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Phylogenetic and morphological resolution of the *Helobdella stagnalis* species-complex (Annelida: Clitellata: Hirudinea)

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Abstract

The glossiphoniid freshwater leech, *Helobdella stagnalis,* was described by Linnaeus 1758 based on common European specimens. The presence of a brown, chitinous scute on the dorsal-anterior surface, as observed on leeches elsewhere in the world, has generally led to the classification of all scute-bearing members of the genus as *H. stagnalis*. Here we describe the morphology and behavior of the type species from Europe, and analyze *H. stagnalis*-like specimens collected worldwide. We present evidence for at least four distinct scute-bearing *Helobdella* species that can be morphologically resolved. Maximum Parsimony (MP) and Bayesian Inference (BI) analyses at the mitochondrial cytochrome *c* oxidase subunit I (COI) locus further supported this notion, with divergence values suggesting a mid-Miocene ancestor of this successful group of cosmopolitan hirudineans. New species, *Helobdella echoensis* **nov. sp.**, *Helobdella eriensis* **nov. sp.**, and *Helobdella serendipitious* **nov. sp.**, are described, based on morphological, anatomical and molecular data. Current distribution patterns of *Helobdella* spp. suggest a robust, global dispersal mechanism, as well as local pockets of endemism.

Key words: leech, Hirudinea, scute, COI

Introduction

The genus of glossiphoniid leech, *Helobdella* (Blanchard 1896), includes >80 freshwater species, more than 45 of which have been described from South America, 12 from North America, and two each from Europe, Africa and Asia (Christoffersen 2009; Lai *et al*. 2009; Moser *et al*. 2013; Kutschera *et al*. 2013; Salas-Montıel *et al*. 2014; Kutschera and Weisblat 2015). Species of *Helobdella* (e.g., *robusta, austinensis)* offer particularly good experimental systems for cell biology and developmental investigations due to their large, robust embryos, which develop rapidly and can be cultured independently from the parent leech (unlike species of *Hirudo;* see Kuo 2009 for review).

Linnaeus (1758) classified a "two-eyed flat leech" found in freshwater ecosystems on every continent, except Antarctica, as *Helobdella stagnalis,* based primarily on a characteristic mound *(aka* scute) found on the dorsal aspect of the 12th and 13th annuli. Thereafter, scute-bearing leeches have routinely been classified as *H. stagnalis,* without fully considering other morphological features, and more recently, DNA phylogeny. *Helobdella stagnalis*-like leeches are relatively small (<2 cm) and dorsoventrally flattened. They have been found abundantly throughout the northern half of the continental United States, Alaska and Canada (Klemm 1982; Sawyer 1986; Chordas *et al*. 1996; Moser *et al*. 2006; Kutschera 2011). Many countries in Europe, Asia, North and South America, and Africa report *H. stagnalis* specimens (Moore 1939; Saglam and Dorucu 2002; Oosthuizen and Siddall 2003; Pfeiffer *et al*. 2004; Oceguera-Figueroa *et al*. 2010b; Gouda 2013; Ben Ahmed *et al*. 2015 a,b). They are typically found under stones, especially in shallow, stagnant and slowly running water, and feed primarily on oligochaites (*Tubifex* sp.), insect larvae (*Chironomus* sp.), and water snails (*Lymnaea* sp.) (Young *et al*. 1993). Tiberti and Gentilli (2010)

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described the first case of parasitism on an anuran amphibian (*Rana temporaria*) by the freshwater leech *H. stagnalis*, in a mountainous area of northwestern Italy.

It has been suggested that *Helobdella modesta* (Verrill, 1872), a nearly indistinguishable leech, should be synonymized under *H. stagnalis* (Klemm 1972, 1982; Sawyer 1986). Later investigations found significant genetic variation between *H. stagnalis* collected from the United Kingdom relative to those from North America, and re-established Verrill's name *Helobdella modesta* for North American species (Siddall *et al*. 2005). Subsequently, *H. modesta* has been defined from different localities of Washington State (Oceguera-Figueroa *et al*. 2010a) and Canada (Davy *et al*. 2009).

Recently, we isolated juvenile leeches contaminating a culture of the aquatic oligochaete, *Lumbriculus variegatus.* Morphological examinations identified the characteristic scute, which would typically mark them as *H. stagnalis.* DNA sequencing of the cyctochrome c oxidase subunit 1 (COI) locus, however, revealed >10% sequence divergence from the formally accepted *H. stagnalis* COI sequence from Europe (GenBank AF329041 and AF116018), suggesting a species-level divergence based on proposed bar-coding criteria (Bely and Weisblat 2006). These observations prompted us to explore further differences in these and related leeches. Here we report that leeches from various geographic regions, with diverse morphologies and divergent DNA sequences, all display scute-like structures in similar positions. We therefore propose that the complex of *H. stagnalis*-like leeches be recognized as separate taxa based on genetic and morphological criteria.

Materials and methods

Specimens. Leech specimens were collected from ponds at Park Schoenfeld, Kassel (Germany), Cooper River (Camden, NJ), the Settle Farm (Canyon Creek, MT), Roseburg Pond (Roseburg, OR), Hadden Lake (Camden, NJ), Echo Lake (Bangor, PA), Lake Redman (York, PA), Farm Pond, (Mt Bethel, PA), Silver Lake, (Gibbsboro, NJ), Greenwich Lake (Greenwich, NJ), Çınar, Çermik, Ergani (Diyarbakır, Turkey), Myvatn, (Iceland) and from a culture of *Lumbriculus variegatus* purchased at Value Pet in Camden, NJ (Table 1). *S*pecimens were transported to Rutgers University (Camden, NJ) or the University of Kassel (Germany), and maintained in glass containers with 0.2% (3g/15L) Instant Ocean© (Aquarium Systems) and non-ionic aquarium salt (1g/15L) at room temperature. Leeches were fed *ad-libitum* with live blackworms, *Lumbriculus variegatus, Chironomus*-larvae, and *Tubifex*-worms. Relevant leech specimens were formally deposited into the collections of the Academy of Natural Sciences, Philadelphia (ANSP), as described below.

Microscopy. External traits of live specimens were observed by stereomicroscopy using a MEIJI (EMZ-TR, Meiji Techno Co. Ltd.). Preserved specimens were dissected dorsally and ventrally, with representative sketches of internal morphology derived directly from the type specimens. For scanning electron microscopy (SEM), pecimens were fixed overnight at 4ºC in paraformaldehyde-picric acid-glutaraldehyde fixative (Ermark and Eakin, 1976), then rinsed several times in distilled water. Following dehydration in an ascending ethanol series, specimens were critical point dried with CO_2, mounted on aluminum stubs with carbon tape, and sputter coated with gold/palladium (Pelco SC-7). Specimens were viewed with a 1450EP SEM (LEO).

DNA extraction and polymerase chain reaction (PCR). Tissue samples (~30 mg) were excised from the caudal sucker of alcohol-preserved specimens with a razor blade. Genomic DNA was extracted using an E.Z.N.A. Tissue DNA Isolation Kit (Omega Bio-tek, Inc.), following the protocol for tissue. PCR reactions contained 1X PCR buffer (50 mM KCl; 10 mM Tris-HCl; 1.5 mM $MgCl_2$), 1.5 mM $MgCl_2$ (supplemental), 0.2 mM dNTPs, 100 pM primers, 1 U Phusion Hot Start II DNA Polymerase (Thermo Scientific), and 50–200 ng total genomic DNA. Gene fragments were PCR-amplified using standard primers (Suppl. Data, Table 1). PCR conditions for COI and 12S rRNA fragments were: 94º C for 1 min followed by 30 cycles of 94º C (30sec), 51º C (30 sec), 72º C (30 sec), and final extension at 72º C for 5 min. Conditions for 18S rRNA were identical, except annealing was at 55º C. Following PCR, samples were electrophoresed on a 1% agarose gel and bands were purified by GeneClean (MP Biomedicals, LLC). DNA sequencing was performed by Genewiz (South Plainfield, NJ) using primers employed for PCR reactions.

Gene alignment and annotation. Sequence data of all *Helobdella* samples were trimmed using BioEdit programe v7.2.5 software and Chromas 2.4.3 (Technelysium), aligned in Clustal-W and Omega (EMBL-EBI), and overlapping sequence fragments were assembled into contigs. Protein coding genes and ribosomal RNAs were

identified using NCBI's BLASTx and BLASTn algorithms, as well as by comparisons to RNA-seq extracted transcripts.

TABLE 1. Leech collection field sites.

Species	Location	Code Number	Coordinates	Elevation (meters)
Helobdella stagnalis L. 1758	Kassel, Germany	HS	51° 19'00" N–9° 30' 00" E	164
Helobdella modesta	Cooper River, NJ	CR	39°55'30.44"N–75°4'59.37"W	2
Helobdella modesta	Hadden Lake, NJ	HLHws1	39°53'8.72"N–75°5'2.83"W	122
Helobdella serendipitous **n. sp.**	Value Pet Rt. 130 Camden NJ	HS	-	-
Helobdella **n. sp.**	Settle Farm Canyon Creek, MT	MONT	46° 49' 52" N–112° 22' 37"W	-
Helobdella echoensis **n. sp.**	Roseburg, OR	OR	43°12'46.43"N–123°20'53.69"W	128
Helobdella echoensis **n. sp.**	Echo Lake, Bangor, PA	EL	40°54'10.45"N–75°8'54.33"W	165
Helobdella echoensis **n. sp.**	Lake Redman, York, PA	YorkPA	39°53'15.43"N–76°41'17.01"W	150
Helobdella **n. sp.**	Farm Pond, Mt. Bethel, PA	FP	40°55'44.40"N–75°10'29.42"W	248
Helobdella eriensis **n. sp.**	Farm Pond, Mt Bethel, PA	FPWS2	40°55'44.40"N–75°10'29.42"W	248
Helobdella modesta	Silver Lake, Gibbsboro, NJ	Sh1	39°50'25.35"N–74°57'42.11"W	29
Helobdella modesta	Silver Lake, Gibbsboro NJ	SH2	39°50'18.53"N–74°57'41.36" W	29
Helobdella modesta	Silver Lake, Gibbsboro NJ	SH3	39°50'17.91" N–74°57'48.84" W	29
Helobdella modesta	Greenwich Lake, Greenwich, NJ	Greenwich	39°48'32.77"N–75°17'13.10"W	0
Helobdella stagnalis	Çınar, Diyarbakır Turkey	12cTS	37°35'22.10"N–40°05'59.37"E	908
Helobdella stagnalis	Çermik, Diyarbakır Turkey	16dTS	37°57'47.14"N–39°19'59.87"E	931
Helobdella stagnalis	Ergani, Diyarbakır Turkey	17bTS	38°15'42.54"N–39°41'32.61"E	888
Helobdella stagnalis	Myvatn, Iceland	Iceland	65°38'21.63"N–16°55'1.00"W	278

Phylogenetic analyses. Individual gene sequences were aligned using Muscle (Edgar 2004) under default parameters, as implemented in Mega version 7 (Kumar *et al*. 2016), with protein coding genes (PGCs) aligned by codons. Statistical analyses to assess phylogenetic informativeness were performed in Mega. For concatenated sequence analyses, PGC stop codons were removed prior to alignment and sequences were joined in-frame. Phylogenetic reconstructions were carried out using Maximum Parsimony (MP) analyses and Bayesian inference (BI). Accession numbers and morphological criteria used in phylogenetic analyses are in Suppl. Data, Tables 3 and 4, respectively.

MP analyses were conducted for COI data. Heuristic searches used 100 replicates of random addition sequences and tree-bisection-reconnection (TBR) branch swapping. Bremer support and clade support using non-parametric bootstrapping with 100 replicates was determined with the Willi Henning Society Edition of Tree analysis using New Technology (TNT; Goloboff *et al*. 2008). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in Nei and Kumar 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Branch lengths were calculated using the average pathway method (Nei and Kumar 2000) and are the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated.

BI analyses were performed on the combined data set (morphological parameters, COI as Nexus format) in MrBayes v. 3.2.1x64 (Ronquist and Huelsenbeck 2003; Ronquist *et al*. 2011) using Bayes-block nexus files created in Mesquite v.3.04 (Maddison and Maddison 2015) from Mega files and morphological parameters of *Helobdella* spp. PartitionFinder v1.1.0 (Lanfear *et al*. 2012) was used to find the best-fit partitioning scheme for each dataset under the following criteria: branch lengths = linked, models = all, model selection = BIC, search = greedy. Protein-coding genes were defined by codon position; the concatenated datasets were further delimited by

individual genes. Each dataset was analyzed partitioned and unpartitioned, using model settings: nst = 6, rates = invgamma. For partitioned analyses, partition parameters were unlinked and the ratepr parameter of the prset command was set to variable to allow rates across partitions to differ. All analyses were run simultaneously with all parameter sets unlinked by partition for two million generations each, sampling every 1,000 generations, with a burn-in achieved by <1,000,000 generations. Setting the burn-in to 500,000 generations left a total of 7,500 trees sampled for assessment of posterior probabilities. Gaps were treated as missing data, and default settings were used for all other parameters.

Results

Reference species Helobdella stagnalis L. 1758 from Europe. About 80 living specimens of *H. stagnalis*, collected in a pond in Germany (Table 2), were maintained in aquaria and investigated. The morphology and pigmentation of these leeches was found to be rather uniform. A representative individual from this population is shown in Figure 1A, B. The leeches are agile predators that, when in search of food (or disturbed), move rapidly. A characteristic feature of *H. stagnalis* is the ability of the animal to contract and elongate, so that the body length can rapidly change by more than 100%. This agility may be interpreted as a co-evolved adaptation to catch highly mobile *Tubifex*-worms and *Chironomus*-larvae, the preferred prey organisms of this glossiphoniid leech. *Helobdella stagnalis* carries its eggs, larvae and juveniles attached to its body, and feeds its young via captured prey organisms. This complex mode of parental care (Fig. 1 A, B) is common for all members of the genus *Helobdella* thus investigated (Kutschera and Wirtz 2001; Kutschera and Weisblat 2015).

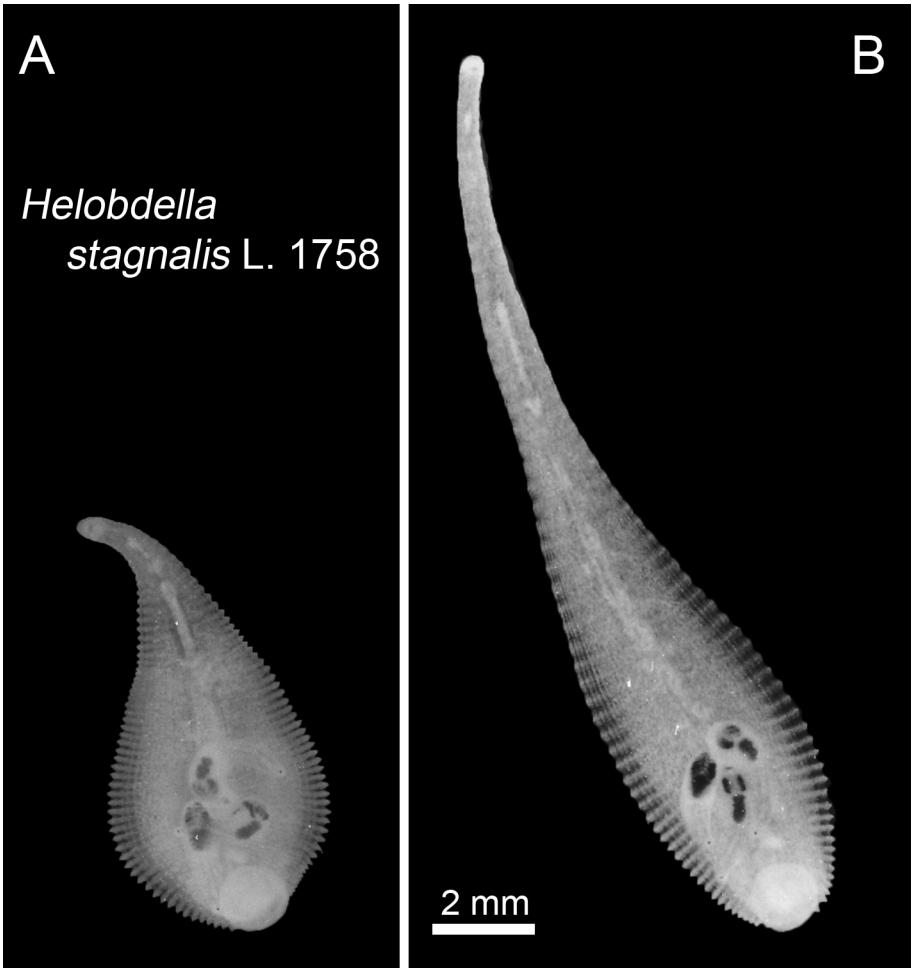

FIGURE 1. Adult specimen of *Helobdella stagnalis* L. 1758, in ventral view, collected in a pond in Kassel, Germany (Central Europe). The photograph shows the same animal in contracted (A) and fully expanded position (B). Note that the leech carries three juveniles attached to its venter. In contrast to the parent, the juvenile leeches have taken up haemolymph from a *Tubieix*-worm that was provided by the adult (feeding behavior).

TABLE 2. *Helobdella* n sp. ZooBank data.

Species	Locality	Accession No (CO1)	12S	Coordinates	Prior species designation	ZooBank Number	Catalogue number
Helobdella echoensis n. sp.	Roseburg, OR, USA	JN692266	MF158972	43°12'46.43"N - 123°20'53.69"W	*Helobdella stagnalis*	LSID: urn:lsid:zoobank.org:act:F55674B6-1767-4F64-ABEF-0347122944E0	
	Echo Lake, PA, USA	JN692267	MF158974	40°54'10.45"N - 75°8'54.33"W	*Helobdella stagnalis*		
	York, PA, USA	JN692268	-	39°53'15.43"N - 76°41'17.01"W	*Helobdella stagnalis*		Holotype: ANSP GI 19496
	Canada	KM612173	-	-	*Helobdella stagnalis*		
Helobdella eriensis n. sp.	FPWS2, Farm Pond, PA, USA	MF158968	-	40°55'44.40"N - 75°10'29.42"W	*Helobdella eriensis* n. sp.	LSID: urn:lsid:zoobank.org:act:3D71AC7D-7422-4767-9A66-A60EA8D0C2A4	Holotype: ANSP GI 19494
	Lake Erie, Toledo, OH, USA	KM196604	-	-	*Helobdella stagnalis*		
Helobdella n. sp.	MONT, Settle Farm Canyon Creek, MT, USA	JN692264	MF158973	46° 49' 52" N - 112° 22' 37"W	*Helobdella stagnalis*	LSID: urn:lsid:zoobank.org:act:72A1A5B1-D440-47C4-A517-0477352D5547	
Helobdella n. sp.	Farm Pond, PA, USA	JN692269	MF158975	40°55'44.40"N - 75°10'29.42"W	*Helobdella stagnalis*	LSID: urn:lsid:zoobank.org:act:91CD93B2-3B58-42C9-B640-9C92FD8827CC	
Helobdella serendipitous n. sp.	Pet shop, NJ, USA	JN692265	MF158971	-	*Helobdella serendipitous* n. sp.	LSID: urn:lsid:zoobank.org:act:D2A31CAB-F69D-413A-AD52-1497EB146753	Holotype: ANSP GI 19495
Helobdella stagnalis	Diyarbakir-Çınar, Turkey	MF150165	-	37°35'22.10"N - 40°05'59.37"E	*Helobdella stagnalis*	LSID: urn:lsid:zoobank.org:act:43012F1D-9824-451F-A839-1F287E01F2E2	Homeotype: ANSP GI 19493
Helobdella modesta	Cooper River @ Rt. 130 in Collingswood, NJ	JN692263	MF158970	39°55'30.44"N - 75°4'59.37"W	*Helobdella modesta*	LSID: urn:lsid:zoobank.org:act:FBFD74DE-57A7-4C98-8514-51559DD2270C	Homeotype: ANSP GI 19497

Morphotypes and their characteristic features. Specimens of *Helobdella* were collected from 16 geographic locations worldwide (Table 2). Leeches were typically found under submerged rocks along the banks of ponds or slow moving rivers. Specimens were scored for morphological characters according to Apakupakul *et al*. (1999), Siddall and Borda (2003), Borda and Siddall (2004a, b), Saglam and Dorucu (2002), Sawyer (1986) and Kutschera and Weisblat (2015) (Suppl. Data, Table 4), with reference to Linnaeus' original 1758-taxon (type species, *Helobdella stagnalis* L. 1758) (Fig. 1A, B).

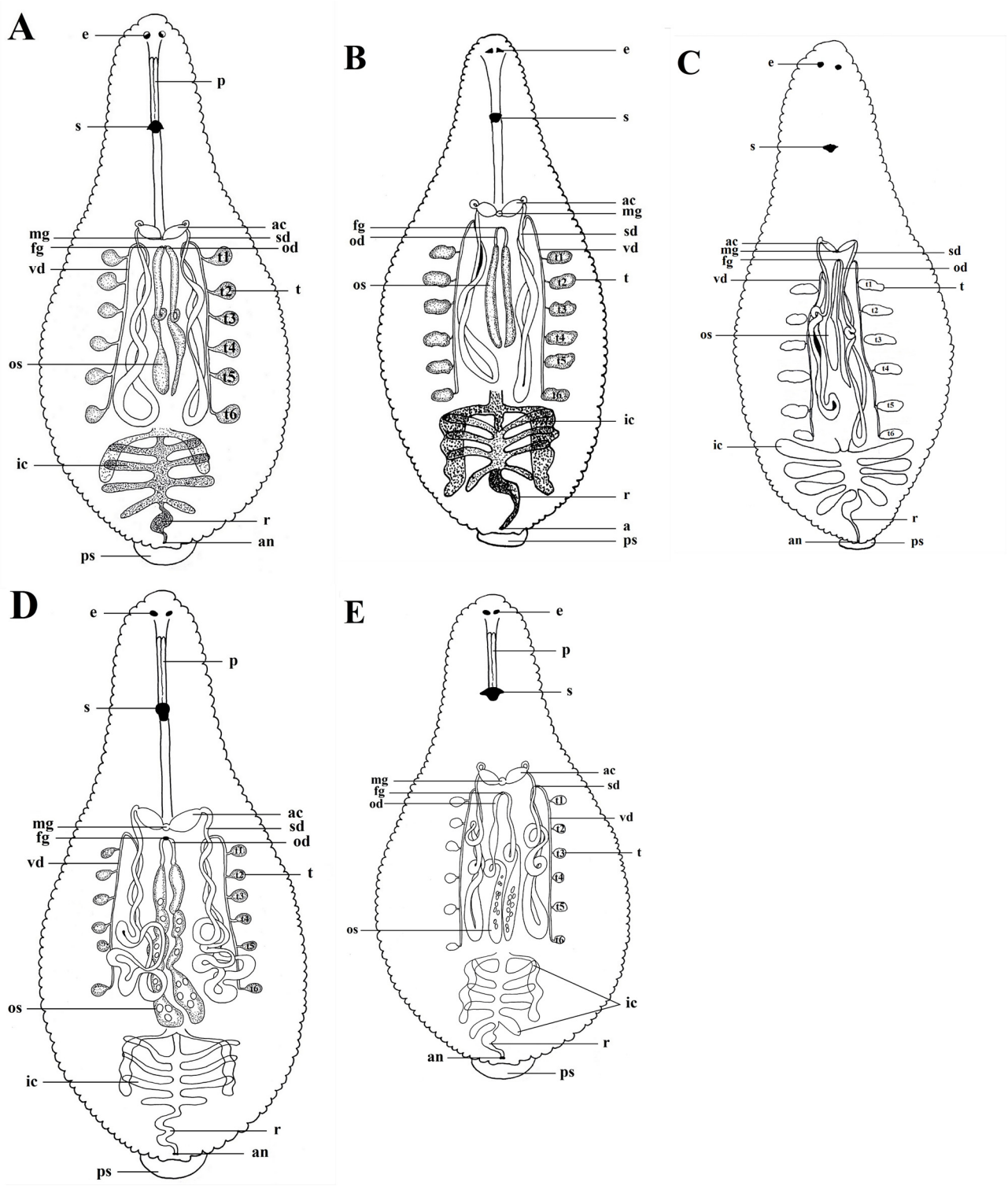

FIGURE 2. Position of reproductive morphology of *Helobdella* species. A, *Helobdella stagnalis*; B, *Helobdella modesta*; C, *Helobdella serendipitous* **n. sp.**; D, *Helobdella eriensis* **n. sp.**; E, *Helobdella echoensis* **n. sp.** ac: atrial cornua; an, anus; e, eyes; fg, female gonopore; ic, intestinal caecum; mg, male gonopore; od, oviduct; os: ovisac; ps, posterior sucker; r, rectum; s, scute; sd, sperm duct; t, testisac, vd. vas deferens.

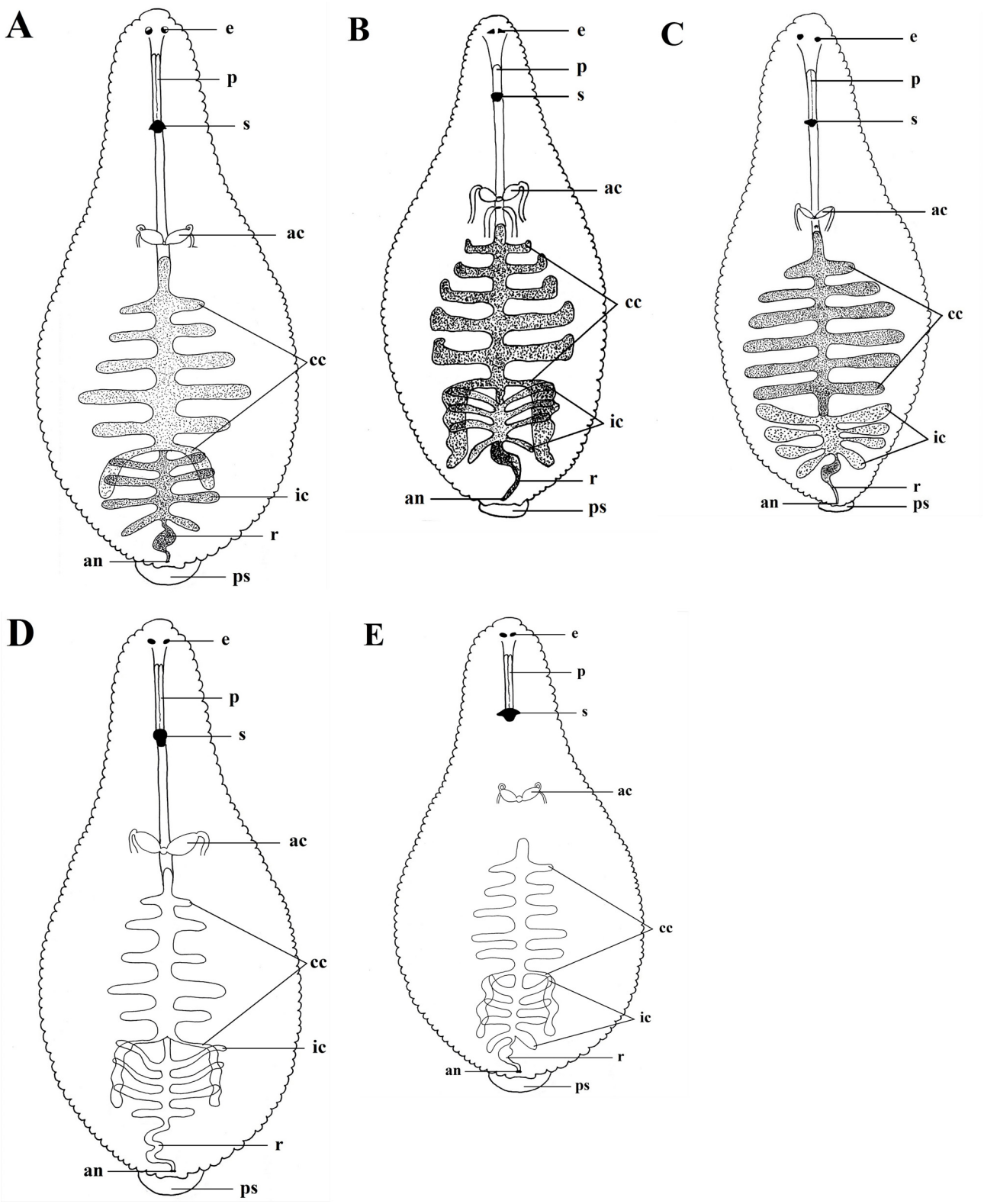

FIGURE 3. Position of digestive system of *Helobdella* species. A, *Helobdella stagnalis*; B, *Helobdella modesta*; C, *Helobdella serendipitous* **n. sp.**; D, *Helobdella eriensis* **n. sp.**; E, *Helobdella echoensis* **n. sp.** ac: atrial cornua; an, anus; cc, corp caecum ; e, eyes; ic, intestinal caecum; p, proboscis; ps, posterior sucker; r, rectum; s, scute.

In this study, five distinct morphotypes were identified, proposed here to comprise respective species-level differences. Distinguishing morphological features for each species include (Suppl. Data, Table 4):

Helobdella stagnalis (Figs. 1A, B; 2A–7A) (Catalogue number: ANSP GI 19493) (LSID:

urn:lsid:zoobank.org:act:43012F1D-9824-451F-A839-1F287E01F2E2)—Dorsal body light-grey or yellowish-grey, sometimes with a greenish tint. Dark spots/dots scattered all over the body, far less numerous along the dorsal median stripe than elsewhere. Thus, an irregular, bright longitudinal line apparent on the dorsal side. Ventral color of mixed creamy-yellow or transparent aureate. Total of 67 annuli. Segments I, II, III uniannulate, IV–V biannulate, mid-bodv segments (VI-XXIII) triannulate, XXIV-XXV biannulate, XXVI-XXVII uniannulate. A redish-brown scute (nuchal plate) settled in dorsal surface of segment VII-a1 (on annulus 12). One pair of rather large, half circular eyes clearly separated, situated on the third annulus (Fig. 7A). Male and female gonopores located between annulus a1/a2 and a2/a3 of XIIth segment, respectively; one annulus between gonopores. Clitellum on XIIth segment. Six pairs of orbicular testisacs intersegmentally arranged at XIII/XIV to XVIII. Ejaculatory bulbs (atrial cornua) open, short and cylindrical, Wide ovisacs, oviduct opens via gonopore into a chamber formed between annuli a2 and a3 of XIIth segment (Figs. 2A, 4A). Six pairs of crop caeca, last pair (6th) directed backwards and short, extends to third intestinal caecum. Four pairs intestinal caecum; first three straight and long, the fourth short and curved downward. Rectum is "S" shaped and short (Figs. 3A, 5A).

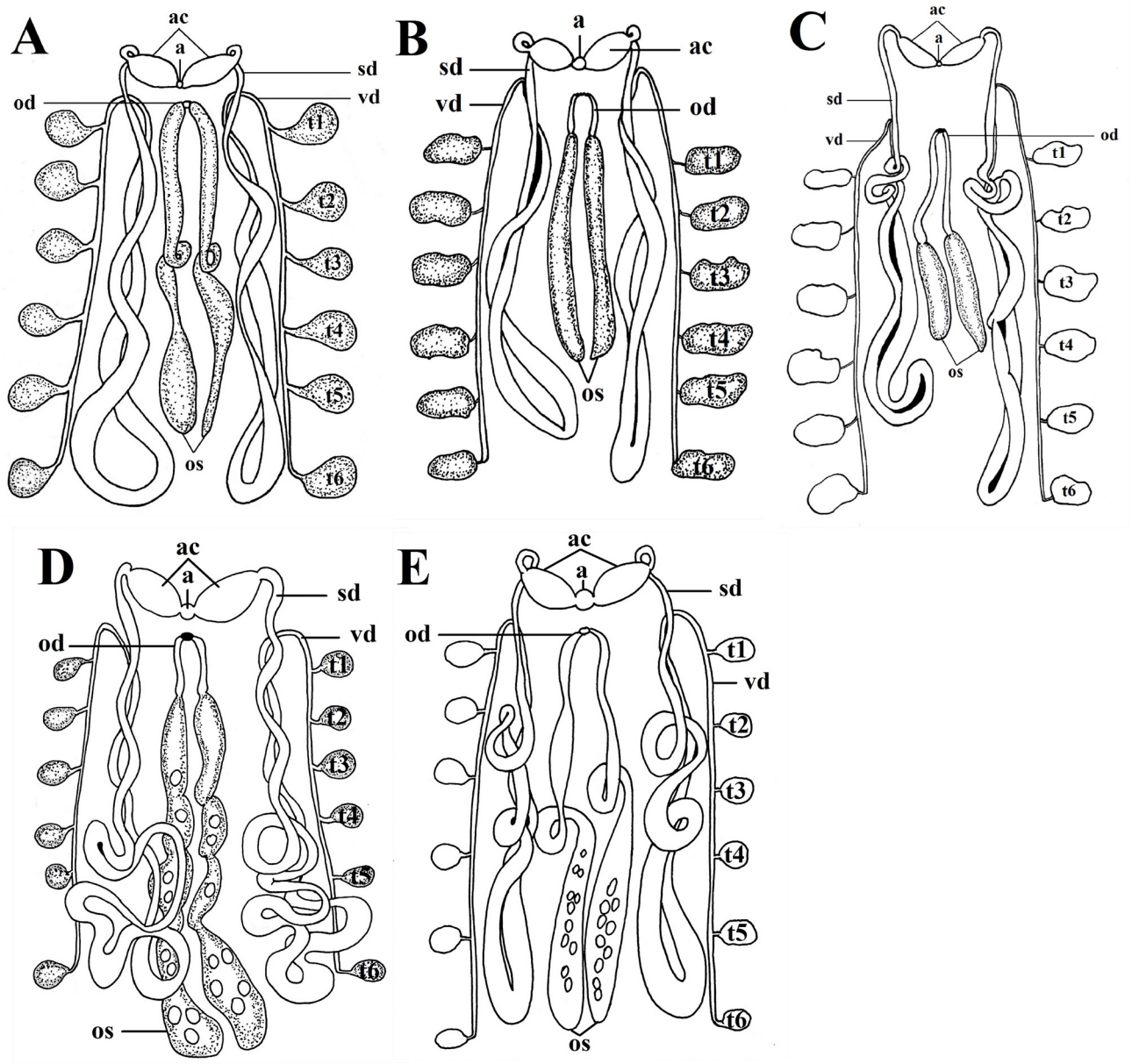

FIGURE 4. Dorsal view of reproductive system of *Helobdella* species. A, *Helobdella stagnalis*; B, *Helobdella modesta*; C, *Helobdella serendipitous* **n. sp.**; D, *Helobdella eriensis* **n. sp.**; E, *Helobdella echoensis* **n. sp.** a, atrium; ac: atrial cornua; os: ovisac; od, oviduct; sd, sperm duct; t, testisac, vd. vas deferens.

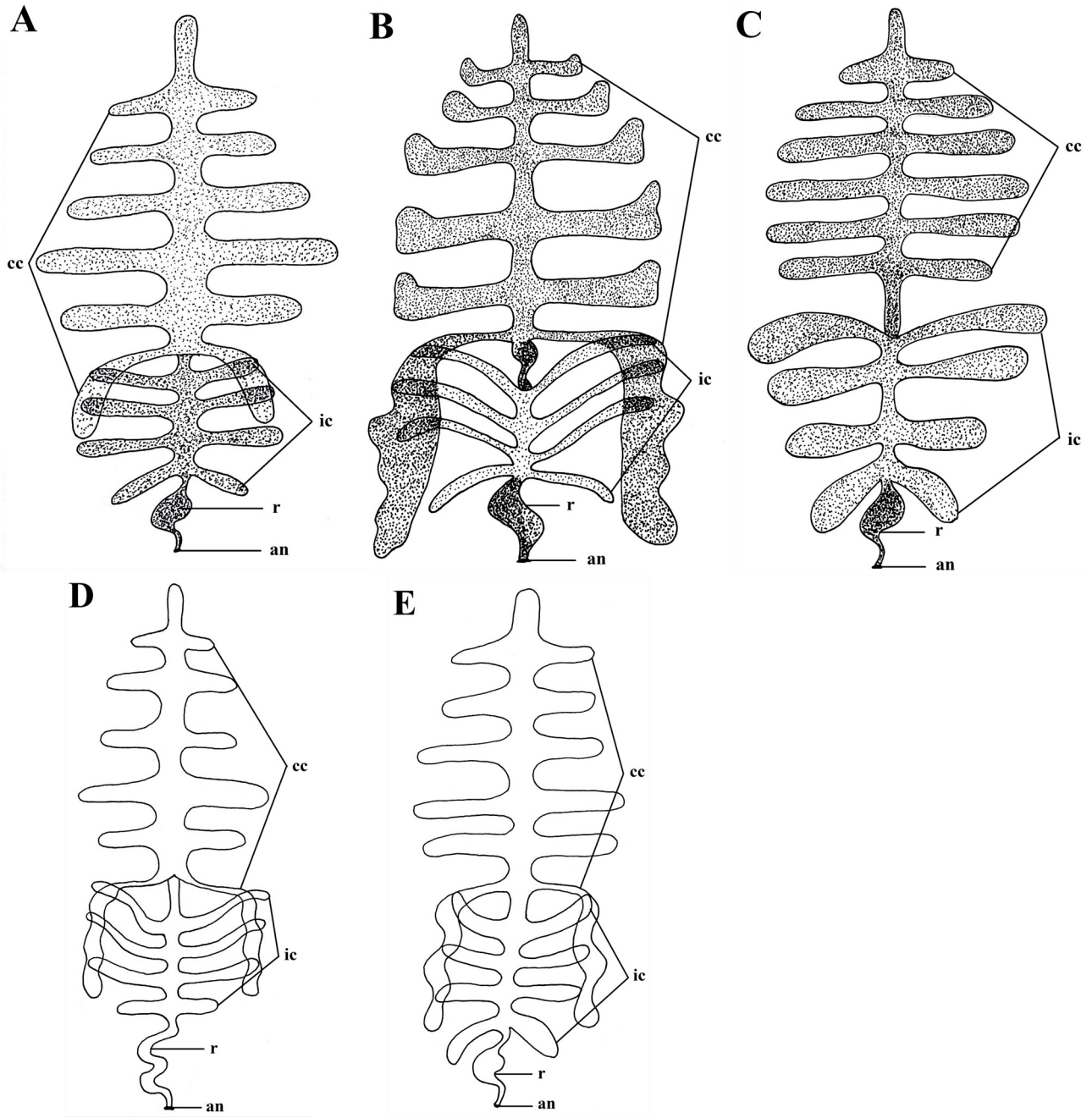

FIGURE 5. Digestive system of *Helobdella* species. A, *Helobdella stagnalis*; B, *Helobdella modesta*; C, *Helobdella serendipitous* **n. sp.**; D, *Helobdella eriensis* **n. sp.**; E, *Helobdella echoensis* **n. sp.** an, anus; cc, corp caecum; ic, intestinal caecum, r, rectum.

Helobdella modesta (Figs. 2B–7B) (LSID: *urn:lsid:zoobank.org:act:FBFD74DE-57A7-4C98-8514-51559DD2270C*)—Dorsal body light to yellowish-grey, sometimes with greenish tint. Dark, circular/ovoid spots scattered all over the body, less numerous along the dorsal median stripe, especially intensive around gonopores. Ventral color creamy-yellow or transparent aureate. Total of 67 annuli. Scute usually settled on 12th annulus (on VII-a1) but sometimes extended up to half of 13th annulus. One pair triangular eyes situated on the third annulus (Fig. 7B). Male and female gonopores located between XI a2/a3 (24th/25th annulus) and XIa3/XIIa1 (25th/26th annulus), respectively; separated by one annulus. Clitellum located between XI-XIIth segments. Six pairs of ovoid, serrated and ruffled testisacs inter-segmentally arranged at XIII to XVIII. Ejaculatory bulbs similiar to *H. stagnalis.* Ovisacs large, connected with long and curled common oviduct. Length of ovisacs and oviduct are almost equal (Figs. 2B, 4B). Six pairs of crop caeca, sixth extends to end of fourth intestinal caecum. Four pairs of thin and curved downward intestinal caeca. Rectum is "S" shaped, longer than that of *H. stagnalis* (Figs. 3B, 5B).

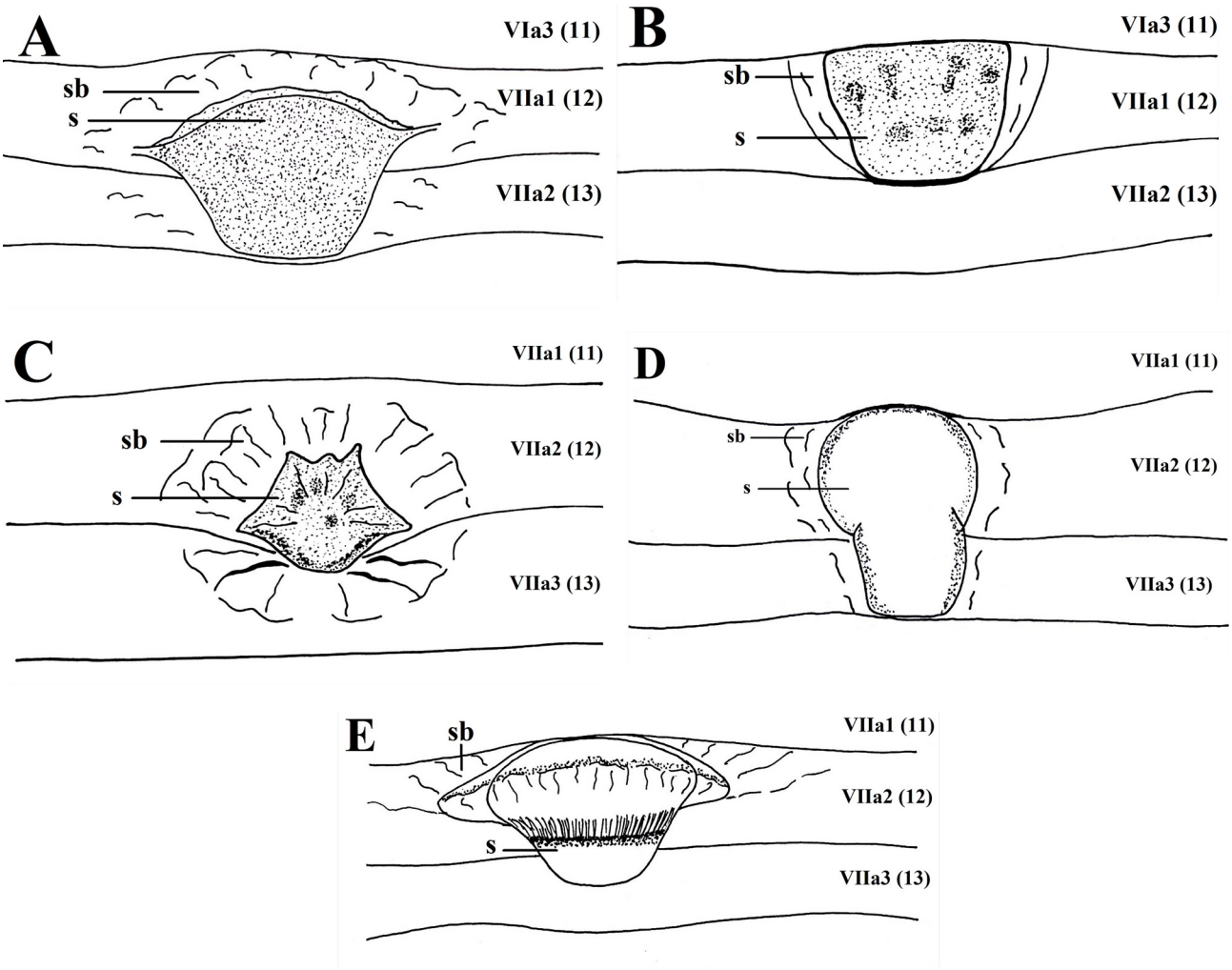

FIGURE 6. View of the scute **(**Nuchal plate**)** of *Helobdella* species. A, *Helobdella stagnalis*; B, *Helobdella modesta*; C, *Helobdella serendipitous* **n. sp.**; D, *Helobdella eriensis* **n. sp.**; E, *Helobdella echoensis* **n. sp.** s, scute; sb: scute base.

Helobdella serendipitious **n. sp.** (Figs. 2C–7C) (Holotype: ANSP GI 19495) (LSID: *urn:lsid:zoobank.org:act:D2A31CAB-F69D-413A-AD52-1497EB146753*)—Adult 11.2–20.3 mm long, 4.5–5.2 mm wide, width of anterior sucker 1.2–2.4 mm, width of posterior sucker 2.0–3.6 mm. Dorsal body light to yellowish-grey, sometimes with greenish tint. Ventral color containing mixed translucent creamy and brown-greenish spots. Total of 66 annuli; I, II, III uniannulate, IV, V biannulate, VI-XXIII triannulate, XXIV biannulate XXV-XXVII uniannulate. One pair of large circular eyes clearly separated on segment III (Fig. 7C). Gonopores located between XI a1/a2 (23th/24th annulus) and XI a2/a3 (24th/25th annulus), respectively. Clitellum on 11th segment. Six pairs of ovoidal, irregular and amorphous testisacs inter-segmentally arranged on XII/XIII–XVII/XVIII. Ejaculatory bulbs moderately longer, elongated ellipsoid, lying at much lower level by the sides of the atrium, connected by slender ejaculatory ducts with sharp turn backwards into atrium in XI. Ovisacs large, connected with long and curled common oviduct. Length of ovisacs and oviduct almost equal. Ovisac and oviduct 2/3 the length of vas deferens (Figs. 2C, 4C). Crop caeca comprising six unbranched pairs. Four pairs thick intestinal caecum curved downward in first and fourth pairs. Rectum short, "S" shaped and similar to that of *H. stagnalis*. (Figs. 3C, 5C).

Helobdella eriensis **n. sp.** (FPWS2-Farm Pond) (Figs. 2D-7D) (Holotype: ANSP GI 19494) (LSID: *urn:lsid:zoobank.org:act:91CD93B2-3B58-42C9-B640-9C92FD8827CC*)—Dorsal body light to yellowish-grey, sometimes with greenish tint. Ventral same as *H. serendipitious* **n. sp.** Total of 67 annul; I, II, III uniannulate, IV, V biannulate, VI-XXIII triannulate, XXV biannulate, XXVI-XXVII uniannulate. One pair of small, elliptical eyes clearly separated on segment III (Fig. 7D). Male and female gonopores between XI a2/a3 (24th/25th annuli) and XIa3/XIIa1 (25th/26th annuli), respectively; clitellum between XI-XIIth segments. Six pairs of testisacs

intersegmentally arranged, located as in *H. modesta*. Ejaculatory bulbs similar to *H. stagnalis*, Ovisacs in XII segment large, connected with long and curled common oviduct. Oviduct shorter than ovisac (Figs. 2D, 4D). Six pairs of crop caeca, 6th directed backwards and short extending up to fourth intestinal caecum. Four pairs of intestinal caecum, first three curved slightly upward and fourth curved downward. Rectum is long with twisted "S" shape (Figs. 3D, 5D).

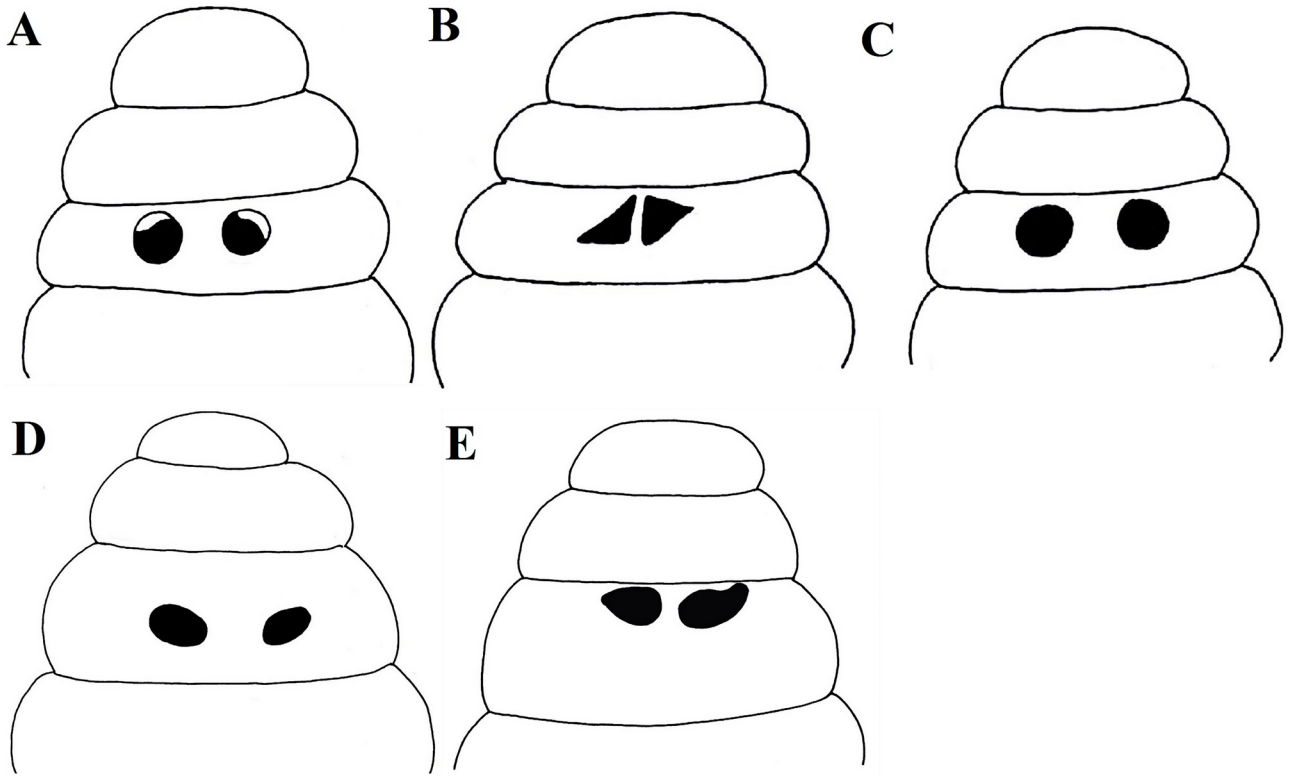

FIGURE 7. Eye position and shape of *Helobdella* species. A, *Helobdella stagnalis*; B, *Helobdella modesta*; C, *Helobdella serendipitous* **n. sp.**; D, *Helobdella eriensis* **n. sp.**; E, *Helobdella echoensis* **n. sp.**

Helobdella echoensis **n. sp.** (York, PA) (Figs. 2E–7E) (Holotype: ANSP GI 19496) (LSID: *urn:lsid:zoobank.org:act:F55674B6-1767-4F64-ABEF-0347122944E0*)—Dorsal body light to yellowish-grey, sometimes with a greenish tint. Black dots on dorsal surface. Ventral coloration and annuli numbers same as *H. serendipitious* **n. sp.** One pair of eyes close to each other on segment III (Fig. 7E). Male and female gonopores in position similar to *H. serendipitious* **n. sp.** Six pairs of testisacs intersegmentally arranged from XII/XIII to XVII/XVIII; shape similar to *H. stagnalis* but smaller. Ejaculatory bulbs (atrial cornua) moderately longer, ovoid-cylindrical, lying at a much lower level by the sides of the atrium, connected by slender ejaculatory ducts with sharp turn backwards into atrium in XIth segment. Ovisacs large, connected with long and curled common oviduct. Oviduct length almost equal to ovisacs (Figs. 2E, 4E). Six pairs crop caeca, 6th directed backwards and short, extending up to fourth intestinal caecum. Four pairs intestinal caecum, first two pairs slightly curved upward, third pair flat and last pair curved downward. Rectum short, "S" shaped, similar *H. stagnalis* (Figs. 3E, 5E).

The dorsal nuchal scute of *H. stagnalis* from Europe has been depicted in a SEM-micrograph by Sawyer (1986, p. 538). Additional SEM identified differences in scute morphology and position between species (Fig. 8). Specifically, the *s*cute *of Helobdella* spp. ranged from rhomboidal to hexagonal, settled on the12th and extending to the 13th annulus The *H. modesta* scute was square-shaped, settled on the 12th annulus (on the VII-a1) and sometimes extending up to half the 13th annulus (Fig. 6B). The *H. serendipitious* scute was pentagonal, settled in the dorsal surface of segment VII-a2 (on annulus 12) (Fig. 6C). The *Helobdella eriensis* ***n. sp.*** (Farm Pond) scute was similar to *H. serendipitious* **n. sp.** but settled in the 12th and up to all of 13th annulus (Fig. 6D). The *Helobdella echoensis* ***n. sp.*** (York, PA) was similar to *H. serendipitious* **n. sp.** but mushroom shaped (Fig. 5E).

Phylogenetic analyses. In a first set of experiments, we sequenced part of the mitochondrial gene cytochrome c oxidase subunit I (COI) from six specimens of *H. stagnalis* L. 1758 (Fig. 1A, B) collected in Germany. These

novel COI-sequences (length ca. 660 bp) were 99% identical with those of *Helobdella stagnalis* from England/UK and France (GenBank AF329041 and AF116018, respectively) (Table 2), indicating that *H. stagnalis* from Central Europe are uniform at the COI-sequence level.

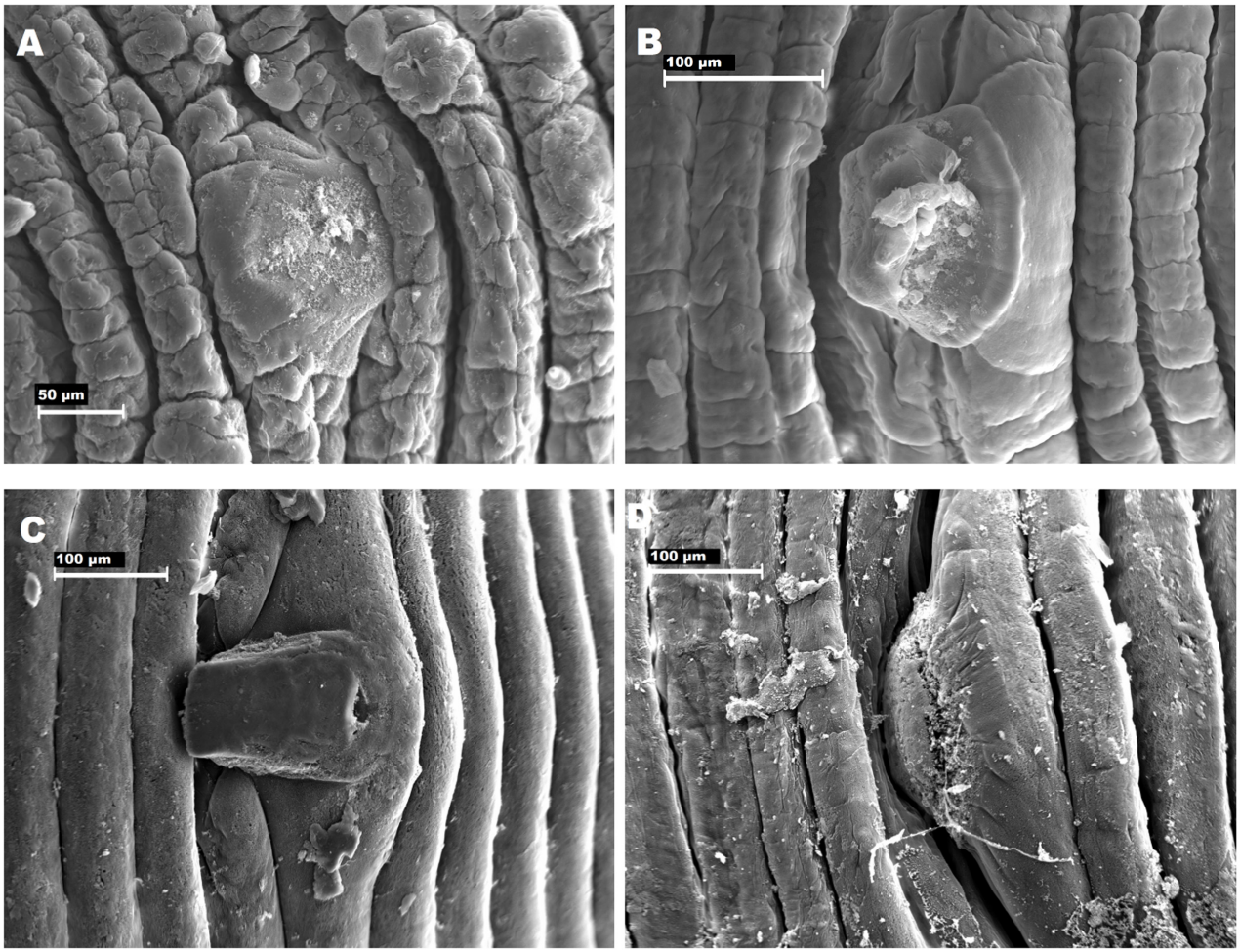

FIGURE 8. Representative scanning electron micrographs of the chitonous plate (scute) of *Helobdella stagnalis*-like species. A, of *Helobdella modesta.* B, *Helobdella serendipitous* **n.sp.** C, *Helobdella* **n. sp.** (Montana). D, *Helobdella* **n. sp.** (Oregon).

DNA sequencing of leech specimens collected worldwide revealed sequence disparity suggestive of cryptic speciation. Specifically, a 574 bp fragment of COI loci varied by at least 12% among the five specimen types considered. To determine the relationship of specimens to other *Helobdella* species, we subjected them to the comparative analysis of COI (cytochrome c oxidase subunit 1) (Fig. 9). Combined COI and morphological analysis contained 35 terminals with 602 aligned characters (28 morphological characters and 574 COI bp; Fig. 10). Collectively, *H. serendipitous* **n. sp.**, formed a basal branch among scute-containing *Helobdella* leeches with strong bootstrap support, while resolution among *H. stagnalis, H. modesta* and *H. californica* lineages were detectable but ambiguous. The distance matrix according to the COI data for some species of *Helobdella* is given in Table 5 (Suppl. Data). Although a number of GenBank sequences were used in the analysis, significant intra-specific differences in populations of *H. stagnalis* (~9–27% at COI) and *H. modesta* (~15–25% at COI) were observed.

Leech specimens first described as *H. stagnalis* (KM196604) in Lake Erie (Toledo, OH, USA) are 100% similar at COI to *Helobdella* species sampled from Farm Pond (Mt Bethel, PA, USA) and differ significantly from other *Helobdella* species (~19–28%) at COI. This is a new species for the genus *Helobdella,* given the name *Helobdella eriensis* **n. sp.** in the current study. Likewise, specimens previously classified as *H. stagnalis* from Roseburg (OR, USA) (JN692266), Echo Lake (PA, USA) (JN692267), York (PA, USA) (JN692268) and Canada (KM612173) are identifed as *Helobdella echoensis* **n. sp.**, differing from other *Helobdella* species by ~9–30% at COI (see Suppl. Data, Table 5).

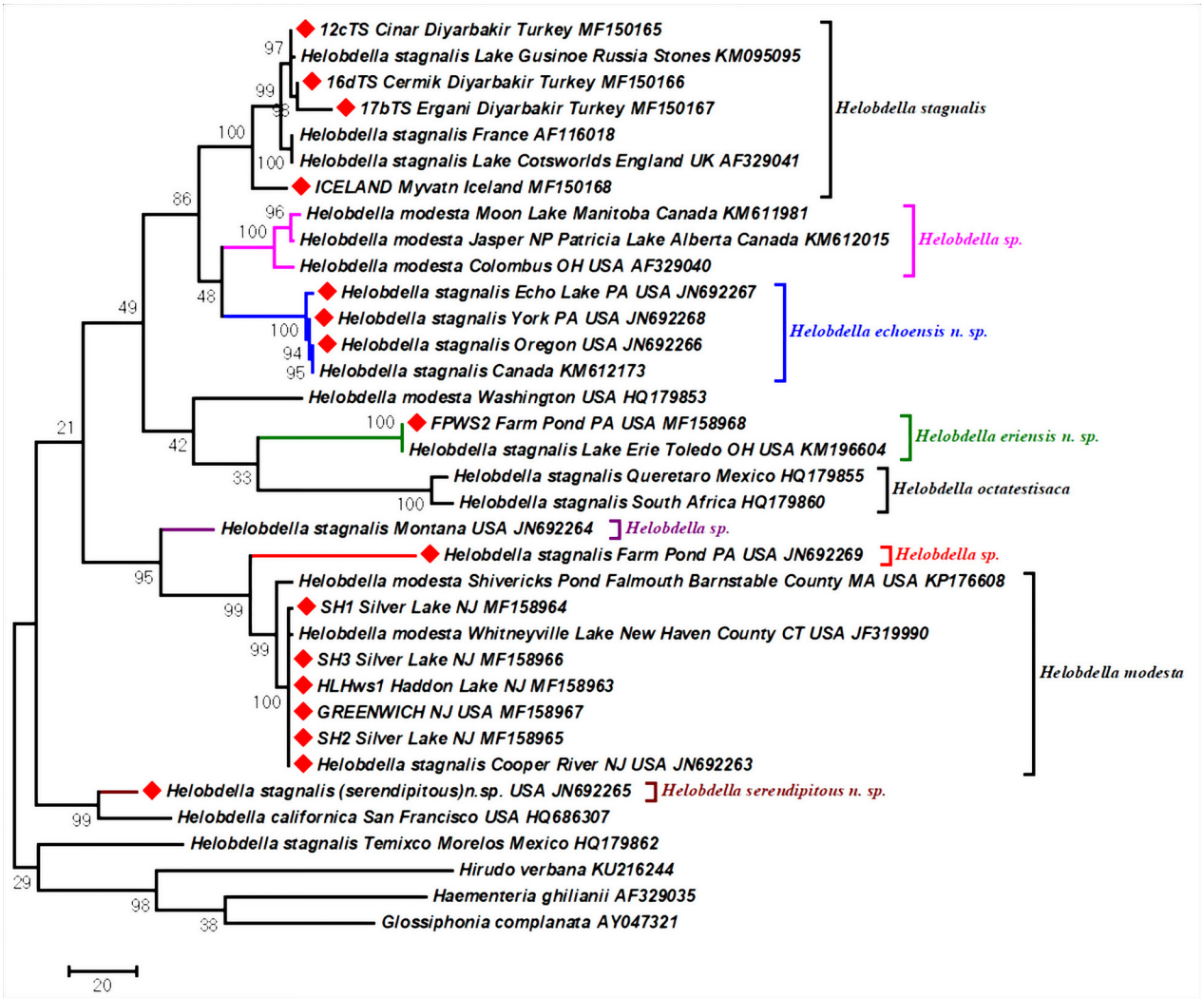

FIGURE 9. Maximum Parsimony phylogenetic analysis of COI mtDNA haplotypes (574 total positions). Tree #1 out of 5 most parsimonious trees (length = 820) is shown. The percentage of replicate trees in which the associated taxa clustered in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). Described species in this study are shown in color, previously unreported sequences are indicated by red diamonds. Remaining sequences were retrieved from GenBank.

Remarks. Despite the fact that *Helobdella serendipitous* **n. sp.** is similar to other *Helobdella* species and *H. californica*, it can be distinguished from its closest relatives using internal and external features. It differs from *H. californica* by black spots on the dorsal and paramedian stripes. Pigment cells are arrayed in *H. californica* as conspicuous longitudinal stripes but *H. serendipitous* **n. sp.**, *H. echoensis* **n. sp.** and *H. eriensis* **n. sp.** have no longitudinal stripes on the dorsal or ventral surface. *Helobdella serendipitous* has a brown, almost pentagon-shaped scute on annuli VII-a2 (on annulus 12). *Helobdella eriensis* has a brown scute with distinct circular (anterior) and square (posterior) shapes combined, while *H. echoensis* scute is mushroom-shaped. The almost square-shaped scute of *H. modesta* is on annulus 12 extending to half of annulus 13. A brown scute is situated on segments V/VI of *H. californica* (Kutschera 1988) and VII (between annuli 12/13) of *H. stagnalis* (Saglam and Dorucu 2002). *Helobdella serendipitous*, *Helobdella eriensis* and *H. stagnalis* (Saglam and Dorucu 2002, Sawyer 1986) have one pair of rather large, clearly separated circular eyes. *Helobdella modesta* has one pair of triangular eyes close to each other. *Helobdella eriensis* has small elliptical eyes clearly separated from each other, while *Helobdella echoensis* has one pair of eyes that are rather close. They are situated on the third annulus, sometimes quite close to its front edge and almost in the furrow separating it from the second annulus. One pair of eyes in *H. californica* is situated on segment II (Kutschera 1988).

The male gonopore is situated on XI a1/a2 (23th/24th annulus) of *H. serendipitous* and *H. echoensis*, XI a2/a3 (24th/25th annulus) of *H. modesta and H. eriensis*, XII a1/a2 of *H. stagnalis* (Saglam and Dorucu, 2002) and XI segment of *H. californica* (Kutschera 1988). The female gonopore is situated on XI a2/a3 (24th/25th annulus) of *H.*

serendipitous and *H. echoensis*, XI a3/XIIa1 (25th/26th annulus) of *H. modesta and H. eriensis*, XII a2/a3 of *H. stagnalis* (Saglam and Dorucu, 2002) and XI segment of *H. californica* (Kutschera 1988). Six pairs of testisacs, inter-segmentally arranged at XII/XIII–XVII/XVIII segments of *H. serendipitous* and *H. echoensis* at XIII to XVIII of *H. modesta and H. eriensis*, at XI11/XIV to XVIII of *H. stagnalis* (Saglam and Dorucu, 2002) and XI to XVII of *H. californica* (Kutschera 1988). The atrial cornua (ejaculatory bulbs) of *H. serendipitous* **n. sp.** and *H. echoensis* is moderately long, ellipsoid, lying at a lower level by the sides of the atrium, connected by slender ejaculatory ducts with a sharp backwards turn into atrium in XI. Atrial cornua of *H. eriensis*, *H. stagnalis* and *H. modesta* is open, short and cylindrical. A complete comparison of morphological traits for *H. stagnalis, H. modesta, H. californica H. echoensis*, *H. eriensis* and *H. serendipitous* **n. sp.** is given in Suppl. Data, Table 4.

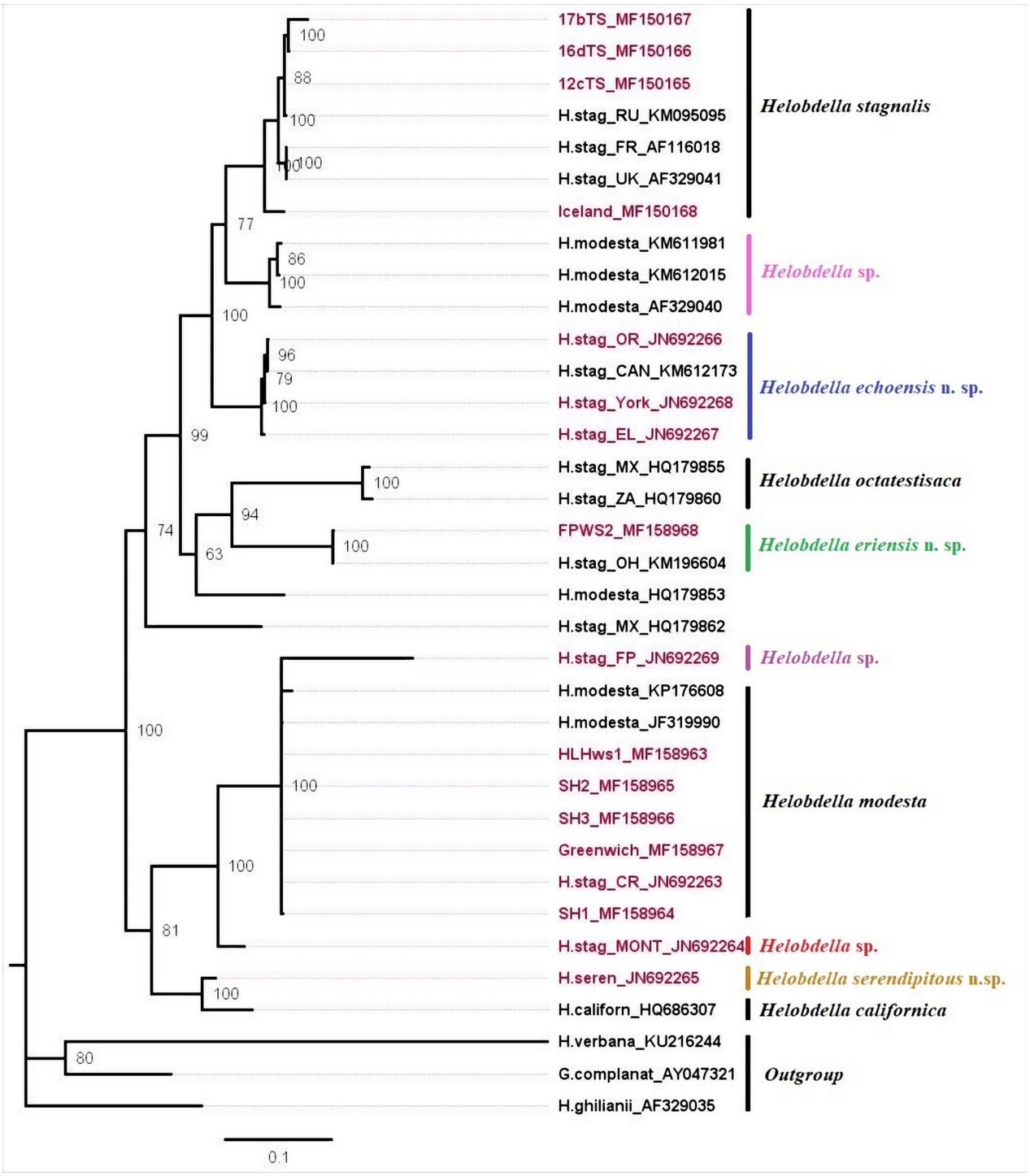

FIGURE 10. Bayesian combined analysis of morphology (28 positions) and COI (574 positions) from *Helobdella stagnalis*-like specimens (602 total positions). Red sequences are from the current study (refer to Table 1); GenBank numbers indicated.

Discussion

Phylogenetic and morphological examinations of geographically distinct populations of *Helobdella stagnalis*-like leeches suggest that the group is monophyletic, morphologically diverse, and likely contains additional members. Historically, considerable taxonomic confusion has surrounded the name *H. stagnalis*. The original species description by Linnaeus in 1758 was based on common European specimens (Fig. 1A, B), and the presence of a conspicuous chitinous scute on the dorsal surface (Moore 1906) inevitably led to a (mis)classification of *H. stagnalis*, which has been considered a cosmopolitan species (Sawyer 1986). For example, a nearly indistinguishable North American leech, described as *Helobdella modesta* (Verrill 1872), was synonymized under *H. stagnalis* (Klemm 1972, 1982; Sawyer 1986), but later *H. stagnalis* and *H. modesta* were again defined as different species (Siddall and Borda 2003; Siddall *et al*. 2005). Thus, *H. modesta* was resurrected as the North American counterpart to European *H. stagnalis* species (Siddall *et al*. 2005). Our results support this designation based on morphological (e.g., eyes, gastric caeca, scute, rectum) and phylogenetic criteria (Figs. 8, 9). Note that the two species are 23–26 % divergent at COI (Suppl. Data, Table 5), suggesting a mid-Miocene (ca. 13 mya) common ancestor of these groups, based on conservative molecular clock estimates (e.g., Dial *et al*. 2012).

Clearly, *H. stagnalis*-like specimens have been misclassified since their original description in 1758, due to their uniform morphology and color and overtly characteristic scute on the dorsal anterior region. Curiously, this feature has no known function and likely represents a vestigial organ/gland possibly associated with mucous secretion and/or adhesion (Sawyer 1986; Moser 1991; Oceguera-Figueroa *et al*. 2010b). Nonetheless, it displays considerable morphological diversity in extant species, ranging from a subtle bulge between annuli to distinctly rhomboidal plates spanning the annuli furrow (see Fig. 8). The relatively small size of this structure, however, precludes its detailed examination in a field setting and this likely has been the cause of much taxonomic confusion (Sawyer 1986).

Phylogenetic groupings of specimens worldwide generally segregate into European and North American clades, with exceptions likely introduced much later through human activity, e.g., in association with the introduction of exotic aquatic plant species (Hughes *et al*. 1998). The putative ancestor to the *H. stagnalis*-like clade developed a few ecologically successful species, one of which spread widely into the Holarctic region, possibly as an accidental passenger on migrating aquatic birds. Two additional species succeeded to spread out of the Neotropical region by some human means (Sket and Trontelj 2008). The geographic diversity occurring in some sub-clades representing identical species (e.g., PA, OR, Canada) indicates a robust dispersal mechanism, which is likely passive (e.g., birds, humans). This would also explain the current distribution of *H. stagnalis* throughout Europe. Certainly, the common diet of *H. stagnalis*-like species, i. e. the haemolymph of oligochaetes, insect-larvae and molluscs (Miller 1929; Klemm 1991; Young *et al*. 1993), as well as their efficient parental care, has permitted these agile predators their successful invasion of aquatic habitats worldwide.

Here we describe the North American leeches *Helobdella echoensis* **n. sp.**, *Helobdella eriensis* **n. sp.** and *Helobdella serendipidous* **n. sp.**, as sister-taxa to *H. californica* (Kutschera 1988), which represent a basal position in the phylogeny of the group (Figs. 9, 10). Importantly, however, our analyses identified other scute-bearing specimens that are resolved by phylogenetic and/or morphological criteria. Thus, the *H. stagnalis*-like clade has proliferated and dispersed rapidly during the Miocene/Pleistocene, making it perhaps the most successful Hirudinid group on Earth. Deeper evolutionary comparisons and review of archived specimens will likely reveal additional undescribed species and interesting biogeographical life histories within the cosmopolitan leech genus *Helobdella* (Blanchard 1896; Sawyer 1986).

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SUPPLEMENTARY TABLE 1. Primers used for PCR amplification and DNA sequencing.

Gene	Primer name	Primer sequence	Reference
18S rDNA	C	5'- CGGTAATTCCAGCTCCAATAG -3'	Apakupakul *et al*. (1999)
	Y	5'-CAGACAAATCGCTCCACCAAC -3'	Apakupakul *et al*. (1999)
12S rDNA	12S-A	5'-AAACTAGGATTAGATACCCTATTAT-3'	Palumbi, 1996
	12S-B	5'-AAGAGCGACGGGCGATGTGT-3'	Simon *et al*., 1990
CO1	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer *et al*. (1994)
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer *et al*. (1994)

SUPPLEMENTARY TABLE 2. Accession numbers used in phylogenetic analyses.

Ingroup Taxon	Locality	Accession No (CO1)	12S
Helobdella modesta	HLHws1, Hadden Lake, NJ, USA	MF158963	-
Helobdella modesta	SH1, Silver Lake, Gibbsboro, NJ, USA	MF158964	-
Helobdella modesta	SH2, Silver Lake, Gibbsboro, NJ, USA	MF158965	-
Helobdella modesta	SH3, Silver Lake, Gibbsboro, NJ, USA	MF158966	-
Helobdella stagnalis	ICELAND Myvatn, Iceland	MF150168	-
Helobdella stagnalis	12cTS, Çınar, Diyarbakır, Turkey	MF150165	-
Helobdella stagnalis	16dTS, Çermik, Diyarbakır, Turkey	MF150166	MF158969
Helobdella stagnalis	17bTS, Ergani, Diyarbakır, Turkey	MF150167	-
Helobdella modesta	GREENWICH, NJ, USA	MF158967	-
Helobdella eriensis **n. sp.**	FPWS2, Farm Pond, PA, USA	MF158968	-
Helobdella eriensis **n. sp.**	Farm Pond, PA, USA	JN692269	MF158975
Helobdella modesta	Cooper River, Camden, NJ, USA	JN692263	MF158970
Helobdella **n. sp.**	MONT, Settle Farm Canyon Creek, MT, USA	JN692264	MF158973
Helobdella serendipitous **n.sp.**	Value Pet shop, NJ, USA	JN692265	MF158971
Helobdella **n. sp.**	Roseburg, OR, USA	JN692266	MF158972
Helobdella stagnalis	Echo Lake, PA, USA	JN692267	MF158974
Helobdella echoensis **n. sp.**	York, PA, USA	JN692268	-
Helobdella stagnalis	Canada	KM612173	-
Helobdella stagnalis	France	AF116018	AY425424
Helobdella stagnalis	Lake Cotsworlds, England, UK,	AF329041	AY336032
Helobdella stagnalis	Park Schoenfeld, Kassel, Germany	AF329041	-
Helobdella stagnalis	Lake Gusinoe, Russia Stones	KM095095	-
Helobdella stagnalis	Queretaro, Mexico	HQ179855	-
Helobdella stagnalis	South Africa	HQ179860	-
Helobdella stagnalis	Temixco, Morelos, Mexico	HQ179862	-
Helobdella stagnalis	Lake Erie,Toledo, OH, USA	KM196604	-
Helobdella californica	San Francisco, CA, USA	HQ686307	-
Helobdella modesta	Colombus, OH, USA.	AF329040	-
Helobdella modesta	Washington, USA	HQ179853	-
Helobdella modesta	Whitneyville Lake, New Haven County, CT, USA	JF319990	-
Helobdella modesta	Riding Mountain NP, Moon Lake, Manitoba, Canada	KM611981	-
Helobdella modesta	Jasper NP, Patricia Lake, Alberta, Canada	KM612015	-
Helobdella modesta	Shivericks Pond, Falmouth, Barnstable County, MA**,** USA	KP176608	-
Outgroup Taxon			
Haementeria ghilianii		AF329035	AY425417
Glossiphonia complanata		AY047321	AF099956
Hirudo verbana		KU216244	KU216249

SUPPLEMENTARY TABLE 3. A matrix of morphological data used in phylogenetic analyses of leeches

Morphological data
Character 1: Pairs eyes: absent (0); one (1); two (2); three (3); four (4); five (5).
Character 2: Position of eyes: one pair per annulus (0); at least two pairs per annulus (1).
Character 3: Testisac arrangement: grapelike clusters (0); five pair (1); six pair (2); ten pair (3).

Character 4: Nephridia: complete in genital somites (0); suppressed in genital somites (1).
Character 5: Pharynx: not protrusible (0); modified into protrusible proboscis (1).
Character 6: Intestine: acaecate (0); caecate (1).
Character 7: Cephalic eyespots: dorsolateral (0); dorsal (1).
Character 8: Intestinal blood sinus: absent (0); present (1).
Character 9: Body shape: dorsoventrally flattened such as tree leaves (0); dorsoventrally slightly flattened (1);
Character 10: Deposition of cocoons: slipped off head (0); secreted ventrally (1).
Character 11: Cocoons: cemented to a substrate (0); not cemented to a substrate (1); attached to the under of leech body (2).
Character 12: Cocoons: without spongy covering (0); with spongy covering (1).
Character 13: Arrangement of salivary tissue: diffuse (0); discrete glands (1).
Character 14: Atria: single bulb (0); bilobed (1).
Character 15: Testisacs: discretely arranged on vasa deferentia (0); hundreds of sacs profusely arranged (1).
Character 16: Ovisacs: tubular (0); spheroid (1).
Character 17: Nephridia: single funnel (0); multiple funnels located in ciliated organ (1).
Character 18: Urinary bladder: absent (0); present (1).
Character 19: Female median reproductive apparatus: simple pocket (0); modified into vaginal tube (1).
Character 20: Epididymes: loosely arranged (0); tightly coiled mass (1).
Character 21: Cocoons: without spongy covering (0); with spongy covering (1).
Character 22: Annuli per somite: three (0); five (1)
Character 23: Location of male gonopore: on ring (0); in furrow (1).
Character 24: Presence of scute (nuchal plate): absent (0); present (1).
Character 25: Last pair of crop caecum: stub (0); long curved (1); short curved (2).
Character 26: Last pairs of crop caeca: unbranched (0); branched (1); very little upward (2).
Character 27: Longitudinal dorsal stripes: absent (0); present (1).
Character 28. The shape of the eye pigment: circular (0); half moon (1); triangle (2); amorphous (3); elipsoid (4).

Taxon		Characters
		12345678901234567890123456 78
Helobdella stagnalis		10201111012001000000000012001
Helobdella californica		10201111012001000000000010110
Helobdella modesta		10201111012001000000000011202
Helobdella serendipitous (**n.sp.** 1)	HS	10201111012001000000000010000
Helobdella eriensis, PA-1 (**n.sp.** 2)	FP	10201111012001000000000012004
Helobdella eriensis, PA-2 (**n.sp.** 3)	FPWS2	10201111012001000000000012004
Roseburg, OR (**n.sp.** 4)	OR	10201111012001000000000012001
Helobdella echoensis, PA (**n.sp.** 4)	EL	10201111012001000000000012001
Lake Redman, York, PA (**n.sp.** 4)	YorkPA	10201111012001000000000012001
Helobdella stagnalis	12cTS	10201111012001000000000012001
Helobdella stagnalis	16dTS	10201111012001000000000012001
Helobdella stagnalis	17bTS	10201111012001000000000012001
Helobdella stagnalis	Iceland	10201111012001000000000012001
Helobdella modesta	CR	10201111012001000000000011202
Helobdella modesta	HLHws1	10201111012001000000000011202
Helobdella modesta	SH1	10201111012001000000000011202
Helobdella modesta	SH2	10201111012001000000000011202
Helobdella modesta	SH3	10201111012001000000000011202
Helobdella modesta	MONT	10201111012001000000000011202
Helobdella modesta	Greenwich	10201111012001000000000011202
Haementeria ghilianii	outgroup	10101111012011000000000--00
Glossiphonia complanata	outgroup	30301111012001000000000--10
Hirudo verbana	outgroup	50310000101100011111111 0--10

SUPPLEMENTARY TABLE 4. Comparison of morphological traits for *Helobdella*

Traits	*Helobdella stagnalis*	*Helobdella modesta*	*Helobdella calfornica*	*Helobdella serendipitous* n. sp.	*Helobdella eriensis* n. sp.	*Helobdella echoensis* n. sp.
GenBank Number (COI)	**MF150166**	**JN692263**	**HQ686307**	**JN692265**	**JN692269**	**JN692268**
Location	Diyarbakır-Turkey	Cooper River	Stow Lake, Golden Gate Park, San Francisco, CA	Value Pet, Rt. 130 Camden NJ	Farm Pond Poconos,PA	Lake Redman, York, PA
Shape of Body	The body is flat and short	The body is flat and short	Body broadest posterior to its middle and tapering gradually toward both ends; dorso-ventrally body very much flattened, especially when leech is at rest.	The body is flat and short	The body is flat and short	The body is flat and large
Color pattern on dorsal	Body light grey or yellowish-grey, sometimes with a greenish tint. Preserved leeches are usually dirty white. Dark (brownish) spots (dots) scattered all over the body are far less numerous along the dorsal median stripe than elsewhere. Thus an irregular bright longitudinal line shows on the dorsal side of the body.	Body light grey or yellowish-grey, sometimes with a greenish tint. Preserved leeches are usually dirty white. Dark (brownish) spots (circular or ovoid) scattered all over the body are far less numerous along the dorsal median stripe than elsewhere. These spots are especially intensive around the male and female gonopore.	The dorsal side is dark grey-black due to the presence of superficial, in or under the epidermis localized,branched assemblies of black pigment cells. The pigment cells are arrayed as conspicuous longitudinal stripes. No papillae present on the dorsal side of the body.	Body light grey or yellowish-grey, sometimes with a greenish tint. Preserved leeches are usually dirty white.	Body light grey or yellowish-grey, sometimes with a greenish tint. Preserved leeches are usually dirty white.	Body light grey or yellowish-grey, sometimes with a greenish tint. Preserved leeches are usually dirty white. It have black dot on the dorsal surface
Color pattern on venter	Colour of body composed of mixed creamy-yellow or transparent aureate.	Colour of body composed of mixed creamy-yellow or transparent aureate.	The ventral side is white, with scattered, irregular arrays of black pigment cells (spots).	The ventral side colour of body composed of mixed translucent creamy and brown-greenish spots	The ventral side colour of body composed of mixed translucent creamy and brown-greenish spots	The ventral side colour of body composed of mixed translucent creamy and brown-greenish spots
pigment pattern	no longitudinal stripes	no longitudinal stripes	arrayed as longitudinal stripes	no longitudinal stripes	no longitudinal stripes	no longitudinal stripes
Number of segments	Totally is 67 annulus. Segments I, II, III uniannulate, IV-V biannulate, mid-body segments (VI-XXIII) triannulate, XXIV-XXV biannulate, XXVI-XXVII uniannulate.	Totally is 67 annulus. Segments I, II, III uniannulate, IV-V biannulate, mid-body segments (VI-XXIII) triannulate, XXIV-XXV biannulate, XXVI-XXVII uniannulate	?	Totally is 66 annulus, I, II, III uniannulate, IV, V biannulate, VI-XXIII triannulate, XXIV biannulate XXV-XXVII uniannulate.	Totally is 67 annulus. I, II, III uniannulate, IV, V biannulate, VI-XXIII triannulate, XXV biannulate, XXVI-XXVII uniannulate.	Totally is 66 annulus, I, II, III uniannulate, IV, V biannulate, VI-XXIII triannulate, XXIV biannulate XXV-XXVII uniannulate.

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SUPPLEMENTARY TABLE 4. (Continued)

Traits	*Helobdella stagnalis*	*Helobdella modesta*	*Helobdella calfornica* (Kutschera, 1988)	*Helobdella serendipitous* **n. sp.**	*Helobdella eriensis* **n. sp.**	*Helobdella echoensis* **n. sp.**
Body length	8.0–13.4 mm	10.0–18.0 mm	Length, at rest: 12–18 mm, fully extended: 25–30 mm.	11.2–20.3 mm	7.02 mm	
Body Width	3.0–4.5 mm	5.0–8.0 mm	Width, at rest: 3–5 mm, fully extended about 2 mm.	4.5–5.2 mm	3.4 mm	
anterior sucker diameter	0.8–1.0 mm	0.8–1.2 mm	?	1.2–2.4 mm	1.3 mm	
posterior sucker diameter	1.5–2.2 mm	2.1–4.5 mm	?	2.0–3.6 mm	2.1 mm	
Scut (Nuchal plate)	Redish-Brown, horny, The scut (chitinoid scute, nuchal plate) on the dorsal surface of segment VII-a1 (between annuli 12). The scute (chitinoid plate) has settled twelfth (annulus VII-between annuli 12). and extended up to all of thirteenth annulus. The shape of the scute is in top circle and in bottom square shape.	The scute has usually settled twelfth annulus (on the VII-a1) but sometime extended up to half of thirteenth annulus. The scute is almost square shape.	a brown scute in the neck (segments V/VI)	A brown scute (nuchal plate) in the neck has settled in dorsal surface of segment VII-a2 (on the annulus 12). The scut is almost pentagon shape.	A brown scute (nuchal plate) in the neck has settled in dorsal surface of segment VII-a2 (on the annulus 12). The scut has settled twelfth and extended up to all of thirteenth annulus. The shape of the scut is in top circle and in bottom square shape.	A brown scute (nuchal plate) in the neck has settled in dorsal surface of segment VII-a2 (on the annulus 12). The scute is almost mushroom shape. The scut has almost extended up to half of thirteenth annulus.
Eyes	*H. stagnalis* has one pair of rather large half circular eyes clearly separated from each other. They are situated on the third annulus, sometimes quite close to its front edge and even almost in the furrow separating it from the second annulus	There is one pair triangular eyes closed to each other. They are situated on the third annulus, sometimes quite close to its front edge and even almost in the furrow separating it from the second annulus.	The head is only slightly wider than the neck with one pair of eyes on segment II.	*H. serendipitious* has one pair of large circular eyes clearly separated from each other situated on III segment.	*Helobdella eriensis* n. sp. has one pair of small elliptical eyes clearly separated from each other situated on III segment.	*Helobdella echoensis* n. sp. has has one pair eyes that is closer to each other, like a bean grains or a water drop situated on III segment.
Number of Annuli at mid of body	VI-XXIII midbody segments and three annulus	VI-XXIII midbody segments and three annulus	three annuli at mid of body	three annuli at mid of body (VI-XXIII)	VI-XXIII triannulate,	three annuli at mid of body (VI-XXIII)
Gonopores	Gonopores separated by one annuli	Gonopores separated by one annuli	Gonopores separated by one annuli	Gonopores separated by one annuli	Gonopores separated by one annuli	Gonopores separated by one annuli
Male gonopor	between annulus al and a2 of segment XII.	XI a2/a3 (24th/25th annulus);	XI segment	XI a1/a2 (23th/24th annulus)	XI a2/a3 (24th/25th annulus);	XI a1/a2 (23th/24th annulus)

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SUPPLEMENTARY TABLE 4. (Continued)

Traits	*Helobdella stagnalis*	*Helobdella modesta*	*Helobdella calfornica* (Kutschera, 1988)	*Helobdella serendipitous* **n. sp.**	*Helobdella eriensis* **n. sp.**	*Helobdella echoensis* **n. sp.**
Female gonopor	XII a2/a3	XIa3/XIIa1 (25th/26th annulus);	XI segment	XI a2/a3 (24th/25th annulus)	XIa3/XIIa1 (25th/26th annulus);	XI a2/a3 (24th/25th annulus)
Clitellum position	XIIth segment,	XI-XIIth segment,	Gonopores separated by 1 annulus and positioned within segment XI. The clitellar glands are localized within the female gonophore.	XIth segment,	XI-XIIth segment,	XIth segment,
Testis	Six pairs of testisacs intersegmentally arranged at XIII/XIV to XVIII.	Six pairs of testisacs inter-segmentally arranged at XIII to XVIII. Shape of Testisacs are ovoidal serrated and ruffled.	Six pairs of testisacs between the lateral crop caeca; extending from segment XI to XVII.	Six pair of testisacs at XII/XIII–XVII/XVIII.	Six pair of testisacs at XIII-XVIII.	Six pair of testisacs at XII/XIII–XVII/XVIII.
Vas deferens	Vas deferens on each side emerges from dorsal body wall in XIII between sixth pairs crop caeca with first pairs intestinal caeca	Vas deferens on each side emerges from dorsal body wall in XIII between sixth pairs crop caeca with first pairs intestinal caeca	?	Vas deferens on each side emerges from dorsal body wall in XIII between sixth pairs crop caeca with first pairs intestinal caeca	Vas deferens on each side emerges from dorsal body wall in XIII between sixth pairs crop caeca with first pairs intestinal caeca	Vas deferens on each side emerges from dorsal body wall in XIII between sixth pairs crop caeca with first pairs intestinal caeca
Atrial cornua	Ejaculatory bulbs (atrial cornua) open into short, cylindrical,	Ejaculatory bulbs (atrial cornua) open into short, cylindrical,	?	Ejaculatory bulbs moderately longer, elongated ellipsoid, lying at a much lower level by the sides of the atrium, connected by slender ejaculatory ducts with a sharp turning backwards into atrium in XI	Ejaculatory bulbs (atrial cornua) open into short, cylindrical,	Ejaculatory bulbs (atrial cornua) moderately longer, ovoid-cylindrical,lying at a much lower level by the sides of the atrium, connected by slender ejaculatory ducts with a sharp turning backwards into atrium in XI.
Atrium	nearly spherical atrium	Small spherical atrium.	?	Small spherical atrium.	Small spherical atrium.	Small spherical atrium.
Ovisacs	Wide ovisac, oviduct opens via gonopore into a chamber formed between annuli a2 and a3 of XII.	Ovisacs in XII, large, connected with long and curled common oviduct. Long of ovisacs and oviduct are almost equal.	Paired ovisacs,	Ovisacs in XII, large, connected with long and curled common oviduct. Long of ovisacs and oviduct are almost equal. Ovisac and oviduct is 2/3 the length of the vas deferens.	Ovisacs in XII segment, large, connected with long and curled common oviduct. Oviduct is shorter than ovisac.	Ovisacs in XII, large, connected with long and curled common oviduct. oviduct Long of ovisacs and are almost equal.

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SUPPLEMENTARY TABLE 4. (Continued)

Traits	***Helobdella stagnalis***	***Helobdella modesta***	***Helobdella calfornica*** (Kutschera, 1988)	***Helobdella serendipitous* n. sp.**	***Helobdella eriensis* n. sp.**	***Helobdella echoensis* n. sp.**
Proboscis	Proboscis present.	Oesophageal tube contains proboscis.	Oesophageal tube contains the eversible proboscis.	Oesophageal tube contains the eversible proboscis.	Oesophageal tube contains proboscis.	Oesophageal tube contains proboscis.
Crop caeca	Crop caeca six pair. Last pair (6^{th}) directed backwards and short. Sixth Corp caeca extends to third intestinal caecum.	Crop caeca are six pairs. Tip of five pairs very little upward, last pair directed backwards and long. Sixth crop caeca extends to end of fourth intestinal caecum.	six branched pairs of crop caeca	Crop caeca is six unbranched pairs.	Crop caeca is six pair. Last pair (6th) directed backwards and short. Sixth Corp caeca extends up to fourth of intestinal caecum.	Crop caeca is six pair. Last pair (6th) directed backwards and short. Sixth Corp caeca extends up to fourth of intestinal caecum.
Intestinal caeca	*H. stagnalis* has four pairs intestinal caecum. The first three pairs are straight and long, the fourth pair are short and curved downward.	*H. modesta* has four pairs of thin and curved downward intestinal caeca.	Four intestinal caeca.	*H. serindipitious* n. sp. has four pairs thick intestinal caecum that curved downward the first and fourth pairs.	The leech has four pairs of intestinal caecum that first three pairs curved slightly curved upward and the fourth pairs curved downward.	This leech has four pairs Intestinal caecum that first two pairs slightly curved upward, third pair flat and the last pair curved downward.
Rectum	Rectum "S" well shaped and short.	The rectum is S shape and light long than rectum of *H. stagnalis*.	The intestine leads into the rectum which opens dorsally	Rectum is short, S shape and similar to rectum of *H. stagnalis*.	The rectum is light long and twisted S shape.	The rectum is short, S shape and similar to rectum of *H. stagnalis*.
Anus	Anus which opens dorsally in the furrow between XXVI (66th annulus) and XXVII (67th annulus).	Anus which opens dorsally in the furrow between XXVI (66th annulus) and XXVII (67th annulus).	?	Anus which opens dorsally in the furrow between XXVI (65th annulus) and XXVII (66th annulus).	Anus which opens dorsally in the furrow between XXVI (66th annulus) and XXVII (67th annulus).	Anus which opens dorsally in the furrow between XXVI (65th annulus) and XXVII (66th annulus).

SUPPLEMENTARY TABLE 5. Pairwise COI divergence distance matrix of *Helobdella* specimens.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
HLHws1- MF158963																	
SH1- MF158964	**0,00**																
SH2- MF158965	**0,00**	**0,00**															
SH3- MF158966	**0,00**	**0,00**	**0,00**														
ICELAND- MF150168	**0,24**	**0,24**	**0,24**	**0,24**													
12cTS- MF150165	**0,23**	**0,23**	**0,23**	**0,23**	**0,04**												
16dTS- MF150166	**0,23**	**0,23**	**0,23**	**0,23**	**0,04**	**0,00**											
17bTS- MF150167	**0,26**	**0,25**	**0,26**	**0,26**	**0,06**	**0,02**	**0,02**										
GREENWICH- MF158967	**0,00**	**0,00**	**0,00**	**0,00**	**0,24**	**0,23**	**0,23**	**0,26**									
FPWS2- MF158968	**0,24**	**0,23**	**0,24**	**0,24**	**0,23**	**0,20**	**0,20**	**0,23**	**0,24**								
H. stagnalis, CR- JN692263	**0,00**	**0,00**	**0,00**	**0,00**	**0,24**	**0,23**	**0,23**	**0,26**	**0,00**	**0,24**							
H. stagnalis, Montana- JN692264	**0,09**	**0,09**	**0,09**	**0,09**	**0,20**	**0,20**	**0,21**	**0,23**	**0,09**	**0,22**	**0,09**						
H. stagnalis (*serendipitous*)- JN692265	**0,19**	**0,19**	**0,19**	**0,19**	**0,19**	**0,20**	**0,20**	**0,23**	**0,19**	**0,21**	**0,19**	**0,13**					
H. stagnalis, Oregon- JN692266	**0,21**	**0,21**	**0,21**	**0,21**	**0,12**	**0,11**	**0,11**	**0,13**	**0,21**	**0,20**	**0,21**	**0,19**	**0,17**				
H. stagnalis, EL- JN692267	**0,21**	**0,21**	**0,21**	**0,21**	**0,11**	**0,10**	**0,11**	**0,13**	**0,21**	**0,20**	**0,21**	**0,20**	**0,17**	**0,01**			
H. stagnalis, York- JN692268	**0,21**	**0,21**	**0,21**	**0,21**	**0,11**	**0,11**	**0,11**	**0,13**	**0,21**	**0,20**	**0,21**	**0,20**	**0,18**	**0,00**	**0,01**		
H. stagnalis, FP- JN692269	**0,12**	**0,13**	**0,12**	**0,12**	**0,33**	**0,32**	**0,33**	**0,36**	**0,12**	**0,28**	**0,12**	**0,21**	**0,29**	**0,30**	**0,30**	**0,30**	
H. stagnalis, Canada- KM612173	**0,21**	**0,21**	**0,21**	**0,21**	**0,12**	**0,11**	**0,11**	**0,13**	**0,21**	**0,20**	**0,21**	**0,19**	**0,17**	**0,00**	**0,01**	**0,00**	**0,30**
H. stagnalis, France- AF116018	**0,22**	**0,22**	**0,22**	**0,22**	**0,04**	**0,01**	**0,01**	**0,03**	**0,22**	**0,20**	**0,22**	**0,20**	**0,20**	**0,12**	**0,11**	**0,12**	**0,32**
H. stagnalis, UK- AF329041	**0,22**	**0,22**	**0,22**	**0,22**	**0,04**	**0,01**	**0,01**	**0,03**	**0,22**	**0,20**	**0,22**	**0,20**	**0,20**	**0,12**	**0,11**	**0,12**	**0,32**
H. stagnalis, Russia- KM095095	**0,23**	**0,22**	**0,23**	**0,23**	**0,04**	**0,00**	**0,01**	**0,02**	**0,23**	**0,20**	**0,23**	**0,20**	**0,20**	**0,11**	**0,11**	**0,11**	**0,31**
H. stagnalis, Mexico- HQ179855	**0,24**	**0,24**	**0,24**	**0,24**	**0,24**	**0,23**	**0,24**	**0,26**	**0,24**	**0,23**	**0,24**	**0,25**	**0,24**	**0,22**	**0,22**	**0,22**	**0,31**
H. stagnalis, South Africa- HQ179860	**0,24**	**0,23**	**0,24**	**0,24**	**0,23**	**0,23**	**0,24**	**0,26**	**0,24**	**0,23**	**0,24**	**0,26**	**0,24**	**0,21**	**0,22**	**0,21**	**0,29**
H. stagnalis, Mexico- HQ179862	**0,22**	**0,22**	**0,22**	**0,22**	**0,20**	**0,20**	**0,21**	**0,23**	**0,22**	**0,24**	**0,22**	**0,20**	**0,18**	**0,18**	**0,18**	**0,18**	**0,29**
H. stagnalis, OH-USA- KM196604	**0,24**	**0,23**	**0,24**	**0,24**	**0,23**	**0,20**	**0,20**	**0,23**	**0,24**	**0,00**	**0,24**	**0,22**	**0,21**	**0,20**	**0,20**	**0,20**	**0,28**
H. californica HQ686307	**0,22**	**0,21**	**0,22**	**0,22**	**0,22**	**0,22**	**0,23**	**0,25**	**0,22**	**0,21**	**0,22**	**0,16**	**0,06**	**0,19**	**0,19**	**0,19**	**0,30**
H. modesta AF329040	**0,24**	**0,24**	**0,24**	**0,24**	**0,10**	**0,09**	**0,09**	**0,11**	**0,24**	**0,20**	**0,24**	**0,20**	**0,18**	**0,09**	**0,09**	**0,09**	**0,34**
H. modesta HQ179853	**0,23**	**0,24**	**0,23**	**0,23**	**0,17**	**0,16**	**0,17**	**0,19**	**0,23**	**0,19**	**0,23**	**0,20**	**0,17**	**0,16**	**0,16**	**0,16**	**0,32**
H. modesta-JF319990	**0,00**	**0,00**	**0,00**	**0,00**	**0,24**	**0,23**	**0,24**	**0,26**	**0,00**	**0,24**	**0,00**	**0,09**	**0,20**	**0,21**	**0,22**	**0,21**	**0,12**
H. modesta KM611981	**0,24**	**0,23**	**0,24**	**0,24**	**0,10**	**0,09**	**0,09**	**0,11**	**0,24**	**0,22**	**0,24**	**0,20**	**0,18**	**0,10**	**0,10**	**0,09**	**0,32**
H. modesta KM612015	**0,24**	**0,23**	**0,24**	**0,24**	**0,09**	**0,08**	**0,09**	**0,10**	**0,24**	**0,22**	**0,24**	**0,20**	**0,18**	**0,09**	**0,09**	**0,09**	**0,34**
H. modesta KP176608	**0,01**	**0,02**	**0,01**	**0,01**	**0,24**	**0,23**	**0,24**	**0,26**	**0,01**	**0,24**	**0,01**	**0,10**	**0,19**	**0,21**	**0,21**	**0,21**	**0,12**
H. ghilianii AF329035	**0,31**	**0,30**	**0,31**	**0,31**	**0,28**	**0,29**	**0,30**	**0,32**	**0,31**	**0,30**	**0,31**	**0,31**	**0,33**	**0,29**	**0,30**	**0,29**	**0,39**
G. complanata AY047321	**0,29**	**0,30**	**0,29**	**0,29**	**0,27**	**0,27**	**0,28**	**0,31**	**0,29**	**0,30**	**0,29**	**0,26**	**0,27**	**0,26**	**0,25**	**0,25**	**0,36**
H. verbana KU216244	**0,46**	**0,45**	**0,46**	**0,46**	**0,42**	**0,45**	**0,46**	**0,50**	**0,46**	**0,56**	**0,46**	**0,45**	**0,40**	**0,42**	**0,43**	**0,42**	**0,60**

……continued on the next page

SUPPLEMENTARY TABLE 5. (Continued)

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
HLHws1- MF158963																	
SH1- MF158964																	
SH2- MF158965																	
SH3- MF158966																	
ICELAND- MF150168																	
12cTS- MF150165																	
16dTS- MF150166																	
17bTS- MF150167																	
GREENWICH- MF158967																	
FPWS2- MF158968																	
H. stagnalis, CR- JN692263																	
H. stagnalis, Montana- JN692264																	
H. stagnalis (*serendipitous*)- JN692265																	
H. stagnalis, Oregon- JN692266																	
H. stagnalis, EL- JN692267																	
H. stagnalis, York- JN692268																	
H. stagnalis, FP- JN692269																	
H. stagnalis, Canada- KM612173																	
H. stagnalis, France- AF116018	**0,12**																
H. stagnalis, UK- AF329041	**0,12**	**0,00**															
H. stagnalis, Russia- KM095095	**0,11**	**0,01**	**0,01**														
H. stagnalis, Mexico- HQ179855	**0,22**	**0,22**	**0,22**	**0,23**													
H. stagnalis, South Africa- HQ179860	**0,21**	**0,22**	**0,22**	**0,23**	**0,02**												
H. stagnalis, Mexico- HQ179862	**0,18**	**0,21**	**0,21**	**0,20**	**0,28**	**0,27**											
H. stagnalis, OH-USA- KM196604	**0,20**	**0,20**	**0,20**	**0,20**	**0,23**	**0,23**	**0,24**										
H. californica HQ686307	**0,19**	**0,22**	**0,22**	**0,23**	**0,25**	**0,24**	**0,19**	**0,21**									
H. modesta AF329040	**0,09**	**0,09**	**0,09**	**0,09**	**0,22**	**0,22**	**0,20**	**0,20**	**0,20**								
H. modesta HQ179853	**0,16**	**0,16**	**0,16**	**0,17**	**0,22**	**0,23**	**0,23**	**0,19**	**0,19**	**0,15**							
H. modesta-JF319990	**0,21**	**0,23**	**0,23**	**0,23**	**0,24**	**0,24**	**0,22**	**0,24**	**0,22**	**0,25**	**0,24**						
H. modesta KM611981	**0,10**	**0,10**	**0,10**	**0,09**	**0,22**	**0,22**	**0,20**	**0,22**	**0,20**	**0,02**	**0,16**	**0,24**					
H. modesta KM612015	**0,09**	**0,09**	**0,09**	**0,08**	**0,22**	**0,23**	**0,20**	**0,22**	**0,20**	**0,02**	**0,16**	**0,24**	**0,01**				
H. modesta KP176608	**0,21**	**0,23**	**0,23**	**0,23**	**0,24**	**0,23**	**0,21**	**0,24**	**0,20**	**0,25**	**0,23**	**0,02**	**0,24**	**0,24**			
H. ghilianii AF329035	**0,29**	**0,29**	**0,29**	**0,29**	**0,34**	**0,33**	**0,32**	**0,30**	**0,33**	**0,32**	**0,33**	**0,31**	**0,30**	**0,31**	**0,29**		
G. complanata AY047321	**0,26**	**0,27**	**0,27**	**0,27**	**0,29**	**0,31**	**0,26**	**0,30**	**0,28**	**0,29**	**0,30**	**0,30**	**0,29**	**0,28**	**0,29**	**0,25**	
H. verbana KU216244	**0,42**	**0,45**	**0,45**	**0,46**	**0,50**	**0,50**	**0,47**	**0,56**	**0,43**	**0,46**	**0,50**	**0,46**	**0,47**	**0,46**	**0,45**	**0,43**	**0,38**