Molecular Characterization and Redescription of *Placobdella nuchalis* Sawyer and Shelley, 1976 (Hirudinida: Glossiphoniidae)

William E. Moser[1, 5], Dennis J. Richardson[2], Charlotte I. Hammond[2], Eric Lazo-Wasem[3], and Anna J. Phillips[4]

[1]Smithsonian Institution, National Museum of Natural History, Department of Invertebrate Zoology, Museum Support Center MRC 534, 4210 Silver Hill Road, Suitland, MD 20746 (e-mail: moserw@si.edu)

[2]Department of Biological Sciences, Quinnipiac University, 275 Mt. Carmel Avenue, Hamden, CT 06518 (e-mail: Dennis.Richardson@quinnipiac.edu and Charlotte.Hammond@quinnipiac.edu)

[3]Division of Invertebrate Zoology, Peabody Museum of Natural History, Yale University, P.O. Box 208118, New Haven, CT 06520 (e-mail: eric.lazo-wasem@yale.edu)

[4]Smithsonian Institution, National Museum of Natural History, Department of Invertebrate Zoology, 10th St and Constitution Ave, NW, Washington, DC 20560-0163 (e-mail: phillipsaj@si.edu)

[5]Corresponding Author

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Abstract: *Placobdella nuchalis* Sawyer and Shelley, 1976 was originally described based on specimens from Four Hole Swamp and a stream near Ashepoo River in South Carolina and Merchant's Millpond and Nine Mile Creek in North Carolina. Leeches collected during August 2014 and August 2015 from Four Hole Swamp (type locality) and Merchant's Millpond facilitated a redescription and molecular characterization of *P. nuchalis*. Two additional specimens of *P. nuchalis* were collected on 10 August 2014 from Nassawango Creek, Maryland representing a state distribution record. *Placobdella nuchalis* has a brownish green dorsum with a dark medial line interrupted three times by yellow/cream spots, a pair of paralateral rows of unpigmented papillae with adjacent dark green papillae and a distinct discoid head. Molecular comparison of CO-I sequence data from a specimen of *P. nuchalis* from Four Hole Swamp (type locality) revealed a 94.5% similarity to a specimen collected from Merchant's Millpond, identical to a specimen collected from Maryland, and 14.2% to 17.5% differences among other species of *Placobdella*.

Placobdella nuchalis was originally described by Sawyer and Shelley (1976) based on free-living specimens from Four Hole Swamp, Orangeburg County, and a stream near Ashepoo River, Colleton County in South Carolina and Merchant's Millpond, Gates County, and Nine Mile Creek, Duplin County in North Carolina. Sawyer and Shelley (1976) designated Four Hole Swamp, Orangeburg County, South Carolina as the type locality. Sawyer and Shelley (1976) also reported *P. nuchalis* from Duke Swamp (Gates County) and Hoggard Mill Swamp (Bertie County) North Carolina. Shelley and Braswell (1981) reported a specimen of *P. nuchalis* from the left opercular flap of a bluegill sunfish, *Lepomis macrochirus* from Tranters Creek, Beaufort/Pitt County line, North Carolina. Additionally, Klemm (1982) reported *P.*

nuchalis from the chin of a channel catfish, *Ictalurus punctatus*, from Kentucky Lake near Paris Landing, Henry County, Tennessee and free-living specimens from the Susquehanna River, Three Mile Island, Dauphin County, Pennsylvania, and the Ogeechee and Canoochee Rivers in Georgia. Klemm (1982) also redetermined a specimen from the eye of a common carp, *Cyprinus carpio*, from the Ohio River in Kentucky, identified by White (1977) as *Placobdella parasitica*, to be *P. nuchalis. Placobdella nuchalis* appears to be a temporary parasite of freshwater fishes, usually collected free-living, and infrequently collected since its original description.

During the course of faunistic surveys of leeches of the eastern United States, specimens of *P. nuchalis* were collected providing a new geographic distribution record and facilitating the molecular characterization and redescription of the species.

Materials and Methods

On 13 August 2015, two specimens of *P. nuchalis* were collected from the type locality, Four Hole Swamp, Orangeburg County, South Carolina (33°21’52”N 080°33’41”W) and on 08 August 2014, five specimens were collected from the paratype locality, Merchant’s Millpond, Gates County, North Carolina (36°25’56”N 076°41’52”W). Molecular characterization was based upon a specimen from the type locality (GenBank XX######; USNM XXXXXXX) and a specimen from Merchant’s Millpond, a paratype locality (GenBank XX######; USNM XXXXXXX). In addition, on 10 August 2014, two free-living specimens of *P. nuchalis* were collected from Nassawango Creek, Worcester County, Maryland (38°15’46”N 075°27’46”W) representing a new geographic distribution record (USNM XXXXXXX and XXXXXXX). Specimens were relaxed, examined, and fixed as described by Moser *et al.* (2006). Terminology for plane shapes follows Clopton (2004). Specimens were deposited in the Smithsonian Institution, National Museum of Natural History (USNM), Washington, District of Columbia and the Peabody Museum of Natural History (YPM IZ), Yale University, New Haven, Connecticut.

DNA Analyses

Molecular analyses were conducted on newly collected material according to Richardson *et al.* (2010) as follows: DNA was isolated from the caudal suckers of individual leeches with the DNeasy Blood & Tissue Kit from Qiagen (Cat. No. 69504), following the protocol given for the purification of total DNA from animal tissues (spin-column). For the proteinase K treatment step, tissue samples were lysed overnight at 56^{o}C. DNA was eluted from the spin columns with 150 µl of buffer.

Polymerase chain reactions (PCR) were prepared using the Illustra PuRe Taq Ready-To-Go PCR beads from GE Health Care (Cat. No. 27-9559-01). Primers were purchased from Invitrogen and were comprised of 2 primers each for cytochrome c oxidase subunit I (CO-I) as specified by Light and Siddall (1999). Specifically the CO-I primers were LCO1490 (5'GGTCAACAAATCATAAAGATATTGG 3') and HCO2198 (5'TAAACTTCAGGGTGACCAAAAAATCA 3'). Final volume of PCR reactions was 25 µl with 2 µl of leech genomic DNA added per reaction. DNA was amplified under the following PCR conditions: 94^{o}C for 5 min.; 35 cycles of (94^{o}C for 30 sec, 50^{o}C for 30 sec, 72^{o}C for 45 sec); 72^{o}C for 7 min. Following PCR, samples were cleaned up using a QIAquick PCR purification kit from Qiagen (Cat. No. 28104). Purified PCR products were sequenced using the HCO2198 primer and the LCO1490 primer for the Cytochrome c oxidase subunit I products by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. The DNA sequences were aligned using Clustal W version 2 (Larkin *et al*. 2007) and checked manually using SeaView 4 (Gouy *et al.* 2010) and then analyzed using PAUP* 4.0b10 (Swofford 2002) and compared to other leech DNA sequences contained within Genbank. Uncorrected p distance was calculated using PAUP*.

Results and Discussion

Specimens collected from Four Hole Swamp, Orangeburg County, South Carolina (holotype locality) and Merchant's Millpond, Gates County, North Carolina (paratype locality) were consistent with the description of *Placobdella nuchalis* by Sawyer and Shelley (1976) and provided the basis for the

redescription and molecular characterization of the species. The redescription is based on the type series (USNM 51529 – 51532) and recently collected specimens from the holotype locality (Four Hole Swamp: USNM XXXXXXX, YPM IZ XXXXXX) and the paratype locality (Merchant's Millpond: USNM XXXXXXX - XXXXXXX, YPM IZ XXXXXX - XXXXXX)(note: USNM XXXXXXX – XXXXXXX were dissected).

Placobdella nuchalis Sawyer and Shelley, 1976
(Figs. 1 – 2)

Diagnosis. External Morphology: Body roughly elliptoid with cephalic region demarcated from the body by nuchal constriction (shallowly to very broadly orbicular discoid head); length of preserved mature specimens 14.6 – 21.0 mm long (n=7), mean 17.2 mm, width 7.5 – 8.4 mm, mean 7.8 mm. Dorsum base color brownish green with yellow/cream and dark green pigmentation and unpigmented stripes from discoid head to caudal sucker. Individual specimens vary from non-papillated to lightly papillated (Figure 1). When present, two pair of yellowish paramedial preanal papillae. Dorsum with a dark medial line interrupted three times by yellow/cream spots; a pair of paralateral rows of unpigmented papillae with adjacent dark green papillae; and a pair of lateral rows of yellow/cream spots. Discoid head with two eyespots (separated by the diameter of an eyespot) in small unpigmented "mask." Caudal sucker orbicular with a diameter of 2.1 – 2.7 mm, mean 2.4 mm, with two rows of spots, and small radiating papillae on lateral margins (appear as ridges). Ventrum unpigmented with two annuli between male and female gonopores.

Alimentary tract: Minute proboscis pore on the rim/lip of the anterior sucker. Blunt-tipped proboscis that is nearly cylindrical and slightly enlarged at base (Figure 2). Near the base of the proboscis, pair of long salivary gland ductals (with salivary cells intersperced in ductal mass) empty into diffuse salivary glands (which extend two second pair of crop ceca) (Figure 2). Slim, flaccid esophagus which extends

from the base of proboscis and with a pair of sac-like mycetomes. Seven pair of diverticulated crop ceca, last pair extending posteriorly and diverticulated into four sections. Four pair of intestinal ceca. Simple rectum opening to anus, located one annulus anteriad of the caudal sucker.

Reproductive system: *Male*. Male gonopore slightly raised. Paired broadly elliptoid atrial cornue extending laterally from male gonopore into robust, loosely coiled muscular ejaculatory ducts, recurving posteriorly to seminal vesicles and narrow vas deferentia connecting to testisacs. Six pair of testisacs, each testisac located between pair of crop ceca. *Female*. Female gonopore simple, opening to pair of bifurcated ovisacs and located within coelomic space that is attached on the ventral body wall. Ovisac length depends on the reproductive condition of the leech.

Molecular comparison of 626 nucleotides of CO-I revealed an intraspecific difference of 5.5% (34 nucleotides) between one specimen of *P. nuchalis* collected from Four Hole Swamp, South Carolina (type locality) (GenBank XX######, USNM XXXXXXX) and a specimen of *P. nuchalis* (GenBank XX######, USNM XXXXXXX) collected from Merchant's Millpond, North Carolina (paratype locality). A specimen of *P. nuchalis* from Maryland (GenBank XX######, USNM XXXXXXX) was identical to the specimen of *P. nuchalis* collected from Four Hole Swamp, South Carolina (GenBank XX######, USNM XXXXXXX). Interspecific differences of 16.9% to 17.5 % (106 to 110 nucleotides) were found between two specimens of *P. nuchalis* (GenBank XX###### - XX######) and one specimen *of P. montifera* (GenBank AY047323; 14.2% to 16.5% differences (89 to 103 nucleotides) between two specimens of *P. nuchalis* (GenBank XX###### - XX######) and five specimens of *Placobdella ornata* (GenBank JQ812128–JQ812132) collected from the type locality (West River, New Haven County, Connecticut); 14.5% to 15.8% differences (91 to 99 nucleotides) were found between two specimens of *P. nuchalis* (GenBank XX###### - XX######) and two specimens of *Placobdella sophieae* (GenBank KF990594–KF990595) collected from Oregon; 14.6% to 17.4% differences (91 to 109 nucleotides) between two specimens of *P.*

nuchalis (GenBank XX###### - XX######) and seven specimens of *Placobdella cryptobranchii* (GenBank KF601755–KF601761) collected from Missouri and Arkansas; 15.9% to 16.4% differences (100 to 103 nucleotides) between two specimens of *P. nuchalis* (GenBank XX###### - XX######) and a specimen of *Placobdella picta* (GenBank AF116020); and 15.6% to 16.7% differences (98 to 105 nucleotides) between two specimens of *P. nuchalis* (GenBank XX###### - XX######) and five specimens of *Placobdella parasitica* collected from the type locality (Minnesota; GenBank KF058895–KF058899). *Placobdella nuchalis* is a distinct species with a 14.2% to 17.5% difference in CO-I sequence data among congeners and a moderate amount of genetic variation (5.5.% difference in CO-I sequence data) between specimens from the type (South Carolina) and paratype (North Carolina) localities.

Placobdella nuchalis does not have a confusing and problematic taxonomic history like some other members of the genus (see Moser *et al*. 2012a, 2012b, 2013, 2014). *Placobdella nuchalis* is distinguished from its congeners by its discoid head. *Placobdella montifera* also has a discoid head but is distinguished from *P. nuchalis* by possession of a more strongly papillated surface (*i.e.* three prominent, dorsal papillar rows that appear as keels or ridges). *Placobdella montifera* also has four pair of preanal papillae. Specimens of *P. nuchalis* are modestly papillated on the dorsal surface. When present, specimens of *P. nuchalis* have two pair of paramedial preanal papillae.

Placobdella nuchalis is infrequently encountered and known from several localities in the coastal plain of North Carolina and South Carolina (including the type locality), and also known from Georgia, Kentucky, Tennessee, and Pennsylvania (Sawyer and Shelley 1976; Klemm 1982, 1985). This study is the first report of *P. nuchalis* from Maryland. Practically nothing is known about the life history of *P. nuchalis*, except that a free-living specimen was found brooding eggs on 14 March 1972 (Sawyer and Shelley 1976). *Placobdella nuchalis* appears to be a temporary blood-feeding leech on fish. Reported hosts to date include *Lepomis macrochirus* (bluegill sunfish), *Ictalurus punctatus* (channel catfish), and

Cyprinus carpio (common carp) (White 1977; Shelley and Braswell 1981; Klemm 1982, 1985). Given the variety of reported hosts, other freshwater fish are potential hosts.

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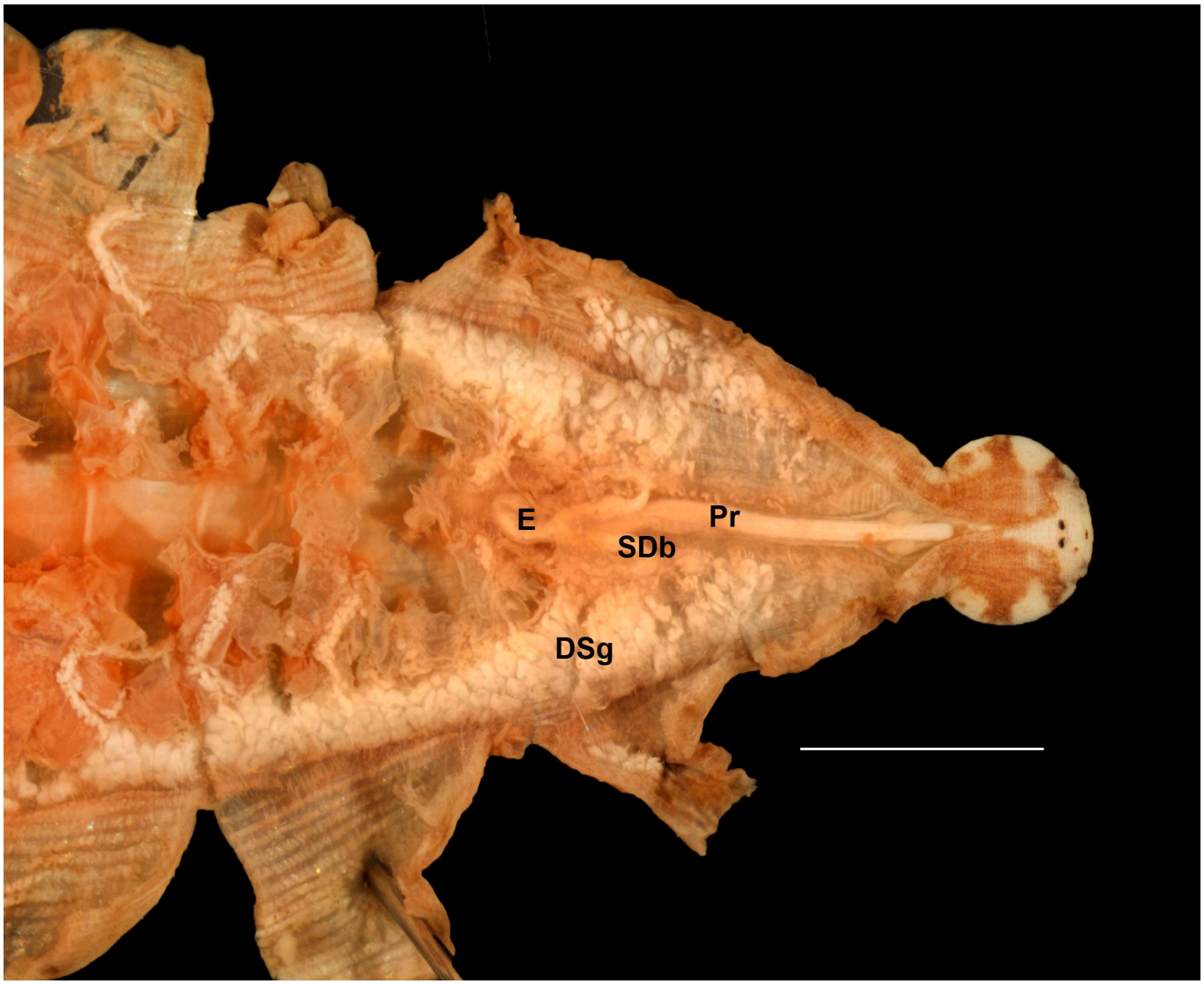
Figure Legends

Figure 1. Living specimens, dorsal surface of *Placobdella nuchalis* from Merchant's Millpond, Gates County , North Carolina, USA. A. USNM XXXXXXX, scale bar equals 3 mm. B. YPM IZ XXXXXX, scale bar equals 3 mm.

Figure 2. Dissected specimen of *Placobdella nuchalis* from Four Hole Swamp, Orangeburg County, South Carolina, USA, showing alimentary system (USNM XXXXXXX). Abbreviations: DSg, diffuse salivary glands; E, esophagus; Pr, Proboscis; SDb, Salivary ductal bundle. Scale bar equals 2 mm.


A
B


E
Pr
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