WAGNER, R. H. 1992. Confidence of paternity and parental effort in Razorbills. Auk 109:556–562.

WESTNEAT, D. F. 1990. Genetic parentage in the Indigo Bunting: A study using DNA fingerprinting. Behavioral Ecology and Sociobiology 27:67–76.

WESTNEAT, D. F., AND P. W. SHERMAN. 1997. Density and extrapair fertilizations in birds: A comparative analysis. Behavioral Ecology and Sociobiology 41:205–215.

WETTON, J. H., R. E. CARTER, D. T. PARKIN, AND D. WALTERS. 1987. Demographic study of a wild House Sparrow population by DNA fingerprinting. Nature 327:147–149.

WITTENBERGER, J. L., AND R. L. TILSON. 1980. The evolution of monogamy: Hypotheses and evidence. Annual Reviews of Ecology and Systematics 11:197–232.

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Adenylate Kinase Intron 5: A New Nuclear Locus for Avian Systematics

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The explosion of use of the polymerase chain reaction (PCR) and direct DNA sequencing in recent years has provided a wealth of new data for avian systematists. Nearly all these sequence data, however, have come from mitochondrial genes, which are inherited as a single unit, typically exhibit strictly matrilineal inheritance, and have other unusual properties (Avise 1991). Although mitochondrial DNA (mtDNA) has well-established advantages for phylogenetic inference (Moore 1995), avian molecular systematists are eager to identify nuclear genes that could provide independent phylogenetic estimates (e.g. Prychitko and Moore 1997, Omland 1999). Most nuclear loci explored to date are slowly evolving protein coding genes and have been useful primarily for resolving deep relationships (e.g. relationships among taxa with divergence times more than 50 Ma ago; Graybeal 1994, but see Lovette and Bermingham 2000). It would clearly be helpful to have at our disposal nuclear DNA sequences that exhibit a faster rate of evolution, making them phylogenetically useful at intermediate and shallow taxonomic levels (e.g. Palumbi 1996, Prychitko and Moore 1997). Nuclear introns, for example, which have recently received attention for their potential utility in population-level studies (e.g. Friesen et al. 1997, Heslewood et al. 1998), may also be very useful for inferring phylogenetic relationships among species. Although these introns typically evolve more quickly than nuclear protein coding regions, they can nevertheless evolve significantly more slowly than protein coding mtDNA, and might therefore be especially helpful in resolving nodes at depths for which the signal from mtDNA is diminished due to saturation.

Despite their potential utility, nuclear genes have so far been little used in sequence-based avian systematics. This is in large part because of a lack of appropriate PCR primers that work well across a broad spectrum of avian groups and that amplify sequences evolving at rates suitable for addressing a range of phylogenetic questions. In the course of a phylogenetic investigation of the pitohuis (J. Dumbacher unpubl. data), we explored the usefulness of a nuclear gene not previously employed for phylogenetic analysis. In this note, we report primers we have designed to amplify this new nuclear marker, intron 5 of the nuclear gene cytosolic adenylate kinase (AK1), and we document both the potential phylogenetic utility of this intron and the very broad taxonomic utility of these primers.

Methods.—PCR primers located in the conserved exon regions flanking AK1 intron 5 (in exon 5 and exon 6) were designed by comparing complete AK1 DNA sequences published for *Gallus* (Suminami et al. 1988) and humans (Matsuura et al. 1989), as well as amino acid sequences for several other taxa. Primer sequences and positions are given in Table 1. The other known members of the well-studied AK gene family differ in sequence so substantially from AK1, including the regions of primer annealing, that we are confident the primers reported here will amplify only AK1 (barring a recent gene duplication within

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TABLE 1. Primers for amplifying AK1 intron 5 from birds. AK5a$^+$ and AK5b$^+$ are located in exon 5 and the remaining three primers are located in exon 6. "Nucleotide position" refers to the position of the primer in the published *Gallus* AK1 sequence (Suminami et al. 1988). Primers AK5a$^+$, AK6d$^-$, and AK6e$^-$ were not used to collect any of the data reported in this paper, but preliminary studies suggest that these primers work well in at least some taxa, so we have included them here.

Primer name	Primer sequence (5′ to 3′)	Nucleotide position
AK5a$^+$	ATGCTGCGGGACGCCATGTTGG	4769 to 4790
AK5b$^+$	ATTGACGGCTACCCTCGCGAGGTG	4820 to 4843
AK6c$^-$	CACCCGCCCGCTGGTCTCTCC	5454 to 5474
AK6d$^-$	GTTCGGTAGCCTTGTAGTACGTCTCC	5507 to 5532
AK6e$^-$	CCTTGTAGAAGGCGATGACGGGTTC	5529 to 5553

lineages). To investigate the taxonomic breadth for which those primers are useful, we used the primer pair AK5b$^+$ and AK6c$^-$ to amplify and sequence approximately 500 to 650 base pairs (bp) of AK1 from representatives of 10 avian families in 7 orders. This amplification product includes intron 5 (ranging in length from about 350 to 500 bp among the taxa we sampled) and 99 bp of flanking exon (27 bp at the 5′ end and 72 bp at the 3′ end, excluding primers).

DNA was extracted using standard phenol–chloroform extractions (Hillis et al. 1996) or using QIAGEN DNeasy Tissue Kits. DNA amplification of AK1 intron 5 was carried out in an MJ Research PTC-200 thermal cycler using the following program: 94°C (10 min)—35 cycles of 92°C (45 s), ramp of 1.5°C/s to 54°C, 54°C (1 min), ramp of 1.5°C/s to 72°C, 72°C (1 min)–72°C (5 min). Our PCR reaction mix contained magnesium chloride (1.5 mM), primers (0.4 μM each), dNTPs (0.2 mM each) and 1 U Perkin Elmer AmpliTaq Gold DNA polymerase (for a 25 μL reaction) with the supplied buffer. PCR products were cleaned using QIAGEN QIAquick PCR Purification Kits and cycle sequenced in both directions using an ABI PRISM cycle sequencing kit. Sequenced products were cleaned using Princeton Separations CentriSep columns and run out on an ABI 373 automated sequencer.

For purposes of comparison, we amplified both AK1 intron 5 (using AK5b$^+$ and AK6c$^-$, as well as several additional primers designed specifically to pachycephalids) and the well-studied mitochondrial gene cytochrome-*b* from 17 individuals representing

TABLE 2. The 7 avian orders and 10 families from which we have amplified and sequenced AK1 intron 5 from one or more species to demonstrate taxonomic breadth of primers. Orders and families follow Gill (1995); scientific and English species names follow the 7th edition of the Checklist of North American Birds (AOU 1998) for species occurring in North or Middle America, and Monroe and Sibley (1993) for all others. The 12 species used for the phylogenetic component of this study are indicated by asterisks.

Order	Family	Species (partial list)
Sphenisciformes	Spheniscidae	Galapagos Penguin (*Spheniscus mendiculus*)
Ciconiiformes	Threskiornithidae	Glossy Ibis (*Plegadis falcinellus*)
Falconiformes	Accipitridae	Rough-legged Hawk (*Buteo lagopus*)
	Falconidae	Aplomado Falcon (*Falco femoralis*)
Gruiformes	Rallidae	White-winged Coot (*Fulica leucoptera*)
		Common Moorhen (*Gallinula chloropus*)
		Virginia Rail (*Rallus limicola*)
		Buff-spotted Flufftail (*Sarothrura elegans*)
Psittaciformes	Psittacidae	Grey Parrot (*Psittacus erithacus*)
Caprimulgiformes	Aegothelidae	Feline Owlet-nightjar (*Aegotheles insignis*)
Passeriformes	Pachycephalidae	Little Shrike-thrush (*Colluricincla megarhyncha*)*
		Golden Whistler (*Pachycephala pectoralis*)*
		Regent Whistler (*Pachycephala schlegelii*)*
		Sclater's Whistler (*Pachycephala soror*)*
		Hooded Pitohui (*Pitohui dichrous*)*
		Variable Pitohui (*Pitohui kirhocephalus*)*
		Mottled Whistler (*Rhagologus leucostigma*)*
	Paradisaeidae	Magnificent Bird-of Paradise (*Cicinnurus magnificus*)*
		Greater Melampitta (*Melampitta gigantea*)*
		Raggiana Bird-of-Paradise (*Paradisaea raggiana*)*
	Emberizidae	Bronzed Cowbird (*Molothrus aeneus*)*
		Brown-headed Cowbird (*Molothrus ater*)*

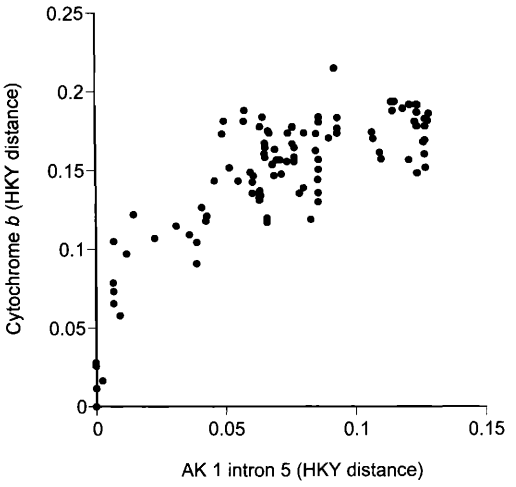


FIG. 1. Cytochrome-*b* pairwise distances versus AK1 intron 5 pairwise distances. Note saturation of cytochrome-*b* relative to AK1. HKY-corrected distances (Hasegawa et al. 1985) were calculated using PAUP* 4.0b4a (Swofford 1999).

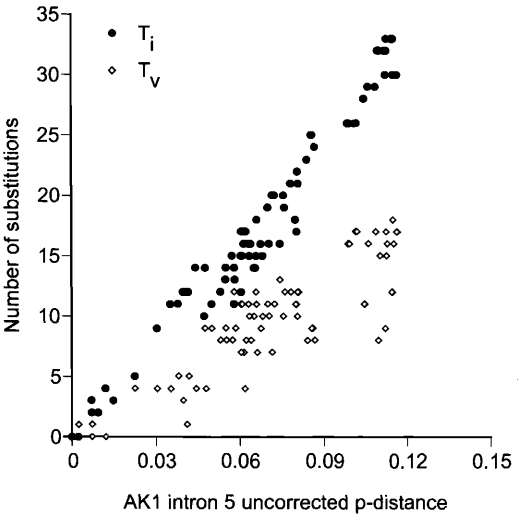
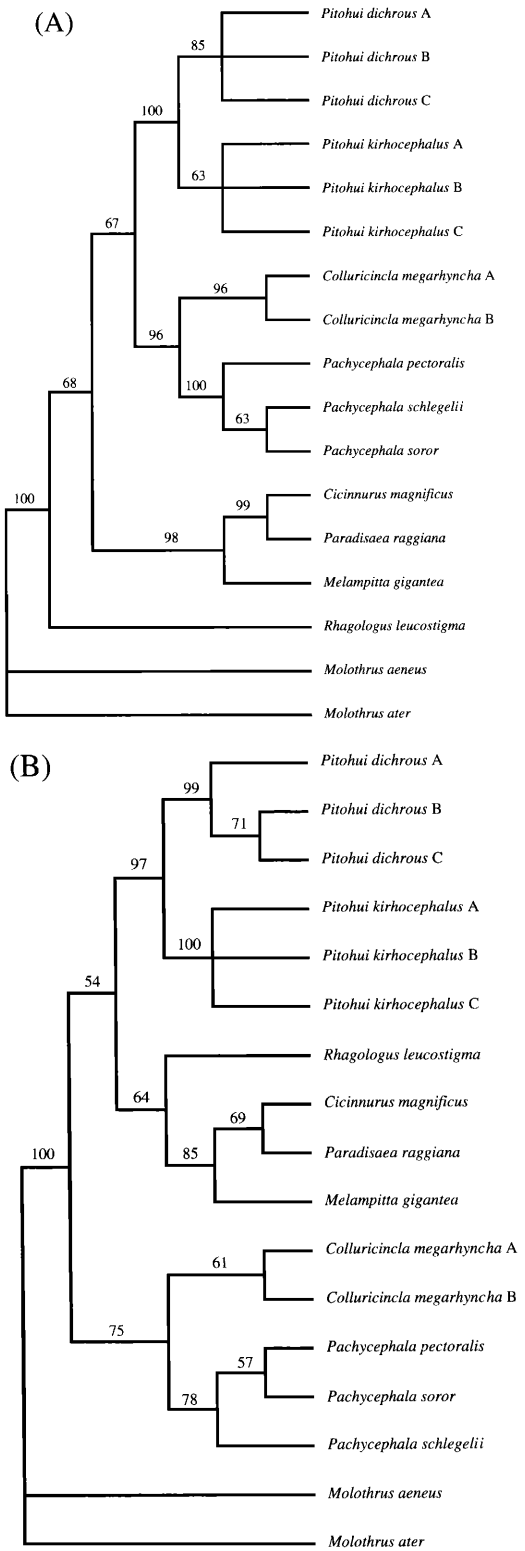


FIG. 2. Transitions (T_i) and transversions (T_v) versus uncorrected pairwise p-distances (T_i + T_v, scaled) for AK1 intron 5. AK1 shows no indication of saturation even at rather deep divergences. Substitutions were tallied and distances calculated using PAUP* 4.0b4a (Swofford 1999).

7 species of Pachycephalidae, 3 species of Paradisaeidae, and (as an outgroup) 2 species of Emberizidae (Table 2). For the analyses presented here we used, wherever possible, 420–440 bp each of both AK1 intron 5 and cytochrome-*b*, but a few sequences are shorter than this. Sequences were easily aligned by eye (Appendix 1). We calculated genetic distances and generated maximum-parsimony trees using PAUP 4.0b4a (Swofford 1999). HKY85 distances were calculated omitting gaps (and other missing or ambiguous positions) from affected pairwise comparisons. Parsimony analyses were performed using the heuristic search option with tree bisection and reconnection (TBR) and 200 random addition sequence replicates. Node support was evaluated using nonparametric bootstrapping (1,000 replicates).

Results.—We amplified and sequenced AK1 intron 5 from one or more representatives of 10 families in 7 avian orders (Table 2; see Appendix 2 for GenBank accession numbers and specimen voucher numbers). Many taxa we attempted to amplify provided clean sequences with no need to redesign primers or gel-purify bands, and the remainder sequenced nicely with minor additional effort. Although double sequence is apparent at a few nucleotide positions (probably due to heterozygosity, but conceivably representing an undetected recent gene duplication), these sites are infrequent and appear not to be a problem for interspecific analyses.

For pachycephalid, paradisaeid, and emberizid sequences, to which we limited our phylogenetic analysis, plotting pairwise distances for cytochrome-*b* versus AK1 intron 5 suggests that for relatively recently diverged taxa, AK1 intron 5 exhibits a substitution rate several times slower than cytochrome-*b*, but this difference is effectively reduced for deeper divergences as cytochrome-*b* saturates relative to AK1 (Fig 1). This saturation indicates the potential for extensive homoplasy that can seriously degrade phylogenetic signal (Griffiths 1997). AK1 shows no indication of saturation even at rather deep divergences (Fig. 2). AK1 intron 5 had fewer variable and parsimony-informative sites than did cytochrome-*b*, but it also exhibited substantially less homoplasy (Table 3). Our parsimony analysis using AK1 intron 5 produces a tree topology that for more recently diverged taxa is similar to that resulting from our analysis using cytochrome-*b*, but there are several substantial disagreements, especially with respect to deeper nodes (Fig. 3). Both AK1 and cytochrome-*b* recover monophyletic species groupings wherever conspecific sequences are included in the data set. They also recover both of the genera that are represented by more than one species (i.e. *Pitohui* and *Pachycephala*, the former clade being recovered with similarly very high bootstrap support by both genes, the latter being supported more consistently by AK1). With respect to relationships among genera, both AK1 and cytochrome-*b* recover the groupings (*Colluricincla* + *Pachycephala*), (*Cicinnurus* + *Paradisaea*), and ((*Cicinnurus* + *Paradisaea*) + *Melampitta*), with AK1 exhibiting in each case substantially higher bootstrap support than does cytochrome-*b*. The AK1 and cytochrome-*b* tree topologies conflict in the



placement of *Rhagologus* and in the relationships among the three included *Pachycephala* species, as well as with respect to deeper relationships among the groups just discussed, but none of these nodes is strongly supported by either gene (this may be a consequence of the relatively sparse taxon sampling in the data set). Resolution of these relationships will be attempted elsewhere in a more comprehensive analysis (J. Dumbacher unpubl. data). Our purpose here is simply to demonstrate that AK1 intron 5 contains substantial phylogenetic signal and warrants further exploration by avian phylogeneticists.

A striking feature of nuclear intron sequences that is rarely seen in sequence from protein coding mitochondrial genes is the presence of insertions and deletions (indels). Indels in introns can be valuable for phylogenetic inference because homoplasy in the form of reversals or convergent gains is very unlikely (however, excessive insertion or deletion activity can make alignment of sequences from distantly related taxa difficult or impossible). We have found parsimony-informative indels in several AK1 intron 5 data sets with which we have been working. In the dataset presented here, there are eight indels (Appendix 1). Five indels distinguish the two emberizids from the other taxa (two 1 bp deletions, one 1 bp insertion, and two 2 bp insertions in the two congeneric emberizids relative to the other taxa), and a sixth indel, a 2 bp deletion, is shared only by the three species of *Pachycephala*. The remaining two indels are unique to single taxa in our small data set, *Cicinnurus* (with a 5 bp insertion relative to the other taxa) and *Paradisea* (with a 68 bp deletion—which overlaps the *Pachycephala* deletion, the *Cicinnurus* insertion, and one of the emberizid insertions—relative to the other taxa). Because the issue of how best to use indels in phylogenetic analyses is not straightforward, in the analysis presented here we have simply treated gaps as missing data, but it seems clear that some of these indels contain useful phylogenetic information.

Discussion.—Avian systematists have recently begun looking toward nuclear genes for at least two reasons. First, different nuclear loci (or classes of loci) may have rates of evolution that make them well suited to addressing phylogenetic questions at different depths of divergence. For example, some slowly evolving protein coding genes may be useful for determining relationships among deeply diverged groups as a consequence of their relatively high sig-

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FIG. 3. Maximum-parsimony trees constructed for the same set of taxa using (A) AK1 intron 5 and (B) cytochrome-*b* (see text for details). Numbers above branches indicate bootstrap support for subtended clades (from 1,000 replicates).

TABLE 3. Proportion of sites variable and proportion of sites parsimony-informative for AK1 intron 5 and cytochrome-*b* sequences included in our analysis, and measures of homoplasy (calculated excluding uninformative sites) for maximum-parsimony trees.

	AK1 intron 5	Cytochrome-*b*
Proportion of sites variable	0.23	0.35
Proportion of sites parsimony-informative	0.17	0.26
Consistency Index	0.89	0.53
Retention Index	0.95	0.65
Rescaled Consistency Index	0.84	0.35

nal-to-noise ratio at those depths (Graybeal 1994, Groth and Barrowclough 1999). In contrast, introns such as AK1 intron 5 may be most useful at intermediate depths—depths at which homoplasy may be a serious problem for rapidly evolving mitochondrial genes, but at which nuclear protein coding genes have accumulated few differences.

A second major reason that systematists are interested in utilizing nuclear sequences is that mtDNA evolves as a single linkage group, and distinguishing individual gene trees from species trees by obtaining multiple independent estimates of phylogenetic histories can be extremely important. For example, although the mitochondrial genome is expected to be generally less prone to lineage sorting problems than are nuclear genes (Moore 1995; but see Hoelzer 1997, Moore 1997), surveying nuclear loci is nevertheless necessary to investigate the possibility of mitochondrial introgression through hybridization when there is a conflict between putative mitochondrial trees and other character sets. Nuclear loci such as AK1 intron 5 are needed to test phylogenies based on mtDNA.

It is well known that selective constraints and rates of evolution across a particular sequence of DNA may be quite heterogeneous. This phenomenon has been thoroughly documented for cytochrome-*b* (Griffiths 1997), and it is possible that analogous heterogeneity occurs even within the noncoding AK1 intron 5 (for example, there may be regions in which indels occur more or less frequently). The analysis we present here is not intended as an exhaustive comparison of patterns of evolution in AK1 intron 5 and cytochrome-*b*, but rather is meant simply to demonstrate the phylogenetic utility of AK1 intron 5 and to encourage avian phylogeneticists to further explore the usefulness of this new marker. With respect to current avian taxonomy, it appears that AK1 may perform well in recovering relationships among intermediate to distantly related congeneric species and among genera (with unexplored potential for examining relationships among families within orders), and the presence of indels that are easily aligned at this level may provide a valuable source of phylogenetic information. However, for inferring relationships among taxa with quite recent divergence times, AK1 intron 5 may evolve too slowly. At higher levels, this intron will probably not be useful for inferring relationships (e.g. among avian orders) because of multiple insertions and deletions, which cause difficulties with sequence alignment among some orders (L. Shapiro unpubl. data).

Progress in the exploration of nuclear genetic markers for avian phylogenetics has been seriously impeded by the lack of widely applicable PCR primers. Given their demonstrated taxonomic versatility and promising phylogenetic utility, the primers described here should prove to be a valuable addition to the toolbox of the avian molecular systematist.

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LITERATURE CITED

AMERICAN ORNITHOLOGISTS' UNION. 1998. Checklist of North American Birds, 7th ed. American Ornithologists' Union, Washington, D.C.

AVISE, J. C. 1991. Ten unorthodox perspectives on evolution prompted by comparative population genetic findings on mitochondrial DNA. Annual Review of Genetics 25:45–69.

FRIESEN, V. L., B. C. CONGDON, H. E. WALSH, AND T. P. BIRT. 1997. Intron variation in Marbled Murrelets detected using analyses of single-stranded conformational polymorphisms. Molecular Ecology 6:1047–1058.

GILL, F. B. 1995. Ornithology, 2nd ed. W. H. Freeman and Company, New York.

GRAYBEAL, A. 1994. Evaluating the phylogenetic utility of genes: A search for genes informative about deep divergences among vertebrates. Systematic Biology 43:174–193.

GRIFFITHS, C. S. 1997. Correlation of functional domains and rates of nucleotide substitution in cytochrome *b*. Molecular Phylogenetics and Evolution 7:352–365.

GROTH, J. G., AND G. F. BARROWCLOUGH. 1999. Basal divergences in birds and the phylogenetic utility of the nuclear RAG-1 gene. Molecular Phylogenetics and Evolution 12:115–123.

HASEGAWA, M., H. KISHINO, AND T. YANO. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22:160–174.

HESLEWOOD, M. M., M. S. ELPHINSTONE, S. C. TIDEMANN, AND P. R. BAVERSTOCK. 1998. Myoglobin intron variation in the Gouldian Finch *Erythrura gouldiae* assessed by temperature gradient gel electrophoresis. Electrophoresis 19:142–151.

HILLIS, D. M., B. K. MABLE, A. LARSON, S. K. DAVIS, AND E. A. ZIMMER. 1996. Nucleic acids IV: Sequencing and cloning. Pages 321–381 *in* Molecular Systematics, 2nd ed. (D. M. Hillis, C. Moritz, and B. K. Mable, Eds.). Sinauer Associates, Sunderland, Massachusetts.

HOELZER, G. A. 1997. Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees revisited. Evolution 51: 622–626.

LOVETTE, I. J. AND E. BERMINGHAM. 2000. *c-mos* variation in songbirds: Molecular evolution, phylogenetic implications, and comparisons with mitochondrial differentiation. Molecular Biology and Evolution 17:1569–1577.

MATSUURA, S., M. IGARASHI, Y. TANIZAWA, M. YAMADA, F. KISHI, T. KAJII, H. FUJII, S. MIWA, M. SAKURAI, AND A. NAKAZAWA. 1989. Human adenylate kinase deficiency associated with hemolytic anemia. A single base substitution affecting solubility and catalytic activity of the cytosolic adenylate kinase. Journal of Biological Chemistry 264:10148–10155.

MONROE, B. L., JR., AND C. G. SIBLEY. 1993. A World Checklist of Birds. Yale University Press, New Haven, Connecticut.

MOORE, W. S. 1995. Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees. Evolution 49:718–726.

MOORE, W. S. 1997. Mitochondrial-gene trees versus nuclear-gene trees, a reply to Hoelzer. Evolution 51:627–629.

OMLAND, K. 1999. Review of "Avian molecular evolution and systematics". Systematic Biology 48: 225–226.

PALUMBI, S. R. 1996. Nucleic acids II: The polymerase chain reaction. Pages 205–247 *in* Molecular Systematics, 2nd ed. (D. M. Hillis, C. Moritz, and B. K. Mable, Eds.). Sinauer Associates, Sunderland, Massachusetts.

PRYCHITKO, T. M., AND W. S. MOORE. 1997. The utility of DNA sequences of an intron from the B-fibrinogen gene in phylogenetic analysis of woodpeckers (Aves: Picidae). Molecular Phylogenetics and Evolution 8:193–204.

SUMINAMI, Y., F. KISHI, T. TORIGOE, AND A. NAKAZAWA. 1988. Structure and complete nucleotide sequence of the gene encoding chicken adenylate kinase. Journal of Biochemistry 103:611–617.

SWOFFORD, D. L. 1999. PAUP* 4.0b4a: Phylogenetic Analysis Using Parsimony. Sinauer Associates, Sunderland, Massachusetts.

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APPENDIX 1. Alignment of AK1 intron 5 sequences for which we calculated genetic distances and constructed parsimony trees. A period in a column indicates that the same nucleotide appears in that position as at the top of the column; a dash indicates a gap.

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                                                                                                                       100
Pitohui dichrous A            CAGTAAAGATCACATCCCAGAAGGGTCTAAAGTGGGCAGCAGGTCCCCCTGCCTCAGGGACAGCCTGTGCCCAGAGCACGGTGGAGGGAGCAGGGTGTT
Pitohui dichrous B            ....................................................................................................
Pitohui dichrous C            ....................................................................................................
Pitohui kirhocephalus A       ...............T...............................................................A....................
Pitohui kirhocephalus B       ...............T...............................................................A....................
Pitohui kirhocephalus C       ...............T...............................................................A....................
Colluricincla megarhyncha A   ...C.....................C.....C...........................................T...A...C.....C..C......C
Colluricincla megarhyncha B   ...C...............N.....C.....C......T..N.................................T...A...T.....C..C......C
Pachycephala pectoralis                          C.G.A.G.....C.....G..........G..........G...............T...A...C.....C..C......C
Pachycephala schlegelii       ...C.....................C.....C.........N......G..........G.............N.T...A...C.....C.GC......C
Pachycephala soror            ...C.....................C.....C................G..........G...............T...A...C.....C..C......C
Rhagologus leucostigma                                    ...C..A............................................AN........C.........C
Cicinnurus magnificus                                         .........N.....................................A.........C.........C
Melampitta gigantea                                                                .................T........A.........C.........C
Paradisaea raggiana           ...CG..........................C...............................................A.......A.C.........C
Molothrus aeneus              ...C.G.........................C.......................-....................Y..A.C.......C.........C
Molothrus ater                ...C.G....G....................C.......................-.......................A.C.......C.........C

                                                                                                                       200
Pitohui dichrous A            CTCAGGCTGACAGAGCATGACAACAGTGCTGACATGATGGTGATGGCAGAGAGCCAGGGCTTGGCCACAGGGTCCTTTTGCCTCCCTCTACACGGCCCAA
Pitohui dichrous B            ....................................................................................................
Pitohui dichrous C            ....................................................................................................
Pitohui kirhocephalus A       ....................................................................................................
Pitohui kirhocephalus B       ....................................................................................................
Pitohui kirhocephalus C       ....................................................................................................
Colluricincla megarhyncha A   .......................GG..................................................C...T..................T.
Colluricincla megarhyncha B   .......................GG..................................................C......................T.
Pachycephala pectoralis       ........................G......G..................A........................C......................T.
Pachycephala schlegelii       ........................G......G........W.........A........................C......................T.
Pachycephala soror            ........................G......G..................A........................C......................T.
Rhagologus leucostigma        ............A........................................................AT....C......................T.
Cicinnurus magnificus         .............T..........G.............................................T....C.........T............T.
Melampitta gigantea           .............T........G...............................................T....C.........T............T.
Paradisaea raggiana           .............T..........G..................C..........................T....C.........T............T.
Molothrus aeneus              ...............TG.....G..........................................TG...T..G-C.......T........T.....T.
Molothrus ater                ...............TG.....G..........................................TG...T..G-C.......T........T.....T.

                                                                                                                       300
Pitohui dichrous A            GAGACCACTGGAACAGTGTCCTGGT-GCCCCCAGACCGATGCAGAGGGAGGCAGAAGATGGTGTGGGGACATCTCTGTGTCCTGGTGG-----CCCCCAG
Pitohui dichrous B            .........................-..............................................................-----.......
Pitohui dichrous C            .........................-..............................................................-----.......
Pitohui kirhocephalus A       .........................-..............................................................-----.......
Pitohui kirhocephalus B       .........................-..............................................................-----.......
Pitohui kirhocephalus C       .........................-..............................................................-----.......
Colluricincla megarhyncha A   .........................-...........A..............................G...................-----.......
Colluricincla megarhyncha B   .........................-...........A..............................G...................-----.......
Pachycephala pectoralis       .........................-...........A........................................--........-----.......
Pachycephala schlegelii       .........................-...........A........................................--........-----.......
Pachycephala soror            .........................-...........A........................................--........-----.......
Rhagologus leucostigma        .........................-.T.........A.A................................................-----.......
Cicinnurus magnificus         ..........A..G...........-...........A....G.......A.........A...............C...........ACAGAG......
Melampitta gigantea           .........................-.....T.....A....G.......A....................C................-----.......
Paradisaea raggiana           .............G...........-...........A....G.......A.........A.............--------------------------
Molothrus aeneus              ...G...G.....G...........G..T........A.A....T.........G..............TG.............C...-----.......
Molothrus ater                ...G...G.....G...........G..T........A.A....T.........G..............TG.............C...-----......N

                                                                                                                       400
Pitohui dichrous A            ACCCATGTGGAGGGAGGC--AGACGGTGGCATGGGGACATCTGTGACCCTCATGCTGACTACCACTACTACC--CCAGACCAAACCAGACCCACACTGAC
Pitohui dichrous B            ..................--....................................................--..........................
Pitohui dichrous C            ..................--....................................................--..........................
Pitohui kirhocephalus A       ..................--....................................................--......................C...
Pitohui kirhocephalus B       ..................--....................................................--......................C...
Pitohui kirhocephalus C       ..................--....................................................--......................C...
Colluricincla megarhyncha A   ..................--...GA...........................................C...--...A.......A..........CAN.
Colluricincla megarhyncha B   ..................--...GA...........................................C...--...A.......A..........CA..
Pachycephala pectoralis       ..................--...G......................................TG..G.C...--...A.......A..........CA..
Pachycephala schlegelii       ..................--...G................................CC...NTGN.G.C...--...A.......A..........CA..
Pachycephala soror            ..................--...G................................C.....TG..G.C...--...A.......A..........CA..
Rhagologus leucostigma        ..............T...--...G.............................A.........G....C...--...A..................CA..
Cicinnurus magnificus         ..................--...GA..............C........T.............TG....C..T--...A......G...........CA..
Melampitta gigantea           ..................--...G........................T..............G....C...--...A......G...........CA..
Paradisaea raggiana           ------------------------------------------......T.............TG....C..T--...A......G...........CA..
Molothrus aeneus              ...........T......AG...G.....TG.........T..................C.....C..C...CC...A...........G......CA..
Molothrus ater               ...........T...R..AG...G.....TG.........T..................C.....C..C...CC...A...........G......CA..
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APPENDIX 1. Continued.

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                                                                    440
Pitohui dichrous A          TCAGTGTGATGACAACTATGTCCGCTCTGTCCATAGATCG
Pitohui dichrous B          ........................................
Pitohui dichrous C          ........................................
Pitohui kirhocephalus A     ........................................
Pitohui kirhocephalus B     ........................................
Pitohui kirhocephalus C     ........................................
Colluricincla megarhyncha A ........G.............C.........C......
Colluricincla megarhyncha B ........G......N.......C.........C....T.
Pachycephala pectoralis     ........G.........C....CS........C......
Pachycephala schlegelii     ........G......N..C....C.........C....T.
Pachycephala soror          ........G.........C....C.........C......
Rhagologus leucostigma      ........G.....G........C.........C......
Cicinnurus magnificus       ........G.............C.........C......
Melampitta gigantea         ........G.............C.........C....T.
Paradisaea raggiana         ........G..........A...C.........C......
Molothrus aeneus            ........G.....G.C.C....C......G..C....Y.
Molothrus ater              ........G.....G.C.C....C......G..C....Y.
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APPENDIX 2. Voucher and accession numbers for tissue samples and DNA sequences used in this study.

Species	Tissue/skin voucher or field number[a]	GenBank accession number[b]
Spheniscus mendiculus	none	AF307894
Plegadis falcinellus	ANSP 3835 (tissue)	AF307896
Buteo lagopus	none	AF307891
Falco femoralis	none	AF307890
Fulica leucoptera	LMS B02782 (tissue), USNM 614599 (skin)	AF307898
Gallinula chloropus	FMNH 364658 (skin)	AF307899
Rallus limicola	ANSP 5478 (tissue), ANSP 187132 (skin)	AF307900
Sarothrura elegans	FMNH 346189 (skin)	AF307902
Psittacus erithacus	none	AF307895
Aegotheles insignis	AM 857[c]	AF307897
Colluricincla megarhyncha A	042-01128[d]	AF308743, AF308760
Colluricincla megarhyncha B	JD93016[e]	AF308744, AF308761
Pachycephala pectoralis	MV1419	AF308745, AF308762
Pachycephala schlegelii	CEM4[f]	AF308746, AF308763
Pachycephala soror	CEM11[f]	AF308747, AF308764
Pitohui dichrous A	051-87052[d]	AF308748, AF308765
Pitohui dichrous B	051-87069[d]	AF308749, AF308766
Pitohui dichrous C	JD93021[e]	AF308750, AF308767
Pitohui kirhocephalus A	062-38831[d]	AF308751, AF308768
Pitohui kirhocephalus B	062-38833[d]	AF308752, AF308769
Pitohui kirhocephalus C	IK01[e]	AF308753, AF308770
Rhagologus leucostigma	CEM19[f]	AF308754, AF308771
Cicinnurus magnificus	ZOO90[g]	AF308755, AF308772
Melampitta gigantea	JD97001MG[e]	AF308756, AF308773
Paradisaea raggiana	ZOO65[g]	AF308757, AF308774
Molothrus aeneus	1142-80938[h]	AF308758, AF308775
Molothrus ater	none	AF308759, AF308776

[a] none = no voucher taken (contact authors for available information); ANSP = Academy of Natural Sciences of Philadelphia; LMS = Laboratory of Molecular Systematics, Smithsonian Institution; USNM = U.S. National Museum of Natural History; FMNH = Field Museum of Natural History (Chicago); MV = Museum of Victoria (Australia).

[b] First GenBank number listed is for adenylate kinase intron 5; second number, if present, is for cytochrome-*b*.

[c] Field number of vouchers that will be accessioned at the Bishop Museum, Honolulu (skin) and the University of Kansas Natural History Museum (tissue).

[d] Band numbers of birds banded under the Australian Bird and Bat Banding Scheme.

[e] Field numbers of vouchers that will be accessioned in the Papua New Guinea National Museum and Art Gallery.

[f] Field numbers of birds caught and released in protected areas.

[g] Zoo band numbers of captive birds at the Wau Ecology Institute.

[h] U.S. Bird Banding Laboratory band number.