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Comparing the population structure of the specialist Butler's gartersnake (*Thamnophis butleri*) and the generalist eastern gartersnake (*Thamnophis sirtalis*) in Ontario and Michigan

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Abstract

Species differing in life history attributes vary in their responses to features within a shared landscape. We evaluated genetic structure of sympatric gartersnake species in Southwestern Ontario, Canada and south-east Michigan, U.S.A., where habitat fragmentation is high due to agriculture and urbanization. We surveyed genetic structure of the habitat specialist, Butler's gartersnake (*Thamnophis butleri*; Cope, 1889) and habitat generalist, eastern gartersnake (*Thamnophis sirtalis sirtalis*; Linnaeus, 1758) using DNA microsatellites. Bayesian clustering, Discriminant Analysis of Principal Components, and pairwise population comparisons revealed genetic differentiation among three major regional clusters of Butler's gartersnake with evidence of further division within one. Genetic clustering of Butler's gartersnake suggest that inhospitable habitat limits dispersal. Eastern gartersnakes showed less structure, with assignment tests implying a single genetic cluster. We found positive significant Mantel's r for both species in the smallest distance class (<15 kms), but significant isolation by distance for *Thamnophis butleri* only. These findings together imply that connectivity for eastern gartersnakes is less impacted by habitat loss and fragmentation or that we were less able to detect their effects. Our study shows the value of multispecies comparisons in studies seeking to understand the underlying causes of genetic structure in natural populations.

38 **Keywords:** population structure, snakes, microsatellites, habitat fragmentation,
39 connectivity, conservation, *Thamnophis butleri*, *Thamnophis sirtalis sirtalis*, Butler's
40 gartersnake, _Eastern gartersnake

Draft

Introduction

A species' ecology (e.g., habitat preferences, dispersal capacity, mating system) shapes population genetic structure (Loveless and Hamrick 1984) and how it will respond to physical, climatological, and biotic variation in landscapes. Human-mediated habitat loss, degradation, and fragmentation can cause fragmentation and isolation of previously contiguous populations or reduce dispersal and gene flow among previously connected populations. This can produce declines in genetic diversity as a consequence of genetic drift and inbreeding, and increases in differentiation among populations. Species ecology thus can impact the magnitude of genetic effects of anthropogenic habitat fragmentation (Schlaepfer et al. 2018).

Reptiles including snakes have been greatly affected by alterations and loss of habitats in North America and globally (Keinath et al. 2017). Snakes make excellent systems for studying the impacts of changes to habitat distribution and fragmentation as they are often tied to particular landscape features like hibernacula, gestation sites and basking sites, can have relatively limited dispersal, and vary markedly in size and ecology (Greene 1997). For example, the western massasauga (*Sistrurus catenatus tergeminus*; Say in James, 1823) occupies a mosaic of fragmented and unfragmented habitat. In areas of greatest habitat continuity, the genetic structure of *Sistrurus catenatus tergeminus* is homogeneous, whereas in areas with fragmented habitat, local populations are genetically differentiated (McCluskey and Bender 2015). Row et al. (2010) found eight distinct genetic clusters in eastern foxsnakes (*Pantherophis gloydi*; Conant 1940) across their geographic range that mapped onto isolated remnant patches of wetland or shoreline habitat. The authors attributed this observation to recent

anthropogenic habitat fragmentation and degradation limiting dispersal. Xuereb et al. (2015), using Approximate Bayesian computation (Beaumont et al. 2002) and genetic clustering methods, found evidence of recent genetic isolation, population declines, and inbreeding in eastern hog-nosed snakes (*Heterodon platirhinos*; Latreille, 1801) in Ontario caused by habitat loss and fragmentation.

Some species are less impacted and better able to persist in anthropogenically-impacted environments than others (Stein et al. 2000; He et al. 2014). In general, species with broad habitat preferences, higher fecundity, and higher propensities for dispersal should be less affected than habitat specialists (e.g. Fahrig 2003; Devictor et al. 2008; DiLeo et al. 2010), and the signature of these differences will be evident in comparisons of demography and genetic population structure (Proctor et al. 2005; Row et al. 2010). Contrasts of co-distributed species with divergent life histories provide a compelling test of these predictions. For example, DiLeo et al. (2010) compared the genetic structure of the eastern gartersnake (*Thamnophis sirtalis sirtalis*) and the much larger *Pantherophis gloydi* across Southern Ontario, and found only the latter, a prairie and grassland specialist, exhibited obvious population clusters.

In this study we quantify the genetic structure of two congeneric species in Southwestern Ontario and adjacent Michigan: *Thamnophis butleri*, a habitat specialist, and *Thamnophis sirtalis sirtalis*, a sympatric generalist. *Thamnophis sirtalis sirtalis* occupies a broad array of habitats spanning forests, wetlands, fallow agricultural fields, open scrub and rocky outcrops across North America (Fig. 1; Harding 1997; Halliday and Blouin-Demers 2015). In contrast, *Thamnophis butleri* occupies remnant landscapes comprising a mix of tallgrass prairie, open grasslands, old fields and

meadows close to wetlands scattered across lower Michigan, northwestern Ohio, northeastern Indiana, and Ontario (Fig. 1; Logier 1939; Planck and Planck 1977; COSEWIC 2010; Environment Canada 2016).

The Canadian range of *Thamnophis butleri* lies entirely within the anthropogenically disturbed landscape of Southwestern Ontario (Kelly 1975; Rowell 2012), including portions of Essex County, Lambton County, and Luther Marsh Wildlife Management Area (Luther Marsh WMA; Fig. 1). *Thamnophis butleri* is now classified as Threatened under the Canadian *Species at Risk Act* (SARA) and as Endangered under Ontario's *Endangered Species Act* (ESA). The species only persists in patches of isolated, remnant habitat and is extirpated from most of its historical range (COSEWIC 2010). Habitat loss in Ontario is estimated to be 60% (COSSARO 2011), much of this caused by replacement of tallgrass prairie and oak savannah habitat preferred by Butler's gartersnake with residential development (COSEWIC 2010).

Thamnophis butleri neither disperses over great distances nor seems to use land changed extensively by human impacts (Freedman and Catling 1979; COSSARO 2011). For example, AMEC (2012, 2013) estimated maximum distances moved within an active season for Butler's gartersnakes in Southwestern Ontario to be between 150 - 380 m. Thus, gene flow is unlikely among the isolated patches of remaining suitable habitat. Using genetic data, Noble et al. (2013) suggest that there may be as many as five local populations within the Canadian range of *Thamnophis butleri*. Based on these differences in habitat preference and dispersal, and this previous research, we predict that the fragmentation of *Thamnophis butleri* will be reflected in greater genetic sub-

structuring and lower genetic diversity when compared to the generalist species *Thamnophis sirtalis sirtalis*. We further predict that *Thamnophis sirtalis sirtalis* will comprise either a single panmictic population or will show patterns consistent with individual-level isolation by distance (Wright 1943; Malécot 1948). Our findings for *Thamnophis butleri* in Ontario might suggest the delineation of designatable units (DUs *sensu* Green 2005) facilitating the development of conservation priorities for the species in Canada.

Materials and methods

Sampling locations and methods

Potential sampling sites were identified using observations from the Natural Heritage Information Centre (NHIC, <https://www.ontario.ca/page/natural-heritage-information-centre>), supplemented with satellite imagery and knowledge of basic habitat preferences for *Thamnophis butleri* and *Thamnophis sirtalis sirtalis* (Harding 1997). Field surveys to collect blood and tissue samples from each species were undertaken in these target areas from 2013 to 2016 (Table 1). Additional samples from 2008, 2009 and 2011 were collected by other researchers, including samples along the Detroit River in Michigan (Table 1, Fig. 2). In Canada, samples were collected from three regions where both species are found: 1) Luther Marsh WMA ($n_{\text{BGS}} = 10$, $n_{\text{EGS}} = 11$); 2) Lambton County ($n_{\text{BGS}} = 94$, $n_{\text{EGS}} = 87$); and 3) Essex County, including select islands in the Detroit River ($n_{\text{BGS}} = 114$, $n_{\text{EGS}} = 40$). We did not obtain samples from Walpole Island, an unceded First Nations reserve known to have both species. We also obtained samples from Michigan from St. Clair, Macomb and Wayne counties along the Great Lakes system, including one island in the Detroit River ($n_{\text{BGS}} = 15$, $n_{\text{EGS}} = 25$).

All field visits occurred during the active season for *Thamnophis* snakes on clear or partly clear days with temperatures between 15-30°C (Peterson et al. 1993). We conducted active searches following the OMNRF (2016) survey protocols for at risk snakes, by walking transects through likely habitat and surveying visually, including checking under cover objects. We also sampled animals that were found dead on roads and had been accidentally killed during lawn mowing.

Live snakes were captured by hand, processed, and released at the point of capture. All snakes were handled as per protocols approved by the Wildlife Animal Care Committee in Ontario (Queen's University Animal Care Committee Protocol 2013-009). We identified the sex of each individual using external tail morphology. Using either a 29-, 30- or 31-gauge, sterile ½ cc insulin syringe, we extracted 50-100 µL of blood from each snake's caudal vein. For small snakes from which blood could not be drawn (typically < 10 g), we clipped < 5 mm from the distal end of the tail. For incidental mortalities, we sampled a 2-3 cm long piece of skeletal muscle with skin. We preserved all samples in 95% ethanol until analysis. Coordinates for each sampling locale were recorded using a Garmin Etrex Legend handheld global positioning system in Universal Transverse Mercator coordinates. We do not include geographic coordinates here as the Butler's gartersnake is a species of conservation concern in Canada.

DNA extraction and microsatellite genotyping

Deoxyribonucleic acid (DNA) was extracted using the QIAGEN DNeasy Blood and Tissue, following the Quick-start Protocol (QIAGEN 2011), with minor modifications. DNA concentrations were quantified using a NanoDrop ND-1000 Spectrophotometer

(NanoDrop, Wilmington, DE, U.S.A.). Extracted DNA was stored in a 20°C freezer until analysis.

We initially chose 22 microsatellite primer pairs designed for various species (*Thamnophis butleri*, *Thamnophis sirtalis sirtalis*, eastern ribbon snake [*Thamnophis sauritus*; Linnaeus, 1766, northern watersnake [*Nerodia sipedon sipedon*; Linnaeus, 1758]] from various publications (McCracken et al. 1999; Prosser et al. 1999; Garner et al. 2002; Garner et al. 2004; Manier and Arnold 2005; Sloss et al. 2012). With a selection of ~50 extracted *Thamnophis butleri* and *Thamnophis sirtalis sirtalis* DNA samples, we ran trial PCRs for all primer pairs (see below). Fifteen primer pairs worked for both species (Table S1). Annealing temperatures for each primer pair were determined using the gradient function of an Eppendorf Mastercycler gradient thermal cycler.

For chosen microsatellites, we ran 2-locus multiplex-PCR reactions containing 10 µM M13-labeled forward primer and unlabeled reverse primer for each locus (Eurofins Genomics, Toronto, ON, Canada for 3Ts primers; Invitrogen, Burlington, ON, Canada for all other primers); for each multiplex, we used 0.2 µL of each primer for the locus with smaller predicted amplicon size, and 0.4 µL for the locus with larger amplicon size. The 10 µL reaction also contained 2 µL of 10 ng/µL genomic DNA, 5.5 µL *Taq* PCR MasterMix (QIAGEN Inc., Toronto, ON, Canada), 1 µL of 1 µM labeled WellRed M13 (Sigma-Aldrich Canada Co., Oakville, ON, Canada), and 0.3 µL RNase-free double-distilled H₂O. Samples were amplified in either a GeneAmp PCR System 2700 or a GeneAmp PCR System 9700 thermal cycler with an initial denaturing step at 95° C for 5 m, 40 cycles with 95° C for 30 s, annealing at temperatures specified in Table S1

for each primer, and extension at 72° C for 45 s, and a final extension at 72° C for 5 m. Microsatellite genotyping was done with a Beckman Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Mississauga, ON, Canada).

For samples where specific microsatellites could not be scored from duplex reactions, we amplified them singly. The protocol was the same as above, except with only one set of primers, and 0.9 µL RNase-free double-distilled H₂O to keep the volume at 10 µL. For individuals with lower DNA concentrations (~ 10 ng/µL) that failed to amplify, we ran a nested PCR using amplified product from the first reaction.

Quantifying population structure

We scored microsatellite genotypes with GeneMarker 2.6.3 (SoftGenetics, State College, PA, U.S.A), assembled the data into separate Microsoft Excel (Microsoft, Mississauga, ON, Canada) files for each species, and binned the fragment sizes using the sort and plot tools. For all loci, and for each species separately, we tested for null alleles, and scoring and typographic errors using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). These tests were done for 12 distinct *Thamnophis butleri* sampling locations, nine of which also had *Thamnophis sirtalis sirtalis* samples. Sampling locations were defined as areas ≤ 10 km in diameter containing at least five *Thamnophis butleri* samples (Table 1, Fig. S1; note exceptions for sparsely-sampled locations, for isolated individuals, and between Aamjiwnaang and North Lambton specifically). *Thamnophis sirtalis sirtalis* samples were grouped into the same locations; if they did not fall within a *Thamnophis butleri* location, they were assigned to the closest sampling locale. We used Arlequin 3.5.2.2 (Excoffier and Lischer 2010) to test

for linkage disequilibrium (LD) between all pairs of loci and for deviations from Hardy-Weinberg Equilibrium (HWE) for all loci, for each location.

We estimated pairwise genetic differentiation between all sampling locale pairs using Wright's F_{ST} (1949) and Slatkin's R_{ST} (1995). To control for Type I errors that can occur with multiple tests, we used both a Bonferroni correction (Rice 1989) and a Benjamini-Yekutieli (B-Y) adjustment (Benjamini and Yekutieli 2001), as recommended by Narum (2006). We also tested for population structure for *Thamnophis butleri* and *Thamnophis sirtalis sirtalis* separately using clustering analysis in the program STRUCTURE (Pritchard et al. 2000) and the program TESS (Chen et al. 2007), which incorporates geographic coordinates from individual sampling locations as prior information. For STRUCTURE analyses, we used the admixture model with a burn-in of 50,000 MCMC steps followed by 100,000 steps with 20 iterations for values of $K = 1$ to $K = 9$. We ran the analyses in STRUCTURE with and without LOCPRIOR (Hubisz et al. 2009), which weights clustering assignment *a priori* using discrete sampling locations. We also assessed the number of discrete genetic clusters in TESS with the same parameters as in STRUCTURE, using the BYM admixture model (Durand et al. 2009) for K values from 2 to $K = 9$. Outputs from STRUCTURE and TESS were summarized using CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) to average the 20 replicates of each K value. Barplots of Q coefficients were constructed using POPHELPER 2.2.0 (Francis 2017) in R 3.3.2 (R Core Team 2016). For STRUCTURE outputs, we estimated the optimal number of clusters using the Evanno et al. (2005) method of determining ΔK , and the mean log likelihood for each value of K , $\ln P(D)$. For TESS outputs, we used the average deviance information criterion (DIC) of each K value, and the overall

representation of membership in each cluster from the Q-matrix. We calculated and plotted the results of all the K -value inference tests in R (R Core Team 2016). Because larger genetic divisions may mask evidence of finer-scale genetic clusters, we re-did our clustering analysis for individual genetic groups diagnosed in the first round of STRUCTURE and TESS analyses that showed evidence of significant admixture or further divisions. We estimated pairwise F_{ST} and R_{ST} for all pairs of genetic clusters identified in our STRUCTURE and TESS results, and used an analysis of molecular variance (AMOVA; Excoffier et al. 1992) to estimate the total genetic variance apportioned among hierarchical sampling levels.

Because clustering analyses make assumptions like Hardy-Weinberg Equilibrium within diagnosed clusters, we also used the model-free discriminant analysis of principal components (DAPC; Jombart et al. 2010) for each species separately, implemented using Adegnet (Jombart 2008) in R. The best-fit values of K were selected using the find.clusters function and the Bayesian information criterion (BIC), with the value of K chosen based on whether the BIC decreased by a negligible amount as K values increased.

Finally, we calculated the Bray-Curtis genetic distance (1-proportion of shared alleles[Dps]) between individuals, which has a relatively lower temporal lag-time in response to barriers to genetic flow (Landguth et al. 2010). We quantified the decline in genetic distance between individuals with increasing geographic distance for each species, using a Mantel's test including the whole dataset (1000 permutations) and within increasing distance classes using a Mantel correlogram (1000 permutations within each of eight equally spaced distance classes) (Legendre and Legendre 2012).

Results

Of 15 loci surveyed, 14 were polymorphic for *Thamnophis butleri* (all but TbuA09) and all were polymorphic for *Thamnophis sirtalis sirtalis*. The average number of alleles per locus varied among the main regions: 3.7 ± 1.7 alleles (BGS) and 7.3 ± 3.4 alleles (EGS) in Luther Marsh; 8.3 ± 4.9 alleles (BGS) and 17.2 ± 9.6 alleles (EGS) in Lambton County; 8.7 ± 4.6 alleles (BGS) and 13.7 ± 7.8 alleles (EGS) in Essex County; and 7.3 ± 3.2 alleles (BGS) and 12.2 ± 6.0 alleles (EGS) in Michigan.

Results from MICRO-CHECKER indicated that there were no scoring or mistyping errors. However, at three loci for *Thamnophis butleri* (Nsμ2, Ts010, Te1Ca3) homozygote excess implied null alleles; these were discarded for all subsequent *Thamnophis butleri* analyses. At six surveyed loci for *Thamnophis sirtalis sirtalis* (Nsμ3, Ts2, 3Ts, TbuA27, TbuA74, TbuB12) there was a possibility of null alleles; these loci were discarded for all subsequent analyses of *Thamnophis sirtalis sirtalis*. As the discarded loci were different for the two species, we ran additional STRUCTURE tests using the six loci (Te1Ca29, TbuA04a, TbuA09, TbuA49, TbuA64, TbuA70) for which we had data for both species.

We found some departures from the HWE for both *Thamnophis butleri* and *Thamnophis sirtalis sirtalis* at most locations (Tables S2, S3). Cluster analyses assume that populations are in HWE, and strong deviations may result in misinterpretation (Falush et al. 2003; Kaeuffer et al. 2007). For HWE tests at each location, experiment-wide error rate (EWR; the probability of at least one test being significant by chance)

were as follows: $EW_{BGS} = 0.46$ and $EW_{EGS} = 0.37$. For all *Thamnophis butleri* loci and eight of nine *Thamnophis sirtalis sirtalis* loci, significant HWE departures occurred at no more than three locations; thus, we retained these loci for STRUCTURE and TESS analyses. The remaining locus in the *Thamnophis sirtalis sirtalis* samples, TbuA70, did not conform to HWE expectations for over half of our sampling locations. We thus performed STRUCTURE tests both with and without TbuA70. HWE-departures occurred most frequently at the Essex locations for *Thamnophis butleri*.

We found some instances of linkage disequilibrium for both *Thamnophis butleri* (Table S4) and *Thamnophis sirtalis sirtalis* (Table S5) between loci at most of the defined locations, although fewer after correction for multiple testing using Bonferroni correction (Rice 1989) and B-Y adjustment (Benjamini and Yekutieli 2001; Tables S4 and S5). Programs like STRUCTURE assume linkage equilibrium and independence among loci (Pritchard et al. 2000). Strong linkage between loci can result in spurious conclusions regarding genetic structure (Kaeuffer et al. 2007), although weak linkage is unlikely to change the overall interpretation (Falush et al. 2003). With multiple testing, the EWR increases (Waples 2015) and apparent statistical significance implying linkage disequilibrium may occur by chance. We found the EWR for all loci combined to be as follows: $EW_{BGS} = 0.97$ and $EW_{EGS} = 0.84$. As no locus pair exhibited disequilibrium at more than three of the 12 locations tested for *Thamnophis butleri* and at no more than two of the nine locations tested for *Thamnophis sirtalis sirtalis*, we retained all locus pairs for our analyses.

Significant pairwise differentiation between *Thamnophis butleri* was evident between many sampled locations (Table S6). Luther Marsh, Aamjiwnaang, and Crystal

Bay were distinct from most other locations for both F_{ST} and R_{ST} , with other Essex and Lambton locations showing significant differentiation for pairwise F_{ST} only. Most locations that were sparsely sampled, including Michigan locations and Belle Isle, were not significantly different from other populations for either metric. Aside from pairwise F_{ST} results that involved Luther Marsh compared to all other locales, pairwise comparisons for *Thamnophis sirtalis sirtalis* (Table S7) did not reveal many significant differences.

Assignment tests for *Thamnophis butleri* using STRUCTURE and TESS revealed strong genetic structure, with four distinct clusters (Figs. 2A, S2 and S3): Luther Marsh WMA, Lambton County, northwestern Essex County, and the remainder of Essex County plus the Detroit River islands (Michigan samples clustered with adjacent Ontario samples; Fig. 2). The barplots of cluster membership from STRUCTURE without LOCPRIOR showed that clusters aligned with sampling locations (Figs. S2 and S4). At $K = 2$, northern Essex appeared as a cluster, as did Lambton and Luther Marsh combined, while south Essex and the Detroit River islands appeared to be a mixture of these two. At $K = 3$, Luther Marsh, south Essex, and the islands fell out into their own cluster, along with some of the northern-most samples from Essex. At $K = 4$, Luther Marsh emerges as its own cluster. For $K \geq 5$, the additional clusters revealed potential genetic differences between the Detroit River islands and evenly split larger populations. The spatial assignment barplots from TESS showed similar patterns, though the clusters tended to be more distinct and Luther Marsh WMA appears as a separate cluster at $K = 3$ (Figs. S3 and S4).

The assessments for number of clusters in *Thamnophis butleri* showed some variation around a single value. From the STRUCTURE results, Evanno's ΔK (Evanno et al. 2005) implies $K = 2$ with some support for $K = 4$ (Fig. S2B). The mean log likelihood plateaued at $K = 4$ (Fig. S2C). For TESS, the deviance information criterion (DIC) began to plateau at $K = 4$ or $K = 5$ (Fig. S3B), with overall membership proportions from the Q-matrix showing strong representation for clusters 1 to 4 (Fig. S3C).

Barplots for *Thamnophis butleri* obtained using LOCPRIOR for analysis including all 12 loci were well-resolved and showed a clear geographic pattern (Fig. S5). Barplots using the six loci common to both species were less well-resolved but suggest the same pattern (Fig. S6). Some admixture is evident in both STRUCTURE and TESS barplots (Figs. S2 and S3) for *Thamnophis butleri* from Essex County. This result is consistent with departures from HWE expectations and linkage disequilibrium evident in samples from northern Essex (Tables S2, S4). As strong levels of structure among populations can mask substructure within a population (Pisa et al. 2015), we re-ran STRUCTURE using only samples from Essex County, including geographically proximate samples from Michigan and the Detroit River islands (Fig. S7). At $K = 2$, the clustering pattern matches the Essex portion of the barplots that include all samples (Figs. 2A, S2 and S3); snakes from Michigan (Wayne County), the Detroit River islands, and localities in southern Essex are part of one cluster, along with individuals from within the city of Windsor in northern Essex, while Lasalle and the Ojibway Prairie Complex are part of a different cluster (Fig. S7). Increasing the number of clusters (K) retains the same basic pattern, but also suggests differences between the islands.

Although neither STRUCTURE nor TESS implied admixture at $K = 4$ within the Lambton County population, STRUCTURE implied some evidence of substructure at higher K values (Fig. S2). We thus ran a STRUCTURE analysis on that subset of samples, including the adjacent Michigan samples. The snakes from this county exhibited weak structure, with some suggestion that individuals from Aamjiwnaang might be genetically distinct from the rest of the snakes in the region (Fig. S8).

Assignment tests of our *Thamnophis sirtalis sirtalis* individuals implied limited genetic structure across their sampled range (Figs. S9 and S10). The barplots from the STRUCTURE runs showed approximately equal contribution from each cluster at all sampling locations for all values of K (Fig. S9). At $K = 2$, only a third of individuals had greater than 60% of their genetic composition (Q) attributed to any one cluster, and those individuals were randomly distributed across the landscape. At $K = 4$, all but five individuals had Q values less than 60% for any one cluster. At all other values of K , no individual had values of Q greater than 60%. In TESS, individuals were assigned almost exclusively to the same cluster (Fig. S10), aside from one individual from southern Lambton County that was consistently suggested to be admixed and a few others exhibiting a similar pattern at higher values of K (two from south Lambton, one from Aamjiwnaang, one from Luther Marsh).

Additional STRUCTURE analyses for *Thamnophis sirtalis sirtalis* yielded comparable results. When using the restricted subset of six loci, Q values were evenly apportioned among K clusters (not shown). The removal of locus TbuA70 due to possible HWE-departure produced similar barplots (not shown). Barplots from the LOCPRIOR model implied some genetic structure, suggesting differentiation between

Essex County and Luther Marsh and individuals from Lambton County and Michigan (Fig. S11). Regardless, in all additional analyses, ΔK was consistently small, and the mean log likelihood plateaued at $K = 1$.

Pairwise values of F_{ST} and R_{ST} identified significant differences among the four *Thamnophis butleri* genetic clusters (Table 2). Pairwise F_{ST} values ranged between 0.06 and 0.23, and pairwise R_{ST} values ranged between 0.06 and 0.52. Essex and Ojibway were the most similar, while Luther Marsh WMA was highly distinct from all other locations. There are no comparable values for *Thamnophis sirtalis sirtalis*, as there was only one inferred genetic cluster.

The AMOVAs were highly significant ($p < 0.00001$). Using the F_{ST} model for *Thamnophis butleri*, 10.3% of the total genetic variation was attributable to differentiation among genetic clusters, 13.4% to among-individuals within clusters, and 76.3% to within-individual variation. Using the R_{ST} model, 30.2% of the total variation comprises differences among clusters, 9.8% among-individuals, and 60.0% within-individuals. Again, there were no results for *Thamnophis sirtalis sirtalis* as it comprises a single cluster.

Results from our DAPC and k.means analysis showed results that were consonant with patterns evident in our cluster analysis for Butler's gartersnake with four clusters (Fig. 3A). For eastern gartersnakes, the DAPC and k.means analysis implied three genetic clusters but these had no geographic coherence (i.e. clusters did not correspond to particular locales or regions; Fig. 3B) and the separation among ellipses was not as marked as in Butler's gartersnake.

The Mantel's test revealed a significant decline in genetic distance with increasing distance for Butler's gartersnakes ($r=0.32$, $p=0.001$), but not eastern gartersnakes ($r=0.03$, $p=0.15$). When testing across distances, we found positive, significant Mantel's r for both species within the first distance class, but the magnitude was much higher for Butler's gartersnakes (Fig. 4). Larger distances classes for Butler's gartersnakes were significantly negative, but not eastern gartersnakes (Fig. 4).

Discussion

We found differences in genetic structure between congeners co-distributed in the heavily fragmented landscape of Southwestern Ontario, with some variation in interpretation depending on the method used to evaluate our microsatellite data. For the habitat specialist *Thamnophis butleri*, we found four genetic clusters using both clustering analyses (Figs. 2A and 2B) and DAPC (Fig. 3A). These corresponded to (1) Luther Marsh WMA, (2) sites within Lambton County, (3) Ojibway and LaSalle in Essex County (Interior - Essex in Fig. 2B), and (4) Essex sites adjacent to the Detroit River, including islands (Riverine - Essex in Fig. 2B). For the habitat generalist, *Thamnophis sirtalis sirtalis*, cluster analyses implied little detectable genetic population structure despite the relatively large spatial scale of our sampling (Figs. S9 and S10), but DAPC analysis appears to show evidence of genetic clusters (Fig. 3B). We compare and explore the possible causes of these genetic patterns and potential implications for conservation below.

Genetic population structure in Butler's gartersnakes

The most coherent interpretation of our analyses for *Thamnophis butleri* is that there are distinct four genetic clusters. The genetic structure that we delineated for *Thamnophis butleri* supports Noble et al. (2013), who also found geographically delineated genetic clusters that coincide with known distribution gaps. We provide a more comprehensive description of genetic connectivity in Southwestern Ontario, particularly within Essex County. Other snake species with disjunct distributions in Ontario have genetic clusters that coincide with known gaps in their distributions as we found for Butler's gartersnake – some of which are caused by local habitat destruction and others which may predate European colonization – e.g., *Pantherophis gloydi* (Row et al. 2010), *Sistrurus catenatus tergeminus* (Chiucchi and Gibbs 2010), and *Heterodon platirhinos* (Xuereb et al. 2015). Essex County appears to contain two genetic clusters for *Thamnophis butleri* despite the distribution appearing geographically contiguous. One cluster ranges the length of the county along the Detroit River, including individuals from the islands (Essex - Riverine cluster in Fig. 2B), and the second lies slightly inland, consisting of snakes from further inland from LaSalle and the Ojibway Prairie Complex (Essex - Inland cluster in Fig. 2B). Prior to European settlement, 83% of the county was covered in wetland (Ducks Unlimited Canada 2010), which would have allowed ready access to leeches, an important prey item for *Thamnophis butleri* prior to the introduction of Palearctic earthworms (Catling and Freedman 1980); there would have been suitable habitat and food available inland for snakes dispersing away from large rivers. Today, the Ojibway Prairie Complex contains some of the most suitable and

extensive *Thamnophis butleri* habitat retained in the region (Tallgrass Ontario 2005). In contrast, connectivity within the more elongated range of the shoreline cluster could be due to passive dispersal by water, as the river's current could have facilitated more substantial downstream movement than the somewhat limited movement patterns normally evident in *Thamnophis butleri* (Macartney et al. 1988). When assigning higher *K* values in STRUCTURE to Essex County individuals (Fig. S7), we found that the more southern Amherstburg individuals appear to fall out as a cluster distinct from rest of the shoreline cluster, which could mean that gene flow occurring up and down the length of the county is low, and that a pattern of isolation by distance is driving the structure of the shoreline population. The genetic differentiation in the Essex region rather puzzlingly implies low to no dispersal between the inland population at Ojibway/LaSalle and the shoreline population. This could be due to a scarcity of available migrants, as density in the shoreline population is apparently quite low. Alternatively, the human development that tends to be associated with water edges and the presence of roadways could have split the inland and riverine populations despite the small spatial scale.

A question that follows from evidence for the genetic clustering of *Thamnophis butleri* in Ontario pertains to its origin. The structure may reflect post-glacial recolonization and historical dispersal into naturally distributed habitat patches; alternatively, and we think more likely, habitat loss caused by European settlement of the area could have destroyed intermediate habitat and impeded gene flow across a now-fragmented range. It is possible that patchy geographical and genetic distribution is the norm for *Thamnophis butleri*, as it is for many specialist species (Habel and Schmitt

2012). Although the generalized global range (Fig. 1) indicates a mostly continuous distribution with only two disjunctions, we know that this is not the case in Ontario. Aside from the three regional populations, there are also several locations where *Thamnophis butleri* has been extirpated. Even in Michigan, the apparent stronghold for the species, sightings of *Thamnophis butleri* are sporadic (Michigan Herp Atlas, <https://www.miherpatlas.org/taxon.php?taxon=84>). *Thamnophis butleri* is a relatively sedentary species (Macartney et al. 1988), and this coupled with relative habitat specialization should result in greater differentiation across fine spatial scales.

Genetic population structure in eastern gartersnakes

Cluster analyses with and without incorporating geographic information imply that *Thamnophis sirtalis sirtalis* consists of a single genetic population across the same area sampled for Butler's gartersnake (Figs. S9 and S10), an observation that is supported by the results of a Mantel's test that showed a significant decline in pair-wise genetic distance with increasing distance only for the smallest distance class. This result is also consistent with DiLeo et al. (2010) who found lack of genetic structure for populations of *Thamnophis sirtalis sirtalis* in Southwestern Ontario (DiLeo et al. 2010). However, eastern gartersnakes are the most common snake species across Southwestern Ontario; the genetically effective population size may be sufficiently large that any signature of reduction in gene flow and divergence among populations via drift might take substantial time to arise. Indeed, in contrast to results from our STRUCTURE and TESS analyses and from Mantel's test, DAPC implies that there are three genetic

clusters in *Thamnophis sirtalis sirtalis* but these do not coincide with any particular sampled region (Fig. 3B). Miller et al. (2020) using simulations showed that, when migration rates are high and groups are not designated a priori, the number of genetic clusters suggested and the placement of individuals within them often can be inaccurate. Thus, we think that lack of obvious geographic associations with these clusters imply that they are not real.

Comparing population structure and genetic diversity in the two gartersnake species

Irrespective of the possibility of some genetic population structure in *Thamnophis sirtalis sirtalis*, our analyses do make clear that the habitat specialist, *Thamnophis butleri*, exhibits well defined genetic clusters (Fig. 2A) and evidence for isolation by distance across geographic distance categories (Fig. 4). In a generalist species like the eastern gartersnake, more subtle genetic population structure reflecting impacts of habitat alteration and fragmentation might only be apparent at larger spatial scales not considered in our study and using the higher resolution and statistical power afforded by larger panels of DNA markers. Regardless, the disparity between the two species is consonant with what we know about habitat preferences and contemporary distributions.

We found no compelling evidence that gene flow is impeded by the major rivers that lie along the international border between Canada and the U.S.A. for either species. Population assignments grouped *Thamnophis butleri* individuals from Michigan and the Detroit River islands with the nearest Ontario cluster, both when considering the

whole area sampled (Figs. S2 and S3) and in the STRUCTURE runs focused on Essex and Lambton counties separately (Figs. S7 and S8, respectively). *Thamnophis sirtalis sirtalis* comprised a uniform cluster that extends across the Detroit and St. Clair Rivers, again suggesting that rivers may not be a strong impediment to gene flow. Indeed, while both species are terrestrial and more often seen on land, both also have been observed swimming (COSEWIC 2010, personal observations), and *Thamnophis butleri* must have colonized the islands in the Detroit River via aquatic dispersal. This might imply that *Thamnophis butleri* from the (unsampled) Walpole Island will align genetically with the geographically proximate Michigan and Ontario samples, putting it within the Lambton County cluster, although this assertion awaits additional sampling and genetic analysis.

Thamnophis butleri consistently contained lower genetic diversity than did its generalist congener, *Thamnophis sirtalis sirtalis*. For every region surveyed and every microsatellite locus, *Thamnophis butleri* had lower allelic richness than *Thamnophis sirtalis sirtalis*. This is common in comparisons of specialists versus generalists (Habel and Schmitt 2012; Janecka et al. 2016), although with exceptions (Brouat et al. 2004). Higher levels of genetic differentiation are often evident for specialist species (DiLeo et al. 2010; Habel and Schmitt 2012), consistent with what we found with *Thamnophis butleri* versus *Thamnophis sirtalis sirtalis*. Lower genetic diversity is not inherently detrimental; specialist species often show local adaption and may not be particularly susceptible to effects of inbreeding, genetic bottlenecks or genetic effects of fluctuations in population size (Habel and Schmitt 2012). It is when environmental conditions change (e.g., habitat degradation) that species with smaller population sizes may be more prone to local extinction (Dennis et al. 2011), and populations with lower levels of

additive genetic variation may not be able adapt to changing environmental circumstances.

Mitochondrial DNA sequencing surveys have revealed that *Thamnophis butleri* in Ontario and Michigan share a single mitochondrial haplotype (Noble et al. 2013; Placyk et al. 2012), with no distinction among the groups that colonized this region of post-glacial North America. The same is also true for *Thamnophis sirtalis sirtalis* (Placyk et al. 2007) in Ontario and eastern Michigan, and for another co-distributed species, *Pantherophis gloydi* (Row et al. 2010). This common pattern is likely due to the sequential founder events that follow glacial retreat (Hewitt 2000), coupled with insufficient time for mutation to have augmented mitochondrial diversity. Recent isolation – particularly as recent as post-European colonization – would not be reflected in mitochondrial patterns.

Conservation implications

Currently, *Thamnophis butleri* is assigned to a single Designatable Unit (COSEWIC 2010), but it may be worth considering assigning individual DUs to each of the three regional populations. These regional populations do meet the criterion of ‘discreteness’ recommended by the Committee on the Status of Endangered Wildlife in Canada for status reports (<https://cosewic.ca/index.php/en-ca/reports/preparing-status-reports/guidelines-recognizing-designatable-units>): “Evidence of genetic distinctiveness including, but not limited to, inherited traits (e.g., morphology, life history, behaviour) and/or neutral genetic markers (e.g. allozymes, DNA microsatellites, DNA restriction fragment length polymorphisms (RFLPs), DNA sequences).” If not DUs then perhaps

these distinct populations should at least be considered management units (MUs) sensu Moritz (1994) and Palsbøll et al. (2007). One other practical reason for designating separate DUs or MUs is that restoring connectivity among these regions is likely impractical. *Thamnophis butleri* only disperse short distances (Carpenter 1952; Freedman and Catling 1979), and disjunctions comprising unsuitable habitat between the regions are simply too large to re-establish connection. While the aforementioned patterns of genetic structure in nuclear markers probably began to arise after European settlement in the 1800s, the major threats facing the species undoubtedly developed conjointly with anthropogenic pressures.

Regardless of whether the genetic structure evident in *Thamnophis butleri* has some origins in natural historical events, human presence has undoubtedly exacerbated the situation. Land conversion of natural habitat, such as grasslands and wetlands, has been an issue since European settlement (Tallgrass Ontario 2005; Ducks Unlimited Canada 2010), as has the suppression of wildfires that create new open habitats to compensate for loss via natural succession (Weakley 1999; Larson 2014). Known locations of recent *Thamnophis butleri* populations have been destroyed for development (COSEWIC 2010). Without conservation efforts targeting habitat preservation and restoration, the species could be eliminated entirely from Ontario. We recommend an approach of improving habitat connectivity within regions. Essex County in particular may benefit from reconnecting local populations, particularly if the genetic differentiation there occurred after European colonization. At Luther Marsh, snakes are unlikely to be threatened by landscape conversion, as the Wildlife Management Area is managed by both the local conservation authority and the Ontario Ministry of Natural

Resources and Forests. However, for *Thamnophis butleri*, small population size and the small area occupied (COSEWIC 2010) could lead to lower genetic diversity, increased susceptibility to stochastic events, and increased probability of local extinction. With low *Thamnophis butleri* density, implementing artificial dispersal and gene flow into Luther Marsh by headstarting juveniles for release may be more beneficial (Conner et al. 2003); this approach has had some success in the rare plains gartersnake, *Thamnophis radix* (King and Stanford 2006), sister species to *Thamnophis butleri* (de Queiroz et al. 2002) and a taxon that shows similar preference for prairie and meadow habitat close to wetlands, streams and swales (Conant et al. 1945).

Conclusions and future directions

Our research quantifies the genetic structure of populations of *Thamnophis butleri* and *Thamnophis sirtalis sirtalis* in Ontario using DNA microsatellites, and highlights the importance of using genetics to understand connectivity and to inform conservation strategies. Because there is apparently little gene flow occurring among genetic populations, the continued destruction (COSEWIC 2010) of *Thamnophis butleri* habitat without any active habitat restoration efforts could result in local extirpation of the species or even its disappearance entirely from Canada. Ours is not the definitive word on the impacts of human-caused habitat degradation on genetic population structure for either species. Additional sampling of *Thamnophis butleri* in the core of the species range in the U.S.A. where suitable habitat may be more extensive would provide context on overall genetic patterns in this geographically restricted species. Because *Thamnophis sirtalis sirtalis* is more continuously distributed in Southwestern

Ontario, future work should include more intensive sampling across the distribution of this species and incorporate landscape genetics approaches to evaluating the relation between genetic patterns, environmental variation, and putative barriers like major roadways. Because the influence of habitat isolation may be subtle, we recommend assessing population structure and diversity using genomics methods (e.g. reduced-representation sequencing) that can provide thousands of genome-wide DNA markers like Single Nucleotide polymorphisms (SNPs) and afford much greater statistical power. Finally, we note that Southern Ontario has 17 extant snake species, varying markedly in size, ecology, and conservation status. While some have rather restricted distributions similar to the Butler's gartersnake (e.g. blue racer, *Coluber constrictor foxii* and queensnake, *Regina septemvittata*), others range more broadly in the southern portion of the province. Of those species that have been studied some show evidence of pronounced genetic subdivisions (e.g. hog-nosed snakes, Xuereb et al. 2015; eastern foxsnakes, Row et al. 2010). We are currently undertaking broad-scale, comparative landscape genomics study across multiple co-distributed snake taxa in Ontario that should provide further insight on the interplay between species' life history, abiotic and biotic variation in landscapes, human-caused habitat fragmentation, and genetic population structure.

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Table 1. Locations designated *a priori* as Butler’s gartersnake (*Thamnophis butleri*) ‘populations’ for genetic analyses, based on groupings of *Thamnophis butleri* samples (≤ 10 km diameter sampling area, ≥ 5 individuals sampled). Single samples were grouped with the closest location. Exceptions to these criteria (e.g., sparsely-sampled locations, barriers determining populations) described below. Eastern gartersnake (*Thamnophis sirtalis sirtalis*) samples were assigned to the closest *Thamnophis butleri* location.

Location	Cause for designation	<i>n</i> _{BGS}	<i>n</i> _{EGS}
Wayne, MI	<ul style="list-style-type: none">• Population diameter: < 1 km (except for one individual, ~ 15 km N, to which Wayne is the closest location)• Due to the small number of samples, Wayne individuals were grouped by their county• Separated from nearest external sample (~ 6 km E) by the Detroit River	7	11
Macomb, MI	<ul style="list-style-type: none">• Population diameter: < 1 km• Due to the small number of samples, Macomb individuals were grouped by their county• ~ 30 km NE to nearest external sample	2	4

St. Clair, MI	<ul style="list-style-type: none"> • Population diameter: < 1 km (except for one individual, ~ 40 km S, to which St. Clair is the closest location) • Due to the small number of samples, St. Clair individuals were grouped by their county • Separated from nearest external sample (~ 4 km E) by the St. Clair River 	3	10
Crystal Bay (island)	<ul style="list-style-type: none"> • Population diameter: < 1 km • Separated from nearest external sample (~ 3 km E) by the Detroit River 	6	n/a
Fighting Island	<ul style="list-style-type: none"> • Population diameter: ~ 3 km • Separated from nearest external sample (~ 5 km E) by the Detroit River 	14	n/a
Belle Isle	<ul style="list-style-type: none"> • Population diameter: < 1 km • Designated as a distinct location due to being an island • Separated from nearest external sample (~ 4 km SW) by the Detroit River 	3	n/a
South Essex	<ul style="list-style-type: none"> • Population diameter: < 1 km (except for one individual, ~ 8 km S, to which South Essex is the closest location) • Separated from nearest external sample (~ 3 km E) by the Detroit River 	9	17

North Essex	<ul style="list-style-type: none"> • Population diameter: ~10 km (except for two individuals, ~ 8 km S and ~ 12 km E respectively, to which North Essex is the closest location without barrier – the Canard River runs across Essex between the locations) • Separated from nearest external sample (~ 4 km NE) by the Detroit River 	85	23
South Lambton	<ul style="list-style-type: none"> • Population diameter: ~10 km • Separated from nearest external sample (~ 11 km W) by the Detroit River 	10	42
Aamjiwnaang (Lambton)	<ul style="list-style-type: none"> • Population diameter: ~ 6 km • ~ 6 km N to nearest external samples (designated as a distinct sample due to the barrier of an industrial plant to the north, and based on anecdotal reports by the Aamjiwnaang First Nation Environmental Department that snakes at this location have distinct ecological patterns) 	42	41
North Lambton	<ul style="list-style-type: none"> • Population diameter: ~ 2.5 km (except for one individual, ~ 16 km E, to which North Lambton is the closest location) • Separated from nearest external sample (~ 4 km) by the St. Clair River • ~ 6 km SW to next nearest external location (see Aamjiwnaang) 	42	4
Luther Marsh	<ul style="list-style-type: none"> • Population diameter: ~ 8 km 	10	11

• ~ 175 km W to nearest external sample

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Table 2. Estimates of pairwise F_{ST} (above diagonal) and R_{ST} (below diagonal) between each of the four genetic clusters determined for Butler’s gartersnake (*Thamnophis butleri*). All pairwise comparisons were significant ($\alpha = 0.05$ without correction for multiple testing (indicated in bold), $\alpha = 0.020$ with a Benjamini-Yekutieli (B-Y) adjustment, $\alpha = 0.0083$ with a Bonferroni correction (§ indicates significance for both BY and Bonferroni corrections).

	LaSalle and Ojibway	Essex and islands (+ adj. MI)	Lambton (+adj. MI)	Luther Marsh
LaSalle and Ojibway	—	0.056 §	0.13 §	0.24 §
Essex and islands (+ adj. MI)	0.060 §	—	0.064 §	0.17 §
Lambton (+ adj. MI)	0.45 §	0.26 §	—	0.23 §
Luther Marsh	0.52 §	0.32 §	0.17 §	—

Figure captions

Figure 1. Map of generalized global ranges of eastern gartersnake (*Thamnophis sirtalis*) and Butler's gartersnake (*Thamnophis butleri*) (IUCN 2016). Maps created using ArcMap (ESRI 2015). Political boundaries source: Esri. "USA States (Generalized)" [Feature Layer]. Scale Not Given. "USA_States_Generalized" June 29, 2013. https://services.arcgis.com/P3ePLMYs2RVChkJx/arcgis/rest/services/USA_States_Generalized/FeatureServer. Esri Canada. "Provinces and Territories of Canada" [Feature Layer]. "Canada_Provinical_boundaries_generalized." November 15, 2013. https://services.arcgis.com/P3ePLMYs2RVChkJx/arcgis/rest/services/USA_States_Generalized/FeatureServer. (May 15, 2019). Waterbodies are drawn from: Esri. "World Waterbodies [Layer Package]. "World Water Bodies." April 27, 2011. <https://www.arcgis.com/home/item.html?id=e750071279bf450cbd510454a80f2e63>. Geographical range boundaries are from Hammerson (2007) and Frost et al. (2015).

Figure 2. (A) Barplot of Butler's gartersnake (*Thamnophis butleri*) population membership proportions assigned by STRUCTURE for $K = 4$ without LOCPRIOR. The barplots are organized southwest to northeast (with Michigan and the islands grouped on the left), and divided by the designated sampling locations (M_W = Wayne, Michigan; M_M = Macomb County, Michigan; M_S = St. Clair County, Michigan; is_{CB} = Crystal Bay; is_{FI} = Fighting Island; is_{BI} = Belle Isle; SE = South Essex County; NE = North Essex County; SL = South Lambton County; A = Aamjiwnaang; NL = North Lambton County; LM = Luther Marsh WMA). Each vertical bar represents a single snake. (B) Sample locations for all Butler's gartersnake (*Thamnophis butleri*) used in this study ($n = 233$)

with coloured polygons indicating the locations of clusters identified in A. C. Sample locations for all eastern gartersnake (*Thamnophis sirtalis sirtalis*) used in this study (n = 163). Maps created using ArcMap (ESRI 2015). Basemap from: Esri. "Topographic" [basemap]. "World Topo Base". March 21, 2016. https://wtb.maptiles.arcgis.com/arcgis/rest/services/World_Topo_Base. Other sources as in Figure 1.

Figure 3. DAPC scatterplots based on the K-means algorithm used to estimate the number of clusters for Butler's gartersnakes (A) and eastern gartersnakes (B). Dots represent individual snakes, and diagnosed clusters are presented in different colors. For eastern gartersnakes, because clusters do not encompass particular geographic regions, select locales are shown including Macomb and St. Clair (Michigan), Ladysmith and Wallaceburg (northeast of Lake St. Clair), and Luther Marsh.

Figure 4. A comparison of Mantel's correlation coefficients for Butler's gartersnake (*Thamnophis butleri*) and eastern gartersnake (*Thamnophis sirtalis sirtalis*) within eight equally spaced distance classes. Significance was determined using 1000 permutations with significantly positive or negative distance classes delineated with a filled symbol.

A

B

C

A

B

Mantel's r

—○— BGS

-△- EGS