**Abstract**

A new species of the tubificine genus *Limnodrilus* is described and COI barcoded from Sulphur Cave and associated springs in Colorado, USA. The habitats are characterized by high sulfide concentrations. The new species, *L. sulphurensis*, is distinguished from all congeners by the elongate, nearly parallel teeth of chaetae in its anterior segments. It has a penis sheath resembling that of *L. profundicola*; consequently, museum specimens and new collections are examined here to resolve some of the taxonomic confusion surrounding that widespread, but uncommon species.

**Key words:** Annelida, Clitellata, Naididae, Tubificinae

**Introduction**

Conspicuous clusters of blood-red worms were observed in Sulphur Cave (Colorado, USA) during a scientific exploration trip in 2008. This cave is characterized by high concentrations of hydrogen sulfide, both in the air and dissolved in the underground stream. Based on morphology and DNA barcoding, these worms were found to be closest to the tubificine genus *Limnodrilus*, but were not attributable to any known species. Although a few common, surface-water species of *Limnodrilus* Claparède, 1862 have been reported from caves (Swayne et al. 2004; Wetzel & Taylor 2001), this genus is not particularly associated with the stygophilic fauna (Creuzé des Châtelliers et al. 2009). This finding of high *Limnodrilus* densities in a cave with high sulfide concentrations is noteworthy; consequently, they are described here as *Limnodrilus sulphurensis* n. sp.

The genus *Limnodrilus* has many unresolved taxonomic issues, which are beyond the scope of the present paper. However, as *L. sulphurensis* resembles the widespread *Limnodrilus profundicola* (Verrill, 1871) in some commonly used characters, we examine new material to clarify the status of the latter. To facilitate future identification of *L. sulphurensis* using DNA barcodes (Hebert et al. 2003), fragments of the COI mtDNA sequences of several specimens, including the holotype, are also provided.

**Methods**

**Site descriptions and habitat measurements.** Sulphur Cave is a short (ca. 20 m), surficial cave in a fragment of late Quaternary (fault-derived) travertine (Madole 1991). It is located south of the Yampa River in the City of Steamboat Springs, in Routt County, Colorado U.S.A (W106.8410°, N 40.4847°) at an elevation of 2,070 m. Geothermal waters emerge from a spring within the cave, and a shallow (ca. 4 cm deep) stream runs the length of
the cave, flowing at 0.2–0.3 m/s. Temperature was 21°C, dissolved oxygen 0.25 ppm, and total dissolved solids (primarily sodium bicarbonate) 3,700 mg/L in the cave spring on 7 July 2007.

Air quality in Sulphur Cave is considered toxic, particularly during summer and autumn. In September 2008, measured levels of H\textsubscript{2}S gas were >200 ppm. In August 2009, H\textsubscript{2}S was measured at 325 ppm (considered not immediately dangerous), but CO\textsubscript{2} was very high, at 20.8%. Total dissolved sulfide within the cave water was measured using a DR5000 spectrophotometer (Hach, Loveland, Colorado, USA) and the methylene blue method (Cline 1969). The dissolved sulfide was as high as 1.48 mg/L where the spring emerges within the cave (pH 6.68, 0.53 mg/L O\textsubscript{2}), and dropped to 1.06 mg/L (pH 7.01, 0.88 mg/L O\textsubscript{2}) as the stream flows into the main cave. High levels of dissolved and atmospheric H\textsubscript{2}S in the cave result in significant gypsum deposits, and support the formation of dense microbial mats on the cave walls and in the cave stream.

*Limnodrilus sulphurensis* n. sp. was the most conspicuous metazoan found within the cave, although the eurytopic ostracod *Heterocypris salina* (Brady) was also present. The worms were not found close to the spring source (where H\textsubscript{2}S levels were the highest), but thrived in dense clusters in the main area of the cave where dissolved H\textsubscript{2}S levels were lower. The guts of worms fixed in the field were filled with a white substance, which molecular analysis demonstrated was consistent with the presence of sulfide oxidizing bacteria (data not shown; Spear *et al.* 2007).

In addition to the cave habitat, *L. sulphurensis* n. sp. was also found in small streams draining two nearby surface springs. Sulphur Cave Spring, just outside the cave, flows at about 0.6 L/s, and after a short (30 m) surface reach, water from the spring enters the cave. Black Sulphur Spring is also on the south side of the Yampa River, about 450 m from the cave entrance. Dissolved oxygen was low at the sources of both springs in July 2007: 0.13 mg/L in Sulphur Cave Spring and 0.005 mg/L in Black Sulphur Spring. Water temperature was 25°C and 24.8°C, and dissolved solids 4260 and 4350 mg/L at Sulphur Cave and Black Sulphur Springs, respectively. Dissolved sulfide was not measured at these spring sites, although a strong H\textsubscript{2}S odor was present during visits to each site.

**Collecting and curation.** In 2008, H\textsubscript{2}S levels were artificially reduced using surface venting to allow human access, but on other occasions, SCBA (Self Contained Breathing Apparatus) was used while collecting the worms from the cave. Due to their high density in the cave stream, clumps of worms were easily collected with forceps. Most specimens were directly fixed in 10% buffered formalin for morphological study, or 100% ethanol for DNA. Some specimens were relaxed in dilute ethanol, followed by fixation in formalin-alcohol-acetic acid (FAA), for histological sections.

Measurements were based on longitudinally dissected or whole-mounted worms dehydrated in an alcohol series, cleared in methyl salicylate, and slide mounted in Canada balsam. Most dissections and whole-mounts were stained in hematoxylin or borax carmine. Thick sections were cut at 7 µm and stained in hematoxylin and eosin. A few specimens were slide mounted in CMC-10™ mountant (Masters Chemical Co.) for comparison with earlier descriptions based on specimens mounted in media such as lactophenol.

Based on characters considered diagnostic within the genus, the new species is morphologically close to a very widespread species, *Limnodrilus profundicola*. Museum specimens, including the *profundicola* holotype, were examined for comparison with the new species. These were supplemented with new *profundicola* material collected in Europe, Japan, and North America (see taxonomy section below).

Specimens were deposited in the following museums: Denver Museum of Nature & Science (DMNS), Denver, Colorado, USA; U.S. National Museum, Smithsonian Institution (USNM), Washington D.C., USA; California Academy of Sciences, Invertebrate Zoology (CASIZ), San Francisco, California, USA; Zoologisches Museum Hamburg (ZMH), Hamburg, Germany; Swedish Museum of Natural History (SMNH), Stockholm, Sweden.

**DNA methods.** Seven ethanol-preserved specimens, 6 from Sulphur Cave and 1 from Black Sulphur Spring, were DNA-barcoded using the mitochondrial cytochrome c oxidase subunit 1 (COI), as described by Martinsson and Erseus (2014); DNA was extracted from a few posterior segments, while the rest of each worm was stained with paracarmine and permanently mounted in Canada balsam on slides (see Erseus 1994), to serve as vouchers. One of the vouchers (DMNS ZE.46275; haplotype #1) was selected as the holotype of the new species. The COI sequences were aligned in ClustalX (Larkin *et al.* 2007), and analyzed by eye. Individual barcodes (Table 2) are identified by Erseus catalog number, i.e. "CE0000". The haplotypes found are deposited in Genbank (NCBI). The sequences were also BLASTed against data in Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and BOLD System ver. 3 (http://www.boldsystems.org/).
Abbreviations used in Figures 1–4: at—atrium, cg—chloragogen tissue, cv—capillary blood vessel, dv—dorsal blood vessel, ed—ejaculatory duct, g—gut, lv—lateral blood vessel, mf—male funnel, mp—male pore, pr—prostate gland, ps—penis sheath, sa—spermathecal ampulla, sd—spermathecal duct, sp—spermathecal pore, vd—vas deferens, vv—ventral blood vessel.

Taxonomy

Limnodrilus sulphurensis Fend, Liu & Erséus n. sp. (Figures 1–3)

Holotype. DMNS ZE.46275, a whole mounted, sexually mature and DNA barcoded specimen; i.e., a small posterior part of the worm was used for DNA extraction. COI barcode = haplotype 1 (CE10482, GenBank KT692957; see also "Barcodes" below).

Type locality. Colorado, Routt Co., City of Steamboat Springs, Sulphur Cave, high H₂S stream in dark zone, W106.841050°, N40.84759°. 11-Apr-2010. Collected by David Steinmann and Fred Luiszer.

Paratypes. All from the type locality. DMNS ZE.46276–46280: 11-Apr-2010, 2 whole-mounts, 3 dissected. SMNH Type 8779, 20-Sep-2008, 1 whole-mounted, mature specimen with COI barcode (CE7491, haplotype 2, GenBank KT692956). USNM 1283518, 18-Aug-2007, 1 whole-mount, mature, with COI barcode (CE7489, haplotype 1); USNM 1283512-1283516, 11-Apr-2010, 2 whole-mounts, 3 dissected; USNM 1283517, 1-Aug-2009, 1 sagittally sectioned. CASIZ 197459, 1-Aug-2009, 1 whole-mount; CASIZ 197460, 1-Aug-2009, 1 whole-mount, 3 dissected. ZMH Ol 154446-15450, 1-Aug-2009, 2 whole-mounts; 11-Apr-2010, 3 dissected. All collected by David Steinmann and Fred Luiszer.

Additional material. The type locality, 18-Aug-2008, SMNH 150175 (CE7486); 20-Sep-2008, SMNH 150176 (CE7490); both whole-mounts with COI barcodes (haplotype 2). 9-Mar-2009, 10 whole-mounts (6 immature), 1 sagittally sectioned, 1-Aug-2009, 5 whole mounts. 11-Apr-2010, 5 whole mounts, 12 dissected. Colorado, Routt Co., Surface spring near Sulphur Cave, 1-Aug-2009, 5 whole mounts. All collected by David Steinmann and Fred Luiszer.

Etymology. Named for the type locality.

Description. Specimens relaxed in dilute alcohol and fixed in FAA 18–25 mm long; formalin-preserved worms more contracted, 12–19 mm. Width 0.52–0.99 mm in V III, to 1.05 mm in XI, tapering in posterior segments. 51–78 (median 64) segments. Prostomium rounded, slightly wider than long in FAA-preserved specimens; broadly conical in formalin-fixed specimens (Fig. 1A). Clitellum weak in all available material, X–XII.

Segments II–VIII or VIIII with 5–11 fully developed chaetae per bundle (median 7 dorsal, 8 ventral), and a similar but indeterminate number of partially developed replacement chaetae, all in a descending, fan-like series (Fig. 1B). Number of chaetae per bundle decreasing posteriorly; by XX 2–3, posterior segments at most 1–2; most specimens with few or no chaetae from about the posterior 1/4 of the worm. All chaetae bifid, sigmoid, with variably developed nodulus at about the distal 1/3 (Fig. 1B–E). Chaetae in anterior part of body (to about X) with teeth approximately equal in length and thickness; teeth long (12–20 µm), nearly straight (never strongly hooked), and nearly perpendicular to the distal part of the shaft (Fig. 3A–C). Chaetae gradually smaller, and teeth shorter and less strongly angled relative to shaft in posterior segments (Figs. 1C–E, 3D). Ventral chaetae absent in XI in mature worms; no modified spermathecal chaetae in X. Chaetal length 84–185 µm in II–X (maximum length in IV–VII); 90–125 in XI–XX; 50–100 µm in posterior segments. In anterior (precitellar) segments, the ventral chaetae about 30% longer and slightly thinner than the corresponding dorsal ones (Fig. 1C vs. 1E).

Pharynx in II–III, weakly differentiated from esophagus; about equally developed dorsally and ventrally. Chloragogen cells dense on gut beginning in VI, with dark inclusions in worms fixed in the field (Fig. 3E,F).

Branches of dorsal blood vessel join to form the ventral vessel at about 2/3. A well-developed supra-intestinal vessel from about 5/6, joining the dorsal vessel in IX; the conjoined "dorsal" vessel curving ventrad along the left side of the gut, and remaining in a lateral or even ventrolateral position until the posterior segments. Dorsal and ventral vessels connected by long, convoluted, commissural vessels in II–VII and in IX–XI. In VIII, commissures
FIGURE 1. Limnodrilus sulphurensis n. sp. from Sulphur Cave, exterior and somatic characters. A. Anterior ends of two whole, unmounted worms. B. Ventral chaetal bundle in IV, from a mature worm (n=19, including 10 partially developed). C. Left to right: two ventral chaetae in V, one from XX, from a mature worm. D. Ventral chaetae (n=13, including 6 partially developed) in V, from a very small, immature worm (diameter 0.3 mm). E. Dorsal chaetae from a mature worm, two each from V, X, and XXX. F. Ventral view of blood vessels in a posterior segment, showing junction of capillary vessels with ventral blood vessel, lateral vessels and (laterally displaced) dorsal blood vessel. G. Lateral view of dorsal and ventral blood vessels, as in Figure 1F.
FIGURE 2. Limnodrilus sulphurensis n. sp. from Sulphur Cave (A–D, F, G) and from Black Sulphur Springs (E), reproductive characters. A. Atrium and penis sheath of an unmated, nearly mature worm. B. Atrium and penis sheath from a mature, mated worm. C. Male duct from a mated worm. D, E. penis sheaths. F. Spermatozeugmata. G. Spermatheca.
shorter and modified as "hearts"; these greatly dilated (to 150 µm), but not obviously muscular, and joining the supra-intestinal vessel instead of the dorsal. A network of fine peripheral, capillary blood vessels visible in posterior segments, extending into the epidermal layer (Fig. 3F); relatively sparse beginning in about XXV, forming a dense network beginning in about XXXV. Branches of capillary vessels coalesce to a short ventrolateral vessel, which joins the ventral vessel near the posterior septum. A second main branch from the capillary blood vessels joins a pair of dorsolateral vessels extending from the dorsal vessel to both sides of the ventral body wall. These dorsolateral vessels join the dorsal vessel near the posterior septum, the one going to the right side passing over the gut; apparently no direct connection to the ventral vessel (Fig. 1F,G).

Nephridia in XIII and many posterior segments; a convoluted duct covered with indistinct, granular cells forming a dense mass, extending ventrally through most of segment, terminating in a short ectal duct, which widens into a small vesicle at the nephriodopore, anterior to the ventral chaetae.

Male ducts and spermathecae paired. Male pores narrow, transverse openings, in line with ventral chaetae, at about the posterior 1/4 of segment XI (Fig. 1A). Spermathecal pores prominent and transverse, in line with ventral chaetae, midway between chaetae in X and anterior septum (9/10). Male funnel 100–120 µm long, either narrowly conical with upper and lower lips about the same length, or with lips laterally spread; projecting into X from 10/11. Vas deferens very long, about 4000–6000 µm, diameter 22–48 µm, generally thinnest in formalin-preserved worms; of nearly uniform diameter and histology (Fig. 2C). Vas deferens composed of a single layer of ciliated epithelium 7–10 µm thick, without obvious muscle layer. Atrium narrowly fusiform, narrowing gradually to vas deferens and ejaculatory ducts; length 240–490 µm, diameter to 65–94 µm ental to prostate attachment, 42–85 µm in ectal part (Fig. 2A–C). Atrial epithelium ciliated, cells granular, irregular, indistinct in ental part; often more regular and less granular in ectal part (Fig. 3H). Atrium joined near midpoint by a single, stalked prostate gland (Figs. 2B, 3G,H); prostate a fan-like cluster of irregular lobes; entire mass 240–380 µm wide; prostatic cells granular and indistinct. Ectal (ejaculatory) duct of atrium 250–480 µm long by 26–38 µm wide, with a thin (5–7 µm), non-ciliated epithelium and indistinct muscle layer about 2–3 µm thick; appearing slightly wrinkled and less evenly cylindrical than vas deferens. Penis without obvious spiral muscles; penis sheath surrounded by a close-fitting sac to 120–240 µm deep, consisting of columnar cells and a thin, indistinct muscle layer. Shaft of cuticular penis sheath nearly tubular, expanded slightly at ends; straight or somewhat curved in slide-mounted specimens (Figs. 2D, 3J); wall thickness up to 3–4 µm. Sheath length 240–410 (median 333) µm; diameter in middle 34–49 µm, 43–82 µm at ental end, 40–60 µm at ectal end (below head); ratio of length to basal width 4.1–5.7 (median 4.9). Ectal end ("head") broad and plate-like, appearing flat or recurved (Fig. 3I,K,L); plate circular and symmetrical, diameter 120–170 µm in fully mature worms. Plate may be indistinct, with very thin cuticle, particularly in unmated specimens (Fig. 2A).

Ectal part of spermathecal duct about 200–300 µm long, thick and pyriform; widest entally, to 121–145 µm thick; ectally narrowing to 80–95 µm (Fig. 2G). Duct composed of an outer, transverse-circular muscle layer, and an irregular epithelium (Fig. 3M,O); lumen narrow ectally, wider entally. Duct surrounded by an irregular layer of cells. Remaining (ental) part of duct narrower (Fig. 3O), but often medially inflated (Fig. 2G); about 60–100 µm long. Spermathecal ampulla nearly spherical to irregular, sacciform. Spermatozeugmata 200–310 µm long, narrow, slightly widened at one end, to 37–55 µm (Figs. 2F, 3N).

Barcodes. Data on the COI barcodes of seven specimens are summarized in Table 2. Six of them were successfully sequenced to the standard length of 658 base pairs (bp), all barcodes covered positions 2–658 in the alignment. Two different haplotypes (Ht 1 and 2 in Table 2) within this interval were recognized, with variation (uncorrected p-distance 0.15%) only in one position (181). When blasting the haplotypes against the BOLD and NCBI/Genbank databases, >80% resemblance was found with several other North American clitellates, including a barcoded worm (about 82 % similar) identified as Limnodrilus hoffmeisteri (Genbank EF089358; http://www.ncbi.nlm.nih.gov/nucleotide/). The closest match (90.21%) was between haplotype 1 and an unidentified "Tubificidae" collected in Manitoba, Canada, and in Early-Release status in BOLD (http://www.boldsystems.org/).

Remarks. The new species clearly belongs to Limnodrilus as defined by Brinkhurst (1971); vas deferens long and ciliated, atrium short with a single large prostate, ejaculatory duct long, leading to a thick, cylindrical penis sheath; genital chaetae absent, somatic chaetae all bifids; spermatozeugmata present in spermathecae; prominent coelomocytes absent.

Limnodrilus sulphurensis is most easily differentiated from congeners by the very long, sharply angled teeth of both ventral and dorsal chaetae in anterior segments. The distinctive chaetae are not restricted to mature worms,
and are consistently seen in very small, immature specimens from Sulphur Cave (Fig. 1D), as well as in worms from nearby surface springs. The large number and continuous progression of developing, replacement chaetae in anterior segments of *L. sulphurensis* are also unusual. Interestingly, Fig. 1A in Smimshchikova (1998) shows several replacement chaetae in anterior ventral bundles of *Limnodrilus dybowskii* (Grube, 1873).

Based on proportions and morphology of the penis sheath, the characters most often used to distinguish *Limnodrilus* species, *L. sulphurensis* appears closest to the widespread *L. profundicola*, and will key to that species using Brinkhurst (1971: 463). It also somewhat resembles the widespread *Limnodrilus udekemianus* Claparède, 1862, the Jamaican *Limnodrilus variesetosus* Brinkhurst, 1979, and a group of endemic Lake Baikal species associated with *L. dybowskii*.

Taxonomy of *L. profundicola* is problematic, due to a very limited original description and lack of useable type material; in the following we attempt to clarify some of these issues with observations on penis sheaths and chaetae in museum specimens and in new collections (see below). Based on new and previously published measurements, the penis sheath is similar in general shape and proportions to that of *L. sulphurensis*, but is smaller (Table 1, Fig. 4), and the terminal expansion ("head") is smaller, but usually more distinct. The *profundicola* atrium is also smaller (Table 1). *Limnodrilus profundicola* has rather ordinary chaetae for the genus, with short, nearly equal teeth. Chaetal bundles have a similar number of well-developed chaetae in the two species (Table 1), although *profundicola* does not have the large number of developing chaetae seen in *L. sulphurensis* (see below; Fig. 1B vs. 4G). Chaetae of *Limnodrilus alpestris* Eisen, 1879, and *Limnodrilus monticola* Eisen, 1879—both taxa that have since been synonymized with *profundicola* (Brinkhurst 1965)—are not well described.

There seems to be good agreement in the literature on the status and diagnostic characters of *L. udekemianus*. Penis sheaths of this species may have similar proportions to those of *L. sulphurensis* (length/width ratio 2–5 [Kennedy 1969], or up to 4 [Timm 2009]), but despite high variability, they are usually illustrated as shorter and broader, gradually expanded, and without a plate-like head (Claparède 1862, Pl. I, Fig. 4; Brinkhurst 1971, Fig. 8.4B; Kennedy 1969, Fig. 9). Nevertheless, the distal end may be variably expanded and reflexed (Dzwillo 1984, Fig. 3; Piquet 1913, Fig. 10a). The anterior chaetae of *L. udekemianus* are considered diagnostic: distal teeth are enlarged, thickened, and sometimes strongly curved, but the proximal teeth are of normal size, differing from those of *L. sulphurensis* (e.g., Kennedy 1969, Fig. 1; Brinkhurst 1965, Fig. 4). Timm (2009) also considers the long, thin posterior end of the body to be distinctive. Penis sheaths of *L. variesetosus* appear quite similar to those of *L. udekemianus*; the anterior ventral chaetae also have a very long distal tooth, but posterior chaetae have a short, thin distal tooth (Brinkhurst 1979, Figs. 1A,E; Rodriguez 2002, Fig. 2B,C).

The male duct of *L. dybowskii*, as illustrated in Fig. 2 of Smimshchikova (1998), appears quite similar to that of *L. sulphurensis*; measurements of *dybowskii* atria and penis sheaths (400–520 and 350 µm, respectively) are also similar. Two other Lake Baikal endemics, *Limnodrilus nitens* (Semernoy, 1982) and *Limnodrilus tendens* (Semernoy, 1982) appear to have similar penial morphology to *dybowskii* (Semernoy 2004, Figs. 117,118); the latter was synonymized with *dybowskii* by Smimshchikova (1998). It should be noted that Semernoy (1982) compared the penis sheath of *L. tendens* to that of *L. helveticus*, and gave similar measurements for *L. nitens*. All three of the Baikal species are distinguished from congeners by enlarged and highly modified chaetae in some anterior ventral (but not dorsal) segments. The enlarged chaetae are fewer in number and simple-pointed (or with small distal tooth), and thus bear little resemblance to those of *L. sulphurensis n. sp.* (Smimshchikova 1998, Figs. 3, 7; Semernoy 2004, Figs. 116, 117, 118).

**The material.** The morphological description is largely based on material collected on three dates in March 2009 (fixed in FAA), August 2009, and April 2010 (fixed in 10% formalin). The March collection included only two mated specimens (only one with developed eggs), a few "nearly mature", and many immature specimens. Penis sheaths in particular were not well developed, and the ectal ends were indistinct. Most measurements used in the description were based on the large series of mature, formalin-fixed worms collected in April 2010. The formalin-fixed material from August contained several mated worms and many immatures.

Some histological differences among collection dates appear related to the fixation method, as formalin-fixed specimens were highly contracted, and tissues appeared shrunken and separated (e.g., Fig. 3K [formalin] vs. 3L [FAA]). Chaetae of specimens mounted in CMC-10 are similar to those in Canada balsam, but teeth appear slightly thicker (Fig. 3B vs. 3A). Penis sheaths have similar length and proportions, although the thinner cuticle of the head may be indistinct in CMC-10.
TABLE 1. Selected measurements of *L. sulphurensis* n. sp. and *L. profundicola* from new material, compared with literature values for *profundicola*. Values are mean and (range), where available.

<table>
<thead>
<tr>
<th>Species/Location</th>
<th>Number of chaetae in V, ventral bundle length in V</th>
<th>Maximum chaeta length in V</th>
<th>Penis sheath length</th>
<th>Penis sheath length/basal width</th>
<th>Penis sheath head width</th>
<th>Atrium length</th>
</tr>
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<tbody>
<tr>
<td><strong>L. sulphurensis n. sp.</strong></td>
<td></td>
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<tr>
<td>Sulphur Cave (n=26–33)</td>
<td>8.1 (4–11)</td>
<td>165 (146–185)</td>
<td>333 (240–410)</td>
<td>4.9 (4.1–5.7)</td>
<td>148 (120–170)</td>
<td>366 (240–492)</td>
</tr>
<tr>
<td><strong>L. profundicola new material</strong></td>
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<tr>
<td>Estonia and European Russia (n=12–16)</td>
<td>6.4 (4–8)</td>
<td>96 (79–110)</td>
<td>191 (156–221)</td>
<td>4.6 (3.8–5.7)</td>
<td>65 (48–77)</td>
<td>171 (150–190)</td>
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<tr>
<td>Kamchatka (Lake Kuritskoe) (n=3)</td>
<td>5.8 (5–7)</td>
<td>114 (103–122)</td>
<td>241 (226–270)</td>
<td>4.9 (4.3–5.3)</td>
<td>66 (58–72)</td>
<td>236 (220–252)</td>
</tr>
<tr>
<td>Japan, Lake Biwa (n=4)</td>
<td>6.3 (6–7)</td>
<td>94 (89–98)</td>
<td>194 (180–221)</td>
<td>4.8 (4.4–5.1)</td>
<td>59 (53–65)</td>
<td>171 (145–190)</td>
</tr>
<tr>
<td>Lake Michigan (n=15–22)</td>
<td>6.1 (4–9)</td>
<td>113 (89–139)</td>
<td>222 (178–270)</td>
<td>4.7 (3.8–5.7)</td>
<td>63 (53–79)</td>
<td>186 (145–252)</td>
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<td><strong>L. profundicola literature values</strong></td>
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<tr>
<td>Sokol’skaya 1972, Kamchatka (as <em>L. helveticus</em>)</td>
<td>(252–340)</td>
<td>(4–6)</td>
<td></td>
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<tr>
<td>Piguet 1913, Switzerland (<em>L. helveticus</em>)</td>
<td>maximum 8</td>
<td>206 (148–264)</td>
<td>5</td>
<td>69</td>
<td></td>
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<tr>
<td>Ohtaka 1992, Japan</td>
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<tr>
<td>Cui <em>et al.</em> 2015, Tibet, China</td>
<td>6–11</td>
<td>290</td>
<td>5</td>
<td>238</td>
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<td>Semenov 2004, Lake Baikal</td>
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<td>(198–234)</td>
<td>(4–5)</td>
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TABLE 2. COI barcodes of 7 specimens of Limnodrilus sulphurensis n. sp., from Sulphur Cave dark zone (SC), Sulphur Cave surface spring (SS) and Black Sulphur Spring (BS). Two haplotypes (Ht #1, #2) are recognized in positions 2-658, i.e. the interval covered by all sequences; 658 base pairs (bp) are the targeted length of this barcode, but first nucleotide is unknown for 1 specimen.

<table>
<thead>
<tr>
<th>GenBank#</th>
<th>Individual#</th>
<th>Length</th>
<th>Ht#</th>
<th>Site</th>
<th>Museum voucher</th>
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<tr>
<td>-</td>
<td>CE7486</td>
<td>658 bp</td>
<td>2</td>
<td>SC, 18 Aug 2008</td>
<td>SMNH 150175</td>
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<tr>
<td>-</td>
<td>CE7489</td>
<td>658 bp</td>
<td>1</td>
<td>SC, 20 Sep 2008</td>
<td>USNM 1283518**</td>
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<tr>
<td>-</td>
<td>CE7490</td>
<td>658 bp</td>
<td>2</td>
<td>SC, 20 Sep 2008</td>
<td>SMNH 150176</td>
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<tr>
<td>KT692956</td>
<td>CE7491</td>
<td>658 bp</td>
<td>2</td>
<td>SC, 20 Sep 2008</td>
<td>SMNH Type 8779**</td>
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<tr>
<td>-</td>
<td>CE10477</td>
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*Holotype (sexually mature); **Paratypes (mature)

Earlier (September 2007 and August 2008) collections included several mature worms, but they had been preserved in strong alcohol, and thus were brittle and contracted. Chaetae were mostly broken, male ducts invisible against the dark chloragogen, and penis sheaths broken and twisted.

Distribution. Other than the cave habitat, L. sulphurensis has only been collected at the adjacent surface spring and at Black Sulphur Spring. The distinctive chaetal morphology allows the attribution of immature specimens, confirmed by reproductive characters in the two mature worms (Fig. 2E, Table 1). DNA, as well as proximity of the three sites, suggests that all are from the same population.

Limnodrilus profundicola (Verrill, 1871)

(Figure 4)

Tubifex profundicola Verrill, 1871, in Smith & Verrill 1871: 451.

Limnodrilus helveticus Piguet, 1913: 134–136, Fig. 8; Chekanovskaya 1962: 316–317, Fig. 153A; Sokol’skaya 1972 (1983 translation): 11.

Limnodrilus profundicola (Verrill). Brinkhurst 1965: 130–131, Fig. 4K–M; Kennedy 1969: 54–58, Figs. 5, 8, Tables 1–3; Brinkhurst 1971: 470, Fig. 8.4D,E; Ohtaka 1992: 34–35, Fig. 3; Steinlechner 1987: 57–63, Figs. 2–4; Timm 1998: 37, Figs. 68, 69; van Haaren & Soors 2013: 180–181, Figs. 235–237; Cui et al. 2015: 526–527, Fig. 7.

?Limnodrilus alpestris Eisen, 1879: 10; Eisen 1886: 896–897, Pl. XII Fig. 11, Pl. XVII Fig. 11, Pl. XIX Fig. 18.

?Limnodrilus monticola: Eisen, 1879: 18; Eisen 1886: 896, Pl. XI, Fig. 10.

Material examined. Museum specimens. USNM 32598, Tubifex profundicola, holotype, 1 slide from Lake Superior. USNM 32597, Green Lake, Wisconsin, 1921, collected by Juday, whole mount, "referred to as ?L. helveticus in notebooks made by F. Smith" (Brinkhurst 1965). USNM 32590, Limnodrilus monticola, syntypes, 3 specimens from Sierra Nevada, California, 1876). USNM 32592, Limnodrilus alpestris, syntypes, 3 specimens from Sierra Nevada, California, 1876. All listed USNM material was examined by Brinkhurst (1965) and identified as Limnodrilus profundicola.


Descriptive notes. Museum specimens: The holotype, USNM 32598, has mostly dissolved since Brinkhurst’s (1965) redescription, with no penis sheath or chaetae visible. USNM 32597 probably represents a different species, as the penis sheaths are about 500 mm long, curved, with length/width ratio over 8. USNM 32590 and 32592 could not be confirmed, as penis sheaths were not visible.
New material: Chaetae are bifid with teeth slightly curved and approximately equal, or the upper tooth slightly longer in anterior segments (Fig. 4G–J). As in most naidid species, chaetae are more numerous and slightly larger in bundles of anterior segments, and there are at most 1 or 2 "replacement" chaetae. Number of chaetae per bundle 4–9 in anterior segments, 2–3 in posterior segments.

Chloragogen tissue is not particularly dark in any of the profundicola specimens. It densely covers the gut beginning in VI, although it may be visible dorsally in V.

Length and proportions of penis sheaths are consistent with published values (Fig. 4A–D, Table 1). The head is always round and symmetrical, well-defined and orthogonal to the straight or slightly curved shaft, and usually somewhat curved back (Fig. 4A–D,K). Musculature is weak or not apparent in the penial sac, and does not show the strong spiral pattern seen in some other Limnodrilus species. Atria appear relatively short for the genus (Fig. 4A–D, Table 1).

The spermathecal ducts appear quite variable in general form. Still, the structure consists of an irregular sac covered with a thick layer of mostly circular muscles, which narrows to a variably expanded duct before joining the ampulla (Fig. 4E,F). The irregular ectal part ranges from globose to narrow within populations, and in some specimens it is similar to the corresponding part in the L. sulphurensis material (cf. Fig. 2G).

Remarks: The original L. profundicola description (Verrill, in Smith & Verrill 1871), based on material from Lake Superior, gives no details on reproductive characters used to distinguish Limnodrilus species. As the holotype and most other museum specimens examined no longer appear useful, diagnostic characters given in more recent accounts are difficult to verify independently. Furthermore, measurements given in Brinkhurst’s (1965) North American key and 1971 redescription may reflect those derived from Britain and continental Europe (Kennedy 1969), rather than toptotypic or even Nearctic populations. The characters given by Brinkhurst (1971) refer to the form of the chaetae ("upper tooth at most a little longer than lower") and then to the proportions of the penis sheaths. Approximate measurements of penis sheaths in Fig. 4 K–M in Brinkhurst (1965) indicate total lengths about 270 µm (for profundicola, presumably based on the holotype), 340 µm for profundicola (as monticola), and 240 µm for profundicola (as helveticus, presumably European). The range of penis sheath length/width values is very broad (2–7) in those references, but the mean (4.5) is similar to other published profundicola values, as well as to the new measurements (Table 1). Kennedy (1969) appears to use the same values, concluding that profundicola is best determined by the circular head at the ectal end of the sheath, which is more broad and flat than that of L. udekemianus, and never forming an asymmetrical hood, as in some forms of L. hoffmeisteri sensu lato.

The description of L. helveticus (Piguet 1913) was more detailed, showing basic morphology of the penis sheath, atria, chaetae, and spermathecae. It should be noted that Sokol’skaya (1972, 1983) questioned Brinkhurst’s synonymsies and preferred to retain helveticus, based on the high variability in penial morphology in the above descriptions, and also because the profundicola spermathecae were not described by either Brinkhurst (1965, 1971) or Kennedy (1969). This apparently refers to the spermathecal duct, which Sokol’skaya (1983, Fig. 6) describes and illustrates as "forming a distal expansion". Piguet (1913, Fig. 8B) described and illustrated a duct with a globular ectal end, and Cui et al. (2015, Fig. 7A) illustrate a sharply expanded spermathecal duct in a specimen from Tibet, attributed to profundicola. Spermathecal ducts were consistently enlarged in all of our new material, but the ectal end was globular in some, sacciform in other specimens (Fig. 4E,F).

The new Nearctic material is from southern Lake Michigan, rather than the type locality in Lake Superior, but still within the Great Lakes region. Size and proportions of the penis sheath and chaetal morphology were reasonably consistent among regions, and comparable to literature values (Table 1), thus supporting Brinkhurst’s (1966) combination of helveticus with profundicola. Penis sheaths of the new material are all similar to illustrations by Brinkhurst (1965, Fig. 4K–M), Piguet (1913, Fig. 10b), van Haaren & Soors (2013, Fig. 237) and Cui et al. (2015, Fig. 7B). Chaetae are typical for the genus; teeth have normal, slight curvature, in contrast to the strongly angled teeth in L. sulphurensis. The nearly equal teeth in new specimens (Fig. 4G–J) generally resemble Fig. 3A–B of Ohtaka (1992), Fig. 68 in Timm (1998) and Fig. 4 in Steinlechner (1987), but contrast slightly with Fig. 8a of Piguet (1913) and Fig. 5 of Kennedy (1969), in which the upper tooth is distinctly longer. The chaetae in anterior bundles show a fan-like orientation, considered characteristic of Palearctic profundicola populations (Fig. 4G; T. Timm, personal communication). The spermathecal duct is expanded (Fig. 4F), consistent with Palearctic populations, and further supporting their combination.
Discussion

Sulfide in limnetic habitats. The impact of dissolved, biogenic hydrogen sulfide in sediments has been frequently investigated, and oxygen supply and exposure to hydrogen sulfide have been found to be key factors in the distribution of marine oligochaetes (Giere & Pfannkuche 1982; Giere 2006). The two factors are ecophysiologically related, as hydrogen sulfide exacerbates the effects of hypoxia ("sulfide dependent anaerobiosis"). Although aquatic annelids, including tubificines, have occasionally been reported from freshwater habitats influenced by sulfide of geothermal origin (Engel 2007, Messina et al. 2015), these organisms do not appear to have been studied in detail. However, it does not appear that the freshwater oligochaete fauna has evolved any of the spectacular adaptations that characterize many marine annelids from hot vents, seeps, and reduced layers of sands (van Dover 2000; Bright & Giere 2005; Levin 2005). In particular, annelids from freshwater habitats have not been reported to have symbioses with "sulfur bacteria". In Sulphur Cave, with its high sulfide concentrations, Limnodrilus sulphurensis n. sp. survives by a combination of anatomical and physiological adaptations. These adaptations, and the possible ecological advantages of living in hypoxic, sulfidic sediments, will be the subject of a future publication (Giere et al., in prep.).

Oligochaetes inNearctic caves. Up to about 150 stygophilic or stygobiont species are listed in Europe (Giani et al. 2001; Creuzé des Châtelliers et al. 2009). In contrast, regional surveys of caves in North America (Wetzel & Taylor 2001; Kathman & Brinkhurst 1984; Cook 1975) have listed about 25 oligochaete taxa, of which only eight can be considered stygophilic or stygobiontic. Strayer (2001) listed 36 microannelids (of which nine were "interstitial specialists") in hyporheic habitats, but other studies of North American hyporheos record few (if any) oligochaetes. Nevertheless, scattered descriptions from more limited collections (Kindred 1918; Rodriguez 1996; Rodriguez & Coates 1996; Erséus 1999) suggest an endemic Nearctic fauna that has been underrepresented in faunal surveys of cave and groundwater habitats.

The three Limnodrilus species listed in the above accounts (L. hoffmeisteri, L. udekemianus and L. cervix Brinkhurst, 1963) are widespread species known primarily from surface waters. Limnodrilus sulphurensis n. sp. has not been found in other regional studies, but it is possible that it has been overlooked in nearby (surface) habitats. The new species has only been collected in Sulphur Cave and in associated springs. Since other aquatic habitats were not studied in the present investigation, we cannot say whether the worm is primarily a stygobiont, or just a stygophile.

Some species of Limnodrilus, as their nearly cosmopolitan distributions suggest, are extremely eurytopic, inhabiting a very wide range of aquatic habitats (e.g., Kennedy 1965). Particularly noteworthy is the ability of some species to thrive in organically enriched habitats due to a tolerance for low dissolved oxygen concentrations. Thus it is not surprising that the dominant aquatic metazoan in Sulphur Cave is a Limnodrilus species. Still, species vary in their tolerance to these conditions (summarized by Rodriguez & Reynoldson 2011), with the extremely tolerant L. hoffmeisteri and the relatively "oligotrophic" L. profundicola perhaps occupying opposite ends of the spectrum.

Diagnostic characters. Using revisions by Brinkhurst (1965, 1971) as a starting point, Limnodrilus species diagnoses are based largely on morphology of chaetae and penis sheaths.

Typical chaetae for the genus are sigmoid bifids, with slightly curved teeth nearly equal in length and thickness, or the upper tooth somewhat longer. Typically, chaetae in anterior bundles are somewhat larger and more numerous than those in posterior segments, and teeth may be more unequal. Nevertheless, several Limnodrilus species have highly modified and diagnostic somatic chaetae in anterior segments. Chaetal modifications include enlarged or giant ventral chaetae in a few segments (L. rubripennis, L. variesetosus, L. grandisetosus Nomura, 1932, L. dybowskii, L. tendens, and L. nitens); upper tooth enlarged and/or sharply curved (L. udekemianus, L. variesetosus); and simple-pointed chaetae (L. dybowskii, L. tendens, and L. simplex). In at least some of these species (L. grandisetosus, L. dybowskii, L. tendens, L. nitens), the enlarged chaetae are reduced in number, typically two or even one per bundle. There does not appear to be any described species with chaetae resembling those of L. sulphurensis n. sp. The occurrence of modified chaetae in dorsal as well as ventral bundles is also distinctive, as is the large number of both partially and fully developed chaetae per bundle.

Although experimental evidence is limited, predictable variations in tubificine chaetae may occur as a function of water chemistry. Presence or development of hair chaetae and pectinations in dorsal bifids varied with salinity, hardness, and/or pH in Ilyodrilus frantzi Brinkhurst, 1965 and Tubifex tubifex (Müller, 1774) (Chapman &
Brinkhurst 1987). Modifications of chaetae in assumed response to pollutants or water chemistry were also shown by Milbrink (1983) in *Potamothrix hammoniensis* (Michaelsen, 1901), and by Loden and Harman (1979) in *Pristina acquiesita* Bourne, 1891 (Pristininae). As such, chaetal morphology in itself may seem questionable for defining a *Limnodrilus* species, particularly in an unusual chemical environment like Sulphur Cave. Still, the unusual form of anterior chaetae in the new species was invariant, and was seen not only in mature worms, but also in very small individuals.

Penis sheaths are a convenient character, as they are rigid structures, visible in most preparations. General proportions (length/width) are commonly given in *Limnodrilus* keys (e.g., Brinkhurst 1965, 1971), yet values vary within and among populations ascribed to a species, providing at most an initial separation (e.g., Kennedy 1969; Ohtaka 1992; Dzwillo 1984; Barbour et al. 1980). As a consequence, some workers (e.g., van Haaren & Soors 2013) consider penial sheath morphology to be of limited use as a diagnostic character for separating *L. profundicola* from *L. hoffmeisteri*. Proportions of both *sulphurensis* and *profundicola* show overlap with ranges given by Brinkhurst (1971) for the ubiquitous and highly variable *L. hoffmeisteri* (L/W 1–141) and *L. udekemianus* (1–4).

The distal end of the penis sheath may expand to form a characteristic "head", a character that seems easier to define than general proportions. In *profundicola*, it is a well-defined flange, nearly circular and orthogonal to the shaft, but typically curved back. In other species it may be triangular, scalloped, or folded over the distal end, but the distal end of the *L. udekemianus* penis sheath is not distinctly modified (Kennedy 1969). The sheath head of most *L. sulphurensis* specimens appears large compared with *L. profundicola* specimens and illustrations. However, the thickness seems quite variable: a wide plate is visible in most specimens (Fig. 3H), but in others the plate is so thin that it is scarcely visible, and the sheath of unmated worms may resemble illustrations of *L. udekemianus* (Fig. 2A).

Other characters. Morphological characters related to the penial musculature, atria, spermathecae, blood vessels and gut have been used to distinguish some *Limnodrilus* species, but are inconsistently described in the literature. The following characters may be difficult to observe in some preparations, but nevertheless should be evaluated in a more comprehensive review of the genus.

Spiral musculature surrounding the penis sheath had been used as a general character for *Limnodrilus* (Brinkhurst 1971, p. 463). Eisen (1886) used this character to separate *Limnodrilus* from *Camptodrilus* Eisen (the latter with spiral musculature). Assuming Brinkhurst’s combinations, this implies that Eisen did not see spiral musculature in *L. profundicola* (as *L. monticola* and *L. alpestris*), *L. udekemianus* (as *L. steigerwaldi* Eisen and *L. ornatus* Eisen), or *L. silvani* Eisen—but did see them in *L. hoffmeisteri* (as *Camptodrilus californicus* Eisen, *C. corallinus* Eisen, and *C. spiralis* Eisen) and *L. claparedeianus* (as *C. igneus* Eisen). This musculature is not apparent in the available *L. profundicola* specimens, or in *L. sulphurensis*, where only faint circular muscles could be seen in a few worms. However, Piguet’s description of *L. helveticus*, synonymized with *L. profundicola* by Brinkhurst (1965) states "Le pénis est entouré d’un épais manchon de muscles annulaires".

Details of the very long, complex male ducts are not consistently given in published descriptions. The atria of *L. profundicola* specimens observed here (Fig. 4A–D) are relatively short compared with some other species (Fend, unpublished). Semernoy (2004, Fig. 115) and Cui et al. (2015, Fig. 7B) also illustrates a rather short atrium for *profundicola* in Lake Baikal and in Tibet, respectively. The thin "ejaculatory duct" of typical *Limnodrilus* seems a good character for the genus, contrasting with the much thicker or undifferentiated ducts of genera such as *Ilyodrilus* or *Varicaetadrilus*.

Spermathecae are not well-described in much of the *Limnodrilus* literature. Ampullae tend to be more or less globular and indeterminate, but structure of the ectal ducts may provide more useful characters. Spermathecal ducts appear to be thickened and irregular, surrounded by a thick circular muscle layer, and variably expanded in the available *L. profundicola* material. The duct has much the same structure in *L. sulphurensis* n. sp., but there it appears thicker, with a more definite form.

Blood vessels are difficult to observe in many preparations. Contrary to some descriptions (e.g., Piguet 1913, in both VIII and IX), large, contractile "hearts" were seen only in VIII in both *L. profundicola* and *L. sulphurensis*, as also stated by Timm (1998) for *profundicola*. Marcus (1942) considered this to be the general case for the genus, and illustrated (Pl. IV, Figs. 11, 12) a similar arrangement of anterior blood vessels for *L. hoffmeisteri*. The arrangement of lateral blood vessels in posterior segments of *sulphurensis* somewhat resembles descriptions of *L. hoffmeisteri* (Nomura 1913, p. 25 as *L. gotoi* Hatai, 1899; Marcus 1942, Pl. IV, Fig. 13); however, the extensive
vascularization in tail segments appears more similar to that of *L. udekemianus* as described by Claparède (1862 Pl. IV, Fig. 1) and Marcus (1942, Pl. IV, Fig. 16), who indicate a network of cutaneous vessels, rather than the single convoluted vessel described for *hoffmeisteri*.

Gut characters are also rarely described in *Limnodrilus* species. Timm (1998) states that *profundicola* has the chloragogen layer beginning in VI, although this is also the case for *L. udekemianus* (Timm 2009). Steinlechner (1987) did not mention the chloragogen layer, but instead used the widening of the gut at 5/6 vs. 4/5 to distinguish *profundicola* from congeners—presumably the transition from pharynx to intestine. The beginning of chloragogen in VI (as opposed to V) was considered the best diagnostic character for *L. profundicola* by van Haaren & Soors (2013, Fig. 235). Dense chloragogen began in VI in all of our material (both *profundicola* and *sulphurensis*), but some sparse cells could usually be seen on the dorsal side of the gut in V.

The future of systematic studies on *Limnodrilus*. The above discussion illustrates some of the current weaknesses in *Limnodrilus* systematics. Morphological variation in some diagnostic characters seems almost endless (e.g., Kennedy 1969, Dzwillo 1984) and the taxonomy of the different species is unlikely to be resolved solely on the basis of analyses of traditional external and internal characters. This is unfortunate, as many of the "species" are commonly encountered in freshwaters and undoubtedly play an important role in ecosystem function and services in many parts of the world.

Recent molecular studies of Clitellata have shown that species boundaries (in the sense of De Queiroz 2007) can be firmly established using a combination of mitochondrial and nuclear DNA data. This has led to the conclusion that cryptic speciation has previously been underestimated in the diversification of various families of this group (e.g., Gustafsson et al. 2009; Kvist et al. 2010; Envall et al. 2012; Matamoros et al. 2012; Martinsson et al. 2013, 2015; Martinsson & Erséus 2014). Interestingly, in several of these cases, re-evaluation of morphological characters has ultimately supported new species delimitations accomplished on a genetic basis. For species of *Limnodrilus*, preliminary results indicate the existence of cryptic taxa (see Erséus & Gustafsson 2009), but more in-depth molecular studies, focusing on species delimitation as well as phylogeny and boundaries of the genus as such, are underway (Liu et al., in preparation).

Although the present paper is largely a morphology-based taxonomic account, we find it important to publish DNA barcodes of our new species. This is to emphasize the need for genetic characterization of (name-bearing) types, as a first important step towards resolving and stabilizing the systematics of *Limnodrilus* as a whole.

Acknowledgements

We are most grateful to Mike Winnell, Tarmo Timm, and Akifumi Ohtaka for providing new specimens of the elusive *L. profundicola*. Bill Moser (U.S. National Museum, Smithsonian Institution) loaned type specimens of *profundicola* and related material; Eric Lazo-Wassem (Yale Peabody Museum) located some of Verrill’s Lake Superior collections; Frank Krell (Denver Museum of Nature & Science) loaned some of the *L. sulphurensis* specimens used in the description. Emelie Lindquist and Urban Olsson assisted in DNA extraction and sequencing. Ralph Brinkhurst, Tarmo Timm and Mark Wetzel reviewed drafts and provided much-needed advice at all stages of this effort.

References


A NEW LIMNODRILUS SPECIES FROM A SULFUR CAVE

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