

Wisconsin Butler's Gartersnake (*Thamnophis butleri*) and Plains Gartersnake (*T. radix*)  
Microsatellite DNA Study Plan

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**Management Issue:**

The Butler's gartersnake (*Thamnophis butleri*) – a diminutive, Great Lakes region wetland specialist - occurs in Wisconsin only as a disjunct population in the greater Milwaukee area in southeast Wisconsin (Rossman *et al.* 1996). This species, hereafter referred to as Butleri, is otherwise found only east of Lake Michigan in a distribution across the eastern half of Michigan and adjacent parts of southwestern Ontario, northeastern Indiana, and northwestern Ohio. Butleri was listed as threatened in Wisconsin in 1997 because of long-term habitat loss in its geographically restricted range (Joppa and Temple 2005, Vogt 1981) and other threats. Butleri presents a management and conservation challenge because it hybridizes with its close relative, the plains gartersnake (*T. radix*). The Eastern plains gartersnake – hereafter referred to as Plains – is not a listed species in Wisconsin but is a species of Special Concern, and inhabits mesic prairies, dry-mesic prairies, and oak savanna with access to nearby water in the form of marshes, ponds, brooks, or rivers (Ernst and Ernst 2003, Vogt 1981). Plains occur south of the Wisconsin Butleri range – as currently understood - in extreme southeastern Wisconsin, northern Illinois, and northwestern Indiana, and west of the Butleri range in south-central Wisconsin and states further west (Fig. 1). A purported Butleri-Plains hybrid zone occurs in southeastern Wisconsin where the two species are sympatric (Figs. 1, 2). The respective ranges of Butleri, Plains, and Butleri-Plains hybrids in this area – and the degree to which these ranges overlap - are not well understood. However, the hybrid zone is thought to encompass Walworth, Kenosha, and Racine counties, and at least the southern portions of Waukesha and Milwaukee counties.

Recent research in Wisconsin (Casper 2008, Fitzpatrick *et al.* 2008) has focused on more precisely delimiting the northern edge of the hybrid range in a narrow, east-west belt across Waukesha and Milwaukee counties referred to as the “indeterminate zone,” or “IZ” (Fig. 2). Bayesian analysis of amplified fragment length polymorphism (AFLP) data suggests that hybrids occur broadly across the IZ. Most of the populations in 11 well-sampled sites ( $\geq 15$  snakes/site) contained hybrids and either or both individuals identified as Butleri or Plains. However, AFLP-based genetic species assignment (Fitzpatrick *et al.* 2008) of the approximately 250 IZ study snakes is not concordant with a morphological species index (Casper 2008). For example, all 17 snakes encountered at Turtle Creek in west-central Walworth County were morphologically identified as strongly Plains (mean “*T. butleri* likeness” score = 0.0735 and maximum = 0.375 – on a 0-1 scale where 1 = “pure” *T. butleri*), whereas a Bayesian analysis of the AFLP data from 15 of the 17 snakes found only three strongly Plains snakes (>90% Plains cluster probability), five strongly Butleri snakes (>90% Butleri cluster probability), and

intermediates (likely hybrids). These results support the inclusion of Walworth County within the hybrid zone, suggest that the hybrid zone may extend further north into Waukesha and Milwaukee counties than previously thought, and also appear to question a basic assumption underlying earlier studies' designs and the regulatory approach for this species: that a Wisconsin Butleri can be conclusively identified as such, based on geographic location or appearance. These observations could, alternatively, be attributable to a sample size limit of the AFLP study - relatively small Butleri and Plains reference sample size (7 Butleri from 6 sites; 8 Plains from 4 sites) - and the study outlined here will employ significantly larger reference site sample sizes from reference sites exclusively outside the known hybrid zone but within the Wisconsin area. (Note that for the purposes of this study the "Wisconsin area" is defined as the region within approximately 150 miles of Milwaukee that includes southern Wisconsin and northern Illinois). This study will develop microsatellite loci, a co-dominant genetic tool frequently applied to conservation genetics questions involving closely related species, to: 1) better elucidate the genetic relationship between Butleri and Plains in Wisconsin, 2) more precisely delimit the Wisconsin ranges of Butleri, Plains and Butleri-Plains hybrids, and 3) if possible, establish physical characteristics (i.e., a morphological index) consistent with genetically identified Butleri, Plains and Butleri-Plains hybrids and, as such, a defensible field method consistent with laboratory molecular identification

### **Proposed Research:**

This research will build on and complement existing Wisconsin Butleri genetics research by developing and applying multiple (goal >12) microsatellite loci derived from well-sampled Wisconsin-area Butleri and Plains populations. This work will also draw on reference material from outside the Wisconsin area (i.e., beyond Wisconsin/northern Illinois). Robust Wisconsin-area reference site sample sets, coupled with reference sample sets from elsewhere in each of the species' ranges, will reveal whether and how Wisconsin Butleri differs from Wisconsin Plains, and will provide additional insight into the relationship between Wisconsin Butleri and Butleri from east of Lake Michigan. If Butleri and Plains are genetically distinguishable in Wisconsin, the resulting, highly spatially resolved distribution map will assist conservation and management of Butleri.

### **General Approach & Justification**

A reference-based analysis of genetic diversity and differences between Butleri and Plains, using a codominant set of molecular genetic markers, will be conducted. This approach will provide 'known' reference samples from which species-diagnostic alleles and/or allele frequency differences can be identified and used for developing a molecular identification approach and/or a molecular hybrid index model.

The principal investigator will develop a robust number (8-16) of diagnostic microsatellite loci from within well-sampled populations (target/site: 30 snakes; minimum: 25) from at least seven Wisconsin Butleri and four Wisconsin/northern Illinois

Plains reference sites. Microsatellite development will follow the established protocols of Glenn and Schable (2005). This effort will also include reference site material from Butleri and Plains reference populations outside of the Wisconsin area, and will ideally include at least three such reference sites for each species. These snakes will be vouchered to the greatest extent possible, so that morphological assessments and other analyses can occur after and if genetic work establishes species integrity. However, at sites where some or all captured snakes are tail-clipped and released because of conservation or other concerns on the part of WDNR, a number of standard objective snake morphometric measures and meristics will be recorded before the snake is released. After microsatellite loci have been developed, they will be applied to extant and future Butleri and Plains tissue and extracted DNA collections to determine the genetic makeup of individual populations across southern Wisconsin and delimit Butleri, Plains, and hybrid ranges. Finally, if genetic analyses support species integrity then the principal investigator will examine whether morphometric measures and meristics from voucher specimens and individuals assessed in the field can be used to reliably distinguish Butleri, Plains, and hybrids in the field.

## **Detailed Approach**

### **I. Microsatellite marker development**

**Initiation:** May 2009

**Timeline:** Cover boards installed in survey sites in May/June 2009. Sample collection and delivery to principal investigator begins in July 2009 and continues through late September. Microsatellite development begins with receipt of first samples (including at least one voucher Butleri from which the microsatellite-enriched libraries will be based), and will be largely completed within **six months** of delivery of bulk sample set (**approximately January 2010**).

WDNR will provide the principal investigator a full sample of Wisconsin-area (Wisconsin and northern Illinois) snakes or tissue from snakes (target sample size/site: n=30; minimum: n=25) from at least seven Butleri and four Plains reference sites for molecular analyses. Reference site selection criteria include (1) high likelihood of containing abundant genetically-pure Butleri or Plains individuals, (2) substantial geographical separation from the suspected zone of hybridization, and (3) geographical isolation from other locations selected for sample collection. Survey protocols and candidate Wisconsin-area reference sites are described below. All suitable individuals obtained during surveys will be vouchered if possible. Vouchers will be euthanized humanely; a small tailsnip (~4 mm) will be taken for subsequent DNA analysis and preserved in pre-labeled 2.0 mL screwtop tubes with 95% EtOH, and following DNA sampling the complete specimen will be fixed in 50% formalin and preserved in 90% EtOH. Where WDNR prohibits vouchering (because of conservation concerns), snakes will be released back into their source sites after tissue samples have been collected and the following standard objective morphological measures and meristics recorded: total

body length, snout-vent length, lateral stripe position, ventral scale count, supralabial scale count, infralabial scale count, head length, head width, rostrum length, and neck width. The principal investigator will house tissue samples and voucher specimens.

In addition, WDNR will obtain a full sample (target sample size/site: n=30; minimum: n=25) of at least three Butleri reference sites and three Plains reference sites from beyond the Wisconsin area (Fig. 1). These Butleri reference sites will be located in the eastern Michigan/northeastern Indiana/northwestern Ohio Butleri range, and the Plains reference sites will be in the eastern Iowa/southeastern Minnesota Plains range. Possible sources include other agencies, investigators, and museum collections.

The final product will consist of a suite of at least 8 microsatellite markers - ideally 12+ markers - capable of amplifying homologous DNA regions of both Butleri and Plains. The markers will be tested against samples from all reference sites to provide the most complete reference genetic diversity assessment possible. Estimates of overall relatedness among *a priori* defined reference samples will be conducted using appropriate genetic analyses including AMOVA, Bayesian structure analysis, and assignment tests.

#### Wisconsin-area Plains reference sample sites

Recent WDNR Bureau of Endangered Resources efforts to assess Plains status and distribution in the state have failed to detect the species in several areas where it has historically occurred, and most Wisconsin sites currently thought to include robust Plains populations lie within the hybrid zone in Walworth, Racine, and Kenosha counties. Plains has historically occurred, and has recently been observed, at sites in Wisconsin west of the putative hybrid zone (Fig. 2, Vogt 1981, Wisconsin Herpetological Atlas, M. Zine, pers. comm.), but the status of these potential reference site populations is less well known and requires initial survey work. Cover boards – objects used to attract and capture gartersnakes - will therefore be deployed at a larger number of sites than will ultimately be required for genetic analyses, as some of these sites will likely fail to yield sufficient Plains numbers. In May/June 2009 cover boards will be placed in sites in Dane County and neighboring counties identified as candidate Plains reference sample sites based on historical occurrences and habitat criteria. Reference Plains samples will also be obtained, if possible, from northern Illinois.

Plains survey protocols will follow general WDNR Butleri survey protocols, except that cover boards will be placed further into upland habitats (i.e. board placement will not be restricted to within 100 ft of wetland line). This will include placement of cover objects (3/4" CDX stock plywood in 32" X 48" pieces) where they have sun exposure for most of the day. Individual board locations will be prepared by removing underlying vegetation via rake and sickle. At least 5 boards will initially be deployed at each site. Boards will be checked primarily in late afternoon (after 4 pm) on days that have reached at least 60 degrees F, and been sunny for several hours. Boards will only be checked in the mornings on rainy days and before 9 am, if it was sunny at 60 degrees F the previous day. Finally, Plains vouchering will proceed cautiously in sites where population size and

status is not known: initially most snakes collected at a site will be measured, and tail-clipped and only a low proportion (~10%) will be vouchered, but the proportion vouchered will be steadily increased if and as data from repeated site visits begin to indicate a robust local population.

NOTE: from approximately day 3 to day 10 snakes may not use board locations due to rotting vegetation remnants, although this time period can be shortened if vegetation is raked and cleared before boards are put down.

#### Wisconsin-area Butleri reference sample sites

Candidate Wisconsin Butleri reference sites are better understood and include two sites – UWM Field Station (Saukville) in west-central Ozaukee County and North Ave/Barker Road (Brookfield) in east-central Waukesha County – where hundreds of cover boards are already in place as part of a Butleri demography study. Butleri tissue from these two sites and five additional sites known to contain robust Butleri populations is expected to easily yield the requisite number of Butleri reference samples.

## **II. Butleri, Plains, and Butleri-Plains hybrid range characterization in southeastern Wisconsin**

**Initiation:** Upon completion of microsatellite development (Task I) and receipt of genetic material from non-reference Wisconsin populations. (*tentative: early 2010*).

**Timeline:** Task scope will be determined largely by WDNR spatial resolution needs (i.e., number and spatial distribution of populations to be investigated). Diagnostic microsatellites are readily and rapidly applied to suitable genetic material, and therefore the genetic structure of individual populations can be assessed within weeks of sample delivery. Consequently, the timeline for this task depends on WDNR determination of task scope and on the rate at which the WDNR can provide robust sample sets to the principal investigator. Analysis of material already obtained from snakes in a number of unknown or geographically intermediate populations, and of samples currently being collected from up to fifteen sites in and near the putative hybrid zone, can begin as soon as diagnostic microsatellites have been developed (task I). Therefore coarse range characterization and assessment of Butleri genetic status in Wisconsin will likely be available in spring 2010, but finer-scale characterization will be based on samples obtained in summer 2010, and will therefore be available in **winter 2010/spring 2011**.

## **III. Assess and further develop morphological Butleri identification schemes**

**Initiation:** This task cannot proceed unless the microsatellite development step (task I) establishes that Butleri, Plains, and hybrids can be reliably genetically distinguished, and until samples from mixed/unknown populations have been examined (task II).

Furthermore, the nature and necessity of this task will be influenced by published results from a large Butleri, Plains, and hybrid morphological dataset currently being analyzed.

**Timeline:** The timeline for this task is fairly short (*tentative*: six months).

This step cannot fully proceed without adequate numbers of both “pure” and hybrid samples, and thus awaits completion of microsatellite development and at least initiation of sampling and testing of mixed and unknown populations (task II). Timeline for completion of this task is **spring/summer 2011**. However, microsatellite markers could be applied to IZ study snake tissue much sooner than that, to help resolve whether the lack of concordance between the AFLP species identification and the existing morphological species index is caused by small AFLP study reference site sample sizes.

**References:**

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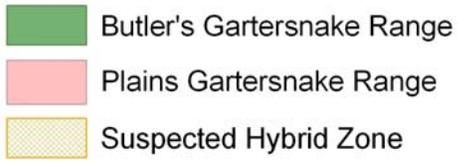
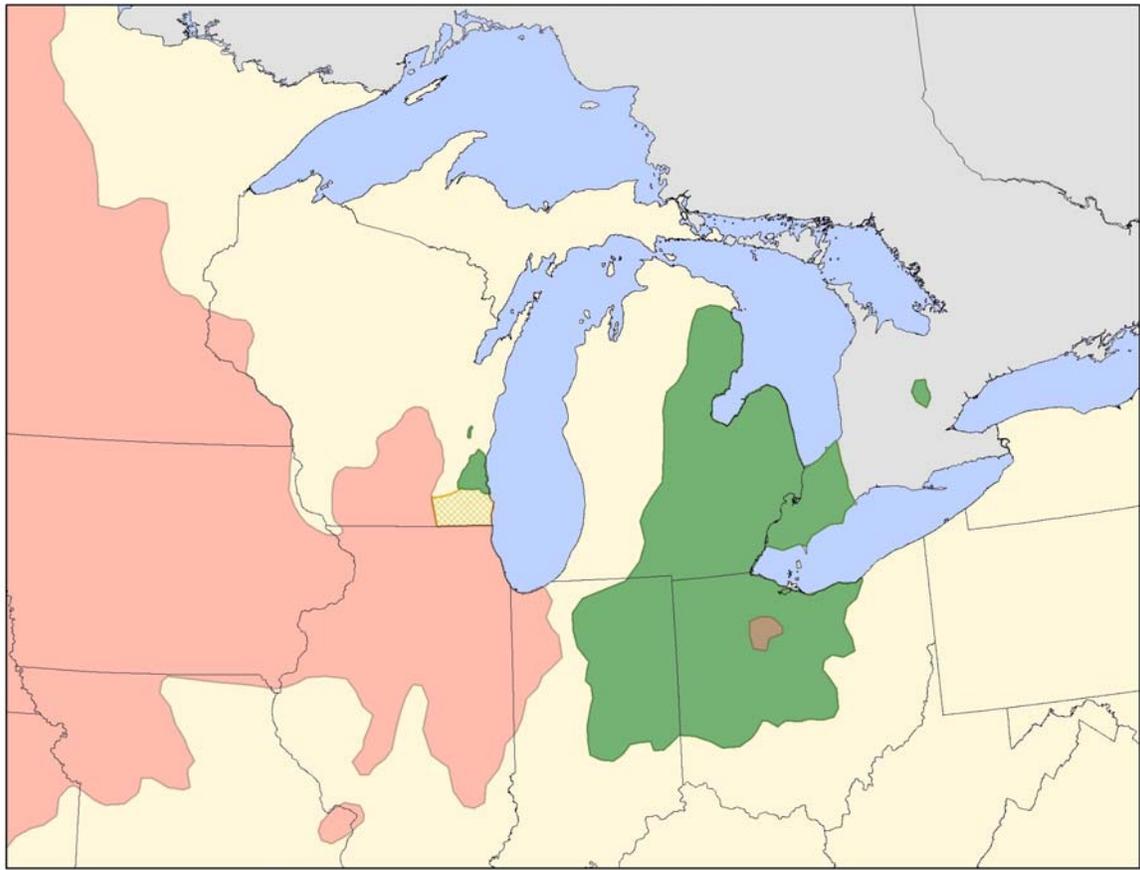


Figure 1. Butleri (*Thamnophis butleri*) and Plains (*T. radix*) ranges in the Great Lakes region, and the suspected hybrid zone in southeastern Wisconsin.

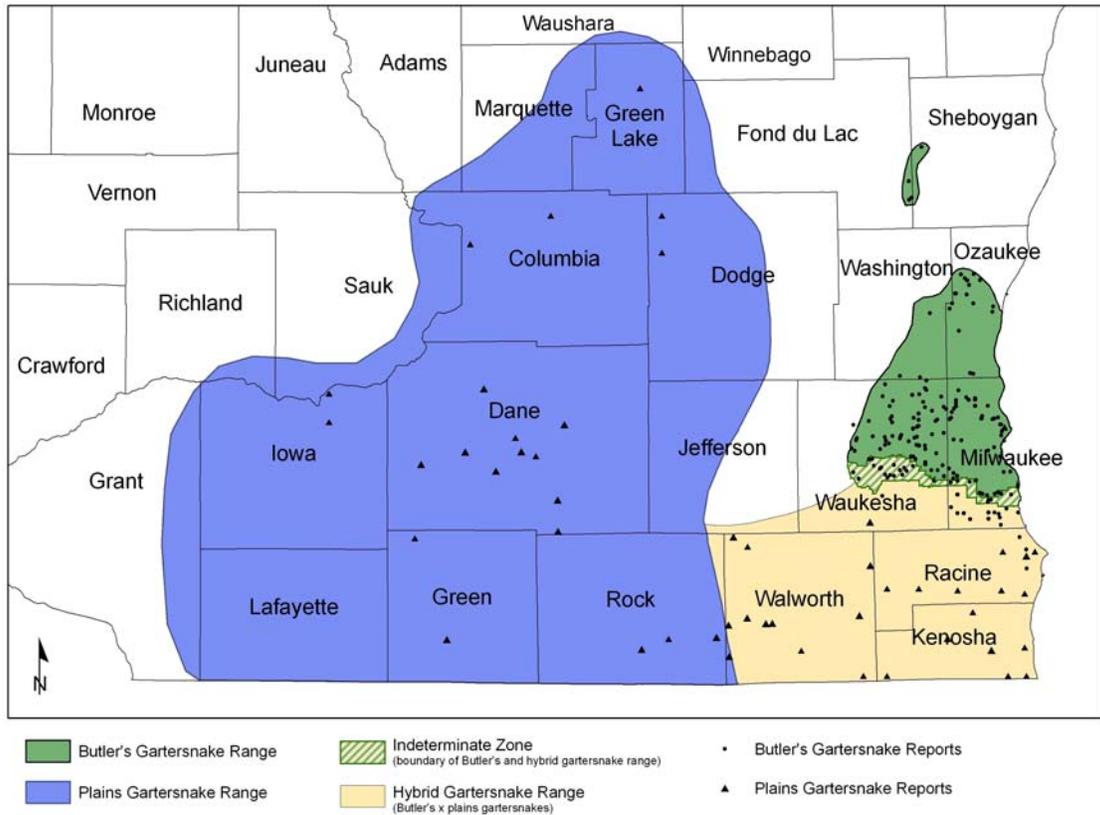


Figure 2. Butleri (*Thamnophis butleri*) and Plains (*T. radix*) ranges and historical occurrences in Wisconsin, and the suspected hybrid zone and “indeterminate zone” (or “IZ”) in southeastern Wisconsin.