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Analysis of the genetic status of populations in the zone of overlap between *Thamnophis butleri* and *Thamnophis radix* based on AFLP analysis

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Executive Summary

Butler's Gartersnake, *Thamnophis butleri*, was described in 1889 (Cope, 1889). This species is closely related to the Plains gartersnake, *Thamnophis radix*, but is ecologically more specialized and narrowly distributed (Rossman, et al. 1996). These two species are distinguished morphologically through color, pattern, scale count, and size differences, except in a narrow hybrid zone in southeastern Wisconsin (Casper, 2003), a finding mirrored by genetic studies (Burghardt et. al 2006; Fitzpatrick et al. 2008). The present study applied AFLP genetic markers to evaluate more precisely the question of whether animals in a subset of populations of special management concern in this putative hybrid zone were genetically mixed or if they contained animals of distinctive Butler's ancestry.

We studied 252 snakes from 15 populations in this area as well as control snakes from each species. All snakes were provided by the WDNR and while sample sizes were limited, we found animals with more than 90% Butler's ancestry in nine populations and animals with more than 75% Butler's ancestry in 12 populations. The populations with no such animals had small sample sizes and no confidence can be given to these negative results. We conclude that animals of primarily Butler's Gartersnake genetic composition occur throughout the entire zone, although the relative density of these animals differs widely.

Introduction

Butler's gartersnake, *Thamnophis butleri* (Cope 1889), is listed as Threatened in Wisconsin and Ontario, Canada, and as Endangered in Indiana. The decline of *T. butleri* is attributed mainly to habitat destruction, with its preferred habitat (i.e., wet meadows and prairies) rapidly being developed for commercial and residential purposes in many areas (Vogt 1981; Joppa and Temple 2005). In Waukesha and Milwaukee counties, conflicts between urban development and habitat conservation have been particularly common. Effective conservation planning has been hampered by lack of data on several important aspects of snake biology. One of the most critical issues arises from difficulties in distinguishing *T. butleri* (the protected taxon) from the Plains Gartersnake, *T. radix* (a close relative with no legal status). This difficulty is a result of their close similarity (Rossman et al. 1996; Alfaro and Arnold 2001; de Queiroz et al. 2002) and the presence of hybrids with intermediate traits (Albright 2001; Casper 2003; Kirby 2005; Fitzpatrick et al. 2008). Here we use DNA markers to evaluate the genetic composition of certain populations with known or suspected hybridization.

Thamnophis butleri and *T. radix* are closely related (Ruthven 1908; Conant 1950), with molecular phylogenetic studies showing several mitochondrial DNA loci to have nearly identical sequences (Alfaro and Arnold 2001; de Queiroz et al. 2002; Burghardt et al. 2006). Rossman et al. (1996) suggested that *T. butleri* is a neotenic (dwarfed) derivative of *T. radix*, which may explain the similarity in genetics between the two, given that any divergence probably occurred relatively recently. Ecologically and behaviorally there are also differences between the two taxa. For example, *T. radix* is a prey generalist and eats earthworms, amphibians, fish, rodents, and even birds. *T. butleri*, in contrast, eats virtually nothing but earthworms and leeches in the wild

although it will often eat fish and amphibians in captivity, further supporting their close relationship (Burghardt, 1969; Halloy & Burghardt, 1990; Rossman et al. 1996).

In previous studies of Wisconsin *T. butleri* and *T. radix*, we have confirmed that the two taxa are very closely related, that they do hybridize where their geographic ranges overlap, and that they maintain distinctiveness in size, shape, and gene frequencies (Burghardt et al. 2006; Fitzpatrick et al. 2008). Burghardt et al. (2006) surveyed mitochondrial DNA variation across both taxa in Wisconsin and neighboring states. They identified a region, dubbed the IZ for "indeterminate zone", where additional sampling was needed to understand the genetic/taxonomic status of the gartersnakes. The IZ largely corresponds to a hybrid zone identified by the occurrence of morphologically intermediate snakes (Casper 2003) and confirmed by DNA analysis showing individuals with mixtures of alleles derived from *T. butleri* and *T. radix* (Fitzpatrick et al. 2008). The IZ in Milwaukee and eastern Waukesha counties is a fairly narrow (2-8 km) east-west band where suitable *T. butleri* habitat occurs. The goal of this study was to assess the genetic composition of gartersnakes at specific sites in the IZ chosen by the WDNR, to assist in conservation planning.

Methods

Sampling

Samples were supplied by WDNR. We received 252 snakes from 15 sites (Table 1; Fig. 1). The target sample size per site was 20 snakes, representing a compromise between statistical rigor and collecting constraints. Reference samples were drawn from our own collections. These included nine *T. butleri* from the northernmost sites we have sampled in Wisconsin and eight *T. radix* from Illinois and southern Wisconsin (Table 1; Fig. 1). The reference samples were

analyzed previously and determined to represent true *T. radix* and *T. butleri* (Fitzpatrick et al. 2008).

Molecular Methods

DNA was extracted from tail tips or scale clips using the DNeasy(R) Tissue Kit (Qiagen). Amplified fragment length polymorphism (AFLP) markers were obtained using the standard protocol (Vos et al. 1995) using four different selective primers (MseCTAG – GATGAGTCCTGAGTAACTAG, MseCTAC – GATGAGTCCTGAGTAACTAC, MseCTTC – GATGAGTCCTGAGTAACTTC, MseCTTG – GATGAGTCCTGAGTAACTTG). Fragment presence-absence was determined by running labeled selective PCR product in an ABI 3100 automated sequencer. Florescent peaks were aligned and called using Peak Scanner 1.0 (Applied Biosystems). Samples were run in 96-well plates with one negative control (reagents with no DNA extraction) per plate. All reference samples were run as positive controls in each plate. We used only AFLP peaks that were present in the reference samples and 100% repeatable across the four analysis plates.

Data Analysis

To estimate the level of *T. butleri* ancestry of each individual in the sample, we first identified ancestry informative markers (AIMs: (Fitzpatrick and Shaffer 2007; Mebert 2008)) and then used Buerkle's (2005) maximum-likelihood algorithm to calculate the ancestry index. We identified AIMs as markers where the difference in band frequency between the reference samples was two-fold or greater and the frequency in one reference sample was ≥ 50%. Fourteen such markers were identified (Table 2). We then used Buerkle's program (hindex) to estimate the fraction of each snake genome that was derived from *T. butleri*.

We used two additional statistical methods to complement the ancestry index estimates from hindex. First, we used the Bayesian algorithm implemented in STRUCTURE 2.2 (Pritchard et al. 2000; Falush et al. 2007) to optimally cluster individuals into two populations following the population genetic criterion that linkage disequilibrium within populations should be minimized. The resulting cluster assignment probabilities estimate the fraction of each individual genome derived from the two hypothetical populations. If the reference samples cluster into different STRUCTURE clusters with high confidence, then the individual ancestry estimates from STRUCTURE should correspond closely to those from hindex.

Second, we took a non-genetic approach by calculating Jaccard's similarity index between each individual and each of the individuals in the reference samples. Jaccard's index uses only shared bands to measure similarity, effectively treating absence of a band as missing data (Gower and Legendre 1986). This is a conservative approach for AFLP data because absence of a band can be a result of variation in reaction conditions or assay sensitivity. In contrast, the genetic methods assume that absence of a band diagnoses an individual as homozygous for absence of a restriction site (Buerkle 2005; Falush et al. 2007), and may therefore be misleading when data have low repeatability or when variation at a single locus results in length polymorphism such that two distinct bands represent alleles of a single locus rather than independent markers (Nei and Li 1979). To classify individuals based on Jaccard's index, we took, for each individual, its average similarity to *T. butleri* (J_B) and its average similarity to *T. radix* (J_R). We used these as two variables to estimate a linear discriminant function for the reference samples and then estimated discriminant scores and classification probabilities for the "unknown" individuals sampled from the IZ sites.

Results

Most of the samples supplied by WDNR were assayed successfully for our ancestry informative markers. A few consistently failed to amplify at the PCR stage, including all five from PCSE, suggesting a problem with the preservation of DNA in those samples.

Estimates of the ancestry index were 0.0 for all reference *T. radix* samples and 1.0 for all reference *T. butleri* samples except for one, estimated at 0.78 (Fig. 2). Linear discriminant analysis classified all reference samples correctly with > 0.9999 classification probability. STRUCTURE clustered all *T. radix* reference samples and one *T. butleri* together. The other *T. butleri* reference samples generally fell into the other cluster (one *T. butleri* reference had about 50% cluster probability). STRUCTURE used no prior information about population origin, whereas the ancestry index was calculated under the explicit assumption that the AFLP band frequencies in the reference samples accurately represent the parental *T. butleri* and *T. radix* gene pools. With respect to classification of animals in the IZ, the three data analyses are concordant but not identical (Figs. 2-4). Linear discriminant analysis tended to classify individuals with high confidence (Fig. 4), but this is likely an artifact of the logistic classification function designed to classify, not to estimate ancestry (Agresti 2002). Clustering based on STRUCTURE resulted in slightly more individuals with very high (>0.90) or very low (<0.10) ancestry relative to the ancestry index from hindex (Figs. 2 and 3). We think the ancestry index from hindex is the most appropriate for the question of ancestry within the IZ given the reference samples.

All of the sites with sample sizes >1 had snakes of hybrid ancestry, but the distribution of ancestry indices varied considerably among sites (Fig. 2, Table 3). Nine of the 14 IZ samples had at least one individual with > 99% estimated *T. butleri* ancestry and 12 of the 14 had at least one individual with > 75% *T. butleri* ancestry (Table 1). The two sites with no individual >75% had

very small sample sizes (4 and 1) and therefore we do not have adequate information to assess the probability that snakes with higher *T. butleri* ancestry exist at those sites. For the sites with larger sample sizes, we can make inferences regarding the expected frequency of unobserved genotypes from confidence limits of the binomial distribution. For example, in the Beloit and Moorland sample of 16 snakes, we observed none with ancestry > 80%. If we assume the number of snakes with ancestry >80% is a binomial random variable, the 95% upper confidence limit on the frequency of such individuals is 0.206 (Agresti 2002). That is, based on the current analysis, we are approximately 95% confident that fewer than 21% of the snakes at Beloit and Moorland have >80% *T. butleri* ancestry.

To address the question of whether the individuals within a site resemble a single randomly mating population, we tested the distribution of the ancestry index within each site against the expected distribution after one generation of random mating. For each sample, we simulated 1000 replicates of random mating between the individuals in the sample to produce 1000 replicate samples equal in size to the observed sample. In all cases, the observed variance of the ancestry index was greater than 99% of the simulated variances (Table 4). Thus, the null hypothesis that each site is a randomly mating population of hybrid origin can be rejected.

Discussion

We used AFLP data to describe the genetic status of 14 sites within the previously identified IZ, or indeterminate zone (Burghardt et al. 2006). We estimated the fraction of each snake's genome that was derived from *T. butleri* (Buerkle 2005; Fitzpatrick et al. 2008). Some samples were predominantly *T. butleri*, some predominantly *T. radix*, and several included individuals with genotypes covering the entire spectrum (Fig. 2). Our results support our earlier

inference that the contact zone between *T. butleri* and *T. radix* should be characterized as a zone of overlap where hybridization occurs, but many individuals of distinctly *T. butleri* or *T. radix* ancestry also coexist (Fitzpatrick et al. 2008).

Specific inferences are limited by sample sizes. This is particularly true with respect to establishing confidence that the protected taxon *T. butleri* does not occur at a particular site. This problem is well known in conservation biology (Krebs 1989) and epidemiology (Dohoo et al. 2003): failing to detect a species in a sample is consistent with that species being rare, but it is impossible to prove that it is absent. Detecting a genotype is similar in many ways to detecting diseased individuals: the sampling unit is the individual organism. If populations are composed of large numbers of individuals, the sampling problem is how many individuals to assay in order to provide an accurate estimate of disease prevalence (Dohoo et al. 2003), or (in our case) the frequency of an allele or genotype (i.e., individuals meeting some level of *T. butleri* ancestry). A common recommendation in wildlife epidemiology is to assay 30-35 individuals for disease or pathogens so that, if none are detected, one can say with 95% confidence that the prevalence of the pathogen is ≤ 10% (Dohoo et al. 2003). On the other hand, when the individuals of interest are rare, the sampling problem is how much surveying effort to expend in order to distinguish rarity from absence (Krebs 1989). Both issues are important for the present study. Budgetary restrictions resulted in target sample sizes (15-20) lower than the usual recommendation for epidemiology. Even so, it was not possible to obtain target sample sizes for several sites (Table 1).

We provide a rough assessment of uncertainty in Table 1 by estimating the upper 95% confidence limit for the proportion of individuals with *T. butleri* ancestry > 90%. For sites with small samples, this number can be large. Even when no individuals with ancestry > 90% were

observed, we can only say with confidence that the true population frequency of such individuals is lower than this 95% UCL. In the absurd case of LRN – where the single snake sampled had an ancestry estimate of 0% *T. butleri* – the true frequency of individuals with 90% *T. butleri* ancestry might be over 97% (Table 1).

Note that PCN and PCS were streamside populations separated by one roadway, across which gene flow undoubtedly occurs (along the stream). Although summary statistics differ slightly between the two samples, there is no statistical support for a genetic difference (Table 3) and the two sites should probably be considered a single breeding population.

We can say with confidence that most sites in the IZ had at least one snake scored as 100% *T. butleri* ancestry. Given this and earlier results, the geographic range of *T. butleri* probably extends through the IZ into Racine County, Wisconsin (Fitzpatrick et al. 2008). The southern portion of this range (southern halves of Waukesha and Milwaukee counties and northern Racine County) overlaps with the range of *T. radix.* The two taxa hybridize in this area of overlap, but they have not merged into a single gene pool (Table 4). Rather, *T. butleri* and *T. radix* maintain distinct gene pools that are linked by hybridization.

Both the stability and geographic limits of this hybridizing but genetically segregated system are unknown. We have postulated that this may be a post-glacial secondary contact zone, where evolution is underway (Burghardt et al. 2006; Fitzpatrick et al. 2008). Sampling more widely in transects perpendicular to the IZ would reveal finer resolution geographic limits. Although beyond the scope of this study, additional information of conservation relevance may be obtained by studying in a comparable manner the genetic composition of *T. radix* and *T. butleri* in Ohio, where they also have been in contiguous populations in recent historic times and

share some mtDNA haplotypes not found in other populations of either species (Burghardt et al. 2006; Burghardt et al. 2001).

Whether or not segregated *T. butleri* and *T. radix* gene pools will persist over time in these hybridizing populations is a more difficult, but important question. The mechanisms maintaining the genetic segregation in these hybridizing populations remain unknown, and in need of investigation. It is not clear if land use (i.e. habitat quality) and conservation practices might affect the genetic dynamics of mixed populations. The conservation value of mixed populations depends on their genetic stability in addition to demographic stability. For example, if *T. radix* genotypes tend to displace *T. butleri*, then sites with predominantly *T. butleri* ancestry might have more potential to contribute to the species recovery. Populations are more isolated in the north (where habitat patches are generally smaller and fewer), and less isolated in the IZ (where habitat patches are generally larger and more numerous). Moreover, unique genetics are concentrated in the IZ (Burghardt et al. 2006). It follows that a conservation focus on the northern populations may better preserve the *T. butleri* genome without hybrid influence, but may have more demographic risk (owing to these more isolated and smaller sites), while a focus on the IZ may have less confidence in preserving the *T. butleri* genome owing to the hybridization issue, but may be more likely to succeed demographically (owing to more, and more connected, habitat availability). Finally, if preserving genetic diversity is important, the IZ populations are where most of the unique alleles are found (Burghardt et al. 2006).

Spatial conservation planning decisions could be more easily informed if a firm relationship between genetic makeup and morphology can be established. This may allow the use of many of the available museum specimens collected decades ago to investigate the stability of the system over time, and would be a more efficient means of evaluating existing populations.

Literature Cited

Agresti, A. 2002. Categorical data analysis, 2nd edition. Wiley & Sons, New York.

Albright, J. D. 2001. Microsatellite DNA markers, multiple paternity, and the inheritance of morphology and behavior in Butler's garter snake (*Thamnophis butleri*). University of Tennessee, Knoxville.

Alfaro, M. E., and S. J. Arnold. 2001. Molecular systematics and evolution of *Regina* and the Thamnophiine snakes. Molecular Phylogenetics and Evolution 21:408-423.

Beissinger, S. R. and D. R. McCullough (editors). 2002. Population viability analysis. The University of Chicago Press, Chicago.

Buerkle, C. A. 2005. Maximum-likelihood estimation of a hybrid index based on molecular markers. Molecular Ecology Notes 5:684-687.

Burghardt, G. M. 1969. Comparative prey-attack studies in newborn snakes of the genus *Thamnophis*. Behaviour 33:77-114.

Burghardt, G. M, Albright, J., McCracken, G. F., Small, R., Placyk, J. S., Quigley, N. Saidek, K. and Bealor, M. 2001. Genetic distinctiveness of *Thamnophis radix* in Ohio. Report to the Ohio Division of Wildlife.

Burghardt, G. M., J. S. J. Placyk, G. S. Casper, R. L. Small, and K. Taylor. 2006. Genetic Structure of Great Lakes Region *Thamnophis butleri* and *Thamnophis radix* based on mtDNA Sequence Data: Conservation Implications for Wisconsin Butler's Gartersnake. Report to the Wisconsin Department of Natural Resources.

Casper, G. S. 2003. Analysis of amphibian and reptile distributions using presence-only data. Ph.D. dissertation. University of Wisconsin, Milwaukee.

Conant, R. 1950. On the taxonomic status of *Thamnophis butleri* (Cope). Bulletin of the Chicago Academy of Sciences 9:71-77.

Cope, E. D. 1889. On the Eutaeniae of southeastern Indiana. Proc. U.S. Natl. Mus. 11:399-401.

de Queiroz, A., R. Lawson, and J. A. Lemos-Espinal. 2002. Phylogenetic relationships of the North American garter snakes (*Thamnophis*) based on four mitochondrial genes: How much DNA sequence is enough? Molecular Phylogenetics and Evolution 22:315-329.

Dohoo, I., W. Martin, and H. Stryhn. 2003. Veterinary Epidemiology Research. AVC, Charlottetown, USA.

Falush, D., M. Stephens, and J. K. Pritchard. 2007. Inference of population structure using multilocus genotype data: Dominant markers and null alleles. Molecular Ecology Notes 7:574-578.

Fitzpatrick, B. M., J. S. Placyk, M. L. Niemiller, G. S. Casper, and G. M. Burghardt. 2008. Distinctiveness in the face of gene flow: Hybridization between specialist and generalist gartersnakes. Molecular Ecology 17:4107-4117.

Fitzpatrick, B. M., and H. B. Shaffer. 2007. Introduction history and habitat variation explain the landscape genetics of hybrid tiger salamanders. Ecological Applications 17:598-608.

Gower, J. C., and P. Legendre. 1986. Metric and Euclidean properties of dissimilarity coefficients. Journal of Classification 3:5-48.

Halloy, M. & Burghardt, G. M. 1990. Ontogeny of fish capture and ingestion in four species of garter snakes (*Thamnophis*). Behaviour 112:299-318.

Joppa, L. N., and S. A. Temple. 2005. Use of upland habitat by Butler's Gartersnake (*Thamnophis butleri*). Bulletin of the Chicago Herpetological Society 40:221-227.

Kirby, L. E. 2005. A comparative study of behavior in neonate garter snakes, *Thamnophis butleri* and *T. radix* (Colubridae), in an area of potential hybridization. M.S. thesis. University of Tennessee, Knoxville.

Krebs, C. J. 1989. Ecological Methodology. Harper Collins, New York, NY.

Mebert, K. 2008. Good species despite massive hybridization: Genetic research on the contact zone between the watersnakes *Nerodia sipedon* and *N. fasciata* in the Carolinas, USA. Molecular Ecology 17:1918-1929.

Nei, M., and W. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences, USA 76:5269-5273.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Rossman, D. A., N. B. Ford, and R. A. Seigel. 1996. The Garter Snakes: Evolution and ecology. University of Oklahoma Press, Norman, OK.

Ruthven, A. G. 1908. Variations and genetic relationships of the gartersnakes. Bulletin of the United States National Museum 61:1-201.

Vogt, R. C. 1981. Natural history of amphibians and reptiles of Wisconsin. Milwaukee Public Museum, Milwaukee.

Vos, P., R Hogers, M Bleeker, M Reijans, T van de Lee, M Hornes, A Friters, J Pot, J Paleman, M. Kuiper, and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23:4407-4414.

Table 1. Summary statistics for each site.

Site	N[a]	mean B[b]	max B[b]	N(>60%)[c]	N(>75%)[c]	N(>90%)[c]	95%UCL(90)[d]
B&M	16 (16)	0.377	0.758	3	1	0	0.206
BF	18 (18)	0.338	1.000	1	1	1	0.273
BSG	21 (21)	0.721	1.000	15	13	9	0.660
CCG	4 (4)	0.471	0.610	1	0	0	0.602
CRS	14 (13)	0.803	1.000	10	7	6	0.749
FP	21 (21)	0.842	1.000	20	14	13	0.819
LRN	1 (1)	0.000	--	--	--	--	0.975
MW	18 (18)	0.609	1.000	10	7	6	0.590
PCN	23 (22)	0.334	1.000	1	1	1	0.228
PCS	23 (23)	0.398	0.823	5	2	0	0.142
PCSE	5 (0)	--	--	--	--	--	--
PP	25 (24)	0.437	1.000	4	4	3	0.324
RNC	20 (18)	0.796	1.000	15	9	7	0.643
TC	17 (15)	0.427	1.000	3	3	3	0.481
WPS	26 (26)	0.441	0.823	6	3	0	0.132

[a] Number of snakes provided by WDNR (number successfully assayed for AFLP markers)

[b] mean B and max B are the mean and maximum of the ancestry index (Buerkle 2005), which estimates the proportion of each snake's genome derived from *T. butleri*.

[c] N(>X%) is the number of individuals in the sample with ancestry index > X%.

[d] 95% upper confidence limit for the estimated proportion of individuals with ancestry index > 90%.

Table 2. AFLP markers scored and their frequencies in the reference samples.

marker	Primer	Size Range (bp)	*T. butleri*	*T. radix*
G131.133	MseCTTC	131 – 133	0.57	0.00
G49.51	""	49 – 51	0.00	0.63
G54.57	""	54 – 57	0.86	0.00
G62.64	""	62 – 64	0.57	0.00
G64.66	""	64 – 66	0.00	0.63
R151.154	MseCTTG	151 – 154	0.14	0.50
R155.160	""	155 – 160	0.71	0.00
R161.163	""	161 – 163	0.00	0.50
R216.220	""	216 – 220	0.71	0.25
R315.318	""	315 – 318	0.00	0.63
Y155.157	MseCTAC	155 – 157	0.14	0.75
Y439.442	""	439 – 442	0.14	0.50
B53.55	MseCTAG	53 – 55	0.14	0.50
B57.58	""	57 – 58	1.00	0.00

Table 3. P-values of pairwise randomization tests of the null hypothesis that each pair of sites has the same average ancestry index (10,000 randomizations of individuals between sites). Bold values are statistically significant (study-wide $\alpha = 0.05$) after sequential Bonferroni adjustment for multiple tests.

	B&M	BF	BSG	CRS	FP	MW	PCN	PCS	PP	RNC	TC
B&M											
BF	0.6656										
BSG	0.0032	0.0013									
CRS	**0.0001**	**0.0000**	0.4406								
FP	**0.0000**	**0.0000**	0.1995	0.6679							
MW	0.0342	0.0174	0.3676	0.0852	0.0216						
PCN	0.5700	0.9500	**0.0002**	**0.0000**	**0.0000**	0.0045					
PCS	0.7600	0.4060	**0.0008**	**0.0001**	**0.0000**	0.0180	0.2994				
PP	0.4962	0.2601	0.0080	**0.0005**	**0.0001**	0.0814	0.1706	0.5840			
RNC	**0.0000**	**0.0000**	0.4176	0.9174	0.5402	0.0578	**0.0000**	**0.0000**	**0.0001**		
TC	0.6556	0.4235	0.0291	0.0029	**0.0001**	0.1518	0.3255	0.7603	0.9262	**0.0010**	
WPS	0.3919	0.1743	0.0027	**0.0001**	**0.0000**	0.0588	0.0998	0.4896	0.9499	**0.0001**	0.8790

Table 4. Tests of the observed variance of the ancestry index against that expected after one generation of random mating.

Site	V(obs)[a]	V(exp)[b]	P[c]
B&M	0.058	0.025	0.000
BF	0.071	0.032	0.000
BSG	0.112	0.051	0.000
CRS	0.048	0.021	0.000
FP	0.063	0.029	0.000
MW	0.122	0.054	0.000
PCN	0.044	0.020	0.001
PCS	0.040	0.018	0.001
PP	0.079	0.036	0.001
RNC	0.037	0.017	0.000
TC	0.131	0.057	0.002
WPS	0.054	0.025	0.000

[a] Observed variance of Buerkle's (2005) ancestry index

[b] Expected variance after one generation of random mating

[c] Fraction of 1000 simulations with variance > V(obs)

Fig. 1. Approximate localities of study sites (black circles). Inset shows broader region with reference *Thamnophis butleri* sites (gray circles) and reference *T. radix* sites (open circles).

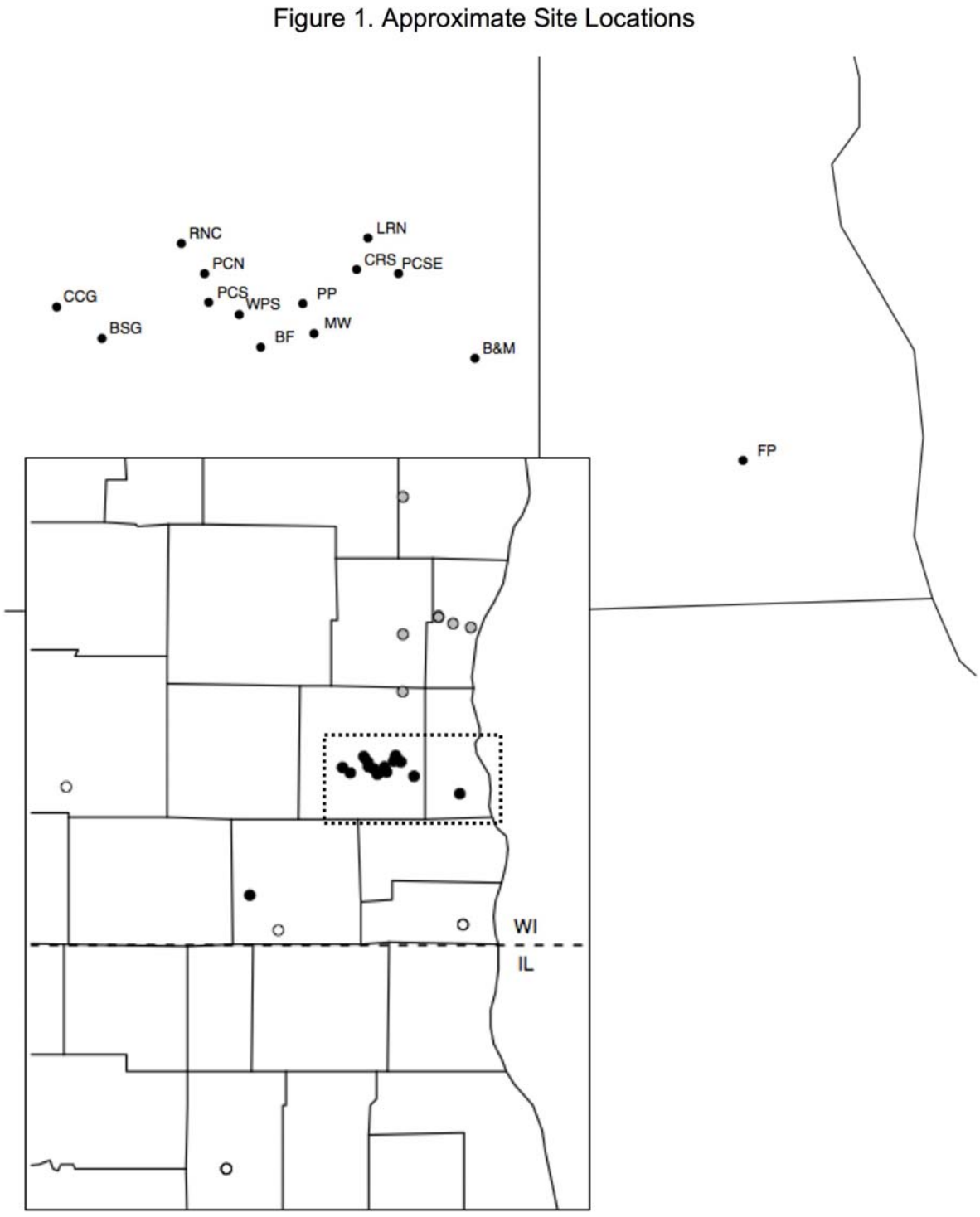

Fig. 2. Distributions of the ancestry index (Buerkle 2005) in the reference samples (A: *T. butleri* in black, *T. radix* in white), and IZ samples (B-O).

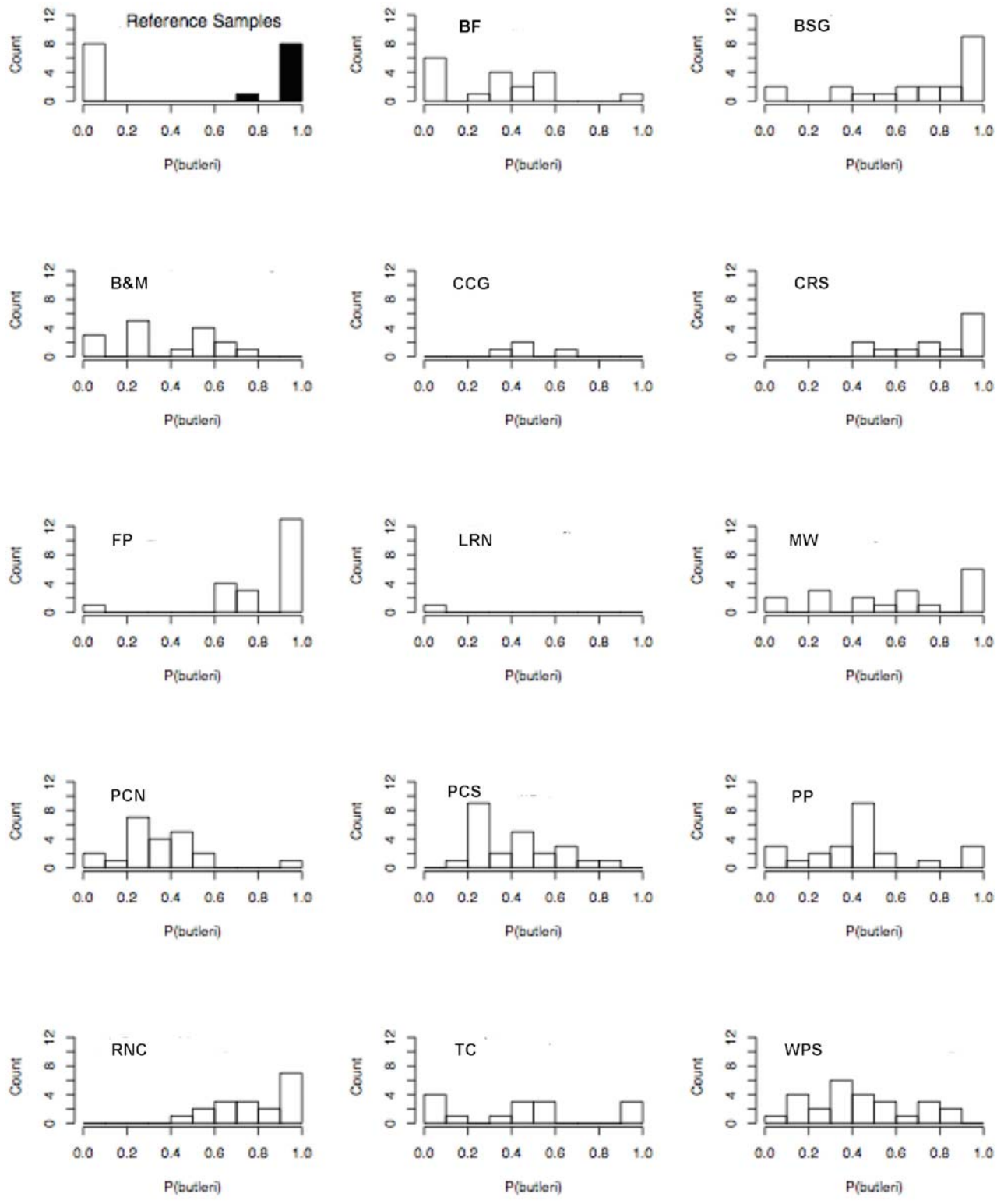

Fig. 3. Distributions of the cluster probability estimated in STRUCTURE (Falush et al. 2007) in the reference samples (A: *T. butleri* in black, *T. radix* in white), and IZ samples (B-O).

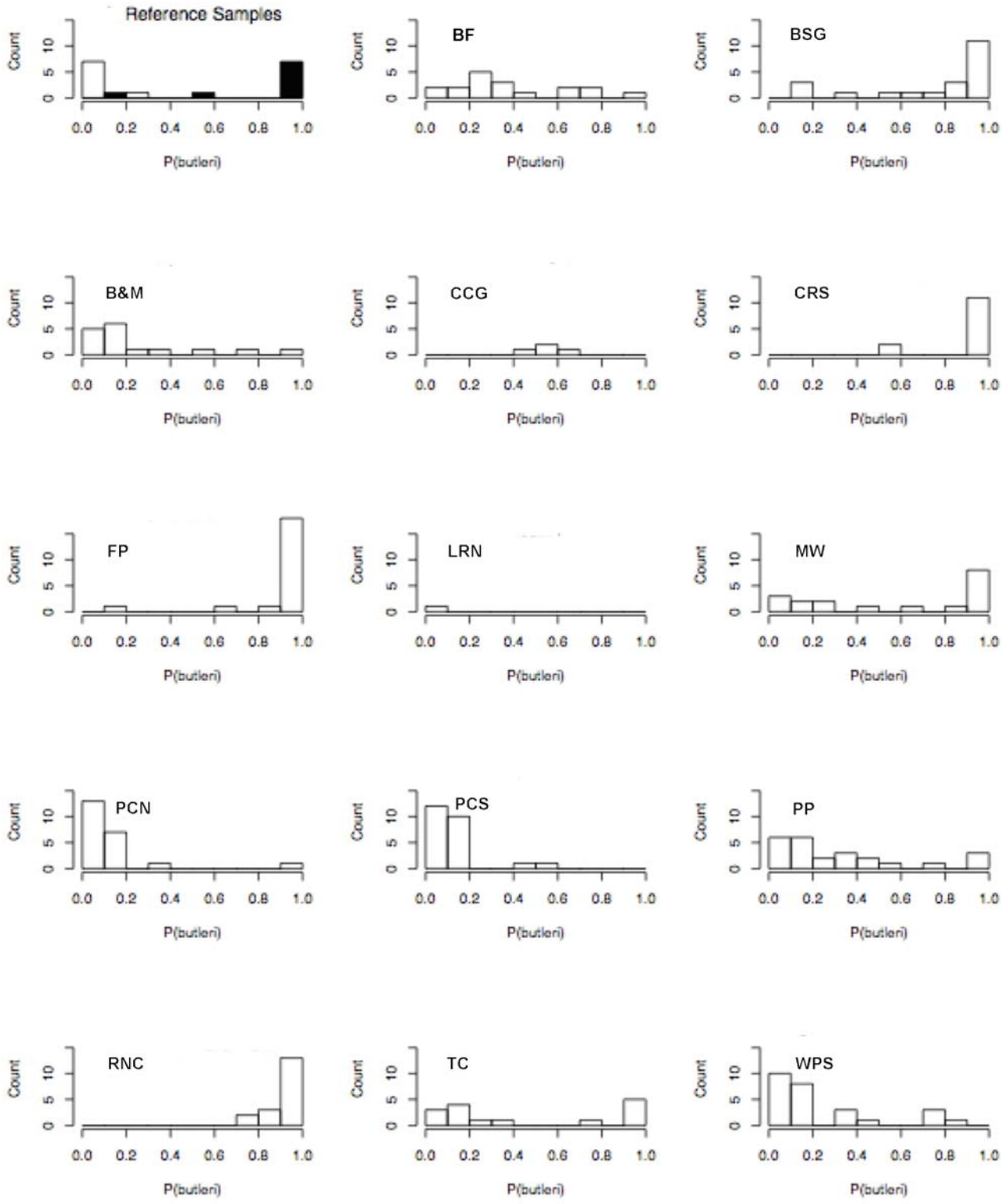

Fig. 4. Distributions of classification probability estimated via linear discriminant analysis of J_B and J_R in the reference samples (A: *T. butleri* in black, *T. radix* in white), and IZ samples (B-O).