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Model-based approach to test hard polytomies in the *Eulaemus* clade of the most diverse South American lizard genus *Liolaemus* (Liolaemini, Squamata)

MELISA OLAVE[1]*, LUCIANO J. AVILA[1], JACK W. SITES Jr[2] and MARIANA MORANDO[1]

[1]*Centro Nacional Patagónico – Consejo Nacional de Investigaciones Científicas y Técnicas (CENPAT-CONICET), Boulevard Almirante Brown 2915, ZC: U9120ACD, Puerto Madryn, Argentina*
[2]*Department of Biology and M. L. Bean Life Science Museum, LSB, Brigham Young University, ZC: 84602, Provo, UT, USA*

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Lack of resolution in a phylogenetic tree is usually represented as a polytomy, and often adding more data (loci and taxa) resolves the species tree. These are the 'soft' polytomies, but in other cases additional data fail to resolve relationships; these are the 'hard' polytomies. This latter case is often interpreted as a simultaneous radiation of lineages in the history of a clade. Although hard polytomies are difficult to address, model-based approaches provide new tools to test these hypotheses. Here, we used a clade of 144 species of the South American lizard clade *Eulaemus* to estimate phylogenies using a traditional concatenated matrix and three species tree methods: *BEAST, BEST, and minimizing deep coalescences (MDC). The different species tree methods recovered largely discordant results, but all resolved the same polytomy (e.g. very short internodes amongst lineages and low nodal support in Bayesian methods). We simulated data sets under eight explicit evolutionary models (including hard polytomies), tested these against empirical data (a total of 14 loci), and found support for two polytomies as the most plausible hypothesis for diversification of this clade. We discuss the performance of these methods and their limitations under the challenging scenario of hard polytomies.

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ADDITIONAL KEYWORDS: deep coalescences – incomplete lineage sorting – phylogeny – species trees.

INTRODUCTION

Estimation of relationships amongst species in an evolutionary context broadly falls within the purview of the discipline of systematics (Knowles & Kubatko, 2010). Although molecular data predominate in the pursuit of estimating evolutionary histories of species, trees estimated from only a few genes may differ from the species tree (Maddison, 1997; Slowinski & Page, 1999). Recent coalescent-based approaches have been developed [e.g. *BEAST: Drummond & Rambaut, 2007; BEST: Liu & Pearl, 2007; BUCKy: Ané *et al*., 2007; minimizing deep coalescence (MDC): Maddison & Knowles, 2006; Than & Nakhleh, 2009; STELLS: Wu, 2012; STEM: Kubatko, Carstens & Knowles, 2009] to accommodate multilocus data for direct estimates of species trees. These analyses may be computationally challenging, but they overcome the idiosyncrasies of individual gene trees or traditional concatenation of genes into 'super matrices'.

Although multilocus phylogenetic studies have increased during the last decade, elucidating the evolutionary history of some relationships remains difficult. Lack of resolution in a phylogenetic tree is usually represented as a polytomy, and although adding more data (loci and taxa) may resolve the species tree in cases of 'soft' polytomies, there are other cases of 'hard' polytomies that cannot be resolved with more data. These hard polytomies identify origins of three or more

*Corresponding author. E-mail: olave@cenpat.edu.ar

branches diverging so closely together in time that few or no derived character states have evolved that clearly signal their order of appearance. In particular cases of rapid simultaneous radiations, additional data will not resolve the polytomies (McCracken & Sorenson, 2005).

In cases of hard polytomies with sufficient time for speciation to be accompanied by postradiation character divergence, individual gene trees may be well resolved but they are expected to show high incongruence with each other because of their independent allelic genealogies and patterns of inheritance and segregation. For example, if we consider three lineages (sp. 1, sp. 2, and sp. 3) sharing a most recent common ancestor (MRCA), then three different resolved topologies are possible: ((sp. 1 + sp. 2) + sp. 3), ((sp. 1 + sp. 3) + sp. 2), and ((sp. 2 + sp. 3) + sp. 1); and it is expected that, under selective neutrality, ~33% of gene trees from independent, polymorphic loci should support each of the three topologies (McCracken & Sorenson, 2005). Individual gene trees may therefore be fully resolved and well supported, and yet provide no signal of a hard polytomy.

Phylogenetic inference for clades characterized by simultaneous rapid radiations is challenging. Leaché & Rannala (2011) tested the performance of different species tree methods (BEST, BUCKy, and STEM) and traditional concatenated analyses (Bayesian and Maximum Parsimony) given different evolutionary scenarios. They found that under challenging scenarios [short internodes (τ) and large population size (N_e)] every method showed low accuracy in recovering the real phylogeny. Thus, given that a hard polytomy fits in a challenging scenario (τ tends to zero), gene tree incongruence owing to stochastic lineage sorting is likely to confound resolution of the species phylogeny (Whitfield & Lockhart, 2007), and different methods are likely to recover different phylogenies.

Some phylogenetic and phylogeographical studies have been based on explicit models of species or population divergence, in which data were simulated under alternative scenarios and statistically compared with real data (e.g. Steele & Storfer, 2006; Carstens & Richards, 2007; Knowles, Carstens & Keat, 2007; Richards, Carstens & Knowles, 2007; Audzijonyte & Vrijenhoek, 2010). Model-based approaches can accommodate complex evolutionary histories involving combinations of processes (e.g. population divergence, admixture, changes in N_e, and stochastic sorting of gene trees) and any number of populations and samples, while also offering a framework for comparing alternative species trees, estimating parameters, and computing bias and precision measures for any given scenario (e.g. Voight *et al*., 2005; Fagundes *et al*., 2007; Cornuet *et al*., 2008; Gray, Huang & Knowles, 2008; Carnaval *et al*., 2009; Hickerson *et al*., 2010; Muster *et al*., 2009). Despite these advantages of using an explicit model and its flexibility for estimating the evolutionary history of poorly known clades, this approach has not been used to test hard polytomies. In this paper we describe a model-based approach to test support for hard polytomies in the evolution of a species-rich clade of South American lizards of the genus *Liolaemus*.

The *Eulaemus* clade is a subgenus within the genus *Liolaemus*, and includes 144 recognized species (those described until the beginning of January 2013). Multiple studies have consistently recovered two large clades within *Eulaemus*, including the *lineomaculatus* and *montanus* sections (Schulte *et al*., 2000; Morando *et al*., 2004; Avila, Morando & Sites, 2006; Abdala, 2007; Fontanella *et al*., 2012). However, at more recent levels of divergence, there is discordance between hypotheses of phylogenetic relationships amongst the main clades within the *montanus* section (122 species). There is general consensus amongst taxonomists in the recognition of the following main clades within *Eulaemus* (Box 1): the *lineomaculatus* section (Schulte *et al*., 2000; 21 species); and several *montanus* section clades, including: the *anomalus* group (Abdala, 2007; seven species); the *montanus* group (Etheridge, 1993; 59 species); the *wiegmannii* group (Etheridge, 1995; 12 species); the *darwinii* group (Etheridge, 1993; 20 species); and the *melanops* series (Fontanella *et al*., 2012; *goestchi* group + *telsen* group, currently 24 species). As our focus here was on resolving relationships amongst the main clades within *Eulaemus*, we included some species from all of these groups, and for some of these we sampled most or all described species: the

Box 1. List of main recognized groups within the *Eulaemus* subgenus, following Etheridge (1993, 1995); Schulte *et al*. (2000); Avila *et al*. (2006); Abdala (2007); Lobo *et al*. (2010); Fontanella *et al*. (2012); Breitman *et al*. (2011, 2012, 2013)

Liolaemus
- *Eulaemus* subgenus (144 species)
  - *lineomaculatus* section (21 species)
  - *montanus* section (122 species)
    - *anomalus* group (seven species)
    - *montanus* group (59 species)
    - *wiegmannii* group (12 species)
    - *darwinii* group (20 species)
    - *melanops* series [= *goestchi* group + *telsen* group (Abdala, 2007); 24 species]
      - *boulengeri* complex (five species)
      - *donosobarrosi* group (five species)
      - *fitzingerii* group (nine species)
      - *rothi* complex (five species)

lineomaculatus section and the *melanops* series (*boulengeri* complex + *rothi* complex + *donosobarrosi* group + *fitzingerii* group; Avila *et al*., 2006), and for others we included only some representative species: *anomalus* group (three species), *wiegmannii* group (seven species), *darwinii* group (seven species), and *montanus* group (seven species); and several candidate species.

Most recently, Fontanella *et al*. (2012) published a phylogenetic tree showing patterns of short internodes and unresolved relationships amongst some of the main clades listed above. Although these authors did not mention a hard polytomy as one possible explanation for their short internodes, this is certainly a viable hypothesis. The fact that this alternative has not been formally proposed makes the genus *Liolaemus* an ideal clade for statistical tests of a simultaneous radiation of lineages. In this study we employed a total of 14 loci to estimate phylogenies using a traditional concatenated matrix, as well as three species tree methods (*BEAST, BEST, and MDC), and tested eight explicit evolutionary models (including hard polytomies models) against empirical data, in order to explain *Eulaemus* evolution history. We discuss the performance of the methods employed and their limitations under this challenging scenario.

MATERIAL AND METHODS

Field sampling and lab work

We included samples used in the taxonomically focused study of Olave *et al*. (2014), which presents the most densely sampled molecular phylogeny of the *Eulaemus* clade currently available, in terms of taxa and loci. Olave *et al*. (2014) focused on the relationships at the species level using species tree methods, whereas here we focused on relationships amongst the main groups by testing explicit alternative models. We included a total of 188 terminals of the subgenera *Eulaemus* and *Liolaemus* (*sensu stricto*), sampled mostly from Argentina, but with a small number from Chile and Brazil. Our ingroup included one to three individuals from 108 described species and 34 candidate species (as defined by Morando, Avila & Sites, 2003) of *Eulaemus*. We used two species of *Liolaemus sensu stricto* as outgroups: *L. petrophilus* and *L. bibronii*.

We included two mitochondrial loci, four anonymous nuclear loci and eight nuclear protein-coding loci, giving a total of 14 loci.

Phylogenetic analyses

We explored the phylogenetic signal of the 14 loci included in the analyses with two different methods. We used a likelihood-mapping algorithm (Strimmer & Von Haeseler, 1997) included in the TREE-PUZZLE software (Schmidt *et al*., 2002), which has been suggested (Whitfield & Lockhart, 2007) as a useful method to evaluate phylogenetic signal in sequence data. We also performed a statistical test developed by Xia *et al*. (2003), as implemented in the DAMBE software (Xia & Xia, 2001), to explore locus informativeness. This method estimates the probability of locus saturation and gives two index values (Iss and Iss.c); when Iss < Iss.c the locus is considered to have phylogenetic signal and thus to be informative for phylogenetic analyses.

We estimated individual gene trees and a concatenated matrix phylogeny using MrBayes v. 3.2 (Ronquist & Huelsenbeck, 2003), and then estimated species trees using three different approaches: MDC using the dynamic programming algorithm implemented in the PhyloNet package (Than & Nakhleh, 2010); *BEAST 1.6.2 (Drummond & Rambaut, 2007); and BEST 2.3.1 (Liu & Pearl, 2007). Some analyses of our full matrix (188 terminals, 14 loci) failed to converge (see Method performance and limitations section below); we therefore only ran the full matrix using traditional concatenated and MDC species tree approaches, and we ran Bayesian species tree estimation methods (i.e. *BEAST and BEST) using a reduced matrix. Based on results of the full matrix analyses, we selected representatives [two species, two individuals per species (Camargo *et al*., 2012)] from each well-supported clade, and implemented *BEAST and BEST analyses on this submatrix (40 individuals representing 20 species). Note that we did not assume the monophyly of each main clade *a priori*, but we tested for this using concatenated and MDC approaches.

Gene trees

We conducted Bayesian analyses with four independent runs and two chains per run for 10×10^6 generations of Markov Chain Monte Carlo (MCMC) and sampled at intervals of 1000 generations with a burn-in of the first 25% generations for each gene alignment. These gene trees were used as the input files to perform MDC analyses.

Eulaemus *phylogeny*

We ran a Bayesian analysis in MrBayes v. 3.2 with the concatenated matrix (14 loci, 188 taxa, 8808 bp) for 10×10^6 generations of MCMC with two independent runs and four chains per run, sampling every 1000 generations with a burn-in of the first 25% generations. After we performed independent runs for each gene tree (Gene trees), we conducted a MDC analysis with this same matrix.

Eulaemus *clade relationships*

Representatives of the main clades were selected from the MDC and concatenated matrices results (188 taxa, 14 loci). We selected two individuals per species, two species per group/complex (for a total of

40 terminals) as follows: (1) the *L. lineomaculatus* section = *L. magellanicus*, *L. baguali*; (2) *L. wiegmannii* group = *L. multimaculatus*, *L. wiegmannii*; (3) *L. darwinii* group = *L. ornatus*, *L. grosseorum*; (4) *L. anomalus* group = *L. lentus*, *L. pseudoanomalus*; (5) *L. montanus* group = *L. andinus*, *L. famatinae*; (6) *L. rothi* complex = *L. rothi*, *L. sagei*; (7) *L. boulengeri* complex = *L. boulengeri*, *L. senguer*; (8) *L. fitzingerii* group = *L. canqueli*, *L. melanops*; (9) *L. donosobarrosi* group = *L. puelche*, *L. donosobarrosi*; and the outgroup = *L. petrophilus*, *L. bibronii*. We ran *BEAST for 500×10^6 generations of MCMC and sampled at intervals of 50 000 generations (burn-in 10%), using 14 loci. We also ran BEST to estimate a species tree using this matrix. We could not obtain high effective sample size (ESS) values in multiple runs with the full data set and different θ values (= 0.3653, calculated from data following BEST 2.3 Manual; and = 0.3; = 0.03; = 0.003; following Leaché & Rannala, 2011; see Method performance and limitations section below). However, we did obtain good ESS values (> 200) using only nuclear genes (12 loci) and four independent runs with two chains per run, 65.5×10^6 generations MCMC, sampling every 1000 generations, θ = 0.3, α = 3, and burn-in 10%. For both Bayesian species tree methods (*BEAST and BEST) we specified 12S and cytochrome *b* (cyt-b) sequences as mitochondrial genes, as well as autosomal and diploid for all nuclear genes.

Divergence times and substitution rates estimations

We obtained a mutation rate for each locus using the *Eulaemus* 'main clades' submatrix and divergence times of each lineage. Following Breitman *et al*. (2011) and Fontanella *et al*. (2012), we calibrated the *Eulaemus* clade using a fossil (Albino, 2008) dated at 20 Mya, to date the divergence between *L.* (*sensu stricto*) and *Eulaemus*, using a lognormal distribution and a standard deviation of 0.13 (24.56–16.01) following the recommendation of Ho (2007). This analysis also estimates the substitution rate of each locus.

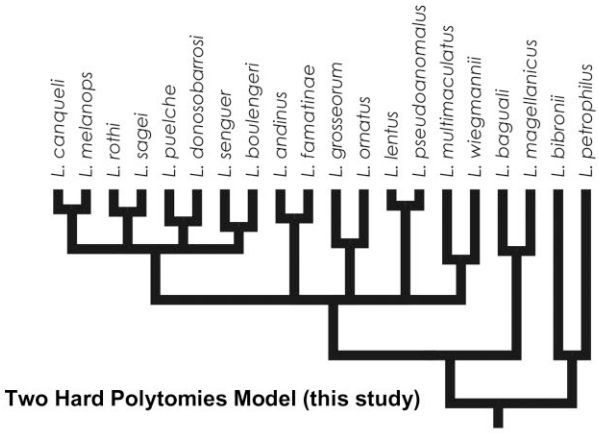
Model-based approach and hypothesis testing

We took phylogenetic hypotheses previously proposed to explain *Eulaemus* evolution (Fig. 1) and used them as models to statistically test the fit of the data against the probability distribution of expected deep coalescence generated by simulated gene trees given each particular model's parameters (Fig. 2). In this context, each of the phylogenetic hypotheses represents a model, and use of this word throughout the paper refers to the set of parameters that comprise a particular hypothesis (i.e. branch length, the topology of a particular phylogenetic tree, and the evolution model for each locus). Thus, eight different models of *Eulaemus* relationships (Fig. 1) were constructed to test alternative hypotheses for diversification histories for this clade. Five of these models were based on published hypotheses: (1) Fontanella *et al*. (2012) (two mtDNA gene regions + two nuclear loci, 2153 bp); (2 and 3) Abdala (2007) [morphology (128 characters) and morphology + mtDNA (1776 bp)]; (4) Avila *et al*. (2006) [three mtDNA gene regions + two nuclear loci, 3287 bp]; and (5) Schulte *et al*. (2000) (a mitochondrial region of 11 fragments, 1710 bp). We also constructed a model (6) based on the *BEAST results obtained here; and then (7) an hypothesis of one hard polytomy (involving all main clades of the *montanus* section), and (8) an hypothesis of two hard polytomies. The oldest of these two hard polytomies includes the *anomalus*, *darwinii*, *montanus*, and *wiegmannii* groups, and *melanops* series main clades, and the younger radiation is within the *melanops* series and includes the *boulengeri*, *rothi*, and *donosobarrosi* complexes and the *fitzingerii* group main clades (Fig. 1).

Models 6 to 8 included lineage divergence times obtained using BEAST (results in Divergence times and rates of evolution). Hard polytomies are dated from nodes where clades coalesce. We used 13 loci in this part of the analysis, including only one mitochondrial (12S) and the 12 nuclear loci (only independent loci are valid for these analyses). We selected the 12S fragment because saturation was detected for cyt-b; although it was corrected for in all phylogenetic analyses by removing the third base position (Results), it was more appropriate to work with 12S for these analyses. Procedures of modelling and hypothesis testing are illustrated in Figure 2.

We used MESQUITE 2.74 (Maddison & Maddison, 2010) to simulate 1000 gene trees for each of the eight models (phylogenetic hypotheses) proposed in Figure 1. These simulated gene trees are those expected based on all the model parameters (Fig. 2, step 1), and were constructed for each of the 13 loci from their respective character evolution models (Table 1).

Figure 1. Models of relationships. Alternative hypotheses tested in this study; the one and two rapid radiation models and the *BEAST topology from this study are also time-calibrated (Divergence times and rates of evolution). Five models were based on previously published topologies: Fontanella *et al*. (2012) (two mitochondrial loci + two nuclear loci, 2153 bp); Abdala (2007) [morphology (128 characters) and morphology + mtDNA (1776 bp)]; Avila *et al*. (2006) (three mitochondrial loci + two nuclear loci, 3287 bp); and Schulte *et al*. (2000) (mtDNA, 1710 bp).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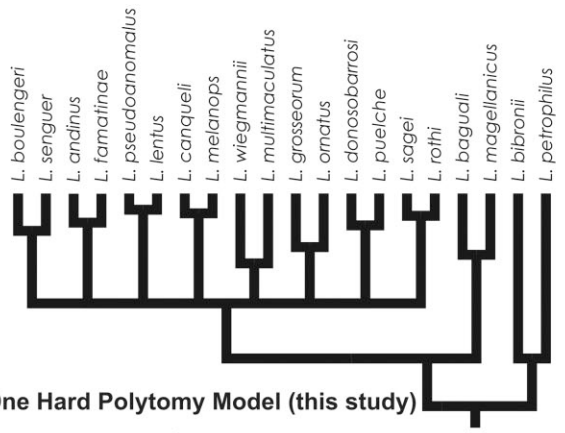
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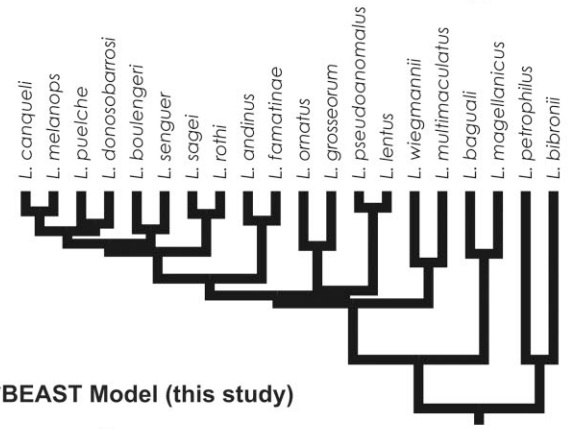
One Hard Polytomy Model (this study)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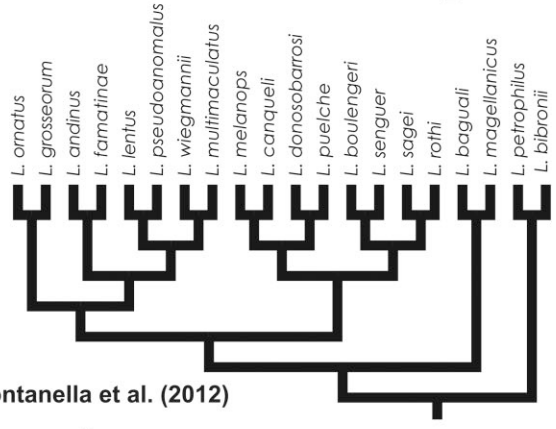
*BEAST Model (this study)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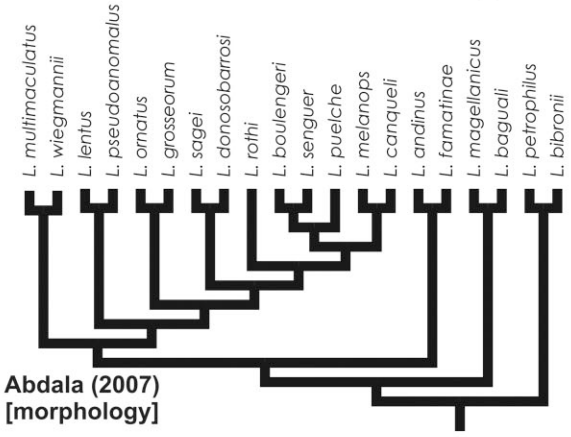
Fontanella et al. (2012)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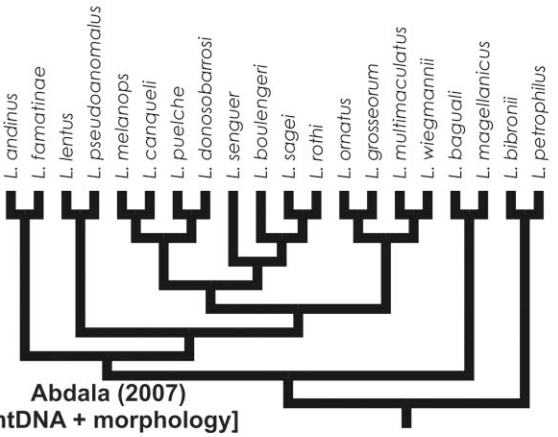
Abdala (2007)
[morphology]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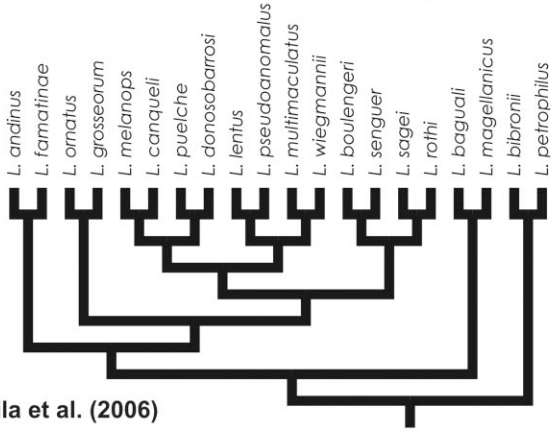
Abdala (2007)
[mtDNA + morphology]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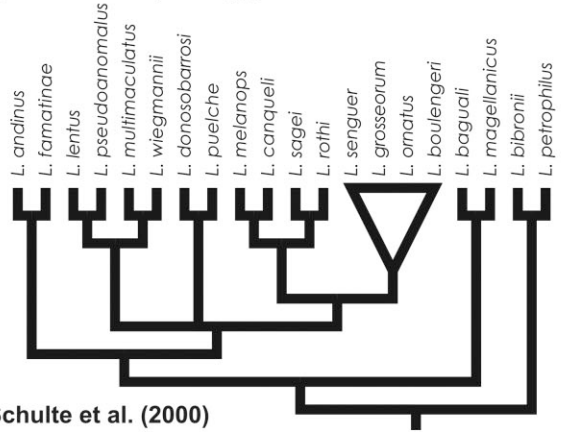
Avila et al. (2006)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Schulte et al. (2000)

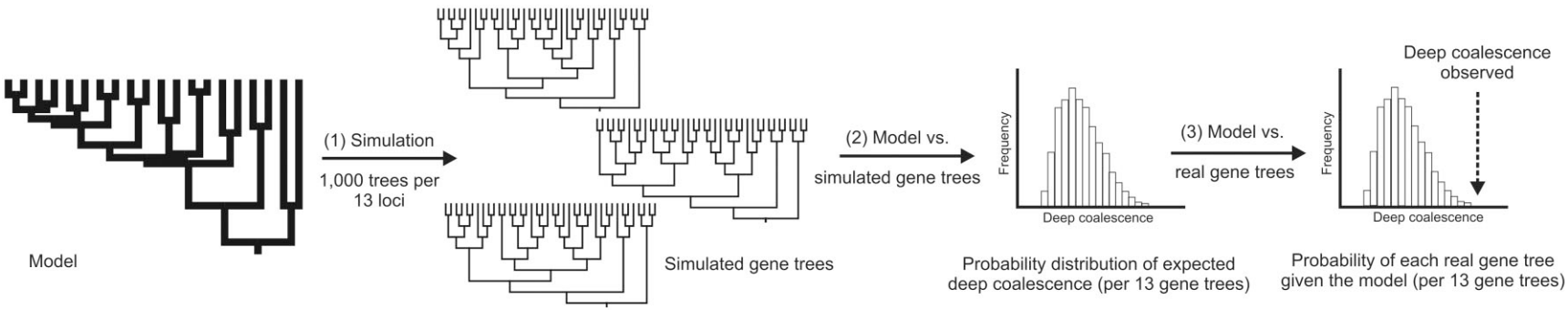

Figure 2. Description of hypothesis-testing procedures. Models were constructed in MESQUITE v. 2.74 and simulations of 1000 gene trees were generated for each of the 13 loci for each of the eight models (step 1). We then compared the simulated trees for each locus against the corresponding model in order to obtain an approximation of a probability distribution of expected deep coalescence events (step 2). Finally, we counted the number of deep coalescences of the real gene tree within the model and obtained the probability of observing that value given the model (step 3). If the empirical data fell outside the 95% confidence interval, then the hypothesized number of deep coalescence events for that gene tree (real data) is not expected under the specified model, thus rejecting that hypothesis.

Table 1. Summary of matrices used. Models of evolution were selected by the corrected Akaike information criterion

Matrix	Total length (bp)	*N*	Mitochondrial loci		ANL				NPCL							
			cyt-b	12S	A1D	A4B	A9C	A12D	CMOS	DNAH3	EXPH5	KIF24	MXRA5	PNN	PRLR	SNCAIP
Full matrices	8808	188	528 bp (1st and 2nd codon position) GTR + I + G	810 bp SYM + G	749 bp HKY + G	759 bp K80 + G	411 bp K80 + G	411 bp HKY + G	481 bp HKY + I + G	639 bp HKY + I + G	811 bp HKY + G	470 bp K80 + G	827 bp HKY + G	887 bp HKY + I + G	431 bp JC	417 bp JC + G
Eulaemus large groups phylogeny	9409	40	528 bp (1st and 2nd codon position) GTR + I + G	820 bp GTR + I + G	777 bp HKY + G	496 bp HKY + G	759 bp HKY + I	803 bp HKY + I + G	531 bp HKY + I + G	536 bp HKY + G	902 bp HKY + G	536 bp HKY + G	849 bp HKY + I	903 bp GTR + G	502 bp HKY + G	468 bp HKY + G

ANL, anonymous nuclear loci; cyt-b, cytochrome *b*; NPCL, nuclear protein-coding loci.

We then compared the 1000 simulated trees for each locus against the corresponding model in order to obtain an approximation of a probability distribution of expected deep coalescence events (Fig. 2, step 2). Deep coalescences represent the source of discord between gene trees and the species tree when the common ancestry of a gene copy at a single locus extends deeper than speciation events. Deep coalescences is calculated as the number of extra lineages by counting the discrete number of differences once the gene tree has been fitted onto the species tree (Maddison, 1997). We compared the number of deep coalescence events of the observed tree (real data) against the probability distribution of deep coalescent events. If the empirical data fell outside of the 95% confidence interval, then the hypothesized number of deep coalescence events for that gene tree (real data) is not expected under the specified model, thus rejecting that hypothesis (Fig. 2, step 3). Full and didactic tutorials to perform these analyses are explained on the webpage http://mesquiteproject.org.

RESULTS

Details of the data matrices and evolution models used in this study are shown in Table 1. Both methods employed revealed that the gene regions used in this study were phylogenetically informative. TREE-PUZZLE results showed that between 65.2 and 93.6% of each locus support the 'tree-likeness' (a well-resolved tree), indicating good phylogenetic signal in our data set. Saturation and phylogenetic signal tests both returned P-values < 0.05, as well as Iss < Iss.c, also indicating no saturation and phylogenetic signal for each locus. The single exception is cyt-b, which is saturated ($P > 0.05$), so we excluded the third base position in all phylogenetic analyses.

In all individual gene tree analyses (not shown) we observed many instances of paraphyly amongst the main clades of *Eulaemus*, with the exception of the *lineomaculatus* section, which was recovered as the sister clade of all other *Eulaemus* clades in almost all individual gene trees.

THE *EULAEMUS* PHYLOGENY (FULL MATRIX)

We recovered nine main clades using both concatenated and MDC analyses (Fig. 3A, B; main clade names: *anomalus* group, *boulengeri* complex, *darwinii* group, *donosobarrosi* complex, *fitzingerii* complex, *lineomaculatus* section, *montanus* group, *rothi* complex, *wiegmannii* group), but relationships amongst these main clades are discordant between methods.

Concatenated analysis (Fig. 3A). The MrBayes analysis of the complete concatenated matrix (188 taxa, 14 loci) recovered all of the traditionally recognized main *Eulaemus* groups with high posterior probabilities (PP = 1). The *lineomaculatus* section was recovered as sister clade of the *montanus* section. The *montanus* section had the following topology: (*montanus* group + (*anomalus* group + *wiegmannii* group)), and its sister clade was resolved as (*darwinii* group + (*rothi* complex, *boulengeri* complex + (*donosobarrosi* group + *fitzingerii* group))). Almost all of these relationships were well supported (PP > 0.95) with the exception of the unresolved positions of the *rothi* and *boulengeri* complexes (PP = 0.77).

MDC (Fig. 3B). Our MDC analysis was also based on the complete data set, and although it recovered the main traditional groups of *Eulaemus* as clades, the majority of relationships amongst these clades are different from those recovered with the concatenation approach. We again recovered the *lineomaculatus* section as the sister group to all other clades; the *anomalus* group was still recovered as the sister clade of the *wiegmannii* group, and this clade is sister to a larger clade with the following topology: ((*donosobarrosi* group + *fitzingerii* group) + ((*darwinii* group + *rothi* complex) + (*montanus* group + *boulengeri* complex))).

EULAEMUS PHYLOGENY

Using a reduced matrix of 40 taxa and 14 loci, the main clades were recovered as monophyletic using every method. However, with the exception of the *lineomaculatus* section, which was recovered as sister clade of the *montanus* section under every method, we found strong discordances amongst phylogenetic estimates of the main clades.

1. Our *BEAST analysis (Fig. 3C) recovered all of the main groups as monophyletic and with high statistical support (PP = 1), except for the *boulengeri* complex and *donosobarrosi* group, which had no support. We again recovered the *L. lineomaculatus* section (PP = 1) as the sister clade to the *L. montanus* section (PP = 1), with strong support (PP = 1). Within the *montanus* section, we recovered the *melanops* series (*rothi* complex + *boulengeri* complex + *fitzingerii* group + *donosobarrosi* group) as a well-supported clade, but relationships amongst these are not resolved (PP < 0.47), and monophyly is strongly supported only for the *fitzingerii* group and the *rothi* complex. The *wiegmannii*, *darwinii*, *anomalus*, and *montanus* groups are strongly supported as monophyletic, but relationships between these and the *melanops* series are not resolved (PP < 0.75).

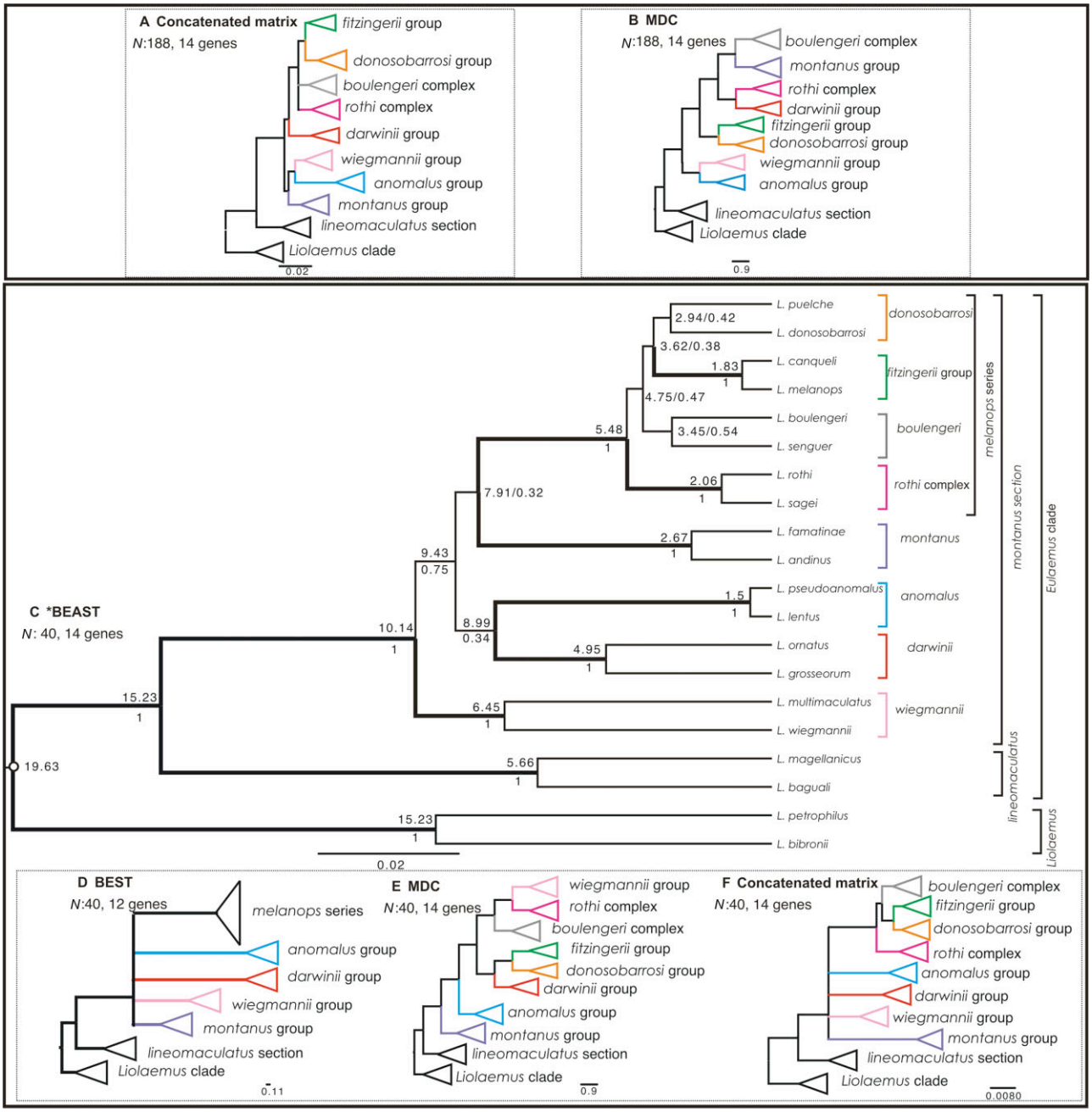

Figure 3. Empirical *Eulaemus* phylogenetic hypotheses. Phylogenetic results using the full matrix of 188 taxa (A, B) and a reduced set of 40 taxa chosen to subsample the largest clades (C to F). A, topology recovered from the concatenated matrix; B, species tree recovered by the minimizing deep coalescence (MDC) approach; C, *BEAST results of partial matrix with representatives of largest clades; values on branches are estimated divergences times (upper) and posterior probability values estimated (lower) or separated by a slash; bold branches represent nodal support > 0.90; D, BEST result; E, MDC result of partial matrix; and F, partial concatenated matrix. In (A), (D), and (F) nodes with support < 0.90 were collapsed. The calibration point for estimation of divergence times is shown with a white circle in the node corresponding to the divergence of the *Eulaemus* and *Liolaemus* clades.

2. BEST (Fig. 3D). The species tree based on 12 nuclear loci obtained with BEST is highly concordant with the one obtained using *BEAST with 14 loci (including the two mitochondrial loci). We recovered the same pattern of strongly supported main clades and the *lineomaculatus* section as the sister clade to all others, which were unresolved.
3. MDC (Fig. 3E). The *lineomaculatus* section is consistently recovered as the sister clade to all others in the *Eulaemus* clade. The *montanus* group is recovered in a basal position within the *montanus* section, and the *anomalus* group was recovered as sister of [((*darwinii* group + (*donosobarrosi* group + *fitzingerii* group)) + ((*boulengeri* complex + (*rothi* complex + *wiegmannii* group))]. This topology differs from the tree recovered by the MDC analysis of the complete data set (Fig. 3B).
4. Concatenated analysis (Fig. 3F). These results are concordant with the *BEAST and BEST trees (Fig. 3C, D), but here we recovered full resolution and higher support values for clades within the *melanops* series (PP = 1 in all cases). We obtained similar no- to low-support values for relationships amongst the *wiegmannii*, *darwinii*, *anomalus*, and *montanus* groups plus the *melanops* series.

Divergence times and rates of evolution

Our time-calibrated BEAST analyses for representatives of the *Eulaemus* clade (Fig. 3C) estimated a divergence time for the subgenera *Eulaemus* and *Liolaemus* at 19.63 Mya, and for the *L. lineomaculatus* clade at 15.23 Mya (11.63–19.33). We estimated that the *L. wiegmannii* group diverged at 10.14 Mya (7.17–13.3), and after this event we detected very short internodes (short times for speciation) for the rest of the groups. For example, the *L. anomalus* and *L. darwinii* groups diverged in less than 0.5 million years later [9.43 Mya (6.73–12.49)]. One and a half million years later the *montanus* group diverged [7.91 Mya (5.4–10.57)], and after 2.5 million years [5.48 Mya (2.19–4.79)] the *melanops* series lineages separated. This pattern of rapid speciation was also found within the *melanops* series. The mutation rates obtained for each locus are presented in Table 2.

Table 2. Mutation rates estimates for each locus in site per million years (s/Myr). Right column shows mean, followed by the SD, and below the highest probability density (HPD) of 95%

Locus	Mutation rate (s/Myr)
cyt-b	0.019355 (± 0.000034639)
	HPD: 0.013099–0.0263359
12S	0.006339 (± 0.0000095782)
	HPD: 0.0042601–0.0084861
CMOS	0.000879215 (± 0.0000025418)
	HPD: 0.00052709–0.0012697
DNAH3	0.00076162 (±0.0000023663)
	HPD: 0.0004473–0.0010827
EXPH5	0.0012955 (± 0.000002806)
	HPD: 0.00085449–0.0017507
KIF24	0.0019021 (± 0.0000035705)
	HPD: 0.0012063–0.0025878
A12D	0.0026373 (± 0.0000061488)
	HPD: 0.0015289–0.0038698
A1D	0.001765 (± 0.00000377775)
	HPD: 0.0010673–0.0025142
A4B	0.0035965 (± 0.000012311)
	HPD: 0.001806–0.0059404
A9C	0.0017753 (± 0.0000032789)
	HPD: 0.001967–0.0024185
MXRA5	0.00077525 (± 0.0000023498)
	HPD: 0.000488–0.0010812
PNN	0.00081714 (± 0.000002345)
	HPD: 0.00052669–0.001122
PRLR	0.00132228 (± 2.9225×10^{-6})
	HPD: 0.00085565–0.0018254
SNCAIP	0.0010351 (± 2.6589×10^{-6})
	HPD: 0.00063883–0.0014966

cyt-b, cytochrome *b*.

Model-based approach and hypothesis testing

The results of hypothesis testing are shown in Figure 4, and although there is some evidence to support each hypothesis, the 'two hard polytomies' model is best supported by the largest data set (nine loci). This hypothesis is the most likely one to explain *Eulaemus* evolution, followed by the one hard polytomy hypothesis (eight loci). Seven loci support the topology proposed by Schulte *et al*. (2000) and the *BEAST topology from this study. We also found that six loci supported the Avila *et al*. (2006) hypothesis, five loci supported both the Fontanella *et al*. (2012) and the mtDNA + morphology topology of Abdala (2007), and four loci supported the morphology-only hypothesis of Abdala (2007).

These results suggest that two hard polytomies is the most likely scenario to describe *Eulaemus* evolution. The pattern with partial support for all of the different proposed phylogenetic hypotheses is also expected under a hard polytomy scenario (McCracken & Sorenson, 2005).

DISCUSSION

Eulaemus phylogeny

Here we used four different methods representing different conceptual approaches to reconstruct phylogenies

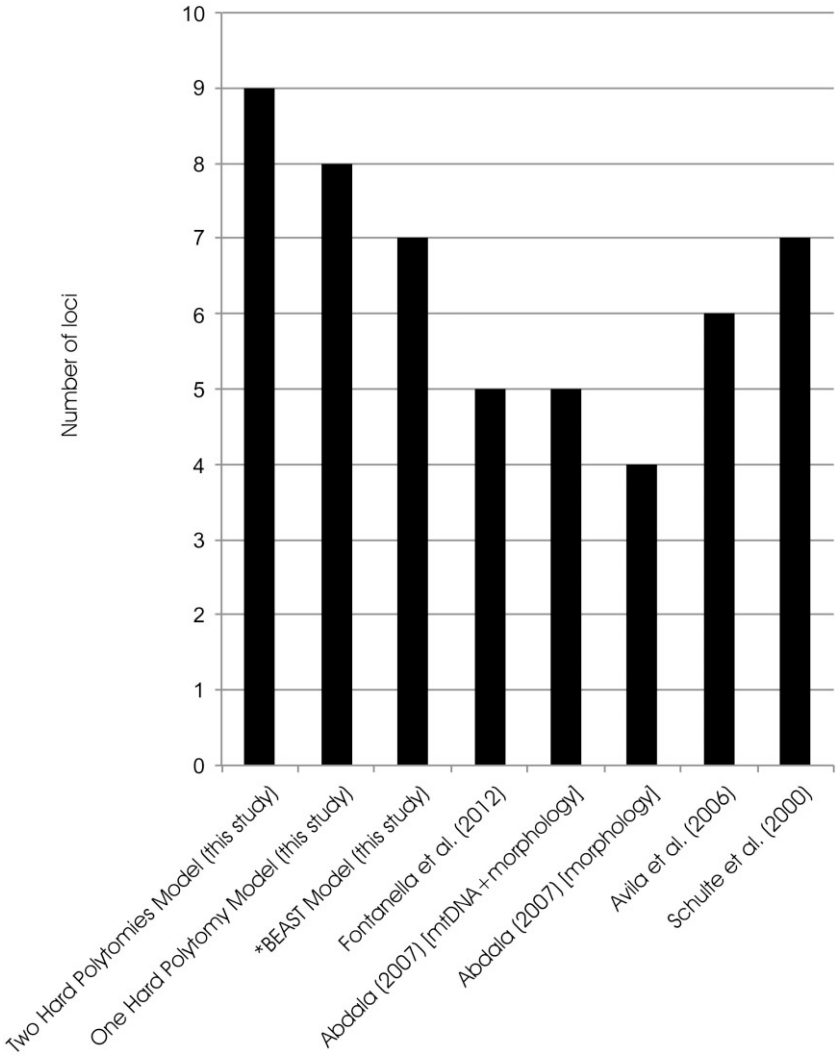

Figure 4. Simulation results. Total number of loci supporting each model after comparisons of the data against 1000 simulated data sets for each locus for each model, and compared with data. We compared the 1000 simulated trees against the model and obtained an approximation of a probability distribution of the deep coalescences expected, and then compared the number of deep coalescences on the observed tree (real data) against the probability distribution. Here, we the show number of loci that fell within the 95% confidence intervals of those probability distributions.

of the subgenus *Eulaemus*. First, we used a complete matrix of 188 terminals and 14 loci (MDC and concatenated analyses), and then selected representatives of the main clades for which we implemented two Bayesian species tree methods (*BEAST and BEST). All of our analyses strongly support the *lineomaculatus* section as the sister clade of the *montanus* section (Fig. 3A-F). We also recovered identical main clades within the *montanus* section with all four methods, with the exception of the paraphyletic *melanops* series' groups in the MDC analysis (Fig. 3E), but these were recovered as monophyletic in the MDC and the concatenated analysis for the full matrix (Fig. 3A, B). However, we did not fully resolve relationships amongst these main clades owing to short, weakly supported internodes, and the same was true for relationships amongst the main clades within the *montanus* section (Fig. 3A, C).

Previously published phylogenetic studies of the genus *Liolaemus* show extensive topological incongruence (e.g. Etheridge, 1995, 2000; Schulte *et al*., 2000; Espinoza, Wiens & Tracy, 2004; Avila *et al*., 2006; Abdala, 2007; Fontanella *et al*., 2012), with few shared strongly supported hypotheses; thus, no consensus has been reached. In this most inclusive study to date, we did not recover a strongly supported and fully resolved phylogeny, and we represent the uncertain relationships as polytomies. Although these polytomies may be re-

solved by adding more data, they may also indicate rapid radiations amongst some clades. However, before arriving at this conclusion, we need to consider that poor branch support can also be the result of: (1) insufficiently informative data; (2) data sets that strongly conflict with one another; (3) inappropriate phylogenetic methods and substitution models; or (4) insufficient data to resolve short branches (Whitfield & Lockhart, 2007).

To address point 1, we explored the informativeness of our data set using likelihood-mapping as well as saturation tests and phylogenetic signal index calculations. These analyses showed that all of the 13 loci included in our empirical analyses and simulations are phylogenetically informative, and although it was not possible to resolve the relationships amongst the main clades within the *montanus* section, our data are informative enough to resolve the oldest divergence (between the *montanus* and *lineomaculatus* sections), as well as providing high levels of resolution within each main clade (results not shown; see Olave *et al*., 2014). This suggests that our data set is sufficiently informative to resolve recent and ancient divergences, probably because we included loci with relatively high (12S and cyt-b), intermediate (KIF24, A12D, A1D, A4B, A9C), and low substitution rates (EXPH5, PRLR, SNCAIP, CMOS, DNAH3, MXRA5, PNN; see details in Table 2). In our experience with other lizard clades, some combination of these or similar loci is usually sufficient to recover well-resolved/well-supported trees (Benavides *et al*., 2009; Sinclair *et al*., 2010; Breitman *et al*., 2011; Camargo *et al*., 2012; Werneck *et al*., 2012). Further, Camargo *et al*. (2012) showed in simulation studies that the accuracy of Bayesian species tree methods is significantly higher when multiple loci of different mutation rates are used. Multiple samples per species are also necessary for successful estimation of species trees in *BEAST and although we had to reduce the number of samples per species, simulation studies show that even two samples per species are sufficient given enough loci (Heled & Drummond, 2010).

To minimize the effects of points 2 (conflicts in data) and 3 (inappropriate methods), we used three recently developed approaches (*BEAST, BEST, and MDC) that accommodate discordance amongst gene trees to estimate species trees. Finally, to address point 4 (insufficient data) we included the largest molecular data set and the most dense species sampling effort (188 terminals, 14 loci) of any phylogenetic study of this genus.

After considering all of these likely causes of poor phylogenetic reconstruction and still not resolving some polytomies, we performed statistical tests of diversification hypotheses within the *montanus* section. We tested two models with one and two hard polytomies (with estimated divergence times of 10.14 and 5.48 Mya, respectively) in the broader context of five models based on different published topologies and a sixth alternative based on our *BEAST results (Fig. 1). We found some support for all these models (Fig. 3), but the strongest support favoured the 'two hard polytomies' model (nine loci), followed by the 'one hard polytomy' model (eight loci). The models based on the Schulte *et al*. (2000) hypothesis and our *BEAST analysis were supported by seven loci, and the Avila *et al*. (2006) topology was supported by six loci.

Hard polytomies are recognized by very short internodes for which by chance every descendent lineage has the same probability of receiving one allele (McCracken & Sorenson, 2005). This implies that when multiple loci are analysed we would expect to find support for different gene tree topologies owing to a stochastic pattern of shared allele sorting amongst lineages. As the length of the internode increases, an increasing proportion of gene trees should become congruent with the species history.

Our tests suggest that two hard polytomies are the most plausible explanation for the history of this clade amongst the eight models evaluated. However, the difference between two and one hard polytomy models is only one locus, and we note that differences probably reflect the uncertainty of a real statistical difference between these results. As the 'two hard polytomies' hypothesis is the most strongly supported, and because we recovered the *melanops* series as monophyletic in most phylogenetic analyses (Fig. 3A, C, D, F) with a longer average speciation time (7.91–5.48 = 2.33 Mya interval), we accept this model as the best working hypothesis. If rapid simultaneous radiations of lineages is the true history for this clade, then the incongruence amongst previously published studies is expected; all of these studies found some well-supported topological differences amongst the main clades regardless of the method or data set used.

Phylogenetic methods are designed to locate dichotomies in trees, and until recently none was appropriate to search for a shared MRCA amongst three or more lineages. Traditional concatenated analyses also tend to inflate nodal support for dichotomies that may not be real (Belfiore, Liang & Moritz, 2008), but model-based approaches now provide new analytical possibilities (Knowles, 2009), and we designed such a test here to shed light on the evolutionary history of the *Eulaemus* clade. Our results suggest that the most plausible species tree for this clade includes two hard polytomies amongst lineages, and describes two events of rapid radiation of lineages in *Eulaemus* history. If true, then we predict that neither the inclusion of species not sampled here, nor the increase in the number of informative loci, will resolve these polytomies (Delsuc,

Brinkmann & Philippe, 2005; Rokas & Carroll, 2006; Whitfield & Lockhart, 2007).

Divergence times and geoclimatic changes

Divergence between the two *Liolaemus* subgenera (*Eulaemus–Liolaemus*) is dated to the Early Miocene (19.63 Mya), close to the beginning of the Andean uplift (~23 Mya; Ramos, 1989). Schulte *et al*. (2000) suggested that this vicariant event promoted divergence between *Eulaemus* and *Liolaemus* (*sensu stricto*). The Andean uplift then may have accelerated, causing a decrease in global temperature and several climate shifts, which probably promoted further diversification, range shifts, and extinctions. Although climatic changes caused the extinction of tropical/subtropical biotas in southern Argentina during this period (Iglesias, Artabe & Morel, 2011), the Middle Miocene later experienced a short climatic optimum with higher global temperatures (Zachos *et al*., 2001), which coincides with our divergence estimates for the *lineomaculatus* and *montanus* sections (15.23 Mya). Ectothermic species, such as lizards are critically sensitive to their ability to regulate body temperature within a narrow temperature range (Labra, Pienaar & Hansen, 2009), because their physiological performance is temperature dependent (Angilletta, Niewiarowski & Navas, 2002). For *Liolaemus* lizards it has been shown that adaptation of thermal preferences to environmental temperatures may happen rapidly although within a relatively narrow range (Labra *et al*., 2009). Thus, if the environmental temperature changes rapidly to either too high or too low, most probably this poses a serious threat to the survival of species as has been shown by Sinervo *et al*. (2010) for very rapid increases in temperature. It is possible that the climatic optimum during the Middle Miocene, may have generated an optimum temperature range for lizards, thus promoting the diversification of the *lineomaculatus* and *montanus* sections.

The earliest *Eulaemus* rapid radiation is dated to the beginning of the Late Miocene (10.14 Mya), a period during which xeric-adapted plants [Asteraceae, Chenopodiaceae, Convolvulaceae, Anacardiaceae (*Schinopsis*), Goodeniaceae, Cyperaceae, Poaceae, Fabaceae, Caesalpinioideae, and Mimosoideae] also increased in abundance and diversity (Iglesias *et al*., 2011). The second radiation within the *Eulaemus* clade is dated to the Miocene–early Pliocene transition at 5.48 Mya. During this time, in addition to strong climatic changes because of glacial cycles, several marine incursions in regions along coastlines, and regional tectonic uplift may have contributed to habitat fragmentation during this period, most probably promoting approximately simultaneous divergences between isolated populations (Rabassa, Coronato & Salemme, 2005). In southern South America, Miocene-to-Pliocene palaeoclimatic and geological events presumably imposed strong selective forces on the evolutionary histories of the southern temperate-adapted vertebrate fauna (Baez & ScillatoYané, 1979; Markgraf, McGlone & Hope, 1995), especially ectothermic species owing to their strong dependence on environmental conditions. This could be the case for *Liolaemus* lizards, perhaps driving the two rapid radiations during environmental temperature cycles that may have acted jointly with specific thermal adaptations within different clades.

Method performance and limitations

We used four different methods to estimate phylogenetic trees: PHYLONET (MDC approach) and MrBayes (concatenated matrix) with a data set of 188 terminals and 14 loci, and *BEAST and BEST using a subsampled matrix of 40 taxa and 14 loci. We found topological incongruence amongst these methods using the same matrix, as well as between the two MDC analyses based on the full matrix and a second with a reduced number of terminals.

Our BEST analyses failed to converge in some cases. We ran analyses on a reduced matrix (40 terminals, 14 loci) using different θ values (0.3653; 0.3; 0.03; 0.003), but failed to obtain MCMC convergence on any of these runs. Many BEST users experience difficulties reaching stationary values when analysing data sets exceeding approximately 50 samples. In some cases, sampling multiple individuals within species is desirable because it increases species tree accuracy (Maddison & Knowles, 2006; Liu *et al*., 2008; Heled & Drummond, 2010; Camargo *et al*., 2012), but too many samples may hinder the convergence of MCMC analyses. Leaché & Rannala (2011) also showed that user-specified θ priors have important influences on convergence, and here we had similar problems but removal of the mitochondrial regions permitted our analyses to reach adequate convergence values. Discordance amongst all gene trees was easily identified by eye, and removing the two most variable regions (cyt-b, 12S) was enough for BEST to accommodate the remaining gene tree incongruences.

By contrast, our *BEAST analyses easily recovered species trees from the smallest matrix (= 40 terminals). This program loads multiple loci and runs MCMC to estimate the posterior distribution of the species tree, and generates posterior samples from a similar model to that implemented in the widely used BEST program. Both programs require user-specified *a priori* individual species associations for all terminals, and errors in these associations have a serious impact on tree topologies. Users should be cautious in making such assessments amongst closely related but poorly delimited species (i.e. species complexes). However,

unlike BEST, *BEAST assumes randomness of the effective population sizes and places a hierarchical prior on them (Kubatko, Gibbs & Bloomquist, 2011). This allows *BEAST to work better given uncertainties of some priors (such as θ values). Further, *BEAST samples the gene trees and the species tree simultaneously, whereas BEST employs a two-stage algorithm; it first finds the marginal posterior estimates (PE) of the gene trees, and then uses an importance sampling correction to transform these marginal estimates into joint PEs. Both algorithms have the same analytical goal of estimating a species tree and associated parameters, but in practice, implementation of *BEAST is more computationally efficient than the BEST program (Kubatko *et al*., 2011). This difference allowed *BEAST to perform better than BEST with our larger data sets: *BEAST could handle 14 loci whereas BEST could not.

However, *BEAST failed to estimate a species tree with 188 terminals and 14 loci. Although this data set should not be too large for the *BEAST algorithm, the large incongruence amongst gene trees combined with the challenging scenario of unresolved polytomies may have confounded the analysis.

The MDC method implements Maddison's (1997) parsimony-based criterion for inferring species trees from gene trees by minimizing the number of extra lineages. The method does not need to specify *a priori* species associations and the output tree is fully resolved, but it is not yet possible to obtain bootstrap (or other) values of nodal support. The principal advantage of this method is that it runs quickly and can handle large data sets.

Than & Nakhleh (2009) showed that the accuracy of a species tree inferred from the MDC approach is higher than 80% when incomplete lineage sorting is low (total depth = 10 N_e), and this improves with increasing numbers of individuals and loci. However, in a worse-case scenario (total branch depth = 1N_e), at least three individuals per species and ten loci (or more than nine individuals and three loci) are needed to obtain the same accuracy. Thus, the MDC algorithm probably performed better with the full matrix (Fig. 3B) in our study than with the reduced matrices (Fig. 3E).

Concatenated analyses of independent loci estimate only a single tree (Degnan & Rosenberg, 2009) by treating the complete matrix as a supergene inherited as a linked block of sequence. By contrast, species trees inference methods take into account genealogical discord rather than forcing loci to conform to a single genealogical history (Hey & Machado, 2003; Wakeley, 2007; Kuhner, 2008). Here, we used MrBayes for tree construction of the complete concatenated matrix and a submatrix including two representatives of 20 species from the main clades (40 terminals, 14 loci). For the case of the full matrix we recovered a well-resolved tree (Fig. 3B), but we also recovered the same pattern of short internodes amongst the large groups of the *montanus* section that we recovered in our Bayesian species tree analyses. When we reduced the number of samples and ran the matrix with representatives of the main clades, we did not obtain this level of resolution (Fig. 3F). Apparently, this drastic reduction in the number of terminals reduced phylogenetic signal in the MrBayes analyses, as reflected in lower nodal support. Here, the concatenated tree shows high resolution within the *melanops* series (Fig. 3F), whereas the *BEAST and BEST analyses do not. Although concatenated data may be useful for a given clade if the species tree does not fall within the 'anomaly zone' of the parameter space (Kubatko & Degnan, 2007; see Smith, Braun & Kimball, 2013, for an empirical evaluation), here we have shown that this method does not accommodate conflicting signals amongst our gene trees (Fig. 3A). The Bayesian species tree method accommodates conflicting signals and down-weights support for those nodes, a result previously described by Belfiore *et al*. (2008).

In general, phylogenetic methods look for dichotomies in trees, and although uncertainty can be estimated with nodal support values, the probability of a hard polytomy is not tested by currently available methods. When a hard polytomy is the real evolutionary history, phylogenetic trees will include short internodes coupled with low nodal support, and conflicting topologies recovered by different methods, even with large and informative data sets. Our study shows all of these signals, and we hypothesize an evolutionary history with two hard polytomies in the *Eulaemus* radiation.

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