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Cryptic species of hairworm parasites revealed by molecular data and crowdsourcing of specimen collections

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ABSTRACT

Recognizing cryptic species promotes a better understanding of biodiversity, systematics, evolutionary biology, and biogeography. When cryptic species are disease-causing organisms, such as parasites, their correct recognition has important implications for the study of epidemiology, disease ecology, and host–parasite relationships. Freshwater nematomorphs (Nematomorpha: Gordiida) or hairworms, are an enigmatic yet fascinating group of parasites that are known to manipulate host behavior to aid transition from the parasitic phase, within terrestrial insects, to the free-living aquatic stage. Hairworm taxonomy has been hampered by a paucity of informative diagnostic characters and it has long been suspected that this group contains numerous cryptic species. Study of single hairworm species over large geographical areas has been difficult due to extremely rare encounters and unreliable methods of collecting adult worms. Here we report that by using crowdsourcing, citizen scientists have collected and submitted samples of *Gordius* cf. *robustus* from throughout its range in North America making its genetic study possible. Combined with our own collections, we examined samples from 28 localities within the USA; despite the collection of numerous hairworms from Canada and Mexico, *G.* cf. *robustus* were not collected outside of the contiguous United States. Mitochondrial CO1 genetic distances revealed that specimens grouped into 8 clades separated by 8–24.3%. In addition, molecular evidence from mitochondrial (CO1 and cytB) and nuclear (partial 28S, ITS1, 5.8S and ITS2) DNA suggests that these 8 clades are distinct species and that this group of species is paraphyletic, since the North American species *G. attoni* and the European species *G. aquaticus* and *G. balticus* group among the *G. robustus* lineages. Furthermore, there was a significant correlation between genetic (CO1) and geographic distance between the 8 *Gordius* species. This study demonstrates the value of involving the general public in biodiversity studies and highlights the feasibility of using the mitochondrial CO1 gene as a taxonomic marker for genetic barcoding and species identification within the phylum Nematomorpha.

1. Introduction

The study and recognition of cryptic species are being increasingly acknowledged as critical parts of modern taxonomic study, and promoting a better understanding of biodiversity, systematics, evolutionary biology, and biogeography. Defined as two or more distinct species classified as a single species (Bickford et al., 2007), cryptic species have now been reported from nearly all major metazoan clades (Pfenninger and Schwenk, 2007). These discoveries have been made possible by modern molecular techniques highlighting genetic differences among morphologically-identical members of previously-recognized species. Discovery of crypsis is particularly important within groups of morphologically simple invertebrates with few taxonomically diagnostic characters. Recent examples of groups in which cryptic species have been found include cnidarians (Schuchert, 2014), marine nematodes (Derycke et al., 2005), freshwater nematodes (Ristau et al., 2013), and parasitic nematodes (Tan et al., 2012).

Although parasitism is thought to represent one of the most successful modes of life (Poulin and Morand, 2000), study of crypsis within parasites has lagged behind their free-living counterparts (Nadler and Ponce de León, 2011; Ponce de León and Nadler, 2010). However, knowing the extent of crypsis among parasites is important since many facets of our understanding of host–parasite relationships are based on the answer to the question of 'who is infected with whom?'. A misunderstanding of the true

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delineation of parasite species may complicate the results of investigations attempting to quantify local parasite adaptation, host-use pattern (host specificity), disease dynamics and can affect diagnostics and control and eradication programs of human and domestic animal parasites (Nadler and Ponce de León, 2011).

Parasites of the phylum Nematomorpha, known as hairworms, are a wide spread group of macroparasites sister to the phylum Nematoda (Bourlat et al., 2008). Hairworms contain few consistent morphological traits useful in the separation of species. One of the most-used specific characters is the ornamentation found on the epicuticle surface of adult worms, such as cuticular plates called areoles (Fig. 1B), and finger- or knob-like projections termed bristles and spines. However, these cuticle structures have been known to vary greatly intraspecifically (de Villalobos and Zanca, 2001; Schmidt-Rhaesa, 2010) or be absent altogether, making morphologically-based taxonomy more difficult in certain groups.

Gordius cf. *robustus*, one of the most commonly reported hairworms in North America, lacks distinct areoles (Fig. 1A and B) and other associated cuticle ornamentations (Schmidt-Rhaesa, 2010; Schmidt-Rhaesa et al., 2003). As a result, taxonomists have used the absence of these cuticle characters as a synapomorphic character to lump specimens together into what we currently consider a single species. The range of *G.* cf. *robustus* extends from Canada, throughout the USA (including Hawaii) and Mexico; additional reports have been made from Venezuela, Trinidad, Paraguay, Chile, Argentina, Brazil, and South Korea (Baek and Noh, 1992). Additionally, across North America *G.* cf. *robustus* has been reported from 12 arthropod species and has the broadest host range for any New World hairworm (Poinar and Weissman, 2004; Schmidt-Rhaesa et al., 2003). Our observations of several North American populations of *G.* cf. *robustus* indicate differences in host use and numerous behavior and life cycle timing (seasonality) differences suggesting that this widely distributed species is comprised of multiple cryptic species.

A major problem in studying hairworms, especially over a wide geographic area, is the difficulty in locating adult worms. A short life span, hiding behavior during mating, species specific variability (host-use, seasonality), and habitat specific variability (amount of rainfall, temperature, etc.) make adult worms problematic to detect and collect (for example see Hanelt et al., 2001). Despite the difficulty in finding worms systematically, their life cycle often leads to encounters with people. Freshwater gordiids infect arthropod hosts, such as crickets, beetles, cockroaches and mantids, and upon maturation manipulate their hosts to provide transportation to water (Biron et al., 2005a,b) where worms are released to begin their free-living, aquatic, adult phase. When manipulated hosts occur around homes, they often end up in sinks, toilets, hot tubs, pet water dishes, and other standing sources of water in or near homes (Hanelt et al., 2005). Upon finding these worms, which are normally around 30 cm long and 2–3 mm thick, people are usually alarmed but their curiosity leads them to use internet search engines to learn about their discovery. Using targeted key words set into our website, we directed internet traffic by people seeking information about hairworms to our website (www.nematomorpha.net). We then recruited these citizen scientists to submit samples to us. In this study, adult worms submitted by citizen scientists and identified as *G.* cf. *robustus* from across the USA and Canada were combined with our collections of *G.* cf. *robustus* from the USA, and *Gordius* spp. from the USA, Mexico, and Nicaragua to investigate the monophyly of *G.* cf. *robustus*. All specimens included in this study were identified with light microscopy and then analyzed phylogenetically using mitochondrial [cytochrome c oxidase subunit 1 (CO1) and cytochrome b (cytB)] and nuclear (partial 28S, ITS1, ITS2, 5.8S) markers to identify highly divergent monophyletic groups.

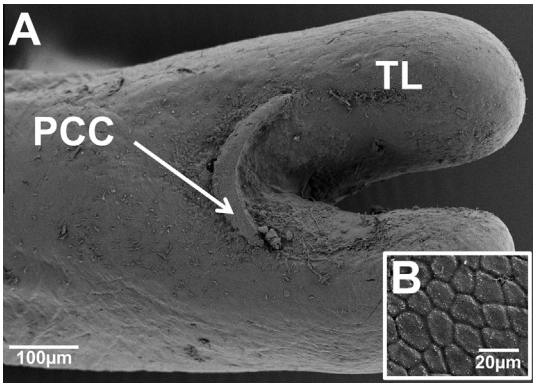

Fig. 1. Scanning electron micrograph of a typical male posterior end of *Gordius* cf. *robustus* (A). PCC: post-cloacal crescent, TL: tail lobe. Note that the cuticle is void of areoles, an example of which is shown from *Gordius attoni* (B).

2. Materials and methods

2.1. Sample collection, processing, and microscopy

Adult worms were collected as free-living adult individuals (Table 1) from various locations across North America. Most worms included in this study were collected by hand by citizen scientists, ranging in age from 12 to 62 years, who first reported them to us though our website (www.nematomorpha.net) and its Report-A-Worm feature. Worms were collected from dog water dishes, toilets, rain-drenched porches, outdoor bird baths, wet lawns, and small streams, and sent alive to us through the United States Postal Service (regular domestic service). Citizen scientists collected 26 adult *G.* cf. *robustus* worms from 14 widely scattered sites within North America. The remaining worms were collected by the authors (and other scientists) by hand, usually from small semi-arid streams, and returned to the laboratory alive. The only exception to this was sample N133, which 'appeared' overnight in a bucket catching rainwater from a leaking roof in the office of one of the authors (BH), in the biology building on the campus of Louisiana State University. The authors collected 24 adult worms from 15 mostly southwestern USA sites and one site each in Mexico, Canada, and Nicaragua. Since most worms were collected in the post-parasitic adult stage, hosts could only be documented for a few populations.

All worms were received alive, and were rinsed in tap water and measured to the nearest millimeter taking the precaution not to stretch worms. Worms were examined with light microscopy and the color and coloration pattern were noted. For all 47 worms identified as *G.* cf. *robustus*, males shared the characteristics of a bilobed posterior end and a postcloacal crescent, and neither sex contained areoles (Fig. 1A) visible with a light microscope. The remaining worms were identified as *G. attoni* based on re-descriptions (Schmidt-Rhaesa, 1997; Schmidt-Rhaesa et al., 2003) or were found to be morphologically distinct from *G.* cf. *robustus*. The anterior, posterior, and midsections of each worm were removed with a razor blade and put into 10% formalin or 70% ethanol for subsequent scanning electron microscopy (SEM) work. The rest of the body was preserved in 100% ethanol and stored at −70 C, for molecular work. Voucher specimens were deposited in the University of New Mexico Museum of Southwestern Biology.

2.2. Molecular work

Total genomic DNA was extracted from each adult worm. For each sample, approximately 0.5–2.0 g of tissue was isolated and