



female worms can often be identified only to genus level by morphology. Claims of non-human infection with *D. medinensis* that are based solely on the morphological features of female worms — especially those from areas that have no, or no recent, history of the endemic transmission of *D. medinensis* — therefore need to be interpreted with great caution.

It would be extremely useful and convenient if female specimens of *Dracunculus* could be identified to species level by molecular characterization. Even a small portion of female worm could be investi-

of the *Dracunculus* specimens from humans consisted of the posterior segments of broken or ruptured female *D. medinensis* (with large numbers of larvae forming sediments at the bottom of many of the collection vials). After the careful decanting off of any ethanol or saline, each sample was washed three times with 0.01 M phosphate-buffered saline (pH 7.2) containing 1 mM EDTA. The sample was then soaked overnight (12–18 h), at 37°C, in 1 ml of the CLS-TC buffer from the FastDNA® kit (Q-BIOgene, Irvine, CA), to facilitate the rehydration of any DNA. Approximately 20 mg of larvae or 2–3 cm of adult worm were used for each DNA extraction. Before processing, each 2- to 3-cm-long piece of adult worm was placed in a Petri dish with 1 ml CLS-TC buffer and cut into small pieces with a disposable scalpel blade. Each sample of larvae or chopped adult worm was then homogenized, with an electric homogenizer, in a 2.0-ml microcentrifuge tube. The FastDNA kit and the FastPrep® cell disruptor (Q-BIOgene) were then used, according to their manufacturer's recommendations, to extract the DNA. The cell disruptor was run, for 20 s at setting 5.0, either once on each sample or, if intact tissue was still visible, twice. Some samples of extracted DNA were found to contain PCR inhibitors and these samples were further purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's recommendations.

C - e3 A                    e3 e3  
 C                    e3 *Dracunculus* 18.                    A  
 e3e3

Extracted DNA was amplified using the custom-made forward primer NEMFG1 (5'-TCT CCG ATT GAT TCT GTC GGC GAT TAT ATG-3'), which matches bases 1–30 of the 18S-rRNA sequence of *Gnathostoma binucleatum* (GenBank accession number Z96946), and the standard, eukaryotic-18S-rRNA reverse primer

CRYPTOR (5'-GCT TGA TCC TTC TGC AGG TTC ACC TAC-3').

Each 50- $\mu$ l PCR reaction mixture consisted of 1 $\times$  buffer (10 mM Tris-HCl, 50 mM KCl and 1.5 mM MgCl<sub>2</sub>, at pH 9.0), 200  $\mu$ mol of each deoxynucleotide triphosphate, 25 pmol of each primer, 2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), and 0.1 or 1  $\mu$ l of DNA. An automated thermocycler (GeneAmp PCR System 9700; Applied Biosystems) was set to give an initial polymerase-activation step of 15 min at 95°C, followed by 45 cycles, each of 30 s at 94°C, 30 s at 60°C and 90 s at 72°C, and then a final extension step for 10 min at 72°C.

e3                    C                    e3  
 The PCR products were resolved by electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized on an ultra-violet trans-illuminator (Fotodyne, Hartland, WI). A 100-bp ladder (Invitrogen, Carlsbad, CA) was used as the size standard.

A e3 e3  
 Amplification products were purified using the StrataPrep® PCR purification kit (Stratagene, La Jolla, CA) according to the instructions from the manufacturer, and eluted in 50  $\mu$ l ultra-violet-irradiated water. The sequencing reactions used to identify the *Dracunculus* species represented by each sample were performed, on both strands of the purified products, using the NEMFG1 and CRYPTOR primers as well as the internal primers NEM1F (5'-CTG CCT TAT CAA CTT TCG ATG-3'), NEM1R (5'-CAT CGA AAG TTG ATA AGG CAG-3'), NEM2F (5'-GCG GTT AAA AAG CTC GTA GTT GG-3'), NEM2R (5'-CCA ACT ACG AGC TTT TTA ACC GC-3'), NEM3F (5'-GCG GCT TAA TTT GAC TCA ACA C-3'), NEM3R (5'-GTG TTG AGT CAA ATT AAG CCG C-3'), NEM4F (5'-CCG GGA CTG AGC CGT TTC GAG-3') and



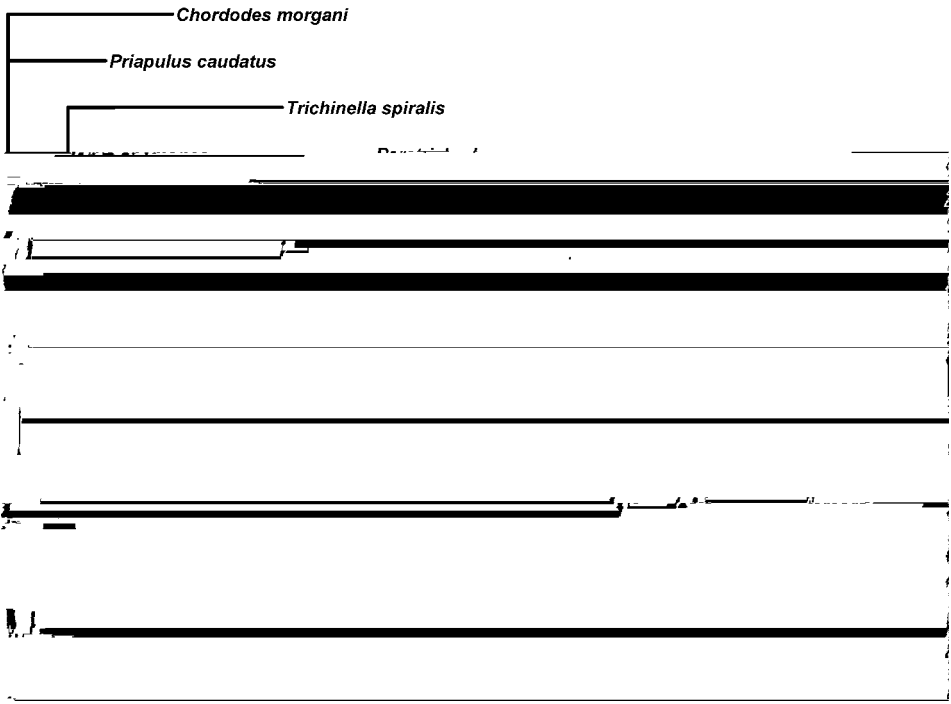


FIG. Phylogenetic tree based on the small-subunit-ribosomal-RNA (18S-rRNA) sequences of *Dracunculus medinensis* and *D. insignis* (as determined in the present study) and those of selected Nematoda (that were already available in the GenBank database). Quartet-puzzling maximum-likelihood results (Strimmer and Haeseler, 1996) are shown, with *Priapulus caudatus* (phylum Priapulida) and *Chordodes morgani* (phylum Nematomorpha) used as

is stopped in more and more areas, the possibility of an unexpected human infection with *Dracunculus* occurring in an area considered dracunculiasis-free increases. The response to each of these unexpected cases will depend partly on the travel history of the patient during the preceding year and partly on the confirmation that the parasite involved is *D. medinensis* and not a zoonotic infection with another species of nematode. The discovery of a viable adult *D. medinensis* in an area categorised as dracunculiasis-free would be cause for considerable concern to the DEP — particularly if the infection could only have been acquired in such an

area — and would necessitate extensive interventions to contain and eventually stop any transmission. If, on the other hand, a suspected case of dracunculiasis was found to be infected only with a species of *Dracunculus* other than *D. medinensis*, there would be no cause for alarm and no public-health interventions would be necessary.

On only three occasions during the last 78 years have cases of human dracunculiasis been reported from countries that have never been known to have the endemic disease: Korea (Hashikura, 1926, 1927), Japan (Kobayashi et al., 1986) and China (Wang et al., 1995). In all three instances

the parasite involved was described as *D. medinensis*, even though the subjects found infected had no history of travel to a country with endemic human dracunculiasis and no further transmission of the infection to other people was observed. These puzzling infections were probably not *D. medinensis* but with a species of *Dracunculus*.

Dracunculoidea. As seen previously (Anderson, 1988; Blaxter et al., 1998), the Ascaridida and Spirurida did not resolve well. It was not possible to identify which group of the spirurids is most closely related to the dracunculids. The phylogenetic relationship of the dracunculids within the Spirurida therefore remains to be elucidated.

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## REFERENCES

- Anderson, R. C. (1988). Nematode transmission patterns. *Journal of Parasitology*, **74**, 30–45.
- Beaver, P. C., Jung, R. C. & Cupp, E. W. (1984). *Clinical Parasitology*, 9th Edn. Philadelphia, PA: Lea & Febiger.
- Blaxter, M. L., de Ley, P., Garey, J. R., Liu, L. X., Scheldeman, P., Vierstraete, A., Vanfleteren, J. R., Mackey, L. Y., Dorris, M., Frisse, L. M., Vida, J. T. & Thomas, W. K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature*, **392**, 71–75.
- Crichton, V. F. J. & Beverley-Burton, M. (1977). Observations on the seasonal prevalence, pathology and transmission of *Dracunculus insignis* (Nematodoidea: Dracunculoidea) in the raccoon (*Procyon lotor* (L.)) in Ontario. *Journal of Wildlife Diseases*, **13**, 273–280.
- Felsenstein, J. (1989). PHYLIP — phylogeny inference package. *Cladistics*, **5**, 164–166.
- Hashikura, T. (1926). A case of *Filaria medinensis* in Chosen (Korea). *Chosen Igakka Zasshi*, **3**, 68.
- Hashikura, T. (1927). One case of *Filaria medinensis* in Korea. *Japanese Medical World*, **7**, 145–146.
- Joseph, S. A. & Kandasamy, S. (1980). On the occurrence of the guinea worm, *Dracunculus medinensis* (Linnaeus, 1758; Galladant 1773) in an Alsatian dog (Tamil Nadu, India). *Cheiron*, **9**, 363–365.
- Kobayashi, A., Katatura, A., Hamada, A., Suzuki, T., Hataba, Y., Tashiro, N. & Yoshida, A. (1986). Human case of dracunculiasis in Japan. *American Journal of Tropical Medicine and Hygiene*, **35**, 159–161.
- Lalitha, C. M. & Anandan, R. (1980). Guinea worm infections in dogs (Tamil Nadu, India). *Cheiron*, **9**, 198–199.
- Litvinov, V. F. & Litvinov, V. P. (1981). Helminths of predatory mammals from eastern Azerbaijan, SSR, USSR. *Parazitologiya*, **13**, 210–223.
- Litvinov, V. F. & Lysenko, A. (1982). Dracunculiasis: history of the discovery of the intermediate host and the eradication of foci of invasion in the USSR. In *Proceedings of a Workshop on Opportunities for Control of Dracunculiasis*, June 16–19, 1982. Washington, DC: National Research Council.
- Moravec, F. & Little, D. (2004). Redescription of *Dracunculus globocephalus* Mackin, 1927 (Nematoda: Dracunculidae), a parasite of the snapping turtle, *Chelydra serpentina*. *Folia Parasitologica*, **51**, 339–345.
- Muhammad, G., Khan, M. Z., Athar, M. & Saqib, A. (2005). Dracunculus infection in a dog during the 'post-eradication' period: the need for a longer period of surveillance. *Annals of Tropical Medicine and Parasitology*, **99**, 105–107.
- Muller, R. (1971). Dracunculus and dracunculiasis. *Advances in Parasitology*, **9**, 73–153.
- Page, R. D. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, **12**, 357–358.
- Strimmer, K. & Haeseler, A. (1996). Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Molecular Biology and Evolution*, **13**, 964–969.
- Swofford, D. L. (1996). PAUP\*, Version 4.0b1. *Phylogenetic Analysis using Parsimony (and Other Methods)*. Sunderland, MA: Sinauer Associates.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673–4680.
- Wang, Z., Jisuan, D. & Wang, X. (1995). Dracunculiasis discovered for the first time in China. *Chinese Journal of Zoonosis*, **11**, 15–17.