Does the Great Lakes basin have novel genotypes of the common reed *Phragmites australis*?

Submitted by

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Abstract

The common reed *Phragmites australis* is present in both native and introduced subspecies throughout North America, and the introduced subspecies is highly invasive. The population of the invasive introduced subspecies in the Midwest is generally thought to be a single genetic lineage. In the following project, difficult to identify stands of *Phragmites* were investigated and evidence was found of more than one genetic lineage in the introduced population. Chloroplast and nuclear gene sequencing results from the project provided evidence that atypical samples can be identified as a different haplotype than the common introduced lineage, with proposed region-specificity to Missaukee County and Blake Lake areas of Michigan. Further studies of other genetic loci are needed for the expansion of knowledge regarding the atypical introduced haplotype, which will allow natural resource managers to treat and control the spread of the species post-identification.

Keywords: Phragmites australis, haplotype, invasive species, genetic sequencing

Introduction

Phragmites australis, or the common reed, is a wetland grass that, in some environments, is considered aggressive. One of the main gaps in research is how to distinguish these aggressive subspecies before they begin to "take over" the environments of other known *Phragmites* species. Past studies have shown that this plant and its counterparts can compete with and disturb other plant species, as well as animals, in the surrounding areas it resides. Distinguishing between a species that is invasive or on the brink of adaptation is not an easy feat, but through genetic research and comparing different haplotypes - or groups of genes passed down from one parental organism of said species - of samples on the molecular scale, one can come to this conclusion, or at least have enough evidence to support one side over the other. While native species and the introduced species are hard to distinguish on morphology alone, through the identification of definitive DNA sequences in the chloroplast of these species, one hopes to easily compare one species to another.

Current research

Lambertini (2016) began working on chloroplast sequencing of *Phragmites*, specifically to determine the likelihood that this species was exhibiting heteroplasmy, or the ability to have more than one genome in a cell or an individual. This is crucial to being able to decide if a species is exhibiting different polymorphisms, or if their genomes are essentially "seeing double" (Lambertini, 2016). While this project examines the atypical properties of unknown haplotype samples, it is unclear if these anomalies are as a result of heteroplasmy. When natural resource managers identify atypical samples and aspire to determine their haplotypes, this is something that should be taken into account as an influential factor in identification, as it may interfere with results. Hybridization is common in many plant species, but with the genus *Phragmites*, this was

not enough to decide what was occurring with its behavior. Along with the native types of *Phragmites* in Northern America, there are eight other non-native types of the species, which often interbreed with the types already present in the environments in which they choose to hybridize (Lambertini, 2016).

James Cronin touches upon the biological changes and possible detrimental effects that pose when trying to take control of an invasive species in his journal article published in 2016. If the introduced *Phragmites* haplotypes were to be taken care of in the way that is seen fit for pest-like species, the much less common native haplotype would be in danger of possible extinction (Cronin, 2016). Because biological control requires such a high level of specificity, more research would need to be done in regards to how to distinguish between introduced and native types in the wild, such as the wetlands environment, before any sort of treatment were to take place. Specificity is key within this issue, as well as this experiment and current research taking place; without the necessary knowledge and distinction between usable primers and materials, no real progress can be made.

A more recent publication was an introduction into the mystery of *Phragmites* is the discovery and study of the Gulf Coast type, which is most prevalent in the southern areas of the United States. In their study, Lambert and his team looked to assess the biogeographical expansion of this type and to compare it to other types of *Phragmites* - such as the *americanus* (native) and haplotype M (introduced). Not only were they observing the behavior of the Gulf Coast type and to see if its biomass would decrease like that of the types prevalent in the East, they found other types of *Phragmites* that were native to Asia (types P and Q) and that were not known to occur in the United States but were found in California (Lambert, 2016).

Up to eleven different haplotypes are present in Northern America, alongside sixteen others that come from all over the world, including Asia and Europe. This information is readily available due to the work of Kristin Saltonstall who, alongside finding the specific primers used for the molecular analyzation of *Phragmites* in this project, was able to create a sort of tree that showed the connections between these different types. By comparing the different types of this plant, Saltonstall was able to determine if they had one or more copies of the indel of interest, which is either an insertion or deletion of bases in the genome of an organism (Saltonstall, 2002). With this information, one can determine if a type of *Phragmites* is either a genotype that is already known to the field of study, or if it is an unknown haplotype that has not yet been discovered in areas of interest, such as Northern America.

In 2014, Saltonstall and her team looked at hybrid plants of *Phragmites* to determine if intraspecific hybridization between native and introduced lineages were resulting in the genetic anomalies we are seeing in current atypical samples (Saltonstall, 2014). When restricted to one loci of interest, these samples displayed both native and introduced properties, suggesting hybridization between the two species and creation of genetically atypical hybrids. Through this work, similar to previous studies, the basis for more than one genetic locus of differences is clear. In-field work of *Phragmites* haplotypes are dependent upon more than one factor to know if a sample is introduced or native, as hybridization between the two species is observed in the wetland regions where these studies are based. In addition to chloroplast DNA being analyzed, Shuo Yu and his research cohort isolated various microsatellite loci for the identification of haplotype differences. While many were hard to identify due to the sensitive and strenuous nature of isolating loci, this study suggests that the diversity of genotypic features can aid in the plant's livelihood, even if it poses a risk to other species ecologically (Yu, 2016). Future studies should focus around

finding more loci to strengthen the proposed haplotype identities, as Saltonstall stated in her publication in 2014.

Project inspiration

Chloroplasts, or the organelles located within plant species that are utilized for photosynthetic purposes, are the main focus of research of *Phragmites*. This organelle is inherited maternally, meaning that it is passed down from generation to generation from the "female" plant. Connections between different haplotypes of *Phragmites* may be drawn through the comparison of chloroplast DNA; the likeness of certain samples may suggest that they were inherited from the same maternal chloroplast DNA, which would prove a genealogical connection.

Using chloroplast DNA, stretches of genetic sequences can be examined through the use of polymerase chain reaction (PCR). PCR is a tool utilized to amplify the amount of sample one has. An exponential growth occurs during this process, leading to a higher concentration of sample that may be tested and further analyzed by the researcher. In PCR, two kinds of primers are required: forward and reverse. These two primers make up a primer pair, which aid in the construction of "new" (or copied) stretches of a given DNA sequence. Since DNA is double-stranded, both primers are essential in copying both strands of the DNA so that a higher concentration of sample may be acquired. This is why it is so important to have the right primer pairs: if inaccuracy happens at this step, the following steps of genetic sequencing will not be as precise as intended.

Different genetic mutations, or indels, can be present within a given species. A specific mutation may often be present in a specific sample or sub-group of samples, which can help in

the comparison of sequences. Nuclear genes, such as NRT2, can be used as a confirmation of haplotype differences among atypical samples of *Phragmites australis*. This gene is present in both native and introduced strands of the plant, with few differences among SNP locations. Comparing base identities at these locations can allow natural resource managers to strengthen their evidence of proposed haplotype identities not just on a chloroplast level, but in addition a nuclear gene level. Not only is *Phragmites australis* allowing for a look into the nature of invasive versus introduced species, research about this reed may lead to even more discoveries, including that of global impact on Earth's organisms. Franziska Eller believes that the common reed may give insight into how global changes play a role in how species evolve and interact with their environments - as well as what kind of adjustments can be made in order to prevent these changes from becoming detrimental towards the species (Eller, 2017). Because of *Phragmites'* stability and overall strength when facing environmental adversity, Eller and her team decided that this species would be the most beneficial to study; not only does this species allow understanding of invasive behaviors, but also how the environment and ecological influences play a role in the species' survival.

To further expand this project and research, gaps in knowledge must be filled, such as the differentiation of contrasting haplotypes of *Phragmites* on a molecular level, as well as making this information readily available to the public so that advancements can be made in preventing introduced species from becoming invasive.

Aims and Objectives

Introduction

When investigating a species and deciding how to approach the likelihood of it eventually becoming invasive, there are several questions and concepts that must be addressed. These include, but are not limited to:

How do we know if a species is considered invasive, or is just introduced? What kind of tools are required to make a change in the effect of this species on the environment it is choosing to invade?

The aims of this project were:

Aims

1. To investigate how species can be identified on the molecular level, through genetic sequencing and analysis.

2. To test the hypothesis that stands of Phragmites identified as atypical by natural resource managers may be genetically distinct from the main population.

Objectives

1. By obtaining different samples of *Phragmites*, such as those showing atypical behaviors and morphological qualities (changes in leaf color, stem height), one can compare each chloroplast sequence to known haplotype sequences. Through that comparison, the hypothesis will be supported regarding the question of whether these new samples are different haplotypes or are exhibiting different biological forms of the known types in Northern America.

Methodology

DNA Purification

Prior to sequencing the chosen samples for this project, some were still in their solid form and had to be broken down to a product of isolated DNA fragments in suspension, or what is called an amplicon. Using a Thermo Scientific MixerMill MM 400, *Phragmites* leaves were degraded to a powder form and treated with a nuclease to further aid in breakdown. Column extraction using the Macherey-Nagel Nucleospin Plant II DNA extraction kit and quantification of DNA with the Thermo Scientific Nanodrop allowed for identification of how much DNA was available within the amplicon.

Sample demographics

Source	Sample ID
Vicki Sawicki - CISMA	M55 & Morey Rd.
Vicki Sawicki - CISMA	Ridgeview Dr.
Vicki Sawicki - CISMA	Gooselake-1
Vicki Sawicki - CISMA	Gooselake-2
Vicki Sawicki - CISMA	Gooselake-3
Joanna Freeland – Trent U	ICP-5 – France
Joanna Freeland – Trent U	Poc1-3 – Quebec
Carolyn Henne – US Forest Service	6-001 – Black Lake, Mason County

Table 1: Sample ID and source of atypical sample

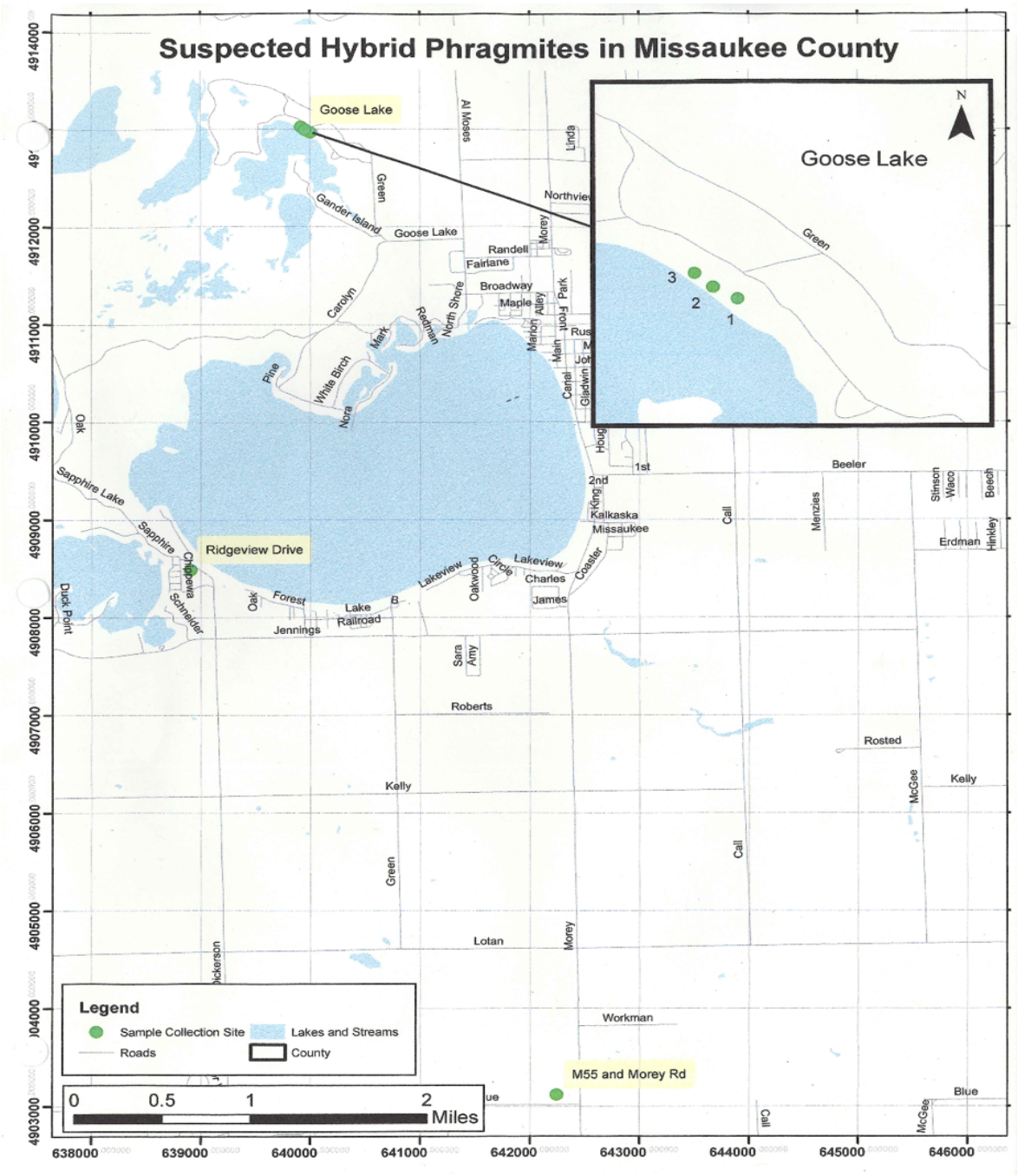

Figure 1: Suspected *Phragmites australis* hybrid map detailing atypical sampling done in Missaukee County, MI. Map courtesy of Vicki Sawicki of North Country CISMA

Preparation & Selection of Primers

As mentioned, both forward and reverse primers are required for a PCR reaction to take place, with one running along the sense direction (5' to 3' end) and the other along the antisense (3' to 5' end), creating an overlap and complete DNA strand. Variants of Kristin Saltonstall's primers were used in these experiments, with new primers designed in June of 2022. By making alternative primers, specificity to atypical samples of interest is achieved.

Chloroplast Sequencing

PCR amplification was carried out according to the program "Phusion" designed with Thermo Scientific Phusion Plus 2x PCR Master Mix.

The Phusion PCR protocol was followed for 20ul or 0.5um total reactions. 10 ul Phusion PCR 2x Master Mix, 2 ul Forward Primer (5uM), 2 ul Reverse Primer (5um) and enough total DNA sample to get 50-100 ng DNA was added in addition to nuclease free water.

Primer	Sequence	PCR	Sequencing	Source	Anneal	Product Size (bp)
trnT(UGU)a	CATTACAAATGCGATGCTCT	X	X	Taberlet 1991	56	900
trnL(UAA)5b	TCTACCGATTTCGCCATATC	X	X	Taberlet 1991	56	900
rbcL	TGTACAAGCTCGTAACGAAGG	X	X	Saltonstall 2001	54	1080
psaI	CTAAGCCTACTAAAGGYACG	X	X	Saltonstall 2001	54	1080
rpL23F	AGGTAGTAGCTGTGAATAGC		X	Saltonstall 2001	54	1080
rpL23R	AGTCGATGGCTATTCACAGC		X	Saltonstall 2001	54	1080
trnT-alt1	CCGATGACCCTCGCATTACA	X	X	This work	54	924
trnL-alt1	TAGCGTCTACCGATTTCGCC	X	X	This work	54	924
rbcL-alt1	GGGTGGCTTTAGAAGCCTGT	X	X	This work	54	1219
psaI-alt1	GAGAAAATTTGGCCCCGTCG	X	X	This work	54	1219

Table 2: Primers used for PCR amplification and sequencing reactions. Any primer with the suffix "alt1" was designed June 6th, 2022 specifically for this project.

Samples were denatured at 98° for 30 seconds, followed by 35 cycles annealing at 98° for 10 seconds, 60° for 10 seconds and 72° for 45 seconds. Extension at 72° was carried out for 5 minutes and then held at 8° until the samples were stored.

PCR product clean-up was made available with the Thermo Scientific ExoSAP-IT Express enzymatic kit, which removes excess primers, buffer, and any other contaminants present in the sequencing solution. Gel electrophoresis to quality control and visually quantify DNA concentration was done with 1.0% agarose gel and GeneRuler 1kb Plus Ladder (Thermo Scientific). Only one band must be present to determine final concentration – if there was more than one band present, contamination may have occurred, and the experiment must be started over. For sequencing, 20ng/ul of the sample must be available.

Sequencing reactions were created to a total of 10ul, with BigDye Terminator Ready Reaction Mix ver1.1 (Thermo Scientific) and 1.6uM of an individual primer. In this stage of testing, samples were separated by primers with 2 tubes per sample and an individual primer assigned to each. Using the protocol BigDye1_1, samples were denatured at 96° for 1 minute, followed by 25 cycles of annealing at 96° for 10 seconds, 50° for 5 seconds, and 60° for 4 minutes extension.

Cycle sequencing cleanup using SAM solution and BigDye XTerminator Solution (Thermo Scientific) was carried out, with samples placed on a shake plate at max speed for 30 minutes and centrifuged at 1000 xg for 2 minutes. Sequencing reactions were analyzed using the SeqStudio Genetic Analyzer by Thermo Scientific according to manufacturer instructions.

Sequence reads are then analyzed post-SeqStudio. Cap3 Sequence Assembly Program (Huang and Madan, 1999) by PRABI was used to make contigs of forward and reverse sequences, which

were then aligned in JalView software (Waterhouse et. al, 2009) with MUSCLE multiple sequence comparison in tandem with known haplotype sequences as provided by Kristin Saltonstall. Using percentage identity by color, known haplotype sequences were narrowed down to specific trnT/trnL or rbcL/psaI haplotypes associated with either composite haplotype M or O. Based upon the pairing of both primer sequences and loci, the haplotype can be identified for that sample.

Nuclear Gene Sequencing - NRT2

Similar to chloroplast sequencing, amplicons were prepared prior to PCR, sample clean-up, sequencing reaction and analysis by the SeqStudio. PCR amplification was done with the protocol “PHRAGED64” and DreamTaq 2x PCR Master Mix from Thermo Scientific.

The PHRAGED64 PCR protocol was followed for 20ul or 0.5 um reactions. 10 ul DreamTaq PCR 2x Master Mix, 2 ul each of AB096061F (5uM) and AB096061R (5um) primers, in addition to enough DNA sample to get 50ng of DNA was mixed with nuclease free water to bring up to 20ul total volume.

Samples were denatured at 94° for 2 minutes, followed by an annealing period of 34 cycles: at 94° for 45 seconds, 64° for 45 seconds and 72° for 1 minute. Samples were then extended at 72° for 2 minutes and held at 12° until storage.

PCR product clean-up was made available with the Thermo Scientific ExoSAP-IT Express enzymatic kit, which removes excess primers, buffer, and any other contaminants present in the sequencing solution. Gel electrophoresis to evaluate purity and estimate the quality of amplicon was compared to Thermo Scientific GeneRuler 1kb Plus DNA ladder. With one band expected to

be present, concentration of the sample can be estimated with the band intensities of the ladder. 20 ng/ul was needed for sequencing of samples.

Sequencing reactions were created to a total of 10ul, with BigDye Terminator Ready Reaction Mix ver3.1 (Thermo Scientific) and 1.6uM of an individual primer. In this stage of testing, samples were separated by primers with 2 tubes per sample and an individual primer assigned to each. Using the protocol BigDye1_1, samples were denatured at 96° for 1 minute, followed by 25 cycles of annealing at 96° for 10 seconds, 50° for 5 seconds, and 60° for 4 minutes extension.

Cycle sequencing cleanup using SAM solution and BigDye XTerminator Solution (Thermo Scientific) was carried out, with samples placed on a shake plate at max speed for 30 minutes and centrifuged at 1000xg for 2 minutes. Sequencing reactions were analyzed using the SeqStudio Genetic Analyzer by Thermo Scientific according to manufacturer instructions.

Sequence reads were then analyzed post-SeqStudio. Cap3 Sequence Assembly Program (Huang and Madan, 1999) by PRABI was used to make contigs of forward and reverse sequences, which are then aligned in JalView software (Waterhouse et. al, 2009) with MUSCLE multiple sequence comparison to determine polymorphisms at genetic loci of interest. Using Excel, these locations were compared with previously identified NRT2 sequences and known haplotype identities to determine if any genetic anomalies were present in proposed haplotype O samples.

Results

Sequence alignment of atypical samples and known samples has allowed the identification of a potential haplotype that is unlike what is normally found in the Great Lakes Basin. Haplotype O is related to Haplotype M, which is commonly found as the introduced identity in this area. However, Haplotype O originates from Europe and is normally found there, while M originates from Europe but can be found across the globe. Using JalView multiple sequence alignment software, haplotypes of atypical samples were identified. Samples of note were Ridgeview Dr., Gooselake-2, Gooselake-3, and Black Lake identified as haplotype O. While samples from France and Quebec showed NRT2 patterns of a haplotype associated with O, their chloroplast results identified them as haplotype M. This is an anomaly as compared to all other atypical samples that were identified as the common introduced form. Identities were compared based on chloroplast sequence alignment and NRT2 nuclear gene alignment.

Chloroplast results

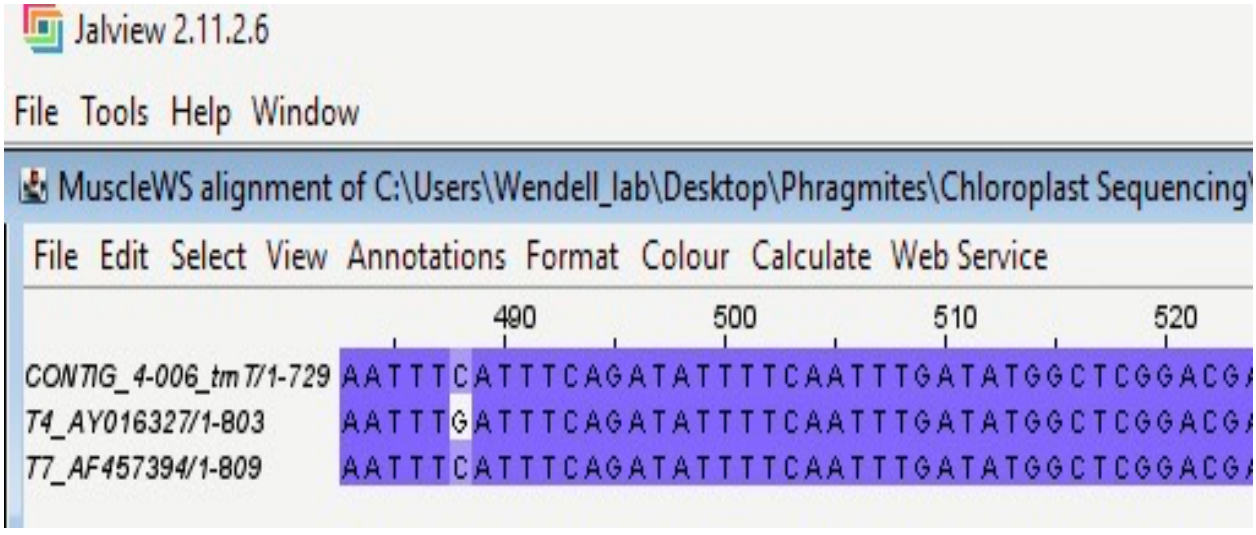

Figure 2: Proposed haplotype O sample 4-006 (Gooselake-2) aligned with trnT/trnL loci identities using JalView and MUSCLE alignment system

Sample 4-006 (Gooselake-2) showed a base change in chloroplast sequence alignment, which motivated the alignment of NRT2 nuclear gene sequences to identify any other anomalies and support the proposed identification of haplotype O. All other proposed haplotype O chloroplast sequences had their original samples sequenced for the NRT2 gene SNPs of interest.

Haplotype identities and associated loci primer pairs

Haplotype Identity	trnT/trnL	rbcL/psaI
M	T4	R4
O	T7	R4
E	T2	R2

Table 3: *Phragmites australis* haplotype identities found in the United States. Haplotype E is representative of the native species, while O and M represent introduced species. Haplotype M is the most common of the introduced to be found in Michigan

Because of the concern that a single base distinguished the differences, and only a small difference was present in the trnT/trnL locus identification for proposed haplotype O atypical samples, additional polymorphisms were hoped to be identified with continued experimentation with NRT2 nuclear gene. As evidence for haplotype differences was proposed on the chloroplast level, nuclear gene sequencing was carried out for additional support in this finding.

NRT2 Results

NRT2 SNP Location	Haplotype 1	Haplotype 2
486	T	C
514	G	C
536	T	C
547	T	C
849	C	G
1185	T	C

Table 4: NRT2 SNP locations and based identities of 2 proposed haplotypes

At these SNP (single-nucleotide polymorphism) locations, homozygous base identities were observed and different across haplotypes, with all other base locations the same identity between haplotypes. These base changes at these SNP locations of interest align with the proposed atypical samples that identify as composite haplotype O, strengthening the evidence of genetic anomalies between them and their known counterparts.

Summary of results

Sample ID	cpDNA Haplotype	NRT2 Haplotype
M55 & Morey Rd.	M	2
Ridgeview Dr.	O	1
Gooselake-1	M	2
Gooselake-2	O	1
Gooselake-3	O	1
France	M	1
Quebec	M	1
Black Lake, MI	O	1

Table 5: Proposed chloroplast (cpDNA) and NRT2 nuclear gene haplotypes of atypical samples from Vicki Sawicki and Carolyn Henne

Samples from France, Quebec, and Black Lake MI had previously been sequenced for their NRT2 data and exhibited a different haplotype than the typical pattern for introduced species. After sequencing their chloroplast DNA, it was determined that the sample from Black Lake followed the pattern of an atypical cpDNA and NRT2 haplotype, with a proposed identification of composite haplotype O. The two other atypical samples were identified as composite haplotype M based on their cpDNA, regardless of their atypical NRT2 data.

Discussion

Sequence alignment and analysis of regions of importance in *Phragmites australis* can help natural resource managers identify typical samples and learn how to treat them. Our results indicate that there may be two different genetic lineages among the invasive subspecies of *Phragmites australis* in Michigan.

The implications of these results are representative of a problem with *Phragmites* identification in Michigan. As our work aimed to identify what haplotype these atypical samples belonged to, we discovered that they showed both novel phenotypic and genotypic properties that set them apart from other introduced forms. However, because of such small differences in their chloroplast genome and base changes in nuclear genes, the answer cannot be definitively given and only proposed strongly. Proposed haplotype O samples near the Missaukee and Blake Lake regions of Michigan seem to be of a region-specific lineage, following the same pattern of differences in both their chloroplast and nuclear genomes.

Future work to be carried out includes identifying other genetic locations where anomalies are present, such as species-specific microsatellites. As we were able to determine more than one genetic location that shows differences between proposed haplotype O and M samples, we can say that our atypical samples are genetically different from the common introduced form present here in the Great Lakes Basin. With the novel identity of proposed haplotype O samples, they appear to be more closely related to each other than to samples of haplotype M. This poses the question of whether these samples truly are haplotype O or are a hybrid species that has yet to be fully analyzed. Further identification of other loci is needed for confirmation.

Biographical Note

My name is Isabella Limbert, and I am a Biology BS major with a cell-molecular specialization. My future goals include becoming a genetic researcher. I hope that my work will make an impact on the world; whether it is through work with innovative genetic research, or if I am just teaching someone something new about genetics, then I know I will have done my job. This project not only gives me the tools on how to display genetic information and ideology to the public, but this opportunity of working in the lab and strengthening my skills is something that I believe will set me apart.

Not only have I had the chance to work with one of my most respected professors, but I also have been given free rein to try out new things in relation to genetics. To try, and to fail, is all part of science, and learning that at such an early stage is helping prepare me for my future. While not everything will turn out to what you thought it would, whether it is a PCR that went bad or a restriction digest that didn't cut, at the end of the day, you're learning something - and that is my goal: to keep learning and living a life of science.

Additional Notes

Special thanks to Dr. Douglas Wendell of Oakland Biological Sciences and the Honors College for the support of this thesis. Thank you to Vicki Sawicki of CISMA Northern County and Carolyn Henne of the US Forest Service for atypical *Phragmites* samples.

References

Cronin, J. T., Kiviat, E., Meyerson, L. A., Bhattarai, G. P., & Allen, W. J. (2016). Biological control of invasive phragmites australis will be detrimental to native P. australis. *Biological Invasions*, *18*(9), 2749–2752. https://doi.org/10.1007/s10530-016-1138-x

Eller, F., Skálová, H., Caplan, J. S., Bhattarai, G. P., Burger, M. K., Cronin, J. T., Guo, W.-Y., Guo, X., Hazelton, E. L., Kettenring, K. M., Lambertini, C., McCormick, M. K., Meyerson, L. A., Mozdzer, T. J., Pyšek, P., Sorrell, B. K., Whigham, D. F., & Brix, H. (2017). Cosmopolitan species as models for ecophysiological responses to global change: The common reed phragmites australis. *Frontiers in Plant Science*, *8*. https://doi.org/10.3389/fpls.2017.01833

Huang, X. and Madan, A. (1999) CAP3: A DNA sequence assembly program

Lambert, A. M., Saltonstall, K., Long, R., & Dudley, T. L. (2016). Biogeography of phragmites australis lineages in the southwestern United States. *Biological Invasions*, *18*(9), 2597–2617. https://doi.org/10.1007/s10530-016-1164-8

Lambertini, C. (2016). Heteroplasmy due to chloroplast paternal leakage: Another insight into phragmites haplotypic diversity in North America. *Biological Invasions*, *18*(9), 2443–2455. https://doi.org/10.1007/s10530-016-1193-3

Lambertini, C., Mendelssohn, I. A., Gustafsson, M. H., Olesen, B., RIIS, T., Sorrell, B. K., & Brix, H. (2012). Tracing the origin of Gulf Coast phragmites (Poaceae): A story of long-distance dispersal and hybridization. *American Journal of Botany*, *99*(3), 538–551. https://doi.org/10.3732/ajb.1100396

Rice, D., Rooth, J., & Stevenson, J. C. (2000). Colonization and expansion of Phragmites Australis in Upper Chesapeake Bay Tidal Marshes. *Wetlands*, *20*(2), 280–299. https://doi.org/10.1672/0277-5212(2000)020[0280:caeopa]2.0.co;2

Saltonstall, K. (2002). Cryptic invasion by a non-native genotype of the common reed, phragmites australis, into North America. *Proceedings of the National Academy of Sciences*, *99*(4), 2445–2449. https://doi.org/10.1073/pnas.032477999

Saltonstall, K., Castillo, H.E. and Blossey, B. (2014), Confirmed field hybridization of native and introduced *Phragmites australis* (Poaceae) in North America. American Journal of Botany, 101: 211-215. https://doi.org/10.3732/ajb.1300298

Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ (2009) Jalview Version 2 - A multiple sequence alignment editor and analysis workbench. Bioinformatics 25 1189-1191.

Yu, S., Zhang, YX., Ren, YL. *et al.* Isolation and characterization of microsatellite loci for *Phragmites australis* . *J Genet* 93 (Suppl 2), 89–92 (2016). https://doi.org/10.1007/s12041-013-0278-3