

USE OF GENETIC SEQUENCING TO DETERMINE THE IDENTITY OF BLACK FLIES (FAMILY: SIMULIIDAE) IN THE SAN PEDRO RIVER (AZ, USA)

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Introduction:

The San Pedro River (Fig.1), located in the southeastern part of Arizona, is home to hundreds of migrating and residential birds, as well as many species of mammals, amphibians, and reptiles who live in the riparian area. The aquatic insects emerging from the river are a food source for the terrestrial consumers in the San Pedro. Insects and invertebrates play a big role as consumers in the San Pedro River riparian food web. The larvae of Simuliidae, a species of blackfly in the order Diptera, serve as a major role in stream ecosystems. By doing this study it can help us determine the health and water quality of the San Pedro River, as well as to be used in a long-term study in the determination of genetic changes within populations and communities occurring due to seasonal and intermittent stream flow. Simuliidae larvae grow in large numbers under favorable conditions in streams, and because larval densities are often so high, considerable amounts of sedimenting faecal pellets increase the local retention of organic material, thus providing nutrition for invertebrates and micro-organisms, as well as potentially fertilizing river margins. (Bjorn, 2004)

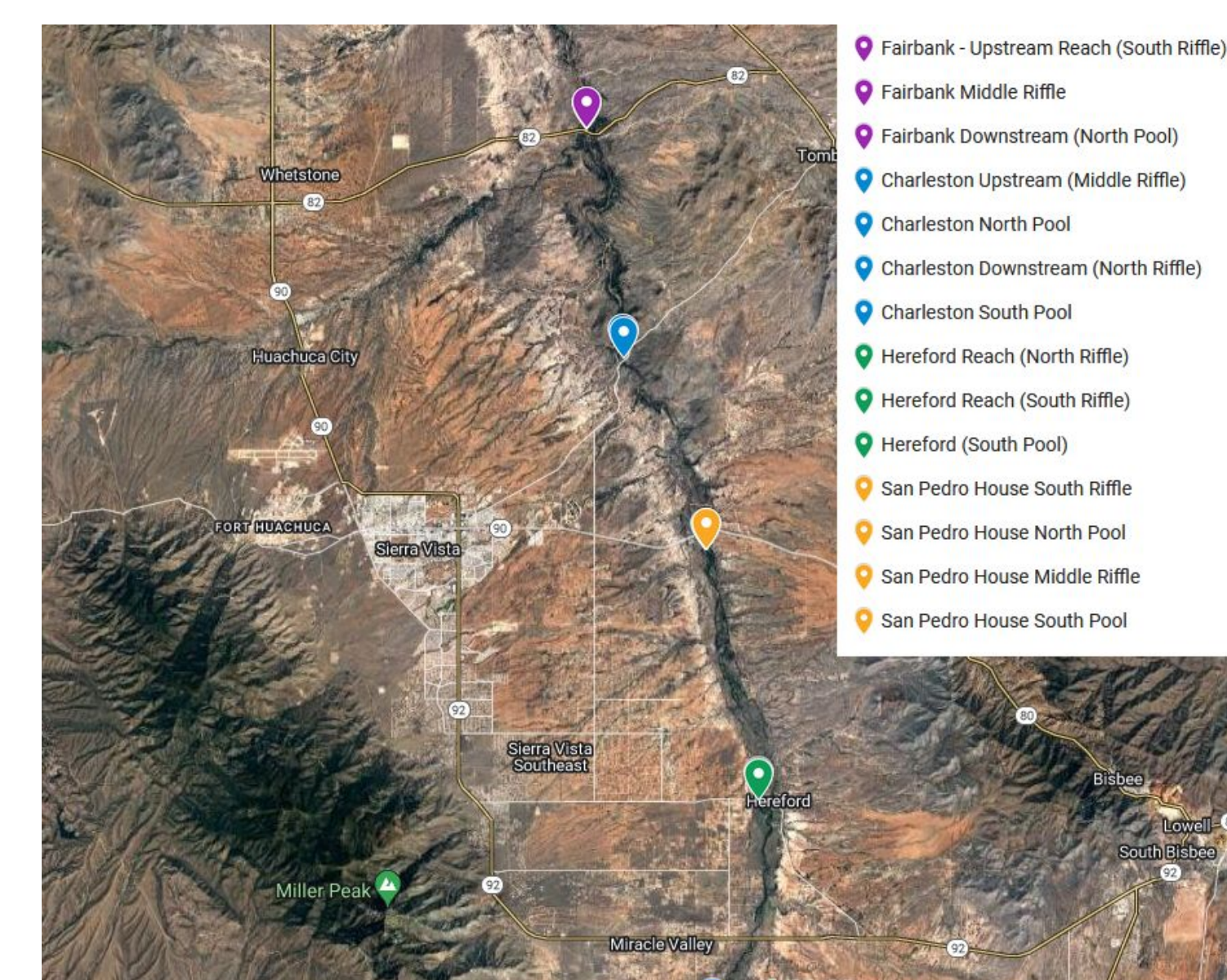


Fig. 1: San Pedro River collection sites with legend

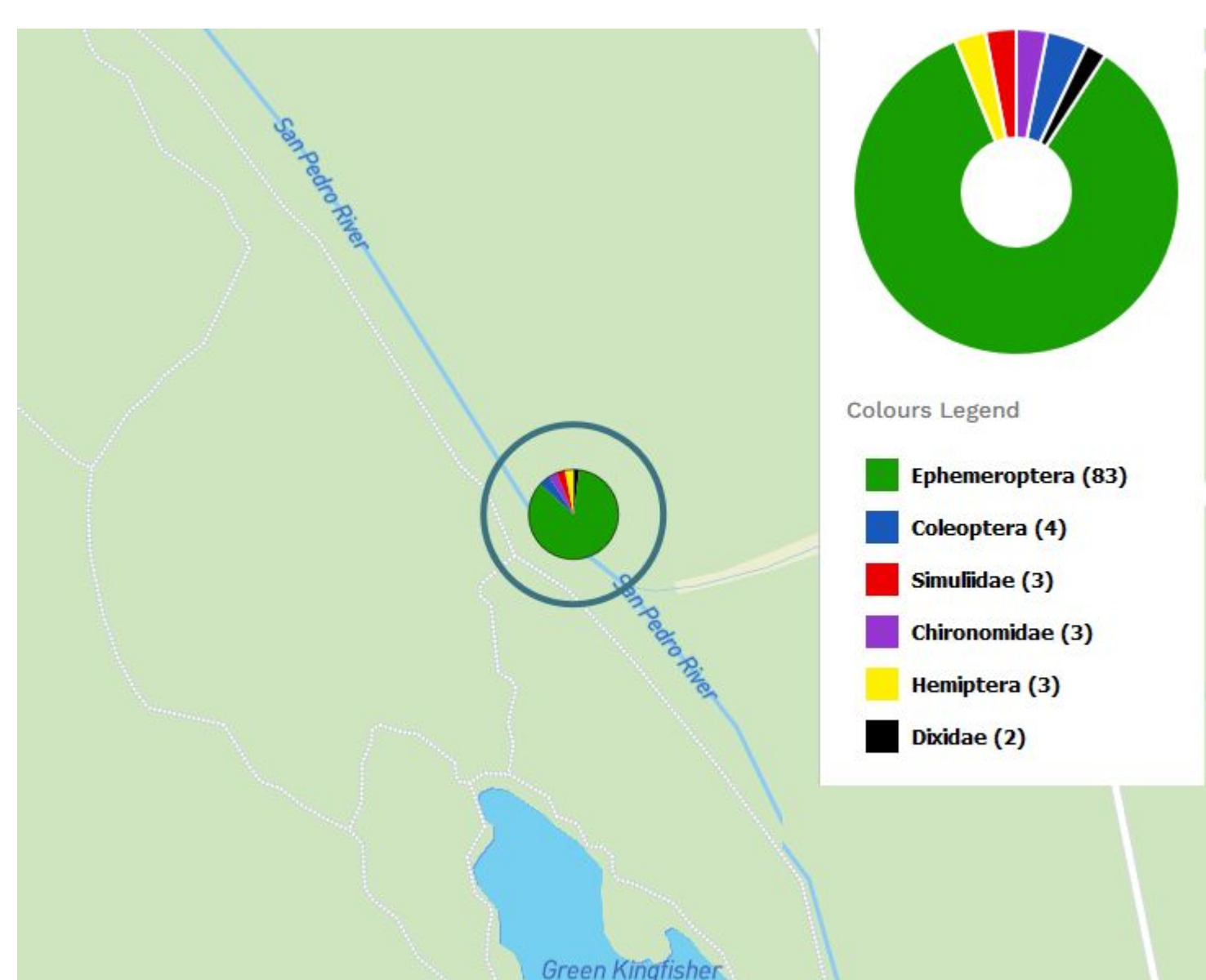


Fig. 2: San Pedro House collection site

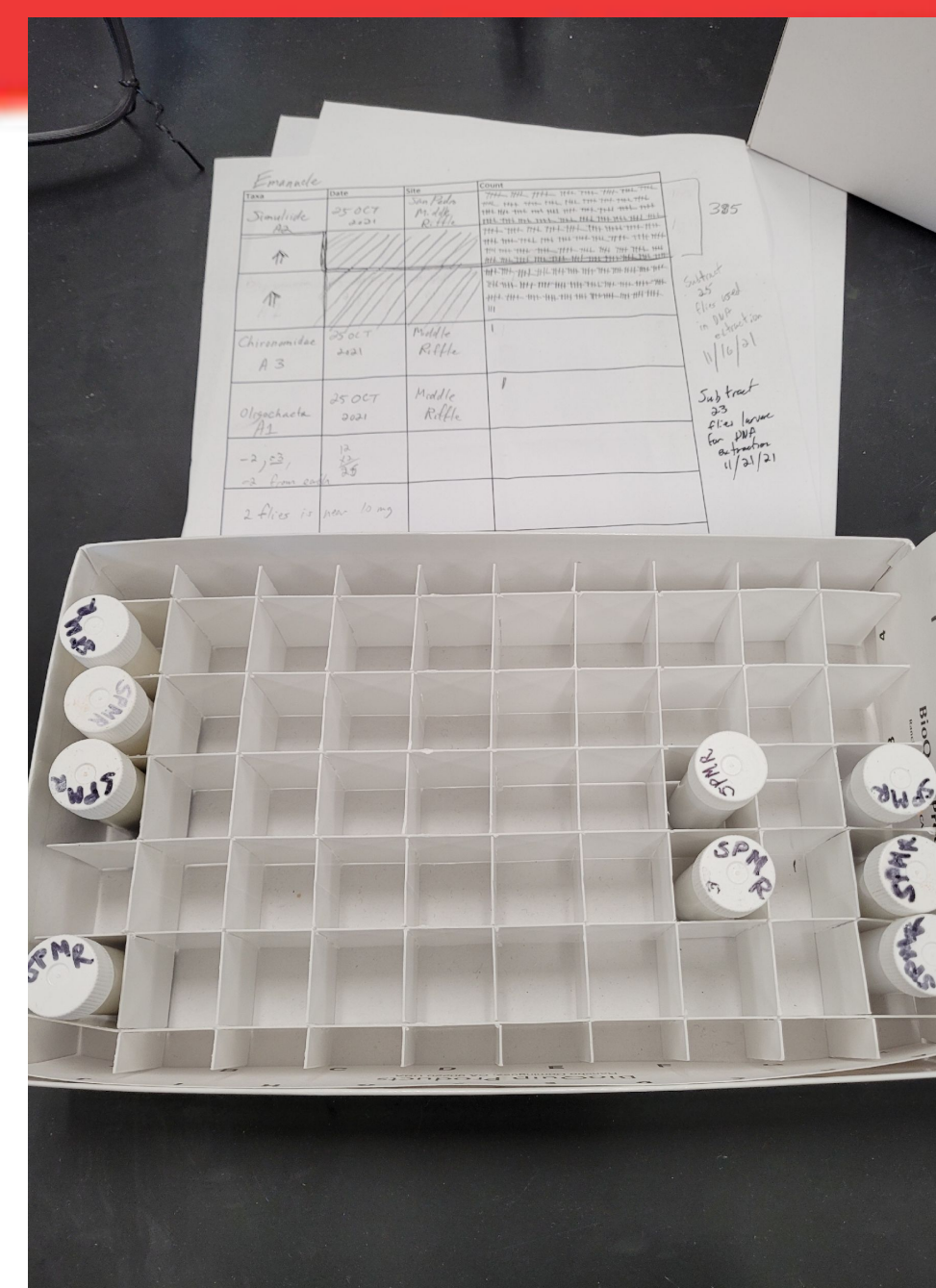
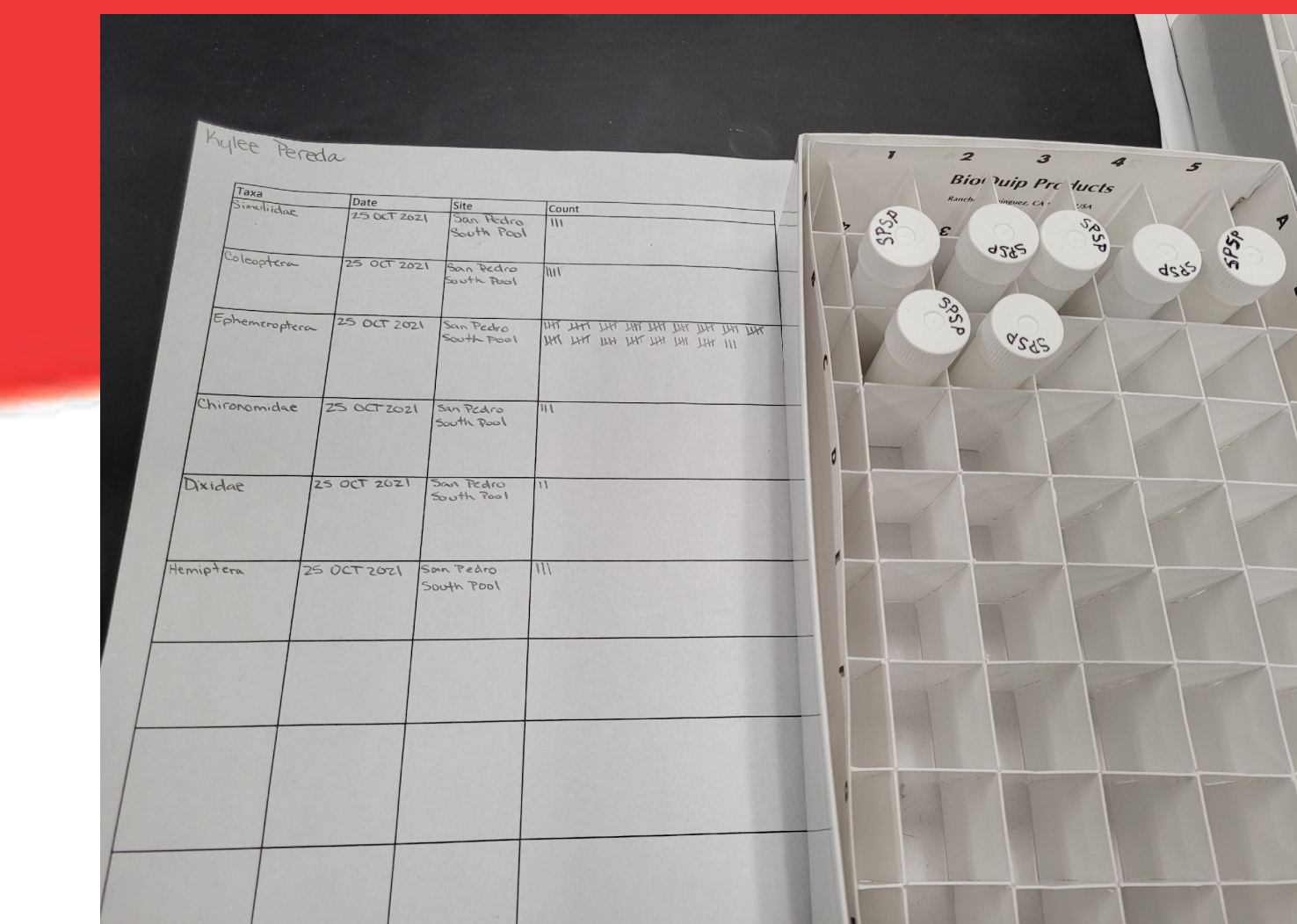


Fig. 4: Samples and counts of the different invertebrates collected from pools and riffles in the San Pedro River

Fig. 3: Microscopic view of the Simuliidae larvae collected at the San Pedro River site

Results:

Our initial BLAST search of the best sequences we obtained showed a species identification of *Simulium paynei* (Fig.5,6,7). The predicted and observed melt curve comparison also confirmed this finding. The predicted melt curve (Fig.8) showed a peak at 82°C and a secondary peak at 85°C. Our observed melt curve (Fig.9) showed similar peaks and therefore provided more evidence of close relationship of our unknown to the *S. paynei*. A phylogenetic tree (Fig. 10) was created with the samples we collected to show the relationship in DNA between them and other Simuliidae species. The tree showed a difference in data compared to our findings with the initial BLAST search. This could be due to the issue of using 3 larvae in order to get enough DNA to do the sequencing, with the possibility of the larva being two different species.

Procedure:

Aquatic invertebrate samples were collected from both riffles and pools from a site in the San Pedro River (Fig.2). In riffles, we used a Surber sampler (30 x 30cm, 1mm mesh) to collect aquatic macroinvertebrates. For pools, we used a D-net (~500µm mesh) with an effort of 10 s/m2. The invertebrate samples were stored and transferred to the lab in a 70% ethanol solution for identification. We used a dichotomous key to identify the samples to the family level. Due to the number of Simuliidae collected, we decided to use them to do DNA sequencing.

The PCR protocol was as follows:

1 min at 96°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min 30 s at 72°C; and finally, 7 min at 72°C

The melt curve protocol:

30 sec at 90°C, 1 min at 60°C, then slowly reach 95°C and hold for 30 sec. A point is taken at every degree change to create the melt curve.

We created a 50-microliter working solution by taking 5 microliters of stock primers and 45 microliters of TE buffer, creating a 10 microMolar concentration of each of the primers. Each PCR reaction was 25 microliters total: consisting of 1 microliter of each primer, 1 microliter of the DNA sample, and 22 microliters of CHAI PCR master mix (*What Is a PCR Master Mix?*).

Quantification of amplicon concentrations were conducted using a Qubit 4 Fluorometer. To clean the PCR product, we used Exo-SAP-IT following the protocols listed for this product. Samples of 10.5 microliters of each product were sent to Eurofins Genomics for sequencing along with primers.

Description	Scientific Name	Max Score	Total Query Cover	E value	Per. Match	Acc. Len	Accession
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1977	100%	0.0	98.06%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8278:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1975	100%	0.0	97.95%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-5099:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1975	100%	0.0	97.95%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-1931:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1975	100%	0.0	97.95%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-2484:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1968	100%	0.0	97.74%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-2484:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1930	100%	0.0	96.77%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4298:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1927	100%	0.0	96.41%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-3173:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1922	100%	0.0	96.45%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1922	100%	0.0	96.45%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1922	100%	0.0	96.45%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1922	100%	0.0	96.45%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1922	100%	0.0	96.45%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1916	100%	0.0	96.29%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1916	100%	0.0	96.29%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4894:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1914	100%	0.0	96.19%	658	KS281223.0

Fig. 5: Blast search for Exo1 sample

Description	Scientific Name	Max Score	Total Query Cover	E value	Per. Match	Acc. Len	Accession
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1929	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8278:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1929	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-5099:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1929	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-1931:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1929	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-2484:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-2484:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4298:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-3173:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4894:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0

Fig. 6: Blast search for Exo2 sample

Description	Scientific Name	Max Score	Total Query Cover	E value	Per. Match	Acc. Len	Accession
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1929	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8278:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1929	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-5099:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1929	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-1931:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1929	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-2484:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-2484:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4298:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-3173:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4738:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-8884:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0
Simulium sp. BOLD:ANE604:couche:18-SCCV08P-4894:cytochrome oxidase subunit 1 (COXI) gene, partial cds, mitochondrial	Simulium sp. BO	1924	100%	0.0	97.49%	658	KS281223.0

Fig. 7: Blast search for Sample 4

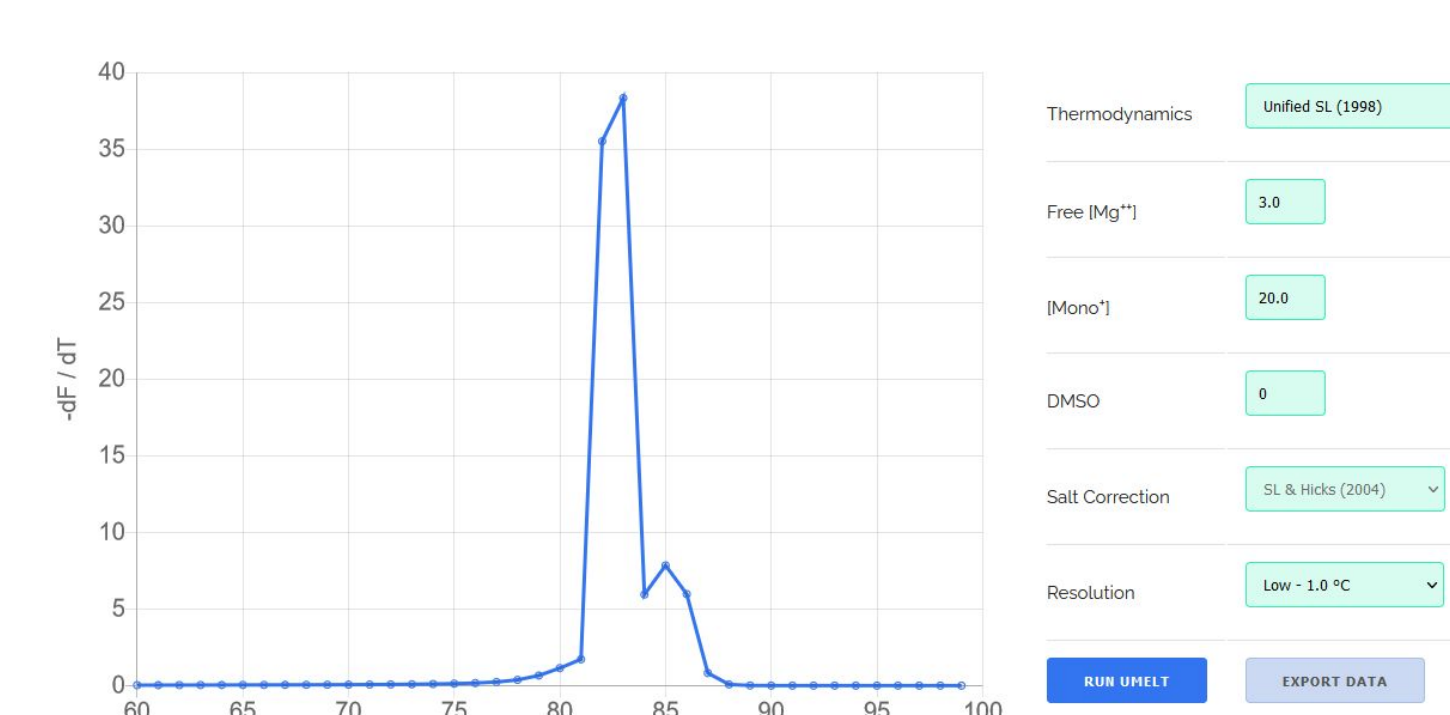


Fig. 8: Predicted melt curve of Simulium paynei (Simulium Paynei Sensu Lato)

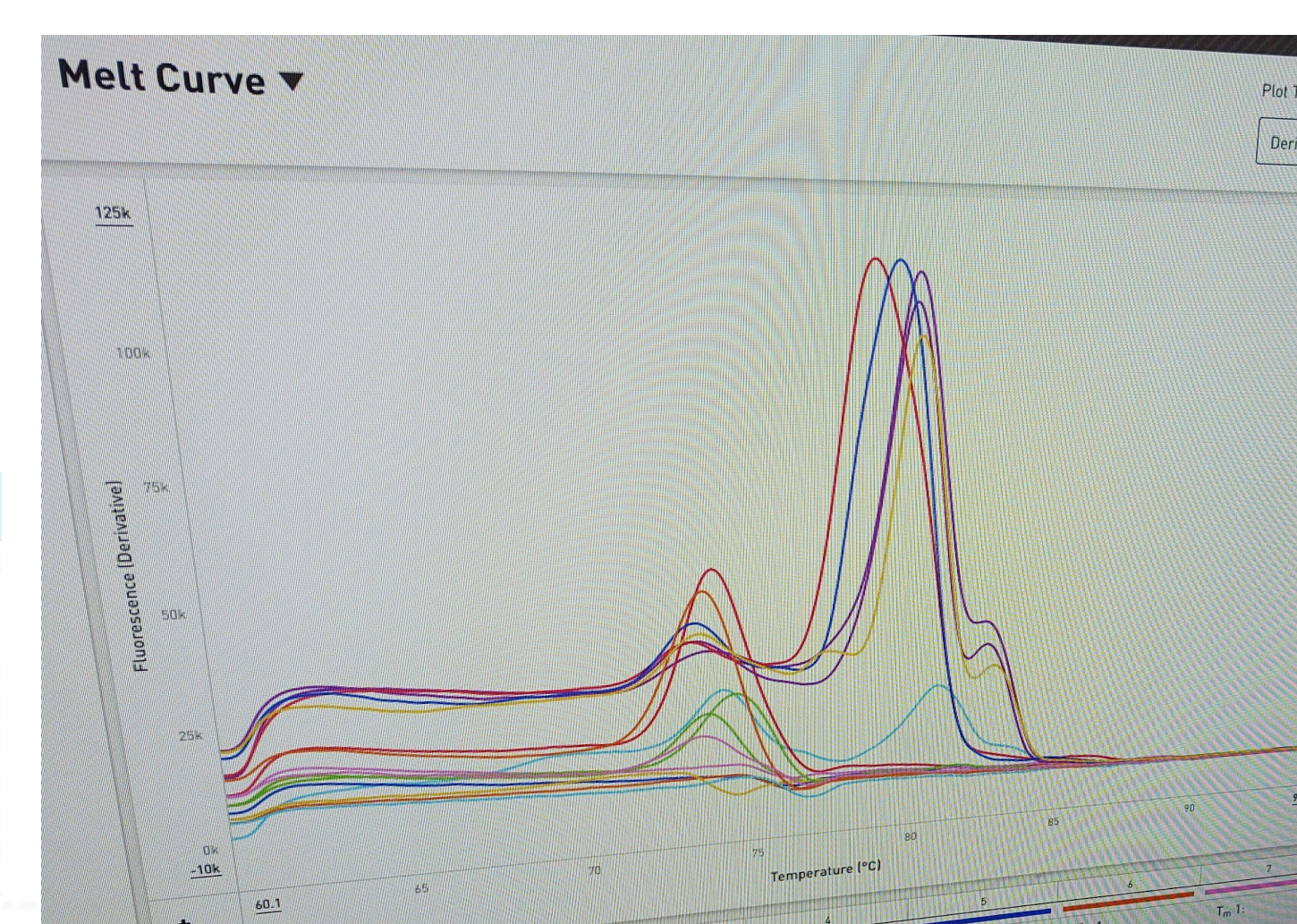


Fig. 9: Observed melt curve of our unknown samples

Future:

Up to this point, taxa identification of the aquatic invertebrates in this study were done to the family level with a dichotomous key. In the future, we will continue this study in determining species of aquatic invertebrates in order to formulate microsatellites to be able to see relations between the populations. We have concluded that when we do this experiment in the future, we would want to use more markers when analyzing the DNA of the samples. In addition to COI, mitochondrial markers COII, cyt b, and ND4, as seen used in Conflitti's thesis paper, would be used to get a more accurate and specific DNA sequence

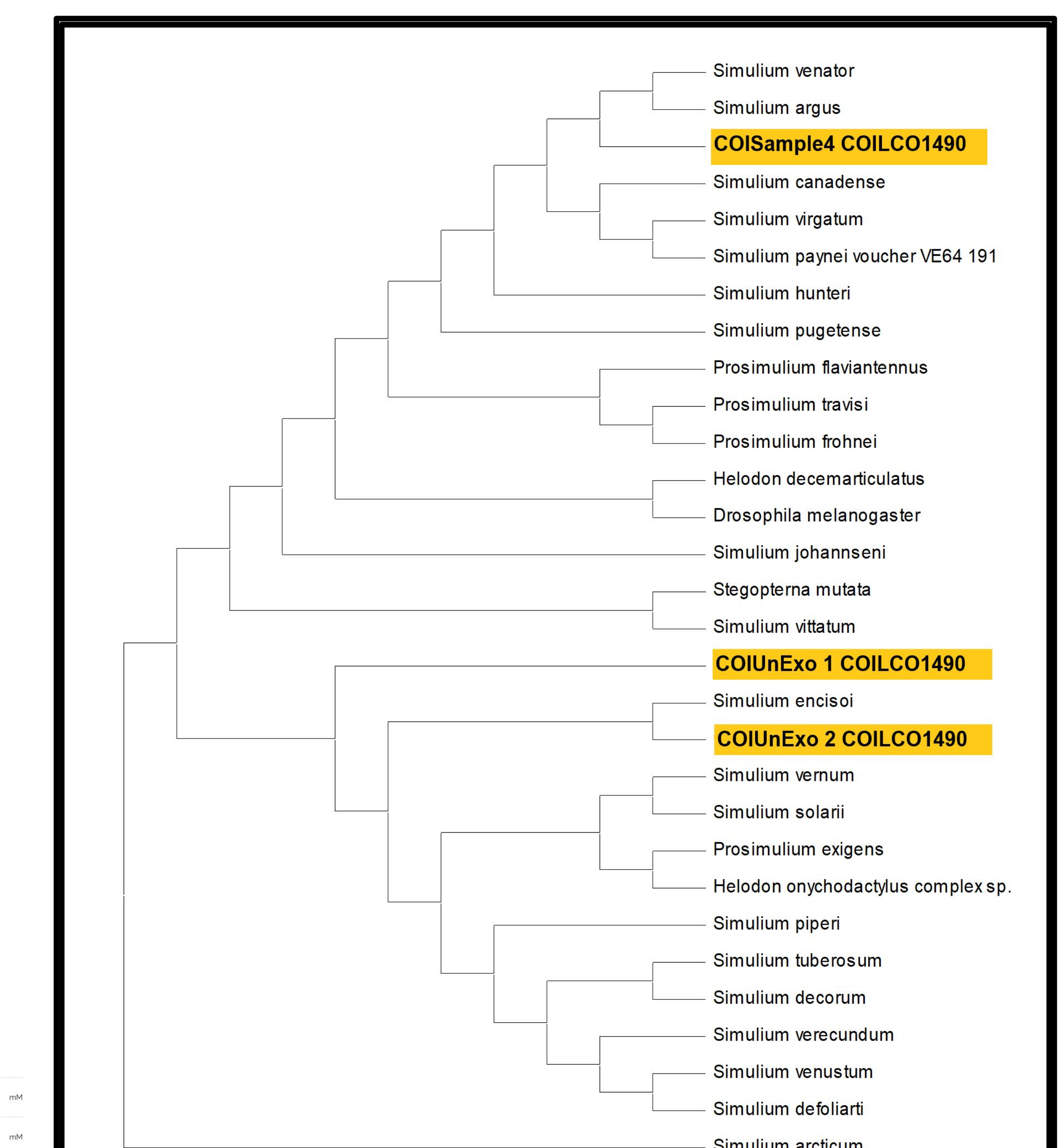


Fig. 10: Phylogeny tree created using the three best DNA sequences we obtained

References:

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- Bjorn Malmqvist, Peter H. Adler, Kalevi Kuusela, Richard W. Merritt & Roger S. Wotton (2004) Black flies in the boreal biome, key organisms in both terrestrial and aquatic environments: A review, Ecoscience, 11:2, 187-200, DOI: 10.1080/11956860.2004.11682824
- Conflitti, Ida M. *Speciation and Species Boundaries in Black Flies (Diptera: Simuliidae)*. 2015. University of Toronto, PhD.
- "Simulium Paynei Sensu Lato LHSMI357-09 Voucher Sim-Canada-454 Cytochro - Nucleotide - NCBI." National Center for Biotechnology Information, U.S. National Library of Medicine, <https://www.ncbi.nlm.nih.gov/nuccore/HQ987825.1?report=fasta>.
- What Is a PCR Master Mix? - Chai. <https://support.chaibio.com/hc/en-us/articles/360004050793-What-is-a-PCR-Master-Mix->.

