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A NEW SPECIES OF *GORDIONUS* (NEMATOMORPHA: GORDIIDA) FROM THE ROCKY MOUNTAINS OF NEW MEXICO

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KEY WORDS

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ABSTRACT

The 19 genera within the phylum Nematomorpha contain approximately 350 species. The cosmopolitan genus *Gordionus* Müller, 1926 contains about 58 species, 6 of which occur in the contiguous United States of America. Recently, 2 new *Gordionus* species were described from high-altitude streams within the southern Rocky Mountains, near Santa Fe, New Mexico. Here we describe another new *Gordionus* species, from a high-altitude stream in the southern Rocky Mountains, from near Taos, New Mexico. The sites consisted of temporary puddles and a small human-made stream at 3,175–3,250-m altitude in aspen/pine woodland. *Gordionus lokeri* n. sp. has 1 areole type, which varies in shape and size between and within body regions. Midbody areoles are elongated, polygonal, or triangular, shingled, with the raised side of the areole serrated. The interareolar space is narrow, containing few bristles. The male cloacal opening is surrounded inside and outside by narrow branching bristles that bifurcate or trifurcate deeply; the furcae then subdivide several times terminally. Adhesive warts are lacking. Genetic data, consisting of partial cytochrome *c* oxidase subunit I (*COI*) gene sequences, clearly separated *G. lokeri* n. sp. from other Nearctic species. This is the third *Gordionus* species described from high-elevation streams in the Rocky Mountains. It appears that this high-altitude habitat represents the preferential niche for numerous species of this genus, and thus future work should focus on describing gordiid diversity in other parts of the Rocky Mountains.

The phylum Nematomorpha is an understudied group taxonomically. The freshwater nematomorphs or gordiids (Nematomorpha: Gordiida), are a group of dioecious and parthenogenetic (Hanelt et al., 2012) parasites, primarily of orthopterans, coleopterans, and mantids (Hanelt et al., 2005). The life cycle of freshwater gordiids is unique (Schmidt-Rhaesa, 2001a; Hanelt and Janovy, 2004a), as juvenile worms mature within insect definitive hosts and manipulate the hosts to seek out and release worms into water. Here, worms mate and lay eggs. Gordiid larvae penetrate and encyst within aquatic insect larvae, which carry cysts to the terrestrial environment (Hanelt et al., 2005; Bolek et al., 2015).

Approximately 350 species of gordiids have been described worldwide from 18 extant and 2 extinct genera (Schmidt-Rhaesa, 2001b; Zanca and Schmidt-Rhaesa, 2008). Despite a recent flurry of taxonomic activity on this group, only about 30 species have been recognized from North America (Schmidt-Rhaesa et al., 2016; Swanteson-Franz et al., 2018). The paucity of diversity within the Nearctic is likely because of a lack of study and sampling effort, and recent new species discoveries in the southern Rocky Mountains and Sky Islands of the American Southwest support this idea (Begay et al., 2012; Swanteson-Franz et al., 2018). To continue our search for new gordiid species, we focused on sampling streams in the Southern Rocky Mountains and discovered another new gordiid in the genus *Gordionus*.

The cosmopolitan genus *Gordionus* contains 58 species (Schmidt-Rhaesa, 1997, 2001b; Begay et al., 2012). Six *Gordionus* species have been reported from the Nearctic. Two species, *Gordionus bilaus* Begay et al., 2012 and *Gordionus lokaaus* Begay et al., 2012, were recently described from high-altitude alpine ecosystems within the Rocky Mountains (Begay et al., 2012). Despite these 2 species being distributed near areas of high human activity (hiking trails, picnic area, ski area parking lot), they seemingly went unnoticed for many years. Those discoveries made us interested in evaluating more remote areas within the Rocky

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Mountains to determine if additional gordiid species could be located. Here we describe a third *Gordionus* species from the southern Rocky Mountains, which was also collected at high altitude.

MATERIALS AND METHODS

Field collections

Worms were collected in the Carson National Forest in the southern Rocky Mountains of Taos County, New Mexico. During mid-June to early July 2006–2011, free-living worms were observed in rain-fed puddles immediately adjacent to the dead end of Forest Road 161 (36.0577, –105.5006, elevation: 3,175 m). However, during the summer of 2011, the U.S. Forest Service improved the end of this road by building a large parking lot necessitating improvements in drainage, thereby removing areas of pooling water; free-living worms have not been seen in the immediate vicinity since. In 2012, worms were found in a nearby small stream crossing the Serpent Lake 19 hiking trail (36.0575, –105.5080, elevation: 3,225 m) about 1 km west of the Serpent Lake Trailhead (Trail 19). The trailhead is located about 0.5 km west of the end of Forest Road 161. The stream, from which worms were collected, was fed by a leak in the La Sierra Ditch, which is an agricultural diversion from the Rito Angostura River. Worms were placed in stream water and brought to the laboratory of the University of New Mexico (UNM), Albuquerque, New Mexico. Some worms were isolated in male–female pairs and in larger groups to stimulate mating and egg-laying behavior.

Biological material and microscopy

In the laboratory, the length and color of the remaining worms were recorded before they were divided with a razor blade into 5 pieces. Established techniques were used to fix and image hairworms (see Bolek et al., 2015; Schmidt-Rhaesa et al., 2016 and references therein). Briefly, 10-mm-long regions of each worm–the anterior, posterior, and midbody–were preserved in 70% ethanol for scanning electron microscopy (SEM) work and stored at room temperature. The 2 remaining pieces of worms were preserved in 100% ethanol for molecular work and stored at –80 C. For SEM, the 3 regions of 70% ethanol-preserved worms were dried using 2 methods. In the first method, samples were placed in acetone and then dried with CO_2 in a CPD-1 critical-point dryer (Denton Vacuum, Moorestown, New Jersey). Many samples processed using this first method collapsed, making some morphological features difficult to visualize. Therefore, a second method was used. In the second method, samples were dried via increasing concentrations of hexamethydisilazane. Tissue samples resulting from both methods were then mounted on stubs with carbon tape and coated with gold–palladium in an EmiTech K950 turbo-pumped vacuum coater with the gold–palladium sputter coater attachment (Quorum Technologies, West Sussex, England). Observations were made and digital images were taken using a JEOL 5800LV SEM at 15kV (JEOL Ltd., Tokyo, Japan).

Molecular methods

Attempts were made to examine the cytochrome *c* oxidase subunit I (*COI*) barcoding region of all samples collected. For all worms, a 0.5–1.0-cm portion of 100% ethanol-preserved tissue was cut into fine pieces and DNA was extracted using the E.Z.N.A.® Mollusc DNA Kit (Omega Bio-Tek, Norcross, Georgia). The extraction was performed following the manufacturer's recommendations, with the following exceptions. First, after cutting ethanol-preserved tissue into small pieces, tissue was dried at room temperature for 1 hr. Second, samples were incubated in buffer ML1 and Proteinase K for up to 4 hr at 60 C to help tissue digest. DNA yield was determined using a NANO DROP 200c spectrophotometer (Thermo Scientific, Waltham, Massachusetts). The *COI* gene was amplified using universal barcoding primer sequences: LCO1490: GGT CAA CAA ATC ATA AAG ATA TTG G, HCO2198: TAA ACT TCA GGG TGA CCA AAA AAT CA (Folmer et al., 1994). In addition, several published and in-hand *Gordionus* sequences were used to design new primers targeting the same region within the *COI* gene: Gordionus-cox1-F1: GCK GTA ATA CCY ATT TTG GT, Gordionus-cox1-R1: TGT TGA AAT ARA ATA GGG TCC C. If both primer sets failed to produce an amplicon, a combination of universal and *Gordionus*-specific primer pairs were run. PCRs were run using GoTaq® Flexi DNA Polymerase (Promega Corp., Madison, Wisconsin), following the manufacturer's protocol. PCR products were analyzed by agarose gel electrophoresis, using 1.0% agarose gels, stained with 0.5% GelRed® nucleic acid stain (Biotium, Hayward, California), and visualized on a UV transilluminator. DNA amplicons were purified by ethanol precipitation and sequenced using the BigDye version 3.1 kit (Applied Biosystems, Foster City, California) on an ABI 3130x sequence analyzer (Applied Biosystems). Both strands of the amplified DNA fragments were sequenced, edited in Sequencer version 5.1 (Gene Codes, Ann Arbor, Michigan) and manually edited for ambiguous base calls. In addition, we also examined the *COI* barcoding regions of several other *Gordionus* spp. (Table I), which were identified using original species descriptions and some subsequent redescriptions (Schmidt-Rhaesa et al., 2003).

Molecular analyses

Partial *COI* sequences were aligned by eye; no sequences contained indels. Previously published sequences from *Gordionus* spp. were included in the analyses with the following GenBank numbers: *Gordionus maori*: KY172788, KY172758, *Gordionus* cf. *chinensis*: AB647252, AB741906. *COI* genetic distances between each pair of samples were calculated using the K2P model (Kimura, 1980) in MEGA X (Kumar et al., 2018). This data set included 449 base pairs. Data were summarized for within and between genetic groups (Table II).

DESCRIPTION

Gordionus lokeri n. sp.

(Figs. 1, 2)

Adult male (n = 13): Length 85–137 mm ($\bar{x}$ = 105 mm); 9 males monochromatically dark brown, 1 uniformly cream brown, 3 with dark brown posterior ends, cream brown/brown midsections and anterior ends. Posterior end with distinctive bifurcating end (Fig. 1A), subterminal, round cloacal opening (Fig. 1B), bristle fields (Fig. 1A, D), and postcloacal cone-like spines (Fig. 1A, C, E). Numerous rows of bristles arranged as 2 distinct bristle fields in collective V-shaped formation (Fig. 1A.) anterior to tail lobe bifurcation. Bristle fields separated by 50 μm

Table I. Collecting location for hairworm samples used in this study.

Species/sample	Accession*	Collection location†	Latitude	Longitude	GenBank accession
Gordionus lokeri n. sp.					
N348L	MSB:PARA:27415	New Mexico	36.058	−105.508	MH349473
N348M	MSB:PARA:27416	New Mexico	36.058	−105.508	MH349470
N348Q	MSB:PARA:27420	New Mexico	36.058	−105.508	MH349471
N348V	MSB:PARA:27425	New Mexico	36.058	−105.508	MH349472
N349J	MSB:PARA:27413	New Mexico	36.058	−105.508	MH349469
N349K	MSB:PARA:27414	New Mexico	36.058	−105.508	MH349468
Outgroups					
Gordionus cf. *violaceus*					
N346A	MSB:PARA:1002	Minnesota	44.087	−92.030	MH349467
N346B	MSB:PARA:1002	Minnesota	44.087	−92.030	MH349466
Gordionus kimberleyae					
NC005A	MSB:PARA:23557	Canada	73.227	−119.577	MH349474
NC001A	MSB:PARA:23660	Canada	73.233	−119.547	MH349475
Gordionus bilaus					
N284A	MSB:PARA:111	New Mexico	35.770	−105.796	JX244253
N284D	MSB:PARA:113	New Mexico	35.770	−105.796	MH349465
Gordionus lokaaus					
N159C	MSB:PARA:109	New Mexico	35.770	−105.796	JX244246
N207A	MSB:PARA:103	New Mexico	35.770	−105.796	JX244245

* Museum of Southwestern Biology, Parasitology Division.
† Within the United States unless otherwise noted.

anteriorly and by 175 μm closer to body bifurcation; end abruptly in line near the beginning of the bifurcation. Bristles elongated, deeply and/or terminally branched (Fig. 1D). Bristles near anterior end of bristle field, shorter and unbranched. Cuticle between bristle fields and below cloacal opening smooth, lacking areoles. Postcloacal spines arranged on ventral and interior surfaces of both tail lobes (Fig. 1A), 5 μm in width, shape variable. Anterior spines dome like (Fig. 1E), posterior spines come to off-center point (Fig. 1C). Smaller simple spines surround larger mound-like postcloacal spines, most numerous on ventral side of tail lobes; also occur on inside of tail lobes, and on ends of tail lobes (Fig. 1C). Round cloacal opening (Fig. 1B) 25 μm in diameter, surrounded by cylindrical spines on outer edge; contains cylindrical spines within interior of opening. Cylindrical spines bifurcating or trifurcating; some spines split into more than 3 parts. Posterior to cloacal opening, 1 or 2 spines greatly expanded and branch terminally numerous times, appearing hand like (Fig. 1B). Cuticle morphology varies greatly along body length, especially along posterior regions with presence of several different areole morphologies. On posterior end, areoles either laterally elongated (Fig. 1F), more narrow and overlapping in sharp shingle-like conformation (Fig. 1G), or arch-like (Fig. 1I). On midbody, areoles elongated, polygonal, and shingle like, containing 1 raised, serrated side (Fig. 1K). Interareolar space narrow, containing few bristles. Areoles in dimple-like depressions in most body regions (Fig. 1G, H).

Adult female ($n = 33$): Length 56–117 mm ($\bar{x} = 77$ mm). Color ranged from monochromatically dark brown, cream brown, and light brown to variations in pattern of those 3 colors along body length, with 1 having grey/dark brown pattern. Unlike in males, areoles much more similar along body length (Fig. 2), appearing as shingled polygons, triangular-shaped (Fig. 2B) and connected to surrounding areoles by thin extensions (Fig. 2A–C); areoles contain raised edge with serrated margin (Fig. 2B). Interareolar space limited, true bristles lacking. Connecting extensions between areoles appear to form interareolar bristles in some areas (Fig. 2B).

Taxonomic summary

Holotype: Male, deposited at the Museum of Southwestern Biology–Parasitology Division, University of New Mexico, Albuquerque, New Mexico, MSB: PARA:27444.

Table II. Intra- and intergroup nucleotide divergence (K2P) of COI amplified from hairworm samples. Values in bold are within species divergences.

Species	n	1	2	3	4	5	6	7
1. *Gordionus lokeri* n. sp.	6	**0.013**						
2. *Gordionus bilaus*	2	0.280	**0.009**					
3. *Gordionus* cf. *violaceus*	2	0.235	0.294	**0.020**				
4. *Gordionus lokaaus*	2	0.390	0.359	0.352	**0.004**			
5. *Gordionus kimberleyae*	2	0.329	0.254	0.291	0.329	**0.002**		
6. *Gordionus maori*	2	0.351	0.346	0.363	0.338	0.391	**0.016**	
7. *Gordionus* cf. *chinensis*	2	0.331	0.339	0.344	0.353	0.373	0.374	**0.002**

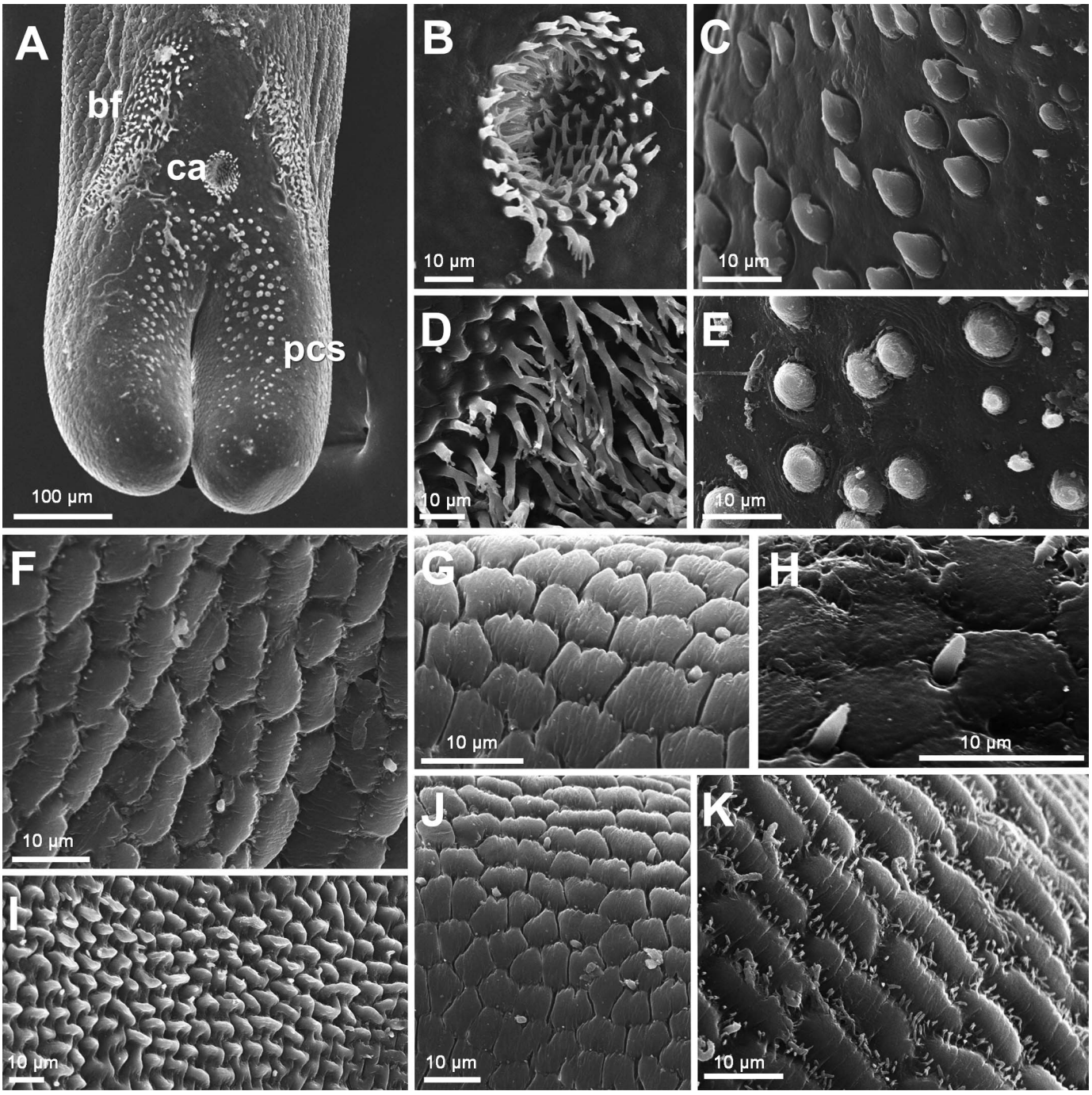

Figure 1. *Gordionus lokeri* n. sp. male. (**A**) Posterior end with bifurcating ends, cloaca (ca), bristle fields (bf), and postcloacal spines (pcs). (**B**) Cloaca surrounded and lined with spines. (**C**) Postcloacal conelike spines on the inside of the tail lobes come to a point. (**D**) Closeup of bristle field showing multiple branching bristles. (**E**) Postcloacal conelike spines are less pointed closer to the cloaca. (**F**) Laterally elongated shinglelike cuticle on posterior end. (**G**) More narrow, but highly serrated areoles also on posterior lateral end. (**H**) Spines between areoles in small dimple-like hollow. (**I**) Archlike areoles running along the midline of the posterior end. (**J**) Variable cuticle on posterior ventral side, showing the change of areoles from a laterally narrow type to a laterally elongated and serrated type. (**K**) Highly elongated areoles in the worm's midbody separated by narrow bristle-filled interareolar space.

Paratypes: Allotype: MSB: PARA:27415. Other paratypes: MSB: PARA:27403–27447.

Distribution: Rocky Mountains, Carson National Forest, Taos County, New Mexico.

Type locality: A stream crossing the Serpent Lake Trail 19, Rocky Mountains, Carson National Forest, Taos County, New Mexico (36.0575, –105.5080) at an elevation of 3,225 m.

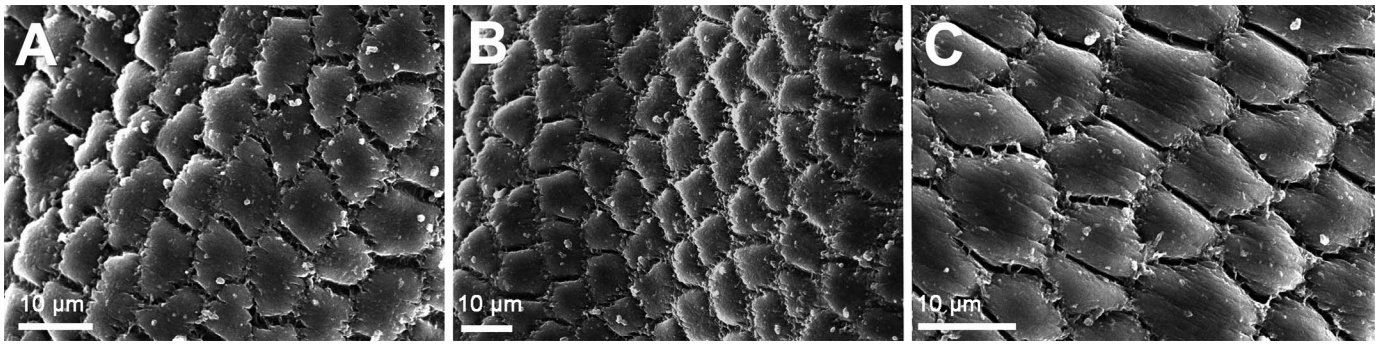

Figure 2. *Gordionus lokeri* n. sp. female cuticle. Near anterior end (**A**), midbody (**B**), and posterior end (**C**).

Bionomics: Worms were collected in the free-living, postparasitic, adult stage, and the paratenic and definitive hosts remain unknown. Adults were collected from mid-June to early July.

Material examined: Of 36 worms (13 males; 33 females) collected, DNA could be amplified and sequences successfully from 6 individuals. For each of these worms, partial *COI* was amplified and sequenced. The anterior, posterior, and midsection of 6 individuals were used for SEM studies.

ZooBank registration: urn:lsid:zoobank.org:act:C0DA1ABC-3AC3-4DFC-AF66-C24DDB936687.

Etymology: The species is named in honor of Dr. Eric S. Loker, for his support of the study of hairworms and contribution to the field of parasitology.

Remarks

Diagnoses and taxonomic comments: Gordonius lokeri resembles *Gordonius violaceus* (Baird, 1853) from Europe, except that the adhesive warts are missing, and areoles have serrated margins (Schmidt-Rhaesa, 2001c). In addition, the midbody areoles of *G. lokeri* are often shingled (Figs. 1K, 2B), whereas midbody areoles in the European *G. violaceus* sit flat, side-by-side. Additionally, the new species resembles *G.* cf. *violaceus* from North America, which has been described to vary geographically. In Massachusetts, populations of *G.* cf. *violaceus*, areoles are serrated, side-by-side, with broad interareolar spaces containing numerous bristles, (Smith, 1991). In midwestern United States populations of *G.* cf. *violaceus*, areoles are round to oval, arranged side-by-side, and lack serrated margins, with broad interareolar spaces containing numerous bristles (Schmidt-Rhaesa et al., 2003). *Gordionus lokeri* n. sp. also contains highly variable-shaped areoles; however, midbody areoles are elongated, polygonal, or triangular, shingled, with the raised side of the areole containing distinct serrations. On the midbody region, interareolar spaces are narrow and contain few bristles (Figs. 1K, 2B). The new species is also distinct from *Gordionus kimberleyae* Ernst, Hanelt, and Buddle, 2016 described from northern Canada, with flat, side-by-side, regularly shaped polygonal areoles, lacking interareolar furrows and bristles.

Laboratory rearing: Despite the success of rearing numerous other hairworm species in the laboratory (Hanelt and Janovy, 2004b; Hanelt et al., 2012; Bolek et al., 2013), repeated attempts to mate *G. lokeri* in the laboratory failed. Worms maintained in the laboratory as pairs or in larger mixed-sex groups were never seen mating and failed to produce eggs. Extensive search of field sites did not reveal eggs or egg strings. This difficulty has been noted in other *Gordionus* species (Begay et al., 2012). Thus, measurements of eggs, larvae, and cysts could not be included in this study.

Molecular analyses: We attempted to amplify all 36 *G. lokeri* worms and dozens of outgroup samples. Of these, 22 never amplified using the universal *COI* primers (658 bp). Six samples amplified using 1 of the universal and 1 of the *Gordionus*-specific primers. Bands, using the forward and reverse *Gordionus*-specific primers (452 bp) were seen from 12 samples. Sequencing also turned out to be a challenge. Ten samples showed double or multiple signal chromatograms for both forward and reverse sequences, indicating the presence of pseudogenes or xenobiotic contamination. Pseudogenes could be identified in 3 cases because of their shortened length and presence of stop codons. All sequences showing these aberrations were excluded from the analysis. In total, only 17% of the samples produced usable sequencing data. However, we were able to amplify and obtain clean sequence data from 6 *G. lokeri* samples and from a few other *Gordionus* species used as outgroups for comparison (Table I). Sequences have been deposited in GenBank (MH349465–MH349475). For our analyses, all sequences were trimmed to a length of 452 bp.

The genetic distance matrix for the partial *COI* gene indicated that the 6 samples of *G. lokeri* were distinct from the *COI* genes sequenced as part of this study or available from GenBank for 6 other *Gordionus* species (Table II). The average intraspecific distance within *G. lokeri* was 1.3%. Interspecific distances between *G. lokeri* and other American *Gordionus* species range from 23.5% to 39%; including 2 species, *G. bilaus* and *G. lokaaus*, which are also endemic to high altitude Rocky Mountain habitats.

DISCUSSION

The genus *Gordionus* was erected by Müller in 1927, to differentiate species of gordiids with adhesive warts from the genus *Parachordodes* which lacks adhesive warts. Adhesive warts, which are only found in males, are canoe-shaped elevated cuticular structures on both sides of the ventral midline and can be located some distance anteriorly to the cloacal opening. However, adhesive warts have become a problematic unifying character for the genus *Gordionus*, because in many species descriptions adhesive warts were rarely noted or described (Schmidt-Rhaesa et al., 2003). Today, taxonomists use the number of areolar types to separate *Gordionus* from *Parachordodes*. Areoles are elevated, plate-like epicuticle surface

structures that clad worms in a single layer (tile-like) or overlapping layers (roof shingle-like). *Gordionus* have a single areolar type (simple), whereas *Parachordodes*, have 2 areolar types including simple and much larger areoles known as superareoles (Schmidt-Rhaesa, 2013).

Although *G. lokeri* lacks adhesive warts and thus does not fit the original definition of the genus *Gordionus*, its single type of areole places it into the more modern definition of the genus. Interestingly, although adhesive warts have been described in *G. violaceus* from Europe, they have never been noted in North American specimens. Despite this significant morphological difference, worms of both phenotypes have been placed into *G. violaceus*. Morphological features separate *G. lokeri* from Nearctic and European samples of *G.* cf. *violaceus*. In addition, the genetic distance of *G. lokeri* from Nearctic samples of *G.* cf. *violaceus* is 23.5%, which is well above the interspecies genetic difference in other hairworm groups (Hanelt et al., 2015).

Gordionus lokeri is the third *Gordionus* species for which mating and nonadult life cycle stages could not be observed. When male and female worms were placed together, they usually formed a loose Gordian knot, but males showed none of the typical behavior observed in other gordiid species during mating. This behavior typically includes males wrapping around the females, and males moving down the female's body to align their cloacae for sperm deposition. Numerous other species, including *Paragordius varius* Leidy 1851, *Paragordius obamai* Hanelt et al., 2012, *Gordius difficilis* Smith 1994, *Gordius* cf. *robustus* Leidy 1870, *Chordodes morgani* Montgomery, 1898, *Chordodes kenyaensis* Bolek, Szmygiel, Kubat, Schmidt-Rhaesa and Hanelt, 2013, *Chordodes janovyi* Bolek, Schmidt-Rhaesa, Hanelt, and Richardson, 2010, and *Neochordodes occidentalis* (Montgomery, 1898) have been brought into the laboratory and observed to mate and produce eggs (Hanelt and Janovy, 2002; Bolek et al., 2010, 2013; Hanelt et al., 2012).

Numerous attempts to find eggs and egg strings in streams and puddles containing worms were unsuccessful. Eggs and egg strings of many of the other gordiid species listed above can usually be found by searching their natural habitat. Failure to observe mating or eggs in nature has also been noted for *G. bilaus* and *G. lokaaus*, both of which also occur in the Rocky Mountains (Begay et al., 2012). It is possible that these high-altitude *Gordionus* species have a unique mating system which may include overwintering and mating during the spring. Additional work is needed to determine the presence of adult worms or egg strings during winter or immediately following the melting of the snowpack.

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