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Indirect Estimates Of Gene Flow And Its Conservation Implications In The Striped Newt (notophthalmus Perstriatus)

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INDIRECT ESTIMATES OF GENE FLOW AND ITS CONSERVATION IMPLICATIONS IN THE STRIPED NEWT (*NOTOPTHALMUS PERSTRIATUS)*

by

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ABSTRACT

This study used indirect methods to estimate patterns of gene flow in a rare salamander species, the striped newt (*Notophthalmus perstriatus*). First, we used combined genetic and ecological methods to determine whether populations that appear to exist in two regions separated by 125 km, exhibited genetic and ecological distinctness such that the regions demarcate separate conservation units. Using mtDNA (*cyt-b*), we found that haplotypes were shared between localities within each region but none were shared between regions. Niche-based distribution modeling revealed significant differences in the ecological setting between the two regions. In combination, the absence of evidence for recent genetic exchange and model-based support for differing ecological conditions utilized by newts between regions provides evidence that eastern and western populations are both distinct and significant. This study suggests a framework to evaluate discreteness and significance among populations for assessment of distinct population segments (DPSs which can be used as a conservation tool for many species. Second, we used microsatellites to characterize patterns of population connectivity, genetic differentiation, and effective population size in *N. perstriatus*. We assessed these patterns by testing several *a priori* hypotheses regarding the influence of gene flow and genetic drift on the distribution of genetic variation among and within populations. Interestingly, several of our results did not conform to our hypotheses. For example, our assessment did not reveal a significant pattern of isolation by distance among populations in this study. Additionally, we found that effective population sizes and genetic diversity of isolated populations were higher than expected. We discuss our results relate to our *a priori* hypotheses and we address the general question of why this species exhibited patterns contrary to what we expected given previous data on this taxon and other studies of similar taxa.

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GENERAL INTRODUCTION

The striped newt (*Notophthalmus perstriatus)* is a rare salamander species endemic to north central Florida and southern Georgia. *Notophthalmus perstriatus* populations have undergone evident declines throughout their range and no longer exist in the type locality in southeastern Georgia (Christman& Means 1992; Johnson 2005). Naturally low relative abundance in concert with documented range-wide declines have caused the striped newt to be considered a rare species (Christman& Means 1992). The United States Fish and Wildlife Service has recently been petitioned to list *N. perstriatus* as a threatened species. As such it is important to develop an understanding of natural processes that contribute to population persistence of this species. Such data can be used by land managers and conservation planners to develop informed conservation strategies.

This species has a complex multi-stage life cycle comprised of obligately aquatic larvae (Johnson 2005). Adults either remain aquatic (i.e. are neotenic) or they metamorphose and move to terrestrial upland habitats (Johnson 2005). Terrestrial adults disperse hundreds of meters from their natal pond spending much of their adult lives in the uplands from where they occasionally disperse to non-natal ponds to breed (Johnson 2005). Their complex life history makes striped newts vulnerable to threats at breeding ponds (e.g., ditching and draining of temporary ponds) as well as in the surrounding uplands (e.g., silviculture practices, fire suppression). Previous studies suggest population persistence is declining throughout its range, owing largely to habitat loss and limited habitat connectivity (Dodd and LaClaire, 1995; Franz and Smith, 1999; Johnson, 2005). Populations of this species appear to occur in two geographic regions (separated by approximately 125 kilometers) with one region consisting of populations located in eastern Florida and Georgia and the other region consisting of populations located in western Florida and Georgia (Dodd and LaClaire, 1995; Franz and Smith, 1999). It appears

that *N. perstriatus* does not occur in the geographic area between eastern and western regions: to date, censuses have not identified *N. perstriatus* within this region (Dodd and LaClaire 1995; Franz and Smith, 1999). Lack of locality data in combination with extensive habitat loss and fragmentation (Dodd and LaClaire 1995) throughout this geographic area suggest that *N. perstriatus* is likely absent from this region.

The first objective of the study was to provide a framework for DPS assessment that efficiently identifies both discreteness and significance and can be implemented across taxa. We combine genetic analyses with niche-based distribution modeling to assess discreteness and significance among populations of the striped newt (*Notophthalmus perstriatus*). Our second objective was to estimate patterns of gene flow among populations and to estimate effective population sizes to identify populations that might be vulnerable to local extinction. Combined, this information is important in the development of focused management and conservation strategies.

CHAPTER ONE: COMBINING GENETIC STRUCTURE AND ECOLOGICAL NICHE MODELING TO ESTABLISH UNITS OF CONSERVATION: A CASE STUDY OF AN IMPERILED SALAMANDER

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Introduction

Conservation focused solely at the species level has the potential to overlook population-level diversity important to a species' evolutionary trajectory. The classification of taxa into units of conservation is a tool used to identify populations requiring integrative conservation planning. Assignment of conservation units facilitates efficient monitoring and management of targeted populations of imperiled species at the appropriate geographic scale (Moritz, 1994a; Fraser and Bernatchez , 2001). However, identifying the smallest meaningful unit of conservation has presented a challenge for biologists and conservation planners. Despite this difficulty, the theory of such operational units has been considered among the most revolutionary ideas resulting from phylogeographic studies at the microevolutionary level (Avise, 2004).

The Endangered Species Act (hereafter the Act), established in 1966, was designed to protect imperiled wildlife at the species level in the United States. In 1978, the Act was amended to include "distinct population segments" (DPS) providing legal protection below the species level to populations of terrestrial vertebrate species and fish (not including Pacific northwest salmonids). The sub-specific DPS classification facilitates efficient management of populations of conservation concern, allowing for application of management only within populations where such actions are

necessary (Pennock and Dimmick, 1997). A sub-specific taxonomic unit must be recognized as a DPS in order for legal conservation action to be taken.

According to the Act, DPSs are assessed based on three criteria: discreteness, significance, and status relative to other populations of the same species. Assessment of discreteness is well-established using morphological or genetic methodology. Indeed, many proposed conservation units exclusively depend on genetic data (i.e., reciprocal monophyly of mtDNA sequence data *and* statistically significant differentiation at nuclear loci; Moritz, 1994b; Zink, 2004). Although genetic data are important to consider in the assessment of conservation units, studies strictly based on genetics may be limited in scope and legal support. With increasing recognition that both genetics and ecology influence evolutionary trajectories, many widely supported conservation units, including DPS, encompass a more integrated approach (Crandall et al., 2000; Fraser and Bernatchez, 2001; Moritz, 2002).

While genetic data are traditionally used to identify discreteness among population segments, significance remains difficult to assess. Some factors considered when assessing significance include: inhabitance of an ecological setting that is atypical for the taxon, extinction of the segment would result in a break in the species distribution, or a population segment represents the only natural occurrence of a species within its native range (Policy regarding recognition of DPS, 1996). There are several methods possible to assess the ecological component of significance. Rader et al. (2005) proposed methods to test for ecological exchangeability based on observed trait differences as a measure of local adaptation (e.g., reciprocal transplants, common-garden experiments). However, this method may be difficult to conduct for many species, especially those that are difficult to collect due to rarity or high vagility. Moreover, conducting reciprocal transplants with imperiled species elicits ethical issues. The DPS designation of "atypical ecological setting" provides an opportunity to

assess the ecology of proposed population segments without requiring the manipulation of the species of interest. In this paper, we use a non-invasive technique, niche-based distribution modeling, to assess significance between two disjunct regions of an imperiled newt. Recently, niche-based distribution modeling has become widely used to model geographic distributions and to compare aspects of the ecological niche for a species between regions and/or continents (Guisan and Thuiller, 2008; Mau-Crimmins et al., 2006; Medley, 2010). While such models clearly don't include every aspect of the niche, they can be used to test hypotheses about differences in environments used by species between regions. In addition, newly developed randomization techniques facilitate the interpretation of observed differences in distribution models. These techniques test whether differences between distribution models (a proxy for the ecological niche) are a result of differences in the overall environment available to the species or if they result from differences in the niche that populations utilize (Warren et al. 2008).

The overall objective of the study was to provide a framework for DPS assessment that efficiently identifies both discreteness and significance and can be implemented across taxa. We combine genetic analyses with niche-based distribution modeling to assess discreteness and significance among populations of a rare salamander species, the striped newt (*Notophthalmus perstriatus*). The striped newt is a poorly understood species endemic to sandhill and scrub habitats of north-central Florida and southern Georgia, USA. This species has a complex multi-stage life cycle comprised of obligately aquatic larvae (Johnson 2005). Adults either remain aquatic (i.e. are neotenic) or they metamorphose and move to terrestrial upland habitats (Johnson 2005). Terrestrial adults disperse hundreds of meters from their natal pond spending much of their adult lives in the uplands from where they occasionally disperse to non-natal ponds to breed (Johnson 2005). Their complex life history makes striped newts vulnerable to threats at breeding ponds (e.g., ditching and

draining of temporary ponds) as well as in the surrounding uplands (e.g., silviculture practices, fire suppression). Previous studies suggest population persistence is declining throughout its range, owing largely to habitat loss and limited habitat connectivity (Dodd and LaClaire, 1995; Franz and Smith, 1999; Johnson, 2005). Populations of this species appear to occur in two geographic regions (separated by approximately 125 kilometers) with one region consisting of populations located in eastern Florida and Georgia and the other region consisting of populations located in western Florida and Georgia (Dodd and LaClaire, 1995; Franz and Smith, 1999). It appears that *N. perstriatus* does not occur in the geographic area between eastern and western regions: to date, censuses have not identified *N. perstriatus* within this region (Dodd and LaClaire 1995; Franz and Smith, 1999). Lack of locality data in combination with extensive habitat loss and fragmentation (Dodd and LaClaire 1995) throughout this geographic area suggest that *N. perstriatus* is likely absent from this region.

Based upon the apparent physical isolation between eastern and western regions, we predicted that the subsequent restricted gene flow would have resulted in genetic divergence and differences in niche utilization between regions. In addition, we predicted that genetic diversity would be lower within western populations than within eastern populations because of low population number and relative isolation in the west. To test these *a priori* predictions, we sought to (1) determine whether eastern and western regions of *N. perstriatus* populations exhibited genetic differentiation using traditional population genetics techniques (mtDNA sequence analysis) and (2) assess ecological differences between regions using niche-based distribution modeling. Our results are discussed with regard to whether populations in eastern and western regions meet the criterion for DPS assessment. We conclude by discussing the conservation implications of our technique for

assessing conservation units in general and how our results impact the conservation status of striped newts.

Methods

Genetic analyses: sample collection

We collected 92 samples from 11 localities throughout the range of *N. perstriatus* (Supplemental Table 1; Figure 1). Samples were collected from March 1999 through March 2000. Additionally, samples from the TAY site were collected in March 2009. We approximated the site locality for one area on private property in St. John's County (site 6, Figure 1) because the owner wishes the location to remain undisclosed. We captured newts with dip nets, seine nets or wire-screen funnel traps. We removed a small section from the distal end of each individual's tail using sterilized scissors, placed the tissue in saturated salt buffer (NaCl; 25mM EDTA, pH 7.5; 20% DMSO; protocol modified from Amos and Hoelzel, 1991), or in DrieRite® Desiccant, and released individuals after the samples were taken.

DNA isolation and sequencing

We isolated total genomic DNA from each sample using standard phenol/chloroform extraction followed by ethanol precipitation and storage in Tris/EDTA buffer (Hillis et al., 1996) or using a Qiagen DNeasy kit. For genetic analysis we used Polymerase Chain Reaction (PCR) to amplify a 585 base-pair fragment of the mitochondrial (mtDNA) cytochrome b (cyt*b*) gene using primers H14447 (Edwards et al., 1991) and MVZ15 (Moritz et al., 1992). Amplifications were carried out in 25-µl reactions containing: 1x buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 0.25 µM each primer, 1 U Taq polymerase (Sigma-Aldrich Corp., St. Louis, MO), and 2-5 ng of template DNA.

PCR conditions were as follows: initial denaturation of 94°C for 3 min, 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and a final extension for 7 min. Positive and negative controls were used in each round of PCR. PCR products were purified with 30,000 MW filters (Millipore, Inc., Bedford, MA) or via ExoSAP-IT (USB Corp., Cleveland, OH). Samples were sequenced at either University of Florida's DNA Sequencing Core or by Nevada Genomics Center on ABI sequencers. We checked chromatograms for base calling and edited sequences using Sequencher version 3.1 (Genes Codes Corp., Ann Arbor, MI).

Data Analysis

We used ClustalW implemented in the software *MEGA* version 4.0 (Tamura et al., 2007) to align the 585 bp fragment of mtDNA cyt-*b* for 92 samples. Genbank sequences for *Notopthalmus viridescens* and *N. meridonalis* were used as outgroups (accession numbers EU88032 and AY691731). We inferred phylogeny using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) using default priors. We tested three different partitioning strategies: no partitioning, first and second codon positions separate from third positions, and all codon positions separate. We used MrModeltest version 2.3 with the Akaike Information Criterion (AIC) (Nylander, 2004) to determine the best evolutionary model for each partition. We ran two Markov-chain Monte Carlo (MCMC) runs with five million generations. Samples were taken every 500th generation. Using Tracer 1.4 (Rambaut and Drummond, 2007), we checked for stationarity and eliminated the first 1,000,000 generations as burnin. We estimated summary statistics and consensus phylograms with nodal posterior probability support from the combination of both runs.

Additionally, we constructed a 95% parsimony haplotype network using TCS version 1.2 (Clement et al., 2000). Haplotype networks enable visualization of the relationship among

haplotypes including internal nodes and are often useful in resolving intraspecific haplotype relationships (Hoffman and Blouin, 2004). For all populations with sample size ≥ 5 we calculated within-population genetic diversity statistics including number of haplotypes, haplotype diversity (*h*) and nucleotide diversity (π) using DnaSP ver. 5 (Librado and Rozas, 2009). To assess differences between eastern and western regions in haplotype and nucleotide diversities we implemented a Welch's two sample t-test in the statistical program R. Finally, we evaluated the effect of sample size on the cumulative number of haplotypes detected for each population using an accumulation curve (i.e. species-area curve) in PC-ORD v5.0. If the shape of the curve flattens, additional samples are unlikely to yield new haplotypes.

Ecological Analyses

Modeling approach

We estimated climatic and habitat aspects of the niche for *N. perstriatus* by generating niche-based distribution models for eastern and western regions using a maximum entropy algorithm implemented in MAXENT software (Phillips et al., 2006). Maximum entropy is a machine-learning technique that predicts species distributions using detailed climatic and environmental datasets together with species occurrence data, and generally performs better than other algorithms in tests of model performance (Elith et al., 2006; Ortega-Huerta & Peterson, 2008). Maximum entropy uses presence-only data to predict the suitability of habitat, and is quite robust to spatial errors in occurrence data (Phillips et al., 2006; Graham et al., 2008).

We calibrated models using known occurrences and six environmental datasets. In addition to using occurrence data from our genetic analyses, we obtained occurrence data for *N. perstriatus* from the Georgia Department of Natural Resources and the Smithsonian National Museum of

Natural History Collection (http://collections.nmnh.si.edu), resulting in a total of ten occurrence points for the western region and 47 points for the eastern region. This occurrence data included all known occurrence points for the western region. Initially, we calibrated models using nine environmental variables. For the final models, we removed three environmental datasets that provided no contribution to the preliminary models, resulting in six environmental variables for calibration: maximum temperature of the warmest month, temperature seasonality, annual precipitation, precipitation of the driest month, and precipitation seasonality from the WORLDCLIM database (Hijmans et al., 2005), and 2001 land cover from the Multi-Resolution Land Characteristics Consortium (29 landcover categories, http://mrlc.gov). We chose a subset of the 19 available WORLDCLIM variables because some of the variables are correlated with one another (Kozak and Wiens, 2006, Rissler and Apodaca, 2007, Shepard and Burbrink, 2008). Thus, we selected variables that represented mean values and also included extremes for both temperature and precipitation because extreme values tend to limit population persistence for the study species. We obtained WORLDCLIM data at 30 arc-second resolution (~1 km^2 per pixel); land-cover data were obtained at 30 m^2 resolution and resampled to 30 arc-second resolution (~ 1 km^2) in ArcGIS version 9.2, (Environmental Systems Research Institute, Redlands CA, USA). We clipped all environmental layers to the study extent and exported them as ASCII grids for use in model development. We used 10,000 points to determine the background distribution, a regularization parameter of 1 for the eastern model and 0.80 for the western model, and a convergence threshold of 1.90.

We used spatial statistics to ensure independence of occurrence locations prior to calibrating models. We first calibrated models with all available occurrence points and calculated spatial autocorrelation on model residuals at multiple distance classes using Moran's I in SAM v3.0 software

(Rangel et al., 2006). Significant spatial autocorrelation was calculated using permutation tests. The distance class at which spatial autocorrelation ceased (i.e. became non-significant), was ~35 km. Thus, we randomly omitted occurrence points from the complete occurrence dataset that were nearer than 35 km from one another. The remaining points were used to calibrate final distribution models (7 points for western region, 10 points for eastern region). This is a substantial reduction from the number of available points in the east, but recent work by Costa et al. (2010) revealed that MAXENT produces accurate predictions with small sample sizes. Moreover, because of the imperiled status of this newt and few known western localities, reducing the number of localities for model training in the eastern region balances analyses between regions.

To determine the optimal extent at which to calibrate models, we ran a suite of models at extents increasing in size from the extent of either eastern or western occurrence points (regional extent) to the extent of all occurrence points combined (full extent). Models calibrated at the regional extent produced models with AUC values ≤ 0.75, even when we reduced the regularization modifier below 1 to improve fit. Thus, final models were calibrated at the full extent. Because random background points are used as pseudo-absences, it is assumed that all occurrence points within the calibration extent that are not included in calibration (e.g. western points for the eastern model) could be a pseudo-absence. However, calibrating in this way also guards against projecting models onto "non-analog climate", a problem recently discussed in the literature as one that can incorrectly predict occurrence probability when projecting models that are calibrated using existing combinations of climate and habitat onto areas having potentially different combinations of such variables (i.e. extrapolation error, Fitzpatrick and Hargrove 2010).

We evaluated model accuracy in two ways. For the eastern model, we used the 10 occurrence points that were > 35 km apart for calibration and used the remainder of all known occurrences (37)

to test the model using binary tests of omission (Phillips et al., 2006). We calculated omission rates as the proportion of test points that were not predicted at a threshold probability equaling the minimum probability of any pixel containing an occurrence point. Because of the limited number of populations in the western region, seven of ten occurrence points were used to generate the model and an omission test on the three remaining points was not useful (i.e. a test using three occurrence points has little power). Thus, we also evaluated model accuracy using the threshold-independent area under the curve (AUC) of the receiver operating characteristic (ROC) plot. AUC is a composite measure of model performance, and compares model fit to that of a random prediction. AUC values range 0-1, where 1 is a perfect fit. Useful models produce AUC values of 0.7-0.9, and models with "good discriminating ability" produce AUC values above 0.9 (Swets, 1988).

Ecological comparisons between regions

We evaluated regional ecological differences in two ways. The first compared entire distributions (our estimate of the regional niche) using niche overlap statistics and the second compared environmental variables at occurrence points using multivariate procedures. We used both procedures because while the multivariate procedures provide an intuitive interpretation, evaluating differences only at known occurrences can bias environmental values towards sampling locations. Comparisons between entire distributions provide a broader estimate of the environment utilized by populations. To compare entire distributions, we used two randomization procedures to compare "niche overlap" between a pair of real models to that between models generated using either randomly generated occurrence points (background similarity) or by randomly assigning identity (eastern or western) to occurrence points (niche identity). Niche identity tests whether models generated for the eastern and western populations are significantly different. For this procedure,

random models are generated by randomly assigning identity (eastern or western) to occurrence points. Background similarity evaluates whether differences detected between models can be explained by underlying environmental differences between regions (i.e. the "background"). This test generates models using points that are randomly generated from all the available pixels in the study area (eastern or western region). We calculated "niche overlap" between all pairs of models using the metric *I* (Warren et al. 2008), which ranges from 0 (no overlap) to 1 (identical). This metric compares probability values for individual pixels between two niche models. For both randomization procedures, 100 overlap values (*I*) were calculated between random models and compared to overlap (*I*) between real models to assess significance. If niche identity indicates a significant difference between models, a significant difference in background similarity would indicate differences in the models were due to differences in the overall environment between regions. Alternatively, if niche identity is significant and background similarity is not significant, then the differences in the models are not due to underlying environmental differences; rather, the differences are due to differences in the niche utilized by populations in each region.

As a supplement to distributional comparisons, we used Principle Components Analysis (PCA) to compare environmental data at occurrence points between regions using PC-ORD 5 (MjM Software, Gleneden Beach, Oregon, USA). We extracted environmental data at each occurrence point in ArcGIS and used Monte Carlo simulations to test whether the PCA ordination was different from a random configuration. We conducted Multi-Response Permutation Procedures (MRPP) in PC-ORD 5 (McCune and Grace, 2002) to test the null hypothesis that environmental data at occurrence points were no different between eastern and western regions. The test statistic for MRPP is A, which quantifies the amount of within-group agreement. A values range from negative values to1; 1 indicates all localities within regions are identical; 0 indicates heterogeneity

within groups is no different from that expected by chance, and values < 0 indicate within-group heterogeneity is less than a random expectation. We evaluated environmental variable importance by correlating each variable with axis scores from the PCA ordination (continuous variables) or using logistic regression (categorical data) as appropriate.

Results

Genetic Analyses

We aligned a 585 bp fragment of the mtDNA cyt*b* gene for 92 samples of *N. perstriatus* collected throughout the species range and identified 27 unique haplotypes (Figs. 2 and 3). Haplotype sequences generated from this study have been submitted to GenBank (accession numbers AF380362, AF380363, and HM804253 - HM804277). Our calculations of cumulative haplotype estimates using PC-ORD support that identified haplotypes are representative of those present within each site even though some sites may have been under-sampled (Supplemental Table 2). For phylogenetic reconstruction, the best fit model identified by AIC for the unpartitioned scheme was HKY+I. The best fit model for the two partition scheme was GTR+G for first and second position and GTR for third position. The best fit models chosen for the three partition scheme were K80+I for position 1, F81 for position 2, and GTR for position 3. Across all models, Bayes factors (Kass and Raftery, 1995) provided very strong support (2 lnB10 > 10) for the two partition model as the best-fit to the dataset and this model was used for phylogeny reconstruction. While the Bayesian phylogeny (Fig. 2) provided support for the *N. perstriatus* samples as a monophyletic group, intraspecific relationships among *N. perstriatus* regions remained unresolved. We did not recover reciprocal monophyly among eastern and western populations.

The 95% statistical parsimony haplotype network generated detailed information regarding relationships among individual haplotypes and haplotype occurrence information per population (Fig. 3). Haplotype C (found only in eastern populations) was identified as the most likely ancestral haplotype given the greatest value for outgroup weight (Clement et al., 2000). Haplotype C was also the most widespread, shared among three of the eleven locations (all eastern region, sites 3, 6, and 8; Fig. 1, Supplemental Table 1), and occurred with the greatest frequency (62.5%) at site 8 in Georgia. Haplotype A was the most abundant haplotype and was found at sites 3 and 4, both in north-central Florida (eastern region). The geographic distribution of the 27 haplotypes (Fig. 1) illustrates the limited number of haplotypes shared among populations throughout the species range. Most sample localities contained endemic haplotypes, and only haplotypes A, B, C, and Y were found at more than one site. Interestingly, shared haplotypes only occurred within regions; that is, we did not uncover any haplotypes shared between western and eastern regions (Fig. 1). Furthermore, the haplotype network (Fig. 3) revealed a pattern suggestive of limited gene flow between regions in that eastern and western haplotypes were not intermingled.

Maximum sequence divergence between haplotypes was 3%; haplotypes S and M were the most divergent differing at 22 nucleotide positions. Estimated average sequence divergence between regions was 0.8%, whereas average sequence divergence within regions was 0.3%. For comparison, the estimated sequence divergence between *N. perstriatus* and *N. meridionalis* was 12.9% and between *N. perstriatus* and *N. viridescens* was 9.3%. Estimates of within population nucleotide diversity (π) ranged from 0 to 0.01324 in the east and from 0 to 0.00757 in the west. Estimates of haplotype diversity (h) ranged from 0.3 to 0.873 in the east and 0 to 0.643 in the west (Table 1). Moreover, regional comparisons of genetic diversity revealed that diversity harbored within populations was

not significantly different between regions (for π: $t = 0.0649$, $df = 5$, $p = 0.9508$; for h: $t = 0.3863$, $df = 4$, $p = 0.7213$).

Ecological Setting

MAXENT produced models with "good discriminating ability" (AUC=0.91 and 0.93, respectively; test omission rate for eastern model=0.03) and predicted different patterns of probability of occurrence between eastern and western regions (Fig. 4). The eastern model predicted high probabilities in the eastern region and into central and south Florida, and an area of low probability flanking western populations (Fig. 4). The western model predicted high probabilities of occurrence over the western region and in a patch along the east Florida coast, and lower probabilities in the eastern region and beyond (Fig. 4). Temperature seasonality was the most important variable for the eastern model (63% of variation explained) followed by precipitation seasonality (16% of variation) and land cover (11% of variation, Table 2). Land cover was the most important variable for the western model (33% of variation explained), followed by precipitation of the driest month (24% of variation) and maximum temperature of the warmest month (23% of variation, Table 2).

Ecological comparisons revealed distinct differences between regions. Niche overlap (I) between eastern and western models was 0.79, and randomization procedures revealed differences between regions were largely due to differences in the environment utilized by newts. Niche identity procedures revealed eastern and western distribution models were significantly different from one another (i.e. niche overlap was higher than random, $p < 0.001$; Table 3). Background similarity tests

showed no significant difference in the overall environment between eastern and western regions (background similarity, $p_{east\_west} = 0.64$, $p_{west\_east} = 0.10$; Table 3).

Principal components analysis revealed an eastern and a western group with minimal overlap based upon environmental data at occurrences (Fig. 5). Two axes were significant (Axis 1 = 53% of overall variance; Axis 2 = 25% of overall variance). Multiple Response Permutation Procedures revealed significant environmental differences between regions ($A = 0.33$, $p = 0.0007$). Correlations between PCA Axis 1 and climatic variables revealed strong associations between mean annual temperature, temperature seasonality, minimum temperature of the coldest quarter, and precipitation seasonality (Table 2). Logistic regression between PCA axis scores and land cover showed no significant associations (Table 2).

Discussion

Conservation Unit Assessment

This study employed a combined approach utilizing genetic analyses and distribution modeling to evaluate discreteness and significance among population segments as a framework to evaluate DPS assessment. This methodology has broad conservation utility as a combined approach that can provide valuable information regarding the feasibility of translocation and is applicable to many taxa. Additionally, niche-based distribution models serve as multi-functional tools, useful not only for evaluating environmental conditions but also assessing habitat factors including suitability which are important for management of endangered taxa. Additionally, this framework has potentially broad applications as a tool for conservation unit assessment in many other species. One highlight of our framework is that it links DPS assessment (a legal, political and scientific term) with contemporary definitions of Evolutionary Significant Units (ESUs; e.g. Crandall et al., 2000) a largely

scientific term by assessing both genetic distinctness and ecological significance which are the primary criteria for identifying both ESUs and DPSs. Our study provides an alternative to ecological exchangeability (i.e. ecological setting) for assessing the ecological component of conservation unit assessment. Ecological exchangeability can be difficult to assess, given the threatened nature of populations undergoing conservation assessment. Use of niche-based distribution modeling provides a non-invasive alternative, and the randomization techniques we employed can serve as a proxy for measuring ecological exchangeability. Moreover, while ESU designation is a widespread and useful conservation tool for identifying populations of concern, ESUs do not provide legal protection under the Act [except as adopted by National Marine Fisheries Service which has defined the ESU as their criterion for DPS (Pennock and Dimmick, 1997)]. Although semantic differences between DPS and ESU are problematic only in the United States, we seek to remove international confusion that arises from the disjunction between intraspecific units described in the literature and the legal policy associated with those units (Haig, 2006). The methodology employed in this study bridges the gap between DPSs and ESUs, broadening the scope of conservation with the flexibility to address both legal and scientific concerns.

How does our methodology work in our case study? Our *a priori* expectation was that restricted gene flow would have resulted in genetic divergence and differences in niche utilization between putatively isolated regions of the striped newt (*N. perstriatus)*. In contrast to our expectations, reciprocal monophyly was not recovered between eastern and western haplotypes. Despite the fact that no clear historical phylogenetic patterns were revealed using the mtDNA *cyt-b* gene, our genetic analysis supports a lack of recent genetic exchange as evidenced by the distribution of haplotypes among populations within and between regions. Namely, haplotypes were shared within regions but eastern and western haplotypes were not intermingled and no haplotypes were

shared between regions. Therefore, *N. perstriatus* population segments comprising eastern and western regions meet the discreteness criteria of DPS assessment based on a lack of contemporary gene flow. Distribution models were significantly different between regions, confirming significant climatic and environmental differences between regions. Thus, the ecological analyses support that *N. perstriatus* population segments within eastern and western regions inhabit different ecological settings and are thereby ecologically significant. Based on our conclusions, population segments in eastern and western regions are both discrete and significant. Therefore, a conservation status assessment should be conducted for each region to determine their current conservation status.

Evolutionary Ecology of N. perstriatus

The combination of genetic and distribution model analyses used in our study provides unique insight into the evolution and ecology of *N. perstriatus*. Genetic analyses did not recover reciprocal monophyly between eastern and western regions. This is likely due to lack of time since populations began to diverge. However, the absence of intermingled haplotypes between regions suggests that recent gene flow among populations does not occur. As one of the fastest-evolving genes, *cyt-b* often provides sufficient information to resolve historical divergences (e.g. Wagner, 2005; Canestrelli et al., 2006; Miller et al., 2006; Sotiropoulos et al., 2008). Our genetic results suggest that observed genetic divergence was recent and not historic. In order to obtain more accurate estimates of contemporary gene flow among populations, different molecular markers (e.g. microsatellites) are likely needed.

Our genetic results were not surprising in light of previous population genetic studies of salamanders that typically reveal patterns of genetic structuring among populations. Studies investigating genetic divergence between populations of salamanders occurring in different regions

typically lead to the designation of separate conservation units including ESUs (Canestrelli et al., 2006; Miller et al., 2006; Sotiropoulos et al., 2008) and Management Units (Lecis and Norris, 2004; Pabijan et al., 2005; Wagner, 2005; Eastman et al., 2007). Indeed, results of other studies have suggested discordance among current taxonomic classification and genetic data identifying cryptic species (Shaffer et al., 2004; Sotiropoulos et al., 2007). Furthermore, population differentiation may reflect historical climatic and geological processes (Kuchta and Tan, 2006) leading to isolation and eventually speciation between some salamander populations (Shepard and Burbrink, 2008). Moreover, natural barriers such as streams, altitude, topography (Marsh et al., 2007; Giordano et al., 2007) in addition to anthropogenic barriers such as roads (Marsh et al., 2008) have been found to limit dispersal resulting in divergence between populations. One aspect of our results that did differ from many of the previously mentioned studies is that we found relatively low levels of differentiation between geographic regions based on mtDNA (*cyt-b*). However, like our study, some salamander populations have managed to retain genetic diversity despite occupation of fragmented habitat (Purrenhage et al., 2009).

While our study identified ecological differences between regions, we did not find sufficient genetic divergence to support splitting eastern and western regions into separate species. In contrast, two recent studies have investigated genetic divergence in combination with ecological differentiation in other species of salamanders and determined that populations comprise multiple species (Kozak and Weins, 2006; Rissler and Apodaca, 2007). Although we identified a lack of shared haplotypes between regions suggesting a lack of contemporary gene flow, the lack of genetic divergence between regions suggests that further corroborating evidence (e.g. based on behavior) would need to be confirmed before eastern and western regions of *N. perstriatus* should be considered separate cryptic species. Moreover, estimates of mean pairwise sequence divergence

within *N. perstriatus* were low compared to maximum diversity estimates described within other species (e.g. 9% sequence divergence within *Taricha torosa* (Tan and Wake ,1995)) versus 3% sequence divergence between haplotypes for *N. perstiatus* (this study).

Distribution models revealed significantly different ecological settings between regions. Niche-based distribution modeling is often used to map out species ranges (e.g. Zhu et al. 2007), but comparisons between models allow hypothesis testing about differences in aspects of the niche between species (e.g., Graham et al. 2004) or between regions or seasons for the same species (e.g. Fitzpatrick et al., 2007; Suárez-Seaone et al., 2008; Medley 2010). Often, comparisons between distributions are made by extracting data from occurrence points and evaluating overlap in multidimensional space (e.g., Gebremedhin et al., 2009), or by assessing how well one model predicts species occurrences in a different region (Pearman, et al., 2008; Broennimann et al., 2007). Our approach went further by evaluating whether differences between models resulted from overall differences in the environment or were a result of differences in the environment utilized by newts. In this way, we were able to show that even while the environment between eastern and western regions was not significantly different, the environment utilized by newts in eastern and western regions was significantly different. These results suggest that transplants between regions to supplement populations may be risky and thus eastern and westerns regions require separate conservation efforts. In addition, the niche based distribution models may provide information regarding the suitability of habitat found in the geographic area between regions. When the eastern and western distribution models were overlaid on a single map (Supplemental Figure 1), it was apparent that this gap region does contain areas of suitable habitat. However, these areas of suitable habitat have a very limited and patchy distribution as compared to the large expanses of continuous suitable habitat found in the eastern and western regions. It is our hypothesis that this patchy

distribution of suitable habitat within this gap area might make dispersal between regions difficult possibly contributing to the apparently disjunct distribution of *N. perstriatus*.

Conservation Implications for N. perstriatus

How does identifying discreteness and significance between eastern and western regions relate to the conservation of striped newts? In recent years, *N. perstriatus* populations have declined throughout their range (Dodd and LaClaire, 1995; Franz and Smith, 1999; Johnson, 2005). Habitat loss, fire suppression, and the naturally patchy distribution of upland habitats (i.e., sandhill and scrub communities) have likely resulted in the fragmented and patchy distribution of the species. Additionally, a complex life history makes striped newts vulnerable to threats at breeding ponds (e.g., ditching and draining of temporary ponds) as well as in the surrounding uplands (e.g., silviculture practices, fire suppression). Densities of striped newts are very low at most sites where they persist (Dodd and LaClaire, 1995; Franz and Smith, 1999). For example, historically large populations in the Florida panhandle have been documented as declining with less than five newts caught from one of the largest clusters of known *N. perstriatus* breeding ponds within the past ten years (1999-2008; R. Means, personal communication). Because of historical declines and current low population densities, the striped newt is currently protected in Georgia as a "threatened" species. Furthermore, it is currently listed as a species of concern in Florida (Christman and Means, 1992) although it has no legal status in the state. The biological status of this species is currently under review by the U.S. Fish and Wildlife Service.

In light of the known conservation concerns for *N. perstriatus*, our study identifies an intensified need for conservation action to help preserve the remaining populations. A conservation status assessment should be conducted for both regions status and may result in subsequent legal

action (listing as separate DPSs). Specifically, western populations are at extreme risk as only nine locations have been recently identified as containing active newt populations. Interestingly, despite the isolation and low number of localities in the west, our analysis of genetic diversity supports that haplotype and nucleotide diversities within populations are not significantly different between regions. High genetic diversity in the western region may be a result of the longevity (Johnson 2005) of *N. perstriatus* individuals. As such, the recent demographic decline is not reflected in the genetic data owing to insufficient time for genetic drift to impact diversity. Thus, it is important that conservation actions be taken in the immediate future to conserve this genetic diversity before it declines. Knowledge of niche characteristics can guide conservation efforts. For example, given the differing niche for each region, individuals translocated between eastern and western regions may produce maladapted offspring (Kirkpatrick and Barton, 1997) and is thus not recommended. Understanding population genetic structure and species ecology additionally aid translocation efforts ensuring that genetically similar individuals are moved between areas with similar environmental conditions to encourage successful establishment. Further research into the population genetics, natural history and ecology of this species is urgently needed to effectively manage and conserve this species.

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CHAPTER TWO: POPULATION GENETIC STRUCTURE AND EFFECTIVE POPULATION SIZE IN THE STRIPED NEWT (*NOTOPHTHALMUS PERSTRIATUS*)

Introduction

Understanding the patterns and processes of gene flow has long been of interest to researchers because many ecological and evolutionary factors are impacted by the movement of individuals and alleles among populations (Hanski& Gilpin 1997). Over time, the combination of both ecological and evolutionary factors can have profound impacts on population genetic structure and even on population persistence. Taken to the extreme, a lack of gene flow combined with development of local adaptations and differences in morphological and physiological traits can lead to population divergence and eventual speciation (Slatkin 1987).Within species, developing an understanding of the influence of population connectivity and genetic structure of sub-divided populations is important for understanding patterns of spatial structure (Moilanen& Nieminen 2002), life history strategies (Taylor& Hellberg 2003), migratory patterns (Webster *et al.* 2002), and species conservation (Mills& Allendorf 1996).

Studies of population genetic structure have focused on the interaction of gene flow and genetic drift and their influence on genetic diversity and differentiation among populations. These studies have generally concluded that spatial arrangement and regional interaction of populations contribute to patterns of gene flow and population genetic structure (Bohonak 1999; Hutchison& Templeton 1999; Slatkin 1985b). These population interactions occur on a continuum of population connectivity (Trenham et al 2001) ranging from panmixia (Wright 1931b), to stepping stone patterns where migrants are shared among neighboring populations (Kimura& Weiss 1964), to metapopulations where asynchronous extinction and re-colonization events occur among patches of regional populations (Hanski& Gilpin 1997). In each case, the process of genetic connectivity has a different effect on the

pattern of genetic structure, allowing for predictions to be made regarding population interactions and connectivity among populations. For panmictic populations, we expect to find a lack of genetic differentiation in combination with high levels of genetic diversity among populations (Wright 1943). If populations exhibit a stepping stone pattern of gene flow we expect to find an increase in genetic differentiation among populations as geographic distance increases (Hutchison& Templeton 1999). In metapopulations, genetic patterns are dependent on how re-colonized populations are founded. If re-colonization occurs with large numbers of individuals from multiple populations then differentiation among populations will be low and genetic diversity within populations will be high (Wade& McCauley 1988). Alternatively, if re-colonization occurs with relatively few founders from few populations, then differentiation among populations might be great but genetic diversity within populations would be low (Wade& McCauley 1988). Testing of these different predictions with empirical examples helps to identify which processes are most common in natural populations.

However, patterns of gene flow are not the only driver influencing patterns of among- and within-population genetic diversity. Effective population size also influences population genetic diversity and differentiation. Effective population size (i.e. N_e (Wright 1931a)) is inversely proportional to within-population genetic diversity and between-population genetic differentiation (Crow& Kimura 1970) and large populations retain diversity, even if isolated. Hence, the interplay between the processes of gene-flow and genetic drift (a product of N_e) produce contemporary patterns of genetic differentiation. When genetic connectivity occurs among populations, gene flow counteracts the effects of genetic drift with migrants moving between natural populations helping to re-introduce genetic variation and prevent fixation of alleles (Mills& Allendorf 1996). However, when small populations are genetically isolated, genetic drift becomes the driving factor and leads to genetic differentiation among populations (Slatkin 1987). Specifically, isolated populations with small N_e are likely to experience increased loss of genetic diversity and evolutionary potential, making them vulnerable to inbreeding and local extinction (Amos&

Balmford 2001). Moreover, genetically and geographically isolated, or peripheral populations, are often genetically and morphologically distinct from more central populations (Lesica& Allendorf 1995) and may provide an important contribution to evolutionary and ecological processes within natural systems (Lammi *et al.* 1999).

In this study, we use 11 polymorphic microsatellite markers to evaluate how contemporary patterns of gene flow and genetic diversity, shedding light on the processes influencing regional population dynamics and effective population size within and among populations of *Notophthalmus perstriatus*, the striped newt. *Notophthalmus perstriatus* is endemic to sandhill and scrub habitats of north central Florida and southern Georgia, USA, and has been documented as declining throughout its range due to habitat loss and limited habitat connectivity (Dodd& LaClaire 1995; Johnson 2005). This species has a complex multi-stage life cycle requiring a matrix of both upland and wetland habitats. *Notophthalmus perstriatus* has a naturally patchy distribution with population localities occurring in both isolated and clustered habitat patches (Johnson 2005). Populations of this species occur in two disjunct geographic regions (separated by approximately 125 km) that demarcate separate Evolutionarily Significant Units (May *et al.* 2011) based on mtDNA variation; one region consists of populations located in eastern Florida and Georgia and the other region consists of populations located in western Florida and Georgia (Dodd and LaClaire, 1995; Franz and Smith, 1999). Interestingly, while there was a lack of haplotypes shared between regions, haplotypes were shared within regions across long distances (approximately 300 km; May et al. 2011). These findings raise the question of whether contemporary gene flow occurs across such distances or if the pattern of variation uncovered reflects historical patterns of variation. The differing scales of habitat connectivity and pond proximity among localities provide a unique setting in which to investigate fine scale patterns of genetic diversity and population connectivity of *N. perstriatus*.

In this study, we sought to answer two main questions: first, were the patterns of genetic differentiation identified in May *et al.* 2011 due to contemporary or historical connectivity of populations; and second, would geographically isolated populations be re-founded if a population became extirpated? To address these questions, we developed and tested hypotheses regarding gene flow, population genetic structure, and effective population sizes of *N. perstriatus*. These hypotheses were based on previous studies of other amphibians with restrictive habitat requirements and limited dispersal abilities as well as previous assessments of effective population sizes in other salamander species. Our first hypothesis focused on testing for a pattern of isolation by distance (IBD; (Wright 1943). Assessment of IBD helps determine whether genetic drift and gene flow are at equilibrium among sub-populations (Hutchison& Templeton 1999). The pattern of IBD characterizes taxa with restricted dispersal abilities, thereby influencing levels of genetic differentiation among sub-populations (Garnier *et al.* 2004). Therefore, we hypothesized that our results would indicate an overall pattern of isolation by distance and restricted gene flow. *Notophthlamus perstriatus* in particular has restrictive habitat requirements and limited dispersal abilities (700 m (Johnson 2005). The pattern of IBD is typical for amphibians in general (Hoffman *et al.* 2004; Newman& Squire 2001; Shaffer *et al.* 2004; Storfer 1999) and previous studies of other newts have demonstrated this pattern among populations (e.g.(Kuchta& Tan 2005; Pabijan *et al.* 2005; Tominaga *et al.* 2010).

Second, because newts tend to have limited dispersal ability (Healy 1975; Johnson 2003) we hypothesized that geographically isolated localities would be highly differentiated from geographically clustered localities. We predicted that isolated localities will exhibit high F_{ST} and represent separate units based on genetic clustering algorithms. Additionally, we hypothesized that geographically isolated localities would harbor lower levels of genetic diversity relative to geographically clustered localities. Due to a combination of factors including complex life history cycles, limited dispersal abilities, and philopatry, newts often exhibit high levels of differentiation among geographically separated

populations. Population genetic studies of multiple salamander species have typically revealed patterns of genetic structuring among populations. In some cases, genetic divergence between populations was sufficient to warrant designation of populations as separate conservation units (Lecis& Norris 2004) and even different species (Shaffer *et al.* 2004). Further, reduced genetic diversity has been found in previous studies investigating isolation populations resulting from founder events and/or reduced population sizes have been found in previous studies investigating isolated populations (Eckstein *et al.* 2006; Lesica& Allendorf 1995; McCommas& Bryant 1990).

Our third hypothesis focused on estimates of effective population sizes for *N. perstriatus* localities. Specifically, we hypothesized that geographically isolated localities would have lower N_e estimates when compared to geographically clustered localities of the same species. Understanding the relationship between genetic connectivity and effective size may play a role in management decisions of this rare species. Effective population size has been estimated for only a limited number of salamander species. In one such study, Gill (1978) estimated the effective population sizes for populations of the sister taxon to *N. perstriatus*, *N. viridescens*, a wide ranging newt species found in North America. Effective population sizes were estimated to be 12-150 from census sizes of 75-544. While effective population size may vary among species, the findings of Gill (1978) are concordant with other similar studies of amphibians which indicate effective sizes are usually fewer than 50 individuals (Phillipsen *et al.* 2011). Based on these data, we also predicted that effective population sizes of each locality would be between 15 – 50 individuals for *N. perstriatus*. We discuss our results in comparison to other studies of gene flow patterns and effective population sizes and how our results might contribute to conservation efforts for *N. perstriatus*.

Methods

Sample Collection

We collected tissue samples from 368 individual from twelve localities (Table 1 and Figure 1) within the eastern region of the range of *N. perstriatus*. For the purpose of this study, we sampled populations that we predicted *a priori* to be isolated from other localities (FSMI, CB, GSF, RSR, and FD) and from populations within strongholds. Strongholds are the few remaining areas containing *N. perstriatus* that have contiguous suitable upland habitat allowing for dispersal among suites of multiple breeding ponds (OR1, OR2, OR3, ONF1, ONF2, ONF3, and ONF4). We captured newts using dip nets, seine nets, or wire screen funnel traps. We removed a small section (1-3 mm) from the distal end of each individual's tail using sterilized scissors, placed the tissue in saturated salt buffer [NaCl; 25mM EDTA, pH 7.5; 20% DMSO; protocol modified from(Amos 1991)], or in DrieRite® desiccant, and released individuals after samples were taken.

DNA Extraction, Microsatellite Development, and Genotyping

We isolated total genomic DNA from each sample using standard phenol/chloroform extraction followed by ethanol precipitation and storage in Tris/EDTA buffer (Hillis 1996) or in DrieRite dessicant®. Microsatellites developed for *N. viridescens* (Croshaw& Glenn 2003) were screened for cross amplification in *N. perstriatus*. All seven successfully cross amplified but only two loci were polymorphic in *N. perstriatus*. To obtain additional loci for *N. perstriatus*, we utilized the microsatellite enrichment protocol summarized in Hoffman *et al.* (2003). First, about 30ng of genomic DNA was cut into smaller pieces using a degenerate oligonucleotide-primed PCR (DOP-PCR). The DOP-PCR product was then enriched using 5'-biotynilated, 3'-amino modified $(GATA)_8$ primers. Hybridized product was separated using streptavidin-coated magnetic beads as explained in Ardren et al. (2002) and the enriched genomic library underwent a second DOP-PCR. Enriched product was then cloned using either TOPO TA Cloning Kit from Invitrogen or QIAGEN Cloning Kit. Colonies were plucked using sterile pipette tips, added to 100µl of H_2O and boiled for 10 minutes to release the plasmid. The T3/T7 procedure (Cabe&

Marshall 2001) was used to screen colonies for colonies. Nine positive colonies were polymorphic and in Hardy-Weinberg Equilibrium (HWE) for a total of 11 polymorphic loci, all were used in this study (Table 2).

Amplifications for all microsatellites were performed in 20μL reactions containing 20-30 ng template DNA, 2μL 10X PCR buffer, 2.5mM $MgCl_2$, 200μM each dNTP, 0.125μM M13-tagged forward primer and 0.5μM reverse primer, 0.5μM fluorescently-labeled M13 primer and 1 Unit of Taq polymerase. PCR amplifications were conducted in a BioRad MyCycler thermocycler (Bio-Rad Laboratories, CA). Cycles started with a denaturing step for 4 minutes at 94ºC, followed by 35 cycles of 94ºC for 30 seconds, annealing temperature (see Table 2) for 30 seconds, and 72ºC for 45 seconds, followed by a final extension at 72ºC for 7 minutes. PCR products were visualized on a 2% agarose gel and genotypes were determined on a CEQ 8000 DNA analyzer or using ABI 3730 DNA Analyzer (University of Arizona, Genetics Core).

Genetic Differentiation

To assess the presence of null allelles and allelic dropout, we used MICRO-CHECKER *v. 2.2.* (Van Oosterhout *et al.* 2004). Additionally, we checked all 11 loci for deviations from HWE and linkage equilibrium (LE) using the Fisher's exact test utilized in GENEPOP *v. 4.0* (Raymond& Rousset 1995) and applied a sequential Bonferroni correction to account for multiple comparisons (Rice 1989). To assess differences of estimates of genetic diversity between isolated and clustered localities (defined *a priori* and *a posteriori*), we implemented a Welch's two sample t-test in the statistical program R. To test for isolation by distance, the relationship between genetic differentiation and geographic distance, a Mantel test was implemented in GENEPOP v. 4.0 (Raymond& Rousset 1995). We estimated global and pairwise genetic distances between populations using the program SPAGeDi *v.* 1.3a (Hardy& Vekemans 2002). We compared F_{ST} and R_{ST} values (95% confidence intervals calculated by jackknifing over loci) with an allele size permutation test to assess which would provide the best estimate of genetic differentiation. This comparison indicates whether genetic drift or stepwise mutation has a greater influence on population differentiation. If mutation plays a greater role than drift, then the R_{ST} value should be significantly larger than F_{ST}.

Population Clustering and Recent Migration

To assess levels of population genetic structure between localities we used a Bayesian clustering analysis (STRUCTURE *v.* 2.3; (Pritchard *et al.* 2000). We used an admixture model with correlated allele frequencies. Prior location information was not included. The number of population clusters was varied from 1 through 12. The following conditions were used: 20 independent runs at each possible *K*, with a burn-in period of 10,000 and MCMC parameters set to 300,000 iterations. The Evanno *et al* (2005) criterion, ΔK, was used to estimate the highest level of population structuring. We hierarchically tested

for population sub-structuring within each inferred cluster until all structure levels were identified (Degner *et al.* 2010).

The modeling algorithm in the program STRUCTURE allows us to visually identify recent migrants, but to take a more analytical approach to detect first generation migrants we employed an assignment test in the program GENECLASS 2 (Piry *et al.* 2004). This program uses Bayesian methods to calculate the probability that each individual belongs to each of the predefined populations. The Rannala and Mountain algorithm (1997) was used with 10,000 simulated individuals and an assignment threshold of $p < 0.05$.

Effective Population Size

To estimate within population N_e and to test whether isolated populations had lower N_e estimates than clustered populations, we estimated N_e using two different methods: first, we used a Bayesian approach implemented using the program ONeSAMP (Tallmon *et al.* 2008). Second, we used an approach based on sibship assignment using the program COLONY (Wang 2009b). For the ONeSAMP estimation we performed the analysis using a prior range of 2-2,000. We estimated effective population size using other priors, however the prior range of 2-2,000 gave us comparable results with the smallest confidence intervals. The COLONY analysis was performed using the full likelihood option and medium length runs. To assess differences of estimates between isolated and clustered localities (defined both *a priori* and *a posteriori*) we implemented a Welch's two sample t-test in the statistical program R.

Results

We obtained multilocus genotypes from a total of 368 individuals representative of the twelve sampling sites (average of 30.7 samples/population; Table 8) for all eleven loci. There was no significant

deviation from expected heterozygosities and after a sequential Bonferroni correction for multiple comparisons all populations conformed to HWE and LE expectations (Table 8). The program MICROCHECKER did not detect scoring errors due to null alleles or allelic dropout.

Genetic Differentiation

Based on the allele size permutation test employed in SPAGeDi, stepwise mutations have contributed to the observed genetic differentiation and so we used R_{ST} estimates of genetic differentiation to estimate population divergence and to test for isolation by distance. The allele size permutation test supported that the observed global R_{ST} was significantly larger (P-value < 0.001) than the permuted R_{ST}. Overall, genetic differentiation was variable among sample sites (global R_{st} = 0.1755). Some sites were highly differentiated while others exhibited low differentiation (Table 9). Our results confirmed our *a priori* prediction that sites FSMI and GSF are greatly differentiated from other localities (average R_{ST} between FSMI and other populations = 0.368; average R_{ST} between GSF and other populations = 0.330 Table 9). However, in contrast to our *a priori* prediction, sites CB, RSR, and FD were only moderately differentiated from the clustered populations (average R_{ST} between each site and all other sites: CB = 0.135; RSR = 0.069; FD = 0.143). We utilized these results for downstream analyses, such that FSMI, GSF, CB, RSR, and FD comprise our *a priori* isolated populations, whereas only FSMI and GSF comprise our *a posteriori* isolated populations. All sites predicted to be clustered showed little to moderate differentiation among populations in which they were clustered (R_{st} estimates ranged from 0.002- 0.331 among ONF1 – ONF4; R_{ST} estimates ranged from 0.027 – 0.284 among ORD 1 – ORD3; Table 9). The relationship between genetic differentiation and geographic distance (Figure 8) was not significant (Mantel R= 0.2034, p-value = 0.061). Furthermore, estimates of genetic diversity were not significantly different between isolated and clustered localities for both *a priori* and *a posteriori*

isolated populations (*a priori* t = 1.1283, df = 8.031, p-value = 0.2918; *a posteriori* t = -2.1426, df = 9.898, p-value = 0.0681).

Population Clustering and Recent Migration

The highest level STRUCTURE analysis (with K varying from one through twelve) revealed that populations grouped into three genetic clusters (Figures 9 and 10). The three population clusters were comprised of (cluster #1) OR1, OR2, and OR3; (cluster #2) CB, ONF 1, ONF 2, ONF 3, ONF 4, FD, RSR; and (cluster #3) FSMI and GSF. Given the geographic distance separating GSF and FSMI, we were concerned that these populations clustered together solely because they were both small isolated populations and hence different than the other two clusters. When only GSF and FSMI were analyzed in STRUCTURE as a separate run, these two localities separated into two distinct clusters showing no admixture (Figure 10). Therefore, GSF and FSMI were excluded from the analysis and the remaining ten localities were run together in STRUCTURE. Here, the result was K=2 with (cluster 1) comprised of OR1, OR2, and OR3 and (cluster 2) comprised of CB, ONF1, ONF2, ONF3, ONF3, FD, and RSR (Figure 3). For each cluster identified in the previous STRUCTURE analysis, we ran an additional STRUCTURE analysis to identify evidence of sub-structure among clusters. Analysis of the cluster 1 found K = 2 with (cluster 1a) comprised of OR1 and OR3 and (cluster 1b) comprised of OR2. Analysis of cluster 2 found K =2 with (cluster 2a) comprised of ONF1, ONF2, and ONF3 and (cluster 2b) comprised of ONF4, CB, FD, and RSR. Further, we ran the populations from cluster 2a together and found K=3 with each ONF locality comprising a separate cluster. We ran the populations from cluster 2b and found K=2 with (cluster 2b.1) comprised of ONF4 and (cluster 2b.2) comprised of CB, FD, and RSR. We ran populations from cluster 2b.2 together and found no further level of substructure among CB, FD, and RSR. After hierarchical analysis, our overall population clustering analysis yielded support for nine discrete population clusters.

The results of the assignment test implemented in GENECLASS2 indicate that migrants are shared between multiple central Florida sites, even between OR3 and RSR which are separated by 113.8 km. The greatest level of migration occurred between OR1 and OR3 and between OR3 and RSR, which both shared four migrants (Table 5). Both pairs FD and RSR and OR2 and CB shared two migrants while localities OR3 and CB, OR1 and CB, OR2 and OR1, OR3 and OR1, each shared a single migrant (Table 10). However, our data also indicated that not all sites have recently given or received migrants. In particular, four sites (FSMI, GSF, ONF2, and ONF3) do not appear to share migrants with any other sampled locations (Table 10).

Effective Population Size

Overall, we found that N_e estimates (Table 8) from ONeSAMP ranged from 31 to 122, with an overall average point estimate among all populations of 61.7; COLONY estimates ranged from 13.8 to 757, with an overall average point estimate of 108. As a cautionary note, sites CB, OR3, and ONF2 had sample sizes between 15 – 17 individuals and so the results from ONeSAMP may not be reliable as they do not meet the program minimum sample size requirements of 20 individuals. We found that the estimates generated from both programs were relatively concordant and not significantly different (i.e. had overlapping 95% confidence intervals) for any population. Our population comparisons revealed that estimates of N_e were not significantly different between isolated (*a priori* mean value COLONY = 72.4, ONeSAMP= 55.6 and *a posteriori* mean value COLONY = 64, ONeSAMP = 38.5) and clustered (*a priori* mean value COLONY = 54, ONeSAMP = 145.5 and *a posteriori* mean value COLONY = 61.2, ONeSAMP = 122) localities (for *a prirori* COLONY: t = -1.074, df = 9.813, p-value = 0.3085, ONeSAMP: t = -0.8638, df = 6.423, p-value = 0.4188; for *a posteriori* COLONY: t = -0.0943, df = 1.26, p = 0.9376, for ONeSAMP: t = 1.1494, df = 9.535, p = 0.2784). With regard to specific site N_e estimates, OR1 had the highest N_e among all populations (122 COLONY, 757 ONeSAMP).

Discussion

In this study, we sought to characterize patterns of population connectivity, genetic differentiation, and effective population size in *N. perstriatus* using microsatellites. We assessed these patterns by testing several *a priori* hypotheses regarding the influence of gene flow and genetic drift on the distribution of genetic variation among and within populations. Interestingly, several of our results did not conform to our hypotheses. For example, our assessment did not reveal a significant pattern of isolation by distance among populations and we found that both effective population sizes and genetic diversity of isolated populations were higher than expected. We discuss below why this species exhibited patterns contrary to what we expected given previous data on this taxon and other studies of similar taxa and we address how these results pertain to our general question of whether the patterns of genetic differentiation were due to contemporary or historical connectivity of populations and whether a geographically isolated population would be re-founded if that population became extirpated.

Hypothesis 1: Our results will indicate an overall significant pattern of isolation by distance. In our *a priori* hypothesis, we predicted that a stepping stone model of migration, where migrants are shared among neighboring populations, would best describe the pattern of connectivity among *N. perstriatus* populations. Our basis here was because this pattern has been observed in other amphibian studies (Monsen& Blouin 2004; Savage *et al.* 2010; Trenham *et al.* 2001), however, it was not supported by our results. Interestingly, our results also do not indicate other characteristic dispersal patterns (e.g. panmixia or metapopulation models). These findings then beg the question of what processes could explain the observed patterns? Based on our estimates of genetic differentiation and population clustering analyses, we found that population genetic structuring occurs among sample localities at strikingly differing scales. Populations in close proximity (OR1 and OR2 separated by 3.5 km) comprised separate genetically distinct clusters and exhibited high levels of genetic differentiation (pairwise R_{ST} = 0.163). Other

localities separated by relatively long distances (e.g. FD and RSR separated by 102.4 km) comprised a single genetic cluster and exhibited low levels of genetic differentiation (FD and RSR pairwise R_{ST} = 0.008). Hence, the differing scales of population sub-structure were largely decoupled from geographic distance indicating that some factor other than geographic distance is acting as a barrier to dispersal between populations.

In contrast, it is possible that isolation by distance has occurred among populations of *N. perstriatus* but remained undetected in our study. The incorporation of landscape features into estimates of isolation by distance might yield a significant pattern. A recent study, May *et al.* (2011) used PCA to compare environmental variables at occurrence points between eastern and western regions of *N. perstriatus*. Results indicated that seasonality of temperature and precipitation specifically are important for occurrence of *N. perstriatus* within the eastern region of their range. The combination of habitat restrictions with observed levels of population structuring and differentiation at varying scales suggest that environmental factors may influence population genetic structure among localities of *N. perstriatus*. Studies of other species have found that incorporating landscape features into estimates of isolation by distance are frequently more informative than isolation by distance alone (Hether *et al.* in review; Giordano *et al.* 2007; Spear *et al.* 2005). Wang (2009a) assessed patterns of gene flow in a study of the black toad and found isolation by distance only became significant when environmental factors including topography and lake barriers were included. Another landscape approach, isolation by resistance, might also provide further insight regarding gene flow patterns among *N. perstriatus* populations. Isolation by resistance uses resistance distance instead of Euclidean distance and may serve as a more appropriate measure of geographic distance because it accounts for both a heterogeneous environment and for range shape, factors which are not considered in the isolation by distance method (McRae 2006). This methodology might be used to better understand how environmental variables might be influencing population genetic structure in *N. perstriatus*.

Hypothesis 2: Geographically isolated localities will be highly differentiated from geographically clustered localities and geographically isolated localities will harbor lower levels of genetic diversity relative to geographically clustered localities. Our results identified genetic differentiation and population substructure among *N. perstriatus* localities sampled in this study. The population clustering analysis revealed nine distinct population clusters indicating limited gene flow among localities. These findings are similar to assessments of population genetic structure in other salamander species which typically reveal high levels of genetic structuring among populations (e.g. Shaffer et al. 2004). For example, Lecis and Norris (2004) assessed genetic differentiation among population clusters of the Sardinian newt (*Euproctus platycephalus*) using mtDNA. Significant levels of genetic differentiation were detected and based on these results it was recommended that these population clusters be designated as separate management units to prevent local extinction.

Interestingly, our investigation of genetic differentiation (population clustering analysis, assignment tests, and estimates of genetic differentiation) did not support our hypothesis that the clusters would group populations in close proximity together and reveal that populations outside the clusters would be genetically unique. Rather, our results confirmed that only two of the five populations we predicted to be isolated indeed occur as genetically isolated populations. The other three *a priori* "isolated" populations (CB, FD, and RSR) grouped together comprising a single cluster despite their being separated by a large geographic distance (CB and RSR are separated by 363.3 kilometers). These results provide further support that currently unidentified variables might play an important role in influencing population connectivity and genetic structure.

In contrast to our hypothesis that the isolated populations would also be depauperate, even the *a posteriori* isolated populations did not harbor lower levels of genetic diversity relative to the clustered localities. Peripheral populations are frequently smaller in size relative to central populations and they often experience lower levels of gene flow (Nei *et al.* 1975). Due to these factors, peripheral populations

are often more prone to the effects of genetic drift resulting in reduced genetic diversity and increased levels of population genetic differentiation (Eckert *et al.* 2008; Hoffman& Blouin 2004; Lesica& Allendorf 1995). These factors then lead to the question of why we saw such high levels of genetic diversity within isolated populations in this study. First, the high levels of diversity present within isolated localities may be a result of large population sizes. Betancourt *et al* (1991) assessed the distribution of genetic diversity in the Pinyon pine tree and discovered a similar pattern of isolated populations having high levels of genetic diversity similar to central populations. They attributed the observed levels of genetic diversity to a considerable initial founding population size. This scenario is a possible explanation for the levels of genetic diversity observed in isolated populations of *N. perstriatus,* that founding populations of isolated localities were substantial and thus populations have maintained genetic diversity over time. The estimates of effective population size are supportive of this hypothesis as they indicate large effective sizes for these localities (FSMI 36 – 51.8 and GSF 25 – 92). In addition to a founding event, isolation may have occurred due to some other factor (fragmentation or environmental change) and initial population size was sufficient to maintain levels of genetic diversity. Second, longevity of individuals is known to be a contributing factor with regard to the maintenance of genetic diversity in peripheral populations. Wagner *et al.*(2011) found that peripheral populations harbored similar levels of genetic diversity as central populations in a species of steppe grass. They attributed these findings to longevity of individuals in addition to large population sizes. Longevity of individuals facilitates overlapping generations in combination with reduced fluctuation of population size, which lessens the effect of genetic drift and maintains levels of genetic diversity (Ellstrand& Elam 1993). *Notophthalmus perstriatus* generally lives for 12 – 15 years and is considered a long-lived species (Dodd 1993) and so longevity may contribute to maintaining genetic diversity in peripheral populations.

Hypothesis 3: Geographically isolated localities will have lower N_e estimates when compared to geographically clustered localities of the same species. We predicted that effective population sizes of each locality will be between 15 – 50

individuals for N. perstriatus. Based on our third hypothesis, we expected effective population sizes of each *N. perstriatus* locality would be between 15-50 individuals. As above, our data seem to reject this hypothesis. Indeed, our estimates indicated higher than expected effective sizes (COLONY ranged from 33-122 and ONeSAMP ranged from 13.8 to 757). One locality specifically (OR1) had high estimates of effective size (122-757) relative to other populations. This locality may exhibit uncharacteristically large N_e because it is located within Ordway-Swisher Biological Station (OR) which contains large areas of undisturbed suitable upland and wetland habitats. Availability of contiguous suitable habitat may result in panmixia among a number of suitable ponds leading to large estimate of effective size. Removal of this locality as an outlier, our mean effective size (COLONY 51.4, and ONeSAMP 54.4) estimates are more similar to estimates found in other salamander studies (Funk *et al.* 1999; Gill 1978). Surprisingly, our estimates for the two geographically isolated localities (GSF and FSMI) did not conform to our hypothesis that they would have lower effective sizes relative to central populations. As with estimates of genetic diversity, estimates of N_e for these populations did not differ significantly from the estimates of more central populations. These findings suggest possible gene flow from unknown localities in addition to larger than expected population sizes.

With these data we can now address our two overarching questions about *N. perstriatus* (i.e. whether the patterns of genetic differentiation were due to contemporary or historical connectivity of populations and whether a geographically isolated population would be re-founded if that population became extirpated). Based on our microsatellite analysis, the genetic connectivity (haplotypes shared between populations within the eastern region) identified by May *et al.* (2011) likely represents historical and not contemporary patterns of population connectivity. Overall, the current study identifies limited gene flow among *N. perstriatus* populations. It is likely that over time environmental conditions have changed and suitable habitat has become increasingly fragmented reducing connectivity among populations. Recently, the upland habitats (sandhill and scrub) to which *Notophthalmus perstriatus* are

restricted have experienced severe loss and fragmentation with north Florida sandhill communities decreasing by as much as 84% over 30 years (Means& Grow 1985). This has likely contributed to a change in historical versus contemporary patterns of gene flow. Consideration of habitat loss and fragmentation leads into our second question of whether locally extirpated populations would likely be re-colonized. Such a pronounced loss of suitable habitat may exacerbate isolation of peripheral populations. Moreover, the combination of habitat fragmentation with observed levels of restricted gene flow indicates that extirpated isolated populations will not likely be re-founded. These findings emphasize the importance of understanding factors contributing population persistence and conservation of *N. perstriatus*.

Overall, the findings of this study indicate that populations in the study area appear to harbor high levels of genetic diversity and that gene flow is restricted among populations of *N. perstriautus*. Multiple factors including land management and preservation of suitable habitat (including uplands and wetlands) are important to maintain natural patterns of gene flow among populations of *N. perstriatus* which is listed as a threatened species in the state of Georgia and is currently under consideration for listing by USFWS. We recommend that intensive survey efforts should be conducted to identify additional localities where *N. perstriatus* may occur (especially near peripheral populations) and to identify areas of suitable habitat which may be important areas for focused efforts on land management and conservation. While we did not find reduced levels of genetic diversity within our isolated populations, there is concern for risk of local extinction of these populations. Based on current data for geographically isolated populations (FSMI and GSF), re-colonization is highly unlikely if local extinction occurs. Identification of additional unknown populations combined with investigation regarding which environmental factors may be most important for population persistence would provide important contributions to a focused management strategy for *N. perstriatus*.

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GENERAL DISCUSSION

This study employed a comprehensive approach to assess patterns of gene flow in a rare and declining species of salamander, the striped newt (*Notophthalmus perstriatus*). First, we used combined ecological (niche modeling) and genetic methods (mtDNA *cytb*) to assess patterns of differentiation at the scale of species range. Using mtDNA (*cyt-b*), we found that haplotypes were shared between localities within each region but none were shared between regions indicating that gene flow had occurred within but not between regions. Additionally, our niche-based distribution models identified significant differences in the ecological setting between eastern and western regions. Considered in combination, the absence of evidence for recent genetic exchange with model-based support for differing ecological conditions utilized by newts between regions provides evidence that eastern and western populations are both distinct and significant. Our findings indicate that eastern and western regions exhibit both ecological and genetic differences which should be considered in development of a focused conservation strategy.

Second, we used genetic methods (microsatellites) to assess fine scale patterns of gene flow. Additionally, we wanted to investigate whether the patterns of genetic differentiation revealed by mtDNA analysis represented historical or contemporary patterns of connectivity. Our results indicated that gene flow among populations is restricted and that unidentified environmental barriers likely contribute to patterns of gene flow. Patterns of gene flow indicated by the mtDNA analysis likely represent historical and not current patterns of gene flow. *Notophthalmus perstriatus* has a complex multi-stage life cycle and is restricted to sandhill and scrub habitats. These habitat restrictions might contribute to the observed patterns of gene flow and population genetic structure. Additionally, microsatellite analysis revealed that two populations within the eastern portion of the species range are genetically isolated relative to remaining populations. Though these populations seem to harbor genetic diversity

and effective population sizes similar to more central populations, these isolated localities should be monitored. It is not likely that these populations would be re-colonized if extirpated based on patterns of limited gene flow in combination with isolation of populations.

This study provides land managers and conservation planners with information regarding ecological setting, genetic differentiation and patterns of gene flow across regions and among populations of *N. perstriatus*. Our results indicate that populations within eastern and western regions exhibit both ecological and genetic differences which should be considered when implementing conservation actions including translocation. Additionally, we have identified that this species exhibits limited gene flow among populations and suggest that environmental factors might influence population connectivity. We found that two populations appear to be genetically isolated. Further surveys should be conducted to identify unknown localities, especially near the isolated peripheral populations. While this was conducted to assess patterns of differentiation and gene flow in a species of salamander, the methodologies we employed have broad utility and are applicable across taxa and can be used to investigate patterns within and among populations of other species.

APPENDIX
TABLES AND FIGURES

Table 1 Estimates of cytb genetic diversity in *Notophthalmus perstriatus*

Site	No. samples	No. haplotypes	Nucleotide Diversity, π (SD)	Haplotype Diversity, *h* (SD)
Eastern Region				
ORA	5	2	0.00103 (0.00030)	0.600 (0.175)
MAR	8	1	0	0
PUT	24	4	0.00165 (0.00081)	0.308 (0.118)
CL1	11	7	0.01324 (0.00215)	0.873 (0.089)
CL2	1	1	na	na
STJ	2	2	na	na
BRY	13	4	0.00127 (0.00037)	0.628 (0.143)
EMM	9	4	0.00123 (0.00032)	0.639 (0.126)
Western Region				
LEO	8	2	0.00757 (0.00316)	0.643 (0.184)
BAK	5	4	0.00103 (0.00030)	0.600 (0.175)
TAY	6	1	0	0

Descriptive statistics comparing genetic variation among sampled populations (SD =standard deviation)

Table 2 Relationship between environmental variables and regional estimates of niche-related characteristics

	Correlation with PCA		Contribution to model	
Variable	Axis 1	Axis 2	West	East
Annual mean temperature*	-0.95	0.14	0	0
Temperature seasonality	0.97	-0.14	0	63.4
Maximum temperature of warmest month	0.16	-0.35	22.8	6.3
Mean temperature of coldest quarter*	-0.97	0.13	0	0
Annual precipitation	-0.50	-0.78	0	0.5
Precipitation of wettest month*	-0.88	-0.33	0	0
Precipitation of driest month	0.07	-0.91	24.1	2.2
Precipitation seasonality	-0.94	0.23	20.0	15.4
land cover	**	**	33.2	11.2

* indicate environmental variables that were removed from final models.

** Logistic regression revealed no significant relationship between land cover and PCA axis 1 ($z = -0.9$, $p = 0.37$) or PCA axis 2 ($z = 1.6$, $p = 0.10$).

Table 3 Results from randomization tests evaluating environmental differences between regions

Statistic	Comparison	p-value
Background similarity	East to West (10 pts.)	0.64
	West to East (7 pts.)	0.10
Niche identity	East vs. West	<0.001

Randomizations were compared to niche overlap (*I*) between eastern and western models ($I = 0.79$). Randomization tests showed the background environment available to newts in east and west regions is similar (background similarity), but that the niche that they utilize differs significantly between regions (niche identity).

Table 4 Locality information for sites from which tissue samples were collected for genetic analysis

Site No.	State	County	No. ponds	Site Name	Latitude	Longitude	Sample Size	Haplotypes
1	FL	Orange	1	ORA	28.77528	81.45583	5	B,H
2	FL	Marion	3	MAR	29.43806	81.78167	8	B
3	FL	Putnam	7	PUT	29.69139	82.00306	24	A,C,R,T
4	FL	Clay	1	CL1	29.84417	81.97944	11	A,E,M,N,O,P,Q
5	FL	Clay	1	CL2	30.10500	81.93083	1	Z
6	FL	St.John's	1	STJ	unavailable	unavailable	2	C,U
7	GA	Bryan	3	BRY	32.02414	81.34694	13	D,I,J,K,L
8	GA	Emanuel	1	EMM	32.52457	82.44464	9	C,G,AA
9	FL	Leon	1	LEO	30.33944	84.32056	8	S,F,V,W
10	GA	Baker	3	BAK	31.41405	84.33932	5	X,Y
11	GA	Taylor	1	TAY	32.57833	84.26944	6	Y

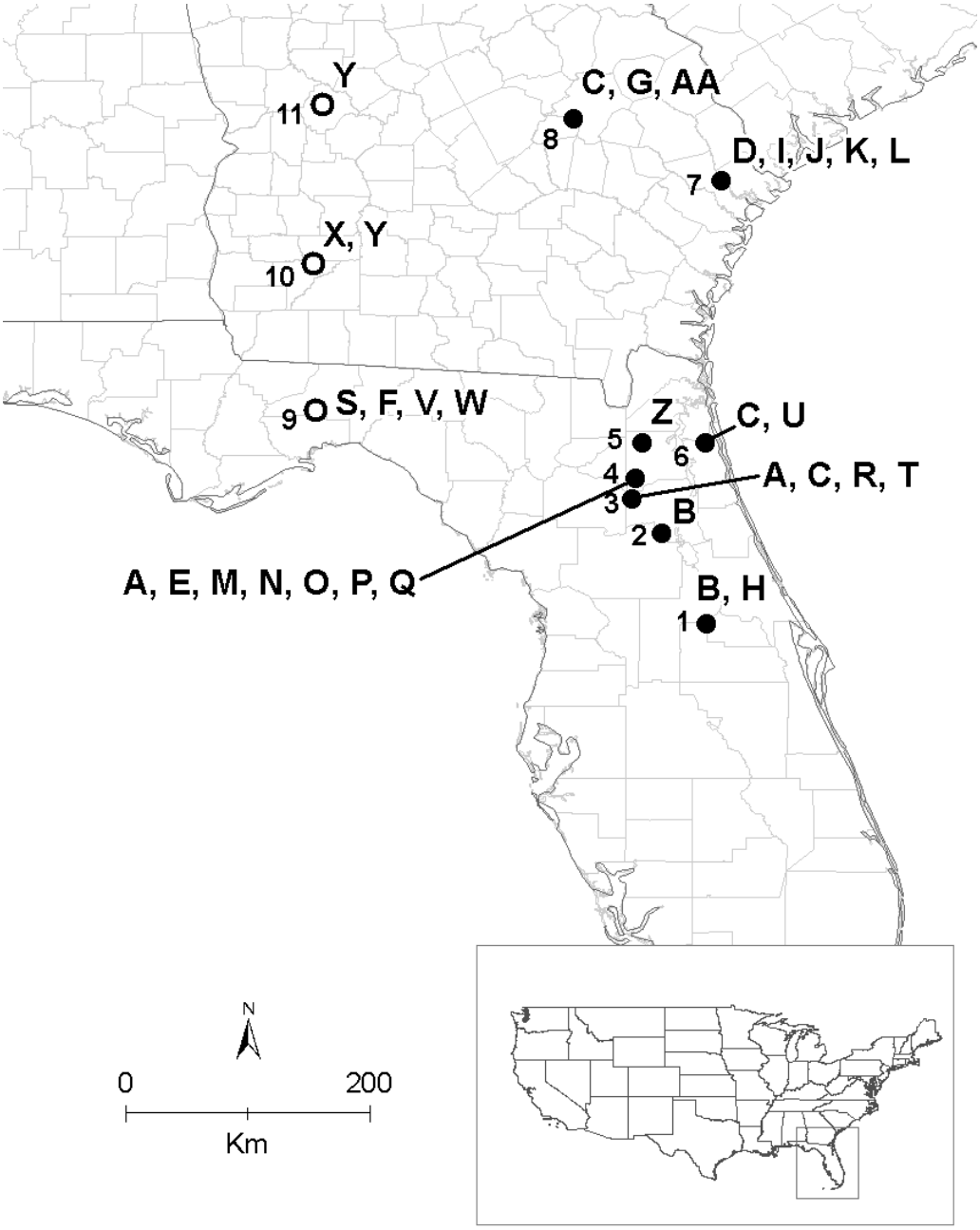
The site numbers and haplotype letters correspond to those depicted in Fig. 1.

Table 5 Actual and estimated numbers of haplotypes for each locality sampled for genetics analysis

Site Name	Actual No. Haplotypes	Estimated No. Haplotypes
Eastern Region		
ORA	2	2
MAR	1	1
PUT	4	5.9
CL1	7	11.5
CL2	1	N/A (matrix too small)
STJ	2	3
BRY	5	7.8
EMM	3	3.9
Western Region		
LEO	4	6.6
BAK	2	2
TAY	1	1

The estimated number of haplotypes represents the number of haplotypes expected at the plateau of an accumulation curve. The species-area accumulation function in PC-ORD v5.0 was used to evaluate expected haplotypes given the sample size for each locality.

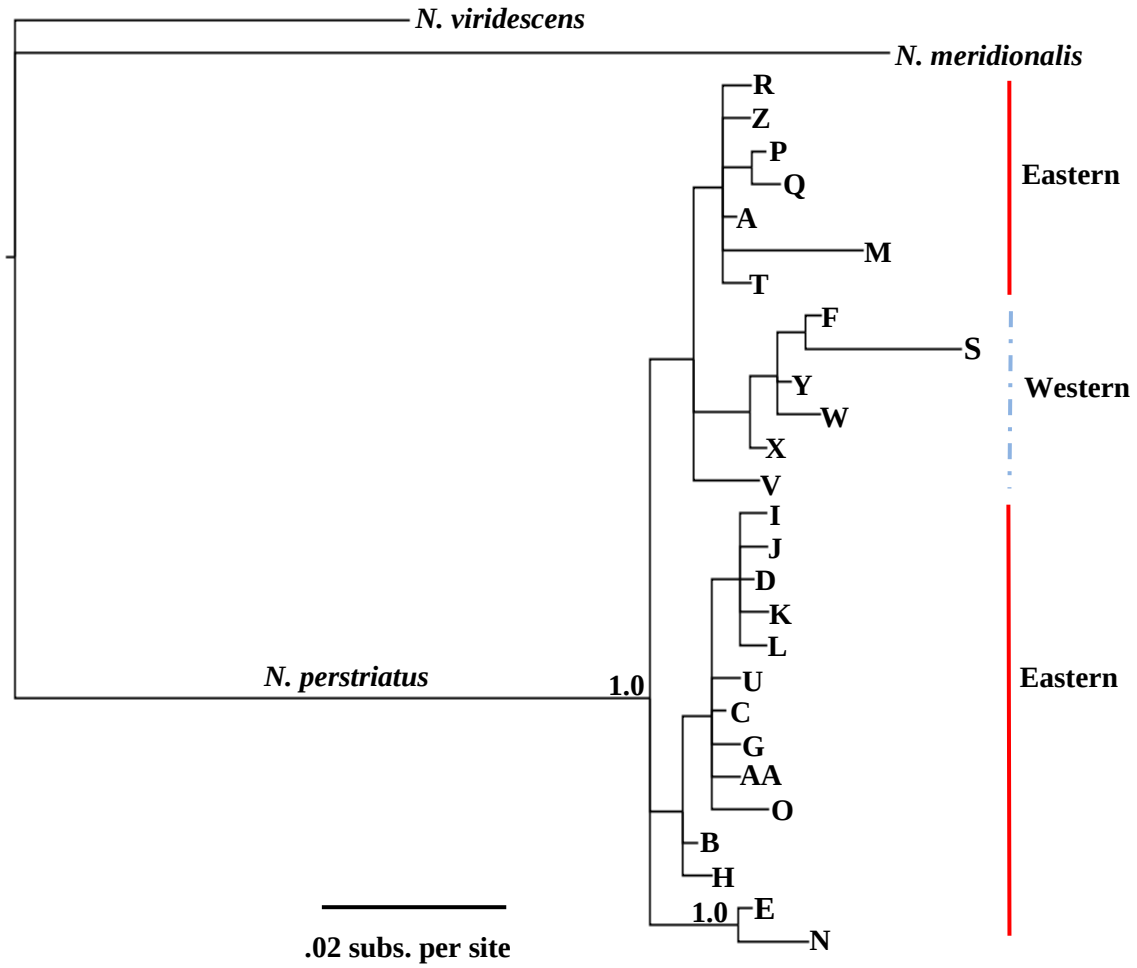
Figure 1 Map of sample localities for genetic analyses



Note that western region populations are denoted with open circles and that eastern region populations are denoted with closed circles. Letters designate haplotypes found within each population.

* Site IDs (numbers) correspond to IDs in Supplemental Table 1

Figure 2 Bayesian phylogeny of N. perstriatus samples



Letters correspond to haplotypes. Numbers represent posterior probabilities at major nodes greater than 0.95. All nodes having posterior probabilities less than 0.5 have been collapsed.

Figure 3 Relationships among haplotypes for N. perstriatus populations

Haplotype network was inferred by statistical parsimony. Circles labeled with white letters represent sampled haplotypes from the eastern region and circles labeled with black letters represent sampled haplotypes from the western region. The letters correspond to the phylogeny in Fig. 2. The size of each circle represents the relative frequency of that haplotype among all samples.

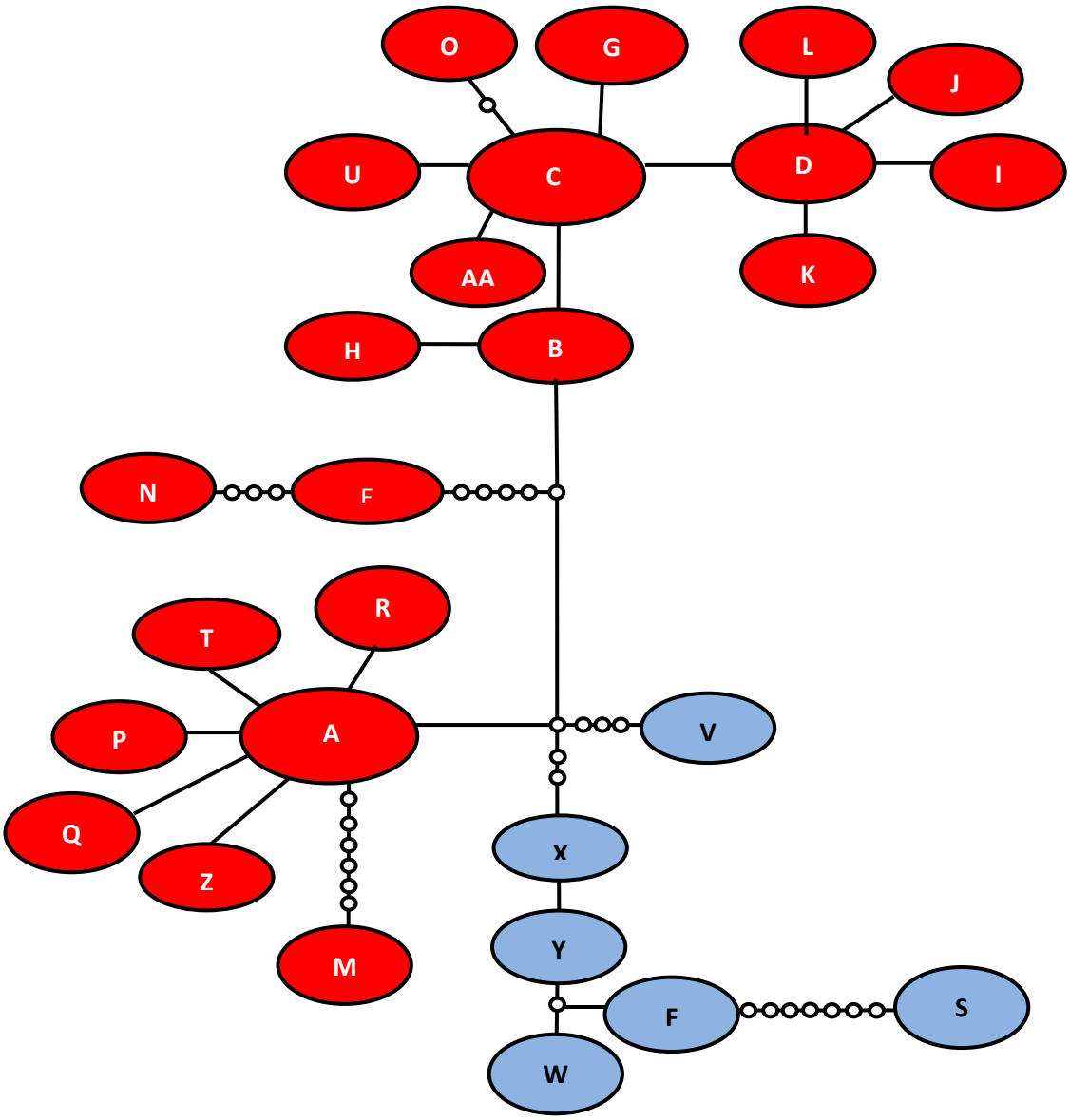
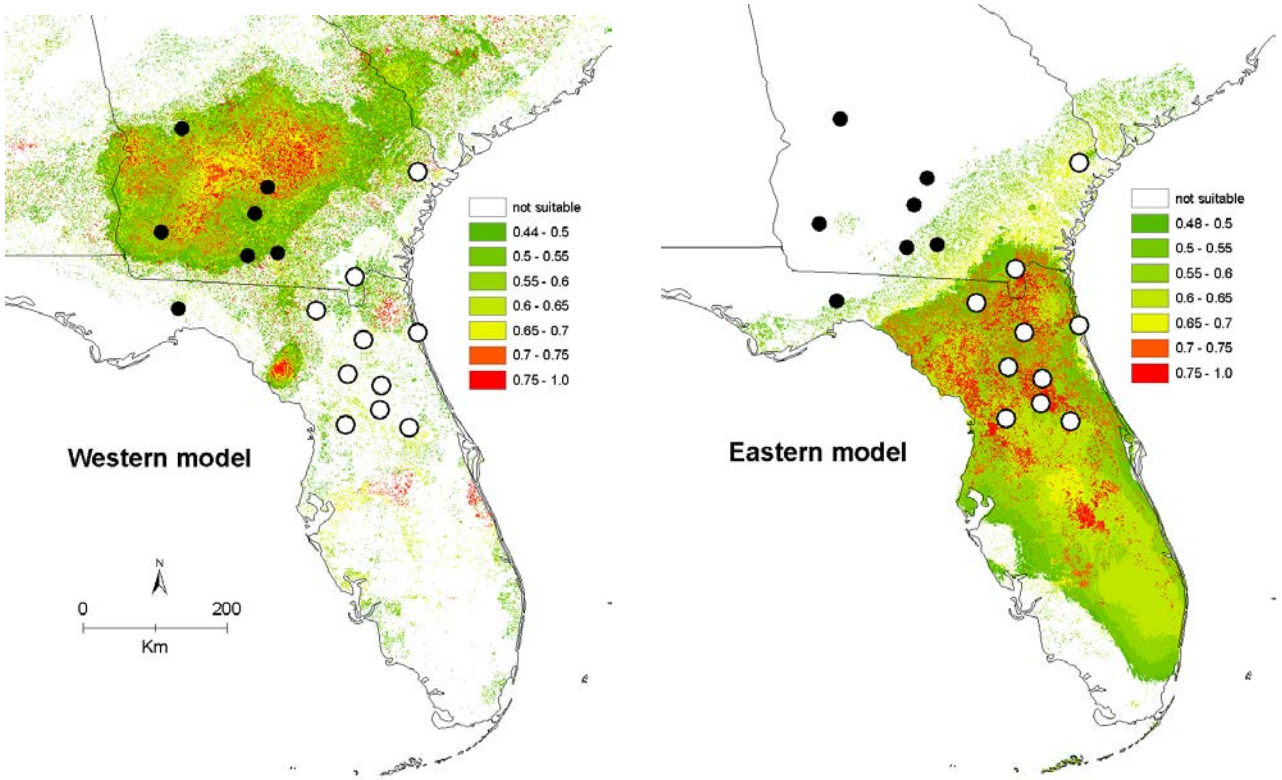


Figure 4 Niche-based distribution models for N. perstriatus



Niche-based distribution models for *N. perstriatus*. Eastern model calibrated using open circles; Western model calibrated using filled circles. Higher probability of occurrence is indicated by warm colors while lower probability of occurrence is represented by cool colors. Areas shown as "not suitable" had a probability of occurrence less than the minimum probability assigned to any occurrence point used to train each model.

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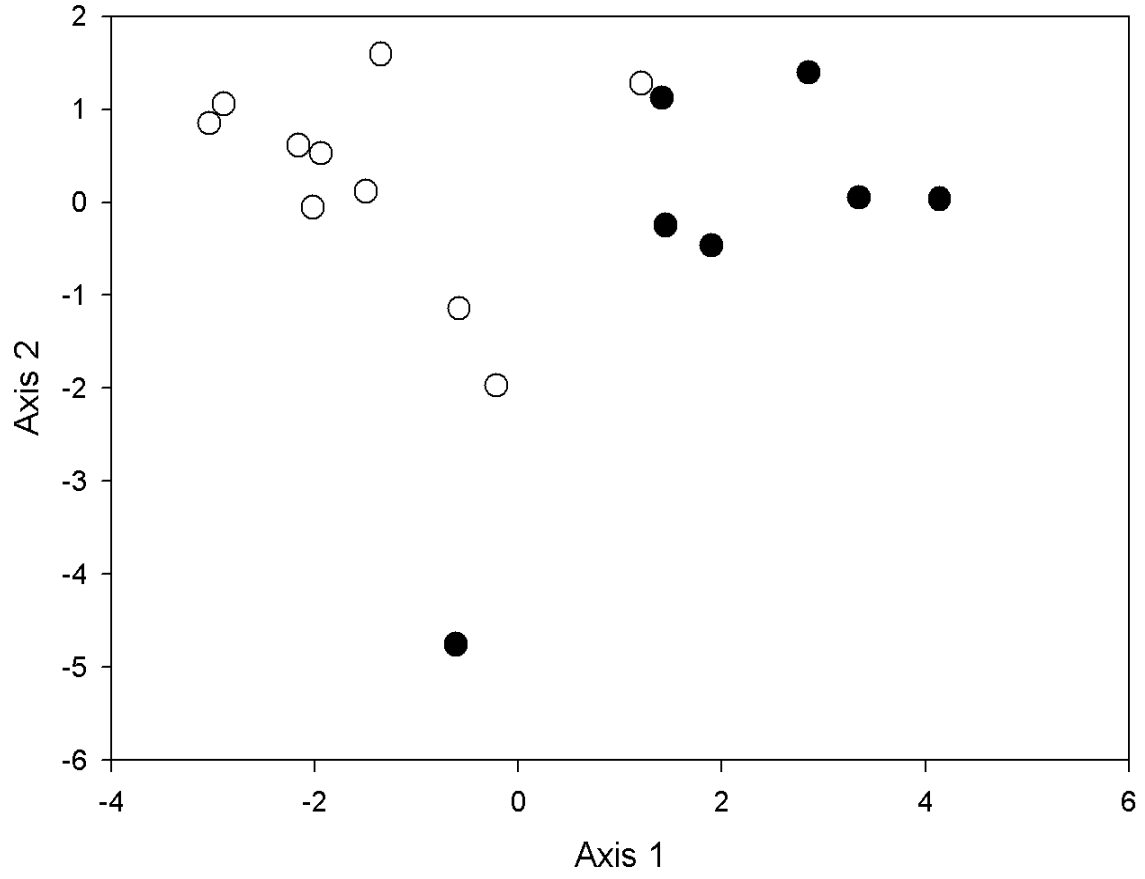
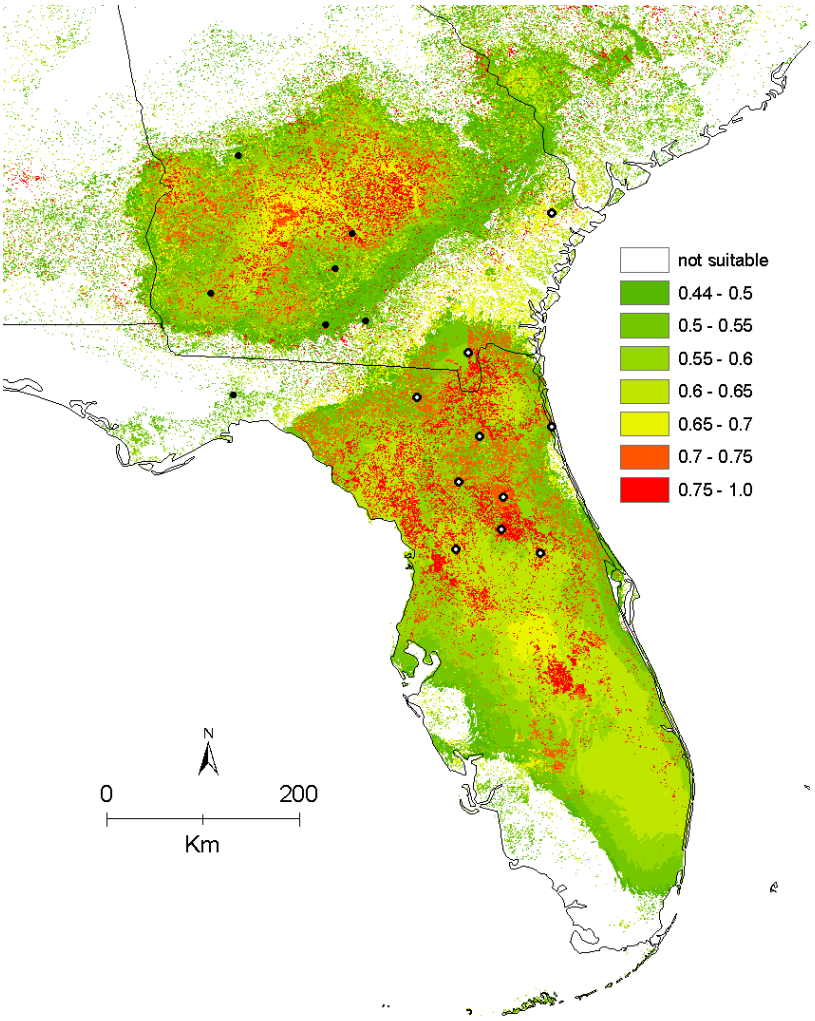
Figure 5 Principal Components Analysis (PCA) ordination of environmental data at N. perstriatus occurrences used for model calibration



Populations in the Eastern region represented by open circles and populations in the Western region represented by filled circles. Two axes were significant (Axis 1 = 53% of overall variance; Axis 2 = 25% of variance). Environmental data at occurrence points were significantly different between regions (MRPP: T = -6.5, A = 0.33, p = 0.0007). Statistical relationships between environmental variables and PCA axes are provided in Table 3

Figure 6 Eastern and western distribution models (Fig. 4) overlain on one another



This view highlights the "gap" area between regions, showing patches of suitable habitat between regions, but little contiguous suitable habitat for inter-region dispersal.

Table 6 Sample locality Information: site names, abbreviations, and geographic coordinates

Site Name	Site Abbreviation	Lat/Long
Fort Stewart Military Installation	FSMI	32.041400, -81.346940
Camp Blanding	CB	29.961389, -81.942500
Ordway Swisher Biological Station site 1	OR 1	29.691667, -82.004444
Ordway Swisher Biological Station site 2	OR 2	29.722222, -81.995556
Ordway Swisher Biological Station site 3	OR 3	29.699444, -81.959167
Goethe State Forest	GSF	29.53475, -82.597861
Ocala National Forest 1	ONF 1	29.383056, -81.793333
Ocala National Forest 2	ONF 2	29.416111, -81.761111
Ocala National Forest 3	ONF 3	29.076667, -81.809722
Ocala National Forest 4	ONF 4	29.058611, -81.560278
Faver-Dykes State Park	FD	29.680833, -81.265833
Rock Springs Run State Park	RSR	28.775280, -81.455833

Table 7 Microsatellite primers designed in this study: annealing temperatures, size ranges, and repeat motif

* Repeat sequence indicated is imperfect

Locus	Direction	Primer sequence	T_a	Size range (bp)	Repeat motif
Nper 25	F	TGTAAAACGACGGCCAGTACCTCTTTTGGGTGTGTGCT	59°C	190 - 304	$(GATA)_{15}$
Nper 25	R	CCATTGCTTAGGTGCTTGGT	59°C		$(GATA)_{15}$
Nper 26	F	TGTAAAACGACGGCCAGTGTCCAGCAAAAGGGTCAGAG	59°C	222 - 288	$*(GATA)_{12}$
Nper 26	R	AGATAGGGCAACGGACAGTG	57-60°C		$*(GATA)_{12}$
Nper 27	F	TGTAAAACGACGGCCAGTCCATTGCTTAGGTGCTTGGT	57-60°C	155 - 307	$(GATA)_{17}$
Nper 27	R	ACCTCTTTTGGGTGTGTGCT	57°C		$(GATA)_{17}$
Nper 28	F	TGTAAAACGACGGCCAGTGGTGGATTCACCAGTGGTTT	57°C	184 - 312	$(GATA)_{10}$
Nper 28	R	GGCTCTGAAGTGCCTGTTTC	57°C		$(GATA)_{10}$
Nper 29	F	TGTAAAACGACGGCCAGTGTGGATTCACCAGTGGCTTT	57°C	211 - 311	$*(GATA)_{11}$
Nper 29	R	ACCTCTTTTGGGTGTGTGCT	57°C		$*(GATA)_{11}$
Nper 30	F	TGTAAAACGACGGCCAGTGGGACACACATTGTGCCATT	59°C	162 - 308	$(GATA)_{18}$
Nper 30	R	CTCCTATGGAAACCCTGACCT	59°C		$(GATA)_{18}$
Nper 3b	F	TGTAAAACGACGGCCAGTGCTAACTCCCAATGCGGATA	51°C	146 - 255	$(GATA)_{13}$
Nper 3b	R	CAGCCCCTTTTTGAGACAGA	51°C		$(GATA)_{13}$
Nper 4	F	TGT AAA ACG ACG GCC AGT GGC TCT GAA GTG CCT GTT TC	51°C	210 - 318	$*(GATA)_{14}$
Nper 4	R	GGT GGA TTC ACC AGT GGT TT	51°C		$*(GATA)_{14}$
Nper 5	F	TGT AAA ACG ACG GCC AGT TTG CCG TGC TCA TAT TTT CA	51°C	232 - 306	$(GATA)_9$
Nper 5	R	TTC ATT CCA CCC CTG GTA AG	51°C		$(GATA)_9$

Table 8 Sample sizes, observed and expected heterozygosities, and effective population sizes

Population	Sample Size	H_E	H_o	N_e Colony	N_e Onesamp (2000)
FSMI	24	0.874	0.954	36	51.8
				(21-69)	(37.5 - 98.6)
CB*	16	0.882	0.932	53	13.8
				(27-180)	(10.7 - 22.8)
OR 1	99	0.892	0.935	122	757
				(93 - 166)	(383.0 - 2385.8)
OR 2	29	0.868	0.959	33	27.2
				(20-59)	(21.1 - 46.2)
OR 3*	17	0.901	0.941	54	15.2
				(28-162)	(10.2 - 34.7)
GSF	23	0.862	0.957	92	25.1
				(50-287)	(19.29 - 39.89)
ONF 1	27	0.838	0.946	40	63.2
				(24-74)	(36.4 - 84.5)
ONF 2*	15	0.844	0.933	30	18
				(15-77)	(10.3 - 28.3)
ONF 3	41	0.887	0.960	68	82.7
				(45-110)	(52.4 - 120.6)
ONF 4	23	0.888	0.940	31	55.4
				(18-62)	(23.8 - 72.4)
FD	31	0.896	0.965	89	124.3
				(55-164)	(78.6 - 174.6)
RSR	24	0.905	0.955	92	62.8
				(54 -240)	(48.4 - 107.7)

* = fewer than the recommended 20 individuals for ONeSAMP

Table 9 Pairwise matrix of geographic distance (km) above the diagonal and genetic distances (R_{ST}) below the diagonal

Pop	FSMI	CB	OR1	OR2	OR3	GSF	ONF1	ONF2	ONF3	ONF4	FD	RSR
FSMI	--	238.1	268.7	265.2	266.9	303.3	298.7	294.6	332.6	332.3	262.6	363.3
CB	0.385	--	30.58	27.08	29.17	79.08	65.9	63.11	99.21	107	77.35	140.1
OR1	0.360	0.116	--	3.504	4.458	59.96	39.93	38.64	70.94	82.51	71.36	115
OR2	0.457	0.142	0.163	--	4.332	61.84	42.48	40.9	74.01	84.99	70.63	117.6
OR3	0.334	0.174	0.027	0.284	--	64.4	38.67	36.87	70.75	81.06	67.01	113.8
GSF	0.558	0.278	0.281	0.264	0.292	--	79.7	82.07	91.84	113.7	129.8	139.4
ONF1	0.443	0.226	0.085	0.331	0.145	0.377	--	4.822	34.11	42.58	60.84	75.12
ONF2	0.374	0.075	0.042	0.182	0.115	0.327	0.106	--	38.04	44.27	56.23	77.18
ONF3	0.288	0.124	0.060	0.222	0.077	0.310	0.090	0.038	--	42.58	74.84	75.12
ONF4	0.389	0.143	0.167	0.169	0.177	0.356	0.173	0.097	0.178	--	74.84	33.1
FD	0.337	0.065	0.186	0.138	0.231	0.287	0.163	0.059	0.122	0.141	--	102.4
RSR	0.119	0.002	0.082	0.035	0.050	0.298	0.052	0.002	0.031	0.082	0.008	--

Table 10 Results of GENECLASS2 assignment tests

	Assigned Population												
Source Population	**FSMI***	**CB**	**OR1**	**OR2**	**OR3**	**GSF***	**ONF1**	**ONF2***	**ONF3***	**ONF4**	**FD**	**RSR**	**N =**
FSMI*	24												24
CB		13	1	2									16
OR1			97	1	1								99
OR2				29									29
OR3		1	4		10							2	17
GSF*						23							23
ONF1				1			26						27
ONF2*								15					15
ONF3*									41				41
ONF4			1							22			23
FD			1								29	1	31
RSR					2						2	20	24

* Indicates populations that neither give nor receive migrants

Figure 7 Map of sample localities of *Notophthalmus perstriatus*

Figure 8 Plot of $R_{ST}/(1- R_{ST})$ over geographic distance (km) among all localities

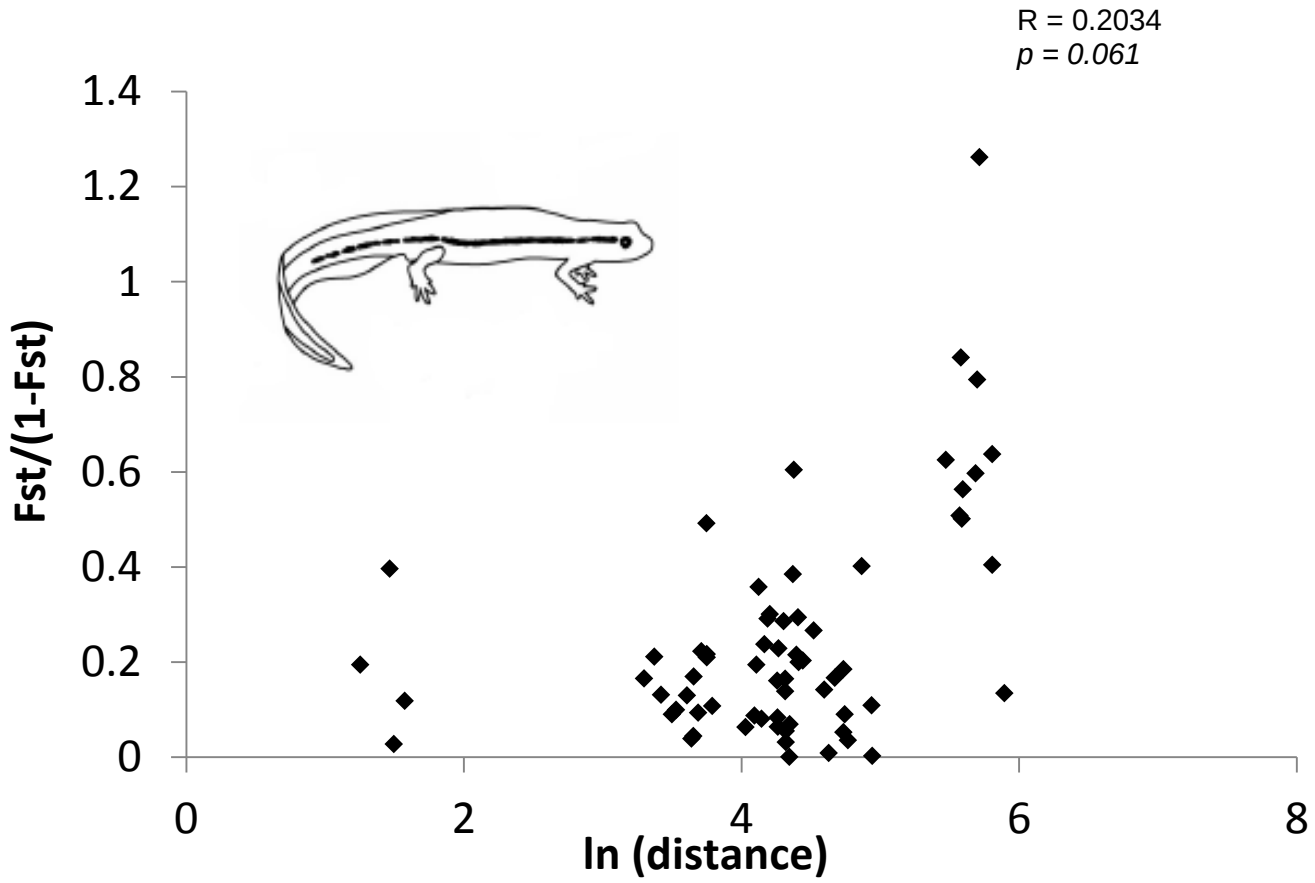


Figure 9 Map of hierarchical clustering analysis

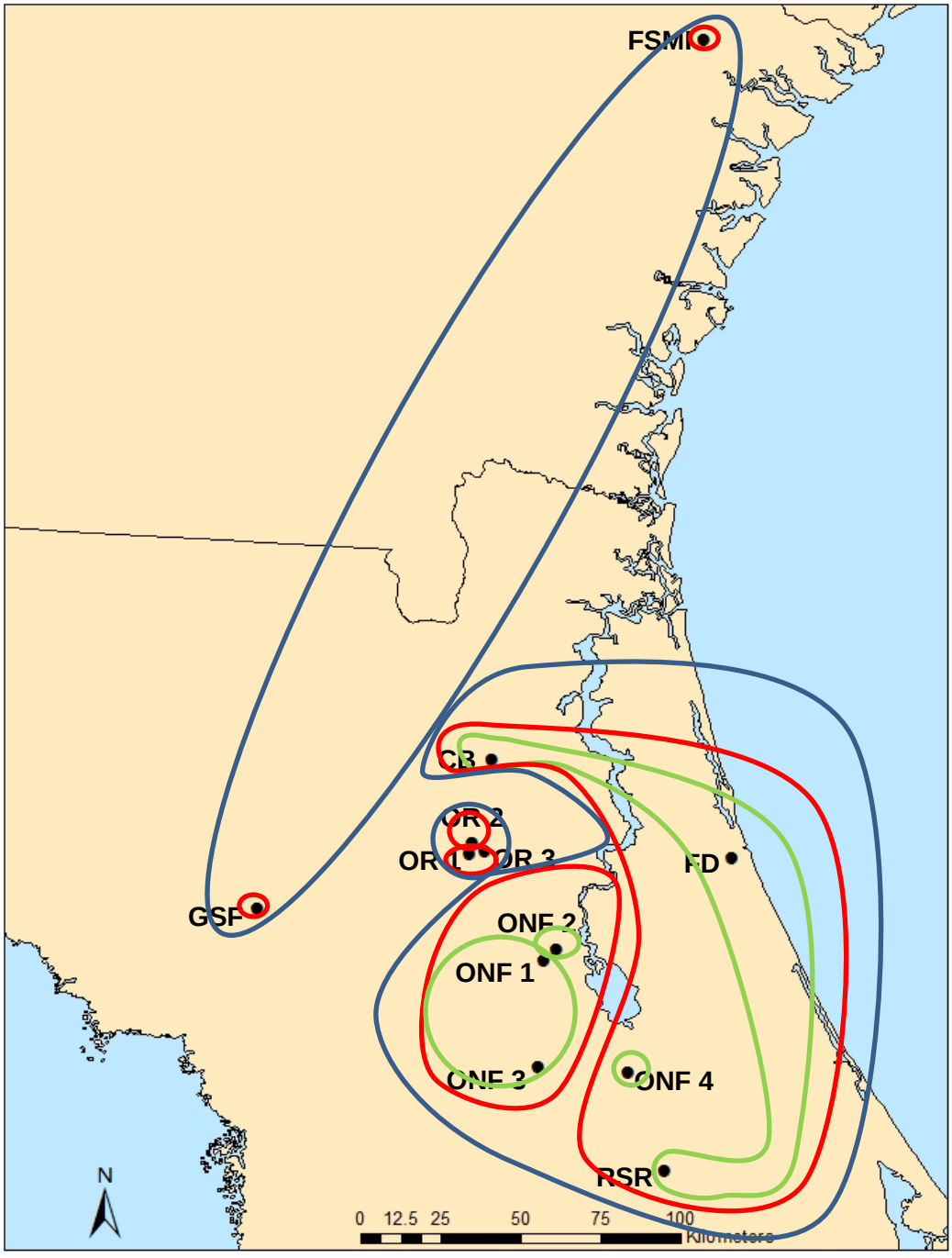
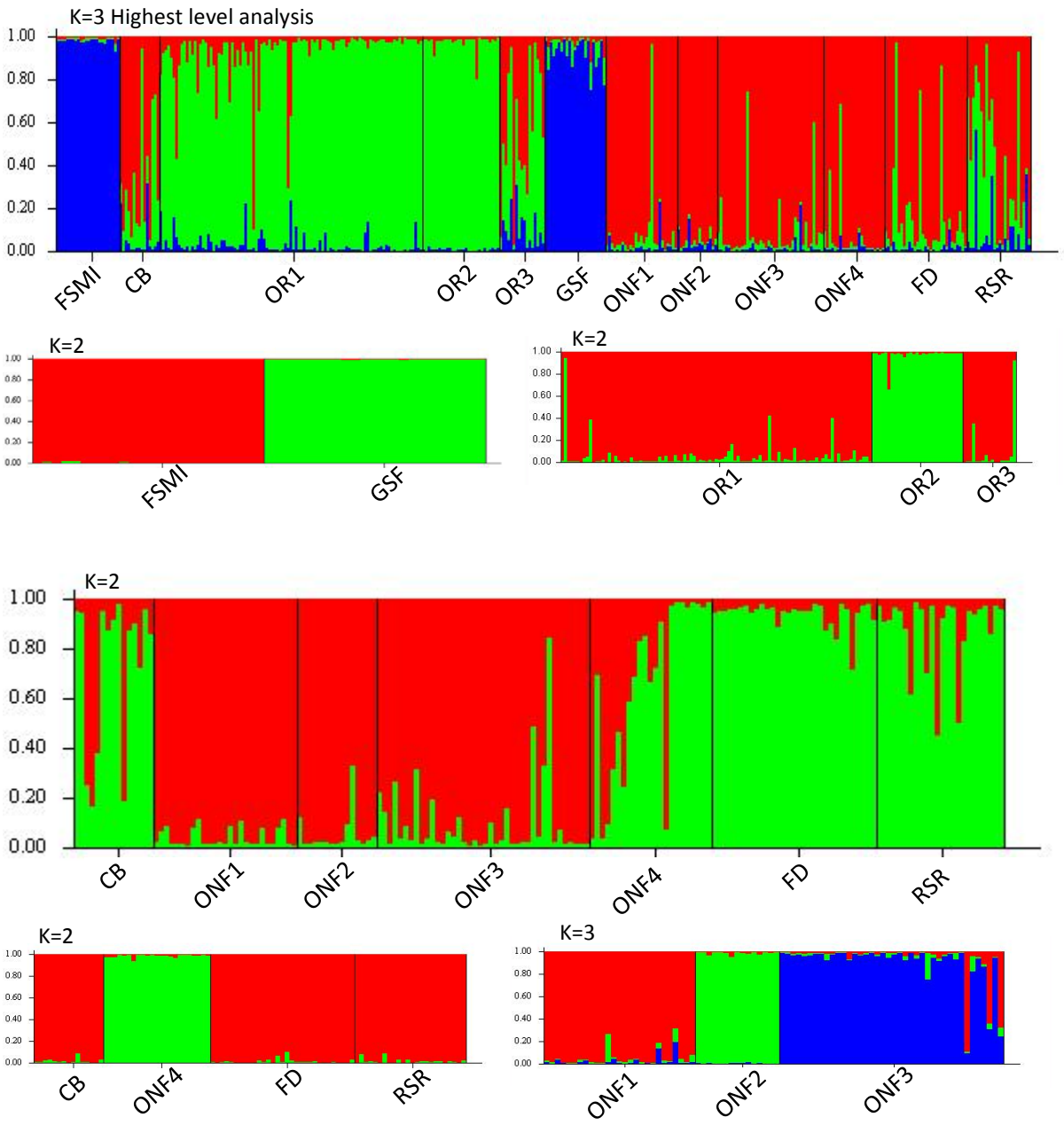


Figure 10 Membership coefficients as estimated in STRUCTURE



Results of hierarchical clustering analysis where each column represents an individual and the membership coefficient for each K is indicated by the relative proportion of each color.