Phylogeny of the avian genus *Pitohui* and the evolution of toxicity in birds

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A B S T R A C T

Bird species in the avian genus *Pitohui* contain potent neurotoxic alkaloids that may be used for defense. The genus comprises multiple species that are endemic to New Guinea and were presumed to belong to the family Pachycephalidae or Colluricinclidae, within the core corvoidea, an ancient Australasian radiation of crow-like birds. In order to understand the evolution of toxicity within the genus *Pitohui*, we sequenced three mitochondrial and two nuclear gene segments and reconstructed a phylogeny of the genus *Pitohui* and its putative relatives. We show that the genus *Pitohui* is polyphyletic, and consists of five different lineages. Using Bayesian ancestral state reconstruction, we estimate that toxicity likely evolved multiple times within this group. Furthermore, because the morphological and behavioral similarity among these poisonous birds appears to have evolved convergently, we hypothesize that this may be a possible example of Müllerian mimicry in birds. The Morningbird of Palau, Micronesia, that has often been included in the genus *Pitohui*, actually belongs in the genus *Pachycephala* and offers an intriguing case of pronounced evolution on a remote oceanic island.

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1. Introduction

The songbird family Pachycephalidae contains about 60 species. Most of these are small insectivorous passerines that inhabit forest interior or edge, but the family also includes the genus *Colluricincla* and *Pitohui*, individuals of which can exceed 100 g and are roughly shrike or jay-like in size, shape, and diet. Most pachycephalid species are found only in New Guinea and Australia, however, some species occur in Indonesia, New Zealand, the Philippines, Solomons, and other Pacific Islands.

About 16 years ago, the family achieved greater notoriety after the report that several *Pitohui* species contained potent defensive neurotoxins (Dumbacher et al., 1992, 2000) in their skins and feathers. The toxins, known as batrachotoxins (BTXs), are among the most toxic natural substances known. BTXs attack voltage-gated sodium channels in nerve and muscle membranes that are highly conserved in complex animals. Available evidence suggests that the toxins can protect them from human (Majnep and Bulmer, 1977; Kocher-Schmid, 1991, 1993; Gorlich, 1995) and ectoparasite (Dumbacher, 1999) enemies. A wide variety of vertebrate and non-vertebrate taxa have been shown to be sensitive to BTXs (Albu-querque et al., 1971), suggesting that *Pitohui* species may be protected from a wider suite of natural enemies.

A report by Dumbacher et al. (2000) reported identical toxins in an unrelated bird, the Blue-capped Ifrita, *Ifrita kowaldi*. Taxonomists have classically treated *Ifrita* as belonging to the core "corvoidea" (sensu (Barker et al., 2004)) but have had difficulty placing it firmly in any particular family. Some authors have suggested Timaliidae (Rand and Gilliard, 1967), Cinclosomatidae (Sibley and Ahlquist, 1990), Orthonychidae (Beehler and Finch, 1985; Beehler et al., 1986), and even Maluridae (Sibley and Ahlquist, 1990), while others hesitate to assign clear affinities (Dickinson, 2003). The discovery of toxins in *Ifrita* (Dumbacher et al., 2000) raises the question whether *Pitohui* and *Ifrita* evolved toxin use independently, or whether toxin use is basal to the core corvoidea.

A phylogeny of the genus *Pitohui* can be used to test whether the evolution of toxicity and bright coloration is associated in pitohuis. Many Pachycephalidae species are brightly colored, including the most toxic *Pitohui* species. Chemically defended species are often brightly colored or conspicuously patterned (aposematically colored). Conspicuous or bright colors may help predators learn and recall past experiences (Guilford, 1986, 1992), they might startle or cause predators to hesitate (Baker and Parker, 1979; Guilford, 1994), or some predators may innately avoid certain conspicuous colors or patterns (Schuler and Hesse, 1985; Lindstrom et al., 1999).

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Six species are currently placed in the genus *Pitohui* (Greenway et al., 1967; Sibley and Ahlquist, 1990), which has been considered monophyletic at least since 1881 (Salvadori, 1881). Toxins have been found in all but one *Pitohui* species, the White-bellied Pitohui, *P. incertus* (Dumbacher, 1997; Dumbacher et al., 2000). Pitohuis are the largest pachycephalids, are similar in size and ecology (with the exception of the Crested Pitohui, *Pitohui cristatus*), and are often gregarious. Long before ornithologists recognized them as poisonous, pitohuis were identified as "leaders" in mixed-species flocks (Diamond, 1987)—the birds that seemed to go where they pleased while the other species followed them. Furthermore, it was noted that birds participating in these flocks converged on similar color patterns. One hypothesis suggests that the mimicry of these mixed-species flocks is Batesian or Müllerian mimicry (Diamond, 1992). A phylogeny for the genus *Pitohui* can also be used to examine whether morphology, ecology, and coloration have evolved convergently or whether they represent shared ancestral characters (Dumbacher and Fleischer, 2001).

Pachycephalid phylogenetics have been partially treated by other researchers (Mayr, 1963; Boles, 1979; Sibley and Ahlquist, 1982, 1990; Dumbacher, 1994; Barker et al., 2004), but these treatments each include only a few of the 12 or so genera suspected to form a monophyletic family. The whistlers in the genus *Pachycephala* (about 33 of about 60 species in the family Pachycephalidae) form the core of the family. Several genera, including *Pitohui* and *Colluricincla* (shrike-thrushes) are widely believed to be familial relatives. However, the remainder of the family traditionally includes several distantly related genera, many of which are monotypic. The relationships of some these genera are poorly known, and even their status as Pachycephalids has been questioned. These include *Falcunculus* (Crested Shrike-tit), *Oreoica* (Bellbird), *Rhagologus* (Mottled Whistler), *Pachycare* (Dwarf Whistler), *Aleadryas* (Rufous-naped Whistler), and *Eulacestoma* (the Wattled Plough-bill). Furthermore, the family Pachycephalidae is fairly closely related to other Australo-Papuan flycatchers, including Monarchidae, Myiagridae, and Rhipiduridae, and each of these also includes several confusing monotypic genera (Boles, 1979). These families appear to be part of a deeper corvid radiation that has been difficult to resolve phylogenetically (Barker et al., 2004).

We have included several groups that are not commonly thought to belong to the Pachycephalidae, and the reasons for including them are discussed below. First, we have included the Greater Melampitta, *Melampitta gigantea*. Although it is currently classified as congeneric to the Lesser Melampitta (*Melampitta lugubris*), these two species differ tremendously in size, ecology, and many other aspects (Diamond, 1983), with *M. gigantea* resembling a large *Pitohui*. Furthermore, the immature *M. gigantea* has a russet back and belly, and thus closely resembles *Pitohui dichrous* in size and coloration (Beehler et al., 1986). Genetic data (Sibley and Ahlquist, 1987, 1990) have argued that *M. lugubris* belongs with birds of paradise (Paradisaidae), but no genetic work has been done testing the relationships of *M. gigantea*. We believe that the placement of *M. gigantea* deserves closer scrutiny (see also Barker et al., 2004).

The other groups appearing in the phylogeny represent putative outgroups to the Pachycephalidae. These include *Melanodryas cucullata* (Hooded Robin), *Petroica multicolor* and *Petroica rosea* (Multicolored and Rose Robin, respectively), *Psophodes olivaceus* (Eastern Whipbird, family Cinclosomatidae), and two bird of paradise representatives, *Cincinnurus magnificus* (Magnificent Bird of Paradise) and *Paradiseae raggiana* (Raggiana Bird of Paradise, family Paradisaeidae).

In this paper, we construct a molecular phylogeny for most genera once suspected of belonging to the Pachycephalidae. We sampled outgroup genera widely but sparsely outside the Pachycephalidae, so it is possible that other genera may correctly belong to Pachycephalidae. We use the phylogeny to test whether toxicity is more likely basal to the common ancestors of all pitohuis and ifritas, or whether it more likely evolved independently in each lineage. We also use the phylogeny to examine the evolution of bright coloration, to test whether bright coloration has co-evolved with toxicity in the corvoidea. In light of the similarities in toxin profiles, flocking, ecology, and appearance, we evaluated the monophyly of *Pitohui* to test whether these similarities might be due to mimetic resemblance (possibly Müllerian mimicry) or shared ancestral characteristics.

2. Materials and methods

2.1. Specimens and taxon sampling

Our taxonomic sampling includes the three major polytypic Pachycephalidae genera (*Colluricincla*, *Pitohui*, *Pachycephala*) and most of the monotypic Pachycephalidae genera (*Rhagologus*, *Aleadryas*, *Eulacestoma*, *Falcunculus*, *Oreoica*), however, our analysis is lacking *Pachycare* and *Hylocitrea*. Potential outgroups include *Petroica*, *Psophodes*, *Cicinnurus*, and *Paradiseae*. Table 1 lists the taxa and relevant museum specimen numbers for all included taxa.

2.2. DNA sequencing protocols

DNA was isolated from fresh tissues using a DNEasy kit (Qiagen), following the manufacturer's recommended protocol. DNA from museum specimens was isolated from a small snippet of toe pad (primarily rare subspecies of *Pitohui kirhocephalus*) using a phenol–chloroform and centrifugal dialysis method (Dumbacher and Fleischer, 2001). All extraction and PCR setup using museum-skin DNA was performed in a dedicated ancient DNA laboratory at Smithsonian National Zoological Park located in a separate building from the main lab. No modern DNA or amplification products are handled in this laboratory, and a number of controls are included in analyses to allow detection of contamination (see Dumbacher and Fleischer, 2001; Sorenson et al., 1999; Cooper et al., 1996 for details on ancient DNA analysis in this facility). Specific protocols for amplification and sequencing of ancient DNA samples can be found elsewhere (Dumbacher and Fleischer, 2001).

Polymerase chain reaction (PCR) was used to amplify three regions of mitochondrial DNA (cytochrome *b*: 766 bases [14893–15659 of the chicken mitochondrial genome], NADH dehydrogenase subunit 2: 806 bases [5261–6066 of the chicken mtDNA], and ATP synthase subunit 8/tRNA-Lys region of approximately 306 bases [bases 8930–9236 of the chicken mtDNA genome (Desjardins and Morais, 1990)]) and two nuclear introns (354 aligned bases of aldolase intron G (Prychitko and Moore, 1997) and 602 aligned bases of adenylate kinase (AK) intron 5 (Shapiro and Dumbacher, 2001)). Because of the degraded nature of the DNA isolated from museum skins, we could often only amplify relatively small segments of DNA (range of 98–347 bp) that were later concatenated. PCR reactions involved standard components and cycling profiles (Dumbacher and Fleischer, 2001), an initial 10 min denaturation at 94 °C before thermocycling up to 45 cycles (profile 92 °C denaturing/45 s, 50 °C annealing/45 s, 72 °C extension/1 min). PCR products were purified and both forward and reverse DNA strands were sequenced using ABI BigDye™ terminator reactions run on an ABI 373-stretch, ABI 377 gel automated sequencer, or on an ABI 3100 capillary sequencer. Genbank numbers for our sequences are EF592218–EF592271 (for cytochrome *b*), EF592427–EF592481 (for ATPase 8), EF592272–EF592324 (for ND2), EF592379–EF592426 (for adenylate kinase intron 5), and EF592325–EF592378 (for aldolase intron G).

Sequences were aligned using Sequencher software (Gene-Codes) where possible. When alignments were questionable or

Table 1
Species and sources of tissues used in this study

Scientific name	Common name	Museum number	Locality
Colluricincla harmonica	Grey Shrike-thrush	NMV: B.19428	Otway Ranges, Victoria, Australia
Colluricincla megarhyncha	Little Shrike-thrush	No Voucher	Wau, Morobe Province, Papua New Guinea
Colluricincla megarhyncha	Little Shrike-thrush	ABBBS 042-01131	Kakoro, Gulf Province, Papua New Guinea
Colluricincla megarhyncha	Little Shrike-thrush	No Voucher	Ohu Village, Madang Province, Papua New Guinea
Colluricincla woodwardi	Sandstone Shrike-thrush	AM O-65824	Musselbrook, Queensland, Australia
Eulacestoma nigripectus	Wattled Plough-bill	KU 91977	Papua New Guinea
Falcunculus frontatus	Crested Shrike-tit	AM O.71375	NSW, Australia
Ifrita kowaldi	Blue-capped Ifrita	No Voucher	Near Kaironk, Madang Province, Papua New Guinea
Melampitta gigantea	Greater Melampitta	PNGNM	OkMa Village, Western Province, Papua New Guinea
Melanodryas cucullata	Hooded Robin	AM O70522	Sunnyside, NSW, Australia
Microeca fascinans	Jacky Winter	AM O 64923	Black Down Tableland, Queensland, Australia
Oreoica gutturalis	Crested Bellbird	AM O 65834	Musselbrook, Queensland, Australia
Oreoica gutturalis	Crested Bellbird	AM O 65838	Musselbrook, Queensland, Australia
Pachycephala lorentzi	Lorentz's Whistler	No Voucher	Near Wau, Morobe Province, Papua New Guinea
Pachycephala olivacea	Olive Whistler	AM O 69044	Wallaby Road, NSW
Pachycephala pectoralis	Golden Whistler	NMV: B.19492	Otway Ranges, Victoria, Australia
Pachycephala rufinucha	Rufous-naped Whistler	No Voucher	Near Wau, Morobe Province, Papua New Guinea
Pachycephala schlegelii	Regent Whistler	No Voucher	Near Wau, Morobe Province, Papua New Guinea
Pachycephala simplex	Grey Whistler	No Voucher	Varirata National Park, Central Province, Papua New Guinea
Pachycephala simplex	Grey Whistler	No Voucher	Varirata National Park, Central Province, Papua New Guinea
Pachycephala soror	Sclater's Whistler	No Voucher	Near Wau, Morobe Province, Papua New Guinea
Pachycephala sp	Whistler (unknown)	No Voucher	Near Wau, Morobe Province, Papua New Guinea
Paradisaea raggiana	Raggiana Bird of Paradise	No Voucher	Wau, Morobe Province, Papua New Guinea
Petroica multicolor	Scarlet Robin	AM O.70523	Gibraltar Range National Park, NSW, Australia
Petroica rosea	Rose Robin	AM O 64996	Meersham Valley/Bagotville, NSW, Australia
Pitohui cristatus	Crested Pitohui	ABBBS 062-20022	Varirata National Park, Central Province, Papua New Guinea
Pitohui cristatus	Crested Pitohui	ABBBS 062-20027	Varirata National Park, Central Province, Papua New Guinea
Pitohui cristatus	Crested Pitohui	ANWC 26733	Tetebedi, Oro Province, Papua New Guinea
Pitohui dichrous	Hooded Pitohui	No Voucher	Baitabag village, Madang Province, Papua New Guinea
Pitohui dichrous	Hooded Pitohui	No Voucher	Nokopo Village, Madang Province, Papua New Guinea
Pitohui dichrous	Hooded Pitohui	No Voucher	Balbe village, Madang Province, Papua New Guinea
Pitohui dichrous	Hooded Pitohui	No Voucher	Kaironk Village, Madang Province, Papua New Guinea
Pitohui ferrugineus	Rusty Pitohui	ABBBS 062-38818	Kakoro Village, Gulf Province, Papua New Guinea
Pitohui ferrugineus	Rusty Pitohui	ABBBS 062-38823	Kakoro Village, Gulf Province, Papua New Guinea
Pitohui ferrugineus	Rusty Pitohui	ABBBS 062-38827	Kakoro Village, Gulf Province, Papua New Guinea
Pitohui incertus	White-bellied Pitohui	ABBBS 051-87111	Ikame, Western Province, Papua New Guinea
Pitohui incertus	White-bellied Pitohui	ABBBS 051-87112	Ikame, Western Province, Papua New Guinea
Pitohui incertus	White-bellied Pitohui	ABBBS 051-87114	Ikame, Western Province, Papua New Guinea
Pitohui kirhocephalus	Variable Pitohui	ABBBS 062-38831	Ikame, Western Province, Papua New Guinea
Pitohui kirhocephalus	Variable Pitohui	ABBBS 062-38833	Ikame, Western Province, Papua New Guinea
Pitohui kirhocephalus	Variable Pitohui	No Voucher	Amazon Bay, Central Province, Papua New Guinea
Pitohui kirhocephalus	Variable Pitohui	No Voucher	Baitabag village, Madang Province, Papua New Guinea
Pitohui kirhocephalus.	Variable Pitohui	AMNH 300941	Waigeo, Irian Jaya, Indonesia
Pitohui kirhocephalus.	Variable Pitohui	AMNH 656386	Waigeo, Irian Jaya, Indonesia
Pitohui kirhocephalus.	Variable Pitohui	AMNH 789695	Batanta Island, West Irian, Indonesia
Pitohui kirhocephalus.	Variable Pitohui	AMNH 789698	Batanta Island, West Irian, Indonesia
Pitohui kirhocephalus	Variable Pitohui	No Voucher	Kakoro Village, Gulf Province, Papua New Guinea
Pitohui kirhocephalus	Variable Pitohui	PNGNM IK01	Ikame, Western Province, Papua New Guinea
Pitohui nigrescens	Black Pitohui	No Voucher	Nokopo Village, Madang Province, Papua New Guinea
Pitohui nigrescens	Black Pitohui	No Voucher	Nokopo Village, Madang Province, Papua New Guinea
Pitohui tenebrosus.	Morningbird	NHM 77.11.17.6	Kubari, Palau
Pitohui tenebrosus.	Morningbird	MVZ 95288	Palau
Psophodes olivaceus	Eastern Whipbird	AM O.71374	Niagra Park, NSW, Australia
Rhagologus leucostigma	Mottled Whistler	KU 95990	Aedo Camp, Simbu, Papua New Guinea
Rhagologus leucostigma	Mottled Whistler	No Voucher	Kubor Ranges, Morobe Province, Papua New Guinea

Stars (∗) indicate "ancient" specimen samples from museum skins. NMV, Natural History Museum of Victoria, Australia, AM, Australian Museum; KU, University of Kansas Natural History Museum; PNGNM, Papua New Guinea National Museum and Art Gallery; ABBBS, Australian Bird and Bat Banding Scheme numbers; AMNH, American Museum of Natural History, New York; NHM, Natural History Museum, Tring, England; MVZ, Museum of Vertebrate Zoology, UC Berkeley.

difficult, sequences were aligned using ClustalX 1.83.1 for Unix and run on MacIntosh G5 in a unix shell (Thompson et al., 1994, 1997) using the multiple alignment mode. This was necessary only for AK and aldolase, both of which contained multiple gaps corresponding to putative insertion–deletion events. For AK, although sequences were obtained for *Microeca fascians*, *Petroica rosea*, and *Petroica multicolor*, these sequences did not clearly align with others in the data matrix, and were thus excluded from the AK analysis. These sequences were nonetheless logged in genbank. Alignments are available through Genbank or from JPD as a nexus file. Because ends were difficult to sequence for some genes for some taxa, some bases near the ends of sequences were excluded from analyses in order to minimize the number of missing bases in the final data matrix.

2.3. Phylogenetic reconstruction and analyses

Sequences were checked to ensure that there were no insertions, deletions, or unexpected stop codons in protein coding regions as such anomalies would be evidence that the sequences might be pseudogenes (i.e., of nuclear rather than mitochondrial origin for mitochondrial sequences). Furthermore, patterns of DNA substitution at codon positions matched those expected for mtDNA coding genes, and thus further supported our belief that

our sequences were of mitochondrial and not nuclear origin (Sorenson and Fleischer, 1996).

Maximum likelihood (ML) analyses were performed using the program PAUP∗4b10 (Swofford, 2000). We used PAUP∗ and the program MODELTEST 3.6 (Posada and Crandall, 1998) to determine the most appropriate model of sequence evolution using a neighbor-joining tree and these genetic data. We additionally examined the fit of site-specific models of evolution using the Akaike Information Criteria. Using the recommended site-specific model of DNA evolution, we performed maximum likelihood tree searches using the successive approximations method (Huelsenbeck, 1998) in PAUP∗ to obtain best-fit tree(s) and parameter estimates (Table 2). Support for particular nodes was assessed using nonparametric bootstrap (Felsenstein, 1985) as implemented in PAUP∗ with 1000 fast-addition bootstrap replicates in a likelihood framework, and 10,000 full heuristic parsimony bootstrap replicates. Since PAUP∗ cannot perform likelihood bootstrap analysis using site-specific rates, we used GTR + I + G model of evolution for bootstrapping, with parameters estimated from the most likely tree.

We also performed Bayesian phylogenetic analyses using Markov Chain Monte Carlo (MCMC) tree searches using the program MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001). Using the recommended model of sequence evolution, we performed two parallel runs of four simultaneous MCMC chains for 10,000,000 generations each, sampling every 500 generations, and discarded results of the first 2000 trees (1,000,000 early generations) as "burnin" (see Section 3). The remaining 18,000 trees per run (36,000 trees total) were used by MrBayes to estimate parameters, parameter variance (Table 2), and posterior probabilities of particular nodes in our phylogenetic reconstruction.

About 10,000 of these MrBayes trees were used to explore probabilities and likelihoods of ancestral character states using the program BayesTraits (Pagel et al., 2004). First, we reconstructed the evolution of toxicity using BayesMultiState in an MCMC framework over several important most-recent common ancestors, including the most-recent common ancestor of all pitohuis and

of all known poisonous species. We adjusted the ratedev parameter to achieve an acceptance rate between approximately 20–40%, set the burnin to a minimum of 100,000 iterations, but increased this to up to 500,000 iterations if there was evidence that the MCMC chain had not yet converged. To explore whether one character state was more likely than another, we used the "fossil" command that fixes the ancestral state, and compared the harmonic mean of the log likelihoods of the end of each run (see BayesTraits manual.) We tested whether the evolution of bright coloration was correlated with the evolution of toxicity in this group using the "dependent" and "independent" discrete options within BayesDiscrete, and comparing the resulting likelihoods of each run (Pagel and Meade, 2006).

3. Results

We obtained high quality DNA from all modern DNA samples. Three taxa were sequenced from museum-skin samples using ancient DNA techniques. These were the Papua, Indonesian representatives of *Pitohui kirhocephalus* (*P. k. pallidus*, *P. k. cerviniventris*) and the Morningbird of Palau (*Pitohui tenebrosus*). These specimens had degraded DNA, but we were able to sequence a portion of each gene for each taxon.

Alignments were relatively straightforward and were performed with the program Sequencher 4.7, although Clustal X 1.83 (Thomsen et al., 1993) was additionally used to assess and refine alignments for adenylate kinase and aldolase intron sequences.

For the phylogenetic analyses, gaps were simply coded as missing data. Although gaps certainly contain additional information, exactly how to code and model the evolution of insertions and deletions is less straight forward and subject to some debate. It was not necessary for us to consider these characters separately in order to obtain a well-supported tree, and so we did not pursue this further.

The total analyzed matrix contained 55 taxa and 3111 aligned characters (bases). Using Akaike Information Criteria (AIC) (Posada and Crandall, 1998; Posada and Buckely, 2004), The program Modeltest chose a GTR plus gamma rates and invariant sites model of evolution (GTR + G + I) as the best-fit model of sequence evolution. We then used AIC to additionally test a variety of partition-specific models of evolution. We considered a two-partition model (mitochondrial DNA and nuclear DNA), a five-partition model (a partition for each sequenced region—three contiguous mitochondrial regions and two nuclear introns), a six-partition model (a partition for each codon-site [1,2,3, and non-coding] for mtDNA and one for each nuclear intron), a seven-partition model (a partition for each gene), and a 15-partition model (a partition for each codon within each gene.) In these analyses, the 15-parameter site-specific model had the highest AIC with a weight of over 0.99%, suggesting that this was the best model of sequence evolution of all the models considered. Subsequent maximum likelihood searches and MrBayes runs used this 15-partition model of sequence evolution. At the request of an anonymous reviewer, we additionally explored the six-partition model (above) in which each codon-site and nuclear intron was independently parameterized using a GTR + G + I model (Nylander et al., 2004). The resulting 95% consensus tree matched that of our 15-parameter model and thus affirmed the robustness of the data signal to variation in our models.

PAUP∗ produced a single maximum likelihood tree (Fig. 1, −ln(likelihood) score = 25525.2048). MrBayes ran two runs for 10,000,000 generations each. We confirmed that the MCMC search reached burnin by the first 1,000,000 generations. Trees were saved every 500 generations thereafter, producing 36,000 trees after burnin for further analysis. All 36,000 trees were combined to produce a single 50% Majority-rule consensus tree in MrBayes.

Table 2
Parameter estimates for GTR plus site-specific rate model of evolution

Parameter	MLE rescaled	Bayesian mean	Credible interval	
			Lower	Upper
TL{all}		2.516857	2.426	2.61
r(A↔C){all}	0.054553381	0.053592	0.047817	0.059799
r(A↔G){all}	0.326717772	0.328629	0.306965	0.351049
r(A↔T){all}	0.041575374	0.041549	0.035439	0.048011
r(C↔G){all}	0.036225283	0.036704	0.029951	0.044139
r(C↔T){all}	0.496229908	0.493954	0.471014	0.516877
r(G↔T){all}	0.044698282	0.045571	0.035891	0.056132
pi(A){all}	0.311965	0.311692	0.297911	0.325622
pi(C){all}	0.30316	0.305884	0.293388	0.318463
pi(G){all}	0.151753	0.149598	0.13919	0.160386
pi(T){all}	0.233121	0.232826	0.221673	0.244196
CO2POS1	0.286201	0.292659	0.169051	0.431291
CO2POS2	0.192148	0.197467	0.104056	0.321357
CO2POS3	2.525978	2.620215	2.084999	3.264503
Tlys	0.749408	0.754679	0.621689	0.912709
A8POS1	1.030115	1.039943	0.871042	1.264273
A8POS2	0.545178	0.536075	0.437412	0.657528
A8POS3	3.349135	3.327608	2.926616	3.74217
CYTBPOS1	0.496251	0.494193	0.431871	0.571086
CYTBPOS2	0.123046	0.118035	0.089662	0.148456
CYTBPOS3	2.974521	3.000189	2.841607	3.177105
ND2POS1	0.864654	0.860578	0.78705	0.95274
ND2POS2	0.198206	0.196486	0.168918	0.25095
ND2POS3	3.691949	3.718343	3.55178	3.904685
Ald	0.268904	0.267384	0.239479	0.302411
AK	0.245338	0.246718	0.216731	0.278477

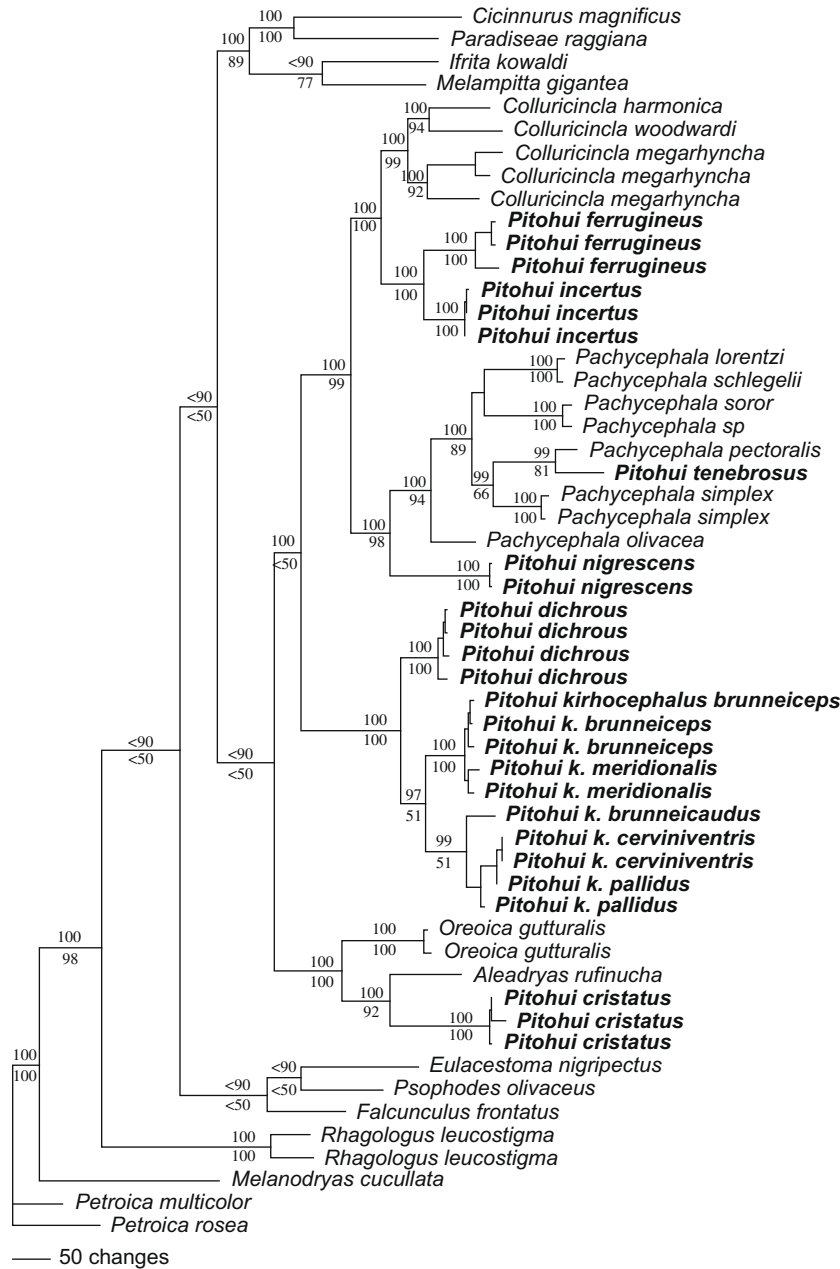The rates estimates show the relative rates for each partition.

Fig. 1. Most likely phylogenetic tree, based upon PAUP* likelihood analysis using a 15-partition model of evolution (see Section 3). Bayesian posterior probabilities from MrBayes 3.1.1 were multiplied by 100 and appear above each node; parsimony bootstrap percentages appear below the node. Taxa currently classified in the genus *Pitohui* are noted in bold.

The topology of the Bayesian consensus tree was completely consistent with the topology of the maximum likelihood tree produced by PAUP* (see Fig. 1). Bayesian posterior probabilities were calculated for each node and are provided in the figure.

Because all sampled taxa belong to the core corvoidea (sensu Barker et al., 2004), we were unable to independently provide an appropriate root for our phylogeny. We therefore rooted the phylogeny consistently with other studies having broader sampling (Sibley and Ahlquist, 1982, 1985, 1987, 1990; Barker et al., 2002, 2004). Once rooted (i.e., with *Petroica* at the base), our results for Pachycephalidae did not conflict with the results of these other studies in any significant ways, although our taxon sampling is very different.

We used the program BayesTraits (Pagel et al., 2004) to explore whether the most-recent common ancestor of pitohuis were toxic or non-toxic. *Ifrita* and all *Pitohui* species except *Pitohui tenebrosus* have been tested for the presence of BTX, as well as several other species (Dumbacher, 1997; Dumbacher et al., 2000). All *Pitohui* species were toxic with the exception of *Pitohui incertus* that was non-toxic for the three individuals tested. *Melampitta gigantea*, *Eulacestoma*, *Pachycephala schlegelii*, and *Rhagologus leucostigma* all tested negative for BTXs. A single *Colluricincla megarhyncha* tested positive for very low toxin levels, while two others failed to show toxins. The toxicity of other species in our phylogeny was unknown, so in the BayesTraits analyses, we coded the remaining untested species as unknown. In likelihood analyses of each Bayesian tree, the most-recent common ancestor of all pitohuis was reconstructed as non-toxic with probability >0.9999. To combine the analyses across trees in an MCMC analysis, the harmonic means of the log likelihoods for toxic vs. non-toxic common

ancestors were compared using likelihood ratio tests. Again, a non-toxic most-recent common ancestor received significantly greater support [harmonic mean likelihoods for toxic (−26.425733) vs. non-toxic (−19.320637), $p > 0.9999$, (Pagel, 1999). We repeated these analyses for several other important nodes, including using the most-recent common ancestor of all poisonous birds (*Pitohui* plus *Ifrita*), which was also estimated to be non-toxic ($p > 0.9999$, see Fig. 2).

We performed a final analysis using BayesTraits to test whether the evolution of bright coloration is correlated with the evolution of toxicity. BayesTraits calculates the likelihoods of two models—one in which bright coloration is dependent upon toxicity, and the other in which the two traits are evolving independently on the tree. We used a likelihood ratio test to compare the harmonic means of the MCMC likelihoods of the two models. The independent model (likelihood = −42.5956) provided a better fit than the dependent model (likelihood = −43.3755), although the support for the independent evolution of bright coloration and toxicity is not statistically significant ($p < 0.212$). Although power is reduced by our lack of toxicity data for some taxa, there is clearly no tight relationship between bright coloration and toxicity in this phylogeny.

4. Discussion

The current classification of the genus *Pitohui* includes at least the six New Guinea species (Greenway et al., 1967; Sibley and Ahlquist, 1990; Sibley and Monroe, 1990; Dickinson, 2003). Even though *Pitohui* has taken different genus names (including *Rectes* (Reichenbach, 1850), *Rhectes* (Salvadori, 1881)) or has been further split (*Pseudorectes* for *P. ferrugineus*, or *Melanorectes* for *P. nigrescens* (Sharpe, 1877)), these species have long been considered congeneric or members of closely related genera. Our phylogeny clearly shows, with strong support, that these six species do not form a monophyletic group. Instead, *P. nigrescens* is allied with the whistlers (genus *Pachycephala*). *Pitohui ferrugineus* and *P. incertus* are allied with the shrike-thrushes (genus *Colluricincla*). *Pitohui cristatus* is sister to *Aleadryas*, and these two are sister to the Crested Bellbird of Australia (*Oreoica gutturalis*). The two remaining *Pitohui* species (*P. dichrous* and *P. kirhocephalus*) form a distinct clade. Based upon data presented here, we are revising the genus *Pitohui* and reviewing the application of taxonomic names, and this will be presented elsewhere.

Pitohui dichrous and *P. kirhocephalus* form a monophyletic group with strong phylogenetic support. These are the most toxic of the batrachotoxin-bearing pitohuis (Dumbacher et al., 1992, 2000; Dumbacher and Fleischer, 2001) as well as the most conspicuously colored species. In the likelihood and Bayesian phylogenies, *P. kirhocephalus* is monophyletic as well, however, the support is poor, and parsimony analyses do not unequivocally support this monophyly. The confusing variation within this species has been long recognized (Beehler et al., 1986) and results from three highly differentiated genetic groups within *P. kirhocephalus* that distinguish the north-coast, south-coast, and West Papuan Island clades (Dumbacher et al., 2004). Other analyses directed at biogeography continue to support further splitting *P. kirhocephalus* (Dumbacher,
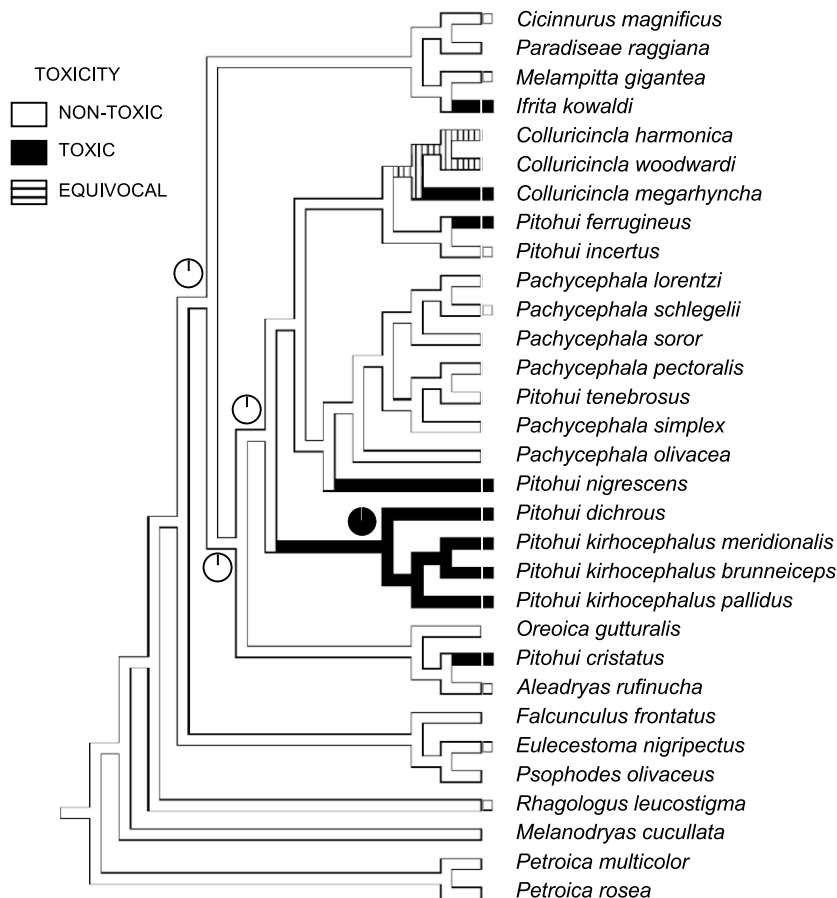

Fig. 2. Summary of BayesTraits analysis of toxin evolution. This partial cladogram roughly depicts the mapping of toxicity onto the *Pitohui* phylogeny. The pie diagrams associated with key nodes denote the relative support for a toxic (black) or non-toxic (white) ancestor.

unpublished data) and the data presented here provide strong support for each of these major groupings. Nonetheless, these groups resemble each other in plumage and behavior, and they form a natural unit. The degree of reproductive isolation among the *P. kirhocephalus* clades is unknown, but the DNA sequence data suggest that the three clades (based on both mtDNA and nuclear DNA sequences) have not been sharing genes for a substantial amount of time. The average pairwise distance among species within this clade (HKY85 distance, mtDNA only) is 6.9%, while the average distance between taxa within this clade and other core pachycephalid taxa is around 20%. We can conclude that *P. dichrous* and *P. kirhocephalus* are quite distantly related to the other Pachycephalidae genera in our phylogeny, including the other species now currently classified in the genus *Pitohui*.

Because our results indicate that the various *Pitohui* taxa are not closely related and are somewhat basal within the core corvoidea, we felt that this suggested that toxicity (or an adaptation allowing the sequestration of toxins) may be more widespread within the group. We attempted to test this specifically by measuring toxicity in several other species, including *Rhagologus*, brightly colored *Pachycephala*, similarly shaped and colored *Melampitta gigantea*, *Colluricincla megarhyncha*, and in *Pomatostomus isidorei* (not examined in this phylogeny). Only *Colluricincla* tested positive for toxins, and only in a single individual that had barely detectable toxin levels (Dumbacher et al., 2000). Most rural New Guineans maintain a tradition of hunting and eating birds, so we additionally surveyed local knowledge from over 16 villages throughout Papua New Guinea. The only birds ever suggested to carry toxins were the pitohuis, *Ifrita*, and in a couple of cases, *Eulacestoma*. By mapping these data on a phylogeny using likelihood and MCMC techniques, our analyses suggested that toxicity evolved several times independently within the core corvoidea, and that toxicity is not ancestral within corvoids. Other data suggest that pitohuis are insensitive to batrachotoxins and that they likely sequester toxins from their diet (Dumbacher et al., 2004). These may be key innovations that allowed toxin sequestration to evolve multiple times, and this warrants future research. Nonetheless, toxicity itself does not appear to be ancestral to corvoids or an important factor in corvoid radiation.

Some pitohuis may be aposematically colored. The colors used by *Pitohui dichrous* and *P. kirhocephalus* are very similar to the burnt orange and black of the monarch butterfly—a toxic butterfly classically considered to be aposematically colored. These two *Pitohui* species are also the two most toxic species, suggesting a correlation between toxicity and aposematism. Many related nontoxic species are also brightly colored—including many *Pachycephala* species (golden whistlers), *Falcunculus*, and birds of paradise. Our tests of the correlated evolution of bright coloration and toxicity argued that bright coloration is not tightly co-evolving with toxicity. This is likely because birds may be brightly colored for a variety of reasons—including sexual selection. As sexually selected species generally have brighter males than females, we can remove much of the effect of sexual selection by reanalyzing the coloration of females (Gotmark, 1994). In our phylogeny, the only species with aposematically colored females are *Pitohui dichrous*, *Pitohui kirhocephalus*, and *Falcunculus*. This analysis provided a somewhat stronger, but still non-significant association of bright coloration and toxicity. The weak association probably resulted because there are several other toxic species that do not have typical aposematic coloration, including the highly toxic *Ifrita*, and the mildly toxic *Pitohui ferrugineus*, *Pitohui cristatus*, and *Pitohui nigrescens*.

Interestingly, the six *Pitohui* species appear to have evolved convergently in a number of important characteristics. First, five of the six species have been shown to carry neurotoxic batrachotoxins in their skin and feathers, with the single exception of *P. incertus* (while in most cases their closest relatives have not). Second, pit-

ohuis are active participants and leaders of mixed-species flocks in New Guinea (Diamond, 1987). These flocking birds tend to be vocal, conspicuous, and gregarious—all characteristics that have been postulated to be associated with toxicity (Guilford, 1986; Sillen-Tullberg and Leimar, 1988; Gagliardo and Guilford, 1993; Riipi et al., 2001). Sister taxa to the pitohuis (*Colluricincla*, *Pachycephala*, *Oreoica*) do not even participate in these mid-sized mixed-species flocks (Diamond, 1987). Third, pitohui species are similar in size, morphology, and ecology—enough so to have been consistently and erroneously classified into a single genus (Stresemann, 1914) by generations of taxonomic ornithologists. Thus, there appears to be general convergent evolution in coloration, size, morphology, and behavior, as well as in toxicity. This independent evolution of overall similarity may function as Müllerian mimicry, where multiple toxic species resemble each other to share the cost of educating predators. None of the pitohui species are as conspicuously colored as *P. dichrous* and *P. kirhocephalus*, but Müllerian mimicry is not expected to produce perfect mimics (Sheppard et al., 1985) and predators are expected to learn general signals when multiple species are toxic (Rowe et al., 2004; Ihalainen et al., 2007).

Mixed-species flocks may also include Batesian mimics. Diamond, (1987) previously noted that several other species (Rufous Babbler, Black Cuckoo-shrike, birds of paradise, etc.) also appear to have converged upon the black and brown colors that are common among flocking birds. As these flocks of birds sift through the under-story foliage, flock members look, and behave similarly. Pitohuis tend to form the leaders of these flocks and tend to be more behaviorally conspicuous in comparison to other species (Diamond, 1987). Thus, the convergence of these other species may be a form of Batesian mimicry (Diamond, 1992), where a non-toxic species resembles toxic species to dupe a predator. These speculations regarding Batesian and Müllerian mimicry require further testing; however, the convergence among "pitohuis" is intriguing and represents one of the best candidates for mimicry in an avian system.

Visual mimicry among pitohuis and other birds implies that these signals evolved for visual predators, and thus implies that the toxins have an anti-predator function. We know that the toxins are strong enough to deter human predators (Salvadori, 1881; Kocher-Schmid, 1991, 1993) and irritate buccal membrane receptors of snakes (unpublished data). We suspect that other vertebrates (mammalian nest predators and hawks) are also sensitive to the toxins and are intelligent enough to learn to avoid pitohuis. This in no way precludes that the toxins have an anti-ectoparasite function as well. Instead, we suggest that the toxins are likely "broad spectrum" poisons that offer a variety of advantages (Dumbacher et al., 2004).

A final interesting finding of our work is that the Morningbird of Palau, *Pitohui tenebrosus*, is actually a whistler, and according to our phylogeny may be closely related to the widely distributed *Pachycephala pectoralis*. Its coloration and size are also quite divergent from *P. pectoralis* despite the relatively close relationship. This suggests that island forms can rapidly and profoundly evolve morphologically when in a novel environment, and it supports the concept of "islands as engines" of evolution (Filardi and Moyle, 2005).

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