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Applicability of selected ribosomal and mitochondrial genetic markers in identification of European bison lungworm: a state of the art review

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Abstract: Large lungworms are nematode parasites of the genus *Dictyocaulus* that can infect wide range of ungulate hosts, including domestic and wild ruminants. They are the causative agents of parasitic bronchitis (husk, dictyocaulosis). Correct diagnosis of lungworm species and better understanding of the transmission patterns of the parasites are crucial in minimising the risk of its cross transmission between wildlife and livestock, and for control of dictyocaulosis. The study was conducted on large lungworms collected from European bison and cervids. The study resulted in several sequences of 18S RNA gene (small subunit) and internal transcribed spacer 2 (ITS2) regions of the ribosomal gene array as well as the mitochondrial (*mt*) cytochrome *c* oxidase subunit 1 (*cox*1) and mitochondrial cytochrome *c* oxidase subunit 3 (*cox*3). The European bison was infected with a distinct genotype of a bovine lungworm, *D. viviparus*. Whereas the high degree of conservation of nuclear rDNA within *Dictyocaulus* taxa was identified, analysis of the mitochondrial *cox*1 sequence data revealed a diverse genetic background and high evolutionary potential within the genus. Additionally, the study revealed that the *cox*3 nucleotide sequences of roe deer lungworm (*D. capreolus*) and of European bison-derived *D. viviparus* were 100% homologous to each other, indicating that the *mt* cox3 gene does not serve as an efficient *mt* marker for systematic, population genetic or molecular epidemiological studies of *Dictyocaulus* spp.

Key words: *Dictyocaulus* lungworm, 18S RNA (small subunit [SSU]), internal transcribed spacer 2 (ITS2), cytochrome *c* oxidase subunit 1 (*cox*1), cytochrome *c* oxidase subunit 3 (*cox*3)

Introduction

Large lungworms of the genus *Dictyocaulus* are the causative agents of parasitic bronchitis (dictyocaulosis) of various ungulate hosts, including domestic (Eysker 1994) and wild ruminants (Gibbons, Höglund 2002; Mahmood *et al*. 2014; Pyziel 2014). The parasitosis results in coughing, nasal discharge, emphysema and pneumonia, and can be fatal in heavily infected individuals (Eysker, van Miltenburg

1988; Panuska 2006). The parasite was detected in European bison, however its systematic position was unclear. Wróblewski (1927) reported infection of European bison with cattle-specific *D. viviparus* and sheep-specific *D. filaria* lungworms. Additionally, Koffman (1942) reported European bison-specific lungworm, *D. bisonis*, whereas Demiaszkiewicz *et al*. (2009) recognized exclusively *D. viviparus* lungworms in investigated individuals. According to Demiaszkiewicz *et al*. (2008) *Dictyocaulus* lungworm is the second most commonly diagnosed parasite of free-roaming and captive European bison in Poland, causing one of the most dangerous parasitosis of bison (Demiaszkiewicz *et al*. 2009).

Investigation of applicability of genetic markers for systematic and molecular epidemiological study on *Dictyocaulus* spp. of wild ruminants was the aim of conducted study.

Materials and Methods

The investigation was conducted between 2011 and 2018 on adult large lungworms of free-roaming and captive European bison culled due to breeding or health reasons. In order to isolate large lugworms from respiratory tract, trachea, bronchi and bronchioles were cut open and immersed into beakers willed with tap water, the sediment allowed to settle to the bottom, and the fluid decanted, after which the sediment was examined. Worms were recovered with the use of dissecting needles under a stereomicroscope and preserved in 70% ethanol for further molecular examination.

Genomic DNA of single lungworm males and was extracted using the Nucleospin tissue DNA extraction kit, according to the manufacturer's protocol.

A partial region of 18S RNA (SSU) and complete ITS2 of the ribosomal gene array, as well as a partial region of the mitochondrial (*mt*) cytochrome *c* oxidase subunit 1 (*cox*1) and of the *mt* cytochrome *c* oxidase subunit 3 (*cox*3) of lungworms were amplified by PCR as described in Pyziel 2014, Pyziel *et al*. 2015, Pyziel *et al*. 2017, Pyziel *et al*. 2018. The amplicons were purified with the Nucleospin gel and PCR clean-up kit and eluted with 30 μl of laboratory-pure PCR water. Purified PCR products were sequenced by Macrogen Europe or Genomed S.A., and then the sequences were assembled into contigs using CLC Main Workbench 7.5 or Contig-Express.

Additionally, Bayesian inference analysis with MrBayes was conducted using combined SSU and ITS2 rDNA sequences (Pyziel *et al*. 2017) and combined *mt cox*1 and *cox*3 sequence data (Pyziel *et al*. 2018).

Results

The partial (1,714 bp) SSU rDNA sequence (GenBank accession: KC771250) of European bison-derived lungworms was identical to *D. viviparus* isolated from cattle

(GenBank accession: AY168856) (Pyziel 2014), whereas they diverged at the complete (463 bp) ITS2 region (2.43–2.60%) (Pyziel 2014, Pyziel *et al*. 2017). Moreover, a slight variation was noted at the ITS2 region among various European bison isolates (0.17%) (Pyziel *et al*. 2017).

A common significant feature of European bison-derived isolates was the occurrence of GAT as a repeating motif within the ITS2 (GenBank accession KF007338-KF007341, KM359411-KM359415) (Pyziel *et al*. 2015), similar to what has been observed in *D. viviparus* from cattle in Germany (single insertion of GAT, GenBank accession U37718) and in *D. viviparus* from cattle in Sweden (absence of GAT, GenBank accession AF105257) (Pyziel *et al*. 2017). Bayesian analysis of rDNA sequence data (SSU combined with ITS2) revealed three strongly supported clades. One of the clusters contained a subclade including *D. viviparus* from both cattle and bison, the other subclade including *D. eckerti* and *D. cervi* from red deer (Pyziel *et al*. 2017).

The analysis of the partial (1,083 bp) *mt cox*1 nucleotide sequences (GenBank accession KT581636) of European bison-derived lungworms and the NCBI reference sequence of *D. viviparus* from cattle (GenBank accession NC_019810) revealed their divergence, reaching 3.20%. Amino acid divergence between them reached 0.48% (Pyziel *et al*. 2017).

In the BI analysis of *cox*1, *D. viviparus* isolates from European bison and cattle were both placed in the same clade with *D. capreolus* from roe deer, as a sister taxon (Pyziel *et al*. 2017).

The partial *mt cox*3 sequences (318–350 bp) of bison (GenBank accession KM359422-KM359429), cattle (GenBank accession DQ299603) and roe deer lungworms (GenBank accession KM374682) were 100% homologous to each other (Pyziel *et al*. 2018). Nucleotide *mt cox*1+*cox*3 sequence variation was 2.59% between *D. viviparus* from European bison and *D. viviparus* from cattle, but only 0.30% between the Swedish and Japanese isolates of *D. viviparus* from cattle (Pyziel *et al*. 2018).

At the amino acid level, 0.45% variation in *cox*1+*cox*3 was observed between *D. viviparus* from European bison and from cattle, but no variation between the Swedish and Japanese isolates of *D. viviparus* from cattle (Pyziel *et al*. 2018).

Bayesian analysis of *mt* sequence data (*cox*1 combined with *cox*3) revealed two strongly supported clades (Fig. 1). One cluster contained a subclade including roe deer lungworm and other including *D. viviparus* from European bison and *D. viviparus* from cattle. *Dictyocaulus viviparus* from cattle was a sister taxon to *D. viviparus* isolated from European bison (Pyziel *et al*. 2018).

Discussion

The results of phylogenetic analysis of the *cox*1 and *cox*1+*cox*3 sequences of large lungworms placed *D. viviparus* of European bison and cattle and *D. capreolus* of roe deer in the same clade (Pyziel *et al*. 2017, Pyziel *et al*. 2018); this finding is in

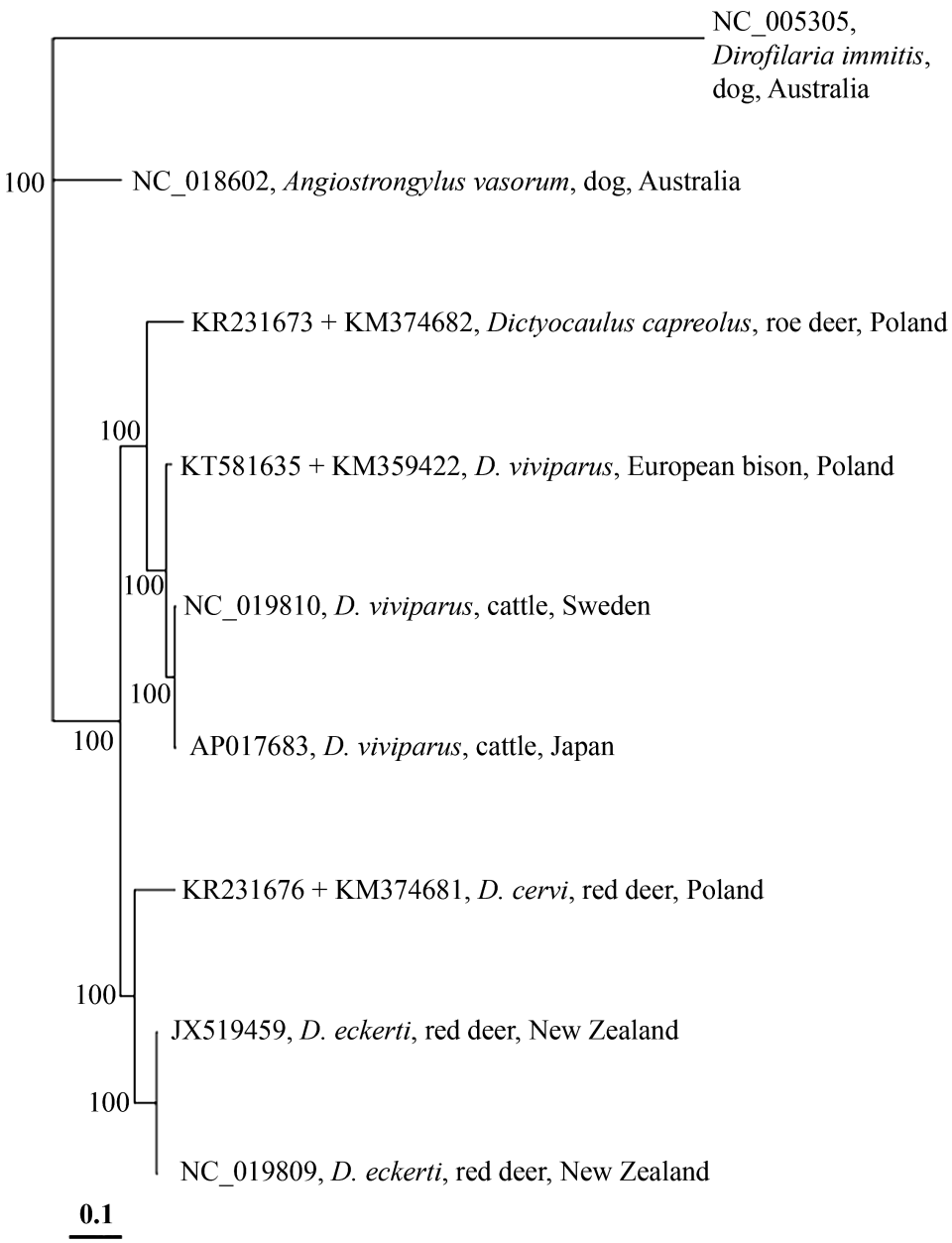

Figure 1. Bayesian analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) combined with the cytochrome *c* oxidase subunit 3 (*cox*3) "codon" data (1,308 base pairs [bp]) of *Dictyocaulus* spp. from wild ruminants, constructed using MrBayes, Bayesian inference method (GTR+G for *cox*1, HKY+G for *cox*3). *Dirofilaria immitis* and *Angiostrongylus vasorum* were used as an outgroup. Analysis was run for 1,000,000 generations. Host, country of origin and GenBank accession number are shown (Pyziel *et al*., 2018).

contrast to those of analysis of ribosomal RNA genes, where roe deer lungworm formed a separate clade (Pyziel *et al*. 2017) or was placed in the same clade as red deer lungworm (Höglund *et al*. 2003).

Presented results endorse the high degree of conservation of nuclear rDNA within *Dictyocaulus* taxa.

The analysis of rDNA sequences gives 100% homology between the large lungworms of European bison and cattle, indicating that they host a common lungworm, *D. viviparus* (Pyziel 2014, Pyziel *et al*. 2015).

At the same time, *mt cox*1 sequences of *Dictyocaulus* spp. show a high degree of intraspecific genetic diversity within *D. viviparus* of European bison and cattle (Pyziel *et al*. 2017).

This makes the *cox*1 a useful marker for resolving distinct population, but it can be also useful to study the evolution of different species (Le *et al*. 2000).

In contrast to the usefulness of the *cox*1 gene, the *cox* 3 gene does not appear to serve as an efficient *mt* marker for systematic, population genetic or molecular epidemiological study of *Dictyocaulus* lungworms. This is due to the results of the *cox*3 sequence data analysis of *Dictyocaulus* spp. that may falsely imply that roe deer and cattle/ European bison are able to share the same species of the lungworm, although it has been experimentally proven that roe deer lungworm is not transmittable to cattle (Divina, Höglund 2002).

According to Blouin (1998) nucleotide sequence variation between distant populations of the same nematode species range 1–3%. Thus, the divergence between cattle and European bison-derived *D. viviparus*, reaching 2.6% for combined *cox*1+*cox*3 sequence data, and 3.2% for the *cox*1 data, are at the edge of the range proposed by Blouin (1998) for isolates of the same species.

Thus, further molecular and morphological investigation is clearly required to find out whether cattle act as a reservoir of *D. viviparus* for red-listed, strictly protected European bison. The possibility, which can represent a threat for European bison reintroduction programmes within Europe, as well as for livestock infected with the wild genotype of the lungworm.

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Przydatność wybranych markerów genetycznych (rybosomalnych i mitochondrialnych) do identyfikacji nicieni płucnych żubra: przegląd dotychczasowego stanu wiedzy

Streszczenie: Nicienie z rodzaju *Dictyocaulus* są stwierdzane u wielu gatunków zwierząt kopytnych, w tym u domowych i dzikich przeżuwaczy. Omawiana grupa pasożytów może powodować objawy chorobowe ze strony układu oddechowego na skutek następczego rozwoju odoskrzelowego, robaczego zapalenia płuc (diktiokauloza). Diktiokauloza stanowi jedną z najgroźniejszych chorób pasożytniczych notowanych u żubrów. Stąd potrzebne jest opracowanie naukowych narzędzi diagnostycznych służących rozpoznaniu cech gatunkowych pasożyta oraz zakresu jego specyficzności żywicielskiej, co pozwoli ograniczać ryzyko zarażeń krzyżowych *Dictyocaulus* oraz zapewni kontrolę szerzenia się zarażeń. Badania przeprowadzano na dorosłych nicieniach płucnych pochodzących od żubrów i jeleniowatych amplifikując i sekwencjonując częściowe lub (rzadziej) kompletne fragmenty DNA

rybosomalnego pasożyta (18S RNA i ITS2) oraz jego DNA mitochondrialnego (*cox*1 i *cox*3). Na podstawie przeprowadzonych analiz stwierdzono, że na poziomie konserwatywnych sekwencji DNA rybosomalnego, izolat pochodzący od żubra nie różni się od pasożyta bydła, *D. viviparus*. Mimo to, nicienie pochodzące od obydwu żywicieli różnią się na poziomie analizowanych sekwencji mitochondrialnego DNA, sugerując występowanie specyficznego dla żubra genotypu *D. viviparus*. Przeprowadzone analizy wykazały największą przydatności markera mitochondrialnego *cox*1 do analiz filogenetycznych w obrębie rodzaju *Dictyocaulus*. Z kolei marker mitochondrialny *cox*3 okazał się zupełnie nieprzydatny w badaniach nad nicieniami płucnymi z rodzaju *Dictyocaulus*, ze względu na wysoki stopień homologii sekwencji *cox*3 dla odrębnych gatunków pasożyta.