Testing species limits of non-echolocating Philippine swiftlets (*Collocalia* spp.) using molecular genetic data

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Swiftlets are small, insectivorous birds that are distributed from the Indian Ocean, through southeast Asia and north Australia, to the Pacific. About 22 species of swiflets nest in caves or other dark places, where they navigate using a crude form of echolocation (Chantler and Driessens 1995). Three additional species, which are incapable of echolocation, do not nest in the dark. The 25 species of swiftlets are considered by many authors to represent the most difficult problem in the taxonomy of birds (e.g., Mayr 1937). This is because swiftlets show extreme morphological similarity, making species limits extremely difficult to decipher. Lee *et al.* (1996) used DNA sequence data to show that morphologically-based species concepts are seriously flawed for swiftlets. They also showed that the echolocating species (*Aerodramus* spp.) are not closely related to the non-echolocating species (*Collocalia* spp.).

Keywords: *Collocalia* spp., **phylogenetic relationships, morphological similarity, genetic divergence, non-echolocating, DNA sequence data, species limits**

Two of the three non-echolocating species occur in the Philippines, where *C. troglodytes* is endemic and *C. esculenta* is represented by four subspecies. One of the latter, *C. e. marginata*, is considered a distinct species by some workers (Sibley and Monroe 1990). We explored species limits of the Philippine *Collocalia* by sequencing the mitochondrial DNA of *C. troglodytes* and two of the *C. esculenta* subspecies. Our results show that *C. troglodytes* and *C. esculenta* differ in their cytochrome b sequences by approximately 9 %, indicating that they speciated at least 4.5 million years ago (assuming a molecular clock). We further show that *C. e. marginata* and *C. e. bagobo*, found mainly on Luzon and Mindanao, respectively, differ in their cytochrome b sequences by 2.2 %, suggesting that they split over a million years ago. The sizeable differences between these subspecies support the specific status of *C. e. marginata*, since the two subspecies are more genetically distinct than are many other sister species pairs of birds. Our results raise the total number of *Collocalia* spp. to four, two of which are endemic to the Philippines. It will be interesting to acquire and sequence tissue from the remaining two Philippine subspecies of *C. esculenta*, in order to resolve further the taxonomy of this difficult, albeit fascinating group of birds.

SWIFTLETS ARE SMALL, INSECTIVOROUS BIRDS THAT ARE DISTRIBUTED FROM THE INDIAN OCEAN, through southeast Asia and north Australia, to the Pacific. About 22 species of swiftlets nest in caves or other dark places, where they navigate using a crude form of echolocation. Three additional species, which are incapable of echolocation, do not nest in the dark. The 25 species of swiftlets are considered by many authors to represent the most difficult problem in the taxonomy of birds because their morphological similarity makes species limits extremely difficult to decipher. Previous molecular work showed that the echolocating species (*Aerodramus* spp.) are not closely related to the non-echolocating species (*Collocalia* spp.). Two of the three non-echolocating species occur in the Philippines, where *C. troglodytes* is endemic, and *C. esculenta* is represented by four subspecies.

Some of the Philippine *C. esculenta* subspecies are considered distinct species by some workers. We explored species limits of Philipppine *Collocalia* by sequencing the mitochondrial DNA of *C. troglodytes* and two of the *C. esculenta* subspecies. Our results show that *C. troglodytes* and *C. esculenta* differ in their cytochrome *b* sequences by approximately 9%, indicating that they speciated at least 4.5 million years ago (assuming a molecular clock). We further show that *C. e. marginata* and *C. e. bagobo* differ in their cyt *b* sequences by 2.2%, suggesting that they split over a million years ago. The sizeable divergence between these subspecies supports the specific status of *C. e. marginata* and *C. e. bagobo*, since the two subspecies are more genetically distinct that are many other species of birds.

Populations of organisms on islands often diverge from mainland populations over evolutionary time. This divergence can lead to speciation. For example, the many islands of Southeast Asia and the South Pacific are home too an impressive radiation of swiftlets (Aves: Collocaliini), which are small, insectivorous birds. Determination of species limits in swiftlets has been difficult because of the extreme morphological similarity of many swiftlets populations (e.g. Mayr 1937). Molecular genetic data have the potential to reveal differences not apparent from morphological data alone. In this paper, we use molecular data to investigate species limits of swiftlets in the genus *Collocalia* from the Philippine Islands.

Table 1. Distribution of Philippine *Collocalia* species and subspecies*.

Taxon	Distribution
Collocalia troglodytes	Banton, Bohol, Catanduanes, Cebu, Dinagat, Gigantes, Guimaras, Leyte Luzon, Marinduque, Masbate, Mindanao Mindoro, Negros, Palawan, Panay Romblon, Samar, Siargao, Sibuyan, Siquijor, Ticao
Collocalia esculenta bagobo	Bongao, Minadanao, Mindoro
Collocalia esculenta isonota	Northern Luzon
Collocalia esculenta marginata	Banton, Bohol, Camiguin Sur, Cebu, Dinagat, Guimaras, Leyte, Central Luzon, Mactan, Masbate, Mindoro, Negros, Panay, Polillo, Romblon, Samar, Siargao, Sibuyan, Tablas
Collocalia esculenta septentrionalis	Babuyan Claro, Calayan, Camiguin Norte, Fuga
Collocalia esculenta undescribed	Palawan

***adapted from Dickinson 1989b**

Swiftlets are small-bodied representatives of the family Apodidae. Members of the genus *Aerodramus* (22 species) nest in caves and navigate using a crude form of echolocation (Chantler and Driessens 1995, Lee *et al.* 1996); six species of *Aerodramus* occur in the Philippines (Dickinson 1989a). In contrast, members of the genus

Collocalia, which do not echolocate, nest near the entrances of caves or under rock overhangs and man-made structures, such as houses (Chantler and Driessens 1995). Two of the three recognized species of *Collocalia* occur in the Philippines. The third species, *C. linchi,* is primarily found in Indonesia. One of the two Philippine species, *C. troglodytes,* is an endemic with no described subspecific variation; it is distributed on all of the large islands in the Philippines (Table 1).

The other species, *C. esculenta,* is found from the Andaman Islands, east through Indonesia, Malaysia and New Guinea, to New Caledonia. This widespread species, which is divided into 31 subspecies (Chantler and Driessens 1995), occurs as far north as the Philippines, which is home to four endemic subspecies: *C. e. bagobo, C. e. isonota, C. e. marginata,* and *C. e. septentrionalis* (Dickinson 1989b). Another undescribed form of *C. esculenta* is endemic to Palawan Island. The species status of the Philippine forms of *C. esculenta* has been debated for years, with some authors recognizing *marginata* (Hackisuka 1934, Sibley and Monroe 1990) and *isonota* (McGregor 1909) as species (reviewed by Dickinson 1989b) by themselves.

For this study, we generated sequences from the mitochondrial cytochrome *b* gene to assess phylogenetic relationships of the two Philippine *Collocalia (troglodytes* and *esculenta)* in order to establish species limits. We used genetic divergences to estimate the timing of speciation events in *Collocalia.* We also assessed genetic variation for two of the four Philippine subspecies of *C. esculenta: C. e. bagobo and* C.e *marginata.* We were unable to study the other two subspecies because of a lack of suitable tissue for genetic analysis.

Methods

We extracted DNA from tissues of *Collocalia troglodytes* (one individual from Sibuyan), *C. esculenta bagobo* (two individuals from Mindanao), and *C. esculenta marginata* (two individuals from Sibuyan) according to the protocol of Johnson and Clayton (in review). We also extracted DNA from representatives of 13 other swift and swiftlets species including *C. esculenta cyanoptila, C. linchi,* and 7 species of *Aerodramus.* Using PCR, we amplified most (1,037 bp) of the mitochondrial cytochrome *b* gene for these samples. We sequenced these PCR products and aligned them using the methods described in Johnson and Clayton (2000). We reconstructed phylogenies using parsimony and PAUP* (Swofford 1999).

We used 10 random sequence addition replicates with TBR branch swapping with equal weighting of characters. We performed bootstrap analyses (1,000 replicates) to assess support for various branches of the tree (Felsenstein 1985). We also compared genetic distances between species, subspecies, and individuals to estimate the timescale of speciation and evaluate species limits in Philippine swiftlets.

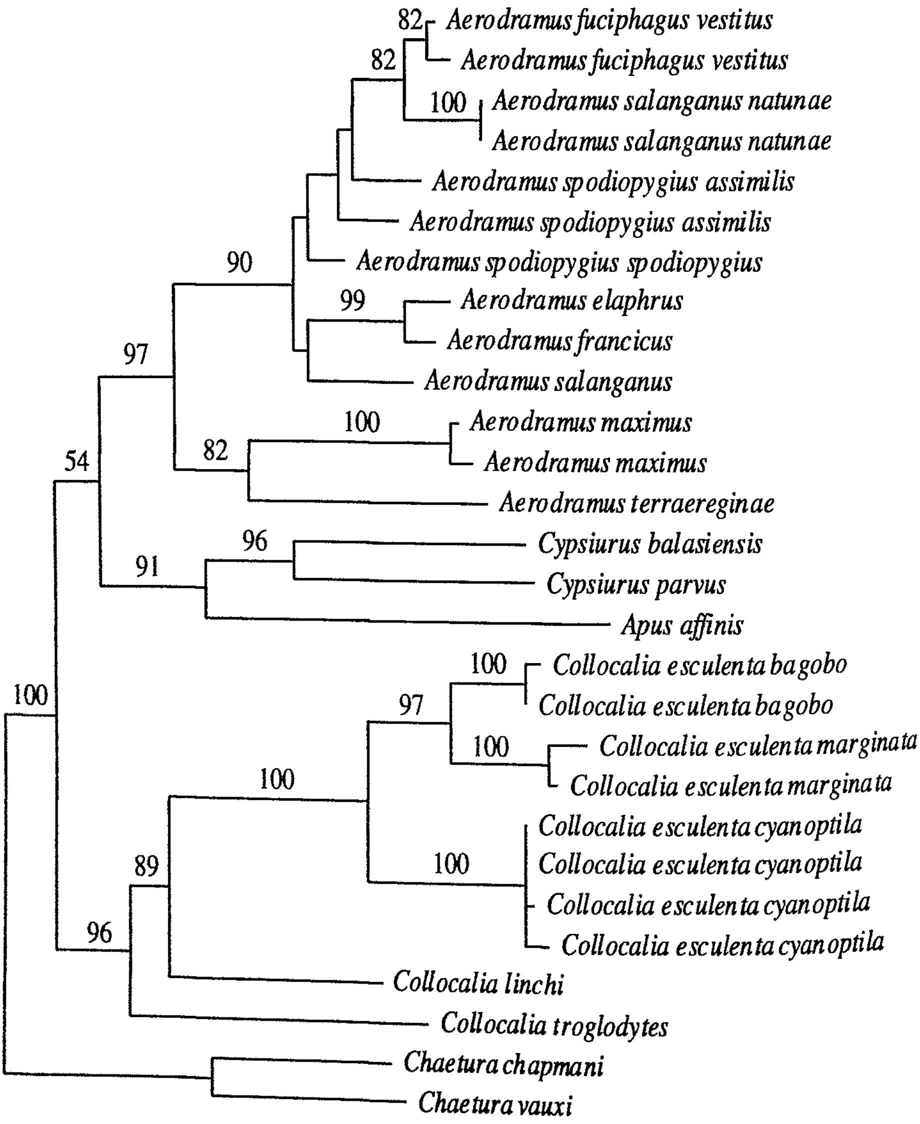

Figure 1. Phylogeny for swifts and swiftlets derived from unordered parsimony of cytochrome *b* sequences (1,037 base pairs), with *Chaetura* used as an outgroup (1 tree, length = 582, consistency index = 0.644, retention index = 0.796). The *C. linchi* clade is based on the 406 bp of Lee et *al.* (1996). Branch lengths are proportional to reconstructed changes under ACCTRAN optimization. Numbers on branches indicate percent bootstrap support from 1,000 replicates. Unlabelled nodes received less than 50% bootstrap support.

Results

The phylogeny shown in Figure 1 is largely congruent with that of Lee *et al.* (1996), which was based on cyt *b* sequences only about a third as long as those generated for this paper. *Collocalia* is clearly monophyletic (96% bootstrap support), as is *Aerodramus* (97%). In addition, as Lee et *al.* (1996) showed, the two swiftlet genera may not be sister taxa, although support for this conclusion is weak (54%). Swiftlet monophyly also cannot be rejected by the Templeton (1983) test ($P = 0.74$). Additional sequencing of swifts could help resolve the question of overall swiftlet monophyly.

Within *Collocalia, C. troglodytes* is considerably divergent from *C.esculenta* (average 9.0% sequence divergence). *C. linchi* is sister to *C. esculenta* and differs from it by 6.0% sequence divergence. Individuals from the same species and subspecies form monophyletic groups. With our limited sample of *Collocalia esculenta* subspecies, the two Philippine subspecies (C. e. *marginata* and C.e. *bagobo)* group together with high bootstrap support (Fig.1). These two subspecies average 4.0% sequence divergence from *C.e.cyanoptila*. The divergence between C.e. *marginata* and C. e. *bagobo* is 2.2%. In contrast, the average divergence between individuals within subspecies of *Collocalia* is only 0.18%.

Discussion

DNA sequence data can help resolve the phylogeny for a group of species, aid in determining species boundaries, and provide an estimate of the timing of speciation events. Sequences from the mitochondrial cytochrome *b* gene show that the two non-echolocating species of Philippine swiftlets do indeed fall within *Collocalia*, being distinct from echolocating *Aerodramus*. Genetic divergence between *Collocalia troglodytes* and the various subspecies of *C. esculenta* suggests that these two lineages diverged from each other over 4.5 million years ago, assuming a molecular clock calibration of 2% per million years for mitochondrial DNA (Klicka and Zink 1997). Sequences of additional subspecies of *Collocalia* are needed to evaluate a geographic scenario for early divergences within *Collocalia*.

Dickinson (1989b) recognized four Philippine subspecies of *Collocalia esculenta*. Within *C. esculenta,* we identified lineages from mitochondrial DNA sequences that correspond to subspecies boundaries. The levels of sequence divergence between subspecies of *Collocalia* are relatively high, similar to or exceeding mitochondrial divergences between species in many other groups of birds. Within the Philippines, we sampled *C. e. bagobo* and *C. e. marginata* , and these two lineages differ by 2.2% sequence divergence. Using the molecular clock calibration, these two subspecies have been genetically isolated for approximately

1.1 million years, which is a very long time for speciation to occur. The monophyly of individuals within each of these subspecies, together with their high divergence, suggests that these two groups should be considered distinct species: *Collocalia bagobo* and *C. marginata.*

While we are unable to evaluate the species status on the basis of genetic information for *C. esculenta septentrionalis, C.* e. *isonata,* and the undescribed Palawan Island form, it is possible that these forms will also show genetic diversification approaching or exceeding that between *C. bagobo* and *C. marginata.* Our results raise the total number of *Collocalia* species to five, three of which are endemic to the Philippines. It will be interesting to acquire and sequence tissue from the remaining Philippine *C. esculenta* subspecies (Table 1) to further resolve the taxonomy of this difficult, albeit interesting group of birds. Given the extreme morphological similarity of many swiftlet species and the relatively high levels of genetic divergence between subspecies, many more cryptic swiftlet species are likely to exist in the numerous islands of Southeast Asia.

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