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Convergence among cave catfishes: long-branch attraction and a Bayesian relative rates test

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Abstract

Convergence has long been of interest to evolutionary biologists. Cave organisms appear to be ideal candidates for studying convergence in morphological, physiological, and developmental traits. Here we report apparent convergence in two cave-catfishes that were described on morphological grounds as congeners: *Prietella phreatophila* and *Prietella lundbergi*. We collected mitochondrial DNA sequence data from 10 species of catfishes, representing five of the seven genera in Ictaluridae, as well as seven species from a broad range of siluriform outgroups. Analysis of the sequence data under parsimony supports a monophyletic *Prietella*. However, both maximum-likelihood and Bayesian analyses support polyphyly of the genus, with *P. lundbergi* sister to *Ictalurus* and *P. phreatophila* sister to *Ameiurus*. The topological difference between parsimony and the other methods appears to result from long-branch attraction between the *Prietella* species. Similarly, the sequence data do not support several other relationships within Ictaluridae supported by morphology. We develop a new Bayesian method for examining variation in molecular rates of evolution across a phylogeny.
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1. Introduction

Distantly related species sometimes share similarities that do not reflect a common ancestry of the respective taxa. These convergent characters are well-documented for both morphological as well as molecular data sets (e.g., Bull et al., 1997; Doolittle, 1994; Gatesy et al., 2003; Stewart et al., 1988). However, despite the fact that convergence of individual character states is commonplace, convergence is rarely extensive enough to overwhelm historical signal in the context of a phylogenetic analysis (Doolittle, 1994; Wiens et al., 2003). In other words, most cases of convergence are clear; the wings of birds and the wings of bats do not mislead systematists to think that birds and bats are sister groups. However, repeated invasions into a markedly distinct environment by a related group of organisms may produce exceptions to this rule. In particular, independent lineages of troglobitic organisms may show convergent changes in a large suite of characters, which in some cases may mislead systematists about the evolutionary relationships of these species (e.g., Christiansen, 1961; Hobbs, 2001; Holsinger, 1967; Wiens et al., 2003).

Convergence on form for a given function provides biologists with opportunities to use comparative techniques to understand the causes and mechanisms that underlie morphological evolution. Examples of massive convergence in morphology thus provide a powerful opportunity for the application of comparative methods. Here we report an example of apparent massive convergence in morphology leading to the placement of two species of small, troglodytic catfish in a single genus (Ictaluridae: *Prietella*).

The family Ictaluridae is the only family of catfishes of North American freshwaters north of south-central México. Approximately 50 extant species in seven genera range from Central America to Canada (Mayden et al., 1992). Although several of the species in this

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family are among the most studied of North American fishes, three highly divergent, hypogean genera remain very poorly known. Two of these genera are monotypic: single species of *Satan* and *Trogloglanis* are found sympatrically in the Edwards Aquifer 300–600 m under the city of San Antonio, Texas, although neither species has been collected in more than two decades (Langecker and Longley, 1993; Longley and Karnei, 1979a,b; Lundberg, 1982). The third hypogean genus is *Prietella*, with two poorly known, allopatric species from northeastern México (Walsh and Gilbert, 1995). Previous morphological studies have indicated the relationships of the genera as *Ictalurus*(*Trogloglanis*(*Ameiurus*((*Noturus* + *Prietella*)(*Satan* + *Pylodictis*)))) (Lundberg, 1992). If this estimated phylogeny is correct, then there have been at least three independent, parallel invasions of underground environments by ictalurids in México and Texas. All of the hypogean species show adaptations or regressions typical of many troglobytic fishes—absence of functional eyes and pigment, lateral line reduction, changes in sensory biology, and related brain morphology and other traits (Culver et al., 1995; Lundberg and PyDaniel, 1994; Poulson, 2001). These features have apparently arisen in parallel at least three times in ictalurids (Langecker and Longley, 1993; Lundberg, 1982).

Prietella phreatophila was described in 1954 from a well at the base of the Sierra de Santa Rosa, near Múzquiz, Coahuila, México (Carranza, 1954), and was later collected from a few spring caves within about 10 km of the type locality and from one locality about 64 km WNW of the type locality (Walsh and Gilbert, 1995). More recently Hendrickson et al. (2001) reported additional localities that significantly extended the known distribution of this species. The *Prietella lundbergi* description was based on a single specimen collected in 1989 from a subsurface thermal spring in southern Tamaulipas, México (Walsh and Gilbert, 1995). Hendrickson et al. (2001) obtained four new specimens referable to this species from an isolated locality about 26 km from the type locality. Utilizing *Prietella* specimens collected by Hendrickson et al. (2001) and subsequent collections of other taxa, we carried out studies designed to contribute new independent data toward improved understanding of the phylogenetic history of the family (especially with regard to the relationships of *Prietella* to the other genera) and to test the hypothesis of monophyly of *Prietella*.

2. Methods and materials

2.1. Sample collection and DNA sequencing

Specimens of *Prietella* were collected from caves at localities indicated in Table 1 and mapped in Fig. 1. Collections were made during April, 1993 and, with the assistance of cave SCUBA divers, in March 1997. All specimens of *P. lundbergi* (locality 6) were frozen in the field in liquid nitrogen. Specimens of *P. phreatophila* were frozen in liquid nitrogen, preserved in 95% ethanol, or transported live to the lab. Specimens of other ictalurids were either obtained from colleagues or collected utilizing nets and electrofishing equipment and transported live or frozen in liquid nitrogen to the lab. All the outgoup taxa were obtained from the aquarium trade.

Table 1
Material analyzed in this study with collection catalog numbers for preserved voucher specimens at Texas Natural History Collection [TNHC—http://www.tmm.utexas.edu/tnhc] (except *A. nebulosus* at Illinois Natural History Survey [INHS - http://www.inhs.uiuc.edu/])

TNHC Numbers	Species	Localities (map number in Fig. 1)
24986	*Prietella phreatophila*	Sotano de Amezcua, Cd. Acuña, Coahuila **(1)**
25759-60	*P. phreatophila*	El Calvillo, Melchor Múzquiz, Coahuila **(2)**
25767-68	*P. lundbergi*	Nacimiento del Río Frío, Gómez Farias, Tamaulipas **(3)**
FTC*	*Ictalurus punctatus*	San Marcos State Fish Hatchery (brood stock), Hays Co., Texas
29350-1	*I. lupus*	San Felipe Creek, Del Rio, Val Verde Co., Texas
25004	*Noturus gyrinus*	Cummins Creek, Colorado Co., Texas
25772	*N. flavus*	Beaver Creek, Trempealeau County, Wisconsin
24985	*N. insignis*	Deep River, Moore Co., North Carolina
25769	*Ameiurus natalis*	Pin Oak Creek, Bastrop Co., Texas
47496	*A. nebulosus*	North Branch (Hudson River Dr.), Saratoga Co., New York
25770	*Pylodictis olivaris*	Pin Oak Creek, Bastrop Co., Texas
25773-4	*Pylodictis olivaris*	Colorado River, Bastrop Co., Texas
29347	*Ancistrus* sp. (Loricariidae)	Aquarium trade
29349	*Krytopterus minor* (Siluridae)	Aquarium trade
29345	*Pseudopimelodus* sp. (Pimelodidae)	Aquarium trade
25771	*Mystus* sp. (Bagridae)	Aquarium trade
29346	*Pangasius sutchi* (Pangasiidae)	Aquarium trade
29348	*Pimelodus pictus* (Pimelodidae)	Aquarium trade
29344	*Synodontis* sp. (Mochokidae)	Aquarium trade

Full locality details available via collection websites. * Frozen tissue voucher specimen from TNHC.

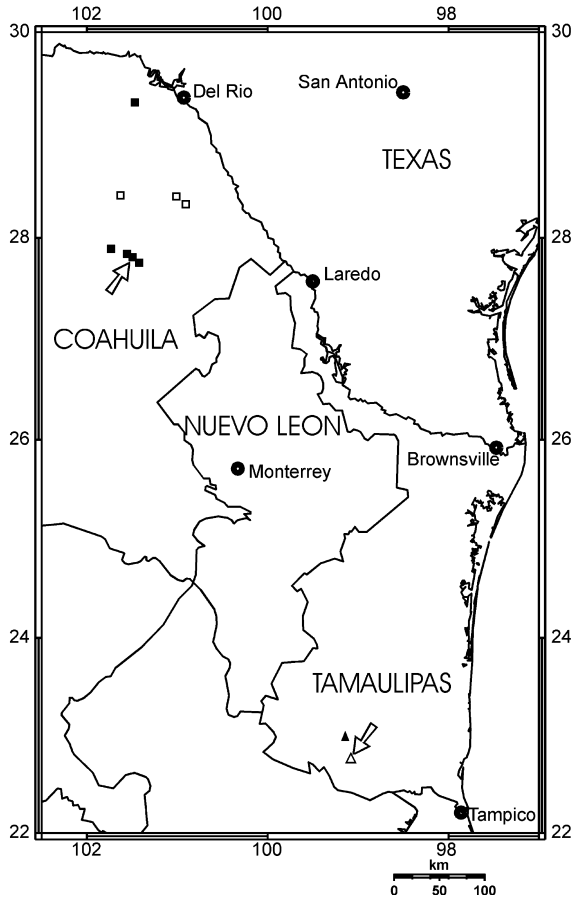

Fig. 1. Map illustrating sample sites for *Prietella* species. Arrows indicate type localities for each species. Rectangles indicate collection sites for *P. phreatophila*. Triangles represent collection sites for *P. lundbergi*. Open symbols indicate sites from which DNA samples were not obtained, but where the species occur (Hendrickson et al., 2001). Numbered localities are described in Table 1.

Vouchers of all specimens used in this study are deposited in the Texas Natural History Collection (TNHC) at The University of Texas at Austin.

Total DNA was extracted from muscle utilizing phenol–chloroform and precipitation in ethyl alcohol or using the DNeasy procedure (Qiagen). Several unsuccessful attempts were made to extract DNA from formalin-preserved specimens of both *Trogloglanis* and *Satan*.

Sequences from two mitochondrial gene regions, the entire cytochrome *b* (cyt*b*) gene plus part of the $tRNA^{pro}$ and a section covering the 12S and 16S ribosomal RNA genes, including $tRNA^{val}$, were obtained using PCR and flourescent sequencing. We amplified the entire cyt*b* gene plus most of tRNA-pro ($\geqslant$1.2 kb) using the primer pair LcGlu-F and LcPro-R. The 12S–16S section ($\geqslant$ 2 kb) was amplified using the primer pair 12L1 and 16H1 (Table 2). These regions correspond to bases 15219–16456 (CytB + tRNA-pro) and bases 1419–3461(12S–16S) in the complete mtDNA sequence of *Ictalurus punctatus* (GenBank Accession No. AF482987). Unfortunately, we could not amplify the full cyt*b* fragments from the two *P. lundbergi* samples (probably because of unique changes in the primer sites). Instead an approximately 800 bp section from the 5′ end of cyt*b* was amplified using the primers Pph01 and CB3H-15560. Using standard PCR protocols (Palumbi, 1996), we amplified each fragment with the following thermal cycles: Initial denaturation—2 min for 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 48 °C, 1 min at 72 °C, and final extension—72 °C for 7 min. Double-stranded sequences for most PCR fragments were obtained using ABI Big Dye thermal cycle sequencing with the PCR primers and other internal primers (Table 2). For the *P. lundbergi* cyt*b* fragment, we only obtained single-stranded sequence using the Pph01 primer. Sequencing products were visualized on an ABI 377 automated sequencer.

Table 2
Primers used for PCR amplification and sequencing

Primer name	Primer sequence (5′ → 3′)
LcPro-R	AATAGTTTAATTAGAATTTTAGCTTT GGGAGC
LcGlu-F	GAACCAATGACTTGAAAAACCACCG TTGT
ICTA cytb 109	TAYAAGGAAACMTGAAAYATTGG
ICTA cytb 161	GTWCAATGAATYTGAGG
Pph01	CCAACAAYGCACTAATTGATC
CB3H-15560	GGCGAATAGGAAATATCATTC
12L1	AAA AAGCTTCAAACTGGGATT AGATACCCCACTAT
12H1	TGACTGCAGAGGGTGACGGGC GGTGTGT
12sm	GGCAAGTCGTAACATGGTAAG
16sa	ATGTTTTTGGTAAACAGGCG
16sh	GCTAGACCATKATGCAAAAGGTA
16sc	GTRGGCCTAAAAGCAGCCAC
16H1	CTCCGGTCTGAACTCAGATCACG TAGG

The range of *P. phreatophila* includes a number of distinct karst systems, yet full sequences were only obtained for two specimens from the same population. To ensure that these specimens represent the variation present in *P. phreatophila*, partial cyt*b* and 12S rRNA gene sequences were obtained from 5 additional specimens collected from three other caves (Table 1). An approximately 800 bp fragment of cyt*b*, between the primers Pph01 and CB3H-15560, and an approximately 500 bp fragment of 12S rRNA, between the primers 12L1 and 12H1, were amplified and sequenced as described above. These sequences have been deposited in GenBank (Accession # AY458864–AY458899).

2.2. Phylogenetic analysis

Cytochrome *b* sequences were aligned manually using inferred codon structure, whereas the structural RNA regions were aligned using Clustal W (Thompson et al., 1994) and then manually adjusted to account for

conserved secondary structure (Cannone et al., 2002; interref http://www.rna.icmb.utexas.edu/urlhttp://www.rna. icmb.utexas.edu/). In a few hyper-variable regions of the structural RNA genes we could not confidently assign positional homology, and these regions were excluded from further analysis (the final alignment is available at http://www.treebase.org, acc ####).

Phylogenies were estimated under parsimony and maximum-likelihood criteria, as implemented in PAUP *(v4.0b8; Swofford, 1998). Parsimony analysis was done by swapping on 100 random stepwise addition trees using tree-bisection-reconnection (TBR) branch swapping. Bootstrap support (Felsenstein, 1985) was established from searches on 1000 pseudo-replicate datasets. For maximum-likelihood analysis, the GTR + Γ + PINVAR model of substitution was identified as the best-fit model using Model Test (v3.06, Posada and Crandall, 1998). Initial model parameters were estimated on the parsimony tree, and then refined by successive approximation (Swofford et al., 1996). Heuristic tree searches, with TBR branch-swapping, were conducted on five stepwise-addition trees (with random taxon addition), and bootstrap support for each clade was established from 100 replicate analyses.

Clade support was also estimated using Bayesian analysis as implemented in MrBayes (v3.0b4; Huelsenbeck and Ronquist, 2001). Model parameters (GTR + Γ + PINVAR) were estimated during the run, with the default values of 4 for the number of chains and 0.2 for the chain temperature parameter. The prior for the shape parameter of the gamma distribution was set to exp(0.8). Default priors were used for all other parameters. Each run was 1×10^6 generations, with sampling every 100 generations, and a burn-in period of 1×10^5 generations. The Bayesian posterior probability (bpp) of each clade was determined as the number of sampled trees, post-burn-in, that contained each observed bipartition (Larget and Simon, 1999). If $\geqslant$95% of the sampled trees contained a given clade, we considered it to be significantly supported by our data (Wilcox et al., 2002).

Given the results of the analysis, we felt it important to determine if the phylogenetic analysis was biased by the data missing from *P. lundbergi* (approximately 13% of the total sequence was missing). Therefore, we conducted a maximum-likelihood analysis, as described above, using only those data that were present for all species. Bayesian posterior probabilities were also estimated for this tree, using the same approach as previously described.

2.3. Hypothesis testing

We used parametric bootstrap analysis to test specific phylogenetic hypotheses (Goldman et al., 2000; Hillis et al., 1996; Huelsenbeck et al., 1996a,b). In parametric bootstrapping, a null model tree (including all nucleotide substitution parameters) is estimated from the original data, and this model is then used to simulate many replicate sets of data. Two tree searches are then conducted on each replicate dataset, once to find the optimal tree and again to find the optimal tree consistent with the null hypothesis. The difference in criterion score under each hypothesis is then determined. The resulting distribution of score differences is then used to asses the significance of a log-likelihood ratio test (LRT) between null and alternative topologies obtained from the original data. We simulated 100 replicate datasets under our null model (Appendix A) and analyzed each replicate under likelihood with the same model of molecular evolution as used in the simulations. TBR searches were performed once per dataset on a random stepwise-addition tree.

2.4. Detecting long-branch attraction

Parsimony and likelihood analyses recovered tree topologies that differed in the placement of two putatively long branches. We used the method developed by Huelsenbeck (1997) to determine if LBA is responsible for parsimony recovering monophyly of the two *Prietella* species. In this analysis, 100 replicate DNA sequence datasets, of the same dimensions as the original dataset, were simulated on two alternative trees using the same GTR + Γ + PINVAR parameters as in the original analysis: the optimal topology with *Prietella* monophyletic, and the ML topology (branches separate). Two heuristic tree searches were then performed on each replicate dataset, once under ML and once under MP. If parsimony is not being significantly misled by LBA, then less than 5% of the trees recovered from data simulated on the ML topology should place the long branches together. Similarly, likelihood should not recover topologies with the long branches separated for datasets simulated on the topology with the long branches monophyletic.

2.5. Bayesian relative rates test

Estimating rates of evolution for specific lineages has primarily been accomplished using a variation of the relative-rates test (Tajima, 1993; Wu and Li, 1985). In these tests, sequence data from an outgroup taxon (a) and two ingroup taxa (b,c) are used to generate pairwise genetic distances (d_{ij}; where i is the taxon 1, j is the taxon 2). These pairwise distances are then used to test the hypothesis that taxa b and c have been diverging from their common ancestor at an equal rate. Although these tests have been used widely, they suffer from several problems. First, if the investigator is interested in the relative rate of evolution of a clade, then clade average distances or multiple tests (with subsequent reductions

in power) are required. Second, pairwise genetic distances, even if calculated using models that account for multiple substitutions, do not account for tree structure, and thus can be very poor estimators of divergence (Swofford et al., 1996). Third, determining if a focal branch deviates significantly from the expectation of equal rates across the tree requires assumptions about the underlying distribution of branch lengths that are difficult to validate. Finally, because pairwise estimates ignore tree structure, the importance of shared history and uncertainty in topology is difficult to assess. In this paper, we present a Bayesian method for assessing branch length variation across a tree that avoids many of these problems.

The posterior probability distribution of lengths for all branches was obtained by saving branch lengths for each sampled tree during a Bayesian tree search (see above for parameter values). For each sampled tree, we then estimated the distance from the most recent common ancestor (MRCA) of our ingroup to each of the terminal taxa by summing branch lengths (Fig. 2). The ingroup MRCA is identified by outgroup comparison, and is simply the ancestral node shared by all ingroup taxa, to the exclusion of the outgroup taxa (Fig. 2). The confidence interval around each distance is the interval within which 95% of the observed distances fall. If the confidence interval around an estimated distance from the MRCA to a given taxon does not overlap with the confidence intervals for the other ingroup taxa, then we can state that the rate of evolution for that taxon is significantly slower or faster than the other taxa (Fig. 2). We conducted the analysis using two different outgroups. In the first analysis, we used all sampled outgroup taxa to identify the MRCA of the ingroup. In the second analysis, we estimated the ancestral sequence of the MRCA for the ingroup, and used this hypothetical ancestor directly as our MRCA. The hypothetical ancestor was estimated from the ML tree under the model of evolution used in the tree search (see above). Compilation of branch length estimates was done using Cadence (v1.08b; written by TPW and available at http://www.biosci.utexas.edu/antisense/).

3. Results

We collected sequences for the mitochondrial genes cytochrome *b* (cyt*b*), tRNA-pro, tRNA-val, 12S rRNA, and 16S rRNA from 18 species of catfish. Entire cyt*b* and tRNA-pro genes were obtained for all specimens except *Prietella lundbergi* and *Ancistrus sp*. For the two *P. lundbergi* samples we could only obtain 583 bp from

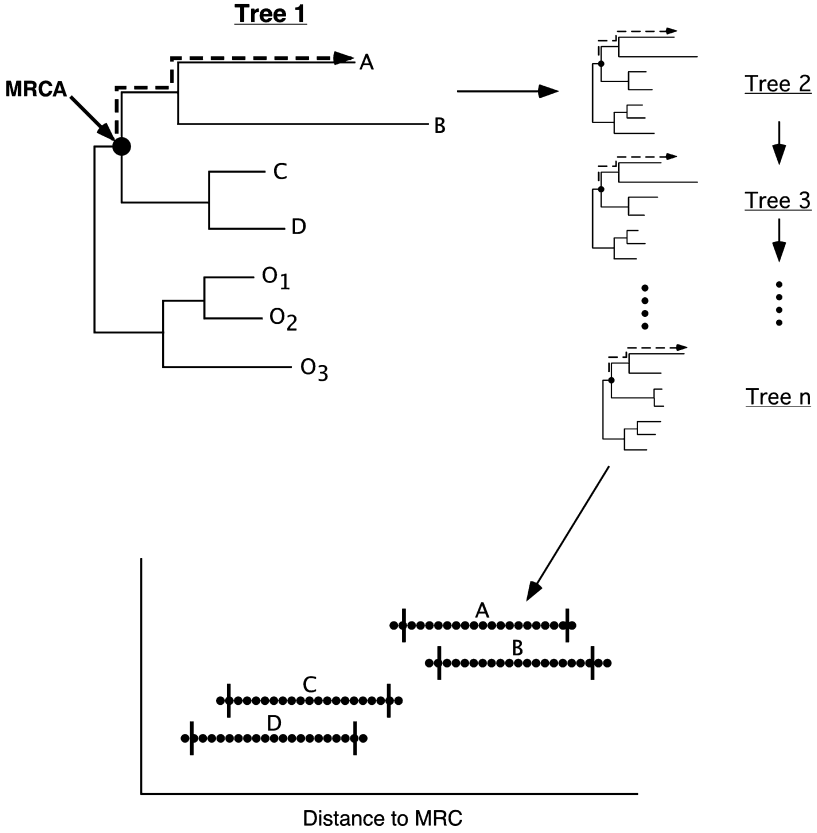

Fig. 2. Illustration of the protocol for the Bayesian relative rates test.

the 5′ end of cyt*b*, and for *Ancistrus* we are missing 116 bp (from 242 to 357 bp) near the 5′ end of the cyt*b* gene. We obtained complete sequence for the 12S–16S gene region (including tRNA-val) for all specimens. It would be optimal to have acquired the complete cyt*b* sequence for *P. lundbergi*. However, after repeated attempts to obtain the full cytb sequence, we ran out of isolated DNA, and obtaining more specimens would have required an expensive and difficult return expedition to the original cave localities.

All sequences were examined for the presence of psuedogenes. BLAST searches conducted for all sequences returned the complete mitochondrial sequence for the catfish *Ictalurus punctatus* as the sequence of highest similarity. Furthermore, highly conserved secondary structural elements were present in all rRNA and tRNA genes sampled. The cyt*b* sequences showed no frameshift or inappropriate stop codon substitutions, and the majority of the substitutions were silent (less than 15% of substitutions were nonsynonymous, even between the most distantly related sequences). Thus, by all appearances the sequences collected represent functional mitochondrial genes.

Sequences for cyt*b*, tRNA-pro, and the 12S–16S region were combined and then aligned. Alignment across the cyt*b* sequence contained no gaps (although several positions could not be resolved), while the structural rRNA regions contained several large indels. We could not confidently determine nucleotide positional homology in several regions of the structural rRNA genes due to considerable length differences among sequences. These regions, encompassing a total of 250 characters in the final alignment, were excluded from further analysis. The final alignment contained 3097 included characters, of which 1094 were variable and 806 were parsimony informative.

Single trees were recovered under maximum-likelihood (ML) and maximum-parsimony (MP) criteria (Fig. 3). Trees recovered under either criterion are similar, in that the Ictaluridae are monophyletic, as are the genera *Ictalurus*, *Noturus*, and *Ameiurus*. In both trees, *Ameiurus* is sister to all other ictalurids and *Pylodictis* is sister to *Noturus*. However, *Prietella* is monophyletic under parsimony, but polyphyletic under likelihood. Under likelihood, *P. phreatophila* is sister to *Ameiurus* ($bpp = 100$) and *P. lundbergi* is sister to *Ictalurus* ($bpp = 43$). Although the Bayesian posterior probability for a sister relationship between *P. lundbergi* and *Ictalurus* is low, no trees sampled from the MCMC chain had *Prietella* monophyletic. When maximum-likelihood analysis is performed using only data common to all sampled taxa, the identical topology is recovered. However, bpp support values for *Pylodictis* + *Noturus*, and the clade containing all ictalurids less *P. phreatophila* + *Ameiurus*, are considerably lower (Fig. 3). These lower values are largely due to instability in the position of *Pylodictis*. As in the analysis with all data included, no trees in the post burn-in MCMC chain had *Prietella* monophyletic. In the parsimony analysis, *P. phreatophila* is monophyletic and sister to a clade containing *Pylodictis*, *Ictalurus*, and *Noturus*. Parsimony bootstrap support for a monophyletic *Prietella* is very low ($npb = 47$) and the relationships among *P. phreatophila*, *Pylodictis*, *Ictalurus*, and *Noturus* are all only weakly supported (Fig. 3).

Given the level of divergence between *P. lundbergi* and *P. phreatophila*, it was important to determine if the populations of *P. phreatophila* identified in earlier surveys (Hendrickson et al., 2001) also represented cryptically diverged species. To examine this possibility, 1038 bp of mtDNA sequence, from regions overlapping cyt*b* and the rRNA genes, was obtained from five

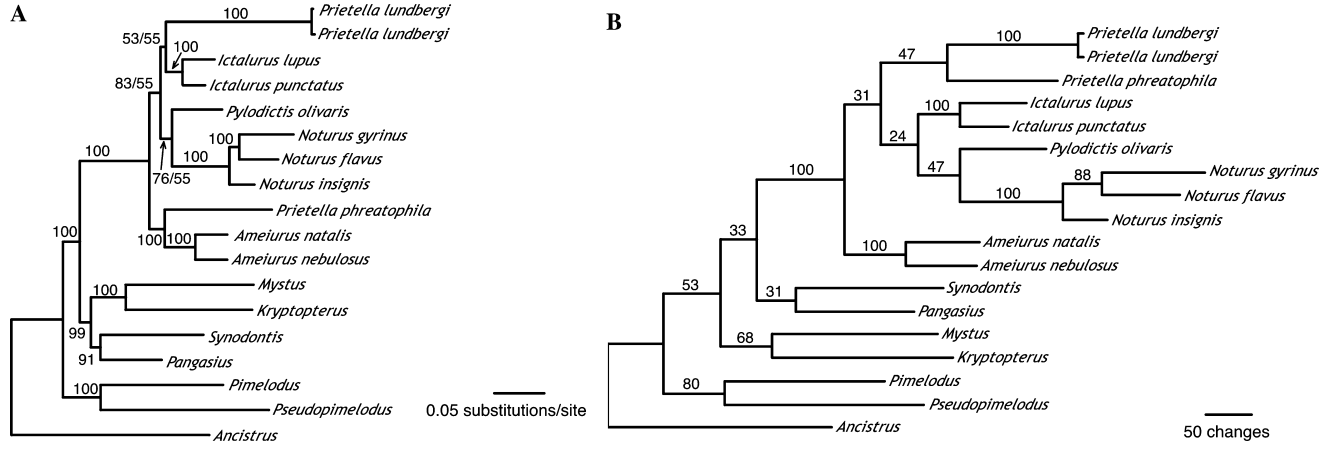

Fig. 3. Phylogenies of Ictaluridae generated from DNA sequences of the 12S and 16S rRNA and cytochrome *b* mitochondrial genes. (A) Topology recovered using Bayesian and maximum-likelihood analysis. Numbers above the branches are Bayesian posterior probabilities (bpp). Where two numbers occur, the first is the bpp obtained using the all data, the second is the bpp obtained excluding positions where *P. lundbergi* has missing data (see Section 2). (B) Topology recovered using maximum parsimony. Numbers above the branches are non-parametric bootstrap support values. See text for details of the phylogenetic analyses.

additional *P. phreatophila* specimens collected from three other caves. Divergences among these specimens plus the specimens of *P. phreatophila* already described ranged from 0.2 to 0.6%. *P. phreatophila* does have an extensive range in northern Mexico (Hendrickson et al., 2001), and our two specimens for which we have complete sequences are representative of the species.

The description of *Prietella lundbergi* (Walsh and Gilbert, 1995) did not include an explicit phylogenetic analysis. However, these authors did present many morphological characters that supported a monophyletic *Prietella* and concluded that the two species, *P. phreatophila* and *P. lundbergi*, formed a sister-species pair that together formed the sister group to the genus *Noturus*. Monophyly of *Prietella* implies either a single invasion of subterranean habitats and attainment of the current distribution of the genus through subterranean connections, or independent cave invasions by a unknown common surface ancestor. Our phylogenetic analysis does not indicate a monophyletic *Prietella*, nor support a basal position for *Ictalurus*, as indicated by the phylogenetic analysis of morphological of Lundberg (1992). We therefore conducted two likelihood ratio tests (LRT, Goldman et al., 2000; Hillis et al., 1996) to determine if our data could reject (a) monophyly of *Prietella* and (b) *Ictalurus* as sister to the other ictalurids. We excluded all outgroup taxa from the analysis of *Prietella* monophyly to reduce the chance of long-branch attraction from our outgroups artificially separating the *Prietella* species. It should be noted that removal of the outgroups does not change the topology of the optimal likelihood tree, relative to the non-monophyly of *Prietella* (data not shown).

Under likelihood, the best tree with *Prietella* monophyletic places the genus as sister to *Ameiurus* (Appendix A), and is 19 log-likelihood units worse than the optimal tree (in which *Prietella* is polyphyletic). Based on our simulated LRT null distribution, monophyly of *Prietella* can be clearly rejected (Fig. 4A, $p < 0.05$). Additionally, *Prietella* monophyly is rejected by the more conservative SH test ($p < 0.001$). The molecular data also reject a basal emergence of *Ictalurus* within Ictaluridae (Fig. 4B, LRT $p < 0.01$).

Our molecular tree differs in one other way from the morphological estimate of Lundberg (1992), which places *Pylodictis* as sister to *Prietella* plus *Noturus*. Our analysis clearly places both species of *Prietella* outside of the *Pylodictis Noturus* clade, but a statistical examination of these contrasting hypotheses is difficult to construct given the polyphyly of *Prietella*.

3.1. Long-branch attraction

When alternative methods recover different trees, it is desirable to understand the causes underlying the discrepancy. One commonly cited reason for incongruence between parsimony and likelihood estimates of phylogeny is long-branch attraction (LBA), in which superimposed changes occurring along a pair of long branches cause parsimony to join them in a group that does not reflect historical relationships (Felsenstein, 1978). When using the method of Huelsenbeck (1997), we found that parsimony recovered a monophyletic *Prietella* for 15% of the datasets simulated along the ML tree (*Prietella* not monophyletic). In contrast, likelihood never recovered a monophyletic *Prietella*. Similarly, likelihood always recovered a monophyletic *Prietella* when used to examine datasets simulated under *Prietella* monophyly (as did parsimony). Therefore, it seems likely that the discrepancy between ML and MP trees results from LBA.

3.2. Relative rates

Inspection of branch lengths on the ML tree (Fig. 3A) appear to indicate considerable variation in rates of molecular evolution among different ictalurid lineages. Indeed, a LRT between a tree constrained to ultrametricity and the optimal ML tree rejects a uniform molecular clock ($LRT = 4.5$, $p < 0.05$). We further

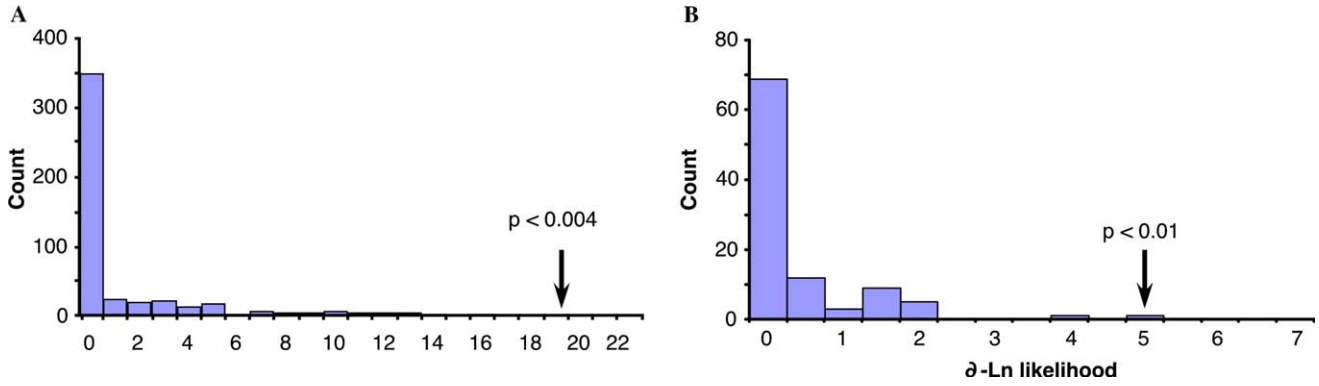

Fig. 4. Null distributions for parametric likelihood-ratio tests (LRT) generated from analysis of simulated sequences. Arrows indicate the observed value of the test statistic and the probability of observing a value at least that large. (A) Distribution for the null hypothesis of *Prietella* monophyly. (B) Distribution for the null hypothesis of a basal *Ictalurus*. See Appendix A for tree descriptions and associated parameters used in simulations.

examined the relative rates of molecular evolution among the included ictalurids using Bayesian analysis (Fig. 5). We used two different approaches for specifying the most recent common ancestor (MRCA) of the ingroup. First, for each Bayesian tree we identified the MRCA as the node connecting the ingroup to the

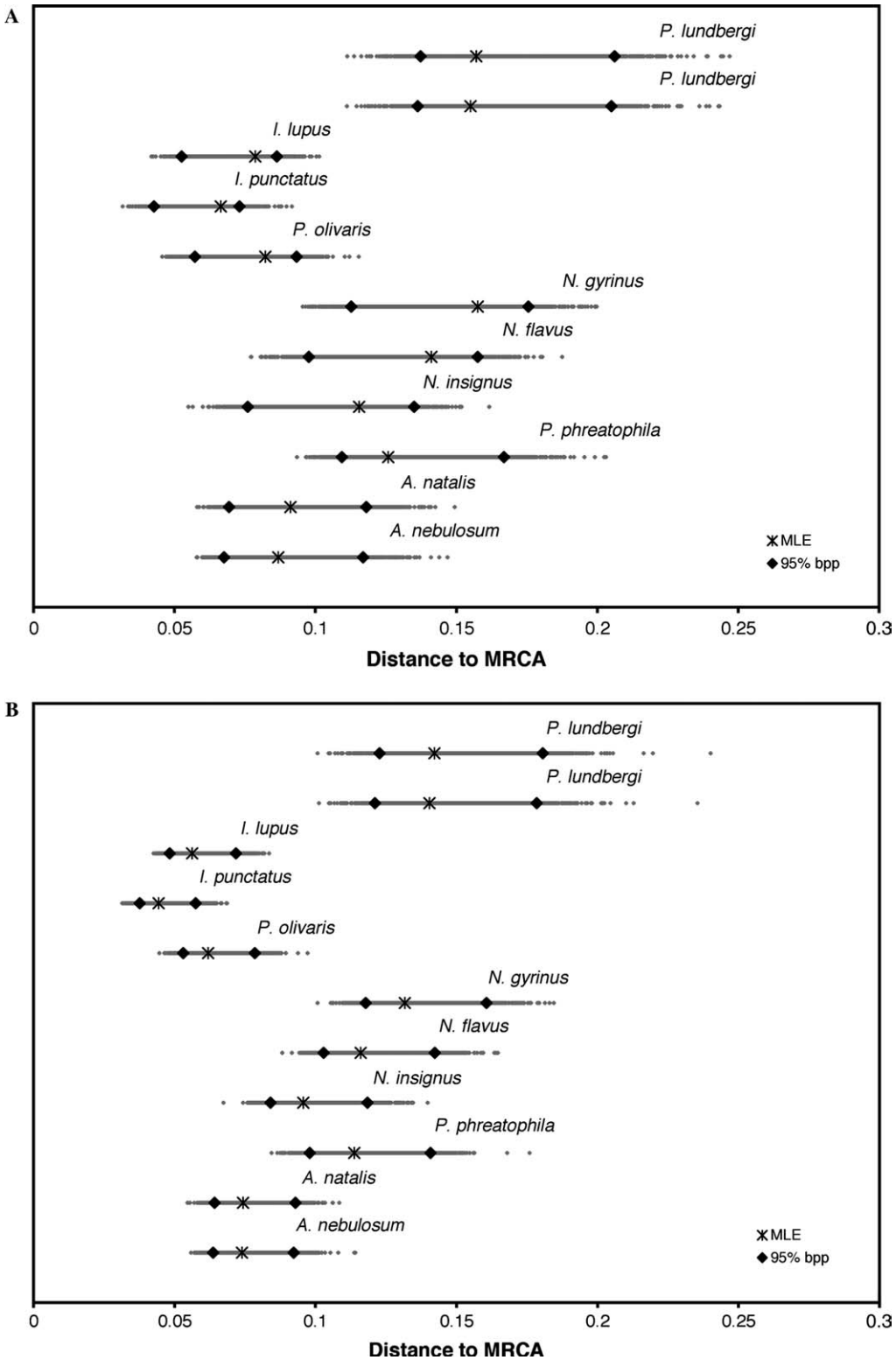

Fig. 5. Distribution of branch lengths from the most recent common ancestor (MRCA) of the included Ictaluridae and the terminal ictalurid taxa. (A) Branch Lengths estimated when the MRCA was identified relative to all included outgroup taxa. (B) When the MRCA was identified as a hypothetical ancestor of the included ictalurids. See Section 2 for further description of analysis.

outgroup. In the second analysis, we specified a hypothetical ancestor as the outgroup. The sequence for the hypothetical ancestor was estimated for the basal node of the ingroup using likelihood and the parameters described above (see phylogenetic analysis and Appendix A). We chose to use a hypothetical ancestor to examine the effect of distant outgroups on the variance in branch lengths estimated during the Bayesian analysis.

In both analyses, considerable variation in rates of evolution are apparent among the included ingroup taxa. *Prietella lundbergi* and *P. phreatophila* have some of the longest branches, whereas their sister taxa (*Ictalurus* and *Ameiurus*, respectively) have some of the shortest branches. The 95% Bayesian confidence interval around the branch lengths became considerably smaller, and rate differences exaggerated, when a hypothetical ancestor was used as the outgroup in the analysis (Fig. 5B). Unfortunately, because of the distribution of relative rates across the tree, it is not possible to determine if the ingroup taxa that have long branches represent an ancestral or derived rate of evolution. Thus, we cannot state whether the rate of molecular evolution in the blind catfish lineages has increased, or that of their close surface relatives has decreased, since they diverged.

4. Discussion

Phylogenetic analysis of ictalurid catfish mitochondrial DNA sequences clearly rejects a monophyletic *Prietella*, with *P. lundbergi* being sister to *Ictalurus* and *P. phreatophila* sister to *Ameiurus* (Fig. 3). Based on morphology, this result is quite surprising. Synapomorphies uniting the two *Prietella* species include: first lepidotrichia of pectoral and rayed dorsal fins segmented and flexible, not forming stiffened spines; dorsal spinelet (first lepidotrichium) absent; anterior cranial fontanelle and anterior portion of posterior cranial fontanelle reduced or nearly occluded by weak sutures between frontals; and extremely reduced lateral line. At least one aspect of morphology of the two blindcats is consistent with the relationships indicated by our molecular data. The emarginate caudal of *P. lundbergi*, reported by Walsh and Gilbert (1995), is consistent with relationship to *Ictalurus*, whereas the much more truncated or rounded caudal of *P. phreatophila* aligns it with *Ameiurus*. The emarginate caudal of *P. lundbergi* is even more pronounced in our specimens than illustrated by Walsh and Gilbert; the shape is very similar to all species of *Ictalurus*, and very unlike the truncated or minimally emarginate caudal of any *Ameiurus* (especially the species of *Ameiurus* that are geographically closest to *P. phreatophila*, namely *A. melas* and *A. natalis*).

Although our molecular data support our conclusions, it is possible that our phylogeny does not actually represent the true species phylogeny. First, it is possible that the mitochondrial DNA of the ancestor of extant ictalurids was polymorphic, and that our tree is the result of lineage sorting. This possibility seems highly unlikely given the ancient nature of such a hypothetical polymorphism and the strong support for the monophyly of the other included ictalurid genera. Second, it is possible that there was hybridization between surface and subterranean taxa, with subsequent introgression of surface mitochondrial DNA. Although certainly possible, this seems unlikely given the level of divergence between surface taxa and their troglobytic sister taxa. However, hybridization of less divergent blind, depigmented cave, and eyed, pigmented surface forms of *Astyanax* has long been invoked (Dowling et al., 2002; Espinasa and Borowsky, 2001; Mitchell et al., 1977; Strecker et al., 2003) to explain evolution of cave populations of this species in caves very near the localities for *P. lundbergi*. Third, it is possible that we have sequenced one or more nuclear pseudogenes of the intended mitochondrial genes, thus making our analysis inappropriate for the question. Analysis of codon structure in the protein coding cytochrome *b* gene, and of conserved secondary structure in the 12S and 16S rRNA genes obtained from *P. lundbergi and P. phreatophila* argues against these sequences representing pseudogenes. Thus, there appears to be strong evidence for two independent invasions of cave habitats in Mexico by ancestors of modern *Ameiurus* and *Ictalurus*, with subsequent convergence in morphology. However, the addition of sequence data from an appropriate nuclear gene would bolster this conclusion.

The independent origins of *P. lundbergi* and *P. phreatophila* are surprising on morphological grounds, but make considerable sense from a biogeographic perspective. There are no known hydrological connections between the karst regions inhabited by *P. lundbergi* and *P. phreatophila*. Evolving cave systems may have progressively, over geologic time, spanned distances as great as 80–100 km in highly karstic areas such as that inhabited by *P. lundbergi* and the cave *Astyanax* of Tamaulipas and San Luis Potosí (Espinasa and Borowsky, 2001). However, extension of such cave systems across the >600 km currently separating the two *Prietella* species is highly improbable. Both species of *Prietella* occur in caves developed in similar Cretaceous limestones deposited along the Tamaulipas peninsula in what is now referred to as the Sierras Tamaulipecas physiographic province that lies immediately east of, and alongside the Sierra Madre Oriental (Humphrey and Díaz, 2003). Despite similarity of the stratigraphy, simple distance and intervening major structural features combine to make subterranean connections between the two species' ranges highly improbable. Midway between the two species is the highly folded, spectacularly sharp bend of the Sierra Madre Oriental from its predominant NW-SE trend to an abrupt east–west

orientation. At many places between the two species a diverse sprinkling of Lower Cenozoic to Upper Tertiary and Quaternary igneous intrusives (Morán-Zenteno, 1994) produces large areas of warm springs. Elevated groundwater temperatures of these areas would seem likely to be barriers to subterranean movements even if connections existed. Therefore, a sister-group relationship between these two taxa would imply independent invasions of separate karst systems by a common surface ancestor. Thus, from this perspective, the independent invasion of karst systems by different ancestral taxa does not seem surprising.

The required distribution of a putative common surface ancestor of both *Prietella* species merits some discussion. *Prietella phreatophila* occurs where extant surface fishes are part of the Rio Grandean fauna that extends considerably further south to include surface drainages that now drain to the Gulf directly (Smith and Miller, 1986). Surface fish faunas in the area of *Prietella lundbergi* are quite distinctive, with far more tropical components (Miller and Smith, 1986). In the Gulf drainages, major groups such as centrarchids and percids have their southern limits well north of *P. lundbergi* in rivers with Rio Grandean affinities, and some clades of cyprinids show this southern limit. Conversely, some *Xiphophorus* and cichlids reach their northern limits in central Tamaulipas. Interestingly, in light of the contrast between our results and those from morphology, in Ictaluridae the genus *Ameiurus*, the clade that our data indicates includes *P. phreatophila*, occurs near *P. phreatophila*. The southern limit of *Ameiurus* is far north of *P. lundbergi* (Smith and Miller, 1986), but both fossil and extant *Ameiurus* are diverse in the US and Canada (Lundberg, 1992). *Prietella lundbergi* lives in aquifers that discharge to the Río Pánuco system, well within the area of México that harbors only one Ictalurid genus—*Ictalurus*, the genus with which our molecular data place *P. lundbergi*. *Ictalurus* is diverse in Mexico, but relatively depauperate to the north (Miller and Smith, 1986).

Although the morphological synapomorphies mentioned earlier that unite the two *Prietella* species have no obvious relationship to the cave environment, overall phenotypic similarity of these two species has clearly been enhanced by adaptation to caves. Generally speaking, the ecology of cave habitats is driven by the availability of surface detritus for food and the absence of light. As a result, troglobytic organisms tend to share a suite of characters, including the loss or reduction of eyes and skin pigment, reduced size, longer generation times, and slower metabolisms relative to their surface ancestors (Culver et al., 1995; Poulson, 2001). Given that many adaptations to cave living are reductions or eliminations of characters that are important for surface organisms, it is perhaps not surprising that *P. lundbergi* and *P. phreatophila* evolved similar morphologies. For example, both *Prietella* species, relative to their surface ancestors, are depigmented, have reduced eyes, smaller body size, and increased body fat. Nonetheless, the degree of similarity is surprising, and may indicate correlations in developmental pathways between characters that respond directly to selection for cave living and other, unselected characters.

Other aspects of our phylogeny are important for an understanding of ictalurid evolution in general. Similar to morphological studies, we uncovered strong support for the monophyly of the genera *Ictalurus*, *Ameiurus* and *Noturus*. Based on morphology, *Pylodictis* was found to be sister to *Prietella* plus *Noturus*. Although neither species of *Prietella* is related to *Noturus* in our molecular analysis, there is some support ($bpp = 76$) for a sister relationship between *Noturus* and *Pylodictis*.

Lundberg's (1992) analysis places *Ictalurus* as basal to all other ictalurids, yet our data reject this hypothesis. Instead, we find that *Ictalurus* + *P. lundbergi* are sister to *Noturus* + *Pylodictis*. In agreement with another recent independent analysis of ictalurid phylogeny based on molecular data (Hardman, 2002), we also find that *Ameiurus* is sister to the remaining ictalurids, although support for this latter hypothesis is weak in our study.

Significant variation in rates of molecular evolution was detected using Bayesian analysis of branch lengths. Both cave taxa show higher rates of evolution than their sister taxa. However, given the taxa sampled, determining if this is a result of an increase in the rate of evolution in the cave taxa or a decrease in their sister taxa is not currently possible. Nonetheless, it is interesting that Hardman (2002) and Hardman and Page (2003) also found (using LRT only) significant rate heterogeneity in cyt*b* and RAG2 among other ictalurids. It is clear from our study that Bayesian analysis of branch lengths is a powerful technique for examining relative rates of molecular evolution. The method has several advantages over other implementations of the test. Most importantly, the test does not rely on a single tree that is assumed correct. Both the topology and the parameters of the model of evolution are free to vary during the analysis, allowing for a robust determination of relative branch lengths. However, the method still suffers from a lack of independence among the estimated relative rates due to shared history. Therefore, this method is most appropriately applied as an exploratory and descriptive analysis of patterns in lineage rate variation. If an a priori hypothesis concerning variation among lineages in rates of evolution is being tested (e.g., homoethermy leads to faster mitochondrial sequence evolution), local-clock or similar LRTs may be more appropriate (Yang and Yoder, 2003). Such tests avoid the problems associated with shared history and lack of independence. However, they require an hypothesis derived independently of the data being analyzed (Yang and Yoder, 2003).

When a phylogenetic method is inconsistent, even infinite amounts of data will lead to an incorrect

phylogeny. Incorrect models of evolution, non-stationarity of model parameters across the lineages of interest, or large variations in branch lengths among lineages can cause methods of phylogenetic estimation to be inconsistent. One of the most frequently cited reasons for inconsistent, or at least incorrect, tree estimates is long-branch attraction (LBA), resulting from chance convergence of characters along long branches, and may be particularly problematic in molecular data where there are maximally four potential states for a given character (Felsenstein, 1978). Of the commonly used phylogenetic methods, parsimony is usually considered to be most susceptible to LBA, since there are only limited, ad hoc, ways of dealing with multiple substitutions at a given character (such as down-weighting sites thought to evolve at higher rates) (Huelsenbeck and Hillis, 1993). In this study, we suspected that the topological differences between the MP and ML trees were due to LBA. Results from simulations indicated that parsimony is favoring the incorrect topology due to LBA. This provides the first example of a case where some analyses of both molecules and morphology mislead for the same reason: spurious convergence.

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Wayne Starnes and R.S. Katula provided *Noturus insignis* and *flavus* specimens, respectively, and Michael Hardman supplied tissues and DNA of *Ameiurus nebulosus*. The San Marcos State Fish Hatchery graciously allowed us to take muscle biopsies from their broodstock of *Ictalurus punctatus*. Grant 54-E-94 from the US Mexico Foundation for Science to DAH supported collections of *Prietella*, which were permitted by the Mexican government under permits A00 700 (2).-06083; A00 700, (2).-06085; A00.700 (2) 00072; A00 700 (2).-00362; DAN-03004; DAN 02748; D000 750.-1576; 150896-213-03; and 041099-213-03, granted by SEDESOL, SEMARNAP, and Secretaria de Relaciones Exteriores. Homero Amezcua and Salvador Martinez Garza permitted collection of *Prietella* samples from caves on their properties. We also thank J. McGuire and three anonymous reviewers for their thoughtful comments on the manuscript, and Z. Yang for clarification of the 'local-clock' LRT. Finally, we thank the Instituto Tecnológico de Ciudad Victoria for having allowed FGDL to carry out the initial laboratory work at University of Texas, Austin.

Appendix A

Model parameters used in simulations (all models are GTR + continuous gamma rate heterogeneity (Γ) + invariant sites (PINVAR)):

1. Maximum-likelihood model estimate on best ML tree (−ln L 17551.94):

Rate matrix:	A → C:	A → G:	A→T:
	4.166	11.104	3.448
		C→G:	C→ T:
		0.557	33.791
			G→ T:
			1.0000

Base frequencies: A:0.3137, C:0.2855, G:0.1830, T:0.2177
Shape parameter for gamma distribution: 0.584
Proportion of invariant sites: 0.454

2. Model estimate on best ML tree (outgroups excluded, −ln L 11373.8):

Rate matrix:	A→C:	A→G:	A→T:
	4.432	21.596	3.105
		C→G:	C→T:
		0.900	45.345
			G→T:
			1.000

Base frequencies: A:0.3033, C:0.2854, G:0.1835, T:0.2278
Shape parameter for gamma distribution: 0.820
Proportion of invariant sites: 0.522

3. Model estimate on *Prietella* monophyly tree (outgroups excluded, −ln L 11403.4):

Rate matrix:	A→C:	A→G:	A→T:
	4.235	19.949	2.924
		C→G:	C→T:
		0.803	41.900
			G→ T:
			1.000

Base frequencies: A:0.3038, C:0.2852, G:0.1830, T:0.2280
Shape parameter for gamma distribution: 0.707
Proportion of invariant sites: 0.505

4. Model estimate on *Ictalurus* basal placement (−ln L 17449.033):

Rate matrix:	A → C:	A → G:	A → T:
	4.101	10.877	3.367
		C → G:	C → T:
		0..533	33.009
			G → T:
			1.000

Base frequencies: A:0.3141, C:0.2851, G:0.1829, T:0.2179
Shape parameter for gamma distribution: 0.456
Proportion of invariant sites: 0.573
Model trees used in LRT tests

1. Test of *Prietella* monophyly (outgroups excluded):
(*Prietella lundbergi*:0, (*Prietella lundbergi*:0.003052, ((((*Ictalurus lupus*:0.031622, *Ictalurus punctatus*:0.022254): 0.025587, (*Pylodictis*:0.052044, ((*Noturus gyrinus*: 0.056880, *flavus*:0.040609):0.006937, *Noturus insignis*: 0.030510) :0.064423):0.012382):0.010114, (*Ameiurus natalis*: 0.034401, *Ameiurus nebulosus*:0.035634):0.054929): 1.731e-08, *Prietella phreatophila*:0.127534):0.144651): 0.001343).
2. Test of *Ictalurus* placement:
(((((*Prietella lundbergi*:0.001714,*Prietella lunberg* i:0.003163):0.141128, (*Pylodictis*:0.049843, ((*Noturus gyrinus*:0.054131, *Noturus flavus*:0.038378):0.008758, *Noturus insignis*:0.025996):0.056373):0.006810):0.004697, (*Prietella phreatophila*:0.104961, (*Ameiurus natalis*: 0.033080, *Ameiurus nebulosus*:0.031962):0.033897): 0.019634):0.002289, (*Ictalurus lupus*:0.031683, *Ictalurus punctatus*:0.020921): 0.020804):0.074983, (((*Mystus*: 0.127155, *Kryptopterus*:0.126190):0.035348, (*Synodontis*:0.101584, *Pangasius*:0.061296):0.009796):0.011180, ((*Pimelodus*:0.122066, *Pseudopimelodus*:0.167630): 0.036567, *Ancistrus*:0.247668): 0.014808)).

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