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An improved phylogeny of the Andean tit-tyrants (Aves, Tyrannidae): More characters trump sophisticated analyses

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ABSTRACT

The phylogeny of the flycatcher genus *Anairetes* was previously inferred using short fragments of mitochondrial DNA and parsimony and distance-based methods. The resulting topology spurred taxonomic revision and influenced understanding of Andean biogeography. More than a decade later, we revisit the phylogeny of *Anairetes* tit-tyrants using more mtDNA characters, seven unlinked loci (three mitochondrial genes, six nuclear loci), more closely related outgroup taxa, partitioned Bayesian analyses, and two coalescent species-tree approaches (Bayesian estimation of species trees, BEST; Bayesian evolutionary analysis by sampling trees, *BEAST). Of these improvements in data and analyses, the fourfold increase in mtDNA characters was both necessary and sufficient to incur a major shift in the topology and near-complete resolution. The species-tree analyses, while theoretically preferable to concatenation or single gene approaches, yielded topologies that were compatible with mtDNA but with weaker statistical resolution at nodes. The previous results that had led to taxonomic and biogeographic reappraisal were refuted, and the current results support the resurrection of the genus *Uromyias* as the sister clade to *Anairetes*. The sister relationship between these two genera corresponds to an ecological dichotomy between a depauperate humid cloud forest clade and a diverse dry-tolerant clade that has diversified along the latitudinal axis of the Andes. The species-tree results and the concatenation results each reaffirm the primacy of mtDNA to provide phylogenetic signal for avian phylogenies at the species and subspecies level. This is due in part to the abundance of informative characters in mtDNA, and in part to its lower effective population size that causes it to more faithfully track the species tree.

1. Introduction

The rise of DNA sequence-based phylogenies over the past two decades has fostered steady improvement in our understanding of avian biogeography and prompted a shift towards a phylogenetic classification of birds. While avian phylogeneticists proceed towards comprehensive taxon sampling there is ample need to revisit phylogenetic hypotheses whose influence may not have been justified by empirical support. In this journal, Roy et al. (1999) provided one of the first comprehensive species-level molecular phylogenies of an avian genus, *Anairetes* (tit-tyrant flycatchers). The resulting topology spurred a taxonomic revision and influenced subsequent researchers with respect to biogeography and avian diversification patterns in the Andes (e.g. Moritz et al., 2000; Webb and Gaston, 2003; Dingle et al., 2006; Boyle and Conway, 2007). In the ensuing decade, numerous technological and analytical advances have improved prospects for accurate estimation of phylogenies, including: (1) primer sequences and refined sequencing technologies to obtain long sequences from multiple unlinked loci (Sehgal and Lovette, 2003; Kimball et al., 2008); (2) increased computational power for tree search, parameter estimation, and bootstrapping (Brownstone and Valletta, 2001); (3) data partitioning and posterior probability estimation using model-based Bayesian analysis (Huelsenbeck and Ronquist, 2001; Huelsenbeck and Crandall, 1997; Huelsenbeck and Rannala, 1997; Brandley et al., 2005); (4) higher-level phylogenetic structure for a substantial portion of extant birds that aids in the selection of appropriate outgroups (e.g. Ohlson et al., 2008; Tello et al., 2009); and (5) coalescent-based analyses of multi-locus datasets (Degnan and Salter, 2005; Drummond and Rambaut, 2007; Liu and Pearl, 2007; Liu, 2008;). Importantly, the latter advance has initiated a shift from multi-locus concatenation to a gene-tree coalescent approach (Edwards, 2009).

Eight species and 17 subspecies are currently recognized in the genus *Anairetes* (Dickinson, 2003). The inclusion of *A. agraphia* and *A. agilis* and subspecies therein (hereforward called *Uromyias*) has been disputed since the early 20th century. Hellmayr (1927) and Lanyon (1988) recognized *A. agraphia* and *A. agilis* as a distinct genus, *Uromyias*, while Smith (1971) and Traylor (1977) recognized

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the two species as members of *Anairetes*. Roy et al. (1999) addressed the phylogenetic placement of *Uromyias* using short mitochondrial DNA fragments (totaling 632 base pairs of ND2 and Cyt b) and techniques available at the time. "*Uromyias agilis*" was recovered nested within *Anairetes* sensu stricto based on parsimony analysis of 342 bp of ND2 sequence and Kimura 2-parameter distances of the combined mtDNA dataset with 54% and <50% bootstrap support, respectively. Neither topology recovered *Uromyias* definitively outside *Anairetes*. Roy et al. (1999) were conservative in interpreting their results because of low resolution at basal nodes, although they stated plainly that "molecular data do not support a monophyletic arrangement of *Anairetes* relative to *Uromyias*." Subsequently, the genus *Uromyias* was merged back to *Anairetes* by major taxonomic authorities (Remsen et al., 2003; Clements, 2004; Del Hoyo et al., 2004; Schulenberg et al., 2007; Gill and Donsker, 2008).

The phylogenetic placement of *Uromyias* (*A. agraphia* and *A. agilis)* has implications for our understanding of the biogeography of the Andes. These two species are the only members of the *Anairetes* group that are restricted to extreme humid habitats. The other continental *Anairetes* species are more tolerant of arid conditions and all occur at high elevations in rain-shadow valleys and on west-facing slopes that are subject to at least seasonal aridity (Fig. 1). These dry-tolerant *Anairetes* species are all sympatric or syntopic with other *Anairetes* species in parts of their distributions, with as many as three species occurring together (e.g. *A. parulus*, *A. reguloides*, and *A. flavirostris* in western Peru). In contrast, the members of *Uromyias* are exclusively allopatric or parapatric with other members of the group. *Uromyias* is the only subclade whose distribution is not primarily south of the equator (*A. parulus aequatorialis* extends to southern Colombia and is the only other taxon in the genus to occur north of the equator). This pattern is apparent in other avian taxa with dry-tolerant montane genera becoming scarce north of the equator, reflecting the humidity gradient along the latitudinal axis of the Andes (e.g. *Asthenes*, *Muscisaxicola*, *Phrygilus*, *Oreotrochilus*, *Geositta*, *Leptasthenura*). If *Uromyias* is nested within *Anairetes* sensu stricto, it implies that the humid cloud-forest specialist clade evolved from a dry-tolerant habitat generalist ancestor. Conversely, if *Uromyias* represents a sister clade it would imply an older ecological dichotomy and a subsequent difference in net diversification, with the 13 dry-tolerant, southern lineages having undergone more morphological, ecological, and lineage diversification than the four humid-restricted taxa.

In this study, we estimate the phylogeny of the genus *Anairetes* using seven loci, 6407 base pairs, partitioned Bayesian analysis, species-tree methods, and appropriate outgroups. By revisiting the Roy et al. (1999) study, we examine the contributions of each technological and analytical advance to changes in the topology

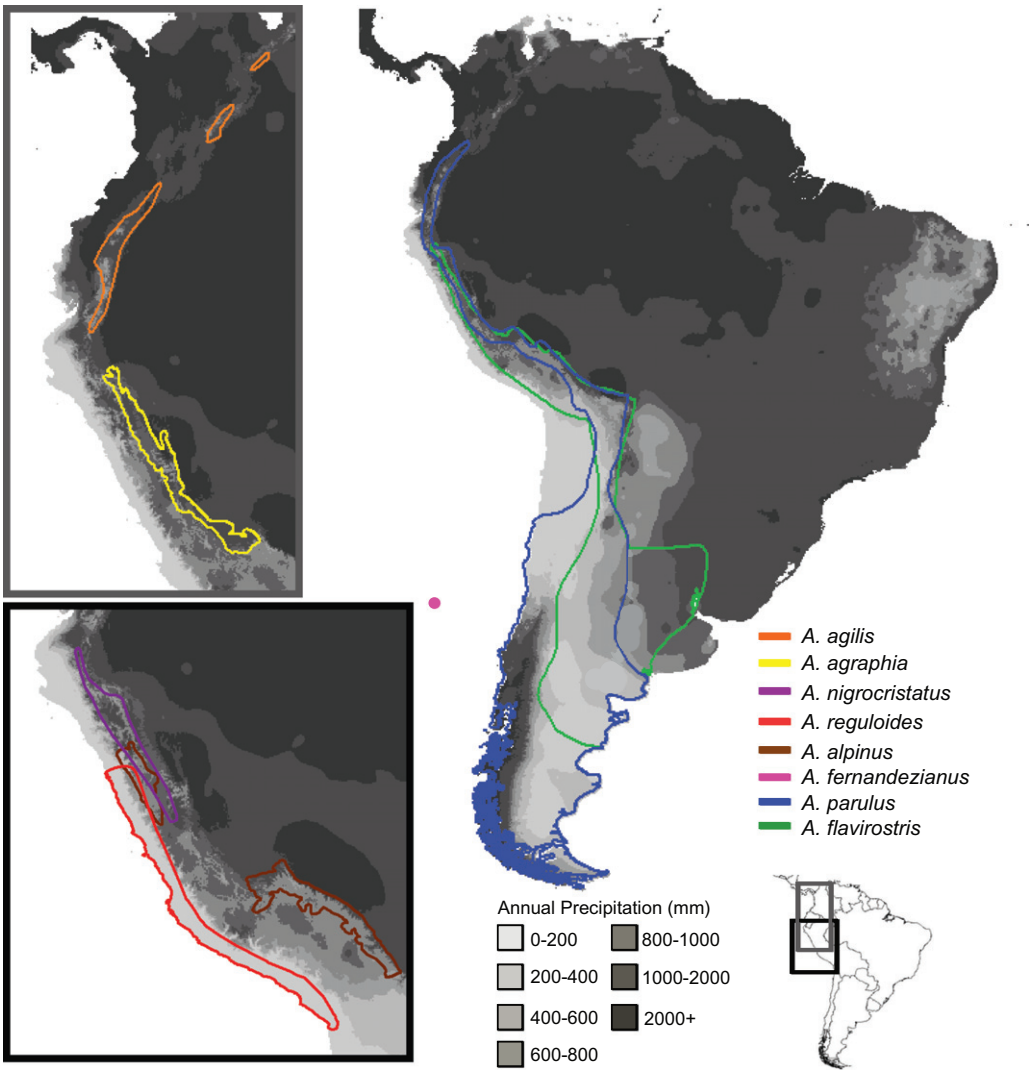

Fig. 1. Species distributions in South America overlaying mean annual precipitation from ~1950 to 2000 (Worldclim v.1.3, 2004).

and its nodal resolution. In particular, we compare the performance of multiple nuclear loci versus mitochondrial DNA and species-tree methods versus concatenation. We aim to confidently place *Uromyias* with regard to *Anairetes* sensu stricto. Accurate placement of *Uromyias* will provide essential framework to better understand the role of dry and humid habitat pressures in shaping the diversification patterns within the group.

2. Materials and methods

2.1. Taxonomic and genomic sampling

We sampled the eight currently recognized species in the genus *Anairetes,* including 14 of the 17 recognized subspecies (Table 1; Dickinson, 2003). We sampled *A. nigrocristatus* and *A. reguloides albiventris* outside of a zone of potential introgression in Ancash, Peru. We were unable to obtain samples of *A. agraphia plengei*, *A. agraphia squamigera*, or endangered *A. alpinus alpinus*. Samples of *A. fernadezianus*, endemic to Robinson Crusoe Island, Chile, were not available; however, mtDNA sequences were available on Genbank (Roy et al., 1999). We selected five outgroup taxa on the basis of recent phylogenies of the Tyrannidae (Ohlson et al., 2008; Tello et al., 2009), including *Culicivora caudacuta*, *Mecocerulus leucophrys*, *Polystictus pectoralis*, *Pseudocolopteryx sclateri*, and *Serpophaga munda*. These five taxa and *Anairetes* comprise a clade within the Elaeniinae assemblage of Tyrannidae with *Mecocerculus leucophrys* positioned basally.

For the majority of samples we obtained DNA sequences for three protein-coding mitochondrial genes [NADH dehydrogenase subunit 2 (ND2), subunit 3 (ND3), cytochrome b (Cyt b)], three autosomal nuclear intron loci [interferon regulatory factor 2 (IRF2), myoglobin intron 2 (Myo2), period homolog 2 (PER2)], two autosomal nuclear exon loci [brain-derived neurotropic factor (BDNF), nerve growth factor (NGF)], and a sex-linked nuclear intron locus [muscle, skeletal, receptor tyrosine kinase (MUSK); (Table 1)]. Nuclear markers were chosen largely based on their location in the chicken genome; we chose unlinked markers on different chromosomes that are known to have different evolutionary rates, selection pressures, and population sizes. Four sequences were obtained from GenBank (*A. agilis*, ND2; *A. fernandezianus*, ND2 and Cyt b; *Serpophaga munda*, Myo2). Sequences for each marker came from the same individual for all other taxa (Table 1). We obtained 112 of 126 possible sequences of the nine genes from the 14 ingroup taxa and 44 of 45 sequences from the five outgroup taxa. The missing sequences were: Cyt b for *Polystictus pectoralis*, PER2 for *A. parulus patagonicus*, ND3 for *A. fernandezianus*, and nuclear sequences for *A. agilis* and *A. fernandezianus* (Table 1). The only *A. agilis* samples available to us were degraded, and we were thus unable to amplify and sequence nuclear loci. Fortunately, the sister relationship of *A. agilis* and *A. agraphia* is uncontroversial and is consistent with mtDNA and all previous taxonomic treatments.

2.2. DNA extraction, PCR, sequencing and alignment

We extracted total DNA from frozen and ethanol-preserved skeletal muscle using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Primers and conditions for PCR amplification for eight of the nine markers were obtained from the literature (see Table 2 for a list of primers used and source). We designed *Anairetes*-specific primers for MUSK, Myo2, and PER2 using Primer 3v.0.4.0 (Rozen and Skaletsky, 2000) from successful *Anairetes sp.* sequences obtained from the literature-specified primers. We used PCR and sequencing protocols described by Johnson et al. (2011). We did not detect evidence of pseudogenes in the mtDNA data. Unambiguous double-peaks in the nuDNA of equal height at the same nucleotide position were coded as ambiguous. We aligned sequences with MUSCLE 3.7 (Edgar, 2004) and we inspected alignments and assigned codon positions using MacClade 4.08 OS X (Maddison and Maddison, 2005).

2.3. Data partitions and model selection

We conducted preliminary Maximum Parsimony and Bayesian Inference analyses separately for each of the mtDNA genes. The mtDNA gene trees showed 100% concordance at the interspecific level and were thus concatenated for further analyses. For Bayesian phylogenetic analyses we identified the best-fitting model for each of the seven loci, with the concatenated mtDNA partitioned by

Table 1
Tissue samples included in this study. Specimen-vouchered museum archived tissues were used when possible. Museums include ANSP: Academy of Natural Sciences, Philadelphia, Pennsylvania, USA; AMNH: American Museum of Natural History, New York, New York, USA; LSUMNS: Louisiana State University Museum of Natural Science, Baton Rouge, Louisiana, USA; MNHN: Museo Nacional de Historia Natural, Santiago, Chile; MSB: Museum of Southwestern Biology, University of New Mexico, Albuquerque, New Mexico, USA; NMR: Swedish Museum of Natural History, Stockholm, Sweden; ZMUC: Zoological Museum, University of Copenhagen, Copenhagen, Denmark. We used Genbank sequences for *A. agilis*, *A. fernandezianus* (Roy et al., 1999), and *S. munda* (Ohlson et al., 2008).

Taxon	Museum and catalog no.	Locality	Accession number
Anairetes agilis	ANSP-18890	Loja, Ecuador	Pending–pending
A. agilis	ZMUC	Loja, Ecuador	AF066998
A. agraphia agraphia	MSB:Bird:34165	Cusco, Peru	Pending–pending
A. alpinus bolivianus	MSB:Bird:27098	Cusco, Peru	Pending–pending
A. fernandezianus	MNHN	Robinson Crusoe Island, Chile	AF066992, AF067001
A. flavirostris arequipae	LSUMNS-B103867	Arequipa, Peru	Pending–pending
A. flavirostris flavirostris	AMNH-10403	Anelo, Argentina	Pending–pending
A. flavirostris cuzcoensis	MSB:Bird:33661	Apurimac, Peru	Pending–pending
A. flavirostris huancabambae	MSB:Bird:34056	Piura, Peru	Pending–pending
A. nigrocristatus	MSB:Bird:36059	Ancash, Peru	Pending–pending
A. paralus parulus	AMNH-12192	Chacabuco, Chile	Pending–pending
A. parulus patagonicus	AMNH-10349	Anelo, Argentina	Pending–pending
A. parulus aequatorialis	LSUMNS-B432	Piura, Peru	Pending–pending
A. reguloides reguloides	MSB:Bird:35064	Tacna, Peru	Pending–pending
A. reguloides albiventris	MSB:Bird:32953	Lima, Peru	Pending–pending
Culicivora caudacuta	LSUMNS-B15410	Santa Cruz, Bolivia	Pending–pending
Mecocerculus leucophrys	LSUMNS-B7476	Amazonas, Venezuela	Pending–pending
Polystictus pectoralis	LSUMNS-B38120	Santa Cruz, Bolivia	Pending–pending
Pseudocolopteryx sclateri	LSUMNS-B7617	Beni, Bolivia	Pending–pending
Serpophaga munda	LSUMNS-B37650	Santa Cruz, Bolivia	Pending–pending
Serpophaga munda	NRM-947171	Alto Paraguay, Paraguay	EU231780

Table 2
Loci included in this study and primers used for PCR amplification and sequencing reactions.

Gene	Length (bp)	Primer name	Sequence (5′–3′)	Source
ND2	1021	L5219	CCCATACCCCGAAAATGATG	Sorenson et al. (1999)
		H6313	CTCTTATTTAAGGCTTTGAAGGC	Sorenson et al. (1999)
ND3	351	L10647	TTYGAAGCMGCMGCMTGATSCTG	Mindell et al. (1998)
		H11151	GATTTGTTGAGCCGAAATCAA	Chesser (1999)
Cytb	1000	L14841	GCTTCCATCCAACATCTCAGCATGATGAAA	Kocher et al. (1989)
		H4a	AAGTGGTAAGTCTTCAGTCTTTGGTTTACAAGACC	Harshman (1996)
BDNF	688	ChickBDNF-5′	ATGACCATCCTTTTCCTTACTATG	Sehgal and Lovette (2003)
		ChickBDNF-3′	TCTTCCCCTTTTAATGGTTAATGTAC	Sehgal and Lovette (2003)
IRF2	642	IRF2.2F	ATGTCTTTGGGTCGGGTTTA	Kimball et al. (2008)
		IRF2.3R	GAAACTGGGCAATTCACACA	Kimball et al. (2008)
Myo2	747	E2F1	GAAGATCTGAAGAAACATGGAGCTA	This study
		E3R1	CAATGACCTTGATAATGACTTCAGA	This study
MUSK	641	MUSK-I3F2	AAATAACCCGACCACCTGTAAA	Kimball et al. (2008)
		MUSK-I3R2	TAGGCACTGCCCAGACTGTT	Kimball et al. (2008)
		AnaMUSK-F	AAATAATAGAAGGCTTAAAGG	This study
		AnaMUSK-R	CTCTGGACATTGTGTATCCTT	This study
NGF	705	AllNGF5′	GGTGCATAGCGTAATGTCCATG	Sehgal and Lovette (2003)
		AllNGF3′	ATAATTTACAGGCTGAGGTAG	Sehgal and Lovette (2003)
PER2	612	PER.9F	CATCTTCAYCCAAATGACAGACC	Kimball et al. (2008)
		PER.10R	CCTGATTGGTGAATAGTCAAAAGG	Kimball et al. (2008)
		AnaPER2-F	TTTCCGAAAATTAATAAGAATG	This study
		AnaPER2-R	CATACTGAAGAACTGAAATGA	This study

codon position (mtDNA codon pos.1, mtDNA codon pos.2 , mtDNA codon pos. 3, BDNF, NGF, IRF2, Myo2, PER2, MUSK), using MrModeltest v2 (Nylander, 2004) with the Akaike information criterion (AIC, Akaike, 1974). Table 3 shows the preferred model for each locus. The concatenated mtDNA was partitioned by codon position to account for different evolutionary rates and selective pressures among position (Table 3; Bull et al., 1993). We did not partition BDNF and NGF by codon position although they are protein-coding genes because limited to no variation was observed in these genes when initially partitioned. For example, BDNF codon position two and NGF codon position one were homogeneous across all study taxa. Non-coding nuclear intron loci were also treated as individual partitions.

2.4. Gene tree analyses

We estimated the mitochondrial topology and each nuclear gene tree with Maximum Parsimony (MP) and Bayesian Inference (BI). The concatenated mtDNA and individual nuclear genes datasets were run independently using the two phylogenetic methods to investigate congruence among loci. We conducted MP analyses in PAUP* 4.0b10 (Swofford, 2002), implementing the branch-and-bound search algorithm with characters equally weighted. We assessed nodal support with 1000 bootstrap pseudoreplicates under a heuristic search, tree bisection-reconnection (TBR) branch swapping, and 100 random sequence additions (Felsenstein, 1985). Bayesian analyses were conducted in MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003) consisting of two replicated runs for each locus with four MCMC chains with default heating (1 cold, 3 heated). Each analysis ran for 15,000,000 generations, sampling every 1000 generations. We assess likelihood stabilization and convergence between runs using Tracer v.1.5 (Rambaut and Drummond, 2009). We discarded the first 25% of trees as burn-in although we achieved split frequencies <0.005 and stationary likelihood values much sooner.

Mitochondrial protein-coding genes show evidence of episodic positive selection or relaxed purifying selection in conjunction

Table 3
Summary of sequence variation among loci and substitution model recommend by MrModeltest under AIC, implemented in Bayesian gene tree and species tree analyses (MrBayes, BEST, *BEAST). Partitioning by codon position for mtDNA was only employed in Bayesian gene tree analyses (MrBayes) and *BEAST specie-tree analyses. Maximum *p*-distance is among ingroup taxa only (*Anairetes*, including *Uromyias*).

Locus	Number of variable sites	Number of parsimony informative characters	Maximum *p*-distance	Substitution model	Base frequency (A, C, G, T)	Number of most parsimonious trees
mtDNA	809	539	0.117	–	–	1
Codon pos. 1	164	93	0.091	GTR + I + G	0.2906, 0.2946, 0.1839, 0.2255	–
Codon pos. 2	51	19	0.043	GTR + I	0.1771, 0.3018, 0.1123, 0.4088	–
Codon pos. 3	594	427	0.282	GTR + I + G	0.3879, 0.3679, 0.0384, 0.2057	–
BDNF	19	7	0.000	HKY + I	0.2755, 0.2632, 0.2745, 0.1867	213
IRF2	30	10	0.009	HKY + I	0.3373, 0.1888, 0.2056, 0.2684	6809
MUSK	51	10	0.017	HKY + I	0.2902, 0.1809, 0.2225, 0.3064	976
Myo2	34	12	0.003	HKY + I	0.2907, 0.2190, 0.2402, 0.2501	180
NGF	8	2	0.000	F81	0.2299, 0.3144, 0.2933, 0.1624	Very large number
PER2	41	8	0.013	HKY + G	0.3207, 0.1378, 0.1657, 0.3758	96339

with changing thermal environments (e.g. Gering et al., 2009). *Anairetes* spans dramatic gradients in elevation and temperature; accordingly, we repeated the mtDNA phylogenetic analysis using only the third codon positions to assess the potential sensitivity of the topology to selection.

In addition to the independent gene tree analyses, we conducted MP and BI analyses of the Roy et al. (1999) mitochondrial dataset, replacing their outgroup, *Stigmatura*, with the more closely related, *Serpophaga munda* (Ohlson et al., 2008; Tello et al., 2009), to test for the effects of appropriate outgroup selection on tree topology.

2.5. Concatenated analyses

Individual genes alone may lack sufficient variation or signal to provide resolution or strong nodal support for bipartitions. Concatenating individual genes can increase phylogenetic signal (if complimentary and not conflicting) and recover bipartitions or increase nodal support not evident in individual gene analyses (Barrett et al., 1991; Chippindale and Wiens, 1994). We conducted MP and BI analyses on two concatenated matrices (total dataset and nuclear dataset alone) under the conditions described in Section 2.4 to investigate the presence or absence of this emergent signal phenomenon within our dataset. We excluded *A. agilis* and *A. fernandezianus* from the total concatenated dataset because they lacked nuclear sequences.

In addition to MP and BI analyses, we also conducted preliminary analyses of individual loci and concatenated datasets using maximum likelihood methods. The resulting topologies were highly consistent with our parsimony and Bayesian analyses regarding the respective dataset.

2.6. Species tree analyses

We independently estimated the species tree for the genus *Anairetes* using two methods: BEST 2.3.1 (Liu, 2008) implemented in MrBayes and *BEAST 1.6.2 (Drummond and Rambaut, 2007). Both methods employ a coalescent model that assumes that any discordance between gene topologies resulted from ancestral polymorphism (i.e. incomplete lineage sorting). BEST reconstructs a species tree from fixed gene trees, while *BEAST simultaneously co-estimates the species tree and gene trees (Heled and Drummond, 2010). For each method, a single species tree was constructed from the seven independent loci (mtDNA, BDNF, IRF2, PER2, MUSK, Myo2, NGF), implementing the recommended substitution models for each locus obtained from MrModeltest. Only taxa with sequence data for every locus were used.

In BEST, a single model (GTR + G + I) was assigned to the mtDNA locus since we could not subpartition by codon position. We conducted two replicated runs in BEST for 300 million generations, sampling every 100,000 generations with four MCMC chains with default heating (1cold, 3 heated). We used a flat-prior distribution of population size (inverse gamma distribution, alpha = 3 and beta = .003) and uniform distributions of mutation rate (bounded values 0.5 and 1.5) as suggested by the authors and previous phylogenetic studies of birds (Brumfield et al., 2008; Liu et al., 2008). We unlinked substitution model parameters between partitions, and we specified the mtDNA locus as haploid and the remaining nuclear loci as diploid. Haploid specification in BEST accounts for the one-fourth smaller effective population size of mtDNA to nuDNA as a consequence of its haploid nature and matrilineal inheritance (Moore, 1995; Liu and Pearl, 2007; Waters et al., 2010).

In *BEAST, the mtDNA was partitioned by codon position and a single model (GTR + G + I) was implemented for each position. We unlinked substitution model parameters between partitions. Nuclear alleles were not phased because populations were represented by single individuals. We formatted our input file using BEAUti 1.7 included in the BEAST software package. We specified a Yule tree prior and a random starting tree for each locus. A Yule speciation process is most appropriate when comparing relationships between species or when populations are represented in the data by single individuals (http://beast.bio.ed.ac.uk/Tree_priors_and_dating; Patterson et al., 2011). We compared strict versus relaxed molecular clock models using a likelihood ratio test and we found no significant departure from a strict clock; thus, a strict molecular clock was implemented in final analyses. In the absence of reliable calibration points (i.e. fossil data), we implemented a fixed mean substitution clock rate of 1.0 as suggested by authors (Drummond et al.,2007). Setting a fixed mean substitution rate is appropriate when the goal of analyses is phylogenetic reconstruction and not divergence dating (Drummond et al., 2007). We conducted two replicated runs in *BEAST for 400 million generations, sampling every 100,000 generations. We used Tracer v.1.5 (Rambaut and Drummond, 2009) to assess likelihood stabilization and convergence for BEST and *BEAST analyses. We discarded the first 50% of trees as burn-in for the two species-tree methods.

3. Results

3.1. Maximum Parsimony and Bayesian Inference

There was no strongly supported conflict between MP and BI analyses of the same dataset for all gene trees and concatenated topologies. We consider strong nodal support as non-parametric bootstrap values >70% and Bayesian posterior probabilities >95%. Nuclear gene trees lacked resolution at the majority of bipartitions (Fig. 2). Myo2 had the highest resolution among nuclear loci, recovering *A. nigrocristatus/reguloides* as sister to *A. alpinus/flavirostris/parulus* with strong support. BDNF and NGF did not recover any nodes with strong support. At strongly supported nodes individual gene trees recovered each species as monophyletic, with one exception: MUSK placed *A. flavirostris huancabambae* basal to *A. alpinus*, making *A. flavirostris* paraphyletic (Fig. 2). This exception was the only strongly supported discordant node among gene trees.

The mtDNA Bayesian tree resolved every *Anairetes* interspecific relationship with posterior probabilities >99% (Fig. 3). The Bayesian tree based on concatenation of all genes (not shown) was identical to the mtDNA Bayesian tree in branching structure and almost identical in nodal support values. This result comports with the substantially higher variation in the mtDNA compared to nuDNA (Table 3). The concatenated nuDNA recovered the same ingroup topology as the mtDNA with the exception: *A. nigrocristatus* sister to *A. reguloides albiventris* to the exclusion of *A. r. reguloides* (Fig. 3b). The resulting paraphyly of *A. reguloides* in the concatenated nuDNA tree was not strongly supported by any individual nuclear gene tree. The concatenated nuDNA tree showed increased resolution over individual nuclear gene trees, including increased nodal support values and novel bipartitions not evident in any individual nuclear gene tree (Fig. 3; evidence of emergent signal).

Contrary to the tree obtained by Roy et al. (1999), both our mtDNA tree and concatenated nuDNA tree place *Uromyias* outside the historical genus *Anairetes*. We recovered two clades that were consistent with the Roy et al. (1999) findings: (1) *A. nigrocristatus/reguloides* and (2) *A. alpinus/flavirostris/fernandezianus/parulus*. However, Roy et al. (1999) placed *A. alpinus* sister to *A. flavirostris* with 67% bootstrap support (Fig. 3c). In our analysis both mitochondrial and nuclear loci strongly support the positioning of *A. alpinus* as sister to the clade containing *A. flavirostris* and *A. parulus/fernandezianus* (Fig. 3). The topology based on the 3rd codon position of mtDNA was the same as that based on the whole mtDNA but with slightly weaker nodal support. This result suggests that

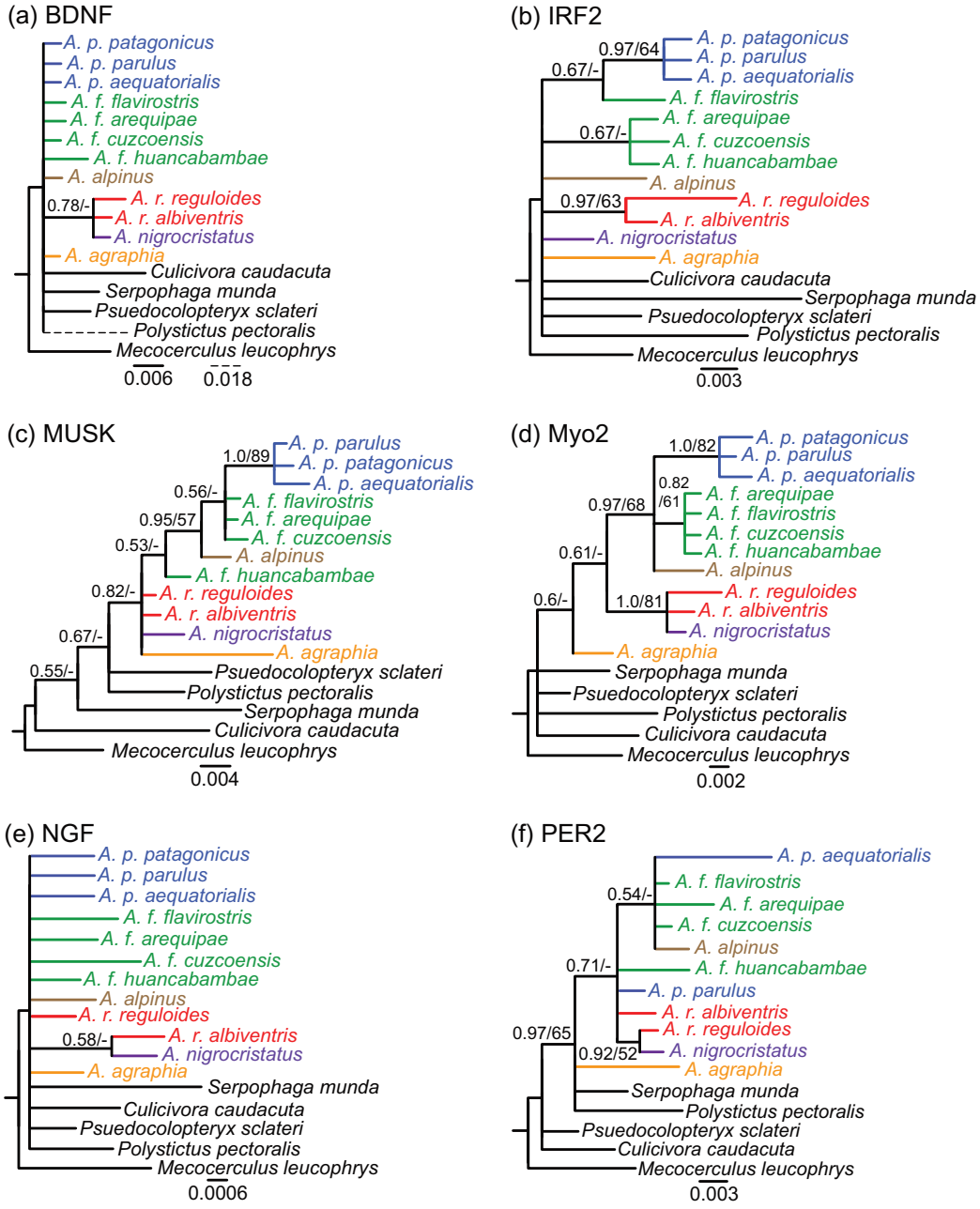

Fig. 2. Gene tree topologies for each nuclear locus obtained from Bayesian Inference and Maximum Parsimony analyses. Values at nodes refer to posterior probability of BI/MP bootstrap. Nodes were collapsed if posterior probabilities were under 50%.

potential selection on mitochondrial coding genes is not driving topology.

Using a more appropriate outgroup with the Roy et al. (1999) mitochondrial dataset apparently caused subtle changes in nodal support under MP and BI, but the overall topology was unchanged from the original. Resolution at internal nodes remained weak and *A. agilis* remained within *Anairetes* sensu stricto.

3.2. Species tree analysis

The species trees produced using BEST and *BEAST strongly supported the majority of interspecific relationships recovered by the mtDNA and concatenated nuDNA (posterior probabilities >95%; Fig. 4). Nodal support values were lower in the species-tree topologies than in the mtDNA and concatenated trees. BEST still recovered seven nodes with strong support while *BEAST recovered six. The species-tree topologies strongly supported the placement of *A. alpinus* sister to *A. flavirostris/parulus* and placed the *A. alpinus/flavirostris/parulus* clade sister to *A. nigrocristatus/reguloides*, corroborating the mtDNA tree and concatenated nuDNA tree, and confirming the monophyly of the core *Anairetes* clade. In the BEST topology, *Uromyias* was not sister to the core *Anairetes* clade, however, it was nested among the outgroup taxa, albeit with weak resolution. We suspect BEST had trouble converging; analysis reached stationary likelihood values, but three of the seven gene trees had split frequencies >0.05 (MUSK = 0.71, Myo2 = 0.72, NGF = 0.56; Hall, 2007). The *BEAST topology showed no strongly supported discordance with the mtDNA tree and recovered *Uromyias* as sister to the core *Anairetes* clade with 100% posterior probability (Figs. 3a and 4b).

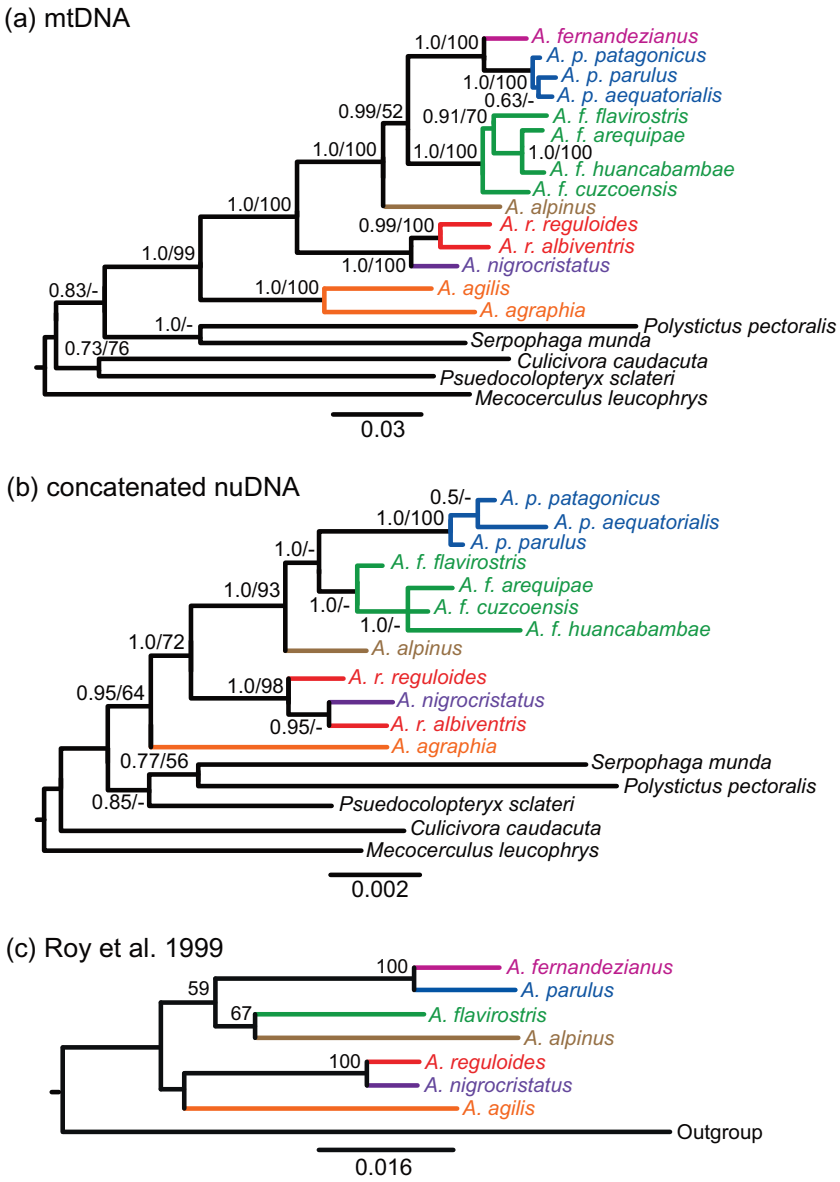

Fig. 3. Mitochondrial genome topology and concatenated nuclear topology obtained from Bayesian Inference and Maximum Parsimony analyses. Nodes were collapsed if posterior probabilities were under 50%. Fig. 1a and b, values at nodes refer to posterior probability of BI/MP bootstrap. Fig. 1c is the topology obtained from 632 bps of mtDNA using neighbor joining of Kimura 2-parameter distances from Roy et al. (1999), values at nodes refer to 500 bootstrap replicates.

4. Discussion

4.1. Technological and analytical advances

4.1.1. Refined sequencing technologies and primer availability

Increased availability of nuclear markers and ease of sequencing allows for the acquisition of long sequences from multiple unlinked loci. Increasing the number of mtDNA characters from those used by Roy et al. (1999) significantly changed tree topology and improved nodal support. *Uromyias* was recovered outside of *Anairetes* sensu stricto, and *A. parulus/fernandezianus* was recovered sister to *A. flavirostris*. The additional mtDNA characters alone led to an increase in the number of interspecific bipartitions with bootstrap values >99% from two to six, out of seven possible. Sequencing multiple unlinked loci provided little increase in resolution in itself (under MP), a result that is likely attributable to limited phylogenetic information in our nuclear loci compared to mtDNA. The maximum p-distance of nuclear loci ranged from 0.000 to 0.017 within ingroup taxa; the maximum *p*-distance of the mtDNA was .117, highlighting the greater levels of divergence in the mitochondrial genome and the limited number of informative nuclear sites (Table 3).

4.1.2. Partitioning and model-based analyses

Partitioning data with model-based analyses recovered more bipartitions and increased nodal support in gene trees and concatenated trees alike. BI (partitioned and model-based) recovered 12 of the 14 strongly supported nodes recovered by MP in individual gene trees and the same four strongly supported nodes in the nuclear concatenation topology, but BI also recovered seven and five additional nodes, respectively. In the six individual nuclear gene trees BI recovered eight strongly supported nodes while MP recovered only three. Parsimony analysis of mtDNA, which had over 44 times the amount of informative variation of any single nuclear locus, recovered 10 of the 12 bipartitions that were strongly supported by the model-based BI analysis of mtDNA, with no conflicts.

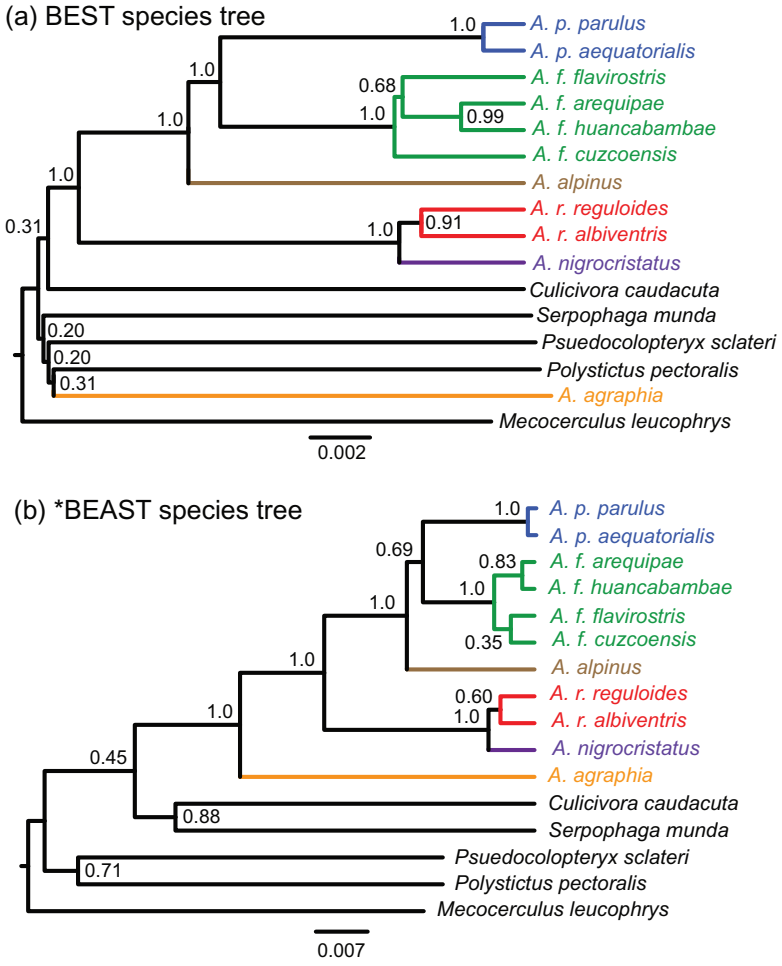

Fig. 4. Species-tree topologies recovered from BEST and *BEAST. Posterior probabilities are labeled at nodes.

Model-based analyses had higher resolution at more bipartitions than MP in both cases, but the improvement over MP was more pronounced for nuclear genes, where phylogenetic information was limited. This observation was unanticipated because we expected that model-based analyses would outperform MP with mtDNA data since it is more likely to be saturated and have homoplastic sites. The difference between MP and BI results for nuclear DNA is difficult to reconcile with the observation that MP estimates are effectively maximum likelihood estimates in the absence of homoplasy (Steel and Penny, 2000).

4.1.3. Appropriate outgroup selection

Recent higher-level phylogenetic structure of genera within Tyrannidae allowed for the closest known outgroups to be selected for this study (Ohlson et al., 2008; Tello et al., 2009). Roy et al. (1999) chose *Stigmatura napensis* as the outgroup based on the morphological classification of tyrannid flycatchers by Lanyon (1988). Ohlson et al. (2008) and Tello et al. (2009) independently recovered five genera that comprise a clade with *Anairetes* to the exclusion of the genus *Stigmatura*. Our re-analyses of the Roy et al. (1999) dataset with more closely related outgroups produced a tree that was consistent with the topology and resolution of Roy et al. (1999) regardless of phylogenetic method.

4.1.4. Coalescent-based analyses of multiple loci

Our species-tree topologies were highly consistent with our mtDNA topology but had weaker nodal support. Our results confirm previous results and predictions that coalescent-based species-tree methods generally have lower statistical confidence than concatenation methods (Belfiore et al., 2008; Thomson et al., 2008; Edwards, 2009). Data "swamping" could potentially cause this pattern, whereby the concatenated topology is biased by one or a few loci that provide the majority of phylogenetic signal, resulting in artificially high nodal support and unrealistic branch lengths (Hillis, 1987; Baker et al., 1998; Edwards, 2009). This phenomenon might explain the results of our total concatenated analysis where the recovered topology was identical to the mtDNA topology. Alternatively, species-tree approaches separately consider the phylogenetic signal from each gene, preventing highly variable loci from "swamping" the tree topology (Hillis, 1987; Baker et al., 1998; Edwards, 2009). Even when gene trees recover identical topologies, significant branch length heterogeneity can produce unexpected and incorrect phylogenetic signal when concatenated (Kolaczkowski and Thornton, 2004; Matsen and Steel, 2007). Edwards (2009) suggests that coalescent-based methods provide more realistic nodal support and branch lengths than concatenation by equally weighting the phylogenetic signal of each locus and explicitly accounting for topological heterogeneity among loci.

BEST and *BEAST take into consideration the one quarter effective population size of mtDNA relative to nuDNA through ploidy specification (Liu and Pearl, 2007; Drummond and Rambaut, 2007). If the mtDNA is not specified as haploid then the mtDNA tree is considered to be just as likely as any nuclear locus to show discordance with the species tree. In BEST, setting small theta values reduces the influence of effective population size on the

resulting species tree by increasing the probability for a speciation event to occur (Liu et al., 2008; Leache and Fujita, 2010), but a uniform small theta does not account for the known differences between mitochondrial and nuclear loci in accurately tracking the species topology. We ran preliminary BEST analysis under the conditions described in Section 2.5 but treated mtDNA as a diploid locus to test the effect of ploidy specification. The topology was highly consistent with our depicted BEST tree (Fig. 3) but had one major conflicting species relationship: *A. reguloides albiventris* was sister to *A. nigrocristatus* to the exclusion of *A. r. reguloides*. This was the same relationship recovered in the nuclear concatenated topology. When the mtDNA was not specified as haploid the resulting topology implied, improbably, that (1) the mtDNA gene tree contained a deep coalescence event, (2) the concatenated nuDNA tracked the correct species tree, and (3) that a morphologically well-defined species is paraphyletic. The latter result is most likely incorrect and highlights the primacy of mtDNA in resolving species and subspecies relationships in birds.

It is important to note that BEST had convergence problems with our dataset. Independent runs in BEST strongly corroborate the topology within *Anairetes* sensu stricto but appear to have trouble resolving basal topology. Convergence problems have been documented in empirical studies of Locustellid warblers (Alstrom et al., 2011), *Zea* maize (Cranston et al., 2009), and *Neodiprion* saw flies (Linnen and Farrell, 2008). These previous studies suggest that increasing the size of datasets may require exceptionally long runs in BEST to reach convergence. Waters et al. (2010) reached convergence after 20 million generations with 11 taxa of *Galaxis* fish and four genes by running six MCMC chains (1 cold, 5 at low heat of 0.1; suggested by Beiko et al., 2006). Increasing the number of heated MCMC chains increases mobility while searching tree space and decreases the probability of getting trapped in local optima. In our study, we initially ran BEST with two MCMC chains. The preliminary analyses resulted in poor convergence. For final analyses we increased the number of MCMC chain from two to four (1 cold, 3 heated). We could not run six MCMC chains as suggested by Beiko et al. (2006) because we lacked sufficient computational time and power. Increasing the number of MCMC chains from two to four helped with convergence, but high split frequency values were still apparent, suggesting that our independent runs had not yet converged.

4.2. Phylogeny of Anairetes

4.2.1. Taxonomy and topology

The placement of *Uromyias* (*A. agraphia* and *A. agilis*) has been debated for decades based on morphological differences and more recently, DNA sequence data (Roy et al., 1999). *A. agraphia* and *A. agilis* were first described as a distinct superspecies within the genus *Anairetes* based on bill and tail morphology (Sclater, 1888; Chapman, 1919). Hellmayr (1927) placed the two species in a new genus, *Uromyias*, based on morphological characters including: a shorter, wider and more depressed bill, more developed rictal bristles, proportionately longer tail, and greater variation between the shortest and longest rectrices. Smith (1971) replaced *A. agraphia* and *A. agilis* to *Anairetes* based on morphological similarity and the premise that ecological differences should not delimit generic boundaries. Traylor (1977) supported dissolving the genus *Uromyias* and argued that the most recently described species, *A. alpinus* (Carriker, 1933), is morphologically intermediate between the two genera. Traylor (1977) argued that the morphological differences between *Uromyias* and *Anairetes* "do not seem of great importance in an otherwise closely related group". Lanyon (1988) supported the validity of the genus *Uromyias* based on cranial morphology; specifically, *Uromyias* has a fully ossified nasal septum and lacks posterior forking in the trabecular plate. Further, Lanyon (1988) argued that posterior forking of the trabecular plate suggests monophyly for an *Anairetes/Serpophaga* clade, excluding *Uromyias*. Roy et al. (1999) provided the first molecular assessment of this group and recovered *Uromyias* nested within *Anairetes*, albeit with weak support. Since Roy et al. (1999), *Uromyias* has been recognized as part of *Anairetes*.

The results of Roy et al. (1999) were empirically inconclusive in placing *Uromyias* with respect to the historically recognized members of *Anairetes*, yet they provided the impetus for dissolving the genus *Uromyias*. In our study, *Uromyias* was independently recovered outside the core *Anairetes* clade by all methods, supporting the validity of two genera and the revival of *Uryomias*. The mtDNA gene tree, concatenated nuDNA tree, and *BEAST species tree strongly support *Uromyias* sister to *Anairetes*, but our BEST species tree lacks basal resolution and nests *Uromyias* among outgroups. Thus, all data sets and analyses point to the placement of *Uromyias* as basal to the core *Anairetes* clade or outside of it. We therefore advocate the resurrection of the genus Uromyias as distinct from *Anairetes*.

We caution that our mtDNA analysis was the only one that included both species of *Uromyias*, and that incomplete lineage sorting in the mitochondrial genome can result in the mtDNA topology being inconsistent with the true species tree. Accordingly, a sizeable mtDNA alignment could lead to high confidence in an "anomalous" gene tree. However, the absence of nuclear sequences for *A. agilis* is not likely to be problematic for three reasons: (1) all evidence from morphology, habitat, distributions, and vocalizations supports the monophyly of *A. agraphia* and *A. agilis*; (2) the branch that subtends *A. agraphia* and *A. agilis* in the mtDNA topology is long, suggesting that incomplete lineage sorting is unlikely; and (3) the high concordance between our mtDNA gene tree and our *BEAST species tree suggests that the mitochondrial genome is tracking the true species tree.

The discordance among our nuclear gene trees indicates incomplete lineage sorting in nuclear loci and rapid diversification of *Anairetes* and its allies, at least relative to the rate of nuDNA sorting (Degnan and Rosenberg, 2009). Given the evidence to date (phenotypic and genotypic) and convergence problems in BEST, the single most credible hypothesis is that *Uromyias* and *Anairetes* are monophyletic and that the mitochondrial tree and *BEAST species tree best reflects interspecific relationships. The mitochondrial genome has an effective population size one-fourth that of autosomal nuclear loci, resulting in a higher probability of tracking the species tree because of a shorter sorting time (Moore, 1995), and there were no apparent convergence issues in our *BEAST analyses. We have no evidence to suspect that these results are affected by potential problems associated with mitochondrial data such as introgression or saturation/homoplasy. The similar level of resolution between MP and BI analyses from our mtDNA dataset suggests limited homoplasy, and the majority of nodes within our mtDNA topology are corroborated by independent nuclear data and the two species-tree analyses.

It is worth noting the emergent signal in our nuclear concatenated topology. Analyses of the concatenated nuDNA not only recovered bipartitions with increased nodal support, but recovered one bipartition not strongly supported by any individual gene tree. This bipartition was in direct conflict with the IRF2 and PER2 gene trees. IRF2 recovered *A. reguloides* as monophyletic with a posterior probability of 0.97, and PER2 recovered *A. reguloides reguloides* sister to *A. nigrocristatus* with posterior probability of 0.92. The concatenated nuDNA topology recovered the other possible resolution of this triad: *A. nigrocristatus* sister to *A. reguloides albiventris* with posterior probability of 0.95. It appears that the individual nuclear genes do not contain sufficient variation to statistically resolve certain bipartitions, but phylogenetic signal becomes evident when they are combined (Barrett et al., 1991;

Chippindale and Wiens, 1994). The observed pattern suggest the two bifurcation events in this triad clade occurred in rapid succession and were accompanied by incomplete lineage sorting at most nuclear loci.

4.2.2. Biogeography

The placement of *Uromyias* outside *Anairetes* implies an ecological dichotomy between humid and dry clades with the dry-tolerant southern *Anairetes* clade more prone to morphological, ecological, and lineage diversification. Members of *Uromyias* are habitat specialists that are restricted to stands of humid, *Chusquea* dominated cloud-forest on the east slope of the Andes (1800–3600 m elevation; Parker and O'Neill, 1980; del Hoyo et al., 2004; Schulenberg et al., 2007). The *Uromyias* clade, containing only two species and four subspecies (Fig. 5; Dickinson, 2003), has undergone little net ecological and lineage diversification compared to *Anairetes* sensu stricto. *A. agraphia* is endemic to Peru and is replaced north of the Marañon Gap by *A. agilis*, whose distribution extends north to Venezuela. Taxa within *Uromyias* exhibit little difference in morphology and habitat preference. At similar montane elevations, members of *Anairetes* sensu stricto occupy seasonally arid rain-shadow valleys and dry west-facing slopes. Since the most recent common ancestor between the two clades, net diversification within *Anairetes* has yielded six species and 13 subspecies, more than three times the diversity of *Uromyias* (Fig. 4). The difference in clade size when considering subspecies taxa is significant by a binomial test ($p = 0.02$); however, we recognize that this level of clade asymmetry is highly likely to occur by chance (Slowinski and Guyer, 1993), and statistically robust comparison of diversification rates between humid-restricted and dry-tolerant lineages will require consideration of numerous co-distributed clades.

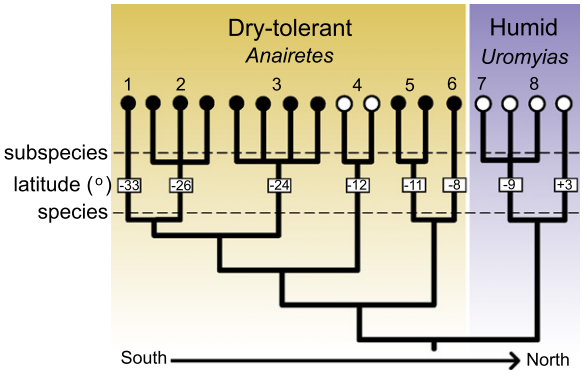

Fig. 5. Net species and subspecies diversification between the dry-tolerant *Anairetes* clade and humid *Uromyias* clade. Latitude = midpoint of species distribution. Black circles = habitat generalists. Open circles = habitat specialists. Species were categorized as generalists if more than one habitat preference is reported in Stotz et al. (1996), if the species occurs in human-modified environments, and/or if the species utilizes non-native flora. (1) *A. fernandezianus*: endemic to Robinson Crusoe Island, Chile, occurs in native montane evergreen forest, gardens, *Eucalyptus*, and other exotic vegetation (Brooke, 1987). (2) *A. parulus*: has the broadest ecological range spanning dry-torn scrub, elfin forest, disturbed humid scrub, *Polylepis* forest, and temperate forest (Cornelius et al., 2000; Jaramillo, 2003; Schulenberg et al., 2007; Lloyd, 2008). (3) *A. flavirostris*: occurs in desert and thorn scrub to semi-humid coastal Lomas and *Polylepis* forest (Jaramillo, 2003; Schulenberg et al., 2007). (4) *A. alpinus*: *Polylepis* specialist. *Polylepis* habitat is considered semi-humid but experiences water stress during their annual 4-month dry season in which relative humidity, atmospheric vapor, and precipitation dramatically decrease (Rada et al., 1996; Braun, 1997). (5) *A. reguloides*: occurs in dry coastal riparian scrub, cultivated hedgerows, arid thorn scrub, semi-arid montane scrub, and *Polylepis* (Fjeldså and Krabbe, 1990; Schulenberg et al., 2007; Pers. obs.). (6) *A. nigrocristatus*: occurs in composite arid montane scrub of *Lupinus* or *Berberis* along rivers and streams, *Polylepis*, and disturbed scrub around pastoral and agricultural fields (Fjeldså and Krabbe, 1990, Pers. obs.). (7 and 8) *A. agraphia* and *A. agilis*, respectively: *Chusquea* bamboo specialists of humid cloud forest (Fjeldså and Krabbe, 1990; Bonier et al., 2008).

Anairetes has undergone more ecological divergence (i.e. niche divergence) than *Uromyias*, as evident by up to three species occurring in sympatry and *A. parulus* and *A. flavirostris* having become migratory in the southern parts of their range. There is one island (strictly lowland) species, *A. ferndandezianus*, and the basal positions of strictly upper montane *A. nigrocristatus* and *A. alpinus* suggest that *A. reguloides*, *A. parulus*, and *A. flavirostris* have secondarily invaded the lowlands only on the extreme dry west slope of the Andes and at southern latitudes. The observed difference in net diversification between *Anairetes* and *Uryomias* may be associated with the dry-tolerant versus humid-restricted dichotomy, but investigation of additional clades that contain dry-tolerant and humid-restricted taxa will be needed to understand whether these disparate ecological pressures generally promote differences in diversification rate in Andean birds. The same ecological dichotomy, reinforced by apparent phylogenetic inertia in humidity-tolerance, is evident in published phylogenies for comparable Neotropical flycatcher clades, including *Elaenia* (Rheindt et al., 2008) and *Muscisaxicola* (Chesser, 2000).

There are two major reasons to predict that ecological generalist lineages will have undergone increased net diversification. First, generalists are more likely to persist through climate shifts and vicariant events due to their broader distributions, larger population sizes (Newman and Pilson, 1997), broader physiological limits (Kellerman et al., 2009), and lower demographic variability (Maliakal-Witt, 2004). Second, taxa on the west slope of the Andes and in rain-shadow valleys generally have broader elevational distributions, and these environments exhibit greater climatic heterogeneity across latitudes, elevations, and seasons (Sarmiento, 1986). Dry-tolerant species such as *Anairetes* that span these large gradients in temperature and elevation may be prone to disruptive selection because of disparate ecological pressures, facilitating diversification during periods of isolation (Sargent and Otto, 2006).

5. Conclusions

In comparing our study with Roy et al. (1999) we can ask what phylogenetic advancement over the past decade has had the most impact on inferring species relationships. The major improvement in resolution was garnered by sequencing nearly fourfold more basepairs of mtDNA. Resolution dramatically increased (from two to six nodes strongly supported at the interspecific level within the *Anairetes* complex), while using the same phylogenetic method (MP) and locus (mtDNA). Each subsequent advancement (partitioned model-based Bayesian analyses, inclusion of multiple unlinked nuclear loci, and species-tree analyses) recovered topologies that were highly consistent with the mtDNA tree under MP (1, 0, and 0 additional resolved nodes, respectively). Importantly, the latter developments provide more robust and easily interpretable empirical support for species relationships, providing an improved platform for taxonomic revision and biogeographic interpretation.

The ongoing shift from the multi-locus concatenation paradigm to a gene-tree coalescent approach allows phylogeneticists to account for conflicting signals due to ancestral polymorphism. BEST and *BEAST recovered a topology that was highly congruent with other methods and should provide a more realistic picture of nodal support and branch lengths (Edwards, 2009). However, our study and other studies report convergence problems between independent runs in BEST. Convergence problems appeared to affect basal topology more than the core *Anairetes* topology, which was strongly corroborated by both runs.

The mtDNA and *BEAST species-tree topologies appear to provide a robust and credible hypothesis for the relationships among *Anairetes* taxa (Figs. 4a and 3b). This leading hypothesis is not

contradicted by any strongly supported nodes for individual nuclear loci and it corroborates the theory that haploid mtDNA is more likely to have correctly tracked the species tree across rapid successive speciation events. *A. agraphia* and *A. agilis* were consistently recovered outside *Anairetes* sensu stricto, supporting the validity of the genus *Uromyias*. The sister relationship of *Uromyias* to *Anairetes* suggests an ecological dichotomy between humid-restricted and dry-tolerant clades, with the latter having undergone more morphological, ecological, and lineage diversification. The general importance of this ecological dichotomy to diversification is a key biogeographical question to be addressed as phylogenies of additional Andean bird groups emerge.

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