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Sky island diversification meets the multispecies coalescent – divergence in the spruce-fir moss spider (*Microhexura montivaga*, Araneae, Mygalomorphae) on the highest peaks of southern Appalachia

MARSHAL HEDIN,* DAVE CARLSON* and FRED COYLE†

*Department of Biology, San Diego State University, San Diego, CA 92182, USA, †PO Box 1935, Cullowhee, NC 28723, USA

Abstract

***Microhexura montivaga* is a miniature tarantula-like spider endemic to the highest peaks of the southern Appalachian mountains and is known only from six allopatric, highly disjunct montane populations. Because of severe declines in spruce-fir forest in the late 20th century, *M. montivaga* was formally listed as a US federally endangered species in 1995. Using DNA sequence data from one mitochondrial and seven nuclear genes, patterns of multigenic genetic divergence were assessed for six montane populations. Independent mitochondrial and nuclear discovery analyses reveal obvious genetic fragmentation both within and among montane populations, with five to seven primary genetic lineages recovered. Multispecies coalescent validation analyses [guide tree and unguided Bayesian Phylogenetics and Phylogeography (BPP), Bayes factor delimitation (BFD)] using nuclear-only data congruently recover six or seven distinct lineages; BFD analyses using combined nuclear plus mitochondrial data favour seven or eight lineages. In stark contrast to this clear genetic fragmentation, a survey of secondary sexual features for available males indicates morphological conservatism across montane populations. While it is certainly possible that morphologically cryptic speciation has occurred in this taxon, this system may alternatively represent a case where extreme population genetic structuring (but not speciation) leads to an oversplitting of lineage diversity by multispecies coalescent methods. Our results have clear conservation implications for this federally endangered taxon and illustrate a methodological issue expected to become more common as genomic-scale data sets are gathered for taxa found in naturally fragmented habitats.**

Keywords: allopatry, Bayes factor delimitation, conservation genetics, montane speciation, population structure, unguided Bayesian Phylogenetics and Phylogeography

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Introduction

Evolutionary biologists have long been interested in evolution on mountains. In mainland North America, conspicuous hotspots for montane diversification include the California Sierra Nevada (Rovito 2010; Schoville & Roderick 2010; Hedin *et al.* 2013), 'sky islands' of the desert southwest (Maddison & McMahon 2000; Derkarabetian *et al.* 2011) and the southern Appalachian mountains (Hedin 1997; Weisrock & Larson 2006; Keith & Hedin 2012). In each of these areas, mountains act as naturally fragmented habitat islands, serve as refugia in the face of climatic variation and/or generate strong ecological gradients. These combinations of genetic and geographic isolation with potential selective differences promote the evolution of both population genetic structure (arrays of geographically distinct populations which are genetically divergent to various degrees) and clear species-level divergences. These naturally allopatric systems also present classic difficulties for speciation

Correspondence: Marshal Hedin, Fax: +1 619 594 5676;
E-mail: mhedin@mail.sdsu.edu

biologists – when allopatry prevails and population structure is ubiquitous, distinguishing population subdivision from speciation is challenging and often 'fuzzy' (Leavitt *et al.* 2007; Bond & Stockman 2008; Keith & Hedin 2012; Satler *et al.* 2013). For example, Hey (2009) pointed out that the null hypothesis for many species delimitation methods is a 'no significant differentiation' model. Because both population subdivision and speciation imply differentiation (i.e. rejection of the null), such methods can mistakenly equate these potentially different evolutionary dynamics (see also Hey & Pinho 2012).

Modern researchers have access to many types of data when investigating the interface between population divergence and speciation, including data derived from morphology, behaviour, ecology and genomes. Assessing nuclear genomic divergence is particularly attractive in naturally fragmented systems because such data represent a common currency for measuring both population genetic structure and speciation, and explicit models are available that *potentially* distinguish population structure from species-level divergence. These include single-locus models (e.g. generalized mixed Yule coalescent model, Pons *et al.* 2006; Fujisawa & Barraclough 2013) and arguably more powerful multilocus models. A plethora of multilocus species delimitation methods have been developed over the past 10 years (O'Meara 2010; Yang & Rannala 2010; Grummer *et al.* 2014). Many empirical studies have been published using such methods (reviewed in Fujita *et al.* 2012; Carstens *et al.* 2013), and this remains an active area of method development (e.g. DISSECT, Jones *et al.* 2014; unguided Bayesian Phylogenetics and Phylogeography (BPP), Yang & Rannala 2014; *BFD, Leaché *et al.* 2014).

Despite this outstanding analytical progress, the performance of multispecies coalescent methods in highly genetically subdivided systems (e.g. taxa inhabiting mountains, islands, caves) has not been extensively explored (Camargo & Sites 2013). A central assumption of many recently developed methods is the neutral coalescent (or something analogous), where gene trees evolve within species according to a no selection, no recombination, panmixia model (Rannala & Yang 2003). For example, the heuristic BROWNIE approaches developed by O'Meara (2010) simultaneously estimate species trees and species limits by assuming unconstrained gene flow within and a lack of gene flow between species. Simulations incorporating population structure tended to result in oversplitting by this method (O'Meara 2010), and the empirical work of Niemiller *et al.* (2012) in naturally fragmented cavefishes hinted at oversplitting, with both allelic and individual sampling inflating species numbers. Another popular method is BPP (Yang & Rannala 2010, 2014; Rannala & Yang 2013) – this method assumes panmixia within species, and has been suggested to potentially oversplit diversity in dispersal-limited taxa (e.g. Niemiller *et al.* 2012; Barley *et al.* 2013; McKay *et al.* 2013; Satler *et al.* 2013). Authors of these methods have acknowledged the inherent difficulties associated with fragmented, allopatric systems (e.g. Zhang *et al.* 2013).

Microhexura montivaga, the spruce-fir moss spider, is a miniature mygalomorph spider (F. Dipluridae) endemic to high-elevation red spruce-Fraser fir forests of the southern Appalachian mountains (Coyle 1981, 1985). Recent survey work (Coyle 2009) indicates that *Microhexura* is distributed as six disjunct montane populations, occupying the Virginia Balsam Mountains (Virginia), Roan Mountain (Tennessee/North Carolina), Grandfather Mountain (NC), the Black Mountains (NC), the Plott Balsam Mountains (NC) and the Great Smoky Mountains (TN/NC) (Fig. 1). Most of these sky island populations are found above 1800 metres and include the highest elevations in eastern North America (e.g. Mt. Mitchell, Black Mountains, NC). Spatial isolation also potentially exists *within* mountain ranges, where spiders build sheet webs underneath bryophyte mats on steep, north-facing rock outcrops (Coyle 2009). These distinct outcrops are often separated by habitats lacking spiders (lower elevations, no spruce-fir forest, no rock outcrops). Because of severe declines in spruce-fir forest in the late 20th century, *M. montivaga* was formally listed as a US federally endangered species in 1995 (Fridell 1994, 2001). This status remains valid today, and while recent survey work (Coyle 2009) has shown that some montane populations include many rock outcrop demes and show comparatively large census population sizes (e.g. Great Smoky and Black Mountain populations), other populations are precariously small, known only from one or a few rock outcrops within a mountain range (e.g. Virginia Balsams, Plott Balsams).

The research presented here was motivated primarily by an interest in documenting multilocus genetic structure both within and among montane *Microhexura* populations, with the goal of using this information to help inform conservation decisions for this endangered species. There are no previous studies of *Microhexura* population structure, and while it is known that other mygalomorphs (e.g. burrow-dwelling trapdoor spiders) display remarkable microgeographic genetic differentiation (e.g. Bond & Stockman 2008; Hedin *et al.* 2013; Satler *et al.* 2013; Castalanelli *et al.* 2014; Opatova & Arnedo 2014), whether such dispersal limitation applies in the small-bodied (adult total length <6 mm), web-building *Microhexura* is currently unknown. A second goal was to explore the use of multilocus species delimitation methods in this naturally fragmented system. The southern Appalachian mountains represent a hotspot for speciation in both arthropods (e.g. Hedin 1997;

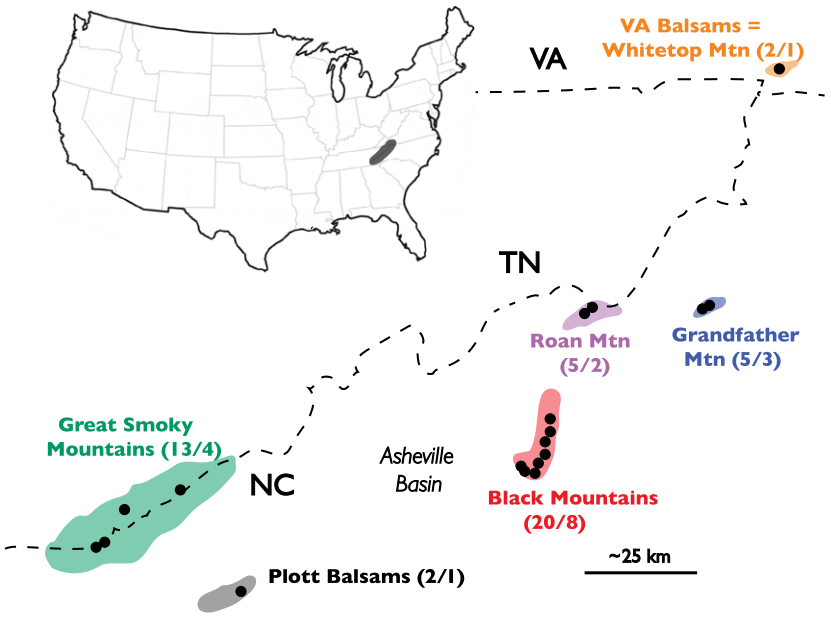

Fig. 1 Geographic distribution of sampled specimens from six primary montane populations. Number of sampled individuals and 'demes' (i.e. separate rock outcrop populations within mountain ranges) also indicated.

Thomas & Hedin 2008; Marek & Bond 2009; Hedin & Thomas 2010; Keith & Hedin 2012) and vertebrates (e.g. Weisrock & Larson 2006; Crespi *et al.* 2010). Although Coyle (1981) did not comment on morphological geographic variation in his revision of *M. montivaga*, it is possible that morphologically cryptic speciation has occurred in this taxon. Conversely, this system may represent a case where extreme population genetic structuring (but not speciation) leads to an oversplitting of lineage diversity by multispecies coalescent methods. While these alternatives are challenging to resolve in any natural system, our results illustrate an empirical issue expected to become increasingly common as genomic-scale data sets are gathered for taxa found in naturally fragmented habitats.

Materials and methods

Morphological study

The morphology of adult male pedipalps and first legs (which possess mating spurs) is often used as a primary character for species delimitation in mygalomorph spiders, including diplurids (e.g. Coyle 1984, 1988, 1995). To assess qualitative morphological divergence across *M. montivaga* populations, all adult males from Grandfather Mountain ($n = 2$) and the Great Smoky Mountains ($n = 8$) were borrowed from the American Museum of Natural History. Males are also known from the Black Mountains (vicinity Mt. Mitchell, including type specimens – Crosby & Bishop 1925; Coyle 1981), but these specimens could not be located at the AMNH. Adult males have never been collected from the Virginia Balsams, Roan Mountain or the Plott Balsams. A subset of specimens was imaged using a Visionary Digital BK Plus system (http://www.visionarydigital.com), including a Canon 5D digital camera, Infinity Optics Long Distance Microscope, P-51 camera controller and FX2 lighting system. Individual images were combined into a composite image using HELICON FOCUS V5.3 and then edited using Adobe Photoshop CS6.

Molecular marker development, sampling, data collection

Mitochondrial and nuclear genetic markers were developed specifically for *Microhexura* using comparative transcriptomic data (Material S1, Supporting information). Genomic DNA was extracted from nondestructively sampled leg tissues, collected under permit by F. Coyle. Forty-seven *M. montivaga* specimens were sampled (Fig. 1, Material S2, Supporting information), plus a specimen of the sister species *M. idahoana* from the Pacific Northwest. Transcriptome data were used to assess whether amplified PCR products were 'on target', but were not used in downstream nuclear analyses as heterozygosity could not be assessed (transcriptomes were derived from multiple individuals, see Material S1, Supporting information).

After multiple iterations of primer testing and preliminary sequencing, one mitochondrial and seven nuclear gene regions were chosen for comprehensive specimen sampling (Table 1; primers, PCR conditions and transcript annotations are provided in Material S3, Supporting information). Amplified PCR products were purified using standard techniques, and Sanger sequenced (2× coverage) at *Macrogen USA*. DNA

Table 1 Gene data summary information

Primer Combo Name	Aligned Length/No. Sequences	Substitution Model	Clock Model	Parsimony Informative Sites	Nucleotide Diversity	Exon or UTR
mtDNA (COI)	1038/47	TN93 (partitioned)	Strict	153	0.048	Exon
B1_E3E4	664/62	HKY+I	Strict	20	0.008	Exon, 3′ UTR
B1_C3C4	943/60	HKY+I	Strict	10	0.004	5′ UTR
B1_D11D12	621/57	TN93 (partitioned)	Strict	23	0.017	Exon
B1_H3H4	432/64	TN93 (partitioned)	Strict	10	0.010	Exon
B2_F3F4	652/54	F81	Relaxed – Lognormal (*Strict used)	9	0.006	No long ORFs, putative noncoding
B2_D3D4	783/54	GTR+I	Strict	17	0.007	No long ORFs, putative noncoding
B2_E7E8	582/58	HKY	Strict	12	0.008	3′ UTR

Parsimony informative sites and nucleotide diversity values calculated using MEGA 6.06 (Tamura *et al.* 2013), in-group data only.

sequences were edited using Geneious Pro (www.geneious.com/) and trimmed to exclude primer sequences. Minor gaps in nuclear sequences were recoded as follows (*data matrices named using arbitrary primer names): E3_E4 matrix, 17-base pair (bp) insertion found in Winter Star and Celo Knob, 6-bp deletion found in Smokies and Plott Balsams each recoded as two-state nucleotide transitions (e.g. A<>G); D11D12 matrix, 3-bp insertion in out-group vs. in-group recoded as a two-state nucleotide transition; B2_D3D4 matrix, 2 separate 2-bp insertions recoded as single two-state nucleotide transitions; B2_E7E8 matrix, single bp indel recoded as a two-state nucleotide transition. Heterozygous nuclear sequences were bioinformatically phased to alleles using the software program PHASE 2.1.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003). SEQPHASE (Flot 2010) was used to convert matrices for input into PHASE. PHASE analyses were conducted using default settings (phase threshold = 90%, 100 iterations, thinning interval = 1, burn-in = 100) and were repeated multiple times to ensure consistent results.

Analytical framework

Our analytical framework included both 'discovery' and 'validation' approaches to delimit species (Ence & Carstens 2011; Carstens & Satler 2013). Mitochondrial gene trees and a mitochondrial Bayesian Poisson tree processes analysis (bPTP, Zhang *et al.* 2013) were used as species 'discovery' methods. Nuclear-only gene trees and nuclear-only clustering results (POFAD, STRUCTURE) were similarly used as independent discovery methods. These various genetic discovery results were combined with geographic criteria (e.g. isolated montane populations as species) to formulate a set of alternative species delimitation hypotheses, which were then statistically compared using Bayes factor delimitation (BFD; Grummer *et al.* 2014) and BPP (Yang & Rannala 2014) 'validation' analyses. Most validation analyses were based on nuclear-only data matrices, although we also conducted BFD analyses using combined nuclear and mitochondrial data.

Discovery analyses

Individual gene trees were estimated using maximum likelihood implemented in the RAxML_GUI (Stamatakis 2006, 2014; Silvestro & Michalak 2012). Analyses included a thorough bootstrap analysis (1000 bootstrap replicates) followed by multiple inferences (100) on alignments. Nuclear gene trees were estimated using an unpartitioned GTR_Γ model; mitochondrial COI data were partitioned by codon position, with the same model applied to each partition.

The mitochondrial RAxML gene tree was used as input in bPTP analyses, implemented on the bPTP server (http://species.h-its.org/ptp/; Zhang *et al.* 2013). PTP is a single-locus species delimitation method using only nucleotide substitution information, implementing a model assuming gene tree branch lengths generated by two independent Poisson process classes (within- and among-species substitution events). Available simulation studies suggest that PTP outperforms GMYC (Pons *et al.* 2006; Fujisawa & Barraclough 2013) for single-locus species delimitations (Zhang *et al.* 2013). Two replicate bPTP analyses were run for 100 000 MCMC generations, with a thinning of 100 and burn-in of 0.1.

Multigenic nuclear genetic distances (uncorrected p-distances from PAUP*, Swofford 2002) among individuals were calculated using POFAD 1.05 (Joly & Bruneau 2006). To eliminate the potentially confounding influence of extreme female-based population structure, mitochondrial data were excluded in POFAD analyses. Also, out-group data were excluded from this analysis.

Analyses were conducted using both standardized matrices (all individual matrices given the same weight) and nonstandardized matrices (more variable matrices with greater weight). Summary distances were used to reconstruct NeighborNet networks in SplitsTree4 (Huson & Bryant 2006).

STRUCTURE detects population structure through the use of allele frequencies, identifying genetically homogeneous clusters of individuals that are in both Hardy–Weinberg and linkage equilibrium (Pritchard *et al.* 2000, 2010). STRUCTURE has also been used to identify putative independently evolving genetic lineages in many studies (e.g. Weisrock *et al.* 2010; Rittmeyer & Austin 2012; Satler *et al.* 2013). Both mitochondrial and out-group data were excluded in STRUCTURE analyses. SNAP Map (Price & Carbone 2005; Aylor *et al.* 2006) and the Mobyle SNAP workbench (Monacell & Cardone 2014) were used to convert DNA sequences to numbered unique alleles (haplotypes) for STRUCTURE input (O'Neill *et al.* 2012). STRUCTURE runs were conducted assuming between 3 and 7 genetic clusters ($K = 3$ through $K = 7$), with each K value replicated three times. Analyses used an admixture model, with a burn-in of 1×10^5 steps (with 1×10^6 MCMC steps after burn-in), and allele frequencies considered independent among populations.

Evanno *et al.* (2005) used simulations to show that the maximal value of the log probability of the data given K ($L(K)$) does not necessarily provide an accurate estimation of K, but sometimes overestimates K. Instead, a statistic called ΔK (= rate of change in log probability of data between successive K values) consistently provided a more accurate estimate of K under the simulation conditions explored. Here, both approaches were considered to identify an optimal K value – estimates from multiple replicates for multiple K values were calculated in Structure Harvester (Earl & vonHoldt 2012). Data were summarized using the *FullSearch* algorithm of CLUMPP (Jakobsson & Rosenberg 2007) and visualized with DISTRUCT (Rosenberg 2004).

Validation analyses

Bayes factor delimitation (BFD; Grummer *et al.* 2014) is a recently developed approach that compares the marginal likelihoods of competing species delimitation hypotheses using Bayes factors. Specifically, this method compares species tree models in which sequences are assigned to differing numbers of lineages (e.g. five species, six species) and chooses the model that best explains the data (Grummer *et al.* 2014). Based on a combination of geography and 'discovery' genetic results (see above), putative *Microhexura* lineages were left separate or combined to generate eight alternative species delimitation hypotheses (Table 2).

A *BEAST species tree (inferred using *BEAST v1.8.0, Drummond *et al.* 2012) was estimated for each alternative hypothesis, using nuclear-only or nuclear plus mitochondrial matrices, without out-groups. *BEAST analyses were performed using 250 000 000 generations, with data saved every 25 000 generations; the first 20% of each run was discarded as burn-in. For each hypothesis, three to five *BEAST replicates were conducted to ensure convergence and assessed using ESS values with TRACER v1.6 (Rambaut *et al.* 2014). Substitution models for individual genes were chosen with JModeltest 2.1.6 (Guindon & Gascuel 2003; Darriba *et al.* 2012) using the

Table 2 Alternative species delimitation hypotheses tested using validation approaches

Hypothesis	Distinct Species (total in parentheses)	Motivation
H_1	Plott Balsams, Smokies, Grandfather_Indian/Attic, Grandfather_Watauga, Whitetop, Roan, Blacks, Blackstock_N **(8)**	Six geographic populations unique, two distinct genetic lineages at Grandfather Mountain and in Black Mountains
H_2	Plott Balsams, Smokies, **Grandfather,** Whitetop, Roan, Blacks, Blackstock_N **(7)**	Six geographic populations unique, two distinct lineages in Black Mountains
H_3	(**Plott Balsams + Smokies**), Grandfather_Indian/Attic, Grandfather_Watauga, Whitetop, Roan, Blacks, Blackstock_N **(7)**	Mitochondrial bPTP
H_4	(**Plott Balsams + Smokies**), **Grandfather,** Whitetop, Roan, Blacks, Blackstock_N **(6)**	POFAD liberal
H_5	(**Plott Balsams + Smokies**), (**Grandfather + Whitetop**), Roan, Blacks, Blackstock_N **(5)**	STRUCTURE $K = 5$
H_6	(**Plott Balsams + Smokies**), Grandfather, Whitetop, Roan, (**Blacks + Blackstock_N**) **(5)**	Geographic populations unique except for Plott Balsams + Smokies
H_7	(**Plott Balsams + Smokies**), (**Grandfather + Whitetop + Blackstock_N**), Roan, Blacks **(4)**	STRUCTURE $K = 4$
H_8	(**Plott Balsams + Smokies**), (**Grandfather + Whitetop** + **Roan + Blacks + Blackstock_N**) **(2)**	Two species on opposite sides of Asheville Basin

Bold values represent collapsed lineages from a preceding hypothesis.

AIC method (Table 1). All *BEAST analyses were conducted on the CIPRES Science Gateway (www.phylo.org; Miller *et al.* 2010). Preliminary results suggested convergence issues when applying a relaxed clock model to the B2_F3F4 data, so a strict clock model was applied for all loci (Table 1). Marginal likelihoods were estimated using path-sampling (PS, Lartillot & Philippe 2006) and stepping-stone (SS, Xie *et al.* 2011) methods, with 100 path steps, a chain length of 100 000 generations and likelihoods saved every 100 generations. Marginal likelihood estimates (MLE) were averaged across replicate runs to generate a single PS and SS value for each hypothesis. Bayes factors were then calculated by taking the difference between the log of the best MLE and the log of other MLEs and multiplying each result by two [i.e. $2*(-\ln_{HypA} - -\ln_{HypB})$]. The significance of Bayes factor results was interpreted following Kass & Raftery (1995), with 2lnBf >10 being considered as 'decisive' support for a hypothesis.

Validation analyses were also performed using BPP v3.0 (Yang & Rannala 2010, 2014). This method utilizes a multispecies coalescent model and Bayesian statistics to delimit species. Traditionally, BPP analyses have relied on a user-specified guide tree to represent a species tree under a highly split delimitation hypothesis. A reversible-jump Markov chain Monte Carlo (rjMCMC) algorithm is then employed to estimate the posterior probability of different delimitation hypotheses by iteratively collapsing or retaining nodes found in the guide tree (Yang & Rannala 2010). More recently, Yang & Rannala (2014) updated BPP to allow for joint inference of species limits and a species tree via a nearest neighbour interchange (NNI) algorithm that is able to significantly change the topology of the input guide tree. We implemented both the traditional (rjMCMC-only) and joint estimation methods (NNI + rjMCMC) using in-group-only nuclear matrices.

For each BPP method, the nuclear-only *BEAST species tree corresponding to a liberal eight-species model (H_1, Table 2) was input as a guide tree. Three combinations of prior values specifying the ancestral population size (θ) and root age (τ_0) were used, following Leaché & Fujita (2010). These correspond to (i) large ancestral population sizes and deep divergences among species ($\theta \sim G(1,10)$ and $\tau_0 \sim G(1,10)$), (ii) small population sizes and shallow divergences ($\theta \sim G(2,2000)$ and $\tau_0 \sim G(2,2000)$) and (iii) large ancestral population sizes and shallow divergences ($\theta \sim G(1,10)$ and $\tau_0 \sim G(2,2000)$). A fourth combination representing small population sizes and intermediate divergence ($\theta \sim G(2,2000)$ and $\tau_0 \sim G(2,1000)$) was also tested following Niemiller *et al.* (2012). For each set of prior combinations, replicate runs were performed using different starting species trees, using both rjMCMC algorithms (0 and 1). Each analysis was run for 50 000 generations, with results sampled every 5 generations; the first 1000 generations of each run were treated as burn-in.

Results

Morphological divergence

We examined all male specimens from the AMNH, and imaged a subset of these. Grandfather and Smokies male specimens are not obviously qualitatively different in detail of the pedipalp and/or modified first leg (Fig. 2, images submitted to Dryad). Although male specimens from the Black Mountains were not available for study, comparisons of imaged specimens to published drawings (Crosby & Bishop 1925 fig. 2; Coyle 1981 fig. 15) do not indicate obvious differences.

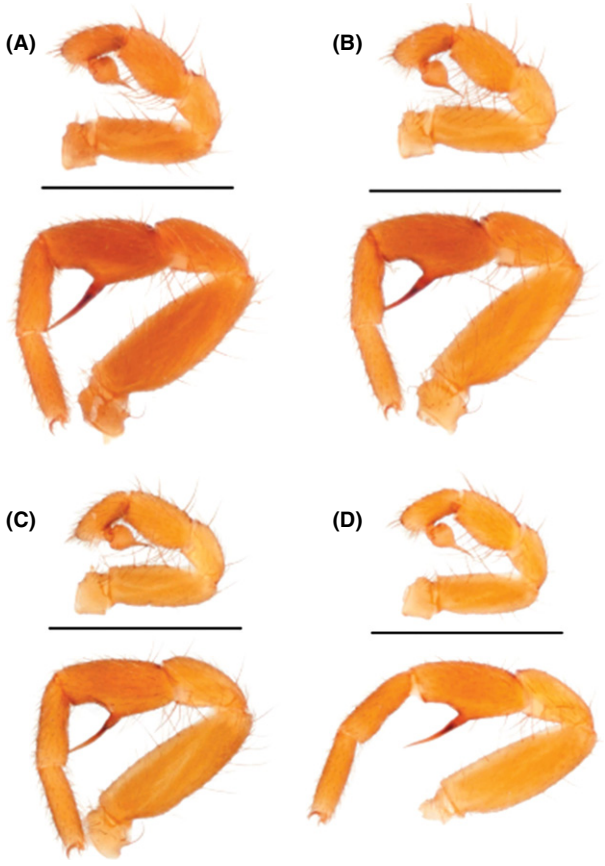

Fig. 2 Digital images of adult male morphology. Top structure in each panel is left pedipalp and bottom structure is left first leg, both in retrolateral view. (A, B) NC: Avery Co., Grandfather Mountain, 17 November 1978, coll. F. Coyle, R. Bruce, J.D. Pittillo; (C, D) NC: Swain Co., just below Clingman's Dome parking lot, Great Smoky Mountains National Park, 18 October, 4 November 1978, coll. F. Coyle. All scale bars = 1 mm.

Mitochondrial discovery analyses

Maximum-likelihood analyses of mitochondrial data (submitted to GenBank, Material S2, Supporting information) show that different montane populations form divergent, well-supported (bootstrap values >70) clades (Fig. 3). An exception includes the Plott Balsam specimens, which are nested within the Smokies mitochondrial clade (Fig. 3). Also, mitochondrial data strongly support high divergence among demes *within* mountain ranges in several instances (Fig. 3). On Roan Mountain, two geographically separate demes are genetically distinct, and on Grandfather Mountain, two rock outcrop demes separated by approximately one kilometre (Indian House Cave_Attic Window vs. Watauga View) form highly divergent mitochondrial clades. In the Black Mountains, almost all rock outcrop demes form distinct mitochondrial microclades, consistent with microgeographic genetic differentiation along the ridgeline of the Blacks (Material S4, Supporting information). A remarkable situation exists in the Blacks at Blackstock Knob, where highly divergent mitochondrial clades (Blackstock_North vs. Blackstock_Northeast) exist on rock outcrops separated by just over 100 m. Sequences from Blackstock_Northeast fall into the Black Mountains mitochondrial clade, whereas sequences from Blackstock_North form a separate divergent mitochondrial clade (Fig. 3, Material S4, Supporting information).

The estimated number of mitochondrial bPTP species is between 7 and 25, with a mean value of 11 species. The conservative number (7) corresponds exactly to primary clades of the RAxML tree (Fig. 3) and implies different species existing on Blackstock_North vs. Blackstock_Northeast rock outcrops (separated by ~ 100 m), and two bPTP species on Grandfather Mountain (Indian

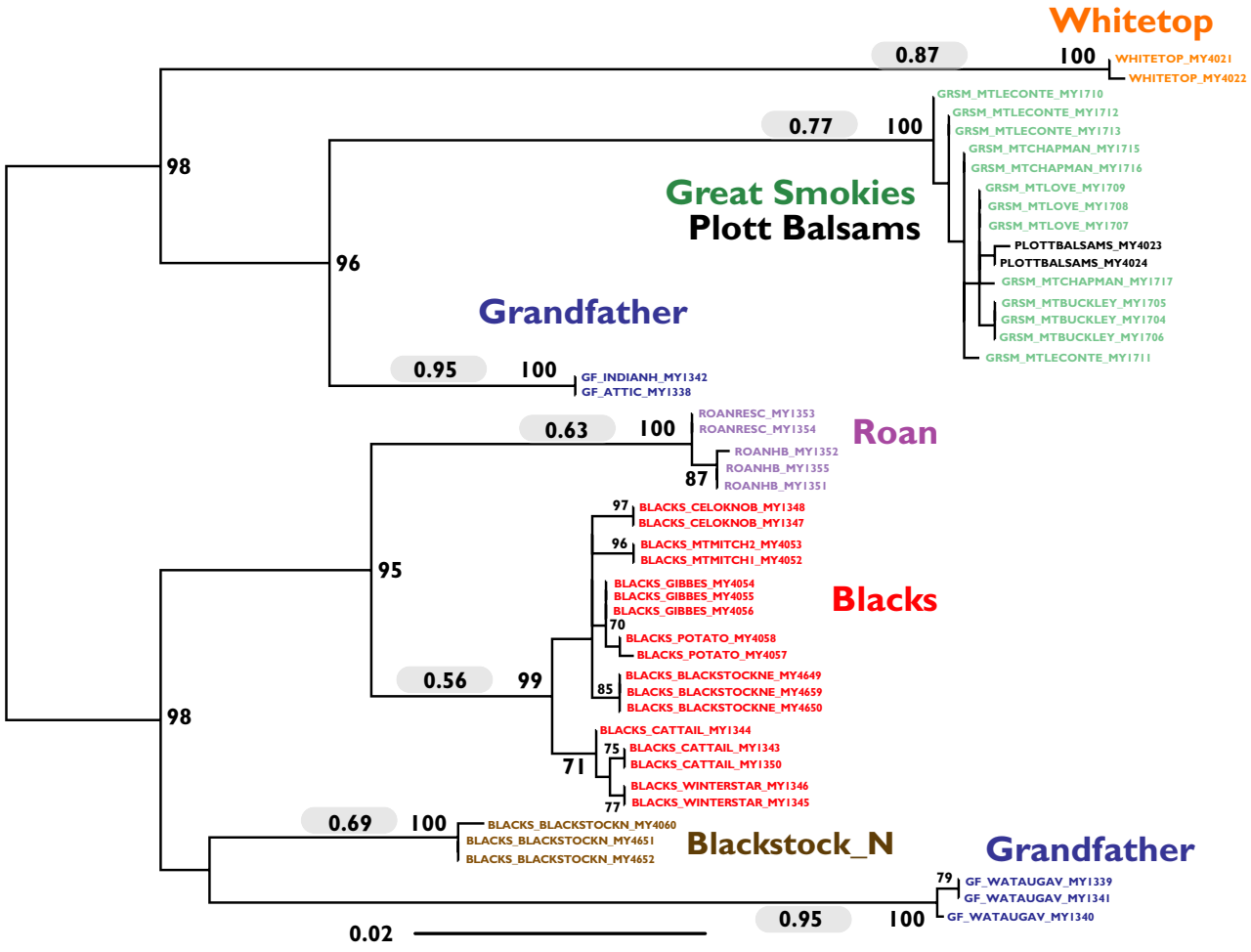

Fig. 3 RA×ML mitochondrial gene tree. Bootstrap values >70 shown on branches; bPTP values in grey boxes. Gene tree arbitrarily rooted at longest internal branch. Colours used to designate montane populations (green = Great Smokies, black = Plott Balsams, dark orange = Whitetop, blue = Grandfather, purple = Roan Mtn., red = Blacks) used for most remaining figures; Blackstock _North deme designated with brown text and used for most remaining figures.

House Cave_Attic Window vs. Watauga View, demes separated by ~ 1 km). The bPTP posterior probability of Plott Balsams separate from Great Smokies is zero.

Nuclear discovery analyses

Data were collected and phased for seven nuclear regions (Material S2, Supporting information), with a total aligned length of 4677 bp (Table 1). Nuclear PCR-amplified Sanger data match transcriptome data (i.e. PCR primers amplified the correct target gene region), and nuclear matrices include minimal missing data (7 nuclear gene matrices × 47 in-group individuals per matrix – 3 total missing sequences, all in the B2_D3_D4 matrix, see Material S2, Supporting information). As expected, each nuclear gene tree is topologically unique (Material S5, Supporting information). Despite this expected gene tree heterogeneity, there is clear phylogenetic signal in the nuclear data (Table 1), and several generalizable patterns are apparent. All nuclear gene trees include Plott Balsam alleles nested within a Smokies clade, with Plott Balsam sequences identical to certain Smokies sequences for all genes. The southwestern Plott Balsam plus Smokies clade is distinct from northeastern populations in most gene trees. Roan Mountain alleles form a clade in 4 of 7 gene trees, Whitetop Mountain alleles form a clade in 4 of 7 gene trees, and Grandfather Mountain alleles from a clade in 2 of 7 gene trees. The genetic relationship between Blackstock_North vs. all other Black Mountains demes (including Blackstock_Northeast) varies from gene to gene, but there is evidence for nuclear genetic divergence of Blackstock_North in several gene trees. Out-group sequences were only available for 3 nuclear genes, and in-group root placement using out-group information varies from gene to gene (Material S5, Supporting information).

Nuclear POFAD results using standardized distances generally correspond to those using nonstandardized distances (Fig. 4). Because POFAD networks do not include quantitative statistical criteria for delineating 'distinct lineages', defining such units is somewhat arbitrary. If geographic criteria are liberally used to recognize discrete genetic units (i.e. montane populations as discrete units), then all montane populations are distinct except for the combined Plott Balsams plus Smokies lineage, which internally shows minimal differentiation. Furthermore, there is POFAD evidence for a nuclear separation between Blackstock_North vs. all other Black Mountains demes, and the northernmost demes in the Black Mountains (Celo Knob, Winter Star) reveal evidence for limited nuclear differentiation.

STRUCTURE analyses recover five genetic clusters, with $K = 5$ including the largest ΔK (478) as estimated using the Evanno method. The maximal value of the log probability of the data given K *(L(K))* also peaks at $K = 5$ (−546.9). Genetic clusters at $K = 5$ correspond to Smokies plus Plott Balsams, Roan Mountain, Black Mountains (except for Blackstock_North), Blackstock_North and a single cluster that includes Grandfather Mountain plus Whitetop Mountain (Fig. 5A). Although $K = 5$ is optimal under the Evanno method, a more conservative $K = 4$ hypothesis was also considered, with a K *(L(K))* value (−568.9) most similar to the preferred K value. The $K = 4$ result includes a single Grandfather + Whitetop + Blackstock_North lineage and suggests mixed ancestry for many individuals (Fig. 5B).

Validation analyses

Marginal likelihood values for alternative species delimitation models assessed using BFD and combined nuclear plus mitochondrial data are reported in Material S6 (Supporting information). Marginal likelihoods favour a seven-species model (H_2), but are very similar to an alternative eight-species (H_1) model (Material S6, Supporting information). Both of these models imply different species on the Plott Balsams vs. the Smokies, despite the fact that we found no evidence for either mitochondrial or nuclear genetic divergence for these allopatric populations (Figs 3–5). Because of these results, and following our general preference for conservative hypotheses, we hereafter emphasize the nuclear-only validation results summarized below.

Bayes factor delimitation marginal likelihood values for alternative species delimitation models assessed using nuclear-only data are shown in Table 3. The favoured result is a seven-species model (H_3) that corresponds to the conservative mitochondrial bPTP hypothesis (Fig. 3), with internal species-level divergence both in the Black Mountains and on Grandfather Mountain, and a combined Plott Balsams plus Smokies lineage. However, this hypothesis does not differ from alternative seven-species (H_2) or six-species (H_4) models at a 'decisive' level of support (2lnBf <10, Table 3). The more conservative six-species model includes separate species on single mountaintops, but with two species in the Blacks, and a single species in the Smokies plus Plott Balsams.

Results of nuclear-only BPP analyses were largely congruent across runs, prior combinations and alternative (0,1) algorithms. Using the rjMCMC-only method, most of the overall posterior probability is split between seven (H_3)- and six (H_4)-species models (Table 4), with higher posterior probabilities for the six-species model under small population size priors. Also, for the seven-species (H_3) model, the nodal 'speciation probability' for separate Grandfather species is always low

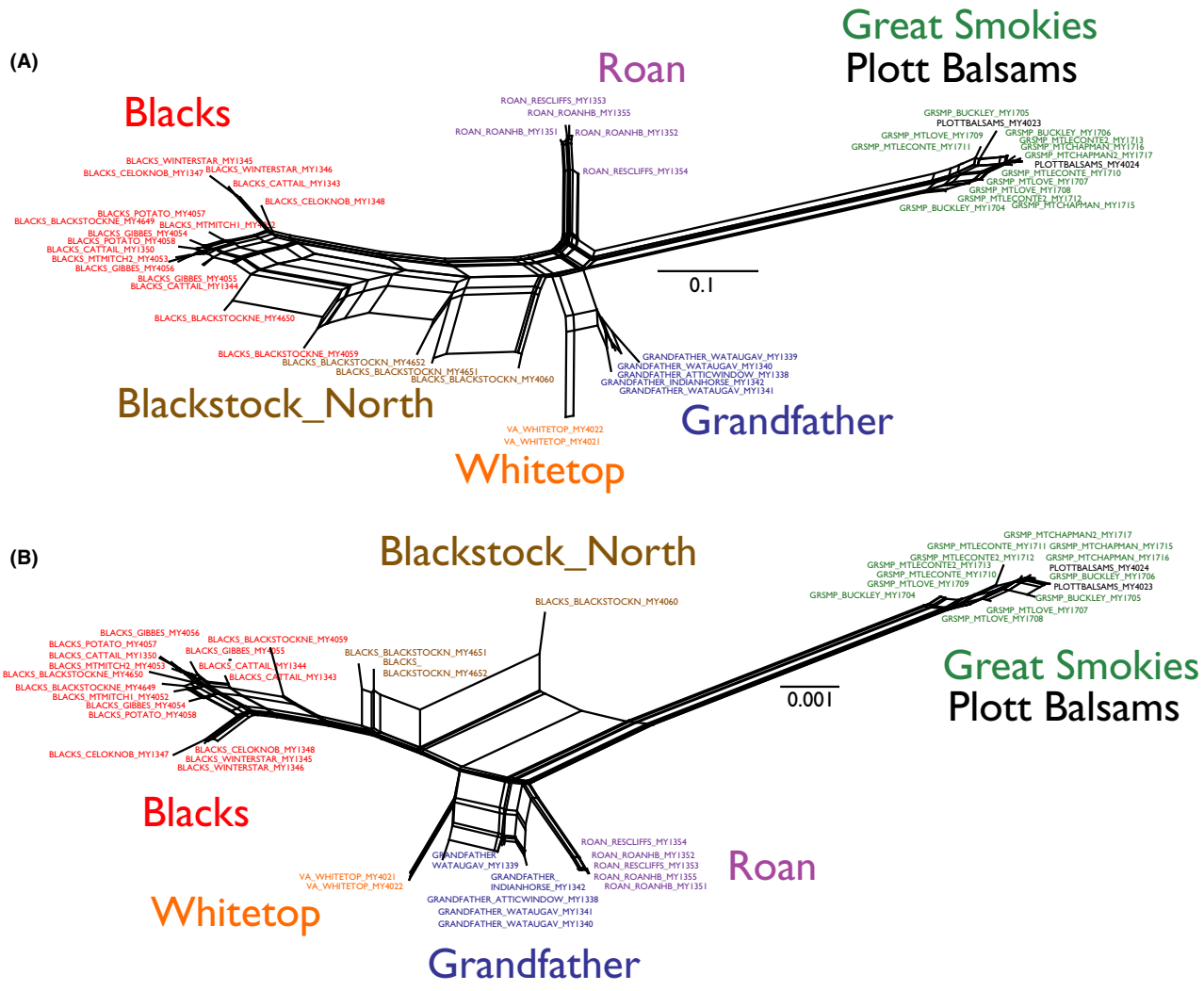

Fig. 4 NeighborNet networks reconstructed using POFAD distances derived from nuclear matrices. (A) Standardized distances. (B) Nonstandardized distances.

(PP < 0.60, Fig. 6A). Results using the NNI+rjMCMC method are generally congruent with those from the rjMCMC-only method, with overall posterior probabilities split between seven-species (H_3) and six-species (H_4) models, and higher posterior probabilities for the six-species model under small population size priors (Table 4).

The nuclear-only *BEAST cloudogram estimated for a preferred six-species (H_4) model (Fig. 6B) shows a primary well-supported split that separates the southwestern Smokies plus Plott Balsam clade from northeastern populations. Biogeographically, this primary division coincides with the low-elevation Asheville Basin barrier, a conspicuous biogeographic barrier in the southern Appalachians (e.g. Crespi *et al.* 2003, 2010; Weisrock & Larson 2006; Thomas & Hedin 2008). Within the northeastern complex, the Blacks plus Blackstock North node is strongly supported. Other nodes in the northeastern complex have low posterior probability values, indicating that even though the northeastern genetic lineages are distinct, phylogenetic relationships among these lineages remain unclear (Fig. 6B).

Discussion

Extreme population subdivision or cryptic speciation in Microhexura?

Two general patterns arise from consideration of revisionary studies of other diplurid mygalomorphs (Coyle 1984, 1988, 1995). First, many of the species delimited by Coyle exhibit intraspecific morphological variation, and second, different described diplurid species are typically *clearly different* in male palpal and/or mating

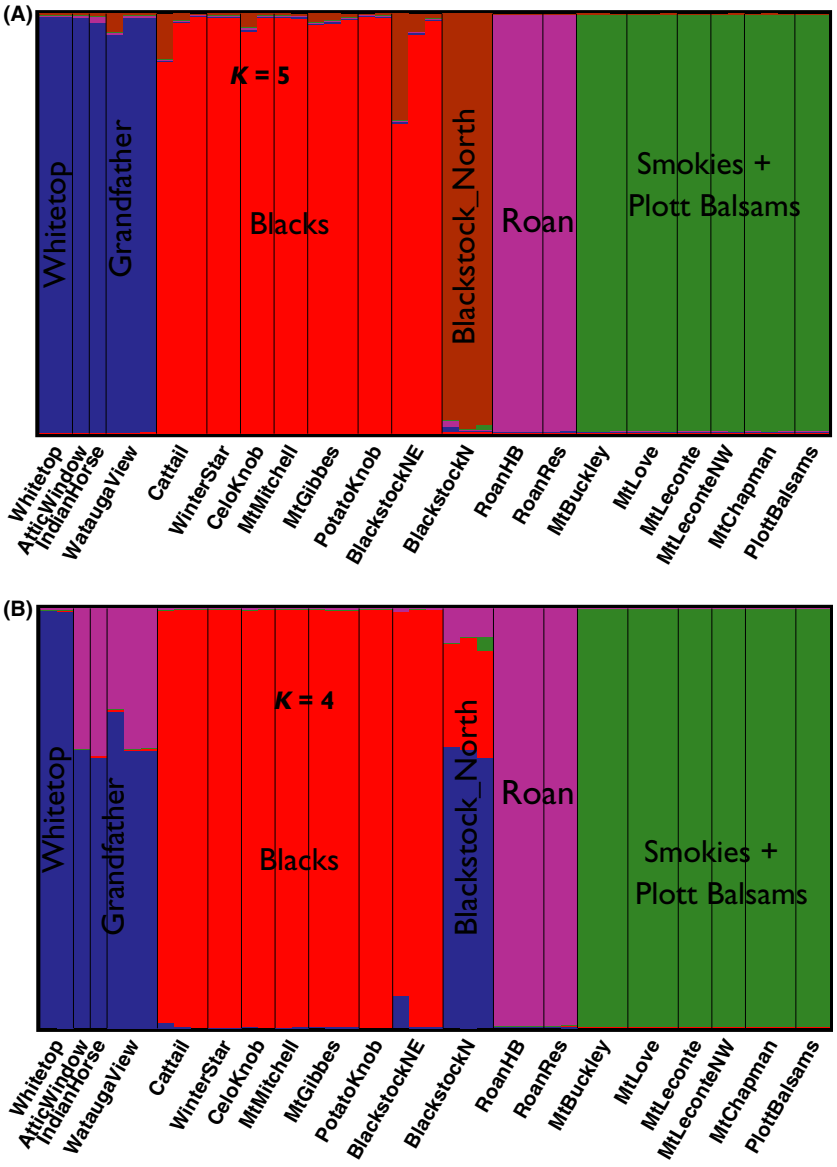

Fig. 5 STRUCTURE graphics (resulting from DISTRUCT) for (A) $K = 5$ and (B) $K = 4$. Each column represents a specimen, grouped by deme of origin. Different colours represent different genetic clusters (K); estimated membership coefficients are proportional to bar colour height.

spur morphology (Coyle 1984, 1988, 1995). We also note that diplurid congeners are typically strictly allopatric (e.g. within *Allothele, Indothele, Andethele*), which implies that allopatry *per se* does not promote genitalic stasis in these spiders. When Coyle revised *Microhexura,* he examined adults of both sexes from three montane populations of *M. montivaga*, including Grandfather Mountain, the Black Mountains (vic. Mt. Mitchell) and the Smokies (Coyle 1981). Coyle did not comment on either male or female geographic morphological variation in *M. montivaga*, but specifically discusses such variation in the sister taxon *M. idahoana* (Coyle 1981; figs 21–43). Although our survey of male *M. montivaga* specimens is consistent with the idea of morphological conservatism in this species, samples sizes are frustratingly small. This situation is challenging to overcome because adult male mygalomorphs are naturally rare, and because *M. montivaga* is federally endangered. We did not survey female morphology for two primary reasons – female morphology is generally less taxonomically informative in mygalomorphs, and in *Microhexur*a specifically, studies of Coyle (1981) indicate morphological conservatism. In referring to *M. montivaga* vs. *M. idahoana* from the Pacific Northwest, Coyle stated that 'if the two . . .species were not allopatric, it would be *difficult to identify the females to species*'. Our results point to a need for the collection and study of males for all genetically distinct populations, and for the examination of alternative character systems in both sexes (e.g. behaviour, chromosomes). However, we stress that the legal status and general rarity of this taxon will make such studies challenging.

Table 3 Marginal likelihood and Bayes factor values for alternative species delimitation hypotheses, *nuclear data only*. Species hypotheses as in Table 2

	Stepping-Stone		Path Sampling	
	ln (Marginal Likelihood)	2ln (Bayes Factor)	ln (Marginal Likelihood)	2ln (Bayes Factor)
H_1 (8 sp)	−7921.95	17.94	−7915.83	16.61
H_2 (7 sp)	−7917.76	9.57	−7912.55	10.04
H_3 (7 sp)	**−7912.98**	N/A	**−7907.53**	N/A
H_4 (6 sp)	−7916.60	7.24	−7910.79	6.53
H_5 (5 sp)	−7921.31	16.67	−7915.99	16.92
H_6 (5 sp)	−7930.29	34.62	−7925.11	35.17
H_7 (4 sp)	−7952.03	78.10	−7946.29	77.52
H_8 (2 sp)	−8102.38	378.80	−8093.81	372.57

Bold values represent collapsed lineages from a preceding hypothesis.

Patterns of genetic fragmentation in *M. montivaga* stand in stark contrast to strong morphological conservatism. Mitochondrial lineages are highly structured within and among montane populations (except for Smokies plus Plott Balsams), and northeast of the Asheville Basin, all mountaintops with multiple sampled rock outcrops show evidence for microgeographic genetic differentiation. For example, in the Black Mountains, specimens from all sampled rock outcrops carry unique COI haplotypes (Material S4, Supporting information). Evidence for genetic divergence also extends to the nuclear genome, and although we prefer a six-lineage hypothesis (Fig. 6), several analyses (e.g. BDF, BPP) recover seven distinct nuclear lineages that correspond exactly to seven divergent mitochondrial clades (Fig. 3). These congruent lineages are not strictly equivalent to montane populations because two distinct genetic lineages exist on Grandfather Mountain and in the Black Mountains, and the Plott Balsam population is not obviously genetically divergent from the Smokies population.

Most genetic studies of mygalomorphs have been conducted on sedentary burrow-dwelling spiders (e.g. trapdoor spiders) and reveal extensive population fragmentation (summarized in Bond *et al.* 2006; Satler *et al.* 2013). Our results for the small-bodied, web-building *Microhexura* suggest that such fragmentation might also characterize mygalomorphs other than trapdoor spiders (see also Arnedo & Ferrandez 2007; Beavis *et al.* 2011; Castalanelli *et al.* 2014; Hamilton *et al.* 2014; Leavitt *et al.* 2015). Additionally, while most prior genetic studies have relied upon mitochondrial data, our results suggest that strong population subdivision in *both* nuclear and mitochondrial genomes may typify mygalomorphs (see also Hedin *et al.* 2013; Satler *et al.* 2013; Leavitt *et al.* 2015). The modern availability of nuclear genomic

Table 4 Results of Bayesian Phylogenetics and Phylogeography analyses. Posterior probability values for six-species (H_4) and seven-species (H_2 and H_3) models shown. Six-species model preferred in all cases except those bolded

			# In-group Species Delimited (PP[a])			
	Priors		Run #, Algorithm Used			
	θ	τ_0	1,0	2,0	1,1	2,1
rjMCMC-only method						
Large pop, shallow div	G (1,10)	G (2,2000)	***H_3* (0.54)**	H_3 (0.47)	H_3 (0.47)	H_3 (0.36)
			H_4 (0.42)	H_4 (0.50)	H_4 (0.53)	H_4 (0.60)
Small pop, shallow div	G (2,2000)	G (2,2000)	H_3 (0.26)	H_3 (0.24)	H_3 (0.24)	H_3 (0.23)
			H_4 (0.70)	H_4 (0.73)	H_4 (0.74)	H_4 (0.75)
Large pop, deep div	G (1,10)	G (1,10)	H_3 (0.35)	H_3 (0.45)	H_3 (0.42)	H_3 (0.40)
			H_4 (0.47)	H_4 (0.55)	H_4 (0.58)	H_4 (0.60)
Small pop, inter div	G (2,2000)	G (2,1000)	H_3 (0.22)	H_3 (0.22)	H_3 (0.23)	H_3 (0.22)
			H_4 (0.75)	H_4 (0.74)	H_4 (0.76)	H_4 (0.76)
NNI+rjMCMC method						
Large pop, shallow div	G (1,10)	G (2,2000)	H_2 (0.11)			H_2 (0.07)
			***H_3* (0.40)**	H_3 (0.41)	H_3 (0.35)	H_3 (0.30)
			H_4 (0.38)	H_4 (0.57)	H_4 (0.65)	H_4 (0.50)
Small pop, shallow div	G (2,2000)	G (2,2000)	H_3 (0.24)	H_3 (0.21)	H_3 (0.22)	H_3 (0.22)
			H_4 (0.73)	H_4 (0.75)	H_4 (0.74)	H_4 (0.75)
Large pop, deep div	G (1,10)	G (1,10)			H_2 (0.27)	
			H_3 (0.34)	H_3 (0.36)	H_3 (0.22)	***H_3* (0.50)**
			H_4 (0.61)	H_4 (0.63)	H_4 (0.32)	H_4 (0.49)
Small pop, inter div	G (2,2000)	G (2,1000)	H_3 (0.18)	H_3 (0.24)	H_3 (0.19)	H_3 (0.19)
			H_4 (0.80)	H_4 (0.73)	H_4 (0.78)	H_4 (0.79)

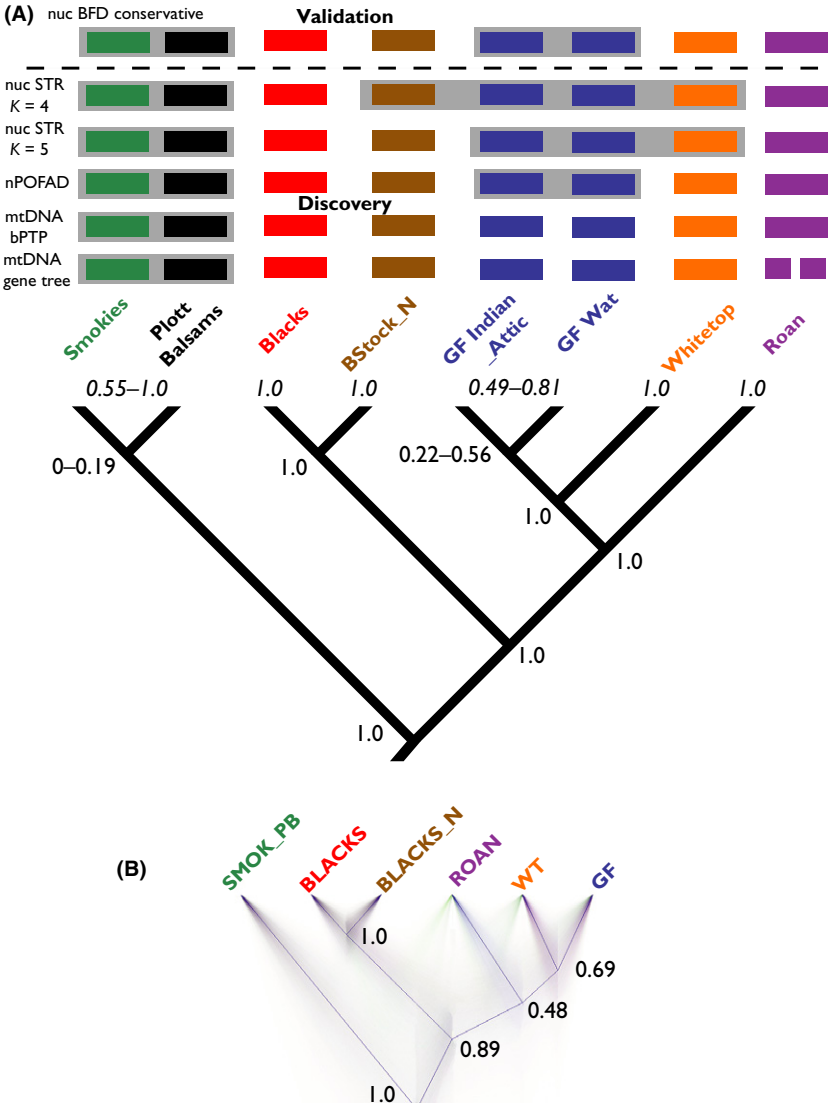

Fig. 6 (A) Bayesian Phylogenetics and Phylogeography guide tree with posterior probability values for species delimitations, summarized across runs, priors and algorithms (0, 1). For the rjMCMC-only method, these values correspond to 'speciation probabilities' at nodes (values NOT italicized). For the NNI+rjMCMC method (italicized values), posterior probabilities are reported for the presence of *single distinct tip lineages* (e.g. PP values for Plott Balsams plus Smokies collapsed as single lineage range from 0.55 to 1.0). (B) Nuclear-only *BEAST species tree estimated for hypothesis four (six species), with posterior probability values at nodes. The posterior distribution of species trees was visualized using DENSITREE v2.0.1 (Bouckaert 2010); the 'root canal' option was chosen to highlight the topology of the MCC tree (in blue), and the next two most frequent topologies are drawn with red and green branches, respectively.

resources for nonmodel taxa will allow this hypothesis to be more broadly tested in the coming years.

Bernardo (2011) defined cryptic species as 'highly evolutionary divergent lineages that share a very similar morphology and which are therefore difficult to distinguish based upon morphological discontinuities'. Appalachian *Microhexura* might include cryptic species, and such a finding in a mygalomorph taxon would certainly not be unexpected (e.g. Bond *et al.* 2001; Cooper *et al.* 2011; Hedin *et al.* 2013; Satler *et al.* 2013; Castalanelli *et al.* 2014; Opatova & Arnedo 2014; Leavitt *et al.* 2015). Cryptic species often share biological features beyond morphological conservatism. For example, microhabitat specialization is also common, with stabilizing selection thus promoting both ecological niche and morphological conservatism (reviewed in Keith & Hedin 2012). Microhabitat specialization clearly applies in *M. montivaga*, as Coyle (2009) found essentially all specimens associated with bryophyte mats on, or at the base of, rock outcrops in high-elevation spruce-fir forest. Searches in other microhabitats or substrates, even in appropriate forests, did not reveal spiders. Ultimately, the biology of cryptic species *narrows* the types of data available to investigate species boundaries. It is for these exact reasons that genetic data have been so valued as a means to discover and objectively delimit cryptic species (Zhang *et al.* 2011; Fujita *et al.* 2012; Niemiller *et al.* 2012).

Multispecies coalescent and the challenge of naturally fragmented systems

Multispecies coalescent methods for genetic species delimitation have been highlighted as statistically rigorous, objective and repeatable (Fujita *et al.* 2012; Camargo & Sites 2013; Carstens *et al.* 2013). However, multispecies

coalescent methods (and genetics-only methods more generally) can fail under certain speciation scenarios, for example if speciation is very recent, or involves strong selection in a small fraction of the genome, with gene flow over the rest of the genome (Fujita *et al.* 2012; Sousa & Hey 2013). These are largely 'too much gene flow' scenarios. As such, authors have recently developed methods that formally integrate multiple lines of evidence in a multispecies coalescent framework (e.g. iBPP, Solís-Lemus *et al.* 2015), consistent with principles of integrative taxonomy (Dayrat 2005; Schlick-Steiner *et al.* 2010; Derkarabetian & Hedin 2014). However, because of the 'data narrowing' problem discussed above, speciation biologists working on cryptic species often are forced to rely on genetics-only delimitations. If such delimitations are biased to mistake population structure for species-level divergence, then 'too little gene flow' also becomes a problem area for multispecies coalescent methods (O'Meara 2010; Niemiller *et al.* 2012; Barley *et al.* 2013; McKay *et al.* 2013).

Hey (2009) argued that many genetic species delimitation approaches are biased towards oversplitting because they confound population structure with speciation. Evidence for this can be seen in the single-locus results presented here (e.g. mean value of 11 bPTP species), and other studies have convincingly shown oversplitting in single-locus GMYC analyses (e.g. Lohse 2009; Keith & Hedin 2012; Miralles & Vences 2013; Satler *et al.* 2013). Multispecies coalescent methods are not immune to oversplitting, for reasons well summarized by Niemiller *et al.* (2012). Low gene flow impacts the coalescent process, as genetic lineages sampled from the same geographic location are more likely to coalesce locally than with lineages from other locations, resulting in more congruence across independent gene trees than is expected under the neutral coalescent (Kuo & Avise 2005). To assess the possibility of false positives (oversplitting), extensive simulations have been conducted for guided BPP (Zhang *et al.* 2011). The most relevant simulations included a linear stepping-stone model with four populations of equal size, with symmetrical migration at variable levels (number of migrants per generation $M = 0.001–100$) constrained to occur among adjacent populations. Under these simulation conditions, two species were inferred (at posterior probability >0.80) only when M values were <1 (using 10 loci). Because the authors essentially equate such low M values with 'speciation' (see below), the authors claim that 'Bayesian inference may be quite robust to complex population structures'. Alternatively, BPP has been suggested to potentially oversplit in complex, low-geneflow empirical systems, including trapdoor spiders (Satler *et al.* 2013), birds (McKay *et al.* (2013), lizards (Barley *et al.* 2013; Miralles & Vences 2013) and plants (Carstens & Satler 2013). These suggestions follow from comparison of BPP results to other lines of evidence and with results from other genetic analyses. False-positive rates have also been assessed via simulation for BFD (Grummer *et al.* 2014), and although oversplitting rates were found to be negligible, these simulations did not explicitly include internal population structure as in Zhang *et al.* (2011). Because BFD is founded on the multispecies coalescent model of *BEAST, which again assumes the neutral coalescent, poor model fit resulting from population structure (see Reid *et al.* 2014) could potentially impact BFD results.

While it remains unclear how many species exist in the *Microhexura montivaga* 'complex', this taxon clearly illustrates an empirical issue that requires additional methodological attention. The multispecies coalescent BPP method has been claimed to delimit biological species, but Zhang *et al.* (2011) acknowledge that 'the method does not take into account whether the ... low migration rate is due to geographical barriers or to intrinsic reproductive isolation' and that 'two allopatric populations that diverge due to neutral drift *without establishment of reproductive barriers* may be inferred to be two species...'. Of course, this is not a problem with the method *per se*, as the method effectively recognizes a low-geneflow threshold (i.e. $M = Nm \ll 1$). In many empirical systems, measuring highly reduced gene flow, in the light of additional data, would indeed provide strong evidence for species status. However, in fragmented systems with naturally constrained gene flow, isolated populations and species are potentially equivalent under this model. Naturally fragmented systems with viscous gene flow are common in nature, and modern access to genomic-scale data sets makes population differentiation ever easier to measure. Unless biologists are willing to recognize all genetically divergent populations as species, there is a clear need to allow and incorporate population structure as a parameter in multispecies coalescent methods (Camargo *et al.* 2012; Niemiller *et al.* 2012; Camargo & Sites 2013; Carstens *et al.* 2013; Leaché *et al.* 2014).

Regional biogeography

Both paleobotanical and genetic data indicate that the current sky island distribution of spruce-fir forests in the southern Appalachians is an evolutionarily recent phenomenon (Delcourt & Delcourt 1984; Potter *et al.* 2008). At the last glacial maximum (LGM) and at multiple glacial maxima earlier in the Pleistocene, these forests existed more expansively and continuously at lower elevations (up to 1000 metres lower), with mostly treeless tundra at the highest elevations (Delcourt & Delcourt

1984). Only recently (8000–4000 years ago) have these forests become restricted to the highest southern Appalachian peaks. Animals restricted to southern Appalachian sky islands, presuming ecological niche conservation through time, are expected to show similar 'recent fragmentation' biogeographic patterns. Although many modern studies have considered regional biogeography (e.g. Weisrock & Larson 2006; Thomas & Hedin 2008; Hedin & Thomas 2010; Keith & Hedin 2012), few have focused on high-elevation spruce-fir specialists. Two notable animal exceptions include pygmy salamanders (*Desmognathus wrighti*, Crespi *et al.* 2003, 2010) and smoky shrews (*Sorex fumeus*, Sipe & Browne 2004). In *Sorex fumeus*, mitochondrial data indicate reduced gene flow across the low-elevation Asheville Basin barrier and between individual northeastern montane populations (e.g. Blacks, Roan Mtn., Grandfather Mtn., Whitetop Mtn.). Although formal dating analyses were not conducted, Sipe & Browne (2004) hypothesized a post-LGM fragmentation model for this taxon.

Genetic patterns in *Desmognathus* are geographically similar to both *Sorex* and *Microhexura*, with populations on opposite sides of the Asheville Basin, and genetically distinct northeastern populations restricted to the Black, Roan, Grandfather and Virginia Balsam mountains (Crespi *et al.* 2003). In this taxon, independent molecular clock analyses of both mitochondrial and nuclear allozyme data suggest Pliocene ages for the primary southwest–northeast Asheville Basin divergence, and more recent studies of ecology, morphology and genetics support the elevation of a distinct species (*D. organi*) northeast of the Asheville Basin (Crespi *et al.* 2010). Genetic data indicate that *D. organi* populations are more isolated and smaller in size compared to southwestern *D. wrighti* populations, and although divergence time estimates for these populations include wide confidence intervals, these estimates appear inconsistent with a post-LGM fragmentation model (mean of 1 million years; Crespi *et al.* 2003). We hypothesize that *Microhexura* divergence times follow a Pliocene/Early Pleistocene model as found in pygmy salamanders. An intriguing implication of this model is that *Microhexura* populations lack complete historical niche conservation, with possible recent (e.g. LGM) habitat occupation at elevations above spruce-fir forests, despite the fact that all modern populations are restricted to these forests (Coyle 2009). We intend to explore these hypotheses further in a separate manuscript.

Conservation recommendations for a federally endangered species complex

The data presented here have important conservation implications, showing that the federally endangered species *M. montivaga* is actually a complex of mostly allopatric, highly distinctive genetic lineages (Fig. 6). Most of these lineages are microendemic, restricted to a single mountain range or even a single rock outcrop. Even if these lineages do not constitute cryptic species, the level of evolutionary independence measured makes them clear candidates for lineage-specific conservation decisions (i.e. as 'management units' or 'evolutionary significant units'). Conservation recommendations for the preferred six-lineage hypothesis are made below.

Virginia Balsams (Whitetop) – This geographically isolated population carries unique genetic diversity in both mitochondrial and nuclear genomes (Fig. 6). Coyle (2009) estimated 13 hectares of suitable habitat on Whitetop Mountain (in pure Spruce forest) and found very low relative spider abundances. Pine Mountain, also part of the Virginia Balsams, includes a spider population that was not sampled in this study. Continued close monitoring is needed at both locations, as loss of the Virginia Balsams population would constitute a considerable loss of unique genetic diversity. Grandfather Mountain – Multiple rock outcrop demes exist on Grandfather Mountain (Coyle 2009). The two demes sampled constitute divergent mitochondrial lineages and show weak evidence for nuclear differentiation in BFD and BPP analyses (Fig. 6). Sampling of additional rock outcrop demes might uncover additional genetic lineages on Grandfather Mountain, and management to maintain all genetically diverse demes within this range should be a priority. Roan Mountain – This allopatric population carries unique genetic diversity in both mitochondrial and nuclear genomes (Fig. 6). Multiple rock outcrop demes exist on Roan Mountain (Coyle 2009); the two sampled demes differ slightly for mitochondrial genes, but are similar in their nuclear genomes. Black Mountains – Multiple rock outcrop demes exist in the Blacks (Coyle 2009). Most of these demes carry unique mitochondrial lineages, consistent with microgeographic genetic differentiation along the ridgeline of the Blacks (Material S4, Supporting information). Management to maintain all genetically diverse demes within this range should be a priority. Blackstock_North – This distinct genetic lineage (Fig. 6) is not strictly geographically isolated, but instead occupies a single rock outcrop at the southwestern edge of the Black Mountains system (Figs 1 and 3). Because the Blackstock_North genetic lineage is only found on a single rock outcrop, this deme should be monitored very closely, and additional rock outcrops in the vicinity should be sampled for this lineage. Again, loss of this single deme would constitute a considerable loss of unique genetic diversity. Great Smokies plus Plott Balsams – This distinct genetic lineage (Fig. 6) occurs southwest of the low-elevation Asheville Basin barrier

and is therefore arguably the most geographically isolated of all lineages. Multiple rock outcrop demes exist in the Smokies (Coyle 2009), but the sampled demes are relatively genetically homogeneous. The geographically isolated Plott Balsams population is essentially genetically identical to the Smokies population. From a genetics perspective, translocation of specimens from the Great Smokies to the Plott Balsams would be justified, which may be required given the very low relative spider abundances and limited habitat (estimated 3 hectares) in the Plott Balsams (Coyle 2009).

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M.H. implemented the study design, directed data collection, conducted analyses and helped in writing the manuscript. D.C. conducted analyses and contributed to writing the manuscript. F.C. collected specimens, assisted in study design and contributed to writing the manuscript.

Data accessibility

Unphased DNA sequences: GenBank Accession nos KR607601–KR607977.

Phased DNA sequence alignments, RAxML.tre files, morphology JPG files, POFAD matrices, STRUCTURE files, transcriptome assemblies: Dryad doi:10.5061/dryad.r87k3.

Illumina data submitted to NCBI Short Read Archive – Mt. Gibbes (SRX652506), Mt. Buckley (SRX1004559).

Marker development information, voucher data, PCR conditions and nuclear gene trees uploaded as online Supporting Information.

Supporting information

Additional supporting information may be found in the online version of this article.

Material S1. Development of Nuclear Markers.

Material S2. Voucher number, location and GenBank information.

Material S3. Primers and gene annotations

Material S4. Figure illustrating microgeographic mitochondrial structuring in the Black Mountains.

Material S5. RAxML maximum likelihood nuclear gene trees.

Material S6. Marginal likelihood and Bayes Factor values for alternative species delimitation hypotheses, based on *combined mitochondrial and nuclear data.*