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Polyphyletic origin of toxic Pitohui birds suggests widespread occurrence of toxicity in corvoid birds

Knud A. Jønsson[1,2,*], Rauri C. K. Bowie[2], Janette A. Norman[3,4,5], Les Christidis[4,5,6] and Jon Fjeldså[1]

[1] *Vertebrate Department, Zoological Museum, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen Ø, Denmark*
[2] *Museum of Vertebrate Zoology and Department of Integrative Biology, 3101 Valley Life Science Building, University of California, Berkeley, CA 94720-3160, USA*
[3] *Sciences Department, Museum Victoria, GPO Box 666, Melbourne, Victoria 3001, Australia*
[4] *Bio21 Institute, and* [5] *Department of Genetics, University of Melbourne, Parkville, Victoria 3052, Australia*
[6] *Division of Research and Collections, Australian Museum, 6 College Street, Sydney, New South Wales 2010, Australia*
**Author for correspondence (kajonsson@snm.ku.dk).*

Pitohui birds from New Guinea have been found to contain a toxin otherwise only found in neotropical poison arrow frogs. Pitohuis have been considered to be monophyletic and thus toxicity is thought to have evolved once in birds. Here, we show that Pitohuis, rather than being a tight-knit group, are polyphyletic and represent several lineages among the corvoid families of passerine birds. This finding demonstrates that the ability to be toxic is widespread among corvoid birds and suggests that additional members of this radiation, comprising more than 700 species, could prove to be toxic. It is postulated that toxic birds ingest the toxin through their insect diet and excrete it through the uropygial gland, from where it is applied to the skin and feathers. Thus, the ability to become toxic is most likely an ancestral condition but variation in diet determines the extent to which toxicity is expressed among corvoid birds. Variability in toxicity levels further suggests that the main function of the toxin is that of a deterrent against ectoparasites and bacterial infection rather than being a defence against predators as initially proposed.

Keywords: Pitohui; toxicity; systematics; batrachotoxin; phylogeny

1. INTRODUCTION

Fifteen years ago, the discovery that New Guinean Pitohui birds contained a toxin in their feathers and muscle tissue received much attention (Dumbacher *et al.* 1992). This toxin was identified as a homobatrachotoxin, a kind of neurotoxic steroidal alkaloid previously known only from the skins of five neotropical frog species in the genus *Phyllobates* (Dendrobatidae; Dumbacher *et al.* 2000). This finding was particularly remarkable because it was the first time that a potentially defensive toxin was identified in a bird. All six species in the genus *Pitohui* were scrutinized, revealing considerable individual variation in toxicity (Dumbacher *et al.* 2000). Two taxa, *Pitohui dichrous* and *P. kirhocephalus*, were particularly toxic; *P. cristatus*, *P. nigrescens* and *P. ferrugineus* were mildly toxic; and no toxins were detected in *P. incertus*. Traces of the toxin were found in a putative close relative of the Pitohuis, *Colluricincla megarhyncha*, but none was found in other putative relatives in the New Guinea region (*Pachycephala schlegelii*, *Rhagologus leucostigma* and *Eulacestoma nigropectus*; Dumbacher *et al.* 2000).

More recently, it was discovered that the birds may obtain the batrachotoxins from their diet, specifically from the poisonous melyrid beetle Choresine (Dumbacher *et al.* 2004). It was also found that the bird *Ifrita kowaldi* contained a similar spectrum of batrachotoxins as *Pitohui*. At the time, however, no reliable phylogenetic framework was available to determine the relationship between *Ifrita* and *Pitohui*, and thus whether the ability to tolerate this toxin in the body is restricted to a specific avian lineage (Dumbacher *et al.* 2004).

By placing Pitohui birds in a broader phylogenetic context, we demonstrate that toxic birds span the phylogeny of the corvoid assemblage of bird families in the Papuan region, and we propose a widespread mechanism to use toxins from the diet as defence, mainly against ectoparasites or dermal infections.

2. MATERIAL AND METHODS

(a) *Taxon sampling, amplification and sequencing*

We obtained sequence data of 26 taxa sampled across the Crown Corvida radiation (table 1) representing all major families and including some aberrant Australo-Papuan taxa that have been suggested to be closely related to *Pitohui*. Three nuclear gene regions, myoglobin intron 2 (Myo2), ornithine decarboxylase (ODC) introns 6–7 and glyceraldehyde-3-phosphodehydrogenase (G3PDH) intron 11, were sequenced. These genes have previously been shown to be useful for resolving phylogenetic relationships in birds (e.g. Irestedt *et al.* 2006; Jønsson *et al.* 2007). For *R. leucostigma*, *I. kowaldi* and *E. nigropectus*, we only obtained sequence data for Myo2.

The combined alignment consists of 1661 bp. For more details of indel length and positions, see the alignments of the individual gene regions deposited in GenBank. For primers, alignment, extractions, amplifications and sequencing procedures, see Irestedt *et al.* (2006) and Jønsson *et al.* (2007).

(b) *Phylogenetic inference*

Owing to the rather low number of insertions in the introns, the combined sequences could easily be aligned. All gaps have been treated as missing data in the analyses. Bayesian inference (BI) and maximum likelihood (ML) were used to determine phylogenetic relationships. Models of nucleotide substitution used in the analyses were selected for each gene individually by applying the Akaike Information Criterion implemented in MrModeltest v. 2.2 (Nylander 2005) in conjunction with PAUP*b10 (Swofford 2001).

Posterior probabilities of trees and parameters in the substitution models were approximated with MCMC and Metropolis coupling using the program MrBayes v. 3.1.1 (Huelsenbeck *et al.* 2001; Ronquist & Huelsenbeck 2003). Analyses were performed for both the individual gene partitions and the combined dataset, where each gene region was unlinked allowing for independent estimation of parameters. The chains for the individual gene partitions and for the combined dataset were all run for 10 million generations, with trees sampled every 100 generations. The trees sampled during the burn-in phase were discarded after checking for convergence and the final inference was made from the concatenated outputs.

The priori selection of nucleotide substitution models suggested that the GTR+Γ model had the best fit for all three gene regions, but as the nucleotide state frequencies and gamma distribution differed between the partitions, we still applied a partitioned analysis of the combined dataset. After discarding the burn-in phase, the inference for the individual genes and the combined dataset were based on a total of 95 000 samples each. The posterior

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Table 1. Taxa used in the study. (Acronyms are AM, Australian Museum, Sydney, Australia; ANWC, Australian National Wildlife Collection, Canberra, Australia; FMNH, Field Museum of Natural History, Chicago, USA; MCSNC, Museo Civico di Storia Naturale di Carmagnola, Italy; MV, Museum Victoria, Melbourne, Australia; NRM, Swedish Museum of Natural History, Stockholm, Sweden; ZMUC Zoological Museum of Copenhagen, Denmark. All samples are vouchered.)

species	voucher/tissue number	origin	G3PDH	ODC	Myo2
Aleadryas rufinucha	NRM543658	New Guinea	EU273375	EU273355	EU273395
Colluricincla harmonica	MV1422	Australia	EU273376	EU273356	EU273396
Colluricincla megarhyncha	MV C391	Australia	EU273377	EU273357	EU273397
Coracina lineata	MV JCW073	Australia	EU273378	EU273358	EU273398
Coracina papuensis	MV C861	Australia	EU273379	EU273359	EU273399
Corcorax melanorhamphos	AM LAB 1059	Australia	EF441214	EF441236	AY064737
Eulacestoma nigropectus	MV B.20041	New Guinea			EU273400
Hylophilus ochraceiceps	ZMUC127900	Ecuador	EU272087	EU272109	EU272100
Ifrita kowaldi	ANWC26890	New Guinea			EU273402
Lalage melanoleuca minor	ZMUC95259	Mindanao	EU273381	EU273361	EU273403
Malurus amabilis	MV C803	Australia	EF441219	EF441241	AY064729
Oriolus chinensis	ZMUC123918	Indonesia	EU273382	EU273362	EU273404
Oriolus flavocinctus	MV1603	Australia	EF441221	EF441243	EF441258
Oriolus oriolus	MCSNC1415	Italy	EF052755	EU273363	EF052766
Orthonyx temminckii	MV B831	Australia	EF441222	EF441244	AY064728
Pachycephala melanura	MV1248	Australia	EU273383	EU273364	EU273405
Pachycephala olivacea	MV1826	Australia	EU273384	EU273365	EU273406
Pachycephala pectoralis	MV3477	Australia	EU273385	EU273366	EU273407
Pachycephala schlegelii	MV E200	New Guinea	EU273386	EU273367	EU273408
Pachycephala simplex	MV E498	New Guinea	EU273387	EU273368	EU273409
Pachycephalopsis hattamensis	NRM552153	New Guinea	EF441224	EF441246	EF441260
Pericrocotus cantonensis	NRM569470	Laos	EU273388	EU273369	EU273410
Pitohui cristatus	MV E061	New Guinea	EU273389	EU273370	EU273411
Pitohui dichrous	MV E545	New Guinea	EU273390	EU273371	EU273412
Pitohui ferrugineus	MV E506	New Guinea	EU273391	EU273372	EU273413
Pitohui kirhocephalus	FMNH 280697	New Guinea	EU273392		EU273414
Pitohui nigrescens	MV E246	New Guinea	EU273393	EU273373	EU273415
Rhagologus leucostigma	ANWC26897	New Guinea			EU273416
Vireo flavoviridis	ZMUC124543	Panama	EU273394	EU273374	EU273417

distribution of topologies is presented as a 50% majority-rule consensus tree from the combined analysis in figure 1.

The trees obtained from the Bayesian analyses of the individual genes (not shown) are topologically congruent overall, and all gene trees support the same relationships for Pitohuis. In fact, there are no topological conflicts that are supported by posterior probabilities above 0.95, and the combined tree is also in good topological agreement with other molecular studies of major relationships among Crown Corvida passerines which includes shrikes, bush-shrikes, butcher-birds, drongos, fantails, monarchs, crows and birds of paradise (Jønsson & Fjeldså 2006*a*).

Maximum-likelihood analyses were performed using Garli v. 0.95 (Zwickl 2006). Five independent analyses were performed using a GTR+I+Γ model and default settings. Nodal support was evaluated with 500 non-parametric bootstrap pseudoreplications. The score of the best likelihood tree ($-\ln L$ 7224.84258) was within 0.025 likelihood units of the best tree recovered in each of the other four runs, suggesting that the five runs had converged. The ML tree topology was almost completely congruent with the BI topology. No differences were found for well-supported nodes.

3. RESULTS AND DISCUSSION

Analyses of the three markers clearly demonstrate that the genus *Pitohui* is highly polyphyletic, with individual species widely dispersed among Crown Corvida lineages (figure 1), which has its centre of origin in the Australo-Papuan region (Jønsson & Fjeldså 2006*b*) and counts more than 700 species worldwide. This suggests that the propensity to assimilate batrachotoxins from dietary sources into feather structures and muscle tissues is widespread among the basal lineages of the Crown Corvida clade of songbirds (figure 1). The two most toxic taxa *P. dichrous* and *P. kirhocephalus* are sister species and closely related to Old World *Oriolus*. *P. nigrescens*, a mildly toxic species, is sister to all true *Pachycephala* whistlers, whereas the mildly toxic *P. cristatus* is closely related to *Aleadryas rufinucha*, a species distantly related to the *Pachycephala* radiation. *P. ferrugineus*, which has often been found to be devoid of batrachotoxins (Dumbacher *et al.* 2004), is closely related to the Australo-Papuan *Colluricincla* shrike-thrushes, which were also demonstrated to have traces of batrachotoxins (Dumbacher *et al.* 2004). *I. kowaldi* is not closely related to any *Pitohui* species.

We find little reason to believe that the toxin is produced de novo but rather that it is assimilated in birds, which, among a wide range of food items, feed on toxic melyrid beetles. We suspect that corvoid birds—notably the caterpillar-loving Campephagidae—eat poisonous insects and have developed a specific ability to tolerate various toxins and excrete them through the uropygial gland. This is probable considering the structural similarity between cholestanol (in the uropygial gland secretions) and one of the batrachotoxins (BTX-A-*cis*-O-cronate; Dan Stærk 2007, personal communication). Furthermore, the uropygial gland secretions of birds are known to contain a high diversity of biocidal/poisonous substances, which are potential agents for defence against ectoparasites, skin fungi, keratin-degrading bacteria, etc. (Jacob 1978; Poulsen 1993).

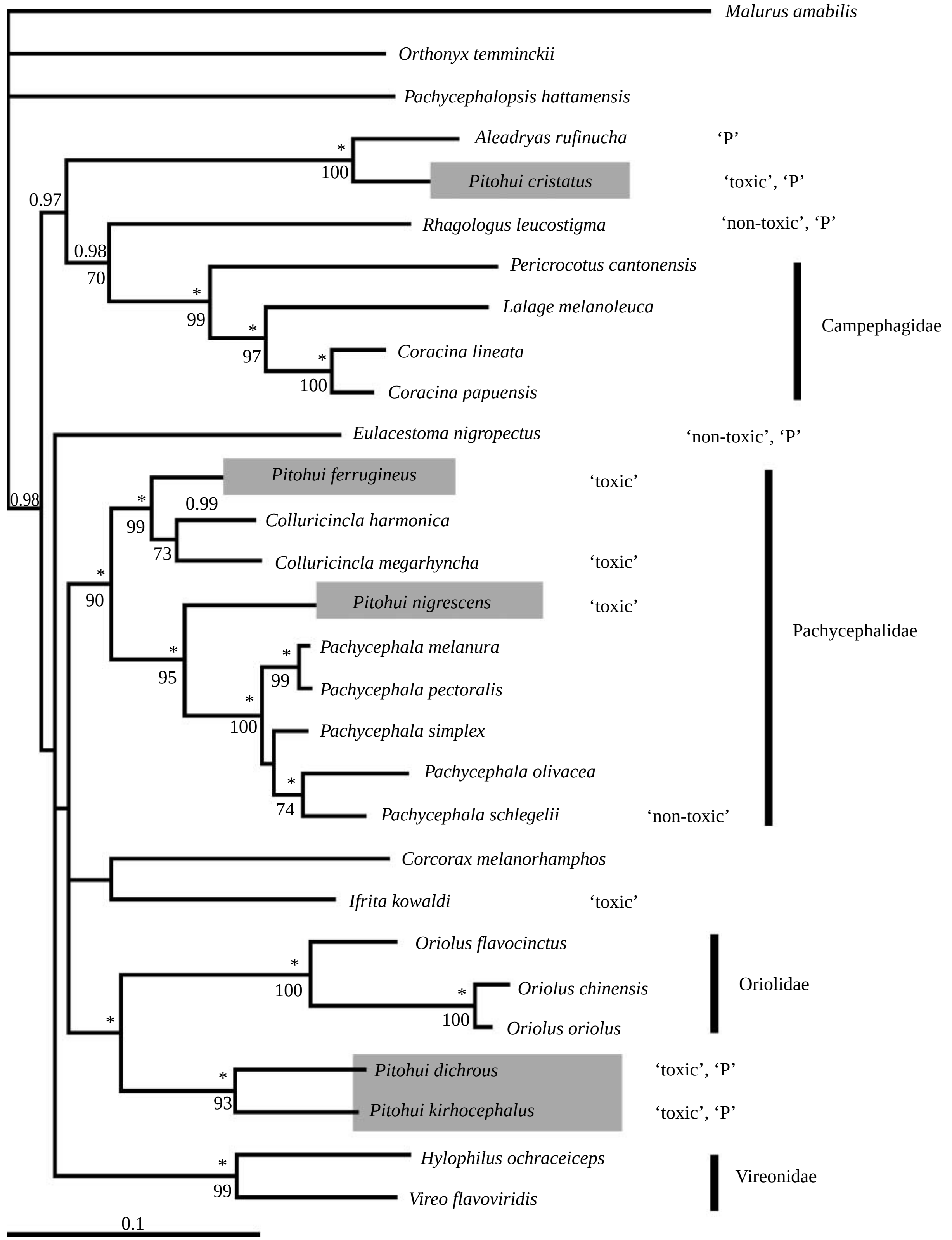

Figure 1. The 50% majority-rule consensus tree of 23 Crown Corvida species obtained from Bayesian analysis of the combined dataset (G3PDH, Myo2 and ODC). Posterior probability values greater than 0.95 are indicated above nodes (asterisk marks 1.00 posterior probabilities) and ML bootstrap support values greater than 70 are indicated below nodes. Family names are indicated to the right; 'P' marks species that have erroneously been referred to as Pachycephalidae in traditional classifications. 'Toxic' denotes species that are known to contain batrachotoxins or traces of batrachotoxins and 'non-toxic' denotes species known not to contain batrachotoxins or traces of batrachotoxins as demonstrated by Dumbacher *et al.* (2000).

Given that batrachotoxins have been recorded in five different lineages of corvoid birds, it seems probable that the ability to 'handle' the toxin is widespread among members of the Crown Corvida, but that there is some variation in diet or the 'risks' associated with being exposed to melyrid beetles as prey. Where melyrid beetles (or other prey containing batrachotoxins) are abundant, it is probable that opportunistically foraging birds such as orioles and cuckoo-shrikes (Poulsen 1993) will feed on these prey items and become toxic. This could go undetected simply because these birds will feed on whatever items are easily accessible and thus toxicity levels are likely to vary in space and time. We predict that with additional field research many more species of corvoid birds will be found to harbour toxins.

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