Genetic Identity of Wisconsin Gartersnakes (*Thamnophis* spp.) Using Microsatellite Genetic Markers

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Abstract

Butler's Gartersnakes (*Thamnophis butleri*) are currently listed by the Wisconsin Department of Natural Resources as state threatened. Several key questions associated with species identity, integrity, and hybridization with other gartersnake species need to be addressed to further refine the species management plan. The objectives of this research were: 1) to determine if genetic markers developed in the initial phase of research could identify discrete genetic groups of Wisconsin gartersnakes, 2) to determine if any or all genetic groups delineated in objective one, were consistent with Butler's, Plains (*T. radix*), and/or Common Gartersnakes (*T. sirtalis*), and 3) to determine if any of the genetic data was consistent with hybridization occurring between gartersnakes in Wisconsin. Snakes were sampled from various Midwestern locations with a focus on sites in Wisconsin. All snakes were photo-vouchered, morphological landmarks were taken, and a tail snip collected for genetic analysis. Genetic data from the previously developed microsatellite markers discriminated three genetic groups from a composite 13-locus dataset (N = 815) using the Bayesian admixture analysis in STRUCTURE v2.3.3. These units were highly consistent with species-groups based on the membership of a small number of known snakes from areas where the species are not thought to co-occur. Using a threshold q-value (proportional genotype) of ≥80%, 498 Butler's, 93 Plains, and 107 Common Gartersnakes were identified in Wisconsin samples; putative hybrid snakes of Butler's x Plains (34), Butler's x Common (8), and a single ambiguous snake were also identified in Wisconsin samples. Levels of divergence among the species groups from Wisconsin were lower than between species groups from other states consistent with either larger than expected Wisconsin population sizes or significant gene flow (introgressive hybridization) having occurred among species. Regardless, levels of divergence and overall integrity of the three groups were such that the presence of three species of gartersnakes in Wisconsin was supported and hybridization, at a minimum between Butler's Gartersnakes and the two other species, was shown to occur.

Introduction

Wisconsin populations of Butler's Gartersnake (*Thamnophis butleri*; BGS) are currently listed by the Wisconsin Department of Natural Resources (WDNR) as state threatened and, as such, are the focus of scientific and regulatory issues. The natural range of the species in Wisconsin is primarily situated in the greater Milwaukee area. The regulatory issues associated with BGS in Wisconsin are further complicated because their primary habitat types are open-canopy wetlands with connected upland open-canopy habitat (WDNR 2005); habitats that are increasingly rare within their range in Wisconsin. The greater Milwaukee area is a focus of growth and development in the state, which leads to fragmentation and eradication of necessary habitat and subsequently, further complicating protection and regulatory efforts.

Effective management and regulation of BGS in Wisconsin relies on accurate and reliable scientific research. Previous research and observations have shown that BGS appears to hybridize with the congeneric Plains Gartersnake (*T. radix*; PGS) (Fitzpatrick et al. 2008). Coupled with difficulties in discriminating the two species – they are closely related based on all available data (de Quieroz et al. 2002) – this suspected hybridization has raised additional issues that need to be addressed to ensure effective regulation and protection. Previous morphological research has shown BGS and PGS at select sites in Wisconsin (especially south-eastern WI) are morphologically more intermediate when compared to results from other sites in the state and in other states (Casper 2008). Despite these results, field identification and discrimination remains problematic and previous research has resulted in key questions related to species identity and integrity as well as whether or not hybridization actually occurs between the two species. Additional complications may exist if hybridization is occurring due to the sympatric presence of another congeneric gartersnake, the Common Gartersnake (*T. sirtalis*; CGS), a prolific species that could also hybridize with BGS and PGS.

Currently, key questions exist that are impediments to further refining the WDNR's BGS management plan. This research directly addresses three key questions:

1. Are there distinct genetic differences between Butler's Gartersnakes (*Thamnophis butleri*) and Plains Gartersnakes (*T. radix*) and/or Common Gartersnakes (*T. sirtalis*)?
2. Are there Butler's Gartersnakes in Wisconsin?

3. Are Butler's Gartersnakes hybridizing with Plains or Common Gartersnakes?

Molecular genetic data using codominant, Mendelian inherited markers can provide valuable insight into species integrity, genetic diversity levels, degree of migration, and levels of hybridization. The use of these markers is preferable because the resulting genetic diversity data directly represent the maternal and paternal lineages thus allowing direct tracking of both putative species involved in suspected hybridization. Previous research (Sloss et al. Submitted) identified a suite of 18 polymorphic microsatellite genetic markers for use in gartersnake issues in Wisconsin. The data showed that discrimination between all three species of Wisconsin gartersnakes, if they exist in the state, should be possible using combinations of these 18 markers. Therefore, the three primary research objectives were:

1. To determine if the genetic markers developed in the initial phase of research could identify discrete genetic groups of Wisconsin gartersnakes.
2. To determine if any or all genetic groups delineated in objective one, were consistent with Butler's, Plains, and/or Common Gartersnakes.
3. To determine if any of the genetic data was consistent with hybridization occurring between gartersnakes in Wisconsin.

Methods

Sample Design

The goal of the study design was to use the most effective and reliable method to answer the aforementioned questions/objectives while minimizing any actual or perceived bias as a result of subjective field identification. To this end, it was determined that an approach using no *a priori* sample identifiers for establishing genetic identity would be preferable. A reference-based approach, where snakes designated as 'type' or reference specimens of each of the three species are used to identify baseline identification data, was rejected because of concerns for accurate and reliable field identification and the reliance on *a priori* identified reference snakes. Instead, a Bayesian admixture detection approach implemented in the software package STRUCTURE (Pritchard et al. 2000; Falush et al. 2003; Falush et al. 2007) was used to predict the number of gartersnake species and the degree of hybridization observed among the samples. In brief, this approach takes a sample of individual genotypes and asks the question, 'How many genetic units

are present in this collection'. Since no *a priori* information is given to the software, no bias in terms of preconceived assignment can be introduced. The result is a prediction of a) how many genetic units occur in the composite sample, and b) the genetic makeup of individual snakes in terms of these genetic units (i.e., are some snakes hybrids?).

Sample Collection

Samples from >900 snakes from various Midwestern locales were collected/obtained (Figure 1). The majority of samples were collected in 2009 by WDNR personnel and contractors with additional samples from museum specimens, the University of Tennessee (samples originally collected by the WDNR for previous genetic research), and from Dr. Rich King, Northern Illinois University (DeKalb, IL). The design goal was to adequately sample Wisconsin locales where Plains-Butler's hybridization was thought to occur and also sites where members of all three species had a high likelihood of being 'pure'. Sites from out of state (non-Wisconsin) were included because PGS and BGS do not co-occur there. All study locations were chosen by the WDNR Endangered Resources personnel.

All WDNR-sampled snakes were photo-vouchered and measured at numerous morphological landmarks for subsequent combined morphological/molecular analysis. All snakes were sampled for genetic analysis by taking a small (50-100 mg) tail clip using aseptic techniques to minimize between-sample contamination. Samples were inserted into prelabeled 2.0 mL microcentrifuge screw-top tubes and preserved with 95-100% non-denatured ethanol. Samples were transferred along with pertinent field data to the Molecular Conservation Genetics Laboratory (MCGL) at the University of Wisconsin-Stevens Point for subsequent genetic analysis.

Genetic Analysis

Genetic analysis was conducted via DNA genotyping of the tail snips using 16 microsatellite loci (Sloss et al. Submitted; Table 1). A standard extraction protocol and amplification protocol was used on all samples. DNA was extracted with an in-house 96-well modification of the Promega Wizard®,[1] Genomic DNA purification kit (Promega Corp., Madison, WI) that included final

[1] Use of tradenames throughout manuscript does not imply endorsement by the U.S. Government.

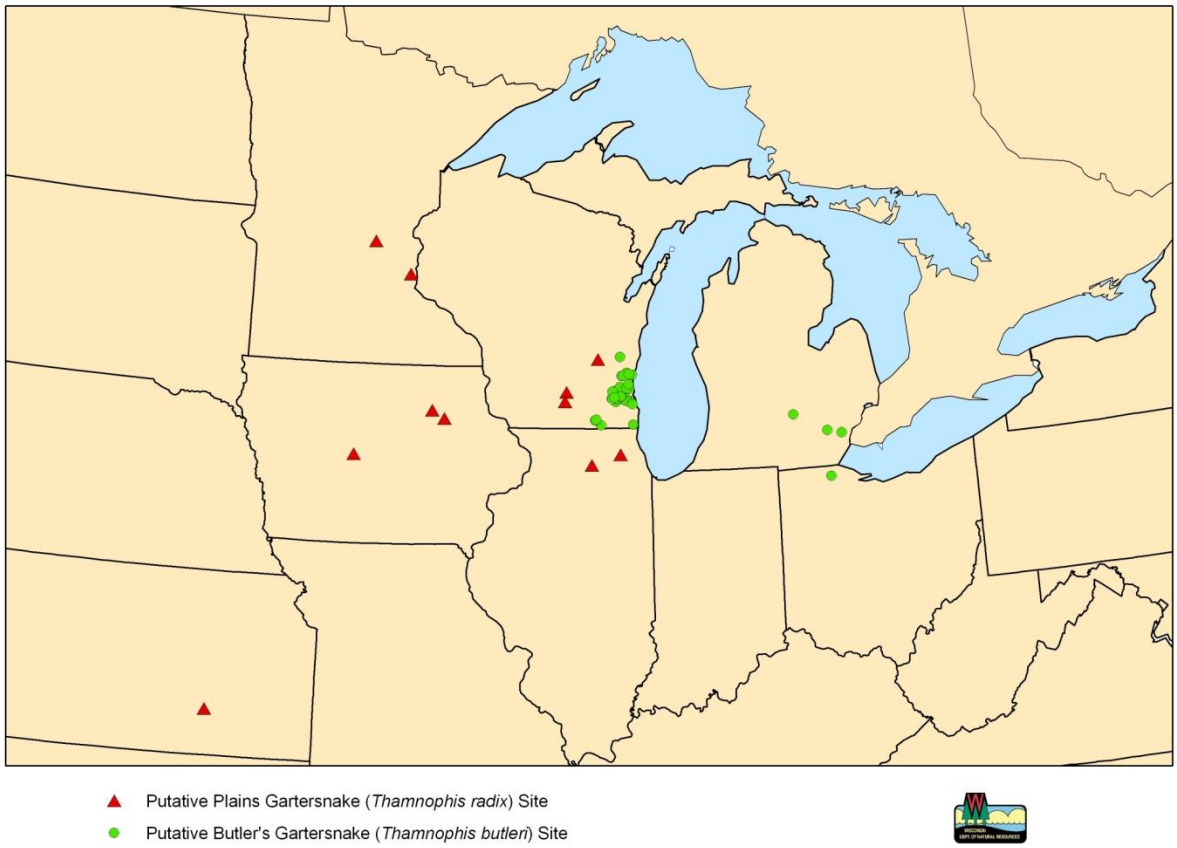


Figure 1. Map of gartersnake sample locales for this study. No sites for CGS are listed, but CGS sampling was limited to Wisconsin and included many of the sites in this figure as well as additional sites in the state.

Table 1. Microsatellite loci used in the present study on gartersnakes, including locus name, fluorescent label, primer sequence, primer concentration (μM each primer), observed size range of alleles (base pairs), and number of alleles observed across all gartersnake samples (N_A). Superscript numbers on locus names denote multiplex reactions (Sloss et al. Submitted).

Locus	Label	Primer Sequence (5'-3')	Primer (μM)	Size Range (bp)	N_A
Tbu A01[1]	6FAM	F: AGTCCACCCACCAGGGAAG R: CACACTGCATAAGGAAAGATGG	0.08	116-130	8
Tbu A03[2]	6FAM	F: TCCAACCTACTTCAAACCTTGG R: CAGGGAGTGCATTACCAAAC	0.08	234-274	17
Tbu A04a[3]	HEX	F: AAGGAGCTTGGGGAATCTTG R: CTGGGAATCTTAGCATTCTGC	0.20	200-206	4
Tbu A09[4]	NED	F: CATCTCAACCAAAGTCGCTTC R: GGATGTTGTGGGGTGTTTTC	0.20	102-116	7
Tbu A27[3]	6FAM	F: AAACTCCAGGGATTTCCAAG R: TGTGTTGCGTGAATACATCC	0.10	216-222	3
Tbu A28[5]	6FAM	F: CAATGTGCAGCGTGGATAAC R: GATAGACAATGGCCGGAATG	0.10	285-482	41
Tbu A49[2]	NED	F: CTTGTAGTTTGGGGGAAAAG R: TTTTCAGAGCTGGATGAAGG	0.40	200-228	15
Tbu A64[6]	NED	F: ACATAGAATGCATCTGGTTGG R: GCCATGCAATCATGTATAAGC	0.20	222-260	19
Tbu A70[7]	6FAM	F: GCCACTTCCACCTAACACAG R: CACACTGGTGAGTTGCTCTG	0.10	150-184	9
Tbu A74[6]	6FAM	F: CTTGGAAATGTCCTGCAATC R: CCCATGCAAGCAATATAACC	0.20	284-310	11
Tbu A92[1]	NED	F: TTTTTGCTTCTGTGCATGAG R: TGTCACAACCCCTGGTATG	0.40	166-198	14
Tbu A95[4]	HEX	F: ACCTTGTTTTATCCGTGTGC R: AATTGCTTAATGTGAGAGAGAC	0.15	132-333	36
Tbu B10[8]	6FAM	F: TCCTCTTTCATTTCCCCTTC R: TGAAATTTTCCCTCCTATACCC	0.10	130-200	13
Tbu B12[5]	NED	F: CTGCTTTTAATCCCATCACC R: AACTGAAAGCCATTCACTGC	0.10	245-549	24
Tbu B19[7]	HEX	F: TGCATACACCACTTCACACC R: CACTCCAACGGTTCTAATGC	0.10	213-221	5
Tbu B38[8]	HEX	F: TCATTTGCCCAAGAATTCAG R: ATTGGGCACCTAGTTTCAGC	0.20	217-273	20

elution with 50 µl TLE. PCR reactions consisted of 10 µL reaction volumes consisting of 1X Fisher PCR Buffer B (Thermo Fisher Scientific, Inc., Waltham, MA), 0.15 mM each dNTP (0.60 mM total), 1.50 mM $MgCl_2$, locus specific primer concentration (Table 2), 0.50 U *Taq* polymerase, and 5-50 ng extracted DNA. All reactions were amplified using a thermal profile of 95°C/5 min followed by 35 cycles of 95 °C/30 s, 55°C/30 s, 72 °C/30 s with a final 7 minute 72 °C extension. Genotype data was collected on an ABI 3730 Automated DNA analyzer (Applied Biosystems, Inc.) with GeneFlo™-625 in-lane standard (Chimerx, Inc., Milwaukee, WI).

A critical objective of this study was determining how many genetic units are present among the sampled snakes. The number of genetic units was estimated from the data without using prior knowledge of snake identification, using the approach of Pritchard et al. (2000) as implemented in the software package STRUCTURE v2.3.3 (Pritchard et al. 2000; Falush et al. 2003; Falush et al. 2007). Standard methods were employed as suggested by the authors of the software, including using an admixture model, varying K (the number of potential genetic units) from 1-6, and performing five replicates of each K with 100,000 burn-ins and 100,000 replicates.

STRUCTURE output was interpreted using a combined approach of the Delta K method of Evanno et al. (2005) and the standard prediction of K based on a plot of the estimated mean ln probability of K [mean ln Pr (K)] with standard deviation. The Delta K of Evanno et al. (2005) predicts the most likely value of K in a given data set based on second order rates of change between successive values of K. The results were visualized as a graph of Delta K values for a given K (1-6) such that a discernible peak compared to the other values would be interpreted as the actual value of K. The ln Pr(K) with standard deviation used a plot of mean ln Pr(K) values and looked for a break in linearity where the values appeared to begin to asymptote. This, coupled with a low standard deviation among values, has been used to predict the most likely value of K for the data (Pritchard et al. 2007). Delta K and mean ln Pr(K) were calculated from the STRUCTURE output using Structure Harvester (Earl 2011).

Once a value for K was identified, individual plots of mean q-values across all five replicates were constructed in Microsoft Excel 2010 (Microsoft Corp., Redmond, WA). Q-values are estimated likelihoods that an individual belongs to a given genetic unit (K). So, the higher a q-

value, the more likely the individual belongs to a given genetic unit. Two values of q were considered for identifying an individual as belonging to a given genetic unit, ≥80% and ≥90%. The ≥80% cutoff was shown as a minimum level of hybrid discrimination in a study examining the genetic detection of hybrids using the program STRUCTURE (Vähä and Primmer 2006) with the use of 12 loci. The higher value (≥90%) was also considered as a more stringent delineation point for membership. Genetic units were correlated to species after analysis by determining where the non-Wisconsin samples of BGS and PGS and the Wisconsin field-identified CGS samples resolved in STRUCTURE analysis. It is important to note the software/input file had no identifiers of individuals other than a generic number code (e.g., TH0001, TH0002, etc.) useful only to the researcher for sample management purposes. All discrimination of site-specific snakes and other specific individual identification (such as that of non-Wisconsin snakes) was completed *after* statistical analysis and had no role in the delineation of genetic units or in validating the results.

Following the delineation of genetic groups, genetic diversity measures including the mean number of alleles per locus (A), the effective number of alleles (A_e; a measure accounting for the evenness of alleles within the overall allelic distribution), observed and expected heterozygosity (H_O and H_E, respectively), and Shannon's information index (Sherwin et al. 2006) were calculated for each group while separating Wisconsin gartersnakes from snakes from other states using Genetic Analysis in Excel v6.41 (GenAlEx 6.41; Peakall and Smouse 2006). To account for differences in diversity solely due to the differences in sample sizes, a rarefaction method of calculating allelic diversity (A_R) was employed using HP-Rare (Kalinowski 2005). Tests for conformance to Hardy-Weinberg equilibrium (HWE) were performed using a chi-square test within GenAlEx 6.41 with sequential Bonferroni correction (Rice 1989) for multiple pairwise comparisons. Pairwise measures of genetic divergence between groups were estimated using F_{ST}, a measure of population differentiation, and testing the value for significant differences from zero using FSTAT v2.9.3.2 with 21,000 permutations (Goudet 1995).

Results

The final genotype data consisted of 13 loci for 815 total snakes (Table 2). Three loci were eliminated from the final dataset because of concerns with inconsistent amplification and

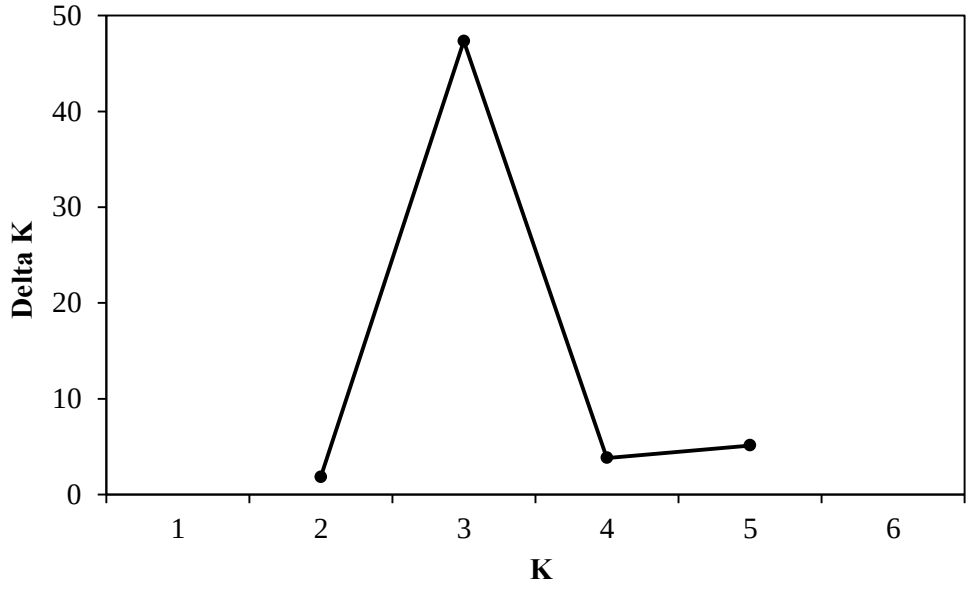
precision in sizing amplified fragments. The primary reason for the lower number of samples was that some samples failed to yield DNA of sufficient quantity and/or quality for amplification of a majority of the microsatellites. This occurs, in our experience, when a combination of DNA quantity (as measured in ng/µl) and quality (as measured via spectrophotometry) results in only the most robust of the PCR reactions producing useable genotype data. The quantity issue can be attributed in large part to insufficient sample size; in many cases the tail snip small (~1 mm) and consisted primarily of scale. The smaller samples may have been diluted when they were placed in ethanol for preservation, and thus failed to yield sufficient DNA. In these instances, the samples were re-extracted (when tissue remained) and attempts were made to collect the remaining data. In some cases, this resulted in at least 7 loci successfully genotyping. If a snake failed to successfully genotype at a minimum of 7 loci, it was excluded from all subsequent analyses. As long as the missing data is not indicative of an allelic state (i.e., null alleles) or inherited in a similar fashion, the presence of missing data should not be an impediment to analysis (Pritchard et al. 2007). STRUCTURE ignores loci with missing data for updating and estimating the q-value for that individual. The result is a more ambiguous q-value for the individual. Of the 815 samples in the final data set, 597 (73.3%) were missing 0-1 locus and 781 (95.8%) were missing no more than 4 loci in their final genotypes.

The STRUCTURE analysis showed strong support for three genetic units (i.e., putative species) in the total data set (Figure 2a, b). The Delta K plot showed a clear peak at K = 3 (Figure 2a). The mean ln probability of K (Figure 2b) showed a break in linearity of increase at K = 3 (mean = -42,887.9) as well as the lowest standard deviation ($SD_{K=3}$ =809.3). The plot of individual q-values for three genetic units (a metric that estimates what proportion of an individual's genotype belongs to a given genetic unit estimated by STRUCTURE), showed the majority of snakes corresponded to one of the three genetic units at a $q \geq 0.80$ (Figure 3, Table 3). According to this criterion, 771 samples (94.6%) were consistent with a minimum genetic requirement for being a 'pure' genetic individual with maximum q-values ≥ 80% and 734 samples (90.1%) met the more stringent maximum q-value ≥ 90% criteria (Table 3). At the 80% threshold, the remaining 5.4% of the samples (44 individuals) represented a combination of potential BGS x PGS hybrids (n = 35) and BGS x CGS hybrids (n = 8) and a single snake that failed to resolve a majority species with the maximum q-value (BGS) being 0.434 (Table 3). When only Wisconsin sampled snakes

Table 2. Samples successfully genotyped for all states included in the study including the number of unique sites per state.

State	Sites	Number of Samples
Wisconsin	54	741
Illinois	2	31
Iowa	3	18
Kansas	1	1
Michigan	3	21
Minnesota	2	2
Ohio	1	1

a)



b)

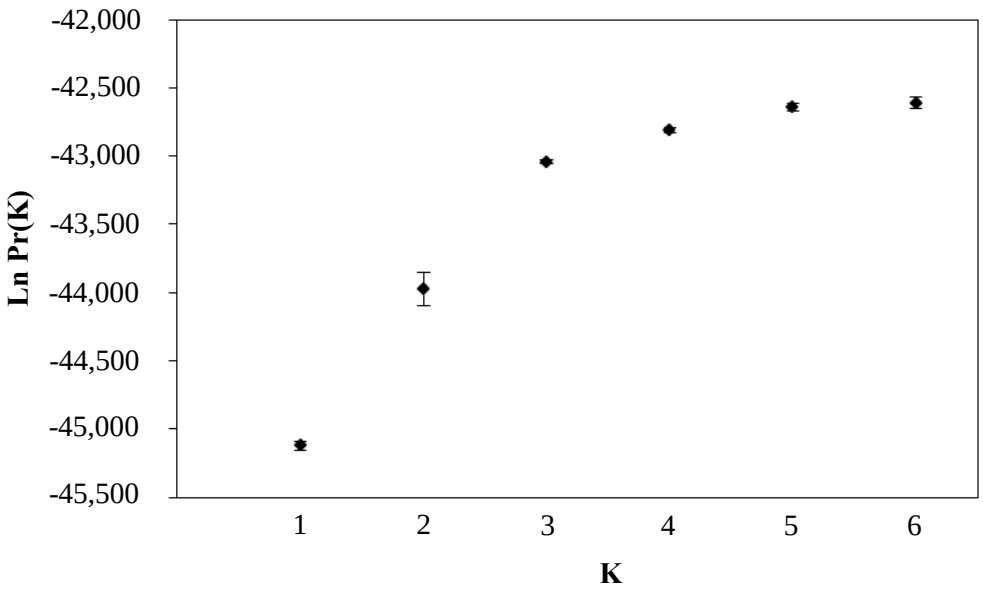


Figure 2. Results of STRUCTURE prediction (summarized across 5 replicates) of the most likely value of genetic units (K) contained in the data. a) Delta K (Evanno et al. 2005) shows the rate of change between successive values of K such that a peak value is interpreted as the 'true' K. For this data, K = 3 shows the most discernible peak. b) Plot of the estimated mean of the ln probability of K [mean ln Pr(K)] with standard deviation. The mean ln Pr(K) shows an asymptotic inflection at K =3 with the lowest standard deviation (809.3) also occurring at K = 3.

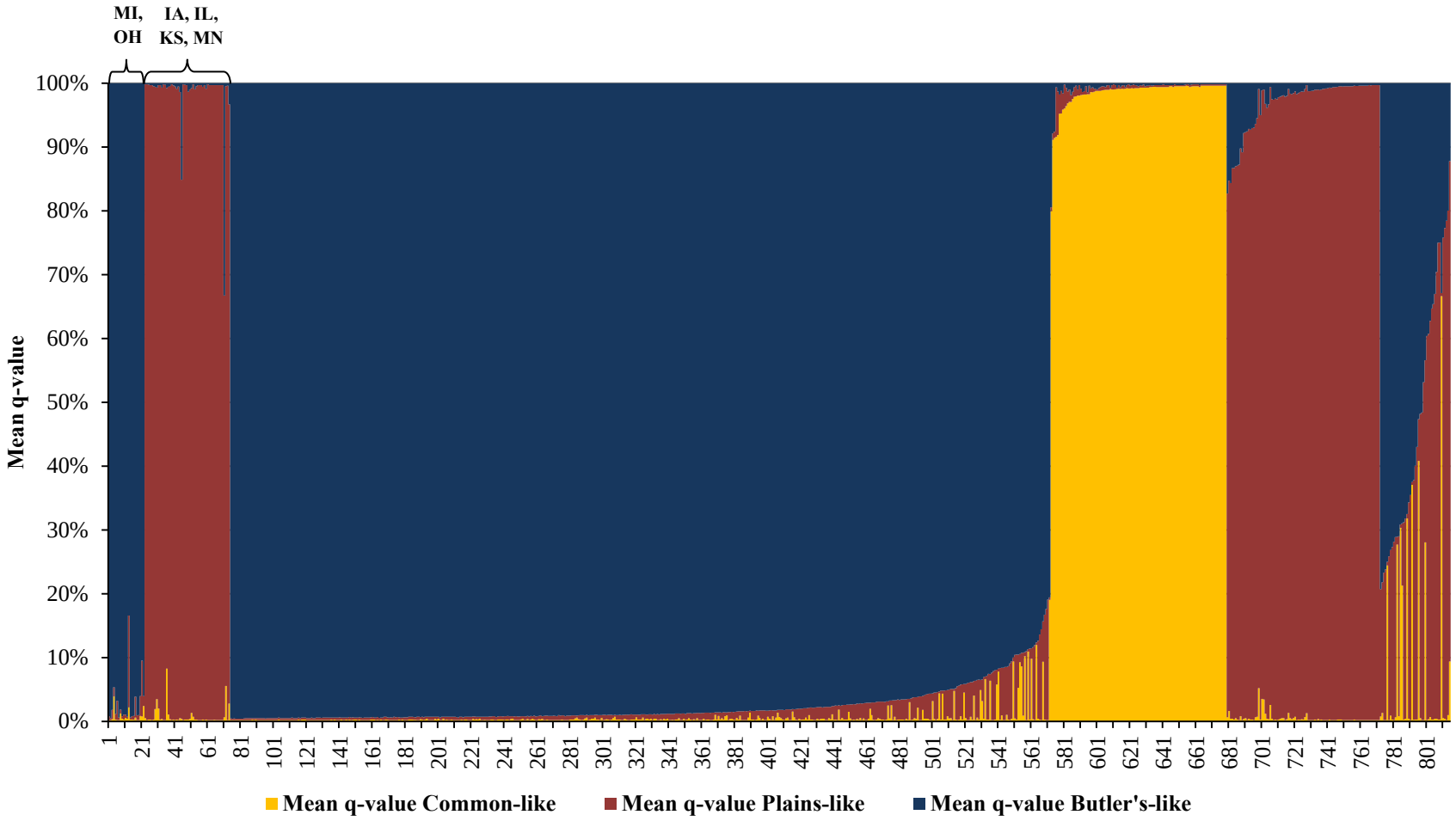


Figure 3. Plot of mean individual q-values from STRUCTURE for all samples included in this study. Samples designated with brackets are 'known' snakes from non-Wisconsin sites. These samples include BGS from Michigan (1-21) and Ohio (22) and PGS from Iowa (23-40), Illinois (41-71), Kansas (72) and Minnesota (73-74). All other snakes are field samples from Wisconsin.

Table 3. Summary of sample identification based on different q-value thresholds. Data is segregated into the inferred species groups following STRUCTURE assignment (Genetic Group), State where samples originated, counts of individuals in each q-value category, and mean and standard deviation (SD) of the highest q-value for each individual in the ≥80% q-value category. Hybrid categories are given as total number of samples (N) within the putative hybrid range (<80% highest q-value), as well as the mean and standard deviation of the BGS-specific q-value. The number of snakes that fell in the more confident 70:30 range of q-values, their mean BGS-specific q-value and standard deviation are also presented.

		q-value					
Genetic Group	**State**	**≥95%**	**≥90%**	**≥80%**	**Mean**	**SD**	
BGS	Wisconsin	437	475	498	0.9767	0.0299	
	Michigan	18	20	21	0.9729	0.0385	
	Ohio	1	1	1	0.9606	---	
	Total	*456*	*496*	*520*	*0.9765*	*0.0302*	
PGS	Wisconsin	72	83	93	0.9647	0.0425	
	Illinois	29	29	30	0.9925	0.0043	
	Iowa	17	17	18	0.9847	0.0213	
	Kansas	0	1	1	0.9400	---	
	Minnesota	1	2	2	0.9663	0.0386	
	Total	*119*	*132*	*144*	*0.9717*	*0.0386*	
CGS	Wisconsin	102	106	107	0.9765	0.0302	
Hybrids		**N**	**Mean**	**SD**	**70:30**	**Mean**	**SD**
Butler's x Plains	Wisconsin	34	0.5120	0.2118	18	0.4974	0.1369
	Illinois	1	0.6610	---	1	0.6610	---
Butler's x Common	Wisconsin	8	0.5854	0.1412	7	0.5675	0.1423
Non-Majority	Wisconsin	1	0.4344	---	1	0.4344	---

were considered (n = 741), the proportions were 94.2% 'pure' species (n = 698) and 5.8% putative hybrids (n = 43). These snakes were designated *putative* hybrids because the maximum q-values for the dominant species ranged from 79.3% - 43.4% with the majority (n = 26) within a 70:30 q-value range (a range close to the expected 50:50 predicted for an F_1). The other >70% snakes (n = 17) should be treated as ambiguous pending additional studies investigating a more specific q-value for discrimination of gartersnakes using this genetic data. Only one instance of a hybrid CGS x PGS snake was observed and that was an individual that failed to meet the 7-locus threshold for final inclusion (data not shown) and was thus not included in the final analysis. Further efforts are underway to confirm/complete that individual's genotype.

The levels of genetic diversity within and among the resolved groups were relatively high yet differed significantly depending on species group. Diversity among all Wisconsin gartersnakes consistently showed WI-BGS with the highest diversity while WI-PGS was consistently the lowest (Table 3). The allelic richness as measured by rarefaction ranged from a high of 16.11 allele/locus (WI-BGS) to a low of 12.56 (WI-PGS), and heterozygosity, Shannon's information Index, and the effective number of alleles (A_e) showed similar trends (Table 4). Furthermore, all Wisconsin gartersnakes showed higher diversity than non-Wisconsin samples even in the sample-size controlled rarefacted allelic richness measures (Table 4). When all Wisconsin snakes of a STRUCTURE-identified species-group were treated as if they were a single population, all three groups failed to conform to HWE with 11, 8, and 8 of the 13 loci failing to conform in BGS, PGS, and CGS, respectively. In contrast, two out of state samples (IA-PGS and MI-BGS) both conformed to HWE at all loci following sequential Bonferroni correction. Levels of divergence as measured by F_{ST} showed all sampled 'pure' groups to be significantly different from each other (Table 5). Interestingly, divergence values between the Wisconsin BGS and Michigan BGS samples (0.1097) were higher than the level of divergence between the BGS and PGS within Wisconsin (0.0741). Across all comparisons, CGS was consistently among the highest divergence values (mean F_{ST} = 0.2099).

Table 4. Summary of diversity statistics for samples with N > 2 including allelic diversity (A), allelic richness following rarefaction with 100 alleles (A_r), effective number of alleles (A_e), Shannon's information index (I), mean number of private alleles/locus (PA), mean numbers of private alleles per locus following rarefaction with 100 total alleles (PA_r), and heterozygosity (observed, H_O and expected, H_E). Genetic group was defined based on the STRUCTURE results for K = 3 with a threshold q-value = 0.80.

Genetic Group	State	A	A_r	A_e	I	PA	PA_r	H_O	H_E
Butler's	Wisconsin	25.31	16.11	9.79	2.16	4.46	1.00	0.662	0.782
	Michigan	7.46	7.46	4.48	1.50	0.39	0.43	0.426	0.686
Plains	Wisconsin	14.08	12.56	6.36	1.96	0.27	0.11	0.619	0.782
	Iowa	8.92	8.92	5.66	1.76	0.47	0.39	0.651	0.766
	Illinois	8.08	8.08	4.38	1.54	0.15	0.09	0.554	0.701
Common	Wisconsin	17.62	15.10	8.18	2.06	4.77	1.55	0.553	0.757

Table 5. Pairwise F_{ST} values (below diagonal) and p-value (above diagonal) for all sampled species groups. All pairwise comparisons were significant following sequential Bonferroni correction.

	MI BGS	WI BGS	WI PGS	IA PGS	IL PGS	WI CGS
MI BGS	***	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
WI BGS	0.1097	***	<0.0001	<0.0001	<0.0001	<0.0001
WI PGS	0.1600	0.0741	***	<0.0001	<0.0001	<0.0001
IA PGS	0.2125	0.1288	0.0515	***	<0.0001	<0.0001
IL PGS	0.2435	0.1479	0.0593	0.0701	***	<0.0001
WI CGS	0.2436	0.1876	0.1901	0.1976	0.2307	***

Discussion

The genetic data collected in this study predicted three genetic units consistent with the contemporary taxonomy of gartersnakes within the sampled area. The use of non-Wisconsin snakes to allow for *post-hoc* identification of these genetic units provided more-or-less clear identification of the units. Without these specimens, the same assignment issues associated with morphological identification of snakes in Wisconsin (Casper 2008) would have made correlating the genetic units to a species designation subjective and reliant on presumed 'type-specimens' of each snake from within the state. Given the contentious nature of field identification because of hybridization and natural range variability, this approach would not have met the target objectives of providing objective delineation of species and detection of hybridization. The analyses showed unequivocally that the microsatellite loci developed previously for this study contained adequate genetic information to distinguish the three species in the state.

A critical decision in this study was setting the threshold q-value to delimit an individual as being a genetically 'pure' individual as opposed to a putative hybrid. A threshold q-value ≥80% was required to consider an individual snake as a pure species based on the work of Vähä and Primmer (2006). Their study showed 12-24 loci and a threshold of 80% was generally sufficient for detecting hybrids at moderate divergence levels. In examining the gartersnake data, two complications to this approach were present. First, the level of differentiation based on F_{ST} between the various designated groups (Table 4) showed F_{ST} levels between Wisconsin BGS and PGS to be 0.0741, lower than the 0.12 level used in Vähä and Primmer (2006). Nevertheless, the efficiency (correctly calling a purebred a purebred and a hybrid a hybrid) in the Vähä and Primmer (2006) study was consistently high (>80%) for F_{ST} values between 0.06 and 0.12 with 12 loci in that study. The error in this case appears to be in erroneously calling some purebred individuals hybrids while the efficiency of hybrid detection increases as the threshold of q-values increases from 0.10 to 0.20. Because of the lower F_{ST} between Wisconsin BGS and PGS, both the 80% and the additional, more stringent (in terms of delineating 'pure' species) 90% criterion were used to examine the amount of potential bias in interpretation of results and provide a more complete picture of the q-value membership values. In reality, the differences between the two criteria were minimal. For example, the use of a 90% criterion versus an 80% criterion resulted in a 4.6% reduction in the number of WI-BGS snakes in the dataset (475 vs. 498), a 10.8%

reduction in WI-PGS (83 vs. 93), and only a 0.9% reduction in the number of WI-CGS snakes (106 vs. 107). The impact was most pronounced in interpreting the percent proportion of putative hybrids observed. This was primarily a result of the relatively small number of observed hybrids resulting in a larger impact when the additional putative hybrids were included. To ensure the highest efficiency in hybrid identification, the 80% criterion was given preference in interpreting hybrid numbers and distribution.

The second issue related to using Vähä and Primmer's (2006) guidance for q-value determination in the gartersnake dataset was the number of loci successfully genotyped per individual. The use of non-lethal sampling of the snakes in this study represented a challenge to the laboratory process. Initially, small tail snips (<4 mm) were being sampled and placed in 95% ethanol. The residual tissue attached or housed inside the snip provides the sample for DNA extraction. Upon processing these samples in the laboratory, it was evident many snips failed to harvest any usable tissue from the snakes; some of the samples were essentially empty scale 'caps'. Scales do not contain DNA. Furthermore, when low quantities or quality of tissue are used for DNA extraction, the subsequent DNA extract is of sub-optimal quality and quantity. The microsatellite markers systems developed by Sloss et al. (Submitted), showed varying success in amplifying DNA samples with extremely low quantity (<5 ng DNA/μL) and low quality (as measured by 260/280 absorbance ratios). Therefore, some samples had incomplete genotypes. The 7-locus threshold was used for inclusion to ensure at least a majority of the loci were successfully genotyped.

Despite these issues, the majority of samples were successfully genotyped and confidently assigned to species or hybrid groups. Of particular concern with the missing loci would be the identification of hybrids. Of the 43 putative Wisconsin hybrid snakes identified via STRUCTURE analysis, 25 snakes had 0 -1 locus missing in their final genotypes (58.1%) thus meeting the minimum 12 loci prescribed by Vähä and Primmer (2006). The allowance of a second missing locus (minimum number of loci = 11) increased the number of snakes to 36 (83.7%). Given the degree of genetic differentiation between the groups and the relatively high discrimination in STRUCTURE q-values, it was decided to allow a larger number of missing loci than in Vähä and Primmer (2006) and likewise, to include the wider range of q-values

(<80%) as *putative* hybrids. Future research should aim to better understand the limits of detection of the current microsatellite data and the impact of missing data on discrimination of genetic heritage of a given snake. Further refinement will be critical in terms of non-lethal sampling to ensure an adequate sample is taken and preserved, refinement of laboratory protocols ensuring the maximum efficiency of DNA extraction, and in the PCR reaction conditions for all loci to ensure more robust results.

Regardless of threshold q-value, the data consistently showed that hybrid gartersnakes exist in Wisconsin. The vast majority of sampled gartersnakes were consistent with pure-species status (94.2% at the 80% criterion). However, 5.8% of all sampled Wisconsin snakes were consistent with the defined hybrid genetic profiles. Hybrid snakes were observed in the Wisconsin samples between BGS and PGS and also between BGS and CGS. Interestingly, all of the observed hybrid crosses occurred between the state-threatened BGS and a more prevalent species (PGS and CGS).

The threat of hybridization to rare and threatened species is well known (Rhymer and Simberloff 1996). Hybridization can be a threat to the genetic integrity of both populations and species. In particular, introgressive hybridization where hybrids continually backcross with pure species and/or each other (creating a 'hybrid swarm') results in the movement of genes from one species into another (Epifanio and Philipp 2000). The end result is the slow erosion of genetic divergence between groups that can exacerbate the problem through the further loss of remaining isolating mechanisms (behavioral, physiological). Environmental disturbance is a primary contributor to introgressive hybridization (Seehausen et al. 2008). Behm et al. (2010) found extensive hybridization among benthic and limnetic Threespine Stickleback (*Gasterosteus aculeatus*) in Enos Lake (British Columbia, CAN) and proposed a loss in ecological postmating isolation in partial response to an invasion of the Signal Crayfish (*Pacifacticus lenisculus*) and a cascading series of habitat and ecological effects. This study, coupled with genetic data on the same phenomenon, found no pure individuals of Threespine Sticklebacks and instead only an introgressed/intermediate stickleback population remaining (Gow et al. 2006). This series of habitat disturbance and ecological change is similar to the situation found in gartersnakes in Wisconsin. The PGS is thought to be a more generalist form, whereas the BGS is more

specialized resulting in ecological separation of the species. The observed degree of genetic divergence between BGS and PGS samples within Wisconsin was significant (F_{ST} = 0.0741, $p < 0.0001$) but more than 20% lower than the estimated divergence between Wisconsin BGS and Michigan BGS (F_{ST} = 0.1097, $p < 0.0001$). The degree of difference in the F_{ST} estimates suggests some level of historical and/or contemporary mixing (partial gene flow/hybridization) has occurred or the population sizes of both species in Wisconsin have stayed larger than currently thought, thus reducing the impact of genetic drift between the species. If this lower observed F_{ST} is the result of introgressive hybridization, the integrity of Wisconsin's BGS may be under immediate threat. Further efforts focused on better representing the Michigan BGS as well as other populations in Indiana, Ohio, and southern Ontario could provide a more appropriate context for evaluating how much mixing, if any, has occurred between Wisconsin BGS and PGS.

Although levels of divergence among groups suggest lower divergence among Wisconsin species, these findings must be taken in context given the restricted number of samples available from outside of the state. It is important to note that the sample size of Michigan BGS snakes was small (n = 8) and from only two counties, whereas Wisconsin BGS (>80% q-value) were both numerous (n = 498) and undoubtedly sampled from more than one distinct population. F_{ST} is a between-subpopulation genetic divergence measure (Wright 1951) and, as such, the treatment of all samples of Wisconsin BGS as being a single subpopulation under this model is likely inaccurate (Jost 2008). Additional genetic structure and within-species divergence is likely to exist in the samples used in this study. Therefore, the observed F_{ST} values should be considered as initial estimates and not in terms of absolute values. Furthermore, there is not an appropriate local comparison for the Michigan BGS (or Iowa/MN PGS) samples where species divergence within an area of sympatry can be compared to determine if landscape level features in Wisconsin correlate to this apparent reduction in divergence between the species. Nevertheless, the heuristic value of examining the observed between-group F_{ST} estimates in this situation is valuable.

Despite the confirmation of hybridization and apparently lower divergence between Wisconsin BGS and PGS, all evidence supports the contemporary, if tenuous, integrity of the species within

the state. The level of divergence discussed previously between Wisconsin BGS and PGS, despite being less than between Wisconsin BGS and Michigan BGS, was still significantly different from zero and consistent with a level of divergence that does not indicate rampant introgressive hybridization. A study examining the genetic diversity and dynamics of two sympatric gartersnake species (the Terrestrial Gartersnake, *T. elegans* and the CGS) showed levels of F_{ST} in multiple pairwise comparisons within each species to be, on average, half to a third lower than the observed values in the current study (mean F = 0.024 and 0.035, respectively; Manier and Arnold 2005). If introgressive hybridization was significant enough to result in a loss of species integrity, the F_{ST} values should not differ from zero. Further, the occurrence of putative hybrids in a subset of locations where the species are likely sympatric suggests site-specific factors, rather than the widespread mixing of the species in the state, are at play. Evaluation of the Bayesian admixture and assignment results confirmed the cohesion/integrity of the species through the resolution of the non-Wisconsin snakes into their respective genetic units.

This study provides a possible solution to one of the central challenges facing BGS management and regulation in Wisconsin – identification and discrimination of BGS versus PGS. The data and analysis put forward in this study show strong discrimination of species, and allow the defensible identification of putative hybrid snakes without relying on subjective or site-specific *a priori* designations. Simultaneous sampling of morphological and site data on all snakes included in this study has provided a wealth of data for further development of field-based identification and co-analysis of morphological and genetic data. Coupled with genetic approaches presented herein, options for discriminate identification using more objective methods are now available and will continue to be refined in the near future.

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Practices (FSP). The FSP process is a peer-review process implemented by the U.S. Geological Survey Office of Science Quality and Integrity. This process includes the external peer-review of all documents submitted for publication or reports (such as this one) in an effort to ensure high quality and sound science practices and interpretation of findings. For more information on the U.S. Geological Survey FSP process please visit http://pubs.usgs.gov/circ/1367/pdf/C1367.pdf. This final report was reviewed and improved through this process by Dr. Wendy Stott (U.S. Geological Survey) and Dr. Gregory Moyer (U.S. Fish and Wildlife Service) as well as several U.S. Geological Survey, University of Wisconsin-Stevens Point, and Wisconsin Department of Natural Resources personnel.

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