Received: 30 August 2017 | Accepted: 25 January 2018

DOI: 10.1111/zsc.12281

ORIGINAL ARTICLE

WILEY Zoologica Scripta

Disintegrating over space and time: Paraphyly and species delimitation in the Wehrle's Salamander complex

Shawn R. Kuchta[1] | **Ashley D. Brown**[1] | **Richard Highton**[2]

[1]Department of Biological Sciences, Ohio Center for Ecology and Evolutionary Studies, Ohio University, Athens, OH, USA

[2]Department of Biology, University of Maryland, College Park, MD, USA

Correspondence
Shawn R. Kuchta, Department of Biological Sciences, Ohio Center for Ecology and Evolutionary Studies, Ohio University, Athens, OH, USA.
Email: kuchta@ohio.edu

Accurate species delimitation is critical for biodiversity studies. However, species complexes characterized by introgression, high levels of population structure and subtle phenotypic differentiation can be challenging to delimit. Here, we report on a molecular systematic investigation of the woodland salamanders *Plethodon wehrlei* and *Plethodon punctatus,* which traditionally have been placed in the *Plethodon wehrlei* species group. To quantify patterns of genetic variation, we collected genetic samples from throughout the range of both species, including 22 individuals from nine populations of *P. punctatus*, and 60 individuals from 26 populations of *P. wehrlei*. From these samples, we sequenced three mtDNA loci (5596 base pairs) and five nuclear loci (3377 base pairs). We inferred time-calibrated gene trees and species trees using BEAST 2.4.6, and we delimited putative species using a Bayesian implementation of the general mixed Yule-coalescent model (bGMYC) and STRUCTURE. Finally, we validated putative species using the multispecies coalescent as implemented in Bayesian Phylogenetics and Phylogeography (BPP). We found substantial phylogeographic diversity in *P. wehrlei*, including multiple geographically cohesive clades and an inferred mitochondrial common ancestor at 11.5 myr (95% HPD: 9.6–13.6 myr) that separated populations formerly assigned to *P. dixi* from all other populations. We also found that *P. punctatus* is deeply nested within *P. wehrlei*, rendering the latter paraphyletic. After discussing the challenges faced by modern species delimitation methods, we recommend retaining *P. punctatus* because it is ecologically and phenotypically distinct. We further recommend that *P. dixi* be recognized as a valid species.

1 | INTRODUCTION

Species are a fundamental category of biological organization, as important for biodiversity studies as the cell is to lower levels of biological integration (Mayr, 1982; de Queiroz, 2005). Nonetheless, species formation is a lengthy and multifaceted process, including the formation of incompletely separated lineages, and a common challenge faced by systematists working on species complexes is how to identify species boundaries given high levels of population structure and discordance among data sets (Kuchta & Wake, 2016; Lumbsch & Leavitt, 2011; Niemiller, Near, & Fitzpatrick, 2011; Tilley et al., 2013). Recent advances in genomics and analytical theory have led to the development of computational methods of identifying putative genetic lineages, which have the key advantage of providing an objective means of species delimitation (Fujita, Leaché, Burbrink, McGuire, & Moritz, 2012; Pritchard, Stephens, & Donnelly, 2000; Rannala & Yang, 2003; Reid & Carstens, 2012; Yang & Rannala, 2014). In particular, models that merge population genetic processes with phylogenetic processes, such as the multispecies coalescent, have resulted in a paradigm shift

in systematics because of their explicit focus on species trees, with gene trees providing estimates of divergence times and effective population sizes in the species tree. By integrating over gene trees, the likelihood of a species delimitation, where the delimitation model is a species tree with nodes signifying speciation events, can also be estimated (Rannala & Yang, 2003; Yang & Rannala, 2014). Whereas in the recent past species delimitation using molecular markers required reciprocal monophyly or fixed differences to diagnose species, models that incorporate the multispecies coalescent can identify evolutionary lineages in the absence of monophyletic gene trees. As sophisticated as these methods are, however, they are simple relative to the complexities of species formation, and complications such as introgression, isolation by distance and population structure present serious challenges that can badly mislead a delimitation (Carstens, Pelletier, Reid, & Satler, 2013; Kuchta, Brown, Converse, & Highton, 2016; Sukumaran & Knowles, 2017).

Salamanders in the family Plethodontidae provide challenging conditions for species delimitation (Tilley et al., 2013). In particular, many terrestrial species are characterized by non-adaptive radiation, or species formation by fragmentation, whereby a species expands its range, then slowly disintegrates into a complex of divergent allopatric or parapatric units (Rundell & Price, 2009). These complexes are typically characterized by high levels of phenotypic and ecological conservatism (Kozak & Wiens, 2010; Wake, Roth, & Wake, 1983), as well as isolation by distance and extreme genetic structure (Kuchta, Parks, & Wake, 2009; Reilly, Corl, & Wake, 2015; Rovito, Parra-Olea, Vásquez-Almazán, Luna-Reyes, & Wake, 2012). Consequently, species boundaries can be contentious. For example, whereas Martínez-Solano, Jockusch, and Wake (2007) regarded *Batrachoseps attenuatus* as a single species, Highton (2014) has argued the complex includes 39 distinct species (see also Highton, 1998; Wake & Schneider, 1998).

Here, we report on a molecular systematic investigation of a complex of salamanders in the genus *Plethodon* (family Plethodontidae). In this genus, largely as a result of genetic studies, the number of species has increased from 16 (Highton, 1962) to 55 (AmphibiaWeb: http://amphibiaweb.org), including many cryptic taxa (Camp & Wooten, 2016; Highton, 1995). Traditionally, *Plethodon* in the eastern United States has been divided into four species groups (Highton, 1962). Three of these groups have been the subject of detailed, large-scale allozyme investigations, which have clarified species boundaries and led to the recognition of many new taxa (reviewed in Highton et al., 2012). The smallest species group, which has not been systematically evaluated using molecular markers, is the *Plethodon wehrlei* group (Highton & Larson, 1979), which at present includes two species: Wehrle's Salamander (*P. wehrlei* Fowler and Dunn 1917) and the Cow Knob Salamander (*Plethodon punctatus* Highton 1972). Unlike many amphibians, *Plethodon* are usually completely terrestrial: there is no aquatic larval stage, and they do not migrate to breeding sites. Moreover, home ranges are small, on the order of a few square metres (Hall & Stafford, 1972; Kleeberger & Werner, 1982). Small home ranges, territoriality and limited vagility promote the accumulation of genetic differences among populations, including high levels of phylogeographic structure (Kuchta, Parks, Mueller, & Wake, 2009; Martínez-Solano et al., 2007; Tilley et al., 2013).

To examine patterns of genetic variation and reveal any cryptic species diversity in the *P. wehrlei* group, we sequenced three mtDNA loci and five nuclear loci. To delimit putative species, we analysed our mtDNA data using a Bayesian implementation of the general mixed Yule-coalescent (bGMYC), and we analysed our nuclear data using the Bayesian clustering program STRUCTURE. In addition, we made a species tree from our nuclear data and tested our delimitations using a method that takes advantage of the multispecies coalescent (BPP) and thus accounts for incomplete lineage sorting. After discussing the limitations of coalescent methods when delimiting species in complexes characterized by high levels of population structure, we review possible taxonomic solutions and recommend *P. dixi* be recognized as a distinct species.

2 | MATERIALS AND METHODS

2.1 | Sampling and laboratory techniques

Blood samples and tail tips were collected from 22 individuals from nine populations of *P. punctatus*, and 60 individuals from 26 populations of *P. wehrlei* (Figure 1). Total genomic DNA was extracted using Qiagen DNeasy Blood and Tissue Kits (Qiagen Corp., Valencia, CA). A total of 8973 base pairs (bp) of DNA were sequenced. Mitochondrial DNA sequence data were collected from the cytochrome *b* gene (Cyt-*b*; 1113 bp), NADH dehydrogenase 2 (ND2; 1041 bp), and most of $tRNA^{trp}$ (65 bp). DNA sequence data were also collected for five nuclear loci: the nuclear exon recombination activating gene 1 (RAG-1; 1168 bp), and the nuclear introns interleukin enhancer-binding factor 3 (ILF3; 254 bp), myosin light chain 2 (MLC2A; 422 bp), glyceraldehyde-3-phosphate dehydrogenase (GAPD; 588 bp) and β-fibrinogen intron 7 (BFI; 945 bp) (Kuchta, Brown, et al., 2016). Most samples were sequenced in both the forward and reverse direction. Primers are provided in Table 1, and sampling details are provided in the Supplementary Materials (Table S1).

Electropherograms for all sequences were viewed using Geneious v7.1 (Kearse et al., 2012), and ambiguous base calls were manually corrected. The phase of heterozygous genotypes was estimated using PHASE v2.1.1 (Stephens, Smith, & Donnelly, 2001). PHASE was run for 1000 iterations, with a thinning interval of two steps and a burn-in of

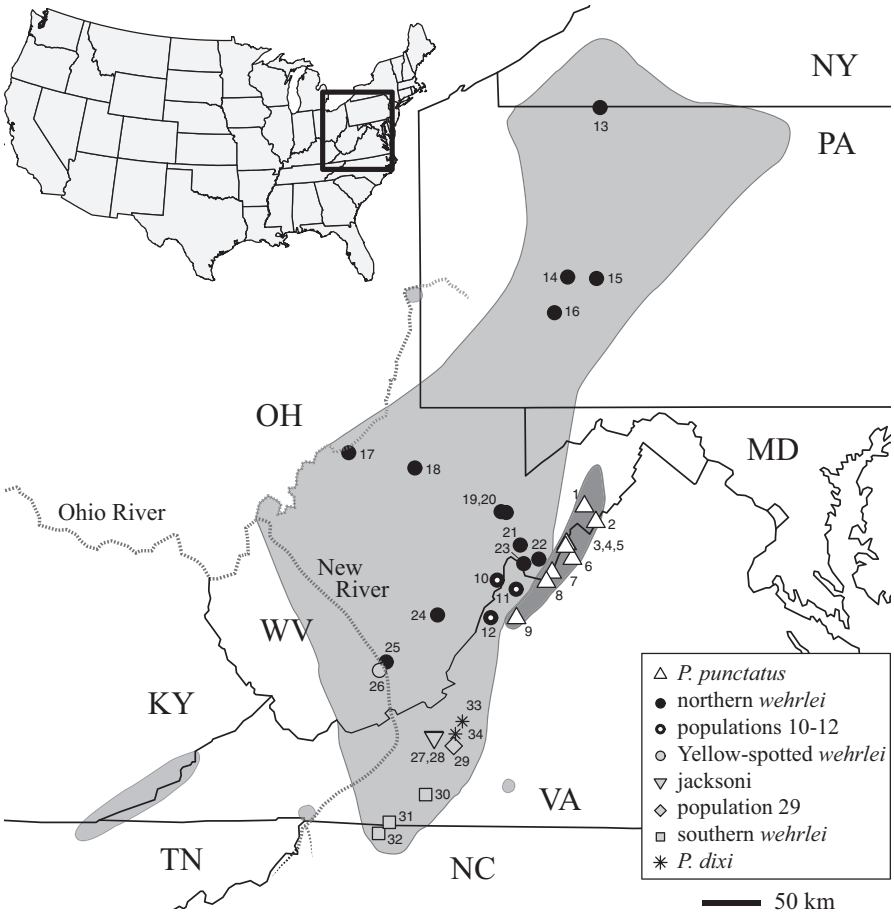

FIGURE 1 Map of the range of *Plethodon wehrlei* and *Plethodon punctatus*. Sample localities are numbered, and match Figure 2 and Table S1. Symbols identify the mtDNA clade of individuals in that population (Figure 2). Dashed lines illustrate the New River and the Ohio River

TABLE 1 PCR primer sequences for loci used in the study

Locus	Primer Name	Nucleotide Sequence 5′ – 3′	Reference
Cyt-*b*	Pglut-F1b	GGTCTGAAAAACCAATGTTGTATTC	Wiens, Engstrom, & Chippindale (2006)
	PThr-R2b	GCCCCCAATTTTGGYTTACAAG	Wiens et al. (2006)
ND2, tRNAtrp	ND2-L4437	AAGCTTTCGGGCCCATACC	Macey, Larson, Ananjeva, Fang, & Papenfuss (1997)
	KND2-R2	AAAGTGTTTGAGTTGCATTCA	Kozak, Weisrock, & Larson (2006)
RAG-1	Rag-1b-F	CTGTCTGGTCTGGTGCAGTCG	Kuchta, Brown, et al. (2016)
	Rag-1a-R	ATTCCCTTCACTCGCCCAAGC	Kuchta, Brown, et al. (2016)
BFI-internal	BFXF	CAGYACTTTYGAYAGAGACAAYGATGG	Sequeira, Ferrand, & Harris (2006)
	BFXR	TTGTACCACCAKCCACCACCRTCTTC	Sequeira et al. (2006)
BFI-external	FIBX7	GGAGANAACAGNACNATGACAATNCAC	Sequeira et al. (2006)
	FIBX8	ATCTNCCATTAGGNTTGGCTGCATGGC	Sequeira et al. (2006)
GAPD	GAPD-F	ACCTTTAATGCGGGTGCTGGCATTGC	Fisher-Reid & Wiens (2011)
	GAPD-R	CATCAAGTCCACAACACGGTTGCTGTA	Fisher-Reid & Wiens (2011)
ILF3	ILF3-F	GATTTCAATCCATTTGCTCTTGC	Fisher-Reid & Wiens (2011)
	ILF3-R	AGGATAAGCCCACCGTTACACTATT	Fisher-Reid & Wiens (2011)
MLC2A	McL2a-F5	TCCAATGTCTTTGCCATGTTCG	Kuchta, Brown, et al. (2016)
	MCL2a-R2	AGTCATCCTTGTCTTTGGCTCC	Kuchta, Brown, et al. (2016)

100 iterations. PCR products exhibiting length heterogeneity due to the presence of indels were phased using Champuru v.1.0 (Flot, 2007). Intragenic recombination was tested for using the difference in sum-of-squares (DSS) test implemented in TOPALi (Milne et al., 2008), including a 10 base pair increment, a window size of 100, and 500 parametric bootstraps. Recombination was not detected at any locus.

Genetic diversity indices were used to compare patterns of differentiation among mtDNA clades. Average uncorrected genetic distances within and between clades were calculated using PAUP* 4.0 b10 (Swofford, 2003). Diversity indices were calculated using DNAsp v.5.10.1 and included the number of segregating sites (*S*), haplotype diversity (*h*), sequence diversity (κ), and nucleotide diversity (π) (Librado & Rozas, 2009).

2.2 | Phylogenetic analyses

Time-calibrated gene trees were inferred using BEAST v.2.4.6 (Drummond & Bouckaert, 2015), including concatenated mitochondrial loci. In BEAST, tree priors assume that all clades are sampled with consistent intensity, which is problematic in phylogeographic analyses because in-group taxa are heavily sampled, but out-group taxa are not. Moreover, a different tree prior often better fits the in-group versus the in-group+out-group taxa. Thus, out-group taxa were not used; rather, BEAST sampled the root position along with all other nodes in the tree (Drummond & Bouckaert, 2015). Analyses of mtDNA from throughout *Plethodon* recovered our in-group as monophyletic (Highton et al., 2012). For nuclear loci, both alleles were included, and identical haplotypes were retained (Drummond & Bouckaert, 2015). For all loci, models of evolution were inferred using jModelTest 2.1.10 (Darriba, Taboada, Doallo, & Posada, 2012), with the best model selected using AICc (Table 2). Multiple preliminary analyses of mtDNA were used to explore a diversity of priors in Tracer v1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). Runs with an exponential tree prior showed a marginal posterior distribution in population growth rate that included zero, indicating a constant population size prior cannot be rejected. In addition, in runs with a relaxed lognormal clock, the coefficients of variation in the clock rates abutted zero, indicating the data were compatible with a strict clock model. Thus, for all gene trees, a constant population size coalescent tree prior and a strict clock model were used. Rates of evolution for each locus were taken from table 3 in Kuchta, Brown, et al. (2016), assuming a plethodontid crown group age of 66 myr (see Shen et al., 2015). Clock models for each locus included lognormal distributions with medians and 95% confidence intervals from table 3 in Kuchta, Brown et al. (2016). In the BEAST analyses, the length of the Markov chain Monte Carlo (MCMC) run was 50 million generations with parameters sampled every 1000 generations, trees sampled every 5000 generations, and a conservative burn-in of 20%. Effective samples sizes (ESS) in all runs were >200. The maximum clade credibility tree with common ancestor node heights was obtained using TreeAnnotator 2.4.6 (Drummond & Bouckaert, 2015).

For comparison with the mtDNA phylogeny, which might not reflect population-level history due to incomplete lineage sorting, natural selection, introgression, and other factors (Ballard & Whitlock, 2004; Kuchta & Tan, 2005), species tree analyses were carried out using our nuclear data in *BEAST v2.4.6 (Drummond & Bouckaert, 2015). Species were defined based on the mtDNA, bGMYC and STRUCTURE analyses. All gene trees were unlinked. Otherwise, analyses in *BEAST were set up as described above, but with an MCMC run of 100 million generations.

2.3 | Delimiting putative historical units

A Bayesian implementation of the general mixed Yule-coalescent model (bGMYC) was first used to assign individuals to species and develop a preliminary set of hypothesized species limits (Reid & Carstens, 2012). Given a single gene tree, the bGMYC infers the transition between population-level (coalescent) processes and species-level (Yule model) processes. bGMYC analyses were run in R v.3.2.4 (R Core Team, 2017) in the eponymous R package (Reid & Carstens, 2012). For the analyses, 500 trees were randomly sampled from the posterior distribution of the BEAST analysis of the mtDNA data set. As bGMYC is prone to over-split trees made using identical alleles (Reid & Carstens, 2012), only the most complete sequences from a set of otherwise identical haplotypes were used. The following options were employed as follows: MCMC = 100,000, burn-in = 50,000, thinning = 200, default scale parameters, default values on the Yule and coalescent rate change priors, and upper and lower bounds on the threshold parameter of 1 and 32, respectively, where 32 was the number of distinct haplotypes in our mtDNA tree.

To complement the bGMYC analysis of our mtDNA data, STRUCTURE v.2.3 was used to infer the number of genotypic clusters in the nuclear data (Pritchard et al., 2000). STRUCTURE assigns individuals to populations by maximizing conformity to Hardy–Weinberg equilibrium while simultaneously minimizing linkage disequilibrium. Unique alleles at each locus were identified using the *haplotype* function in the *haplotypes* package in R (Aktas, 2017), and these alleles (not sequences) were used as input in STRUCTURE. STRUCTURE was run from $K = 1$ to $K = 15$ populations, with each value of K replicated ten times with randomly generated starting seeds. Each MCMC run consisted of 500,000 iterations, with the first 100,000

TABLE 2 Summary of sampling and genetic diversity for mtDNA (cytochrome b, ND2, tRNA-trp) and average values across all five nuclear loci (BFI, GAPD, ILF3, MLC2A, RAG-1). Calculations for individual loci, as well as models of evolution used in phylogenetic analyses for each locus, are available in the supplementary materials (Table S10)

Locus	Group	Length[a]	Sequences[a]	Pops	Sites	Haplotypes	*h*	π	κ
mtDNA	All clades	1858	59	35	354	29	0.94	0.0399	74.15
	P. punctatus	2004	15	9	18	6	0.57	0.0024	4.82
	Populations 10-12	2044	3	3	2	3	1.00	0.0007	1.33
	Yellow-spotted *wehrlei*	2116	4	2	1	2	0.50	0.0002	0.50
	jacksoni	2192	3	2	2	3	1.00	0.0006	1.33
	Southern *wehrlei*	2072	5	3	7	3	0.80	0.0016	4.00
	Northern *wehrlei*	1866	22	13	51	9	0.78	0.0084	15.65
	Population 29	2153	2	1	1	2	1.00	0.0005	1.00
	dixi	2123	5	2	4	3	0.70	0.0009	2.00
Nuclear DNA	All clades	1106.2	104.8	32	24.6	14.4	0.67	0.0090	3.69
	P. punctatus	642.2	32.8	9	1.2	2	0.14	0.0005	0.16
	Populations 10-12	517.75	4.5	1.8	4.75	2.25	0.49	0.0073	2.73
	Yellow-spotted *wehrlei*	641.2	5.6	2	2.8	1.4	0.25	0.0048	1.69
	jacksoni	717.75	5.5	1.4	0.75	1.75	0.31	0.0007	0.34
	Southern *wehrlei*	625.2	8	2.4	1	2	0.29	0.0011	0.39
	Northern *wehrlei*	585	39.2	12.4	8	5.2	0.41	0.0055	1.74
	Population 29	652.8	4	1	1.2	2	0.53	0.0011	0.71
	dixi	613	6.8	2	8.6	4.6	0.83	0.0098	3.93

Diversity indices: (i) haplotype diversity, *h*, the probability that two randomly selected haplotypes are different from each other; (ii) nucleotide diversity, π, the average number of nucleotide differences per site between two sequences; (iii) sequence diversity, κ, the average number of nucleotide differences between paired sequences (Nei, 1987).

[a]Because sites with missing data are excluded from calcuations, relatively short sequences were first removed from the data set. The sample size is larger for nuclear data because individuals are diploid. All sequences were used in our phylogenetic analyses, and thus, our sample sizes for the phylogeographic analyses were larger (Table S1).

discarded as burn-in. The admixture model, an inferred α, and fixed $\lambda = 1$ were used, and sampling localities were used as priors. STRUCTURE results were collated, analysed and visualized using Structure Harvester v.0.6.94 (Earl & vonHoldt, 2011), CLUMPP v.1.1.2 (Jakobsson & Rosenberg, 2007) and DISTRUCT v.1.1 (Rosenberg, 2003). To choose a value for K, log likelihoods and ΔK were examined (Evanno, Regnaut, & Goudet, 2005; Pritchard et al., 2000). As ΔK recovers the basal level of structure, this procedure was replicated iteratively within clusters to detect substructure (Janes et al., 2017).

The validity of delimitations inferred using the bGMYC (mtDNA data) and STRUCTURE (nuclear data) was tested using Bayesian Phylogenetics and Phylogeography (BPP) v.3.3 (Yang & Rannala, 2014). This program accounts for incomplete lineage sorting (ILS) using the multispecies coalescent, and phylogenetic uncertainty is accommodated by branch swapping on the input species tree. Because the mitochondrial genome does not always reflect population-level history, yet due to its variability can dominate analyses, and for comparison with our mtDNA clades and bGMYC results, the BPP analyses included only nuclear loci. After several exploratory analyses, population size parameters (θ) were assigned the gamma prior G(10, 1000), while divergence time at the root of the species tree (τ) was assigned the gamma prior G(5, 1500). All other divergence time parameters were assigned a Dirichlet prior, and algorithm 0 with a fine-tune parameter (ε) of 10 was used. Each species delimitation model was assigned equal prior probability. For the MCMC, after a burn-in of 50,000 generations, samples were collected every five generations until 100,000 samples were obtained. The analysis was run three times to confirm consistency among runs.

2.4 | Introgression vs. incomplete lineage sorting

All of our gene trees had different topologies, which can be caused by hybridization/introgression, ILS, or other processes. To test for introgression as a cause of discordance, JML v1.03 (Joly, 2012) was used. This program uses posterior predictive checking to determine whether the minimum genetic distance between two sequences is smaller than expected given only ILS. All loci were examined separately. For the input species tree, 1025 post-burn-in species trees from a posterior distribution generated using *BEAST v1.8.0 (Heled & Drummond, 2010) were used. Version 1.8 of *BEAST was used because JML was designed to be used with this version. Species tree analyses were inferred as described above, except that both mtDNA and nuclear DNA were used; for this analysis, nuclear loci were unlinked, while mitochondrial loci were linked. The maximum clade credibility tree from this analysis did not differ from the species tree generated using only nuclear data at any statistically supported node. Analyses also included the piecewise constant for the population size model, as recommended by the author of JML. Alpha (α) was set to 0.05.

3 | RESULTS

3.1 | Gene trees

Substantial phylogeographic diversity was recovered in our mtDNA gene tree (Figure 2), especially among populations at the southern portion of the range of *P. wehrlei*. All the major clades in our mtDNA tree constituted geographically cohesive sets of populations and were strongly supported (posterior probability = 1), with one exception (pp = 0.83). The basal split in the tree was dated at 11.5 myr (95% HPD: 9.6–13.6 myr), and separated populations 33 and 34 from the rest. We call these populations the dixi clade because they are within the range of a previously described taxon, *P. dixi* (Pope & Fowler, 1949).

The next split in the tree was dated at 5.1 myr (4.4–5.9 myr) and separated the southernmost populations from the rest. This clade included two subclades, a "southern *wehrlei"* clade and haplotypes from population 29. Population 29 was not associated with southern *wehrlei* in our analyses of nuclear data (see below).

Two other groups of populations in the south also formed geographically cohesive clades. The first clade included populations 27 and 28, which are ~1 km from each other in Blacksburg, Montgomery Co, VA. This is the type locality for a previously described taxon, *P. jacksoni* (Newman, 1954); thus, we call this the jacksoni clade. Our analyses indicated this clade diverged from populations to the north 4.6 myr ago (3.7–5.2 myr). North-west of jacksoni is population 26, west of the New River in Raleigh Co, WV. At this locality, we collected several individuals with yellow spots on the dorsum, a rare phenotype first described by Cupp and Towles (1983). Our analyses suggested the yellow-spotted *wehrlei* diverged from populations to the north 3.0 myr ago (2.4–3.6 myr).

The rest of the phylogeny included two clades found at the northern end of the distribution. The first clade, dated at 1.0 myr ago (0.7–1.2 myr), extended from southeastern WV northward into PA, and included four geographically cohesive subclades (Figure 2). We call this clade "northern *wehrlei*." The eastern-most subclade comes into close contact with *P. punctatus*; however, despite considerable effort, a contact zone has never been found (R. Highton, personal observation). The second divergent clade was also dated at 1.0 myr (0.7–1.2) and included two subclades: *P. punctatus* and a clade of three populations with a *P. wehrlei* phenotype (populations 10–12). The *P. punctatus* clade is dated at 0.4 myr (0.3–0.6 myr). As *P. punctatus* is deeply nested within *P. wehrlei,* this renders *P. wehrlei* paraphyletic.

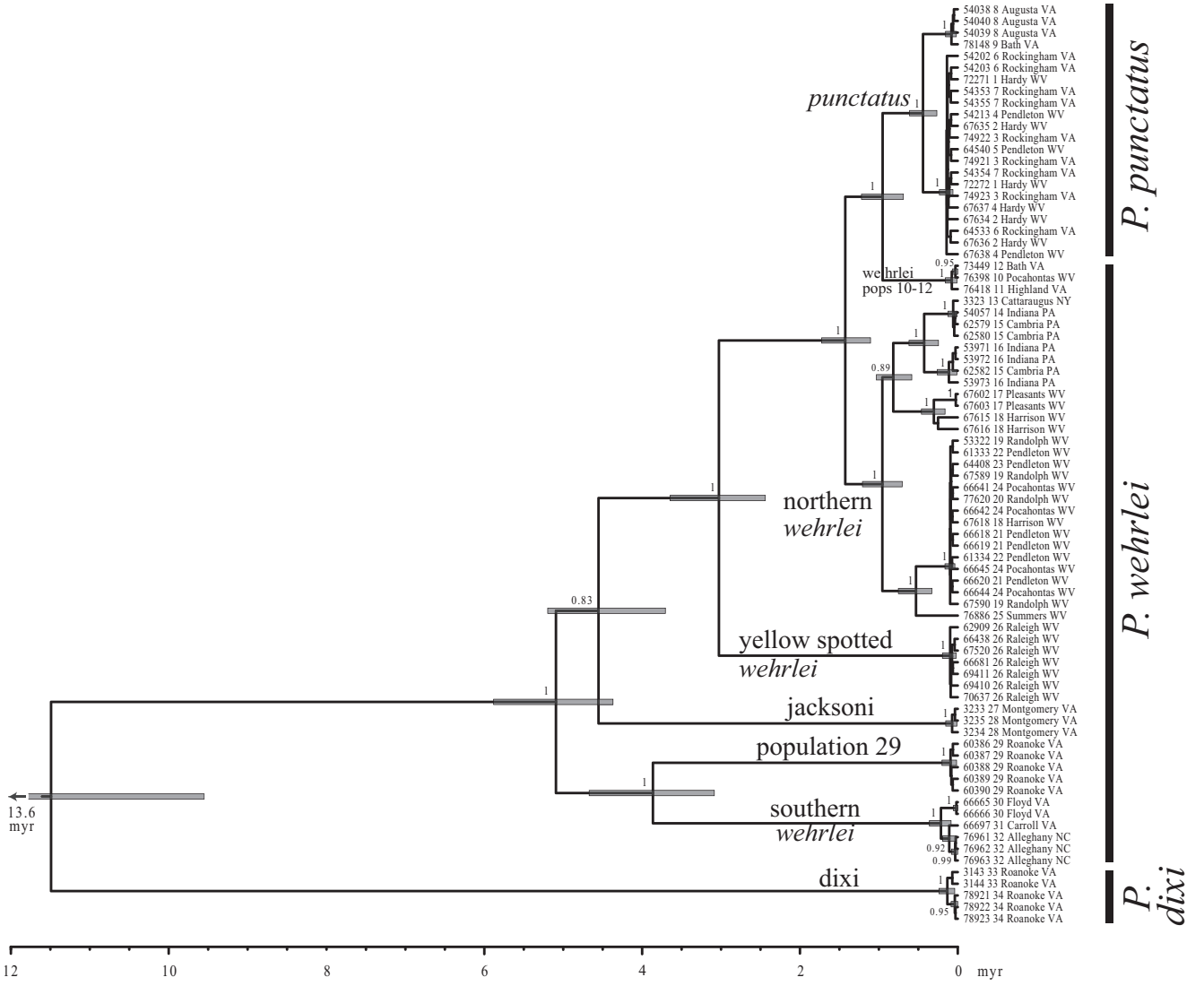

FIGURE 2 Bayesian maximum clade credibility tree inferred using concatenated mtDNA sequence data (Cyt-*b,* ND2, $tRNA^{trp}$). Taxon labels include specimen identification number (Table S1), population numbers from Figure 1, and county plus state information. Numbers adjacent to nodes are posterior probabilities (pp), and bars indicate 95% highest posterior densities for node ages. For visual clarity, many pp values and error bars were removed near the tips of the tree. The vertical bars to the right of the tree illustrate taxonomy, including *P. dixi*, which we elevate to specific status in this study

Phylogenies for our five nuclear loci are presented in the Supplementary Materials (Figs S1–S5). As is typical of nuclear loci, all were less resolved than our mtDNA tree, though much more structure was evident than we found in *P. kentucki* and *P. cinereus* using the same loci (Kuchta, Brown, et al., 2016; Radomski, Kuchta, Brown, Hantak, & Highton, 2018). In general, southern populations were the most divergent. Populations of dixi were recovered as sister to the remainder of the samples with RAG-1 (matching our mtDNA tree), with all other southern populations forming a sister clade (pp = 0.99). For BFI, populations belonging to dixi connected into the base of the tree, and for GAPD, dixi was sister to southern *wehrlei* + jacksoni. For most loci, jacksoni was not distinct from southern *wehrlei* (ILF3, MLC2A, RAG-1), though jacksoni formed a clade using GAPD (pp = 1). The populations belonging to *P. punctatus* did not form a clade at any nuclear locus; rather, these populations tended to be closely related and paraphyletic with respect to northern *wehrlei* populations.

3.2 | Genetic diversity

Matrices of genetic divergence within and between mtDNA groups for all loci are presented in the Supplementary Materials (Tables S2–S9). Divergence of mitochondrial haplotypes within groups was low (all < 0.1%), but between-group differentiation was substantially higher (Table S4). Populations of dixi were the most divergent at ≥9% different

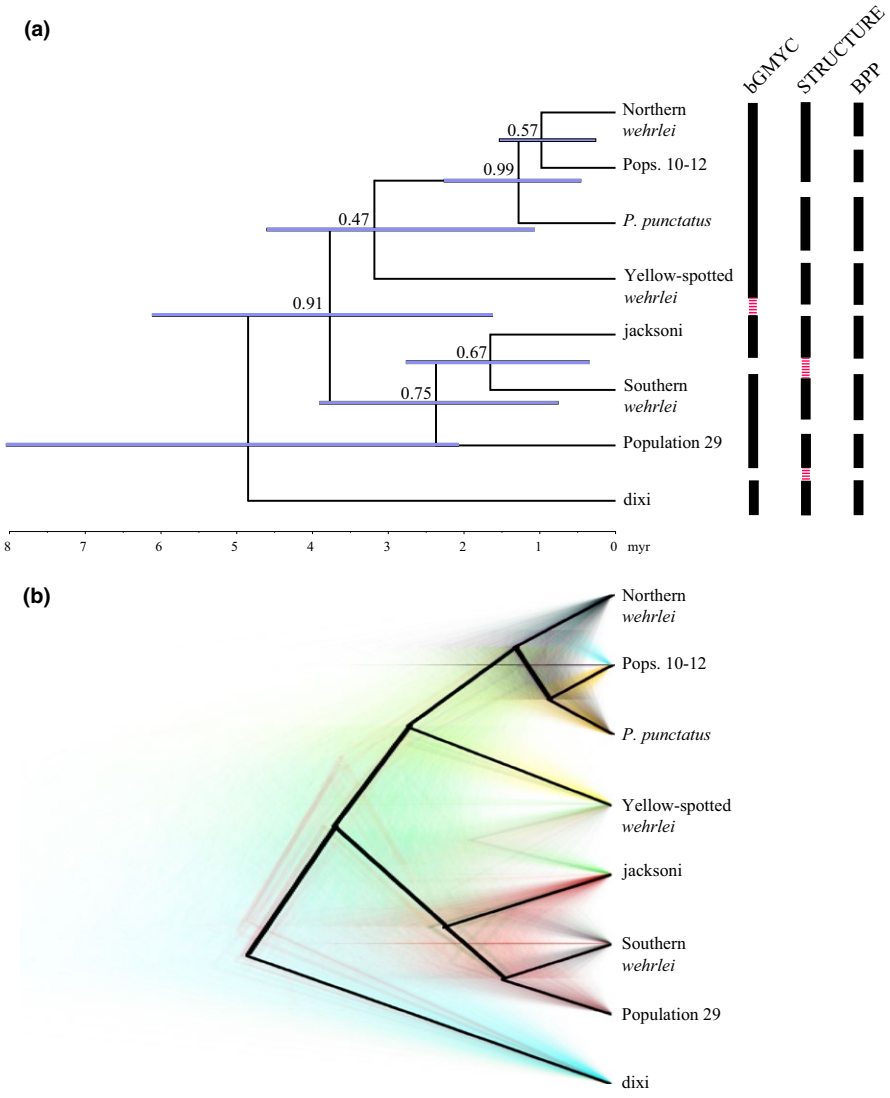

FIGURE 3 Species trees inferred using nuclear loci: (a) Species tree from *BEAST v.2.4.6. To the right are the putative species delimited using bGMYC, STRUCTURE and BPP. Dark lines connect species, and dashed lines connect relatively weakly supported species (for bGMYC, $P < 0.10$; for STRUCTURE, see Figure 4 and text). (b) DensiTree diagram illustrating the 10,000 trees in our posterior distribution of species trees. The thick black line illustrates the consensus tree with the highest clade support (the "root canal"). The timeline at the bottom of (a) also applies to the divergence time estimates in (b)

from all other groups, while the southern clades (jacksoni, southern *wehrlei*, population 29) were ~5–6% divergent from each other. Yellow-spotted *wehrlei* were ≤4% different from groups to the north. *Plethodon punctatus* and northern *wehrlei* were 2.3% divergent from each other. Populations 10–12, which are considered *P. wehrlei*, were more similar to *P. punctatus* than to northern *wehrlei* (1.3% vs. 1.9%).

Divergences were lower for nuclear loci than for mtDNA loci. Between groups, genetic distances ranged from zero to 3.8%, while within-group differentiation averaged <2%, except for MLC2A, which averaged up to 2.8% divergent.

Similar patterns were revealed using measures of genetic diversity, with mtDNA more divergent than the nuclear loci (Table 2). For example, sequence diversity (κ) was 74.15 for the mtDNA haplotypes, but averaged 3.69 across the nuclear loci. Divergence within groups was lower. For instance, κ within groups ranged from 0.05 to 4.82 for mtDNA, but ranged from 0.16 to 3.93 for nuclear DNA. The exception is northern *wehrlei*, which exhibited relatively high values for mtDNA ($\kappa = 15.65$). For the nuclear loci, the dixi group had the highest levels of genetic variation. See the Supplementary Materials (Table S10) for levels of diversity at each locus separately.

3.3 | Species tree

We used the mtDNA phylogeny (Figure 2) to define a priori species for species tree analyses using nuclear data in BEAST v.2.4.6 (Drummond & Bouckaert, 2015). The basal split in our species tree was dated at 4.9 myr (2.1–8.0 myr), with other splits occurring from ~1 to 4 myr ago (Figure 3a). These ages were substantially younger than inferences made from mtDNA (Figure 2). Most clades in the maximum clade credibility species tree had low posterior probabilities, though the topology resembled the mtDNA tree (*c.f.,* Figures 2, 3). The basal split separated dixi from all other groups. A clade including *P. punctatus*, northern *wehrlei* and populations 10–12 was well supported (pp=0.99), but in contrast with the mtDNA tree, populations 10–12 were sister to northern *wehrlei*, not *P. punctatus*, though with weak support (pp = 0.57). A visualization of the posterior distribution

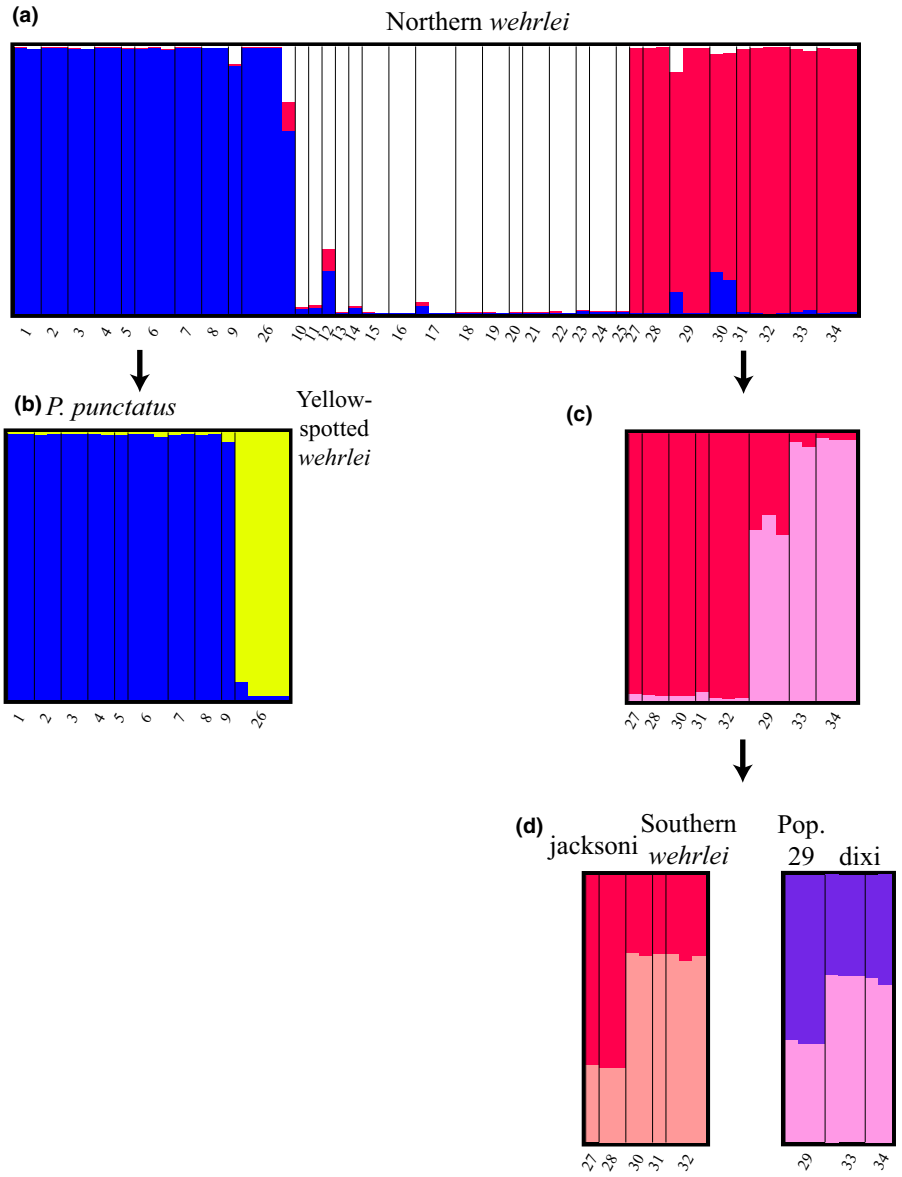

FIGURE 4 STRUCTURE results using nuclear sequence data coded as haplotypes. (a) Results for the entire data set for $\Delta K = 3$ (Evanno et al., 2005). The central group corresponds with northern *wehrlei* and includes no substructure. The left group includes two subgroups, *Plethodon punctatus* and the yellow-spotted *wehrlei* (b). The right group includes two subgroups (c), each of which includes two subgroups (d), including jacksoni + southern *wehrlei* (left), and population 29 + dixi (right). Numbers below each panel are population numbers, and correspond with Figures 1, 2 and Table S1 [Colour figure can be viewed at wileyonlinelibrary.com]

of species trees (Figure 3b) using DensiTree (Bouckaert, 2010) showed that the dixi group and the clade including northern *wehrlei* + populations 10–12 + *P. punctatus* were present in most topologies. By contrast, yellow-spotted *wehrlei*, jacksoni, population 29 and southern *wehrlei* were joined by a diversity of topologies.

3.4 | Delimitation and validation of putative species

The bGMYC method delimits putative species by identifying the transition between population-level (coalescent) processes and species-level (Yule model) processes. We applied this model to our mtDNA data. At $P = .05$, three putative species were identified: (1) dixi; (2) southern *wehrlei* + population 29; and (3) everything else. At $P = .10$, jacksoni was also recognized as distinct (Figure 3a).

For our nuclear loci, plots of the estimated log probability of the data for replicated STRUCTURE analyses revealed a peak Ln Pr(X|K) at $K = 5$, with a decrease in likelihood (and an increase in variance) at $K > 5$ (Fig. S6a). By contrast, the Evanno method (Evanno et al., 2005) revealed a peak at $\Delta K = 3$ (Fig. S6b). This discrepancy was likely due to hierarchical structure in the data (Converse et al., 2015; Evanno et al., 2005; Janes et al., 2017). We first analysed our data for $K = 3$ and found three clearly defined clusters (Figure 4a). One cluster corresponded with northern *wehrlei* + populations 10–12, and no substructure was found within this cluster. The second cluster included populations belonging to *P. punctatus* + yellow-spotted *wehrlei*, and when we looked for substructure within this cluster, we recovered these two groups as distinct (Figure 4b). The third cluster included all southern populations. Substructure within this cluster separated jacksoni + southern *wehrlei* from dixi + population 29

(Figure 4c). Looking within these clusters, we found weak to modest support for separating jacksoni from southern *wehrlei*, and dixi from population 29 (Figure 4d). In both cases, there were signs of either admixture or poor statistical discrimination; more data are needed to distinguish between these two explanations. No further substructure was found in either group. In summary, by testing for substructure within clusters, the Evanno method recovered a total of seven clusters.

We validated putative species using BPP with our nuclear data. Replicate runs of BPP produced consistent results, suggesting that the MCMC chains were well mixed. The delimitation with the highest posterior probability (pp = 0.99) supported the recognition of all eight groups as distinct species (Figure 3a). This result was consistently found using a variety of priors and starting conditions.

3.5 | Introgression vs. incomplete lineage sorting

Coalescent simulations in JML failed to detect introgression for any locus except MLC2A. At MLC2A, there was evidence of introgression between population 29 and *P. punctatus*, dixi, and northern *wehrlei*. JML also detected introgression between dixi and *P. punctatus,* and between dixi and northern *wehrlei*. While our other loci, including mtDNA, did not provide evidence of introgression, in many cases a minimal recorded genetic distance of zero was not significant at $P < .05$. Thus, the power of this test was low for many comparisons.

4 | DISCUSSION

4.1 | Phylogeography

In this study, we uncovered large amounts of phylogeographic diversity in the *P. wehrlei* group. Much of this diversity is in the southern portion of the range, in agreement with phylogeographic analyses of other taxa in the region (Bonett et al., 2007; Herman & Bouzat, 2016; Soltis, Morris, McLachlan, Manos, & Soltis, 2006). Our analyses recovered two groups in the south that correspond with previously described species. The basal splits in our mtDNA gene tree and our species tree separated populations we call the dixi group from all other groups (Figure 2). The dixi group includes samples from Dixie Caverns, Roanoke Co, VA, which is the type locality for a taxon that was previously described as *P. dixi* (Pope & Fowler, 1949), but was sunk into *P. wehrlei* by Highton (1962) because phenotypic differences were considered too limited. West of dixi are two samples from Blacksburg, Montgomery Co, VA. These are from near the type locality of the former species *P. jacksoni* (Newman, 1954), which was also sunk into *P. wehrlei* by Highton (1962). More work is needed to circumscribe the geographic distributions of both dixi and jacksoni, though both are likely to have relatively restricted distributions (Figure 1).

North-west of jacksoni is population 26, west of the New River in Raleigh Co, WV (Figure 1). This population is distinctive because all individuals collected there possessed yellow spots on their dorsum, a phenotype that was first reported from Kentucky (Cupp & Towles, 1983). Discovery of the yellow-spotted phenotype west of the New River in West Virginia represents a significant range extension. Whether population 26 is closely related to disjunct yellow-spotted populations to the west, or is instead an ecotype or recurring mutation, requires more data. The extreme rarity of the yellow-spotted phenotype hampers studies of its evolutionary history.

East of the New River, ~9 km northeast of population 26, is population 25, which is most closely related to northern populations. The finding that the New River is a phylogeographic divide is consistent with other studies that have found it to be an important biogeographic barrier in the region (Berendzen, Simons, & Wood, 2003; Highton, 1999; Hocutt, Jenkins, & Stuaffer, 1986; Kuchta, Haughey, Wynn, Jacobs, & Highton, 2016). Despite its name, the New River may be one of the oldest rivers in the world, and it flows in the same valley as the ancient and extinct Teays River, a former major drainage in eastern North America that flowed westward out of the central Appalachians across the Midwestern United States into the Old Mississippi (Hocutt et al., 1986).

Perhaps our most striking result is that *P. punctatus* is nested within *P. wehrlei*, rendering the latter species paraphyletic. While many species of *Plethodon* can be difficult to identify, *P. punctatus* was described on the basis of phenotypic and ecological differences (Highton, 1972), and its distinctiveness has never been questioned. Relative to *P. wehrlei*, *P. punctatus* reaches larger sizes, has a larger modal number of trunk vertebrae (19 vs. 18), a darker ground colour, and white or yellow flecks on the sides, legs and cheeks. Unlike *P. wehrlei*, it lacks red, orange or yellow dorsal spots or brassy flecking (Highton, 1972).

Phylogeographic patterns revealed by analysis of mtDNA were largely corroborated by our nuclear data. The important exceptions were as follows:

1. The affinities of population 29 remain unclear. Our mtDNA phylogeny recovered population 29 as sister to southern *wehrlei*, while STRUCTURE associated population 29 with dixi, and our species tree recovered population 29 as sister to a clade that included southern *wehrlei* + jacksoni.
2. Our mtDNA phylogeny recovered jacksoni as most closely related to populations to the north, whereas in our species tree jacksoni was more closely related to southern populations. In STRUCTURE, jacksoni was only

moderately divergent from southern *wehrlei*, suggesting either introgression or a lack of confidence in group assignments.

3. In our mtDNA tree, *P. punctatus* was sister to populations 10–12, while STRUCTURE and our species tree recovered populations 10–12 as more closely related to northern *wehrlei*. We favour the results of the nuclear studies because populations 10–12 are located on the eastern border of northern *wehrlei* and have a *P. wehrlei* phenotype. The discordance between our mtDNA and nuclear analyses suggests an ancient introgression event moved mtDNA from *P. punctatus* into populations 10–12; however, when we tested for introgression using JML, mtDNA introgression was not detected.
4. In our species tree, clade ages were roughly one-half to one-third as old as in our mtDNA phylogeny. For example, the basal split for mtDNA was inferred to be 11.5 myr, whereas the same split was 4.9 myr in our species tree. This difference may reflect the fact that ancestral polymorphism can lead to substantial overestimates of divergence times, as coalescent events must predate (or be contemporaneous with) population/species divergence (Edwards & Beerli, 2000). Another possible cause of younger divergence times in species trees compared to gene trees is introgressive hybridization, which can lead to species tree "compression" when speciation events are forced to follow coalescent events (Kuchta, Brown, et al., 2016; Leaché, 2009). We tested for introgression in our data using JML and detected evidence of introgression only at MLC2A. One needs to be careful when using JML for exploratory tests of introgression because of the large number of comparisons performed, but the current results suggest that introgression may have compressed the time scale of our species tree.

4.2 | Species delimitation

Over the last decade, debate has shifted away from species concepts to the evidence and methods used to demarcate species boundaries (Carstens et al., 2013; Rannala, 2015; Sites & Marshall, 2003). At the same time, an increasing number of biologists, especially systematists and phylogeographers, have adopted the framework of the unified species concept (also commonly referred to as the General Lineage Concept; de Queiroz, 1998, 2005). Under the unified species concept, species are segments of independently evolving metapopulation-level evolutionary lineages. Moreover, lineage independence is the only necessary property of species, though there is value in quantifying differentiation in important biological features, such as behaviour, morphology or reproduction (Dayrat, 2005; Sites & Marshall, 2003). Alongside the unified species concept, there has been a concordant effort to develop objective, computational methods for identifying independent lineages from multilocus genetic data (Knowles & Carstens, 2007; Rannala, 2015; Yang & Rannala, 2014). These methods are particularly appealing to those doing investigations of species complexes characterized by allopatry, parapatry, morphological stasis, or complex patterns of phenotypic variation (Hotaling et al., 2016; Lumbsch & Leavitt, 2011; Ruane, Bryson, Pyron, & Burbrink, 2014; Singh et al., 2015).

In this study, we searched for independent genetic lineages using bGMYC and STRUCTURE, and we tested these delimitations using BPP (Figure 3a). The most conservative results were provided by bGMYC, which delimited three species that included dixi, southern *wehrlei* + population 29 and the rest of the mtDNA groups. There was modest support for recognizing jacksoni as well. STRUCTURE delimited seven clusters, including most of the major clades in our mtDNA phylogeny, though there were signs of introgression or weak divergence between some groups. BPP provided the most liberal delimitation, recognizing all putative species as distinct, including separating populations 10–12 from northern *wehrlei*.

We carried out these delimitations in the spirit of exploration and discovery. Darwinian species formation is a lengthy and convoluted process (Kuchta & Wake, 2016; Mallet, 2008), and all methods of species delimitation come with assumptions that are easily and often violated (Carstens et al., 2013). For instance, Sukumaran and Knowles (2017) have recently shown that methods of species delimitation that employ the multispecies coalescent, such as BPP, identify population structure, not species, because such models assume instantaneous species formation and a lack of genetic structure (see also Barley, Brown, & Thomson, 2018). Similarly, STRUCTURE assumes no isolation by distance or population structure (Meirmans, 2012). The problem of high levels of genetic structure is particularly acute in terrestrial plethodontid salamanders, which are territorial and exhibit very limited vagility (Kuchta, Parks, Mueller, et al. 2009; Martínez-Solano et al., 2007; Tilley et al., 2013). At the same time, a high degree of phenotypic and ecological conservatism is also common (Kozak & Wiens, 2010; Wake et al., 1983). In such situations, species formation is often achieved by fragmentation, or non-adaptive radiation, whereby species originate and expand their range, then slowly disintegrate on the landscape (Rundell & Price, 2009; Wake, 2017). Introgressive hybridization from repeated instances of isolation followed by secondary contact (even the anastomosis of formerly isolated lineages) can further complicate the interpretation of genetic data. It is likely that methods of species delimitation that do not account for introgressive hybridization, isolation by distance, and population structure will find such complexes to be a worst-case scenario. An advantage of explicit mathematical models of species delimitation is that they precisely identify what assumptions were made in making

a delimitation. However, no models accommodate the severe assumptions imposed by species formation by fragmentation: all models make simplifying assumptions that can lead them to fail when faced with such complex patterns (Carstens et al., 2013). For example, Kuchta, Brown, et al. (2016) showed in the Cumberland Plateau Salamander (*P. kentucki*) that mutually exclusive "species" were identified using different approaches to delimitation, and that different subsamples of the same data set led to conflicting results. For these reasons, while we find the burgeoning field of species delimitation exciting, we stress that caution is warranted (Barley et al., 2018; Martínez-Solano et al., 2007; Sukumaran & Knowles, 2017).

4.3 | Taxonomic implications

In our analyses, the most distinctive group was the dixi group, which was recovered as sister to the rest of *P. wehrlei* and *P. punctatus* in our mtDNA tree and our nuclear DNA species tree, with age estimates of 11.5 myr (mtDNA) and 4.9 myr (nuclear species tree). While these age estimates differ, both are old. All three methods of species delimitation we employed also recovered the dixi group as distinct. In their original description of *P. dixi*, Pope and Fowler (1949) observed that individuals of this species were covered with bronzy mottling and dotted with small, light flecks, which were usually present on the head and were more numerous laterally and on the tail. Red dorsal spots, which are a variable character in *P. wehrlei*, were rare or absent. By contrast, population 29 of *P. wehrlei* (Poor Mountain, ~10 km south of Dixie Caverns) has a mean of 4.8 dorsal red spots ($N = 125$; Highton, 2018). Given the phenotypic and genetic distinctiveness of the dixi populations, we propose that the original taxonomy of Pope and Fowler (1949) be followed and *P. dixi* be recognized as a distinct species. The type locality of *P. dixi* is Dixie Caverns, Roanoke Co., VA, and the type locality of *P. wehrlei* is Indiana Co., PA.

The recognition of *P. dixi* still leaves *P. wehrlei* paraphyletic with respect to *P. punctatus*. The status of *P. punctatus* is important in part because it is recognized as a rare or sensitive species by the George Washington National Forest, the Virginia Department of Game and Inland Fisheries, and West Virginia Wildlife Diversity Program (Mitchell & Pauley, 2005). If one demands species be monophyletic for mtDNA, there are three solutions. The first is to sink *P. punctatus* into *P. wehrlei*. However, the status of *P. punctatus* has never been questioned, as it is allopatric from *P. wehrlei* and is ecologically and phenotypically distinct (Highton, 1972). The second solution is to further split *P. wehrlei*. This approach is complicated by discordances between the mtDNA and nuclear data, unless one chooses to split *P. wehrlei* rather finely. The third solution is to recognize the biological reality of paraphyletic species (Crisp & Chandler, 1996; Funk & Omland, 2003). When a new species originates, especially if it originates relatively quickly or on the periphery of another species' range, the ancestral taxon will usually be rendered paraphyletic, or even multiply paraphyletic. Over time, gene trees are expected to transition back to monophyly (Baum & Shaw, 1995). However, rates of lineage sorting are dependent upon the rate of genetic drift, which is a function of effective population size and rates of migration among populations (Wakeley, 1999). Consequently, coalescence will often be slow in dispersal-limited organisms with high population sizes, such as woodland salamanders, and reciprocal monophyly at a locus may not be achieved before further population fragmentation events occur. For this reason, both *P. wehrlei* and *P. punctatus* can be valid species even if *P. wehrlei* is paraphyletic.

Given the amount of genetic diversity in *P. wehrlei*, recognition of *P. dixi* is a modest taxonomic change. To further evaluate taxonomy and patterns of genetic variation, we are currently collecting next-generation sequencing data from throughout the range of *P. wehrlei*, *P. punctatus* and *P. dixi*. Further work should also examine patterns of coloration and morphology in the *P. wehrlei* complex, and test the independence of *P. dixi* with respect to the jacksoni group, the southern *wehrlei* group, and population 29. In addition to taxonomic evaluation, further study of the phylogeographic history of the *P. wehrlei* complex stands to inform us about how range fragmentation—the disintegration of a lineage over space and time—contributes to the build-up of species diversity (Kuchta & Wake, 2016; Rundell & Price, 2009).

ACKNOWLEDGEMENTS

We thank Kaylee Soellner for her assistance in the DNA laboratory. Research was conducted under Ohio University IACUC 12-L-050. Permits were obtained from the Virginia Department of Game and Inland Fisheries (015603, 048037), the West Virginia Division of Natural Resources (2013.115), and the Pennsylvania Fish and Boat Commission (CID: 031793417). We thank Maggie Hantak and two anonymous reviewers for their insightful and careful comments, which greatly improved the manuscript.

ORCID


Shawn R. Kuchta http://orcid.org/0000-0003-0502-0150

REFERENCES

Aktas, C. (2017). haplotypes: haplotype inference and statistical analysis of genetic variation. R package version 1.0. https://CRAN.R-project.org/package=haplotypes.

Ballard, J. W. O., & Whitlock, M. C. (2004). The incomplete natural history of mitochondria. *Molecular Ecology*, *13*, 729–744.

Barley, A. J., Brown, J. M., & Thomson, R. C. (2018). Impact of model violations on the inference of species boundaries under the multispecies coalescent. *Systematic Biology*, *67*, 269–284.

Baum, D. A., & Shaw, K. L. (1995). Genealogical perspectives on the species problem. *Monographs in Systematic Botany from the Missouri Botanical Garden*, *53*, 289–303.

Berendzen, P. B., Simons, A. M., & Wood, R. M. (2003). Phylogeography of the northern hogsucker, *Hypentelium nigricans* (Teleostei: Cypriniformes): Genetic evidence for the existence of the ancient Teays River. *Journal of Biogeography*, *30*, 1139–1152.

Bonett, R. M., Kozak, K. H., Vieites, D. R., Bare, A., Wooten, J. A., & Trauth, S. E. (2007). The importance of comparative phylogeography in diagnosing introduced species: A lesson from the seal salamander, *Desmognathus monticola*. *BMC Ecology*, *7*, 7.

Bouckaert, R. R. (2010). DensiTree: Making sense of sets of phylogenetic trees. *Bioinformatics*, *26*, 1372–1373.

Camp, C. D., & Wooten, J. A. (2016). Hidden in plain sight: Cryptic diversity in the Plethodontidae. *Copeia*, *104*, 111–117.

Carstens, B. C., Pelletier, T. A., Reid, N. M., & Satler, J. D. (2013). How to fail at species delimitation. *Molecular Ecology*, *22*, 4369–4383.

Converse, P. E., Kuchta, S. R., Roosenburg, W. M., Henry, P. F. P., Haramis, G. M., & King, T. L. (2015). Spatiotemporal analysis of gene flow in Chesapeake Bay Diamondback Terrapins (*Malaclemys terrapin*). *Molecular Ecology*, *24*, 5864–5876.

Crisp, M. D., & Chandler, G. T. (1996). Paraphyletic species. *Telopea*, *6*, 813–838.

Cupp, P. V. J., & Towles, D. T. (1983). A new variant of *Plethodon wehrlei* in Kentucky and West Virginia. *Transactions of the Kentucky Academy of Science*, *44*, 157–158.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: More models, new heuristics and parallel computing. *Nature Methods*, *9*, 772.

Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society*, *85*, 407–415.

Drummond, A. J., & Bouckaert, R. R. (2015). *Bayesian evolutionary analysis with BEAST*. Cambridge, UK: Cambridge University Press.

Earl, D. A., & vonHoldt, B. M. (2011). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, *4*, 359–361.

Edwards, S. V., & Beerli, P. (2000). Gene divergence, population divergence, and the variance in coalescence time in phylogeographic studies. *Evolution*, *54*, 1839–1854.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology*, *14*, 2611–2620.

Fisher-Reid, M. C., & Wiens, J. J. (2011). What are the consequences of combining nuclear and mitochondrial data for phylogenetic analysis? Lessons from *Plethodon* salamanders and 13 other vertebrate clades. *BMC Evolutionary Biology*, *11*, 300.

Flot, J.-F. (2007). Champuru 1.0: A computer software for unraveling mixtures of two DNA sequences of unequal lengths. *Molecular Ecology Notes*, *7*, 974–977.

Fujita, M. K., Leaché, A. D., Burbrink, F. T., McGuire, J. A., & Moritz, C. (2012). Coalescent-based species delimitation in an integrative taxonomy. *Trends in Ecology & Evolution*, *27*, 480–488.

Funk, D. J., & Omland, K. E. (2003). Species-level paraphyly and polyphyly: Frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, *34*, 397–423.

Hall, R. J., & Stafford, D. P. (1972). Studies in the life history of Wehrle's Salamander, *Plethodon wehrlei*. *Herpetologica*, *28*, 300–309.

Heled, J., & Drummond, A. J. (2010). Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution*, *27*, 570–580.

Herman, T. A., & Bouzat, J. L. (2016). Range-wide phylogeography of the four-toed salamander: Out of Appalachia and into the glacial aftermath. *Journal of Biogeography*, *43*, 666–678.

Highton, R. (1962). Revision of North American salamanders of the genus *Plethodon*. *Bulletin of the Florida State Museum*, *6*, 235–367.

Highton, R. (1972). Distributional interactions among eastern North American salamanders of the genus *Plethodon*. In P. C. Holt (Ed.), *The distributional history of the biota of the southern Appalachians. Part III: Vertebrates* (pp. 139–188). Research Division Monograph. Blacksburg, VA: Virginia Polytechnic Institute and State University.

Highton, R. (1995). Speciation in eastern North American salamanders of the genus *Plethodon*. *Annual Review of Ecology, Evolution, and Systematics*, *26*, 579–600.

Highton, R. (1998). Is *Ensatina eschscholtzii* a ring-species? *Herpetologica*, *54*, 254–278.

Highton, R. (1999). Geographic protein variation and speciation in the salamanders of the *Plethodon cinereus* group with the description of two new species. *Herpetologica*, *55*, 43–90.

Highton, R. (2014). Detecting cryptic species in phylogeographic studies: Speciation in the California Slender Salamander, *Batrachoseps attenuatus*. *Molecular Phylogenetics and Evolution*, *71*, 127–141.

Highton, R. (2018). Speciation in the *Plethodon wehrlei* group. Unpublished data.

Highton, R., Hastings, A. P., Palmer, C., Watts, R., Hass, C. A., Culver, M., & Arnold, S. J. (2012). Concurrent speciation in the eastern woodland salamanders (genus *Plethodon*): DNA sequences of the complete albumin nuclear and partial mitochondrial 12s genes. *Molecular Phylogenetics and Evolution*, *63*, 278–290.

Highton, R., & Larson, A. (1979). The genetic relationships of the salamanders of the genus *Plethodon*. *Systematic Biology*, *28*, 579–599.

Hocutt, C. H., Jenkins, R. E., & Stuaffer, J. R. J. (1986). Zoogeography of the fishes of the central Appalachians and central Atlantic coastal plain. In C. H. Hocutt, E. O. Wiley (Eds), *The zoogeography of North American freshwater fishes* (pp. 161–211). New York: John Wiley & Sons.

Hotaling, S., Foley, M. E., Lawrence, N. M., Bocanegra, J., Blanco, M. B., Rasoloarison, R., & Weisrock, D. W. (2016). Species discovery and validation in a cryptic radiation of endangered primates: Coalescent-based species delimitation in Madagascar's mouse lemurs. *Molecular Ecology*, *25*, 2029–2045.

Jakobsson, M., & Rosenberg, N. A. (2007). CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics*, *23*, 1801–1806.

Janes, J. K., Miller, J. M., Dupuis, J. R., Malenfant, R. M., Gorrell, J. C., Cullingham, C. I., & Andrew, R. L. (2017). The K = 2 conundrum. *Molecular Ecology*, *26*, 3594–3602.

Joly, S. (2012). JML: Testing hybridization from species trees. *Molecular Ecology Resources*, *12*, 179–184.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., … Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*, 1647–1649.

Kleeberger, S. R., & Werner, J. K. (1982). Home range and homing behavior of *Plethodon cinereus* in northern Michigan. *Copeia*, *1982*, 409–415.

Knowles, L. L., & Carstens, B. (2007). Delimiting species without monophyletic gene trees. *Systematic Biology*, *56*, 887–895.

Kozak, K. H., Weisrock, D. W., & Larson, A. (2006). Rapid lineage accumulation in a non-adaptive radiation: Phylogenetic analysis of diversification rates in eastern North American woodland salamanders (Plethodontidae: Plethodon). *Proceedings of the Royal Society B*, *273*, 530–546.

Kozak, K. H., & Wiens, J. J. (2010). Niche conservatism drives elevational diversity patterns in Appalachian salamanders. *American Naturalist*, *176*, 40–54.

Kuchta, S. R., Brown, A. D., Converse, P. E., & Highton, R. (2016). Multilocus phylogeography and species delimitation in the Cumberland Plateau Salamander, *Plethodon kentucki*: Incongruence among data sets and methods. *PLoS ONE*, *11*, e0150022.

Kuchta, S. R., Haughey, M., Wynn, A. H., Jacobs, J. F., & Highton, R. (2016). Ancient river systems and phylogeographical structure in the spring salamander, *Gyrinophilus porphyriticus*. *Journal of Biogeography*, *43*, 639–652.

Kuchta, S. R., Parks, D. S., Mueller, R. L., & Wake, D. B. (2009). Closing the ring: Historical biogeography of the salamander ring species *Ensatina eschscholtzii*. *Journal of Biogeography*, *36*, 982–995.

Kuchta, S. R., Parks, D. S., & Wake, D. B. (2009). Pronounced phylogeographic structure on a small spatial scale: Geomorphological evolution and lineage history in the salamander ring species *Ensatina eschscholtzii* in central coastal California. *Molecular Phylogenetics and Evolution*, *50*, 240–255.

Kuchta, S. R., & Tan, A. M. (2005). Isolation by distance and post-glacial range expansion in the rough-skinned newt, *Taricha granulosa*. *Molecular Ecology*, *14*, 225–244.

Kuchta, S. R., & Wake, D. B. (2016). Wherefore and whither the ring species? *Copeia*, *104*, 189–201.

Leaché, A. D. (2009). Species tree discordance traces to phylogeographic clade boundaries in North American fence lizards (*Sceloporus*). *Systematic Biology*, *58*, 547–559.

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*, 1451–1452.

Lumbsch, H. T., & Leavitt, S. D. (2011). Goodbye morphology? A paradigm shift in the delimitation of species in lichenized fungi. *Fungal Diversity*, *50*, 59–72.

Macey, J. R., Larson, A., Ananjeva, N. B., Fang, Z., & Papenfuss, T. J. (1997). Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. *Molecular Biology and Evolution*, *14*, 91–104.

Mallet, J. (2008). Mayr's view of Darwin: Was Darwin wrong about speciation? *Biological Journal of the Linnean Society*, *95*, 3–16.

Martínez-Solano, I., Jockusch, E. L., & Wake, D. B. (2007). Extreme population subdivision throughout a continuous range: Phylogeography of *Batrachoseps attenuatus* (Caudata: Plethodontidae) in western North America. *Molecular Ecology*, *16*, 4335–4355.

Mayr, E. (1982). *The growth of biological thought: diversity, evolution, and inheritance*. Cambridge, MA: The Belknap Press of Harvard University Press.

Meirmans, P. G. (2012). The trouble with isolation by distance. *Molecular Ecology*, *21*, 2839–2846.

Milne, I., Lindner, D., Bayer, M., Husmeier, D., McGuire, G., Marshall, D. F., & Wright, F. (2008). TOPALi v2: A rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. *Bioinformatics*, *25*, 126–127.

Mitchell, J. C., & Pauley, T. K. (2005). *Plethodon punctatus*. In M. J. Lannoo (Ed.), *Amphibian declines: The conservation status of United States species* (pp. 834–835). Berkeley, CA: University of California Press.

Nei, M. (1987). *Molecular evolutionary genetics*. New York, NY: Columbia University Press.

Newman, W. B. (1954). A new plethodontid salamander from southwestern Virginia. *Herpetologica*, *10*, 9–14.

Niemiller, M. L., Near, T. J., & Fitzpatrick, B. M. (2011). Delimiting species using multilocus data: Diagnosing cryptic diversity in the southern cavefish, *Typhlichthys subterraneus* (Teleostei: Amblyopsidae). *Evolution*, *66*, 846–866.

Pope, C. H., & Fowler, J. A. (1949). A new species of salamander (*Plethodon*) from southwestern Virginia. *Natural History Miscellanea Chicago Academy of Sciences*, *47*, 1–4.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*, 945–959.

de Queiroz, K. (1998). The general lineage concept of species, species criteria, and the process of speciation: A conceptual unification and terminological recommendations. In D. J. Howard, & S. H. Berlocher (Eds.), *Endless forms: Species and speciation* (pp. 57–75). New York, NY: Oxford University Press.

de Queiroz, K. (2005). Ernst Mayr and the modern concept of species. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 6600–6607.

R Core Team (2017). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. URL http://www.R-project.org/.

Radomski, T., Kuchta, S. R., Brown, A. D., Hantak, M. H., & Highton, R. (2018). Testing the ecological causes of niche conservatism: Biogeography of the Eastern Red-backed Salamander (*Plethodon cinereus*). Unpublished data.

Rambaut, A., Suchard, M. A., Xie, D., & Drummond, A. J. (2014). Tracer v1.6, Available from http://tree.bio.ed.ac.uk/software/tracer/

Rannala, B. (2015). The art and science of species delimitation. *Current Zoology*, *61*, 846–853.

Rannala, B., & Yang, Z. (2003). Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. *Genetics*, *164*, 1645–1656.

Reid, N. M., & Carstens, B. C. (2012). Phylogenetic estimation error can decrease the accuracy of species delimitation: A Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology*, *12*, 196.

Reilly, S. B., Corl, A., & Wake, D. B. (2015). An integrative approach to phylogeography: Investigating the effects of ancient seaways, climate, and historical geology on multi-locus phylogeographic boundaries of the Arboreal Salamander (*Aneides lugubris*). *BMC Evolutionary Biology*, *15*, 241.

Rosenberg, N. A. (2003). Distruct: A program for the graphical display of population structure. *Molecular Ecology Resources*, *4*, 137–138.

Rovito, S. M., Parra-Olea, G., Vásquez-Almazán, C. R., Luna-Reyes, R., & Wake, D. B. (2012). Deep divergences and extensive phylogeographic structure in a clade of lowland tropical salamanders. *BMC Evolutionary Biology*, *12*, 255.

Ruane, S., Bryson, R. W., Pyron, R. A., & Burbrink, F. T. (2014). Coalescent species delimitation in milksnakes (genus *Lampropeltis*) and impacts on phylogenetic comparative analyses. *Systematic Biology*, *63*, 231–250.

Rundell, R. J., & Price, T. D. (2009). Adaptive radiation, nonadaptive radiation, ecological speciation and nonecological speciation. *Trends in Ecology & Evolution*, *24*, 394–399.

Sequeira, F., Ferrand, N., & Harris, D. J. (2006). Assessing the phylogenetic signal of the nuclear b-Fibrinogen intron 7 in salamandrids (Amphibia: Salamandridae). *Amphibia-Reptilia*, *27*, 409–418.

Shen, X.-X., Liang, D., Chen, M.-Y., Mao, R.-L., Wake, D. B., & Zhang, P. (2015). Enlarged multilocus data set provides surprisingly younger time of origin for the Plethodontidae, the largest family of salamanders. *Systematic Biology*, *65*, 66–81.

Singh, G., Dal Grande, F., Divakar, P. K., Otte, J., Leavitt, S. D., Szczepanska, K., … Schmitt, I. (2015). Coalescent-based species delimitation approach uncovers high cryptic diversity in the cosmopolitan lichen-forming fungal genus *Protoparmelia* (Lecanorales, Ascomycota). *PLoS ONE*, *10*, e0124625.

Sites, J. W. Jr, & Marshall, J. C. (2003). Operational criteria for delimiting species. *Annual Review of Ecology, Evolution, and Systematics*, *35*, 199–227.

Soltis, D. E., Morris, A. B., McLachlan, J. S., Manos, P. S., & Soltis, P. S. (2006). Comparative phylogeography of unglaciated eastern North America. *Molecular Ecology*, *15*, 4261–4293.

Stephens, M., Smith, N. J., & Donnelly, P. (2001). A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics*, *68*, 978–989.

Sukumaran, J., & Knowles, L. L. (2017). Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences of the United States of America*, *114*, 1607–1612.

Swofford, D. L. (2003). *PAUP*: Phylogenetic Analysis using Parsimony (*and other methods), version 4.0b.10*. Sunderland, MA: Sinauer Associates.

Tilley, S. G., Bernardo, J., Katz, L. A., López, L., Devon Roll, J., Eriksen, R. L., … Crandall, K. A. (2013). Failed species, innominate forms, and the vain search for species limits: Cryptic diversity in dusky salamanders (*Desmognathus*) of eastern Tennessee. *Ecology and Evolution*, *3*, 2547–2567.

Wake, D. B. (2017). Persistent plethodontid themes: Species, phylogenies, and biogeography. *Herpetologica*, *73*, 242–251.

Wake, D. B., Roth, G., & Wake, M. H. (1983). On the problem of stasis in organismal evolution. *Journal of Theoretical Biology*, *101*, 211–224.

Wake, D. B., & Schneider, C. J. (1998). Taxonomy of the plethodontid salamander genus *Ensatina*. *Herpetologica*, *54*, 279–298.

Wakeley, J. (1999). Nonequilibrium migration in human history. *Genetics*, *153*, 1863–1871.

Wiens, J. J., Engstrom, T. N., & Chippindale, P. T. (2006). Rapid diversification, incomplete isolation, and the "speciation clock" in North American salamanders (genus *Plethodon*): Testing the hybrid swarm hypothesis of rapid radiation. *Evolution*, *60*, 2585–2603.

Yang, Z., & Rannala, B. (2014). Unguided species delimitation using DNA sequence data from multiple loci. *Molecular Biology and Evolution*, *31*, 3125–3135.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Kuchta SR, Brown AD, Highton R. Disintegrating over space and time: Paraphyly and species delimitation in the Wehrle's Salamander complex. *Zool Scr*. 2018;47:285–299. https://doi.org/10.1111/zsc.12281