Molecular systematics and species limits in the Philippine fantails (Aves: *Rhipidura*)

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ABSTRACT

Islands have long-attracted scientists because of their relatively simple biotas and stark geographic boundaries. However, for many islands and archipelagos, this simplicity may be overstated because of methodological and conceptual limitations when these biotas were described. One archipelago that has received relatively little recent attention is the Philippine islands. Although much of its biota was documented long ago, taxonomic revision and evolutionary study has been surprisingly scarce, and only a few molecular phylogenetic studies are beginning to appear. We present a molecular phylogeny and taxonomic revision for the Philippine fantails (Aves: *Rhipidura*) using nuclear and mitochondrial DNA sequences. Our results suggest that current taxonomy underestimates diversity in the group. Some morphologically distinct subspecies warrant species status, whereas one was indistinguishable genetically and morphologically and should not be retained. A few taxa require additional sampling for thorough taxonomic assessment. Patterns of diversity within Philippine *Rhipidura* mostly corroborate predictions of the Pleistocene aggregate island complex (PAIC) hypothesis, in which diversity is expected to be partitioned by deep water channels separating Pleistocene aggregate islands rather than by current islands. Substantial structure within PAIC clades indicates that additional drivers of diversification should be considered.

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1. Introduction

Fantails (genus *Rhipidura*) are active, insectivorous birds widely distributed in forests from southern Asia to Australia and southwestern Pacific Islands (Boles, 2006). With the removal of "*Rhipidura*" (*Chelidorhynx*) *hypoxantha*, which was recently shown not to belong to the Rhipiduridae (Nyári et al., 2009; Fuchs et al., 2009); the genus is composed of approximately 44 species that exhibit fairly uniform behaviors and overall morphology. Nyári et al. (2009) produced a phylogenetic hypothesis in which the three Philippine endemic species (*Rhipidura cyaniceps, R. superciliaris*, and *R. nigrocinnamomea*) formed a clade. Each of these endemic species exhibits morphological differentiation across the archipelago, as indicated by the number of described subspecies (Dickinson et al., 1991). *R. cyaniceps* has four subspecies distributed in the northern and central Philippines (Luzon, Tablas, Negros and Panay Islands), whereas *R. superciliaris* has a complimentary distribution of three subspecies in Mindanao, Basilan and the Eastern Visayan group (Samar, Leyte and Bohol). Both of these species are unique in the genus in that they have blue color in the plumage, which led early ornithologists to allocate them to the subgenus *Cyanonympha* (Boles, 2006).

The third endemic species is *R. nigrocinnamomea*, with two subspecies restricted to the highlands of Mindanao (Reis and Kennedy, 1999). This species shows a characteristic fantail plumage, with black head and cinnamon body and tail, which led taxonomists to group it with an otherwise New Guinea group of montane forests species in the subgenus *Howeavis* (Boles, 2006).

The fourth species of fantail in the Philippines, *Rhipidura javanica*, is a widespread species ranging from southern Myanmar and southern Vietnam through the Greater Sunda Islands, the westernmost Lesser Sundas, and the Philippines (including Palawan), where the endemic subspecies *R. j. nigritorquis* is present. In the published phylogeny (Nyári et al., 2009) this species was not closely related to the endemic Philippine clade, but rather to a clade of widespread species composed mainly of black and white-plumaged taxa, thus suggesting an independent colonization event into the Philippine islands. The Nyári et al. (2009) phylogeny included samples only from Borneo (subspecies *javanica*), but a recent study included denser sampling within the species and recovered deep genetic differentiation between samples from Southeast Asia and the Philippines (Lohman et al., 2010).

The Philippine Archipelago has had a dynamic geological history that likely influenced the evolution of its biota. The present shape and contour of the islands is a consequence of tectonic activity throughout the Tertiary (Hall, 2002) and Pleistocene sea-level changes (Heaney, 1991). Tectonic movements beginning in the early Eocene, about 50 Ma, fueled the *de novo* formation of the

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Philippine archipelago by moving most of the Proto-Philippines westward towards their current position. By 10 Ma, this tectonic movement caused a collision of the Philippine Sea plate against the Eurasian margin. In contrast, Palawan and Mindoro originated as continental fragments that rifted from the SE Asian mainland and rafted across the South China Sea to their present positions by 5 Ma. After this period of major tectonic movements, Pleistocene sea-level changes may have played a substantial role in shaping modern Philippine biota (Heaney, 1986; Voris, 2000; Hall, 2002). Sea level fluctuation promoted periodic coalescence of modern Philippine islands into larger islands called Pleistocene Aggregate Island Complexes (PAIC, Brown and Diesmos, 2002), which may have facilitated dispersal and gene flow among currently isolated islands. Six major PAICs are recognized (Greater Luzon, Greater Negros–Panay, Greater Mindanao, Greater Palawan, Greater Mindoro, and Greater Sulu), separated by deep-water channels such that each PAIC was isolated from all other PAICs (Heaney, 1985). Greater Palawan was probably connected to the Greater Sundas via Borneo during the lowest sea-level stands in the Pleistocene, thus allowing for interchange of biotas between those landmasses. Palawan is variously viewed as an extension of the Sunda fauna, a center of differentiation, or a dispersal corridor between the Philippines and Greater Sunda Islands (Delacour and Mayr, 1946; Inger, 1954; Brown and Alcala, 1970; Diamond and Gilpin, 1983; Heaney, 1986; Dickinson et al., 1991; Brown and Guttman, 2002; Esselstyn et al., 2004, 2010; Siler et al., 2010). It is hypothesized that sea-level fluctuation allowed repeated dispersal and isolation of elements of the Philippine biota, producing the current diversity patterns (Heaney, 1986), and allowing for the colonization of the Philippines by various taxa that show affinities to the Greater Sundas, Sulawesi, and Eurasia (Dickinson et al., 1991; Jones and Kennedy, 2008; Oliveros and Moyle, 2010).

Cycles of dispersal and isolation caused by sea-level fluctuation have been implicated as the main drivers of diversification in the Philippine archipelago. Although much of the Philippine avifauna was described by the early 1900s (e.g. Dickerson, 1928), it remains one of the most poorly known in the world (Kennedy et al., 2000). This is surprising, because the Philippines is a biodiversity "hotspot" (Myers et al., 2000) and contains approximately 556 bird species, of which 183 are endemic (Myers et al., 2000; Kennedy et al., 2000). Philippine birds have been the subject of few taxonomic revisions (see Peterson, 2006), but recent molecular work suggests that the number of Philippine bird species may be higher than previously thought (e.g. Sheldon et al., 2009; Lohman et al., 2010; Oliveros and Moyle, 2010), increasing the significance of the Philippine archipelago as a center for diversification in Southeast Asia.

We explored the relationships of all *Rhipidura* species and subspecies inhabiting the Philippine archipelago through a molecular phylogenetic approach. Monophyly of the Philippine endemic *Rhipidura* is strongly supported (Nyári et al., 2009); however relationships among Philippine species received relatively low support values. We addressed this question by expanding the sampling within this clade, including key populations from throughout the archipelago. Results of the phylogenetic analysis were used to assess species limits within the Philippine *Rhipidura*, some of which were first described as full species, only to be merged in subsequent taxonomic revisions (Delacour and Mayr, 1946; Peters, 1934-1979). We also used the phylogenetic hypothesis as a framework to discuss a biogeographic scenario for the Philippine *Rhipidura*.

2. Methods

All recognized species and all subspecies of *Rhipidura* fantails found in the Philippine islands were included in this study

(Table 1). Multiple individuals from dispersed geographic localities were included for most subspecies in order to assess more thoroughly patterns of differentiation within species (Table 1, Fig. 1). All of the samples are represented by voucher specimens in the ornithological collections of the Biodiversity Institute of the University of Kansas, the Field Museum of Natural History in Chicago, and the Cincinnati Museum Center.

Representatives from each of the major clades recovered in Nyári et al. (2009) were included in the study to reassess monophyly of the Philippine endemics and root the ingroup. The Pygmy Drongo *Chaetorhynchus papuensis* was designated as an outgroup to all of the fantails, because this species has been identified as a close relative of the genus (Barker et al., 2004; Irestedt et al., 2008; Nyári et al., 2009).

2.1. DNA sequencing

We extracted complete genomic DNA from frozen or ethanol-preserved tissues using proteinase K digestion following manufacturer's protocols (DNeasy; Qiagen, http://www.qiagen.com/). We selected molecular markers that have been good indicators of relationships within the genus (Nyári et al., 2009). These included the entire second and third subunits of nicotinamide adenine dinucleotide dehydrogenase (ND2, 1041 bp; ND3, 351 bp), which were amplified with primers L5215 and H6313 (Sorenson et al., 1999) and L10755-H11151 (Chesser, 1999) respectively. We used internal primers ND2-SWH (Sheldon et al., 2005), H5766 (Sorenson et al., 1999) and 487L (5′-GCCYTRGGRGGATGAATRGG-3′; provided by C. Oliveros) for ND2. Additionally, the fifth intron of the nuclear gene Beta-Fibrinogen (FIB5 575 bp) was amplified using primers FIB5 and FIB6 (Marini and Hackett, 2002).

Genomic DNA was amplified using 5-prime*Taq* DNA polymerase™ using standard PCR thermocycling protocols, and visualized in agarose gels stained with ethidium bromide. PCR products were cleaned with ExoSAP-IT™ (GE Healthcare Corp.) and then cycle-sequenced with ABI Prisma BigDye™ v3.1 terminator chemistry using the same primers as in the PCR thermocycling. Cycle-sequenced products were purified with ethanol precipitation protocols and sequenced on an ABI 3730 automated sequencer. Sequences were examined and aligned by eye in Sequencher 4.9 (GeneCodes Corp.), and the nuclear intron was further inspected for insertions and deletions (indels), which were adjusted as necessary.

2.2. Phylogenetic analysis

Initial separate analyses conducted on the nuclear and mitochondrial genes showed different levels of support for the terminal branches, with both datasets maintaining the Philippine *Rhipidura* monophyly with relatively good support (Suppl. Figs. 1 and 2). The main differences were at shallow levels, where only the mtDNA recovered strong support for clades (Suppl. Fig. 1) corresponding to taxa distributed within PAICs (e.g. *R. cyaniceps*) as well as multiple clades within a PAIC (*R. cyaniceps* and *R. superciliaris*). The topology of the nDNA dataset recovered only three monophyletic lineages at deeper levels with good support, although relationships of the internal branches in those clades are not resolved (Suppl. Fig. 2), which is expected in slow mutation rate genes (see Zink and Barrowclough, 2008). We combined results of both analyses into a single analysis, as nuclear genes are generally observed to provide greater resolving power (especially at deeper taxonomic levels), show lower levels of homoplasy, and provide greater bootstrap support (see Lin and Danforth, 2004).

We reconstructed phylogenetic relationships based on the concatenated dataset and individual genes with Maximum Likelihood analysis (ML) as implemented in RAxML 7.0.3 (Stamatakis, 2006),