



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 north-western 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 parapatry 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 descendants. 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. welleri* 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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