

# RAPID DIVERSIFICATION, INCOMPLETE ISOLATION, AND THE “SPECIATION CLOCK” IN NORTH AMERICAN SALAMANDERS (GENUS *PLETHODON*): TESTING THE HYBRID SWARM HYPOTHESIS OF RAPID RADIATION

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**Abstract.**—The history of life has been marked by several spectacular radiations, in which many lineages arise over a short period of time. A possible consequence of such rapid splitting in the recent past is that the intrinsic barriers that prevent gene flow between many species may have too little time to develop fully, leading to extensive hybridization among recently evolved lineages. The salamander genus *Plethodon* in eastern North America has been proposed as a possible example of this scenario, but without explicit statistical tests. In this paper, we present a nearly comprehensive phylogeny for the 45 extant species of eastern *Plethodon*, based on DNA sequences of mitochondrial (two genes, 1335 base pairs) and nuclear genes (two genes, up to 3481 base pairs). We then use this phylogeny to examine rates and patterns of diversification and hybridization. We find significantly rapid diversification within the *glutinosus* species group. Examining patterns of natural hybridization in light of the phylogeny shows considerable hybridization within this clade, including introgression between species that are morphologically distinct and distantly related. Reproductive isolation increases over time and may be very weak among the most recently diverged species. These results suggest that the origin of species and the evolution of intrinsic reproductive isolating mechanisms, rather than being synonymous, may be decoupled in some cases (i.e., rapid origin of lineages outstrips the “speciation clock”). In contrast to the conclusions of a recent review of adaptive radiation and hybridization, we suggest that extensive hybridization sometimes may be a consequence, rather than a cause, of rapid diversification.

**Key words.**—Amphibians, diversification, hybridization, phylogeny, reproductive isolation, salamanders, speciation.

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Many clades contain pulses of rapid radiation, in which large numbers of new species arise over a short period of time. These rapid radiations may be adaptive (involving ecological differentiation of the species), nonadaptive, or some combination of the two (Schluter 2000). One potential consequence of rapid radiations in the recent past is that there may be too little time for effective intrinsic prezygotic and postzygotic isolating mechanisms to have evolved, leaving these species subject to introgressive hybridization (i.e., hybridization in which there is actual exchange of genes between species and not merely production of inviable or infertile offspring; Seehausen 2004). For example, the Galapagos finches have provided a classic example of adaptive radiation (reviewed by Grant and Grant 2002). They also represent one of the fastest radiations of species known, in terms of the absolute number of species produced per unit time (but lagging behind the Rift Lake cichlids; Table 12.1 of Coyne and Orr 2004). Recent studies have documented frequent hybridization among some species of Darwin’s finches, hybridization which may have important adaptive consequences (e.g., Grant and Grant 1992; Grant et al. 2005). Similarly, studies of Rift Lake cichlids suggest that these species may genetically swamp each other when changes in water visibility impair visual premating isolating mechanisms (Seehausen et al. 1997). There is also widespread gene flow

among species of Hawaiian crickets of the genus *Laupala* (Shaw 2002), the fastest known radiation of arthropods (Mendelson and Shaw 2005). All three radiations (finches, fish, and crickets) appear to have occurred relatively recently.

In an intriguing review article, Seehausen (2004) summarized many cases of hybridization among species generated by adaptive radiations. He suggested that hybridization was an important factor driving these rapid radiations, in large part because hybridization can generate new variability and novel phenotypes for selection to act upon (i.e., transgressive segregation). Seehausen’s hypothesis was motivated (in part) by the observation of strongly supported incongruence between gene genealogies at the base of rapid radiations, possibly indicating ancient hybridization events. Although this “hybrid swarm” hypothesis of rapid radiation may apply in many cases, we suggest that in other cases widespread hybridization may be a consequence of rapid, recent radiation, rather than a cause of it. A fundamental difference between these hypotheses is that the former predicts introgressive hybridization at the phylogenetic base of the radiation, whereas the latter predicts introgression among the tips. However, these two hypotheses need not always be mutually exclusive (e.g., ancient hybridization in a clade might lead to accelerated diversification rates, which then leads to extensive hybridization among the rapidly generated species).

The idea that rapid, recent diversification may sometimes produce incompletely isolated lineages is consistent with studies showing that intrinsic reproductive isolation increases gradually over time (i.e., the “speciation clock”) and is

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weakest among the most recently diverged species (e.g., Coyne and Orr 1989, 1997, 2004; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002; Mendelson 2003; Bolnick and Near 2005). However, the relationship between rapid diversification and incomplete reproductive isolation may also depend on geographic patterns of species overlap (i.e., sympatric species seem to become reproductively isolated more rapidly, possibly through reinforcement; Coyne and Orr 2004), whether the radiation is adaptive or nonadaptive (i.e., ecological divergence may hasten reproductive isolation; Schluter 2000), and other factors.

The salamander genus *Plethodon* in eastern North America may offer an example of a rapid, recent radiation of incompletely isolated lineages. In a review of his extensive systematic research on *Plethodon*, Highton (1995) suggested that there was a rapid burst of speciation about 5 million years ago, which produced most of the approximately 35 species in eastern North America recognized at that time (22 of these species belong to a single putative clade, the *glutinosus* species group). Highton (1995; see also Highton and Peabody 2000) summarized the occurrence of many hybrid zones between these species, and suggested that “the amount of divergence between many species of large eastern *Plethodon* has not been sufficient to result in complete reproductive isolation between many recently differentiated species” (p. 592) and that “reproductive isolation may take several million years to evolve and has not yet been completed between a number of pairs of species” (p. 595). If these inferences are correct, the eastern *Plethodon* would represent a rapid and recent radiation of species on par with the Galapagos finches and might support the hypothesis that recently generated species are especially prone to hybridization. However, these basic patterns require further verification. For example, Highton (1995) provided neither a rigorous phylogeny nor any statistical tests of these hypotheses.

In this study, we test Highton’s hypotheses utilizing a nearly comprehensive phylogeny for *Plethodon* in eastern North America based on nuclear and mitochondrial DNA sequences and using new methods for testing dates and rates of diversification and the evolution of reproductive isolation over time. Several previous studies have addressed relationships among sets of *Plethodon* species using allozymes (e.g., Highton and Larson 1979; Highton 1989; Highton and Peabody 2000) and mitochondrial DNA sequences (e.g., Mahoney 2001; Sites et al. 2004; Weisrock et al. 2005), but most included only a limited number of species. A recent study addressed phylogenetic relationships and diversification rates of most eastern *Plethodon* species (Kozak et al. 2006), and found evidence for rapid diversification in the group. However, that study only included mitochondrial data, found only weak support for most deeper nodes in the phylogeny, and did not address the evolution of reproductive isolation, genealogical discordance, or the potential relationship between hybridization and rapid radiation.

Here we address the following questions. (1) What are the phylogenetic relationships among species of eastern *Plethodon*? (2) What is the temporal scale over which these species and clades arose (i.e., did most species arise in the last five million years)? (3) Is there evidence for a significant increase in speciation rates within eastern *Plethodon* in the recent past?

(4) How are patterns of natural hybridization in eastern *Plethodon* related to phylogenetic distance and time? In other words, is there widespread introgression among very recently diverged species, as predicted by Highton (1995)? Many previous studies have examined the rate of evolution of reproductive isolation using levels of genetic divergence as a proxy for time and implicitly assuming a strict molecular clock (e.g., Coyne and Orr 1989, 1997; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002; Mendelson 2003), but few have considered patterns of isolation using rigorous estimates of time (e.g., Bolnick and Near 2005). (5) Is there strongly supported incongruence between gene genealogies at the base of rapidly speciating *Plethodon* clades, as predicted by Seehausen’s (2004) hybrid swarm hypothesis?

The genus *Plethodon* consists of 55 currently recognized species (AmphibiaWeb 2006). The genus is confined to the United States and southern Canada. Most species occur in eastern North America (especially the Appalachian Mountains), although there are eight species in the mesic Pacific Northwest and a single species in the mountains of northern New Mexico (AmphibiaWeb 2006). All *Plethodon* are direct developing, terrestrial, and generally feed on insects and other small invertebrates (Petranka 1998).

## MATERIALS AND METHODS

### Taxonomy

We follow current taxonomy in recognizing 55 species of *Plethodon* (AmphibiaWeb 2006). Many species of *Plethodon* have been recognized based on allozymes (i.e., diagnostic alleles, levels of genetic distance, and clustering of populations on a tree), coloration, and body proportions by Highton (1989) and Highton and Peabody (2000), and their recognition was justified based on appeals to the biological species concept. Although some authors have voiced concerns about the distinctness of some species recognized by Highton (e.g., Petranka 1998) because some are morphologically cryptic and some hybridize, no alternate taxonomy has been proposed. Highton’s taxonomy has been adopted by many other researchers (e.g., Watts et al. 2004; Palmer et al. 2005; Weisrock et al. 2005; Kozak et al. 2006), field guides (e.g., Conant and Collins 1991), and major web-based summaries of amphibian taxonomy, including the Global Amphibian Assessment web-site (<http://www.globalamphibians.org>; IUCN et al. 2004), and Amphibian Species of the World, version 3.0 (Frost 2004). Furthermore, as we describe in the Discussion, the distinctness of most of these species is also supported by our results. We differ from standard taxonomy only in that we treat *P. oconalufee* and *P. tayahalee* as separate terminal units, to allow the analysis to address their distinctness. Similarly, we also treat *P. longicrus* and *P. yonahlossee* as separate taxa, given that their taxonomic status has been controversial (Petranka 1998). However, our analyses of diversification rate are robust to consideration of these pairs of taxa as distinct or synonymous (see below).

### Sampling of Taxa

Recent work on higher-level plethodontid relationships shows that *Plethodon* is monophyletic with respect to other

plethodontine genera (Chippindale et al. 2004) despite previous uncertainty (e.g., Mahoney 2001). Furthermore, many previous authors have suggested that the *Plethodon* of eastern North America form a monophyletic group (e.g., Highton and Larson 1979; Mahoney 2001; Chippindale et al. 2004). We included representatives of all currently recognized and extant species of *Plethodon* in eastern North America, with the exception of the recently described *P. sherando* (Highton 2004). Note that *P. ainsworthi* is considered to be extinct (IUCN et al. 2004), and is known only from two formalin-preserved specimens. For most species, we included at least two individuals. A few geographically restricted species were represented by a single individual, and some geographically widespread species were represented by up to six. Five species of western *Plethodon* were also included. The specimens examined and their localities are listed in Appendix 1 (see Supplementary Material available online at: <http://dx.doi.org/10.1554/06-138.1.s1>).

We also included representatives of 12 genera of plethodontids as outgroups, using data from Chippindale et al. (2004). Two genera of plethodontines (*Hydromantes* and *Karsenia*) were not included in that study, but they clearly are outside of *Plethodon* and their exclusion should have no impact on our results (Min et al. 2005).

#### Molecular Data

We sequenced portions of two mitochondrial genes (cytochrome *b*; *cyt-b* and NADH dehydrogenase subunit 4; ND4) and one nuclear gene (recombination-activating gene 1; RAG-1) for at least one representative of almost all included taxa. These mitochondrial genes have proven useful in previous species-level analyses of plethodontids (e.g., Mahoney 2001; Chippindale et al. 2004; Sites et al. 2004). The segment of RAG-1 we utilized appears to have considerable resolving power at many phylogenetic scales in salamanders, including within plethodontid genera (e.g., *Eurycea*, *Desmognathus*, *Plethodon*; Chippindale et al. 2004; Min et al. 2005). To obtain additional information from the nuclear genome, we also sequenced two regions of the nuclear triose phosphate isomerase (TPI) gene for single individuals of 29 taxa, including 26 species of eastern *Plethodon*. One region spans the end of exon 2 through nearly all of intron 4, and the other extends from the middle of intron 5 through the beginning of exon 7. Basic properties of these datasets are described in Table 1.

DNA was extracted from frozen and alcohol preserved tissues using standard protocols. Targeted sequences were amplified using the polymerase chain reaction (PCR). Primers are listed in Table 2. Polymerase chain reaction products for *cyt b*, ND4, and RAG-1 were purified and then sequenced using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA). PCR products for TPI were gel-purified, cloned using TOPO-TA kits (Invitrogen, Carlsbad, CA), and a minimum of two clones per fragment were sequenced on a LiCor 4200L long-read sequencer (LI-COR Biosciences, Lincoln, NE). Some sequences were further checked by sequencing PCR products on an ABI 377 automated sequencer. Sequences were edited using Sequence Navigator (vers. 1.0.1, Applied Biosystems) or Sequencher

TABLE 1. Basic properties of the gene regions analyzed in this study. To make the datasets maximally comparable, the numbers of variable and parsimony-informative characters are based on the combined dataset (no more than 77 taxa) with no more than a single individual per species.

Gene (dataset)	Total number of characters	Variable characters	Parsimony-informative characters
Cytochrome <i>b</i>	649	373	335
ND4	686	441	397
Combined mtDNA	1335	814	732
RAG-1	1467	521	349
TPI (sequence)	1943	569	211
TPI (indels)	71	71	24
TPI (combined)	2014	640	235
Combined nuclear	3481	1161	584
All data combined	4816	1975	1316

(vers. 4.2, GeneCodes Corp., Ann Arbor, MI). Gene regions for *cyt b*, ND4, and RAG-1 were protein coding and generally lacked indels, and alignment was therefore unambiguous. Sequences for TPI consisted primarily (~75%) of intron regions; alignment using the pairwise alignment tool in MacClade version 4.02 (Maddison and Maddison 2001) generally was straightforward, although minor manual adjustments were employed. GenBank numbers for all sequences used are provided in Appendix 2 (see Supplementary Material available online at: <http://dx.doi.org/10.1554/06-138.1.s2>).

Reanalysis of the allozyme data of Highton and collaborators would be useful, but was not feasible for this study. There are no comprehensive allozyme datasets for eastern *Plethodon*, and combining data from different studies is problematic (alleles typically cannot be homologized between studies). Furthermore, parsimony analysis of the most taxonomically comprehensive dataset (Highton 1989, table 4; based on the most common allele in each species) yields almost no phylogenetic resolution (J. J. Wiens, unpubl. data).

#### Phylogenetic Analysis

Phylogenetic trees were reconstructed using parsimony and Bayesian methods and with separate and combined analyses of the four genes. However, our primary estimate of phylogeny was based on the partitioned Bayesian analysis of the combined data, and the other analyses were largely for exploratory purposes. We consider the best estimate of phylogeny to come from combined analysis of all the data (i.e., using the largest sample of characters), but taking into consideration clades that are strongly supported and incongruent (Wiens 1998a). We favor Bayesian analysis because it is model based and generally similar to maximum likelihood, but can easily accommodate different models and model parameters within a combined dataset (e.g., Nylander et al. 2004), can estimate phylogenies for large numbers of taxa, and provides measures of clade support that may be somewhat easier to interpret than bootstrap values (e.g., Wilcox et al. 2002; Alfaro et al. 2003; Erixon et al. 2003; Huelsenbeck and Rannala 2004).

We first performed a separate analysis of each of the four genes using parsimony and Bayesian methods. Parsimony analyses were implemented with PAUP\* version 4.0b10

TABLE 2. Oligonucleotide primers used in this study. Most primers reported as “this study” originally were used by Chippindale et al. (2004), but were not reported in that paper. TPI was sequenced unidirectionally but multiple times per sample for confirmation.

Primer	Sequence (5'–3')	Source
<i>cytochrome b</i>		
PGIudg2	GGTCTGAAAACCAATGTTGTATTC	this study
PThrR1	GCCCCAATTTTGGTTTACAAG	this study
Pcyt 659R	TGTATGAGAAGTATGGGTGRAATG	this study
Pcyt b 589 R	GTTTCATGTAGGAARAGGAGGTG	this study
Pcytb 419F	GTYCTCCCATGAGGACAAATATC	this study
MVZ16 (R)	AAATAGGAARTATCAYTCTGGTTTRAT	Moritz et al. (1992)
<i>ND4</i>		
ND4 (F)	CACCTATGACTACCAAAGCTCATGTAGAAGC	Arévalo et al. (1994)
Ephist	TCRTTTTTAGGGTCACRGCCTAG	this study
Phist	TTTTYTAGGRTCACRGCCTA	this study
PND4-350-F	ATGAACGAAACACATAGCCGAAC	this study
PND4-490-R	ATGGTTCAAGGGGATCAGTTA	this study
<i>RAG-1</i>		
R1FVEB	AGYCARTAYCAYAARATGTA	Ventakesh et al. (2001)
RP1184R	CATCTTCCGTGCAAAGTTTCC	this study
RP1485R	GTGGTGCTTCAGAACATCCTCC	this study
RAG1F	CAYTGYGAYATHGGNAAYGC	Greenhalgh et al. (1994)
RP1093R	TACGCCATCATCTTCCGTGC	this study
RP1032R	TTCTTCTCAAGTGCTTGTGCG	this study
RS6R	TGCTATRARNGGGCTCAAGATGG	this study
RP816F	AGAACCTGGAGCGCTATGAGATGTGGCG	this study
RP835F	GGCGGTCCAACCTCGCACCATGAG	this study
<i>TPI</i>		
TPI EXON2-9F	GGAGCCTTCACYGGNGAGATG	this study
TPI EXON7-14R	GATCCCCGACNARRAANCRTCCAGRTCAGGYTG	3' extension of TPI 3' primer of Nikoh et al. (1997)

(Swofford 2002), utilizing heuristic searches with tree-bisection-reconnection (TBR) branch swapping and 1000 random-taxon-addition sequence replicates. Support for individual clades was evaluated using bootstrapping (Felsenstein 1985) with 500 bootstrap pseudoreplicates and 10 random-addition sequence replicates per bootstrap pseudoreplicate, with TBR branch swapping. Clades with bootstrap values  $\geq 70\%$  were considered to be strongly supported for our purposes, following Hillis and Bull (1993; but see their extensive caveats).

Bayesian analyses were implemented using MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001). Prior to the Bayesian analysis of each gene, we identified the best-fitting model of DNA sequence evolution for each gene using hierarchical likelihood-ratio tests and the Akaike Information Criterion, as implemented in MrModeltest version 2.0 (Nylander 2004). Both approaches concurred that the best fitting model for cytochrome *b*, ND4, and RAG1 is GTR + I +  $\Gamma$  (general time reversible [Rodriguez et al. 1990] with a proportion of sites invariable [Gu et al. 1995] and rates at other sites varying according to a gamma distribution [Yang 1994]). We then tested whether recognizing partitions within each of these genes (i.e., for different codon positions) improved the fit of the model to the data using comparison of Bayes factors (see Nylander et al. 2004). Codon-position partitions were significantly supported for all three genes.

For TPI, the best fitting model is GTR +  $\Gamma$  (using both approaches), and we did not recognize partitions for different codon positions because most of the sequenced region consists of introns. Given that most of the sequence was non-

coding, there was considerable length variation within the gene. We coded putative insertions and deletions for phylogenetic analysis following the general method outlined by Simmons and Ochoterena (2000), coding gaps as present or absent, and treating overlapping gaps of varying length as different character states. Because current versions of MrBayes do not allow for complex ordering of character states, gaps of different lengths were generally treated as unordered, unless length differences could all be explained by length changes to only one end (i.e., 5' or 3'), in which case the character was ordered based on length of the gap. Indel characters were included in the Bayesian analyses along with the TPI sequences using the Mk +  $\Gamma$  model of Lewis (2001). TPI sequences were partitioned into intron and exon regions, and this partitioning was significantly supported over an unpartitioned analysis based on comparison of Bayes factors.

Each Bayesian analysis used two replicate searches with several million generations each (see below), sampling once every 1,000 generations. Plots of log-likelihoods over time were examined for stationarity (i.e., likelihood values stabilized over time) and trees generated prior to achieving stationarity were discarded as burn-in. As additional tests for stationarity, we evaluated whether separate analyses converged on similar harmonic mean log-likelihoods, topologies, and clade posterior probabilities (for the putative postburn-in trees). Each analysis used four chains and default priors (i.e., Dirichlet for substitution rates and state frequencies; uniform for the gamma shape parameter and proportion of invariable sites; all topologies equally likely a priori; branch

TABLE 3. Estimated ages of major clades within *Plethodon*, based on penalized-likelihood analysis of the four genes combined, followed by the standard deviation and range among 160 Bayesian trees. Because r8s requires a fixed age for at least one clade in the tree (e.g., the root) and the age of Plethodontidae is uncertain, three different root ages were used (50, 66, and 85 mya).

Clade	Estimated age of clade (mya)		
	Root age = 50	Root age = 66	Root age = 85
<i>Plethodon</i>	34.53 ± 1.72 (30.84–39.00)	45.22 ± 2.27 (40.26–51.14)	58.65 ± 2.92 (52.35–66.23)
Western <i>Plethodon</i>	30.45 ± 1.79 (25.47–35.29)	39.55 ± 2.38 (32.98–45.90)	51.65 ± 3.05 (43.18–59.88)
Eastern <i>Plethodon</i>	18.96 ± 1.17 (16.59–22.78)	25.12 ± 1.46 (22.14–29.93)	32.25 ± 1.97 (28.24–38.68)
<i>cinereus</i> group	11.53 ± 1.01 (9.32–14.37)	15.17 ± 1.24 (12.30–18.55)	19.58 ± 1.70 (15.84–24.30)
<i>welleri-wehrlei</i> group	13.31 ± 0.98 (11.62–16.77)	17.83 ± 1.21 (15.56–21.94)	22.67 ± 1.65 (19.78–28.46)
<i>glutinosus</i> group	8.43 ± 0.70 (7.01–10.75)	11.40 ± 0.85 (9.59–14.03)	14.38 ± 1.18 (11.97–18.22)
<i>ouachitae</i> complex	5.54 ± 0.50 (4.64–7.27)	7.59 ± 0.60 (6.35–9.52)	9.47 ± 0.84 (7.92–12.33)
Clade A ( <i>glutinosus</i> group)	4.38 ± 0.45 (3.28–5.73)	6.05 ± 0.55 (4.60–7.68)	7.49 ± 0.76 (5.62–9.74)
Clade B ( <i>glutinosus</i> group)	4.81 ± 0.44 (3.70–6.29)	6.62 ± 0.53 (5.12–8.41)	8.22 ± 0.73 (6.33–10.71)

lengths unconstrained:exponential). The phylogeny for each dataset was estimated from the majority-rule consensus of the pooled postburn-in trees from the two replicate analyses. Clades with Bayesian posterior probabilities ( $Pp$ )  $\geq 0.95$  were considered strongly supported (Wilcox et al. 2002; Alfaro et al. 2003; Erixon et al. 2003; Huelsenbeck and Rannala 2004).

Bayesian analyses of the mitochondrial and RAG-1 genes alone (105–140 taxa) used  $3.0 \times 10^6$  generations, and stationarity was achieved in less than 100,000 generations. Analyses of TPI alone and the combined nuclear data (29 taxa) used  $2.0 \times 10^6$  generations, and stationarity was again achieved in less than 100,000 generations. Analyses of the combined nuclear and mitochondrial data (four genes, 77 taxa) initially used  $3.0 \times 10^6$  generations. However, we found that stationarity was achieved relatively slowly for this data set ( $\sim 1.5$  million generations), and we performed three additional analyses using  $6.0 \times 10^6$  generations. Two of these converged on nearly identical log likelihoods between  $1.5 \times 10^6$  and  $2.0 \times 10^6$  generations, and these pooled data were used as our estimate of phylogeny.

The final estimate of *Plethodon* relationships was based on a combined analysis including all four genes. We found little or no strongly supported incongruence between the two mitochondrial genes, between the mitochondrial genes and RAG-1, or between TPI and RAG-1. However, there was some strongly supported incongruence between the TPI data (and the combined nuclear data) and the combined mitochondrial data, which is discussed in the Results.

The combined analysis of all four genes used only a single individual per species, so that the estimate of phylogeny used for analyses of diversification and hybridization represented each species as a single lineage. In most cases, deleting redundant individuals was straightforward and could be done arbitrarily (i.e., when all the sampled individuals of a species form a clade). In a few other cases (i.e., *P. chlorobryonis*, *P. glutinosus*, *P. metcalfi*, *P. shermani*) individuals appeared in disparate locations in the phylogeny, presumably because of introgression, particularly in the mtDNA tree (e.g., Weisrock et al. 2005). In these cases, we chose representative individuals for each species based on their concordant placement in the mtDNA tree, nuclear gene trees, and/or the allozyme distance trees of Highton and Peabody (2000; with each allozyme locus representing an independent gene within the nuclear genome). Thus, each representative of each species should combine only the “correct” genes for that species,

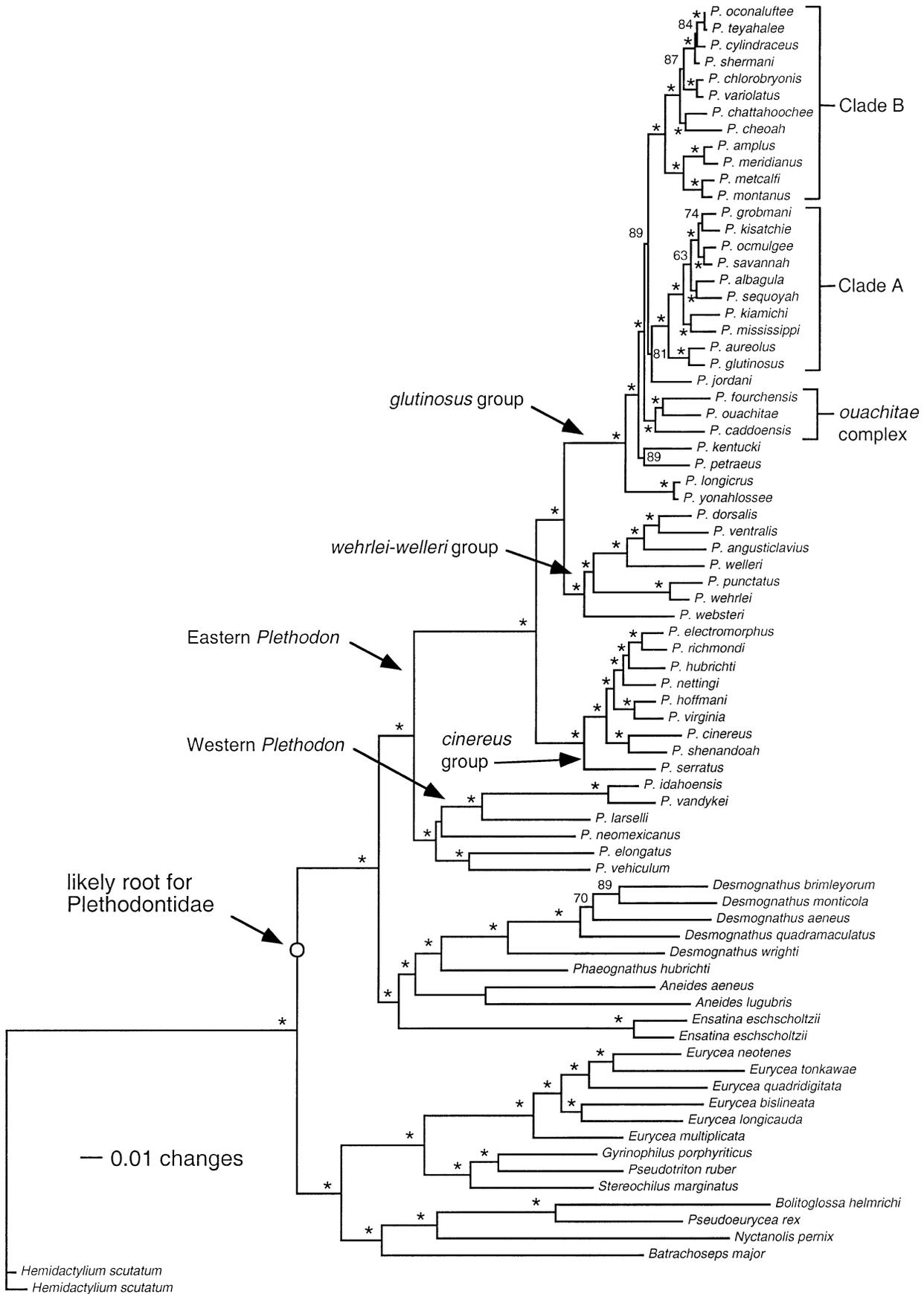
rather than including some genes that are artifacts of introgressive hybridization.

Due to difficulty in amplification, TPI data were available for only 26 of the 46 species of eastern *Plethodon*, and the combined dataset included missing data for several taxa for TPI. Simulations (Wiens 1998b) and analyses of empirical datasets (Wiens et al. 2005a) suggest that adding sets of characters scored for only some taxa can often be helpful (e.g., improve phylogenetic accuracy) despite missing data cells. Although it would be preferable to have TPI data for all species, analyses of this initial dataset showed considerable congruence between TPI and other datasets for the major clades of eastern *Plethodon*, but relatively weak support for relationships within the problematic *glutinosus* group (suggesting that sequencing additional taxa for this gene would be relatively uninformative).

#### Analysis of Divergence Dates

Absolute dates of divergence were estimated using penalized likelihood (PL; Sanderson 2002) as implemented in r8s (vers. 1.6 for Unix; Sanderson 2003). This “relaxed” molecular clock method allows for different rates of evolution in different clades, while minimizing hypothesized changes in rates across the tree (particularly between closely related species). Penalized-likelihood analyses used the topology and branch lengths from the combined, partitioned Bayesian analysis of all four nuclear and mitochondrial genes. Triose phosphate isomerase data were available for only 26 species of eastern *Plethodon*. Analyses using only RAG-1 and the mitochondrial genes gave very similar age estimates (e.g., estimates for all nodes in Table 3 were within 1 million years), given that these analyses yield very similar topologies and branch lengths.

Although the fossil record of plethodontids is not extensive, we used calibration points from the relevant fossil taxa to constrain the minimum ages of the following four nodes. (1) The most recent common ancestor (MRCA) of *Plethodon* originated at least 19 million years ago (mya). Tihen and Wake (1981) reported fossil vertebrae of *Plethodon* and *Aneides* from the Arikareean (Lower Miocene) of Montana. One of the *Plethodon* vertebra was considered to be “very similar” to that of modern *Plethodon* from western North America. We therefore tentatively consider this vertebra as representing a lineage that evolved after the split between the



modern eastern and western *Plethodon* clades. The Arikareean extends from 19 to 30 mya (Barnosky 2001), and thus we consider *Plethodon* to be at least 19 million years old; (2) MRCA of *Aneides*, *Desmognathus*, and *Phaeognathus*, originated at least 19 mya. Given the presence of an *Aneides* vertebra in the Arikareean period (Tihen and Wake 1981), the MRCA of the clade containing modern *Aneides* must be at least 19 mya; and (3) MRCA of *Aneides*, originated at least five mya. A fossil was identified as *Aneides lugubris* from the late Miocene (Hemphillian) by Clark (1985), who estimated its age at five million years. Thus, the MRCA of the two species of *Aneides* sampled (*A. aeneus* and *A. lugubris*) is at least this old; (4) MRCA of bolitoglossines originated at least 5 mya. Clark (1985) reported a fossil *Batrachoseps* from the late Miocene (Hemphillian) of California, estimated to be five million years old. Given that our analyses show *Batrachoseps* as the sister group of all other included bolitoglossines, the MRCA of bolitoglossines must be at least this old. In our results, we found that the estimated ages of these four clades were all considerably older than the ages of these fossils (at least 50% older), which suggests that any potential errors associated with these fossil taxa (e.g., misidentification) have not strongly influenced the results.

Penalized-likelihood also requires the specification of an estimated age for at least one clade (rather than just a constraint on the minimum age). The age of Plethodontidae is uncertain; therefore we used three potential dates. Chippindale et al. (2004) performed a penalized-likelihood analysis based on the slow-evolving RAG-1 gene alone that included representatives of almost all salamander families (all but Hynobiidae), three fossil calibration points within Plethodontidae (basically equivalent to the first, second, and fourth above) and three additional fossil calibration points outside of Plethodontidae, and two possible ages for the root of salamanders based on paleontological data (160 mya and 250 mya). These analyses yielded estimates for the MRCA of extant Plethodontidae of 49.7 mya (using 160 mya) and 84.8 (using 250 mya). This general range of estimated dates has been confirmed by more recent analyses using additional taxa and additional fossil calibration points (J. J. Wiens, unpubl. data). Furthermore, there is strong evidence that Amphiumidae is the sister group of Plethodontidae (e.g., Chippindale et al. 2004; Wiens et al. 2005b). The oldest known amphiumid fossil (*Proamphiuma cretacea*) is late Maastrichtian or early Paleocene, and thus approximately 66 million years old (Gardner 2003); the split between Plethodontidae and Amphiumidae must be at least this old (but this does not necessarily mean that the MRCA of extant Plethodontidae must be this old). We performed PL analyses using ages for the MRCA or “crown group” of extant Plethodontidae of 50, 66, and 85 mya. We excluded *Hemidactylium* from the r8s

analyses to facilitate rooting the tree; this taxon is only distantly related to *Plethodon* and its exclusion should have no impact on the results.

Penalized-likelihood analyses were implemented using the TN (truncated Newton) algorithm. We used cross-validated assessment to select the best-fitting smoothing parameter, with values from  $10^0$  to  $10^4$  in exponential increments of 0.5. Two replicate cross-validation analyses were run for each root age. These analyses showed that the optimal smoothing parameter is 1.0 using a root age of 50 million, and 3.16 using root ages of 66 and 85 million years. To assess confidence in our estimates of clade ages, we reestimated these ages using 160 trees from the combined Bayesian analysis of four genes (i.e., sampling one tree every 50,000 generations from among the eight million postburn-in trees), repeated the penalized-likelihood analysis on each tree using the three root ages (but without retesting the smoothing parameter for each root age), and summarized the range and standard deviation using the “profile” command in r8s.

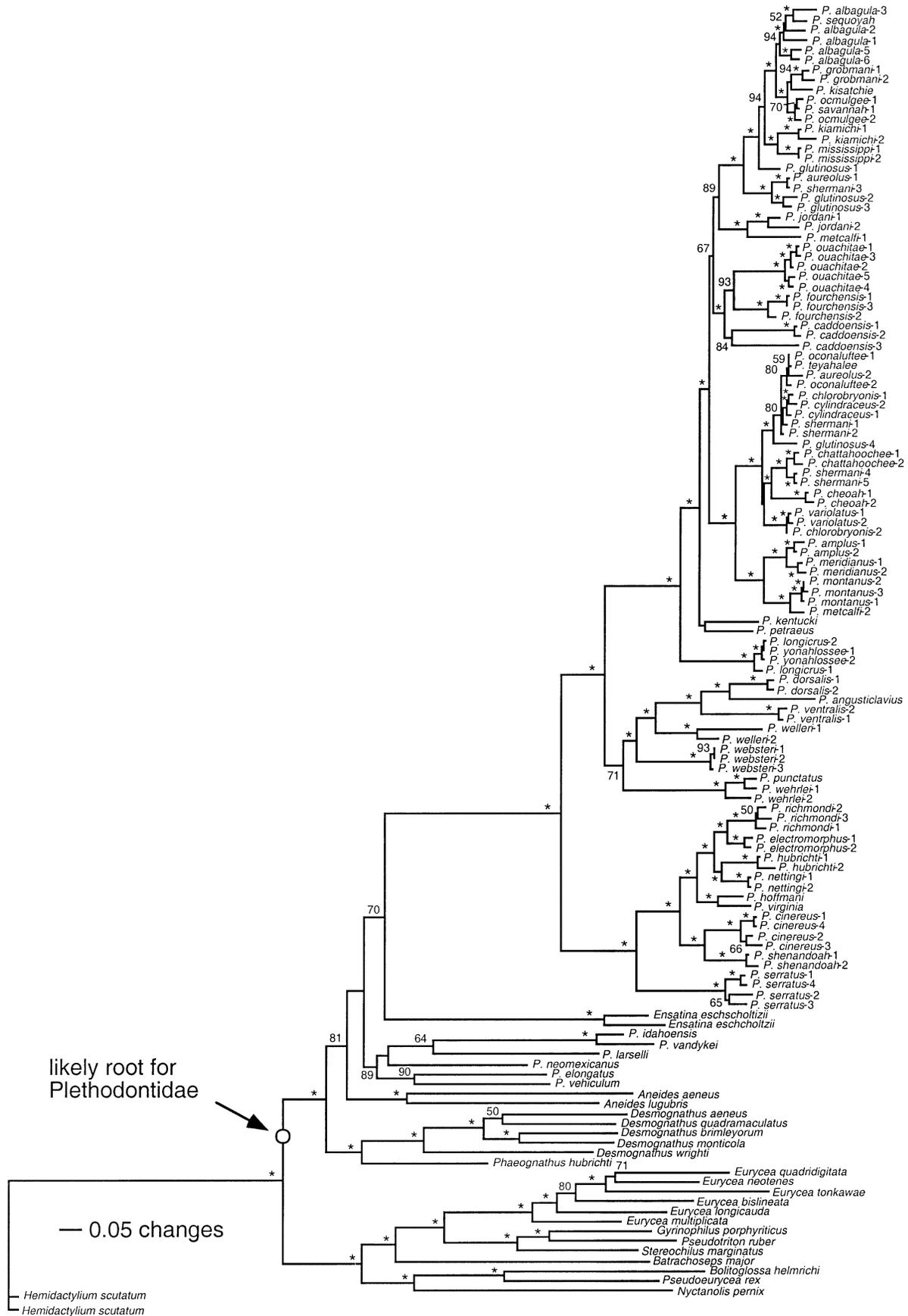
#### Rate of Diversification

Highton (1995) hypothesized that there was a rapid burst of speciation in eastern *Plethodon* during the Pliocene. We tested for significant departures from constant rates of speciation and extinction across the phylogeny of *Plethodon* using the relative cladogenesis statistic ( $P_k$ ) of Nee et al. (1994), as implemented in the program End-Epi, version 1.0 (Rambaut et al. 1997). The method calculates the probability that a clade that originated at a given time ( $t$ ) will have a given number of species ( $k$ ) at the present time (relative to the total number of extant species in the group as a whole), given a phylogeny with branch lengths and assuming a birth-death model with constant rates. The relative cladogenesis statistic is particularly useful relative to other methods (e.g., lineages-through-time plots) in that it identifies specific nodes that deviate from the constant rates model.

The input tree for this analysis was the chronogram from the PL analysis, using branch lengths from the Bayesian analysis of the combined nuclear and mitochondrial data and including only one individual per species. In our phylogenetic analyses, our sampling of species was nearly comprehensive within eastern *Plethodon*, but included relatively few species of other genera. To avoid biasing the results with this uneven sampling, we included only *Plethodon* in the actual calculations of  $P_k$ . We also performed analyses including only eastern *Plethodon*. We used chronograms for all three root ages for Plethodontidae, and all three gave very similar results for the analysis of diversification rates. We also reran the analyses after deleting five *Plethodon* taxa of questionable status (*P. longicrus*, *P. oconaluftee*, *P. savannah*, *P. variol-*

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FIG. 1. Phylogeny of *Plethodon* and other plethodontid salamanders based on a combined, partitioned Bayesian analysis of the mitochondrial *cyt b* and ND4 genes and the nuclear RAG-1 and TPI genes. Branches are drawn proportional to estimated branch lengths. Asterisks adjacent to branches indicate  $P_p \geq 0.95$  and numbers indicate  $P_p > 0.49$  and  $< 0.95$ .  $P_p < 0.50$  are not shown ( $P_p$  multiplied by 100 on tree). Because only plethodontids are included, the tree is unrooted. Previous studies (e.g., Chippindale et al. 2004) and unpublished data (Chippindale and Wiens, unpubl. data) suggest that the root is mostly likely between Plethodontinae and the common ancestor of a clade consisting of Hemidactylinae (*Hemidactylium*), Bolitoglossinae, and Spelerpinae. Some terminal taxa represent the combination of gene sequences from different individuals, and individual voucher specimens are not shown.



atus, and *P. sequoyah*; see Discussion). Deletion of these taxa had no impact on the results.

Diversification may be considered rapid based on comparisons within a group of interest (as above) or when compared to rates in other groups. Coyne and Orr (2004) compared putative speciation rates across many different groups, using an index called the NDI or net diversification interval (the average time between the origin of a new lineage and when that lineage branches again). The NDI was estimated as  $t/\ln(N_t)$ , where  $t$  is the age of the MRCA of the clade and  $N_t$  is the number of species in that clade. We estimated NDI for a group of species that was found to be rapidly evolving using the relative cladogenesis statistic and compared the estimated NDI value to those summarized by Coyne and Orr (2004; their table 12.1).

#### *Patterns of Hybridization and the ‘‘Speciation Clock’’*

Previous studies have found evidence of hybridization between many pairs of sympatric or parapatric species in the *glutinosus* group, whereas other pairs of species coexist without apparent introgression (e.g., Highton 1995; Highton and Peabody 2000; Weisrock et al. 2005). Highton and Peabody (2000) summarized patterns of species interactions in the *glutinosus* group and found very little difference between the average genetic distance of species that hybridize and those that do not. However, simply comparing the average ages of species pairs that hybridize and those that do not is somewhat problematic; the species pairs are not independent data points because a single species may be involved in several pairs and because all species share phylogenetic history to different degrees. Furthermore, their levels of genetic divergence are potentially influenced by introgression between them.

As an alternate approach, we used the data on species interactions to test for a relationship between reproductive isolation and time (i.e., the ‘‘speciation clock,’’ following Coyne and Orr 1989 and subsequent authors), using a new method designed to minimize potential problems of nonindependence due to phylogeny and use of single species in multiple-species comparisons (Bolnick and Near 2005). We obtained data on the interactions of sympatric species pairs from Highton and Peabody (2000) with the addition of *P. chatahoochee* and *P. chlorobryonis* (from Highton 1995). These authors inferred introgressive hybridization based primarily on allozyme data (with supplemental information from morphology in some cases), and many of their hypothesized inferences of introgression are also supported by mtDNA (Weisrock et al. 2005; this study, Fig. 2). Their sampling of species was confined to 17 species in the *glutinosus* group, includes only species occurring in southeastern North America (although this is where the majority of species occur), and only includes species interactions that have been the subject of focused study.

The 27 sympatric (or partially sympatric) species pairs

were each given an index of overall reproductive isolation. Species pairs that occur in sympatry without hybridization were given a score of 10 (most reproductively isolated), whereas those that show extensive zones of introgressive hybridization were given a score of one (least isolated). Some species were difficult to characterize as undergoing extensive hybridization or not. These included five species pairs in which the species generally remain distinct in sympatry but show some evidence of rare hybridization and two species pairs in which the species hybridize at some sympatric localities but do not hybridize at others. Both types of cases were given an intermediate isolation score of five.

All 27 species pairs spanned one of eight nodes on the preferred phylogeny (Fig. 5). In other words, if a line is drawn connecting each of the 27 species pairs, all of these lines pass through only eight nodes on the tree. These eight nodes were the basic units in the analysis of isolation versus time. For time, we used the estimated ages of these nodes from the PL analyses (see above), assuming 66 mya as the root age for Plethodontidae. The choice of absolute age for Plethodontidae should have little or no impact on our comparisons of the relative ages of species pairs.

We calculated the weighted mean isolation of each node using equation 5 of Bolnick and Near (2005), which averages the isolation indices of the species pairs at each node and weights them based on the phylogeny, branch lengths, and their independence from other species pairs (i.e., species pairs that include species that are included in many other species pairs are given less weight). We then performed linear regression analysis of the mean isolation score for the node versus the age of the node.

We also compared qualitatively the number of speciation events that separate hybridizing and nonhybridizing pairs of sympatric species (i.e., the smallest number of splits separating a given pair of species, but not including the splitting of their MRCA). Thus, given a phylogeny of four species ((A,B),(C,D)), we would count two speciation events separating species A and D. This index provides a measure of the phylogenetic relatedness of species that is not strictly related to time.

We acknowledge that our analysis of isolation over time lacks the rigorous, quantitative estimates of reproductive isolation found in many other studies of the ‘‘speciation clock’’ (e.g., Coyne and Orr 1989, 1997; Mendelson 2003). Nevertheless, even coarse patterns of natural introgression should be relevant to studies of the evolution of overall reproductive isolation, and to the relationship between introgression and rapid diversification.

#### *Genealogical Discordance and Rapid Radiation*

Seehausen (2004) predicted that there should be strongly supported incongruence between trees based on different genes (particularly nuclear and mitochondrial) for the basal

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FIG. 2. Phylogeny of *Plethodon* and other plethodontid salamanders based on a combined, partitioned Bayesian analysis of the mitochondrial *cyt b* and ND4 genes. Branches are drawn proportional to estimated branch lengths. Asterisks adjacent to branches indicate  $P_p \geq 0.95$  and numbers indicate  $P_p > 0.49$  and  $< 0.95$ .  $P_p < 0.50$  are not shown ( $P_p$  multiplied by 100 on tree). Because only plethodontids are included, the tree is unrooted. Voucher specimens for numbered individuals are listed in Appendix 1 available online.



lineages of a rapid radiation. This incongruence may indicate ancient hybridization events that are not detectable based on current patterns of introgression among extant species. We predict that the incongruence should involve the most basal clade or clades showing significant deviations from constant rates of speciation and extinction, based on the relative cladogenesis statistic ( $P_k$ ) described above. The incongruence should also be strongly supported by the conflicting genes (Bayesian  $P_p \geq 0.95$ ), given that discordance may be an artifact of failure to estimate the gene genealogy correctly (i.e., because of homoplasy and/or insufficient variation). We also caution that strongly supported discordance between genes may have many other causes apart from introgression, including incomplete lineage sorting (which may be especially common in recent radiations). We do not utilize overall measures of dataset congruence given that we are interested in the specific location of strongly supported incongruence between datasets (which such methods cannot address), and given that the sensitivity of such methods to topologically localized incongruence in species-rich datasets remains largely unknown.

## RESULTS

### Phylogenetic Relationships

We consider the combined, partitioned, Bayesian analysis of all four genes (Fig. 1) to be the best estimate of phylogeny, and we discuss the results of the separate analyses of different genes in light of this phylogeny (combined mitochondrial DNA [mtDNA], Fig. 2; RAG-1 alone, Fig. 3; TPI and combined nuclear data for 29 taxa, Fig. 4). Based on the combined-data tree (Fig. 1), the species of *Plethodon* form a monophyletic group. Our results support the basal split between eastern and western *Plethodon* species (Highton 1995) and Highton's (1995) general arrangement of species into species groups within eastern *Plethodon*. The *cinereus* group is the sister taxon to all other eastern *Plethodon*. The *welleri* and *wehrlei* species groups together form a monophyletic group, which is the sister taxon of the *glutinosus* group. However, monophyly of the *welleri* group is not supported in the combined analysis, and we refer to the two groups collectively as the *wehrlei-welleri* group hereafter. The *glutinosus* group contains all the remaining species of eastern *Plethodon*. The nuclear and mtDNA data are strongly concordant with regard to these basal clades within eastern *Plethodon*. Specifically, separate analyses of the combined mtDNA data (Fig. 2), RAG-1 (Fig. 3), and TPI (Fig. 4) concur that the *cinereus* group is the sister taxon of the remaining species, that the *wehrlei-welleri* group is the sister taxon of the *glutinosus* group, and that each of these groups is monophyletic.

Within the *glutinosus* group, many of the divergences are extremely shallow and many clades are weakly supported (especially in the trees from RAG-1 and TPI; Figs. 3 and 4). In the combined analysis, *P. yonahlossee* and *P. longicrus*

make up the sister group of the remaining species. *Plethodon kentucki* and *P. petraeus* are weakly supported as sister taxa, and there is strong support for placing these species as the sister group of the remaining species (Fig. 1). The mtDNA data and nuclear genes are concordant in showing that *P. yonahlossee* (and its sister taxon, *P. longicrus*) are at the base of the *glutinosus* group, and that *P. kentucki* and *P. petraeus* are basal as well (although TPI data are lacking for these two species).

The remaining species fall into three strongly supported clades in the combined analysis (Fig. 1), with the exception of *P. jordani*. One clade corresponds to the *ouachitae* complex of Highton (*P. caddoensis*, *P. fourchensis*, and *P. ouachitae*), and this clade is weakly supported as the sister group of the other three clades in the combined analysis. The *ouachitae* complex is supported as monophyletic by separate analyses of the mtDNA, RAG-1, and TPI (but lacking data for *P. fourchensis*). The TPI data show strong support for placing the *ouachitae* complex as the sister group of the remaining species of the *glutinosus* group (Fig. 4), and this is not strongly discordant with the RAG-1 or combined mtDNA.

The second clade (Clade A hereafter) includes 10 species of the *glutinosus* complex (*P. albagula*, *P. aureolus*, *P. glutinosus*, *P. grobmani*, *P. kiamichi*, *P. mississippi*, *P. ocmulgee*, *P. savannah*, *P. sequoyah*). The third clade (Clade B) includes six species of the *jordani* complex (*P. amplus*, *P. cheoah*, *P. meridianus*, *P. metcalfi*, *P. montanus*, *P. shermani*), and five species of the *glutinosus* complex (*P. chattahoochee*, *P. chlorobryonis*, *P. cylindraceus*, *P. oconaluftee*, *P. teyahalee*). *Plethodon jordani* is not assigned to either of these clades, but is weakly supported as the sister taxon of Clade A.

Clades A and B are each strongly supported by mtDNA data (Fig. 2). However, some species that are represented by multiple individuals appear in both clades (including *P. aureolus*, *P. glutinosus*, and *P. shermani*) suggesting introgression between species in these two clades (see also Highton 1995; Highton and Peabody 2000; Weisrock et al. 2005). The RAG-1 data (Fig. 3) show moderately weak support for a clade including seven of the 10 species of Clade A (*P. albagula*, *P. grobmani*, *P. kiamichi*, *P. kisatchie*, *P. ocmulgee*, *P. savannah*, *P. sequoyah*; RAG-1 data are lacking for *P. mississippi*). The RAG-1 and TPI data both show strong support for placing *P. albagula* with *P. sequoyah* (as in the mtDNA tree), and the TPI data show strong support for placing *P. glutinosus* with *P. grobmani* (Fig. 4). The RAG-1 data (Fig. 3) also show very weak support for a clade including eight of 11 species of Clade B (*P. amplus*, *P. cheoah*, *P. cylindraceus*, *P. metcalfi*, *P. montanus*, *P. oconaluftee*, *P. shermani*, *P. teyahalee*). The TPI data (Fig. 4) show strong support for a clade including three species of Clade B (*P. chlorobryonis*, *P. cylindraceus*, *P. teyahalee*), but also including *P. savannah* of Clade A. Despite some concordance, neither Clade A or Clade B is supported by the nuclear genes (either alone or together), although much of the discordance is only

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Fig. 3. Phylogeny of *Plethodon* and other plethodontid salamanders based on a Bayesian analysis of the nuclear RAG-1 gene. Branches are drawn proportional to estimated branch lengths. Asterisks adjacent to branches indicate  $P_p \geq 0.95$  and numbers indicate  $P_p > 0.49$  and  $< 0.95$ .  $P_p < 0.50$  are not shown ( $P_p$  multiplied by 100 on tree).

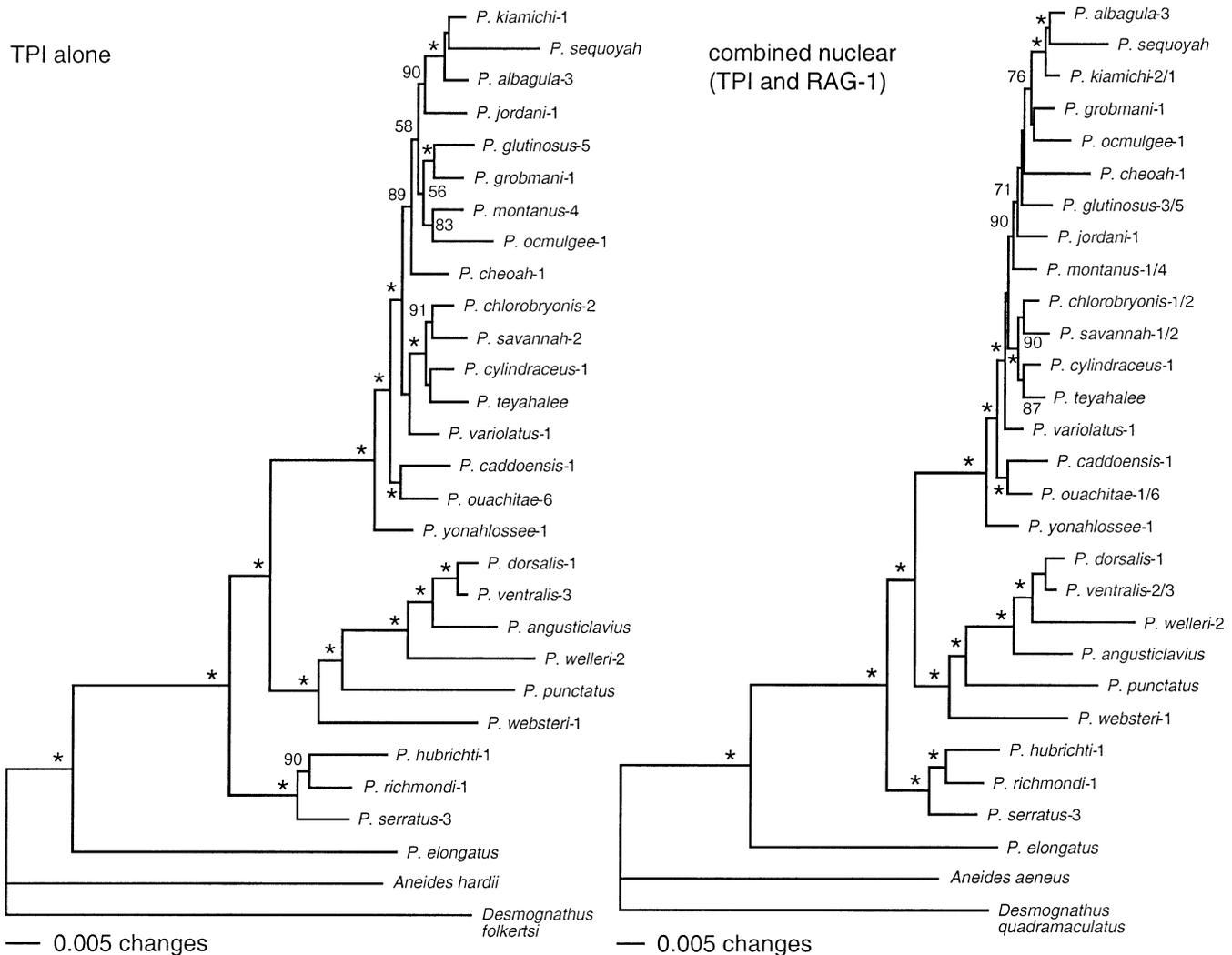


FIG. 4. Phylogeny of *Plethodon* and other plethodontid salamanders based on a Bayesian analysis of the nuclear TPI gene (left) and the combined TPI and RAG-1 genes. Branches are drawn proportional to estimated branch lengths. Asterisks adjacent to branches indicate  $Pp \geq 0.95$  and numbers indicate  $Pp > 0.49$  and  $< 0.95$ .  $Pp < 0.50$  are not shown ( $Pp$  multiplied by 100 on tree). Because only plethodontids are included, the tree is unrooted. Voucher specimens for numbered individuals are listed in Appendix 1 available online. For the combined-data tree, some species were represented by the combination of data from a different individual for each gene; in these cases the source of each gene is indicated (RAG-1/TPI).

weakly supported. In the RAG-1 tree (Fig. 3) many putatively conspecific individuals appear in disparate locations on the phylogeny, which may reflect hybridization, incomplete lineage sorting, or insufficient sampling of characters.

#### Timing of Diversification

Estimated ages of major clades are summarized in Table 3, and a chronogram is shown in Fig. 5. These estimates vary

depending on the assumed age of Plethodontidae, but (as expected) these estimates become increasingly similar for more recent clades. Regardless of the root age used, all species groups of eastern *Plethodon* originated well before the beginning of the Pliocene (5.3 mya), contra Highton (1995). Nevertheless, the species-rich *glutinosus* group (30 species of 54 in the genus) is relatively recent (8–14 million years old).

FIG. 5. Chronogram for *Plethodon* and related plethodontid salamanders, showing estimated ages of clades and also those that differ significantly from a constant-rates birth-death model using the relative cladogenesis statistic ( $Pk$ ). The chronogram was based on a penalized-likelihood analysis using the topology and branch lengths from the combined, partitioned Bayesian analysis of the four genes (Fig. 1) and a root age of 66 million years.  $Pk$  was calculated using only eastern *Plethodon*, for which species-level sampling is nearly complete. Nodes indicated with circles and numbers are those used to determine the relationship between reproductive isolation and time, and asterisks indicate those 17 species for which data on reproductive interactions (i.e., hybridization vs. sympatry without introgression) are available.

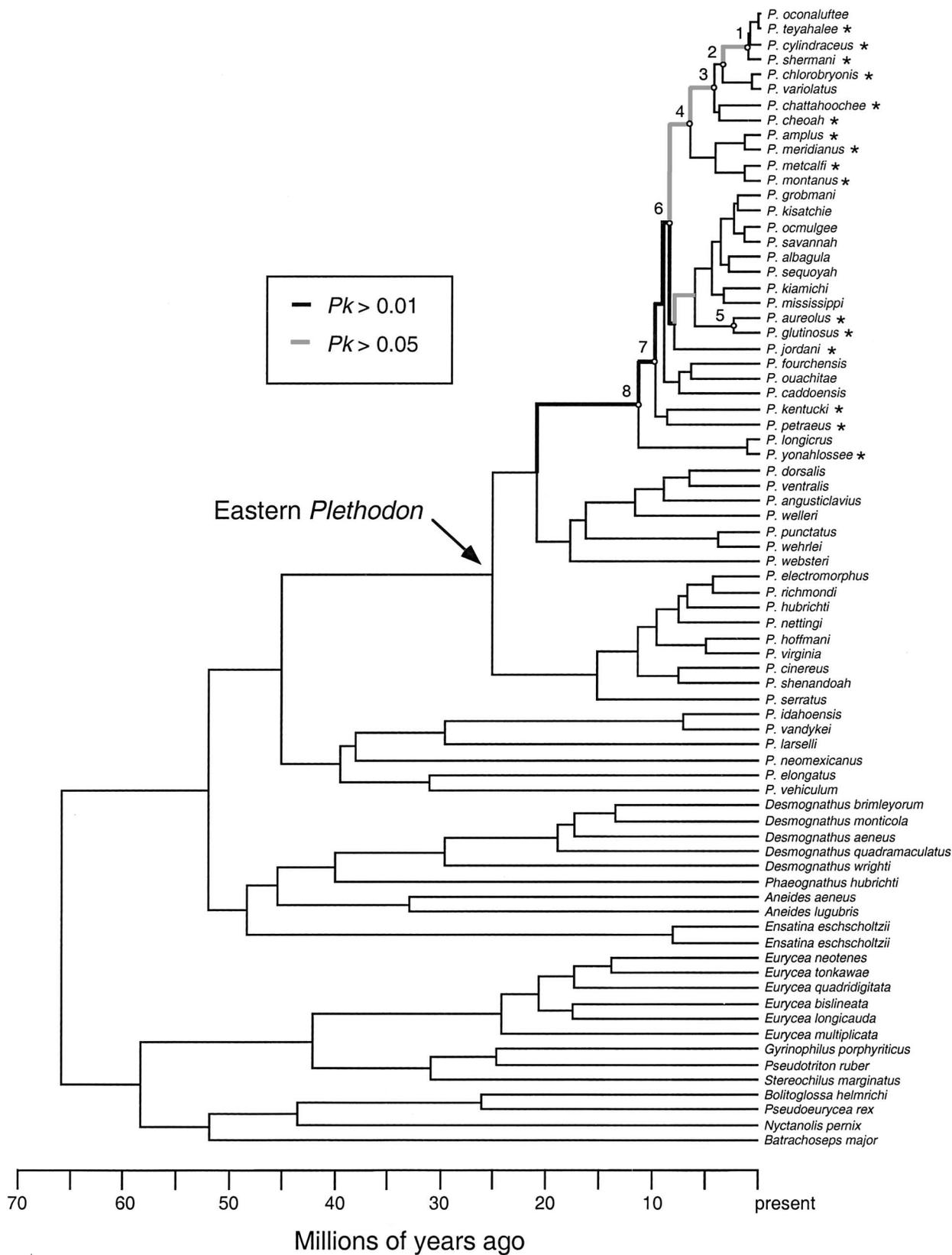


TABLE 4. Reported instances of natural hybridization and of sympatry without hybridization in the *Plethodon glutinosus* group, summarized from Highton (1995) and Highton and Peabody (2000). Asterisks denote pairs that hybridize at some sites and are sympatric without hybridization at others. Rare hybridization indicates that hybrids were not actually observed, but that alleles usually diagnostic of one species were observed in the other. Ages of the most recent common ancestor (MRCA) of each pair and the number of splits separating the species are based on the chronogram in Figure 5.

Species-pair	Age of MRCA	Number splits
<b>Hybridizing</b>		
<i>aureolus-shermani</i>	8.57	7
<i>chatahoochee-chlorobryonis</i>	4.36	3
<i>chatahoochee-shermani</i>	4.36	3
<i>chatahoochee-teyahalee</i>	4.36	5
<i>chlorobryonis-cylindraceus</i>	3.47	3
<i>chlorobryonis-metcalfi</i>	6.62	5
<i>chlorobryonis-teyahalee</i>	3.47	4
<i>cylindraceus-glutinosus</i>	8.57	8
<i>glutinosus-teyahalee</i>	8.57	9
<i>jordani-metcalfi</i>	8.57	4
<i>jordani-teyahalee*</i>	8.57	7
<i>metcalfi-teyahalee*</i>	6.62	7
<i>shermani-teyahalee</i>	1.19	2
<b>Sympatric, no hybrids</b>		
<i>amplus-cylindraceus</i>	6.62	6
<i>amplus-yonahlossee</i>	11.40	7
<i>aureolus-glutinosus</i>	2.46	0
<i>aureolus-teyahalee</i>	8.57	9
<i>cylindraceus-meridianus</i>	6.62	6
<i>cylindraceus-metcalfi</i>	6.62	6
<i>cylindraceus-montanus</i>	6.62	6
<i>cylindraceus-yonahlossee</i>	11.40	9
<i>montanus-yonahlossee</i>	11.40	7
<b>Sympatric, rare hybrids</b>		
<i>cheoah-teyahalee</i>	4.36	5
<i>glutinosus-montanus</i>	8.57	6
<i>glutinosus-kentucky</i>	9.79	6
<i>glutinosus-petraeus</i>	9.79	6
<i>glutinosus-yonahlossee</i>	11.40	7

#### Rates of Diversification

The results support the hypothesis that there has been unusually rapid speciation in eastern *Plethodon*, particularly within the *glutinosus* species group (Fig. 5). Testing for deviation from the constant-rates birth-death model using only eastern *Plethodon* (which are sampled almost completely), the common ancestor of the *glutinosus* group shows a highly significant deviation ( $Pk < 0.01$ ) as do the basal splits within this group (including the ancestors of Clade A and Clade B), but no other clades do. If the analysis is rerun including western *Plethodon* (only 6 of 8 species sampled), then all branches within eastern *Plethodon* that include the *glutinosus* group show highly significant deviation, but no clades outside the *glutinosus* group do (i.e., western *Plethodon* and the *cinereus* and *wehrlei-welleri* groups). Results (not shown) are similar using different chronograms and deleting questionable taxa.

The *glutinosus* group has 30 putative species and an NDI of 2.4785, 3.3518, or 4.2279 million years, depending on the root age assumed for Plethodontidae (50, 66, and 85 million years, respectively). These estimates are shorter than the average interval of 6.5 million years across all 84 clades of

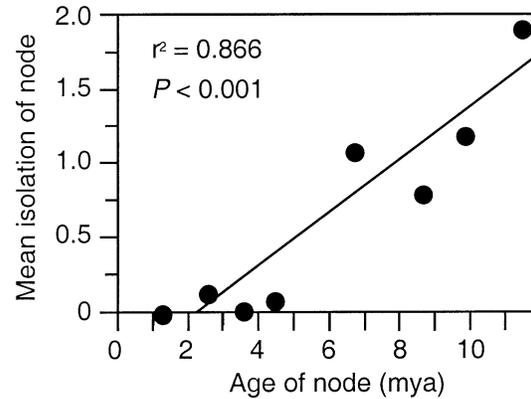


FIG. 6. Relationship between reproductive isolation and time in the *Plethodon glutinosus* group. Reproductive isolation is based on the presence or absence of introgressive hybridization among 27 species pairs. Each data point represents the weighted average reproductive isolation for the species pairs connected by that node and the estimated age of the node based on penalized likelihood (see Fig. 5 for species, nodes, and dates).

plants and animals reported by Coyne and Orr (2004). However, they are also considerably longer than that for the classic examples of rapid speciation in vertebrates, namely the African cichlids (0.004–0.4 my) and Galapagos finches (0.8–1.1 my). We did not find this group to be as rapidly speciating as implied by Highton (1995) because our PL analyses found the group to be roughly twice as old.

#### Patterns of Hybridization and Sympatry

Patterns of hybridization and sympatry in species pairs of the *glutinosus* group relative to the age and phylogenetic distance of the species are summarized in Table 4. Using 66 mya as a root age for Plethodontidae, the results suggest that there is widespread introgressive hybridization among species that are several million years old (mean = 5.95 mya; range = 1.19–8.57 my;  $n = 13$  species pairs) and relatively distantly related (mean = 5.15 speciation events; range = 2–9;  $n = 13$ ). Species pairs that are sympatric with no known or rare hybridization are similar in age (mean = 8.26 mya; range = 2.46–11.40 mya;  $n = 14$ ) and phylogenetic distance (mean = 6.14 speciation events; range = 0–9;  $n = 14$ ) to those that hybridize. Nevertheless, the regression analysis using a weighted measure of isolation (Fig. 6) shows a strong relationship between time and evolution of reproductive isolation ( $r^2 = 0.866$ ;  $P = 0.0008$ ).

Overall, there are multiple instances of hybridization between species of the *glutinosus* group that last shared a common ancestor 6.3–10.7 mya (depending upon the root age for Plethodontidae) and rare hybridization between species (e.g., *P. glutinosus* and *P. yonahlossee*) separated for 8.4–14.4 million years. However, there is extensive sympatry between species of the *glutinosus* group and the *cinereus* and *wehrlei-welleri* groups, and no recorded instances of hybridization between species in different species groups (Petranka 1998). Thus, there are no reported instances of natural hybridization between species of *Plethodon* separated for more than 15.7–26.8 million years (i.e., the age of the MRCA of the *glutinosus* and *wehrlei-welleri* groups).

### *Genealogical Discordance and Rapid Radiation*

Overall, our results for *Plethodon* do not support Seehausen's (2004) "hybrid swarm" hypothesis of genealogical discordance at the base of rapid radiations. We found significantly high rates of diversification in the *glutinosus* group, beginning with the basal lineages of the group. However, we found only three instances of strongly supported incongruence between gene genealogies in this group, which are fundamentally disagreements between TPI and the mtDNA data over the phylogenetic placement of three species from Clade A (*P. grobmani*, *P. kiamichi*, and *P. savannah*). All three of these species are relatively recent and there is no strongly supported incongruence involving the basal lineages of the *glutinosus* group. The nuclear and mitochondrial trees are generally concordant at the base of the *glutinosus* group (Figs. 2, 3, and 4), which suggests that the lack of strong incongruence is not merely an artifact of weak phylogenetic signal.

### DISCUSSION

Overall, our results are consistent with the hypothesis that incomplete isolation of lineages may be a consequence of rapid and recent diversification, as initially suggested by Highton (1995). Our results support Highton's hypothesis that there has been rapid diversification in a recent clade of eastern *Plethodon* (the *glutinosus* group), relative to the expectation of constant rates of birth and death of species over time (see also Kozak et al. 2006). Many of these rapidly generated species are incompletely isolated, in that there is introgressive hybridization among many species within the *glutinosus* group. These introgressing species include many that are distantly related and morphologically distinct. We also show that overall reproductive isolation increases over time in the *glutinosus* group, suggesting that many of the rapidly generated lineages may simply be too young to have evolved effective isolating mechanisms. Furthermore, we find no evidence of genealogical discordance at the base of the rapidly diversifying *glutinosus* group, in contrast to the expectations of Seehausen's (2004) hybrid swarm hypothesis for rapid radiations.

### *Causes of Rapid Diversification*

What might have caused the increased diversification rate in the *glutinosus* group? Under the classic model of adaptive radiation, rapid speciation is associated with adaptive phenotypic change, divergent ecological specialization, and competition (reviewed by Schluter 2000). This model seems unlikely to apply fully to the *glutinosus* group, for several reasons (see also Kozak et al. 2006). In general, species in this group share similar overall morphology, microhabitat usage, and diet; they are relatively large bodied, terrestrial and forest dwelling, and feed on small invertebrates (Petranka 1998). The morphological similarity of many species is evidenced by the fact that, until recently, nearly half of the species in this group were taxonomically unrecognized and were considered geographic variants of only two species (*P. glutinosus* and *P. jordani*). Specifically, the species *P. albagula*, *P. chattahoochee*, *P. chlorobryonis*, *P. cylindraceus*, *P. grobmani*, *P. kiamichi*, *P. kisatchie*, *P. mississippi*, *P. ocmulgee*, *P. savan-*

*nah*, and *P. variolatus* were considered conspecific with *P. glutinosus* (commonly referred to as the "glutinosus complex") whereas *P. amplus*, *P. cheoah*, *P. meridianus*, *P. metcalfi*, *P. montanus*, and *P. shermani* were considered conspecific with *P. jordani* (referred to as "jordani complex"). In general, species in the group are distributed allopatrically or parapatrically, particularly within the former *glutinosus* and *jordani* complexes (although our analyses show that these complexes are not monophyletic; see also Highton and Peabody 2000). Overall, the *glutinosus* group seems unlikely to be an adaptive radiation in the classic sense.

On the other hand, adaptation to different climatic regimes (e.g., high and low elevation forests) might contribute to diversification in this group. This type of adaptive diversification might leave little obvious trace in the morphology, microhabitat, or diet. Within the *glutinosus* group, many species are confined to montane forests (e.g., *P. amplus*, *P. caddoensis*, *P. cheoah*, *P. fourchensis*, *P. jordani*, *P. kentucky*, *P. kiamichi*, *P. meridianus*, *P. metcalfi*, *P. montanus*, *P. ouachitae*, *P. petraeus*, *P. shermani*, *P. yonahlossee*; Highton 1989; Petranka 1998), whereas other species occur at lower elevations (e.g., *P. chlorobryonis*, *P. grobmani*, *P. kisatchie*, *P. mississippi*, *P. ocmulgee*, *P. savannah*, *P. variolatus*; Highton 1989). Repeated shifts between highland and lowland specialization, as well as isolation of montane habitats by the spread of lowland habitats during periods of climate change (i.e., lineage splitting through niche conservatism; Wiens 2004a) might both be important in diversification in this group. A recent study using ecological niche modeling shows that niche conservatism seems to drive allopatric isolation and speciation in many montane plethodontid salamanders in eastern North America (Kozak and Wiens 2006).

Differentiation in sexually selected characters used in species recognition is thought to be important in rapid speciation in many groups, including Rift Lake cichlids (e.g., Seehausen and Van Alphen 1999) and Hawaiian crickets (e.g., Mendelson and Shaw 2005). Intriguingly, there is a shift in sexual signaling systems within *Plethodon* that coincides with the shift in diversification rates. The *glutinosus* group has a derived "olfactory" transmission system for delivery of courtship pheromones, in which the male has an enlarged mental gland that is slapped onto the snout of the female to transfer the secretions from this gland (Palmer et al. 2005). In contrast, other *Plethodon* and other plethodontids are characterized by the "vaccination" delivery system, in which the male has enlarged premaxillary teeth that are used to abrade the female's skin; the male then rubs his mental gland secretions into the abraded site (Watts et al. 2004). Species of the *wehrlei-welleri* group are intermediate between the two modes (Palmer et al. 2005). In general, chemical cues are known to be important in courtship, species recognition, and prezygotic isolation in the *glutinosus* group (e.g., Dawley 1984, 1986). A change in species recognition systems might also be relevant to the widespread hybridization between species in the *glutinosus* group. However, we do not know of a direct mechanism that ties this change in signaling system to increased diversification rates, and there is some question as to whether courtship pheromones are important in species recognition in the *glutinosus* group (Rollman et al. 2003; but note that

the *glutinosus* group is the clade in which there is extensive hybridization). This is an area in need of further study.

#### Rapid Diversification versus Taxonomic Artifact

An alternative explanation for the seemingly rapid diversification within eastern *Plethodon* is that it is simply a taxonomic artifact, and that many so-called species are not distinct. This hypothesis might also explain the frequent introgression between putative species in this group. In fact, recognition of some species within the *glutinosus* group has been controversial, with some authors declining to follow Highton's (1989) proposed changes (e.g., Petranka 1998). However, several lines of evidence, both old and new, support recognition of most species in the *glutinosus* group.

First, some species are readily distinguished morphologically, using characters such as size and coloration, including *P. petraeus* and *P. yonahlossee* (Petranka 1998). Twelve species are morphologically very similar to each other, and have previously been recognized as a single species (*P. glutinosus*). Some of these may possibly be distinguished from each other by subtle differences in coloration and morphometrics (e.g., Highton 1989; Carr 1996), but regardless, these 12 species are (collectively) readily distinguished from most other species in the group. Similarly, all seven species of the *jordani* complex can be easily distinguished from other species in the *glutinosus* group, and many species of the *jordani* complex are morphologically distinct from each other (e.g., some are black with red cheeks, others black with red legs; Highton and Peabody 2000).

Second, many species in the *glutinosus* group are sympatric but without any evidence of hybridization (Table 4); these pairs of species are similar in age and phylogenetic distance to those that hybridize and those that are morphologically cryptic. Third, all of the taxa recognized, including those that are morphologically cryptic, form distinct clusters in distance analyses of allozyme data based on extensive population-level sampling (e.g., Highton 1989; Highton and Peabody 2000). These allozyme-based analyses were used by Highton to recognize many species. Although Highton's approach has been challenged by some authors (e.g., Frost and Hillis 1990; Petranka 1998), no alternate analyses or interpretations of the allozyme data have been proposed, and even these authors agreed that *P. glutinosus* (as recognized before Highton 1989) consisted of multiple species.

Fourth, many of the morphologically cryptic taxa appear as phylogenetically distinct based on mtDNA sequence data (Fig. 2). For example, the species of the *glutinosus* complex fall into two distinct clades (Clades A and B, Fig. 1), and therefore many species of the complex are not closely related to each other. In Clade B, species of the *glutinosus* complex interdigitate among species of the *jordani* complex (Fig. 1), and only two morphologically cryptic species appear as sister taxa (*P. chlorobryonis* and *P. variolatus*). Clade A contains nine species of the morphologically cryptic *glutinosus* complex, most of which are closely related.

Fifth, some of the taxa in Clade A that are morphologically cryptic and closely related based on our phylogeny appear to be allopatric and geographically distant from each other (see maps in Highton 1989; Conant and Collins 1991), mak-

ing current gene flow and conspecificity seem very unlikely. These include the sister species *P. kiamichi*-*P. mississippi* and *P. grobmani*-*P. kisatchie* (Fig. 1). The sister species in these pairs are not only geographically distant, but much of the geographic region separating the species of each pair is occupied by other morphologically and ecologically similar species of the *glutinosus* complex, suggesting that future sympatry of these species pairs may be unlikely.

We acknowledge the possibility that some pairs of species in this group may prove to be conspecific upon further investigation (e.g., *P. ocmulgee* and *P. savannah*, *P. albagula* and *P. sequoyah*, *P. chlorobryonis* and *P. variolatus*), but we consider it unlikely that the number of species in the *glutinosus* group has been substantially overestimated. Furthermore, when the five taxa we consider most questionable are deleted from the analyses of diversification rates (*P. longicrus*, *P. oconaluftee*, *P. savannah*, *P. sequoyah*, *P. variolatus*) the results are essentially identical to those including all species (i.e., the *glutinosus* group shows a significant increase in diversification rates).

#### Patterns of Hybridization: Implications for Speciation

Within the *glutinosus* group, there is widespread introgressive hybridization among species, including those that are relatively distantly related (Table 4). In some cases, interspecific gene flow appears to be relatively extensive (e.g., Weisrock et al. 2005) and some species hybridize with multiple species (e.g., *P. teyahalee* seemingly hybridizes with six different species, *P. chlorobryonis* with four, and *P. chatahoochee* and *P. shermani* with three each). Many of the pairs of species that hybridize are morphologically distinct from each other, and have long been recognized as separate species in the *glutinosus* and *jordani* species complexes. For example, *P. chatahoochee* (*glutinosus* complex) hybridizes with *P. shermani* (*jordani* complex) and *P. chlorobryonis* (*glutinosus* complex) hybridizes with *P. metcalfi* (*jordani* complex). We also show that reproductive isolation appears to increase gradually over time within the group, and is very weak among the youngest species (Fig. 6).

These patterns suggest that the evolution of intrinsic reproductive isolating mechanisms may lag behind the origin of new lineages in this group. Based on our phylogeny and published range maps (e.g., Petranka 1998; Highton and Peabody 2000), many sister species within the *glutinosus* group are allopatric (*chatahoochee-cheoah*, *amplus-meridianus*, *grobmani-kisatchie*, *kiamichi-mississippi*, *fourchensis-ouachitae*, *kentucky-petraeus*) or geographically abutting (*albagula-sequoyah*, *chlorobryonis-variolatus*, *metcalfi-montanus*, *ocmulgee-savannah*). If species often arise in allopatry, then the evolution of intrinsic barriers to reproduction are not necessary for the origin of these lineages (i.e., species become reproductively isolated through geographic barriers instead). These intrinsic reproductive barriers seem to have evolved in some lineages but not others. The decoupling of lineage origin from the evolution of intrinsic reproductive isolation is somewhat problematic if one equates speciation with the evolution of complete reproductive isolation between populations; in the *glutinosus* group, one would be forced to consider distantly related and morphologically distinct taxa

to be conspecific. The decoupling of lineage origin and intrinsic isolation may be less problematic if one equates speciation with the origin of new lineages (for a discussion of the evolutionary species concept in speciation research see Wiens 2004b). Many other studies have also found that intrinsic isolating mechanisms may be limited or absent between relatively young species (i.e., Coyne and Orr 1997, 2004; Mendelson 2003) and that natural hybridization can occur frequently between distantly related species in some groups (e.g., leopard frogs; Hillis 1988). Our findings suggest the usefulness of adopting a lineage-based species concept, especially in clades with incomplete reproductive isolation. Nevertheless, the results also reinforce the importance of research programs that seek to understand why species that arise in allopatry remain distinct in sympatry, the traditional focus of speciation research based on the biological species concept (Coyne and Orr 2004).

#### *Hybridization and Rapid Radiation*

Seehausen (2004) has proposed that hybridization may be a cause of rapid radiation, in that transgressive segregation creates new functional complexes upon which selection can act. We suggest that in some cases hybridization may instead be a consequence of rapid, recent speciation, because there has been too little time for intrinsic barriers to gene flow to evolve (i.e., the origin of new lineages outstrips the biological “speciation clock”). This pattern may be exacerbated in cases (such as eastern *Plethodon*) in which there seems to be relatively little morphological and ecological differentiation among species (i.e., nonadaptive radiation; Kozak et al. 2006). Of course, the critical distinction between Seehausen’s (2004) hypothesis and the scenario described here is that he postulates critical hybridization events immediately *prior* to the adaptive radiation, whereas under our hypothesis most hybridization occurs *after* the rapid proliferation of species.

Was there hybridization at the base of the *glutinosus* group? This seems very unlikely. We found no strongly supported discordance between the nuclear and mitochondrial data at the base of the *glutinosus* group (an important prediction in Seehausen’s scenario), and many clades that are congruent among datasets. Instead, there is extensive hybridization among the younger lineages of the *glutinosus* group, as evidenced by allozyme data (Highton and Peabody 2000), mtDNA sequences (Weisrock et al. 2005; this study), and possibly by incongruence between TPI and mtDNA genealogies.

We acknowledge that we have only provided a single potential case study of the scenario that we propose (i.e., rapid, recent diversification leading to extensive hybridization). Rigorously testing this hypothesis will require comparisons among clades to determine whether the frequency of hybridization between species (i.e., number of hybridizing species pairs relative to the overall number of species) is higher in groups where speciation is more rapid. Unfortunately, the overall frequency of hybridization is difficult to estimate within *Plethodon* because many species are morphologically cryptic (making hybrid zones hard to detect without more detailed geographic sampling). Nevertheless, future studies that document extensive hybridization among species in a

recent radiation should at least consider the possibility that introgression may be a consequence of rapid diversification, rather than a cause.

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