

Morphological and molecular characterisation of *Paratrophurus bursifer* (Loof, 1960) Siddiqi, 1971 (Nematoda: Tylenchida) from Belgium

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Accepted for publication 23 May 2017

Summary. *Paratrophurus bursifer* is reported from a forest in Belgium for the first time. This species differs from all other tylenchids by a peculiar female tail shape, which has a distal hook-shaped part with bursa formed by lateral fields. Molecular characterisation of *P. bursifer* using the D2-D3 expansion segments of 28S rRNA and the ITS rRNA gene sequences are given. Molecular phylogenetic analysis revealed that this species clustered within the genus *Bitylenchus*. The Shimodaira-Hasegawa test of alternative topologies using two rRNA gene sequence datasets did not support the validity of the genus *Paratrophurus*.

Key words: 28S rRNA, *Bitylenchus*, phylogeny, ITS rRNA.

Paratrophurus bursifer (Loof, 1960) Siddiqi, 1971 was originally described from The Netherlands. The species clearly differs from all other Telotylenchidae by a peculiar tail shape in females. The female tail is constricted ventrally near the middle, distal part hook-shaped, with bursa formed by lateral fields. From this unusual female nematode feature, the name for this species was derived (Loof, 1960). *Paratrophurus bursifer* has been considered as a very rare species by Andr ssy (1991) because it has been found only in a few locations; however, the study by Sturhan and Liřkov (2004) revealed this species at many sites in Germany. These authors also reported that this species was distributed in eight European countries in woodland and along river banks with trees in wet soils, with soil types ranging from sandy to loamy. Although *P. bursifer* is morphologically well characterised, the taxonomic position of this species is still controversial. Siddiqi (1971, 2000), Andr ssy (2007) and Geraert (2011) placed this species in the genus *Paratrophurus*, whereas Loof (2001) Sturhan and Liřkov (2004) and Sturhan (2014) believed that it should be returned to the genus *Tylenchorhynchus* in which it was originally placed (Loof, 1960).

In the summer of 2016, several specimens of *P. bursifer* were found in a soil sample collected in a forest near Merelbeke, Belgium. The present study presents a morphological and molecular characterisation of this nematode.

MATERIALS AND METHODS

The sample with *P. bursifer* was collected from Gentbos, Merelbeke, Belgium (GPS: 50°58'15.3" N, 3°45'13.2" E). Nematode specimens were extracted from soil using the rapid centrifugal-flotation method (Jenkins, 1964), killed by heating and temporarily mounted onto slides. Light micrographs were taken with an automatic Infinity 2 camera attached to a compound Olympus BX51 microscope equipped with a Nomarski interference contrast.

DNA was extracted from several specimens using the proteinase K protocol. DNA extraction, PCR and cloning protocols were as described by Tanha Maafi *et al.* (2003). The following primer sets were used for PCR: the forward D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and the reverse D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Subbotin *et al.*, 2006) for amplification of the D2-D3 expansion segments of 28S rRNA

gene and the forward TW81 (5'-GTTTCCGTAGGT GAACCTGC-3') and the reverse AB28 (5'-ATATG CTTAAGTTCAGCGGGT-3') primers (Tanha Maafi *et al.*, 2003) for amplification of the ITS rRNA gene. The new sequences were submitted to the GenBank database under accession numbers: KY940402 and KY940403 as indicated in the phylogenetic trees.

The new sequences for the D2-D3 of 28S rRNA and ITS rRNA were aligned using Clustal X 1.83 (Thompson *et al.*, 1997) with default parameters with their corresponding published gene sequences of Telotylenchidae (Subbotin *et al.*, 2006; Ghaderi *et al.*, 2014; Handoo *et al.*, 2014 and others). Outgroup taxa for each dataset were chosen based on previously published data (Subbotin *et al.*, 2006; Handoo *et al.*, 2014). Sequence datasets were analysed with maximum likelihood (ML) using PAUP* 4b10 (Swofford, 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best-fitting model of DNA evolution was obtained using jModelTest 2 (Darriba

et al., 2012) with the Akaike information criterion (AIC). BI analysis for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 1000 generations. After discarding burn-in samples, other trees were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. For testing of alternative topologies we used the Shimodaira-Hasegawa (SH) test as implemented in PAUP.

RESULTS AND DISCUSSION

The finding of *P. bursifer* in Belgium is the first report of the species in this country. The specimens were found from a soil sample collected from a forest near Ghent. Sturhan and Lišková (2004) reported this species in eight European countries

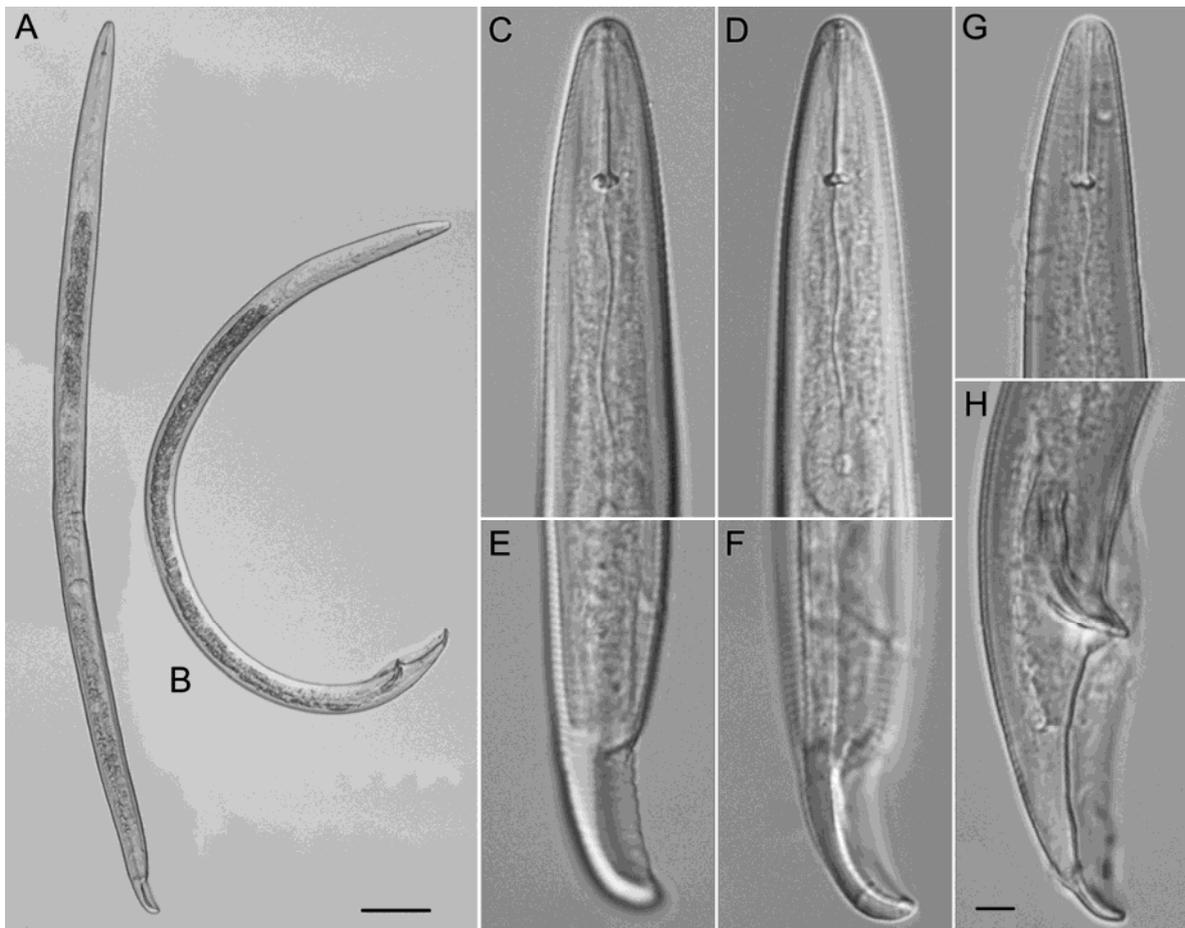


Fig. 1. Light micrographs of *Paratrophurus bursifer*. A: female; B: male; C, D: anterior end of females; E, F: posterior end of females; G: anterior end of male; H: posterior end of male. Scale bars: A, B – 45 μ m; C-H – 5 μ m.

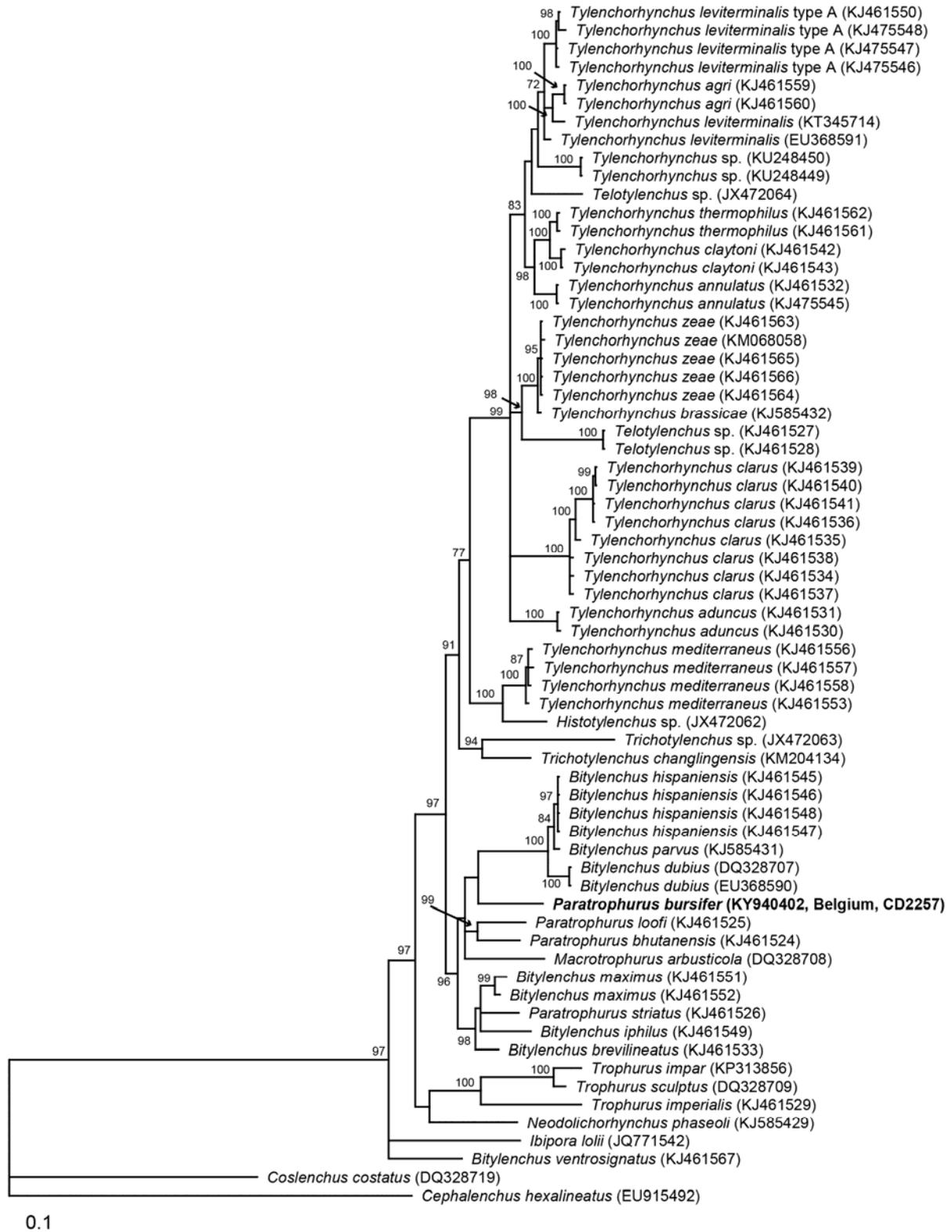


Fig. 2. Phylogenetic relationships within Telotylenchinae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal or more than 70% are given for appropriate clades. Original sequences are indicated by bold font.

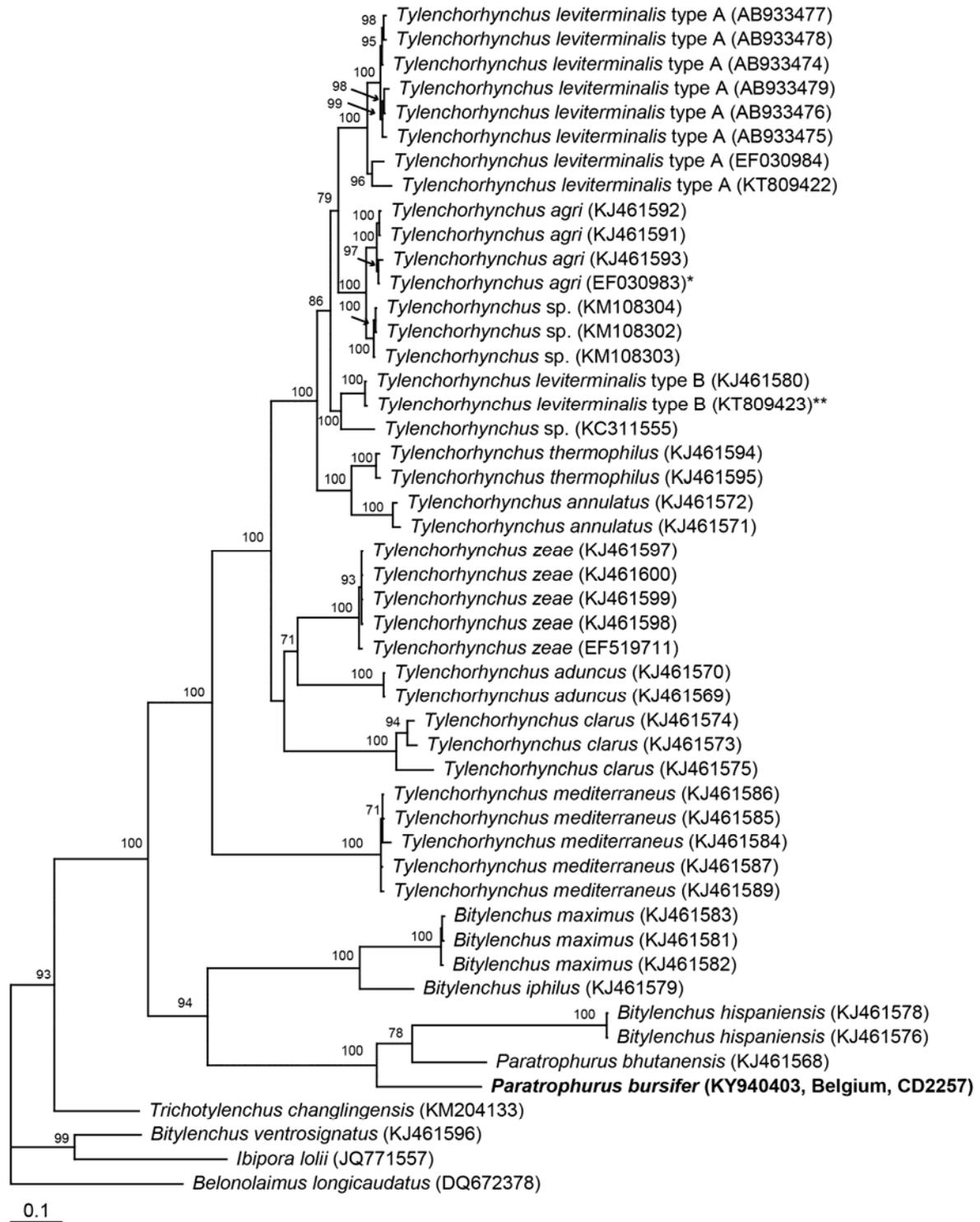


Fig. 3. Phylogenetic relationships within Telotylenchinae: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the ITS rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal or more than 70% are given for appropriate clades. Original sequences are indicated by bold font. * – originally identified as *Tylenchorhynchus annulatus* by Chen *et al.* (2006); ** – identified as *Tylenchorhynchus* sp. by Yu *et al.* (unpublished; GenBank).

Table 1. Results of Shimodaira-Hasegawa test of tree topologies and alternative phylogenetic hypotheses.

Hypothesis	Gene	D2-D3 of 28S rRNA			ITS rRNA		
		-ln L	Δ ln L	P	-ln L	Δ ln L	P
ML best tree		7659.144	best	–	12242.163	best	–
<i>Paratrophurus</i> species constrained as a monophyletic group within <i>Bitylenchus</i>		7689.799	30.655	0.008*	12244.567	2.403	0.546
<i>Paratrophurus</i> species constrained as a monophyletic group outside <i>Bitylenchus</i> (without <i>B. ventrosignatus</i>)		7692.730	33.586	0.003*	12284.848	42.685	0.000*

Note: * $P < 0.05$.

(United Kingdom, Spain, The Netherlands, Germany, Switzerland, Slovak Republic, Hungary and Bulgaria). Our literature analysis revealed additional records of *P. bursifer* in Georgia (Eliava *et al.*, 2002) and Poland (Chalanska & Skwiercz, 2011). Thus, the presently known area of distribution of this species includes eleven European countries. This species was also identified by Mulvey (1961) in Canada from an interception of imported shrub and evergreen soil from The Netherlands.

Morphology and morphometrics of females and males for the Belgian population of *P. bursifer* (Fig. 1) fit well with the original description (Loof, 1960) and descriptions of German and Slovakian populations provided by Sturhan and Lišková (2004).

Females of *P. bursifer* from Belgium (n = 7): L = 666 ± 25.2 (632-700) μm ; a = 28.9 ± 0.8 (28.0-30.1); b = 4.9 ± 0.1 (4.7-5.1); c = 14.0 ± 0.6 (13.0-14.9); V = 54.4 ± 1.2 (52.5-56.1); stylet length = 24.2 ± 0.9 (23.1-25.6) μm ; distance from anterior end to: median pharynx bulb valve = 72.3 ± 2.7 (70.0-77.5) μm , excretory pore = 103 ± 6.9 (95-115) μm ; pharynx length = 134.2 ± 6.2 (125-142) μm ; tail length = 47.5 ± 2.2 (45.0-51.2) μm .

Males of *P. bursifer* from Belgium (n = 5): L = 636 ± 26.5 (592-662) μm ; a = 28.4 ± 1.4 (26.3-30.1); b = 4.8 ± 0.2 (4.4-5.1); stylet = 24.2 ± 0.6 (23.7-25.0) μm ; distance from anterior end to: median pharynx bulb valve = 71.5 ± 2.2 (70.0-75) μm ; excretory pore = 102 ± 2.0 (100-105) μm ; pharynx length = 132.5 ± 5.3 (125-140) μm ; gubernaculum = 13.5 ± 1.0 (12.5-15.0) μm ; spicules = 27.2 ± 1.0 (25.5-28.7) μm .

Phylogenetic positions of *P. bursifer* within the Telotylenchinae obtained from the BI analysis of the D2-D3 of 28S rRNA gene sequences and the ITS rRNA gene sequences are given in Figures 2 and 3, respectively. The BI and ML tree (not shown) topologies were generally congruent. In the partial 28S rRNA gene phylogenetic tree, *P. bursifer* is nested within the *Bitylenchus-Paratrophurus-*

Macrotrophurus clade and has sister relationships with *B. hispaniensis* and *B. dubius*, although with a low statistical support. In the ITS rRNA gene tree, *P. bursifer* is clustered within the *Bitylenchus-Paratrophurus* clade and has sister relationships with *B. hispaniensis* and *P. bhutanensis* with a high support. In this study, the two alternative hypotheses of placement of the genus *Paratrophurus* with *Bitylenchus* were tested using SH test. The SH tests for the D2-D3 of 28S rRNA dataset did not accept the hypothesis of monophyly for the genus *Paratrophurus*, which makes it different from the testing results provided by Handoo *et al.* (2014). Moreover, the SH test for the ITS rRNA dataset did not also accept the hypothesis when *Paratrophurus* species constrained as a monophyletic group outside the genus *Bitylenchus* with excluding *B. ventrosignatus*. Thus, the validity of the genus *Paratrophurus* is not supported from our results. However, we propose to keep *P. bursifer* in the genus *Paratrophurus* until further studies have been done with comprehensive phylogenetic analysis, including other species presently considered as representatives of *Paratrophurus* and detailed testing of position of this genus within Telotylenchidae.

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E. Consoli, J. Akanwari and S.A. Subbotin. Морфологическая и молекулярная характеристика *Paratrophurus bursifer* (Loof, 1960) Siddiqi, 1971 (Nematoda: Tylenchida) из Бельгии.

Резюме. В лесной почве, впервые в Бельгии, обнаружен вид *Paratrophurus bursifer*. Этот вид отличается от других тиленхид особой формой хвостового конца самок, на котором имеется дистальная крючковидная часть с образованной латеральными полями структурой, напоминающей бурсу. Получены нуклеотидные последовательности D2-D3 сегмента 28S рРНК и ITS рРНК. Молекулярно-филогенетический анализ показал, что данный вид образует единую группу с родом *Bitylenchus*. Тест Шимодайра-Хасегава с оценкой альтернативных топологий по этим двум участкам рибосомальной РНК не подтвердил валидности рода *Paratrophurus*.
