

**MITOCHONDRIAL COI IN PHYLOGENETIC RELATIONSHIPS OF
LAIMAPHELENCHUS BELGRADIENSIS (NEMATODA: APHELENCHOIDIDAE)**

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Oro V., N. Milovanović, V. Petrović, B. Nikolić, J. Blagojević (2015): *Mitochondrial COI in phylogenetic relationships of Laimaphelenchus belgradiensis (nematoda: Aphelenchoididae)*.- Genetika, Vol 47, No. 3,909 -916.

Nematodes of the genus *Laimaphelenchus* are small and tiny organisms. Some parts of their body are measured in nanometers. The identification and classification of such organisms is a complex task. Previously, the major source of classification was morphology based on anatomical characters and measurements. Nowadays, this approach is supplemented by: “nano-morphology” based on scanning electron microscopy and molecular data and phylogeny, resulting in molecular systematics. *Laimaphelenchus belgradiensis* was recently described species. Since *cytochrome c oxidase subunit I* gene was successful in DNA based species diagnosis, it was chosen as a molecular marker to infer phylogeny of the newly discovered species. Phylogenetic relationships were based on Bayesian inference, the pairwise distances and the content of nitrogenous bases. The great genetic diversity was observed among close and distant species.

Key words: *Laimaphelenchus belgradiensis*, *mCOI*, phylogeny

INTRODUCTION

Nematodes of the genus *Laimaphelenchus* Fuchs, 1947 belong to Aphelenchids, a group of small and diverse nematode species. They can be plant parasitic, mycophagous, predators or associated with insects. They are among the smallest organisms in the nematode world. Some parts of their body such as the tail accessory organs (finger-like protrusions) are measured in nanometers. A new species of *Laimaphelenchus* was recently described (ORO, 2015). *Laimaphelenchus belgradiensis* was found on the black pine in Belgrade showing the symptoms similar to the Pine wilt disease. The Pine wilt nematode - *Bursaphelenchus xylophilus* Steiner & Buhner, 1934 (NICKLE, 1981) is a quarantine species which in Europe still has limited distribution and is found only in Portugal and Spain (EPPO, 2013). In spite of the fact that both genera belong to the same family and are similar in some aspects, results revealed that a new species is not of quarantine concern. The identification and classification of such small organisms is a complex task. The

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systematics of Aphelenchids, especially the higher systematics was regarded as tentative and was subject to different interpretations (HUNT, 2008; HODDA, 2011). In the past, the major source of classification was morphology based on anatomical characters and measurements (based on optical microscopy). Nowadays, this approach is supplemented by: “nano-morphology” based on scanning electron microscopy revealing structures and morphological patterns unknown to standard microscopy and molecular data and phylogeny, resulting in molecular systematics confirming or opposing the previous morpho-metric approach.

The work of HEBERT *et al.*, (2003) suggested that a DNA-based identification system, founded on the mitochondrial gene, *cytochrome c oxidase subunit I (COI)*, can aid the resolution of the millions of animal species. This success in species diagnosis reflects both the high rates of sequence change at *COI* in most animal groups and constraints on intraspecific mitochondrial DNA divergence arising, at least in part, through selective sweeps mediated via interactions with the nuclear genome.

Since *cytochrome c oxidase* is best suited to deeper lineage phylogeny such as affinities between genera (PEAT, 2010), the mitochondrial *COI* was chosen as a molecular marker to infer phylogeny of the newly discovered species.

Beside molecular identification and characterization of some Tylenchid nematodes from our country (GRUJIĆ, 2010; ORO *et al.*, 2010; ORO and ORO RADOVANOVIĆ, 2012; ORO *et al.*, 2014), there have not been similar records on Aphelenchids.

MATERIALS AND METHODS

The collected specimens of nematodes were used for DNA extraction with a Dneasy blood & tissue kit (Qiagen). The mitochondrial *COI* marker was obtained with COI-F1 and COI-R2 primers and protocol according to ZHAO *et al.*, (2008). The newly obtained sequence was deposited in the GenBank database under accession number KF881747. Phylogenetic analyses were carried out using selected sequences from GenBank (Table 1). *Ditylenchus* sp. was chosen as an outgroup. The sequences were aligned with ClustalW. The genetic distances among 29 nematode species were calculated using pairwise distances of Mega 4 (TAMURA *et al.*, 2007). The Bayesian phylogenetic analysis was performed using (GTR+I+G) as nucleotide evolution model within MrBayes 3.1.2. (HUELSENBECK and RONQUIST, 2005). The dendrogram obtained by Bayesian inference was created by 1 400 000 generations of MCMC (Markov Chain Monte Carlo), with the frequency of the sample of 100 and burnin function of 2 800. The posterior probabilities more than 50% were shown for each appropriate clade.

RESULTS

Investigation of 550 molecular characters of the 29 selected nematode species revealed 223 conserved sites, 326 variable sites, 251 parsimony informative sites and 75 singletons. The content of nitrogenous bases (shown in Table 1) varied within the same species and among close and distant species. In comparison with the ITS region of potato cyst nematodes that had the higher percentage of guanine and thymine (ORO and ORO RADOVANOVIĆ, 2012), the mitochondrial *COI* in most of Aphelenchid species, had the highest content of thymine and adenine. The content of cytosine was the lowest. The only representative of Tylenchid species - *Ditylenchus* sp. had the highest content of thymine (35.4%) and guanine (24.3%), while the content of adenine was the lowest (17.7%) in its mCOI. Within the genus of *Laimaphelenchus*, the thymine content varied from 41.6% (*L. preissi*) to 46% (*L. belgradiensis*) and adenine content

was 21.2% (*L. belgradiensis*) to 24.6% (*L. preissi*), while the content of other nitrogenous bases were similar. The content of thymine in *Devibursaphelenchus* sp. was 42% while in *D. eproctatus* was 49.6%. Also, adenine content for these species varied: 19.5% (*D. eproctatus*) - 27.6% (*Devibursaphelenchus* sp.). Between *L. belgradiensis* and its closely related genus *Aphelenchoides*, the greatest discrepancy occurred in the adenine: 21.2-25.4% and guanine content: 20.8-17.7% respectively. The greatest divergence was observed between the two distant species: *Ektaphelenchus* sp. and *Ditylenchus* sp. in all nitrogenous bases. The content of purine bases ranged as follows: adenine, 17.7-21.9%, guanine, 24.3-17.9% and pyrimidine bases varied: cytosine, 22.6-10.0% and thymine (uracil), 35.4-50.2%, respectively.

Tab. 1 List of selected *Aphelenchid* species with accession numbers and content of nitrogenous bases

No.	Acc. No.	Species name	T(U)%	C%	A%	G%
1.	EU287593	<i>Aphelenchoides</i> sp.	46.5	10.4	25.4	17.7
2.	AY508072	<i>Aphelenchoides besseyi</i>	42.7	12.0	25.5	19.7
3.	AB067761	<i>Aphelenchoides fragariae</i>	41.7	14.6	20.2	23.5
4.	GU367869	<i>Aphelenchoides ritzemabosi</i>	44.2	13.7	24.1	18.1
5.	AB252222	<i>Aphelenchoides xylocopae</i>	45.8	10.4	24.6	19.2
6.	AY508038	<i>Bursaphelenchus borealis</i>	42.3	10.9	28.5	18.2
7.	AY508055	<i>Bursaphelenchus gerberae</i>	46.0	10.8	27.0	16.2
8.	AY508048	<i>Bursaphelenchus hylobianum</i>	40.9	13.7	24.5	21.0
9.	AB634849	<i>Bursaphelenchus mucronatus</i>	45.3	10.6	27.4	16.6
10.	AY508058	<i>Bursaphelenchus paracorneolus</i>	40.7	15.1	23.2	21.0
11.	AY508059	<i>Bursaphelenchus poligraphi</i>	42.9	10.8	27.4	19.0
12.	HQ699854	<i>Bursaphelenchus populi</i>	44.7	12.4	26.3	16.6
13.	AY508065	<i>Bursaphelenchus sextentati</i>	43.8	10.8	27.6	17.9
14.	JF317251	<i>Bursaphelenchus xylophilus</i>	42.2	12.2	27.9	17.7
15.	KC154091	<i>Devibursaphelenchus</i> sp.	42.0	12.6	27.6	17.9
16.	JN122013	<i>Devibursaphelenchus eproctatus</i>	49.6	10.8	19.5	20.1
17.	JX979197	<i>Ektaphelenchus</i> sp.	50.2	10.0	21.9	17.9
18.	AB368531	<i>Ektaphelenchus obtusus</i>	45.4	13.5	23.5	17.5
19.	KF881747	<i>Laimaphelenchus belgradiensis</i>	46.0	12.0	21.2	20.8
20.	EU287592	<i>Laimaphelenchus heidelbergi</i>	46.0	11.9	21.5	20.6
21.	EU287594	<i>Laimaphelenchus preissii</i>	41.6	11.7	24.6	22.1
22.	AB971165	<i>Pseudaphelenchus</i> sp.	42.7	14.1	21.2	22.1
23.	AB971168	<i>Pseudaphelenchus</i> sp.	40.9	13.9	22.3	23.0
24.	JN377733	<i>Ruehmaphelenchus digitulus</i>	42.3	10.6	29.9	17.2
25.	HM151002	<i>Schistonchus</i> sp.	47.6	10.0	22.8	19.5
26.	GQ849474	<i>Schistonchus hirtus</i>	46.0	10.4	23.9	19.7
27.	FN564939	<i>Schistonchus caprifici</i>	47.4	10.8	20.4	21.4
28.	KC951996	<i>Sheraphelenchus entomophagus</i>	42.2	13.9	24.3	19.7
29.	KF612018	<i>Ditylenchus</i> sp.	35.4	22.6	17.7	24.3

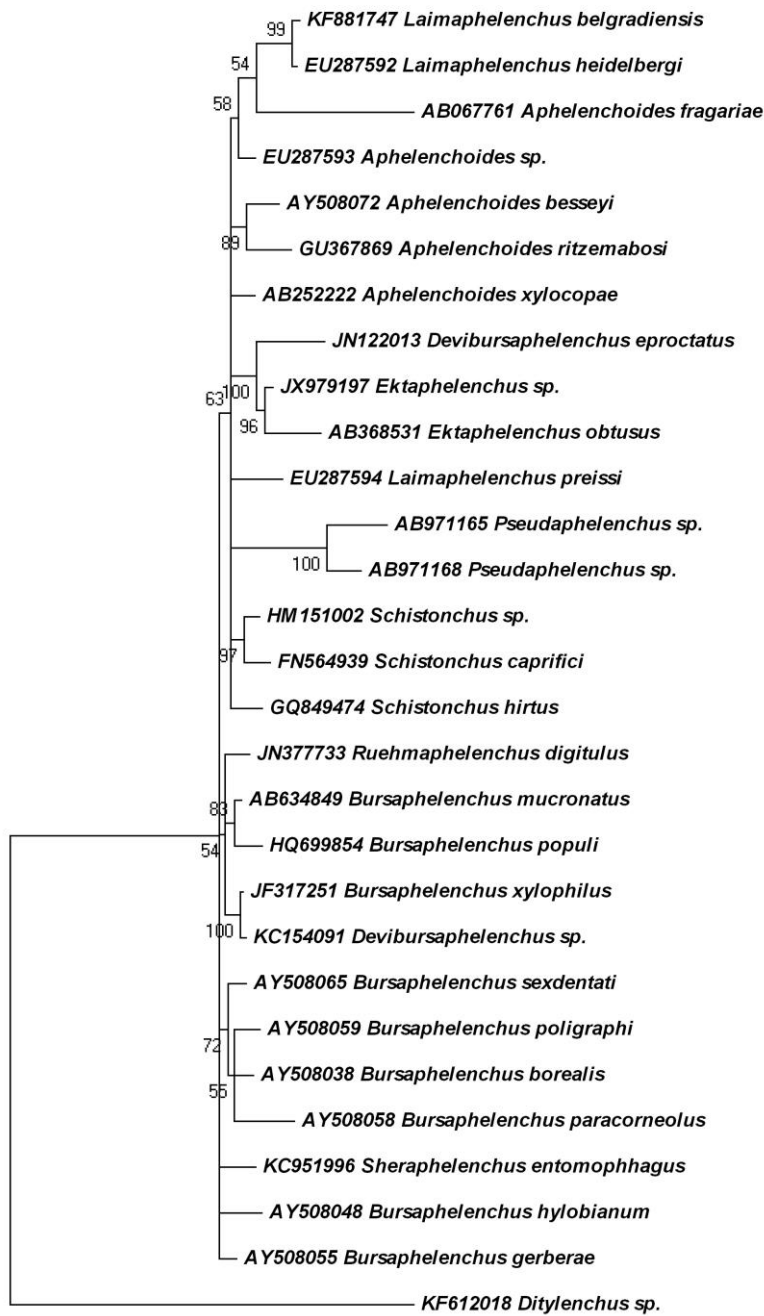
Pairwise distances were used as matrix for creating a 29X29 table (Table 2) representing percentage differences in nucleotides among selected species. In relation to *L. belgradiensis*,

distances varied from 3.3% for the closest *L. heidelbergi* to 38.7% for *Ditylenchus* species. Generally, the least difference was 2.6% between *Devibursaphelenchus* sp. and *Bursaphelenchus xylophilus*, while the greatest difference 40.4% was observed between *Ditylenchus* sp. on one side and *Bursaphelenchus mucronatus* or *Ruehmaphelenchus digitulus* on the other side. The overall divergence among the species was high. Almost 60% of nucleotides were variable.

Tab. 2 Divergence among investigated Aphelenchid species calculated from pairwise distances

SPP.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.
1.																												
2.	16.0																											
3.	22.6	26.8																										
4.	16.3	15.8	26.2																									
5.	10.3	16.5	24.4	15.4																								
6.	13.4	16.0	27.2	17.8	15.2																							
7.	11.9	17.8	25.5	17.6	12.8	12.8																						
8.	20.4	19.5	29.5	20.6	19.8	17.1	18.9																					
9.	13.0	15.6	27.2	17.1	13.9	11.0	9.2	17.1																				
10.	18.2	20.9	27.7	20.7	19.1	16.3	15.6	20.9	15.8																			
11.	14.5	16.9	27.7	18.2	13.8	10.8	12.8	17.6	13.6	17.4																		
12.	15.4	16.9	27.3	15.1	14.1	13.4	11.4	16.9	9.2	16.2	13.0																	
13.	15.1	16.5	27.2	17.3	14.7	10.3	9.5	18.9	11.7	16.2	11.7	12.1																
14.	15.2	16.0	28.1	17.3	15.8	13.6	11.7	16.5	8.6	15.2	15.1	13.0	12.7															
15.	15.6	17.8	29.0	17.4	16.0	13.9	11.7	17.3	9.2	16.2	14.9	12.8	13.2	2.6														
16.	18.4	21.7	27.3	21.1	19.6	22.4	19.5	23.1	18.4	23.5	22.2	20.0	19.8	20.7	21.1													
17.	12.8	18.1	25.7	17.4	13.4	16.3	12.7	21.8	13.9	20.4	15.6	15.2	15.6	17.1	16.7	14.5												
18.	19.6	21.8	28.1	21.8	19.5	21.1	18.9	24.4	18.1	24.2	21.1	18.2	20.2	20.6	21.1	19.3	13.0											
19.	14.5	17.1	23.3	17.6	14.9	17.3	16.7	19.8	18.0	21.3	17.8	17.1	18.5	19.1	19.8	20.1	17.1	21.6										
20.	14.3	17.4	24.4	17.1	15.2	16.3	16.7	20.0	17.6	22.4	17.8	16.5	18.2	19.8	19.8	21.1	16.7	21.3	3.3									
21.	15.8	15.6	27.0	19.3	16.9	16.3	17.3	22.8	17.3	20.4	18.7	18.7	16.2	18.7	20.0	22.6	19.1	21.8	18.2	18.7								
22.	24.6	23.9	29.2	24.4	22.8	24.8	21.7	27.9	23.7	27.5	24.6	23.9	23.1	25.7	26.1	27.2	23.7	27.0	25.1	25.7	24.8							
23.	21.7	24.0	30.5	26.6	23.1	22.4	20.9	27.0	21.3	23.7	24.8	23.7	23.3	23.3	23.3	27.0	23.3	26.1	23.7	23.7	21.8	18.2						
24.	13.8	16.3	27.9	17.1	14.3	11.0	13.0	17.4	10.1	29.1	14.1	12.7	11.6	10.7	11.2	21.1	15.8	20.4	19.1	18.9	18.3	23.7	22.0					
25.	13.6	16.0	25.1	16.1	12.5	16.5	14.9	19.8	12.8	20.2	15.4	13.8	15.2	16.7	16.5	17.1	12.8	17.6	14.9	13.9	15.8	22.2	22.2	14.7				
26.	14.1	15.6	27.5	18.0	13.8	17.6	15.8	21.1	14.7	20.6	17.1	15.6	15.6	17.8	18.1	16.7	13.8	19.3	16.5	16.0	17.1	24.6	22.4	15.6	11.9			
27.	14.1	15.6	23.9	16.7	13.8	17.3	15.2	20.9	16.2	20.4	17.3	17.3	17.3	18.0	18.7	18.7	14.3	19.3	14.9	15.4	15.6	22.9	21.7	16.9	9.36	14.5		
28.	15.2	18.5	26.2	19.1	15.1	15.2	14.9	18.7	14.9	19.8	15.1	16.3	15.4	13.9	15.1	24.4	18.4	21.3	17.8	17.4	18.5	23.3	23.5	15.1	16.5	18.9	17.3	
29.	37.6	40.2	39.6	39.1	38.9	39.8	38.9	38.2	40.4	39.1	39.8	37.8	39.3	39.3	40.2	38.4	39.1	38.7	38.7	38.7	38.0	41.1	38.9	40.4	39.3	38.9	37.8	37.2

The dendrogram based on Bayesian inference (Figure 1) provided the most phylogenetically informative dataset. The Neighbour Joining and Maximum Likelihood methods were tested as well but dendrograms could not resolve interspecific relationships with reliable probability values (results are not shown). The obtained phylogenetic tree of Aphelenchids revealed two distinct clades. In the first clade there are *Laimaphelenchus*, *Aphelenchoides*, *Ektaphelenchus*, *Schistonchus*, *Pseudaphelenchus* and a *Devibursaphelenchus* species. The other clade is consisted of all *Bursaphelenchus* species with the exception of *Devibursaphelenchus* sp. and *Ruehmaphelenchus digitulus*. Within the first clade, several subclades were observed: *L. belgradiensis* and *L. heidelbergi* formed a subclade with *Aphelenchoides* spp., *A. besseyi* and *A. ritzemabosi* clustered in a separate subclade, *D. eproctatus* grouped with *Ektaphelenchus* spp., the two *Pseudaphelenchus* species presented a distinct subclade and *Schistonchus* spp. formed the last subclade.



H
0.05

DISCUSSION

The pairwise distances based on sequence differences, show the great level of variations, varying more than 10 times. Unusually large percentage of variations is expressed among closely related species. The difference of 3.3 % between *L. belgradiensis* and its closest relative *L. heidelbergi* is in congruence with difference between some closely related Tylenchids (ORO and ORO RADOVANOVIĆ, 2012). While 18.7% is surprisingly large percentage of deviation between *L. belgradiensis* and *L. preissi*, as they are congeneric species. In addition, the content of adenine and thymine of the two species was quite different. The percentages of differences between *L. belgradiensis* and its sister genus *Aphelenchoides* varied from 14.5 to 23.3%. The content of their adenine was incongruent. The percentages of differences within *Aphelenchoides* spp. were also high. They varied from 10.3% between *A. xylocopae* and *Aphelenchoides* sp. to 26.8% between *A. fragariae* and *A. besseyi*, both plant parasitic nematodes. The diversity of *Schistonchus* spp. ranged between 14.9 and 16.5% in relation to *L. belgradiensis*. The content of their purine and pyrimidine bases was similar. The percentages of differences within *Schistonchus* spp. varied from 9.4 between *S. caprifici* and *Schistonchus* sp. to 14.5 between *S. caprifici* and *S. hirtus*. The genera like *Sheraphelenchus*, *Devibursaphelenchus* and *Ruehmaphelenchus* were different 17.8, 20 and 19% from *L. belgradiensis* respectively. The greatest difference among these species in the sense of the presence of adenine occurred between *L. belgradiensis* and *R. digitulus* (21.2-29.9%). The difference between *Devibursaphelenchus* sp. and *D. eproctatus* was 21.1%. The percentages of divergence between *Ektaphelenchus* spp. and *L. belgradiensis* were between 17.1 and 21.6. The percentages of their purine and pyrimidine bases were similar. The difference between *Ektaphelenchus* sp. and *E. obtusus* was 13.0%.

Bursaphelenchus species were diverse from *L. belgradiensis* 16.7% in case of *B. gerberae* and 21.3% for *B. paracorneolus* as the most different *Bursaphelenchus* species. Within *Bursaphelenchus* spp., the percentages of differences varied from 8.6 (between *B. xylophilus* and *B. mucronatus*) to 20.9 (between *B. paracorneolus* and *B. hylobianum*). *Pseudaphelenchus* spp. were the most distant (23.7-25.1%) in relation to *L. belgradiensis* and all other species. *Laimaphelenchus belgradiensis* had more thymine but less guanine in comparison with *Pseudaphelenchus* spp. The difference between the two *Pseudaphelenchus* sp. was 18%. Among 28 Aphelenchids, the greatest difference occurred between *Pseudaphelenchus* sp. and *A. fragariae*: 30.5%.

The cladogram based on Bayesian inference clustered species of *Laimaphelenchus*, *Aphelenchoides*, *Ektaphelenchus*, *Schistonchus*, *Pseudaphelenchus* and *Devibursaphelenchus* which represent different subfamilies (HUNT, 2008) or families (HODDA, 2011) as monophyletic. *Bursaphelenchus* species created the other branch of the phylogenetic tree suggesting polyphyletic (paraphyletic) origin. The two exceptions in the *Bursaphelenchus* clade were *Devibursaphelenchus* sp. and *Ruehmaphelenchus digitulus* which were genetically closer to the *Bursaphelenchus* than to the *Laimaphelenchus* clade. The content of nitrogenous bases was more similar to that of *Bursaphelenchus* spp. suggesting they might be members of the latter genus. The broader species sampling is needed to infer more phylogenetic conclusions.

There are still many gaps in our knowledge about these species and their high *COI* sequence diversity. Does it mean they evolved long ago from one another? Or it means that something caused the accelerated evolution? Does it mean high mitochondrial recombination events? Or it simply means that many other species do exist waiting to be discovered?

ACKNOWLEDGMENT

This study was supported by the Ministry of Education, Science and Technical Development Grants TR 31018 and III 46007.

Received May 15ND, 2015

Accepted October 20th, 2015

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MITOHONDRIJALNI COI U FILOGENETSKIM ODNOSIMA *LAIMAPHELENCHUS BELGRADIENSIS* (NEMATODA: APHELENCHOIDIDAE)

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Izvod

Nematode roda *Laimaphelenchus* su sitni i nežni organizmi. Neki delovi njihovog tela se mere nanometrима. Identifikacija i klasifikacija takvih organizama je kompleksan zadatak. Ranije, glavni izvor klasifikacije je bila morfologija koja se zasnivala na anatomskim karakteristikama i merenjima. Danas, ovaj pristup se dopunjava "nano-morfologijom" koja se bazira na scanning elektronskoj mikroskopiji i molekularnim podacima i filogeniji koji imaju za rezultat molekularnu sistematiku. *Laimaphelenchus belgradiensis* je skoro opisana vrsta. Pošto je citohrom *c oksidaza I* gen bio uspešan u DNK dijagnozi vrsta, izabran je kao molekularni marker za izvođenje filogenije novootkrivene vrste. Filogenetski odnosi su bazirani na Bajesovoj inferenciji, p-distancama i sadržaju azotnih baza. Veliki genetički diverzitet je primećen između bliskih i udaljenih vrsta.

Primljeno 15.V. 2015.

Odobreno 20. X. 2015.