MOLECULAR VARIATION AND BIOGEOGRAPHY OF THE COMMON NORTH AMERICAN TURTLE LEECH, *PLACOBDELLA PARASITICA*

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ABSTRACT

Placobdella parasitica (Say, 1824) is one of the most commonly encountered turtle leeches in North America. Molecular analysis of individuals of *P. parasitica* representing various populations throughout its range in North America utilizing the cytochrome *c* oxidase subunit I gene, revealed the presence of 9 distinct groups: 1. *P. parasitica sensu stricto* containing members, including the neotype specimens, occurring broadly throughout the central United States westward from the Mississippi River to the Rocky Mountains and throughout southern Ontario, Canada and the upper mid-Western United States as far east as New York State, 2. West Virginia, 3. Mississippi/Alabama, 4. Northeast including New York, Massachusetts, and Vermont, 5. New England including Rhode Island, Massachusetts, and Connecticut, 6. North Carolina/West Virginia, 7. South Carolina, 8. Tennessee, 9. Florida. Both neighbor-joining and maximum likelihood analyses recovered an east-west split along the Appalachian Mountains with groups 1–3 clustering together and groups 4–9 clustering separately, with the exception of group 8 (Tennessee) that placed with the eastern groups. Group 1 includes specimens from a broad geographic distribution, yet with relatively low genetic variation, a pattern observed in other glossiphoniid species in North America. The group of with members east of the Applachian Mountains are more tightly clustered by locality. This leech species is known to parasitize several turtle species, including *Chrysemys picta*, the painted turtle that originated in the central Gulf Coast region and dispersed northward representing a recolonization following Pleistocene glaciation. The neighbor-joining tree and pairwise distance data could suggest that *P. parasitica* has a similar phylogeographic pattern and dispersal history with its turtle hosts. In view of the morphological uniformity exhibited among the various groups, *P. parasitica* is provisionally considered to be a widely distributed, molecularly variable species.

Key Words: *Placobdella parasitica,* biogeography, COI, Turtle leeches, *Chrysemys,* Testudines, Rhynchobdellida, Hirudinea, Glossiphoniidae, Glossiphoniiformes

INTRODUCTION

Placobdella parasitica (Say, 1824) is one of most commonly encountered turtle leeches in North America found on a wide range of turtle hosts (Sawyer, 1972; Moser et al., 2005). Moser et al. (2013) provided a redescription, molecular characterization, and designation of a neotype of *P. parasitica* based on specimens collected from the type locality of Lily Lake, Waseca County, Minnesota, USA. Moser et al. (2013) also noted the high degree of variability in external dorsal morphology exhibited by *P. parasitica* stating that coloration of the dorsal surface ranged from simple to elaborate and that the dorsum was usually smooth but that in some specimens, numerous sensillae were noted.

In the course of studies on the turtle leeches of New England (Richardson et al., 2015), many individuals of *P. parasitica* were collected. Subsequent molecular analysis of the cytochrome *c* oxidase subunit I (COI) gene of specimens of *P. parasitica* revealed differences in 47-51 of 658 nucleotides (7.2-7.8%) relative to specimens from the type locality (Minnesota). Although specimens of *P. parasitica* from New England exhibited a range in external morphological variability similar to that exhibited by specimens from the type locality (Figures. 1-2), no consistent differences in internal or external anatomy were detected between specimens from the two populations. The genetic differences between these New England and Minnesota populations prompted a broader molecular survey of *P. parasitica* to more fully characterize molecular variation exhibited by this species across its distribution in North America.

MATERIALS AND METHODS

Specimens of *P. parasitica*, collected from 2013 – 2019 at various localities throughout the United States east of the Rocky Mountains (Table 1 and Figure 1) were subjected to molecular analysis according to Richardson et al. (2010) as follows:

Purification of DNA was accomplished using the DNeasy Blood and Tissue Kit from Qiagen (Cat. No. 69504). For the proteinase K treatment step, tissue samples were lysed overnight at 56°C. Subsequently, DNA was eluted from the spin columns.

Polymerase chain reactions were prepared using the Illustra PuRe Taq Ready-To-Go PCR beads from GE Health Care (Cat. No. 27-9559-01). The COI primers used were as specified by Folmer et al. (1994) and Light and Siddall (1999). Amplification of DNA occurred under the following conditions: 94°C for 5 min; 35 cycles of (94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec); 72°C for 7 min. Samples were cleaned using a QIAquick PCR purification kit from Qiagen (Cat. No. 28104). Purified products were sent to the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University for sequencing.

Chromatograms were edited and assembled in Geneious Prime 2019.2.3–2020.1.2. All novel molecular sequences were deposited in GenBank (see Table 1). Additionally, COI sequences of specimens collected from Ontario, Canada were acquired from GenBank for inclusion in molecular comparisons (Siddall and Burreson, 1998; Oceguera-Figueroa et al., 2016; de Carle et al., 2017; deWaard, J.R., unpublished). Multiple sequence alignments were carried out with the MAFFT multiple sequence alignment plugin in Geneious Prime (Katoh and Standley, 2013) applying default settings. The alignment was checked by eye for gaps and the sequences were translated to amino acids as an independent assessment of

sequence quality. Pairwise (uncorrected 'p') sequence distances also were calculated in Geneious Prime. The neighbor joining tree (NJ) was generated using SeaView version 4 (Guoy et al., 2010) using the Jukes-Cantor model including 500 bootstrap replicates. The data was partitioned by codon position for a total of three partitions. PartitionFinder2 was used to estimate the optimal model of nucleotide evolution and best partitioning scheme, testing each codon position as a separate partition under the 'user' algorithm (Lanfear, et al., 2016; Guindon, et al., 2010). Estimation of substitution models by codon position was carried out using ModelFinder within IQTREE (Kalyaanamoorthy et al., 2017) resulting in the following models as best fit by partition by the BIC: first codon position = F81+F, second codon position = TPM2+F+G4, and third codon position =TNe+G4). Maximum likelihood analysis was performed using IQTREE 1.6.12 (Nguyen, et al., 2015) using the models suggested for each unlinked partition, the *-spp* option that allowed each partition to have its own evolutionary rate, and included 1000 ultrafast bootstraps (UFBOOT2) approximations (Hoang et al., 2018). *Helobdella bowermani*, *Helobdella modesta*, and *Helobdella octatestisaca* were designated as outgroups. Trees were visualized in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and edited with Adobe Illustrator CC.

Representative specimens were deposited in the Division of Invertebrate Zoology, Peabody Museum of Natural History (YPM IZ), Yale University, New Haven, Connecticut, the Smithsonian Institution, National Museum of Natural History, Department of Invertebrate Zoology (USNM), and the North Carolina Museum of Natural Sciences (NCSM).

RESULTS

Molecular analysis of 52 specimens from 21 states in the United States of America and 5 specimens from Ontario, Canada representing the assemblage of leeches embodied by the current concept of *P. parasitica* as clarified by Moser et al. (2013), revealed the presence of 9 well-supported lineages (Figs. 4 and 5) exhibiting molecular differences of 0–2.7% difference within groups and 1.6 – 9.4% between groups in COI nucleotide sequences (Table 2). Labelled groups were defined by well-supported clades (BS greater than or equal to 80) in the NJ tree with p-distance values of less than or equal to 2.7% between members of the same group. Henceforth, the 9 groups shall be referred to the following names (and numbers) with no intention of taxonomic inference: 1. *P. parasitica sensu stricto* (including specimens collected from the type locality), 2. West Virginia, 3. Mississippi/Alabama, 4. Northeast, 5. New England, 6. North Carolina/West Virginia, 7. South Carolina, 8. Tennessee, 9. Florida. Collection locations of individual group members are indicated in Table 1 and Figures 3-5.

The molecular dataset included 57 individuals (52 of *P. parasitica*, three of *Helobdella* species as outgroups) and 658 aligned characters. The log-likelihood of the topology produced by the ML analysis was 2626.836 and the topology is depicted in Figure 5. The numbered groups are composed of the same members in the ML and NJ trees. Both ML and NJ analyses recovered *P. parasitica sensu stricto* (NJ BS = 96, ML BS = 63) with groups 2 and 3 containing members from west of the Appalachian mountains (NJ BS = 98, ML BS = 91). The NJ tree recovered two primary clades with reciprocal monophyly, one included groups 1 – 3 and members west of the Appalachian Mountains, and the other containing groups 4 – 9 and members east of the mountain range. Interestingly, group 8 from western Tennessee placed within the eastern group. The ML tree recovered the western group (groups 1–3; ML BS = 91) as reciprocally monophyletic to a subset of the eastern group containing groups 4, 5, and 6 (ML BS = 100). The placement of groups 7, 8,

and 9 differed between NJ and ML analyses. In the ML analysis, groups 7, 8, and 9 place as paraphyletic lineages at the base of the tree (group 7 + groups 1–6 ML BS = 50, group 8 + groups 1–6, 7, 9 ML BS =100, group 9 + groups 1–7 ML BS = 76). Both trees have strong support for each of groups 3, 5, and 6 (NJ BS=100, ML BS=100 for each group), and the monophyly of groups 4, 5, and 6 (NJ BS=100, ML BS=100).

DISCUSSION

The NJ and ML trees recovered two well-supported clades, one composed of specimens west of the Appalachian Mountains (groups 1–3) and a subset of specimens east of the mountain range (groups 4– 6). Groups 7, 8, 9 from South Carolina, western Tennessee, and Florida, respectively, placed near each other and clustered with the eastern group in the NJ analysis, but their placement in each tree was poorly supported. Sequences of members of group 1 were relatively uniform (group 1: 0–2.7% difference) despite being collected across a broad geographic range from the Gulf Coast north to Minnesota and Ontario. This is in contrast to specimens collected east of the Appalachians that cluster with other individuals from similar geographic regions with strong support. This geographic structure in the tree could indicate eastern and western distinction within the species, but thorough morphological comparison did not reveal any obvious differences. Molecular phylogenetic analyses indicate that gene flow has been interrupted between the eastern (groups 1–3) and western groups (groups 4–9) with the Appalachian Mountains possibly acting as a geographic barrier. East of the Appalachian Mountains, a phylogeographic pattern is recovered but with modest support that we attribute to using a single mitochondrial gene fragment.

It is curious to note lower genetic distances between specimens at a higher latitudes (1.6–2.9% between groups 4, 5, and 6, and 3.0–4.4% between groups 1 and 2) than specimens collected in southern regions (4.8–6.0% between groups 1 and 3, and 5.4% between groups 7 and 9) on both sides of the mountain range. This could indicate that the populations in the south are more established and with longer histories than northern populations, possibly a result of recolonization of the northern regions post-glaciation via their turtle hosts. Less than 20,000 years have elapsed since aquatic life was extirpated from much of northern North America, including New England and the upper Midwest extending south to present day southern Missouri, during the last ice age. Thus, the aquatic fauna of these regions must be of recent colonization, subsequent to the final period of the Pleistocene Ice Age, the Wisconsonian glaciation. If the Southern Atlantic and Central Gulf Coast regions represent the origins of *P. parasitica* that dispersed north with their turtle hosts, then these areas might also have served as refugia for the leeches, and presumably at least some of the species of turtles serving as hosts during the most recent glacial advance and maximum.

Turtles dispersing north subsequent to glacial retreat would have been accompanied by their parasites and commensals, and there is a general expectation that recent evolutionary signatures among host lineages will be mirrored among the lineages of their parasites.

Placobdella parasitica is a generalist parasite of freshwater turtles (Moser, 1995; Watermolen, 1996; Moser et al., 2013) and of the known hosts parasitized by this leech species, *C. picta* and *C. serpentina* are the most vagile. There has been a general consensus that major events sundering populations of turtles and other aquatic organisms occurred during the last glacial advance and are coincident with present-day rivers such as the

Apalachicola, Tombidgee, and Mississippi (Soltis et al., 2006). The turtles themselves display varying patterns of spatially-explicit genetic structure and inferred historical relationships among lineages. Some, such as *Apalone* species, *Sternotherus* species, *Trachemys scripta*, *Deirochelys reticularia*, and *Macroclemys temminckii* (all species known to host *P. parasitica*), show substantial degrees of genetic isolation between eastern Gulf, western Gulf, and/or Atlantic coast populations (Walker et al., 1995; Walker et al., 1997; Avise et al., 1992; Walker and Avise, 1998; Roman et al., 1999; Weisrock and Janzen, 2000). These patterns are broadly consistent with the pattern for *P. parasitica* observed herein. However, other studies show different, or no, clear genetic divergence associated with geographical locale, such as for the common snapping turtle, *Chelydra serpentina* (Walker et al., 1998). Since members of these turtle species and others are competent hosts for *P. parasitica*, any one turtle species would not be the driving influence of patterns of isolation and range expansion that would inexorably lead to the pattern observed for just one of their many parasites.

If turtles are the primary mechanism of leech dispersal, then it might also be true that any phylogeographical pattern evident in the former will be replicated in the latter. Reid et al. (2019) evaluated evidence for the existence of multiple hypothesized refugia for painted turtles (*Chrysemys picta*), including Gulf Coast (roughly eastern Texas to Alabama) and East Coast (roughly North Carolina to Georgia) refugia. They used environmental niche models (ENMs) to evaluate habitat suitability over time and found that present-day habitats for painted turtles show almost no overlap with those at the last glacial maximum, and that there were no habitats available in the present U.S.A. at the last glacial maximum that could have supported turtles with habitat requirements identical to present-day *C. dorsalis* (=*C. picta dorsalis*), the southern lineage of painted turtles. Furthermore, their analyses, which included nuclear, mitochondrial, and microsatellite data, indicated that the best explanation for the genetic diversity in painted turtles was divergence during range expansion originating from a single refugium coincident with the modern-day southeastern U.S.A. rather than isolation and divergence in multiple allopatric refugia during glacial advance. This is most consistent with our ML tree, wherein a group of specimens from southern localities is basal to the rest of the tree, including those both east of and west of the Appalachian Mountains farther north.

Starkey et al. (2003) suggested that there were at least two dispersal events of painted turtles into the northern areas of the continent following glacial retreat, one into New England and one into the upper Midwest. This is most consistent with the results of the NJ analysis in the present investigation, in which there appears to be two geographical starting points of northern recolonization separated by the Appalachian Mountains and the Mobile River Basin.

Recent studies of the genetic variability of turtle leeches from the interior regions of North America have also uncovered far less variation than expected. Mack et al. (2019) found very low variation in COI sequences in *Placobdella rugosa* from Quebec, Canada west to Saskatchewan, and Nebraska, U.S.A. The only meaningful distinction was between a broadly western group of clades and those in the east, within which there was almost no phylogenetic signal. This mirrors what Mack and Kvist (2019) found for *Glossiphonia elegans* across much of the same territory.

The results of Mack et al. (2019) and Mack and Kvist (2019) are similar to the current findings of *P. parasitica sensu stricto* (group 1) with low genetic variability for COI

(0–2.7% difference) yet a broad distribution throughout the Midwestern United States into southeastern Canada, relative to the more narrow distributions of the other clusters in the south, southeast and eastern seaboard (groups 4-9). Based on the models provided by Bartlein et al. (1998), Starkey et al. (2003) suggested that a period of extreme aridification associated with the retreat of the Laurentide ice sheets peaking about 14,000 years ago may have had a deleterious effect on the aquatic fauna along a line extending roughly from present day Chicago, Illinois southward to Houston, Texas. This may have led to the lack of genetic variation of *Chrysemys* throughout the Great Plains. Likewise, these conditions may have contributed to the large contiguously uninterrupted range and genetic similarity of the *P. parasitica sensu stricto* (group 1).

Mack et al. (2019) suggested that the lack of genetic structure of *P. rugosa* is the result of the high dispersal potential of the turtle hosts as well as the rapid reintroduction of turtle hosts and their leeches to northern regions following glaciation. The lack of phylogeographical structure of *P. rugosa* and of *P. parasitica sensu stricto* (group 1) is consistent with rapid dispersal, after the most recent glacial retreat, to recolonize northern areas. More established populations of these species with longer histories would be expected to display higher genetic variability, as we observed in southern and eastern groups of *P. parasitica*.

Additional sampling and the addition of nuclear DNA sequence data will facilitate further resolution of the relationships between these groups. In view of the relative morphological uniformity exhibited among the various groups, *P. parasitica* is provisionally considered to be a widely distributed, molecularly variable species.

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FIGURE CAPTIONS

Figure 1. Images of living specimens of *Placobdella parasitica* from Connecticut, illustrating the diversity of dorsal pigmentation. 2A. YPM IZ 058137. 2B. YPM IZ 058131. 2C. YPM IZ 058239. 2D. YPM IZ 058136. 2E. YPM IZ 058236. 2F. YPM IZ 058138. 2G. YPM IZ 058130. 2H. YPM IZ 43251.

Figure 2. Images of living specimens of *Placobdella parasitica* from Minnesota (type locality), illustrating the diversity of dorsal pigmentation. 2A. YPM IZ 058091. 2B. YPM IZ 058092. 2C. YPM IZ 058093. 2D. YPM IZ 058094. 2E. YPM IZ 058095. 2F. YPM IZ 058096. 2G. USNM 1207630. 2H. USNM 1207632 (from Moser et al., 2013).

Figure 3. Map showing approximate collection localities of numbered group members (see Table 1) of *Placobdella parasitica* utilized in this study, relative to the Appalachian mountains. Western Group (1-3) in red and Eastern Group in blue (4-9).

Figure 4. Neighbor joining tree (NJ) of *Placobdella parasitica* based on mitochondrial COI sequence data. Bootstrap values above 70 are labeled at internodes. The scale represents genetic distances in substitutions per nucleotide. GenBank accession number and locality is provided for each specimen of *P. parasitica*.

Figure 5. Maximum Likelihood phylogeny (ln*L* = -2626.836) *of Placobdella parasitica* based on mitochondrial COI sequence data partitioned by codon position and with identical sequences removed. Maximum Likelihood bootstrap values above 70 are shown at internodes. The scale represents the number of nucleotide substitutions per site. GenBank accession number and locality is provided for each specimen of *P. parasitica*. Branches are drawn proportional to the amount of change.