Systematics and Phylogenetics
Research Article

ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., 67 (3), 2015: 345-350

Morphological and Molecular Study of *Laimaphelenchus penardi* (Steiner, 1914) Filipjev & Schuurmans Stekhoven, 1941 (Nematoda: Aphelenchoididae) from Iran

Kourosh Azizi, Ali Eskandari*, Ramezan Asghari

Department of Plant Protection, Faculty of Agriculture, University of Zanjan, 45371-38791, Zanjan, Iran;
E-mail: eskandari.a@znu.ac.ir

Abstract: During a nematode survey on bark and wood collected from oak trees from Western Iran, Lorestan Province, Zagros Mountains; one species of the genus *Laimaphelenchus* was isolated, described and illustrated herein as *L. penardi.* It is characterised by ventrally arcuate body; cephalic region set off with six labial sectors of equal width; two incisures in lateral field; vulva having a flap, post-uterine sac extending for about half the vulva-anus distance (PUS=99-139 μm), vagina having a thin cuticular wall; tail conoid with a single stalk-like terminus and four pedunculate tubercles. Phylogenetic analysis, using sequences of the D2/D3 expansion segment of 28S rRNA, placed *L. penardi* in a well-supported monophyletic clade with *L. persicus*, *L. preissii* and *Aphelenchoides* sp., and suggested that *L. penardi* and *L. persicus* are the most closely related species.

Keywords. Aphelenchoididae, 28S rRNA, *Quercus* spp., first record

Introduction

The genus *Laimaphelenchus* Fuchs, 1937 includes 15 valid species (Asghari *et al*. 2012). These species are not pathogenic and are apparently feeding on fungi, moss, algae and lichen; they are associated with conifers and are found also in tunnels of wood boring insects (Hunt 1993). Records of *Laimaphelenchus* spp.in Iran are few. Only two species, i.e. and *L. persicus* Asghari, Pourjam, Heydari & Zhao, 2012 and *L. deconincki* Elmiligy & Geraert, 1972, were described and reported from the bark of *Pinus sylvestris* L. and *Taxus baccata* L. trees, respectively, from the Caspian region from Iran (Asghari *et al.* 2012, asghari & Eskandari 2014). The aim of this study was to provide additional data on molecular and morphological characteristics of *L. penardi* from Western Iran.

Material and Methods

***Nematode materials*.** Samples were collected in October 2013 from the Lorestan province of Iran from bark and dead wood or weakened *Quercus* spp., having many bark beetle galleries. Using a hatchet, bark and wood samples were cut into small pieces (no more than 2 cm wide), for extraction. Nematodes were extracted from pieces using the tray method (Whitehead & Heming 1965) over two days at 25°C. The recovered specimens were collected using 400 mesh (37 μm aperture) sieves, fixed by adding FGA 4:1:1 (formaldehyde, glycerine, acetic acid) and then processed to anhydrous glycerine using De Grisse's (1969) method. Morphological studies were made on both living and fixed material using a light microscope (Olympus BH2). Measurements and photomicrographs were done using a *DinoCapture camera;*

*d*rawings were made using a drawing tube attached to the microscope and were redrawn using Adobe® Photoshop 7.0 ® ME software.

For molecular analysis, a single nematode specimen was hand-picked and transferred into distilled water. Its identity was confirmed using a light microscope. Then specimen was put in 50 µl of AE buffer (10mM Tris-Cl, 0.5mM EDTA; pH9.0) and crushed into multiple pieces on a microscope slide with a pipette tip. DNA samples were stored at -20°C until used as a PCR template. Primers used for amplification of D2/D3 expansion segment of 28S rRNA were: D2A (5'–ACAAGTACCGTGAGGGAAAGTTG–3') as forward and D3B (5'–TCGGAAGGAACCAGCTACTA–3') as reverse primer (Nunn 1992). PCR amplification was performed in a final volume of 25 µl containing 1 µl MgCl2, 1 µl dNTPs, 3 µl of each primer (10 pmol µl-1), 2.5 µl of 10× buffer (100 mM Tris-HCl, 500 mM KCl, pH8.4), 0.4 U Taq DNA polymerase (CinnaGen, Tehran, Iran), 3 µl of template DNA and 10.1 µl ddH2O. The cycling program consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing for 45 s at 55°C, and extension at 72°C for 2 min, with a final elongation step for 10 min at 72°C (Ye *et al*. 2007). After the completion of PCR, the products were separated electrophoretically using a 1% agarose gel cast in TEB buffer, stained with Ethidium Bromide and photographed under UV light.

Twenty three LSU published sequences from GenBank were included in our phylogenetic analysis. Nematode species and GenBank accession numbers are listed for each taxon in the phylogenetic trees, if known. DNA sequences were aligned in ClustalX implemented in MEGA version 5.0 (Tamura *et al.* 2011) using default parameter values and jModeltest v.0.1.1 (Posada 2008) to select the best AIC model. Bayesian analyses were conducted in MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003) according to the evolutionary model and parameters suggested by jModeltest. Four MCMC chains for 10^6 generations under the best-fit model GTR + G were run. We started analysis from a random topology and used a temperature of 0.2 a burning of 10% of trees and a thinning interval of 1,000. Markov Chain Monte Carlo methods were used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees using the 50% majority rule. The trees were rooted using sequences of *Aphelenchus avenae*.

Laimaphelenchus penardi (Steiner, 1914) Filipjev & Schuurmans Stekhoven, 1941

(Figs 1, 2)

Measurements. See Table 1.

Description. Female: Body ventrally arcuate, or in spiral shape when killed using heat. Body annules 1-1.5 µm wide at mid-body. Two (probably three ?) incisures visible in lateral field, the third incisure not easily observed under light microscope. Cephalic region rounded, offset, wider than body at base, 3-4 µm high and 6.5-7 µm wide, with six labial sectors of equal width, separated by pairs of well-marked ribs (Figs. 1B,C and 2G,H.); cephalic framework poorly developed. Stylet slender with basal swellings. Pharynx with a typical aphelenchoid bulb, rounded to oval, 16-20 µm long, 12.5-14.5 µm wide with crescentic valves in the middle, located 60-69 µm from the anterior end. Hemizonid not seen. Nerve ring anterior to excretory pore and posterior to the oesophago-intestinal junction, at 84-94 µm from the head end. Excretory pore conspicuous, at 93-106 µm from the anterior end. Pharyngeal glands overlapping the intestine on dorsal side, extending for 75-124 µm. Reproductive

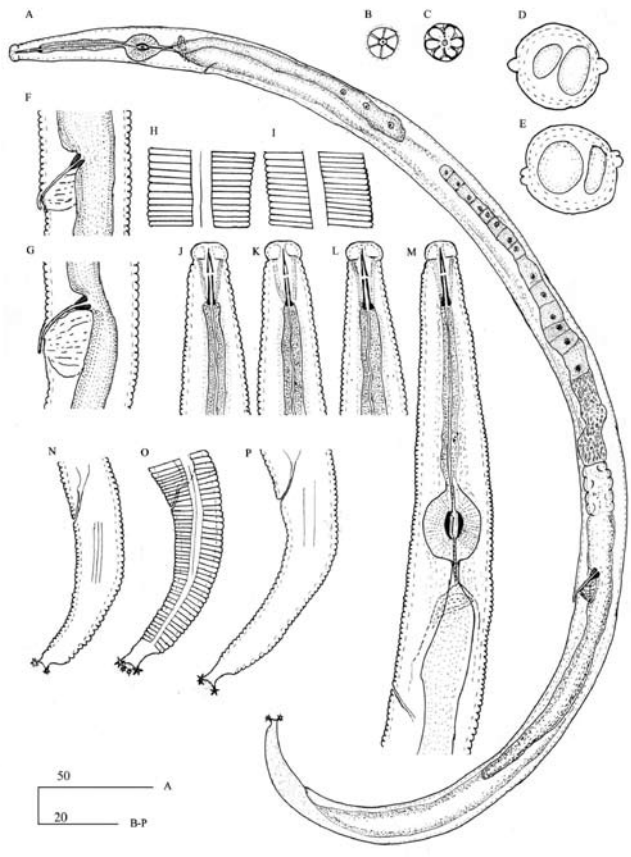

Fig. 1. Line drawings of *Laimaphelenchus penardi* females from Iran. (A) General view of body; (B, C) *En-face* view of lips showing six equal-sized lips and ribs; (D, E) Cross section of mid-body; (F, G) Vulva region; (H, I) lateral field; (J-L) Head and stylet; (M) Pharyngeal region; (N-P) Tail variation

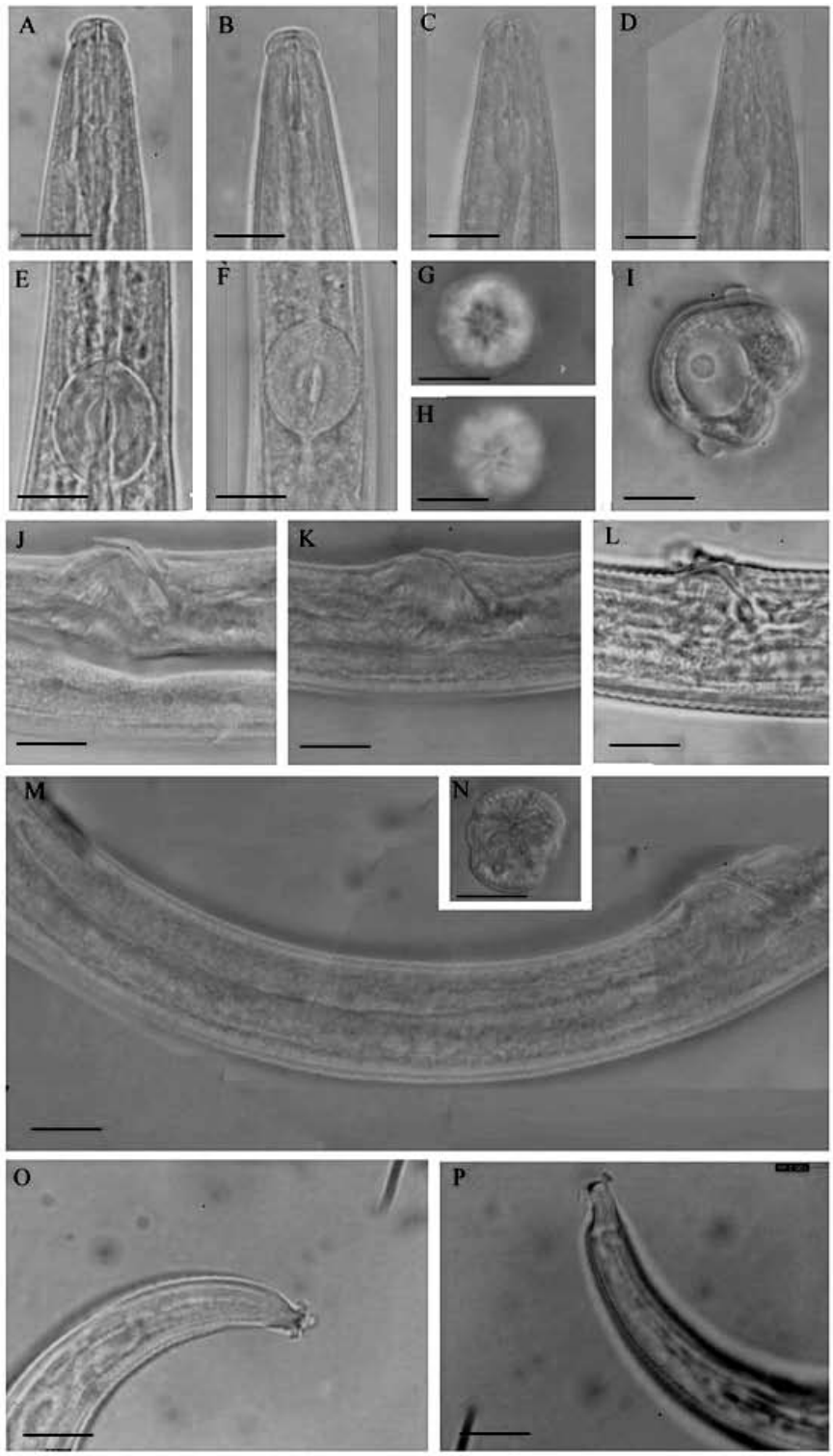

Fig. 2. Photomicrographs of *Laimaphelenchus penardi* from Iran. (A-D) Head and stylet; (E, F) Median bulb; (G,H) *En-face* view of lip region; (I, N) Cross section of mid-body; (J-L) Variation of vaginal sclerotisation; (M) Post-uterine sac; (O, P) Tails and tubercles. Scale bars = 10 µm

system mono-prodelphic, with outstretched ovary and long post-uterine sac, the latter occupying 41-60% of distance from vulva to anus (Figs. 1A and 2M); oocytes in one row, spermatheca empty. Anterior vulval lip well developed, overlapping the vulval slit, of equal thickness over almost all of its length. Refringent muscular bundles, surrounding the vagina thin (Figs. 1F,H and 2J-L). Tail ventrally curved, conoid with a single stalk-like terminus and with four pedunculate tubercles.

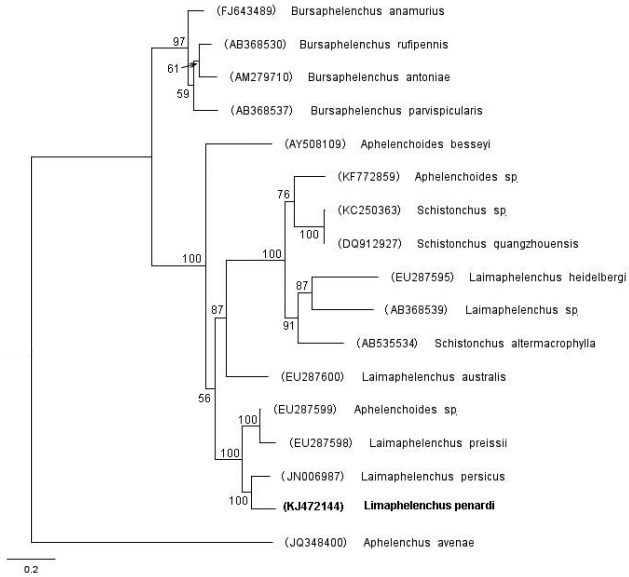

Fig. 3. Bayesian tree inferred from LSU gene DNA sequences. Posterior probabilities exceeding 50% are given on appropriate clades. Nematode species and GenBank numbers are listed for each taxon

Male: Not found

Diagnosis of Iranian population. Females of *L. penardi* were characterised by a set off lip region; lateral fields with two or three incisures; anterior vulval lip well developed, overlying the vulvar slit; vagina having a thin cuticular tube; long post-uterine sac, occupying one half of distance from vulva to anus and tail with an offset terminus, having four clearly pedunculate tubercles.

Locality and Habitat. Zagros Mountains, Lorestan Province, Iran; bark and beetle galleries of dead or weakened oak trees *Quercus* spp.

Remarks. *Laimaphelenchus penardi* has been originally described by Steiner (1914) as *Aphelenchus* Bastian, 1865. The Iranian specimens agree well for most of the morphometrics and morphological aspects with the original description. We found small differences in body length (695-870 *vs* 570 µm), c' value (3-3.7 *vs* 2.5-3), absence of male and longer tails (34-46 *vs* 28-35 µm) in comparison with the French population described by Baujard

Table 1. Morphometrics of the females of *Laimaphelenchus penardi* collected from oak tree, Lorestan Province, Iran and its comparison with other populations (all measurements are in µm)

Characters	Iranian population	Steiner (1914)	Massey (1974)	Baujard (1981)
n	10	-	-	24
L	758 ± 60 (695-870)	570	700-800	720 (640-810)
a	33.3 ± 3.0 (29.3-37.8)	30	30	34 (29-44)
b	9.9 ± 0.8 (9.0-11.4)	10.6	8.8-9.2	10 (9-11)
b'	4.2 ± 0.5 (3.7-5.0)	-	-	-
c	18.4 ± 2.1 (16.0-21.3)	20	18.4-20	22 (19-26)
c'	3.4 ± 0.3 (3.0-3.7)	-	-	2.7 (2.5-3)
V	65.9 ± 1.2 (64.7-67.4)	66	68-70	66 (63-68)
Stylet	13.6 ± 0.4 (13.0-14.0)	-	14-15	13 (13-14)
Conus	5.9 ± 0.5 (5.0-6.5)	-	-	-
m (conus/stylet %)	43.0 ± 2.0 (41.4-45.8)	-	-	-
Median bulb	64.2 ± 3.2 (60.0-69.0)	-	-	61 (55-68)
Head cardia	76.6 ± 3.9 (70.0-80.5)	-	-	-
Oesophageal glands end	184.9 ± 20.0 (153.0-207.0)	-	-	-
Overlaping	102.4 ± 18.7 (75.0-124.0)	-	-	112 (92-132)
MB	83.9 ± 3.5 (77.8-87.5)	-	-	-
Excretory pore	99.6 ± 4.1 (93.5-106.0)	-	-	99 (88-106)
Nerve ring	87.9 ± 4.5 (84.0-94.0)	-	-	-
Head-vulva	499.3 ± 40.2 (450.0-573.0)	-	-	-
Head-anus	716.7 ± 59.8 (653.3-827.9)	-	-	-
Vulva-anus	217.4 ± 22.2 (194.0-254.9)	-	-	204 (179-243)
Tail length	41.5 ± 4.0 (34.0-46.0)	-	-	32 (28-35)
Body width	22.8 ± 1.9 (20.8-26.0)	-	-	-
Vulva body width	22.8 ± 1.7 (20.8-25.0)	-	-	-
Anal body width	12.3 ± 0.8 (11.3-13.5)	-	-	-
Pus	114.1 ± 13.6 (99.0-138.6)	-	-	126 (72-173)
Pus/Bw	5.0 ± 0.7 (4.1-5.8)	-	-	6 (3.6-8.6)
pus/L%	15.1 ± 1.3 (13.0-16.5)	-	-	-
pus/v-a%	52.7 ± 5.8 (44.1-60.3)	-	-	60 (34-77)

(1981). Further they show differences in V value (64.7-67.4 *vs* 68-70 μm), and absence of male in comparison with population described by Massey (1974).

Data on the precise number of incisures in the lateral field of this species are controversial. Massey (1974) noted the presence of two incisures, while Baujard (1981) examined paratypes and observed three incisures, with the inner one barely visible by light microscopy. We observed a pattern similar to Baujard's (1981) observations. was

Iranian individuals of *L. penardi* can be easily distinguished from two other species reported from Iran *(L. persicus* and *L. deconincki*). F:rom *L. persicus*, they can be differentiated by having a longer stylet (13-14.2 *vs* 10-11.5 μm), higher b value (9-11.4 *vs* 6.1-7.4), number of incisures in lateral field (2 or 3 *vs* 4) and shape of the vaginal sclerotisation (thin *vs* thick) (Asghari *et al*. 2012). From *L. deconincki*, they can be distinguished by longer post-uterine sac (99-138 *vs* 22-50 μm) and shape of the cuticle surrounding the vagina (thin *vs* thick) (Elmiligy & Geraert 1972; asghari & Eskandari 2014).

This is the first record of *L. penardi* from Iran.

DNA Characterisation

Alignment of the D2-D3 of 28S rRNA gene contained 23 sequences and was 769 bp in length. As inferred from the topology of the majority rule 50 consensus Bayesian tree (Fig. 3) using *Aphelenchus avenae* as the outgroup, suggested that: 1) the genus *Laimaphelenchus* was in two separate clades, close to *Aphelenchoides* and *Schistonchus;* 2) *Laimaphelenchus* and *Aphelenchoides* were paraphyletic in our molecular analyses. These results were also obtained during other studies (Zhao *et al.* 2008, Ryss *et al*. 2013, Asghari & Eskandari 2014, Maleita *et. al*. 2014).

Laimaphelenchus penardi has a well-supported monophyletic clade with *L. persicus*, *L. preissii* and *Aphelenchoides* sp., and a highly supported (Posterior probabilities = 100%) sister relation with *L. persicus*. When compared genetically with the two closest species, *L. penardi* (accession number KJ472144) differed from *L. persicus* (accession number JN006987) by 13% (96 nt in 710 bp,) and from *L. preissii* (accession number EU287598) by 20% (151nt in 729 bp).

Acknowledgements: The authors would like to thank the Lorestan University for technical assistance.

References

Asghari R., A. Eskandari 2014. Morphological and molecular characterization of *Laimaphelenchus deconincki* (Nematoda: Aphelenchoididae) based on specimens from Iran. – *Biologia*, **69** (9): 1172-1178.

Asghari R., E. Pourjam, R. Heydari and Z. Q. Zhao 2012. *Laimaphelenchus persicus* n. sp. (Nematoda: Aphelenchoididae) from Iran. – *Zootaxa*, **3325**: 59-67.

Baujard P. 1981. Revue taxonomique du genre *Laimaphelenchus* Fuchs, 1937 et description de *Laimaphelenchus pini* n. sp. – *Revue de Nématologie*, **4**: 75-92.

De Grisse A. 1969. Redescription ou modifications de quelques techniques utilisées dans l'étude des nématodes phytoparasitaires. – *Meded Rijksfak Landbouwwet Gent*, **34**: 351-369.

Elmiligy I., E. Geraert 1971. *Laimaphelenchus deconincki* n. sp. (Nematoda: Tylenchida). – *Biologisch Jaarboek*, **39**: 145-149.

Filipjev I., J. J. Schuurmans Stekhoven 1941. A Manual of Agricultural Helminthology. Leiden, Netherlands (E.J: Brill). 878 p.

Fuchs A. 1937. Neue parasitische und halbparasitische Nematoden bei Borkenkäfern und einige andere Nematoden. – *Zoologische Jahrbucher, Abteilung fur Systematik, Oekologie and Geographie der Tiere*, **70**: 291-380.

Hunt D. 1993. Aphelenchida, Longidoridae and Trichodoridae: Their Systematics and Bionomics. Wallingford, UK(CAB International). 352 p.

Maleita C., S. R. Costa and I. Abrantes 2014. First report of *Laimaphelenchus heidelbergi* (Nematoda: Aphelenchoididae) in Europe. *Forest Pathology*. (In press).

Massey C. 1974. Biology and Taxonomy of Nematode Parasites and Associates of Bark Beetles in the United States. US Department of Agricultural Forest Agricultural Service, Handbook, No. 446. Washington, D.C. 233 p.

Nunn G. 1992. Nematode Molecular Evolution. Ph.D. thesis. University of Nottingham, U.K. 228 p.

Posada D. 2008. jModel Test: Phylogenetic model averaging. – *Molecular Biology and Evolution*, **25**: 1253-1256.

Ronquist F., J. Huelsenbeck 2003. MR BAYES: Bayesian inference of phylogenetic trees. – *Bioinformatics*, **19**: 1572-1574.

Ryss A. Y., M. A. McClure, C. Nischwitz, C. Dhiman and S. A. Subbotin 2013. Redescription of *Robustodorus megadorus* with molecular characterization and analysis of its phylogenetic position within the family Aphelenchoididae. – *Journal of Nematology*, **45**(4): 237-252.

Steiner G. 1914. Freilebende nematoden aus der schweiz. – *Archiv fur Hydrobiologie und Planktonkunde*, **9**: 259-276

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. – *Molecular Biology and Evolution*, **28**: 2731-2739.

Whitehead A., J. Hemming 1965. A comparison of some quan-

titative methods of extracting vermiform nematodes from soil. – *Annals of Applied Biology*, **55**: 25-38.

Ye W., R. M. Giblin-Davis, H. Braasch, K. Morris and K. W. Thomas 2007. Phylogenetic relationships among *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. – *Molecular Phylogenetics and Evolution*, **43**: 1185-1197.

Zhao Z. Q., W. Ye, R. M. Giblin-Davis, D. Li, K. W. Thomas, K. A. Davies and I. T. Riley 2008. Morphological and molecular analysis of six aphelenchoidoids from Australian conifers and their relationship to *Bursaphelenchus* (fuchs, 1937). – *Nematology*, **10** (5): 663-678.

Received: 23.09.2014
Accepted: 23.06.2015