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Rajnish Sharma
University of Saskatchewan, ras863@mail.usask.ca

Peter C. Thompson
Beltsville Agricultural Research Center

Eric P. Hoberg
University of New Mexico, Albuquerque

W. Brad Scandrett
Saskatoon Laboratory

Kelly Konecsni
Saskatoon Laboratory

See next page for additional authors

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Hiding in plain sight: discovery and phylogeography of a cryptic species of *Trichinella* (Nematoda: Trichinellidae) in wolverine (*Gulo gulo*)

Rajnish Sharma [a,*,1], Peter C. Thompson [b,1], Eric P. Hoberg [c], W. Brad Scandrett [d], Kelly Konecsni [d], N. Jane Harms [e], Piia M. Kukka [e], Thomas S. Jung [e,f], Brett Elkin [g], Robert Mulders [g], Nicholas C. Larter [h], Marsha Branigan [i], Jodie Pongracz [i], Brent Wagner [a], Pratap Kafle [a], Vladislav A. Lobanov [d], Benjamin M. Rosenthal [b,2], Emily J. Jenkins [a,2]

[a] Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK S7N 5B4, Canada
[b] USDA-Agricultural Research Service, Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center, 10300 Baltimore Avenue, Beltsville, MD 20705, USA
[c] Museum of Southwestern Biology and Department of Biology, University of New Mexico, Albuquerque, NM 87131-0001, USA
[d] Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, Saskatoon Laboratory, 116 Veterinary Road, Saskatoon, SK S7N 2R3, Canada
[e] Department of Environment, Government of Yukon, P.O. Box 2703, Whitehorse, YT Y1A 2C6, Canada
[f] Department of Renewable Resources, University of Alberta, 751 General Services Building, Edmonton, AB, T6G 2H1, Canada
[g] Department of Environment and Natural Resources, Government of the Northwest Territories, 600, 5102-50th Avenue, Yellowknife, NT X1A 3S8, Canada
[h] Department of Environment and Natural Resources, Government of the Northwest Territories, P.O. Box 240, Fort Simpson, NT X0E 0N0, Canada
[i] Department of Environment and Natural Resources, Government of the Northwest Territories, P.O. Box 2749, Inuvik, NT X0E 0T0, Canada

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ABSTRACT

Understanding parasite diversity and distribution is essential in managing the potential impact of parasitic diseases in animals and people. Imperfect diagnostic methods, however, may conceal cryptic species. Here, we report the discovery and phylogeography of a previously unrecognized species of *Trichinella* in wolverine (*Gulo gulo*) from northwestern Canada that was indistinguishable from *T. nativa* using the standard multiplex PCR assay based on the expansion segment 5 (ESV) of ribosomal DNA. The novel genotype, designated as T13, was discovered when sequencing the mitochondrial genome. Phylogenetic analyses of the mitochondrial genome and of 15 concatenated single copy orthologs of nuclear DNA indicated a common ancestor for the encapsulated clade is shared by a subclade containing *Trichinella spiralis* and *Trichinella nelsoni,* and a subclade containing T13 and remaining taxa: T12 + (T2 + T6) + [(T5 + T9) + (T3 + T8)]. Of 95 individual hosts from 12 species of mammalian carnivores from northwestern Canada from which larvae were identified as *T. nativa* on multiplex PCR, only wolverines were infected with T13 (14 of 42 individuals). These infections were single or mixed with *T. nativa* and/or T6. Visual examination and motility testing confirmed that T13 is encapsulated and likely freeze-tolerant. We developed a new Polymerase Chain Reaction-Restriction Fragment Length Polymorphism which unequivocally distinguishes between T13 and *T. nativa*. We propose *Trichinella chanchalensis* n. sp. for T13, based on significant genetic divergence from other species of *Trichinella* and broad-based sampling of the *Trichinella* genome. Exploration of Alaskan and Siberian isolates may contribute to further resolution of a phylogeographically complex history for species of *Trichinella* across Beringia, including *Trichinella chanchalensis* n. sp. (T13).

1. Introduction

The zoonotic nematode *Trichinella* was considered a monotypic genus, recognizing only *Trichinella spiralis*, until 1972 (Pozio et al., 2009). Thereafter, researchers using different methods (e.g., biology, alloenzymes, crossbreeding, biogeography and molecular markers) recognized, differentiated and described a series of species and genotypes. These *Trichinella* taxa lacked distinctive morphological features, and there was no singular clear proposal for establishing species limits within the *Trichinella* genus. By 1972, three species were recognized in the genus, and eight more species and genotypes were discovered between 1992 and 2008 (Britov and Boev, 1972; Garkavi, 1972; Pozio et al., 1992; Nagano et al.,

* Corresponding author.
E-mail address: ras863@mail.usask.ca (R. Sharma).
[1] These authors contributed equally.
[2] These senior co-authors contributed equally.

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1999; Pozio et al., 1999, 2002; Pozio and La Rosa, 2000; Krivokapich et al., 2008). Currently, 12 distinct lineages of *Trichinella* are grouped into encapsulated and non-encapsulated forms based on the presence or absence of a collagen layer surrounding the nurse cell containing the infective L1 (Zarlenga et al., 2006; Pozio and Zarlenga, 2013). This subdivision has been corroborated by subsequent molecular phylogenetic analyses (Zarlenga et al., 2006; Pozio and Zarlenga, 2013; Korhonen et al., 2016).

The encapsulated clade includes six named species, *T. spiralis* (T1), *Trichinella nativa* (T2), *Trichinella britovi* (T3), *Trichinella murrelli* (T5), *Trichinella nelsoni* (T7), and *Trichinella patagoniensis* (T12); and three unnamed genotypes, *Trichinella* T6, T8, and T9. The non-encapsulated clade includes three species: *Trichinella pseudospiralis* (T4), *Trichinella papuae* (T10) and *Trichinella zimbabwensis* (T11) (Pozio and Zarlenga, 2013). All species and genotypes of *Trichinella* are zoonotic, circulating among mammals; additionally, *T. pseudospiralis* has also been found in birds of prey and scavengers, and *T. papuae* and *T. zimbabwensis* in reptiles. *Trichinella spiralis* and *T. pseudospiralis* are globally distributed (La Rosa et al., 2003a; Rosenthal et al., 2008), whereas the other species are geographically restricted. For example, *T. nativa* is restricted to the Holarctic region, with its southern boundary between the isotherms −5 and −4 °C in January (Pozio and Zarlenga, 2005). Besides geographical distribution, taxa of *Trichinella* vary in other characteristics including their host specificity and resistance to freezing (Pozio and Murrell, 2006).

In North America, five taxa of *Trichinella* (*T. spiralis*, *T. nativa*, *T. pseudospiralis*, *T. murrelli* and *Trichinella* T6) have been found in terrestrial vertebrates (Gajadhar and Forbes, 2010; Jenkins et al., 2013). *Trichinella spiralis* has been eradicated from commercial swine in Canada and is rarely reported in non-commercial swine and wildlife (Appleyard et al., 1998; Gajadhar and Forbes, 2010; Newman, 2014). *Trichinella pseudospiralis* and *T. murrelli* have rarely been reported in wild animals from Canada (Gajadhar and Forbes 2010; Sharma et al., 2019). *Trichinella nativa* and *Trichinella* T6 are the most common species in free-ranging mammals (e.g., Mustelidae, Canidae, Felidae, Odobenidae and Ursidae) from Canada, with reported prevalence varying from 1% to 77% (Gajadhar and Forbes, 2010; Jenkins et al., 2013). These species of *Trichinella* are sympatric geographically and within individual hosts, including gray wolves (*Canis lupus*) (La Rosa et al., 2003b) and wolverines (Reichard et al., 2008).

Controversy over the ability of *T. nativa* and *Trichinella* T6 to hybridize led us to further genetically characterize larvae of T2 and T6 obtained in 338 wolverines from the Yukon, identified using a multiplex PCR (Zarlenga et al., 1999). While confirming multiplex genotyping by DNA sequencing, we discovered an undescribed cryptic species of *Trichinella*. Herein, we describe the larval morphology and molecular diagnostics for this previously unrecognized lineage and explore phylogeny, geography, and host range of this proposed species.

2. Material and methods

2.1. Recovery and identification of Trichinella spp. larvae for whole genome sequencing

Larvae of *Trichinella* spp. were recovered from previously frozen wolverine muscle tissue by the double separatory funnel digestion method (Forbes and Gajadhar, 1999). DNA was extracted from pools of 15 larvae from an individual wolverine (17WV049) using Promega DNA IQ kits (Promega, Madison, WI, USA) as per the manufacturer's instructions. Multiplex PCR was performed to identify species/genotype (Zarlenga et al., 1999).

2.2. Whole genome sequencing

Only a single isolate (17WV049) was subjected to whole genome sequencing. To sequence both nuclear and mitochondrial loci, genomic DNA were prepared for paired-end next-generation sequencing on the Illumina MiSeq platform. DNA was quantified on a Qubit fluorometer (ThermoFisher, Waltham, MA, USA), as per the manufacturer's instructions for high sensitivity assays. Sequencing libraries were prepared using Nextera DNA Flex library kits (Illumina Inc., San Diego, CA, USA) according to the manufacturer's recommendations. Briefly, DNA was fragmented by transposase and linked to Illumina specific adapters (see manufacturer's specifications). Labeled fragments were amplified by PCR with the following conditions: initial denaturation at 98 °C for 3 min, followed by six cycles of 98 °C, 40 s; 62 °C, 30 s; 68 °C, 2 min, with a final extension of 68 °C for 1 min. Final libraries were obtained by cleaning the amplified DNA with Solid Phase Reversible Immobilization (SPRI) beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Libraries were quantified on a Qubit fluorometer and diluted to 8 picomolar, and run on an Illumina MiSeq. Initial library quality was determined by generating 150 bp paired-end reads on a MiSeq v2 nano kit. In order to provide adequate coverage of the entire genome, libraries were then subjected to a paired-end 300 bp MiSeq V3 run. Reads were pooled and analyzed using Geneious ®11.1.5 (BioMatters, Ltd., New Zealand).

2.3. Data analysis

2.3.1. De novo assembly

Paired-end reads were trimmed to have no bases lower than Q20 (<1 in 100 chance of an error) and no more than two bases with Q-scores lower than Q30 (<1 in 1000 chance of an error) prior to assembly. We performed de novo assembly of the whole genome sequencing (WGS) reads using Geneious Assembler with medium–low sensitivity in Geneious v.11.1.5. The mitochondrial genome was identified based on BLAST searches of contigs against complete mitochondrial genomes of 12 taxa of *Trichinella* downloaded from GenBank (Mohandas et al., 2014). Reads were also mapped to the chromosomal reference of *T. spiralis* (Thompson et al., 2017) using standard Geneious assembler (sensitivity: medium–low/fast) to reach consensus.

2.3.2. Annotation

Annotations were transferred from mitochondrial genomes of *T. nativa* (GenBank Accession no: N 025752; Mohandas et al., 2014) and listed chromosomes of *T. spiralis* (Mitreva et al., 2011; Thompson et al., 2017) to the cryptic species of *Trichinella*.

2.3.3. Phylogenetic analysis

Phylogenetic trees were generated for both mitochondrial and nuclear DNA sequences from all *Trichinella* taxa. For comparison of nuclear genomes, 15 single-copy orthologs (SCO) present in all *Trichinella* taxa were identified (Korhonen et al., 2016). DNA sequences from all 15 SCOs were concatenated for each taxon, and aligned using MAFFT v.7.388 (Katoh and Standley, 2013) as implemented in Geneious v.11.1.5. A total of 62,334 nucleotides were present in the final alignment. Mitochondrial sequences were aligned separately, as they have a different mode of inheritance. JModelTest was used to determine the best substitution model for tree building. Phylogenetic trees were built using maximum likelihood (PHYML with GTR substitution model; Guindon et al., 2010), and neighbor-joining algorithms (as implemented in Geneious tree builder with the HKY substitution model) to determine the evolutionary relationships among new and known taxa of *Trichinella*. In order to confirm the relationships in the concate-

nated nuclear and mitochondrial trees, we also evaluated the relationships of all taxa using 15 SCOs individually.

2.4. Geographical distribution and host range of cryptic species

2.4.1. Isolates of Trichinella nativa as classified by multiplex PCR

Larvae or extracted DNA of *Trichinella* that had been identified as *T. nativa* using multiplex PCR (Zarlenga et al., 1999) were obtained from 95 animals including 12 species of mammalian carnivores (Table 1) from Canada; 37 were archived larvae at the Canadian Food Inspection Agency (CFIA), six were from Arctic foxes (*Vulpes lagopus*) from the Northwest Territories, 41 were from wolverines collected from the Yukon and Northwest Territories, and 11 were from red foxes (*V. vulpes*) from Québec (Bachand et al., 2018).

In conjunction with the Yukon government, we legally obtained wolverine carcasses from licensed trappers as a by-product of their legal harvest activities (Kukka et al., 2017). Export permits were obtained from the relevant government wildlife management agencies to ship samples out of the territory. All animal use (including Arctic and Red fox) was considered Level A Category of Invasiveness (animals were harvested for reasons other than research) and therefore exempt from review by the Animal Research Ethics Board at the University of Saskatchewan, Canada in accordance with guidelines of the Canadian Council on Animal Care.

For those samples from which DNA had not been previously extracted, DNA was extracted from five individual larvae using Proteinase K (Zarlenga et al., 1999; Scandrett et al., 2018).

2.4.2. Mitochondrial PCR-restriction fragment length polymorphism (PCR-RFLP)

To differentiate the cryptic species of *Trichinella* from *T. nativa*, we designed a novel PCR-Restriction Fragment Length Polymorphism (RFLP) assay. The PCR targeted the mitochondrial cytochrome b (Cytb) gene using the primer pair Cytb-JLF199 (5′-ATACGAGAAGTAAAATTTGG-3′) and Cytb JLR1093 (5′-TCATT GAATAGAGTGTGGTT-3′), which were designed using the Primer3 plug-in (Rozen and Skaletsky, 2000) for Geneious v.11.1.5 Kearse et al. (2012). Each reaction contained 1X AmpliTaq Gold 360 master mix (Applied Biosystems, CA, USA), 1 µl of each primer, 3 µl of GC enhancer (Applied Biosystems, CA, USA), and 3 µl of genomic DNA in a 35 µl reaction. PCR was conducted in a thermocycler (Bio-Rad, USA) with the following conditions: 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min, then a final extension step at 72 °C for 10 min. Amplified PCR products of approximately 920 bp were digested using *Bsa*I (New England Biolabs, Whitby, ON, Canada) and *Bgl*II (New England Biolabs) in buffer NEB 3.1 (New England Biolabs) at 37 °C for 2 h. T2, T6, and T13 were differentiated by separating these digestion products via electrophoresis in a 2% agarose gel under ultraviolet (UV) light using the Gel Doc system (Alpha Innotech Alpha Imager digital imaging system; Fig. 1). DNA from these same three taxa of *Trichinella* was used as positive controls in each run of the multiplex PCR and PCR-RFLP, as well as a no-DNA negative control.

2.5. Motility, viability, and encapsulation status of larvae

For 14 wolverine samples positive for T13 based on RFLP, additional larvae were isolated from frozen tongues to assess morphology and motility after freezing at −20 °C for 9 months to 8 years. Briefly, tissues were digested using the double separatory funnel digestion method (Forbes and Gajadhar, 1999) and larvae were examined under a dissecting microscope after incubating in a Petri dish at 37 °C for 30 min. Motile larvae were counted, and the number expressed as a percentage of the total number of larvae examined. Based on morphology, coiled and 'C' (comma) shaped larvae were classified as alive or dead, respectively. Small samples of thawed tissues were also compressed between glass plates (compressoria) and examined for the presence of a capsule surrounding larvae in situ.

2.6. Morphometrics of muscle larvae of T13

Muscle larvae were recovered by digesting tissues (Forbes and Gajadhar, 1999) of wolverines with single species infections of T13, based on initial investigation of recovered isolates by mPCR and mitochondrial PCR-RFLP (Table 1). Larvae (n = 58) were placed individually on a glass slide in a drop of tap water (50 µL) and heat fixed by gently passing the slide over the Bunsen burner flame. Larvae which had assumed a "C" shape were examined in detail under a bright-field microscope (Olympus BX51) and morphometric measurements were done with the imaging software (CellSens;

Table 1
Host, geographic location, and identification (based on multiplex PCR and mitochondrial PCR+RFLP) of *Trichinella nativa* (T2) and *Trichinella chanchalensis* (T13).

Animal Host	N[a]	Location[b] (n[c])	Designation based on							
			Multiplex PCR		Mitochondrial PCR + RFLP[d]					
			T2	T2 + T6	T2	T6	T13	T2 + T6	T2 + T13	T6 + T13
Wolverine (*Gulo gulo*)	42	NT (27), YT (15)	39	3	23	1	11	4	2	1
Wolf (*Canis lupus*)	8	NL (3), NT (5)	8	0	8	0	0	0	0	0
Walrus (*Odobenus rosmarus*)	1	QC (1)	1	0	1	0	0	0	0	0
Raccoon (*Procyon lotor*)	1	QC (1)	1	0	1	0	0	0	0	0
Red Fox (*Vulpes vulpes*)	19	NT (3), QC (16)	19	0	17	0	0	2	0	0
Polar Bear (*Ursus maritimus*)	3	NT (2), QC (1)	3	0	3	0	0	0	0	0
Mountain Lion (*Puma concolor*)	9	BC (8), AB (1)	7	2	4	1	0	4	0	0
Grizzly Bear (*Ursus arctos*)	1	BC (1)	1	0	1	0	0	0	0	0
Fisher (*Pekania pennant*)	2	BC (2)	2	0	2	0	0	0	0	0
Coyote (*Canis latrans*)	1	QC (1)	1	0	1	0	0	0	0	0
Black Bear (*Ursus americanus*)	1	NT (1)	1	0	0	0	0	1	0	0
Bear[e] (*Ursus* spp.)	1	BC (1)	1	0	1	0	0	0	0	0
Arctic Fox (*Vulpes lagopus*)	6	NT (6)	6	0	6	0	0	0	0	0
Total	95	NT (44), QC (20), YT (15), BC (12), NL (3), AB (1)	90	5	68	2	11	11	2	1

[a] *N*, total number of animals tested.
[b] Names of provinces and territories in Canada: AB, Alberta; BC, British Columbia; NL, Newfoundland and Labrador; NT, Northwest Territories; YT, Yukon; QC, Québec.
[c] *n*, number of animals from a respective location.
[d] RFLP, Restriction Fragment Length Polymorphism.
[e] Species unknown.

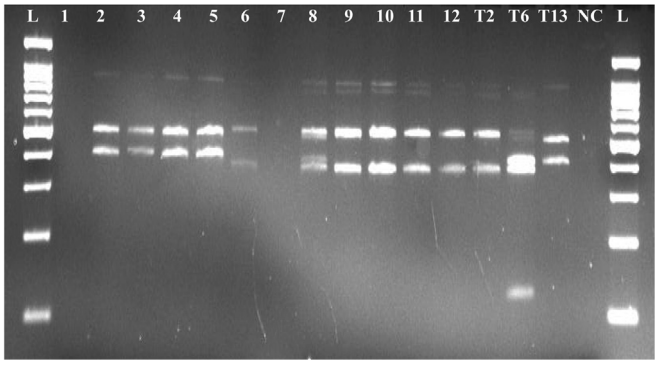

Fig. 1. Restriction fragment length polymorphism in larvae designated as *Trichinella nativa* using a conventional multiplex PCR assay. The last three lanes show restriction fragments resulting from the amplification and digestion of *T. nativa* (T2), *Trichinella* T6, and the newly recognized species (*Trichinella chanchalensis* n.sp.; T13). RFLP thus demonstrated isolates in lanes 2–5 to be the new species, and those in lanes 6, 8–12 to be *T. nativa;* Lanes L have 100 bp ladder.

Olympus Soft Imaging Solutions). The following morphometric parameters were evaluated: total body length, width at mid-body, length of oesophagus, length of stichosome, length of genital primordium, length of rectum, and distance from posterior margin of genital primordium to posterior extremity of the body. Gender for individual muscle larvae was determined by the position of intestine, intestinal bulb, and length of rectum, as per Belosevic and Dick (1980) and Britov (1982). Male larvae had (1) intestinal bulb close to the convex surface; (2) intestine crossing the gonad from the convex to the concave surface; and (3) length of rectum 40–50 µm. Female larvae had (1) intestinal bulb close to the concave surface; (2) intestine on the convex surface and/or intestine crossing the gonad from the convex to the concave surface; and (3) length of rectum 20–30 µm. If rectal length fell between 30 and 40 µm, gender was decided based on the other two criteria (position of intestine and intestinal bulb). After molecular characterization, we retained morphological data only for larvae confirmed as T13 using PCR-RFLP.

2.7. Archival

Syntypes, voucher specimens, and symbiotypes (archived host specimens from the type and additional localities from which syntypes and vouchers were designated) (Frey et al., 1992; Dunnum et al., 2017) were deposited and accessioned in multiple museum collections in Canada, the USA, and Italy.

3. Results

3.1. Mitochondrial genome characterization

Whole genome shotgun sequencing generated 7,901,532 reads that were used for sequence assembly. Of these, 135,528 were mitochondrial sequences. Following assembly, average read depth was 1973.5 in the coding regions of the mitochondrial DNA. The complete coding region of the mitochondrial genome of the new taxon of *Trichinella* was 14,072 bp, and G + C content was 34.4%. Similar to other taxa of *Trichinella*, the mitochondrial genome of the new species has 13 protein-coding genes (*atp6*, *atp8*, *nad1-6*, *nad4L*, *cox1-3*, *cytb*), two rRNA genes (*rrnS* and *rrnL*) and 22 tRNA genes (size ranged from 53 to 75 bp). Repetitive regions outside of coding regions were difficult to assemble and not analyzed further. Nevertheless, the tRNA for Lysine was associated with the origin of replication found within the repetitive regions of the mitochondrial genome.

3.2. Trichinella T13 is a distinct, archaic species, not closely related to T2 and T6

Phylogenetic analysis differentiated encapsulated from non-encapsulated clades, as has previously been observed. The monophyletic encapsulated clade shared a common ancestor, with a subclade containing *T. spiralis* + *T. nelsoni* and a subclade that diagnosed relationships for the remaining taxa. Discovery of a previously unrecognized and putative cryptic taxon of *Trichinella*, designated here as T13, was consistently supported (bootstrap values = 92–100%). Within the encapsulated clade, T13 did not group with either T1 or T7, but was placed basally in the subclade containing T12 + (T2 + T6) + ((T5 + T9) + (T3 + T8)). Phylogenetic trees constructed from both the mitochondrial genome (Fig. 2) and 15 concatenated SCOs (rooted (Fig. 3)) showed a similar topologies, with a consistent position for the cryptic lineage of *Trichinella*. Similar phylogenetic relationships were demonstrated when trees were built individually for SCOs (Supplementary Fig. S1). Phylogenies consistently indicated a basal divergence for the previously unrecognized taxon as the sister lineage to a larger assemblage of *Trichinella* in Eurasia and the Western Hemisphere (excluding *T. spiralis* and *T. nelsoni*). Furthermore, it is notable that T13 was not the sister taxon, nor closely related to, the geographically sympatric *T. nativa* and T6.

3.3. Mitochondrial PCR-RFLP can be used to differentiate T2 and T13

PCR targeting the Cytb gene of mtDNA produced a 920 bp band in reference isolates of T2, T6, and T13. Digesting these products produced distinct banding patterns: from *T. nativa* (T2), bands of approximately 537 and 377 bp; from T6, three bands of approximately 407, 377 and 130 bp; and from T13, two bands of approximately 507 and 407 bp (Fig. 1). Using the multiplex PCR (Table 1), of the 95 presumed *T. nativa* isolates 68 were confirmed as *T. nativa* by the new PCR-RFLP, two were T6, 11 had mitochondrial haplotypes characteristic of both *T. nativa* and *Trichinella* T6 (mixed infections), and 14 were T13 (all in wolverine hosts). Single infections of T13 were detected in 11 of 14 wolverines, mixed infections of T13 and *T. nativa* were detected in two of 14, and T13 and T6 in one wolverine (Table 1). Infections of T13 were more frequent among wolverines from the Yukon (10 of 15) than from the Northwest Territories (four of 27). Harvest location, sex, and age of the T13 positive wolverines are shown in Table 2.

3.4. T13 larvae were resistant to freezing

Between 1 and 135 larvae per gram of muscle tissue were identified in wolverines infected with T13. Most larvae were deemed alive based on their coiled morphology. In two samples (IDs: 17WV014 and 17WV064), 47% (28/60) and 58% (35/60) of the larvae were motile (Table 2). Compression showed that T13 is encapsulated (Fig. 4).

3.5. Morphology of larvae of T13 were indistinguishable from other species

Of 58 measured larvae, PCR did not amplify for 11; of 47 amplified DNA samples, 42 were identified as T13 and five as T2 (*T. nativa*). Of 42 T13 larvae, 16 male larvae and one female larva had rectal length < 30 µm and >40 µm, respectively; we excluded these and retained measurements for 25 larvae (18 female and seven male). Morphological characteristics together with average measurements of the muscle larvae are provided in Table 3. Female larvae were longer than male larvae (Fig. 5). Overall, the morphometric parameters of T13 were comparable to other species of *Trichinella* available in the literature (Supplementary Table S1).

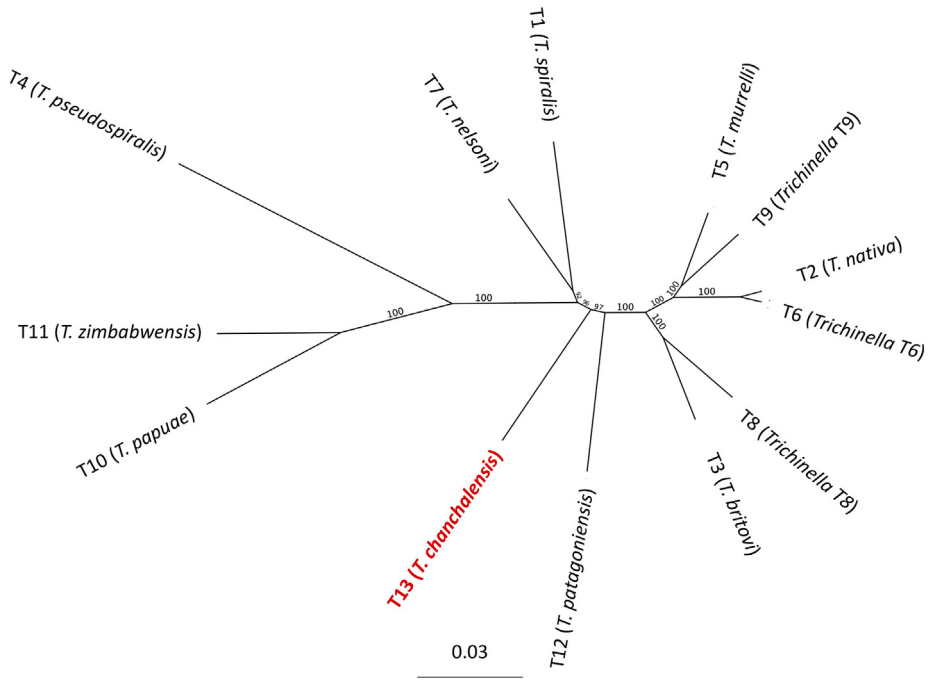

Fig. 2. Relationships among the described species of *Trichinella* (T1–T12) and *Trichinella chanchalensis* n.sp. (T13) based on analyses of mitochondrial nucleotide sequences using neighbor joining and maximum likelihood algorithms.

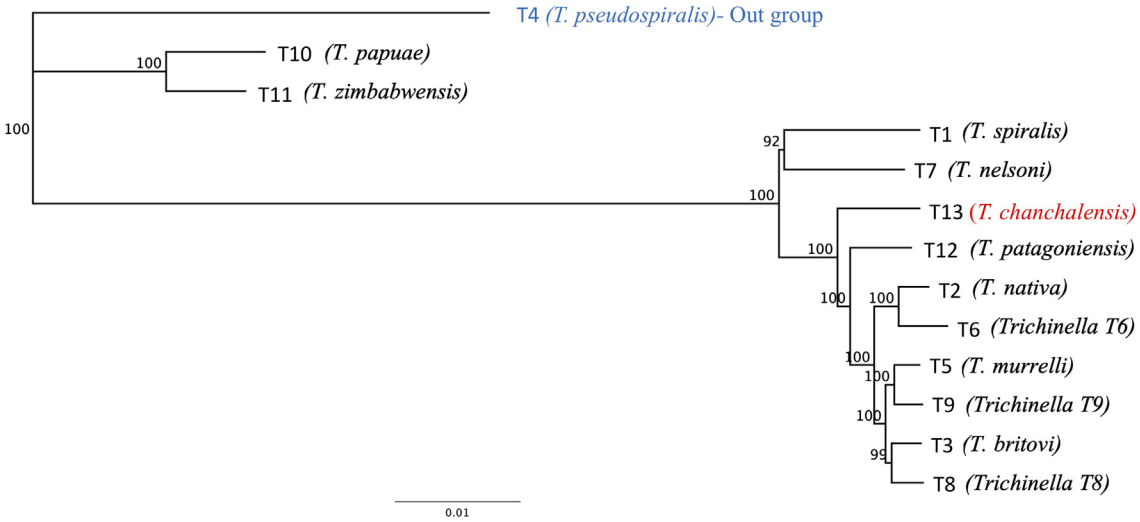

Fig. 3. Relationships among the described and cryptic species of *Trichinella* based on analyses of nucleotide sequences of 15 concatenated single copy orthologs using neighbor joining and maximum likelihood algorithms (rooted; *Trichinella pseudospiralis* as an outgroup (blue text); *Trichinella chanchalensis* (red text).

3.6. Taxonomic summary

Trichinella chanchalensis n. sp. Sharma, Thompson, Rosenthal, Hoberg and Jenkins.

Biological features: Encapsulated first-stage larvae (L1) in host musculature, likely freeze-tolerant.

Molecular features: Whole genome (GenBank Accession number **VOSH00000000)** and mitochondrial sequence (**MK547536**) of DNA extracted from L1 from a wolverine (Field ID: 17WV049).

Morphological features: Average total length of muscle larvae, 877 μm (896–946 μm) for males and 1024 μm (range: 910–1136 μm) for females.

Type host: Wolverine (*Gulo gulo*), field ID 17WV049, 1 year old male harvested on 23 February 2017 by trapper, carcass collected by T.S Jung and P.M Kukka. Additional hosts: Wolverines (Field IDs: 15WV013 and 15WV026) were harvested on 13 December 2014 and 28 February 2015, carcasses collected by T.S Jung and P.M Kukka. Wolverine (Field ID: WV2011-093) was harvested on 17 February 2011, samples collected by R R R. Mulders.

Other host species: Currently unknown.

Type locality: Specimens in the designated syntype series originated from a wolverine (field ID 17WV049) harvested at Ch'anchàłen Mountain, near the Village of Old Crow, Yukon, Canada (ca., 67° 51′ 55.808″ N and 140° 32′ 16.883″ W). Additional localities: sites near Old Crow, Yukon, Canada (67.56885°, −139.83770°), a Vuntut Gwitchin First Nation community. Specimens from wolverines (Field IDs: 15WV013; 15WV026; and WV2011-093) originated from the Yukon Plateau North (Yukon),

Table 2
Host demographic details, identification, and larval characteristics (intensity, morphology, and motility) of *Trichinella chanchalensis* n. sp. (T13) in wolverines in northwestern Canada.

Territory	Harvest Location	Animal ID	Age (years)	Sex	Multi PCR[a]	PCR-RFLP[d]	LPG[e]	L/D[f]	Motility (%)
Yukon	Old Crow Flats	14WV098	1	M	T2[b]	T13	6.9	7.3	0%
		17WV049[i]	1	M	T2 + T6[c]	T13 + T2	8.5	41.5	NP[h]
	Mackenzie Mountains	15WV026[j]	2	F	T2 + T6	T13 + T6	58.5	4.5	NP
	Yukon Plateau North	15WV013	3	M	T2	T13	7.1	NDL[g]	0%
	Yukon Plateau North/Selwyn Mountains	17WV014	1	M	T2	T13 + T2	5.4	NDL	47% (28/60)
	Yukon Plateau Central	15WV038	6	F	T2	T13	1.0	0.7	0%
	Pelly Mountains	14WV043	1	F	T2	T13	8.0	79.0	0%
	Ruby Ranges	14WV040	1	M	T2	T13	85.5	19.9	0%
		17WV064	7	M	T2	T13	32.5	18.1	58% (35/60)
	Yukon Southern Lakes	14WV026	2	M	T2	T13	134.6	133.6	0%
Northwest Territories	Inuvik	IN11/26	1	M	T2	T13	15.4	NDL	0%
	Dehcho	W10-112	8	F	T2	T13	6.9	4.8	0%
		WV11-093	2	M	T2	T13	12.8	1.5	NP
	South Slave	W10-013	9	M	T2	T13 + T2	19.8	3.0	NP

[a] multiPCR, multiplex PCR based on nuclear locus (Zarlenga et al., 1999).
[b] T2, *Trichinella nativa*.
[c] T6, *Trichinella* T6.
[d] PCR-RFLP, PCR Restriction Fragment Length Polymorphism to distinguish T2, T6, and T13 based on the mitochondrial genome.
[e] LPG, larvae per gram of processed muscle tissue.
[f] L/D, ratio of the number of live (coiled) to dead (comma shaped) larvae.
[g] NDL, no dead larvae found.
[h] NP, not performed.
[i] Four larvae were T2 (nuclear) + T13 (mitochondrial); one larva was T6 + T2.
[j] Three larvae were T2 (nuclear) + T13 (mitochondrial); the other two larvae were T2 + T6, and T6 + T6.

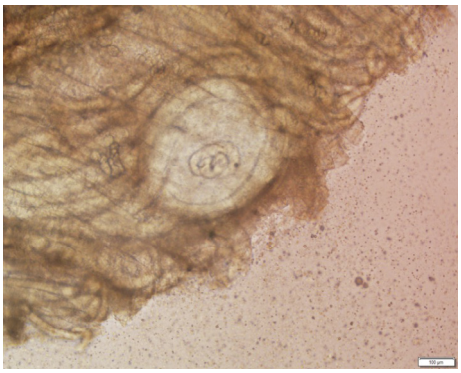

Fig. 4. Encapsulated larva of *Trichinella chanchalensis* (T13) in the tongue of a wolverine (*Gulo gulo*).

Table 3
Morphometric characteristics of the muscle-dwelling L1 of *Trichinella chanchalensis* n. sp. (T13).

Characteristics	Measurement ± S.D. (Range), µm	
	Male larva (n = 18)	Female larva (n = 7)
Total length	877 ± 56 (896–946)	1024 ± 66 (910–1136)
Width at mid-body	30 ± 2 (26–33)	32 ± 3 (28–38)
Length of oesophagus	167 ± 17 (134–183)	168 ± 19 (121–196)
Length of stichosome	436 ± 41 (391–505)	434 ± 34 (393–495)
Length of genital primordium	198 ± 24 (167–246)	187 ± 18 (153–219)
Length of rectum	44 ± 16 (31–79)	26 ± 4 (21–32)
Distance from posterior margin of the genital primordium to tail tip	61 ± 7 (51–75)	65 ± 14 (44–100)

Mackenzie Mountains (Yukon) and Dehcho (Northwest Territories), respectively.

Distribution: northwestern Canada.

Specimens deposited: Multiple specimens of L1 extracted from musculature in an infection demonstrated to be dominated by *T. chanchalensis* in the type host from the type locality (17WV049) were fixed in 95% ethanol and deposited as a series of syntypes in the Canadian Museum of Nature (CMN), Ottawa, Canada (CMNPA 2019-0234). Specimens of *T. nativa* and T6 may rarely occur in these syntype samples; consequently, low levels of coinfection involving multiple species of *Trichinella* cannot be excluded from any host individual. The syntypes were collected from the Old Crow ecoregion which varies from 325 to 610 m asl with an average elevation of 327 m and mean January temperatures of −30 °C. Additional specimens (L1 larvae in 95% ethanol) from wolverines at multiple geographic localities (field collection numbers-15WV013 (CMN No: CMNPA 2019-0233); 15WV026 (CMN No: CMNPA 2019-0235) and WV2011-093 (CMN No: CMNPA 2019-0232)) are recognized as a series of vouchers; some may constitute mixed-species infections with *Trichinella* T6 and T2. Voucher specimens of L1 stored in 95% ethanol from these hosts were deposited at CMN (CMNPA 2019-0232-35); the Division of Parasitology (MSB-PARA), Museum of Southwestern Biology (MSB), Albuquerque, New Mexico, USA (MSB: Para: 29,131 (field ID WV2011-093), MSB: Para: 29,132 (field ID 15WV013) and MSB: Para: 29,133 (field ID 15WV026)) and the International *Trichinella* Reference Centre, Istituto Superiore di Sanita, Italy (ISS 7582 (field ID 15WV013), ISS 7583 (field ID WV2011-093) and ISS 7584 (field ID 15WV026)).

Etymology: The species is named *Trichinella chanchalensis* for the type locality of collection, Ch'anchàłen, a mountain near the Village of Old Crow, Yukon, Canada.

Symbiotype specimen: Wolverine (*Gulo gulo*) from type locality, represented by tongue tissue (field collection 17WV049) was deposited in the CMN (CMN Accession No. A2019.0124). Skull, skeleton and tissue were archived in the MSB Division of Mammals (MSB: Mamm: 292,371 (Field ID 15WV026) and MSB: Mamm: 304681 (Field ID WV2011-093)).

Other symbiotypes deposited:

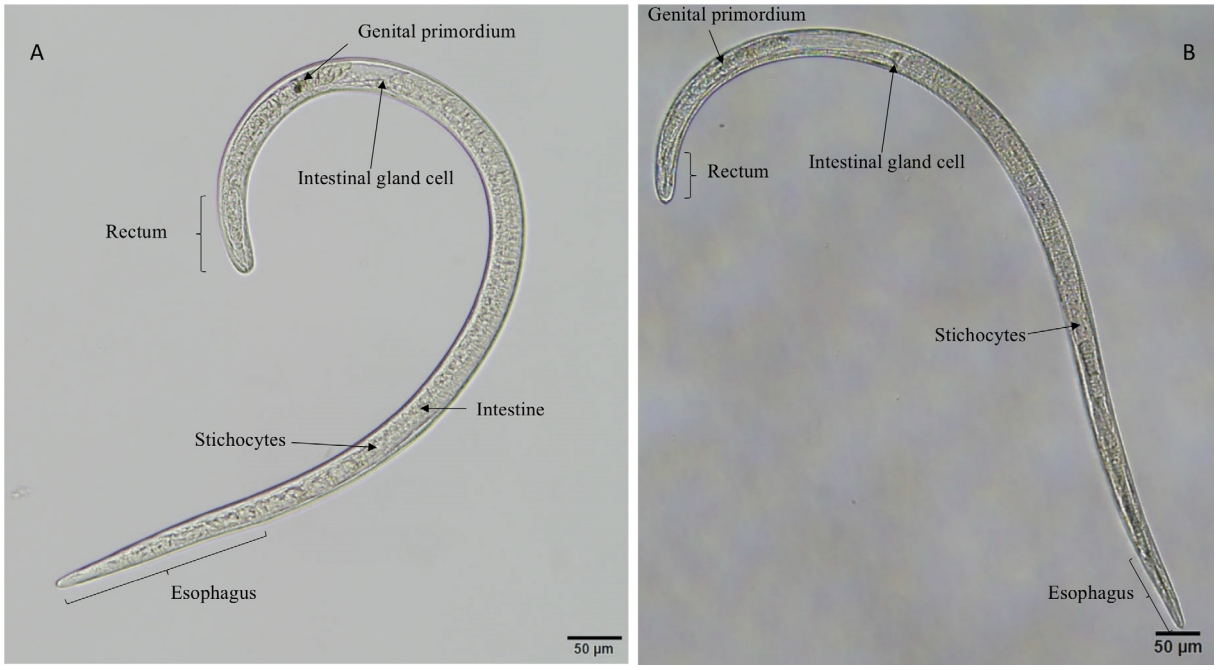

Fig. 5. Muscle larvae, L1s of *Trichinella chanchalensis* (T13). (A) Male, (B) female.

MSB: Mamm: 292,371 (Field ID 15WV026); Canada: Yukon Territory (64.53523°/−137.88819°). 28 February 2015. Collected by T.S. Jung and P.M. Kukka

MSB: Mamm: 304681 (Field ID WV2011-093); Locality: Canada; Northwest Territories, Dehcho Region, Trout Lake area (60.583°/−121.317°). 17 February 2011. Collected by R. Mulders.

4. Discussion

We describe a previously unrecognized cryptic species, proposed as *Trichinella chanchalensis* n. sp. (also designated as T13), in wolverines from northwestern Canada. This taxon is differentiated from congeners based on molecular sequence data and comparative phylogenetic criteria. Specimens of this cryptic taxon were initially identified as *T. nativa* based on the location of bands on a multiplex, conventional PCR used for identification of isolates within the genus. Despite sharing the same band position as *T. nativa* on the multiplex PCR, *T. chanchalensis* is genetically discrete and not closely related to any currently recognized species or genotype in North America or Eurasia (Figs. 2 and 3). *Trichinella chanchalensis* (T13) does not share a direct common ancestor with other taxa of parasites that occur in sympatry from high latitudes (i.e., T6 and *T. nativa*), nor to the exclusion of other encapsulated taxa. Discovery and characterization of *T. chanchalensis* constitutes the thirteenth taxon designated in the last 50 years, and the first since recognition and naming of *T. patagoniensis* (Garkavi, 1972; Krivokapich et al., 2008; Pozio et al., 2009). This previously unknown taxon in wolverine is recognized by distinct phylogenetic placement based on its complete mitochondrial genome, as well as from 15 single-copy nuclear genes. Both nuclear and mitochondrial data provide compelling evidence that the newly discovered *T. chanchalensis*, sequenced in its entirety from just one isolate, is markedly distinct from any other known type of *Trichinella* (native to the Arctic or elsewhere). Following recent precedents employing phylogenetic criteria to delimit morphologically similar species in the genus (Zarlenga et al., 2006; Pozio et al., 2009; Korhonen et al., 2016), we have (for the first known time) provided a draft genome sequence when first naming a previously unrecognized species of *Trichinella*.

Following the advent of molecular-based approaches, taxonomic designation within *Trichinella* has generally rested on relatively few markers: cytochrome c-oxidase subunit I (COI) to designate *Trichinella* T9 (Nagano et al., 1999); the expansion segment 5 (expansion segment V (ESV) of nuclear ribosomal DNA) to delineate *T. papuae* (Pozio et al., 1999); COI, ESV, and mitochondrial-large subunit ribosomal (mt-lsr) DNA to distinguish *T. zimbabwensis* (Pozio et al., 2002); and cytochrome c-COI, ESV and 5S ribosomal DNA intergenic spacer region (5S ISR) to recognize *T. patagoniensis* (Krivokapich et al., 2008, Krivokapich et al., 2012). Here, we applied maximum likelihood estimation and neighbor-joining algorithms to determine the relationship among *T. chanchalensis* and all currently described taxa of *Trichinella*. All trees constructed were consistent in topology and with substantial nodal support (bootstrap value = 92–100 in the tree reconstructed from variation in the combined SCOs, and mitochondrial genome). When reconstructing phylogenies from each of the 15 SCOs individually, similar topologies were demonstrated.

Trichinella chanchalensis, Trichinella T6, and *T. nativa* occur in sympatry geographically and in the same host species (wolverine). Sympatry of *Trichinella* T6 and *T. nativa* has been previously reported in wildlife from Alaska and in wolverines from Nunavut, Canada (La Rosa et al., 2003b; Reichard et al., 2008). Mixed infections could result either from concurrent or successive exposures. With the discovery of *T. chanchalensis*, wolverine across a relatively limited region in northwestern North America may now host multiple taxa of *Trichinella* (*Trichinella* T6, *T. nativa*, *T. pseudospiralis* and *T. chanchalensis*) (Sharma et al., 2018, 2019), a tribute to their non-selective diet that includes carnivory, scavenging, and cannibalism.

The multiplex PCR has been widely adopted as a standard diagnostic approach, and has broad application within food safety and regulatory contexts. It is inexpensive and rapid, and targets internal transcribed spacers ITS1 and ITS2, and the ESV region of the rRNA (Zarlenga et al., 1999). However, the 127 bp size band indicative of *T. nativa* is also shared by *T. chanchalensis*. Prior identifications (and reports) of *T. nativa* may have been similarly

inaccurate. This serves as a reminder that any diagnostic method may not recognize cryptic variation. Ideally, multiple loci or larger portions of the genome should be used to characterize diversity. Other taxa may await discovery when subjected to more intensive genetic characterization, which is needed given that larvae of species of *Trichinella* cannot be reliably differentiated based on their morphology (Zarlenga et al., 1999; Zarlenga and La Rosa, 2000). We have included larval measurements in this study to fulfill requirements for designating a new species; however, larval morphology and morphometrics cannot definitively identify taxa of *Trichinella*, and adult nematodes are almost never detected, nor relevant for epidemiological or surveillance purposes. The PCR-RFLP developed in this study can unequivocally differentiate among *T. nativa*, *T. chanchalensis*, and *Trichinella* T6. Further studies are required to design new primers for multiplex PCR or a new PCR methodology to differentiate all species of *Trichinella,* and best practices should include sequencing to confirm taxa of *Trichinella* isolates. This could represent a substantial portion of any mitochondrial gene; for example the cytochrome oxidase subunit 1 (cox1), which is easy to amplify, well characterized within the genus, and at which all named species have differed by at least 4% at the nucleotide level. Therefore, sequencing 300 bp of cox1 should be adequate to differentiate any two species of *Trichinella*, with the exception of *T. nativa* and *Trichinella* genotype T6, which differ by only 1% at the cox1 locus.

While interspecific differences are generally detectable, intraspecific variation is minimal within species of *Trichinella*; the larval cohorts of other species of *Trichinella* derived from a given animal have generally proven to be highly inbred siblings (La Rosa et al., 2012, 2018). This is also likely to be true for *T. chanchalensis*; in the sequenced isolate, two single nucleotide polymorphisms (SNPs) were detected in 14,000 nucleotides of coding mitochondrial DNA sequenced with high confidence. Unless these are sequence errors, the sequenced cohort was derived from more than one mother, each minimally distinguishable from the other. The true diversity among isolates of this species may be higher, but in no case have members of a given species of *Trichinella* differed by more than 1% in mitochondrial genes. Nuclear heterozygosity was detected in this isolate at a rate of approximately three per 10,000 sequenced bases. This modest level of heterozygosity may merely affirm natural inbreeding. Future isolates will need to be explored in order to understand the extent and distribution of genetic variation within *T. chanchalensis*.

Our phylogenetic analysis and compressorium examination of muscle tissue placed *T. chanchalensis* in the encapsulated clade. Moreover, this species of *Trichinella* appears to resist freezing, based on the observation that almost all of the positive animals had tightly coiled larvae despite being frozen at −20 °C for months to years prior to processing, and undergoing multiple freeze–thaw cycles. Motile larvae were recovered from tissues frozen for 9 months to 8 years. However, animal infectivity studies are required to confirm the viability, reproductive capacity index (RCI), and pathogenicity of *T. chanchalensis*, relative to other species of *Trichinella*.

Discovery of *T. chanchalensis* has significance for our understanding of the evolutionary and biogeographic history of encapsulated species of *Trichinella* (Zarlenga et al., 2006; Pozio et al., 2009; Hoberg et al., 2012a; Korhonen et al., 2016). Our current analyses corroborate those based previously on 12 complete draft genomes and the general, fully-resolved tree topology for relationships among named species and genotypes within the encapsulated *Trichinella* (Korhonen et al., 2016). In these analyses, a common ancestor for the encapsulated clade is shared by a subclade containing *T. spiralis* and *T. nelsoni,* and a subclade of remaining taxa, which now contains *T. chanchalensis* (T13). Tree topology is consistent with a complex history of episodic expansion and geographic colonization during the diversification of this assemblage linking Eurasia with Africa, and secondarily Eurasia with the Nearctic and the Neotropical regions (Hoberg and Brooks, 2008; Hoberg et al., 2012a; Korhonen et al., 2016). The history of faunal assembly across the Holarctic, with the addition of *T. chanchalensis*, is temporally deep, extending from the Miocene and involving multiple and independent events of colonization across Beringia through the Quaternary (Hoberg et al., 2012a; Korhonen et al., 2016).

Our analyses place T13 as the sister of a subclade including *T. patagoniensis* and the remaining encapsulated species (*T. nativa,* T6, *T. britovi*, T8, *T. murrelli*, and T9). Thus, *T. chanchalensis* represents an apparently archaic lineage which emerged early in the diversification of encapsulated taxa of *Trichinella*, even before *T. patagoniensis,* which likely diverged 6–10 million years ago in the Neotropical region following expansion from Eurasia and subsequent isolation (Pozio et al., 2009; Korhonen et al., 2016). The origin of *T. chanchalensis* pre-dates the divergence of *T. patagoniensis* and indicates that *T. chanchalensis* represents a major and long-standing component of *Trichinella* biodiversity in the Holarctic.

We hypothesize a Eurasian/Beringian origin for *T. chanchalensis* based on its current distribution in northwestern Canada and knowledge of historical glaciation. Initial geographic limits for a parasite-host assemblage linking Eurasia and the northwestern Nearctic were constrained by continental glaciation that defined the periphery of East Beringia during the Pleistocene and as late as the Last Glacial Maximum (LGM) near 18–20 kilo years ago (KYA) (Hoberg et al., 2012a, 2017; Cook et al 2017). Although *T. chanchalensis* is an ancient species, probably arising in Eurasia, it has likely only been in northwestern Canada and Alaska for <12–15 KY, following deglaciation. Our observation of a relatively restricted host (wolverine) and geographic range is consistent with post-glacial expansion into the present Yukon and Northwest Territories. The structure of mustelid diversity and that of other potential carnivoran hosts (e.g., ursids and canids) for species of *Trichinella,* together with a broader parasite assemblage, reflects a complex and extended history for episodic expansion, isolation and development of faunal mosaics linking Eurasia and the Nearctic through the late Tertiary and Quaternary (Hoberg et al., 2012a, b; Korhonen et al., 2016).

Wolverine are distributed across the Holarctic, with fossil and molecular evidence suggesting origins in North America during the Miocene (Kurtén and Anderson, 1980; Tomasik and Cook, 2005; Koepfli et al., 2008). Wolverine occurred in Beringia and south of the Laurentide/Cordilleran continental glaciers in North America during phases of the final glaciation (Wisconsonian). Contemporary populations in the Nearctic appear to have originated in Beringia, reflecting a history of rapid post-glacial expansion to the east and south following the LGM into the Holocene (Tomasik and Cook, 2005). If limited to wolverine as hosts, as indicated by the current findings, this history may explain a circumscribed geographic range for *T. chanchalensis.* Further studies of Alaskan and Siberian isolates of *Trichinella,* and broader sampling in carnivores in North America, are required to test this hypothesis and resolve this complex history. In the current study, *T. chanchalensis* was more frequently encountered in wolverine in the Yukon, which is further west than the Northwest Territories (Fig. 6). Although data are currently limited, this distribution is consistent with a geographic signature of post-glacial expansion from Eastern Beringia. Sampling bias may account for some of this apparent difference, as more wolverines were tested than any other host species, and further studies are needed to verify that *T. chanchalensis* is truly restricted to wolverines in northwestern Canada. As well, isolates of *T. nativa* were obtained by artificial digestion of tongue tissues. Although most species of *Trichinella* have predilections for tongue, diaphragm, and leg muscles in wild animals (Kapel et al., 1994, 1995; Mikkonen et al., 2001; Sharma et al., 2018), we do not know

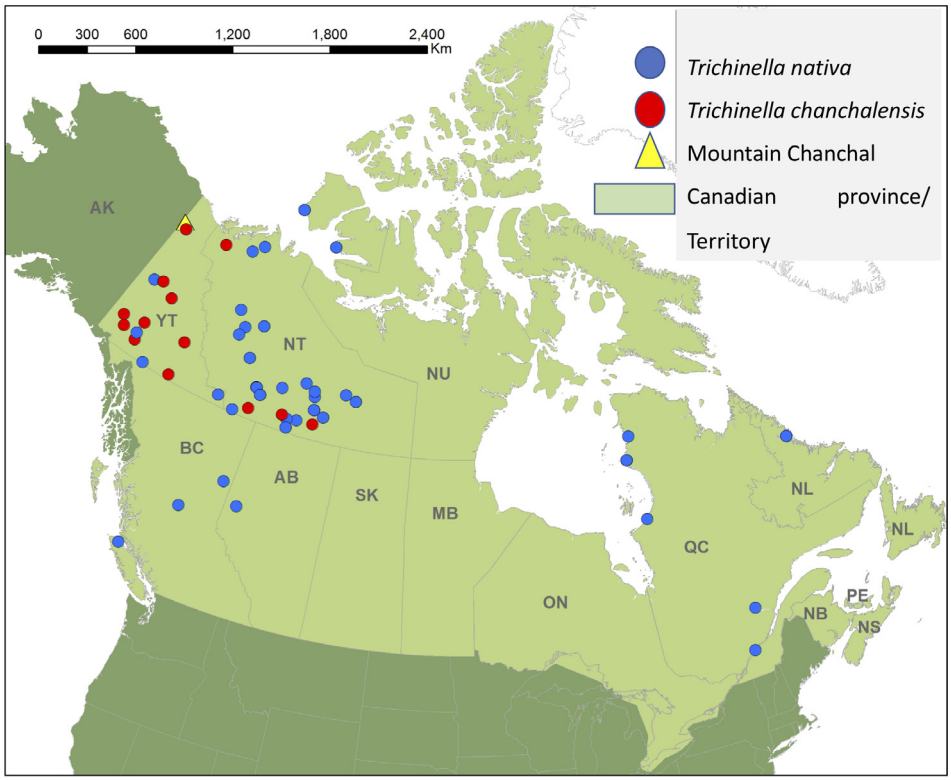

Fig. 6. Geographic distribution of *Trichinella chanchalensis* (T13) and *Trichinella nativa* (T2) in the current study in Canada. Exact coordinates were available only for the wolverines from Northwest Territories. Where exact coordinates were not available, verbatim coordinates were used.

the predilection sites for *T. chanchalensis* in mammalian hosts. This could account for our lack of detection of *T. chanchalensis* in mammalian hosts other than wolverines, as well as the low prevalence in wolverine relative to other taxa of *Trichinella*.

There are distinct host-parasite associations for some taxa of *Trichinella*, but many species have broad host ranges in space and time, driven by opportunity (events of ecological disruption, often driven by climate, and episodic expansion with geographic colonization) and capacity to utilize host resources (ecological fitting) (Agosta et al., 2010; Araujo et al., 2015). Over millions of years, species of *Trichinella* have diversified according to patterns of host and geographic colonization (Zarlenga et al., 2006; Hoberg and Brooks, 2008). Ecological fitting, host colonization, and oscillation involving trends in specialization and generalization may be apparent in the relationship between *T. chanchalensis* and its hosts: wolverine, and possibly other mustelids and carnivorans (Brooks et al., 2019).

In summary, we describe and differentiate a cryptic species of *Trichinella,* designated as *T. chanchalensis* or T13, that appears to be ancient, and not closely related to any other known members of the encapsulated clade from North America. Our current results indicate that *T. chanchalensis* is host and geographically restricted to wolverines from northwestern Canada. Further characterization of Canadian, Alaskan, and Russian isolates is necessary to better characterize current host diversity and geographic distribution, and ultimately to understand the history and origins of this new species. Discovery of *T. chanchalensis* contributes to a deeper understanding of the history of episodic expansion, geographic and host colonization between Eurasia and North America through Beringia. In phylogenetic analyses, *T. chanchalensis* was the putative sister of a subclade of encapsulated *Trichinella* containing *T. patagoniensis* and the remaining species of the encapsulated group, excluding *T. spiralis* and *T. nelsoni*. Our findings demonstrate that the current multiplex PCR, universally deployed as the global standard to distinguish among known genotypes and species of *Trichinella,* lacks the sensitivity needed to recognize otherwise cryptic diversity. We recommend application of a newly developed PCR-RFLP, sequencing methodologies, and/or other molecular or protein-based technologies to confirm the identity of *Trichinella* isolates, and suggest that deeper genetic characterization may yet reveal other cryptic forms of this important genus of zoonotic parasites with global distribution.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2020.01.003.

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