TESTING FOR ISOLATION IN FLORIDA RACCOONS (*PROCYON LOTOR*) USING PHYLOGENETICS AND POPULATION GENETIC STRUCTURE

by

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ABSTRACT

Molecular data are useful in determining if populations are isolated and for species delimitation. Researchers and managers currently recognize five subspecies of raccoons (*Procyon lotor*) in Florida, based largely on perceived geographic isolation due to the island ranges of four subspecies. In this study, I provide the first estimate of phylogenetic relationships and population divergences within Florida raccoons using a molecular dataset. I analyze the mitochondrial control region, cytochrome *b* gene, and eight nuclear microsatellite loci to test two hypotheses: 1) the five, morphologically and geographically-defined subspecies of raccoon in Florida represent genetically distinct populations and (2) due to differing range sizes and habitat variation between island and mainland subspecies, the four island populations should exhibit reduced levels of genetic diversity and smaller effective population sizes compared to the mainland population. My results indicate no evidence of historical differentiation between the subspecies, but suggest a recent restriction of gene flow among three clusters of raccoons. The three clusters do not correlate to traditional geographies for subspecies identification. I provide evidence of reduced genetic diversity in island populations of raccoons compared to their mainland counterparts. These data stress the importance of using multiple lines of evidence when naming taxa to avoid misinforming the taxonomy.

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INTRODUCTION

The occurrence of geographic barriers (e.g. bodies of water, mountain ranges, or canyons) may restrict gene flow and cause once-contiguous populations to become separated. Once populations have been separated, the restricted gene flow, over time, causes populations to diverge owing to genetic drift and potential differential selection in the separate habitats (Templeton 1981). These evolutionary processes often lead to physiological and morphological differences, and subsequent genetic differentiation, between the once panmictic populations. There are many different ways to identify these distinct populations (e.g. species, subspecies, distinct population segment, etc.) and all are useful and common in biology to determine levels of distinction whether used for taxonomy, management, systematics, or ecological studies. However, distinctness is often assumed without rigorous analyses or data collection. When populations appear to be morphologically or geographically distinct, isolation (and genetic distinction) is usually assumed and taxa are named based on that superficial distinction without verifying whether the nomenclature reflects the evolutionary history, thus the taxonomy is misinformed (Ryder 1986; Zink 2004).

There is a trend towards using genetic data to determine existence of evolutionary lineages, breeding populations, and genetic diversity to add support in determining taxon distinction. This is because problems arise when using only one line of evidence (e.g. morphology) to determine taxon differentiation and are exacerbated when researchers assume that historic taxonomy reflects separately evolving lineages. Indeed, it is now well established that morphological or geographic differentiation does not necessarily equate to patterns of genetic differentiation (e.g. Burbrink et al. 2000; Zink 2004; Degner et al. 2007). Therefore,

using multiple lines of evidence, including estimates of gene flow, play an important role in determining if populations are isolated and how taxa should be named.

Insular populations, in particular, are often assumed to represent distinct populations as a result of their apparent geographic isolation and, in many cases, morphological distinction. For example, multiple species of treeshrews on different islands in Indonesia were named as separate species or subspecies based on geographic isolation (Sargis et al. 2014). However, a subsequent rigorous morphological study used multivariate analyses of skull and hand measurements of these treeshrews to determine that the initial geographically defined taxonomy was not supported (Sargis et al. 2014). Supplementing geographic and morphological data with genetics can aid in strengthening evidence of isolation, especially when the initial study was based on superficial evidence for describing geographic and morphological distinction. Furness et al. (2010) compared mitochondrial data (mtDNA) to the geographic and morphological data used to define four island subspecies of common eider duck from the Shetland archipelago and found that the two data sets did not match and suggested that one of the named subspecies be classified within a different subspecies group. Additionally, a genetic study on morphologically recognized *Podarcis* lizards occurring in mainland Greece and adjacent islands examined a segment of the mtDNA cytochrome *b* gene and revealed that the subspecies are paraphyletic, i.e. the molecular data did not correspond to morphological data (Poulakakis et al. 2003). Indeed, genetic identification of discrete evolutionary lineages is important for aiding conservation efforts to maintain the evolutionary trajectory of isolated lineages, especially on islands where classification discrepancies often occur. Other studies have found confounding lines of evidence between molecular data and taxonomy of birds, and mammal species on islands (e.g. Robertson et al. 2011; Eldridge et al. 2014). These studies are just a few of the studies showing the

necessity of incorporating thorough analyses using multiple lines of evidence to determine whether or not assumed isolation actually exists before naming and describing new taxa.

In addition to the high occurrence of morphological and geographic differentiation between island and mainland populations, insular populations also tend to exhibit smaller population sizes, reduced levels of genetic diversity, and are at greater risk of extinction owing to this lack of diversity (Allendorf and Luikart 2007). Determining the levels of genetic diversity is important when comparing and contrasting island and mainland populations of a species because low genetic diversity may restrict the ability of populations to adapt and persist in altering environments (Frankham et al. 2010). For example, New Zealand populations of the tuatara exhibit low genetic diversity and small population sizes increasing their risk of extinction (Hay et al. 2003). Similarly, Eldridge et al. (1999) found reduced fitness of island populations of the black-footed rock-wallaby, compared to mainland populations, due to low levels of genetic variation.

Raccoons (*Procyon spp.*) provide an ideal study system in which to investigate questions related to taxonomic and phylogenetic congruence in accordance with island subspecies and their genetic diversity. There are more than 50 named types (i.e. species or subspecies) of raccoons ranging from Central Canada, across North and South America, to the southern Amazon, and the current taxonomy is not well-supported (Helgen and Wilson 2003). In particular, within the state of Florida, there are currently five recognized raccoon subspecies: *P. l. elucus* (mainland Florida raccoon; Bangs 1898), *P. l. inesperatus* (Matecumbe Bay raccoon; Nelson 1930), *P. l. auspicatus* (Key Vaca raccoon; Nelson 1930), *P. l. incautus* (Torch Key raccoon; Nelson 1930), and *P. l. marinus* (Ten Thousand Islands raccoon; Nelson 1930). Four of these subspecies reside exclusively on islands in south Florida (Figure 1). These island subspecies, described by Nelson

(1930), were delimited based on geographic and morphological characters such as skull shape, size, and pelage coloration (Table 1). Nelson (1930) also included average quantitative measurements to help delineate subspecies, such as: weight, total length, length of tail vertebrae, hind foot, skull, and condylobasal, zygomatic breadth, interorbital breadth, least width palatal shelf, and upper canine-molariform tooth row. However, there are a few problems associated with Nelson's (1930) delimitation method: 1) there is large overlap in the morphological characters that demarcate subspecies, 2) although some quantitative measurements are used, the morphological characteristics are largely subjective and qualitative, 3) sample sizes used by Nelson (1930) to differentiate subspecies were small and inconsistent for both quantitative (e.g. four to eight specimens) and qualitative (e.g. 12 to 20) characteristics, and 4) geographic isolation is used to assume reproductive isolation. Lazell Jr. (1989) attempted to replicate Nelson's (1930) morphological measurements, but obtained contradictory results and suggested that there were only three subspecies in Florida: *P. l. elucus* (the mainland raccoon), *P. l. marinus* (the Ten Thousand Islands raccoon), and *P. l. auspicatus* (the Key raccoon). Additionally, Zeveloff (2002) stated that *P. l. marinus* range not only includes the Ten Thousand Islands, but part of the mainland ranging from Cape Sable northwest to the southwest edge of Lake Okeechobee.

The overlap in morphological characters between subspecies makes it challenging to differentiate between subspecies if geographic location of the sample is unknown (Lotze and Anderson 1979), and the inconsistency of morphological studies illuminates the need to find alternative, independent lines of evidence in which to base differentiation. This is especially important given that studies have suggested eradication of raccoons in specific areas where they may have negative impacts on endangered species (e.g. Sea turtles and Lower Keys marsh

rabbits) (Garmestani and Percival 2005; Schmidt et al. 2010). Genetic data have been useful in raccoons for identifying patterns of gene flow and differentiation (e.g. Cullingham et al. 2008; Cullingham et al. 2009; Dharmarajan et al. 2009; Castillo et al. 2010; Santonastaso et al. 2012; Kyle et al. 2014). Most of these studies are focused on the spread of the rabies disease. However, four of the studies used genetic markers to help understand a general pattern of population substructure. Cullingham et al. (2008) used mtDNA and tested for genetic evidence of four named subspecies, but the data only supported evidence for three subspecies and the authors suggested the use of only two names (*P. l. elucus* and *P. l. lotor*) to describe the subspecies they examined. Three more recent studies used microsatellite markers to assess structure and found evidence of two genetic clusters within different sampled regions located in the eastern US (Cullingham et al. 2009; Santonastaso et al. 2012; Kyle et al. 2014). A fourth microsatellite study tested 29 different neighborhoods in Chicago and found no evidence for structuring (Dharmarajan et al. 2009) across this microgeographic scale. Despite efforts to clarify the relationships among raccoon subspecies, no genetic work has been conducted with regard to Florida subspecies of raccoon.

In this study, I used genetic data to elucidate if Nelson's (1930) nomenclature of Florida raccoons is congruent with their evolutionary history and contemporary genetic structure. I used the mtDNA control region (CR), cytochrome *b* (cyt *b*) gene, and variation present in eight nuclear microsatellite loci to test two hypotheses pertaining to raccoon evolutionary history. First, if the seawater isolating these islands acts as a barrier to gene flow for raccoons, then Nelson's (1930) naming should correctly reflect raccoon evolutionary history and we should find reciprocal monophyly of mtDNA and genetic differentiation of microsatellite markers between all five Florida raccoon subspecies. However, I predict that mtDNA and microsatellite data will

show incongruences with the current naming. Support for this prediction is based on the preponderance of evidence that suggests that raccoons exhibit both substantial natural and artificial gene flow. Raccoons have high natural dispersal abilities (Helgen et al. 2008) and have been documented swimming across more than 500 meters of seawater (Lazell Jr. 1989). Furthermore, raccoons have home ranges varying from 5.1 ha (Lotze and Anderson 1979) to 49 ha (Urban 1970), depending on population density and openness of the habitat (Prange et al. 2003). Additionally, artificial gene flow has been documented via human-aided translocations, especially for hunting (Lotze and Anderson 1979; Kennedy and Lindsay 1984). My second hypothesis is that if the sample sites on islands follow the typical island trend, then they will exhibit reduced levels of genetic diversity and lower effective population sizes compared to mainland sites. I predict that this trend will be demonstrated regardless of whether island sites comprise unique subspecies. This prediction is supported by studies of different taxa that have shown lower levels of genetic diversity and effective population sizes in island populations versus their mainland counterparts (e.g. Hay et al. 2003; Boessenkool et al. 2007; White and Searle 2007). In particular, other studies of Keys endemic taxa show reduced diversity and effective population size relative to mainland sister taxa (Ellsworth et al. 1994; Tursi et al. 2012). Finally, I discuss these results in light of general patterns of discordance between taxonomy and evolutionary history and the implications of such discordance with regard to population management.

METHODS

Sampling

In order to test whether current raccoon nomenclature is congruent with the molecular phylogeny, I obtained a total of 173 samples from eight sampling localities throughout mainland Florida and the Florida Keys to represent the five currently named subspecies of raccoons that occur in Florida, USA (Figure 1). Sample collection was completed haphazardly throughout the range of each subspecies with the help of pest control companies, parks, and taxidermists throughout Florida who collected samples via live-trapping or the collection of road-kills (by taking either hair samples or ear clips) and storing them in tubes filled with Drie-rite™ desiccant, as a preservative. In the Lower Keys (Big Pine Key to Key West), I collected a total of 23 samples representing the putative subspecies of *P. l. incautus*. Additionally, I acquired five samples from the Middle Keys (*P. l. auspicatus*), 24 from Key Biscayne, and 18 from throughout the remainder of the Upper Keys (collected from Key Largo to Lower Matecumbe Key), both of which represent *P. l. inesperatus*, and 13 samples from Ten Thousand Islands (*P. l. marinus*). Samples of the putative mainland subspecies, *P. l. elucus*, included 84 samples from three mainland sites (Miami=35, Central Florida=24, Tampa=25) and 6 singleton samples scattered throughout mainland Florida. For the sake of this study, I based subspecies classifications on geography, since I could not adequately evaluate morphologies from roadkill, hair, or tissue samples.

Genetic Data Collection

All tissue samples were extracted using either the Qiagen DNeasy Blood and Tissue kit or a Serapure Bead (Rohland and Reich 2012) extraction method. I extracted DNA from hair follicles using six to eleven follicles with the Qiagen DNeasy Blood and Tissue kit following modifications suggested by Tursi et al. (2012) in which the samples were placed in a water bath for 4 – 4.5 hours to ensure a maximum yield of DNA without degradation (due to the low DNA yield of hair follicles) and I eluted each sample twice with 50µL of water heated to 70°C.

To evaluate the evolutionary history of Florida raccoons, I amplified two mitochondrial genes, control region (CR) and cytochrome *b* (cyt *b*), by conducting polymerase chain reactions (PCRs) with DNA from each individual. For all individuals, I used the forward primer L15997 (Ward et al. 1991) and the reverse primer H00651 (Kocher et al. 1989) to achieve full coverage of the CR (~1300bp). I performed PCR amplifications for the CR in a 20 µL reaction using 1 µL of 5-50ng/µL sample DNA, 2 µL of 10x PCR buffer, 1.6 µL of 25mM $MgCl_2$, 1.6 µL of 10 mM of dNTPs, 1 µL of 10 µM of each primer, and 0.2 µL of Taq DNA polymerase. Amplifications proceeded as follows: initial denaturation of 95°C for 5 min., 30 cycles of 94°C for 30 s, annealing at 60°C for 30 s, and 72°C for 30 s, followed by a final extension period at 72°C for 2 min and then held at 10°C (Cullingham et al. 2008). Likely the result of DNA degradation, some samples failed to amplify well with the L15997/H00651 primer pair. To compensate for degradation of certain samples, I replaced the original reverse primer with an internal reverse primer, PLO-CRL1 (Cullingham et al. 2008), using the same PCR protocol as above, to supplement sequence data. For cyt *b* amplification, I used the primers MTCB-F and MTCB-R, which were designed for mammals and previously tested on *P. lotor* (Naidu et al. 2012). I

performed cyt *b* PCR amplifications in a 20 µL reaction using 1 µL of 5-50ng/µL sample DNA, 2 µL of 10x PCR buffer, 2 µL of 25mM $MgCl_2$, 1.6 µL of 10 mM of dNTPs, 0.2 µL of DMSO, 1 µL of 10 µM of each primer, and 0.2 µL of Taq DNA polymerase with and initial denaturation step of 95°C for 10 min., 35 cycles of 95°C for 45 s, annealing between 57°C and 53°C for 1 min, and 72°C for 2 min, followed by a final extension period at 72°C for 10 min and then held at 10°C (Naidu et al. 2012). Once I ran the mitochondrial PCR products on an agarose gel to test for accurate amplification I cleaned them either using the shrimp alkaline phosphatase and exonuclease I (i.e. Exo-SAP purification) method or they were cleaned at the University of Arizona Genetics Core (UAGC) or at Eurofins Genomics when sent for sequencing. I edited CR and cyt *b* sequences in Sequencher v5.1 (Gene Codes Inc., Ann Arbor, MI, USA) and aligned them using the ClustalW method in MEGA6 (Tamura et al. 2011).

I also amplified the DNA from each individual at eight microsatellite loci (PLO-M15, PLO-M17, PLO-M2, PLO-M20, PLO2-117, PLO2-14, PLO-M3, PLO3-86) developed and optimized for *P. lotor* by Cullingham et al. (2006). I performed microsatellite PCR amplifications in a 15 µL reaction using 1.5 µL of 5-50ng/µL sample DNA, 1.5 µL of 10x PCR buffer, 0.975 µL of 25mM $MgCl_2$, 1.2 µL of 10 mM of dNTPs, 0.75 µL of florescent dye, 0.375 µL of 10 µM of each primer, and 0.15 µL of Taq polymerase, edited from Cullingham et al. (2006) with an initial denaturation step of 95°C for 5 min., 30 cycles of 95°C for 30 s, annealing between 60°C and 55°C for 1 min, and 72°C for 1 min, followed by a final extension period at 72°C for 7 min and then held at 10°C. I ran the PCR product on an agarose gel to test for accurate amplification and subsequently sent the PCR product to UAGC for genotyping. Genotypes were scored in GeneMarker v2.6.3 (SoftGenetics, LLC).

Statistical Analyses

Phylogenetic Reconstruction

Due to the hypervariable nature of the CR and the inability to estimate homology within this region, a 450bp fragment was discarded from analysis. Since cyt *b* is a coding gene, I confirmed that the sequences did not contain any stop codons, which could indicate the amplification of a pseudogene, by translating sequences to amino acids. After concatenating the trimmed CR and cyt *b* sequences, I eliminated individuals from downstream analyses that did not have sequence for both genes, which left me with 108 full sequences. I ran Partition Finder with unique haplotypes (Lanfear et al. 2012) to determine which partitioning scheme and models of evolution would be the most informative to create a phylogeny. The four partitions are defined as: the CR, and each codon position of cyt *b*. To create a graphical depiction of the evolutionary relationships of Florida raccoons, I constructed a Bayesian phylogeny of my Florida raccoon samples with MrBayes v3.2.2 (Ronquist et al. 2012), using each unique haplotype only once and including two GenBank *P. lotor* samples (accession numbers: AB291073 and AB297804) from outside of Florida as outgroups. Conditions for MrBayes included two independent runs of 5×10^6 generations with the first 10,000 trees discarded as burn-in. I also analyzed the MrBayes output data in Tracer v1.5 (Clement et al. 2000) to confirm stationarity and sufficient sampling of the posterior. Finally, I used TCS v1.21 (Clement et al. 2000) to build a haplotype network to determine the relationships among haplotypes that may be too similar to exhibit strong nodal support within the phylogenetic reconstruction with MrBayes.

Genetic differentiation and gene flow

To determine whether the eight microsatellite markers conform to the expectations of neutral markers, I calculated deviation from Hardy-Weinberg Equilibrium (HWE) using Fischer Exact Tests in the program GenePop v4.2.1 (Rousset 2008) with a sequential Bonferroni correction (Rice 1989) to account for multiple comparisons. Given that the data indicated no consistent patterns deviating from HWE (see Results), I used all sample sites and all loci in downstream analyses.

In order to evaluate whether Nelson's (1930) subspecies represent distinct genetic clusters, I used GenePop to estimate global F_{ST} as well as pairwise F_{ST} values among all sample sites and used STRUCTURE v2.3.4 (Pritchard et al. 2000), a Bayesian-based clustering method for multilocus data, to determine the number of clusters (K) supported by the data. I completed 10 runs for each K value from 1 to 11 with a burn-in period of 100000 iterations. To determine the number of clusters for all STRUCTURE runs, I used Structure Harvester (Earl and vonHoldt 2012). I also tested for substructure within each population using STRUCTURE to identify if additional clusters could be identified within the clusters identified from the initial screen (Degner et al. 2010).

I tested for a pattern of isolation by distance (IBD) using the genetic distances/similarities function in the isolation by distance web service v3.23 (Jensen et al. 2005), which uses Mantel tests with 10,000 randomizations, to determine if limited dispersal across space was detected. Due to the non-linear arrangement of sample sites in this study, I used log-transformed geographic distances for this correlation analysis. Additionally, in order to determine whether my modified regional groupings better described the genetic structure than groupings identified by

Nelson (1930), I conducted an analysis of molecular variance (AMOVA; Excoffier et al. 1992) in GenAlEx v6.5 (Peakall and Smouse 2006, 2012) using two different groupings (i.e. *a priori* and *a posteriori*; see Results). Furthermore, I ran BayesAss v3 (Wilson and Rannala 2003), which uses Markov chain Monte Carlo resampling techniques, with 3,000,000 iterations at a sampling frequency of 2,000 and a burn-in of 999,999, to estimate recent migration rates between all pairs of sample sites.

Genetic diversity and effective population size

In order to evaluate whether levels of genetic diversity and effective population size on island sample sites were lower than sites on the mainland, I estimated nucleotide diversity (π) and gene diversity (h) of mtDNA variation, allelic richness (AR) and expected heterozygosity (H_E) of microsatellite variation, and effective population size (N_e), for each sample site. I determined π and h using the concatenated cyt *b* and CR dataset in DnaSP v5.10 (Librado and Rozas 2009) and I calculated AR and H_E with Fstat v1.2 (Goudet 1995). I performed Welch's t-tests in R (R Core Team 2013), for both π and h, to identify significant differences between mainland and island geographic sites of mtDNA diversity. For differences in microsatellite genetic diversity between mainland and island sites, I ran a two-way analysis of variance (ANOVA) in R (R Core Team 2013). Additionally, I estimated effective population size for each sample site using ONeSAMP (Tallmon et al. 2008) with priors for effective population size set from 2 (minimum) to 500 (maximum). I then compared the 95% confidence intervals between each site to determine whether significant differences were detected in effective population size between mainland and island populations.

RESULTS

Phylogenetic Reconstruction

My phylogenetic analysis included a total of 1991 base pairs (bp), consisting of 851 bp of trimmed CR and the complete cyt *b* gene (1140 bp) sequenced for 108 individuals throughout Florida (*P. l. elucus* = 52, *P. l. marinus* = 12, *P. l. inesperatus* = 24, *P. l. auspicatus* = 5, and *P. l. incautus* = 15). From these 108 samples, I identified 37 unique haplotypes defined by 64 variable sites, 36 of which were parsimony informative (Table 2). I created the final phylogenetic tree with the maximum partition setup (i.e. 4 partitions: CR and each codon position of cyt *b*) and the best model of DNA evolution for each partition was: HKY+I+G, K80+I, F81, and HKY, respectively (Kimura 1980; Felsenstein 1981; Hasegawa et al. 1985). Well-supported clades (> 95% posterior probability) in the phylogenetic tree uncovered paraphyly of each subspecies except *P. l. marinus* (Figure 2), refuting the hypothesis that the subspecies named by Nelson (1930) represent monophyletic clades.

In order to build the 95% statistical parsimony haplotype network, I removed a total of 15 bp from the ends of sequence fragments to create equal fragment sizes for all samples. In correspondence with the phylogenetic tree, the haplotype network (Figure 3) did not reveal any support for distinct haplogroups differentiating the subspecies defined by Nelson (1930), except possibly *P. l. marinus*. Haplotypes 14 and 15 are the only haplotypes found in Ten Thousand Islands (*P. l. marinus*) and these haplotypes are not shared among any other subspecies. Moreover, these two haplotypes are separated by a single mutation and are four mutations from the next most closely related haplotype (H13). In addition to a lack of monophyly among subspecies, many haplotypes are shared between pairs of described subspecies. H13 is a shared

haplotype between *P. l. incautus* (Lower Keys) and *P. l. auspicatus* (Middle Keys). H1 is shared between *P. l. elucus* (Miami) and *P. l. incautus* (Lower Keys), and H6 is shared between *P. l. inesperatus* (Upper Keys) and *P. l. incautus* (Lower Keys). Due to H36 being too different (31 steps), it did not fall within the 95% probability limit achieved at 19 steps and fewer. Therefore, haplotype H36 (putative *P. l. elucus* collected in Central Florida) was not included in Figure 3.

Genetic differentiation and gene flow

I successfully genotyped 168/173 samples for all eight microsatellite loci. Fifty-five of 56 locus-sample site comparisons conformed to HWE expectations after a Bonferroni correction. The one comparison that was out of HWE equilibrium was Miami at PLO-M17 ($p = 0.0002$). However, since there was no overall pattern of locus by sample site out of HWE, all loci and sites were included in downstream analyses. With all sample sites included, we found that the Bayesian algorithm in STRUCTURE identified $K = 3$ as the highest level of genetic structure. The three regions of genetic structure did not reveal a split between the mainland and island sample sites or the five putative subspecies. Instead, they support a mainland Florida (including Ten Thousand Islands) population, a Florida Keys (excluding Key Biscayne) population, and a Key Biscayne population (Figure 6). Further STRUCTURE assessment within each of these three genetic clusters resulted in a $K = 1$ for all analyses, indicating no evidence of additional substructure. It is important to note that since I had a sample size of five for the Middle Keys, these individuals were grouped with Upper Keys (excluding Key Biscayne) for further microsatellite analyses. This grouping was informed by the findings from STRUCTURE. Overall, with regard to differentiation among sample sites, global F_{ST} was moderate (0.066)

among all sites. Pairwise F_{ST} values were significant and ranged from 0.009 (between Central Florida and Miami) to 0.15 (between Key Biscayne and Middle/Upper Keys). Interestingly, Key Biscayne had moderate to high F_{ST} values between all sample sites, ranging from 0.09 to 0.15 (Table 3).

Additionally, I found no evidence of a correlation between genetic distance and geographic distances (IBD) among all sample sites (r = -0.1977, p = 0.7545). However, I did find that the modified populations (i.e. clusters) defined by STRUCTURE better described the pattern of genetic structuring found in Florida. Here, two AMOVA's were run using *a priori* groups (defined by Nelson (1930)) and *a posteriori* groups (as defined by the STRUCTURE analysis of this study). The highest amount of genetic variation for both runs was found within sample sites (Table 4). However, variance among regions increased from 0% to 5% variance explained after differentiating the sites to match the three clusters that STRUCTURE demonstrated (i.e. *a posteriori* grouping), showing that the genetic regions identified by this study better explained patterns of isolation (Table 4). Migration rates calculated from BayesASS v3 tended to show high assignment back to home sites (0.6795 – 0.9255) and low assignment between sample sites. Two exceptions to this pattern suggested evidence for migration from Miami to three different locations (Tampa, Central Florida, and Ten Thousand Islands) and from the Middle/Upper Keys to the Lower Keys (Table 5) confirming the general contemporary patterns of genetic differentiation uncovered by STRUCTURE.

Genetic diversity and effective population size

I tested for statistically significant differences in genetic diversity between mainland and island sites, representative of the four island sample sites (e.g. Ten Thousand Islands, Key Biscayne, Middle/Upper Keys, and Lower Keys) versus the three mainland sample sites (Tampa, Central FL, and Miami). Overall, average nucleotide diversity (π) was 0.00372 in the mainland geographic sites as compared to the island sites that averaged 0.00138 (Table 2). The average estimates for gene diversity (*h*) between mainland and island sites were 0.863 and 0.405, respectively (Table 2). However, the results from running the Welch's t-test demonstrate that π was not significantly different between mainland and island sites (t = -1.5451, df = 3.765, p = 0.2016), whereas *h* was significantly greater in the mainland sites than the island sites (t = -3.899, df = 4.059, p = 0.0171). I estimated average allelic richness (*AR*) of microsatellites in mainland geographic sites at 8.768 and in islands at 7.298 (Table 2). Additionally, expected heterozygosity (H_E) in mainland averaged 0.84 and island geographic sites averaged 0.78. The two-way ANOVA results indicated that mainland genetic diversity is significantly higher than island diversity for both *AR* and H_E (*AR*: p = 5.55e-05, Figure 4; H_E: p = 0.00586, Figure 5). Finally, effective population size estimates between island and mainland geographic sites exhibited no significant differences based on pairwise comparisons of the 95% confidence intervals.

DISCUSSION

Researchers commonly assign subspecies names to morphological variants within species, especially when these differentiated morphological populations occur in unique locations, such as islands. In this study I employed genetic analyses to evaluate evolutionary histories, patterns of differentiation, and genetic diversity in the mainland Florida raccoon (*P. l. elucus*) and its four island sister subspecies (*P. l. marinus*, *P. l. inesperatus*, *P. l. auspicatus*, and *P. l. incautus*) to evaluate whether the current nomenclature (described in 1930) corresponds to the evolutionary history of these raccoons. This study provides evidence for the discordance between earlier subspecies designations based on morphology and geography and the evolutionary history elucidated here. In accordance with my predictions, my results do not demonstrate genetic support for Nelson's (1930) taxonomy, but do loosely support the typical island trend in which island sites display reduced levels of genetic diversity and small effective population sizes. Overall, these data shed new light on the evolutionary history of *P. lotor* subspecies, and the consequences of incongruences between taxonomy and phylogeny.

Contemporary genetic structure

I found three genetic patterns that would have been undetectable without the genetic analyses employed by this study: evidence of distinct groups, the presence of recent gene flow between the mainland and island sites, and evidence of long-distance dispersal. First, I found molecular evidence for two genetically distinct island groups using microsatellite data: Key Biscayne and all other Florida Keys. Surprisingly, the Key Biscayne sample site is a single distinct population. This Key Biscayne population displayed the highest amount of pairwise

differentiation (F_{ST} = 0.09 to 0.15), especially compared to the Upper Keys sample site (F_{ST} = 0.15) of the same named subspecies (*P. l. inesperatus*). In contrast, the remaining Keys all grouped together as a single cluster. These data suggest that high gene flow exists throughout the Keys (excluding Key Biscayne) and that there is restricted gene flow between all Keys and the mainland. Initially, this pattern of high gene flow within the Keys, but restriction of gene flow to and from Key Biscayne, seems inconsistent with their geographic arrangement in that both regions (Keys and Key Biscayne) are separated from the mainland by seawater and connected only by bridges with abundant traffic. However, artificial connectivity may help to explain some of the connectivity between the Key Largo and Key West. In 1912, a railway was built to enable easy transportation spanning the Keys (excluding Key Biscayne). This railway stopped functioning in 1935, when parts of it were destroyed by hurricanes, but the structure has remained largely intact (Wilkinson 2011). Interestingly, raccoons have been seen travelling on the deserted railway (personal observation). Additionally, the current overseas highway, was completed in 1938 creating a second (albeit more dangerous) route between islands. In contrast, there is only a single route connecting Key Biscayne to the mainland and this bridge opened for travel in 1947. Overall, the connectivity from mainland to Key Biscayne appears more difficult for raccoon travel compared to connectivity throughout the rest of the Keys and may contribute to the contemporary pattern of high gene flow within the Keys and restricted gene flow to and from the mainland.

Second, in contrast to the patterns described above, the presence of contemporary gene flow between Ten Thousand Islands and all mainland sample sites is surprising if seawater provides a barrier to gene flow. The high gene flow between the Ten Thousand Islands site and mainland sites begs the question of why Ten Thousand Islands exhibits a genetic pattern

different than the other island sites. Here, it is likely the natural formation of the islands that provides insight into the genetic patterns. The Ten Thousand Islands were formed by the build-up of peat and oyster beds over time (Hoffmeister 1974), whereas the Keys were formed during the glacial retreat coupled with rising sea level which isolated the islands from the mainland (Lazell Jr. 1989). These differences in origin cause alterations in how the islands are contemporaneously separated from the mainland. The Keys are disjoint, with about 3000 meters of seawater between mainland and Keys. In contrast, the Ten Thousand Islands are separated from the mainland by small waterways, which raccoons are likely able to cross (Lazell Jr. 1989).

The third interesting genetic pattern that I discovered was the presence of an individual, collected from Central Florida, that exhibited a haplotype (i.e. H36) that was more than twice as divergent (at 1.5% uncorrected sequence divergence) as the next most divergent haplotype (H12). In comparison with the haplotype groups of Cullingham (2008), this sample most closely grouped within Cullingham's lineage II, a lineage generally found in the Mid-Western United States. There are two likely explanations for the occurrence of this sample in central Florida: artificial translocation or natural long-distance dispersal. In finding a genetic outlier like H36, we need to consider that human interferences may obscure our interpretations of genetic data. Indeed, forced migrations have been documented, especially when raccoon hunting was a popular past-time (Lotze and Anderson 1979; Kennedy and Lindsay 1984) and even in recent years as raccoons are often seen as nuisance animals and are trapped and relocated. Alternatively, H36 could be a rare long-distance disperser. Natural long distance dispersal has been documented previously in raccoons, with individuals recorded as traveling over 200 km (Zeveloff 2002). Although we cannot rule out natural dispersal, it seems that the distance

traveled in this case (approximately 3000km to the Mid-Western United States) increases the likelihood that this individual was an artificial transplant.

Contemporary versus historic differentiation

Many studies utilize one type of molecular marker which may be problematic if there are differences between recent and historical patterns of divergence. Historical divergence may go undetected if using only microsatellite markers, because microsatellites tend to reveal contemporary patterns of gene flow. Whereas, contemporary patterns of gene flow may be undetectable when using only mtDNA, because mtDNA tends to uncover patterns of evolutionary history. For example, a phylogenetic study conducted on the subspecies status of mainland and island populations of the Indochinese box turtle, exclusively utilized mtDNA markers and found that the island population is not genetically divergent from the mainland populations of Laos and Vietnam (Stuart and Parham 2004). Although this study has strong evidence for historic gene flow, it is still possible that more contemporary patterns of genetic differentiation exist, but went undetected. The differences between mtDNA and microsatellite patterns of gene flow can be used to determine if populations exhibit historic versus contemporary genetic isolation, providing information on the degree of population divergence (Crandall et al. 2000).

By comparing and contrasting the data from different molecular markers, I was able to evaluate differences of contemporary versus historic patterns of gene flow. In the case of island populations, seawater may act as a barrier for dispersal, isolating these island populations from the mainland, therefore allowing genetic differentiation to accumulate over time. Given that it

typically takes about $4N_e$ generations to reach reciprocal monophyly (Neigel and Avise 1986), any populations that were completely isolated and have an N_e less than 1000 (assuming shortest time since isolation) should exhibit reciprocal monophyly. Since, the Florida Keys have been isolated from the mainland for about 6,000 to 10,000 years (Lazell Jr. 1989) and the Ten Thousand Islands formed about 4,000 years ago (Hoffmeister 1974; Randazzo and Jones 1997), I would expect a pattern of historic differentiation. Yet, all of the patterns of island isolation discussed in the previous section are based on contemporary estimates of gene flow (i.e. microsatellites). When we evaluate evolutionary history via mtDNA variation, the data tend towards a lack of monophyly between clades with haplotypes shared among subspecies, suggesting historic panmixia.

One exception to this pattern is the mtDNA structure found in Ten Thousand Islands. Despite evidence for recent gene flow connecting Ten Thousand Islands (*P. l. marinus*) to the mainland, mtDNA haplotypes from this site are not shared with other sites and form a monophyletic Ten Thousand Islands clade. This is interesting because no other sites display a pattern of historic isolation, even those that demonstrate contemporary divergence. Three possible explanations for the signal of historic isolation in Ten Thousand Islands exist. First, this site was historically isolated, leading to a monophyletic lineage present on this island group. However, in more contemporary time gene flow has increased causing Ten Thousand Islands to be more similar to the mainland. This explanation is not likely given that it would require one-way gene flow from Ten Thousand Islands to the mainland, as no mainland haplotypes occur on this island group. Second, genetic structure is influenced by sex-biased dispersal. A study on sex-biased dispersal in red deer revealed that estimates of population structure were eight times higher when using mtDNA as opposed to microsatellite markers, indicating male-biased

dispersal (Perez-Espona et al. 2010). Male raccoons disperse earlier (within year one) and further (18.9 km on average) than females who tend to be philopatric and remain in their natal areas (Zeveloff 2002), which is typical of mammals (Greenwood 1980). If a male raccoon travels to or from Ten Thousand Islands and reproduces, this will not be detected by mtDNA since mitochondria are maternally inherited. The problem with this explanation is that it does not address why other populations exhibit patterns of panmixia for mtDNA. Third, Ten Thousand Islands is actually isolated from the mainland. This explanation is similar to the first in that the Ten Thousand Islands were indeed isolated, but here my evidence of contemporary gene flow between mainland and Ten Thousand Islands is called into question. Specifically, since I was only able to genotype 13 individuals from Ten Thousand Islands, these individuals may be too few to be distinguished from the 80 mainland individuals in the substructure analysis. As with the previous explanations, this one also does not seem likely. Specifically, it seems unlikely that F_{ST} between Ten Thousand Islands and the other mainland sites would be so low (or migration rates so high) if Ten Thousand Islands was truly isolated. With regard to Crandall et al. (2000), the population divergence among all populations falls into "Case 8" – treat as a single population, so long as there is no evidence of recent ecological (or historical) divergence. However, if there is evidence of recent ecological divergence, this divergence changes the management recommendation for the mainland and Keys populations. For example, these populations would then fall under "Case 5" – treat as distinct populations, instead of "Case 8". In contrast, in order for the Ten Thousand Islands site to be deemed distinct, we would need to posit evidence for the lack of both recent genetic and ecological exchangeability. This would cause the Ten Thousand Islands population to be treated as a separate species ("Case 2"). I suggest that further research be conducted to obtain evidence to better understand whether sites exhibit

ecological exchangeability and whether greater sample sizes at Ten Thousand Islands would reveal a contemporary genetic pattern different than I observed.

Genetic diversity and effective population size

Species living in sympatry to the Keys population of raccoons have been found to exhibit reduced genetic diversity and lower effective population sizes compared to mainland counterparts. Genetic patterns of these species: Key deer (Villanova 2015), Lower Keys marsh rabbit (Tursi et al. 2012), and silver rice rat (Indorf and Gaines 2013), lead me to predict that I would find evidence to support this trend even though levels of genetic divergence of Florida raccoons do not support Nelson's (1930) current subspecies naming. The island sites do show the typical pattern of reduced genetic diversity, which may be a factor of founder effect or a population bottleneck (Mayr 1970; Maruyama and Fuerst 1985). Patterns of lower genetic diversity are usually coupled with smaller effective population sizes, but interestingly, my data does not show a trend of significantly lower N_E values on islands compared to mainland sites. Overall the effective population size estimates were low for all sites. One exception was the Miami site, which had high effective population size estimates and high unidirectional migration rates to Tampa (0.2114), Central Florida (0.2538), and Ten Thousand Islands (0.2032), suggesting that this site may be a source “population” of raccoons to the rest of mainland Florida.

Subspecific naming

Why is the naming of taxa so important? Scientists need to be cautious so they do not split species unnecessarily, thus wasting effort and funds on widespread and abundant taxa (e.g. American puma: Culver et al. 2000; willow flycatcher: Zink 2015) or lump species that are actually distinct and denying protection from taxa in need of support (e.g. Kemp's Ridley sea turtle: Bowen and Avise 1996).

Overall, the results of my study do not support the current subspecies naming of Florida raccoons based on historic and contemporary patterns of genetic structure. These data provide adequate evidence to suggest two revisions in the current taxonomy. First, the use of the Ten Thousand Islands raccoon subspecies (*P. l. marinus*) should be discontinued and synonymized with *P. l. elucus*, as there is no evidence of differentiation from the mainland. The Keys group (excluding Key Biscayne) should be synonymized to *P. l. auspicatus*, as suggested by Lazell Jr. (1989) in a study using blood protein analyses and supported by microsatellite data in this study. The Key Biscayne population must be further studied to determine whether it is distinct enough to warrant management of these genetic variants and should keep its current name (*P. l. inesperatus*) for the time being. This would leave Florida with three raccoon subspecies: the Florida raccoon (*P. l. elucus*), the Keys raccoon (*P. l. auspicatus*), and the Key Biscayne raccoon (*P. l. inesperatus*). While my genetic data do not support Nelson's (1930) taxonomy, the evidence for revised taxonomy would benefit from a thorough morphological assessment since Nelson's (1930) morphological accounts overlap in their descriptions and have been met with difficulty when other researchers have tried to reanalyze them.

Similar studies are being used to revise taxonomies, across multiple taxa, using genetic information to guide the accuracy of naming. For example, Burbrink et al. (2000) also called for a taxonomic revision in light of the evolutionary history they uncovered in a study of North American rat snakes in which the named subspecies were mixed throughout the phylogeny and did not exhibit reciprocal monophyly. Moreover, islands appear especially problematic owing to the geographic isolation they exhibit relative to mainland taxa. A recent study used genomic scans of flightless Caribbean crickets across the Virgin Islands and showed population level divergences between island populations without patterns of long-term isolation, and suggested that all populations be considered the same species (Papadopoulou and Knowles 2015). On the contrary, not all studies find incongruences. For example, another island study found taxonomy and phylogeny congruence by illustrating that the evolutionary histories corroborated the species status of Philippine forest mice using nuclear and mitochondrial genes because they grouped into well-supported monophyletic clades (Justiniano et al. 2015).

Conservation Implications

Identifying differentiation among island populations enables resource managers to make informed decisions with regard to controlling nuisance populations of *P. lotor*. Zeveloff (2002) stated that it is extremely difficult to devise conservation plans when there is uncertainty about the classification of the focal species. Here, our focal species (*P. lotor*) is not endangered, but rather is considered a pest that may have direct negative impacts on sympatric endangered species in different areas of their range. They are known to raid sea turtle nests for the consumption of eggs, and a recent study also found that Torch Key raccoons (*P. l. incautus*) are a

threat to the Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*) by limiting their persistence and recovery due to predation (Schmidt et al. 2010). Based on these data, it has been suggested that managers should consider removal or eradication of *P. lotor* from turtle nesting beaches (Garmestani and Percival 2005) and even the entire Lower Keys (Schmidt et al. 2010). According to my data, the Ten Thousand Islands site is part of the mainland population and can be managed as such. Sea turtles are not known to nest on Key Biscayne, so this population does not pose a major issue with regard to the conservation of sea turtles. Lastly, although the Lower Keys site is grouped with the rest of the Keys sites, they are different from other Florida populations and should not be completely eradicated, but do not require special protection.

APPENDIX A: FIGURES

Figure 1. Map of eight sample localities in Florida, including six scattered singleton samples (S1-S6), and the putative subspecies names for each location. Numbers in parentheses indicate number of samples per site.

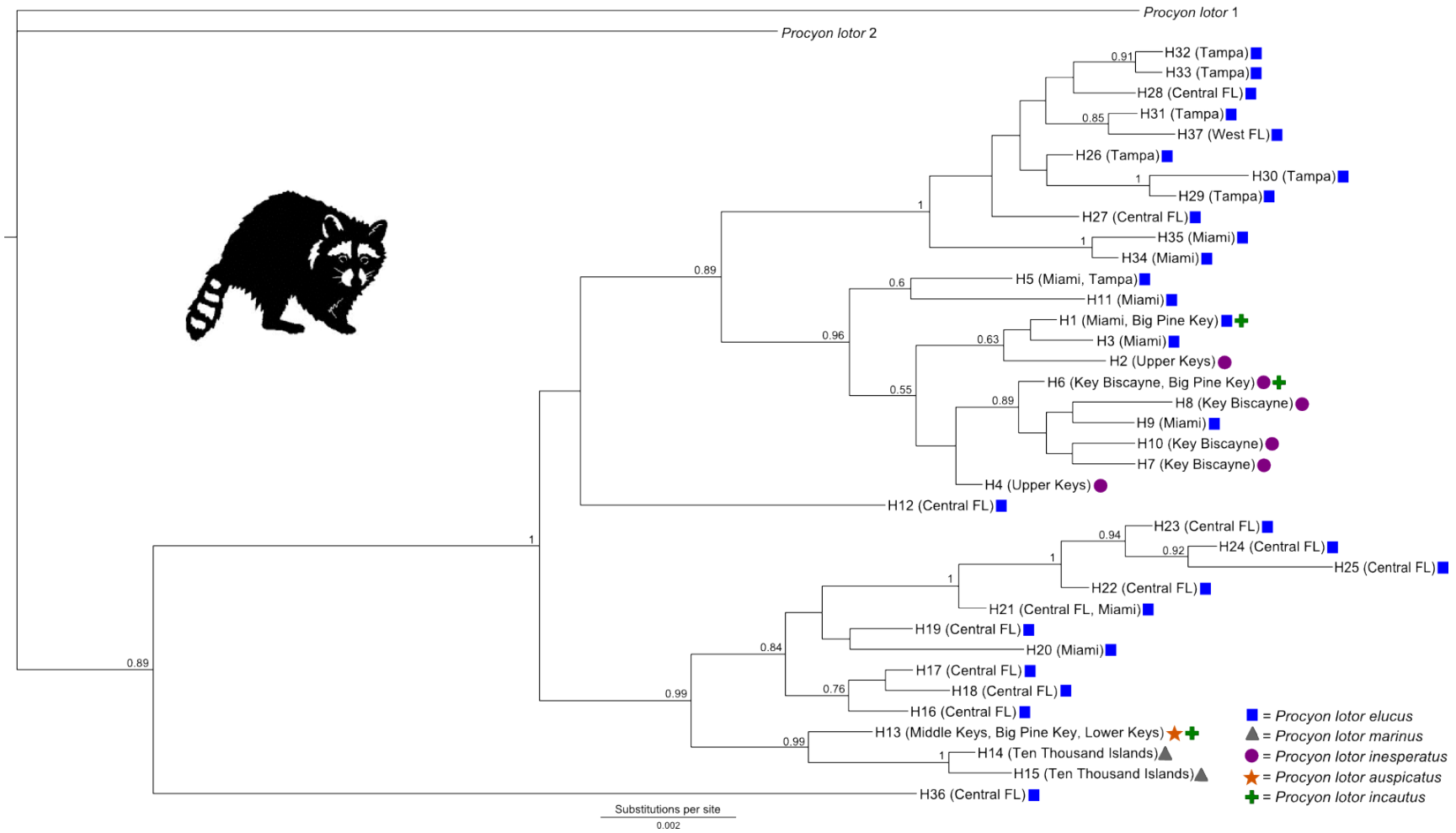

Figure 2. Concatenated mitochondrial control region and cytochrome *b* haplotype phylogeny generated in MrBayes v3.2.2, with node posterior probabilities (*Pp*) ≥ 0.5. Haplotype labels correspond to haplotypes in Figure 3.

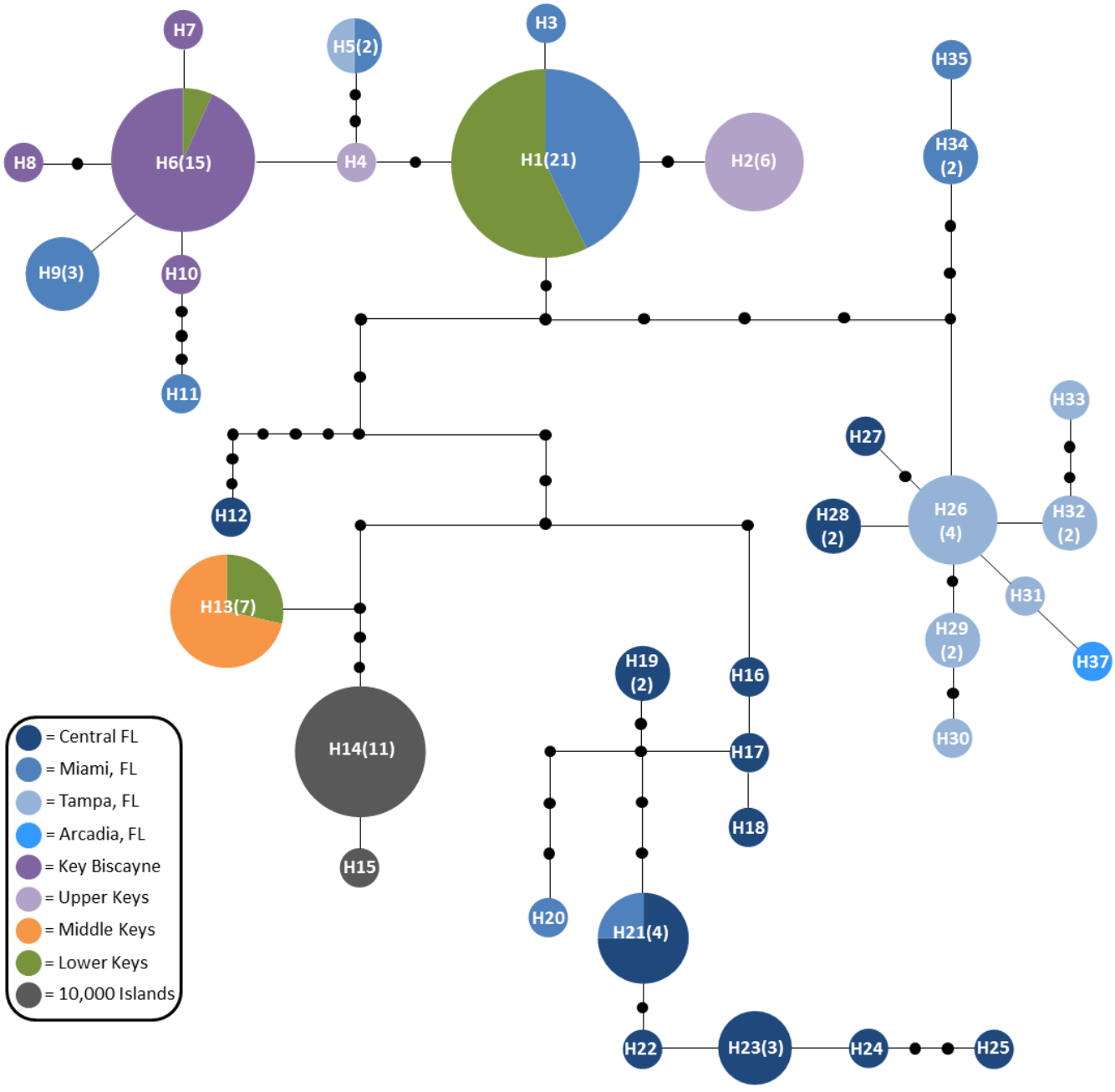

Figure 3. 95% Parsimony network of concatenated mitochondrial control region and cytochrome *b* haplotypes generated in TCS v1.21. Circles represent distinct haplotypes; pie sizes and numbers in parentheses indicate the number of samples with that haplotype, no number indicates one individual; and colors represent subspecies: blue = *P. l. elucus*, purple = *P. l. inesperatus*, orange = *P. l. auspicatus*, green = *P. l. incautus*, and gray = *P. l. marinus*.

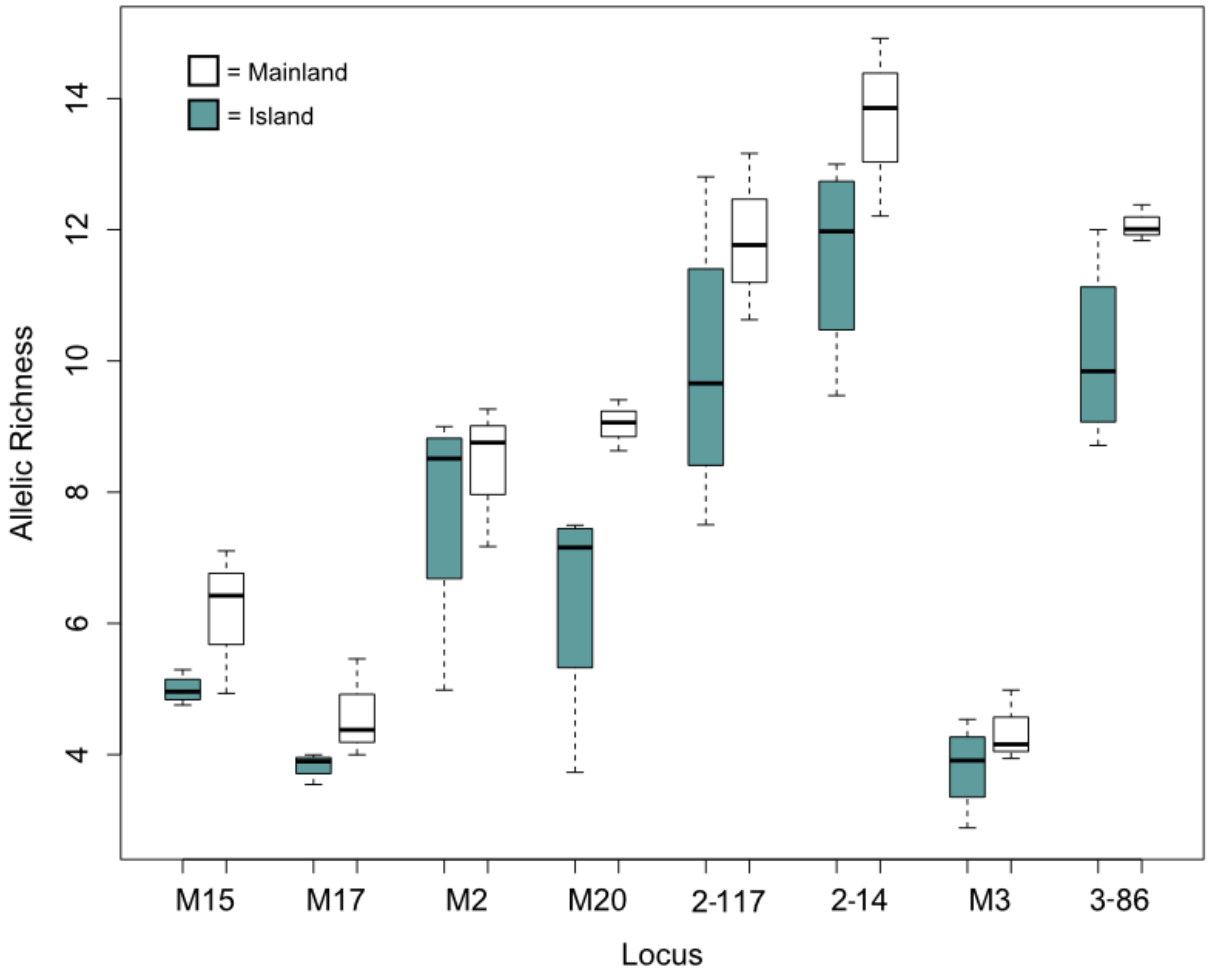

Figure 4. Two-way ANOVA boxplot of allelic richness comparing levels of genetic diversity in mainland and island geographic sites for all eight microsatellite loci. Prefix “PLO” was removed from the locus names.

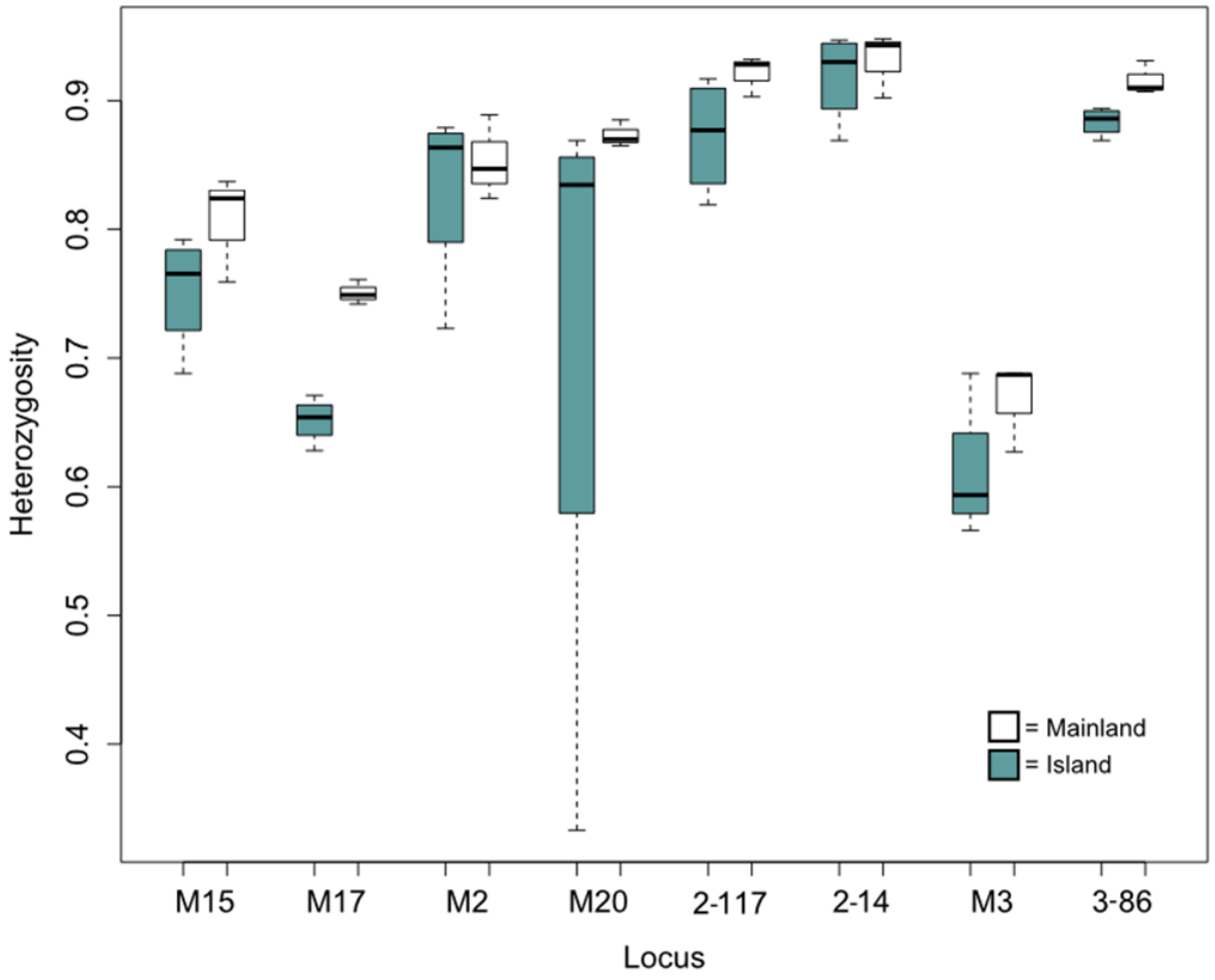

Figure 5. Two-way ANOVA boxplot of heterozygosity to compare levels of genetic diversity in mainland and island geographic sites for all eight microsatellite loci. Prefix “PLO” was removed from the locus names.

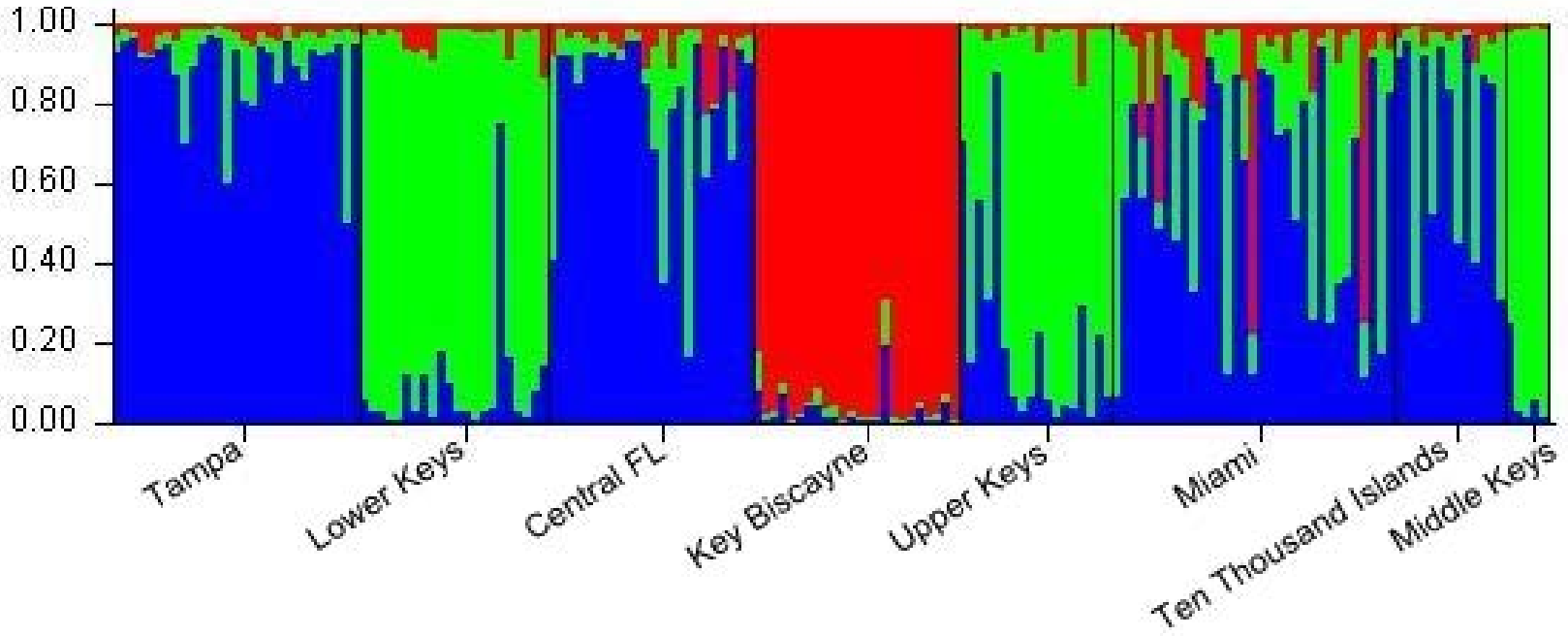

Figure 6. Output of the STRUCTURE analysis with K=3. The output shows structure between the Keys (green), Key Biscayne (red), and mainland (blue), with no structure between Ten Thousand Islands and the mainland.

APPENDIX B: TABLES

Table 1. Summary of the ranges and morphological characters of the Florida subspecies of *Procyon lotor*.

Subspecies Classification	Common Name	Range	Morphological Traits	Citation
Procyon lotor elucus	Mainland Florida Raccoon	Peninsular Florida and extreme southern Georgia	Medium sized, dark colored	Bangs (1898)
Procyon lotor marinus	Ten Thousand Islands Raccoon	Ten Thousand Islands group (South of Naples to Shark River	Small, more restricted mask, depressed frontal skull	Nelson (1930)
Procyon lotor inesperatus	Matacumbe Bay Raccoon	Upper Keys group (Virginia Key to Lower Matecumbe Key)	Small, grey, more restricted mask, small hind foot, depressed frontal skull	Nelson (1930)
Procyon lotor auspicatus	Key Vaca Raccoon	Middle Keys group (Long Key to Knights Key)	Very small, pale, depressed frontal skull	Nelson (1930)
Procyon lotor incautus	Torch Key Raccoon	Lower Keys group (No Name Key to Key West)	Small, palest, smaller molariform teeth, elevated frontal skull, greater interorbital compression	Nelson (1930)

Table 2. Basic diversity statistics and effective population size estimates of mainland and island geographic sites of *Procyon lotor* in Florida. Basic diversity statistics shows number of individuals used (*n*), number of haplotypes, number of segregating sites, nucleotide diversity (π), haplotype diversity (*h*), allelic richness (*AR*), heterozygosity (H_E), and standard deviation (SD) for each estimate. Effective population size (N_e) results show estimates based on approximate Bayesian computation using a max prior of 500 with 95% credibility limits.

	Mitochondrial Diversity					Microsatellite Diversity			
Location	*n*	No. of haplotypes	No. of segregating sites	π (SD)	*h* (SD)	*n*	*AR* (SD)	H_E (SD)	N_e (ABC, 500)
Mainland									
Tampa	13	6	13	0.00145 (0.00063)	0.821 (0.082)	25	8.9865 (0.08)	0.848 (0.08)	42.88 (32.25-69.77)
Central FL	20	14	49	0.00576 (0.00117)	0.958 (0.028)	24	8.86825 (3.72)	0.8495 (0.09)	17.67 (14.34-24.28)
Miami	21	10	32	0.00396 (0.00077)	0.810 (0.080)	31	8.450625 (3.95)	0.82225 (0.11)	75.05 (54.71-141.01)
Island									
Ten Th Islands	12	2	1	0.00008 (0.00007)	0.167 (0.134)	13	7.989625 (3.55)	0.81325 (0.13)	23.75 (18.17-42.44)
Key Biscayne	17	4	4	0.00024 (0.00012)	0.331 (0.143)	24	5.99025 (2.97)	0.698375 (0.19)	32.94 (25.73-55.59)
Mid/Up Keys	13	4	15	0.00379 (0.00050)	0.679 (0.089)	23	7.619125 (3.11)	0.798875 (0.12)	30.08 (23.24-57.55)
Lower Keys	16	4	10	0.00142 (0.00059)	0.442 (0.145)	22	7.59325 (3.12)	0.8095 (0.1)	22.60 (18.45-37.63)

Table 3. Pairwise estimates of F_{ST} between sample sites. Key Biscayne has the highest amount of differentiation when compared to all other sites. There is little to no differentiation among mainland sites, including Ten Thousand Islands, except between Ten Thousand Islands and Tampa. Within the Keys, there is little differentiation between sites. All estimates of F_{ST} are significantly greater than zero.

Population	**Tampa**	**Lower Keys**	**Central FL**	**Key Biscayne**	**Miami**	**Ten Th Islands**
Lower Keys	0.0590					
Central FL	0.0200	0.0407				
Key Biscayne	0.1165	0.1355	0.1055			
Miami	0.0369	0.0315	0.0099	0.0983		
Ten Thousand Islands	0.0528	0.0527	0.0374	0.1293	0.0195	
Middle/Upper Keys	0.0545	0.0290	0.0352	0.1510	0.0338	0.0391

Table 4. Data generated from an AMOVA showing that regional groupings with Ten Thousand Islands included in the mainland and Key Biscayne separate from the rest of the Upper Keys (*a posteriori* groups based on the STRUCTURE results) were more informative in explaining the amount of genetic variation present among the regions than the *a priori* subspecies groupings.

	Source of variation	**df**	**Sum of squares**	**Percentage of variation**
a priori	Among regions	3	37.768	0
	Among sites within regions	3	41.187	6
	Within sample sites	303	976.758	93
a posteriori	Among regions	2	46.137	5
	Among sites within regions	4	32.818	3
	Within sample sites	303	976.758	92

Table 5. Migration rates (**m**) between all sample sites estimated with BayesAss v3. Standard deviations for all distributions were <0.05 except the three italicized values. Migration rates greater than 0.1 are bolded. Values along the diagonal are proportions of individuals derived from the source site each generation. Sites from which individuals migrated are listed in the columns and sites that from which each individual was sampled are listed in the rows.

	Tampa	Lower Keys	Central FL	Key Biscayne	Miami	Ten Th Islands	Mid/Up Keys
Tampa	**0.7185**	0.0119	0.0124	0.0121	**0.2114**	0.011	0.0227
Lower Keys	0.0099	**0.6795**	0.0096	0.0101	0.0474	0.0099	***0.2335***
Central FL	0.0112	0.0101	**0.6802**	0.0178	**0.2538**	0.0161	0.0108
Key Biscayne	0.0107	0.0108	0.0111	**0.9255**	0.0192	0.0111	0.0116
Miami	0.0099	0.0094	0.0099	0.0363	**0.898**	0.0097	0.0268
Ten Thousand Islands	0.013	0.0137	0.0162	0.0141	**0.2032**	**0.6987**	0.041
Middle/Upper Keys	0.0119	0.0128	0.0146	0.0135	*0.0987*	0.0171	***0.8314***

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