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Differential sensitivity of myeloid and lymphoid cell populations to apoptosis in peritoneal cavity of mice with model larval *Mesocestoides vogae* infection

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Summary

The metacestode stage of the tapeworm *Mesocestoides vogae* (*M. vogae*) has the ability of asexual growth in the peritoneal cavity of rodents and other intermediate hosts without restriction. Early immunological events have decisive role in the establishment of infection. In the present study we investigated the kinetic of myeloid and lymphoid cell populations and the proportions of cells undergoing apoptosis in peritoneal cavities of mice within the first month after oral infection with *M. vogae* larvae. Proportions of cell phenotypes and apoptotic cells were examined by flow cytometry and by microscopical analysis of cells following May/Grünwald staining and fluorescent stain Hoechst 33234, respectively. Total numbers of peritoneal cells increased and their distribution changed towards accumulation of myelo-monocytic cell lineage in the account of reduced proportions of lymphoid cells. CD4+ T cell subpopulations were more abundant than CD8+ and their proportions elevated within two weeks post infection (p.i.) which was followed by a significant decline. Expression level of CD11c marker on myelo-monocytic cells revealed phenotype heterogeneity and proportions of cells with low and medium expression elevated from day 14 p.i. along with concurrent very low presence of $CD11c^{high}$ phenotype. Lymphoid cell population was highly resistant to apoptosis but elevated proportions of myeloid cells were in early/late stage of apoptosis. Apoptosis was detected in a higher number of adherent cells from day 14 p.i. onwards as evidenced by nuclear fluorescent staining. By contrast, cells adherent to larvae, mostly macrophages and eosinophils, did not have fragmented nuclei. Our data demonstrated that apoptosis did not account for diminished population of peritoneal lymphoid cells and substantial proportions of myeloid cells seem to be more susceptible to apoptotic turnover in peritoneal cavity of mice with ongoing *M. vogae* infection, suggesting their important role in the host-parasite interactions.

Keywords: *Mesocestoides vogae* larvae; mouse; peritoneal cells; apoptosis

Introduction

Mesocestoides vogae (syn. *M. corti*) infection in rodents has gained increasing acceptance as a model for proliferating larval cestode infections in immunological and chemotherapeutic studies. The metacestode stage, known as a tetrathyridium, multiplies asexually by anterio-posterior fission and budding mostly in the peritoneal cavities of mice or rats (Specht & Voge, 1965). Larvae also invade the livers of the hosts, causing parenchymal destruction what ultimately leads to the initiation of fibrogenesis and enclosing larvae in the granulomatous fibrous capsules (Specht & Widmer, 1972; Hrčková *et al*., 2010). After oral infection of highly

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susceptible ICR strain of mice, larvae appear in the peritoneal cavity as early as on day 3 post infection (p.i.), triggering early pro-inflammatory immune response with larvicidal potential (Vendelova *et al*., 2015). Laboratory mice seem to be very suitable animals for establishment of the infection by both oral and intraperitoneal route, what however, influences the kinetics of immune response in the peritoneal cavity (PerC) (White el al., 1982). It was showed that the local immune response in PerC of ICR strain, examined on mRNA levels, is characterized by minimal IFN-γ production up to day 7 p.i., transient IL-4 elevation, followed by a dominant IL-10 production. Beside recruited eosinophils, parasite-elicited monocytes/macrophages attained the characteristic of alternatively activated macrophages (which also been called M2 myeloid cells (Martinez *et al*., 2009), based on the high expression of mRNA for Fizz-1, YM1 and Arg-1proteins (Vendelova *et al*., 2015; Horsnell & Brombacher, 2010).

Differentiation of M2 cells is induced by IL4/IL-13 cytokines through the IL-4Rα (Brombacher *et al*., 2009) that also favor T helper (Th) 2 cell polarization. Intraperitoneal injection of live and in lesser extent heat-killed *M. corti* larvae, elicited a significant recruitment of CD11c+ cells up to day 7 p.i. (Vendelova *et al*., 2016b).

Resident peritoneal cells in various strains of mice also contain B and T lymphocytes (Composto *et al*., 2011). Lymphocytes were observed in low numbers in PerC of C57BL/6 strain of mice on cytospin preparations within onset of *M. corti* infection (O´Connel *et al*., 2009). A key feature of helminth infections is an early induction of strong Th2-biased immune response in their hosts. After stimulation, CD4+T cells differentiate to distinct subsets characterized by their functions and cytokine profiles (Th1 vs. Th2 cells). Increasing number of studies report that excreted/secreted products from helminths play the crucial role in promoting Th2 response and inducing immunosuppression in the chronic stage of infections (Rodriguez-Sosa; 2002; Vuitton & Gottstein, 2010; Vendelova *et al*., 2016a; Vendelova *et al*., 2016b). Multiple types of immunosuppressive cells operate in the immune system, including CD4+Foxp3+ regulatory T cells, B cells and macrophages which dampen Th2 immunity towards T regulatory type (Maizels *et al*., 2012, Gordon & Martinez, 2010). In avoiding severe peritoneal inflammation and liver pathology with the fatal consequences for the hosts, fast growing *M. vogae* infection has to promote early down-regulation of Th2 type of immunity (Vendelova *et al*., 2015). IL-4, a canonical Th2 cytokine involved also in liver fibrotic response to invading tetrathyridia, was shown to be the essential cytokine in the regulation of *M. corti* infection in C57BL/6 mice (Rawat *et al*., 2003) and its absence resulted in augmented proliferation of larvae. Numerous studies showed that immune response to flatworm infections is regulated specifically in different periods of infection and developmental stages by parasite-derived molecules (Maizels, 2009; Voehringer *et al*., 2004; Vuitton & Gottstein, 2010) employing also programmed cell death - apoptosis (Nono *et al*., 2012; Spotin *et al*., 2012; Zepeda *et al*., 2010). Apoptotic processes can be divided into extrinsic and intrinsic pathways. The extrinsic pathway involves the members of TNF-α and TNF-α receptor family of proteins and down-stream activation of the caspases cascade. The key feature of the intrinsic apoptotic pathway is the breakdown in the integrity of the mitochondrial outer membrane. Early apoptotic events include exposure of phosphatidylserine on the cell surface, whereas morphological changes associated with the later stage is nuclear and chromosomal fragmentation, chromatin condensation, cell shrinkage followed by the formation of apoptotic bodies (Wyllie, 1997). Thus the selective elimination of a certain cell type might be a way by which flatworms are able to maintain the suitable immunological settings and reduced harmful pathology.

The aim of present study was to investigate the kinetics of cellular immune response in peritoneal cavity of ICR strain of mice infected with proliferating tetrathyridia of *M. vogae*. We also assessed the kinetics of apoptosis in lymphoid and myeloid cell populations as well as in cells adherent to the larval surface within the first month of infection.

Material and Methods

Parasites and infection

Infection with tetrathyridia of *M. vogae* is maintained by intraperitoneal passage through ICR-strain of mice at the animal facilities of Institute of Parasitology of the Slovak Academy of Sciences under pathogen-free conditions. Prior to initiation of the experiment, tetrathyridia were recovered from the peritoneal cavity of a mouse with long-term infection (3 months). In the experiment, male mice of the same strain aged 8weeks were orally inoculated with 65±2 tetrathyridia in 0.2ml of sterile PBS by the oral gavage.

Peritoneal exudate cells

Mice were sacrificed by cervical dislocation and peritoneal exudates cells (PEC) were collected by washing the peritoneal cavity with RPMI medium (Biochrom-Merck, Germany) containing 2mM of stable glutamine and supplemented with 10 % heat-inactivated bovine fetal serum (Biochrom-Merck, Germany), 100U/ml penicillin, 100µg/ ml streptomycin, 10µg/ml gentamicin and 2.5µg/ml amphotericin B (complete medium, CM) (all from Sigma-Aldrich, St. Louis, USA). The PEC suspension was washed with LPS-free Dulbecco phosphate buffered saline (DPBS), re-suspended in CM and counted. Total cell numbers obtained from PC of each mouse was enumerated. For preparation of cell smears of PEC, approximately 0.1×10^6 cells from PerC of infected mice were re-suspended in DPBS, placed on glass slides and fixed in 70 % methanol. Viability of the cells was more than 95 % as determined by trypan blue exclusion. Cell suspensions were used for phenotypic analysis and level of apoptosis performed by flow cytometry. In addition, part of cell suspensions were left to adhere onto glass slides which were placed into quadriPERM® chambers (Sarstedt, Germany) for 3 h at 37°C, 5 % CO_2 in CM allowing cells to adherence. After washing, the non-adherent cells were collected and used to

prepare cell smears. Both cell preparations were used to assess apoptosis by morphological criteria.

Isolation of cells attached to larval surface
To investigate whether PEC are adhered to the larval surface, larvae isolated from peritoneal cavities of individual mice were properly washed several times in PBS, placed into complete medium and incubated for 2 h at 37 °C with shaking. Medium was collected, centrifuged and cell pellets were isolated. Then larval adherent cells isolated on indicated time points after infection were pooled and cell smears were prepared. Slides were stained with either May- Grünwald/Giemsa solutions or Hoechst 33342 (ThermoFisher Scientific, USA) for morphological detection of apoptotic cells.

Flow cytometry
Single-cell suspension of PEC isolated from intact and infected mice were prepared as described above. Cells were re-suspended in CM (0.5×10^6 cells/100µl) and stained with anti-mouse monoclonal antibodies: anti-CD4-FITC (clone GK1.5), anti-CD8-PE (clone 53–6.7), anti CD 11c (PerCP Cyanine 5.5). Isotype-matched monoclonal antibodies (isotype IgG2b and isotype IgG2a and IgM) were used as controls (all antibodies were from eBioscience, Germany). Cell samples were incubated with antibodies in the dark for 30 min at room temperature and washed twice with FACS buffer. Finally, cells were re-suspended in 100µl of FACS buffer. Phenotypic analysis was performed on a FACS Canto (Becton Dickinson Biosciences, USA) and data were analyzed using FACS Diva software.

Annexin V/propidium iodide apoptosis assay
Cell suspensions isolated from peritoneal cavities of mice were also used to study stages of apoptotic process in addition of phenotypic analysis. Cells were re-suspended in CM (1×10^6 cells/100µl) and placed to ice until the staining. Apoptosis was detected with BD Pharmingen Annexin V-FITC Apoptosis Detection Kit (APO Alert Annexin V, Clon Tech, California, USA) according to manufacturer's instructions. Briefly, treated cells were centrifuged for 10 min at 1500 rpm to remove CM. Later, the cells were washed and resuspended in 200 µl of the binding buffer. Apoptotic cells were detected after their staining with 5 µl of Annexin V and 10 µl of propidium iodide solution. Cells were then incubated at room temperature in the dark for 15 min. Analysis was performed by flow cytometry.

May-Grünwald staining of cell smears
Slides with cell smears and adherent cells were stained with May-Grünwald/ Giemsa solutions according to the standard procedure. The stained cells were then observed under the light microscope (Olympus, Prague, Czech Republic) and analysis of cell types was done at 1000 x magnification.

Detection of apoptosis on cell smears
Morphological changes of cells undergoing late stage of apoptosis can be detected by condensed chromatic and/or fragmented nuclei after staining of cell nuclei with DNA-specific dye Hoechst 33342 (ThermoFisher Scientific, USA). The final dye concentration was 2 µg/ml in PBS. Cell smears were fixed with 4 % formaldehyde for 10 min at room temperature and washed with PBS. Cells were then incubated with Hoechst dye for 20 min at room temperature in dark. The stained cells were observed under a fluorescence microscope (Leica DM4000 B, Germany) and cells with above mentioned morphological changes were counted. At least 200 total cells were counted on slides with cells isolated from 3 mice for each time-point. Finally proportions (%) were calculated and are expressed as mean ± SD.

Statistical analysis
Data obtained from individual analyses for indicated number of samples were finally calculated as mean ± SD. Results were analyzed either by one-way ANOVA followed by Tukey's post- hoc test. In the grouped analyses two-way ANOVA and Sidak's post-hoc test were applied. Data were evaluated using GraphPad Prism (version 7) (GraphPad Software, Inc., San Diego, CA, USA) and differences were regarded as significant when $p<0.05$.

Ethical Approval and/or Informed Consent

The experiment was carried out according to the guidelines for the care and use of experimental animals No. 289/2003 and approved by State Veterinary and Food Administration of the Slovak Republic under project No. 3871/15-221d.

Results

Larval proliferation is associated with accumulation of myeloid cell types in the peritoneal cavity
Metacestodes of *M. vogae* extensively reproduce within the mouse liver and PerC (Specht and Voge, 1965; Specht and Widmer, 1972) and the first larvae appeared in PerC already on day 3 p.i. (personal observations). Flow cytometry analysis revealed that with progressing infection distribution of cell types has been dramatically changed towards accumulation of myelo-monocytic cell lineage in the account of reduced proportions of lymphoid cell types (Fig. 1A, C). We showed that the total cell numbers in PerC gradually increased as the infection progressed and reached $64.8 \pm 18.1 \times 10^6$ cells on day 28 p.i. (Fig. 1B). Analysis of stained cell smears confirmed that the proportions of lymphocytes gradually decreased and recruited myelo-monocytic cell fraction comprised mixture of macrophages cells having different staining pattern and size, multinucleated giant cells as well as eosinophils, and in the lesser extent, neutrophils (Fig. 1D).

Proportions of CD4 +/CD8+ T lymphocytes are differentially regulated during onset of infection

The general feature during establishment of tissue-dwelling helminth infections is proliferation of parasite-specific T cells which display highly polarized Th2 cytokine profiles. We next analysed the proportions of T cell subpopulations by flow cytometry. As depicted in Fig. 2, CD4+ cells were more abundant population than CD8+ in PerC of healthy ICR mice. Their proportions elevated within two weeks p.i. what was followed by a significant decline. Proportions of CD8+ cells did not change significantly up to day 28 p.i., although total lymphoid population increased (not shown) as is depicted from a massive accumulation of inflammatory cells. We assume that the composition of lymphoid population has been changed from day 14 p.i. towards elevated numbers of other lymphoid cells, probably B cells and NK cells.

Differential expression level of CD11c marker on myeloid cells during onset of infection

CD11c cell surface molecule is an important molecule in regulating immune responses and its expression depends on cell type

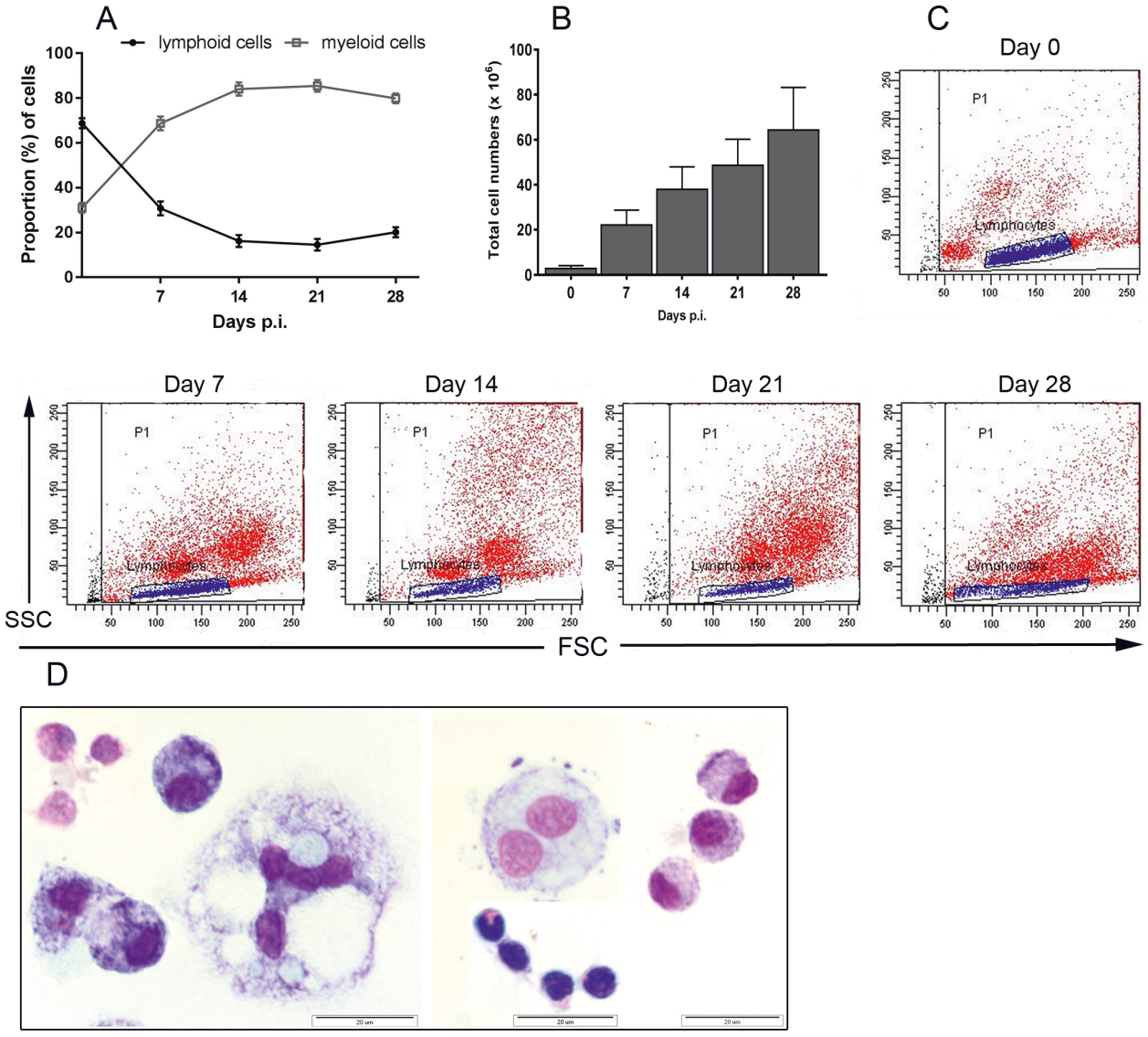

Fig. 1. *Mesocestoides vogae* infection is associated with accumulation of myeloid cell in the peritoneal cavity of infected mice. ICR mice were infected orally with 65 ± 2 tetrathyridia. At the indicated time points, proportions of lymphoid and myeloid cells (A) and total cell numbers (B) in the peritoneal cavities of healthy and infected mice were assessed by flow cytometry. Data are present as the means ± SD. (C) Representative dot plot shows FSC and SSC gating of total peritoneal cells. (D) Representative images of May-Grünwald/Giemsa-stained cell smears prepared from peritoneal fluid of infected mice. The images show populations of myeloid cells including giant cell, granulocytes and lymphoid cells. Original magnification = 1000 x; Scale bar: = 20 µm.

and state of activation (Singh-Jasuja *et al*., 2013). In the present study myelo-monocytic cells from PerC of intact and infected mice were assessed for the expression level of this marker showing surface phenotype heterogeneity after gating on cells designated as CD11c ^{low}, CD11c^{medium} and CD11c^{high} (Fig. 3 A, B). Cell phenotype CD11c^{low} was dominant in intact mice and in infected mice up to day 7 p.i. During this period CD11c^{medium} cells formed smaller population, what was enlarged by day 28 p.i. representing 40.1 ± 4.0 %. High expression of CD11c is considered to be more restricted to mature dendritic cells (Lai *et al*., 1998), but in our study CD11c^{high} phenotype did not form discrete cell population, neither in intact peritoneal cells, nor in peritoneal exudate cells from infected mice, and represented very low population through examined period. Moderate elevation of this phenotype was detected on day 14 p.i. (16.8 ± 1.1 %).

Myeloid cells are more susceptible to apoptosis than lymphoid cells

Helminth parasites evolved several strategies in inducing immunosuppression and increasing body of evidence propose apoptosis as an effective mechanism in reducing viability of a certain cell populations or their complete removal to avoid immune attack (Solano *et al*., 2006, Serradell *et al*., 2007, Zepeda *et al*., 2010). To investigate which cell type is more resistant to apoptosis, PEC were stained with annexin V and propidium iodide. Gating strategy involved separate analysis of lymphoid and myeloid cell populations (Fig. 4A) followed by discrimination of three populations: live cells, cells in the early stage of apoptosis and cells in late stage including dead cells. Interestingly, nearly all lymphoid cells retained their full viability through the examined period and dead cells were not detected (Fig. 4 B). In contrast, phenotypically heterogeneous myeloid population underwent programmed cell death at a various levels (Fig. 4C). In PerC of intact mice more than 80 % of these cells were live and small proportions were in the early and late apoptosis, respectively. With progressing infection proportions of live cells diminished, whereas percentage of early apoptotic cells increased, most notably from day 14 p.i. From this day, elevated number of cells in late stage of apoptosis was recorded. As shown by images from cell distribution analyses based on size (FSC) and granularity (SSC) (Fig. 4D upper panel), live cells were co-localized with macrophages and early apoptotic cells were situated at positions where granulocytes occur, probably eosinophils or other granular cells. Late apoptotic cells formed mixed population of giant cells and large granular cells, which were stained intensively with acidic stains giving light blue/pink appearance on the cell smears (see Fig.1D).

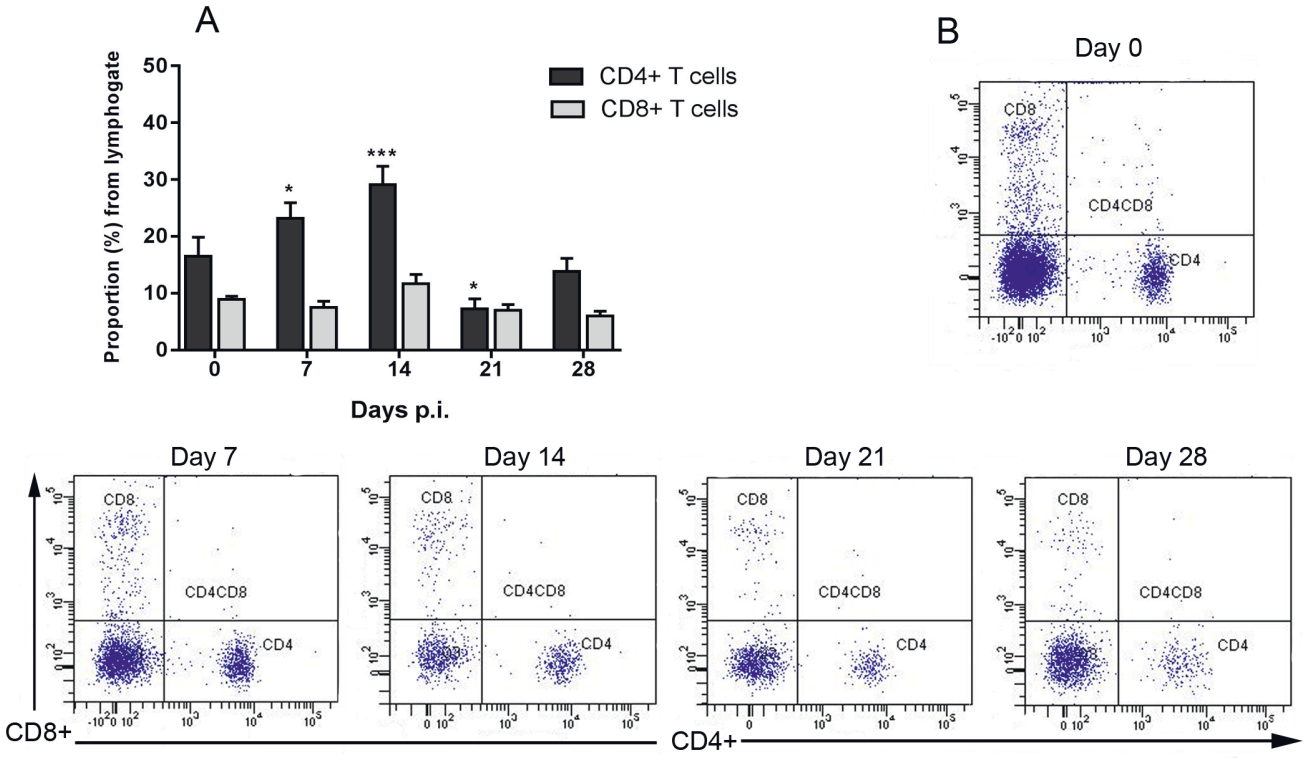

Fig. 2. Proportions of CD4 +/CD8+ T lymphocytes in peritoneal cavity are differentially regulated during onset of *Mesocestoides vogae* infection. (A) Peritoneal cells isolated from healthy and infected mice analyzed for CD4 and CD8 from lymphogate by flow cytometry. Data are expressed as the means ± SD. Values significantly different from healthy mice are indicated as: * $p < 0.05$, *** $p < 0.001$. Representative dot plots (lower panel) show the expression of CD4/CD8 (B) on peritoneal cells.

Adherent myeloid cells are more sensitive to late apoptosis than non-adherent cells

Cells which enter apoptotic process can be distinguished from healthy ones morphologically after visualisation of their nuclei with fluorescent nuclear dye. Adherence to plastic or glass surface is believed to be property of monocytes/macrophages and some subpopulation of dendritic cells. To investigate apoptosis morphologically, we prepared adherent and non-adherent cell fractions on slides, which were stained either with May-Grünwald/Giemsa solutions and or with fluorescent dye Hoechst 33342. Cells with condensed chromatin were considered to be in the early stage, whereas cells with fragmented nuclei were apparently in late stage. Fig. 5A shows proportions of late stage apoptotic cells detected for adherent and non-adherent cells, using fluorescent microscopy. Adherent cells comprised majority of cells with monocyte/macrophage appearance but also smaller cells, probably granulocytes (Fig. 5C, arrows). In agreement with results obtained by flow cytometry, the percentage of adherent apoptotic cells increased by day 7 p.i. and after a drop on day 14 p.i., their proportions gradually increased up to day 28 p.i. (Fig. 5A, B). Only minor population of non-adherent cells, comprising mostly lymphocytes and small proportions of granulocytes, underwent late apoptosis and peak was found on day 21 p.i. (Fig. 5 A, D).

Mixed populations of myeloid cells were adhered to larval surface

We next tested whether myeloid cells from infected mice can adhere to larval surface and if they undergo apoptosis. We first analysed cell smears after May-Grünwald/Giemsa staining and counted individual cell type. We detected two populations of macrophages based on affinity to basophilic and acidophilic stains and these cells were dominant cells adhered on the larval tegument (Fig. 6 A, B). The presence of granulocytes, mainly eosinophils, was gradually augmented within the first month of infection. Lymphocytes were absent and other cell types having bigger nuclei and several cytoplasmic extensions were found in lower numbers. Fluorescent staining of nuclei did not show cells in late stage of apoptosis, however small proportions of cells appeared to have condensed chromatin (Fig. 6 D, arrowheads).

Discussion

Murine peritoneal cavity has served immunologists as the site to study inflammation *in vivo* and as the source of activated (elicited) myeloid cells for *in vitro* studies. It is also interesting anatomical niche to study host-parasite interactions during infection with larval stage of cestodes, for example metacestodes of *M. corti* (syn. *M. vogae*) (Jenkins *et al*., 1991; Hrčková & Velebný, 1997; Hrčková *et*

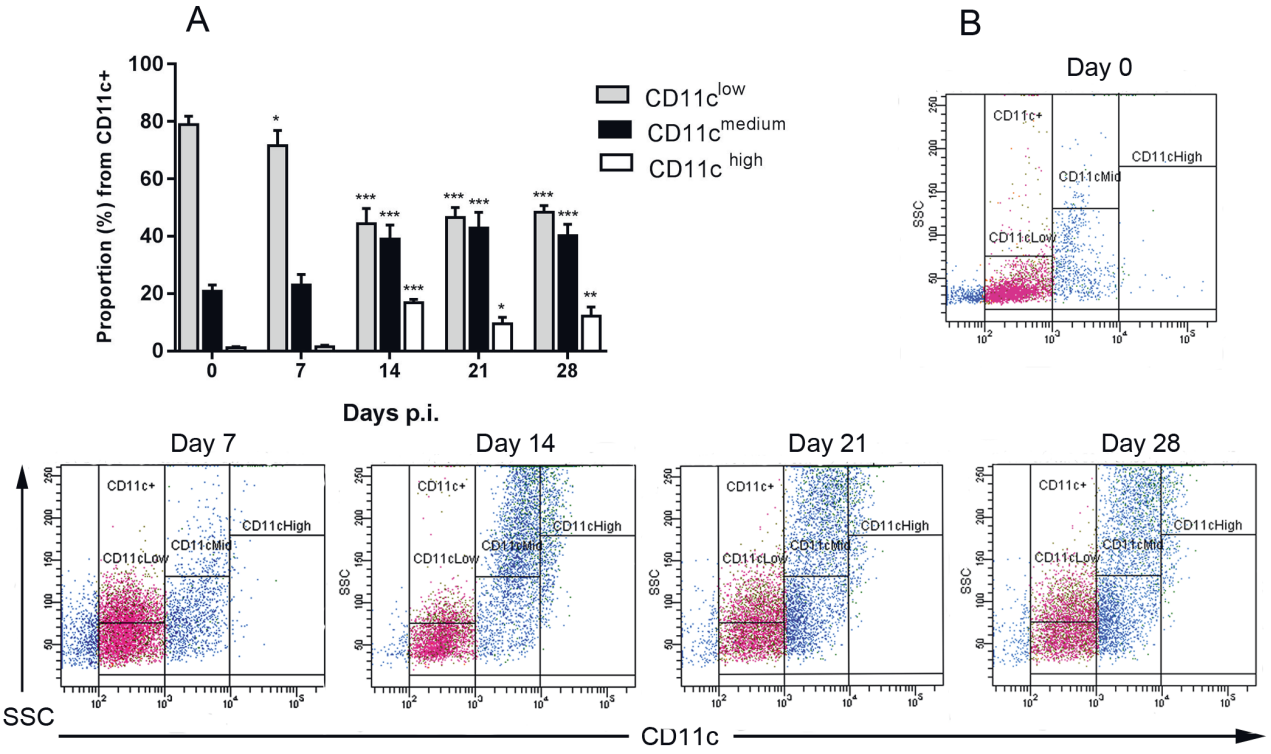

Fig. 3. Myeloid peritoneal cells differentially express CD11c marker during *Mesocestoides vogae* infection. (A) Peritoneal cells isolated from healthy and infected mice analyzed for CD11c from total cells by flow cytometry. Data are expressed as the means ± SD. Values significantly different from healthy mice are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Representative dot plots (lower panel) show the expression of CD11c (B) on peritoneal cells.

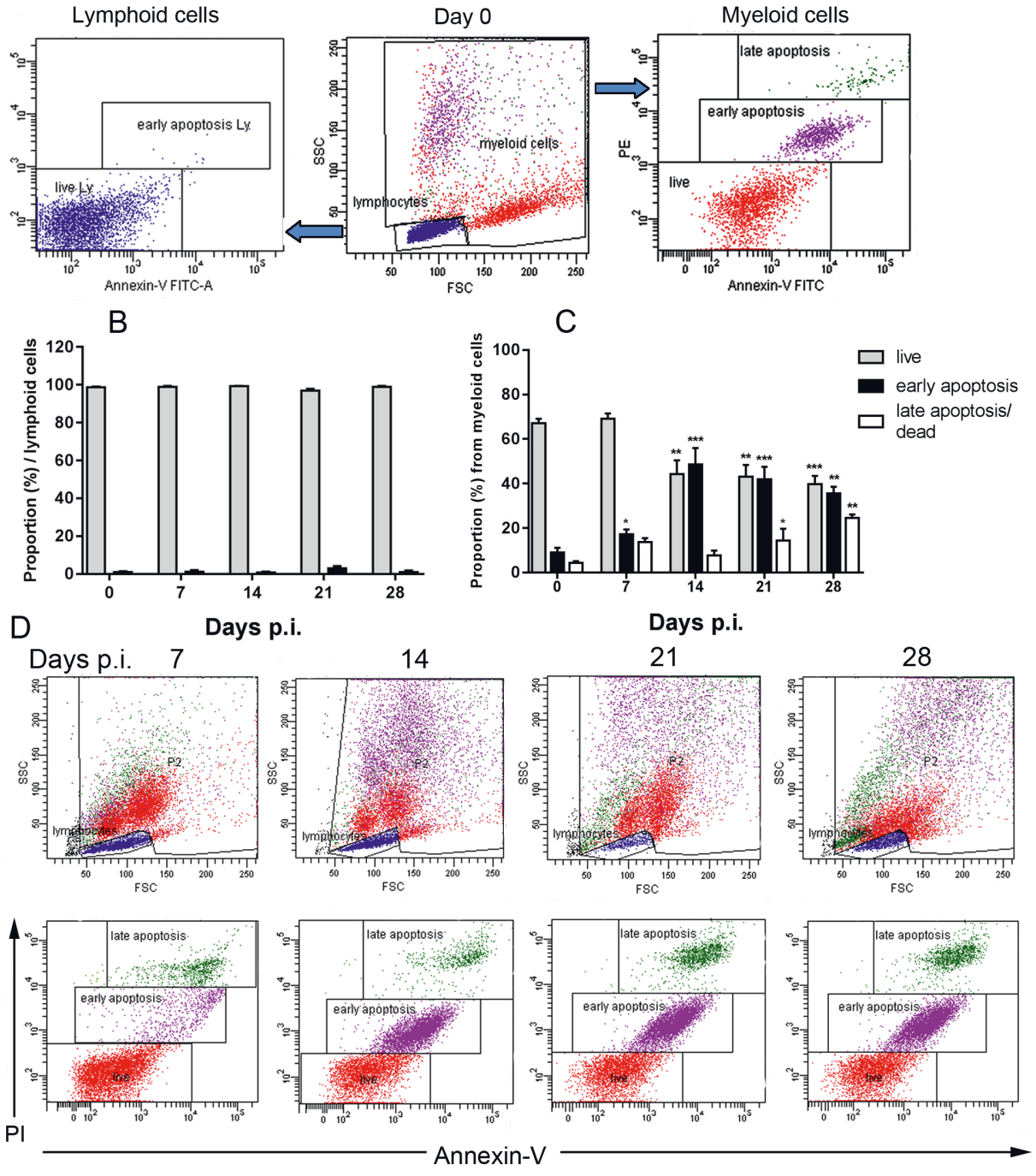

Fig. 4. Myeloid cell subpopulation is more susceptible to apoptosis than lymphoid cells. Analysis of apoptosis performed using an Annexin V-FITC Apoptosis Detection Kit. (A) Gating strategy used to identify live/apoptotic cells in both lymphoid (left) or myeloid (right) subpopulations of peritoneal cells. Graph displays proportion of live, early apoptotic and late apoptotic cells (n = 4 mice) within lymfoid (B) or myeloid (C) peritoneal cell subpopulations. Data are expressed as the means ± SD. Values significantly different from healthy mice are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D)

al., 2006; O´Connell *et al*., 2009; Vendelova *et al*., 2015), *Taenia crassiceps* (Zepeda *et al*., 2010) and *Echinococcus granulosus* (Mourglia- Ettlin *et al*., 2011). They can persist and grow in their host for a long period of time eliciting intensive inflammatory reactions with severe pathological changes. Interestingly, proliferative metacestode stages of *Mesocestoides* sp. were also documented in peritoneal and pleural cavities of dogs (Toplu *et al*., 2004; Boyce *et al*., 2011) and cats (Eleni *et al*., 2007; Lanteri *et al*., 2017), although carnivores represent the definitive hosts in which tapeworm reach adult stage in the gut. There is no immunopathological restriction of parasite growth in terms of enclosing metacestodes in the fibrous capsules, thus the events occurring during the early stages of metacestode infections in peritoneum seem to be important for their establishment. The reasons why parasitic infections persist lie in the complex interactions that have evolved between parasite evasion tactics and host countermeasures. Better understanding of immunological processes and their regulation could help to design more specific and effective therapeutic intervention. In the present study, we first focussed on the kinetic changes in the proportions of lymphoid and myeloid inflammatory cells within the first month of *M. vogae* infection in susceptible ICR strain of mice. We found the massive recruitment of myeloid cells from day 7 p.i., what accounted for 68.2 ± 3.08 % from total PEC (approximately 60 million) on day 28 p.i. Although proportions of lymphoid populations declined, their total numbers elevated, but in the lesser extent than numbers of myeloid cells (data not shown). The similar massive inflammation in the peritoneal cavity of C56BL/6 mice following intraperitoneal infection with *M. corti* larvae were reported

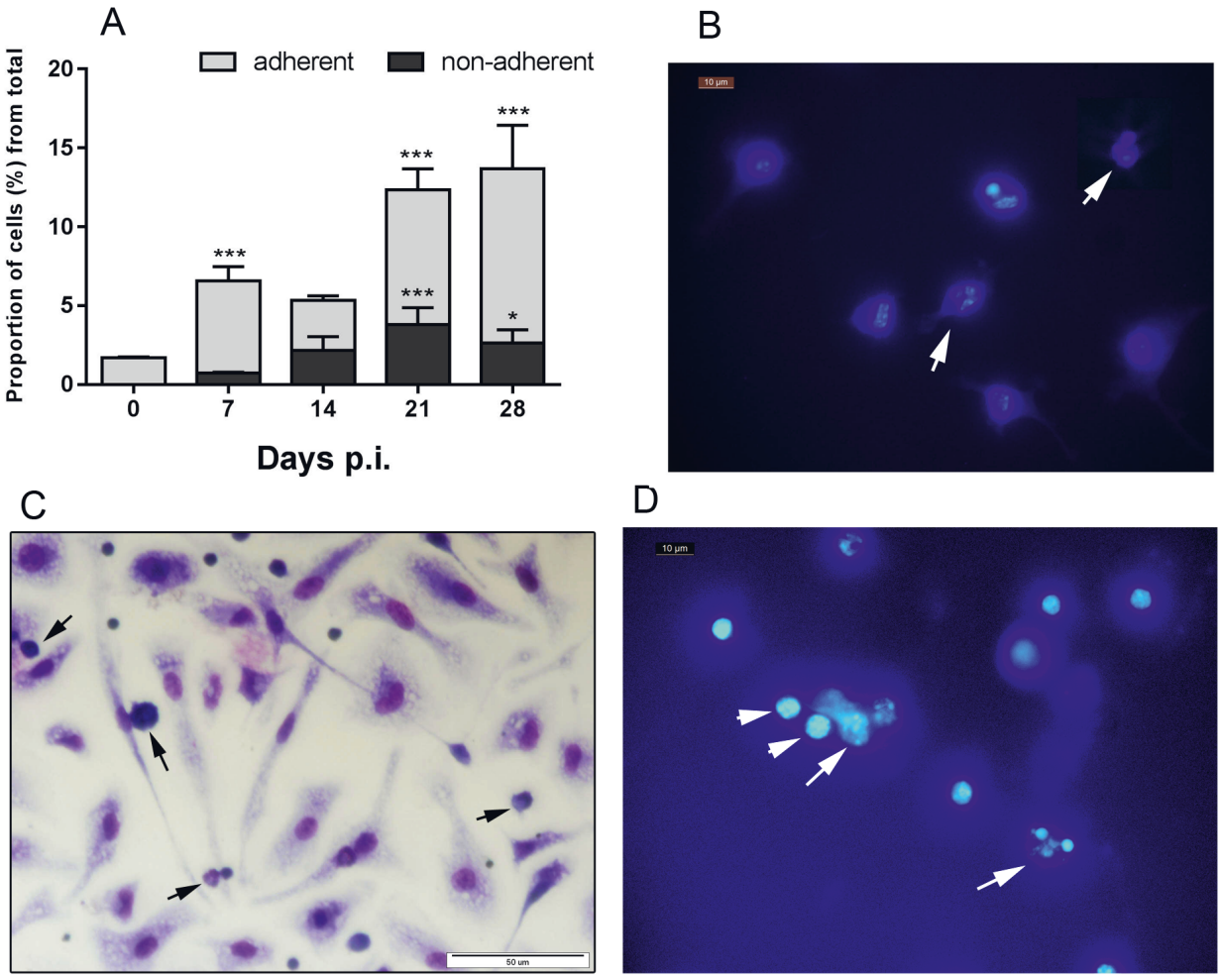

Fig. 5. Adherent fractions of myeloid peritoneal cells are more sensitive to late apoptosis in later stage of *Mesocestoides vogae* infection. Peritoneal cells isolated at the indicated time points, and proportions of apoptotic cell in adherent and non-adherent fraction assessed by fluorescent microscopy (A). Data are expressed as the means ± SD. Values significantly different from healthy mice are indicated as: * $p < 0.05$, *** $p < 0.001$. Adherent cells were stained with May-Grünwald/Giemsa solutions (C). Morphological changes of adherent cells undergoing late stage of apoptosis (arrows) and condensation of chromatin (arrowheads) were detected after staining of cell nuclei with DNA-specific dye Hoechst 33342 (B, D).

by O´Connell *et al*. (2009), although this strain was shown to be the most resistant out of six inbred strains of mice (White *et al*., 1982). We observed several phenotypically different inflammatory myeloid cells by means of morphological evaluation and flow cytometry with increasing proportion of large granular cells, macrophage-like cells and multinucleated giant cells. In previous studies we showed that majority of these cells attained the genetic signature of alternatively activated macrophages upon direct effect of excretory/secretory (ES) larval products (Vendelova *et al*., 2015). These cells had dampened phagocytic ability and production of superoxide anions (Hrčková *et al*., 2016; Velebný *et al*., 2010).

CD4+ T cell subsets have critical role in the regulation of adaptive immunity, since they provide essential help for both cytotoxic CD8+ T cells and humoral response. Flow cytometry analysis in our experiments revealed that the proportions of CD4+ T helper cells out of total lymphoid cells elevated up to day 14 p.i. and dropped thereafter, whereas proportions of CD8+ cells were only moderately modified. We assume that from day 14 p.i. other lymphoid populations like B cells, NK and NKT cells accounted for elevated total numbers of lymphoid cells, as we showed recently that on day 25 p.i. NK cells represented approximately 26 % of lymphoid cells in PerC of ICR mice (Mačák Kubašková *et al*., 2018).

In the study of Mourglia-Ettlin *et al*. (2011) on infection with *E. granulosus* in Balb/c mice authors demonstrated that immune response in PerC elicited at early stage (by day 9 p.i.) has biphasic behavior with initial promotion of Th1 towards Th2 type. Regarding peritoneal lymphoid cells, NK cells showed a substantial increase and predominance of CD4+ cells was observed. The massive recruitment of inflammatory cells was seen after intraperitoneal infection of Balb/c mice strain with *Taenia crassiceps* metaces-

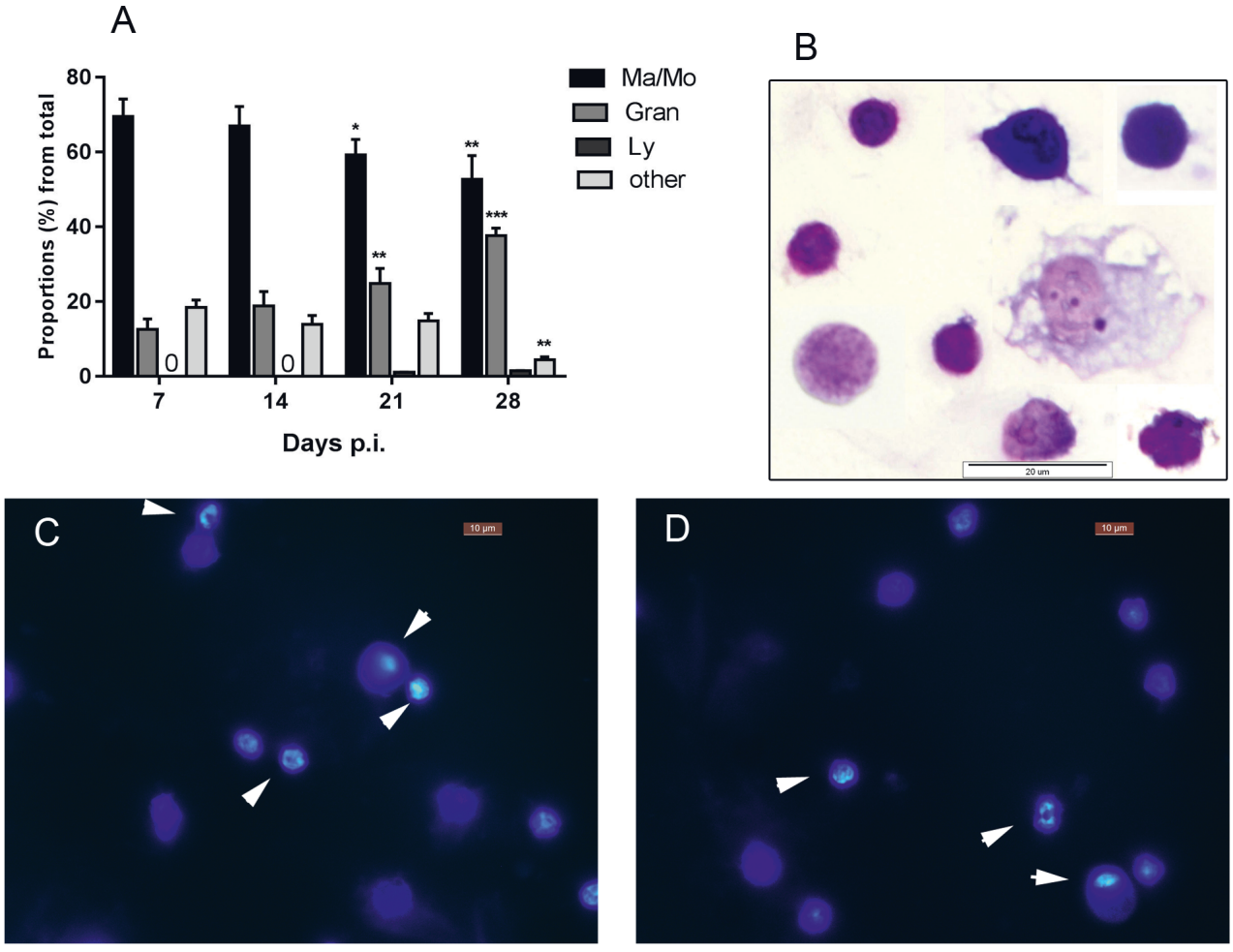

Fig. 6. Mixed populations of myeloid cells adhered to *Mesocestoides vogae* larval surface. Larvae isolated from peritoneal cavities of mice and incubated in complete medium for 3h. Then adherent cells pooled and cell smears prepared. Graph (A) depict proportion of subpopulation of adherent cells – monocyte/macrophages (Mo/Ma), granulocytes (Gran), lymphocytes (Ly) (A). Data are expressed as the means ± SD. Values significantly different from healthy mice are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Adherent cells stained with May-Grünwald/Giemsa solutions (B). Morphological changes of cells undergoing late stage of apoptosis detected after staining of cell nuclei with DNA-specific dye Hoechst 33342 (C, D). Representative images of cell smears showing cells with condensed chromatin (arrowheads).

todes within 16 days of examination, but the proportions of T and B lymphocytes gradually decreased as detected by flow cytometry. These studies indicate that interactions between host immune system and metacestodes seem to be parasite species-specific to a certain extent.

In peritoneal cavity of healthy mice, two macrophage subsets coexist which differ from each other in the expression of surface markers CD11b, F4/80 and MHCII and only so-called small type with high expression of MHCII predominates after LPS stimulation. This population derives from blood monocytes and differentiates to mature macrophages upon stimulation (Eid Bou Ghosn *et al*., 2010). Transmembrane protein CD11c is also expressed on myeloid-derived cells of which mature monocytes, macrophages and granulocytes have, in general, lower expression than have mature un-stimulated dendritic cells (Lai *et al*., 1998). We therefore examined CD11c expression level on myeloid cells during onset of peritoneal *M. vogae* infection by flow cytometry. We found decline of cells with low expression up day 14 p.i. when population of cells with medium expression started to elevate. We suppose that on this day the shift to Th2 response occurs and $CD11c^{medium}$ cells are predominantly alternatively activated macrophages as on this time point the rapid elevation of IL-4 mRNA levels in PEC in the same model infection was found (Vendelova *et al*., 2015). High expression of CD11c is assigned to dendritic cells. However, Singh-Jasuja *et al*. (2013) showed that CD11c marker on dendritic cells is down-regulated upon cell activation through Toll-like receptor triggering. Maturation of naïve dendritic cells was shown to be impaired after exposure to ES antigens of *E. multilocularis* (Nono *et al*., 2012) and *T. crassiceps* (Terrazas *et al*., 2010*) in vitro* resulting in Th2-biasing ability. In line with these reports, Smith *et al*. (2011) demonstrated that $CD11c^{low}$ non-plasmacytoid dendritic cell expand during chronic nematode infection inducing lower levels of antigen-specific CD4+ cells. Moreover expanded peritoneal macrophages (F4/80+phenotype) during later stage of *T. crassiceps* infection (M2 type) possessed a poor ability to induce antigen-specific proliferation of CD4+ T lymphocytes. In particular, very low proportions of $CD11c^{high}$ dendritic cells in PerC were found in our study. Collectively, we assume that highly decreased proportions of CD4+ T cells in PerC of *M. vogae* are the result of their down-regulated proliferation upon interaction with M2 macrophages and suppressive dendritic cells.

Apoptosis is highly regulated process of cell removal as opposite to necrosis and is also involved in regulation of host-parasite interactions (James & Green, 2004). To survive within their hosts, parasitic protozoa and helminthes demonstrate the ability to modulate host apoptosis pathways to their own advantage. In our study, we showed that peritoneal lymphoid population is resistant to early apoptosis and cell death. Similar resistance of lymphocytes in PerC with *T. crassiceps* infection was reported by Zepeda *et al*. (2010). In contrast, substantial proportions of recruited myeloid cells occurred in early and late stage of apoptotic process from day 14 p.i. when larval proliferation is accelerated (Vendelova *et al*., 2015; personal observation). Assessing size and granularity of cells by flow cytometry, cells undergoing early apoptosis represented highly granular population with medium to large size, possibly immature granulocytes and macrophages. Proportions of dead cells elevated by day 28 p.i. and they were of reduced size and lower granularity. Eosinophils represent the main granulocyte population in peritoneal cavity with *M. corti* (Johnson *et al*., 1979) or *T. crassiceps* infection, and significant level of apoptosis in eosinophils in comparison with macrophages was demonstrated within 16 days p.i. (Zepeda *et al*. 2010). E/S products of helminthes play the role in cell-specific apoptosis what is also dependent on tissue localization of parasites. There is no information regarding the effects of *M. vogae* E/S antigens on apoptosis initiation neither in the peritoneal cavity nor in the infected liver, but this effect can be suggested based on following studies. Nono *et al*. (2012) showed that E/S products of *E. multilocullaris* induced death of bone-marrow derived dendritic cells via apoptosis. Apoptosis of naïve rat peritoneal eosinophils was initiated by E/S products from trematode *Fasciola hepatica in vitro* by caspase-dependent mechanism (Serradell *et al*., 2007). Adherence is basic feature of macrophages and monocytes and adherent macrophages act as scavengers of late apoptotic cells. On the other hand, non-adherent macrophages may have growth-promoting role and could rescue early apoptotic cells (Selvarajan *et al*., 2011). We therefore analyzed whether adherent and non-adherent PEC undergo apoptosis by assessing morphological changes of cell nuclei. Adherent cells comprised mostly monocytes and macrophages but also granulocytes (Fig. 5 B). Late apoptosis was detected in the small portion of non-adherent cells, probably granulocytes, and other types of myeloid cells as lymphoid cells were shown to be resistant to apoptosis. In the adherent cell fraction, fragmentation of cell nuclei, as definitive sign of cell death, was seen in an increased level from day 21 p.i. in correlation with flow cytometry analysis. Scavenger receptors are responsible for scavenger function of macrophages and increased expression of these receptors was demonstrated in adherent macrophages (Selvarajan *et al*., 2011). It is possible that *M. vogae* larval-derived E/S products induce apoptosis in selected populations of macrophages, mainly adherent fraction, and eosinophils in order to ameliorate cytotoxicity and pathology in PerC. It is well known that macrophages tend to adhere to antibody-opsonized surface of flatworms as a part of antibody-mediated cellular cytotoxicity process. In our study, populations of larvae-attached cells comprised mainly macrophages but also other cell types, including eosinophils and large granular cells with compact nuclei, possibly NK cells. Neither cell type isolated from larval surface underwent late apoptosis although condensation of nuclear chromatin in some cells indicated on the early events. Attachment of eosinophils and partially neutrophils to *M. corti* larvae in PerC of rats was reported by Cook *et al*. (1988) showing down-regulation of Fc receptors for antibodies on these cells, but not the complement receptors.

Conclusion

In PerC of ICR mice *M. vogae* larval infection elicited the massive recruitment of inflammatory cells and their distribution changed towards accumulation of myelo-monocytic cell lineage in the account of reduced proportions of lymphoid cells. Our data demonstrated that apoptosis did not account for diminished population of peritoneal lymphoid cells, mostly CD4+T cells. Gradually elevated populations of myeloid cells with low and medium expression of CD11c became dominant cells and substantial proportions of myeloid cells were subjected to apoptotic turnover in peritoneal cavity of mice. Moreover, peritoneal adherent cells were seen in late apoptosis by contrast to cells adhered to larval surface, suggesting that cell-specific apoptosis is regulated by metacestodes to create permissive environment.

Acknowledgement

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Conflict of interests

Authors state no conflict of interest

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Determination of *Echinococcus granulosus* genotypes in livestock slaughtered in Shush County, Southwest Iran using PCR-RFLP

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Summary

Echinococosis is a zoonotic disease caused by the larval stages of *Echinococcus* spp. that occurs in most parts of the world. Herein, we aimed to evaluate the genotypes of isolated hydatid cysts from slaughtered animals in Shush county, southwestern Iran. Totally, 96 hydatid cysts were collected, including 11 buffaloes, 13 cattle, 12 goat and 60 sheep. The PCR was done by a primer pair (BDI and 4s) to amplify ITS1 fragment. Four restriction endonucleases including AluI, HpaII, RsaI, and TaqI were used for RFLP products and enzymatic reactions were electrophoresed. Finally, twenty PCR products were sent for sequencing and phylogenetic tree was drawn with MEGA6. Molecular identification of 96 hydatid cysts demonstrated a distinctive 1000 bp fragment in all samples from four animal hosts. RFLP analysis showed similar digestion patterns in all samples. AluI digestion yielded 800 bp and 200 bp fragments, HpaII digestion made 700 bp and 300 bp fragments and RsaI digestion entailed 655 and 345segments. Moreover, TaqI rendered no digestion pattern on rDNA-ITS1 region. Additionally, *E. granulosus* sensu stricto (G1-3 complex) was the prevailing genotype in all livestock samples, according to PCR-RFLP and sequencing analyses.

Keywords: *Echinococcus granulosus*; genotypes; livestock; Shush County; PCR-RFLP

Introduction

As an ancient zoonotic parasitosis, cystic echinococcosis (CE) is still a landmark neglected tropical disease around the globe, rendered by *Echinococcus granulosus* (*E. granulosus*) larval stage (Cardona & Carmena, 2013; Ito & Budke, 2017; Moro, P. & Schantz, 2009). The endemicity of CE is appointed to various parts of the world, including South America, the Middle East, and Mediterranean zone, northeastern Africa as well as Australia (Ito & Budke, 2017; Rojas *et al.*, 2014). Iran is considered as an endemic area for CE in the Middle East region, specifically owing to traditional animal husbandry and availability of abattoir wastes to dogs (Rokni, 2009). Based on slaughterhouse investigations, the animal prevalence rates in hyperendemic areas ranges from 20 % to 95 %. Also, it is estimated that US$ 3 billion is considered for case treatment and livestock losses (Who, 2017). Being a cyclozoonosis, the continuity of the *E. granulosus* life cycle is relied on the ecological interactions between domestic/wild canid populations (definitive hosts) and ungulates (intermediate hosts) in the natural environment. In this regard, there exist two ecological cycles of the hydatidosis: the domestic cycle frequently found in countries with sheep and cattle farming, and sylvatic cycle involving feral carnivores and wild herbivores (Carmena & Cardona, 2014; Otero-Abad & Torgerson, 2013). Although rare, aberrant human infections may occur via accidental ingestion of parasite eggs shed in dog feces in communities with poor sanitation practices (Rokni, 2009; Torgerson *et al.*, 2002).

From phenotypic characters and gene sequences standpoint, *E.*

* – corresponding author

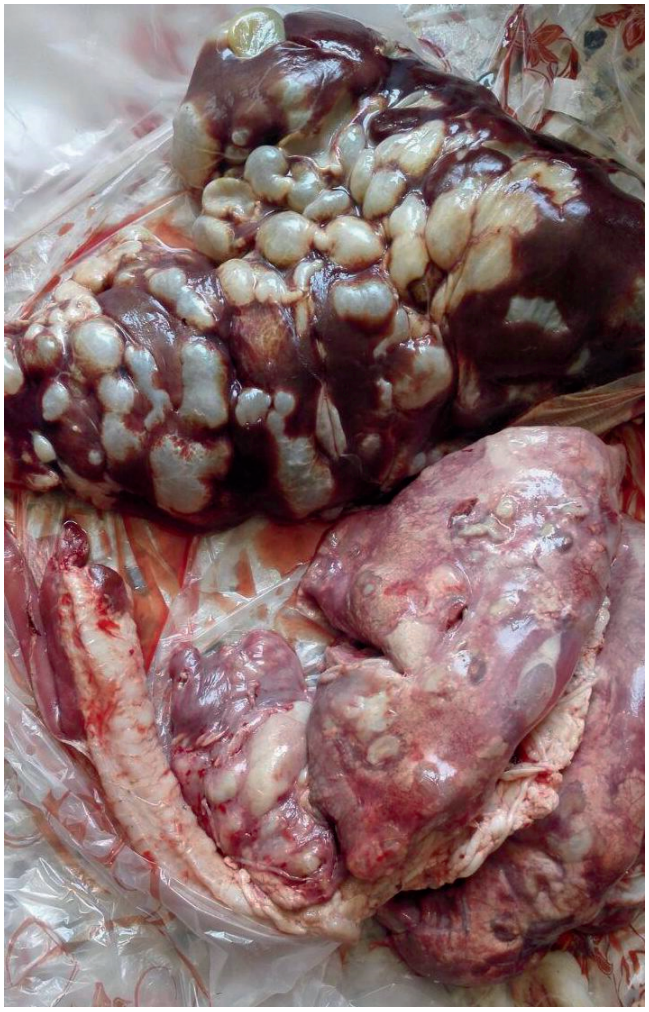

Fig. 1. Collected hydatid cyst samples from slaughtered livestock in Shush abattoir.

granulosus sensu lato is currently divided into genotypes G1– G10 of which *E. granulosus* sensu stricto (G1-3 complex) are the most frequently implicated genotypes in human infections. *E. felidis* (the former 'lion strain'), *E.equinus* (the 'horse strain', G4), *E. ortleppi* (the'cattle strain', G5), *E. canadensis* and the latter species, as recognized here, shows the highest diversity and is composed of the 'camel strain', G6, the 'pig strain', G7,and two 'cervid strains', G8 and G10 (Kinkar *et al.*, 2017; Moazeni-Bistgani *et al.*, 2013). G1 is the most eminent and exclusive cause of human and animal infection, cycling between dogs and sheep in vast pastoral lands globally; however, phylogenetic evidence demonstrate that other animals such as goat, cattle, and camel, also, act as minor intermediate hosts for this genotype (Lymbery, 2017). So far, many molecular tests have been used to discern the genotypic characteristics of *E. granulosus* (Ito & Budke, 2017). Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) is a verified molecular diagnostic for genotype detection, based on sequence-specific endonucleases (Bowles & Mcmanus, 1993; Dousti *et al.*, 2013; Mcmanus, 2002). Alterations in the genetic variants of *E. granulosus* populations, their host diversity, and public health importance would emphasize the molecular discernment of *E. granulosus* genotypes (Craig *et al.*, 2007; Moazeni-Bistgani , Taghipoor *et al.*, 2013; Siles-Lucas & Gottstein, 2001). Herein, we investigated the prevalence of *E. granulosus* genotypes in slaughtered livestock of Shush city, Khuzestan province, Southwest of Iran.

Materials and Methods

Study area and sample collection

Shush city is located in Khuzestan province, Southwest of Iran, have 3,577 km^2 area and dry and hot weather, with air temperatures ranging +1 and +53°C. During March - November 2017, a total number of 96 hydatid cysts were collected from slaughtered livestock at Shush abattoir, including 11 buffaloes, 13 cattle, 12 goats and 60 sheep (Fig. 1). Protoscolices of *E. granulosus* were removed from hydatid cyst contents and prepared as previously described by Smyth et al. and Balbinotti et al. (Balbinotti *et al.*, 2012; Smyth & Davies, 1974) Briefly, the hydatid cysts were aspirated and examined microscopically for cyst fertility. Protoscoleces that aspirated from fertile cyst were rinsed multiple times with sterile 0.9 % sodium chloride solution and maintained in 70 % ethanol for molecular purposes.

DNA extraction and PCR amplification

At first, all stored samples were washed twice with PBS to remove ethanol. The DNA extraction procedure was accomplished using PrimePrep genomic DNA isolation kit from tissue (GeNet Bio, South Korea) based on the manufacturer's protocol and the genomic DNA was kept at -20°C for PCR reaction. The concentration of each DNA sample was measured by NanoDrop (Thermo, USA) evaluation at A260.In each sample, an Internal Transcribed

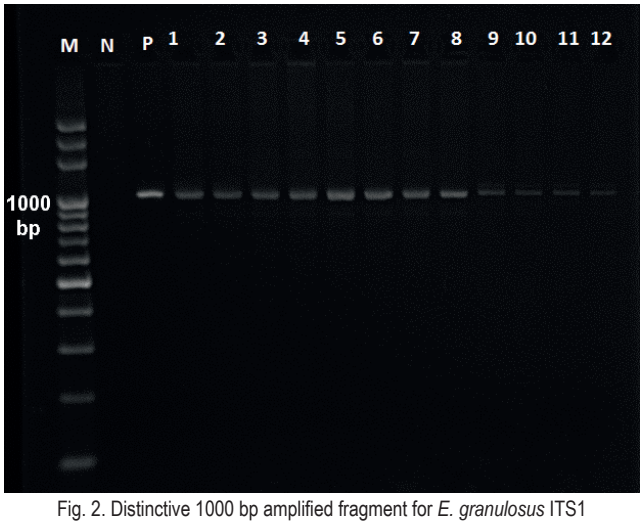

Fig. 2. Distinctive 1000 bp amplified fragment for *E. granulosus* ITS1 in conventional PCR.

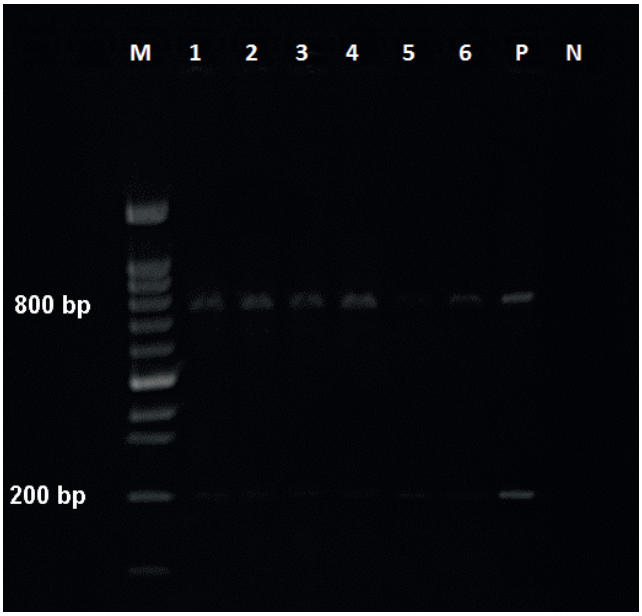

Fig. 3. Enzymatic digestion with AluI. Lanes 1,2: sheep; lanes 3,4: cattle; lane 5: buffalo; lane 6: goat; lanes 7 and 8: positive and negative controls, respectively.

Spacer 1 (ITS1) fragment was amplified by conventional PCR, using a specific primer pair:
BDI (5′-GTCCTAACAAGGTTTCCGTA-3′) for the 18S region.
4S (5′-TCTAGCGTTCGAA(G/A)TGTCGATG-3′) for the 5.8S region.
A 25 µl PCR mixture was prepared for each sample, containing 12.5 µl of Master Mix (Ampliqon, Denmark), 3 µl of extracted DNA, ten pmol of each primer and 6.5 µl of sterilized water. The following PCR program was carried out in an automated thermo cycler (FlexCycler, Analytik Jena, Germany): an initial denaturation step at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec, followed by a final extension step at 72°C for 5 min. Finally, the PCR products were separated by 1.5 % agarose gel electrophoresis and displayed under ultraviolet (UV) illumination.

Enzymatic digestion
To perform PCR-RFLP, four specific restriction endonucleases, enclosing AluI (AG/CT), HpaII (C/CGG) (Jena Bioscience, Jena, Germany), RsaI (GT/AC) and TaqI (T/CGA) (Thermo Scientific, Massachusetts, United States) were used. In case of digestion with RsaI and TaqI, the procedure was done in 50 µl reaction mixture, including 5 µl of the universal buffer, one µg of pure DNA or PCR product, 10 U of enzyme and PCR-grade water for the rest. The prepared mixtures were incubated in a thermocycler, being set to 37°C for RsaI and 65°C for TaqI, both for 2 hours. Also the digestion mixture for AluI and HpaII included Tango buffer (2 µl), PCR product (10 µl), enzyme (1-2 µl) and nuclease-free water (18 µl), which incubated at 37°C for an hour. All digested products were visualized using 2 % agarose gel electrophoresis and under UV condition.

Sequencing and phylogenetic analysis
Twenty PCR products were submitted to be sequenced by an ABI-3730XL capillary machine (Macrogen Inc., South Korea) in two directions using forward and reverse primers. The sequencing results were interpreted and compared to other GenBank registered sequences using the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments was exerted by ClustalX and Bioedit software to align and compare obtained nucleotide sequences. Neighbor-joining approach using MEGA 6 bioinformatics software was used to create the phylogenetic tree.

Ethical Approval and/or Informed Consent

The study protocol No: IR.AJUMS.REC.1396.410 was approved by the Ethics Committee on Research in School of Medicine, Ahvaz Jundishapur University of Medical Sciences

Results

In total, 96 hydatid cyst isolates were collected from slaughtered domestic animals of Shush abattoir. Molecular identification of hydatid cysts by conventional PCR showed a distinctive 1000 base pair (bp) fragment in all samples from four animal hosts (Fig. 2). The PCR-RFLP analysis of ITS1 segment of *E. granulosus* cysts demonstrated similar digestion patterns in all samples. AluI digestion yielded 800 bp and 200 bp fragments (Fig. 3), HpaII digestion made 700 bp and 300 bp fragments (Fig. 4), and RsaI digestion entailed 655 bp and 345 bp segments (Fig. 5). Moreover, TaqI rendered no digestion pattern on rDNA-ITS1 region (Fig. 6). Also, molecular sequencing disclosed that all 20 hydatid cysts were *E. granulosus* sensu stricto genotype and no other strains were discovered.

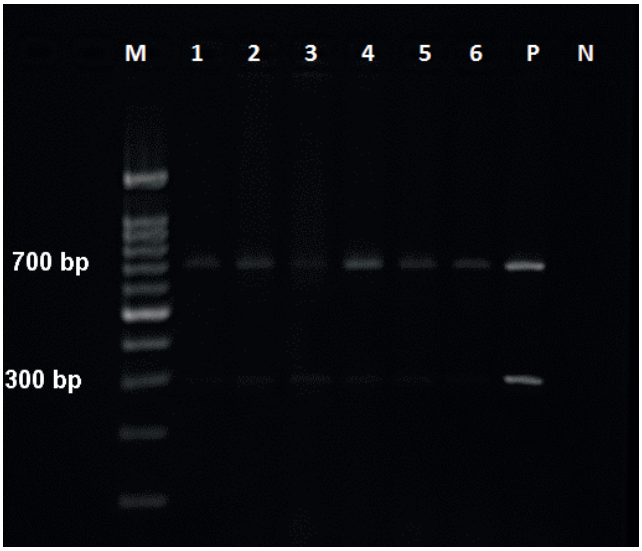

Fig. 4. Enzymatic digestion with HpaII. Lanes 1,2: sheep; lanes 3,4: cattle; lane 5: buffalo; lane 6: goat; lanes 7 and 8: positive and negative controls, respectively.

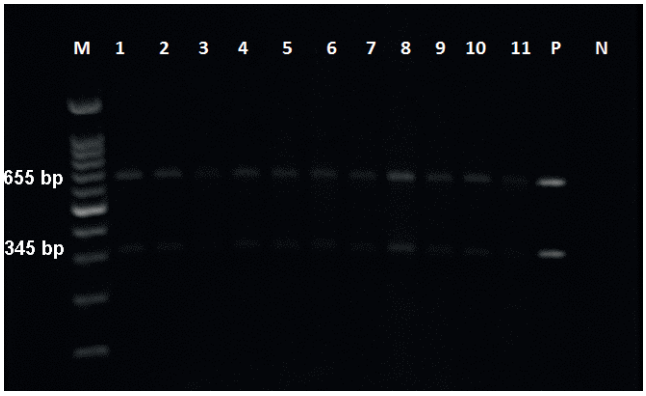

Fig. 5. Enzymatic digestion with RsaI. Lanes 1-4: sheep; lanes 5-7: cattle; lanes 8, 9: buffalo; lanes 10, 11: goat; lanes 12 and 13: positive and negative controls, respectively.

Discussion

Hydatidosis is a major public health issue in endemic countries such as Iran (Rokni, 2009). Determination of variations in *E. granulosus* genotypes is an interesting standpoint of hydatidosis research, to better understand ecological processes and life cycle patterns. The accurate taxonomy of *E. granulosus* isolates can be revealed by a wide array of molecular approaches (Carmena & Cardona, 2014; Cucher *et al.*, 2015; Mcmanus & Bowles, 1996). The ITS segment of rDNA, particularly ITS1, has been proved to be an impressive genetic marker for *Echinococcus* genotyping studies (Fadakar *et al.*, 2015; Siles-Lucas *et al.*, 2017). Concerning Bowels *et al.*, a 1000 bp band was observed in all 96 specimens by amplification of rDNA-ITS1 fragment, highlighting the *E. granulosus* identity (Bowles *et al.*, 1995; Bowles & Mcmanus, 1993). Additionally, *E. granulosus* sensu stricto was the prevailing

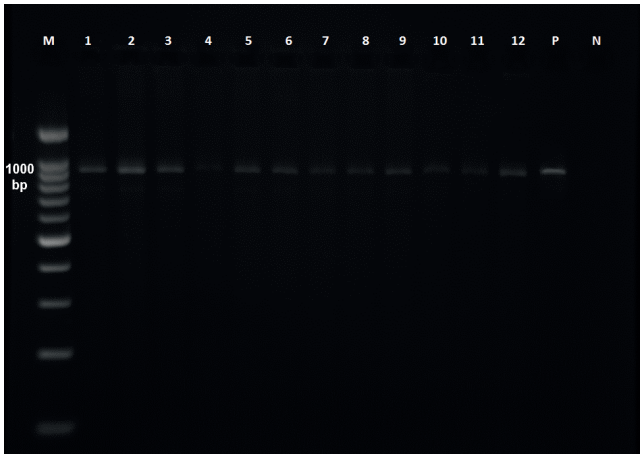

Fig. 6. Enzymatic digestion with TaqI. Lanes 1-4: sheep; lanes 5-7: cattle; lanes 8, 9: buffalo; lanes 10, 11: goat; lanes 12 and 13: positive and negative controls, respectively.

genotype in all livestock samples, according to PCR-RFLP and sequencing analyses. Reportedly, previous investigations across the country have demonstrated the sheep strain as the major detected genotype of CE in production animals (Ahmadi & Dalimi, 2006; Harandi *et al.*, 2002; Moghaddas *et al.*, 2015; Pezeshki *et al.*, 2013; Pour *et al.*, 2011; Sharbatkhori *et al.*, 2010; Sharbatkhori *et al.*, 2016; Zhang *et al.*, 1998). Khademvatan *et al.* reported G1 as the only cause of hydatid cyst in 329 examined livestock (sheep, cattle, and goat) of southwestern Iran, using PCR-RFLP with AluI, MspI and RsaI restriction endonucleases (Khademvatan *et al.*, 2013). Based on a genotyping study in Isfahan province, animal hydatid cysts from sheep, camel, cattle, and goat were characterized using PCR-RFLP with the same primers and enzymes as our study; it was demonstrated that the sheep strain is the most prevalent isolate among these animals (Shahnazi *et al.*, 2011). In a neighbor province, hydatid isolates were gathered from sheep, goat, and cattle; likewise ours, the authors used TaqI, AluI, RsaI and HpaII and showed G1 as the frequent strain (Parsa *et al.*, 2011). In contrast with our study that used ITS1, Sharbatkhori *et al.* used cytochrome C oxidase subunit 1 (*cox1*) and NADH dehydrogenase 1 (*nad1*) mitochondrial genes for sequencing and detected G1 (78.3 %) in all animal hosts such as sheep, goat, cattle, camel and buffalo in Golestan province (Sharbatkhori , Tanzifi *et al.*, 2016). Our results are compatible with these findings. Also, there exist convincing molecular proofs all over the globe suggesting G1 infection of sheep, cattle, goat, and buffalo, enclosing investigations in Iraq (Hammad *et al.*, 2018), Pakistan (Latif *et al.*, 2010), India (Singh *et al.*, 2012), Argentina (Andresiuk *et al.*, 2013), Ethiopia (Tigre *et al.*, 2016) and Greece (Chaligiannis *et al.*, 2015). The current investigation likewise several studies (Khademvatan , Yousefi *et al.*, 2013; Moro, P. L. *et al.*, 2009; Parsa , Haghpanah *et al.*, 2011; Pour , Hosseini *et al.*, 2011; Sharbatkhori , Tanzifi *et al.*, 2016; Varcasia *et al.*, 2006) have also isolated G1 from other animals rather than sheep, and goat, such as cattle/buffalo, suggesting the dominance of this genotype in these animals. Although, it is noticeable that G1 frequently renders infertile hydatid cysts in cattle (Hüttner & Romig, 2009). This finding also emphasizes the interaction of different ecological cycles of CE and warrants further researches to disclose the epidemiology and transmission dynamics of hydatidosis in Iran.

Regarding our results, *E. granulosus* sensu stricto was the predominant genotype in slaughtered sheep, goat, cattle and buffalo of this area, southwestern Iran, similar to other parts of the country. This strain is supposed as the most known genotype of *E. granulosus* with potential serious sequela in humans. Obtained results from this study would shed light for local and nation-wide CE preventive measures, consisting of improved diagnostics and better therapeutics.

Conflict of Interest Statement

We declare that we have no conflict of interest.

Acknowledgments

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The impact of acids approved for use in foods on the vitality of *Haemonchus contortus* and *Strongyloides papillosus* (Nematoda) larvae

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Article info

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Summary

The laboratory experiment described in this article evaluated the death rate of larvae of *Haemonchus contortus* (Rudolphi, 1803) nematodes of the Strongylida order and *Strongyloides papillosus* (Wedl, 1856) of the Rhabditida order under the impact of different concentrations of 8 flavouring acids and source materials approved for use in and on foods and in medicine (formic, wine, benzoic, salicylic, stearic, kojic, aminoacetic, succinic acids). Minimum LD_{50} for third stage larvae of (L_3) *S. papillosus* was observed with salicylic and wine acids, for L_3 *H. contortus* larvae – with formic acid. Minimum impact on all studied stages of development of nematodes was caused by stearic, kojic, aminoacetic and succinic acids: larvae did not die in the course of one day even at 1 % concentration of these substances. The best parameters of LD_{50} were observed for benzoic and formic acid. Further experiments on flavouring acids and source materials approved for use in and on foods and in medicines, and also their compounds, will contribute to developing preparations with a stronger impact on nematode larvae – parasites of the digestive tract of vertebrate animals and humans.
Keywords: nematodes of ungulates; flavouring acids; death rate of larvae

Introduction

Achieving high quality livestock production requires following the rules of maintenance conditions. Important factors are the animals' diet and measures for preventing infections (Zazharska *et al*., 2018). One of the most common animal diseases around the world is considered to be helminthiasis. Annually it causes great losses in livestock production and great economic losses. An increasing amount of scientific research is being conducted on preventing and treating agricultural parasites. Farming uses synthetic anthelmintic preparations. Highly popular are broad spectrum anthelmintics: Albendazole**,** Fenbendazole, Ivermectin preparations (Belœil *et al*., 2003; Faye *et al*., 2003; Fthenakis *et al*., 2005; Veneziano *et al*., 2004; Charlier *et al*., 2007; Cringoli *et al*., 2008).

Also, research on the relative antiparasitic properties of plants is conducted all around the world (Rahmann & Seip, 2006; Burke *et al*., 2009; Lu *et al*., 2010). Plant- based medical preparations against helminths show encouraging results. Rahmann and Seip (2006) suggest a list of plants with anthelmintic properties: black cumin, black walnut, boundary tree, common mugwort, common wormwood, crucifers, custard tree, eucalyptus, Eurasian wormwood, fargara, fennel, fern, fumitory garlic, Gambian mahagony, goosefoot, Indian lilac, kamala tree, neem tree, papaya, pinkroot, pumpkin, pyrethrum, sacred basil, southern wormwood, tansy, tarragon, wild carrot, and wild ginger.

A number of authors have studied the impact of sainfoins on the nematodes of animals: feeding sainfoins to goats reduces the number of eggs of *Trichostrongylus* sp. in the animals' feces (Paolini *et al*., 2005). Ferreira *et al*. (2011) described the impact of crude alcoholic extracts of *Artemisia annua*, *A. absinthium*, *Asimi-*

na triloba, and *Fumaria officinalis* on trematodes in laboratory conditions *in vitro*.

Earlier, we tested against nematodes the following flavourings and source materials approved for use in and on foods: p-Anisaldehyde, Benzaldehyde, γ-Undecalactone, Cinnamaldehyde, Ethyl acetate, Benzyl acetate, α-Terpineol, Benzyl alcohol, Citral, L-Linalool, β-Ionone, Citronellol, Acetoin, D-Limonene (Boyko & Brygadyrenko, 2019). The experiments showed that minimum LD_{50} for L_3 *S. papillosus* were achieved using Cinnamaldehyde, α-Terpineol and Benzyl alcohol, for L_{1-2} *S. papillosus* – using Benzyl alcohol, Cinnamaldehyde, L-Linalool and Benzyl acetate, for L_3 *H. contortus* – using γ-Undecalactone and Cinnamaldehyde. Lowest indicators of LD_{50} (mg/l) against *Strongyloides ransomi* Schwartz and Alicata, 1930 were observed using Benzaldehyde (Boyko & Brygadyrenko, 2017b). When invasive eggs of *A. suum*, were exposed to Cinnamaldehyde, benzoic acid (E210, Codex Alimentarius) and methylparaben (E218, Codex Alimentarius) at 1 % concentration, we determined the lowest parameters of LD50 for benzoic acid (Boyko & Brygadyrenko, 2017a).

Strongyloidiasis and haemonchosis are some of the commonest helminthiases of *Ruminantia*. The development of *Strongyloides* spp. and *Haemonchus* spp. up to the invasive stage takes place in the external medium. The animals become infested with invasive larvae through intake of food and water. Nematodes of *Strongyloides* spp. have two generations. They consist of parasitic and free-living individuals, and therefore can breed both in the host`s organism and in the external environment. At the same time, larvae of *Strongyloides* spp. can penetrate the host organism not only by the alimentary route, but also through the skin. These peculiarities of the life cycle of *Strongyloides* spp. can lead to intensive infection of animals. In cases of parasitisation of the gastrointestinal tract by *Strongyloides* spp., *Haemonchus* spp., and also other species of Strongylida, a decrease in weight and productivity parameters of animals, heightened sensitivity to other diseases, and at higher level of infestation – death of animals have been observed by numerous researchers (Kabasa et al., 1999; Vercruysse et al. 2001; Peter *et al*., 2015; Kobayashi & Horii, 2008; Besier et al., 2016; Boyko et al., 2016; Flores-Pérez et al., 2019).

We found no information on the impact of the acids on helminths of the class Nematoda, which parasite in mammals. The research presented in this article is aimed at defining the vitality level of larvae of *Haemonchus contortus* (Rudolphi, 1803) and *Strongyloides papillosus* (Wedl, 1856) nematodes, parasites of ungulates, under the impact of flavouring acids and source materials approved for use in and on foods.

Material and Methods

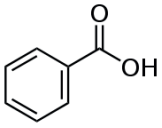
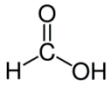
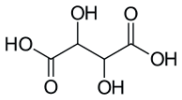
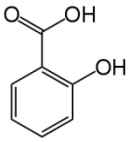
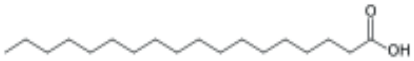
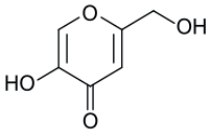
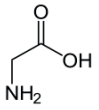
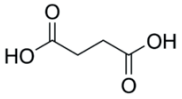
The samples of ungulates' feces were obtained in Dnipropetrovsk Oblast, from the clinic of Dnipro National Agricultural-Economic University in 2017. At a temperature regime of +22…+24 °C they were delivered to the laboratory of the Department of Parasitology and Veterinary-Sanitary Examination. The larvae of nematodes of goats' digestive tracts were cultivated in the conditions of thermostat during 8-days at the temperature of +22…+24 °C. The cultivation obtained third stage (L_3) *H. contortus* larvae and first, second and third stage (L_1, L_2, L_3) *S. papillosus* larvae (Van Wyk *et al*. 2004; Van Wyk & Mayhew 2013; Boyko *et al*., 2016). The larvae were obtained using the Baermann test (Zajac & Conboy, 2011). 4 ml of water with larvae was centrifuged for 4 minutes with 1,500 rotations per minute. The centrifuged sediment of liquid with nematode larvae (0.1 ml) was put in plastic test tubes of 1.5 ml capacity. The solutions of acids were added, and the tubes were left in a thermostat for 24 hours at the temperature of +22…+24 °C.

The larvae were exposed to the impact of formic, wine, benzoic, salicylic, stearic, kojic, aminoacetic, succinic acids – flavourings and source materials approved for use in and on foods and in medicines. Three concentrations of the substances were used in eightfold replication for every variant of the experiment (Table 1).

Benzoic acid is a substance of average toxicity. LD_{50} (median dose) of benzoic acid for laboratory animals (intravenous administration to rats) equals 1700 mg/kg, for cats – 300 mg/kg (Bedford & Clarke, 1972; Jakimowska, 1961). Formic acid has low toxicity. According to the classification of the European Union (Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances), concentration of formic acid not higher than 10 % has an irritating effect, over 10 % - corrosive. LD_{50} (median dose) of formic acid for laboratory animals equals 700, 1100, 4000 mg/kg for mice, rats and dogs respectively (oral) (Von Oettingen, 1960, Montgomery, 2000). Wine acid (Tartaric acid) is used in the food industry as a E334 additive (Codex Alimentarius). LD_{50} is about 5.3 g/kg for rabbits, and 4.4 g/kg for mice (Maga, Tu, 1995). Salicylic acid is toxic for humans only in high doses. LD_{50} (median dose) of salicylic acid for laboratory animals (mice, intravenous) is 184 mg/kg (Ozawa *et al*., 1971). Stearic acid is broadly used in cosmetics. LD_{50} (median dose) of stearic acid for laboratory animals is 21.5 mg/kg (rats, intravenous), 23 mg/kg (mice, intravenous) (Oro & Wretlind, 1961). Kojic acid is broadly used in the food industry, cosmetology, and also medicine. Aminoacetic acid is also used widely. It is used in the food industry (E640), for preparing pharmaceutical preparations, fodders for animals. LD_{50} (median dose) of aminoacetic acid is 7930, mg/kg (rats, oral) (Wypych, 2016). As a food additive and dietary supplement, succinic acid is generally recognized as safe by the U.S. Food and Drug Administration. This acid is used in the food industry as a E363 additive. Also it is used for obtaining medical preparations. LD_{50} is 2702mg/kg for laboratory animals (mice, intraperitoneal) (Domingo *et al*., 1990).

The statistical analysis of the results was performed through a set of Statistica 8.0 (StatSoft Inc., USA).On the figures is shown the median, 25 % and 75 % quartiles, minimum and maximum values. LD_{50} is expressed as a %: average (x) ± standard deviation (SD).

Table 1. Usage and properties of acids used for defining the vitality level of *H. contortus* and *S. papillosus* larvae

The name of substance	Chemical formula	Structural formula	Usage
benzoic acid (E_{210})	$C_7H_6O_2$		used for preserving food products; used in medicine for treating skin diseases, as dermatic antiseptic and fungicidal preparation
formic acid (E_{236})	CH_2O_2		as a preserving and antibacterial agent in preparation of fodder; against parasites of bees
wine acid (E_{334})	$C_4H_6O_6$		used in food production and medicine
salicylic acid	$C_7H_6O_3$		has slight antiseptic, irritating and keratolytic properties, used in medicine in the content of unguents and solutions for treating skin diseases
stearic acid	$C_{18}H_{36}O_2$		is one of the main components of soap, is included in many cosmetic preparations
kojic acid	$C_6H_6O_4$		is a reaction inhibitor of formation of melanin, and also is used in food production and cosmetology for preserving or changing colour of substances, is a component of antibiotics, insecticides and pesticides; has an anti-inflammatory, bactericidal, insecticidal and fungicidal effect
aminoacetic acid (E_{640})	$C_2H_5NO_2$		for preparing buffer solutions, synthesis of peptides, hippuric and amino-hippuric acids, as a complexing reagent; used for obtaining fertilizers, nitrates of cellulose, colourings, sulfur acid, etc.; used in the food industry
succinic acid (E_{363})	$C_4H_6O_4$		used for obtaining medical preparations, in particular, Chinotilinum; also used in the food industry

Ethical Approval and/or Informed Consent

This work does not involve human or experimentation with animals.

Results

The best results were shown by benzoic and formic acids. A total of 100 % of L_3 *H. contortus* and L_1, L_2, L_3 *S. papillosus* larvae died at 1 % concentration of both solutions (Fig. 1 a, b). But about 100 % of L_3 *S. papillosus* and L_3 *H. contortus* L_1, L_2 *S* survived in the next (0.01 %) concentration of benzoic acid. L_1, L_2 *S. papillosus* were less resistant to 0.01 % concentration of benzoic acid – we observed around 70 % viable individuals at this concentration. Under the impact of 0.01 % concentration of formic acid, 100 % of invasive larvae of (L_3 *H. contortus* and L_3 *S. papillosus*) nematodes and 50 % of L_1, L_2 *S. papillosus* survived. Lower concentrations of these acids showed no positive effect, vitality of larvae of all stages remained at 100 %.

Stearic acid was not effective against nematodes *S. papillosus* and *H. contortus*. Under different concentrations of this acid, 100 % of L_3 survived. Only L_2 *S. Papillosus* were affected by 1 % concentration of stearic acid: only 10 % of individuals remained alive. The impact of the next concentration (0.01 %) provided no positive effect: 80 % of L_1, L_2 *S. papillosus* (Fig. 1 c) survived.

The study of the impact of succinic (Fig. 1 d) and aminoacetic acids (Fig. 2 a) also showed a negative anthelmintic effect. About 80 % of L_3 *S. papillosus* and 100 % L_3 *H. contortus* remained alive

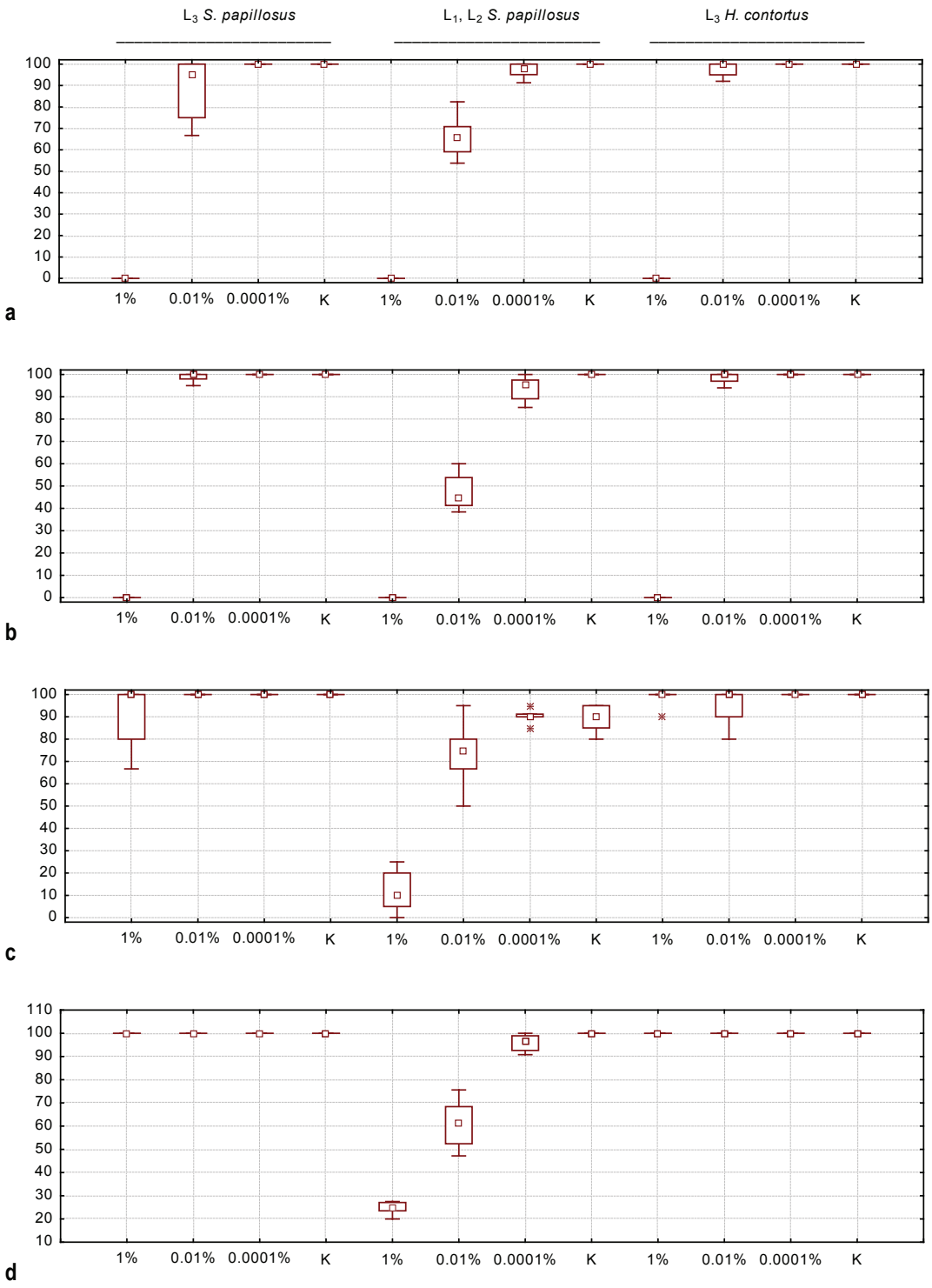


Fig. 1. The impact of benzoic (a), formic (b), stearic (c) and succinic (d) acid on vitality of larvae of nematodes of ruminants: the ordinate axis indicates the percentage of living nematode larvae in the course of the 24-hour experiment; the abscissa axis indicates the concentration of the solution's active substance (%); (K) control, where the concentration of the active substance is 0%; (L_3) invasive larvae of *S. papillosus* or *H. contortus*; (L_1, L_2) non-invasive larvae of *S. papillosus*; the small square in the centre corresponds to the median, the lower and upper edge of the large rectangle corresponds to first and third quartiles, respectively, the vertical segments, directed upward and downward from the rectangles, correspond to minimum and maximum values (n = 8)

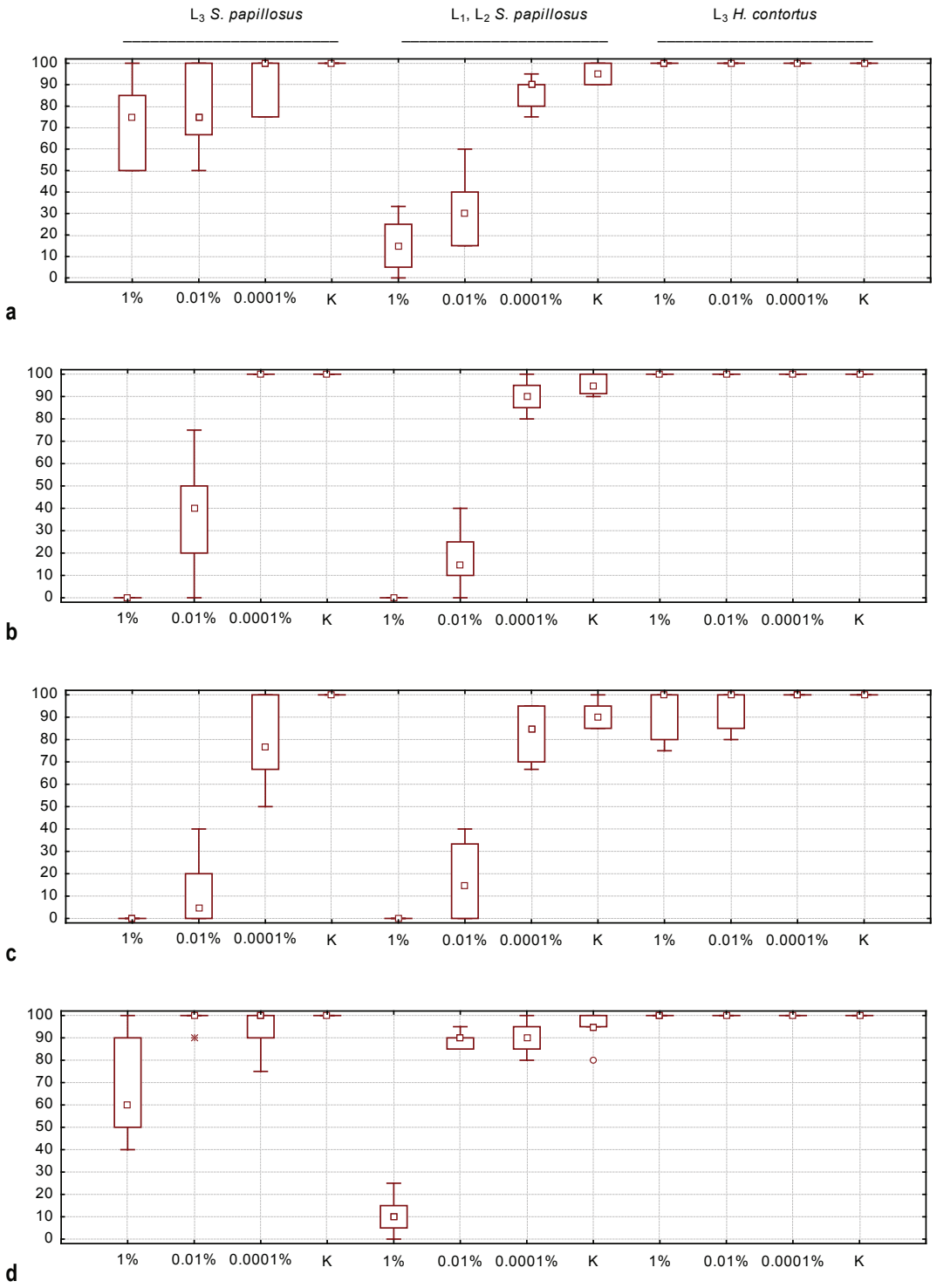


Fig. 2. The effect of aminoacetic (a), wine (b), salicylic (c) and kojic (d) acid on vitality of larvae of nematodes of ruminants': explanations see in Fig. 1.

Table 2. LD_{50} (%, x ± SD) for *S. papillosus* and *H. contortus* larvae in laboratory experiment during 24 hours; (–) the experiment did not achieve death of 50 % of the larvae (over 1 % concentration is needed)

Substance	*S. papillosus*, L_3	*S. papillosus*, $L_1 + L_2$	*H. contortus*, L_3
benzoic acid	0.18 ± 0.14	0.07 ± 0.04	0.52 ± 0.18
formic acid	0.47 ± 0.29	0.008 ± 0.007	0.41 ± 0.32
wine acid	0.008 ± 0.005	0.006 ± 0.004	–
salicylic acid	0.0010 ± 0.0006	0.0009 ± 0.0006	–
stearic acid	–	0.09 ± 0.03	–
kojic acid	–	0.08 ± 0.03	–
aminoacetic acid	–	0.006 ± 0.004	–
succinic acid	–	0.08 ± 0.03	–

under the impact of aminoacetic acid. 100 % of all invasive L_3 *S. papillosus* and L_3 *H. contortus*) survived in 1 % solution of succinic acid. L_1, L_2 *S. papillosus* were less resistant to the acids. Only 15 % and 25 % of the larvae withstood 1 % solution of aminoacetic and succinic acids respectively. The next concentration of aminoacetic acid (0.01 %) also caused positive results only with L_1, L_2 *S. papillosus*: about 70 % of the larvae died. In 0.01 % solution of succinic acid, the larvae were more resistant: around 60 % survived. Smaller concentrations provided no positive effect against larvae of L_1, L_2 L_3 nematodes of the studied species.

Wine acid showed a less significant nematocidal effect. None of the L_3 *H. contortus* died even in 1 % concentration of this substance. By contrast, the larvae of *S. papillosus* of all stages of development died at this concentration. But the next concentration of the wine acid solution (0.01 %) caused mortality of only 40 % of L_3 *S. papillosus* and 15 % of L_3 *S. papillosus*. 100 % L_3 of both studied species of nematodes survived at 0.0001 % concentration of this acid (Fig. 2 b).

Exposure to 1 % solution of salicylic acid caused death of only *S. papillosus* larvae. No more than 20 % of the larvae were able to withstand 0.01 % solution. Exposing *S. papillosus* larvae to 0.0001 % concentration of salicylic acid did not lead to positive results: more than 70 % of the larvae remained alive. L_3 *H. contortus* larvae were found to be the most resistant to different concentrations of this acid (Fig. 2 c).

Similar results were obtained after using kojic acid. Over 60 % of L_3 *S. papillosus* and L_3 *H. contortus* survived in 1 % solution of this acid. Non-invasive larvae also appeared to be less resistant to the acid: vitality of L_1, L_2 *S. papillosus* larvae was only 10 % at such concentration. Over 90 % of the larvae of the studied nematode species survived at 0.01 % concentration of kojic acid (Fig. 2 d).

Thus, the best LD_{50} indicators were observed for benzoic and formic acids. These acids caused death of all studied species of nematode larvae. Stearic, kojic, aminoacetic and succinic acids did not demonstrate a significant impact on the nematode larvae. They increased mortality of only L_1, L_2 *S. papillosus* (Table 2).

Discussion

Therefore, flavourings and source materials approved for use in and on foods and acids used in medicine and cosmetology are capable of having a significant effect on vitality of larvae of nematodes of ruminants (*S. papillosus*, *H. contortus*). Currently, scientists are closely studying natural factors which are unfavourable for parasites of agricultural animals and plants. The question of using anthelmintic substances of non-synthetic origin against agricultural pests is becoming increasingly relevant. The issues we are interested in, the impacts of flavouring acids and source materials approved for use in and on foods and acids used in medicine and cosmetology, are being reported in the scientific literature. Positive results in using acids against nematodes were also achieved by Browning *et al*. (2004). They described the nematocidal properties of butyric acid, which is obtained through fermentation of organic substances by anaerobic soil bacteria. A 2-day incubation in sand amended with 0.88 mg/g butyric acid reduced plant parasitic and fungivorous nematodes by 84–100 % as compared to untreated controls. The species compound of nematodes is highly significant. Significant decrease in the number of some species requires using 0.88 mg butyric acid / g of sand, others (*Steinernema*) require 8.8 mg butyric acid / g of sand in order to effect a significant decline (85 %). Therefore, when formic and benzoic acids are used, much lower concentrations are needed for eliminating nematode larvae (0.01 g/ml) than for butyric acid. Using the gaseous phase of butyric acid against plant nematodes in a 7-day incubation period showed similar results. The vapour from a 0.1 M solution reduced plant-parasitic and fungivorous nematodes by 89–96 % while the vapour from a 1 M solution of butyric acid reduced entomogenous nematodes by 94–99 %.

Browning *et al*. (2006) have also studied in laboratory conditions the nematocidal properties of butyric acid on fungal and nematode endoparasites of strawberries. Drenching strawberry plants infested with *Pratylenchus penetrans* with butyric acid (0.1 and 1 M) reduced nematode densities by 98 – 100 %. The results of their

research prove the hypothesis that butyric acid is an alternative to synthetic substances.

Sahebani *et al*. (2011) in their research mentioned the impact of β-aminobutyric acid on nematodes of gherkin roots (*Meloidogyne javanica*). They presume that this acid is capable of improving protection reactions in gherkin roots.

Sources from the literature contain a large amount of information on the impact of the studied substances on nematode parasites of mammals, insects, and plants. *Moslemi et al*. (2016) observed salicylic acid to demonstrate inhibitory effects against *Meloidogyne javanica*, a nematode of plants, by inhibiting its reproduction in tomato plants. Nematocidal activity of kojic acid was determined by Kim *et al*. (2016) in the course of a study of its impact on *Meloidogyne incognita*, a parasite of agricultural plants. Our results were negative regarding the mortality of nematode larvae exposed to kojic acid. However, in their experiments, Kim *et al*. (2016) determined a much higher concentration of kojic acid for eliminating nematodes compared to the concentration we used in our experiment. Accordingly, the researchers observed death of 60 % of nematodes at concentration of 333.3 mg/ml. By contrast, in our experiments, concentrations higher than 10 g/l were not used. However, even such a concentration led to similar results for its effect on non-invasive larvae (around 40 % of larvae died).

One of the most widely used substances against parasites currently is formic acid. We also observed positive results against larvae of nematodes of animals: even 1 % solution caused death of L_1, L_2 L_3 of all studied species of nematodes. Although we found no data in the literature on the relative impact of this acid on vitality of mature nematodes, their larvae and eggs, it is often used against Acari parasites of bees (Underwood & Currie, 2003; Underwood & Currie, 2007). According to Underwood *et al*. (2003), even 0.08 and 0.16 mg/l doses of formic acid is efficient against the Acari *Varroa destructor* at a temperature of over 5 °C. Nonetheless, the highest medical efficiency was observed for a dose of 0.16 mg/l at the temperature of 35 °C. In treatment of coccidiosis, one may use compounds including formic, acetic, propionic, succinic, glycolic, lactic, malic, tartaric, citric, ascorbic, maleic, pyruvic and other acids (Muzi & Rahman, 2005). We obtained slightly different results for succinic and tartaric acids. Though L_1, L_2 *S. papillosus* were also exposed to their impact, L_3 *S. papillosus* and L_3 *H. contortus* were resistant to succinic acid, and L_3 *H. contortus* to both succinic and tartaric acids.

Benzoic acid – carboxylic acid of aromatic compound (E210 Codex Alimentarius) is used as a powerful antiseptic and fungicide. It is included in preservatives – food additives, such as E211 – Sodium Benzoate, E212 – Potassium Benzoate, E213 – Calcium Dibenzoate and others (Beerse *et al*., 2001; Amborabe *et al*., 2002; Joshi *et al*., 2008). The results of our study also prove the nematocidal activity of this acid in relation to larvae of nematodes of animals. A 1 % solution can cause death of larvae of *S. papillosus* and *H. contortus* nematodes.

Conclusion

Using food additives, including acids against parasites of animals and humans is one of the new directions in veterinary medicine and biology. Periodic addition to fodder of these substances with nematocidal properties can manage the intensity of helminth infection. Therefore, it is possible for farmers to maintain dairy and meat products at a high level without using anthelmintic preparations of synthetic origin, farmers can. Further experiments can lead to development of preparations containing formic and benzoic acids.

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First record of *Stephanostomum sp.* Looss, 1899 (Digenea: Acanthocolpidae) metacercariae parasitising the pleasure oyster *Crassostrea corteziensis* (Hertlein) from the Mexican Pacific coast

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Summary

The aim of this investigation was to identify the parasites present in the largely understudied pleasure oyster *Crassostrea corteziensis* in Sinaloa state in the northwestern Mexican Pacific coast. Inspection of twenty-eight oysters collected on "Ceuta" lagoon revealed the presence of the digenean *Stephanostomum sp*. (Digenea: Acanthocolpidae) cysts. Metacercariae were found encapsulated and embedded in the digestive gland and mantle tissue of oysters. The prevalence of infection revealed that 84.6 % were infected, the abundance was 13.62, with a mean intensity of 16.09 per host. The members of this genus are characterized by a double crown of spines in the cephalic region surrounding the buccal opening of the worm. Significantly, we report the first incidence of the digenean *Stephanostomum* sp of the family Acanthocolpidae parasitizing *Crassostrea corteziensis*. Further we report that this bivalve is now considered a new intermediate host, and the northwestern Mexican Pacific coast is a new geographical distribution area for this digenean. The findings contribute to our understanding of the biology, biodiversity and host preference of these parasites, with implications for health risks posed by human consumption of the pleasure oyster.
Keywords: bivalve; digenean; trematode; parasites; helminth; oysters; Mexico

Introduction

There are some species of digenens that are known to employ mollusks and use them as definitive or final, intermediate or paratenic hosts for unidentified reasons. Digeneans are one of the most common groups of helminths found parasitising vertebrates. In Mexico, around 503 species have been reported in nearly 440 of the 4,697 species of vertebrates known and a total of 153 digenean species are endemic within Mexican territory (Perez-Ponce de Leon, 2001). Digeneans have a complex life cycle, involving one or two, but rarely more, intermediate hosts prior to infecting the definitive host. Many invertebrates may function as intermediate hosts for a wide variety of parasites. Bivalve mollusks play a key role as hosts for larval stages of digeneans, mainly trematodes (Lasiak, 1992; Ukong, 2007; Thieltges *et al.,* 2006). Mollusk infection may occur through ingestion of the eggs or penetration by free-swimming larvae called miriacidia. Inside the first intermediate host, several different larval stages (i.e., sporocyst, redia, and cercaria) are formed by asexual reproduction. After emerging from the animal tissue, the larvae of trematodes (cercariae) may find a suitable secondary intermediate host i.e., a crustacean by transmission thru a trophic link (metacercariae-cyst) (Gomez del Prado *et al.,* 2003; Grano-Maldonado & Alvarez-Cadena, 2010) or a definitive host by active penetration until it reaches a vertebrate host. The mollusk-trematode model system which has been extensively studied is the host-parasite *Biomphalaria-Schistosoma mansoni.*

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According to Lasiak (1992), the larvae of the bucephalid trematode sporocysts are found between the mantle tissue and visceral mass of intertidal mytilid bivalves from Chile. In Brazil, Ceuta & Boehs (2012) reported that some parasites found in the mangrove mussel *Mytella guyanensis* (Lamarck, 1819) showed *Rickettsia*-like organisms, *Nematopsis* sp. (Apicomplexa), including a turbellarian, sporocysts and metacercariae of *Bucephalus* sp., and metacestodes of *Tylocephalum* sp. in the gills, mantle, and digestive gland. In Mexico, in a coastal lagoon of the southern Gulf of Mexico, Aguirre-Macedo *et al.,* (2007) performed a parasite survey of the oyster *Crassostrea virginica* Gmelin, 1791. These authors found two protozoan (*Nematopsis prytherchi* and *Perkinsus marinus*), and 4 helminth species (*Urastoma cyprinae, Proctoeces maculatus, Bucephalus sp., Tylocephalum sp.*). However, in the Mexican Pacific coast, information about the parasite fauna of the pleasure oyster is almost absent.

The study of parasites in intermediate hosts is important for human health. For example, the host-parasite *Biomphalaria-Schistosoma mansoni* is responsible for human schistosomiasis (Hung *et al.,* 2013; Hotez *et al.,* 2014; Keiser & Utzinger, 2009). Metacercarie *Clinostomum complanatum* has been mainly found in fish *P. pardalis* in the southwest of Mexico (Rodriguez-Santiago *et al.,* 2016), and members of this species have been found attached to the human larynx and pharynx after eating raw seafood (Witenberg, 1944; García *et al*., 2007; Park *et al.,* 2009). Thus, the consumption of raw fish and bivalves such as oysters potentially poses a health risk and should be explored further, motivating this study of parasites in the much understudied mollusk: the pleasure oyster *Crassostrea corteziensis* (Hertlein, 1951).

In the Sinaloa coast in northwest Mexico, the culture of the pleasure oyster is emerging as an important commercial alternative to the Pacific oyster *Crassostrea gigas* for oyster consumers. This mollusk is actively being cultured by local coastal communities for local consumption and commercial market (Chávez-Villalba *et al.,* 2010; Chavez-Villalba 2014; Chávez-Villalba & Aragón-Noriega, 2015). Research of oysters in the Pacific coast has focused on histopathological surveys revealing the presence of hypertrophied gametes, rickettsiales-like prokaryotes, the protozoan *Perkinsus marinus*, *Nematopsis* sp., *Ancistrocoma*, *Sphenophrya*-like ciliates, a turbellarian *Urastoma* sp., hyperparasite of the rickettsiales-like and copepods *Pseudomyicola spinosus and Modiolicola gracilis* (Cruz Flores & Cáceres- Martínez, 2016; Caceres-Martinez *et al.,* 1996, 1998, 1999, 2005, 2010, 2012, 2015, 2016; Costa *et al.,* 2013; Da Silva *et al.,* 2012, 2016; Dantas-Neto *et al.,* 2015; Pinho *et al.,* 2013).

The aim of this investigation was to identify for the first time the helminth parasites present in the pleasure oyster *Crassostrea corteziensis* in Sinaloa state in the northwestern Mexican Pacific coast to increase the current parasite life cycle and biological knowledge. Further research should consider the potential transmission to humans through consumption of this bivalve.

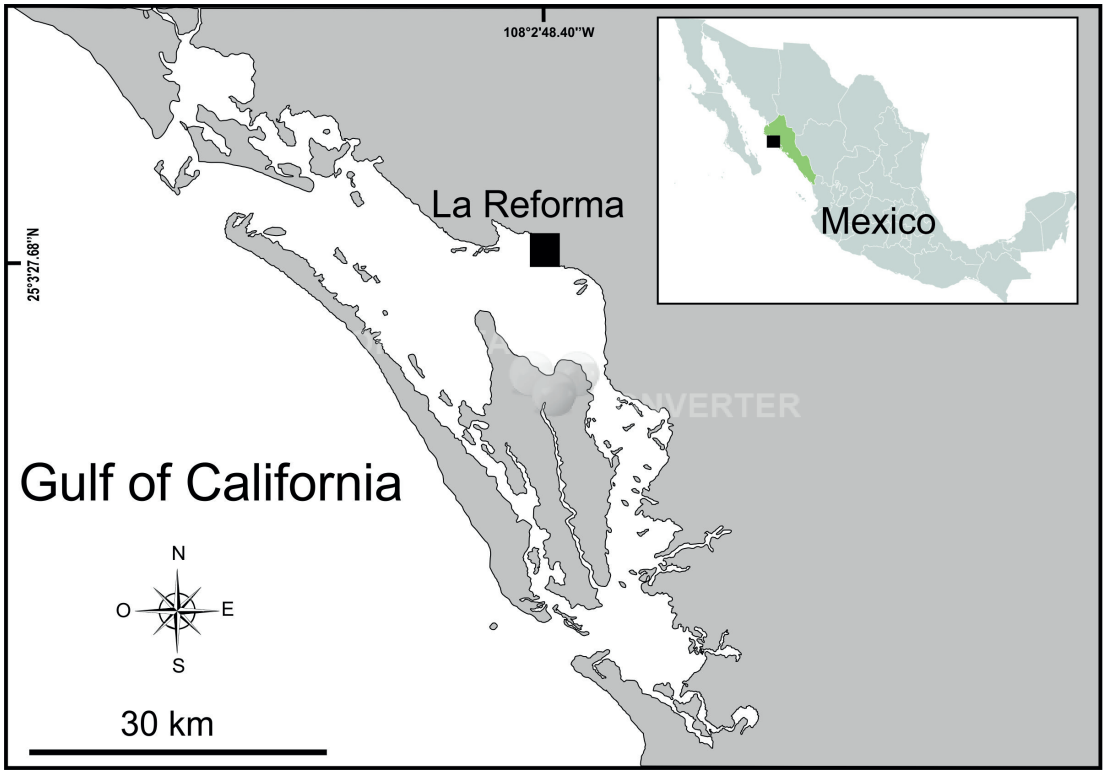

Fig. 1. Location of the collecting area of the pleasure oyster *Crassostrea corteziensis* on coast of Sinaloa, Mexico.

Materials and Methods

Source of parasites

Twenty-eight pleasure oysters *Crassostrea corteziensis* (7.4 ± 1.05 cm average length) were collected by hand from intertidal mudflats close to the mangrove roots from a local fish cooperative in 'Ceuta coastal lagoon, la Reforma' in the State of Sinaloa on the northwest Pacific coast of Mexico (25° 3'27.68"N 108° 2'48.40"W) (Fig. 1). The oysters were transported live to the Microalgae laboratory facilities at the Marine Science Faculty, Autonomous University of Sinaloa. Upon arrival, the organisms were place individually in 2L aquaria supplied with filtered flow-through seawater and constant aeration. The oysters were fed daily with the microalgae *Thalassiosira weissflogii* (Grunow) around 241, 300 cel/ml for five days when all were sacrificed. Animal care and handling were carried out in accordance with Mexican laws (NOM-033-ZOO-1995). The oysters were dissected and organs were carefully separated on individual petri dishes with clean and filtered seawater then reviewed by compression between two 10 cm^2 glass slides under a stereomicroscope (LEICA MZ 9.5, Wetzlar, Germany). Cysts were found in the mantle and digestive gland. The metacercariae were extracted from the cysts using small needles. The parasites were then mounted with a coverslip to flatten the specimens. AFA (a mixture of 85 ml of ethanol, 25 ml of formaldehyde, and 5 ml of acetic acid) was added drop by drop to the edge of the coverslip to fix the parasites, and then they were made transparent with glycerin and mounted. Further observations were done with an optic microscope (LEICA DMLB 10, Wetzlar, Germany) for better resolution. In order to perform morphological descriptions, the parasites were observed under an optical microscope (100 x/oil immersion magnification).

The preparations were made for each parasite for their identification to the lowest taxonomical level. The taxonomic identification of the metacercariae found in this study was based on the work of previous studies (Bray & Cribb, 2003; 2004; 2006; 2008; Bray *et al.,* 2007).

Ethical approval and/or Informed Consent

Animal care and handling were carried out in accordance with institutional guidelines according to Mexican laws (NOM-033-ZOO-1995).

Results

A total of 354 metacercariae cysts recovered from the 28 oysters host (Figs. 1 – 3). The parasite was identified as a *Stephanostomum* Looss, 1899 (Digenea:Acanthocolpidae) by several charac-

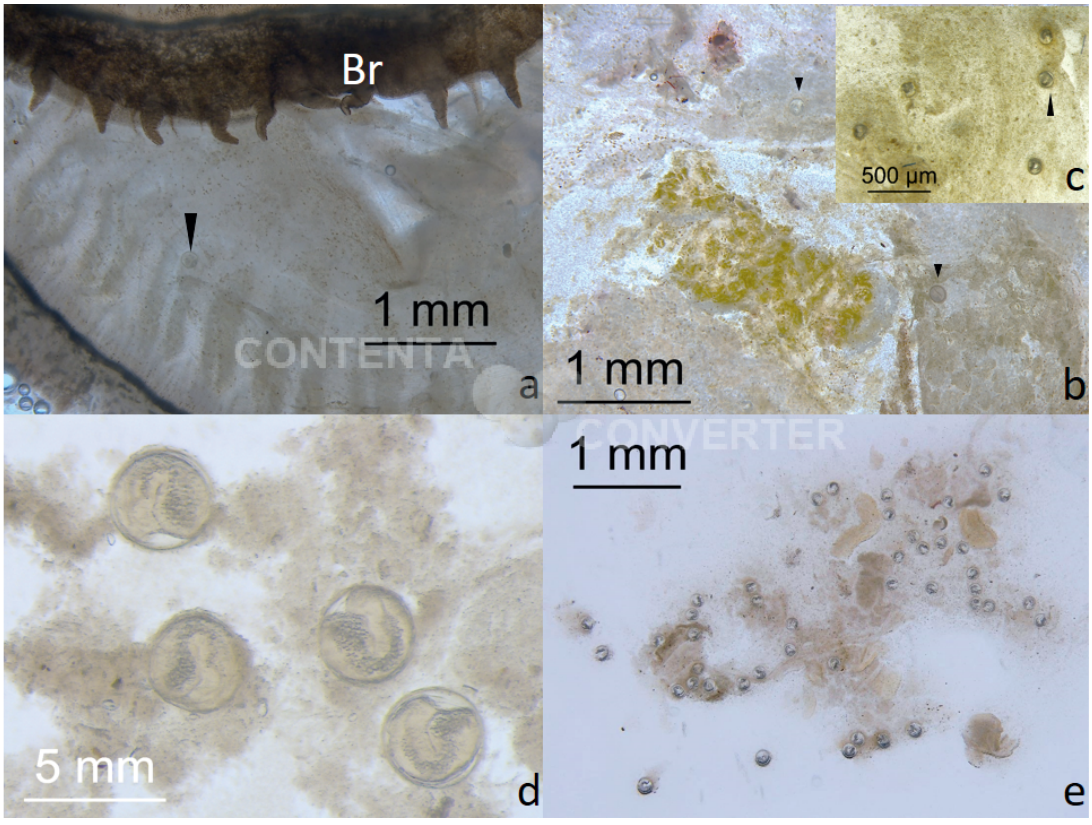

Fig. 2. *Stephanostomum* spp. (Digenea) from the pleasure oyster *Crassostrea corteziensis*. a-b) Cyst in the digestive gland. d-e) Cyst in the mantle.

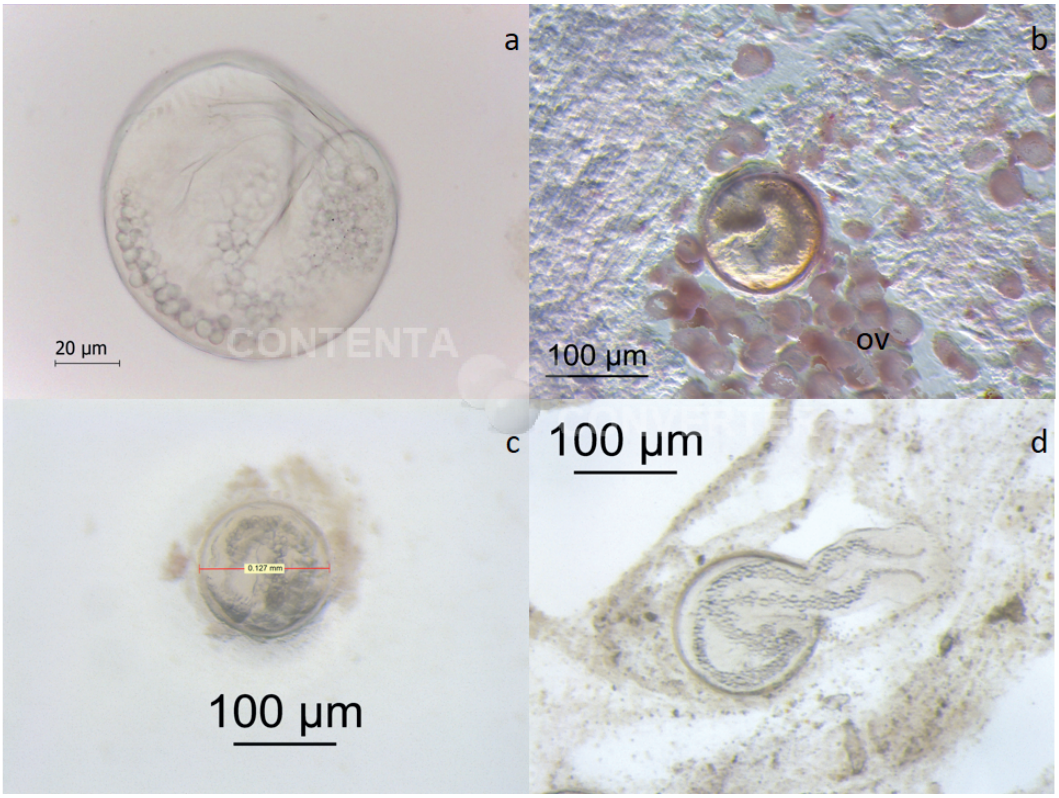

Fig. 3. Metacercariae *Stephanostomum* spp. (Digenea) were found encapsulated and embedded in the digestive gland and mantle tissue of oysters *Crassostrea corteziensis* a-d) individual cysts located in the mantle. b) cyst located close to the gonad, d) opened cyst to reveal the metacercariae.

teristics, particularly the double crown of spines in the cephalic region surrounding the buccal opening of the parasite (Fig. 3). Exemplars were registered (catalogue number: 10630) in the CNHE-IBUNAM. The prevalence was 84 %, and the abundance was 13. The mean intensity was16 per host and the number of parasites per host varied from 1 to 56.

Taxonomic Remarks

Parasite: *Stephanostomum* sp. Looss, 1899 (Digenea: Acanthocolpidae)
Type-host: the pleasure oyster *Crassostrea corteziensis* (Hertlein) (Molluska: Bivalvia)
Habitat: Intertidal mudflats
Site of infection: mantle and digestive gland
Type-locality: Ceuta coastal lagoon Sinaloa, México 25°3'27.68"N 108° 2'48.40"W
Date collection: November 2017
Deposition of specimens: CNHE-IBUNAM- 10630

Recent and extensive studies (Bartoli & Bray, 2001; Saoud *et al.,* 2002, Bray & Cribb, 2003; Bartoli & Bray, 2004) point out that *Stephanostomum* sp. Looss, 1899 is an extremely large genus. It is composed of 112 species nominally distributed around the world that infect a considerable number of teleost fishes, particularly those of temperate waters (Bartoli & Bray, 2001). Cribb *et al.,* (2001) revised the overall pattern of specificity of trematodes in fishes and first intermediate hosts. The acanthocolpid fauna of fishes from Australian and South Pacific waters is very well known. Cribb *et al.,* (2001) inferred that this family Acanthocolpidae is consistent with the overall mollusk pattern. Agreeing with Barnet et al., (2010) who performed and extensive review, the success of this genus is marked by the extensive range of fishes (over 70 families) that are infected as adults. Species of *Stephanostomum* reported from eastern Australian waters include *S. adlardi*, *S. aaravi, S. bicoronatum, S. carangi, S. cobia, S. ditrematis, S. hawaiiense, S. lamothei, S. madhaviae, S. nyoomwa, S. pagrosomi, S. petimba, S. talakitok, S. tantabiddii, S. tupatupa,* and *S. cf. uku*. Barnet *et al.* (2010) reported that there are nine families of trematodes that routinely infect bivalves as first intermediate hosts and a few others (e.g. Lepocreadiidae, Hemiuridae) that infect them exceptionally. However, far more host-parasite records may need to be accumulated for better understanding of the relationships in the Acanthocolpidae group. Our results agree with Barnet *et al*., (2010) and Cribb *et al.,* (2002) who reported that i) *Stephanostomum* was the second largest genus of trematodes of fishes, ii) the genus also appears to have low specificity for first intermediate hosts, and iii) given the current size of the genus there are clearly many more intermediate hosts to be found within and beyond the mollusk. In the present study, *Stephanostomum* was found in the pleasure oyster *Crassostrea corteziensis* in the northwest of the Pacific coast of Mexico. In Mexico, the species of this type of digenan is widely

distributed in marine fish, according with Lamothe-Argumedo *et al.,* (1997) where eight species that parasitize fish of different families have been recorded (*S. californicum* Manter and Van Cleave, 1951 in the intestine of *Genyonemus Californucum* California, USA; *S. casum* was collected from the intestine of *Microlepidotus brevipinnis* in Chamela Bay, Jalisco, *S. dentatum* (Linton, 1910) Linton, 1940 of the intestine of *Balistes polylepis* and *Paralicthys californicus* of Baja California; *S. ditrematis* collected in the stomach of *Seriola dorsalis* captured on the Partida Island; Baja California Sur; *S. hispidum* (Yamaguti, 1934) of *Caranx hippos* in Manzanillo, Colima; *S. megacephalum* Manter, 1940 collected in the intestine of *Caranx hippos* in the Bay of Chamela, Jalisco; *S. tenue* Linton 1898 collected from the *Selar crumenophthalmus* fish from Puerto Vallarta, Jalisco in members of the Lutjanidae family (Garcia-Vargas, 2010).

The members of this genus are characterized by a double crown of spines in the cephalic region surrounding the buccal opening of the worm. Bray *et al.,* (2005) pointed out that the characteristic with the highest taxonomic load to separate the species of this genus is the number of spines that surround the mouth, but this is not a regular pattern. The identification for species level could not be carried out as the parasites in our study displayed a greater number of circumoral spines 63 (58-64, arranged in oral 28-32 and 30 – 32 suboral). Additionally, molecular identification of these parasites merits further investigation to better determine accurate taxonomic status.

We found that the pleasure oyster acts as an intermediate host having infective stages of *Stephanostomum* sp. Looss, 1899 (Digenea:Acanthocolpidae) parasites. Members of the genus *Stephanostomum* have been studied comprehensively in Australia (Bray & Cribb, 2003; 2004, 2004a; 2005; 2008; Bray *et al.,* 2007) where adult stages were present in fishes of the family Carangidae and Scombrids. Based on our contribution in which we identified the parasitic larval stages metacercarie existing in *Crassostrea cortezensis,* further research should consider the potential transmission to humans through consumption of this bivalve.

Discussion

This study is the first report identifying the metacercariae of *Stephanostomum* species, a digenean of the family Acanthocolpidae, parasitising the pleasure oyster *Crassostrea corteziensis* (Hertlein) from the Mexican Pacific Coast. According to Caceres-Martinez *et al.,* (2016) other pathogens like *Perkinsus marinus* seems to represent a more significant risk for the health of pleasure oysters than do other parasites, and surveillance and control of these parasites are needed for the development of pleasure oyster culture. However, in our study the majority of parasitised oysters appeared

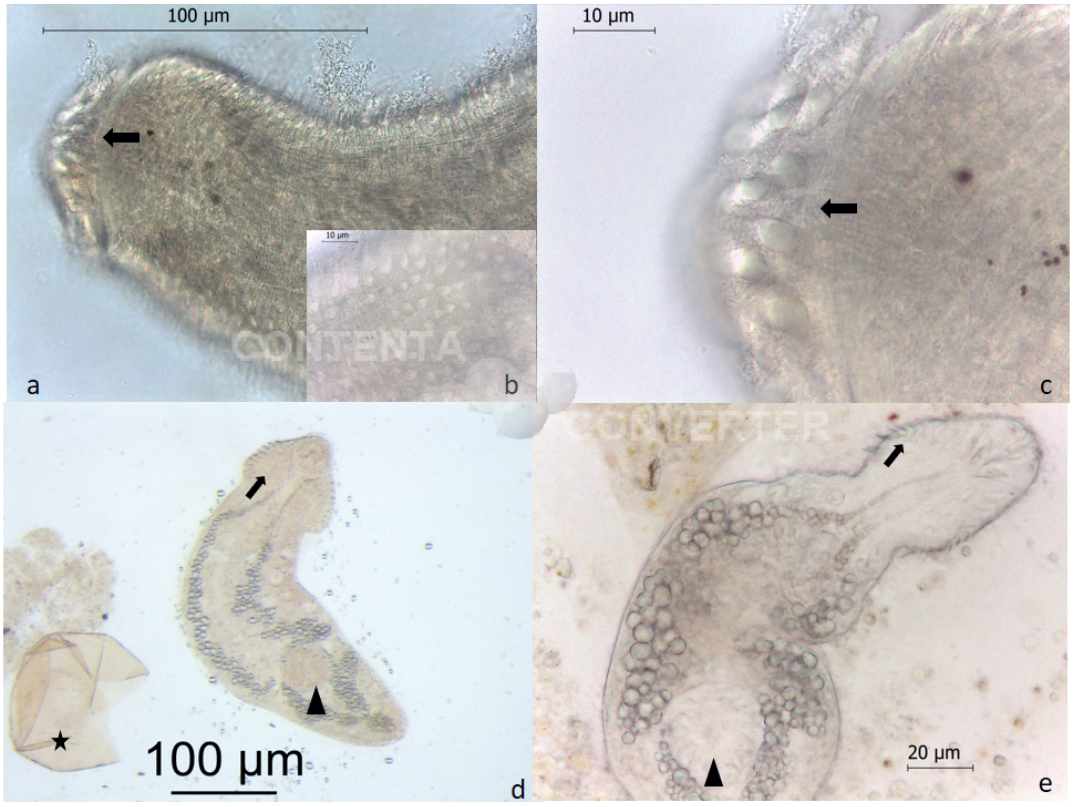

Fig. 4. Microphotography using an optical microscope of cyst released metacercariae of *Stephanostomum* spp. (Digenea). a-d) noticed the a double crown of spines in the cephalic region (arrow) surrounding the buccal opening of the worm; d-e) acetabulum (arrow head), empty cyst (star).

to be free of infection and no apparent destruction of the host's gonadal tissue, digestive or branchial damage was observed. In the parasitological study performed by Aguirre-Macedo et al. (2007) the prevalence and mean abundances for the protozoa and helminths varied widely between locations but were generally below 50 %. *Nematopsis prytherchi* and the *Tylocephalum sp*. were the most prevalent species (>60 %). *Perkinsus marinus* was present in oysters of eight coastal lagoons and had low prevalence (<30 %) in almost all samples. All identified protozoa and helminths are widely distributed in the Gulf of Mexico and are common oyster parasites. Only *P. marinus* and the *Bucephalus sp*. were associated with damage to host tissues. In our study the prevalence of *Stephanostomum* species infection was 84.6 %, the abundance was 13.62 and the mean intensity of 16.09 per host. These results revealed a high percentage of parasitism in the zone, this is alarming since the typical consumption of this animal is uncooked. It is important to mention that most human-parasite disease transmission is mainly associated with the consumption of raw seafood dishes i.e., sushi, sashimi, ceviche (Kuchta *et al.,* 2005ab). Most of the species potentially pathogenic to human are pathogenic mostly in larval stage, i.e., L1 and L3 in nematodes, plerocercoid in cestodes and metacercariae in digenan trematodes (Rodriguez-Santiago *et al.,* 2016). Consequently, the ingestion of bivalves such as oysters as a source of raw food may have a health risk factor and should be explored further.

Barnet *et al.,* (2010) reported in the case of other *Stephanostomum* spp. cercariae from mollusks *(*Gastropoda*:* Nassariidae*)* in Australia. Their results suggest that parasite location in the host was similar to our findings (Digestive gland, gonads) and the habitat of bivalve collection of intertidal mudflats was similar with a prevalence of emergence: 0.57 % (10 of 1766 *Nassarius dorsatus*), 0.21 % (4 of 1908 *Nassarius olivaceus*). In our study, the parasite *Stephanostomum sp.*was also present in a costal lagoon in Sinaloa state in quantities greater than 84.6 %. With respect to the digenetic trematodes, the presence of metacercariae observed in our study reveals that bivalves may serve as potentially primary and secondary intermediate hosts at the same time.

A limitation of this study is that molecular techniques could not be used because the samples were preserved on formalin. Future comparison of the 18S ribosomal RNA gene (or other conserved genes) sequence of the parasite collected from pleasure oysters should be conducted. There is no doubt, nonetheless, that infections by parasites have major consequences for species of host in natural conditions and must consequently be considered as a fundamental factor within any system of aquaculture.

Conflicts of Interest

Authors have no potential conflict of interest pertaining to this submission to *Helminthologia*.

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First report of segmented fi lamentous bacteria associated with *Rhigonema* sp. (Nematoda: Rhigonematidae) dwelling in hindgut of *Riukiaria* sp. (Diplopoda: Xystodesmidae)

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Summary

We morphologically and molecularly characterized segmented fi lamentous bacteria (SFB) associated with *Rhigonema* sp. nematodes in millipede hindguts. Seventy-three *Riukiaria* sp. millipedes were collected from a broad-leaf forest in Japan, and nematodes were excised from the millipede's hindguts. The occurrence rate of SFB associated with nematodes was 24 % (10/41) for males, 47 % (14/30) for females, and 100 % (2/2) for juveniles. Genomic DNA was extracted from four SFB-rich nematode heads, and we obtained 40 bacterial clones via analysis of nearly full-length 16S rDNA gene sequences. At the phylum level, Firmicutes, Proteobacteria, and Verrucomicrobia accounted for 55 %, 40 %, and 5 % of SFB, respectively. In Firmicutes, Clostridiaceae (28 %) and Lachnospiraceae (15 %) were the dominant groups. Our sequences were divided into seven and three subclades between Firmicutes and Proteobacteria in the phylogenetic tree. In the Firmicutes clade, eight sequences were classifi ed as Lachnospiraceae with a bootstrap value >83 %. A phylogenetic tree involving known uncultured Lachnospiraceae sequences characterized the phylogenetic position of SFB associated with nematodes. Our results suggest that the association of SFB with nematode bodies was probably incidental and that SFB are not always present in millipede hindguts. Our bacterial groups corresponded to those of arthropod hindgut, and SFB associated with nematodes were inferred to belong to Lachnospiraceae. Because the Lachnospiraceae sequences obtained in this study showed specifi c lineages that differed from all the known deposited sequence data, these groups may be unique to *Riukiaria* sp.

Keywords: Firmicutes; Lachnospiraceae; phylogenetic analysis; morphology; 16S rDNA

Introduction

Guts of many arthropods (e.g., termites, cockroaches, and millipedes) are inhabited by segmented fi lamentous bacteria (SFB), which are also referred to as "long segmented fi lamentous structures" (Margulis *et al.*, 1998; Thompson *et al.*, 2012). In the guts, these Gram-positive endospore-forming microbes are attached to the epithelial walls (Klaasen *et al.*, 1992). Although SFB are diffi cult to culture *in vitro*, this group is well known as common constituents of gut microbes (Krecek *et al.*, 1987; Brune & Dietrich, 2015). Moreover, SFB were suggested to play a crucial role in host immune function through the coordination of T-cell responses (Ivanov *et al.*, 2009).

Among soil-dwelling arthropods, nematodes have frequently been isolated from the guts of termites (Carta & Osbrink, 2005), cockroaches (Ozawa *et al.*, 2014), and millipedes (Hunt & Moore, 1995; Morffe & Hasegawa, 2017). SFB-like organisms were reported to be associated with the gut parasite nematodes *Rhigonema* spp.

* – corresponding author

Table 1. Numbers of millipede *Riukiaria* sp., occurrence frequency of nematode *Rhigonema* sp., and segmented filamentous bacteria (SFB) associated with nematodes in millipede hindguts.

Sex	Observed numbers	Body length (mm)	Occurrence frequency of nematodes [b]					SFB associated with nematodes (%)
			0	1 – 10	11 – 20	21 – 30	>30	
Male	41	34.0 ± 0.4 [a]	4 (10 % [c])	17 (41 %)	13 (32 %)	6 (15 %)	1 (2 %)	24 [d]
Female	30	36.5 ± 0.5	0	3 (10 %)	7 (23 %)	19 (64 %)	1 (3 %)	47
Juvenile	2	19.5 ± 2.5	0	0	1 (50 %)	1 (50 %)	0	100

[a] Data shown are mean ± SE.
[b] Numbers in parentheses indicate relative abundance. The frequency distribution of nematode abundances was significantly different between male and female millipedes (χ^2-test, χ = 21.164, df = 4, P < 0.001), but there was no significant difference between the sexes with regard to occurrence rate of SFB associated with nematodes (χ^2-test, χ = 2.911, df = 1, P = 0.08). Data on juveniles were not used in these analyses owing to the small sample size.
[c] Frequency of nematode individuals at each stage (%) = (number of nematodes in a certain range) / (all nematodes observed in hindguts of millipedes) × 100.
[d] SFB associated with nematode at each stage (%) = (occurrence frequency of SFB attached nematodes) / (observed hindguts of millipedes) × 100.

in millipede hindgut as early as the 19th century (Leidy, 1853). The SFB associated with *Rhigonema* spp. were also confirmed in hindguts of *Riukiaria* spp. (Xystodesmidae) in Japan (Kanzaki *et al.*, 2016). However, the frequency of occurrence and phylogenetic position of SFB associated with *Rhigonema* sp. in millipede hindguts has not been investigated in detail.

The purpose of this study was to clarify the occurrence rate and phylogenetic position of SFB associated with nematodes. For this, we collected millipedes in a broad-leaf forest and characterized SFB associated with hindgut-dwelling nematodes by both morphological and molecular approaches.

Materials and Methods

Adults and juveniles of *Riukiaria* sp. were collected at Hinokuma Park in Saga Prefecture (34°33'N, 130°35'E), Japan, in June 2018 (Fig. 1a). At the site, *Cinnamomum camphora*, *Quercus aliena*, *Quercus serrata*, and *Schoepfia jasminodora* of various ages were patchily distributed, and their broad-leaf litter covered the ground (Fig. 1b). Annual precipitation and annual mean temperature in June 2018, as recorded at the nearest weather station in Saga (8 km from the site), were 291 mm and 24.1 °C, respectively.

The millipedes were brought back to the laboratory alive and stored at 20 °C for less than a month, until extraction of nematodes. The hypopygium of *Riukiaria* sp. was dissected with fine tweezers, and the hindgut was pulled out and excised. *Rhigonema* sp. individuals were isolated from the hindgut of juvenile, male, and female millipedes in distilled water. All the *Rhigonema* nematodes retrieved from each millipede's hindgut were transferred into a Petri dish filled with distilled water. For each millipede, the numbers of *Rhigonema* sp. with and without SFB were counted under a stereomicroscope (SZX16, Olympus, Tokyo, Japan; maximum magnification ×115).

We performed a χ^2-test to ascertain whether the frequencies of occurrence of nematodes and SFB associated with nematodes in guts differed significantly between male and female millipedes. We also performed a Pearson's correlation test to assess the relationship between the number of nematodes per millipede and

Fig. 1. Location of sampling site in Japan and a collected millipede. (a) The gray circle indicates the sampling location. Bar = 100 km. (b) View of a sampled broad-leaf forest stand. (c) *Riukiaria* sp. collected beneath the litter layer on the ground.

the number of nematodes associated with SFB. All data analyses were performed in R 3.3.2 (R Core Team, 2016), and the significance level was set at $P < 0.05$.

Extracted nematodes were observed under a light microscope (BX53, Olympus; ×100–400) for detailed observation of SFB. The nematodes were Gram stained with crystal violet (Wako, Osaka, Japan) for the presence of SFB, then photographed (TIFF format, 1360 × 1024 pixels) with a digital camera (DP70, Olympus) connected to the microscope.

Genomic DNA was extracted from four SFB-rich nematode heads using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). To obtain nearly the full length of 16S rDNA, the extracted DNAs were amplified using Tks Gflex (Takara, Shiga, Japan) with the primer pair 9f (GAGTTTGATCCTGGCTCAG; Yoon *et al.*, 1997) and 1541r (AAGGAGGTGATCCAGCCG; Sato *et al*., 2004). Thermal conditions were one cycle of 94 °C for 1 min, followed by 30 cycles of 98 °C for 10 sec, 55 °C for 30 sec, and 72 °C for 90 sec. Positive PCR products were cloned using the TA-Enhancer Cloning Kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Sixteen successfully inserted white colonies were picked up per nematode head part. Selected colonies were further amplified with Tks Gflex and the primer pair 9f and 1541r. The thermal cycle program comprised 25 cycles of 98 °C for 10 sec, 55 °C for 30 sec, and 72.0 °C for 90 sec. When PCR amplicons were successfully produced, they were purified by using ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix, Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. Purified DNAs were labeled by using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and then sequenced by using an ABI 3730 sequencer (Applied Biosystems) with one of the three primers – 9f, 1541r, or 533f (GTGCCAGCAGCCGCGGTAA; Weisburg *et al.*, 1991) – to obtain full-length 16S rDNAs.

Obtained sequences were adjusted manually in MEGA v. 7 software (Kumar *et al*., 2016; https://www.megasoftware.net/), and sequences of the same sample read by different primers were assembled into a longer sequence whenever possible. Forty sequences were deposited in the DDBJ under accession numbers LC462722–LC462761. Sequences were compared with deposited sequences and identified to the nearest taxon using pairwise searches with the basic local alignment search tool (BLAST) (Altschul *et al*., 1997; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

To estimate the phylogenetic positions of the bacteria, we constructed phylogenetic trees by using the maximum likelihood

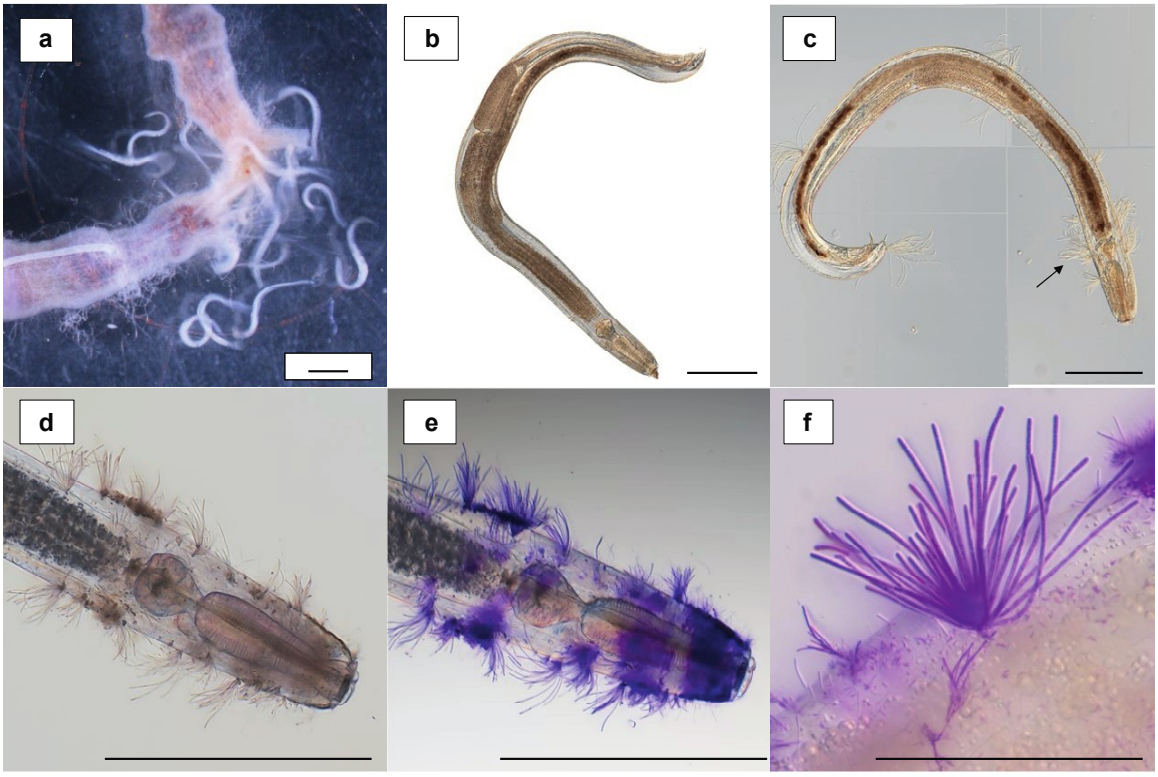

Fig. 2. Micrographs of *Rhigonema* sp. isolated from millipede guts. Images show whole individual and enlarged head part. (a) *Rhigonema* sp. crawl out from a millipede hindgut. (b) *Rhigonema* sp. without segmented filamentous bacteria (SFB). (c) *Rhigonema* sp. with SFB (arrow). (d) Enlarged head part of *Rhigonema* sp. with associated SFB. (e) Gram staining with crystal violet of associated SFB at head part. (f) SFB attached to nematode body surface. Bars represent (a) 1 mm, (b) 500 µm, and (f) 100 µm.

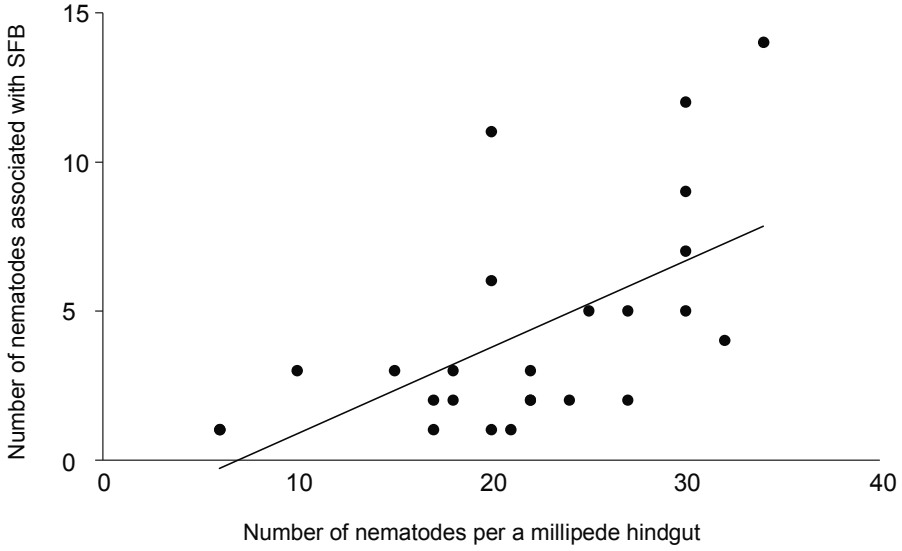

Fig. 3. Correlation between number of *Rhigonema* sp. nematodes per *Riukiaria* sp. millipede hindgut and number of nematodes associated with segmented filamentous bacteria (SFB), for those millipedes that had at least one nematode associated with SFB (n = 25, Pearson's correlation test, r = 0.593, P = 0.0017).

method in MEGA. For the default substitution model, we selected General Time Reversible (GTR) with Gamma-distributed rates plus Invariant sites (G+I), which had the smallest Akaike's information criterion (AIC) and Bayesian information criterion (BIC) based on the model selection implemented in MEGA. To draw the trees, we referred to the clone library of bacterial sequences derived from the cockroach *Shefordella lateralis* (Schauer *et al*., 2012), the termite *Reticulitermes santonensis*, and the millipede *Tachypodoiulus niger* (Thompson *et al*., 2012) deposited in GenBank. The sequences were aligned in MAFFT v. 7 software (Katoh & Standley, 2013; http://mafft.cbrc.jp/alignment/software/) with default settings. In addition, we used the closest sequences of named family

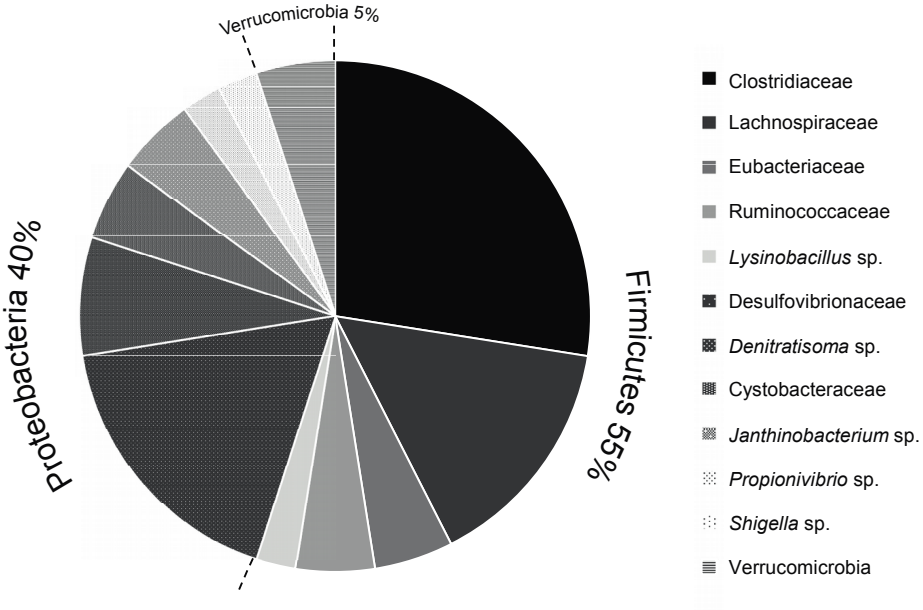

Fig. 4. Occurrence rates (%) of bacterial taxa detected from segmented filamentous bacteria–enriched *Rhigonema* sp. heads in hindgut of *Riukiaria* sp. (40 clones).

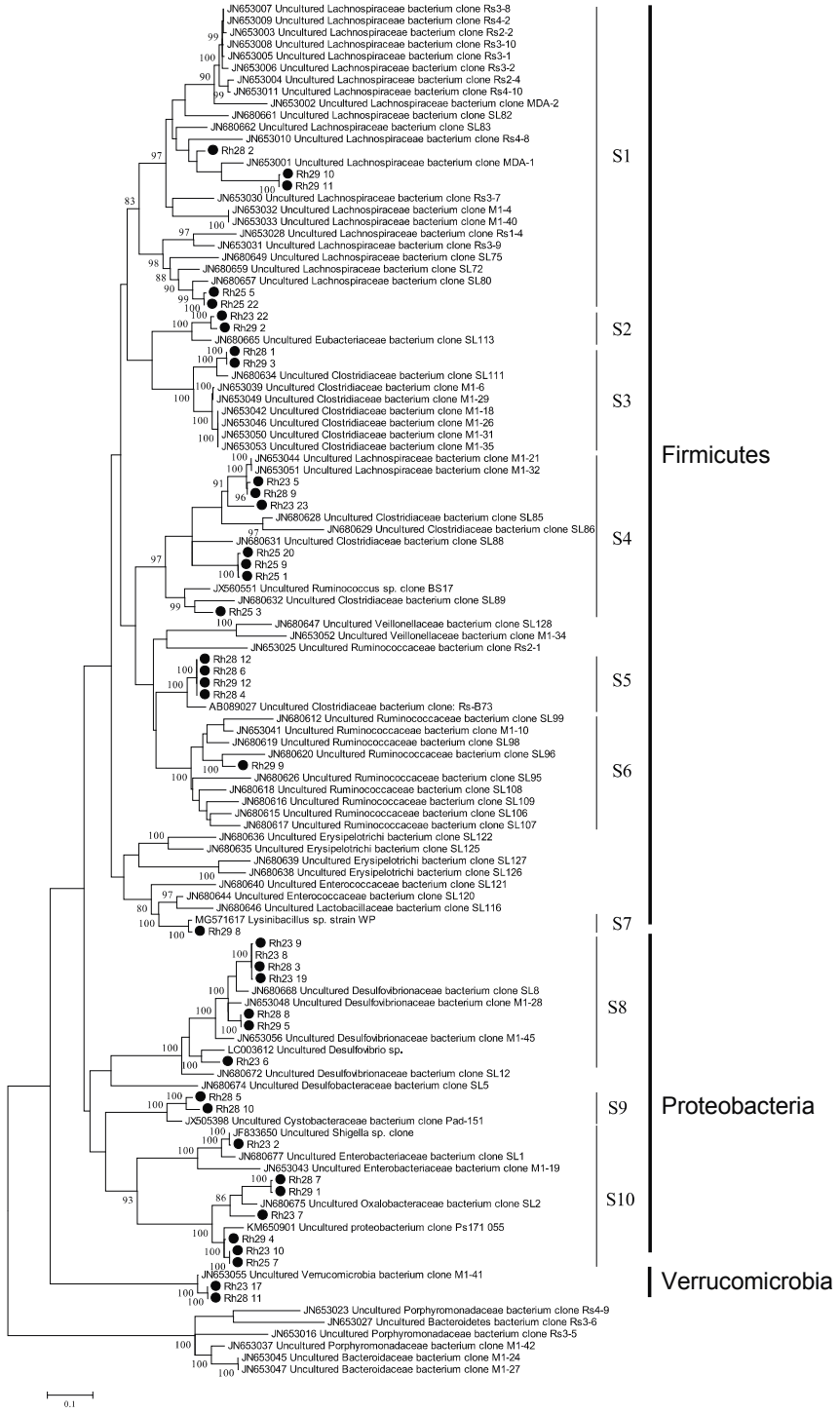

Fig. 5. A maximum likelihood phylogenetic tree of segmented filamentous bacteria (SFB) associated with *Rhigonema* sp. based on partial 16S rDNA sequences. The substitution model was GTR with G+I sites (AIC = 33224.486, BIC = 35454.324). We used clone libraries of 76 bacterial sequences as ingroups derived from the cockroach *Shefordella lateralis* (Schauer *et al*., 2012), termite *Reticulitermes santonensis*, and millipede *Tachypodoiulus niger* (Thompson *et al*., 2012) deposited in GenBank. No outgroups were used. Values ≥80% on branches indicate confidence limits estimated by bootstrap analysis with 1000 replicates.

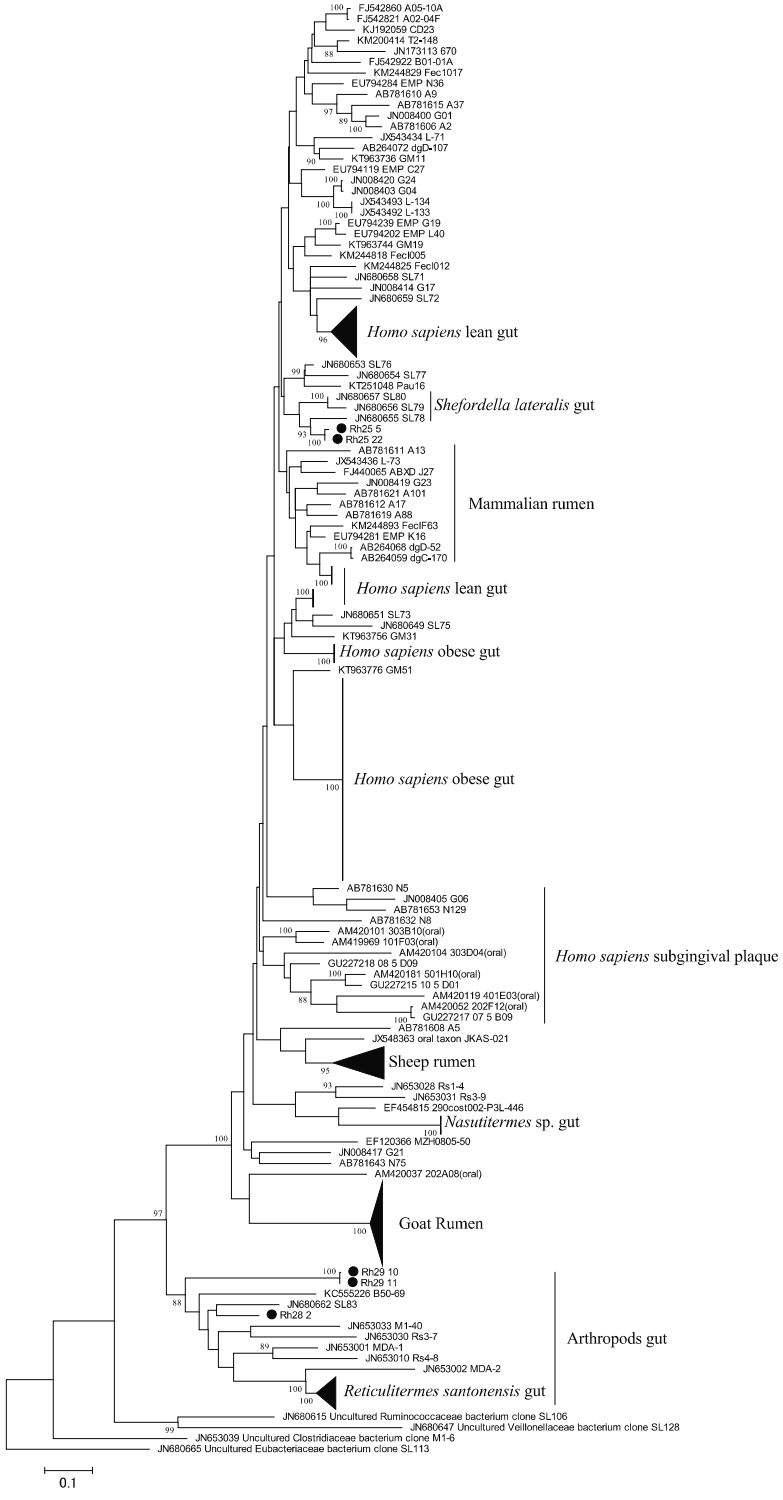

Fig. 6. A maximum likelihood phylogenetic tree of associated Lachnospiraceae groups of subclade S1 based on partial 16S rDNA sequences. The substitution model was GTR with G+I sites (AIC = 53520.724, BIC = 57505.700). Known sequences of 181 uncultured Lachnospiraceae with nearly the full length of 16S rDNA deposited in GenBank were used as ingroup sequences. Clostridiaceae (JN653039), Eubacteriaceae (JN680665), Ruminococcaceae (JN680615), and Veillonellaceae (JN680647) were used as outgroups. Values ≥80% on branches indicate confidence limits estimated by bootstrap analysis with 1000 replicates.

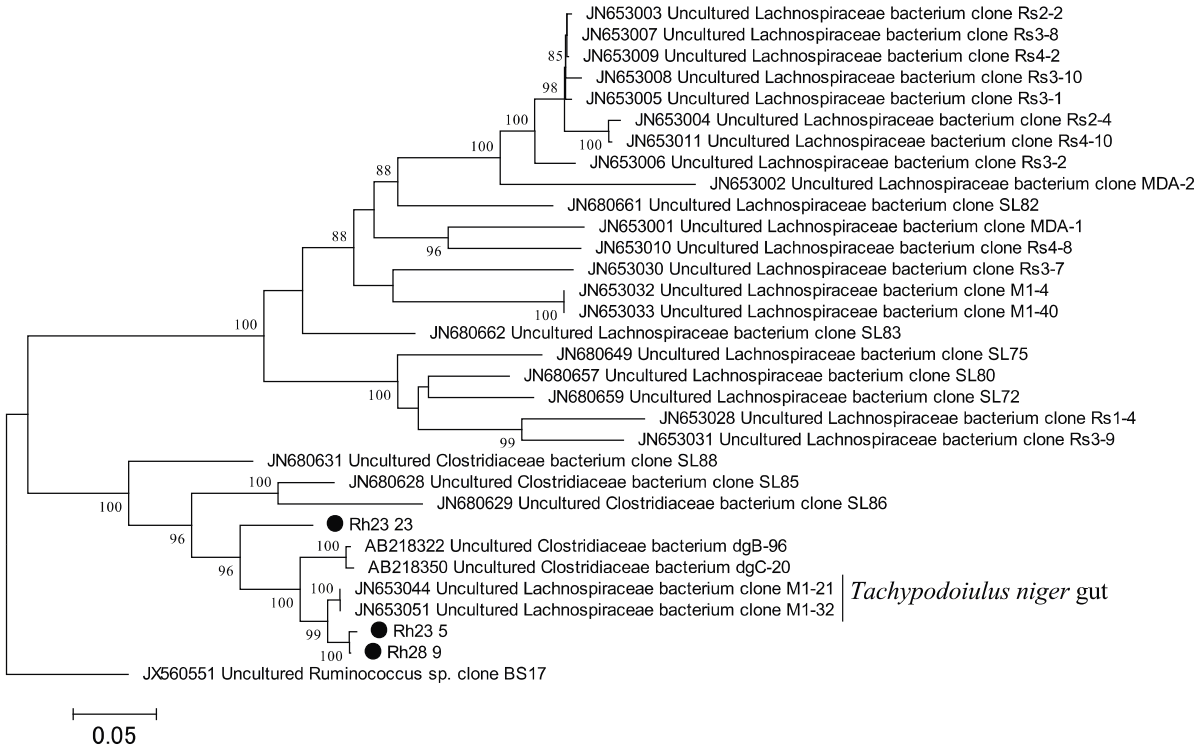

Fig. 7. A maximum likelihood phylogenetic tree of associated Lachnospiraceae groups of subclade S4 based on partial 16S rDNA sequences. The substitution model was GTR with G+I sites (AIC = 20633.765, BIC = 21242.547). As ingroup sequences, we used known sequences of 26 uncultured Lachnospiraceae with nearly the full length of 16S rDNA derived from the cockroach *Shefordella lateralis* (Schauer et al., 2012), termite *Reticulitermes santonensis*, and millipede *Tachypodoiulus niger* (Thompson et al., 2012) deposited in GenBank. *Ruminocccus* sp. (JX560551) was used as the outgroup. Values ≥80% on branches indicate confidence limits estimated by bootstrap analysis with 1000 replicates.

or genus in GenBank identified by using pairwise BLAST searches as in-group sequences. To increase the resolution of phylogenetic positions, the sequences that were tentatively assigned to Lachnospiraceae (see Results) were re-analyzed by the maximum likelihood method as above involving all the known uncultured Lachnospiraceae sequences with nearly the full length of 16S rDNA deposited in GenBank. For the default substitution model, we selected GTR with G+I, which had the smallest AIC and BIC based on the model selection in MEGA. For the Lachnospiraceae tree, Clostridiaceae (JN653039), Eubacteriaceae (JN680665), Ruminococcaceae (JN680615), and Veillonellaceae (JN680647) in subclade S1 (see Fig. 6), *Ruminocccus* sp. (JX560551 in subclade S4 (see Fig. 7) were used as outgroups. The reliability of all tree topologies was evaluated by 1000 bootstrap resamplings (Felsenstein, 1985).

Ethical Approval and/or Informed Consent

The research related to animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

The microscopy of *Rhigonema* sp. demonstrated that SFB attached to the nematode body (Fig. 2a – d), and the parts associated with SFB were clearly stained purple with the crystal violet (Fig. 2e, f). The occurrence rates of *Rhigonema* sp. in millipede hindguts were 90 % (37/41) in males, 100 % (30/30) in females, and 100 % (2/2) in juveniles (Table 1). The highest frequency of *Rhigonema* sp. per hindgut was 41 % for males harboring 1 – 10 individuals and 64 % for females harboring 21 – 30 individuals. The relative abundance of nematodes was significantly different between male and female millipedes (Table 1, χ^2-test, $\chi = 21.164$, df = 4, $P < 0.001$). The occurrence rate of SFB associated with nematodes was 24 % (10/41) for males, 47 % (14/30) for females, and 100 % (2/2) for juveniles, and there was no significant difference between sexes (Table 1, χ^2-test, $\chi = 2.911$, df = 1, $P = 0.08$). We noted a significant positive correlation between the number of nematodes per millipede and the number of nematodes associated with SFB (Fig. 3, Pearson's correlation test, $n = 25$, $r = 0.593$, $P = 0.0017$).

Since 6 clones were identified as *Escherichia coli* (negative con-

trol) by BLAST search, they were excluded. DNA sequences were obtained from 69 % (40/58) of clones. At the phylum level, Firmicutes, Proteobacteria and Verrucomicrobia accounted for 55 %, 40 %, and 5 %, respectively (Fig. 4). In the Firmicutes, Clostridiaceae (28 %) and Lachnospiraceae (15 %) were the dominant groups, whereas Desulfovibrionaceae (18 %) dominated the Proteobacteria. These clones were used for the construction of a maximum likelihood tree with known, named sequences (Fig. 5). Our sequences were affiliated with taxonomic clades (Firmicutes, Proteobacteria, or Verrucomicrobia) in the tree. Among them, Firmicutes and Proteobacteria harbored seven and three subclades, respectively, with bootstrap (BS) values greater than 83 % (Fig. 5). The subclade S1 formed with Lachnospiraceae lineages, and two of five sequences (Rh25_5, Rh25_22) were close to uncultured Lachnospiraceae derived from *S. lateralis* (JN680655), with a 93 % BS value (Fig. 6). On the other hand, clones of Rh28_2, Rh29_10, and Rh29_11 formed a clade with an 88 % BS value. In a tree retrieved from the S4 clade, three of our sequences were positioned differently from most Lachnospiraceae sequences, with 100 % BS values (Fig. 7). The tree was an admix lineage of Clostridiaceae and Lachnospiraceae, and three sequences (Rh23_5, Rh23_23, Rh28_9) were nested within Clostridiaceae groups neighboring Lachnospiraceae clones, with a 99 % BS value.

Discussion

Our results clearly showed that most nematodes in millipede hindguts were associated with SFB. This study is the first to reveal the identity of SFB associated with nematodes by using molecular methods. Moreover, our bacterial sequences were clustered with known data derived from arthropod guts at high BS values. On the basis of these results, we discuss the occurrence frequency and phylogenetic position of SFB associated with nematodes.

Rhigonema spp. are assumed to be common in hindguts of *Riukiaria* spp. in Japan (Kanzaki *et al*., 2016), and the frequency of occurrence of the nematode in millipede guts was high in both sexes (Table 1). However, the number of nematodes per millipede gut was significantly greater in females than males. Females feed more actively than males in order to obtain more nutrients for oviposition (Boggs, 1981), so nutritional levels likely differ between sexes, as reflected in the population size of nematodes in their guts. The occurrence rate of SFB associated with nematodes was less than 50 % for both sexes. Moreover, the number of nematodes per millipede hindgut and the number of nematodes associated with SFB were significantly and positively correlated. Thus, the SFB density in millipede guts might depend on the population size of parasitizing nematodes.

Microscopy of *Rhigonema* sp. revealed that associating SFB were Gram-positive, formed endospores, and attached to the body of nematodes. These morphological characters agree well with previous reports (Klaasen *et al*., 1992). Leidy (1853) observed a SFB associated with *Rhigonema infectum*, *Thelastoma attenuatum* and *Aorurus agilis* in the hindgut of the millipede *Narceus annularis* (summarized in Sayre & Starr, 1988). Further, more recently, *Blatta orientalis*, *Leidynema* spp. nematodes were found to be covered with SFB in the hindgut of black cockroach, *Blatta orientalis* (Dr. Sergei Spiridonov, pers. comm.). Thus, SFB might generally associate with nematodes harbored within arthropod hindguts.

The most representative phyla in this study were Firmicutes and Proteobacteria, which were also dominant in the hindgut of Japanese cockroach, *Periplaneta japonica* (Vicente *et al*., 2018). The most frequent taxa were Clostridiaceae and Lachnospiraceae, which are anaerobic and unculturable taxa known to be an abundant group in arthropod guts (Engel & Moran, 2013; Vicente *et al*., 2018). Our sequence data were nested with known anaerobic bacteria in gut conditions of millipedes, termites, and cockroaches with a high BS probability (>80 % BS, Fig. 5). Common bacterial groups in arthropods' hindguts were attached to nematode head parts. The SFB in the hindgut of the termite *R. santonensis* and the millipede *T. niger* were classified phylogenetically to a group of Lachnospiraceae (Thompson *et al*., 2012). Lachnospiraceae were common in the hindgut of arthropods, such as termites, cockroaches, and millipedes, and were attached to their gut walls (Thompson *et al*., 2012). In the Lachnospiraceae tree in subclade S1, our sequences were clearly separated from other known sequences (100 % BS, Fig. 6). Moreover, the refined tree of subclade S4 was constructed by admixed sequences of both Clostridiaceae and Lachnospiraceae; our sequences were nested within the former taxon but clearly differed from most members of the latter taxon (Fig. 7). The phylum Firmicutes included representatives of common gut bacteria, with most belonging to the family Lachnospiraceae, and highly specific lineages associated with the hindgut cuticle of arthropods (Brune & Dietrich, 2015). Since the sequences obtained in this study also showed specific lineages differing from all the known deposited sequence data, these groups might be unique to *Riukiaria* sp. In addition, one of our obtained sequences was not placed with any known taxa, implying that the sequence is from an as-yet-unknown taxon. The Lachnospiraceae lineages were distributed among various locations and suggested to include cryptic species, indicating that the phylogenetic relationship within this group should be further investigated.

Our morphological and molecular analyses helped to characterize the SFB associated with nematodes within millipede hindguts in Japan. The association of SFB with nematode bodies was probably incidental and did not occur in all millipede hindguts. While the function of SFB in arthropod guts remains unknown, Ivanov *et al*. (2009) reported their crucial role in a host immune function through the coordination of T-cell responses. The distribution and functional significance of nematode-associated SFB remain unclear. To resolve these issues, in future research we plan to observe the ultrafine structure of SFB-associated parts in nematodes.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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A new root-knot nematode, *Meloidogyne moensi* n. sp. (Nematoda: Meloidogynidae), parasitizing Robusta coffee from Western Highlands, Vietnam

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Summary

A new root-knot nematode, parasitizing Robusta coffee in Dak Lak Province, Western Highlands of Vietnam, is described as *Meloidogyne moensi* n. sp. Morphological and molecular analyses demonstrated that this species differs clearly from other previously described root-knot nematodes. Morphologically, the new species is characterized by a swollen body of females with a small posterior protuberance that elongated from ovoid to saccate; perineal patterns with smooth striae, continuous and low dorsal arch; lateral lines marked as a faint space or linear depression at junction of the dorsal and ventral striate; distinct phasmids; perivulval region free of striae; visible and wide tail terminus surrounding by concentric circles of striae; medial lips of females in dumbbell-shaped and slightly raised above lateral lips; female stylet is normally straight with posteriorly sloping stylet knobs; lip region of second stage juvenile (J2) is not annulated; medial lips and labial disc of J2 formed dumbbell shape; lateral lips are large and triangular; tail of J2 is conoid with rounded unstriated tail tip; distinct phasmids and hyaline; dilated rectum. *Meloidogyne moensi* n. sp. is most similar to *M. africana*, *M. ottersoni* by prominent posterior protuberance. Results of molecular analysis of rDNA sequences including the D2–D3 expansion regions of 28S rDNA, COI, and partial COII/16S rRNA of mitochondrial DNA support for the new species status.
Keywords: new species; coffee; root-knot nematode; Vietnam

Introduction

Coffee is one of the most important crops worldwide because of its economic value (Campos & Villain, 2005; Waller *et al*., 2007). According to Wiryadiputra and Tran (2008), Vietnamese Robusta coffee growing area is one of the biggest Robusta coffee growing areas in the world. The Vietnamese coffee growing area is about 500000 hectares, and most of them are located in Western Highlands of Vietnam (Trinh *et al*., 2009). The quality of coffee plantations is crucial since any imperfection can affect the outcome (Wiryadiputra & Tran, 2008). Several factors challenge the coffee production such as soil fertility, varietal choice, and most importantly is the presence of pathogens. One of the biotic agents that threatens coffee production is root-knot nematode. Root-knot nematodes, *Meloidogyne* spp., are distributed worldwide and parasitize a wide range of economically important crops, including coffee, that cause serious damages (Campos & Villain, 2005; Perry *et al*., 2009; Souza, 2008; Waller *et al*., 2007). They are obligate parasites on the roots of thousands of plant species, including monocotyledonous and dicotyledonous, herbaceous, and woody plants (Perry *et al*., 2009). Root-knot nematode infection causes root galls, shoot chlorosis, stunted growth, nutrient deficiencies,

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and secondary infections by other pathogens (Souza, 2008).

To date, eighteen species of the genus *Meloidogyne* have been reported in the world, namely *M. exigua* Goldi, 1887, *M. africana* Whitehead, 1960, *M. arabicida* Lopez, 1989, *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. coffeicola* Lordello and Zamith, 1960, *M. daklakensis* Trinh, Le, Nguyen, Nguyen, Liebanas and Nguyen, 2018, *M. decalineata* Whitehead, 1968, *M. hapla* Chitwood, 1949, *M. incognita* (Kofoid and White, 1919) Chitwood, 1949, *M. inornata* Lordello, 1956, *M. izalcoensis* Carneiro, Almeida, Gomes and Hernandez, 2005, *M. javanica* (Treub, 1885) Chitwood, 1949, *M. kikuyensis* de Grisse, 1960, *M. konaensis* Eisenback, Bernard and Schmitt, 1994, *M. mayaguensis* Rammah and Hirschmann, 1988, *M. megadora* Whitehead, 1968, M. oteifae Elmiligy, 1968, *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos and Almeida, 1996 (Souza, 2008; Trinh *et al*., 2018). Root-knot nematodes are considered the most damaging plant parasitic nematode on coffee in Vietnam (Trinh *et al*., 2009; 2013; Wiryadiputra & Tran, 2008). The coffee associated species, *M. incognita*, has been mainly documented in the country (Bell *et al*., 2018; Nguyen & Nguyen, 2000; Trinh *et al*., 2009; 2013; Wiryadiputra & Tran, 2008). Subsequently, four other *Meloidogyne* species have also been found in coffee plantations in Vietnam, namely *M. exigua, M. coffeicola, M. hapla*, and *M. daklakensis* (Bell *et al*., 2018; Trinh *et al*., 2013; Trinh *et al*., 2018).

Identification of root-knot nematodes species is becoming more important for designing an effective nematode management practices (Perry & Moens, 2013). Root-knot nematode species are normally identified using morphological features and morphometrics of the female, the second-stage juveniles, and the male such as the structure of the labial region, stylet, basal knobs, and the perineal patterns of mature females (Blok & Powers, 2009; Eisenback, 1985). Molecular approaches, that have been using in identification of *Meloidogyne* species, can strongly support morphological approaches (Carneiro *et al*., 2014; Charchar *et al*., 2008; Janssen *et al*., 2017; Hummrey-Pereira *et al*., 2014; Subbotin *et al*., 2006; Trinh *et al*., 2018).

During a survey on coffee in Dak Lak Provinces, Western Highlands of Vietnam, one small unknown root-knot nematode species with a posterior protuberance was collected from unusual galls from replanted coffee plantation. The survey revealed high infection rates of coffee roots caused by a root-knot nematode species which may pose a potential threat to coffee. Therefore, the objectives of this study were to describe the new root-knot nematode species, *Meloidogyne moensi* n. sp., using light microscopy and Scanning Election Microscopy (SEM) observations; assess the diagnostic value of morphological and molecular characters.

Material and Methods

Nematode population

Nematodes were extracted from root galls and soil samples of the rhizosphere of Robusta coffee that were collected from Cu M'gar District, Dak Lak Province, Western Highlands, Vietnam.

Morphological characterization

Second-stage juveniles and males were extracted from soil samples using the decanting and modified Baermann tray method (Whitehead & Hemming, 1965). Measurements were made on permanent slides by heat-killed nematodes after a fixation by TAF and ethanol-glycerin dehydration according to the method that was described by De Grisse, 1969. Females were dissected from root tissues under a stereo-microscope, using a scalpel and forceps. Perineal patterns of mature females were prepared following Hartman & Sasser (1985) and mounted in lactophenol.

Light microscopy (LM)

All slides were observed under the compound light microscope Carl Zeiss Axio Lab.A1 (Carl Zeiss AG, Oberkochen, Germany). Measurements and pictures were taken using ZEN lite software with the support of ZEISS Axiocam ERc5s digital camera (Carl Zeiss AG, Oberkochen, Germany). Pencil drawings were obtained using an Olympus BX51 DIC Microscope equipped with a digital camera and a drawing tube. Illustrations were made using Illustrator CS3 based on pencil drawing, microphotographs, and SEM pics. Identification was based on the diagnostic key of Eisenback (1985), Hewlett & Tarjan (1983), Jepson (1987), Karssen (2002), and Kazachenko & Mukhina (2013). Furthermore, a comparison with other recently described species was also accomplished (Humphreys *et al*., 2014; Tao *et al*., 2017; Trinh *et al*., 2018).

Scanning electron microscopy (SEM)

After the examination and identification, some specimens in good condition were selected for observation under SEM following the protocol of Nguyen *et al*. (2017). The nematodes were hydrated in distilled water, dehydrated in a graded ethanol and acetone series, critical point dried, coated with gold, and observed using a Zeiss Merlin Scanning Electron Microscope (Carl Zeiss AG, Oberkochen, Germany).

Molecular characterization

DNA was extracted from individual second-stage juveniles using the protocol of Holterman *et al*. (2009). The DNA extracted crude was kept at -70 °C until it was used. DNA crude of each specimen was amplified using the following sets of primers: for D2–D3 expansion regions of 28S rDNA,, forward primer D2-5'-ACAAGTACCGTGAGGGAAAGTTG-3' and reverse primer D3-5'-TCCTCGGAAGGAACCAGCTACTA-3' were used (De Ley *et al*., 1999); for the mtDNA cytochrome c oxidase I (COI) genes, primers Cox1F/Cox1R (Cox1-5'-TGGTCATCCTGAAGTTTATG-3'/Cox1R-5'-CTACAACATAATAAGTATCATG-3') were used (Kiewnick *et al*., 2014); for the mtDNA cytochrome c oxidase II (COII) genes, primers C2F3 (5'-GGTCAATGTTCAGAAATTTGTGG-3') and 1108 (5-TACCTTTGACCAATCACGCT-3') were used (Powers and Harris, 1993). The PCR mixtures contained 25 µl Hotstat PCR Mas-

Table 1. Morphometrics indices of the females, males and second–stage juveniles (J2) of *Meloidogyne moensi* n. sp.
All measurements are in µm and in the form: mean ± SD (range).

Character	Haplotype (Female)	Paratypes		
		Female	Male	Second – stage juveniles (J2)
n	1	10	10	20
L	741.4	588 ± 133 (345 – 751)	1198 ± 57 (1103 – 1266)	464.1 ± 21.1 (428 – 449)
a	2.3	2.16 ± 0.64 (1.3 – 3.8)	35.7 ± 6.8 (26.8 – 44.8)	37.6 ± 1.8 (35 – 42)
b	–	–	11.7 ± 0.8 (10.4 – 13)	6 ± 0.5 (5.6 – 7.6)
b’	9.0	6.1 ± 1.96 (3.3 – 9.3)	5.3 ± 0.3 (4.7 – 5.7)	3.2 ± 0.2 (3.0 – 3.6)
c	–	–	113.6 ± 46.5 (74.4 – 205)	12.3 ± 1.7 (9.4 – 15.1)
c’	–	–	0.5 ± 0.1 (0.3 – 0.6)	4.4 ± 0.5 (3.6 – 5.4)
Percentage excretory pore	4.3	6.8 ± 2.4 (4.3 – 10.9)	11.2 ± 1.2 9.3 – 12.8	15.6 ± 0.7 14.4 – 17.3
Maximum body diameter	327.6	287 ± 73 (149 – 367)	34.7 ± 6.4 (27 – 42.7)	12.3 ± 0.6 (11.5 – 13.6)
Neck length	242.1	200 ± 52 (96.0 – 267)	–	–
Ratio of body length to length of neck	3.1	2.94 ± 2.5 (2.8 – 3.5)	–	–
Lip length	6.3	7.4 ± 1.1 (6.2 – 10.2)	9.8 ± 0.6 (9 – 10.8)	3.4 ± 0.4 (2.8 – 4.4)
Lip width	2.9		4 ± 0.3 (3.5 – 4.5)	3.8 ± 0.5 (2.8 – 4.4)
Stylet length	14.5	16 ± 1.4 (14.3 – 19.1)	15.4 ± 0.7 (14 – 16.2)	10.4 ± 0.7 (9.0 – 12)
Stylet knob width	–	–	4 ± 0.4 (3.4 – 4.7)	–
Stylet knob height	–	–	2.3 ± 0.5 (2 – 2.7)	–
DGO	5.1	6.0 ± 1.0 (4.6 – 7.8)	3.9 ± 0.7 (3 – 4.8)	3.6 ± 0.4 (2.9 – 4.1)
Head end to posterior end of metacorpus	82.7	99.3 ± 11.7 (80.2 – 112.3)	72.8 ± 4.3 (67 – 80.3)	52.1 ± 1.6 (49 – 54.6)
Metacorpus width	33.7	33.0 ± 6.8 (25.9 – 44.4)	8.7 ± 0.6 (8 – 10)	–

Metacorpus length	44.1	42.4 ± 6.8 (32.9 – 54.2)	13.2 ± 1.5 (10.8 – 15)	
Metacorpus valve length	13.8	12.4 ± 1.1 (10.4 – 13.8)	–	–
Metacorpus valve width	9.4	9.6 ± .9 (8.0 – 11.7)	–	–
Distance from anterior end to excretory pore	34.4	37.9 ± 10.8 (27.9 – 61.5)	134.4 ± 11.2 (116 – 148)	72.7 ± 4.2 (67.7 – 85.1)
Distance from anterior end to nerve ring	–	–	91.4 ± 4.8 (84 – 99.2)	63.1 ± 2.7 (58.5 – 66.8)
Distance from anterior end to pharyngeal–intestinal valve	–	–	102 ± 6.2 (93.2 – 113.8)	76.2 ± 4.5 (62.7 – 80.9)
Distance from anterior end to end of pharyngeal gland lobe	–	–	224.2 ± 13.5 (194 – 236)	142 ± 6.9 (128.9 – 150.8)
Vulva–anus distance	11.6	12.2 ± 2.6 (8.7 – 16.2)	–	–
Vulva slit length	21.5	18.8 ± 2.2 (16 – 24.4)	–	–
Phasmid distance	13.7	15.7 ± 2.4 (12 – 19)	–	–
Tail length	–	–	11.8 ± 3.5 (5.9 – 16.6)	38.2 ± 5.0 (32.2 – 48.8)
Anal body diameter	–		23 ± 2.5 (19.5 – 27)	8.5 ± 0.8 (6.5 – 9.7)
Spicule length	–	–	23.8 ± 2.6 (21.1 – 28.0)	–
Spicule width	–	–	3 ± 0.4 (2.5 – 3.7)	–
Gerber length	–	–	11.3 ± 0.7 (10.2 – 12)	–
Gerber width	–	–	1.7 ± 0.4 (1 – 2.1)	–
Phasmids from tail terminus	–	–	7.8 ± 2.7 (3.9 – 11.5)	–
Testis length	–	–	664 ± 789 (538 – 747)	–
Hyaline tail terminus	–	–	–	22.9 ± 3.9 (17.9 – 30.9)

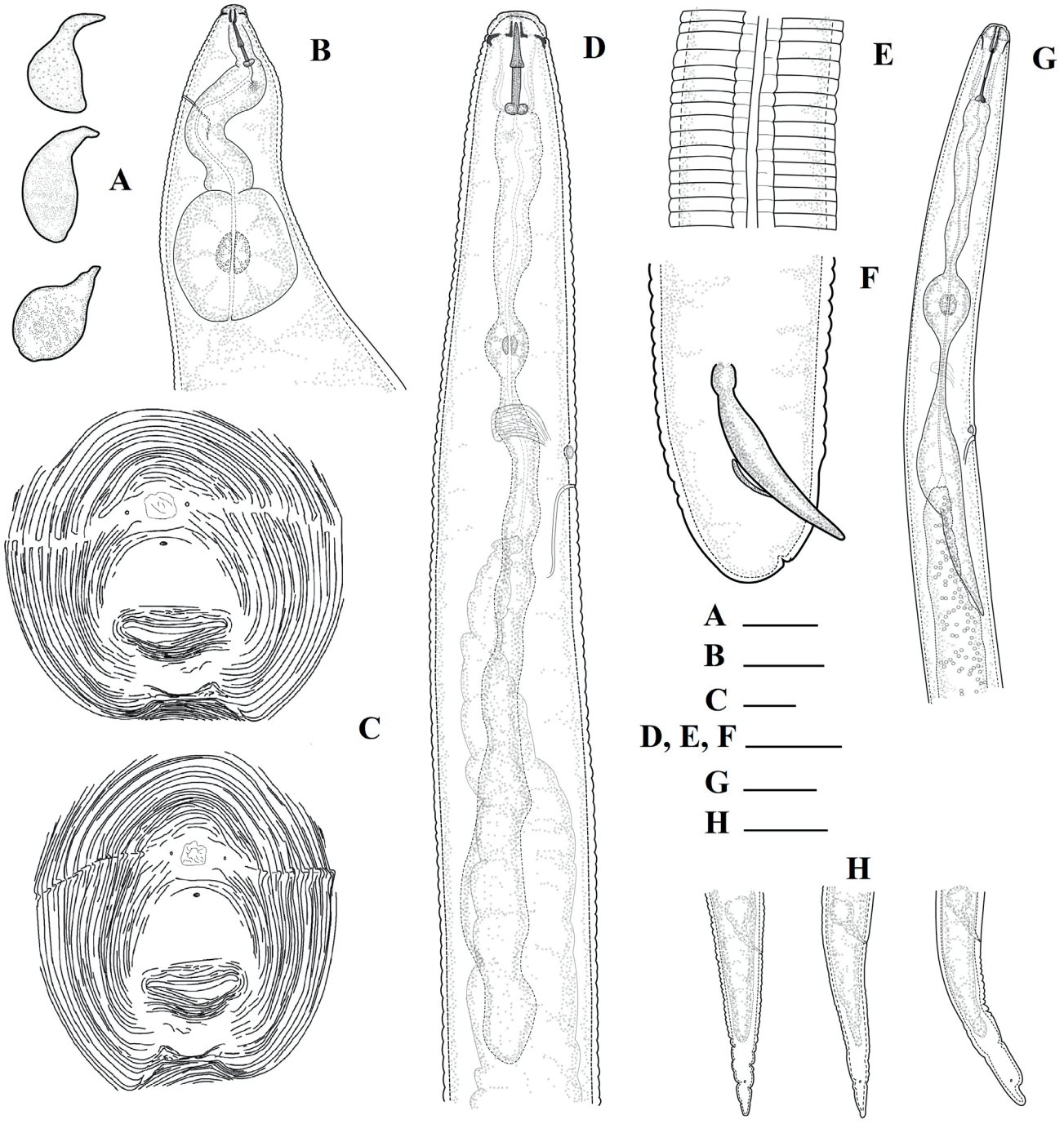

Fig. 1. Line drawing of *Meloidogyne moensi* n. sp. Female: (a) entire body variation; (b) anterior end; (c) perineal pattern. Male: (d) anterior region; (e) lateral field; (f) tail region. Second–stage juvenile: (g) anterior region; (h) tail variation. (Scale bar: (a): 200 µm; (b, h): 20 µm; (c): 25 µm; (d, e, f): 15 µm; (g): 10 µm).

termix (Promega, USA), 1 µM forward and reverse primers, 5 µl crude DNA-extract and sterile water up to a volume of 50 µl. The PCR reaction started at 95 °C in 5 minute, followed by 35 cycles of 96 °C in 35 seconds; 50 °C in 30 seconds; 72 °C in 45 seconds and the last step was 72 °C in 3 minute. After electrophoresis of 5 µl PCR product on 1.5 % agarose gel with TAE buffer (1h, 100V), the gel was stained with Gelred for 10 min and photographed under UV light. Sequence alignments were manually edited using ChromasPro software (ChromasPro 1.7.5, Technelysium Pty Ltd, Tewantin QLD, Australia). The datasets of D2-D3 expansion seg-

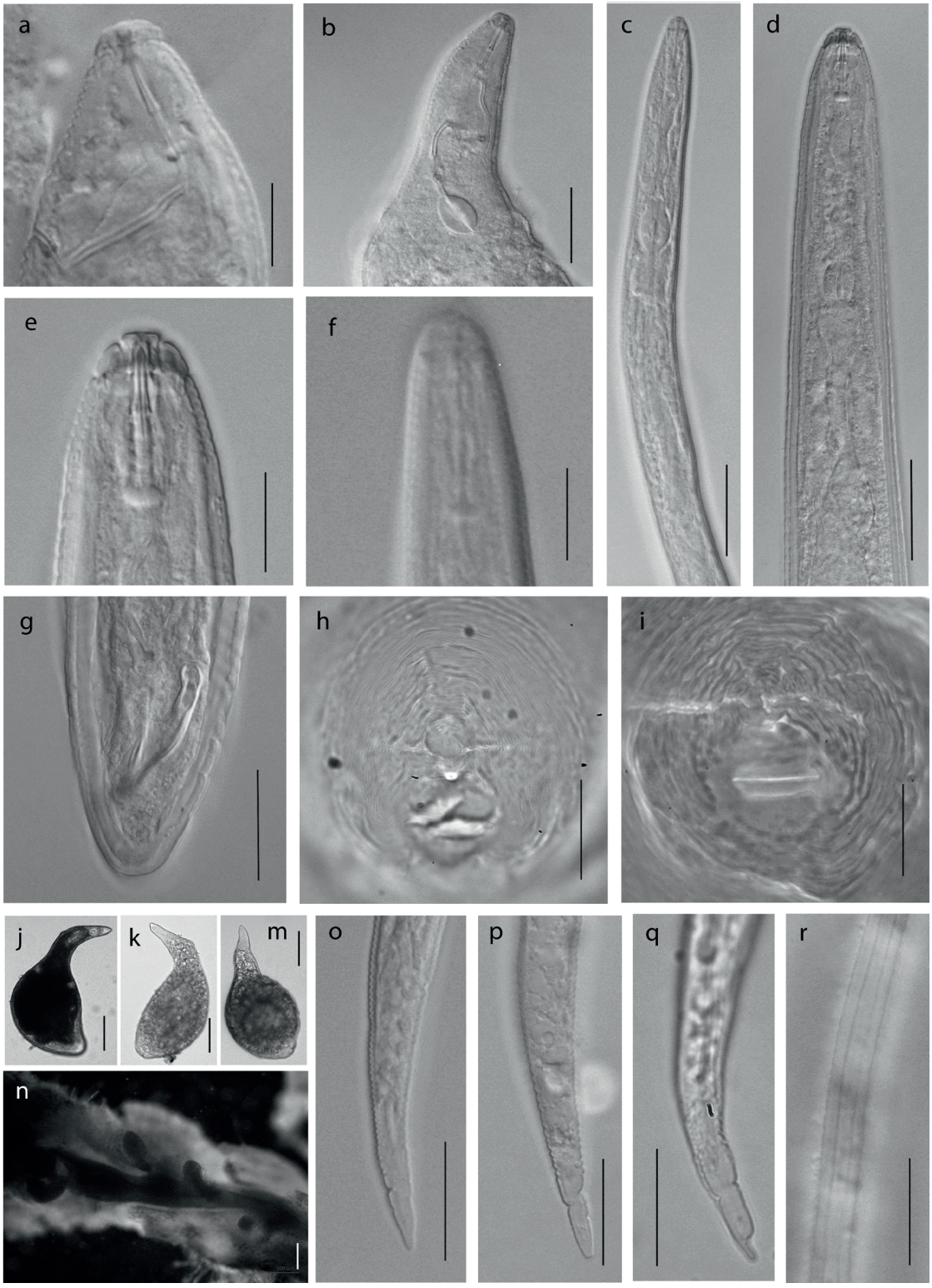

Fig. 2. Light microphotographs of *Meloidogyne moensi* n. sp. Female: (a) anterior end; (h, i) perineal patterns; (j, k, m) entire body variation. Male: (d, e) anterior end; (g) tail region. Second stage juvenile: (c, f) anterior end; (r) lateral field; (o–q) tail variation. (n) stained root shows females inside. (Scale bar: (f) 5 µm; (a, e, g, o–r) 10 µm; (b, c, d, h, i) 20 µm).

ment of 28S rDNA, COI, and COII/16S–rRNA of mtDNA sequences were blasted on GenBank. The most similar sequences and recently added sequences of *Meloidogyne* species were selected for phylogenetic analyses. The sequence alignments were created using ClustalW in BioEdit v7.2.0 (Hall, 1999). The phylogenetic trees were built using Maximum Likelihood (ML) method in MEGA 6 software (Tamura *et al*., 2013). The best-fit models of base substitution were evaluated using MODELTEST in MEGA 6.0. *Radopholus similis* (JN091964) and *Subanguina radicicola* (LT714119) were chosen as outgroup for D2–D3 expansion regions of 28S rDNA sequences; *Pratylenchus zeae* (KU522440) was chosen as outgroup for COI of mitochondrial DNA sequences; *Pratylenchus vulnus* (NC020434) was chosen as outgroup for COII/16S–rRNA of mtDNA sequences.

Ethical Approval and/or Informed Consent

The conducted research is neither related to human nor animals use.

Results

Meloidogyne moensi n. sp. (Table 1, Fig. 1 – 3)
Type material: Vietnam: ♀ Holotype (IEBR-Nema–5229/1), paratypes: 10♂, 10♀♀ and 20 juveniles (IEBR-Nema–5229/2–7) are deposited in the Nematode Collection of Nematology department in Institute of Ecology and Biological Resources (IEBR), Vietnam Academy of Sciences and Technology (VAST). Five female paratypes are deposited in Ghent University, K.L. Ledeganckstraat 35, Ghent, Belgium. The D2D3, COI and COII/16S–rDNA sequences are deposited in GenBank under accession number: (MF429805, MG825431), (MG825430, MG825432) and (MF429804, KY084500), respectively.

Type host: Robusta coffee *(Coffea canephora* Pierre ex A. Froehner)

Site of infection: roots

Type locality: Western Highlands, Dak Lak Province with GPS coordinates 12°47'06N and 108°05'44' E.

Pathology: *Meloidogyne moensi* n. sp. causes inconspicuous galls or slight swellings on coffee roots (Fig. 2n). The presence of the nematodes was detected by the distortion of root system preventing absorption of nutrients which leads to stunting and chlorosis of the host plant. Coffee roots infected by *Meloidogyne moensi* n. sp. had relatively small galls (1 – 2 mm diam.). Each gall contained several females with egg mass inside the gall (Figs. 2n).

Etymology. The species is named after Prof. Dr. Maurice Moens as a recognization to his valuable help for nematology in Vietnam.

Description

Meloidogyne moensi n. sp.

Females. Body swollen with a small posterior protuberance, pearly white varying in shape, elongated from ovoid to saccate (Fig. 1a,2j, k, m). Neck prominent, bent at various angles to body (Figs. 1b,2b). Lip region slightly set off from rest of body (Fig. 2a); stoma slit-like, located in prominent ovoid pre-stomatal cavity, surrounded by pit-like openings of six inner labial sensilla; labial disc round, slightly raised above medial lips (Figs. 3a, b); labial cap and medial lips slightly raised above lateral lips; medial lips dumbbell-shaped (in SEM); lateral lips large, fused laterally with lip region; amphidial apertures oval shaped, located between labial disc and lateral lips. Cephalic framework strong, hexaradiate (Fig. 1b). Stylet short; cone base triangular and wider than shaft (Figs. 1 b and 2 a); stylet tip normally straight, sometimes slightly curved dorsally (Figs. 1b and 2 a); stylet knobs three, oval and sloping posteriorly. Distance from base of stylet to dorsal pharyngeal gland orifice (DGO) 4.6 – 7.8 µm long. Secretory-excretory pore located at level of procorpus, posterior to stylet knobs. Metacorpus rounded or oval, with oval-shaped valve; pharyngeal glands with one large dorsal lobe, variable in shape, position and size. Perineal pattern round to oval with continuous, smooth, distinct striae; lateral field marked as a faint space or linear depression junction of dorsal and ventral striate (Figs. 1 l.c; 2h, i and 3d, e); dorsal arch low, rounded, covering distinct vulva and tail terminus; phasmids large, distinct; vulva slit centrally located at unstriated area, nearly as wide as vulva-anus distance; perivulval region free of striae; tail tip visible, wide surrounded by concentric circles of striae; ventral striae concave, often free of striae (Fig. 1c, 2b, c, 5e, f).
Males. Body vermiform 1103 – 1266 µm long, anterior end tapering, posterior end bluntly rounded. Body annuli large, distinct. Lateral fields areolated with three incisures beginning near level of stylet knobs, two additional incisures starting near level of metacorpus (Figs. 1e and 3i). Head caps high and rounded, consisting of a large labial and two post-labial annuli, sometime with incomplete annuli (LM) (Figs. 1d, 2d, e). Lip region continuous to body; stoma slit-like, located in ovoid to hexagonal cavity, surrounded by pit-like openings of six inner labial sensilla; subventral and subdorsal lips fused to form median lips, each lips with two cephalic sensilla; lateral lips large, triangular, lower than labial disc and medial lips; posterior edge of one or both lateral lips separated with lip region, crescent-shaped (Fig. 3f); amphidial apertures elongated, located between labial disc and lateral lips. Stylet robust 14 – 16.2 µm long; cone pointed, smaller than shaft, slightly increasing in diameter posteriorly; knobs rounded, reniform, distinctly indented, backwardly sloping, transversely elongated, merging gradually with shaft (Figs. 1d and 2d, e). Distance from base of stylet to DGO 3-4.8 µm long. Procorpus distinctly outlined; metacorpus ovoid, with a strong valve apparatus. Secretory-excretory pore distinct, located four to six annuli posterior to hemizonid. Testis one,

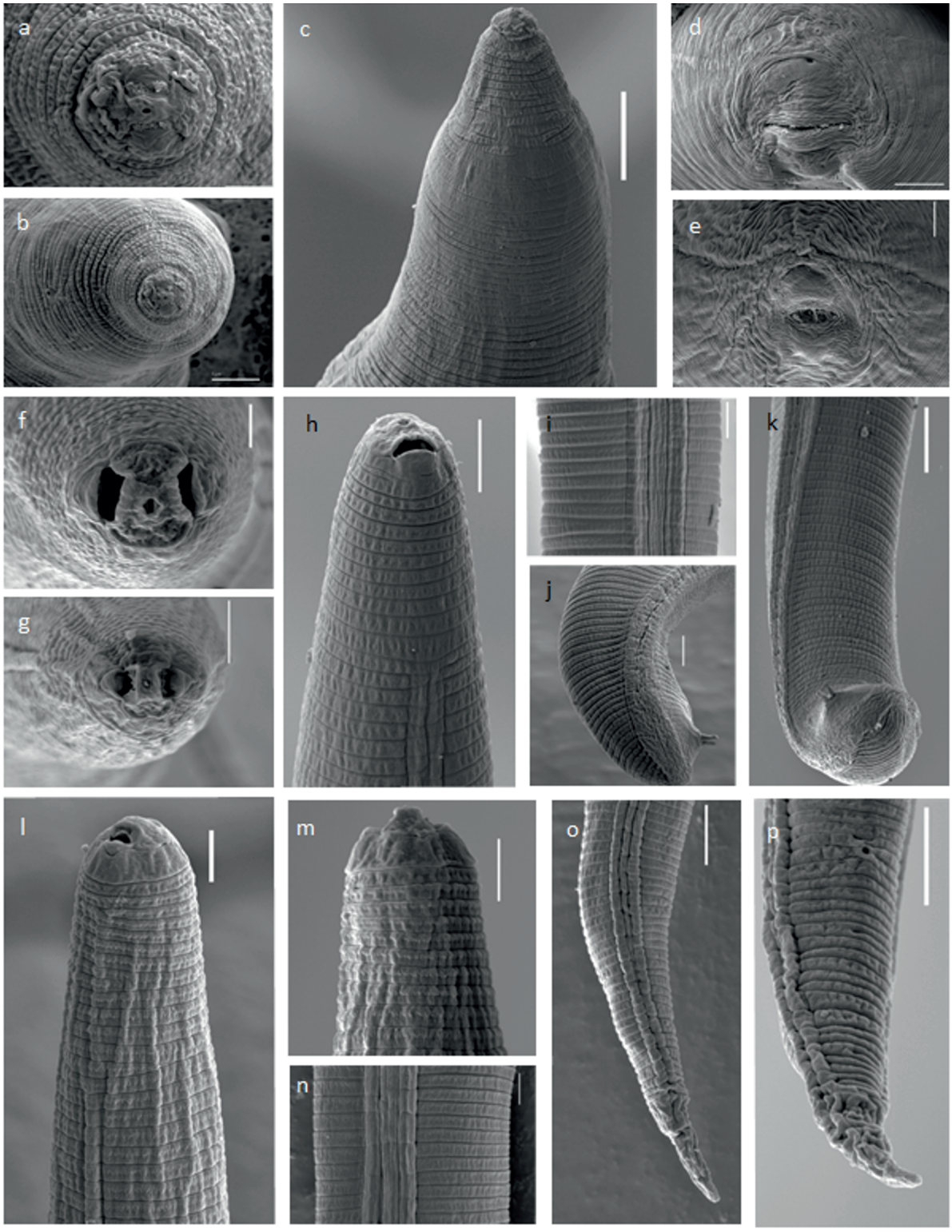

Fig. 3. Scanning electron microphotographs of Meloidogyne moensi n. sp. Female: (a, b) enface view; (c) anterior end, lateral view; (d, e) perineal pattern. Male: (f) enface view; (h) anterior end, lateral view; (i) lateral field; (j) tail region, lateral view; (k) tail region, ventral view. Second–stage juvenile: (g) enface view; (l) anterior end, lateral view; (m) anterior end, ventral view; (n) lateral field; (o) tail region, lateral view; (p) tail region, ventral view. (Scale bar: (a, f, l, m, n) 2 µm; (b, h, i, j, o,p) 5 µm; (c, d, e, g, k) 10 µm).

occupying 58 % body cavity; spicules slightly curved ventrally with bluntly rounded terminus (Figs. 1f and 2g); gubernaculum short, crescent-shaped. Tail short; phasmids distinct, located at cloacal aperture level.

Second-stage juveniles (J2). Body slender, tapering to an elongated tail. Body annuli distinct, but fine. Lateral fields start near level of procorpus as two lines, third line start near metacorpus and quickly devide into four lines running entire length of body before gradually decrease to two lines that end near hyaline of tail; lateral field areolated whole body (Fig. 3n). Lip region narrower than body, weak and slightly set off (Figs. 1g and 2f). Under SEM, prestoma opening rounded, surrounded by small, pore-like openings of six inner labial sensilla; medial lips and labial disc dumbbell-shaped; lateral lips large, triangular, lower than labial disc and medial lips; cephalic sensilla not seen; amphidial apertures elongated, located between labial disc and lateral lips (Fig. 3g); lip region not annulated (Figs. 1g, 2h, 3l, m). Stylet slender; cone weakly expanding at junction with shaft; knobs small, oval shaped and backwardly sloping (Figs. 1g, 2o, p, q, r). Distance from stylet knobs to dorsal

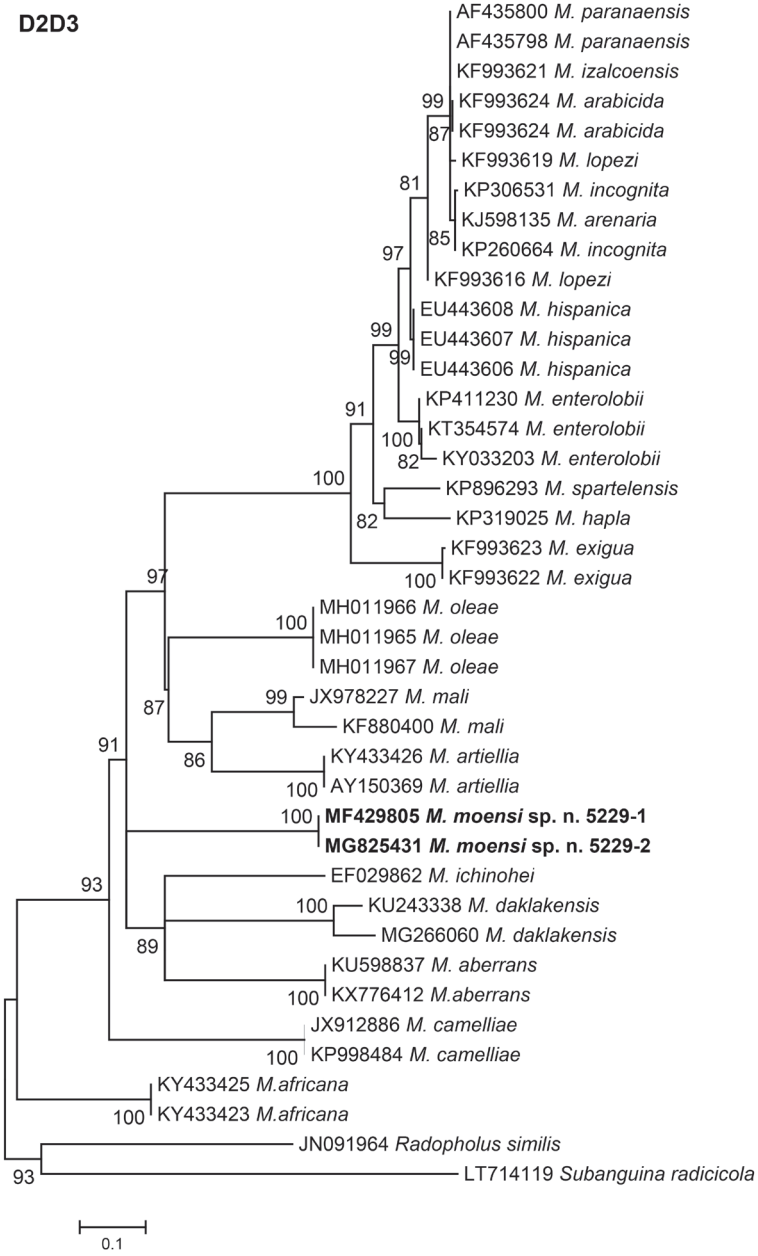

Fig. 4. Phylogenetic relationships of *Meloidogyne moensi* n. sp. with other *Meloidogyne* spp., based on D2-D3 of 28S–rDNA sequences using GTR+G model. Numbers on the left of nodes are bootstrap values for 1000 replications

gland orifice 2.9-4.1 µm long. Procorpus faintly outlined; metacorpus broadly oval, valve large and heavily sclerotized; isthmus clearly defined; pharyngo-intestinal junction located posterior to level of secretory-excretory pore; gland lobe variable in length overlapping intestine ventrally. Secretory-excretory pore located posterior to hemizonid. Tail conoid with rounded unstriated tail tip (Figs. 1h, 3o, p); hyaline clearly defined (Fig. 2 o, p, q, r); rectum dilated; phasmids small, distinct.

Differential diagnosis

Females of *Meloidogyne moensi* n. sp. are characterized by the following characteristics: body swollen with a small posterior protuberance, elongated from ovoid to saccate; perineal patterns are rounded with smooth striae; dorsal arch is continuous and low; lateral lines appearing as a faint, discontinuous, or linear depression at junction of the dorsal and ventral striate; phasmids are distinct; perivulval region is free of striae; tail tip is wide, surrounded by concentric circles of striae; stylet tip is normally straight; stylet

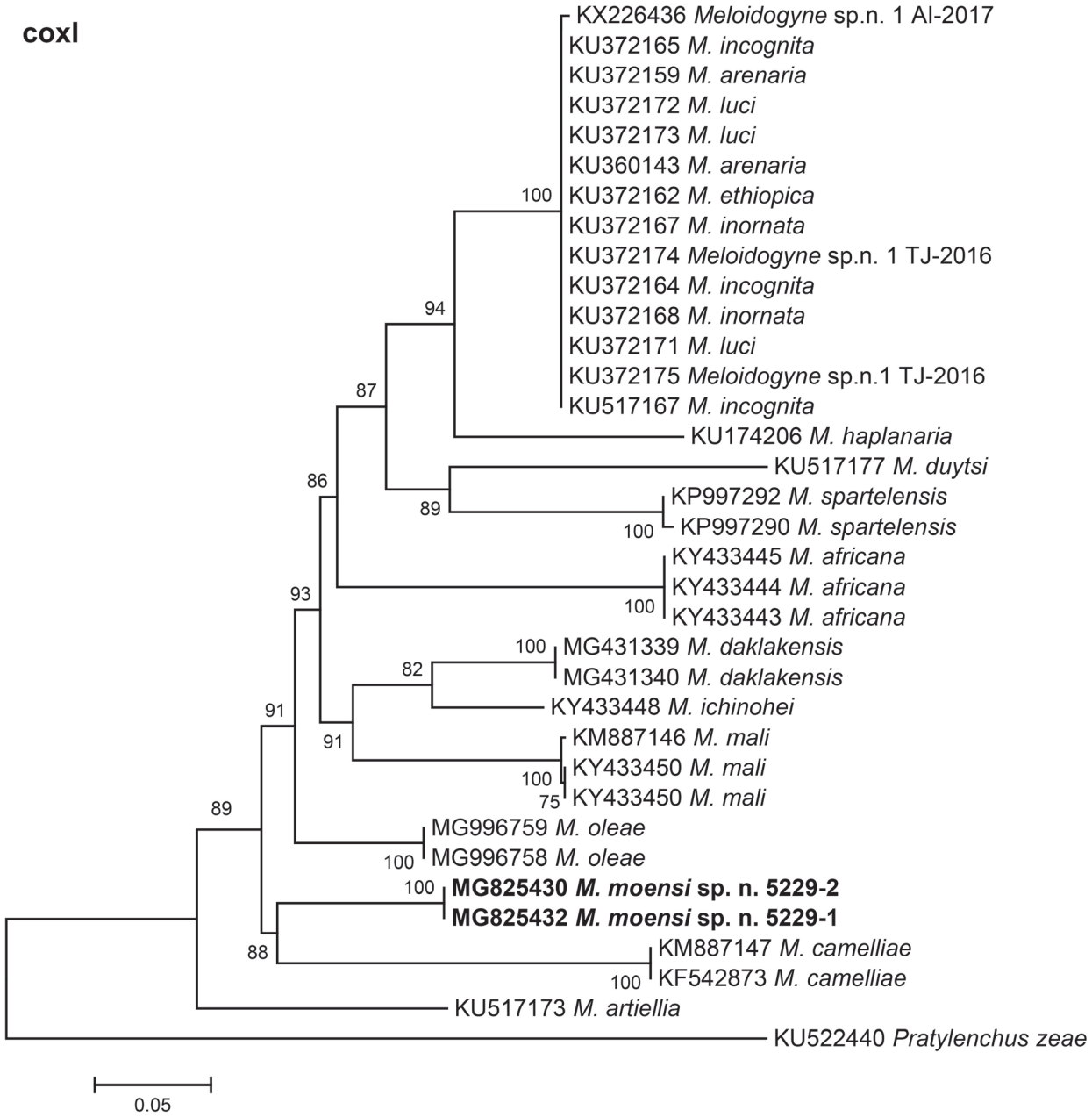

Fig. 5. Phylogenetic relationships of *Meloidogyne moensi* n. sp. with other *Meloidogyne* spp., based on COI sequences using TN93+G+I model. Numbers on the left of nodes are bootstrap values for 1000 replications

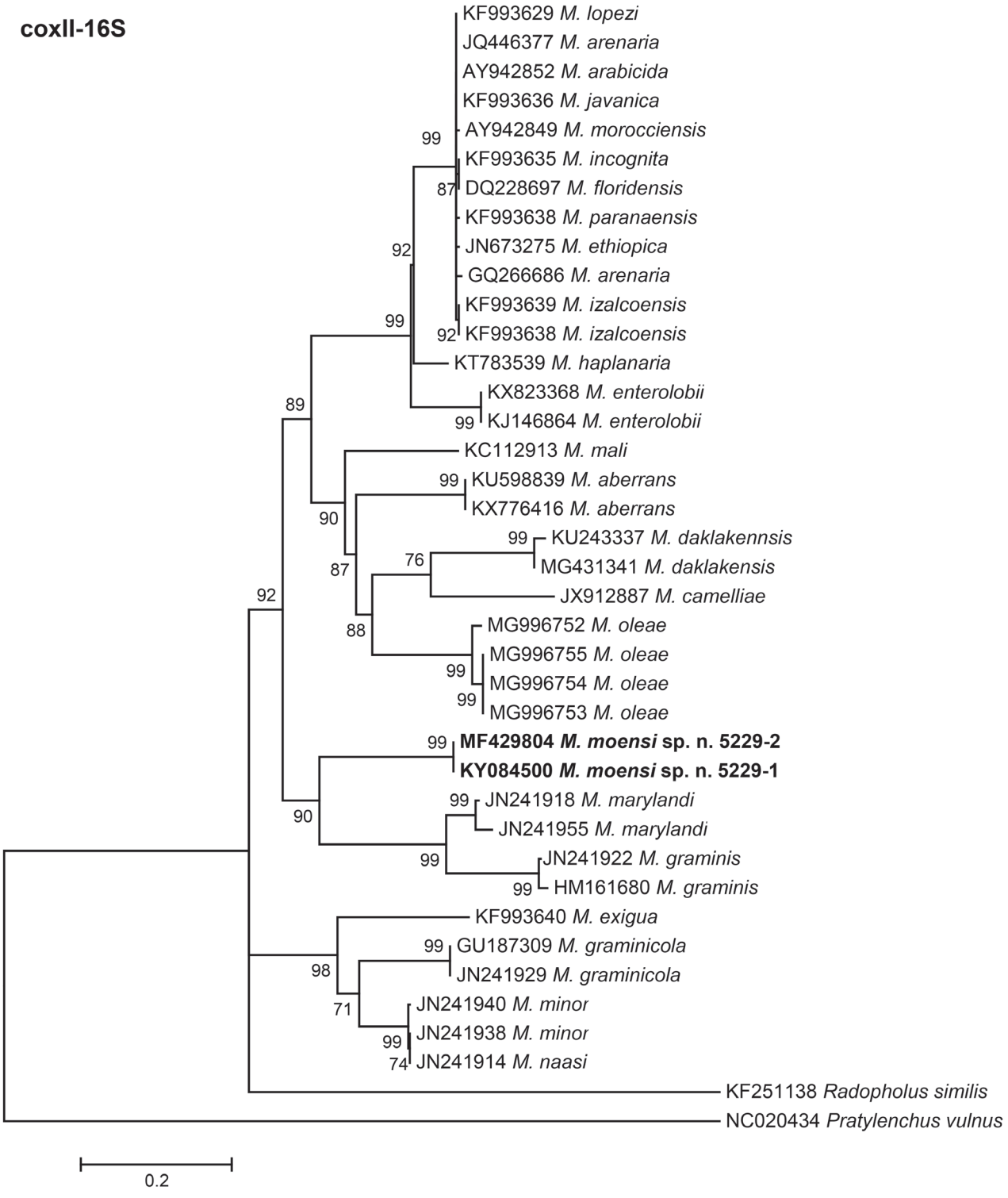

Fig. 6. Phylogenetic relationships of *Meloidogyne moensi* n. sp. with other *Meloidogyne* spp., based on COII–16S rRNA sequences using HKY+G+I model. Numbers on the left of nodes are bootstrap values for 1000 replications.

knobs are oval-shaped and slope posteriorly. Second stage juveniles are characterized by lip region is not annulated; medial lips and labial disc fused to form dumbbell-shape; lateral lips are large, triangular, and lower than labial disc and medial lips; tail is conoid with rounded unstriated tail terminus; hyaline is clearly defined; dilated rectum; distinct phasmids.

Meloidogyne moensi n. sp. is most similar to *M. acronea* Coetzee, 1956, *M. aberrans* Tao, Xu, Yuan, Wang, Lin, Zhuo & Liao, 2017, *M. africana* Whitehead, 1959, *M. graminis* Sledge and Golden, 1964) Whitehead, 1968, *M. ichinohei* Araki, 1992, *M. marylandi* Jepson and Golden, 1987, *M. megadora* Whitehead, 1968; and *M. ottersoni* Thorne, 1969 by prominent posterior protuberance and perineal patterns belong to group 3 (except for *M. graminis* that belong to group 5) according to Jepson (1987). However, they can be distinguished from each other by the following features.

Meloidogyne moensi n. sp. differs from *M. acronea* Coetzee, 1956 (Coetzee & Helen, 1965) in the females by having perineal patterns not obscure vs extremely obscure, shorter body length, longer stylet length, smaller maximum body diameter; in the male by having shorter body length, shorter stylet length, larger "b" ratio, shorter spicule length; in the second-stage juveniles by having shorter body length, larger "a" ratio, larger "c" ratio (Table 2).

Meloidogyne moensi n. sp. differs from *M. aberrans* Tao, Xu, Yuan, Wang, Lin, Zhuo, & Liao, 2017 in the females by having striae in perineal pattern (distinct and continuous vs extremely faint and broken), phasmids distinct vs phasmids not visible, shorter body length, smaller maximum body diameter, longer stylet length, shorter vulva-anus distance, smaller vulva slit length; in the males by having shorter body length, shorter stylet length, smaller c value, shorter spicule length; in the juveniles by having shorter stylet length, larger "a" ratio, larger "b" ratio, larger "c" ratio (Table 2). *Meloidogyne moensi* n. sp. also differs from *M. aberrans* by D2-D3 of 28S rDNA sequences (25 % different), and *COII/16S–rRNA* sequences (37 % different).

Meloidogyne moensi n. sp. differs from *M. africana* Whitehead, 1959 (Jassen *et al*., 2017) in the females by having lateral field structure (lateral lines marked as a faint space or linear depression junction of the dorsal and ventral striate vs lateral fields are unmarked by incisures, but they present tiny, disordered striae), smaller maximum body diameter, longer stylet length; in the males by having smaller DGO, larger "a" ratio, smaller spicule length; in the second-stage juvenile by having smaller body width, smaller stylet length, and larger "a" ratio, shorter tail length (Table 2). *Meloidogyne moensi* n. sp. differs from *M. africana* by D2-D3 of 28S rDNA sequences (23 % different), and *COI* sequences (20 % different).

Meloidogyne moensi n. sp. differs from *M. graminis* (Sledge and Golden, 1964) Whitehead, 1968, in the females by having longer stylet (14.3 – 19.1 vs 11.7 – 13.44 µm), lip region slightly set off vs not distinctly set off, cephalic framework strong vs cephalic framework indistinct, excretory pore located behind from stylet knobs vs located about on a level with knobs, perineal patterns with smooth striae, low arch vs perineal pattern with coarse striae, high arch, perineal patterns belong to group 3 vs group 5 according to Jepson (1987); in the males by having shorter body length (1103 – 1266 vs 1275 – 1734 µm), shorter stylet (14 – 16.2 vs 17.92 – 19.00 µm); larger "b" ratio (10.4 – 13 vs 6.42 – 8.2); in second–stage juveniles by having larger "a", "b" and "c" ratio (35 – 42 vs 28.77 – 33.99), 6 ± 0.5 (5.6 – 7.6 vs 2.10 – 2.95); (9.4 – 15.1 vs 5.71 – 6.78), respectively. *Meloidogyne moensi* n. sp. can be distinguished from *M. graminis* by D2-D3 of 28S rDNA sequences (27 % different), and *COII/16S–rRNA* sequences (35 % different).

Meloidogyne moensi n. sp. differs from *M. ichinohei* Araki, 1992 in the females by having perineal patterns with smooth and continuous striae vs striae extremely faint, broken and somewhat spaced, phasmids distinct vs phasmids small and obscure, lateral lines marked as a faint space or linear depression junction of the dorsal and ventral striate vs lateral lines absent, sometimes with lines of particles instead. smaller body length (345 – 751 vs 618.8 – 1018.8 µm), longer stylet length, shorter vulva-anus distance, shorter vulva slit length; males have smaller body length, shorter DGO; second-stage juveniles have smaller body width, shorter DGO (Table 2). *Meloidogyne moensi* n. sp. is different from *M. ichinohei* by D2-D3 of 28S rDNA sequences (19 % different), and *COI* sequences (15 % different).

Meloidogyne moensi n. sp. differs from *M. marylandi* Jepson and Golden, 1987 in the females by having perineal pattern with low dorsal arch vs squared dorsal arch, striae are smooth, continuous and closely spaced vs striae are coarse, wavy, and widely spaced, smaller body length (345 – 751 vs 525.1 – 923.2 µm), longer stylet length (14.3 – 19.1 vs 14.2 – 14.8 µm), longer DGO (4.6 – 7.8 vs 3.5 – 4.7 µm); the second-stage juveniles have smaller maximum body diameter (11.5 – 13.6 vs 13.6 – 17.1 µm), larger "a" ratio (35 – 42 *vs* 25.2 – 33.1), and shorter tail length (32.2 – 48.8 *vs* 52.5 – 68.4 µm). *Meloidogyne moensi* n. sp. also differs from *M. myrylandi* by D2-D3 of 28S rDNA sequences (27 % different), and *COII/16S–rRNA* sequences (30 % different).

Meloidogyne moensi n. sp. differs from *M. megadora* Whitehead, 1968 (Maleita *et al*., 2016) in the females by having perineal pattern with closely spaced, continuous, and smooth striae vs faint, often broken, widely spaced, shorter female body length, smaller female body width, shorter vulva-anus distance and vulva slit length; juveniles of *M. moensi* n. sp. differs from *M. megadora* by having longer DGO, larger "a" and "c" ratio. Males of *M. moensi* n. sp. differs from *M. megadora* by shorter stylet length, and shorter spicule length (Table 2).

Meloidogyne moensi n. sp. differs from *M. ottersoni* Thorne 1969 in the females by having perineal pattern with concave ventral striae vs perineal pattern with simple rounded striae, longer stylet length; in the juveniles by having smaller stylet length, larger "a" and "c" ratio (Table 2).

Table 2. Morphological differences between some *Meloidogyne* species closely related to *Meloidogyne moensi* n.sp.
All measurements are in µm and in the form: mean ± SD (range).

Character	*M. moensi* n.sp.	*M. acronea* (Coetzee, 1956; Coetzee & Helen 1965)	*M. aberrans* (Tao *et al*., 2017)	*M. africana* (Janssen *et al*., 2017)	*M. ichinohei* (Araki, 1992)	*M. megadora* (Maleita *et al.,* 2016)	*M. ottersoni* (Thorne 1969)
Females							
Body length	588 ± 133 (345 – 751)	840 – 950	938.1 ± 91.7 (806.2 – 1119.1)	615 ± 95 (400 – 700)	796.1 ± 114.48 (618.8 – 1018.8)	1118.3 ± 203.1 (830 – 1520)	390 – 520
Length neck	200 ± 52 (96 – 267)	–	282.8 ± 57.8 (184.4±378.0)	217 ± 48.1 (120 – 300)	195.7 ± 45.2 (135 – 246)	205.3 ± 42.7 (110.0 – 300.0)	–
Ratio body length/ length of neck	2.9 ± 2.5 (2.8 – 3.5)	–	–	2.9 ± 0.4 (2.3 – 3.8)	–	–	–
Max. body diam.	287 ± 73 (149 – 367)	400 – 600	581.6 ± 78.5 (441.3 – 712.6)	375 ± 59 (300 – 540)	393.2 ± 93.27 (300 – 575)	569.5 ± 81.2 (440 – 750)	180 – 320
Stylet length	16 ± 1.4 (14.3 – 19.1)	11 – 13	14.5 ± 0.6 (13.6 – 15.5)	14.3 ± 0.8 (13.0 – 15.5)	12.3+0.77 (11 – 13.6)	15.4 ± 0.9 (14.0 – 16.5)	10-12
DGO	6 ± 1 (4.6 – 7.8)	4	4.5 ± 0.5 (3.7 – 5.8)	5.7 ± 0.8 (4.5 – 7.0)	5.4 ± 0.88 (3.8 – 6.8)	5.8 ± 0.6 (5.0 – 6.5)	–
Vulva – anus distance	12.2 ± 2.6 (8.7 – 16.2)	–	23.1 ± 2.5 (17.8 – 27.1)	–	25.1 ± 4.53 (14.1 – 29.6)	41.5 ± 6.2 (30.0 – 51.0)	–
Vulva slit length	18.8 ± 2.2 (16 – 24.4)	–	33.6 ± 4.4 (23.7 – 41.1)	–	34.2 ± 6.2 (25.5 – 46.1)	41.5 ± 6.2 (30.0 – 51.0)	–
Perineal pattern	Round to oval, smooth striae, continuous, low dorsal arch, Lateral lines marked as a faint space or linear depression junction of the dorsal and ventral striate. Phasmids distinct. Perivulval region free of striae. Tail terminus visible, surrounded by concentric circles of striae.	Perineal pattern extremely obscure Phasmids not observed.	Oval, striae extremely faint, broken. Vulva slit wider than vulva-anus distance. Anus fold visible in several specimens. Phasmid not visible.	Low dorsal arch, phasmids positioned adjacent to tail terminus, start of the lateral field of variable width and composed of irregular striae, perineal pattern on a raised perineum as a consequence of a clear protuberance, and vulva surrounded by circles of striae.	The area around anus covered by the fold of the tail tip. Perineal pattern rounded; striae extremely faint, broken and somewhat spaced. Vulva slit like, without surrounding striae; phasmids small, obscure. Dorsal arch low and rounded; lateral lines absent.	Rounded, composed of fine, wavy striae, often broken, low and rounded dorsal arch. Anal fold. Phasmids distinct. Vulva was wider than interphasmidial distance. Fine, broken striations between vulva and anus. lateral field marked by breaks in the striae. Concentric circles of striae formed a distinct raised tail pattern.	Simple rounded striae, usually on a slight terminal elevation. Anal opening visible from a lateral view and rarely from ventral.
Enzyme phenotypes (Est; Mdh)	–	–	S2; N1	AF2; H1	–	Me3; Me1	–

Juveniles							
Body length	464 ± 21 (428 – 449)	440 – 460	451.7 ± 17.4 (419.2 – 473.8)	422 ± 39 (352 – 536)	469 ± 20 (413.1 – 524.3)	434.5 ± 27.6 (370.0 – 485.0)	430 – 500
Body width	12.3 ± 0.6 (11.5 – 13.6)	14.6	14.7 ± 0.2 (14.4 – 15.2)	16.8 ± 2.3 (14.0 – 22.0)	15 ± 0.45 (14.1 – 16)	–	–
Stylet length	10.4 ± 0.7 (9 – 12)	10	16.3 ± 0.3 (15.9 – 16.8)	11.5 ± 0.5 (10.5 – 12.5)	11.3 ± 0.68 (9.7 – 12.9)	12.5 ± 1.0 (11.0 – 15.0)	13 – 15
DGO	3.6 ± 0.4 (2.9 – 4.1)	3	3.3 ± 0.3 (3 – 3.9)	4 ± 0.6 (3 – 5.5)	5.2 ± 0.6 (3.6 – 6.4)	4.1 ± 0.5 (3.5 – 5.0)	4
a	37.6 ± 1.8 (35 – 42)	32	30.7 ± 1.3 (28.9 – 32.7)	25.5 ± 3.1 (19.5 – 31.1)	31.3 ± 1.7 (27.1 – 34.9)	27.1 ± 2.2 (23.3 – 32.3)	23 – 30
b	6 ± 0.5 (5.6 – 7.6)	5.8	4.3 ± 0.3 (3.8 – 5)	–	9.2 ± 0.68 (7.4 – 9.3)	–	–
b’	3.2 ± 0.2 (3.0 – 3.6)	–	2.4 ± 0.1 (2.2 – 2.5)	–	–	–	–
c	12.3 ± 1.7 (9.4 – 15.1)	9.2	8.5 ± 0.4 (7.9 – 9.3)	10.1 ± 1 (7.8 – 12.7)	8.8 ± 0.82 (7.0 – 12.1)	7.5 ± 0.8 (5.8 – 9.5)	–
c’	4.4 ± 0.5 (3.6 – 5.4)	–	5.7 ± 0.4 (4.9 – 6.2)	4.1 ± 0.3 (3.5 – 4.7)	5.3 ± 0.68 (3.7 – 6.8)	–	–
Tail length	38.2 ± 5.0 (32.2 – 48.8)	–		42.1 ± 1.9 (39 – 46)	54.2 ± 5.98 (37.5 – 69.6)	–	–
Males							
Body length	1198 ± 57 (1103 – 1266)	1600	1882.2 ± 162.7 (1701.5 – 2162.6)	1285 ± 245 (816 – 1750)	1516 (1450.8 – 1581)	2280.3 ± 369.3 (1700 – 2720)	900 – 1000
Stylet length	15.4 ± 0.7 (14 – 16.2)	16 – 18	18.9 ± 0.6 (18.2 – 19.6)	15.7 ± 1.1 (14.0 – 18.0)	17 (16.6 – 17.4)	21.3 ± 1.1 (18.0 – 23.0)	14 – 16
DGO	3.9 ± 0.7 (3 – 4.8)	–	4.6 ± 0.6 (3.8 – 5.3)	5 ± 0.4 (4 – 6)	6.2 (6.1 – 6.9)	6.1 ± 1.2 (4.0 – 8.0)	–
a	35.7 ± 6.8 (26.8 – 44.8)	38	34.6 ± 2.7 (29.8 – 37)	26.0 ± 4 (19.2 – 34.3)	35.5 (32.7 – 38.3)	49.3 ± 9.4 (35.5 – 67.4)	34
b	11.7 ± 0.8 (10.4 – 13)	16	–	–	–	–	–
c	113.6 ± 46.5 (74.4 – 205)	138 – 150	202.2 ± 27.4 (167.1 – 240.3)	–	113.9 (113.3 – 114.5)	161.4 ± 31.5 (97.5 – 237.0)	–
Spicule length	23.8 ± 2.6 (21 – 28)	32 – 34	31.5 ± 5 (22.7 – 36.8)	26.5 ± 2.3 (24 – 31)	33.3 (32.9 – 33.7)	38.5 ± 3.3 (30.0 – 43.0)	19 – 23

Molecular characterization

D2-D3 of 28S rDNA

Length of two D2-D3 of 28S rDNA sequences of *M. moensi* n. sp. were 788 bp (%T = 28.1; %A = 23.2; %G = 25.8; %C = 22.9). These sequences are 100 % identical. The D2–D3 of 28S rDNA sequences of *Meloidogyne moensi* n. sp. are most similar to that in *M. africanca* (KY433425), *M. ichinohei* (EF029862) and *M. aberrans* (KX776412) (84 %, 80 %, and 79 % similar, respectively). Phylogenetic relationship between related *Meloidogyne* species showed that *M. africana* was a sister of the clade formed by all *Meloidogyne* species. The sequences of *Meloidogyne moensi* n. sp. were distinctly formed a separate clade with 100 % bootstrap support. *Meloidogyne moensi* n. sp. has a sister relationship to *M. paranaensis*, *M. izalcoensis*, *M. arabicida*, *M. lopezi*, *M. hispanica*, *M. enterolobii*, *M. spartelensis*, *M. hapla*, *M. exigua*, *M. olae*, *M. mali*, *M. artiellia*, *M. ichinohei*, *M. daklakensis*, and *M. aberrans* with 91 % bootstrap support (Fig. 4).

COI mtDNA

Two obtained COI sequences were 100 % similar, 562 bp long (%T = 47.7; %A=28.3; %G=15.8; %C=8.2). The blast result on Genbank showed that the new species is most similar to *M. mali* (KM887146; KY433450), *M. ichinohei* (KY433448), and *M. africana* (KY433444) (86 %, 82 %, and 80 % similar, respectively). The phylogenetic tree COI sequences showed that the sequences of *M. moensi* n. sp. formed a separate clade with 100 % bootstrap support. The sequences of *Meloidogyne moensi* n. sp. have a sister relationship to *M. camelliae* (88 % bootstrap support) (Fig. 5).

COII/16S–rRNA

The obtained sequences of the region between the 3'end of COII gene and the 5'portion of 16S rRNA region was 526 bp (%T=54; %A=34; %G=8; %C=4.1). These sequences are 100 % identical. The COII/16S–rRNA sequences of *Meloidogyne moensi* n. sp. were blasted using NCBI database, and the result showed that the COII/16S–rRNA sequences of *Meloidogyne moensi* n. sp. were 74-80 % similar compared to other *Meloidogyne* species. The sequences of *Meloidogyne moensi* n. sp. are most similar to *M. marylandi* (JN241955.1; JN241918.1), *M. graminis* (JN241922.1), and *M. aberrans* (KU598839) (80 %, 75 %, and 70 % similar, respectively). The phylogenetic tree was created based on COII/16S–rRNA sequences showed a sister relationship of *M. moensi* n. sp. to *M. marylandi* and *M. graminis* with 90 % bootstrap support. The sequences of *Meloidogyne moensi* n. sp. formed a separate clade with 99 % bootstrap support (Fig. 6).

Discussion

In this study, a polyphasic approach including morphological observations, morphometry, and phylogeny of mitochondrial and ribosome DNA sequences were used to describe a new root-knot nematode from Robusta coffee in Western Highlands ofVietnam.

At the morphological level, body shape and size of females are useful to separate *M. moensi* n. sp. from species with prominent posterior protuberance, *viz. M. acronea*, *M. aberrans*, *M. africana*, *M. megadora*, *M. ichinohei* and *M. ottersoni*. According to Jepson (1987), perineal pattern characterisations are useful to separate *Meloidogyne* species into sub-group. The perineal pattern of this new species belongs to group 3 according to Jepson (1987) that can be clearly separated from other species in other groups. Measurements of the new species were also well-supported to differentiate this species from others (Jepson, 1987; Karssen, 2002; Perry *et al*., 2009).

Molecular characters are important tools for identification of nematodes, remains essential for accurate diagnosis of root-knot nematodes (Perry & Moens, 2013). Furthermore, DNA-based methods can be applied to various stages in nematode development, separate individual species from mixed populations, and DNA voucher specimens, that have been stored for several years, can still be used efficiently (Onkendi & Moleleki, 2013). The most commonly used sequences, such as 18S, 28S, and ITS rDNA or *COI* mtDNA regions, have been proven to be insufficient to distinguish some closely-related *Meloidogyne* species, making isozyme electrophoresis a prevalent choice in root-knot nematode identification, especially for the tropical group (Eisenback & Triantaphyllou, 1985; Janssen *et al*., 2016). Nonetheless, the technique based on biochemical diagnosis, that is reliant on isozyme profiles, is only applicable to young adult females of *Meloidogyne* spp., and many results from different studies implied that polymorphic enzyme profiles exist. Additionally, the isozyme profiles are not available to all the valid species, making the identification using isozyme profiles more difficult (Esbenshade & Triantaphyllou, 1985; Perry *et al*., 2009; Janssen *et al*., 2016). It is predicted that biochemical-based techniques, such as isozyme electrophoresis, will soon be replaced by PCR-based methods for many applications due to rapidly reducing cost, improvement of molecular techniques, and the finer resolution (Perry *et al*., 2009). Although isozyme charaterisation of the new species is not provided in this study due to the lack of facilities in Vietnam, as well as a degradation of machines for studying isozyme in our partner network; molecular analyses, morphological, and morphometric of *M. moensi.* n. sp. clearly define a new species that is distinct from all other *Meloidogyne* species.

Meloidogyne species that have a prominent posterior protuberance with an elevated perineum was previously assigned to the genus *Hypsoperine* (Sledge & Golden, 1964). Later on, *Hypsoperine* was considered as a junior synonym of *Meloidogyne* (Araki, 1992; Eisenback & Triantaphyllou, 1991; Jepson 1987; Luc & Fortuner, 1988; Siddiqi, 2000; Whitehead, 1968). The phylogenetic tree inferred from 18S sequences also showed that *Hypsoperine* was a junior synonym of *Meloidogyne* (Plantard *et al.*, 2007). Our phylogenetic trees also placed those *Meloidogyne* species with a prominent posterior protuberance and elevated perineum, including *M. moensis* n. sp., *M. aberrans*, *M. graminis*, *M. ichinohei*, and *M. africana M. megadora* together with all *Meloidogyne* species.

However, these species did not form a monophyletic group. In addition, *M. moensis* n. sp. is neither related to the *exigua-chitwoodi-fallax* group nor to the *arenaria-javanica-incognita* group.
The new species *Meloidogyne moensi* n. sp. was found on coffee, bringing the total number of coffee parasitic root-knot nematodes to 19 species in the world. This species was isolated from many samples of coffee with the symptoms such as root galls, stunting, and chlorosis; these symptoms implied a serious pathogen caused by the new root-knot nematode that need to be attended to prevent an outbreak in coffee growing areas.

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Conflict of Interest

Authors stated no conflict of interest.

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Research Note

Human dirofilariasis in Bulgaria between 2009 and 2018

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Article info

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Summary

Autochthonous *Dirofilaria repens* infections have been found in dogs and other carnivores in most European countries. In the same countries, reports of human dirofilariasis are becoming increasingly prevalent. We present 18 cases of people infected with *D. repens* for a 10-year period traced in our hospital. The data was collected from the observations and tests of all 18 patients from the whole country treated at the Specialized Hospital for Infectious and Parasitic Diseases in Sofia in the period 2009 – 2018. We used a morphological method, serology test and Knott's method for microfilariae. The patients were 11 to 74 years of age, 12 female and 6 male. In most cases, patients have subcutaneous nodules or face, eyelid and eyes localization. The trend of increasing incidence in Bulgaria continues, with age and sex distribution and localization of the larva being similar to those in other European countries.

Keywords: *Dirofilaria repens;* dirofilariasis; dogs; subcutaneous nodules

Introduction

The most common mosquito-borne nematodiasis in Europe is dirofilariasis. Two types of parasites are etiological agents in Europe: *Dirofilaria immitis* and *Dirofilaria (Nochtiella) repens*. Reservoirs of both parasites are predominantly dogs and wild-living carnivores, and vectors are mosquitoes of order *Diptera* (Angelov & Vuchev, 2010; Cancrini & Gabrielli, 2007). In Bulgaria and neighbouring countries are found several mosquito species capable to transmit dirofilaria: *Anopheles maculipennis* complex, *Culex pipiens*, *Coquillettidia richiardii* and *Aedes vexans* (Mikov *et al*., 2011; Pudar *et al*., 2018; Tomazatos *et al*., 2018).

D. immitis localizes in the pulmonary artery of dogs and wild predators and causes severe disease. Infections in people are rare in Europe, but not in North America (Miterpáková *et al*., 2018).

Dirofilaria repens typically causes a milder, subcutaneous infection in dogs, wild predators, and more rarely in cats. It is the major parasite causing dirofilariasis in humans, with an increasing number of cases reported in Europe. In dogs and wild predators, the parasite is localized in the subcutaneous tissues, and the microfilariae circulate in the bloodstream. Microfilaraemic dogs are the most important reservoir of infection (Genchi & Kramer, 2017). In humans, the larvae rarely reach sexual maturity, and most often migrate into the subcutaneous tissue, rarely localizing in an organ (Poppert *et al*., 2009; Kłudkowska *et al*., 2018). In the past ten years, *D. repens* has been described as a typical example of an emerging pathogen, since it is affecting more and more regions in Europe. This phenomenon is typical of most vector borne zoonotic diseases and is explained mainly with warmer climate.

Autochthonous cases of dirofilariasis in dogs have been reported in almost the entire southern part of the Old World, most of all are the Mediterranean countries and Central Europe (Sassnau *et al*., 2013; Muro *et al*., 1999).

In Bulgaria, prevalence of both parasites has been confirmed in

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dogs and wild predators – *D. immitis* was found in 5 dogs of 40 tested and in 4 foxes of 78 tested. 8.6 % of the dogs tested and almost 9 % of the jackals tested were microfilaraemic (Panayotova-Pencheva *et al*., 2016). In another study in Sofia, 240 dogs were tested, 15 of which were infected with *D. immitis*, 12 with *D. repens* and 6 with both parasites (Konstantinov, 2017).
In Bulgaria, all reported cases of infected people are only with *D. repens*. For a period of 39 years, 1973-2011, Harizanov et al. summarized 47 cases in different centers. We summarize 18 cases of people infected with *D. repens* for a period of 10 years (Harizanov *et al*., 2014).

Material and Methods

The data was collected from the observations and tests of all 18 patients from the whole country treated at the Specialized Hospital for Infectious and Parasitic Diseases in Sofia in the period 2009-2018. 8 out of 28 regions (Sofia-city, Blagoevgrad, Kardzhali, Burgas, Pleven, Stara Zagora, Ruse, Varna) were covered by place of residence. Most of the cases were reported in Sofia-city (7) and Blagoevgrad (4).
Patients were classified by demographic characteristics and by clinical and diagnostic data. Morphological methods were used for species determination of the parasite - macroscopic in the case of a preserved worm and microscopic, using a histological preparation (Heidari *et al*., 2015). In some cases, serological methods were used commercial ELISA kit (Bordier Affinity Products SA, Crissier, Switzerland) and Knott's method for microfilariae. Full blood count were performed in all patients, with follow-up of eosinophil levels.

Ethical Approval and/or Informed Consent

The study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of the hospital. Most of the patients were referred by their family doctors or by surgeons after removal of the worm.

Results

Demographic characteristics
In a 10-year period, 2009-2018, we observed and described 18 patients with dirofilariasis (Table 1). The patients were 11 to 74 years of age, 12 female and 6 male, only one patient was a child – an 11-year-old boy with epididymal dirofilariasis. This is the first reported case of a child affected by dirofilariasis in Bulgaria (Velev *et al*., 2019).

Clinical data
In most cases, patients have subcutaneous nodules - the dead and calcified larva. Before it dies, the larva can move through the subcutaneous tissue or submucosa, causing unpleasant sensations. The patient can see the moving larva if it is in a visible place or feel the formed parasitic nodule. Most commonly, the patient seeks medical attention because of finding a calcified nodule.

Table 1. *Dirofilaria repens*-infections in humans registered in Sofia hospital between 2009 and 2018.

№	Year of diagnosis	Gender	Age	Place (v=village; c=city)	Localization
1	2009	m	26	c. Sofia	Subcutaneus – upper limb
2	2010	f	64	c. Varna	Subcutaneus – eyelid
3	2010	f	34	v. Strumyani, c. Blagoevgrad	Subcutaneus – chest
4	2011	f	52	v. Obnova,c. Pleven	Eye
5	2012	m	48	c. Sofia	Subcutaneous-lower limb
6	2013	f	30	v. Belchin, c. Sofia	Lymph node
7	2013	f	55	v. Sozopol,c. Burgas	Subcutaneus – upper limb
8	2015	f	23	c. Vraca	Subcutaneus – eyelid
9	2015	m	44	v. Volvo,c. Ruse	Eye
10	2016	f	74	c. Sofia	Subcutaneus – upper limb
11	2016	f	61	v. Kravino, c. Stara Zagora	Subcutaneus – face
12	2016	f	29	c. Blagoevgrad	Eye
13	2016	f	35	c. Sofia	Subcutaneus – upper limb
14	2017	m	37	c. Sofia	Oral cavity
15	2017	f	72	v. Gulia, c. Kurdzali	Eye
16	2017	m	61	c. Blagoevgrad	Subcutaneous—lower limb
17	2018	m	11	v. Gotse Delchev, c. Blagoevgrad	Testis
18	2018	f	67	c. Sofia	Subcutaneus – upper limb

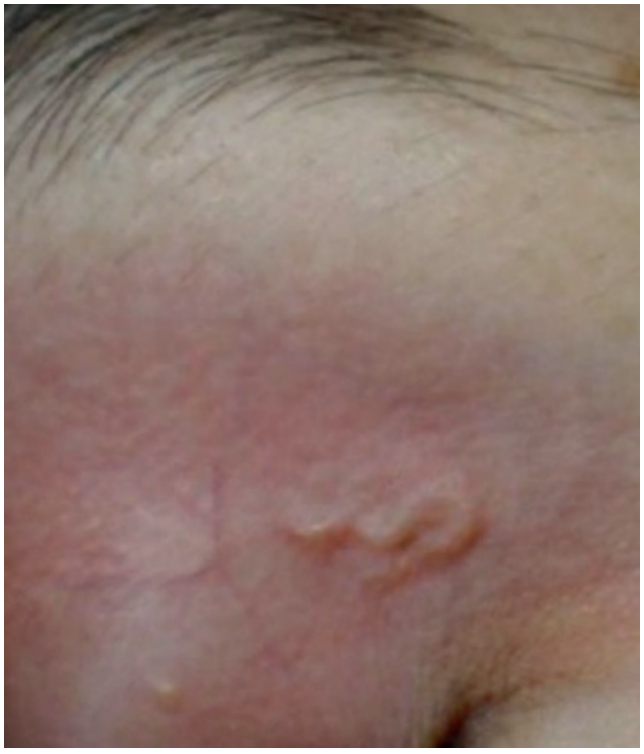

Fig. 1. Subcutaneous localization of Dirofilaria repens in an eyelid.

Only in ocular dirofilariasis, the larva remains intact subconjunctivally, does not calcify, and is usually easily noticed. Numerous subcutaneous and organ localizations have been described in the literature - lungs, oral cavity, reproductive organs, lymph nodes. In most of the cases described by us localization was subcutaneous - 11 (61.1 %), in 9 of these 11 cases, the larva was localized in the upper part of the body - subcutaneous tissue of the upper limb, face, eyelid (Fig. 1). Next was subconjunctival localization - in 4 (22.2 %) cases. In two of these cases, the larva was freely moving. The onset in all four patients manifested with blurred vision of the affected eye, photophobia, irritation, conjunctival injection and tearing. In one patient, the localization was in the oral cavity, and the larva located bucally was extirpated alive (Fig. 2a, b, c). We also described one case of lymph node localization and one case of epididymal localization. Besides local swelling and a subcutaneous nodule found upon palpation, in these individual cases, the patients had no symptoms. In one of the patients with subcutaneous localization of the larva in the area of the right mamilla, we observed a transient maculo-papular rash covering the upper half of the trunk, which, in our opinion, can be explained with antigenic irritation by the parasite.

Diagnostics

In all 18 patients, histological preparations of the extirpated nodules or whole larva were made, and the diagnosis was based on the morphological characteristics of the parasite. In one of our patients, with localization in the oral cavity, a whole living larva was also extirpated, which was subject to macroscopic diagnostics as well. Attention should be paid to the longitudinal ridges of the cuticle, which is a characteristic morphological feature of *D. repens.* In one case, with histologically proven subcutaneous dirofilariasis in a 34-year-old woman, we performed a serological test with a commercial ELISA kit, Bordier Affinity Products SA, Switzerland, but the result was negative. In two patients, one of them with a larva in an inguinal lymph node, we performed testing of blood samples for microfilariae using a Knott's method, but both cases were negative.

In literature, recommendations are made to measure the total IgE level and eosinophilia if dirofilariasis is suspected (Kłudkowska *et al*., 2018; Ermakova *et al*., 2017). In all 18 patients, we analysed complete blood count, but only in the patient with a living larva extirpated from the oral cavity, we found elevated eosinophil levels reaching 31 %.

Treatment

All 18 patients underwent surgical treatment with extirpation of the larva or parts of it, before or after our definitive diagnosis. No one was treated with anthelmintic medication and there is no evidence of relapse of the disease.

Discussion

The trend of increasing the incidence of human dirofilariasis in the European countries, and, in particular, in Bulgaria, continues. In the period 1973 – 2011, Harizanov *et al*. (2014) summarized 47 cases from the whole country, and in the period 2009 – 2018, we described 18 cases from only one clinical center. The distribution by age group does not differ significantly from that described in other European countries, nor from the previous large study in Bulgaria. This is our first case in a child, as these cases are rare in Europe, but in countries like Sri Lanka they are not uncommon (Dissanaike *et al*., 1997). In our study, the female sex predominates, and this trend is described by most authors, with no satisfactory explanation suggested so far. The fact that most of the patients are in Sofia-city and Blagoevgrad regions is probably due to the proximity of our clinical center, but the fact that Blagoevgrad is usually the warmest area in Bulgaria is likely to matter. In most cases, patients have no subjective symptoms except in the case of a moving larva or ocular localization. Due to the absence of specific symptoms, the initial diagnoses may be various - lymphadenitis, lipoma, infectious or allergic conjunctivitis. The definitive diagnosis is made after removal of the worm and macroscopic or histological assessment. In the only case in which we were able to perform a serological diagnosis, we obtained a negative result, which, along with other literature data, makes us reserved regarding this diagnostic method (Genchi & Kramer, 2017; Pampiglione & Rivasi, 2000). In the literature examined, we found several published cases of microfilaraemia in humans (Poppert *et al*., 2009; Kłudkowska *et al*., 2018). We performed two tests for microfilaraemia in blood

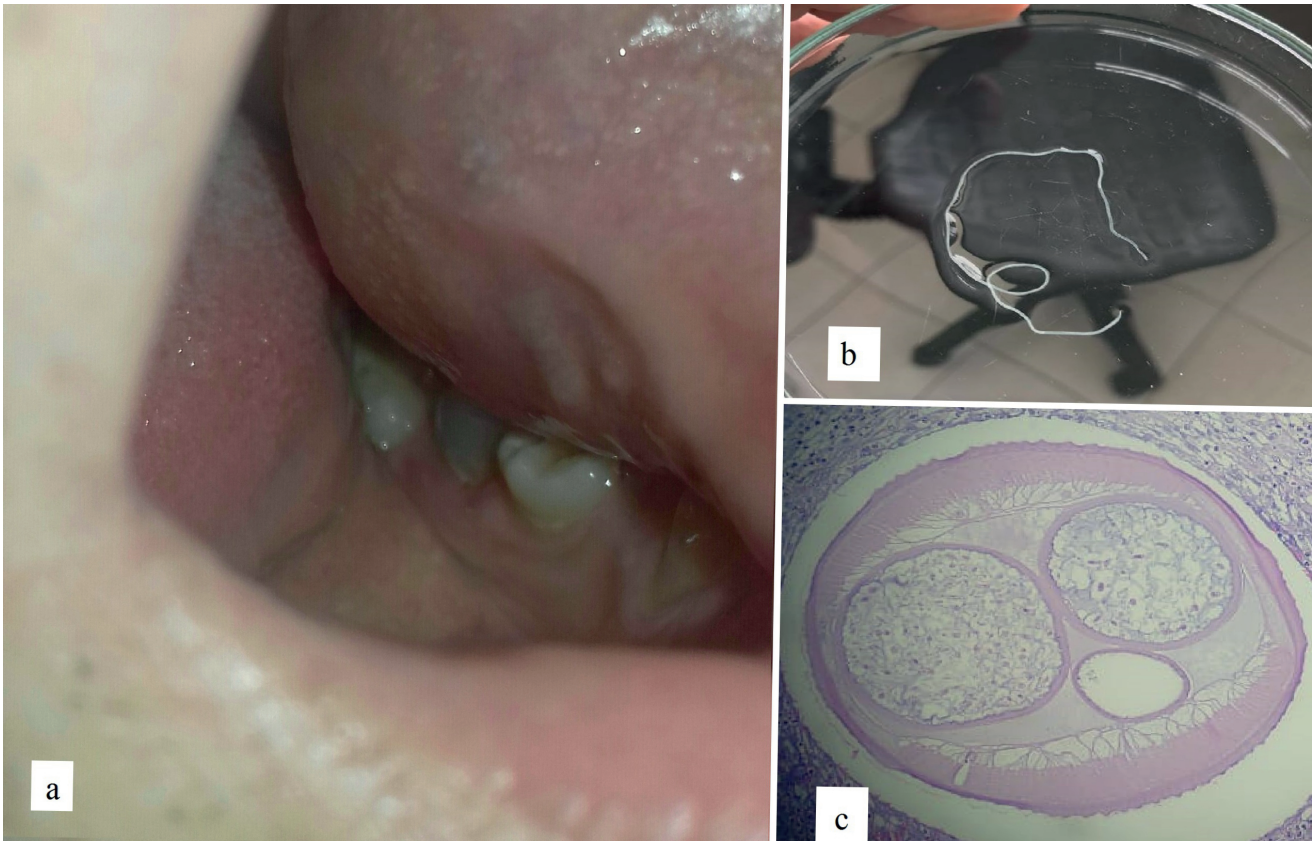

Fig. 2. *Dirofilaria repens* located in oral cavity (a). Extracted living parasite (b). Cross section of the extracted worm (c).

samples, one in a patient with lymph node localization, but the results obtained were negative.
Regarding the therapy, like other authors, we think surgical removal of the larva is sufficient.

Conclusion

We summarized the cases of dirofilariasis in patients at one clinical center in a ten-year-period. The trend of increasing incidence in Bulgaria continues, with age and sex distribution and localization of the larva being similar to those in other European countries. The increasing incidence of dirofilariasis can be explained both by the warmer climate and by the large number of stray dog in Bulgaria. Dirofilariasis is already endemic in most Southern and Central European countries and is an increasingly important clinical and social problem. Such summaries of clinical cases help to draw the attention of both medical doctors and veterinarians in Europe for active search of this disease.

Conflict of Interest

The authors report no conflict of interests.

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Research Note

The first description of *Setaria tundra* (Issaitshikoff & Rajewskaya, 1928) in roe deer from Croatia

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Summary

Genus *Setaria*, Viborg 1795, comprises 46 species that parasitize in the peritoneal cavity of Artiodactyla, Perissodactyla and Hyracoidea. The majority of these infections pass unnoticed, but occasionally they can induce severe peritonitis or neurological signs in aberrant hosts and, rarely, even in humans. In this paper we describe for the first time the finding of *Setaria tundra* in roe deer in Croatia. We examined 45 roe deer and determined the presence of *Setaria* nematodes in 24.4% of samples, which were subsequently diagnosed as *Setaria tundra* using molecular methods.
Keywords: roe deer; *Setaria tundra*; vector-borne disease; Croatia

Introduction

Filarioidea, the superfamily of nematodes comprises two families, *Filariidae* and *Onchocercidae*. The latter encompasses eight subfamilies: *Waltonellinae, Setariinae, Oswaldofilariinae, Icosellinae, Splendidofilariinae, Lemdaninae, Onchocercinae* and *Dirofilariinae* (Taylor *et al*., 2005). Presently, the genus *Setaria*, Viborg, 1795, contains 46 species that parasitize in the peritoneal cavity of Artiodactyla, Perissodactyla and Hyracoidea. The nematodes from *Setaria* genus have an indirect life cycle that includes mosquitoes (*Culicidae* family) and flies (*Haematobia* spp.) as vectors (vector-borne disease). Czajka *et al*. (2012) screened mosquitoes in Germany for filarial parasites using RT PCR, and found minimum prevalence rates of up to 24 infected per 1000 mosquitoes, which were attributed mainly to *Setaria tundra* infection. In the final hosts adult females that inhabit the peritoneal cavity produce large numbers of microfilariae each day (in thousands) which end up in the blood (Nelson, 1966), and are taken up by the vectors. Within 2 – 3 weeks in the vector, microfilariae become infective (L3) and are ready to be transferred to the final hosts (Anderson, 2000).

Usually *Setaria* sp. are not associated with clinical disease and therefore go undetected, unless the animals are submitted for necropsy. For example, Richter (1959) described findings of 43 nematodes in the peritoneal cavity of one roe deer (*Capreolus capreolus*), while Bednarski *et al*. (2010) reported finding 14 adult worms in the peritoneal cavity of a female roe deer, and in both cases the animals were without any notable signs of harmful effects. In rare circumstances, presence of adult worms in the peritoneal cavity can result in mild or even severe peritonitis. Such outbreaks of severe peritonitis were recorded in the ninety-seventies in reindeer (*Rangifer tarandus*) in Sweden and Norway, and in the period between 2003 to 2005 in reindeer and moose (*Alces alces*) in Finland, leading to significant economic losses (Rehbinder *et al*., 1975; Laaksonen *et al*., 2007). Occasionally, in aberrant hosts such as horses, goats or sheep, larvae have been known to migrate to the central nervous system, where they can induce severe neurological signs (Sundar and D'Souza, 2015). Panaitescu *et al*. (1999) reported four cases of human infection with *Setaria labiatopapillosa* in Romania.

In Croatia, the first scientific paper reporting the presence of *Setaria* sp. in different hosts dates back to as long ago as 1933 (Babić, 1933). Later, Mikačić (1941) described *S. labiatopapillosa* in cattle,

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and Richter (1959) pointed out the same species with a prevalence of 34 % in roe deer. However, due to the fact we have mentioned that these parasites rarely cause clinical disease, knowledge of their presence in Croatia is still limited. The goal of this research was to determine the present day presence of *Setaria* sp. in roe deer, using morphological and molecular methods.

Material and Methods

A total of 45 complete carcasses or whole digestive systems of roe deer were analysed at the University of Zagreb, Faculty of Veterinary Medicine, following the regular implementation of game management plans. The animals originated from two types of habitats: lowland (Zagreb County, n=28; Međimurska County, n=6; Bjelovarsko-bilogorska County, n=1; Sisačko-moslavačka County, n=1) and hilly/mountain (Nature Park Medvednica, n=9). Each sample was thoroughly analysed for free nematodes on the surface of the intestines, fore-stomachs or liver. The nematodes collected were counted, washed with physiological saline, and cleared with lactophenol to analyse their morphological characteristics according to recent morphological keys (Nikander *et al*., 2007), and then stored in 96 % alcohol. DNA isolation was performed using a commercial Genomic DNA Purification Kit (Wizard®, Promega) following the manufacturer's instructions. Subunit 1 of the cytochrome oxidase gene (Cox1) was amplified using cox1int F (5'-TGATTGGTGGTTTTGGTAA-'3) and cox1int R (5'-ATAAGTACGAGTATCAATATC-3') primers (Casiraghi, 2001). PCR reaction was executed in 25 µL suspension containing 2 µL of DNA, 0.25 µL of primers, 0.25 µL of nucleotides, 1.5 nM $MgCl_2$, and 1.25 U Promega GoTaq G2 Hot Start polymerase. Initial denaturation was performed at 95°C for 2 min, followed by 1 min cycles (n=35) at 94°C, and then 1 min at 52°C and 1 min at 72°C. Final extension was performed at 72°C for 5 min.
The PCR products obtained were sent to Macrogen Inc. (Amsterdam, Netherlands) for sequencing. The sequences were compared with those in GeneBank using a nucleotide blast tool.

Ethical Approval and/or Informed Consent

The research was performed under the approval of the Ethical Committee (Class: 640-01/14-305/16; No. 251-61-01/139-14-27)

Results and Discussion

Setaria nematodes were detected in 11 roe deer samples (prevalence 24.44 %) (Table 1) but we did not observe any gross lesions that could be attributable to *Setaria* sp. infection. According to the locality, the highest prevalence was in Zagrebačka County (P=25 %, n=7), followed by Medvednica Nature Park (P=22 %, n=2), and Međimurska County (P=17 %, n=1). Two other localities contributed with one sample each and should not be observed alone. The infection rate of nematodes per animal ranged from 1 to 24 (18 males, 77 females; 95 nematodes overall in 11 animals). Morphological analysis revealed typical *Setaria tundra* (Fig. 1) morphology (i.e. oval peribuccal crown with two elevations and without lateral lips; in femal's caudolateral appendages and the tip of the tail with a knob containing pores and grooves; caudal end containing 11 paired, one unpaired papillae and unequal spicules). DNA was successfully isolated from all samples and a 514 bp subunit of cox1 gene was amplified. Comparison with sequences archived in the GeneBank revealed that the analysed nematodes belong to the species *Setaria tundra*. Sequences from this research were deposited in the GeneBank under the accession number MH590581 – MH590586.
Recently, the number of reports describing this parasite in roe deer and mosquitoes in Europe has been increasing (Rehbein *et al*., 2001; Favia *et al*., 2003; Ferri *et al*., 2009; Laaksonen *et al*., 2009; Czajka *et al*., 2012; Kowal *et al*., 2013; Masny *et al*., 2013; Kemensei *et al*., 2015; Zittra *et al*., 2015; Angelone-Alasaad *et al*., 2016; Enemark *et al*., 2017). After the findings of Yanchev (1973), our results present the second confirmation of *S. tundra* in southeast Europe.The published prevalence in roe deer varies, from minimal, i.e. 9.4 % in Poland (Kowal *et al*., 2013), to as high as 40.1 % in Finland (Laaksonen *et al*., 2009.). Our findings of 24.4 % fit into this range. The fact that the prevalence of positive roe deer is rather high, with a wide area of distribution, and the fact that no gross lesions associated with *Setaria* infection were observed, means that roe deer are an important natural host and potential long-distance carrier of *S. tundra* (Laaksonen *et al*., 2009).
Another important question is whether the distribution of *S. tundra* started to spread due to climatic changes which favour the vectors, or due to the increase in the number of suitable hosts (namely roe deer), or was it simply previously mistakenly diagnosed as another species from the Setariinae subfamily? Proper species identifica-

Table 1. Number of samples positive on *Setaria* sp. according to location of sampling

Location/County	N	Positive	P%
Zagreb	9	2	22 %
Zagrebačka	28	7	25 %
Bjelovarsko – bilogorska	1	1	100 %
Sisačko – moslavačka	1	0	0 %
Međimurska	6	1	17 %

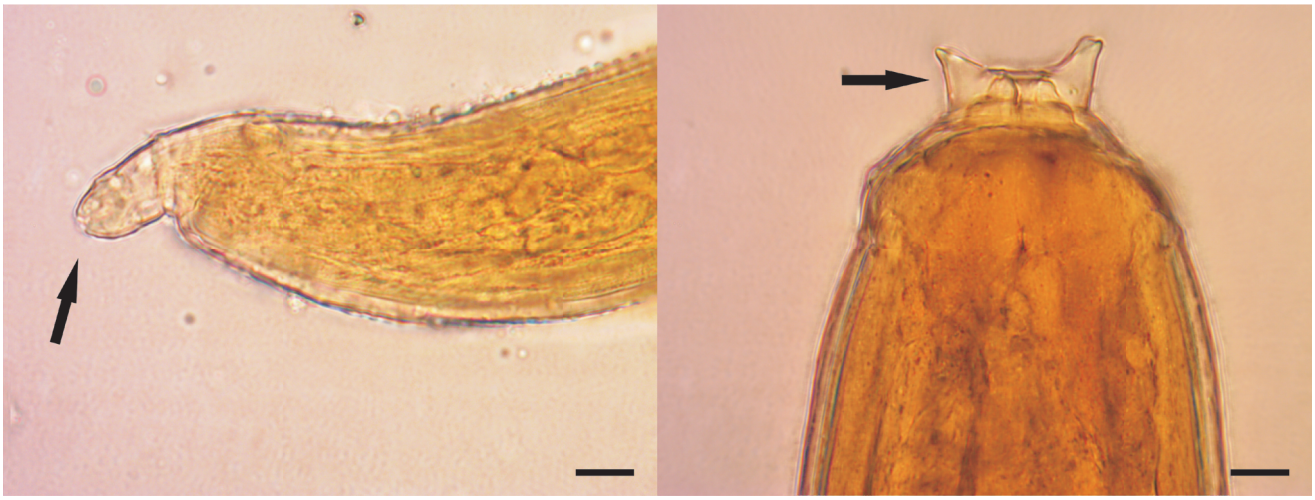

Fig. 1. *Setaria tundra*. The left part of the image shows tip of the tail with pores and grooves, without thorns (arrow). The right side points to an oval peribuccal crown with two elevations and without lateral lips (arrow). Scale bar – 10µm.

tion is of special importance for biologists and taxonomists, but also for medical and veterinary professionals due to the potential health implications. The traditional way to identify *Setaria* species was to use their morphological characteristics. However, the similar morphological keys of different *Setaria* lineages can weaken species identification, requiring application of molecular methods to confirm traditional tools (Yatawara *et al*., 2007; Ferri *et al*., 2009; Laaksonen *et al*., 2009). *S. tundra* differs from *S. labiatopapillosa* mainly in the appearance of the female peribuccal crown with the elongated mouth opening and cuticularized lateral lips (Yeh, 1959). However, according to Yeh (1959), the tails of female *S. tundra* and *S. labiatopapillosa* look very much alike, complicating proper identification. Later on, over history, many controversies about these two and other species have arisen, where authors described old species as new ones (reviewed in Nikander *et al*., 2007). On the molecular level, the difference between these two species is more pronounced and was shown in the phylogenetic study of *Setaria cervi* in Italy, which revealed that *S. labiatopapillosa* belong to one clade together with *S. cervi* and *S. digitata*, which is well separated from the one containing *S. tundra* and *S. equina* (Alasaad *et al*., 2012). In Croatia, from the first finding and over the past decades, the only previous detailed description available is from *S. labiatopapillosa* in cattle (Mikačić, 1941), where the author, besides the presence of lateral lips, clearly describes the thorn structures on the tail knob which were at that time also attributed as a characteristic of *S. tundra.* Interestingly, in our survey, among the 77 females, we did not find any thorned knobs (thorned morphotype). Eighteen years later, in his paper Richter recorded and described some parasitic fauna (without protozoa) in 47 roe deer. Among them, he pointed out findings of *Setaria labiatopapillosa* in 34 % of roe deer with one animal harbouring 43 nematodes in the abdominal cavity (Richter, 1959). Unfortunately, no detailed description or images were presented in the article, leaving us no possibility to re-examine his findings. A similar problem was encountered by Enemark Larsen *et al*. (2017) when discussing the findings of *S. transcaucausica* in Denmark (Korsholm, 1988). Therefore, the initial thought is that *S. tundra* might have been mistakenly diagnosed as *S. labiatopapillosa*. On the other hand, due to the controversial history and doubtful validity of setarian species in different and even the same mammalian species (Nikander *et al*., 2007), there is also a possibility that roe deer may harbour more than one species of *Setaria.* Since the description of *S. labiatopapillosa* in roe deer is indeed rare, this leaves us no other choice than to continue the survey of setarian species in roe deer and also cattle as type hosts for *S. labiatopapillosa*. Finally, there is also a possibility that *S. labiatopapillosa* was correctly diagnosed in roe deer, but now this species is very rare or even no longer exists in roe deer due to extrusion with *S. tundra* which seems to be increasingly prevalent in roe deer in Europe.

Acknowledgement

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Research Note

Results on search for the broad fish tapeworm *Dibothriocephalus latus* (Linnaeus, 1758), (syn. *Diphyllobothrium latum*) (Cestoda: Diphyllobothriidea), in the Danube River

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Summary

Diphyllobothriosis is a fish-borne parasitic zoonosis caused by so-called "broad tapeworms" or "fish tapeworms" of different genera of the order Diphyllobothriidea. *Dibothriocephalus latus* (Linnaeus 1758), (syn. *Diphyllobothrium latum)*, is a medically important type species of the genus, whose occurrence in various European regions is either regular, e.g. in the Alpine lakes region, or occasional and sporadic, e. g. in the Danube River region. For the latter, data on the detection of *D. latus* plerocercoids in the second intermediate fish host (European perch *Perca fluviatilis*), as well as in definitive hosts (human and dog), in which infection was directly linked to the consumption of infected fish from the Danube, were published more than 50 years ago. In order to assess the current situation, we aimed to find out whether *D. latus* is present in the natural environment of the Danube River. In total, 700 perch from five sampling sites in the Slovak part of the Danube River were examined. Plerocercoids were not detected in any fish examined, which leads to the conclusion that *D. latus* is currently not present in the studied aquatic environment.
Keywords: European perch; plerocercoid; diphyllobothriosis; fish-borne zoonosis

Introduction

Diphyllobothriosis, caused by so-called "broad tapeworms" or "fish tapeworms" of different genera of the order Diphyllobothriidea, is a significant fish-borne parasitic zoonosis responsible for about 20 million human infections worldwide (Chai *et al*., 2005). The type species of the recently resurrected genus *Dibothriocephalus* is the medically important tapeworm *Dibothriocephalus latus* (Linnaeus 1758), (syn. *Diphyllobothrium latum*) (Waeschenbach *et al*., 2017). It utilizes copepods as the first intermediate hosts and freshwater fish (in Europe mainly European perch *Perca fluviatilis*, Northern pike *Esox lucius* and burbot *Lota lota*) as the second intermediate hosts. Humans and carnivorous mammals serve as definitive hosts. Humans can be infected with *D. latus* plerocercoids by eating raw or undercooked fish. The infection can be either autochthonous (the infected fish originated from local rivers or lakes) or imported (the infection was acquired by consumption of fish during a stay abroad). The Food and Agricultural Organization (FAO) of the United Nations and the World Health Organization (WHO) included diphyllobothriosis on the list of 24 medically and veterinary important fish-borne parasites (Robertson *et al*., 2013). Understandably, knowledge on the occurrence and geographic distribution of *D. latus*, its circulation in the natural environment, and determination of possible risks of infection are crucial.

Based on the frequency of occurrence of *D. latus* in Europe, there are regions with frequent or relatively frequent occurrence, areas with sporadic or imported cases and countries where diphyllobothriosis has not been reported. Frequent occurrence of the parasite in humans, as well as in the natural environment, has been recorded e.g. in the Alpine lakes region, including Switzerland,

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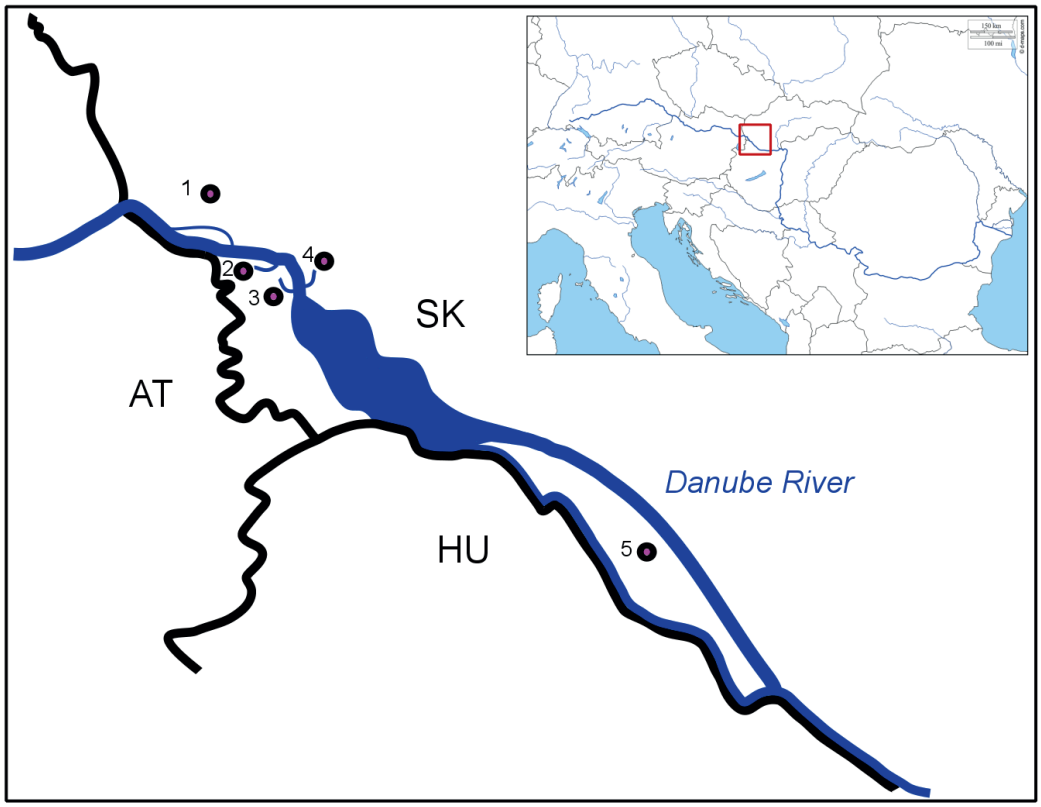

Fig. 1. Schematic representation of the entire flow of the Danube River (right upper corner) and graphical scheme of sampling sites; (1) Karloveské river arm; (2) Starohájske river arm; (3) Jarovecké river arm; (4) Biskupické river arm; (5) pond Bodíky-materiálová jama B. Abbreviations: SK, Slovakia; HU, Hungary; AT, Austria.

eastern France and northern Italy (Yera *et al*., 2008; Wicht *et al*., 2010; Dupoy-Camet, 2015; Gustinelli *et al*., 2016). Regions with sporadic occurrence are localities with imported human cases, e.g. Czech Republic (Vlková *et al*., 2007) and Romania (Stanciu *et al*., 2009), and regions with the occasional findings of the tapeworm in fish, e.g. Ireland (Harris & Hickey, 1945), Serbia (Petrovic, 1972 cited in Djikanovic *et al*., 2012) and the Danube River (Roman, 1955).

The Danube River plays a notable role in spreading various parasitic and infectious diseases of fish, since it is shared by 10 European countries (Germany, Austria, Slovakia, Hungary, Croatia, Serbia, Romania, Bulgaria, Moldova and Ukraine), making it the most international river basin in the world (Liska, 2015). According to several studies, the middle Danube (the part of the river between Slovakia and Serbia/Romania) certainly represents a great risk for native fish communities due to the presence of different pathogenic parasites, such as the nematode *Anguillicola crassus* or the rosette agent *Sphaerothecum destruens* (Gozlan *et al*., 2005, 2009). Concerning diphyllobothriosis, only a few published papers have addressed either a direct presence of larval stages (plerocercoids) of *D. latus* in fish from the Danube River (Roman 1955) or documented autochthonous *D. latus* infections linked with the consumption of raw perch from the Danube in humans (Čatár *et al*., 1967) and dogs (Supperer & Wenzel, 1967). These data were published more than 50 years ago and can be classified as sporadic. The presence of *D. latus* in the Slovak part of the Danube River was mentioned in other papers dealing with summary data on tapeworms in Slovakia (Macko *et al*., 1993, Moravec, 2001) and a review of fish parasites in Slovakia (Žitňan, 1982). However, these publications referred to a single human case report published by Čatár *et al*. (1967), and examinations of fish have never been performed.

Several other papers have been published on *D. latus* occurrence in the Danube countries (e.g. Serbia, Romania and Moldova). However, majority of those data were either not based on direct observations of the authors, or proper taxonomic identification, details on location of plerocercoid in fish and geographic locality were missing. Plerocercoids of *D. latus* were found in pikes in the Danube Delta district called Mila in Romania and from the Prut River (tributary to the Danube River) in Moldova (von Bondsdorff, 1977). Djikanovic *et al*. (2012) listed occurrence of *D. latus* in perch and salmonid *Salmo letnica* from Serbia (data originally published as a conference abstract by Petrovic, 1972). However, precise geographic locality was not provided, therefore, this finding cannot be reliably related to the Danube River. Another paper dealing with *D. latus* in perch in Romania was published by Goga *et al*. (2014); based on the figures of the tapeworm, this finding seems to be a misidentification.

The aim of the current work was to find out whether *D. latus* is present in the natural environment of the Danube River in order to assess the current situation. Since relevant data were published more than a half-century ago, and due to medical importance of diphyllobothriosis, up-to-date data on the occurrence of *D. latus* in the Danube River are needed.

Material and Methods

Parasitological examinations of 700 European perch from four arms of the Slovak part of the Danube River and one artificial pond filled with water from the Danube (Fig. 1) were carried out in autumn 2017 (October) and spring-summer 2018 (April and June). In particular, 86 fish originated from the Karloveské river arm (48° 8'46.08"N, 17° 3'50.33"E); 314 perch were examined from the Starohájske river arm (48° 6'11.50"N, 17° 7'56.19"E); 119 fish came from the Jarovecké river arm (48° 4'32.34"N, 17° 8'23.90"E); 98 were examined from the Biskupické river arm (48° 5'15.45"N, 17° 9'44.21"E); and 83 perch were caught in the pond called Bodíky-materiálová jama B (47°56'26.66"N, 17°25'42.55"E). Fish were provided to us under a commercial service by certified fishermen. Considering that a significant majority of plerocercoids collected from perch have been found in the fillet (Gustinelli *et al*., 2016), all fish were examined by detailed filleting of the entire musculature. In addition, the peritoneal cavity, intestine, liver and other abdominal organs were also checked.

Ethical Approval and/or Informed Consent

The research related to animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results and Discussion

As the result of our examinations, no *D. latus* plerocercoids were found in any of the 700 fish from five studied localities of the Danube River in Slovakia. Instead, the nematode *Eustrongylides* spp. and the fluke *Clinostomum complanatum* were found in the musculature. In addition, the tapeworm *Proteocephalus percae* was detected in the perch intestine and larvae of the tapeworm *Triaenophorus nodulosus* were isolated from the liver cysts (details not provided).
In general, determination of plerocercoids in fish or adult tapeworms in definitive hosts provides direct evidence on the occurrence of *D. latus* in the local environment. The oldest data on the presence of *D. latus* were published by Roman (1955); the author listed the detection of a single *D. latus* plerocercoid from the intestine of a European perch from the Danube Delta in Romania. However, the information provided by Roman (1955) is very brief, lacking details on morphological description or any other more species-specific data. Moreover, *D. latus* is preferably localized in the upper mass of the musculature of the European perch, especially in the dorsal muscles (Gustinelli *et al*., 2016).
Later, Supperer and Wenzel (1967) reported on a dog fed with fish from the Danube River being infected with *D. latus*. In the same year, a detailed and well documented human case report was provided by Čatár *et al*. (1967). A local fisherman, who declared regular eating of raw perch caught in the Slovak part of the Danube River, indicated gastro-intestinal problems. After medical examination, diagnosis and relevant anthelmintic treatment, a 7.5 m long *D. latus* tapeworm was released from the patient. Thanks to the detailed case report and epidemiological anamnesis of this particular human infection, the source of infection was detected, and it was overwhelmingly linked with the patient's habit of consuming raw perch caught in the Danube River.
Taking into consideration a medical importance and zoonotic potential of *D. latus*, it is rather incomprehensible, why no recent reliable data on its occurrence in fish from the Danube River have been published. Did the infection persist in this local environment but somehow manage to escape the attention of local ichthyologists and parasitologists? Or, was the prevalence of the infection so low that diphyllobothriosis was overlooked and not recorded? Finally, what was the reason for possible disappearance of *D. latus* from the Danube River?
Concerning the ecological and biological conditions crucial for maintaining the life cycle of *D. latus*, apart from a presence of a dominant second intermediate fish host (European perch) also the first intermediate host (Copepoda) (Vadadi-Fülöp, 2009) is present in the Danube River. However, *D. latus* plerocercoids have been more frequently found in fish coming from backwater, mainly lakes e.g. Alpine lakes of northern Italy (Gustinelli *et al*., 2016), Switzerland (Wicht *et al*., 2010), eastern France (Yera *et al*., 2008) and rarely in fish originating from rivers, e.g. the Kokemänjoki River in Finland (Wikgren, 1963). The explanation for why some Alpine lakes represent localities with ongoing detection of *D. latus* plerocercoids in perch and adult tapeworms in humans is the ineffective sewage treatment systems leading to the contamination of *D. latus* eggs shed by infected humans, and possibly also other mammals. In addition, consumption of a local raw fish delicacy called carpaccio results in the persistence of human *D. latus* cases in the Alpine lakes region (Gustinelli *et al*., 2016). It is evident that human play important role in maintaining the life cycle of *D. latus* in the Alpine region. On the contrary, the Danube River represents a more dynamic biotope with possible barriers for the maintenance of the life cycle stages of *D. latus*. The absence of *D. latus* in the Danube River is also supported by latest preliminary data on dissection of 32 European perch from the Danube Delta in Romania, where no *D. latus* plerocercoids have been found (M. Oros and D. Barčák, Institute of Parasitology, SAS, personal communication).
In conclusion, the localities with old findings of *D. latus*, sporadic detection of the parasite, imported human cases and random determination in the natural environment, had to be considered carefully and deserve further attention for revealing the actual epidemiological and ecological situation. Based on the amount of

fish investigated in our study, *D. latus* is very probably not present in the middle part of the Danube River. Novel data on ichthyoparasitological surveys, further examinations and epidemiological studies are needed in order to create the latest distribution map of *D. latus* in Europe.

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Conflict of interest

Authors declare no conflict of interest.

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Research Note

Helminths of the Eurasian marsh frog, *Pelophylax ridibundus* (Pallas, 1771) (Anura: Ranidae), from the Shiraz region, southwestern Iran

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Summary

Fourty seven specimens of *Pelophylax ridibundus* were collected in the vicinity of Shiraz, Fars Province, Iran in 1972. Fourteen helminth species were found, eight digeneans (*Diplodiscus subclavatus, Halipegus alhaussaini, Haematoloechus similis, Codonocephalus urniger,* and four species of metacercariae) and 6 nematodes (*Cosmocerca ornata, Rhabdias bufonis, Abbreviata* sp., *Eustrongylides* sp., Onchocercidae gen. sp. and one species of larval nematodes). Of these, only six are adults, while 8 are in their larval stage. The most prevalent helminths were the metacercariae of *Codonocephalus urniger* (61.7%) and the larvae *Abbreviata* sp. (55.32%). The adults with the highest prevalence are the digenean *Halipegus alhaussaini*, and the nematode *Cosmocerca ornata* (34% in both cases).
Keywords: Amphibians; Platyhelminthes; Nematoda; Parasites

Introduction

Helminths of Iranian amphibians have been scarcely studied. Of the 14 recognized anuran species inhabiting this country (Safaei-Mahroo *et al.,* 2015), only 5 have been examined for helminths: *Bufotes variabilis* Pallas, 1769 (recorded as *Bufo viridis* Laurenti, 1768), *Hyla savignyi* Audouin, 1827 (recorded as *Hyla arborea savignyi*), *Pelophylax ridibundus* (Pallas, 1771), *Rana macrocnemis* Boulenger, 1885 (reported as *Rana camerani* Boulenger, 1886) and *R. pseudodalmatina* Eiselt and Schmidtler, 1971 (reported as *R. macrocnemis pseudodalmatina*) (Combes & Knoepffler, 1972; Massoud & Farahnak, 1994; Mashaii, 1999, 2005; Mashaii *et al.,* 2000, 2008; Rakhshandehroo *et al.,* 2017) (Table 1). The marsh frog *Pelophylax ridibundus* is considered to be widely distributed in Iran, and it has been the most extensively studied species from the helminthological perspective (Combes & Knoepffler, 1972; Mashaii, 1999, 2005; Mashaii *et al.,* 2000, 2008) (Table 1), nevertheless, the specific identity of the marsh frogs in Iran has been recently questioned based on molecular evidence (Pesarakloo *et al.,* 2017). The goal of this study is to contribute to the knowledge of the helminth fauna of *Pelophylax ridibundus* of Iran.

Materials and Methods

Unidentified helminths recovered from 47 specimens of *Pelophylax ridibundus* (Pallas, 1771) (but see Pesarakloo *et al.,* 2017) collected in the vicinity of Shiraz, Fars Province, Iran in 1972, were borrowed from the Canadian Museum of Nature Parasites Collection in 2016 by the author. Platyhelminthes were stained with Mayer's paracarmine or Gomori´s trichrome, dehydrated, cleared in methyl salicylate, and mounted in Canada balsam. Some specimens were permanently mounted between cover slips and held in Cobb slides. Nematodes were cleared with Amann´s lactophenol and mounted in temporary slides for microscopical study. For

Table 1. Helminth record of amphibians of Iran. A = adult; L = larvae; bc = body cavity; i = intestine; k = kidneys; li = liver; lu = lungs; me = mesenteries; mo = mouth cavity floor; mu = muscle; o = ovary; r = rectum; t = testes; u = urinary bladder. 1 = Combes and Knoepffler, 1972; 2 = Massoud and Farahnak, 1994; 3 = Mashaii, 1999; 4 = Mashaii *et al*., 2000; 5 = Mashaii, 2005; 6 = Mashaii *et al*., 2008; 7 = Rakhshandehroo *et al*., 2017.

Host species	Helminth species	Stage	Habitat	Locality	Reference
Bufotes variabilis	Monogenea				
	Polystoma viridis Euzet, Combes and Batchvarov, 1974	A	u	Khouzestan, W Iran	5
		A	u	Semnan, NE Iran	6
		A	u	Fars, SW Iran	7
	Digenea				
	Haplometra cylindracea (Zeder, 1800) Looss, 1899	A	lu	Khouzestan, W Iran	5
	Cestoda				
	Nematotaenia dispar (Goeze, 1782)	A	i	Khouzestan, W Iran	5
	Lühe, 1899	A	i	Semnan, NE Iran	6
	Diplopylidium acanthotetra Parona, 1886	A	i	Khouzestan, W Iran	5
	Nematoda				
	Aplectana sp.	A	r	Khouzestan, W Iran	5
	Cosmocerca commutata Diesing, 1851	A	r	Khouzestan, W Iran	5
	Cosmocerca ornata Diesing, 1861	A	r	Khouzestan, W Iran	5
	Oswaldocruzia sp.*	A	i	Fars, SW Iran	7
	Rhabdias bufonis Schrank, 1788	A	lu	Khouzestan, W Iran	5
		A	lu	Semnan, NE Iran	6
Hyla savignyi	Monogenea				
	Polystoma viridis	A	u	Khouzestan, W Iran	5
	Cestoda				
	Nematotaenia dispar	A	i	Khouzestan, W Iran	5
		A	i	Fars, SW Iran	7
	Nematoda				
	Aplectana sp.	A	r	Khouzestan, W Iran	5
Pelophylax ridibundus	Digenea				
	Diplodiscus subclavatus (Goeze, 1782)	A	r	Anzali, NW Iran	4
	Gorgodera dollfusi Pigulevsky, 1945	A	u	Anzali, NW Iran	4
	Gorgodera microovata Fuhrmann, 1924	A	u	Anzali, NW Iran	1
	Haematoloechus breviansa (Sudarikov, 1950)	A	lu	Anzali, NW Iran	4
	Haematoloechus similis (Looss, 1899)	A	lu	Anzali, NW Iran	1
		A	lu	Chaharmahal and Bakhtiari, W Iran	3
		A	lu	Anzali, NW Iran	4
		A	lu	Semnan, NE Iran	6
	Haematoloechus variegatus (Rudolphi, 1819)	A	lu	Anzali, NW Iran	1
	Opisthioglyphe ranae Frohlich, 1791	A	i	Anzali, NW Iran	1
		A	i	Anzali, NW Iran	4
	Pleurogenoides medians (Olsson, 1876)	A	i	Aghbaba, NE Iran	4
	Prosotocus confusus (Loss, 1894)	A	i	Anzali and Astara, NW Iran	4
	Prosotocus fuelleborni Travassos, 1930	A	i	Anzali, NW Iran	1
	Codonocephalus ridibundus (Rudolphi, 1819) Luhe, 1909	L	o, k, mu, me	Chaharmahal and Bakhtiari, W Iran	3
		L	bc, me	Anzali and Astara, NW Iran	4
		L	o, k, mu, me	Khouzestan, W Iran	5
	Encyclometra colubrimurorum (Rudolphi, 1819)	L	li	Anzali, NW Iran	4
	Nematoda				
	Aplectana sp.	A	r	Khouzestan, W Iran	5
	Cosmocerca ornata	A	r	Astara, NW Iran	4
	Acanthocephala				
	Acanthocephalus ranae Schrank, 1788	A	i	Anzali, NW Iran	1
Rana macrocnemis	Digenea				
	Dolichosaccus rastelus (Olson, 1876) Travassos, 1930	A	i	Chaharmahal and Bakhtiari, W Iran	3
Rana pseudodalmatina	Digenea				
	Haplometra cylindracea	A	lu	Khouzestan, W Iran	5
	Nematoda				
	Oswaldocruzia filiformis Goeze, 1782	A	i	Semnan, NE Iran	6
Not specified	Digenea				
	Heterophidae	L	–	Khouzestan, W Iran	2

* This specimens were recorded as *Rhabdias bufonis* by Rakhshandehroo et al. (2017), but according to their drawings and the habitat of the helminths, they correspond to *Oswaldocruzia* sp.

the identification of specimens, specialized literature (Anderson *et al.,* 2009; Gibson *et al.,* 2002; Prudhoe & Bray, 1982) and original descriptions were used. Host nomenclature follows Frost (2018). Helminth specimens were deposited in the Canadian Museum of Nature Parasites Collection, 1740 Pink Road, Gatineau, Quebec, Canada, with the Accession numbers CMNPA2019-0001 - CNMPA2019-0207.

Ethical Approval and/or Informed Consent

This article does not contain any studies with human participants or animals by any of the authors.

Results and Discussion

Fourteen helminth species were found in *Pelophylax ridibundus* in the Shiraz region, eight digeneans and 6 nematodes (Table 2). Of these, only six are adults, while 8 are in their larval stage. The most prevalent helminths were the metacercariae of *Codonocephalus urniger*, found in diverse organs of 61.7 % of the examined frogs, followed by the larvae of the nematode *Abbreviata* sp., present in the stomach wall of 55.32 % of the hosts. The adults with the highest prevalence are the digenean *Halipegus alhaussaini* in the stomach, and the nematode *Cosmocerca ornata* in the rectum, in 34 % of the frogs.

Diplodiscus subclavatus (Goeze, 1782) (Digenea: Paramphistomidae) has been recorded in *P. ridibundus* in the North East of Iran (Mashaii *et al.,* 2000), and in a variety of anuran hosts in Europe and Africa (Amin *et al.,* 2012; Bakhoum *et al.,* 2011; Düşen & Öz, 2006; Düşen *et al.,* 2009, 2010; Galeano *et al.,* 1996; Grabda-Kazubska, 1980; Herczeg *et al.,* 2016; Honer, 1961; Kir *et al.,* 2001; Oğuz *et al.,* 1994; Salami-Cadoux & DeGregorio, 1976; Yildirimhan *et al.,* 1996, 2005, 2012; Yildirimhan & Incedogan, 2013). The life cycle of species of this genus include a freshwater snail of the family Planorbidae as intermediate host; cercariae encyst on diverse surfaces including the skin of tadpoles. Frogs get infected through ingestion of encysted metacercariae, grazing on surfaces in their tadpole stage, or during moulting, when they eat their cast-off skin. The metacercariae mature in the rectum of the frog (Herber, 1939; Yamaguti, 1975).

Halipegus alhaussaini Saoud and Roshdy, 1970 (Digenea: Derogenidae) was described from *Rana esculenta* Linnaeus, 1758 (=*Pelophylax ridibundus*) in Al-Basrah, Southwestern Iraq (Saoud & Roshdy, 1970) and has never been recorded afterwards. This species differs from other *Halipegus* species in the region by the small body size (2.47 – 2.61 mm), the postacetabular distribution of the vitelline glands, and the length of the egg filament (twice the length of the egg length). This is the first report of this species in Iran. First and second intermediate hosts of *Halipegus* spp. are freshwater snails and arthropods (copepods and ostracods) respectively; tadpoles get the infection when they eat infected copepods or ostracods and helminths mature in the upper digestive tract after metamorphoses (Yamaguti, 1975).

Haematoloechus similis (Looss, 1899) (Digenea: Haematoloechi-

Table 2. Helminths of *Pelophylax ridibundus* from the Shiraz region, southwestern Iran; n = 47.

	Habitat	Stage	Prevalence
Digenea			
Diplodiscus subclavatus	Rectum	Adult	21.28
Halipegus alhaussaini	Stomach, anterior intestine	Adult	34.04
Haematoloechus similis	Lungs	Adult	23.40
Codonocephalus urniger	Ovary, testes, mouth cavity floor, muscle, kidneys	Metacercariae	61.7
Diplostomidae (Neodiplostomulum type)	Kidneys	Metacercariae	12.77
Diplostomidae (Diplostomulum type)	Liver	Metacrecariae	4.26
Strigeidae (Tetracotyle type)	Ovary	Metacercariae	8.51
Digenea	Muscle, heart, mesenteries, urinary bladder	Metacercariae	14.89
Nematoda			
Cosmocerca ornata	Rectum	Adults	34.04
Rhabdias bufonis	Lungs	Adults	38.30
Abbreviata sp.	Stomach walls	Larvae	55.32
Eustrongylides sp.	Mesenteries	Larvae	10.64
Onchocercidae	Body cavity	Juvenile	2.13
Nematoda	Intestine wall, mesenteries, testes	Larvae	8.51

Table 3. Comparative helminth record of *Rana ridibunda* in different regions of Iran.

	Combes and Knoepffler, 1972	Mashaii, 1999	Mashaii et al., 2000	Mashaii, 2005	Mashaii et al., 2008	This study
	Anzali, NW Iran	Chaharmahal and Bakhtiari, W Iran	Anzali, NW Iran	Khouzestan, W Iran	Semnan, NE Iran	Shiraz, SW Iran
Digenea						
Diplodiscus subclavatus			X			X
Gorgodera dollfusi			X			
Gorgodera microovata	X					
Halipegus ahaussaini						X
Haematoloechus breviansa			X			
Haematoloechus similis	X	X	X		X	X
Haematoloechus variegatus	X					
Opisthioglyphe ranae	X		X			
Pleurogenoides medians			X			
Prosotocus confusus			X			
Prosotocus fuelleborni	X					
Codonocephalus ridibundus		X	X	X		X
Encyclometra colubrimurorum			X			
Neodiplostomulum						X
Diplostomulum						X
Tetracotyle						X
Digenea Metacercariae						X
Nematoda						
Aplectana sp.				X		
Cosmocerca ornata			X			X
Rhabdias bufonis						X
Abbreviata sp.						X
Eustrongylides sp.						X
Onchocercidae						X
Nematoda larvae						X
Acanthocephala						
Acanthocephalus ranae	X					

dae) has been recorded in *P. ridibundus* in the North East of Iran (Combes & Knoepffler, 1972) and in several species of *Pelophylax* and *Rana* in Europe: *P. kl. esculentus* Linnaeus, 1758 (Bailenger & Chanseau 1954; Bjelic–Cabrilo *et al*. 2009; Chikhlaev *et al*. 2009; Looss, 1899; Odening, 1960; Prokopic & Krivanec, 1974); *P. ridibundus* (Odening, 1960; Romanova & Matveeva 2010; Saeed *et al.,* 2007), *Rana arvalis* Nilsson, 1842 (Odening 1960; Tkach *et al*. 2000); *R. temporaria* Linnaeus, 1758 (Chikhlyaev & Ruchin 2014; Odening, 1960); *Rana* sp. (Travassos & Darriba 1930). *Haematoloechus similis* first intermediate host is the snail *Planorbis planorbis* Linnaeus, 1758; dragonfly nymphs act as second intermediate hosts and frogs are infected through the ingestion of infected dragonflies (Grabda, 1960).

Codonocephalus urniger has been recorded as metacercariae in marsh frogs in Europe and Middle East (Amin *et al.,* 2012; Dollfus & Patay, 1956; Düşen & Öz, 2006; Murvandize *et al.,* 2008; Saeed *et al.,* 2007; Yildirimhan *et al.,* 1996, 2005). Particularly in Iran, *C. codonocephalus* has been recorded parasitizing *P. ridibundus* in the northeast and in the southwest (Mashaii, 1999, 2005; Mashaii *et al.,* 2000). The life cycle of this species involves the snail *Stagnicola palustris* Müller, 1774 as first intermediate host, marsh frogs as second intermediate hosts and ardeiform birds as definitive hosts (Kostadinova, 1993; Niewiadomska, 1964; Prudhoe & Bray, 1982).

We found three types of metacercariae of Diplostomoidea: Neodiplostomum type, Diplostomulum type (Diplostomidae), and Tetracotyle type (Strigeidae), being the first record of this kind of metacercariae in *P. ridibundus* in this region. These helminth species may use aquatic birds or mammals as definitive hosts (Prudhoe & Bray, 1982).

Metacercariae infecting heart, muscle, mesenteries and urinary bladder were impossible to identify due to their lack of diagnostic characters, and poor preservation conditions.

Cosmocerca ornata is widely distributed in Europe, Asia and Africa in various anuran and some lizard species (Aisien *et al.,* 2004; Amin *et al.,* 2012; Baker, 1981; Bursey & Goldberg, 2011; Düşen, 2007, 2011, 2012; Düşen & Oğuz, 2010; Düzen & Yaka, 2014; Düşen *et al.,* 2009, 2010; Galeano *et al.,* 1990; Galli *et al.,* 2001; Kirillov & Kirillova, 2016; Moravec & Barus, 1990; Moravec *et al.,* 1987; Norval *et al.,* 2013; Sey & Al-Ghaith, 2000; Schad et al., 1960; Yildirimhan & Karadeniz, 2007; Yildirimhan *et al.,* 2005, 2006a, 2006b, 2006c, 2009); it has been recorded in *Bufotes variabilis* and *Pelophylax ridibundus* in the South West and North East of Iran (Mashaii, 2005; Mashaii *et al.,* 2000). *Cosmocerca* spp. have a direct life cycle; eggs are released to the environment with the faeces, larvae hatch in the soil and infect definitive hosts through skin penetration (Anderson, 2000).

Rhabdias bufonis has a palearctic geographic distribution and has been recorded in numerous anuran species, *Bombina bombina* (Yildirimhan *et al.,* 2001); *Bufo bufo* (Düşen, 2011; Yildirimhan *et al.,* 1997; Yildirimhan & Karadeniz, 2007); *Bufo (Pseudepidalea) viridis* (Düşen, 2011; Yildirimhan, 1999); *Rana camerani* (Yildirimhan *et al.,* 2006c); *R. dalmatina* (Düşen *et al.,* 2009); *R. macrocnemis* (Yildirimhan *et al.,* 1996); *R. ridibunda* (Düşen & Öz, 2006; Kir *et al.,* 2001; Sağlam & Arikan, 2006; Yildirimhan *et al.,* 1996; 1997); *Pelodytes caucasicus* (Yildirimhan *et al.,* 2009). Nevertheless, Kuzmin (2013) considers that it is not unlikely that this is a complex of cryptic species; in Iran it has been recorded only in *Bufotes variabilis* (Mashaii, 2005; Mashaii *et al.,* 2008). *Rhabdias bufonis* life cycle shows a free gonochoristic generation and a generation of parasitic hermaphrodites; amphibians get the infection by skin penetration or ingestion of larvae (Kuzmin, 2013).

Abbreviata sp. larvae are commonly found encapsulated in the stomach walls of amphibian and reptiles (Anderson, 2000), but this is the first record in Iran. In the Middle East, larvae of *Abbreviata* sp. have been recorded in *P. ridibundus* and *Hyla orientalis* Bedriaga 1890 in Turkey (Düzen & Öz, 2006; Düzen & Yaka, 2014; Heckmann *et al.,* 2010), and in *Ophisaurus apodus* Daudin, 1803 (Lacertilia) and *Coluber jugularis* Linnaeus, 1758 (Serpentes) in Georgia (Murvandize *et al.,* 2008). The adults of *Abbreviata adonisi* Sulahian and Schacher, 2009 were described from the lizard *Agama stellio* in Lebanon (Sulahian & Schacher, 1968). The life cycle of nematodes in the genus *Abbreviata* includes an arthropod first intermediate host, amphibians and small reptiles as paratenic hosts and the majority of species use reptiles as definitive hosts (Anderson, 2000; Gafurov *et al.,* 1970; King *et al.,* 2013).

Adult *Eustrongylides* inhabit the proventriculus of aquatic birds, whereas the infective larval stage is found in the tissues and body cavity of fishes, amphibians and reptiles (Anderson, 2000). This is the first record of *Eustrongylides* sp. larvae in amphibians in Iran, although they have been previously recorded in anurans and fish from the Middle East region (Düşen & Öz, 2006; Sağlam & Arikan, 2006; Sattari *et al.,* 2002; Yildirimhan *et al.,* 2005)

Onchocercid nematodes that parasitize amphibians belong to the subfamilies Icosiellinae, Waltonellinae or Driofilariinae. As adults they live in the body cavity and mesenteries, females release the larvae (microfilariae) into the blood stream and these are taken by hematophagous vectors, which transmit them to other host after some development (Anderson, 2000). Specimens in this study were collected in a juvenile phase and were poorly preserved, which prevented the identification to a lower level. This is the first record of an onchocercid nematode of amphibians from Iran.

Nematode larvae encysted in the intestine wall, testes and mesenteries of marsh frogs were impossible to identify because their lack of diagnostic characters and poor preservation conditions.

The helminth record of the marsh frogs of the Shiraz Region studied herein presents the highest number of species compared to studies performed in other regions (14 species in this study vs 1 to 10 species in other studies) (Table 3). The second richest helminth record is the one from frogs in Anzali (Mashaii, 2005) with ten species, with the difference that most of those species were adults (8 species), while in our study only 6 species were adults and 8 species were larval stages, many of them parasites of birds in their adult stage. This indicates the presence of abundant aquatic birds

in the area at the time of collection and the important role that these frogs played in those birds diet.

Conflict of Interest

Author states no conflict of interest.

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Research Note

Quantifying spirorchiid eggs in splenic histological samples from green turtles

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Summary

The present study proposes a new methodology for the quantification of parasite eggs in animal tissue. Quantification of parasites are important to understand epidemiology of spirorchiid infections in sea turtles, however different methodologies for quantifying Spirorchiidae eggs in turtle tissues have been used. The most representative way to quantify Spirorchiidae burdens in tissues is counting eggs / g of tissue, however, this method is very laborious. As an alternative, we propose quantifying number of Spirorchiidae eggs/ area of tissue on a microscope slide. We compared this method to number of eggs / slide, a common metric of egg burden in turtle tissues. Both methods correlated well with eggs / g with eggs/mm^2 of tissue having better correlation.
Keywords: *Chelonia mydas*; helminth; pathology

Introduction

The green turtle, *Chelonia mydas,* is distributed worldwide, occurring from tropical regions to temperate zones. The green turtle forages in coastal habitats (Hirth, 1997) and according to Seminoff (2004), is listed as endangered or near-threatened in portions of its range. In Hawaii, green turtles have been recovering since protection was established in the early 1970s (Balazs & Chaloupka, 2004).

Spirorchiidae are a group of parasitic trematodes found in the circulatory system of marine and freshwater turtles (Platt, 2002) comprising 96 species in 22 genera of which ten are unique to sea turtles (WoRMS, 2018). Spirorchiidae infections are often associated with stranding and mortality of sea turtles worldwide and tend to be more severe in debilitated turtles (Stacy *et al*., 2010). However, the actual impact of these parasites on the health of these animals is not entirely known (Goldberg *et al*., 2013). Adult helminths infect the vascular system, primarily the heart, as well as vessels of various internal organs and mesenteries. There, they copulate and oviposit, causing vasculitis, parasitic granulomas and thromboses (Aguirre *et al*., 1998). Commonly affected tissues are the gastrointestinal tract, liver, spleen, lung and central nervous system (Glazebrook & Campbell, 1981); however, Goodchild and Dennis (1967) found that the spleen is the organ of *Chrysemys picta* most commonly infected by Spirorchiidae eggs, and similar observations were made by Flint *et al*. (2009), in green turtles. The spleen is also heavily infected in *C. caretta* (Ribeiro *et al*., 2017).

The detection of infections caused by Spirorchiidae in turtles is usually made during necropsy, when adult helminths or eggs can be observed grossly or microscopically (Work *et al*., 2005). Parasites can be identified through morphometric analysis of adult helminths, and more recently molecular means (Chapman *et al*., 2017). However, decomposition is a limiting factor in the detection of these parasites at necropsy, because adults are difficult to detect in decomposed tissues. In contrast, parasite eggs and their shells are more numerous in tissues and, relative to adults,

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more resistant to degradation thereby acting as a more durable indicator of infestation (Chen *et al*., 2012). Other studies have detected turtles infected with Spirorchiidae eggs in the absence of the adult worm, which seems to confirm this impression (Aguirre *et al*., 1998; Santoro *et al*., 2007; Flint *et al*., 2010).

The lesions caused by Spirorchiidae eggs are well described and are characterized by the formation of granulomas with giant cell infiltrations (Wolke *et al*., 1982; Gordon *et al*., 1998; Work *et al*., 2005; Flint *et al*., 2009; Flint *et al*., 2010; Marchiori *et al*., 2017; Ribeiro *et al*., 2017; Santoro *et al*., 2017; Werneck *et al*., 2017). Severe forms of the disease are generally associated with other problems such as fibropapillomatosis, coccidiosis and generalized infections with Gram-negative bacteria (Gordon *et al*., 1998; Raidal *et al*., 1998), but few studies exist attempting to relate parasite burdens to health outcomes. One exception is a study showing a negative relationship between tissue spirorchiid egg burden and body condition in green turtles from Hawaii (Work et al. 2005). In cases of intestinal impaction, it was observed that in most cases, animals were concomitantly infected with moderate to severe burdens of spirorchids (Flint *et al*., 2010). When found in low amounts, Spirorchiidae infections are considered incidental for healthy populations of sea turtles (Flint *et al*., 2010).

Different methodologies for quantifying Spirorchiidae eggs in turtle tissues have been used. Goodchild and Dennis (1967) proposed an egg counting by histological section, regardless of the area, a method also used by Ribeiro *et al*. (2017). Stacy *et al*. (2010) created subjective scores for infection intensity of Spirorchiidae eggs, ranging from 0 to 3 according to the amount of egg masses seen in tissue sections. Flint *et al*. (2010) presented a similar methodology, scoring severity of spirorchiid infection from 0 to 3 (absent, mild, moderate or severe) based on presence of <5 or >5 eggs per 20X microscope field. This methodology was also used by Santoro *et al*. (2017) and Werneck *et al*. (2017). Whilst useful empirically, none of these methods relate egg counts to actual area or mass of tissue examined, making cross-study comparisons difficult. A more rigorous assessment of parasite burden was done by quantifying eggs per gram of spleen (Work *et al*., 2005), a technique adapted from quantifying spirorchiid eggs in tumors (Dailey & Morris, 1995). Whilst quantifying eggs per unit weight of tissue might yield more robust results, quantifying eggs/g of tissue is laborious, requiring tissue digestion and extensive filtration. Quantifying eggs in tissue sections would be an attractive alternative as this is less laborious, however, these counts would need to be validated to a more stringent sampling regimen (e.g. eggs/g). Here we investigated how egg burden relativized to surface area of tissues related to eggs/g of spleen (GOS) using tissues from stranded Hawaiian green turtles.

Material and Methods

Tissues for this study originated from turtles stranded in Hawaii with various conditions in Hawaii (Work *et al*., 2004). Forty randomly selected formalin- fixed spleens that had previously been quantified for egg burdens (Work *et al*., 2005) were selected for this study. Spleens were embedded in paraffin, sectioned at 5 um, and stained with hematoxylin and eosin. Tissue sections (one section per individual) were examined under light microscopy and number of eggs in the section was counted. The slides were then scanned, and Image J (Schneider *et al*., 2012) was used to calculate the area of tissue quantified (mm^2). This allowed calculation of number of eggs/mm^2 of tissues which was then related to eggs/GOS (Work *et al*., 2005) using linear regression.

Ethical Approval and/or Informed Consent

National Oceanic and Atmospheric Administration National Marine Fisheries Permit 16865, US Fish and Wildlife Service endangered species permit BRD-VETAGENT-8, and CITES permit 17US105568/9.

Results

Means of 243.4 ± 238.7 (2.0 - 1014.0) eggs per section and 1.7 ± 1.9 (0.0 - 7.7) eggs per mm^2 were found in the splenic tissues

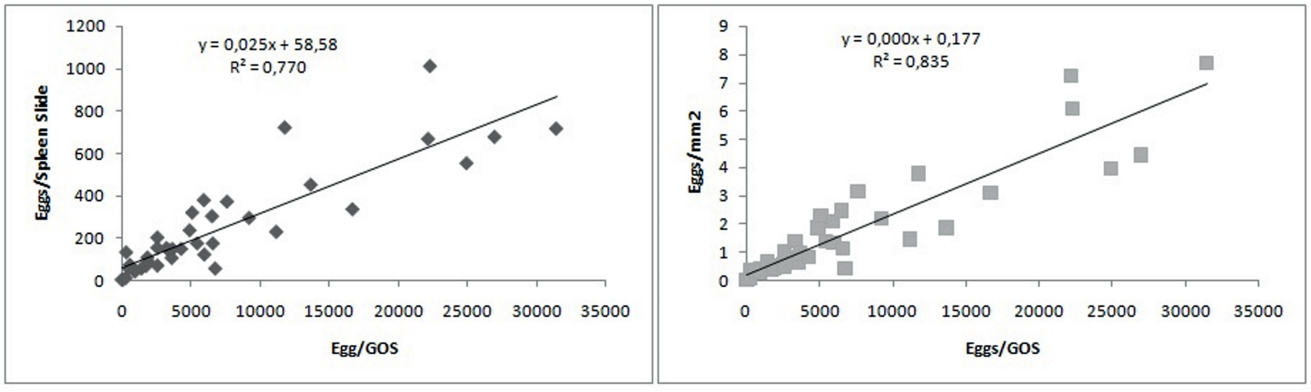

Fig. 1. Linear regression of eggs/gram of spleen (GOS) vs eggs/spleen section (left) or eggs/mm^2 of tissue section (right).

analyzed. The area of tissues examined ranged from 87,126 to 375,842 mm². Eggs/section correlated less robustly (r^2=0.770) to eggs/GOS than eggs/mm^2 (r^2 = 0.835) (Fig. 1).

Discussion

Quantifying eggs/tissue (Goodchild & Dennis, 1967) provides reasonably good correlation with egg burdens assessed as eggs/GOS. However, this method suffers from a lack of standardization in terms of accounting for amount of tissue examined. As amount of tissues placed on slides will vary between studies, this could lead to increased variation and complications when trying to compare egg burdens between studies. In contrast, the correlation between eggs/GOS and eggs/mm^2 was higher (0.85), but the difference between the two correlations was not statistically significant, so theoretically either method could be used to quantify tissue egg burden in green turtles.

The advantage of standardizing egg burden to tissue area is that it allows for more robust interstudy comparisons by accounting for different amounts of tissues used between studies. It also has the added advantage of reducing subjectivity inherent in more qualitative approaches (Stacy *et al*., 2010; Flint *et al*., 2010). Finally, it is less laborious than quantifying eggs/GOS. We thus recommend this technique for those interested in quantifying tissue spirorchiid egg burdens in green turtles.

Conflict of Interest Statement

No financial or personal relationship between the authors and other people or organizations have inappropriately influenced (biased) this work.

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