Molecular Ecology (2005) **14**, 1457–1472 doi: 10.1111/j.1365-294X.2005.02524.x

Phylogeographic analysis of mitochondrial gene flow and introgression in the salamander, *Plethodon shermani*

D. W. WEISROCK, K. H. KOZAK and A. LARSON
Department of Biology, Box 1137, Washington University, Saint Louis, MO 63130

Abstract

***Plethodon shermani* comprises a series of geographically disjunct populations occupying high-elevation mountain isolates. These populations hybridize at their borders with salamanders of the *Plethodon glutinosus* species complex, and past range expansions inferred from Pleistocene climatic cycles may have increased the possible genetic interactions between *P. shermani* and species of the *P. glutinosus* complex. Because mitochondrial DNA haplotypes often show introgression across species borders, we survey mtDNA variation for evidence of past and ongoing genetic interactions between *P. shermani*, its close relative *Plethodon cheoah*, and species of the *P. glutinosus* complex. Ongoing hybridization with the *P. glutinosus*-complex species *Plethodon teyahalee* is accompanied by extensive mitochondrial introgression in some Unicoi populations of *P. shermani*, but it has little genetic impact on *P. shermani* populations outside hybrid zones at three other isolates (Tusquitee, Wayah Bald, Standing Indian). Some Unicoi populations of *P. shermani* exhibit mtDNA evidence of past hybridization with diverse lineages from *P. aureolus* and *P. glutinosus*. The Tusquitee isolate of *P. shermani* is also characterized by mtDNA haplotypes most closely related to *Plethodon aureolus* and *P. glutinosus*, presumably introduced by past genetic contact with these species or with introgressed populations of Unicoi *P. shermani*. The mtDNA variation in sampled populations of the Wayah Bald and Standing Indian isolates of *P. shermani* appears largely unaffected by ongoing hybridization. Principal components analyses of allozymic data indicate that *P. shermani* isolates collectively form a genetically homogeneous unit clearly demarcated from species with which they have had current or past genetic interactions. Rapid mtDNA introgression associated with transient contacts between *P. shermani* and other species permits a fine-level resolution of evolutionary lineages not evident from allozymic data.**

Keywords: allozymes, hybridization, introgression, mitochondrial DNA, phylogeny, salamander

Received 16 November 2004; revision accepted 26 January 2005

Introduction

The magnitude of gene flow that occurs between previously isolated populations upon secondary contact depends on multiple factors including degree of sexual isolation, the adaptive and ecological properties of the diverged populations, and the fitness of hybrid offspring (Moore 1977; Barton & Hewitt 1985; Barton 2001). Patterns of gene flow between previously separated populations (i.e. introgression *sensu* Anderson 1949) may reflect not only current genetic exchange but intermittent past hybridization. Limited hybridization at the borders of two species may be sufficient to permit mitochondrial haplotypes from one species to spread by introgression through the other one (e.g. Tegelström 1987; Wilson & Bernatchez 1998; Durand *et al*. 2000; Shimizu & Ueshima 2000; Gerber *et al*. 2001; Sota *et al*. 2001; Walton *et al*. 2001; Masta *et al*. 2002; Redenbach & Taylor 2002; Alves *et al*. 2003).

A history of range shifts and repeated bouts of secondary contact likely fits many species influenced directly or indirectly by glacial advances of the Pleistocene (Hewitt 2000). This scenario suggests that many diverged populations or closely related species may have experienced historical hybridization resulting in introgressive gene flow. A

Correspondence: D. W. Weisrock, Present address: T. H. Morgan School of Biological Sciences, University of Kentucky, Lexington, Kentucky 40506; Fax: (859) 257– 1717; E-mail: weisrock@uky.edu

propensity for mtDNA haplotypes to introgress (Funk & Omland 2003), relative to nuclear markers, may provide an informative record of the history of hybridization between closely related species. By reconstructing the genealogical history of mtDNA haplotypes we may gain insights into the history of genetic exchanges not evident in nuclear markers.

In this study, we investigate the evolutionary history of hybridization among salamander species of the genus *Plethodon* from the southern Appalachian Mountains using both mtDNA sequence data and allozymes. One of these species, *Plethodon shermani*, is a member of the *Plethodon jordani* species complex (Table 1), a group of seven high-elevation species spread across a number of mountain isolates. *P. shermani* occurs in four mountain isolates in the southern Appalachians (Fig. 1) and has a colour morphology distinct from other sympatric and parapatric species of *Plethodon*. In three isolates it has a black body with conspicuous red legs. In the Unicoi Mountains it rarely has red colouration on legs but has lateral white to yellow spots. The other focal species of this study are southern Appalachian members of the *Plethodon glutinosus* species complex (Table 1). Species of the *P. glutinosus* complex generally have larger bodies than those of the *P. jordani* complex, lack conspicuous red colouration, and have varying degrees of lateral and dorsal spotting. Species of the *P. glutinosus* complex occur at a range of elevations from sympatry with high-elevation *P. jordani*-complex species down to sea level; some of these species have well-documented hybrid contacts with *P. shermani* (Table 2; Highton & Henry 1970; Peabody 1978; Highton 1983, 1995; Hairston *et al*. 1992; Highton & Peabody 2000). The most extensive current hybridization occurs between *Plethodon teyahalee* and *P. shermani*, which contact along the periphery of all four mountain isolates of *P. shermani*, and which replace each other along an elevational gradient (Highton & Henry 1970; Hairston 1987). Species of the *P. glutinosus* complex to which we refer have been diagnosed as biological species, and allozyme data appear to support these groupings (Highton *et al*. 1989; Highton & Peabody 2000). Further analyses of both allozyme and mtDNA data support a number of *P. jordani*-complex species as being evolutionarily distinct population-level lineages deserving recognition as phylogenetic species (Weisrock 2003); here we test this hypothesis for geographical isolates of *P. shermani* with particular attention to their genetic interactions with members of the *P. glutinosus* species complex.

Hairston *et al*. (1992) suggest from a long-term morphological study of hybridization between *P. shermani* and

Table 1 The three *Plethodon* species complexes involved in this study (Highton *et al*. 1989; Highton & Peabody 2000). *Plethodon shermani*, the focal species of this study, is part of the *Plethodon jordani* complex but shows genetic interactions with members of the *Plethodon glutinosus* complex. The *Plethodon ouachitae* complex, *Plethodon petraeus* and *Plethodon yonahlossee* are used as outgroups

Plethodon glutinosus complex	*P. albagula*, *P. aureolus*, *P. chattahoochee*, *P. chlorobryonis*, *P. cylindraceus*, *P. glutinosus*, *P. grobmani*, *P. kentucki*, *P. ocmulgee*, *P. savannah*, and *P. teyahalee*
Plethodon jordani complex	*P. amplus*, *P. cheoah*, *P. jordani*, *P. meridianus*, *P. metcalfi*, *P. montanus*, and *P. shermani*
Plethodon ouachitae complex	*P. caddoensis*, *P. fourchensis*, and *P. ouachitae*

Table 2 Descriptions of known geographical and genetic interactions between species of the *Plethodon jordani* species complex (*Plethodon cheoah* and *Plethodon shermani*) and species of the *Plethodon glutinosus* species complex in the Nantahala and Unicoi mountains of the southern Appalachians

Species involved	Geographic contact	Extent of hybridization
P. cheoah–P. teyahalee	sympatric	Maybe occasional hybridization as evidenced by a single morphologically and allozymically intermediate individual found at Stecoah Gap (Highton & Peabody 2000).
P. shermani–P. teyahalee	parapatric	Extensive hybridization occurs along the periphery of all *P. shermani* isolates. Areas between the Standing Indian, Tusquitee, and Wayah isolates are considered hybrid swarms (Highton & Henry 1970; Hairston 1993; Highton & Peabody 2000).
P. shermani–P. chattahoochee	parapatric	Contact is between *P. shermani* in the southern portion of the Standing Indian isolate and *P. chattahoochee* near the GA-NC border and results in a potentially wide hybrid zone (Highton & Peabody 2000).
P. shermani–P. aureolus	parapatric	A wide hybrid zone occurs in the northern portion of the Unicoi isolate (Highton 1983; Highton & Peabody 2000).

P. teyahalee that hybrid contact in the Standing Indian Mountains (and potentially the other three isolates) is relatively recent, potentially a result of lumbering in the early part of the 20th century. An asymmetric movement of *P. teyahalee*-like phenotypic traits into the range of *P. shermani* indicates recent genetic introgression into *P. shermani*. Allozyme analysis of transects through *P. shermani*–*P. teyahalee* contacts in all four mountain isolates is consistent with this hypothesis (Peabody 1978). Although no fixed differences are detected between these two species, large frequency differences occur at a number of inferred allozyme loci. This pattern may reflect recent contact between these two species. The lack of fixed allelic differences could result from recent introgression and may foreshadow an eventual merger of these once-isolated groups. Alternatively, hybrid contact between *P. shermani* and *P. teyahalee* may be an older and ongoing process in which the parental species persist as separate lineages despite limited gene flow at their edges. Highton & Peabody (2000) hypothesize that an ancestral population of *P. shermani* in the Snowbird Mountains of North Carolina has been swamped by past hybridization with *P. teyahalee*. Furthermore, mtDNA introgression facilitated by historical range expansions is a common phylogeographical pattern detected in many other species of the *P. jordani* complex (Weisrock 2003), suggesting that the morphological and allozymic patterns seen in *P. shermani* may not be caused solely by recent secondary contact with species of the *P. glutinosus* complex.

In this study, we synthesize results from phylogeographical analyses of newly reported mtDNA sequence data with reanalysis of allozyme data to evaluate the effects of hybridization and introgression between *P. shermani* and species of the *P. glutinosus* complex. We show through phylogenetic analyses that hybridization has had different effects on mtDNA introgression across the spatial distribution and temporal duration of *P. shermani*. Past hybridization has caused significant introgression of mtDNA haplotypes from the *P. glutinosus* species complex into the Tusquitee and Unicoi isolates of *P. shermani*, whereas the Standing Indian and Wayah isolates exhibit limited evidence of mtDNA introgression. Because of substantial mitochondrial genomic introgression, at least three distinct population lineages can be diagnosed within *P. shermani*. In contrast, ordination-based analyses of allozyme data group *P. shermani* populations as a unit distinct from all southern Appalachian species of the *P. glutinosus* complex. Given the extensive patterns of historical hybridization revealed by mtDNA haplotypes and the significant differentiation of *P. shermani* revealed by the allozyme data, we hypothesize that hybridization between *P. shermani* and species of the *P. glutinosus* complex is not strictly a recent phenomenon and that both intrinsic and extrinsic factors may limit the amount of genetic exchange among species. This work emphasizes the importance of continued usage of mitochondrial haplotype information in phylogeographical studies of congeneric species (Funk & Omland 2003), but also highlights the importance of nuclear–mitochondrial comparisons.

Materials and methods

Population sampling

Tissue samples were used from 18 populations of *Plethodon shermani* ($n = 95$) (Fig. 1, Appendix I). Three of these populations (70 and 74 from the Standing Indian isolate and 73 from the Wayah isolate) were the same populations used in the allozyme study of Highton & Peabody (2000). Three populations from the Unicoi isolate (71, 75, and 79) were provided by R. Highton and putatively exist outside any hybrid zones inferred from allozyme evidence. The remaining populations were field sampled with an effort to collect away from or on the *P. shermani* end of known hybrid zones (Fig. 2). Some populations of *P. shermani* were collected in areas of potential contact with *Plethodon teyahalee* and exhibited morphological patterns of hybridization, typically reduced red colouration in the legs and presence of lateral white spotting. These populations were given a species category according to the taxon they most closely resembled; however, the population number is marked with an 'H' to indicate potential hybridization. This criterion was difficult to apply to specimens from the Unicoi isolate because *P. shermani* populations at this isolate normally lack red leg colouration and have lateral white spotting. *P. shermani* contacts both *Plethodon aureolus* and *P. teyahalee* at the Unicoi isolate. All collections from the Unicoi isolate were made from high-elevation sites considered *P. shermani* populations from allozyme evidence (Peabody 1978; Highton 1983; Highton & Peabody 2000). Furthermore, all *P. shermani* collected in the Unicoi isolate were nearly uniform in morphology and did not contain the dorsal spotting potentially indicative of *P. aureolus* and *P. teyahalee*.

All three populations of *Plethodon cheoah* used by Weisrock (2003) were included ($n = 13$; Fig. 1; Appendix I). Color morphology, allozymes, and mtDNA data all indicate a close phylogenetic relationship between *P. cheoah* and *P. shermani*, but *P. cheoah* is completely sympatric with *P. teyahalee* with minimal evidence of hybridization, making it an important comparison. Representatives of the major clades identified for the five remaining species of the *Plethodon jordani* complex also were included (Weisrock 2003). In addition, a sequence from a single individual of *P. jordani* used by Weisrock (2003) (haplotype jordani-11 from population 89) was included because of its potential involvement in hybridization between *P. jordani* and *P. shermani*.

Within-population sampling in the *P. glutinosus* complex was generally not as high as in *P. shermani* locales but

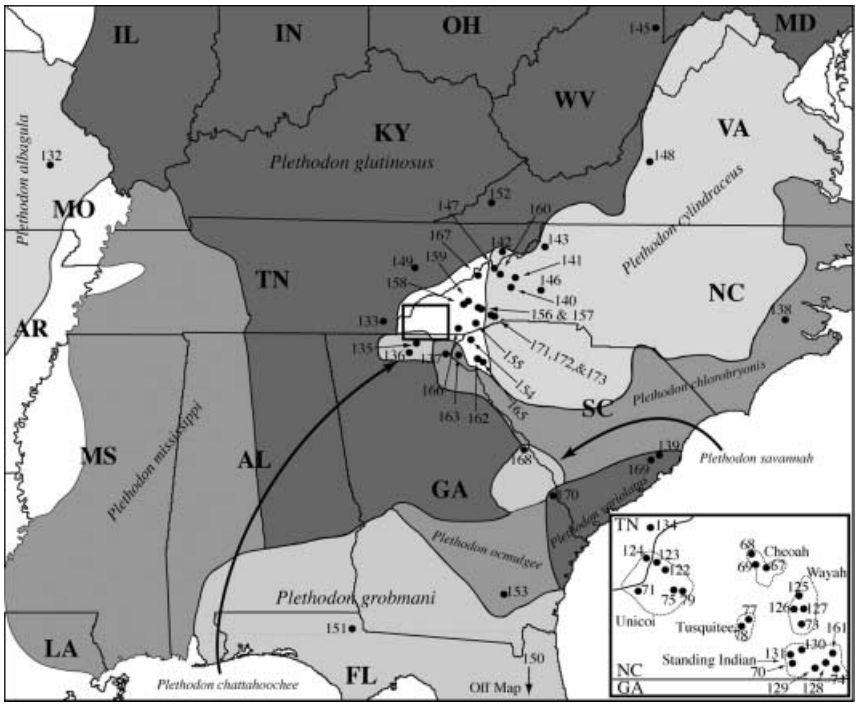


Fig. 1 Populations sampled in this study. Alternating shadings signify the approximate distribution of species of the *Plethodon glutinosus* complex. The central white area approximates the distribution of *Plethodon teyahalee*. *Plethodon aureolus* has a very small range sympatric with both *P. glutinosus* and *P. teyahalee* and is not shaded on the map. The square box highlights the distribution of *Plethodon shermani* (Standing Indian, Tusquitee, Unicoi, and Wayah isolates) and *Plethodon cheoah* (Cheoah isolate), and dotted lines circumscribe their general high-elevation distributions. Numbers correspond to those listed in Appendix I.

covered a wide geographical range for most species. We sampled multiple populations of *P. teyahalee* and *Plethodon cylindraceus* because *P. teyahalee* may have originated from past hybridization between *P. cylindraceus* and *P. shermani* (Highton *et al.* 1989; Hairston 1993). Twelve populations were sampled from the putative range of *P. teyahalee* ($n = 23$), and 11 populations were sampled from the putative range of *P. cylindraceus* ($n = 27$). In areas where these species may contact we used the morphological characteristics described by Highton *et al.* (1989) to discriminate species (small dorsal spots and reduced lateral spotting for *P. teyahalee* vs. large dorsal spots and abundant lateral spotting for *P. cylindraceus*). Sampling for the remaining *P. glutinosus*-complex species of the southern Appalachians (*P. aureolus*, *Plethodon chattahoochee*, *Plethodon chlorobryonis*, *P. glutinosus*, and *Plethodon kentucki*) was limited to one to three populations. Sequences were included for additional species of the *P. glutinosus* complex (*Plethdon albagula*, *Plethodon grobmani*, *Plethodon ocmulgee*, and *Plethodon savannah*). Sequences from the three species of the *Plethodon ouachitae* complex (*Plethodon caddoensis*, *Plethodon fourchensis*, and *P. ouachitae*), *Plethodon yonahlossee* and *Plethodon petraeus* were used as outgroups. *Plethodon glutinosus*-complex samples were obtained through personal field collections and donations by R. Highton.

Laboratory work

DNA extraction, polymerase chain reaction (PCR), and sequencing methods were performed as in Weisrock *et al.* (2001) with the exception that most sequencing reactions were performed using a BigDye Terminator Ready Reaction kit (Perkin-Elmer) and run on either an ABI (PE Applied Biosystems) 373A automated DNA sequencer or an MJ Research BaseStation. Amplification and sequencing of the *ND2* to $tRNA^{Ala}$ mtDNA genic region utilized the forward $tRNA^{Met}$ primer, *L4437* (Macey *et al.* 1997), and the reverse $tRNA^{Asn}$ primer *H5692* (Weisrock *et al.* 2001). Additionally, an internal forward *ND2* primer (5′-TGACAAAANCTNGCCCC-3′) was designed and used for sequencing. The 3′ position of the latter primer anneals close to the midpoint of the *ND2* gene and corresponds to position 5195 of the *Mertensiella luschani* mitochondrial genome (Zardoya & Meyer 2001). All haplotype sequences used in this study have been deposited in GenBank with the Accession nos AY874875–AY874879, AY874897, and AY874996–AY875088.

Sequence alignment

Sequence data from individual reactions were assembled into a contiguous fragment using SEQMAN II version 4.0 (DNASTAR). Assembled contigs were manually aligned in PAUP* version 4.0b10 (Swofford 2002). No regions of ambiguous alignment were present. Length variation was minor and limited to $tRNA^{Ala}$. The protein-coding portion of each sequence was translated into amino acids using the program MACCLADE version 4.0 (Maddison & Maddison 2000). Protein-coding sequence was scanned for premature stop codons, and inferred tRNA sequences were folded into secondary structures following the model of

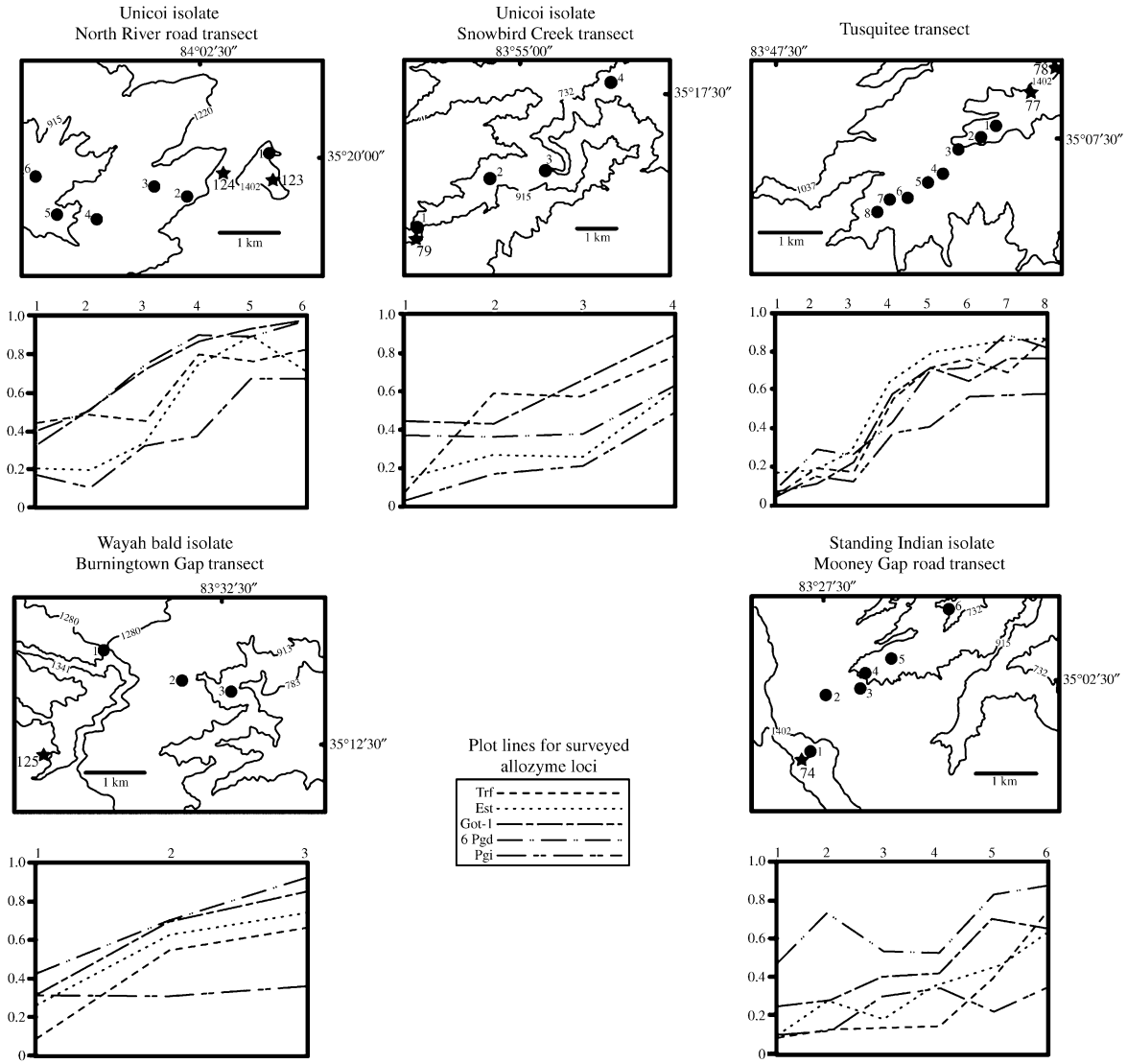


Fig. 2 Geographic and allele-frequency plots used in the hybrid transect work of Peabody (1978). Elevations are given in metres above sea level. Filled circles represent sampling locations used by Peabody (1978) starting in the geographical range of *Plethodon shermani* and moving into *Plethodon teyahalee*. Stars represent the relative position of some of the sampled localities used in the current study.

Kumazawa & Nishida (1993) to confirm authenticity of the mtDNA sequences.

Hierarchical phylogeny estimation

We analysed the haplotypic data using parsimony and Bayesian optimality criteria. Parsimony analyses were conducted in PAUP* version 4.0b10 using a heuristic search with 10 random-addition replicates and tree-bisection–reconnection (TBR) branch swapping. Gaps were treated as missing data. One thousand bootstrap (BP) replicates were performed using similar conditions except that the maximum number of trees saved at each random-addition replicate was set to 1000. Decay-index (DI) values were calculated in PAUP using constraint trees generated in TREEROT version 2.0 (Sorenson 1999).

Bayesian phylogenetic analysis was performed using the parallel-processor version of MRBAYES version 3.0 (Huelsenbeck & Ronquist 2001; Altekar *et al*. 2004) run on a Silicon Graphics Origin 2000 through the Washington University Center for Scientific Parallel Computing (http://harpo.wustl.edu). Eight Markov chains were used with the temperature profile at the default setting of 0.2. The best-fit evolutionary model used was determined through likelihood-ratio tests as implemented in MODELTEST version 3.06 (Posada & Crandall 1998). Uniform priors were used for all parameter estimates and random trees were used to begin each Markov chain. A molecular clock was

not enforced. One million generations were run with tree samplings taken every 1000 generations. Sampled trees were parsed with MRBAYES to construct a phylogram based upon mean branch lengths and with PAUP* to calculate the posterior probabilities (PP) of all branches using a majority-rule consensus tree. All saved trees from generations prior to a stationary –ln likelihood (–lnL) value were discarded. To account for the possibility that the analysis may not be converging upon the optimal posterior distribution, three additional runs were performed using identical conditions. Mean Bayesian parameter estimates for the substitution matrix, base frequencies, gamma shape parameter, and proportion of invariant sites were calculated and used to calculate maximum-likelihood corrected sequence divergences in PAUP*.

Multivariate analysis of allozyme data

Principal components analysis (PCA) of the allozyme data of Highton & Peabody (2000) was used to provide a nonhierarchical view of populations in multidimensional space (Lessa 1990; De Queiroz & Good 1997). Analyses were performed on two subsets of the data including (i) populations from *P. shermani* and populations from species of the *P. glutinosus* complex that are known to hybridize with *P. shermani* or to show mtDNA patterns of hybridization with *P. shermani*, and (ii) only populations from *P. shermani* and *P. teyahalee*. PCA was performed on a matrix of populations and allelic frequencies. The data set of Highton & Peabody (2000) had many more alleles than populations; therefore, any allelic class that did not exceed 5% frequency in at least one population was removed. The first data set contained 87 populations and 104 allelic classes from 20 inferred loci. The second data set contained 31 populations and 73 allelic classes from 18 inferred loci. PCA was performed on the correlation matrix using the program JMP version 3.2.1. Principal component (PC) scores were calculated and used to plot populations in ordination space. A multivariate discriminant analysis was used to determine whether populations could be correctly assigned to a prior classification (species) based upon their PC scores. Analysis of variance (ANOVA) was used to determine which PCs showed significant differences using species categories as treatment effects. To determine whether species categories were significantly different from each other at a particular PC, a Tukey–Kramer honestly significant difference test was used to account for multiple comparisons (Kramer 1956).

Results

Mitochondrial DNA sequence data

Mitochondrial DNA sequences reported here are combined with sequences from Weisrock (2003) to form a data set of 214 individual sequences. This data set contains 105 different haplotypes. Haplotypic information for *Plethodon cheoah*, *Plethodon shermani*, and *Plethodon glutinosus*-complex species is presented in Appendix II. The haplotypic data set contains 530 variable sites of which 396 are parsimony informative.

Likelihood-ratio tests favour the general time-reversible model of evolution with some sites being invariable and rate variation following a gamma distribution (GTR + I + Γ). We observe low guanine base frequencies (average Bayesian-estimated base frequencies across haplotypes: A = 37%, C = 25%, G = 9%, T = 28.0%). Bayesian estimates of substitution-rate parameters indicate high transition rates relative to transversions (A⇔C = 1.38, A⇔G = 20.83, A⇔T = 0.68, C⇔G = 1.11, C⇔T = 9.56, G⇔T = 1). The gamma-shape parameter and proportion of invariant sites are estimated at 0.9 and 0.31, respectively. Reconstructed secondary structures from the tRNA genes and the lack of premature stop codons in the *ND2* gene suggest that homologous mitochondrial genes are being compared.

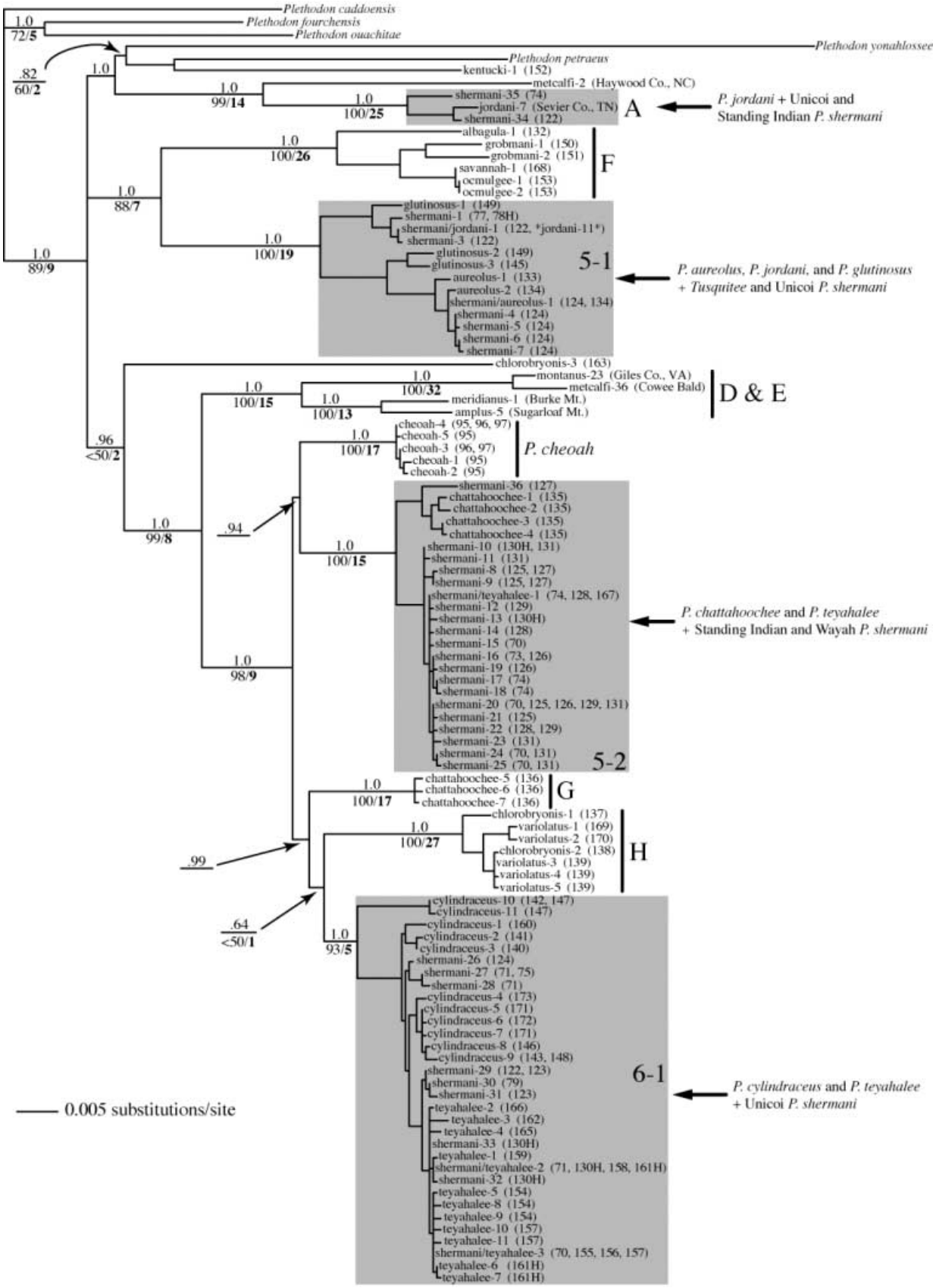
Hierarchical phylogenetic results

Parsimony and Bayesian analyses produce similar phylogenetic groupings of haplotypes (Fig. 3). Parsimony analysis produces 12 trees of 1429 steps in length. The mean –lnL of the Bayesian posterior distribution is 8903.70 (± 18.67).

Haplotypes sampled from *P. shermani* form four separate clades in the tree. Three of these clades are identified in the Bayesian phylogram (Fig. 3) as 5-1, 5-2, and 6-1, labelled according to their highest nesting level in a nested haplotype network analysis (Weisrock 2003). The fourth clade is labelled clade 'A' following Weisrock (2003) for the majority of haplotypes sampled from *P. jordani*.

Clade 5-1 is strongly supported (PP = 1.0; BP = 100%; DI = 19) and contains haplotypes sampled from *P. shermani* populations 77 and 78 in the Tusquitee isolate and populations 122 and 124 in the Unicoi isolate. Phylogenetically

Fig. 3 Phylogram based on mean branch lengths of trees sampled from the Bayesian posterior distribution. Numbers above branches indicate the posterior probability of a clade. Numbers below branches represent bootstrap (before slash) and decay indices (after slash) for clades concordantly resolved in parsimony analysis. The four lightly shaded boxes encompass clades containing haplotypes sampled from *Plethodon shermani*. Clade labels are from the haplotype-network analysis of Weisrock (2003), and a short description of the geographical distribution of haplotypes within each *P. shermani* clade is provided. Clades A, and D and E are labelled following the nomenclature of Weisrock (2003). Clade A includes most haplotypes sampled from populations of the red-cheeked salamander, *Plethodon jordani*. Clades D and E contain haplotypes sampled from populations of *Plethodon amplus*, *Plethodon meridianus*, *Plethodon metcalfi*, and *Plethodon montanus*. Additional clades containing haplotypes sampled solely from species of the *Plethodon glutinosus* complex are labelled as clades F, G, and H.


Plethodon caddoensis
Plethodon fourchensis
Plethodon ouachitae
Plethodon yonahlossee
Plethodon petraeus
kentucki-1 (152)
metcalfi-2 (Haywood Co., NC)
shermani-35 (74)
jordani-7 (Sevier Co., TN)
shermani-34 (122)
A
P. jordani + Unicoi and Standing Indian P. shermani
albagula-1 (132)
grobmani-1 (150)
grobmani-2 (151)
savannah-1 (168)
ocmulgee-1 (153)
ocmulgee-2 (153)
F
glutinosus-1 (149)
shermani-1 (77, 78H)
shermani/jordani-1 (122, *jordani-11*)
shermani-3 (122)
glutinosus-2 (149)
glutinosus-3 (145)
aureolus-1 (133)
aureolus-2 (134)
shermani/aureolus-1 (124, 134)
shermani-4 (124)
shermani-5 (124)
shermani-6 (124)
shermani-7 (124)
5-1
P. aureolus, P. jordani, and P. glutinosus + Tusquitee and Unicoi P. shermani
chlorobryonis-3 (163)
montanus-23 (Giles Co., VA)
metcalfi-36 (Cowee Bald)
meridianus-1 (Burke Mt.)
amplus-5 (Sugarloaf Mt.)
D & E
cheoah-4 (95, 96, 97)
cheoah-5 (95)
cheoah-3 (96, 97)
cheoah-1 (95)
cheoah-2 (95)
P. cheoah
shermani-36 (127)
chattahoochee-1 (135)
chattahoochee-2 (135)
chattahoochee-3 (135)
chattahoochee-4 (135)
shermani-10 (130H, 131)
shermani-11 (131)
shermani-8 (125, 127)
shermani-9 (125, 127)
shermani/teyahalee-1 (74, 128, 167)
shermani-12 (129)
shermani-13 (130H)
shermani-14 (128)
shermani-15 (70)
shermani-16 (73, 126)
shermani-19 (126)
shermani-17 (74)
shermani-18 (74)
shermani-20 (70, 125, 126, 129, 131)
shermani-21 (125)
shermani-22 (128, 129)
shermani-23 (131)
shermani-24 (70, 131)
shermani-25 (70, 131)
5-2
P. chattahoochee and P. teyahalee + Standing Indian and Wayah P. shermani
chattahoochee-5 (136)
chattahoochee-6 (136)
chattahoochee-7 (136)
G
chlorobryonis-1 (137)
variolatus-1 (169)
variolatus-2 (170)
chlorobryonis-2 (138)
variolatus-3 (139)
variolatus-4 (139)
variolatus-5 (139)
H
cylindraceus-10 (142, 147)
cylindraceus-11 (147)
cylindraceus-1 (160)
cylindraceus-2 (141)
cylindraceus-3 (140)
shermani-26 (124)
shermani-27 (71, 75)
shermani-28 (71)
cylindraceus-4 (173)
cylindraceus-5 (171)
cylindraceus-6 (172)
cylindraceus-7 (171)
cylindraceus-8 (146)
cylindraceus-9 (143, 148)
shermani-29 (122, 123)
shermani-30 (79)
shermani-31 (123)
teyahalee-2 (166)
teyahalee-3 (162)
teyahalee-4 (165)
shermani-33 (130H)
teyahalee-1 (159)
shermani/teyahalee-2 (71, 130H, 158, 161H)
shermani-32 (130H)
teyahalee-5 (154)
teyahalee-8 (154)
teyahalee-9 (154)
teyahalee-10 (157)
teyahalee-11 (157)
shermani/teyahalee-3 (70, 155, 156, 157)
teyahalee-6 (161H)
teyahalee-7 (161H)
6-1
P. cylindraceus and P. teyahalee + Unicoi P. shermani
0.005 substitutions/site

associated with these haplotypes are haplotypes sampled from populations of *P. glutinosus* and *Plethodon aureolus* and a single haplotype sampled from *Plethodon jordani* in the Great Smoky Mountains. Average corrected sequence divergence within 5-1 is 2.65%. Two haplotypes are shared between some of these species, suggesting recent gene flow. (i) Shermani/aureolus-1 is found in individuals of *P. aureolus* from population 134 and *P. shermani* from population 124. Population 124 is found south of the putative northern limit of *P. shermani* in the Unicoi isolate as defined by allozyme data (Highton 1983), but not far removed from a known hybrid zone between these two species along Sassafras Ridge in Tennessee. (ii) Shermani/jordani-1 is found in individuals from population 122 of *P. shermani* in the Unicoi isolate and a single individual from population 89 of *P. jordani* (haplotype reported as jordani-11 in Weisrock 2003).

Clade 5-2 is strongly supported (PP = 1.0; BP = 100%; DI = 15) and is composed of almost all haplotypes sampled from populations of *P. shermani* in the Wayah and Standing Indian isolates, as well as haplotypes sampled from *Plethodon teyahalee* population 167 and *Plethodon chattahoochee* population 135. All individuals from *P. chattahoochee* population 135 have haplotypes that are nested with a haplotype from *P. shermani* population 127, together forming the relatively divergent sister group to a clade containing most haplotypes sampled from the Wayah and Standing Indian isolates of *P. shermani*. Additionally, a single individual from population 167 of *P. teyahalee* has a haplotype identical to those found in *P. shermani* populations 74 and 128 of the Standing Indian isolate. Average corrected sequence divergence within clade 5-2 is 0.84%.

Clade 6-1 is moderately supported in the parsimony analysis (BP = 93%; DI = 5) and strongly supported in the Bayesian analysis (PP = 1.0). It is composed of all haplotypes sampled from *P. cylindraceus*, most haplotypes sampled from *P. teyahalee*, and haplotypes sampled from *P. shermani* in the Unicoi (populations 71, 75, 79, 122, 123 and 124) and Standing Indian (populations 70 and 130) isolates. Clade 6-1's haplotype distribution among species has the most complicated pattern of all *P. shermani* haplotype clades. *Plethodon cylindraceus* does not share haplotypes with any other species. Two haplotypes are found in both *P. shermani* and *P. teyahalee*. Shermani/teyahalee-2 is found in two populations of *P. teyahalee* (158 and 161) and two populations in two different isolates of *P. shermani* (71 and 130). Shermani/teyahalee-3 is found in three different populations of *P. teyahalee* (155, 156, and 167) and a single population of *P. shermani* in the Standing Indian isolate (70). Most *P. shermani* individuals sampled from populations 71, 75, 79, and 123 in the Unicoi isolate have haplotypes that nest within clade 6-1. None of these haplotypes are found in any sampled population of *P. teyahalee*, yet their placement in clade 6-1 suggests that they may have resulted from an old hybridization event with a *P. cylindraceus*/*P. teyahalee* ancestor. It is noteworthy that a single individual of *P. shermani* from population 124, which is dominated by mtDNA haplotypes from clade 5-1, has a haplotype (shermani-26) from clade 6-1. Average corrected sequence divergence within 6-1 is 0.98%.

The fourth clade containing haplotypes sampled from *P. shermani* is strongly supported (PP = 1.0; BP = 100%; DI = 25) and contains a representative haplotype from *P. jordani* of the Great Smoky Mountains (designated 'clade A' in Fig. 3). The *P. jordani* haplotype is a relatively common haplotype found in three different sampled populations of *P. jordani* (Weisrock 2003). Haplotype shermani-34 is sampled from a single individual from population 122 in the Unicoi isolate, and haplotype shermani-35 was sampled from a single individual in population 74 of the Standing Indian isolate. Average corrected sequence divergence among haplotypes in this clade is 1.82%.

All haplotypes sampled from *P. cheoah* form a strongly supported monophyletic group (PP = 1.0; BP = 100%; DI = 17). The exact phylogenetic position of this branch is relatively uncertain in the parsimony analysis but is placed as the sister group to clade 5-2 in the Bayesian analysis with fairly high support (PP = 0.94). Levels of corrected sequence divergence within the *P. cheoah* clade are low (0.23%). Average corrected sequence divergence between the *P. cheoah* clade and clade 5-2 is 5.36%.

Some additional relationships involving haplotypes sampled from the *P. glutinosus* complex are noteworthy. (i) Haplotype clade 5-1 is the sister group to a strongly supported clade of haplotypes sampled from *Plethodon albagula*, *Plethodon grobmani*, *Plethodon ocmulgee*, and *Plethodon savannah* (clade F in Fig. 3; PP = 1.0; BP = 100%; DI = 26). The branch uniting clade F and clade 5-1 is strongly supported by both analyses (PP = 1.0; BP = 100%; DI = 7). Average corrected sequence divergence between clades F and 5-1 is 12.23%. (ii) Clade 6-1 is placed in a more inclusive clade with two other strongly supported groupings of haplotypes. One of these groups contains all haplotypes sampled from *Plethodon chattahoochee* population 136 (clade G in Fig. 3). The other group contains all sampled haplotypes from *Plethodon variolatus* and haplotypes sampled from two populations of *P. chlorobryonis* (clade H in Fig. 3); the grouping of these three clades is poorly supported by parsimony analysis but receives a high posterior probability in the Bayesian analysis (PP = 0.99). Relationships among these three clades are poorly supported in all analyses. Finally (iii) both individuals sampled from *P. chlorobryonis* population 163 contained a haplotype (chlorobryonis-3) placed in a very divergent position from all other haplotypes sampled from *P. chlorobryonis*. Phylogenetic placement of chlorobryonis-3 is relatively uncertain in the parsimony analysis, but Bayesian analysis strongly supports it as the sister lineage to a clade containing clades

Table 3 Principal components showing significant differences among treatment effects (species categories) in a one-way ANOVA using the allozyme frequency data of Highton & Peabody (2000). The upper part of the table summarizes an analysis using data from *Plethodon aureolus*, *P. chattahoochee*, *P. cylindraceus*, *P. glutinosus*, *P. shermani*, and *P. teyahalee*. The lower part of the table summarizes an analysis using data only from *P. shermani* and *P. teyahalee*

Principal component	Eigenvalue (% variation)	ANOVA F (*P* value)	Alleles loading ≥ 0.5 or = −0.5
PC1	13.57(13.04)	402.81 (< 0.001)	*Alb c* (−0.744), *Alb d* (0.766), *Est e* (−0.875), *Est p* (0.668), *Est t* (0.714), *Got-1 h* (−0.878), *Got-1 j* (0.907), *Ldh* (*muscle*) *g* (0.829), *Ldh (muscle) h* (−0.881), *6-Pgd b* (−0.840), *6-Pgd d* (0.862), *Pgi c* (−0.664), *Pgi g* (0.658), *Pt-2 a* (0.580), *Pt-2 b* (−0.778), *Trf a* (−0.744), *Trf o* (0.566)
PC2	12.15 (11.69)	147.75 (< 0.001)	*Est c* (0.629), *Est g* (0.701), *Est k* (0.589), *Est x* (0.579), *Est ii* (0.536), *a-Gpd b* (−0.581), a-*Gpd e* (0.581), *Idh-1 b* (−0.654), *Idh-1 c* (0.726), *Idh-1 e* (0.580), *Lap a* (−0.776), *Lap c* (0.780), *Ldh* (*heart*) *h* (0.686), *Pep c* (0.792), *Pep d* (−0.789), *6-Pgd f* (0.618), *Pt-2 a* (−0.538), *Pt-2 c* (0.853)
PC3	8.03 (7.73)	82.32 (< 0.001)	*Est r* (0.530), *Idh-1 b* (−0.516), *Idh-1 e* (0.573), *6-Pgd c* (0.590), *Mdh-2 d* (−0.526), *Mdh-2 f* (0.513), *Trf k* (0.571)
PC4	6.67 (6.41)	130.34 (< 0.001)	*Fum a* (0.531), *Idh-2 c* (−0.655), *Idh-2 d* (0.669), *Ldh (muscle) f* (0.798), *Pt-1 b* (−0.764), *Pt-1 c* (0.764), *Pt-2 d* (0.806),
PC5	4.97 (4.78)	28.69 (< 0.001)	*Est j* (0.672), *Mdh-1 a* (0.519), *Mdh-1 b* (−0.519), *Pgi c* (−0.507), *Pgi g* (0.509), *Trf a* (−0.539), *Trf I* (0.669),
PC6	4.48 (4.31)	2.29 (0.05)	*Pgm c* (−0.555), *Pgm d* (0.543),
PC1	20.08 (27.14)	260.94 (< 0.001)	*Est d* (0.588), *Est e* (−0.972), *Est g* (0.518), *Est h* (0.738), *Est j* (0.544), *Est p* (0.532), *Est r* (0.655), *Est t* (0.552), *Est x* (0.510), *Got-1 h* (−0.937), *Got-1 j* (0.866), *Got-1 L* (0.659), *Idh-1 b* (−0.839), *Idh-1 e* (0.840), *Mdh-2 d* (−0.626), *Mdh-2 f* (0.626), *6-Pgd b* (−0.858), *6-Pgd c* (0.752), *6-Pgd d* (0.656), *Pgi c* (−0.839), *Pgi g* (0.839), *Pgm a* (0.591), *Pgm c* (−0.585), *Pt-2 b* (−0.642), *Pt-2 c* (0.539), *Pt-2 e* (0.540), *Trf a* (−0.930), *Trf d* (0.718), *Trf f* (0.776), *Trf g* (0.616), *Trf h* (0.684), *Trf k* (0.853), *Trf l* (0.622)

D & E, G, H, *P. cheoah*, 5-2, and 6-1. Chlorobryonis-3 has 15.08% average corrected sequence divergence from members of its sister group.

Principal components analysis of allozyme data

Principal components analysis of allozyme data of Highton & Peabody (2000) for *P. shermani* and species of the *P. glutinosus* complex that are known to hybridize with *P. shermani* or show mtDNA patterns of hybridization with *P. shermani* produce a first, second, and third PC with eigenvalues of 13.57, 12.15, and 8.04, respectively, which account for 13.04%, 11.69%, and 7.73% of the total variation in the data set, respectively (Table 3). The first 13 PC axes explain more variation in allele frequencies than expected by chance according to a broken-stick distribution (Legendre & Legendre 1998); however, only the first six PCs produce scores that can significantly differentiate at least one of the species using a univariate ANOVA (Table 3). Using scores from the first six PCs, a discriminant-function analysis assigns each population correctly to its *a priori* defined species category with a probability of one. Plots involving the first two PCs produce three major groupings (Fig. 4). One group contains all populations from *P. aureolus* and *P. glutinosus*. A second group contains all populations from *P. chattahoochee*. The third group contains all populations of *P. shermani*, *P. cylindraceus*, and *P. teyahalee*. The combination of populations from *P. shermani*, *P. cylindraceus*, and *P. teyahalee* can be significantly differentiated from the combination of populations from *P. aureolus*, *P. chattahoochee*, and *P. glutinosus* in PC1 using a Tukey–Kramer test (results not shown). A number of alleles have high loadings on this PC; however, few alleles are completely diagnostic of these groupings. The most diagnostic of all alleles is allele *h* of the inferred locus *Ldh* (muscle). This allele is fixed or in very high frequency in nearly every population of

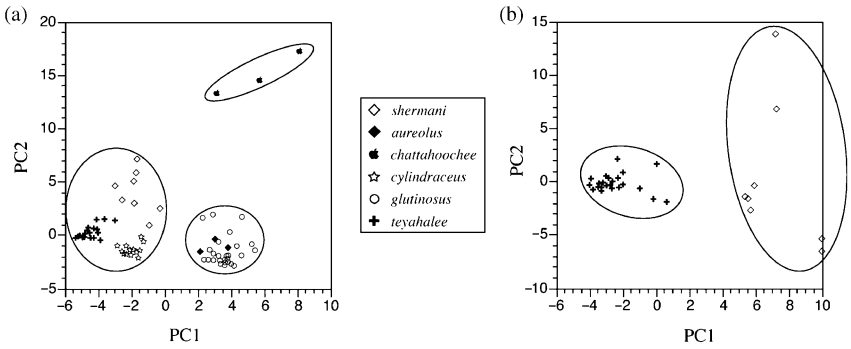


Fig. 4 Ordination plots of the first two principal components of allozyme data of Highton & Peabody (2000) for (a) *Plethodon shermani* and species of the *Plethodon glutinosus* species complex that are known to hybridize or show mtDNA patterns of hybridization with *P. shermani* and (b) only *P. shermani* and *Plethodon teyahalee*. Ellipses denote distinct groupings of populations in ordination space.

P. shermani, *P. cylindraceus*, and *P. teyahalee* and is absent or low in frequency in all populations of *P. glutinosus* and *P. aureolus*, but it has very high frequency in two of the three populations of *P. chattahoochee*. However, *P. chattahoochee* can be distinguished easily on PC2 and diagnosed by fixation or high frequencies of *Pep* allele *c*, which is absent from the other five species.

An additional PCA limited to data from *P. shermani* and *P. teyahalee* is unable to explain a large portion of the variance in the allozyme data. The first 10 PC axes explain more variation than expected by chance according to a broken-stick distribution. The first, second, and third PCs have eigenvalues of 20.08, 11.37, and 6.52, respectively, which explain 27.14%, 15.37%, and 8.82% of the total variation in the data set, respectively. Ordination of the first two PCs produces two groupings of populations corresponding to the two individual species (Fig. 4). These two groupings can be statistically differentiated from each other only using scores from PC1 in an ANOVA (Table 3). No fixed differences exist between these two species. Many inferred loci exhibit high loadings on PC1 and the highest-loading alleles are those used in the transect studies of Peabody (1978). *Est* allele *e* is found in fixation or high frequency in all populations of *P. teyahalee* and is absent or low in frequency in all populations of *P. shermani*. A similar pattern occurs for allele *b* of the protein *Idh-1* and allele a of the protein *Trf*. *Got-1* allele *l* is found in low to moderate frequencies in populations of *P. shermani* and is absent from almost all populations of *P. teyahalee*.

Discussion

Hybridization and introgression

Mitochondrial DNA haplotype variation in *Plethodon shermani* and associated species of the *Plethodon glutinosus* complex provides insight into their evolutionary history not available through analysis of allozyme allele frequencies alone. The presence in *P. shermani* of mtDNA haplotypes from four divergent clades provides evidence for a history of hybrid interactions with *Plethodon jordani* and numerous species of the *P. glutinosus* complex. This pattern is sharply contrasted with the PCA of the allozyme data, which groups populations from all four mountain isolates of *P. shermani* together in multidimensional space and significantly differentiates this cluster from all other included species. Differential sorting of ancestral mtDNA polymorphism across isolates of *P. shermani* is not likely to explain these patterns. In some cases, haplotypes are shared between geographically disjunct species that are otherwise characterized by deeply divergent mitochondrial lineages. These patterns would not be expected under a process of stochastic sorting of ancestral polymorphism; consequently, introgressive hybridization is a more likely explanation.

Hybridization has had varied effects on *P. shermani* depending on its geographical mountain isolate and in some cases provides a significant contrast to the effects of hybridization on the nuclear gene pool as indicated by allozyme data. We summarize these findings in the succeeding discussion.

Tusquitee isolate

Two different scenarios may explain the mtDNA patterns resolved in populations of *P. shermani* from the Tusquitee isolate. If the haplotypes in clade 5-1 are derived from the *P. glutinosus*-complex species, *Plethodon aureolus* and *P. glutinosus*, then past hybridization may have produced introgression of mtDNA haplotypes from these species into the Tusquitee isolate of *P. shermani*. Alternatively, ancestral Tusquitee populations may have had historical genetic contact with ancestral, but already-introgressed Unicoi populations, thus transferring mtDNA haplotypes characteristic of the species *P. aureolus* and *P. glutinosus*. In either case, these results are surprising considering that the Tusquitee isolate is surrounded by a hybrid zone with *Plethodon teyahalee*. Although only two populations of *P. shermani* are sampled from the Tusquitee isolate, one is sampled outside a known hybrid zone with *P. teyahalee* and is less than 2 km from a putatively nonhybrid population used by Highton & Peabody (2000). Animals collected from these two populations have high levels of red colouration on their legs and no indication of phenotypic traits resembling any of the *P. glutinosus*-complex species.

Mitochondrial introgression in this isolate must represent some form of past genetic contact because no current contact exists between Tusquitee *P. shermani* and Unicoi *P. shermani* or with *P. aureolus* or *P. glutinosus*. The latter two species get no closer to Tusquitee *P. shermani* than just west of the Unicoi Mountains in eastern Tennessee, nearly 40 km away. The mtDNA patterns seen in Tusquitee *P. shermani* indicate that historical range changes likely explain the patterns of mtDNA introgression.

In contrast to the mtDNA introgression in the Tusquitee isolate, allozyme allele frequencies from within the isolate are more characteristic of other *P. shermani* isolates (Fig. 4; Highton & Peabody 2000). The populations used in Highton & Peabody (2000) were specifically chosen to reduce the potential effects of hybrid interactions with *P. teyahalee*. However, these data provide little evidence for hybridization with *P. aureolus* and *P. glutinosus*. Allozyme analysis of an elevational transect from a population within the Tusquitee isolate down to the lower-elevation range of *P. teyahalee* produces a narrow cline of allele frequencies at multiple loci from high frequencies of alleles more characteristic of *P. shermani* to high frequencies of alleles more characteristic of *P. teyahalee* (Fig. 2; Peabody 1978). This allozyme pattern indicates hybridization between these two species, and greater mtDNA sampling closer to areas of current secondary contact may reveal patterns of mitochondrial introgressive hybridization with *P. teyahalee*. Nonetheless, the contrasting patterns of mtDNA and allozyme variation reveal a complicated history of hybridization in the Tusquitee *P. shermani*, where historical range shifts and gene flow have led to substantial introgression of the mtDNA characteristic of a different species (*P. aureolus*/*P. glutinosus*) than the one with which it currently hybridizes (*P. teyahalee*). Furthermore, the allozyme data (Fig. 4; Peabody 1978; Highton & Peabody 2000) indicate that historical contact was sufficiently limited that no substantial introgression is evident for nuclear markers characteristic of *P. aureolus*/*P. glutinosus*.

Unicoi isolate

Introgressive hybridization has made a substantial impact also on mtDNA variation of *P. shermani* in the Unicoi isolate. All individuals sampled in this isolate contain mtDNA haplotypes associated with other species in the *P. jordani* and *P. glutinosus* complexes. This result is not surprising given the resemblance between the *P. glutinosus* complex and Unicoi *P. shermani* in colour morphology. In the northern portion of the isolate, most individuals sampled from population 124 have haplotypes from clade 5-1, and one individual shares a haplotype with an individual of *P. aureolus* from population 134. None of the individuals sampled from population 124 shows any evidence of dorsal spotting that would suggest hybridization between *P. aureolus* and *P. shermani*; however, Highton (1983) notes that individuals in the northeastern range of *P. aureolus* have little or no dorsal spotting, which would limit morphological diagnosis of hybridization. Nonetheless, this population is within the Unicoi range of *P. shermani* as indicated by allozyme variation (Fig. 2; Highton 1983; Peabody 1978).

Most *P. shermani* individuals from the remaining populations in the Unicoi isolate have mtDNA haplotypes from group 6-1, which characterizes *P. glutinosus*-complex species *P. cylindraceus* and *P. teyahalee*. This result is not surprising given the extensive hybridization that occurs between *P. shermani* and *P. teyahalee* around the entire periphery of the Unicoi isolate (Table 2). Unlike *P. teyahalee*, *Plethodon cylindraceus* does not share any haplotypes with *P. shermani*, indicating a lack of recent genetic exchange with *P. shermani*. However, a number of *P. shermani* haplotypes are intermediate between *P. cylindraceus* haplotypes and *P. teyahalee* haplotypes (Fig. 3), suggesting past hybridization between *P. shermani* and a *P. cylindraceus–P. teyahalee* ancestor. Unicoi populations of *P. shermani* also show relatively recent genetic interactions with *P. jordani* because many individuals sampled from population 122 have mtDNA haplotypes shared or closely related to haplotypes sequenced from *Plethodon jordani*. In addition, some of these haplotypes are in clade 5-1, indicating a more complicated pattern of historical gene flow involving populations of *P. aureolus*/*P. glutinosus*.

Allozyme alleles characteristic of *P. shermani* throughout its range occur in moderate to high frequency in the Unicoi isolate (Peabody 1978; Highton & Peabody 2000). The lack of fixed allelic differences in allozymes between *P. shermani* and *P. teyahalee* may represent extensive hybridization between these two species. However, the allozymic patterns are in sharp contrast to the complete mtDNA introgression of Unicoi *P. shermani*. As in the Tusquitee isolate, the history of hybridization in the Unicoi isolate has likely involved historical reproductive contacts with other species. These contacts have been facilitated by past range expansions out of their currently isolated distributions and have produced patterns of mtDNA and allozyme variation similar to that found in the Tusquitee isolate: complete mtDNA introgression of haplotypes associated with species of the *P. glutinosus* complex, but maintenance of high allele frequencies of nuclear-encoded proteins that characterize *P. shermani* as a whole.

Standing Indian and Wayah isolates

In contrast to the Tusquitee and Unicoi isolates, the mitochondrial genetic integrity of the Standing Indian and Wayah isolates appears complete despite extensive hybridization around the periphery of both isolates with *P. teyahalee* and past contact with other species of the

P. glutinosus complex. Almost all individuals of *P. shermani* sampled from these two isolates contain haplotypes that form clade 5-2 with the exceptions being from populations 70 and 130, the latter of which appears to be a hybrid population with *P. teyahalee*. While mitochondrial introgression in the Tusquitee and Unicoi isolates has typically revealed directional patterns of gene flow from *P. glutinosus*-complex species into *P. shermani* isolates, the opposite trend is evident in the Standing Indian and Wayah isolates. An individual of *P. teyahalee* from population 167, nearly 100 km to the northeast, shares a haplotype found in *P. shermani* individuals in the Standing Indian and Wayah isolates, and all haplotypes from a population of *Plethodon chattahoochee* 40 km to the southwest of the Wayah isolate have a close relationship to a haplotype sampled only from *P. shermani* population 127 in the Wayah isolate. It is interesting to note that a single individual sampled from *P. shermani* population 74 has a haplotype more closely related to those of *P. jordani* in the Great Smoky Mountains nearly 65 km to the northwest. As in the other isolates, the large geographical distances separating the populations of *P. chattahoochee*, *P. jordani*, *P. shermani*, and *P. teyahalee* described here indicate past hybridization involving historical range shifts.

The discordant patterns of variation in the mtDNA and allozyme data are challenging to explain. It is possible that current hybridization between *P. shermani* and *P. teyahalee* in the Standing Indian and Wayah isolates is of very recent origin, as proposed by Hairston *et al.* (1992), whereas hybridization in the Tusquitee and Unicoi isolates has a much longer duration. However, the mtDNA data reveal a long history of hybridization between all isolates of *P. shermani* and species of the *P. glutinosus* complex much older than current distributional interactions. It therefore seems unlikely that hybridization at any isolate would have been confined to postlogging habitat disturbance in the last 100 years (Hairston *et al*. 1992).

Variation in the evolution of sexual isolation among isolates of *P. shermani* may explain the different patterns of hybrid introgression. Laboratory studies of reproductive isolation between *P. shermani* and *P. teyahalee* in and around the Standing Indian isolate indicate some sexual isolation between allopatric populations of these species (Reagan 1992). Sexual isolation is only partial, however, and no sexual isolation is evident between *P. shermani* and hybrids of the two species. However, sexual isolation may be sufficient to have limited gene flow between these species in the Standing Indian and Wayah isolates, while a lesser extent of sexual isolation in the Tusquitee and Unicoi isolates of *P. shermani* could account for their extreme mtDNA introgression.

Courtship pheromones are an important factor in plethodontid reproduction (Arnold 1977; Houck 1986), and the evolution of sexual isolation among species in the *P. jordani* and *P. glutinosus* complexes may be tied to the evolution of male-delivered pheromones affecting female receptivity (Rollman *et al*. 1999). Experimental studies show that pheromone delivery significantly reduces the time needed to reach different stages of the courtship process (Houck & Reagan 1990; Houck *et al*. 1998). Comparative studies of populations from *P. shermani* document significant interpopulation differences in the types and amounts of isoforms of proteins found in pheromone extracts (Rollman *et al*. 2000), providing a potential basis for differing levels of sexual isolation among isolates of *P. shermani*.

Hybridization and species delimitation

Plethodon shermani presents a unique situation among species of the *P. jordani* complex in which mitochondrial haplotypic variation diagnoses population-level lineages that cannot be distinguished by allozyme variation. Weisrock (2003) demonstrates that comparable analyses of mtDNA sequence data and allozyme data concordantly diagnose the other six species of the *P. jordani* complex as phylogenetic species (*sensu* Cracraft 1989). In contrast, two of the four mountain isolates of *P. shermani* have been completely introgressed by mtDNA haplotypes from the *P. glutinosus* complex. Because the introgressed haplotypes are confined to individual isolates, this introgression probably followed the last genetic contact between these isolates and other isolates of *P. shermani*. Mitochondrial introgression in this case permits genetic diagnosis of the separate evolutionary histories of the Unicoi and Tusquitee isolates relative to other populations of *P. shermani*, a finding not apparent from analyses of allozymic data. Although the mitochondrial DNA data diagnose these separate population lineages, sequence divergence between their mtDNA haplotypes would overestimate the evolutionary separation of the lineages, which was probably very recent given their lack of allozymic differentiation.

The Standing Indian and Wayah isolates form a population-level lineage (*sensu* De Queiroz 1999) diagnosed by mtDNA haplotypes of group 5-2. Hybridization with *P. teyahalee* at the geographical borders of this lineage has been too limited to erase genetic evidence of its separate evolutionary history. The overwhelming majority of individuals sampled from the Standing Indian and Wayah isolates contain haplotypes from group 5-2, and only two populations of non-*P. shermani* species have haplotypes from this group. Furthermore, Bayesian analysis places group 5-2 as the sister clade to the other red-legged *Plethodon* species, *Plethodon cheoah*. There is very little evidence of hybridization between *P. cheoah* and any member of the *P. glutinosus* complex (Highton & Peabody 2000), suggesting that introgression has not clouded its mtDNA history. Moreover, the sister-group relationship of the two red-legged species

is not surprising, given that no other species of eastern *Plethodon* have this colour pattern.

PCA of allozyme data differentiates all populations of *P. shermani* from all sampled populations of hybridizing species of the *P. glutinosus* complex. A separate analysis restricted to populations of *P. shermani* and *P. teyahalee* further demonstrates their genetic differentiation and reveals a number of alleles that have significant loadings on the first PC axis and can discriminate these two species. These results suggest that although all isolates of *P. shermani* have had some hybrid interactions with members of the *P. glutinosus* complex at some time during their evolutionary histories, and that this hybridization sometimes has produced introgression of mitochondrial haplotypes, this hybridization has been limited to the geographical borders of species and in each case was too limited to produce a genetic merging between *P. shermani* populations and any *P. glutinosus*-group populations.

Acknowledgements

This work was supported by an NSF grant DDIG-0105066 to DWW and AL and a grant-in aid from the Highlands Biological Station, Highlands, NC to DWW. Special thanks go to R. Highton for support of this project, assistance in the field, and comments on an earlier draft of this manuscript. Two anonymous reviewers provided suggestions that improved the paper. D.K. Weisrock provided valuable assistance during field work.

References

Altekar G, Dwarkadas S, Huelsenbeck JP, Ronquist F (2004) Parallel metropolis coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. *Bioinformatics*, **20**, 407–415.

Alves PC, Ferrand N, Suchentrunk F, Harris DJ (2003) Ancient introgression of *Lepus timidus* mtDNA into *L. granatensis* and *L. europaeus* in the Iberian Peninsula. *Molecular Phylogenetics and Evolution*, **27**, 70–80.

Anderson E (1949) *Introgressive Hybridization*. Wiley, New York.

Arnold SJ (1977) The evolution of courtship behavior in New World salamanders with some comments on Old World salamanders. In: *The Reproductive Biology of Amphibians* (eds Taylor DH, Guttman SI), pp. 141–183. Plenum Publishers, New York.

Barton NH (2001) The role of hybridization in evolution. *Molecular Ecology*, **10**, 551–568.

Barton NH, Hewitt G (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**, 113–148.

Cracraft J (1989) Speciation and its ontology: the empirical consequences of alternative concepts for understanding patterns and processes of differentiation. In: *Speciation and its Consequences* (eds Otte D, Endler JA), pp. 28–59. Sinauer Associates, Sunderland, Massachusetts.

De Queiroz K (1999) The general lineage concept of species and the defining properties of the species category. In: *Species: New Interdisciplinary Essays* (ed. Wilson RA), pp. 49–89. MIT Press, Cambridge, Massachusetts.

De Queiroz K, Good DA (1997) Phenetic clustering in biology: a critique. *Quarterly Review of Biology*, **72**, 3–30.

Durand JD, Unlu E, Doadrio I, Pipoyan S, Templeton AR (2000) Origin, radiation, dispersion, and allopatric hybridization in the chub *Leuciscus cephalus*. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **267**, 1687–1697.

Funk DJ, Omland KE (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.

Gerber AS, Tibbets A, Dowling TE (2001) The role of introgressive hybridization in the evolution of the *Gila robusta* complex (Teleostei: Cyprinidae). *Evolution*, **55**, 2028–2039.

Hairston NG (1987) *Community Ecology and Salamander Guilds*. Cambridge University Press, Cambridge, UK.

Hairston NG (1993) On the validity of the name *Plethodon teyahalee* as applied to a member of the *Plethodon glutinosus* complex (Caudata, Plethodontidae) – a new name. *Brimleyana*, **18**, 65–69.

Hairston NG, Wiley RH, Smith CK, Kneidel KA (1992) The dynamics of two hybrid zones in Appalachian salamanders of the genus *Plethodon*. *Evolution*, **46**, 930–938.

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.

Highton R (1983) A new species of woodland salamander of the *Plethodon glutinosus* group from the southern Appalachian Mountains. *Brimleyana*, **9**, 1–20.

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

Highton R, Henry SA (1970) Evolutionary interactions between species of North American salamanders of the genus *Plethodon*. *Evolutionary Biology*, **4**, 211–256.

Highton R, Peabody RB (2000) Geographic protein variation and speciation in salamanders of the *Plethodon jordani* and *Plethodon glutinosus* complexes in the southern Appalachian Mountains with the description of four new species. In: *The Biology of Plethodontid Salamanders* (eds Bruce RC, Jaeger R, Houck LD), pp. 31–93. Kluwer Academic/Plenum Publishers, New York.

Highton R, Maha GC, Maxson LR (1989) Biochemical evolution in the slimy salamanders of the *Plethodon glutinosus* complex in the eastern United States. *Illinois Biological Monographs*, **57**, 1–78.

Houck LD (1986) The evolution of salamander courtship pheromones. In: *Chemical Signals in Vertebrates IV* (eds Duvall D, Müller-Schwarze D, Silverstein RM), pp. 173–190. Plenum Press, New York.

Houck LD, Reagan NL (1990) Male courtship pheromones increase female receptivity in a plethodontid salamander. *Animal Behaviour*, **39**, 729–734.

Houck LD, Bell AM, Reagan-Wallin NL, Feldhoff RC (1998) Effects of experimental delivery of male courtship pheromones on the timing of courtship in a terrestrial salamander, *Plethodon jordani* (Caudata: Plethodontidae). *Copeia*, **1998**, 214–219.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754–755.

Kramer CY (1956) Extension of multiple range tests to group mean with unequal numbers of replications. *Biometrics*, **12**, 309–310.

Kumazawa Y, Nishida M (1993) Sequence evolution of mitochondrial transfer RNA genes and deep-branch animal phylogenetics. *Journal of Molecular Evolution*, **37**, 380–398.

Legendre P, Legendre L (1998) *Numerical Ecology*, 2nd edn. Elsevier, Amsterdam.

Lessa EP (1990) Multidimensional analysis of geographic structure. *Systematic Zoology*, **39**, 242–252.

Macey JR, Larson A, Ananjeva NB, Fang ZL, Papenfuss TJ (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.

Maddison WP, Maddison DR (2000) MACCLADE *4: Analysis of Phylogeny and Character Evolution*. Sinauer Associates, Sunderland, Massachusetts.

Masta SE, Sullivan BK, Lamb T, Routman EJ (2002) Molecular systematics, hybridization, and phylogeography of the *Bufo americanus* complex in eastern North America. *Molecular Phylogenetics and Evolution*, **24**, 302–314.

Moore WS (1977) An evaluation of narrow hybrid zones in vertebrates. *Quarterly Review of Biology*, **52**, 263–277.

Peabody RB (1978) Electrophoretic analysis of geographic variation of two Appalachian salamanders, *Plethodon jordani* and *Plethodon glutinosus*. PhD Thesis, University of Maryland, College Park, Maryland.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Reagan NL (1992) Evolution of sexual isolation in salamanders of the genus *Plethodon*. PhD Thesis, University of Chicago, Chicago.

Redenbach Z, Taylor EB (2002) Evidence for historical introgression along a contact zone between two species of char (Pisces: Salmonidae) in northwestern North America. *Evolution*, **56**, 1021–1035.

Rollman SM, Houck LD, Feldhoff RC (1999) Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. *Science*, **285**, 1907–1909.

Rollman SM, Houck LD, Feldhoff RC (2000) Population variation in salamander courtship pheromones. *Journal of Chemical Ecology*, **26**, 2713–2724.

Shimizu Y, Ueshima R (2000) Historical biogeography and interspecific mtDNA introgression in *Euhadra peliomphala* (the Japanese land snail). *Heredity*, **85**, 84–96.

Sorenson MD (1999) TREEROT, *Version 2*. Boston University, Boston, Massachusetts.

Sota T, Ishikawa R, Ujiie M, Kusumoto F, Vogler AP (2001) Extensive trans-species mitochondrial polymorphisms in the carabid beetles *Carabus* subgenus *Ohomopterus* caused by repeated introgressive hybridization. *Molecular Ecology*, **10**, 2833–2847.

Swofford DL (2002) PAUP*: *Phylogenetic Analysis Using Parsimony (*and Other Methods)*, *Version 4*. Sinauer Associates, Sunderland, Massachusetts.

Tegelström H (1987) Transfer of mitochondrial DNA from the northern red-backed vole (*Clethionomys rutilus*) to the bank vole (*C. glareolus*). *Journal of Molecular Evolution*, **24**, 218–227.

Walton C, Handley JM, Collins FH *et al.* (2001) Genetic population structure and introgression in *Anopheles dirus* mosquitoes in Southeast Asia. *Molecular Ecology*, **10**, 569–580.

Weisrock DW (2003) Molecular phylogenetic studies of speciation and phylogeny in salamanders: macroevolutionary and microevolutionary perspectives. PhD Dissertation. Washington University, St. Louis, Missouri.

Weisrock DW, Macey JR, Ugurtas IH, Larson A, Papenfuss TJ (2001) Molecular phylogenetics and historical biogeography among salamandrids of the 'true' salamander clade: rapid branching of numerous highly divergent lineages in *Mertensiella luschani* associated with the rise of Anatolia. *Molecular Phylogenetics and Evolution*, **18**, 434–448.

Wilson CC, Bernatchez L (1998) The ghost of hybrids past: fixation of arctic charr (*Salvelinus alpinus*) mitochondrial DNA in an introgressed population of lake trout (*S. namaycush*). *Molecular Ecology*, **7**, 127–132.

Zardoya R, Meyer A (2001) On the origin of and phylogenetic relationships among living amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 7380–7383.

This work represents a chapter of David Weisrock's PhD thesis, the whole of which focused on studying patterns of lineage diversification at both macroevolutionary and microevolutionary levels. His research continues in the development and application of genome-wide molecular markers to questions of lineage diversification and species delimitation. Kenneth Kozak is a PhD candidate at Washington University. His research applies comparative phylogenetic approaches to study the build-up of species and ecomorphological diversity in North American plethodontid salamanders. Dr Allan Larson is Professor of Biology at Washington University. His research specialty is molecular phylogenetics and evolution of vertebrates, particularly lizards and salamanders.

Appendix I

Geographic information for salamanders used in this study. Population numbers are as presented by Weisrock (2003). Numbers marked with an H represent potentially hybrid populations based on morphology. County and state labels followed by an *h* number in parentheses signify a sampling population used in Highton *et al*. (1989). Population information for haplotypes included from *Plethodon amplus*, *Plethodon jordani*, *Plethodon meridianus*, *Plethodon metcalfi*, and *Plethodon montanus* as well as outgroup haplotypes can be found in Weisrock (2003)

Population no.	Species	*n*	Latitude (N), longitude (W)	Isolate	County, State*
67	*Plethodon cheoah*	5	35°19′30″, 83°40′42″	Cheoah	Graham-Swain, NC
68	*Plethodon cheoah*	3	35°21′27″, 83°43′08″	Cheoah	Graham, NC
69	*Plethodon cheoah*	5	35°20′03″, 83°42′15″	Cheoah	Graham, NC
70	*Plethodon shermani*	8	35°02′20″, 83°33′08″	Standing Indian	Clay-Macon, NC
71	*Plethodon shermani*	3	35°14′24″, 84°03′07″	Unicoi	Cherokee, NC
73	*Plethodon shermani*	1	35°09′44″, 83°35′00″	Wayah	Macon, NC
74	*Plethodon shermani*	5	35°01′48″, 83°27′44″	Standing Indian	Macon, NC
75	*Plethodon shermani*	3	35°15′17″, 83°57′36″	Unicoi	Graham, NC
77H	*Plethodon shermani*	5	35°09′03″, 83 °45′38″	Tusquitee	Clay, NC
78	*Plethodon shermani*	4	35°09′12″, 83 °45′04″	Tusquitee	Clay, NC
79	*Plethodon shermani*	5	35°15′52″, 83°56′18″	Unicoi	Graham, NC
122	*Plethodon shermani*	6	35°19′14″, 83°58′57″	Unicoi	Graham, NC
123	*Plethodon shermani*	3	35°19′29″, 84°01′58″	Unicoi	Graham, NC
124	*Plethodon shermani*	9	35°19′35″, 84°02′15″	Unicoi	Monroe, TN
125	*Plethodon shermani*	7	35°12′14″, 83°35′37″	Wayah	Macon, NC
126	*Plethodon shermani*	4	35°10′27″, 83°36′26″	Wayah	Macon, NC
127	*Plethodon shermani*	5	35°10′38″, 83°33′58″	Wayah	Macon, NC
128	*Plethodon shermani*	6	35°02′03″, 83°28′50″	Standing Indian	Macon, NC
129	*Plethodon shermani*	4	35°02′33″, 83°30′21″	Standing Indian	Macon, NC
130H	*Plethodon shermani*	7	35°05′30″, 83°31′29″	Standing Indian	Macon, NC
131	*Plethodon shermani*	10	35°03′41″, 83°34′04″	Standing Indian	Clay-Macon, NC
132	*Plethodon albagula*	1	37°33 37″, 90°40′16″		Iron, MO (H118)
133	*Plethodon aureolus*	5	35°11′33″, 84°29′43″		Polk, TN (H131)
134	*Plethodon aureolus*	2	35°27′45″, 84°01′37″		Monroe, TN (H130)
135	*Plethodon chattahoochee*	5	34°52′21″, 83°48′31″		Towns, GA (H9)
136	*Plethodon chattahoochee*	4	34°39′10″, 84°08′20″		Union, GA (H7)
137	*Plethodon chlorobryonis*	3	34°42′40″, 83°24′19″		Rabun, GA
138	*Plethodon chlorobryonis*	1	35°17′19″, 77°07′39″		Craven, NC (H14)
139	*Plethodon variolatus*	4	33°14′41″, 79°31′14″		Berkeley, SC
140	*Plethodon cylindraceus*	3	35°42′21″, 82°15′12″		Yancey, NC
141	*Plethodon cylindraceus*	1	35°48′05″, 82°09′23″		Yancey-McDowell, NC
142	*Plethodon cylindraceus*	11	36°10′28″, 82°17′56″		Unicoi, TN
143	*Plethodon cylindraceus*	1	36°16′02″, 81°41′31″		Watauga, NC
145	*Plethodon glutinosus*	5	39°27′24″, 79°31′08″		Preston, WV (H57)
146	*Plethodon cylindraceus*	2	35°41′43″, 81°43′02″		Burke, NC (H111)
147	*Plethodon cylindraceus*	3	36°00′30″, 82°36′32″		Madison-Unicoi, TN-NC(H113)
148	*Plethodon cylindraceus*	1	37°29′28″, 79°32′56″		Bedford, VA
149	*Plethodon glutinosus*	3	35°53′30″, 83°57′09″		Knox, TN
150	*Plethodon grobmani*	1	0.5 mi NE of Silver Springs		Marion, FL
151	*Plethodon grobmani*	1	30°49′25″, 85°18′15″		Jackson, FL (H102)
152	*Plethodon kentucki*	3	36°53′42″, 82°37′58″		Wise, VA (H132)
153	*Plethodon ocmulgee*	2	31°26′31″, 82°25′53″		Bacon, GA (H30)
154	*Plethodon teyahalee*	2	34°57′00″, 83°01′00″		Oconee, SC
155	*Plethodon teyahalee*	1	35°07′30″, 82°55′41″		Transylvania, NC
156	*Plethodon teyahalee*	1	35°17′33″, 82°47′55″		Transylvania, NC
157	*Plethodon teyahalee*	3	35°18′12″, 82°49′36″		Transylvania, NC
158	*Plethodon teyahalee*	1	35°19′31″, 83°06′18″		Jackson, NC
159	*Plethodon teyahalee*	3	35°19′59″, 83°05′30″		Jackson, NC
160	*Plethodon cylindraceus*	1	35°53′21″, 82°35′14″		Madison, NC
161H	*Plethodon teyahalee*	3	35°03′52″, 83°27′46″		Macon, NC
162	*Plethodon teyahalee*	1	34°41′00″, 82°50′00″		Pickens, SC
163	*Plethodon chlorobryonis*	2	34°51′00″, 83°13′00″		Oconee, SC

Appendix I *Continued*

Population no.	Species	*n*	Latitude (N), longitude (W)	Isolate	County, State*
165	*Plethodon teyahalee*	1	34°39′00″, 82°43′00″		Anderson, SC
166	*Plethodon teyahalee*	2	35°06′20″, 83°17′05″		Macon, NC (H3)
167	*Plethodon teyahalee*	3	35°48′50″, 82°56′58″		Madison, NC
168	*Plethodon savannah*	1	33°19′48″, 82°03′49″		Richmond, GA (H128)
169	*Plethodon variolatus*	1	33°08′00″, 79°47′06″		Berkeley, SC (H27)
170	*Plethodon variolatus*	1	32°34′02″, 81°20′52″		Effingham, GA (H29)
171	*Plethodon cylindraceus*	2	35°11′14″, 82°40′05″		Transylvania, NC
172	*Plethodon cylindraceus*	1	35°10′34″, 82°34′24″		Henderson, NC
173	*Plethodon cylindraceus*	1	35°10′56″, 82°33′24″		Henderson, NC

Appendix II

Haplotypes sequenced in this study. Haplotypes are named according to the species in which they are found. The haplotype name is the same as those presented in all trees and networks. The population (or populations) number from which each haplotype was found is given followed by the number of individuals (n) in parentheses. Haplotypes from outgroups and *Plethodon amplus*, *Plethodon jordani*, *Plethodon meridianus*, *Plethodon metcalfi*, and *Plethodon montanus* are presented in Weisrock (2003)

Haplotype	Population (*n*)	Haplotype	Population (*n*)	Haplotype	Population (*n*)
cheoah-1	95(1)	shermani-22	128(2); 129(1)	cylindraceus-3	140(3)
cheoah-2	95(1)	shermani-23	131(1)	cylindraceus-4	173(1)
cheoah-3	96(1); 97(1)	shermani-24	70(1); 131(1)	cylindraceus-5	171(1)
cheoah-4	95(2); 96(2); 97(4)	shermani-25	70(2); 131(4)	cylindraceus-6	172(1)
cheaoh-5	95(1)	shermani-26	124(1)	cylindraceus-7	171(1)
she/aur-1	124(1); 134(1)	shermani-27	71(1)	cylindraceus-8	146(2)
she/jor-1	89(1); 122(3)	shermani-28	71 (1)	cylindraceus-9	143(1); 148(1)
she/tey-1	74(1); 128(3); 167(1)	shermani-29	122(1); 123(2)	cylindraceus-10	147(2); 142(11)
she/tey-2	71(1); 130(1); 158(1); 159 (2); 161(1)	shermani-30	79(5)	cylindraceus-11	147(1)
she/tey-3	70(1); 155(1); 156(1); 167(2)	shermani-31	123(1)	grobmani-1	150(1)
shermani-1	77(5); 78(5)	shermani-32	130(2)	grobmani-2	151(1)
shermani-3	122(1)	shermani-33	130(1)	glutinosus-1	149(2)
shermani-4	124(4)	shermani-34	122(1)	glutinosus-2	149(1)
shermani-5	124(1)	shermani-35	74(1)	glutinosus-3	145(3)
shermani-6	124(1)	shermani-36	127(1)	kentucki-1	152(3)
shermani-7	124(1)	albagula-1	132(1)	ocmulgee-1	153(1)
shermani-8	125(4); 127(2)	aureolus-1	133(5)	ocmulgee-2	153(1)
shermani-9	125(1); 127(2)	aureolus-2	134(1)	savannah-1	168(1)
shermani-10	130(2); 131(2)	chattahoochee-1	135(2)	teyahalee-1	159(1)
shermani-11	131(1)	chattahoochee-2	135(1)	teyahalee-2	166(2)
shermani-12	129(1)	chattahoochee-3	135(1)	teyahalee-3	162(1)
shermani-13	130(1)	chattahoochee-4	135(1)	teyahalee-4	165(1)
shermani-14	128(1)	chattahoochee-5	136(2)	teyahalee-5	154(1)
shermani-15	70(2)	chattahoochee-6	136(1)	teyahalee-6	161(1)
shermani-16	73(1); 126(2)	chattahoochee-7	136(1)	teyahalee-7	161(1)
shermani-17	74(2)	chlorobryonis-1	137(3)	teyahalee-8	154(1)
shermani-18	74(1)	chlorobryonis-2	138(1)	teyahalee-9	154(1)
shermani-19	126(1)	chlorobryonis-3	163(2)	teyahalee-10	157(1)
shermani-20	70(2); 125(1); 126(1); 129(2); 131(1)	cylindraceus-1	160(1)	teyehalee-11	157(2)
shermani-21	125(1)	cylindraceus-2	141(1)		