and insoluble proteins and shape the cuticle through inter- and intra-molecular crosslinks [10, 15, 16]. Genes coding for insoluble cuticular proteins were identified in several nematodes, i.e., *C. elegans* [17, 18], *Meloidogyne ariiellia* [19], *A. lumbricoidea* [20], *Brugia pahangi*, and *Brugia malayi* [21]. These genes were called cut-1 to cut-6. Cut-1 encodes a secreted protein of 423 amino acids with a zona pellucida (ZP) domain and is expressed in the dauer larva of *C. elegans* in a ribbon lying beneath the alae [22]. In filariae, CUT-1 is restricted to the median layer of the cuticles [21]. ZP domains are also identified in cuticins encoded by cut-3 to cut-6 [23]. In contrast, cut-2 of *C. elegans* has no ZP domain and is transcribed in all stages before molting [18]. The CUT-2 protein, composed of 215 amino acids, has been immunolocalized in the cortical layers of the cuticle, but not in the electron-dense epicuticle [22]. The protein contains several short repeats, also found in structural proteins of the cuticles or eggshells of various insects [18]. All insoluble cuticular proteins are crosslinked via di- and tri-tyrosines [16, 18, 24–26].

The electron-dense layer at the exterior boundary of nematode cuticles is defined as the epicuticle [27]. Using *A. suum*, Fujimoto and Kanaya [24] manually separated the cuticles from the body carcass. Cuticular collagens were removed through digestion with bacterial collagenase. They termed the remaining insoluble material cuticlin, having high contents of proline and alanine. Electron microscopy of the isolated material showed the presence of electron-dense structures [24], typical for the epicuticle. We showed that polyclonal antibodies prepared against the isolated insoluble material of *A. suum* reacted with the cortical outermost epicuticular layers of *A. suum* and filariae [25, 28]. Immunoscreening of an *A. suum* cDNA library with a monoclonal antibody specific for the insoluble fraction allowed the isolation of a cDNA clone with an incomplete coding region of 644 nucleotides (NCBI GenBank accession number X92101.1) [29]. The deduced amino acid sequence had six repetitive peptide motifs containing three tyrosine residues each. As described by Fujimoto for his cuticlin, the protein is rich in proline and alanine residues [24, 29]. A fusion protein was produced to raise specific antibodies, and the epitope’s presence within the epicuticular layer of *A. suum* was confirmed [29]. Additional cDNA and genomic clones were isolated, showing the typical nucleotide repeat patterns. The partial gene was named *Asu-epicut1* [30] underlining that its encoded protein is in the epicuticle. The early data suggested that the 5' and 3' parts of the cDNA clones would
belong to two different exons separated by an intron in the gene. No full-length gene structure could be inferred before genomic sequencing.

Significant technological advances recently allowed the complete genome of *A. suum* to be sequenced [31]. We reverified, completed, and identified the *Asu-epicut1* sequences using this new database. Using *Asu-epicut1* as a query, we identified two additional epicuticular genes, *Asu-epic-2* and *Asu-epic-3*. Homologous proteins are found in over 50 nematode species of three different nematode clades. The possible role and mode of interaction of epicuticulins with cuticular collagens are discussed based on their typical intrinsically disordered protein characteristics, tyrosine motifs, and cysteine residues. Finally, the relationship of this new category of epicuticular proteins with proteins present in Arthropoda is elucidated.

**Material and methods**

The cDNA (X92101.2, 31B1A; AJ408885.1, C1; AJ408886.1, C2; AJ408871.1, C3) and genomic clones (AJ408881.1, G1; AJ408889.1, G2; AJ408890.1, G3), coding for *A. suum* epicuticulins, were compared through alignments using Jalview [32]. Based on their conserved nucleotide sequence (aagaggaa) (Fig 2), Blasts were carried out on the following databases: NCBI (https://blast.ncbi.nlm.nih.gov/ Blast.cgi), Nucleotide collection and whole-genome shotgun contigs (wgs) of *A. suum*; WormbaseParasite [33] and the EMBL-EBI services [34]. The *A. suum* TSA sequences JI177333, JI176387, JI178090, and JI180250, were used in their reversed complementary version (rc). All former *A. suum* epicuticulin sequences were called “epicut”. In agreement with the nomenclature directives of Wormbase the new epicuticulin genes are termed “epic”. The complete *Asu-epic-1* gene was identified through blasts using the 5’ part of 55 nucleotides of cDNA/TSA clones starting with ATG and the 3’ part formed of the repeat-containing region (S1 and S2 Figs). The *Asu-epic-1* and *Asu-epic-2* nucleotide sequence data reported are available in the Third-Party Annotation Section of the DDBJ/ENA/GenBank databases under the accession number TPA: BK061342 and TPA: BK061351. Additional data are deposited in the Dryad, Dataset, https://doi.org/10.5061/dryad.fttdz08vs.

Protein Blasts were carried out mainly on the protein knowledge platform UniProt [35] and the same platforms as the nucleotide sequence Blast. Multiple or pairwise nucleotide or protein sequence alignments were carried out using the EMBL-EBI services: Clustal Omega for multiple alignments and Needle or Matcher for pairwise alignments. Radar was used to automatically align protein repeats and Phobius to identify signal peptides [34]. The Multiple sequence viewer [36] was used to compare nucleotide and protein repeat sequences. Wormbase was the authoritative data source for the *C. elegans* genes [37]. Expasy services (https://www.expasy.org/) [38] were applied for the in-depth study of the epicuticulins: STRING [39] and Compute pI/MW [40]. The presence of intrinsically disordered regions in the proteins was calculated using the IUPred2A tool (https://iupred2a.elte.hu/) [41], and the characteristics of the IDPs were analyzed using CIDER [42]. For the prediction of MoRFs, we used the software tool MoRFchibi | Gsponer Lab (ubc.ca) [43]. The *C. elegans* collagen database, CeColDB (http://CeColDB.permalink.cc/), was used to analyze the structure of cuticular collagens.

**Results**

**Identification of the first *A. suum* epicuticular gene, *Asu-epic-1***

Blast with the *A. suum* cDNA AJ408887 resulted in thirty nucleotide matches with > 95% nucleotide identity to the query, comprising cDNA sequences (including our previously isolated cDNA clones: X92101, AJ408885, AJ408886, AJ40887, Fig 1), TSA sequences, and Expressed Sequence Tag (EST) sequences (S1 Table).
Only six entries had a 5' start codon (S1 Fig). Two TSA sequences (JI176387rc and J178090rc) have the ATG initiation codon, an unrepeated sequence stretch, followed by six repeats. While JI176387rc ends with the termination codon, the J178090rc sequence does not. Otherwise, the two are 100% identical. The other four EST fragments (BI781775, BI781825, BI782671, and BI781932) also contain the correct initiation codon. Complementary to the cDNA/TSA sequences, thirteen different genomic sequences with more than 95% nucleotide identities to the query were identified in three genome databases. Three were, as expected, the genomic clones already described (Fig 1). The most recent genomic sequence was from the *A. suum* isolate RED_2019 chromosome 6, whole genome shotgun sequencing project (PRJNA62057) [31] with a 99.2% nucleotide identity to the query. The sequence JI176387rc was considered the appropriate mRNA (Fig 1 and S1 Fig) from which to build a gene model (S2 Fig). The proposed gene is localized on the negative strand of chromosome VI and comprises two exons, separated by an intron of 1593 nucleotides (Fig 1 and S2 Fig). The *Asu-epic-1* gene starts with a signal sequence of 60 nucleotides followed by seven TRs varying in length between 147 and 153 nucleotides, and the last repeat ends with a stop codon (Fig 2).

The seven repeats were aligned, and specific nucleotide differences were detected (Fig 2). For example, an adenine replaces a thymidine in position 120 of repeat five. These patterns helped manually optimize the automatically generated alignments of one TSA and four cDNA sequences. They were individually compared with the identified *Asu-epic-1* gene (Fig 3), and the presence of the specific nucleotide patterns in the diverse clones was confirmed.

TR1 and TR2 of all sequences are identical to the gene except for one difference in four cDNA sequences (substituting GC by CG in positions 61–62 within TR 1). All sequences end with a termination codon, indicating that they are complete and that their repeat numbers (six-seven) are defined. AJ408887 is the only cDNA sequence containing seven repeats as *Asu-epic-1*. However, the TR4 is duplicated while TR5 is missing. In repeats three to five, all sequences showed variations produced by Indels.

**Fig 1. Sequence comparison of different *Asu-epic-1* clones.** Formerly characterized genomic (G) and cDNA clones (C) are aligned with the TSA sequence JI176387.1rc and the complete *Asu-epic-1* gene of *A. suum*. Exons 1 and 2 are indicated as light and darker blue boxes or lines. The intron and untranslated regions are indicated as a gray box and gray lines with the number of nucleotides, respectively. The GenBank accession numbers, the initial designation used for the sequence submission [29, 30], and the sequence length (bases, b) are given.

https://doi.org/10.1371/journal.pone.0274751.g001
Properties of the deduced Asu-EPIC-1 protein

The deduced protein of Asu-EPIC-1 has 373 amino acids (37.64 kDa) and an estimated isoelectric point of 4.28 (calculated with Compute pI/MW). It starts with a signal peptide of 20 amino acids (S3 Fig). Asu-EPIC-1 has seven TRs with a length of 51 (repeats 1 and 3–6) and 49 amino acids (repeats 2 and 7). The aligned amino acid sequences of the repeats showed high conservation of the N- (KRNAYGDEP) and C-terminal (VESSGKYR) parts, including two of the three tyrosine residues. Some amino acid variations are present between these two conserved parts, mainly in TR1 and TR2 (Fig 2). Repeat 2 lacks two amino acids, has only two tyrosine residues, and contains more variations (8) than the other repeats. Repeat 7 ends with the third tyrosine (Fig 2). Importantly, the different deletions and insertions found in several clones (Fig 3) did not change the reading frame and did not result in amino acid variations (S3...
Only one substitution (arginine instead of lysine) at the end of repeat six was found in JI176387rc, and the nucleotide differences in repeat one of the four cDNA sequences replaced the alanine with arginine at position 41 of the first protein repeat (R instead of A) (S3 Fig).

The deduced Asu-EPIC-1 protein is an intrinsically disordered protein (IDP), as shown by computational analysis (Fig 4A), with 73.2% of the amino acids having disorder-promoting characteristics (Fig 4D). The hydrophobicity plot indicates six hydrophilic regions formed each by the four amino acids RKKR or RRKR with scores of up to -2.989 (Fig 4B). The hydropathy pattern confirms the hydrophobicity profile with six blocks of hydrophilic amino acid stretches of RKKRNN (Fig 4E). In the first repeat, the four hydrophilic amino acids KRNN confer less hydrophilic property than the six hydrophilic amino acids of the other repeats. Since Asu-EPIC-1 is predicted as an IDP, it would not fold into a defined or fixed three-dimensional structure but changes its form according to potential interacting partners. Seven molecular recognition features (MoRFs) are effectively detectable in Asu-EPIC-1 (Fig 4C). The last questionable MoRF starting at position 353 (QAPAPI) differs from the others and does not include tyrosine. The presence of three tyrosine residues per TR (except TR2 with two tyrosine residues) and two in each MoRF points to their crucial role in the assembly of the epicuticlin structure. The conserved tyrosine motif GYR at the end of each repeat is an annotated Pfam motif (PF02756) found in various cuticular IDPs of insects. The protein B4IZ60 of Drosophila grimshawi was selected to compare the architecture of these motifs with the tyrosine motifs of Asu-EPIC-1 (Fig 5).

Fig 4. Physicochemical properties of Asu-EPIC-1. A) The protein has a highly intrinsically disordered profile as predicted by IUPred2A [41]. The ANCHOR2 score of 0.65 is a threshold indicative of intrinsically disordered regions. B) Hydrophobicity plot, according to Kyte & Doolittle, shows six hydrophilic regions. C) Identification of seven putative molecular recognition features (MoRFs) (blue regions) using the MoRFchibi SYSTEM [43]. The cut-off level is set at 0.72 (blue line). The first six MoRFs are the parts of the conserved amino acid regions, and each contains two of the three tyrosine residues (SGYRKKRNNAY). The last MoRF is associated with the sequence QAPAPI. D) The distribution and proportion of disorder-promoting amino acids (in red) versus more order-promoting amino acids (blue) [44]. E) Distribution of the hydropathy pattern in the TRs according to Pommié et al. [45].

https://doi.org/10.1371/journal.pone.0274751.g004
The repeats of both species have an almost identical length of 51 amino acids, and both proteins are IDPs and have a signal peptide. The fifteen TRs of the insect protein have four tyrosine residues per repeat. Three are organized in the two Pfam annotated motifs YLP and GYR \[40\]. In both proteins, the motifs YGD and YLP are at the same N-terminal position of each repeat. The following tyrosine residue is in the GYR motif or a variant (GYA, resp GYQY). The last tyrosine residue is present in the C-terminal region of the repeats within the GYR motif in Asu-EPIC-1 and within the not annotated KYR motif in \textit{D. grimshawi} repeats.

**Dentification of additional nematode epicuticulins**

Blasts with parts of the Asu-EPIC-1 sequence as a query on the SIB-UniProtKB and the NCBI protein databases rendered over one hundred unidentified epicuticular protein sequences belonging to 54 different nematode species of the class Chromadoria (Spirurina, clade III; Tylenchina, clade IV; Rhabditina, clade V) (S2 Table). The number of repeats varies from one (in several species) to maximally 33 repeats in \textit{T. canis} (A0A0B2V0B7). Common to all
sequences is the presence of the two conserved epicuticlin amino acid motifs GYR and YGD (Fig 6).

All these putative epicuticlin proteins are IDP and have alanine and proline-rich patterns. Some, but not all of them, have a signal peptide. The number and structure of TRs vary within a species, between species, and between the three clades. The repeats' length ranges from 49–71 amino acids. Several nematode species have two to three distinct epicuticlin proteins (Fig 6 and S2 Table), which differ in their amino acid sequences, the number, and length of the repeats, and the number of tyrosines (two to four) per repeat. Many species of clade V have two different epicuticlin proteins, one of which contains a cysteine localized between the conserved regions (Fig 6 and S2 Table). No cysteine was found in the epicuticular repeats of the other nematodes. Besides EPIC-1, two different epicuticlin proteins, named EPIC-2 and EPIC-3, were identified in the databanks of A. suum, A. lumbricoides, and T. canis (see below for more details). An appropriate and consistent overall classification of the epicuticlin is at present impossible. The epicuticular proteins of seven nematode species are presented in more detail to illustrate their differences within the clades III to V. The widely used model nematode C. elegans is included in clade V.

Clade III Spirurina: A. suum, A. lumbricoides and T. canis

Asu-EPIC-2 protein is encoded by the gene Asu-epic-2, located on chromosome III (isolate RED_2019) in the position 10824598–10825857 of the negative strand. The deduced protein has 419 residues (42.79 kDa) with an estimated isoelectric point of pH 4.10. It consists of an NH2-terminal unrepeated stretch of five amino acids followed by eight TRs. The seven first repeats consist of 52 amino acids and the last one of 50 amino acids. Each repeat contains only two tyrosine residues (Fig 6). The highly conserved tyrosine regions differ from Asu-EPIC-1 by five amino acids (underlined) KRNNQYGDEP and VQASGYRR. The third A. suum
epicuticlin sequence was identified in a genomic DNA fragment annotated AgB12_g028 (on scaffold AgB12:654,298–657,498 reverse strand). This sequence, named Asu-epic-3, is present on chromosome VI of isolate RED_2019, whole genome shotgun sequence (position 809306–810340 negative strand). The AgB12_g028_t01 transcript codes for a protein of 405 amino acids with an isoelectric point of pH 3.98. The deduced Asu-EPIC-3 sequence has a signal peptide followed by five TRs of varying length (48 to 61 amino acids) with three tyrosine residues per repeat, except repeat 1, which contains only two tyrosine residues. The amino acid sequence motifs differ from the other two epicuticins in the conserved tyrosine-containing regions (KRNNEYGDEP and EIEASYGRK) (Fig 6 and Dryad, Dataset, https://doi.org/10.5061/dryad.fttdz08vs). In the human parasite A. lumbricoides, a closely related species to A. suum [48], three different proteins (Uniprot accessions A0A0M3I7G7; A0A0M3IF25; A0A0M3I7G8), sharing high similarity with the epicuticins of A. suum were identified (Fig 6; S2 Table). Compared to their homologous in A. suum, Alu-EPIC-1 lacks one repeat (probably repeat 5). Alu-EPIC-2 has only two repeats, and Alu-EPIC-3 has one repeat more than Asu-EPIC-3. Different epicuticlin sequences were also identified in T. canis (S2 Table). Some of these epicuticins have up to 33 repeats (e.g., A0A0B2UZ77 and A0A0B2V0B7). Three epicuticins have the characteristics typically found in the A. suum species. Tca-EPIC-1 (A0A183TVS5) has a repeat length of up to 51 amino acids and up to three tyrosine residues per repeat. Tca-EPIC-2 (A0A183V326) has three repeats of 54 amino acids and only two tyrosine per repeat. Tca-EPIC-3 has up to 61 amino acids and three tyrosine per repeat (Fig 6).

Clade IV Tylenchina: Steinernema glaseri

S. glaseri is a representative species of the clade Tylenchina where only two different epicuticins could be identified (A0A118ANA2 and A0A117YLQ6). Both have a signal peptide followed by six repeats with 50 to 56 amino acids. A0A118ANA2 has two tyrosines per repeat, and A0A117YLQ6 has three tyrosines. (Fig 6 and S2 Table).

Clade V Rhabditina: Ancylostoma caninum, Pristionchus pacificus, and C. elegans

Two different epicuticlin sequences were found in A. caninum (A0A368GWQ4 and A0A368GWR3) with nine and four repeats, respectively (Fig 6). A0A368GWQ4 has the particularity that one cysteine per repeat is present between the two tyrosine motifs. The last repeat has no cysteine. A0A368GWR3 has up to four tyrosines per repeat (Fig 6). The two epicuticins of P. pacificus differ in the repeat number and length: A0A2A6C2N0 has six repeats of 52 to 69 amino acids, and H3DHI4 has thirteen repeats of 51 to 81 amino acids. The tyrosine numbers are different as well, ranging from two to five. No cysteine residues were found in the repeats of these epicuticins.

In the best-studied nematode C. elegans, epicuticins have not yet been published in peer-reviewed journals. A previous PCR screen in a C. elegans library recovered sequences resembling Asu-epic-1 in genes F11E6.3 (Q9U3J8) and K08D12.6 (Q8MXU8) [30] (S2 Table). F11E6.3 gene is localized on chromosome IV (position 17470514–17471920, negative strand) and consists of two exons transcribed into a pre-messenger of 1199 nucleotides, containing a coding region of 1038 nucleotides. The encoded protein Q9U3J8 comprises 345 amino acids with an estimated molecular weight of 35.2 kD and an isoelectric point of 4.02. It contains six TRs with 53–58 amino acids (Fig 6). Each repeat includes the two tyrosine motifs within the N- and C-terminal conserved regions (KRQAQNSYGDEA and DAGYYS). Cysteine residues are absent in the repeats of this epicuticlin. The second epicuticlin, gene K08D12.6, is localized on chromosome IV (positions 1720790–1723072, forward strand). The deduced protein...
Q8MXU8 has 668 amino acids, an estimated molecular weight of 62.8 kD, and an isoelectric point of 4.21. It contains ten TRs varying between 57 and 70 amino acids (Fig 6). The conserved motifs comprising two tyrosine residues (KRNAYGDGEQVT and DSGYRS) differ from those of Q9U3J8. This epicuticulin contains one cysteine per repeat, except repeat eight. These two C. elegans epicuticulins are expressed in the larval stages (L1-L4) and in young adults with aggregate expression estimates of mean FPKM values (Fragments Per Kilobase of transcript per Million fragments mapped) from 119 to 590 in Q9U3J8 and from 96 to 486 for Q8MXU8. In dauer larvae and embryos, the expression is low.

STRING analysis of the epicuticulin Q8MXU8 indicated a co-expression and homology with the epicuticulin Q9U3J8 (Fig 7).

CELE_F46F2.3 and CELE_T04F8.8 are annotated as uncharacterized IDPs. The first, featuring 137 amino acids and 13 tyrosine residues, is expressed in the hypodermis. The second protein of 165 amino acids with 16 tyrosine residues is rich in proline. The location of its expression is unknown. Co-expressed cuticular collagens belong to the cuticular collagen

![STRING analysis diagram](https://doi.org/10.1371/journal.pone.0274751.g007)

**Fig 7.** Scheme of STRING analysis of the C. elegans epicuticulin Q8MXU8 (K08D12.6) to show potential interactions of this epicuticulin with the second epicuticulin Q9U3J8 (F11E6.3), cuticular collagens, and other proteins rich in tyrosine. The red-colored node represents the query protein, and the other nodes are the first shell of interactors. Co-expression and homology analysis are indicated in the table. Each protein-protein interaction is annotated with 'scores', which are confidence indicators. All scores rank from 0 to 1, with 1 being the highest possible confidence. A score of 0.5 indicates that roughly every second interaction might be erroneous (i.e., a false positive) [39].

| Predicted Functional Partners of Cel-EPIC-x (K08D12.6): | Coexpression | Homology | Score |
|---------------------------------------------------------|-------------|----------|-------|
| F11E6.3 Protein F11E6.3 (F11E6.3) mRNA, complete cds   | +           | +        | 0.53  |
| col-42 COLlagen; Protein Y69H2.14 (Y69H2.14) mRNA, complete cds | +           |          | 0.526 |
| col-103 COLlagen; Protein COL-103 (col-103) mRNA, complete cds | +           |          | 0.523 |
| col-160 COLlagen; Protein COL-160 (col-160) mRNA, complete cds | +           |          | 0.52  |
| col-98 COLlagen; Protein COL-98 (col-98) mRNA, complete cds | +           |          | 0.515 |
| col-93 COLlagen; Protein COL-93 (col-93) mRNA, complete cds | +           |          | 0.487 |
| col-80 Putative cuticle collagen 80                       | +           |          | 0.467 |
| col-142 COLlagen; Protein COL-142 (col-142) mRNA, complete cds | +           |          | 0.463 |
| T04F8.8 Protein T04F8.8, isoform b (T04F8.8) mRNA, complete cds | +           |          | 0.461 |
| F46F2.3 Protein F46F2.3 (F46F2.3) mRNA, complete cds    | +           |          | 0.454 |
clusters C (COL-142 and COL-93) or D [49]. As collagens are potential first interactors with epicuticlin, we looked for collagens potentially featuring YGD and GYR motifs. The collagens indicated in the String analysis had no such motifs. Among the 173 cuticular collagen proteins available in the *C. elegans* matrisome database [49], sixteen had one or two of these motifs (Dryad (https://doi.org/10.5061/dryad.fttdz08vs). The collagen DPY—10 of cluster B13 was the only one with YGD and GYR motifs (Fig 8).

A schematic representation of the two *C. elegans* epicuticlin Q9U3J8 and Q8MXU8 and the cuticular collagen DPY—10. The Uniprot accession numbers, in parentheses, the Wormbase accession, and the total amino acid numbers are shown. The three protein sequences are drawn to scale. The TRs in the two epicuticlins are indicated as blue boxes with the amino acid numbers. The GYR and YGD motifs are shown as orange and green boxes, respectively. The positions of the cysteine residue in the epicuticlin Q8MXU8 and the eight cysteine residues, organized in three clusters (red triangles) in the collagen, are marked with red triangles. In the collagen, the three collagen (GXY)n regions (green boxes), the two tyrosine motifs, and thirteen tyrosine residues (yellow circles) are indicated.

https://doi.org/10.1371/journal.pone.0274751.g008

Discussion

*A. suum*, a crucial starting point for the identification of epicuticlin

The isolation and purification of sufficient insoluble cuticular material is easy using *A. suum*, with a size of up to 20 cm [24]. Modified procedures were used to isolate insoluble fractions, for example, of the cuticles of *C. elegans* [15], *A. viteae* [50], and *B. pahangi* [51]. However, the insolubility and the small amounts of material obtained hampered their further biochemical characterization. Yet, $^{125}$I labeling methods demonstrated that a significant part of the labeled tyrosine-containing proteins remained in the insoluble epicuticul fraction [52, 53]. Immunogold and lectin-gold techniques showed no reaction with the epicuticular outer surface [54]. However, specific antibodies against the isolated and purified insoluble epicuticular residue of *A. suum* allowed the isolation of a cDNA clone, X92101, encoding repetitive motifs rich in alanine, proline, and tyrosine, typical characteristics found in the epicuticle [29]. Due to the repetitive nature, the sequencing of the cDNA clone remained incomplete. This sequencing problem is also observed in several genomic and cDNA sequences deposited in databanks by different laboratories. For example, scaffold data (GCA 18702v3; PRJNA62057 or Asc-Suum_1.0; PRJNA80881) are incomplete in the assumed epicuticlin repeat regions (for example gene ID AgB12_g029 and GS_02605). Using ExoIII/Mung Bean nuclease allowed us to
overcome these problems [30] before its use for sequencing long stretches of repetitive DNA [55]. However, only the recent publication of the fully assembled chromosomes of A. suum (GCA 1343314v1) [31] made it possible to identify now the complete Asu-epic-1 gene.

Characteristics of the epicuticlin genes

The nucleotide repeats in the Asu-epic-1 gene match with repeats of all previously identified sequences, although some differences appeared, such as nucleotide substitutions and variations in the repeat numbers. The identification of the unique nucleotide patterns allowed us to achieve an optimal manual alignment and comparison of the diverse epicuticlin repeat sequences. The variations in the repeat number in the cDNA and the genomic PCR products are mostly related to insertion/deletion (Indel) incidences since no alternative splicing is involved, and only a single copy of the gene was identified in the genome. Moreover, the fact that the epicuticlin genes are intrinsically disordered proteins and a higher Indel rate was described in disordered domain repeats [56] reinforces our suggestion concerning slippage of polymerases and Indel generations. Recombination events, such as unequal crossing over and gene conversion, may additionally lead to contractions and expansions of TR sequences by which repeat-containing haplotypes rapidly evolve. Similar nucleotide patterns are present in the nucleotide sequences of epicuticlin genes of other nematodes, but they have not been analyzed.

Asu-epic-1 and Asu-epic-3 are relatively closely located on chromosome VI, and Asu-epic-2 is on chromosome III. However, the two C. elegans epicuticlin genes (F11E6.3 and K08D12.6) are at the opposite ends of chromosome IV. Wang et al. [31] compared the chromosomes of both species, and a significant part of genes present on chromosome VI of A. suum have their homologs on chromosome IV of C. elegans. The exact evolutionary chromosomal relationship of the epicuticlin genes in both species remains to be shown. We could not detect epicuticlin sequences in Dorylaimia (Clade I) and Enoplia (Clade II). It is possibly due to incomplete sequencing data or the presence of another category of epicuticular proteins in these nematodes. Additional epicuticlin genes might be detected in several nematode species through an in-depth analysis of the available databanks and future whole-genome sequencing projects.

All identified epicuticlin genes are characterized by varying numbers of TRs. The number of repeats in Asu-epic-1 and the two C. elegans epicuticlin genes was ascertained through the available DNA gene sequences. However, the repeat numbers of epicuticlin-like sequences of many other species should be re-examined. The very high numbers of TRs (>30) found in the sequences of T. canis (KHN74524.1 and KHN74525.1) were surprising. However, we found identical sequences to these proteins (VDM24067.1 and VDM24068.1), which have only nine and three TRs, respectively. KHN74524.1 (32 TRs) and VDM24067.1 (9 TRs) are hypothetical proteins deposited in databanks of two different bioprojects (PRJEB533 and PRJNA248777) carried out by two different research groups. Despite the difference in the repeat numbers, their gene sequences (with the flanking, upstream, and downstream untranslated regions) are practically identical. The discrepancy in the repeat numbers could be due to the sequencing and assembly difficulties associated with the TRs. Again, more accurate sequencing/assembly data are needed to determine the actual repeat number.

Epicuticlin genes are expressed in different larval stages of the model nematode C. elegans

The cuticle of nematodes is newly synthesized during the four molting processes from L1—L4 larval stages to the adult stage [57]. The complexity of the processes involved has been recently reviewed in C. elegans [58]. Correct temporal expression of the genes and the synthesis of cuticular proteins are crucial. While oscillating gene expression has been shown for the
cuticular collagen genes of *C. elegans* [59], it is not known whether the epicuticlin expression follows a similar temporal oscillation pattern. However, it is evident that the expression levels of the *C. elegans* epicuticlin genes F11E6.3 and K08D12.6 increase significantly through all larval stages to young adults. At the same time, it is absent or low in the early embryo and dauer stages. F11E6.3, K08D12.6 and K08D12.7 were recently annotated in Wormbase as *epic-1, epic-2 and epic-3*. Since the epicuticlin genes are involved in building the outermost electron-dense layer of the cuticle, we propose that their synthesis and secretion start somewhat before the collagens are synthesized. The recently described role of TMEM131 proteins for the cargo collagen secretion in *C. elegans* [60] will undoubtedly help to understand the secretion machinery and let us suggest that a similar cargo procedure may secrete the epicuticlin.

**Epicuticlin, as intrinsically disordered proteins with tandem repeats and molecular recognition features, are prime candidates as nucleation sites for the formation of the cuticle**

The presumed Asu-EPIC-1 protein corresponds in its biochemical properties to the insoluble residue originally described by Fujimoto and Kanaya [24]. Several attempts to produce recombinant epicuticlin proteins using a wide variety of different expression systems were unsuccessful. So far, the only expression was possible via the production of a fusion protein using the 26-kDa GST tag from *Schistosoma japonicum* [61] subcloned into the plasmid vector pGEX [29]. This failure might be associated with the intrinsic characteristics of the epicuticlin since other recombinant nematode proteins were simultaneously successfully produced [62].

All the analyzed epicuticlin are predicted to be IDPs, apart from their signal peptide. Intrinsically disordered proteins (IDP) have gained increasing attention during the last decade [44, 63, 64]. Their identification as weak polyampholytes, showing structures of globule and tadpole-like proteins [42], could point to their crucial role in assembling the cuticles. The combination of intrinsically disordered regions (IDR) and tandem repeats is frequent, and Delucchi et al. [65] defined four categories of TRs, according to the repeat unit length. Repeats with 15 or more residues were called domain repeats, representing 30% of all predicted TR. The association of TR with intrinsic disorder allowed the distinction of four types of overlaps. The epicuticlin are IDPs with tandem repeats of the category of domain repeats (> 15 residues) and belong to the fraction of proteins where IDR and TR cover the whole protein length. The analyzed epicuticlin sequences are predicted to have molecular recognition features (MoRFs), short protein-binding regions that undergo disorder-to-order transitions upon binding appropriate protein partners [66, 67]. We propose that these properties of epicuticlin enable them to be disorder-enriched hubs for protein-protein linking [65, 68] in the cuticle assembly. They could play essential roles in interacting with other epicuticlin, different cuticular collagens, cuticlin, and glycoproteins. The STRING analysis indicates that the cuticular collagens are privileged partners for such interactions, probably due to tyrosine and cysteine clusters. Based on the rotary shadowing of collagen molecules, the length of cuticular collagens of *A. suum* was calculated to be 45 nm [69]. The theoretical size of Asu-EPIC-1, assuming a linear protein with 470 amino acids, would be around 112 nm. Interaction of two canonical collagen units with one epicuticlin protein is theoretically possible via the proline, tyrosine, or cysteine residues.

**Roles of conserved motifs of epicuticlin and their homologs in arthropods**

The epicuticlin contain between six and ten repeats. Whether this represents a fixed pattern is not clear. If the number of repeats should be vital in a structural crosslinking pattern, a specific number of repeats could be expected to perform a given function. Remarkably, one tyrosine is
present in the two conserved motifs of all epicuticlins of all nematode species. One or more additional tyrosine residues is/are present in the repeats outside the two conserved motifs in several epicuticlins. Each of these additional tyrosine residues is present within a potential not-annotated motif. In general, tyrosine residues are well known to be involved in crosslinking processes [18, 70]. In A. suum, dityrosine was identified in the hydrolysate of cuticlin [71] and the cuticle of larvae of H. contortus [72]. Marti [25] isolated di-, tri-, and iso- trityrosine from the hydrolyzed cuticlin of A. suum and showed a characteristic blue fluorescence of cuticlin under UV excitation at 254 nm, typical for dityrosine. Specific antibodies raised against dityrosine labeled the electron-dense layers of the epicuticlin, confirming the presence of dityrosine in the insoluble epicuticular structures. The tyrosine residues identified in the conserved epicuticlin motifs GYR and YGD are the prime sites in forming dityrosine and linking epicuticlins and other cuticular proteins. The role of the 29-kDa glycoprotein (gp29) identified in the cuticles of filarial nematodes [13] in catalyzing the formation of the crosslinking residues should be further analyzed. Several nematode species of the clade V have two different epicuticlin types, distinguished via the presence or absence of cysteine residues in the repeats. The cysteines are localized between the conserved motifs GYR and YGD. We propose that they could also be involved in the crosslinking with cysteines in the cuticular collagens.

The conserved Pfam annotated (PF02756) GYR motif is present in cuticular protein families of arthropods [46, 73]. Twelve cuticular protein (CP) families were defined by Willis [74], and they were suggested to be involved in the arthropod cuticle assembly. Willis mentioned: “All of these protein families appear to be restricted to arthropods...” and “Furthermore, given the similar cuticle construction throughout the Éc dyszoa (Schmidt-Rhaesa et al., 1998), it is intriguing that the arthropods seem to have adopted so many unique configurations for their CPs”. Detecting the GYR and YGD motifs in the nematode epicuticlins made it possible to compare them with the motifs in the Tweedle and CPR families of arthropods [46]. For the first time, the presence of similarities between cuticular proteins of arthropods and nematodes is confirmed. Whereas in arthropods, motif GYR frequently is located within the N-terminal region and the motif YLP within the C-terminal region, the selected D. grimshawi cuticular protein B4IZ60 has in each repeat first the motif YLP, followed by the motif GYR. The role of the additional tyrosine residue motifs in arthropod and nematode proteins remains to be clarified. New ways are now opened to study the formation of the cuticular structures using proteomics and matrisomic approaches [49]. A combination of diverse genetic and molecular techniques well established for Drosophila and Caenorhabditis model organisms will be helpful.

**Outlook**

Innovative ways to produce epicuticlins are needed. Recombinant epicuticlins would be especially useful for future applications in the biomaterial sciences, as shown for recombinant resilin [75] and elastin [76]. The potential of the highly organized characteristics of epicuticlins is evident, for example, as biosynthetic polymers in medical applications, such as in skin healing procedures. Of general scientific interest is the clarification of the synthetic machinery leading to the formation of the complex extracellular matrix of the cuticle of nematodes and arthropods. In general, even if the role of repetitive sequences is more and more recognized, this study underlines their importance.

**Supporting information**

**S1 Fig.** Clustal alignment of the 5’ regions of Asu-epic-1 genomic and cDNA sequences. (DOCX)
S2 Fig. Scheme of the identification of the complete Asu-epic-I gene of *A. suum.*
(DOCX)

S3 Fig. Comparative alignment of the amino acid sequence of Asu-EPIC-1 with sequences encoded by four cDNA and one TSA clone.
(DOCX)

S1 Table. BLASTN results with query AJ408887, an *A. suum* partial mRNA.
(DOCX)

S2 Table. Epicuticlin sequences detected in different nematode species.
(DOCX)

**Acknowledgments**

We would particularly thank all the former Ph.D., MSc students, and technicians who contributed to elaborating some of the initial data.

**Author Contributions**

**Conceptualization:** Bruno Betschart, Marco Bisoffi, Ferial Alaeddine.

**Data curation:** Bruno Betschart.

**Formal analysis:** Bruno Betschart, Ferial Alaeddine.

**Funding acquisition:** Bruno Betschart.

**Investigation:** Bruno Betschart, Marco Bisoffi, Ferial Alaeddine.

**Methodology:** Bruno Betschart.

**Project administration:** Bruno Betschart.

**Supervision:** Bruno Betschart.

**Validation:** Bruno Betschart.

**Writing – original draft:** Bruno Betschart, Marco Bisoffi, Ferial Alaeddine.

**Writing – review & editing:** Marco Bisoffi, Ferial Alaeddine.

**References**

1. Bongers T, Ferris H, Bongers T, Ferris H, Bongers T, Ferris H, et al. Nematode community structure as a bioindicator in environmental monitoring. Trends in Ecology & Evolution. 1999; 14: 224–228. https://doi.org/10.1016/s0169-5347(98)01583-3 PMID: 10354624

2. Nematode. Wikipedia. 2021. https://en.wikipedia.org/w/index.php?title=Nematode&oldid=1034700811

3. Chitwood BG, Chitwood MBH, Christenson RO. Introduction to Nematology. University Park Press; 1974.

4. Maggenti A. General Nematology. Springer Science & Business Media; 2012.

5. Bird AF, Bird J. The Structure of Nematodes. Academic Press; 2012.

6. Cox GN. Molecular and Biochemical Aspects of Nematode Collagens. The Journal of Parasitology. 1992; 78: 1–15. https://doi.org/10.2307/3283678 PMID: 1738051

7. Proudfoot L, Kusel JR, Smith HV, Harnett W, Worms MJ, Kennedy MW. Rapid changes in the surface of parasitic nematodes during transition from pre- to post-parasitic forms. Parasitology. 1993; 107: 107–117. https://doi.org/10.1017/s0031182000079464 PMID: 8365993

8. Spiegel Y, McClure MA. The Surface Coat of Plant-Parasitic Nematodes: Chemical Composition, Origin, and Biological Role—A Review. J Nematol. 1995; 27: 127–134. PMID: 19277272
New insoluble epicuticular proteins identified in nematode cuticles

9. Kennedy MW, Harnett W. Parasitic Nematodes: Molecular Biology, Biochemistry, and Immunology. CABI; 2001.
10. Page AP, Johnstone U. The cuticle. WormBook. The C. elegans Research Community; 2007. http://www.wormbook.org
11. Cipollo JF, Awad AM, Costello CE, Hirschberg CB. srf-3, a Mutant of Caenorhabditis elegans, Resistant to Bacterial Infection and to Biofilm Binding, Is Deficient in Glycoconjugates *. Journal of Biological Chemistry. 2004; 279: 52893–52903. https://doi.org/10.1074/jbc.M409557200 PMID: 15452127
12. Höflich J, Berninsone P, Göbel C, Gravato-Nobre MJ, Libby BJ, Darby C, et al. Loss of srf-3-encoded Nucleotide Sugar Transporter Activity in Caenorhabditis elegans Alters Surface Antigenicity and Prevents Bacterial Adherence *. Journal of Biological Chemistry. 2004; 279: 30440–30448. https://doi.org/10.1074/jbc.M402429200 PMID: 15123614
13. Cookson E, Blaxter ML, Selkirk ME. Identification of the major soluble cuticular glycoprotein of lymphatic filarial nematode parasites (gp29) as a secretory homolog of glutathione peroxidase. Proc Natl Acad Sci USA. 1992; 89: 5837–5841. https://doi.org/10.1073/pnas.89.13.5837 PMID: 1631065
14. Blaxter ML, Page AP, Rudin W, Maizels RM. Nematode surface coats: actively evading immunity. Parasitology Today. 1992; 8: 243–247. https://doi.org/10.1016/0169-4838(92)90126-m PMID: 15463630
15. Cox GN, Kusch M, Edgar RS. Cuticle of Caenorhabditis elegans: its isolation and partial characterization. Journal of Cell Biology. 1981; 90: 7–17. https://doi.org/10.1083/jcb.90.1.7 PMID: 7251677
16. Fetterer RH, Rhoads ML, Urban JF. Synthesis of Tyrosine-Derived Cross-Links in Ascaris suum Cuticular Proteins. The Journal of Parasitology. 1993; 79: 160. https://doi.org/10.2307/3283502 PMID: 8459325
17. Sebastiano M, Lassandro F, Bazzicalupo P. cut-1 a Caenorhabditis elegans gene coding for a dauer-specific noncollagenous component of the cuticle. Developmental Biology. 1991; 146: 519–530. https://doi.org/10.1016/0012-1606(91)90253-y PMID: 1864469
18. Lassandro F, Sebastiano M, Zei F, Bazzicalupo P. The role of dityrosine formation in the crosslinking of CUT-2, the product of a second cuticlin gene of Caenorhabditis elegans. Molecular and Biochemical Parasitology. 1994; 65: 147–159. https://doi.org/10.1016/0166-6851(94)90123-6 PMID: 7935621
19. De Giorgi C, De Luca F, Lamberti F. A silent trans-splicing signal in the cuticlin-encoding gene of the plant-parasitic nematode Meloidogyne artiellia. Gene. 1996; 170: 261–265. https://doi.org/10.1016/0378-1119(95)00894-2 PMID: 8666256
20. Timinouni M, Bazzicalupo P. cut-1-like genes of Ascaris lumbricoides. Gene. 1997; 193: 81–87. https://doi.org/10.1016/s0378-1119(97)00070-5 PMID: 10413052
21. Lewis E, Hunter SJ, Tettey L, Pavia Nunes C, Bazzicalupo P, Devaney E. cut-1-like genes are present in the filarial nematodes, Brugia pahangi and Brugia malayi, and, as in other nematodes, code for components of the cuticle. Molecular and Biochemical Parasitology. 1999; 101: 173–183. https://doi.org/10.1016/s0166-6851(99)00070-5 PMID: 10413052
22. Ristoratore F, Cermola M, Nola M, Bazzicalupo P, Favre R. Ultrastructural immuno-localization of CUT-1 and CUT-2 antigenic sites in the cuticles of the nematode Caenorhabditis elegans. J Submicrosc Cytol Pathol. 1994; 26: 437–443. PMID: 8087805
23. Sapio MR, Hilliard MA, Cermola M, Favre R, Bazzicalupo P. The Zona Pellucida domain containing proteins, CUT-1, CUT-3 and CUT-5, play essential roles in the development of the larval alae in Caenorhabditis elegans. Developmental Biology. 2005; 282: 231–245. https://doi.org/10.1016/j.ydbio.2005.03.011 PMID: 15936343
24. Fujimoto D, Kanaya S. Cuticlin: a noncollagen structural protein from Ascaris cuticle. Arch Biochem Biophys. 1973; 157: 1–6. https://doi.org/10.1016/0003-9861(73)90382-0 PMID: 4352055
25. Marti S. Biochemische und immunologische Charakterisierung von Cuticlin, einem Strukturprotein aus der Kutikula von Ascaris suum. PhD Thesis, Basel. 1991.
26. Parise G, Bazzicalupo P. Assembly of nematode cuticle: role of hydrophobic interactions in CUT-2 cross-linking. Biochimica et Biophysica Acta (BBA)—Protein Structure and Molecular Enzymology. 1997; 1337: 295–301. https://doi.org/10.1016/s0167-4838(96)00178-1 PMID: 9048907
27. Riddle DL, Blumenthal T, Meyer BJ, Priess JR. The Nematode Surface. C. elegans II. 2nd edition. Cold Spring Harbor Laboratory Press; 1997. https://www.ncbi.nlm.nih.gov/books/NBK20022/
28. Betschart B, Marti S, Glaser M. Antibodies against the cuticlin of Ascaris suum cross-react with epicuticular structures of filarial parasites. Acta Trop. 1990; 47: 331–338. https://doi.org/10.1016/0001-706x(90)90034-w PMID: 1978533
29. Bisoffi M, Marti S, Betschart B. Repetitive peptide motifs in the cuticlin of Ascaris suum. Molecular and Biochemical Parasitology. 1996; 80: 55–64. https://doi.org/10.1016/0166-6851(96)02668-0 PMID: 8885222
30. Alaeddine F. Etudes moléculaires de la surface des nématodes. PhD Thesis, Neuchâtel. 2000.
31. Wang J, Veronezi GMB, Kang Y, Zagoskin M, O’Toole ET, Davis RE. Comprehensive Chromosome End Remodeling during Programmed DNA Elimination. Current Biology. 2020; 30: 3397–3413.e4. https://doi.org/10.1016/j.cub.2020.06.058 PMID: 32679104

32. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics. 2009; 25: 1189–1191. https://doi.org/10.1093/bioinformatics/btp130 PMID: 19151095

33. Howe KL, Bolt BJ, Shafie M, Kersey P, Berriman M. WormBase ParaSite—a comprehensive resource for helminth genomics. Molecular and Biochemical Parasitology. 2017; 215: 2–10. https://doi.org/10.1016/j.molbiopara.2016.11.005 PMID: 27899279

34. The EMBL-EBI search and sequence analysis tools APIs in 2019.—Abstract—Europe PMC. [cited 14 Mar 2021]. http://europepmc.org/article/MED/30976793

35. The UniProt Consortium. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Research. 2021; 49: D480–D489. https://doi.org/10.1093/nar/gkaa1100 PMID: 33237286

36. The NCBI Multiple Sequence Alignment Viewer 1.22.0. [cited 20 Jul 2022]. https://www.ncbi.nlm.nih.gov/projects/msaviewer/

37. Harris TW, Arnaboldi V, Cain S, Chan J, Chen WJ, Cho J, et al. WormBase: a modern Model Organism Information Resource. Nucleic Acids Research. 2020; 48: D762–D767. https://doi.org/10.1093/nar/gkz920 PMID: 31642470

38. Artimo P, Jonnala gedda M, Arnold K, Baratin D, Csardi G, de Castro E, et al. ExPASy: SIB bioinformatics resource portal. Nucleic Acids Res. 2012; 40: W597–603. https://doi.org/10.1093/nar/gks400 PMID: 22661580

39. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 2019; 47: D607–D613. https://doi.org/10.1093/nar/gky1131 PMID: 30476243

40. Walker JM, editor. The Proteomics Protocols Handbook. Humana Press; 2005. https://doi.org/10.1385/1592598900

41. Erdős G, Dosztányi Z. Analyzing Protein Disorder with IUPred2A. Current Protocols in Bioinformatics. 2020; 70: e99. https://doi.org/10.1002/cpbi.99 PMID: 32237272

42. Holehouse AS, Das RK, Ahad JN, Richardson MOG, Pappu RV. CIDER: Resources to Analyze Sequence-Ensemble Relationships of Intrinsically Disordered Proteins. Biophysical Journal. 2017; 112: 16–21. https://doi.org/10.1016/j.bpj.2016.11.3200 PMID: 28076807

43. Malhis N, Jacobson M, Gspone J. MoRFchi bi SYSTEM: software tools for the identification of MoRFs in protein sequences. Nucleic Acids Research. 2016; 44: W488–W493. https://doi.org/10.1093/nar/gkw409 PMID: 27174932

44. Uversky VN. Intrinsically Disordered Proteins and Their “Mysterious” (Meta)Physics. Front Phys. 2019; 7. https://doi.org/10.3389/fphy.2019.00010

45. Pommiez C, Levadoux S, Sabatier R, Lefranc G, Lefranc M-P. IMGT standardised criteria for statistical analysis of immunoglobulin V-REGION amino acid properties. J Mol Recognit. 2004; 17: 17–32. https://doi.org/10.1002/jmr.647 PMID: 14872534

46. Cornman RS. The Distribution of GYR- and YLP-Like Motifs in Drosophila Suggests a General Role in Cuticle Assembly and Other Protein-Protein Interactions. Brembs B, editor. PLoS ONE. 2010; 5: e12536. https://doi.org/10.1371/journal.pone.0012536 PMID: 20824096

47. Liu W, Xie Y, Ma J, Luo X, Nie P, Zuo Z, et al. IBS: an illustrator for the presentation and visualization of biological sequences. Bioinformatics. 2015; 31: 3359–3361. https://doi.org/10.1093/bioinformatics/btv362 PMID: 26069263

48. Easton A, Gao S, Lawton SP, Bennuru S, Khan A, Dahlstrom E, et al. Molecular evidence of hybridization between pig and human Ascaris indicates an interbred species complex infecting humans. Harris NL, Soldati-Favre D, Brindley PJ, editors. eLife. 2020; 9: e61562. https://doi.org/10.7554/eLife.61562 PMID: 33155980

49. Teuscher AC, Jongsmia E, Davis MN, Statzer C, Gebauer JM, Naba A, et al. The in-silico characterization of the Caenorhabditis elegans matrisome and proposal of a novel collagen classification. Matrix Biology Plus. 2019; 1: 100001. https://doi.org/10.1016/j.mblplus.2018.11.001 PMID: 33543001

50. Betschart B, Rudin W, Weiss N. The isolation and immunogenicity of the cuticle of Dipetalonema vitaeae (Filaroidae). Z Parasitenkd. 1985; 71: 87–95. https://doi.org/10.1007/BF00932922 PMID: 3885606

51. Devaney E, Betschart B, Rudin W. The analysis of the 30 kDa antigen of Brugia pahangi and its interaction with the cuticle: A short review. Acta Tropica. 1990; 47: 365–372. https://doi.org/10.1016/0001-706x(90)90037-z PMID: 1978536
52. Betschart B, Jenkins JM. Distribution of iodinated proteins in Dipetalonema viteae after surface labeling. Mol Biochem Parasitol. 1987; 22: 1–8. https://doi.org/10.1016/0166-6851(87)90063-6 PMID: 3807948

53. Baschong W, Tanner M, Betschart B, Rudin W, Weiss N. Dipetalonema viteae: Extraction and immunogenicity of cuticular antigens from female worms. Experimental Parasitology. 1982; 53: 262–269. https://doi.org/10.1016/0014-4894(82)90068-6 PMID: 7037442

54. Rudin W. Comparison of the cuticular structure of parasitic nematodes recognized by immunocytochemical and lectin binding studies. Acta Tropica. 1990; 47: 255–268. https://doi.org/10.1016/0001-706x(90)90027-w PMID: 1978526

55. De Bustos A, Cuadrado A, Jouve N. Sequencing of long stretches of repetitive DNA. Sci Rep. 2016; 6: 36665. https://doi.org/10.1038/srep36665 PMID: 27819354

56. Schüler A, Bornberg-Bauer E. Evolution of Protein Domain Repeats in Metazoa. Molecular Biology and Evolution. 2016; 33: 3170–3182. https://doi.org/10.1093/molbev/msw194 PMID: 27671125

57. Bird AF. Growth and moulting in nematodes: moulting and development of the hatched larva of Rotylenchulus reniformis. Parasitology. 1990; 107–120. https://doi.org/10.1017/S0031182000001165 PMID: 1978526

58. Lažetić V, Fay DS. Molting in C. elegans. Worm. 2017; 6. https://doi.org/10.1080/21624054.2017.1330246 PMID: 28702275

59. Johnstone IL, Barry JD. Temporal reiteration of a precise gene expression pattern during nematode development. EMBO J. 1996; 15: 3633–3639. PMID: 8670866

60. Zhang Z, Bai M, Barbosa GO, Chen A, Wei Y, Luo S, et al. Broadly conserved roles of TMEM131 family proteins in intracellular collagen assembly and secretory cargo trafficking. Science Advances. 2020; 6: eaay7667. https://doi.org/10.1126/sciadv.aay7667 PMID: 32095531

61. Azzouz, Fayçal. Identification moléculaire d’une chitinase CHT-1, sa localisation et son rôle chez le nématode ‘Caenorhabditis elegans. Thèse. 2001. https://unine.swisscovery.slsp.ch/permalink/41LSLP_UNE/177trdk/alma991007578719705517

62. van der Lee R, Buljan M, Lang B, Weatheritt RJ, Daughdrill GW, Dunker AK, et al. Classification of Intrinsically Disordered Regions and Proteins. Chem Rev. 2014; 114: 6589–6631. https://doi.org/10.1021/cr400525m PMID: 24773235

63. Katuwapawala A, Peng Z, Yang J, Kurgan L. Computational Prediction of MoRFs, Short Disorder-to-order Transitioning Protein Binding Regions. Computational and Structural Biotechnology Journal. 2019; 17: 454–462. https://doi.org/10.1016/j.csbi.2019.03.013 PMID: 3107871

64. Hu G, Wu Z, Uversky VN, Kurgan L. Functional Analysis of Human Hub Proteins and Their Interactors Involved in the Intrinsically Disorder-Enhanced Interactions. Int J Mol Sci. 2017; 18. https://doi.org/10.3390/ijms18122761 PMID: 29257115

65. Betschart B, Wysk K. Analysis of the cuticular collagens of Ascaris suum. Acta tropica. 1990. https://doi.org/10.1016/0001-706x(90)90031-4 PMID: 1978530

66. Kumaki Y. Side Chain-Side Chain Interactions of Arginine with Tyrosine and Aspartic Acid in Arg/Gly/Tyr-Rich Domains within Plant Glycine-Rich RNA Binding Proteins. Journal of Biochemistry. 2004; 136: 29–37. https://doi.org/10.1093/jb/mvh091 PMID: 15269237

67. Fujimoto D. Occurrence of dityrosine in cuticlin, a structural protein from Ascaris cuticle. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry. 1978; 51: 205–207. https://doi.org/10.1016/0305-0491(78)90209-6

68. Fetterer RH, Rhoads ML. Tyrosine-Derived Cross-Linking Amino Acids in the Sheath of Haemonchus contortus Infective Larvae. The Journal of Parasitology. 1990; 76: 619–624. https://doi.org/10.2307/3282970 PMID: 2213404

69. Andersen SO. Are structural proteins in insect cuticles dominated by intrinsically disordered regions? Insect Biochemistry and Molecular Biology. 2011; 41: 620–627. https://doi.org/10.1016/j.ibmb.2011.03.015 PMID: 21477652
74. Willis JH. Structural cuticular proteins from arthropods: annotation, nomenclature, and sequence characteristics in the genomics era. Insect Biochem Mol Biol. 2010; 40: 189–204. https://doi.org/10.1016/j.ibmb.2010.02.001 PMID: 20171281

75. Mikael PE, Udangawa R, Sorci M, Cress B, Shtein Z, Belfort G, et al. Production and Characterization of Recombinant Collagen-Binding Resilin Nanocomposite for Regenerative Medicine Applications. Regen Eng Transl Med. 2019; 5: 362–372. https://doi.org/10.1007/s40883-019-00092-8

76. Acosta S, Quintanilla-Sierra L, Mbundi L, Rebolo V, Rodríguez-Cabello JC. Elastin-Like Recombinamers: Deconstructing and Recapitulating the Functionality of Extracellular Matrix Proteins Using Recombinant Protein Polymers. Advanced Functional Materials. 2020; 30: 1909050. https://doi.org/10.1002/adfm.201909050