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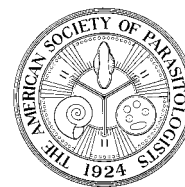
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## DRACUNCULUS SPECIES IN MESO-MAMMALS FROM GEORGIA, UNITED STATES, AND IMPLICATIONS FOR THE GUINEA WORM ERADICATION PROGRAM IN CHAD, AFRICA

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### KEY WORDS ABSTRACT

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Transmission

The prevalence and diversity of parasitic nematodes in wildlife have been well studied for certain species, yet for others considerable gaps in knowledge exist. The parasitic nematode *Dracunculus insignis* infects North American wildlife, and past research on this species has led to an increased understanding of the potential host diversity and transmission of the closely related human Guinea worm, *Dracunculus medinensis* (which is currently the focus of a global eradication program). Many definitive hosts have been documented for *D. insignis*; however, the life cycle has been studied only in laboratories, and only a single phylogenetic study has been conducted on *D. insignis* (from Canada). The goals of the present study were to investigate the prevalence of infections with *Dracunculus* species among wildlife at a single site (Di-Lane plantation) in the southeastern United States, evaluate the genetic diversity of parasites at this site, and investigate potential paratenic hosts that may be involved in transmission. Over 3 yr, we sampled 228 meso-mammals, reporting an overall prevalence of infection with *Dracunculus insignis* of 20% (46/228). Amphibians and fish were sampled in the same geographic area as infected meso-mammals. *Dracunculus insignis* third-stage larvae were recovered from 2 different species of amphibians, but all fish sampled were negative. Phylogenetic analysis of the partial cytochrome *c* oxidase I (*COI*) gene showed very little diversity of *Dracunculus* at Di-Lane; however, we did recover a single nematode from a Virginia opossum (*Didelphis virginiana*) that falls outside of the *D. insignis* clade, more closely aligns with *Dracunculus lutrae*, and may represent an undescribed species. This work documents the occurrence of *D. insignis* in frogs, a potential transmission pathway for *D. insignis* at a single geographic site in nature. When applied to the global Guinea Worm Eradication Program, and Chad, Africa, in particular, this work increases our knowledge of the potential role of aquatic animals in the transmission of *Dracunculus* species and informs on potential intervention strategies that may be applied to the eradication of Guinea worm in Africa.

*Dracunculus* spp. are subcutaneous nematode parasites (Family Dracunculidae, Order Spirurida) of numerous reptile and mammalian species. Although natural history data are limited for reptile-infecting species, considerable data are available for *Dracunculus medinensis*, which infects people and causes Guinea worm disease in Africa. Considerable research also has been conducted on *Dracunculus insignis*, which infects wildlife species such as raccoons (*Procyon lotor*), mink (*Neovison vison*), skunks (*Mephitis mephitis*), weasels (*Mustela* spp.), and North American river otters (*Lontra canadensis*) in North America (Crites, 1963; Crichton and Beverly-Burton, 1974; Anderson, 2000; Cleveland et

al., 2018; Williams et al., 2018). *Dracunculus* infections have been documented in a variety of hosts from multiple regions of North America; however, identification in most studies was limited to the morphology of female worms, which cannot be reliably identified to species without genetic characterization. Although male worms are morphologically distinguishable, they are rarely detected (Cleveland et al., 2018). The only previous study that examined the host range of *D. insignis* (confirmed by sequence analysis) was conducted in Canada and found that *D. insignis* recovered from raccoons, mink, fishers (*Martes pennanti*), and river otters had minimal sequence divergence among hosts,



supporting the hypothesis that *D. insignis* is a host generalist (Elsasser et al., 2009). By contrast, *Dracunculus lutrae* was found to be a host specialist for river otters but exhibited a high degree of genetic diversity, and several individual otters were infected with multiple parasite lineages (Elsasser et al., 2009).

*Dracunculus insignis* has been used as a model parasite for eradication efforts of *D. medinensis* because of its close genetic relationship, the presumed similarities in life cycles, the ability to acquire local specimens, and a history of successful experimental infections (Beverly-Burton and Crichton, 1976; Eberhard et al., 1988; Eberhard and Brandt, 1995; Elsasser et al., 2009). The effectiveness of the eradication program is limited by the state of knowledge on the life cycle of *D. medinensis*. For example, the epidemiology of *D. medinensis* has recognizably changed (Eberhard et al., 2014). Since 2012, there has been an increase in the number of peri-domestic dog and cat infections in Chad, Ethiopia, and Mali in Africa (Eberhard et al., 2014; Molyneux and Sankara, 2017). The geographic distribution and increasing number of animal infections led to the hypothesis that aquatic paratenic hosts (e.g., fish and amphibians) may play a role in the transmission of *D. medinensis*. This was recently supported by experimental work and the detection of a *D. medinensis* third-stage larvae (L3s) in a wild-caught frog in Chad (Eberhard et al., 2014, 2016a, 2016b; Cleveland et al., 2017). However, little work has been done on the natural cycle of transmission of *D. medinensis* in Chad among dogs, and no research has been conducted on potential susceptible wildlife. Understanding the natural history and genetic diversity of *D. insignis* in wildlife in North America may assist the eradication efforts for *D. medinensis* because, if amphibians are a reliable source of infection to definitive hosts, then this information can provide valuable insight into devising and implementing interventions to prevent infections of *D. medinensis* in Chadian dogs and cats.

One purpose of this study was to better understand the prevalence and genetic diversity of *Dracunculus* spp. in various meso-mammals—raccoons, opossums (*Didelphis virginiana*), coyotes (*Canis latrans*), bobcats (*Lynx rufus*), and armadillos (*Dasypus novemcinctus*)—at a single site in Georgia. To date, no multi-host surveys for *Dracunculus* spp. have been conducted in the southeastern United States, and the single study that investigated the genetic diversity of *D. insignis* was conducted on worms collected from throughout Ontario, Canada (Elsasser et al., 2009). In addition, little work has been done examining the natural transmission cycle. Although it has been hypothesized that *Dracunculus* can use aquatic paratenic host(s) (likely an amphibian), no natural infections of paratenic hosts have been reported for *D. insignis* and only limited frog species infected with *D. medinensis* (Crichton and Beverly-Burton, 1977; Anderson, 2000; Eberhard et al., 2016a, 2016b, Cleveland et al., 2019). Thus, we conducted surveillance of local fish and amphibians for *D. insignis* to identify any paratenic or transport hosts potentially involved in transmission at a site with suspected persistence of the parasite in raccoons.

## MATERIALS AND METHODS

### Sampling site

Di-Lane plantation is a 3,278-ha wildlife management area located in Burke County, Georgia (32°58'12.2"N, 82°03'34.0"W) managed by the Georgia Department of Natural Resources for

early successional habitat with an emphasis on bobwhite quail (*Colinus virginianus*). In addition to habitat management, supplemental feeding and predator control programs currently are being conducted in an effort to increase bobwhite quail populations.

### Animal collection

**Meso-mammals:** Predator removal at Di-Lane plantation was conducted by USDA APHIS Wildlife Services, and we examined the following adult animals (based on size) captured from 2015 to 2017: raccoons, opossums, coyotes, bobcats, and armadillos. In each year of trapping, 120 live capture traps (Tomahawk Live-Trap Company, Tomahawk, Wisconsin) and 120 double coil spring offset jaw foot-hold traps (MB-550, Minnesota Trapline Products Inc., Pennock, Minnesota) were used during 2 trapping sessions occurring from late February to mid-March and from mid-May to June (between 14 and 21 trapping nights per session). Trapping sessions were either right before or during the peak time of emergence of female *D. insignis*, an important consideration for retrieval of subcutaneous worms because it allows for easier detection of patent infections and determination of whether the females have been mated, are gravid, and may be sustaining the life cycle of *Dracunculus* spp. at Di-Lane plantation (Cleveland et al., 2018). Each trap was checked beginning at sunrise. Captured animals were euthanized following the American Veterinary Medical Association's guidelines for humane euthanasia of animals (Leary et al., 2013). The examination of animals for pathogens was included in a protocol reviewed and approved by University of Georgia, Institutional Animal Care and Use Committee (UGA IACUC) (A2018 02-010).

**Paratenic hosts:** Amphibians and fish were caught at several permanent ponds at Di-Lane plantation using dip nets in shallow areas (1 m or less) March–June of 2016 and 2017. Animal choice was based on proximity to shallow areas of ponds that would be accessible to meso-mammals during hunting or scavenging events, rather than amphibians and fish that occur in water deeper than 1 m and were unlikely to be caught by meso-mammals. Each animal was euthanized via cervical dislocation, identified to species, eviscerated, and skinned. The remaining muscle tissue was bluntly dissected, facilitating release and recovery of any *D. insignis* larvae present as described previously (Eberhard et al. 2016a, 2016b; Cleveland et al., 2019). Tissue was placed in Petri dishes with Dulbecco's phosphate buffered saline (DPBS) for a minimum of 4 hr until microscopy was conducted for larval detection (Eberhard and Brandt, 1995). Capture and sampling of aquatic hosts were reviewed and approved by UGA's IACUC (A2018 02-010).

**Parasite collection and identification:** Meso-mammals were necropsied immediately or were frozen at  $-20^{\circ}\text{C}$  until they were necropsied. Peritoneal cavities and subcutaneous tissues were examined for parasites. Subcutaneous parasites were stored in either 70% ethanol (EtOH) immediately or in DPBS overnight to allow L3s to exit for use in experimental infection trials (Cleveland et al., 2017). After the recovery of larvae, nematodes were stored in 70% EtOH for subsequent molecular analyses. Adult females were classified as *Dracunculus* sp. based on general characteristics such as location in the definitive host, size, morphology, and the presence of larvae (Anderson, 2000; Cleveland et al., 2018).

**Table I.** Prevalence of adult female *Dracunculus* in meso-mammals sampled at Di-Lane plantation, Waynesboro, Georgia, during 2015–2017.

Year of sampling	Raccoon ( <i>Procyon lotor</i> )		Virginia opossum ( <i>Didelphis virginiana</i> )	
	Male	Female	Male	Female
2015	58.8% (10/17)	0% (0/1)	NA*	NA*
2016	45% (18/40)	8.3% (1/12)	33.3% (5/15)	0% (0/25)
2017	20.7% (6/29)	13% (3/23)	5.6% (1/18)	8.7% (2/23)
Overall Prevalence	39.5% (34/86)	11.1% (4/36)	18.2% (6/33)	4.2% (2/48)
Total prevalence	31.1% (38/122)		9.9% (8/81)	

\* NA = Not available.

After tissues from amphibians and fish were soaked, the DPBS was examined for larvae. Any larvae exhibiting gross morphologic similarity to *Dracunculus* spp. were removed, assessed under a compound microscope for the presence of a blunt, trifid tail, and then placed individually in 1.5 ml microcentrifuge tubes with 70% EtOH for molecular characterization. A generalized linear model was performed to analyze the relationship between binary infection status (0 = negative, 1 = infected) with *Dracunculus* species and the independent variables of species (raccoon or opossum) and sex (male or female). All analyses were performed in R (R Core Team 2019).

**Molecular characterization:** Adult nematodes were removed from EtOH, and several small (1–2 mm) pieces were placed into a microcentrifuge tube, which was left open for 12 hr to allow EtOH to evaporate. Suspect larvae were processed in the same way as adult nematodes except whole larvae were extracted and the *18S* gene was amplified as described by Bimi et al. (2005). DNA was extracted from adult nematodes and larvae using a commercial DNA extraction kit (DNeasy, Qiagen, Valencia, California) following the manufacturer's instructions for tissue. The partial cytochrome *c* oxidase I (*COI*) gene was amplified using a cocktail of 6 M13-tagged primers as described (Prosser et al., 2013). Amplicons were purified from a 0.8% agarose gel stained with gel red (Biotium Inc., Hayward, California) using a commercial gel-purification kit (Qiagen). Purified amplicons were bi-directionally sequenced at the University of Georgia Genomics Facility (Athens, Georgia). Chromatograms were analyzed in Geneious R7 (Auckland, New Zealand), and consensus sequences were generated and compared to sequences in the GenBank database. Sequences were aligned using ClustalW (Thompson et al., 2002) in MEGA. Phylogenetic trees were constructed in MEGA X using maximum-likelihood algorithms with partial deletion and 1,000 bootstrap iterations (Kumar et al., 2018). Sequences from this study were deposited into GenBank (accession nos. MK085893–MK085902). For comparison, sequences from *D. medinensis* (AP017682, HQ216219), *D. lutrae* from Canada (EU646594, EU646593, EU646600, EU646602), and *D. insignis* from Canada (EU646534, EU646535, EU646559, EU646569) were obtained from GenBank and included in the phylogenetic analysis with *Philometroides sanguinensis* (NC024931) and *Procamallanus slomei* (MG948463) as outgroups to root the tree.

## RESULTS

### Meso-mammals

A total of 228 meso-mammals were sampled for infection with *Dracunculus* (Table I), of which 46 were infected with subcuta-

neous nematodes grossly identified as *Dracunculus* species. The highest prevalence was noted in raccoons (31%, 38/122, 95% CI 23–40%). Prevalence in male raccoons is 39.5% (95% CI 30–51%), and in female raccoons 11% (95% CI 4–26%). Opossums had the second-highest prevalence (9.9%, 8/81, 95% CI 5%–18%), for male opossums 18% (95% CI 8–35%) and for female opossums 4.2% (95% CI 0.7–14%). Sample sizes were low for coyotes ( $n = 11$ ), bobcats ( $n = 7$ ), and armadillos ( $n = 7$ ), and all individuals were negative. A total of 90 female *Dracunculus* were recovered, 77 from raccoons and 13 from opossums. Of the 90 recovered specimens, 71% (55/77) were found in the hind limbs of raccoons, and 54% (7/13) were on hind limbs of the opossums. We recovered 5 female *Dracunculus* from the abdominal (3) and pectoral (2) musculature of opossums. Male *Dracunculus* spp. were not recovered during this study.

The generalized linear model showed that the probability of being infected with *Dracunculus* species was significantly higher among raccoons ( $z = 3.38$ ,  $P < 0.001$ ) and males ( $z = 3.01$ ,  $P < 0.01$ ) (full model outputs presented in Suppl. Table S1) compared to opossums and females, and males of both species were more frequently infected than females. However, the highest worm burden was recorded in a female raccoon ( $n = 9$ ), and female raccoons had a higher average worm burden and range (4.3,  $n = 1–9$ , respectively) compared to male raccoons (average 2.7 worms, range  $n = 1–7$ ). Male opossums had an average of 2.2 worms with a range of 1–5, whereas we recovered only single *Dracunculus* female worms from female opossums.

### Paratenic hosts

During the peak transmission seasons of 2 yr (March–June 2015/2016) of *Dracunculus* species in Georgia, we sampled 68 frogs representing 5 species (Table II). *Dracunculus* larvae were detected in 2 species, *Rana* [*Lithobates*] *catesbeiana* (6/43) and *Rana* [*Lithobates*] *sphenocephala* (5/11). The intensity was generally low (mean of 1.6 and 15, respectively) but 1 *R. [Lithobates] sphenocephala* harbored 45 larvae. A single larva from each positive frog was confirmed to be *D. insignis* by sequence analysis of the *18S* gene and was 99% similar to *D. insignis*. We also sampled 68 *Centrarchus macropterus* (Flier sunfish) from ponds on Di-Lane plantation, and all were negative for *Dracunculus* larvae.

### Molecular and phylogenetic analyses

A total of 50/90 adult female nematodes recovered from definitive hosts (38 raccoons, 12 opossums) yielded useable sequence via Sanger sequencing and were genetically character-

**Table II.** Prevalence of *Dracunculus insignis* third-stage larvae (L3) recovered from fish and amphibians at Di-Lane plantation, Waynesboro, Georgia during 2015–2017.

Host	Species	No. sampled	Prevalence (positive/total)	No. larvae (range, mean)
Fish	<i>Centrarchus macropterus</i> (Flier sunfish)	68	0% (0/68)	NA*
Amphibians	<i>Rana catesbeiana</i> (Bullfrog)	43	14% (6/43)	10 (1-4, 1.6)
	<i>Rana sphenoccephala</i> (Southern leopard frog)	11	45% (5/11)	61 (2-45, 15)
	<i>Acris crepitans</i> (Northern cricket frog)	3	0% (0/3)	NA
	<i>Hyla cinerea</i> (Green treefrog)	7	0% (0/7)	NA
	<i>Psuedacris crucifer</i> (Spring peeper)	4	0% (0/4)	NA

\* NA = Not available.

ized. All were confirmed to be *Dracunculus* species. The maximum-likelihood phylogenetic tree revealed no geographic or host clustering of *D. insignis* sequences. Sequences from all but 1 worm from Di-Lane were 99.9% similar to one another and *D. insignis* sequences derived in various hosts from Canada (Elsasser et al., 2009). A single worm from an opossum (MK085893) was only 92% similar to *D. insignis* and 91% similar to *D. lutrae* (our study and from Elsasser et al. [2009]). The unique *Dracunculus* sequence from the opossum clustered with other sequences of *D. lutrae* outside of the *D. insignis* clade (Fig. 1).

## DISCUSSION

*Dracunculus insignis* has been well documented in raccoons across a wide geographic area of North America (Crichton and Beverly-Burton, 1974, 1977; Cleveland et al., 2018); however, detailed investigation into the range of susceptible wildlife, associated phylogenetic relationships, and the role of paratenic and transport hosts in the transmission cycle at endemic locations had yet to be investigated. Our study supports the role of raccoons as the most common definitive host for *D. insignis* (Crichton and Beverly-Burton, 1974) but also indicates the potential involvement of opossums in supporting sylvatic transmission. The diet of opossums is similar to raccoons (McManus, 1974; Kasparian et al., 2002), therefore transmission of *D. insignis* via consumption of a potential paratenic host could occur. Our finding of *D. insignis* in *R. [Lithobates] catesbeiana* and *R. [Lithobates] sphenoccephala* provides further support that paratenic hosts may be important in the life cycle of *Dracunculus* spp. as revealed by experimental studies and a recent finding in Chad (Eberhard et al., 2016a, 2016b; Cleveland et al., 2017).

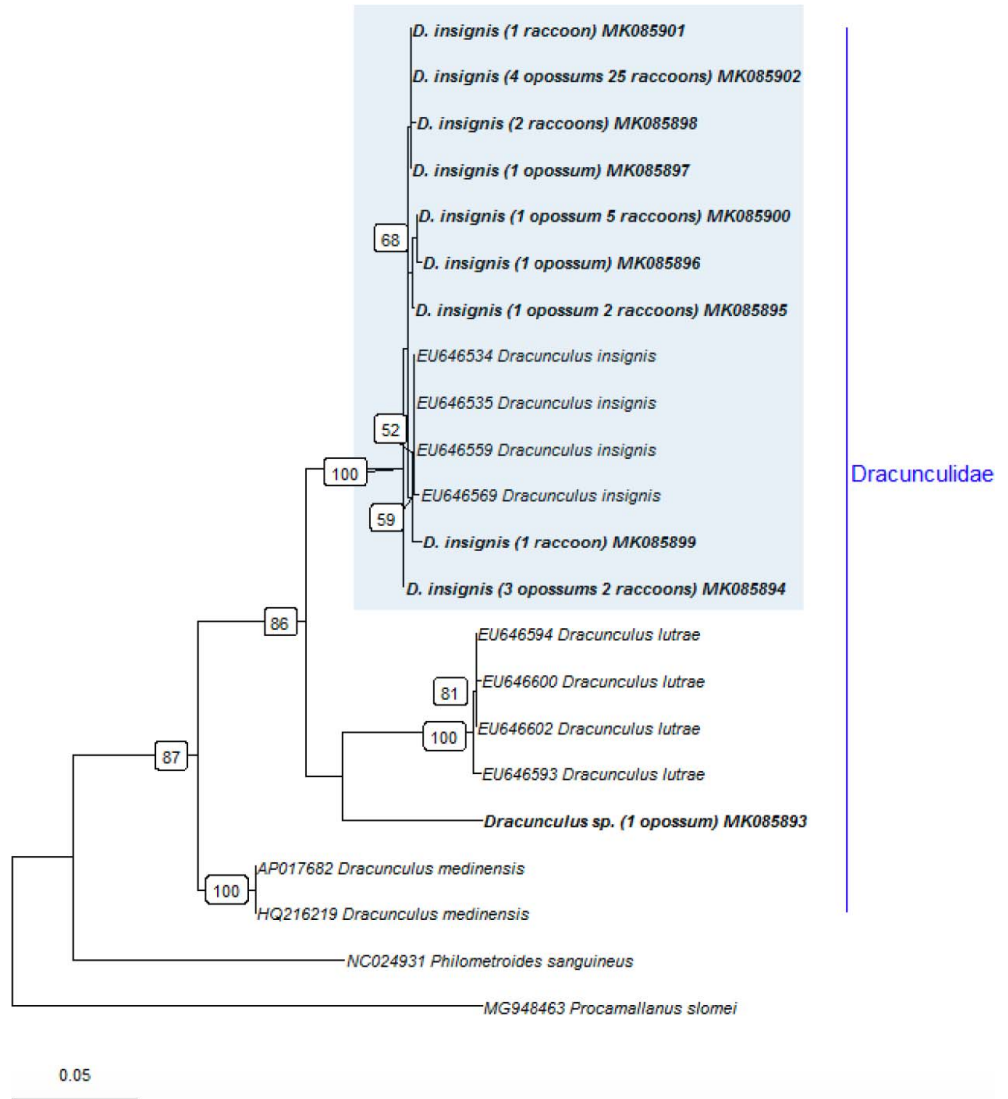
The significant difference between the prevalence of infection in male versus female animals could be a result of behavioral differences during the seasonality of sampling (spring); females of both raccoons and opossums are with young during these sampling periods and may have decreased movement while increasing localized foraging bouts. Additionally, female raccoons exhibit greater site fidelity than their male counterparts (Gehrt and Fritzell, 1998), which could limit the use of multiple water sources across the landscape and may explain the lower prevalence of infection among female raccoons in this study. Male raccoons and opossums often have larger territories than females (Holmes and Sanderson, 1965; Lotze and Anderson, 1979; Gehrt and Fritzell, 1998) and could be foraging more broadly across the landscape. Finally, the relationship between raccoons and water is supported by the documented behavior of

raccoons utilizing water sources to “wash” their prey, resulting in raccoons at water sources and in proximity to amphibian populations and potential predation of amphibians (Lyall-Watson, 1963; Lotze and Anderson, 1979).

We found no infection in coyotes and bobcats; however, sample sizes were low. Further work is needed to determine if these animals are possibly unrecognized hosts of *D. insignis* (Cleveland et al., 2018). A recent review of *Dracunculus* infections in domestic dogs and cats highlights that canids and felids are susceptible to infection (Williams et al., 2018). Furthermore, given the diversity of known hosts for *D. insignis*, it would seem that infection with *D. insignis* in coyotes and bobcats is possible and warrants further investigation. Phylogenetic analysis of *Dracunculus* species recovered from raccoons and opossums showed little sequence divergence, reiterating the role of *D. insignis* as a host generalist.

The life cycle of *D. medinensis* was first determined in 1871 (Fedchenko, 1871), and since that time it was considered a human parasite with only rare spill-over events to animals. However, the changing epidemiology in Chad led to the suggestion that aquatic hosts could be involved based on previous experimental data, indicating tadpoles (*Rana [Lithobates] pipiens*, *Rana [Lithobates] clamitans*, *R. [Lithobates] catesbeiana*) are susceptible to infection with *D. insignis* (Crichton and Beverly-Burton 1977; Eberhard and Brandt, 1995; Eberhard et al., 2014). Despite these experimental data, a published report has documented a single frog in Chad that was found infected with *D. medinensis* (Eberhard et al., 2016a), and similar findings in 2 additional frog species in Chad have been documented (Cleveland et al., 2019). Our finding of *D. insignis* L3s in 11 frogs provides support that paratenic hosts may be involved in the life cycle of *Dracunculus* spp. (Eberhard and Brandt, 1995; Anderson, 2000). The high number of L3s ( $n = 45$ ) recovered from the tissue of a single frog from Di-Lane may also partially explain high worm burdens that may occur in some raccoons and opossums. The intensity of infection was generally low among frogs in this study, however, and further work is required to better appreciate the relationship between *D. insignis*, frogs, and definitive hosts (Table I).

In contrast to frogs, we did not find any larvae in tissues of the sampled fish species *C. macropterus*. These fish were caught near the shore in the same water bodies as the sampled amphibians and were the only species readily caught via the use of dip nets. It is possible that there are other fish species available to and consumed by meso-mammals at Di-Lane plantation; however, we were not able to catch or sample any other species. The lack of natural infection supports previous experimental work highlighting the difficulty of experimentally infecting fish (Crichton and



**Figure 1.** Genetic relationships of 50 *Dracunculus* adult females from raccoons (n = 38) and Virginia opossums (n = 12) from Di-Lane plantation (Georgia) compared with other *Dracunculus* spp. based on partial cytochrome *c* oxidase subunit 1 gene sequences. The text in bold in the figure represents specimens analyzed in this study. The shaded portion indicates specimens that fall into the *Dracunculus insignis* clade. Color version available online.

Beverly-Burton, 1977). However, experimental work does show that fish may have a role as transport hosts instead of paratenic hosts (Cleveland et al., 2017). The ability of a fish to act as a transport host could be a result of the short transit time of larvae and copepods (the intermediate host) through fish gastrointestinal (GI) tracts or the narrow probability of detection of *D. insignis* L3s within short intervals of fish predating upon infected copepods. For example, previous experimental work has shown that beyond 3 hr from initial ingestion, various species of fish will either digest copepods and larvae or they will pass through the GI system (Cleveland et al., 2017). To date, no fish investigated in the wild has had a true infection in musculature with *D. insignis* L3s. The role of fish in the transmission of infection to definitive hosts continues to be an important research topic due to the high numbers of fish that are consumed by humans, dogs, and cats in Chad and may be supporting transmission despite eradication efforts.

One purpose of this study was to investigate the diversity of hosts that may be infected with *D. insignis* at a single geographic site and to evaluate the genetic differences of *D. insignis* recovered from those infected animals. Overall, we found infections in 2/5

species (raccoons and opossums) and that there was very little genetic difference among the female *D. insignis* recovered. However, a single recovered worm (OPO MK085893) was not similar to either of the 2 described mammalian *Dracunculus* species in North America (Fig. 1). This raises numerous questions, including the host range, geographic range, and life cycle of this parasite, highlighting the need for increased molecular characterization work on parasites, especially those like *Dracunculus* for which few morphological features on adult female worms are useful for distinguishing species. The results of our work and the new influx of publicly available sequences represent a significant contribution to a rather understudied group of parasites of increasing interest and importance. It is our hope that future findings can illustrate the hidden diversity and natural history of the *Dracunculus* genus.

Finally, we recovered *D. insignis* L3s from 2 species of amphibians (*R. catesbeiana* and *R. sphenoccephala*) that may be acting as a common food source among raccoons and opossums (Lotze and Anderson, 1979; Kasparian et al, 2002) and potentially supporting transmission of *D. insignis* at Di-Lane plantation. When placed in the context of Guinea worm eradication in Chad,

it is important to appreciate that sylvatic transmission of *D. medinensis* may be occurring in wildlife, and this could be responsible for the increasing incidence and prevalence of infections in dogs and cats despite comprehensive intervention strategies. The finding of naturally occurring paratenic hosts for *D. insignis* coupled with the previous report of a naturally infected frog in Chad (Eberhard et al., 2016a) show that transmission to dogs, cats, and wildlife in Chad may be possible outside of the classical route of ingestion of infected copepods from drinking water. If eradication of *D. medinensis* is to be achieved, consideration of a wildlife reservoir with potential transmission routes similar to that of *D. insignis* must be considered.

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