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Massive occurrence and identification of the nematode *Alloionema appendiculatum* Schneider (Rhabditida: Alloionematidae) found in Arionidae slugs in Slovenia

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ABSTRACT

In the period from June to October 2008 we collected 500 slugs from the genus *Arion* in the area of Ljubljana and Prekmurje (Slovenia). By means of dissection we determined the presence of parasitic nematodes in slug cadavers. Identification of the nematodes was made by a molecular technique (PCR). In these slugs we did not find the parasitic nematode *Phasmarhabditis hermaphrodita*, however the presence of *Alloionema appendiculatum* in larger quantities was confirmed. The most infected was a Spanish slug, *Arion lusitanicus*. In Petri dishes younger slugs showed a satisfactory mortality rate already on the fourth day after the application of the nematode suspension. Unfortunately, we can not confirm with certainly that the nematode *A. appendiculatum* undergoes a complete life cycle in *A. lusitanicus*, which is otherwise typical for *Phasmarhabditis hermaphrodita*.

Key words: *Alloionema appendiculatum*, *Arion lusitanicus*, parasitic nematodes

IZVLEČEK

ŠTEVILČNI POJAV IN IDENTIFIKACIJA OGORČICE *Alloionema appendiculatum* Schneider (Rhabditida: Alloionematidae) V LAZARJIH (Arionidae) V SLOVENIJI

V obdobju od junija do oktobra 2008 smo na območju Ljubljane in Prekmurja nabrali 500 polžev iz rodu *Arion*. Polže smo secirali in ugotavljali zastopanost ogorčic v njihovem telesu. Identifikacija ogorčic je bila opravljena z molekulsko tehniko (PCR). V nobenem polžu nismo našli parazitske ogorčice *Phasmarhabditis hermaphrodita*, medtem ko smo zastopanost ogorčice *Alloionema appendiculatum*, predvsem v predstavnikih vrste *Arion lusitanicus*, potrdili v večjem številu. V petrijevkah so mladi polži pokazali zadovoljivo stopnjo smrtnosti že četrti dan po nanosu suspenzije ogorčic. Žal pa z našo raziskavo ne moremo potrditi, da ogorčica *A. appendiculatum* v polžu *A. lusitanicus* razvije popolni parazitski razvojni krog, kot je to značilno za ogorčico *P. hermaphrodita*.

Ključne besede: *Alloionema appendiculatum*, *Arion lusitanicus*, parazitske ogorčice polžev

1 INTRODUCTION

More than 25,000 species of nematodes have been described up to the present, of which around 4,000 are marine free-living nematodes, 6,000 terrestrial free-living nematodes, 12,000 parasitic nematodes of vertebrates, and 3,500 parasitic nematodes of invertebrates (Poulin and Morand, 2000; Hugot *et al*., 2001). Hitherto, in terrestrial slugs nematodes from eight families have been determined. Among them are parasitic species (of the order Rhabditida) which spend their complete life cycle inside slugs, as well as nematodes (of the order Strongylida) which use slugs as intermediate hosts (Grewal *et al*., 2003).

When compared with entomopathogenic nematodes - the natural enemies of insects (Kaya and Gaugler, 1993) – these nematodes, which are related to slugs, are poorly studied (Wilson and Grewal, 2005). However, among those which have been studied up to now, specificity to

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host species as well as diversity among individual species of nematodes are known. Slug-parasitic nematodes were found in all organs of the body cavity and also on foot muscles. Infection mechanisms are different, from entering through the body wall (infective juveniles of nematodes which are found in the soil) to the transference of infective juveniles between slugs during mating. It is well known that slug-parasitic nematodes can be an important biotic factor which balances the slug population in the natural environment (Grewal *et al*., 2003). The most intensively studied species of slug-parasitic nematode is *Phasmarhabditis hermaphrodita* (Schneider) (Rhabditidae), and in some countries this species is also used in the biological control of slugs (Wilson *et al*., 1993ab). The natural hosts of *P. hermaphrodita* are numerous slug species which can also be found in Slovenia: *Deroceras leave* (Müller), *D. reticulatum* (Müller), *Arion distinctus* Mabille, *A. lusitanicus* Mabille, *A. silvaticus* Lohmander, *Tandonia budapestensis* (Hazay) and *T. sowerbyi* (de Férussac) (Vaupotič and Velkovrh, 2002; Morand *et al*., 2004).

Alloionema appendiculatum Schneider is classified into the family Alloionematidae and it consists of two genera, *Rhabitophanes* Fuchs, the species of which are most frequently associated with insects, and the genus *Alloionema* Schneider, whose only representative, *A. appendiculatum*, is linked with gastropod molluscs (Morand *et al*., 2004). The former species was discovered for the first time in 1859 (Schneider). Its presence has hitherto been confirmed in Europe and Australia (Cabaret *et al*., 1988). In Slovenia this species was confirmed as early as in the 1970s, when Hržič studied the nematological fauna of Slovenia. The nematode was found in the soils of a vineyard situated in the Primorska region of Slovenia (Hržič, 1969; Urek *et al*., 2003). With our research we also wanted to determine if in Slovenia this nematode species is also present in slugs, as they are becoming increasingly more important pest organisms of some cultivated plants (Barker, 2001).

2 MATERIAL AND METHODS

In September and October 2008 we collected 500 slugs in the area of Prekmurje and Ljubljana from the genus *Arion* (Arionidae). We dissected the slugs and with electronic magnification searched for the possible presence of nematodes inside them. We confirmed the presence of nematodes in the slugs collected in Ljubljana. With a pipette we separated the nematodes from the slug tissue and cleaned them according to the prescribed protocol (Riddle 1988). We infected new slugs with the obtained nematode suspension. We put 1 ml of a nematode suspension with a concentration of 300 IJ/ml into a Petri dish (diameter = 9 cm) and added 5 smaller slugs of the species *Arion lusitanicus* Mabille. After 4 days the slugs died and we dried the dead individuals at air temperature for 10 days. On the 10th day we placed the dried cadavers into a so-called White trap (Bedding and Akhurst 1975) in order to separate the nematodes from the dead slugs. The nematodes started to leave the cadavers on the 4th day after being exposed to the White trap. We washed the nematodes according to a procedure using a centrifuge and a 5 % concentration of sodium hypochlorate. The aim of this process was to obtain infective juveniles from the suspension. The nematode suspension was sent to the Institute of Biological and Environmental Sciences, University of Aberdeen, for further analysis.

DNA was extracted using a QIAmp DNA Mini Kit. The 18S gene was amplified using a combination of universal primer pairs: G18S4, 26R, 24F, and 18P (Blaxter et al. 1998). An additional primer pair, 22F, and a newly designed primer, 1080R (TCC-TGG-TGG-TGC-CCT-TCC-GTC-AAT-TTC), were also used to ensure complete coverage of the 18S region (J. Ross, unpubl.). PCR cycling parameters involved primary denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 60 s, 55°C for 90 s, and 72°C for 2 min. Post-amplification extension occurred at 72°C for 10 min (J. Ross, unpubl.). The PCR products were visualized on a 1% agarose gel and cleaned using a Qiagen QIAquick® PCR Purification Kit (fig. 1). Sequencing was carried out using an ABI3730 sequencer. The sequences were assembled using Sequencer 4.1 (Genes Codes Corp. Ann Arbor, Michigan, USA). The results were then compared with the GenBank Database, (http://www.ncbi.nlm.nih.gov/), using the BLASTn search tool (Altschul *et al*. 1990). The *Alloionema appendiculatum* sequence was deposited in the NCBI GenBank under FJ665982.

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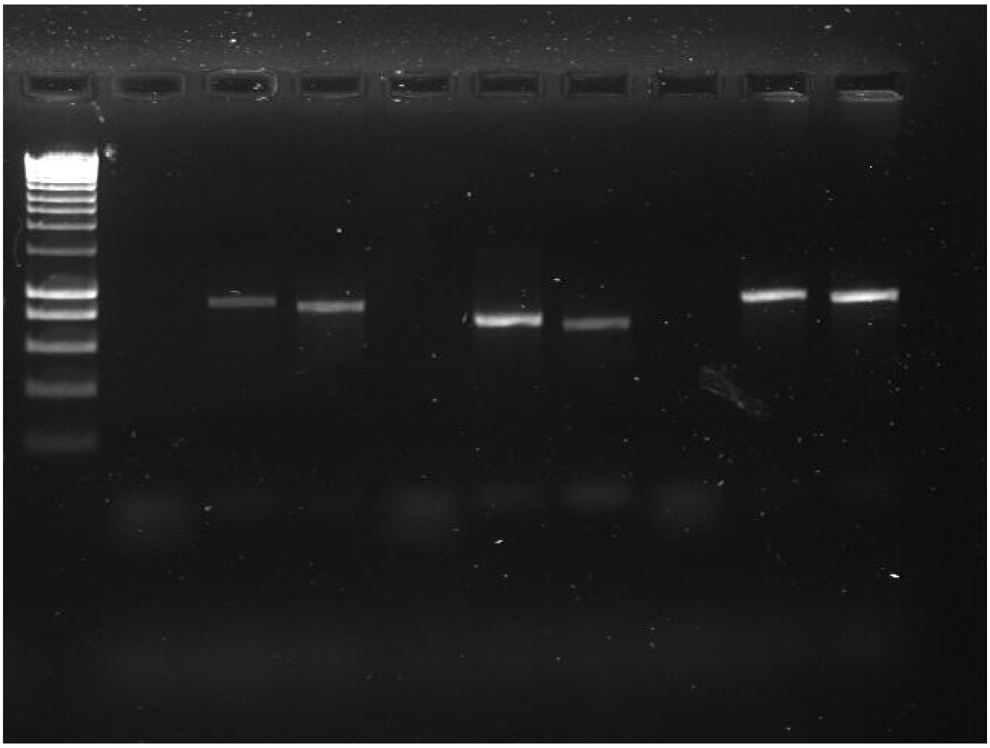

Figure 1: 1% TAE buffered agarose gel, in the 1st lane; GeneRuler 100 bp DNA Hyperladder (Fermentas), in the 3rd, 6th, and 9th lane; the PCR product of *Phasmarhabditis hermaphrodita* (Becker Underwood commercial strain), which was used as a positive control, using the primer pair specified in the text; the 4th, 7th and 10th lane: the PCR product of the sample slug nematode – *Alloionema appendiculatum*.

3 RESULTS AND DISCUSSION

The genetic studies proved that this nematode species is *Alloionema appendiculatum* Schneider (1859). The closest matches to this *Alloionema appendiculatum* sequence were Rhabditoid sp. (DQ531722.1) (Qvarnstrom *et al*., 2007) with 92% identity over 89% coverage, followed by *Strongyloides ratti* (U81581.1) (Frisse *et al*., unpubl.) with 87% identity over 100% coverage (Fig. 2). The ITS1-5.8S-ITS2 region, including the partial 18S and 28S rRNA genes (flanked by the above primers) of the Slovenian isolate of *A. appendiculatum* is 1425 bp long (fig. 2).

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FJ665982  1    AACTGAGGTAATTCTTGAGCTAATACACGC--T--T--TA-ATGCCACATT-CGTGGTGC  52
U81581    86   ..............................T-.--.--.T-..A.......-A.......  138

FJ665982  53   GTTTATTTGACTAGA-C-A---T-C-A-TA-T-T-GGTTGACTCAAAATATCCTTGCT-A  100
U81581    139  ..........T..A.C.-.---.---T-.T-.-.C...................C...-G  187

FJ665982  101  ATTTTTTTTTTA-AAAAT-ATGCCGTATGAGTATCTGCTTTATCAACTTTCGATGGTAGG  158
DQ531722  1                                                        ......  6
U81581    188  .....G..AC.--....C-..A.......T.......G......................  244

FJ665982  159  GTATTGGCCTACCATGG-TGTTGACGGATAACGGAGAATTAGGGTTCGACTCCGGAGAGG  217
DQ531722  7    .................-.A........................................  65
U81581    245  .................T..-.......................................  303

FJ665982  218  GAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGAAAATTACCCAATCT  277
DQ531722  66   ..........................................................T.  125
U81581    304  ..........................................................T.  363
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FJ665982  278   TAG-TT-AAAGGAGGTAGTGACGAAAAATGACAAAG-C-AA-CACT-TT-TTAAG-T-GT  328
DQ531722  126   ...-..-...A.........................C.-.T-G...-..-..--.T.-A.  176
U81581    364   ...-..-...A.....................-..C-.A..-T.T.A..-..T..-.AT.  416

FJ665982  329   TGTTATTGGAAATCTTGC-AA-CCTAAAT-A-GTTGCTTGGTAAAGGAGAGGGCCAGTCT  384
DQ531722  177   G................T-.G-TG.....-.-AC.A........................  232
U81581    417   ..-G...A........-.-..GTT.....A.-C...-...........A.....A.....  471

FJ665982  385   GGTGCCAGCAGCCGCGGTAATACCAGCTCTCCAAGTGCATAAAATGATTGTTGTGGTTAA  444
DQ531722  233   ............................................................  292
U81581    472   ............................T...............................  531

FJ665982  445   AAAGCTCGTA-GTTGGATT-T----ATTG---AATA-A-G--T-TCA-GA-G---T-C--  482
DQ531722  293   ..........-........-.----....---.G..-.G.--.-.-.-.T-.T--.-G--  331
U81581    532   ..........-......-.-.----.-.A---..G.TT-.--.G.A.T..-.CA-.-.--  572

FJ665982  483   --A-T-GT-A-T-ATG-GCT--T--C--TGA-A-AT-G-TT---CA-TA-C--T-TT--T  514
DQ531722  332   --.C.-..-G-.---.A-A.A--.--.--.-.-.C..-.C..AAAT.T..-G--.A..--.  369
U81581    573   --T-.-.G-.-.GT.A-TT.--.--.A-.T.CC-..-C-.----.-..T-T--.-..--.  606

FJ665982  515   -G-C---A-A---TT-AG---C-A-AT-A-A-T--T-GC-CT-GTTACTTTGAATAAATC  551
DQ531722  370   -T-T---T-T---..A.A---.G.-.A-.-.-A--.-A.-..-.................  408
U81581    607   AT-T---.T.---..-..---A-.-..A.T.-.AA.-AA-..-.................  649

FJ665982  552   AGAGTGTTCAAA--A--CA-G---TC-A---T-ATGAC-TG-A--AT-A-T-CCTAGCAT  591
DQ531722  409   ............--.--..-.---..-.---.-.....-..-.--..-.-.-........  448
U81581    650   ....G.......--C--..G.CA-.T-.---.-...-.T..-T--..GG-.--.......  692

FJ665982  592   GGAATAACACTATA-GAAGTATATG-TAGTGCGGTGTTTCA-C--ACA-T-TACTTCATG  644
DQ531722  449   ..............-.......-.T-.....T.........TT--.-.-A-.........  500
U81581    693   ..............-...--.-.A.T.....-T.-......-.---T.-.C.TT......  741

FJ665982  645   GTTAATA-GAAACAAACGGGGGCATTCGTATCGATACGTTAGAGGTGAAATTCTTGGACC  703
DQ531722  501   .......-....................................................  559
U81581    742   A......-.G.......................C..........................  800

FJ665982  704   GTATCGAGACGTCCTACTGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGA  763
DQ531722  560   ............................................................  619
U81581    801   ...G........................................................  860

FJ665982  764   AAGTTAGAGGTTCGAAGGCGATCAGATACCGCCCTAGTTCTAACCGTAAACTATGCCTAC  823
DQ531722  620   ...................................................G........  679
U81581    861   ............................................................  920

FJ665982  824   TAGATGTTTG-A-TGGTA-AT-GTA-AAT-TA-TTA-TTGA-GCATCTTCTCGGAAACGA  874
DQ531722  680   .......A..-.-.A...-..-...-...-..-...-...T-..................  730
U81581    921   ...G...A..A.-.--..-T.A..--T..-A.-.A.-..T.T...C..............  971

FJ665982  875   AAGTCTTTCGGTTCCGGGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGGAATTGACGGA  934
DQ531722  731   ............................................................  790
U81581    972   ............................................................  1031

FJ665982  935   AGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTCACCC  994
DQ531722  791   ............................................................  850
U81581    1032  .....................................N......................  1091

FJ665982  995   GGGCCGGACA-TAAG-AAGGATTGACAGATTTAGAGCTCTTTCAAGATTTTA-TGGTTGG  1051
DQ531722  851   ..........-CG.T-......AA.........T........TT......CG-.......  907
U81581    1092  ..........C..T--...............G.T..........T.....-.G.......  1148

FJ665982  1052  TGGTGCATGGCCGTTCTTAGTTCGTGGATATGATTTGTCTGGTTGATTCCGATAACGAAC  1111
DQ531722  908   ............................................................  967
U81581    1149  ..........................................................G.  1208
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FJ665982  1112   GAGACTGTTAA-T-T-TAT-T---T---T-AC--AC-G-TGGGGG-ATTC-T-T--A-A-  1149
DQ531722  968    .......C...-.-A-...-.---.---.-.G--.T-A-.ACATT-...TA.-.--G-.-  1006
U81581    1209   ......T...--.G.-...-A---.---.-.A--.T---.ATATT-...T-.-.T-.-.-  1246

FJ665982  1150   -TCC---T--T-TA-ACGGTT--AATAA-ATGATTAGT-GA-TGTTTAA-TCACTTGAGA  1195
DQ531722  1007   -.AG---.--GA..T..--.--....T-.........-.GC.-....G-..........  1052
U81581    1247   -.AA---.A-.-..-.TT-..--.....C.-.....A.A.--.......C.-.T......  1292

FJ665982  1196   AAGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCTGGGGCTGCACGCGCGCTACAATG  1255
DQ531722  1053   .G..........................................................  1112
U81581    1293   G.....G.............A...............C.......................  1352

FJ665982  1256   TAGTGAGCA-CTATGTTCCTGATTC-GAGAGGATTGGGTAAACCTTGAAAGCATTACGTA  1313
DQ531722  1113   .........T.-.............-..................................  1170
U81581    1353   ......T..-T..........T..A-....TA.A.........A......A.........  1410

FJ665982  1314   ACTGGGAGTGTAACTTGCAATTATGTTACATGAACGAGGAATTCCAAGTAAACGTGAA-T  1372
DQ531722  1171   .......................................................C...-.  1229
U81581    1411   ..........A................T...........................-..G.  1469

FJ665982  1373   CATTAG-TTCACATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTG  1425
DQ531722  1230   ......-...G...........................................  1282
U81581    1470   ......C..-............................................  1522
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Figure 2: Multiple sequence alignment of the ITS rDNA region (including partial fragments of the 18S and 28S rRNA genes) of 3 nematode species. Code FJ665982 corresponds to the Slovenian isolate of *Alloionema appendiculatum*. Codes DQ531722 and U81581 refer to the Rhabditoid sp. and *Strongyloides ratti*, respectively.

4 CONCLUSIONS

For the nematode *Alloionema appendiculatum* it is acknowledge that it has a free-living and parasitic life cycle (Cabaret and Morand, 1990). In the parasitic part, the larvae of the third larval stage (L3) enter into the slug's body through its foot, where they moult and undergo a fourth larval stage (L4) and which stays encapsulated in foot muscles. The encysted larvae engorge (mechanism unknown) and then exit the slug and molt into immature adults (L5), which are free-living (Morand and Daguzan, 1986). Adult nematodes which undergo a series of molts in the parasitic life cycle are two to three times longer than those (from 1 to 2 mm) which grow in a free-living life cycle.

The nematode *A. appendiculatum* has hitherto been found in different species of slugs; *Deroceras* sp. (Schneider, 1859), *Arion ater* (Schuuramns – Stekhoven, 1950), *A. circumscriptus* (Mengert, 1953), *A. intermedius* (Cabaret *et al*., 1988), *A. silvaticus* (Morand and Bonnet, 1989), *A. subfuscus* (Cabaret and Morand, 1990), *Cantareus aspersus* (Charwat and Davies 1999), and *Prietocella barbara* (Morand, 2004). Research studies have not yet found a natural host in which the nematode *A. appendiculatum* undergoes a complete life cycle and which causes it to have a higher mortality rate (Morand *et al*., 2004). Morand and Daguzan (1986) report that the nematode *A. appendiculatum* does not appear very frequently in natural slug populations, meanwhile its presence in snails which have been bred is more common (Cabaret *et al*., 1988).

A research study which was conducted by Cabaret and Morand (1990) indicates that a positive interaction exists between the nematodes *Muellerius capillaris* (Strongylida: Strongyloididae) and *A. appendiculatum*, as with the species *M. capillaris* previously infected slugs attain a higher mortality rate when infected with *A. appendiculatum* afterwards. The application of *A. appendiculatum* in practice might be carried out in soils where a larger number of nematodes from the family Strongyloididae is present and for which it is known that slugs are only intermediate hosts (Grewal *et al*., 2003). But for now, no such co-infection has been discovered (Morand *et al*., 2004).

This is the first large-scale record of the mentioned nematode in slugs from the genus *Arion*, among which *Arion lusitanicus* Mabille predominated in terms of the number of individuals. Younger slugs of the mentioned species showed a satisfactory mortality rate already on the fourth day of the nematode suspension application in Petri dishes. But because these results are merely preliminary, we can not yet confirm for certain that the nematode *A. appendiculatum* is capable of developing a complete life cycle inside the slug *A. lusitanicus*, although this is common for *Phasmarhabditis hermaphrodita* (Wilson *et al*., 1993a). The presence of *P. hermaphrodita* has not yet been confirmed in Slovenia, thus only chemical plant protection products with limacide activity can be used to control slugs and snails (Ozimič *et al*., 2007).

Due to the fact that the Regulations on Biological Plant Protection prohibit the introduction of exotic organisms into the natural environment, we will continue our research on the presence of slug-parasitic nematodes. Possible future identification of the nematode *P. hermaphrodita* could entail an alternative to chemical limacides, as due to the milder winters and humid summers, slugs and snails are increasingly important pests on cultivated plants in Slovenia as well as in many other countries (Kozowski *et al*., 2006; Grubišić *et al*., 2007).

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