Developing environmental DNA assays for non-invasive detection of *Martes pennanti* (Fisher) populations

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Background

- Fishers (*Martes pennanti*) are a mammal found exclusively in North America, and can be found throughout New York State (NYS) as well as parts of the Pacific Northwestern region of the U.S.[1]
- Fishers are habitat-specialists that require relatively specific conditions in order to persist.[2]
- Fishers occur at naturally low densities making encounters rare, and thus they are a difficult animal to study.
- The population in NYS has remained stable, and they are increasing their range into central New York as well as parts of Pennsylvania.[1]
- In the Northwestern U.S., populations are declining as deforestation and habitat fragmentation take place.[2]
- Most Fisher populations are monitored by camera traps or reported sightings from the public.[1,3]
- Environmental DNA (eDNA) is trace DNA that is found in the environment in soil or water.
  - eDNA is often degraded and is not always recent
  - eDNA is used to detect many species, including those that are rare like the Fisher.[4]

Objectives

- Develop an eDNA assay for Fisher detection.
- Determine if detection rate of the Fisher differs between primers that vary in amplicon size.

General Methods

- Developed two primers sets
  - Primer Set One (PS1) should amplify a 267 base pair (bp) fragment of Fisher DNA.
  - Primer Set Two (PS2) should amplify a 124 bp fragment of Fisher DNA.
- Conducted an *in silico via* Primer-BLAST analysis of PS1 and PS2 against other mammals in the same family to determine if non-specific amplification was expected[5].
- Created a polymerase chain reaction (PCR) master mix using a $MgCl_2$ concentration of 1.5 mM for PS1 and tested with positive DNA samples of the Fisher, American Marten (*Martes americana*), and River Otter (*Lontra canadensis*).
- Multiple PCR master mixes were created for PS2 that included different concentrations of $MgCl_2$ as well as dimethyl sulfoxide (DMSO) and run with the same positives.
- Ran 2% agarose gels that contained PCR products and ethidium bromide for 35 minutes at 100 volts to visualize amplicons under ultraviolet light.

Preliminary Results

1) *In silico* analysis of PS1 and PS2 determined both primers may amplify the DNA of other species besides Fisher.

- The forward and reverse primers in Table 1 and Table 2 are specific sequences from Fisher DNA, and the bold and underlined nucleotides are locations in the primers that identify sequence variation among species.
- PS1 was predicted to amplify a PCR product of 267 bps in length for the Fisher and Marten.
- PS2 was predicted to amplify a PCR product size for Fisher DNA of 124 bps in length, and Ermine (Mustela erminea) DNA of 206 bps in length.

Table 1. PS1 *in silico* analysis using Primer-BLAST[5]

Common name	Scientific name	Forward Primer	Reverse Primer	PCR product size
Fisher	Martes pennanti	CATCCCTTACATCGG**A**ACCAACC	ATTATGAGAACTAGGACTAGG	267
American Marten	Martes americana	CATCCC**T**TACATCGGAACCA**A**CC	ATTATGAG**AA**CTAGGA**C**TAGG	267
Ermine	Mustela erminea			No Amplifcation
Long-Tailed Weasel	Mustela frenata			No Amplifcation
American Mink	Neovison vison			No Amplifcation
North American River Otter	Lontra canadensis			No Amplifcation

Table 2. PS2 *in silico* analysis using Primer-BLAST[5]

Common name	Scientific name	Forward Primer	Reverse Primer	PCR product size
Fisher	Martes pennanti	GACTATTAG**C**AGC**T**GACCTCC	GGTATGAAGACTAGGAGAATTGCG	124
American Marten	Martes americana			No Amplification
Ermine	Mustela erminea	G**A**C**TA**T**T**AGCAGCTGACCT**CC**	GGTATGAAGACTAGGAGAATTGCG	206
Long-Tailed Weasel	Mustela frenata			No Amplification
American Mink	Neovison vison			No Amplification
North American River Otter	Lontra canadensis			No Amplification

2) Visualization of the PCR products for both PS1 and PS2 with the same master mix concentrations resulted in PS1 amplifying only Fisher DNA and PS2 amplifying both Fisher and Marten DNA.

- PS1 successfully amplified only Fisher DNA at 267 bps with a lower $MgCl_2$ concentration of 1.5 mM.
- PS2 amplified Fisher DNA and Marten DNA, contrary to the expected results based on the *in silico* analysis.

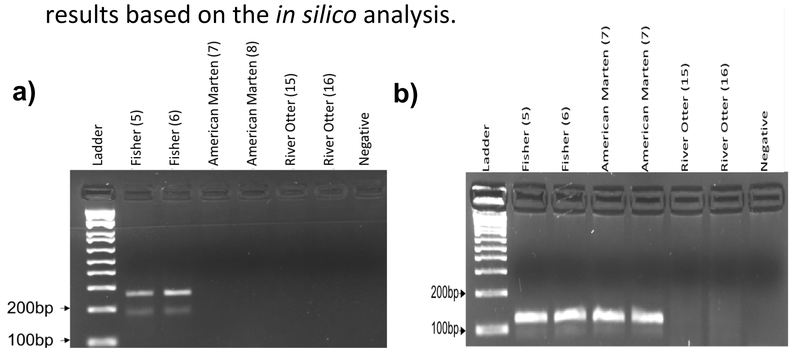

Figure 1a) Gel image of Fisher, Marten, and River Otter DNA with $MgCl_2$ concentration of 1.5 mM for PS1. Figure 1b) Gel image of Fisher, Marten, and River Otter DNA with $MgCl_2$ concentration of 1.5 mM for PS2.

3) Altering PCR master mix conditions and thermal cycling conditions for PS2 did not resolve non-specific amplification.

- Lowering $MgCl_2$ to 1 mM did not reduce non-specific amplification.
- Altering the annealing temperature and the duration of various PCR steps during three-step PCRs and two-step PCR protocols did not resolve the non-specific amplification issue.
- DMSO can help to prevent non-specific binding.
  - Different concentrations of DMSO (3%, 5%, 7%, and 9%) were tested against Fisher and Marten DNA samples.
  - Figure 2 suggests that DMSO has a slight effect on the amplification of Marten DNA but did not resolve non-specific amplification altogether.

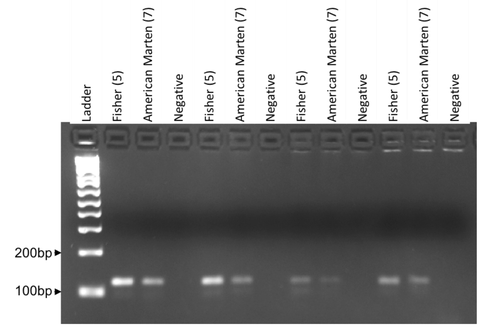

Figure 2. Gel image of Fisher DNA, Marten DNA, and non-template control (negative) for 3%, 5%, 7%, and 9% DMSO concentrations (from left to right).

Discussion and Future Work

- *In silico* analysis predicted that PS1 may amplify with Marten DNA in addition to Fisher DNA.
  - By lowering the $MgCl_2$ concentration we were able to amplify only Fisher DNA.
- *In silico* analysis of PS2 suggested that Marten DNA would not amplify, however *in vitro* tests clearly show that it does, despite multiple alterations to the PCR protocol.
  - Results suggest that non-specific amplification issues with PS2 can not be resolved.
- Future work on this project will:
  - Develop a new primer that will amplify ~130 bp product using MEGA[6] and Primer-BLAST[5].
  - Both primer sets will be used to detect Fisher in 118 eDNA samples collected at Rice Creek Field Station from 2018 and 2019.
  - Detection rates for the Fisher will be compared between the two primer sets using logistic regression.

Figure 3. Martes Pennanti (Fisher)

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References

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