

Resolving the tips of the Tree of Life: How much mitochondrial data do we need?

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Key Words: Amphibia, Bayesian analysis, *Eurycea*, mitochondrial genomes, parsimony,
phylogeny reconstruction, sequence evolution, Tree of Life

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Mitochondrial (mt) DNA sequences are used extensively to reconstruct evolutionary relationships among recently diverged animals, and have constituted the most widely used markers for species- and generic-level relationships for the last decade or more. However, most studies to date have employed relatively small portions of the mt-genome. In contrast, complete mt-genomes primarily have been used to investigate deep divergences, including several studies of the amount of mt sequence necessary to recover ancient relationships. We sequenced and analyzed 24 complete mt-genomes from a group of salamander species exhibiting divergences typical of those in many species-level studies. We present the first comprehensive investigation of the amount of mt sequence data necessary to consistently recover the mt-genome tree at this level, using parsimony and Bayesian methods. Both methods of phylogenetic analysis revealed extremely similar results. A surprising number of well supported, yet conflicting, relationships were found in trees based on fragments less than ~2000 nucleotides (nt), typical of the vast majority of the thousands of mt-based studies published to date. Large amounts of data (11,500+ nt) were necessary to consistently recover the whole mt-genome tree. Some relationships consistently were recovered with fragments of all sizes, but many nodes required the majority of the mt-genome to stabilize, particularly those associated with short internal branches. Although moderate amounts of data (2000 -3000 nt) were adequate to recover mt-based relationships for which most nodes were congruent with the whole mt-genome tree, many thousands of nucleotides were necessary to resolve rapid bursts of evolution. Recent advances in genomics are making collection of large amounts of sequence data highly feasible, and our results provide the basis for comparative studies

of other closely related groups to optimize mt sequence sampling and phylogenetic resolution at the "tips" of the Tree of Life.

Introduction

The ongoing genomic revolution is providing evolutionary biologists with a tremendous number of characters for reconstruction of the Tree of Life (e.g., Pollock 2002; Rokas et al. 2003; Chen, Orti, and Meyer 2004; Cracraft and Donoghue 2004). This includes mitochondrial (mt) DNA sequences, which are widely used for phylogenetic analyses (particularly in animals), and play a central role in evolutionary, population genetic, biodiversity, and conservation studies. Animal mt-genomes typically contain 37 genes and offer the advantages of rapid evolution, a general lack of recombination, and relative ease of PCR amplification (Ballard and Whitlock 2004). For recently diverged organisms, the relatively short coalescence time of mtDNA allows efficient reconstruction of relationships at the "tips" of the tree, whereas numerous nuclear sequences may be necessary to achieve accurate resolution at this scale (Moore 1995; Palumbi, Cipriano, and Matthew 2001; Wiens and Penkrot 2002). However, the question of how many sequence characters are necessary to reliably recover relationships remains open, and, surprisingly, for closely related organisms, little is known regarding phylogenetic congruence (agreement in tree topology) of subsets of the mt-genome with that of the entire molecule. The majority of completely sequenced animal mt-genomes represent either distantly related lineages (e.g., major vertebrate groups) or conspecific model organisms (e.g., humans), providing researchers with a rich source of data for exploring sequence evolution and relationships at higher levels (Harrison et al. 2004; Helfenbein et al. 2004; Macey et al. 2004; Mueller et al. 2004; San Mauro, Garcia-Paris, and Zardoya 2004) or within species (Ballard 2000a; Ingman et al. 2000). Yet, many

workers employ mtDNA sequences to investigate questions between these extremes of evolutionary divergence, and generally use only a small fraction of the mt-genome, typically < 2,000 nucleotides (nt) (fig. 1). This phenomenon is largely historical, as collecting lengthy sequences was extremely challenging until recently. In most systems, mtDNA is inherited as a single linkage unit (Scheffler 1999), so a tree based on a subset of the genome often is assumed to be representative of the whole locus. Despite evidence of phylogenetic incongruence across the mt-genome from studies of distantly related lineages (Cao et al. 1994; Cummings et al. 1995; Russo, Takezaki, and Nei 1996; Zardoya and Meyer 1996; Simmons and Miya 2004) use of relatively short mt-sequences remains common in studies of recently diverged organisms.

Here we provide the first investigation of the amount of mtDNA sequence data necessary to consistently recover a tree identical to that based on the entire mt-genome, in a group of closely related species. We collected 24 complete mt-sequences (GenBank #####-#####), 19 from members of the *Eurycea multiplicata* complex, a morphologically diverse radiation of plethodontid salamanders endemic to the Interior Highlands of North America (Bonett and Chippindale 2004), plus five from closely related species. This complex (our focal group) diverged from all other *Eurycea* at most 24 million years ago (MYA) and the deepest divergence within the group is roughly 4-9 MYA (see online Supplemental Material). Thus, divergences within the focal group likely are relatively recent (Chippindale et al. 2004), and typical of what many researchers encounter in species-level studies. We compare maximum parsimony (MP) and Bayesian (BA)-based phylogenies of 150 fragments drawn randomly from across the

genome and compare them to the mt-genome tree. These comparisons are used to determine how much data are necessary to converge on the topology revealed by the complete data set, and we identify phylogenetic conflicts among fragments of different sizes drawn from throughout the mt-genome. Further, we present a regression analysis of the amount of data required to consistently recover each node in the tree vs. the length of its subtending branch. We use the results to draw inferences about the amount of mt sequence needed resolve relationships involving a range of times between cladogenetic events. Neither ultimate utility of data types (mt vs. nuclear DNA) nor relative performances of methods for phylogeny reconstruction is intended to be the focus of this article. Instead, we ask, using a very large empirical data set, (a) how much mt sequence do we need to achieve a topology congruent with the whole mt-genome tree; (b) can use of short mt sequences positively mislead phylogenetic analyses; and (c) how sensitive are short internal branches to varying amounts of data, especially within a recently diverged group of species?

Materials and Methods

Sampling

The complete mitochondrial (mt) genomes of 19 salamanders from the *Eurycea multiplicata* complex (the focal group), four closely related species of *Eurycea*, and *Pseudotriton montanus* were sequenced (Appendix 1). There currently are only three named species within the focal group (*E. multiplicata*, *E. spelaeus*, and *E. tynerensis*); however, taxonomic revisions in progress indicate that each of these should be split into

multiple species (Bonett and Chippindale 2004). The 19 focal group specimens represent the full spectrum of mt-divergence within this group (Bonett & Chippindale 2004). Non-focal group species represent each of the other four major lineages of *Eurycea* (*E. cirrigera*, *E. lucifuga*, *E. cf. neotenes*, and *E. quadridigitata*) and a member of the lineage sister to *Eurycea* (*Pseudotriton montanus*; Chippindale et al. 2004; Mueller et al. 2004). Vouchers and tissues were deposited in the collection of the University of Texas at Arlington (UTA) Amphibian and Reptile Biodiversity Center.

Whole Mitochondrial Genome Sequencing and Alignment

Qiagen DNeasy extraction kits were used to isolate DNA from frozen tissues. Mitochondrial genomes were primarily amplified in two pieces (~ 9 – 12 kb), from *rrnS* or *rrnL* to *nad4* or *trnL2* and from *cox3* to *rrnL*, using *rTth* Long PCR (Applied Biosystems). Primer sequences are available from RMB and JRM. PCR products were run through a Hydroshear (Gene Machines) to shear products into ~ 1 to 2 kb fragments. Fragments were blunt-ended using T4 DNA polymerase, and Klenow enzyme, then electrophoresed in 1% agarose gels adjacent to DNA ladders for size selection. Fragments were excised from the gel and extracted using Qiagen Gel Extraction kits. Fragments were ligated into plasmid vectors (pUC18) using Fast-Link ligation kits (Epicentre Technologies), applying standard protocols, and the resulting plasmids were used to transform *E. coli* via electroporation. Colonies were grown on LB/Amp/X-gal plates.

Ninety-six to 384 white colonies were picked from each plate using a QBot robot (Genetix). Rolling circle amplification (RCA; [Dean et al. 2001; Hawkins, Detter, and Richardson 2002]) was used to amplify plasmids using TempliPhi kits (Amersham Biosciences), pUC18 and ET terminators. Amplifications were cleaned using SPRI technology (Elkin et al. 2002) and sequenced on an ABI 3730xl or MegaBACE 4000 capillary sequencer.

All sequences from a given PCR product were aligned using Phrap. Alignments were imported into SequencherTM v. 4.1 (Gene Codes Corp.) for editing and to create a consensus sequence for each PCR product. Consensus sequences from a given genome were overlapped and aligned in MacVector (Accelrys) to complete each mitochondrial genome sequence and subsequently were annotated for genic boundaries. Protein coding sequences were translated to amino acids and checked in MacClade (Maddison and Maddison 2001). Regions encoding tRNAs were compared to previously published tRNA secondary structural models to determine homology (Kumazawa and Nishida 1993; Macey and Verma 1997; Zardoya and Meyer 2000). The 24 mt-genomes ranged from 16,564 to 17,049 nt. The genomes were composed of ~ 1,480 nt of tRNAs, ~ 11,330 nt of protein coding genes, ~ 2,500 nt of ribosomal genes, and ~ 1250 – 1750 nt of interspersed non-coding sequence and control region. A few regions of ambiguous alignment were excluded from the alignment using MacClade (Maddison and Maddison 2001). In total 58 nucleotide (nt) tRNAs, 116 nt of protein coding genes, 394 nt of ribosomal genes, 75 nt of interspersed non-coding sequence, and the entire control region were excluded. The

final alignment of the 24 mt-genome sequences, after removal of these very fast-evolving regions of uncertain alignment (e.g., the Control Region), is 14,824 nt in length.

Fragment selection and phylogenetic analyses

The beginning of *trnF* was used as position one in the alignment of all mitochondrial genomes. Random starting points of each fragment were selected from position one at the beginning of *trnF* to 14,824 nt minus the length of the random fragment. Initially, we randomly selected 100 fragments of contiguous sequence ranging from 500 to 14,824 nt to estimate how much data are necessary to consistently recover the same topology as the mt-genome tree (i.e., converge on a single tree). To more fully explore phylogenetic congruence among small sequences (typical of those used by most investigators) we randomly selected an additional 50 fragments of contiguous sequence of approximately 500 to 2,000 nt from across the mt-genome.

The complete data set and the 150 randomly selected fragments were analyzed via maximum-parsimony and Bayesian analyses. Unweighted maximum-parsimony analyses were performed in PAUP* v. 4.0b10 (Swofford 2001) using heuristic searches with 100 random-taxon-addition replicates. For each analysis, the shortest tree or strict consensus of all shortest trees were saved. Only *P. montanus* was designated as the outgroup, so that monophyly of the *E. multiplicata* complex with respect to other major lineages of *Eurycea* could be tested. Non-parametric bootstrapping (Felsenstein 1985) (1000 pseudoreplicates, 10 random taxon-addition-replicates per pseudoreplicate) was used to assess support for each node. For Bayesian analyses, Modeltest v. 3.06 (Posada and

Crandall 1998) was used to determine the most appropriate model of nucleotide substitution for each of the 37 genes (partitions), (Appendix 2). In a few instances adjacent tRNA genes had the same or a similar model. We included these in the same partition, so the whole data set ultimately contained 30 partitions, the most that can currently be handled by MrBayes 3.0 (Huelsenbeck and Ronquist 2001). Fragments that included any of the 30 partitions were also partitioned accordingly and the same models as used for the corresponding partitions were applied. Partitioned Bayesian analyses (all partitions unlinked) implemented via MrBayes 3.0 were run with four chains and uniform priors for 4.05 million generations (with the first 50,000 discarded as burn-in; stationarity of likelihoods achieved before this point in all analyses). The resulting 50% majority-rule consensus of the 40,000 post burn-in trees, sampled every 100 generations, was computed in PAUP* (Swofford 2001). The resulting topologies for the focal group were completely congruent for both parsimony and Bayesian analyses with the exception of one shallow node, which we collapsed to a trichotomy for the purposes of this investigation, leaving 17 focal group nodes. Herein we refer to this tree as the mt-genome tree (fig. 2; table 1).

Measures of Congruence and Phylogenetic Stability

To assess the relationship between phylogenetic congruence and mt-sequence length we compared the number of nodes in each of the phylogenies based on analyses of random fragments that were congruent versus incongruent with the mt-genome topology (considering only the 17 focal group nodes). We also counted the number of nodes at or

above two levels of support for each analysis, using non-parametric bootstrap values for parsimony analyses and posterior probabilities for Bayesian analyses. To evaluate support we counted nodes at or above two different levels of confidence. For maximum–parsimony bootstrap we considered values $\geq 70\%$ (Hillis and Bull 1993; but see their caveats) and $\geq 95\%$ (Felsenstein and Kishino 1993). Bayesian posterior probabilities follow a true probability distribution (Ranalla and Yang 1996; Huelsenbeck and Ronquist 2001; Alfaro, Zoller, and Lutzoni 2003), so we counted number of nodes $\geq 90\%$ and $\geq 95\%$, to provide estimates that reflect a 10% and 5% error rate, respectively. In a few trees the focal group was not monophyletic. In nearly all of these cases one or more of the non-focal group *Eurycea* were more closely related to either the *E. multiplicata* clade or the *E. spelaeus* + *E. tynerensis* clade than these clades were to one another. Since we were not examining the relationships among the non-focal group species, we counted any instance of focal group non-monophyly as one incongruent node, and we used the support value from the clade that most strongly supported non-monophyly.

We measured the amount of sequence data necessary to achieve phylogenetic “stability” (i.e. the sequence length beyond which all analyses consistently recover the tree with resolution and topology identical to that of the mt-genome tree) using both Bayesian and parsimony analyses. We also determined the amount of data necessary for each of the 17 nodes to reach “stability” (i.e., the sequence length beyond which all fragments recovered a given relationship with resolution and topology identical to that of the mt-genome tree).

Bayesian branch lengths for the tree based on the whole data set, averaged across all 40,000 trees (representing 4 million generations sampled every 100 generations), were determined in MrBayes 3.0 (Huelsenbeck and Ronquist 2001). For both Bayesian and maximum-parsimony we performed regression analyses of log-transformed stability point for each node versus the log of the branch length subtending the node. Using SAS v. 8.2 (SAS institute Inc.), 1000 Markov Chain Monte Carlo (MCMC) random iterations were used to create a random distribution of r^2 values based on the original data. We regressed parsimony stability points on the Bayesian branch lengths because the parsimony model inherently underestimates branch lengths, especially the longest ones. We found the regressions of the log of parsimony stability points on the log of parsimony branch lengths, using both accelerated transformation (ACCTRAN) and delayed transformation (DELTRAN), to show the same relationship.

Results and Discussion

In total, analyses of the 150 fragments recovered 28 alternative topologies using MP and 35 using BA. The alternate topologies involved more than half (9 out of 17) of the nodes in our comparison. Some topologies were substantially different than the mt-genome tree, with as many as seven incongruent nodes (fig. 3). Alternate topologies primarily were obtained from analyses of small fragments (< 2000 nt). Notably, every alternate topology, except the mt-genome tree, was represented among the trees based on the 63 “small” fragments in this study. Therefore, analyses of small fragments produced results that were both inconsistent with each another and the mt-genome tree at this scale

of divergence, even though all of the fragments are derived from the same locus. The minimum amount of sequence needed to recover a topology including 17 nodes congruent with the mt-genome tree was 2,899 nt with MP and 5,107 with BA. However, many trees based on even longer fragments disagree with the mt-genome tree. Even though the number of incongruent nodes per tree decreased, in analyses of fragments greater than 2000 nt, these conflicts still involved several (5 of the 17) different nodes (fig 2). We found that a surprisingly large amount of data was necessary to consistently recover the mt-genome topology, at least 11,593 nt under both MP and BA.

In general, support for nodes congruent with the mt-genome tree increased with increasing sequence length. However, analyses using less than ~8,000 nt (about half of the mt-genome) frequently assigned high support values to nodes that were incongruent with the mt-genome tree. In total, across trees based on all randomly sampled fragments, there were 165 nodes incongruent with the mt-genome tree in MP and 195 in BA. Under MP bootstrapping 25.4% (42 / 165) of incongruent nodes had values between 70 and 94 %, but only 0.6% (1 / 165) were $\geq 95\%$. For BA, 36.5% (71 / 195) of incongruent nodes had posterior probabilities between 90% - 94%, and 24.6% (48 / 195) were $\geq 95\%$. Regardless of whether our mt-genome tree most accurately reflects the evolutionary history of this molecule, our analyses show moderate to strong support (under widely used criteria) for several conflicting relationships. This underscores the fact that even for close relationships a phylogeny based on a small portion of the mt-genome may not be representative of the whole.

Here, we demonstrate high levels of incongruence among fragments from throughout the mt-genome (a single locus), incorporating portions of all genes. The conflicting signal in our data likely results from differences, in the tempo and mode of evolution, across the mt-genome (Cao et al. 1994; Cummings et al. 1995; Zardoya and Meyer 1996; Mattern 2004). However, our results suggest that phylogenetic incongruence is not localized to any particular, clearly identifiable sequence region, warranting caution both in choice of gene and length of sequence. Recently, authors advocate that given the amount of available whole genomic data, better models of sequence evolution can, and should be, developed for phylogenetics (Phillips, Delsuc, and Penny 2004). Data sets, such as the one presented here, will allow models of mitochondrial sequence evolution to be optimized for phylogenetics of closely related species. Moreover, regions of the mt-genome with very complex histories (that are too difficult to model) can be identified and potentially excluded from analyses. Which genes provide the best estimate of the mt-genome tree at this scale of divergence in these salamanders, and in a diversity of other animal groups, currently is being investigated.

Short internodes (internal branches) in phylogenetic trees generally indicate rapid cladogenesis, confounding recovery of relationships due to the limited time for accumulation of informative nucleotide changes (DeFilippis and Moore 2000; Murphy et al. 2001). Eight of the 17 nodes were recovered using all fragments of all lengths (fig. 2), but several nodes (29%; 5 out of 17) in different and often distant parts of the tree, all of which involve the shortest internodes, required fragments of considerably longer than 2,000 nt to stabilize (i.e., to consistently agree with the mt-genome tree). We found a

strong negative correlation between increasing internode length and amount of sequence necessary for phylogenetic stability (MP: $r^2 = 0.7807$, $p < 0.001$; BA: $r^2 = 0.6223$, $p < 0.001$; fig. 4). This result is consistent with results from simulations (Alfaro et al. 2003), and is expected, as a short branch reflects few character changes. The empirical perspective presented here, also indicates that short internodes are truly problematic and require extensive data for consistent and accurate resolution.

Thus far, use of whole mt-genomes in phylogenetic studies that focus on closely related species have been extremely rare (Ballard 2000b). We find that the typical amount of mt-DNA data used to reconstruct relationships may yield misleading results at this scale of divergence, especially when lineage splitting has occurred rapidly. The rapid evolution of mitochondrial genes (with considerably higher rates of mutation than the fastest known nuclear loci; Moriyama and Powell [1997]), provides a large number of variable characters for reconstructing species-level relationships. Although a moderate amount of data may be enough to get a rough estimate of species-level phylogeny, more data than previously expected may be necessary to accurately recover relationships involving rapid cladogenetic events. Encouragingly, advances in molecular biology and the genomic revolution are making collection of large amounts of sequence increasingly practical. Collection of more complete mt-genomes of closely related taxa will increase our understanding of evolution of the whole molecule at this scale of divergence and is essential for phylogeny estimation. These approaches show tremendous promise for recovery of relationships at the very tips of the Tree of Life.

Supplemental Material

Methods of dating estimates and journal survey.

Appendix 2 & 3.

Acknowledgements

We thank M. Gerson, O. Idris, K. Irwin, L. Irwin, W. Van Devender, and J. Wiens for assistance in the field, and D. Engle, J. Froula, M. Fourcade, K. Ivanova, J. Kuehl, and R. Mueller for lab assistance. P. McGuigan and B. Shirazi enabled use of the Distributed and Parallel Computing Cluster (DPCC) (partially funded by NSF grant EIA-0216500). Primary funding was provided by NSF DDIG to PTC and RMB (DEB-0129242), NSF grant to PTC (DEB-0206730), and ASIH Gaige Award to RMB. This work (LBNL-55130) was performed under the auspices of the U.S. Department of Energy, Office of Biological and Environmental Research, under contract No. DE-AC03-76SF00098 with the University of California, Lawrence Berkeley National Laboratory.

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Figure 1. Histograms of the amount of DNA sequence data applied in 200 “species-level” studies of animals published in 2004. (A) Amount of mt-DNA used in the 200 studies. 36% (72 of 200) of the studies also included nuclear DNA sequence data. (B) Total amount of DNA sequence data used in all 200 studies. For details of this survey see online Supplemental Material.

Figure 2. Mt-genome tree based on 14,824 nt showing the consensus of the maximum-parsimony and Bayesian analyses. Numbers designate the 17 focal group nodes that were considered in congruence tests. All but one node in the focal group agree; this portion of the tree is collapsed to a trichotomy. This tree was recovered in analyses of all fragments $\geq 11,593$ nt. For support values see table 1. Branch lengths were averaged from across 40,000 post-burn-in Bayesian trees, based on 4 million generations sampled every 100 generations. Nodes 2, 4, 6, 7, 10, 12, and 16 were recovered in analyses of all sized fragments that were sampled. Nodes 1, 5, 8, 9, 14, 15, 17, highlighted with an asterisk, required more than 1500 nucleotides to stabilize (table 1).

Figure 3. Comparison of the topologies of phylogenetic trees produced when based on 150 randomly selected mtDNA sequence fragments of varying length. The number of nodes found to be congruent and incongruent with the whole mtDNA phylogeny are separately plotted for parsimony (A) and Bayesian (B) analyses, in each case considering confidence measure thresholds (maximum-parsimony nonparametric bootstrap values $\geq 70\%$ and $\geq 95\%$, Bayesian posterior probabilities $\geq 90\%$ and $\geq 95\%$). Lines demark the minimum sequence length for which complete congruence with the mt-genome tree was achieved (but not necessarily with complete resolution) and the minimum sequence length for which resolution and topology are identical to that of the mt-genome tree. Congruence and stability points are identical in BA.

Figure 4. Regression plot showing strong negative correlation between log internode length and log phylogenetic stability (number of nucleotides necessary to consistently recover a given relationship) for MP and BA.

Appendix 1. Specimens examined.

Species	Museum #	GenBank #	Locality
<i>Eurycea tynnerensis</i> 1	UTA 56373	#####	AR: Searcy Co.
<i>Eurycea tynnerensis</i> 2	UTA 56375	#####	AR: White Co.
<i>Eurycea tynnerensis</i> 3	UTA 56380	#####	AR: Searcy Co.
<i>Eurycea tynnerensis</i> 4	UTA 56384	#####	AR: Crawford Co.
<i>Eurycea tynnerensis</i> 5	UTA 53860	#####	OK: Cherokee Co.
<i>Eurycea tynnerensis</i> 6	UTA 56406	#####	OK: Mayes Co.
<i>Eurycea tynnerensis</i> 7	UTA 56387	#####	AR: Benton Co.
<i>Eurycea tynnerensis</i> 8	UTA 56404	#####	OK: Sequoyah Co.
<i>Eurycea tynnerensis</i> 9	UTA 56402	#####	OK: Sequoyah Co.
<i>Eurycea spelaesus</i> 1	UTA 53846	#####	OK: Mayes Co.
<i>Eurycea spelaesus</i> 2	UTA 56360	#####	AR: Benton Co.
<i>Eurycea spelaesus</i> 3	UTA 56361	#####	AR: Madison Co.
<i>Eurycea spelaesus</i> 4	UTA 56673	#####	MO: Pulaski Co.
<i>Eurycea multiplicata</i> 1	UTA 56353	#####	AR: Saline Co.
<i>Eurycea multiplicata</i> 2	UTA 56350	#####	AR: Saline Co.
<i>Eurycea multiplicata</i> 3	UTA 56354	#####	AR: Montgomery Co.
<i>Eurycea multiplicata</i> 4	UTA 56359	#####	AR: Logan Co.
<i>Eurycea multiplicata</i> 5	UTA 56367	#####	OK: Choctaw Co.
<i>Eurycea multiplicata</i> 6	UTA 56369	#####	OK: Atoka Co.
<i>Eurycea cirrigera</i>	UTA 56672	#####	FL.
<i>Eurycea lucifuga</i>	UTA 53867	#####	AR: Independence Co.
<i>Eurycea</i> cf. <i>neotenes</i>	UTA 56674	#####	TX: Comal Co.
<i>Eurycea quadridigitata</i>	UTA 56675	#####	FL.
<i>Pseudotriton montanus</i>	UTA 56676	#####	SC: Laurens Co.

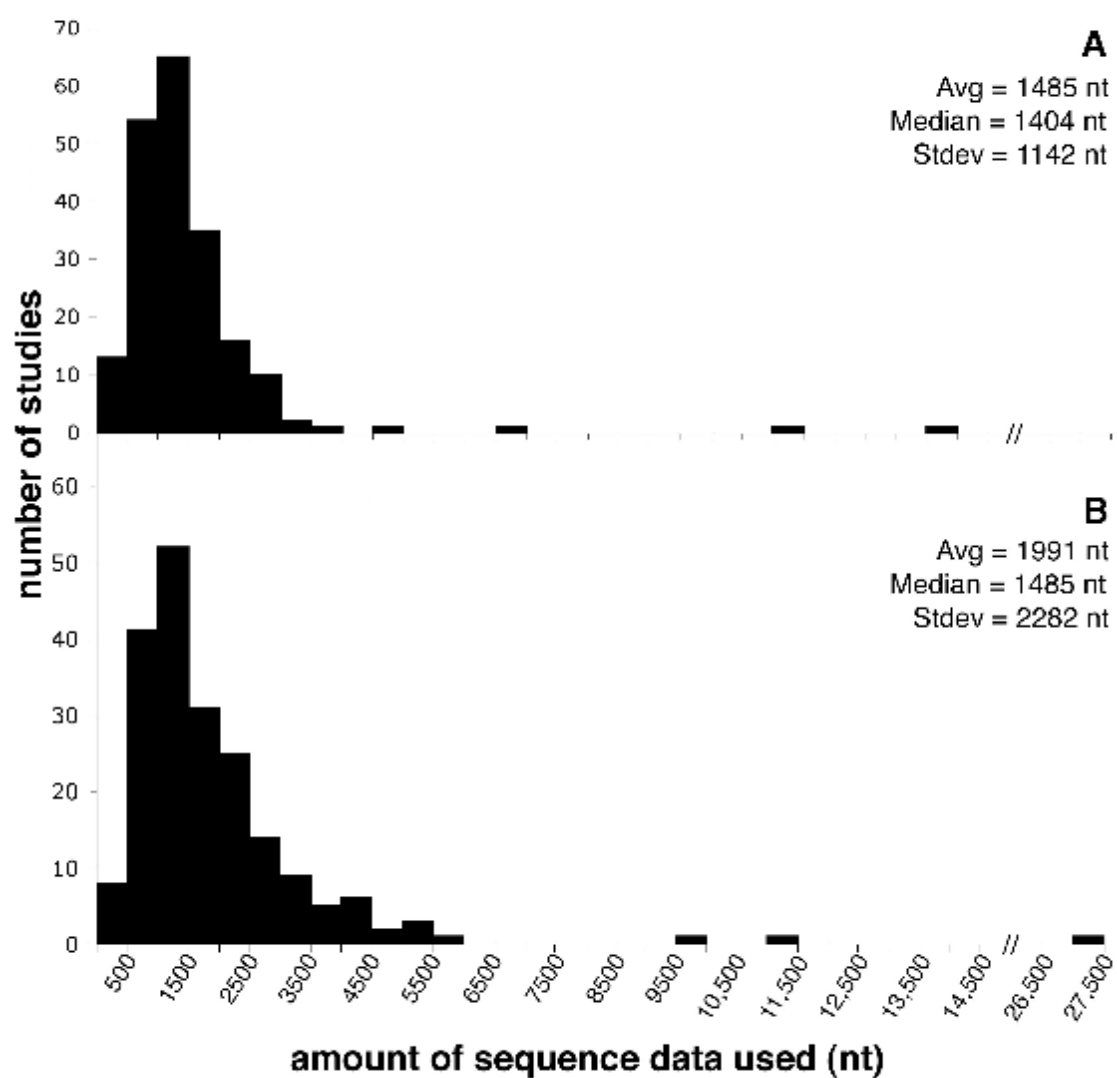


Figure 1.

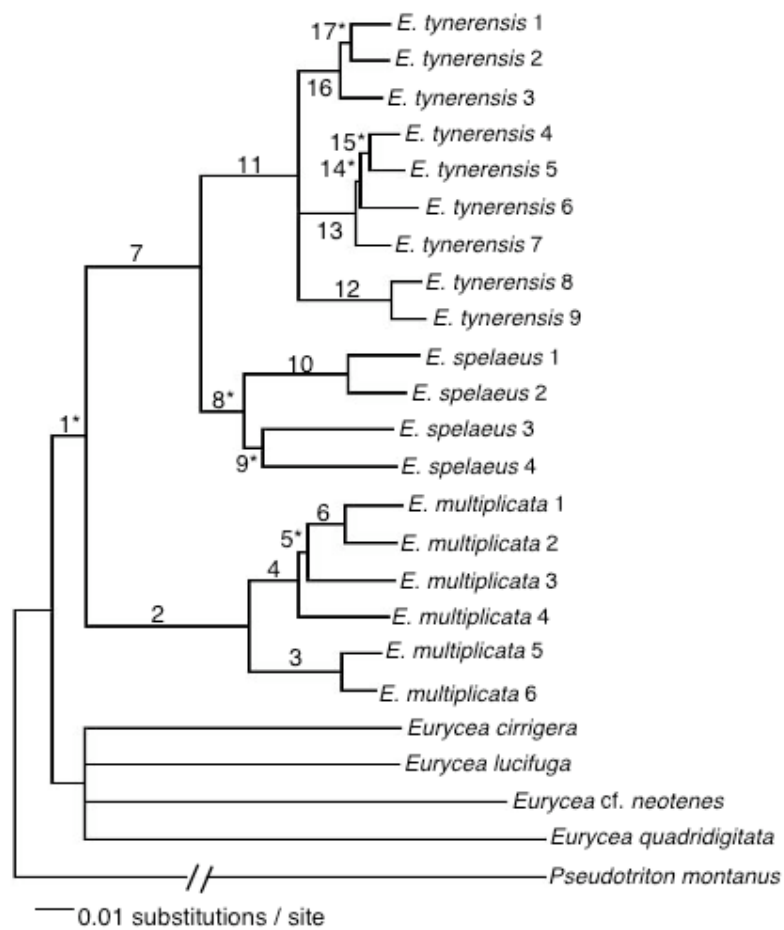


Figure 2.

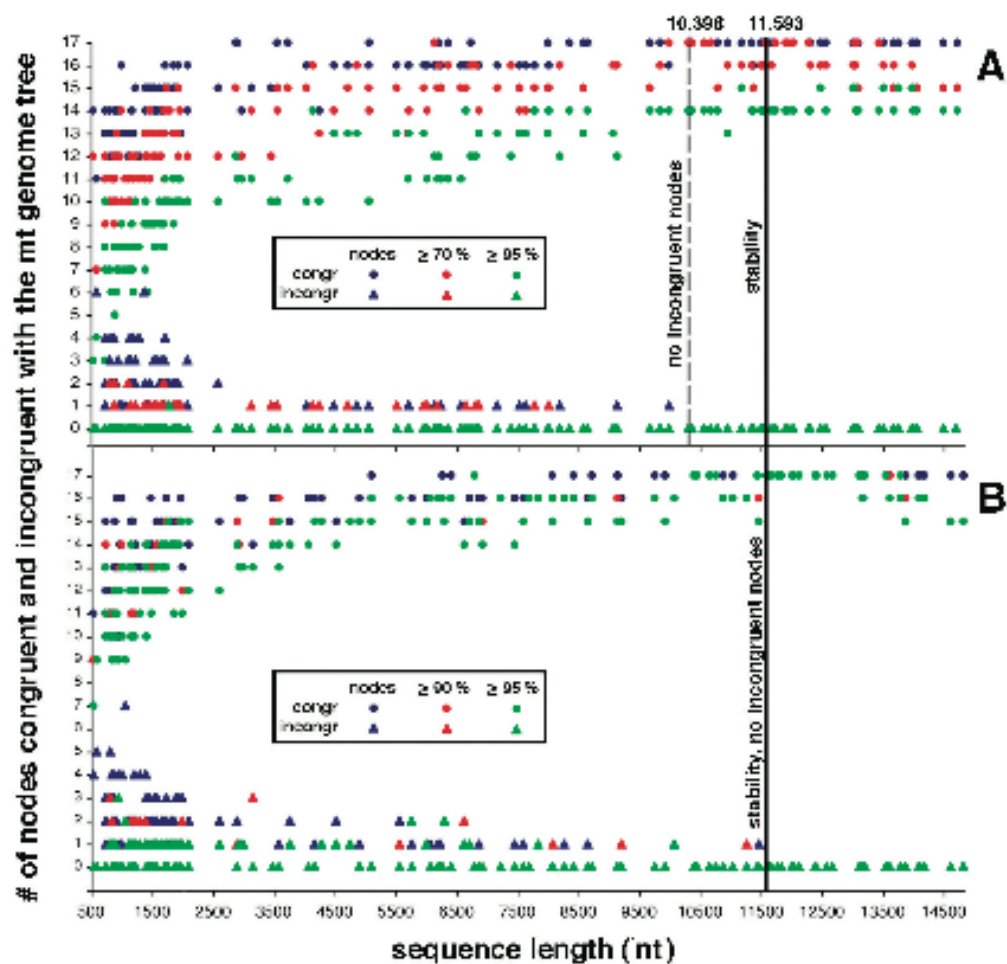


Figure 3.

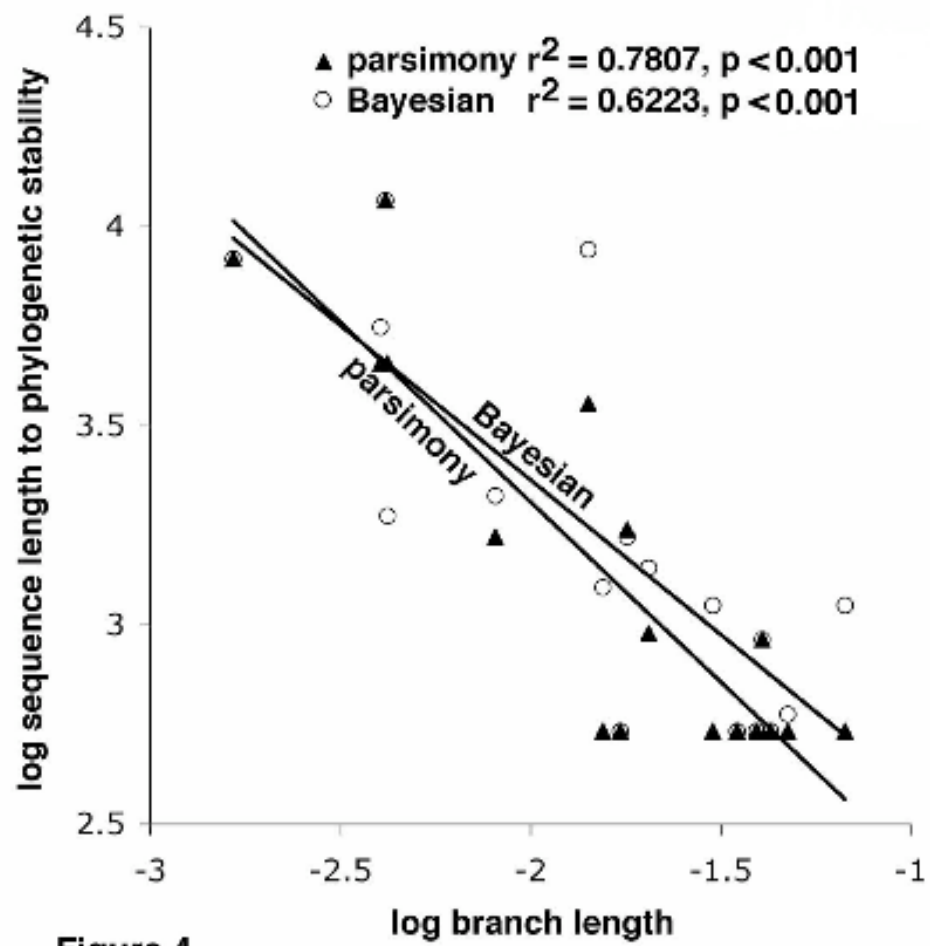


Table 1**Support values and stability points for each node.**

Node #	Maximun Parsimony			Bayesian		
	Bootstrap mt-genome tree	Highest bootstrap for node	Stability point for node	Post. prob. mt-genome tree	Highest post. prob. for node	Stability point for node
1	100	100	3586	100	100	8722
2	100	100	539	100	100	1118
3	100	100	539	100	100	539
4	100	100	539	100	100	1118
5	61	95	11593	87	100	11593
6	100	100	539	100	100	1237
7	100	100	539	100	100	595
8	100	100	1730	100	100	1658
9	100	100	1658	100	100	2101
10	100	100	539	100	100	539
11	100	100	917	100	100	917
12	100	100	539	100	100	539
13	100	100	949	100	100	1387
14	63	90	8266	84	100	8266
15	100	100	4524	100	100	1872
16	100	100	539	100	100	539
17	93	97	4524	100	100	5566

Supplemental Material: Bonett et al.

EXPERIMENTAL PROCEDURES

Divergence Time Estimates

Time of divergence of the ancestor of the *E. multiplicata* complex from that of other *Eurycea* was estimated using the penalized likelihood method of Sanderson (2002), implemented via the Unix version of r8s (Sanderson 2003). The methodology and data set used were identical to those of Chippindale et al. (2004). Rough estimates of the age of the split between the two major clades in the *E. multiplicata* complex (Ozark vs. Ouachita) were made by comparison to the deepest divisions in the central Texas *Eurycea* (Chippindale 2000; Chippindale et al. 2000; Hillis et al. 2001; Chippindale et al. 2004), which appear to have occurred over a similar time frame.

Penalized likelihood analyses (Sanderson 2002; 2003) indicate that the ancestor of the *E. multiplicata* complex diverged from that of all other *Eurycea* at most 24 MYA, and the deepest divergence within our focal group is roughly 4-9 MYA. Thus, divergences within our focal group likely are relatively recent, and comparable to those in many groups of closely related animals that have been studied using mtDNA sequences.

SEQUENCE SURVEY

To estimate the amount of mtDNA sequence data currently being applied to species-level phylogenies of animals, we surveyed studies published in 2004 in the journals *Mol. Phylogenet. Evol.*, *Mol. Ecol.*, *Syst. Biol.*, *Biol. J. Linn. Soc.*, *Evolution*, *Proc. R. Soc. London Ser. B*, and *J. Mol. Evol.* We counted the amount of mtDNA

sequence data (nt) that was used in all studies that met the following criteria. The study had to involve the reconstruction of a new phylogeny (including at least some new data) that included multiple (three or more) animal species that are classified in the same genus, not including the outgroup taxon or taxa. We did not include studies that initially focused on intraspecific relationships, even if results ultimately indicate the presence of multiple species within samples. We also counted the amount of nuclear DNA sequence data used in these studies (if any) to estimate the total amount of DNA sequence currently being used in typical studies of closely-related animals. Of 200 studies: 83.5 % used < 2,000 nt of mtDNA, average = 1,485 nt, median = 1,142 nt, Stdev. = 1,404 nt (fig 1; Appendix 3). Of these phylogenies, 36 % also included nuclear DNA sequence data. Considering total DNA sequence applied, 66 % used < 2,000 nt, average = 1,991 nt, median = 1,485 nt, Stdev = 2,282 nt (Appendix 3).

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Appendix 2. Model of sequence evolution for each gene chosen by Modeltest (Posada and Crandall 1998). The order of genes is as they occur in the mt-genome. * indicates adjacent tRNAs that were combined in one partition and a two parameter + Γ model was applied in MrBayes.

Gene	Model
<i>trnF</i>	K80 + Γ
<i>rrnS</i>	TrN + I + Γ
<i>trnV</i>	K80 + Γ
<i>rrnL</i>	TrN + I + Γ
<i>trnL1</i>	K80 + Γ
<i>nad1</i>	HKY + I + Γ
<i>trnI</i>	K80
<i>trnQ</i> *	K80 + Γ
<i>trnM</i> *	K80 + Γ
<i>nad2</i>	TVM + I + Γ
<i>trnW</i> *	K80 + Γ
<i>trnA</i> *	K80 + Γ
<i>trnN</i> *	HKY + Γ
<i>trnC</i>	K80
<i>trnY</i>	K80 + Γ
<i>cox1</i>	HKY + I + Γ
<i>trnS2</i> *	K80 + Γ
<i>trnD</i> *	HKY + Γ
<i>cox2</i>	HKY + I + Γ
<i>trnK</i>	TrNef + Γ

<i>atp8</i>	TIM + I + Γ
<i>atp6</i>	TVM + I + Γ
<i>cox3</i>	HKY + I + Γ
<i>trnQ</i>	K80 + Γ
<i>nad3</i>	HKY + I + Γ
<i>trnR</i>	HKY
<i>nad4L</i>	HKY + Γ
<i>nad4</i>	HKY + I + Γ
<i>trnH</i> *	K80 + Γ
<i>trnS1</i> *	K80 + Γ
<i>trnL2</i> *	K80 + Γ
<i>nad5</i>	HKY + I + Γ
<i>nad6</i>	GTR + I + Γ
<i>trnE</i>	K80 + Γ
<i>cob</i>	TrN + I + Γ
<i>trnT</i> *	K80 + Γ
<i>trnP</i> *	K80 + Γ

Appendix 3. Sequence survey.

Reference	MtDNA sequence (nt)	NucDNA sequence (nt)	Total DNA sequence (nt)
Abbot and Withgott 2004	~2,500	~2,880	5,380
Appleton, McKenzie, and Christidis 2004	1,037	0	1,037
Austin, Arnold, and Jones 2004	1,086	374	1,460
Baker et al. 2004	1268	0	1,268
Balke, Ribera, and Vogler 2004	1575	0	1,575
Banford, Bermingham, and Collette 2004	2647	1042	3,689
Becerra 2004	1,464	408	1,872
Bell et al. 2004	1,330	0	1,330
Bellwood, van Herwerden, and Konow 2004	942	0	942
Bely and Wray 2004	1,224	0	1,224
Bickham et al. 2004	1140	0	1,140
Bonett and Chippindale 2004	1,818	0	1,818
Borda and Siddall 2004	~930	~2,700	3,630
Burns and Naoki 2004	1473	0	1,473
Burridge and Smolenski 2004	806	0	806
Cabrero-Sañudo and Zardoya 2004	1210	0	1,210
Caletka and McAllister 2004	967	0	967
Campbell et al. 2004	1262	0	1,262
Carranza et al. 2004	1075	0	1,075

Casey et al. 2004	1,141	0	1,141
Castoe, Doan, and Parkinson 2004	1402	408	1,810
Chapco and Litzenberger 2004	1,716	0	1,716
Chapple and Keogh 2004	696	0	696
Chapple, Keogh, and Hutchinson 2004	1196	648	1,844
Chen et al. 2004	891	1,497	2,388
Chinn and Gemmell 2004	405	0	405
Chippindale et al. 2004	1473	1525	2,998
Clements et al. 2004	1120	1218	2,338
Cook, Runck, and Conroy 2004	1,143	0	1,143
Cook and Gullan 2004	~270	~1000	1,270
Crespi and Fulton 2004	~14,000	~ 13:000	27,000
Crochet et al. 2004	1,454	0	1,454
Croucher, Oxford, and Searle 2004	868	0	868
Crow, Kanamoto, and Bernardi 2004	863	1150	2,013
Cunningham and Cherry 2004	~2050	~600	2,650
Daniels, Hamer, and Rogers 2004	913	~1500	2,413
Degnan et al. 2004	1,677	1,448	3,125
Dick et al. 2004	550	0	550
Doadrio and Carmona 2004	1140	0	1,140
Doadrio and Domínguez 2004	1,140	0	1,140
Driskell and Christidis 2004	2996	547	3,543

Duda and Palumbi 2004	~450	~1100	1,550
Engstrom, Shaffer, and McCord 2004	~1870	1063	2,933
Evans et al. 2004	2335	0	2,335
Faivovich et al. 2004	~2800	0	2,800
Faulkes et al. 2004	1,975	0	1,975
Favret and Voegtlin 2004	678	965	1,643
Franck et al. 2004	432	0	432
Fromhage, Vences, and Veith 2004	900	0	900
Gallardo et al. 2004	936	0	936
Garb, González, and Gillespie 2004	428	0	428
Gaubert et al. 2004	1,140	0	1,140
Gittenberger, Piel, and Groenenberg 2004	616	0	616
Glaubrecht and Köhler 2004	1504	0	1,504
Gómez-Zurita 2004	1238	1139	2,377
Goropashnaya, Fedorov, and Pamilo 2004	2051	0	2,051
Grande et al. 2004	1034	1279	2,313
Groombridge et al. 2004	799	0	799
Grosjean, Vences, and Dubois 2004	~1,887	~700	2,587
Hardman 2004	1,139	733	1,872
Harris et al. 2004	887	0	887
Harrison 2004	1,247	0	1,247
Hassanin and Ropiquet 2004	1722	339	2,061

Herron, Castoe, and Parkinson 2004	1140	0	1,140
Hershler and Liu 2004	1149	0	1,149
Hodges and Zamudio 2004	2510	32	2,510
Holland and Hadfield 2004	663	0	663
Hrbek et al. 2004	1141	0	1,141
Hughes and Vogler 2004	1316	1198	2,514
Hurt 2004	658	0	658
Hulsey et al. 2004	1,137	0	1,137
Huyse, Van Houdt, and Volckaert 2004	980	614	1,594
Ikejima et al. 2004	3198	0	3,198
Inbar, Wink, and Wool 2004	1952	0	1,952
Ingram, Burda, and Honeycutt 2004	1050	986	2,036
Jaarola et al. 2004	1140	0	1,140
Janda, Folková, and Zrzav 2004	587	0	587
Jordal, Kirkendall, and Harkestad 2004	675	828	1,503
Jordal and Hewitt 2004	946	1614	2,560
Joseph et al. 2004	842	0	842
Jousselin, van Noort, and Greeff 2004	469	480	949
Juste et al. 2004	1063	0	1,063
Kandul et al. 2004	1969	1195	3,164
Kankare and Shaw 2004	~2150	~250	2,400
Kawakita et al. 2004	1325	1263	2,588

Kennedy and Spencer 2004	1,756	0	1,756
Kergoat, Delobel, and Silvain 2004	2206	0	2,206
Ketmaier et al. 2004	1140	0	1,140
Kirkendale and Meyer 2004	1132	0	1,132
Kizirian et al. 2004	1949	0	1,949
Klanten et al. 2004	913	419	1,332
Koblmüller, Salzburger, and Sturmbauer 2004	1,814	0	1,814
Köhler et al. 2004	924	0	924
Kojima, Fujikura, and Okutani 2004	501	0	501
Kotlik, Bogutskaya, and Ekmekçi 2004	1,141	0	1,141
Kronauer, Hölldobler, and Gadau 2004	1534	0	1,534
Kvist et al. 2004	578	0	578
Lavery et al. 2004	888	0	888
Lavrenchenko et al. 2004	902	0	902
Le Goff-Vitry, Rogers, and Baglow 2004	565	0	565
Lijtmaer et al. 2004	498	0	498
Lin, Danforth, and Wood 2004	2235	373	2,608
Liu et al. 2004	1140	0	1,140
Lörz and Held 2004	553	0	553
Luo et al. 2004	1143	0	1,143
Mabuchi, Nakabo, and Nishida 2004	1442	0	1,442
Macey et al. 2004	11,946	0	11,946

Machordom and Macpherson 2004	1203	0	1,203
Mahoney 2004	1,734	0	1,734
Malhotra and Thorpe 2004	2403	0	2,403
Matthee, Tilbury, and Townsend 2004	1346	2799	4,145
Matthee et al. 2004	1882	3601	5,483
Megens et al. 2004	1778	393	2,171
Melville, Schulte, and Larson 2004	1646	0	1,646
Michel-Salzat, Cameron, and Oliveira 2004	1324	1175	2,499
Morando et al. 2004	713	0	713
Moriarty and Cannatella 2004	2,333	0	2,333
Morrison, Rios, and Duffy 2004	1,067	0	1,067
Moyle 2004	1,045	938	1,983
Munasinghe, Burrridge, and Austin 2004	910	0	910
Mundy and Helbig 2004	203	0	203
Near, Pesavento, and Cheng 2004	1764	0	1,764
Near, Bolnick, and Wainwright 2004	1047	~1200	2,247
Near and Benard 2004	2187	0	2,187
Niehuis and Wägele 2004	900	0	900
Norris et al. 2004	2031	0	2,031
Orrell and Carpenter 2004	1761	0	1,761
Overton and Rhoads 2004	1920	591	2,511
Page et al. 2004	853	347	1,200

Park et al. 2004	~780	~750	1,530
Parra-Olea, García-París, and Wake 2004	1,196	0	1,196
Passamonti, Mantovani, and Scali 2004	639	0	639
Pauly, Hillis, and Cannatella 2004	2370	0	2,370
Peng, He, and Zhang 2004	1138	0	1,138
Percy, Page, and Cronk 2004	961	0	961
Pérez-Losada et al. 2004	2502	3099	5,601
Pérez-Losada, Høeg, and Crandall 2004	872	3999	4,871
Pfeiffer, Brenig, and Kutschera 2004	663	0	663
Pinou et al. 2004	613	0	613
Pitra et al. 2004	1140	0	1,140
Ponniah and Hughes 2004	1037	0	1,037
Quek et al. 2004	565	0	565
Quenouille, Bermingham, and Planes 2004	1,989	1,500	3,489
Ren et al. 2004	432	0	432
Ribas and Miyaki 2004	2,029	0	2,029
Ribera and Vogler 2004	~1,300	0	1,300
Ribera, Nilsson, and Vogler 2004	1,300	0	1,300
Rüber et al. 2004	1140	1984	3,124
Rüber et al. 2004	2969	1479	4,448
Rundell, Holland, and Cowie 2004	663	0	663
Russello and Amato 2004	1,507	1,653	3,160

Rychel et al. 2004	2815	~1150	3,965
Schwarz et al. 2004	1698	457	2,155
Scott, Keogh, and Whiting 2004	864	0	864
Shaffer et al. 2004	397	0	397
Shimabukuro-Dias et al. 2004	1570	0	1,570
Sloss et al. 2004	2109	0	2,109
Smith and Wheeler 2004	~2000	~1400	3,400
Sorenson, Balakrishnan, and Payne 2004	6,844	2,908	9,752
Sorenson, Balakrishnan, and Payne 2004	1,527	0	1,527
Sparks 2004	1,193	0	1,193
Sparks and Smith 2004	~2600	~1700	4,300
Steinke, Albrecht, and Pfenninger 2004	906	1091	1,997
Stenson, Thorpe, and Malhotra 2004	1005	6	1,005
Stuart and Parham 2004	1,790	0	1,790
Su et al. 2004	1,069	0	1,069
Sullivan et al. 2004	1140	60	1,140
Suzuki et al. 2004	1990	2154	4,144
SwigoImageová and Kjer 2004	~900	~500	1,400
Teske, Cherry, and Matthee 2004	1,484	828	2,312
Therriault et al. 2004	987	0	987
Thomas, Wills, and Székely 2004	1,143	0	1,143
Tolley et al. 2004	1,418	0	1,418

Turon and López-Legentil 2004	617	0	617
Uit de Weerd, Piel, and Gittenberger 2004	~1300	~1250	2,550
Uva et al. 2004	667	382	1,049
Vences et al. 2004	~2,500	~350	2,850
Vences et al. 2004	391	0	391
Vila and Björklund 2004	~1,750	0	1,750
Villesen et al. 2004	984	0	984
von Rintelen et al. 2004	1498	0	1,498
Waters, O'Loughlin, and Roy 2004a	4,904	0	4,904
Waters, O'Loughlin, and Roy 2004b	3661	1437	5,098
Webb et al. 2004	1,057	0	1,057
Webster, Thomas, and McCormack 2004	334	581	915
Weckstein 2004	379	347	726
Weiblen 2004	1,774	0	1,774
Westerman, Loke, and Springer 2004	1,374	0	1,374
Wilcox et al. 2004	3097	0	3,097
Wildman et al. 2004	896	0	896
Williams and Reid 2004	1536	1413	2,949
Wilson, Glaubrecht, and Meyer 2004	1,021	0	1,021
Ye et al. 2004	1,590	0	1,590
Yoder and Yang 2004	1,824	2,603	4,427
Yu et al. 2004a	810	0	810

Yu et al. 2004b		1942	2287	4,229
Zakharov, Caterino, and Sperling 2004		2293	995	3,288
Zakharov et al. 2004		2285	1644	3,929
Zaldívar-Riverón et al. 2004		797	0	797
Zigler and Lessios 2004		640	~1700	2,340
	Average	1,485	506	1,991
	Median	1,142	0	1,485
	Stdev.	1,404	1,175	2,282