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Does behavior reflect phylogeny in swiftlets (Aves: Apodidae)? A test using cytochrome *b* mitochondrial DNA sequences

(molecular systematics/nest structure/echolocation/birds)

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ABSTRACT **Swiftlets are small insectivorous birds, many of which nest in caves and are known to echolocate. Due to a lack of distinguishing morphological characters, the taxonomy of swiftlets is primarily based on the presence or absence of echolocating ability, together with nest characters. To test the reliability of these behavioral characters, we constructed an independent phylogeny using cytochrome *b* mitochondrial DNA sequences from swiftlets and their relatives. This phylogeny is broadly consistent with the higher classification of swifts but does not support the monophyly of swiftlets. Echolocating swiftlets (*Aerodramus*) and the nonecholocating "giant swiftlet" (*Hydrochous gigas*) group together, but the remaining nonecholocating swiftlets belonging to *Collocalia* are not sister taxa to these swiftlets. While echolocation may be a synapomorphy of *Aerodramus* (perhaps secondarily lost in *Hydrochous*), no character of *Aerodramus* nests showed a statistically significant fit to the molecular phylogeny, indicating that nest characters are not phylogenetically reliable in this group.**

As expressed by Mayr (1), "Every author who has ever worked with these small swiftlets of the Indo-Australian region will contend that their classification presents the most difficult problem in the taxonomy of birds." Swiftlets (collocaliini) are small aerial insectivorous birds that are distributed from the Indian Ocean, through Southeast Asia and North Australia, to the Pacific. Most species nest in caves, often in total darkness, and are capable of echolocation, an ability found elsewhere among birds only in the neotropical oilbird, *Steatornis caripensis* (2). Some species of swiftlets are also known for the commercial value of their nests, which are used to make "bird's-nest soup." Increasing demand is threatening the survival of these economically important species (3). Mayr's opinion (1) that swiftlets represent avian taxonomy's greatest challenge stems from the extreme degree of morphological similarity among some species of swiftlets. This is reflected by the chaotic state of swiftlet classification, which suffers from an abundance of races [Peters lists 85 (4)] that have frequently been assigned to different species by different authors (5). Developing a phylogeny-based classification is an urgent priority because lack of consensus hampers attempts to list swiftlet species in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (3).

The paucity of distinguishing morphological characters among swiftlets has led to a reliance on behavioral characters, such as echolocation and nest structure, to arrange taxa. Brooke (6) placed echolocating species in the genus *Aerodramus*, separate from nonecholocating species, which remained in the genus *Collocalia*. Although recently disputed (7–9), this split has some support from morphology: *Aerodramus* species weigh an average of 14 g and have dull plumage, whereas *Collocalia* species average 6.5 g and have glossy plumage. The genus *Hydrochous* (6) contains a single large-bodied (37 g) species (*Hydrochous gigas*) that is intermediate with dull plumage but no echolocation. *H. gigas* is unique among swiftlets in nesting behind or near waterfalls (10, 11) like some New World swifts (Cypseloidinae) (12).

Swiftlet nests are constructed with salivary "glue" and may or may not incorporate other materials such as vegetation or feathers (Fig. 1). Variation in nest composition has been used to discriminate between swiftlet taxa and to infer evolutionary relationships (13, 14). However, nest structure may be environmentally plastic (15), raising questions about the utility of nest characters in swiftlet taxonomy (8).

Behavioral characters are often viewed as less reliable than morphological characters on the assumption that they are more prone to homoplasy (16). However, recent studies (17–21) show that behavioral characters contain useful phylogenetic information. Hence, the reliance of swiftlet taxonomists on these kinds of characters is not unreasonable *a priori*. Nest structure, in particular, has been shown to be tightly linked to evolutionary history in the case of swallows (19) and may be a useful character for other birds, such as swiftlets (16). However, testing whether these characters are in fact reliable indicators of phylogeny requires independent information on swiftlet relationships.

To obtain such information, we sequenced 406 bp of the cytochrome *b* gene of mtDNA [corresponding to positions 15,303–15,708 of the chicken mtDNA sequence (22)] from a series of swiftlets and, as outgroups, a number of other swifts and a hummingbird. Our study addressed three main questions: (*i*) Are swiftlets monophyletic? (*ii*) Are the genera *Collocalia* and *Aerodramus* monophyletic? (*iii*) What is the relationship of *H. gigas* to the other swiftlets? Answers to these questions should allow us to evaluate whether echolocation is a synapomorphy of *Aerodramus* and whether nest structure reflects phylogeny.

MATERIALS AND METHODS

Samples. A hummingbird (Trochiliformes: *Phaethornis superciliosus*; GenBank accession no. U50037) was used as an outgroup for the Apodiformes, based on evidence from DNA–DNA hybridization studies (23, 24). Tissue or blood samples from swifts and swiftlets (Table 1) were obtained from several institutions (acknowledgements) or collected in the field by D.H.C. Tissue samples were frozen immediately in liquid nitrogen, and blood samples were diluted in a preservative solution (2% SDS/50 mM EDTA/50 mM Tris) and frozen as

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Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U49981–U50039).
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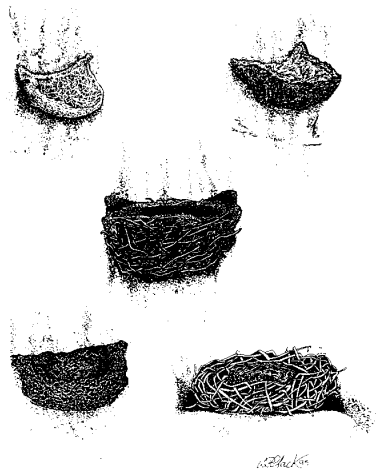

FIG. 1. Swiftlet nests showing species-specific variation in characters used for swiftlet taxonomy. Illustrations are of voucher nests collected from populations also sampled for DNA work. (*Upper Left*) *A. fuciphagus vestitus* nest made entirely of, and vertically supported by, salivary "glue" (*A. fuciphagus* is the only species with a pure saliva nest). (*Upper Right*) *A. maximus* nest made of saliva and feathers and vertically supported. (*Middle*) *A. brevirostris* nest made of saliva, feathers, and vegetation and vertically supported. (*Lower Left*) *A. spodiopygius assimilis* nest made of saliva and vegetation and vertically supported. (*Lower Right*) *A. sawtelli* nest made of saliva and vegetation and supported by a horizontal surface.

soon as possible. At least one voucher specimen and nest were collected from most localities. In the case of cryptic taxa in multispecies colonies (*Aerodramus fuciphagus vestitus*, *Aerodramus fuciphagus germani*, and *Aerodramus salanganus*), samples were collected from birds taken directly off nests.

Molecular Data Collection. Genomic DNA was extracted using the protocol of Boom *et al.* (25) with the following modifications: 2–5 μl of blood/tissue was incubated for 10 min at room temperature in 60 μl of L6 lysis buffer (25), and the DNA was extracted from this by using the Geneclean II kit (Bio 101).

Degenerate primers were designed from avian sequences in GenBank. The primer sequences are, for PCR amplification, L15302 (5′-GTAGGATATGTCCTNCCHTGAGG-3′) and H15709 (5′-GGCATATGCGAATARGAARTATCA-3′) and, for sequencing, H15541 (5′-KGGGTGGAANGGRA-TTTTRTC-3′) and L15430 (5′-CCCACATTNACYCGNT-TYT-3′). The prefixes to the DNA sequences, L and H, refer to the light and heavy strand, respectively, and the numbers refer to the position of the 3′ end of the primer according to the chicken mtDNA sequence (22).

Double-stranded PCR amplifications with biotinylated primers were performed in 25 μl containing 1–3 μl of extracted DNA, 50 ng of each primer, all four dNTPs (each at 40 μM), 2.5 μl of 10× *Taq* buffer with 1.5 mM $MgCl_2$ and 0.94 unit of *Taq* polymerase. Negative controls were used and all PCR mixtures were covered with 25 μl of mineral oil. The reaction began with denaturation (94°C for 1 min) followed by 30 amplification cycles of annealing (54°C for 1 min), template extension (72°C for 1 min), and denaturation (92°C for 1 min). A final annealing and extension step of 54°C for 5 min and 72°C for 5 min completed the reaction.

Single-stranded PCR products bound to streptavidin-coated paramagnetic beads (Dynal, Great Neck, NY) were sequenced directly by using the dideoxynucleotide chain-termination method (26). Two internal sequencing primers produced overlapping sequences spanning the entire amplified DNA fragment [sequencing kits: Sequenase 2 (USB) and *Taq*Track (Promega)]. The PCR products of some samples were cloned into pCR-Script SK(+) (Stratagene) or pUC18 plasmid (Amersham). Both strands were sequenced to verify the accuracy of the direct sequencing strategy.

Table 1. Taxa (collecting localities) sequenced

Apodiformes taxa	No. of individuals sequenced	GenBank accession no.
Family Hemiprocnidae (treeswifts)		
Hemiprocne mystacea (New Guinea)	1	U50036
Family Apodidae (typical swifts)		
Subfamily Cypseloidinae		
Cypseloides niger (California)	2	U50033
Streptoprocne zonaris (Peru)	2	U50038
Subfamily Apodinae		
Tribe Chaeturini		
Chaetura vauxi (Oregon)	2	U50029
Tribe Apodini		
Apus nipalensis (Sabah, Malaysia)	1	U50001
Apus apus (Oxford, UK)	2	U49981
Cypsiurus balasiensis (Peninsular Malaysia)	2	U50031
Tribe Collocaliini		
Hydrochous gigas (Java, Indonesia)	1	U50035
Collocalia esculenta races:		
C. e. bagobo (Mindanao, Philippines)	2	U50018
C. e. cyanoptila (Sabah, Malaysia)	4	U50020
C. e. marginata (Sibuyan, Philippines)	1	U50026
Collocalia linchi (Java, Indonesia)	2	U50024
Collocalia troglodytes (Sibuyan, Philippines)	2	U50027
Aerodramus elaphrus (Seychelles)	4	U49986
Aerodramus francicus (Mauritius)	3	U49989
Aerodramus spodiopygius races:		
A. s. assimilis (Suva and Vanua Balavu, Fiji)	4	U50002 U50009
A. s. spodiopygius (Western Samoa)	2	U50013
Aerodramus terraereginae (Queensland, Australia)	2	U50015
Aerodramus brevirostris (Java, Indonesia)	1	U50017
Aerodramus salanganus (Sabah* and Balambangan†, Malaysia)	5	U50004 U50006
Aerodramus bartschi (introduced to Hawaii from Guam)	2	U49983
Aerodramus sawtelli (Atiu, Cook Islands)	2	U50011
Aerodramus maximus (Sabah* and Balambangan†, Malaysia)	5	U49996
Aerodramus fuciphagus races:		
A. f. vestitus (Sabah*, Malaysia)	3	U49992 U49994
A. f. germani (Balambangan†, Malaysia)	1	U49995

Classification follows Chantler and Driessens (7), except for generic nomenclature, which follows Brooke (6). Generic abbreviations: *C. Collocalia*; *A. Aerodramus*. GenBank accession numbers are for sequences used in analyses.
*Gomantong Caves.
†Island 20 km off North Borneo coast.

Table 2. Base composition for each codon position for the 29 sequences analyzed

Codon	% of total bases			
	A	C	G	T
1	26.2 ± 0.8	31.3 ± 0.8	21.8 ± 0.6	20.6 ± 0.7
2	20.7 ± 0.3	30.6 ± 0.5	10.5 ± 0.4	38.1 ± 0.6
3	36.2 ± 2.0	52.7 ± 3.9	3.6 ± 1.9	7.6 ± 3.9

Data are the mean ± SD.

DNA Sequence Analyses. DNA sequences were aligned by using the Genetics Computer Group package (27). To control for DNA contamination in the samples, more than one individual per taxon was sequenced for a total of 59 sequences. Since most of the sequences from the same taxa were identical or nearly identical, those forming monophyletic groups in initial analyses were reduced to one representative per taxon, resulting in a data set of 29 sequences. GenBank accession numbers of sequences used are given in Table 1. The maximum likelihood estimates were computed using FASTDNAML version 1.0.6 (28) and maximum parsimony trees were constructed with PAUP (29). Likelihood difference tests and bootstrap analyses were conducted using PHYLIP (30). Construction of constraint trees and nest character analyses were performed using MACCLADE (31).

RESULTS

Of the 406 sites, 248 were invariant and of the remaining sites, 103 were phylogenetically informative (two or more taxa shared a variant character state). The base composition at the third codon position showed a strong bias with 89% A or C and very few instances of G (Table 2). This pattern is typical of avian mtDNA (22, 32, 33). The strong base composition bias against G and, to a lesser extent, T and the tendency for saturation of transitional changes has prompted some workers (33) to discard information from the third position. However, we retained all data for analysis because third position T ⇌ C transitions in this data set were not saturated, and discarding these sites would remove 86% of the potentially phylogenetically informative variation.

Unweighted maximum parsimony analysis using a heuristic search (tree–bisection–resection) produced 104 trees, 408 steps long (consistency index = 0.54; retention index = 0.55). Using MACCLADE (31), an average transition/transversion ratio of 2.2 was calculated over all trees. Maximum likelihood analysis with a transition/transversion ratio of 2, empirical base frequencies, and global rearrangement produced a tree (Fig. 2) with a log likelihood of −2661.82. This tree, which is similar to the consensus parsimony tree, has a length of 411 steps under parsimony (3 steps longer than the most parsimonious trees) but is not significantly worse under a likelihood difference test (34) (log likelihood difference = −4.70 ± 11.91). Trees constructed using neighbor joining (35) (maximum likelihood distances) showed only minor differences from the maximum parsimony and maximum likelihood trees, with the exception that the two representatives of the Cypseloidinae, *Streptoprocne* and *Cypseloides*, were grouped together.

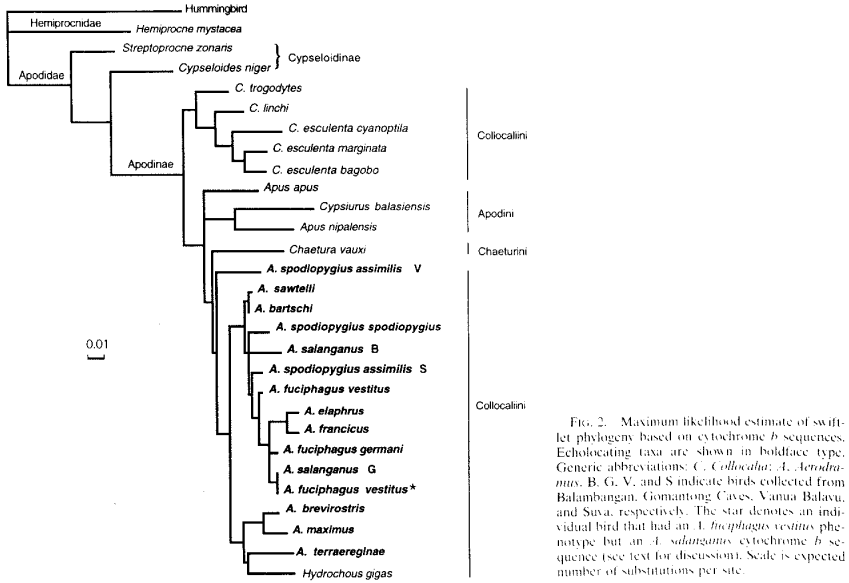

FIG. 2. Maximum likelihood estimate of swiftlet phylogeny based on cytochrome *b* sequences. Echolocating taxa are shown in boldface type. Generic abbreviations: *C. Collocalia*; *A. Aerodramus*. B, G, V, and S indicate birds collected from Balambangan, Gomantong Caves, Vanua Balavu, and Suva, respectively. The star denotes an individual bird that had an *A. fuciphagus vestitus* phenotype but an *A. salanganus* cytochrome *b* sequence (see text for discussion). Scale is expected number of substitutions per site.

The robustness of our results was evaluated by bootstrap analysis of the sequence data (36) and computing neighbor joining trees from maximum likelihood distance matrices computed for each of the 1000 replicates. In the resulting bootstrap consensus tree (Fig. 3), the basal nodes corresponding to the Apodidae (95%), the Cypseloidinae (82%), and the Apodinae (96%) all have good support, as does the genus *Collocalia* (85%). However, some relationships within the Apodini, Chaeturini, and remaining Collocaliini are less robust. Examination of the bootstrap trees revealed that much of this ambiguity was due to uncertainty in the placement of *Apus apus*, *Apus nipalensis*, *Cypsiurus*, and *Chaetura*. These taxa are unstable and tend to "wander" over the tree, reducing bootstrap values for nodes that might otherwise have good support. The majority-rule consensus tree used to summarize the results of bootstrap analysis (36) is particularly sensitive to taxa that have very different positions on different trees (37). For example, pruning the four unstable taxa from the 1000 bootstrap trees and recomputing the consensus tree increased the bootstrap value for the node representing the ancestor of the *Aerodramus* and *Hydrochous* from 19% to 80% (Fig. 3).

While *Hydrochous* is closely related to *Aerodramus*, its exact relationship is uncertain. All most parsimonious trees placed *Hydrochous* in various positions within *Aerodramus*, never as sister taxon to that genus, and only 13 of 1000 bootstrap trees (1.3%) have *Hydrochous* and *Aerodramus* as sister taxa. The suggestion that *Hydrochous* may be an Old World representative of the Cypseloidinae (11) can clearly be rejected (log likelihood difference between the tree in Fig. 2 and the tree constrained to have *Hydrochous* in Cypseloidinae = −51.9 ± 22.3).

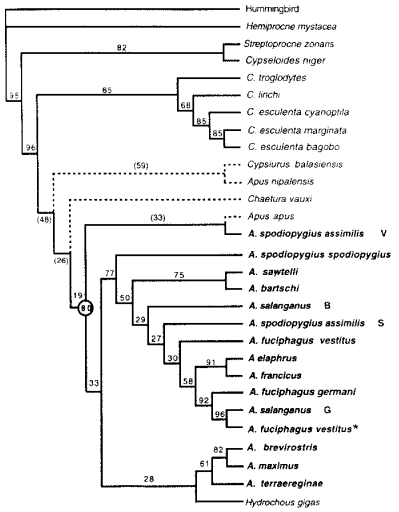

FIG. 3. Bootstrap consensus tree for 1000 replicates computed using neighbor joining of maximum likelihood distances. Numbers on each branch represent percentage of trees containing that branch. Conventions are as in Fig. 2. When the Apodini and Chaeturini species (Fig. 2) are pruned from the 1000 bootstrap trees (indicated by dashed branches and bootstrap values in parentheses) and the consensus tree is recomputed, the bootstrap value for the node grouping *Aerodramus* − *Hydrochous* (circle) increases from 19 to 80. Bootstrap values for other nodes did not change markedly (<5%). This indicates that uncertainty in the placement of the Apodini and Chaeturini taxa deflates the bootstrap value for the common ancestor of the *Aerodramus* + *Hydrochous* taxa.

In some cases, previously recognized species boundaries are not supported. The mtDNA samples from different populations of two taxa (*A. salanganus* and *A. spodiopygius assimilis*) do not cluster as monophyletic groups (Fig. 2). Given the instability of swiftlet species and subspecies boundaries, this is not entirely surprising, although it is possible that the discordance reflects lineage sorting or past hybridization events (38). In one case there is evidence for introgression of mtDNA between species: the sequence of one *A. fuciphagus vestitus* individual from Gomantong Caves is identical to that of three *A. salanganus* individuals from the same site, but different from two other *A. fuciphagus vestitus* individuals from that site (Fig. 2 and Table 1). The individual in question was collected directly from a saliva "white" nest characteristic of *A. fuciphagus* (Fig. 1 *Upper Left*); *A. salanganus* builds "mossy" nests of saliva and vegetation (similar to Fig. 1 *Lower Left*). Identification of the individual's dried skin by an experienced swiftlet researcher confirmed it to be a specimen of *A. fuciphagus vestitus* (D. M. Tompkins, personal communication). As a final check, we sequenced mtDNA from the dried skin itself and found that sequence to be identical to the original sequence, thus ruling out the possibility of mislabeling or other mistakes. Determining whether incongruence between taxon boundaries in *A. salanganus* and *A. spodiopygius assimilis* is the result of past instances of introgression will require information from nuclear genes (38).

Given the independent molecular phylogeny for swiftlets, we can evaluate the phylogenetic value of nest characters. We evaluated four traits: mode of support, presence of feathers in the wall of the nest, presence of vegetation in the nest, and proximity of nests (Fig. 4). With one exception, the characters were scored directly from voucher nests collected from the same populations of *Aerodramus* spp. that were sampled for the DNA work (Fig. 1); *Hydrochous* characters were scored from a published description of the nest (11). If the molecular data and nest data contain phylogenetic information, then the nest data should be congruent with the molecular phylogeny. Using the shuffle command in MACCLADE (31), we compared the number of steps required to optimize each nest character onto the maximum likelihood tree (Fig. 2), with the distribution of steps obtained from 100 random reassignments of the nest character states. In every case, the fit between nest character and the molecular phylogeny was no better than one would expect by chance ($P = 0.30, 0.22, 1.00$, and 0.96, respectively). When the analysis was repeated with the consensus tree for the 104 trees obtained using parsimony [interpreting polytomies as uncertainties in resolution (39)], the only trait congruent with the tree ($P = 0.03$) was presence of feathers.

DISCUSSION

The use of behavioral traits as phylogenetic characters has received increasing support in recent years (16–21). Although nest structure and placement are species specific behavioral traits, our study suggests that these traits are unlikely to be phylogenetically informative in swiftlets. Consistency between nest building behavior and our molecular phylogeny is not evident. The only character displaying congruence with the molecular phylogeny is the use of feathers in the walls of the nest, but this congruence depends on the position of *H. gigas*, which is uncertain. Hence, the caution expressed by some authors (8) regarding the value of swiftlet nest characters is justified. Our results contrast with those of Winkler and Sheldon (19), who demonstrated pronounced congruence of nest characters with a DNA–DNA hybridization phylogeny of swallows.

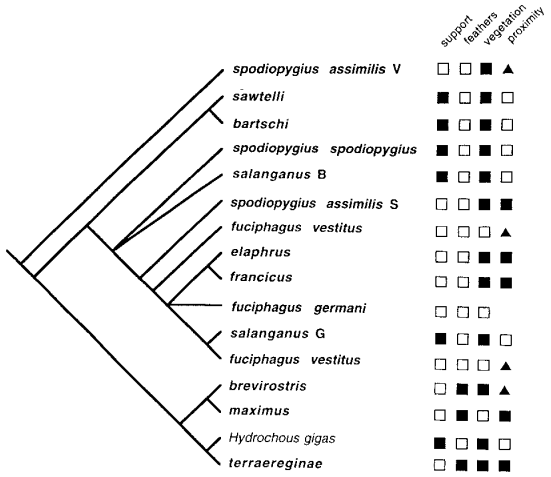

FIG. 4. Distribution of four characters of *Aerodramus* and *Hydrochous* nests on the maximum likelihood tree shown in Fig. 2. Characters and their states are as follows: primary mode of nest support, salivary "glue" (open box) versus horizontal surface (solid box); presence (solid box) or absence (open box) of feathers embedded in nest wall; presence (solid box) or absence (open box) of vegetation in nest; proximity of nests, separated by >1 m (open box), loosely clustered (solid triangle), or tightly clustered with many nests touching (solid box). Only a single nest of *A. f. germani* was observed so that nest proximity is not scored for this taxon. Conventions are as in Fig. 2.

Phylogenetically poor characters can still have taxonomic value. For example, the cryptic swiftlet species *A. fuciphagus*, *A. salanganus*, and *A. maximus* all breed in the same caves in Borneo and are difficult, if not impossible, to identify in the field on the basis of morphological criteria alone. However, *A. fuciphagus* builds a nest of pure saliva (Fig. 1 *Upper Left*), *A. maximus* builds a nest of saliva and feathers (Fig. 1 *Upper Right*), and *A. salanganus* builds a nest of vegetation and saliva (like Fig. 1 *Lower Left*) (13), making it possible to discriminate among the three species on the basis of nest characters (Fig. 4). Reliable identification of these species in the field is critical for the implementation of proposed swiftlet conservation policies (40).

Echolocation, in contrast to nest building behavior, shows better agreement with our molecularly derived phylogeny and is a useful behavioral character for delineating the genus *Aerodramus*. The most parsimonious interpretation of the history of echolocation is that it arose once at the base of the *Aerodramus* clade and was secondarily lost in *H. gigas*, although Medway and Pye (14) have argued that loss of echolocation is highly unlikely. The position of *H. gigas* on the molecular tree is uncertain and so further data are required for a more robust estimate of its placement and, thus, a better understanding of the evolution of echolocation. Within the echolocating taxa, detailed comparisons of clicks (14, 41) could provide additional phylogenetic information.

In summary, our results raise questions about the utility of behavioral (nest) characters for making phylogenetic inferences about swiftlets, contrary to past practice (13). On the other hand, we confirm that echolocation is a behavioral trait that is well correlated with morphological and molecular differences between genera (6). Our data suggest the need for caution when using behavioral characters to reconstruct phylogenies. Behavioral traits, like all characters, may or may not inform us about evolutionary relationships. Comparison with additional independent data sets will serve to further test the utility of behavioral traits in this, and other, groups of animals.

TAXONOMIC CONCLUSIONS

The results of this study allow us to comment on the classification of swiftlets at a number of taxonomic levels. First, Brooke's (42) higher classification of swifts is supported by our molecular phylogeny (Figs. 2 and 3). Robust bootstrap values support the families Apodidae and Hemiprocnidae and the subfamilies Cypseloidinae and Apodinae. The only discrepancy in our analyses is whether the Cypseloidinae are monophyletic (compare Figs. 2 and 3).

At the tribal level, our molecular data strongly suggest that the Collocaliini are not monophyletic. This conclusion is supported by the maximum likelihood analysis (Fig. 2), which places the Chaeturini and Apodini taxa between the two swiftlet (Collocaliini) genera. Only 36 of the 1000 bootstrap trees (3.6%) had a monophyletic Collocaliini. On the other hand, the node grouping the Apodini and Chaeturini with *Aerodramus*, to the exclusion of *Collocalia*, is poorly supported in the bootstrap tree (48%). Although our molecular data indicate that Collocaliini may not be monophyletic, monophyly cannot be completely ruled out. Evidence in support of a Collocaliini clade is provided by ectoparasitic lice (Insecta: Phthiraptera). The genera *Collocalia*, *Aerodramus*, and *Hydrochous* are all parasitized by the louse subgenus *Collodennyus* (genus: *Dennyus*), which is found on no other host (43). The Apodini are host to a different subgenus of *Dennyus* (44). This argument presumes that the lice have cospeciated with their hosts, an hypothesis that we are currently testing with lice collected during this study.

At the generic level, monophyly of *Collocalia* is well supported by the molecular data. However, whether *Aerodramus*

(+ *Hydrochous*) is monophyletic (as suggested by the parsimony and maximum likelihood analyses) depends on the relationships of the Apodini and Chaeturini, which in some bootstrap trees intrude into *Aerodramus*. Resolution of this question could be facilitated by future analysis of more taxa and longer sequences.

Specific relationships within the *Aerodramus* clade are ambiguous and some existing species boundaries are not consistent with the molecular phylogeny. This has important implications for conservation efforts, as the genetic limits of morphospecies are, therefore, uncertain (3). More extensive phylogenetic work on swiftlets is clearly needed. Characters derived from morphology, molecules, and behavior all have the potential to shed light on various levels of swiftlet classification.

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