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A Molecular Phylogenetic Survey of the Nightjars and Allies (Caprimulgiformes) with Special Emphasis on the Potoos (Nyctibiidae)

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A 656-pb fragment of the mitochondrial cytochrome *b* gene was sequenced for six species of Central and South American potoos (genus *Nyctibius*, Nyctibiidae) as well as for selected representatives of all other caprimulgiform families. Sequence divergence among potoos was much higher (11.1–16.2%) than has typically been observed among congeneric species of birds, suggesting that the species of *Nyctibius* are quite old. Divergence among families was also quite high (13.7–21.8%), confirming recent DNA–DNA hybridization studies. Such high genetic divergences in a functionally constrained gene like cyt *b* indicate that many of those sites which are free to vary will have experienced multiple substitutions. We therefore performed phylogenetic analysis using parsimony under a variety of weighting schemes intended to reduce the effect of multiple substitutions. The monophyly of all the traditional caprimulgiform families was confirmed and a number of new hypotheses of relationship emerged. From our analysis, it appears that the oilbird (*Steatornis*) is an ancient member of the order, and it is not closely related to the potoos. We also note the close link between Aegothelidae and Caprimulgidae, and the basal position of the Podargidae/Batrachostomidae clade in the phylogeny. These results are in accordance with several classical works of the first half of the century. The relationships of the various *Nyctibius* species to each other have not been fully resolved; however, a close link between *N. leucopterus* and *N. maculosus* appears to be highly probable from our data.

INTRODUCTION

The potoos (Nyctibiidae) form a small but distinctive family of night birds endemic to the Neotropical Region. While potoos occur in most areas of humid forest from Mexico to Argentina, their secretive habits make them exceedingly difficult to study. Consequently, potoos are among the most poorly known of all birds. Recent fieldwork in South America has yielded new information on vocalizations and life history of several little known taxa, clarifying species boundaries and providing new material for anatomical and molecular studies (Remsen and Traylor, 1983; Schulenberg *et al.*, 1984; Parker *et al.*, 1985; Cohn-Haft, 1993). Here, we use DNA sequence data to assess phylogenetic relationships among potoos. A second goal of the present work is to determine the position of the Nyctibiidae in caprimulgiform systematics, as well as to understand the structure of this order in general.

While the nightjars and their allies (Caprimulgiformes) are now generally considered monophyletic, both their position in avian classification and the structure of the group have been discussed extensively since the 19th century. Historically, caprimulgiforms have been thought to be related to the owls, swifts, cuckoos, rollers, motmots, jacamars, puffbirds, or trogons, among others (see Sibley and Ahlquist, 1990 for a detailed summary). In recent literature, a close relationship to owls is advocated by Sibley and Ahlquist (1990), while Cracraft (1981) proposed a swift–hummingbird clade as the sister-taxon. The DNA hybridization data of Bleiweiss *et al.* (1994) indicated that owls and caprimulgiforms form a clade, which is sister to the swift–hummingbird clade. The diversity of the Caprimulgiformes has been noted both at the anatomical level (Hoff, 1966) and at the molecular level (Sibley and Ahlquist, 1990). The latter authors remark (p. 418) that "the subgroups of Caprimulgi are far more divergent from one another than most classifications have suggested" and further (p. 149) "clearly, the Caprimulgi have a large amount of genetic diversity concealed by a conserved external appearance."

Although formal phylogenetic hypotheses for Caprimulgiformes are scarce (Sheldon and Bledsoe, 1993), varied schemes of relationships of the different capri-

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Sequence data from this article have been deposited with the GenBank Data Library under Accession No. X95764–X95781.

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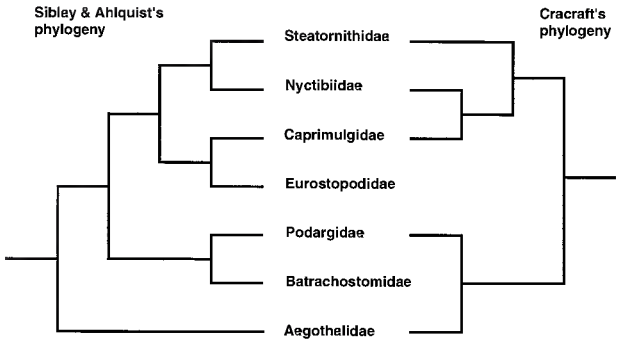

FIG. 1. Recent hypotheses of relationships of the Caprimulgiformes as proposed by Cracraft (1981) and Sibley and Ahlquist (1990).

mulgiform subgroups to each other have been proposed. Common propositions include treatment of *Steatornis* in a monotypic family, a main division between *Podargus* and its allies and *Caprimulgus* and its allies, and possibly a close relationship between *Caprimulgus* and *Nyctibius* (various authors, as reported in Sibley and Ahlquist, 1990). In a myological survey, Hoff (1966) concluded that the Nyctibiidae are very different from all other Caprimulgiformes and that *Aegotheles* shows more specialization in the direction of owls than any other caprimulgiform. More recently, Cracraft (1981) proposed a classification based on phylogenetic analysis of morphological characters in which he placed the Aegothelidae and the Podargidae in one suborder and *Steatornis,* the Caprimulgidae, and the Nyctibiidae in another, with the last two taxa as sister-groups (Fig. 1). In their revision of avian systematics based on DNA–DNA hybridization, Sibley and Ahlquist (1990) reached a different conclusion: They found the Aegothelidae to be the sister-group of all other Caprimulgiformes, the Nyctibiidae to be related to *Steatornis,* and *Podargus* and *Eurostopodus* to be fairly distant within the Caprimulgi (Fig. 1). However, Sibley and Ahlquist's hypotheses of relationship are equivocal because a different type of analysis mostly on the same data gave a tree which is incongruent with their primary analysis. In their Fitch tree (p. 819), *Aegotheles* is the sister-group of a clade grouping *Podargus* and *Steatornis,* all of them being the sister-group of the Caprimulgidae. Both Cracraft's (1981) and Sibley and Ahlquist's (1990) phylogenies have been criticized (Olson, 1982; Cracraft, 1992; Lanyon, 1992; Mindell, 1992; Harshman, 1994).

To our knowledge, the only published hypotheses of relationship among potoo species are the possibility of a close link between *Nyctibius maculosus* and *N. leucopterus* suggested by Chapman (1926) and the contradictory position of Schulenberg *et al.* (1984) who favor a *N. maculosus–N. griseus* grouping. The only other works dealing with potoo relationships are based on acoustic analyses. Davis (1973, 1978) believed that there were at least four cryptic species subsumed in *N. griseus,* and felt that *N. grandis* and *N. griseus* (*sensu stricto*) belong to clearly distinct species groups.

As a probe of potoo phylogeny, we chose to sequence a portion of the mitochondrial cytochrome *b* gene (cyt *b*). Cyt *b* has become popular for molecular systematics due to the development of "universal" primers (Kocher *et al.,* 1989), which allow relatively easy amplifications via the polymerase chain reaction (PCR). This gene has been used extensively for phylogenetic studies at a range of taxonomic levels (e.g., Edwards and Wilson, 1990; Meyer and Wilson, 1990; Smith *et al.,* 1991; Edwards *et al.,* 1991; Graybeal, 1993; Helm-Bychowski and Cracraft, 1993). The rapid rate of evolution of cyt *b* (and mtDNA in general) makes it most useful for studies of relatively closely related organisms (Brown, 1985; Li and Graur, 1991; Hillis and Huelsenbeck, 1992; Graybeal, 1994). Therefore, the choice of cyt *b* for the study of Nyctibiidae seemed appropriate, as all potoos belong to a single genus and genetic divergence is usually low within genera of birds (Kessler and Avise, 1985; Edwards *et al.,* 1991; Smith *et al.,* 1991; Birt-Friesen *et al.,* 1992; Richman and Price, 1992; Lanyon, 1994).

MATERIALS AND METHODS

Species Investigated

Frozen tissue samples were available from six of the seven known species of potoos and from several representatives of all other caprimulgiform families. A list of the taxa we have examined and related information is given in Table 1. We used the chicken cyt *b* sequence of Desjardins and Morais (1990) as an outgroup for phylogenetic analysis. The supraspecific nomenclature of Sibley and Monroe (1990) will be followed throughout this paper as a matter of convenience, because it is the most recent comprehensive treatment of the group.

Methods

Extraction of DNA. Tissue was homogenized in a buffer containing 10 mM Tris, 100 mM Na_2EDTA, 100 mM NaCl, and 0.5% SDS, pH 8.0, and digested for at least 3 h with 200 μg/ml proteinase K at 55°C. RNase was added to a final concentration of 50 μg/ml and the solution was incubated for 30 min, after which it was extracted twice with phenol/chloroform (50/50, v/v). The DNA was precipitated by addition of two volumes of 95% ethanol and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0). Alternatively, DNA from some samples was obtained through a Chelex extraction (Walsh *et al.,* 1991).

PCR conditions. Amplifications were carried out in 50- or 100-μl reaction volumes using 2.5 units *Taq* poly-

TABLE 1

Name and Origin of Taxa Examined[a]

Family and species	Origin	Tissue specimen number	Number of sequenced clones (L/H strands)
Nyctibiidae			
Nyctibius aethereus	Peru	LSU B10877	5 (4/1)
Nyctibius bracteatus	Peru	LSU B4509	3 (3/0)
Nyctibius grandis	Bolivia	LSU B15415	3 (3/0)
Nyctibius griseus	Panama	USNM B00493	3 (3/0)
Nyctibius leucopterus	Brazil	USNM B00031	4 (2/2)
	Brazil	LSU B20315	1 (1/0)
Nyctibius maculosus	Peru	LSU B271	3 (2/1)
	Peru	LSU B1825	1 (0/1)
Aegothelidae			
Aegotheles albertisi	PNG	MV E044	2 (1/1)
Aegotheles bennettii	PNG	MV E636	4 (2/2)
Aegotheles cristatus	Australia	MV W0191	4 (2/2)
Eurostopodidae			
Eurostopodus mystacalis	Australia	MV JWC129	2 (1/1)
Eurostopodus papuensis	PNG	MV E660	2 (2/0)
Batrachostomidae			
Batrachostomus cornutus	Borneo	CGS 2350	2 (1/1)
Podargidae			
Podargus ocellatus	Australia	MV C363	4 (2/2)
	Australia	MV C332	4 (2/2)
Podargus papuensis	Australia	MV C876	1 (1/0)
Caprimulgidae			
Caprimulgus longirostris	Peru	PA 13-7.5.87	[b]
Chordeiles rupestris	Ecuador	ANSP T2755	4 (2/2)
Phalaenoptilus nuttallii	United States	USNM B00084	2 (1/1)
Steatornithidae			
Steatornis caripensis	Venezuela	LSU B7472	3 (3/0)
	Venezuela	LSU B20984	2 (1/1)

Note. Abbreviations: PNG, Papua New Guinea; USNM, Laboratory of Molecular Systematics, Smithsonian Institution, Washington, DC; LSU, Louisiana State University, Baton Rouge, LA; MV, Museum of Victoria, Victoria, Australia; PA, Peter Arctander, Copenhagen; ANSP, Academy of Natural Science, Philadelphia, PA; CGS, Charles G. Sibley, San Francisco, CA.

[a] The classification used here is that of Sibley and Monroe (1990).

[b] Sequence determined by Peter Arctander.

merase (Promega); 1 mM each primer; 1.5 mM $MgCl_2$; 200 μM each dNTP; and 0.2–2 ng/μl genomic DNA (or 2 μl of Chelex DNA extract) in 1× *Taq* polymerase buffer (5% glycerol, 5 mM Tris–HCl, pH 8.0, 10 mM NaCl, 0.01 mM EDTA, 0.1 mM DTT, 0.1% Triton ×-100 final concentrations) supplied by Promega. Templates were denaturated at 94° for 2 min and then submitted to 30 to 36 cycles of the following conditions: 94° (40 s to 1 min); 48–51° (1 to 1.5 min); 68–72° (2.5–3.5 min), final extension 7 min at 68–72°. We used the primers L14841 (Kocher *et al.,* 1989) and H15498 (5'-AAACTGC AGGGAATAAAGTTATCTGGGTCTC-3') [same identification system as Kocher *et al.* (1989)] to which we added *Hin*dIII (L14841) and *Pst*I (H15498) recognition sites at the 5' end. The fragment generated was 722 bp long (656 bp of unknown sequence).

Cloning. PCR products were digested with *Hin*dIII and *Pst*I as per manufacturer recommendations, then ligated into similarly digested pBluescript (Stratagene) phagemids (KS+ and KS−) using T4 ligase (New England Biolabs). XL1-Blue *Escherichia coli* (Stratagene) were transformed with the ligation product by heat-shock (Sambrook *et al.,* 1989) or by electroporation with a BRL Gene Pulser electroporator according to Dower *et al.* (1988). Recombinant plasmids were identified by color selection after growth on LB plates containing IPTG and X-GAL. Plasmid minipreps were performed and the size of the insert was checked by agarose gel electrophoresis. Single-stranded phagemid molecules suitable for sequencing were produced according to manufacturer's recommendations (Stratagene).

Sequencing. The Sequenase II kit (USB) was used to perform dideoxy sequencing reactions on the single stranded DNA. Sequencing was performed using universal primers USB No. 70705 for pBluescript (KS+) and BRL 8050SA for pBluescript (KS−), as well as internal primers H15149 (Kocher *et al.,* 1989) and L15173 (5'-TGAGGACAAATATCATTCTGAGGGG-3') or L15174 (5'-CCATGAGGACAAATATCATTCTGAGG TGC-3') (newly designed) for the annealing reactions. Both strands were sequenced for most taxa, including at least one species in each family (Table 1).

Parsimony Analyses

Options in PAUP 3.0s (Swofford, 1991) were as follows: Multistate characters were treated as an uncertainty, accelerated optimization selected, shortcuts in stepmatrix allowed, only informative characters were used in the analysis. For each heuristic search, 20 replications were run. Other search settings were: swap on minimal trees only, trees added by random stepwise addition, seed of the first replicate equals 1, TBR swapping, collapse yes, mulpars yes, steepest descent no.

RESULTS

Sequences

We sequenced a 656-bp portion of cyt *b* from 6 of the 7 known species of potoos and from several representatives of all other caprimulgiform families (Fig. 2). This section codes for 218 amino acids, corresponding to residues 34 to 251 of the chicken (*Gallus*) sequence of Desjardins and Morais (1990). Because chicken was used as an outgroup for our phylogenetic analysis, we have adopted their numbering system. Sequences generated from single-stranded clones were easily and unambiguously readable, and several clones were sequenced for most species. The alignment of the sequences using GCG (Devereux, 1989) was unambiguous. No insertion/deletion events were observed, and no stop codon appeared.

Some sequence variation among clones derived from the same individual was observed. Each such case was studied carefully and many clones were resequenced to eliminate sequencing artifacts and reading errors.

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                          1         2         3         4         5
                 12345678901234567890123456789012345678901234567890
Chicken          TTTCGGCTCCCTATTAGCAGTCTGCCTCATGACCCAAATCCTCACCGGCC
Steatornis       C..........CC...GCA.......A.CA..G........A.....A.
N. aethereus     C..T..A..A..CC...GCA.....T.A..C..A........A.......
N. bracteatus    ......A..A..CC...GCA.......A..T..A.....T..A.......
N. grandis       C.....A.....CC...GCA....TT.A.CC..T..........T....
N. griseus       C..T..A..A..TC...GTA.T.....A..T..A........A..T....
N. leucopterus   C..T..G..G..CC...GTA.......AGCA..A........A......T
N. maculosus     C..T..A..A..CC...GTA.....T.AG.A..A........A.......
Phalaenoptilus   C.....A..T..CC...GGA.....T.A..A.........T.A.....A.
Caprimulgus      C..T..A.....GC...G.A.......A..A..........A..T..AT
Chordeiles       C.....T.....T....G.A.......A..A..A........A.....G.
Eu. papuensis    C.....A..T..CC...GCA.......A..AR.A......T.A.......
Eu. mystacalis   C..T.....A..CC...GTA.......A..A..A......T.A.......
Ae. albertisi    C..T..G.....CC...GCA........GCA..A.....T..A..T....
Ae. bennettii    C.....A..T..CC...GTA.......TGCA..A........A..T....
Ae. cristatus    C.....A..T..CC...GCA.......TGCA..A........A.......
P. ocellatus     C........A...C.T.GCA.......AC.A.TA......T.A.......
P. papuensis     C.....T..A...C.C.GCA.......AT.A.TA......T.A.......
Batrachostomus   C........A..TC.C.GTA.T.....AT.A..G.....TACA.......

                                                                  1
                          6         7         8         9         0
                 12345678901234567890123456789012345678901234567890
Chicken          TACTACTAGCCATGCACTACACAGCAGACACATCCCTAGCCTTCTCCTCC
Steatornis       .G..C........A.........C..T......A.......T.....A..A
N. aethereus     .....A.......A.........T..T......A.T..G..A.....T...
N. bracteatus    .....T.G..T..A......T..C..C......A.............G...
N. grandis       .T...........A.........T..C..T...A....G.....T..A..A
N. griseus       ..........T..A.........C..C......A.......A.....A..A
N. leucopterus   ....C.....A..A.....T..C..C......A.......A..T..A..A
N. maculosus     ....C.....T..A.........T..T.....GA.......A..T..G..A
Phalaenoptilus   ....CT.......A.........Y.........A..T.......T......
Caprimulgus      ....T........A.....T..C.........A.............T...
Chordeiles       ....C.......CA.........C.....T..TA..........T......
Eu. papuensis    .....T.......A.........C..C..T...A.......A.....T..A
Eu. mystacalis   .C.....................C..T..T...A..T....A........A
Ae. albertisi    ..........T.....................A.T............A...
Ae. bennettii    ..........T..A.........C..G......A.............A...
Ae. cristatus    ..........T..A.........C.........A.............A...
P. ocellatus     ............GCT.....T..T..T......A....T..T..TA....A
P. papuensis     ............GCC.....T..C.........A....C..T...A.T..A
Batrachostomus   .T..G.....A..A..T..T..T.........A.T..C.....TAT....

                                                                  1
                          1         2         3         4         5
                 12345678901234567890123456789012345678901234567890
Chicken          GTAGCCCACACTTGCCGGAACGTACAATACGGCTGACTCATCCGGAATCT
Steatornis       ...........A.....A..T..................A.....C..C..
N. aethereus     ..T........A.....A.....................A....AT..C..
N. bracteatus    ..C........A..T..A.....G.....T......T.A.....C..C..
N. grandis       ..T........A..T..AG.......G..T......T.A..T..T..C..
N. griseus       ..TA.......A.....A..................T.A..T..C..C..
N. leucopterus   ..T.......TA.....AG.......G.....T...T.A..T..T..C..
N. maculosus     ..T..T..T..A.....AG.T..............A.A..T..C..C..
Phalaenoptilus   ..C..T.....A.....A..T..............A.....C.....
Caprimulgus      ..G........C.....A........G..T......T.AC....C..C..
Chordeiles       ..C........C.....A........G.........T.A.....C..C..
Eu. papuensis    ..C.....T..A..T..A..T..C...Y........T.A.....C..C..
Eu. mystacalis   ........T..A..T..A..T..C..G.........T.A.....A..C..
Ae. albertisi    ..C.....T..A.....A........G.....T.....A.....A..CA.
Ae. bennettii    ..T........A..T..A..T.....G.....T...T.A.....A..CA.
Ae. cristatus    ..T.....T..A.....A........G...........A.....A...A.
P. ocellatus     A.C........A.....A..T......C.........A.....C..C..
P. papuensis     A.T........A..T..A.........C.T.......A........CT.
Batrachostomus   ACC........A.....A..T..C.....T........A..A..A..C..
```

FIG. 2. 656-bp sequence of 18 caprimulgiform taxa for the cyt *b* gene aligned with the chicken sequence of Desjardins and Morais (1990). Dots indicate a match with the chicken sequence. The first base corresponds to position 14991 of Desjardins and Morais (1990). R denotes A or G, K denotes G or T, and Y denotes C or T. Ambiguities represent actual differences between clones that may be due to *Taq* polymerase error or microheteroplasmy (see Results).

```
                                                                      2
                          6         7         8         9         0
                 12345678901234567890123456789012345678901234567890
Chicken          CCACGCAAACGGCGCCTCATTCTTCTTCATCTGTATCTTCCTTCACATCG
Steatornis       T..........A..T..........T..T.......A...A.......
N. aethereus     T..T........A..T..C.....................A...A.....T.
N. bracteatus    A..........A..A.....................T.A...A.....T.
N. grandis       A..T........A.....T...........T..C....A...A.......
N. griseus       A..........A.................T.......A.........T.
N. leucopterus   A..T........A..............T..T.....T.A...A.......
N. maculosus     A..........A..A.....T........T.....T.A...A.......
Phalaenoptilus   T..T........A.....................C..T.A...........
Caprimulgus      ...T........A........T...........CG.A.A...C.....T.
Chordeiles       ...T.....T..A..A........T.............A...G.......
Eu. papuensis    ............A..T..................C....A.........T.
Eu. mystacalis   T..T........A..............T........T.AT..C.....T.
Ae. albertisi    A..T........A..T..C.........T........T.A...A.......
Ae. bennettii    A..T........A..T..C............T..C..T.A.........T.
Ae. cristatus    A..T........A.....C............T..C..T.A...........
P. ocellatus     A..T........A........T............G.......C..T..T.
P. papuensis     A..T.....T..A..T.....................T..C.......
Batrachostomus   A.....G.....A.....G..T..................A...A.......

                                                                      2
                          1         2         3         4         5
                 12345678901234567890123456789012345678901234567890
Chicken          GACGAGGCCTATACTACGGCTCCTACCTCTACAAGGAAACCTGAAACACA
Steatornis       .......A..C.....T...........T..................T...
N. aethereus     .......A..T...........A.T...A.....A.....T.........
N. bracteatus    .......A..T...........A.T...A.T...A...........T...
N. grandis       .......A..C.....T.....A.T...G..T..A...........T..C
N. griseus       .......A..T...........A.T.........A........G.....C
N. leucopterus   .......A..............A........T...................
N. maculosus     .......A........T...........A..............G......
Phalaenoptilus   ..........T...........A...........A...............
Caprimulgus      ......................A.....A.....A...........T...
Chordeiles       ....T..T........T..................A...............
Eu. papuensis    .......AT.G........T......T.A.TT..A...............
Eu. mystacalis   .......A.....T..............A.T.R.A...............
Ae. albertisi    .......AT.T.....T...........A..................T...
Ae. bennettii    .......AT.C..................A..T..A...............
Ae. cristatus    .......AT.C..............T..A..T..A...............
P. ocellatus     .......A...........T..G...T.A.....A.....T......T...
P. papuensis     .......A.....T.....T..A...T.A.....A.....T......T..
Batrachostomus   .G...........T..T..A..............A.....T.........

                                                                      3
                          6         7         8         9         0
                 12345678901234567890123456789012345678901234567890
Chicken          GGAGTAATCCTCCTCCTCACACTCATAGCCACCGCCTTTGTGGGCTATGT
Steatornis       .....T........A..T..C..A.....A..T.....C..A..A.....
N. aethereus     ..G...........A..T..T..A.....T...........A..A..C..
N. bracteatus    ..GA.CT.......A.....C..A.....T..A.....C..A..A.....
N. grandis       .....C........A..A..C..A........A.....C..A.....C..
N. griseus       ........T.....A.....C..A.....T..A........A..T..C..
N. leucopterus   .....G........A..T..C..A........A........A..T.....
N. maculosus     ..G.....T.....A.....C..A.....T..A........A..A..C..
Phalaenoptilus   .....T........A.....C..A.....A..T..T..C..A........
Caprimulgus      .....TG.......G.....C..A.....A..T..T..C..A........
Chordeiles       .....C........A.....C..A.....A...........A..T..C..
Eu. papuensis    .....T...........G..C........A..T.....C..A..A..C..
Eu. mystacalis   .....T.....A..A..A..C..T.....A..T.....C..A..G..C..
Ae. albertisi    .....C..T.....A..G..C..A.....A..T.....C..A..A.....
Ae. bennettii    .....C..T.....A..AG.C..A.....A..T........A..A..C..
Ae. cristatus    .....C..T...T.A..G..CT.A.....A..T.....C..A..A.....
P. ocellatus     ...A.C..T..T..TA....C..G.....A..A..A..C..A..G..C..
P. papuensis     ...A.C..T..T...A....C..A.....A..A..A..C..A..A..C..
Batrachostomus   ...A.C.....A........C........A..A.....C..A..A..C..
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FIG. 2—*Continued*

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                                                                      3
                    1         2         3         4         5
                    12345678901234567890123456789012345678901234567890
Chicken             TCTCCCATGGGGCCAAATATCATTCTGAGGGGCCACCGTTATCACAAACC
Steatornis          C..A.....A..A..G.....C..........T..A..C.....C....
N. aethereus        .T.A.....A..G........T........T..T..A..C.....T...T
N. bracteatus       C..A.....A..A.....G..........C.....A..C.....C....
N. grandis          C..G.....A..A..G.....C........T.....A........C....
N. griseus          C..A.....A..A..................T.....A..C.....C....
N. leucopterus      C..A.....A..A..................T.....A.....T..C....
N. maculosus        C..T.....A..A.....G..........T..T..A..C.....C....
Phalaenoptilus      .T.A.....A..A........C........C.....A..C.....C....
Caprimulgus         C..A.....A..A.....................T..A.....T..C..T.
Chordeiles          CT.A........A..G..G..............T..A.....T..C....
Eu. papuensis       CT.A........A........T........A.....A..C.....C...T
Eu. mystacalis      C..A.....A..A........C..........T.....C.....C.R..
Ae. albertisi       CT.A.....A..A....................T..A..C.....C....
Ae. bennettii       C..A.....A..A..................A.....A........C..T.
Ae. cristatus       C..A.....A..A..................A..T..G........C....
P. ocellatus        C..A.....A..A........C.....G.....T..A........C....
P. papuensis        C..A.....A..A.....G..C..........T..A..C.....C....
Batrachostomus      A..A..T..A..A........C........C.....A..C..T.......

                                                                      4
                    6         7         8         9         0
                    12345678901234567890123456789012345678901234567890
Chicken             TATTCTCAGCAATTCCCTACATTGGACACACCCTAGTAGAGTGAGCCTGA
Steatornis          .......G..C.....A..T..C..C..A...........A..G..G...
N. aethereus        ....T.....C.....T........C..G...........A.....T...
N. bracteatus       ..........C..C..A..T..C..C..A...........A.........
N. grandis          .......T..T.....T..T.....T..A...........A..G......
N. griseus          .......T..C..C..A........C..A...T....C..A..G..T...
N. leucopterus      .......C..C..C..T........T..A...T.G.....A..G......
N. maculosus        .......T..T..C........C..T..A........T..A.........
Phalaenoptilus      ..........T..C..A.....C..C..A.....T.....A.....A...
Caprimulgus         ..........T..C..A........C..A.....C.....A.....A..G
Chordeiles          .C.........C.....A........C..A...........A.....A...
Eu. papuensis       .......C..C..C...........C..A...A.C.....A.........
Eu. mystacalis      ..........CC.C.....T.....C..A...A.C.....A.....T...
Ae. albertisi       .......C..C..C..A........C..A..AA.C.....A..K......
Ae. bennettii       .......T..C..C..T........T..G..AA.C..............
Ae. cristatus       .......T..C..C...........C..A..AA.C.....A.........
P. ocellatus        ..........T..C..A..T..C..C..A.A...G.....A.....A...
P. papuensis        ..........T..C..A.....C..C..A.A.........A.....A...
Batrachostomus      ..........C..C.....T.....C..AG..Y.T.....A...A.A...

                                                                      4
                    1         2         3         4         5
                    12345678901234567890123456789012345678901234567890
Chicken             GGGGGATTTTCAGTCGACAACCCAACCCTTACCCGATTCTTCGCTTTACA
Steatornis          ........C.....A........C..T..A........T..T..CC....
N. aethereus        ..A.....C..T..A..T.....C..A..A...........CC....
N. bracteatus       ..A..C........A.....T..C..G..A...........T..CC.T..
N. grandis          ..C.....C..C..A........T..A..C........T.....CC....
N. griseus          ..T.....C.....A........C..A..A..A...........CC....
N. leucopterus      ..C..C..C.....A........T..AT.A..T...........CC....
N. maculosus        ..C.....C.....A........C..A..A..T...........C.....
Phalaenoptilus      ........C..C..A........C..A..A........T.....CC....
Caprimulgus         ..T.....C..C..A........C..AT.A...........T...C....
Chordeiles          ..A........T..A........C..A..A.....C........CC....
Eu. papuensis       ........C.....A...........AT.A..A.....T.....CC....
Eu. mystacalis      ..A...........A..T........AT.A..T........T..CC....
Ae. albertisi       ..T.....C.....A..T..T.....A..A..T...........CC....
Ae. bennettii       ..C...........A..T........A..A..T...........C.....
Ae. cristatus       ..T.....C.....A..T........A..A..T...........CC....
P. ocellatus        ..T..C..C..T..A...........AT.A.....C......A.CC....
P. papuensis        ..C..C..C..T..A...........AT.A.....C..T...A.CC....
Batrachostomus      ..T.....C..C..A...........AT.A...........A.C.....
```

FIG. 2—*Continued*

```
                                                                      5
                         6         7         8         9         0
                1234567890123456789012345678901234567890123456789012345678890
Chicken         CTTCCTCCTCCCCTTTGCAATCGCAGGTATTACTATCATCCACCTCACCTTCCTACACGAAT
Steatornis      ......G.....A..CAT.........C..C..CT.A.....................T.....
N. aethereus    ...T..T........CAT...TA.....C.A..CT.A...........A.....T.....G.
N. bracteatus   ..............C.....T.....AC.A..AT.A.....................T.....
N. grandis      ..............CATG..T.....CC.....G.A........T..T.....C.....T.....
N. griseus      ...T..A........CAT...T.....CC.A..CT.A.....T.....T.....T.....T.....
N. leucopterus  T.....A........CATT..T.....CC.A..CC.AG.....................T.....
N. maculosus    ......A..T.....CATC..T...A..C.A..CT.AG....T.....T.....T.....T.....
Phalaenoptilus  ......A.....A..CAT...T.....CC.C..CC....T.....A..A.....T.....T.....
Caprimulgus     T...T.A.....A..CAT.........C.C..CT.A........A..T.....T.....T.....
Chordeiles      ............A...AT.........CC.C..CC....T.....A..A.....T.....T.....
Eu. papuensis   ...T..A.....G..CAT......T...C....CC.AG.........A.....T.....T.....
Eu. mystacalis  ............A..C.T......T..CC.....T.AG.........A.....T.....T.....
Ae. albertisi   .........T..A..CAT...T.....CC.C..GC.A.....T.....T.....T.....T.....
Ae. bennettii   .........T..A..CAT...T.....CC.C..AC.....................T.....T.....
Ae. cristatus   .........T..A..CAT...T.....CC.C..GC.T..........T.....T.....T.....
P. ocellatus    ............A...TT....A.C...C.C.....A..........A.....T.....T.....
P. papuensis    ............A...TT....A.C..CC.C.....A..........A.....T.....T.....
Batrachostomus  T.....T..G..A..CATCC.TA..A.CC.C..C..A..T.....................T.....

                                                                      5
                         1         2         3         4         5
                1234567890123456789012345678901234567890123456789012345678890
Chicken         TCCTACACGAATCAGGCTCAAACAACCCCCTAGGCATCTCATCCGACTCTTCCTACACGAAT
Steatornis      ....T..........A.....................TA.G...AG..G.....T.....T.....
N. aethereus    ....T........G..............T....TTG.GT........GC.....T.....T.....
N. bracteatus   ....C.....................................GT.......T.GC.....T.....
N. grandis      ....C........C.....G........A.....T...A.G..A....G.....T.....T.....
N. griseus      ....C..T.....G.............TT....T..TGT...A....G.....T.....T.....
N. leucopterus  ....T.........................T........TGT...TA...GC.....T.....T.....
N. maculosus    ....T..T.....................T........TGT....A...GC.....T.....T.....
Phalaenoptilus  ....T..............................A...GT...A....G.....T.....T.....
Caprimulgus     ....C...........A..................A...ATG..AA...GC.....T.....T.....
Chordeiles      .T..C.........C.....................A..TGT....A...GC.....T.....T.....
Eu. papuensis   .T..C.....G..C.....C..............A...GT...AA...G.....T.....T.....
Eu. mystacalis  ....C......A..........T.....TT....A...AT...A..T.GC.....T.....T.....
Ae. albertisi   .T..C..T..................G........AG.AATT..AA...G.....T.....T.....
Ae. bennettii   ....C..............................A..AGT...AA...G.....T.....T.....
Ae. cristatus   ....C........G.....................AG.AGT...AA...G.....T.....T.....
P. ocellatus    ....T........T..G..T........A......C.TC....AA...G.....T.....T.....
P. papuensis    ....T..T..G..C..A...........AT.....C..C....AA.T.G.....T.....T.....
Batrachostomus  ....C.....G..T..A...........A........G.....A....C.....T.....T.....

                                                                      6
                         6         7         8         9         0
                1234567890123456789012345678901234567890123456789012345678890
Chicken         GACAAAATTCCATTTCACCCATACTACTCCTTCAAAGACATTCTGGGCTTCCTACACGAAT
Steatornis      ........C...........T........A..A.....T..C.....T.....T.....T.....
N. aethereus    ........C.....C.....C..T.T......A........C..A..T..T.....T.....T.....
N. bracteatus   ..............C.....C....T....C.A........CC..A.....T.....T.....T.....
N. grandis      ..T.....C.....C.....C....T....C.A........C..A.....T.....T.....T.....
N. griseus      ........C.....C.....C....T....C.A...........A.....T.....T.....T.....
N. leucopterus  ..T.....C.....C.....C....T....C.A........CT.A..T..T.....T.....T.....
N. maculosus    ........C.....C.....C....T....C.A........C..A..T..T.....T.....T.....
Phalaenoptilus  ........C.....C.....C....T....C.T...........A.....T.....T.....T.....
Caprimulgus     ..............C.....C....T.....A.....T.....A.....T.....T.....T.....
Chordeiles      ..T.....C.....C.....C....T...TC.A...........A..T..T.....T.....T.....
Eu. papuensis   ..T.....C.....C..Y..T....TTA.TC.A.....T..C..A..A..T.....T.....T.....
Eu. mystacalis  ........C.....C.....C....T.A.TC.A........C..A..A..T.....T.....T.....
Ae. albertisi   ........C..T..C..T..C....T....A.A........C..A..T..T.....T.....T.....
Ae. bennettii   ........C..C..C.....C....T....ACA........C..A..T..T.....T.....T.....
Ae. cristatus   ........C..C..C.....C....T....ACA........C..A..T..T.....T.....T.....
P. ocellatus    ........C.....C..........T.....ACA.....TG.A..A.....T.....T.....T.....
P. papuensis    ........C.....C..........T...AACA.....TG.C..A..TC.....T.....T.....T.....
Batrachostomus  ..............C.....T....T......A........CAT.A.....T.....T.....T.....
```

FIG. 2—*Continued*

```
                                                                      6
                          1         2         3         4         5
                 12345678901234567890123456789012345678901234567890
Chicken          AACTCTCATACTCACCCCATTCCTAACACTAGCCCTATTCTCCCCCAACC
Steatornis       T.TA..T......CT...CC.AAC...C..T...T.......A.......
N. aethereus     C..AA.T.....T....T...AAC...CT................T..TT
N. bracteatus    TG.AA......CTG...TGC.AAC...C..T...................
N. grandis       C.TAA.T......G...T.C.AAC...CT.....................T
N. griseus       C.TAA.......TG..AT.C.AAC...CT....T.....T.....T....
N. leucopterus   C.TAA.T.....T...AT.C.AAC...C..................A....
N. maculosus     C.TAGC......TG.A.TTC.AA....C...A.............A....
Phalaenoptilus   CG.A..T......CT....C.AA.G...T.....A....T.....A....
Caprimulgus      TT...........CT....C.AA.......................T...T
Chordeiles       CT.A.........CT....C.AA...........A..........A....
Eu. papuensis    CG.A........ACT......AAC...T..C...........A..T....
Eu. mystacalis   C.TA..T..GT.ACTT...C.AAC...C..T...T.......A..A....
Ae. albertisi    C..AT........CT...CC.AA.G.TC...........A...G......A
Ae. bennettii    T.TAT........CT...CC.AA....T...........T..T......A
Ae. cristatus    C..AT.......TCT...CC.AA....C.....A........T......A
P. ocellatus     T..A..TT.....CTT...C.TAC.T.C...A..........A..A....
P. papuensis     T.TA..TT.....CT...CC..AC.T.C...A.......TA.A..A...T
Batrachostomus   T..C..TG.T...CTTAT.C.AAC.G.C.....TT.......A..T....
```

```
                      6
                      5
                 123456
Chicken          TCCTAG
Steatornis       ......
N. aethereus     .G....
N. bracteatus    .G....
N. grandis       .A....
N. griseus       .G....
N. leucopterus   .GT...
N. maculosus     .G....
Phalaenoptilus   .A....
Caprimulgus      .A....
Chordeiles       .A....
Eu. papuensis    .T....
Eu. mystacalis   .A....
Ae. albertisi    .G....
Ae. bennettii    .A....
Ae. cristatus    .A....
P. ocellatus     ..T.G.
P. papuensis     ..T.G.
Batrachostomus   ......
```

FIG. 2—*Continued*

Such differences amounted to roughly 1.5 variable bases per 1000 bases sequenced. Possible sources of this variation include *Taq* polymerase errors during PCR amplification and microheteroplasmy (perhaps due to somatic mutation) in the mitochondrial DNA template. In cases where we had sequenced more than two clones for an individual, there was never more than a single clone with a variant base at a given position, and we used the predominant base in phylogenetic analyses. In cases where we had sequenced only two clones for an individual, we treated variable positions (total of eight in five taxa) as ambiguous (Fig. 2). In three of these cases, there was no other variation among taxa, so the ambiguous base was phylogenetically uninformative for parsimony methods.

Sequence Analysis

Distances. The raw sequence divergence among potoo cyt *b* genes varies from 11.1 to 16.2% (Table 2), which is notably higher than within other genera in our study (5.9–12.8%). Sequence divergence ranges from 13.7 to 21.8% between families. In pairwise comparisons, the ratio of transitions (TS) to transversions (TV) is always greater than 2 within potoos (mean TS/TV = 2.94) and within other genera, but never reaches this value between families, where the mean TS/TV = 1.42 (Table 2). A plot of sequence divergence against TS/TV ratio for each pairwise comparison (Fig. 3) indicates that readily substitutable sites are essentially saturated with transitions at the family level but not at the genus level.

Intraspecific variation. We sequenced four clones (two on each strand) from two specimens of *Podargus ocellatus* to assess the amount of intraspecific variation. No differences were observed between clones from these individuals. Clones from two specimens of *N. leucopterus* and *Steatornis* were also identical. There are

TABLE 2

Pairwise Comparisons among Studied Taxa

	N.AET	N.BRA	N.GRA	N.GRI	N.LEU	N.MAC	A.ALB	A.BEN	A.CRI
N. aethereus	—	73–23	76–27	57–25	76–27	69–29	68–47	72–43	65–44
N. bracteatus	0.143	—	76–28	62–22	76–24	67–26	66–42	65–40	65–41
N. grandis	0.154	0.159	—	64–22	59–28	74–32	74–42	62–38	64–39
N. griseus	0.122	0.128	0.131	—	60–14	53–20	61–44	55–40	53–41
N. leucopterus	0.154	0.152	0.133	0.113	—	61–12	55–46	50–42	52–43
N. maculosus	0.146	0.142	0.162	0.111	0.111	—	60–44	58–40	58–41
Ae. albertisi	0.171	0.163	0.175	0.159	0.152	0.157	—	58–8	44–9
Ae. bennettii	0.172	0.160	0.152	0.145	0.140	0.149	0.099	—	38–1
Ae. cristatus	0.163	0.162	0.157	0.143	0.145	0.151	0.079	0.059	—
Eu. mystacalis	0.172	0.162	0.180	0.163	0.163	0.172	0.165	0.155	0.155
Eu. papuensis	0.163	0.163	0.177	0.155	0.169	0.177	0.163	0.146	0.143
Batrachostomus	0.212	0.198	0.215	0.201	0.198	0.204	0.218	0.218	0.212
P. ocellatus	0.204	0.198	0.206	0.203	0.203	0.200	0.207	0.206	0.195
P. papuensis	0.203	0.200	0.207	0.204	0.210	0.204	0.206	0.198	0.195
Caprimulgus	0.175	0.157	0.169	0.160	0.166	0.183	0.163	0.159	0.154
Chordeiles	0.166	0.165	0.169	0.159	0.157	0.163	0.160	0.155	0.152
Phalaenoptilus	0.151	0.151	0.155	0.143	0.159	0.159	0.159	0.148	0.139
Steatornis	0.168	0.154	0.151	0.160	0.155	0.157	0.146	0.160	0.155
	E.MYS	E.PAP	BATR	P.OCE	P.PAP	CAPR	CHOR	PHAL	STEA
N. aethereus	71–44	66–43	82–59	80–56	75–60	72–45	67–44	64–37	73–39
N. bracteatus	59–47	61–46	70–60	73–57	70–61	57–46	63–45	57–42	65–36
N. grandis	69–49	72–44	87–54	82–53	79–57	67–44	62–49	62–40	57–42
N. griseus	58–49	60–42	72–60	76–57	73–61	63–42	55–49	56–38	67–38
N. leucopterus	60–47	67–44	78–52	78–55	79–59	67–42	60–43	66–38	66–36
N. maculosus	66–47	72–44	76–58	72–59	76–61	74–46	62–45	60–44	67–36
Ae. albertisi	66–43	69–40	84–60	74–61	75–59	64–44	57–49	61–44	57–40
Ae. bennettii	63–39	60–36	85–58	76–59	73–57	64–40	57–45	59–38	65–40
Ae. cristatus	62–40	57–37	80–59	68–60	70–58	60–41	54–46	52–39	61–41
Eu. mystacalis	—	67–17	80–53	65–54	67–56	62–47	59–48	59–43	59–39
Eu. papuensis	0.128	—	84–54	76–49	71–55	63–44	54–47	52–40	67–36
Batrachostomus	0.203	0.210	—	69–49	79–51	72–56	75–57	67–58	73–52
P. ocellatus	0.181	0.189	0.180	—	48–8	62–55	55–56	63–55	63–51
P. papuensis	0.188	0.191	0.198	0.085	—	72–57	67–58	68–57	66–51
Caprimulgus	0.166	0.163	0.195	0.178	0.197	—	60–25	60–18	64–34
Chordeiles	0.163	0.154	0.201	0.186	0.191	0.130	—	57–19	65–35
Phalaenoptilus	0.155	0.140	0.186	0.178	0.191	0.119	0.116	—	58–32
Steatornis	0.149	0.157	0.191	0.174	0.178	0.149	0.152	0.137	—

Note. Above diagonal: transitions–transversions. Below diagonal: proportional sequence divergence (uncorrected).

few other data available on the intraspecific variation in avian cyt *b*. Edwards and Wilson (1990) report a 0 to 3.9% sequence divergence on a short fragment of this gene between specimens from *Pomatostomus temporalis* belonging to clearly distinct lineages. For other species of *Pomatostomus,* the within species divergence averaged 1%, and the same value is reported for several blackbird species (Lanyon, 1994) and for a shrike (Smith *et al.,* 1991). Intraspecific divergence is similar in murres (up to 2.4%, Birt-Friesen *et al.,* 1992) and dunlins (1.6%, Wenink *et al.,* 1993). These values, together with the very high sequence divergence between species reported in this paper, indicate that intraspecific variation has not had a significant influence on our interspecific comparisons.

Codon usage, amino acid composition, and strand specificity. Codon usage and amino acid composition were compared in the 19 analyzed sequences. No peculiarities were observed at this level. As is usual for mtDNA, a heavy strand and a light strand can be recognized (Friesen *et al.,* 1993 and references therein). The heavy/light ratio of G varies between 2.13 and 2.5 in our sequences, which is comparable to that for human mtDNA (Brown, 1985). The G + C content, which varies between 44 and 48% in our sequences, is also normal for birds (Brown, 1985; Jermiin *et al.,* 1994).

Variability along the gene. The cyt *b* gene is known to comprise distinct regions that accumulate mutations at different rates. Most variation occurs in the transmembrane segments of the molecule, especially in the region spanning residues 210–250 (Irwin *et al.,* 1991; Degli Esposti *et al.,* 1993). We calculated the expected position of the transmembrane segments for our se-

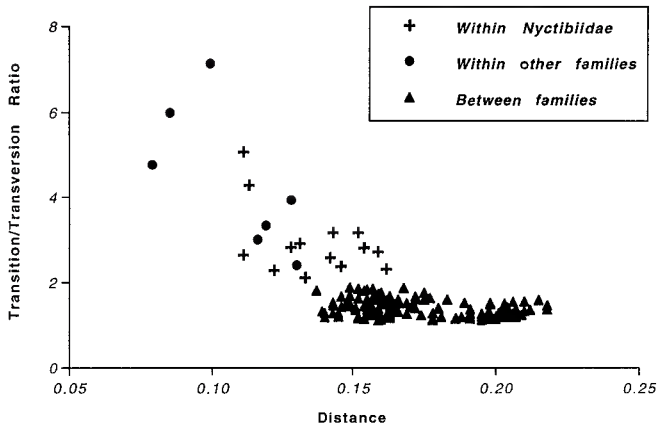

FIG. 3. Transition/transversion ratio plotted against proportional sequence divergence within Nyctibiidae, within other families, and among families.

quences using the program ALOM of the IDEAS package (Klein *et al.,* 1985; Kanehisa, 1986) and found them to be at the same location as reported in the widely accepted 8-domain model (Howell, 1989; Degli Esposti *et al.,* 1993) (Fig. 4). Transmembrane domains 1, 3, 5, and 6 are very hydrophobic and hence predicted with high confidence; domain 2 and extramembrane domain 4 are weaker. Most of the observed variation at the amino

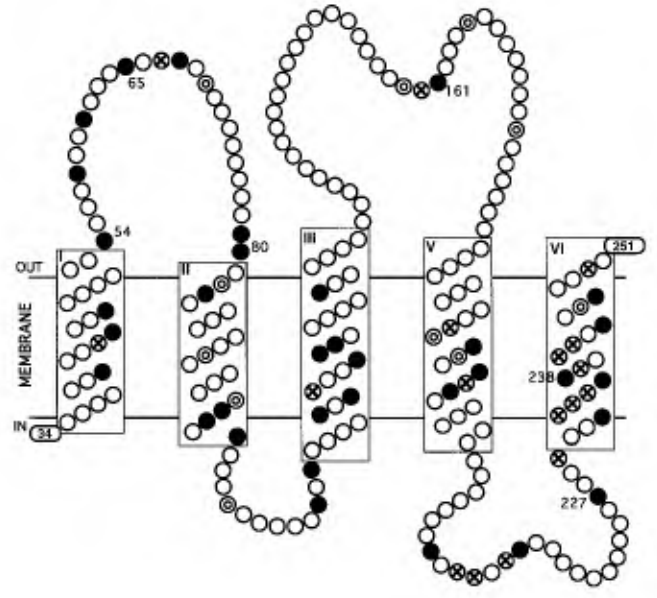

FIG. 4. Model of cytochrome b (modified from Howell (1989) and Irwin *et al.* (1991)). Square boxes represent the transmembrane segments. Each circle represents one amino acid residue. Only the sequenced portion of the gene is shown. Residues found to hypervariable both in the present study and the work of Irwin *et al.* are marked with an X. Those found hypervariable in the paper by Irwin *et al.* but not in the present study are in black. Those found hypervariable in this paper but not in that of Irwin *et al.* are in black and their position number is indicated. The new hypervariable sites found after combination of both data sets are shown with two concentric circles.

acid level lies within these domains, especially in domains 5 and 6 (Fig. 5). However, extramembrane domain 4 is highly conserved as noted by Howell and Gilbert (1988).

The number of sequences obtained in this work allowed us to compare the distribution of the variability along the molecule to the distributions reported by Howell (1989) for a broad range of organisms, and of Irwin *et al.* (1991) for various mammals. We consider a residue to be hypervariable if we observed it in 3 or more amino acid states. For our 19 sequences, we found 43 such residues. Most of these residues were also considered hypervariable by Irwin *et al.* (1991); however, 6 (54, 65, 80, 161, 227, 238) (Fig. 4) were considered conservative by these authors. One (80) is also in a segment found to be very conserved by Howell (1989). Two of them (80 and 161) lie within the Q_0 redox center. If we combine the present data with those of Irwin *et al.* (1991), 11 new residues emerge as hypervariable sites (i.e., more than 3 states) in the enlarged data set (Fig. 4). Most of them are located within the hydrophobic transmembrane segment. However, some belong to the extramembrane part of the gene and some (82, 173) are in the most conserved domain of Howell (1989). This is in accordance with Howell (1993), who states that even the most conserved regions may show variable residues when phylogenetically distant organisms are compared.

Comparison of our data with those of Irwin *et al.* (1991) allows some other observations concerning the variability of the amino acid residues. Of special interest is the conservation in each data set of several marker residues. For example, residue 62 is L without exception in our work and T in the mammal data set. These amino acids belong to different classes according to French and Robson (1983). Other marker positions for the two groups (belonging to different class of amino acids) are positions 90 (F in birds, I or M in mammals), 151 (F in birds, L in mammals), and 159 (H or Q in birds, T in mammals). Another interesting residue is 111, which is always K in birds and is very diverse in mammals.

Phylogenetic Analysis

We conducted two phylogenetic analyses using parsimony: one on all our taxa and one on the potoos alone. This two-step approach allowed us to obtain exact solutions for the potoos as the number of taxa was low enough to run exhaustive searches. These analyses were performed using PAUP 3.0s (Swofford, 1991).

Caprimulgiformes. The chicken sequence (Desjardins and Morais, 1990) was used as the outgroup in our analysis of the Caprimulgiformes. The number of taxa in the analysis (19, including outgroup) allowed only heuristic searches. Options used in our searches are given under Methods. We first performed a straightforward maximum parsimony search with no weighting of the data. There were 243 informative characters in this

```
                    3    4         5         6         7         8         9         1         1
                    4    0         0         0         0         0         0         0         1
                                                                                     0         0
                    ==========                                                  ==========
Chicken             FGSLLAVCLMIQILTGLLLAMHYTADTSLAFSSVAHTCRNVQYGWLIRNLHANGASFFFICIFLHIGRGLYYGSYLY
Steatornis          .....GI..T.................T.....................................Y.............
N. aethereus        .....GI..I........M........T.....................H.....................Y.....F..
N. bracteatus       .....GI..I.................T.....................................Y.....F.F
N. grandis          .....GI..T.................T..........D..........................Y.....F..
N. griseus          .....GI..I.................T......T..............................Y.....F..
N. leucopterus      .....GI..A.................T........M..D.........................Y.............
N. maculosus        .....GI..V.................T..........D.....M....................Y.............
Phalaenoptilus      .....GI....................T.....................................Y.............
Caprimulgus         .....GI....................T.....................L...............VY.............
Chordeiles          .....GI..............T.....T.....................................Y.............
Eu. papuensis       .....GI....................T.....................................Y.............F
Eu. mystacalis      .....GI....................T.....................................Y.............F
Ae. albertisi       .....GI..A.................T.....................M...............Y......F.......
Ae. bennettii       .....GI..A.................T.....................M...............Y......F.......
Ae. cristatus       .....GI..A.................T.....................M...............Y......F.......
P. ocellatus        .....GI..LM.........A......T...T.I.........H.....................V.............
P. papuensis        .....GI..LM.........A......T...T.I.........H.....................................
Batrachostomus      .....GI..L...T.............T...I.T.............M.................Y.............

                              1         1         1         1         1         1         1
                              2         3         4         5         6         7         8
                              0         0         0         0         0         0         0
                    ==========================================================================
Chicken             KETWNTGVILLLTLMATAFVGYVLPWGQMSFWGATVITNLFSAIPYIGHTLVEWAWGGFSVDNPTLTRFFALHFLLP
Steatornis          ..........................................................Q.....................
N. aethereus        ..........................................................Q.....................
N. bracteatus       .......IF..................................................Q.....................
N. grandis          ..........................................................Q.....................
N. griseus          ..........................................................Q.....................
N. leucopterus      ..........................................................Q.....................
N. maculosus        ..........................................................Q.....................
Phalaenoptilus      ..........................................................Q.....................
Caprimulgus         ........V.................................................Q.....................
Chordeiles          ..........................................................Q.....................
Eu. papuensis       ..........................................................Q.I...................
Eu. mystacalis      ..................................................L.....Q.I...................
Ae. albertisi       ..........................................................Q.I...................
Ae. bennettii       ............A.............................................Q.I...................
Ae. cristatus       ..........................................................Q.I...................
P. ocellatus        .......I...I..............................................QN..................T.....
P. papuensis        .....S.I...I..............................................QN..................T.....
Batrachostomus      .......I..................................................QAF...T..............T.....

                    1         2         2         2         2         2         2
                    9         0         1         2         3         4         5
                    0         0         0         0         0         0         1
                    ==========  ==========  ==========  ==========  ==========  ==========
Chicken             FAIAGITIIHLTFLHESGSNNPLGISSDSDKIPFHPYYSFKDILGLTLMLTPFLTLALFSPNLL
Steatornis          .M.....L.................T.SC.........L.....FM...L.LT..........
N. aethereus        .M.T.L.L.................CV..C........F.L.....F.I...LLT.........
N. bracteatus       .....L.L.................V..C........F.L..T..FAI.PALLT..........
N. grandis          .M...L.V.................T..C........F.L.....FMI..ALLT..........
N. griseus          .M...L.L.................V..C........F.L.....FMI..AMLT..........
N. leucopterus      .I...L.LV................V.NC........F.L.....FMI...MLT..........
N. maculosus        .I..SL.LV................V.NC........F.L.....FMA..ALLM..T.......
Phalaenoptilus      .M...L.L.................V..C........F.L.....FA...L.LM...M......
Caprimulgus         .M...L.L.................M.NC........F.L.....FS...L.LM..........
Chordeiles          .M...L.L.................V.NC........F.L.....FS...L.LM...M......
Eu. papuensis       .M...L.LV................V.NC........FTL.....FA...L.LT..........
Eu. mystacalis      .V...L.LV.......T.........M..C........FTL.....FM...L.LT..........
Ae. albertisi       .M...L.L.............K...VI.NC........F.M.....F.F..L.LMI...Y...M.
Ae. bennettii       .M...L.L.................MV.NC........F.T.....FMF..L.LM........M.
Ae. cristatus       .M...L.L.................VV.NC........F.T.....F.F..L.LM........M.
P. ocellatus        .L.T.L.M.................LP.NC........F.T..V..F..L.L.LTS.T.......
P. papuensis        .L.T.L.M.................LP.NC........F.T..V...M.L.L.LTS.T..T..F.
Batrachostomus      .ILTSL.M.................A.N.........F.L..T..F..V.LMLTA.........
```

FIG. 5. Predicted amino acid sequence of residues 34–251 of cyt b from 18 caprimulgiform taxa aligned on the chicken sequence. Numbers follow Desjardins and Morais (1990). Transmembrane domains are overlined, and the extramembrane domain is overlined with a dotted line.

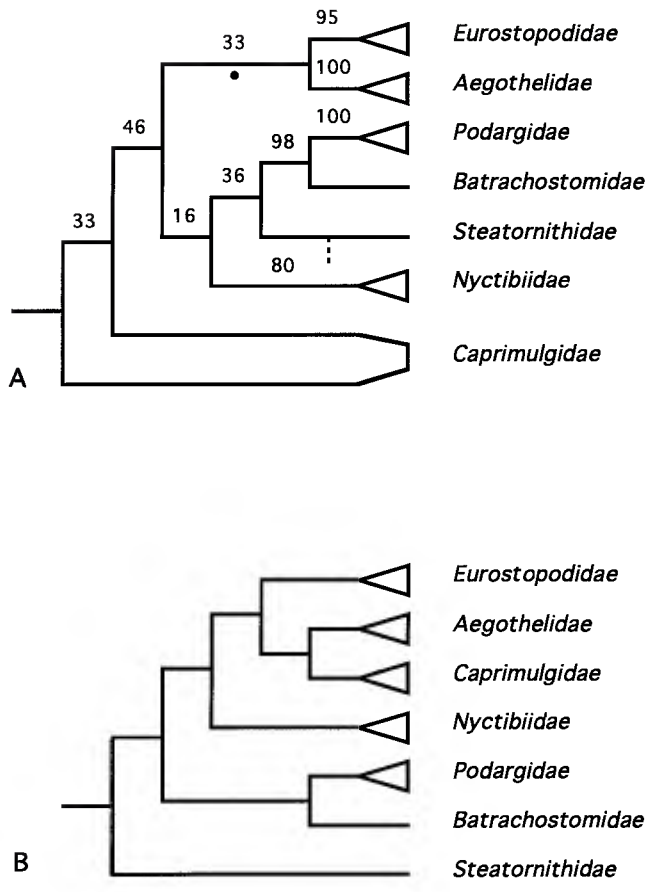

FIG. 6. Relationships among the caprimulgiform families based on parsimony analysis of cyt *b* sequence data. (A) No character-weighting. Numbers above branches are bootstrap values. Each bootstrap was submitted to five heuristic replications with random sequence addition. One tree was kept at each step. Dotted line, position of the root when outgroup (chicken) is added with a conservative assumption. (·) Branch collapsing in the 50% majority rule consensus tree when third position transitions are removed from the analysis. (B) Relationships with various character-weighting patterns (see text).

analysis; 51, 17, and 175 in first, second, and third codon positions, respectively. This search produced a single shortest tree (length 956, CI 0.41, Fig. 6A). The skewness of the tree distribution is high ($g_1 = -0.937$, estimated from 100,000 random trees), indicating strong phylogenetic signal in the data set. However, values from 100 bootstrap replications showed poor support for most of the nodes of this tree (Fig. 6A). We also looked at the next shortest trees; there are 5 of them of length 958 (CI 0.41). A 60% majority rule tree is almost identical to the shortest tree with the exception of the branch marked by a dot (Fig. 6A) collapsing in a trichotomy. The *Batrachostomus–Podargus* link is supported by all 6 trees, but the strict consensus tree lacks any other suprafamilial structure.

In the next step of the analysis, we tried to minimize the influence of positions which were the most susceptible to multiple hits. In a first attempt, we excluded third position transitions from the analysis. These changes are synonymous in most cases and therefore are susceptible to accumulate quickly with the risk of becoming randomized, especially when a large amount of change between taxa has accumulated as in this study. In this configuration, 11 shortest trees of length 453 (CI 0.48) were found by PAUP. The strict consensus tree lacks any suprafamilial structure; however, the 50% majority rule tree is similar to the first tree shown (Fig. 6A), except that the sister-group relationship of Nyctibiidae with Podargidae–Batrachostomidae–Steatornithidae collapses, and internal relationships within Nyctibiidae and Caprimulgidae are different.

Curiously, when the chicken sequence is taken as the outgroup, a position which is not in doubt, the foregoing analyses indicate that the traditional family Caprimulgidae is polyphyletic. This result seems improbable on morphological grounds and because the low sequence divergence between the three caprimulgid taxa (mean = 12.2%) and the high TS/TV ratio (mean = 2.9) indicate that these taxa are relatively closely related (Table 2).

We then weighted the three codon positions in inverse proportion to the number of informative sites in that position (first: 3; second: 10; third: 1). Two most parsimonious trees (L 1592, CI 0.44) varying only within the Nyctibiidae were found in this case (Fig. 6B). They are radically different from the previous results. *Steatornis* appears as the sister-group of all caprimulgiforms, the Caprimulgidae are related to the Aegothelidae, and the Podargidae branch just before *Steatornis.* If we combine this weighting with a suppression of the transitions in third position, a single and almost identical tree (L 1083, CI 0.49) is found (the only difference being within the Nyctibiidae). Various other weightings of the first and second positions (2:4:1; 2:2:1; 5:5:1) give very similar results with the exception of the Aegothelidae branching alternatively with the Eurostopodidae or the Caprimulgidae. A transversion parsimony analysis including only the first and second codon positions confirmed the basal position of *Steatornis,* but did not help to resolve the relations between the other families. Finally, an analysis run on the amino acid sequence of all these taxa gave 719 most parsimonious trees of length 114 with a CI of 0.70 (43 informative positions). Although almost completely unresolved among families, both a strict and a 50% majority rule consensus tree are consistent with the tree in Fig. 6B. The position of *Steatornis,* in particular, is again the same (tree not shown).

From this analysis, two apparently distinct hypotheses of relationship seem to emerge. However, this distinction may be due to a rooting artifact. The two trees (Figs. 6A and 6B) are very similar topologically if the root of the tree in Fig. 6A is shifted to the *Steatornis* branch (Fig. 6A, dotted line). It appears that, when no

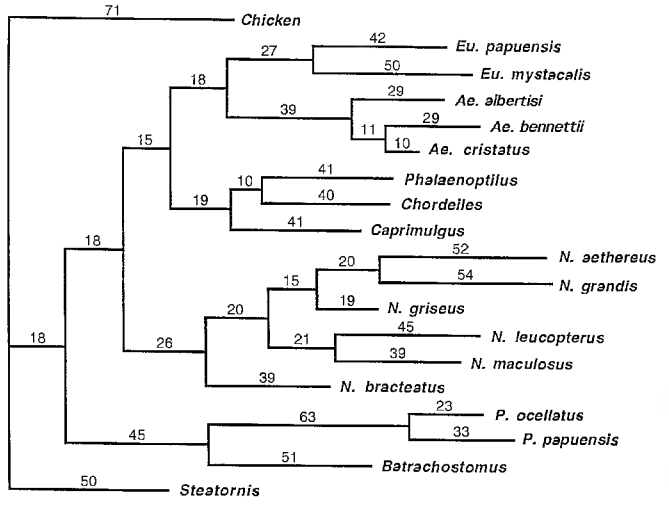

FIG. 7. Example of phylogram with branch lengths indicated above each branch (weighting scheme in this case 2/2/1; other weighting schemes do not change significantly the proportion of lengths between internodal and terminal branches).

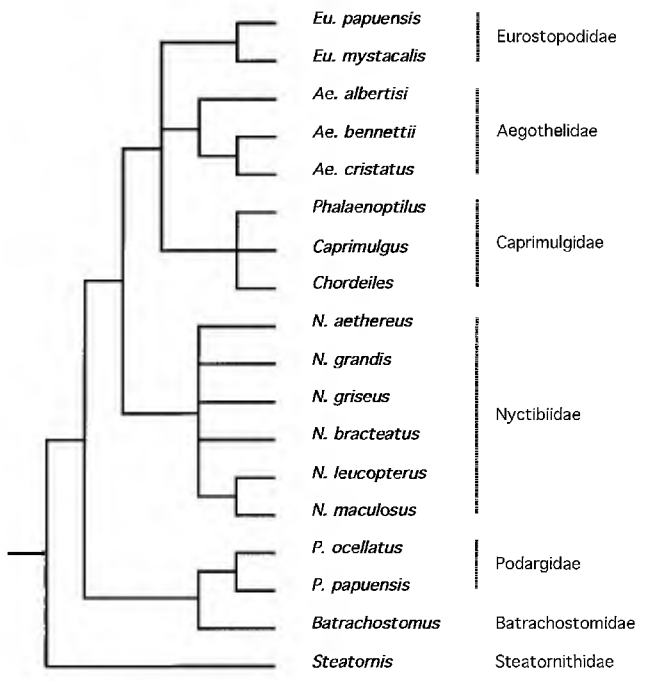

FIG. 8. Best estimate of the relationships of 18 caprimulgiform taxa based on parsimony analysis of 656 bp of cyt *b* sequence data.

weighting is applied (as for the tree in Fig. 6A), the rooting is unreliable due to outgroup saturation. We explored this phenomenon by doing the analysis, without any weighting, on all taxa except the chicken. Then the tree structure was frozen as a backbone constraint in PAUP and the outgroup (i.e., root) was attached using a more conservative assumption (like a 3/10/1 weighting or another of the schemes used above). Invariably, the chicken sequence rooted the tree on the *Steatornis* branch, yielding a topology identical to the tree in Fig. 6B. This suggests that *Steatornis* really is the most basal taxon within the Caprimulgiformes. This result is further supported by the examination of the branch-length distribution in the tree. As shown in Fig. 7, internodal distances are not particularly short as compared to terminal branch lengths, suggesting that considerable evolution has occurred along most branches in the tree.

By trying various other weightings, we determined what weight was sufficient to switch the root from the Caprimulgidae branch (Topology 6A) to the *Steatornis* branch (Topology 6B). As described above, equal weighting or ignoring third position transitions gave the 6A topology. Weightings of 1/2/1 or 1/3/1 gave the same result. 2/1/1 and 1/4/1 gave both topologies as equally parsimonious; 3/1/1, 1/5/1, 2/2/1, 2/4/1, 5/5/1, and 3/10/1 gave the 6B topology. A minimal weight of 3 for the first position, 5 for the second, or 2 for both is therefore necessary to root the tree on the *Steatornis* branch using the chicken sequence as the sole outgroup, provided that no other weight is applied to the ingroup. These weightings do not seem unreasonable given the distance to the outgroup (which averages 20.2%) and the concomitant probability of multiple changes at third positions. As a last test, in order to decrease the possible influence of a long outgroup branch, we ran all the above analyses again using *Steatornis* as a functional outgroup. In this case, 228 informative sites were retained: 49 in first, 14 in second, and 165 in third position. All of these analyses gave results compatible with the tree in Fig. 8. Our best estimate of the relationships within the Caprimulgiformes using parsimony methods on the current cyt *b* data set is therefore summarized in Fig. 8.

Several observations can be made on our results to this point. First, the usually recognized families are strongly supported. The potoos, especially, are always monophyletic. Furthermore, *N. leucopterus* and *N. maculosus* are always found as sister-species. This will be seen in more detail below. *Batrachostomus* is always found as sister-group of the Podargidae, and the *Batrachostomus–Podargus* clade is highly (practically always) supported. Surprisingly, *Phalaenoptilus* consistently (but not always) groups with *Chordeiles* instead of *Caprimulgus*.

Potoos. The choice of an appropriate outgroup is an issue for parsimony analysis within potoos, as there is no agreement in the prior literature on the sister-group of the Nyctibiidae. Therefore, we ran our analysis with the potential outgroups determined in our study of the whole order. A clade grouping the Caprimulgidae, the Eurostopodidae, and the Aegothelidae appeared to be the sister-group of the potoos in our first analysis. We therefore chose (arbitrarily) one member from each

of these families and grouped them to form the outgroup for *Nyctibius*. This method has several advantages. It avoids an arbitrary choice of a single taxon, which might not be the closest relative of the Nyctibiidae. It increases the number of outgroup taxa, which is an advantage in parsimony analysis as the importance of long branches is reduced (Swofford and Olsen, 1990). It is convenient because exact solutions in parsimony searches can be obtained with nine taxa. *Caprimulgus longirostris, Eurostopodus mystacalis,* and *Aegotheles cristatus* were retained as outgroups for this part of the study. There were 133 informative sites in this data set, with 19, 7, and 107 in first, second, and third positions, respectively. For this data set, we conducted exhaustive searches, using only informative positions in the sequences and ran 1000 bootstrap replications at any time when that kind of analysis made sense (practically, each time that no character weighting was set up in the analysis profile).

The level of sequence divergence between *Nyctibius* species (11–16%) and the clear predominance of transitions over transversions in this group indicate that most variable positions will contain phylogenetic signal for this analysis. Therefore, a first run was conducted with all positions included and weighted equally. This analysis gave us three equally parsimonious trees 363 steps long (CI 0.53) whose strict consensus is given in Fig. 9A and whose majority rule consensus is given in Fig. 9B. However, results of 1000 bootstrap replications (Fig. 9B) showed that, except for the *leucopterus/maculosus* clade, support for each node is poor. Furthermore, the skewness of tree-length distribution in this analysis ($g_1 = -0.348$), while significant, is rather low (Hillis and Huelsenbeck, 1992). To test the robustness of this phylogeny, we conducted several more analyses on the same data set. In the first analysis, we weighted the characters according to their position in the codon with the 2/2/1 scheme. The same tree as in Fig. 9B was obtained. Heavier weightings of the first and/or second positions (such as 2/4/1; 5/5/1 or 6/15/1) all gave a single shortest tree (Fig. 9C). Tree length distribution skewness in these cases is below the critical value given by Hillis and Huelsenbeck (1992). This tree is different from the previous one. However, rerooting on the *bracteatus* branch reveals that the topologies of trees in Fig. 9B and 9C are close, the only difference being the grouping of *N. griseus* with the *aethereus/grandis* clade in one case and with the *leucopterus/maculosus* one in the other. A parsimony analysis on these data using the protein sequences (14 informative positions) gave seven trees of length 36 and CI 0.72. Their strict consensus (not shown) gave a tree consistent with the one of Fig. 9C.

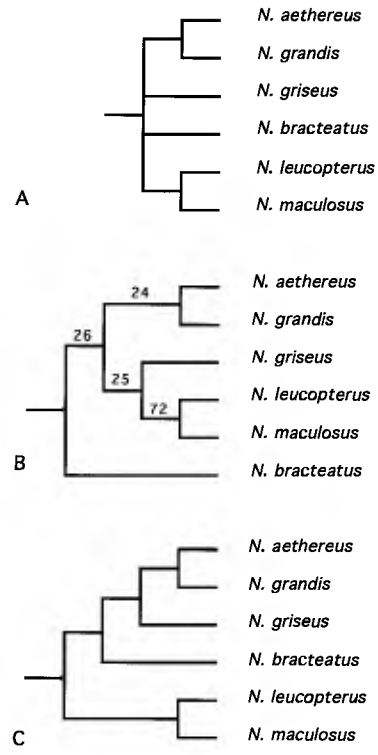

FIG. 9. Relationships among six *Nyctibius* species. (A) Strict consensus of three most parsimonious trees (363 steps long, CI 0.53) found with no character-weighting. (B) Majority rule consensus of the same three shortest trees as in (A). Numbers above branches are bootstrap values. (C) Single shortest tree when various character-weighting schemes are applied (see text). All analyses were performed with *Eurostopodus mystacalis, Aegotheles cristatus,* and *Caprimulgus longirostris* constrained as outgroups to root the tree.

Caprimulgidae. It is interesting to note that the relationships within Caprimulgidae were highly unstable in our previous analysis. In order to test them more thoroughly, we ran several analyses with a restricted data set composed of *Caprimulgus, Phalaenoptilus,* and *Chordeiles* as an ingroup and two *Eurostopodus* and three *Aegotheles* as outgroups (these taxa formed the sister-group of the Caprimulgidae in our previous analysis). The shortest tree obtained with this data set (no weighting) groups *Phalaenoptilus* and *Caprimulgus* as expected. However, this node is supported by a low bootstrap value (45 vs 30% for *Phalaenoptilus/Chordeiles* and 26% for *Caprimulgus/Chordeiles*). Moderate weightings such as 2/2/1 or 2/4/1 as well as a weight of 0.5 attributed to third position transitions against transversions yield the *Phalaenoptilus/Caprimulgus* tree as equally parsimonious with the tree grouping *Phalaenoptilus* and *Chordeiles*. Further decrease of the third position transition weight or increase of the first and second position weight yields the nonorthodox *Phalaenoptilus/Chordeiles* tree alone. Our data therefore do not clearly support a *Phalaenoptilus/Caprimulgus* clade against a *Phalaenoptilus/Chordeiles* clade.

Aegotheles. Three species of *Aegotheles* were included in this study. Two of them, *A. albertisi* and *A.*

bennettii, are from Papua New Guinea and smaller islands, while *A. cristatus* is found in both Papua New Guinea and Australia. We have studied in detail the relationship of these three species as shown by their cyt *b* sequence, using three Caprimulgidae and two *Eurostopodus* as outgroups. A simple analysis without weighting, which seems appropriate in the case of such close taxa in a single genus (less than 10% sequence divergence), gives *A. bennettii* and *A. cristatus* as being most closely related (bootstrap support 69%). Almost any kind of light weighting confirms this pattern, although the tree with *A. albertisi* and *A. cristatus* as sister-taxa is most parsimonious with some weighting schemes (e.g., 5/5/1).

DISCUSSION

At both the familial and the specific levels, phylogenetic results inferred from our cyt *b* sequences offer unequal support for the different nodes. In the interfamilial analysis, the monophyly of most families and the *Podargus/Batrachostomus* clade are well supported. The position of *Steatornis* as the earliest branching group seems secure, but would benefit from addition to the analysis of closer sister-taxa to the Caprimulgiformes as outgroups. Owls, hummingbirds, and swifts are likely candidates (Cracraft, 1981; Sibley and Ahlquist, 1990; Bleiweiss *et al.,* 1994). However, because our original aim was to investigate potoo relationships, we have used only the previously published chicken sequence to root the entire caprimulgiform tree. Figure 8 represents our best estimate of caprimulgiform relationships based on the current cyt *b* data set. It includes all those nodes which are stable in the analyses presented herein. However, since the bootstrap support for some basal nodes is low (Fig. 6A), those nodes should still be considered tentative. The general topology of the family tree is therefore rather strong with the exception of the link of Aegothelidae with Caprimulgidae or Eurostopodidae, which is unclear, and internal relationships within Caprimulgidae, which are unstable.

Our results corroborate neither Cracraft's (1981) nor Sibley and Ahlquist's (1990) proposed patterns of relationships (Fig. 1). In particular, the basal position of *Steatornis* is not recognized by these authors. None of the other groups of Cracraft's phylogeny appear in our analysis. Our results are a little closer to propositions of Sibley and Ahlquist. They suggest a sister-group relationship of Caprimulgidae and Eurostopodidae with Nyctibiidae and of all these families with the *Podargus/Batrachostomus* clade. The main differences are in the positions of the Aegothelidae and Steatornithidae.

While not presented in the form of a phylogenetic tree, the results of Hoff (1966) can be partially compared to ours. However, we do not confirm most of the conclusions of this author. We do not find the closest relative of the potoos to be *Podargus,* nor do we find a close relationship between *Batrachostomus* and the *Aegothelidae*. We also do not place the Podargidae and Aegothelidae as the most primitive of the Caprimulgiformes. Actually, our results strengthen the propositions of some earlier ornithologists. The traditional view of *Steatornis* in a separate family (or subfamily) has been advocated by Peters (1940), Verheihen (1956), and many others before them. More generally, the division of Caprimulgiformes in four groups: *Steatornis,* Podargidae (including *Batrachostomus*), *Aegotheles,* and Caprimulgidae (including *Eurostopodus*) was proposed by Beddard in 1886. Wetmore (1918) also placed *Nyctibius* "in between" the Podargidae and the Caprimulgidae, which is consistent with our results.

For potoos, good support is found for the clade grouping *N. maculosus* and *N. leucopterus.* Therefore, we concur with the proposition of Chapman (1926), who suggested that *N. maculosus* is an Andean relative of *leucopterus.* However, the relationships among the other species in *Nyctibius* are not as clear. The position of *bracteatus* is uncertain and the placement of *griseus* is, at best, marginally supported. There is some support for a *N. aethereus/N. grandis* clade. Thus, the best resolution of potoo relationships based on these data (Fig. 9B) is in accordance with Davis' (1978) suggestion that *N. griseus* and *N. grandis* are not closely related.

The fact that the long-established Caprimulginae/Chordeilinae taxa are not clearly supported is surprising. This can be seen as either a lack of resolution of the cyt *b* data in this particular case, or an indication that the proposition of Sibley and Ahlquist (1990), that the different genera in the Caprimulginae may deserve a higher categorical status, is correct. Within *Aegotheles,* we note the close link between *A. bennettii* and *A. cristatus.*

Use of cyt b *in This Case*

We chose to study cyt *b* on the assumption that this rapidly evolving gene would be suitable for the study of species relationships within the genus *Nyctibius,* as it has proven to be for other avian genera (Lanyon, 1994). The poor resolution we have obtained at this level is due to a surprisingly great genetic distance between potoos species, perhaps coupled with a relatively rapid radiation of these birds. Distance values between *Nyctibius* species are notably higher than those reported for cyt *b* in other genera of birds. Intrageneric distances fall in the range 3 to 7% in *Molothrus* (S. M. Lanyon, personal communication), and can reach 10% in other blackbird genera (Lanyon, 1994). A similar divergence (6.2%) was noted between congeneric murres (Birt-Friesen *et al.,* 1992), cranes (Krajewski and Fetzner, 1994), and various other bird genera (Kessler and Avise, 1985). Higher values were published for babblers of the genus *Pomatostomus* (Edwards and Wilson, 1990; Edwards *et al.,* 1991), shrikes of the ge-

nus *Laniarius* (Smith *et al.,* 1991), titmice of the genus *Parus* (Gill and Slikas, 1992), and warblers of the genus *Phylloscopus* (Richman and Price, 1992), but, in all these studies, intrageneric divergences fell in the range 5 to 13%. The portion of cyt *b* sequenced is not identical in all these studies, so some variation is expected due to higher or lower degrees of conservation in different regions of the gene. However, the values for *Nyctibius* still seem remarkably high.

The unexpected consequence of this situation is that, in this case, the cyt *b* gene allows somewhat better resolution at a higher level of relationships. However, the extent of divergence reached in these analyses (up to 22%) approaches the limit of phylogenetic usefulness of the gene due to strong saturation effects. A better resolution of potoo relationships might be achieved by adding more data or by sequencing a more slowly evolving gene.

Taxonomic Implications

Although our aim is not to propose changes in taxonomy, our results can reinforce some suggestions. First, the Nyctibiidae form a clearly distinct and monophyletic clade, unambiguously separated from other Caprimulgiformes. Furthermore, members of this group are more distant from one another than is usual in the other families of the order. A recent allozyme electrophoresis survey demonstrates that genetic divergence among potoo species is large in the nuclear genome as well (Brumfield *et al.,* in press). This high genetic diversity probably means that the species of potoos are quite old. It would be desirable to reflect this diversity in the classification of the group, possibly by elevating some species groups to the generic level. Unfortunately, poor resolution of the phylogeny makes delineation of monophyletic genera guesswork at the moment.

Second, we note that while *Batrachostomus* and *Podargus* always are very strongly related in the phylogenetic analyses, their cyt *b* sequences are actually quite distinct. They have high sequence divergence (mean = 0.19; Table 2), low TS/TV ratios (mean = 1.5; Table 2) and there are many amino acid replacements (Fig. 5). These data probably indicate that the genera diverged a long time ago and lend credence to the proposal of Sibley and Ahlquist (1990) to erect a separate family Batrachostomidae. It would be useful to examine more species from these genera, however.

Third, the cyt *b* data also confirm the distinctiveness of *Eurostopodus* from other genera traditionally included in Caprimulgidae. The two species of *Eurostopodus* we examined are always clearly separated from the Caprimulgidae in our analyses and are more clearly distinct from the Caprimulginae (*Caprimulgus* and *Phalaenoptilus*) than is *Chordeiles.* The relatively high sequence divergence (mean = 0.16; Table 2) and low TS/TV ratios (mean = 1.3; Table 2) between *Eurostopodus* and the three caprimulgid genera probably indicate that the lineages are quite old. If age is a desirable criterion for determining categorical rank, the proposal of Sibley and Ahlquist (1990) to treat *Eurostopodus* as a separate family has merit. It must be noted, however, that examination of the many other species and genera in this group might complicate (and may clarify!) the final picture of their relationships.

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