



Developing a Standard Operating Procedure for Species Identifications of Dragonfly Larvae Using DNA Barcoding

Standard Operating Procedure, Version 1.0

Megan Little^{1*}, Karen James^{2*}, Michael Marion^{2*}, Hamish Grieg¹, and Sarah J. Nelson^{1*+}

¹ University of Maine
Orono, ME 04469

⁺ Contact author: snelson@outdoors.org

² MDI Biological Laboratory
Bar Harbor, ME 04609

*Affiliations at the time of SOP completion.

This report is available in digital format from [NPS Data Store](#).

Please cite this publication as:

Little, M., K. James, M. Marion, H. Grieg, and S.J. Nelson. 2022. Developing a standard operating procedure for species identifications of dragonfly larvae using DNA barcoding: Standard Operating Procedure, version 1.0. Air Resources Division, National Park Service, Denver, Colorado.

April 2022

U.S. Department of the Interior
National Park Service
Air Resources Division
Denver, Colorado

Contents

	Page
Revision History Log.....	iii
Acknowledgments.....	iii
1.0 Introduction.....	1
1.1 Preparing the Tissue Samples	2
1.1.1 Materials and Equipment.....	2
1.1.2 Procedure.....	2
1.2 DNA Extraction	3
1.2.1 Materials and Equipment:.....	3
1.2.2 Procedure:.....	3
1.3 PCR.....	4
1.3.1 Materials and Equipment:.....	4
1.3.2 Procedure:.....	4
1.4 Gel Electrophoresis	5
1.4.1 Materials and Equipment:.....	5
1.4.2 Procedure:.....	5
1.5 Sample Clean-Up and Sequencing at the University of Maine’s DNA Sequencing Facility	6
1.5.1 Materials and Equipment:.....	6
1.5.2 Procedure:.....	6
1.6 Sample Identification	7
1.6.1 Procedure:.....	7
1.7 Literature Cited	8

Revision History Log

The following table lists all edits and amendments to this document since the original publication date. Information entered in the log must be complete and concise. Users of this standard operating procedure will promptly notify the Dragonfly Mercury Project (DMP) Project Manager at the National Park Service and/or the U.S. Geological Survey about recommended and required changes. The project manager must review and incorporate all changes, complete the revision history log, and change the date and version number on the title page and in the header of the document file.

Revision History Log:

Previous Version #	Revision Date	Author (with title and affiliation)	Location in Document and Concise Description of Revision	Reason for Change	New Version #

Add rows as needed for each change or set of changes tied to an updated version number.

Acknowledgments

University of Maine faculty research funds to Sarah Nelson and Hamish Greig supported the DNA Barcoding effort. The Senator George J. Mitchell Center for Sustainability Solutions and The MDI Biological Laboratory provided laboratory space and support for this project. We thank Kat Ko of the Appalachian Mountain Club and National Park Service for assistance with report formatting. Two peer reviewers representing both the NPS and the University of Colorado – Boulder provided constructive feedback that improved this report. The United States Geological Survey and the National Park Service are core collaborators in the Dragonfly Mercury Project, which provided the dragonfly samples used in this research. Staff from the NPS, its partners, and over 824 citizen scientists collected samples across the US in 2014.

1.0 Introduction

Mercury (Hg) is a global pollutant that threatens aquatic resources across the US, even in seemingly pristine watersheds within national parks. Within a water body, Hg (as methylmercury, MeHg, the more toxic form) becomes entrained in food chains and accumulates in fish, with health implications for humans and wildlife. Concentrations of mercury (Hg) in fish and other biota exceed human and wildlife health thresholds in approximately 50% of US lake fish, and over one million river miles, including many national parks across the United States. Consequently, fish consumption advisories exist in all 50 US states and its territories (US EPA 2011).

Efforts by resource managers to focus conservation work in areas of highest risk to humans, fish, and wildlife are hampered by significant variability in mercury concentrations from site to site—even among neighboring lakes, streams, or wetlands. We are developing the use of dragonfly larvae as bio-sentinels that indicate watersheds most at risk for Hg accumulation in fish and other wildlife. Dragonfly larvae (*Odonata: Anisoptera*) are aquatic macroinvertebrates that meet the requirements of being effective bio-sentinels of Hg risk in aquatic ecosystems: they are widespread in diverse freshwater habitats across the US, where they are key components of aquatic food webs; contain nearly all (~93%) of their Hg as MeHg (Haines et al. 2003, Gorski et al. 2003); and exhibit site fidelity, (each individual remains in the same waterbody throughout its aquatic life). The application of bio-sentinel organisms has emerged as an important monitoring and research tool that can provide a better-integrated indicator of Hg variation among locations than water, and are more appropriate proxies for human and wildlife risk (Knights et al. 2005, Simonin et al. 2008, Flanagan Pritz et al. 2014; see go.nps.gov/dragonflymercury).

However, our interpretation of Hg results is hindered by the potentially confounding influence of dragonfly species identity. We are most interested in understanding species because there are important differences among species in two key aspects that influence Hg bioaccumulation: 1) diet, which directly controls the magnification of Hg with increasing trophic level; and 2) the length of aquatic life stage, which influences the lifetime accumulation of Hg. DNA barcoding provides a promising new approach to species identification, where the DNA sequence of difficult-to-identify larval dragonflies are matched to global DNA databases based on adult dragonflies that are more often studied and collected, and much more easily identified with visually-based taxonomy.

DNA-assisted species identification (“DNA barcoding” and related techniques) involves extracting, amplifying, and finally obtaining the nucleotide sequence of (“sequencing”) a short stretch of a specimen’s DNA (for animal species, part of the mitochondrial *CO1* gene is used) from a biological specimen. This query sequence is then compared to a reference library of *CO1* sequences (“DNA barcodes”) located in the Barcode of Life Data Systems (BOLD) database (<http://boldsystems.org>) and linked to voucher specimens that have been identified by professional taxonomic specialists. The outcome is a DNA-assisted species identification, that is, a species-level name for the query specimen, inferred solely from its DNA.

Refer to the associated dragonfly larvae DNA barcoding narrative (Nelson et al. 2021) for details on the development of this SOP and comparative visual identifications, along with species identities.

1.1 Preparing the Tissue Samples

1.1.1 Materials and Equipment

- Preserved dragonflies¹
- Glass petri dish
- Kimwipes®
- Forceps
- Scissors
- 1.5 mL microcentrifuge tubes
- Flame source (Bunsen burner, lighter, etc.)



Figure 1. Setup for dragonfly dissections.

1.1.2 Procedure

- 1) Sterilize all instruments and the glass petri dish by passing them over a flame before beginning the dissections.
- 2) Working with one dragonfly at a time and using the setup shown in Figure 1, cut all the legs off one side of the dragonfly using the scissors.
- 3) Place all legs into one microcentrifuge tube labeled with the sample ID and date. If not moving directly to part C, freeze sample in -20°C freezer.
- 4) Sterilize all instruments used in the dissection, including the glass dish. Replace the Kimwipe® on the work surface.
- 5) Repeat procedure for all dragonflies being analyzed.

¹ Dragonflies may be preserved by either freezing or submersion in 95% ethanol.

1.2 DNA Extraction

1.2.1 Materials and Equipment:

- Qiagen DNeasy Blood and Tissue Kit
- 1.5 mL microcentrifuge tubes
- Heat block or oven
- Microcentrifuge
- Vortex
- Pipettes and sterile, disposable pipette tips (ideally filter tips)
- Disposable plastic pestles

1.2.2 Procedure:

- 1) Add 180 μ L of Buffer ATL and 20 μ L Proteinase K to each sample and vortex for 5 seconds. Incubate overnight at 55°C.
- 2) Vortex each sample for 15 seconds, add 200 μ L Buffer AL, and vortex again. Incubate at 55°C for 20 minutes.
- 3) Add 200 μ L of ethanol to each sample and vortex.
- 4) Working with each sample separately, pipette the entire mixture volume into a DNeasy Mini Spin Column placed in a 2 mL collection tube. Centrifuge at 8000 rpm for 1 minute in a microcentrifuge.
- 5) Discard the flow-through and replace the collection tube. Pipette 500 μ L Buffer AW1 into the column and centrifuge at 8000 rpm for 1 minute.
- 6) Discard the flow-through and replace the collection tube. Pipette 500 μ L Buffer AW2 into the column and centrifuge at 14,000 rpm for 3 minutes.
- 7) Discard the flow-through and discard the collection tube, placing the column into a 1.5 mL microcentrifuge tube.
- 8) Pipette 100 μ L Buffer AE into the column and incubate at room temperature for 1 minute. Centrifuge at 8000 rpm for 1 minute to elute the DNA.
- 9) Pipette 50 μ L of Buffer AE into the column and incubate at 55°C for 5 minutes. Centrifuge at 8000 rpm for 1 minute to elute the remaining DNA.
- 10) Discard the column and quantify the DNA using the nanodrop.
- 11) Store the purified DNA in a -20°C freezer if not moving directly to part D.

1.3 PCR

1.3.1 Materials and Equipment:

- OdoF1_t1 (5' – TGT AAA ACG ACG GCC AGT ATT CAA CHA ATC ATA ARG ATA TTG G) and OdoR1_t1 (5' – CAG GAA ACA GCT ATG ACT AAA CTT CTG GAT GYC CRA ARA AYC A) primers
- Sigma RedTaq ReadyMix
- Purified DNA
- Microcentrifuge or mini-microcentrifuge
- Pipettes and sterile, disposable pipette tips (ideally filter tips)
- Thermocycler

1.3.2 Procedure:

- 1) Use <http://www.idtdna.com/Calc/resuspension> to calculate how much nuclease-free water is needed to resuspend your new primers to form a 100 μ M stock solution. Make an aliquot of 10 μ M working solution.
- 2) Make your master-mix in a 1.5 mL microcentrifuge tube using the following component volumes per reaction, including a negative control and 2 extra reactions: 12.5 μ L Sigma RedTaq ReadyMix, 10.5 μ L nuclease-free water, 0.5 μ L forward primer, and 0.5 μ L reverse primer. Vortex then spin mixture in mini-microcentrifuge, then keep on ice.
- 3) Add 24 μ L of master-mix to 1 0.2 mL PCR tube per reaction.
- 4) Add 1 μ L of DNA to each tube. Use 1 μ L nuclease-free water for the negative control.
- 5) Spin tubes in the mini-microcentrifuge then place them in the thermal cycler. Run samples on an amplification program which should have the following conditions:
 - a) 16 cycles of the following:
 - i. 95°C for 10 seconds
 - ii. 62°C for 30 seconds, -1° per cycle
 - iii. 72°C for 60 seconds
 - b) 25 cycles of the following:
 - i. 95°C for 10 seconds
 - ii. 46°C for 30 seconds
 - iii. 72°C for 60 seconds
 - c) Final hold at 4°C
- 6) Samples can be frozen in a -20°C freezer if not moving directly on to part E.

1.4 Gel Electrophoresis

1.4.1 Materials and Equipment:

- PCR products
- Invitrogen Low DNA Mass Ladder
- Pipettes and sterile, disposable pipette tips (ideally filter tips)
- Large 1.5% Agarose Gel (Maximum 40 wells):
 - 150 mL TBE Buffer
 - 2.25 g Agarose
 - 2.5 μ L Ethidium Bromide
- Small 1.5% Agarose Gel (Maximum 24 wells):
 - 60 mL TBE Buffer
 - 0.9 g Agarose
 - 2.0 μ L Ethidium Bromide

1.4.2 Procedure:

- 1) Combine TBE buffer and agarose in a flask.
- 2) Heat in a microwave in 30 or 60 second intervals depending on if making a small or large gel. Heat until mixture boils and appears clear.
- 3) Cool flask until it can be touched for three seconds on any side.
- 4) Add ethidium bromide and gently swirl mixture. Pour into gel rig. Let sit at least half an hour.
- 5) Pipette 5 μ L of Invitrogen Low DNA Mass Ladder into the first well. Pipette 15 μ L of each PCR product into its own well, making sure to include the negative control.
- 6) Run the gel for 30 minutes at voltage specified above then view using a UV transilluminator with a mounted camera. If you do not see bands for your PCR products, go back and troubleshoot your PCR. If your PCR products show bands, you can proceed.
- 7) Samples can be frozen in a -20°C freezer if not moving directly on to part F.

1.5 Sample Clean-Up and Sequencing at the University of Maine's DNA Sequencing Facility

1.5.1 Materials and Equipment:

- Affymetrix ExoSAP-IT
- 0.2 mL microcentrifuge tubes
- 10 μ M PCR primers
- Pipettes and sterile, disposable pipette tips (ideally filter tips)
- UV Transilluminator

1.5.2 Procedure:

- 1) Add 2 μ L of Affymetrix ExoSAP-IT to one 0.2 mL microcentrifuge tube for each PCR product. Keep tubes on ice.
- 2) Add 5 μ L of a PCR product to each tube and spin briefly in a mini-microcentrifuge.
- 3) Place tubes in a thermal cycler and run on the ExoSAP program. The product can either be frozen in a -20°C freezer or you can proceed.
- 4) In a new 0.2 mL microcentrifuge tube, dilute 1 μ L of each PCR product with enough nuclease-free water to yield a final DNA concentration of 10 ng/ μ L.
- 5) Briefly spin tubes in the mini-microcentrifuge and deliver to the sequencing lab.

1.6 Sample Identification

1.6.1 Procedure:

- 1) Once sequence files returned, typically in “.abi” format, open them in a DNA editing software (e.g. 4Peaks for Mac OSX) then:
 - a) Visually scan sequences for sequence quality.
 - b) Trim the ends to remove poor sequence quality.
 - c) For sequences from the reverse primer, “Flip Sequence” (reverse complement).
 - d) Highlight and copy (ctrl-c) sequence.
 - e) Go to the Barcode of Life Database (<http://boldsystems.org>) and click on the “Identification” tab.
- 2) While under the “Animal Identification [CO1]” tab, paste (ctrl-v) your sequence into the text box.
- 3) If no match is found, repeat selecting less stringent search options.
- 4) Check that the search results for the forward and reverse sequences from each specimen match.

Note: All standard lab safety procedures should be followed throughout, including, but not limited to, use of safety glasses or goggles, gloves, and other personal protective equipment; the separation of the sterilizing flame from areas in which ethanol is being used; and storage of reagents and chemicals in an appropriate manner.

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