Comparative analysis of nematode defaecation motor program

Georgy A. Slivko-Koltchik[1], Victor P. Kuznetsov[1], Kirill V. Mikhailov[1, 2], Dmitry A. Voronov[1,2] and Yuri V. Panchin[1, 2]

[1]Institute for Information Transmission Problems of Russian Academy of Sciences, Moscow, Russia
[2]A.N. Belozersky Institute of Physico-Chemical Biology MSU, Moscow, Russia
e-mail:s.koltchik@gmail.com

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Summary. The nervous system controls most rhythmic behaviours, with one remarkable exception. The defaecation motor program (DMP) in *C. elegans* is initiated by intestinal cells' pacemaker without participation of the nervous system. Our study on *Heterorhabditis megidis* and *Enoplus brevis* demonstrates that DMP machinery is not a unique feature of *C. elegans*. In *C. elegans* the role of several genes important for DMP has been experimentally studied. PBO-5/PBO-6 proton-gated ion channel mediates the signal transduction from gut cells to body muscles. We found homologues for this protein in other nematodes and demonstrated pH sensitivity of *E. brevis* body muscles. These and other DMP hallmark proteins NLP-40, AEX-2 and EXP-1 are also found in *E. brevis* and other species but not outside nematodes. Some nematodes lack individual proteins of DMP toolkit. For example, mermithids with degenerated digestive systems and with no through gut lost them all. Different nematode species possess the same non-nervous rhythmical DMP mechanism that evolved in the nematode common ancestor.

Key words: *Caenorhabditis elegans*, *Enoplus brevis*, *Heterorhabditis megidis*, nematode evolution.

Rhythmic activity plays an important role in physiology of various species. The period of rhythmic oscillations in different systems can vary from seconds to years resulting in a great variety of mechanisms for the generation and adjustment of physiological rhythms. Rhythmic model system studies are especially important as they allow in depth understanding of the mechanisms of the system functioning. One of the examples is the defaecation in nematodes, which has been studied in detail in the model species *Caenorhabditis elegans*. The defaecation motor program (DMP) is represented by the coordinated events occurring in the midgut, some muscle cells, and several neurons (Avery & Thomas, 1997). The defaecation process consists of three consecutive series of muscle contractions that occur every 45 s in adult feeding hermaphrodites in the presence of food: posterior body wall muscle contraction (pBoc), anterior body wall muscle contraction (aBoc) and expulsion step (Exp) (Thomas, 1990; Liu & Thomas, 1994). pBoc is a well-studied process, while mechanisms for aBoc and Exp are incompletely examined. DMP is primarily controlled by the mid-gut cells, which can autonomously generate rhythmical oscillations of calcium concentration in the cytoplasm (Espelt *et al.*, 2005; Teramoto & Iwasaki, 2006). The concentration of calcium initially increases in the posterior cells, and then a wave of high calcium concentration is propagated from the posterior to anterior midgut. This wave temporally coordinates the consecutive acts of defaecation: pBoc, aBoc and Exp. In this process, one of the main mechanisms of signal transmission relies on the body cavity acidification: cells of the midgut pump out protons that act on the muscle cells as a transmitter (Beg *et al.*, 2008; Pfeiffer *et al.*, 2008). In a species related to *C. elegans*, *Heterorhabditis megidis*, DMP is accompanied by periodical fluctuations of the membrane electrical potential in the intestinal cells, which are also synchronised through gap junctions (Kuznetsov *et al.*, 2017). So, in these Rhabditida species, *C. elegans* and *H. megidis*, the endoderm cells act like brain central pattern generator interneurons, and similarly to neurons these intestinal cells can be synchronised along the cellular network *via* electrical synapses (gap junctions) (Kuznetsov *et al.*, 2016).

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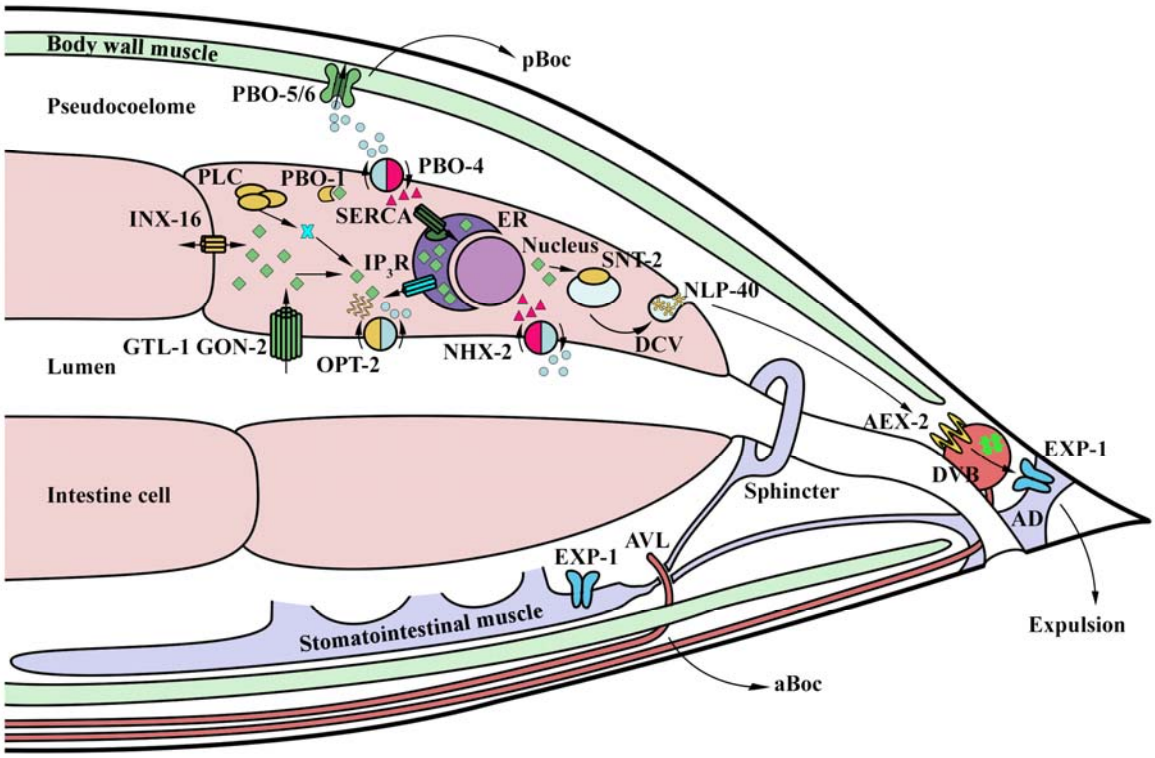

Fig. 1. Schematic diagram of the posterior part of *C. elegans* nematode body with the midgut, surrounding muscles and neurons shows the main players in signalling pathways involved in the defaecation behaviour. IP_3 (inositol trisphosphate) (cyan cross) and Ca^{2+} ions (green rhomb) play the key role in the initiation, regulation and propagation of the oscillatory process (Dal Santo *et al.*, 1999). IP_3Rs (inositol trisphosphate receptors) are activated by IP_3 that forms by hydrolysis of phospholipid PIP_2 (phosphatidylinositol 4, 5-bisphosphate) by phospholipase C (PLC) located in the plasma membrane. Calcium wave is initiated by Ca^{2+} ion release from the endoplasmic reticulum triggered by IP_3Rs (Baylis & Vázquez-Manrique, 2012). Wave propagates from the posterior to anterior via gap junctions (INX-16) (Peters *et al.*, 2007). TRPM channels GTL-1/GON-2 (Kwan *et al.*, 2008) increase the intracellular concentration of Ca^{2+}. To keep the process cycled the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) restores Ca^{2+} to the baseline levels (Hoon Cho *et al.*, 2000). PBO-1, a calcium binding protein, coordinates Na^+ (magenta triangle) and H^+ (grey circle) ion exchange by PBO-4/NHX-7 with cyclic calcium oscillations (Beg *et al.*, 2008). PBO-4/NHX-7 anion exchanger transfers protons to pseudocoelome and sodium into the cell through the basolateral membrane. On the apical side, NHX-2 ion exchanger transfers protons to lumen and sodium into the cell through the basolateral membrane (Pfeiffer *et al.*, 2008). It is coupled with a high capacity oligopeptide transporter OPT-2/PEPT-1 that absorbs dipeptides (yellow waves) and protons from the lumen (Wagner *et al.*, 2011). PBO-4/NHX-7 proton afflux from the gut cells activates PBO-5/PBO-6 proton-gated ion channel heterodimer, which depolarises the posterior body wall muscles and causes their contraction (pBoc). aBoc is the most incompletely studied event in this process. It is known that killing AVL neuron causes strong defects of the aBoc step (Wang & Sieburth, 2013). Although AVL is GABAergic neuron, GABA dysfunction mutants have a normal aBoc step. Thus anterior body muscles are controlled by AVL motoneurons but do not require GABA in the pathway from Ca^{2+} wave initiation to muscle depolarisation. Synaptotagmin (SNT-2) localised on the dense core vesicles (DCV) being bound to calcium promote the release of neuropeptide-like protein NLP-40 from the basolateral surface of intestinal cells (Zhao & Schafer, 2013). Secreted NLP-40 activates AEX-2/GPCR receptor on AVL and DVB neurons. cAMP signalling pathway inside the neuron leads to a calcium influx, neuron excitation and GABA release (green octothorpe) (Wang *et al.*, 2013). Excitatory GABA receptor EXP-1, located on enteric muscles (electrically coupled *via* gap junctions (Altun *et al.*, 2009) anal sphincter, anal depressor (AD) and stomato-intestinal muscles), binds GABA and causes contraction. This results in the final expulsion DMP step.

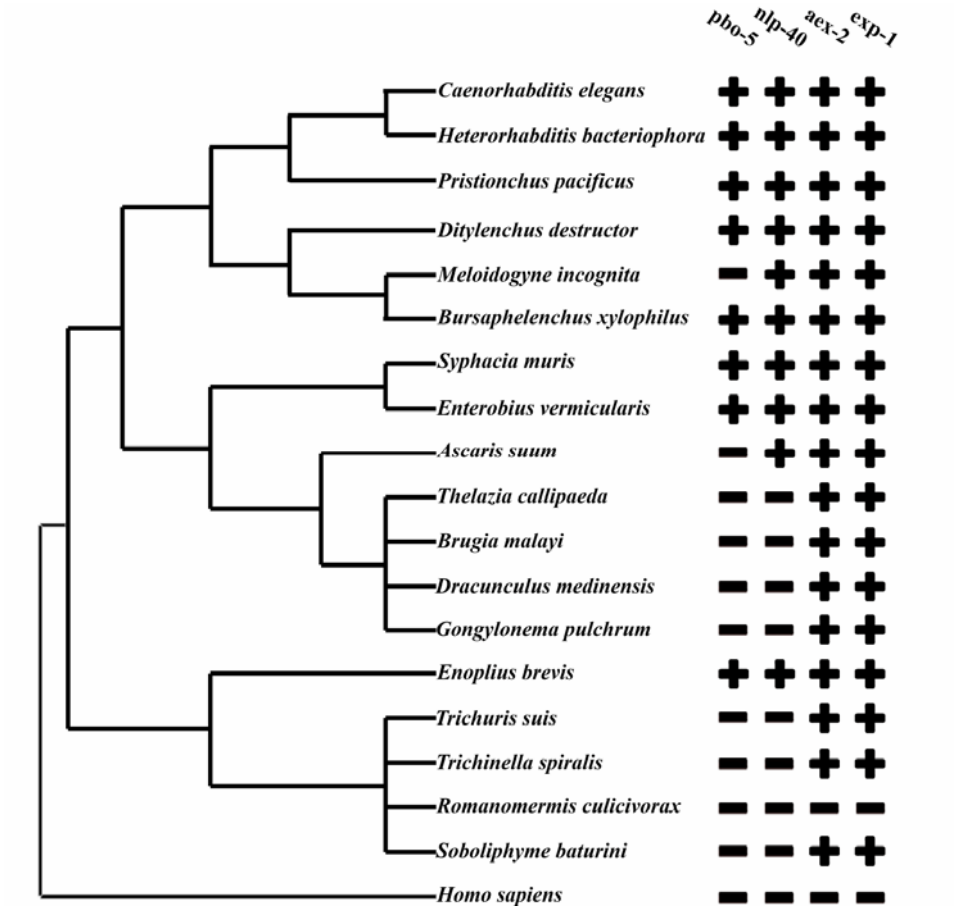

Fig. 2. Cladogram (Blaxter *et al.*, 1998) of nematode species. *Homo sapiens* is an outgroup. Presence or absence of four hallmark proteins involved in the nematode defaecation process in various species is shown on the right.

It remained unclear if the defaecation mechanism is a unique feature of the investigated representatives of Rhabditida or exists in other nematodes. In the previous paper we described membrane potential cycling using intracellular electrophysiological techniques in the midgut cells of a non-rhabditid nematode *Enoplus brevis* (Enoplida) (Slivko-Koltchik *et al.*, 2018) resembling DMP oscillations in *C. elegans* and *H. megidis*. We also found that, similarly to *C. elegans* and *H. megidis*, the gut cells in *E. brevis*, are electrically connected through gap junctions. Here we compare molecular elements of the DMP machinery in phylogenetically distant species, including *E. brevis* and consider its evolutionary origin in nematodes.

At present, the roles of several dozen genes important for DMP in *C. elegans* have been experimentally studied (Branicky & Hekimi, 2006) (Fig. 1). In combination with the availability of different nematode species genomic sequences, this makes it possible to study the evolution of the DMP within the whole Nematoda phylum.

We have analysed four genes *aex-2*, *nlp-40*, *pbo-5/6* and *exp-1*, specifically important for *C. elegans* DMP execution. The presence of true orthologues of these genes in other species can indicate the presence of DMP in these animals. We conducted the bidirectional best hits search (BBH) (Overbeek *et al.*, 1999) for the selected genes among 18 currently available complete genomes of the Nematoda phylum and carried out phylogenetic analysis of a wide superfamily of the Cys-loop ligand-gated ion channels including PBO-5/6 and EXP-1 proteins (Jaiteh *et al.*, 2016).

Predicted expression of PBO-5/6 in *E. brevis* was confirmed in physiological experiments that demonstrate body muscle pH sensitivity in this species.

MATERIAL AND METHODS

Identification of proteins homologous to the *C. elegans* DMP related genes was performed by sequence similarity search using the BLASTP program and the WormBase ParaSite

(https://parasite.wormbase.org) and NCBI protein databases (https://www.ncbi.nlm.nih.gov/). In the case of nematode *E. brevis* we assembled the available transcriptomic data (ERR660661 from the NCBI BioProject PRJEB7588) with Trinity software (Grabherr *et al.*, 2013) and extracted translated ORF from transcripts with TransDecoder (Haas *et al.*, 2013). The orthology of proteins found by BLASTP search seeded with *C. elegans* DMP related genes was tested by bidirectional best hits (BBH) method (Overbeek *et al.*, 1999) that identifies the pairs of genes in two different genomes that are more similar to each other than either is to any other gene in another genome. If the reciprocal best BLAST hit coincided with the initially used *C. elegans* protein, both proteins were considered orthologous. This procedure was used for four proteins searched in 18 various species (Fig. 2). For receptor's phylogenetic tree, selected nematode sequences and *H. sapiens* receptors (Beg & Jorgensen, 2003) were used. This protein collection was aligned by MUSCLE program (Edgar, 2004) and the phylogenetic tree was constructed by the neighbour-joining algorithm.

Enoplus brevis were extracted from sandy littoral at the White Sea biological station of the Russian Academy of Sciences "Kartesh" in the summer of 2017 and 2018 and kept in seawater at 4°C. The adult *Romanomermis culicivorax* were harvested from larvae of chironomids (lake flies) obtained at the local pet store. All physiological experiments were carried out at room temperature (30°C). The posterior fragments of worms containing the body muscles were cut out using fine forceps and scissors, placed in a Petri dish with a bottom coated by silicone rubber, and fastened by thin metal needles or braces, fixing one point and allowing the loose end to move freely. As a bath media for *E. brevis* we used Millipore-filtered sterile sea water (buffered with 1mM HEPES to pH 7); for *R. culicivorax* we used Hanks solution (137 mM NaCl, 5.4 mM KCl, 0.25 mM, Na_2HPO_4, 0.44 mM KH_2PO, 1.3 mM CaCl, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$; 1mM HEPES; pH = 7.4). To study the action of acetylcholine (ACh) on the posterior body wall muscles, the ACh was added to Petri dish with the final concentration of 10^{-6} M. To study the effects of pH changes, bath media in Petri dish was replaced by the same media buffered to pH 6. The muscle contractions were video recorded. To visualise the contractions, 30 s video fragments were split to image sequences and uploaded into FIJI image analysis tool (Dobretsov *et al.*, 2017). Total images were acquired by Z-projection algorithm (z-time) with the maximum intensity parameter.

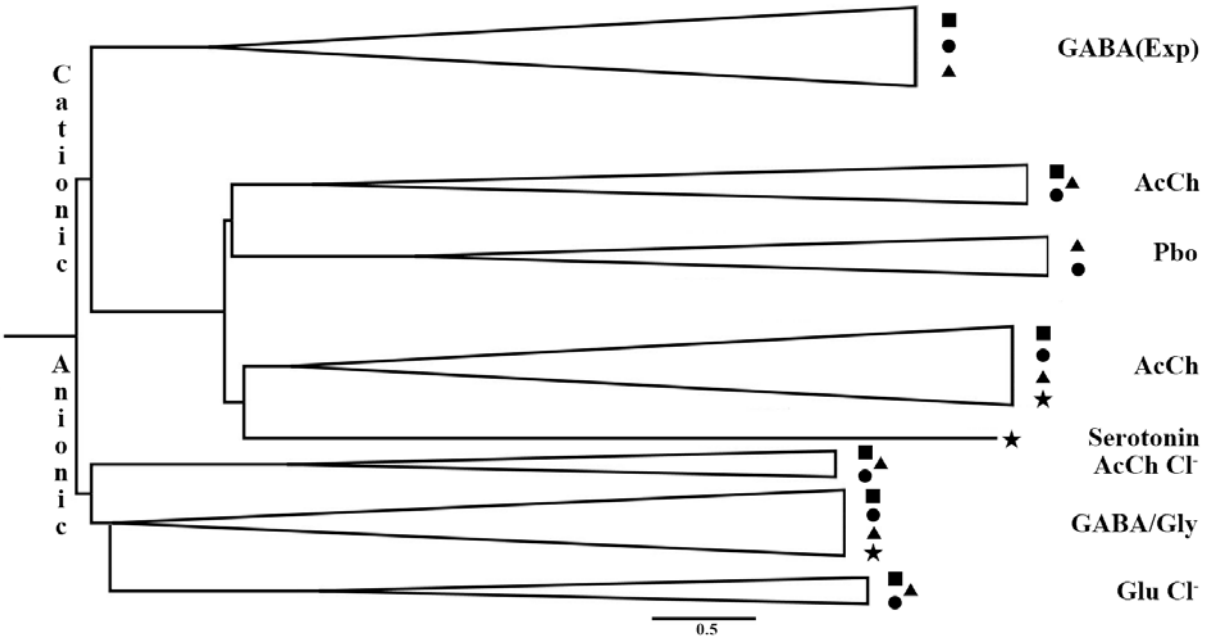

Fig. 3. Phylogeny tree of Cys-loop receptors in nematodes species with *Homo sapiens* outgroup. Only nematodes with the presence of either *exp-1* or *pbo-5* were selected. Star – *Homo sapiens*. Circle – *Caenorhabditis elegans*. Triangle – *Heterorhabditis bacteriophora*, *Pristionchus pacificus*, *Ditylenchus destructor*, *Bursaphelenchus xylophilius*, *Syphacia muris*, *Enterobius vermiculari*, *Enoplius brevis* (have both *pbo-5* and *exp-1*). Square – *Meloidogyne incognita*, *Ascaris suum*, *Thelazia callipaeda*, *Brugia malayi*, *Dracunculus medinensis*, *Gongylonema pulchurum*, *Trichuris suis*, *Trichinella spiralis*, *Soboliphyme baturini* (with *exp-1* but without *pbo-5* gene).

RESULTS AND DISCUSSION

BLAST search seeded by *C. elegans* orthologues showed that two of the four genes from the selected DMP hallmark genes, namely, *aex-2* and its ligand *nlp-40*, were found only among nematodes (less than E-value 10^{-87}) and were not found in species outside Nematoda species (more than E-value 10^{-10}). The other two genes, *pbo-5* and *exp-1*, have homologues with various functions from both inside and outside the phylum Nematoda. To search for the true orthologues of these genes among the all animals, we used the bidirectional best hits method (BBH) that confirmed the presence of *pbo-5* and *exp-1* in several nematode species. It is known that the BBH in some cases can lead to misinterpretations and therefore it requires verification using phylogenetic analysis (Dick *et al.*, 2017). The *pbo-5* and *exp-1* genes belong to the widespread class of Cys-loop ligand-gated ion channels such as nicotinic acetylcholine, GABA, glycine, glutamate and serotonin ionotropic receptors; *exp-1* encodes unusual excitatory GABA receptors (Beg & Jorgensen, 2003). In Cys-loop family tree, *exp-1* from *C. elegans* clusters together with its putative orthologues found by BLAST and BBH search in all studied nematodes with the exception of *R. culicivorax*. At the same time, this phylogenetic tree confirms the presence of *C. elegans pbo-5* homologues in eight nematode species, including *E. brevis*.

The results of our search for DMP related genes within 18 nematodes with sequenced genomes are presented in the Fig. 2. Eight species have all four genes under consideration *aex-2*, *nlp-40*, *pbo-5* and *exp-1*; two species do not have *pbo-5* and have *nlp-40*, seven species do not have *pbo-5* and *nlp-40*, and one lacks all four DMP genes. Since the studied genes are found in phylogenetically distant species, including *E. brevis*, which is located at the root of the phylogenetic tree of Nematoda (Blaxter *et al.*, 1998) , it can be assumed that the common ancestor of Nematoda had all four hallmark DMP genes. Further, in the course of evolution some species lost some of these genes, while the *R. culicivorax*, which is a parasitic mermithid nematode with degenerated digestive systems and with no through gut, lost them all.

The presence of *C. elegans pbo-5/6* orthologues

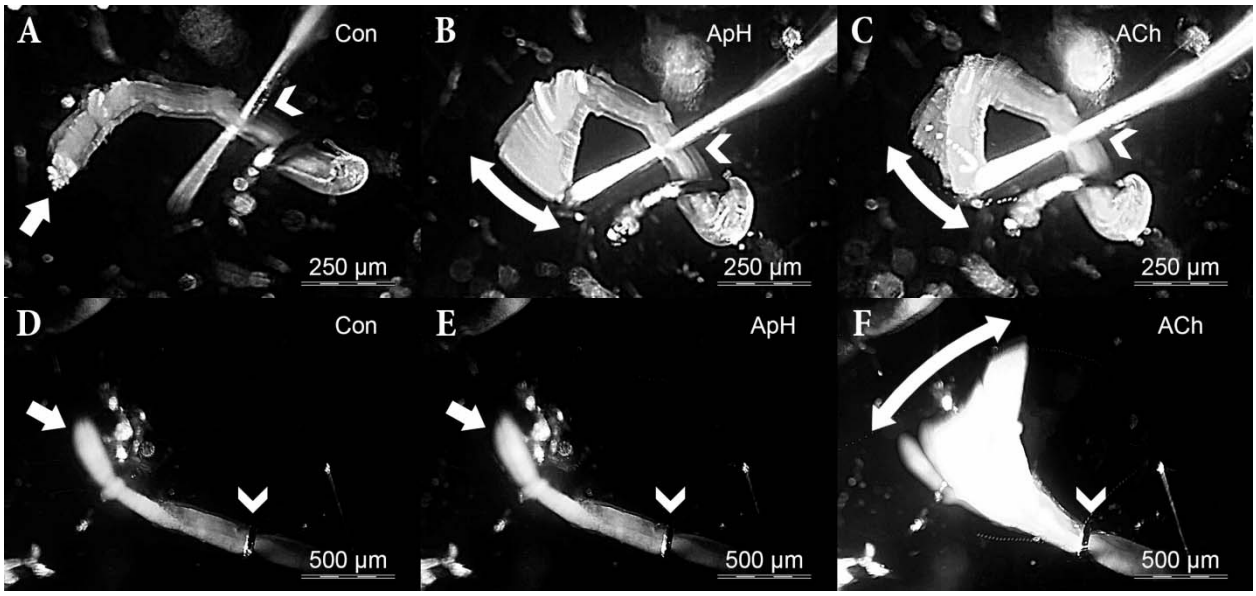

Fig. 4. Effects of acetylcholine (ACh) and acidification (ApH) on body muscle contraction in *Enoplus brevis* (A, B, C) and *Romanomermis culicivorax* (D, E, F) nematodes. Preparations of posterior body with exposed muscles were fastened in chamber by thin metal needles or braces (white arrowhead), fixing one point and allowing the loose end (white arrow) to move freely. To visualise the preparation movement video recordings were taken during the entire experiment; 30 s fragments were split into frames and image stacks were downloaded to the FIJI image analysation tool and Z-projected (z-time) with the maximum intensity parameter. Motion (shown by double arrows) appear as a sequence of the preparation images during 30 s time period. Plain bath medium applied in control (Con) experiments (A, D) produced no effect. Application of acidified medium (pH 6) activated *E. brevis* muscle and had no effect in *R. culicivorax*. In the positive control, ACh (10-6M) application produced muscle contraction in both species (C, F). The sequence of the same experimental procedures: control – ApH – ACh with intermediate washouts was repeated with the same results for four times for both nematode species.

in *E. brevis* implies that its muscle cells are pH sensitive and they will contract in response to external medium acidification. By contrast, in *R. culicivorax*, which lacks *pbo-5/6* genes, the muscles will not respond to pH changes. At the same time, muscle cells of both species will be able to respond to acetylcholine application in the same way due to nicotinic acetylcholine receptors' conservation in all nematode species (Figs 2 & 3). We have developed a preparation where cuticle and hypoderm were removed from the p art of the nematode body, hence muscle cells were exposed to physiological solution and muscle contractions were recorded by a video camera. In the experiments presented in Fig. 4 acidification of the medium in the vicinity of the posterior body muscles caused contraction in *E. brevis* (Fig. 4B), while *R. culicivorax* muscle cells showed no response (Fig. 4E). However, the application of acetylcholine to the medium resulted in contraction of the posterior muscles in both *E. brevis* and *R. culicivorax* (Fig. 4C, F). The muscle contraction was not caused by a mechanical effect of the media replacement in the experimental chamber, given that during control replacements the contraction has not occurred (Fig. 4A, D). Thus, our data from physiological experiments confirms the results of bioinformatics research for DMP related genes in nematodes.

Previously in the physiological experiments we have demonstrated the presence of a specific rhythmic activity associated with DMP in the midgut of *E. brevis* and *H. megidis* (Kuznetsov *et al.*, 2017; Slivko-Koltchik *et al.*, 2018). New bioinformatics and physiological study reported here confirms that the intricate DMP machinery first discovered in *C. elegans* is not a unique feature of this species but is a nematode-specific biological creation that evolved in the common ancestor of nematodes.

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Г.А. Сливко-Кольчик, В. Кузнецов, К. Михайлов, Д.А. Воронов и Ю.В. Панчин. Сравнительный анализ моторной программы дефекации нематод.

Резюме. Обычно ритмические формы поведения у животных управляются нервной системой. Но есть одно исключение. Дефекационная моторная программа (ДМП) нематоды *C. elegans* управляется пейсмекером из кишечных клеток без участия нервной системы. Наши предыдущие исследования на нематодах *Heterorhabditis megidis* и *Enoplus brevis* показали, что механизм ДМП не является уникальной особенностью вида *C. elegans*. Роль нескольких генов, необходимых для организации ДМП, подробно изучена. Протон-зависимые ионные каналы PBO-5/PBO-6 являются важными посредниками при передаче сигнала от кишечника к мышечным клеткам тела. Мы нашли гомологи этого белка в других нематодах и показали чувствительность мышц нематоды *E. brevis* к изменению уровня pH. PBO-5/PBO-6 и белки NLP-40, AEX-2 и EXP-1 были обнаружены нами в геноме *E. brevis* и у других нематод, но не у видов за пределами типа Nematoda. Некоторые представители типа потеряли определенные белки, необходимые для ДМП. Например, все перечисленные гены потеряны у мермитид – паразитов с дегенерированной пищеварительной системой, не имеющих сквозной кишки. Различные виды нематод имеют сходный, независимый от нервной системы механизм ДМП, возникший у общего предка всех нематод.