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Broad geographic sampling and DNA barcoding do not support the presence of *Helobdella stagnalis* (Linnaeus, 1758) (Clitellata: Glossiphoniidae) in North America

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Abstract

The description of *Helobdella stagnalis* (Linnaeus, 1758) has emphasized the presence of a nuchal, chitinous scute located on the dorsal surface in the first third of the body as the diagnostic character for the species. Historically, identifications of species of *Helobdella* have relied heavily on this character and, as a result, *Helobdella stagnalis* has been reported from an inordinately broad geographic range, including Europe, Asia, Africa, North America, and South America. In addition to a few earlier investigations, a recent analysis showed that great genetic distances (orders of magnitude greater than previous estimations of intraspecific divergence in leeches) are present between scute-bearing specimens identified as *H. stagnalis* from Europe and North America, implying that *H. stagnalis* does not occur in North America. The present study expands the geographic boundaries of taxon sampling for both European and North American taxa, and re-examines the phylogenetic relationships and cytochrome *c* oxidase subunit I (COI) variation within scute-bearing species of the genus *Helobdella*. Our analyses include specimens putatively identified as "*Helobdella stagnalis*" from Sweden, Norway, Iceland, England, France, Italy, Slovenia, Turkey, Russia, and Iran, as well as numerous localities covering Canada and the USA. Our results corroborate previous studies in that European and west Asian specimens form a clade, including the neotype, which is separate from North American taxa. To alleviate future taxonomic confusion, we redescribe *H. stagnalis* and designate a neotype from the inferred type locality. The designation of a neotype stabilizes the taxonomy of scute-bearing leeches of the genus *Helobdella* and enables us to definitively correct erroneous identifications reported in previous studies. We also note that at least four lineages of scute-bearing, North American species of *Helobdella* lack formal descriptions.

Key words: *Helobdella stagnalis,* neotype, redescription, DNA barcoding, leech.

Introduction

Helobdella stagnalis (Linnaeus, 1758), the type species of the genus *Helobdella* Blanchard, 1896, is an important component of freshwater ecosystems and has been recorded from various localities in Europe, Asia, Africa, North America, and South America. (Davies *et al.* 1979). Whereas species of this genus are known to feed on snails, oligochaetes and insect larvae, a few dubious records exist reporting its parasitism on frogs and salamanders (Moore 1899; Ringuelet 1949; Sciacchitano 1967; Sawyer 1986; Tiberti & Gentilli 2010; Arslan & Oktener 2012; Zimic 2015). Notably, however, these latter records provide no evidence of feeding behavior.

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Historically, the main diagnostic feature of *H. stagnalis* has been the presence of a conspicuous chitinous scute on the dorsal surface of the anterior region of the body (Sawyer 1986). Vexingly, this character is shared by several other *Helobdella* species described and/or recorded from North America, South America and Asia (*e.g.* Verrill 1872; Siddall 2001a; Oceguera-Figueroa *et al.* 2005; Iwama & Arruda 2016; Saglam *et al.* 2018). Distinguishing between these species is difficult, owing to their cryptic, or pseudo-cryptic, nature (*sensu* Kawauchi & Giribet 2014; Achurra *et al.* 2015), as well as the lack of detailed morphological descriptions for structures other than the presence of the chitinous scute. In North America, a species of *Helobdella* with a nuchal scute was described as *Helobdella modesta* (Verrill, 1872) however, its taxonomic status has been variable and Sawyer (1986) considered it as a junior synonym of *H. stagnalis*. The original description of *H. stagnalis* itself omits information regarding many important morphological details (Linnaeus 1758). Although Linnaeus's description is presumably based on specimens collected from a locality in Uppsala, Sweden, no type specimens were designated. As a result, the name *H. stagnalis* has been assigned, based solely on the presence of the chitinous nucal scute, to numerous specimens collected from across Europe, Asia, Africa, North America and South America without further morphological or molecular investigation.

Due to the difficulty inherent in accurately identifying species of *Helobdella* based on morphological characters (*e.g.* Bely & Weisblat 2006; Siddall & Borda 2003; Siddall & Budinoff 2005), molecular data have become imperative in recognizing species groups. On the basis of molecular phylogenetic analyses, Light and Siddall (1999) were the first to point out a possible separation between North American and European scute-bearing specimens identified as *H. stagnalis*. Later, Siddall and Borda (2003) reported an average uncorrected *p*-distance of 8% at the mitochondrial cytochrome *c* oxidase subunit I (COI) locus between representatives of *H. stagnalis* from Europe and North America. Based on this evidence, the authors suggested that scute-bearing specimens from the two continents should be treated as a separate species, and reerected the name *Helobdella modesta* (Verrill, 1872) for North American representatives. Subsequently, these results were corroborated by Moser *et al.* (2011), that found similar levels of molecular genetic distance when comparing specimens from the type locality of *H. modesta* with specimens of *H. stagnalis* from Europe.

In Asia, *H. stagnalis* has been recorded from several localities. The description of the morphologically similar *Helobdella octatestisaca* Lai, Chang & Chen, 2009 from Taiwan added confusion to the taxonomic status of *Helobdella stagnalis*, and raised questions about previous records in this continent. To further complicate matters, *H. octatestisaca* has subsequently been recorded from Mexico and the United States (Oceguera-Figueroa *et al.*, 2010; Richardson *et al.*, 2017). Recently, Saglam *et al.* (2018) described three scute-bearing species of *Helobdella* from North America, and identified three additional clades needing taxonomic circumscription. All these species are superficially similar to specimens of *H. stagnalis* from Europe, but exhibit some differences in internal morphology. The authors also demonstrated a clear separation between North American species and European *H. stagnalis* based on molecular data; this reaffirms the notion that *H. stagnalis* does not exist in North America. Unfortunately, Saglam *et al.* (2018) did not include extensive sampling of European and North American localities, nor did they designate a neotype from their European samples, likely due to the lack of sampling from the inferred type locality.

Despite past efforts to elucidate the taxonomic status of *H. stagnalis*, sparse geographic sampling of Europe, Asia, and North America, in combination with the absence of type material for the species, make it difficult to identify this and other species of the genus *Helobdella*. The aims of this paper are to stabilize the taxonomy of scute-bearing species and to facilitate future identifications of scute-bearing species of *Helobdella*. We herein redescribe and designate a neotype for *H. stagnalis* under the article 75.1 of the International Code of Zoological Nomenclature (ICZN) (ICZN 1999). We examine the morphological and molecular characteristics of scute-bearing species from across Europe, North America, South America and Asia. In particular, we perform phylogenetic and distance analyses using a large data set of COI sequences, combining previously-published data with new sampling efforts to determine the systematic position of *H. stagnalis*, uncover erroneous identifications of *H. stagnalis* in the literature, and explore the genetic distinctiveness within and between the various species of *Helobdella*.

Methods

Collection of specimens. Most of the specimens of *Helobdella* from Sweden and Norway were collected by CE between 2010 and 2017 as part of nationwide inventories of the clitellate fauna in both countries (*i.e.* Norwegian Taxonomy Initiative, and Swedish Taxonomy Initiative); specimens from Jonsvatn Lake, Sör-Trondelag, Norway, were collected by Torbjörn Ekrem and Karstein Hårsaker. Leeches, generally as part of bulk samples of benthic fauna, were preserved in 75–95% ethanol in the field and, subsequently, a tissue sample from each worm was stored in 95% ethanol. Vouchers for these specimens are deposited in the collections of the Swedish Museum of Natural History (SMNH), Stockholm; the University Museum of Bergen (ZMBN), Norway; and the NTNU University Museum (NTNU-VM), Trondheim, Norway. Specimens from Uppsala, Sweden, were collected in June 2016 by Lotta Calmerfalk, Melvin Calmerfalk and Charlotte Calmerfalk Kvist. These leeches were relaxed in 10–15% ethanol and fixed in 95% ethanol. Voucher specimens are deposited at the Royal Ontario Museum (ROM), Canada, and the neotype is deposited at SMNH. Numerous North American specimens were collected between 2015 and 2018 by MES, DdC, SK, Don Stacey and Charlotte Calmerfalk Kvist. Specimens from Slovenia were collected in 2016 by SK, Charlotte Calmerfalk Kvist and Peter Trontelj. These specimens were relaxed by gradual addition of 70% ethanol to pond water. After relaxation, the leeches were fixed in 95% ethanol. Voucher specimens for North American and Slovenian samples are deposited at the ROM, the American Museum of Natural History (AMNH) and the National Museum of Natural History (USNM), USA. Information about new material included in this study is provided in Table 1.

Investigation of external morphology was conducted using a Leica Wild M10 dissecting microscope (Leica Microsystems, Richmond Hill, ON, Canada). For internal morphology, specimens were dissected ventrally, studied under the same dissecting microscope, and photographed using a Keyence VHX-5000 microscope and camera system (Keyence Corporation, Mississauga, ON, Canada).

DNA extraction and sequencing. DNA was extracted from tissue of the posterior suckers of the leeches using Qiagen DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturers protocol, and mitochondrial COI sequences were amplified using the primers LCO1490 (5'-GGTCAACAAARCARAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994). The 25µl Polymerase Chain Reactions (PCR) consisted of 16.35 µl ddH_2O, 2.5 µl buffer, 2.5 µl $MgCl_2$, 1 µl of each primer, 0.56 µl dNTPs, 0.1 µl TAQ polymerase and 1 µl of DNA template. PCR amplifications used the following thermoprofile: an initial denaturation step at 94°C (1 min.), followed by 5 cycles of 94°C (30 sec.), 40°C (40 sec.), 72°C (1 min.), then 35 cycles of 94°C (30 sec.), 46°C (40 sec.), 72°C (1 min.) and a final extension at 72°C (5 min.) for all samples. Successful amplifications (checked by Gel Electrophoresis on a 1% agarose gel) were purified with a solution of 1.5 µl ddH_2O and 0.5 µl ExoSAP-IT (Affymetrix, Santa Clara, CA) following the manufacturers protocol. Cycle sequencing reactions used 4 µl ddH_2O, 0.5 µl ABI Big Dye Terminator ver. 3.1, 2 µl of primer at 10 µM, 0.5 µl BigDye 5x Sequencing Buffer (Applied Biosystems, Carlsbad, CA) and 3 µl purified PCR products. Ethanol precipitation was then performed, and samples were sequenced on an ABI PRISM 3730 (Applied Biosystems, Carlsbad, CA) at the AMNH or ROM.

For the Scandinavian specimens, DNA was extracted using Epicentre's QuickExtract DNA Extraction Solution 1.0 (Lucigen, Wisconsin, USA), and COI was sequenced by Eurofins MWG Operon (Ebersberg, Germany); gene amplification followed the protocol given above.

Alignment and phylogenetic analyses. A total of 195 COI sequences were used in the analyses, 106 of these were newly generated for the present study. New sequences were assembled and edited using Geneious ver. 9.0.4 (Kearse *et al.* 2012); all new sequences are deposited in GenBank under accession numbers MN071246–MN071352 (Table 1). To supplement the dataset, sequences from both North America and Europe labeled *Helobdella stagnalis* were downloaded from GenBank; we also included representative COI sequences for each species of *Helobdella* present in the database (Table 2). Importantly, the resulting dataset includes all sequences used by Saglam *et al.*(2018). The online version of MAFFT ver. 7 (Katoh & Standley 2013) was employed for multiple sequence alignment, using default parameters, with search strategy set to automatic.

A parsimony analysis was performed in TNT ver. 1.5 (Goloboff *et al.* 2008) using a new technology search for 1000 replications with five rounds of the ratchet and three rounds of tree fusing, stipulating that the shortest tree be encountered 10 times before terminating each round (xmult: replic 1000 ratchet 5 fusing 3 hits 10). Parsimony bootstrap support (PBS) values were estimated in TNT using 1000 replicates and the same settings as mentioned above. Prior to maximum likelihood (ML) analysis, PartitionFinder ver. 1.1.1 (Lanfear *et al.* 2012) was used to determine the best fitting model of nucleotide evolution and the optimal partitioning scheme of the data, considering each codon position. ML analysis was conducted using RaxML ver. 8 (Stamatakis 2014) on the XSEDE server on the CIPRES platform (Miller *et al.* 2010). For this, 1000 iterations were conducted, each with 25 initial GAMMA rate categories that were allowed to fluctuate independently for each partition, and final optimization with four

TABLE 1. New specimens used in this study and their respective collection locality, catalog number and COI GenBank accession number ("COI").

Species Names	Autorship of name	Locality	Country	Coordinates	Collection catalog number	COI
Helobdella stagnalis	**(Linnaeus, 1758)**	**Lake Trehörningen, Uppland**	**Sweden**	**59°50.60000' 17°52.97167'**	**SMNH Type-9177**	**MN071246**
Helobdella stagnalis	(Linnaeus, 1758)	Lake Trehörningen, Uppland	Sweden	59°50.60000' 17°52.97167'	ROMIZI11710	MN071247
Helobdella stagnalis	(Linnaeus, 1758)	Pisovarina	Slovenia	46°06.67000' 14°30.64667'	ROMIZI11752	MN071248
Helobdella stagnalis	(Linnaeus, 1758)	Västergötland	Sweden	57°41.02833' 11°56.82667'	SMNH 177725	MN071249
Helobdella stagnalis	(Linnaeus, 1758)	Västergötland	Sweden	57°41.02833' 11°56.82667'	SMNH 177726	MN071250
Helobdella stagnalis	(Linnaeus, 1758)	Bohuslän	Sweden	58°54.01833' 11°13.56667'	SMNH 177727	MN071251
Helobdella stagnalis	(Linnaeus, 1758)	Västergötland	Sweden	57°41.02833' 11°56.82667'	SMNH 177728	MN071252
Helobdella stagnalis	(Linnaeus, 1758)	Akershus, Norway	Norway	59°41.63333' 10°44.12333'	ZMBN 128838	MN071253
Helobdella stagnalis	(Linnaeus, 1758)	Närke	Sweden	59°14.17167' 15°34.05667'	SMNH 177729	MN071254
Helobdella stagnalis	(Linnaeus, 1758)	Östfold	Norway	59°07.86000' 11°29.28667'	ZMBN 128983	MN071255
Helobdella stagnalis	(Linnaeus, 1758)	Östfold	Norway	59°07.86000' 11°29.28667'	ZMBN 128984	MN071256
Helobdella stagnalis	(Linnaeus, 1758)	Västergötland	Sweden	57°55.99167' 12°30.04167'	SMNH 177730	MN071257
Helobdella stagnalis	(Linnaeus, 1758)	Oppland	Norway	61°39.57000' 8°08.82667'	ZMBN 129300	MN071258
Helobdella stagnalis	(Linnaeus, 1758)	Oppland	Norway	61°39.57000' 8°08.82667'	ZMBN 129301	MN071259
Helobdella stagnalis	(Linnaeus, 1758)	Oppland	Norway	61°43.24833' 8°23.60333'	ZMBN 129298	MN071260
Helobdella stagnalis	(Linnaeus, 1758)	Bohuslän	Sweden	58°50.70667' 11°11.59833'	SMNH 177731	MN071261
Helobdella stagnalis	(Linnaeus, 1758)	Västergötland	Sweden	57°51.54000' 12°26.34000'	SMNH 177732	MN071262
Helobdella stagnalis	(Linnaeus, 1758)	Telemark	Norway	59°43.53000' 9°21.36667'	ZMBN 129064	MN071263
Helobdella stagnalis	(Linnaeus, 1758)	Buskerud	Norway	60°46.16333' 9°00.16167'	ZMBN 128756	MN071264
Helobdella stagnalis	(Linnaeus, 1758)	Hordaland	Norway	60°28.86000' 5°22.08667'	ZMBN 128923	MN071265
Helobdella stagnalis	(Linnaeus, 1758)	Hordaland	Norway	60°28.86000' 5°22.08667'	ZMBN 128924	MN071266
Helobdella stagnalis	(Linnaeus, 1758)	Sör-Tröndelag	Norway	63°23.74167' 10°33.22167'	NTNU-VM 203836	MN071267
Helobdella stagnalis	(Linnaeus, 1758)	Möre og Romsdal	Norway	63°01.80667' 8°01.51167'	ZMBN 129174	MN071268
Helobdella stagnalis	(Linnaeus, 1758)	Möre og Romsdal	Norway	62°55.95667' 7°12.72667'	ZMBN 129216	MN071269
Helobdella stagnalis	(Linnaeus, 1758)	Nordland	Norway	69°15.33667' 16°04.18167'	ZMBN 129349	MN071270
Helobdella stagnalis	(Linnaeus, 1758)	Nordland	Norway	69°15.33667' 16°04.10333'	ZMBN 129352	MN071271
Helobdella stagnalis	(Linnaeus, 1758)	Möre og Romsdal	Norway	63°01.80667' 8°01.51167'	ZMBN 129173	MN071272
Helobdella stagnalis	(Linnaeus, 1758)	Sogn og Fjordane	Norway	61°43.01333' 6°43.01333'	ZMBN 129314	MN071273
Helobdella stagnalis	(Linnaeus, 1758)	Sogn og Fjordane	Norway	61°56.37000' 6°29.52667	ZMBN 129306	MN071274
Helobdella stagnalis	(Linnaeus, 1758)	Sogn og Fjordane	Norway	61°56.37000' 6°29.52667	ZMBN 129307	MN071275

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TABLE 1. (Continued)

Species Names	Autorship of name	Locality	Country	Coordinates	Collection catalog number	COI
Helobdella stagnalis	(Linnaeus, 1758)	Sogn og Fjordane	Norway	61°56.37000’ 6°29.52667’	ZMBN 129308	MN071276
Helobdella stagnalis	(Linnaeus, 1758)	Sogn og Fjordane	Norway	61°43.01333’ 6°43.01333’	ZMBN 129315	MN071277 MN071277
Helobdella stagnalis	(Linnaeus, 1758)	Oppland	Norway	61°50.71833’ 9°21.63667’	ZMBN 129243	MN071278
Helobdella stagnalis	(Linnaeus, 1758)	Närke	Sweden	59°14.17167’ 15°34.05667’	SMNH 177733	MN071279
Helobdella stagnalis	(Linnaeus, 1758)	Nordland	Norway	68°32.20167’ 17°15.71333’	ZMBN 129351	MN071280
Helobdella stagnalis	(Linnaeus, 1758)	Telemark	Norway	59°43.53000’ 9°21.36667’	ZMBN 129063	MN071281
Helobdella stagnalis	(Linnaeus, 1758)	Sör-Tröndelag	Norway	63°23.74167’ 10°33.22167’	ZMBN 203843	MN071282
Helobdella stagnalis	(Linnaeus, 1758)	Västergötland	Sweden	57°59.84333’ 12°35.19667’	SMNH 177734	MN071283
Helobdella echoensis	Saglam *et al.*, 2018	Big Creek Lake, Iowa	United States	41°49.91667’ -93°45.36667’	ROMIZI10509	MN071284
Helobdella echoensis	Saglam *et al.*, 2018	Moore Lake, Ontario	Canada	46°17.55000’ -78°52.71833’	ROMIZI10259	MN071285
Helobdella echoensis	Saglam *et al.*, 2018	Hazel Creek, Manitoba	Canada	49°52.82667’ -96°14.22000’	ROMIZI11502	MN071286
Helobdella echoensis	Saglam *et al.*, 2018	Eagle Lake, Minnesota	United States	44°12.41333’ -93°53.38500’	ROMIZI10496	MN071287
Helobdella echoensis	Saglam *et al.*, 2018	Tooeys Lake, Ontario	Canada	45°20.45833’ -77°01.36500’	ROMIZI10337	MN071288
Helobdella echoensis	Saglam *et al.*, 2018	Jackfish Lake, Alberta	Canada	52°30.10833’ -115°33.51667’	ROMIZI11365	MN071289
Helobdella echoensis	Saglam *et al.*, 2018	Tributary to Lake McConaughy, Nebraska	United States	41°16.97167’ -101°49.26333’	ROMIZI10576	MN071290
Helobdella echoensis	Saglam *et al.*, 2018	Tributary to Lake McConaughy, Nebraska	United States	47°52.48667’ -92°08.44500’	ROMIZI10574	MN071291
Helobdella echoensis	Saglam *et al.*, 2018	Mohonk Lake, New York	United States	41°46.07667’ -74°09.31333’	AMNH-IZC 00331459	MN071292
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°43.62667’ -74°14.25833’	AMNH-IZC 00331460	MN071293
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°43.66000’ -74°14.23667’	AMNH-IZC 00331460	MN071294
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°46.07667’ -74°09.31333’	AMNH-IZC 00331460	MN071295
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°46.07667’ -74°09.31333’	AMNH-IZC 00331460	MN071296
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°46.07667’ -74°09.31333’	AMNH-IZC 00331460	MN071297
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°46.07667’ -74°09.31333’	AMNH-IZC 00331460	MN071298
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°43.66000’ -74°14.23667’	AMNH-IZC 00331460	MN071299
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°43.66000’ -74°14.23667’	AMNH-IZC 00331460	MN071300
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°43.66000’ -74°14.23667’	AMNH-IZC 00331460	MN071301
Helobdella echoensis	Saglam *et al.*, 2018	Minnewaska Lake, New York	United States	41°43.62667’ -74°14.25833’	AMNH-IZC 00331460	MN071302

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TABLE 1. (Continued)

Species Names	Autorship of name	Locality	Country	Coordinates	Collection catalog number	COI
Helobdella echoensis	Saglam *et al*., 2018	Minnewaska Lake, New York	United States	41°43.66000' -74°14.23667'	AMNH-IZC 00331460	MN071303
Helobdella echoensis	Saglam *et al*., 2018	Minnewaska Lake, New York	United States	41°46. 07667' -74°09.31333'	AMNH-IZC 00331460	MN071304
Helobdella echoensis	Saglam *et al*., 2018	Minnewaska Lake, New York	United States	41°43.62667' -74°14.25833'	AMNH-IZC 00331460	MN071305
Helobdella echoensis	Saglam *et al*., 2018	Minnewaska Lake, New York	United States	41°43.66000' -74°14.23667'	AMNH-IZC 00331460	MN071306
Helobdella echoensis	Saglam *et al*., 2018	Minnewaska Lake, New York	United States	41°43.66000' -74°14.23667'	AMNH-IZC 00331460	MN071307
Helobdella echoensis	Saglam *et al*., 2018	Minnewaska Lake, New York	United States	41°43.62667' -74°14.25833'	AMNH-IZC 00331460	MN071308
Helobdella echoensis	Saglam *et al*., 2018	Minnewaska Lake, New York	United States	41°43.66000' -74°14.23667'	AMNH-IZC 00331460	MN071309
Helobdella echoensis	Saglam *et al*., 2018	Minnewaska Lake, New York	United States	41°46.07667' -74°09.31333'	AMNH-IZC 00331460	MN071310
Helobdella eriensis	Saglam *et al*., 2018	Skannatati Lake, New York	United States	41°14.50833' -74°06.16500'	AMNH-IZC 00331461	MN071311
Helobdella eriensis	Saglam *et al*., 2018	Moraine Lake, New York	United States	42°51.55842' -75°30.75300'	AMNH-IZC 00331462	MN071312
Helobdella eriensis	Saglam *et al*., 2018	Stoney Pond, New York	United States	42°52.90128' -75°42.79806'	AMNH-IZC 00331463	MN071313
Helobdella modesta	(Verrill, 1872)	Lake Como, New York	United States	42°40.44167' -76°17.87167'	AMNH-IZC 00331464	MN071314
Helobdella modesta	(Verrill, 1872)	Rochester, New York	United States		USNM-SZOO 0069	MN071315
Helobdella modesta	(Verrill, 1872)	Three Rivers Wildlife Management Area, New York	United States	43°12.57833' -76°19.54000'	AMNH-IZC 00331465	MN071316
Helobdella modesta	(Verrill, 1872)	Beaver Lake, New York	United States	43°10. 82833' -76°24.30833'	AMNH-IZC 00331466	MN071317
Helobdella modesta	(Verrill, 1872)	Beaver Lake, New York	United States	43°10. 82833' -76°24.30833'	AMNH-IZC 00331466	MN071318
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Gamboa	Panama	09°06.71333' -79°41.27167'	ROMIZI12957	MN071319
Helobdella sp. 2		Nevens Ranch, Nebraska	United States	41°11.56500' -101°18.20000'	ROMIZI10528	MN071320
Helobdella sp. 2		Swan Lake, Arthur County, Nebraska	United States	41°44.25667' -101°28.47000'	ROMIZI10549	MN071321
Helobdella sp. 3		Alberta	Canada	54°37.91667' 111°38.38333'	ROMIZI11568	MN071322
Helobdella sp. 5		Big Island Lake, Alberta	Canada	53°29.62333' -113°11.83167'	ROMIZI11226	MN071323
Helobdella sp. 5		Restoule Lake, Ontario	Canada	46°03.63667 -79°45.93833'	ROMIZI10241	MN071324
Helobdella sp. 5		Ranger Lake	Canada	46°25.77333' -88°34.13667'	ROMIZI10134	MN071325
Helobdella sp. 5		Mijinemungshing Lake, Ontario	Canada	47°42.16667' -84°43.81667'	ROMIZI10093	MN071326
Helobdella sp. 5		Fish Lake, Alberta	Canada	52°27.21333' -116°08.72167'	ROMIZI11376	MN071327
Helobdella sp. 5		Roy H. Park, New York	United States		AMNH-IZC 00331467	MN071328
Helobdella sp. 6		Pipestone Creek, Saskatchewan	Canada	50°04.55667' -101°42.45667'	ROMIZI11417	MN071329
Helobdella sp. 6		Lake Lac La Biche, Alberta	Canada	54°53.82667' -112°02.37167'	ROMIZI11325	MN071330

......*continued on the next page*

TABLE 1. (Continued)

Species Names	Autorship of name	Locality	Country	Coordinates	Collection catalog number	COI
Helobdella sp. 6		Elk Water Lake, Cypress Hill, Alberta	Canada	49°39.68833’ -110°17.37667’	ROMIZI11242	MN071331
Helobdella sp. 6		Lakeland Privincial Park, Alberta	Canada	54°46.66500’ -111°42.66000’	ROMIZI11314	MN071332
Helobdella sp. 6		Big Island Lake, Alberta	Canada	53°29.62333’ -113°11.83167’	ROMIZI11261	MN071333
Helobdella sp. 6		Muir Lake, Alberta	Canada	53°37.68000’ -113°57.39333’	ROMIZI11354	MN071334
Helobdella sp. 6		Whitegoat Lake, Alberta	Canada	52°12.20667’ -116°28.81833’	ROMIZI11371	MN071335
Helobdella sp. 6		Paul Lake, British Columbia	Canada	50°44.09167’ -120°07.84667’	ROMIZI11223	MN071336
Helobdella sp. 6		Lake Winnipeg, Manitoba	Canada	50°43.37167’ -96°32.34833’	ROMIZI11540	MN071337
Helobdella sp. 6		Goldeye Lake, Alberta	Canada	54°37.91667’ -111°38.38333’	ROMIZI11382	MN071338
Helobdella sp. 6		Anglin Lake, Alberta	Canada	54°46.66500’ -111°42.66000’	ROMIZI11445	MN071339
Helobdella sp. 6		Cypress Lake, Saskatchewan	Canada	49°28.35167’ -109°34.19833’	ROMIZI11487	MN071340
Helobdella sp. 6		Chump Lake, Alberta	Canada	54°39.05167’ -112°33.68167’	ROMIZI11336	MN071341
Helobdella sp. 6		Long Lake, Alberta	Canada	54°26.59500’ -112°45.96833’	ROMIZI11283	MN071342
Helobdella elongata	(Castle, 1900)	Canopus Lake, New York	United States	41°28.30667’ -73°49.53667’	AMNH-IZC 00331468	MN071343
Helobdella elongata	(Castle, 1900)	Canopus Lake, New York	United States	41°28.30667’ -73°49.53667’	AMNH-IZC 00331468	MN071346
Helobdella elongata	(Castle, 1900)	Canopus Lake, New York	United States	41°28.30667’ -73°49.53667’	AMNH-IZC 00331468	MN071347
Helobdella lineata	(Verrill, 1874)	Madison Lake, New York	United States	42°54.16896’ -75°31.16790’	AMNH-IZC 00331469	MN071348
Helobdella robusta	Shankland, Bissen & Weisblat, 1992	John Allen Pond, New York	United States		AMNH-IZC 331458	MN071349
Helobdella robusta	Shankland, Bissen & Weisblat, 1992	John Allen Pond, New York	United States		AMNH-IZC 331458	MN071350
Helobdella sp. 1		Saskatchewan	Canada	49°50.33500’ -102°18.63167’	ROMIZI11391	MN071351
Helobdella lineata		Alberta	Canada	53°29.62333’ -113°11.83167’	ROMIZI11255	MN071352

TABLE 2. Sequences from previous studies included in the phylogenetic analyses and their respective collection locality and COI GenBank accession number ("COI").

Species name	Authorship of name	Locality	Country	Reference	COI
Helobdella stagnalis	(Linnaeus, 1758)	Ficuzza, Sicily	Italy	Marrone *et al.* (2016)	KT382519
Helobdella stagnalis	(Linnaeus, 1758)	Ficuzza, Sicily	Italy	Marrone *et al.* (2016)	KT382518
Helobdella stagnalis	(Linnaeus, 1758)	Ficuzza, Sicily	Italy	Marrone *et al.* (2016)	KT382520
Helobdella stagnalis	(Linnaeus, 1758)	Ficuzza, Sicily	Italy	Marrone *et al.* (2016)	KT363868
Helobdella stagnalis	(Linnaeus, 1758)	Ficuzza, Sicily	Italy	Marrone *et al.* (2016)	KT382517
Helobdella stagnalis	(Linnaeus, 1758)	Múvatn	Iceland	Saglam *et al.* (2018)	MF150168
Helobdella stagnalis	(Linnaeus, 1758)	Çermik, Diyarbakýr	Turkey	Saglam *et al.* (2018)	MF150166
Helobdella stagnalis	(Linnaeus, 1758)	Diyarbakýr	Turkey	Saglam *et al.* (2018)	MF150167
Helobdella stagnalis	(Linnaeus, 1758)	Çýnar, Diyarbakýr,	Turkey	Saglam *et al.* (2018)	MF150165
Helobdella stagnalis	(Linnaeus, 1758)	Lake Gusinoe	Russia	Kaygorodova *et al.* (2014)	KM095095
Helobdella stagnalis	(Linnaeus, 1758)	Lake Gusinoe	Russia	Kaygorodova *et al.* (2014)	KM095096
Helobdella stagnalis	(Linnaeus, 1758)		Iran		KY989485
Helobdella stagnalis	(Linnaeus, 1758)	Cotswolds	England	Siddal & Borda (2003)	AF329041
Helobdella stagnalis	(Linnaeus, 1758)		France	Apakupakul *et al.* (1999)	AF116018
Helobdella atli	Oceguera-Figueroa & León-Règagnon, 2005	Aljojuca, D. F.	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179850
Helobdella atli	Oceguera-Figueroa & León-Règagnon, 2006	Totolcingo, Tlaxcala	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179851
Helobdella atli	Oceguera-Figueroa & León-Règagnon, 2007	Xochimilco, D. F.	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179852
Helobdella austinensis	Kutschera *et al.*, 2013	UC Berkeley, California	United States	Kutschera *et al.* (2013)	KC812736
Helobdella bolivianita	Siddall, 2001b	Laguna Volcan	Bolivia	Siddal & Borda (2003)	AF329053
Helobdella bowermani	Moser *et al.*, 2013	Upper Klamath Lake, Klamath County, Oregon	United States	Moser *et al.* (2013)	KF683192
Helobdella bowermani	Moser *et al.*, 2013	Upper Klamath Lake, Klamath County, Oregon	United States	Moser *et al.* (2013)	KF683194
Helobdella bowermani	Moser *et al.*, 2013	Upper Klamath Lake, Klamath County, Oregon	United States	Moser *et al.* (2013)	KF683193

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TABLE 2. (Continued)

Species name	Authorship of name	Locality	Country	Reference	COI
Helobdella californica	Kutschera, 1988	Golden Gate Park, San Francisco, California	United States	Kutschera (2011)	HQ686307
Helobdella echoensis	Saglam *et al.*, 2018	Echo Lake, Pennsylvania	United States	Saglam *et al.* (2018)	JN692267
Helobdella echoensis	Saglam *et al.*, 2018	Roseburg, Oregon	United States	Saglam *et al.* (2018)	JN692266
Helobdella echoensis	Saglam *et al.*, 2018			De Carle *et al.* (2017)	MF067147
Helobdella echoensis	Saglam *et al.*, 2018	Dog Lake, British Columbia	Canada		KM612173
Helobdella echoensis	Saglam *et al.*, 2018	York, Pennsylvania	United States	Saglam *et al.* (2018)	JN692268
Helobdella eriensis	Saglam *et al.*, 2018	Maumee River, Toledo, Ohio	United States		KM196604
Helobdella modesta	(Verrill, 1872)	Barnstable County, Massachusetts	United States	Richardson *et al.* (2015)	KP176608
Helobdella modesta	(Verrill, 1872)	Silver Lakem Gibbsboro, New Jersey	United States	Saglam *et al.* (2018)	MF158964
Helobdella modesta	(Verrill, 1872)	Silver Lakem Gibbsboro, New Jersey	United States	Saglam *et al.* (2018)	MF158965
Helobdella modesta	(Verrill, 1872)	Silver Lakem Gibbsboro, New Jersey	United States	Saglam *et al.* (2018)	MF158966
Helobdella modesta	(Verrill, 1872)	Hadden Lake, New Jersey	United States	Saglam *et al.* (2018)	MF158963
Helobdella modesta	(Verrill, 1872)	Greenwhich, New Jersey	United States	Saglam *et al.* (2018)	MF158967
Helobdella modesta	(Verrill, 1872)	Whitneyville Lake, New Haven County, Connectcut	United States	Moser *et al.* (2011)	JF319990
Helobdella modesta	(Verrill, 1872)	Cooper River, New Jersey	United States	Saglam *et al.* (2018)	JN692263
Helobdella modesta	(Verrill, 1872)	Whitneyville Lake, New Haven County, Connectcut	United States	Moser *et al.* (2011)	JF319989
Helobdella modesta	(Verrill, 1872)	Farm Pond, Pennsylvania	United States	Saglam *et al.* (2018)	JN692269
Helobdella modesta	(Verrill, 1872)	Whitneyville Lake, New Haven County, Connectcut	United States	Moser *et al.* (2011)	JF319992
Helobdella modesta	(Verrill, 1872)	West River, New Haven County, Con-nectcut	United States	Moser *et al.* (2011)	JF319994
Helobdella modesta	(Verrill, 1872)	Whitneyville Lake, New Haven County, Connectcut	United States	Moser *et al.* (2011)	JF319988
Helobdella modesta	(Verrill, 1872)	Whitneyville Lake, New Haven County, Connectcut	United States	Moser *et al.* (2011)	JF319991

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TABLE 2. (Continued)

Species name	Authorship of name	Locality	Country	Reference	COI
Helobdella modesta	(Verrill, 1872)	West River, New Haven County, Con-nectcut	United States	Moser *et al.* (2011)	JF319996
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Taipei	Taiwan	Lai *et al.* (2009)	FJ000342
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Taipei	Taiwan	Lai *et al.* (2009)	FJ000344
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Taipei	Taiwan	Lai *et al.* (2009)	FJ000348
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Taipei	Taiwan	Lai *et al.* (2009)	FJ000346
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Taipei	Taiwan	Lai *et al.* (2009)	FJ000345
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Taipei	Taiwan	Lai *et al.* (2009)	FJ000343
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Taipei	Taiwan	Lai *et al.* (2009)	FJ000347
Helobdella octatestisaca	Lai, Chang & Chen, 2009		South Africa	Oceguera-Figueroa *et al.* (2010)	HQ179860
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Tabasco	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179859
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Guanajuato	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179858
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Queretaro	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179855
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Ameca, Jalisco	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179856
Helobdella octatestisaca	Lai, Chang & Chen, 2009	Hidalgo	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179857
Helobdella ringueleti	Siddall, 2001	Madidi	Bolivia	Siddal & Borda (2003)	AF329051
Helobdella serendipitous	Saglam *et al.*, 2018	New Jersey	United States	Saglam *et al.* (2018)	JN692265
Helobdella sp. 4		New Jersey	United States	Saglam *et al.* (2018)	JN692264
Helobdella sp. 5		Washington	United States	Oceguera-Figueroa *et al.* (2010)	HQ179854
Helobdella sp. 5		Washington	United States	Oceguera-Figueroa *et al.* (2010)	HQ179853
Helobdella sp. 5			United States	Deiner *et al.* (2013)	KF000141
Helobdella sp. 5			United States	Deiner *et al.* (2013)	KF000158
Helobdella sp. 6		Patricia Lake, Alberta	Canada		KM612015
Helobdella sp. 6		Columbus, Ohio	United States	Siddal & Borda (2003)	AF329040
Helobdella sp. 6		Moon Lake, Manitoba	Canada		KM611981
Helobdella temiscoensis	Salas-Montiel et al, 2014	Temixco, Morelos	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179862
Helobdella temiscoensis	Salas-Montiel at al., 2014	Temixco, Morelos	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179861
Haementeria lopezi	Oceguera-Figueroa, 2006	Jalisco	Mexico	Oceguera-Figueroa *et al.* (2012)	JN850919

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TABLE 2. (Continued)

Species name	Authorship of name	Locality	Country	Reference	COI
Helobdella europaea	Kutschera, 1987	Castellon, Valencia	Spain	Reyes-Prieto *et al.* (2014)	KC904243
Helobdella fusca	(Castle, 1900)	Douglas Lake, Michigan	United States	Siddal & Borda (2003)	AF329038
Helobdella elongata	(Castle, 1900)	Michigan	United States	Siddal & Borda (2003)	AF329045
Helobdella elongata	(Castle, 1900)	Jalisco	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179863
Helobdella lineata	(Verrill, 1874)	Douglas Lake, Michigan	United States	Siddal & Borda (2003)	AF329039
Helobdella melananus	Lai, Chang & Chen, 2009	Taipei	Taiwan	Lai *et al.* (2009)	FJ000353
Helobdella michaelseni	Blanchard, 1900		Chile	Siddal & Borda (2003)	AF536824
Helobdella papilata	(Moore, 1952)	Lake Huron, Michigan	United States	Siddal & Borda (2003)	AF329042
Helobdella paranensis	(Oka, 1930)	Arroyo Aspinas	Uruguay	Siddal & Borda (2003)	AF329037
Helobdella pichipanan	Siddall & Borda, 2004	Lago Chico	Chile	Siddal *et al.* (2005)	AY962456
Helobdella robusta	Shankland, Bissen & Weisblat, 1992	Sacramento, California	United States	Bely & Weisblat (2006)	DQ995301
Helobdella robusta	Shankland, Bissen & Weisblat, 1992	Sacramento, California	United States	Bely & Weisblat (2006)	DQ995302
Helobdella simplex	(Moore, 1911)			Moser *et al.* (2013)	KF683197
Helobdella socimulcensis	(Caballero, 1931)	Guanajuato	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179870
Helobdella Sorojchi	Siddall, 2001	Madidi	Bolivia	Siddal & Borda (2003)	AF329050
Helobdella sp. 1		San Luis Potosí	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179865
Helobdella transversa	Sawyer, 1972	Cheboygan State Park, Michigan	United States	Siddal & Borda (2003)	AF329044
Helobdella triserialis	Blanchard, 1849	Laguna Volcan	Bolivia	Siddal & Borda (2003)	AF329054
Helobdella virginiae	Oceguera-Figueroa, 2007	Catemaco, Veracruz	Mexico	Oceguera-Figueroa *et al.* (2010)	HQ179864

GAMMA shape categories (raxmlHPC-HYBRID -T 4 -n Helobdella_stagnalis -s infile.txt -m GTRGAMMA -p 12345 -q part.txt -f a -N 1000 -x 12345). Likelihood bootstrap support (LBS) was estimated using 1000 replicates of the rapid bootstrap algorithm with the same settings as above. Following previous phylogenetic analyses (*e.g.* Oceguera-Figueroa 2012), *Haementeria lopezi* Oceguera-Figueroa, 2006 was used to root all trees.

In addition, uncorrected *p*-distances between COI sequences for all taxa were calculated in MEGA ver. 7.0.21 (Kumar *et al.* 2016) with complete deletion of missing data, uniform rates among sites, and using the bootstrap method to estimate standard errors. For the sake of estimating intraspecific versus interspecific divergences, sequences were placed in putative species groups in accordance with the clades recovered by the phylogenetic analyses, and average distances were calculated.

Taxonomy

Helobdella stagnalis (Linnaeus, 1758)

(Figures 1, 2)

(Synonyms modified from Harding 1930)
Hirudo stagnalis Linnaeus, 1758; Pennant *et al.* 1768; Turton 1807; Dalyell 1853.
Glossophora stagnalis (Linnaeus): Johnson 1817.
Glossiphonia stagnalis (Linnaeus): Blanchard 1894; Scharff 1898; Evans 1905.
Erpobdella stagnalis (Linnaeus): Templeton 1936.
Hirudo bioculata Bergman, 1757; Müller 1774; Müller 1776; Carena 1820.
Clepsine bioculata (Bergman): Savigny 1809; Carena 1820.
Erpobdella bioculata (Bergman): Lamarck 1838.
Glossophora bioculata (Bergman): Johnson 1817.
Helluo bioculata (Bergman): Oken 1815.
Helobdella bioculata (Bergman): Bayer 1898.
?*Hirudo puligera*: Daudin, 1800.
?*Hirudo stagnorum*: Derheims, 1825.
?*Clepsine sowerbyi*: Monquin-Tandon, 1846.
?*Glossiphonia circulans*: Monquin-Tandon, 1846.

Neotype. SMNH Type-9177, fixed and stored in 95% ethanol, dissected, collected in October 2016 from north side of Lake Trehörningen (59 50.600'N; 17 52.971'E), Uppsala, Uppsala County, Uppland province, Sweden.

Other material examined. One non-dissected specimen (ROMIZ I11708) and one dissected specimen (ROMIZ I11710) from same locality and date as the neotype.

Diagnosis. One pair of circular or semicircular shaped eyespots; dorsal nuchal scute at 12/13, VIII a2/a3; no metameric pigmentation; papillae, sensillae, tubercles all absent; gonopores separated by one primary annulus, male gonopore at XII a1/a2, female gonopore at XII a2/a3; mid-body somites triannulate (a1+a2+a3) on both dorsum and venter; diffuse salivary glands; 5–6 pairs of digitiform crop caeca, last pair forming post caeca; 6 pairs of testisacs at interganglionic intervals.

Body shape. Length 4.8–5 mm; maximum width 1.7–1.8 mm; body sublanceolate (Fig. 1A, B), dorsoventrally flattened; anterior sucker well developed, somewhat triangular with the mouth pore located on the anterior border; head somewhat triangular; posterior sucker circular, ventrally directed, not pedunculated, maximum width ±1.4 mm.

External morphology. Body yellowish, with scattered black chromatophores, no metameric pigmentation on either dorsum or venter; dorsal pair of paramedial lines; papillae and sensillae absent (Fig. 1A–C); one pair of eyespots on somite III (Fig. 1E), circular or semicircular in shape, well-separated from each other; dorsal nuchal scute at VIII a2/a3, oval (Fig. 1F); male gonopore at XII a1/a2, female gonopore at XII a2/a3, separated by one annulus (Fig. 1E); somites I–III: uniannulate; somites IV–V: biannulate; somites VI–XXIII: triannulate, (a1+a2+a3) on both dorsum and venter; somites XXIV–XXV: biannulate; somites XXVI–XXVII: uniannulate; one post anal annulus.

Internal morphology. Pharynx eversible (proboscis in leech literature), J-shaped at base in fixed specimen, base at XIV; one pair of diffuse salivary glands extending from XII/XIII–XIV/XV (Fig. 2); linear salivary ducts, no descendant or ascendant portion, connected to base of pharynx at XIII/XIV; esophagus short, limited to XIV; 5 pairs of simple, digitiform crop caeca (Fig. 1D), first pair at XV, last pair forming post caeca from XIX extending to

XXIV; 4 pairs of simple intestinal caeca; male atrium located anterior to ganglia XII (Fig. 1B); atrial cornua laterally directed; ejaculatory ducts forming descendant and ascendant portions (Fig. 2), descendant portion restricted to XII–XIV, ascendant portion from XIV to XI/XII; 6 pairs of testisacs; first pair at XIII/XIV (Fig. 2), last pair at XVIII/XIX; simple ovisacs extending from XII to XV (Fig. 1B).

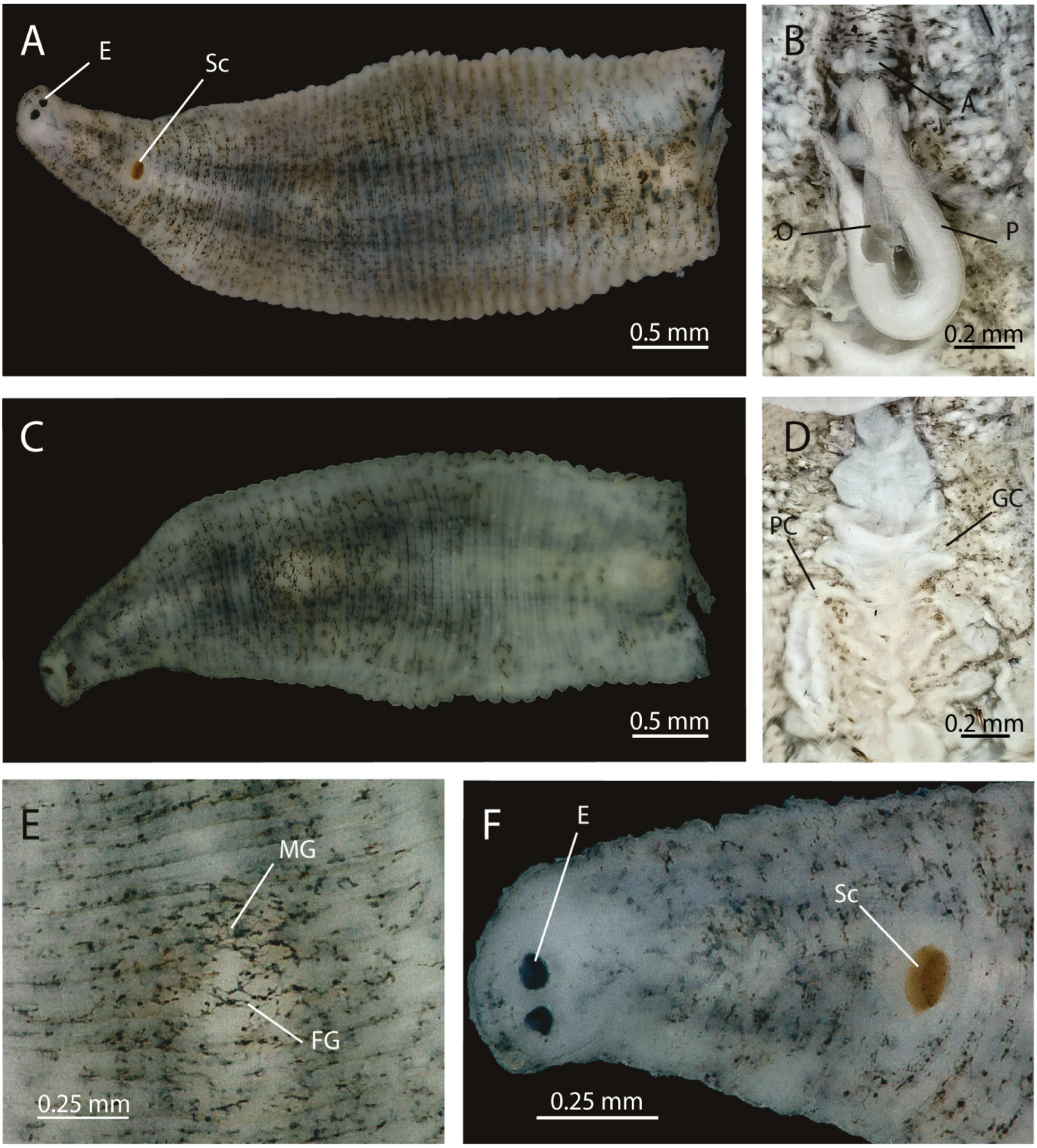

FIGURE 1. ***Helobdella stagnalis*** **(Linnaeus, 1758).** Neotype (SMNH Type-9177) and non-type (ROMIZ I11710). **A.** Dorsal view (SMNH Type-9177). **B.** Dissection of the genital region (ROMIZ I11710). **C.** Ventral view (SMNH Type-9177). **D.** Dissection of median region of the body (ROMIZ I11710). **E.** Ventral surface of the genital region (SMNH Type-9177). **F.** Dorsal view of the nuchal region (SMNH Type-9177). Abbreviations: A, atrium; E, eyespots; FG, female gonopore; GC, gastric caeca; MG, male gonopore; O, ovisacs; P, pharynx; PC, post caeca; Sc, nuchal scute.

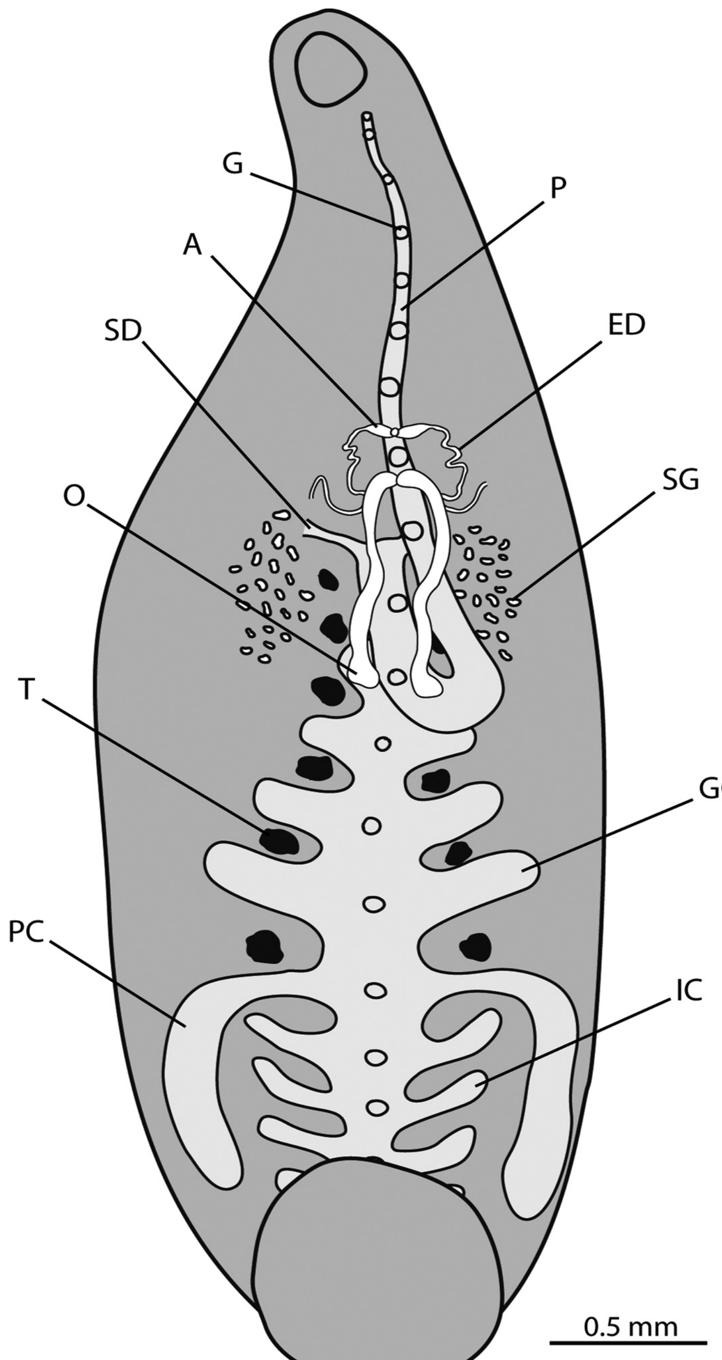

FIGURE 2. *Helobdella stagnalis* (Linnaeus, 1758). Internal morphology based on a specimen from the type locality (ROMIZ I11710). Abbreviations: A, atrium; ED, ejaculatory ducts; G, ganglia; GC, gastric caeca; IC, intestinal caeca; O, ovisacs; P, pharynx; PC, post caeca; SD, salivary ducts; SG, salivary glands; T, testisacs.

Remarks. Linnaeus did not assign type specimens for *H. stagnalis*, nor did he indicate the type locality. Kvist *et al.* (2010), in their designation of a neotype for *Hirudo medicinalis* (Linnaeus, 1758), assumed that specimens were collected from Uppsala, Sweden, based on Linnaeus's residency and intense research activity in the area (Reid 2009). Following this assumption, we herein infer the type locality for *H. stagnalis* to be Uppsala, Sweden as required by article 75.3.6 of the ICZN (ICZN 1999). In his description of *H. stagnalis,* Linnaeus mentions only a few, non-diagnostic characters such as body shape and color. According to Linnaeus (1758), this species is flat, blackish and the abdomen is greyish. Although subsequent records of *H. stagnalis* included information on size, pigmentation, and feeding behavior, the diagnostic character for the species was, for a long time, the presence of only two eyespots (Dalyell 1853; Johnson 1817b; Müller 1774, 1776; Turton 1807). The chitinous scute was first described by Savigny (1809), and was rarely incorporated into *H. stagnalis* redescriptions until the late 1800s (Blanchard 1894; Monquin-Tandon 1846). The material that formed the basis for these redescriptions cannot be compared to our recently collected samples. Nonetheless, the neotype of *H. stagnalis* designated here possesses two eyespots and a chitinous scute, the two diagnostic features used since Blanchard (1894) and Monquin-Tandon (1846), that differentiates this specimen from all other leech species recorded in Europe, as required by article 75.3.5 of the ICZN (ICZN 1999).

In overall internal and external appearance, our specimens resemble those described by Saglam *et al*. (2018) as *Helobdella stagnalis* collected in Europe. However, our specimens differ minutely in the number of crop caeca and the proportional size of the ejaculatory ducts and ovisacs. Similar intraspecific variation is well documented in other species of the genus (Shankland *et al.* 1992; Iwama & Arruda 2016). Another important difference is the J-shape formed by the base of the pharynx in the neotype specimen. This characteristic is not usually reported by other studies and might be due to differences in fixation methods. Several scute-bearing species of *Helobdella* can easily be confused with *H. stagnalis s.str.*. For example, *Helobdella octatestisaca* is only differentiated from *H. stagnalis s.str.* by the number of testisacs (*H. stagnalis* = 6 pairs, *H. octotestisaca* = 4 pairs) (Lai *et al.* 2009). Further, some morphological differences between *Helobdella temiscoensis* Salas-Montiel, Phillips, Pérez-Ponce de León & Oceguera-Figueroa, 2014 and specimens of *H. stagnalis s.str.* include the proportional size of the ejaculatory ducts, extending from XVII to XII in *H. temiscoensis* (Salas-Montiel *et al.* 2014); note that Saglam *et al.* (2018) suggest that some specimens of *H. stagnalis* possess ejaculatory ducts of similar proportion to *H. temiscoensis*. Furthermore, both atrial cornuae are anteriorly directed in *H. temiscoensis*, whereas in *H. stagnalis s.str.* these structures are laterally directed. Also, the nuchal scute is triangular in *H. temiscoensis* and ovoid in *H. stagnalis s.str.*. By contrast, *Helobdella atli* Oceguera-Figueroa & Len-Rgagnon, 2005; *Helobdella serendipitous* Saglam, Kutschera, Sauders, Saidel, Balombini & Shain, 2018; and *Helobdella californica* Kutschera, 1988 are easily differentiated from *H. stagnalis* by their absence of post caeca (Kutschera 1988; Oceguera-Figueroa & León-Règagnon 2005), contrary to the well developed post caeca of *H. stagnalis s.str.*. Also, *Helobdella bowermani* Moser, Fend, Richardson, Hammond, Lazo-Wasem, Govedich & Gullo, 2013 presents both external and internal differences when compared to *H. stagnalis s.str.*: for example, while no dorsal papillation is present in *H. stagnalis s.str.*, a single dorsal median longitudinal row of papillae is present in *H. bowermani* (Moser *et al.* 2013). The detailed morphological analysis of *Helobdella modesta,* performed by Saglam *et al.* (2018), suggests that this species differs from *H. stagnalis* in the shape of the crop caeca: the crop caeca are digitiform in *H. stagnalis s.str.,* but exhibit an anteriorly directed tip in *H. modesta*. Saglam *et al.* (2018) also suggest that there is variation in the position of gonopores between scute-bearing species. In *H. modesta,* gonopores are located at XI a2/a3 and XI a3/XIIa1, and the same structures are present at XII a1/a2 and XII a2/a3 in *H. stagnalis s.str.*. Moreover, the positioning of the male and female gonopores exhibited by *H. modesta* is also shared by *Helobdella serendipitous* Saglam, Kutschera, Saunders, Saidel, Balombini & Shain, 2018, *Helobdella eriensis* Saglam, Kutschera, Saunders, Saidel, Balombini & Shain, 2018 and *Helobdella echoensis* Saglam, Kutschera, Saunders, Saidel, Balombini & Shain, 2018 (see Saglam *et al.* 2018). The 6th pair of gastric caeca in *H. serendipitous* does not form post caeca, as in *H. stagnalis s.str.*. The shape of the nuchal scute differs slightly between *H. stagnalis s.str.*, *H. serendipitous,* and *H. echoensis*: the nuchal scute is ovoid in *H. stagnalis*, is pentagonal in *H. serendipitous*, and forms two lobes in *H. eriensis* (Saglam *et al.* 2018).

Phylogenetic analyses and COI distances

The final alignment included 658 aligned sites and was devoid of internal gaps. The maximum likelihood analysis resulted in a tree with a log likelihood of -100106.195003 (Fig. 3). Parsimony analysis resulted in 174 most parsimonious trees with 2,184 steps each (Appendix). By and large, the topologies for the trees are similar, with some minor exceptions.

Scute-bearing specimens from Sweden, Norway, Iceland, England, France, Italy, Slovenia, Turkey, Russia, and Iran form a clade (*H. stagnalis s.str.*) with high bootstrap support in both parsimony and ML analyses (100% PBS, 91% LBS). In both analyses (Fig. 3, Appendix), this clade places as the sister group to a clade comprised of two North American lineages (92% LBS, 97% PBS). The latter clade (monophyletic, but poorly supported: PBS and LBS <75%) consists of the recently described *H. echoensis* (monophyletic with 100% PBS, 92% LBS) and an

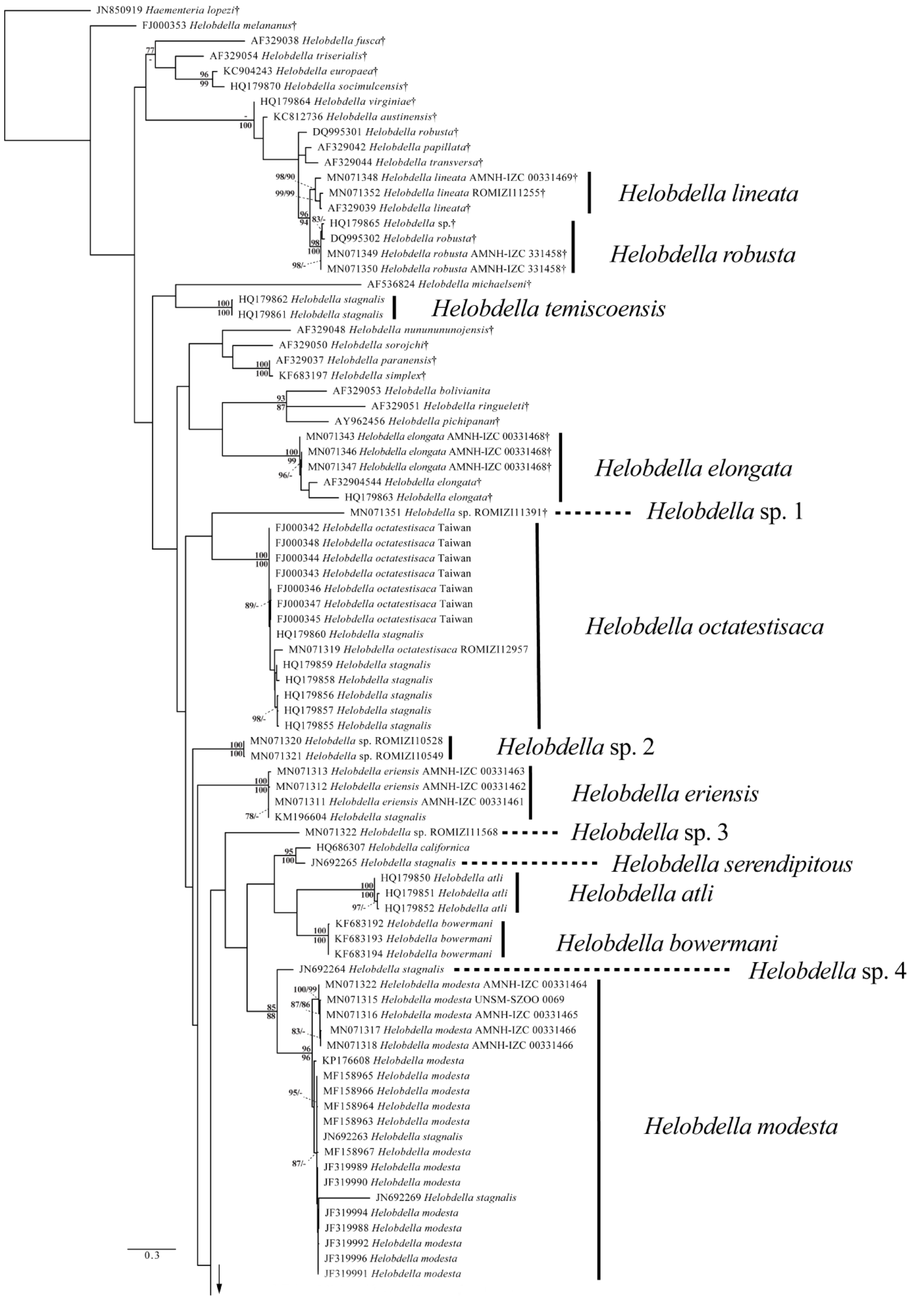

FIGURE 3. Best scoring maximum likelihood tree recovered from the analysis of the COI alignment (*lnL* = -100106.195003). Likelihood bootstrap support values and parsimony bootstrap support values are denoted above and below each node, respectively. Branches are drawn proportionate to the amount of change and GenBank accession numbers are noted before each taxon. Taxon names on tip labels of GenBank sequences are based on identifications made by previous studies. The neotype specimen of *H. stagnalis* is indicated in bold. Dagger (†) indicates non-scute-bearing specimens.

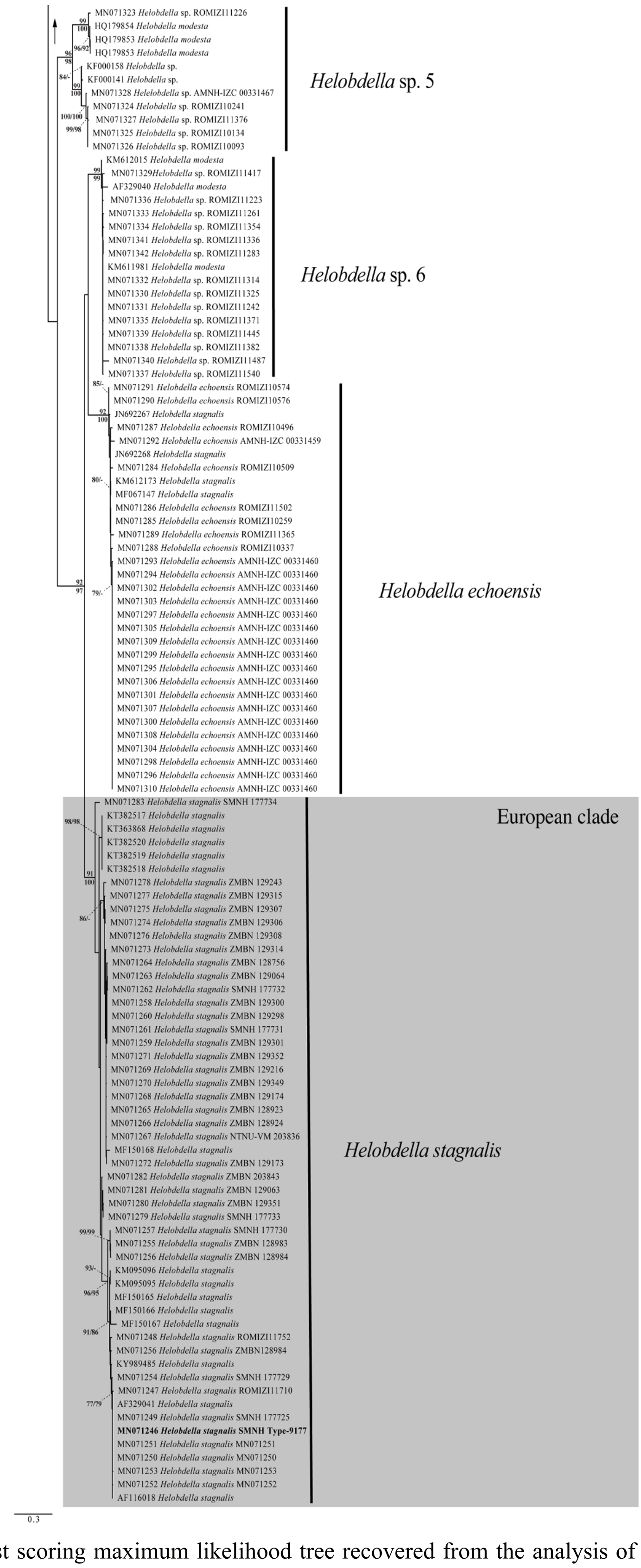

FIGURE 3 (Continued). Best scoring maximum likelihood tree recovered from the analysis of the COI alignment (*lnL* = -100106.195003). Likelihood bootstrap support values and parsimony bootstrap support values are denoted above and below each node, respectively. Branches are drawn proportionate to the amount of change and GenBank accession numbers are noted before each taxon. Taxon names on tip labels of GenBank sequences are based on identifications made by previous studies. The neotype specimen of *H. stagnalis* is indicated in bold. Dagger (†) indicates non-scute-bearing specimens.

undescribed species (*Helobdella* sp. 6; monophyletic with 99% PBS and LBS). The *H. echoensis* clade includes specimens from the midwest region of the U.S., as well as the Canadian provinces of Alberta, Manitoba, and Ontario. The clade comprising *Helobdella* sp. 6 consists of leeches from Ohio (USA) and various localities throughout Canada. Species-level clades of scute-bearing taxa are generally well supported in our analyses, except for that of *H. californica* and *H. serendipitous*. These two species together form a clade with high support values (98% LBS, 96% PBS), but with short branch lengths. However, the small sample size included in our analyses prevents any conclusion about the taxonomic status of these two species. Additionally, five other undescribed species (*Helobdella* spp. 1–5) are present in our analyses with high species-level clade support (>75% PBS and LBS). The monophyly of scute-bearing *Helobdella* species is not supported by the present study, due to the placement of seven non-scute-bearing species within the scute-bearing taxa: *Helobdella michaelseni* Blanchard, 1900; *Helobdella nununununojensis* Siddall, 2001a; *Helobdella sorojchi* Siddall, 2001a *Helobdella paranensis* (Oka, 1930); *Helobdella simplex* (Moore, 1911); *Helobdella elongata* Castle, 1900; and *Helobdella* sp. 1.

The average uncorrected *p*-distance and standard error for the COI locus between *H. stagnalis s.str.* and *Helobdella* sp. 6 + *H. echoensis* is 8.3% ±1.1. For comparison with previous estimations (Siddall & Borda 2003; Moser *et al.* 2011), the average COI distance between *H. stagnalis s.str.* and *H. modesta s.str.* is 18.2% ±1.6.

Discussion

By combining broad geographic sampling of scute-bearing *Helobdella* specimens from across Europe and North America with previously generated sequences from European, North American, South American, and Asian specimens, we corroborate the finding of Saglam *et al.* (2018) that *H. stagnalis* is not present in North America. Our analyses demonstrate that European and west Asian specimens of scute-bearing leeches in the genus *Helobdella* form a clade (*H. stagnalis s. str*. clade), which includes individuals from the inferred type locality of *H. stagnalis*—see also Kvist *et al.* (2010) for discussion on the inference of type locality for *Hirudo medicinalis* (Linnaeus, 1758). Several studies have found high genetic variation between specimens previously identified as *H. stagnalis* from Europe and North America, but the identity of *H. stagnalis s. str.* could not be unambiguously determined, since all previous studies lacked analyses of type material (Siddall & Borda 2003; Moser *et al.* 2011; Saglam *et al.* 2018). The present phylogenetic analyses demonstrate that many records in the literature and public databases given as "*H. stagnalis*", in reality refer to other taxa. The redescription in the present study, and the assignment of a COI-barcoded neotype for *H. stagnalis s. str.*, will not only ameliorate the taxonomic confusion regarding scute-bearing species from these continents, but also add stability to future taxonomic studies of scute-bearing leeches of this genus that are not well morphologically differentiated. Our analyses also show that, despite the efforts of Saglam *et al.* (2018), at least four lineages (*Helobdella* sp. 2, *H.* sp. 3, *H.* sp. 5 and *H.* sp. 6) of North American, scute-bearing *Helobdella* lack formal descriptions. The identity of *Helobdella* sp. 4 is less clear: although it is situated on a relatively long branch, it is possible that it falls within a standard range of intraspecific genetic variation when compared to *Helobdella modesta* (Fig. 3).

Previous studies have recorded *H. stagnalis* from several countries, with identifications relying heavily on the presence of a nuchal scute (Moore 1899; Šapkarev 1970; Chandra & Mahajan 1976; Ringuelet 1985; Arslan & Oktener 2012). *Helobdella modesta,* also described based solely on the presence of a nuchal scute, was described in 1872 from North American samples (Verrill 1872), and this species was later synonymized with *H. stagnalis* (Moore 1899). However, recent studies have demonstrated distinct genetic differentiation between scute-bearing specimens from Europe and North America. Siddall and Borda (2003) recovered 8% and 10.3% uncorrected *p*-distance in the COI and ND1 loci, respectively, between scute-bearing specimens from the two continents. This was the basis for the authors' resurrection of the name *H. modesta* for scute-bearing species from North America. Later, Moser *et al.* (2011) found 16.6%–17.1% and 17.4%–20.9% genetic distance in COI and ND1, respectively, between specimens of *H. modesta* from the type locality (New Haven County, Connecticut, USA) and the scute-bearing specimens from Ohio, USA used by Siddall and Borda (2003) and Light and Siddall (1999). As a result of this considerable divergence, Saglam *et al.* (2018) erected a new species, *H. echoensis*, to accommodate the samples used by Siddall and Borda (2003) and Light and Siddall (1999), as well as those used in their own study. In addition, Saglam *et al.* (2018) described *H. eriensis* and *H. serendipitous* from North America, on the basis of the distinct molecular and morphological profiles of these species: COI distances exceeded 9% between each of these species and any other species in their dataset. *Helobdella stagnalis* has also been recorded from South America, and so has *Helobdella adiastola* Ringuelet, 1972, another scute-bearing species (Cordero 1937; Ringuelet 1972, 1985; Iwama & Arruda 2016). As neither *H. adiastola* nor *H. stagnalis* from South America was available for consideration in the present study, their taxonomic status is still unknown.

Despite the fact that, historically, most authors (Moore 1899, 1939; Verrill 1874) did not separate the various scute-bearing species from different geographical regions, recent morphological examinations have found that many lineages show differences in the position of gonopores, in the shape and size of the chitinous scute, and in the numbers of gastric caeca and testisacs. (Kutschera 1988; Oceguera-Figueroa & Leon-Regagnon 2005; Lai *et al.* 2009; Moser *et al.* 2013; Salas-Montiel *et al.* 2014; Beresic-Perrins *et al.* 2017; Saglam *et al.* 2018). Many of these morphological differences were established subsequent to the genetic differences that were used to flag the specimens as putatively different species (*e.g.* Siddall & Borda 2003; Oceguera-Figueroa *et al.* 2010). In addition to the species described by Saglam *et al.* (2018), other *H. stagnalis*-like species have been recently described, but these seem to possess morphological characters that set them apart from *H. stagnalis*. For example, *H. octatestisaca* is externally similar to *H. stagnalis* but, internally, this species possesses four pairs of testisacs instead of the five or six pairs possessed by other scute-bearing *Helobdella* species (Lai *et al.* 2009). Oceguera-Figueroa *et al.* (2010) found representatives of *H. octatestisaca* in Mexico and, based on the low genetic variation among individuals of the species (0.4%), suggested that it was recently introduced in both, Mexico and Taiwan from an unknown locality, probably from South Americia. This hypothesis was first proposed by Lai *et al.* (2009), as the authors noted that this species was not present in previous scientific collections or reports of the well-documented local fauna. To further illustrate the complication of identifying non-descript species of *Helobdella,* the Mexican scute-bearing species *Helobdella atli* was mistaken for *H. adiastola* by Ringuelet (1985), despite the fact that *H. atli* does not possess post caeca (Oceguera-Figueroa & Leon-Regagnon 2005; Salas-Montiel *et al.* 2014). *Helobdella temiscoensis* cannot be morphologically differentiated from many of the *H. stagnalis*-like species, but forms an independent clade in phylogenetic analyses (Oceguera-Figueroa *et al.* 2010; Salas-Montiel *et al.* 2014; Fig. 3); and *Helobdella californica* is only differentiated from its sister species, *H. serendipitous,* by the presence of one pair of dark longitudinal lines on the dorsal surface. To this end, the results presented here underscore the utility of molecular data for species-level identifications within *Helobdella*. For maximum rigor, future studies should encompass both morphological and molecular data to identify and circumscribe species of this largely non-descript, vexing genus.

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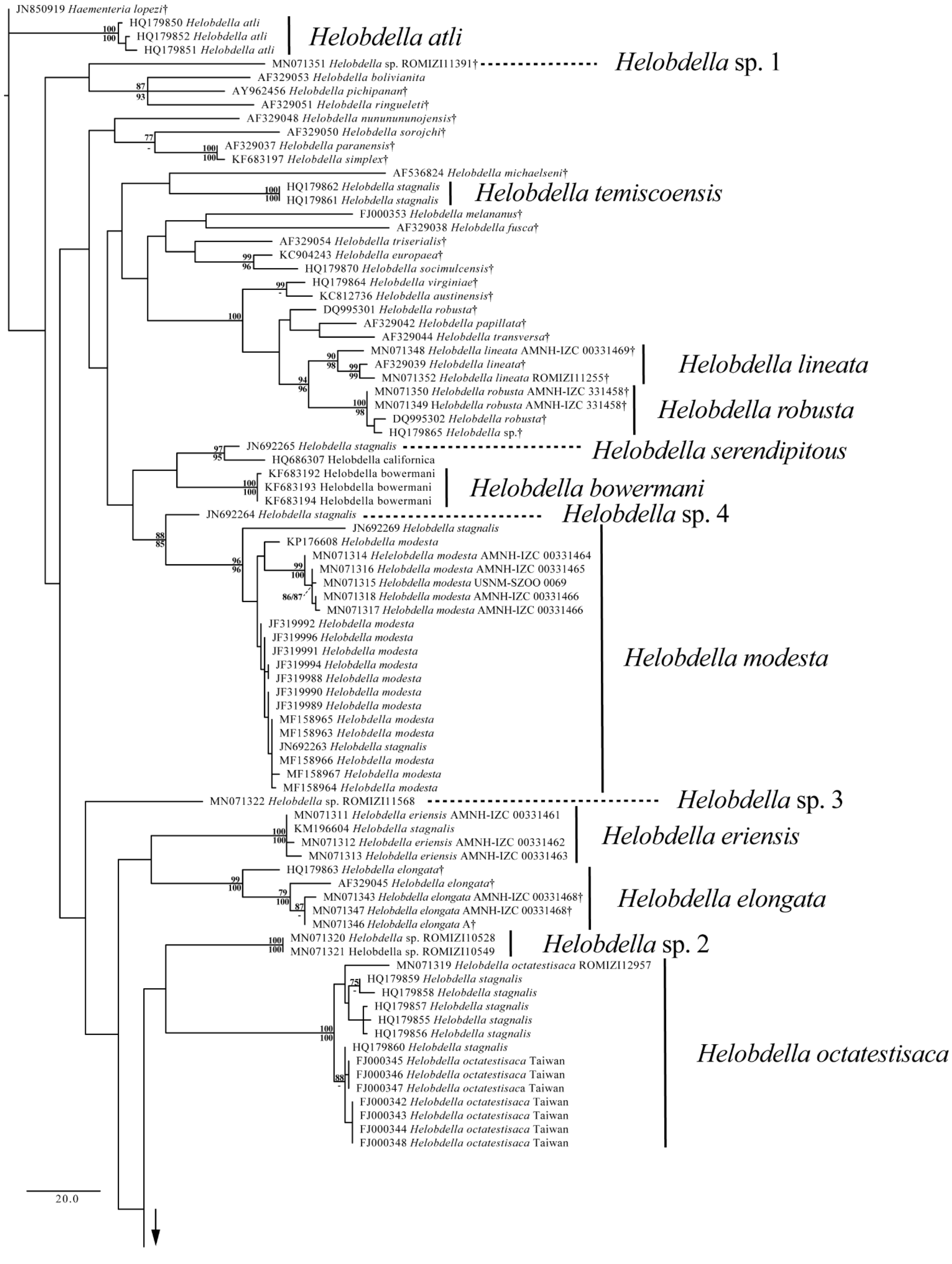
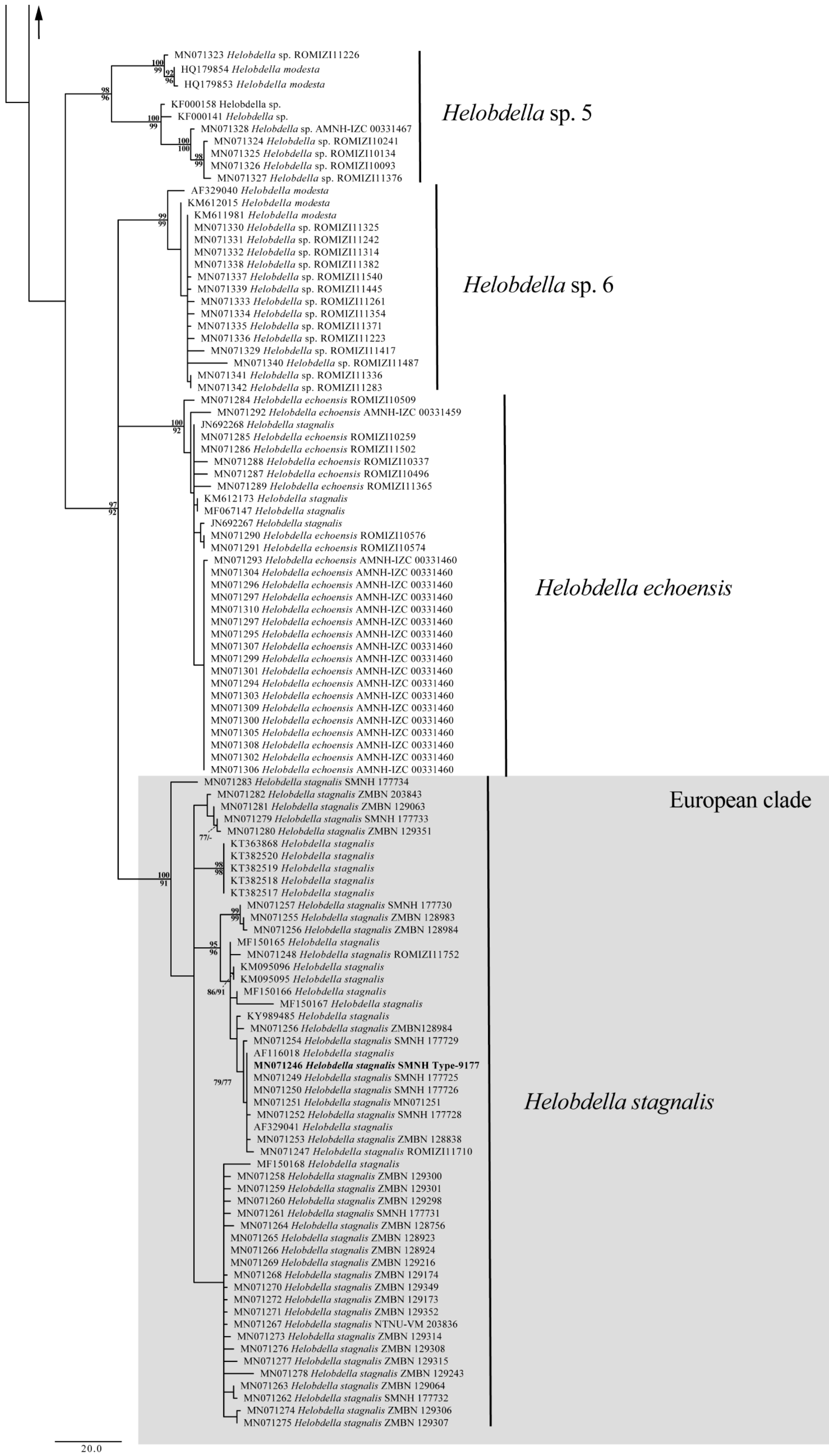


APPENDIX (Part A). Strict consensus of the most parsimonious trees, recovered from the analysis of the COI alignment (2,184 steps). Parsimony bootstrap support values and likelihood bootstrap support values are denoted above and below each node, respectively. Branches are drawn proportionate to the amount of change and GenBank accession number are noted before each taxon. Taxon names on tip labels of GenBank sequences are based on identifications made by previous studies. Neotype specimen of *H. stagnalis* is indicated in bold. Dagger (†) indicates non-scute-bearing specimens.



APPENDIX (Part B). Strict consensus of the most parsimonious trees, recovered from the analysis of the COI alignment (2,184 steps). Parsimony bootstrap support values and likelihood bootstrap support values are denoted above and below each node, respectively. Branches are drawn proportionate to the amount of change and GenBank accession number are noted before each taxon. Taxon names on tip labels of GenBank sequences are based on identifications made by previous studies. Neotype specimen of *H. stagnalis* is indicated in bold. Dagger (†) indicates non-scute-bearing specimens.