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 over-night (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 re-hydration 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 elec-tric homogenizer, in a 2.0-ml microcentri-fuge 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 acces-sion number Z96946), and the standard, eukaryotic-18S-rRNA reverse primer

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

Each 50-μl PCR reaction mixture con-sisted of 1× buffer (10 mM Tris-HCl, 50 mM KCl and 1.5 mM MgCl$_2$, at pH 9.0), 200 μmol 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 μl of DNA. An automated thermo-cycler (GeneAmp PCR System 9700; Applied Biosystems) was set to give an initial poly-merase-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 ➤-

The PCR products were resolved by elec-trophoresis in 1% agarose gel, stained with ethidium bromide, and visualized on an ultra-violet trans-illuminator (Fotodyne, Hartland, WI). A 100-bp ladder (Invit-rogen, 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 μl 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

*Chordodes morgani*

*Priapulus caudatus*

*Trichinella spiralis*
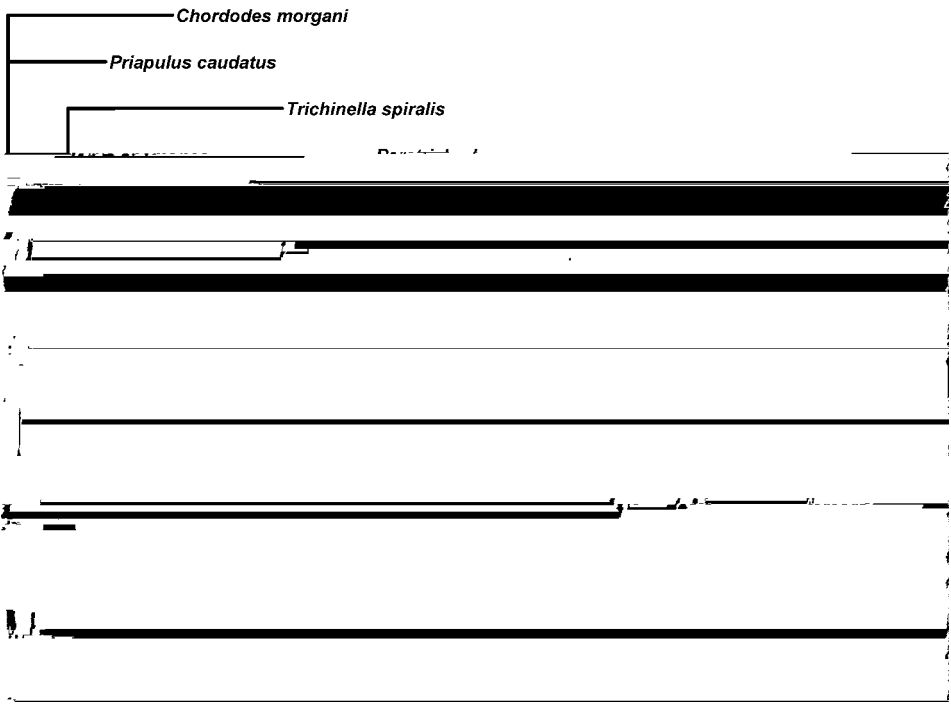
FIG. Phylogenetic tree based on the small-subunit-ribosomal-RNA (18S-rRNA) sequences of Dracunculus medinensis and D. insignis (as determined in the present study) and those of selected Nematoda (that were already available in the GenBank database). Quartet-puzzling maximum-likelihood results (Strimmer 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 puz-
zling infections were probably not D.
medinensis but with a species of DracuncuT/F37wesis

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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