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Integrating phylogenomic and morphological data to assess candidate species-delimitation models in brown and red-bellied snakes (*Storeria*)

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Systematics at the species level is still marked by theoretical and empirical tensions amongst the desires to identify geographical lineages, delimit species, and estimate their relationships. These goals are often confounded because each relies, at least to some extent, on the others being known. However, recently developed methods can simultaneously address all three. Furthermore, next-generation genomic sequencing allows us to generate large-scale molecular data sets to examine variation within species at a fine scale. Finally, a renaissance in morphological species validation allows us to integrate historical species definitions with coalescent models for species delimitation. Here, we investigate the applicability of these methods in an empirical case, in the Nearctic snake genus *Storeria*. Integrating trait data into species delimitation reduces the number of species delimited from molecular data alone. Whereas molecular data support eight distinct species-level lineages, including morphological data reduces this to four. The taxa *Storeria dekayi*, *Storeria occipitomaculata*, *Storeria storerioides*, and *Storeria victa* are considered distinct, monotypic species, with no subspecies recognized. We highlight the need for careful assessment of species delimitation, combining both computational genetic methods as well as traditional character-based descriptions. It is now possible to identify phylogeographical lineages, delimit species using molecular and morphological data, and estimate their relationships in a single coherent set of analyses. Moving forward, this will allow for more rapid and objective assessments of cryptic diversity at the species level.

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INTRODUCTION

Systematics at the species level represents the intersection of population genetics, demography, and phylogeography, along with phylogenetics, speciation, and species delimitation (Avise *et al.*, 1987; Hickerson *et al.*, 2010). Major goals include identifying geographical genetic variation and delimiting species (Fujita *et al.*, 2012; Carstens *et al.*, 2013), and inference of demographic processes before, during, and after speciation (Gutenkunst *et al.*, 2009; Brandley *et al.*, 2010). These goals are intercalated with empirical issues such as coalescent variation amongst genealogical histories (Edwards & Beerli, 2000; Arbogast *et al.*, 2002), and theoretical questions about how to delimit biodiversity despite cryptic speciation and continuing gene flow between species (Bickford *et al.*, 2007; Knowles & Carstens, 2007; de Queiroz, 2007; Nosil, 2008).

A historical limitation for untangling these processes and answering these questions has been the amount of genetic data available for inference (Avise, 2009). Although studies of model organisms are able to conduct inferences using hundreds of loci and individuals (Rosenberg *et al.*, 2002), most species-level studies in groups such as amphibians

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and reptiles have sampled large numbers of individuals (Lemmon *et al.*, 2007; Pyron & Burbrink, 2009), but typically only a few loci, usually a few mitochondrial genes or nuclear exons (Burbrink *et al.*, 2011; Myers *et al.*, 2013). In a recent review of species-delimitation methods (Carstens *et al.*, 2013), all studies used < 21 loci, whereas next-generation approaches can yield hundreds or thousands of markers (McCormack *et al.*, 2013). Sequence data from hundreds of loci, representing hundreds of thousands of base pairs and thousands of single nucleotide polymorphisms (SNPs), should allow powerful resolution of many evolutionary problems (Lemmon & Lemmon, 2013).

Even with this amount of data, there are still questions regarding how species are recognized (see Hey, 2009). Coalescent analyses of large genetic data sets provide a powerful tool for delimiting distinct population clusters, which some authors have defined as species (Leaché & Fujita, 2010). However, the practical application and historical theory of species delimitation still necessitate a rigorous nomenclatural and taxonomic definition of species, including character-based descriptions (Bauer *et al.*, 2011; Fujita & Leaché, 2011).

Thus, an integrative taxonomy considering genomic variation as well as morphological characters is desirable (Fujita *et al.*, 2012; Burbrink & Guiher, 2015). It is possible that genetic methods using only molecular data may 'over split' divergent populations into candidate species (Hedin, Carlson & Coyle, 2015). This may occur for distantly related populations with many fixed genetic differences, when those taxa would not be recognized as 'good species' under most traditional species concepts (see de Queiroz, 1998; Hey, 2009). Such populations may lack morphologically diagnostic differences, and may exhibit hybridization or gene flow.

Fortunately, integrative methods to address many of these questions have recently become available that accommodate both genome-scale molecular data and trait data. These include methods for species delimitation that can accommodate hundreds of loci (Yang & Rannala, 2010), methods that can simultaneously estimate species trees and species assignment (Yang & Rannala, 2014), and methods that can incorporate morphological traits into coalescent species delimitation (Solis-Lemus, Knowles & Ane, 2015). These methods have yet to be used in combination for a phylogenomic data set in an empirical setting. At least three main questions exist: (1) are these methods computationally tractable when used with hundreds of loci; (2) are the results affected heavily by the priors; and (3) does the addition of morphological data alter the conclusions from coalescent delimitation based on molecular data alone?

Here, we investigate the efficacy of these approaches for inferring phylogeographical structure and delimiting phylogeographical species in the brown and red-bellied snakes (Natricinae: *Storeria*). In an unusual situation for North American snakes, the two species are highly similar in terms of ecomorphology, distribution, life history, and habitats, and are sympatric throughout the majority of their ranges (Ernst & Ernst, 2003). They are small, typically < 30 cm in total length, terrestrial snakes that feed primarily on earthworms and other invertebrates (Ernst & Ernst, 2003). They are often highly abundant around human habitations, in leaf litter or piles of debris (Conant & Collins, 1998). They also occur across least two major geographical regions associated with divergence in snakes (Soltis *et al.*, 2006; Pyron & Burbrink, 2010); the Mississippi River (both) and the Florida Peninsula (*Storeria dekayi*). We hypothesized that they will both exhibit similar genetic divergence across these features.

We addressed these hypotheses with a broad geographical sampling from both species, plus the relict Mexican congener *Storeria storerioides*. Using the anchored phylogenomics approach, we generated a large-scale genetic data set containing hundreds of loci, thousands of SNPs, and hundreds of thousands of total base pairs (Lemmon, Emme & Lemmon, 2012), which we analysed in conjunction with several key morphological traits. We evaluated the usefulness of combining morphological and molecular data with methods for species delimitation that are tractable for data sets of this size, i.e. many samples, hundreds of loci, and several traits (Yang & Rannala, 2010, 2014; Solis-Lemus *et al.*, 2015).

We found extensive genetic variation within *Storeria*, and re-delimited species based on both morphological and coalescent-based species delimitation. Integrating morphological data into coalescent species-delimitation reduced the number of candidate species from 8 to 4. Future studies sampling more Central American populations or using additional traits may increase this number. We offer some perspectives for future analyses of genome-scale data for phylogeography. In particular, there is still a conceptual and empirical gap between the theoretical and philosophical underpinnings of species concepts and the analytical methods used to delimit species. We suggest that integrating morphological data into species-delimitation analyses is the best approach, using programs such as iBPP, and predict that such integrative approaches will help clarify many confusing taxonomic situations (see Fujita *et al.*, 2012).

MATERIAL AND METHODS

Taxonomic Status

The number of species in the genus *Storeria* is currently unclear. The Mexican brown snake, *S. storerioides,* is restricted to high elevations (> 2500 m) in the central and western Mexican plateau, and is diagnosed morphologically by the presence of a loreal scale, seven supralabials, two preoculars, three postoculars, and dorsal scales in 15 rows at midbody (Cope, 1865). The brown snake, *S. dekayi*, occurs from Guatemala to the eastern USA and Canada, and is diagnosable by the absence of a loreal, and the presence of seven supralabials, one preocular, two postoculars, and dorsal scales in 17 rows at midbody (Ernst & Ernst, 2003). There are at least eight recognized subspecies of *S. dekayi*: *S. d. anomala*, *S. d. dekayi*, *S. d. limnetes*, *S. d. temporalineata*, *S. d. texana*, *S. d. tropica*, *S. d. victa*, and *S. d. wrightorum* (see Wallach, Williams & Boundy, 2014). The Central American populations have been recognized as a distinct species (*Storeria 'tropica'*) by some authors (Cope, 1885; Anderson, 1961). These subspecies are primarily defined by qualitative variation in colour pattern rather than fixed morphological differences (e.g. the loreal, supralabial, preocular, postocular, or dorsal-scale characters that differentiate the other species), and smoothly intergrade across huge geographical areas (Trapido, 1944; Anderson, 1961; Sabath & Sabath, 1969; Ernst & Ernst, 2003). The Florida brown snake, *S. d. victa*, has dorsal scales in 15 rows at midbody, and is sometimes considered a distinct species from *S. dekayi* (Christman, 1980; see Crother *et al.*, 2012).

The red-bellied snake, *Storeria occipitomaculata*, ranges from north-eastern Mexico to the eastern USA and Canada, and is diagnosable by the absence of a loreal, and the presence of six supralabials, two preoculars, two postoculars, and dorsal scales in 15 rows at midbody. Three subspecies are recognized, the northern (*S. o. occipitomaculata*), Florida (*S. o. obscura*), and Black Hills (*S. o. pahasapae*) red-bellied snakes, but these, too, are based primarily on ambiguously defined colour-pattern differences and have no clear geographical separation (Ernst & Ernst, 2003). A small, relict population apparently allied to *S. occipitomaculata* in north-eastern Mexico is sometimes recognized as *Storeria hidalgoensis* (Wallach *et al.*, 2014), but is not diagnosable morphologically from *S. occipitomaculata* (Taylor, 1942), and may not represent a valid species. All scalation characters are also occasionally subject to rare individual variation (Ernst & Ernst, 2003).

Thus, based on different taxonomic authorities (i.e. Crother *et al.*, 2012; Wallach *et al.*, 2014; Uetz & Hošek, 2015), one could choose to recognize as many as five (*S. dekayi*, *S. hidalgoensis*, *S. occipitomaculata*, *S. storerioides*, and *S. victa*) or as few as three (*S. dekayi*, *S. occipitomaculata*, and *S. storerioides*). Within each of these populations, there may also be cryptic variation associated with known geographical barriers to gene flow, such as the Mississippi River (Soltis *et al.*, 2006; Pyron & Burbrink, 2010). Without a systematic review and species-delimitation analysis, species diversity in *Storeria* is ambiguous. We aimed to diagnose and delimit species-level variation coherently using both genetic and morphological data to produce an integrative taxonomy for the group (Fujita *et al.*, 2012), while accounting for issues such as gene flow between populations that can hamper such inferences (Burbrink & Guiher, 2015).

Genetic Sampling

We sampled specimens from throughout the range of *S. dekayi* and *S. occipitomaculata* through fieldwork and museum loans (Supporting Information Appendix S1). We obtained material for 27 *S. dekayi*, 15 *S. occipitomaculata*, and one *S. storerioides*, along with outgroups *Regina rigida* (Natricinae) and *Diadophis punctatus* (Dipsadinae). Although this sampling does not allow us to test monophyly of *Storeria* directly, the genus is supported by multilocus and genomic data sets presented by previous authors (Pyron *et al.*, 2014; McVay, Flores-Villela & Carstens, 2015).

Additional sampling could possibly have improved geographical coverage, but was limited primarily by the time and funding available for next-generation genomic sequencing. Although *Storeria* are typically highly abundant, and one could easily collect hundreds or thousands throughout their range, this density of sampling is not typically required for a high-quality phylogeographical assessment. Our sampling contains representatives from within the putative ranges of all *S. occipitomaculata* subspecies (except *S. 'o.' hidalgoensis*), and all *S. dekayi* subspecies except *S. d. anomala, S. d. temporalineata*, and *S. d. 'tropica'*. The missing subspecies are all geographically restricted, and we thus sampled populations from throughout the majority of the geographical ranges and subspecific variation of both species.

Library preparation, enrichment, sequencing, and read assembly were performed at the Center for Anchored Phylogenomics (www.anchoredphylogeny.com) following the protocol outlined in Lemmon *et al.* (2012), Rokyta *et al.* (2012), and Pyron *et al.* (2014). The 45 indexed libraries were pooled with equal concentration in two sets of ~23 samples and enriched using the Anchored Hybrid Enrichment Kit for

Vertebrates (version 1). The enriched pools were then pooled with equal concentration and sequenced on two Illumina HiSeq 2000 paired-end 100-bp sequencing lanes, with 8 bp indexing. Reads passing the Casava quality filter were processed following Prum *et al.* (2015). In short, overlapping reads were merged following Rokyta *et al.* (2012), reads were assembled using *Anolis carolinensis* references derived from the version 1 Vertebrate Anchor design, alleles were phased using read overlap information in a Bayesian statistical framework, orthology was assessed using sequence similarity, alignments were performed by MAFFT (v. 7.023b; Katoh, 2013), and alignments were trimmed/masked to remove ambiguously aligned regions. Methodological details and scripts are given in Dryad Repository doi:10.5061/dryad.51v22. After bioinformatics processing and quality control, we obtained data from 322 loci, totalling 227 911 bp, with 3.9% missing data ('-') or ambiguous bases ('N').

As an initial estimate of population structure, we performed a maximum likelihood (ML) analysis on the concatenated alleles (90 terminals from 45 individuals). We used RAxML v. 8.0.6 with the rapid bootstrapping algorithm for 100 replicates (Stamatakis, Hoover & Rougemont, 2008), partitioned by locus and using the General Time Reversible + gamma (GTRGAMMA) model (Stamatakis, 2006). Strictly speaking, concatenating alleles across loci is not biologically informative, because we do not have information on the linkage groups (i.e. parental chromosomes) of each allele. However, in the absence of gene flow and incomplete lineage sorting, we expect mutation to be the only process driving divergence between alleles, and thus each allele should be each other's closest relative, regardless of linkage. If sets of alleles are not each other's closest relative, it indicates that at least some loci in the arbitrary linkage group have experienced gene flow or incomplete lineage sorting. Above the individual level, this analysis will allow us to observe geographical clusters of alleles, much like in traditional phylogeographical analyses of unphased, concatenated loci (Brito & Edwards, 2009; Ruane *et al.*, 2014). There is thus little conceptual difference between this approach, and the traditional strategy of treating heterozygosity as ambiguity, and creating an ML tree containing 45 terminals by collapsing alleles across loci, which would yield essentially identical results.

Genetic Species Delimitation

We followed recent protocols for genetic species delimitation, the aims of which are to identify well-supported, geographically defined genetic clusters, estimate relationships between them, and evaluate whether they represent distinct species (Sites & Marshall, 2004; Fujita *et al.*, 2012). One consideration is the interface between species delimitation and phylogenetic inference (O'Meara, 2010). Accurate inference of species trees typically requires assignment of individuals to species a priori, whereas this very assignment is usually one of the primary goals of phylogeographical inference (Fujita *et al.*, 2012). In cases where a large number of cryptic taxa are likely to be present (e.g. Barley *et al.*, 2013), an analysis of the raw data (e.g. using traditional concatenation methods) will probably be necessary to serve as a preliminary identifier of geographical population clusters to construct a basic guide-tree (Leaché & Fujita, 2010). In our case, we predicted existing phylogeographical structure based on previous biogeographical studies: divergence across the Florida Peninsula and Mississippi River (Soltis *et al.*, 2006; Pyron & Burbrink, 2010).

First, we observed if there were reciprocally monophyletic clades distributed across these barriers using the ML tree described above, as has historically been done for qualitative phylogeographical analyses. We examined the tree to determine if the sampled individuals of each species were monophyletic, and then if the alleles of each species exhibited geographical differentiation across the Mississippi River or Florida Peninsula. We then determined if there was any genetic structure concordant with current subspecies definitions. This is difficult, as the subspecies as currently defined are geographically ambiguous, with no clear demarcations and broad zones of putative intergradation (Trapido, 1944; Conant & Collins, 1998).

Second, we objectively evaluated geographical clustering and admixture of lineages using discriminant analysis of principal components (DAPC) in the R package 'adegenet' (Jombart, 2008; Jombart *et al.*, 2008). The DAPC approach conducts a principal components analysis on the matrix of allele frequencies in the individuals, and uses discriminant function analysis to identify maximum separation between genetic groups (Jombart, Devillard & Balloux, 2010). The appropriate number of groups is then chosen as the one that minimizes the Bayesian information criterion score across groupings. The method also calculates posterior probabilities for group membership, indicating potential admixture within individuals. This approach is similar in concept to other clustering algorithms such as STRUCTURAMA (Huelsenbeck & Andolfatto, 2007), but has the benefits of making no population-genetic assumptions such as Hardy–Weinberg equilibrium, and handling massive data sets with little computational cost.

Third, we evaluated the distinctiveness of the potential geographical species clusters using coalescent-based species-validation methods. We conducted

this analysis using the program BP&P v. 3.1 (Rannala & Yang, 2003; Yang & Rannala, 2010, 2014). This program includes an algorithm (A11) that simultaneously estimates species trees and species-delimitation models, based on a priori assignments of individuals to a maximum possible number of potential population clusters. As the geographical clade assignments match the DAPC clusters almost exactly (see Results), we used the eight-species model and ML topology for the starting values. Thus, we could estimate up to eight species, with a freely estimated topology for those eight species. We included all 322 loci for both alleles by increasing the *numloci* parameter in the source code.

Following Leaché & Fujita (2010), we implemented three different combinations of priors for ancestral population size (θ) and the root age (τ_0). In BP&P, both priors are assigned a gamma $\Gamma(\alpha, \beta)$ distribution, and thus we parameterized these priors for: very large ancestral populations and deep divergences, $\theta \sim \Gamma(1, 10)$ and $\tau_0 \sim \Gamma(1, 10)$; small ancestral population size and shallow divergences, $\theta \sim \Gamma(2, 2000)$ and $\tau_0 \sim \Gamma(2, 2000)$; and a more conservative prior combination that accounts for large ancestral population sizes and recent divergences, $\theta \sim \Gamma(1, 10)$ and $\tau_0 \sim \Gamma(2, 2000)$, which may be the most biologically realistic scenario (Myers *et al.*, 2013). The other divergence time parameters are assigned the Dirichlet prior (Yang & Rannala, 2010: equation 2). We used a uniform rooted tree-prior on the species-tree topology (Prior 1), and estimated per-locus rate variation using the Dirichlet prior. We ran each set of priors for 50 000 generations, with a burn-in of 1000, and a sampling frequency of once every 50 generations (1000 samples). Each analysis was run twice to confirm consistency between runs.

Integrative Species Validation

Ideally, species delimitation should be based on more than genetic variability alone, and recently developed models allow us to integrate morphological traits with multispecies coalescent models (Solis-Lemus *et al.*, 2015). We used the program iBPP v. 2.1.2 to integrate trait variation into the genetic framework for species delimitation described above, analysing the phylogenomic and trait data sets simultaneously. This method uses the original Bayesian Phylogenetics & Phylogeography (BP&P) model for coalescent species delimitation (Yang & Rannala, 2010), conditioned on a Brownian motion (BM) model of trait evolution. We used the species-tree topology from BP&P v. 3.1 as the guide tree, with eight candidate species (see Results).

We coded the five scale counts traditionally used to delimit species in *Storeria* as described above: loreal, supralabial, preocular, postocular, and midbody dorsal scale rows. As described above, these are essentially fixed in all populations within the four currently recognized species (*S. dekayi*, *S. occipitomaculata*, *S. storerioides*, and *S. victa*), with only very rare individual variation (see Trapido, 1944; Ernst & Ernst, 2003). We confirmed this with additional measurements (Supporting Information Appendix S1). Thus, we treated them as fixed within samples from these populations, with each individual given the same coding within lineages. The BM model used by iBPP is applicable to discrete meristic characters such as scale count, as their distribution can typically be approximated by a normally distributed variance over time (C. Ané, pers. comm.).

We followed the original authors (Solis-Lemus *et al.*, 2015) in placing priors of $\theta \sim \Gamma(2, 400)$ and $\tau_0 \sim \Gamma(2, 400)$, with a uniform prior on the BM control parameters ν and κQ We used the species delimitation algorithm 0 with $\varepsilon = 15$ (Yang & Rannala, 2010), and default fine-tuning parameters. We ran five chains for 10 000 generations sampled every ten generations, with 1000 generations burn-in. Each chain was started from a one-species model (all internal nodes collapsed) to ensure that the chains were mixing adequately and converging on the same result by only adding in strongly supported nodes from the guide tree to the final model. This allowed us to test whether the full species-tree/species-delimitation model from BP&P v. 3.1 is supported by the trait data typically used to diagnose species boundaries in the genus. We did not use other continuous traits such as snout–vent length, ventral, or subcaudal scale counts, because these are known to be sexually dimorphic in snakes, which may affects the iBPP algorithm in uncertain ways, particularly if sampling of sexes is uneven (Solis-Lemus *et al.*, 2015).

RESULTS

Genetic Diversity

As a genus, *Storeria* and all species therein are strongly supported as monophyletic in the ML tree (Fig. 1A). We found strong support for *S. storerioides* + (*S. dekayi* + *S. occipitomaculata*). Almost all allele sets (1 and 2 from each individual) are monophyletic. Within each species, geographical differentiation is present, concordant with both known barriers to gene flow, and to some extent, subspecific differentiation. Using these qualitative criteria, we identify a Florida Peninsula clade of *S. dekayi* (i.e. *S. d. victa*), and clades east and west of the Mississippi River. Structuring is also extensive in *S. occipitomaculata*, with northern (i.e. *S. o. occipitomaculata*)

and southern (i.e. *S. o. obscura*) clades east of the Mississippi, and a Black Hills clade (i.e. *S. o. pahasapae*) and a 'central' clade of *S. o. occipitomaculata* west of the river.

Thus, a species-tree analysis based on this interpretation would have eight terminals: *S. storerioides*, *S. o. obscura*, 'northern' *S. o. occipitomaculata*, 'central' *S. o. occipitomaculata*, *S. o. pahasapae*, *S. victa*, 'east' *S. dekayi*, and 'west' *S. dekayi*. Note, however, that we are using these subspecific names derived from the geographical origin of the sample, and not any discrete morphological traits defining each subspecific group, as no traits are known that diagnose the subspecies. Rather, many of the samples of each 'subspecies' originate from putative intergrade zones (see Conant & Collins, 1998), based on clinal variation in subjective characters related primarily to the extent of pigmentation on the cephalic and dorsal scales (see Trapido, 1944).

Remarkably, the results from the DAPC clustering are nearly identical (Fig. 1C). The grouping with the lowest BIC score contains nine clusters including *Diadophis* and *Regina*, with seven *Storeria* clusters. The only difference from the geographical clusters is that the 'central' clade of *S. o. occipitomaculata* is grouped with the 'northern' clade of *S. o. occipitomaculata*, and *S. o. occipitomaculata* thus forms a single clade. Note that this result is derived independently of any a priori information on the geographical location or assignment of individuals. This suggests that the geographical clades are robust descriptors of the genetic diversity within the group.

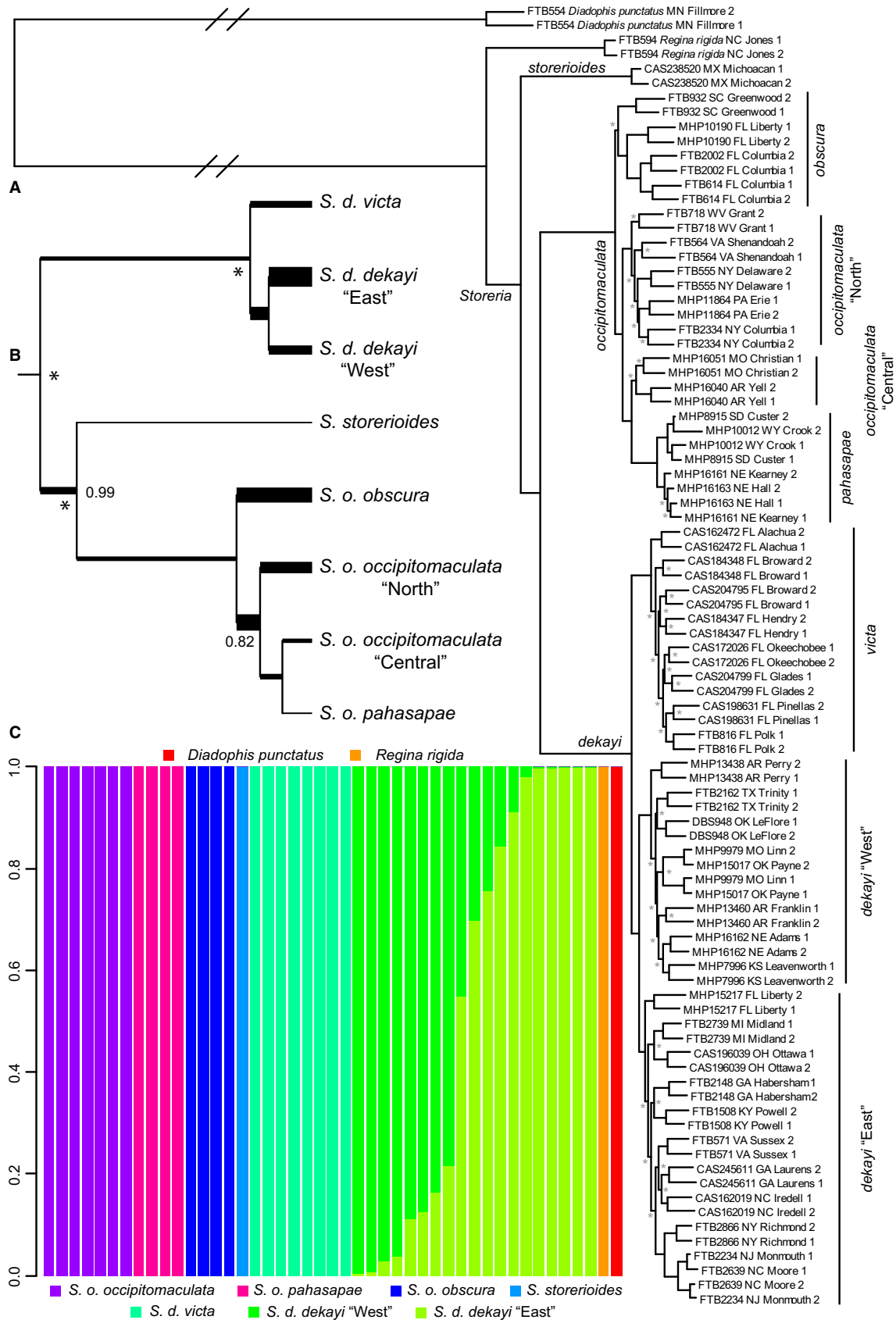
In subsequent analyses, we used the eight-species model to allow for the greatest amount of genetic diversity to be captured by the species-delimitation algorithms. As both the species tree and species-delimitation model are estimated by BP&P, the seven-species arrangement (in which 'central' *S. o. occipitomaculata* and 'northern' *S. o. occipitomaculata* are sister lineages and considered the same species) is a submodel of the eight-species arrangement, and thus could still be estimated as the correct model. Therefore, the eight-species model of maximal diversity is the most conservative descriptor of maximum potential geographical divergence.

Coalescent Species Validation

The two independent runs of BP&P v. 3.1 converged on identical results for the species tree and delimitation models (Fig. 1B). The eight-species model is supported at 100%, whereas the majority species-tree topology is supported at 81%. The minority topology (18%) is nearly identical, the only difference being a reversal in the position of 'north' *S. o. occipitomaculata* and *S. o. obscura* (Fig. 1B). Examining the output confirms that these samples occurred early in one of the runs before it converged on the final result, suggesting that both were at stationarity when sampling the majority topology (Fig. 1B). The species tree also shows a sister-group relationship between *S. storerioides* and *S. occipitomaculata*, in contrast to the concatenated ML tree, but confirming recent species-tree analyses based on more limited sampling of individuals and loci (McVay *et al.*, 2015). Contrastingly, our recent analysis of a larger snake clade (Colubroidea) containing one specimen each of *S. dekayi*, *S. occipitomaculata*, and *S. storerioides* sampled for 333 loci recovered a weakly supported sister-group relationship between *S. dekayi* and *S. occipitomaculata*, to the exclusion of *S. storerioides* (Pyron *et al.*, 2014), as in the concatenated ML analysis reported above.

The runs using prior densities of $\theta \sim \Gamma(1, 10)$ and $\tau_0 \sim \Gamma(1, 10)$, indicating large ancestral population sizes and ancient divergences, also supported the eight-species model at 100%. For the species tree, these priors yielded 75% support for a similar result as the main run, in which *S. o. pahasapae* was the sister lineage to the *S. o. occipitomaculata* (i.e. 'north' + 'central') clades. The minority topology was the same as the main result described above, supported at 21%. By contrast, the prior densities indicating small ancestral population sizes and shallow divergences, $\theta \sim \Gamma(2, 2000)$ and $\tau_0 \sim \Gamma(2, 2000)$, yielded 96% support for the main result described above.

Figure 1. A, maximum likelihood phylogeny of 45 *Storeria* samples and outgroups, phased into two sets of alleles concatenated across loci. Asterisks indicate nodes that are *not* supported > 95% bootstrap support; all unmarked nodes are supported at > 95%. B, species tree from BP&P v. 3.1 analysis of the 322-locus data set, showing the eight-species model, with branch widths proportional to effective population size, posterior probabilities (unmarked nodes are supported at 100%), and asterisks indicating nodes that are also supported in the iBPP v. 2.1.2 molecular + morphological analysis. C, assignment probabilities to the seven-species model from discriminant analysis of principal components clustering of individuals, showing strong association of most individuals with their geographical genetic cluster, but assignment uncertainty potentially representing admixture between 'east' and 'west' *Storeria dekayi*.



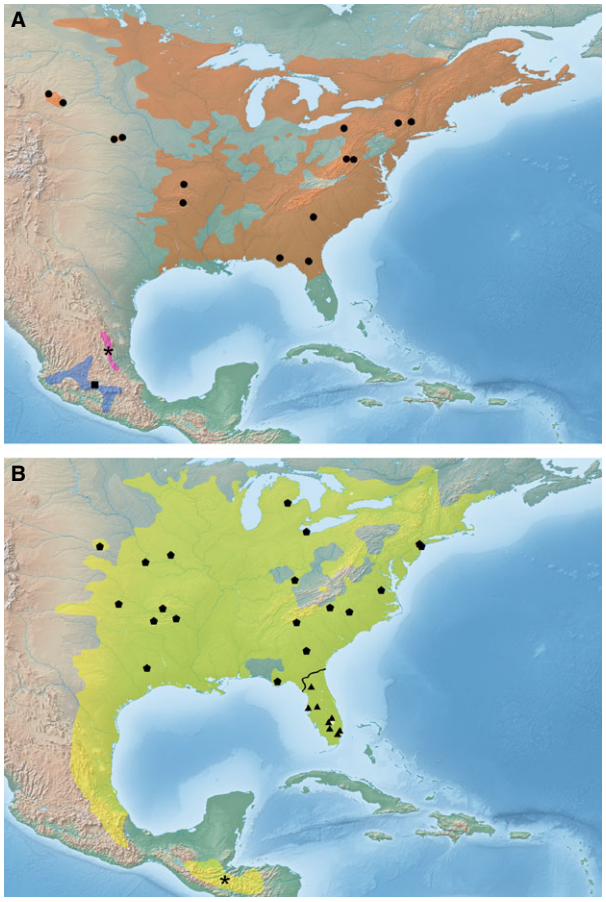


Figure 2. Map of *Storeria* ranges and sampling locations, showing geographical extent of populations, and range of former species with respect to re-delimited taxa. A, previous geographical extent of *Storeria occipitomaculata* is shown in red; circles indicate sampling localities. The asterisk indicates the sample of *Storeria* '*hidalgoensis*' examined for morphology, with the range of this subpopulation, now considered part of *S. occipitomaculata*, indicated in pink. The range of *Storeria storerioides* is indicated in blue, with the sampling locality indicated by a square. B, previous geographical extent of *Storeria dekayi* is shown in yellow, with pentagons indicating sampling localities of *S. dekayi*, triangles indicating *Storeria victa*, and a line drawn to approximate the range boundary. The asterisk in the Central American population indicates the collection location of the specimen of *Storeria* '*tropica*' examined for morphology, which is now considered part of *S. dekayi*.

Morphological Species Validation

The addition of five morphological traits to the 322 anchored loci supports a reduced species-delimitation model with fewer candidate species. The five iBPP v. 2.1.2 runs all converged at 100% support on a four-species topology (Fig. 1B), collapsing the 'east' and 'west' clades of *S. dekayi*, and all clades of *S. occipitomaculata,* into single species. This yields a species-delimitation model that recognizes the four morphologically distinct lineages as species (Fig. 2), with strong molecular and morphological support: *S. dekayi*, *S. occipitomaculata*, *S. storerioides*, and *S. victa.* We include *S. 'tropica'* in *S. dekayi*, and *S. 'hidalgoensis'* in *S. occipitomaculata* (see Discussion; Fig. 2). Gene flow is thus more common in the intraspecific lineages ('east' and 'west') of *S. dekayi*, and apparently more limited amongst the geographically distant and occasionally allopatric (*S. o. pahasapae*) lineages of *S. occipitomaculata* (Fig. 2).

DISCUSSION

Cryptic Species-Level Diversity

Both *S. dekayi* and *S. occipitomaculata* exhibit extensive phylogeographical diversity and distinct population clusters (Figs 1,2). The deepest divergences within species track the well-known geographical barriers to gene flow posed by the Mississippi River and Florida Peninsula (Soltis *et al.*, 2006; Pyron & Burbrink, 2010). The presence of *S. dekayi* and *S. occipitomaculata* populations in Mexico and Central America suggests that both species ranged more extensively in tropical and subtropical regions historically. Fragmentation and extirpation in those areas were presumably driven by Pliocene and Pleistocene glacial cycles, as in numerous other snake species (Pyron & Burbrink, 2009; Burbrink *et al.*, 2011; Myers *et al.*, 2013). These hypothesized drivers of speciation can be tested in future studies using demographic and ecological methods for modelling divergence (Lemmon & Lemmon, 2008; Knowles & Alvarado-Serrano, 2010).

Overall, the phylogeographical patterns in *Storeria* are not particularly remarkable, and are shared with numerous other organisms from plants to birds (Soltis *et al.*, 2006). What is remarkable, however, is the intersection between the patterns of phylogeographical diversity, scalation, colour-pattern, and species-delimitation models. Our results highlight several persistent issues regarding how species are recognized when combining genomic data and morphological traits (Bauer *et al.*, 2011; Fujita & Leaché, 2011; Fujita *et al.*, 2012). The deep genetic divergences that we observe are not unexpected given the great geographical distances spanned by the populations. The lack of observed admixture between adjacent lineages may simply reflect a lack of sampling from hybrid zones, which can be examined in future studies.

Importantly, we find extensive genetic diversity that is not tracked by the morphological characters

historically used to delimit species in the group. Nor do they track, at least not closely, the qualitative variation in colour pattern historically used to delimit subspecies. A recent study also used genomic data to estimate phylogeographical structure in a group of birds with a Holarctic distribution (Mason & Taylor, 2015). They also found that extensive colour-pattern differentiation was not reflected in genomic divergence, but represented polygenic expression patterns of a small subset of genes, and that morphologically distinct populations represented larger panmictic species (Lifjeld, 2015). Their results are concordant with ours in finding that qualitative variation in traits such as colour pattern may be extensive, yet unrelated to underlying speciation patterns, a common pattern in other snakes (Burbrink, Lawson & Slowinski, 2000; Cox & Rabosky, 2013). Small segments of the genome, such as the melanocortin 1 receptor (MC1R) locus (Rosenblum, Hoekstra & Nachman, 2004; Cox, Rabosky & Chippindale, 2013), may have drastic effects on colour pattern that are not reflective of population differentiation, necessitating careful consideration of the morphological traits used to delimit species.

Species Delimitation and Taxonomy

The genus *Storeria* has been systematically reviewed in the past (Trapido, 1944), and geographical variation in key morphological characters is well known. Taxonomic judgment is thus required to reconcile the species-delimitation analyses with the existing nomenclature. Our genetic and morphological species-delimitation models are concordant in supporting a four-species taxonomy that is congruent with diagnostic characters historically used to delimit species.

We find that *S. storerioides* (Cope, 1865) is clearly a distinct, valid species, and is diagnosed by the presence of a loreal. Within *S. occipitomaculata*, several subspecies are currently recognized (*S. o. obscura*, *S. occipitomaculata*, and *S. o. pahasapae*) that are not unambiguously diagnosable by fixed morphological differences and intergrade extensively throughout their ranges (Ernst & Ernst, 2003). As these are also not concordant with any geographically recognizable genetic clusters, we synonymize them with *S. occipitomaculata*. Similarly, we also synonymize *S.* '*hidalgoensis*' with *S. occipitomaculata* following previous authors (Trapido, 1944), as this population is not diagnosable by any fixed morphological differences. We thus recognize *S. occipitomaculata* (Storer, 1839) as a monotypic, polymorphic species (exhibiting both red and grey phases found rangewide), diagnosed by six supralabials.

Within *S. dekayi*, the Florida Peninsula population (*S.* '*d.*' *victa*) is clearly distinct, and we restore the original description of *S. victa* (Hay, 1892), which has been treated as a subspecies by some recent authors (Ernst & Ernst, 2003; Wallach *et al.*, 2014). The presence of 15 dorsal scale rows in this population distinguishes it from *S. dekayi*, which has 17 (Ernst & Ernst, 2003). The consistency of this character has been confirmed throughout the Florida Peninsula (Christman, 1980), defining the range of this species to be the Florida Peninsula east of the Suwanee River and north to the Okefenokee Swamp in Georgia. The presence of seven supralabials distinguishes it from *S. occipitomaculata*, which has six (Trapido, 1944).

We treat all remaining populations as a monotypic polymorphic species *S. dekayi* (Holbrook, 1839), synonymizing the previously defined subspecies *S. d. anomala*, *S. d. dekayi*, *S. d. limnetes*, *S. d. temporalineata*, *S. d. texana*, *S. d. tropica*, and *S. d. wrightorum*. These subspecies were all primarily defined by qualitative variation in colour pattern (e.g. pigmentation on labial scales) rather than fixed morphological differences (i.e. the loreal, supralabial, preocular, postocular, or dorsal scale characters that differentiate the other species), and smoothly intergrade across huge geographical areas (Trapido, 1944; Anderson, 1961; Sabath & Sabath, 1969; Ernst & Ernst, 2003). This precludes any meaningful application of the 'subspecies' rank for these populations (see Burbrink *et al.*, 2000).

Integrative Species Delimitation

Genome sequencing allow for hundreds or thousands of loci to be sequenced for many individuals, quickly and cheaply (Lemmon & Lemmon, 2013; McCormack *et al.*, 2013). Data sets of this magnitude drastically exceed the computational limits of some commonly used programs, particularly those that rely on full multispecies coalescent analysis (O'Neill *et al.*, 2013). However, the volume of data (informative sites) means that higher-level summary approaches are likely to be powerful alternatives in many cases (Jombart, 2008). Here, we demonstrate that simple techniques such as DAPC-based population clustering (Jombart *et al.*, 2010) can provide a robust first-pass assessment of phylogeographical diversity and species delimitation (e.g. Ruane *et al.*, 2014). Coalescent-based species-delimitation approaches are tractable using Bayesian methods for data sets of this magnitude (Yang & Rannala, 2010). The ability to estimate species trees and delimitation models quickly using genomic and trait data should allow for easier biodiversity discovery and species delimitation in the future (Fujita *et al.*, 2012; Pyron, 2015).

This brings up an issue that has received little attention in most empirical analyses, that of the disconnect between species concepts and species-delimitation methods (Sites & Marshall, 2004; Carstens *et al.*, 2013). Despite the massive historical literature on species concepts (de Queiroz, 1998), a given algorithm is not necessarily diagnosing a biological, phylogenetic, or evolutionary (etc.) species per se. Approaches such as BP&P (Yang & Rannala, 2010) are essentially diagnosing distinct populations with regard to the multispecies coalescent, which may or may not correspond to full species. This is not a result of inadequate data or methods, but a consequence of the philosophically variable nature of species concepts.

As examined here, *Storeria* provides a good example of the tension amongst purely genetic, purely morphological, and integrative taxonomic strategies. The results from the phylogenetic, clustering, and species-validation analyses performed here support the existence of eight potentially species-level lineages. However, not all of these can be diagnosed by the traditional scalation characters that have been used to define species in the group. Integrating these data using a model that assesses coalescent genealogical variation as well as continuous trait variation (Solis-Lemus *et al.*, 2015) yields a four-species delimitation model that is congruent with key diagnostic morphological characters and historical taxonomy. Although many studies consider previously defined morphological character states when delimiting species based on genetic data, this is usually implicit rather than quantitatively evaluated (Burbrink *et al.*, 2011; Camargo *et al.*, 2012; Leaché & Fujita, 2010; Lemmon *et al.*, 2007; Myers *et al.*, 2013; Ruane *et al.,* 2014).

Here, as in other cases such as *Hemidactylus* geckos from West Africa or Appalachian spiders (Leaché & Fujita, 2010; Bauer *et al.*, 2011; Fujita & Leaché, 2011; Hedin *et al.*, 2015), it is not surprising that geographically separated populations may show strong genetic differentiation, owing to local fixation of SNPs simply from isolation by distance. A tendency for coalescent-based genetic species-delimitation methods to over-split may also result from the confounding influence of extreme population genetic structuring across smaller geographical scales (Hedin *et al.*, 2015; see Hey, 2009), Contrastingly, highly visible phenotypic differences (such as colour-pattern variation) may accumulate because of a small number of point mutations in otherwise homogeneous populations (Cox & Rabosky, 2013; Mason & Taylor, 2015). This may be the case within both *S. dekayi* and *S. occipitomaculata*, which exhibit extensive colour-pattern variation geographically (Trapido, 1944; Ernst & Ernst, 2003) that does not track observed genetic differentiation of local populations.

Whenever possible, empirical species delimitation should include explicit reference to both the previously defined morphological character states traditionally used to diagnose the focal species (Bauer *et al.*, 2011) as well as coalescent genetic variation amongst lineages (Fujita *et al.*, 2012). These states can then be defined with reference to the candidate taxa (Wiens & Penkrot, 2002), and their distribution within and amongst the potential species can then guide the final choices made for species delimitation (Solis-Lemus *et al.*, 2015), while also providing character-based diagnoses for those species. This provides a simple metric for objective species delimitation in an integrative framework (Fujita & Leaché, 2011; Fujita *et al.*, 2012), while preserving traditional morphological descriptions (Wiens & Penkrot, 2002; Bauer *et al.*, 2011). An important future consideration will be the choice of traits.

An important question for future studies will thus be how traits are identified, selected, and measured. For comparison, the anchored loci represent the maximum set of homologous loci that have been identified under minimum criteria for length and variability (Lemmon *et al.*, 2012). Contrastingly, morphological traits are often chosen subjectively, from a limited set of variable characters (e.g. body sizes, limb lengths, scale counts) that are observed, often qualitatively, to be variable within populations a priori. Here, we only used discrete meristic traits, historically used for species delimitation, that are not sexually dimorphic or subject to allometric or ontogenetic variation (Ernst & Ernst, 2003), to avoid these confounding influences. However, future analyses of this type may consider other candidate traits such as quantitative measurements of colour pattern or multidimensional body shape, which may also be appropriate if they show meaningful variation amongst populations (Solis-Lemus *et al.*, 2015).

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DATA ACCESSIBILITY

All data, code, results, and methods are available at Dryad doi:10.5061/dryad.51v22.

AUTHOR CONTRIBUTIONS

RAP gathered the samples, analyzed the data, and wrote the manuscript. CRH contributed to data collection and edited the manuscript. FWH contributed to data collection and edited the manuscript. EML contributed to data collection and edited the manuscript. ARL developed tools for data collection, performed bioinformatic analysis of sequence data, and edited the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1. Sample localities, accession numbers, and other material examined.