MAGNETIC CAPTURE HYBRIDIZATION OF AMPHIBIAN MITOCHONDRIAL GENOMES

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

A --- Adenine

ATP --- Adenosine Triphosphate

Bd --- *Batrachochytrium dendrobatidis*

bp --- base pairs

BSA --- bovine serum albumin

C --- Cytosine

DNA --- Deoxyribonucleic Acid

DT --- DreamTaq

EDTA --- Ethylenediaminetetraacetic acid

Eb --- Eleutherodactylus marnockii

G --- Guanine

Lb --- Lithobates berlandieri

$MgCl_2$ --- magnesium chloride

mtDNA --- mitochondrial DNA (*see above*)

NaCl --- sodium chloride

nucDNA --- nuclear DNA (*see above*)

PCR --- Polymerase Chain Reaction

SDS --- sodium dodecyl sulfate

SSC --- saline sodium citrate

tDNA --- total DNA *(see above)*

NCBI --- National Center for Biotechnology Information

T --- Thymine

WGA --- Whole Genome Amplification

ABSTRACT

Analysis of mitochondrial DNA (mtDNA) provides useful insights into population dynamics of target organisms, and efficient extraction of this DNA is pivotal to ensuring the reliability of subsequent reactions. When extracted from organic tissue, resulting solutions contain a mixture of genomic and mitochondrial DNA, and separation of these two molecules usually requires the use of an ultracentrifuge; because many amphibian organisms of interest are found in tropical regions of the world without ready access to the required machinery, a new, more portable, method of selectively isolating mitochondrial genomes from complex mixtures to facilitate whole mitochondrial genome sequencing is proposed here. Conserved sequences in the 12S and 16S regions can be targeted by a uniquely designed biotinylated probe that will form a complex with magnetic beads covalently coupled to streptavidin. This research shows that the complex can be selectively filtered from solution through magnetic capture, and heat treatment then dissociates the probe complex from the mtDNA. The resulting solution contains samples suitable for downstream reactions to expand the knowledge of population dynamics as they relate to the emerging threat of chytridiomycosis in Anuran populations.

I. Introduction

Frogs, species of the order *Anura*, represent a key component of the stability of terrestrial and aquatic ecosystems dispersed throughout most major continents. As tadpoles, they consume and control the algal mass of the body of water to which they are limited, maintaining a delicate equilibrium with a capacity to host a diverse set of organisms. Once mature, tadpoles metamorphose and increase their range of distribution by adapting an ability to conduct oxygen exchange outside of water, as well as exchanging their thick, swimming-enhancing tail for muscular legs more aptly suited for terrestrial mobility. These frogs help to control insect populations, and, by proxy, the populations of the pathogens and diseases which they carry; in turn, frogs comprise a portion of the food sources regularly utilized by the birds and other predators one degree of consumption higher in the food chain (Hocking & Babbitt 2014).

In addition to gas exchange, amphibians conduct biomolecular exchange through their skin, making them particularly susceptible to subtle changes in their habitat (Malvin, 1988; Bentley & Yorio, 1976). As such, certain taxa are regarded as indicator species of their respective environments. Because certain harmful compounds, such as the phenols common to cleaning products and pesticides, are so easily absorbed through the thin membrane required for efficient gas exchange in amphibians, the population dynamics of frogs act as relatively easily traceable precursors to upcoming stability trends among other species in that habitat in response to select chemical contaminants. Additionally, frogs may be particularly sensitive to certain pollutants since they tend to spend a majority of their life in water, where toxins like water-soluble pesticides are most transmissible (Welsh and Olliver, 1998; Kerby et al., 2010).

One notable threat to frog populations of increasingly pressing concern is the discovery and characterization of the fungus *Batrachochytrium dendrobatidis* (*Bd)*, a species belonging to the early-diverged Chytridiomycota phylum. One theory posits that the global panzootic lineage of this pathogen originated in the Asian continent centuries prior to its discovery in the 1990s (O'hanlon et al., 2018). This fungus is capable of utilizing the keratin found in frog skin as a growing medium, interfering with the amphibian's ability to conduct gas exchange, which induces stress in the individual afflicted by chytridiomycosis. Ultimately, frogs often succumb to cardiac arrest at the hands of infection (Berger et al., 1998). Likely due to mutual pressures exerted over the span of an evolutionary timescale, amphibian species in the Asian continent are believed to possess some level of immunity to the advances of *Bd*, with frog populations in Asia remaining relatively stable despite the prevalence of the fungus (Swei et. al, 2011).

However, in recent decades, an interest in human exploration and emphasis on facilitated trade relationships among previously disparate parts of the world have allowed *Bd* spores, once doomed to perish on trade ships sequestered to sea for months at a time, to survive now expedited intercontinental trips. As such, an unforeseen consequence of improved connectivity between landmasses was the introduction of spores to new, susceptible continents and populations of naïve amphibians that lacked the evolutionary defenses to avoid infection. Since *Bd* targets the keratin utilized by frogs as a general dermal component, the pathogen is capable of infecting a pool of amphibian organisms, rather than a species— as is traditional of other diseases— and has proven to be an effective killer, with the latest studies reporting a marked decline in ~500 amphibian species (Scheele et al., 2019). Understanding amphibian population dynamics would

provide a key piece of knowledge in further characterizing this pathogen-host interaction and possibly prevent further extinctions.

Deoxyribonucleic acid, or DNA, has been identified as the chief molecule dictating genetic inheritance. Arranged in a double helix conformation and composed of a phosphate backbone supporting a long series of four distinct nucleotides paired to a complement nucleotide on the opposing strand, DNA is comprised of genes which code for the proteins that regulate not only phenotypic expression, but the metabolic and biochemical processes that form the basis of life (Levitt, 1983). DNA can be furthermore parsed into two categories: nuclear DNA (nucDNA) and mitochondrial DNA (mtDNA)—the former of which is linear, and the latter circular. Though nucDNA is housed in the nucleus of a eukaryotic cell, consisting of roughly 1 to 10 billion bp (base pairs) in amphibians, mtDNA is considerably shorter in length, measuring at approximately 17k bp in amphibians.

As suggested in the endosymbiosis theory, mitochondria, the organelles predominantly responsible for adenosine triphosphate (ATP) production, may possess a genome unique from the rest of the cell due to their ancient origins as bacteria that were once autonomous from what would become modern eukaryotic cells. Because the ancestral mitochondrion was capable of using oxygen as a vital component of efficient energy production via cellular respiration, rather than digest the foreign organism, the eukaryotic cell may have engulfed and integrated it into its own cellular processes and developed a closely intertwined mutualistic symbiosis. Though the two are now two components of a single cell, the mitochondrion has retained its unique genome after generations of duplication and progeny (Gray, Burger & Lang 1999).

As opposed to nucDNA, which is biparentally inherited, mtDNA is matrilineal in nature. Progeny of two reproducing individuals possess an exact copy of the mother's mtDNA, despite the fact that males possess a copy of their own distinct, maternally inherited mitochondrial genome. Because inheritance of mtDNA is a direct transfer between generations and does not display the confounding factor of recombination, mtDNA can be used as a valuable source of information from which researchers may more accurately extrapolate family lineages. By sequencing and analyzing mtDNA data, population dynamics of ancestral groups can be catalogued and provide valuable context for currently observed dynamics. This information can be used to frame the discussion surrounding modern complications faced by contemporary populations (Avise, 1986).

Through modern techniques, extraction of total DNA (tDNA) from organic tissues results in solutions containing not only the target mitochondrial genome, but nucDNA fragments, excess primers, and other genetic sequences that may confound sequencing results. The presence of these molecules may kinetically hinder the completion of downstream sequencing reactions from which population-based data can be generated, as well as confounds attribution of conclusions and patterns exclusively to mtDNA. As such, modern methods of mtDNA purification require ultracentrifugation, a process that employs use of a high-powered centrifuge (Hornig-Do et al., 2009). When placed in an ultracentrifuge, the contents of the sample separate into bands according to molecular weight, and mtDNA can be selectively extracted for downstream reactions; however, the process of ultracentrifugation is not only relatively expensive, but lacks portability, and presents a barrier to overcome when collecting tissue samples during fieldwork, as access to a lab and the proper machinery is limited.

Probes are artificially generated nucleotide sequences which are designed to bond to complementary sequences contained within a DNA strand. Probes are designed to exploit the preferential pairing of nucleotides— since adenine (A) couples with thymine (T) and cytosine (C) with guanine (G), respectively, probe sequences designed to be complements of a region of DNA will anneal to their target region and hybridize with the DNA strand. The probe, often 100-1000 bases long to ensure specificity of binding, can be modified to include a fluorescent tag that can be detected by sequencing technology post-hybridization. Probes are often employed to check if a particular region of DNA expresses known genes of interest. A probe is designed as a complement to the target gene, and introduced to the DNA sample. If the gene is present along the DNA strand, then the probe will hybridize with the DNA and emit a detectable fluorescent signal that confirms the presence of the target sequence (Lathe, 1985).

Alternatively, DNA probes can be fitted with biotin in lieu of a fluorescent tag (biotinylation). Biotin is a small molecule, and as such, has no measurable steric hindrance on the behavior of the attached compound (Manuelidis, Langer-Safer & Ward, 1982). Furthermore, biotin has a strong affinity for streptavidin, an association which acts with strength on an order just shy of a covalent bond. Magnetic beads coated in streptavidin, when added to a solution containing biotinylated molecules, will associate with each other and from a complex that acts as one unit (Tagle, Swaroop, Lovett & Collins, 1993). Though the exploitation of biotin-streptavidin affinity has been used to isolate biotinylated molecules of interest, including nucDNA fragments, the same concept has potential to aid in the capture of whole mitochondrial genomes (Hornig-Do et al., 2009).

Mitochondrial genomes of organisms are widely available on internet databases, such as on the genome database of the National Center for Biotechnology Information (NCBI). If the genomes of several Anuran species are collected, and the frequently targeted 12S and 16S regions compiled, aligned, and assembled, then the resulting data could be analyzed for conserved sequences (Stojavonic et al., 1999). These sequences, unique to Anurans yet still conserved across the differing genomes of each species, represent regions of interest to be targeted by a probe. The probes, designed as complements to the identified sequences, can be modified to include a biotin molecule, and will hybridize selectively to the mtDNA, forming an mtDNA-biotinylated probe hybrid. The hybrid can then be subsequently exposed to streptavidin-coated magnetic beads, which will associate to the biotin molecule and form a larger mtDNA-biotinylated probe and magnetic bead complex.

When exposed to a magnetic gradient, the complex will migrate to the side of the tube containing the solution, and the supernatant liquid, containing all unwanted DNA sequences, can be eluted out of the tube, leaving behind only the mtDNA-containing complex of interest. The contents of the tube can then be resuspended, the beads decoupled, and the probes disassociated from the mtDNA strand with a heat treatment. The resulting solution would, in theory, contain isolated whole mitochondrial genomes suitable for downstream reactions (Rodriguez, Longo & Zamudio 2012). Proposed in this project is a method for magnetic capture of mitochondrial amphibian genomes based on the concepts outlined above, resulting in a method that has potential for efficient next-generation sequencing of amphibian mitochondrial genomes at the population level while circumventing the need for ultracentrifugation.

II. Materials and Methods

1) Probe Design

Both 12S and 16S mitochondrial region sequences from 71 species (listed in **Table 1**) were collected from NCBI and imported into Geneious 2, where 43 were successfully assembled to produce 16 contigs. Once aligned, the sequences were examined and two sequences conserved across species were identified for probe candidacy. After modification with a biotin molecule attachment and glycol spacer, the resulting probes were designated as H_16S (5'-/BTEG/AGGCGATGTTAAACAGGCG-3') and L_16S (5'-/BiotinTEG/CCYACGTGATCTGAGTTCAGACCGGAGTAATCCAGGTC-3') for the heavy (probe 1) and light (probe 2) strands, respectively.

2) Buffer and Prerequisite Reagent Preparation

To begin, 50 mL of 2X binding and washing (B&W) Buffer were prepared according to the Dynabeads® M-270 and C1 Streptavidin (Invitrogen) manufacturer's protocol; 50 mL of a 10 mM Tris-HCl and 1mM EDTA solution were prepared by diluting 0.5 mL of concentrated Tris-EDTA (1.0 M Tris/0.1 M EDTA) in 50 mL of nuclease-free (N.F.) water, and 5.85 g of NaCl were then added to yield a 2M NaCl solution.

A 20X saline sodium citrate (SSC) was prepared by dissolving 8.77 g of NaCl and 4.41 g of trisodium citrate (300mM) in 40 mL of N.F. water. The resulting solution was adjusted dropwise to a pH of 7.0 with 1M HCl, and the quantity was adjusted to a final volume of 50 mL with N.F. water. The resulting solution was sterilized via syringe pore filtration. A reserve of 12X SSC solution was prepared by diluting 30 mL of the 20X SSC solution to 50 mL with N.F. water.

A 1% w/v solution of sodium dodecyl sulfate (SDS) was prepared by dissolving 0.5 g of SDS in 50 mL of N.F. water.

3) Probe Hybridization

Probes were hydrated and diluted to a 10 mM working concentration using N.F. water. Then 10 μL of each tDNA sample were placed in each well of a 0.2 mL strip tube, and combined with 15 μL of 12X SSC, 1.5 μL of N.F. water, 1.0 μL of bovine serum albumin (BSA), and 0.5 μL each of probe 1 and probe 2. Denaturation was initially allowed to take place at 98 ºC for 15 minutes but was later extended to 30 minutes. Hybridization of the probes was initially allowed to occur at 40 ºC for 1 hour, but was raised to 50 ºC for 2 hours in subsequent iterations. The reaction was allowed to cool to room temperature before proceeding.

4) Magnetic Bead Coupling, mtDNA Extraction, and Decoupling

To perform bead-biotin coupling, 10 μL of magnetic beads, washed and resuspended in B&W buffer per the manufacturer's instructions, were added to 30 μL of each hybridization reaction and incubated at 45 ºC for 30 minutes in a ThermoMixer with constant vortexing at ~1400 rpm, then allowed to cool to room temperature. The tubes were then exposed to a magnet, and the supernatant was eluted into separate tubes, labeled as nucDNA, and reserved. The magnetic beads were resuspended in 20 μL of Elution Buffer, vortexed, and then incubated at 95 ºC for 10 minutes to decouple beads. The tubes were once again exposed to a magnet, and the supernatant was eluted into tubes labeled mtDNA. A visual overview of the methods described in steps 3 and 4 can be seen in **Fig. 2**.

5) PCR and Gel Electrophoresis Verification

After magnetic capture protocol, a polymerase chain reaction (PCR) was performed by combining 0.5 µL of tDNA, mtDNA, or nucDNA samples with 6.25 µL of DreamTaq (DT), 0.125 µL of forward primer dgLC01490 and 0.125 µL of reverse primer dgHC02198, 0.5 µL of $MgCl_2$, 3.875 µL of N.F. water, and 1.125 µL of BSA in a tube. The contents were then initially denatured for 2 minutes at 95 ºC and then taken through 33 cycles of denaturation at 95 ºC for 30 seconds, annealing at 46 ºC for 30 seconds, and extension at 72 ºC for 45 seconds. The contents were then held at 72 ºC for ten minutes and allowed to cool to room temperature. Gel electrophoresis analysis was performed on raw tDNA, mtDNA, and nucDNA samples on a 1% agarose gel, and all PCR products were visualized on a 2% agarose gel. **Fig. 1** summarizes a workflow of methods described in steps 1-5.

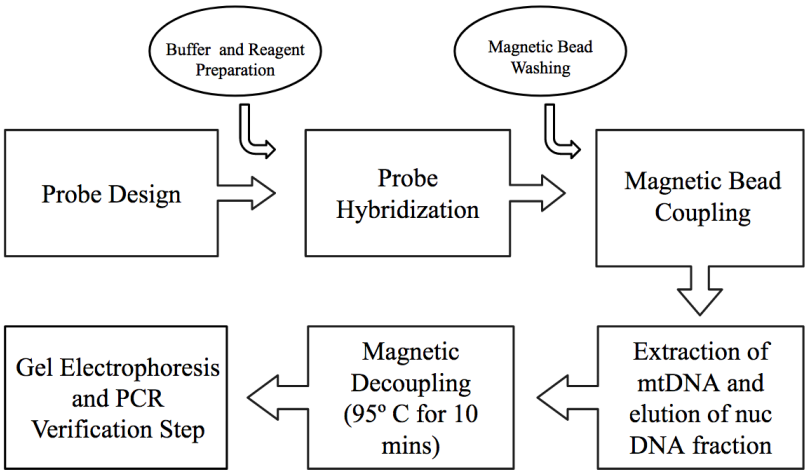


Figure 1: Workflow of magnetic capture hybridization protocol.

Table 1: All organisms successfully assembled and aligned by Geneious 2. *In total, 71 mitochondrial genomes were analyzed, but 28 were not assembled by the software.*

Contig	NCBI Ref Seq	Family	Organism
1	KX686108	Ranidae	*Rana catesbeiana*
	NC_027236	Ranidae	*Rana sylvatica*
	NC_030042	Ranidae	*Rana amurensis*
	AB761266	Ranidae	*Rana okinavana*
	EF196679	Ranidae	*Rana plancyi*
2	DQ275350	Bufonidae	*Bufo gargarizans*
	KT223827	Bufonidae	*Bufotes raddei*
	KR136211	Bufonidae	*Bufo stejnegeri*
	AB303363	Bufonidae	*Bufo japonicus*
3	JX564857	Centrolenidae	*Espadarana prosoblepon*
	JX564891	Rhinodermatidae	*Rhinoderma darwinii*
	KY962391	Bufonidae	*Melanophryniscus moreirae*
4	JX181763	Dicroglossidae	*Hoplobatrachus rugulosus*
	AP011543	Dicroglossidae	*Hoplobatrachus tigerinus*
	AP011544	Dicroglossidae	*Euphlyctis hexadactylus*
5	MG264891	Dendrobatidae	*Anomaloglossus dewynteri*
	MG264893	Dendrobatidae	*Anomaloglossus surinamensis*
	KU958559	Dendrobatidae	*Anomaloglossus baeobatrachus*
6	AB303949	Hylidae	*Hyla japonica*
	KT964710	Hylidae	*Hyla ussurensis*
	AY458593	Hylidae	*Hyla chinensis*
7	KT878719	Microhylidae	*Kaloula rugifera*
	MG962359	Microhylidae	*Kaloula verrucosa*
	JQ692869	Microhylidae	*Kaloula borealis*
8	JX893183	Bombinatoridae	*Bombina lichuanensis*
	JX893181	Bombinatoridae	*Bombina maxima*
	AY458591	Bombinatoridae	*Bombina fortinuptialis*
9	KU096847	Megophorydae	*Oreolalax lichuanensis*
	KX615450	Megophorydae	*Scutiger ningshanensis*
10	KY962392	Dendrobatidae	*Hyloxalus subpunctatus*
	KY962393	Dendrobatidae	*Phyllobates terribilis*
11	NC_026789	Dicroglossidae	*Nanorana parkeri*
	KY594708	Dicroglossidae	*Nanorana ventripunctata*
12	MH141597	Mantellidae	*Mantella baroni*
	AB212225	Mantellidae	*Mantella madagascariensis*
13	AY957562	Bombinatoridae	*Bombina orientalis*
	JX893175	Bombinatoridae	*Bombina veriegata*
14	KX854020	Hylidae	*Dryophytes suweonensis*
	KP212702	Hylidae	*Hyla tsinlingensis*
15	KT285802	Microhylidae	*Microhyla butleri*
	NC_024547	Microhylidae	*Microhyla pulchra*
16	KM035412	Rhacophoridae	*Rhacophorus dennysi*
	AB202078	Rhacophoridae	*Rhacophorus schlegelii*

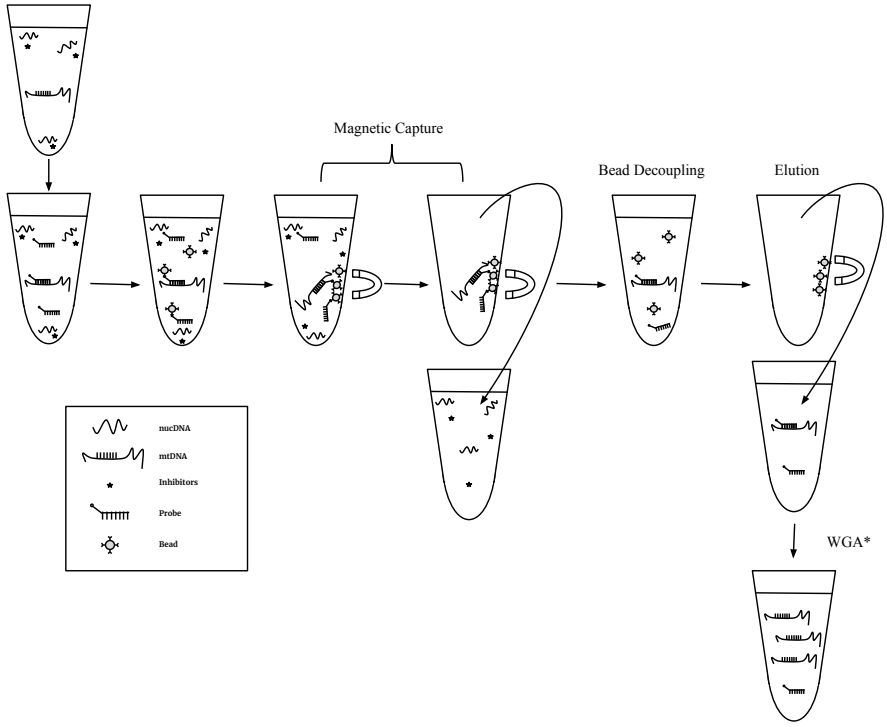


Figure 2: Visual summation of protocol per sample*. Whole genome analysis (WGA) is not performed in the scope of this experiment, but would be a possible addition to the protocol prior to downstream reactions. Figure modified from Rodriguez, Longo, and Zamudio 2012.*

III. Results and Discussion

Initial Run

An initial trial run using the samples SFM 944, SFM 945, SFM 982, and SFM 946 with a hybridization time of 30 minutes was conducted and yielded the results observed in **Fig. 3**. Pre-PCR, the tDNA samples show a band confirming the presence of both mtDNA and nucDNA in the sample, with sample SFM 945 (lane 4) displaying the darkest band and therefore the highest concentrations of component DNA. Conversely,

sample SFM 982 displayed the lightest band, implying a relatively lower concentration of tDNA in the sample. The magnetically separated nuclear fraction of sample SFM 945 displayed some slight smearing, implying a low concentration of DNA present in the nuclear fragment.

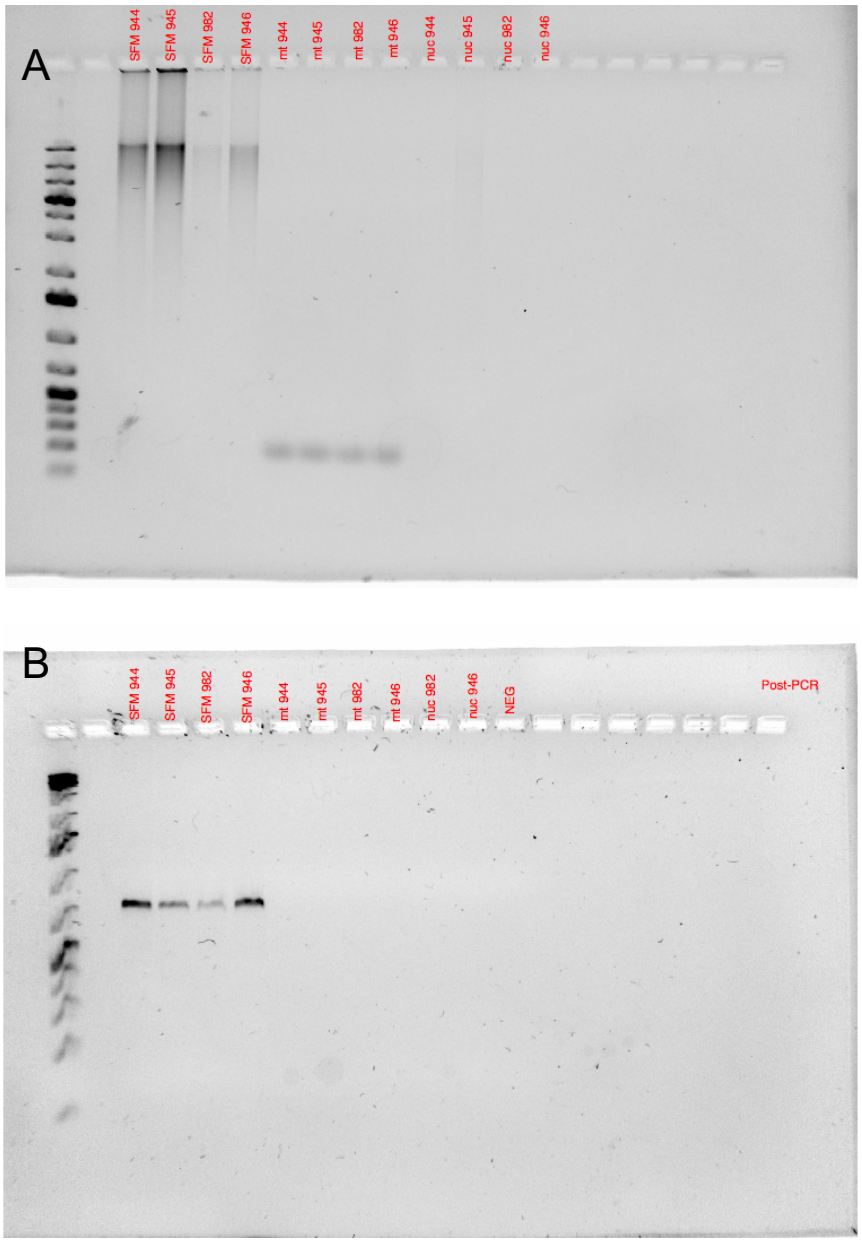


Figure 3: Gel Electrophoresis of magnetic capture products before (A) and after PCR (B). *Dynabeads M-270 Streptavidin were used and hybridization temperature was 45 ºC*

for an initial trial run of 30 minutes.

All other samples appear to display no bands pertaining to nuclear or mitochondrial DNA; small bands are observed under the mitochondrial samples— though these bands raise concerns of possible probe dimerization, analysis of the probe sequences, shown in **Table 2**, shows that both probes are at low risk of either self or cross dimerization. Therefore, these bands likely pertain to unincorporated probes, as the added mass provided by the glycol spacer and biotin head may cause the probes to produce this banding pattern.

Table 2: Properties of probe designs. *Data generated by ThermoFisher Scientific online analysis tools.*

Name	Sequence	CG %	Length	Self-Dimers	Cross Dimers
L_16S	ccyacgtgatctgagttcagaccggagtaatccaggtc	53.9	38	NA	NA
H_16S	aggcgatgtttttggtaaacaggc	45.8	24	NA	

Additionally, a PCR performed on extracts obtained through magnetic capture using mtDNA-specific primers failed to yield successful amplification of mitochondrial genes, with bands only appearing on tDNA samples and neither fractions. Despite a low predicted likelihood, dimerization of probes may still be preventing them from forming mtDNA-probe hybrids. However, a lack of appropriate bands in magnetically separated fractions may be due instead to insufficient hybridization time since the protocol was expedited for the initial run.

Complete Run

The experiment was repeated with a hybridization time of 1 hour, constituting a complete run, and yielded the following results.

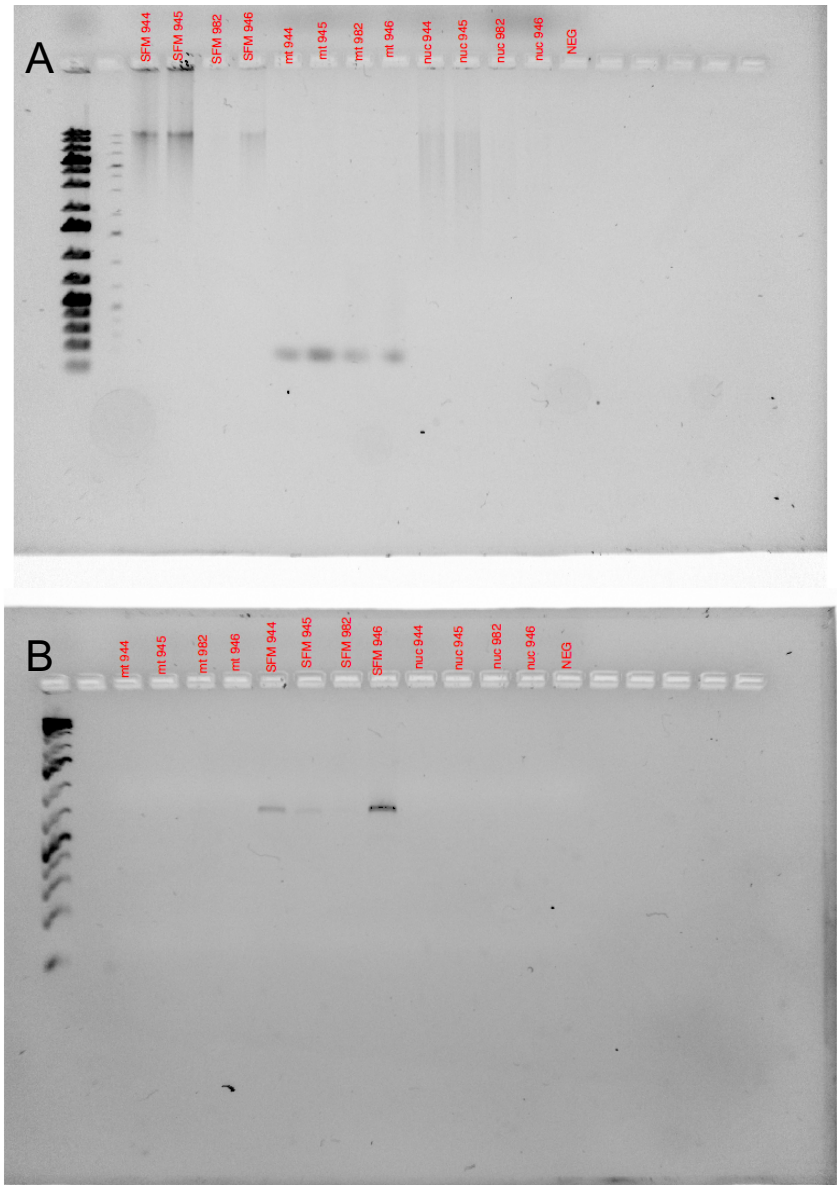


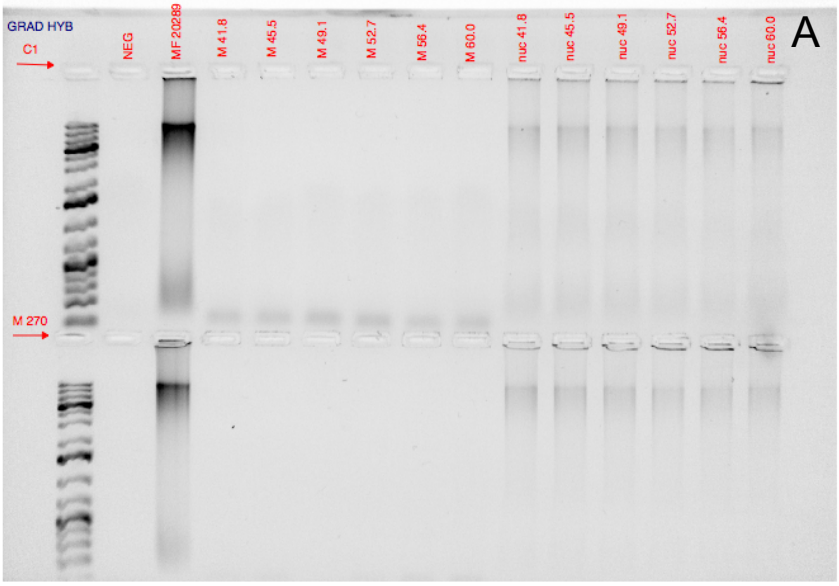
Figure 4: Gel Electrophoresis of products before (A) and after PCR (B) with 1-hour hybridization step*. Dynabeads M-270 Streptavidin were used and hybridization temperature was 45 ºC.*

Despite a longer hybridization time, the results did not change drastically from the first trial, as shown in **Fig. 4**. tDNA samples yielded the appropriate bands to indicate both nucDNA and mtDNA (with the exception of sample SFM 982), as well as displayed successful amplification through PCR, but no magnetically separated mitochondrial fractions showed presence of mtDNA. However, slight bands in nuclear fragments indicate a low concentration of DNA in those extracts. Under the assumption that magnetic separation was unsuccessful, the nuclear 'fragment' should contain both nucDNA and mtDNA; however, a lack of observed amplification post PCR may be due to the steric hindrances presented by the wide array of molecules present in the nuclear fragments.

Hybridization Under a Temperature Gradient

Because a longer hybridization time still failed to produce results indicating successful magnetic capture, the temperature at which the probes were allowed to bind was reconsidered. As shown in Table 2**,** the GC% of the probes designed for this experiment was relatively high, with roughly 54% and 46% for the light and heavy strands, respectively. Because GC bonds are triple bonded, and stronger than the double bonds holding together AT groupings, it is possible that a higher hybridization temperature was needed for the probes to be successful. To determine if temperature plays a significant role in the success of probes, the hybridization step was conducted along a temperature gradient ranging from 41.8-60 ºC across 6 samples (increments of

roughly ~3.7 ºC). The protocol was then carried out as before with a new sample, MF 20289, and the magnetically captured fractions underwent PCR amplification with the same mitochondrial primers. Additionally, a second gradient hybridization was performed using Dynabeads C1 Streptavidin in lieu of the M-270s to rule out the possibility that the bead type used in previous trials was the source of error.



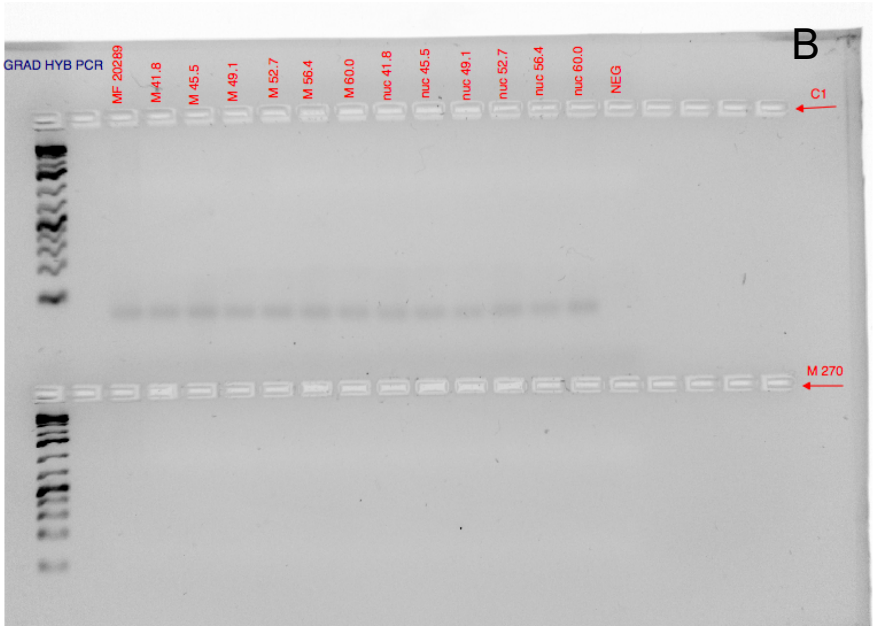


Figure 5: Gel Electrophoresis of products before (A) and after PCR (B) under a temperature gradient and with vairbale bead types*. Dynabeads M-270 and C1 Streptavidin were used and hybridization temperature was varied from 41.8-60 ºC for 1 hour.*

The gradient gels shown in **Fig. 5** indicate that the hybridization temperature, at least within the tested range, does not greatly impact the success of the probes and is ultimately not a reason for failed mtDNA extraction. The nuclear fragments display light banding, indicative of the presence of DNA, which the mitochondrial samples appear to lack. Subsequent PCR analysis confirms the absence of mtDNA in the mitochondrial extracts. Since the post-PCR gel yielded no band for the tDNA sample, it either indicates that this sample may not be suitable for use, or that the PCR reaction was improperly conducted; regardless, the following trials returned to using samples from the SFM line of extracts.

Extended Hybridization Time

After temperature was ruled out as the source of error, the factor of hybridization time was revisited. Though hybridization had previously been allowed to occur for 1 hour per trial, new trials were conducted with hybridization allowed to occur for 5 and 24 hour increments. These trials were conducted with the samples SFM 986, SFM 1152, SFM 1158, and SFM 1160.

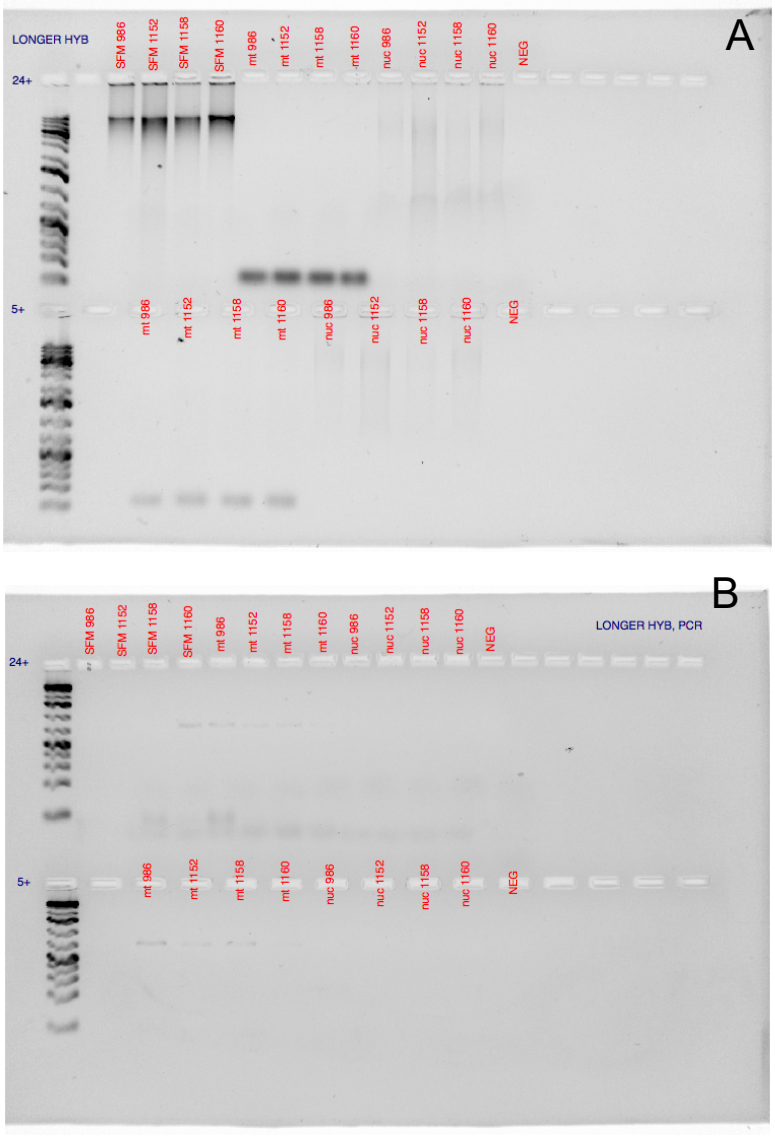


Figure 6: Gel Electrophoresis of products before (A) and after PCR (B) with extended hybridization times*. Dynabeads C1 Streptavidin were used and hybridization temperature was 40 ºC for 24+ hours (top) and 5 hours (bottom).*

As shown in **Fig. 6**, though mtDNA extract samples in the pre-PCR gel seem to display no banding associated with mtDNA, the post-PCR results show succesful amplification in some instances, both in the 5 and 24 hour trials. This is the first indication that the magnetic capture method shows some signs of success— successful

amplification with mitochondrial primers confirms the presence, however faint, of mtDNA in the mitochondrial extracts. Because some amplification was observed, part of the source of error may have been due to the original SFM line of samples not containing tDNA in high enough concentrations to reflect the presence of mtDNA in the extracts.

Fresh Extracts under Variable Conditions

For this reason, new tDNA extracts were obtained from two more recently collected species: one extract collected from *Eleutherodactylus marnockii* (*Eb*), and one from *Lithobates berlandieri* (*Lb*). These extracts were used to conduct another trial, which also tested the efficacy of using each probe individually as opposed to in tandem, as well as tests the impact of tDNA concentration in the success of magnetic capture. The protocol was followed as before, except using an *Eb* sample where hybridization only occurred with probe 1 (L_16S), one *Eb* sample where hybridization occurred only with probe 2 (H_16S), one sample where hybridization occurred with both probes but used 20 µL of *Eb* tDNA as opposed to 10 µL, and one sample that used 40 µL of *Eb* tDNA. The same four trial conditions were conducted with *Lb* samples. Additionally, the C1 beads were used for this trial, and the hybridization temperature was raised to 50 ºC. Most notably, initial denaturing was extended from 15 minutes to 30 minutes.

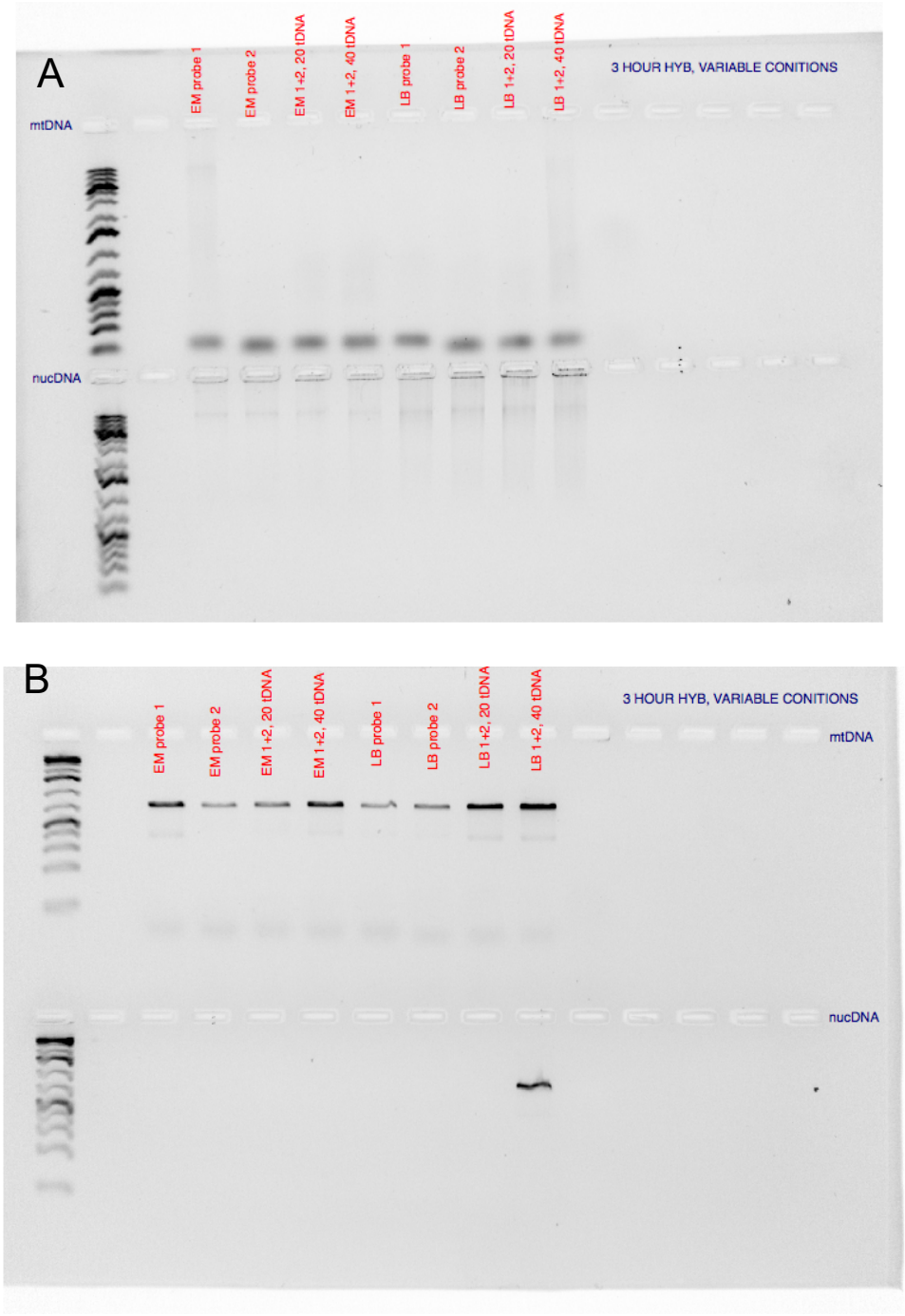


Figure 7: Gel Electrophoresis of freshly extracted products before (A) and after PCR (B) under variable probe combinations and initial tDNA. *Dynabeads C1 Streptavidin were used and hybridization temperature was 50 ºC for 2 hours; samples collected from Eb and Lb.*

The pre-PCR gels shown in **Fig. 7** show faint banding patterns, indicating the presence of mtDNA and nucDNA in the extracted fractions. More notably, post-PCR gels indicate successful amplification in the mitochondrial extracts, supporting successful isolation of mtDNA through magnetic capture. A successful trial may be in part due to fresh samples with a higher concentration of tDNA, to the increased denaturation time— which, if previously insufficient, would prevent proper probe hybridization under any set of conditions— or a combination of these two factors, in addition to the extended 2-hour hybridization time. Of note, the nuclear *Lb* fraction using both probes and an initial volume of 40 µL of tDNA (row 2, lane 10) shows PCR amplification, which could be attributed to errors propagating at one or multiple steps of the protocol. Most likely, contamination between fractions may have occurred while conducting the magnetic separation, or DNA concentrations were high enough that probe saturation occurred.

Extended Denaturation Time versus Fresh Extracts

To determine whether the success of this run was due to fresh samples or to a longer denaturation time, a follow-up trial was conducted using samples from the original SFM line of samples, an extended (30 minute) denaturation time, and a 2 hour hybridization time at 50 ºC.

Fig. 8 shows successful PCR amplification in 3 out of 4 magnetically extracted mtDNA fragments. Though the resulting bands are light, successful amplification in a majority of samples indicates that the longer denaturation time played a key role in the proper probe hybridization prerequisite to successful magnetic extraction. However, because the bands reflect low concentrations of mtDNA in the extracts, and one mtDNA sample failed to amplify entirely, a combination of fresh extracts with a higher

concentration of tDNA and a longer denaturation time remains the set of conditions most likely to yield successful results.

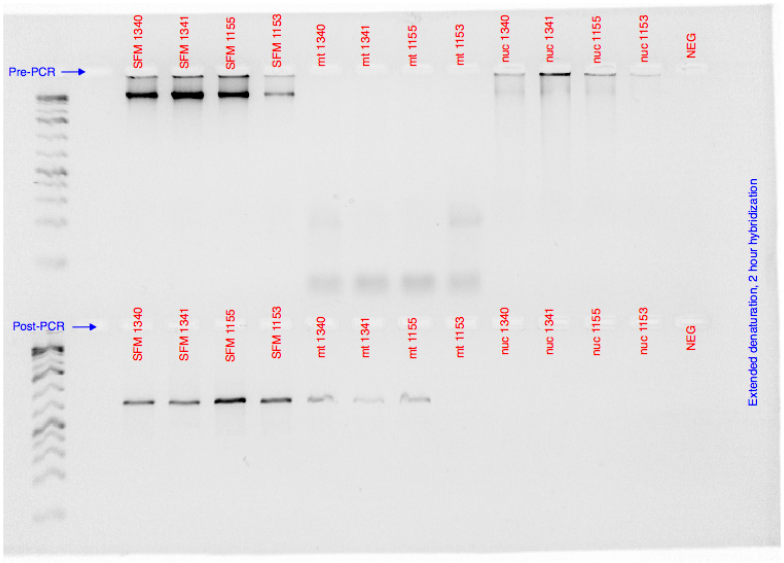


Figure 8: Gel Electrophoresis of SFM line of products before (top row) and after PCR (bottom row) after an extended denaturation. *Denaturation occurred for 30 minutes, while hybridization occurred for 2 hours at 50 ºC.*

In further testing, an important area of research would be to determine the stringency of the probes. The probes may be better suited for certain species over others, and testing for specificity to order may prove to be worthwhile. Should the probes successfully isolate non-amphibian genomes, this could prove troublesome when doing extractions in the field, as the chance for contamination with other organic material may result in confounding results. Additionally, amplifying both the mtDNA and nucDNA fragments with nuclear primers would determine if the fragments contain successfully segregated DNA samples. Furthermore, though this method shows promise after further

testing and refinement, an alternative to this method of whole mitochondrial isolation is to use a PCR-based method that uses primer sets attempting to amplify long fragments of mitochondrial DNA. When primer sets targeting large regions of the mitochondrial genome are used in tandem, a whole mitochondrial genome composite can be generated from the resulting long fragments.

The applications of this mitochondrial genome isolation technique may be well suited for whole genome analysis of amphibian hosts. The mitochondrial genomes are viable for a host of downstream analyses, with NGS library preparation being of notable interest. This method shows potential for efficient upward scaling; if conducted on plates as batches containing samples collected from multiple organisms, the isolations can undergo WGA and be taken directly to Illumina sequencing and library preparation, allowing for streamlined data acquisition at the population level.

IV. References

Avise, J. C. (1986). Mitochondrial DNA and the evolutionary genetics of higher animals. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *312*(1154), 325-342.

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., ... & Hines, H. B. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences*, *95*(15), 9031-9036.

Bentley, P. J., & Yorio, T. (1976). The passive permeability of the skin of anuran amphibia: a comparison of frogs (Rana pipiens) and toads (Bufo marinus). *The Journal of physiology*, *261*(3), 603-615.

Gray, M. W., Burger, G., & Lang, B. F. (1999). Mitochondrial evolution. *Science*, *283*(5407), 1476-1481.

Hocking, D. J., & Babbitt, K. J. (2014). Amphibian contributions to ecosystem services. *Herpetological Conservation and Biology*.

Hornig-Do, H. T., Günther, G., Bust, M., Lehnartz, P., Bosio, A., & Wiesner, R. J. (2009). Isolation of functional pure mitochondria by superparamagnetic microbeads. *Analytical biochemistry*, *389*(1), 1-5.

Kerby, J. L., Richards-Hrdlicka, K. L., Storfer, A., & Skelly, D. K. (2010). An examination of amphibian sensitivity to environmental contaminants: are amphibians poor canaries?. *Ecology letters*, *13*(1), 60-67.

Lathe, R. (1985). Synthetic oligonucleotide probes deduced from amino acid sequence data: theoretical and practical considerations. *Journal of molecular biology*, *183*(1), 1-12.

Levitt, M. (1983, January). Computer simulation of DNA double-helix dynamics. In *Cold Spring Harbor symposia on quantitative biology* (Vol. 47, pp. 251-262). Cold Spring Harbor Laboratory Press.

Malvin, G. M. (1988). Microvascular regulation of cutaneous gas exchange in amphibians. *American Zoologist*, *28*(3), 999-1007

Manuelidis, L., Langer-Safer, P. R., & Ward, D. C. (1982). High-resolution mapping of satellite DNA using biotin-labeled DNA probes. *The Journal of cell biology*, *95*(2), 619-625.

O'hanlon, S. J., Rieux, A., Farrer, R. A., Rosa, G. M., Waldman, B., Bataille, A., ... & Martin, M. D. (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. Science, 360(6389), 621-627.

Rodriguez, D., Longo, A. V., & Zamudio, K. R. (2012). Magnetic capture hybridization of Batrachochytrium dendrobatidis genomic DNA. *Journal of microbiological methods*, *90*(3), 156-159.

Scheele, B. C., Pasmans, F., Skerratt, L. F., Berger, L., Masrtel, A., Beukema, W., … Canessa, S. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science, 363*(6434), 1459-1463.

Stojanovic, N., Florea, L., Riemer, C., Gumucio, D., Slightom, J., Goodman, M., ... & Hardison, R. (1999). Comparison of five methods for finding conserved

sequences in multiple alignments of gene regulatory regions. *Nucleic Acids Research*, *27*(19), 3899-3910.

Swei, A., Rowley, J. J., Rödder, D., Diesmos, M. L., Diesmos, A. C., Briggs, C. J., ... & Han, B. (2011). Is chytridiomycosis an emerging infectious disease in Asia?. *PloS one*, *6*(8), e23179.

Tagle, D. A., Swaroop, M., Lovett, M., & Collins, F. S. (1993). Magnetic bead capture of expressed sequences encoded within large genomic segments.

Welsh Jr, H. H., & Ollivier, L. M. (1998). Stream amphibians as indicators of ecosystem stress: a case study from California's redwoods. *Ecological Applications*, *8*(4), 1118-1132.