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Concurrent speciation in the eastern woodland salamanders (Genus *Plethodon*): DNA sequences of the complete albumin nuclear and partial mitochondrial 12s genes

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ABSTRACT

Salamanders of the North American plethodontid genus *Plethodon* are important model organisms in a variety of studies that depend on a phylogenetic framework (e.g., chemical communication, ecological competition, life histories, hybridization, and speciation), and consequently their systematics has been intensively investigated over several decades. Nevertheless, we lack a synthesis of relationships among the species. In the analyses reported here we use new DNA sequence data from the complete nuclear albumin gene (1818 bp) and the 12s mitochondrial gene (355 bp), as well as published data for four other genes (Wiens et al., 2006), up to a total of 6989 bp, to infer relationships. We relate these results to past systematic work based on morphology, allozymes, and DNA sequences. Although basal relationships show a strong consensus across studies, many terminal relationships remain in flux despite substantial sequencing and other molecular and morphological studies. This systematic instability appears to be a consequence of contemporaneous bursts of speciation in the late Miocene and Pliocene, yielding many closely related extant species in each of the four eastern species groups. Therefore we conclude that many relationships are likely to remain poorly resolved in the face of additional sequencing efforts. On the other hand, the current classification of the 45 eastern species into four species groups is supported. The *Plethodon cinereus* group (10 species) is the sister group to the clade comprising the other three groups, but these latter groups (*Plethodon glutinosus* [28 species], *Plethodon welleri* [5 species], and *Plethodon wehrlei* [2 species]) probably diverged from each other at approximately the same time.

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1. Introduction

The Woodland Salamander genus *Plethodon* is the largest North American salamander genus with 55 recognized species. Before recent declines (Highton, 2005), populations of some of its species were the most common vertebrates in forests of eastern and northwestern North America (e.g., Burton and Likens, 1975). *Plethodon* belongs to the lungless family Plethodontidae and is largely terrestrial, lacking the aquatic larval stage characteristic of most amphibians. The genus has a long history that dates back at least to the Eocene Epoch (Highton and Larson, 1979; Vieites et al., 2007, 2011). Cryptic species are common in *Plethodon* and, primarily as a result of allozyme studies, the number of species in the genus has increased from 16 (Highton, 1962) based on morphology, to the 55 species currently recognized. To reconstruct the phylogeny of *Plethodon*, workers have analyzed variation in morphology and distributional patterns (Dunn, 1926; Grobman, 1944; Highton, 1962, 1972; Wake, 1966), allozymes (Highton and Larson, 1979; Highton, 1991, 1993, 1995), immunology (Maxson et al., 1979; Hass et al., 1992), DNA hybridization (Mizuno and Macgregor, 1974); and DNA sequence variation (Mahoney, 2001; Sites et al., 2004; Kozak et al., 2005; Palmer et al., 2005; Weisrock et al., 2005; Weisrock and Larson, 2006; Wiens et al., 2006; Shepard and Burbrink, 2008, 2009, 2011; Chatfield et al., 2010).

Interspecific morphological variation in the genus *Plethodon* provides few characters that contain phylogenetic information. Skeletal novelties are rare and quantitative traits such as body size, coloration, number of teeth, and body proportions vary too continuously to be optimal for cladistic analysis. The morphological similarity of some of the genetically highly divergent species of *Plethodon* is unusual considering the long geographic separation

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of the two major clades (eastern and western) since the Eocene Epoch, estimated as about 42 mya (Highton and Larson, 1979; Maxson and Maxson, 1979). For example, the distantly related eastern *Plethodon cinereus* and western *Plethodon vehiculum* not only are similar in size and proportions but even share the same dorsal color morph polymorphism (red striped and unstriped morphs). The species of both groups have 14 pairs of chromosomes, but the amount of DNA in the western species is much larger than that of the eastern species (Mizuno and Macgregor, 1974; Larson, 1984). Mahoney (2001) discussed the taxonomic status of the two major clades and the possible paraphyly of *Plethodon* with what had long been considered its sister genus *Aneides* (Wake, 1966). More recent DNA comparisons have indicated that *Aneides* is not the sister genus of *Plethodon* (Chippindale et al., 2004; Mueller et al., 2004; Macey, 2005; Mueller and Boore, 2005; Vieites et al., 2007), but it nevertheless serves as a satisfactory outgroup for *Plethodon*. Recently Vieites et al. (2011) have divided the genus *Plethodon* into two subgenera: *Plethodon* for the eastern clade and *Hightonia* for the western clade.

Highton and Larson (1979) suggested that there are four species groups in both the eastern and western clades, a conclusion supported by subsequent molecular studies. In eastern *Plethodon*, there are two groups of large-sized species (*Plethodon glutinosus* and *Plethodon wehrlei* groups) and two groups of small-sized species (*P. cinereus* and *Plethodon welleri* groups). Most comprehensive molecular studies to date indicate that the *P. cinereus* group is the sister group to a clade comprising all other eastern *Plethodon*. However, relationships among the other three groups are problematic. In addition, the affinities of many species within the species groups are not clear.

A remarkable number of speciation events occurred in the four eastern species groups during the warm, dry climates of the late Miocene and Pliocene epochs (Highton and Larson, 1979; Highton, 1995). Only five clades of eastern *Plethodon* that were present in the Miocene have known living descendents. Three of these clades are the ancestors of the *P. glutinosus*, *P. wehrlei*, and *P. cinereus* groups, and two clades now classified within the *P. welleri* group include the ancestor of *Plethodon websteri*, and a clade containing the ancestor of the four remaining species of the *P. welleri* group. Speciation in the late Miocene and Pliocene has yielded 28 known species of the *P. glutinosus* group, two species of the *P. wehrlei* group, and the 10 species of the *P. cinereus* group. The two clades in the *P. welleri* group had diverged much earlier, probably in the late Oligocene or early Miocene. One of these is the ancestor of *P. websteri*, and speciation in the other clade has produced four species (*Plethodon angusticlavius*, *Plethodon dorsalis*, *Plethodon ventralis*, *P. welleri*). *P. websteri* still resembles the other four species of the *P. welleri* group morphologically and has been placed in that group ever since it was discovered (Larson and Highton, 1978; Highton, 1979). It so closely resembles *P. angusticlavius*, *P. dorsalis*, and *P. ventralis* that prior to allozyme studies the four were recognized as a single species. In a phylogeny based on allozyme comparisons (Highton and Larson, 1979), *P. websteri* is the closest relative of the other four species of the *P. well*eri group.

The present distribution of most of the known eastern *Plethodon* species includes a highland area. Prolonged dry periods of the Pliocene may have limited forests to higher elevations. Considerable evidence indicates that grasslands were widespread at low elevations in eastern North America for long arid periods during the Pliocene (Edwards et al., 2010; Rea, 1994; Stanley, 1989; Van Valkenburgh and Janis, 1993). Highton (1995) hypothesized that allopatric speciation occurred contemporaneously in each of the eastern species groups because of subdivision of populations and isolation in various mountain ranges of eastern North America. No doubt, because of the cyclical nature of climatic changes in the late Cenozoic, ranges of diverging taxa probably have both restricted and expanded, the latter making possible secondary contacts and frequent hybridization events, which continue today (Highton and Peabody, 2000). If the hypothesis of multiple simultaneous speciation is correct, it might be expected that there would be numerous polytomies within species groups and that statistical support for the topology of peripheral branches of the tree would be low. However, Wiens et al. (2006), in a study of *Plethodon* based on DNA sequences of four genes, provided several trees, all with high statistical support for most nodes, and concluded that the trees were so robust that they could be used to test evolutionary hypotheses of rates and patterns of diversification and hybridization, as well as to infer phylogenetic relationships within species groups. To reconstruct phylogeny they used Bayesian trees with posterior probabilities to test for statistical support. However, posterior probabilities are known to be inflated (Simmons et al., 2004; Suzuki et al., 2002). Since the results of allozyme and immunological studies, as well as those based on DNA sequence analyses reported in this paper, differ from those of Wiens et al. (2006), a review of the evidence for the relationships of the species in eastern *Plethodon* is warranted.

Two of us (MC, RH) first attempted to reconstruct the phylogeny of eastern *Plethodon* by sequencing up to 355 bp of the 12s mitochondrial gene in 45 eastern species. The one missing species is *Plethodon ainsworthi* (Lazell, 1998). It is known only from two specimens collected in 1962, and no additional individuals of this species have been found since. Its relationships are not known, although it most resembles the *P. glutinosus* group on the basis of its 17 trunk vertebrae. Our study did little to clarify the details of species relationships within species groups, so we sequenced the entire nuclear albumin gene (1818 bp, 606 amino acids) in 42 of the eastern species (the four missing species are *P. ainsworthi*, *Plethodon fourchensis*, *Plethodon ocmulgee*, and *Plethodon shenandoah*). Results of both of these studies are reported in this paper, and these data are combined with those of the four genes sequenced by Wiens et al. (2006) for a new analysis.

The trees resulting from allozyme studies of *Plethodon* cited above are estimates of species trees because the analysis is of the populations being sampled, not those based on the DNA sequences of any one gene. When the DNA sequence from a single gene is used to estimate a phylogeny the result is a gene tree (gene genealogy) which may or may not be a good estimate of the species tree (organismal history) (Arbogast et al., 2002). To attempt better estimates of this history, workers doing sequencing now usually try to include sequences of several genes. We hoped the inclusion of sequences from six genes might obtain a better tree for estimating the evolutionary history of eastern *Plethodon*.

The taxonomy of *Plethodon* may be confusing because the genus has not been formally subdivided into taxonomic groups below the subgeneric level. In the *P. glutinosus* group, there are four species (*Plethodon aureolus*, *Plethodon kentucki*, *Plethodon petraeus*, and *Plethodon yonahlossee*) that, based on allozyme data, are not closely related to three species complexes. Each of these complexes comprises morphologically similar species with parapatric distributions: the *P. glutinosus* complex, widely distributed in eastern United States (14 species), the *Plethodon jordani* complex of the southern Appalachian Mountains (seven species), and the *Plethodon ouachitae* complex of the Ouachita Mountains (three species). Prior to the allozyme studies of Highton (1989) and Highton and Peabody (2000), each of the first two complexes was long considered a single species. However, none of the allozyme or DNA sequence studies have supported the hypothesis that either of these complexes is monophyletic. On the other hand, the monophyly of the *P. ouachitae* complex has been supported by all comprehensive molecular studies (Duncan and Highton, 1979;

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Table 1
Localities for specimens used in 12s sequencing. MVZ refers to Museum of Vertebrate Zoology, University of California, Berkeley.

Species	RH Number	State	County/Parrish	Latitude			Longitude		
Eastern Plethodon									
P. glutinosus group									
albagula (1)	65802	OK	Stone	35	59	05	92	16	02
albagula (2)	71791	TX	Hays	29	56	27	97	54	14
amplus	58004	NC	Henderson	35	29	42	82	20	08
aureolus	50879	TN	Polk	35	11	33	84	29	43
caddoensis	58705	AR	Montgomery	34	22	35	93	52	45
chattahoochee	57589	GA	Union	34	39	10	84	08	20
cheoah	64835	NC	Graham-Swain	35	19	30	83	40	52
chlorobryonis (1)	70503	GA	Wilkes	33	40	05	82	53	05
chlorobryonis (2)	67213	NC	Columbus	34	19	55	78	52	36
cylindraceus	55779	VA	Pittsylvania	36	34	13	79	26	06
fourchensis	72308	AR	Scott	34	41	30	93	56	30
glutinosus (1)	68311	GA	Walker	34	39	46	85	21	50
glutinosus (2)		IL	Union			Little River Canyon			
glutinosus (3)	65366	KY	Harlan	36	56	01	83	11	51
glutinosus (4)	66537	NJ	Union	40	40	42	74	23	10
grobmani	64013	FL	Marion	29	13	15	82	02	30
jordani	57577	NC-TN	Swain-Sevier	35	36	34	83	26	50
kentucki	70639	WV	Raleigh	37	44	02	80	55	00
kiamichi	58699	OK	Le Flore	34	36	55	94	29	50
kisatchie	65986	LA	Grant	31	43	15	92	28	02
meridianus	73801	NC	Burke	35	39	27	81	41	55
metcalfi	68040	NC	Haywood	35	21	43	85	55	32
mississippi	65838	MS	Tishomingo	34	36	38	88	11	56
montanus	73849	NC	Buncombe	35	37	20	82	49	48
ocmulgee	61449	GA	Bulloch	32	23	03	81	49	59
ouachitae (1)	58643	OK	Le Flore	34	40	48	94	36	40
ouachitae (2)	58651	OK	Le Flore	34	42	45	94	40	45
ouachitae (3)	58656	OK	Le Flore	34	37	40	94	48	43
ouachitae (4)	58688	OK	Le Flore	34	36	55	94	29	50
ouachitae (5)	74770	OK	Latimer	34	46	03	95	05	54
petraeus	74484	GA	Walker	34	39	50	85	22	10
savannah	65808	GA	Richmond	33	19	48	82	03	49
sequoyah	71206	OK	McCurtain	34	07	29	94	40	15
shermani (1)	74841	NC	Clay-Macon	35	02	20	83	33	08
shermani (2)	73899	NC	Graham	35	15	17	83	57	36
teyahalee	68028	NC	Haywood	35	21	43	85	55	32
variolatus	56618	SC	Berkeley	33	08	00	79	47	06
yonahlossee (1)	72129	NC-TN	Mitchell-Unicoi	36	06	36	82	21	40
yonahlossee (2)	68356	NC	Henderson	36	27	48	82	19	24
P. wehrlei group									
punctatus	64534	WV	Pendleton	38	41	36	79	05	44
wehrlei (1)	56910	KY	Letcher	37	04	31	82	59	46
wehrlei (2)	62577	PA	Cambria	40	42	07	78	48	08
wehrlei (3)	70637	WV	Raleigh	37	44	02	80	55	00
wehrlei (4)	60385	VA	Montgomery	36	47	22	80	27	34
P. welleri group									
angusticlavius (1)	74743	AR	Pope	35	38	28	93	04	03
angusticlavius (2)	74735	AR	Stone	35	57	14	92	05	02
dorsalis	72216	KY	Jefferson	38	16	35	85	41	30
ventralis	72055	GA	Walker	35	39	46	85	21	50
websteri	74570	AL	Etowah	34	04	06	86	18	43
welleri	72415	TN	Johnson	36	24	00	81	57	47
P. cinereus group									
cinereus (I)	74437	NC-TN	Madison-Unicoi	35	57	15	82	33	39
cinereus (II)	57216	VA	Washington	36	50	13	82	05	15
cinereus (III)	65054	NY	Tomkins	42	19	55	76	39	34
cinereus (IV)	66732	WV	Pocahontas-Randolph	38	9	15	80	21	03
electromorphus	64308	WV	Mason	38	59	18	81	58	47
hoffmani (1)	74756	VA	Bath	38	05	55	79	50	54
hoffmani (2)	53760	MD	Washington	38	36	34	79	50	38
hoffmani (3)	62171	PA	Armstrong	40	44	34	79	32	09
hubrichti	72139	VA	Botetourt	37	31	43	79	32	58
nettingi	66735	WV	Pocahontas-Randolph	38	36	34	79	50	38
richmondi	52928	WV	Cabell	38	24	13	82	26	03
serratus (2)	67410	GA	White	34	32	08	83	41	50
sherando	78930	VA	Augusta	37	55	09	79	04	00
virginia	64530	WV	Pendleton	37	31	43	79	32	58
Outgroups									
Aneides aeneus (1):	59175	AL	Marion	34	08	11	87	49	54
Aneides aeneus (2)	61345	NC	Henderson	35	27	20	82	17	50
Aneides aeneus (3)	72813	NC	Macon	35	01	57	83	13	28

(*continued on next page*)

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Table 1 (*continued*)

Species	RH Number	State	County/Parrish	Latitude			Longitude		
Aneides aeneus (4)	63659	OH	Adams	38	39	32	83	21	25
Aneides aeneus (5)	65786	TN	Marion	34	59	24	85	36	40
Ambystoma maculatum	MVZ 144934	NC	Wake	35	43	42	78	46	47
Ambystoma texanum	MVZ 144954	KS	Douglas	35	54	41	95	13	23

Highton and Larson, 1979; Shepard and Burbrink, 2008, 2009, 2011).

2. Materials and methods

2.1. 12s gene

The locality data for salamanders used in 12s sequencing are listed in Table 1. The samples were from the frozen tissue collection of R. Highton at the University of Maryland, except for the two *Ambystoma*, which are from the University of California at Berkeley. DNA from red blood cells of 68 individuals of 45 species of eastern *Plethodon* (all but *P. ainsworthi*) was obtained from frozen samples maintained at −60 °C or below. Multiple individuals of some species with extensive ranges or with significant allozyme or morphological variation were sequenced. Four *P. glutinosus* and two *Plethodon albagula* are from distant localities within their large ranges. Two *Plethodon shermani* represent forms that differ in coloration from the (1) Nantahala Mountains and (2) Unicoi Mountains of North Carolina and Tennessee. Five *P. ouachitae* represent populations from each of five isolates identified by morphological (Blair and Lindsay, 1965) and allozyme differences (Duncan and Highton, 1979), and confirmed by DNA variation (Shepard and Burbrink, 2008). The pair of *Plethodon yonahlossee* represents two color variants: the typical form (1) and the Bat Cave form (2). The latter was named *Plethodon longicrus* by Adler and Dennis (1962), but is no longer recognized as a separate species. The four *P. wehrlei* represent two each of the yellow-spotted form reported by Cupp and Towles (1983) from West Virginia and Kentucky, and the typical form from Pennsylvania and Virginia. Four *P. cinereus* represent each of the four genetically differentiated groups detected in an allozyme study by Hass (1985) (her groups I–IV). Two *Plethodon chlorobryonis* represent the typical coastal North Carolina variant and the northeast Georgia variant. Two *P. angusticlavius* are from Pope and Stone counties, Arkansas, and three *Plethodon hoffmani* are from Maryland, Pennsylvania, and Virginia. We used as outgroups two species of ambystomatid salamanders (*Ambystoma maculatum* and *Ambystoma texanum*, GenBank accession nos. AF217182-3), five geographically widespread green salamanders (*Aneides aeneus*), an eastern species of a closely related genus, and two western *Plethodon* from the *P. elongatus* group (*P. elongatus* and *P. stormi*). DNA was extracted from blood samples using a proteinase K digestion and phenol–chloroform protocol (Saitour et al., 1989). Resulting DNA was resuspended in TLE buffer and stored at 4 °C. PCR amplification was performed using 12S primers from Kocher et al. (1989), 12S fwd: AAAAAGCTTCAAACTGGGATTAGATACC CCACTAT and 12S-rev: TGACTGCAGAGGGTGACGGGCGGTGTGT. PCR reactions were performed using 50 ng of genomic DNA in the presence of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.16 mg/ml BSA, 1 μM of each primer, and 1 unit Taq polymerase enzyme in a volume of 10 μl. Thermocycling conditions consisted of 0.5 min denaturation at 94 °C, 1.5 min annealing at 51 °C, and 1 min extension at 72 °C for 30 cycles. Resulting PCR products were visualized on a 2% agarose gel in TBE buffer. DNA sequences will be provided to GenBank.

2.2. Albumin gene

Locality data for the 62 eastern *Plethodon* used in albumin sequencing are given in Table 2. The samples were in the frozen tissue collection of R. Highton except for those with SA (S. Arnold) numbers. There are two individuals from the same locality for six species: *P. aureolus*, *P. cheoah*, *P. dorsalis*, *P. hoffmani*, *P. metcalfi*, *P. welleri*, and individuals from multiple localities of five species: *P. albagula* (Oklahoma, Texas), *P. cinereus* (Hass, 1985, groups I, II, IV), *P. chlorobryonis* (Georgia, North Carolina, South Carolina), *P. glutinosus* (Georgia, Maryland), and *Plethodon metcalfi* (North Carolina, Tennessee). DNA sequences will be provided to GenBank.

Complimentary DNA was produced from mRNA expressed in liver tissue. Total RNA was extracted from liver tissue with Trizol® reagent (Invitrogen [Carlsbad CA] #15596-026), redissolved in 30 μl RNase-free water and stored at −80 °C. First-strand 3′RACE-ready cDNA was synthesized from liver mRNA (ImpromII™ Reverse Transcription System (Promega [Madison WI] #A3800) using 1 μg cDNA cloning primer per reaction (Integrated DNA Technologies [Coralville IA]; see Table 3 for primer sequences). ISA 1854–1875 bp region of the albumin gene was PCR-amplified in two overlapping fragments (fragment 1:912–924 bp; fragment 2:990–999 bp; Table 3). The following PCR conditions were used to amplify both fragments: 95 °C for 2 min, 30 s (initial annealing), then 35 cycles of (1) denaturing (95 °C, 30 s), (2) annealing (69 °C, 30 s) and (3) extension (72 °C, 1 min), with a final extension of 72 °C for 7 min. A proofreading DNA polymerase, (Easy-A® High-Fidelity PCR Cloning Enzyme (Stratagene [La Jolla CA] #600402), was used to minimize polymerase error. PCR products were excised from a 1.5% agarose gel, purified (QIAquick Gel Extraction Kit, Qiagen [Valencia CA] #28706) and either: (1) direct sequenced (Nevada Genomics Center, University of Nevada, Reno, NV) or, (2) cloned using the pGEM®-T Easy Vector System (Promega #A1380). When cloned, a minimum of four clones per albumin fragment per individual was sequenced using universal primers and a specific sequencing primer (SEQ; Table 3).

2.3. Phylogenetic analysis

All DNA sequences were aligned with the MEGA program (Tamura et al., 2007). MEGA was used also to compute genetic distances using the Jukes–Cantor (1969) method, and to perform phylogenetic analysis using the Maximum Likelihood method with model selection obtained for the 12s tree (General time reversible model) and the albumin tree (Tamura 3-parameter model) as recommended by the jModelTest (Posada, 2008; Guindon and Gascuel, 2003). Other trees calculated by MEGA are the unweighted pair-group method using arithmetic averages (UPGMA; Sokal and Michener, 1958), neighbor-joining method (NJ, Saitou and Nei, 1987), minimum-evolution method (ME, Rzhetsky and Nei, 1992), and maximum-parsimony method (MP) (Eck and Dayhoff, 1966; Fitch, 1977). MEGA also was used to calculate the percentage of replicate trees in which the associated taxa clustered together at each node using the bootstrap method (Felsenstein, 1985), with 2000 replicate trees for the maximum likelihood method and 10,000 trees for the other four methods.

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Table 2
Locality data for specimens used in albumin sequencing. SA refers to Steven J. Arnold numbers.

Species	RH Number	State	County/Parish	Latitude			Longitude		
Eastern Plethodon									
P. glutinosus Group									
albagula (1)	71954	TX	Travis	30	17	32	97	47	19
albagula (2)	75488	OK	Adair	35	50	13	94	39	20
amplus	75114	NC	Henderson	35	27	50	82	19	21
aureolus (*n* = 2)	SA 16767, 16770	TN	Monroe	35	27	29	84	01	24
caddoensis	74949	AR	Montgomery	34	26	30	93	53	25
chattahoochee	76620	GA	Towns	34	52	21	83	48	31
cheoah (*n* = 2)	SA 12500, 12501	NC	Graham	35	21	30	83	43	04
chlorobryonis (1)	75247	NC	Craven	35	17	19	77	07	39
chlorobryonis (2)	75026	GA	Wilkes	33	40	05	82	53	06
chlorobryonis (3)	77257	SC	McCormick	34	01	47	82	23	55
cylindraceus	77280	TN	Johnson	36	29	51	81	53	13
glutinosus (2)	77365	GA	Henry	33	29	53	84	11	03
glutinosus (1)	77131	MD	Frederick	39	37	50	77	28	17
glutinosus (3)	75685	KY	Bell	36	38	53	83	50	58
grobmani	74674	AL	Houston	30	59	56	85	24	23
jordani	SA 13431	TN	Sevier	35	36	34	83	26	50
kentucki	SA 17020	VA	Wise	36	53	25	83	20	10
kiamichi	75453	OK	Le Flore	34	36	55	94	29	50
kisatchie	75459	LA	Grant	31	43	15	92	28	02
meridianus	75111	NC	Burke	35	39	27	81	41	55
metcalfi (*n* = 2)	SA 12445, 12448	NC	Macon	35	19	40	83	20	10
mississippi	75438	MS	Scott	32	24	37	89	29	02
montanus (1)	74908	NC-TN	Mitchell-Unicoi	36	06	36	82	21	40
montanus (2)	SA 12480	NC	Madison	35	50	24	82	57	11
ouachitae (1)	74881	OK	Le Flore	34	36	55	94	29	50
ouachitae (2)	74868	OK	Le Flore	34	36	55	94	37	52
ouachitae (3)	75468	OK	Le Flore	34	40	48	94	36	40
ouachitae (4)	SA 36284	OK	Le Flore	34	47	50	94	54	29
ouachitae (5)	74942	OK	Latimer	34	46	03	95	05	54
petraeus	74484	GA	Walker	34	39	50	85	22	10
savannah	74937	GA	Richmond	33	19	48	82	03	49
sequoyah	74653	OK	McCurtain	34	07	29	94	40	15
shermani	SA 12886	NC	Macon	35	10	48	83	33	38
teyahalee	SA 12692	NC	Madison	35	50	24	82	57	11
variolatus	75229	SC	Jasper	32	36	14	80	54	08
yonahlossee	SA 51313	NC	Yancey	35	44	38	82	12	51
P. wehrlei group									
punctatus	74919	WV	Pendleton	38	41	36	79	05	44
wehrlei (1)	77620	WV	Randolph	38	56	09	79	43	52
wehrlei (3)	74967	WV	Raleigh	37	44	02	80	55	00
wehrlei (2)	SA 20662	VA	Floyd	36	47	38	80	28	00
P. welleri group									
angusticlavius	74866	AR	Stone	35	59	05	92	16	02
dorsalis (*n* = 2)	SA 20667, 20670	IN	Parke	39	53	00	87	12	08
ventralis	75559	AL	Jefferson	33	43	40	86	49	08
websteri	75563	AL	Jefferson	33	43	40	86	49	08
welleri (*n* = 2)	74885-86	NC-TN	Mitchell-Unicoi	36	06	36	82	21	40
P. cinereus group									
cinereus (I)	78913	NC-TN	Mitchell-Unicoi	36	06	36	82	21	40
cinereus (II)	SA 22208	VA	Giles	37	22	02	80	31	56
cinereus (IV)	78920	WV	Pocahontas	38	09	15	80	21	03
electromorphus	75915	WV	Gilmer	38	52	34	80	51	03
hoffmani (*n* = 2)	74755-56	VA	Bath	38	05	55	79	50	54
hubrichti	75146	VA	Bedford	37	29	03	79	32	56
nettingi	30672	WV	Randolph	38	56	10	79	41	27
richmondi	SA 17023	VA	Wise	36	53	42	82	37	58
serratus	77372	GA	Henry	33	29	53	84	11	03
sherando	79219	VA	Augusta	37	55	09	79	04	00
virginia	74958	WV	Hardy	38	54	56	78	56	07
Outgroups									
stormi	SA 21161	CA	Siskiyou	41	59	09	123	11	36
vehiculum	SA 19856	OR	Benton	44	29	49	123	34	05
Ambystoma maculatum	AF 217183	GenBank							
Ambystoma texanum	AF 217182	GenBank							

3. Results

3.1. Sequence divergence in the 12s Gene

Using Tajima–Nei (1984) distances, the percent sequence divergence of 12s sequences indicates little overlap between the range of variation of comparisons between (1) the two outgroup *Ambystoma* species and the 47 *Plethodon* species (range, 18.7–30.9%); (2) the 62 eastern and the two western *Plethodon* (range 13.9–19.9%), (3) the 10 species of the *P. cinereus* group and the 35 other species of eastern *Plethodon* (range 7.6–16.8%), and (4) comparisons within and among the species of the three remaining clades of eastern

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Table 3
Sequence information for primers used in this study. In addition to PCR primers, sequences for the cDNA cloning primer (used to make 3′RACE-ready cDNA) and a specific sequencing primer (SEQ) are also listed.

Albumin fragment	Forward/reverse	Primer sequence
Albumin F1	Forward	5′-AATTCGGCACGAGCAGACATGAAGTGGG-3′
Albumin F1	Reverse	5′-ACCGTTCAAYGAYGKSCTTCTCACAGCA-3′
Albumin F2	Forward	5′-TGCATGKMRGACAGGCTGGCTCTCACC-3′
Albumin F2	Reverse	5′-GGCCACGCGTCGACTAGTAC-3′
cDNA cloning primer	n/a	5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTV-3′
SEQ	n/a	5′-AAGATAAGCTTACATGTCTAG-3′

Plethodon (*glutinosus*, *wehrlei*, and *welleri* groups), (range 0–11.0%). In the maximum likelihood gene tree (Fig. 1), the ancient differentiation of the eastern and western *Plethodon* agree with the findings of previous DNA sequencing studies (Mahoney, 2001; Sites et al., 2004; Palmer et al., 2005; Wiens et al., 2006), and other molecular studies using allozymes (Highton and Larson, 1979) and immunology (Maxson et al., 1979). The *P. cinereus* group is the sister group to a clade comprising the other three eastern groups, which receives 99% bootstrap support. Within eastern *Plethodon*, only 9 of the 65 nodes on the tree are supported at the 95% level. Eleven eastern species are represented by more than one individual, but only four of these within-species comparisons are supported at the 95% level: all 5 *P. ouachitae*, 2 of 3 *P. wehrlei*, and the pairs of *P. yonahlossee* and *Plethodon serratus*. The five other nodes within eastern *Plethodon* with 95% or higher bootstrap support indicate that: (1) *P. metcalfi* and *Plethodon montanus* are sister species, (2) *P. dorsalis* and *P. ventralis* are sister species, (3) *P. hoffmani* and *Plethodon virginia* are sister species, (4) the two species of the *P. wehrlei* group are a monophyletic group, and (5) the 10 species of the *P. cinereus* group form a monophyletic group.

Despite the low bootstrap support for the remaining nodes, the topology of the tree places 44 of the 45 species of eastern *Plethodon* into the four species groups that have been supported by morphological and molecular studies (Highton, 1962; Highton and Larson, 1979). In most molecular studies, the *P. cinereus* group is usually the sister group to the other three groups of eastern *Plethodon.* The only eastern species that does not cluster within its presently recognized species group is *P. websteri*, which clusters within the *P. glutinosus* group instead of the *P. welleri* group. The similarity of the 12s mitochondrial sequences of *P. websteri* to those of the *P. glutinosus* group is unexpected on the basis of analysis of morphology, allozymes, and immunology of the eastern species cited above. *P. websteri* is the sister group to three other species groups in the albumin tree (see below) and its position is variable in the several trees in Wiens et al. (2006). A contamination or misidentification problem with the *P. websteri* 12s DNA sample is ruled out because we sequenced the 12s gene of another individual from a different Alabama county, and it has the same sequence as the one reported here (see below). Moreover, DNA sequences for the nuclear genes encoding a courtship pheromone indicate that *P. websteri* is more similar to other species of the *P. welleri* group than it is to the *P. glutinosus* group (Palmer et al., 2005). The amino acid sequence of albumin in *P. websteri* is more similar to those of the *P. welleri* group (6.4–6.6%) than it is to that of the *P. glutinosus* group (7.5–8.4%). Therefore no evidence suggests that hybridization with a *P. glutinosus* ancestor transferred sequences of the nuclear genes to *P. websteri*. Possibly hybridization or a lateral transfer of the mitochondria from the ancestor of the *P. glutinosus* group to the ancestral *P. websteri* lineage occurred after the latter species separated from the ancestral line of the other species of the *P. welleri* group, transferring a mitochondrial genome from the proto-*P. glutinosus* group lineage to the ancestor of *P. websteri*.

Since the 12s sequence data do not support many of the relationships indicated in previous morphological and molecular studies attempting to reconstruct the phylogeny of eastern *Plethodon*, we tried to obtain a more highly supported DNA sequence-based phylogeny by sequencing a complete nuclear gene, albumin, with 1818 nucleotides.

3.2. Sequence divergence in the albumin gene

The number of bp in the albumin gene has remained very stable during the evolution of *Plethodon* as indicated by the comparison of the number of nucleotides in the genus (eastern and western *Plethodon*) with albumin sequences of the two ambystomatid species. All eastern *Plethodon* have albumins with 1818 bp, except for 19 eastern species of the *P. glutinosus* species group that are missing 12 bp at sites 22–33 in the remaining eastern species. The two outgroups have shorter indels. The two western species (*P. elongatus* and *P. stormi*) have a 3 bp indel at sites 26–28, while the two *Ambystoma* have an indel at sites 48–53 of the complete *Plethodon* albumin sequence. The indel present in the *P. glutinosus* group is in five of the seven species of the *P. jordani* complex, 13 of the 14 species of the *P. glutinosus* complex, and *P. aureolus*. Two other species of the *P. jordani* complex (*P. montanus*, *P. metcalfi*), one species of the *P. glutinosus* complex (*P. cylindraceus*), and five other species of the *P. glutinosus* species group (*P. caddoensis*, *P. kentucki*, *P. ouachitae*, *P. petraeus*, *P. yonahlossee*) all have the complete eastern *Plethodon* albumin sequence, which is probably the ancestral condition.

The percent sequence divergence of albumin DNA indicates little or no overlap between the range of variation in comparisons between (1) the two outgroup *Ambystoma* species compared to the 64 *Plethodon*: mean = 51%, range, 50–52%; (2) the 62 eastern compared to the two western *Plethodon*: mean%SD = 24%, range 21–27%; (3) the *P. cinereus* group (represented by nine of its ten known species, all but *P. shenandoah*), compared to the species of the three other groups of eastern *Plethodon*: mean = 12%, range 10–14%, and (4) comparisons within and between species of the three remaining clades of eastern *Plethodon* (P. *glutinosus*, *P. wehrlei*, and *P. welleri* groups): 1–10%. The ancient differentiation of the eastern and western *Plethodon* and the position of the *P. cinereus* group outside a clade comprising the other three groups of eastern *Plethodon* agree with the 12s findings and those of previous molecular studies cited above.

In the maximum likelihood albumin gene tree (Fig. 2 is a 70% consensus tree), the eastern *Plethodon* are monophyletic with 100% bootstrap support. Within the eastern *Plethodon* only 13 nodes are supported at the 95% level, which include eight of the 13 within-species comparisons (*P. aureolus*, *P. cheoah*, *P. cinereus*, *P. dorsalis*, *P. kentucki*, *P. ouachitae*, *P. welleri*, *P. wehrlei*). Some comparisons within four species (two *P. albagula*, three *P. chlorobryonis*, two *P. glutinosus*, and two *P. metcalfi*) do not cluster together in the tree. Two individuals of the *P. wehrlei* group from West Virginia cluster together with 98% bootstrap support, but they cluster with *Plethodon punctatus* (91% support) before the three cluster with the third *P. wehrlei* from Virginia. With 100% bootstrap support, all 26 of the species within the *P. glutinosus* group cluster as a monophyletic group (*P. ocmulgee* and *P. fourchensis* were not sequenced), as do nine species of the *P. cinereus* group (*P. shenandoah* was not se-

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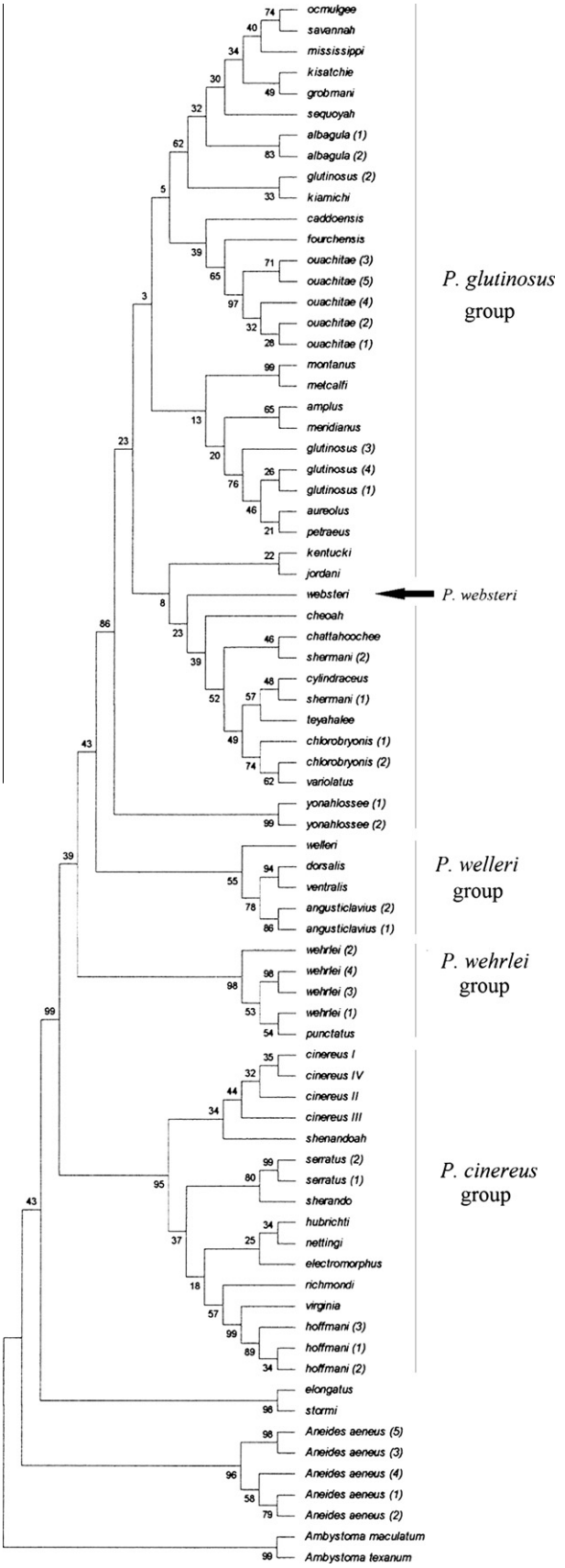

Fig. 1. Maximum likelihood tree for 12s sequence data with support values for each node, based on 2000 bootstrap trees.

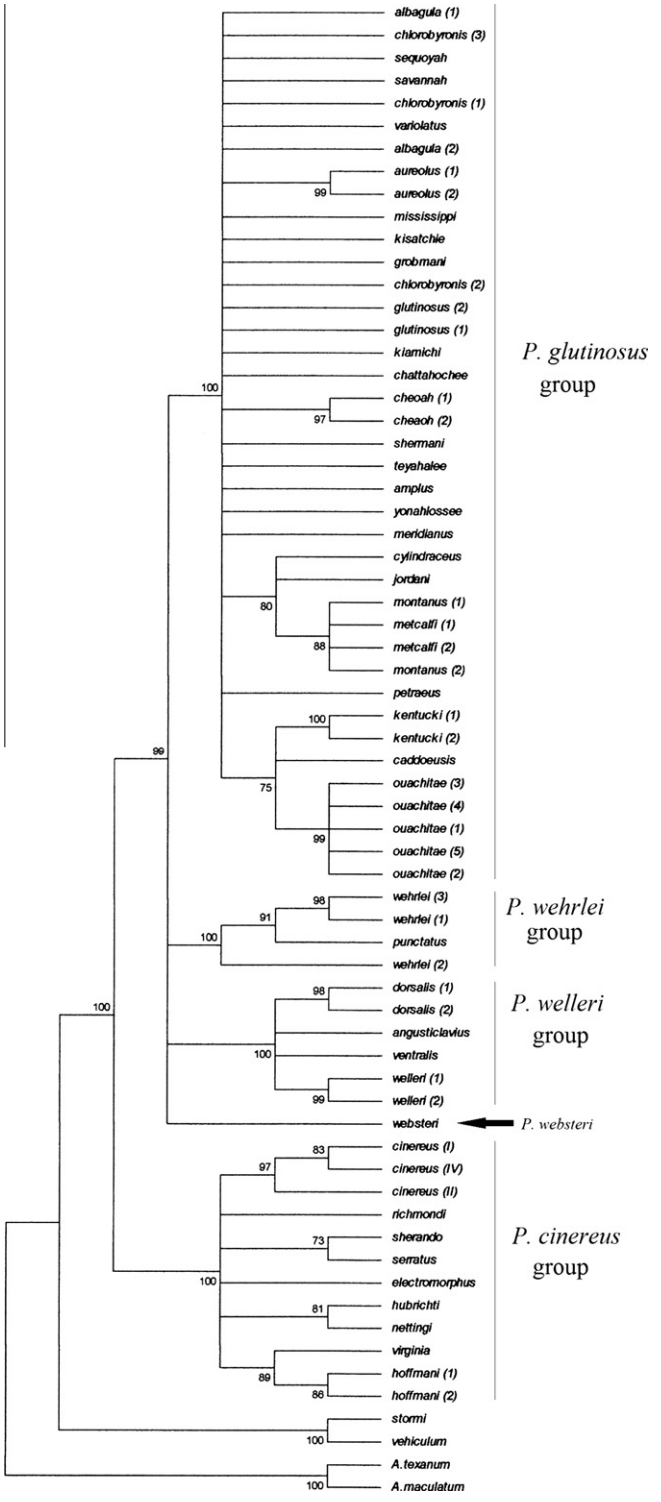

Fig. 2. 70% consensus maximum likelihood tree for albumin sequence data with support values for each node based on 2000 bootstrap trees.

quenced). In the other trees (not shown), the *P. welleri* and *P. wehrlei* groups are most closely related in the NJ tree, and *P. websteri* lies outside the clade comprising these two groups and the P. *glutinosus* group, not within the *P. glutinosus* group as it is in the 12s gene tree. The remaining four species of the *P. welleri* group make up a monophyletic group with 100% bootstrap support. The last node with high (99%) bootstrap support indicates that the *P. cinereus* group is the sister group to the other three groups of eastern *Pleth-*

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odon. In the 70% consensus tree only 22 nodes have bootstrap support at that level.

The divergence of albumin proteins also shows little or no overlap between the range of variation of comparisons between (1) the two outgroup *Ambystoma* species and the 64 *Plethodon* have percent amino acid differences: (mean = 70%, range, 66–73%); (2) the 62 eastern and two western *Plethodon* sequences (mean = 26%, range 22–29%; (3) the *P. cinereus* group and the remaining species of eastern *Plethodon* (mean = 9%, range 7–12%), (4) comparisons within and among the three other species groups of eastern *Plethodon* (*glutinosus*, *wehrlei*, and *welleri* groups) (0–8%). The ancient differentiation of the eastern and western *Plethodon* and the basal position of the *P. cinereus* group in relation to the other three groups of eastern *Plethodon* agree with the 12s findings and those of previous molecular studies cited above.

3.3. Combined sequences of 12s and albumin genes

The 12s sequences for 45 eastern species of *Plethodon* were combined with the albumin sequences of 42 species (not shown). The trees estimating the phylogeny based on these concatenated sequences are all similar in topology at basal levels to those based on a single gene, but no improvement in the number of bootstrap supported nodes is apparent.

3.4. Combined sequences of all six genes (⩽6989 bp)

The different trees estimating the phylogeny based on the six genes (the mitochondrial genes: 12s, cytochrome-b, and ND4; and the nuclear genes: albumin, RAG-1, and TPI, up to 6989 bp) are all quite similar in topology at basal levels. A neighbor joining tree for the six genes has only nine nodes within the eastern *Plethodon* with better than 95% bootstrap support, fewer than seen in the two gene trees (Figs. 1 and 2). Different populations within four species (*P. ouachitae*, *P. yonahlossee*, *P. wehrlei*, *P. serratus*) are supported at that level, as are the *P. wehrlei* and *P. cinereus* groups. *Plethodon montanus* and *P. metcalfi* are the only sister species in the tree that are supported. *P. websteri* clusters within the *P. glutinosus* group with 93% bootstrap support, and *P. shenandoah* clusters within the polyphyletic *P. cinereus*, but with low bootstrap support.

Using Tajima–Nei distances, the ranges of the percent sequence divergence for the six genes are: (1) between the two outgroup *Ambystoma* species and the 68 *Plethodon* (18–27%); (2) between the five samples of *A. aeneus* and the 66 *Plethodon* (11–21%), (3) between the two western *Plethodon* and the 64 eastern *Plethodon* (14–20%), and (4) between the 16 samples of the 10 species of the *P. cinereus* group and the 50 other eastern *Plethodon* of the *P. glutinosus*, *P. wehrlei*, and *P. welleri* groups (8–17%). The range of sequence divergences between *P. websteri* and the remaining four species of the *P. welleri* group is 5–9%, and among five samples of the two species of the *P. wehrlei* group is 8–14%, while those between the *P. glutinosus* group and the *P. wehrlei* groups are 10–14%. Those of *P. websteri* to the four other species of the *P. welleri* group are 6–11%. Comparisons of *P. websteri* to the *P. glutinosus* group are lower (2–6%) than are comparisons of *P. websteri* to the other species of the *P. welleri* group (5–9%) or the *P. wehrlei* group (11–12%).

4. Discussion

4.1. Taxonomy

The studies of the DNA sequences of *Plethodon* make it one the most studied of North American amphibians. This is the sixth study to include representatives from all four of the eastern species groups. Parts of five different mtDNA and four different nuclear genes have been sequenced. The studies include: Mahoney (2001), ND4 and tRNA genes, (21 species, 13 eastern); Sites et al. (2004), cytochrome-b, (14 species, all eastern); Palmer et al. (2005), two plethodontid receptivity factor genes (28 species, 27 eastern); Kozak et al. (2005), complete ND2 and tRNA genes (46 species, 44 eastern); Wiens et al. (2006), cytochrome-b, ND4, RAG-1, TPI genes, (50 species, 44 eastern); 12s gene and complete albumin gene (47 species, 45 eastern) (this study). In addition, there are five other mtDNA sequence analyses, all of the *P. glutinosus* group, of the cytochrome-b, ND2, ND4, and tRNA genes: Shepard and Burbrink (2008, 2009, 2011) (five species); Weisrock and Larson (2006) (19 species); and Weisrock et al. (2005) (23 species). Instead of gene trees converging on well-supported phylogenies within species groups, the results are disappointing.

Trees estimating the phylogeny of the included species are provided in all of the above mentioned studies. Instead of a broad consensus across studies for many nodes, only 10 clades in the eastern *Plethodon* tree are highly supported. These are: (1) the eastern *Plethodon* (45 species) are a monophyletic group; (2) the *P. cinereus* group (10 species) is the sister group to clade consisting of the other three groups of eastern *Plethodon*; (3) the *P. wehrlei* (two species) group is monophyletic; (4–7) four species of the *P. welleri* group have the relationships: (((*dorsalis*, *ventralis*) *angusticlavius*) *welleri*), (8) the *P. ouachitae* complex (three species) is a monophyletic group; (9) *P. hoffmani* and *P. virginia* are sister species, and (10) *P. serratus* and *Plethodon sherando* are sister species (the recently described *P. sherando* is included only in this study). With only 10 nodes supported consistently, the other 34 nodes in the 45-species tree of eastern *Plethodon* represent relationships that remain in doubt despite the considerable DNA sequencing effort. Since the ten strongly (>95%) supported relationships of the DNA sequence studies were previously indicated in morphological, allozyme, and immunological studies, the DNA sequencing studies (including this one) have made few advances in our understanding of the details of the phylogeny of eastern *Plethodon*.

Some additional nodes that are less strongly supported in one or more of the DNA sequence studies also agree with previous genetic studies, but the relationships of many species are still not clear. The two species groups that are not included in the 10 highly supported nodes are the *P. glutinosus* and *P. welleri* groups, although some sequence studies indicate that the groups are monophyletic at the 98–100% bootstrap level. A major problem is that one species, *P. websteri*, sometimes clusters within the *P. glutinosus* group, although in other studies it is closest to the other four species of the *P. welleri* group, the *P. wehrlei* group, or to both of the latter groups. If *P. websteri* had been omitted from all studies, all trees would show that both the *P. glutinosus* and *P. welleri* groups are monophyletic groups. The relationship of all the species of the *P. welleri* group (including *P. websteri*) is supported by morphological, allozyme, and immunological data. Morphology unquestionably indicates that *P. websteri* is a member of the *P. welleri* group.

A second problem with the DNA sequence data of *Plethodon* is the frequency of non-monophyly of within-species comparisons when multiple geographic samples are included in the studies. Since the monophyly of almost all species is strongly supported by morphological and allozyme evidence as well as geographic cohesiveness, the indications of non-monophyly of some species in gene trees obtained from sequence data may indicate that variation within the species in some genes has not yet coalesced within each species. Three examples are:

(1) *P. cinereus* and *P. shenandoah* differ consistently in a number of morphological characters (Highton and Worthington, 1967), yet sometimes they are not sorted into monophyletic

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species in DNA trees. The latter species is found in a limited area (in three northwest facing talus slopes on three of the highest mountains of Shenandoah National Park, Virginia). On one of these mountains (The Pinnacle) it is sympatric with *P. cinereus* throughout the isolate with no morphological evidence of hybridization. In the other two isolates the two species overlap mainly at the periphery of the isolates with little or no evidence of hybridization except at one site at the edge of the Hawksbill isolate. Clearly these are two biological species.

(2) The similarity of the 12s mitochondrial DNA of *P. glutinosus* and *P. aureolus* is unexpected in light of the amount of protein divergence between the two species (range of Nei *D* [Nei, 1972] = 0.22–0.42, Highton and Peabody, 2000; mean *D* = 0.31, Highton, 1989). The two species are largely parapatric, but have been taken together at a single site in Polk County, Tennessee (Highton, 1984) without evidence of hybridization. They differ in size and show a complete difference at one locus and have two loci with fixed differences in 24 protein loci evaluated electrophoretically. Yet the 12s haplotypes of *P. aureolus* from Tennessee and *P. glutinosus* from Illinois are identical, although three other *P. glutinosus* samples from Georgia, New Jersey, and Kentucky have haplotypes differing in only 1–2 bp. We also sequenced the 12s gene in single individuals from two other populations of *P. aureolus* and found they differed from the one used in this study by only two and five bp (unpublished data). These comparisons indicate that there is little geographic variation within both species. The similarity in the 12s sequences is unexpected because in studies based on allozyme data, *P. aureolus* is not closely related to *P. glutinosus*. The two species differ morphologically (Highton, 1984; Carr, 1996) and there is no evidence of present hybridization between the two species at the one known site where they are sympatric. The estimate of the time of their divergence based on allozyme data is >4 my. Since the ranges of these species are parapatric, there is a possibility that the 12s mtDNA of *P. aureolus* was derived from that of *P. glutinosus* by hybridization. On the other hand, the presence of the same haplotype in Illinois *P. glutinosus* and Tennessee *P. aureolus* might be due to extremely slow rates of 12s evolution in both species. There is little chance of a laboratory error because the Illinois *P. glutinosus* was never in our possession and was sequenced in Allan Larson's laboratory at Washington University.

(3) Another example of a problem with the 12s mt DNA sequence data is the similarity of *P. websteri* to the members of the *P. glutinosus* group (Fig. 1). We sequenced the mtDNA of a second individual of *P. websteri* from a different locality (in Blount County, Alabama) and found it had the same haplotype as the one in this study from Jefferson County, Alabama. Morphological and all other DNA sequencing studies (including our albumin analysis) do not place *P. websteri* in the *P. glutinosus* group, so we doubt that *P. websteri* and the *P. glutinosus* group form a clade.

A different taxonomic problem in the *P. wehrlei* group raises the possibility that unrecognized species are still to be found in eastern *Plethodon*. We sequenced the albumin gene in three geographic samples and the 12s gene in four widely separated populations of *P. wehrlei*. An allozyme study of this group has not yet been completed. Very different geographic color phenotypes occur in *P. wehrlei* (Cupp and Towles, 1983; Highton, 1987). All known individuals from the Appalachian Plateau physiographic province west of the New River of southwestern West Virginia, southeastern Kentucky, and northeastern Tennessee possess large dorsal yellow spots. In the remainder of the range of *P. wehrlei*, the yellow spots are absent, although some populations have smaller red dorsal spots. None of the 12s or albumin trees indicate consistent sequence differences between the yellow-spotted form and the northern form of *P. wehrlei*, even though they reveal considerable sequence variation among other populations. Our DNA sequence data indicate that the sample of *P. wehrlei* from the Blue Ridge physiographic province of southwestern Virginia is quite different from other populations of the *P. wehrlei* group. The latter cluster more closely with *P. punctatus*, suggesting that as currently recognized *P. wehrlei* may be a non-monophyletic species. If the southwestern Virginia populations of *P. wehrlei* represent a different species, there are two available names currently in the synonymy of *P. wehrlei* (*Plethodon dixi* Pope and Fowler, 1949, and *Plethodon jacksoni* Newman, 1954).

The taxonomic interpretations of Wiens et al. (2006) suggest other instances in which DNA data may be misleading:

(1) Because the mtDNA sequences of *P. ocmulgee* and *Plethodon savannah* show little differentiation, Wiens et al. (2006) question the recognition of these taxa as separate species. The two species are also similar in all trees calculated from 12s sequences where they cluster as sister species. Yet the two nuclear genes sequenced by Wiens et al. (2006) show considerable differentiation between these two species, and they do not cluster together as sister species in their two nuclear gene trees (we did not sequence the albumin of *P. ocmulgee*). The allozymes of these two species are quite different, and consequently they are widely separated in the trees in Highton (1989). It is not useful to question the validity of species on the basis of the sequences of one or two genes without considering other available sequence data, as well as previous morphological and molecular studies, especially when data on nuclear genes in their own study indicate that these two species are genetically quite different.

(2) Wiens et al. (2006) also questioned the recognition of *P. albagula* and *P. sequoyah* as separate species. *Plethodon sequoyah* is nested within several populations of *P. albagula* in their tree based on the sequences of two mtDNA genes, but the two are not always sister species in their other trees, nor are they sister species on our 12s and albumin gene trees. These two species also have several allozyme differences and are not closely related in the tree in Highton (1989).

(3) Wiens et al. (2006) questioned the recognition of *P. chlorobryonis* and *P. variolatus* as separate species on the basis of similarities in their mtDNA sequences, but these two species do not cluster together in their trees based on nuclear gene trees. However, these parapatric species cluster as sister species in a tree based on allozyme data (Highton, 1989).

These examples illustrate the need to take all available data into account when suggesting taxonomic relationships. Previous morphological and genetic studies should not be ignored in favor of conclusions based on a limited number of DNA sequences in gene trees and statistical analyses that are based on inflated support probabilities. Larger geographic representation of each species would also be useful in attaining well-supported phylogenies for use in testing taxonomic and phylogeographic hypotheses.

Wiens et al. (2006) questioned the validity of one species in another species pair that they recognized: *P. teyahalee* and *P. ocona-luftee*. No taxonomist has ever suggested that these were separate species; there is only a disagreement about which name to use for the species (Hairston, 1993; Highton and Peabody, 2000). Thus, Wiens et al. (2006) compared two individuals of the same species but gave them different species names. They further

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compounded their error by regarding one specimen as a *P. teyahalee* when it actually came from a site near the type locality of *P. oconaluftee*, and by listing their other specimen as a *P. oconaluftee* when it came from near the type locality of *P. teyahalee*.

Another pair of species (*P. yonahlossee* and *P. longicrus*) recognized by Wiens et al. (2006) were regarded as distinct species by Adler and Dennis (1962). However, Guttman et al. (1978) showed that their allozymes are similar, and found that intergradation occurs between the two forms. Ever since, taxonomists have not recognized *P. longicrus* as a valid species.

There are similar problems in the topology of trees based on DNA variation in *Plethodon* in other sequence studies. For example, in the Weisrock et al. (2005) analysis of mitochondrial sequence variation in populations of the *P. jordani* complex, several species appear not to be monophyletic (*P. shermani*, *P. metcalfi*, *P. teyahalee*), probably due to lineage assortment in the mitochondrial gene and/or hybridization among species. These cases are probably not indications that the present taxonomy is incorrect, because these problems do not occur in the species trees of ten allozyme studies that have been published on eastern salamanders of the genus *Plethodon* (Duncan and Highton, 1979; Highton, 1984, 1989, 1997, 1999, 2009; Highton and MacGregor, 1983; Highton and Peabody, 2000; Highton and Webster, 1976; Larson and Highton, 1978). All of the species in these allozyme studies are based on series of salamanders from multiple populations of each species. Only one sample (population 92 in Highton, 1989) of the 592 populations included in the above ten studies (omitting hybrid populations) fails to cluster in UPGMA trees within a monophyletic group that includes all the other samples of its own species. Moreover, in the allozyme studies, almost all species are made up of contiguous geographic units. There may be some circularity to the consistent finding of monophyly of species in allozyme studies in which some of the species were first discovered as a result of allozyme variation. However, all these species differ considerably genetically, many pairs are sympatric with no evidence of hybridization, and many pairs also have been shown to be independently evolving from each other for long periods as indicated by Good and Wake (1992) comparisons and the distributions of genetic distances (Highton, 1998, 1999, 2000, 2009). Moreover, the concordance of species limits based on morphological and allozyme variation is general in the parapatric pairs of morphologically distinct species of *Plethodon*, (e.g., compare the ranges of members of the *P. glutinosus* complex in Highton, 1972, with that in Highton, 1989, and Highton and Peabody, 2000). In allozyme studies, credible hypotheses of the geographic location of species boundaries are often in agreement with geological history and barriers to gene flow in *Plethodon* (large rivers, mountain ranges, or uninhabited lowland areas (Highton, 1989, 1997, 1999, 2009). All of these results argue that, with the present methodology of interpreting sequence data, allozyme and morphological data should not be ignored in the interpretation of DNA sequence results.

A final example illustrates the point that failure to take other types of studies into account may cause workers to fail to appreciate that strong phylogenetic signals in different data sets tend to be in agreement. Shepard and Burbrink (2008) studied geographic variation in two mt DNA genes of 55 samples of *P. ouachitae* and concluded that "Our results provide new insight into sky island diversifications in a previously unstudied region." Yet in their paper they cite studies on geographic variation in color pattern throughout the known range of *P. ouachitae* in the Ouachita Mountains by Blair and Lindsay (1965), as well an allozyme analysis of protein variation by Duncan and Highton (1979). Both of the latter studies revealed essentially the same geographic patterns of isolation as found by Shepard and Burbrink (2008), indicating that the diversification in the area had indeed been successfully studied earlier. Blair and Lindsay's morphological study found three groups within *P. ouachitae*, while Duncan and Highton found five groups. The sequence study by Shepard and Burbrink confirmed the same five geographic groups, but they reported two additional groups of haplotypes in the analysis of their mt DNA sequences, although their Fig. 1 shows that neither is restricted to a single geographic isolate. Thus, it is more likely that five rather than seven geographic isolates are present in *P. ouachitae*. On the other hand, using the same approach that they applied to *P. ouachitae* on *P. fourchensis* and *P. caddoensis*, Shepard and Burbrink (2009, 2011) discovered previously unknown patterns of geographic subdivision that the earlier studies had not revealed.

4.2. Phylogeny and speciation

The difficulties encountered in attempting to elucidate the evolutionary relationships within species groups of *Plethodon* based on DNA sequence data are not surprising given the timing of speciation within these groups. The hypothesis of a late Miocene and Pliocene burst of speciation (Highton and Larson, 1979; Highton, 1995) suggests that most of the species within species groups of eastern *Plethodon* were isolated in various mountain ranges during the warm dry periods of the Pliocene Epoch (5.3–1.8 mya), because during that time forests were replaced by grasslands in many lowland areas in eastern North America. Molecular clock estimates for the beginning of speciation based on albumin immunological distances and allozyme genetic distances within the four eastern species groups (except for *P. websteri*) are mostly within the Pliocene or late Miocene Epochs and none are >10 my. Most estimates for the initiation of speciation are in the Pliocene. These generally agree with divergence times based on DNA sequence data in Wiens et al. (2006). The similarities in the amount of genetic divergence among so many of the species of eastern *Plethodon* may make it extremely difficult to obtain a dichotomous species phylogeny no matter how many genes are sequenced. Thus far, allozymes may have sometimes provided better tree topologies than presently available sequence data in revealing species relationships in this genus because of the larger number of genes studied (22–29), the larger number of populations sampled within most species, the large sample sizes (usually 10–30 individuals per sample), and the use of population data based on allele frequencies, which is one of population lineages and not of gene lineages. In contrast, DNA sequence studies are sometimes based on only one or a few genes, populations per species, and individuals per population.

Nevertheless, some patterns of variation revealed by the allozyme and DNA sequencing methods agree and support previous taxonomic conclusions. An example is the three species of the *P. ouachitae* complex (*P. caddoensis*, *P. fourchensis*, and *P. ouachitae*), endemic to the Ouachita Mountains of Arkansas and Oklahoma, which cluster as a monophyletic group within the *P. glutinosus* group in all studies based on morphology and molecular analyses. These species probably were derived from a common ancestor that had been isolated in the Ouachita Mountains for some time before later speciation by subdivision and long periods of isolation occurred in three isolated mountains resulting in the three living species. They may also have occasionally and intermittently exchanged genes with each other during interludes of wetter and/or cooler climates during the Pliocene and Pleistocene Epochs, as *P. fourchensis* and *P. ouachitae* do at the present time in a narrow hybrid zone on Fourche Mountain (Blair and Lindsay, 1965; Duncan and Highton, 1979, Shepard et al., 2011).

4.3. Species groups

All species of eastern *Plethodon* form a monophyletic group, as shown by morphological, allozyme, DNA hybridization, and immu-

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nological studies. This conclusion is also supported in all published DNA sequence studies and the new DNA sequence data in this study.

The *P. cinereus* group is the sister group to a clade comprising the other three groups of eastern *Plethodon* in all molecular studies. Sequence data do not clarify the phylogenetic relationships of the other three species groups. The ancient separation of the *P. cinereus* group is also shown in trees based on allozyme data (81% bootstrap support; Highton, 1991), immunological data (Maxson and Maxson, 1979; Hass et al., 1992) and DNA sequence data of Mahoney (2001), Chippindale et al. (2004), Kozak et al. (2005), Palmer et al. (2005), Vieites et al. (2007), and this study. The relationships of the other three groups are problematic. Morphological studies, based on size and body proportions, indicate that these characteristics of the *P. glutinosus* and *P. wehrlei* groups are derived, while the *P. welleri* group retains the ancestral size, coloration, and body proportions of the *P. cinereus* group. The DNA sequencing studies are inconclusive in that some indicate that the *P. welleri* and *P. wehrlei* groups are closely related, intermediate between the *P. glutinosus* and *P. cinereus* groups (Wiens et al., 2006; Palmer et al., 2005), while others do not (Mahoney, 2001; Kozak et al., 2005). Mahoney (2001) obtained conflicting arrangements depending on the method of tree construction, but her trees included only one species in each of the *P. wehrlei* and *P. welleri* groups. These conflicting results would not be surprising if the three groups (and *P. websteri* as well) diverged from each other at about the same time.

4.4. P. cinereus group

The *P. cinereus* group consists of 10 species (Highton, 1999, 2004). The range of the widely distributed *P. cinereus* overlaps eight of the other species of the group. *Plethodon serratus* has a subdivided range and is the only species that does not occur sympatrically with any other member of the group, whereas *P. cinereus* occurs sympatrically with the other eight species. The *P. cinereus* group may also be morphologically the most primitive of the eastern *Plethodon*. The eastern *P. cinereus* and the western *P. vehiculum* are very similar in appearance, even possessing the same striped-unstriped dorsal color pattern polymorphism. The shape of the mental gland of all species in the *P. cinereus* group is distinct from that of all other eastern *Plethodon* (Highton, 1962). The group also retains ancestral courtship behavior (Palmer et al., 2005) and associated dental modifications in the premaxillary teeth of males (Highton, 1962). An allozyme study by Hass (1985) revealed extensive geographic genetic variation within the widely distributed *P. cinereus*. All four groups of *P. cinereus* are included in the 12s analysis, but representatives of only three groups were sequenced for albumin.

The phylogeny of the 10 species of the *P. cinereus* group remains poorly resolved, with partially different arrangements in the allozyme (Highton, 1999a), mitochondrial DNA trees (Mahoney, 2001; Sites et al., 2004; Wiens et al., 2006), and nuclear DNA trees (Palmer et al., 2005; Wiens et al., 2006; our albumin data). Morphology would indicate that *P. hoffmani* and *P. virginia* are sister species, and all the DNA sequence comparisons support that conclusion, as do allozyme studies (Highton, 1999, 2009). Morphology and allozyme data indicate that *Plethodon richmondi* and *Plethodon electromorphus* are sister species, but this monophyly is not supported by some DNA trees. Morphology indicates that *P. cinereus*, *P. sherando*, and *P. serratus* are very similar, and the latter two species cluster as sister species on our DNA trees. They also cluster as sister species in allozyme comparisons (Highton, 2004).

4.5. P. welleri group

Except for the unstable position of *P. websteri* in the trees based on 12s data (see above), the remaining four species of the *P. welleri* group cluster with the same topology as in the allozyme tree (Larson and Highton, 1978). Two sister sibling species (*P. ventralis* and *P. dorsalis*) were recognized taxonomically as a single species until Highton (1997). They hybridize in a narrow hybrid zone in Lincoln County, Kentucky. *P.* is morphologically distinct and is the sister clade to one consisting of the three species *P. angusticlavius*, *P. dorsalis*, and *P. ventralis* in all molecular studies.

4.6. P. wehrlei group

In the *P. wehrlei* group, five populations are represented, four *P. wehrlei* and one *P. punctatus*. In several DNA trees the single *P. punctatus* population clusters within *P. wehrlei*. The two species were originally recognized as a result of differences in coloration and number of vertebrae (Highton, 1972), and there are fixed or complete differences between single samples of the two species at four of 29 allozyme loci (Highton and Larson, 1979). The reason that *P. wehrlei* is not monophyletic is that the southern populations of this species are more different genetically from both northern *P. wehrlei* and *P. punctatus* than the two species are from each other, as discussed above.

4.7. P. glutinosus group

Except for the inclusion of *P. websteri* within the *P. glutinosus* group in the 12s trees discussed above, this group is monophyletic in all DNA trees. In allozyme trees, the most different species are usually *P. yonahlossee*, *P. petraeus*, *P. kentucki*, *P. aureolus*, *P. savannah*, *P. cheoah*, and the three species of the *P. ouachitae* complex. The members of both the *P. glutinosus* (14 species) and *P. jordani* (seven species) complexes do not cluster as monophyletic groups in allozyme and mtDNA trees, suggesting that these complexes may not be natural groups. This is surprising in light of the parapatric distributional patterns within each complex, and the morphological and ecological similarities of the species within the two complexes. Additional studies on the relationships of these species are needed as it seems likely that in spite of numerous molecular data sets, existing data are not appropriate to address the question of the phylogenetic relationships among the species in the *P. glutinosus* group. This may be due to the contemporaneous isolation of most of the species in the *P. glutinosus* and *P. jordani* complexes.

5. Conclusions

In the eastern *Plethodon*, a clade with 46 living species, there probably were contemporaneous bursts of speciation in the late Miocene and Pliocene which produced many closely related extant species in each of the four species groups. We believe that this history is the cause of variability among different DNA studies of systematic relationships, particularly within the *P. glutinosus* and *P. cinereus* species groups. Our analysis of the available evidence indicates that many relationships remain unresolved within these species groups of eastern *Plethodon*. This lack of resolution may be expected because many speciation events were contemporaneous. Nevertheless, some recent studies based on DNA sequences have reported high support values for relationships which were used to test evolutionary hypotheses. Our analysis indicates that the highly-resolved *Plethodon* phylogeny seen in some DNA studies is an illusion that has two primary sources. (1) Because some recent studies have based their conclusions on Bayesian trees, investigators have not appreciated that the support values (posterior probabilities) exaggerate statistical support for nodes. (2) Some recent studies have often failed to compare their results with the large body of systematic work that is available for the genus *Plethodon*. That body of work includes extensive analyses based on distribu-

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tional patterns, morphology, allozymes, immunology, and DNA hybridization, as well as DNA sequences.

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