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ALLOZYME DIVERGENCE AND PHYLOGENETIC RELATIONSHIPS WITHIN THE STRIGIFORMES[1]

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Abstract. Multilocus protein electrophoresis was used to study the phylogenetic relationships among seven species of Strigiformes. Genetic divergence was quantified by Nei's unbiased standard genetic distance, and dendrograms were computed both with distance and parsimony methods. *Caprimulgus europaeus* was used an an outgroup. The average genetic distance between *Tyto alba* and the Strigidae was $\bar{D} = 1.49$; among the Strigidae was $\bar{D} = 0.88$. Dendrograms computed with distance methods, with or without assuming a molecular clock, as well as the character state matrix tree, support the following phylogenetic relationships: Tytonidae and Strigidae are very divergent lineages; *Athene* is ancestral to most of the modern Strigidae and is related to *Strix*; *Bubo* is related to *Otus*; *Asio otus* and *Asio flammeus* show high genetic distance. These findings have been discussed taking into account both DNA-DNA hybridization data and the fossil record. A tentative calibration of the molecular clock is used to date divergence times among the studied taxa.

Key words: Strigiformes; allozyme electrophoresis; phylogenetic relationships; protein polymorphism; genetic divergence.

INTRODUCTION

The order Strigiformes is currently subdivided into two living families: Tytonidae, with two genera and 10 species, and Strigidae, with 22 genera and 109–143 species depending on the classification used by different authors (Clark et al. 1978, Wetmore 1960, Storer 1971). Both taxonomy and phylogenetic relationships of the Strigiformes are uncertain, and different opinions have been expressed (for a detailed review, see Sibley and Ahlquist 1972).

Only two papers based on modern phylogenetic methods have been published so far. Cracraft (1981) proposed an avian classification based on a phylogenetic (cladistic) approach. He recommended that owls be considered a superfamily (Strigoidea, with the single family Strigidae, including *Tyto*) within the order Falconiformes.

A different opinion was expressed by Sibley et al. (1988) on the basis of a distance analysis of DNA-DNA hybridization data. In their classification, Falconiformes and Strigiformes appear distantly related. The order Strigiformes was divided into three suborders (Strigi, Aegotheli and Caprimulgi).

The intra-familial phylogenetic relationships have not been studied enough to allow identification of the evolutionary patterns. Unpublished DNA-DNA hybridization data (Sibley, pers. comm.) indicate in the Strigidae the existence of an ancestral cluster of genera (*Speotyto, Athene, Glaucidium, Ninox, Aegolius*) that are followed by a more recent radiation including *Asio* and *Otus* (quite distantly related), *Bubo, Nyctea, Ketupa,* and *Strix*. The karyologic studies by Belterman and De Boer (1984) indicate some groups with similar chromosomes (i.e., *Otus, Bubo, Nyctea* and *Ketupa*), but do not allow clear identification of phylogenetic relationships among them.

Multilocus protein electrophoresis is a useful tool to investigate the phylogenetic relationships at higher taxonomic level in birds (Randi and Spina 1987, Lanyon and Zink 1987) by taking advantage of the short genetic distances among avian taxa as compared to most non-avian taxa (Avise and Aquadro 1982). Although the reliability of the dendrograms derived from genetic distance matrices suffers from theoretical (Lanyon 1988) as well as from empirical (Randi et al. 1989) limitations, its effective range of application has not been yet determined.

In this paper we use multilocus protein electrophoresis in an attempt to clarify phylogenetic relationships among seven strigiform species, using *Caprimulgus europaeus* as an outgroup. Relationships were assessed by means of several

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different analytical approaches and were compared both with DNA-DNA hybridization and fossil data.

MATERIALS AND METHODS

Liver, heart, and kidneys were collected within a few hours of death from the following specimens: Tytonidae: *Tyto alba* (Barn Owl; $n = 19$); Strigidae: *Athene noctua* (Little Owl; $n = 17$), *Strix aluco* (Tawny Owl; $n = 16$), *Otus scops* (Common Scops Owl; $n = 2$), *Asio otus* (Long-eared Owl; $n = 18$), *Asio flammeus* (Short-eared Owl; $n = 3$), *Bubo bubo* (Eagle Owl; $n = 2$); Caprimulgiformes: *Caprimulgus europaeus* (European Nightjar; $n = 1$). All samples derive from dead birds obtained by the Raptor Rehabilitation Center of the "Lega Italiana per la Protezione degli Uccelli" (LIPU, Parma).

Tissues were homogenized in 0.01 M Tris/HCl (+ 0.001 M β-mercaptoethanol), pH 7.5, centrifuged at 12,000 rpm for 15 min, the supernatant was then collected and stored at −80°C. Vertical polyacrylamide gels (7.5% acrylamide concentration) were used. Fourteen enzyme systems and seven non-enzymatic heart proteins, encoding 29 presumptive genetic loci, were examined using the following electrophoretic conditions (multiple proteins are designated with numbers starting from the most anodal locus): 1) discontinuous Tris/Glycine, pH 8.3 (Davis 1964): mannose phosphate isomerase (MPI, 5.3.1.8.), superoxide dismutase (SOD-1, -2, 2.1.15.1.), adenylate kinase (AK, 2.7.4.3.), peroxidase (POX-2, 1.11.1.7.), lactate dehydrogenase (LDH-1, -2, 1.1.1.27.), malate dehydrogenase (MDH-1, -2, 1.1.1.37.), albumin and unidentified non-enzymatic heart proteins (ALB, PT-1 through PT-6); 2) discontinuous Tris/Glycine, pH 8.5 (Jolley and Allen 1965): glucose phosphate isomerase (GPI, 5.3.1.9.), phosphoglucomutase (PGM-1, 2.7.5.1.), aspartate aminotransferase (AAT-1, 2.6.1.1.), malic enzyme (ME-1, 1.1.1.40.); 3) Citrate/Morpholine, pH 7.5 (Clayton and Tretiak 1972): creatine kinase (CK-1, -2, 2.7.3.2.), acid phosphatase (ACP-1, -2, 3.1.3.2.); 4) Lithium hydroxide, pH 8.2 (Ferguson 1980): leucine-alanine dipeptidase (LA, 3.4.11.); 5) Tris/Borate, pH 8.9 (Studier 1973): esterase (EST-1, -2, -3, 3.1.1.1.). Staining recipes were adapted from Harris and Hopkinson (1976).

Electromorphs at each locus were coded by their mobility and designated with letters, "a" being the most anodal. The program BIOSYS-1 (Swofford and Selander 1989) was used to compute genetic distance matrices and dendrograms using the unweighted pair-group method UPGMA (Sneath and Sokal 1973) with the constraint of a molecular clock and the distance Wagner procedure (Swofford 1981) without the constraint of a molecular clock.

Phylogenetic trees were computed after coding alleles as present or absent. Forty-five phylogenetically informative alleles (shared by two or more taxa, italicized in Table 1) were analyzed by the program HENNIG86 (Farris 1988) with the IE algorithm to find the most parsimonious trees.

Unrooted trees have been rooted using *Caprimulgus europaeus* as an outgroup by assuming, as a working hypothesis, that the Caprimulgiformes is the sister lineage of the Strigiformes. This is supported by DNA-DNA hybridization results (Sibley et al. 1988; Sibley, pers. comm.).

RESULTS

The distribution of the electromorphs in the seven species of Strigiformes and the allelic frequencies at the polymorphic loci are shown in Table 1. The extent of genetic divergence appears to be high in the Strigiformes. Only one, the mitochondrial MDH (MDH-2), out of 29 loci was monomorphic among species. Genetic divergence is quantified by Nei's unbiased standard genetic distances (Table 2). The average genetic distance between *C. europaeus* and the Strigiformes is $\bar{D} = 3.09$ between *T. alba* and the Strigidae is $\bar{D} = 1.49$, and among the Strigidae is $\bar{D} = 0.88$. These distances are higher than usually reported at correspondent taxonomic levels in other birds (Barrowclough 1980, Gutierrez et al. 1983, Lanyon and Zink 1987). It should be noted, however, that each study used a different set of loci and distance measures are strongly dependent on locus choice. UPGMA and Wagner dendrograms are shown in Figures 1a and 1b. Both reflect the traditional taxonomic relationships between *T. alba* and the Strigidae. Within the Strigidae, *A. noctua* is distantly and equally related to two groups. The first one comprises the genera *Strix* and *Asio* and the second one links *Otus* with *Bubo*. A set of topologically identical UPGMA dendrograms have been obtained by using different distance measures (Rogers 1972, Rogers as modified by Wright 1978, Nei 1972, Cavalli-Sforza and Edwards 1967).

Different criteria of fitting and species input

TABLE 1. Distribution of the electromorphs and allelic frequencies at the polymorphic loci in seven species of Strigiformes and in the outgroup *C. europaeus*. Allele frequencies equal 1.0 unless otherwise noted. Abbreviations: *T. alba* = T.a.; *A. noctua* = A.n.; *S. aluco* = S.a.; *O. scops* = O.s.; *A. otus* = A.o.; *A. flammeus* = A.f.; *B. bubo* = B.b.; *C. europaeus* = C.e.

Locus	Species							
	T.a.	A.n.	S.a.	O.s.	A.o.	A.f.	B.b.	C.e.
MPI	a	b	*c*	c	c	c	c	d
SOD-1	*e*	e	*b*	c	b	b	a	d
SOD-2	c	b	*c*	c	c	c	c	a
AK	*a*	a	a	a	a	a	a	b
POX-2	*c*	c	c	*b*	*a*	a	b	d
LDH-1	a	h	g	c	e	b	d	f
LDH-2	f	g	h	c	e	b	d	a
GPI	*d*	c (0.94) e (0.06)	*b*	b	a (0.07) b (0.93)	b	b	d
CK-1	d	a (0.94) *b* (0.06)	*a*	c	a (0.94) b (0.06)	a	a	e
CK-2	e	*c* (0.60) *d* (0.40)	c (0.60) d (0.40)	a	c	c	c	b
ACP-1	b	*c*	c	c	c	c	c	a
ACP-2	a	*c*	c	c	c	c	c	b
PGM-1	d	b	*a*	a	a	a	a	c
LA	d	b	f	e	h	g	c	a
AAT-1	a (0.20) *b* (0.80)	d	b	b	b	b	b	c
ME-1	f (0.10) h (0.90)	*g*	g (0.25) i (0.75)	a	b (0.35) c (0.65)	c	d	e
EST-1	*d*	d (0.05) e (0.95)	d	*f*	a (0.07) b (0.93)	*a*	f	c
EST-2	a	*h*	h	d	*f* (0.02) g (0.98)	*e* (0.34) f (0.66)	c (0.50) e (0.50)	b
EST-3	d	c	*e*	e	*b*	b	e	a
MDH-1	*a*	a	a	a	a	a	a	b
MDH-2	a	a	a	a	a	a	a	a
ALB	b	*d*	d	f	e	*c*	c	a
PT-1	*c*	c	a	c	*b* (0.03) c (0.97)	c (0.34) b (0.66)	b (0.34) c (0.66)	d
PT-2	*e*	e	a	c	*d*	d	b	f
PT-3	*b*	*c*	c	d	c	*a*	a	b
PT-4	*d*	*c*	d	a	c	*b*	b	f
PT-5	f	d	*c*	*a*	c	b	a	e
PT-6	a	c	*b*	*d*	b	d	d	e

sequence (Swofford 1981) have been used to search for the best fitted Wagner tree. All the methods produced topologies similar to the tree shown in Figure 1b. *T. alba* is the sister lineage of the Strigidae. *A. noctua,* followed by *S. aluco,* are in lineages ancestral to the genera *Asio, Bubo* and *Otus.* Similar results are obtained after rooting the trees at midpoint of the greatest patristic distance. Branch lengths in Figure 1b show that without the constraint of a molecular clock, the rates of protein changing appear to be quite regular along the lineages. *O. scops* is exceptional and seems to have accumulated genetic divergence at a higher rate than on average.

Three equivalent parsimony trees (Figs. 2a, b, c) have been obtained by HENNIG86 using discrete state coding of characters. When *C. europaeus* is used to root the trees, *T. alba* is the sister lineage of the Strigidae. Within the Strigidae, three clusters consistently can be identified: *A. noctua* and *S. aluco,* the two species of the genus *Asio,* and *Bubo* and *Otus.* This method cannot resolve the relations of ancestrality among clusters. It is equally parsimonious to place *Strix* and *Athene* ancestral to the other two clusters or to place the other two clusters as ancestors. The consensus tree (Fig. 2d), therefore, shows a trichotomic relation among the three clusters.

TABLE 2. Nei's (1978) standard unbiased distances (below the diagonal) and identities (above), among seven species of Strigiformes and the outgruop *C. europaeus*. Abbreviations as in Table 1.

Species	T.a.	A.n.	S.a.	O.s.	A.o.	A.f.	B.b.	C.e.
T.a.	—	0.249	0.276	0.202	0.204	0.185	0.186	0.070
A.n.	1.354	—	0.418	0.245	0.372	0.285	0.297	0.070
S.a.	1.254	0.923	—	0.420	0.587	0.493	0.493	0.105
O.s.	1.567	1.527	0.918	—	0.417	0.440	0.594	0.103
A.o.	1.553	1.048	0.560	0.926	—	0.632	0.491	0.103
A.f.	1.669	1.364	0.764	0.885	0.466	—	0.660	0.130
B.b.	1.589	1.304	0.762	0.554	0.759	0.493	—	0.107
C.e.	2.245	3.318	3.316	3.332	3.314	2.802	3.311	—

DISCUSSION

The phylogenetic relationships of the Strigiformes have long been debated. Owls have been alternatively considered to be related to the Falconiformes or to the Caprimulgiformes (Sibley and Ahlquist 1972). Only scanty information derived from modern genetic and phylogenetic studies are available to support the former or latter opinions. Cracraft (1981) used a cladistic approach and concluded that owls are a superfamily of the order Falconiformes. DNA-DNA hybridization (Sibley et al. 1988) suggested the following different phylogeny. The Strigi, Aegoteli, and Caprimulgi must be considered suborders of the order Strigiformes, while the diurnal raptors are distantly related and placed (infraorder Falconides) within the order Ciconiiformes.

Electrophoretic data support Sibley et al.'s (1988) conclusions. Qualitative patterns of egg-white proteins indicate affinities between Strigiformes and Caprimulgiformes (Sibley and Ahlquist 1972). Kuroda et al. (1982) studied the evolution of mitochondria (mtMDH) in birds and showed that Strigiformes and Caprimulgiformes shared the same electromorph. (mtMDH electrophoretic mobility was 200 as compared to the standard value of 100 for *Anas platyrhynchos*, while the mtMDH mobility of the Falconiformes was 140.)

The ancestry of the Strigiformes could not be further clarified either by karyology (Belterman and De Boer 1984) or the fossil record (Olson 1985). Recent investigations (Mourer-Chauvirè 1989) show that the Caprimulgiformes were highly diversified in the Paleogene. They apparently belong to an early phase of the evolution of birds and were probably antecedent or coeval of the first strigiforms.

The results presented in this study are not aimed to address the controversial problem of determining the closest phylogenetic relatives of the Strigiformes. The average genetic distance between *C. europaeus* and the Strigiformes ($\bar{D}$ = 3.09) is very large and few alleles are shared between the two orders (Table 1). When this level of genetic divergence is reached, the resolutive power of multilocus protein electrophoresis is reduced. Nevertheless, *C. europaeus* can work as a functional outgroup for the Strigiformes, since it shares two alleles (GPI "d," H-PT-3 "b") with *T. alba*, but no one with the Strigidae species we have studied.

Phylogenetic relationships within the Strigiformes have been studied mainly with traditional qualitative taxonomic methods (Mikkola 1983). Exceptions are the still unpublished and therefore largely undiscussed DNA-DNA hybridization studies by Sibley and co-workers (pers. comm.). Their results indicate the existence of large genetic distances between *Tyto* and the Strigidae (DTH50 = 13.6). Concordant information comes out from karyological studies (Capanna et al. 1987, Belterman and De Boer 1984). Diploid number is 2n = 92 in the Tytonidae, while average 2n = 82 in the Strigidae. The karyotype of the Tytonidae is peculiar for having all acrocentric chromosomes with no clear distinction between micro- and macro-chromosomes. Belterman and De Boer (1984) were unable to derive a phylogeny of the Strigidae using karyological information. Some groups with similar karyotype were proposed, but similarity does not mean phylogenetic relationship, since homology between similar chromosomes has not been demonstrated and independent origin of similar karyotypes is a possible event in birds.

Egg-white protein electrophoresis (Sibley and

Ahlquist 1972) suggests that *Tyto* differs from the Strigidae, although the two groups appear to be closely related. Mosher et al. (1982) studied serum protein electrophoresis by using a phenetic distance measure and only four species: *Strix* and *Otus* were found to be more similar than *Asio* and *Aegolius*.

Our multilocus protein electrophoresis data indicate the existence of great genetic divergence between *Tyto* and the Strigidae (average genetic distance $\bar{D} = 1.49$). Both distance and parsimony methods produced dendrograms supporting the current view of the Tytonidae as sister lineage of the Strigidae. The relationships within the Strigidae are depicted by two different topologies. In the first one, obtained by the distance methods, the genus *Athene* is ancestral to all the other genera; *Strix* is ancestral to the genus *Asio*; *Otus* and *Bubo* are sister genera. In the second one, obtained by the parsimony method, the relations of ancestrality among the clusters (*Athene* with *Strix*; *Asio otus* with *Asio flammeus, Otus* with *Bubo*) are not resolved.

The rather poor literature on phylogenetic relationships within the Strigidae makes it difficult to compare our findings with other kinds of data. However, some concordant results are worth noting. Some species belonging to the genera *Otus* and *Bubo* show similar chromosomes, probably directly derived from the same presumed ancestral karyotype (Belterman and De Boer 1984). In a study of vocal taxonomy of the genus *Otus*, Van Der Weyden (1975) believes that the sonogram of *Bubo bubo* closely resembles those of some species of the genus *Otus*. Our protein electrophoresis results support the point of view of a phylogenetic relatedness of the genera *Bubo* and *Otus*, notwithstanding the large differences in size, probably due to adaptive specialization and to natural selection on quantitative anatomic and morphologic traits. The genus *Athene* is ancestral to most of the Strigidae, as resulting from DNA-DNA hybridization data (Sibley, pers. comm.), in agreement with our protein electrophoresis distance dendrograms.

Some moderate variation in the rates of protein evolution along the lineages is apparent when comparing UPGMA (Fig. 1a) and Wagner (Fig. 1b) dendrograms. Rate heterogeneity could result as a consequence of both methodological or biological reasons. In birds the relation between genetic distance and divergence time is no longer linear for $\bar{D}$ values larger than about 1.4. Consequently, the genetic distances between *Tyto* and the Strigidae could be significantly underestimated, and this could produce apparent rate heterogeneity. Moreover, some lineages could accumulate mutations at a higher rate than others. It could be the case of the apparent high rate of protein change in *Otus scops* (Fig. 1b). Within the limitations due to variability in the rates of protein evolution and to the sampling errors associated with any measure of genetic distance, the molecular clock can be calibrated using the average rate of protein evolution after the divergence time.

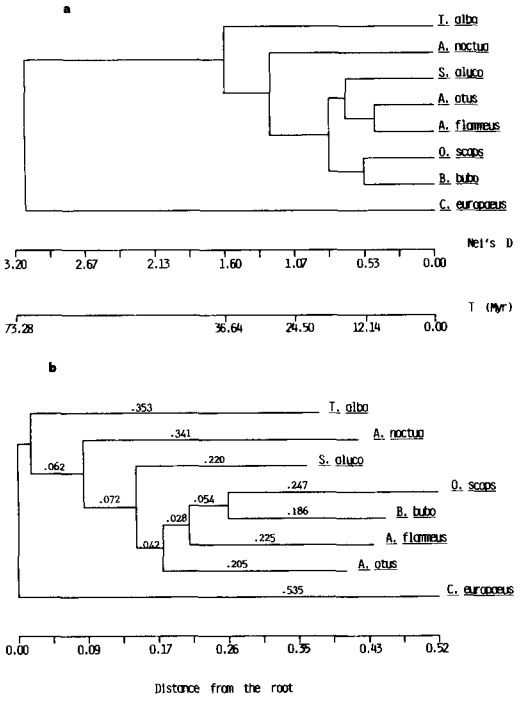

FIGURE 1. Dendrograms showing the clustering of seven Strigiformes based on Nei's genetic distances (Table 1). *Caprimulgus europaeus* is used as an outgroup. (a) UPGMA; cophenetic correlation = 0.968. (b) Wagner method; cophenetic correlation = 0.974.

We use the relation: 1 Nei's $\bar{D}$ = 23 million years (Myr), obtained from phylogenetic studies on Galliformes and other birds (Randi et al. 1989). In Figure 1a, this time scale is compared with available fossil data (Olson 1985, Mourer-Chauviré 1987). This comparison suggests that the separation between Tytonidae and Strigidae must be more ancient than 40 million years ago, and that the evolution of the modern Strigidae is not older than the lower Miocene (Mourer-

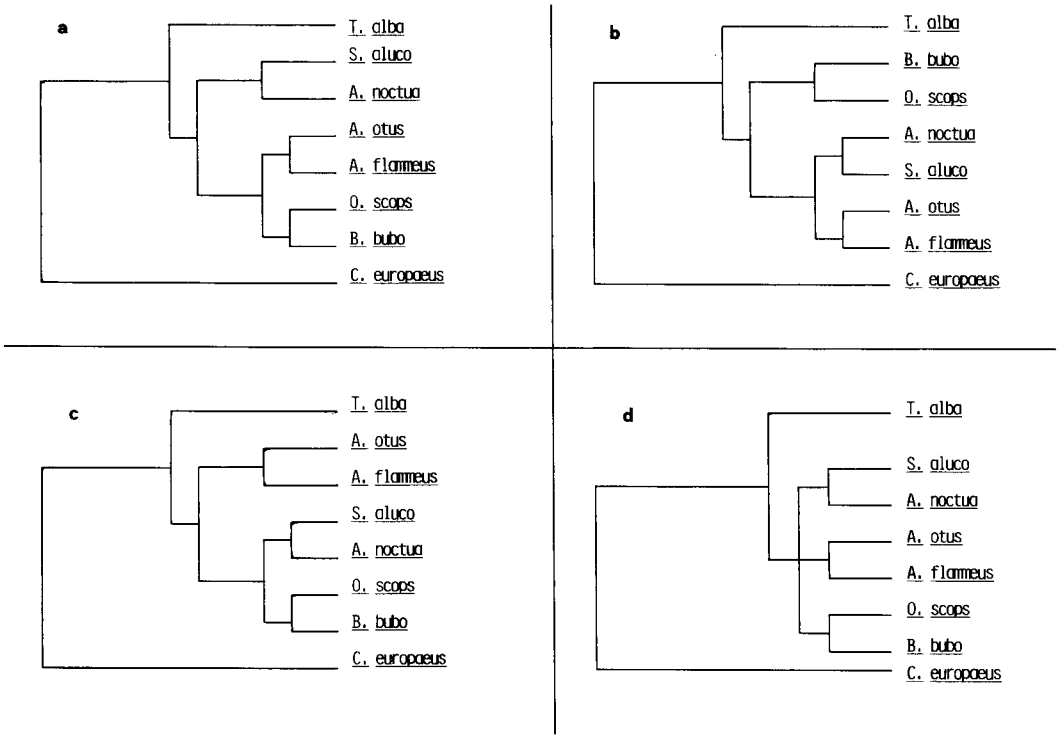

FIGURE 2. HENNIG86 parsimony trees (a, b, c). Strict Nelson consensus (d) of the parsimony trees. Tree length = 71; consistency index = 0.63.

Chauvirè, pers. comm.). Our estimated divergence times are probably low when the separation between the Strigidae and the Tytonidae is considered (34.35 Myr), but they are in agreement with the fossil record of the modern Strigidae (25 Myr).

The phylogenetic relationships among the seven species of Strigiformes we studied lead to the following conclusions. The genetic distance between Caprimulgiformes and Strigiformes is very large, but the few shared alleles allow the use of *C. europaeus* as a functional outgroup to root the strigiform dendrograms. The current taxonomic subdivision of the Strigiformes into two families (or subfamilies), Tytonidae and Strigidae, is supported on genetic basis. *Athene* belongs to an old lineage, originated about 25 Myr, and could be ancestral to most of the modern Strigidae. *Athene* and *Strix,* as well as *Bubo* and *Otus,* are related genera. The genetic distance between *A. otus* and *A. flammeus* is unusually large ($\bar{D}$ = 0.46) for congeneric bird species (Avise and Aquadro 1982) and suggests that phylogenetic relationships should be more deeply investigated in this group of birds.

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