DNA BARCODING

Species identification of North American guinea worms (Nematoda: *Dracunculus*) with DNA barcoding

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Abstract

Dracunculus insignis **is a nematode parasite that infects the subcutaneous tissues of mammals such as raccoon (***Procyon lotor***), mink (***Neovison vison***) and fisher (***Martes pennanti***).** ***D. lutrae***, **a morphologically similar species, has only been recovered from the otter (***Lontra canadensis***). Species identification of these two North American guinea worms has only been achieved by morphology of males and host identity. As a result, where only female specimens are present, accurate identifications are not possible. To date, specimens recovered from otter have been assumed to be** ***D. lutrae***, **while those from all other hosts are assumed to be** ***D. insignis***. **This study uses DNA barcoding to differentiate between these two North American dracunculoids. Our results show that** ***D. insignis*** **is a 'true' generalist, showing little sequence divergence regardless of host association, although our studies did validate its occurrence in a new host — the otter. Interestingly, specimens of the host specialist,** ***D. lutrae***, **showed some sequence divergence, although it was low. The finding of** ***D. insignis*** **in otter substantiates the need to supplement morphology-based methods in providing species identifications for certain dracunculoids.**

Keywords: COI, DNA barcoding, nematode, parasite

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Introduction

The importance of accurately and efficiently identifying biological diversity is becoming ever more evident. However, morphological approaches to species identification can produce incorrect identifications due to phenotypic plasticity, genetic variability, morphologically cryptic taxa and differences in particular life stages or gender (Hebert *et al.* 2003a). Recent studies propose that DNA barcoding will often circumvent these problems (Hebert *et al.* 2003a; Savolainen *et al.* 2003). The barcoding method uses a short, standardized genetic marker as a tool for identification — a 658-bp region of the mitochondrial cytochrome *c* oxidase I (COI) gene is the barcode standard for the animal kingdom (Hebert *et al.* 2003a, b). Most eukaryotic cells contain mitochondria, and in comparison to the nuclear genome, mtDNA lacks introns, is less exposed to recombination,

has a haploid mode of inheritance and has a relatively high mutation rate (Saccone *et al.* 1999). This results in significant variance in mtDNA sequences between species and a comparatively small variance within species.

Taxonomists have begun using DNA barcodes as a 'triage' tool for sorting specimens into groups, of which some will belong to known species and others will be new to science. DNA barcoding also allows the identification of specimens in those cases where morphological features are missing (in the case of immature, partial or damaged specimens) or misleading (as in sexually dimorphic species) and as a supplement to other taxonomic data sets to aid the delimitation of species boundaries (Schindel & Miller 2005). For example, a recent DNA barcoding study (Hebert *et al.* 2004) found the common, Neotropical skipper butterfly (*Astraptes fulgerator*), previously thought to be a single species, is a complex of 10 species. This method may prove to be especially attractive for taxonomists identifying parasites whose morphological identifications are often difficult (Besansky *et al.* 2003; Powers 2004).

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Currently, the identification of many nematode parasites is based on a combination of host use and morphology (Anderson 2000). For example, the guinea worms, members of the genus *Dracunculus* infecting mammals, are identified using morphological features that are only present in males, which are rarely available for study (Anderson 2000). Consequently, the identification of *Dracunculus* species based on morphology has proven complex (Muller 1971; Bimi *et al*. 2005; Wijova *et al*. 2005) and identifications are frequently questioned because of inadequate descriptions and/or morphological similarities (Muller 1971; Wijova *et al*. 2005). For example, Beverley-Burton & Crichton (1973, 1976) found that it was impossible to morphologically distinguish the human guinea worm, *D. medinensis* (Linnaeus 1758), from *D. insignis* (Leidy 1858) of North American mammals such as mink (*Neovison vison*), raccoon (*Procyon lotor*) and fisher (*Martes pennanti*). In fact, based on cross-infection experiments, they suggested that *D. medinensis* and *D. insignis* might simply be different physiological 'strains' of a single species. However, Bimi *et al*. (2005) and Wijova *et al*. (2005) have recently shown that it is possible to distinguish *D. medinensis* from both *D. insignis* and from the reptile-infecting *D. oesophageus* based on 18S rRNA gene sequences.

To date, species identification of the North American mammal-infecting guinea worms, *D. lutrae* and *D. insignis*, has only been achieved by morphology. *D. lutrae* has only been recovered from otter (*Lontra canadensis*) and is distinguished from other mammal-infecting species of *Dracunculus* by the greater length of males, greater length of spicules and gubernaculum, presence of only three pairs of preanal papillae, and the arrangement of papillae in two transverse rows immediately posterior to the anus (Crichton & Beverley-Burton 1973). However, in cases where males are lacking, specimens are identified by host alone (Bimi *et al*. 2005; Wijova *et al*. 2005). Furthermore, the role of phenotypic plasticity in these two species has not been examined.

Studies have found that due to the close association between parasites and their hosts, many 'generalist' parasites become specialized on different host species, often resulting in host race formation (Jaenike 1993; McCoy *et al*. 2001). Mayr (1970) defined host races as 'noninterbreeding sympatric populations that differ in biological characteristics but not, or scarcely, in morphology' and suggested they were prevented from interbreeding by preference for different food plants or other hosts. Jaenike (1981) distinguished host races from sympatric host-associated sibling species by adding that 'if gene flow among two or more populations was restricted solely or primarily because of differential host preference, then these would constitute host races'. He argues that if this basis for reproductive isolation were not present, host races would fuse into a single panmictic population, whereas sibling species would maintain their separate genetic identities. As Anderson & Jaenike (1997)

point out, our understanding of the epidemiology of many parasitic diseases is severely hampered by the existence of morphologically identical cryptic species and host races. Recently, the DNA barcoding initiative is proving to be a useful tool when investigating host specialization or host races (Smith *et al*. 2007).

This study attempts to differentiate between the two North American guinea worm species, *D. lutrae* and *D. insignis*, and investigate possible host-related genetic divergence in *D. insignis* by sequencing a gene region that is less conserved than 18S rRNA. In the case of *D. insignis*, significant host-associated genetic differences based on DNA barcoding of the COI gene may shed light on the presence of host specialization or host races. If this generalist parasite has become specialized to a specific host, it is expected that the parasite will show high levels of sequence divergence with respect to the host species.

Methods

Samples

Fisher (*Martes pennanti*), mink (*Neovison vison*), raccoon (*Procyon lotor*) and otter (*Lontra canadensis*) carcasses were collected during the fur harvests of 2005–2006 and 2006–2007 from licensed Ontario fur trappers on registered trap lines in northeastern, southern and southeastern Ontario, Canada and frozen until dissection (< 6 months at –18 °C).

During necropsies for guinea worm, external surfaces of all superficial muscles, intermuscular areas of the legs and feet, connective tissue beneath the latissimus dorsi and of the inguinal and axillary regions, and internal surfaces of the abdominal and pelvic cavities of each animal were examined. The prevalence of infection was high — 20/33 mink, 18/31 raccoon, 20/51 fisher and 20/21 otter were infected with guinea worm. Guinea worms were counted, sexed, measured with digital calipers (± 0.1 mm), weighed using an ACCULAB scale (± 0.001 g), and preserved in 70% ethanol. Tissue from one specimen of *D. lutrae* from each of 20 individual otter hosts and one specimen of *D. insignis* from each of 20 individual fisher, 18 individual raccoon and 20 individual mink specimens were sequenced at the Canadian Centre for DNA Barcoding. As well, seven additional guinea worms from one of the infected otters and seven from one of the infected mink were included for within-host comparisons. Prior to submission, male specimens from fisher ($n = 2$), mink ($n = 1$), raccoon ($n = 2$) and otter ($n = 11$) were identified to the species level according to Crichton & Beverley-Burton (1973). Female guinea worms from fisher ($n = 18$), mink ($n = 26$), raccoon ($n = 15$) and otter ($n = 16$) and 1 specimen of unknown sex from raccoon were classified to the species level according to their host. Specimens were deposited in the Canadian National Collection of Nematodes, Ottawa, Ontario, Canada.
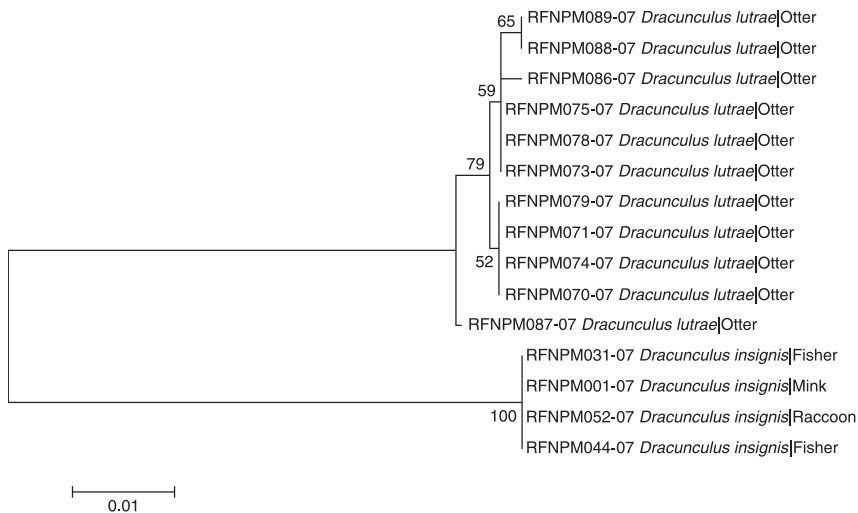
Fig. 1 Neighbour-joining tree of Kimura 2-parameter (K2P) distances of COI *Dracunculus* sequences, with BOLD identifiers, from male specimens (*n* = 15).

DNA extraction, amplification and sequencing

DNA extracts were prepared by placing 1–2 cm of ethanol-preserved tissue from each specimen directly into 96-well plates containing lysis buffer and proteinase K. DNA extraction employed a glass fibre protocol (Ivanova *et al.* 2006). The 658-bp target region of COI was amplified by polymerase chain reaction (PCR); each 12.5 μL PCR mixture included 6.25 μL of 10% trehalose, 1.25 μL 10× PCR buffer, 0.625 μL (2.5 mM) MgCl$_2$, 0.125 μL (10 μM) each oligonucleotide primer, 0.625 μL (10 mM) dNTPs, 0.625 μL *Taq* polymerase and 4 μL H$_2$O + template DNA (Hajibabaei *et al.* 2005). PCRs were run at the following thermal cycle conditions: 1 min at 94 °C, followed by five cycles of 30 s at 94 °C, 40 s at 50 °C, and 1 min at 72 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 55 °C, and 1 min at 72 °C, and finally, 10 min at 72 °C. The standard invertebrate primer pair LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) with HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) was used (Folmer *et al.* 1994). Additionally, all primers were tailed with standard flanking sequences (M13F: 5′-TGTAAAACGACGGCCAGT-3′; M13R: 5′-CAGGAAACAGCTATGAC-3′) to allow subsequent sequencing with M13 primers. PCR products were visualized in a 96-well E-Gel (Invitrogen), and sequenced (forward and reverse reads using M13F and M13R primers) on an ABI 3730 (Hajibabaei *et al.* 2005). Sequences were assembled and edited using SeqScape software (Applied Biosystems) and entered into the Barcode of Life Data Systems (BOLD) database (Ratnasingham S, Hebert PDN; 2007); sequences were also deposited in GenBank with the Accession nos EU646534–EU646615.

Data analysis

Sequences and original trace files are available in the 'Nematode Parasites of Canadian Mammals' project on the BOLD database (www.barcodinglife.org) and on GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). Sequences were aligned using the ClustalW (Thompson *et al.* 1994) module with MEGA version 4.0 (Tamura *et al.* 2007). The Kimura 2-parameter (K2P) model of base substitution (Kimura 1980) was used as a simple measure of pairwise sequence distances. To visualize these distances, a neighbour-joining (NJ) tree with bootstrap analysis (500 replications) of K2P sequence distances showing intraspecific and interspecific variation was created using MEGA. K2P sequence divergences at both levels were determined using the 'Distance Summary' tool on BOLD.

Results

In total, 82 of 92 specimens were sequenced successfully; all reads were between 496 and 660 bp in length. The shorter sequences were due to a small number of chromatograms showing low-quality signal at one end or another; the ambiguous bases were trimmed so that only high-quality data were used. Alignment was straightforward as the sequences represent protein-coding genes containing no insertions or deletions which would alter the reading frame. Aligned sequences are available directly as a download from the BOLD project file. An NJ tree of K2P distances of COI *Dracunculus* sequences from male *D. insignis* and *D. lutrae* specimens shows that the two species are clearly separated (Fig. 1) with a mean interspecific sequence divergence of 9.82% (*n* = 15).

An NJ tree of K2P distances of COI *Dracunculus* sequences from male and female specimens shows that, regardless of sex, the two species are clearly separated (Fig. 2). When considering both sexes, the mean K2P sequence distance between *Dracunculus insignis* and *D. lutrae* was 9.64%. Two female specimens obtained from two separate otter hosts yielded sequences that group with specimens recovered from fisher, raccoon or mink rather than with other specimens

RFNPM033-07 *Dracunculus insignis*|Fisher
RFNPM016-07 *Dracunculus insignis*|Mink
RFNPM021-07 *Dracunculus insignis*|Mink
RFNPM029-07 *Dracunculus insignis*|Fisher
RFNPM008-07 *Dracunculus insignis*|Mink
RFNPM084-07 *Dracunculus insignis*|Otter*
RFNPM018-07 *Dracunculus insignis*|Mink
RFNPM015-07 *Dracunculus insignis*|Mink
RFNPM047-07 *Dracunculus insignis*|Fisher
RFNPM002-07 *Dracunculus insignis*|Mink
RFNPM024-07 *Dracunculus insignis*|Mink
RFNPM036-07 *Dracunculus insignis*|Fisher
RFNPM017-07 *Dracunculus insignis*|Mink
RFNPM064-07 *Dracunculus insignis*|Raccoon
RFNPM045-07 *Dracunculus insignis*|Fisher
RFNPM063-07 *Dracunculus insignis*|Raccoon
RFNPM046-07 *Dracunculus insignis*|Fisher
RFNPM014-07 *Dracunculus insignis*|Mink
RFNPM051-07 *Dracunculus insignis*|Raccoon
RFNPM040-07 *Dracunculus insignis*|Fisher
RFNPM026-07 *Dracunculus insignis*|Mink
RFNPM004-07 *Dracunculus insignis*|Mink
RFNPM085-07 *Dracunculus insignis*|Otter*
RFNPM035-07 *Dracunculus insignis*|Fisher
RFNPM013-07 *Dracunculus insignis*|Mink
RFNPM019-07 *Dracunculus insignis*|Mink
RFNPM039-07 *Dracunculus insignis*|Fisher
RFNPM009-07 *Dracunculus insignis*|Mink
RFNPM005-07 *Dracunculus insignis*|Mink
RFNPM025-07 *Dracunculus insignis*|Mink
RFNPM011-07 *Dracunculus insignis*|Mink
RFNPM003-07 *Dracunculus insignis*|Mink
RFNPM067-07 *Dracunculus insignis*|Raccoon
RFNPM038-07 *Dracunculus insignis*|Fisher
RFNPM037-07 *Dracunculus insignis*|Fisher
RFNPM060-07 *Dracunculus insignis*|Raccoon
RFNPM044-07 *Dracunculus insignis*|Fisher
RFNPM006-07 *Dracunculus insignis*|Mink
RFNPM061-07 *Dracunculus insignis*|Raccoon
RFNPM027-07 *Dracunculus insignis*|Mink
RFNPM056-07 *Dracunculus insignis*|Raccoon
RFNPM052-07 *Dracunculus insignis*|Raccoon
RFNPM031-07 *Dracunculus insignis*|Fisher
RFNPM054-07 *Dracunculus insignis*|Raccoon
RFNPM020-07 *Dracunculus insignis*|Mink
RFNPM001-07 *Dracunculus insignis*|Mink
RFNPM030-07 *Dracunculus insignis*|Fisher
RFNPM065-07 *Dracunculus insignis*|Raccoon
RFNPM012-07 *Dracunculus insignis*|Mink
RFNPM034-07 *Dracunculus insignis*|Fisher
RFNPM062-07 *Dracunculus insignis*|Raccoon
RFNPM022-07 *Dracunculus insignis*|Mink
RFNPM010-07 *Dracunculus insignis*|Mink
RFNPM057-07 *Dracunculus insignis*|Raccoon
RFNPM042-07 *Dracunculus insignis*|Fisher
RFNPM053-07 *Dracunculus insignis*|Raccoon
RFNPM023-07 *Dracunculus insignis*|Mink
RFNPM066-07 *Dracunculus insignis*|Raccoon
RFNPM055-07 *Dracunculus insignis*|Raccoon
RFNPM094-07 *Dracunculus lutrae*|Otter
RFNPM090-07 *Dracunculus lutrae*|Otter
RFNPM068-07 *Dracunculus lutrae*|Otter
RFNPM087-07 *Dracunculus lutrae*|Otter
RFNPM080-07 *Dracunculus lutrae*|Otter
RFNPM079-07 *Dracunculus lutrae*|Otter
RFNPM072-07 *Dracunculus lutrae*|Otter
RFNPM071-07 *Dracunculus lutrae*|Otter
RFNPM074-07 *Dracunculus lutrae*|Otter
RFNPM070-07 *Dracunculus lutrae*|Otter
RFNPM089-07 *Dracunculus lutrae*|Otter
RFNPM088-07 *Dracunculus lutrae*|Otter
RFNPM091-07 *Dracunculus lutrae*|Otter
RFNPM092-07 *Dracunculus lutrae*|Otter
RFNPM077-07 *Dracunculus lutrae*|Otter
RFNPM082-07 *Dracunculus lutrae*|Otter
RFNPM075-07 *Dracunculus lutrae*|Otter
RFNPM081-07 *Dracunculus lutrae*|Otter
RFNPM073-07 *Dracunculus lutrae*|Otter
RFNPM076-07 *Dracunculus lutrae*|Otter
RFNPM069-07 *Dracunculus lutrae*|Otter
RFNPM086-07 *Dracunculus lutrae*|Otter
RFNPM078-07 *Dracunculus lutrae*|Otter

0.01

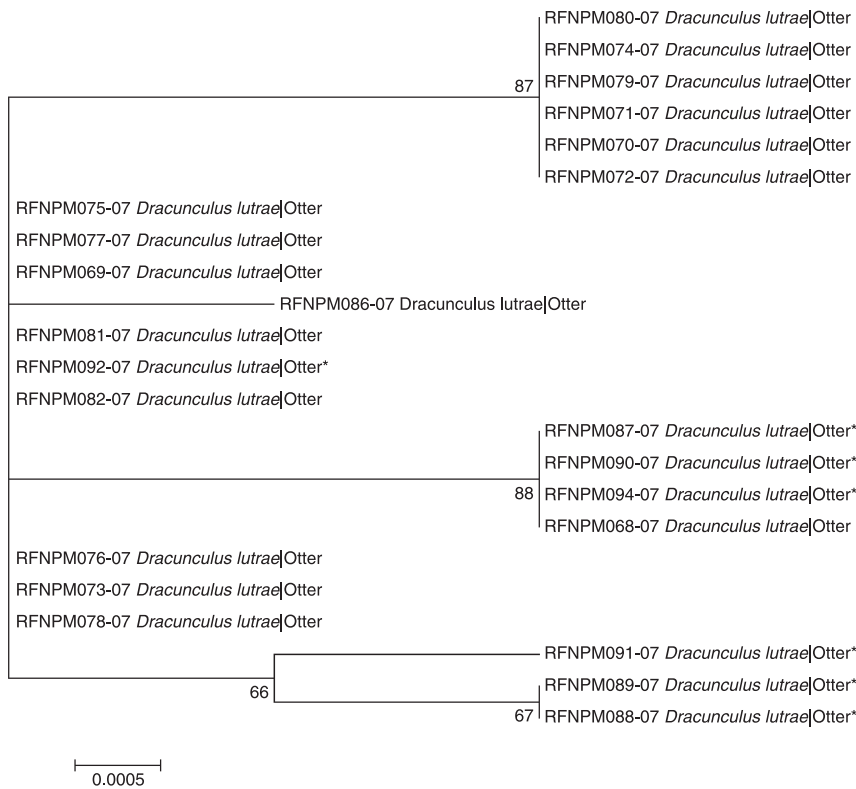from otter (Fig. 2). These two specimens are considered as *D. insignis* for further analyses.

Despite their recovery from four different hosts, specimens of *D. insignis* showed very little sequence variation; intraspecific divergence averaged just 0.02% and the maximum divergence value between any pair of individuals was 0.31%, representing just two nucleotide changes (Table 1). Intraspecific variation was higher in *D. lutrae*, averaging 0.33% and reaching a maximum pair-wise divergence of 0.62%. The very low levels of sequence divergence in *D. insignis* prevented any analysis of the number of separate infections in single hosts. However, by analysing multiple nematodes from one otter (Fig. 3), it was possible to show that it had experienced at least two infections.

Discussion

Although methods commonly associated with phylogenetics (e.g. K2P divergences, NJ trees) were used for analysis of patterns of sequence divergence, it should be noted that the purpose of this study was not phylogenetic reconstruction but species discrimination. DNA barcoding reliably differentiated *Dracunculus insignis* and *D. lutrae* as the two species were represented by distinct, nonoverlapping clusters of sequences in the NJ tree. Two specimens from separate otter hosts yielded sequences that grouped with *D. insignis* rather than with *D. lutrae*. Reports have indicated that *D. insignis* is capable of infecting otter, although none of the cases reported provide descriptions or accurate identification of the species (see Kimber & Kollias 2000). This study is the first to report *D. insignis* infections in otter with certainty.

In each of these two otter hosts, only one female guinea worm was recovered. However, because both specimens were gravid females, these cases were viable infections. The fact that no males were present, although gravid females were, is not uncommon. From inoculation experiments of ferrets (*Mustela putorius furo*) with *D. insignis*, Brandt & Eberhard (1990) found that the recovery of female worms was at least twice as great as for male worms. In one group of 10 animals infected with approximately 100 third-stage *D. insignis* larvae, 29 (66%) worms recovered were female and 15 (34%) were male (Brandt & Eberhard 1990). In a second group of 21 ferrets inoculated with 50 third-stage larvae, 83 (90%) were female and nine (10%) were male (Brandt & Eberhard 1990). In a separate experiment, Brandt & Eberhard (1991) found that of 19 *D. insignis* recovered from seven of 10 infected ferrets, 74% were gravid females, 10% were immature females and 16% were males with only

Fig. 2 Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of COI *Dracunculus* sequences with BOLD identifiers (*n* = 82). Asterisks indicate specimens from otter that yielded *D. insignis* sequences.

RFNPM080-07 *Dracunculus lutrae*|Otter
RFNPM074-07 *Dracunculus lutrae*|Otter
87 RFNPM079-07 *Dracunculus lutrae*|Otter
RFNPM071-07 *Dracunculus lutrae*|Otter
RFNPM070-07 *Dracunculus lutrae*|Otter
RFNPM072-07 *Dracunculus lutrae*|Otter
RFNPM075-07 *Dracunculus lutrae*|Otter
RFNPM077-07 *Dracunculus lutrae*|Otter
RFNPM069-07 *Dracunculus lutrae*|Otter
RFNPM086-07 Dracunculus lutrae|Otter
RFNPM081-07 *Dracunculus lutrae*|Otter
RFNPM092-07 *Dracunculus lutrae*|Otter*
RFNPM082-07 *Dracunculus lutrae*|Otter
RFNPM087-07 *Dracunculus lutrae*|Otter*
RFNPM090-07 *Dracunculus lutrae*|Otter*
88 RFNPM094-07 *Dracunculus lutrae*|Otter*
RFNPM068-07 *Dracunculus lutrae*|Otter
RFNPM076-07 *Dracunculus lutrae*|Otter
RFNPM073-07 *Dracunculus lutrae*|Otter
RFNPM078-07 *Dracunculus lutrae*|Otter
RFNPM091-07 *Dracunculus lutrae*|Otter*
66 RFNPM089-07 *Dracunculus lutrae*|Otter*
67 RFNPM088-07 *Dracunculus lutrae*|Otter*

0.0005

Fig. 3 Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of COI *Dracunculus lutrae* sequences ($n = 23$) with bold identifiers. Asterisks indicate multiple nematodes from one individual otter.

Table 1 COI sequence divergence (K2P) within *Dracunculus* species

Species	COI sequence divergence (K2P)				
	Mean	SE	Minimum	Maximum	N
Dracunculus insignis	0.021	0.001	0	0.325	59
Dracunculus lutrae	0.327	0.013	0	0.621	23

two animals harbouring both male and gravid female worms. As Brandt & Eberhard (1990) point out, mature females are large and difficult to overlook, whereas males are small and could easily be missed. It has also been suggested that *Dracunculus* males die after mating and are re-absorbed by the host (Crichton 1972), therefore, the difference in recovery rates of male and female worms may be reflected by this (Brandt & Eberhard 1990). Eberhard *et al*. (1988) did, however, recover living male worms at 200 days of age in experimentally infected ferrets, indicating that not all male worms die shortly after mating. In our study, DNA barcoding was able to reveal that otter are not only infected with *D. lutrae*, but also with *D. insignis*. Without male specimens present in an infected otter, it would not be possible to confidently determine species based on morphology; however, DNA barcoding would permit species assignment.

Barcoding is also likely to be an effective tool for differentiating other members of the *Dracunculus* genus, such as *D. medinensis,* as well as potentially identifying other life-cycle stages. For example, it is suggested that transmission of *D. insignis* and *D. lutrae* from the intermediate to definitive host may involve a paratenic host (Eberhard & Brandt 1995). However, neither naturally infected intermediate hosts nor naturally infected paratenic hosts have been identified. In addition, there are occasional reports of human dracunculiasis in countries that are thought to be free of *D. medinensis* (Bimi *et al*. 2005). Bimi *et al*. (2005) report that these infections could have involved species of *Dracunculus*, other than *D. medinensis,* that had been acquired by humans when they inadvertently ingested a paratenic host of another species of the parasite; DNA barcoding may well answer such questions.

Individuals of the *D. insignis* group showed little sequence divergence regardless of the source host, indicating that this species is a 'true' generalist that parasitizes at least four mammal species, including the otter. Interestingly, despite its host specialization, *D. lutrae* included several separate mitochondrial lineages. Individual nematodes from a single otter had high sequence divergence (Fig. 3), indicating that this individual was infected on several occasions. This result suggests that *D. lutrae* is both genetically diverse and panmictic, differing in this regard from the common presumption that genetic differentiation is occurring among parasites from different hosts (Price 1980).

Acknowledgements

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