Îlet Chancel *I. delicatissima* Final Genetic Report: Data preparation and analyses conducted by the Welch lab at Mississippi State University

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Overview

Within this genetic report, I summarize the methodology and results of the work conducted on samples received by the Welch lab from the French West Indies in 2021. The samples received were mostly comprised of dorsal spine clips collected from *I. delicatissima* on Îlet Chancel and Martinique. Upon arrival to the lab, dorsal spines were stored in vials containing ethanol within a -20 °C freezer. Before DNA could be extracted from samples, one to two cm segments were cut from each sample with a razor tool and forceps. The bench space was thoroughly cleaned with ethanol-soaked wipes between each dissection to limit cross contamination between samples. Pieces of spine clips were then placed into 2 mL vials along with four to five Steel Zinc Plated Shots and 200 to 300 mL of TE buffer (1000 mL TE, ph 8.0: 2 mL 0.5 M EDTA solution, 10 mL 1M Tris solution, brought to volume with ddH2O), depending on sample size. Buckshot was soaked in EtOH before placed in vials to eliminate potential contamination. Tubes, which now contained dorsal spine segments, buckshot, and TE buffer, were then placed into a Fisher Scientific Bead Mill 24 where samples were milled until a homogenous, semiliquid consistency was achieved (~10 minutes). Milled samples were then centrifuged to allow sample debris to collect in the bottom of the vial. Milled spine samples, along with any remaining TE buffer, were loaded into individual Maxwell® 16 Tissue DNA Purification kits where DNA extraction was conducted via the manufacturer's protocol (~45 minutes/16 samples). Upon completion of the purification step, DNA samples were then loaded into a 1% agarose gel along with loading dye to determine if extractions were successful via banding patterns before conducting further analyses.

Of the 136 *I. delicatissima* samples received, only 117 were able to have DNA successfully extracted, and the quality of these extractions varied. The lower success rate of achieving extractions with high concentrations of DNA could be due to multiple factors, including sample type, storage of sample, or extraction error. However, it is probable that the type of samples collected for genetic analyses impacted the DNA purification process. It is well documented that blood samples stored in lysis buffer are the optimum sample type for large quantity, population-wide genetic analyses due to the lysing of nuclei and isolation of DNA, which increases DNA yield and minimizes DNA degradation (Creel et al. 2003, Seutin et al. 1991, Kilpatrick 2002, Psifidi et al. 2015). Unfortunately, dorsal spines, and other non-invasive sampling techniques, do not always offer the same opportunity of optimum DNA extraction or amplification (Broquet et al. 2007, Smith & Wang 2014, Adams et al. 2019). Additionally, many of the collected spines seemed to have been clipped right above the dorsal tissue where the spine protrudes from the skin, which eliminates contact with the tissue and avoids potential injury. While this method can ensure less stress or pain for the animal, it also limits the amount of live epithelial tissue or blood that is collected. Many studies have recorded success in extracting DNA from genetic material such as shed skin, feathers, and buccal swabs (Rajpoot et al. 2021, Horváth et al. 2005, Schulte et al. 2011). However, these studies are predominately conducted by research labs that specialize in projects where forensic techniques are paired with historical or environmental sample preparation (Waits & Paetkau 2005, Beja-Pereira et al. 2009). Therefore, these labs have optimized their methodologies to ensure that DNA can be extracted from such sensitive samples with little

contamination (e.g., not using the same scalpel or tweezers to collect samples, preparing samples and DNA extractions in a 'pre-PCR' room completely free of PCR products, digesting samples for a minimum of 12 hours in proteinase K, using highly species-specific primers, etc.) (Carroll et al. 2018, Taberlet et al. 1996, Morin et al. 2001). The work conducted in the Welch lab has traditionally focused on projects with high sample volumes and the utilization of cross-species molecular markers where blood samples are preferred and provide clearer results. Therefore, the work conducted on this project was our best attempt at using the provided samples to complete a population-wide genetic analysis of *I. delicatissima* on Îlet Chancel using the tools and methodologies available to us here at MSU.

As stated previously, 117 of the original 136 samples were able to be genotyped across 22 nuclear microsatellite markers (Table 1). The mitochondrial DNA of 14 samples were also sequenced. Both the microsatellite and mitochondrial markers were derived from other genetic studies for this species as well as a prior genetic assessment performed by Genindexe (Valette et al. 2013, Martin-Judson et al. 2018, Vuillaume et al. 2015, van den Burg et al. 2018, Moss et al. 2017, Malone et al. 2000). Of the 22 microsatellite markers, 14 amplified reliably across most of the individuals and were used to calculate genetic indices (e.g., heterozygosity values, F-statistics, allele frequencies, AMOVA, etc.). The remaining eight were removed from calculations since they were unable to amplify product in more than 25% of the samples. While adequate data was obtained to conduct these calculations, many of the markers still failed to amplify successfully within some individuals resulting in multiple missing data points. A possible reason for these issues could be allelic dropout, which can increase in frequency as DNA concentration decreases (Broquet & Petit 2004, Gagneux et al. 2008). Due to this, the values presented here are merely an initial assessment of the genetic information of this population and could change once additional genotype data is acquired. Additionally, interpretations of this data are limited.

Microsatellite Analyses & Results

A total of 22 microsatellite markers were evaluated in this study, and polymerase chain reaction (PCR) protocols specified in Schuelke 2000 were used to amplify DNA segments at the specific marker sites. A 1% agarose gel was used to verify successful PCR amplification. Fragment analysis was conducted by the Cornell Institute of Biotechnology where microsatellites were sized by an Applied BioSystems 3730XL instrument using the LIZ500 size standard. The raw allelic data received was then scored by eye with PeakScanner™ v 1.0 (Applied Biosystems). Allelic ranges were cross-examined with other published *I. delicatissima* microsatellite data to ensure scored alleles fell within the size range of the species (van den Burg et al. 2021). Genetic indices were calculated within the genetic software programs Structure v.2.3.4 and GenAlEx v.6.5 (Pritchard etal. 2000, Peakall & Smouse 2012). Additional subpopulation structuring and the plotting of Îlet Chancel GPS coordinates were completed in R (R Core Team 2020). Similar studies within other taxa were assessed to ensure the most appropriate and informative genetic parameters were being calculated for this study (Latch et al. 2008, Trizio et al. 2005, Colosimo et al. 2014).

After analyzing the microsatellite data, eight (i.e., D105, IgdL4, IgdL5, IgdL8, IgdL17, IgdL20, IgdL23, and IgdL24) of the initial 22 microsatellites were removed for amplifying in less than 25% of the individuals. The number of alleles for the 14 remaining loci ranged from 1 to 11 (Table 1). All loci were able to be compared to previously published data to confirm that scored alleles were within the range of *I. delicatissima* for each marker. Markers of considerable interest were the species-specific primers (i.e., IgdL), published by Valette et al. 2015. All nine IgdL

primers had alleles that were identified within other *Iguana* populations in the Lesser Antilles by van den Burg et al. 2021 (Table 1). Some of the alleles scored for these primers, according to Figure 2b (van den Burg et al. 2021), have only been previously documented in non-*delicatissima* individuals (Table 1). While this was unexpected and potentially problematic considering the threat of hybridization between *I. delicatissima* and *I. iguana* on other islands, the purity of the Îlet Chancel population may not need to be immediately questioned. Due to the previously addressed issues of sample quality and DNA extraction success in this project, there is potential for error in PCR amplification and fragment analysis despite the efforts taken to optimize all purification procedures. Differences in allele size binning due to an absence of molecular calibration between laboratories could have also impacted these genotypes. Additionally, it is presumed that invasive *I. iguana* individuals have yet to be introduced to Îlet Chancel. Many of the other islands where hybridization is considered an immediate threat to the viability of pure *I. delicatissima* populations have well-documented evidence that non-native iguanas are present (Vuillaume et al. 2015, van den Burg et al. 2018, Pounder et al. 2020).

To assess the distribution of molecular variation across the Îlet Chancel population, an AMOVA was conducted with the genotypes (n_{loci} = 14) of the 117 individuals. Individuals were sorted into two different populations, East or West, according to the GPS coordinates recorded when the sample was collected (Figure 1). The highest proportion of molecular variation, 53%, was attributed to the difference in genotypes among individuals (Figure 2). Variation across the genotype of a single individual attributed to 40% of the molecular variation, and the remaining 7% was a result of differences among the East and West 'populations' (Figure 2). F-statistics (i.e., F_{st}, F_{is}, and F_{it}), observed and expected heterozygosity (H_o and H_e, respectively), and fixation indices were also calculated (Table 2). The F_{st} and fixation index (0.070 and 0.058, respectively) suggest that there is very little population differentiation due to genetic structuring on Îlet Chancel. The inbreeding coefficient, F_{is}, (0.566) is moderate and could imply that inbreeding is influencing the rates of observed heterozygosity (0.389), which is lower than the expected heterozygosity (0.420), in this isolated population. However, as stated previously, the interpretation of these values is limited and could be impacted by the difficulties in PCR amplification due to low DNA yields. Additional samples should be collected and analyzed to further investigate the threat of inbreeding and hybridization to the Îlet Chancel *I. delicatissima* population. Molecular calibrations between laboratories would also improve our ability to accurately interpret these results by standardizing fragment analysis protocols and allele binning. This would ultimately allow for more precise comparisons of genotypes between varying populations of *I. delicatissima* and their affiliated research groups.

Mitochondrial DNA Analyses & Results

The mitochondrial NADH dehydrogenase subunit 4 (ND4) of 14 individuals was sequenced with primer pairs and PCR protocols following previous studies (Malone et al. 2000). The two primer sequences, forward and reverse respectively, were 5'-ACTCCTCAGTAAGCCACATAGG-3' and 5'-ACACCTCTCGGTTTGCAAGAA-3'. Chromatogram files were edited and aligned (SEQUENCHER v5.3; Gene Codes Corp., Ann Arbor), and geographic origin and haplotype assignments were determined using similarity matches against GenBank. All sequenced haplotypes aligned with 100% similarity to a haplotype previously published in Martin et al. 2015 (Table 3). This haplotype was found in only three samples that also originated from Îlet Chancel of Martinique. The finding of a single pre-published

mitochondrial haplotype further emphasizes the lack of genetic diversity and population structure on Îlet Chancel. Additionally, since all individuals sequenced in this study were assigned an *I. delicatissima* mitochondrial haplotype, the likelihood of concurring hybridization between native and non-native iguanas on Îlet Chancel is limited in conjunction with the microsatellite results.

The number of individuals successfully sequenced at the mitochondrial locus was far less than anticipated. When screening all 117 samples with various primers, many of the samples were unable to amplify at any annealing temperature. These failed optimizations reflect the similar problems faced when screening for microsatellite fragment analysis. Ultimately, 21 samples generated enough viable PCR product to be sent for Sanger sequencing. Once data was received, base pair assignments were checked, and final sequences with low assignment probabilities were trimmed (i.e., base pair assignments were unclear for a large portion of the sequence and could not be confidently determined), only 14 sequences remained that were of decent quality to BLAST in GenBank for haplotype assignments. However, due to the extremely low haplotype diversity across the various *I. delicatissima* populations (Martin et al. 2015), it is possible that there is only a single mitochondrial haplotype found in the Îlet Chancel iguanas. To fully determine the genetic diversity of the Îlet Chancel population, additional samples should be screened. These additional samples should consist of high-quality genetic material, preferentially blood samples, to ensure optimum DNA extraction and PCR amplification across both microsatellite and mitochondrial primers.

Tables and Figures

Table 1. List of microsatellite markers (n = 14), allele number (N_a), and allele sizes found in this study. The alleles in green have been recorded previously in only non-*delicatissima* samples (van den Burg et al. 2021, Figure 2b). The allele in blue was not documented in any *delicatissima* or non-*delicatissima* samples (van den Burg et al. 2021, Figure 3c). All remaining alleles in black have been documented as occurring in native *delicatissima* samples from prior studies.

Microsatellite Marker	N_a	Allele Sizes	Microsatellite Marker	N_a	Allele Sizes
Z13	5	269, 271, 282, 284, 286	IgdL6	5	**242**, **244**, 246, **248**, **250**
Z148	Monomorphic	116	IgdL11	5	250, **254**, 268, 270, 278
CCSTE02	5	286, 290, 292, 294, 296	IgdL12	5	178, 180, 186, 188, 194
CYC177	3	237, 240, 243	IgdL14	8	**184**, 186, 188, 190, 192, **194**, **196**, **198**
D110	11	215, 221, 225, 229, 233, 235, 237, 241, 245, 249, 253	IgdL16	5	**174**, 176, **178**, 180, 182
IgdL2	Monomorphic	212	IgdL18	2	198, 200
IgdL3	3	252, 256, **260**	IgdL19	2	**254**, **260**

Table 2. List of mean values, standard errors, and p-values for the following calculated genetic indices: F-statistics (i.e., F_{st}, F_{is}, and F_{it}), observed and expected heterozygosity (H_o and H_e, respectively), and the fixation index (F).

Genetic Index	Mean Value	Standard Error or p-value
F_{st}	0.070	p = 0.001
F_{is}	0.566	p = 0.001
F_{it}	0.596	p = 0.001
H_o	0.389	SE = 0.060
H_e	0.420	SE = 0.051
F	0.058	SE = 0.088

Table 3. List of the Îlet Chancel *I. delicatissima* samples (n = 14) that were used for mitochondrial DNA sequencing and analysis. This table also includes the haplotype ID and corresponding GenBank Accession number that aligned to each Îlet Chancel sequence. The delicatissima_D haplotype was originally published in Martin et al. 2015.

Iguana ID	Haplotype ID	GenBank Accession #	Iguana ID	Haplotype ID	GenBank Accession #
A3	delicatissima_D	KJ561224.1	A36	delicatissima_D	KJ561224.1
A4	delicatissima_D	KJ561224.1	A37	delicatissima_D	KJ561224.1
A11	delicatissima_D	KJ561224.1	A41	delicatissima_D	KJ561224.1
A12	delicatissima_D	KJ561224.1	A42	delicatissima_D	KJ561224.1
A15	delicatissima_D	KJ561224.1	A43	delicatissima_D	KJ561224.1
A18	delicatissima_D	KJ561224.1	B25	delicatissima_D	KJ561224.1
A25	delicatissima_D	KJ561224.1	B29	delicatissima_D	KJ561224.1

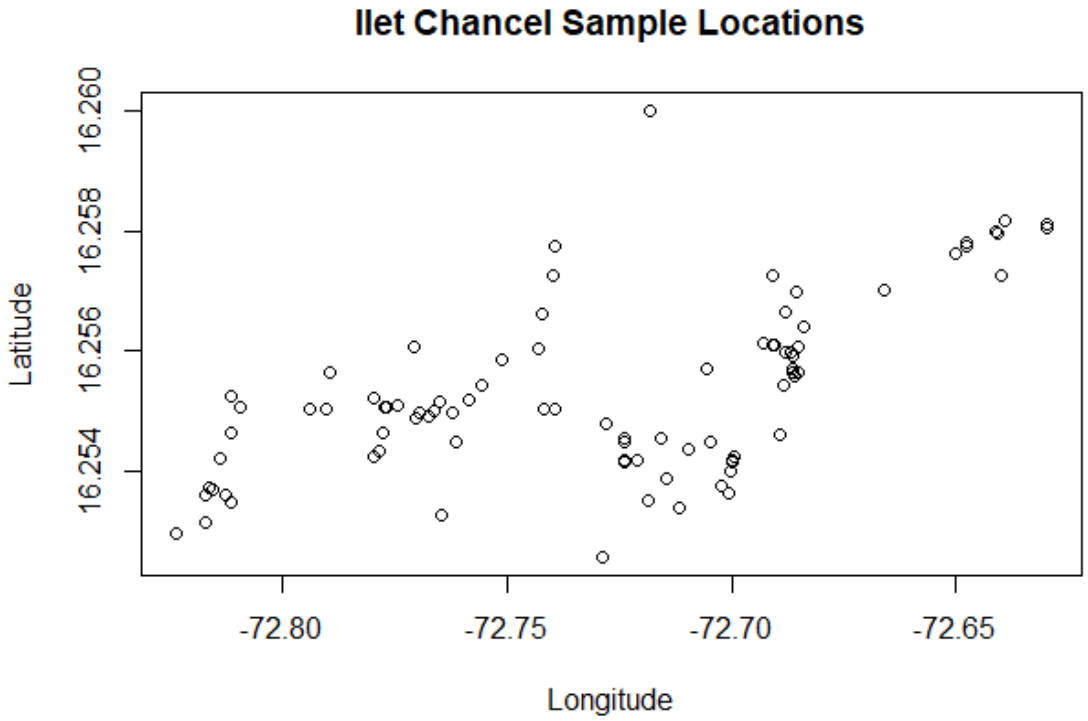

Figure 1. Plot of GPS coordinates of Îlet Chancel individuals. This plot was used to assign individuals to East and West subpopulations for analysis in GenAlEx and Structure.

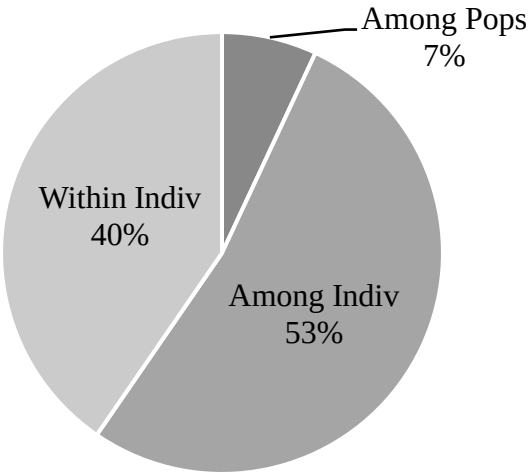

Figure 2. Percentages of Molecular Variance (AMOVA). The AMOVA was conducted with the 14 loci from Table 1 and the 117 *I. delicatissima* samples from Îlet Chancel with individuals separated into East or West subpopulations according to GPS coordinates. There is very little variation amongst this population (i.e., 7% molecular variation among populations), and the majority of the molecular variation (i.e., 53% among individuals) is due to the differences in allele sizes between the various Îlet Chancel samples.

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