

# Phylogenetic evidence for colour pattern convergence in toxic pitohuis: Müllerian mimicry in birds?

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Bird species in the genus *Pitohui* are chemically defended by a potent neurotoxic alkaloid in their skin and feathers. The two most toxic pitohui species, the hooded pitohui (*Pitohui dichrous*) and the variable pitohui (*Pitohui kirhocephalus*), are sometimes strikingly patterned and, in certain portions of their geographical ranges, both species share a nearly identical colour pattern, whereas in other areas they do not. Müllerian mimicry (the mutual resemblance of two chemically defended prey species) is common in some other animal groups and *Pitohui* birds have been suggested as one of the most likely cases in birds. Here, we examine pitohui plumage evolution in the context of a well-supported molecular phylogeny and use a maximum likelihood approach to test for convergent evolution in coloration. We show that the 'mimetic' phenotype is ancestral to both species and that the resemblance in most races is better explained by a shared ancestry. One large clade of *P. kirhocephalus* lost this mimetic phenotype early in their evolution and one race nested deep within this clade appears to have re-evolved this phenotype. These latter findings are consistent with the hypothesis that Müllerian mimicry is driving the evolution for a similar colour pattern between *P. dichrous*, but only in this one clade of *P. kirhocephalus*.

**Keywords:** *Pitohui*; toxic birds; molecular systematics; mitochondrial DNA; Müllerian mimicry; New Guinea

## 1. INTRODUCTION

Bird species in the endemic New Guinea genus *Pitohui* contain potent defensive toxins in their skin and feathers (Dumbacher *et al.* 1992, 2000). Evidence suggests that these toxins may defend pitohuis from human hunters (Majnep & Bulmer 1977; Kocher-Schmid 1991, 1993), as well as ectoparasites (Mouritsen & Madsen 1994; Dumbacher 1999). Pitohui toxins belong to the well-known batrachotoxin family of compounds, which attack voltage-dependent sodium channels (Daly & Spande 1986). Because most higher animals have these sodium channels and because the channels are highly conserved, it is expected that these toxins could defend against a wide variety of natural predators, including other visual predators such as hawks and some snakes.

The genus *Pitohui* contains six species, of which *Pitohui kirhocephalus* and *Pitohui dichrous* are significantly more toxic than the others (Dumbacher 1997; Dumbacher *et al.* 2000). *Pitohui dichrous* sometimes carries enough toxin to irritate buccal membranes and cause sneezing and watery eyes if merely handled (Salvadori 1881; Kocher-Schmid 1991). *Pitohui kirhocephalus* and *P. dichrous* also have bolder plumage patterns; *P. dichrous* has a brick-red belly and back that contrasts with a jet-black head, wings and tail. Contrasting colour patterns are common among toxic animals and are often thought to serve as warning or 'aposematic' signals for visually orientated predators (Cott 1940; Owen 1980).

The plumage coloration of *P. kirhocephalus* varies tremendously across its geographic range and as many as 20 morphologically diagnosable subspecies are recognized

(Mayr 1941; Rand & Gilliard 1967). Four different *P. kirhocephalus* subspecies in northwest New Guinea, southeast New Guinea and on the Wandammen Peninsula almost perfectly resemble the colour pattern of *P. dichrous* (figure 1). This colour pattern is a brick-red belly and back that contrasts with a jet-black head, wings and tail and is hereafter called the 'type 1' plumage pattern. Intervening *P. kirhocephalus* races can have strikingly different colour patterns from *P. dichrous*, for example some have grey or brown hoods and a lighter back, some have a more tawny breast and belly plumage, some are shades of light grey–brown throughout and still others are different shades of bright ferruginous throughout (figure 1). From their distribution it appears that multiple *P. kirhocephalus* races could have independently evolved the type 1 plumage pattern via selection for Müllerian mimicry of *P. dichrous* (Diamond 1992; Dumbacher *et al.* 1992). Müllerian mimicry is the mutual resemblance of multiple, chemically defended prey species (Sheppard *et al.* 1985), in which the mimics benefit by sharing the cost of educating predators (Müller 1879). Müllerian mimicry is common in insects, such as butterflies, and pitohuis may represent a very rare case of Müllerian mimicry in birds (Dumbacher & Pruett-Jones 1996). However, an alternative hypothesis is that the type 1 plumage pattern resemblance is a historical artefact due to a shared primitive colour pattern (Dumbacher *et al.* 1992).

Performing direct tests for colour pattern selection and Müllerian mimicry between *P. kirhocephalus* and *P. dichrous* is currently impossible because pitohuis' natural habitat is remote and the crucial portions of their ranges are currently inaccessible. However, we can use a phylogenetic approach for examining colour pattern evolution in *P. kirhocephalus* and *P. dichrous*. If the type 1 colour pattern

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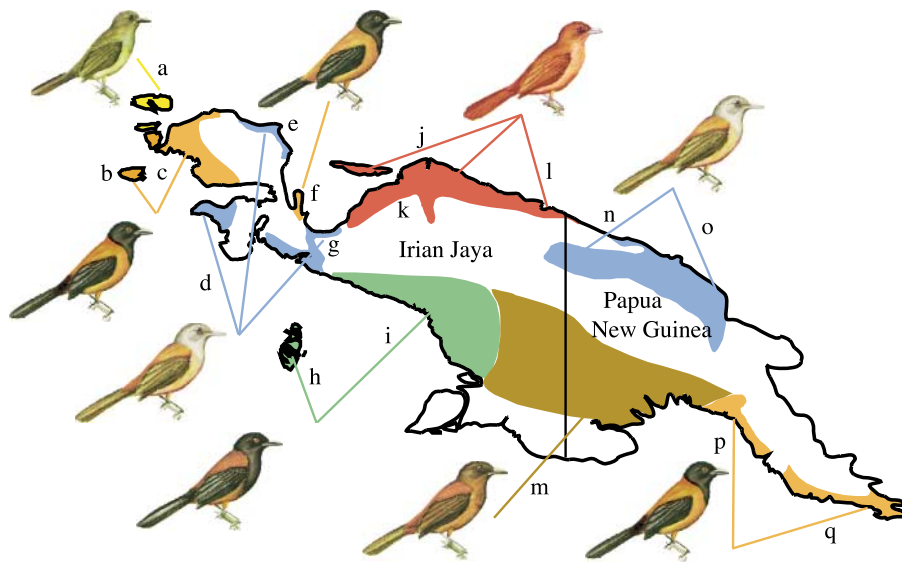


Figure 1. Map of *Pitohui kirhocephalus* subspecific ranges shown with *P. kirhocephalus* phenotypes. This unusual ‘checkerboard’ distribution of plumage types has puzzled ornithologists (Beehler *et al.* 1986), yet *P. kirhocephalus* is usually treated as a single species on the basis of similarity in their song and habits, clinal variation between some races and because no two races have been found in sympatry. *Pitohui dichrous* shows little geographical variation throughout its range and its plumage pattern is identical to that of *P. k. dohertyi* (f) found on the Wandammen Peninsula. The subspecies sampled for this study are marked with letters. ‘Type 1’ phenotypes (ranges shown in orange and green in b, c, f, h, i, p, q) are considered potential mimics of *P. dichrous*. The pitohui images were taken from Beehler *et al.* (1996). (© 1986 Princeton University Press.)

is pleisiomorphic or ancestral for the potentially mimetic subspecies, then this provides no phylogenetic evidence for Müllerian mimicry. Alternatively, if the type 1 colour pattern is derived multiple times from non-black-hooded ancestors, then this pattern is consistent with Müllerian mimicry and is taken as phylogenetic evidence for mimicry.

Here, we use DNA sequence data from two mitochondrial genes and two nuclear gene introns in order to reconstruct a phylogenetic hypothesis for *P. dichrous* and *P. kirhocephalus*. We sampled *P. kirhocephalus* tissue from 10 recently collected Papua New Guinea individuals and an additional 27 museum skins. Analyses of the entire *Pitohui* genus show that the *P. dichrous* and *P. kirhocephalus* races form a clade (J. P. Dumbacher and R. C. Fleischer, unpublished data). We therefore included 10 *P. dichrous* in order to help polarize characters within *P. kirhocephalus*, and we used two *Pitohui incertus* (white-bellied pitohui) individuals in order to root the tree.

**2. MATERIAL AND METHODS**

We measured pitohui plumage colour by matching their head, back, wings, belly, tail and rump colours to those of standard colour charts and translated their colours into Munsell notation (Smithe 1975). We illustrate the colour and pattern similarity by plotting the value of head colour against the contrast of head colour and back colour in figure 2. Those *P. kirhocephalus* races that cluster with *P. dichrous* have type 1 plumage patterns and are considered potential mimics in our analyses. It is important to note that the type 1 races are also similar in their overall plumage colour and plumage pattern throughout the body and not just on the hood and back. This is perhaps best illustrated by noting that local New Guinean hunters are renowned for their ability to distinguish between

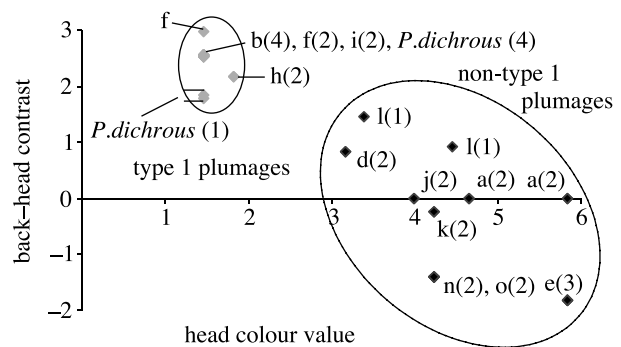


Figure 2. Head colour value versus back-head contrast of pitohui plumage. The Munsell ‘value’ measures brightness, with zero being pure black (‘jet black’, 1.46) and 10 being pure white. Back-head contrast is measured as the Munsell back colour value minus the head value. Letters reference *Pitohui kirhocephalus* subspecies from figures 1 and 3 and numbers in parentheses indicate the number of individuals of each subspecies represented by each point. Note that type-1-patterned subspecies clearly cluster with *Pitohui dichrous*.

bird species (Diamond 1966, 1972), yet in many places we visited, local New Guineans did not distinguish between type 1 *P. kirhocephalus* and *P. dichrous* species (Herowana Village, Eastern Highlands Province, Bonua Village, Central Province and Kakoro Village, Gulf Province, Papua New Guinea). In areas where the two species overlapped but were not similar, the local people invariably recognized them as distinct species.

**(a) DNA isolation and sequencing**

DNA was isolated from museum skins using standard ancient DNA techniques in a DNA isolation laboratory in which no modern DNA or post-polymerase chain reaction (PCR) samples

are handled (Cooper *et al.* 1996; Sorenson *et al.* 1999). A small toe pad fragment (2 mm × 5 mm × 0.5 mm) was digested overnight at 56 °C in lysis buffer (1 mM Tris, EDTA, DTT and proteinase k, pH 8.0), phenol and chloroform extracted and dialysed in centrifuge-assisted Centricon<sup>®</sup> tubes (Amicon, Beverly, MA, USA). Existing primers were modified slightly in order to amplify a 307 bp region in cytochrome *b* (Kocher *et al.* 1989) and a 196 bp mitochondrial DNA region that includes portions of tRNA-lysine and ATPase8 genes (L9051 and H9241) (Greenberg *et al.* 1998; Sorenson *et al.* 1999). We also amplified a 109 bp fragment of aldolase intron G (primers ald2f, 5'-ATCCAGGAGAACGCCAACAC-3' and ald3r, 5'-AACCCCTCACAGGGATTCTCC-3') and a 211 bp fragment of adenylate kinase intron 5 (primers AK5178f, 5'-CTCTACACGGCCCAA GAGAC-3' and AK5291r, 5'-GGGGCGATCTATGGACAG-3'). PCR reactions were prepared in a DNA-free hood and a single PCR reaction was run using Perkin-Elmer (Foster City, CA, USA) TaqGold DNA polymerase (10 min at 94 °C before the cycling began and then 45 cycles at 92 °C for 60 s, 50 °C for 45 s and 72 °C for 45 s denaturing, annealing and extension temperatures, respectively). The PCR products were purified and sequenced using dideoxy chain termination using recommended Applied Biosystems Inc. (Foster City, CA, USA) protocols and sequence reactions were cleaned using sephadex resin and run on an ABI 373-stretch automated sequencer.

### (b) Phylogeny and ancestral character state reconstruction

Sequences were aligned and edited with SEQUENCHER 3.1.1 software (GeneCode, Ann Arbor, MI, USA). Sequences were exported in Nexus format to MACCLADE 4.0 (Maddison & Maddison 2000) in which sequences from different genes were concatenated for each individual. The complete DNA data matrix was analysed using PAUP\* 4.0 (Swofford 1999). We used maximum-likelihood heuristic searches with a two-parameter model of sequence evolution (Hasegawa *et al.* 1985). We used the successive approximations method (Huelsenbeck 1998) in order to obtain a single best-fit tree and parameter estimate (see figure 3) (transition: transversion ratio = 5.097). We accepted this tree as the best working hypothesis for the relationships of *P. kirhocephalus* subspecies.

We then reconstructed the evolution of the type 1 phenotype onto this phylogeny using a maximum-likelihood approach with the computer program DISCRETE 4.0 (Pagel 1999a,b). Because nothing is known about the genetic basis for colour pattern in pitohuis, we made no starting assumptions and used the phylogeny for estimating ancestral states and evolutionary parameters. DISCRETE requires a fully resolved dichotomous tree, so unresolved nodes were arbitrarily resolved for analyses. DISCRETE estimated the probabilities of gains ( $\alpha$ ) and losses ( $\beta$ ) without restrictions and also by restricting to  $\alpha = \beta$ . The estimates of gains and losses were very similar to each other and to estimates when  $\alpha$  was restricted to equal  $\beta$ . The one-parameter model (e.g. setting  $\alpha = \beta$ ) did not lead to a significant reduction in likelihood when compared to the unrestricted two-parameter model (maximum-likelihood ratio test, LR = 0.075 561 and  $p = 0.783$ ), so all subsequent analyses used the simpler one-parameter model (Pagel 1999b). We used the method outlined by Mooers & Schluter (1999) for estimating ancestral character states using DISCRETE 4.0. Relative support for ancestral character state estimations is based upon 'local' likelihood estimates (*sensu* Pagel 1999). We compared the relative support based upon 'global' likelihoods as given by PAUP\* 4.0. For this

data set, estimates of relative support are similar using either global or local methods, but we report estimates based upon local likelihoods because they are more conservative.

### 3. RESULTS

Our phylogenetic analysis yielded a single most likely tree (figure 3). When rooting with *P. incertus*, our best tree contained a monophyletic *P. kirhocephalus*, but bootstrap support for this node was weak (47.1%). The phylogeny identified three highly divergent *P. kirhocephalus* clades: a north-coast clade (bootstrap support 97.6%), a south-coast clade (93.8%) and a West-Papuan Island clade (99.9%). The average HKY85-corrected (Hasegawa *et al.* 1985) pairwise distances for cytochrome *b* are 0.077 between the north-coast and south-coast clades, and 0.086 between the north-coast and West-Papuan Island clades, indicating a deep phylogenetic divergence between these groups. By assuming a passerine molecular clock rate of 0.016 sequence divergence per million years (Myr) for this portion of cytochrome *b* (Tarr & Fleischer 1993; Fleischer *et al.* 1998) and by subtracting the average within-clade pairwise distances (Wilson *et al.* 1885; Nei 1987), we can estimate approximately the time that these clades diverged. The estimated divergence of the north-coast and south-coast clades is 0.077 between (−0.017% within clades). Dividing this by 0.016 per Myr results in an age of divergence of *ca.* 3.7 Myr. For the north-coast/West-Papuan Island split we estimate *ca.* 4.6 Myr. Thus, these three major clades probably formed *ca.* 3.5–4.6 Myr ago.

We reconstructed the evolution of the type 1 phenotype using this phylogeny. Our analysis suggested that the probabilities of gains and losses of type 1 plumage were approximately equal ( $\alpha/\beta = 0.825$ ) and that the differences were not statistically significant (maximum-likelihood ratio test, d.f. = 1 and  $p = 0.783$ ). Because most type 1 *P. kirhocephalus* subspecies belonged to the south-coast clade, our analysis suggests that the ancestor of the south-coast clade also had the type 1 plumage pattern (relative support 0.999 for type 1 plumage). Therefore, the resemblance between *P. dichrous* and the south-coast *P. kirhocephalus* is best explained by shared ancestry rather than by convergent evolution.

However, in the north-coast and West-Papuan Island clades, 10 of the 11 *P. kirhocephalus* subspecies have non-type 1 plumage with the exception of the type 1 subspecies *Pitohui kirhocephalus dohertyi*, which is found on the Wandammen Peninsula (figure 1f). The relative support for non-type 1 plumage in the ancestor of both groups is 0.541. Our analyses indicate that the ancestor of the north-coast clade had non-type 1 plumage (relative support *ca.* 1.0). This *P. k. dohertyi* subspecies is phylogenetically nested deep within the north-coast clade. These maximum-likelihood analyses indicate that the immediate ancestors of *P. k. dohertyi* had non-type 1 plumage patterns and that *P. k. dohertyi* recently re-evolved the type 1 phenotype after it had been lost some 3.5–6 Myr ago.

In order to test whether the tree topology significantly supported the secondary gain of the type 1 phenotype in the north-coast clade, we searched for the next shortest tree that did not require additional losses or gains of the type 1 phenotype. The best alternative tree placed

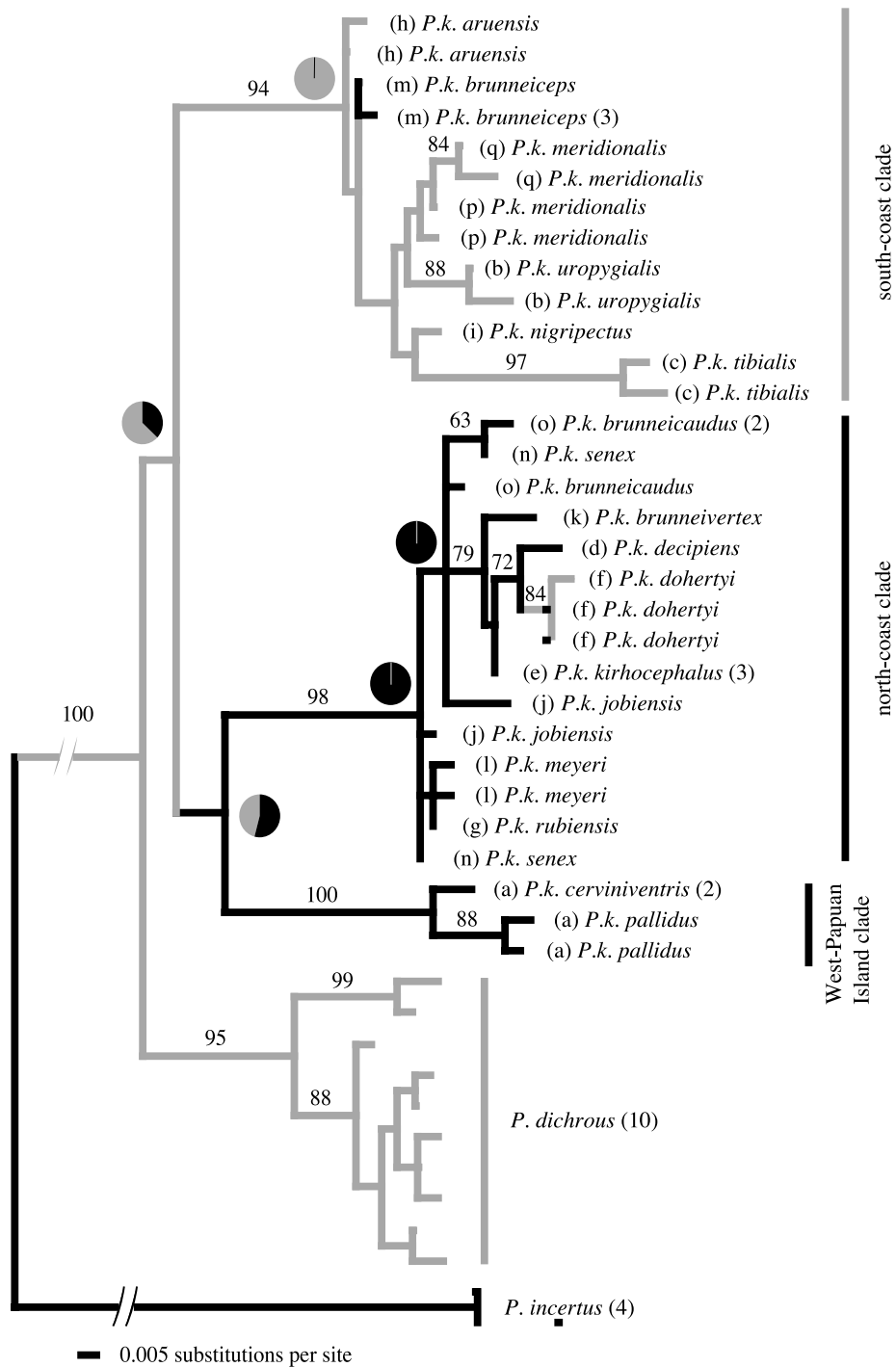


Figure 3. The total evidence maximum-likelihood phylogram showing the relationships of *Pitohui kirhocephalus* and *Pitohui dichrous*. Branches shorter than  $10^{-8}$  were collapsed to polytomies. Numbers above branches represent bootstrap support in 1000 maximum-likelihood, fast-addition bootstrap replications performed by PAUP\*. The letters preceding taxon names correspond to the taxon ranges in figure 1. The most parsimonious plumage character state reconstruction is shown, with type 1 branches shown in grey and non-type 1 branches shown in black. Maximum-likelihood relative support for character state reconstructions is shown with pie diagrams drawn adjacent to the ancestors of important clades. Numbers in parentheses indicate the number of individuals sequenced with identical haplotypes.

*P. k. dohertyi* as the sister taxon to the north-coast clade and was significantly longer (five additional steps) (Templeton non-parametric test,  $p < 0.0253$ ). We therefore conclude that both the tree topology and the character reconstructions indicate convergent evolution between *P. k. dohertyi* and *P. dichrous*. Thus, phylogenetic evidence refutes the null hypothesis that plumage similarity is

merely a result of shared ancestral character states in *P. k. dohertyi*.

By contrast, our data indicate no convergent evolution between *P. dichrous* and *P. kirhocephalus* in the south-coast clade. *Pitohui kirhocephalus meridionalis* and *Pitohui kirhocephalus uropygialis* look remarkably similar to each other and to *P. dichrous*, yet they are found at opposite ends of the

island, at least 2000 km apart. Our data suggest that *P. k. meridionalis* and *P. k. uropygialis* are closely related and possibly sister taxa, and have an average HKY85-corrected pairwise distance (Hasegawa *et al.* 1985) of only 0.012 in cytochrome *b*. A historical long-distance dispersal event better explains their unusual distribution.

When considering evidence of long-distance dispersal in the south-coast clade, we were concerned that dispersal and gene introgression might account for the type 1 plumage of *P. k. dohertyi*. However, three independently segregating genetic loci provided no evidence of gene flow from the south-coast clade. Mitochondrial genes, as well as phylogenetically informative transitions in each nuclear gene intron (adenylate kinase (C↔T) and aldolase (G↔A)), clearly identify *P. k. dohertyi* as a member of the north-coast clade and suggest that the type 1 phenotype evolved secondarily from non-type 1 ancestors.

#### 4. DISCUSSION

Our phylogenetic evidence argues that one major clade of *P. kirhocephalus* type 1 subspecies evolved their plumage pattern from type 1-patterned ancestors. In these cases, our phylogeny therefore provides no evidence of convergent evolution or support for Müllerian mimicry.

Our data for the one remaining type 1 subspecies *P. k. dohertyi* are more consistent with the hypothesis of Müllerian mimicry. For Müllerian mimicry to be a tenable explanation for the convergent evolution of the type 1 phenotype in *P. k. dohertyi*, two additional conditions must be met. First, both species must carry defensive chemicals. In every locality from which they have been sampled (Dumbacher 1997; Dumbacher *et al.* 2000), both *P. dichrous* and *P. kirhocephalus* carry potent alkaloid toxins, although their toxin concentrations vary greatly. Second, the ranges of *P. dichrous* and *P. kirhocephalus* must overlap. Although the two species have somewhat different altitudinal distributions, they are expected to overlap broadly from *ca.* 500 to 1100 m altitude (Rand & Gilliard 1967; Beehler *et al.* 1986; Coates 1990). Where collecting trips have been made, *P. dichrous* and *P. kirhocephalus* are often sympatric. For example, museum specimens at the American Museum of Natural History and Academy Of Natural Sciences of Philadelphia document sympatry in the Weyland Mountains, along the Idenburg River, Cyclops Mountains, Hollandia, Ifaar Lake Sentani, Adelbert Mountains, Madang, Huon Peninsula, Herowana Village, Karimui and So'obo. We also estimate that over 50% of the Wandammen Peninsula, where *P. k. dohertyi* is found, lies in this middle altitude range from 500 to 1100 m and *P. dichrous* has also been collected on the Wandammen Peninsula. We therefore expect that these two species have a broad overlap in this region.

Six of the 20 *P. kirhocephalus* subspecies resemble *P. dichrous* to a reasonable degree. Of those six, we only found strong phylogenetic evidence that refutes the null hypothesis of resemblance due to shared ancestry in *P. k. dohertyi*. Although this is not conclusive evidence for Müllerian mimicry, these data are consistent with the hypothesis that Müllerian mimicry helped drive plumage evolution on the Wandammen Peninsula. Although our data are consistent with the mimicry hypothesis, we suggest caution for several reasons. First, this is not a

direct test of mimicry. We are currently unable to perform the difficult tests that are necessary for implying direct selection, particularly in many of the remote and politically sensitive localities where pitohuis live. However, we can offer that the most promising area to conduct such studies would be on the Wandammen Peninsula where *P. k. dohertyi* and *P. dichrous* live sympatrically. Second, as nothing is known of the genetic basis of their colour pattern, it is possible that some other factor, genetic or ecological, may be responsible for the reversal to the type 1 phenotype in *P. k. dohertyi*. Third, multiple *P. kirhocephalus* subspecies show sexual plumage dichromatism, primarily in the south-coast clade and females have a slightly lighter head, wings and tail. It is possible that both type 1 and non-type 1 phenotypes were retained in the ancestors of *P. k. dohertyi* species through dichromatism, which would affect our character reconstruction in a way that is difficult to predict. Fourth, although we found no phylogenetic evidence for mimicry in the south-coast clade, selection for mimicry may still be strong and may be important for maintaining the colour resemblance over several million years. Again, direct studies of selection will be necessary in order to test this hypothesis.

Convergent evolution explained less of the overall geographical variation in *P. kirhocephalus* than we predicted. Our data imply that multiple factors may be working concurrently in order to produce the extreme morphological and genetic variation in this group. These factors include vicariance events (such as the formation of the central mountain ranges which isolated the north-coast and south-coast clades) and long-distance dispersal (which appears to have distributed the sister subspecies *P. k. meridionalis* and *P. k. uropygialis* to opposite ends of the island).

Why is *Pitohui* mimicry not more common in New Guinea and why has the north-coast clade lost the type 1 plumage? We hypothesize that variation in their toxin concentrations, the degree of sympatry between *Pitohui* species and variation in predator guilds may all affect the strength of selection for bright coloration and mimicry. For example, in surveys along the north coast near Madang Town (Dumbacher 1997), *P. dichrous* was less toxic than *Pitohui kirhocephalus brunneicaudus* so there may be no selective advantage in *P. kirhocephalus* resembling *P. dichrous*. In the nearby Finisterre Mountains, where *P. dichrous* was highly toxic, *P. kirhocephalus* did not extend into the mountains, so the two species were not sympatric. In localities where visually hunting predators are rare, selection might be too weak for driving plumage mimicry despite the high toxicity of models.

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