2016

# Using Cleaved Amplified Polymorphic Sequence (CAPS) Genetic Markers to Determine the Extent of Hybridization between Castilleja affinis and Castilleja mollis as a Mechanism for Adapting to Climate Change on Santa Rosa Island

Elizabeth Medford
*Scripps College*

# Using Cleaved Amplified Polymorphic Sequence (CAPS) Genetic Markers to Determine the Extent of Hybridization between *Castilleja affinis and Castilleja mollis* as a Mechanism for Adapting to Climate Change on Santa Rosa Island

A Thesis Presented by
Lizzie Medford

To the W.M. Keck Science Department
Of Claremont McKenna, Pitzer, and Scripps Colleges
In partial fulfillment of
The degree of Bachelor of Arts

Senior Thesis in Environmental Analysis
May 2016

## Table of Contents

**Abstract:**

Hybridization, the process of interbreeding between individuals of different species, is one method by which plants and animals adapt to a changing environment. One example of such adaptation through hybridization may be occurring on the California Channel Islands with two species of *Castilleja*. While United State Geological Survey (USGS) researchers have been studying the populations of *Castilleja affinis* and *Castilleja mollis* to determine if hybridization is occurring on Santa Rosa Island since the early 1990s, up until this point primarily overt phenotypic characteristics have been used to differentiate between the two species. Genetic methods of differentiation were adopted to confirm that hybridization is in fact occurring on the island, possibly in response to climate change. Hybrids may be expanding into areas once occupied by pure *C. mollis*, because they might carry some of C. affinis' traits like an ability to survive warmer, drier climates as parts of the island are starting to become warmer and drier. In this study, I have developed a cleaved amplified polymorphic sequences (CAPS) marker based on internal transcribed spacer (ITS) regions to differentiate between the two species and hybrids and have applied these CAPS markers to genotype DNA samples isolated from 132 individuals. This protocol was used to determine the extent of hybridization on Santa Rosa Island in conjunction with ongoing surveys conducted by the USGS. Work focused on genotyping previously collected samples from two main sites on the island, which allowed confirmation that patterns observed based on phenotype in the field are supported by genetic data. In the future, findings will link genetic type with survivorship and growth data, to test whether hybrids perform differently than pure *C. mollis*. Broadly, this will determine if the two species are in fact hybridizing as a method for adapting to climate change, the most severe threat to Channel Island biodiversity.

# Introduction:

Biodiversity hotspots are places that have an unusually high number of different species with more species per square kilometer than most other areas (CNPS, 2012). California is one of the 25 most biologically diverse places on the planet and is the only biodiversity hotspot in North America (CNPS, 2012). The eight Channel Islands, which are located between 20 km and 98 km off the southern California Coast, are an important component of California's biodiversity hotspot. The US National Park Service manages five of the islands: Anacapa, Santa Cruz, Santa Rosa, San Miguel, and Santa Barbara. The Channel Islands are a part of the California Floristic Province, which is an area within California that is especially biodiverse with high levels of plant endemism (Critical Ecosystem Partnership Fund, 2016). Besides high plant species diversity, the islands also contain diverse marine ecosystems and bird populations (National Parks Service, 2016).


**Figure 1.** Map of The Channel Islands off the coast of southern California

Despite high levels of biodiversity and endemism on the islands, they face many dire threats ranging from grazing to climate change. The Channel Islands face issues associated with being isolated as islands such as having low genetic diversity. Conservation concerns are often exacerbated on islands because islands experience higher rates of species extinction than mainland ecosystems (McEachern *et al.*, 2009). Plants on the California Channel Islands have also been subject to a variety of human caused pressures. The islands have been shaped by both modern and ancient land uses including Native American hunting

and gathering, commercial ranching, fishing, national defense, tourism, and conservation (Rick, 2014). From feral animals like pigs and rats, to intensive grazing pressure from herbivores, the native and endemic plants of Santa Rosa Island have faced many threats to their existence. Native Americans strongly influenced the Channel Islands with fire to clear vegetation (Erlandson and Rick 2010). Overgrazing, drought, and flawed management practices led to widespread erosion, the introduction and spread of exotic plant species, and the disappearance of native flora and fauna (Johnson DL 1980, Corry and McEachern 2009). Elimination of cattle in 1998, and deer and elk in 2011, have resulted in improved habitat conditions on the island (McEachern, 2014). Reversing the extensive and well-documented ecological effects of the ranching era is the primary focus of current restoration efforts on the Channel Islands. In recent decades, managers have removed most introduced herbivores (e.g., sheep, cattle, pigs, deer, goats, elk), and dramatic vegetation changes have followed the release from herbivory (Cohen et al. 2009).

While many threats to biodiversity on the Channel Islands have been addressed, another major concern is the impact of anthropogenic climate change. Anthropogenic effects on the climate have led to sustained warming of global temperatures over the last three decades, and current models predict substantial future changes in rainfall, temperature and other parts of the global climate system (IPCC 2013). Specifically, the consequences that are likely to have an impact on the Channel Islands are warming ocean temperatures, ocean acidification, sea level rise, changing weather patterns, and warming air temperatures (National Parks Service, 2010). For many species on the Channel Islands, changes in precipitation patterns and fog cover as well as temperature increase will cause their habitat to contract to only the coolest, moistest areas of the islands (McEachern, 2009).

When dealing with climate change, populations can either move to track their ideal temperature, remain in the same range and adapt to the higher temperature, or go locally extinct (Corlett, 2013). Migration therefore, is one option for responding in the face of climate change or other rapid changes that organisms may face. However, many studies have found that species will have to migrate much faster than historic rates to keep up with rates of temperature and precipitation change (Aitken, 2008). Additionally, not all organisms can migrate as well as others. For example, unlike mobile animals, plants rely on pollination and seed dispersal to shift their ranges and therefore require population distributional changes rather than individual movement. Besides the individual species' characteristics however, the structure of the landscape also determines mobility. For example, the presence of natural barriers like mountain ranges or bodies of water will determine whether a species can migrate or not. Researchers found that in Central America, the division of two niches by the Isthmus of Tehuantepec caused the conservatism of ecological niches across moderate periods of evolutionary time by preventing migration (Peterson et al. 1999). Artificial fragmentation of habitats through urbanization or deforestation similarly determines mobility. Therefore, in many parts of the world these barriers to migration will prevent species from migrating at a sufficient rate to keep pace with climate change (Pearson, 2003). However, migration is not the only options that species have for adapting to rapid changes in climate. For example, studies show that plants and animals can alter the timing of spring events such as flowering, egg laying, or migrating when spring is warmer (Veder, 2013).

In order to persist in a rapidly changing climate, besides the ability to migrate without physical barriers, species also need adequate time and adequate genetic diversity in their populations. A lack of appropriate genetic variation can constrain rapid evolution

because genetic diversity helps organisms cope with environmental variability. Specifically, subtle differences among individuals increase the probability that some individuals, and not others, will survive to reproduce. Differences among individuals are determined at least partly by genotype, meaning that in variable environments a broader range of genetic variation will be necessary to persist (Tuljapurkar 1989; Tilman 1999). When populations have been experiencing stable environmental conditions for a long time, they are likely to undergo stabilizing selection and lose genetic variation (Rice, 2009). For long-lived species and poor dispersers, rapid evolution will be difficult since intergenerational selection as well as selection at expanding range margins is required for evolutionary processes to take effect (Pearson, 2003). Meaning species that have a small dispersal range will have smaller range margins at which genetic selection can take place. In genetically homogenous populations it is less likely that any individuals will have a mutation necessary to withstand higher temperatures or retain more moisture. In addition to genetic diversity, populations need adequate time to adapt to changes in climate. One study suggests that some species of the major tetrapod clades may have to evolve at least 10,000 times faster than they have in the past to cope with the degree of climate warming that is projected in the next 100 years (Quintero, 2013). For example, the annual mean temperature was found to increase at a rate that is $2.30*10^{10}$ times faster than plethodontid salamanders' climatic niche evolution will occur (Quintero, 2013). Mean precipitation was found to change at a rate that is at most $8.12*10^{13}$ time greater than the rate at which Mustelid mammals will be able to adapt. This means that even with sufficient genetic diversity, populations may not be able to evolve fast enough to keep up with climate change.

Recent research suggests that many populations of organisms in fact may have limited genetic variation for responding to selection with increasing temperatures (Quintero, 2013). For example, a study of 13 marine invertebrates from the Antarctic Peninsula found the highest temperature of acclimation that could be tolerated over a period of months was 1ºC to 6ºC (Peck et al., 2009) For the brittle star *Ophinotus victoriae* however, acclimation to temperatures of only 2-3º C is not possible. The authors conclude that if these upper temperature limits reflect the genetically fixed abilities of these animals to tolerate high temperatures, some species will face higher risks from climate change (Somero, 2010). Another study mentions that it is often assumed that populations have abundant genetic variation in quantitative traits for adaptation but that this is on the basis of studies of genetic variation in generalist model species with broad distributions (Hoffmann, 2003). In a study of the Australian tropical rainforest fly *Drosophila birchii* researchers found that despite high levels of genetic variation for morphology, parent-offspring comparisons indicate low heritable variation for the trait of desiccation resistance (Hoffmann, 2003). Therefore, low levels of genetic variation, or genetic variation unrelated to traits that are needed to adapt to climate change, provide an indication of whether rapid evolutionary adaptation is likely and without genetic diversity species are unlikely to adapt to climate change (Hoffman, 2011). However, some studies have shown that genetic variation allows for local adaptation to climatic conditions in fitness-related traits including traits related to physiological limits as well as phenological timing (Hoffman, 2011). Additionally, in a paper on managing microevolution, Rice et al. (2003) suggests that adaptation can occur on much shorter timescales than originally thought by biologists. This paper also suggests that the adaptive

potential of species could be used in conservation and restoration efforts that are faced with rapid environmental change (Rice, 2003).

In addition to migrating when possible or adapting when genetic diversity is sufficient, one specific type of evolution for adapting to climate change is hybridization. Hybridization is the process of interbreeding between individuals of different species (interspecific hybridization) or genetically divergent individuals from the same species (intraspecific hybridization). Plants hybridize much more frequently and successfully than animals do (Grant, 1971). This often occurs between closely related species that have recently overlapping ranges because climate change has caused their ranges to shift. As some species spread under favorable conditions, new contact zones arise between related species, increasing the likelihood of hybridization (Hoffman, 2011). While it may seem like having another adaptation option would be a positive outcome for many species, hybridization between closely related taxa due to climate change is often regarded as a negative outcome for conservation. While, in some cases hybridization, through hybrid vigor, leads to an increase in fitness, superior levels of biomass, stature, growth rate, and/or fertility it also leads to the loss of rare species (Chen, 2010). Not only is genetic diversity lost when one species' genome is replaced by the hybrid, but it has also been found that fitness declines after hybridization over many generations (Hoffman, 2011). Further, hybridization can dilute or genetically assimilate the native genotype leaving no "pure" natives (Huxel, 1999). For example, "pure"' native Pecos pupfish may no longer exist due to introgression with an introduced bait fish, the sheepshead minnow (Echelle and Connor, 1989). Hybridization is especially a threat to populations that are becoming small and peripheral with climate change, like Channel Island endemic plants.

There are few studies showing that hybridization in plants is happening in response to climate change. However, we know this mechanism is plausible and that rapid evolution can happen due to hybridization because it has been documented in invasive species (Ellstrand, 2006). This is therefore sometimes a concern with climate change because native species are often more vulnerable to invasions of non-native species when stressed by drought or high temperatures. Often, invasive species hybridize with native species and thereby become better suited for their new habitat and out compete the native species they hybridized with (Hovick, 2014). One study shows that in a remarkable number of cases, hybridization precedes the emergence of successful invasive populations (Ellstrand, 2000). For example, Abbott observed that interspecific hybridization has often served as a stimulus for the evolution of entirely new, and sometimes invasive, species (Abbott, 1992). However, besides being a conservation threat, the adaptation methods of hybridization may prove effective in the future for conservation efforts in a changing climate. For example, one recently introduced idea for conservation methods is intentional hybridization of related species, called "genetic rescue," to increase genetic diversity and therefore decrease extinction risk (Whiteley, 2015). Genetic rescue shows that hybridization can quickly introduce genetic variation into a population, and lead to new phenotypes.

**Figure 2A. (left)**: *Castilleja affinis ssp. Affinis*

**Figure 2B. (right)**: *Castilleja mollis*

In summary, hybridization is one important way that plants in particular might acquire new genetic variation that would allow rapid adaptive evolution to climate change. This mechanism

might be especially important in rare species that have limited ranges and few possibilities for migration, such as island endemics. However, there are not many studies showing this sort of rapid evolution in progress, and most of those are for invasive species and not rare native endemics. One major barrier to looking for patterns of hybridization is the challenge of genetically identifying hybrids.

In this study, we developed methods for distinguishing hybrids and pure line individuals for a Channel Islands endemic plant, *Castilleja mollis* (Figure 2B.), and a common close relative that grows in sympatry in adjacent habitat, *Castilleja affinis* (Figure 2A.). *Castilleja mollis* is found only in two distinct subpopulations on Santa Rosa Island at Carrington Point and Jaw Gulch. The total population size of *C. mollis* is less than 1,500 individuals as of 2006 (McEchern *et. al.*, 2009). This species occurs mostly at low elevation on coastal cliffs that experience heavy fog cover in the summer. In contrast, *Castilleja affinis* is a common species both on Santa Rosa Island and in mainland California. On Santa Rosa, this plant occurs in warmer, drier habitat at higher elevations. Starting in the late 1990s, US Geological Survey and National Park Service biologists monitoring *C. mollis* began to notice increasing numbers of potential hybrids between *C. mollis* and *C. affinis* in what had previously been pure stands of *C. mollis*. These potential hybrid phenotypes appear to be moving downslope into some cooler, lower elevation habitat that in the past has supported only *C. mollis*. One hypothesis for this pattern is that hotter and drier conditions during recent droughts have favored hybrid individuals. However as mentioned before, the ability to adapt rapidly to environmental changes depends on the presence of substantial heritable genetic variation. For *Castilleja mollis*, dwelling on the Channel Islands, this is an

issue because of the known general lack of genetic diversity in small and isolated plant populations (Ellstrand, 1993).

**Genetic Identification of Species**

The broader question this project explores is the role of hybridization as one possible way organisms adapt to anthropogenic climate change. Specifically, this project aims to determine the extent of *Castilleja* hybrid individuals on Santa Rosa Island by developing genetic markers and applying them to collected samples. A major problem that this project solves is that of ambiguity in hybrid versus pure species identification. In the past, identification of *C. mollis* versus *C. affinis* was based solely on phenotype, which is less accurate because plant phenotypes can be variable depending on short-term environmental conditions (Chegou, 2011). Often times the methods for using physical characteristics to differentiate related species can cause a higher probability of error in identification (Chegou, 2011). A more dependable and less error-prone method involves identification using genetic information. I therefore created a methodology for identifying *Castilleja* hybrids genotypically to differentiate between the two *Castilleja* species and the hybrid individuals. Specifically, we aimed to use single nucleotide polymorphisms (SNPs) to make a genetic marker that would differentiate between the species (Figure 3.). SNPs represent differences in a single nucleotide and are therefore useful for genotyping (Shastry, 2009).

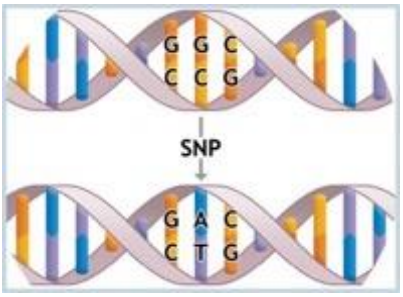


**Figure 3.** Single Nucleotide Polymorphism

I then developed a cleaved amplified polymorphic sequence (CAPS) marker, which detect DNA polymorphisms using PCR based techniques (Figure 4.). These markers detect differences between genotypes by differentiating between single nucleotide polymorphisms (SNP) to measure genetic variation between members of a species (NCBI).

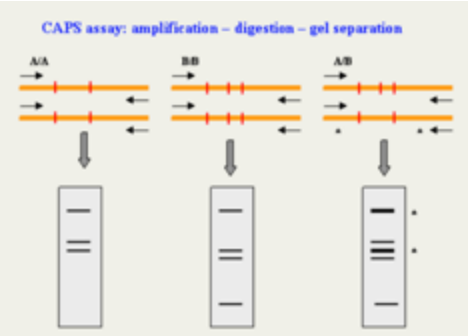


**Figure 4**. Cleaved Amplified Polymorphic Sequence marker digestion and on a gel

Internal transcribed spacer (ITS) sequences (Figure 5.), which are regions within the ribosomal transcript that show more variation than the ribosomal RNA sequences, are typically used for species identification (Schoch, 2011). Researchers have shown that the ITS region has the highest probability of successful identification for the broadest range of species (Schoch, 2011). The length and sequences of ITS regions of rDNA vary. Their small size (600-700bp) and high copy number (up to 30,000 per cell) enable easy amplification of ITS regions making them ideal for PCR primers (Sharma, 2000). Mutation of the ITS regions is frequent with a mean of 35 variants per species. However, three of the most abundant variants make up 91% of all ITS copies for ITS 2. (Song, 2012).

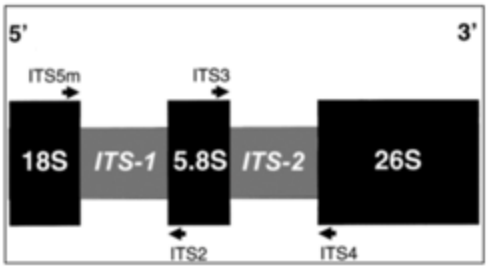


**Figure 5.** The three coding and two internal transcribed spacer regions of the nuclear ribosomal DNA repeat unit of a typical angiosperm. Arrows indicate approximate locations of the four primers used for PCR amplification.

Aside from running as many previously collected samples through our protocol, we are also developing a new technique that could be faster and more economical than the technique we

have used so far. This technique is called high resolution melting (HRM). It is performed on double stranded DNA samples and involves using DNA amplified using PCR techniques. The region that is amplified is known as the amplicon. After PCR amplification, the amplicon is treated with heat in a precise warming protocol from around 50º C up to around 95º C. During this warming process, the melting temperature of the amplicon is reached and the two strands of DNA separate (Liew, 2003). HRM monitors this process of separating the strands of DNA in real-time using a fluorescent dye. HRM uses intercalating dyes that have a unique property where they bind specifically to double-stranded DNA and when they are bound they fluoresce brightly. In the absence of double stranded DNA, they have nothing to bind to and they only fluoresce at a low level. At the beginning of the HRM analysis there is a high level of fluorescence in the sample because of the many copies of the amplicon. As the sample is heated up and the two strands of the DNA melt apart, presence of double stranded DNA decreases and thus fluorescence is reduced. The HRM machine has a camera that plots fluorescence in a melt curve, showing the level of fluorescence compared to temperature (Liew, 2004). Therefore, HRM is a cost effective way to process many samples in a short amount of time. Genetic differences can be easily seen as different curves making genetic differentiation clear. However, HRM is a more sensitive technique than the protocol previously used and future work should focus on optimizing this technique with the *Castilleja* samples.

This project focuses on genotyping previously collected *C. mollis* and *C. affinis* using genetic markers to study broad patterns in population. This data will allow researchers to confirm that patterns of hybridization observed in the field are supported by genetic data. Then USGS researchers will be able to link genetic type with survivorship and growth data,

to test whether hybrids perform differently than pure *C. mollis*. Other research questions involved in this project relate to the degree to which *C. mollis* and *C. affinis* hybridization differs across the island, if the degree of hybridization relates in some way to population trends, and if there is evidence that hybridization is increasing over time. Future research includes comparing population trends to temperature trends and determining if hybrid plants survive hot years more than pure *C. mollis* to connect hybridization to changes in climate and biodiversity loss.

# Methodology

### *Plant Material*

The United States Geological Survey (USGS) has been monitoring the *Castilleja mollis* populations on Santa Rosa Island since the early 1990s. *Castilleja mollis* grows on terraces at Jaw Gulch and bluffs at Carrington Point. These sites are about 14 km apart on the north side of Santa Rosa (Figure 15). The Carrington Point population is adjacent to much denser populations of *C. affinis* than the one at Jaw Gulch, and the majority of hypothesis hybrids have been observed at Carrington Point. This project used tissue samples previously collected in 2012 from *Castilleja mollis, Castilleja affinis ssp. affinis* and apparent hybrids in and around the *C. mollis* populations at Jaw Gulch and Carrington Point (Figure 15 and Figure 16). In 2013-14, researchers again collected tissue samples from tagged individuals in 9 demography monitoring plots, six at Carrington Point and another 3 at Jaw Gulch.

### *DNA Extraction*

The genomic DNA was extracted from frozen leaves using the CTAB method (Lukowitz *et al*. 2000). In brief, approximately 0.5g of leaf tissue was ground with a pestle in a 1.5 mL tube after which 700 µl of 2X CTAB Buffer (100mM Tris-HCL, 1.4M NaCl, 20mM EDTA, 2%CTAB) was added. Samples were immediately vortexed and incubated in a 65ºC water bath for 30 minutes. After incubation samples were then spun in a microcentrifuge for 1 minute at maximum speed before transferring the supernatant to a new tube. 700 µl chloroform was then added to the supernatant and samples were vortexed thoroughly before being spun in the microcentrifuge for 5 minutes at maximum speed to

separate the phases. 600 µl of the aqueous (upper) phase was then transferred to a new tube and equal volume (600 µl) isopropanol added. The samples were then spun for 5 minutes at maximum speed to pellet the DNA. The supernatant was decanted and the pellet was washed twice with 1 mL 70% ethanol. The ethanol was pipetted off before the samples were dried and resuspended in 75 µl TE buffer (10mM Tris, 1mM EDTA, pH 8.0).

***Primer Design for Internal Transcribed Spacer***

We used established primers previously shown to amplify from the ITS region from plants (Prince, 2010). These primers (CGATTGAATGGTCCGGTGAAG, AGGACGCTTCTACAGACTACAA) produced amplicons both from *C. affinis* and *C. mollis*. PCR products were run on a 1% agarose gel and then purified using Qiagen gel extraction kit (https://www.qiagen.com/us/) and submitted for Sanger sequencing to Operon (http://www.operon.com). SNPs were identified using Sequencher (https://www.genecodes.com) (Note: primer design was completed prior to fall 2015).

***CAPS Marker Amplification and Digest***

We used CAPS markers to differentiate between CAAF, CAMO, and hybrid samples. A polymorphic rDNA region was amplified using forward and reverse primers 18SF and 26SF. (CGATTGAATGGTCCGGTGAAG, AGGACGCTTCTACAGACTACAA) For a 10 µl reaction, 1.0 µl forward primer (10 µM) and 1.0 µl reverse primer (10 µM) were added to 2.5 µl Milli Q water, 5 µl 2X GoTaq mix (www.promega.com), and 0.5 µl genomic DNA. The PCR program consisted of 90ºC for 2 minutes, followed by 34 cycles of 94ºC for 20 seconds, 55ºC for 20 seconds, and 72ºC for 1

minute. The amplified DNA was then treated with the restriction enzyme BsrBI. For a 20 µl enzyme digest, 1 µl *MbiI* (isoschizomer of *BsrBI*) was added to 2 µl 10X tango buffer, 12 µl nuclease free water, and 5 µl of the PCR amplified DNA then incubated for two hours at 37ºC. The digests were then separated and visualized on a 1.0% agarose TAE gel with ethidium bromide. This protocol was repeated for 132 samples and images of the 1.0% electrophoresis gel results were recorded using the UVP BioSpectrum Imaging System (http://www.uvp.com).

***DNA Precipitation***

For the thirteen samples from plot 10 DNA precipitation was performed to increase the quality of the DNA as well as for the samples used for High Resolution Melt (HRM) curve analysis. DNA volume was adjusted to 250 µl and an equal volume (250 µl) of chloroform was added. Samples were vortexed and spun at maximum speed in a centrifuge. The aqueous (top) layer was removed. Then, 1/10 volume (25 µl) of 3M NaOAc, pH 5.8, is added as well as equal volume (~250 µl) room temperature isopropanol. The sample was vortexed and then centrifuged at maximum speed for 15 minutes after which the supernatant was carefully decanted. The sample was then washed twice by adding 1 ml of room temperature 70% ethanol, centrifuging at maximum speed for 1 minute, and decanting off the supernatant. The pellet was then air-dried for 5-20 minutes before being re-dissolved in a TE buffer (see above). DNA quality was assessed with the Nanodrop (http://www.nanodrop.com/Default.aspx). (See Appendix B for DNA quality)

### *High Resolution Melting*

Genomic DNA samples were diluted to 10 ng/µl, and for a 20 µl reaction 1 µl DNA was added to 10 µl 2X Precision Melt Supermix, 7 µl water, 1 µM forward primer and 1 µl reverse primer (Appendix A). Samples were then placed in the Bio-Rad CFX96 system for PCR amplification and HRM analysis consisting of 95ºC for 2 minutes, followed by 40 cycles of 95ºC for 10 seconds, 60ºC for 30 seconds, and 72ºC for 30 seconds. This was followed by one cycle of 95ºC for 30 seconds, 60ºC for 1 minute, and 65ºC to 95ºC for 10 seconds per step in 0.2 or 0.1ºC increments. (See primer table in Appendix E)

### *Sample Processing*

First the protocol for cleaving samples using the enzyme treatment was applied to known *C. affinis* and *C. mollis* samples to ensure that the protocol worked consistently. Once it was determined that the protocol did consistently differentiate between the pure species, the protocol was applied to hybrid samples that we created by combining the extracted *C. affinis* and *C. mollis* DNA. The synthesized hybrids were treated and then run out on a gel as the other samples had been (Lanes 9 and 10). The first few rounds of samples were known to be pure *C. affinis* or pure *C. mollis* and were used to test the effectiveness of the primers and of the CAPS marker enzyme digest. Next, tested samples were unknown in terms of species and are identified by their sample numbers.

## Results

The final gel consisted of both treated and untreated *C. affinis*, *C. mollis*, and hybrid samples (Figure 6.). This is the ideal result for our protocol: that there will be two bans for *C. mollis* because it is being cleaved, one ban for *C. affinis* because it is not being cleaved, and three bans for the hybrid samples because it has both cleaved and uncleaved DNA. BsrBI discriminated between CAMO and CAAF alleles located between base pairs (bp) 340 and 360. For the protocol that was used in this project, enzyme treatment was used to differentially cleave the amplified genetic material of our samples so that these cuts appeared on the electrophoresis gel and the samples could be differentiated genetically and visually.

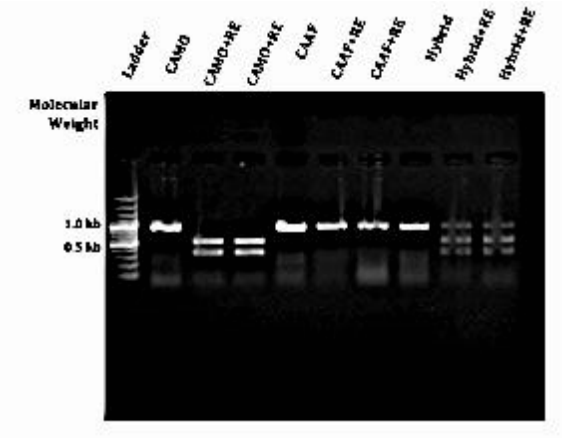


**Figure 6**. Cleaved Amplified Polymorphic Sequence markers differentiate between CAMO and CAAF. Agarose gel electrophoresis amplified 5.8S rDNA sequences cut or uncut with restriction enzyme BsrBI. "+RE" identifies samples cut with BsrBI restriction enzyme

Results fall into two categories: samples tested over the course of CAPS marker development (Table 1) and samples tested during implementation of the CAPS marker protocol (Table 2). Sample numbers have the following format: 13-1-21 with the first number (13) corresponding to the year the sample was collected, in this case 2013. The second number (1) corresponds to the plot from which the sample was collected, in this case plot 1. The final number corresponds to the identification number for that sample, in this case sample number 21 of plot 1 collected in 2013. All sample results found in the table correspond to an electrophoresis gel images found on pages 23-25.

**Table 1**. Identified plant samples confirmed by CAPS marker

| Sample Number or Label | Date Analyzed | Result |
|---|---|---|
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 4 | 12/12/15 | *C. affinis* |
| CAAF near plot 2 | 12/12/15 | *C. affinis* |
| CAMO near plot 8 | 12/12/15 | *C. mollis* |
| CAMO near plot 8 | 12/12/15 | *C. mollis* |
| CAMO near plot 8 | 12/12/15 | *C. mollis* |
| CAMO near plot 8 | 12/12/15 | *C. mollis* |
| CAMO near plot 8 | 12/12/15 | *C. mollis* |
| CAMO near plot 9 | 12/12/15 | *C. mollis* |
| CAMO near plot 9 | 12/12/15 | *C. mollis* |
| CAMO near plot 9 | 12/12/15 | *C. mollis* |
| CAMO near plot 9 | 12/12/15 | *C. mollis* |
| CAMO near plot 9 | 12/12/15 | *C. mollis* |
| CAAF | 4/8/14 | *C. affinis* |
| CAAF | 4/8/14 | *C. affinis* |
| CAAF | 4/8/14 | *C. affinis* |
| CAAF | 4/8/14 | *C. affinis* |
| CAAF | 4/8/14 | *C. affinis* |
| CAAF | 4/8/14 | *C. affinis* |
| CAAF | 4/8/14 | *C. affinis* |
| CAAF | 4/8/14 | *C. affinis* |
| CAAF | 4/29/14 | *C. affinis* |
| CAAF | 4/29/14 | *C. affinis* |
| CAAF | 4/29/14 | *C. affinis* |
| CAAF | 4/29/14 | *C. affinis* |

| | | |
|---|---|---|
| CAMO | 4/29/14 | *C. mollis* |
| CAMO | 4/29/14 | *C. mollis* |
| CAMO | 4/29/14 | Inconclusive |
| CAMO | 4/29/14 | *C. mollis* |
| CAAF near plot 2 | 11/4/14 | *C. affinis* |
| CAAF near plot 2 | 11/4/14 | *C. affinis* |
| CAAF near plot 2 | 11/4/14 | *C. affinis* |
| CAAF near plot 2 | 11/4/14 | *C. affinis* |
| CAAF near plot 2 | 11/4/14 | *C. affinis* |
| CAAF near plot 2 | 11/4/14 | *C. affinis* |
| CAAF near plot 4 | 11/4/14 | *C. affinis* |
| CAAF near plot 4 | 11/4/14 | *C. affinis* |
| CAAF near plot 4 | 11/4/14 | *C. affinis* |
| CAAF near plot 4 | 11/4/14 | *C. affinis* |
| CAMO near plot 8 | 11/4/14 | *C. mollis* |
| CAMO near plot 8 | 11/4/14 | *C. mollis* |
| CAMO near plot 8 | 11/4/14 | *C. mollis* |
| CAMO near plot 8 | 11/4/14 | *C. mollis* |
| CAMO near plot 8 | 11/4/14 | *C. mollis* |
| CAMO near plot 8 | 11/4/14 | *C. mollis* |
| CAMO near plot 9 | 11/4/14 | *C. mollis* |
| CAMO near plot 9 | 11/4/14 | *C. mollis* |
| CAMO near plot 9 | 11/4/14 | *C. mollis* |
| CAMO near plot 9 | 11/4/14 | *C. mollis* |

**Table 2.** Plant samples analyzed by CAPS marker

| Sample Number | Location | Date Analyzed | Result |
|---|---|---|---|
| 1776 | Jaw Gulch | 10/17/15 | *C. mollis* |
| 12/9/10 | Jaw Gulch | 10/17/15 | *C. mollis* |
| 1778 | Jaw Gulch | 10/17/15 | *C. mollis* |
| 12/9/04 | Jaw Gulch | 10/17/15 | *C. mollis* |
| 13-9-6 | Jaw Gulch | 10/17/15 | Inconclusive |
| 12/9/12 | Jaw Gulch | 10/17/15 | Inconclusive |
| 1228 | Carrington Point | 11/13/15 | *C. mollis* |
| 13-5-03 | Carrington Point | 11/13/15 | *C. mollis* |
| 967 | Carrington Point | 11/13/15 | Inconclusive |
| 956 | Carrington Point | 11/13/15 | Inconclusive |
| 1227 | Carrington Point | 11/13/15 | Inconclusive |
| 12/5/29 | Carrington Point | 11/13/15 | Inconclusive |
| 13-5-02 | Carrington Point | 11/13/15 | Inconclusive |
| 13-5-03 | Carrington Point | 11/13/15 | Inconclusive |
| 1432 | Carrington Point | 2/13/16 | Inconclusive |
| 1001 | Carrington Point | 2/13/16 | Inconclusive |
| 1003 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/01 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/02 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/07 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/19 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/24 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/30 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/32 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/45 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/49 | Carrington Point | 2/13/16 | Inconclusive |
| 11/1/57 | Carrington Point | 2/13/16 | Inconclusive |

Most of the samples from Jaw Gulch, which includes plots 7-9, are *C. mollis* (Table 2). Specifically, samples from plots 8 and 9 that were labeled as *C. mollis* were in fact pure *C. mollis* when testing using our CAPS marker protocol. Some *C. mollis* samples, without a tag number, were also tested and found to be *C. mollis.* Samples from plot 9 and plot 5 not labeled with species were found to be *C. mollis.* We also found that the samples from Carrington Point, plots 1-6, consist of both *C. affinis* and *C. mollis*. The samples labeled as *C. affinis* near plots 2 and 4 were found to in fact be *C. affinis* after genetic analysis.

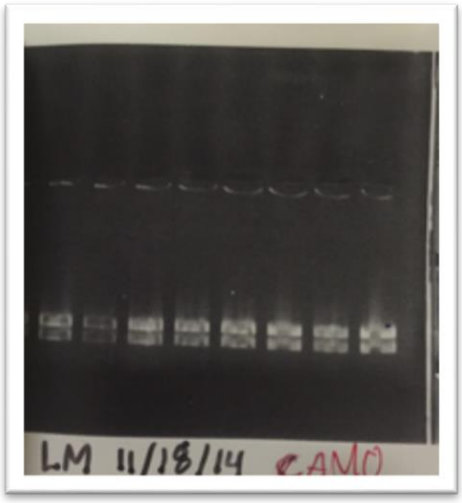


**Figure 7**. Electrophoresis gel of *C. mollis* samples

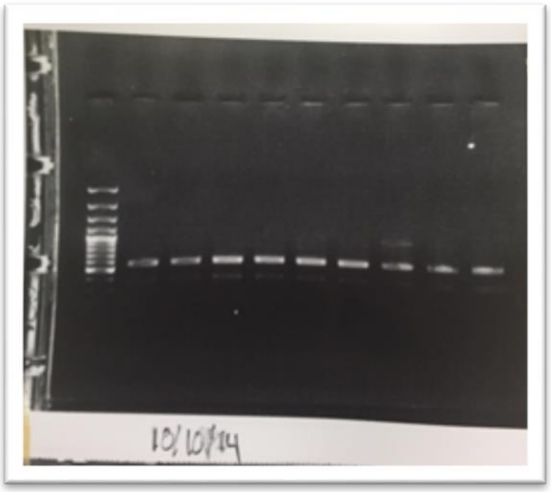


**Figure 8**. Electrophoresis gel of *C. affinis* samples

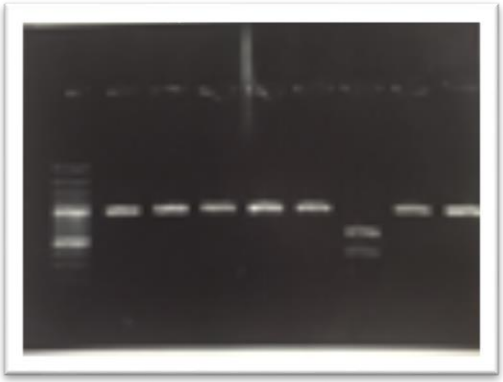

**Figure 9**. Electrophoresis gel of *C. affinis* and *C. mollis* samples

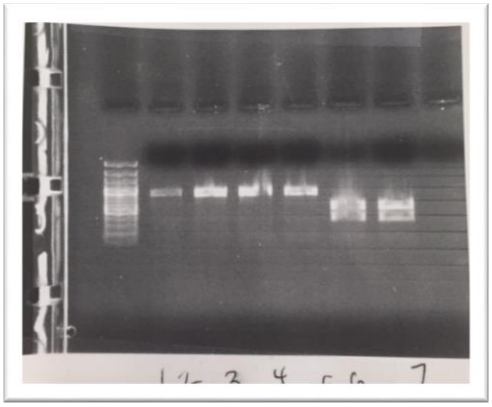

**Figure 10**. Electrophoresis gel of *C. affinis* and *C. mollis* samples

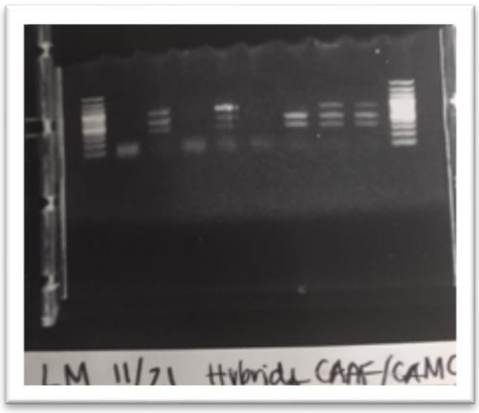


**Figure 11**. Electrophoresis gel of *C. mollis* samples

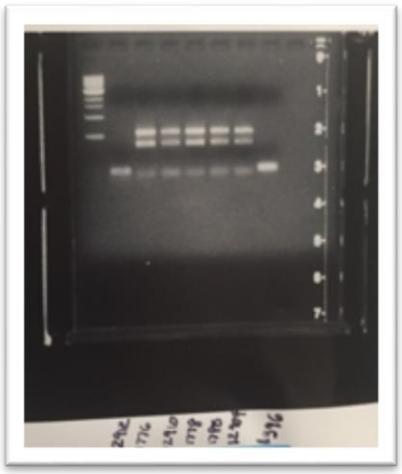

**Figure 12**. Electrophoresis gel of *C. mollis* samples

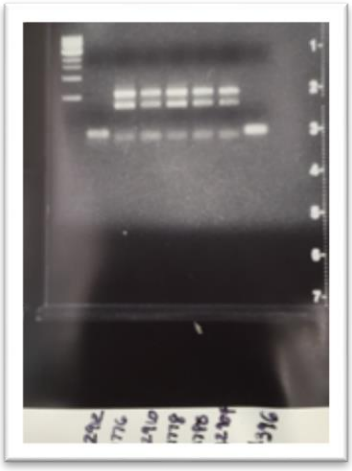

**Figure 13**. Electrophoresis gel of *C. mollis* samples

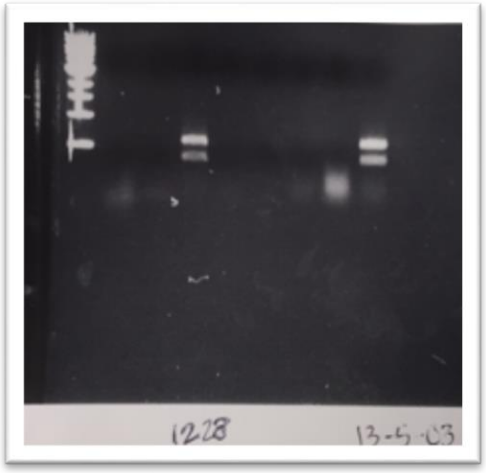


**Figure 14**. Electrophoresis gel of *C. mollis* samples

Once samples were being processes I ran into the issue of understanding the meaning of extra bands on our gel pictures that did not show up in the past. We thought that this faint lower band might imply that the samples are hybrids. By running a gel with the cleaved and un-cleaved DNA next to each other we were able to see that in fact we were not dealing with hybrids but instead have some DNA appearing on the bottom of the gel. This was non specific amplification rather than evidence of hybrids. In the spring we also ran into the issue of contaminated samples and unclean DNA. 78% of our CAPS marker processed samples were inconclusive likely because of contamination. Specifically, the samples from plot 10 had grit inside the tubes, which our protocol cannot adapt to. This caused there to be no bands when we ran the samples out on a gel.

The other set of results we generated were through High Resolution Melting (HRM), which is the second protocol that we developed for genotyping faster and more specifically. We were able to identify many sets of forward and reverse primers for HRM analysis.

Among the primers that we tested, the pairs that worked the best were A/B and D/E (See Appendix E). Using these primers, we were able to produce a few sets of melt curves.

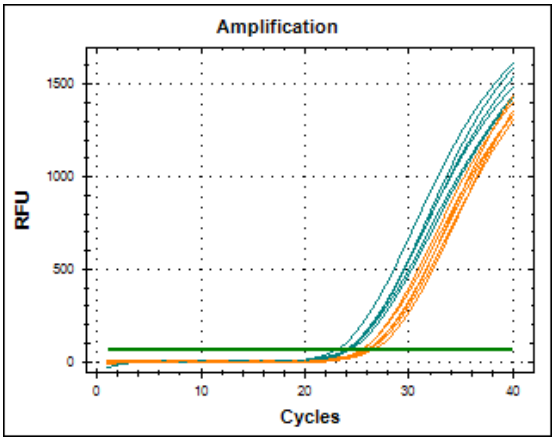


**Figure 15**. High Resolution Amplification Curve with *C. affinis* in blue and *C. mollis* in orange

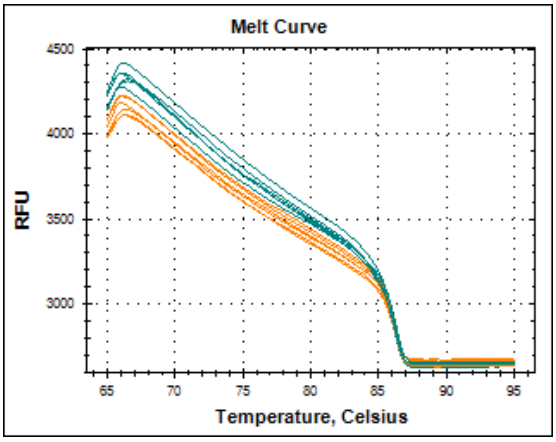


**Figure 16**. High Resolution Melt Curve with *C. affinis* in blue and *C. mollis* in orange

The best curves were produced when using A/B and D/E primers along with pure *C. affinis* and pure *C. mollis* DNA which was purified and diluted. The best amplification curve (Figure 15) and melt curve (Figure 16) show that *C. mollis* and *C. affinis* samples have different melt curves and that the curves are distinctly grouped by species as we would like. While the two species' curves are grouped together there is little distinction between the values of the two species. Applying this to unknown samples it would be impossible to distinguish between the two species or hybrids. However, achieving melt curves where the two species are grouped together does show that there is potential for future application of HRM analysis to this project.

## Discussion:

Our results support the observational data collected in the past by USGS researchers. They observed that since the 1990s, at Jaw Gulch, *C. mollis* has disappeared from the most inland, upslope parts of its habitat. At Carrington Point, *C. mollis* has contracted and expanded laterally along the coastal margin, but has not lost or gained ground upslope and inland.

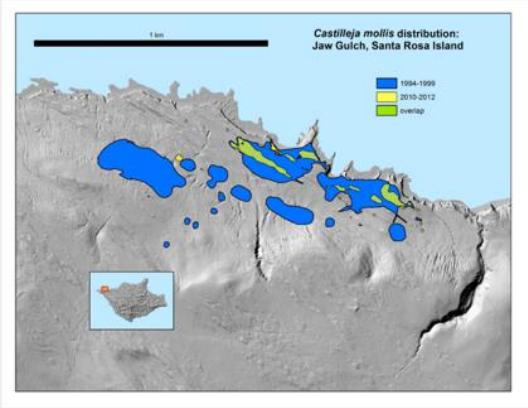


**Figure 17a**. *C. mollis* distribution at Jaw Gulch

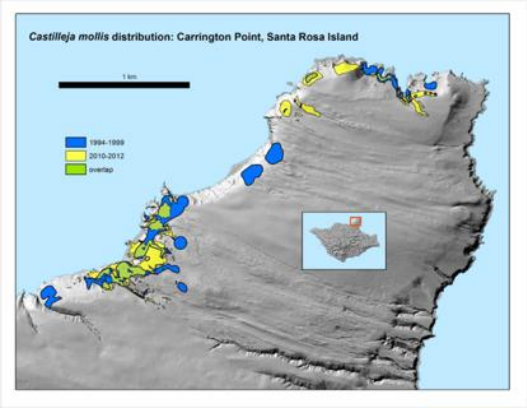


**Figure 17b**. *C. mollis* distribution at Carrington Point

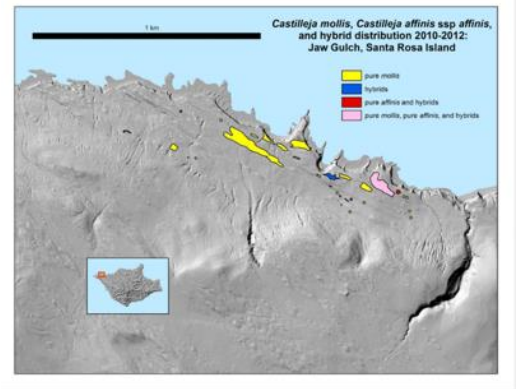


**Figure 18a**. *C. mollis*, *C. affinis*, and hybrid populations at Jaw Gulch

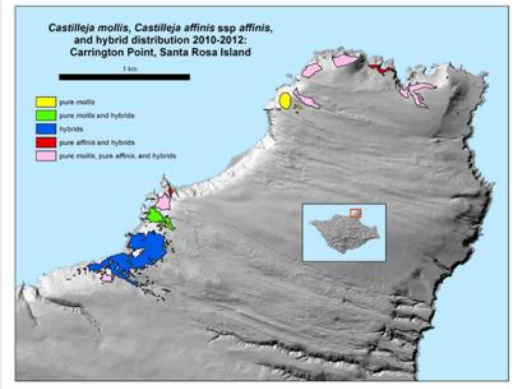


**Figure 18b**. *C. mollis*, *C. affinis*, and hybrid populations at Carrington Point

According to past observational data, at Jaw Gulch, most area is occupied by "pure" *C. mollis*, but several *C. affinis* ssp. *affinis* individuals and hybrids are mixed in at the

western edge of the population. Additionally, at Carrington Point, most of the *C. mollis* are growing intermixed with hybrids and also some *C. affinis* ssp. *affinis* (McEachern, 2015).

Our results support the conclusions that Jaw Gulch is still contains pure *C. mollis* individuals (samples 1776, 1778, 1780, 12910, 1396). Since our samples from the Jaw Gulch area from plots 8 and 9 were all found to be *C. mollis* we can conclude that the observational data was correct in saying that Jaw Gulch is occupied by pure *C. mollis* plants. Our results about samples from plots 5 show that pure *C. mollis* is also still present at Carrington Point as seen in samples 1228 and 13-5-03. Our results from plots 2 and 4 show that there are also populations of pure *C. affinis* in the Carrington Point plots. We did not encounter any samples that were hybrids of the two pure species. This is likely because we were testing samples from the less hybridized parts of each plot to be sure that the CAPS marker worked on pure samples. To make more specific conclusions about where pure species and hybrids are at the two sites further analysis of samples is needed. Once more of the samples are analyzed it will be possible to determine how correct the observation data is and which *C. mollis* populations are most at risk for extinction.

To address the issue of contaminated samples we concluded that the plant tissue is collected at the end of the flowering season in early summer when the plants are beginning to die back. This means that the tissue may be partially decomposed or dying when it is collected. To resolve the issues that we experienced with contaminated samples, plant tissue should be collected earlier in the growing season to ensure that the tissue has not started to decay. Also, there are DNA purification techniques that can reduce contamination issues and improve the quality of samples.

### *High Resolution Melting*

As the results show, we found a CAPS marker that works well with the two pure species and with created hybrids. While this protocol has worked well, to make the process of genotyping each sample quicker and achieve a higher throughput we are also developing a protocol using High Resolution Melting (HRM). We were able to get some conclusive melt curves using HRM analysis but further work should focus on analyzing the melt curves produced to determine if HRM will be a viable way to genetically differentiate between the two pure species and hybrid individuals. We did not have any conclusive HRM results for hybrids and therefore cannot be sure that this method would work for hybrids. Mostly, future HRM work with *Castilleja* should focus on determining if the melt curves are significantly different for the two pure species.

In summary, the CAPS marker enzyme digest protocol successfully differentiates between pure species and hybrids. Due to the limited number of samples processed, we can only conclude that there are still pure *C. mollis* populations at Jaw Gulch and that there are also pure *C. mollis* individuals at Carrington Point. We cannot make any conclusions about the abundance of either pure species or of hybrid until more samples are processed and a larger sample size is reached. High Resolution Melting is another option for analyzing the remaining samples that may save time and money. However, using HRM for the *Castilleja* project requires more work to be sure that this method will differentiate between the species successfully and consistently. Many research questions remain unanswered including: the degree to which *C. mollis* and *C. affinis* hybridization differs across the island, if the degree of hybridization relates in some way to population trends, and if there is evidence that hybridization is increasing over time. Future research should include genotyping as many of

the remaining samples as possible and collecting more samples to process. Future research should also involve comparing population trends to temperature trends and determining if hybrid plants survive hot years more than pure *C. mollis* to connect hybridization to changes in climate and biodiversity loss.

## References:


Abbott, R. (1992) Trends in Ecology and Evolution. 7**,** 401–405

Aitken, S., Yeaman, S. (2008) Adaptation, migration or extirpation: climate change outcomes for tree populations. Evolutionary Applications: 1752-4563.

Anderson, E. & Stebbins, G. L. (1954) Evolution 8**,** 378–388.

Brennan, A., Woodward, G. (2014) Hybridization due to changing species distributions: adding problems or solutions to conservation of biodiversity during global change? Evolutionary Ecological Research 16: 1-39.

Chegou, N., Hoek, K. (2011) Tuberculosis Assays: Past, Present and Future. Expert Review Anti-infective Therapy 9(4): 457-469.

Chen, J. (2010). Molecular mechanisms of polyploidy and hybrid vigor. Trends in Plant Science 15(2): 57.

Cohen. B., Cory, C., (2009). A spatial database of Santa Cruz Island vegetation. Proceedings of the Seventh California Islands Symposium. Institute for Wildlife Studies: 229–244.

Corlett, R., Westcott, D. (2013) Will plant movements keep up with climate change? Trend in Ecology and Evolution 28(8): 482-488.

Corry P, McEachern K. (2009). Patterns in post-grazing vegetation changes among species and environments, San Miguel and Santa Barbara Islands. Proceedings of the Seventh California Islands Symposium. Institute for Wildlife Studies. 201–214

De La Vega, F., Lazaruk, K. (2005) Assessment of two flexible and compatible SNP genotyping platforms: TaqMan® SNP Genotyping Assays and the SNPlexTM Genotyping System. Mutation Research 573: 111-135.

Echelle, A.A., Connor, P.J., 1989. Rapid, geographically extensive genetic introgression after secondary contact between two pupfish species (*Cyprinodon*, Cyprinodontidae). Evolution 43: 717-727.

Ellstrand, N. C., Elam, D. R.. (1993). Population Genetic Consequences of Small Population Size: Implications for Plant Conservation. Annual Review of Ecology and Systematics, 24, 217–242.

Ellstrand, N., Schierenbeck, K. (2000) Hybridization as a stimulus for the evolution of invasiveness in plants. PNAS 97(13): 7043-7050.

Erlandson, J., Rick, T., (2010) Archaeology meets marine ecology: The antiquity of maritime cultures and human impacts on marine fisheries and ecosystems. Annual Review of Marine Science 2: 231–251.

Garroway, C., Bowman, J. (2010) Climate change induced hybridization in flying squirrels. Global Change Biology 16: 113-121.

Grant, Verne. *Plant Speciation.* New York: Columbia University Press, 1971.

Hampe, J., Wollstein, A., (2001) Assessment of two flexible and compatible SNP genotyping platforms: TaqMan® SNP Genotyping Assays and the SNPlexTM Genotyping System. Bioinformatics Applications Note 17 (72001): 654-655.

Hoffmann, A., Sgro, C. (2011) Climate change and evolutionary adaptation. Nature 470: 479-485.

Hoffmann, A., Hallas, R. (2003). Low Potential for Climate Stress Adaptation in a Rainfroest Drosophila Species. Science 301(5629): 100-102).

Hovick, S., Whitney, K. (2014). Hybridization is associated with increased fecundity and size in invasive taxa: meta-analytic support for the hybridization-invasion

hypothesis. Ecology Letters 17(1): 1464-1477.

Huxel, G. (1999). Rapid displacement of native species by invasive species: effects of hybridization. Biological Conservation 89(2): 143-152.

International Panel on Climate Change (2013). Summary for Policymakers. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Johnson,. (1980). Episodic vegetation stripping, soil erosion, and landscape modification in prehistoric and recent historic time, San Miguel Island, California. The California Channel Islands: Proceedings of a Multidisciplinary Symposium. Santa Barbara Museum of Natural History: 103–121.

Liew, M., Pryor, R., (2003) Genotyping of Single-Nucleotide Polymorphisms by High-Resolution Melting of Small Amplicons. Clinical Chemistry 50(7): 1156-1164.

Lombardo, C. A., & Faulkner, K. R. (2000). Eradication of feral pigs (Sus scrofa) from Santa Rosa Island, Channel Islands National Park, California. In Proceedings of the Fifth California Islands Symposium, Santa Barbara Museum of Natural History.

Lukowitz *et al*. (2000) Positional Cloning In Arabidopsis. Plan Physiology 123(3): 795-806.

McEachern, A., Thomson, D. (2009) Climate Alters Response of an Endemic Island Plant to Removal of Invasive Herbivores. Ecological Applications 19 (6): 1574-1584.

McEachern, A. (2015) "Is hybridization between an endemic and a widespread native plant

cause for conservation concern? Soft-leaved island paintbrush (*Castilleja mollis*) on Santa Rosa Island." Poster for the California Native Plant Society Meeting January 2015.

Muhlfeld, C., Kovach, R. (2014) Invasive hybridization in a threatened species is accelerated by climate change. Nature Climate Change 4: 620-624.

National Parks Service. (2010) Planning for Global Climate Change. Channel Islands National Park California.

NCBI (2015) Cleaved Amplified Polymorphic Sequence. Probe Database.

Nordfors, L., Jansson, M. (2002) Large-Scale Genotyping of Single Nucleotide Polymorphisms by Pyrosequencing™ and Validation Against the 5¢Nuclease (TaqMan®) Assay. Human Mutation 19: 395-401.

Parmesan, C. (2006). Ecological and Evolutionary Responses to Recent Climate Change. Annual Review of Ecology, Evolution, and Systematics 37: 637-669.

Pearson, R. (2003). Predicting the impacts of climate change on the distribution of species: are bioclimate envelope models useful? Global Ecology & Biogeography 12: 361–371.

Peck, L. S., Massey, A., Thorne, M. A. and Clark, M. S. (2009). Lack of acclimation in Ophionotus victoriae: brittle stars are not fish. Polar Biol. 32: 399-402.

Peterson A. T., Soberon J., Sanchez-Cordero V. 1999 Conservatism of ecological niches in evolutionary time. Science 285, 1265–1267.

Porter, (2010). Adapt, Migrate, or Die.

Quintero, I., Wiens, J. (2013). Rates of projected climate change dramatically exceed past

rates of climatic niche evolution among vertebrate species. Ecology Letters 16(8): 1095–1103.

Rice, K., Emery, N. (2003) Managing Microevolution: Restoration in the Face of Global Change. Frontiers in Ecology and the Environment 1(9): 469-478.

Rick, T., Sillett, S. (2014) Ecological Change on California's Channel Islands from the Pleistocene to the Anthropocene. BioScience: 1-13.

Sharma, S., Rustgi, S., Balyan, H. (2000) Internal transcribed spacer (ITS) sequences of ribosomal DNA of wild barley and their comparison with ITS sequences in common wheat. Barley Genetics Newsletter 32: 38-45.

Shastry, B. (2009) SNPs: impact on gene function and phenotype. Methods in Molecular Biology 578: 3-22.

Shavrukov, Y. (2014) Cleaved Amplified Polymorphic Sequences (CAPS) Markers in Plant Biology. Australian Centre for Plant Functional Genomics, University of Adelaide.

Somero, G. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. The Journal of Experimental Biology 213: 912-920.

Song, J., Shi, L., Li, D. (2012). Extensive Pyrosequencing Reveals Frequent Intra Genomic Variations of Internal Transcribed Spacer Regions of Nuclear Ribosomal DNA. NCBI 7(8).

Strong, D., Ayres, D. (2015) Ecological and Evolutionary Misadventures of *Spartina*. Evolution and Ecology 44: 389-410.

Theurillat, J., Guisan, A. (2001) Potential Impact of Climate Change on Vegetation in the European Alps: A Review. Climatic Change 50: 77-109.

Thomas, C., Cameron, A. (2004) Extinction risk from Climate Change. Nature 427:145-148.

Tilman, D. (1999) The ecological consequences of changes in biodiversity: a search for general principles. Ecology 80:1455-1474.

Tuljapurkar, S. (1989) An uncertain life: Demography in random environments. Theoretical Population Biology 35:227-294.

Veder, O., Bouwhuis, S. (2013). Quantitative Assessment of the Importance of Phenotypic Plasticity in Adaptation to Climate Change in Wild Bird Populations. PLOS Biology.

Whiteley, A., Fitzpatrick, S. (2015) Genetic Rescue to the Rescue. Trends in Ecology & Evolution 30(1): 42-49.

## Appendix A: Primer Design

| Location | Species | Sequence | Enzyme |
|---|---|---|---|
| 150-180 | CAAF: | GCACAATCAGATTGGGCCGTT | Hpy188I 5bp |
| | CAMO: | GCACAATCAGTTTGGGCCGTT | none |
| 190-210 | CAAF: | TGCCCGATGTGCGACTCGAAT | none |
| | CAMO: | TGCCCGATGTTCGACTCGAAT | TaqI 4bp |
| 200- 230 | CAAF: | CGAATCGCCAAGACAGCACGA | MwoI |
| | CAMO: | CGAATCGCCACGACAGCACGA | MwoI |
| **340-360** | **CAAF:** | **CCCTTGCGGTGCGGAAGGAA** | **none** |
| | **CAMO:** | **CCCTTGCGGAGCGGACGGGA** | **BsrBI 6bp $63.00/1000units** |
| 345-365 | CAAF: | GCGGTGCGGAAGGAATGGGGG | HpyAV |
| | CAMO: | GCGGAGCGGACGGGATGGGGG | none |
| 348-368 | CAAF: | GTGCGGAAGGAATGGGGGACG | none |
| | CAMO: | GAGCGGACGGGATGGGGGACG | BccI and BsCl |

## Appendix B: DNA Precipitation

| | Before Precipitation: | | After Precipitation: | | |
|---|---|---|---|---|---|
| Sample | 260/280 | 260/230 | Concentration (ng/µl) | 260/280 | 260/230 |
| 1A | 2.08 | 1.46 | 210.5 | 1.88 | 1.89 |
| lA | 2.21 | 1.37 | 670.7 | 1.98 | 2.18 |
| 7M | 2.38 | 1.04 | 190 | 1.98 | 1.98 |
| 8M | 2.04 | 0.7 | 163 | 1.84 | 1.46 |

## Appendix C: Protocols

<u>1 M Tris-HCl (500 mL)</u>

1. Add 400 mL of MilliQ water into a beaker with stir bar
2. Add 60.5 g Tris base and stir until dissolved

note: the powdered form of Tris base is different than Tris-HCl, make sure you are using the right one or adjust your calculations accordingly

3. Adjust pH to 8.0 with concentrated HCl (will take approx. 21 mL HCl)—add slowly
4. Adjust final volume with MilliQ water to 500 mL in graduated cylinder
5. Store in glass bottle

0.5 M EDTA (1000 mL)

1. Add 700 mL distilled water to a beaker with stir bar
2. Add 186.1 g Na2EDTA . 2H2O (ethylenediamine tetraacetic acid, disodium salt dehydrate)
3. Stir until dissolved (this will take a while. You may need to start adding the NaOH before the EDTA dissolves, but it WILL dissolve after pH reaches 8.0)
4. Adjust pH to 8.0 with 10 M NaOH (~50 mL)
5. Adjust final volume with MilliQ water to 1000 mL in graduated cyclinder.
6. Autoclave and store at room temperature

5 M NaCl (500 mL)

1. Add 350 mL MilliQ water to a beaker with stir bar
2. Add 146 g NaCl and stir until dissolved
3. Adjust volume to 500 mL with MilliQ water
4. Pour into glass bottles and autoclave

10% (w/v) CTAB (250 mL)

1. Add 150 mL MilliQ water to glass bottle with stir bar
2. Add 25 g CTAB and stir for 15 minutes
3. Put bottle in 65ºC bath and let sit until CTAB dissolves
4. Adjust volume to 250 mL with MilliQ water

Note: do NOT autoclave

CTAB Genomic DNA Extraction

1. Grind leaf tissue with mortar/pestle (aim for about 100mg of tissue).

*young leaves work best

2. Add 500 uL 2X CTAB Buffer (made fresh, see below), mix well with leaf tissue by inverting several times (do not vortex). Incubate in 65oC water bath for 30 minutes to two hours, mix occasionally. Cool on bench for a few minutes when done.
3. Spin sample for 1 minute at maximum speed, and then transfer supernatant to new tube.
4. Add 500 uL chloroform and vortex thoroughly (do in hood).

*Wear gloves and do this step in the fume hood. Dispose of chloroform tips and tubes in the appropriate container in the satellite accumulation area.

5. Spin tubes in microcentrifuge for 5 minutes at maximum speed (13K rpm). Check to see afterwards that the phases are separated.
6. Transfer 400 uL of the aqueous phase (upper phase here) to a new microfuge tube.

*Dispose of old tubes in the chemical fume hood.

7. Add 400 uL isopropanol and mix by inverting several times, wait five minutes.

8. Spin tubes in microcentrifuge for 5 minutes at 13K rpm to pellet the DNA. A white pellet should be visible after this step.
9. Pour off the supernatant, make sure the white pellet sticks to the bottom of the tube.
10. Add 1 mL 70% ethanol to each tube, mix by inverting several times. Spin in microcentrifuge for 3 minutes at 13K rpm.
11. Gently pour off supernatant, make sure pellet sticks to the tube. Repeat steps 9 and 10 two more times.
12. Use a pipet to gently remove any remaining ethanol. Dry pellet briefly in speed-vac. Check every 5 to 10 minutes. Do not dry longer than necessary.
13. Gently resuspend pellet in 50 uL TE buffer.

PCR amplification master mix
40µl GoTaq
8 µl F primer (18SF)
8 µl R primer (26SF)
20 µl milli q water
*9.5 µl mater mix and .5ml DNA into each tube for PCR

Enzyme cleavage master mix
168 µl nuclease free water
28 µl 10X tango buffer
14 µl MbiI (BsrBI)
*15 µl master mix in each tube and 5ml amplified DNA

## Appendix D: HMR Protocol for precision melt supermix on Bio-Rad's CFX96

| Cycling Step | Temperature | Time | # Cycles | Additional Comments |
|---|---|---|---|---|
| **Initial DNA denaturation** | 95°C | 2 min | 1 | Complete activation of hot-start polymerase. |
| **2-step or 3-step PCR Cycling** | | | | |
| **Denaturation** | 95°C | 10 sec | 40–45 | A 3-step run protocol is recommended for amplicons >200 bp or GC-rich targets. |
| **Annealing/extension (+ plate read)** | 60°C (= primer Tm) | 30 sec | | |
| **Extension + plate read (optional)** | 72°C | 30 sec | | |
| **High Resolution Melting Analysis** | | | | |
| **Heteroduplex formation** | 95°C | 30 sec | 1 | |
| | 60°C | 1 min | 1 | |
| **High resolution melting + plate read** | 65–95°C (in 0.2°C increments) | 10 sec/step | 1 | A narrower melting range (amplicon Tm +/- 5°C) can reduce total run times. |

## Appendix E: Primer Sequences

| Id | Sequence 5'--> 3' | Primer |
|---|---|---|
| 18SF | CGATTGAATGGTCCGGTGAAG | 18SF |
| 26S | AGGACGCTTCTACAGACTACAA | 26S |
| A | TTAAACTCAGCGGGTGATCC | SNP1_Primer_F1 |
| B | GTCACGACAAGTGGTGGTTG | SNP1_Primer_R1 |
| C | CGACGCACGTCACGACAAGT | SNP1_Primer_R2 |
| D | TTAAACTCAGCGGGTGATCC | SNP2_Primer_F1 |
| E | GTCACGACAAGTGGTGGTTG | SNP2_Primer_R1 |
| F | CGACTCACGTCACGACAAGT | SNP2_Primer_R2 |
| G | ACTTGTCGTGACGTGTGTCA | SNP3_Primer_F1 |
| H | AATTGCAGAATCCCGTGAAC | SNP3_Primer_R1 |
| I | CCGTGAACCATCGAGTCTTT | SNP3_Primer_R2 |