Final Report for Illinois Wildlife Preservation Fund Contract
IDNR 11-017W
Genetic Variation in Populations of the Four-toed Salamander at the Middle Fork State Fish and Wildlife Area

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Summary

Understanding and estimating gene flow between populations of *H. scutatum* may be vital to the conservation of this species. A total of 153 tissue samples were collected from three subpopulations of *H. scutatum* at the Middlefork State Fish and Wildlife Area and 28 microsatellite loci from four salamander species were screened for their potential usefulness. Of these, only 7 proved useful for this project. A subsample of individuals from each subpopulation were genotyped and MICROCHECKER v.2.2.3 3 (Oosterhout et al. 2004) and GENEPOP v3.4 (Raymond & Rousset, 1995) and were used to check for scoring errors, null alleles, and large allele dropout and to test the assumptions of Hardy-Weinberg equilibrium and linkage equilibrium respectively. GENEPOP v3.4 was also used to calculate the inbreeding coefficient, F_{IS}, for each population and GENEALEX v6.5 (Peakall and Smouse 2012) was used to evaluate genetic diversity by calculating the allelic diversity and heterozygosity for each population. These subpopulations of *H. scutatum* may be useful as a model for future restoration sites for this and other similar salamander species.

Project Objectives

1. Collect tissue samples from individuals at each site.
2. Estimate genetic variation within each population.

Introduction

Globally amphibians are declining at a greater rate than other vertebrate taxa (Alford & Richards 1999, Barinaga 1990). In Illinois alone, eight of the 41 native amphibian species are listed as endangered or threatened and an additional six species have been identified as conservation priorities (Illinois Wildlife Action Plan 2005). The long term survival of any species depends on its ability to tolerate or adapt to changes in its environment. Commonly, such changes involve short-term natural processes, such as seasonal weather changes, and species in temperate climates evolved phenotypic plasticity to respond to these environmental fluctuations. Directional shifts of environmental conditions, such as lower average temperatures towards colder climate or increased precipitation, generally occurred slowly and species could track and adapt to these long-term changes. But human impacts with long-term consequences, including habitat fragmentation or destruction, introduction of exotic species, and environmental pollution, have occurred with increasing frequency and at an accelerated rate and tested the ability of species to adapt.

The capacity or evolutionary flexibility of a species to cope with new environmental challenges depends on the genetic diversity present in the population. As populations decrease in size and become increasingly isolated, alleles are lost due to genetic drift, and inbreeding as a result of decreased genetic diversity can become a serious problem. Because of their limited lifetime dispersal, plethodontid salamanders are especially vulnerable to habitat fragmentation resulting in isolated populations and decreased genetic diversity. Microsatellites are especially useful for assessing genetic diversity because they are highly polymorphic, can be examined with PCR-based techniques, and are relatively inexpensive.

The four-toed salamander, *Hemidactylium scutatum,* is a plethodontid species with a large geographic distribution ranging as far north as Nova Scotia, south to Florida,

and as far west as Oklahoma and Missouri (Petranka 1998). The distribution of this species is unusual among plethodontids, however, in that it is characterized by patchy occurrence in the southern and western sections of its range. Even in the continuous portions of its distribution *H. scutatum* populations tend to centralize around patches of suitable habitat due to the specific breeding requirements of this species. An understanding of the genetic variation present in the remaining Illinois populations of *H. scutatum* may be important for the future management and conservation decisions for this state-threatened species and for other plethodontids with limited dispersal capabilities.

The objectives of this study are to collect tissue samples from and estimate genetic variation within populations of *H. scutatum* in the Middle Fork State Fish and Wildlife Area. Based on these results, the level of inbreeding within the populations will be determined. These findings will be important for future assessments of the health of *H. scutatum* populations within Illinois and may be used to advise future management and conservation decisions.

Materials and Methods

I conducted visual encounter surveys for *H. scutatum* at three localities (Northern Marsh, Sweet Flag Marsh, and Silvery Seep) in the Middle Fork State Fish and Wildlife Area in Vermilion County, IL from March – November of 2009, 2010, 2011, and 2012. For each capture, I recorded the GPS location, salamander gender and size, and photographed the underside of the individual for re-capture identification purposes. Small tail clips (1-5 mm) were collected from individuals with a mass larger than 0.18g using sterilized clippers and stored in EtOH at -80°C. The target sample size for each breeding pool was 30 tissue samples. I isolated whole genomic DNA from tail clips using a Qiagen DNEasy Extraction kit following the manufacture's protocol with the exception that tissue samples were digested overnight in proteinase K.

I screened 28 microsatellite loci from four salamander species for their potential usefulness in amplification of target DNA and assessing gene flow in in *H. scutatum*. Seven of these loci (HS3a, HS3b, HS5, HS7, HS8, HS14, and HS15) were developed for *H. scutatum* by the Reid Harris lab at James Madison University. The remaining loci were initially designed for other salamander species, including 11 for *Plethodon elongatus* (PE0, PE1, PE3, PE4, PE5, PE7, PE8, PE9, PE10, PE11, and PE12; Degross et al. 2004), seven for *P. cinereus* (PC1, PC2, PC3, PC4, PC5, PC6, and PC7; Connors & Cabe 2003), and three for *Dicamptodon tenebrosus* (DT4, DT5, and DT8; Curtis & Taylor 2001). Initial screening was assessed using gel electrophoresis and successful primers were assigned to fluorescent dyes. To further assess and examine the usefulness of these loci in terms of polymorphism, loci specificity, and signal strength and to genotype individuals using these loci, fragment analysis of resulting PCR products was conducted on an Applied Biosystems 3730xl Analyzer at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois. An internal size standard (Liz 500) was included with each sample to determine fragment length. I scored alleles using GENEMAPPER v3.5 and genotypes compiled in an excel spreadsheet.

Genotype scoring errors, the presence of null alleles, and the occurrence of large allele dropout were assessed via MICROCHECKER v2.2.3 (Oosterhout et al. 2004). I used GENEPOP v3.4 (Raymond and Rousset 1995) to test each locus for Hardy-Weinberg equilibrium and linkage disequilibrium and to estimate the inbreeding coefficient, F_{IS}, for

each population. I used GENEALEX v6.5 (Peakall and Smouse 2012) to evaluate genetic diversity by calculating the allelic diversity and heterozygosity for each population.

Results

A total of 153 unique tissue samples (Table 1) were collected from *H. scutatum* at three distinct locations in the Middlefork State Fish and Wildlife Area (Figure 1). Of the 28 microsatellite loci screened, 20 initially amplified via gel electrophoresis (Figure 2, Table 2) and were subsequently assigned to a fluorescent dye. When screened using fragment analysis at the Keck Center, eight of these loci, HS3a, HS5, HS7, HS8, HS14, HS15, PC1, and PC2, demonstrated variability and appeared to be potentially useful in assessing the gene flow and population genetics of *H. scutatum* and were used in genetic analyses. The remaining 12 loci, HS3b, PE0, PE3, PE7, PE8, PE9, PE10, PE11, PC5, DT4, DT5, and DT8, had additional scoring problems, including inconsistent or weak amplification and artifacts. As a result, I excluded these from this analysis, but additional optimization could potentially render these loci useful in future studies of *H. scutatum*. Because amplification issues sometimes resulted in incomplete genotypes, a subset of tissue samples with the most complete genotypes were chosen for each location to be used in the genetic analysis (Table 3). While no large allele dropout was detected for any of the eight loci, a homozygote excess, possibly suggestive of null alleles, was found at locus HS7 in the Sweet Flag Marsh population. As a result, this was the only marker/population combination for which a violation of Hardy-Weinberg equilibrium was indicated (Table 4). HS7 was also involved in the only marker pair/population combination (HS7 & PC1/Silvery Seep) for which linkage disequilibrium was suggested (Table 5). Because a test analysis run without HS7 resulted in no effective difference in results and because the p-value for the linkage disequilibrium test was not highly significant, HS7 was included in subsequent analyses. The number of observed alleles and number of effective alleles found at each locus in each population are shown in Table 6 and a summary of the allelic diversity per population, including mean number of alleles, mean number of effective alleles, mean number of rare alleles, and mean number of private alleles, is illustrated in Figure 3. While the mean number of alleles ranged from 2.750 (Northern Marsh) to 8.500 (Sweet Flag Marsh), the mean effective numbers of alleles in each population were relatively similar and ranged only from 2.433 (Northern Marsh) to 3.782 (Silvery Seep). The genetic diversity as measured by heterozygosity, and the inbreeding coefficients, FIS, for each population are shown in Table 7 and Table 8 respectively.

Discussion

While the goal of at least 30 tissue samples was achieved for both the Sweet Flag Marsh and the Silvery Seep populations, obtaining a sufficient samples size from the Northern Marsh was challenging. While this may be an indication of low population density, the drought-like conditions of the 2012 sampling season probably contributed to the difficulty in finding individuals.

Microsatellite cross-amplification is notoriously difficult in amphibians as a result of their large genome size (Garner 2002). Because of this, it is not surprising that so many of the loci from species other than *H. scutatum* failed to amplify initially or were not able to be scored. Despite this, the results obtained using the reduced set of seven loci

should be relatively robust as a number of other studies have successfully used similar numbers of microsatellites to assess salamander population structure (i.e. 6 loci (Cabe et al. 2007), 7 loci (Giordano et al 2007, Noël et al. 2007), and 8 loci (Spear et al. 2005, Purrenhage et al. 2009)).

Because the observed levels of heterozygosity were greater than the expected levels of heterozygosity in every population and because large numbers of alleles were found for each locus in each population, there appears to be no inbreeding and relatively high levels of genetic diversity for this species in these locations. Similarly, all values for F_{IS} were very close to zero, suggesting that no more inbreeding is occurring than would be expected by chance. While high levels of genetic diversity could be caused by a high mutation rate, other research suggests that these three "populations" are actually all part of one larger population and that gene flow may readily occur between them or has occurred between them in the recent past (Berkey unpubl. data).

Although *H. scutatum* is state threatened in Illinois, the relatively high levels of expected heterozygosity and effectively zero values for F_{IS} in each population found in this study suggests that these subpopulations are healthy in terms of genetic diversity and inbreeding. Because this species is of conservation concern, this site could be used as a model for future restoration efforts for *H. scutatum* and other similar salamander species and the levels of heterozygosity found here could serve as a baseline data to assess future management needs.

Table 1. Results of Visual Encounter Surveys

Population	**Individuals Encountered**	**No. of Tissue Samples**
Northern Marsh	3	2
Sweet Flag Marsh	178	101
Silvery Seep	61	50
Total	242	153

Table 2. Amplification and Potential Usefulness of Screened Microsatellites

Species	GenBank #	Locus	Citation	Initial Screening Results	Final Usefulness
Hemidactylium scutatum	N/A	HS3a	McGrath (1996)	+	+
	N/A	HS3b		+	Ambiguous
	N/A	HS5	Schrecengost (1998)	+	+
	N/A	HS7		+	+
	N/A	HS8		+	+
	N/A	HS14		+	+
	N/A	HS15	Reid (1994)	+	+
Plethodon elongatus	AY532595	PE0	Degross et al. (2004)	+	Ambiguous
	AY532596	PE1		-	-
	AY532597	PE3		+	Ambiguous
	AY532598	PE4		-	-
	AY532599	PE5		-	-
	AY532600	PE7		+	Ambiguous
	AY532601	PE8		+	Ambiguous
	AY532602	PE9		+	-
	AY532603	PE10		+	-
	AY532604	PE11		+	-
	AY532605	PE12		-	-
P. cinereus	AY151377	PC1	Connors & Cabe (2003)	+	+
	AY151374	PC2		+	+
	AY151380	PC3		-	-
	AY151379	PC4		-	-
	AY151372	PC5		+	-

	AY151376	PC6		-	-
	AY151373	PC7		-	-
Dicamptodon tenebrosus	AF149305	DT4	Curtis & Taylor (2001)	+	Ambiguous
	AF150725	DT5		+	Ambiguous
	AF150728	DT8		+	Ambiguous

Table 3. Final Samples Sizes used in Genetic Analysis

Population	**Sample Size**
Northern Marsh	2
Sweet Flag Marsh	53
Silvery Seep	31
Total	86

Table 4. Results of Hardy-Weinberg Tests (H1=heterozygote deficient)

Population	**Locus**	**P-value***
Northern Marsh	HS14	1.0000
	HS15	N/A**
	HS3b	1.0000
	HS5	0.3333
	HS7	1.0000
	HS8	1.0000
	PC1	N/A**
	PC2	N/A**
Sweet Flag Marsh	HS14	0.0149
	HS15	0.4538
	HS3b	0.5702
	HS5	0.1485
	HS7	**0.0000**
	HS8	0.6447
	PC1	0.7858
	PC2	0.1245
Silvery Seep	HS14	0.3191
	HS15	0.4905
	HS3b	0.0933
	HS5	0.8633
	HS7	0.1006
	HS8	0.0118
	PC1	0.1325
	PC2	0.3064

*To reduce the chance of Type I error due to multiple comparisons, the p-value needed for significance is $p<0.00208$ (Bonferroni correction).
**Not available due to small sample size.

Table 5. Results of Tests for Linkage Disequilibrium

Population*	Locus #1	Locus #2	P-value**
SF	HS14	HS15	0.505803
SF	HS14	HS3b	0.381346
SF	HS15	HS3b	0.292471
SF	HS14	HS5	0.537894
SF	HS15	HS5	0.784105
SF	HS3b	HS5	0.597005
SF	HS14	HS7	0.359231
SF	HS15	HS7	0.50257
SF	HS3b	HS7	0.917335
SF	HS5	HS7	0.624627
SF	HS14	HS8	0.034469
SF	HS15	HS8	0.221889
SF	HS3b	HS8	0.060098
SF	HS5	HS8	0.463774
SF	HS7	HS8	0.144583
SF	HS14	PC1	0.593275
SF	HS15	PC1	0.210328
SF	HS3b	PC1	0.056382
SF	HS5	PC1	0.407901
SF	HS7	PC1	0.370698
SF	HS8	PC1	0.582916
SF	HS14	PC2	0.843522
SF	HS15	PC2	0.221361
SF	HS3b	PC2	0.429495
SF	HS5	PC2	0.579298
SF	HS7	PC2	0.058932
SF	HS8	PC2	0.349058
SF	PC1	PC2	0.291652
SS	HS14	HS15	1.000000
SS	HS14	HS3b	0.678051
SS	HS15	HS3b	0.848423
SS	HS14	HS5	0.362071
SS	HS15	HS5	0.548622
SS	HS3b	HS5	0.276448
SS	HS14	HS7	0.656383
SS	HS15	HS7	0.697767
SS	HS3b	HS7	0.929021
SS	HS5	HS7	0.372291
SS	HS14	HS8	0.97592
SS	HS15	HS8	0.903865
SS	HS3b	HS8	0.358231
SS	HS5	HS8	0.078494
SS	HS7	HS8	0.496616

SS	HS14	PC1	0.715394
SS	HS15	PC1	0.65083
SS	HS3b	PC1	0.64202
SS	HS5	PC1	0.292805
SS	**HS7**	**PC1**	**0.000293**
SS	HS8	PC1	0.585925
SS	HS14	PC2	0.361297
SS	HS15	PC2	0.642569
SS	HS3b	PC2	0.479241
SS	HS5	PC2	0.772166
SS	HS7	PC2	0.744442
SS	HS8	PC2	0.416838
SS	PC1	PC2	0.438524

*P-values for Northern Marsh population not available due to small sample size.

**To reduce the chance of Type I error due to multiple comparisons, the p-value needed for significance is $p<0.000595$ (Bonferroni correction).

Table 6. Allelic Diversity per Population

Population	Locus	Sample Size (N)	No. Alleles (Na)	No. Effective Alleles (Ne)
Northern Marsh	HS14	2	3.000	2.667
	HS15	2	2.000	1.600
	HS3b	2	3.000	2.667
	HS5	2	3.000	2.667
	HS7	2	4.000	4.000
	HS8	2	3.000	2.667
	PC1	2	2.000	1.600
	PC2	2	2.000	1.600
Sweet Flag Marsh	HS14	53	6.000	2.746
	HS15	53	15.000	3.192
	HS3b	53	8.000	3.806
	HS5	53	9.000	4.594
	HS7	53	14.000	3.249
	HS8	53	8.000	4.379
	PC1	53	2.000	1.994
	PC2	53	6.000	2.117
Silvery Seep	HS14	31	6.000	3.348
	HS15	31	14.000	5.555
	HS3b	31	10.000	2.696
	HS5	31	8.000	5.414
	HS7	31	14.000	4.398
	HS8	31	7.000	4.449
	PC1	31	2.000	1.875
	PC2	31	5.000	2.522

Table 7. Genetic Diversity as Measured by Heterozygosity

Population		**Observed Het.**	**Expected Het.**	**Unbiased Expected Het.**
Northern Marsh	Mean	0.750	0.547	0.729
	SE	0.094	0.052	0.070
Sweet Flag Marsh	Mean	0.667	0.666	0.673
	SE	0.050	0.038	0.038
Silvery Seep	Mean	0.734	0.698	0.709
	SE	0.036	0.044	0.045
All Populations	Mean	0.717	0.637	0.704
	SE	0.037	0.028	0.029

Table 8. Inbreeding Coefficients (F_{IS}) for Each Population

Population	**F_{IS}**
Northern Marsh	-0.0435
Sweet Flag Marsh	0.0081
Silvery Seep	-0.0351

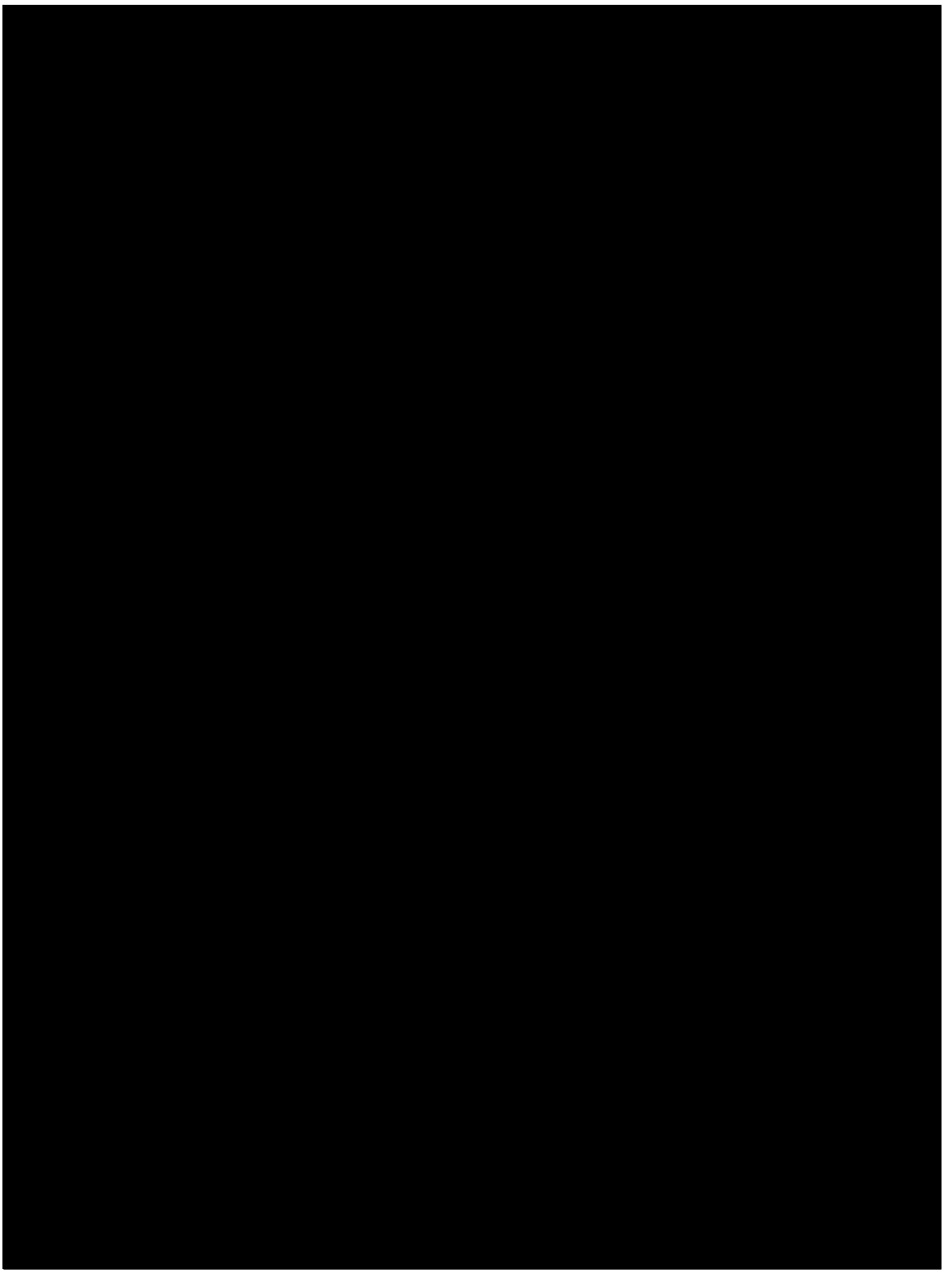
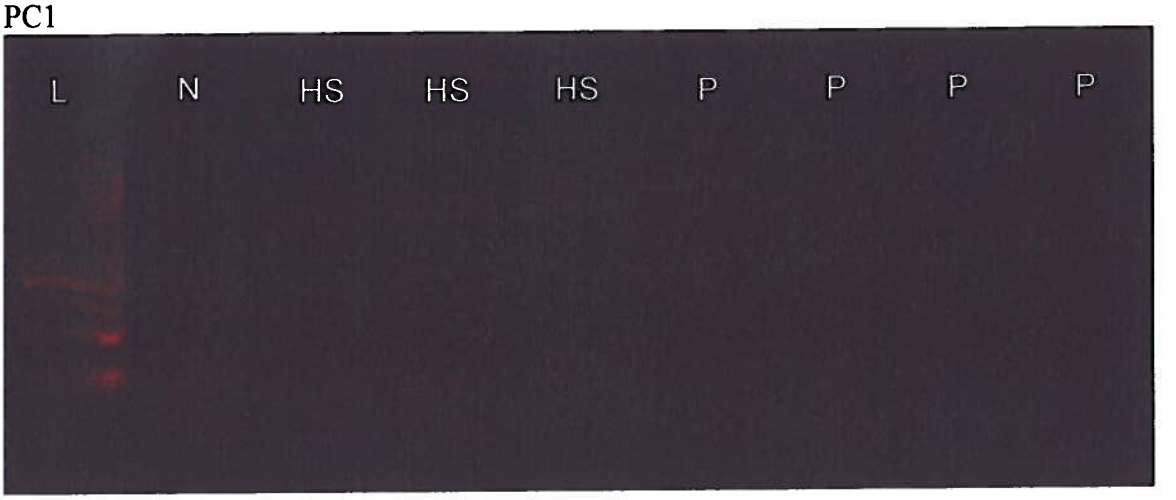
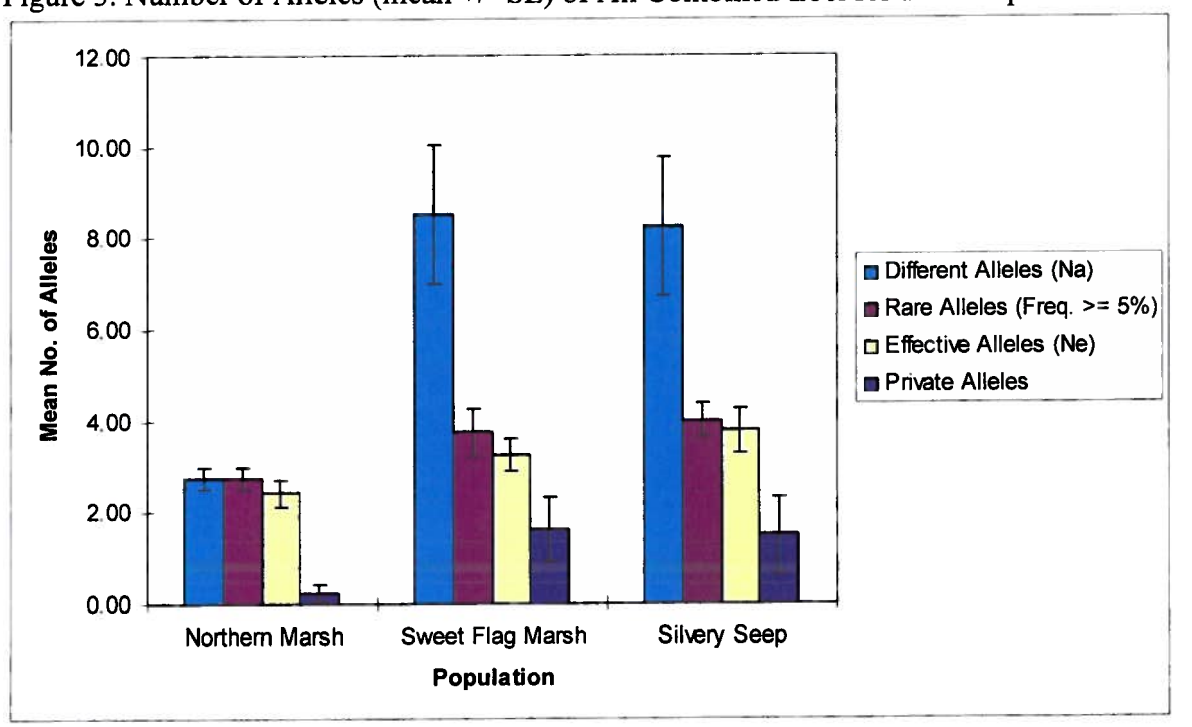


Figure 2. Example Gel Photo Illustrating Successful Cross-Amplification of Locus PC1



L = 50bp Ladder
N = Negative Control (No DNA)
HS = Cross-amplification (*H. scutatum* DNA)
P = Positive Control (*Plethodon cinereus* (original target species for PC1) DNA)

Figure 3. Number of Alleles (mean +/- SE) of All Combined Loci for Each Population



Digital Images

Image 1. *H. scutatum* adult on the forest floor.

Image 2. Group of *H. scutatum* found under a log.

Image 3. Ventral view of *H. scutatum*

Image 4. *H. scutatum* nest

Image 5. Researcher measuring *H. scutatum* individual

Literature Cited

Alford, R. A. and S. J. Richards. 1999. Global amphibian declines: a problem in applied ecology. Annual Review of Ecology and Systematics 30:133-165.

Barinaga, M. 1990. Where have all the froggies gone? Science 1990 247(4946):1033-1034.

Cabe, P. R., R. B. Page, T. J. Hanlon, M. E. Aldrich, L. Connors, and D. M. Marsh. 2007. Fine-scale population differentiation and gene flow in a terrestrial salamander (*Plethodon cinereus*) living in continuous habitat. Heredity 98:53-60.

Carstens, B. C., J. D. Degenhardt, A. L. Stevenson, and J. Sullivan. 2005. Accounting for coalescent stochasticity in testing phylogeographical hypotheses: modeling Pleistocene population structure in the Idaho giant salamander *Dicamptodon aterrimus*. Molecular Ecology 14:255-265.

Connors, L. M. and P. R. Cabe. 2003. Isolation of dinucleotide microsatellite loci from red-backed salamander (*Plethodon cinereus*). Mol. Ecol. Notes 3:131-133.

Curtis, J. M. R. and E. B. Taylor. 2001. Isolation and characterization of microsatellite loci in the Pacific giant salamander *Dicamptodon tenebrosus*. Mol. Ecol. Notes 9:116-118.

Curtis, J. M. R. and E. B. Taylor. 2003. The genetic structure of coastal giant salamanders (*Dicamptodon tenebrosus*) in a managed forest. Biological Conservation 115:45-54.

Degross, D. J., L. S. Mead, and S. J. Arnold. 2004. Novel tetranucleotide microsatellite markers from the Del Norte salamander (*Plethodon elongates*) with application to its sister species the Siskiyou Mountain salamander (*P. stormi*). Mol. Ecol. Notes 4:352-354.

Garner, T. W. J. 2002. Genome size and microsatellites: the effect of nuclear size on amplification potential. Genome 45:212-215.

Giordano, A. R., B. J. Ridenhour, and A. Storfer. 2007. The influence of altitude and topography on genetic structure in the long-toed salamander (*Ambystoma macrodactulym*). Molecular Ecology 16:1625-1637.

Illinois Wildlife Action Plan. 2005.

Larson, A., D. B. Wake, and K.P. Yanev. 1984. Measuring gene flow among populations having high levels of genetic fragmentation. Genetics 106:293-308.

McGrath, K. M. 1996. Analysis of trinucleotide microsatellite DNA in the genome of the four-toed salamander, *Hemidactylium scutatum*. Undergraduate Thesis: James Madison University.

Niemiller, M. L., B. M. Fitzpatrick, and B.T. Miller. 2008. Recent divergence with gene flow in Tennessee cave salamander (Plethodontidae: *Gyrinophilus*) inferred from gene genealogies. Molecular Ecology 17:2258-2275.

Noël, S. M. Ouellet, P. Galois, and F. -J. Lapointe. 2007. Impact of urban fragmentation on the genetic structure of the eastern red-backed salamander. Conservation Genetics 8:599-606.

Oosterhout, C. V., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molec. Ecol. Notes 4:535-538.

Peakall, R. and P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28:2537-2539.

Petranka, J. W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, D.C., USA

Phillips, C. A. 1991. Geographic distribution: *Hemidactylium scutatum*. Herpetological Review 22:133.

Purrenhage, J. L., P. H. Niewiarowski, and F. B.-G. Moore. 2009. Population structure of spotted salamanders (*Ambystoma maculatum*) in a fragmented landscape. Molecular Ecology, 18: 235–247.

Raymond, M. and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Heredity 86:248-249.

Reid, L. A. 1994. Characterization of a microsatellite DNA locus in the four-toed salamander, *Hemidactylium scutatum*. Undergraduate Thesis: James Madison University.

Schneider, S., D. Roessli, and L. Excoffier. 2001. Arlequin: A software for population genetics data analysis, 2.0 (2001 update). http://anthro.unige.ch/arlequin

Schrecengost, A. C. 1998. The isolation and characterization of genetic markers for use in a kinship study of the four-toed salamander. Undergraduate Thesis: James Madison University.

Spear, S. F., C. R. Peterson, M. D. Matocq, and A. Storfer. 2005. Landscape genetics of the blotched tiger salamander (*Ambysoma tigrinum melanostictum*). Molecular Ecology 14:2553-2564.

Storfer, A. and A. Sih. 1998. Gene flow and ineffective antipredator behavior in a stream-breeding salamander. Evolution 52:558-565.

Storfer, A. 1999. Gene flow and population subdivision in the streamside salamander, *Ambystoma barbouri*. Copeia 1999:174-181.

Storfer, A., J. Cross, V. Rush, and J. Caruso. 1999. Adaptive coloration and gene flow as a constraint to local adaptation in the streamside salamander, *Ambystoma barbouri*. Evolution 53:889-898.

Templeton, A. R., E. Routman, and C. A. Phillips. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. Genetics 140:767-782.

Wallis, G. P. and J. W. Arntzen. Mitochondrial-DNA variation in the crested newt superspecies: limited cytoplasmic gene flow among species. Evolution 43:88-104.

Weisrock, D.W., K.H. Kozak, and A. Larson. 2005. Phylogeographic analysis of mitochondrial gene flow and introgression in the salamander, *Plethodon shermani*. Molecular Ecology 14:1457-1472.

Zamudio, K. R. and A. M. Wieczorek. 2007. Fine-scale spatial genetic structure and dispersal among spotted salamander (*Ambystoma maculatum*) breeding populations. Molecular Ecology 16:257-274.

Appendix I. Supplies Purchased*	
Description	**Cost**
UIUC Life Sciences Storeroom – Laboratory Supplies (i.e. Pipette tips, gloves, PCR Reagents, DNA Extraction Kits)	555.09
Integrated DNA Technologies - Microsatellite Primers	848.80
VWR International, Inc. – Misc. Laboratory Supplies	89.11
Grainger – Electronic Balance (x1)	75.02
Fisher Scientific – Laboratory Reagents	35.77
Denville Scientific, Inc. – Cryovials (x1 case)	135.89
DOT Scientific, Inc. – PCR Strip Tubes (x120 strips)	78.50
Total	1818.18

*Additional supplies and contractual services at the UIUC Keck Center paid for by other funding sources.

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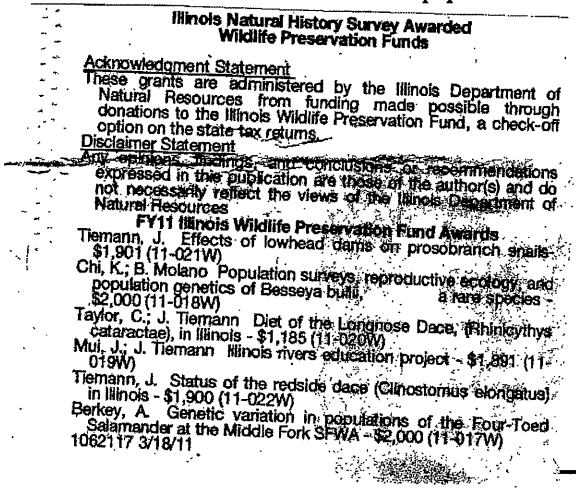

Illinois Natural History Survey Awarded Wildlife Preservation Funds

Acknowledgment Statement
These grants are administered by the Illinois Department of Natural Resources from funding made possible through donations to the Illinois Wildlife Preservation Fund, a check-off option on the state tax returns.

Disclaimer Statement
Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the Illinois Department of Natural Resources

FY11 Illinois Wildlife Preservation Fund Awards

Tiemann, J. Effects of lowhead dams on prosobranch snails - $1,901 (11-021W)

Chi, K.; B. Molano Population surveys, reproductive ecology, and population genetics of Besseya bullii, a rare species - $2,000 (11-018W)

Taylor, C.; J. Tiemann Diet of the Longnose Dace, (Rhinichthys cataractae), in Illinois - $1,185 (11-020W)

Mui, J.; J. Tiemann Illinois rivers education project - $1,891 (11-019W)

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Berkey, A. Genetic variation in populations of the Four-Toed Salamander at the Middle Fork SFWA - $2,000 (11-017W)

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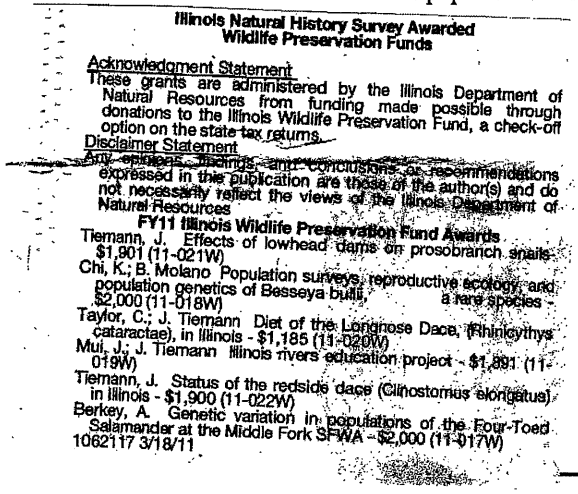

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