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Integration of CE-SSCP in Genetic Analysis: Fabrication and Optimization of the Polymer Matrix

An Undergraduate Honors College Thesis

in the

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College of Engineering
University of Arkansas
Fayetteville, AR

by

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April 29, 2011

Abstract

Genetic analysis has become an essential aspect in the field of biological research. Genetic material contains an abundance of information that can be harnessed, from the pathological study of diseases to the identification of algal species used for alternative fuel. The detection of genetic species and/or their mutated counterparts is an approach that still warrants improvement, however. Capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) offers a rapid and highly-reproducible means to detect alterations in genetic material. In this project, its implementation in the field of genetic studies as a way to detect mutations in hereditary diseases and identifying species via genetic coding was done by optimizing the molecular weight of the polymer medium. The polymer, polydimethyl acrylamide (PDMA), was fabricated and characterized at different polymer molecular weights. The fabricated polymer was then used in order to detect mutations in a model DNA and to genetically identify between two algae species. The polymer molecular weights of 3.4, 1.8, and 0.26 MDa were used in order to determine how varying polymer molecular weights affect the resolution of the data. The data shows that decreasing polymer molecular weight decreases the resolution of the peaks that resulted from the CE. It was found that all the mutations were consistently detected at 1.8 MDa polymer molecular weights, and genetic identification can be done with all three polymer weights. Thus for detection of mutations in a clinical setting, a recommended polymer weight of 1.8 MDa should be used. For identification between species of algae, lower polymer molecular weights can be used since it results in a shorter elution time.

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Introduction

Genetic analysis has become an essential aspect in the field of biological research. Genetic material contains an abundance of information that can be harnessed, from the pathological study of diseases to the identification of algal species used for alternative fuel. The detection of genetic species and/or their mutated counterparts is an approach that still warrants improvement, however. Thus in the past decade, such methods have progressed from brute force genetic sequencing and the time-intensive gel electrophoresis to its contemporary counterparts. Capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) offers a rapid and highly-reproducible means to detect alterations in genetic material. This technique has been developed for over ten years and has proven to be as effective, if not more, in the field of analytical genetics.

The detection of mutations, in order to explain the development of diseases, is a novel approach in analytical medicine. Multiple mutations in genetic coding have been theorized to account for the majority of the genetic risk in type 2 diabetes mellitus (T2DM). Thus using CE-SSCP as a means to detect mobility shifts produced by multiple conformations of single-stranded DNA carries great potential. Integrating CE-SSCP into the clinical setting, and moving its use from conceptual research to applicable diagnostic medicine, calls for the development and optimization CE-SSCP system that will allow for rapid diagnosis of a patient's genes.

In the same manner, CE-SSCP can also be used to differentiate between different species of algae. The many species of algae holds great potential for genetic variability that, when further studied, could result in the genetic identification of algae cultures that will result in high yields of fuel at low capital costs. This study involves optimizing the CE-SSCP system in order to differentiate strains of similar plant DNA extracted from both cultured and environmental samples of algae.

Therefore this project will focus on the optimization of CE-SSCP system by characterizing the polymer medium used for both applications. In the first case, CE-SSCP will be focused on the detection of mutations in human DNA. In the second case, CE-SSCP will focus on differentiating between similar genetic material from different species of algae.

Genetic Analysis

Single-strand conformation polymorphism analysis

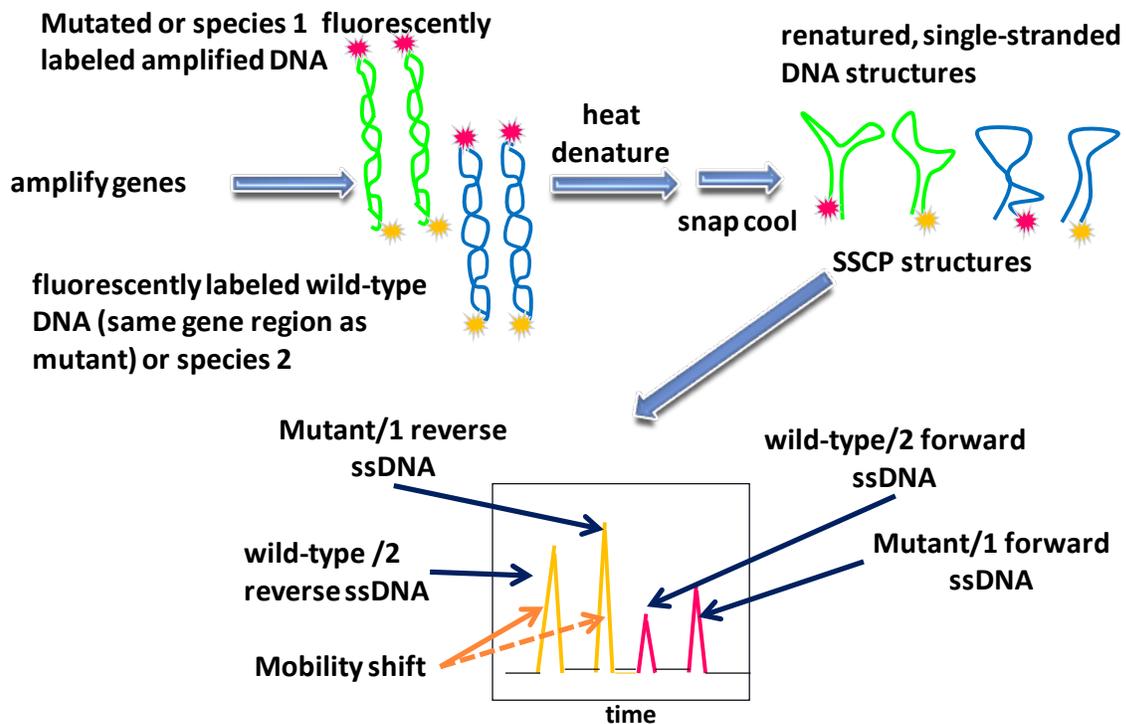


Figure 1 - Artistic rendition of single-stand conformation polymorphism. Credit to Dr. Christa Hestekin.

The detection of mutations by the analysis of the mobility of conformed single-stranded DNA was first developed by Orita on genomic DNA (Orita et al., 1989). Single-strand conformation polymorphism by capillary electrophoresis is a nonspecific yet reproducible and efficient method in analyzing DNA (Arakawa et al., 1996). This method is preceded by amplification of the DNA by polymerase chain reaction (Ren, 2000).

The DNA samples for this type of analysis are first PCR amplified. The control (wild-type) and/or experimental PCR products are then combined with a buffer, denatured, and then immediately snap-cooled on ice. As shown in Figure 1, this immediate decrease in temperature causes the ssDNA to fold into its unique conformation (Hestekin, 2006).

This method rests on the idea that single-strand DNA (ssDNA) fold into unique conformations dependent on its nucleotide sequence, due to the differences in intramolecular interaction of the bases. The conformation affects the mobility of the DNA structure in a medium such as polyacrylamide gel (Ren, 2000). Single-stranded DNA migrating at different rates indicate the presence of a mutation, since a mutation affects the way the strand conforms. The sensitivity of this technique is thus dependent on how the type of mutation causes a specific conformation in the ssDNA and how its conformation affects its electrophoretic mobility. There is no known theory that could accurately predict DNA conformations and how mutations conform, thus CE-SSCP cannot be predicted physicochemically (Hayashi, 1992).

Capillary Electrophoresis

Capillary electrophoresis (CE) is a technological leap from slab-gel electrophoresis. It requires smaller sample amounts and has excellent sensitivity (Ren, 2000). It is this characteristic that renders CE-SSCP as rapid and high-throughput. In capillary electrophoresis, DNA molecules are separated with the

use of fused silica capillaries filled with polymer matrix and electrophoresis buffer. The DNA molecules are injected into the capillaries and are detected with the absorption of UV light or laser-induced fluorescence as it passes under the detector (Andersen et al., 2003).

The most notable advantage of capillary electrophoresis is its ability to dissipate Joule heat. Slab gel electrophoresis is limited by the excessive heating of the medium due to the current applied to it. The low surface-to-volume ratio (~ 1.3) of a slab gel limits the Joule heat dissipated, and hence limiting the voltage to ranges of approximate 15-40 V/cm. The high surface-to-volume ratio of capillary electrophoresis (20-200) allows for electric fields to operate at 800 V/cm (Landers, 2008).

CE also allows for precise temperature control, which is key in keeping the unique conformations of the ssDNA. Additionally, CE is an automated process—unlike slab gel electrophoresis, a time-consuming technique that, when compared to CE, results in poor reproducibility (Ren, 2000; Landers, 2008). The use of replaceable polymer solution, such as polydimethylacrylamide (PDMA), as separation media has also greatly optimized the CE method. This more convenient medium is not only effective but is also commercially available (Ren, 2000).

Electrophoretic Mobility

The polymer medium is necessary in order to elute different species of DNA according to their size and conformation. The theory of SSCP is that the conformations that arise from the difference in genetic coding would dictate the elution time of the single strand DNA. Using polymer mediums such as polyacrylamide allows for the electrophoretic mobility of the SSCP structure to become dependent on its size.

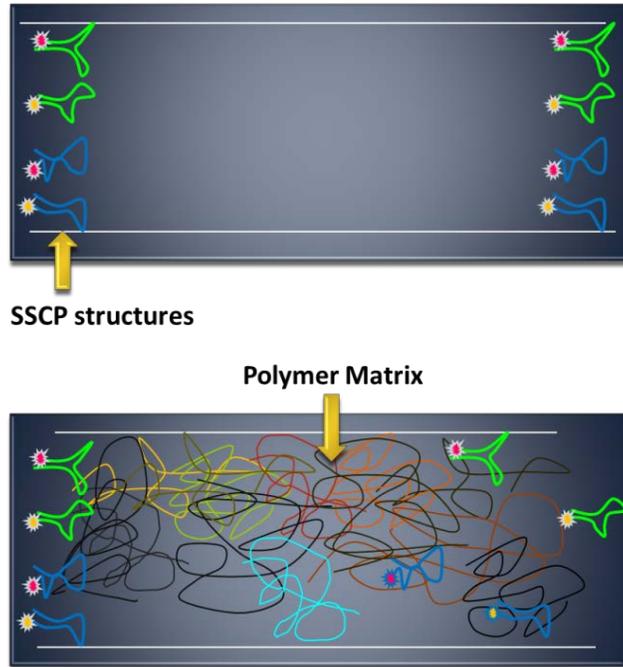


Figure 2 - Rendition of the purpose of the polymer matrix in CE. Credit to Dr. Christa Hestekin.

In this case, the electrophoretic mobility, or the rate of migration over a given electric field of a charged species, can be approximated by the Debye-Huckel-Henry Theory:

$$\mu = \frac{q}{6\pi\eta r} \quad (2)$$

where q is the charge of the particle, η is the viscosity of the buffer, and r is the Stokes' particle radius, which can be assumed as a geometric sphere in order to simplify the system. In this approximation, the mobility of the particle is assumed to be the ratio between the ionic charge of the particle and the frictional forces in the buffer (Landers, 2008). The polymer viscosity is directly related to the molecular weight and radius of the medium.

Electroosmotic Flow

The polymer coating is also necessary in order to suppress electroosmotic flow. Electroosmotic flow occurs when the potential across a capillary tube causes bulk motion of the substance. Due to the silica groups on the surface of the substrate, the application of an electric field pulls the mobile layer towards the cathode. Generally, electroosmotic flow is advantageous in differentiating between neutral

and positively charged species (Landers, 2008). DNA on the other hand must flow towards the anode, hence EOF is counterproductive in this study and is suppressed by coating the capillary wall with polymer. A polymer such as PDMA is typically of a neutral charge and acts as a stable dynamic coating that reduces the effects of electroosmotic flow to insignificance (Ren et al., 1999).

Fabrication

Polymer, such as polydimethyl acrylamide (PDMA), is fabricated by the repeated addition of its respective monomer (dimethyl acrylamide). Free-radical addition polymerization starts with an initiator that spontaneously produces a free radical ($R \cdot$) at a rate constant of k_d . Initiators for polymerization are often organic peroxides or azo compounds, such as azobisisobutyronitrile.



When the initiator produces the first free-radical, the unshared electron attracts another electron on the monomer (M) at a rate constant (k_a), starting a chain-reaction that consists of the producing of the free-radical on the ever growing chain of polymer (P_x). This then leads into a propagation reaction for the length of the polymer.



The rate constant (k_p) of the growing chain is assumed to be constant for the purposes of this study.

Thus it is assumed that the reactivity of the chain is independent of its current length.

Termination occurs by one of two ways. Combination terminations involve two chains of polymer free-radicals attaching to each other.



The second, disproportionation, involves the transfer of a proton from another separate chain, inducing a double bond on the end carbon of that chain.



In the case of this study, the proton can be abstracted from a good proton donor such as isopropyl alcohol. Thus in the fabrication of the polymers in this project, IPA is often used to control the lengths of the PDMA chain.



Characterization, Optimization, and Previous Studies

Characterization of the polymer is performed via multi-angle light-scattering analysis (MALS). It is based on the concept of how light, when shone on a substance either continues on its original direction or is scattered. This scattering can be analyzed and studied in order to determine the physical properties of the substance. Light scattering allows for the measurement of the molecular mass, size (also radius of gyration), second virial coefficient, and translational diffusion. Light is often linearly polarized--its electric field moves in a linear direction. Thus when this oscillating electric field hits the positive and negative charges of a particle, the amount scattered is determined by the substance's polarizability. If the wavelength of the light used exceeds the physical dimensions of the particle, the charges produce a dipole field, scattered in a direction that is typically perpendicular to the plane of polarization. (Wyatt, 1996)

After the polymer is characterized according to molecular weight and radius, it is then used in CE-SSCP in order to determine what size is optimum for the system currently being tested. In order to determine the efficiency of the polymer's ability to separate between genetic species, the resolution of the resulting peaks is analyzed with the following equation:

$$R = \frac{2|t_1 - t_2|}{t_1 + t_2} \quad (7)$$

where t_1 (1st species) is the elution time of the species against which a different species of a different elution time t_2 (2nd species) is tested. Thus, in terms of optimization, increasing resolution is often countered by an increase in processing time.

It has been observed in previous experiments that the resolution (R) has a correlation with polymer molecular weight. In more recent studies, such as Kourkine et al., moderately short-chain linear polyacrylamide polymer (~600 kDa) has produced optimum results for short DNA fragments (<200 base pairs) of the *p53* gene. These authors have determined that using this polymer in their system resulted in 93% sensitivity in detecting mutations. (Kourkine et al., 2002)

Blends of differing polymer molecular weight of PDMA have been tested as well. Hert et al. observed that similar resolutions were achieved at differing blends of both high (3.5 MDa) and low (770 kDa) molar mass polymers, totaled to a 5% overall w/v polymer blend. They have observed that the resolution of DNA fragments of 40-150 bases is highly dependent on just the overall or total concentration of the polymer matrix in solution (in this case the total of 5% w/v of polymer was used), and not the polymer molecular weight. However, when looking at the separation of DNA fragments greater than 150 bases, the resolution is greatly determined by the amount of high molecular weight polymer, which has been observed in previous literature as well (Bunz et al., 1996). Nevertheless, Hert et al. countered that though higher polymer molecular weight increases the resolution of larger DNA fragments, differentiation is still achievable with polymer blends that only contain 1% w/v of the 770 kDa polymer.

In this project, the CE trend between molecular weight and resolution will be extended to different types of DNA—more specifically the determination of different types of mutations (transition, insertions, and deletions) in a model (bacterial) DNA and the differentiation of genetic material from different species of algae.

Materials and Methods

Polymer synthesis

In this project, polymer was synthesized with more than one method. Table 1 summarizes the difference in protocols used. The difference in protocols was utilized in order to vary and control the resulting molecular size of the polymer. N,N-Dimethyl acrylamide monomer solution was dissolved in deionized water in order to make a 4% (vol) solution.

Table 1 - Table of different protocols for fabricating polymer.

Protocol #	Chain initiator	Chain terminator	Size of polymer (MDa)
1	0.5 mL V-50/100 mL mix	Pre-bubble addition of isopropyl alcohol (2.5, 5.0, 7.0 mL IPA/100 mL)	~0.26-1.8
2	0.5 mL V-50/100 mL mix	Post-bubble addition of isopropyl alcohol (0.25 and 0.5 mL IPA/100 mL mix)	~3.7-3.8
3	0.5 mL V-50/100 mL mix	None	~3.4-3.7
4	0.5 mL of both APS and TEMED/100 mL mix	IPA 3.1 mL/100 mL	~3.88

In **protocol 1**, a varied amount (see chart) of isopropyl alcohol (IPA) was added for every 100 mL of monomer-mixture after the bubbling period right before the removal of oxygen via nitrogen bubbling through the mixture for at least 3 hours. In **protocol 2**, the IPA was added with the V-50 chain initiator (2,2'-azobis(2methylpropionamidine)dihydrochloride) after the 3-hour bubbling. Whereas in **protocol 3**, IPA was not added at all and the polymer mixture was allowed to bubble with nitrogen gas for 3 hours after dissolution of the monomer in the water. After the addition of V-50, the polymer is allowed to react in a 47°C for 4 hours in an anaerobic atmosphere. For **protocol 4**, 3.1 mL of IPA was added for every 100 mL of polymer mix and bubbled with nitrogen for 30 minutes in a water bath set at 50°C. The

initiator used was a 10% APS- 10% TEMED (wt) solution, added after the bubbling. The solution was allowed to react for 1.5 hours.

After polymerization, the polymer was dialyzed in dialysis membrane in 10 washes, with at least 5 hours between washes. The polymer was then transferred into 50 mL tubes, frozen at -80 °C for 48 hours, and lyophilized for 72 hours. The polymer was then stored in room temperature until use in capillary electrophoresis or characterization.

Measurement of PDMA Molecular Weight and Radius

The characterization of the polymer was performed through multi-angle light scattering analysis (MALS) with the DAWN® HELEOS light scatterer (Wyatt Technologies) and the Astra software provided. A 5 mg/mL sample of the fabricated polymer was made with deionized water and then diluted to five concentrations: 1, 0.75, 0.5, 0.25, and 0.1 mg/mL. The DAWN® HELEOS was first flushed with 10 mL of 10% ethanol and 10 mL of deionized water at the beginning of each experiment. Afterwards, the ASTRA program is accessed and prepped in order to run the concentrations. Deionized water was used as a 3-5 minute baseline for the experiment and then the differing concentrations were injected into the DAWN® HELEOS in ascending order. Each concentration was allowed to run on the module until a steady signal was obtained on the Astra program. The molecular weight and the root-mean square radius (RMS) were obtained for each fabricated polymer.

DNA Amplification

For the mutation detection part of this project, model DNA samples (*M. Vanbaalinii* culture) were isolated using an UltraExtraction Tissue kit (MOBIO Laboratories). For the genetic identification part, DNA was extracted from cultured algae species (*ectocarpus* and *oedogonium*) obtained from Carolina Biologicals. Both sets of DNA were then amplified via PCR, with fluorescent primers FAM and HEX. The GoTaq® PCR Core Systems Kit (PROMEGA) was used and the following thermocycle protocol was applied:

Table 2 - PCR protocol used

95 °C	2 min	1 cycle	Initial Denaturation
94 °C	45 seconds	35 cycles	Denaturation
50 °C	45 seconds	35 cycles	Annealing
74 °C	45 seconds	35 cycles	Extension
74 °C	20 minutes	1 cycle	Final Extension
4 °C		Hold	Storage

Single Strand Conformation Polymorphism through Capillary Electrophoresis

The capillary electrophoresis module used was the ABI 3130 genetic analyzer from Applied Biosystems (36 cm long capillaries, dye set G5). The polymer is prepared at 3.5% (wt) made with running buffer (10X solution consists of 54 g tris base, 27.5 boric acid, 20 mL EDTA (0.5 M pH 8.0) totaling up to 1 L volume in MilliQ water). The fluorescently labeled DNA was then diluted to 1 ng/uL with 10 mM Tris-HCl (pH 7.8) buffer. Ten uL of the solution is placed in each well of the CE plate. The DNA was then denatured at 95 °C for 3 minutes and then snap cooled on ice for 3 minutes. The CE plate is then placed in the tray module of the ABI 3130. The samples were run at 25 °C, injection time of 15 seconds, voltage of 15 kV, and a run time of 60 minutes.

Results and Discussion

Detection of Mutations

The detection of mutations via CE-SSCP is a straight forward process. In a clinical setting, the genetic material in questions will be analyzed with a model “wild type” sample in order to see any variation from the “wild type’s” fingerprint on an electropherogram. This variation signals the presence of a mutation in the analyte. In Figure 4, such examples of the variation caused by the presence of the mutation can be seen in the last two graphs. The differences in peak structure and number of peaks in the last two graphs, from the uppermost wild type graph, are an indication that there are alterations present in the sample.

Three different mutations were used: insertions, transitions, and deletions. Insertions are when the mutation is caused by the addition of an extra base pair in the genetic coding. Deletion is the removal of a base pair, and a transition is a specific replacement of a purine base pair to a different purine base pair or pyrimidine to pyrimidine base pair. These, in theory, should cause unique conformations according to the type of mutation and where the mutation occurred. It should be noted that in this study, the DNA used had a length of ~182 base pairs.

Figure 4 is a collection of model electropherograms that shows the elution times of different types of mutation. Originally, two different sets of data were collected for each electropherogram—one for the forward ssDNA strand and one for the reverse. This study will only look at the forward strand, which was tagged with FAM fluorescent primers.

Figure 4 shows that using 3.4 MDa polymer medium results in elution times past 20 minutes. The red circles in the uppermost graph indicate the original wild type peaks. In the CE runs of the 3.4 MDa

polymer, the insertion mutation samples resulted in inconsistent peaks, thus it was not used in this study. Examples of how the insertion mutation manifests in peaks can be seen in Figures 5 and 6.

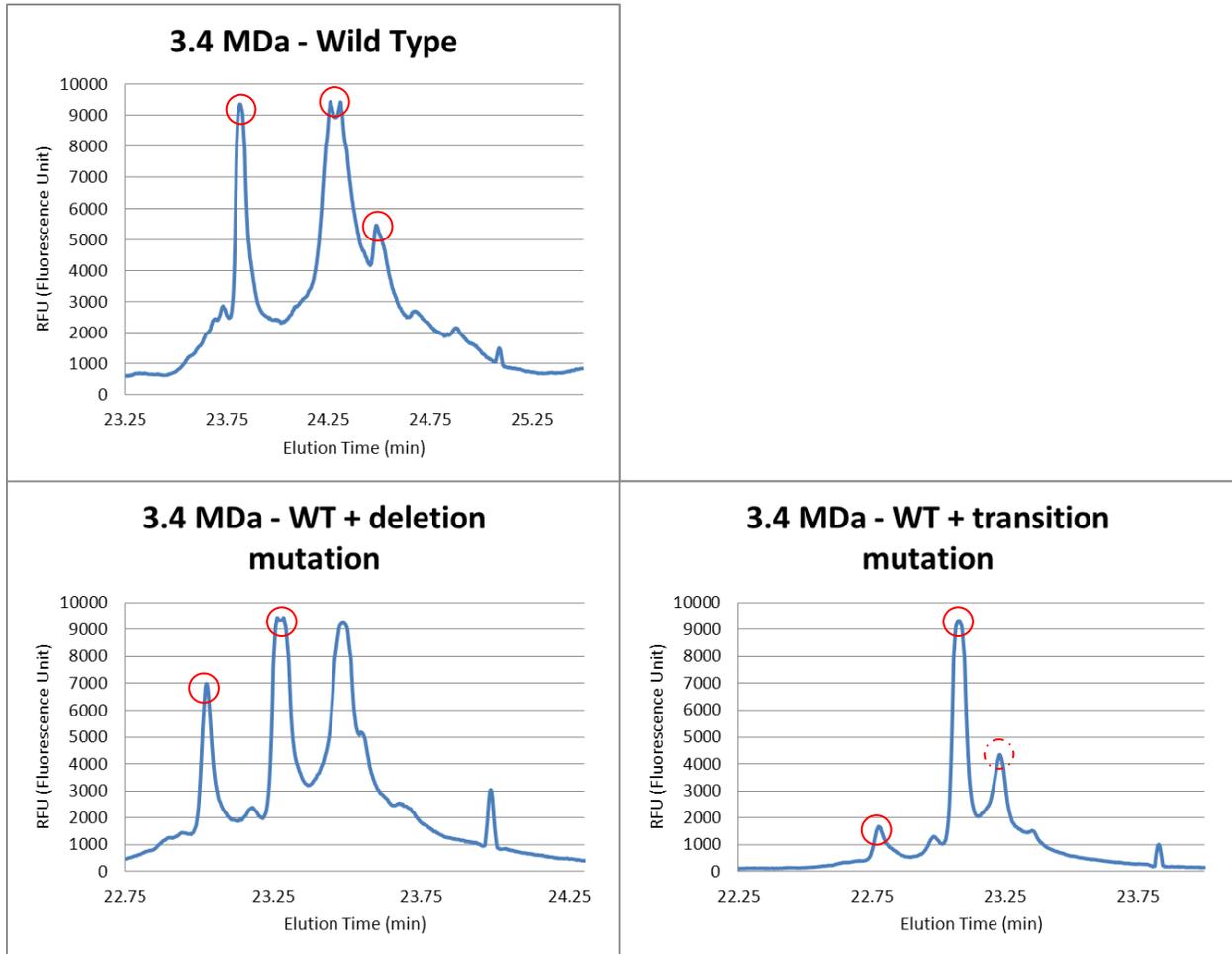


Figure 3 - Electropherograms of samples of wild type and different types of mutations in 3.4 MDa polymer.

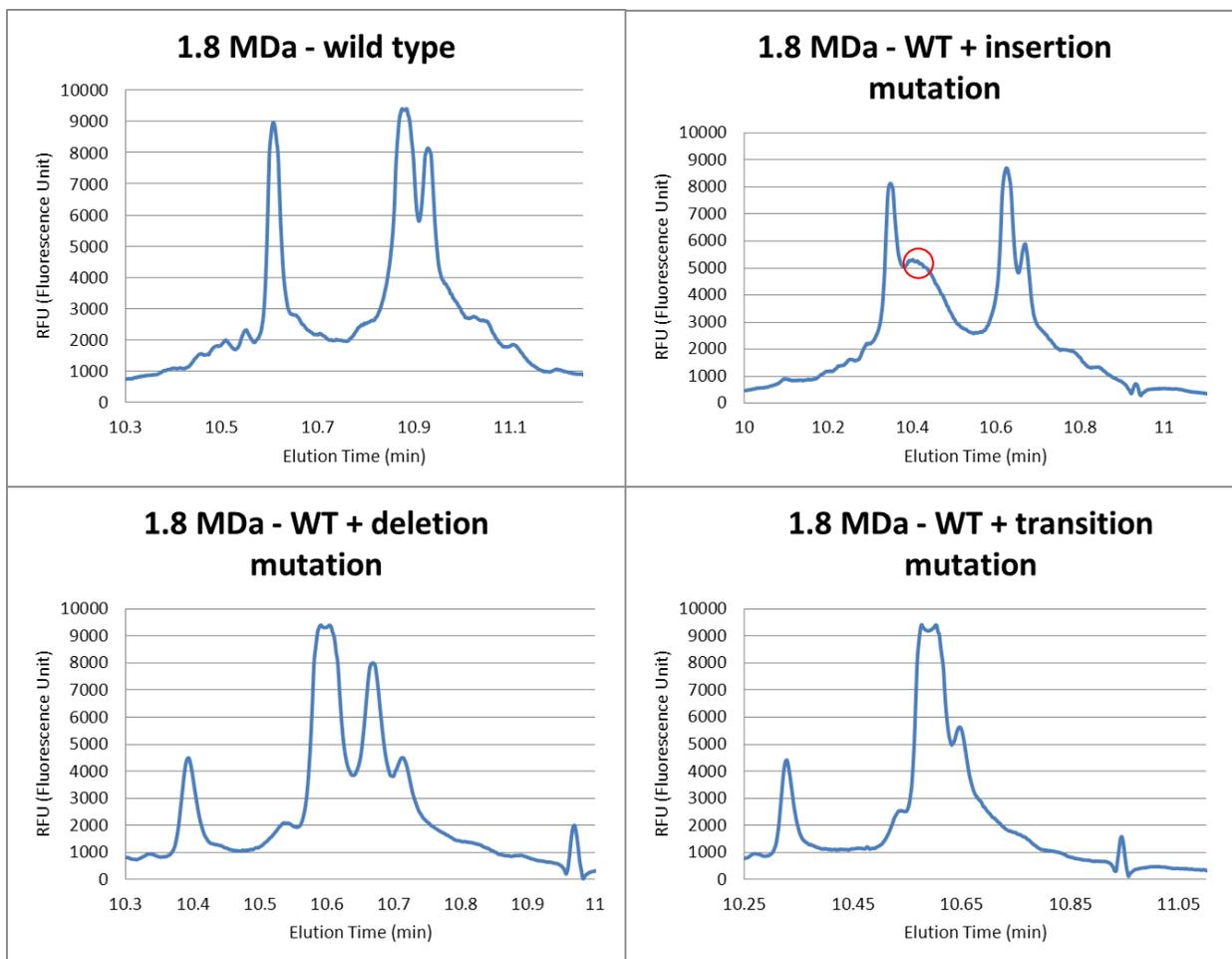


Figure 4 – Electropherograms of samples of wild type and different types of mutations in 1.8 MDa polymer.

In the 1.8 MDa polymer, the elution times of the samples were ~10 minutes. This significant difference in time shows that there is an optimized polymer molecular weight. In this case however, there is less of a time difference between the peaks. The insertion mutation shows as a shoulder in the first wild type peak as indicated by the red circle on Figure 5.

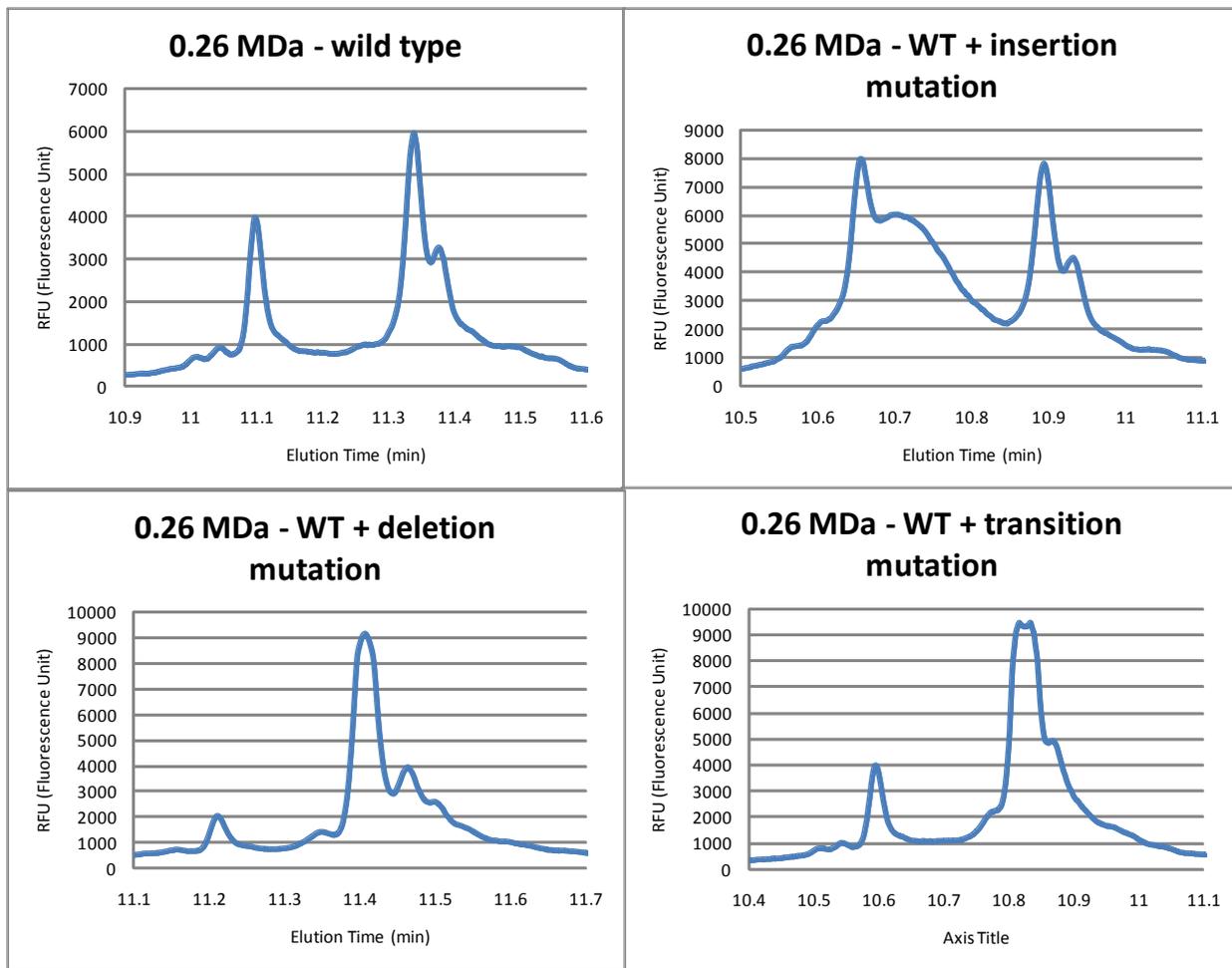


Figure 5 - Electropherograms of samples of wild type and different types of mutations in 0.26 MDa polymer.

Figure 6 shows a less resolved version of Figures 4 and 5.

As seen in some of the graphs, there exists a double-peak for the second wild type peak in both the wild-type graph and some of the mutation graphs. It is unclear whether this double-peak is wild-type, since it is not consistent. Thus a way to determine whether this peak is wild type genetic material, spiking the sample (wherein twice the amount of the concentration of the wild type) would be used in the wild-type + mutation runs.

Wild Type on Peakfit – 3.4 MDa polymer

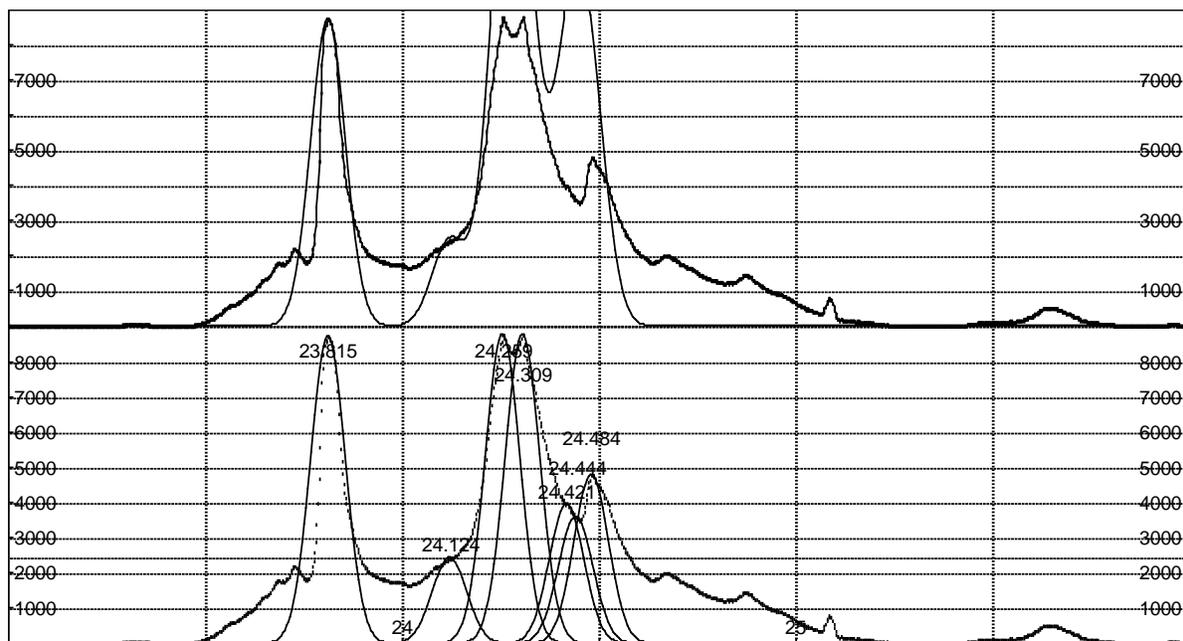


Figure 6 - Peakfit program visualization of fitting peaks onto the wild type graph resulting from using 3.4 MDa polymer.

In order to determine the resolution of the results, the wild type peaks must first be identified in the transition, insertion, and deletion results. Once wild-type peak is labeled as a peak of reference, the other mutation peaks are then compared back to this reference point. Peakfit® is used to determine the exact time of elution for each peak. It assigns a single width to all the peaks in the graph. Thus, as shown in Figure 7, all the fitted peaks are assigned one width value.

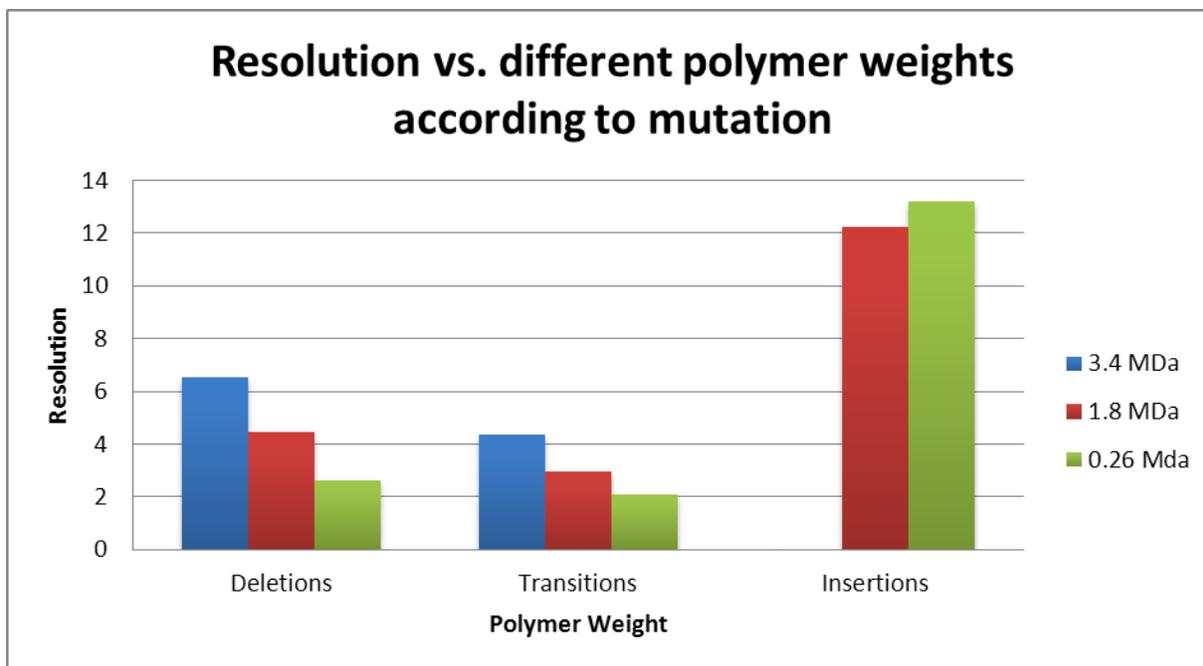


Figure 7 - Comparative resolutions of different polymer molecular weights in detecting mutations.

The observed trend of resolution as it relates to the polymer molecular weight is that as polymer molecular weight increases, so does the resolution. However, that only applies to deletions and transitions in this study. For insertions, no mutation was consistently detected in 3.4 MDa.

Statistical analyses result, without including insertion data, in the following significant p-values.

Table 3 - Table of p-values between polymers.		
3.4 MDa vs. 1.8 MDa	1.8 MDa vs. 0.26 MDa	3.4 MDa vs. 0.26 MDa
p = 0.0358	p = 0.0249	p = 0.0002

The affect of the polymer molecular weight on the resolution of the different peaks is therefore significant.

Shorter elution times are preferred but, as this study shows, at the cost of resolution. In this study, it was observed, however, that the shortest elution time was at 1.8 MDa. As Kourkine et al. suggests, the optimal polymer molecular weight for base pair lengths should be around ~600 kDa for a different type of polymer.

Species Identification

The identification of different species in a sample can be done visually. A difference in peak width or even in peak height can indicate the presence of a different species, but specifically identifying a certain species requires obtaining a “fingerprint” electropherogram of the species. In the case of this project, since only two different species were used, ectocarpus and oedogonium, identification is not complicated.

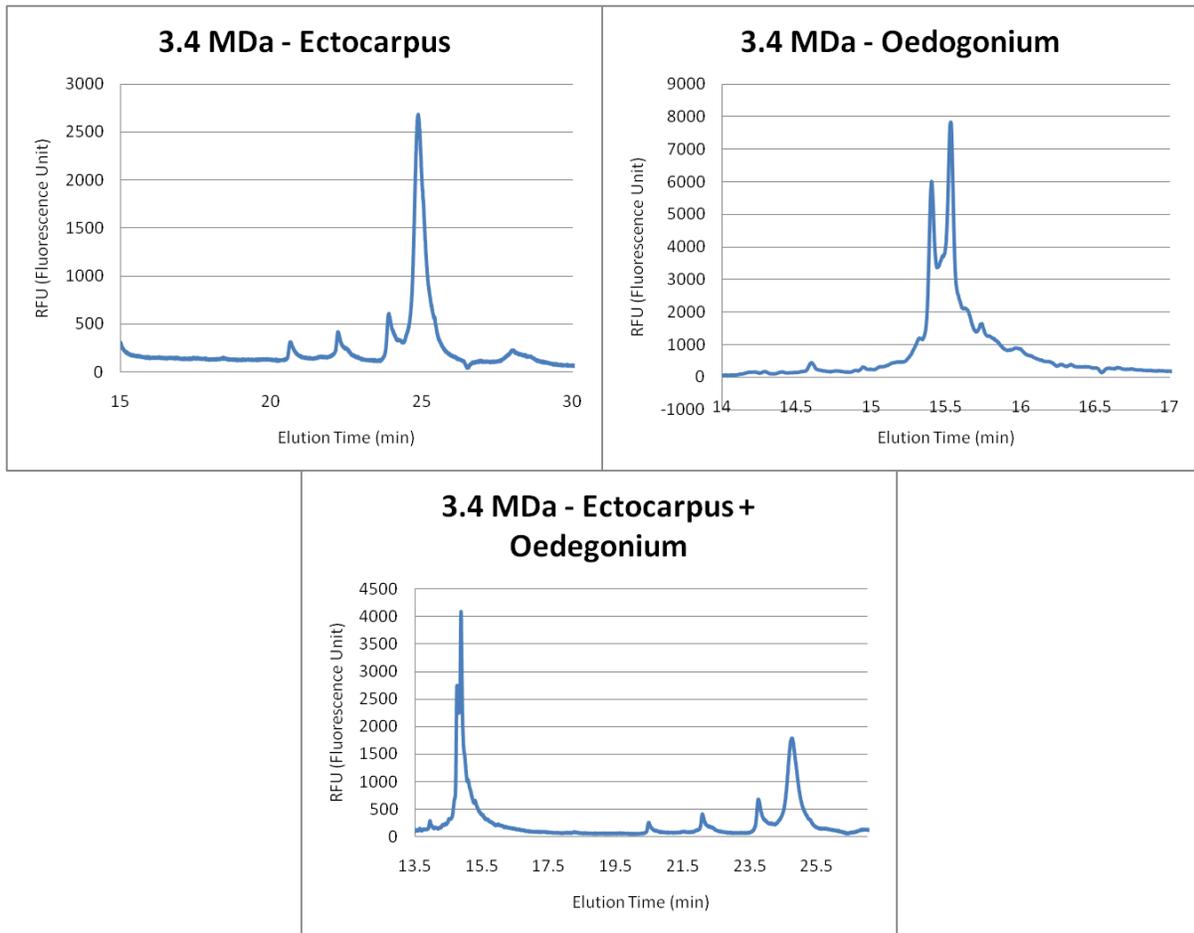


Figure 8 - Electropherograms of algae species at 3.4 MDa.

The peaks for ectocarpus and oedogonium have significant differences. At 3.4 MDa polymer molecular weight, this difference in peaks can allow for identification since the elution times of both species are very different—ectocarpus at ~15 minutes and oedogonium at ~24 minutes. The elution

times of both species in the combination sample shows that there is a difference of about 0.5 minute. The RFU for the ectocarpus is found to be an order of magnitude less than that of the oedogonium.

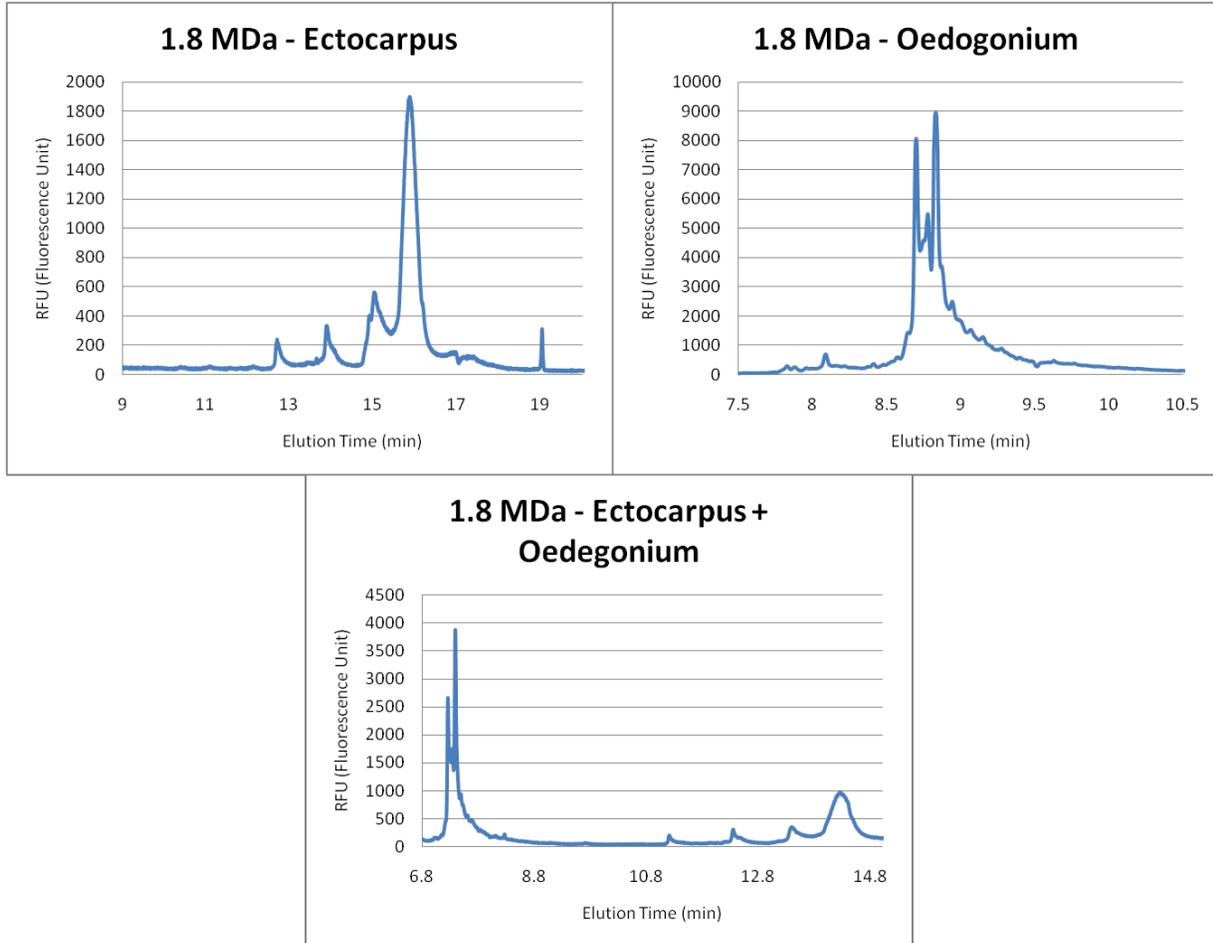


Figure 9 - Electropherograms of algae species at 1.8 MDa.

At 1.8 MDa, the elution times are reduced to ~13 minutes for ectocarpus and ~8.5 minutes for oedogonium. For the combination of both, the elution times of both species have differed by about 1-1.5 minutes, which might be due to the fact that the interaction between the analyte species and the polymer is more likely to produce a slightly different elution time.

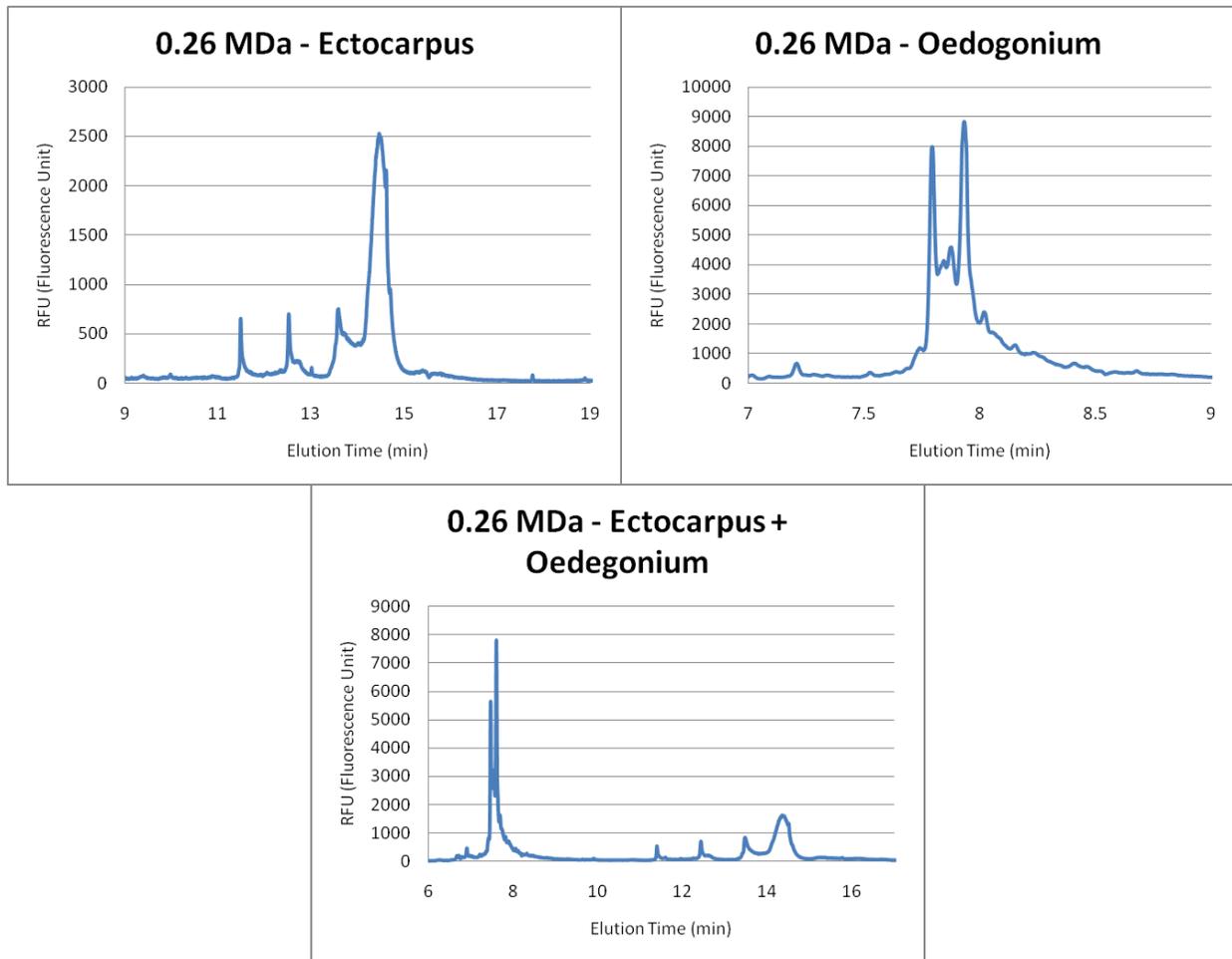


Figure 10 - Electropherograms of algae species at 0.26 MDa.

In 0.26 MDa polymer, the elution times in the combination graph are almost equal to the individualized runs. The resulting elutions are ~11 minutes for ectocarpus and ~7.75 minutes in oedogonium.

This trend of decreasing elution time as polymer molecular weight decreases is consistent with algal DNA. This study with two species, it was shown that there is a distinct pattern for the elution of the sample DNA material. The usage of this technique as a means to identify different species is thus highly possible.

Conclusions

CE-SSCP is shown to be an efficient method of detecting mutations. The trend of using smaller polymer molecular weights to decrease analysis time, should this technique be applied in a clinical setting, can be implemented and optimized further. A polymer molecular weight of 1.8 MDa is therefore recommended since it is the polymer molecular weight that allows for the best resolution and detection of all the mutations in this study. In this project, a model DNA of ~182 base pairs was used to simulate the behavior of the T2DM hereditary genes. To extend this study, it is recommended that DNA from a diabetic patient be used, along with the polymer that Kourkine et al. determined to be optimum for ~200 base pairs (LPA polymer, 0.6 MDa) .

In the identification of different algae species, CE-SSCP holds great potential in differentiating the genetic material from any number of species. In order to validate and quantify its efficiency in such a purpose, an additional third species should be used. In this project, genetic material from both the ectocarpus and oedogonium algae was used. A resolution study can also be completed with the algae data in order to find the optimized polymer molecular weight. However, the correlation between decreasing polymer molecular weights inducing a decrease in resolution did not hinder the differentiation between species. If in any case, identification between just these two species is necessary, even lower polymer molecular weights can be used. For the purpose of visual identification, there is enough distinction between the used oedogonium and ectocarpus SSCP conforms that result in a wide range of elution times.

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