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A footworm in the door: revising *Onchocerca* phylogeny with previously unknown cryptic species in wild North American ungulates ☆

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ABSTRACT

Onchocerca is an important genus of vector-borne filarial nematodes that infect both humans and animals worldwide. Many *Onchocerca* spp., most of medical and veterinary health relevance, are the focus of a variety of diagnostic and molecular research. However, despite the importance of these parasites, there is growing evidence of previously unexplored genetic diversity of these nematodes, particularly among wild ungulate hosts in North America. These understudied parasites prevent us from comprehending the evolutionary history of the genus *Onchocerca*, monitoring potential One Health threats, and improving our filarioid diagnostic capabilities. In order to fill these knowledge gaps, we identified five uncharacterized *Onchocerca* lineages and compared them with other well-known filarioid species using single and concatenated gene regions (i.e., *nd5*, *cox1*, 12S, 18S, 28S, *hsp70*, *MyoHC*, *rbp1*). Phylogenetic analyses revealed that the novel *Onchocerca* lineages of wild North American ungulates segregate into two clades. One clade comprised *Onchocerca* lineages II, IV, and V and other species found mainly in domestic animals and humans, and the second comprised *Onchocerca* lineages I and III and other species from a variety of hosts including cervids, bovids, and equids. The formation of two clearly separate clades supports the idea of at least two independent expansion events of ancestral *Onchocerca* spp. into the North American continent via the Bering land bridge. Cophylogenetic analysis shows evidence of ancestral *Onchocerca* spp. of Bovidae host-switching to wild Cervidae and giving rise to the novel *Onchocerca* spp. Lastly, pairwise analysis confirms informative molecular markers of diagnostic relevance in both mitochondrial and nuclear gene regions of filarioid nematodes. The overall information provides greater context to the genus *Onchocerca* and emphasizes the need to discover, characterize, and monitor neglected parasites, especially those of wildlife origin.

☆ Note: Supplementary data associated with this article.

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1. Introduction

Onchocerca Diesing, 1841 is a globally distributed genus of parasitic nematodes comprising 37 known species (Lefoulon et al., 2015, 2017). These parasites are known to infect mammalian hosts including Artiodactyla (e.g., Bovidae, Camelidae, Cervidae, and Suidae), Carnivora (e.g., Canidae and Felidae), Primates (e.g., Hominidae), and Perissodactyla (e.g., Equidae) (Anderson, 2000). Similar to other genera within the Family Onchocercidae (Spirurida), species of *Onchocerca* are transmitted by blood-sucking dipteran vectors (Anderson, 2000). Specifically, black flies (Simuliidae) and biting midges (Ceratopogonidae) have been demonstrated to be competent vectors for such filarial nematodes (Anderson, 2000). *Onchocerca* infections are well known for their relevance to human, veterinary, and wildlife health. Perhaps the most widely known *Onchocerca* spp. is the agent known for causing river blindness in humans, or *Onchocerca volvulus* (Leuckart, 1893). River blindness is a human disease that afflicts over 110 million people in total in both Africa and South America (World Health Organization, 2014). In addition, *Onchocerca lupi* Rodonaja, 1967, is zoonotic and the causative agent of ocular onchocercosis in domestic dogs and cats, and wild canids of North America and Europe (Labelle et al., 2011, 2013; Verocai et al., 2016, 2021; Lefoulon et al., 2017; McLean et al., 2017; Roe et al., 2020).

Since *Onchocerca* spp. can have medical and ecological consequences, there is great scientific interest in using their molecular data for diagnosis, xenomonitoring, and guiding decisions of mass drug administration (Bain, 1981; Krueger et al., 2007; Osei-Atweneboana et al., 2011; World Health Organization, 2016; Pilotte et al., 2017). This need also led Lefoulon et al. (2017) to provide one of the most recent comprehensive investigations on the evolutionary history of *Onchocerca*. However, there still remain critical gaps that hinder our full understanding of *Onchocerca* biodiversity. In fact, investigations exploring this diversity in North America have been noticeably scarce despite complex faunal colonization and distribution.

From the late Pliocene to Holocene, the Bering land bridge, the former northern intercontinental connection between the Palearctic and Nearctic, has experienced periods of biotic accessibility driven by climatic and environmental factors. The geographic expansion and contraction of Eurasian mammals colonizing the Americas through time and space have thus propagated a mosaic distribution and diversification of host-parasite assemblages (Hoberg et al., 2012). One would therefore expect more cryptic *Onchocerca* biodiversity in North America and there is now mounting evidence in wild ungulate hosts that it is much richer than previously thought (Verocai et al., 2012; McFrederick et al., 2013; Verocai et al., 2018b; Kulpa et al., 2021; Benedict et al., 2023). This pattern of underestimated *Onchocerca* spp. richness is not uncommon and has been noted in other wild ungulates from eastern Asia (Yagi et al., 1994; Uni et al., 2007, 2015, 2020).

The formerly named *Onchocerca cervipedis* Wehr & Dikmans, 1935, or what is frequently referred to as the "foot worm" or "leg worm", is a common filarial parasite of wild ungulates in North America. The infection has been reported to cause sores and hoof damage in ungulates, but little is known about its clinical or ecological significance (De Nio and West, 1942; Benedict et al., 2023). However, other filarial worms of North America are known to cause serious pathology in wild ungulates such as peritonitis (Kutz et al., 2014; Verocai et al., 2024) and damage to blood vessels (Madden et al., 1991; Henningsen et al., 2012; LeVan et al., 2013). From the early 1930s to 2012, cases have been reported in several Cervidae hosts: white-tailed deer *Odocoileus virginianus* (Zimmermann, 1780); mule deer *Odocoileus hemionus* (Rafinesque, 1817); moose *Alces americanus* Clinton, 1822; elk or wapiti *Cervus canadensis* Erxleben, 1777; caribou *Rangifer tarandus* (Linnaeus, 1758); and the non-cervid pronghorn host, *Antilocapra americana* (Ord, 1815) (Dikmans, 1933; Rush, 1935; Annereaux, 1941; De Nio and West, 1942; Cowan, 1946; Herman and Bischoff, 1946; Caballero, 1954; Ritcey and Edwards, 1958; Williams and Babero, 1958; Low, 1976; Samuel et al., 1976; Carreno et al., 2001; Verocai et al., 2012; Mariyam Thomas, 2023). However, since 2012, molecular investigations began to uncover multiple *Onchocerca* spp. in a variety of cervid hosts native to North America. Previous publications have referred collectively to these uncharacterized *Onchocerca* as a species complex to highlight the lengthy misclassification of many species into one (Verocai et al., 2012, 2018b; McFrederick et al., 2013; Kulpa et al., 2021). Therefore, all previous reports of *O. cervipedis* across the Americas, including ungulate hosts and vector associations, require a comprehensive reevaluation.

In order to shed light on this cryptic *Onchocerca* biodiversity among wild North American ungulates, we collected and evaluated both cervid skin comprising microfilariae and adult worm samples, and then utilized a multi-locus approach to elucidate their relationship within the genus *Onchocerca*. Phylogenetic analysis was used to classify *Onchocerca* samples into lineages with a corresponding numerical notation (e.g., *Onchocerca* lineage I). This analysis comprised eight molecular markers (three mitochondrial; five nuclear) that were concatenated in a variety of ways to not only provide a robust evolutionary history of the genus *Onchocerca,* but to also fill gaps that have been missing in previous analyses. We also investigated each molecular marker's ability to be used as a diagnostic tool or inform evolutionary questions in the field of parasitology.

2. Materials and methods

Onchocerca adult worms or skin tissues comprising possible *Onchocerca* microfilariae were collected from cervid hosts spanning across North America (Table 1; Supplementary Table S1). This includes newly acquired samples from the United States in Alaska, Maryland, New York, West Virginia, Wyoming, and from Canada in Newfoundland and Labrador. All five cervid host species were included in this study, but we were unable to collect samples from the non-cervid host, pronghorn. Additionally, formerly extracted *Onchocerca* DNA from previous studies (Verocai et al., 2012, 2018b; Kulpa et al., 2021; Benedict et al., 2023) were used in this study for comparative analysis. *Onchocerca* microfilariae of cervids are known to dwell within the legs of moose (Pledger, 1978; Benedict et al., 2023) and the muzzle (Jensen et al., 1982) and pinnae of mule and white-tailed deer (Hibler, 1965; Beaudoin et al., 1970; Robbins and Clark, 1978; McFrederick et al., 2013). Newly obtained samples were collected in two ways from post-mortem cervids: adult worms were either physically recovered, often from the distal extremities, or the ear skin from the pinnae was removed from the cervid host. Generally, the ear tissue appeared healthy with no noticeable lesions. After collection, adult worms were fixed in 70% ethanol tubes and skin tissues were kept in − 80 °C freezers.

Genomic DNA was extracted from adult worms (n = 17) and host ear skin tissues potentially containing microfilariae (n = 63) via a Qiagen DNeasy© Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. This extracted DNA was then evaluated using conventional PCRs (cPCRs) that targeted eight partial gene regions of filarial nematodes. This sequencing includes three mitochondrial genes: NADH dehydrogenase subunit 5 (*nd5*), 12S small subunit ribosomal DNA (12S) and cytochrome oxidase subunit I (*cox1*); and five nuclear genes: 18S ssrRNA (18S), 28S lsrRNA (28S), myosin heavy chain (*MyoHC*),

Table 1
Biological samples that were acquired and subjected to molecular analysis at Texas A&M University, USA. All samples originated from a free-ranging cervid mammal host in either Canada (CA) or the United States (USA). *Onchocerca* DNA was amplified from whole adult worms or via ear or leg skin biopsies comprising microfilariae (*n* = number of samples collected).

Sample origin	Sample type	Host	Organization or institute
Newfoundland and Labrador, CA	Adult worms (*n* = 1)	*Rangifer tarandus*	Newfoundland and Labrador Department of Fisheries, Forestry and Agriculture
(Kenai Peninsula) (Interior) (Southeast Panhandle) Alaska, USA [a]	1. Leg skin (*n* = 17) [a] 2. Adult worms (*n* = 5) 3. Adult worms (*n* = 1)	1. *Alces alces* [a] 2. *Alces alces* 3. *Odocoileus hemionus*	1. Alaska Department of Fish & Game [a] 2. Alaska Department of Fish & Game 3. Alaska Department of Fish & Game
California, USA [a]	Black fly vectors [a]	*Odocoileus hemionus* [a]	1. San Gabriel Valley Mosquito & Vector Control District [a] 2. Greater Los Angeles County Vector Control District [a] 3. Lake County Vector Control District [a]
Maryland, USA	Adult worms (*n* = 3)	*Odocoileus virginianus*	Southeastern Cooperative Wildlife Disease Study
New York, USA	1. Ear skin (*n* = 15) 2. Adult worms (*n* = 1)	*Odocoileus virginianus*	Cornell University
West Virginia, USA	Adult worms (*n* = 7)	*Odocoileus virginianus*	West Virginia Division of Natural Resources
Wyoming, USA	1. Ear skin (*n* = 18) 2. Ear skin (*n* = 14) 3. Ear skin (n = 16)	1. *Cervus canadensis* 2. *Odocoileus hemionus* 3. *Odocoileus virginianus*	1. US Fish & Wildlife Service 2. Wyoming Game and Fish Department 3. Wyoming Game and Fish Department

[a] Denotes samples from previous studies (Benedict et al., 2023; Kulpa et al., 2021; Verocai et al., 2018b).

RNA polymerase II large subunit (*rbp1*), and 70 kDa heat shock proteins (*hsp70*). Each unique primer comprised its own cycling conditions that have been previously established (Morales-Hojas et al., 2006; Lefoulon et al., 2015). Moreover, archived *Onchocerca* DNA (see Table 1) underwent cPCR for gene regions that have no prior record in GenBank. All PCR products were then processed through agarose gel to reveal the presence of amplicons. A Cycle Pure ENZA kit (Omega Bio-Tek, Norcross, GA, USA) was used to purify DNA using the manufacturer's protocol and then Sanger sequenced using the same primers. Representative sequences (*n* = 3) of the eight gene regions in each *Onchocerca* lineage were submitted to the GenBank database (NCBI) (Supplementary Table S2).

Filarioid sequences were individually trimmed and aligned for each gene region (*nd5*, *cox1*, 12S, 18S, 28S, *hsp70*, *MyoHC*, *rbp1*) in MEGA X 10.1 (Kumar et al., 2018) using MUSCLE or v0.5 ProAlign software (Löytynoja and Milinkovitch, 2003). Alignment data for each gene region has been made permanently available through Mendeley Data (https://doi.org/10.17632/yxr5gtgwjt.1). Previously published *Onchocerca* sequences were downloaded from GenBank and aligned with the newly generated sequences for inclusion in phylogenetic analyses. After alignment, MEGA X 10.1 was used for corrected pairwise distance analyses (Tamura and Nei, 1993) (Supplementary Table S3-S8) and the subsequent data was used to create violin plots (Fig. 1) through the ggplot2 package in Rstudio (Wickham, 2011; Racine, 2012). SequenceMatrix (Vaidya et al., 2011) was used to concatenate gene regions and a length difference (ILD) test (Farris et al., 1995) was performed in PAUP* v4.0a (Sinauer Associates, Sunderland, MA, USA) to assess homogeneity between partitions. Prior to phylogenetic analysis, evolutionary models were generated using both MEGA X 10.1 (Kumar et al., 2018) and IQ-Tree v2.2.2.7 (Nguyen et al., 2015) (Supplementary Table S9). Of the eight sequencing targets, we concatenated seven and excluded *nd5* due to limited data (Fig. 2). Additional concatenations were performed and included mitochondrial markers (*cox1*, 12S) (Supplementary Fig. S1), nuclear markers (18S, 28S, *hsp70*, *MyoHC*, *rbp1*) (Supplementary Fig. S2), protein-encoding markers (*cox1*, *hsp70*, *MyoHC*, *rbp1*) (Supplementary Fig. S3), and rRNA markers (12S, 18S, 28S) (Supplementary Fig. S4).

Individual and concatenated gene sequences were both used for phylogenetic analyses. For each, maximum likelihood (ML) and Bayesian inference (BI) approaches were performed using RAxML v8.2 (Stamatakis, 2014) and MrBayes V3.2.7a (Ronquist et al., 2012), respectively, via the Cyberinfrastructure for Phylogenetic Research (CIPRES) web portal (https://www.phylo.org). Outgroups used in phylogenetic analyses were in the Family Onchocercidae and included: *Icosiella neglecta* (Diesing, 1851) from amphibians, *Oswaldofilaria chabaudi* Pereira, Souza and Bain, 2010 from lizards, and *Setaria labiatopapillosa* (Alessandrini, 1848) from bovids. Tree topologies were edited in FigTree v1.4.4 (https://tree.bio.ed.ac.uk/software/figtree/).

To reconstruct co-evolutionary scenarios between *Onchocerca* parasites and their vertebrate hosts (Table 2; Supplementary Table S10) Jane 4.0 (Conow et al., 2010) was used, which finds the most probable co-evolutionary scenario based on stipulated parameters and cost events. This software is based on the event-cost method, which models and assigns costs to evolutionary events (host switches, duplication, losses, and failure to diverge). This method allows reconstruction of the co-evolutionary scenario that minimizes the total cost of these events. The default values were used to have more direct comparisons to the most robust phylogenetic framework for *Onchocerca* (Lefoulon et al., 2017). As recommended, population numbers were higher than generation numbers (10,000 and 3,000, respectively) when used for analysis (Conow et al., 2010).

3. Results and discussion

Filarioid DNA of the eight molecular markers was successfully amplified for 100% of adult worm specimens (17/17) and 52.4% of cervid ear skin samples (33/63). This included positive amplifications of filarioid DNA of white-tailed deer ear skin from New York (12/15; 80%) and Wyoming (12/16; 75%), USA, and mule deer ear skin from Wyoming (9/14; 47.4%). Filarioid DNA was not found in elk ear skin samples (0/18; 0%). Four notable patterns are apparent in results of a multi-locus phylogenetic analysis that included 17 known filarioid species (Supplementary Table S2). First, all analyzed *Onchocerca* spp., including the five uncharacterized *Onchocerca* lineages, are grouped in one monophyletic clade (Fig. 2) validating our assumption that each grouped lineage belongs to the genus *Onchocerca*.

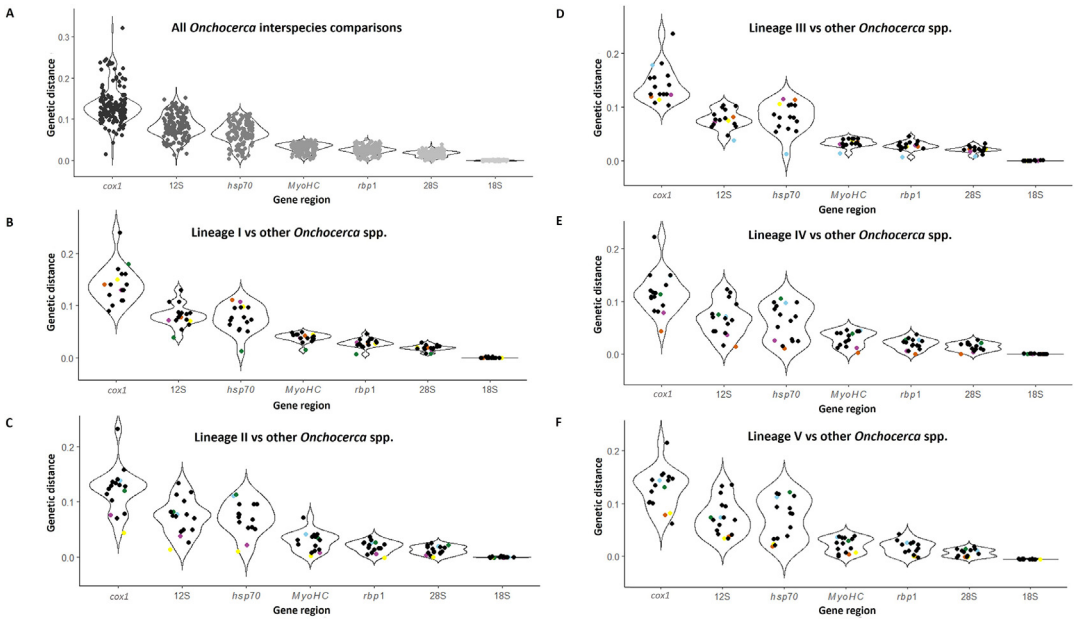

Fig. 1. Violin plots reflect the genetic diversity of various *Onchocerca* spp. at seven different genetic regions (i.e., *cox1*, 12S, 18S, 28S, *hsp70*, *MyoHC*, and *rbp1*). (A) Each point represents an interspecies comparison between all 17–18 *Onchocerca* spp./lineages (depending on genetic region) that were used for analysis or (B-F) a comparison of an *Onchocerca* lineage (n = 1) with other species/lineages. Black dots represent *Onchocerca* spp. comparisons and colored dots represent an *Onchocerca* lineage (blue = I, red = II, green = III, yellow = IV, purple = V). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

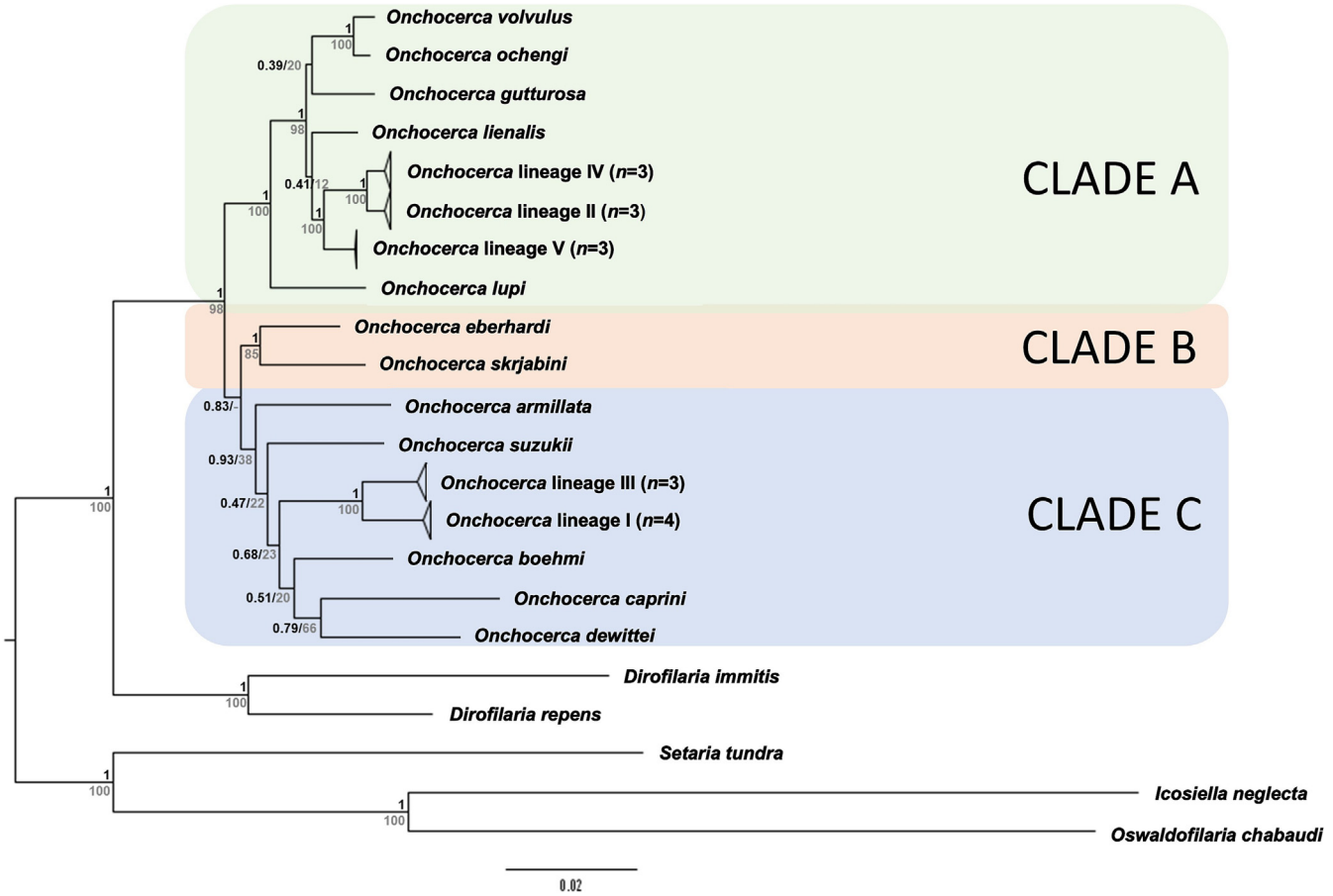

Fig. 2. Phylogenetic tree depicting the relationship of 22 different filarioid species using seven concatenated genetic markers (*cox1*, 12S, 18S, 28S, *hsp70*, *MyoHC*, and *rbp1*). The 17 *Onchocerca* spp. in this tree are separated into three possible clades which are designated Clades A, B, and C. Phylogenetic analysis is an amalgamation of using a Maximum likelihood approach with denoted bootstrap support (gray) and a Bayesian inference with posterior probabilities (black) approach. Filarial species *Oswaldofilaria chabaudi*, *Icosiella neglecta* and *Setaria labiatopapillosa* were used as outgroups.

Table 2
Onchocerca lineages found to date with corresponding geographic distribution and hosts. Prior to 2012, all *Onchocerca* diagnosed cases found in North American ungulates were classified as *Onchocerca cervipedis*. Molecular analysis has revealed distinguishable *Onchocerca* lineages that have yet to be described. Until morphological description, none of these lineages had been elevated to species status.

Onchocerca spp.	Sample origins	Known hosts	References
***Onchocerca* lineage I**	(Interior) Alaska, USA	*Alces alces*	Verocai et al., 2012
	(Southeast Panhandle) Alaska, USA [a]	*Odocoileus hemionus* [a]	(Unpublished data)
	Northwest Territories, CA	*Alces alces*	Lefoulon et al., 2017; Verocai et al., 2012
***Onchocerca* lineage II**	New York, USA	*Odocoileus virginianus*	McFrederick et al., 2013; (Unpublished data)
	Newfoundland and Labrador, CA [a]	*Rangifer tarandus*	(Unpublished data)
	Northwest Territories, CA	*Rangifer tarandus*	Mariyam Thomas, A., 2023
***Onchocerca* lineage III**	Maryland, USA [a]	*Odocoileus virginianus* [a]	(Unpublished data)
	New York, USA [a]		
	West Virginia, USA [a]		
***Onchocerca* lineage IV**	California, USA	*Odocoileus hemionus*	Kulpa et al., 2021; Verocai et al., 2018b
	Wyoming, USA [a]	*O. hemionus* [a]; *O. virginianus* [a]	(Unpublished data)
***Onchocerca* lineage V**	(Kenai Peninsula) Alaska, USA	*Alces alces*	Benedict et al., 2023

CA, Canada; USA, United States.
[a] Denotes newly identified locations and hosts from this study.

Second, the uncharacterized *Onchocerca* specimens form five separate reciprocal monophyletic clades (Fig. 2) that comprise values of high confidence (100% bootstrap support; 1.00 posterior probabilities). This reciprocal monophyly is shown across concatenated gene regions including mitochondrial, nuclear, and protein-encoding markers. Reciprocal monophyly is a useful criterion of species delimitation (De Queiroz, 2007) and thus supports our hypothesis that there are five distinct *Onchocerca* spp. associated with wild North American ungulates. Pairwise data comparisons unequivocally support four of these lineages as separate species, and we believe there is enough evidence to merit the recognition of a fifth by separating *Onchocerca* lineages II and IV. For example, the *cox1* region shows an average *Onchocerca* interspecies diversity of 12.26% (6.34–23.09%) and 12.22% (7.76–22.66%) for lineages II and IV, respectively, and 0.34% (0.00–0.51%) for intraspecies diversity. However, between these two lineages, there is an average pairwise difference of 3.94% (3.45–4.65%) (Fig. 2; Supplementary Table S3). While certainly both these populations exhibit a close pairwise relationship, there are no intraspecies comparisons that eclipse 0.51% in our analysis. We also know from Lefoulon et al. (2017) that some outlier interspecies comparisons can fall below 2.00% (e.g., *O. volvulus* and *Onchocerca ochengi*) and the majority of other interspecies comparisons begin at approximately 4.50%, relatively close to our 3.94% average. Gonçalves et al. (2021) report that *cox1* intraspecies comparisons range from 0.50-3.50%, just below our comparison of *Onchocerca* lineages II and IV. Complete mitogenome sequencing, a powerful tool that has provided resolution to other cryptic nematode relationships (Cháves-González et al., 2022), could provide greater context to these cryptic lineages. Table 2 categorizes these five separate lineages more formally, including their origins and host-parasite assemblages.

Third, within the *Onchocerca* clade, two to three distinct groups are regularly formed in analysis (Fig. 2). The first group, which we henceforth refer to as Clade A, has strong support present across most molecular markers and includes *Onchocerca* lineages II, IV, and V, together with those found in other ungulates (e.g., *O. ochengi* Bwangamoi, 1969, *Onchocerca lienalis* (Stiles, 1892) and *Onchocerca gutturosa* Neumann, 1910), carnivore (e.g., *O. lupi*), and human hosts (e.g., *O. volvulus*). The second group, or Clade B, is formed between two *Onchocerca* spp. with Cervidae host species, *Onchocerca eberhardi* Uni and Bain, 2007 and *Onchocerca skrjabini* Ruklyadev, 1964. However, this grouping is more strongly supported in nuclear or protein-encoding molecular markers (Supplementary Figs. S2 and S3) rather than mitochondrial (Supplementary Fig. S1). This finding is consistent with the clade seen in Lefoulon et al. (2017) with the notable exclusion of *Onchocerca flexuosa* (Weld, 1856) which did not have all molecular markers available for concatenation analyses. Interestingly, all three of these *Onchocerca* spp. (*O. eberhardi*, *O. skrjabini*, and *O. flexuosa*) form host-parasite assemblages with mammals from the Cervidae family. Despite this fact, no uncharacterized *Onchocerca* lineage, which are all known to infect North American cervids, is closely related. The remaining group, Clade C, has only weak support revealed in several nuclear markers (Supplementary Fig. S2). Nonetheless, within this group, uncharacterized *Onchocerca* lineages I and III are shown to be closely related to each other in both nuclear and mitochondrial markers. All three *Onchocerca* clades formed from our concatenated phylogenetic analysis comprise definitive hosts within Cervidae (Fig. 1). This was not the case in prior analyses which lacked cryptic *Onchocerca* diversity, and it is the only family of mammalian hosts to occupy all three clades. Considering this information, the most parsimonious co-evolution scenario for *Onchocerca*-host assemblages, or what would entail the fewest possible host switching events, would involve descent from former ancestral Cervidae hosts.

Lastly, *Onchocerca* lineages II, IV, and V of Clade A are not closely related to *Onchocerca* lineages I and III of Clade C (Fig. 2; Supplementary Table S3) and provide evidence of, at least, two separate geographic migration events of these *Onchocerca* spp. into the North American continent via the Bering land bridge. These diverse host-parasite assemblages with geographic mosaic patterns are best explained by the Stockholm Paradigm (Brooks and Hoberg, 2007; Hoberg and Brooks, 2008, 2010; Brooks et al., 2019), which postulates that parasite specialists can become parasite generalists in the face of environmental perturbation across space and time, such as what is seen with climate change (Kutz et al., 2014; Dobson et al., 2015). As a result, host-parasite assemblages can undergo cyclical taxon pulses that set in motion the mosaic coevolutionary landscape we face when investigating the North American *Onchocerca* spp. of wild cervids (Fig. 3). Prior research has shown this pattern in other parasitic species including certain groups of tapeworms (Haas et al., 2020) and nematodes (Verocai

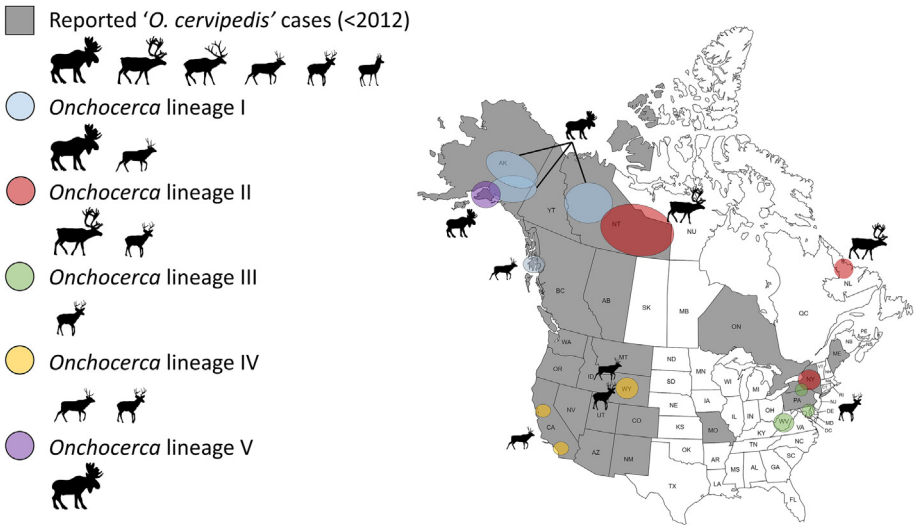

Fig. 3. North American map depicting *Onchocerca cervipedis* case reports prior to 2012 (gray) and the samples comprising *Onchocerca* spp. that have been uncovered since 2012 (blue, red, green, yellow, and purple circles). Each color corresponds to an *Onchocerca* lineage and the circle to the general area where it was found in (e.g., blue circles represent the area where *Onchocerca* lineage I was found). In addition, silhouettes of mammals represent definitive hosts that are now associated with each lineage. From left-to-right the mammal silhouettes in the "Reported from '*O. cervipedis*' cases (<2012)" section include: moose, caribou, elk, mule deer, white-tailed deer, and pronghorn. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2018a, 2020). This framework also helps explain the diversity of infected host species we see, including the capability for lineages to infect more than one type of cervid. While it does appear, as predicted, there is greater *Onchocerca* spp. diversity in North American wild cervids, it is not due to host-specific lineage strains as speculated (McFrederick et al., 2013). At this time, at least three of the five *Onchocerca* genetic lineages associated with wild North American ungulates are known to infect multiple cervid host species including caribou, moose, mule deer, and white-tailed deer (Table 2; Fig. 3; Supplementary Table S5). On the other hand, there is evidence lineage V only infect genetically isolated moose of the Kenai Peninsula (Wilson et al., 2015). Elk ear samples were not positive for *Onchocerca* DNA in this study. While it is possible that elk are not a definitive host for any *Onchocerca* lineage, contrary to a previous report (De Nio and West, 1942), these results could be from many limiting factors such as inadequate sample size, population selection (e.g., age class, geographic origin), or microfilariae dwelling in a different anatomic location besides ears.

The phylogenetic separation of *Onchocerca* lineages II, IV, and V of Clade A and the *Onchocerca* lineages I and III of Clade C represents a geographic isolation of deep time and space (Fig. 2). However, each group comprises complex host-parasite assemblages that obscure the historical biogeography of each separate lineage. Three out of the four host species confirmed to be infected with these cryptic *Onchocerca* spp. have had a presence in North America for millions of years: both ancestors of *Odocoileus* Rafinesque, 1832 and *Rangifer* C.H. Smith, 1827 expanded to North America by 4–5 and 1–2 million years ago, respectively (Cronin, 1989; Harington, 1999; Feldhamer et al., 2003; Gilbert et al., 2006; Verocai et al., 2020). On the other hand, *Alces alces* (Linnaeus, 1758) migrated to the Nearctic from the Palearctic around the late Pleistocene, or about 10,000 years ago (Feldhamer et al., 2003). Both groups from Clades A and C share *Odocoileus* spp. and *A. alces* hosts, with also *R. tarandus* being a definitive host of *Onchocerca* lineage II in Clade A. We also do not know which, if any, of these ungulates are the primary host for each *Onchocerca* lineage. In other words, we cannot speculate on which group of *Onchocerca* lineages first expanded to the Nearctic and when this expansion event occurred. However, we hypothesize that one of these *Onchocerca* groups was permanently, or at least intermittently, isolated in Eurasia while the other spread through the Americas.

Our molecular data underline that morphological analyses of this group of *Onchocerca* spp. needs to be readdressed. Morphological studies of this group remain inconsistent, and this could be due to several factors. For one, there are morphological discrepancies in the scientific literature and a consensus description of *O. cervipedis* has not been published to date (Wehr and Dikmans, 1935; Caballero, 1945). We hypothesize that there are numerous reports attributed to *O. cervipedis* that were likely based on the assumption that this was the only *Onchocerca* sp. known to parasitize wild North American ungulates. Furthermore, with the discovery of cryptic *Onchocerca* diversity in wild North American cervids, it is not possible to ascertain which of the five lineages, if any, was originally described as *O. cervipedis*. For these reasons, a redescription of *O. cervipedis* sensu stricto is required to confirm its validity. However, we hypothesize the original description of *O. cervipedis* is based on *Onchocerca* lineage IV. The original specimens were described from *O. virginianus* and *O. hemionus* hosts in Montana, USA and British Columbia, Canada, respectively. Both locations are geographically close to the area where *Onchocerca* lineage IV DNA was found in Wyoming and comprise the same host mammals. While these *Onchocerca* lineages may appear grossly similar to each other, it is possible that minor morphological features or unexamined ultrastructural differences exist between lineages or lineage groups. This could explain why discrepancies exist in the literature (Wehr and Dikmans, 1935; Caballero, 1945). This prospect is reminiscent of anatomical differences recently found between *O. skrjabini* subpopulations of red deer (*Cervus elaphus* L.) from Switzerland and dwarf goats (*Capra hircus* L.) from Japan (Yagi et al., 1994; Manzanell et al., 2022). It is noteworthy that *O. skrjabini* is primarily associated with cervid hosts, and goats are likely incidental hosts; therefore, this could have partially contributed to morphological discrepancies (Manzanell et al., 2022).

Interestingly, if we compare our molecular phylogenetic topology of the genus *Onchocerca* with evolutionary hypotheses developed using morphological traits of *Onchocerca* (Bain et al., 1976; Bain, 1981), the uncharacterized *Onchocerca* spp. of wild North American cervids are found in two distinct groups: one clade that is comprised mostly of morphological ancestral species (i.e., *Onchocerca* lineages I and III) and the other clade that is comprised mostly of derived species (i.e., *Onchocerca* lineages II, IV, and V). The derived clade comprises nearly all domesticated host species

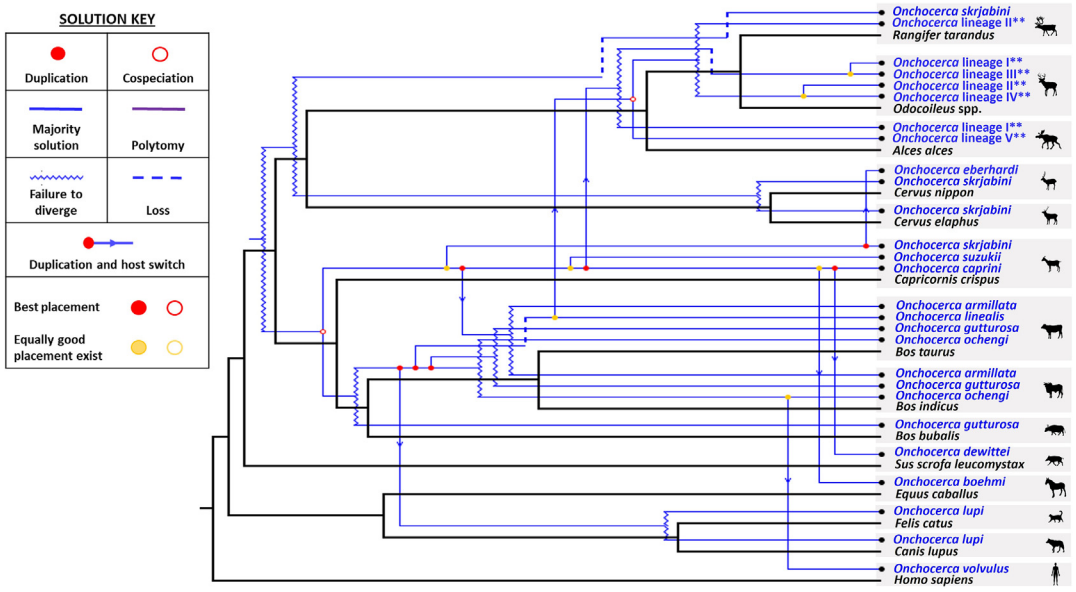

Fig. 4. Cophylogenetic reconstruction using known Onchocerca spp. and their definitive vertebrate hosts. Each event (e.g., cospeciation, duplication, duplication with host switch, loss, and failure to diverge) was assigned the default cost parameters except for duplication then host switching ("co-speciation" event = 0 cost; "duplication" event = 1; "loss" event = 1; "duplication then host switching" event = 2) for analysis in Jane 4.0 software (Conow et al., 2010). This figure represents one of six different isomorphic solutions representing the lowest cost scenarios (n = 36) using a population of 10,000 and a generation of 3,000.

(e.g., cattle, dogs, and cats) and it has been proposed that the process of domestication led to parasite host switching events with more recent *Onchocerca* speciation (Lefoulon et al., 2017). However, our co-evolutionary analysis reveals that other factors likely influenced diversification. Moreover, these morphological descriptions are based on the features of adult nematode specimens. It has been suggested that traits of the larval stages such as infective L3s could be more informative for evolutionary studies (Chabaud and Bain, 1994). The L3 stage, however, is often not accessible, limiting most morphological studies to adults or microfilariae.

When using co-evolutionary analyses with default parameters, there were six distinctly possible solutions (Fig. 4; Supplementary Figs. S5-S9). All solutions reveal two consistent patterns regarding the *Onchocerca* lineages of wild North American cervids. Firstly, co-evolutionary analysis showed *O. lienalis* ancestors of bovids host-switching to wild cervids (4/6; 66%), to give rise to *Onchocerca* lineages II, IV, and V (bootstrap support = 100) (Fig. 4; Supplementary Figs. S5-S7). This adds complexity to the idea that domestication was the major driver for host-switching and speciation that we see in Clade A. However, it is possible that this host-switching was the result of increased wild and domestic animal contact through human influence, leading to greater chances of parasite transmission between the two groups. Secondly, *Onchocerca* lineages I and III arose from *O. caprini* ancestors, a nematode of Japanese serows (*Capricornis crispus* (Swinhoe 1870)) (bootstrap support = 100) (Fig. 4; Supplementary Figs. S5-S9). This host-switching event was not shown in former studies looking at '*O. cervipedis*' (i.e., *Onchocerca* lineage I), but is a plausible scenario considering their similar adult worm morphology (e.g., external labial/cephalic papillae, esophagus, vulva position, and female cuticle) and weak co-evolutionary patterns of filariae-*Wolbachia* associations (Lefoulon et al., 2017).

It should be noted, Clade A (Fig. 2) encompasses many of the *Onchocerca* spp. that have been reported to infect humans (e.g., *O. gutturosa, O. lienalis*, and *O. lupi*) or are definitive hosts for their life cycle (e.g., *O. volvulus*) (Cambra-Pellejà et al., 2020). The only Clade A species that has not been reported to infect humans in is *O. ochengi* and the zoonotic potential of these three newly revealed *Onchocerca* lineages is unknown. Therefore, thorough documentation and characterization of *Onchocerca* diversity should be considered a public health concern.

The seven molecular markers used in this study were selected based on availability of sequence data from previous studies (Lefoulon et al., 2015, 2017), which facilitated phylogenetic analysis to investigate the evolutionary history of the genus *Onchocerca* with a focus on novel lineages uncovered from wild North American cervids. When comparing each genetic region with interspecies genetic diversity (Fig. 2), mitochondrial markers (e.g., *cox1*, 12S) have far greater genetic distance values than nuclear markers (e.g., 18S, 28S, *hsp70*, *MyoHC*, and *rbp1*) and comprise more overall variability. Given that mtDNA evolves more rapidly than nuclear DNA, this finding is expected (Hwang and Kim, 1999; Le et al., 2000; Blouin, 2002; Allio et al., 2017; Chan et al., 2021). Further, maternal heritability of mtDNA and restricted recombination help elucidate evolutionary history (Hurst and Jiggins, 2005). Moreover, because mtDNA, particularly *cox1*, is such an important diagnostic tool to parasitologists and researchers, there is an abundance of mitochondrial sequences available in genetic databases for comparison (Mejías-Alpízar et al., 2024). Despite these advantages, mtDNA is not without its problems. Nuclear copies of mtDNA (i.e., nuclear-mitochondrial DNA segments, NUMTs), or DNA from the mitochondria that has been integrated in the nuclear DNA, can be amplified via cPCR, which can cause misleading results (Bensasson et al., 2001). Additionally, direct and/or indirect selection pressures can disrupt the mitochondria as a neutral model (Ballard and Whitlock, 2004). Thus, it could be difficult interpreting results using molecular markers from one source (i.e., nuclear or mitochondrial DNA).

Despite lower *Onchocerca* interspecies genetic distance in nuclear compared with mitochondrial markers, nuclear DNA can still provide meaningful information regarding taxonomic resolution. Congruence in mitochondrial and nuclear phylogenetic analyses can bolster support in evolutionary tree topology (Kern et al., 2020). Nuclear markers are generally most useful when teasing apart organisms of higher taxonomic order, and minute nucleotide variations can offer enough evidence to highlight interspecies differences. This is best exemplified with a marker such as 18S rRNA which has miniscule genetic distances with minimal ranges

(0.00–0.16%) (Fig. 1). Still, this is not true for all nuclear markers. For example, *hsp70* has far greater genetic distances and ranges (2.23–11.48%) (Fig. 1). These two genetic regions exemplify how marker choice can hinge on the diagnostic or research question at hand. For example, 18S rRNA is not a particularly useful marker for deduction of the evolutionary history of the genus *Onchocerca* and arguably should be reserved for diagnostic scenarios.

Regardless of the molecular markers used (i.e., nuclear, nuclear ribosomal, mitochondrial) variation within and among gene region (s) was noticed (Fig. 1; Supplementary Table S3). For example, *Onchocerca* lineages I and III have closer genetic distances to each other (i.e., lower) compared with any of the other three lineages in five of the six gene regions (excluding 18S rRNA due to low interspecific variation). However, based on *cox1*, lineages I and III have the highest genetic distance compared with each other versus the other three lineages (Fig. 1). The use of multiple mtDNA and nDNA markers will mitigate the effects of a single gene region variability to provide enhanced resolution to species relatedness and evolutionary history. Moreover, mitogenome analysis could be a robust tool to mitigate gene variability problems, and eliminate other historic analysis challenges such as NUMTs (Yilmaz et al., 2016; Crainey et al., 2018).

Despite the global importance of the genus *Onchocerca* and mounting evidence of its cryptic diversity, knowledge regarding North American *Onchocerca* diversity remains sparse. To better understand North American filarial diversity, we sequenced novel *Onchocerca* spp. and used a robust, multi-gene phylogenetic approach for genus-level comparisons. Our analysis supports four or five separate species and groups two or three of these *Onchocerca* lineages in Clade A and two *Onchocerca* lineages in Clade C. Based on *Onchocerca* lineages II and IV being accepted as separate species, the number of valid *Onchocerca* spp. would be as high as 42 species. This includes the delineation of *Onchocerca dewittei* from *Onchocerca japonica* into separate species, the recently uncovered *Onchocerca borneensis*, and the reclassification of *Loxodontofilaria caprini* to *Onchocerca caprini* (Bain et al., 2013; Lefoulon et al., 2015, 2017; Uni et al., 2020).

Clade A is mostly comprised of parasites that infect domestic animals or humans and the host-switching events from ancestral bovids to wild cervids resolved by cophylogenetic analyses obscure the degree to which domestication influences parasite speciation. In addition, the distant groupings of *Onchocerca* lineages provide evidence of at least two distinct migration events across the Bering land bridge. Lastly, we reveal mitochondrial (e.g., *cox1* and 12S) and nuclear markers (e.g., *hsp70*) that, when concatenated together, are best utilized to understand evolutionary questions. A further reevaluation using molecular clock analysis on complete mitogenomes could provide further clarity to the complicated historical biogeography of *Onchocerca* spp. in North American wild cervids (Hoberg and Klassen, 2002; Galbreath et al., 2020). Our findings also support a call for a redescription of the original '*O. cervipedis*' species in conjunction with the morphological examination of the type material for *O. cervipedis sensu* Wehr & Dikmans, 1935., morphological description of adult and microfilariae of all five *Onchocerca* lineages, and a reevaluation of all novel *Onchocerca* spp. vectors and hosts, taking into consideration potential differences in anatomic location of adult nematodes amongst hosts.

CRediT authorship contribution statement

Matthew R. Kulpa: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Emilie Lefoulon:** Writing – review & editing, Methodology. **Kimberlee B. Beckmen:** Writing – review & editing, Resources. **Samantha E. Allen:** Writing – review & editing, Resources. **Jennifer Malmberg:** Writing – review & editing, Resources. **John A. Crouse:** Writing – review & editing, Resources. **Daniel P. Thompson:** Writing – review & editing, Resources. **Bridgett M. Benedict:** Writing – review & editing, Resources. **Dayna A. Goldsmith:** Writing – review & editing, Resources. **Sara McCarthy:** Writing – review & editing, Resources. **Lee C. Jones:** Writing – review & editing, Resources. **Michael J. Yabsley:** Writing – review & editing, Resources. **James M. Crum:** Investigation, Writing – review & editing. **Susan J. Kutz:** Writing – review & editing, Resources. **Guilherme G. Verocai:** Writing – review & editing, Supervision, Conceptualization.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2024.10.006.

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