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# Contributions to the Biodiversity and Biogeography of the Genus Varestrongylus Bhalerao, 1932 (Nematoda: Protostrongylidae), Lungworms of Ungulates, with Emphasis on a New Nearctic Species

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# Contributions to the Biodiversity and Biogeography of the Genus *Varestrongylus* Bhalerao, 1932 (Nematoda: Protostrongylidae), Lungworms of Ungulates, with Emphasis on a New Nearctic Species

by

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## Abstract

Explorations of parasite biodiversity and biogeography are essential for elucidating evolutionary and ecological patterns and processes that shaped the biosphere. Among relevant nematode parasites of ungulates are species within the family Protostrongylidae Leiper, 1926 (Strongylida: Metastrongyloidea), which often cause pulmonary, muscular or neurological diseases in their hosts. The finding of a novel protostrongylid species infecting North American ungulates in 2007 demonstrated that the knowledge of the biodiversity of this group remained incomplete, despite the substantial advances in recent decades. Through my thesis research I focused on developing and understanding the biodiversity, phylogenetic relationships, and biogeography of the genus *Varestrongylus* Bhalerao, 1932 (Nematoda: Protostrongylidae), lungworms of cervids and caprine bovids. To do this, I first explored the validity of existing species, and have resurrected and redescribed *Varestrongylus alces* Demidova & Naumitscheva, 1953 of the Eurasian moose from Europe. Secondly, I described *Varestrongylus eleguneniensis* Verocai, Kutz, Simard & Hoberg, 2014. This new species primarily infects caribou, but also muskoxen and, rarely moose across a vast range in North America. By exploring the relationships among *Varestrongylus* species, I have developed a phylogenetic hypothesis for the genus and for co-evolution and biogeography of this host-parasite assemblage. *Varestrongylus* has a complex history, characterized by multiple events of host-switching and geographic expansion. Moreover, through a geographically wide assessment of the distribution of *V. eleguneniensis* in caribou, muskoxen and moose populations across North America I have established baselines for future studies monitoring the geographic distribution of a lungworm species. This work also provides a foundation for understanding historical and current processes affecting the biogeography of host-

parasite assemblages. The present work is an original contribution to the field by adding to the present knowledge on taxonomy and biodiversity of this relevant group of parasites, to the general understanding of the formation of host-parasite assemblages, and the colonization of North America by both parasites and hosts. Ultimately, by understanding the historical and contemporary processes that have lead to current biogeography of *V. eleguneniensis,* this work can be used for predicting future trends on the distribution, host-associations and biogeography of *V. eleguneniensis* under a scenario of rapid climate change.

## Preface

The following manuscripts have been published in peer-reviewed journals. Guilherme G. Verocai collected and analysed the data, interpreted the results and wrote the papers with guidance from his supervisor, selected members of his supervisory committee and other collaborators. All co-authors contributed technically and intellectually to the content of the papers, and provided critical comments prior to publication. Written permission for reproduction of the scientific articles in its entirety for this thesis has been obtained from the publishers and all co-authors.

Verocai, G.G., Hoberg, E. P., Vikøren, T., Handeland, K., Ytrehus, B., Rezansoff, A. M., Davidson, R. K., Gilleard, J. S., Kutz, S. J., 2014, Resurrection and redescription of *Varestrongylus alces* (Nematoda; Protostrongylidae), a lungworm of the Eurasian moose (*Alces alces*), with report on associated pathology. Parasites & Vectors 7, 557 [Chapter Two]

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I extend my sincere thankfulness to all other members of my supervisory committee, Drs John Gilleard, Alessandro Massolo, and Sean Rogers. Thank you very much for contributing your diverse expertise to this project, for providing me with much valued guidance, and broadening my horizons. A special thanks to John, for all your guidance and mentorship in my career development and plans.

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*I dedicate this thesis to my family that from far away gave me the means and support for me to get here and conclude this work.*

*My parents: Waldyr de Souza Verocai Filho and Rosângela Gomes Verocai.*

*My sisters: Flávia Gomes Verocai, Marcela Gomes Verocai, Renata Gomes Verocai.*

*My nephew: Victor Rieschi Verocai .*

*My grandmothers: Iris Côrtes Verocai and Maria Eunice Gomes da Silva.*

*My late grandfathers: Waldyr de Souza Verocai and Wilmon Gomes da Silva.*

*Among many people, these were without a doubt the most crucial for the formation of my character…*

*… and to the parasites*

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## List of Symbols, Abbreviations and Nomenclature

| Symbol | Definition |
|---|---|
| CI | Consistency index |
| DSL | Dorsal-spined larvae |
| ICZN | International Code for Zoological Nomenclature |
| IUCN | International Union for Conservation of Nature |
| IH | Intermediate host |
| L1 | First-stage larvae |
| L2 | Second-stage larvae |
| L3 | Third-stage larvae |
| LPG | Larvae per gram of feces |
| MPT | Most parsimonious tree |
| USNPC | United States National Parasite Collection |

To know is to know that you know nothing. That is the meaning of true knowledge.

- Socrates

# Chapter One: **INTRODUCTION**

## 1.1 Background

### *1.1.1 Parasites biodiversity and biogeography*

Parasitic species account for over 50% of global biodiversity, encompassing all kingdoms proving not only the diverse nature of parasites, but also the success of the parasitic life-style (Dobson et al., 2008; Poulin and Morand, 2004). Parasitism, as a complex evolutionary adaptation, emerged independently across all kingdoms, including Animalia. Despite their fascinating nature, great diversity, and important ecological roles, parasites generally have not been adequately considered in the context of biodiversity (Dobson et al., 2008; Hoberg et al., 2013; Poulin, 2014; Poulin and Morand, 2004). A deep understanding of global parasite biodiversity is fundamental for us to elucidate evolutionary and ecological patterns and processes that shaped the biosphere, and substantially contributes to different fields such as conservation biology, disease ecology, ecosystem health, and human and veterinary medicine (Brooks et al., 2014; Hoberg et al., 2014; Hoberg et al., 2008; Polley and Thompson, 2009; Poulin, 2014).

Of special interest in studying such intricate inter-specific relationships are species within the diverse and ubiquitous Phylum Nematoda. Nematoda encompasses organisms that are free-living and parasitic on plant and animal hosts, including some that share the same Class or Order, which emphasizes that the parasitic life-style independently evolved across and within nematode lineages (Blaxter et al., 1998; Gilabert and Wasmuth, 2013). More specifically, the focus of my work is nematode parasites of mammals within the Family Protostrongylidae Leiper, 1926 (Strongylida: Metastrongylina: Metastrongyloidea), which will be later assessed in detail.

The complexities that shaped the biodiversity of host species, also and likely in an even more intricate fashion, also molded the diversity of parasitic species. This is because parasitic

species depend - to different extents - on their hosts (i.e., living on or inside their bodies, depending on hosts' resources) and, therefore, have followed their hosts in the successive events of geographic expansion and retraction. These events contributed to isolation of populations and, often concurrently, created zones of contact of parasites with other potential hosts. Across space and time, these patterns and processes determined diversification, extinctions, and the appearance of novel host-parasite assemblages (Hoberg and Brooks, 2008; Hoberg and Brooks, 2010, 2013).

Until recent decades, the vicariance paradigm prevailed as a model for exploring and explaining the patterns and processes that structured the global biota, including parasites. This concept sustains that a new species is the result of the permanent geographic separation and isolation of a given subpopulation that, across time, differentiated from the original population (allopatric speciation). Recently, however, this paradigm has been challenged by the integration of several hypotheses that better describe the complex structure of faunas based on empirical data across various taxonomic groups of metazoan parasites, Hoberg and Brooks (Hoberg and Brooks, 2008; Hoberg and Brooks, 2010) suggested that vicariance alone has limited explanatory power for the understanding of parasite biodiversity and host-parasite assemblages. The current fauna is instead, a complex mosaic assembled across space and time by various interacting factors.

As alternative (and complementary) hypotheses to explain parasite biodiversity, new concepts were brought to the discipline of parasitology. In detail, these included ecological fitting, the oscillation hypotheses, the geographic mosaic theory of coevolution, and the taxon pulse hypothesis, which altogether composes the 'Stockholm Paradigm' (Agosta et al., 2010; Hoberg et al., 2014; Hoberg and Brooks, 2008; Hoberg and Brooks, 2010, 2013). Ecological

fitting is the process where an organism colonizes and persists in new environments, uses novel resources or forms novel associations with other species (host) as a result of pre-existing traits carried by itself at the time they encounter the novel conditions. In a parasitological perspective, ecological fitting determines the initial host range of a species, and also the intrinsic potential of a parasitic species to colonize new hosts (Agosta et al., 2010; Hoberg and Brooks, 2008). The oscillation hypothesis consists of episodes of increasing host range alternated with isolation of parasite subpopulations in particular hosts, leading to speciation (Hoberg and Brooks, 2008; Hoberg and Brooks, 2010). The geographic mosaic of coevolution defines the microevolutionary dynamics among new assemblages of interacting host and parasite species, in which an ancestral generalist parasite (i.e.; multi-host or resource generalist) may evolve/diversify into a new specialist parasitic species (e.g.; single host or resource specialist) (Hoberg and Brooks, 2008; Hoberg and Brooks, 2010). Finally, the taxon pulse hypothesis added the realistic complexity of the cyclical existence and breakdown of geographic barriers facilitating events of expansion and isolation of both host and parasite species, with multiple consequences to the biota, from extinction to diversification (Hoberg and Brooks, 2008; Hoberg and Brooks, 2010, 2013).

Generally, historical associations of hosts and parasites can be elucidated by interactions among the processes described above. Together, these interactions created opportunities for episodes of host-switching (followed by speciation) and host-colonization (not followed by speciation, but a wider host range), generating the mosaic structure of currently recognized host-parasite assemblages (Hoberg, 2010; Hoberg and Brooks, 2008; Hoberg and Brooks, 2010; Hoberg et al., 2012b). Such processes naturally occurred across geological, evolutionary and historical time, and are in fact dynamic and continuous. These processes took millions of years to happen, and were mainly dependent on time, geography and climate. Humanity accelerated the

pace of transformations, directly by translocating domestic and wild species into new regions, transforming habitats, decimating species, or and indirectly through impacts on climate change (Hoberg et al., 2014; Hoberg et al., 2012b).

Because of their complex histories and intimate association with hosts, the study of biodiversity and biogeography of parasites is relevant for understanding overall evolutionary processes through which parasites diversified, for elucidating the intricate histories of host species, and also for guiding future studies on biodiversity prospecting and monitoring parasite distribution. Nematodes of the Family Protostrongylidae Leiper, 1926, because of their complex life-cycle and responsiveness to climate, serve as a great model for understanding historical and current processes affecting the biogeography of host-parasite assemblages, and ultimately, for predicting future trends under a scenario of rapid climate change (Hoberg and Brooks, 2008; Hoberg et al., 2012b; Kutz et al., 2012a; Kutz et al., 2014; Kutz et al., 2009; Molnár et al., 2013). Arctic and sub-Arctic ecosystems are relatively simple systems with lower biodiversity and are less impacted by direct anthropogenic perturbations when contrasted to southern ecosystems, and therefore, have been suggested as a good model for understanding the influence of climate change on parasitic and other infectious diseases in more complex systems, such as temperate and tropical regions (Kutz et al., 2014; Kutz et al., 2009).

### 1.2 The study system: the Family Protostrongylidae Leiper, 1926

Throughout this thesis, my focus is on parasitic nematodes of mammals within the family Protostrongylidae, with emphasis on the pulmonary nematodes within the protostrongylid genus *Varestrongylus* Bhalerao, 1932.

Nematodes within the family Protostrongylidae are important and often pathogenic parasites of ungulates and lagomorphs globally (Anderson, 2000; Lankester, 2001), and are more diverse across the northern hemisphere. Species of four subfamilies within this family are known to infect ungulate hosts: Muelleriinae Skrjabin, 1933; Elaphostrongylinae Boev and Shulz, 1950, Varestrongylinae Boev, 1968; and Protostrongylinae Kamensky, 1905 (Carreno and Hoberg, 1999). Different genera and species within these subfamilies infecting ungulates will be either studied or discussed within the thesis. In contrast, only species within Protostrongylinae infect lagomorphs, and these will not be the focus of this work.

Adults of some protostrongylid species inhabit the respiratory tract of ungulates and may lead to parasitic pneumonia and respiratory disease. Adults of other species reside in skeletal muscles causing myositis, affecting gait and body condition; or the central nervous system causing debilitating neurological disease (Anderson, 2000; Boev, 1975; Lankester, 2001). For all protostrongylid species the first stage larvae may cause significant lung pathology. As previously speculated, animals infected with protostrongylids may be more prone to predation (Jenkins et al., 2005b; Kutz et al., 2012a). Protostrongylids have indirect life cycles requiring gastropod intermediate hosts for development. For lung-dwelling species, eggs produced by the females hatch in the parenchyma or airways, releasing first-stage larvae (L1) that move up the respiratory tree, are swallowed and passed in the feces. Adults of tissue-dwelling species deposit eggs in the blood stream and these are transported to the lungs. There, L1 hatch and break into the alveoli, and follow the same route as larvae of pulmonary species. Once released in the feces, the L1 must enter a suitable gastropod intermediate host in which the larvae can develop to the infective third stage. Mammalian hosts become infected when they accidentally ingest gastropods harbouring the infective third-stage larvae (L3) (Anderson, 2000; Lankester, 2001) or L3 that

have spontaneously emerged from the gastropods and are present in the environment (Kutz et al., 2000, 2001b).

Additional information on protostrongylid species infecting wild ungulates of high latitudes of North America can be found in a review article by Kutz and colleagues (2012a).

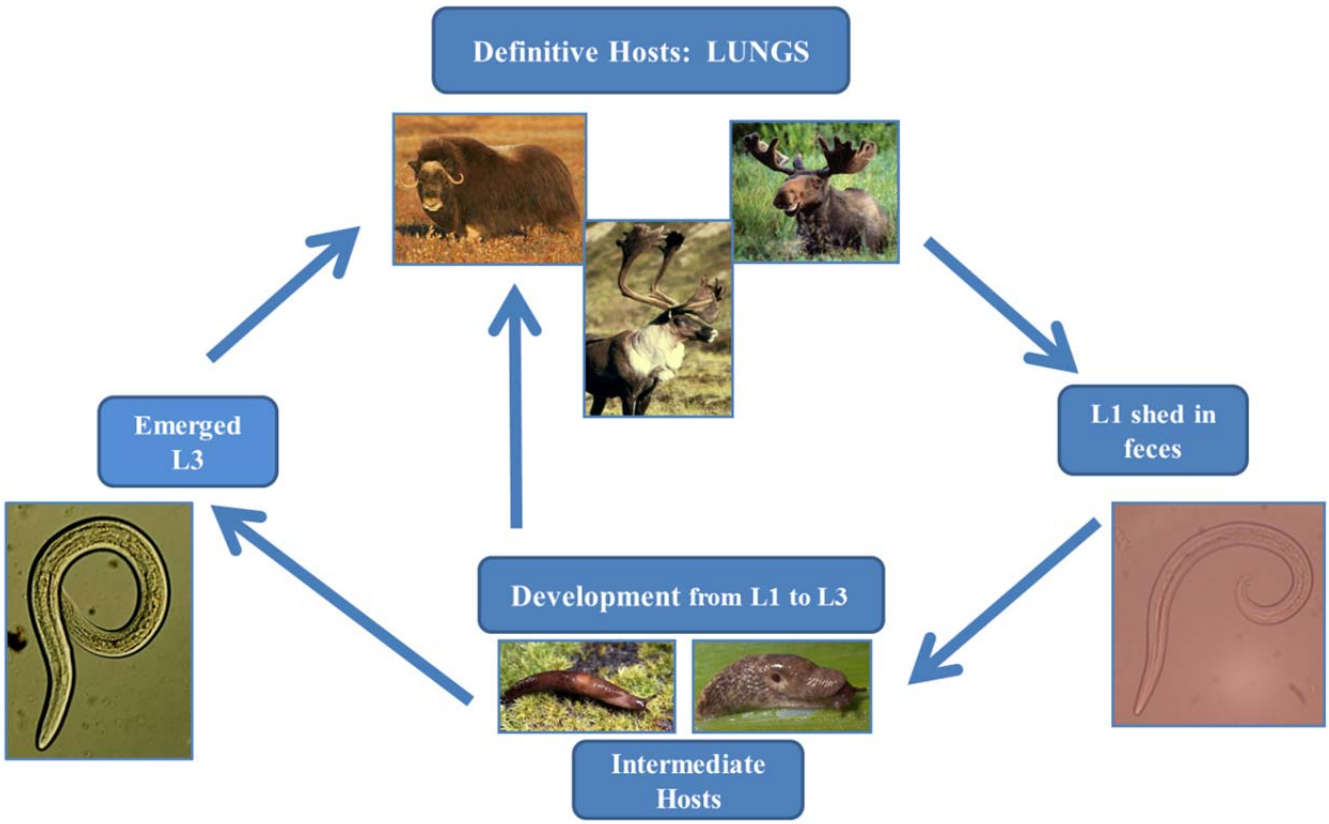


**Figure 1.1.** Example of the indirect life-cycle of the protostrongylid lungworm, based on *Varestrongylus eleguneniensis*, species described in Chapter 3.

### 1.3 Project Rationale and Problem Defined

In the last two decades there have been significant advances in the knowledge of the biodiversity of protostrongylid parasites. Among these advances are the description of a new genus and species and findings of putative undescribed species, demonstration of numerous novel host-associations, and relevant insights on historical and current biogeography of species within this group in the Americas (Asmundsson et al., 2008; Carreno et al., 2012; Carreno et al., 2001; Hoberg et al., 2002; Hoberg et al., 1995; Jenkins et al., 2005a; Kutz et al., 2007; Kutz et al., 2013a; Kutz et al., 2001c). The majority of this body of knowledge comprises the history and discovery of protostrongylids infecting ungulates at high latitudes of North America. The first significant discovery that triggered much of these advances was the finding and description of a novel protostrongylid genus and species within the subfamily Muelleriinae, *Umingmakstrongylus pallikuukensis* Hoberg, Polley, Gunn & Nishi, 1995 a lungworm specific to muskoxen (Gunn and Wobeser, 1993; Hoberg et al., 1995). This initial finding attracted great interest of scientists for understanding the biodiversity of protostrongylids, and also Arctic biodiversity as a whole (Hoberg et al., 2012b; Hoberg et al., 2013; Kutz et al., 2012a). Apart from *U. pallikuukensis*, other relatively well studied protostrongylid species were the protostrongyline lungworm *Protostrongylus stilesi* (Dikmans, 1931), and the elaphostrongyline muscle-worms *Parelaphostrongylus andersoni* Prestwood, 1972, and *Parelaphostrongylus odocoilei* (Hobmaier & Hobmaier, 1934). A much greater geographic distribution and host associations were recognized for these three species (Asmundsson et al., 2008; Hoberg et al., 2002; Jenkins et al., 2005a; Jenkins et al., 2005b; Kutz et al., 2007; Kutz et al., 2001c; Mortenson et al., 2006). In addition, as protostrongylids have a complex life-cycle, they are of particular interest for understanding the response of parasites to changing climatic conditions in an extreme

environment such as the Arctic, including parasite development and dynamics, and modification of their distributions (Jenkins et al., 2006; Kutz et al., 2005; Molnár et al., 2013).

As a serendipitous consequence of the series of studies on protostrongylids of northern North America, yet another species was discovered. This putative new species had a broad geographic distribution and host range across the continent (Kutz et al., 2007). This novel parasite was molecularly characterized based on larvae from the feces, and was most similar to *Varestrongylus alpenae* (Dikmans, 1935), a lungworm of white-tailed deer, *Odocoileus virginianus* (Zimmermann, 1780). At that time, however, no taxonomical description was provided since adult parasites were not isolated. Based on larval recovery and sequencing, however, it was reported across a broad geographic range, extending throughout the Nearctic, including five Canadian provinces or territories: Newfoundland and Labrador, Quebec, Nunavut, Northwest Territories and Yukon, as well as Alaska, USA, and was found in woodland, barrenground and Grant's caribou, both muskox subspecies, and moose (*Alces alces gigas* Miller, 1899) (Kutz et al., 2007). The discovery of this new parasite species prompted further work, including a formal taxonomic description of the novel species, and a better definition of its host associations, geographic distribution, and understanding its biogeography, which became the topic of my thesis.

### *1.3.1 The Subfamily Varestrongylinae Boev, 1968*

The subfamily Varestrongylinae was erected by Boev in 1968 (Boev, 1968), in his revision of the subfamily Capreocaulinae, in which the genus *Varestrongylus* Bhalerao, 1932 was enlarged by reducing the genera *Capreocaulus* Schulz & Kadenazy, 1948 and *Leptostrongylus* Dougherty & Goble, 1946 to synonyms of the former, and thus transferring their species to it (Boev, 1975)(Boev, 1975). For the following decades the subfamily comprised

three genera: *Varestrongylus*, *Pneumostrongylus* Mönnig, 1932, and *Pneumocaulus* Schulz and Andreeva, 1948 (Boev, 1975; Carreno and Hoberg, 1999), all infecting the lungs of ungulate hosts. After a comprehensive genus-level phylogenetic analysis of the family Protostrongylidae based on morphological characters, Carreno and Hoberg (Carreno and Hoberg, 1999), suggested the exclusion of *Pneumocaulus*. Therefore, currently, this subfamily comprises only two genera: *Varestrongylus* from cervids and caprines across Eurasia and North America, and *Pneumostrongylus*, with two species infecting African ruminants. However, the relationships among species within the two varestrongyline genera remain unclear, as a species-level phylogenetic analysis by the same authors suggested that *Varestrongylus* was paraphyletic relative to *Pneumostrongylus*, although this may have reflected the non-inclusion of some attributes directly relevant for the Varestrongylinae and for the former taxon.

### *1.3.2 The genus Varestrongylus Bhalerao, 1932*

Within the Family Protostrongylidae, the genus *Varestrongylus* is the second most taxonomically diverse genus, only after *Protostrongylus* Kamensky, 1905 (Boev, 1975), and includes lungworms of wild and domestic ungulates, more specifically cervids and caprine bovids. Adults are found strictly in the parenchyma and bronchi of the lungs of their hosts, and are of variable veterinary importance. Many species cause verminous pneumonia, as a result of focal changes in the lungs of affected hosts (Bhatia and Pande, 1960; Boev, 1975; Panayotova-Pencheva, 2008; Petrosyan, 1963; Stroh and Schmid, 1938). Their life-cycles follow the generalities for the Family depicted in Figure 1.1. Morphologically, the genus is diagnosed mainly by copulatory structures of males and females (Boev, 1975; Carreno and Hoberg, 1999), but exceptions and variations in other characters are numerous.

The relatively neglected lungworm genus *Varestrongylus* does not have the same economic importance in animal production or evident impacts on wildlife health and conservation as related species within the subfamilies Protostrongylinae Kamensky, 1905; Muelleriinae Skrjabin, 1950 and Elaphostrongylinae Boev & Schulz, 1950. Therefore, the study of *Varestrongylus* species has been often overshadowed by these co-existing related protostrongylids. In addition, the delicate nature of specimens, obscure localization in the host lungs (often not causing gross lesions), and associations with hosts distributed in areas that are difficult to access or that are protected species of conservation relevance, may have also contributed to the scarcity of studies on *Varestrongylus* species.

Prior to my thesis work, the generic biodiversity of *Varestrongylus* comprised eight valid species, of which seven are native to Eurasia and a single one is native to North America (Boev, 1975; Liu, 1989; Liu, 1984). Previous phylogenetic analyses and review of generic biogeography support an origin in the Palearctic, current Eurasia (centre of diversity of Pecoran hosts, in particular cervids and caprine bovids), with subsequent expansion into other ecozones (Carreno and Hoberg, 1999; Hoberg et al., 2012) (for a better understanding of biogeographic ecozones see: Figure 1 of Appendix I). Also, the same authors postulate cervids as ancestral hosts for *Varestrongylus*. However, since the last comprehensive revision of the genus *Varestrongylus* (Boev, 1975), two additional species have been described from domestic and wild caprine hosts in China (Liu, 1989; Liu, 1984). Thus, an emended diagnosis for the genus was pending, in order to account for the significant morphological variations among species.

Furthermore, given the diversity within *Varestrongylus*, and its unstable taxonomic history, the validity of another species needed to be investigated. This is the case of *Varestrongylus alces* Demidova & Naumitscheva, 1953, from Eurasian moose (*Alces alces* L.),

considered in the last revision of the genus as a junior-synonym of *Varestrongylus capreoli* Stroh & Schmidt 1938 in European roe deer (*Capreolus capreolus* (L.)), due to a poorly detailed description (Boev, 1975; Demidova and Naumitscheva, 1953). This potential erroneous synonymy was impeding the appreciation of the actual diversity within the genus, and needed to be addressed prior to the recognition and description of the novel North American species.

Also limited is the current understanding on the origins and phylogenetic relationships within *Varestrongylus*, as the most recent assessment of the Protostrongylidae only included three of its valid species. Possibly due to this misrepresentation of the intra-generic diversity, *Varestrongylus* was considered paraphyletic, as *Pneumostrongylus* was included within the *Varestrongylus* clade (Carreno and Hoberg, 1999). Therefore, questions on the validity and priority of these two genera arose, also because both were proposed in 1932. Because of the current scenario, a comprehensive assessment and phylogenetic inference of species within *Varestrongylus* is essential, in a broader context within the family. By understanding the relationship among species, a framework was built to investigate their host-associations and historical biogeography, linking to general processes that shaped biodiversity and host-parasite assemblages.

### 1.4 Thesis Aims and Chapters: A Brief Overview

My doctoral thesis aimed to advance the current knowledge of the biodiversity of Protostrongylidae with a specific emphasis on *Varestrongylus*, and to explore phylogenetic relationships among *Varestrongylus* species in order to develop hypotheses for speciation, co-evolution and biogeography of this host-parasite assemblage. Moreover, my goals were to enhance our understanding of historical and current processes affecting the biogeography of

*Varestrongylus* and that of its hosts, and ultimately, provide a foundation for predicting future trends under scenarios of accelerated climate change. My work is an original contribution to the field, adding to the present knowledge on taxonomy and biodiversity of this relevant group of parasites, and to the general understanding of the formation of host-parasite assemblages, and the colonization of North America by both parasites and hosts.

**Chapter 2** reassesses the taxonomic validity of a *Varestrongylus* species. Previous work erroneously considered *V. alces*, a parasite of the lungs of the Eurasian moose (*Alces alces* L.), as a synonym of *V. capreoli* in roe deer *Capreolus capreolus*. In this chapter, *V. alces* is resurrected and redescribed using integrated molecular and morphological evidence. This chapter contributes to the knowledge on the biodiversity of the genus, provides a discussion on the historical and current biogeography of the species, and also reports the pathology associated with the infection of the lungs of the Eurasian moose.

**Chapter 3** presents the taxonomic description of new a species within the genus *Varestrongylus* - *Varestrongylus eleguneniensis* Verocai, Kutz, Simard & Hoberg, 2014. Similar to the previous chapter, I have used integrated approaches in parasitology which permitted to differentiate this new species from all of the previously existing species of *Varestrongylus*. Within this chapter, the generic diagnosis for *Varestrongylus* is emended, and the historical biogeography of the new species is discussed. A contribution is made to the biodiversity and taxonomy of this lungworm genus and its intricate host and geographic associations, which are further explored in the following chapter.

**Chapter 4** explores the broader diversity of species within *Varestrongylus*, their geographic and host associations, and host-parasite historical biogeography. The primary phylogenetic analysis is based on morphological characters of all ten valid species within the genus. Further, the phylogenetic inference for the genus is contrasted to the phylogeny of their ungulate hosts, revealing a complex history involving events of host-switching and geographic expansion. Secondary bouts of host colonization occurred both in natural systems and systems altered by anthropogenic influence in the recent past and present. The exploration of the histories of the diversity within genus *Varestrongylus* is paralleled with the complexities of the formation of faunas, and host-parasite assemblages through space and time.

**Chapters 5** deals with the current geographic distribution of the newly described 'caribou lungworm', *V. eleguneniensis*, in wild ungulates across North America. Here, my goal was to better define the distribution of this species, through geographically extensive parasitological assessment of caribou, muskox and moose fecal samples. This resulted in a substantial expansion on the knowledge of the range of *V. eleguneniensis*, which is spread from coast to coast. In addition, relevant information on the distribution of the muscle-dwelling elaphostrongyline *P. andersoni* is provided (and also in Appendix A; Verocai et al., 2013). Surveys such as this are fundamental baselines for monitoring and assessing changing patterns of geographic distributions under a regime of accelerated climate change and other anthropogenic factors including introductions and translocations related to management. Results from this chapter and related studies from our research group suggest the caribou lungworm is undergoing a northward range shift, influenced by climate change and human pressure since the European colonization of North America. Also, the combination of past and present information on this

host(s)-parasite assemblage provides a framework to postulate hypotheses on the future biogeography and persistence of this lungworm in different scenarios.

Finally, **Chapter 6** summarizes my doctoral research, which provided a comprehensive understanding of the biodiversity, phylogenetic relationships, historical biogeography, and host and geographic associations of the genus *Varestrongylus*. Also, I highlight that integrated approaches in parasitology should be applied to better understand the past, present and future of biogeography host-parasites assemblages.

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Chapter Two: **RESURRECTION AND REDESCRIPTION OF *VARESTRONGYLUS ALCES* (NEMATODA: PROTOSTRONGYLIDAE), A LUNGWORM OF THE EURASIAN MOOSE (*ALCES ALCES*), WITH REPORT ON ASSOCIATED PATHOLOGY**

## 2.1 Abstract

*Varestrongylus alces*, a lungworm in Eurasian moose from Europe has been considered a junior synonym of *Varestrongylus capreoli*, in European roe deer, due to a poorly detailed morphological description and the absence of a type-series. Specimens used in the redescription were collected from lesions in the lungs of Eurasian moose, from Vestby, Norway. Specimens were described based on comparative morphology and integrated approaches. Molecular identification was based on PCR, cloning and sequencing of the ITS-2 region of the nuclear ribosomal DNA. Phylogenetic analysis compared *V. alces* ITS-2 sequences to these of other *Varestrongylus* species and other protostrongylids. *Varestrongylus alces* is resurrected for protostrongylid nematodes of Eurasian moose from Europe. *Varestrongylus alces* causes firm nodular lesions that are clearly differentiated from the adjacent lung tissue. Histologically, lesions are restricted to the parenchyma with adult, egg and larval parasites surrounded by multinucleated giant cells, macrophages, eosinophilic granulocytes, and lymphocytes. The species is valid and distinct from others referred to *Varestrongylus*, and should be separated from *V. capreoli*. Morphologically, *V. alces* can be distinguished from other species by characters in the males that include a distally bifurcated gubernaculum, arched denticulate crura, spicules that are equal in length and relatively short, and a dorsal ray that is elongate and bifurcated. Females have a well-developed provagina, and are very similar to those of *V. capreoli*. Morphometrics of first-stage larvae largely overlap with those of other *Varestrongylus*. Sequences of the ITS-2 region strongly support mutual independence of *V. alces*, *V.* cf. *capreoli*, and the yet undescribed species of *Varestrongylus* from North American ungulates. These three taxa form a well-supported crown-clade as the putative sister of *V. alpenae*. The association of *V. alces* and *Alces*

or its ancestors is discussed in light of host and parasite phylogeny and host historical biogeography. *Varestrongylus alces* is a valid species, and should be considered distinct from *V. capreoli*. Phylogenetic relationships among *Varestrongylus* spp. from Eurasia and North America are complex and consistent with faunal assembly involving recurrent events of geographic expansion, host switching and subsequent speciation.

## 2.2 Introduction

The Family Protostrongylidae Leiper, 1926 (Metastrongylina) is comprised of six subfamilies: Protostrongylinae Kamensky, 1905; Muelleriinae Skrjabin, 1933; Elaphostrongylinae Boev & Shulz, 1950; Neostrongylinae Boev & Shulz, 1950; Skrjabincaulinae Boev & Sulimov, 1963; and Varestrongylinae Boev, 1968 (Boev, 1975; Carreno and Hoberg, 1999). Representative species of all subfamilies occur in the Palaearctic, and are often pathogenic parasites of Artiodactyla, especially cervids and caprines, and Lagomorpha. Adult nematodes of species within Varestrongylinae, including those within the genus *Varestrongylus* Bhalerao, 1932, reside in the lung parenchyma, bronchi and bronchioles of their hosts, and cause verminous pneumonia (Bhatia and Pande, 1960; Petrosyan, 1963; Stroh and Schmid, 1938). Similar to other protostrongylids, definitive hosts are infected by *Varestrongylus* spp. through ingestion of infective third-stage larvae (L3) contained within gastropod intermediate hosts (IH) or, possibly, L3 that have emerged from the gastropods (Anderson, 2000; Kutz et al., 2000; Lankester, 2001).

The majority of species within *Varestrongylus* are endemic to Eurasia, which is the centre of diversity for this genus and their hosts (Carreno and Hoberg, 1999; Hernández-Fernández and Vrba, 2005; Hoberg et al., 2012b; Kurtén and Anderson, 1980). Currently, the Eurasian biodiversity of *Varestrongylus* includes seven species, infecting an array of hosts within Bovidae (Caprinae) and Cervidae (Cervinae and Odocoileinae or Capreolinae *sensu* (Grubb, 2005)): *Varestrongylus sagittatus* (Mueller 1890), *Varestrongylus pneumonicus* Bhalerao, 1932, *Varestrongylus capreoli* (Stroh & Schmid, 1938), *Varestrongylus capricola* Sarwar, 1944, *Varestrongylus tuvae* (Boev & Sulimov, 1963), *Varestrongylus qinghaiensis* Liu, 1984 and

*Varestrongylus longispiculatus* Liu, 1989 (Anderson, 2000; Boev, 1975; Liu, 1989; Liu, 1984). This Eurasian fauna is significantly richer when contrasted with the diversity *Varestrongylus* in the Nearctic which, to date, includes only one described species, *Varestrongylus alpenae* (Dikmans 1935), and an as yet undescribed taxon that is known from sequence data and first stage larvae (Kutz et al., 2007; Verocai et al., 2011).

Not surprisingly, given its diverse nature, the taxonomic history for this genus has been markedly unstable, with several taxa having inconsistently been reduced as junior synonyms (Boev, 1968, 1975; Dougherty, 1945; Dougherty and Goble, 1946). One such example is *V. alces*, originally described in the Eurasian moose (also known as Eurasian elk) (*Alces alces* L.) from Russia (Demidova and Naumitscheva, 1953). *Varestrongylus alces* was later synonymized with *V. capreoli* in European roe deer (*Capreolus capreolus* (L.) (Boev, 1975). Synonymy was primarily due to a vague, poorly illustrated description and assumptions about host distributions for these parasites, confounded by the absence of a designated type series deposited in a museum collection (Boev, 1975; Demidova and Naumitscheva, 1953; Arseny Makarikov, pers. comm.)

Despite apparent taxonomic confusion around the validity of *V. alces*, many authors continued to report this varestrongyline, usually as an incidental finding under various names including *V. capreoli*, *V. alces*, *Bicaulus alces or* '*Bicaulus alcis*' (sic). These identifications do not appear to have been confirmed through careful morphological examination, nor were these survey collections accompanied by voucher specimens in a recognized repository (Dróżdż, 1966; Handeland and Gibbons, 2001; Järvis, 1995; Nilsson, 1971; Stéen et al., 1998; Stuve, 1986, 1987). An additional factor that might have drawn attention away from *V. alces* was the description of the pathogenic, *Elaphostrongylus alces* Stéen, Chabaud & Rehbinder (Stéen et al.,

1989). This meningeal nematode, which shares its host and geographic range with *V. alces*, has irrefutable veterinary importance, causing neurologic disease in affected hosts, and commonly occurs in co-infections with its less pathogenic, pulmonary relative *V. alces* (Stéen et al., 1997; Stéen et al., 1989); additionally both species have dorsal-spined first stage larvae that would be largely indistinguishable.

Herein, using combined morphological and molecular approaches, we resurrect and redescribe *V. alces*, a protostrongylid lungworm in Eurasian moose. A proposal for designation of a neotype specimen and an associated series is presented. We report associated gross and histopathological findings, and comment on phylogenetic relationships among selected *Varestrongylus* species, their host-associations and biogeography.

## 2.3 Methods

### *2.3.1 Specimen collection*

Lungs of 13 Eurasian moose were examined for the presence of lungworms at the wildlife unit of the Norwegian Veterinary Institute (NVI), Oslo between October and December, 2011. All animals were harvested in the municipality of Vestby (59°30′N, 10°40′E), County of Akershus, East Norway Region, Norway.

Additional varestrongyline specimens, attributable to *V. capreoli* (hereafter named *V.* cf. *capreoli*) were recovered from lungs of two European roe deer at the NVI, an adult male and a female calf, from the same region.

Lungs from Eurasian moose and roe deer were examined for lungworms. Gross lesions consistent with *Varestrongylus* infection were removed, placed in saline solution, and finely

dissected to isolate adult nematodes. All intact worms or fragments of anterior and posterior extremities were collected, identified by gender, and stored in tagged vials containing 70% ethanol. The lung samples were also flushed with saline in order to isolate larvae and eggs. These were fixed in steaming 70% ethanol.

### *2.3.2 Morphological identification*

Adult specimens and fragments containing relevant morphological characters were mounted and cleared in phenol-alcohol, and examined under differential interference contrast microscopy (Table 1). In the redescription, measurements are in micrometers unless specified otherwise, and are presented with the numbers of adult male, female and larval nematodes examined (n=), and the range is followed by the mean ± 1 SD in parentheses. Adult specimens of other species of *Varestrongylus* were mounted and cleared in phenol-alcohol and examined microscopically. These included some species in potential sympatry with *V. alces*, and other prominent taxa in cervids (Table 2.1).

**Table 2.1.** Lungworm material collected and/or used in the study

| USNPC* | *Varestrongylus* species | Host | Country | Specimens | GenBank** |
|---|---|---|---|---|---|
| 106331 | *V. alces* Demidova & Naumitscheva 1953 | *Alces alces*[a] | Norway | 1♂ | KJ452181-83 |
| 106332 | *V. alces* | *A. alces*[a] | Norway | 1♂ | KJ452188-90 |
| 106333 | *V. alces* | *A. alces*[a] | Norway | 3♀ | NA |
| 106334 | *V. alces* | *A. alces*[a] | Norway | DSL | NA |
| 106335 | *V. alces* | *A. alces*[b] | Norway | 1♂, 2♀ | NA |
| 106336 | *V. alces* | *A. alces*[c] | Norway | 2♂, 3♀ | NA |
| 106337 | *V. alces* | *A. alces*[d] | Norway | 1♂ (neotype) | NA |
| 106338 | *V. alces* | *A. alces*[d] | Norway | 2♂, 3♀ | NA |
| 106339 | *V. alces* | *A. alces*[d] | Norway | ♀ | KJ452195-96 |
| 106340 | *V. alces* | *A. alces*[d] | Norway | ♂[g] | KJ452191-94 |
| NA | *V. alces* | *A. alces*[d] | Norway | fragment | KJ452184-87 |
| 106341 | *V.* cf. *capreoli* | *Capreolus capreolus*[e] | Norway | 6♂,5♀ | NA |
| 106342 | *V.* cf. *capreoli* | *C. capreolus*[e] | Norway | 1♀ | KJ452177-80 |
| 106343 | *V.* cf. *capreoli* | *C. capreolus*[e] | Norway | 1♀ | NA |
| 106344 | *V.* cf. *capreoli* | *C. capreolus*[f] | Norway | 1♀, DSL | NA |
| NA | *V.* cf. *capreoli* | *C. capreolus*[e] | Norway | fragment | KJ452174-76 |
| 104105 | *V. sagittatus* (Mueller 1890) | *Cervus elaphus* | Bulgaria | 1♂ | KJ439592-95 |
| 104105 | *V. sagittatus* | *C. elaphus* | Bulgaria | 1♀ | KJ439596-99 |

*Museum accession numbers; Additional host information (Eurasian moose): a. V-376, yearling female; b.V-377, yearling female; c.V-383, adult female; d. V-456, yearling male. Roe deer - e. V-379, adult male; f. V-510, adult female; g. broken specimen, not used for morphometry. ** Number of ITS-2 sequences varies according to number of clones yielded from DNA lysates of each individual worm.

Lungworm material collected and/or used in the study, with information on host and origin, and matching accession numbers for specimens deposited at the United States National Parasite Collection (USNPC) and sequences at the internal transcribed spacer-2 locus of the nuclear ribosomal DNA (ITS-2) deposited in GenBank.

**Table 2.2.** Additional *Varestrongylus* specimens from the United States National Parasite Collection (USNPC) morphologically examined.

| USNPC * | *Varestrongylus* species | Host | Locality | Specimens |
|---|---|---|---|---|
| 34066 | *V. alpenae* (Dikmans 1935) | *Odocoileus virginianus* | Michigan, USA | 1♂ (holotype) |
| 78599 | *V. alpenae* | *O. virginianus* | Alberta, Canada | 2♂, 1♀ |
| 37833 | *V. pneumonicus* Bhalerao, 1932[a] | *Ovis aries* | Alma-Ata, Kazakhstan | 1♂ |
| 37834 | *V. pneumonicus*[a] | *O. aries* | Alma-Ata, Kazakhstan | 1♂ |
| 45106 | *V. pneumonicus*[b] | *O. aries* | Lanchow, China | 2♂, 2♀ |
| 37851 | *V. sagittatus* (Mueller 1890)[c] | *Cervus elaphus* | Altai Mtns., Kazakhstan | 1♀ |
| 37855 | *V. sagittatus* | *C. elaphus* | Altai Mtns., Kazakhstan | 1♂ |
| 89171 | *V. sagittatus* | *C. elaphus* | Altai Region, Russia | 1♂, 1♀ |

*Museum accession numbers; [a] referred as *Bicaulus schulzi* (Boev and Wolf 1938); [b] referred as *V. sinicus* Dikmans 1945; [c] referred as *Bicaulus sagittatus* (Mueller 1890).

Eggs and first-stage dorsal-spined larvae (DSL) recovered from the lungs of one Eurasian moose (V-376) were microscopically examined. Measurements are in micrometers.

Specimens of *V.* cf. *capreoli* and *V. sagittatus* (Table 1), collected respectively from the lungs of European roe deer from Norway (by the authors) and the European red deer (*Cervus*

*elaphus*) in Bulgaria (by M. S. Panayotova-Pencheva), were processed for molecular-based comparisons according to methodology described below; sequences produced for both species were included in the phylogenetic analysis.

### *2.3.3 Gross and histopathology*

Gross pathologic changes in Eurasian moose lungs were documented during necropsy and dissection. Sections of fresh lung tissue were collected from one Eurasian moose (V-456), fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin (H&E) and van Gieson (VG) for histological examination.

### *2.3.4 Molecular analyses*

2.3.4.1 DNA extraction and amplification

Genomic DNA (gDNA) was extracted from small fragments of adult nematodes in 0.2 mL tubes containing 5 μL of deionized water and 25 μL of lysis buffer (0.5 mg/mL of proteinase K, 10× PCR buffer). The following DNA extraction protocol was used: tubes containing adult worm fragments were incubated at 60°C for 60 min, 65°C for 60 min, then at 95°C for 15 min. Extracted DNA was diluted 1:10. For species identification, a PCR was performed using primers NC1 (5′-ACG TCT GGT TCA GGG TTG TT-302B9) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) targeting the ITS-2 region of the nuclear ribosomal DNA (Kutz et al., 2007; Verocai et al., 2013). PCR amplification was performed in 40 μL reactions containing: 20.4 μL of water, 8 μL of 10× PCR buffer + $MgCl_2$, 0.8 μL of 10 mmol dNTPs, 4 μL (10 μM) of each primer, 0.4 μL of bovine serum albumin, 0.4 μL of *Taq* Phusion HF DNA polymerase, and 2 μL of DNA template. The amplification conditions used were an initial 2 min denaturation at 98°C, followed by 35 cycles of 98°C for 10 s, 52.5°C for 30 s, and 72°C for 30 s. A final extension phase of 72°C for 5 min was followed by cooling to 10°C (Verocai et al., 2013).

2.3.4.2 Cloning and sequencing

PCR products were gel-purified using e.Z.N.A MicroElute® Gel Extraction Kit (Omega Biotek) following the manufacturer's protocol. All 40 μL of the reactions were used. Gel-purified DNA was eluted in 15 μL nuclease free water. Gel purified DNA amplicons were then ligated using CloneJET PCR Cloning Kit according to manufacturer's instructions and transformed into Subcloning Efficiency™ DH5α™ Competent Cells. After overnight incubation on standard LB agar bacterial plates with 100 μg/mL ampicillin, four colonies were randomly selected from plates of each individual, and re-colonized in 3 mL LB broth. After a second overnight incubation these cultures were centrifuged to attain bacterial pellets, for which and plasmid DNA was prepared using e.Z.N.A Plasmid Mini-Kit I (Omega Biotek). Plasmid DNA isolates were then sequenced using NC1 and NC2 primers on BigDye Terminator Cycle Sequencing platform (Applied Biosystems).

2.3.4.3 Sequence analysis

A total of 31 clonal sequences representing 9 individuals (16 clones from 5 *V. alces* specimens, 7 clones from 2 *V.* cf. *capreoli*, 8 clones from 2 *V. sagittatus* individuals) passed quality control and were included in the analysis using Geneious Pro (Drummond et al., 2011). Once fully processed the 31 clones were realigned to attain pairwise distances among clones, and other protostrongylid ITS-2 sequences available in GenBank.

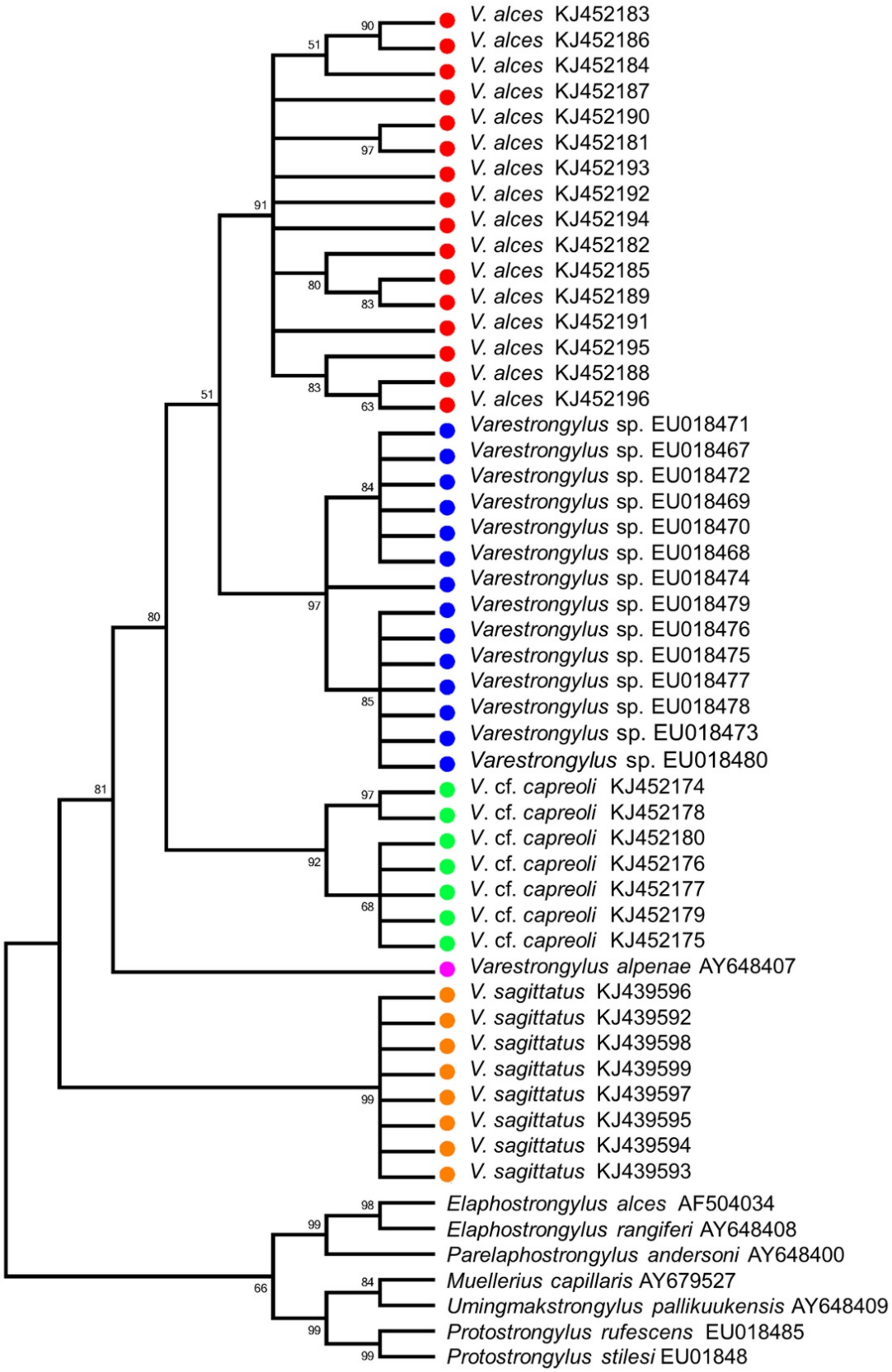
2.3.4.4 Phylogenetic analysis

Cloned ITS-2 sequences produced in this study for *V. alces*, *V.* cf. *capreoli* and *V. sagittatus* were compared to those of *V. alpenae*, and an undescribed species of *Varestrongylus* in wild North American ungulates (Kutz et al., 2007). Broader comparisons involved other

protostrongylids examined in prior studies (e.g., Kutz et al., 2007) with sequence data obtained from GenBank including representatives of Elaphostrongylinae (*E. alces, E. rangiferi* and *P. andersoni*), Muelleriinae (*Muellerius capillaris* (Mueller 1889), *Cystocaulus ocreatus* (Railliet & Henry, 1908), and *Umingmakstrongylus pallikuukensis* Hoberg, Polley, Gunn & Nishi, 1995) and Protostrongylinae (*Protostrongylus rufescens* (Leuckart, 1965) and *Protostrongylus stilesi* Dikmans, 1931) (accession numbers in Figure 2.1). Sequences were aligned using PRANK, a probabilistic multiple alignment program available through the European Bioinformatics Institute (http://www.ebi.ac.uk/goldman-srv/prank). Aligned sites were not filtered by posterior probability. Phylogenetic reconstruction analysis was performed using the maximum parsimony (MP) method in MEGA 5.2 (Tamura et al., 2011), with gaps treated as complete deletion (100%), sub-tree pruning regrafting as MP search model, and 5,000 bootstrap replicates.

Intra- and interspecific pairwise similarity was calculated for ITS-2 sequences of six different *Varestrongylus* spp., including the sequenced clones, using the distance matrix generated by Geneious Pro (Drummond et al., 2011).

Specimens of *V.* cf. *capreoli* and *V. sagittatus* (Table 2.1), collected respectively from the lungs of European roe deer from Norway (by the authors) and the European red deer (*Cervus elaphus*) in Bulgaria (by M. S. Panayotova-Pencheva), were processed for molecular-based comparisons according to methodology described below; sequences produced for both species were included in the phylogenetic analysis.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V. alces KJ452186
V. alces KJ452184
V. alces KJ452187
V. alces KJ452190
V. alces KJ452181
V. alces KJ452193
V. alces KJ452192
V. alces KJ452194
V. alces KJ452182
V. alces KJ452185
V. alces KJ452189
V. alces KJ452191
V. alces KJ452195
V. alces KJ452188
V. alces KJ452196
Varestrongylus sp. EU018471
Varestrongylus sp. EU018467
Varestrongylus sp. EU018472
Varestrongylus sp. EU018469
Varestrongylus sp. EU018470
Varestrongylus sp. EU018468
Varestrongylus sp. EU018474
Varestrongylus sp. EU018479
Varestrongylus sp. EU018476
Varestrongylus sp. EU018475
Varestrongylus sp. EU018477
Varestrongylus sp. EU018478
Varestrongylus sp. EU018473
Varestrongylus sp. EU018480
V. cf. capreoli KJ452174
V. cf. capreoli KJ452178
V. cf. capreoli KJ452180
V. cf. capreoli KJ452176
V. cf. capreoli KJ452177
V. cf. capreoli KJ452179
V. cf. capreoli KJ452175
Varestrongylus alpenae AY648407
V. sagittatus KJ439596
V. sagittatus KJ439592
V. sagittatus KJ439598
V. sagittatus KJ439599
V. sagittatus KJ439597
V. sagittatus KJ439595
V. sagittatus KJ439594
V. sagittatus KJ439593
Elaphostrongylus alces AF504034
Elaphostrongylus rangiferi AY648408
Parelaphostrongylus andersoni AY648400
Muellerius capillaris AY679527
Umingmakstrongylus pallikuukensis AY648409
Protostrongylus rufescens EU018485
Protostrongylus stilesi EU01848

**Figure 2.1.** Phylogenetic relationships among *Varestrongylus* species and other Protostrongylidae. Most-parsimonious tree depicting the independence of *Varestrongylus alces* and other *Varestrongylus* species, and the reciprocal monophyly of sequences within each. The bootstrap consensus tree inferred from 5,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5,000 replicates) shown next to the branches (Tamura et al., 2011).

## 2.4 Results

Nematode specimens used for this redescription of *V. alces* were isolated from the lungs of four (30.8%, n = 13) Eurasian moose. Infected hosts were: an adult female (V-383), two yearling females (V-376, V-377) and a yearling male (V-456).

### *2.4.1 Redescription*

***Varestrongylus alces* Demidova & Naumitscheva, 1953**

Syn.: *Bicaulus alces* (Demidova & Naumitscheva, 1953) Boev, 1957; *Varestrongylus capreoli* (in part., *sensu* Boev, 1975)

2.4.1.1 General description

Figures: 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8 and 2.1.

Protostrongylidae, Varestrongylinae, thin and minute nematodes, reddish brown prior to fixation with delicate, transversally striated cuticle. Cephalic extremity bluntly rounded. Buccal opening surrounded by four papillae. Esophagus cylindrical, clavate, broader at base, and poorly demarcated in muscular and glandular sections. Nerve ring indistinct, located at anterior or middle third of esophagus. Diminutive cervical papillae and excretory pore located at middle or posterior third of esophagus, always posterior to nerve ring.

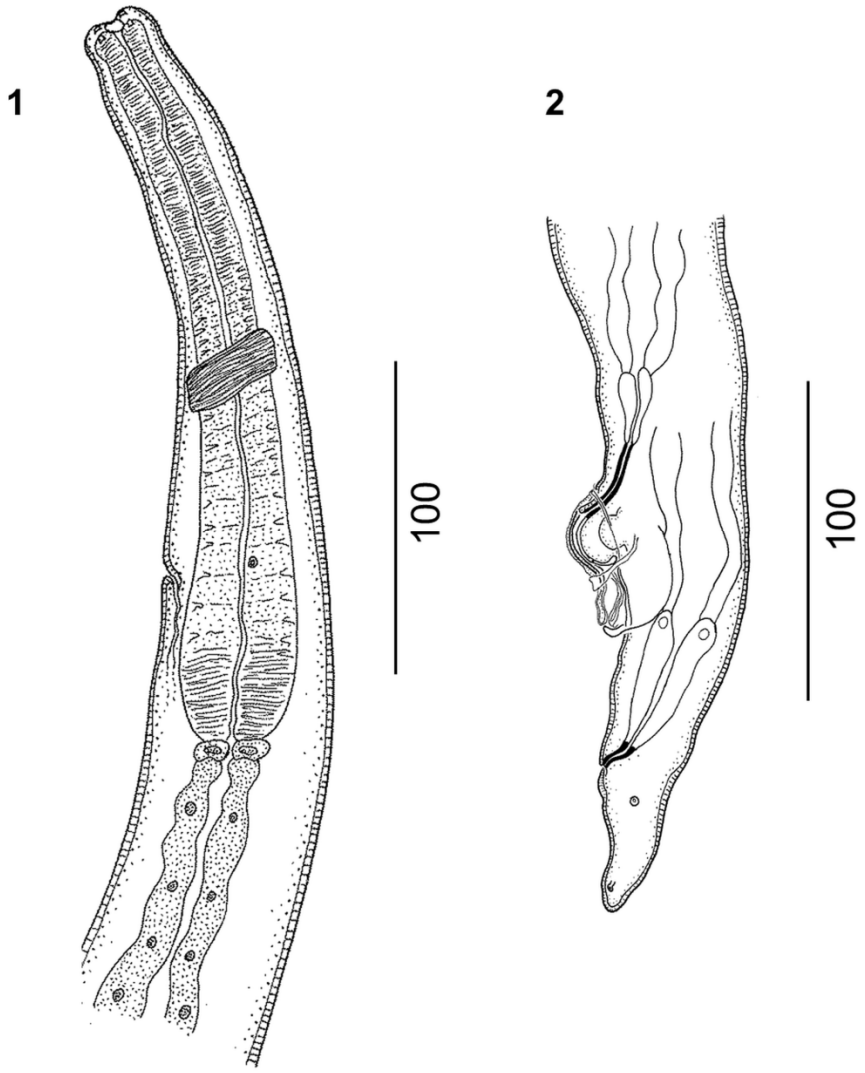

**Figure 2.2.** *Varestrongylus alces*. Female. **1.** Cephalic extremity of a female specimen at ventral view. **2.** Caudal extremity of a female specimen at lateral view, showing a developed provagina.

2.4.1.2 Males

Based on specimens in four Eurasian moose: six intact males, including neotype, and three fragments containing caudal extremities. Total length (n = 6) 11.36–14.7 mm (12.97 ± 2.01); maximum width (n = 6) 68.5–80 (74.2 ± 4.97). Esophagus (n = 5) 250–272 (264.5 ± 8.95) long, 32–37 (33.9 ± 2.19) wide, (n = 5) 1.6–2.3% (2.0 ± 0.28%) of body length. Body width at esophagus (n = 5) 53.8–61.9 (33.9 ± 2.19). Nerve-ring (n = 5) 68–89.65 (81.8 ± 9.04), cervical papillae (n = 3) 201–207 (203.4 ± 3.19), and excretory pore (n = 3) 208–230.3 (221.8 ± 9.83) from cephalic extremity. Copulatory bursa rounded, with indistinct dorsal lobe. Bursal rays approaching, rarely attaining margin of bursa. Body width at bursa (n = 7) 42–56 (48.3 ± 7.36), bursa length (n = 6) 75–90 (84.3 ± 5.86), bursa width (n = 3) 125–160 (140 ± 18.03). Ventro-ventral and latero-ventral rays equal, parallel, arising from common stalk, directed anteriad and isolated, tips of rays distally separate. Lateral rays arising from common base; externo-lateral elongate, attaining bursal margin, isolated from medio- and postero-lateral rays. Externo-lateral and medio-lateral rays of equal length. Medio-lateral rays long, postero-lateral rays reduced, with tips separate from near to less than half of common stalk. Externo-dorsal rays long, origins independent from base of dorsal ray. Dorsal ray elongate (n = 7) 18–30 (24.5 ± 3.65) long, (n = 8) 11.41–15 (12.9 ± 1.51) wide at base. Dorsal ray bifurcate near middle third (n = 5) 12–17 (14.2 ± 1.79) from base, (n = 4) 40–58.3% (51.5 ± 7.95%) of its length. Spicules tubular, equal, symmetrical, yellowish brown, (n = 8) 138.55–163 (153.3 ± 7.31) long, anterior portion short, strongly chitinized, without distal split; prominent bilateral alae with prominent ridges and trabeculae, originating in first third of spicule length from anterior extremity. Alae spatulate,

prominent, extending to distal termination of spicule tips. Gubernaculum lacking capitulum, thin, arched, elongate, (n = 8) 65–83.13 (76.6 ± 7.06) composed of single corpus and paired crurae. Unpaired anterior corpus (n = 8) 38–49 (44 ± 4.34), bifurcate distally into two lateral legs near mid-third (n = 8) 24–39.12 (32.6 ± 5.09); distal tips prominent, arched ventrally, joined by delicate membrane, located between, slightly ventral to paired denticulate plates of crurae. Denticulate plates of crurae (n = 8) 15–25 (19.5 ± 2.91) long, ‘triangular’ to trapezoid, slightly twisted along longitudinal axis, each with five odontoid processes. Tooth-like structures vary in size, ventrally becoming prominent, overall conferring triangular aspect to crurae. Telamon plates poorly developed, triangular in lateral view, located ventrally to posterior extremity of gubernaculum.

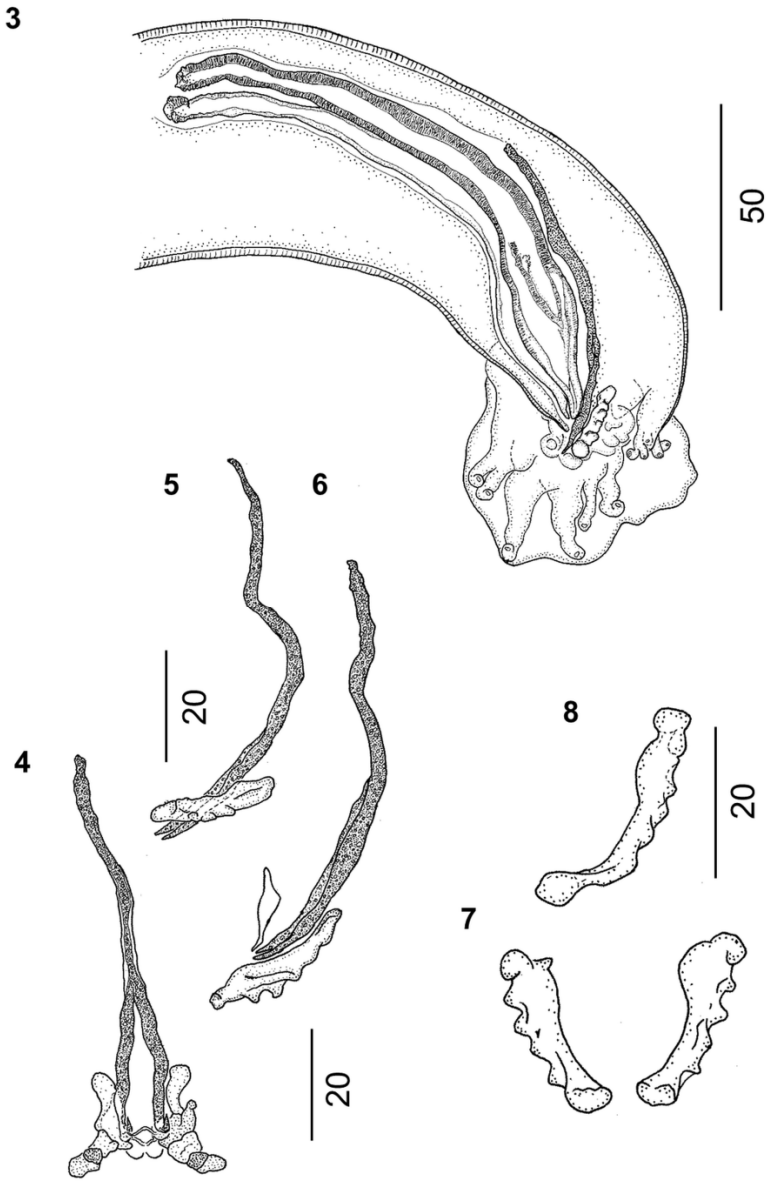


**Figure 2.3.** *Varestrongylus alces*. Male. 3. Caudal extremity of a male specimen at lateral view showing spicule, partially covering gubernaculum and denticulate plates of crura and copulatory bursa; **4.** Ventral view of bifurcate gubernaculum; **5, 6.** Lateral view of gubernaculum and denticulate plates of crura, note triangular telamon plate in 6. **7.** Ventral view of paired denticulate plates of crura. **8.** Lateral view of a denticulate plate of crura.

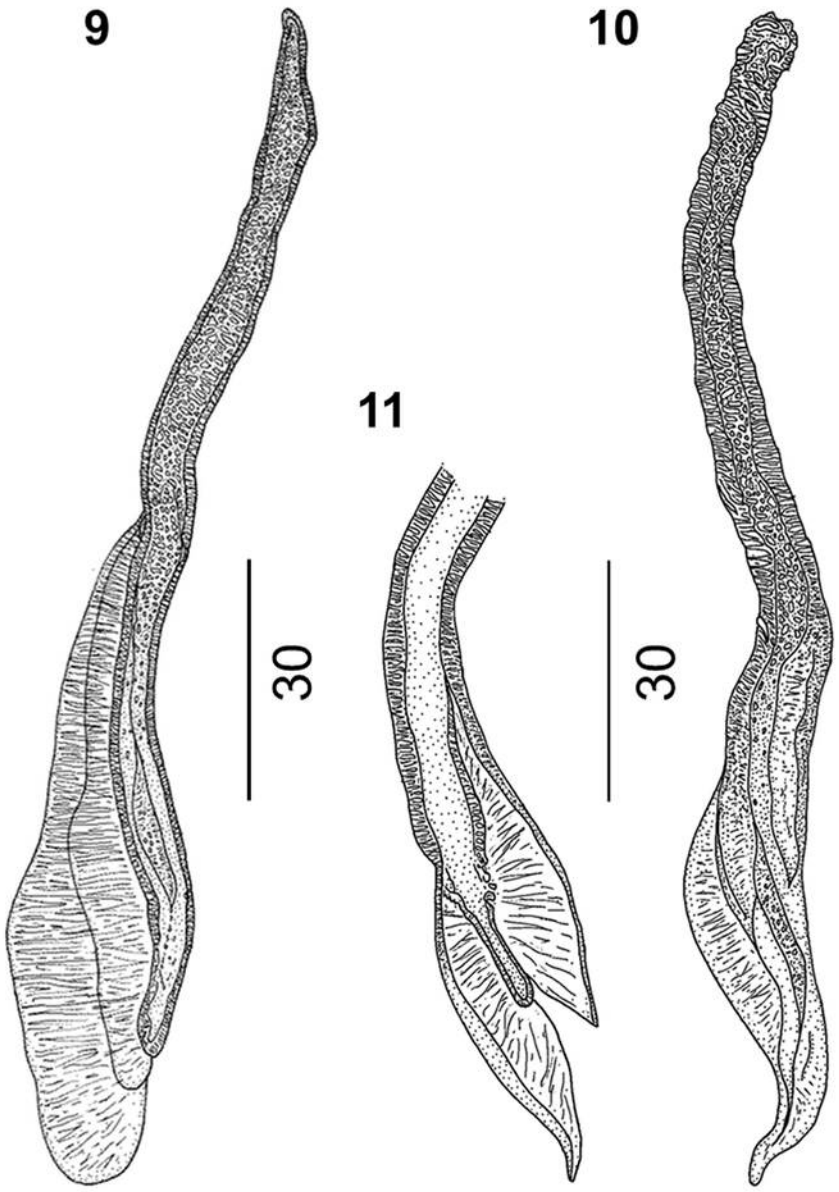


**Figure 2.4.** *Varestrongylus alces*. Male, spicules. **9.** Dorsal view, note prominent alae and spatulate shape. **10.** Lateral view. **11.** Ventral view of spicule distal end.

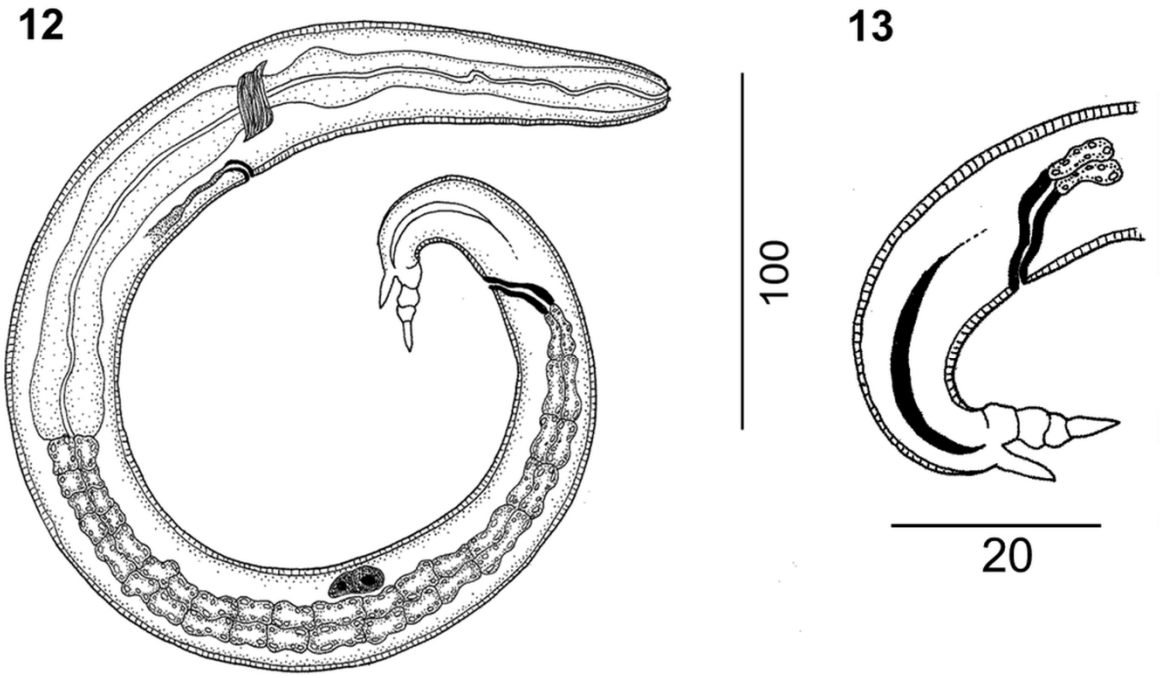


**Figure 2.5.** *Varestrongylus alces*. First-stage larva (DSL). **12.** DSL at lateral view. **13.** Detail on caudal extremity, note dorsal spine and tail extremity composed by three segments.

2.4.1.3 Females

Based on four intact females, one cephalic and seven caudal extremities. Total length (n = 4) 16.25–21.52 mm (18.3 ± 2.3); maximum width (n = 9) 73–102 (86.0 ± 9.9). Esophagus (n = 5) 270–310 (289 ± 14.71) long and 30–42 (36.7 ± 4.32) wide at base, and (n = 4) 1.3–1.7% (1.6 ± 0.2%) of body length. Nerve-ring (n = 5) 86–97 (91.6 ± 4.33), cervical papillae (n = 3) 150–180, excretory pore (n = 5) 159–220 (190.4 ± 29.11) from cephalic extremity. Uteri paired, prodelphic; sphincter at end of uterine limbs (n = 7) 21.19–35.86 (31.8 ± 3.96) long. Vagina voluminous (n = 8) 702.2–961.42 (846.4 ± 94.94) long, subdivided in vagina uterina (n = 8)

637–889.7 (779.2 ± 93.82) and vagina vera (n = 10) 63.27–71.72 (66.8 ± 2.7) connected by sphincter. Vulval aperture on solid knob-like protuberance; cuticular fold extending ventrally across protuberance from anterior lip of vulva; body width at vulva (n = 12) 45.64–69 (56 ± 7.31). Provagina well developed with a hood-like fold extending ventrally across prominent genital protuberance. Peri-vulval pores disposed bilaterally at level of vulva. Anus in the mid-third of distance between vulva and tail tip; distance vulva-anus (n = 11) 70.1–104 (87.3 ± 10.12); vulva-tail (n = 11) 107.58–146 (131.9 ± 12.77). Tail conical (n = 11) 34.23–50.53 (44.5 ± 4.65) with lateral phasmids near apex.

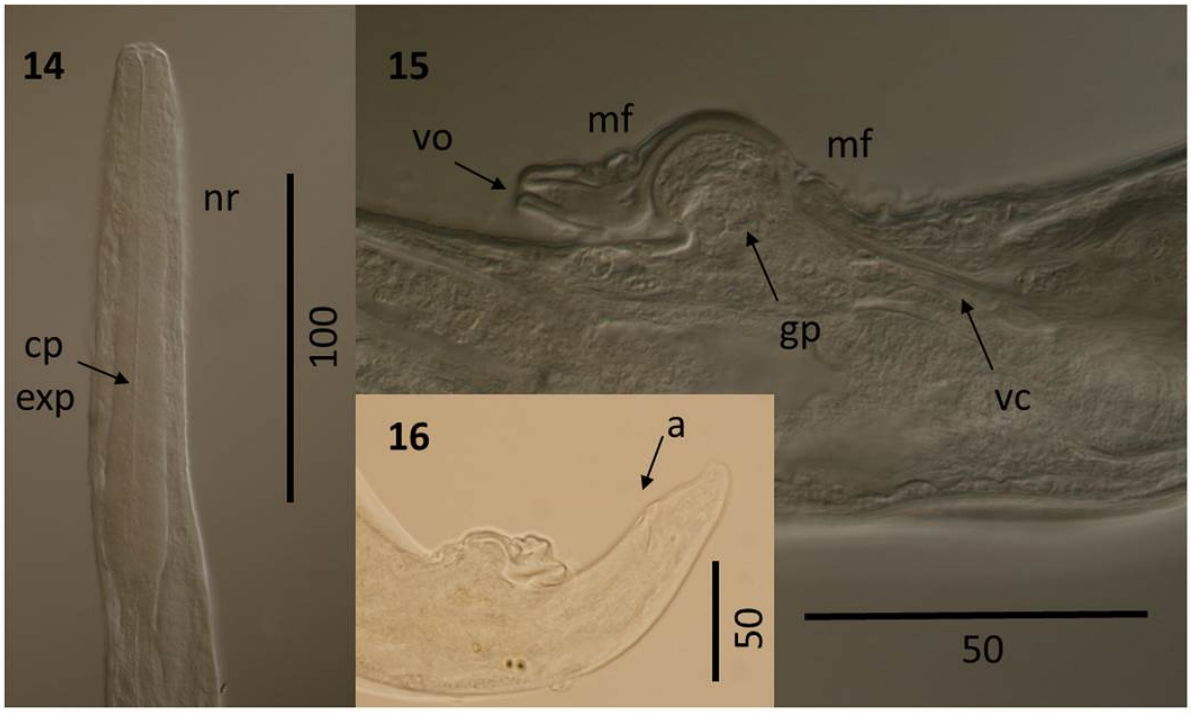


**Figure 2.6.** *Varestrongylus alces*. Female. 14. Cephalic extremity at ventral view: claviform esophagus, cervical papillae (cp), excretory pore (exp), and nerve ring (nr) (64×). **15.** Caudal extremity at lateral view: developed provagina with membranous folds (mf), genital

protuberance (gp), vaginal opening (vo), and vaginal canal (vc) (100×). **16.** Caudal extremity at lateral view, slightly ventral: anus (a), and conical tail tip (100×).

2.4.1.4 Immature stages

***First-stage larvae (DSL)*:** Based on 15 larvae from the lungs of an Eurasian moose. Total length 221.5–373.7 (268.6 ± 40.81). Maximum body width 12.2–29.6 (20.1 ± 5.94). Esophagus 111.6–182.5 (132.2 ± 15.92), 41.2–55.5% (46.4 ± 3.85%) of body length, maximum width at base 6.19–15.7 (10.7 ± 3.51). Body width at esophageal base 10.9–29.6 (19.5 ± 5.95). Nerve-ring 64.5–86.3 (74.1 ± 5.26), excretory pore 70.5–88.9 (78.8 ± 5.33) posterior to cephalic extremity. Genital primordium 145.6–250.6 (202.3 ± 30.69), from anterior end, 54.7–79% (70.7 ± 6.04%) of body length from anterior. Anus-tip of tail spike 34.4–40.4 (37.3 ± 3.03), Anus-insertion of tail spike 19.2–30.3 (26.9 ± 2.95), Tail spike 9.7–12.4 (10.4 ± 0.68) in length with three prominent folds; dorsal spine 2.8–3.5 (3.1 ± 0.24). ***Eggs*:** Spherical to ovoid with delicate, smooth shell (n = 20); 55.2–66.5 (61. 9 ± 3.51) long, 46.2– 63.0 (55.2 ± 6.14) in width.

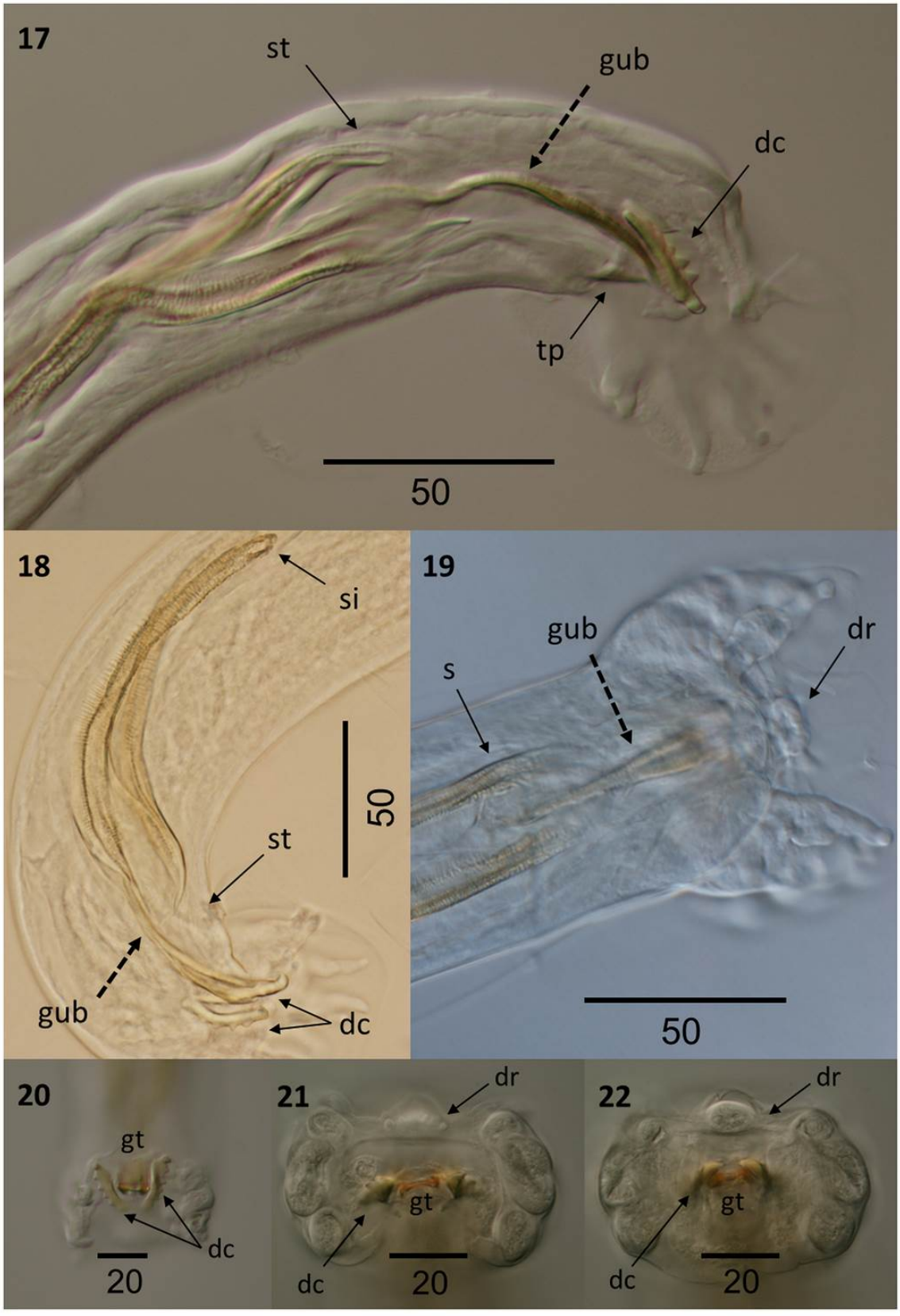
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**Figure 2.7.** *Varestrongylus alces*. Male. **17.** Caudal extremity of a male specimen at dorsal view: arched bifurcate gubernaculum (gub), spatulate spicule tips (st), denticulate plates of crura (dc) and triangular telamon plate (tp) (64×). **18.** Caudal extremity of a male specimen at lateral view: spicule insertion (si) and spatulate tips (st), bifurcate gubernaculum (gub), and paired denticulate plates of crura (dc) (100×). **19.** Caudal extremity of a male specimen at ventral view: distal end of spicules (s), bifurcate gubernaculum (gub), and dorsal ray (dr) (40×). **20.** Caudal extremity of a male specimen at ventral view: denticulate plates of crura (dc), and tip of gubernaculum (gt) (64×). **21, 22.** Detail of male caudal extremity at caudal view: dorsal ray (dr), denticulate crura (dc), and tip of gubernaculum fused by delicate membrane (tg) (160×).

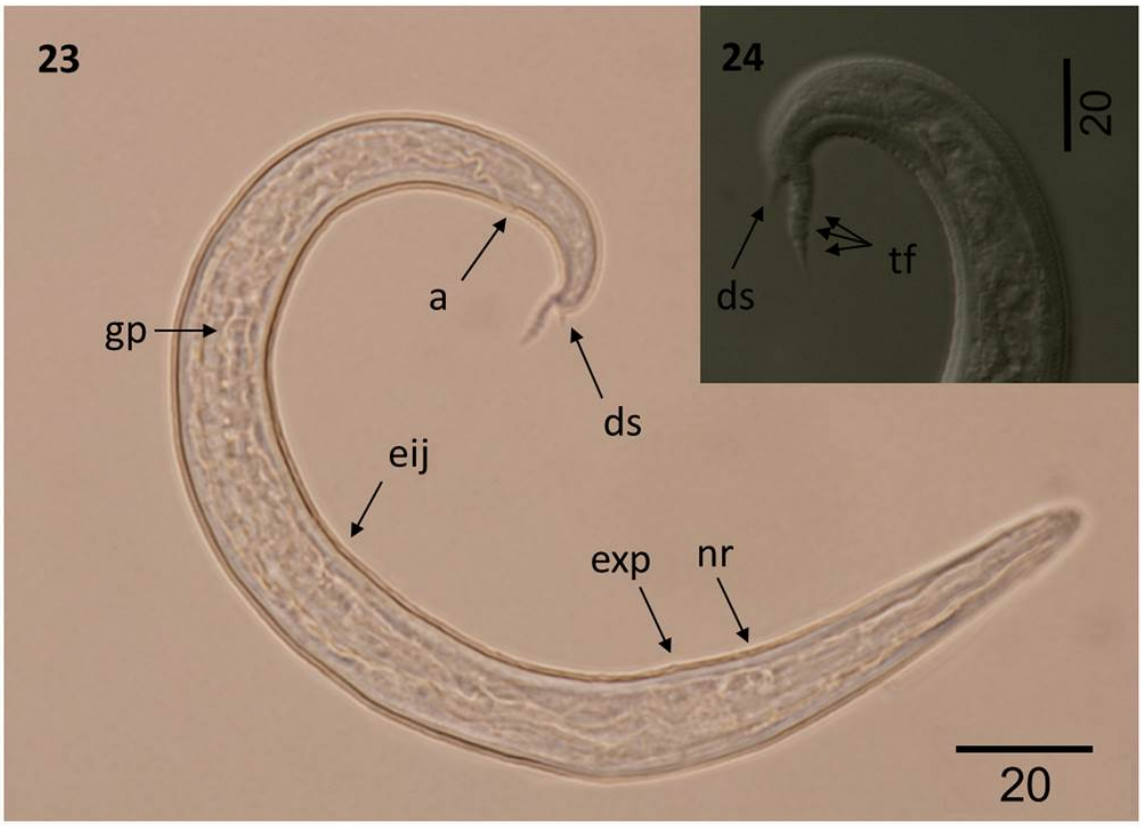


**Figure 2.8.** *Varestrongylus alces*. First-stage larva (DSL). **23.** DSL at lateral view (100×): nerve ring (nr), excretory pore (exp), esophageal-intestinal junction (eij), genital primordium (gp), anus (a) and dorsal spine (ds). **24.** Detail of tail, showing dorsal spine (ds) and the three tail folds (tf) (100×).

### *2.4.2 Taxonomic summary*

***Type-host***

Eurasian moose (*Alces alces*). Other common name: Eurasian elk.

***Habitat***

Adult males and females in terminal bronchioles and alveoli of lungs based on recovery of specimens through dissection of lesions.

***Type-locality***

Original type-locality: Moscow Region, Russia. Additional locality for designated Neotype: Vestby Municipality, Akerhus County, Eastern Norway, Norway (present study). Also known from areas of Sweden, Finland, Poland, and Estonia.

***Specimens***

Neotype male from type host and new designated locality (59°30′N, 10°40′E) collected from lungs of a young male Eurasian moose (V-456) by S. Kutz and others in Norway, USNPC 106337. Voucher specimens collected from the same host, USNPC 106338–106340, and from three other hosts: a young female (V-376), USNPC 106331–106334 (including DSL material); another young female (V-377), USNPC 106335; and an adult female (V-383), USNPC 106336; all from the same locality.

### *2.4.3 Differential diagnosis*

*Varestrongylus alces* is resurrected based on morphological and molecular character data; and, therefore, this valid taxon must be separated from *V. capreoli.* A neotype is designated herein because name-bearing types were not identified or deposited at the time of the original description (Demidova and Naumitscheva, 1953) and are apparently absent in Russian museum collections (A. Makarikov, pers. comm.). This proposal is consistent with and based on the

provisions specified in Article 75, Chapter 16 of the International Code for Zoological Nomenclature (2012), with the intent of clarifying the taxonomic status of *V. alces* within the genus.

Consistent with the current generic diagnosis, males of *V. alces* possess a prominent gubernaculum with paired denticulate plates of the crurae disposed slightly lateral, dorsal and distal to the split corpus or legs, and a typical configuration of bursal rays; and females have a well-developed provagina.

Among males, specimens of *V. alces* are readily distinguished by the dimensions and structure of spicules (138.6–163 μm). Spicules of *V. alces* are substantially shorter than those typical of 'the large spicule group': *V. alpenae*, *V. capricola*, *V. longispiculatus*, *V. pneumonicus*, *V. qinghaiensis*, *V. sagittatus* and *V. tuvae* (all > 300 μm, except *V capricola* whose spicules are approximately 250 μm). Similarly, the gubernaculum (65–83 μm) of *V. alces* is much smaller than that of the aforementioned species (all > 100 μm).

Among the Varestrongylinae, *V. alces* is most similar to *V. capreoli* (and *V.* cf. *capreoli*, which is identical to *V. capreoli* but for one character and, therefore, will be mentioned again for comparative matters in this exception) and these two species characterize the small-spicule forms currently known within the genus. Nevertheless, males of *V. alces* differ from those of *V. capreoli* by dimensions of the spicule and gubernaculum as well as several other characters. The conformation of the gubernaculum is the most noticeable difference between *V. alces* and *V. capreoli*; both have a bifurcate corpus, but in the latter, the legs are fused by a transparent membrane that is not observed in the former. In addition, the gubernaculum of *V. alces* does not have a capitulum (head). In contrast, different authors, including the original description (Stroh

and Schmid, 1938) and works cited in the most recent revision of the genus (Boev, 1975), regard the presence of a distinctive capitulum of the gubernaculum with two acute ventrally directed projections as typical in *V. capreoli*. Variation, however, may be evident in this attribute as specimens, referred to *V.* cf. *capreoli* in roe deer from the present study lacked a capitulum, suggesting a more extensive series of male nematodes should be evaluated for this character. Among additional characters, spicules of *V. alces* and *V. capreoli* are comparable in length, and morphologically very similar. For both, the alae originate in the first third and extend slightly beyond the distal extremity of each spicule. However, the distal ends of the spicules of *V. alces* are more spatulate than in *V. capreoli*. The denticulate plates of the crurae differ in shape, being slightly twisted and conferring an arched appearance in *V. alces,* with both plates together resembling a horseshoe (Figure 7). In contrast, in *V. capreoli*, the denticulate plates of the crurae are triangular, and more parallel to each other, resembling "Hermes' wings". Numbers of denticulate processes in these plates also differ, with *V. alces* having 5 and *V. capreoli* having 3 prominent teeth. The copulatory bursa of *V. alces* is dorsally notched with an indistinct dorsal lobe, whereas the bursa of *V. capreoli* is bi-lobate. A series of subtle differences are also observed in the morphology and disposition of the bursal rays. The dorsal ray in *V. alces* is slightly elongate and bifurcate near its mid-length as opposed to *V. capreoli,* in which the dorsal ray is reduced and rounded, yet still distinguishable. In *V. alces,* the externo-dorsal ray originates independently from the lateral rays, unlike in *V. capreoli*. Ventral rays of both species originate from a common stalk but this is distally split in *V. alces,* whereas it is split near its base in *V. capreoli*.

Measurements for multiple characters overlap between the two species, including some characters that are distinguishable based on morphology (Table 2.3), but this may be because of the wide range in measurements previously reported for *V. capreoli* in Boev (1975).

**Table 2.3.** Comparative morphometry of males of *Varestrongylus alces* and *V. capreoli*

| **Characters** | ***V. alces*[a]** | ***V. alces*[b]** | ***V. capreoli*[c]** | ***V.* cf. *capreoli*[a]** |
|---|---|---|---|---|
| **Total length** | 11.4–14.7 (12.9 ± 2.01) | 5–6 | 5.3–13.5 | 7.1–8.9 (7.9 ± 0.88) |
| **Maximum width** | 68.5–80 (74.2 ± 4.97) | 65 | 32–68 | 42–44 (43.5 ± 1.00) |
| **Esophagus**[§] | 250–272 (264.5 ± 8.95) | 146 | 90–146 | 227–239 (232 ± 5.10) |
| **Esophagus base width** | 32–37 (33.9 ± 2.19) | 36 | – | 20–36 (24.6 ± 6.47) |
| **Body width at esophagus** | 53.8–61.9 (56.3 ± 3.38) | – | – | 33–60 (40.4 ± 11.10) |
| **Nerve-ring**[§] | 68–89.7 (81.8 ± 9.04) | – | – | 70–81 (76.3 ± 5.60) |
| **Cervical papillae**[§] | 201–207 (203.4 ± 3.19) | – | – | 163[*] |
| **Excretory pore**[§] | 208–230.3 (221.8 ± 9.83) | – | – | 166–201 (180.5 ± 14.71) |
| **Spicules** | 138.6–163 (152.3 ± 7.31) | 150–166 | 129–160 | 134–152 (138.3 ± 7.03) |
| **Gubernaculum** | 65–83.13 (76.58 ± 7.06) | – | 70–86 | 70–92 (81.8 ± 8.14) |
| **Gubernaculum head** | Absent | Absent | Present 8–14 | Absent |
| **Gubernaculum corpus** | 38–49 (43.9 ± 4.34) | – | NA | 30–38 (32.8 ± 3.77) |

| | | | | |
|---|---|---|---|---|
| **Gubernaculum crura** | 24–39.12 (32.6 ± 5.09) | – | NA | 32–56 (46.5 ± 10.25) |
| **Crura denticulate piece** | 15–25 (19. 5 ± 2.91) | – | 18–30 | 21–25 (23.2 ± 1.47) |
| **Body width at bursa** | 42–56 (48.3 ± 7.36) | – | – | 33–37 (34.5 ± 1.38) |
| **Bursa width** | 125–160 (140 ± 18.03) | – | – | NA |
| **Bursa length** | 75–90 (84.3 ± 5.9) | – | – | NA |
| **Dorsal ray length** | 18–30 (24.5 ± 3.65) | – | NA | 6–10 (8.6 ± 1.79) |
| **Dorsal ray base** | 11.4–15 (12.9 ± 1.51) | | NA | 7.5–12.5 (9.2 ± 2.06) |

[a] Present study; [b] Original description (Demidova and Naumitscheva, 1953); [c] Original description (Stroh and Schmid, 1938), plus additional information compiled in Boev (1975).

§ Measurements from anterior end; *Single measurement.

Range of measurements are given followed by mean and standard deviation. Total length in millimeters (mm), and all other measurements are in micrometers (μm).

Among females, the size and shape of the provagina is not always a useful character for discriminating among species of *Varestrongylus*. For instance, the provagina of *V. alces* and *V. capreoli* (and *V.* cf. *capreoli*) is morphologically identical. Similarly, the ranges for maximum body width, and distances between vulva and tip of tail, and anus and tip of tail (tail) for *V. alces* and those for *V. capreoli* largely overlap

Table 2.4). In contrast when comparing to the roe deer material, identified as *V.* cf. *capreoli*, these measurements, as well as body width at vulva and distance between vulva and anus, are wider or longer in those of *V. alces*. Nevertheless, morphological species identification solely based on female specimens remains challenging.

**Table 2.4.** Comparative morphometry of females of *Varestrongylus alces* and *V. capreoli*

| **Characters** | ***V. alces*[a]** | ***V. alces*[b]** | ***V. capreoli*[c]** | ***V.* cf. *capreoli*[a]** |
|---|---|---|---|---|
| **Total length** | 16.3–21.5 (18.3 ± 2.3) | 11.1–11.5 | 9.41–15 | 17.93[*] |
| **Maximum width** | 73–102 (86.0 ± 9.9) | 75–95 | 38–95 | 48.9–52.2 (50.5 ± 2.31) |
| **Esophagus**[§] | 270–310 (289 ± 14.71) | – | 122–290 | 196–242.9 (225.0 ± 20.21) |
| **Esophagus base width** | 30–42 (36.7 ± 4.32) | – | – | 21.9–27.7 (23.8 ± 2.60) |
| **Body width at esophagus base** | 57–67 (61.1 ± 4.56) | – | – | 31–40.8 (35.3 ± 5.06) |
| **Nerve-ring**[§] | 86–97 (91.6 ± 4.33) | – | 72–90 | 55.4–65.2 (60.7 ± 4.49) |
| **Cervical papillae**[§] | 150–180 (163.3 ± 15.28) | – | – | 185.82[*] |
| **Excretory pore**[§] | 159–220 (190.4 ± 29.11) | – | 86–186 | 171.5–190.8 (183.4 ± 10.37) |
| **Tail** | 34.2–50.5 (44.5 ± 4.65) | – | 34–78 | 31–40.8 (37.2 ± 3.47) |
| **Vulva-anus** | 70.1–104 (87.3 ± 10.1) | – | – | 57.1–73.4 (64.3 ± 6.62) |
| **Vulva-tail** | 107.6–146 (131.9 ± 12.77) | 122 | 90–144 | 91–114.1 (101.6 ± 8.42) |
| **Width at vulva** | 45.6–69 (56 ± 7.31) | – | – | 32.2–35.9 (33.4 ± 1.42) |

| | | | | |
|---|---|---|---|---|
| **Vagina** | 702.2–961.42 (846.41 ± 94.94) | – | – | 467* |
| **Vagina Vera** | 63.3–71.7 (66.8 ± 2.70) | – | – | 73.4–91.3 (77.4 ± 6.90) |
| **Vagina Uterina** | 637–889.7 (779.2 ± 93.82) | – | – | 391.2* |
| **Sphincter** | 21.2–35.9 (31.8 ± 3.96) | – | – | 24.45* |
| **Eggs Length†** | 55.2–66.5 (61.9 ± 3.51) | 78 | 56–78 | NA |
| **Eggs Width†** | 46.2–63.0 (55.6 ± 6.14) | – | 37–45 | NA |

[a] Present study; [b] Original description (Demidova and Naumitscheva, 1953); [c] Original description (Stroh and Schmid, 1938), plus additional information compiled in Boev (1975).

§ Measurements from anterior end; †Eggs collected from lungs of infected Eurasian moose, not inside female uteri; *Single measurements. Range of measurements are given followed by mean and standard deviation. Total length in millimeters (mm), and all other measurements are in micrometers (μm).

***First-stage larvae (DSL):*** Comparisons among *Varestrongylus* species DSL are provided in Table 2.5. Comparisons with other members of the Family Protostrongylidae that occur in the same host or which may have overlapping geographic distributions were also included. In general, most of the characteristics overlap in measurement. The wide range for total length of *V. alces* in our study, especially the lower values, may be attributable to the pulmonary origin (vs. feces) and the fact that lungs were frozen before dissection, and collection and preservation of DSL material. Co-infections with *V. alces* and *E. alces* are common; however DSL of *E. alces* and other *Elaphostrongylus* species appear to be consistently longer than those of *V. alces* (Table 2.5).

**Table 2.5.** Comparative morphometrics of first-stage larvae (DSL) of *Varestrongylus* and of Elaphostrongylinae sympatric with *V. alces*.

| **Characters** | ***V. alces*** [a,b] **(n = 15)** | ***V. capreoli*** [c] | ***V. sagittatus*** [d] | ***V. sagittatus*** [e] | ***Varestrongylus* sp.** [f1] **(n = 10)** | ***Varestrongylus* sp.** [f2] **(n = 20)** | ***V. alpenae*** [g] | ***E. alces*** [h] **(n = 30)** | ***E. cervi*** [i] **(n = 30)** | ***E. rangiferi*** [j] **(n = 15)** |
|---|---|---|---|---|---|---|---|---|---|---|
| Total length | 221.5–373.7 | 255–341 | 260–305 | 268.8–295.7 | 281–374 | 348–400 | 310–380 | 377–445 | 392–445 | 381–490 |
| | (286.6 ± 40.81) | (227–260) | (233–305) | (281 ± 11.9) | (329) | (377) | | (417 ± 16) | (420 ± 13) | (426) |
| Nerve-ring[§] | 64.5–86.3 | – | – | – | – | 78–107 | 85–93 | 83–106 | 106–125 | 95–130 |
| | (74.1 ± 5.3) | | | | | (97) | | (90 ± 16) | (114 ± 5) | (110) |
| Excretory pore[§] | 67.5–88.9 | – | 81–84 | 77–122.9 | 71–105 | 92–107 | 85–93 | 104–132 | 104–121 | 97–125 |
| | (78.8 ± 5.33) | | | (96±17.5) | (84.5) | (102) | | (112 ± 7) | (111 ± 4) | (109) |
| Esophagus[§] | 111.6–182.5 | 70–83 | 115–151 | 134.4–161.3 | 88–155 | 151–180 | 155–180 | 173–236 | 175–206 | 163–230 |
| | (132.2±15.92) | (120–140) | (124) | (147±15.9) | (128) | (168) | | (188 ± 12) | (187 ± 7) | (191) |
| Esophagus/Total length (%) | 41.2–55.5 | – | – | – | 28–46 | 43–46 | 47–50 | – | – | – |
| | (46.3±3.85) | | | | (38) | (45) | | | | |
| Esophagus base width | 6.2–15.7 | – | – | – | 8–15.5 | 9–15 | – | – | – | – |
| | (10.7 ± 3.51) | | | | (10) | (12) | | | | |
| Body at esophagus base | 10.9–29.6 | – | – | – | – | – | – | – | – | – |
| | (19.5 ± 5.95) | | | | | | | | | |
| Max body width | 12.2–29.6 | 10–17 | 14–17 | 13.2–16.9 | 16–23 | 17–20 | 15–17 | 17–21 | 17–22 | 17–24 |
| | (20.1± 5.94) | (11–14) | (14) | (15± 1.1) | (19.5) | (18) | | (19 ± 1) | (19 ± 1) | (20) |
| Genital primordium[§] | 145.6–250.6 | – | 179–201 | 154–249.6 | 173–224 | 218–273 | 195–242 | 204–289 | 253–288 | 245–325 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (202.3 ± 30.69) | | | (197±25.1) | (206) | (244) | | (262 ± 16) | (270 ± 10) | (267) |
| Genital primordium/Total length (%) | 69.3–72.9 (70.7±6.04) | – | – | – | 62–64 | 61–68 | 63–64 | – | – | – |
| | | | | | (63) | (65) | | | | |
| Tail length | 28.6–39.4 | 28–32 | 25–31 | 24.64–29.28 | 31–42 | 32–41 | – | 32–49 | 37–47 | 32–53 |
| | (36.4 ± 2.95) | | | (28± 1.63) | (35) | (38) | | (42 ± 5) | (43 ± 3) | (44) |
| Tail spike | 9.8–12.4 | 8 | (9–10) | 9.2–10.78 | 8–11 | 6–12 | data not given | data not given | data not given | data not given |
| | (10.4 ± 0.68) | | | (9.6± 0.7) | (9) | (9) | | | | |
| Dorsal spine | 2.8–3.5 | 2 | data not given | data not given | 1.6–3 | data not given | data not given | data not given | data not given | data not given |
| | (3.1 ± 0.24) | | | | (2) | | | | | |

[a] Present study – DSL recovered from lung washes and fixed in 70% ethanol and measured at 1000× magnification. The wide range for total length, especially the lower values might be attributable to the pulmonary origin (vs. feces) and fixation method;

[b] only measurements available in the original description [20], were total length, 305–441 μm and maximum width, 12 μm;

[c] Combined sources compiled in [1], origin (lungs/feces) or fixation method not mentioned;

[d] Combined sources compiled in [1], recovered from lungs, fixation method not mentioned;

[e] DSL recovered from feces of red deer from the Vitinya wildlife-breeding station in the west Balkan Mountains, Bulgaria, not fixed and measured after iodine staining [45];

[f] Undescribed *Varestrongylus* species found in caribou, muskoxen and moose across northern North America [14]. DSL recovered from feces of muskoxen from: (f1) Nunavik Region, Quebec, Canada, fixed in 70% ethanol and measured at 1600× magnification, (f2) near Aklavik, Northwest Territories, Canada, heat-relaxed in water and measured at 400× magnification;

[g] *V. alpenae* DSL extracted from white-tailed deer feces, New York, USA in [45].

[h] DSL recovered from feces of experimentally infected Eurasian elk, material was heat-relaxed in water and measured at 1000× magnification [8];

[i] DSL recovered from feces of experimentally infected red deer, material was heat-relaxed in water and measured at 1000× magnification [8];

[j] DSL recovered from feces of woodland caribou from Newfoundland, Canada. Material was heat-relaxed in water, magnification not mentioned [8].

[§] Measurements from anterior end.

Range of measurements are given followed by mean and standard deviation. Measurements are given in micrometers (μm).

**Molecular identification and phylogenetic comparisons**

All ITS-2 sequences generated were deposited in GenBank under accession numbers: KJ452181–96 for *V. alces* of Eurasian moose; KJ452174–80 for *V.* cf. *capreoli* of European roe deer; and KJ439592–98 for *V. sagittatus* isolates in red deer from Bulgaria and are accompanied by vouchers specimens deposited in the USNPC (Table 2.1). Intra-individual ITS-2 sequence polymorphisms were found for all three *Varestrongylus* species evaluated. The ranges of pairwise similarity among individuals, within species, and between the five *Varestrongylus* species are provided in Table 2.6.

**Table 2.6.** ITS-2 pairwise identity among *Varestrongylus* species and individuals, including intra-individual variability

| ***Varestrongylus* species** | ***V. alces*** * | ***V.* cf. *capreoli*** * | ***Varestrongylus* sp.** | ***V. alpenae*** ** | ***V. sagittatus*** * |
|---|---|---|---|---|---|
| ***Varestrongylus alces*** | 71.7–99.5 | – | – | – | – |
| | (87.14 ± 6.46) | | | | |
| ***Varestrongylus* cf. *capreoli*** | 64.8–89.6 | 78.1–100 | – | – | – |
| | (78.76 ± 4.73) | (92.85 ± 8.12) | | | |
| ***Varestrongylus* sp.** | 64.9–87.1 | 74.9–84.9 | 94.7–100 | – | – |
| | (78.25 ± 4.63) | (82.06 ± 1.91) | (97.37 ± 1.73) | | |
| ***Varestrongylus alpenae*** | 57.2–72.8 | 64.6–72.5 | 72.4–74.7 | 100** | – |
| | (63.9 ± 6.5) | (63.25 ± 3.65) | (74.35 ± 0.92) | | |
| ***Varestrongylus sagittatus*** | 42.1–58.7 | 50.3–61.2 | 55.4–58.8 | 50.8–53.5 | 87–100 |
| | (51.92 ± 3.24) | (58.33 ± 2.23) | (57.47 ± 0.76) | (52.35 ± 0.45) | (92.65 ± 5.24) |

* Including clones of the same nematode specimen;**single sequence.

Range, average and standard deviation are given.

The alignment of 53 ITS-2 sequences of 12 Protostrongylidae taxa resulted in a dataset of 210 characters. The strict consensus of the three most-parsimonious trees had a length of 271 steps, a consistency index of 0.73, and yielded five monophyletic groups of *Varestrongylus*, each matching pre-determined taxa at representing discrete species. The MP analysis of ITS-2 sequences (Figure 2.1) strongly support the reciprocal monophyly of *V. alces* isolates (91% bootstrap support), and hence independence from *V.* cf. *capreoli*, and by extrapolation, from *V. capreoli* (*sensu* Stroh and Schmid, 1938). Clonal sequences of *V.* cf. *capreoli* (92%) and *V. sagittatus* (99%) also formed strongly supported monophyletic clades, confirming their validity as independent taxa. Moreover, the DSL-derived ITS-2 sequences for an undescribed *Varestrongylus* strongly supported recognition of a previously unknown species and confirmed its placement within the genus (97%) (Kutz et al., 2007; Verocai et al., 2011).

*Varestrongylus alces* formed a well-supported clade with this undescribed Nearctic species and *V.* cf. *capreoli* (80%), but relationships among these three species were equivocal. A sister relationship of *V. alpenae* to the clade formed by *V. alces*, *V.* cf. *capreoli* and the undescribed North American species was also well supported (81%). *Varestrongylus sagittatus*, a parasite of Cervinae, is sister for a clade formed by the four *Varestrongylus* species parasitic in Odocoileinae cervids (Figure 2.1). Sequences from species within the subfamilies Elaphostrongylinae (99%), Muelleriinae (84%) and Protostrongylinae (99%) also formed well supported clades.

### *2.4.4 Pathology*

2.4.4.1 Gross pathology

Grossly, lesions in Eurasian moose lungs were well defined, tan to pale and firm nodular lesions that ranged in size from a few millimetres to 2–3 cm in diameter. These were mostly seen subpleurally, but could also be found deeper in the lung tissue (Figure 2.9). Most lesions were found in the caudo-dorsal region of the diaphragmatic lobes. Lesions were clearly demarcated against adjacent normal lung tissue.

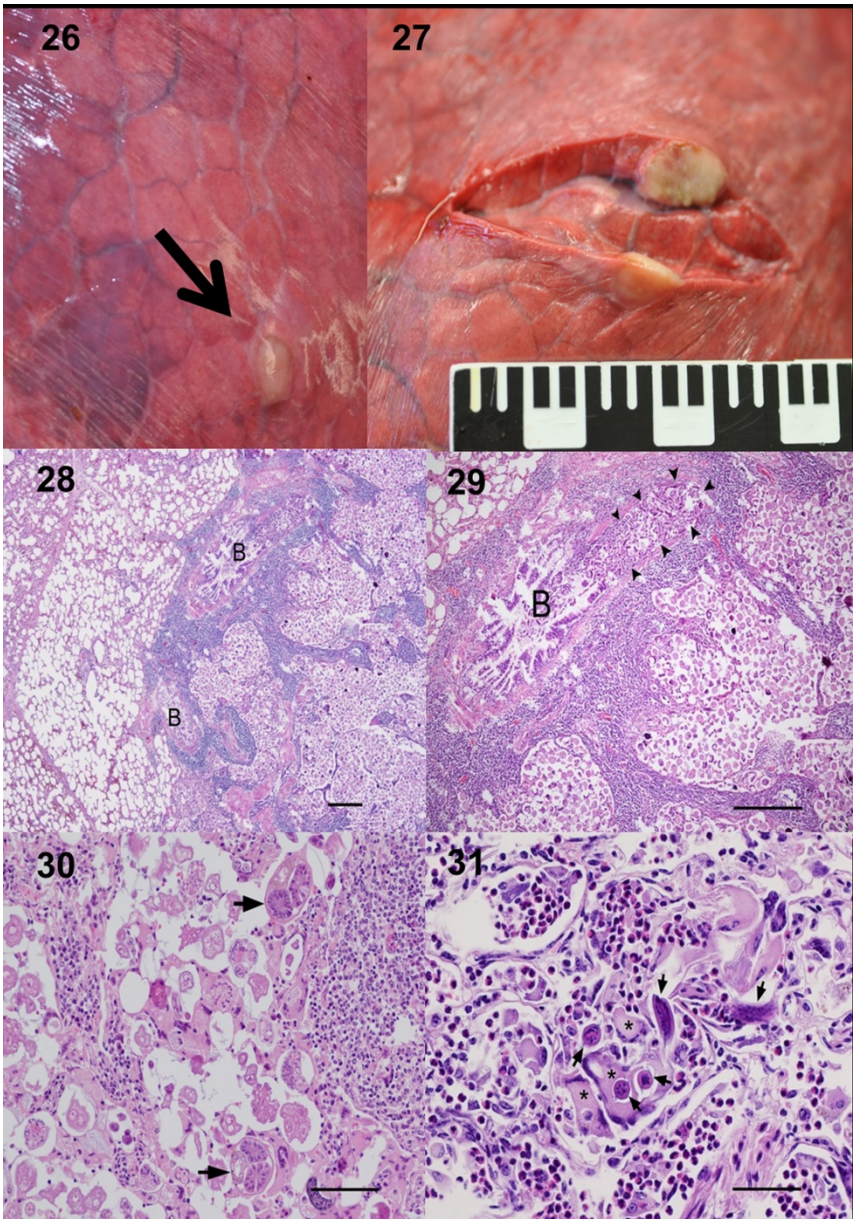

**Figure 2.9.** Gross and histopathological changes in lungs of Eurasian moose infected with *Varestrongylus alces*. **26, 27**. Gross lesion seen from lung surface during gross examination (arrow), typical of varestrongylosis (**26**), and sectioned lesion (≈1.5 cm) (**27**). **28–31.** Histological sections (H&E). **28.** Part of the nodule is seen to the right, consisting mainly of large amounts of eggs, larvae and inflammatory cells, whereas normal, slightly emphysematous tissue is seen to the left. Scale-bar: 500 μm. **29.** A close up of **28** showing to the left a large bronchiole (B) with epithelial hyperplasia and peri-bronchiolar lymphocytic inflammation that has large amounts of larvae in the lumen (area surrounded by arrowheads). To the right numerous eggs and larvae are filling up the alveolar space with rupture of alveolar septa and infiltration of inflammatory cells, mainly interstitially. Scale-bar: 500 μm. **30.** Cross sections of adult nematodes (arrows) in the alveolar lumen surrounded by large amounts of eggs and some larvae with scattered multinucleated giant cells. Scale-bar: 100 μm. **31.** First-stage larvae (arrows) partly engulfed and surrounded by giant cells (*), some macrophages and numerous eosinophilic granulocytes. Scale-bar: 50 μm.

2.4.4.2 Histopathological findings

Histological examination revealed acute to sub-acute focal verminous pneumonia restricted to one or a few neighboring lobules (Figure 2.9). Within the affected lobules, large numbers of eggs and larvae, some of them degenerated and mineralized, were filling up the alveolar lumen with rupture of alveolar septa. Numerous larvae were also seen in the lumen of some of the surrounding large bronchioles (Figure 2.9). Scattered cross sections of adult nematodes were found in the alveoli (Figure 2.9). Reactive changes included infiltration of variable amounts of multinucleated giant cells, macrophages, eosinophilic granulocytes and

lymphocytes (Figure 2.9). Marked interstitial infiltrations of inflammatory cells, dominated by lymphocytes and macrophages, were evident around bronchioles and vessels and in the remaining alveolar septa surrounding islands of ruptured alveoli filled with eggs and larvae. Bronchioles with larvae in the lumen had mild hyperplasia of the epithelium and inflammation of the wall. The overlying pleura and the interlobular septa showed variable degree of fibrosis and infiltration of inflammatory cells dominated by lymphocytes.

In adjacent tissue, a few scattered eggs and larvae in the alveolar lumen with little reactive changes (microgranulomas) were seen, as typically found in *E. alces* infection (Handeland and Gibbons, 2001).

## 2.5 Discussion

### *2.5.1 Species identity*

*Varestrongylus alces* is a valid species based on combined morphological and molecular evidence, corroborating the findings of the original species description (Demidova and Naumitscheva, 1953) and, therefore, should be separated from *V. capreoli*, as postulated in the last revision of the genus (Boev, 1975). Given that the types were either never deposited in a Russian museum repository (there is no indication in the original description), or have been subsequently lost, we propose designation of neotype for *V. alces*. Such a proposal serves to clearly validate the species, distinguishing this taxon among its congeners, and establishes stability in the current nomenclature for this group of nematodes.

As for many taxa within Protostrongylidae, and especially within the genus *Varestrongylus*, the taxonomic history of *V. alces* has been confusing (Boev, 1975). Despite the

widely accepted synonymy with *V. capreoli*, a few authors have continued to use *V. alces* as a valid taxon, however, without emphasizing its dubious taxonomic status and not focusing on aspects of its life history. Others did not follow the proposed revision at the generic level made by Boev (1968), in which *Capreocaulus* Schulz & Kadenazy, 1948 and *Bicaulus* Schulz & Boev, 1940, were regarded as junior synonyms of *Varestrongylus.* Adding to the confusion, studies that disregarded the species-level synonymy have placed both species in two separate genera: *Capreocaulus* for *V. capreoli* (as *Capreocaulus capreoli* (Stroh & Schmid, 1938) Schulz & Kadenazy, 1948) (Dróżdż, 1966; Järvis, 1995; Nilsson, 1971) and *Bicaulus* Schulz & Boev, 1940 for *V. alces* (as *Bicaulus alces* (Demidova & Naumitscheva, 1953) Boev, 1957 or *B. alcis* (sic) (Stéen et al., 1998; Stéen et al., 1993). Such inconsistencies reinforced our need to resolve the taxonomy and the possible synonymy or independence of *V. alces* and *V. capreoli* (Boev, 1975), given recognition of an unknown taxon in related hosts from North America.

### *2.5.2 Molecular findings*

Sequences at the ITS-2 locus of *V. alces* formed a strongly supported monophyletic group, and were distinct from those of *V.* cf. *capreoli*, and all *Varestrongylus* species from which sequences were available. According to the most parsimonious tree, *V. alces* is the sister taxon of *V.* cf. *capreoli*. These two species form a well-supported clade with the undescribed *Varestrongylus* from the Nearctic, and are more distantly related to *V. alpenae* and *V. sagittatus*. The multiple sequences of *V.* cf. *capreoli, V. sagittatus* (clones from this study), and the undescribed Nearctic species (from (Kutz et al., 2007)) also formed strong monophyletic clades, supporting species identity. In the only previous attempt to apply molecular or genetic data in comparisons of *Varestrongylus* isolates from *Alces* and *Capreolus* hosts (Stéen et al., 1993),

protein band patterns and their protein isoeletric points were used to distinguish protostrongylid larvae from different host sources. Isolates attributable to *V. alces* were closely related, but not identical, to those of *V. capreoli* (referred as *C. capreoli*) when contrasted to larval isolates from muskoxen and elaphostrongylines in Eurasian moose and reindeer, consistent with our findings in the present study.

The intra-individual ITS-2 variability we found for *V. alces*, *V.* cf. *capreoli* and *V. sagittatus* is not surprising as it is a multi-copy gene (Gasser, 2006). In fact, variability at this region has been demonstrated in members of the Family Protostrongylidae and the undescribed Nearctic *Varestrongylus* species (Kutz et al., 2007), and the intra-species diversity is expected to increase with the number of individual worms and clones sequenced. Intra-individual variability in multi-copy genes, such as the ITS-2 region, appears to be common in parasitic nematode species and other organisms, and it may indicate incomplete rDNA repeat homogenization within these species (Gasser, 2006). Similar patterns have been reported for *Nematodirus battus* Crofton & Thomas 1951 (Nadler et al., 2000), as well as for various other gastrointestinal strongylid species infecting domestic and wild mammals (Gasser et al., 1998; Heise et al., 1999; Nadler et al., 2013; Stevenson et al., 1995, 1996; Troell et al., 2003). Conversely, all clones from the two *V. sagittatus* specimens showed minimal variability within and between specimens.

### *2.5.3 Pathology and significance*

Gross and histopathological pathological lesions found in *V. alces*-infected moose in the present study resembled those described for several other congeneric species, such as *V. capreoli* (Stroh and Schmid, 1938; Švarc and Pajersky, 1990), *V. pneumonicus* (Bhatia and Pande, 1960), *V.*

*alpenae* (Cheatum, 1949), and *V. sagittatus* (Panayotova-Pencheva, 2008), and previous reports for *V. alces* (Petrosyan, 1963; Stuve, 1986, 1987). Since adult *Varestrongylus* are often found in small bronchioles, infection is generally associated with focal or multi-focal pneumonia, most often in the diaphragmatic lobes (Boev, 1975; Cheatum, 1949), as opposed to the diffuse interstitial pneumonia typical of the non-pulmonary protostrongylids (i.e., elaphostrongylines), where larvae and eggs are disseminated into the lungs via blood stream (Jenkins et al., 2007). Perhaps, the similar pulmonary pathology caused by *V. capreoli* in European roe deer (Boev, 1975; Stroh and Schmid, 1938; Švarc and Pajersky, 1990; S. Kutz, unpubl obs.) may have influenced Boev and other Russian parasitologists in making *V. alces* a junior synonym of the former, together with the previously mentioned reasons.

*Varestrongylus alces* is a common parasite in moose in Norway, with reported prevalence ranging from 8% to 26% (Stuve, 1986, 1987). The infection occurs as an incidental autopsy finding in moose dying from various causes and has never been associated with disease in moose in this country. It could, however, be speculated that heavy *V. alces* infection may predispose the lungs to secondary bacterial infections. This could also be the case if this parasite occurs in combination with *Dictyocaulus* and *E. alces*, as observed in at least two animals in the present study. Co-infection of *V. alces* and *E. alces* appears to be relatively common in moose both in Norway (Stuve, 1986, 1987), and other European countries (Dróżdż, 1966; Nilsson, 1971). Parasitism by multiple species of lungworms and/or extra-pulmonary protostrongylids may produce cumulative or synergistic deleterious effects, as suggested in cases of co-infection in different host-parasite systems (Jenkins et al., 2007; Kutz et al., 2013a; Kutz et al., 2012a; Panayotova-Pencheva and Alexandrov, 2010).

### *2.5.4 Biogeography – past and present*

*Varestrongylus alces* appears to be geographically restricted to the Palaearctic. To date, the parasite has been recognized in *A. alces* from at least six European countries: Poland (Dróżdż, 1966), Norway (Stuve, 1986; 1987; present study), Sweden (Nilsson, 1971), Finland (cited in (Stéen et al., 1998)), Estonia (Järvis, 1995), and areas of western Russia (Demidova and Naumitscheva, 1953; Petrosyan, 1963). *Varestrongylus alces* has not been reported from subspecies of *A. americanus* in eastern Russia, although the search effort for the parasite is not known. In North America, despite reasonably extensive fecal surveys of North American moose in northern Canada and Alaska, it has not been found (revised in Kutz et al. (2012a); G. Verocai, unpubl. data). In the absence of extensive geographic and host sampling, we can look to host and parasite phylogeny and host historical biogeography to develop and explore hypotheses about the geographic distribution and host associations for *V. alces*.

Recent genetic evidence supports that *Alces* comprises two extant species: *A. alces,* referred as Siberian moose or elk as per the International Union for Conservation of Nature (IUCN) (Geist et al., 2008), in Central Russia to Europe, and *Alces americanus* (Clinton 1822), referred as moose, in eastern Asia and North America (Hundertmark et al., 2003; Hundertmark et al., 2002; Kurtén and Anderson, 1980; Wilson and Reeder, 2005) (or two major genetic clades, but only different subspecies (Hundertmark et al., 2002)). The contemporary distribution of *Alces* is a result of complex historical patterns of geographic expansion and retraction, and isolation. *Alces* survived the glaciations of the Pleistocene in multiple refugia south of the ice-sheets, as supported by fossil records within Europe and Asia (Breda and Marchetti, 2005;

Niedziałkowska et al., 2014; Sommer and Nadachowski, 2006). Throughout the Pleistocene and early Holocene, the distribution of *A. alces* in Europe was considerably broader, comprising many countries of western and central Europe, as per fossil and sub-fossil findings. After recession of the continental ice-sheet, *A. alces* recolonized much of the previously glaciated regions of Eastern Europe, Fennoscandia and Russia, and concomitantly went extinct in areas of Western and Central Europe (Hewitt, 1999; Schmölcke and Zachos, 2005). In more recent times, *A. alces* was nearly extirpated in Europe and only recolonized its current range after World War II. This population bottleneck, followed by recent geographic expansion, resulted in the low genetic diversity seen among extant populations (Niedziałkowska et al., 2014). Nonetheless, *V. alces* seems to have persisted in regions represented by the different genetic clades reported in this study, and potentially recolonized suitable areas from Fennoscandia and eastern Europe, extending eastwards to the Central Russian Federation, Kazakhstan, Northern China and Mongolia (Wilson and Reeder, 2005) along with its definitive host.

Siberian moose in Eastern Asia are conspecific to the North American moose subspecies. Historical processes that shaped this divergence and lead to speciation within *Alces* may explain the absence of *V. alces* in *A. americanus* from Eastern Asia and, consequently, North America, to where *Alces* expanded and colonized only during the Late Pleistocene (Hoberg et al., 2012b; Kurtén and Anderson, 1980). If the association with *Alces* is evolutionarily deep, *V. alces* or its ancestor may have been lost in *A. americanus* populations due to ecological factors. Alternatively, in case of a more shallow association, *V. alces* or its ancestor could have host-switched, and established in *A. alces* after isolation, and allopatric speciation when this host was in sympatry with other cervids associated with *Varestrongylus* species**.**

Current literature and the knowledge of the historical biogeography of *Alces* and other ungulates may support an exclusive contemporary association of *V. alces* to the Eurasian moose; potentially suggesting a deep historical association with this host. Historically, during the Pleistocene, *Alces* was sympatric with other cervids including *Cervus elaphus*, *Capreolus capreolus*, and *Rangifer tarandus* (Azzaroli et al., 1988; Sommer and Nadachowski, 2006) in several temperate refugia within Eurasia. Coincidentally, all these cervids are hosts for other *Varestrongylus* species in Eurasia or North America. Additionally, this historical host sympatry indicates the long-term co-existence of different *Varestrongylus*/Cervidae assemblages, which support an early diversification within the parasite genus, perhaps congruent to ungulate diversification. Alternatively, this extensive sympatry and diversity of the mammalian megafauna may also have facilitated the occurrence of host-switching among ungulate hosts. Glacial cycles during the Pleistocene caused the extinction of multiple elements of this mammalian community and promoted isolation in different refugia, also altering patterns of sympatry of cervid hosts of *Varestrongylus* species, as mentioned above. As proposed by Hoberg et al. (1995), increased allopatry and host extinction events could have: (i) resulted in lowered diversity in certain parasite groups, as in the monospecific genus *Umingmakstrongylus* Hoberg, Polley, Gunn & Nishi, 1995, or (ii) constituted a determinant of post-glacial isolation and allopatric speciation of certain parasites, which may be the case of the diverse genus *Varestrongylus*.

This apparent absence from North American moose is perhaps not surprising, as there is no overlap between Eurasian and Nearctic protostrongylid fauna, with the exception of cases where there have been anthropogenic introductions (Boev, 1975; Hoberg et al., 2012b; Lankester, 2001; Verocai et al., 2013). Nevertheless, North American moose are incidental hosts

for many protostrongylids: *Orthostrongylus macrotis* (Dikmans 1931) Dougherty and Goble 1946 (Samuel et al., 1976), *Parelaphostrongylus tenuis* (Dougherty, 1945) (Lankester et al., 2007), *Parelaphostrongylus andersoni* Prestwood 1971, the introduced Eurasian protostrongylid *E. rangiferi* (Mitskevitch 1960) (Lankester and Fong, 1998), and the undescribed Nearctic *Varestrongylus* species (Kutz et al., 2007).

Whether *V. alces* is exclusively associated with *Alces* or if other contemporary sympatric cervids may serve as suitable hosts is still unclear. Herein, we consider previous reports of *V. capreoli* in Eurasian moose suspect, and more likely to be *V. alces,* as specimens were not confirmed by morphological or molecular identification; vouchers do not exist in museum collections from these surveys. Future studies should use combined morphological and molecular approaches to unequivocally diagnose *V. alces* and *V. capreoli,* and further assess their host specificity, especially in areas of sympatry. Further it is critical that any field collections be accompanied by deposition of specimens which make it possible to apply integrated approaches to assessments of parasite diversity (Hoberg et al., 2009).

There is a relatively close genetic association of *V. alces* to the undescribed, multi-host, *Varestrongylus* species whose putative primary host is the caribou and appears to be geographically restricted to the Nearctic (Kutz et al., 2007; Kutz et al., 2013a). This may suggest its potential infectivity to other hosts, in particular reindeer. Recently, pulmonary lesions compatible with those caused by *Varestrongylus* species were found in reindeer in Finland (Antti Oksanen, pers. comm.). In regions of Fennoscandia and Russia, the geographic range of the Eurasian moose overlaps with reindeer and it is conceivable that *V. alces* can persist in both hosts. Alternatively, the lesions may be associated with infection by the newly described

*Varestrongylus* sp. from North American caribou despite no gross pulmonary lesions have been ever observed caribou or muskoxen examined for this lungworm species (Kutz et al., 2007; Verocai et al., 2011), or could be caused by yet another cryptic species of *Varestrongylus* circulating in Eurasian reindeer.

### *2.5.5* Varestrongylus *cf.* capreoli – V. capreoli *as a species complex?*

In our study, the male specimens recovered from lungs of roe deer were largely consistent with *V. capreoli* (*sensu* Stroh & Schmid, 1938) but differed based on one structural character, the absence of a capitulum/head of the gubernaculum. Such remarkable intra-specific morphological variations have not previously been described for *Varestrongylus* species or other protostrongylids (Boev, 1975). This morphological difference was consistent across specimens and led us to identify these as *V.* cf. *capreoli.* In *V. capreoli*, the head of gubernaculum in males is considered not only as a diagnostic feature, but as an autapomorphy of this species, that is, a unique feature not shared within *Varestrongylus*, potentially due to an independent evolutionary trajectory (speciation) and, therefore, has been considered of phylogenetic relevance (Carreno and Hoberg, 1999). Notably, we did not have access to any archival material of *V. capreoli*, and could not verify the original description of the capitulum. Morphologically, females of *V.* cf. *capreoli* are virtually indistinguishable from either *V. capreoli*, and *V. alces.* The wide range of measurements reported for *V. capreoli* (Boev, 1975) could be hiding either a species complex or simply represent intra-specific variability (i.e. the existence of morphotypes or lineages in males). However, as in the case of *V. alces*, morphologically similar species could have been equivocally identified as, or arbitrarily synonymized with, *V. capreoli*. Additionally, supporting the potential existence of a species complex within *V. capreoli* is its apparent broad host range,

as it has been reported in sympatric caprine hosts: the mouflon (*Ovis aries*) in Czech Republic and domestic goats in the Swiss Alps (Bouvier and Hörning, 1963; Erhardova, 1957). These reports may be equivocal and are yet to be confirmed. Conversely, the recent finding of *Varestrongylus* species that infects caribou, muskoxen (caprine) and rarely moose in North America (Kutz et al., 2007), could support a potentially wide host range for *V. capreoli*.

To address this emerging question, *V. capreoli*-like material of cervid and caprine hosts from the type locality (Bavaria, Germany) and other Eurasian regions should be assessed by combined morphological and molecular approaches. A first step would be to retrieve ITS-2 sequences of male *V. capreoli* that possess the capitulum of the gubernaculum for comparative analysis, and later evaluate multiple genetic markers. In this way, it would be possible to determine if these different morphological features are only intra-specific variation or if there is a cryptic *Varestrongylus* species in roe deer from Norway and other areas of Fennoscandia, reflecting perhaps a more recent event of geographic isolation of parasite populations and speciation within the same host. In recent history, after periods of population fluctuations, roe deer in Fennoscandia were reduced to less than 100 individuals concentrated in the southernmost part of the Scandinavian Peninsula (Southern Scandia, Sweden) (Andersen et al., 2004; Ekman, 1919). From the 1850's onwards roe population expanded, and now occupies most of Norway, Sweden and Finland (Andersen et al., 2004). This recent and drastic host population bottleneck could have resulted in the genetic drift of this heritable polymorphic gubernaculum in males of *V. capreoli* in the region, or alternatively, this polymorphism may be attributed to natural selection.

### *2.5.6 Final remarks*

Further comprehensive investigation targeting *Varestrongylus* hosts in Eurasia and North America (i.e. cervids and caprines) in conjunction with a systematic reassessment of the taxonomic status of dubious taxa through integrated classical and molecular methods in parasitology may reveal an even richer hidden biodiversity within *Varestrongylus*. Consequently, such investigation would give us a better understanding on the historical biogeography and relationships among the species within the genus, their associations with different ungulate hosts, and, ultimately, provide valuable insights on the historical biogeography of ungulate species.

The use of appropriate molecular markers for species-level identification is a powerful tool for discriminating valid species among cryptic species complexes (Gasser, 2006; Nadler and Pérez-Ponce de León, 2011; Pérez-Ponce de León and Nadler, 2010). In this study, molecular analysis, combined with classical methods, assisted us in re-examining the taxonomic status of a valid species erroneously reduced as a junior-synonym. In addition to their irrefutable similar morphology, other factors that led to this synonymy were the incomplete description and the absence of species types, or vouchers, deposited in a museum collection, hence the importance of specimen deposition (Hoberg, 2002). Molecular information is relatively scarce for members of the genus *Varestrongylus*, and there is a need to produce new data for species, and ideally, this should be done concurrently from specimens with matching morphologic identification, (i.e. adults). After that, larvae confirmed as belonging to a given species could be used to assess its geographic and host ranges, and may provide relevant material for studies on the species historical biogeography and phylogeography, in conjunction with the history of host-parasite assemblages.

## 2.6 Conclusions

*Varestrongylus alces* is a valid species, and should be considered separate from *V. capreoli*. Phylogenetic relationships among *Varestrongylus* species from Eurasia and North America are complex and consistent with faunal assembly involving recurrent events of geographic expansion and host switching and subsequent speciation.

## 2.7 Competing interests

The authors declare that they have no competing interests.

## 2.8 Authors' contributions

GGV led the study and preparation of the manuscript. SJK and EPH oversaw the study and manuscript preparation. SJK, RKD, BY, and KH collected specimens for the study. TV and KH were responsible for the pathology. GGV and EPH did morphological redescription of the species. GGV, AMR and JSG carried out the molecular analysis. All authors critically revised and approved the final manuscript.

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# Chapter Three: *VARESTRONGYLUS ELEGUNENIENSIS* SP. N. (NEMATODA: PROTOSTRONGYLIDAE): A WIDESPREAD, MULTI-HOST LUNGWORM OF WILD NORTH AMERICAN UNGULATES, WITH AN EMENDED DIAGNOSIS FOR THE GENUS AND EXPLORATIONS OF BIOGEOGRAPHY

## 3.1 Abstract

A putative new species of *Varestrongylus* has been recently recognized in wild North American ungulates based on the ITS-2 sequences of larvae isolated from feces during a wide geographic survey. No taxonomic description was provided, as adult specimens were not examined. Lungworm specimens were collected in the terminal bronchioles of muskoxen from Quebec, and a woodland caribou from central Alberta, Canada. The L3 stage was recovered from experimentally infected slugs (*Deroceras* spp.). Description of specimens was based on comparative morphology and integrated approaches. Molecular identity was determined by PCR and sequencing of the ITS-2 region of the nuclear ribosomal DNA, and compared to other protostrongylids. *Varestrongylus eleguneniensis* sp. n. is established for a recently discovered protostrongylid nematode found in caribou (*Rangifer tarandus*), muskoxen (*Ovibos moschatus*) and moose (*Alces americanus*); hosts that collectively occupy an extensive geographic range across northern North America. Adults of *Varestrongylus eleguneniensis* are distinguished from congeners by a combination of characters in males (distally bifurcate gubernaculum, relatively short equal spicules not split distally, a strongly elongate and bifurcate dorsal ray, and an undivided copulatory bursa) and females (reduced provagina with hood-like fold extending ventrally across prominent genital protuberance). Third-stage larvae resemble those found among other species in the genus. The genus *Varestrongylus* is emended to account for the structure of the dorsal ray characteristic of *V. eleguneniensis*, *V. alpenae*, *V. alces* and *V. longispiculatus*. Herein we describe and name *V. eleguneniensis*, a pulmonary protostrongylid with *Rangifer tarandus* as a primary definitive host, and which secondarily infects muskoxen and moose in areas of sympatry. Biogeographic history for *V. eleguneniensis* and *V. alpenae*, the

only two endemic species of *Varestrongylus* known from North America, appears consistent with independent events of geographic expansion with cervid hosts from Eurasia into North America during the late Pliocene and Quaternary.

## 3.2 Introduction

Nematodes of the Family Protostrongylidae Leiper, 1926 are characteristic and often pathogenic parasites among Bovidae, particularly Caprinae, and Cervidae (Artiodactyla) across the Holarctic (comprised by the Palaearctic and Nearctic regions) (Boev, 1975; Carreno and Hoberg, 1999; Lankester, 2001), and less frequently in tropical regions of the southern hemisphere (Canaris and Gardner, 2003; Carreno et al., 2012). The Nearctic protostrongylid fauna includes genera and species, partitioned among four subfamilies, occurring in domestic and free-ranging ungulates and lagomorphs: Protostrongylinae Kamensky, 1905; Muelleriinae Skrjabin, 1933; Elaphostrongylinae Boev & Shulz, 1950; and Varestrongylinae Boev, 1968 (e.g., Carreno and Hoberg, 1999). Species within the Protostrongylinae, Muelleriinae, and Varestrongylinae are strictly pulmonary parasites, with adult nematodes residing in the bronchi, bronchioles, or lung parenchyma, whereas those of the Elaphostrongylinae are found in the skeletal musculature or central nervous system of their hosts. Although the protostrongylid assemblage of Nearctic ungulates was thought to be well defined, there is a growing body of knowledge regarding their biodiversity with new insights about historical processes, host range, geographic distribution and faunal structure (Asmundsson et al., 2008; Hoberg et al., 2012b; Hoberg et al., 2002; Hoberg et al., 1995; Jenkins et al., 2005a; Kutz et al., 2007; Kutz et al., 2013a; Kutz et al., 2012a; Kutz et al., 2001c; Mortenson et al., 2006; Verocai et al., 2013).

Within the Varestrongylinae, the genus *Varestrongylus* Bhalerao, 1932 is of special interest due to its complex taxonomic history (Boev, 1975; Dougherty, 1945; Dougherty and Goble, 1946). Currently, there are nine nominal species considered valid within the genus, with free-ranging cervids and wild and domestic caprine bovids as the main hosts (Boev, 1975; Liu,

1989; Liu, 1984; Verocai et al., 2014a). The center of diversity for the genus is Eurasia (Grubb, 2005), where eight species have been described.

Until recently, *Varestrongylus alpenae* (Dikmans, 1935) a lungworm in the white-tailed deer, *Odocoileus virginianus* (Zimmermann), occurring at temperate and subtropical regions of southern, eastern and central North America (Cheatum, 1949, 1951; Dikmans, 1935; Gray et al., 1985; Kutz et al., 2007; O'Roke and Cheatum, 1952; Prestwood and Pursglove, 1974), was recognized as the sole species of *Varestrongylus* endemic in North America. However, during the last decade molecular-based survey of high latitude protostrongylids in North America resulted in the detection of a putative second Nearctic species of *Varestrongylus* (Kutz et al., 2007). This parasite was distinguished from other protostrongylids based on sequences of the internal transcribed spacer region-2 (ITS-2) of the nuclear ribosomal DNA (rDNA) derived from first-stage dorsal-spined larvae (DSL) extracted from ungulates feces (Kutz et al., 2007). Although sequence data suggested its placement within the genus *Varestrongylus*, considerable divergence was demonstrated relative to *V. alpenae* (Kutz et al., 2007).

At the time of the original discovery (Kutz et al., 2007), field collections indicated a broad geographic range, extending throughout the northern Nearctic, encompassing five Canadian provinces and territories: mainland Labrador and Newfoundland, Quebec (QC), Nunavut (NU), Northwest Territories and the Yukon, as well as Alaska, USA (Kutz et al., 2007). More recently, this undescribed species was found on Victoria Island in the Canadian Arctic, demonstrating that its distribution is not restricted to mainland North America (Kutz et al., 2013a). The host range of this new species is remarkably broad, with natural infections detected in caribou of three subspecies: the woodland caribou, *Rangifer tarandus caribou* (Gmelin), the

barrenground caribou, *Rangifer tarandus groenlandicus* (Borowski), and the Grant's caribou, *Rangifer tarandus granti*; muskoxen of two subspecies, *Ovibos moschatus moschatus* (Zimmermann) and *Ovibos moschatus wardi* Lyddeker; and one subspecies of moose, *A. americanus gigas* Miller (Kutz et al., 2007; Kutz et al., 2013a; Kutz et al., 2012a; Verocai, Kutz, unpublished data).

Protostrongylids have an indirect life-cycle, requiring gastropods as intermediate hosts (IH) (Anderson, 2000; Boev, 1975; Lankester, 2001). To date, the only known naturally infected IH is the meadow slug *Deroceras laeve* (Müller, 1774) (Kutz et al., 2007). This species is widely distributed across northern North America (Pilsbry, 1946) and is believed to be the main intermediate host for several protostrongylids (Kutz et al., 2012a).

Recent collections of adult nematodes from the bronchioles of caribou and muskoxen now allow a complete description and series of comparisons to characterize this previously unknown species. In this study we propose the establishment of *Varestrongylus eleguneniensis* sp. n. for this geographically wide-spread, multi-host protostrongylid lungworm occurring across northern North America. Further, we provide an emended diagnosis for the genus *Varestrongylus*, and explore the historical biogeography of these nematodes in the Nearctic.

## 3.3 Methods

### *3.3.1 Taxonomic criteria*

Host taxonomic classification follows Grubb (2005) and Hernández-Fernández and Vrba (2005). Parasite taxonomy is largely consistent with the latest revision (Boev, 1975) and the most recent phylogenetic hypothesis for the family Protostrongylidae (Carreno and Hoberg, 1999).

### *3.3.2 Collection*

#### 3.3.2.1 *Muskoxen*

Muskoxen were harvested from a free-ranging population in Nunavik, QC. These animals were introduced in 1967 from Ellesmere Island, NU to Old Fort Chimo located across the Koksoak River, near Kuujjuaq, for farming purposes, mainly for qiviut production (muskox wool). The farm was shut down and animals were released between 1973 and 1983, and their descendants are currently distributed throughout much of the Ungava Peninsula in northern QC extending eastwards into Labrador (Chubbs and Brazil, 2007; Le Hénaff and Crête, 1989). All muskoxen examined in this study were harvested through either subsistence or sport hunting regulated by the Ministry of Natural Resources and Fauna of Quebec (Ministère des Ressources Naturelles et de la Faune du Québec). Hunters submitted selected samples of harvested muskoxen to the Nunavik Research Centre (NRC), Makivik Corporation, located in Kuujjuaq, for general health and food safety assessment (zoonotic diseases and contaminants). Nematodes were isolated from the lungs of two muskoxen harvested near the town of Tasiujaq, QC in late March, 2010: an adult female (Om-01-2010, March 20th, 58°44′51″N, 70°02′06″W) and an adult male (Om-02-2010, March 28th, 58°44′10″N, 69°34′18″W). Another adult female (Om-10-2010) found dead on 31 December, 2009 near Kuujjuaq (58°06′24″N, 68°23′55″W) was collected by NRC personal and kept frozen until necropsy. Additionally, a single female nematode, collected from an adult male muskox (Om-10-2007) hunted near Kuujjuaq (58°45′00″N, 68°33′29″W) on March 21st, 2007, was preserved in 70% ethanol by M. Simard, and later identified as *Varestrongylus*, and also used for the species description.

3.3.2.2 Caribou

Additional adult nematodes were collected from the lungs of an adult male woodland caribou belonging to the Cold Lake herd, Alberta, Canada. The animal was found dead, likely killed by collision with motor vehicle, at 55°2′25″N, 110°34′5″W, near the border with Saskatchewan. The carcass was collected by the Fish and Wildlife Division (FWD) of the Alberta Sustainable Resource Development under the Alberta Wildlife Research Permit no. 48549.

### *3.3.3 Fecal analyses*

Fecal samples of the three muskoxen and the woodland caribou were collected and kept frozen at −20°C until analyses. Samples were analyzed for the presence of protostrongylid DSL using the modified beaker Baermann technique (Forrester and Lankester, 1997) at the NRC and the University of Calgary, prior to lung dissection.

### *3.3.4 Lung dissection*

Lungs were thawed and individually processed. Briefly, they were first grossly examined for pathology and presence of nematodes. A first wash was done with the lungs still intact by flushing tap water into the trachea and then pouring the fluid from the lungs back through a 75 μm mesh sieve. Material retained on the sieve was put in Petri dishes and examined under a stereomicroscope for the presence of nematodes. The entire bronchial tree was then dissected with repeated washing of the exposed airways and pulmonary tissue through the sieve. Material retained in the sieve was examined as described above. All intact nematodes or fragments were collected, identified by gender, and stored in 70% ethanol.

### *3.3.5 Morphological identification*

3.3.5.1 Nematodes examined

*Adults*. Specimens and fragments of adult nematodes were mounted and cleared in either lactophenol or phenol-alcohol, and examined under light microscopy with differential interference optics. Photomicrographs were prepared with a Nikon DX 1200 digital camera and a Zeiss Axiophot microscope. Line drawings were prepared with the use of a drawing tube. Throughout the descriptions, measurements are given in micrometres (μm) unless specified otherwise, and are presented with the numbers of adult male, female or larval (DSL and third-stage larvae, L3) nematodes examined (n=), and the range is followed by the mean ± 1 SD within parentheses. *First-stage larvae.* DSL from feces were recovered from muskox Om-02-10 using the modified beaker Baermann technique (Forrester and Lankester, 1997). Isolated live DSL were killed in steaming 70% ethanol/glycerine solution (19:1), and held for further evaluation. Species identity was confirmed by molecular sequencing prior to the use of DSL to establish experimental infections in slug intermediate hosts (see below). *Third-stage larvae.* DSL for infection of slugs *D. laeve* and *Deroceras reticulatum* were isolated from feces of the same muskox population at Nunavik, QC (UC-133, representative DSL ITS-2 sequence in Table 1). The slugs were experimentally exposed to 300 DSL/slug as per (Kutz et al., 1999; Kutz et al., 2001b). Gastropods were maintained in Petri dishes for a day and transferred to plastic containers with an autoclaved soil/vermiculite mix kept at 20°C, making sure that there was constant moisture, and provided with food (lettuce and carrot). Slugs were killed between 18–21 or 50–60 days post exposure (*D. laeve* and *D. reticulatum*, respectively), cut into small pieces,

and digested in HCl/pepsin solution (Hoberg et al., 1995; Kutz et al., 2001b). Material was analyzed under a dissecting microscope, and all L3 recovered were preserved in 70% ethanol.

**Table 3.1.**Summary of collected specimens of *Varestrongylus eleguneniensis* sp. n. from muskoxen (*Ovibos moschatus wardi*) from Nunavik Region, Quebec, Canada, and woodland caribou (*Rangifer tarandus caribou*) from Alberta, Canada

| Animal ID | Species | Sex | LPG[a] | Coordinates | Locality | Number of adult specimens | | | | Accession Numbers | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Males | Male fragments | Females | Female fragments | USNPC | GenBank (ITS-2) |
| Om-10-07 | *O. m. wardi* | Male | - | 58° 45′00″N 68° 33′29″ W | Kuujjuaq, QC | 0 | 0 | 1 | 0 | 103743 (♀) | – |
| Om-01-10 | *O. m. wardi* | Female | 6 | 58° 44′51″N 70° 02′06″W | Tasiujaq, QC | 1* | 0 | 0 | 0 | 103740 (♂)* | JQ478746 |
| Om-02-10 | *O. m. wardi* | Male | 25 | 58° 44′10″N 69° 34′18″W | Tasiujaq, QC | 3 | 1 tail | 1** | 1 head, | 103741 (♀)** 103742 (♂,♀)[†] | – |
| | | | | | | | | | 3 tails | 103748 (♀) 103749 (♀) | JQ478649 (♂) JQ478647 JQ478648 |
| Om-10-10 | *O. m. wardi* | Female | 0.4 | 58°45′00″N 68° 33′29″W | Kuujjuaq, QC | 4 | 1 tail | 2 | 1 tail | 103744 (♀,♂)[†] | JQ478644 (♀) JQ478645 (♂) |
| UC178-2 | *R. t. caribou* | Male | 0.4 | 55° 2′25″N 110° 34′5″W | Cold Lake, AB | 1[§] | 1 head, | 0 | 0 | 105697 (♂) | JX115007 |
| | | | | | | | 1 tail | | | 105698 (♂) 105699 (♂) | – |
| | | | | | | | | | | 105700 (♂)[§] DSL[§§] | JX115007 |
| | | | | | | | | | | | – |
| | | | | | | | | | | | JQ478651[§§] |

All these specimens were used for the taxonomical description (holotype, allotype and paratypes), with matching accession numbers at the United States National Parasite collection (USNPC) and for sequences at the second internal transcript spacer (ITS-2) region at the nuclear ribosomal DNA deposited at GenBank.

[a] Protostrongylid first-stage larvae (DSL) per gram of feces; *Holotype; ** Allotype; [†] Multiple vials containing males and females (intact specimens or fragments), USNPC103742 also contains a vial with DSL; [§] Additional immature male not included in the description, but accessioned at the USNPC as a voucher; [§§]Additional sequence from DSL extracted from host feces, not accessioned at USNPC.

**Table 3.2.** Comparative morphometry of males of all valid species within the genus *Varestrongylus*

| **Species / Characters** | ***V. eleguneniensis*** | | | ***V. alpenae***[a] | ***V. alces***[b] | ***V. capreoli***[c] | ***V. capricola***[d] | ***V. longispiculatus***[e] | ***V. pneumonicus***[f] | ***V. qinghaiensis***[g] | ***V. sagittatus***[h] | ***V. tuvae***[i] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Range** | **ex muskox** | **ex caribou** | | | | | | | | | |
| Total length | 8.8–14.7 | 8.8–12.28 | 11.7–14.7 | 13–15 | 11.36–14.7 | 5.3–13.5 | 15–18 | 14.47–17.19 | 14–24 | 9–12.2 | 14.5-33.8 | nd |
| Maximum width | 78–147 | 78–147 | 84–106 | _ | 68.46–80 | 32–68 | 140 | 62–75 | 165–200 | 54–94 | 112–189 | 174 |
| Esophagus[§] | 247–395 | 247–377 | 365–395 | 230–250 | 250–272 | 90–146 | 275 | 294–349 | 310–390 | 257–338 | 260-360 | _ |
| Esophagus base width | 44–62 | 44–60 | 52–62 | _ | 32–37 | _ | 50 | 27–39 | _ | _ | 51 | _ |
| Body width at esophagus base | 70–91 | 70–91 | 82 | 60–65 | 53.8–61.9 | _ | _ | _ | _ | 46–79 | _ | _ |
| Nerve-ring[§] | 87–196 | 87–143 | 182–196 | _ | 68–89.7 | _ | _ | 78–99 | 170–180 | _ | 160 | _ |
| Cervical papillae | 151–215 | 151–215 | 184–200 | _ | 201–207 | _ | _ | _ | _ | _ | _ | _ |
| Excretory pore[§] | 147–242 | 147–242 | 216–234 | 190–200 | 208–230.3 | _ | _ | 135–189 | – | _ | 240 | _ |
| Spicule (right) | 105–148 | 105–140 | 135–148 | 375 | 138.6–163 | 129–160 | 250–280 | 232–282 | 290–570 | 80–116.5 | 325–433.8 | 361 |
| Spicule (left) | Equal | Equal | Equal | Equal | Equal | Equal | Equal | Sub-equal, 280–314 | Equal | Sub-equal, 107.4–160.9 | Equal | Equal |
| Gubernaculum | 60–86 | 60–86 | 67–72 | 145–170* | 65–83.1 | 70–86 | 150 | 114–147 | 138–165 | 104–139 | 128–176.6 | 200 |
| Gubernaculum head | Absent | Absent | Absent | Absent | Absent | 8–14 | Absent | Absent | Absent | Absent | Absent | Absent |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gubernaculum corpus | 32–57 | 32–57 | 37–42 | 95–110 | 38–49 | – | _ | _ | _ | _ | _ | _ |
| Gubernaculum crura | 23–36 | 23–36 | 30 | 50–60 | 24–39.1 | – | _ | _ | _ | _ | _ | _ |
| Crura denticulate piece | 15–25 | 15–25 | 17–23 | >50 | 15–25 | 18–30 | 25 | 27–29 | 36–40 | 27.2–35 | 33–53.8 | NA |
| Body width at bursa | 50–75 | 50–75 | 58–65 | 90–100 | 42–56 | _ | _ | _ | – | 49–62 | 82 | nd |
| Bursa width | 95–135 | 95–135 | 110–116 | 350 | 125–160 | _ | _ | _ | 165–220 | _ | _ | _ |
| Bursa length | 65–91 | 65–91 | 80–87 | 90 | 75–90 | _ | _ | _ | 140–165 | _ | _ | _ |
| Dorsal ray length | 23–39 | 23–39 | 23–36 | 15 | 18–30 | NA | NA | 18–27 | NA | NA | NA | NA |
| Dorsal ray base | 21–31 | 21–31 | 26–27 | 10 | 11.4–15 | NA | NA | NA | NA | NA | NA | NA |

Total length in millimeters (mm), and all other measurements are in micrometers (μm).

[a] *V. alpenae*: original description (Dikmans, 1935); [b] *V.* alces, according to (Verocai et al., 2014a); [c] *V. capreoli*: original description (Stroh and Schmid, 1938), plus additional information compiled in (Boev, 1975); [d] *V. capricola*: Measurements from original description cited in (Boev, 1975); [e] *V. longispiculatus*: Measurements from original description (Liu, 1989); [f] *V.* pneumonicus: Data from the original description (Bhalerao, 1932), and additional data from (Bhatia and Pande, 1960) and (Boev, 1975); [g] *V. qinhaiensis*: Measurements from (Liu, 1984); [h] *V. sagittatus*: Combined measurements from Measurements from cited in (Boev, 1975; Stroh and Schmid, 1938); [i] *V. tuvae*: Measurements from original description cited in (Boev, 1975); § Measurements from anterior end; ; nd = never determined; NA = not applicable.

**Table 3.3.** Comparative morphometry of females of all valid species within the genus *Varestrongylus*

| **Characters** | ***V. eleguneniensis*** | ***V. alpenae***[a] | ***V. alces***[b] | ***V. capreoli***[c] | ***V. capricola***[d] | ***V. longispiculatus***[e] | ***V. pneumonicus***[f] | ***V. qinghaiensis***[g] | ***V. sagittatus***[i] | ***V. tuvae***[j] |
|---|---|---|---|---|---|---|---|---|---|---|
| Total length | 18.41–21.27 | 20 | 16.25–21.52 | 9.4–15 | nd | 44. 6–51.8 | 19.6–31 | 13–18 | 22–61 | >11 |
| Maximum width | 108–195 | – | 73–102 | 38–95 | 140 | 90–120 | 100–190 | 64–119 | 170–300 | 204 |
| Esophagus[§] | 265–337 | – | 270–310 | 122–290 | 400 | 289–365 | 360–400 | 275–320 | 260–360 | – |
| Esophagus base | 39–64 | – | 30–42 | – | 65 | 42–66 | – | 28.7–37 | 51 | – |
| Body at esophagus | 75–107 | – | 57–67 | – | – | – | – | – | – | – |
| Nerve-ring[§] | 63–156 | – | 86–97 | 72–90 | – | 87–96 | 190–250 | – | 160 | – |
| Cervical papillae | 189–217 | – | 150–180 | – | – | – | – | – | – | – |
| Excretory pore[§] | 154–237 | – | 159–220 | 180–186 | – | 198–228 | – | – | 240 | – |
| Tail | 39–55 | 70–75 | 34–51 | 34–78 | 50 | 66–93 | 40–60 | 37–65 | 90–112 | 128–149 |
| Vulva-anus | 99–166 | 150–160 | 70.1–104 | | – | – | 40–60 | 64–92 | – | – |
| Vulva-tail | 143–215 | 90 | 108 –146 | 90–144 | – | 150–188 | 80–120 | 101–157 | 180–225 | 255–362 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Width at vulva | 57–90 | 100 | 46–69 | – | – | – | – | – | – | 162–200 |
| Vagina | 377–711 | 550–600 | 702–961 | – | – | – | – | – | – | – |
| Eggs length† | 60–78† | 60–90 | 55–67 | 56–78 | 65-75 | 54–63 | 57–80 | 86–91 | 78 | – |
| Eggs width† | 57–74† | 25–35 | 46–63 | 37–45 | 30-40 | 27–30 | 30–43 | 12–34 | 48 | – |

Total length in millimeters (mm), and all other measurements are in micrometers (μm).

[a] *V. alpenae*: original description (Dikmans, 1935); [b] *V.* alces, according to (Verocai et al., 2014a); [c] *V. capreoli*: original description (Stroh and Schmid, 1938), plus additional information compiled in (Boev, 1975); [d] *V. capricola*: Measurements from original description cited in (Boev, 1975); [e] *V. longispiculatus*: Measurements from original description (Liu, 1989); [f] *V.* pneumonicus: Data from the original description (Bhalerao, 1932), and additional data from (Bhatia and Pande, 1960) and (Boev, 1975); [g] *V. qinhaiensis*: Measurements from (Liu, 1984); [h] *V. sagittatus*: Combined measurements from Measurements from cited in (Boev, 1975; Stroh and Schmid, 1938); [i] *V. tuvae*: Measurements from original description cited in (Boev, 1975); § measurements from anterior end; † eggs collected from lungs of infected caribou, not inside female uteri; nd = never determined.

### *3.3.6 Molecular analyses*

3.3.6.1 DNA extraction and amplification

Adult nematode fragments were recovered and subsampled from each of the three muskoxen and the caribou. These fragments were individually transferred into wells prior to DNA extraction. Of these, eight fragments (6 from the 3 muskoxen, and 2 from the caribou) had matching caudal extremities or caudal and cephalic extremities used in the morphological description of the species, and, therefore, are part of the type-series (holotype and paratypes) deposited in the United States National Parasite Collection (USNPC), Agricultural Research Service, USDA, Beltsville, MD, USA (see Table 3.1). In accordance with section 8.5 of the ICZN's International Code of Zoological Nomenclature, details of the new species have been submitted to the ZooBank under the life science identifier (LSID) zoobank.org:pub:0E9BC9BC-EE4F-461E-9000-37FCFEB4C71F.

Genomic DNA (gDNA) was extracted from 2–4 mm nematode fragments in 2 mL tubes containing 5 µL of deionized water. To each tube was added 25 µL of lysis buffer (0.5 mg/mL of proteinase K, 10× PCR buffer). DNA extraction followed the following protocol: tubes containing adult worm fragments were incubated at 60°C for 60 min, 65°C for 60 min, then at 95°C for 15 min. The lysis was repeated, after the addition of 1 µL of proteinase K (20 mg/mL) in each well, following the same protocol. Extracted DNA was diluted 1:10. For species identification, a PCR modified from (Kutz et al., 2007) was performed using primers NC1 (5′-ACG TCT GGT TCA GGG TTG TT-3′) and NC2 (5′- TTA GTT TCT TTT CCT CCG CT-3′) targeting the ITS-2 region of rDNA. PCR amplification was performed in 40 µL reactions containing: 20.4 µL of water, 8 µL of 10× PCR buffer + $MgCl_2$, 0.8 µL of 10 mmol dNTPs, 4 µL (10 µM) of each primer, 0.4 µL of bovine serum albumin, 0.4 µL of *Taq* Phusion HF DNA polymerase, and 2 µL of DNA template. The amplification conditions used

were an initial 2 min denaturation at 98°C, followed by 35 cycles of 98°C for 10 s, 52.5°C for 30 s, and 72°C for 30 s, annealing. A final extension phase of 72°C for 5 min was followed by cooling to 10°C.

PCR products were cleaned using ExoSAP-it® and sequenced directly using NC1 and NC2 primers using BigDye Terminator Cycle Sequencing (Applied Biosystems).

3.3.6.2 Molecular identification of larvae

Specific identity of first-stage larvae used for gastropod infection (L3 description) and from the woodland caribou (UC178-2) was also based on PCR and sequencing of the ITS-2 region of the nuclear ribosomal DNA. DNA lysis was performed as described above, and PCR was done using the same NC1 and NC2 primers. Each in 20 μL reactions contained 10.2 μL of water, 4 μL of 5× PCR buffer + Mg, 0.4 μL of 10 mmol dNTPs, 2 μL (10 μM) of each primer, 0.2 μL of *Taq* Phusion HF DNA polymerase, 0.2 μL of bovine serum albumine (20 mg/mL), and 1 μL of DNA template. The amplification conditions used were the same as described above. PCR products were also cleaned and sequenced as previously described, and sequences analyzed accordingly. Representative sequences were deposited in GenBank.

3.3.6.3 Sequence analyses

Direct sequences at the ITS-2 locus of adults and larvae of *V. eleguneniensis* produced in the present study were edited using FinchTV 1.4.0 (Geospiza Inc.) and MEGA version 5 (Tamura et al., 2011). ITS-2 sequences were compared with those of putative *V. eleguneniensis* sequences (Kutz et al., 2007; Kutz et al., 2013a), and those for other protostrongylids from eight genera and 13 species represented in the original finding of this new species (Kutz et al., 2007) and available from GenBank: *Varestrongylus alpenae* (AY648407), *Parelaphostrongylus andersoni* (AF504030), *Parelaphostrongylus odocoilei* (AF504031), *Parelaphostrongylus tenuis* (Dougherty, 1945) (AF504029), *Elaphostrongylus*

*rangiferi* (Mitskevitch, 1960) (EU018482, AF504033), *Elaphostrongylus alces* Stéen, Chabaud & Rehbinder, 1989 (AF504034), *Elaphostrongylus cervi* Cameron, 1931 (AF504026), *Umingmakstrongylus pallikuukensis* Hoberg, Polley, Gunn & Nishi, 1995 (AY648409), *Muellerius capillaris* (Mueller, 1889) (AY679527), *Cystocaulus ocreatus* (Railliet & Henry, 1908) (EU018481), *Orthostrongylus macrotis* (Dikmans, 1931) (EU018483), *Protostrongylus stilesi* Dikmans, 1931 (EU018484), and *Protostrongylus rufescens* (Leuckart, 1965) (EU018485). Sequences were then aligned using PRANK, a probabilistic multiple alignment program available through the European Bioinformatics Institute (http://www.ebi.ac.uk/goldman-srv/prank).

The maximum identity of the *V. eleguneniensis* ITS-2 sequences (including range, average and SD) obtained in this study and those previously produced for this species (Kutz et al., 2007; Kutz et al., 2013a) was calculated using the pairwise distance matrix produced by Geneious (Drummond et al., 2011). In order to test the hypothesis of conspecificity of isolates, including the holotype and paratypes for *V. eleguneniensis*, and selected sequences from [12] (n = 8) and [15] (n = 2), an unrooted neighbour-joining tree was designed in Geneious [37], using the substitution model HKY, with gaps treated as complete deletion, and 5,000 bootstrap replicates, including also the sequences of all protostrongylid species mentioned above.

### *3.3.7 Other specimens examined*

Specimens of *Varestrongylus alces* Demidova & Naumitscheva, 1953, *V. alpenae*, *Varestrongylus pneumonicus* Bhalerao, 1932, *Varestrongylus sagittatus* (Mueller, 1890), and specimens attributable to *Varestrongylus capreoli* (Stroh & Schmid, 1938) (referred as *Varestrongylus* cf. *capreoli* in Verocai et al. (2014a)) available in the USNPC were examined and compared to those of the undescribed species during the development of the

morphological description (see Table one from (Verocai et al., 2014a)). Representative specimens of other species were not immediately available for comparison.

### *3.3.8 Community consultation*

The first DSL confirmed as belonging to this novel protostrongylid species were isolated from feces of a barrenground caribou belonging to the Bluenose East herd that ranges in the Sahtu Settlement Area (SSA), NT (Kutz et al., 2007) and vicinities. Therefore, we returned to three communities in the SSA: Deline (Délînê), Fort Good Hope (Rádeyîlîkóé), and Colville Lake (K'áhbamítúé), where Sahtu Dene elders and hunters were consulted on naming the parasite species in their language. The name proposed was based on the North Slavey (Sahtúot'ınę Yatı́) language, which belongs to the northwestern Canada group of the Northern Athabaskan language family (Cook and Flynn, 2008).

## 3.4 Results

### *3.4.1 Lung and fecal analyses, and specimens examined*

No gross pulmonary lesions indicative of parasite infection were observed in the three muskoxen and one caribou. Nine entire male and four entire female nematodes and four male fragments and five female fragments containing relevant morphological characteristics were recovered from four muskoxen and the woodland caribou (Table 3.1). Also, numerous DSL and eggs containing different developmental stages (from morula to fully developed DSL) were found in lung washes. Results for larvae per gram (LPG) of feces for the muskoxen and the woodland caribou are provided in Table 3.1.

### *3.4.2 Molecular findings*

Genetic identity (similarity) based on ITS-2 sequences from 10 adult nematodes from this study and DSL belonging to the putative species of *Varestrongylus* discovered and reported by Kutz et al. (2007; 2013a) was 95–100% (98.7 ± 1.4). The neighbor-joining tree supports the conspecificity of these widespread populations of *Varestrongylus* represented by adults and larval parasites (Figure 3.1). Further, reciprocal monophyly is demonstrated relative to the putative sister species within *Varestrongylus* (99.3 bootstrap support), and homologous sequences are distinct among other species of *Varestrongylus* where molecular data are available (see Verocai et al., 2014a; present study). The independent nature of *Varestrongylus* among related protostongylids and within the subfamilies Elaphostrongylinae, Muelleriinae, and Protostrongylinae is confirmed. Sequences from adult nematodes were deposited in the GenBank under accession numbers: JQ478644–49 for muskox isolates and for JX115006–07 for caribou isolates; all of these are linked to vouchers in the type series in the USNPC (see Table 3.1). Representative ITS-2 sequences were deposited for DSL used for gastropod infection and from the same caribou (JQ478650 and JQ478651, respectively).

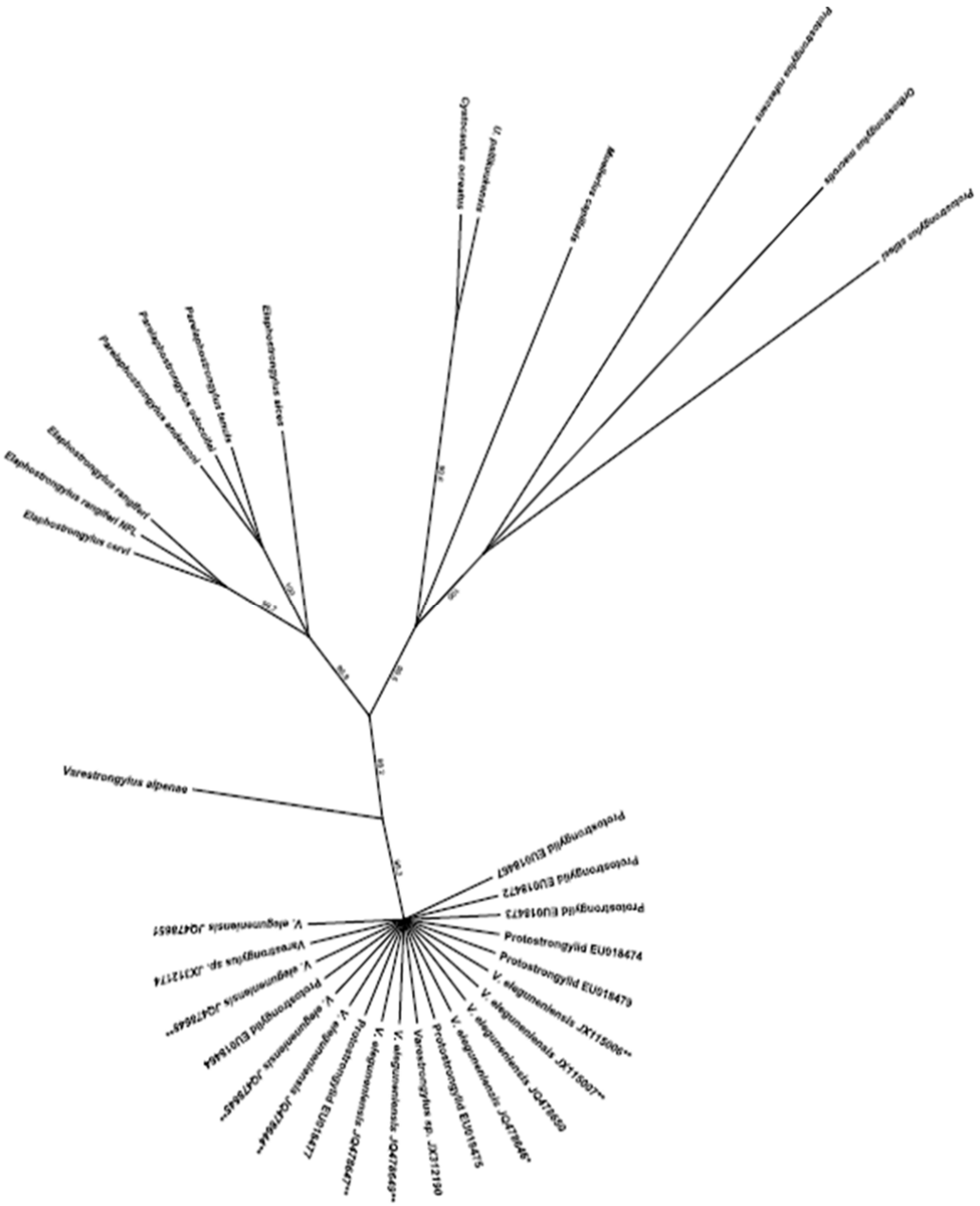


**Figure 3.1.** Unrooted neighbor-joining tree of the ITS-2 region, demonstrating reciprocal monophyly of *Varestrongylus eleguneniensis*. Unrooted neighbor-joining tree based on HKY distances at the ITS-2 region of the nuclear ribosomal DNA, demonstrating the identity of adult and larval specimens of *V. eleguneniensis* produced in the current study and those previously attributed to an unknown species of *Varestrongylus* at high altitudes of North America. Selected sequences shown in this tree represent adults, L1 and L3 of *V.*

*eleguneniensis* from the current study, L1 'Protostrongylid' from Kutz et al. (2007) and L1 of '*Varestrongylus* sp.' from Kutz et al. (2013). Sequences at the ITS-2 locus for other genera and species of protostrongylids included those used in the original comparisons by Kutz et al. (2007); GenBank accession numbers in Methods). Bootstraps (5,000) values are only shown for branches with over 95% support. Superscript (*) refers to the holotype and (**) to paratypes of *V. eleguneniensis* deposited at the United States National Parasite Collection (see Table 3.1).

### *3.4.3 Description*

***Varestrongylus eleguneniensis* sp. n.**

3.4.3.1 General description

Figures: 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, and 3.8.

Protostrongylidae, Varestrongylinae, minuscule, thread-like nematodes, reddish-brown prior to fixation, with delicate cuticle marked by transverse striations. Cephalic extremity bluntly rounded. Buccal aperture surrounded by four papillae. Esophagus cylindrical, clavate, broadening at base, poorly demarcated into muscular and glandular regions. Nerve ring indistinct, located in anterior third or mid-third of esophagus; minuscule cervical papillae and excretory pore usually situated posterior to nerve ring, in middle or posterior third of esophagus.

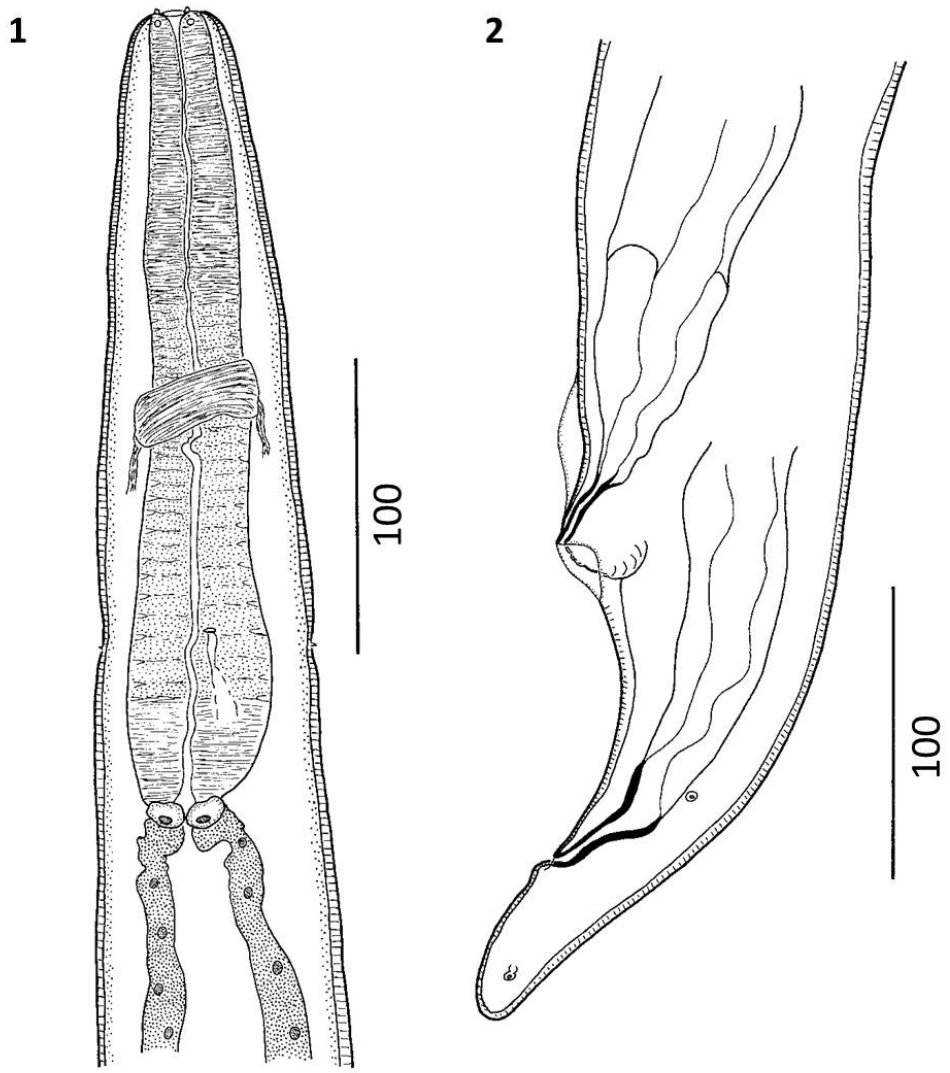


**Figure 3.2.** *Varestrongylus eleguneniensis* sp. n. female. **1.** Cephalic extremity at ventral view. **2.** Caudal extremity at lateral view, note poorly developed provagina.

3.4.3.2 Males

Based on specimens in muskoxen (8 intact males, including holotype, and two posterior end fragments) and caribou (one intact male, one anterior end, and one posterior end). Total length (n = 10) 8.8–14.7 mm (11.2 ± 1.64); maximum width (n = 10) 78–147 (102.7 ± 18.08) attained approximately at mid-body. Esophagus (n = 9) 247–395 (325.7 ± 51.23) long, 44–62 (50.9 ± 6.35) wide, (n = 8) 2.8–3.5% (2.9 ± 0.46%) of body length. Nerve-ring (n = 7) 87–196 (142.1 ± 37.11), cervical papillae (n = 4) 151–215 (187.5 ± 27.43), excretory pore (n = 7) 147–242 (202.3 ± 37.27) from cephalic extremity. Copulatory bursa rounded, lacking distinct lobes, slightly notched at posterior margin. Bursal rays approaching, but rarely attaining margin of bursa. Body width at bursa (n = 12) 50–75 (59.2 ± 6.90), bursa length (n = 11) 65–91 (79.5 ± 7.27), bursa width (n = 11) 95–135 (110.2 ± 9.77). Ventroventral and lateroventral rays equal, arising from common stalk, parallel to one another, tips of rays distally separate, and directed anteriad and isolated from others. Lateral rays arising from common base; externo-lateral elongate, reaching margin and isolated from medio- and postero-lateral rays. Medio-lateral ray long and postero-lateral ray reduced, tips separate from near middle or less than half of common stalk. Externo-dorsal rays long, origins independent from base of dorsal ray. Dorsal ray elongate (n = 9) 23–39 (29.2 ± 6.10) long, (n = 8) 21–31 (26.8 ± 3.11) wide at base. Dorsal ray bifurcated near middle or posterior third (n = 9) 11–26 (17.9 ± 5.67) from base, representing (n = 9) 47–78% (60.6 ± 13%) of dorsal ray length; bifurcation with a single papilla, each of two branches containing two pedunculate papillae, disposed on postero-ventral margin. Spicules equal, symmetrical, yellowish brown (n = 12) 105–148 (126.8 ± 12.62) in length; prominent paired alae arising in anterior third (determined from capitulum) extending to near distal spicule tip; alae strongly trabeculate through most of length, with trabeculae becoming indistinct distally. Shaft of spicule not split, tip blunt rounded at extremity, claw-like at lateral-view. Gubernaculum

lacking capitulum, composed of bifurcate corpus with paired legs and paired denticulate plates of crura. Corpus thin, arched, elongate (n = 11) 60–86 (73.4 ± 6.95) in total length; composed unpaired anterior portion (body) (n = 11) 32–57 (42.1 ± 6.66) long, bifurcating in two lateral legs (n = 11) 23–36 (31.3 ± 4.27) long, near mid-length; distal tips of legs of gubernaculum situated slightly ventral and medial between denticulate plates of crura. Crurae plates (n = 11) 15–25 (19.5 ± 2.91) long, each with 4–5 denticulate processes (usually five), often not equally distributed in individual specimens; axis of plates slightly twisted. Denticulate crurae with delicate wing-like expansions extending anteriad from proximal end; appear joined ventrally (across corpus split/legs) by relatively narrow hyaline band of tissue. Post-cloacal papilliform protuberances situated antero-ventral to cloaca, disposed ventrally to base of dorsal ray. Telamon present, bar-like at lateral view and poorly developed.

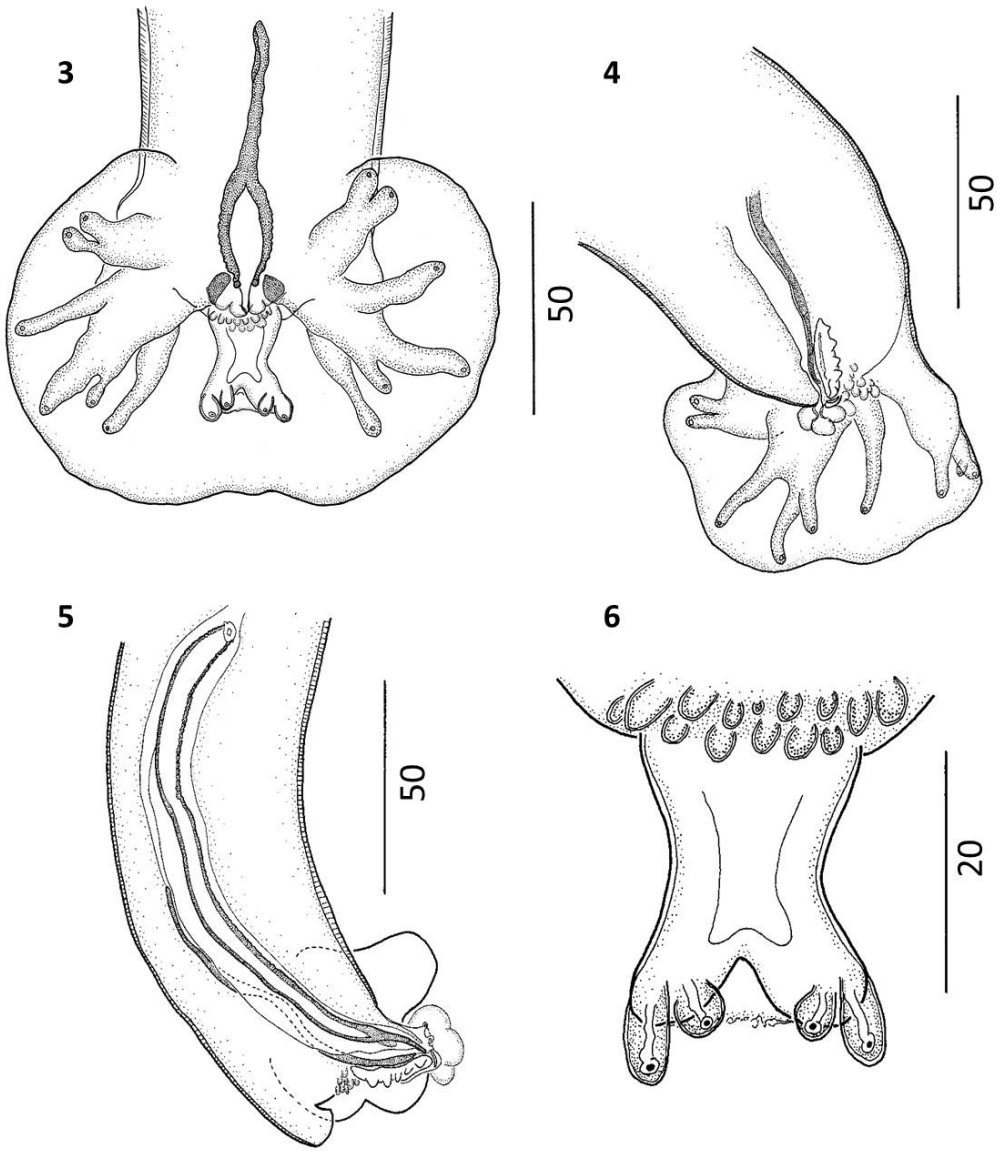


**Figure 3.3.** *Varestrongylus eleguneniensis* sp. n. male, caudal extremity. **4.** Ventral view, note the dorsally notched copulatory bursa and the disposition of bursal rays, and bifurcate gubernaculum. **5.** Ventro-lateral view, note the denticulate plate of crura, and genital protuberances. **6.** Lateral view: spicule, partially covering gubernaculum, denticulate plates of crura. **7.** Ventral view, detail on the elongate, bifurcate dorsal ray, and genital protuberances.

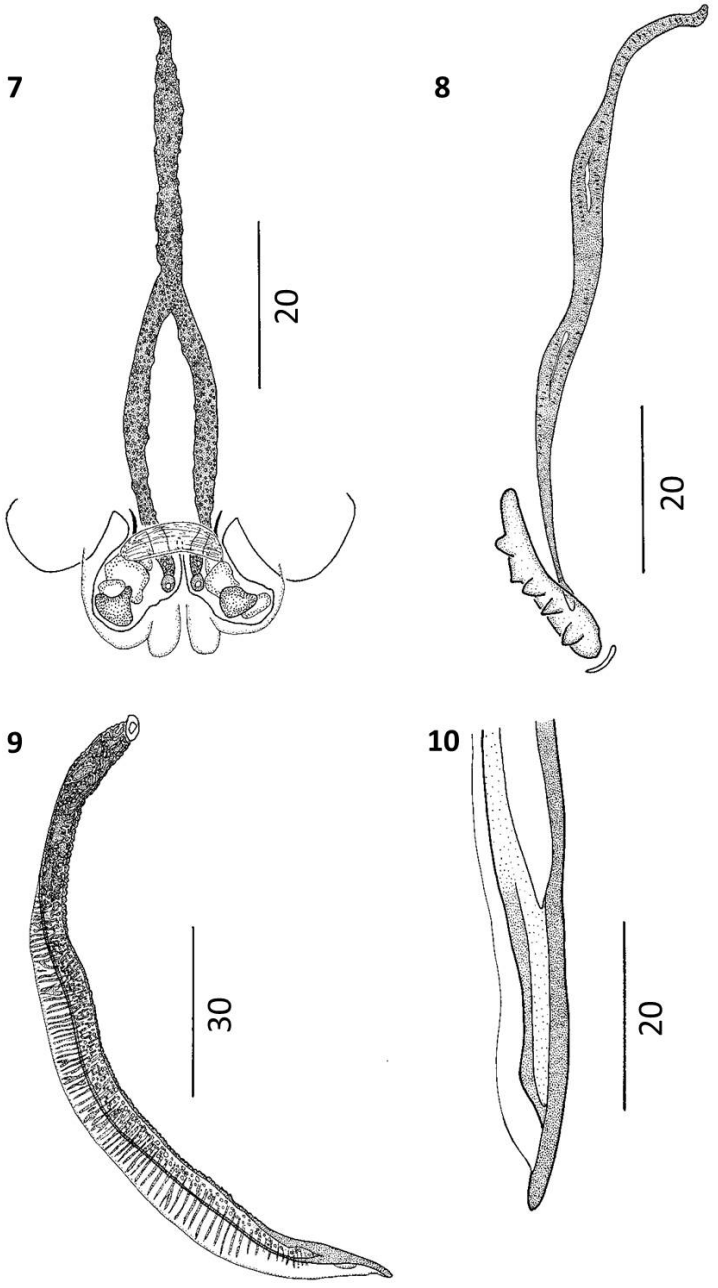


**Figure 3.4.** *Varestrongylus eleguneniensis* sp. n. male. 8. Ventral view of gubernaculum and denticulate plates of crura. **9.** Lateral view of gubernaculum and denticulate plates of crura, and poorly developed telamon plate. **10.** Lateral view of spicule; **11.** Lateral view of spicule tip, non-split and ending in a finger-like projection.

3.4.3.3 Females

Based on specimens from muskoxen (allotype, three additional entire females, and cephalic or caudal extremities). Total length (n = 3) 18.4–21.3 mm (19.4 ± 1.63); maximum width (n = 4) 108–195 (147.3 ± 36.09). Esophagus (n = 4) 265–332 (313.8 ± 28.89) long and 39–64 (49.8 ± 9.04) wide, (n = 3) 1.2–1.8% (1.6 ± 0.32) of body length. Nerve-ring (n = 3) 63–156 (118.7 ± 49.14), cervical papillae (n = 2) 189–217, excretory pore (n = 2) 154–237 from cephalic extremity. Uteri paired, prodelphic; sphincter at termination of uterine limbs (n = 4) 52–57 (54.5 ± 2.89) long. Vagina uterina voluminous, (n = 4) 260–598 (468.8 ± 154.39) long, extending posteriad from sphincter (n = 4) 52–57 (54.5 ± 2.89), vagina vera (n = 4) 62–117 (99 ± 25.26) in length. Vulvar aperture on solid knob-like protuberance; body width at vulva (n = 7) 57–78 (68.4 ± 11.86). Provagina reduced with hood-like fold extending from anterior lip of vulva ventrally across prominent genital protuberance. Perivulval pores situated bilaterally at level of vulva and genital protuberance. Anus in middle to distal third between vulva and tail tip; distance vulva-anus (n = 7) 99–166 (131.3 ± 22.38); vulva-tail (n = 7) 143–215 (178.6 ± 24.76). Tail conical (n = 8) 39–55 (46.3 ± 6.39) long, with lateral phasmids near apex.

3.4.3.4 Immature Stages

*Ova*: Eggs, as determined in lung washes from caribou UC-178-2, spherical to ovoid with delicate, smooth shell (n = 20) 60–78 (68.4 ± 5.39) long by 57–74 (64.9 ± 5.06) wide.

*First-stage larvae*: Body slender, often coiled in life, with paired lateral alae extending from near cephalic extremity to near anus. Tail composed of three segments defined by prominent folds; dorsal spine at level of insertion of the proximal tail fold; tail tip with acutely pointed terminal spike. Meristic data provided in Kutz's original findings (Kutz et al., 2007), and compared in Table five of Verocai et al. (2014).

*Second-stage larvae*: Transitional larval stage, non-diagnostic, characterized by variation in developmental attributes relative to age of infection in intermediate host. Usually cuticle of L1 retained; interior of body often appearing vacuolated.

*Third-stage larvae*: Based on 21 fully developed L3 recovered from digested *D. laeve*. Total length (n = 21) 453–540 (497 ± 25.95). Cephalic extremity with papillae surrounding oral aperture. Buccal cavity with prominent, paired stylet-like structures (n = 5) 7.5–8.5 (7.9 ± 0.42). Esophagus claviform (n = 20) 151–210 (178 ± 14.12) long and 14–21 (16.4 ± 2.14) wide, with bulbous formation in anterior; (n = 20) 30–42% (35.9 ± 3.1) of body length. Body width at esophageal base (n = 20) 23–40 (29.8 ± 4.5). Nerve-ring (n = 20) 71–94 (83.8 ± 5.66) in anterior half of esophagus, excretory pore (n = 19) 92–119 (105.5 ± 6.6), and genital primordium (n = 7) 288–400 (349.4 ± 46.7), from cephalic extremity. Tail (n = 21) 25–34 (29.4 ± 3.5) in length, with spike-like protuberance located ventrally on tip (n = 21) 2–5 (3 ± 0.88), structurally variable, ranging from bluntly rounded to slightly elongate and acute.

Additional data based on four fully developed L3 recovered from digested *D. reticulatum*. Total length (n = 4) 451–541 (491 ± 37.34). Esophagus (n = 4) 163–187 (180.3 ± 11.53) long and 15–18 (16.3 ± 1.26) wide, (n = 4) 35–38% (36.8 ± 1.5) of body length. Body width at oesophageal base (n = 4) 31–34 (31.8 ± 1.5). Nerve-ring (n = 2) 72–85 (78.5 ± 9.19), excretory pore (n = 4) 90–108 (101.8 ± 8.5), and genital primordium (n = 3) 301–384 (336.6 ± 42.85), from cephalic extremity. Tail (n = 4) 26–31 (29.8 ± 2.5) in length, with spike-like protuberance located ventrally on tip (n = 4) 2.5–4 (3.3 ± 0.61).

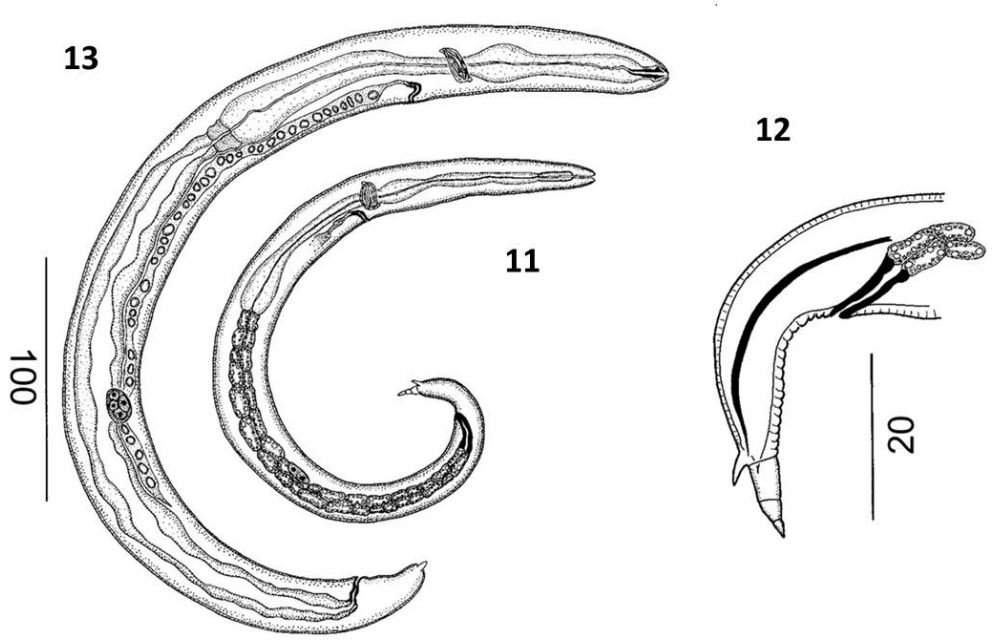


**Figure 3.5.** *Varestrongylus eleguneniensis* sp. n. larval stages. **12.** First-stage larva (L1 or dorsal spined-larva, DSL) at lateral view. **13.** Detail on caudal extremity of the First-stage larva (dorsal spined-larva) at lateral view. **14.** Third-stage larva (L3) at lateral view.

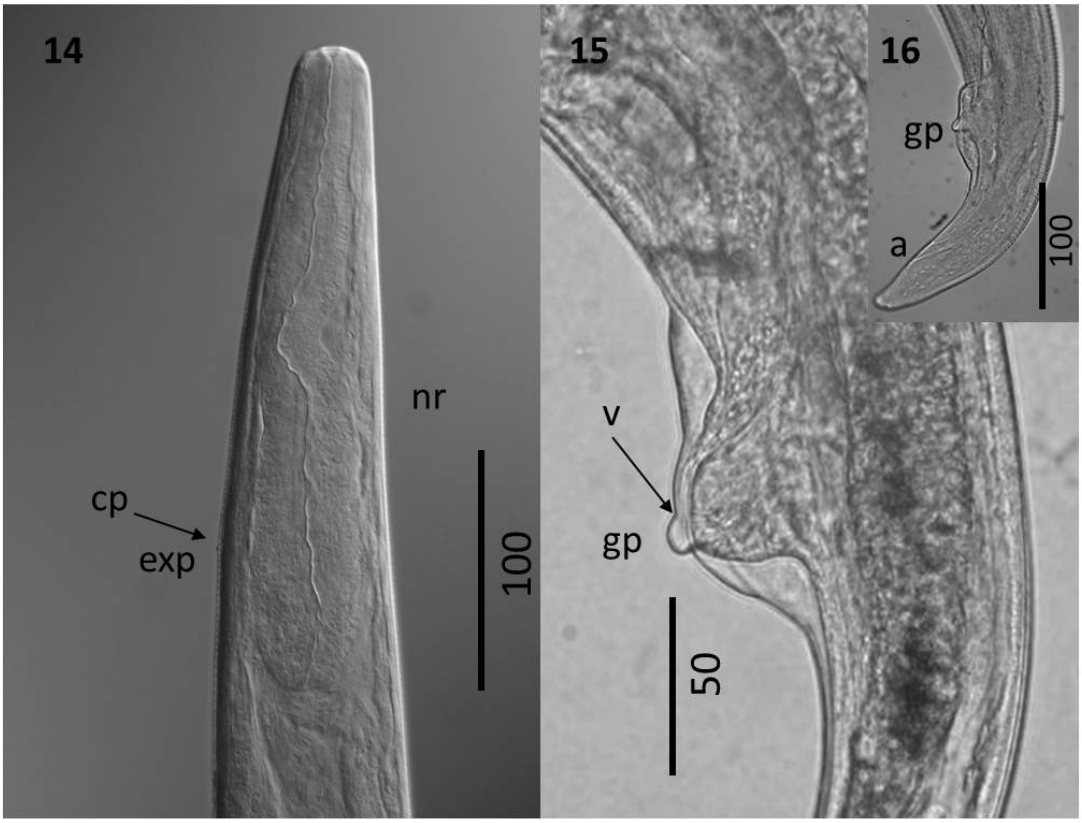


**Figure 3.6.** *Varestrongylus eleguneniensis* sp. n. female. 15. Cephalic extremity of a female specimen at ventral view, note the claviform esophagus, nerve-ring (nr), and cervical papillae (cp), excretory pore (ep) located at posterior third of esophagus (40×).**16.** Caudal extremity of a female specimen at lateral view, note the poorly developed provagina, genital protuberance (gp) and vaginal opening (v), (40×).**17.** Caudal extremity of a female specimen at lateral view, showing the anus (a), and the conical tail (40×).

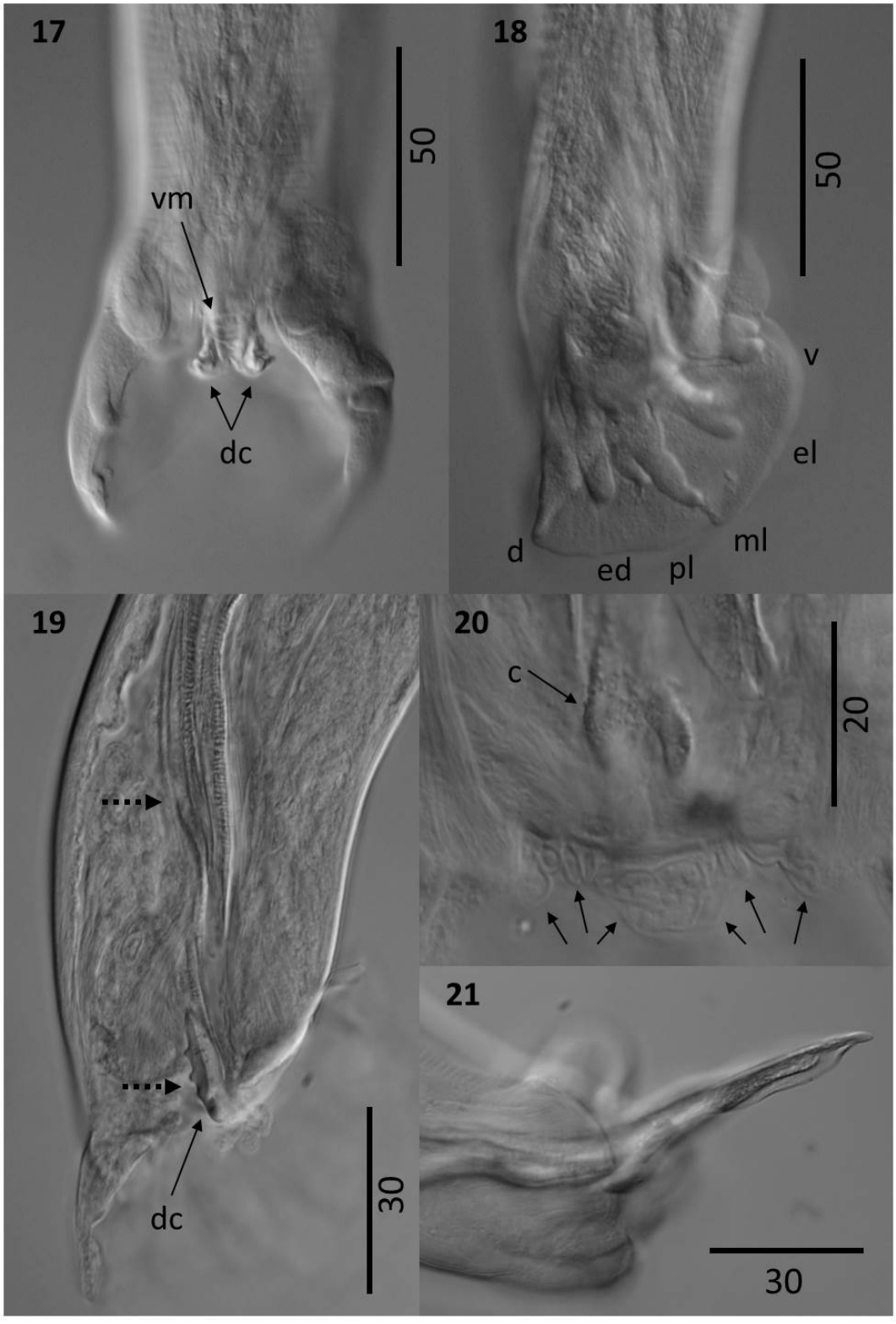
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**Figure 3.7.** *Varestrongylus eleguneniensis* sp. n. male. **18.** Caudal extremity ventral view: cuticular striations, denticulate plates of crura (dc), (40×). **19.** Caudal extremity at lateral view showing disposition of the bursal rays: ventral (v), externo-lateral (el), medio-lateral (ml), postero-lateral (pl), externo-dorsal (ed), dorsal (d) (40×). **20.** Caudal extremity at lateral view: distal portion of spicule and gubernaculum, and gubernaculum (dashed arrows) and denticulate plates of crura (dc), (100×). **21.** Detail on caudal extremity of a male specimen: crura (c), and genital protuberances (arrows) (160×). **22.** Lateral view of the tip of protruded spicule, non-split and ending in a finger-like projection (100×).

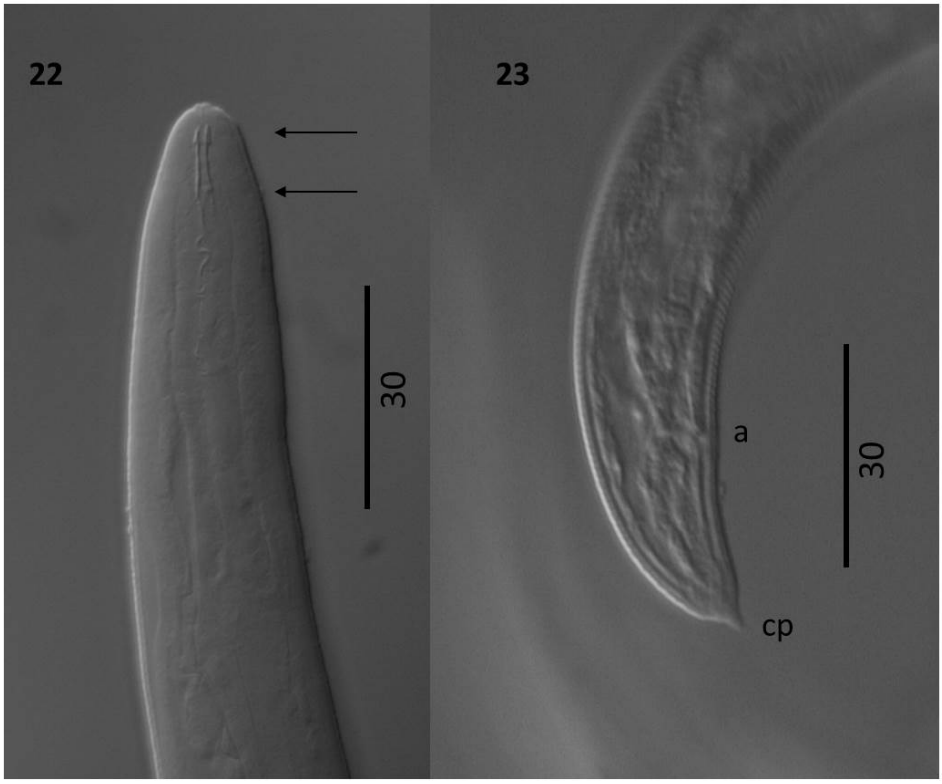


**Figure 3.8.** *Varestrongylus eleguneniensis* sp. n. third-stage larva (L3). **23.** Cephalic extremity of L3 at dorsal view: buccal structures, stylets (arrows), and anterior part of esophagus (100×). **24.** Caudal extremity of L3 at lateral view: detail on the cuticular striations, anus (a), and tail spike (ts) or caudal protuberance (64×).

### *3.4.4 Taxonomic summary*

***Type-host:*** Muskox, *Ovibos moschatus wardi* (Lyddeker, 1900).

***Additional hosts:*** Woodland caribou, *Rangifer tarandus caribou* (Gmelin, 1788). Also known in *Rangifer tarandus groenlandicus* (Borowski, 1780), *Rangifer tarandus grantii* (Allen, 1902), *Alces americanus gigas* Miller 1899, and *Ovibos moschatus moschatus* (Zimmermann, 1780) (in part based on sequence comparisons from Kutz et al. (2007)).

***Intermediate hosts:*** Natural gastropod intermediate host: *Deroceras laeve* (Müller, 1774) (Kutz et al., 2007). Experimental intermediate host: *Deroceras reticulatum* (Müller, 1774).

***Predilection site:*** Adult males and females occur in terminal bronchioles and alveoli of lungs based on recovery of few intact worms from repeated tracheal washes, and dissection of minute bronchi.

***Type-locality:*** Tasiujaq, Nunavik Region, Quebec, Canada (58°44′51″N, 70° 02′ 06″W).

***Additional localities:*** Near Tasiujaq and type locality (58° 44′ 10″N; 69° 34′ 18″W); near Kuujjuaq, Nunavik Region, Quebec (58° 45′ 00″N; 68° 33′29″W); and the Cold Lake Air Weapons Range, Alberta, Canada (55° 2′ 25″N; 110° 34′5″W) (type series). Also known to be widely distributed across high latitudes of North America based on prior sampling of DSL (see Table two in Kutz et al. (2007)).

***Type-specimens:*** *Adult specimens*: Holotype male from type-host and locality on 7 April 2010 collected by G. Verocai, S. Kutz and M. Simard, USNPC 103740 (GenBank JQ478646). Allotype female on 8 April 2010 collected by G. Verocai, S. Kutz and M. Simard from *O. m. wardi* near type-locality adjacent to Tasiujaq, Quebec, USNPC 103741. Paratypes

include males and females in host Om-10-2007 (1 vial), USNPC 103743 collected by M. Simard on 21 March 2007 at Kuujjuaq, Quebec; Om-02-2010 (9 vials), USNPC 103742 (GenBank JQ478649), 103748 (GenBank JQ478647), and 103749 (GenBank JQ478648); and Om-10-2010 (7 vials), USNPC 103744 (GenBank JQ478644-5), collected by G. Verocai, S. Kutz and M. Simard in *O. m. wardi* in Kuujjuaq in animals from Tasiujaq and Kuujjuaq (Table 1), respectively, Nunavik Region, Quebec, on April 7th and 8th , 2010. Additional paratypes from woodland caribou (UC178-2) from the Cold Lake herd, Alberta, found road killed in November 23rd 2010 (Bob McClymont, Alberta FWD), collected by G. Verocai on October, 2011, USNPC 105697 (GenBank JX115006), 105698, and 105699 (GenBank J115007). *First-stage larvae*: Paratypes in Om-02-2010 under accession number USNPC 103742. Additional paratypes used for DSL morphometric and molecular data in [12] are available under the following accession numbers: *O. m. wardi*, Nunavik, QC, USNPC 98648–98652 (GenBank EU018464, 018465, 018479); and *R. t. granti*, North Alaska Peninsula, AK, USNPC 10585, 10586, and 10589 (GenBank EU018478). *Third-stage larvae*: L3 paratypes recovered from experimentally infected terrestrial slug *D. reticulatum*, in September 2008 by J. Ouellet, USNPC 103745 (matching DSL: USNPC103747), and March 2010 by G. Verocai, USNPC 103746 (GenBank JQ478650); and experimentally infected terrestrial slug *D. laeve* by G. Verocai in September 2011, USNPC 107784.

***Etymology:*** The specific name, "*eleguneniensis"* denotes the extensive geographic distribution across northern North America for this protostrongylid. The derivation is from the North Slavey language spoken by the Dene people from the Sahtu Settlement Area, NT, Canada, in which "elegu" means cold and "nene" means land. The term "elegunene" is used by them to refer to the northern lands. This specific name is in recognition of over a decade of collaboration with the Sahtu Dene supporting wildlife health and parasitology research in northern Canada (Brook et al., 2009).

### *3.4.5 Differential diagnosis*

In the context of our studies, we accept the validity of the Varestrongylinae and placement of the genus *Varestrongylus* within this restricted group of protostrongylids (see Boev, 1975; Carreno and Hoberg, 1999). Following the characterization of *V. eleguneniensis* the genus now contains ten valid species of lungworms typical of caprine bovids and cervids across the Holarctic (Boev, 1975; Kutz et al., 2007; Liu, 1989; Liu, 1984; Verocai et al., 2014a). Species are mainly diagnosed by the structure of the copulatory bursa and its rays, and configuration of the spicules and gubernaculum (corpus and plate-like paired crurae) in males and by the structure of provagina in females (Boev, 1975; Dougherty, 1945; Dougherty and Goble, 1946). *Varestrongylus eleguneniensi*s is established based on an integration of comparative morphological and molecular criteria for adults and larval parasites.

*Adult nematodes*: Consistent with the current generic diagnosis, males of *V. eleguneniensis* possess prominent, paired denticulate plates of the crurae disposed slightly lateral, dorsal and distal to the legs of the gubernaculum, and a configuration of bursal rays typical to the genus. In females there is a reduced provagina.

*Males*: Among males (Table 3.2), *V. eleguneniensis* is immediately distinguished by the dimensions of its miniscule spicules from: *V. alpenae*; *Varestrongylus capricola* Sarwar, 1944; *Varestrongylus longispiculatus* Liu, 1989; *V. pneumonicus*; *Varestrongylus qinghaiensis* Liu, 1984; *V. sagittatus*; and *Varestrongylus tuvae* (Boev & Sulimov, 1963); we refer to these seven species as ‘long-spicule forms’ (Verocai et al., 2014a). In contrast, spicules of *V. eleguneniensis* are small (105–148 μm) compared to > 200 μm for all of the above species. Further, the diminutive, split gubernaculum (60–86 μm) of *V. eleguneniensis* contrasts with the solid, rod-like corpus typical of *V. capricola*, *V. longispiculatus*, *V.*

*pneumonicus* and *V. tuvae*. The split gubernaculum in other long-spicule forms of *Varestrongylus* (*V. alpenae*, *V. qinghaiensis* and *V. sagittatus)*, has legs that are usually fused by a poorly cuticularized membrane. *Varestrongylus eleguneniensis* is distinguished from all others by the configuration of the denticulate plates of crurae (e.g., Boev, 1975; Liu, 1989; Liu, 1984).

Specimens of *V. eleguneniensis* are further characterized and distinct from congeners based on a combination of attributes of the male genital system and copulatory bursa. The conformation of the gubernaculum allows recognition of two groups in the genus: (i) species in which the corpus is distally bifurcate with an evident separation of anterior (corpus) and posterior (legs) regions (*V. eleguneniensis, V. alces, V. alpenae, V. capreoli* and *V. qinghaiensis*); or (ii) where the corpus is entire, and usually rod-like, without distal legs (*V. pneumonicus, V. sagittatus, V. capricola, V. tuvae* and *V. longispiculatus*). Further, the distal extremity of the spicules can be either: (i) entire (*V. eleguneniensis, V. capreoli, V. alces, V. longispiculatus* and *V. qinghaiensis*); or (ii) branched (*V. pneumonicus, V. sagittatus, V. alpenae, V. capricola* and*V. tuvae*). In general, in species of *Varestrongylus* spicules are equal in length, except for those in *V. qinghaiensis* and *V. longispiculatus,* whose spicules are sub-equal. Species can also be divided in two groups based on length of the dorsal ray: (i) where the dorsal ray is short, rounded and often indistinct (*V. pneumonicus, V. sagittatus, V. capreoli, V. capricola, V. tuvae* and *V. qinghaiensis*); or (ii) where the dorsal ray is elongate with prominent papilliform structures (*V. eleguneniensis, V. alces, V. alpenae* and *V. longispiculatus*).

Although males of *V. eleguneniensis* are immediately distinguished from seven congeneric species based on spicule length, specimens are most similar to those of *V. alces* and *V. capreoli* in the overall dimensions of the spicules, and structure and dimensions of the

gubernaculum. Collectively, these three species characterize the small-spicule forms within the genus. Although equivalent in length, the morphology of the spicule tips and alae effectively distinguish *V. eleguneniensis* from *V. capreoli* and *V. alces*. Spicules are distally entire in the three species, but for *V. eleguneniensis* the paired spicule alae taper distally, are not inflated, and do not reach the apex of the spicule tip; in *V. alces*, a somewhat spatulate condition is apparent distally (Verocai et al., 2014a). The form of the denticulate plates of the crurae is an additional diagnostic feature among these three species. In *V. eleguneniensis* and *V. alces* the relatively "stocky" plates are twisted on the longitudinal axis and have a similar number of denticulate processes, 4–5 in the former and always 5 in the latter; plates in *V. alces* contrast with *V. eleguneniensis* in being strongly arched in dorso-ventral view. In contrast to these species, *V. capreoli* (and specimens identified as *V.* cf. *capreoli*) has strongly triangular plates armed with four acute, prominent teeth. Telamon plates of *V. eleguneniensis* and *V. capreoli* are bar-like and poorly developed, whereas in *V. alces*, these are slightly more developed and triangular in lateral view. The bursa of *V. eleguneniensis* and *V. alces* is dorsally notched with an indistinct dorsal lobe, differing from *V. capreoli*, in which it is weakly bi-lobed. Also, the relative disposition of bursal rays in *V. eleguneniensis* is comparable to that of *V. alces*, but differs from that of *V. capreoli*: the dorsal ray in *V. alces* is slightly elongate and bifurcate instead of short and rounded and the externo-dorsal lateral rays originate independently from each other. Ventral rays in the three species originate from a common stalk, but are distally split in *V. eleguneniensis* and *V. alces*, and basally split in *V. capreoli*.

*Females*: Females of *V. elegunieniensis* differ from *V. capreoli* and *V. alces*, and all other valid species of *Varestrongylus*, by having a strongly reduced provagina (Table 3.3). The only other species with a reduced provagina is *V. capricola*, although the relative degree of development exceeds that observed in *V. eleguneniensis*. Among other members of

*Varestrongylus*, the well-developed provagina (at different degrees) is a membranous, tubular structure extending posterior from the vulva along the ventral aspect of the tail anterior to the anus. Eggs are expelled from the vulva after and are released posterior to the genital protuberance through this series of tubular membranes.

*Identification of DSL and L3 stages*: Attributes of DSL and L3 stages, but not the L2, were considered because these are the larval stages of diagnostic relevance. We compared morphometric features of both stages to these of other protostrongylids that may occur in a similar spectrum of hosts, or which potentially may be sympatric with *V. eleguneniensis* in North America.

The characteristic dorsal-spine readily separates *V. eleguneniensis* DSL from L1 of species within the Subfamily Protostrongylinae (including known or potentially sympatric *Pr. stilesi*, *Protostrongylus rushi* Dikmans, 1937, and *O. macrotis*), but it is shared among all genera/species within the Subfamilies Elaphostrongylinae and Muelleriinae (Carreno and Hoberg, 1999; Kutz et al., 2007). The morphometry of DSL of *V. eleguneniensis* may overlap not only with other *Varestrongylus* species as previously mentioned, but also with those of other genera within Elaphostrongylinae and Muelleriinae, although larvae of *V. eleguneniesis* are generally shorter than those of the sympatric *Parelaphostrongylus* spp., and *U. pallikuukensis* (Kutz et al., 2007; Kutz et al., 2013a; Lankester, 2001). DSL of *V. eleguneniensis* are also shorter than those of *Elaphostrongylus* spp. (Lankester, 2001; Lankester et al., 1998; Verocai et al., 2014a). The importance of differentiating these two is uncertain as it is unclear if the new species occurs in sympatry with *E. rangiferi* in Newfoundland, Eastern Canada, where this Eurasian protostrongylid has been introduced (Lankester and Fong, 1989, 1998).

The L3 stage of *V. eleguneniensis* appears to be similar to those of species within *Varestrongylus* and Elaphostrongylinae, in which all have a caudal protuberance. Despite this similarity, L3 of elaphostrongylines that geographically co-occur with *V. eleguneniensis (Parelaphostrongylus* spp. and, potentially, *E. rangiferi*) are generally double the size of these of *V. eleguneniensis*, and larval total length may be useful for identification at the generic level (i.e., *Varestrongylus*) (see Table 3.4; (Ballantyne and Samuel, 1984; Lankester, 2001; Lankester and Hauta, 1989)). L3 of *V. eleguneniensis* can easily be distinguished from those of species within Muelleriinae, in particular the sympatric *U. pallikuukensis*, in which the rounded tail lacks a caudal protuberance (Hoberg et al., 1995; Kutz et al., 1999). Further, L3 of *V. eleguneniensis* appear undistinguishable from those of *V. alpenae*, although the geographic range of the latter is not well characterized, and it is not certain if these congeners may occur in sympatry or are characterized by parapatric ranges in North America. Morphometric data of L3's should be cautiously interpreted since intra-specific variation exists mainly related to age of larva (i.e., early, intermediate, or late stage, e.g., Kutz et al. .(2001b)), but also may reflect variation related to development in different gastropod species.

**Table 3.4.** Comparative morphometrics of L3 of *Varestrongylus eleguneniensis* and selected Protostrongylidae species (Varestrongylinae, Elaphostrongylinae, Muellerinae)

| **Characters** | ***V. eleguneniensis*[a1]** **(n = 7–21)** | ***V. eleguneniensis*[a2]** **(n = 4)** | ***V. alpenae*[b]** **(n = 18)** | ***P. andersoni*[c]** **(n = 10)** | ***P. odocoilei*[c]** **(n = 10)** | ***P. tenuis*[c]** **(n = 10)** | ***E. rangiferi*[d]** **(n = 15)** | ***U. pallikuukensis*[e]** **(n = 10)** | ***U. pallikuukensis*[f]** **(n = 29)** |
|---|---|---|---|---|---|---|---|---|---|
| Total length | 453–540 (497 ± 25.95) | 451–541 (491 ± 37.34) | 434-515 (480 ± 20) | 911–1,085 (1,003) | 738–977 (890) | 1,100–1,323 (1,200) | 937–1,041 (1,004) | 514–600 (560 ± 33.64) | 545–691 (648 ± 35) |
| Esophagus[§] | 151–210 (178 ± 14.12) | 163–187 (180.3 ± 11.53) | – | 322–412 (365) | 282–399 (323) | 412–521 (463) | 338–421 (381) | 181–214 (200 ± 11.71) | 201–263 (233 ± 13) |
| Esophagus base width | 14–21 (16.4 ± 2.14) | 15–18 (16.3 ± 1.26) | – | – | – | – | – | – | 18–35 (23 ± 3.1) |
| Body at esophagus base | 23–40 (29.8 ± 4.5) | 31–34 (31.8 ± 1.5) | 26–30 (28 ± 2) | 36–43* (40) | 36–52* (44) | 47–62* (54) | 42–49 (46) | 39–60 (47 ± 7) | 42–46 (44 ± 1.8) |
| Nerve-ring[§] | 71–94 (83.8 ± 5.66) | 72–85 (78.5 ± 9.19) | – | 123–130 (128) | 135–154 (141) | 152–174 (165) | 120–150 (139) | 93–106 (99 ± 4.23) | 83–118 (107 ± 6.7) |
| Excretory pore[§] | 92–119 (105.5 ± 6.6) | 90–108 (101.8 ± 8.5) | – | – | – | – | 138–163 (153) | 109–127 (118 ± 5.01) | 104–146 (130 ± 7.9) |
| Genital primordium[§] | 288–400 (349.4 ± 46.7) | 301–384 (336.6 ± 42.85) | – | 651–738 (697) | 521–586 (561) | 694–846 (749) | 574–648 (615) | 318–388 (361 ± 22.74) | 316–432 (402 ± 26) |
| Tail | 25–34 (29.4 ± 3.5) | 26–31 (29.8 ± 2.5) | – | 32–43 (36) | 45–50 (47) | 48–64 (53) | 40–70 (52) | 26–34 (31 ± 2.88) | 26–34 (31 ± 2.88) |
| Tail protuberance | 2–5 (3 ± 0.88) | 2.5–4 (3.3 ± 0.61) | – | – | – | – | – | Not present | Not present |

All measurements are given in micrometers (μm).

[a1] Present study, L3 from experimentally infected *Deroceras laeve.* Measurements in variable number of larval specimens (n = 21: Total length, Tail, Tail protuberance; n = 20: Esophagus, Esophagus base width, Body at esophagus base, Nerve-ring; n = 19: Excretory pore; n = 7: Genital primordium);

[a2] Present study, L3 from experimentally infected *Deroceras reticulatum*;

[b] From experimentally infected White-tailed and Mule deer using *Webbhelix multineata* (syn. *Triodopsis multineata)* and *N. albolabris* as IH (Gray et al., 1985);

[c] From Ballantyne and Samuel (1984) using *W. multineata* as IH. Larval sources: *P tenuis* from White-tailed deer, Rachelwood Wildlife Research Preserve, Pennsylvania; *P. odocoilei* from Mule deer, Jasper National Park, Alberta; *P. andersoni* from White-tailed deer, southeastern BC;

[d] L1 of caribou from Newfoundland (as *Elaphostrongylus cervi*), cited in Kuutz et al. (2007);

[e] From Hoberg et al. (1995) using *D. reticulatum* as IH. Source of L1: muskoxen from Nunavut;

[f] Larvae grown in *D. laeve* as IH. Measurements from 'late' and emerged L3 are included (Kutz et al., 2001b). Source of L1: experimentally infected muskoxen (Kutz et al., 1999).

§ Measurements from anterior end.

Dashes represent measurements that were not determined, despite of the presence of the character in larvae of the species.

* Body width measured at intersection of esophagus and intestine.

### *3.4.6 Emended diagnosis of* **Varestrongylus** *Bhalerao, 1932*

Details from the current study and description of *V. eleguneniensis* along with the recent resurrection of *V. alces* (Verocai et al., 2014a) have made it necessary to propose an emended diagnosis for the genus in order to accommodate adequate recognition of some morphological attributes typical of *Varestrongylus.* The need for an emended diagnosis reflects inconsistency in prior descriptions and the names applied to designate some structural features (Anderson, 2000; Bhalerao, 1932; Boev, 1975; Verocai et al., 2014a). We, hereby, emend the generic diagnosis of *Varestrongylus* as follows:

*Varestrongylus* Bhalerao, 1932 (syn. *Strongylus* Müller, 1780 (in part., *sensu* Mueller 1891)); *Protostrongylus* Kamensky, 1905 (in part.); *Synthetocaulus* Railliet & Henry, 1907 (in part.); *Bicaulus* Schulz & Boev, 1940; *Leptostrongylus* Dougherty & Goble, 1946; *Capreocaulus* Schulz & Kadenazy, 1948, *Cystocaulus* Boev, 1950 (in part.).

Metastrongyloidea: Protostrongylidae: Varestrongylinae. Male: dorsal ray of copulatory bursa short or elongate, sometimes almost reduced (*V. capreoli*, *V. tuvae*); apex sometimes bifurcate (*V. alpenae*, *V. longispiculatus*, *V. eleguneniensis*) with variable number of small papillae. Postero-lateral rays of bursa considerably shorter than medio-lateral rays. Telamon plates variable: complex structure (*V. tuvae*), poorly developed, or absent (*V. qinghaiensis*). Spicules of filamentous composition, distally entire or bifurcate, provided with alae and lacking manubrium. Capitulum of gubernaculum present (*V. capreoli sensu stricto*) or absent. Body of gubernaculum in form of long, narrow, and usually colorless structure, entire or distally bifurcate as distinct legs. Paired plates of crurae of gubernaculum (sometimes referred as feet) independent

of body or fused by hyaline membrane and in form of two structures with odontoid processes on their edges (denticules) (except in *V. tuvae*, which has smooth feet). Female: provagina always present; reduced (*V. eleguneniensis, V. capricola*) or well-developed; tail conical, pointed, often acute. First-stage larva with dorsal spine on tail insertion (DSL), tip of tail kinked and composed by three segments defined by transverse folds. Third-stage larvae possess morphologically variable caudal protuberance. Parasites of lungs in Cervidae: *Cervus* and *Dama* (Cervinae), and *Capreolus, Alces, Rangifer, Odocoileus* (Odocoileinae)*;* and Bovidae (Caprinae): *Ovis*, *Pseudois*, *Capra*, *Ovibos*, and *Budorcas. Valid species: V. sagittatus* (Mueller, 1890) Dougherty, 1945; *V. pneumonicus* Bhalerao, 1932; *V. alpenae* (Dikmans, 1935) Dougherty, 1945; *V. capreoli* (Stroh & Schmid, 1938) Dougherty, 1945; *V. capricola* Sarwar, 1944; *Varestrongylus alces* Demidova & Naumitscheva, 1953; *V. tuvae* (Boev & Sulimov, 1963) Boev, 1968; *Varestrongylus qinghaiensis* Liu, 1984; *Varestrongylus longispiculatus* Liu, 1989; *V. eleguneniensis* (present study).

### 3.5 Discussion

*Varestrongylus eleguneniensis* sp. n. is the first protostrongylid ‘true lungworm’ to be described in caribou and is also found in muskoxen and, less often, moose from boreal to Arctic environs of North America, with exception of High Arctic islands of the Canadian Archipelago and Greenland. The description of this species corroborates the discovery of a previously unknown protostrongylid circulating in ungulates across high latitudes of North America (Kutz et al., 2007). This new taxon is clearly distinct from other protostrongylids, varestrongylines, and species of *Varestrongylus* on the basis of morphological attributes of adult males and females. Molecular sequence data confirm the conspecificity of adult and larval parasites, and provide

further differentiation among the small-spicule forms known in the genus (Kutz et al., 2007; Kutz et al., 2013a; Verocai et al., 2014a). Current knowledge of geographic distribution and host associations across an extensive range in the northern Nearctic, combined with phylogenetic analysis based on morphological characters (G. Verocai, E.P. Hoberg, unpublished), suggests that caribou (i.e., subspecies of *R. tarandus* native to North America) may be the primary host for *V. eleguneniensis*, as cervids are considered the ancestral hosts for the genus *Varestrongylus* (Hoberg et al., 2012b)

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### *3.5.1 Host distribution*

*Varestrongylus eleguneniensis* is the only known protostrongylid lungworm associated with subspecies of *Rangifer*, and appears to be restricted to the Nearctic. Previous studies have reported only elaphostrongyline protostrongylids in Eurasian reindeer (e.g., Boev, 1975; Halvorsen, 1986; Kontrimavichus et al., 1976), and subspecies of caribou or introduced semi-domesticated reindeer from North America (Ball et al., 2001; Boev, 1975; Dikmans, 1939; Dougherty, 1945; Hadwen, 1922; Lankester and Fong, 1989, 1998; Lankester and Hauta, 1989; Verocai et al., 2013). Prior to the advent of molecular-based diagnostics it is likely that DSL of *V. eleguneniensis* were identified as *P. andersoni* in caribou herds across Canada (Kutz et al., 2007; Lankester and Fong, 1989, 1998; Lankester and Hauta, 1989). These two protostrongylids occur in sympatry and cases of mixed infections have been reported (Kutz et al., 2007; Kutz et al., 2012a).

In muskoxen, lung-dwelling protostrongylids were unknown until relatively recently (Hoberg et al., 2002; Hoberg et al., 1995). Muskoxen at high latitudes of North America are infected with *U. pallikuukensis* and *Pr. stilesi*, the latter of which is considered to be a result of

independent events of host-switching from Dall's sheep (*Ovis dalli dalli* Nelson) in areas of sympatry in Northwest Territories, Yukon and Alaska (Hoberg et al., 2002; G. Verocai, L. Adams, S. Kutz, unpublished obs.). All confirmed records of *V. eleguneniensis* in muskoxen come from populations sympatric to caribou, either through: (i) long-term sympatry as it occurs in the core of muskox range in mainland central Canadian Arctic (Kutz et al., 2007; Kutz et al., 2013a); (ii) recent translocation events in the 20th century with traceable origins (i.e. Ellesmere Island and Greenland, where protostrongylids have not been detected in caribou nor muskoxen) (Kutz et al., 2007; present study; Kutz et al., 2012a); or (iii) occur in areas that lungworms until recent years, due to environmental conditions, could not complete their life cycle, and establish (Kutz et al., 2013a). These findings reinforce the hypothesis that this previously unrecognized species has the caribou as its ancestral host. However, given the high prevalence of *V. eleguneniensis* found in some muskox populations sympatric with infected caribou (Kutz et al., 2007; Kutz et al., 2013a; G. Verocai, M. Simard and S. Kutz, unpublished obs.), we predict that this parasite could be maintained in muskoxen in the absence of caribou.

In contrast to caribou and muskoxen, until now, the Yukon-Alaska moose (*Alces americanus gigas* Miller, 1899) had never been reported as host of protostrongylid lungworms. Pulmonary protostrongylid parasites appear to be rare in other moose subspecies from the Nearctic, although, in southern latitudes, there has been an isolated report of *O. macrotis* in naturally infected *Alces americanus andersoni* Peterson 1952 (Samuel et al., 1976; G. Verocai, C. Kashivakura and S. Kutz, unpublished obs.). Moose in Newfoundland are believed to be infected with *P. andersoni* (Ball et al., 2001; Lankester and Fong, 1998), along with *E. rangiferi*, but larval identity was not confirmed by molecular techniques and nor were adult worms

recovered. Similar to other lungworms, we consider the findings of *V. eleguneniensis* in moose to be relatively isolated and indicative of incidental infections (Kutz et al., 2007).

Available survey data suggest that *V. eleguneniensis* is restricted to the Nearctic, and records for protostrongylids in reindeer, Eurasian moose (*Alces alces* L.), or introduced muskoxen in Eurasia, involve other genera and species. In the Palearctic, only the tissue-dwelling *E. rangiferi* has been recognized in populations of reindeer (*R. t. tarandus* L., *R. t. fennicus* Lönnberg, *R. t. platyrhynchus* Vrolik) (Boev, 1975). For instance, there have been reports of *E. rangiferi* as far east as Buryatia, near Lake Baikal (Kontrimavichus et al., 1976). Yet, studies on reindeer in the Russian Far East are scarce. The irrefutable veterinary importance of *E. rangiferi* may have led researchers to overlook the potential presence of a small and obscure, pulmonary protostrongylid species such as *V. eleguneniensis* that are associated with significant gross pathology. However, lesions apparently characteristic of infections by *Varestrongylus* sp. have recently been observed in semi-domesticated reindeer (*R. t. tarandus*) from north-central Finland, but the parasite species involved has not been identified (A. Oksanen and S. Laaksonen, pers. comm., 2010).

The Eurasian moose, congeneric with the North American moose, is recognized as a primary and potentially the only host for *V. alces*, in Russia, Poland, and Fennoscandia (Demidova and Naumitscheva, 1953; Dróżdż, 1966; Nilsson, 1971; Petrosyan, 1963; Stéen et al., 1998; Stuve, 1986, 1987). Presence of *V. eleguneniensis* in this host is unlikely, as the parasite seems to only incidentally infect moose in Alaska, and has not been found in assessed moose populations in northern Canada (Kutz et al., 2007). For introduced muskoxen in Norway and Sweden it is also unlikely that *V. eleguneniensis* is present. These animals were originally

introduced from Greenland, a location where protostrongylids have not previously been detected in caribou or muskoxen (Kutz et al., 2012a). Dorsal-spined larvae have been reported in muskoxen from Norway and Sweden but are most certainly acquired locally (Alendal and Helle, 1983), and DSL and adult specimens from the Norwegian population were identified as *M. capillaris* (Davidson et al., 2014). More extensive field collections are required to completely resolve faunal diversity and host associations for protostrongylids in the northern Palearctic.

### *3.5.2 Pathology and significance*

*Varestrongylus eleguneniensis* does not appear to cause substantial pulmonary pathology in infected hosts. No gross lesions were observed in any of the muskox and caribou lungs examined in the present study. Also, previously, despite careful examination, our group failed to find lesions in over 50 caribou lungs and over 100 muskoxen lungs from areas that we now know are in the geographic range of *V. eleguneniensis* (Kutz et al., 2007). Little is known about its pathology and impact on infected ungulates, although light parasitic pneumonia was histologically demonstrated in muskoxen from the same source population (M. Simard, S. Lair, A. Dallaire, Pers. comm.). Further investigations and histological examination of infected lungs are warranted. In contrast, other species of *Varestrongylus*, such as *V. alces* (Petrosyan, 1963; Verocai et al., 2014a), *V. capreoli* (Stroh and Schmid, 1938), *V. alpenae* (Cheatum, 1949, 1951), and *V. pneumonicus* (Bhatia and Pande, 1960), are known to cause gross lesions, and histopathologic changes.

Co-infections of *V. eleguneniensis* with *P. andersoni* in caribou (G. Verocai, S. Kutz, unpublished data) and *U. pallikuukensis* in muskoxen (Kutz et al., 2013a) occur, and could have additive effects on the hosts. Co-infections with other protostrongylids that overlap in host and

geographic range, such as *P. odocoilei* in caribou and *Pr. stilesi* in muskoxen have not been reported. Additionally, another lungworm, *Dictyocaulus eckerti* Skrjabin, 1931, occurs in caribou and muskox populations across the distribution of *V. eleguneniensis* (Kutz et al., 2007), and at least one muskox evaluated in this study was co-infected (G. Verocai, M. Simard, S. Kutz, unpublished obs.).

### *3.5.3 Explorations on historical biogeography*

Species of *Varestrongylus* are known from ungulates of the families Cervidae (six species in Cervinae and Odocoileinae (=Capreolinae)) and Bovidae (four species in Caprinae) in Eurasia and North America (Anderson, 2000; Boev, 1975; Verocai et al., 2014a; present study). Eurasia is the center of diversity for both these ungulate groups. The modern tribes of Caprinae and Cervinae originated in Central Asia near 14.7-14.5 Ma (millions of years ago) during the middle Miocene; and Odocoileinae diversified between 11.0-10.0 Ma around the middle and late Miocene boundary (Hernández-Fernández and Vrba, 2005; Kurtén and Anderson, 1980). Coincidentally, Eurasia is the center of diversity for species within *Varestrongylus,* as well as for other members of Protostrongylidae and a substantially broader strongylate nematode fauna in artiodactyls (Hoberg, 2005; Hoberg et al., 2012a; Hoberg et al., 2012b; Hoberg et al., 1995). As a generality, Eurasian biodiversity or species richness among ungulate nematodes considerably exceeds that observed in the Nearctic (Hoberg et al., 2012b). Geographically, *Varestrongylus* is characterized by eight species endemic to Eurasia and the western Palearctic (Boev, 1975; Verocai et al., 2014a) in contrast to *V. alpenae* and *V. eleguneniensis* which have distributions restricted to North America (Dikmans, 1935; present study).

The formation of the contemporary North American fauna involved expansion from Eurasia, with successive waves of invasion and geographic colonization during the late Pliocene and Quaternary across the Bering Land Bridge (Hoberg and Brooks, 2008; Hoberg et al., 2012b; Hoberg et al., 2003; Kurtén and Anderson, 1980; Shafer et al., 2010). A consequence of these episodic processes has been the development of an extensive faunal mosaic coinciding with asynchronous arrival and recurrent establishment of particular ungulate groups and their associated parasite faunas (Hoberg, 2005, 2010; Hoberg et al., 2012a; Hoberg et al., 2012b). During these independent events of geographic colonization, the hosts of the two Nearctic species of *Varestrongylus* entered North America and expanded across much of the continent. The ancestors of *O. virginianus,* the only known host of *V. alpenae*, reached the Nearctic around 4 Ma, whereas the three known hosts of *V. eleguneniensis* invaded and became established in North America in more recent times. Current evidence based on field surveys including collections of adult parasites, fecal examination and sequencing, and studies documenting the distribution of *P. andersoni*, *P. tenuis*, *P. odocoilei*, *Protostrongylus coburni* Dikmans, 1935, *O. macrotis* and *V. alpenae* among species of *Odocoileus* have not revealed the presence of *V. eleguneniensis* in relatively southern host populations (Anderson, 2000; Asmundsson et al., 2008; Dikmans, 1935; Hoberg et al., 2012b; Lankester, 2001; Mortenson et al., 2006).

*Rangifer* is a Beringian endemic, and first arrived to North America approximately 2 Ma, but multiple events of expansion and retraction followed during glacial-interglacial cycles of the Pleistocene, with secondary isolation of *Rangifer* north and south of the Nearctic continental glaciers (Banfield, 1961; Flagstad and Røed, 2003; Weckworth et al., 2012). From Beringia, *Rangifer* also expanded westwards through the Palearctic, resulting in its present Holarctic distribution (Banfield, 1961; Flagstad and Røed, 2003; McDevitt et al., 2009b; Weckworth et al.,

2012). In contrast, the other two hosts of *V. eleguneniensis* only became established in North America in shallower time. *Ovibos* (as *Ovibos moschatus*) expanded into Beringia around 900–700 Ka (thousands of years ago) and, similarly to *Rangifer,* occurred in isolated populations both in Beringia and environs south of the ice-sheets during the Pleistocene. Currently, natural muskox populations only occur in North America. The species became extinct in the Palearctic in the Holocene (Campos et al., 2010; Hoberg et al., 2012b; Kurtén and Anderson, 1980). *Alces,* as a late Pleistocene migrant to the Nearctic, entered the Nearctic only 14–11 Ka, with subsequent eastwards and southwards expansion after the recession of the continental ice (Hundertmark et al., 2003; Hundertmark et al., 2002; Kurtén and Anderson, 1980).

Historical biogeography of these host-parasite assemblages and development of associations of these parasites with cervids and caprines is complex and can be initially considered in the context of phylogenetic inference among the protostrongylids and species of *Varestrongylus.* An ancestral association with cervids has been proposed for *Varestrongylus* (Hoberg et al., 2012b), which is supported by the host-associations of the Elaphostrongylinae, the sister group of Varestrongylinae (Carreno and Hoberg, 1999), also primarily parasites of cervids (Boev, 1975; Carreno et al., 2012; Carreno and Lankester, 1994; Lankester, 2001). Concurrently, this supports a primary association of *V. eleguneniensis* with caribou and secondary host switching to muskoxen and moose in zones of relatively recent to very recent contact, proposed by Hoberg et al. (2012b). This primary association is further supported by the current geographic distribution of *V. eleguneniensis*, which virtually mirrors that of caribou in North America. Further, an ancient association with *Rangifer* may indicate that *V. eleguneniensis* may also have been a Beringian endemic during the late Pliocene, but since subsequently multiple expansion events of *Rangifer* have occurred it is impossible to estimate with precision

when *V. eleguneniensis* first arrived in the continent (Hoberg and Brooks, 2008; Hoberg et al., 2012b).

A limited phylogenetic analysis (based on ITS-2) among five species of *Varestrongylus* suggests that *V. eleguneniesis* is genetically closer to *V. alces* and *V. capreoli* (Verocai et al., 2014a). This putative association of *V. eleguneniensis* with these Eurasian species, as opposed to the only other Nearctic species, *V. alpenae*, appears consistent with at least two independent events of host-parasite invasion from Eurasia, involving Beringia, to the Nearctic during the late Pliocene and Quaternary. The morphological similarities of the two Eurasian species and *V. eleguneniensis*, which collectively form the 'short-spicule' group within *Varestrongylus*, further support their relationship (see also Verocai et al. (2014a)). Therefore, we hypothesize that two distinct *Varestrongylus* species crossed Beringia and reached the Nearctic from Eurasia: with *V. alpenae* and *V. eleguneniensis*, or their ancestors, invading North America along with *Odocoileus* and *Rangifer* hosts, respectively. Based on these empirical data, a primary association of *V. eleguneniensis* with muskoxen, a caprine host would not be predicted; its presence in muskoxen is likely the result of several independent host switching events in areas of sympatry with infected caribou, including recent events linked to translocations and introductions (see Host Distribution section above). Also, a primary association with muskoxen would be considerably shallow in time; and perhaps, more difficult considering the strong population bottlenecks and extinctions across its range (Campos et al., 2010; Gunn and Adamczewski, 1998). Contrasting with this distribution, muskoxen are the only recognized hosts for an otherwise relictual protostrongylid species, *U. pallikuukensis* (Hoberg et al., 1995; Kutz et al., 2001a). Besides its very recent invasion of the Nearctic, infection of moose with *V.*

*eleguneniensis* is rare and incidental, and is only reported in areas of sympatry with caribou (Kutz et al., 2007).

Considering finer scale geographic and host associations, genetic studies on *Rangifer* distinguish two main lineages of caribou in the Nearctic, the North-American *Rangifer* lineage (NAL), which ranged during glacial maxima south of the Laurentide and Cordilleran ice-sheets, isolated from the Beringian-Eurasian lineage (BEL) vastly distributed from Europe to Beringia (Banfield, 1961; Flagstad and Røed, 2003; Klütsch et al., 2012; McDevitt et al., 2009b; Weckworth et al., 2012).The ancient association of *V. eleguneniensis* with *Rangifer* and this host's intricate historical biogeography allow us to articulate testable hypotheses on the historical biogeography and phylogeography of this novel lungworm species: (i) the parasite was maintained within BEL caribou and restricted to Beringia, and expanded along with caribou eastwards and southwards, colonizing NAL populations; (ii) the parasite was maintained both within BEL in Beringia and within NAL south of ice sheets, and expanded geographically with both lineages; (iii) the parasite was maintained within NAL, solely south of the ice sheets in one or multiple refugia and expanded northwards with NAL caribou and, later, colonized and expanded with BEL populations. Future studies on the population genetics of *V. eleguneniensis*, involving geographically extensive sampling across its vast range in North America, may reveal genetic signatures compatible to such events of expansion and/or isolation within a single or multiple refugia.

### 3.6 Conclusions

Herein we have described and named *V. eleguneniensis*, a pulmonary protostrongylid with *Rangifer* as a primary definitive host; that secondarily infects muskoxen and moose in areas of sympatry. The parasite appears to be geographically restricted to North America; however there is a lack of surveys for pulmonary protostrongylids in *Rangifer* from Eurasia, including western Beringia. Detailed investigations for the presence of *V. eleguneniensis*, its close relative *V. alces*, or another *Varestrongylus* in reindeer from the Palearctic remain necessary. The biogeographic history for two endemic species of *Varestrongylus* known from North America appears consistent with events of parasite invasion with cervid hosts from Eurasia into North America during the late Pliocene and Quaternary. The putative ancient association with *Rangifer* hosts could be investigated through the phylogeography of *V. eleguneniensis*, which may provide new insights on caribou historical biogeography and the history of colonization of the Nearctic by host-parasite assemblages.

### 3.7 Competing interests

The authors declare that they have no competing interests.

### 3.8 Authors' contributions

GGV lead the study and preparation of the manuscript. GGV, SJK and MS collected the parasite specimens in the field and lab. SJK and EPH oversaw the study. GGV and EPH did

morphological description of specimens. GGV carried out the molecular genetic study. All authors critically revised and approved the final manuscript.

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# Chapter Four: **THE GENUS *VARESTRONGYLUS* BHALERAO, 1932 (NEMATODA: PROTOSTRONGYLIDAE), LUNGWORMS OF UNGULATES: PHYLOGENETIC INFERENCE OF RELATIONSHIPS AMONG SPECIES BASED ON MORPHOLOGY, WITH AN EXPLORATION OF BIOGEOGRAPHY AND HOST-ASSOCIATIONS**

## 4.1 Abstract

*Varestrongylus* Bhalerao, 1932 comprises ten valid lungworm species in wild and domestic ungulates from Eurasia and North America. Here we present a phylogenetic hypothesis based on morphological characters in a broader context for the family Protostrongylidae, and discuss species relationships, host-associations and historical biogeography. Phylogenetic analysis of 25 morphological characters among the ten valid species of *Varestrongylus* resulted in one fully resolved most parsimonious tree (61 steps; consistency index = 0.672, retention index = 0.722, and consistency index excluding uninformative characters= 0.667). *Varestrongylus* forms a monophyletic clade and is the sister of *Pneumostrongylus*, supporting the subfamily Varestrongylinae. Monophyly for *Varestrongylus* is diagnosed by six unequivocal synapomorphies, all associated with structural characters of the copulatory system of males. Species of *Varestrongylus* have complex histories with respect to host and geographic associations: (1) *Varestrongylus* has origins in Eurasia with independent expansions into bordering ecozones; (2) cervids are ancestral hosts; (3) the caprine-associated *V. pneumonicus* is basal and a result of an independent host-switching event; (4) secondary diversification, linked to sequential and independent events of host switching, occurred within cervids (*V. sagittatus* + *V. tuvae*; *V. alpenae*; and *V. capreoli*, *V. alces* +*V. eleguneniensis* ); (5) at least two additional host-switching events into caprines occurred, followed or not by diversification (*V. qinghaiensis* + *V. longispiculatus*; *V. capricola*, respectively); (6) two independent events of geographic expansion into North America with cervids in the late Pliocene and early Pleistocene are postulated (*V. alpenae*, *V. eleguneniensis*), congruent with existing molecular evidence. Episodes of geographic and host colonization, often in relation to climate variation and habitat perturbation have dominated the history of diversification among species of *Varestrongylus*. Highlighted is the

continuing importance of phylogenetic assessment based on morphological characters as a foundation to explore the structure of the biosphere in space and time.

## 4.2 Introduction

*Varestrongylus* Bhalerao 1932 (Nematoda; Protostrongylidae) currently comprises ten valid species of lungworms parasitic in the small and large bronchioles of wild and domestic ungulates within the families Bovidae and Cervidae (Anderson, 2000; Boev, 1968, 1975; Dougherty and Goble, 1946). The genus is a relatively speciose group, and diversity among other Protostrongylidae Leiper, 1926 is only exceeded by *Protostrongylus* Kamensky, 1905 which encompasses about thirty nominal species. Species of *Varestrongylus* have variable veterinary importance, but many are responsible for pathological changes in the lungs of affected hosts, leading to focal verminous pneumonia known as varestrongylosis (Panayotova-Pencheva, 2007; Petrosyan, 1963; Stroh and Schmid, 1938; Verocai et al., 2014a). All species have indirect life cycles, requiring a gastropod intermediate host in which dorsal-spined first-stage larvae shed in ungulate feces develop to the infective third-stage (Anderson, 2000; Boev, 1975).

Among eight species distributed in Eurasia, four are primarily associated with Cervidae and are distributed among Cervinae and Odocoileinae (=Capreolinae *sensu* (Grubb, 2005): *Varestrongylus alces* Demidova & Naumitscheva, 1953 in Eurasian elk (*Alces alces*); *Varestrongylus capreoli* (Stroh & Schmid, 1938) in roe deer (*Capreolus capreolus*); *Varestrongylus sagittatus* (Mueller, 1890) primarily in red deer (*Cervus elaphus*) but also in fallow and Sika deer (*Dama dama* and *Cervus nippon*, respectively); and *Varestrongylus tuvae* (Boev & Sulimov, 1963) in Siberian wapiti (*Cervus canadensis sibiricus*). Elsewhere in Asia, four other species infect caprine bovids: *Varestrongylus pneumonicus* Bhalerao, 1932 and *Varestrongylus capricola* Sarwar, 1944 in wild and domestic caprines; *Varestrongylus qinghaiensis* Liu, 1984 reported only in domestic sheep; and *Varestrongylus longispiculatus* Liu, 1989 in takin (*Budorcas taxicolor*). The North American *Varestrongylus* fauna comprises two

species infecting odocoileine cervids: *Varestrongylus alpenae* (Dikmans, 1935) in white-tailed deer (*Odocoileus virginianus*), and *Varestrongylus eleguneniensis* Verocai, Kutz, Simard & Hoberg, 2014 primarily in caribou (*Rangifer tarandus* sspp.), but also common in muskoxen (*Ovibos moschatus* sspp.) and rarely in moose (*Alces americanus gigas*).

The taxonomy of *Varestrongylus* has been remarkably confused and convoluted. For example, at different times *V. sagittatus*, and its synonyms, have been allocated among five different genera, and *V. capreoli* has been placed in three genera other than *Varestrongylus* (Boev, 1975; Verocai et al., 2014a). Since the generic revision by Boev (1975) three additional species have been described, and another species resurrected (Liu, 1989; Liu, 1984; Verocai et al., 2014a; Verocai et al., 2014b). This prompted Verocai et al. (2014b) to emend the generic diagnosis to accommodate morphological features and variations of these species described in the last three decades. Morphologically, the genus is diagnosed mainly by copulatory structures of males and females (Boev, 1975; Dougherty, 1949 ; Dougherty and Goble, 1946), but exceptions are numerous, which partially explains the creation of many synonymous genera and the 'taxonomic mobility' of many species in the absence of an explicit phylogenetic hypothesis for the group.

There have been no comprehensive phylogenetic interpretations for the evolution of this genus and relationships among species, although a few *Varestrongylus* species (*V. sagittatus*, *V. pneumonicus*, and *V. alpenae*) were included in a generic-level analysis for the Protostrongylidae (Carreno and Hoberg, 1999). In those analyses, *Varestrongylus* was considered paraphyletic relative to *Pneumostrongylus* Mönnig, 1932 although this may have reflected absence of a comprehensive framework for species diversity and exclusion of some structural attributes and putative synapomorphies otherwise directly relevant among species-groups in Varestrongylinae

Boev, 1968. Carreno and Hoberg (1999) proposed a modified taxonomy for Varestrongylinae, retaining the subfamily for *Varestrongylus* and *Pneumostrongylus*, but with the exclusion of *Pneumocaulus* Schulz and Andreeva, 1948.

The only existing molecular-based comparisons encompass five out of the ten valid species of *Varestrongylus* and the analysis is limited to a single nuclear ribosomal locus (internal transcribed spacer region-2 or ITS-2). This analysis diagnosed a well-supported clade including, *V. alpenae, V. alces*, *V. eleguneniensis,* and specimens attributable to *V. capreoli* (referred as *V.* cf. *capreoli*) and a basal position for *V. sagittatus.* Comprehensive phylogenetic inference could not be achieved given the lack of access to appropriate specimens representing other species (Verocai et al., 2014a).

A combination of factors hinders a comprehensive assessment of species of *Varestrongylus* based on either morphological or molecular attributes. Among these are: (1) a necessity to collect adult worms, rather than larvae, which limits the potential for large scale survey and inventory (but see Kutz et al., 2007); (2) the delicate nature of specimens (e.g. descriptions are often based on fragmented specimens; no report of intact specimens of *V. tuvae*); (3) collectively, species which occupy an extensive geographic range across Eurasia and North America and often occur in remote areas logistically difficult to access (e.g., Himalayan region, Arctic) creating challenges for sample acquisition; and (4) conservation status of certain ungulate species which limits field collections and hinders the acquisition of adult specimens, which can only be collected through the post-mortem examination of infected hosts (e.g., *V. longispiculatus*). Broad-based comparative studies of these and other protostrongylids are further complicated by absence of archival specimens in museum collections for many species that would be appropriate for concurrent morphological and molecular analyses. Additionally, when

these nematodes receive attention, studies are often restricted to a single species of lungworm among cervids or bovids (i.e.; considerable focus on elaphostrongylines and protostrongylines), and do not contribute to explorations or discovery of diversity.

Consequently, in the absence of representative specimens of all valid species, comparative morphology constitutes an important source of information in this diverse genus. New field collections are required to provide materials suitable for molecular-based and integrative approaches. In the interim, detailed assessment of structural attributes provides a pathway to develop a reasonably comprehensive estimate of phylogeny among species of *Varestrongylus*.

Herein, we develop a phylogenetic hypothesis for species of *Varestrongylus* as inferred by morphological characters. The phylogenetic hypothesis forms the basis for confirming species validity and for explorations of putative host associations and biogeographic history in this Holarctic assemblage of ungulates and parasites. Through this work, we aim to create the foundations for future studies on this understudied lungworm genus.

## 4.3 Materials and methods

### *4.3.1 Phylogenetic analyses*

Phylogenetic analysis involved morphological characters among adult nematodes representing the ten currently recognized species of *Varestrongylus* (Table 4.1). Characters were derived from direct study of available specimens (Table 4.2), along with data from the literature, including original descriptions (Bhalerao, 1932; Bhatia and Pande, 1960; Boev, 1975; Demidova and Naumitscheva, 1953; Liu, 1989; Liu, 1984; Sarwar, 1955; Verocai et al., 2014a; Verocai et al., 2014b). Standard methods for comparative morphology among species within

*Varestrongylus* were based on the literature, including the last revision for the genus (Boev, 1975), analysis completed during phylogenetic assessments of Protostrongylidae (Carreno and Hoberg, 1999), along with more recent studies (Verocai et al., 2014a; Verocai et al., 2014b).

Characters included morphological attributes of both male and female nematodes. We identified 25 attributes suitable for analysis and phylogenetic reconstruction among species of *Varestrongylus*, including 14 that were binary (two states) and 11 multistate (three or more states) characters. Polarity was estimated based on the taxonomic or functional outgroups (Watrous and Wheeler, 1981). The principal taxonomic outgroups constituted 2 higher taxa including composites constructed based on an estimation of ancestral characters and states among multiple species within each group for the subfamilies Protostrongylinae (a distant outgroup) and Elaphostrongylinae (a close outgroup and together with Muelleriinae, the putative sister of *Varestrongylus* + *Pneumostrongylus* based on Carreno and Hoberg (1999)). In addition both species in the putative sister-group, *Pneumostrongylus* were included: *Pneumostrongylus calcaratus* Mönnig, 1932 and *Pneumostrongylus cornigerus* Ortlepp, 1962 (Mönnig, 1932; Ortlepp., 1962).

**Table 4.1.** Valid species of *Varestrongylus* Bhalerao, 1932, with their host and geographic associations

| ***Varestrongylus* species** | **Host(s)** | **Geographic Distribution** |
|---|---|---|
| *V. eleguneniensis* Verocai, Kutz, Simard & Hoberg, 2014 | Caribou (*Rangifer tarandus* sspp.) | Canada and Alaska (USA) |
| | Muskox (*Ovibos moschatus* sspp.) | |
| | Moose (*Alces americanus gigas*) | |
| *V. alpenae* (Dikmans, 1935) Dougherty, 1945* | White-tailed deer (*Odocoileus virginianus*) | Eastern Canada and USA |
| *V. capreoli* (Stroh & Schmid, 1938) Dougherty, 1945* | Roe deer (*Capreolus capreolus*)[‡] | Europe |
| *V. alces* Demidova & Naumitscheva, 1953 | Eurasian Elk (*Alces alces*) | Eurasia |
| *V. capricola* Sarwar, 1944 | Wild & Domestic Sheep and Goats (*Ovis aries* and | India and Pakistan |
| | *Capra hircus*) | |
| *V. pneumonicus* Bhalerao, 1932 | Wild & Domestic Sheep and Goats | India and Pakistan |
| | (*Ovis* spp., *Capra hircus, Pseudois*) | |
| *V. sagittatus* (Mueller, 1890) Dougherty, 1945* | Red deer, Maral, Fallow Deer | Europe |
| | (*Cervus elaphus* sspp., *Dama dama*) | |
| *V. tuvae* (Boev & Sulimov, 1963) Boev, 1968 | Siberian wapiti (*Cervus canadensis sibiricus*) | Russia |
| *V. qinghaiensis* Liu, 1984 | Sheep (*O. aries*) | China |

| | | |
|---|---|---|
| *V. longispiculatus* Liu, 1989 | Takin (*Budorcas taxicolor*) | China |

| **Outgroups** | | |
|---|---|---|
| Protostrongylinae Kamensky, 1905 | Bovidae: Caprinae, Cervidae, Lagomorpha | Eurasia, Africa, North America |
| Elaphostrongylinae Boev and Schulz, 1950 | Cervidae and Caprinae | Eurasia, Americas |
| *Pneumostrongylus calcaratus* Mönnig, 1932 | Bovidae: Aepycerontinae, Alcelaphinae, Antilopinae | Africa |
| *Pn. cornigerus* Ortlepp, 1962 | Bovidae: Aepycerontinae, Antilopinae | Africa |

[‡] Reports of *V. capreoli* in mouflon (*Ovis musimon*) in Czech Republic (Erhardova, 1957) and domestic goat (*Capra aegagrus hircus* L.) in Switzerland (Bouvier and Hörning, 1963) may be equivocal, and require further verification.

Outgroup taxa were selected based on relationships established in previous phylogenetic analyses within Protostrongylidae and Metastrongyloidea (Carreno and Hoberg, 1999; Carreno and Nadler, 2003; Verocai et al., 2014a). In cases where a simple comparison to a taxonomic outgroup did not resolve polarity, decisions were derived with reference to functional outgroups (Watrous and Wheeler, 1981). Functional outgroups were established based on topology and relationships diagnosed in preliminary analyses, and generally represented putative basal taxa of the ingroup.

The data matrix (Table 4.2) was analyzed using the software Phylogenetic Analysis Using Parsimony (PAUP 4.0 beta version 10) (Swofford, 2002). Morphological character states were coded as unordered. Primary analyses used the following options: branch and bound, characters with equal weights, addseq= furthest, and accelerated transformation. The result is shown as a single most parsimonious (MP) tree with associated statistics (number of steps; consistency index, CI; and retention index, RI). Further analysis included jackknife resampling as implemented by PAUP with 1,000 replicates using both branch-and-bound and heuristic searches, and presentation as strict consensus trees. Character changes, including putative synapomorphies, are mapped onto the phylogenetic hypothesis allowing discussion of character evolution and support of phylogenetic structure and topology. Support indices are also shown in the MP tree. Bremer decay indices were calculated with sequential analyses in PAUP and were used to examine node support for putative relationships in this study (Bremer, 1994). The results from these analyses provide the foundations for confirmation of species status, and discussions about host and geographic associations and historical biogeography.

**Table 4.2.**Character matrix for the genus *Varestrongylus* and outgroups, containing 25 morphological characters included in the phylogenetic analysis

| **Characters** / **Taxon** | **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** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Protostrongylinae* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Elaphostrongylinae* | 3 | 0,1 | 0,2 | 1 | 0 | 0 | 0 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0,1 | 1 | 0 | 0 | 1 | 0 | 0 |
| *Pn. calcaratus* | 0 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| *Pn. cornigerus* | 0 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| *V. eleguneniensis* | 2 | 0 | 2 | 1 | 1 | 0 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 2 | 1 | 1 |
| *V. alces* | 1 | 0 | 2 | 1 | 1 | 0 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 2 | 1 | 1 |
| *V. capreoli* | 1 | 0 | 2 | 0 | 2 | 0 | 1 | 1 | 2 | 3 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 2 | 1 | 1 |
| *V. alpenae* | 1 | 0 | 2 | 1 | 2 | 0 | 2 | 1 | 3 | 1 | 2 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 |
| *V. capricola* | 2 | 1 | 2 | 1 | 2 | 0 | 2 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 |
| *V. sagittatus* | 1 | 0 | 2 | 1 | 2 | 1 | 2 | 1 | 4 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 1 | 1 |
| *V. tuvae* | 1 | 0 | 1 | 1 | 0 | 1 | 2 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 1 | 1 |
| *V. qinghaiensis* | 1 | 1 | 2 | 1 | 2 | 2 | 2 | 2 | 1 | 3 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 2 | 1 | 2 | 1 | 1 |
| *V. longispiculatus* | 0 | 1 | 2 | 1 | 2 | 2 | 2 | 1 | 4 | 1 | 2 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 2 | 1 | 2 | 1 | 1 |

| | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *V. pneumonicus* | 0 | 1 | 2 | 1 | 1 | 0 | 2 | 1 | 3 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 |

* Composite of species within Protostrongylinae and Elaphostrongylinae; *Pn. = Pneumostrongylus.*

### *4.3.2 Character Coding for analyses among species of* **Varestrongylus**

**1. Provagina structure. Multistate.** 0= Tubular, well developed extending to near caudal extremity; 1= Tubular, well developed, extending no greater than half tail length from vulva; 2= Reduced to obscure hood-like fold; 3= Absent.

**2. Gubernaculum (I) corpus form. Multistate.** 0= Corpus and legs continuous, prominent, split distally; 1= Corpus prominent, not split distally; 2= Corpus narrow, indistinct, triangular distally.

**3. Gubernaculum (II) corpus and crurae. Multistate.** 0= Feet fused to corpus, with distinct crurae fused to corpus, modified structures, prominent, strongly cuticularized, smooth, recurved distally; 1= Distinct paired crura plates absent, modifications of distal gubernaculum as ventro-distal shield; 2= Distinct paired crura plates well developed, denticulate.

**4. Gubernaculum (III) capitulum. Binary.** 0= Present; 1= Absent.

**5. Gubernaculum (IV) structure of plates of the crura. Multistate.** This character is considered only in the context of those species in which paired denticulate plates are present. 0= Denticulate plates lacking; 1= Rectangular or trapezoidal in shape; 2= Strongly triangular.

**6. Gubernaculum: structure of proximal extremity. Multistate.** 0= narrow proximal region; 1= Expanded proximal region; 2= Split proximal region.

**7. Gubernaculum length. Multistate.** Functional outgroup: Elaphostrongylinae. 0= Short compact, with expansion distally; 1= Short (<100μm) narrow through length and distally; 2= Long (> 100 μm), narrow through length.

**8. Telamon plates: degree of development. Multistate.** 0= Strongly developed, complex; 1= Reduced, but present; 2= Absent.

**9. Telamon plates: shape and relative position. Multistate.** 0= Complex, lateral, ventral plates developed; 1= Absent; 2= Bar-like, vestigial ventral plate; 3= Arcuate lateral plates developed; 4= Heart shaped, ventral plate.

**10. Copulatory bursa: form. Multistate.** 0= Undivided, round; 1= Notched dorsally, with indistinct dorsal lobe; 2= Strongly bilobate; 3= Weakly lobate.

**11. Dorsal Ray: structure. Multistate.** 0= Rounded, indistinct, with prominent papillae; 1= Irregular; 2= Stalk-like, distinctly bifurcate.

**12. Dorsal Ray length. Binary.** 0= Short, not strongly developed; 1= Elongate.

**13. Dorsal Ray symmetry. Binary.** Functional outgroup: Elaphostrongylinae. 0= Asymmetrical; 1= Symmetric.

**14. Externodorsal ray: origin (proximity) of base. Binary.** 0= Base of externo-dorsal in proximity to lateral rays; 1= Base of exteno-dorsal independent.

**15. Bursal Rays, relative length postero-lateral and medio-lateral rays. Binary.** 0= Rays of equal length; 1= Postero-lateral shorter than medio-lateral.

**16. Bursal Rays, relative length medio-lateral and externo-lateral rays. Binary.** 0= Medio-lateral longer than externo-lateral; 1= Medio-lateral and externo-lateral of equal length.

**17. Bursal Rays, ventral ray structure. Binary.** 0= Common stalk, split near base; 1= Common stalk, split distally.

**18. Ventral rays, relative length. Binary.** 0= Ventral rays of equal length; 1= Ventro-ventral short.

**19. Bursal Rays, disposition of ventral rays. Binary.** 0= Latero-ventral ray parallel to ventro-ventral ray throughout its length; 1= Latero-ventral ray strongly recurved.

**20. Spicule, branch structure distally. Binary.** 0= Branched; 1= Unbranched.

**21. Spicule length (absolute). Multistate.** 0= Medium in length (200-600μm); 1= Short in length (> 170 μm); 2= Very long (>600 μm).

**22. Spicule length, equality. Binary.** 0= Spicules equal in length; 1= Spicules subequal.

**23. Spicule, structure of shaft. Binary.** Functional outgroup: *Pneumostrongylus*. 0= With distinct joint defining a distal manubrium; 1= Compact spicules lacking manubrium; 2= Filamentous spicules lacking manubrium.

**24. Excretory pore, relative position. Binary.** Functional outgroup: Elaphostrongylinae. 0= Excretory pore at level, or anterior to nerve ring; 1= Posterior to nerve ring, near base of esophagus.

**25. Female tail, structure. Binary.** Functional outgroup: Elaphostrongylinae. 0= Blunt; 1= Acute, pointed.

### *4.3.3 Host inference and Geography*

Historical relationships among host-group taxa and geographic associations for species of *Varestrongylus* were examined by mapping and optimization onto the parasite phylogeny using MacClade 4.0 (Maddison and Maddison, 2000). For further analysis, the outgroup Protostrongylinae (associated with Caprinae) was pruned from the tree as it has no direct implications for understanding host and geographic associations within *Varestrongylus* and Varestrongylinae (see also structure of generic relationships postulated in Carreno and Hoberg (1999).

Associations for parasites and hosts were also examined by mapping relative to a phylogenetic hypothesis for Pecoran ungulates across a taxonomic hierarchy for families, subfamilies and tribes (excluding Giraffidae and Antilocapridae) (see Grubb, 2005; Hernández-

Fernández and Vrba, 2005). Detailed generic and species-level relationships within Cervidae and Caprinae were based on phylogenetic hypotheses from works by Gilbert et al. (2006) and Yang et al. (2013), respectively. Timeframes for the history of host-parasite assemblages are designated as Ma (millions of years ago) and Ka (thousands of years ago). Historical and current data for hosts, including origin, patterns of range expansion and anthropogenic translocations until modern times are derived from the primary literature and reviews (e.g., Grubb, 2005; Kurtén and Anderson, 1980). The conservation status of host species is consistent with the International Union for Conservation of Nature (IUCN) Red List (www.iucnredlist.org) (Wilson and Reeder, 2005).

## 4.4 Results

### *4.4.1 Phylogenetic diagnosis for Varestrongylus*

#### 4.4.1.1 Phylogenetic relationships

Primary phylogenetic analysis resulted in one fully resolved most parsimonious tree (MPT) (61 steps; consistency index = 0.672, retention index = 0.722, and consistency index excluding uninformative characters= 0.667) (Figure 4.1). Homoplasy was associated with 13 characters, and among these, 12 attributes (1-3, 5, 8-12, 14, 17, 20) demonstrated parallelism/convergence and 3 were influenced by reversal (1,4,12) (Figure 4.2). Diagnostics for individual characters are shown in Table 4.3. Based on CI values for individual attributes and states (Table 4.3), characters 3, 6, 7, 13, 15, 16, 18, 19, 21-25 were entirely consistent (CI=100%), whereas characters 12 and 17 had CI values lower than 50%.

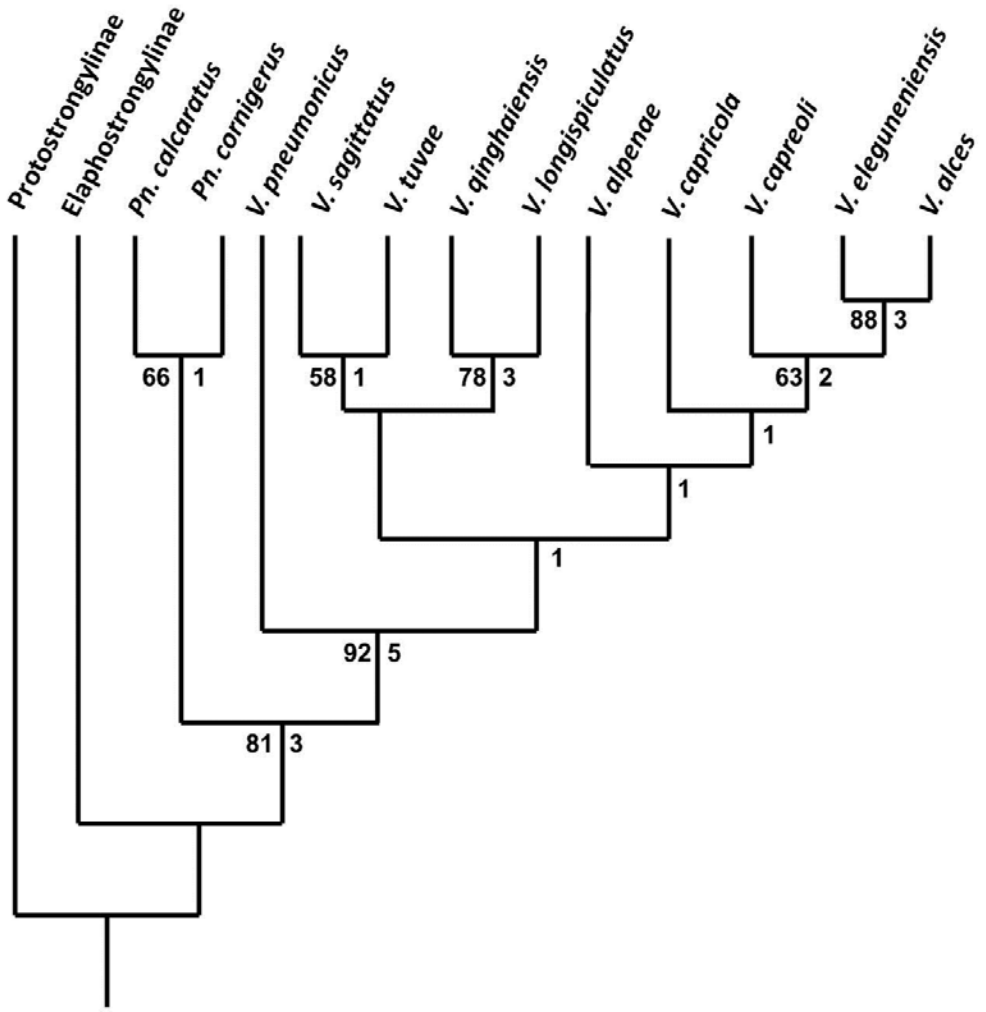


**Figure 4.1.** Phylogenetic relationships for species within the genus *Varestrongylus* Bhalerao, 1932 as inferred by parsimony analysis of morphological characters. Represented is the single most parsimonious tree (CI = 0.672, 61 steps). Values shown to the left of each node are jackknife resampling as a percentage based on 1,000 replicates. Values shown to the right of each node are those for Bremer decay indices, as steps required to collapse the tree. Monophyly of *Varestrongylus* was strongly supported (5 steps, and 92%).

4.4.1.2 Relationships among *Varestrongylus*

Species of *Varestrongylus* form a clade separate from those partitioned in *Pneumostrongylus*, and collectively, these taxa constitute the Varestrongylinae which is diagnosed by 3 unambiguous synapomorphies (chars. 17, 24, 25) (Figure 4.2). Monophyly for *Varestrongylus* was diagnosed by six unequivocal synapomorphies: distinct, denticulate and well developed paired crura plates (character 3); length of gubernaculum (character 7); reduced telamon plates (character 8); shape of telamon plates (character 9); relative length of postero-lateral and medio-lateral bursal rays (character 15) and the filamentous constitution of the spicule shaft (character 23) (Figure 4.2).

Within the genus, *Varestrongylus pneumonicus* is basal, and sister to all other species, which form two subclades (I and II). Subclade I contains *V. sagittatus* + *V. tuvae* and *V. qinghaiensis* + *V. longispiculatus*, and is diagnosed by two unambiguous synapomorphies (chars. 9, 18). Subclade II contains *V. alpenae*, *V. capricola*, *V. capreoli* and *V. eleguneniensis* + *V. alces*, and is also diagnosed by two unambiguous synapomorphies (characters 12, 17) (Figure 4.2).

Jackknife resampling and Bremer decay indices revealed strong support for monophyly of the genus *Varestrongylus* (92%, and 5, respectively), and moderate to strong support for Varestrongylinae (81%, 3) (Figure 4.1). Although the most parsimonious tree was stable, low to moderate jackknife or Bremer support characterized most nodes (≥50%, 1-2) other than those diagnosing sister-species relationships for *V. qinghaiensis* + *V. longispiculatus* (78%, 3) and *V. eleguneniensis* + *V. alces* (88%, 3) (Figure 4.1).

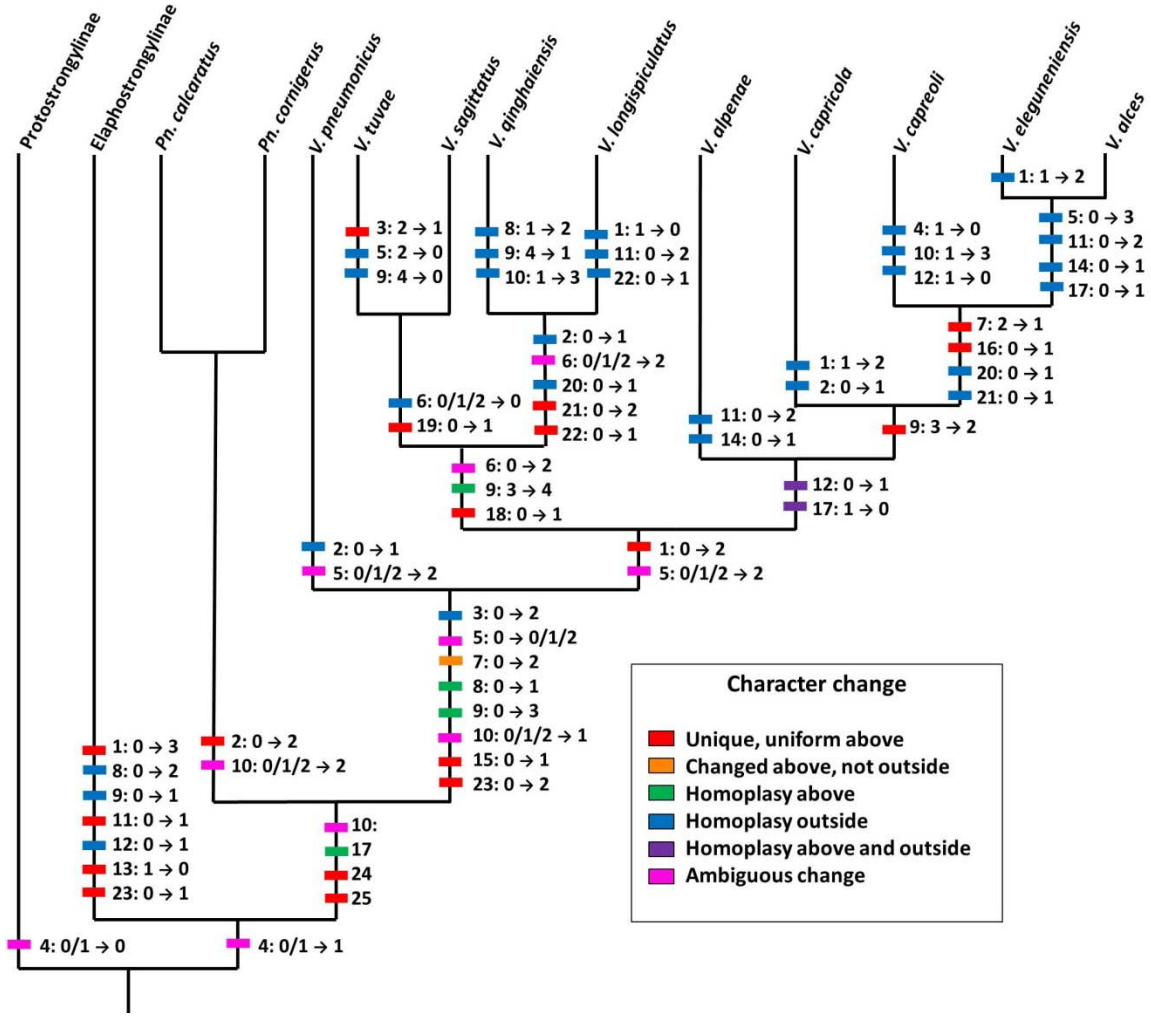


**Figure 4.2.** Phylogenetic relationships for species within *Varestrongylus* Bhalerao, 1932 as inferred by parsimony analysis of morphological characters. Character change have been mapped directly onto the single most parsimonious tree with MacClade 4.0 (Maddision and Maddison, 2000). Note the eight synapomorphies, with six unequivocal, for phylogenetic diagnosis of *Varestrongylus*.

**Table 4.3.**Consistency index and number of steps on the single most parsimonious tree for the 25 characters used in the phylogenetic analysis for *Varestrongylus*

| Character | Tree steps | CI |
|---|---|---|
| **1** | 5 | 0.600 |
| **2** | 4 | 0.500 |
| **3** | 2 | 1.000 |
| **4** | 2 | 0.500 |
| **5** | 4 | 0.500 |
| **6** | 2 | 1.000 |
| **7** | 2 | 1.000 |
| **8** | 3 | 0.677 |
| **9** | 6 | 0.677 |
| **10** | 4 | 0.750 |
| **11** | 4 | 0.500 |
| **12** | 4 | 0.250 |
| **13** | 1 | 1.000 |
| **14** | 2 | 0.500 |
| **15** | 1 | 1.000 |
| **16** | 1 | 1.000 |
| **17** | 3 | 0.333 |
| **18** | 1 | 1.000 |
| **19** | 1 | 1.000 |
| **20** | 2 | 0.500 |
| **21** | 2 | 1.000 |
| **22** | 1 | 1.000 |
| **23** | 2 | 1.000 |
| **24** | 1 | 1.000 |
| **25** | 1 | 1.000 |

### *4.4.2 Parasite-host coevolution and historical biogeography*

Host and geographic associations were explored through a process of reciprocal mapping, with ungulate taxa mapped directly on the phylogenetic hypothesis for *Varestrongylus* and Varestrongylinae and with species of *Varestrongylus* examined relative to host phylogeny (Figures 4.3-4.6). General host and geographic distributions for Elaphostrongylinae, and Varestrongylinae, with inclusive species of *Pneumostrongylus* and *Varestrongylus*, are outlined and form the basis for an examination of history relative to phylogeny (Table 4.1). Further, tanglegrams were used to explore the direct relationships for host and parasite phylogeny and as a basis for postulating a history for development of relationships over time (Figure 4.5, 4.6).

Mapping and optimization of hosts, host groups, and geography on the fully resolved parasite tree with MacClade diagnoses a basal association for Elaphostrongyline + Varestrongylinae among Cervidae and origins in Eurasia (Figures 4.3, 4.4). Host associations within Varestrongylinae are complex, with species of *Pneumostrongylus* occurring among Bovidae and specifically in Aepycerontinae, Antilopinae and Alcelaphinae (and few reports from Hippotraginae) in Africa, whereas species of *Varestrongylus* occur among genera and species of Cervidae and Caprinae across the Holarctic with geographic partitions evident in Eurasia and the Nearctic (Figures 4.3, 4.4). These broader relationships are compatible with initial diversification of Varestrongylines among cervids, and a single episode of host switching to bovids that radiated in Africa accounting for species of *Pneumostrongylus*. Subsequent divergence among species of *Varestrongylus* reflects limited cospeciation, and is dominated by bouts of host switching from cervid to caprine hosts, and among cervids primarily in the Palearctic.

Direct comparison of phylogenetic hypotheses for *Varestrongylus* and pecoran ruminants through a tangle-gram (Figs 4.5, 4.6) reveals considerable incongruence, supporting a history of colonization. Biodiversity within *Varestrongylus* is limited to hosts of two families, with

Cervidae represented by Cervinae (Cervini, but not Muntiacini) and Capreolinae/Odocoileinae (Odocoileini, Capreolini, Rangiferini, and Alceini) and Bovidae represented only by Caprinae (primarily with Caprini and Rupicaprini, and secondarily Ovibovini). Four of the ten species of *Varestrongylus* have been reported from field-based collections involving free-ranging populations of multiple host species. Overall patterns support a speciation process linked to host-switching among Cervidae and Caprinae.

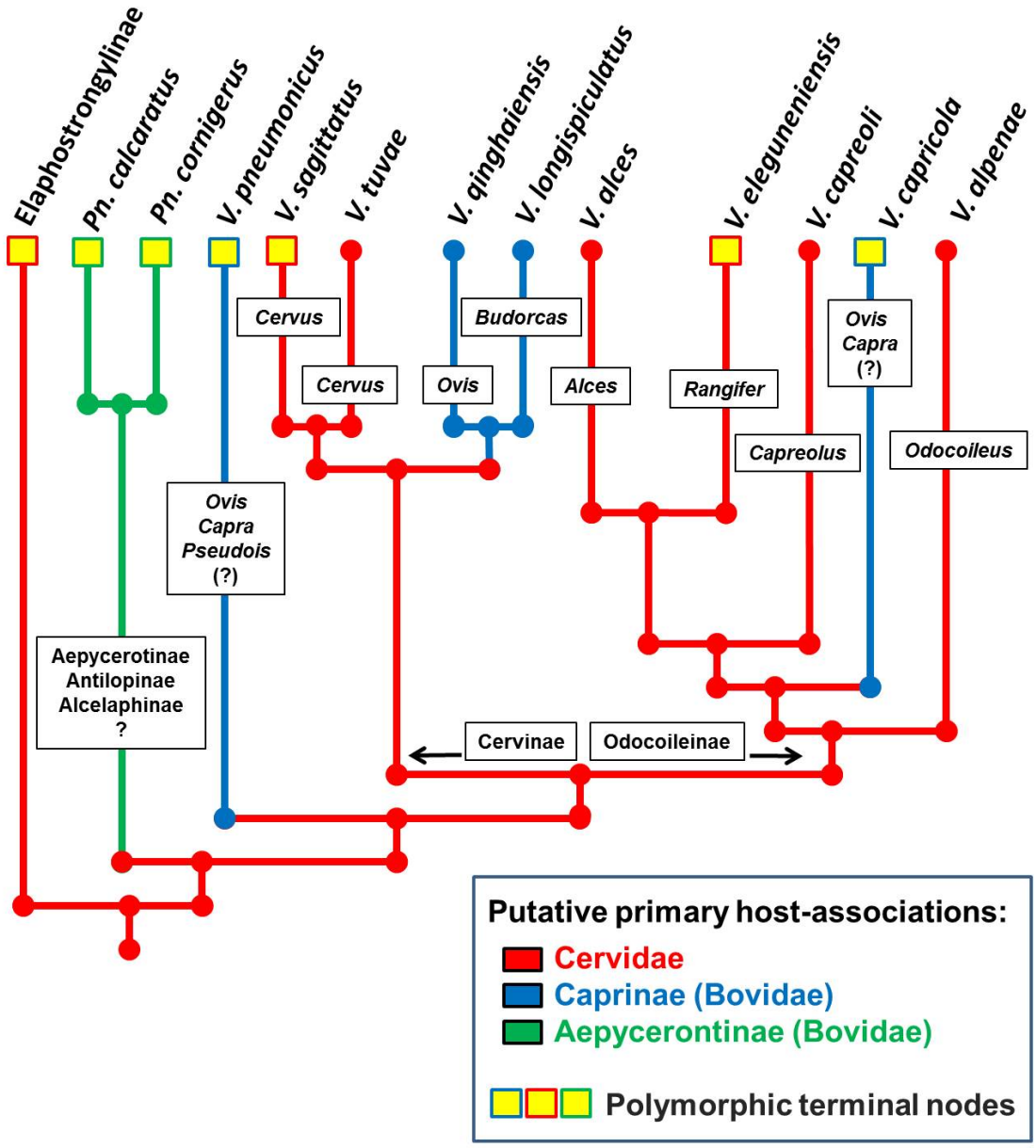


**Figure 4.3.** Phylogenetic hypothesis for *Varestrongylus* Bhalerao, 1932 species, showing distribution of definitive host associations on the parasite cladogram (CI = 0.672, 61 steps). Note the Cervidae ancestral association of Varestrongylinae, considering Elaphostrongylinae as its sister-group, and *Varestrongylus*.

In summary, *Varestrongylus* has origins in the Palearctic and an ancestral association with Cervidae, but a relationship with particular subfamilies cannot be specified unequivocally. In this context, *V. pneumonicus* represents the outcome of a host-switch to caprines although the ancestral hosts, among species of *Ovis*, *Capra* and *Pseudois*, remain undetermined.

Subsequent radiation occurred within an assemblage of cervids resulting in origins of 2 primary subclades (Figures 4.1-4.4). Subclade I is formed by the Cervinae-associated *V. sagittatus* + *V. tuvae* as parasites in species of *Cervus* (Cervini), and a branch formed by *V. qinghaiensis* + *V. longispiculatus* that represents host colonization of Caprinae (*Ovis* (Caprini) and *Budorcas* (Rupicaprini)) in Eurasia. Subclade II is formed by species primarily associated with Capreolinae (Odocoileinae) cervids (*V. alpenae*, *V. capricola*, *V. capreoli*, and *V. alces* + *V. eleguneniensis*) in Eurasia and the Nearctic Among the 5 species relegated to Subclade II, *V. capricola* occurs in caprines (*Capra* and *Ovis*), indicative of an episode of host colonization. Geographically, two of the caprine-associated species, *V. pneumonicus* and *V. capricola* may also demonstrate a pattern of geographic colonization and expansion from Eurasia into the Indian-Oriental region.

Other terminal nodes (species) are also polymorphic regarding host and/or geographic associations (Figures 4.3, 4.4). *Varestrongylus sagittatus* with a putative primary association with *Cervus elaphus* has colonized at least two other sympatric hosts, within the same subfamily, tribe and/or genus: *Dama dama* and *Cervus nippon*. Another species of *Varestrongylus* primarily associated with a cervid host is *V. eleguneniensis*, which has host-switched within the same subfamily, *Alces americanus gigas* (Capreolinae; Alceini) and to a bovid, *Ovibos moschatus* (Caprinae; Ovibovini) (Figure 4.3). Species distributed in the Nearctic, *V. eleguneniensis* and *V.*

*alpenae*, are more closely related to other *Varestrongylus* and thus may indicate independent episodes of geographic colonization from Eurasia into North America with cervid hosts.

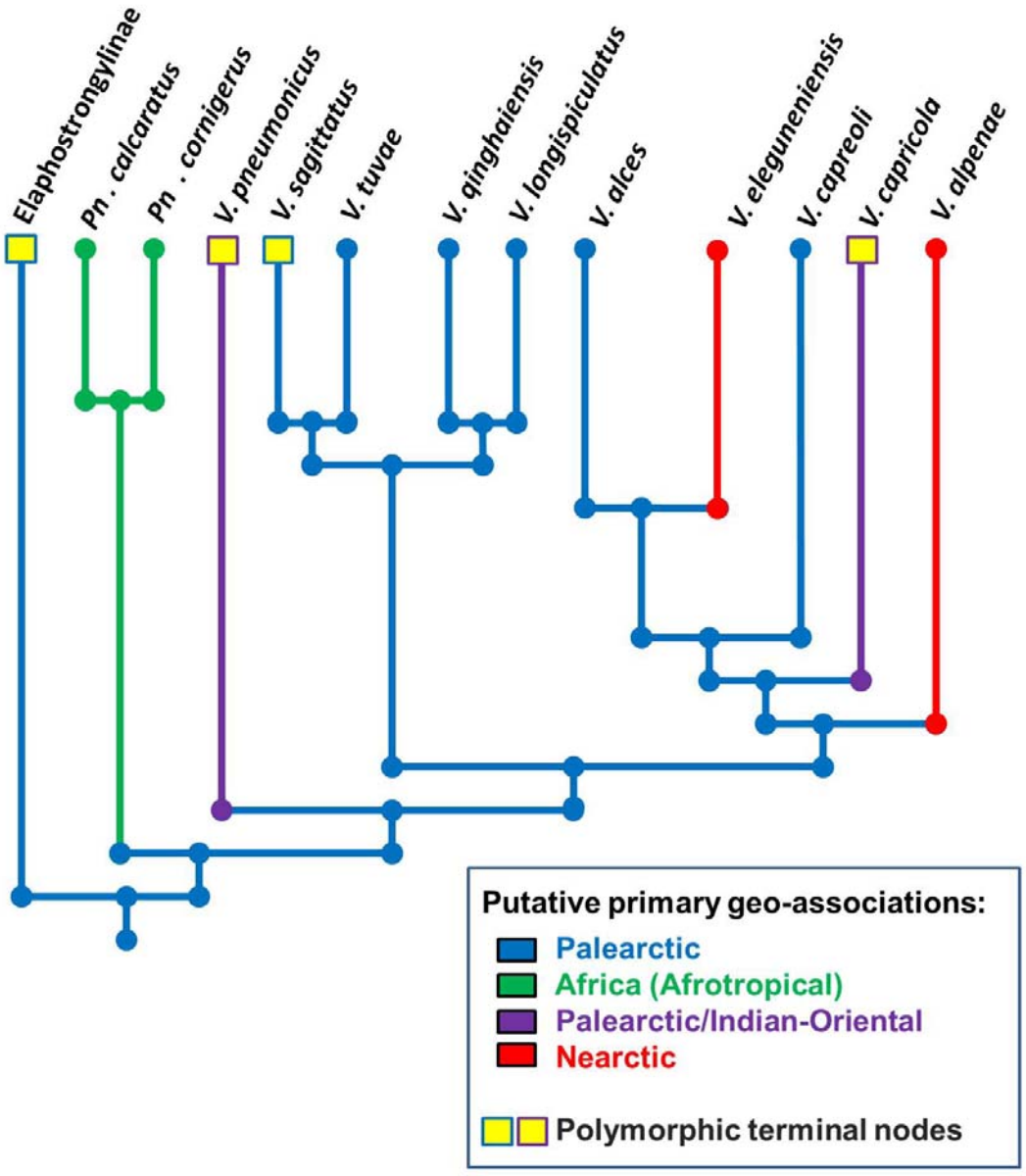


**Figure 4.4.** Phylogenetic hypothesis for *Varestrongylus* Bhalerao, 1932 species, showing their geographic associations on the parasite cladogram (CI = 0.672, 61 steps). Note the origins of Varestrongylinae and *Varestrongylus* within Eurasia, with subsequent events of expansion into the African, the Indian (2 independent events), and the Neartcic (2 independent events) biogeographic ecozones.

## 4.5 Discussion

### *4.5.1 Phylogenetic diagnosis for* Varestrongylus

Phylogenetic analyses of morphological attributes among species of *Varestrongylus* and *Pneumostrongylus* consistently diagnose monophyly for Varestrongylinae. Morphological attributes considered in the current analysis further support recognition of Varestrongylinae, with exclusion of *Pneumocaulus*, from the subfamily as advocated by Carreno and Hoberg (1999). Phylogenetic reconstruction among protostrongylids based on morphological or molecular character data have supported a putative sister relationship of Varestrongylinae with Elaphostrongylinae + Muelleriinae (Carreno and Hoberg, 1999; Verocai et al., 2014a). Further, Protostrongylinae are generally considered among crown groups of the Protostrongylidae (Carreno and Hoberg, 1999) or as a sister-group of Muelleriinae within a clade that includes Elaphostrongylinae (Carreno et al. 2012), although some number of generic level taxa and putative subfamilies remain absent from most analyses. These relationships, confirmed in the current study, constituted the basis for identifying near and distant outgroups to explore relationships for the Varestrongylinae and *Varestrongylus* in the current analyses.

Monophyly for *Varestrongylus* and *Pneumostrongylus* is corroborated by the current analysis. Our results further support the validity of the former genus, encompassing all six species listed by Boev (1975) plus the four additional species that have been described or resurrected thereafter. The present analysis focusing on species relationships among *Varestrongylus* contrasts with a more inclusive reconstruction of the Protostrongylidae in which *Varestrongylus* was considered paraphyletic (Carreno and Hoberg, 1999). These authors,

included three species of *Varestrongylus*, considered in the context of the family Protostrongylidae, placing *Pn. calcaratus* within a subclade diagnosing the former genus.

Monophyly for *Varestrongylus* is consistent with the taxonomic revisions by Boev (Boev, 1968, 1975). These taxonomic proposals reduced 3 genera as junior synonyms of *Varestrongylus* (e.g., *Leptostrongylus* Dougherty & Goble, 1946; *Bicaulus* Schulz & Boev, 1940; *Capreocaulus* Schulz & Kadenazy, 1948) a conclusion consistent with the species-level phylogeny. Several valid species of *Varestrongylus* had at various times been allocated in these three genera. For example, *Leptostrongylus* had been proposed for *V. capreoli* and *V. alpenae* based on the elongate nature of the dorsal ray, an attribute not typical of the former species (Dougherty and Goble, 1946). In the present analysis, however, this character was shown to be present in most species in Subclade II - *V. alpenae, V. eleguneniensis, V. alces*; but also shared by *V. longispiculatus* from Subclade I. Further, species once referred to *Bicaulus* (*V. tuvae*, *V. alces, V. capricola, V. pneumonicus= B. schulzi*) are not an inclusive monophyletic group and are similarly distributed in both subclades. Morphological characters used to distinguished *Bicaulus* (e.g., obtusely round proximal end of gubernaculum, large dentate crura plates, short dorsal ray) were later accommodated within the relatively broad variations for *Varestrongylus* in the generic diagnosis provided in Boev (1975), and Anderson (2000). More recently, the confusing taxonomy of *Varestrongylus* and the broad morphological variation among species in conjunction with the addition of new species in the last decade made necessary an emendation of the diagnosis for the genus by Verocai et al. (2014b).

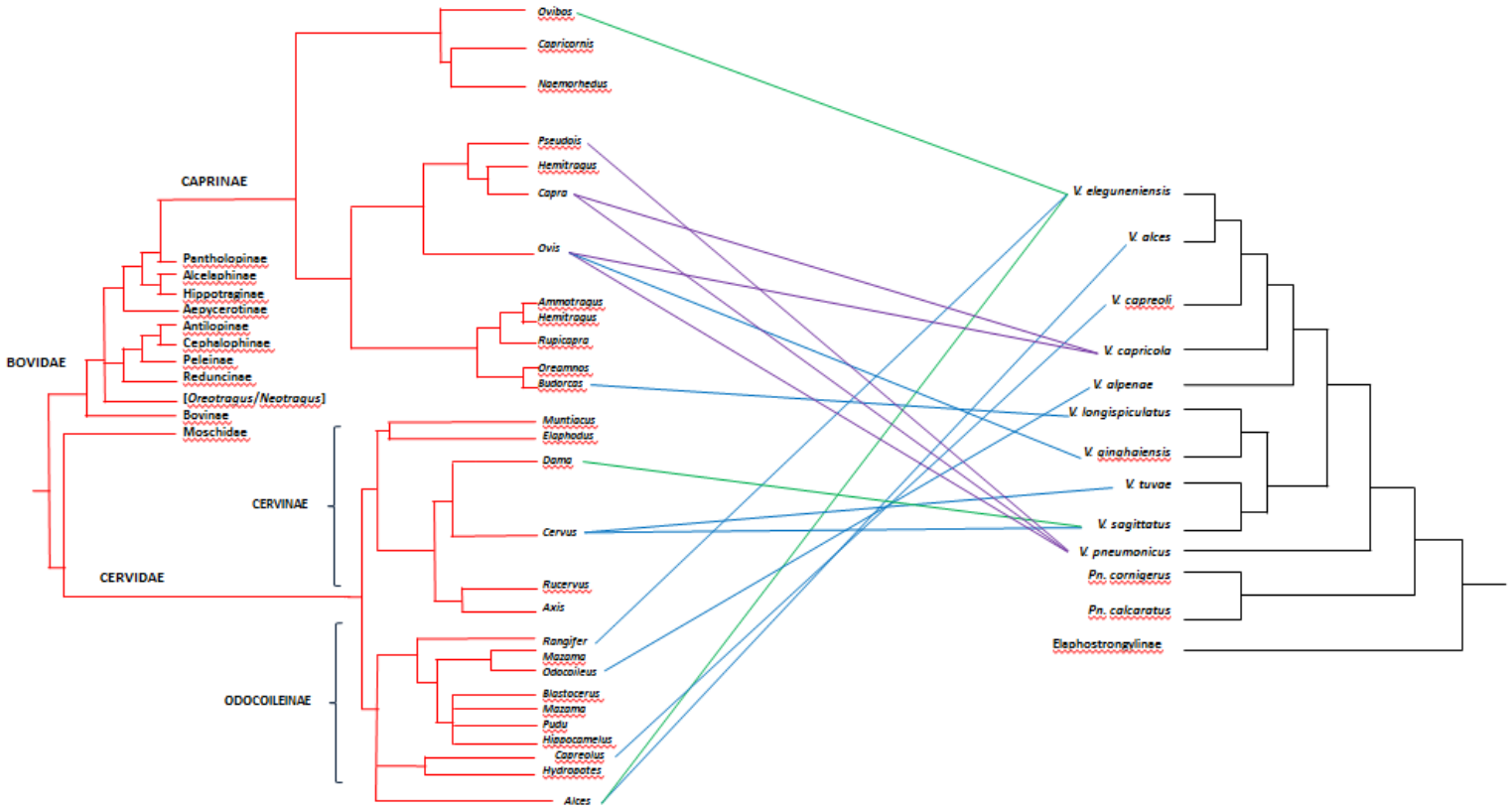


**Figure 4.5.** Tangle-gram comparing the phylogeny of *Varestrongylus* (Figure 4.1), and that of ungulate hosts at generic level, depicting host-associations of *Varestrongylus species*, where blue lines indicate well-supported primary associations, purple lines indicate ambiguous primary associations, and green lines indicate secondary associations (host-colonization). High hierarchy host relationships of hosts follow Hernandez-Fernandez and Vrba (2005). Fine-scale relationships of genera within Cervidae follow Gilbert et al. (2006), and within Caprinae follow Yang et al. (2013).

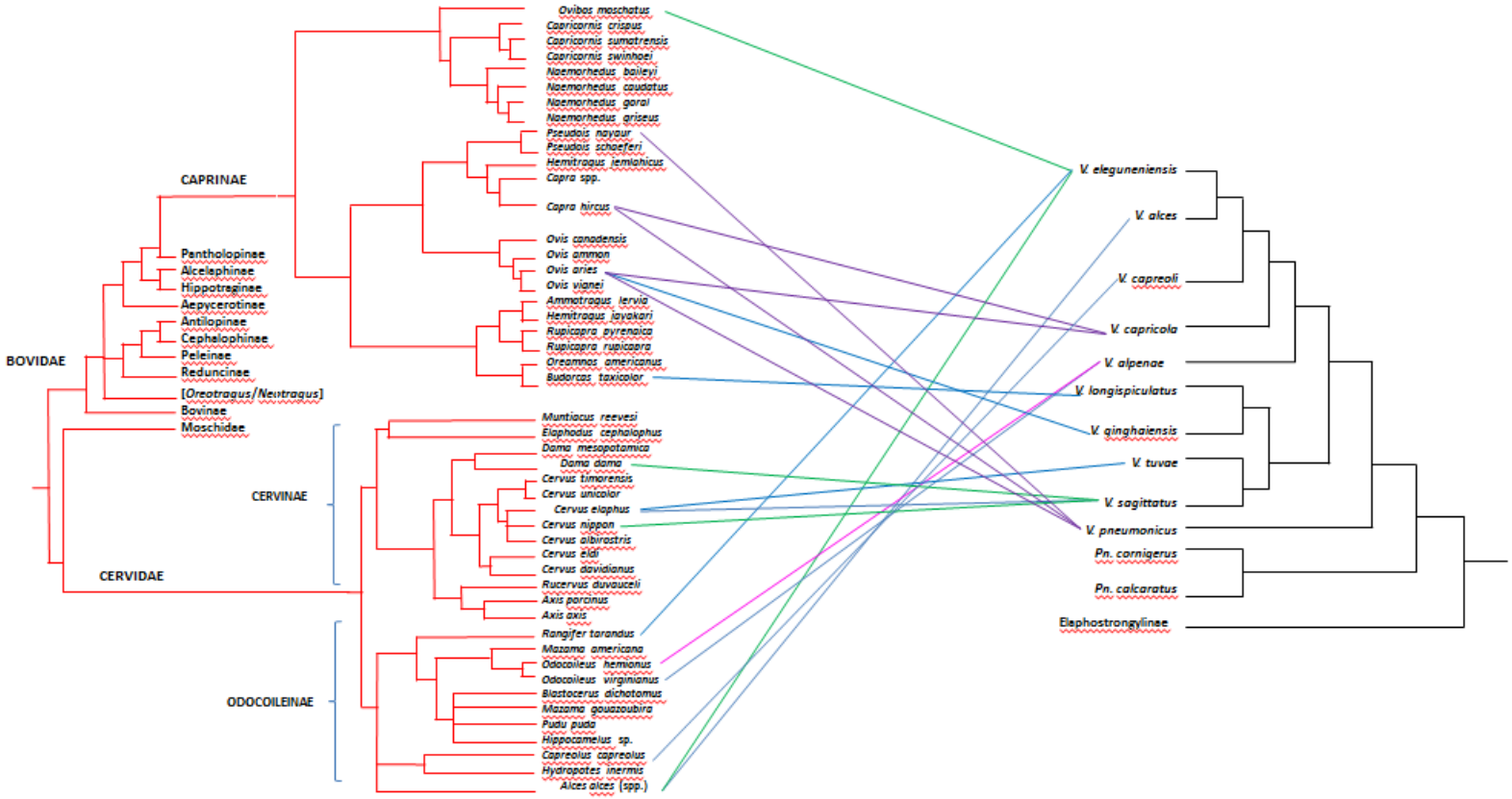


**Figure 4.6.** Tangle-gram comparing the phylogeny of *Varestrongylus* (Figure 4.1), and that of ungulate hosts at species level, depicting host-associations of *Varestrongylus species*. Blue lines indicate well-supported primary associations, purple lines indicate ambiguous primary associations, green lines indicate secondary associations (host-colonization), and the pink line indicates successful established of patent infection experimentally. High hierarchy host relationships of hosts follow Hernandez-Fernandez and Vrba (2005). Fine-scale relationships of genera within Cervidae follow Gilbert et al. (2006), and within Caprinae follow Yang et al. (2013).

### *4.5.2 Historical biogeography and host associations of Varestrongylinae*

An ancestral host-association of the Varestrongylinae among cervids appears corroborated (Carreno and Hoberg, 1999; Carreno and Lankester, 1994; Hoberg et al., 2012b) (Figures 4.1-4.4). Initial diversification of Varestrongylinae among Cervidae in Eurasia appears coincidental with the center of diversification of this ruminant group, which emerged around 20 Ma during the early Miocene (Hernández-Fernández and Vrba, 2005).

The sister of *Varestrongylus*, *Pneumostrongylus* reflects diversification following a single event of host colonization from cervids to antelopes and related bovids within Eurasia in the early Miocene, prior to their expansion into Africa; geographic colonization in Africa was initiated in the middle Miocene and extended into the Pliocene from 16 to 3 Ma (Gentry, 2000a, b; Hernández-Fernández and Vrba, 2005). The current broad host ranges among the species of *Pneumostrongylus* are similar to those described for the abomasal parasites *Haemonchus* spp. among African ruminants. In both cases, rich biodiversity of sympatric ungulates, the overlap of ecological niches among these grazing species and drivers related to climate shifts and habitat disruption may have served as determinants for episodes of host colonization (Hoberg and Brooks, 2008; Hoberg et al., 2004).

In contrast to *Pneumostrongylus*, diversity within *Varestrongylus* is considerably greater, although the history for diversification also appears strongly linked to episodic processes related to climate and environmental change during the late Miocene through the Quaternary (Figures 4.1-4.6). Species of *Varestrongylus* have complex histories with respect to host and geographic associations: (1) *Varestrongylus* has origins in Eurasia with radiation coinciding with episodic and independent events of geographic colonization into adjacent ecozones; (2) cervids are ancestral hosts; (3) the caprine-associated *V. pneumonicus* is basal and can be linked to independent host-switching from cervids; (4) secondary diversification, emerging from

sequential and independent events of host switching, occurred within cervids (*V. sagittatus* + *V. tuvae; V. alpenae; V. capreoli*, and *V. alces* +*V. eleguneniensis*); (5) at least two additional host-switching events into caprines occurred in Eurasia (*V. qinghaiensis* + *V. longispiculatus; V. capricola*, respectively); (6) events of expansion and geographic colonization into the Indian-Oriental region may account for the distributions of *V. pneumonicus* and *V. capricola*; and (7) two independent events of geographic expansion into North America with cervids in the late Pliocene and early Pleistocene are postulated (*V. alpenae*, *V. eleguneniensis*), the latter concordant with existing molecular evidence (Verocai et al., 2014a).

### *4.5.3 A Detailed History of Varestrongylus*

Mapping the current host-associations and biogeography of *Varestrongylus* species onto our phylogenetic hypothesis for the genus unequivocally revealed an ancestral association with cervid hosts in the Palearctic corroborating previous hypotheses for coevolution and biogeography (Carreno and Hoberg, 1999; Hoberg et al., 2012b). Thus, the geographic origin and centre of diversification for the genus *Varestrongylus* coincide with the centre of diversity for their primary cervid and secondary caprine hosts during the Miocene (Hoberg et al., 2012b; Kurtén and Anderson, 1980; Pitra et al., 2004; Prothero and Foss, 2007). The Palearctic origin of *Varestrongylus* is further supported by considerably greater species-richness which characterizes the Eurasian fauna relative to diversity recognized in the Nearctic (Boev, 1975; Hoberg et al., 2012b; Verocai et al., 2014a; Verocai et al., 2014b; present work).

Based on a taxon-limited analysis, Carreno and Hoberg (1999) postulated at least one host-switching event into Caprinae from Cervidae within *Varestrongylus*. Inclusion of all valid species of *Varestrongylus* in the analysis revealed a more complex and nuanced history of host

and geographic-association (Figures-4.3-4.6). A hypothesis constrained by tree topology indicates at least three independent events of colonization and subsequent parasite speciation among Caprinae over the evolutionary history of this ungulate group: (1) the basal species, *V. pneumonicus*; (2) in Subclade I, *V. qinghaiensis* + *V. longispiculatus*; (3) in Subclade II, *V. capricola*. Diversification within Caprinae extends to approximately 14.5 Ma, in the middle Miocene, and the emergence of the tribes Caprini and Rupicaprini, around 11.3 and 8.8 Ma, respectively (Hernández-Fernández and Vrba, 2005). Nearctic species within both of these caprine tribes lack primary or secondary associations with *Varestrongylus* species (but do have associations with *Protostrongylus* and *Parelaphostrongylus*) (see Kutz et al., 2012a).

Temporal limits and duration for *Varestrongylus* may extend to the early Miocene (or late Oligocene) coincidental with initial radiation of cervids (Gilbert et al., 2006; Hernández-Fernández and Vrba, 2005), a hypothesis that requires examination within the broader context of radiation for the Protostrongylidae. Subsequent host colonization from a cervid source into caprines with parasite speciation during the middle or late Miocene may account for the origin of *V. pneumonicus* (Figures 4.3, 4.4). This parasite is associated with three caprine genera, suggesting that multiple but temporally shallow events of colonization (i.e., not associated with speciation) have occurred among an assemblage of sympatric hosts. Additionally, the current biogeography of *V. pneumonicus* and *V. capricola* (Table 4.1) indicates a contemporary range in the Indian ecoregion (type locality for the former) and areas of the Palearctic (e.g., Kazakhstan and China); a Palearctic origin of the genus may constitute evidence for geographic expansion into the Indian subcontinent (Figures 4.3, 4.4). Host-switching and subsequent parasite speciation in carpines is also evident in the distributions of *V. qinghaiensis* and *V. longispiculatus* which occur in Rupicaprini and Caprini from China (Figures 4.3, 4.4).

### *4.5.4 Further Explorations of Subclade I*

Diversification occurred in the Palearctic among cervids during the middle Miocene, where both *Varestrongylus* subclades appeared, coincidental with the emergence and radiation of the Cervinae and Odocoileinae (Gilbert et al., 2006; Hernández-Fernández and Vrba, 2005) (Figures 4.1-4.6). In Subclade I, *V. sagittatus* and *V. tuvae* are sister-species primarily associated with *Cervus* (Cervinae; Cervini) and the former, has a wide range extending from Europe to western Eurasia (Palearctic). *Varestrongylus sagittatus* appears primarily associated with red deer (*Cervus elaphus*) but also occurs in fallow deer (*D. dama*) and sika deer (*C. nippon*). Historically, both red and fallow deer were present across Europe in glacial times; however, the range of the latter species became restricted in the Middle-East and Eastern Mediteranean region of Europe in the early Holocene (Chapman and Chapman, 1980). Distributional data suggest that populations of *V. sagittatus* in fallow and sika deer constitute contemporary events of colonization from red deer related to anthropogenic translocation and establishment. Numerous reports of this lungworm in red deer, in areas where fallow or sika deer are absent (e.g., Russia and Kazakhstan), may support this contention. Archaeological data indicate that fallow deer were reintroduced by the Romans and possibly other European peoples and spread across the continent through the centuries (Masseti et al., 2006; Sykes et al., 2011), becoming once again sympatric with red deer populations. Further studies in regions of sympatry or allopatry between red deer and fallow deer could assist in elucidating primary host associations and direction of colonization events. Nevertheless, the association of *V. sagittatus* with both red and fallow deer may represent an early example of human-mediated host colonization by a parasite, followed by anthropogenic translocation. Sympatry of red and sika deer is rather recent and due to introduction of later cervid for hunting or farming purposes in regions of Europe, where

currently there are a few free-living populations, and hybridized ones (Borkovcová et al., 2009; McDevitt et al., 2009a).

At present, *V. sagittatus* can also be found in the Australian ecoregion. The parasite was introduced to New Zealand coincidental with several host translocations initially for hunting purposes in the mid-1800's and more recently, for farming purposes and is an important agro-economic activity in the country (Hunnam, 2011; Mason, 1994). Interestingly, the main source of red deer (but also fallow deer and some other species) in New Zealand were parks in England and feral populations in Scotland (reviewed in Hunnam, 2011), from where there are no reports of *V. sagittatus.* This lack of data on parasite distribution might be due to the veterinary relevance of the elaphostrongyline *Elaphostrongylus cervi* Cameron, 1931 reported from the United Kingdom (Cameron, 1931) and New Zealand (Mason, 1994). Such a situation may parallel the obscurity of *V. alces* in areas of symparty with *Elaphostrongylus alces* Steen, Chabaud & Rehbinder, 1989 in Eurasian elk (moose) as suggested by Verocai et al. (2014a). Nevertheless, the presence of *V. sagittatus* in New Zealand suggests a great capacity for invasion and establishment of this species in a new environment, potentially associated with ecological fitting and multi-level host switching requiring the presence of suitable gastropod intermediate hosts (either native or introduced species) (Hoberg and Brooks, 2015). To date, this seems to be the only instance in which a species of *Varestrongylus* has been introduced and established along with its host into a new continent (and ecozone).

Absence of broad-based data for the distribution of *V. tuvae*, only reported from Eastern Eurasia in Siberian Wapiti (*Cervus canadensis*), limits our understanding of its associations. A more extensive range might be anticipated across eastern Siberia, Mongolia and northern China within the taxonomically confusing *Cervus*-group in eastern Eurasia (Ludt et al. 2004, Pitra et al.

2004; Groves, 2006). Data from comprehensive survey, however, are not available and *V. tuvae* has not been demonstrated among any other species of *Cervus* or among species of related genera of Cervini (e.g., Pryadko, 1976). *Varestrongylus tuvae* apparently failed to colonize North America during the Pleistocene, as subspecies of *C. canadensis* in the Nearctic are not primarily associated with any protostrongylid species (Anderson, 2000; Boev, 1975).

Aside from the cervid parasites in Subclade I, two sister-species, *V. qinghaiensis* and *V. longispiculatus,* are associated with host switching and possible geographic expansion (Figures 4.3, 4.4). Host-switching and speciation may have occurred within refugia, where Rupicaprini and Caprini were sympatric. Knowledge of the biogeography of both species is limited to their original descriptions, and therefore further research should be carried out to understand their actual geographic and host ranges. Perhaps, *V. qinghaiensis* may also infect domestic sheep or other wild caprines, and *V. longispiculatus* may be associated with other takin subspecies across the mountain ranges that separate the Palearctic and Indian ecotones. Considering the type locality of *V. longispiculatus*, the type host subspecies is likely the golden takin, *B. t. bedfordi* (see Li et al., 2003). Fossil records suggest that *Budorcas* emerged in northern China during the late Pliocene and expanded southwards until the early Pleistocene (Li et al., 2003; Li, 2006; Neas and Hoffmann, 1987). As suggested by the phylogeographic pattern found by Li et al. (2003) the current takin subspecies (*B. t. bedfordi, B. t. taxicolor, B. t. tibetana,* and possibly *B. t. whitei*) resulted from isolation of populations during the post-Pleistocene. Currently, there is no evidence of other takin subspecies serving as hosts for *V. longispiculatus* or any other species of *Varestrongylus*. Further assessments of parasite diversity among takin may be limited as all subspecies are listed as either endangered or vulnerable in the IUCN Red List (Song et al., 2008). However, studies of the health status and parasite biodiversity of these caprines, including host

and geographic ranges of *V. longispiculatus* and its potential impacts, would be a relevant conservation effort.

### *4.5.5 Further Explorations of Subclade II*

Species of Varestrongylus diagnosed in Subclade II include *V. alpenae*, *V. capricola*, *V. capreoli* and *V. alces* + *V. eleguneniensis* (Figures 4.1-4.6); all except *V. capricola* are parasites in cervid hosts (Odocoileinae) (Table 4.1). The only caprine-associated species within this subclade, *V. capricola* may represent a host-switching event from Odocoileinae hosts in sympatry. To date, *V. capricola* has been poorly studied and is only reported in domestic caprines (*Capra hircus* and *Ovis aries*) from the Indian ecozone (Boev, 1975; Sarwar, 1947, 1955). Host-switching between domestic sheep and goat is apparently common and likely anthropogenically mediated. No studies have been performed to assess potential host-association of *V. capricola* with other genera or species within Caprini (*Capra*, *Ovis*, *Pseudois*, *Hemitragus*) in Eurasia and the Indian subcontinent.

Remaining species in this subclade are strongly associated with the Odocoileinae, with geographic distribution limited either to the Palearctic (*V. capreoli*, *V. alces*) or to the Nearctic (*V. alpenae*, *V. eleguneniensis*). Each of the four Odocoileinae-associated species of *Varestrongylus* primarily infects hosts from a different tribe within this subfamily (Alceini, Rangiferini, Capreolini and Odocoileini) (Figures 4.4, 4.6) The crown group of species constituting *V. capreoli*, *V. alces* and *V. eleguneniensis* are morphologically similar and constitute the “small-spicule” forms recognized in *Varestrongylus* in contrast to the structurally discordant *V. alpenae* (Figure 4.1).

A history of cervid lungworms in this subclade is consistent with episodic expansion within Eurasia and from Eurasia into the Nearctic and some level of host switching coinciding with glacial-interglacial cycles extending from the late Pliocene through the Quaternary (Figure 4.4). Temporally circumscribed and independent events of geographic colonization from Eurasia into North America account for the contemporary distributions of *V. alpenae* (limited to *Odocoileus virginianus*) and *V. eleguneniensis* (*Rangifer tarandus* with secondary colonization of *Ovibos moschatus* and *Alces americanus*). The phyogenetic independence of *V. alpenae* and *V. eleguneniensis* and a complex history of geographic colonization and invasion is corroborated by molecular phylogenetic analyses (Verocai et al., 2014a). This history is also strongly concordant with the historical biogeography of the primary host genera (*Odocoileus* and *Rangifer*, respectively) which reached the Nearctic at different times in the late Pliocene, and at least for *Rangifer*, in multiple waves that extended into the Pleistocene (Banfield, 1961; Kurtén and Anderson, 1980; Weckworth et al., 2012).

The two Palearctic species remaining in this subclade, *V. capreoli* and *V. alces* are essentially found in western and central Eurasia, and seem to virtually mirror the current biogeography of their hosts. However, Eurasian moose and Eurasian roe deer populations have substantially oscillated in geography and numbers since the Pleistocene and throughout the Holocene until modern times (Andersen et al., 2004; Niedziałkowska et al., 2014), likely influencing current distributions for parasites. Further discussion on the historical biogeography of *V. alces* and, to a lesser extent *V. capreoli*, can be found in Verocai et al. (2014a). It is also unknown if the Eastern or Siberian roe deer, *Capreolus pygarcus* Pallas, is associated with *V. capreoli* (or any *Varestrongylus*). Nonetheless this would substantially expand the potential geographic range of the species eastwards. Putative cryptic diversity (or a morpho-variant) has

been recently described for males of *V. capreoli* (reported as *V.* cf. *capreoli*) in Eurasian roe deer from Norway, and this matter should be further explored by combined classical and molecular approaches (Verocai et al., 2014a). The relationship among the four Odocoileini-associated species (*V. alces*, *V. eleguneniensis*, *V. capreoli*, and *V. alpenae*) also corroborates the limited phylogenetic information for *Varestrongylus* (Verocai et al., 2014a). In these molecular-based analyses a close relationship is diagnosed among the three small spicule species: *V. alces*, *V. eleguneniensis*, and specimens attributable to *V. capreoli* (as *V.* cf. *capreoli*), with *V. alpenae* as sister to this clade. In this same analysis *V. sagittatus* appeared to be basal to the clade formed by the Odocoileini-associated species, in the absence of genetic data for broader diversity in *Varestrongylus*.

Historical biogeographic hypotheses for *V. alces*, *V. capreoli* and the crown sister-species were investigated and discussed in Verocai et al. (2014a). Only *Alces* from the Eastern Palearctic are hosts for *V. alces* (Demidova and Naumitscheva, 1953; Dróżdż, 1966; Handeland and Gibbons, 2001; Järvis, 1995; Nilsson, 1971; Petrosyan, 1963; Stéen et al., 1998; Stuve, 1986, 1987; Verocai et al., 2014a), with no reports from the Western Palearctic or the Neartic, where the genus entered and established only around the late Pleistocene/Holocene boundary (Hoberg et al., 2012b; Hundertmark et al., 2003; Hundertmark et al., 2002; Kurtén and Anderson, 1980). This is supported by the East/West phylogeographic pattern for the host genus, often separated in two species: *A. alces* and *A. americanus*, from Eastern Siberia China and North America, consistent with a complex history (Hundertmark et al., 2003; Hundertmark et al., 2002; Niedziałkowska et al., 2014). The current range of *V. alces* seems to be directly associated with the northeastward range shift of its host within Eurasia over the past 3Ma over the Quaternary (Breda and Marchetti, 2005).

### *4.5.6 Contemporary Host-associations*

*Varestrongylus* species are commonly exclusively associated with a single host species or multiple hosts within the same taxonomic group (i.e., Caprinae and Cervidae). The most notable exception, *Varestrongylus eleguneniensis*, is the only species that unequivocally infects both cervid and caprine hosts (Kutz et al., 2007; Kutz et al., 2013a; Kutz et al., 2012a; Verocai et al., 2014b). Our results support the primary association of this species with *R. tarandus*, with multiple events of colonization of sympatric *Ovibos moschatus* and *Alces americanus* (Kutz et al., 2007; Verocai et al., 2014b). Independent events driving colonization of *O. moschatus* include anthropogenic episodes following translocation and reintroduction (Kutz et al., 2007; Verocai et al., 2014b), and climate-mediated parasite establishment into new regions with subsequent host colonization (Hoberg and Brooks, 2015; Kutz et al., 2007; Kutz et al., 2013a; Verocai et al., 2014b).

The biogeography of caprine-associated species (*V. pneumonicus*, *V. capricola*, and also *V. qinghaiensis*), as for many other nematodes associated with domestic animals, has been influenced by continuing anthropogenic translocation of hosts and parasites. Human-related geographic colonization for assemblages of hosts and parasites extends from near 11 ka, coinciding with the development of agriculture and domestication of caprines in the Fertile Crescent, to the present (Driscoll et al., 2009; Hoberg, 2010; Hoberg and Brooks, 2013). Translocations, introductions and establishment have been idiosyncratic in contributing to the mosaic assembly of ungulate parasite faunas (Hoberg, 2010; Hoberg and Brooks, 2013). These species of *Varestrongylus* apparently did not spread to Europe or North America along with other protostrongylids (*Muellerius capillaris*, *Protostrongylus rufescens*) (Cardoso and Oliveira, 1993; Duarte and Miranda, 1984; Ezenwa et al., 2010; Pybus and Shave, 1984) and strongylids

(Hoberg, 2010; Hoberg et al., 2004; Vicente et al., 1997) among domestic caprines. Relatively restricted distributions for *V. pneumonicus, V. capricola,* and *V. qinghaiensis* are compatible with an archaic association among relictual caprine populations isolated in refugia in Central and/or South Asia.

Reports of *V. capreoli* infecting wild and domestic caprines in Europe remain dubious and require further confirmation (e.g., mouflon (*Ovis musimon*) in Czech Republic (Erhardova, 1957) and domestic goat in Switzerland (Bouvier and Hörning, 1963). Verocai et al. (2014a) found morphological variance within *V. capreoli* males of Norway in comparison to other descriptions (Boev, 1975; Stroh and Schmid, 1938), and discussed the potential existence of a species complex, but further integrated classical and molecular studies remain necessary. Other multi-host *Varestrongylus* species have a more phylogenetic or restricted host-range, infecting sympatric genera or species within a single tribe.

### *4.5.7 Faunal structure and history*

The historical biogeography of the subfamily Varestrongylinae and the genus *Varestrongylus* reflect the generalities of faunal assembly and mosaic structure for phylogenetically and temporally disparate host and parasite taxa at macroevolutionary scales across the Holarctic (Hoberg and Brooks, 2008; Hoberg and Brooks, 2010, 2013; Hoberg et al., 2012b). These complex histories that shaped contemporary mosaic faunas involved episodic geographic expansion, isolation and in some cases, shifts and reduction in ranges, determined by climatic fluctuations during the glaciations of the late Pliocene and Pleistocene (Hoberg and Brooks, 2015; Hoberg et al., 2012b). Specific for the genera within Varestrongylinae, expansion events for host-parasite assemblages extended to the Afrotropical ecozone for *Pneumostrongylus*, and the Indian and Nearctic ecozones for members of *Varestrongylus*.

More specifically, the history of *Varestrongylus* is largely concordant with that defined across an array of parasite taxa associated with assemblages of pecoran ruminants and other mammalian groups (e.g., Lagomorphs, Rodents, Carnivorans). For instance, an ancestral center of origin and diversity within Eurasia, with subsequent expansion into other regions at different timeframes is supported by the downstream gradient of biodiversity between the Palearctic and Nearctic (e.g., Hoberg et al., 2012b). This is also valid for other Metastrongylina, but also strongylids within Trichostrongylina (Ostertagiinae and Nematodirinae) (Carreno and Hoberg, 1999; Hoberg, 2005; Hoberg et al., 2012b; Hoberg et al., 2005), among other metazoan parasites. Like many of these parasite genera, *Varestrongylus* exhibits a Holarctic distribution (Boev, 1975; Hoberg et al., 2012b; Verocai et al., 2014a; Verocai et al., 2014b). Comparing histories of protostrongylid genera of different subfamilies, only *Protostrongylus*, the most diverse protostrongylid genus, has a contemporary distribution across the Holarctic with historical expansions into Africa (this without considering contemporary parasite movement with domestic hosts). Among the Muelleriinae and Elaphostrongylinae neither genus nor species naturally occur in both the Palearctic and the Nearctic, also apart from anthropogenic introductions (e.g. *M. capillaris* in the Americas and *E. rangiferi* in Newfoundland) (Hoberg et al., 2012b). Much of the contemporary Nearctic fauna was established in relatively shallow evolutionary time during the Pliocene and throughout the Pleistocene (Hoberg et al., 2012b; Shafer et al., 2010; Waltari et al., 2007). This faunal assembly that includes mammals within Carnivora, Lagomorpha, Rodentia, and Artiodactyls underwent waves of invasions through the Beringian nexus strongly dictated by climate. Along with their hosts, a diverse collection of parasites became established in North America, and continued the dynamic processes of faunal formation, including periods of expansion and isolation, host-switching, speciation, and extinction. Such Nearctic invasion

included at least two *Varestrongylus* species, *V. eleguneniensis* and *V. alpenae,* or their predecessors, which most likely came with *Rangifer* and *Odocoileus* hosts.

Species of *Varestrongylus* (and other Varestrongylinae) seem to have failed to expand into the Neotropics, in historical or contemporary times, or at least were not able to establish and persist until the present in South American cervids, including these of the genera *Blastocerus* and *Mazama*. In contrast, within Protostrongylidae, *P. tenuis* has been reported as far south as Costa Rica (Carreno et al., 2001) and at least one putative native elaphostrongyline species occurs in South America (Carreno et al., 2012). Similar to varestrongylines, there have been no reports of species within Protostrongylinae or Muelleriinae in the neotropical ungulate fauna, apart from those introduced with caprine livestock.

### 4.6 Conclusion

Herein, we provided the first comprehensive phylogenetic hypothesis for the genus *Varestrongylus,* a core component of the parasite fauna of pecoran ruminants. Its rich biodiversity and the recent advances in defining its taxonomy, systematics and biogeography suggest that more remains uncovered. Future work should focus on the redescription of poorly described species (such as *V. capricola*), the biology and ecology and the geographic and host range of poorly studied species, and the veterinary importance of those species associated with domestic, game and threatened ungulate hosts. The archival deposition of representative specimens in museum collections is extremely necessary so parasitologists can have access to such materials for future studies (Brooks et al., 2014; Hoberg, 2002; Hoberg et al., 2014; Hoberg et al., 2009). Moreover, the genetic characterization of all valid species lacking molecular information would provide a deeper understanding of relationships among *Varestrongylus*

species and host-parasite coevolution. The combination of robust morphological and molecular data is paramount for critically assessing the outcomes of phylogenetic inference presented in the current analyses. A clear or unequivocal definition of species diversity and relationships are the necessary foundation for developing robust and testable hypotheses for historical biogeography among Caprinae and Cervidae. A more comprehensive picture of past and current trends on *Varestrongylus*-ungulate assemblages are an instrument for better understanding faunal formation and host parasite coevolution, and can also be used in prospecting for parasite biodiversity and in targeted survey for novel and cryptic species. Robust definitions of diversity are in part the baselines that are necessary to recognize and anticipate the effects of accelerating climate and environmental change with the understanding that what we reveal about the past can inform us about processes in the present and the future.

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# Chapter Five: **THE BIOGEOGRAPHY OF THE CARIBOU LUNGWORM, *VARESTRONGYLUS ELEGUNENIENSIS* (NEMATODA: PROTOSTRONGYLIDAE): THE PRESENT AS A REFLECTION OF THE PAST, AND AN INDICATOR OF AN UNCERTAIN FUTURE**

## 5.1 Abstract

*Varestrongylus eleguneniensis* (Nematoda; Protostrongylidae) is a recently described lungworm species that infects caribou (*Rangifer tarandus* sspp.), muskox (*Ovibos moschatus* sspp.) and moose (*Alces americanus* sspp.) across Northern North America. Initially, the species was characterized molecularly based on larvae recovered from feces of these ungulates at high latitudes of the continent's mainland, and was known to occupy a wide range extending from Alaska (AK), USA, to the Labrador Peninsula, Canada. Further work found the species in a low Arctic island and also in the boreal forest of Alberta (AB), Canada, extending its already vast distribution. My objective was to better define the distribution of this species, through geographically extensive parasitological assessment of caribou, muskox and moose fecal samples. Herein, I further explore the geographic distribution of the caribou lungworm, *V. eleguneniensis*, through geographically extensive sampling of its three host species and discuss its present biogeography. I analyzed caribou samples (n=1,525) of three subspecies: woodland (*R. t. caribou*), Grant's (*R. t. granti*), and barrenground (*R. t. groenlandicus*) distributed in across Canada, AK, and Greenland. In addition, we analyzed muskox samples (n=159) from AK, Northwest Territories (NT), Nunavut and Quebec, and moose samples (n=264) from AK, NT and AB. The presence of lungworm larvae in fecal samples was assessed by the modified beaker Baermann technique. Species identity of individual larvae was determined by PCR and sequencing of the ITS-2 region of the nuclear ribosomal DNA or the COI region of the mitochondrial DNA. *Varestrongylus eleguneniensis* is widely distributed across caribou range in North America, and infect sympatric muskoxen and, rarely, moose. Overall, 364 (24.5%) caribou, 117 (73.6%) muskoxen and 9 (3.7%) moose samples were positive for protostrongylid DSL. A total of 74 DSL of 44 caribou samples from 30 populations were identified as *V.*

*eleguneniensis*, whereas 238 DSL of 94 caribou samples from 37 herds were identified as *P. andersoni*. *Elaphostrongylus rangiferi* was the only species found in Newfoundland caribou. Muskoxen introduced to AK and QC were infected by *V. eleguneniensis*. Only a single Alaskan moose was found positive for *V. eleguneniensis*, but moose from AK (n=4), NT (n=1), and AB (n=1) were positive for *P. andersoni*. This study provides a substantial expansion of the knowledge of the distribution and biogeography of *V. eleguneniensis* and may serve as a strong baseline for monitoring current range and predicting future biogeographic scenarios under accelerating perturbation.

## 5.2 Introduction

The biogeography of a parasite species is directly influenced by the distribution of its host (definitive and intermediate) species, and as such is the product of complex processes that have occurred through space and time. Climatic fluctuations, for example, are among the primary drivers that have shaped current host biodiversity and have served as determinants of presently recognized host-parasite associations (Hoberg, 2010; Hoberg and Brooks, 2008; 2013, 2015). The dynamic processes driving host-parasite associations and biogeography are not only part of history, but continue to act across the biosphere today (Altizer et al., 2013; Hoberg et al., 2012b; Kutz et al., 2012a; Kutz et al., 2009; Parmesan and Yohe, 2003). In fact, processes that took millennia or thousands of years are now occurring at a much faster pace in a world influenced by direct and indirect anthropogenic drivers, including climatic perturbations, landscape modifications and animal movement, impacting both host and parasite biogeography (Hoberg and Brooks, 2008; Hoberg and Brooks, 2010, 2015). Understanding of processes that formed host-parasite associations and biogeography in evolutionary and ecological time provides a pathway to explore the implications of accelerating environmental perturbation today (Hoberg and Brooks, 2015; Hoberg et al., 2012b; Kutz et al., 2014).

Caribou (*Rangifer tarandus* sspp.) is an iconic species in North America, where it ranges from the boreal forests of Canada and USA to the islands of the High Arctic, and Greenland. Across the continent, numerous caribou populations of different subspecies and ecotypes are facing declines, and their recovery and sustainability are uncertain (Gustine et al., 2014; Hervieux et al., 2013; Vors and Boyce, 2009), raising serious concerns on the species conservation and long-term persistence (COSEWIC, 2011; Festa-Bianchet et al., 2011). Despite

the conservation importance of caribou, parasite biodiversity in these keystone ungulates has been relatively understudied (reviewed in Kutz et al., 2012a).

Among parasites in caribou, nematodes of the Family Protostrongylidae are of concern as potential pathogens that can influence morbidity, mortality and host demographics (Kutz et al., 2012a; Lankester, 2001). Depending on the species, adult protostrongylids inhabit the respiratory tract, skeletal muscles or the central nervous system of their definitive hosts causing parasitic pneumonia and/or debilitating muscular or neurological disease (Anderson, 2000; Kutz et al., 2012a; Lankester, 2001). The recent discovery of a new, wide-spread, protostrongylid lungworm, *Varestrongylus eleguneniensis* Verocai, Kutz, Simard & Hoberg, 2014, has reinforced the need for a comprehensive assessment of protostrongylid biodiversity in caribou as a basis for understanding the complexities of this host-parasite assemblage (Kutz et al., 2007; Kutz et al., 2012a; Verocai et al., 2014b). In addition to caribou, *V. eleguneniensis* also infects muskoxen (*Ovibos moschatus*) and, incidentally, moose (*Alces americanus*) (Kutz et al., 2007; Kutz et al., 2013a; Kutz et al., 2012a; Verocai et al., 2014b). Initially, the parasite was found only at high latitudes of mainland North America (Kutz et al., 2007); however it was also reported from the boreal forests of Canada (Verocai et al., 2014b). Moreover, there is recent evidence of establishment and ongoing northward range expansion on an Arctic Island in a caribou-muskox system (Hoberg and Brooks, 2015; Kutz et al., 2013a). These early advances of knowledge around this lungworm species lead us to further investigate its biogeography at a finer scale focusing on caribou from Alaska to Greenland, but also sympatric muskox and moose populations across the continent.

Herein, I explore the biogeography of the caribou lungworm, *V. eleguneniensis*, through geographically extensive sampling across its primary host range. I discuss the complexities of

this dynamic system in light of historical processes and current trends in host populations, ongoing range shifts, and potential future scenarios. In addition, I also provide substantial information on the biogeography of the muscleworm, *Parelaphostrongylus andersoni* Prestwood, 1972, as well as opportunistic findings on other protostrongylids.

## 5.3 Materials and Methods

### *5.3.1 Sample acquisition*

Fecal samples of caribou (Table 5.1) and muskoxen from across Canada, Alaska and Greenland (Table 5.2) and moose (Table 5.3) from western Canada and Alaska were acquired opportunistically through a wide network of collaborators with research, government, and co-management institutions at regional, provincial, territorial and state levels. Acquisition and use of samples were covered under Animal Care Committee of the Faculty of Veterinary Medicine of the University of Calgary, permit # AC13-0121. For caribou, there has been much controversy in the different classifications of caribou across its range in North America. Therefore, I opted to use the currently accepted subspecies, but also included the 'ecotypes' in which a herd was allocated, according to Festa-Bianchet et al. (2011). The sampling methods varied among sources: tundra collections (by helicopter), capture of animals for collaring and monitoring, capturing animals for translocation purpose, scat detection dogs (e.g.; Wasser et al., 2011), animal observation, and also from animals harvested for research or by aboriginal hunters (e.g.; Kutz et al., 2013b). Where major geographic gaps in sampling existed, increased efforts were made to purposefully collect samples.

### *5.3.2 Fecal analyses*

Fecal samples of all three wild ungulate species were evaluated for the presence of protostrongylid dorsal-spined larvae (DSL) using the beaker Baermann technique (Forrester and Lankester, 1997; Verocai et al., 2013). For caribou and muskoxen, approximately 5g feces were used for each Baermann, and for moose, because a single pellet often weighed up to 5g, we used approximately 5 to 10g. Larvae from each positive host were quantified and stored in water and frozen at -20 ºC.

**Table 5.1.** Total caribou (*Rangifer tarandus* sspp.) fecal samples included in the study: information on subspecies and origin, and Baermann results (DSL prevalence). Molecular identification of DSL was based on sequences of the ITS-2 region of the nuclear ribosomal DNA

| **Caribou subspecies** | **N** | **DSL (%)** | ***V. ele.* (DSL/host)** | ***P. and.* (DSL/host)** |
|---|---|---|---|---|
| ***R. t. granti*** | 203 | 54 (26.6) | 13; 8 | 77; 32 |
| ***R. t. groenlandicus*** | 383 | 96 (25.1) | 10; 8 | 5; 3 |
| ***R. t. caribou*** | 939 | 221* (23.5) | 69; 36 | 228; 81 |
| **Total** | **1,525** | **371 (24.3%)** | **92; 52** | **310; 116** |

* Also animals infected with *Elaphostrongylus rangiferi* (Newfoundland caribou herds) and undertermined *Parelaphostrongylus* species (some British Columbia woodland caribou herds).

**Table 5.2.** Grant's caribou (*Rangifer tarandus granti*) fecal samples included in the study: information on subspecies and origin, and Baermann results (DSL prevalence). Molecular identification of DSL was based on sequences of the ITS-2 region of the nuclear ribosomal DNA

| ***R. t. granti*** | **Ecotype** | **Geographic Range** | **Month, Year** | **N** | **DSL (%)** | ***V. ele.* (DSL/host)** | ***P. and.* (DSL/host)** |
|---|---|---|---|---|---|---|---|
| Western Arctic | Migratory Tundra | AK | Sept, 2007 | 11 | 1 (9) | - | 6; 2 |
| | | | Sept, 2010 | 9 | 2 (22.2) | - | 4; 1 |
| | | | June, 2011 | 1 | 0 | - | - |
| Teshekpuk | Migratory Tundra | AK | June, 2010 | 39 | 10 (25.6) | - | 13; 6 |
| | | | June, 2011 | 21 | 9 (42.9) | 1; 1* | 9; 5* |
| Central Arctic | Migratory Tundra | AK | June, 2010 | 14 | 2 (14.3) | 1; 1 | 6; 1 |
| | | | April, 2011 | 16 | 9 (56.3) | - | 14; 7 |
| Porcupine | Migratory Tundra | AK-YT-NT | Sept, 2008 | 13 | 1 (7.7) | 2; 1 | - |
| | | | Sept, 2009 | 10 | 1 (10) | - | 1; 1 |
| Forty Mile | Mountain | AK-YT | May, 2011 | 8 | 6 | 2; 1 | 8; 4 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Oct, 2011 | 7 | 1 (14.3) | - | - |
| | | | May, 2012 | 14 | 1 (7.1) | - | - |
| Nelchina | Mountain | AK | April, 2011 | 19 | 3 (15.8) | - | 7; 3 |
| | | | Oct, 2011 | 5 | 1 (20) | 1;1* | 1;1* |
| Delta | Mountain | AK | April, 2011 | **13** | 7 (53.8) | 6 ; 3 | 5; 2 |
| Denali | Mountain | AK | March, 2011 | **3** | 1 (33.3) | - | 3 ; 1 |
| White Mountain | Mountain | AK | April, 2011 | **1** | 0 | - | - |
| **TOTAL** | | | | **203** | **54 (26.6)** | **13; 8** | **77; 32** |
| ***R. t. granti*** | | | | | | | |

**Table 5.3.** Barrenground caribou (*Rangifer tarandus groenlandicus*) fecal samples included in the study: information on subspecies and origin, and Baermann results (DSL prevalence). Molecular identification of DSL was based on sequences of the ITS-2 region of the nuclear ribosomal DNA

| ***R. t. groenlandicus*** | **Ecotype** | **Geographic Range** | **Month, Year** | **N** | **DSL (%)** | ***V. ele.* (DSL/host)** | ***P. and.* (DSL/host)** |
|---|---|---|---|---|---|---|---|
| Bluenose West, Tuktoyaktuk Peninsula and Cape Bathurst | Migratory Tundra | NT | May-July, 2009 | 112 | 35 (31.3) | 2; 2 | 4; 2 |
| Bathurst | Migratory Tundra | NT-NU | Aug-Sept, 2008 | 33 | 11 (33.3) | - | - |
| | | | Sept, 2008 | 26 | 1 (4) | - | - |
| | | | Spring, 2009 | 34 | 4 (11.8) | 2; 1 | 1; 1 |
| Bluenose East | Migratory Tundra | NT-NU | Feb, 2009 | 18 | 8 (17.8) | - | - |
| Ahiak | Migratory Tundra | NU | March, 2009 | 36 | 21 (58.3) | 2; 2 | - |
| Beverly-Qamanirjuaq | Migratory Tundra | NT-NU-MB | March, 2009 | 23 | 15 (65.2) | 3; 2 | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Dolphin-and-Union | Migratory Tundra | NU-NT | Aug, 2011 | 8 | 1 (12.5) | 1; 1 | - |
| Akia-Manitsoq | NA | Greenland | March-April, 2008 | 47 | 0 (0) | - | - |
| Kangerlussuaq-Sisimiut | NA | Greenland | March, 2009 | 50 | 0 (0) | - | - |
| **TOTAL** | | | | **383** | **96 (25.1)** | **10; 8** | **5; 3** |
| ***R. t. groenlandicus*** | | | | | | | |

**Table 5.4.** Woodland caribou (*Rangifer tarandus caribou*) fecal samples included in the study: information on subspecies and origin, and Baermann results (DSL prevalence). Molecular identification of DSL was based on sequences of the ITS-2 region of the nuclear ribosomal DNA.

| ***R. t. caribou*** | **Ecotype** | **Geographic Range** | **Month, Year** | **N** | **DSL (%)** | ***V. ele.*** **(DSL/host)** | ***P. and.*** **(DSL/host)** |
|---|---|---|---|---|---|---|---|
| South Nahanni | Mountain | YT/NT | Oct, 2008 | 17 | 2 (12) | 1; 1 | - |
| Aishihik | Mountain | YT | Oct, 2011 | 4 | 0 (0) | - | - |
| Laberge | Mountain | YT | Oct, 2011 | 4 | 0 (0) | - | - |
| Finlayson | Mountain | YT | Oct, 2011 | 2 | 0 (0) | - | - |
| Coal River | Mountain | YT/NT | Oct, 2011 | 2 | 0 (0) | - | - |
| | | | Nov, 2012 | 1 | 1 (100) | 1; 1 | |
| Level-Kawdy | Mountain | BC | Oct, 2011 | 20 | 1 (5) | - | 1; 1 |
| | | | Feb-March, 2012 | 23 | 1 (4.3) | 5; 1 | - |
| | | | March, 2012 | 5 | 0 (0) | - | - |
| | | | April, 2012 | 4 | 0 | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Liard Plateau | Mountain | YT-BC | Dec, 2010 | 15 | 1 (6.7) | 1; 1 | - |
| Telkwa | Mountain | BC | | 5 | 0 | - | - |
| | | | | 5 | 1 (20) [P] | - | - |
| Kennedy Siding | Mountain | BC | | 8 | 1 (12.5) [P] | - | - |
| Moberly | Mountain | BC | March, 2011 | 5 | 0 | - | - |
| | | | March, 2012 | 1 | 1 (100) | 1 ; 1 | - |
| Quintette | Mountain | BC | March, 2010 | 3 | 1 (33) | 1 ; 1 | - |
| | | | March, 2011 | 1 | 0 | - | - |
| Narraway | Mountain | BC-AB | Jan, 2009 | 1 | 0 | - | - |
| | | | Dec, 2009 | 2 | 0 | - | - |
| | | | March, 2010 | 3 | 0 | - | - |
| A La Peche | Mountain | AB | March, 2010 | 5 | 1 (20) | - | - |
| North Jasper | Mountain | AB | July, 2009 | 28 | 2 () | - | - |
| | | | June, 2011 | 12 | 0 | - | - |
| North Banff | Mountain | AB | March, 2009 | 2 | 0 | - | - |
| Bakerville | Mountain | BC | March, 2011 | 18 | 0 | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Purcells South | Mountain | BC | April, 2012 | 1 | 1(100)[P] | - | - |
| Calendar | Boreal Forest | BC | Jan-Feb, 2008 | 16 | 4 (25) | - | 6; 2 |
| | | | March, 2010 | 1 | 0 (0) | - | - |
| Maxhamish | Boreal Forest | BC | March, 2010 | 3 | 0 (0) | - | - |
| Snake-Sahtaneh | Boreal Forest | BC | April, 2008 | 1 | 0 (0) | - | - |
| | | | Nov, 2008 | 6 | 2 (33.3) | - | 8; 2 |
| | | | March, 2009 | 1 | 1 (100) | - | 1; 1 |
| | | | March, 2010 | 4 | 1 (25) | - | - |
| Chinchaga | Boreal Forest | BC-AB | Feb, 2003 | 8 | 5 (62.5) | 2; 1* | 5 ,3* |
| | | | March, 2010 | 3 | 1 (33.3) | - | 2; 1 |
| | | | Feb, 2012 | 3 | 1 (33.3) | - | 4; 1 |
| Little Smoky | Boreal Forest | AB | Nov, 2010 | 1 | 1 (100) | 3;1 | - |
| Slave Lake | Boreal Forest | AB | Feb, 2003 | 2 | 1 (50) | - | 2; 1 |
| Caribou Mountain | Boreal Forest | AB | Feb, 2001 | 9 | 2 (22.2) | - | 4; 1 |
| Red Earth | Boreal Forest | AB | Feb, 2000 | 13 | 2 (15.4) | 3; 1* | 1; 1* |
| | | | Feb, 2004 | 8 | 2 (25) | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| WSAR (not specified) | Boreal Forest | AB | Feb, 2003 | 7 | 0(0) | - | - |
| WSAR - Wabasca | Boreal Forest | AB | Feb, 2000 | 11 | 2 (18.2) | - | 3; 1 |
| ESAR (not specified) | Boreal Forest | AB | Feb, 2001 | 13 | 1 (7.7) | - | - |
| | | | | 5 | 1 (20) | - | 1; 1 |
| ESAR - Algar | Boreal Forest | AB | Feb-March, 2009 | 50 | 10 (20) | 12 ; 6 | - |
| ESAR – Egg Pony | Boreal Forest | AB | Feb-March, 2009 | 48 | 17 (35.4) | 5 ; 3* | 7; 5* |
| Cold Lake | Boreal Forest | AB-SK | Feb, 2001 | 9 | 3 (33.3) | 2; 1 | 2;1 |
| | | | Feb, 2003 | 5 | 1 (20) | - | 2; 1 |
| | | | Feb, 2004 | 4 | 0 (0) | - | - |
| | | | Nov, 2010 | 1 | 1 (100) | 2; 1 | - |
| Smoothstone-Wapeweka | Boreal Forest | SK | Feb, 2013 | 30 | 14 (46.7) | 6; 2 | 18; 4 |
| The Bog | Boreal Forest | MB | Feb, 2007 | 20 | 6 (30) | - | 11; 6 |
| Northern Interlake | Boreal Forest | MB | Jan, 2009 | 30 | 0 (0) | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Norway House | Boreal Forest | MB | Jan, 2012 | 30 | 21 (70) | - | 17 ; 5 [§] |
| Cape Churchill | Migratory Tundra | MB | March, 2010 | 22 | 9 (40.9) | - | 24 ; 5 |
| Pen Island | Migratory Tundra | MB-ON | March, 2012 | 20 | 7 (28.6) | - | 22 ; 6 |
| Auden | Boreal Forest | ON | April, 2010 | 41 | 9 (22) | 5; 3 | 10; 4 |
| | | | April, 2012 | 9 | 3 (33.3) | - | - |
| Pickle Lake | Boreal Forest | ON | April, 2010 | 38 | 5 (13.7) | 2; 1 | 8; 3 |
| | | | April, 2012 | 7 | 0 (0) | - | - |
| Cochrane | Boreal Forest | ON | April, 2010 | 31 | 7 (22.6) | 6; 5 | 5; 2 |
| | | | April, 2012 | 6 | 0 (0) | - | - |
| Manicouagan | Boreal Forest | QC | March, 2009 | 3 | 1 (33.3) | - | 2; 1 |
| | | | April, 2011 | 3 | 0 (0) | - | - |
| Lac Joseph | Boreal Forest | QC | March, 1998 | 7 | 2 (28.6) | - | 1; 1 |
| Romaine | Boreal Forest | QC | Feb, 2011 | 7 | 0 (0) | - | - |
| | | | March, 2011 | 8 | 3 (35.7) | 2; 1 | 3; 2 |
| Saguenay South | Boreal Forest | QC | March, 2011 | 5 | 2 (40) | - | 3; 2 |
| Leaf River | Migratory Tundra | QC | Oct, 2008 | 1 | 0 (0) | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Winter 2009 | 31 | 8 (25.8) | - | - |
| | | | Spring 2010 | 15 | 3 (20) | - | - |
| | | | Summer 2010 | 30 | 6 (20) | 5; 2 | 8; 3 |
| | | | May, 2011 | 10 | 3 () | - | 7; 4 |
| George River | Migratory Tundra | QC-NL | Winter 2009 | 29 | 2 (6.9) | - | - |
| | | | Spring 2010 | 15 | 8 (53) | - | - |
| | | | Summer 2010 | 30 | 11(36.7) | 4; 2 | 5; 2 |
| Grey River | Boreal Forest | NL | Jan, 2009 | 1 | 1 (100)[E] | - | - |
| Lapoile | Boreal Forest | NL | Jan, 2009 | 3 | 0 | - | - |
| Buchans | Boreal Forest | NL | April, 2009 | 2 | 1 (50) [E] | - | - |
| Topsails | Boreal Forest | NL | March 2010 | 2 | 1 (50) [E] | - | - |
| Gregory | Boreal Forest | NL | March 2010 | 2 | 1 (50) [E] | - | - |
| Gros Morne | Boreal Forest | NL | Feb, 2011 | 1 | 0 | - | - |
| Gaspésie | Mountain | QC | Feb, 2013 | 19 | 6 (32) | - | 23; 6 [§] |
| | | | Feb-Mar, 2014 | 13 | 3 (23.1) | - | 12; 3 [§] |
| **TOTAL** | | | | **939** | **221 (23.5)** | **69; 36** | **228; 81** |

| |
|---|
| ***R.t.caribou*** |

*: Co-infections of *V. eleguneniensis* and *P. andersoni*; *E:* DSL identified as *Elaphostrongylus rangiferi*, *P:* DSL identified as *Parelaphostrongylus* sp., but species not determined.

**Table 5.5.** Muskox fecal samples included in the study: information on subspecies and origin, and Baermann results (DSL prevalence). Molecular identification of *Varestrongylus eleguneniensis* larvae were determined based on sequences of the mitochondrial cytochrome c oxidase subunit 1 (COI)

| **Subspecies/ Range** | **Region/ Game Unit** | **Month, Year** | **N** | **DSL (%)** | ***V. ele.* (DSL; host)** | **Comments** |
|---|---|---|---|---|---|---|
| ***Ovibos m. wardi*** | | | | | | |
| **Native** | | | | | | |
| Greenland | West Greenland | April, 2009 | 6 | 0 (0) | - | - |
| Nunavut | Ellesmere Isl. | July-Aug, 2010 | 4 | 0 (0) | - | - |
| **Introduced** | | | | | | |
| Alaska | GMU 22C | April, 2010 | 1 | 0 (0) | - | - |
| | GMU 26B | June, 2010 | 1 | 0 (0) | - | - |
| | GMU 23 | March, 2011 | 6 | 6 (100) | 6; 6 | All also + for *P. stilesi* |
| | GMU 22 E | March, 2011 | 4 | 4 (100) | 4; 4 | |
| | GMU 26B | March, 2011 | 5 | 0 (0) | 0 | All adults |
| | GMU 22E | March, 2011 | 3 | 2 (66.7) | 2 | Adult Females |
| | GMU 23 | March, 2011 | 2 | 0 (0) | 0 | - |
| | GMU unknown | March, 2011 | 1 | 0 (0) | 0 | - |
| | GMU 22D | April, 2011 | 13 | 13 (100) | NA | All adults |

| | | | | | | |
|---|---|---|---|---|---|---|
| | GMU 20B | April, 2011 | 1 | 0 (0) | - | + for *P. stilesi* |
| Quebec[*] | Nunavik | Winter, 2008 | 15 | 13 (86.7) | + | Previous work |
| | | April, 2009 | 7 | 5 (71) | + | Previous work |
| | | Dec, 2009 | 1 | 1 (100) | + | (Verocai et al. 2014) |
| | | Jan, 2010[*] | 2 | 2 (100) | + | (Verocai et al. 2014) |
| | | March, 2010 | 2 | 2 (100) | + | (Verocai et al. 2014) |
| | | April, 2011[*] | 20 | 14 (70) | 9; 9 | All adults |
| **Total *O. m. wardi*** | | | **94** | **64 (68)** | **19; 19** | |
| ***Ovibos m. moschatus*** | | | | | | |
| **Native[§]** | | | | | | |
| Nunavut | Kugluktuk | May-Aug 2007 | 57 | 47 (82.5) | NA[†] | *U. pallikuukensis* range |
| Northwest Territories | Sahtu | Feb, 2011 | 8 | 8 (100) | NA[†] | *U. pallikuukensis* range |
| **Total *O. m. moschatus*** | | | **65** | **55 (84.6)** | **NA[†]** | |
| **TOTAL *O. moschatus*** | | | **159** | **117 (73.6)** | **19; 19** | |

* Additional fecal samples of Nunavik muskox were collected by helicopter on the tundra in April, 2010 and April, 2011. All herds examined were positive for *V. eleguneniensis*. Material of these tundra collections along with material from the January, 2010 collection and of the April, 2011 experimental hunt were used for experimental infections of reindeer and muskoxen for elucidating the life cycle of the species (Kafle, Sullivan, Verocai, and Kutz, manuscript in preparation).

[§] Additional hundreds of muskoxen fecal samples from Victoria Island, shared by Nunavut and the Northwest Territories were also analyzed. Larvae of both *V. eleguneniensis* and *U. pallikuukensis* were isolated and sequenced at the ITS-2 region, including a case of co-infection by the two protostrongylid species. Results are published in Kutz et al. (2013).

+ = Indicates that the identity of larvae from these animals was confirmed by sequencing of the ITS-2 region instead of COI. Material from these collections (adult and larval nematodes) were used for the taxonomic description of the species (Verocai et al. 2014, Chapter 3), and therefore consist in the type series of *V. eleguneniensis*.

[†] DSL not sequenced because animals from these areas were already known to be infected by *V. eleguneniensis* as per Kutz et al. (2007). Also these populations are largely sympatric with infected barrenground caribou herds (Kutz et al. 2007, 2013; Present Study).

**Table 5.6.** Moose fecal samples included in the study: information on subspecies and origin, and Baermann results (DSL prevalence). Molecular identification of DSL was based on sequences of the ITS-2 region of the nuclear ribosomal DNA

| **Subspecies/ Range** | **Region/Game Management Unit** | **Month, Year** | **N** | **DSL (%)** | ***V. ele.* (DSL; host)** | ***P. and.* (DSL; host)** | **Comments** |
|---|---|---|---|---|---|---|---|
| ***Alces a. gigas*** | | | | | | | |
| **Alaska** | GMU 22C | April, 2010 | 30 | 2 (6.7) | - | - | Not determined. All 10mo. males |
| | GMU 26A | April, 2010 | 20 | 0 | - | - | All adult females |
| | GMU 20A | April, 2010 | 2 | 0 | - | - | No info |
| | GMU 20D | April, 2010 | 2 | 0 | - | - | No info |
| | MRC | Aug, 2010 | 2 | 0 | - | - | All calves |
| | GMU 16A/14B | Aug, 2010 | 1 | 0 | - | - | Female calf |
| | GMU 20B | Oct, 2010 | 1 | 0 | - | - | Male calf |
| | GMU 14 | Jan-Feb, 2011 | 3 | 0 | - | - | All calves |
| | GMU 20C | March, 2011 | 18 | 0 | - | - | All 10mo calves |
| | GMU 24B | April, 2011 | 34 | 1 (2.9) | - | - | Not determined. All adult females |
| | GMU 15 | May, 2011 | 1 | 0 | - | - | Adult female |
| | GMU 20A | Oct, 2011 | 36 | 3 (8.3) | - | 3; 2 | All adult females |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | GMU 11 | Oct, 2011 | 9 | 2 (22.2) | 1; 1 | 5; 1 | All Adults |
| | GMU 12 | Oct, 2011 | 6 | 1 (16.7) | - | 2; 1 | Adult female |
| | GMU 9E | Oct, 2011 | 1 | 0 | - | - | Adult female |
| | GMU 20A | March, 2012 | 34 | 0 | - | - | All calves |
| | GMU 20D | March, 2012 | 32 | 0 | - | - | All calves |
| | GMU 20C | March, 2012 | 13 | 0 | - | - | All adult females |
| **Total *A. a. gigas*** | | | **245** | **9 (3.7)** | **1; 1** | **10; 4** | |
| ***Alces a. andersoni*** | | | | | | | |
| **Northwest Territories** | Sahtu | March, 2009 | 8 | 0 | - | - | |
| | Sahtu | 2010/2011 | 9 | 1 (11) | - | 1; 1 | Adult |
| **Alberta** | Peace River | 2011 | 2 | 1 (50) | - | 1; 1 | Adult Male** |
| **Total *A. a. andersoni*** | | | **19** | **2 (10.5)** | | **2; 2** | |
| **TOTAL *A. americanus* sspp.** | | | **264** | **11 (4.2)** | **1; 1** | **12; 6** | |

** The other animal, a yearling male was infected by *Orthostrongylus macrotis*.

### *5.3.3 Molecular identification*

5.3.3.1 Sampling criteria

My primary goal was to determine presence of *V. eleguneniensis* in a herd, subspecies or location. My general study design for the molecular confirmation of the species identity of DSL of infected animals was to initially subsample up to five DSL from five positive animals of each population, however, this number varied for several reasons. In some cases more DSL were tested because several turned out to be *P. andersoni* before I found *V. eleguneniensis*. In other cases fewer larvae were sequenced. For example, once an animal was confirmed positive for *V. eleguneniensis*, no other animal from that herd needed to be sampled, unless samples of several animals were processed in the same day. For some populations, few larvae were recovered and/or the number of animals sampled or positive was less than five; this is particularly true for moose and some caribou populations. Also, because of our previous knowledge of the presence of *V. eleguneniensis* in a given caribou or muskox population, I may have opted to not sequence material from DSL-positive animals. In muskoxen from Alaska and Quebec, I sequenced larvae from a greater number of animals (1 DSL per animal, of 9 or 10 animals) as a preliminary study for assessing the genetic diversity of *V. eleguneniensis* (data not shown). DSL of some muskox populations and larvae of non-DSL protostrongylids found in Baermanns were either identified by DNA sequences by the same process described below or allocated to the species known to occur in certain population/region (including sympatry with caribou or Dall's sheep, and the known range of *U. pallikuukensis*).

5.2.3.2 Molecular analyses

DSL isolated from feces were initially collected, and placed individually in 0.2mL tubes containing 5µL of deionized $H_2O$, and subsequently frozen at -80 ºC for approximately 15 minutes. Genomic DNA (gDNA) of individual DSL was extracted in tubes containing 25µL of lysis buffer (0.5mg/mL of proteinase K, 10x PCR buffer) was added and incubated at 65 ºC for 60 min followed by 95 ºC for 15 min. DNA lysate was diluted 1:5 in DNase, RNase free deionized $H_2O$ and stored at -20 ºC until PCR (Verocai et al., 2013).

***ITS-2:*** PCR of individual DSL was performed using primers NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) targeting the ITS-2 region of rRNA gene according to Verocai et al. (2013). Each 20µL reaction consisted in 10.2µL of sterile dd$H_2O$, 4µL of 5x PCR buffer + $MgCl_2$, 0.4µL of 10mmol dNTPs, 2µL (10µM) of each primer, 0.2µL of *Taq* Phusion HF DNA polymerase, 0.2µL of bovine serum albumin (20mg/mL), and 1µL of diluted DNA lysate was added. The amplification conditions used were an initial 2min denaturation at 98 ºC, followed by 35 cycles of 98 ºC for 10s, 52.5 ºC for 30s, and 72 ºC for 30s. A final extension of 72 ºC for 5min was followed by cooling to 4 ºC.

***COX-1:*** This marker was used only for DSL of muskox populations. PCR of individual DSL was performed using primers PtCOI-F (5'-GGTTGGAGAGTTCTAATCATAAAGA-3′) and VeCOI-R (5′-CAACAGTATACATATGGT GAGCC-3′) targeting the COX-1 region of the mitochondrial DNA. These primers and the PCR conditions to follow were previously designed and optimized for *V. eleguneniensis* by collaborators at the United States National Parasite Collection (USNPC; Ingrid Asmundsson, Art Abrams, EPH). Each 20µL reaction consisted in

13.5μL of sterile dd$H_2O$, 2μL of 5x PCR buffer, 0.8 μL $MgCl_2$, 0.8μL of 10mmol dNTPs, 0.4μL (10μM) of each primer, 0.1μL of *Taq* Platinum DNA polymerase, and 2μL of diluted DNA lysate was added. The amplification conditions used were an initial 2min denaturation at 94 ºC, followed by 38 cycles of 94 ºC for 20s, 52.5 ºC for 30s, and 68 ºC for 40s. A final extension phase of 7min at 68 ºC was followed by cooling to 10 ºC.

5.3.3.2 Sequencing and sequence analysis

DNA templates for direct sequencing of the ITS-2 region were cleaned using ExoSAP-it® or column purified using e.Z.N.A MicroElute® Cycle Pure Kit (Omega Biotek) following the manufacturers' protocols. Amplicons were sequenced from both ends using the same primers used for PCR amplification for each region with BigDye Terminator Cycle Sequencing (Applied Biosystems). Sequences of complete ITS-2 and partial COX-1 were edited using FinchTV 1.4.0 and MEGA 6.0 (Drummond et al. 2011).

## 5.4 Results

### *5.4.1 Caribou*

I tested a total of 1,525 caribou fecal samples encompassing 69 populations of three subspecies: Grant's (*R. t. granti*), barrenground (*R. t. groenlandicus*), and woodland (*R. t. caribou*), distributed from Alaska to Newfoundland Island, including every Canadian Province and Territory where caribou still occur, and Greenland (Tables 5.1-5.4). These populations covered three of the four caribou ecotypes: migratory tundra, mountain, and boreal forest. There is no ecotype designation for Greenland caribou populations. Of the total caribou samples, 364

(24.5%) were positive for protostrongylid DSL (Tables 5.1-5.4). These DSL-positive caribou were distributed across 57 herds encompassing the three subspecies and three ecotypes, from Alaska to Newfoundland. Information on DSL prevalence per subspecies and number of infected populations can also be found in Tables 5.1-5.4.

*Varestrongylus eleguneniensis* was found in caribou populations from Alaska to Quebec/Labrador, including new geographic records for the parasite in herds across North America (see Tables 5.1-5.4). More specifically, *V. eleguneniensis* (74 sequenced DSL) was found in 44 caribou of 30 herds of all subspecies and ecotypes. *Parelaphostrongylus andersoni* (238 sequenced DSL) was found in 94 caribou, distributed in 37 herds, and also in all subspecies and ecotypes. Co-infections of *V. eleguneniensis* and *P. andersoni* were diagnosed in 6 animals of different populations, including the three subspecies and ecotypes. As my sampling design was not optimal to look for mixed infections, I cannot assure if co-infections are rare or simply missed. *Elaphostrongylus rangiferi* was restricted to the island of Newfoundland, Province of Newfoundland and Labrador, and I report this parasite for the first time in the Gregory herd (Table 5.4). DSL (12) in four animals of three woodland caribou populations from BC (mountain ecotype) were determined to be *Parelaphostrongylus*, but could not be assigned to species (incomplete or noisy sequence, or ambiguous SNPs at sites relevant for the discriminaton between *P. andersoni* and *P. odocoilei*) (Table 5.4). Additionally, DSL from two woodland caribou populations of Alberta (mountain ecotype) could not be determined because of loss of DSL and PCR and/or sequencing failure.

DSL of both *V. eleguneniensis* and *P. andersoni* were detected in adult females and males, yearlings, and calves across the seasons, regardless of caribou subspecies or ecotype (in a wide variation of larval output, information not provided). Additional data on a few individual

caribou is worth mentioning. A DSL-negative caribou recaptured one year later was found infected with *V. eleguneniensis*. Similarly, another DSL-negative female recaptured two years later was then infected by *P. andersoni*, and yet another one was found infected by *P. andersoni* in two captures two years apart.

### *5.4.2 Muskoxen*

A total of 144 fecal samples were acquired from populations of the two subspecies: *Ovibos moschatus moschatus* and *Ovibos moschatus wardi* from Alaska, Northwest Territories, Nunavut, Quebec and Greenland (Table 5.5). For this latter subspecies, there were samples included from native (NU, Greenland) and anthropogenically introduced populations (Alaska and Quebec). DSL were found in 104 samples (72.2%) from endemic and introduced populations of the two subspecies. In most regions, DSL prevalence ranged from approximately 70% up to 100% (Table 5.5). *Varestrongylus eleguneniensis* was confirmed in *O. m. wardi* populations from Quebec and Alaska, with 19 larvae sequenced from 19 different animals. Larval output in muskoxen from these two areas ranged from just over 1 to 355.6 LPG, and moderate to high counts were frequent.

DSL in populations of *O. m. moschatus* from mainland Northwest Territories and Nunavut were not identified because *V. eleguneniensis* has previously been identified in these populations Kutz et al. (2007). These come from areas where *U. pallikuukensis* co-occurs with *V. eleguneniensis* (Central Canadian Arctic) and are reported elsewhere (Kutz et al., 2013a). *Protostrongylus stilesi* was found in an Alaskan muskox population, in co-infections with *V. eleguneniensis*.

### *5.4.3 Moose*

We tested 264 moose samples from Alaska, Northwest Territories and Alberta, encompassing populations of two of the four subspecies occurring in North America: the Yukon-Alaska moose, *Alces americanus gigas*, and the Western moose, *Alces americanus andersoni* (Table 5.6).

Among moose, DSL were found two subspecies: *A. a. gigas* from Alaska, and *A. a. andersoni* from the Northwest Territories and Alberta. Due to the subspecific diversity and great distances between sampling locations, findings will be treated separately (Table 5.6).

Regarding the Alaskan samples, 9 out of 245 (3.7%) moose were found positive for DSL. All DSL-positive moose were adults. Of these, five DSL had their identity confirmed, with *V. eleguneniensis* being confirmed from a single adult female (GMU11), and *P. andersoni* confirmed in four animals (a male from GMUs 11, a female from GMU 12, and a female from 20A). Larval counts were lower than 1 LPG and species identity could not be confirmed from four animals either because of larval loss or failure in the molecular processing (from DNA extraction to sequencing).

A single adult male moose from Northwest Territories was found infected with DSL (1/17), which turned out to be *P. andersoni.* From the two animals sampled from Alberta, an adult male was positive for *P. andersoni*, and the other animal, a male yearling, was infected with the protostrongyline *Orthostrongylus macrotis* (Dikmans 1931) Dougherty and Goble, whose larvae do not possess a dorsal spine (Carreno and Hoberg, 1999).

## 5.5 Discussion

### *5.5.1 General findings on V. eleguneniensis*

The geographic distribution of *V. eleguneniensis* is largely concordant with that of caribou, although absent from some locations. The presence of this lungworm species across caribou populations of three different subspecies and ecotypes is further confirmed, corroborating preliminary observations based on less extensive sampling regimes (Kutz et al., 2007; Kutz et al., 2013a; Verocai et al., 2014b). My findings support the wide geographic range previously demonstrated for *V. eleguneniensis* by these authors and further expand it to areas of northern and interior Alaska, and across the boreal forests of Canada, including the Provinces of British Columbia, Saskatchewan, Ontario, and Quebec, and multiple areas of Alberta. As suggested by Kutz et al. (2007) and Verocai et al. (2014b), multiple studies that relied only on the morphological appearance of larval protostrongylids in caribou prior to the original finding and recent description of *V. eleguneniensis*, should be reconsidered. Current data documenting the known range of *V. eleguneniensis* indicates considerable spatial overlap with two elaphostrongyline muscle-worms also reported in caribou, *P. andersoni* and *P. odocoilei* (Ball et al., 2001; Gray and Samuel, 1986; Jenkins et al., 2005a; Johnson et al., 2010; Kutz et al., 2007; Lankester et al., 1976; Lankester and Fong, 1989, 1998; Lankester and Hauta, 1989). It is noteworthy, however, to emphasize that some of these studies have not applied molecular techniques for confirmation of the protostrongylid DSL species identity.

The presence of *V. eleguneniensis* across a vast distribution encompassing different caribou subspecies and ecotypes further supports a primary association with caribou (Kutz et al., 2007; Verocai et al., 2014a; Verocai et al., 2014b). Currently, there is a lack of evidence of *V.*

*eleguneniensis* infection in other cervids in its southern boreal range, although this apparent absence has yet to be adequately explored. I postulate a continuous distribution for *V. eleguneniensis* across partially overlapping populations of caribou. This includes the large migratory herds in the Arctic and Subarctic, whose winter range may overlap with range of non-migratory caribou, and those who undergo short migrations belonging to the boreal forest and mountain ecotypes. The largely overlapping distribution of the caribou-*V. eleguneniensis* assemblage may be a product of a concomitant historical geographic colonization by this assemblage after recession of the continental ice, or initial geographic colonization by the host, with subsequent colonization by the parasite, followed by its spread across much of the host's range (Asmundsson et al., 2008; Hoberg et al., 2012b; Hoberg et al., 2008; Kutz et al., 2007; Kutz et al., 2012a). Further pieces for this mosaic are the independent events of colonization of new hosts – muskoxen and moose in sympatry with geographically distinct caribou populations (Hoberg, 2010; Hoberg et al., 2012b; Kutz et al., 2007; Kutz et al., 2013a; Verocai et al., 2014b; present study).

### *5.5.2 Absence of* Varestrongylus eleguneniensis *in caribou herds*

I did not detect *V. eleguneniensis* in 38 caribou populations. Given the opportunistic nature of my sampling, despite geographically extensive and often relatively site intensive field collections, the true absence of *V. eleguneniensis* in many of the assessed caribou populations cannot be confirmed. Nevertheless, for some of these herds, various historical and current factors linked to the environment, and the definitive and intermediate host history and ecology, may explain the apparent absence.

#### 5.5.2.1 Greenland and Peary caribou

My work provided further evidence for absence of *V. eleguneniensis* in Greenland caribou, corroborating with previous assumptions (Kutz et al., 2012a). In this case, absence may be a result of either parasite loss during or after colonization, or historical absence, i.e. the founder caribou that colonized the region were never infected with the parasite ("missing the boat"). With respect to parasite loss, this may have been because of host and/or parasite populations being too low to maintain the life-cycle, absence or low numbers of gastropods, or (episodic or constant) unfavorable environmental conditions. A previous study by Steele et al. (2013) hypothesized that the common abomasal nematode *Ostertagia gruehneri* was absent from one of these two caribou herds because of parasite loss during geographic colonisation of the region by caribou approximately 4,000–7,000 years ago. True absence of *V. eleguneniensis* is also likely for Peary caribou (not sampled in this study) as previously suggested due to climate and probable absence of gastropods on islands of the High Arctic and Greenland (Kutz et al., 2012a). In fact, there is evidence that the caribou lungworm only recently established in muskoxen on Victoria Island, Nunavut, having the Dolphin and Union caribou herd as source, because of increasingly permissive climatic conditions for parasite development (Hoberg and Brooks, 2015; Kutz et al., 2013a). Perhaps, the establishment of *V. eleguneniensis* on the island and its continued expansion northwards (P. Kafle and S. Kutz, unpublished data), may facilitate the exposure of Peary caribou to this lungworm in the future.

#### 5.5.2.2 Newfoundland caribou

*Varestrongylus eleguneniensis* was not found in Newfoundland caribou. These caribou were insufficiently sampled in the present study (n=11), however, and need further assessment.

Previous studies on protostrongylid species infecting Newfoundland caribou have relied solely on morphometrics for identification of DSL (Ball et al., 2001; Lankester and Fong, 1998; Lankester and Northcott, 1979), and also were conducted prior to the discovery and description of *V. eleguneniensis* (Kutz et al., 2007; Verocai et al., 2014b). There is considerable overlap in DSL measurement range between *V. eleguneniensis* [281-400µm, as per (Kutz et al., 2007), and 355-394µm as per (Kafle et al., *In prep.*)] and *P. andersoni* [308-382µm, as per (Prestwood, 1972)], and to a lesser extent with *E. rangiferi* [381-490 µm, as per (Lankester and Northcott, 1979) from caribou with identity of adults confirmed]. Therefore, it is possible that DSL of *V. eleguneniensis* were misidentified among these of *P. andersoni* and *E. rangiferi*, and this minute lungworm may in fact be present in Newfoundland caribou. Conversely, a potential absence of *V. eleguneniensis* on this island could be due to: historical absence within caribou of the North American lineage (NAL) that first colonized the island, or parasite loss after colonization of the island around 12-20 Ka (Yannic et al., 2014). Alternatively, absence of *V. eleguneniensis* could be derived from recent competition with other two protostrongylid species, *P. andersoni* and the introduced *E. rangiferi*, confirmed on the island, although such competition has not been demonstrated elsewhere.

5.5.2.3 Gaspésie caribou

Also, *V. eleguneniensis* appears to be absent from the Gaspésie herd of Quebec, for which a high percentage of the total population was sampled (60% of approximately 80 caribou). If true, the absence of *V. eleguneniensis* may be explained by either historical absence in founder caribou or loss in modern or recent times. This population, which originates from the NAL caribou lineage, has suffered drastic reduction in modern times and is currently the only

remaining caribou herd south of the Saint Lawrence River (COSEWIC, 2011; Festa-Bianchet et al., 2011). This loss of connectivity with other caribou (isolation) is a result of extinctions and habitat fragmentation in the last centuries, and may have impacted the persistence of *V. eleguneniensis*, if once present. In contrast, *P. andersoni* seems to have persisted in this herd, which is parapatric with white-tailed deer populations that could have assisted in sustaining this muscle-worm.

Despite a relatively robust sampling (n=122), *V. eleguneniensis* was not found in any of the five sampled Manitoban herds, including three of the boreal forest ecotype and two of the migratory tundra ecotype. However, the parasite is common in other allopatric and parapatric woodland caribou populations, including populations in the neighboring provinces of Ontario and Saskatchewan (Festa-Bianchet et al., 2011). In fact, one of the Manitoba caribou populations, Norway House, had the highest prevalence of DSL seen among woodland caribou populations (70%), but these all sequenced as *P. andersoni*. This high DSL prevalence may have masked the presence of *V. eleguneniensis* in this herd, as we only sequenced larvae (n=17) from five out of 21 DSL-shedding caribou. Regardless of the apparent absence of *V. eleguneniensis* in the sampled woodland caribou populations in Manitoba, the parasite is certainly present in Northern areas of Province, as the barrenground herds Beverly and Qamanirjuaq are infected (Kutz et al., 2007, present study). The winter range of Qamanirjuaq herd partially overlaps with Cape Churchill caribou, whose range partially overlaps with the Pen Island herd. Therefore, this may support a potential presence of *V. eleguneniensis* in these herds, despite unrecognized in the present study. Infection of these two herds would depend, though, on DSL surviving in the environment over winter. However, as biological aspects of this parasite, including larval survival and transmission, have not been fully elucidated, such assumptions remain to be tested.

### *5.5.3 Muskoxen*

*Varestrongylus eleguneniensis* was commonly found in muskox populations, corroborating previous knowledge (Kutz et al., 2007; Kutz et al., 2013a; Kutz et al., 2012a; Verocai et al., 2014b). It is probable that *V. eleguneniensis* originated in caribou and subsequently colonized muskoxen on a variety of different time scales. In the central Canadian Arctic, native *O. m. moschatus* have co-existed with caribou for millennia, and, therefore, this host colonization event has historical roots and may be prior to or coincidental with the recolonization of these areas by the two species after recession of the Cordilleran and Laurentide continental ice sheets in the late Pleistocene entering into the early Holocene. In this area, muskoxen are also infected with the cyst-forming protostrongylid lungworm *U. pallikuukensis*, a relictual species with an ancient association with muskoxen (Hoberg et al., 1995; Hoberg et al., 2008; Kutz et al., 2007; Kutz et al., 2005). The recent colonization events of translocated muskox populations by *V. eleguneniensis* were briefly discussed by Verocai et al. (2014b). The presence of *V. eleguneniensis* in Nunavik muskoxen (originally from Ellesmere Island) was previously reported by Kutz et al. (2007) and Verocai et al. (2014), however, for the first time the lungworm is confirmed in the two sympatric caribou herds (Rivière-aux-Feuilles and River George), suggesting their potential role as source for muskox infection. Similarly, the presence of *V. eleguneniensis* in muskox populations in Alaska (originally from Greenland) can be explained by their sympatry with the Western Arctic caribou herd, and to a lesser extent with the Teshekpuk herd. Through our caribou sampling we could only find *V. eleguneniensis* in the Teshekpuk herd, and probably due to our low sampling of Western Arctic herd, we could not confirm the presence of the parasite in it. However, because of the presence in sympatric muskoxen, the presence of *V. eleguneniensis* in the Western Arctic herd may be extrapolated. In Alaska, natural populations of

*O. m. moschatus* existed until complete extirpation in the late 1800s (Paul, 2009). These muskoxen were likely to be historically infected by *V. eleguneniensis*, similar to their relatives in the Central Canadian Arctic. Based on this knowledge, we can predict future events of colonization in natural and translocated muskox populations of both subspecies, in areas of sympatry with caribou.

### *5.5.4 Moose*

My work confirms that *V. eleguneniensis* is rare in moose, and that this parasite may be a spill-over from sympatric caribou and not likely to persist in moose in the absence of caribou, as previously suggested (Kutz et al., 2007). The only moose that I found infected by *V. eleguneniensis* was also from Alaska, likely sympatric with caribou herds from Interior Alaska (e.g. Chisana, Nelchina or Mentasta). However, only *P. andersoni* has been found in two of these caribou herds (Kutz et al., 2007; present study), while the remaining herd has not been assessed for the presence of protostrongylids. Despite being an isolated finding, the presence of *V. eleguneniensis* in moose from this region indicates that this parasite is likely present in sympatric caribou herds, which act as source for infection of moose.

For the first time, I also confirmed *P. andersoni* infections in two moose subspecies, the Yukon-Alaska moose (*A. a. alces*) and the western moose (*A. a. andersoni*) from NT and Alberta. All these moose are sympatric with either barrenground or woodland caribou populations, some infected by *P. andersoni* (Kutz et al., 2007; present study), indicating their potential source for moose. Previously, the only unconfirmed reports of *P. andersoni* in moose came from the eastern moose (*A. a. americana*) in Newfoundland (Lankester and Fong, 1998). In

addition, my finding of *O. macrotis* in a yearling moose from Peace River, AB, is likely the northernmost record for the species (Samuel et al., 1976).

### *5.5.5* Parelaphostrongylus andersoni *in caribou*

My widespread findings of *P. andersoni* across caribou range corroborate previous assumptions of an extensive geographic distribution across northern North America (Asmundsson et al., 2008; Hoberg et al., 2008; Kutz et al., 2007; Lankester, 2001). Since the first discovery of *P. andersoni* in caribou from Ontario and Quebec by Lankester and Hauta (1989), knowledge of the distribution of this host-parasite assemblage has increased dramatically (Kutz et al., 2007; Lankester and Fong, 1989). The contributions my work now provide a finer-scale picture of its presence across caribou range, including various new herd and geographic records for this parasite. For most of the caribou range, *P. andersoni* and *V. eleguneniensis* co-occurred, however, *P. andersoni* was more commonly confirmed from DSL-infected caribou, and possibly due to sampling bias it was present in some herds where *V. eleguneniensis* was not sequenced, similar to the findings of Kutz et al. (2007). However, the opportunistic sampling strategies of both these studies do not allow for a powerful comparison of distribution or prevalence of these two protostrongylids. In addition, even though our sampling strategy was not designed specifically to diagnose co-infections, *P. andersoni* and *V. eleguneniensis* co-infections were determined in caribou of different subspecies and ecotypes. To date, there has been no evidence whether or not dual infections by these protostrongylids have additive or synergistic impacts on caribou health, but this hypothesis was postulated by Kutz et al. (2012a).

Absence of *P. andersoni* in muskoxen is well supported by my study and the literature (Hoberg et al., 2002; Hoberg et al., 1995; Kutz et al., 2007; Kutz et al., 2013a; Kutz et al.,

2012a), despite the fact that muskoxen are sympatric with infected caribou populations across much of their range. To date, *P. andersoni* has never been reported from any caprine hosts (Kutz et al., 2012a; Lankester, 2001), but its close relative, *P. odocoilei,* is found in Dall's and bighorn sheep. Currently, though, the range of muskoxen and *P. odocoilei* do not overlap (Jenkins et al., 2005a; Jenkins et al., 2006; Kutz et al., 2004; Kutz et al., 2001c), as in its northern range, Dall's sheep sympatric with muskoxen only harbour *P. stilesi* (Hoberg et al., 2002; Jenkins et al., 2006; Kutz et al., 2012a; Kutz et al., 2001c).

I did identify *P. odocoilei* in this study, but only in woodland caribou of three British Columbia populations, however, its presence in other populations cannot be ruled out without further sequencing. The two previous reports of this parasite in caribou seem to be incidental cases in areas of sympatry with *Odocoileus* and wild caprines in western Canada (Gray and Samuel, 1986; Jenkins et al., 2005a).

### *5.5.6 Insights on the biology and epidemiology of protostrongylids in caribou*

Although not designed specifically to address the ecology of protostrongylids in their ungulate hosts, my research has provided additional insights on the biology and epidemiology *V. elegueneiensis* and *P. andersoni*. I provide strong evidence that DSL of both parasites are shed by caribou of all age classes, both sexes, and during all seasons. Previous studies on *P. andersoni* in caribou based on the experimental infection of a single calf suggested a short patency, with a sharp decline in larval output (Lankester and Hauta, 1989), but longer patency was achieved in infected white-tailed deer (Nettles and Prestwood, 1976; Pybus and Samuel, 1981). Studies on experimental infection of captive wild ungulates with protostrongylids are challenging and logistically difficult, and often include only a few individuals. Therefore, although informative,

their results should be cautiously interpreted as these will likely not simulate natural infection conditions (e.g., infective dose, constant exposure to infection), and often do not investigate the life-cycle in different suitable hosts. Moreover, Ball et al. (2001) in attempting to determine caribou age by fecal pellet size suggested that *P. andersoni* was shed only by calves and yearlings caribou in Newfoundland, a paradigm that is contradicted by my findings of this parasite in adult caribou and moose.

Prevalence of *V. eleguneniensis* and larval output in muskox populations are commonly very high, suggesting that the parasite could persist in muskoxen in the absence of caribou. Findings of *V. eleguneniensis* and *P. andersoni* in moose were scarce and associated with very low larval output. Therefore, it is unlikely that moose contributes substantially to the transmission and maintenance of either species (low prevalence, low LPG), and could not support parasite persistence in the absence of alternate hosts (e.g., caribou/muskoxen or caribou/deer, respectively).

### *5.5.7 Synthesis – linking the past, present and future of* V. eleguneniensis

Establishing the current biogeography and the host associations of *V. eleguneniensis*, and indirectly of *P. andersoni*, allow us to make inferences about the past. The distribution of any parasitic species is a reflection the distribution of its host(s), but current host ranges have been shaped by complex processes across historical times until the present. Therefore, the study of the distribution of such host-parasite assemblages must take into consideration the deep past, the recent past and the numerous variables that are affecting these systems at present time (Hoberg et al., 2014; Hoberg and Brooks, 2008; Hoberg and Brooks, 2010, 2013; Hoberg et al., 2012b; Kutz et al., 2012a; Kutz et al., 2014; Kutz et al., 2009).

5.5.7.1 The Past

Understanding the complex history and biogeography of *Varestrongylus eleguneniensis* is fundamental to critically assess its presence and potential absence in determined caribou subspecies (e.g., *R. t. pearyi*) or populations. A historically deep association of *V. eleguneniensis* and *Rangifer* has been supported by the phylogenetic inference for *Varestrongylus*, presented in Chapter 4, and by the limited molecular phylogenetic data (Verocai et al., 2014a; Verocai et al., 2014b). Likely, this association existed prior to the multiple waves of expansion of this ungulate into the Nearctic across a wide window of 2 to 3 million years during the Pleistocene (Banfield, 1961; Flagstad and Røed, 2003; Weckworth et al., 2012). Therefore, *V. eleguneniensis* may be, in fact, a Beringian endemic as the genus *Rangifer* (Hoberg et al., 2012b; Kurtén and Anderson, 1980; Weckworth et al., 2012; Yannic et al., 2014). However, the biogeographic history of caribou is also very complex and the species survived the Glacial Maxima in multiple refugia, north and south of the continental ice-sheets, as did many other components of the current Nearctic fauna (Banfield, 1961; Hoberg et al., 2012b; Klütsch et al., 2012; Shafer et al., 2010; Yannic et al., 2014). My sampling across the vast *V. eleguneniensis* range has generated material to further investigate the three hypotheses postulated by Verocai et al. (2014), in which population genetics of the parasite may support one of the three hypotheses: i) the parasite distribution was restricted to Beringia with caribou of the Beringian-Eurasian lineage, and expanded eastwards and southwards across the continent after deglaciation (just over 10ka), ii) the parasite distribution was restricted to the south of the continental ice in caribou of the North American lineage and later expanded northwards, or iii) the parasite was present in caribou populations north and south of the icesheets and expanded in all directions until covering its current range. Further, satellite-hypotheses of multiple refugia within refugia may arise after a

powerful study on the genetic diversity and signatures of *V. eleguneniensis* populations, and may be an indicator of caribou population genetics and phylogeography. Nevertheless, as highlighted by Verocai et al. (2014b), there is still a need for a broader assessment for the presence of *V. eleguneniensis* in Eurasian reindeer, which ranges from Fennoscandia to Eastern Russia. If *V. eleguneniensis* is present across most of *Rangifer* range, novel hypotheses for its historical biogeography will have to be articulated, and tested.

5.5.7.2 The Present

***In the northern range***

Overall, the recovery and sustainability of caribou populations across the continent is uncertain (Festa-Bianchet et al., 2011; Gustine et al., 2014; Hervieux et al., 2013; Vors and Boyce, 2009), raising serious concerns about the species conservation and long-term persistence. Currently, caribou's northern range is facing unprecedented, fast, and ongoing changes that may also impact the geographic distribution and host-associations for *V. eleguneniensis*. In fact, it has been recently demonstrated that *V. eleguneniensis* is undergoing a northward range expansion, as climate possibly became permissive to its establishment on an Arctic Island, where it was likely seasonally present within caribou but could not complete its cycle in resident muskox populations (Hoberg and Brooks, 2015; Kutz et al., 2013a). Moreover, the anthropogenic introduction of muskoxen into multiple areas within the range of the caribou-*V. eleguneniensis* assemblage may impact the population dynamics of this lungworm species, as another suitable host is contributing to the environmental contamination with larvae of the parasite. Therefore, an increased infection pressure by *V. eleguneniensis* may be expected in these two-host areas (Kutz et al., 2007; Kutz et al., 2013a; Kutz et al., 2012a; Verocai et al., 2014b).

***In the southern range (dynamic process: recent past and present)***

Across their southern range, woodland caribou have suffered considerable geographic retraction that began during European colonization and has continued to present (Banfield, 1961; COSEWIC, 2011; Festa-Bianchet et al., 2011). Currently, in this region, where *V. eleguneniensis* is thought to have the caribou as its only epidemiologically relevant definitive host (i.e., considering moose infections incidental), direct and indirect anthropogenic pressures including exploitation of renewable (logging, eco-tourism) and non-renewable natural resources (mining and oil and gas industries) continue to profoundly impact caribou, including an event of local extinction and unlikely long-term persistence of multiple herds (Festa-Bianchet et al., 2011; Hebblewhite et al., 2010; Hervieux et al., 2013). I postulate that along with the ongoing range retraction of caribou, the range of *V. eleguneniensis* is also continuously retracting northwards.

5.5.7.3 Range Shift and the future

Combining the current knowledge and predictions on host populations, the geographic range of *V. eleguneniensis* is gradually shifting northwards. At the southern limit of its range, where caribou are considered to be the only suitable host, *V. eleguneniensis* is expected to parallel the range retraction of caribou. At the northern limit of its range the lungworm persists in a multi-host caribou/muskox system, and this,, together with increasingly permissive climatic conditions, is likely to facilitate its ongoing northward expansion (Kutz et al., 2013a; Kafle, Kutz, Leclerc, unpublished data). I predict that the range shift of *V. eleguneniensis* may continue into the next decades and centuries, and perhaps, the long-term persistence of this lungworm will be only possible in its northern range. This northern expansion and potential persistence of *V. eleguneniensis*, is supported by empirical data, predictions, and quantitative modelling for

protostrongylid species under changing climatic conditions (Hoberg and Brooks, 2013, 2015; Kutz et al., 2014; Kutz et al., 2005; Kutz et al., 2009; Molnár et al., 2013).

## 5.6 Conclusion

*Varestrongylus eleguneniensis* is widely distributed across caribou range in North America. The substantial expansion of the knowledge of the biogeography of *V. eleguneniensis* provided by this study provides a strong baseline for monitoring current range and predicting future biogeographic scenarios under accelerating perturbation. The biogeography of *V. eleguneniensis* is a result of an intricate historical association with caribou, independent events of colonization of alternate hosts, and ongoing climatic and anthropogenic perturbations. Together, these will likely continue to influence the dynamic biogeography of this lungworm species, which may be a powerful model for studying impacts of climate and people on complex faunal assemblages.

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# Chapter Six: **GENERAL DISCUSSION**

## ***VARESTRONGYLUS* LUNGWORMS: MORE DIVERSE THAN WE THOUGHT, MORE COMPLEX THAN IT SEEMED**

## 6.1 Overview of main findings

Through this thesis research, the knowledge on the biodiversity of species within the genus *Varestrongylus* substantially increased, and more broadly that of the family Protostrongylidae. Prior to this work, combining the latest revision of the genus (Boev, 1975) and the taxonomic description of two additional species infecting caprines in China (Liu, 1989; Liu, 1984), the genus *Varestrongylus* comprised eight nominal species. Of these, seven are native to Eurasia and a single one native to North America (Boev, 1975; Liu, 1989; Liu, 1984). After my contributions to the subject (Verocai et al., 2014a; Verocai et al., 2014b; Chapters 2 and 3, respectively) we now know that the genus *Varestrongylus* is composed of ten species, eight from Eurasian cervids and caprine bovids, and the remaining two primarily in cervids and, secondarily, in caprine bovids of North America. This increased knowledge of the generic diversity highlighted the need for providing an emended diagnosis for the genus, allowing the allocation of the ten valid species of *Varestrongylus* (Verocai et al., 2014b). This also reinforces the need of an up-to-date review on the genus. The major contributions of my thesis work to understanding the diversity, historical and current biogeography, and host-parasite associations within *Varestrongylus* are briefly highlighted below.

The first major contribution of my work was the resurrection of *V. alces*, lungworm of the Eurasian moose. Prior to my work, the main and authoritative resource in the literature around the genus *Varestrongylus* regarded *V. alces* as a junior synonym of *V. capreoli* based on the poorly detailed original taxonomic description of the former (Boev, 1975). Now, we have unequivocally separated *V. alces* from *V. capreoli* by means of integrated classical and molecular approaches. The resurrection of *V. alces* corroborates the work of many Scandinavian

parasitologists who continued to accept the species validity, regardless of the synonymy proposed by Boev (1975). We assumed that all previous reports of *V. capreoli*, and their synonyms in Eurasian moose were in fact due to *V. alces*, however, there is still need for further verifying the host-specificity of both species in contact zones of their hosts. Interestingly, male specimens of *V. capreoli* isolated from roe deer in Norway are morphologically distinct from the original description of the species, an issue that deserves further investigation in order to confirm if it is only a morphological variation or cryptic diversity. Moreover, the limited, but informative molecular data on species of *Varestrongylus* provided supported that the resurrected *V. alces* was also separate from the putative new North America. *Varestrongylus* species, with different host and geographic ranges. The clear separation of these lungworm taxa, which presumably do not share any host species, are informative for wildlife managers and pathologists, and should be taken into consideration in management and conservation efforts. In addition, the limited molecular data presented suggested complex relationships among *Varestrongylus* species from Eurasia and North America, and their hosts, involving recurrent events of geographic expansion and host switching and subsequent speciation, further explored in Chapters 3, 4, and 5, consistent with faunal assembly.

The second major contribution of my work, the topic of Chapter 3, was the taxonomic description of a novel *Varestrongylus* species, *V. eleguneniensis*. Prior to my work, Kutz et al., (2007) demonstrated the existence of a novel, undescribed protostrongylid species infecting wild North American ungulates but no taxonomic description nor placement could be provided. This formal recognition of a new species within *Varestrongylus* is the latest advance in the knowledge of the generic biodiversity, occurring a quarter of a century after the last description of a new species (Liu, 1989).

Through chapters 3 and 5, I provide substantial advances to the historical and present biogeography of *V. eleguneniensis*, supporting its primary association with caribou, and secondary colonization of muskoxen and moose in sympatric regions. My geographic extensive surveys for *V. eleguneniensis*, together with complementary work by Kutz et al. (2007; 2013a; 2012a) have shown a very broad geographic distribution of this lungworm, extending across North America's Arctic, subarctic and boreal forest to at least one island of the Arctic Archipelago. The information generated by these studies constitutes a critical baseline for monitoring the distribution of this species. This, combined with the hypotheses of historical biogeography for the species, can inform predictions for future responses under the current climate change scenarios and continued anthropogenic influence. In addition to describing the geographic distribution of *V. eleguneniensis*, I was also able to expand our knowledge on the distribution of other protostrongylids infecting wild and semi-domesticated ungulates in North America, in particular that of *P. andersoni*. The distribution of muscle-worm species is relatively well-studied (Asmundsson et al., 2008; Hoberg et al., 2008; Kutz et al., 2007; Lankester, 2001; Lankester and Hauta, 1989; Verocai et al., 2013; Chapter 5), and its northern range vastly overlaps with that of *V. eleguneniensis* (Kutz et al., 2007; Kutz et al., 2012a; Verocai et al., 2014b; Chapter 5).

Another major contribution of my work is the first comprehensive phylogenetic hypothesis for the genus *Varestrongylus*, which allowed me to explore the relationships among species within the genus (Chapter 4). This framework permitted the exploration of the biogeography of species, and their association with ungulate hosts and biogeographic ecozones, historically and in the present. The history of the genus *Varestrongylus* is marked by multiple events of independent geographic expansion and host-switching, exemplified by at least three

events from cervid to caprine hosts from which the four caprine-associated species have originated (*V. pneumonicus, V. capricola, V. longispiculatus* and *V. qinghaiensis*). Initial diversification of *Varestrongylus* occurred within Eurasia, which is the centre of diversification of cervids and caprine bovids, and coincidentally of various parasite groups associated with these hosts (Hoberg et al., 2012b). From Eurasia, independent events of expansion of *Varestrongylus* occurred across the mountainous areas of central and northern South Asia into the bordering Indian region and, eventually across the Beringian nexus into the Nearctic. In fact, the current North American fauna is composed mainly by both host and parasite species (and their descendants) that crossed this bridge or survived within Beringia during glaciations, and later invaded the continent (Hoberg and Brooks, 2010; Hoberg et al., 2012b; Shafer et al., 2010; Waltari et al., 2007). My work shows that at least two independent invasions of ungulate-*Varestrongylus* assemblages into North America occurred during the late Pliocene and Pleistocene. Recognizing these events of invasion and establishment of host-parasite assemblages is foundational for understanding the evolutionary and historical processes that shaped biodiversity within the genus and their hosts, and permit us to contrast these histories to that of other parasite groups and host-parasite assemblages.

### 6.2 Challenges and limitations

Apart from the focused sampling to obtain adult nematodes from the lungs of ungulates for the redescription and description of the two *Varestrongylus* species, my work largely relied on opportunistic sampling of host feces through a wide network of collaborators across North America, and Europe. This opportunistic sampling strategy gave us a broad knowledge on the

presence of *V. eleguneniensis* or other protostrongylids; however it was inadequate to determine true absence of these parasites within host populations. Yet, hypotheses on potential absence were postulated for several populations according to general knowledge on host biogeography and conservation status.

Given the vast geographic area that I wanted to sample, I relied heavily on a wide collaborative network of researchers, hunters, and wildlife biologists and veterinarians with government. For the most part this was very successful; however, parasites and pathogens were not always on the radar for some researchers and managers. Nevertheless, we succeeded in explaining the relevance of our work by highlighting the scarcity and patchiness of information on parasite fauna of caribou, in particular of woodland caribou from boreal forest and mountain ecotypes.

As for the sampling strategy for sequencing of protostrongylid DSL, a few aspects may have limited the information gained with my work. Firstly, across several host populations the number of DSL-positive animals was lower than my initial experimental design. Secondly, the low larval counts in many hosts (especially some caribou and all moose) also affected the planned strategy, because in many instances DSL were lost or the DNA extraction, PCR or sequencing did not yield satisfactory results. Sometimes, because of the unavailability of leftover fecal samples of many hosts, it was not possible to obtain additional larvae. Regarding caribou samples, the wide overlap with *P. andersoni* also influenced the study logistics because this species tended to be more common and perhaps is more prolific than *V. eleguneniensis*, forcing the sampling of more animals of certain herds to determine the presence of the latter species. Unfortunately, even if additional material of DSL-positive hosts was available for some populations, sequencing of a higher number of larvae collected from extra hosts was not always

possible. However, a substantial amount of larval material is archived and may be useful for studies on diagnostics and parasite population genetics.

Cutting edge molecular diagnostic techniques such as high throughput sequencing have been recently developed and can be optimized for the use in studies of the biogeography of protostrongylids of wild North American ungulates, as these more practical for such large-scale studies and are able to detect co-infections by two or more species, even when in disparate proportions (i.e., Avramenko, Gilleard, unpublished data). A practical reason for the potential success of this high throughput sequencing approach is that it relies on the ITS-2 region of the nuclear ribosomal DNA, region which has been characterized for most prostostrongylid species infecting wild ungulates across North America (apart from the understudied *Protostrongylus rushi* and *Protostrongylus frosti* from caprine hosts in the western portion of the continent, and *Protostrongylus coburni* in white-tailed deer from the Northeastern USA).

### *6.2.1 Availability of specimens for phylogenetic analysis*

Access to and availability of adult and larval material for several species within the genus was limited limiting the molecular phylogenetic comparison to the available *Varestrongylus* material. For genetics, specimens of only four species were available: *V. alces, V. eleguneniensis, V.* cf. *capreoli,* and *V. sagittatus.* The only species previously sequenced were *V. alpenae* and *V. eleguneniensis*. For morphological comparisons, apart from material of the four species included in the genetic study, we only had access to physical specimens of *V. alpenae* and *V. pneumonicus,* deposited in the USNPC. Material for both morphology and genetic comparisons was not available for the four other species, and therefore, previously described morphological characters were the only informative way for a comprehensive inference of the

phylogeny of the genus, as we had access to the original descriptions and some additional literature on these missing taxa (Boev, 1975; Liu, 1989; Liu, 1984; Chapter 4).

Although challenging, explorations of the phylogenetic relationships of *Varestrongylus* species using multiple genetic markers, or other technologies should be pursued in the future, as it could confirm or contest hypotheses articulated in my work, and perhaps reveal more intricate interspecific relationships and histories.

## 6.3 Broader context – *Varestrongylus* as a component of the biosphere

### *6.3.1 Biodiversity*

Despite the high percentage of known parasitic species, much more remains to be discovered (Brooks et al., 2014; Dobson et al., 2008; Hoberg et al., 2014; Poulin, 2014). My work is an example of discovery of parasite biodiversity, and similar to multiple other studies, began with the serendipitous discovery of a parasite that simply did not match genetically with other related protostrongylid species (Kutz et al., 2007). A core approach of my work was to take this discovery of a novel *Varestrongylus* species and apply integrated approaches, including taxonomy and systematics, phylogeny and historical biogeography of their ungulate hosts to get us to the point of knowledge today where we have hypotheses for evolution and historical biogeography of the new species, as well as for its congeners. Nevertheless, major gaps in knowledge remain, and include the definition of host and geographic ranges, life-cycle, and broader explorations on its ecology and epidemiology, including impacts on individual hosts and populations.

### *6.3.2 Integrative approaches in Parasitology*

The integration of classical and molecular approaches is fundamental for defining biodiversity of parasite species (Hoberg et al., 2014; Hoberg et al., 2008). Alone, classical methods, including taxonomy and systematics and comparative morphology, are not always reliable across multiple parasite groups, as the existence of cryptic and polymorphic species has caused, and still causes, considerable taxonomic confusion (Hoberg et al., 2014; Nadler and Pérez-Ponce de León, 2011; Pérez-Ponce de León and Nadler, 2010). Examples of cryptic and polymorphic species across parasitic nematodes are abundant, in particular for the bursate nematodes of the Order Strongylida (Blouin, 2002; Catalano et al., 2015; Hoberg et al., 2014; Hoberg et al., 1999; Stevenson et al., 1996; Verocai et al., 2014a). In contrast, molecular approaches alone may demonstrate that the taxon in question is indeed different from others, but does not characterize it as a valid nominal species, according to the International Code for Zoological Nomenclature (International Commission on Zoological Nomenclature, 2012). Therefore, ideally, parasitologists should consider these approaches as complementary and not interchangeable.

### *6.3.3 Phylogenetic framework for exploring parasite biodiversity*

The integration of classical and molecular methods is the foundation for taxonomic decisions regarding a given new taxon (Hoberg et al., 2014). Generally, these decisions should be taken by considering morphological and molecular characters, which will serve for developing hypotheses for the historical biogeography of a novel species. These biogeographic explorations help us in understanding not only the history of the species, but also their host and geographic associations, and ultimately may provide insights on host history and faunal

formation across time. Altogether, this new knowledge and derived hypotheses serve to connect the new taxon to the biosphere, as a new piece of an intricate mosaic. Therefore, the phylogenetic framework for parasite and host assemblages (at varied taxonomic levels) constitutes a powerful tool for the prospection of hidden biodiversity. This way, scientists can direct themselves to the most likely hosts that could share a given species or harbour a closely related, unrecognized species.

### *6.3.4 Large-scale surveys on parasite biodiversity: archives and baselines for monitoring*

Broad surveys on parasite diversity across vast geographic scales can provide us with the current status of the parasite fauna of a host species. This baseline data informs about biodiversity, species distribution and host associations, but can also inform on ecological indicators such as parasite species richness, parasite abundance, among others (Bush et al., 1997). Long-term and continuous surveys also provide epidemiological information on different parasite species that is foundational for understanding parasite and disease dynamics.

Wide geographic surveys of parasite biodiversity and health assessment of wild ungulates, such as those described in this thesis, can provide baselines, and must be considered in conservation biology, including decisions and policy around global animal trade and movement of domestic and wild species (introductions, reintroductions, and translocations) (Deem et al., 2001; Hoberg et al., 2014; Kutz et al., 2012a; Kutz et al., 2009). This comprehensive knowledge on parasite biodiversity can be used to predict host colonization and the emergence of diseases in both native and introduced host species (e.g., muskoxen introduced into caribou range may be colonized by *V. elegueniensis*). Knowledge of biodiversity, faunal structure and host-parasite interactions, linking deep and present histories is essential for understanding ecological

responses to accelerating perturbations, including climate change and direct anthropogenic pressure. Therefore, actions can be taken towards understanding, mitigating and resolving climatic and anthropogenic impacts on the host-parasite systems and the biosphere (Hoberg et al., 2014; Hoberg et al., 2008; Kutz et al., 2014; Kutz et al., 2009).

### *6.3.5 Parasite surveys on endangered species: non-invasive sampling, archives and secondary use*

Geographically wide surveys on wildlife pathogens and parasites, in particular those affecting threatened species or populations, largely rely on non-invasive sampling methods. Non-invasive sampling for the extraction of developmental stages of parasites (e.g., eggs, larvae) in conjunction with identification based on morphological and molecular means are proven as reliable tools to elucidate the biodiversity and distribution of various pathogens and parasite groups, including nematodes (deBruyn, 2010; Jenkins et al., 2005a; Kutz et al., 2007; Polley and Thompson, 2009). My research in addition to the integrated approaches above mentioned, relied on tissue and fecal samples from hunted animals (barrenground caribou, moose, muskoxen) acquired through collaborating subsistence hunters, but the vast majority of woodland caribou samples, which included various threatened populations, relied on non-invasive (or minimally invasive) methods for collection of fecal samples. These were collected through animal observation, ground collection within caribou range, using scat-detection dogs (e.g.; Wasser et al., 2011), but also animals that were captured and immobilized for placing radio-telemetry collars. Of these, many samples had been archived frozen at -20 °C for up to 8 years prior to larval extraction. Although larvae of many protostrongylid species survive long-term periods frozen, larval counts may drop over the years, and may also be affected by freeze-thaw cycles

(Kutz et al., 2012a). Nevertheless, for the purpose of studying the protostrongylid diversity, it seems worthwhile to analyze archival fecal samples of ungulates, as often the acquisition of fresh samples is not possible. Many other fecal samples were 'recycled' after the removal of the outer mucosal layer of fecal pellets for the extraction of hosts' DNA (e.g.; Ball et al., 2010; Klütsch et al., 2012). A study demonstrated that most DSL of the elaphostrongyline *Parelaphostrongylus tenuis* are found on and within this mucous layer of the fecal pellets of white-tailed deer (Forrester and Lankester, 1997). Therefore, the removal of mucus for DNA extraction may have interfered in the subsequent extraction of protostrongylid DSL through Baermann (including caribou populations from Manitoba, Northern Ontario, and Saskatchewan). However, there have been no studies assessing the specific localization of larvae of *V. eleguneniensis* in the fecal pellet of its different hosts.

Collections and archives of parasite specimens are fundamental resources for the scientific community, beyond the discipline of parasitology (Brooks et al., 2014; Hoberg et al., 2014; Hoberg et al., 2009; Hoberg et al., 2008), as they serve as basis for an inventory of parasite biodiversity, and material for studying comparative morphology, population genetics and phylogenetic relationships within and among parasite taxa (Criscione et al., 2011; Gilabert and Wasmuth, 2013; Hoberg, 2002; Hoberg et al., 2014; Hoberg et al., 2008). In addition, parasite material gathered from these wide geographic surveys across the range of their host(s) is ideal for explorations on the phylogeography of parasite species and can also be informative for elucidating host history (Avise et al., 1987; Criscione and Blouin, 2007; Criscione et al., 2005; Koehler et al., 2009; Nieberding et al., 2004; Nieberding et al., 2008; Nieberding and Olivieri, 2007).

## 6.4 Future perspectives

### *6.4.1 Taxonomy and biodiversity of Varestrongylus*

The majority of species within *Varestrongylus* remain largely understudied. Among the knowledge gaps, there are taxonomic issues, such as the need for the redescription of *V. capricola* (Boev, 1975; Sarwar, 1955) and assessing the actual identity of 'morphotypes' within *V. capreoli* (Verocai et al., 2014a). Also, multiple species require further investigation on host-associations (including all caprine-related species, and dubious information on *V. capreoli*), and biogeography, including *V. tuvae*, *V. longispiculatus*, and even *V. alpenae*, which currently shows a patchy distribution across eastern North America.

Incongruence between parasite and host phylogenies (Chapter 4), and scarce studies on the protostrongylid fauna (and often overall parasite fauna) associated with numerous cervid and caprine species, may suggest the potential for a hidden protostrongylid biodiversity. The recent finding of *V. eleguneniensis* in a high profile and relatively well-studied host, supports the necessity of evaluating the parasitic biodiversity of ungulate hosts.

### *6.4.2 Knowledge gaps*

In spite of the recent advances on the diversity of the genus *Varestrongylus* and the overall contributions of the present work there are numerous knowledge gaps around most species within this genus. Further research is required on host and geographic range and ecology of multiple *Varestrongylus* species, as well as the impact they may have on host health at both individual and herd level.

Taxonomically, there is a need for a redescription of adults of *V. capricola*, as stressed in Chapter 4, and previously by Boev (1975). In addition, in regards to larval stages, description of DSL is lacking for *V. tuvae* and *V. capricola*, and the description of L3 is lacking for *V. tuvae, V. alces, V. qinghaiensis, V. longispiculatus*, and *V. capricola*.

The dated comprehensive revision by Boev (1975) highlights the need for an updated generic revision for *Varestrongylus*. Specific points that need further investigations on the two species central to do this thesis are detailed below.

### *6.4.3 Varestrongylus alces*

There have been no studies on the life-history and ecology of *V. alces*. The association with *A. alces* from Western Eurasia is assumed, but subspecies of *A. americanus* from Eastern Eurasia remain to be tested for the presence of *Varestrongylus* species. Further, there is a need for investigating if *V. alces* is actually host specific, or it may infect sympatric cervid species or even muskoxen introduced to Norway and Sweden (but see Davidson et al., 2014). Along with intensive assessment for host specificity and range, a better delineation of the geographic distribution of *V. alces* would be achieved. Together, more comprehensive host and geographic associations would be the basis for testing the historical biogeographic hypotheses articulated in Chapter 2.

Although infection by *V. alces*, and many congeners, cause significant lesions in the lungs of their hosts, we still do not understand impacts on health of the individual hosts (clinical disease), nor herd level impacts (population dynamics). Therefore, further studies combining pathology, experimental infections, and field observations remain necessary.

### *6.4.4 Varestrongylus eleguneniensis*

Intensive studies around the most recently discovered species, *V. eleguneniensis*, resulted in a fairly well-defined host and geographic range (Kutz et al., 2007; Kutz et al., 2013a; Verocai et al., 2014b). However, a geographically extensive and site intensive assessment of reindeer across Eurasia is necessary to determine if *Varestrongylus*, either *V. eleguneniensis*, or another species, is present. The presence of *V. eleguneniensis* in Eurasia would contest the hypotheses on its historical biogeography framed in this work, and add much complexity to this taxon and its association to *Rangifer*. Nevertheless, the geographically-wide sampling of *V. eleguneniensis* in the present study is of extreme importance for initial explorations on the population genetics and phylogeography of the species and would be useful for testing the biogeographical hypotheses proposed in my thesis work.

Our research group has performed initial investigations on the life-cycle of *V. eleguneniensis* through experimental infections of captive reindeer and a muskox (Kafle, Sullivan, Verocai and Kutz, manuscript in preparation). In addition to basic life-cycle parameters and host suitability, more detailed studies on the influence of temperature on larval development of *Varestrongylus* are required. These are essential for feeding mathematical models for predicting responses under climate change, such as what have been done for other protostrongylid species of high latitudes (Jenkins et al., 2006; Kutz et al., 2013a; Kutz et al., 2014; Kutz et al., 2005; Molnár et al., 2013).

Moreover, a way for anticipating future changes in the biogeography of *V. eleguneniensis*, and potential colonization of novel hosts after the breakdown of ecological barriers due to climatic and anthropogenic perturbations would be through assessing suitability of potential additional hosts that are or might become increasingly sympatric with caribou (e.g.,

northward expansion of mule and white-tailed deer). Therefore, experimental infection trials of captive *Odocoileus* spp. and Dall's sheep and surveys using high throughput sequencing of protostrongylid larvae of natural populations of *Odocoileus* spp. and Dall's sheep sympatric with caribou, could reveal a broader host range and new instances of host colonization.

### *6.4.5 Monitoring changes*

Protostrongylids are particularly sensitive to temperature and climatic perturbations, in particular for the survival of larval environmental stages and larval development within gastropod intermediate hosts (Kutz et al., 2012a; Kutz et al., 2005; Kutz et al., 2009; Molnár et al., 2013). Species of *Varestrongylus* are native to cool climates, from temperate to arctic environments, including mountainous regions at lower latitudes (e.g., Himalayans, and adjacent regions of Central Asia and China) (Bhalerao, 1932; Bhatia and Pande, 1960; Boev, 1975; Liu, 1989; Liu, 1984). Evidence that *V. eleguneniensis* is experiencing a northern range shift congruent with recent climate warming (Kutz et al., 2013a), highlights the need for monitoring its range, but also suggests that other *Varestrongylus* species deserve attention. With changing parasite dynamics, there is potential for a higher pressure for infection and, subsequent, disease emergence. Many *Varestrongylus* species are known to have some impact on their hosts, and are considered of veterinary and economical importance for game cervids in Europe, and perhaps of domestic caprines in Asia (including the multi-host *V. pneumonicus*). Some species of *Varestrongylus* may have conservation relevance. For instance, subspecies of takin, host of *V. longispiculatus*, are listed as endangered or vulnerable by the IUCN red list. Studies on parasite biodiversity and diseases are negligible, reinforcing the need for better understanding of potential

impacts of this lungworm on host health, and also to define associations with the different host subspecies.

### *6.4.6 Testing the phylogenetic hypothesis for Varestrongylus*

Through my research I have defined the biodiversity within the genus *Varestrongylus* and proposed a phylogenetic framework for assessing the biogeography and host-associations of species within the genus. It is now necessary to test the phylogenetic hypothesis for the genus using molecular tools, but this will require the (challenging) acquisition of specimens of all species. Thereafter, genetic work should target multiple informative loci and/or next generation sequencing which generates impressive genetic information. Currently, the complete mitochondrial genome is available for certain Protostrongylidae, and may assist in primer designing, and serve as comparison and the basis for annotating the mitochondrial genome of *Varestrongylus* species (Jabbar et al., 2013).

## 6.5 Conclusion

My research has provided a more comprehensive understanding of the biodiversity, phylogenetic relationships, historical biogeography, and host and geographic associations of the genus *Varestrongylus*. It has contributed to the body of knowledge of the diversity and biogeography of protostrongylid parasites, and the parasite fauna of wild and domestic ungulate hosts within the families Cervidae and Bovidae. The complex histories that shaped the biodiversity, biogeographic and host associations of species of *Varestrongylus* highlight that current recognized mosaic faunas were formed by the interactions of multiple processes and

patterns including cyclical geographic expansions and retraction of host-parasite assemblages through space and time, facilitating events of host-switching and species diversification. Currently, direct and indirect anthropogenic perturbations are continuously influencing the biosphere, emphasizing the dynamic and ongoing nature of the processes that shape the biodiversity. The present unprecedented regime of climatic perturbations has been proven to be actively impacting the distribution and dynamics of parasites and host species. The integration of taxonomic, historical biogeographic and ecological knowledge is a powerful tool for the deciphering the past, understanding the present, and predicting future changes in complex host-parasite systems globally.

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# Appendix

# APPENDIX I

# MAP OF BIOGEOGRAPHIC ECOZONES

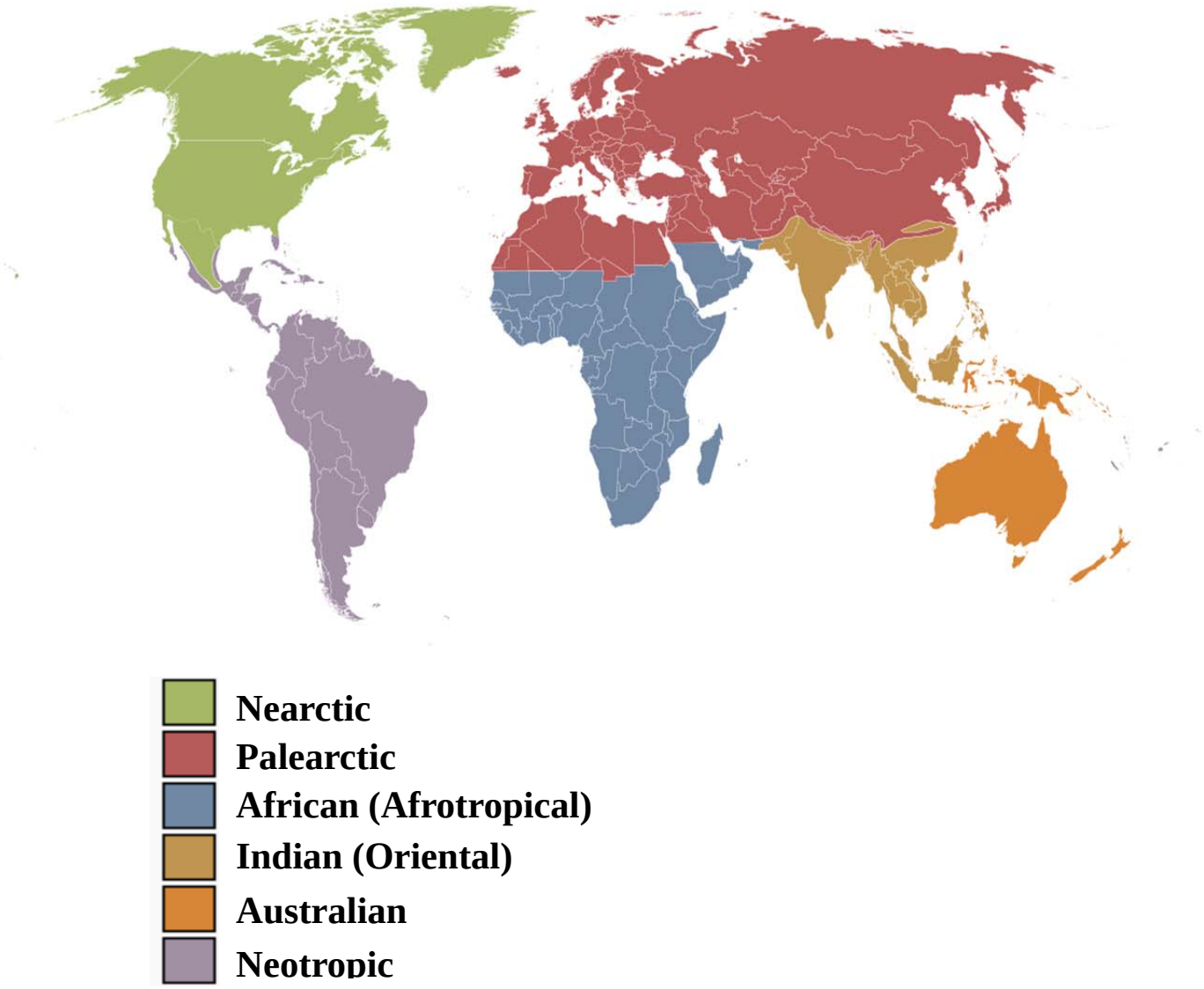


**Figure 1.** Biogeographic ecozones represented on a geopolitical map. Source: "Ecozones" by carol - Ecozones and Image:BlankMap-World6, compact.svg by User:Lokal_Profil.. Licensed under CC BY-SA 3.0 via Wikimedia Commons – http://commons.wikimedia.org/wik i/File:Ecozones.svg#/media/File:Ecozones.svg

# APPENDIX II

## PHOTOMICROGRAPHS OF SPECIMENS OF *VARESTRONGYLUS* AVAILABLE AT THE UNITED STATES NATIONAL PARASITE COLLECTION EXAMINED FOR CHAPTERS 2, 3 AND 4

**Figure 1.** *Varestrongylus pneumonicus* – Caudal extremity of a female specimen. Note the well-developed provagina.

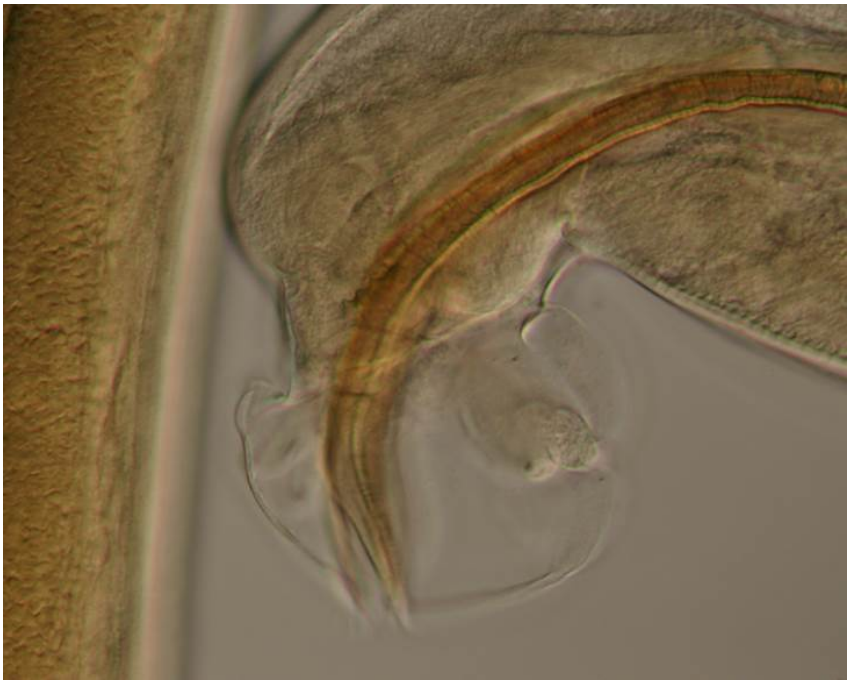

**Figure 2.** *Varestrongylus pneumonicus* – Caudal extremity of a male specimen. Lateral view of denticulate crura and protruded spicules.

**Figure 3.** *Varestrongylus sagittatus* – Caudal extremity of a female specimen. Note the well- developed provagina.

**Figure 4.** *Varestrongylus sagittatus*– Caudal extremity of a male specimen. Lateral view of denticulate crura, long protruded spicules and copulatory bursa.

**Figure 5.** *Varestrongylus sagittatus*– Caudal extremity of a male specimen. Ventral view of gubernaculum, denticulate crura and protruded spicules.

**Figure 6.** *Varestrongylus alpenae* – Cephalic extremity of a female specimen.

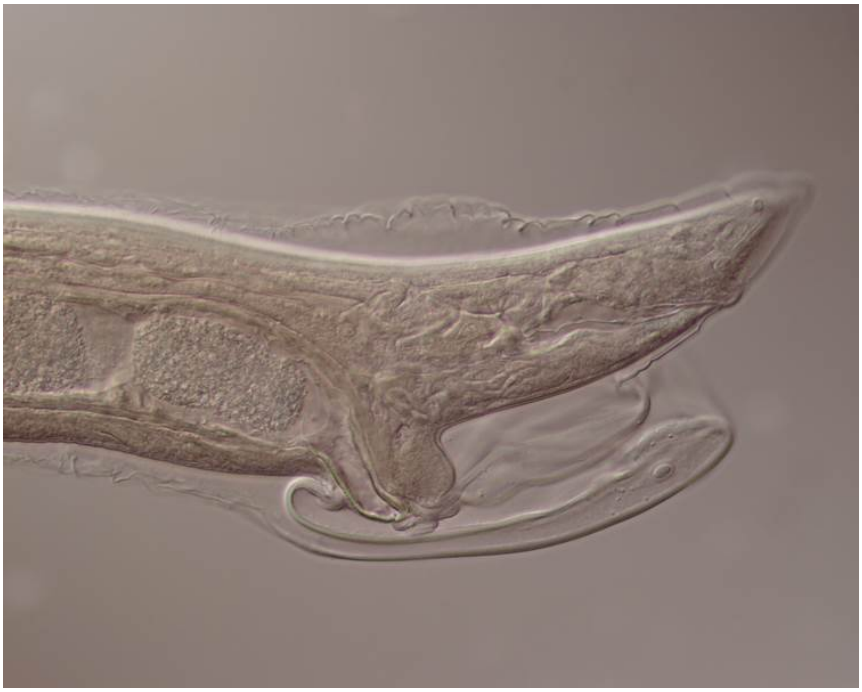

**Figure 7.** *Varestrongylus alpenae* – Caudal extremity of a female specimen. Note well-developed membranous provagina.

**Figure 8.** *Varestrongylus alpenae* – Caudal extremity of a male specimen.Note triangular denticulate plate of crura and long, robust spicules.

**Figure 9.** *Varestrongylus alpenae* – Caudal extremity of a male specimen. Ventral view of gubernaculum, denticulate plates of crura and robust spicules.

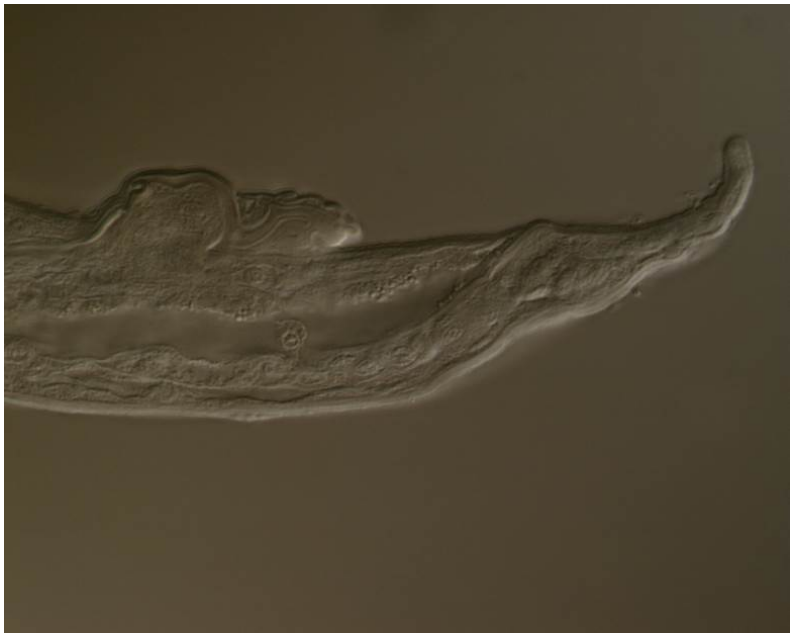

**Figure 10.** *Varestrongylus* cf. *capreoli* – Caudal extremity of a female specimen, which is similar to that of *V. alces*, including the shape of the provagina, but narrower.

**Figure 11.** *Varestrongylus* cf. *capreoli* – Caudal extremity of a male specimen at lateral view. Note the absence of a strong capitulum expected in specimens of *V. capreoli*.

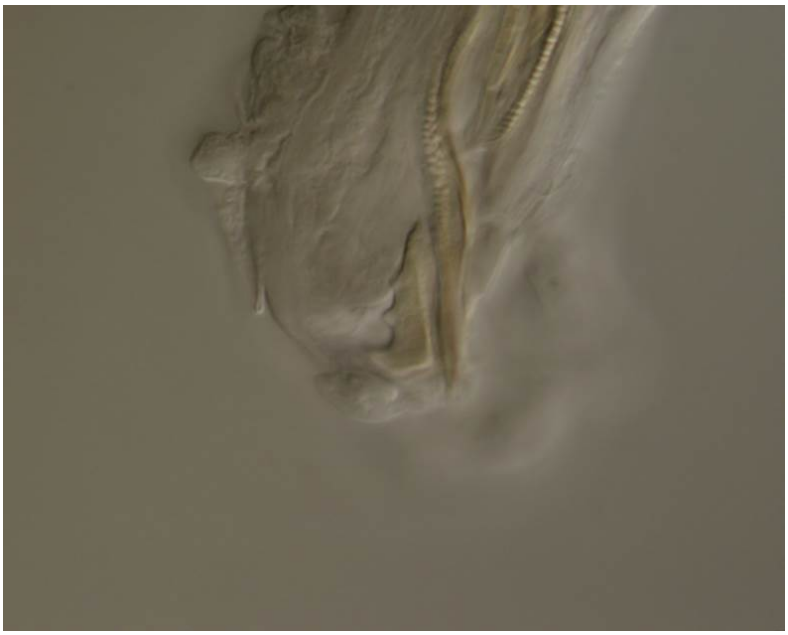

**Figure 12.** *Varestrongylus* cf. *capreoli* – Caudal extremity of a male specimen at lateral view. Note the strongly triangular denticulate plate of crura, with few teeth, which is one the main differences between males of *V. capreoli* and *V. alces*.

# APPENDIX III

## RELEVANT MAPS FOR CHAPTER 5

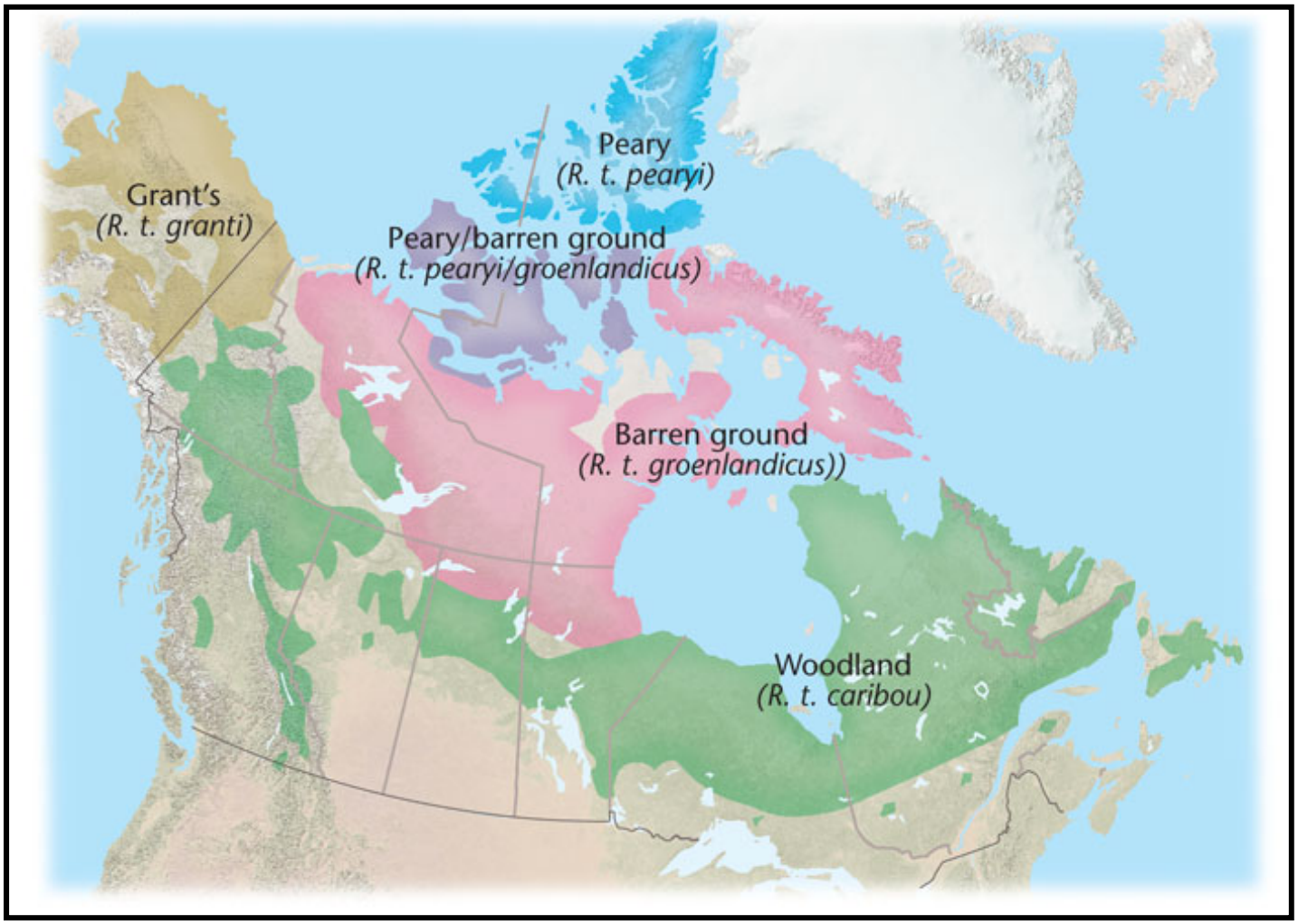


**Figure 1.** Extant caribou (*Rangifer tarandus*) subspecies after Banfield (1961). Extracted from COSEWIC (2011) Draft Report 'Designatable Units for Caribou in Canada'

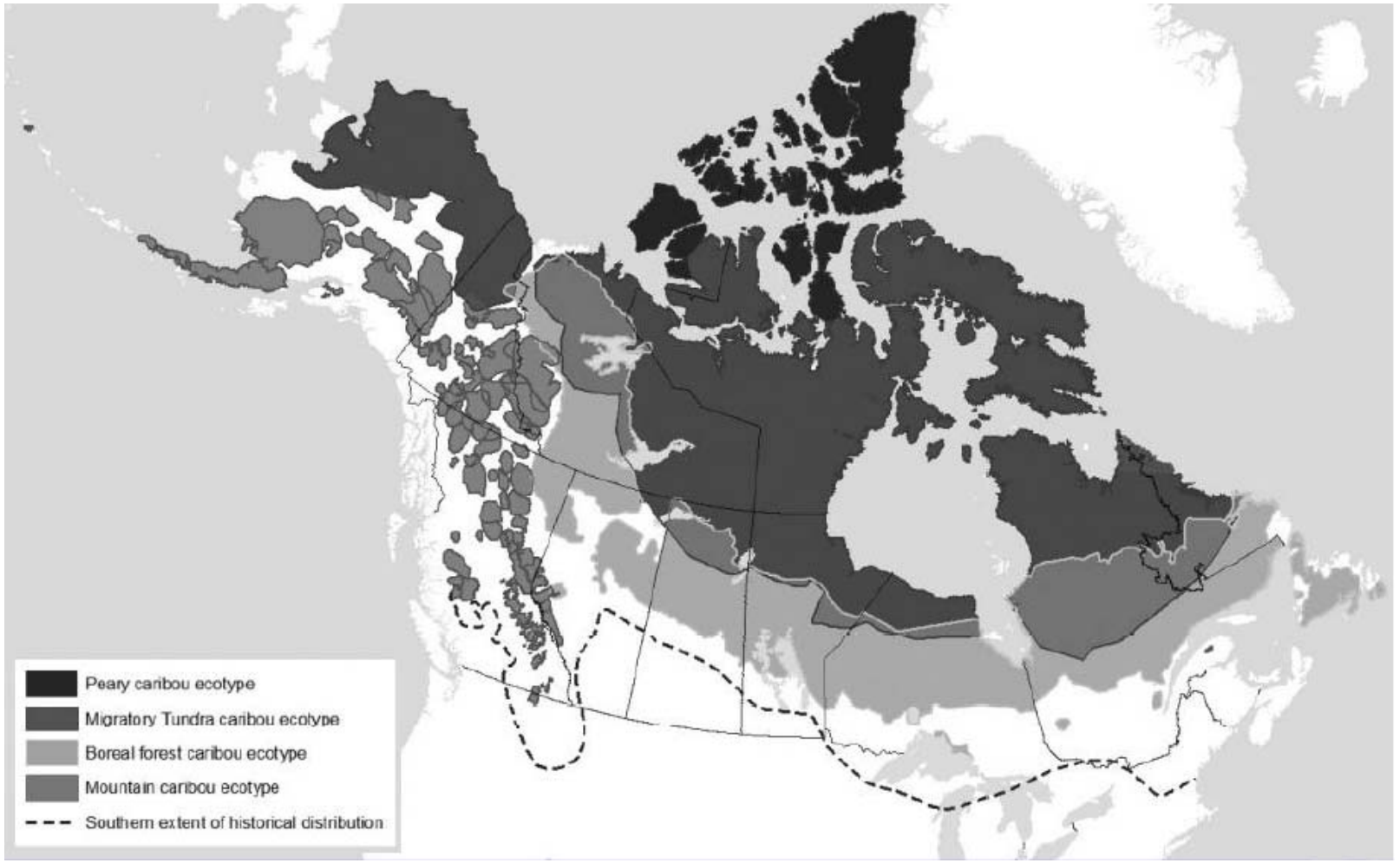


**Figure 2.** Distribution of ecotypes of caribou (*Rangifer tarandus*) as discussed in this review. The spatial overlap between the Boreal ecotype and the Migratory Tundra and Mountain ecotypes is indicated by intermediate shading. Source: Festa-Bianchet et al. 2011, modified from map 2.11 of Hummel and Ray (2008).

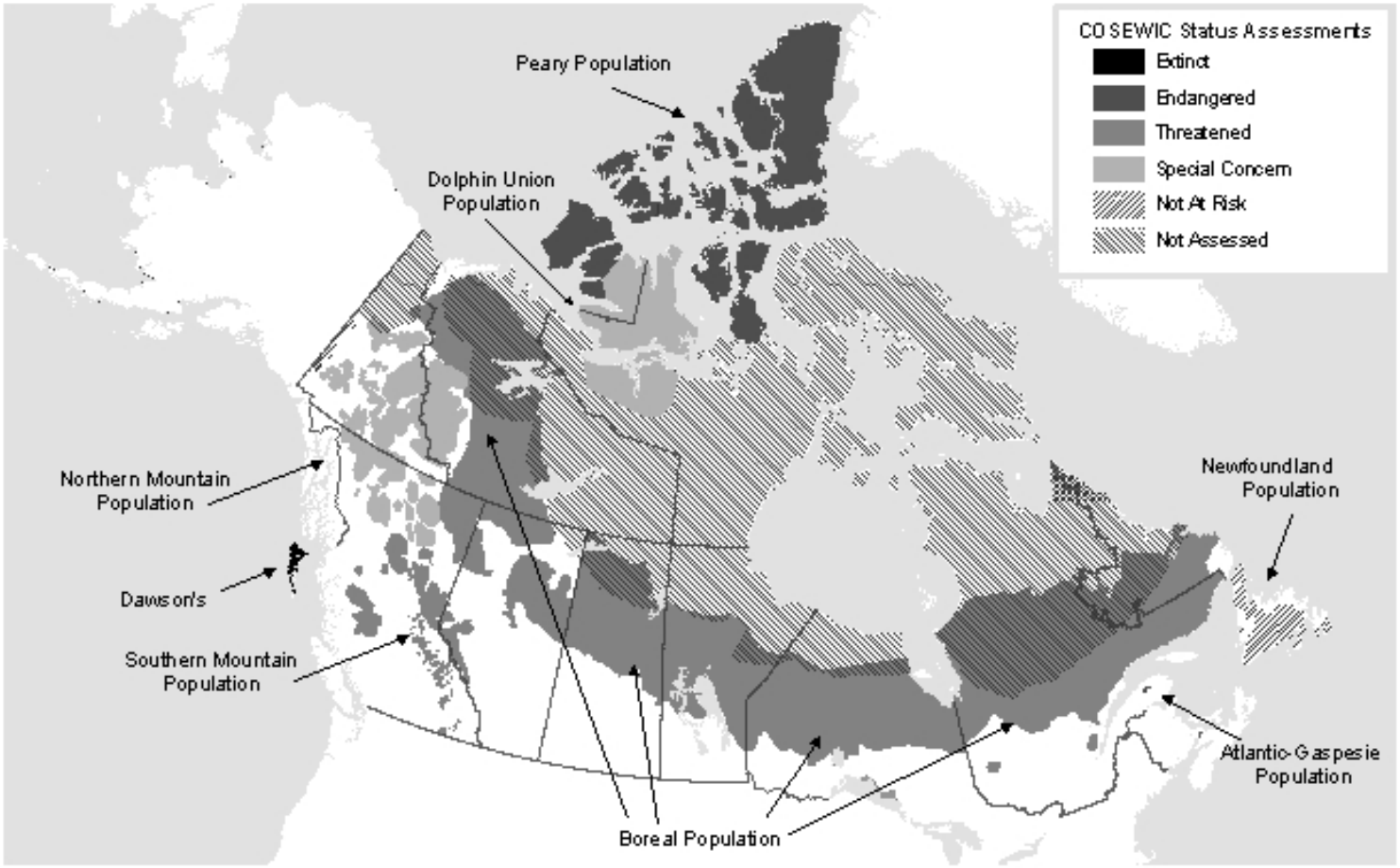


**Figure 3.** "Nationally Significant Populations" (predecessor of Designatable Unit) of caribou arising from COSEWIC status assessments (2000, 2002, and 2004). Extracted from COSEWIC (2011) Draft Report 'Designatable Units for Caribou in Canada'.

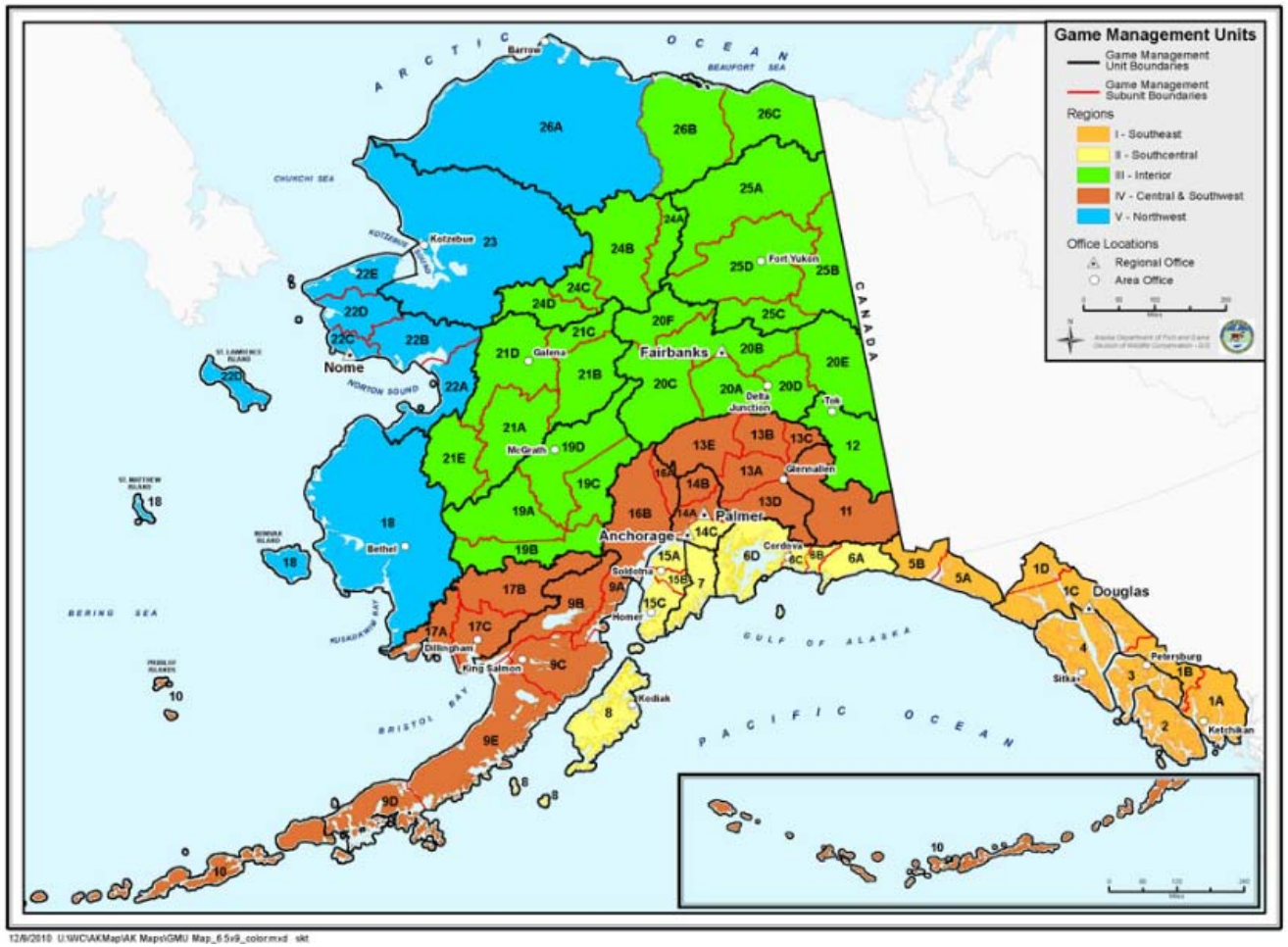


**Figure 5.** Game Management Units of Alaska, USA.

## APPENDIX IV

# A NEARCTIC PARASITE IN A PALEARCTIC HOST: *PARELAPHOSTRONGYLUS ANDERSONI* (NEMATODA; PROTOSTRONGYLIDAE) INFECTING SEMI-DOMESTICATED REINDEER IN ALASKA

**Authors:** Guilherme G. Verocai, Manigandan Lejeune, Greg L. Finstad, and Susan J. Kutz

International Journal for Parasitology: Parasites and Wildlife; v.2, p.119-123, 2013.

**Abstract**

*Parelaphostrongylus andersoni* is a muscle-dwelling protostrongylid nematode that infects caribou and white-tailed deer across North America, and can cause significant muscular and pulmonary pathology in these species. We collected fecal samples from 44 semi-domesticated reindeer (*Rangifer tarandus tarandus)* from the Kakarak herd of western Seward Peninsula, Alaska, USA. This herd has no record of historical contact, and extremely limited possibility of contemporary contact, with native Grant's caribou (*R. t. granti)* of the Western Arctic herd. Samples were processed using the Baermann technique, and 22.7% (n=10) were positive for Protostrongylidae dorsal-spined larvae (DSL). Genomic DNA extracted from individual DSL of all ten positive reindeer (total of 48 DSL) was amplified by PCR targeting the ITS-2 region of ribosomal RNA. Forty of 48 DSL were successfully sequenced and confirmed as *P. andersoni* and one representative sequence for each positive host was deposited in GenBank. No other protostrongylids, including *Varestrongylus* sp., presumed to be widespread across caribou range, and *Elaphostrongylus rangiferi,* which could have been introduced with reindeer from Eurasia, were detected in these samples. *Parelaphostrongylus andersoni* is likely widespread among introduced reindeer in Alaska, potentially causing subtle but deleterious effects with negative economic impacts on commercial herding activities.

**Introduction**

*Parelaphostrongylus andersoni* Prestwood, 1972 is a muscle-dwelling nematode of the family Protostrongylidae Leiper, 1926 that infects caribou (*Rangifer tarandus* sspp.) and white-

tailed deer (*Odocoileus virginianus* sspp.) across North America (Asmundsson et al., 2008; Lankester, 2001). White-tailed deer are considered the primary host, and caribou were likely colonized in zones of contact during the Pleistocene (Carreno and Lankester, 1994). The life cycle of *P. andersoni* is complex and, as typical for all protostrongylids, requires gastropod intermediate hosts for development of larvae from first to the infective third stage (Anderson, 2000). Adult parasites reside in skeletal muscles and can cause significant muscular pathology that compromises the mobility of animals, whereas eggs and larvae can cause significant pulmonary disease. Disease linked to muscular or pulmonary pathology may lead to increased predation (Lankester and Hauta, 1989; Nettles and Prestwood, 1976; Pybus and Samuel, 1981).

*Parelaphostrongylus andersoni* has been confirmed in three caribou subspecies: Grant's (*R. t. granti*), barren-ground (*R. t. groenlandicus*), and woodland (*R. t. caribou*), from Alaska to eastern Canada, including Newfoundland (Ball et al., 2001; Kutz et al., 2007; Lankester and Fong, 1998; Lankester and Hauta, 1989). It is generally accepted that *P. andersoni* has a virtually continuous distribution across caribou range in mainland North America and Newfoundland (Hoberg et al., 2008; Lankester and Hauta, 1989). Despite fairly extensive investigation based on combined fecal analyses and molecular diagnostics, this species has never been detected in sympatric ungulates such as muskoxen (*Ovibos moschatus*), moose (*Alces americanus*), and Dall's sheep (*Ovis dalli*) (Jenkins et al., 2005a; Kutz et al., 2007), suggesting host fidelity to *Rangifer* at high latitudes of the Nearctic. In fact, unequivocal species level identification of protostrongylids relies on morphologic and/or molecular identification of adult specimens, or molecular identification of larvae of morphologically indistinguishable species (e.g., protostrongylids within the genera of *Parelaphostrongylus, Elaphostrongylus, Varestrongylus,*

*Umingmakstrongylus, Muellerius*, that produce larvae provided with a dorsal spine) recovered from feces (Jenkins et al., 2005a; Kutz et al., 2007).

The history of introductions of Eurasian reindeer, *Rangifer tarandus tarandus*, to North America begins in 1892, when animals from Chukotka, eastern Russia, were translocated to Seward Peninsula, western Alaska, to establish semi-domestic herds (Finstad et al., 2006). The intent was to provide the local aboriginal people, the Inupiat, with a reliable source of meat that would complement caribou hunting and, yet more significant, the reduced harvest of marine mammals (primary source of protein) negatively impacted by whaling industry. Cyclical fluctuations in caribou populations and varying migratory paths for these ungulates meant they could not be depended on as a constant source of food, and in fact, caribou virtually disappeared from most of the Seward Peninsula during the mid-1800s (Finstad et al., 2006). Today on Seward Peninsula, reindeer are maintained as commercial livestock using modern management practices (Finstad et al., 2006; Oleson, 2005).

The knowledge of biodiversity, distribution and impacts, of protostrongylid faunas in native North American ungulates has increased substantially throughout the last decades (see Hoberg et al., 2008; Kutz et al., 2012b); however, no attention has been given to free-ranging introduced ungulates such as the Eurasian reindeer. In the few parasitological studies that have been done on semi-domesticated reindeer in North America, protostrongylids were not reported (Choquette et al., 1957; Dikmans, 1939; Hadwen, 1922). In this paper we provide the first report of a native North American protostrongylid, the muscle-worm *P. andersoni*, in a herd of introduced semi-domesticated reindeer from Seward Peninsula, Alaska, USA, and discuss the broader significance of host movements and colonization for structuring the protostrongylid fauna of ungulates in North America.

## Material and Methods

### *Study population*

The Kakarak reindeer herd was established in Alaska from the original reindeer introductions when 1,280 animals were brought from Russia between 1882 and 1901(Finstad *et al.*, 2006). This herd ranges on the western portion of the Seward Peninsula, approximately between 65º 16'N and 64º 35'N, and 165º 56'W and 164º 37'W (Figure 1), near Teller, Alaska, and has recently been the most productive herd on the peninsula. Unofficial counts in 2012 by the Reindeer Herders Association estimated the herd size at 5,000 reindeer. Animals are free-ranging and brought into corral systems once or twice a year for handling (marking of calves and castration of adult males, vaccinations and other veterinary services), as well as for harvest of antler velvet, but anti-parasitic treatment has not been administered in the last 15 years.

The Kakarak reindeer herd has had no known historical contact with native caribou. In the 1990s the Western Arctic caribou herd (WAH), which historically migrated on the far eastern part of the peninsula began expanding its range westwards (Figure 1). This resulted in significant loss for many reindeer herders as their animals would join the migration of the WAH caribou herd (Finstad et al., 2006; Rattenbury et al., 2009) (Figure 1). To prevent contact with caribou and subsequent loss of reindeer to the caribou herds, for the last decade the Kakarak reindeer have been intensively herded and held in a restricted area on the western portion of their historical range near the town of Teller (G. Finstad, Unpublished data).

**Figure 1.** Semi-domesticated reindeer of the Kakarak herd, kept on the western portion of the Seward Peninsula, Alaska, USA (left). Dorsal-spined larvae identified as *Parelaphotrongylus andersoni* isolated from Kakarak reindeer (right).

***Fecal sampling and analyses***

Fresh fecal samples (n=44) from reindeer of the Kakarak herd were collected from the ground. Sampled groups consisted mainly of adult females and few adult males and calves. Approximately 5g of feces per animal were evaluated for the presence of protostrongylid dorsal-spined larvae (DSL) using a modification of the beaker Baermann technique (Forrester and Lankester, 1997). Larvae from each positive host were collected individually in a 0.2mL tubes containing 5µL of deionized $H_2O$ and stored frozen at -20º C.

**6.2.3 *Molecular identification***

Genomic DNA (gDNA) lysate was prepared from 48 larvae from all ten DSL-positive reindeer (3-5 DSL per positive animal). Briefly, to each tube containing a DSL, 25µL of lysis buffer (0.5mg/mL of proteinase K, 10x PCR buffer) was added and incubated at 65ºC for 60 min followed by 95ºC for 15 min. DNA lysate was diluted 1:5 in DNase, RNase free deionized $H_2O$ and stored at -20$^0$C until further use. For PCR, a protocol modified from Kutz *et al.* (2007) was

performed using primers NC1 (5′-ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) targeting the ITS-2 region of rRNA gene. For a 20µL PCR reaction: 10.2µL of sterile dd$H_2O$, 4µL of 5x PCR buffer + $MgCl_2$, 0.4µL of 10mmol dNTPs, 2µL (10µM) of each primer, 0.2µL of *Taq* Phusion HF DNA polymerase, 0.2µL of bovine serum albumin (20mg/mL), and 1µL of diluted DNA lysate was added. The amplification conditions used were an initial 2min denaturation at 98ºC, followed by 35 cycles of 98ºC for 10s, 52.5ºC for 30s, and 72ºC for 30s. A final extension of 72ºC for 5min was followed by cooling to 4ºC. Reagent-only reactions were used as negative controls. Amplicons of 40 DSL were cleaned using ExoSAP-it® and sequenced directly using NC2 primer to obtain partial ITS-2 sequence. To obtain complete ITS-2 sequence, PCR was repeated with DNA template of ten DSL (one from each DSL-positive animal) out of the 40 whose ITS-2 amplification was initially successful. Amplicons thus obtained were sequenced from both ends using primers NC1 and NC2 with BigDye Terminator Cycle Sequencing (Applied Biosystems).

Sequences (3-5 per each DSL-positive animal) were edited using FinchTV 1.4.0 and MEGA version 5 (Tamura *et al.*, 2011). BLAST searches were used to compare the resulting sequences to ITS-2 rRNA sequences available in GenBank, and aligned by Clustal W with MEGA version 5 (Tamura *et al.*, 2011).

**Results**

Ten reindeer fecal samples (22.7%; n=44) were positive for DSL, with the counts of larvae per gram of feces ranging from approximately 0.2 to 50. Partial and complete sequences for the ITS-2 of all 40 DSL were confirmed as *P. andersoni* based on BLAST analysis with

sequences from Kutz et al. (2007). Complete ITS-2 sequences (460 bp) of ten DSL were deposited in GenBank under the accession numbers: JQ 946524 to JQ 946533.

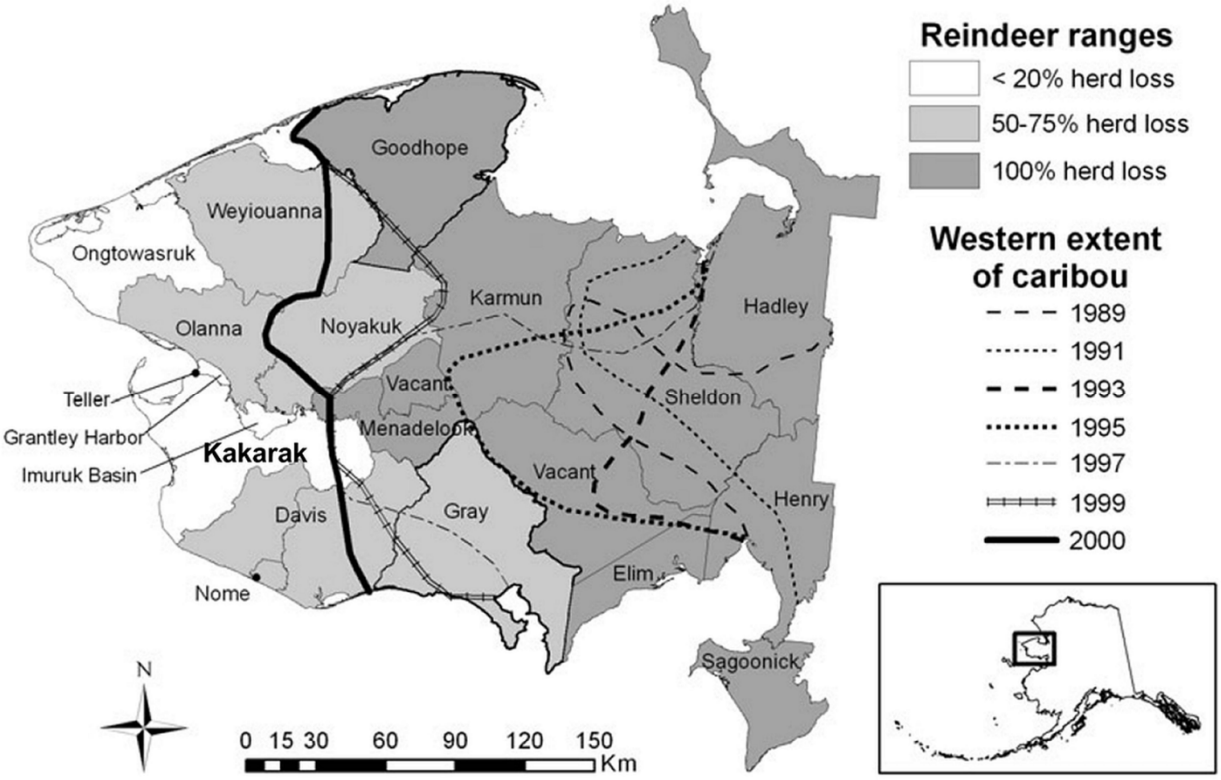


**Figure 2.** Map of the Seward Peninsula, Alaska, showing semi-domesticated reindeer herd ranges including the Kakarak herd (bold), and reindeer herd loss to Western Arctic caribou herd; and the western limits of caribou migration from 1989-2000 (modified from Finstad et al. 2006 and Rattenbury et al. 2009).

**Discussion**

**General findings**

We document for the first time, the occurrence of *P. andersoni* in semi-domesticated reindeer, demonstrating infections with a Nearctic protostrongylid in an introduced Palearctic host. The Kakarak reindeer herd has no historical or contemporary contact or shared range use with the WAH caribou, thus direct transmission from caribou to these reindeer is unlikely (Figure 1). Rather, the establishment of *P. andersoni* in the Kakarak herd may have occurred through sequential events of host colonization, not requiring direct contact with the WAH. This hypothesis is supported by evidence of gene flow between native caribou and introduced reindeer, demonstrated by the reciprocal occurrence of hybrids distributed among caribou and reindeer herds (Mager, 2012). This suggests that seasonal sympatry has occurred and may have facilitated parasite transmission from caribou to reindeer. Temporal overlap (seasonal sympatry) between caribou and reindeer, or between different reindeer herds, however, is not necessary for parasite establishment and transmission between herds. Host-switching could occur through ingestion of gastropods infected with *P. andersoni* L3 if grazing occurs in an area where an infected herd was present months, or even the year before, as suggested by (Hoberg et al., 2002) for other protostrongylid-ungulates system. Thus, in the course of over a hundred years of reindeer residence on Seward Peninsula, Alaska, *P. andersoni* may have been transferred from the Western Arctic caribou to other sympatric reindeer herds, and secondarily expanding through those herds and to adjacent herds with overlapping ranges, until reaching the westernmost areas of the Seward Peninsula.

Alternatively, reindeer movement among herds occurs either by animal exchange between herds (i.e. loan of animals through a program launched by the Bureau of Indian Affairs for new herders to start or build up his herd. These were to pay it back by loaning same number

of animals to next herder in line when the herd increased) or herd admixture via natural animal movement, thus infected reindeer originating from herds with direct contact with WAH may have entered the Kakarak herd on multiple occasions (Finstad *et al.*, 2006). For instance, reindeer from the adjacent Davis (Figure 1) herd have left home range and commingled with the Kakarak herd around 2002-2004 based on our satellite telemetry data of radio-collared reindeer. Also, there is evidence of Noyakuk reindeer (Figure 1), which commingles extensively with WAH, mixing with Karakak animals in several occasions between 2000 and 2009 (G. Finstad, Unpublished data). The fact that the Kakarak herd has not been treated with anti-parasitic drugs for over a decade may have facilitated the establishment of *P. andersoni*, especially if this is a recent parasite introduction event. Similarly, other intensive herd management strategies may have facilitated parasite establishment. During the last decade the Kakarak reindeer herd has been maintained at very high density in a restricted area to avoid animal loss to WAH. This has resulted in overgrazing of many lichen ranges and may also have facilitated the establishment and spread of parasites within the herd. Other reindeer herds in Alaska that have contemporary or historical contact with the WAH caribou are also likely to be infected with *P. andersoni*.

**Significance of *P. andersoni***

Understanding the potential impacts of *P. andersoni* on meat quality and individual and herd productivity is important. Helminth parasitism in muscles can be a common cause for discarding meat and may lead to secondary bacterial infection to damaged tissues (Kutz et al., 2009; Rehbinder, 1990). To date, however, according to the Reindeer Herders Association, parasitism hasn't been identified as a major cause of meat condemnation for animals of the Kakarak herd or any other Alaskan reindeer herd. Protostrongylid nematodes may also have

subtle, but substantial impacts on individual animals, negatively affecting growth and survival (Jenkins et al., 2005b; Kutz et al., 2012b). In addition, as suggested by Kutz et al., (2012b), co-infections with other pulmonary nematodes potentially present in this herd, such as *Varestrongylus* and *Dictyocaulus,* might result in more severe cases of verminous pneumonia, because of additive or synergistic effects.

The reindeer industry in Alaska is an important economic activity, and has been severely impacted due to animal loss to the WAH caribou herd, estimated at over $16 million in 1990-2000s (Carlson, 2005; Finstad et al., 2006; Rattenbury et al., 2009). In addition to this direct impact, native caribou may have indirect impacts on reindeer health through pathogen spillover. For example, the impacts of *P. andersoni* on individual animals may translate to poor herd productivity and economic losses. Such effects are likely to be amplified under intensive herding conditions and current climate change scenarios where abundance and impacts of protostrongylid parasites in northern ungulate hosts are predicted to increase (Ball et al., 2001; Handeland and Slettbakk, 1994; Kutz et al., 2009). Adaptive management of herders, such as range management and use of anthelminthic drugs, may be helpful. However, a handful of studies on anthelmintic efficacy against protostrongylids species in wild North American ungulates suggests that deworming may be met with variable success (Jenkins et al., 2005b; Samuel and Gray, 1988).

**Other protostrongylids of *Rangifer* in North America**

Three other protostrongylid species are reported from *Rangifer* in North America. Two are native to the Nearctic, *Varestrongylus* sp. and *Parelaphostrongylus odocoilei* (Hobmaier & Hobmaier, 1934), and the third, *Elaphostrongylus rangiferi* (Mitskevich, 1960), an introduced Palearctic species (Anderson, 2000; Kutz et al., 2012b; Lankester, 2001).

An as yet undescribed species of *Varestrongylus* that appears to be widespread across caribou range in North America (Kutz et al., 2007; Verocai et al., 2011), and occurs in sympatry with *P. andersoni*, was not found in our study. This apparent absence, however, may reflect insufficient sample size as opposed to true absence (e.g. Kutz et al., 2007).

In contrast, the occurrence of *P. odocoilei* is rather unlikely in the region. This species has only been reported twice in *Rangifer*, and in both cases in woodland caribou, from Alberta (Gray and Samuel, 1986) and the Northwest Territories (Jenkins et al., 2005a). In Alaska, its distribution in Dall's sheep and Mountain goats (*Oreamnus americanus*) is restricted to the interior and southeastern regions, (Hoberg et al., 2008; Jenkins et al., 2005a). It may also be present in sympatric Sitka black-tailed deer (*Odocoileus hemionus sitkensis*), but unequivocal reports are still pending.

Host translocation, introduction, and range expansion are common causes of pathogen host-switching, including parasitic nematodes (e.g. Hoberg, 2010). A classic example for a protostrongylid nematode introduction is that of *E. rangiferi.* A major concern of the introduction of Eurasian reindeer and contact with North American caribou was the introduction of infectious diseases, in particular the Eurasian meningeal-worm, *E. rangiferi* (Lankester and Fong, 1989). This parasite was introduced to North America in 1908 with the introduction of Norwegian reindeer to the island of Newfoundland. It is now established and widespread across many native caribou herds, and causes significant outbreaks of neurological disease (Ball et al., 2001; Lankester and Fong, 1989, 1998). Despite several other reindeer introductions to North America (Lankester and Fong, 1989), *E. rangiferi* has not been reported outside of Newfoundland (Kutz et al., 2012).

On the Seward Peninsula, reindeer may have lost their protostrongylid species, *E. rangiferi*, at the time of their introductions in the late 1890s and early 1900s. This pattern would be similar to that documented in other introduced populations of reindeer on Iceland (Gunðmundsdóttir, 2006) and South Georgia Island in the South Atlantic Ocean (Leader-Williams, 1980). In these cases, loss of *E. rangiferi* may be attributed to absence within hosts at time of introduction, insufficient parasite populations or host population density to maintain transmission, unsuitable climatic conditions, and/or absence of essential gastropod intermediate hosts. Loss of *E. rangiferi* from introduced reindeer herds is consistent with the general processes of parasite loss in introduced host populations demonstrated by Torchin et al. (2003) across a taxonomically wide host range. The parasite fauna of an introduced host population is generally reduced in comparison to populations from where they are native, and the process of acquiring parasite species native to the new regions is rather common, although it will depend in a variety of factors (Torchin et al., 2003).

Nevertheless, the apparent absence of *E. rangiferi* in Alaskan reindeer or caribou cannot be assured. Logistical and financial constraints make it challenging to document the occurrence (or absence) of parasites in remote areas of the Arctic (Kutz et al., 2007). Active surveillance for protostrongylids (fecal sampling and comprehensive post mortem neurological examinations) has been limited on the Seward Peninsula and adjacent areas of Alaska. Fecal analysis based only on morphological identification of larvae has poor specificity because larvae of the three known genera of protostrongylids in *Rangifer* spp. are morphologically indistinguishable. Thus, subsequent DNA sequencing is essential to identify species present and this has not been done previously in these regions. Conversely, passive surveillance may not be particularly sensitive. Semi-domesticated reindeer herds have little contact with people, free-ranging over vast and

poorly populated areas, and are handled or tracked a few times per year (Finstad et al., 2006).Animals with neurological disease are likely to be predated relatively quickly and their carcasses would be consumed shortly after death and thus not detected by the infrequent human presence in the area.

Limiting the potential for introductions of *E. rangiferi* to Canada (other than Newfoundland) is that most of the reindeer introductions were unsuccessful (Godkin, 1986; Lankester and Fong, 1989; Scotter, 1972). The only extant semi-domesticated reindeer herd (Tuktoyaktuk Peninsula, NT) was established in 1935 after a 7 year 'trek' from western Alaska (Godkin, 1986). It is probable that because of the directional movement over time, these animals would have lost most of their parasites, including *E. rangiferi*, if present originally. The Tuktoyaktuk Peninsula reindeer herds is periodically sympatric with wild caribou and thus may also harbour protostrongylids common to caribou in the region, such as *P. andersoni* and *Varestrongylus* sp. (Kutz et al., 2007; Kutz et al., 2012b).

**Host switching among northern ungulates**

Although reindeer and the caribou share many parasite species, such as gastrointestinal nematodes (e.g. *Ostertagia gruehneri* and *Marshallagia marshalli*) (Bye and Halvorsen, 1983; Halvorsen, 1986; Kutz et al., 2012b; Stien et al., 2002), these two conspecific hosts have a divergent native Protostrongylid fauna (Kutz et al., 2012b; Lankester, 2001). This makes this parasite group an interesting system to investigate parasite exchange among wild ungulates. In fact, host switching is a rather common phenomena in protostrongylid-ungulate systems in North America (Hoberg, 2010; Hoberg et al., 2012b). In an evolutionary time scale, we have as an

example *P. andersoni* passing from deer (*Odocoileus* sp.) to caribou (Carreno and Lankester, 1994). In more recent times *Protostrongylus stilesi* Dikmans, 1931, has colonized muskoxen sympatric with Dall's sheep (Hoberg et al., 2002). In both these cases, as in our study, the introduced population acquired a novel pathogen from the native host. In contrast, native bighorn sheep (*Ovis canadensis*) acquired *Muellerius capillaris* (Mueller, 1889), from domestic caprine primary hosts (Ezenwa et al. 2010). In all these scenarios, breakdown in barriers for ecological isolation resulted in successful parasite invasion and subsequent establishment of new hosts-parasite associations (Hoberg et al., 2012b; Kutz et al., 2012b). For *P. andersoni*, the conspecificity of the native (caribou) and the introduced (reindeer) hosts, not surprisingly, did not pose a barrier for parasite transfer. Similarly, ecological conditions on the Seward Peninsula, including gastropod intermediate host availability, climate, and habitat use (herd management), were suitable for maintenance of *P.andersoni.*

**Conclusion**

*Parelaphostrongylus andersoni,* a Nearctic protostrongylid nematode, occurs in semi-domesticated reindeer; a Palearctic host introduced to western Alaska, and might also infect other reindeer herds in Alaska and Canada. The occurrence of this parasite in reindeer may drive disease and diminished productivity for infected animals and thus can have detrimental impacts for individual animals and commercial herds. Molecular tools permitting high throughput analyses of larvae of morphologically indistinguishable species will greatly facilitate broader biodiversity assessment and significantly contribute to management of both semi-domesticated reindeer and wild caribou.

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