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First report of nematodes *Parasitylenchus bifurcatus* Poinar & Steenberg, 2012 parasitizing multicolored Asian lady beetle *Harmonia axyridis* (Pallas, 1773) in Slovenia

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ABSTRACT

In years 2015 and 2016 around 200 specimens of ladybird species *Harmonia axyridis* were collected in Slovenia. Ladybirds were dissected and checked under stereomicroscope for presence of nematodes. Nematodes were discovered in samples of ladybirds *H. axyridis* collected at Brdo pri Lukovici and Trboje. The incidence of parasitized ladybirds was around 30 %. Nematodes were analysed morphometrically and the characteristic features of *Parasitylenchus bifurcatus* nematodes were observed: straight stylet lacking basal thickenings, a bursa and a forked tail tip in the vermiform females and juvenile males. The identity was confirmed with 18S rDNA region sequence (acc. no. LT629306 and LT629307) which showed high similarity (>99.9 % nucleotide identity) to the *P. bifurcatus* sequences in the public domain. This is a first report of *P. bifurcatus* nematode species found parasitizing ladybird *Harmonia axyridis* in Slovenia. The species compromises fecundity of its host and has therefore a potential to be used as a biological control agent to control high abundance of invasive *H. axyridis* ladybirds.

Key words: parasitic nematodes; ladybirds beetles; *Parasitylenchus bifurcatus; Harmonia axyridis*; harlequin ladybird; biological control

IZVLEČEK

PRVA NAJDBA OGORČIC *PARASITYLENCHUS BIFURCATUS* POINAR & STEENBERG, 2012, PARAZITOV PISANE POLONICE *HARMONIA AXYRIDIS* (PALLAS, 1773) V SLOVENIJI

V letih 2015 in 2016 smo v Sloveniji nabrali okrog 200 osebkov pisane polonice *Harmonia axyridis.* Pikapolonice smo secirali in pod lupo preverili prisotnost parazitskih ogorčic. Ogorčice so bile prisotne v pikapolonicah nabranih na Brdu pri Lukovici in v Trbojah. Stopnja parazitiranih pikapolonic je bila okrog 30 %. Morfometrična analiza je razkrila karakteristične znake ogorčic vrste *Parasitylenchus bifurcatus*: ravno bodalo brez zadebelitev pri osnovi, burso in razcepljeno konico repa pri črvastih samicah in ličinkah. Identiteto najdenih ogorčic smo potrdili z določitvijo nukleotidnega zaporedja odseka 18S rDNA (št. v javni bazi: LT629306 in LT629307), ki kaže veliko podobnost z zaporedji vrste *P. bifurcatus* dostopnimi v javnih bazah (več kot 99,9 odstotna enakost). Gre za prvo najdbo vrste *P. bifurcatus,* parazita pisane polonice *Harmonia axyridis,* v Sloveniji. Ogorčice vrste *P. bifurcatus* zmanjšajo rodnost svojega gostitelja in so kot take potencialni biotični agens za nadzor invazivne vrste pikapolonice *H. axyridis*.

Ključne besede: parazitske ogorčice; *Parasitylenchus bifurcatus*; pikapolonice; pisana polonica; *Harmonia axyridis*; biotično varstvo

1 INTRODUCTION

The multicolored Asian lady beetle, *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae) originating from Asia has been applied as a biocontrol agent to control aphids and other harmful insects in orchards, vineyards, greenhouses, crop fields, and gardens (Koch 2003, Poinar and Steenberg 2012). It has been introduced to North America several times in the beginning of 20th century, to Eastern Europe in 1960s and become commercially available as a biological control agent in 1980s in the Western Europe. While its' ravenous appetite resulted in efficient biological control of harmful organisms, no one predicted that

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establishment of this introduced species in the natural environment will result in rapid spread and build-up of large populations endangering biodiversity of native insects as it feeds on numerous species of insects. It is also a fruit pest since it feeds and aggregates on apples, pears and grapes. It endangers wine production as wine produced from grape clusters containing adult beetles has an unpleasant flavour and odour. Further, it is a nuisance for humans since they try to overwinter in homes, release haemolymph from their legs when agitated (reflex bleed) leading to unpleasant odours and stains, and even occasionally bites humans and cause allergic reactions (Kovach, 2004).

Because of the above mentioned reasons the European and Mediterranean Plant Protection Organization (EPPO) has removed *Harmonia axyridis* from the list of recommended biological control agents in 2009 (EPPO, 2016). Multicolored Asian lady beetle has rapidly spread even in the countries where it has not been purposely introduced (e.g. Austria, Denmark, Great Britain) (Brown et al., 2008). The first observation of *H. axyridis* in natural environment in Slovenia dates back to 2007 (Polak, 2013). There is no known evidence that *H. axyridis* was used for biological control purposes in Slovenia. Its spread in Slovenia may result from (illegally) imported specimens escaped from greenhouses or gardens or it has spread naturally from the neighbouring countries, possibly from Austria. Since then, multicolored Asian lady beetle has become widely spread in Slovenia (Laznik et al., 2012, Polak, 2013).

The multicolored Asian lady beetle has a great reproductive and expansion potential (Polak, 2013). Furthermore, the successful invasion of this species can be attributed to extreme resistance of this species to diseases and parasites that attack other ladybirds. In Slovenia chemical control of multicolored Asian lady beetle is limited as there is no registered pesticide for controlling this species in agricultural production. However biocides can be used for the species control in household environments which are not intended for crop production. Therefore researchers have allocated their efforts in the search of natural enemies of multicolored Asian lady beetle that could reduce their numbers (Raak-van den Berg et al., 2014). Several natural enemies attacking adult multicolored Asian lady beetle have been reported, among them the nematode *Parasitylenchus bifurcatus* Poinar and Steenberg, 2012 (Tylenchida, Hexatylina: Iontonchioidea, Parasitylenchidae), entomopathogenic fungus *Hesperomyces virescens* Thaxt. (Ascomycota: Laboulbeniomycetes: Laboulbeniales), ectoparasitic mite *Coccipolipus hippodamiae* (McDaniel and Morrill, 1969) (Acarina: Podapolipidae), insect parasitoid *Dinocampus coccinellae* (Schrank, 1802) (Hymenoptera: Braconidae) and bacteria of the genus *Spiroplasma* (Majerus et al., 1999, Raak-van den Berg et al., 2014, Haelewaters et al., 2017).

Adult multicolored Asian lady beetles have been found parasitized by nematodes in Denmark in 2009 and the parasitic nematode was subsequently described as a new nematode species *Parasitylenchus bifurcatus* (Harding et al., 2011, Poinar and Steenberg, 2012). Diagnostic morphological characters of *Parasitylenchus bifurcatus* are a straight stylet lacking basal thickenings, an excretory pore opening at the level of or somewhat posterior to the nerve ring and a gubernaculum, a narrow bursa in the males and a forked tail tip in the vermiform (infective) females and juvenile males. The characteristic cleft tail tip giving rise also to the name of this new species separates it clearly from a similar species *Parasitylenchus coccinellinae* Iperti and van Waerebeke. Molecular marker often used as a character for nematode identification, the sequence of nearly full length SSU rDNA is available in public domain (Raak-van den Berg et al., 2014).

Different developmental stages of *P. bifurcatus*, first generation parasitic females, subsequent generation parasitic females, vermiform (infective) females, males and juvenile nematodes, occurred together in the body cavity of both female and male *H. axyridis* (Harding et al., 2011, Poinar and Steenberg, 2012, Raak-van den Berg et al., 2014). The prevalence of infected adult ladybirds collected in nature ranged from 2 - 47 % and increased up to 60 % when field-collected ladybirds were incubated in the laboratory for 30 days (Poinar and Steenberg, 2012, Haelewaters et al., 2017). These high rates of parasitism imply that *P. bifurcatus* is a potential biological control agent of *H. axyridis* as parasitism results in depletion of the fat body and partial or complete atrophy of the reproductive organs of the host. Further, *P. bifurcatus* parasitism has been shown as significant mortality factor of *H. axyridis* in the laboratory conditions (Poinar and Steenberg, 2012) and strong association between female failure to reproduce and infection with *P. bifurcatus* has been documented (Raak-van den Berg et al., 2014). Interestingly, *P. bifurcatus* parasitizes only adult host, while larvae and pupae are not included in its life cycle. With several subsequent generations of nematodes within the host, the numbers of nematodes can reach several hundred or even thousand juveniles. It is not documented how *P. bifurcatus* finds and enters uninfected adult beetles, but it is proposed that infective vermiform females with thick cuticle leave infected ladybirds while these are aggregated and enter other uninfected ones (Poinar and Steenberg, 2012).

The origin of *P. bifurcatus* infection in *H. axyridis* is unknown. One possibility is that it could have arrived with *H. axyridis* from Asia or North America, while the other possibility is that infection was acquired from endemic European ladybirds (Poinar and Steenberg, 2012, Raak-van den Berg et al., 2014).

So far, *P. bifurcatus* infecting *H. axyridis* has been documented from Denmark (Poinar and Steenberg, 2012), the Netherlands (Raak-van den Berg et al., 2014), the Czech Republic and Poland (Haelewaters et al., 2017), Kentucky and West Virginia in USA (Tove Steenberg, personal communication, May 28, 2015).

The objective of this study was to examine *H. axyridis* in Slovenia and to check whether it is parasitized by nematodes.

2 MATERIALS AND METHODS

2.1 Samples

In years 2015 and 2016 around 200 samples of adult ladybird species *Harmonia axyridis* from six different locations in Slovenia were collected and identified using morphological keys and species descriptions (Reitter, 1911; Freude et al., 1967; Chapin and Brou 1991; Riedel and Bastian, 2005; Polak, 2013). Ladybirds were dissected one to four days after sampling and individual nematodes were collected under the binocular in 0.9 % NaCl solution. The nematodes for morphometrical analysis were heat killed (at 65 ˚C), fixed in 4 % formalin and transferred on slides for further examination and measurements. Nematodes for molecular analysis were placed into 1 µl of dH_20 in 1.5 ml tubes and stored at -20 ^oC until isolation of DNA.

2.2 Morphomerics

Morphometric analysis encompassing measurements of common nematode body features was performed on 10 fixed nematode specimens of each nematode life stage using Nikon TiE microscope.

2.3 Molecular identification

2.3.1 DNA extraction

DNA was extracted from twenty nematodes. The Promega Genomic DNA Wizard purification kit (Madison, WI, USA) was used after a slight modification of the manufacturer's instructions. Extracted DNA was diluted in 20 µl of dH_20.

2.3.2 PCR amplification

For the amplification of 18S rDNA fragment, two set of primers, 1096F and 1912R, and 1813F and 2646R described by Holterman et al. (2006) were used. Both primers sets give overlapping sequences and together produce an 18 rDNA gene sequence of approximately 1.600 bp. PCR reactions contained 1 µl of isolated DNA, 1x GoTaq buffer (Promega), 1.5 mM MgCl2, 2.5 mM of each of the dNTPs, 1 µM of each of the primers, 1 U GoTaq Flexi DNA Polymerase (Promega) and distilled water up to 25 µl. The amplification was carried out in a thermal cycler Veriti (Applied Biosystems) using the amplification program as described by Holterman et al. (2006). Electrophoresis was performed on a 1 % TBE agarose gel to detect and inspect the amplified DNA product.

2.3.3 Cloning

PCR products were cloned using a pGEM®-T Easy vector kit (Promega) and transformed into competent cells of *E. coli* JM109 (Promega) according to the manufacturer's instructions. White colonies were selected in the blue/white colour screening for further analysis.

2.3.4 Isolation of plasmid DNA and sequencing

Selected clones were grown in 5 ml of LB medium with ampicillin (150 µg/ml) incubated overnight at 37 °C on a rotation shaker at 300 rpm. Overnight cultures were centrifuged at 2.700 rcf for 10 min, and the pellet used for isolation of plasmid DNA by GeneJet Plasmid Miniprep (Thermo Scientific) according to the manufacturer's instructions. The isolated plasmids were sequenced by Macrogen Inc. (Korea) using universal primers SP6 and T7.

2.3.5 Sequence analysis

DNA sequences were assembled using the computer software BioEdit v. 7.0.5.2 (Hall 1999). NCBI blastn suite was used to determine sequence similarity to other sequences in the public domain.

3 RESULTS AND DISCUSSION

In year 2015 none of the dissected ladybirds' specimens exhibited nematode parasites. In 2016 parasitic nematodes were recovered from *H. axyridis* collected at two locations, Brdo pri Lukovici and Trboje. The incidence of parasitized ladybirds from the samples was around 30%, which is in accordance with previous studies finding up to 35 % incidence in Denmark (Poinar and Steenberg, 2012). Even higher incidence up to 47 % was reported from the Czech Republic, (Haelewaters et al., 2017), while lower incidence was reported for the Netherlands (Raak-van den Berg et al., 2014).

Three different nematode life/developmental stages within the individual host were found, including subsequent generation parasitic females, vermiform (infective) females and males (Figure 1). First generation parasitic females were not encountered in analysed samples. Numbers of nematodes found in ladybirds varied, but up to several hundreds of nematodes could be found in some specimens. Contemporaneity of different developmental stages within a host and large nematode populations within a single beetle have been observed before (Poinar and Steenberg, 2012).

Common nematode body features were measured in morphometric analysis. The nematodes extracted from ladybirds were identified as *P. bifurcatus* (Table 1, Figure 1). The characteristic observed features of *P. bifurcatus* nematodes were straight stylet lacking basal thickenings, a narrow bursa and a forked tail tip in the vermiform females and juvenile males. Subsequent generation parasitic females from Trboje had smaller body length of 886.4 µm (782.0-1098.0) and body width of 72.3 µm (59.0-81.0) compared to 1300 µm (920 - 1600) and 195 µm (158-271) of *P. bifurcatus* nematodes from Denmark, respectively. All the other morphometrical characters were in the range of *P. bifurcatus* species description (Poinar and Steenberg, 2012). It can therefore be concluded that *P. bifurcatus* species morphometric characters of subsequent generation parasitic females may have somewhat higher variability than previously reported.

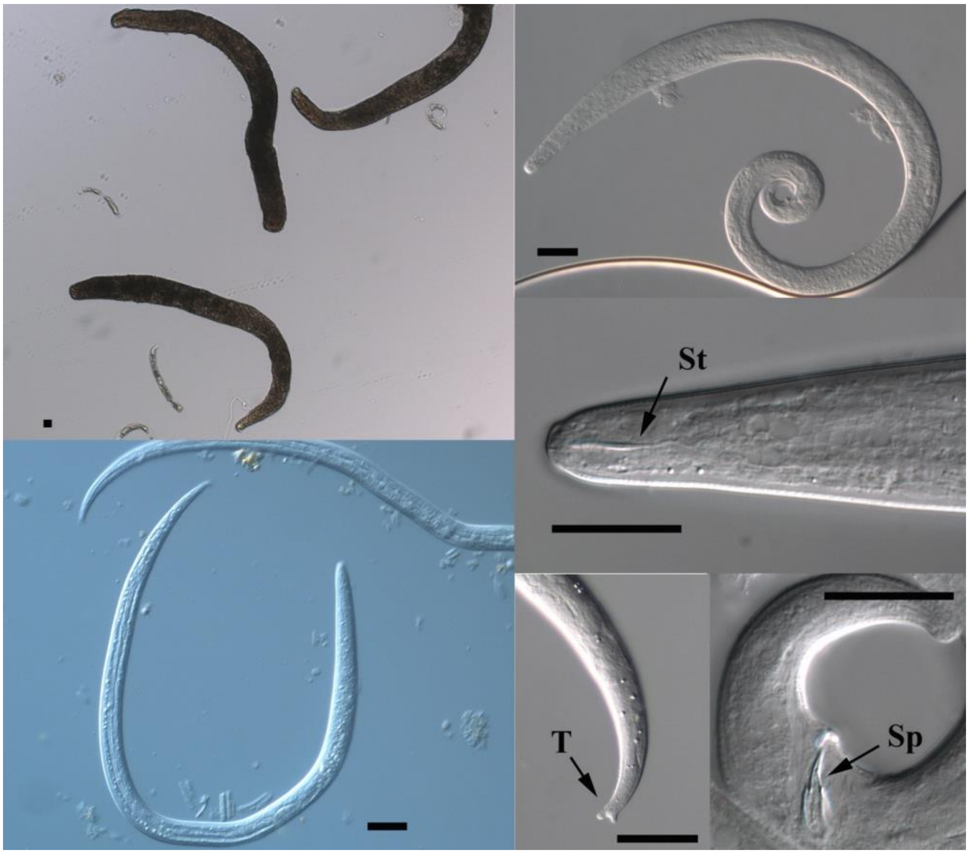

Figure 1: Different developmental stages of *Parasitylenchus bifurcatus* nematodes, parasites of *Harmonia axyridis* ladybirds. Arrows indicating straight stylet lacking basal thickenings (St), spicule (Sp) and a forked tail tip (T). Scale bars = 20µm

Table 1: Morphometric characters of subsequent generation parasitic females, vermiform (infective) females and males of *P. bifurcatus* nematodes isolated from *H. axyridis* from Trboje. All measurements are in µm, presented as mean ± standard deviation, with the range in parentheses

Character	Subsequent generation parasitic females (n = 10)	Vermiform (infective) females (n = 10)	Males (n=10)
Body length	886.4 ± 124.2 (782.0-1098.0)	646.9 ± 60.5 (573.0-752.0)	605.6 ± 58.8 (530.0-689.0)
Body width	72.3 ± 8.1 (59.0-81.0)	16.8 ± 1.6 (14.4-18.6)	21.2 ± 3.3 (17.9-25.8)
Stylet length	13.3 ± 0.5 (12.7-13.9)	12.6 ± 1.4 (10.6-14.2)	9.4 ± 0.3 (9.1-9.7)
Tail length	32.3 ± 1.3 (30.9-34.0)	37.8 ± 2.9 (35.2-41.0)	38.3 ± 4.6 (33.0-44.2)
Tail width	37.1 ± 3.3 (34.2-41.0)	10.7 ± 1.1 (9.5-11.8)	15.7 ± 1.6 (13.7-17.6)
Distance from head to excretory pore	219.3 ± 13.0 (206.5-234.0)	108.5 ± 3.5 (101.5-114.0)	103.6 ± 9.5 (90.9-117.0)
Vulva position %	93.9 ± 0.8 (93.0-94.8)	87.5 ± 0.8 (86.2-88.0)	-
Spicule length	-	-	11.8 ± 0.5 (11.3-12.2)
Spicule width at base	-	-	3.7 ± 0.3 (3.4-4.0)
Gubernaculum length	-	-	4.1 ± 0.4 (3.8-4.8)
Bursa length	-	-	8.6 ± 0.9 (7.3-9.2)

Two sequences were determined from nematodes extracted from the sample of *Harmonia axyridis* from Brdo, Slovenia. Two determined sequences of 18S rDNA region are 1.623 bp long and differ among them in 1 bp position. Both determined sequences belong to *P. bifurcatus* based on a high similarity to the *P. bifurcatus* sequences in the public domain determined with NCBI BlastN tool (99.94 % identity to *P. bifurcatus* sequence with acc. no. KC875397.1). Sequences obtained in this study were deposited at the European Nucleotide Archive, EMBL Nucleotide Sequence Database with the accession numbers LT629306 and LT629307. While no sequences are available for the species *P. coccinellinae* Iperti and Waerebeke to assess the phylogenetic relationship of these two related species, the bifurcated tail has not been reported for *P. coccinellinae* (Iperti and van Waerebeke, 1968, Poinar and Steenberg, 2012). Therefore, based on morphological and molecular characters this is a first report of nematodes *P. bifurcatus* parasitizing multicolored Asian lady beetles *H. axyridis* in Slovenia.

4 CONCLUSIONS

Multicolored Asian lady beetles collected in Slovenia in 2016 were found to be parasitized by nematode *P. bifurcatus.* Identification was confirmed with both, morphological and molecular characters.

Nematode species *P. bifurcatus* compromises fecundity of its host and has therefore a potential to be used as a biological control agent to control high abundance of invasive ladybirds of *H. axyridis* species. However, future studies on biology, possible transmission and effect of this parasitic nematode for native species of ladybirds are needed before any practical application. It is critical that the introduced biological control agent does not become pest themselves as has happened in the past with the intentional introduction of *H. axyridis* into North America and Europe. To our knowlede *P. bifurcatus* is at the moment not used anywhere to manage *H. axyridis*, but studies needed for justification and approval of such use are in progress (T. Steenberg, personal communication, December 1, 2015).

In EU there is no unified federal legislation regulating use of biological control agents (BCA) (Loomans, 2015). Some EU member states have national regulations, which are based on international standards, but are implemented in different ways (Hunt et al., 2011 cited in Loomans, 2015). Introduction of exotic BCA is covered in the following international acts: the International Plant Protection Convention, the Plant Protection Product Acts and the Convention on Biodiversity (Loomans, 2015). In Slovenia the use of biological control agent is regulated with the Plant Health Act (Zakon o zdravstvenem varstvu rastlin, Uradni list RS št. 62/07- uradno prečiščeno besedilo, 36/10 in 40/14 - ZIN-B). Detailed procedures for introduction, cultivation, trade and use of invertebrate BCA are subject to the Rules on biological control of plant pests (Pravilnik o biotičnem varstvu rastlin, Uradni list RS, št. 45/06, 28.4.2006). BCA are classified in two lists: the List of indigenous and the List of non-indigenous species of invertebrate organisms for biological control. The lists are maintained by the Administration based on the EPPO list (PM6/3) and on the basis of the results of researches, professional or scientific articles. Provisions of these regulations do not apply for the introduction and use of microorganisms for biological control. They are regulated by legislation in the field of plant protection products and are subject to different risk assessment in the registration procedure. Microorganisms are regulated by EU Regulation (EC) No. 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market or equivalent regulations. *P. bifurcatus* is not included on the lists of approved BCA in Slovenia. Further studies on safety and efficacy would be needed to include *P. bifurcatus* on this list.

Use of parasitic nematodes in plant protection is an accepted practice. Entomopathogenic nematodes are widely used as biocontrol agents to control several harmuful insect species in integrated pest management programs and slug-parasitic nematodes are used to control certain slugs and snails. Both groups are available as comercial biocontrol products and have been used for decades. Further, there are several studies exploring potential of predatory nematodes to control plant parasitic nematodes and potential of fungal-feeding nematodes for the control of soilborne plant pathogens (Grewal et al., 2005).

Although *P. bifurcatus* is a parasite of an insect species it is not an entomopathogenic nematode. Entomopathogenic nematodes kill their hosts relatively quickly (typically within 24–48 h of infection) with the help of their associated bacteria. On the other hand, *P. bifurcatus* is a typical parasite which can co-exist with its host for an extended time without killing it, but it does cause depletion of fat body as well as partial or complete atrophy of the insect’s reproductive organs (Poinar and Steenberg, 2012, Haelewaters et al., 2017). As discussed above, *P. bifurcatus* may have potential as a biocontrol agent of *H. axyridis*.

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