Molecular Phylogeny and Morphological Homoplasy in Fruitbats

Yolanda Álvarez, Javier Juste B.,*† Enrique Tabares,* Amando Garrido-Pertierra,* Carlos Ibáñez,† and José M. Bautista**

*Departamento de Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain; and †Estación Biológica de Doñana (CSIC), Seville, Spain

The present study evaluates the evolutionary framework of the Old World fruitbats based on the cytochrome *b* and 16S rRNA mitochondrial gene sequences from a wide range of taxa. Phylogenetic analyses indicated that morphology-based subfamilies and most suprageneric groups are nonnatural assemblages. They also support the existence of an endemic African clade of fruitbats. The discrepancy between the evolutionary relationships yielded by molecular and morphological data sets may be, at least in part, explained by the recurrent retention of primitive morphology (*Rousettus*-like) across different lineages. The maintenance of primitive characters in different groups of flying foxes, as well as morphological convergence in nectar-feeding bats and possibly also in short-muzzle bats, may have led to high levels of homoplasy, resulting in misleading taxonomic arrangements. This may be particularly so with respect to high taxonomic levels based on morphological characters.

Introduction

Fruitbats, or Megachiroptera, form one of the two suborders within the Chiroptera. Generally of medium to large size, these bats are characterized by the fact that they feed on nectar and/or fruit juices, lack an echolocation system (except for the genus *Rousettus*), and are found throughout the Old World tropics. Their distribution is more restricted to tropical regions than is that of the insectivorous bats of the suborder Microchiroptera (Hill and Smith 1984). From a remarkably modern perspective, Andersen (1912) first established evolutionary relationships within the Megachiroptera based on in-depth knowledge of bat morphology. His taxonomic arrangement is still the main evolutionary framework reference for the relationships among the approximately 200 species of fruitbats described (e.g., Koopman 1994). The whole group forms one family divided into three subfamilies; the nectar- and pollen-feeding Macroglossinae, the monotypic and aberrant Harpyonycterinae, and the Pteropodinae, or flying foxes. Each subfamily is further divided into different sections (fig. 1). The internal consistency of this traditional morphological classification has recently been validated by applying cladistic methods to discrete morphological characters (Springer, Hollar, and Kirsch 1995).

Andersen's (1912) general evolutionary framework has recently been reviewed at the molecular level by restriction fragment length polymorphism (RFLP) analysis (Colgan and Flannery 1995), single-copy DNA hybridization (Kirsch et al. 1995), and mitochondrial DNA sequencing (Hollar and Springer 1997). Although these molecular approaches have focused mainly on the Australasian species, they unanimously suggest that the subfamily which comprises all the nectar-feeding bats is not a natural assemblage. They also propose a geographically sound endemic clade which includes the African species (Kirsch et al. 1995; Hollar and Springer 1997).

The differences in the evolutionary relationships among fruitbats suggested by molecular and morphological data are outstanding (Springer, Hollar, and Kirsch 1995; Kirsch and Lapointe 1997). Conflict levels between data sets are higher than are those reported for any other group of animals (Springer, Hollar, and Kirsch 1995). Independent data sets are required to resolve discrepancies between competing evolutionary hypotheses (Hillis 1987), since a high level of congruence is considered to reflect strong support for a particular evolutionary reconstruction. Evolutionary arrangements corresponding to the different molecular-based phylogenies are highly concordant and are preferred to the traditional morphology-based evolutionary picture (Springer, Hollar, and Kirsch 1995). However, molecular reconstructions do not always agree (e.g., relationships within rousettine and cynopterine), and further information is required to clarify the origins of, and relationships within, the suborder Megachiroptera (Colgan and Flannery 1995), particularly for the African species.

This paper explores the evolutionary relationships among fruitbats by comparative analysis of the mitochondrial cytochrome *b* and 16S rRNA gene sequences of a wide range of African and Australasian taxa. Special emphasis is placed, however, on the African taxa. This paper also contrasts the validity and structure of a proposed African clade of fruitbats with the traditional morphological classification. The investigation also contributes to our understanding of the dynamics of the morphological variation from molecular topologies (Graur 1993) underlying the conflicting morphological and molecular results.

Materials and Methods

DNA samples were obtained from 28 Megachiroptera representing all of the fruitbat subfamilies (with the exception of the Harpyonycterinae) and all of the traditionally defined African groups. DNA extraction was performed using phenol/chloroform followed by isopropanol precipitation (at −20°C) according established methods (Maniatis, Fritsch, and Sambrook 1989). Some

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Address for correspondence and reprints: José M. Bautista, Departamento de Bioquímica y Biología Molecular IV, Universidad Complutense de Madrid, Facultad de Veterinaria, 28040 Madrid, Spain. E-mail: bauchem@eucmax.sim.ucm.es.

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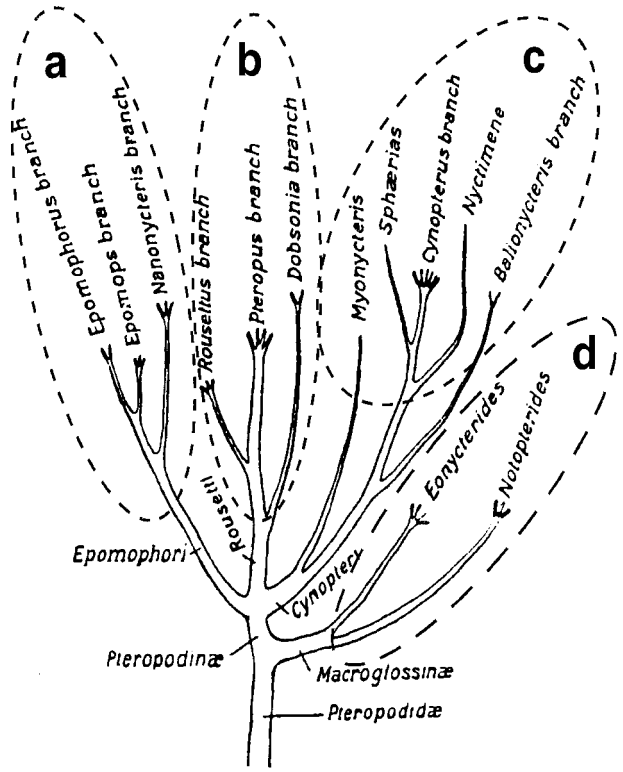

FIG. 1.—Traditional taxonomic arrangement of fruitbats at the suprageneric level excluding the Harpyonycterinae (modified from Andersen 1912). Based on morphological similarities, Andersen assigned the following to the same Pteropodinae subfamily: (*a*) an African section (epomophorine), (*b*) a mixed African and Australasian section (rousettine), and (*c*) an Australasian section (cynopterine) including, however, the African *Myonycteris.* The subfamily Macroglossinae of (*d*) specialized nectar-feeding bats only includes Australasian species with the exception of the sole African representative, *Megaloglossus.*

tissue samples from old museum specimens were rehydrated prior to DNA extraction as described by Smith et al. (1987). Small DNA fragments were removed using size exclusion columns (CHROMA SPIN 1000, CLONTECH). DNA was examined by electrophoresis in 0.8% agarose. PCR amplification and sequencing of the mitochondrial cytochrome *b* (402 bp) and 16S rRNA (550 bp) gene fragments was performed according to procedures described elsewhere (Zardoya, Garrido-Pertierra, and Bautista 1995). The generic primers H15149 (Kocher et al. 1989) plus L14722 (5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′) and 16 Sar-L plus 16 Sbr-H (Palumbi 1991) were used for PCR amplification of the cytochrome *b* and 16S rRNA sequences, respectively. Both forward and reverse strands were sequenced for each gene fragment. New sequences were deposited in the GenBank database (accession numbers AF044604–AF044665). The resultant sequences were aligned with Pileup (Genetics Computer Group 1994) using default parameters and were visually inspected for regions of mistaken alignment. Protein-coding fragments were translated to check codons. Noncoding fragments (16S rRNA) were examined for gaps/insertions. These were never longer than 2 bp and were considered missing data. No modification of alignments was required. Separate and combined phylogenetic analyses were performed using these gene fragments. For each species, the 5′ end of the 16S rRNA sequence was joined to the 3′ end of the cytochrome *b* fragment in a single sequence after testing for congruence between the data sets.

Transition/transversion (ts/tv) ratios were calculated by quartet puzzling (Strimmer and von Haeseler 1996) with 10,000 puzzling steps and the ''slow-exact'' option of parameter estimates. Final ts/tv values were estimated by optimizing the logarithms of maximum likelihood.

Phylogenetic hypotheses were established by the following phylogenetic procedures: minimum evolution, maximum likelihood, and maximum parsimony. All analyses were performed with PAUP 4d64 (Swofford 1998) using full heuristic searches by tree bisection reconnection (TBR) branch-swapping. The analyses were performed with single and multiple outgroups, and topologies were assessed by bootstrapping. The three Microchiroptera *Rhinolophus hipposideros, Nycteris thebaica,* and *Saccopteryx bilineata* were used as the triple outgroup. *Rhinolophus hipposideros* was used as the single outgroup. Minimum-evolution trees were obtained using both the Kimura (1980) two-parameter and Tamura-Nei (1993) distance models. Maximum-likelihood analyses were performed according to the HKY85 evolution model, and the α shape parameter of a Γ distribution for unequal rates among sites was estimated from the data using four categories. Maximum-parsimony trees were constructed using equal and differential weighting models. Nucleotides were differentially weighted 1/0 (zero was assigned to invariable positions or to a mutation occurring in a single nonoutgroup taxon). The decay index statistic (AutDecay, version 3.0, by T. Eriksson and N. Wilkström) was calculated for the parsimony topology. The strengths of particular nodes were evaluated using the Templeton, Crandall, and Sing (1992) and Kishino-Hasegawa (1989) tests for the parsimony and maximum-likelihood topologies, respectively.

Results and Discussion

Gene Sequences and Phylogenetic Reconstruction

The estimated ts/tv ratios for the 31 taxa analyzed (28 Megachiroptera and 3 Microchiroptera) were 2.5 and 1.7 for the cytochrome *b* and 16S rRNA gene sequences, respectively, and 2.2 when these sequences were joined. These relatively low ts/tv values—particularly among Australasian species—which reflect multiple-hit and saturation phenomena, suggest that estimates of divergence times should be cautious. Ratios corresponding to closely related taxa were much higher (e.g., 5.3 for the *Rousettus* group in the combined-genes analysis). The use of a single outgroup (*R. hipposideros*) in the analysis of combined genes led to an expected slight increase in values (2.4). These analyses yielded topologies equivalent to those of the triple outgroup, although in this case, three most- parsimonious trees were

Table 1
Comparisons of Bootstrap Support (After 1,000 Iterations) on Common Nodes in the Topologies Resulting from Minimum Evolution and Maximum Parsimony

	Minimum Evolution				Maximum Parsimony			
Node	Cyt*b* K2P	16S K2P	Cyt*b* + 16S T-N ($\alpha = 0.2$)	Cyt*b* + 16S K2P	Cyt*b* Uw	16S Uw	Cyt*b* + 16S W	Cyt*b* + 16S Uw
(*a*) African *Rousettus* clade	100	100	80	100	100	95	100	100 (3)
(*b*) Epomophorine clade	92	<50	78	92	76	<50	78	77 (5)
(*c*) Myonycterine clade	87	60	91	96	83	60	96	95 (7)
(*d*) African clade (epomophorine + myonycterine)	82	93	83	99	66	<50	98	98 (8)
(*e*) African clade + *Eonycteris*	NP	98	<50	69	NP	89	52	51 (1)
(*f*) African clade + *Eonycteris* + *Rousettus.*	<50	79	<50	72	<50	77	77	77 (14)

Note.—The distance models are based on the Kimura two-parameter (K2P) and the Tamura-Nei (T-N) model with heterogeneity variation among sites ($\alpha = 0.2$). The maximum-parsimony models consider weighted (W) and unweighted (Uw) characters. The topologies are independently based on the cytochrome *b* (402 bp), the 16S rRNA (550 bp), and the combined (Cyt*b* + 16S) sequences. Numbers in parentheses are the calculated decay indices. Minimum-evolution tree scores were 2.27787, 1.25955, and 1.52996, respectively. Maximum-parsimony tree parameters were as follows: Cyt*b*—eight trees retained with a length of 954, 165 informative characters, consistency index (CI) = 0.33, retention index (RI) = 0.40; 16S rRNA—three trees retained with a length of 781, 151 informative characters, CI = 0.39, RI = 0.54; Cyt*b* + 16S—one tree retained with a length of 1,744, 315 informative characters, CI = 0.35, RI = 0.46. Letters in parentheses indicate the positions of the nodes in the maximum-likelihood topology shown in figure 2, and "NP" indicates nodes not present in the analyses.

retained, whereas only one was retained using the multiple outgroup.

For the minimum-evolution hypotheses, both the Tamura-Nei distance model (using a gamma distribution shape parameter of 0.2) and the Kimura two-parameter model yielded similar topologies. However, the Tamura-Nei distance trees showed similar bootstrap support on the nodes when combined sequences were used (table 1). Neither differential weighting of the 16S rRNA sequence nor the assignment of three different categories to codon positions on the cytochrome *b* sequence improved their phylogenetic signals, as shown by the resolution of the resultant parsimony- and maximum-likelihood-based trees. The elimination the third codon positions on the cytochrome *b* sequence, to avoid a possible saturation problem, was to the detriment of the phylogenetic signal (24 trees were retained in the parsimony analysis). Finally, taking into account the heterogeneity rates across sites ($\alpha = 0.2$) in the maximum-likelihood analysis led to topologies consistent with the hypothesis obtained using the HKY85 model. However, bootstrap support for the nodes was reduced. These preliminary analyses led us to base our evolutionary inferences on the multiple-outgroup models, minimum evolution using Kimura two-parameter distances, parsimony using equally weighted characters, and maximum-likelihood analyses with equal substitution rates.

Although mtDNA can be considered an evolutionary unit, its genes do not all evolve at the same rate. The types of mutation and the probabilities of a mutation occurring are different for protein-coding and noncoding regions (Kumar 1996). Differences are shown even among protein-coding mitochondrial genes, leading to variation in phylogenetic performance (Zardoya and Meyer 1996). In the present analyses, the two genes showed different evolutionary patterns. Nucleotide substitutions in cytochrome *b* occurred throughout the alignments, whereas variations in the 16S rRNA sequences were restricted to 150 bp (between positions 240 and 390). Despite this, phylogenetic arrangements based on the independent analysis of these two mitochondrial gene sequences yielded highly concordant topologies, with only slight differences in the bootstrap support for the main nodes (table 1). The 16S rRNA fragment showed higher basal branching support, particularly among the African groups (e.g., the node connecting the African clade + *Eonycteris* + *Rousettus*; see table 1).

When two independent gene sequences are joined in a single matrix, the phylogenetically informative positions of each sequence show a cumulative effect in addition to the dispersion and contrast of background noise (Miyamoto et al. 1994). For our data set, both sequences showed high compatibility ($P = 0.24$ in a partition-homogeneity test). The data matrix corresponding to the combined mitochondrial sequences yielded topologies that were in close agreement with those of the independent data sets. Moreover, when the information corresponding to each gene was added, congruence among the topologies improved and bootstrap support increased (table 1). Taking into account all of the substitutions in the 966-character data set, the different evolutionary models yielded the same single best topology with a consistent pattern of taxa groupings shown in the maximum-likelihood-based hypothesis (fig. 2).

Phylogenetic Inferences

The evolutionary picture suggested by the present analyses conflicts with the traditional subdivisions of the Megachiroptera (fig. 1). The subfamily Macroglossinae (nectar- and pollen-feeding bats) is confirmed as a nonnatural assemblage, with nectar-feeding species scattered across the trees. The topology constraining Macroglossinae monophyly required 118 additional steps in the parsimony analysis and was also significantly rejected against our maximum-likelihood topology, as shown by the Templeton and Kishino-Hasegawa tests, respectively (table 2). This conclusion is highly supported by a comparative study of reproductive tracts in the Macroglossinae (Hood 1989) and by other molecular approaches (Colgan and Flannery 1995; Kirsch et al.

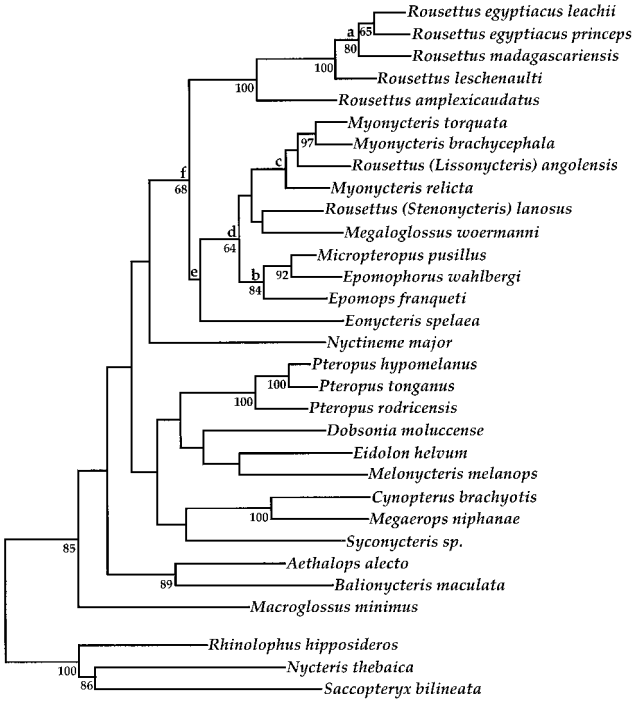

FIG. 2.—Phylogenetic relationships among fruitbats based on mitochondrial cytochrome *b* and 16S rRNA gene sequence data (966 aligned sites). This single maximum- likelihood tree was obtained using the HKY85 model. Ln likelihood = −9,928.21694. Bootstrap values over the nodes are based on 100 replicates. See table 1 for description and bootstrap support values on the nodes (*a*–*f*) according to the minimum- evolution and parsimony models.

1995; Hollar and Springer 1997). The morphological modifications in the skull and the masticatory apparatus toward specialized nectar- and pollen-feeding habits—characters used to link the species—have been described as an illustrative example of morphological convergence (Kirsch et al. 1995). In fact, the shift to nectarivory seems to have occurred independently on several occasions throughout the evolution of fruitbats (Kirsch and Lapointe 1997).

The African epomophorine section (fig. 1*a*) defined by Andersen (1912) is the only group within the Pteropodinae subfamily supported by our topology (fig. 2*b*), since species of the Australasian cynopterine section (*Myonycteris, Cynopterus, Balionycteris, Megaerops,* and *Nyctineme*) were spread over the branches (fig. 2). Similarly, the taxa comprising Andersen's rousettine section (fig. 1*b*)—particularly *Rousettus* and *Pteropus*—are distantly spaced, ruling out their previously proposed sister relationship. Both the Templeton and the Kishino-Hasegawa tests show our patterns to be significantly better than those constraining the monophyly of the cynopterine and rousettine groups (table 2).

The present topologies clearly support an African monophyletic clade linked to the *Rousettus,* as suggested by other studies (Kirsch et al. 1995; Hollar and Springer 1997). Moreover, the present findings suggest that the African clade is formed by two main groups (fig. 2): the traditional epomophorine section of Andersen (1912), clearly supported by bootstrap values, and the more recently proposed assemblage, the myonycterine section (Lawrence and Novick 1963) (fig. 2*c*). The African fruitbats *Rousettus* (*Lissonycteris*) *angolensis* and, more unexpectedly, *Rousettus* (*Stenonycteris*) *lanosus* are shown to be connected to the myonycterine clade. The latter are considered subgenera of *Rousettus* due to their morphological characters (Andersen 1912) but cluster far apart from *Rousettus* in the topologies (fig. 2). Contrary to all general classifications (e.g., Koopman 1994), the African endemic genus *Eidolon* was not shown to be included in this African clade, nor was it found to be closely related to the *Rousettus* (fig. 2). The clustering of this unique fruitbat far away from the other African species is supported by other molecular and morphological attributes and adds a novel perspective to the reconstruction of the origin of African fruitbats.

Morphological Evolution

From the African epomophorine to the Australasian pteropodine, a ''*Rousettus*-like'' morphology characterizes many species and confers a foxlike appearance upon the bats known as flying foxes. This phenotype includes a relatively long and narrow muzzle, big eyes, and forward-pointed ears. The skull is typically elongated, with the craniofacial axis almost horizontal and the braincase slightly vaulted and raised above the face line. Nectar-feeding bats typically share a ''*Macroglossus*-like'' skull morphology characterized by a long and narrow muzzle coupled with a delicate mandible and weakened dentition. A third general ''*Cynopterus*-like''

Table 2
Additional Steps, Differences in Likelihood Logarithms, Statistics, and Probability Values of the Tests Comparing Trees from Our Study Against the Alternative Morphologically Based Topologies: (*a*) Constraining Nectar-Feeding Bats (Macroglossinae) to a Monophyletic Group; (*b*) Constraining the Short-Muzzled Group of Bats (Cynopterine) to Monophyly; (*c*) Constraining the Foxlike Group of Bats to Monophyly; and (*d*) with Constraint Assuming a Closer Relationship of the Endemic African Group to the *Rousettine* Section Defined by Andersen (1912)

	Templeton's Test				Kishino-Hasegawa Test			
Constraint	Δ Length	SD	*z*	*P*	Δ ln	SD	*T*	*P*
(*a*) Macroglossinae + other fruitbats . . .	118	14.27574	7.5987	<0.0001	280.24646	42.49847	6.5943	<0.0001
(*b*) Cynopterine + other fruitbats	63	10.63054	5.1761	<0.0001	204.00209	32.25112	6.3254	<0.0001
(*c*) Rousettine + other fruitbats.	29	7.62821	3.4427	0.0006	94.48296	28.92264	3.2667	0.0011
(*d*) Myonycterine + rousettine.	12	4.45775	2.3520	0.0187	44.87796	14.72624	3.0475	0.0024

NOTE.—Significance level at $P < 0.05$. All the probabilities are significant after a sequential alpha-level Bonferroni adjustment for multiple comparisons.

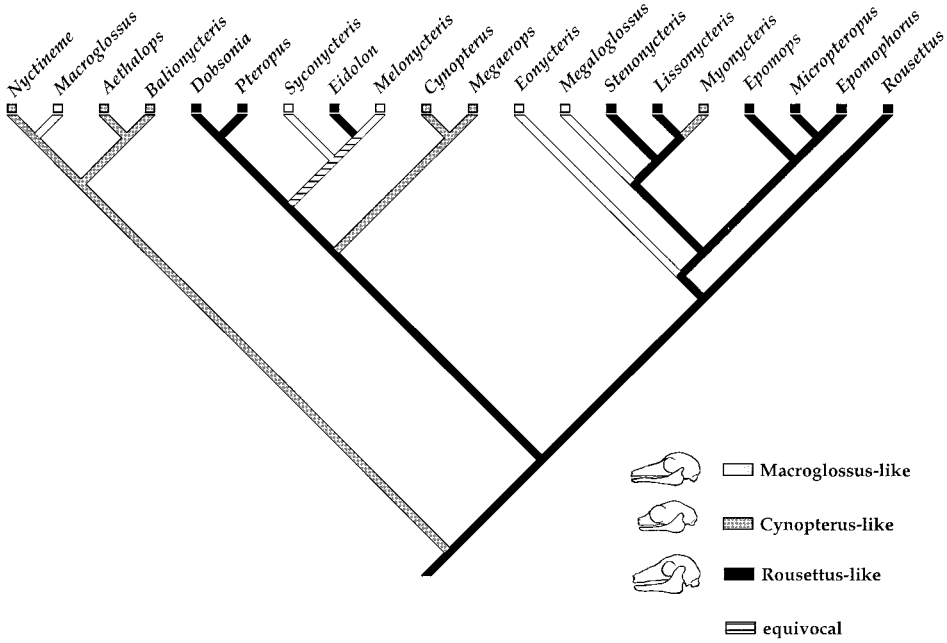

FIG. 3.—Schematic scenario of evolution patterns of skull characters in African fruitbats using MacClade, version 3.07 (Maddison and Maddison 1993), on the maximum- parsimony topology, considering a nonspecialized *Rousettus*-like phenotype presumably basal to all fruitbats (Springer, Hollar, and Kirsch 1995). *Rousettus*-like, *Macroglossus*-like, and *Cynopterus*-like morphotypes are considered unordered character states. The nonspecialized *Rousettus*-like phenotype has independently evolved toward aptness for nectar- and pollen-feeding habits in the African *Megaloglossus* and in other lineages (Kirsch and Lapointe 1997), resulting in convergent morphologies with elongated rostrum, long tongue, and weak dentition and mandibulae. Similarly, another convergence toward shortened faces has produced similar morphologies (*Cynopterus*-like) in the African *Myonycteris* as well as in different Asian groups. The persistence of the primitive *Rousettus*-like morphology and the convergence of the *Macroglossus*-like and *Cynpoterus*-like morphologies have led morphologists to cluster similar species in nonmonophyletic clades such as the Macroglossinae subfamily and the rousettine and cynopterine sections.

skull morphology among the Old-World fruitbats is, in contrast, characterized by a short rostrum and a slightly deflected facial axis (Andersen 1912).

The spread of the *Rousettus*-like morphology could indicate a convergent and polyphyletic origin resembling that of the nectar-feeding phenotype of the Macroglossinae (Kirsch and Lapointe 1997). Alternatively, the success of a nonspecialized primitive morphology (Kirsch et al. 1995) may serve to explain this recurrent phenotype. In fact, most African fruitbats show this *Rousettus*-like, poorly specialized general cranial morphology. Dentition, for instance, is not particularly modified, showing few departures from a simple and cuspless morphology apt for crushing pulpy fruit (Hill and Smith 1984) in typically broad diets and rather generalist feeding habits (Fleming 1982; Banack 1998). Moreover, nonspecialized fruit and nectar feeding has been put forward as the possible primitive condition in the evolution of feeding strategies in fruitbats (Gillette 1975; Kirsch et al. 1995). The unmodified premaxillae, rostrum, palate, and braincase clearly point to the *Rousettus*-like morphology as the most primitive among fruitbats (Andersen 1912), and it has been shown that this morphotype requires the fewest changes with respect to a presumed common ancestor to all fruitbats (Springer, Hollar, and Kirsch 1995).

Decoupled morphological and molecular evolution rates are common in nature (Patterson, Williams, and Humphries 1993) particularly with respect to speciation and morphological changes (e.g., cichlid fishes [Sturmbauer and Meyer 1992], skinks [Bruna, Fisher, and Case 1996], or salamanders [Larson 1989]). Although morphological differentiation during speciation is apparent in fruitbats, it seems that the recurrence of the primitive and nonspecialized *Rousettus*-like morphology has been favored across the different evolutionary lineages. Indeed, it is maintained in the majority of steps (15 times) when the three general morphotypes are mapped (fig. 3) on the maximum-parsimony topology (at genus level) using MacClade (Maddison and Maddison 1993). The retention of these primitive characters in different lineages implies a high level of morphological homoplasy, which would explain the inaccuracies detected in the traditional taxonomic arrangements of the African genera *Eidolon, Lissonycteris,* and *Stenonycteris,* all sharing the typical *Rousettus*-like skull morphology. In our admittedly simplified morphological reconstruction (fig. 3), the *Macroglossus*-like morphotype appears in at least four independent events, and the *Cynopterus*-like morphotype appears in three. The latter morphotype has a less clear functional interpretation than the former and may have a structural origin (*sensu* Wake 1991) attributable to the modification of a developmental program. In fact, all *Cynopterus*-like fruitbats are medium or small sized. A shortened muzzle is also present in most of the small taxa (e.g., *Scotonycteris* or *Casinycteris*) within the *Rousettus*-like derived epomophorine. In this context, a structural relationship between a shift toward a smaller size and a reduction of the rostrum would ex-

plain the phylogenetic noise of this morphological character.

The close agreement between the classical species definitions and the molecular identification of these taxa validates the traditional taxonomic conclusions at the lower levels. However, at the more inclusive levels, it would appear that the decrease in the number of synapomorphies in addition to the increase in plesiomorphies due to evolutionary convergence, as in case of the nectar-feeding fruitbats (*Macroglossus*-like morphotype) and probably in that of the short-muzzled bats (*Cynopterus*-like morphotype), plus the persistence of a successful primitive morphology (*Rousettus*-like morphotype), have led traditional taxonomists to the incorrect clustering of taxa.

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