



Reconstructing the phylogeny of “*Buarremon*” brush-finches and near relatives (Aves, Emberizidae) from individual gene trees

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ABSTRACT

Gene trees are often assumed to be equivalent to species trees, but processes such as incomplete lineage sorting can generate incongruence among gene topologies and analyzing multilocus data in concatenated matrices can be prone to systematic errors. Accordingly, a variety of new methods have been developed to estimate species trees using multilocus data sets. Here, we apply some of these methods to reconstruct the phylogeny of *Buarremon* and near relatives, a group in which phylogenetic analyses of mitochondrial DNA sequences produced results that were inconsistent with relationships implied by a taxonomy based on variation in external phenotype. Gene genealogies obtained for seven loci (one mitochondrial, six nuclear) were varied, with some supporting and some rejecting the monophyly of *Buarremon*. Overall, our species-tree analyses tended to support a monophyletic *Buarremon*, but due to lack of congruence between methodologies, resolution of the phylogeny of this group remains uncertain. More generally, our study indicates that the number of individuals sampled can have an important effect on phylogenetic reconstruction, that the use of seven markers does not guarantee obtaining a strongly-supported species tree, and that methods for species-tree reconstruction can produce different results using the same data; these are important considerations for researchers using these new phylogenetic approaches in other systems.

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1. Introduction

The accurate reconstruction of phylogenetic relationships among species using molecular data can be complicated for a variety of reasons (Carstens and Knowles, 2007; Brumfield et al., 2008; Degnan and Rosenberg, 2009). One obstacle is the stochastic sorting of ancestral polymorphisms following species divergence at deep or shallow levels of the phylogeny, which can result in discordant topologies between gene and species trees (Pamilo and Nei, 1988; Takahata, 1989; Maddison and Knowles, 2006; McCormack et al., 2009). Furthermore, if reproductive isolation between taxa is not complete, then gene flow can cause incongruent topologies across genes (Meng and Kubatko, 2009). In addition, processes of gene duplication (Fitch, 1970) and horizontal transfer (Cummins, 1994) can also complicate the traditional assumption that gene trees always reflect species trees (Nichols, 2001). Some of these problems are particularly acute when phylogenies are reconstructed from single-locus datasets (e.g. mitochondrial DNA; Jennings and Edwards, 2005).

The ability to obtain sequence data from multiple loci across taxa is one of the major recent breakthroughs in molecular system-

atics, and has brought with it new opportunities and challenges for phylogeny reconstruction (Brito and Edwards, 2009; Knowles, 2010; Knowles and Kubatko, 2010). To date, the most common approach used in multilocus phylogenetics is concatenation of data using supermatrices, an approach assuming that all gene trees have the same topology (Rokas et al., 2003; Philippe et al., 2009). However, this approach might be positively misleading because of the existence of anomalous gene trees (i.e. gene trees that are more likely than the tree matching the species tree; Degnan and Rosenberg, 2006; Liu and Edwards, 2009). In addition, obtaining well-supported trees consistent with the true phylogeny using concatenated data might require a large number of loci in comparison to other, novel methods for species-tree reconstruction (Edwards et al., 2007; see below). Another often employed approach for phylogeny reconstruction from multilocus data is to construct consensus trees based on genealogies obtained independently for each locus (De Queiroz, 1993), but this requires a greater number of genes than concatenation to obtain a similarly supported tree (Gadagkar et al., 2005) and is also prone to be positively misleading as the number of genes increases when anomalous gene trees exist (Degnan et al., 2009). Owing to these limitations, developing alternatives to concatenation and consensus methods for the reconstruction of robust species trees has become an important priority.

Novel analytical tools have allowed a movement towards multilocus methodologies for phylogenetic reconstruction more robust

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than concatenation or consensus methods (Jennings and Edwards, 2005; Carling and Brumfield, 2008; Degnan and Rosenberg, 2009; Knowles, 2009, 2010). For example, one can attempt to reconcile gene trees contained within species trees by minimizing the number of deep coalescences, i.e., the coalescence of two gene copies that predates a particular speciation event (Maddison, 1997; Maddison and Knowles, 2006; Leaché, 2009). Alternatively, the BEST (Bayesian Estimation of Species Trees) method estimates the joint posterior distribution of gene trees for each locus and uses the resulting joint posterior distribution of gene trees to approximate the Bayesian posterior distribution of the species tree based on coalescent theory (Liu and Pearl, 2007; Edwards et al., 2007). Yet another alternative is to calculate the probability of a genealogy given a species tree under the coalescent (Degnan and Salter, 2005). Despite these developments, as the field of multilocus phylogenetics is still maturing, studies using species-tree approaches with empirical data are scarce (Brumfield et al., 2008; Liu et al., 2008; Linnen and Farrell, 2008; Hird et al., 2010; Waters et al., 2010). In this study, we use three methods of species-tree reconstruction based on multilocus data to revisit an empirical phylogenetic question of interest in systematic ornithology. In so doing, we come across some practical issues related to the effect of taxon sampling and the variation in results among methods that should be of interest to developers and users of such methods.

The genus *Buarremon* (Aves, Emberizidae), as traditionally defined, consists of three morphologically similar species: *Buarremon torquatus*, *Buarremon brunneinucha*, and *Buarremon virenticeps*. However, a recent study rejected the monophyly of the genus. Based on analyses involving sequences of four mitochondrial genes (ND2, cyt b, ATPase 6, ATPase 8) and two nuclear introns (ACOI and MUSK), the clade formed by representatives of multiple populations of *B. torquatus* was recovered as sister to the monophyletic genus *Arremon*, whereas *B. brunneinucha* and *B. virenticeps* formed a clade that was sister to a clade formed by species in the genus *Lysurus* albeit with low support (Cadena et al., 2007; Fig. 1a). This result was unexpected considering the overall phenotypic similarity of *Buarremon* taxa, but it led to the merging of the three genera in an expanded genus *Arremon* (Remsen et al., 2010). However, the results of this study were supported mainly by mitochondrial DNA sequences and the deep internodes of the mitochondrial topologies were notably shorter than the terminal branches. The existence of short internal branches can lead to retention of ancestral polymorphisms, representing one of the most difficult scenarios for inferring phylogenies from single-locus data sets owing the high stochasticity in gene sorting. Under such scenarios, mitochondrial DNA can reveal trees with good nodal support that are incongruent with the species tree (Carling and Brumfield, 2008; Leaché, 2009; McCormack et al., 2009). In addition, the mitochondrial DNA topology was at least partly inconsistent with the topologies of two nuclear introns (Cadena et al., 2007). Thus, in this study we revisit the relationships of *Buarremon* and related genera by reconstructing the species tree based on phylogenetic analyses of sequences from multiple loci. We discuss the implications of our results in relation to challenges in sampling design and in the use of different methods, which might be common to other studies seeking to reconstruct species trees using multilocus sequence data.

2. Materials and methods

2.1. Sampling, PCR amplification and sequencing

We obtained frozen tissue samples from the collections of Instituto Alexander von Humboldt (IAvH) and the Banco de Tejidos of the Museo de Historia Natural, Universidad de los Andes (ANDES-BT) for a single individual of four focal taxa (*B. torquatus* (IAvH-

1145), *B. brunneinucha* (ANDES-BT-0120), *Arremon schelegeli* (ANDES-BT-0016) and *Lysurus castaneiceps* (IAvH-CT-825)) and one outgroup (*Atlapetes latinuchus* (ANDES-BT-0130), a valid strategy considering these species are strongly supported monophyletic groups (Cadena et al., 2007). Note we did not include *B. virenticeps* in our study because this species was nested with strong support within a clade formed by populations of *B. brunneinucha* in phylogenetic analyses of mitochondrial and nuclear DNA sequences (Cadena et al., 2007). Therefore, the inclusion of this taxon (and of populations of *B. torquatus* that likely merit species status; Cadena and Cuervo, 2010) was not necessary to address the monophyly of *Buarremon*. The important question in this regard is whether the *brunneinucha-virenticeps* clade and the *torquatus* clade form a monophyletic group to the exclusion of the *Lysurus* and *Arremon* clades.

Total DNA was extracted from all samples using a DNeasy tissue kit (QIAGEN, Valencia, CA), following the manufacturer's protocol. Subsequently, we amplified six nuclear introns (four autosomal, two z-linked) and one mitochondrial protein-coding gene (Table 1) using primers published by Slade et al. (1993), Sorenson et al. (1999), and Kimball et al. (2009). The concentrations and conditions used for PCR were those described by Cadena et al. (2007). Amplicons were cleaned using Exosap IT (USB corporation, Cleveland, Ohio) and then sequenced in both directions. Resulting chromatographs were assembled in Geneious Basic 4.02. (Drummond et al., 2007). In cases where double peaks of equal height were detected in the sequence, the site was considered ambiguous (i.e. we did not attempt to phase haplotypes because sites with double peaks were scarce and because we had data for a single individual per species, which impeded haplotype estimations).

2.2. Alignment and conventional phylogenetic analyses

Sequences were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in Geneious (Drummond et al., 2007) and edited manually. Intralocus recombination was tested using the program RecombiTEST (Piganeau et al., 2004). Genealogies were reconstructed individually for each locus using maximum likelihood (ML) and Bayesian inference (BI) methods, and we also conducted analyses using a concatenated matrix that included sequences of all seven genes and a partitioned matrix specifying a substitution model for each of the seven loci. For each analysis, we implemented the model of nucleotide substitution selected as the best-fit to the data (Table 1) based on the Akaike Information Criterion using ModelTest 3.7 for ML (Posada and Crandall, 1998) and MrModelTest 2.3 for BI (Nylander, 2004). Branch-and-bound searches were conducted using PAUP* 4.0b10 (Swofford, 2002) for ML analyses; nodal support was assessed with 1000 ML heuristic bootstrap replicates with tree bisection-reconnection (TBR) branch swapping. Bayesian analyses were conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and consisted of two runs of four MCMC chains for 15,000,000 generations sampled every 1000 generations; the first 25% of the trees sampled was discarded as burn-in.

We used the program Tracer v.1.4.1 (Rambaut and Drummond, 2007) to evaluate sampling of the tree and parameter space in Bayesian analyses. Because plots of number of generations vs. likelihood showed stabilization, effective sample sizes for all parameters was always greater than 200, and the average standard deviation of split frequencies across runs was less than 0.002 in all the analyses, chains likely sampled the posterior distributions adequately. To assess convergence of MCMC runs, we plotted posterior probabilities of clades as a function of generation number and compared results of different runs by plotting the posterior probabilities of all splits for paired runs using AWTY (Wilgenbusch et al., 2004).

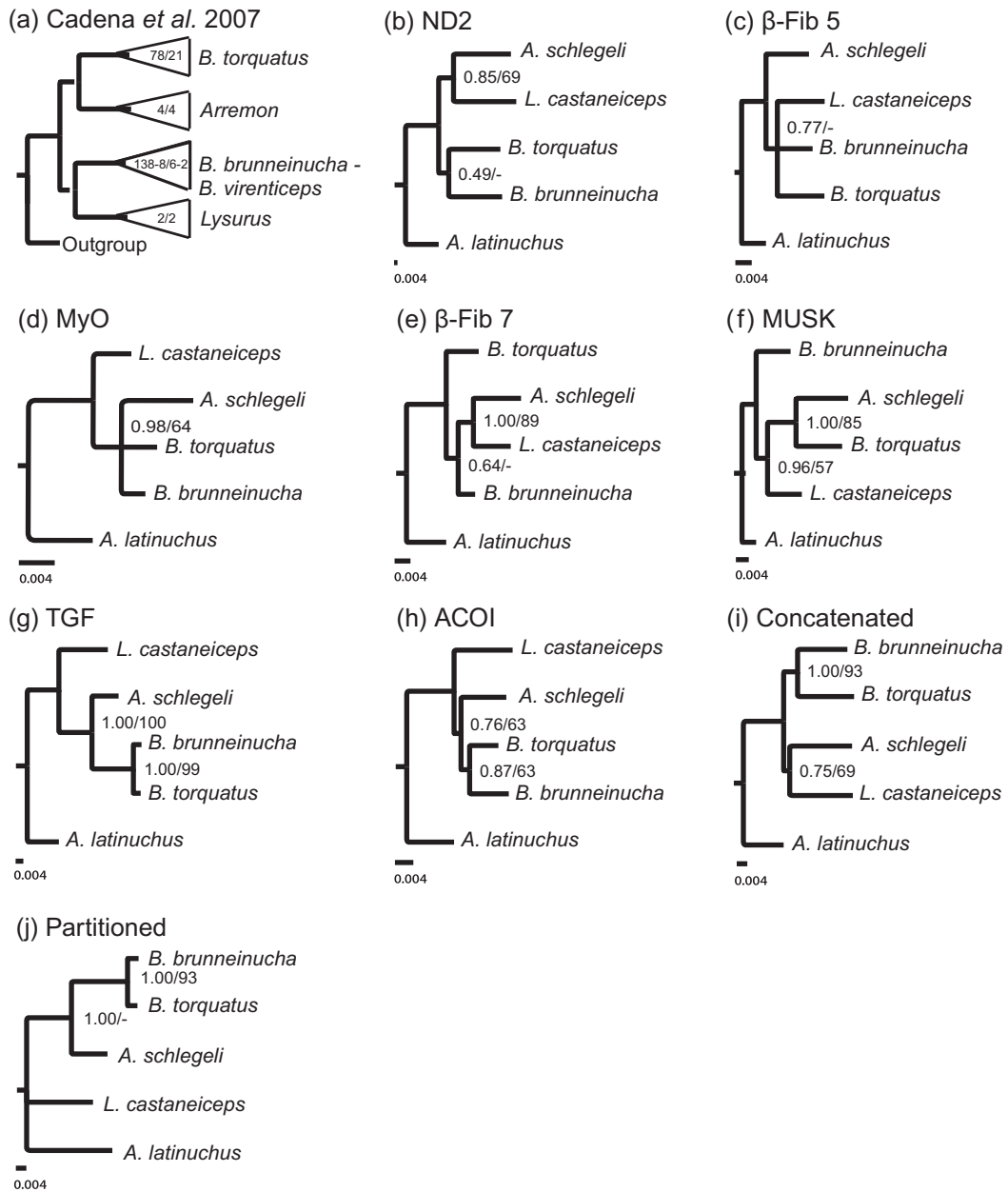


Fig. 1. Gene tree topologies obtained for each locus and for the analyses of the concatenated and partitioned matrix. Fig. 1a is a schematic topology of the results obtained for Cadena et al. (2007), values within the triangles are indicating the sampling for: ND2/Concatenated matrix of four mitochondrial DNA. From 1b–j, value *s* in the nodes correspond to posterior probability of IB/ML bootstrap.

Table 1

Summary of sequence variation and substitution models determined according to the AIC for different loci employed in phylogenetic analyses.

Locus	Length (pb)	Number of parsimony informative characters	Substitution model ^a	Substitution model ^b	Base frequency ^c	Accession number
βFib5	601	0	TVM + I	GTR + I	0.2966, 0.1779, 0.211, 0.3144	HQ537396–400
MyO	740	1	HKY	HKY	0.2686, 0.2374, 0.2191, 0.274	HQ537411–415
βFib7	998	4	TVM	GTR	0.3128, 0.1829, 0.1657, 0.338	HQ537401–405
MUSK	563	7	TVM	GTR	0.2792, 0.1712, 0.2221, 0.327	HQ537406–410
ACOI	992	2	TrN	HKY	0.2587, 0.1735, 0.208, 0.3598	HQ537391–395
TGF	546	12	GTR	GTR	0.2419, 0.2216, 0.2269, 0.309	HQ537421–425
ND2	1026	99	TrN + I	HKY + G	0.3004, 0.3727, 0.0984, 0.228	HQ537416–420
Conc	5456	125	TVM + I	GTR + I	0.2801, 0.2222, 0.187, 0.3107	

Conc = Concatenated sequences of all seven loci.

^a Substitution model in ModelTest.

^b Substitution model in MrModelTest.

^c A,C,G,T.

2.3. Minimizing deep coalescence

We inferred a species tree by reconciling each of the individual gene trees obtained from ML and BI analyses described above with the 15 possible rooted evolutionary histories for the four ingroup taxa by minimizing deep coalescence events using the program Mesquite (Maddison and Maddison, 2004; Maddison and Knowles, 2006). The total cost of each species tree was estimated summing the number of deep coalescent events over the seven genealogies; the selected species tree was the topology with the lowest score of coalescent steps summed across all genealogies.

2.4. Probabilities of gene trees given the species tree

We calculated the probability of each gene genealogy given all possible species trees - 15 species trees for 4 taxa - using COAL (Degnan and Salter, 2005). For this analysis, we considered five different internal:terminal branch length ratios (RIT) for the species trees: (1) RIT 1:1 with length (coalescence units) = 1.0, (2) RIT 1:1 with length = 0.5, (3) RIT 1:1 with length = 0.2, (4) RIT 1:100 where branch 1 has length 0.01 and other branches have length 1.0, and (5) RIT 1:100 where branch $n - 2$ (n is the number of taxa) has length 0.01 and other branches have length 1.0. We chose these options available in COAL to represent different evolutionary scenarios with internal and terminal branches of equal length (1–3), short internal branches (4) and short terminal branches (5). Because COAL requires fully resolved bifurcating topologies and the forced resolution of polytomies can bias results (Carling and Brumfield, 2008), genes resulting in unresolved topologies (MyO and β -Fib 5, see below) were not included in this analysis.

2.5. Bayesian estimation of species tree

The joint posterior distribution of gene trees and the species tree was estimated in BEST 1.6 (Liu et al., 2008). For our analysis, we employed commonly-used flat priors recommended by the authors (Liu et al., 2008), which were applied in an earlier phylogenetic study of birds (Brumfield et al., 2008): inverse gamma distribution with $\alpha = 3$ and $\beta = 0.003$, and a uniform distribution with bounded values of 0.5 and 1.5, for the prior distribution of population size and mutation rate, respectively. Three runs of two MCMC chains were performed for 200,000,000 generations with a sampling frequency of 20,000; the first 25% of trees was discarded as burn-in. Chain stationarity was assessed using Tracer v.1.4. based on plots of number of generations vs. likelihood and estimates of effective sample sizes for parameters, and convergence across runs was examined using AWTY as described above. We did not observe changes in posterior probabilities after 20 million generations and results of independent runs were remarkably similar to each other, indicating convergence.

3. Results

3.1. Conventional phylogenetic analyses

No recombination events were detected for any of the seven loci. All six nuclear loci exhibited few informative characters (Table 1), which led to unresolved gene trees in some cases (e.g. β -Fib 5 and MyO; Fig. 1c and d). However, several gene trees recovered clades with good branch support (Bayesian posterior probabilities >0.95 and maximum likelihood bootstrap values >85%). Some of these suggested paraphyly of *Buarremon* (β -Fib 7 and MUSK; Fig. 1e and f) and others supported its monophyly (TGF, ACOI; Fig. 1g and h). The mitochondrial locus (ND2) also supported monophyly of *Buarremon* (Fig. 1b), a result contrary to that ob-

tained by Cadena et al. (2007; Fig. 1a) for the same locus with a more extensive sample of individuals, but this result was not strongly supported in our analyses (see below). Some of the loci that supported the monophyly of *Buarremon* did so in different ways (e.g. *Lysurus* was found as sister to *Buarremon* in the ACOI tree, whereas TGF placed *Arremon* and *Buarremon* as sister groups) and with varying support (0.87–1.00 posterior probability and 63–99% ML bootstrap). Likewise, gene genealogies that recovered a paraphyletic *Buarremon* (e.g. β -Fib 7 and MUSK) differed topologically. Despite the variation across loci in gene-tree topologies, a partition homogeneity test did not reveal significant inconsistency in phylogenetic signal across loci. The concatenated and partitioned analyses using data from all loci showed strong support for the monophyly of *Buarremon*, but recovered different relationships of *Buarremon* with *Lysurus* and *Arremon* (Fig. 1i and j).

3.2. Species tree analysis

Buarremon was consistently recovered as a monophyletic group using the three methodologies for species-tree reconstruction. In particular, the species tree obtained by minimizing deep coalescences was the topology that placed a clade formed by *L. castaneiceps* and *A. schlegeli* as sister to a monophyletic *Buarremon* (six deep coalescence events), followed by the topology that placed *A. schlegeli* sister to a monophyletic *Buarremon* (seven deep coalescence events; Table 2). In contrast, the topology showing *Buarremon* as a paraphyletic group (Cadena et al., 2007) required 11 deep coalescence events. The topology with *A. schlegeli* sister to a monophyletic *Buarremon* was the species tree with the highest average probability of genealogies according to all replicates with different RITs in COAL (the highest value was recovered with RITs of 1:1 and 1.0; Table 3). This topology was also the species tree reconstructed using the Bayesian analysis in BEST, which supported the monophyly of *Buarremon* with a posterior probability of 0.82 (Fig. 2).

4. Discussion

The monophyly of *Buarremon* based on traditional taxonomy was brought into question by Cadena et al. (2007) based on phylogenetic analyses of sequences of four mitochondrial loci. This result was partly inconsistent with one of the nuclear gene trees reported by them, but not with another. Based on sequence data from additional loci collected from a small number of individuals, we here found that other nuclear genes recovered different topologies from those reported by these authors, some of which had good support. Because there is only one species history, the variation in gene genealogies demonstrates that in *Buarremon* and near relatives, phylogenetic reconstructions based on single-locus data sets can result in erroneous inferences of evolutionary relationships.

Incongruence between gene-tree topologies is often an indication of incomplete lineage sorting resulting from rapid radiations, a pattern frequently seen in phylogenies where internal branches are short in comparison to terminal branches (Degnan and Rosenberg, 2009). However, in the case of *Buarremon* and allies, the results of COAL show the highest average probability for a topology lacking short internodes (i.e. RIT of 1:1 and branch lengths of 1.0; Table 3). This suggests that a possible source of incongruence between gene genealogies in our study system could be the existence of populations with large effective sizes, in which ancestral polymorphism was retained despite relatively long intervals between speciation events.

In contrast to results of the earlier study based on conventional phylogenetic analyses of a few genes (Cadena et al., 2007), three different methods for species-tree reconstruction from multilocus

Table 2
Coalescence cost required to reconcile gene-tree topologies with the fifteen possible species trees.

Species tree	Genealogies															
	βFib5		MyO		βFib7		MUSK		ACOI		TGF		ND2		Total score	
	ML	BI	ML	BI	ML	BI	ML	BI	ML	BI	ML	BI	ML	BI	ML	BI
((A sch,(B bru,B tor)),L cas,A lat)	0	0	0	0	3	3	2	2	1	1	0	0	1	1	7	7
((B tor,(B bru,A sch)),L cas,A lat)	0	0	0	0	3	3	2	2	2	2	1	1	2	2	10	10
((B bru,(B tor,A sch)),L cas,A lat)	0	0	0	0	3	3	1	1	2	2	1	1	2	2	9	9
((L cas,(B tor,B bru)),A sch,A lat)	0	0	1	1	3	3	3	3	0	0	1	1	1	1	9	9
((B tor,(L cas,B bru)),A sch,A lat)	0	0	2	2	3	3	3	3	1	1	2	2	2	2	13	13
((B bru,(L cas,B tor)),A sch,A lat)	0	0	2	2	3	3	3	3	1	1	2	2	2	2	13	13
((L cas,(B bru,A sch)),B tor,A lat)	0	0	1	1	1	1	3	3	3	3	3	3	2	1	13	12
((A sch,(B bru,L cas)),B tor,A lat)	0	0	2	2	1	1	3	3	3	3	3	3	2	1	14	13
((B bru,(A sch,L cas)),B tor,A lat)	0	0	2	2	0	0	3	3	3	3	3	3	1	0	12	11
((L cas,(A sch,B tor)),B bru,A lat)	0	0	1	1	2	2	0	0	3	3	3	3	2	1	11	10
((A sch,(L cas,B tor)),B bru,A lat)	0	0	2	2	2	2	1	1	3	3	3	3	2	1	13	12
((B tor,(L cas,A sch)),B bru,A lat)	0	0	2	2	1	1	1	1	3	3	3	3	1	0	11	10
((L cas,A sch),(B tor,B bru)),A lat)	0	0	1	1	1	1	2	2	1	1	1	1	0	0	6	6
((L cas,B tor),(A sch,B bru)),A lat)	0	0	1	1	2	2	2	2	2	2	2	2	2	2	11	11
((L cas,B bru),(A sch,B tor)),A lat)	0	0	1	1	2	2	1	1	2	2	2	2	2	2	10	10

Bold numbers indicate the lowest values of coalescence cost for each genealogy.

Total is the sum across genealogies for each species tree.

Abbreviations: A sch (Arremon schlegeli); B tor (Buarremon torquatus); B bru (Buarremon brunneinucha); L cas (Lysurus castaneiceps) and A lat (Atlapetes latinuchus).

Table 3
Probabilities of gene trees given the 15 possible species trees with RIT of 1:1 and branch lengths of 1.0.

Species tree	MyO	βFib7	ACOI	TGF	ND2	Average
((A sch,(B bru,B tor)),L cas,A lat)	0.057	0.001	0.063	0.408	0.063	0.118
((B tor,(B bru,A sch)),L cas,A lat)	0.057	0.001	0.013	0.057	0.014	0.028
((B bru,(B tor,A sch)),L cas,A lat)	0.408	0.001	0.013	0.057	0.014	0.098
((L cas,(B tor,B bru)),A sch,A lat)	0.001	0.001	0.408	0.063	0.063	0.107
((B tor,(L cas,B bru)),A sch,A lat)	0.001	0.001	0.057	0.013	0.014	0.017
((B bru,(L cas,B tor)),A sch,A lat)	0.001	0.001	0.057	0.013	0.014	0.017
((L cas,(B bru,A sch)),B tor,A lat)	0.001	0.057	0.001	0.001	0.014	0.014
((A sch,(B bru,L cas)),B tor,A lat)	0.001	0.057	0.001	0.001	0.014	0.014
((B bru,(A sch,L cas)),B tor,A lat)	0.001	0.408	0.001	0.001	0.063	0.095
((L cas,(A sch,B tor)),B bru,A lat)	0.063	0.013	0.001	0.001	0.014	0.018
((A sch,(L cas,B tor)),B bru,A lat)	0.013	0.013	0.001	0.001	0.014	0.008
((B tor,(L cas,A sch)),B bru,A lat)	0.013	0.063	0.001	0.001	0.063	0.028
((L cas,A sch),(B tor,B bru)),A lat)	0.004	0.054	0.054	0.054	0.410	0.115
((L cas,B tor),(A sch,B bru)),A lat)	0.004	0.004	0.004	0.004	0.009	0.005
((L cas,B bru),(A sch,B tor)),A lat)	0.054	0.004	0.004	0.004	0.009	0.015

Bold numbers indicate the highest values of probability.

Abbreviations: A sch (Arremon schlegeli); B tor (Buarremon torquatus); B bru (Buarremon brunneinucha); L cas (Lysurus castaneiceps) and A lat (Atlapetes latinuchus).

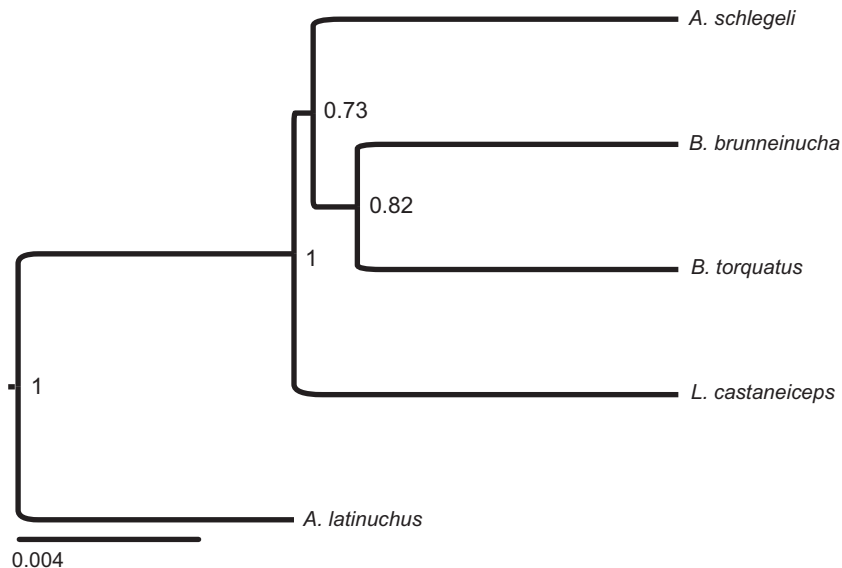


Fig. 2. Species tree reconstructed with BEST. Posterior probability values on nodes were obtained in analysis with 200,000,000 generations.

data consistently recovered *Buarremon* as a monophyletic group in the present study. Therefore, the mitochondrial DNA topology obtained by Cadena et al. (2007) might be an anomalous gene tree or might incorrectly reflect relationships owing to stochastic sorting along short branches deep in the tree (Degnan and Rosenberg, 2006; Degnan et al., 2009), and the true species tree could be one that is consistent with traditional taxonomy based on external phenotypic similarity. However, the relationships among our in-group taxa varied among different approaches and support was highly variable (see below), demonstrating the difficulty of resolving the phylogeny of this group with certainty.

An important difference between the design of our study and that of Cadena et al. (2007), in addition to the number of loci used, is the number of individuals sequenced per locus. Cadena et al. (2007) generated sequences of one mitochondrial gene (ND2) for 238 samples, including all the subspecies of *B. brunneinucha*, 13 of 14 subspecies of *B. torquatus*, eight individuals of *B. virenticeps* and one individual for three species of *Arremon* and two of *Lysurus*. In addition, they sequenced three mitochondrial loci (cyt b, ATPase 6, ATPase 8) for 43 individuals, and two nuclear loci (ACOI and MUSK) for 22 individuals. Conversely, in our study we analyzed sequences of seven genes for a single individual per species, which we considered justifiable considering that all the lineages that they represent were recovered as strongly supported monophyletic groups that have evolved in isolation for a considerable period of time in the earlier study (Cadena et al., 2007). In scenarios such as this one, the number of individuals per taxon included in analyses is not expected to strongly influence phylogenetic reconstructions (Leaché, 2009). In addition, because all the information required to infer species trees is contained in the pattern of gene lineage coalescence across multiple loci, adding loci rather than individuals is more likely to improve phylogenetic accuracy when reciprocal monophyly has been achieved (Maddison and Knowles, 2006; Knowles, 2010). We note that because individual loci had limited variation, some researchers would advise excluding them from analyses and replacing them with more variable loci. However, the influence of data quality on the accuracy of species-tree methods remains to be fully examined (Huang et al., 2010), and removing loci with few informative characters from species-tree analyses is not recommended because this could lead to ascertainment bias in the estimation of effective population sizes (Wakeley et al., 2001), a parameter on which methods based on coalescence (e.g. BEST) rely to evaluate the probability of alternative species trees (Knowles, 2010).

Our analyses suggest that the density of sampling has an important effect on the conventional reconstruction of gene genealogies: one of the most surprising results from our analyses is that the ND2 topology we recovered (which was weakly supported; Fig. 1b) and the topology reconstructed by Cadena et al. (2007) for this same gene and for a concatenated set of four mitochondrial loci, were incongruent. However, when we analyzed the five sequences considered in this study together with the ND2 data of Cadena et al. (2007), the results (tree not shown) matched those of the earlier study (i.e. supported the paraphyly of *Buarremon*) and not those recovered here for these five individual sequences. An explanation for this result could be that parameter estimation for model-based analyses can be difficult and thus lead to erroneous conclusions with a small sample of sequences (Huelsenbeck et al., 1994; Lewis, 1998). In any event, this unexpected result suggests that limited sampling may introduce bias in analyses, and because ND2 is the most variable locus in our data set, analyses concerning multilocus data are likely to be influenced strongly by variation in this gene and their results should be interpreted cautiously.

Phylogenetics is moving from the individual reconstruction of gene genealogies to a new paradigm where the explicit reconstruc-

tion of species trees is a fundamental aim (Edwards et al., 2007; Knowles, 2009). Amid this transition, empirical analyses of real data obtained from non-model organisms, such as those presented in this study, are valuable to highlight the promises of new approaches and to help identify potential pitfalls in their application. In addition to the issues related to number of individuals sampled which we described above, our analyses show that the number of loci suggested as sufficient to resolve species trees with accuracy under some simulation conditions (e.g. eight loci, Edwards et al., 2007) might be insufficient in cases such as the *Buarremon* and allies radiation (see also Brumfield et al., 2008). Our analyses also demonstrate that the same data can lead to different inferences of evolutionary relationships when analyzed with different methods for species-tree reconstruction. Although all three methods for species-tree reconstruction consistently recovered *Buarremon* as a monophyletic group (Fig. 2, Tables 2 and 3), relationships among taxa varied among different approaches. Specifically, MDC recovered a species tree in which *Lysurus* and *Arremon* form a clade that is sister to a monophyletic *Buarremon*, whereas BEST and COAL placed *Buarremon* as sister to *Arremon*, with *Lysurus* sister to the clade formed by these two. We note, however, that differences between these topologies in terms of the number of coalescent events are minor owing to the small number of individuals in the sample (i.e. 6 vs. 7 deep coalescent events). Other studies have reported different results using different methods for species-tree reconstruction on the same data set (McCormack et al., 2009), implying (1) that more work involving simulations and analyses of empirical data is necessary to better understand the sources of discrepancy between alternative approaches that use data in different ways, and (2) that users of methods for species-tree reconstruction should examine the robustness of their inferences across different analytical approaches.

In conclusion, although *Buarremon* was consistently reconstructed as a monophyletic group by our multilocus approach, considerable incongruence was observed across analyses, indicating the difficulty of achieving phylogenetic resolution in this group. Adding molecular data in the way of additional sequences from individual loci (Spinks et al., 2009) or from genome-wide surveys of SNP variation generated using high-throughput technologies (cf. Decker et al., 2009) may allow for increased resolution in the future, but the uncertainty existing at present leads us to recommend that the traditional genera *Buarremon*, *Arremon* and *Lysurus* be maintained in an expanded genus *Arremon* as suggested by Cadena et al. (2007), the monophyly of which seems clear.

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