The biology of an invasive plant species, *Phragmites australis*

a thesis by
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Contents

Abstract: This paper examines the evolutionary history of an invasive plant species. We used molecular markers to examine levels of genetic diversity among North American populations of the common reed *Phragmites australis* in order to determine whether the species is indigenous to North America or was introduced to the continent after European colonization. We sequenced the 45S rDNA ITS fragments for two individuals of *P. australis* and found 0.305% divergence between the sequences. We compared both sequences to a related grass and found 13.5% divergence, indicating that the variation within *P. australis* is significant. Further seqeuncing of members of this species will provide a more reliable assessment of polymorphism among North American populations and will allow us to reconstruct its evolutionary history.

Introduction

Invasive plants are a frequent source of headaches to naturalists, forest ecologists, wildlife managers, and even gardeners. Invasive species are those organisms that will aggressively colonize a new habitat to the exclusion of native species, commonly known as weeds. They pose a threat to biodiversity because they often displace native species. Recently, a member of the grass family, *Phragmites australis*, has come to the attention of North American scientists and naturalists because it exhibits certain ecological traits common to invasives and is expanding its current range.

The common reed, *Phragmites australis*, is prominent in the flora of New York City and the surrounding region. In North America, it is found as far north as northern Quebec (Gervais et al., 1993) and as far south as the Carolinas, and some maps document it as having a nearly worldwide distribution (Holm et al., 1977). *P. australis* is known to be native to the wetlands of the Danube River and other parts of Europe, however there is still debate as to whether a species or subspecies is native to the United States. The debate centers around whether *P. australis* was here before European settlers colonized North America. If so, then mention of it should appear in the records of pre-Columbian peoples or the early European explorers. If not, then a logical hypothesis would be that it was brought here either purposefully or by accident by European settlers.

Soil core evidence is equivocal. Loeb (1998) took pollen cores from Hunter Island, New York, in the Bronx, and found no evidence of *P. australis* before about 1980. Other pollen core records show that it may have been present along the Hudson River during Pre-Columbian times (D. Peteet, 1998). Thus the debate over its origins is still open to investigation.

Invasives can be exotic or native. Knowing whether or not *P. australis* is indigenous to

North America will influence how forest managers approach controlling its spread. Other evidence such as historical records and the results of molecular assays of the plant's evolutionary history will help answer this question.

One feature typical of invasives is that they are habitat generalists, meaning that they will colonize a diversity of habitat types (Meffe and Carroll, 1997). *Phragmites australis* most often grows in fresh and saltwater marshes, but it has also been observed along roadsides both in ditches and on embankments, at the edge of lowland forests, around ponds, and at bogs in upstate New York. Thus *P. australis* is capable of colonizing a wide variety of habitats, ranging from saturated wetlands to the drier soils of embankments, and grows in both full sun and slightly shaded environments. In addition, it has a broad climatic range (Holm et al., 1977).

Certain reproductive traits are often a good indicator that a plant is an invasive species. *Phragmites australis* possesses several reproductive traits common to potent invaders. It reproduces clonally by vegetative spread, and it also produces seeds, but it is unknown whether its seeds will germinate outside the laboratory (Gervais et al., 1993). This leads researchers to raise interesting questions about the species's reproductive biology. Do individuals in a given accession arise from one vegetative ramet, or several, and did these develop clonally or from seed? How does *P. australis* disperse across large distances? Is it outcrossing or selfing, and if selfing, does it hybridize with other species or subspecies, and can we observe hybridization at the molecular level? Discovering answers to these questions will illuminate our understanding of *P. australis*' role in ecological communities.

Phragmites australis has steadily increased its rate of spread in the United States. Several sources, including the Nature Conservancy, include it on their lists of weedy or invasive species (Randall, 1993). This study examines the nature of *P. australis*' recent proliferation. Is it a native or introduced species, and what factors have allowed it to spread to the point of becoming problematic? The study tests two alternate hypotheses: 1) *P. australis* is a native species which has benefited from extensive anthropogenic disturbances to the Northeastern United States' ecosystems, or 2) *P. australis* was introduced to the United States during Post-Columbian times and thrives here for the same reasons many exotic species become weeds.

This study uses nuclear and chloroplast DNA markers to assess the degree of genetic diversity among local populations of *Phragmites australis* in an attempt to reconstruct its

evolutionary history. Evidence of low diversity would suggest that a species is new to a region and has had little time to evolve, that a species was introduced at a single point of entry, or that the species reproduces clonally. High diversity, in contrast, would suggest long standing in the community with time for populations to evolve and diversify, reproduction by seed, or, if the species is an introduction, several points of entry. Using molecular markers to estimate diversity will allow us to determine whether North American populations stem from one or several introductions, or whether the species has evolved in a pattern similar to other native species.

Since *Phragmites australis* exhibits several requisite ecological traits of a successful invader, we expect to find genetic traits that correspond to these observations. Therefore, we expect to see relatively low genetic diversity. Some common ecological traits include clonal reproduction, favoring a diversity of habitats, and being a pioneer species–one of the first species to colonize a new area (Meffe and Carroll, 1997). By measuring levels of diversity among populations, we hope to show that *P. australis*' biological characteristics mirror its ecological behavior.

Background

Indigenous and exotic invasive species

An invasive plant is one which colonizes a habitat to the exclusion of native species. Invasives present a threat to biodiversity because they are capable of outcompeting and thereby eliminating native species, especially rare and threatened species (Meffe and Carroll, 1997). Invasives can be either native or introduced species. An example of a particularly potent invader is *Lythrum salicaria*, or purple loosestrife. Introduced from Europe, this plant has no natural competitors or predators in the United States. It alters the chemical and physical environment within its new habitat, usually freshwater wetlands, such that native species can no longer occupy the space. It also grows faster and more voraciously than native species because it does not have to fight off herbivores. Eventually it outcompetes other wetland herbaceous species and continues to dominate the vegetative community.

Exotics like *L. salicaria* are species which are found naturally in another environment, but which have dispersed to the local habitat. Often human movements bring exotics from a remote

continent either intentionally or as hitch-hikers (Wagner, 1993). Frequently plants are imported for cultivation, but then they "escape" by dispersing a few seeds to a wild habitat. *Alliaria petiolata*, or garlic mustard weed, was brought to the United States from Europe because it is a tasty salad ingredient. People commonly grew it in backyard gardens in the 19th century. Unfortunately this biennial is now seen in large patches along roadsides and at the forest exterior.

Exotic species become invasives when the new climate, soil conditions, hydrology, and physical environment match those of their original habitat. In addition, they typically have few if any biotic barriers to success in a new region (Meffe and Carroll, 1997). Since they did not evolve in their new habitat, there are no natural competitors or predators to control their success. Local species either cannot or do not adapt to their presence. Thus invasive exotics often grow without any biotic checks to their proliferation.

Native invasives originally evolved in the local area. They have natural predators and competitors, and originally they held a stable position in the local environment. However, if conditions change such that a native species gains a more prominent position in an ecosystem, it can become an invasive. Such changes can be biotic, as when a key competitor is lost to the region, or they can be abiotic changes such as flooding or fire. Anthropogenic disturbances allow indigenous species to disperse to new communities where they were not formerly prominent in the flora (Wagner, 1993). An indigenous species can also become an invasive if natural conditions change. For example, if seeds are blown to a forest where the species is not common, and these seeds germinate and grow, the plant has the potential to become an important player in the local community. Certain types of habitats are considered "invadable" because they have been weakened by changes to the community structure. If an area's natural diversity is low or it has recently suffered a fire or similar disturbance, it is considered invadable. A plant can easily become an invasive in these types of communities (Meffe and Carroll, 1997).

The human factor and disturbed habitats

Certain habitats are more vulnerable than others to invasions. These include areas that are low in biodiversity, communities with simple food webs, early successional communities, and disturbed areas. Island communities are also highly susceptible to invasions (Meffe and Caroll, 1997). Disturbed habitats suffer a higher rate of invasions for several reasons. Fragmented

habitats have a large surface to area ratio; that is, the area within a given fragment is small relative to its perimeter. This means that for a smaller piece of land, there is a greater ratio of potential points of entry than for a larger habitat. The borders of fragmented areas are often highly trafficked. Roads are frequently implicated in destructive habitat fragmentations. A new road is problematic because the new edge or exposure offers weed species a pathway into a forest, wetland, or field. Also, a roadway brings more human traffic to the area, which increases the risk that a foreign seed will be carried to the vicinity.

Recently human movements have coincided with an increase in the spread of invasives worldwide. Wagner (1993) argues that human movements affect the rate at which invasions occur. He writes that although invasions themselves are natural phenomena, the rate at which they have taken place in this century is not natural. Wagner characterizes humans as a "superinvader"; their presence has led to changes in natural environments and ecosystems, which in turn have led to changes in global species composition, perhaps inadvertantly selecting for weeds and pests.

In the northeastern United States, this century has brought a rapid increase in the number of disturbed habitats. This means not simply the destruction of existing natural areas, but fragmentation as well. If *P. australis* is a native species, then its recent proliferation may be a function of increased rates of disturbance to local habitats. In addition, the greater New York area is vulnerable to invasion because of a relatively low diversity of native flora. Again, loss of diversity is in large part a result of human activity. Habitat destruction and fragmentation is stressful to rare and endangered species because they lose the physical environment necessary for survival, but also because it allows invasions to occur at a higher rate, which is itself a source of biodiversity loss. The effects of anthropogenic disturbances, the introduction of invasives, and habitat loss are compounded when they work in tandem to reduce the strength of native communities.

Some naturalists and residents of New York City argue that any green is better than none, even if the source is foreign species. There are numerous examples of exotics that thrive in New York because they were cultivated or because they are particularly adapted to a highly disturbed habitat. *Ailanthus altissima*, commonly known as tree of heaven, is a tree that can be found growing out of cracks in the sidewalk. It is one of the few trees that grows abundantly in this city, yet it was originally imported here from East Asia. Other imports that now thrive here are *Acer*

platanoides (Norway maple), *Taraxacum officinale* (dandelions) and *Lythrum salicaria* (purple loosestrife). Norway maple is displacing the native sugar maple (*Acer saccharum*), and loosestrife is a threat to freshwater wetlands. Yet these species are often used to argue that without imported plants and trees, New York would be entirely concrete and metal.

Similarly, *Phragmites australis* grows around the Central Park reservoir, at Jamaica Bay in Queens, and at ponds in small parks like Riverside and Morningside. A pamphlet distributed at Gateway National Park in Queens describes the ways *P. australis* benefits both city kids, who often see no other sign of nature on a daily basis, and the city's ecological landscape, which lacks an abundance of green. We can therefore argue that *P. australis* is an important component of the urban environment. Unfortunately it is spreading rapidly in areas that are less disturbed and in which native diversity is still largely intact. Parks such as Black Rock Forest and Harriman State Park, which are located within an hour's drive of New York City, are at a higher risk of invasion than more distant parks. Both parks already harbor multiple accessions of the common reed. At these sites, preservation of native flora is a more immediate goal than simply maintaining "greenness," as in New York City.

A molecular approach to invasiveness

Molecular markers are short regions of the chromosome (500-1000 base pairs) which can be used to compare evolutionary trends among species. A scientist can amplify and sequence one or a few regions of the genome for multiple plants, then compare the sequence data among all the different organisms. Theoretically, species which are most closely related will have the most similar sequences. One can measure the evolutionary "distance" between any two species based on the number of base pair differences they have within one molecular marker region.

Numerous genetic studies have taken advantage of the wealth of knowledge that exists about the genomes of certain species to design these types of evolutionary studies. The results can be used to confirm taxonomical relationships that were originally set up using morphological traits. Also, a researcher can use molecular markers to identify the origins of species radiations, where several species arise from one original founder species. An example of a radiation is Darwin's finches in the Galapagos Islands. The dominant theory is that these species all evolved from one original mainland species. Molecular marker studies of the finches have confirmed this hypothesis

(Weiner 1994).

In plant systematics, one can use molecular markers to identify the founder species for a set of related species growing in one region. Also, one can study a single species and look for variation within that species that corresponds to geographical barriers, historical trends, or climate changes. This type of study can also help determine how much polymorphism exists within a species.

Our study attempts to use two regions, one located on the nuclear genome and one found on the chloroplast genome, to identify trends in the evolution of local populations of *Phragmites australis*. We propose that if *P. australis* is an introduced species, then North American populations should show a pattern of diversification that traces back to one or a few points of entry. Assuming that *P. australis* is an introduction, then it has had a relatively short time to diversify from the original founder population, and overall genetic diversity among populations should be relatively low. Conversely, if *P. australis* is native to North America, then it should show a higher amount of diversity among local populations as evidence of its long standing in the community. However, measures of genetic diversity are frequently influenced by other factors, such as the organism's primary mechanism of reproduction, therefore one should be careful to compare results with species that have similar reproductive biology and ecological traits.

For the purposes of this study, we selected the 45S internally transcribed spacers (ITS) rDNA region on the nuclear genome (Baldwin et al., 1995), the trnL-F spacer and introns (Taberlet et al., 1991), and trnS-T and trnH-K spacers on the chloroplast (Demesure et al., 1995). Spacers and introns are non-coding regions of DNA. Because mutations that arise in non-coding regions tend to be preserved with greater frequency than mutations in coding regions, the former exhibit higher levels of variation. When mutations in the coding regions cause translation errors, they are usually corrected by the cell's own regualtory mechanisms. Alternatively, the non-functional protein product causes a lethal reaction whereby either the cell or the entire organism dies. In either case the mutation is not transferred to the next generation. Mutations in non-coding regions are rarely lethal to a cell, therefore they are more frequently passed to the offspring.

The ITS region is particularly suited to the purposes of this study. The 18S and 26S flanking regions (Figure 1) are highly conserved among taxa (Baldwin et al., 1995). This means that amplification is relatively easy. The length of the spacer regions does not vary substantially

among taxa so that aligning sequences among taxa is not difficult. At the same time, the individual nucleotide sequences are variable, so that one can detect patterns of diversity among species. Baldwin et al. (1995), write, "DNA sequences of these spacers might be readily alignable across taxa, yet sufficiently variable to allow resolution of lower level phylogenetic questions in angiosperms."

The purpose of this study is to use nuclear and chloroplast molecular markers to assess the degree of diversity among local populations of *Phragmites australis*, thereby providing evidence for its status as either an introduced or native invasive.

Methods

Field work

Experimenters visited several local sites where *Phragmites australis* is abundant, including Jamaica Bay, New York, Island Beach, New Jersey, Harriman State Park, New York, and Black Rock Forest, New York. In addition, samples were taken from Myrtle Beach, South Carolina. By comparing the level of diversity among the New York and New Jersey sites with that of the South Carolina samples, one can determine whether variation seen locally is significant among United States populations as a whole.

To collect tissue samples, experimenters clipped one to three leaves from multiple plants in each stand of *P. australis*. For each location, vouchers were made by pressing an entire plant between newspaper and blotting paper in a standard plant press. The samples were numbered and labelled, and each sample was recorded with a short description of the location from which it was collected. The tissue samples were stored in sealable plastic bags at -80C until needed for extraction.

DNA extraction

The extraction procedure used (Bult et al., 1992) was adapted for use on grasses and herbaceous species after a trial and error series that yielded scattered successes (Appendix 1). The most significant modification we made to Bult's protocol was the grinding procedure. Grasses and

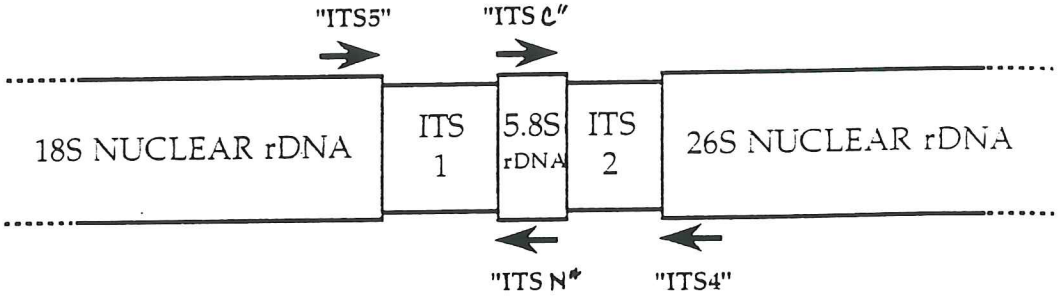
some perennials yielded sufficient quantities of DNA, while other species were more problematic. Successful extractions seemed to result when the structure of a species' leaf tissue was suited to the grinding procedure.

Ultimately the procedure we used required the following steps. Leaf tissue was taken from an individual plant and placed in a 2.0 mL Eppendorf tube that had been cooled in liquid nitrogen. The tissue itself was then soaked in liquid nitrogen and ground using plastic grinding tools (Fisher, Chicago, Illinois) until it was a fine powder. Next, CTAB solution (6% CTAB, 0.7M NaCl, 50mM Tris-HCl, and 10mM EDTA) was warmed to 60C and 1 ml of CTAB was added to each tube. In addition we added 1.0 µl of β-mercaptoethanol to prevent the tissue from oxidizing. The tubes soaked in a water bath at 60C for 30 minutes. Every 5 minutes the tubes were agitated. After 30 minutes, we added 500 µl of 24:1 chloroform:isoamyl alcohol (CI) to each tube. The tubes were gently inverted several times, and were centrifuged at high speed for 5-10 minutes to separate the dissolved nucleic acids from other dissolved plant tissue material. The upper aqueous layer contained the nucleic acids. It was aliquoted into a 1.5 ml Eppendorf tube, and the waste was discarded. To the nucleic acids, we added 1.0 ml of 100% ethanol or isopropyl alcohol and 10 µl 5M NaCl. These were stored at -20C for at least ten minutes to allow the DNA to precipitate out of solution. The precipitate was pelleted by centrifugation at high speed for 5 minutes, the aqueous solution discarded, and the pellet resuspended in 300 µl TE buffer. Two additional precipitations further purified the DNA of any remaining dissolved tissue. The first used 200 µL of 7.5M NH_3OAc and 1.0 ml ethanol. Then the nucleic acids were pelleted again and resuspended in 300 µl 1x TE buffer. The final precipitation used 42 µl 3M NaOAc and 1.0 ml ethanol. After centrifuging the tubes, we added 70% ethanol to cleanse the tubes of any residual salts, and resuspended the DNA in 100 µl 1x TE buffer. The success of these suspensions was tested against an uncut sample of λ DNA on a 1% agarose gel stained with ethidium bromide.

Amplification of target regions

Successful DNA extractions were amplified using the Polymerase Chain Reaction (PCR) on regions of both the chloroplast and nuclear genomes (Appendix 2). For the plastid genome, the reaction targeted the trnL-F spacer and introns (Taberlet et al., 1991) and trnS-T and trn H-K spacers (Demesure et al., 1995) using primers published in these papers. In the nuclear genome, we amplified the 45S rDNA internally transcribed spacers (ITS) using primers specified in Baldwin et al. (1995) (Figure 1).

FIGURE 1. Organization of the ITS region. Arrows indicate the approximate location of primer start sites. The ITS 1 and ITS 2 regions are approximately 250 bp long each. The 5.8S region is approx. 163 bp long. Adapted from Baldwin et al., 1995.

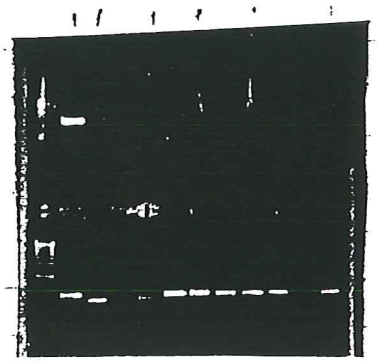


For each sample, reactions began with a 3-minute denaturation followed by 30 cycles of 30-second denaturations at 94C, 1-minute primer annealing at 49C, and 1-minute primer extension at 72C. A final extension segment of 5 minutes ended the reaction sequence, and the reactions were stored at -20C until purification. Reaction success was tested against a 1kb ladder on a 1% agarose gel stained with Ethidium Bromide (Figure 2). Failed reactions may be attributed to several factors, including variable primer integrity, and quantity and quality of the DNA extracted.

Purification following the Qiagen QIAquick-spin PCR purification kit procedure (Qiagen Inc., Valencia, California; Appendix 3) cleaned the reactions of excess primers, enzymes, and buffers. During the purification process it is possible to lose the reaction product, therefore, these

were also tested against a 1kb ladder on a 1% agarose gel. Cleaned product was stored at -20C in 25 µL of 10mM Tris-HCl.

FIGURE 2. Successful amplification of the ITS region. Lane 1, row 1 contains PCR product for a sample of *Phragmites australis*, GV001 ITS.



Sequencing and analysis of molecular markers

Amplification products were sequenced using ABI Prism DyeTerminator^{R} chemistry (Applied Biosystems Inc., Foster City, California). To approximately 2-4 µl of reaction product we added 2 µL primer solution and 4 µL FS pre-mix. Each reaction was covered with 1-2 drops of mineral oil and exposed to the following reaction sequence: 2 min denaturation at 100C, then 25 cycles of 1 min at 94C, 1 min primer annealing at 50C, and 4 min primer extension at 60C. Sequence reactions were cleaned over Centri-Sep Sephadex columns (Princeton Separations, Princeton, New Jersey), vacuum dried, and stored at -80C for no more than two weeks.

Automated sequencing, including pouring and loading a polyacrylamide gel, followed the protocol for ABI Prism sequencing technology (Applied Biosystems Inc., Foster City, California). The sequencing hardware uses a laser to scan the polyacrylamide gel. Sequence reactions pass down the gel across the laser's beam, and terminal bases are tagged with markers that fluoresce

when hit with the laser. The software program interprets these pseudocolor signals as nucleotide bases. For example, where the laser picks up a blue signal, the software reads cytosine (C). Similarly, a red signal is read as thymine (T), a green signal is adenine (A), and black is guanine (G). Appendix 4 depicts a printout of a successful sequence.

The reactions often lose their integrity after about 300 bases (Appendix 4), therefore the printouts are only partial sequences. To obtain a complete sequence of 700 base pairs, one must join together sequence data compiled using multiple start points. For this reason, the ITS amplifications are sequenced using four primers (Figure 2). Each primer begins replication at a different point along the fragment. Where one primer breaks down, another picks up the gap. Thus, overlap among primers assures good results because it allows one to check one sequence's results against another, where all other variables are presumably the same.

The sequence fragments are joined to form contigs and edited using Sequencher^{R} (GeneCodes, Ann Arbor, Michigan). One can then compare completed sequences among individual plants (Appendix 5). The PAUP program performs this analysis (Dave Swofford, Smithsonian Institution). It identifies point mutations by looking for base pair differences between two individuals. The program tallies the total number of base pair differences and gives a measure of genetic diversity as the percent difference between two sequences.

Results

We obtained successful sequences for two samples of *Phragmites australis* (Appendix 5). There were 655 overlapping base pairs between the two samples. Sequence analysis yielded seven base pair differences between them. Of these mutations, three were insertions or deletions (indels), two were base pair substitutions, and two were of unknown origin. One base pair substitution replaced what was originally a T with a C. The other substitution replaced a G with an A. Both of these substitutions are called transitions, where a purine is replace by a purine or a pyrimidine is replaced by a pyrimidine. Transitions are more common than transversions, where a purine replaces a pyrimidine, because the purines have similar molecular structures, as do the pyrimidines. A purine is more likely to slip into the place of another purine than that of a pyrimidine because of the structural similarities.

A difference of seven bases amounts to 0.305% sequence devergence (Table 1). We compared each *P. australis* sequence to a sequence from an individual of a related grass, *Zea mays* (corn). *Z. mays* and *P. australis* are both in the family Poaceae. In comparing *P. australis* to a species outside its own genus, we obtained a measure of 13.5% sequence divergence between either one of the *P. australis* samples and the *Z. mays* sequence. Within a single species, 0.305% diversity is significant when compared to only 13.5% diversity between genera of the same family.

TABLE 1. Uncorrected ("p") distance matrix

7205 ITS	-	-	-
7152 ITS	0.00305	-	-
Zea mays ITS	0.13538	0.13409	-

The *P. australis* sequences we obtained correspond well with other species in the Poaceae family for ITS fragment length and G-C content (Table 2). Our total fragment length was 655 base pairs. Other Poaceae sequences also average around 600-650 base pairs (Baldwin et al., 1995). The excess we picked up is due to carry-over into the adjacent 18S and 26S regions. Table 2 shows that G-C content for the two samples of *P. australis* was about 60%. Comparing these values to *Z. mays*, which is also about 60%, shows that there are no major anomalies in this species and that our results are reliable. Both ITS length and G-C content correspond to the results of ITS sequences done on other grasses (Baldwin et al., 1995).

TABLE 2. Base frequencies

Taxon	A	C	G	T	# sites
7205 ITS	0.20619	0.28399	0.30287	0.20695	662
7152 ITS	0.21304	0.27971	0.30145	0.20580	690
Zea mays ITS	0.19739	0.33605	0.31158	0.15498	613
Mean	0.20585	0.29873	0.30509	0.19033	655

To further confirm the reliability of these results, we performed a Chi-square test of homogeneity for all three sequences (Table 3). This test measures the observed numbers of each nucleotide base against what would be the expected numbers based on previously obtained values. Again, the results we obtained correspond to expected values.

Two mutations were of unknown nature. In Appendix 5 these are marked with a "+" instead of a dot. For each, there is an N or a Y where there should be an A, G, T, or C. This means that the computer could not recognize any one base, nor did it recognize a space or deletion. To compensate for basecalling errors, the program labels these bases "null" and marks them with an N or a Y. There may actually be a mutation at these points, or the base may be the same as the first sequence. By sequencing multiple samples of the species in question, researchers can compensate for these common mishaps.

TABLE 3. Chi-square test of homogeneity of base frequencies across taxa.

Taxon		A	C	G	T
7205 ITS	O	136.5	188	200.5	137
	E	136.27	197.76	201.97	126
7152 ITS	O	147	193	208	142
	E	142.04	206.12	210.51	131.33
Zea mays ITS	O	121	206	191	95
	E	126.19	183.12	187.02	116.67

$\chi^2 = 10.541$ (df = 6), P = 0.104

Discussion

Molecular markers

We obtained two complete ITS sequences and found a low level of variation between them. There were no successful sequences for the chloroplast DNA markers. To reiterate, low variation suggests that a species is relatively new to a region, that it reproduces clonally, or that it colonized a region via one point of entry. The data we obtained are insufficient to support either of our original hypotheses. Two complete sequences are not enough to determine whether the New York

area harbors populations of *P. australis* that are low in genetic diversity. However, the results obtained herein are substantial enough to verify that this topic merits further research. We obtained a measure of diversity meaningful enough to suggest that there is some polymorphism within local populations. Previous ITS studies done on grasses confirm that our fragment lengths are within the expected range, that the G-C content is normal for members of the Poaceae family, and that the numbers of each nucleotide base correspond to expected values.

Future research would entail sequencing many more samples of *P. australis* and building a larger database. Ultimately, one can conduct crosswise comparisons to analyze diversity both within single accessions and among stands at locations in New York City, Harriman State Park, Black Rock Forest, and other local sites. The goal would be to find a set of relationships among the different accessions that will illuminate the origins and pattern of *P. australis*' evolutionary history. Since measures of diversity can be complicated by factors such as reproductive biology, analysis will also need to include a comparison of these results to the diversity observed within a species that has similar biological and ecological characteristics.

Figure 3 is an example of the type of information phylogenetic studies yield. Presumably, one can reconstruct evolutionary patterns based on phylogenetic data. The diagram depicts what is termed a "tree." Organisms which are most closely related appear on the map with short "limbs" connecting them, while more distantly related groups are indicated by longer limbs. The tree demonstrates that this genus contains two major groups of species. The group in the Northeastern United States contains two species, while the Gulf of Mexico harbors one major group which contains three species more closely related to each other than any is to the Northeastern species. This group also shares its origins with a closely related species in the Caribbean Islands. The relationships depicted in Figure 3 are among a genus of beetles, however the same methods can be applied to a group of any type of organism. Thus the tree diagram has been applied with equal efficacy to families of frogs and subspecies of plants.

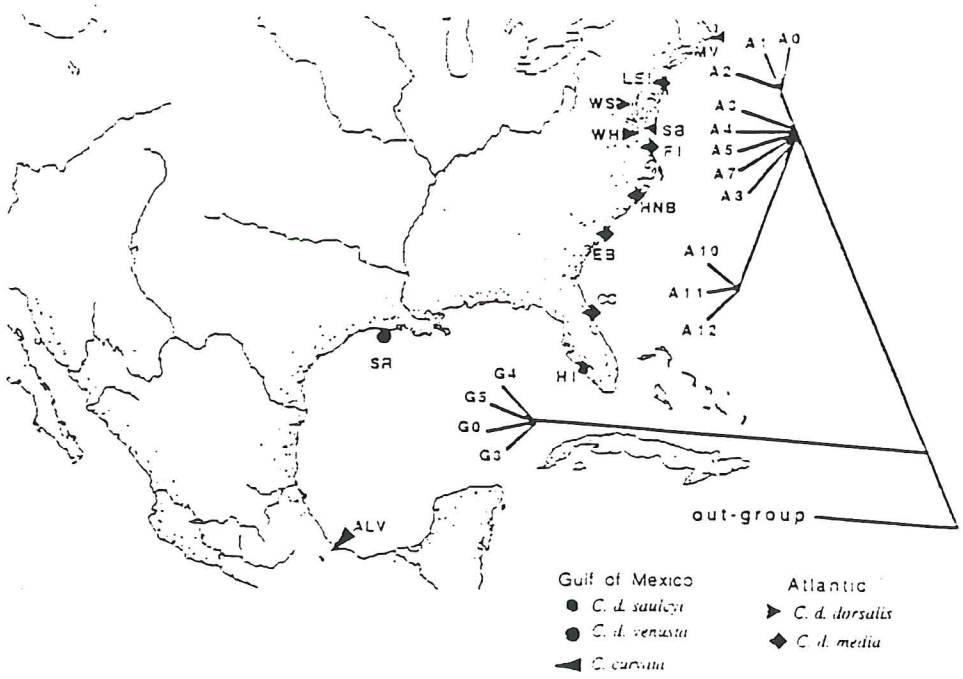
Polyploidy and hybridization

We began with two hypotheses. The first stated that *Phragmites australis* is a native species which has become an invasive because of anthropogenic disturbances to the local habitat. The second hypothesis stated that *P. australis* was introduced from Europe and has spread because

there are few natural checks in place to control its proliferation. A third explanation has surfaced over the course of this study. Often, heterozygotes are more prolific than homozygotes of the same species. This phenomenon is termed hybrid vigor (Li and Graur, 1991). Research has shown that polyploids of a given species are more likely to become invasive than diploids of the same species (Rejmanek, 1996). Dufresne (1995) writes, "The combination of two or more divergent genomes often leads to high heterozygosity levels in polyploids which may endow them with novel ecological tolerance and explain their wider distribution."

Li and Graur (1991) also note that the monocots tend to have a higher rate of polyploidy than dicots. *P. australis* is a monocot. It is possible that three strains of *P. australis* exist. One is native to Europe, the second is native to North America, and a third is a hybrid of the two. If it exists, a hybrid would be a likely candidate for the source of invasive populations of *P. australis*.

FIGURE 3. Tree diagram illustrating the evolutionary relationships within a genus of beetle. G0, G3, G4, and G5 are more closely related to each other than any of these species is to a species of group A.



Hybridization does not always imply polyploidy, nor does polyploidy necessarily result from hybridization. Organisms can be autopolyploid, where a failure of chromosome disjunction after DNA replication results in daughter cells with duplicate sets of chromosomes. Hybridization can result in the introduction of new alleles without necessarily requiring chromosome duplications.

To test the hybridization hypothesis, one can use both molecular markers and simple chromosome counts. Chromosome counts entail examining the chromatin under a microscope and counting the number of sets of chromosomes. If some individuals show diploidy while others show tetraploidy and/or triploidy, for example, then the species expresses polyploidy in some strains. Evidence of hybridization would appear in a molecular marker survey. One would sequence samples from Europe and North America. If one sees more diversity between these two groups than between either group and a third, intermediate group, then that is evidence that hybridization has occurred. Chloroplast DNA markers would be better suited to a study of hybridization since they are not affected by polyploidy.

Theories of invasive plant species

Research has found generalizations about invasive species to be unreliable (Blossey and Notzold, 1995 and Rejmanek, 1996). Nevertheless, certain trends seem to withstand scrutiny. In general, plants tend to be more vigorous, to grow taller, and to produce more seeds in alien environments than in their native habitat. Blossey and Notzold (1995) found that the added growth and seed production stem from a change in resource availability within individual plants whereby more photosynthate is diverted to secondary metabolisms. They propose a theory of the evolution of increased competitive ability, or EICA, which states that in an area free of herbivory, plants evolve increased competitive ability either by generating more plant biomass or more seeds. Their study focuses on *Lythrum salicaria*, which like *Phragmites australis* is native to European wetlands and is now invading North America. Unlike *P. australis*, however, loosestrife is a dicot and it reproduces primarily by seed. As predicted by the EICA theory, loosestrife grown from seeds collected from areas of low herbivore pressure showed more vegetative growth than plants grown from seeds produced in areas of high herbivore pressure. A similar study of *P. australis* would be a useful addition to current knowledge about its ecology.

Ellstrand (1996) examined patterns of invasiveness within taxa and found that certain families and genera are more likely than others to produce invasive species. In particular, he found that outcrossing perennials with reproductive modes such as vegetative spread, which stabilizes hybridity, will show a higher than average rate of invasiveness. Species of Poaceae (the grasses) were shown to hybridize in habitats in the British Isles, the mountains of the northwestern United States, the Great Plains, and in Scandinavia. Outcrossing allows a species greater opportunity to hybridize by accidental contact with a related species. Being a perennial affords longer seasonal exposure and therefore a greater likelihood of being observed and identified as a hybrid. Clonal reproduction tends to stabilize hybridization, at least within populations. *Phragmites australis* is an outcrossing perennial, and it reproduces clonally, thus it is a good candidate for hybridization.

Ellstrand also found that having these characteristics does not guarantee that a plant will become an invasive. Many species which are introduced to a habitat and which fit all the predictive criteria do not hybridize and do not become invasives. Thus we cannot use this model to make fool-proof predictions, but it is a useful guide for deciding whether to consider a species to be problematic and to regulate its spread appropriately.

Some scientists have tried to generate a comprehensive theory of seed plant invasiveness which could be used to predict which species will eventually become invasives and which will settle into a more inconspicuous position in a community. Again, no universal predictors exist, but certain traits are common to many invasives. These include small seed size, a short interval between seed crops, a short juvenile period in the life cycle, a broad native range, and a small genome size (which seems to correlate with small seeds). Species of exotic genera are more often invasive thatn new species of a native genus. Rejmanek's theory ultimately combines these factors and explains how they are interrelated (1996). Studies of *P. australis*' ecology have confirmed that it has many of these traits.

Studies of the ecology of *Phragmites australis*

One study that focused on *Phragmites australis* found it to be highly problematic along the St. Lawrence River in Quebec (Gervais et al., 1993). The report described *P. australis* growing along roadsides in Quebec. Its authors warned of the risk of its being introduced to water reservoir basins because it seems to possess the taxonomic and cytologic traits of problem populations in the

United States.

Investigators studied three aspects of the biology of *Phragmites australis*, including chromosome number, geographic distribution, and reproductive biology. They found that chromosome numbers ranged from 2n=42 to 2n=59 among Quebec populations. Northern populations had lower chromosome numbers and floral development was faster, probably as an adaptation to the shortened growing season in northern Quebec. In general, populations with higher chromosome numbers were less developed, while seed production varied somewhat among populations with high and low chromosome numbers (Gervais et al., 1993).

Gervais and his colleagues found it difficult to determine the chromosome number for individual plants because the chromosomes were small, variable in size, and had a tendency to agglutinate. Nonetheless, observing them at metaphase I of meiosis, they found evidence of aneuploidy, including variation within single colonies of the reed.

Several hypotheses can explain the observed aneuploidy. Several rhizomes could give rise to each colony, so that there would be variation within one colony. Each colony could arise from one rhizome which gives rise to seed-bearing plants, and the seeds would then germinate to produce genetically diverse offspring. Or, each colony could arise from one rhizome, and within individual plants, tissue types exhibit a mosaic of chromosome numbers. The variation observed within a single population could arise from variation within individual plants, among individual plants, or among groups of individuals.

The researchers mapped sites where *P. australis* was observed prior to 1950 against sites where it has been observed since then. The former amounted to 26 sites, the latter to 140. Thus its distribution has increased fivefold in the last 50 years. The authors agree that *P. australis* is a problematic species. "A comparison of the current distribution with that before 1950 can give us an indication of the species's aggressiveness" (Gervais et al., 1993; my translation). The authors attribute the reed's vigorous recent spread to roads, ditches, the transport of materials along autoroutes, and to dispersal by water. They note that disturbances to established stands could promote the spread of rhizomes, and that soil disturbances might favor vegetative spread of the reed by creating hospitable terrain (Gervais et al., 1993). With this information in mind, management efforts need to be careful to assure that treatments do not do more harm than good to local ecosystems.

Seeds were harvested from different stands of the reed and grown in the lab to test their viability. They germinated, but were weak and grew slowly. In the field, seeds are produced in less than 1% of flowers, and by autumn, most of these are still immature. In this study seeds were produced in less than 5% of flowers on average, although a few scattered communities had 10.75% to 59.12% of flowers producing seeds. Most of these are attacked by a smut, *Claviceps microcephala*, and as stated above, the rest germinate slowly (Gervais et al., 1993). Ultimately, the authors conclude that *P. australis* reproduces primarily by vegetative spread and secondarily by seed. Since the seeds demonstrated some viability in the lab, it is possible that *P. australis* spreads over large distances by seed. One model to explain its reproductive biology is that it first establishes a colony via vegetative reproduction. Only a small percentage of flowers within that stand produce seeds because seeds are not necessary to sustain the population. However, a few seeds disperse to distant sites where they found new colonies.

Other studies have yielded information about the physiology of *Phragmites australis*. Gries (1990) found that the reed will colonize a lake shore up to a depth of 2 meters. It has a low rate of 0_2 consumption and a low 0_2 saturation point, therefore it can tolerate the partially flooded conditions of moderate depths. Armstrong (1992) examined the use of *P. australis* to build wetlands in the United Kingdom. These wetlands were constructed to treat pollution, specifically domestic, industrial, and agricultural sewage. Armstrong studied the reed's ability to tolerate toxic conditions via rhizosphere aeration and rhizosphere oxidation.

An interesting study conducted at the Delta Marsh in Manitoba demonstrated that *Phragmites australis* is undoubtedly an invasive species. Thompson and Shay (1989) examined the response of *P. australis* communities to seasonal burning. They found that summer burnings successfully controlled an overabundance of *P. australis*, while fall and spring burnings were less effective. They note that where seasonal burning practices have been abandoned, the reed's robustness has increased. Over the course of their study, Thompson and Shay found evidence that *P. australis* is an aggressive colonizer of freshwater wetlands:

> *Phragmites australis* can place strong competitive constraints on understory species because of shading effects and accumulation of litter. As *P. australis* dominance is negatively related to species richness, changes in the performance of *P. australis*

> with season of burn may be reflected in altered species numbers and substory abundances.

(Thompson and Shay, 1989).

Phragmites australis seems to utilize several mechanisms to invade a community. It produces a fluctuating biomass from year to year, depending on water levels and agricultural runoff. This prevents less abundant species from adjusting to stable competitive conditions. *P. australis* produces an abundance of leaf litter which prevents the seeds of other wild species from germinating. The authors found that *P. australis* accounts for 90% of the aboveground biomass in unburned communities. A few vigorous perennials manage to survive by vegetative growth. The common reed also reduces light intensity near the surface because it blocks the sun. This prevents small plants and other perennials from forming buds at the soil surface (Thompson and Shay, 1989).

Seed bank response to burning is unpredictable. Burns precipitate changes in the water chemistry and soil structure that may adversely affect other wetland species. On the other hand, summer burns tended to reduce *Phragmites australis*' aboveground biomass and, by thinning the canopy that the reed creates, to increase the community's overall species richness (Thompson and Shay, 1989). The authors conclude that summer burning may slow competitive exclusion and help maintain species diversity.

Methods for the control of invasive species

Seasonal burning is one of several methods by which wildlife managers attempt to control the spread of invasive species. Other options are biological controls, chemicals such as herbicides and fungicides, manual removal of the species, and strict monitoring of transport vessels at points of entry.

Wagner (1993) evaluates several of these options. He argues that biological control is unpredictable and therefore risky. With biological control, wildlife managers import a natural predator of the invasive species in order to control its proliferation "naturally." There is always the possibility that the control agent will escape and become a problem species, therefore biological control efforts require extensive study before the program is implemented and careful monitoring

of their success afterwards. This renders them impractical as well as risky since the time spent monitoring the technique often outlasts the time required for the invasive species to become dominant in its new community (Wagner, 1993).

Uprooting plants is risky because it can trigger them to grow back more voraciously. In some cases uprooting triggers the seeds to sprout. It also causes shoots of vegetative ramets to grow aggressively. Thus the organism returns to its former dominance, colonizing the area more aggressively than it did before the disturbance took place (Thompson and Shay, 1989; Wagner, 1993).

Another option Wagner suggests is genetic control. This would entail splicing in genes that retard reproduction or block resistance to certain diseases or chemicals. The ethical and philosophical issues that underlie this proposal indicate that it will not pass public approval without a struggle.

The United States Department of Agriculture has an acute interest in controlling and eliminating invasive plants, insects, and microorganisms that pose a threat to agricultural crops. They have implemented a multidimensional system of control that operates at the local, state, national, and international levels. The USDA Animal and Plant Health Inspection Service (APHIS) Program requires that imports be guaranteed pest-free. Inspection occurs at the port of import and the port of export. Within the United States, the program promotes early detection of infestations, and uses biological, chemical and mechanical control mechanisms to contain and eliminate invasive species (Westbrooks, 1993). As of 1993, APHIS did not list *Phragmites australis* as as a "federal noxious weed."

The Nature Conservancy does include *P. australis* on its list of invasive plant species (Randall, 1993). The Nature Conservancy operates at a local level by purchasing lands in order to protect them under its stewardship program. Control of invasive species varies from one preserve to the next depending on the species, the nature of its spread, and the available control mechanisms. Their program treats an area as delicately as possible, using biological controls only under specific conditions. The general philosophy is not to do more harm than good.

Controlling invasive plants is a complicated and frustrating endeavor. It requires that an understanding of the important ecological, reproductive and genetic traits of the organism. In addition, control efforts must not disturb the habitat in a way that will eliminate native species.

This can happen if herbicides are sprayed carelessly or, as in the case of the Delta Marsh in Manitoba, if burning alters the chemical substrate so that native species cannot move back into their former niches. Thus, one must also understand the dynamics of local ecosystems.

Phragmites australis has fewer predators in North America than in Europe. This suggests that biological control is an option. Manual removal, on the other hand, is clearly not an option, as Gervais et al. (1993) indicated. As with any other invasive species, successfully managing *P. australis* will require careful study of its response to different control techniques.

Recommendations

Ecological studies (Gervais et al., 1993; Thompson and Shay, 1989) clearly demonstrate that North American strains of *Phragmites australis* exhibit features typical of other invasive species. However, as mentioned above, plants that exhibit these traits do not always become pests. In many cases they integrate unobtrusively into a new community. Park managers, scientists, and agricultural agencies need to examine the wealth of knowledge that exists on *P. australis*' role in wetland ecosystems and to determine whether or not it poses a threat to native biodiversity. In particular, further study of *P. australis*' molecular biology will help interested parties understand the nature of the common reed's recent increase in its rate of spread. Studies of polyploidy and hybridization will contribute to our understanding of *P. australis*' evolutionary history. This knowledge will aid conservation efforts. Knowing the amount of polymorphism within a species is important to understanding how it will respond to different control treatments. Highly polymorphous species will adapt more quickly to biological and chemical controls. Polymorphism will also influence which strains maintain dominance in local ecosystems and which are destroyed. We must be careful not to destroy the less harmful strains of *P. australis* while promoting the spread of weedy strains. Lastly, wildlife managers need to consider the effects that biological, chemical, and physical pest controls will have on other species in the community. We must be careful, in our effort to correct the mistakes of the past, not to cause more problems in our environment.

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Appendix 1: DNA Extraction Procedure

Recipe

50mM Tris-HCl	2.5 ml 2M Tris-HCl stock
0.7M NaCl	4.091 g NaCl
10mM EDTA	2.0 ml 0.5M EDTA stock
6% hexadecyltrimethylammonium bromide (CTAB)	6.0 g CTAB
dH20	bring to 100 ml final volume

1. Incubate CTAB and heat water bath to 60C prior to beginning extraction.

2. Pour approximately 1000 ml liquid nitrogen into a cold-safe container such as a styrofoam shipping carton. Label 2 ml Eppendorf tubes and place in plastic lab rack. Place rack, with tubes, in liquid nitrogen so that tubes can cool before adding plant tissue to them.

3. Place plant tissue in cold Eppendorf tubes. Pour liquid nitrogen into tubes, being careful not to splash it as this could contaminate samples in adjacent tubes. Grind tissue using plastic douncers. You may need to repeat this step several times until tissue grinds readily into a fine greenish-white powder.

4. Add 500 µl warm CTAB to each tube. Add 1.0 µl B-mercaptoethanol. Add another 500 µl CTAB. Mix well, close tubes, and place tubes in warm water bath for 30 min. Invert tubes every 5 min to ensure proper mixing of plant tissue and CTAB solution.

5. After incubation, remove tubes and add 500 µl 24:1 chloroform:isoamyl alcohol (CI) solution. Close tubes, invert several times, and gently open tube to release pressure. Balance the tubes with CI and centrifuge at high speed for 5 min.

6. Remove the upper aqueous layer and transfer to a clean 1.5 mL Eppendorf tube. Add 1 ml of 100% ethanol or isopropyl alcohol and 10 µl of 5M NaCl solution. Invert several times to mix well. Allow to precipitate at -20C for 10 min or longer.

7. Centrifuge at high speed for 5 min to pellet the nucleid acid precipitate. Gently discard the supernatant and allow any alcohol to evaporate. Do not allow the pellet to completely dry out as resuspension may be difficult.

8. Resuspend the pellet in 300 µl of 1x TE solution. Add 200 µl 7.5M NH_3OAc. To this add 1ml of 100% ethanol or isopropyl alcohol. Close tubes and invert several times. Precipitate at -20C for 10 min. or longer.

9. Centrifuge at high speed for 5-10 min. Discard the supernatant and resuspend the pellet again in 300 µl 1x TE. Add 50 µl 2.5M NaOAc and 1 ml ethanol or isopropyl alcohol. Precipitate at -20 for at least 10 min.

10. Centrifuge at high speed 5-10 min. Gently discard supernatant. Fill the tube with 70% ethanol and let sit a few minutes to a few hours to dissolve salts. Spin again at high speed 5-10 min in case pellet has dislodged. Pour off the ethanol. Allow samples to air dry. Resuspend in 100 µl 1x TE. Store at -20 C until needed.

Appendix 3: PCR Purification Protocol*

Using **QIAquick-spin PCR Purification Kit**, purify the amplified product of residual primers, nucleotides, and polymerases.

1. Add 5 volumes of PB buffer to the completed PCR product (for 50 µl product add 250 µl buffer), mix well, and place in a labelled quick spin column. The spin column should rest in a 2 ml collection tube.

2. Centrifuge at high speed 30-60 s.

3. Drain flow-through from collection tube.

4. Add 750 µL buffer PE to spin column. Centrifuge at high speed 30-60 s. Drain flow through. Spin again to remove any remaining solution from spin column.

5. Transfer spin column to a labelled 1.5 ml Eppendorf tube. Add 25 µl Tris-HCl to column to elute the DNA. Centrifuge 30-60 s. Discard spin columns and store purified product at -20 C until needed.

* adapted from **QIAquick-spin** protocol (Qiagen Inc., Valencia, CA)

Model 377
Version 2.1.1

18•OE11

OE11
Lane 18

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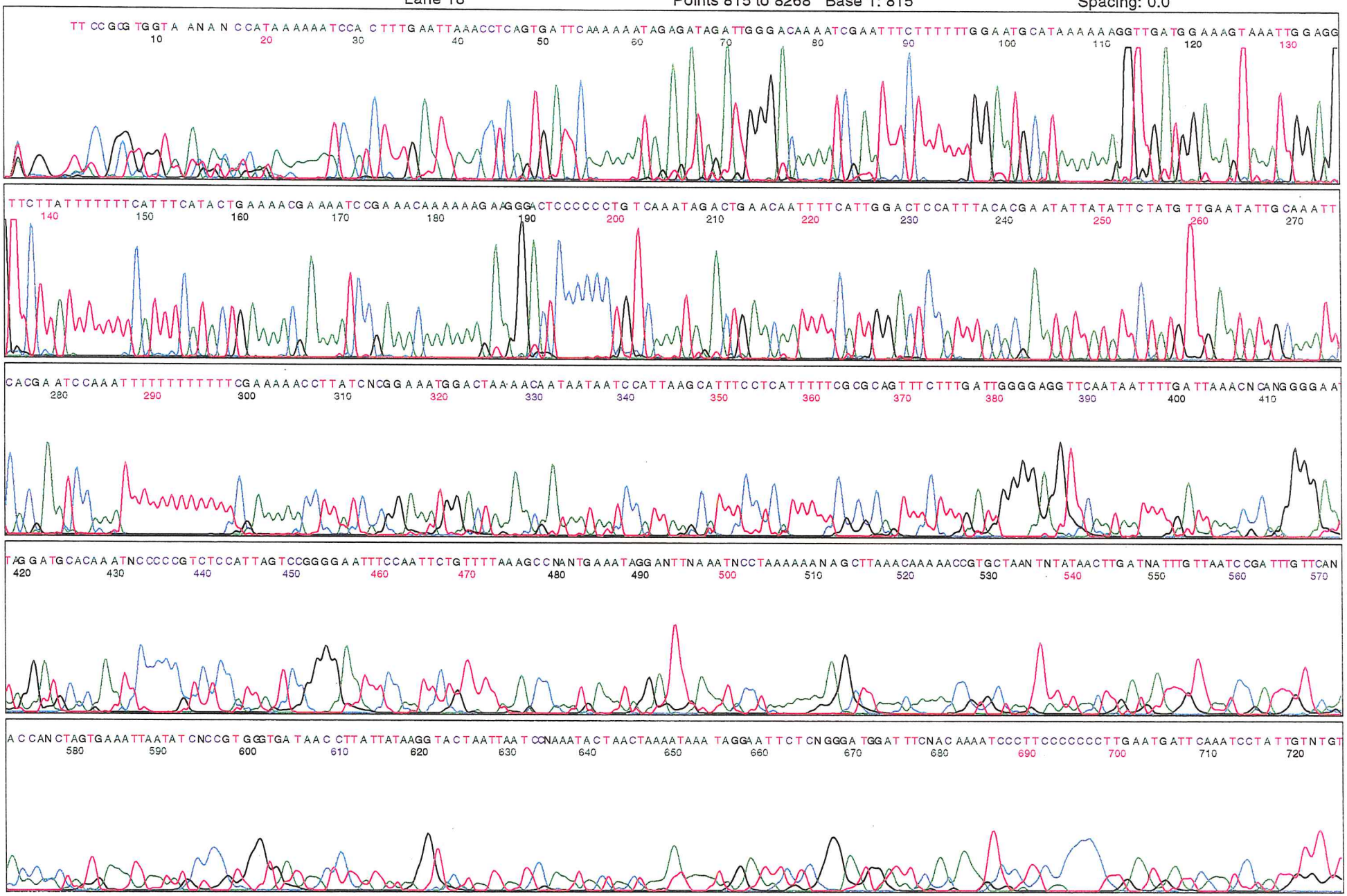
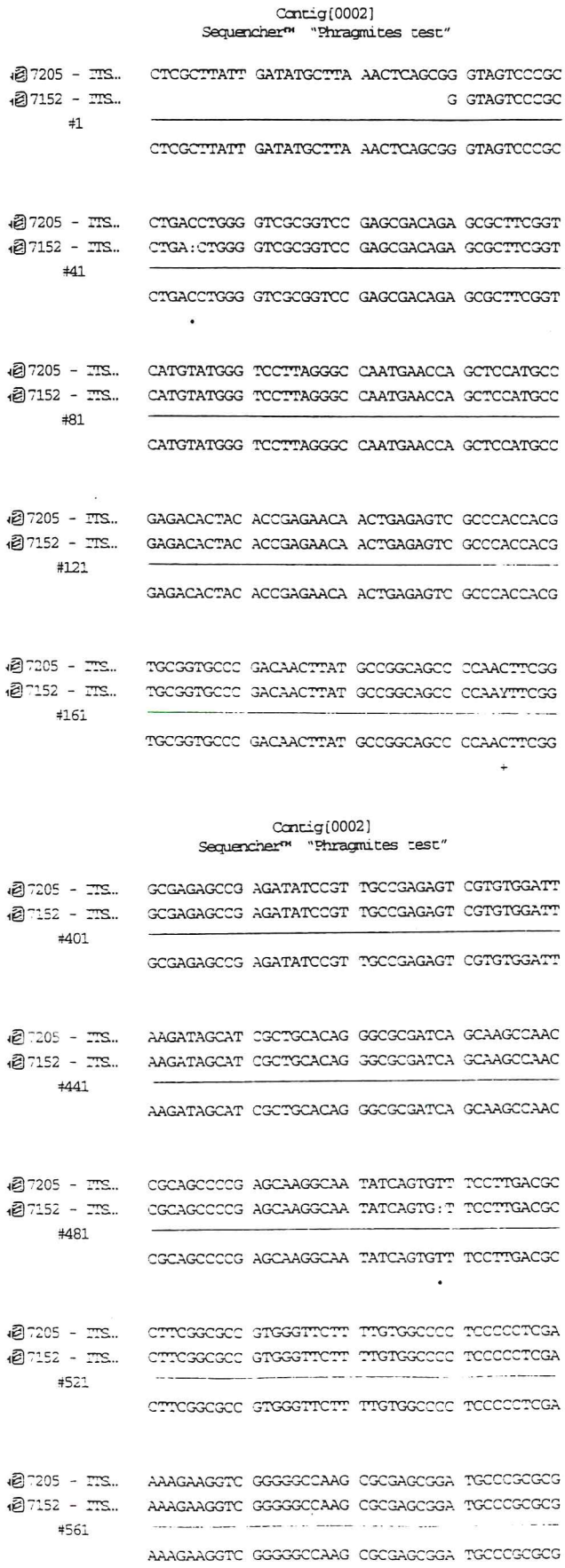


Appendix 5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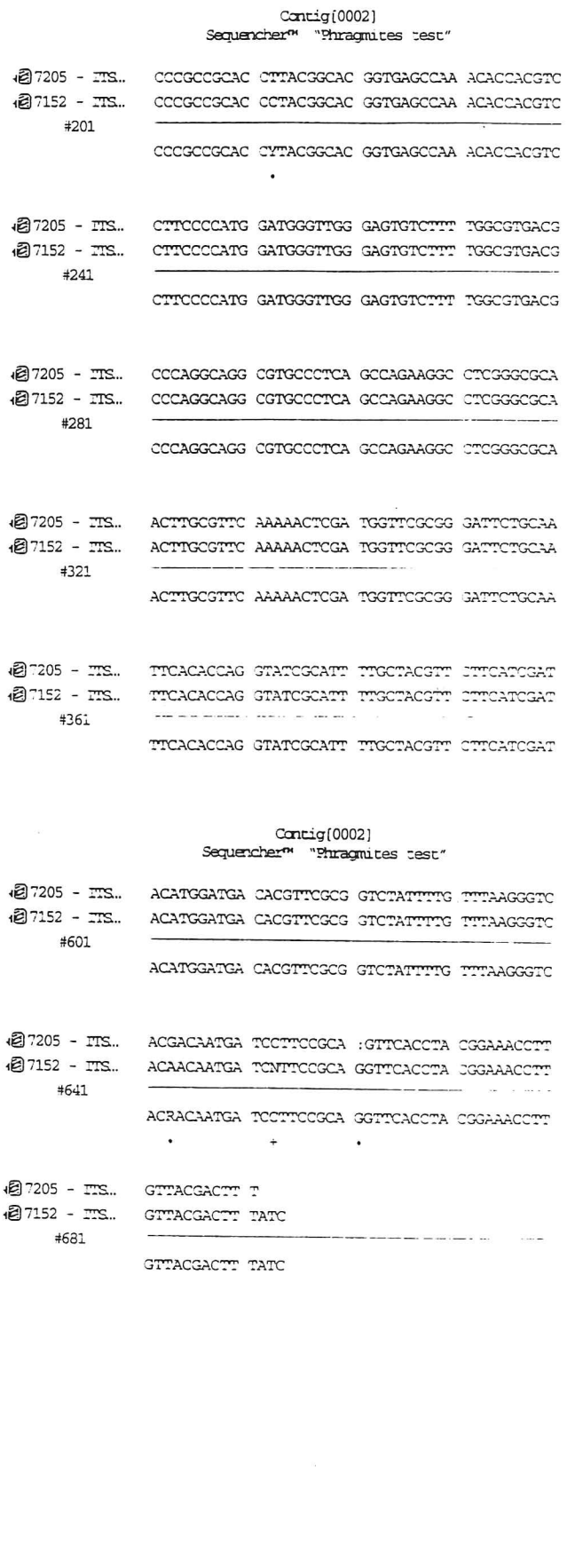
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