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DNA barcoding and identification of intermediate slug hosts in the framework of an epidemiological survey in Germany

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INTRODUCTION

Recently, metastrongyloid lungworms that infect canids, felids and humans, have gained special attention due to their increasing prevalence in domestic and wildlife populations. *Angiostrongylus vasorum*, the notorious canid lungworm species that can cause severe systemic bleeding disorders in domestic dogs, are being spread by means of the parasite's most common intermediate host, viz. slugs. Nevertheless, there are only a few studies on the prevalence of metastrongyloid lungworms in their natural intermediate host populations in Europe.

The Barcoding facility for Organisms and tissues of Policy Concern (BopCo project – a Belgian federal in-kind contribution to LifeWatch) aims at developing a virtual laboratory, expertise forum and databank network facilitating the identification of biological samples of policy concern for Belgium and Europe. As such, the purpose of the present contribution is a DNA-based validation of slug host samples that were collected by the Institute of Parasitology of the Justus Liebig University Giessen (Germany), where for the first time an epidemiological study was conducted to assess the prevalence of lungworm species in natural slug populations in Germany.

MATERIAL, METHODS AND RESULTS

SAMPLING

Samples were collected in autumn and winter 2014 and in spring and summer 2015 (Table 1). In total, four different sites in Germany (two in Hesse and two in Rhineland - Palatinate) were sampled.

Total number of slugs	2701
Percentage of missing values	15% (397/2701)
Percentage of samples based on morphological ID	85% (2304/2701)
Number of slugs that required additional DNA-based validation, after morphological ID	19
Prevalence of slug samples that revealed positive for lungworm larvae	6.4% (174/2701)
Prevalence of slug samples that revealed positive for *A. vasorum*	4.7 % (127/2701)

Table 1 : Partitioning of slug samples collected throughout one year in areas that were previously shown as hyperendemic for *Angiostrongylus vasorum* infections in foxes

mtDNA SEQUENCING

Pieces of slug head tissue were used to extract DNA. This DNA was used to amplify two mtDNA gene fragments: the COI barcode region (LCO1490/HCO2198) and the 16S ribosomal rDNA (16Sar/16Sbr). Positive PCR products were purified and sequenced in both directions.

SEQUENCE SIMILARITIES

Sequences were corrected and trimmed. Samples were identified with blastn in GenBank (16S) and IDS-BOLD in BOLD (COI).

The new sequences were most similar to reference sequences of ***Arion vulgaris*** (= ***Arion lusitanicus*** **Auct.**) (Arionidae) and ***Deroceras reticulatum*** (Agriolimacidae) (Table 2 and 3).

Species identifications were consistent between 16S and COI.

NEIGHBOR-JOINING TREES

NJ-trees (pairwise K2P distances, 1000 BS) were inferred for 16S and COI separately. Each database included all generated sequences, as well as a selected number of sequences of congeneric species retrieved from online repositories GenBank and BOLD (depending on the amount of what was available in the repositories). This resulted in 21 and 63 sequences, representing 13 and 34 species, respectively for 16S and COI. The clustering results confirmed the ***Arion vulgaris*** and ***Deroceras reticulatum*** identifications (Figs. 1, 2 and 3).

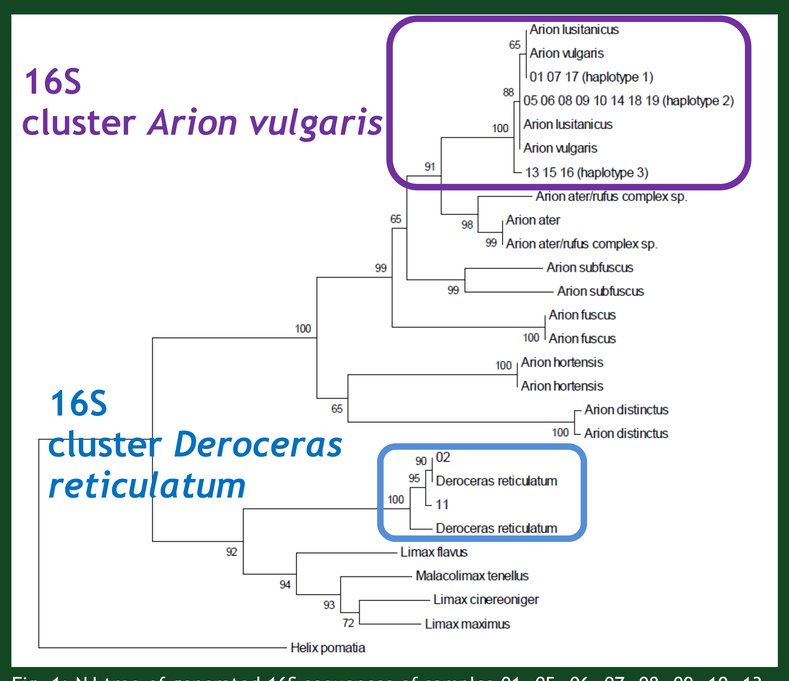

Fig. 1: NJ-tree of generated 16S sequences of samples 01, 05, 06, 07, 08, 09, 10, 13, 14, 15, 16 17, 18 and 19 that clustered with *Arion vulgaris* sequences from GenBank and of samples 02 and 11 that clustered with *Deroceras reticulatum* sequences from GenBank

16S					
Sample number	blastn - GenBank	Accession	Query cover (%)	E-value	Sequence identity (%)
01	*Arion vulgaris*	KP835188.1	99	0	99
02	*Deroceras reticulatum*	FJ896814.1	100	0	99
05	*Arion vulgaris*	KP835188.1	99	0	99
06	*Arion vulgaris*	KP835188.1	99	0	99
07	*Arion vulgaris*	KP835188.1	99	0	99
08	*Arion vulgaris*	KP835188.1	99	0	99
09	*Arion vulgaris*	KP835188.1	98	0	99
10	*Arion vulgaris*	KP835188.1	99	0	99
11	*Deroceras reticulatum*	AF238045.1	100	0	99
13	*Arion vulgaris*	KP835188.1	99	0	98
14	*Arion vulgaris*	KP835188.1	99	0	99
15	*Arion vulgaris*	KP835188.1	99	0	98
16	*Arion vulgaris*	KP835188.1	99	0	98
17	*Arion vulgaris*	KP835188.1	99	0	99
18	*Arion vulgaris*	KP835188.1	97	0	99
19	*Arion vulgaris*	KP835188.1	99	0	99

Table 2: Blastn (Basic Local Alignment Search Tool for nucleotides) results from GenBank for the 16S sequences

COI			
Sample number	IDS-BOLD - BOLD	GenBank Accession	Similarity (%)
01	*Arion lusitanicus*	EU734825	100
02	*Deroceras reticulatum*	KF894378	100
05	*Arion lusitanicus*	EU734824	100
06	*Arion lusitanicus*	EU734824	100
07	*Arion lusitanicus*	EU734825	100
09	*Arion lusitanicus*	EU734828	100
11	*Deroceras reticulatum*	KM611812	99.85
13	*Arion lusitanicus*	EU734827	99.04
14	*Arion lusitanicus*	AY987894	100
15	*Arion lusitanicus*	EU734827	99.04
16	*Arion lusitanicus*	EU734827	99.04
17	*Arion lusitanicus*	EU734828	99.83
18	*Arion lusitanicus*	EU734828	100
19	*Arion lusitanicus*	EU734828	100

Table 3: IDS-BOLD (Identification System) results from BOLD for the COI sequences

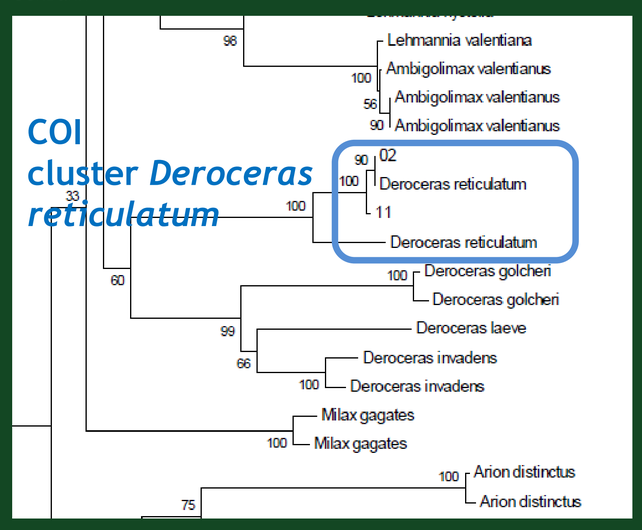

Fig. 2: Detail of NJ-tree of generated COI sequences of samples 02 and 11 that clustered with *Deroceras retiulatum* sequences retrieved from BOLD

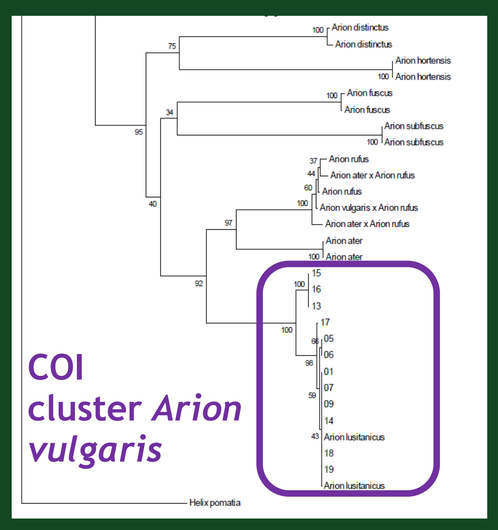

Fig. 3: Detail of NJ-tree of generated COI sequences of samples 01, 05, 06, 07, 09, 13, 14, 15, 16, 17, 18 and 19 that clustered with *Arion vulgaris* sequences retrieved from BOLD

Adult and juvenile specimens of *Arion vulgaris* © Malin Lange, Institute of Parasitology JLU Giessen

Adult specimen of *Deroceras reticulatum* © Malin Lange, Institute of Parasitology JLU Giessen

DISCUSSION

COI and 16S sequences (16 sequences for 16S and 14 sequences for COI) were generated for 16 slug specimens. The samples were identified as belonging to either *Arion vulgaris* (N = 14) or *Deroceras reticulatum* (N = 2). *Arion vulgaris* (N = 1587) was the most abundant slug species found in the current epidemiological study, followed by *Deroceras reticulatum* (N = 699).