The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2013, **108**, 889–900. With 4 figures

Rampant polyphyly indicates cryptic diversity in a clade of Neotropical flycatchers (Aves: Tyrannidae)

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Received 2 June 2012; revised 5 September 2012, 19 January 2013; accepted for publication 19 January 2013

Polyphyletic arrangements in DNA phylogenies are often indicators of cryptic species diversity masked by erroneous taxonomic treatments that are frequently based on morphological data. Although mitochondrial (mt)DNA polyphyly is detected relatively rarely in phylogenetic studies, it has recently been found in a variety of tyrant-flycatcher (Tyrannidae) groups. In the present study, we provide a DNA phylogeny for a mitochondrial and a nuclear locus with a complete species sampling in *Zimmerius* flycatchers, showing that the genus is characterized by multiple mtDNA polyphyly. Based on phylogenetic and life-history information, we suggest the elevation of a number of taxa to species status, leading to a doubling of *Zimmerius* species-level diversity compared to taxonomic treatments conducted before 2001. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **108**, 889–900.

ADDITIONAL KEYWORDS: Amazonia – Andes – *improbus* – *petersi* – tyrannids – tyrannulets.

INTRODUCTION

Birds are commonly considered as the best-known animal class. Even compared with other vertebrate groups, the species-level taxonomy of birds has been remarkably stable (Mayr, 1946). However, this view has been seriously challenged by a recent accumulation of natural history information and the integration of museum research with molecular systematics approaches in various highly diverse tropical radiations. One of the most prominent of these radiations is the tyrant-flycatcher family Tyrannidae, which occupies a wide variety of niches in the New World and is one of the three most diverse bird families (Ridgely & Tudor, 1994; Dickinson, 2003; Clements *et al*., 2011). Increasing knowledge of flycatcher vocalizations has led to a redefinition of many species boundaries (Browning, 1993; Reynard, Garrido & Sutton, 1993; Zimmer & Whittaker, 2000) and has facilitated the discovery of undescribed species (Schulenberg & Parker, 1997; Coopmans & Krabbe, 2000; Álvarez Alonso & Whitney, 2001; Zimmer, Whittaker & Oren, 2001; Lane *et al*., 2007). Similarly, molecular phylogenies of tyrannid genera have revealed deep divergences within so-called species and paraphyletic or polyphyletic arrangements (Chesser, 2000; Joseph *et al*., 2003; Joseph & Wilke, 2004; Rheindt, Christidis & Norman, 2008c, 2009; Rheindt, Norman & Christidis, 2008a, b; Rheindt *et al*., 2009). A recurring theme has been the agreement of vocal and molecular data on new species boundaries where previous phenotypic data, the bedrock of traditional flycatcher classification, had disagreed (Rheindt *et al*., 2008a).

In the present study, we analyzed the evolutionary history of *Zimmerius* (Traylor, 1977), comprising a genus of at least nine currently recognized species from South and Central America (Traylor, 1979; Remsen *et al*., 2012) inhabiting a variety of lowland and montane forest habitats and largely specializing

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in mistletoe feeding. Rheindt *et al*. (2008a) presented a phylogeny based on mitochondrial (mt)DNA spanning six previously recognized species of *Zimmerius* tyrannulets, indicating a high level of unexpected cryptic diversity. In particular, they showed that two traditionally recognized species (*Zimmerius gracilipes* and *Zimmerius chrysops*) each comprised two groups that are not each other's closest relatives in the mtDNA phylogeny. This result agreed closely with bioacoustic information indicating that *Z. gracilipes* and *Z. chrysops* each consisted of more than one vocal lineage. Consequently, Rheindt *et al*. (2008a) proposed that vocal-genetic lineages constitute cryptic species or (in the case of an unnamed population of *Zimmerius chrysops chrysops* identical in song to *Zimmerius viridiflavus*) that species boundaries should be redrawn according to mtDNA and vocal information.

Cryptic species diversity in *Zimmerius* may further exceed previous suggestions based on molecular data (Rheindt *et al*., 2008a), particularly in widespread taxa with disjunct populations. For example, the taxa *improbus* and *petersi*, long considered subspecies of *Zimmerius vilissimus* but unsampled by Rheindt *et al*. (2008a), have been proposed to constitute a different species based on distinct sound recordings available for *petersi* (Ridgely & Tudor, 1994; Donegan *et al*., 2010). Complete taxon sampling and comprehensive geographical coverage are crucial for understanding the full extent of cryptic species-level diversity (Johnson, 2001; Braun & Kimball, 2002) in tyrant-flycatchers and other suboscine passerines. In the present study, we have expanded on the molecular study of Rheindt *et al*. (2008a) in two ways: (1) we have included all the remaining currently recognized species and a number of distinct subspecies and geographically isolated populations, increasing the sampling to 15 out of 17 *Zimmerius* taxa recognized by Traylor (1979) or subsequently described; (2) we present the results of nuclear DNA analysis in addition to mtDNA data.

MATERIAL AND METHODS

Sampling and laboratory techniques

Identity, collection localities, and museum voucher numbers of all sequences used in the present study are listed in Table 1. We sequenced two loci: (1) the complete mitochondrial NADH dehydrogenase subunit 2 gene including an adjacent stretch of partial sequence of tRNA-Met (hereafter referred to as ND2) and (2) the nuclear Fibrinogen intron 5 (Fib5). GenBank sequences were available for 16 *Zimmerius* individuals in ND2 and for 13 out of these in Fib5 (Table 1) (Rheindt *et al*., 2008a). We generated new sequences for 30 individuals in ND2 and for 29 out of these in Fib5 (GenBank accession numbers: JX568901–JX568929 and JX568930–JX568959). Laboratory procedures and sequence alignment were conducted *sensu* Rheindt, Norman & Christidis (2008d). Sequence alignment was generally straightforward, and three minor indels of 3–6 bp length in Fib5 were not considered for analysis because they added no phylogenetic information. Heterozygous sites in Fib5 were labelled as ambiguities. As outgroups, we used *Camptostoma obsoletum* (GenBank accession numbers ND2: EF501888; Fib5 EF501847; Rheindt *et al*., 2008d) and *Phyllomyias uropygialis* (ND2: DQ294567; Fib5: DQ294479; Tello & Bates, 2007) because they have been shown to be nested in the same clade of elaeniine flycatchers as *Zimmerius* (Rheindt *et al*., 2008d; Tello *et al*., 2009).

Phylogenetic analysis

All mtDNA sequences were checked for functionality and stop codons using an open reading frame finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Phylogenetic analysis was conducted on Fib5, ND2, and on the concatenated dataset combining the two loci. We employed maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods using PAUP* 4.0b10 (Swofford, 2002) and MrBayes, version 3.1.2 (Ronquist & Huelsenbeck, 2003), respectively. For heuristic ML and MP searches, we ran PAUP's tree bisection–reconnection method for tree-swapping by stepwise addition using a random addition sequence. Support for individual nodes was estimated through heuristic bootstrap resampling (1000 replicates in MP; 100 replicates in ML but no ML analysis for Fib5). In the MrBayes analyses, we conducted four runs for each dataset. Each run consisted of four chains (one hot, three cold) and was carried out over 10 000 000 generations (in the ND2 or concatenated datasets) or 8 000 000 generations (in the Fib5 dataset), sampling trees every 100 generations for the evaluation of posterior probabilities. Likelihood versus generation plots were inspected in TRACER, version 1.4.1 (Rambaut & Drummond, 2008) to determine how many generations each run required to reach a likelihood plateau. In this fashion, we determined that a burn-in of 10% is appropriate for most runs, except for two out of four Fib5 runs, in which the burn-in had to be adjusted to 36%. We evaluated convergence using TRACER, making sure that Bayesian runs reached an effective sample size greater than 200 at burn-in.

We used the Akaike information criterion as implemented in jModelTest (Posada, 2008) to assess the best fit among 88 different evolutionary models for ND2 and Fib5. We incorporated the parameters of

Table 1. Sample information, including institutions, voucher numbers, and collection localities

Taxon	Sample	Collection locality
Zimmerius cinereicapilla	LSUMZ B44642	Peru: San Martín, Abra Patricia
Zimmerius cinereicapilla	LSUMZ B44671	Peru: San Martín, Abra Patricia
Zimmerius cinereicapilla	LSUMZ B44681	Peru: San Martín, Abra Patricia
Zimmerius villarejoi	LSUMZ B44108	Peru: San Martín, Quebrada Upaquihua
Zimmerius villarejoi	LSUMZ B44220	Peru: San Martín, Quebrada Upaquihua
Zimmerius villarejoi	LSUMZ B44227	Peru: San Martín, Quebrada Upaquihua
*Zimmerius acer**	USNM B11347	Guyana
*Zimmerius acer**	ANSP 6189	Guyana: Iwokrama Reserve, approximately 5 km by road south-west Kurupukari
Zimmerius [vilissimus] vilissimus	MBM UNLV	Guatemala: Quetzaltenango (voucher number not available)
*Zimmerius albigularis**	LSUMZ B11973	Ecuador: Esmeraldas, El Placer, approximately 670 m
Zimmerius [vilissimus] parvus	LSUMZ B72149	Costa Rica: Limón, Tuba Creek
*Zimmerius [vilissimus] parvus**	LSUMZ B26529	Panama: Colón, 17 km by road north-west Gamboa, Río Agua Salud
*Zimmerius [vilissimus] parvus**	ANSP 3714	Panama: Cobachón
Zimmerius [vilissimus] i. improbus	COP AMC1083	Venezuela: Táchira, PN Páramos El Batallón y La Negra
Zimmerius [vilissimus] i. improbus	COP AMC1087	Venezuela: Táchira, PN Páramos El Batallón y La Negra
Zimmerius [vilissimus] improbus tamae	IAvH JM900	Colombia: Norte de Santander, PN Tamá
Zimmerius [vilissimus] improbus tamae	IAvH BT 1839	Colombia: Norte de Santander, Cucutilla
Zimmerius [vilissimus] improbus (subsp. nov.)	ICN JPL246	Colombia: Cesar, Serranía de Perijá
Zimmerius [vilissimus] improbus (subsp. nov.)	ICN AMC1030	Colombia: Cesar, Serranía de Perijá
Zimmerius [vilissimus] improbus (subsp. nov.)	ICN 36459	Colombia: Magdalena, Santa Marta
Zimmerius [vilissimus] improbus (subsp. nov.)	ICN 36483	Colombia: Magdalena, Santa Marta
Zimmerius [vilissimus] petersi	AMNH DOT5019	Venezuela: Aragua, El Junquito-Colonia Tovar Road. 2300 m (collected in 1996)
Zimmerius gracilipes gracilipes	IAvH BT 1052	Colombia: Caquetá, Río Cuñaré
*Zimmerius gracilipes gracilipes**	ANSP 3283	Ecuador: Zancudo Cocha, 200 m
Zimmerius gracilipes gracilipes	FMNH 457371	Brazil: Amazonas, Maraã, Lago Cumapá
Zimmerius gracilipes gracilipes	FMNH 457372	Brazil: Amazonas, Japurá, Rio Acanaui
*Zimmerius gracilipes gilvus**	LSUMZ B9489	Bolivia: Pando, Nicolas Suárez, 12 km by road south of Cobija
*Zimmerius bolivianus**	LSUMZ B22772	Bolivia: La Paz, Prov. B. Saavedra, Cerro Asunta Pata
*Zimmerius viridiflavus viridiflavus**	LSUMZ B8009	Peru: Pasco, Playa Pampa, 8 km north-west Cushi on trail to Chaglla
*Zimmerius viridiflavus viridiflavus**	LSUMZ B1749	Peru: Pasco, Sta Cruz, approximately 9 km south-south-east Oxapampa
*Zimmerius viridiflavus flavidifrons**	ANSP 5157	Ecuador: El Oro, 10 km east El Limón
Zimmerius viridiflavus (subsp. nov.)	LSUMZ B44038	Peru: San Martín, Abra Patricia
Zimmerius viridiflavus (subsp. nov.)	LSUMZ B44091	Peru: San Martín, Abra Patricia
Zimmerius viridiflavus (subsp. nov.)	LSUMZ B44213	Peru: San Martín, Abra Patricia
Zimmerius viridiflavus (subsp. nov.)	LSUMZ B44344	Peru: San Martín, Abra Patricia
Zimmerius viridiflavus (subsp. nov.)*	LSUMZ B5598	Peru: San Martín, 15 km by trail north-east Jirillo towards Balsapuerto
*Zimmerius chrysops chrysops**	LSUMZ B33191	Peru: Cajamarca, 3 km north-north-east San José de Lourdes
*Zimmerius chrysops chrysops**	LSUMZ B33214	Peru: Cajamarca, 3 km north-north-east San José de Lourdes
*Zimmerius chrysops chrysops**	LSUMZ B34835	Peru: Cajamarca, Cordillera del Cóndor, Picorana
*Zimmerius chrysops chrysops**	ZMUC 125512	Ecuador: Zamora-Chinchipe, Cordillera del Cóndor, Chinapintza
Zimmerius chrysops chrysops	LSUMZ B30030	Ecuador: Carchi, 5 km east Maldonado
Zimmerius chrysops chrysops	IAvH BT 7392	Colombia: Huila, Palestina
Zimmerius chrysops chrysops	ICN JVR4476	Colombia: Meta, El Calvario
Zimmerius chrysops chrysops	ICN AMC1166	Colombia: Antioquia, Sabaneta
Zimmerius chrysops chrysops (now: ~~*Zimmerius minimus*~~)	COP AMC1297	Venezuela: Táchira, Río Chiquito
Zimmerius chrysops chrysops (now: ~~*Z. minimus*~~)	COP AMC1264	Venezuela: Táchira, PN Chorro El Indio

An asterisk (*) indicates samples sourced from Rheindt *et al*. (2008a). Institutional abbreviations: LSUMZ – Louisiana State University Museum of Natural Science (Baton Rouge); ZMUC – Natural History Museum of Denmark (Copenhagen); ANSP – Academy of Natural Sciences in Philadelphia; USNM – Smithsonian National Museum of Natural History (Washington, D.C.); FMNH – Field Museum of Natural History (Chicago); ICN – Instituto de Ciencias Naturales at the Universidad Nacional de Colombia (Bogotá); IAvH – Instituto Alexander von Humboldt (Bogotá); COP – Colección Ornitológica Phelps (Caracas); AMNH – American Museum of Natural History (New York); MBM – Marjorie Barrick Museum of Natural History (Las Vegas).

each model, as given by jModelTest, into our ML runs in PAUP. In our Bayesian runs, we only specified the number of substitution types and the basic model for among-site rate variation (e.g. gamma-distributed or equal rate variation) as given by jModelTest. In Bayesian analysis, there is a moderate computational penalty associated with estimating parameters as opposed to fixing them prior to analysis (Ronquist & Huelsenbeck, 2003). Therefore, we allowed MrBayes to estimate the particular parameters of the evolutionary model (such as base frequencies, the rate matrix, and the gamma shape parameter value).

We used the ND2 model in the ML analysis for the concatenated dataset because ND2 is the larger of the two data partitions. However, we subjected the Fib5 model to a shorter test run of ten bootstrap replicates (not shown) to make sure that strongly supported parts of the tree topology do not differ between both models. The models of both partitions (ND2 and Fib5) were used in combination and all parameters were unlinked among partitions in the MrBayes analysis of the concatenated dataset.

We tested whether the phylogenetic signal between ND2 and Fib5 is in conflict by conducting a partition homogeneity test (Farris *et al*., 1994) as implemented in PAUP using 100 bootstrap replicates. Because we found a highly supported topological difference between the ND2 and Fib5 datasets, we subjected the ND2 sequences to Shimodaira–Hasegawa testing (Shimodaira & Hasegawa, 1999) as implemented in the package 'Ape' in conjunction with R software (Ihaka & Gentleman, 1996), using 10 000 bootstrap replicates with model parameters adjusted to the ND2 model chosen by jModelTest. Standardizing branch lengths in all trees, we specifically tested whether the topology of the most-likely tree is a significantly better fit than two alternative topologies as suggested by traditional taxonomy and the Fib5 data. We computed uncorrected p-divergences between selected taxa using MEGA, version 5.05 (Tamura *et al*., 2011), applying pairwise deletion where the nucleotide was unknown in one member of the comparison.

RESULTS

All mtDNA sequences appeared fully functional and did not display any unexpected stop codons. Table 2 provides details on the model type and parameters determined by jModelTest for both ND2 and Fib5. A partition homogeneity test (Farris *et al*., 1994) indicated significant conflict in phylogenetic signal between Fib5 and ND2 ($P = 0.010$). We therefore present the results of each locus separately, as well as combined, in a concatenated dataset. There were no topological differences, and branch support values were generally very similar or identical among MP, ML, and Bayesian analyses within each dataset (ND2, Fib5, concatenated).

The ND2 tree exhibited high to maximum branch support values for most nodes (Fig. 1). It revealed that *Zimmerius villarejoi* and *Zimmerius cinereicapilla*, comprising two species that had previously not been included in molecular studies, form a well-supported monophyletic group that is sister to the remainder of the genus (Fig. 1). It also showed a polyphyletic arrangement for taxa that had formerly been considered members of a polytypic *Z. vilissimus*, with *Zimmerius [v.] petersi* and *Zimmerius [v.] improbus* from northern South America emerging in parts of the tree distant from each other, as well as from the two predominantly Central American members of *Z. vilissimus* (i.e. *parvus* and nominate *vilissimus*). Sequence divergences among all three groups (*petersi*, *improbus*, and *vilissimus*/*parvus*) were high (approximately 10%; Table 3) relative to intraspecific divergences in other *Zimmerius* flycatchers (Rheindt *et al*., 2008a). Our sampling of *Z. [v.] improbus* revealed pronounced population-genetic structuring (Fig. 1, Table 3). The two taxa *vilissimus* and *parvus* emerged in a well-supported 'trans-Andean' clade that also included *Zimmerius albigularis* from southwestern Colombia and western Ecuador, although there was no strong evidence on whether *vilissimus* and *parvus* were sister to each other or were paraphyletic with respect to *Z. albigularis*. Sequence divergences among the three trans-Andean groups (*Z. albigularis*,

Table 2. Details on the evolutionary models as computed by jModelTest

Model parameters	ND2	Fib5
Number of substitution types	6	6
Base frequencies (A, C, G)	0.318, 0.325, 0.079	0.299, 0.168, 0.217
Rate matrix (AC, AG, AT, CG, CT)	0.444, 20.874, 1, 0.444, 10.558	2.298, 3.916, 1, 2.298, 3.916
Gamma shape	0.229	0.213
Number of categories to divide discrete approximation of gamma distribution	4	4
Proportion of invariable sites	0	0

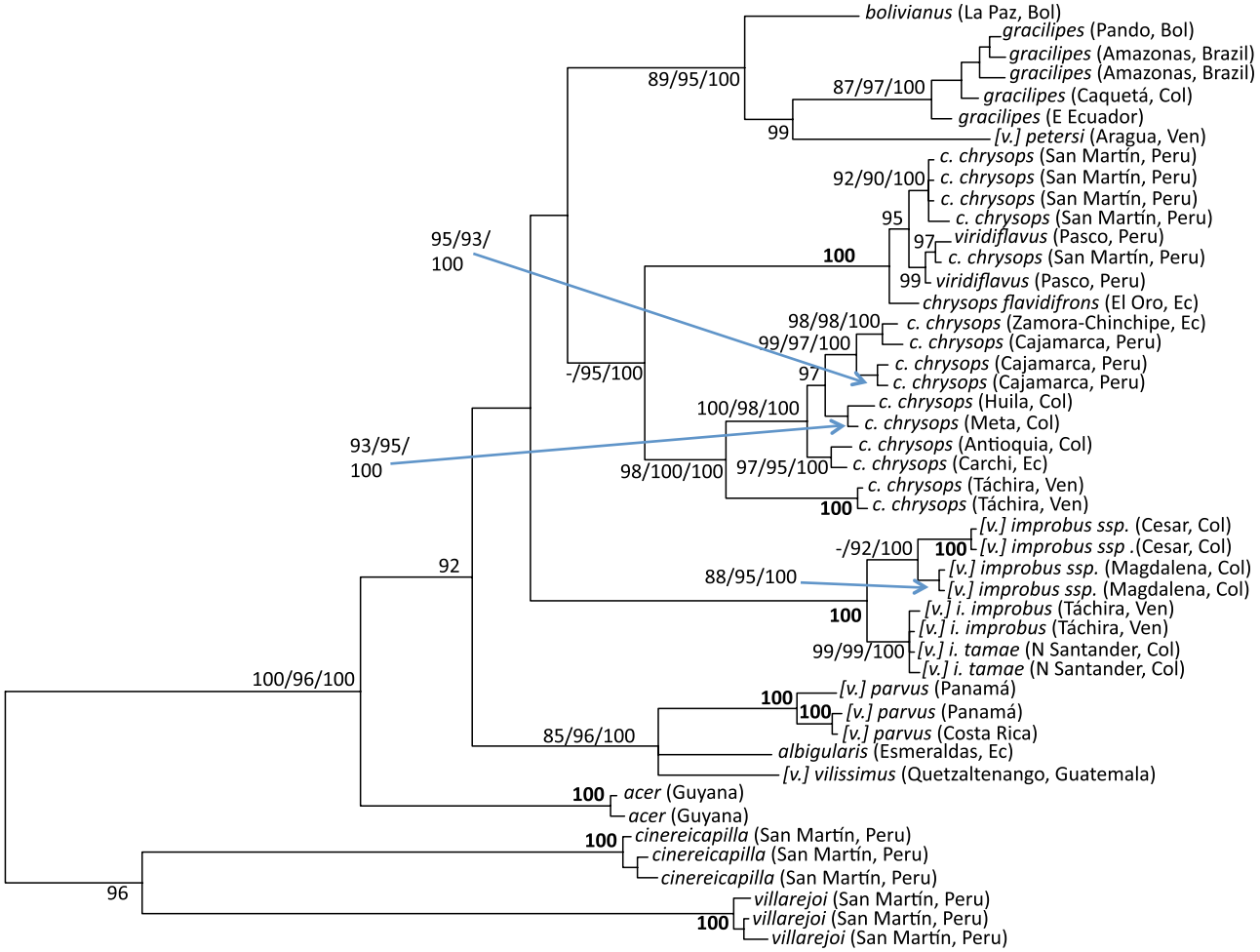

Figure 1. Bayesian tree topology of the mitochondrial DNA (= ND2) dataset (outgroup not shown). Nomenclature *sensu* Remsen *et al*. (2012) [i.e. populations from San Martín (Peru) shown by Rheindt *et al*. (2008a) to belong to *Zimmerius viridiflavus* are here labelled '*Zimmerius chrysops*' (south)]. Branch support is given in the order: parsimony bootstrap/maximum likelihood bootstrap/Bayesian posterior probabilities. Only significant branch support is given, here defined as > 90 (for Bayesian) or > 85 (for parsimony/likelihood). A bold '100' indicates maximum branch support for all three analytical modes. Where only one nonbold number is given, it refers to Bayesian support and implies that likelihood and parsimony support were not significant.

Z. [v.] vilissimus, and *Zimmerius [v.] parvus*) were equal and comparatively deep (approximately 4.5%; Table 3), indicating that the two Central American forms are likely to have been separated from each other for at least as long as from *Z. albigularis*. The ND2 tree also revealed extensive population-genetic structure within *Z. chrysops* (*sensu* Rheindt *et al*., 2008a; or northern *Z. chrysops*, *sensu* Remsen *et al*., 2012), with samples from separate mountain regions in the Andes of Colombia and Venezuela having unusually deep divergences from other individuals of this group (Fig. 1, Table 3).

On account of the lower phylogenetic signal of Fib5 in *Zimmerius* (Rheindt *et al*., 2008a), the intron tree was much less well resolved than the mtDNA tree (Fig. 2). There were only five well-supported groupings in the Fib5 tree, four of which were in agreement with the ND2 tree: (1) a monophyletic *Z. cinereicapilla*; (2) a monophyletic *Z. villarejoi* (with a well-supported sub-clade uniting two out of three *villarejoi* individuals that had not received significant support by ND2); (3) a monophyletic clade uniting *Z. villarejoi* and *Z. cinereicapilla*; and (4) a monophyletic *Z. [v.] improbus*. As in the mtDNA tree, the more slowly evolving nuclear intron indicates that *Z. villarejoi* and *Z. cinereicapilla* are the most distinct members of the genus. The fifth grouping that was highly supported by Fib5 (Fig. 2) includes *Z. [v.] improbus* and

Table 3. Uncorrected p-divergences of the ND2 sequence between selected taxon pairs

Taxon pair	Uncorrected divergence range
petersi versus *improbus* (including *tamae*)	10.3–11.0%
improbus (including *tamae*) versus *vilissimus*/*parvus*	6.7–10.8%
petersi versus *vilissimus*/*parvus*	9.1–10.4%
parvus versus *vilissimus*	4.5–4.7%
parvus versus *albigularis*	4.5–4.7%
vilissimus versus *albigularis*	4.4%
within *parvus*	0.1–1.3%
within *improbus* (including *tamae*)	0–2.4%
petersi versus *gracilipes*	3.3–6.2%
within *viridiflavus* (including southern *chrysops*	0–1.2%
within (northern) *chrysops*	0.2–4.7%

Z. [v.] petersi as members of a monophyletic group, an arrangement that is significantly contradicted by the ND2 tree (Fig. 1).

The concatenated tree was very similar in topology and branch support to the ND2 tree (Fig. 3), reflecting the fact that ND2 proportionally contributed more sequence data than Fib5.

A Shimodaira–Hasegawa test on the ND2 dataset found that a phylogeny constrained to keep *Z. vilissimus* (*s.l.*, i.e. including *parvus*, *improbus*, and *petersi*) monophyletic is a significantly poorer fit than the most-likely tree ($P < 0.00001$). Similarly, an ND2 phylogeny constrained to keep only *petersi* and *improbus* monophyletic (as strongly supported by the Fib5 dataset) is also a significantly poorer fit than the most likely tree ($P < 0.00001$).

DISCUSSION

Differentiation within *Z. c. chrysops*

As shown by Rheindt *et al*. (2008a), the *Z. viridiflavus* superspecies exhibits deep genetic differentiation (Figs 1, 3), with a southern *Z. viridiflavus* (incl. 'southern *chrysops*' *sensu* Remsen *et al*., 2012) and a northern *Z. chrysops* occupying opposite sides of the North Peruvian Low, a prominent biogeographical barrier in the Andes (Parker *et al*., 1985; Gutiérrez-Pinto *et al*., 2012). The southern species (i.e. *Z. viridiflavus*) is discussed in greater detail by Rheindt *et al*. (2008a) and is not considered further here, although the addition of six individuals from the northernmost parts of the range of northern *Z. c. chrysops* (*sensu* Rheindt *et al*., 2008a) further revealed deep levels of differentiation between Venezuelan populations and those from further south (Figs 1, 3), with Venezuelan birds (from western Táchira) differing by approximately 4.7% mtDNA divergence (Table 3).

Preliminary, unpublished inspection of calls (not dawn songs) may indicate that the genetically distinct Venezuelan clade is in fact referable to the taxon *minimus* from the Santa Marta range in Colombia (F. E. Rheindt, pers. observ.). However, both *minimus* and the coastal Venezuelan taxon *cumanensis* remain genetically unsampled. Formal vocal analysis and genetic sampling of birds from the Santa Marta mountains are desirable before such a treatment should be accepted. Until then, *Z. chrysops* should be treated as a potentially polytypic species containing the taxa *minimus* and *cumanensis* besides the nominate subspecies.

Polyphyly of *Z. vilissimus*

Our expanded sampling regime and the Shimodaira–Hasegawa test revealed a three- to four-fold polyphyly in the *Z. vilissimus* complex, implying that three taxa currently subsumed under *Z. vilissimus* could potentially be elevated to species level (*parvus*, *improbus*, *petersi*; Figs 1, 3; Table 3). The four distinct taxa that make up the *Z. vilissimus* complex inhabit a variety of habitats in disjunct geographical areas, from lowland and montane forests in Central America to the cloud forests of the Andes and the coastal mountains of northern Colombia and Venezuela (Fig. 4). Based on superficial comparisons of vocalizations between two of these populations, Ridgely & Tudor (1994) and Donegan *et al*. (2010) [see also Proposal 441 in Remsen *et al*. (2012)] suggested that the complex be divided into a Central American (*Z. vilissimus*) and a South American species (*Z. improbus*). However, previous vocal comparisons did not include samples of the topotypical populations (i.e. nominate *improbus* or *vilissimus*). Our molecular data indicate that the situation is more complex. The mtDNA phylogeny renders the constituent taxa of both *Z. improbus* and *Z. vilissimus* (*sensu* Donegan *et al*., 2010) as paraphyletic or polyphyletic, indicating that additional cryptic diversity may be involved and that taxonomic decisions should preferably be informed by a comprehensive sampling regime.

Taxonomic implications for the taxa *improbus* and petersi

The rampant polyphyly within '*Z. vilissimus*' allows for a reappraisal of species-level boundaries. As suggested by previous proposals (proposal 441 in Ridgely & Tudor, 1994; Donegan *et al*., 2010; Remsen *et al*., 2012), South American *petersi* and *improbus* should

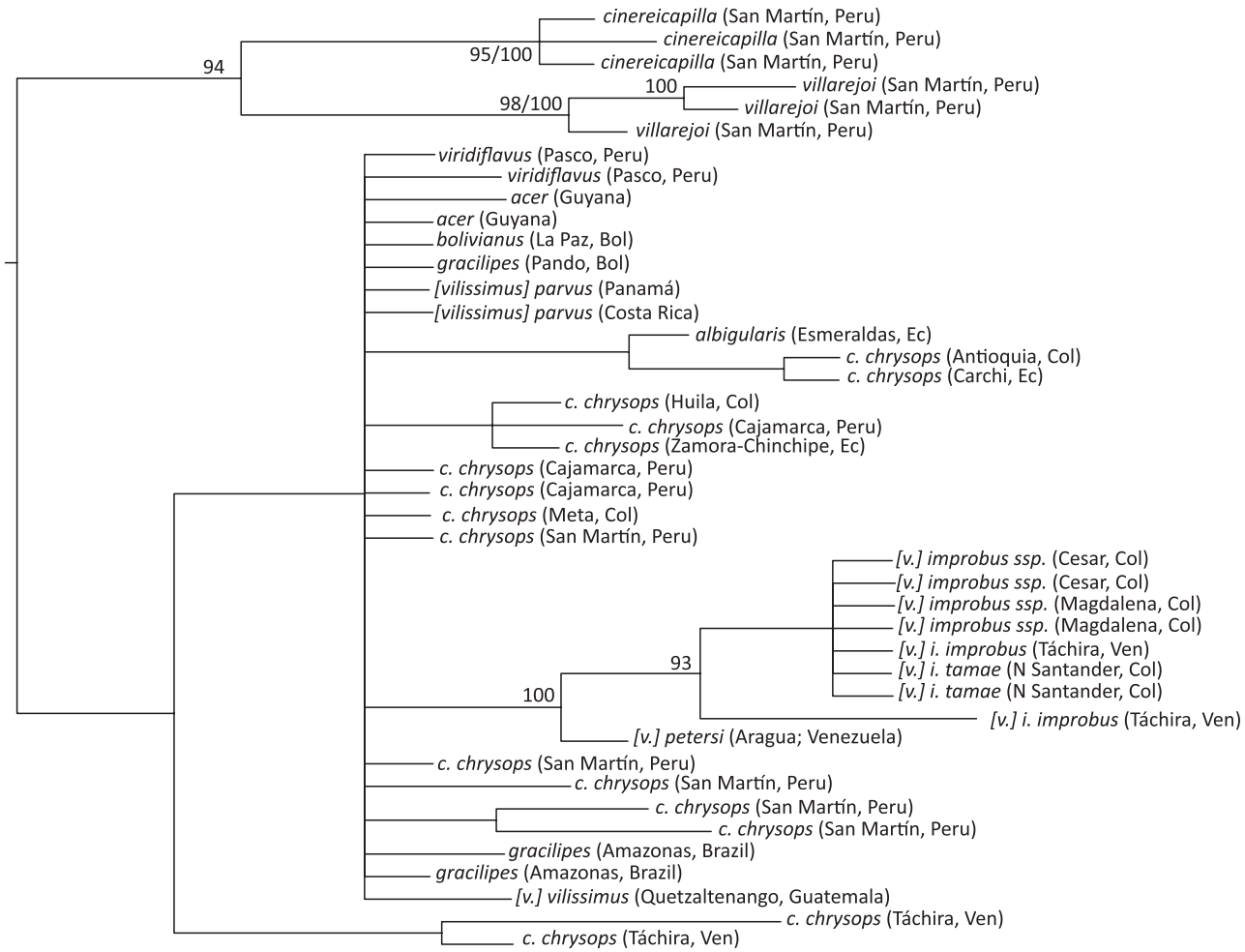

Figure 2. Bayesian tree topology of the Fib5 nuclear intron dataset (outgroup not shown). Nomenclature *sensu* Remsen *et al*. (2012) [i.e. populations from San Martín (Peru) shown by Rheindt *et al*. (2008a) to belong to *Zimmerius viridiflavus* are here labelled '*Zimmerius chrysops*' (south)]. Branch support is given in the order: parsimony bootstrap/Bayesian posterior probabilities. Only significant branch support is given, here defined as > 90 (for Bayesian) or > 85 (for parsimony). Where only one number is given, it refers to Bayesian support and implies that parsimony support was not significant.

no longer be considered conspecific with Central American *Z. vilissimus*. However, our mtDNA data go further in suggesting that *petersi* and *improbus* may not be closely related to each other either. A Shimodaira–Hasegawa test of mtDNA sequences reveals that a monophyletic clade containing *petersi* and *improbus* is a significantly poorer fit to the data than the tree topology in Figure 1. The Fib5 data, in contrast, placed *petersi* and *improbus* as divergent sister taxa. The concatenated tree resembled the ND2 tree presumably because the ND2 signal swamped out that of Fib5.

With only one specimen of *petersi* available to us, we cannot determine conclusively whether the mtDNA or the Fib5 sequence captures the true species history. Multiple polymerase chain reaction amplifications and sequencing of this individual produced the same results, suggesting it is not a laboratory artefact. ML and MP analyses of a partial ND2 alignment in which *petersi* is removed (data not shown) resulted in identical topologies and similar branch support values as those shown in Figure 1, which increases confidence in this sequence not being artefactual. We cannot reject other potential sources of incongruence, such as mitochondrial introgression (Rheindt & Edwards, 2011) or gene paralogy, although we emphasize that the lack of unexpected stop codons in the ND2 sequence makes it less likely that this is a nonfunctional pseudogene.

At a minimum, the available evidence indicates *petersi* is divergent genetically and phenotypically

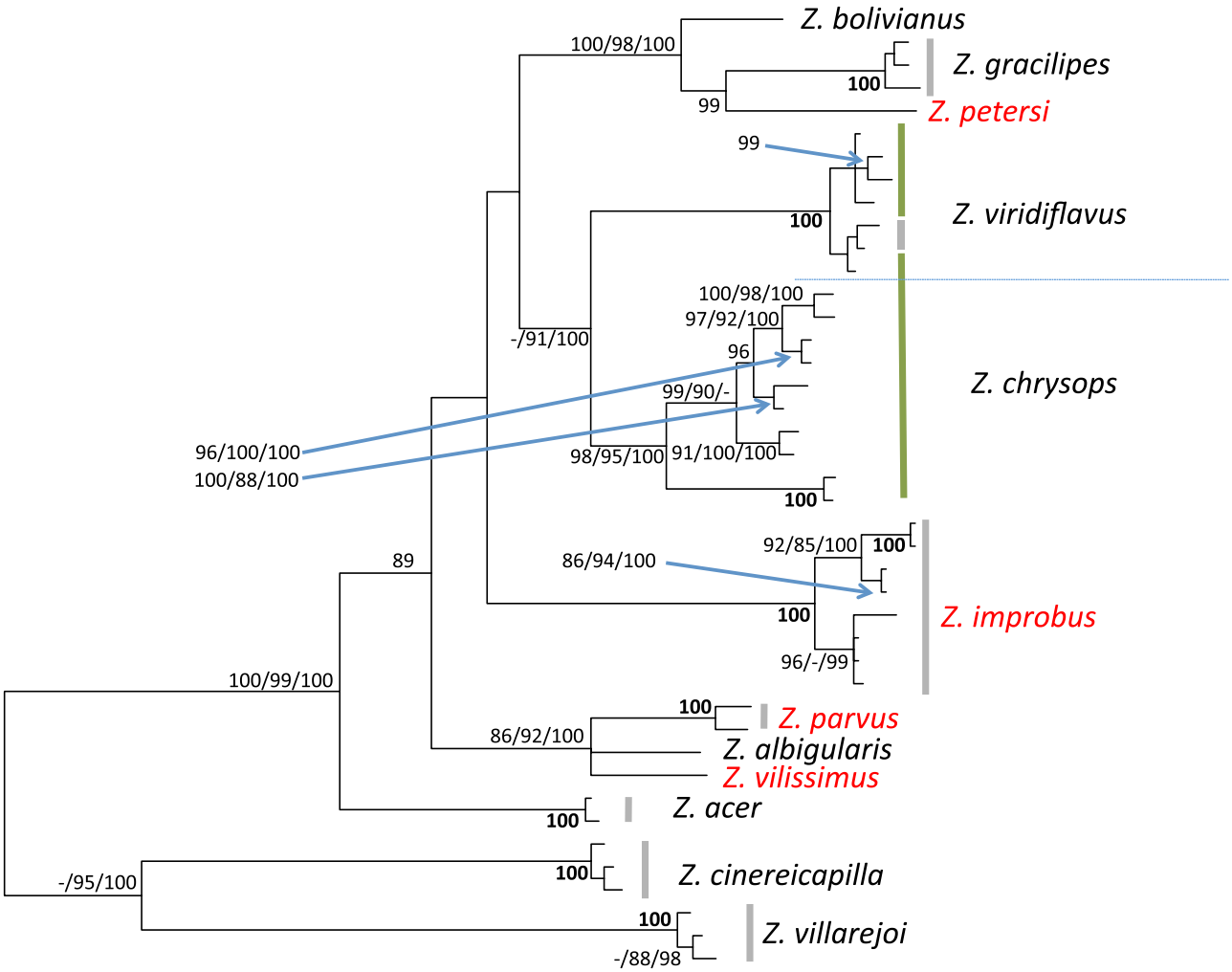

Figure 3. Bayesian tree topology of the concatenated dataset (outgroup not shown). Nomenclature is based on the taxonomic recommendations of the present study. The green vertical bar marks the former *Zimmerius chrysops* (*sensu* Remsen *et al.*, 2012). Taxa formerly subsumed under *Zimmerius vilissimus* are given in red. Branch support is given in the order: parsimony bootstrap/maximum likelihood bootstrap/Bayesian posterior probabilities. Only significant branch support is given, here defined as > 90 (for Bayesian) or > 85 (for parsimony/likelihood). A bold '100' indicates maximum branch support for all three analytical modes. Where only one nonbold number is given, it refers to Bayesian support and implies that likelihood and parsimony support were not significant.

from *improbus* at a level comparable to other species level taxa. Recently obtained sound recordings of *improbus* from the Mérida cordillera and the Serranía de Perijá are distinct from the better-known songs of *petersi* (A. M. Cuervo, unpubl. data). The taxon *petersi* was originally described as a monotypic species and displays striking differences in head and underparts coloration compared to *improbus*, exceeding those found between long-recognized *Zimmerius* species (Restall, Rodner & Lentino, 2007; A. M. Cuervo and F. E. Rheindt, pers. observ.). We advocate that *petersi* of the coastal range of Venezuela be recognized as a monotypic species, 'Venezuelan Tyrannulet' *Z. petersi*, distinct from *improbus* from the Mérida Andes of Venezuela and the Eastern Cordillera, Serranía de Perijá, and Santa Marta Range in Colombia. Corroboration of the exact species relationship of *petersi* will have to await additional sampling.

Equally, although to a lesser degree, there is pronounced population-genetic differentiation within *Z. improbus* (*s.s.*, i.e. not including the taxon *petersi*) with up to 2.4% divergence among samples (Figs 1, 3; Table 3). The three clades within *Z. improbus* correspond well with geography; one is strictly Andean from the Mérida cordillera and the northern part of the eastern Andes and includes the taxa *improbus* and *tamae*, whereas the other two are restricted to the Serranía de Perijá and the Santa Marta range.

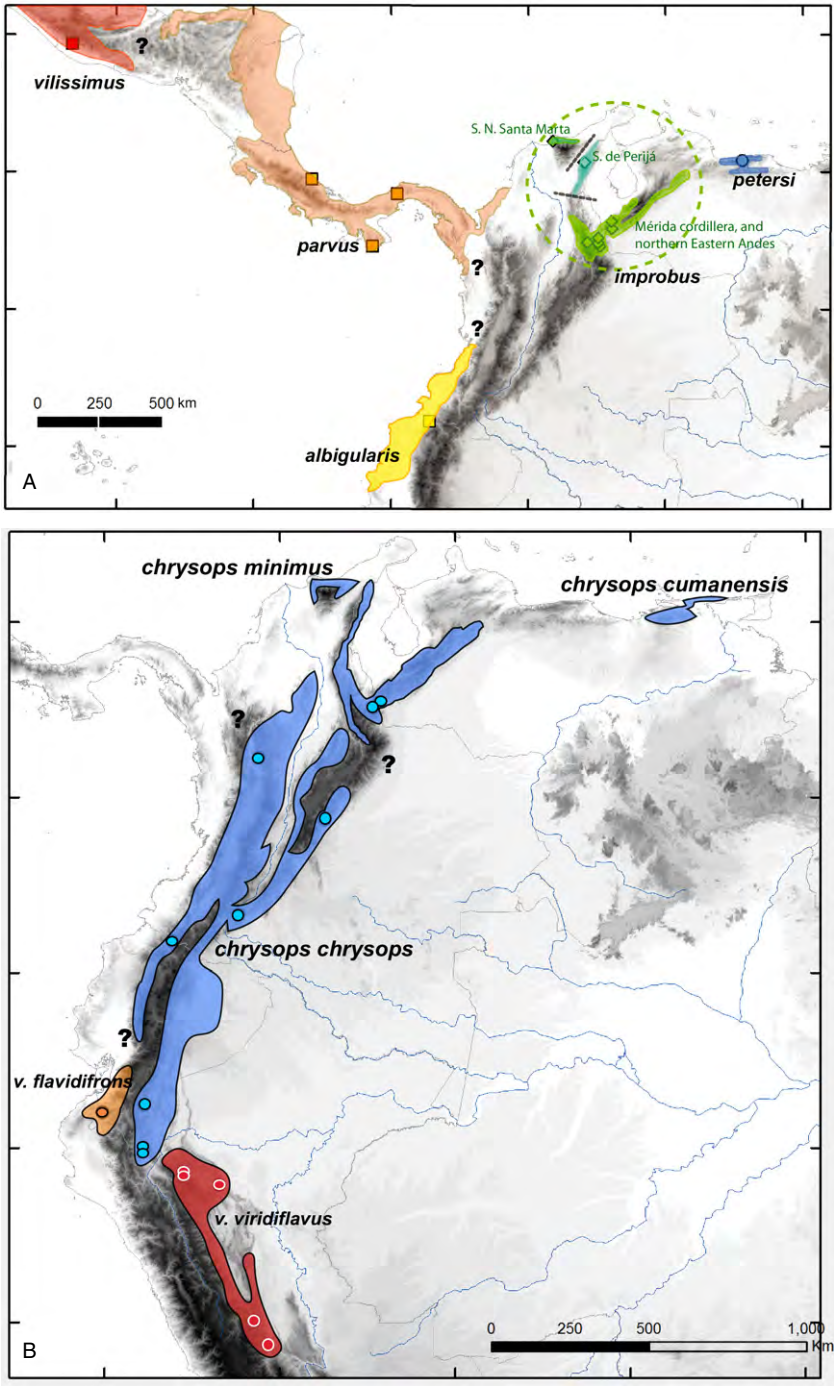

Figure 4. Distribution maps of species complexes discussed. Genetic sample points are indicated with circles, diamonds or squares. A, *Zimmerius albigularis* plus all taxa formerly included in the *Zimmerius vilissimus*. B, the *Zimmerius viridiflavus* complex. Nomenclature follows the taxonomic recommendations of the present study.

The taxon *tamae* is undifferentiated mitochondrially (Fig. 1) and may not be distinct phenotypically, whereas the populations from Santa Marta and Serranía de Perijá are unnamed, fairly deeply diverged and perhaps phenotypically distinct (A. M. Cuervo and M. Lentino, unpubl. data). Additional research using more individuals, molecular markers, and vocal and phenotypic data may well reveal that the

populations from Perijá and Santa Marta are well-differentiated subspecies, if not close to the species threshold.

Taxonomic implications for the taxa *vilissimus* and *parvus*

Most recent taxonomic treatments have united *parvus* and *vilissimus* in a single species (*Z. vilissimus*). We sequenced three individuals of the former and obtained a single (partially incomplete) ND2 sequence of the latter. They form a clade of trans-Andean *Zimmerius* tyrannulets that also includes *Z. albigularis* of the Chocó foothills of western Colombia and Ecuador, a species that has formerly been treated as conspecific with *Z. chrysops* (Rheindt *et al*., 2008a; Remsen *et al*., 2012). The tree topology (Figs 1, 3) may indicate a paraphyletic *Z. vilissimus* (*s.l.*, i.e. including *parvus*) with respect to *Z. albigularis*, although branch support for paraphyly is not strong. However, we note that: (1) sequence divergences among all three members of this trans-Andean clade are equal and relatively deep (approximately 4.5%; Table 3) compared to intra-specific divergences of other *Zimmerius* species (Rheindt *et al*., 2008a); (2) *vilissimus* and *parvus* are distinct in a number of morphological traits (Cory & Hellmayr, 1927); and (3) their call notes may be diagnosable (F. E. Rheindt, pers. observ.). We propose that *parvus* be elevated to species level as 'Mistletoe Tyrannulet' *Z. parvus*, although further specimen collection (especially in the central Chocó in Colombia), voice recordings from topotypical localities, and complementary molecular analysis would be desirable to confirm range limits and species boundaries.

Biogeographical considerations

The two major clades of *Zimmerius* may have been subject to different rates of diversification. The species *Z. villarejoi* and *Z. cinereicapilla* are two old, rare, highly divergent species. They share several distinctive traits otherwise absent in the genus (Álvarez Alonso & Whitney, 2001), including a relictual, patchy distribution and a tight ecological specialization for particular forest habitats in the eastern Andes (patchy, tall cloud forest) and Amazonia (white-sand forests). In the larger *Zimmerius* clade, species are more generalist and more widespread throughout Neotropical montane and lowland rainforests. Ecological specialization and extinction probability may have accounted for the disparate species diversity presently observed between the two major *Zimmerius* clades.

Rheindt *et al*. (2009) showed that tyrant-flycatcher mtDNA appears to evolve at a rate approximately consistent with the widely used avian mtDNA clock (approximately 2% divergence/million years). Applying this rate to the equal divergences amongst the three members of the trans-Andean clade (*albigularis*, *vilissimus*, and *parvus*; Table 3) results in a split at approximately 2–2.5 Mya. This timing is concordant with a colonization event of Central America just approximately 1 Myr after the putative closure of the Isthmus of Panama at 3.5 Mya, although new evidence suggests the closure may have been 15 Myr earlier than this (Farris *et al*., 2011; Montes *et al*., 2012).

The deep genetic structure among *Zimmerius* populations from separate northern South American mountain ranges suggests a long history of isolation. Phylogenetic and phylogeographical studies of other Andean bird complexes reveal similarly high levels of genetic differentiation in the Andes (Cadena & Cuervo, 2010; Chaves & Smith, 2011; Gutiérrez-Pinto *et al*., 2012), including cryptic species-level differentiation (Isler *et al*., 2012; d'Horta *et al*., 2013). However, *Zimmerius* tyrannulets stand out on account of their extremely confusing and subtle phenotypic variation, which has misled traditional taxonomists and has resulted in multiple instances of polyphyly being discovered in the last few years (Rheindt *et al*., 2008a; present study). A combination of new discoveries in the field (Álvarez Alonso & Whitney, 2001) and molecular–vocal comparisons (Rheindt *et al*., 2008a; present study) has resulted in a doubling of the number of recognized *Zimmerius* species from six (before 2011) to twelve. Based on the recent unpublished discovery of a new *Zimmerius* taxon in Brazilian Amazonia (B. Whitney, pers. comm.) as well as the lack of molecular sampling for a further 3–4 named populations, we consider it possible that the number of recognized *Zimmerius* species will increase further in the future.

ACKNOWLEDGEMENTS

We thank Scott Edwards for providing the laboratory infrastructure enabling the sequencing of some of the samples and for his helpful comments and advice throughout the project. We are indebted to the following collections managers and curators for their generous tissue loans: J. Maley, D. Dittmann, and V. Remsen (LSUMZ); D. Willard, S. Hackett, and J. Bates (FMNH); J. Klicka (MBM); T. Trombone, J. Cracraft, and P. Sweet (AMNH); G. Stiles and J. P. López (ICN); D. López (IAvH); and M. Lentino, J. Pérez-Emán, and J. Miranda (COP). Field assistance was provided by the GEMA group at the Instituto Alexander von Humboldt (S. Sierra, F. Forero, and M. Álvarez), C. Leal, N. Gutiérrez-Pinto,

J. Miranda, K. Certuche, Y. López, J. Márquez, J. E. Avendaño, J. P. López, O. Marín, J. Botero, and C. Sánchez. We are grateful to local people, staff at national parks, and government authorities for supporting our research. We thank Allison Shultz for helping us with the application of the Shimodaira–Hasegawa test in R software. Two anonymous reviewers are acknowledged for their helpful comments. This work was partially funded by the Lewis and Clark Exploration Fund, Society of Systematic Biologists, Society of Integrative and Comparative Biology, F. M. Chapman Memorial Fund, American Ornithologists' Union, Wilson Ornithological Society, Idea Wild, NSF grants DEB-0910285 (to A.M.C. and R.T.B.) and DEB-0841729 (to R.T.B.), and a DAAD (German Academic Exchange Program) postdoctoral fellowship (to F.E.R.).

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