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Multilocus phylogeny of a cryptic radiation of Afrotropical long-fingered bats (Chiroptera, Miniopteridae)

Terrence C. Demos[1] | **Paul W. Webala**[2] | **Holly L. Lutz**[1] | **Julian C. Kerbis Peterhans**[1,3] | **Steven M. Goodman**[1,4] | **Natalia Cortés-Delgado**[1,5] | **Michael Bartonjo**[6] | **Bruce D. Patterson**[1]

[1]Integrative Research Center, Field Museum of Natural History, Chicago, IL, USA

[2]Department of Forestry and Wildlife Management, Maasai Mara University, Narok, Kenya

[3]College of Arts and Sciences, Roosevelt University, Chicago, IL, USA

[4]Association Vahatra, Antananarivo, Madagascar

[5]Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL, USA

[6]Mammalogy Section, National Museums of Kenya, Nairobi, Kenya

Correspondence
Terrence C. Demos, Integrative Resource Center, Field Museum of Natural History, 1400 S. Lake Shore Drive, Chicago, IL, 60605 USA.
Email: tdemos@fieldmuseum.org

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Abstract

The Old World bat family Miniopteridae comprises only the genus *Miniopterus*, which includes 20 currently recognized species from the Afrotropical realm and 15 species from Eurasia and Australasia. Since 2003, the number of recognized *Miniopterus* species has grown from 19 to 35, with most newly described species endemic to Madagascar and the Comoros Archipelago. We investigated genetic variation, phylogenetic relationships and clade membership in *Miniopterus* focusing on Afrotropical taxa. We generated mitochondrial cytochrome-*b* (cyt-*b*) and nuclear intron data (five genes) from 352 vouchered individuals collected at 78 georeferenced localities. Including 99 additional mitochondrial sequences from GenBank, we analysed a total of 25 recognized species. Mitochondrial genetic distances among cyt-*b*-supported clades averaged 9.3%, representing as many as five undescribed species. Multilocus coalescent delimitation strongly supported the genetic isolation of eight of nine tested unnamed clades. A large number of sampled clades in sub-Saharan Africa are distributed wholly or partly in East Africa (nine of 13 clades), suggesting that *Miniopterus* diversity has been grossly underestimated. Although 25 of 27 cyt-*b* and 23 of 25 nuclear gene tree lineages from the Afrotropics were strongly supported as monophyletic, a majority of deep nodes were poorly resolved in phylogenetic analyses. Long terminal branches subtending short backbone internodes in the phylogenetic analyses suggest a rapid radiation model of diversification. This hypothesis needs to be tested using more phylogenetically informative data.

KEYWORDS

Afrotropics, Madagascar, *Miniopterus*, phylogenetics, species tree, taxonomy

1 | INTRODUCTION

Bats of the family Miniopteridae, all in the genus *Miniopterus*, include 35 currently recognized species; 20 of these occur in the Afrotropical realm, including seven in sub-Saharan Africa, 12 on Madagascar (two also extending to Comoros) and one on São Tomé Island (mammaldiversity.org). The remaining 15 species range across the Palearctic from Western

Europe to Japan, and extend south through the Indomalayan and Australasian realms. Formerly included as a sub-family of Vespertilionidae (Simmons, 2005), Miniopteridae has subsequently been recognized as a distinct family (Miller-Butterworth et al., 2007). All members of the genus are characterized by a uniquely elongated second phalanx of the third finger that also allows the wing to 'bend' back on itself. These features account for the vernacular names 'long-fingered' and 'bent-wing' bats.

The phylogenetic relationships and species limits of bats of the genus *Miniopterus* remain poorly understood due to limited geographic and population-level sampling and to weak topological support for intra-generic relationships (Amador, Moyers Arévalo, Almeida, Catalano, & Giannini, 2018; Miller-Butterworth et al., 2007; Shi & Rabosky, 2015). The apparent morphological uniformity exhibited by the genus has hindered consensus on the classification and species limits of its members (Christidis, Goodman, Naughton, & Appleton, 2014; Corbet & Hill, 1992; Goodman, Maminirina, Bradman, Christidis, & Appleton, 2009; Peterson, Eger, & Mitchell, 1995).

One result has been stasis in the recognition of undescribed *Miniopterus* diversity: only a single new species of Afrotropical *Miniopterus* described between 1936 and 1995. More recently, there has been a dramatic increase in the number of recognized taxa, with as many as 19 additional species recognized since 2005 (Figure 1; Simmons, 2005; mammaldiversity.org). *Miniopterus* is now considered so diverse on Madagascar that the 12 currently recognized species endemic to that island (two also shared with the Comoros) have been considered an adaptive radiation (Christidis et al., 2014). However, progress in parsing the cryptic diversity in the genus has been much slower elsewhere. In sub-Saharan Africa, since 1936, only one new species has been described and two elevated from synonymy: *Miniopterus mossambicus* and *M.* (*natalensis*) *arenarius* (Monadjem, Goodman, Stanley, & Appleton, 2013) and *M.* (*minor*) *newtoni* (Juste, Fernández, Fa, Masefield, & Ibáñez, 2007).

The four most comprehensive phylogenetic studies of *Miniopterus* all inferred support for a monophyletic Afrotropical group. Mitochondrial analyses of cytochrome-*b* (cyt-*b*) allied this group with the Palearctic species *Miniopterus schreibersii* and sister to other Asiatic and Australasian taxa (Christidis et al., 2014; Miller-Butterworth, Eick, Jacobs, Schoeman, & Harley, 2005), whereas analyses that also included nuclear loci recovered the Afrotropical clade as sister to a cohesive Palearctic + Indomalayan + Australasian clade (Amador et al., 2018; Shi & Rabosky, 2015). The phylogenies of Miller-Butterworth et al. (2005) and Christidis et al. (2014) both included three named and one unnamed Afrotropical species; although relationships were mostly unresolved in their analyses, most species were supported as monophyletic. The multilocus phylogenetic analysis of Shi and Rabosky (2015) included 23 *Miniopterus* species and supported a sister relationship of their *Miniopterus inflatus* to the remaining three African and nine Malagasy species analysed. The monophyly of the Madagascar species was poorly supported as were most nodes within the Afrotropical group. Recently, Amador et al. (2018) included 20 *Miniopterus* species in a fossil-dated multilocus phylogenetic analysis that also mostly failed to resolve relationships within the clade. *Miniopterus natalensis* from southern Africa was recovered

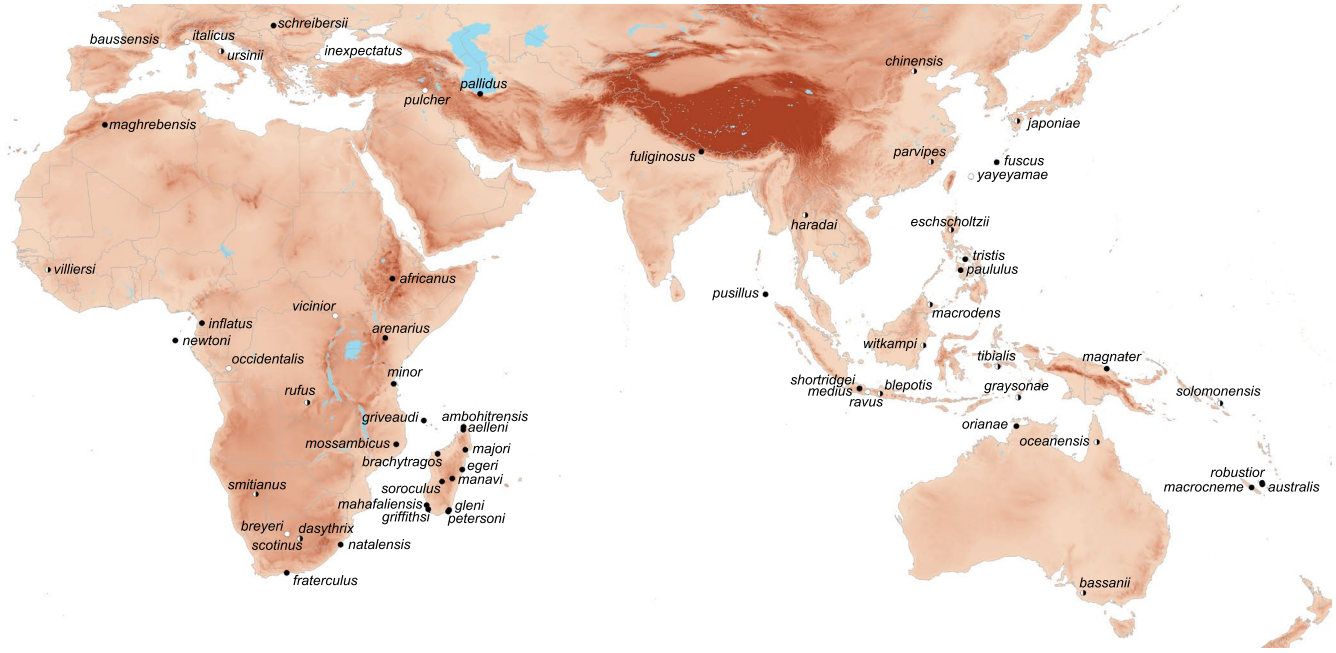

FIGURE 1 Type localities for species-group names applied to populations of *Miniopterus*. Names represent the specific epithets of valid species (filled circles), subspecies (half-filled circles) and synonyms (open circles)

as nested within a poorly supported Madagascar + Comoros clade.

The ambiguous and conflicting phylogenetic relationships inferred by earlier studies underscore the necessity for a more comprehensive molecular systematic analysis of the evolutionary relationships of Afrotropical *Miniopterus*, one with population-level sampling. Of the two prior phylogenetic studies focused on Afrotropical *Miniopterus*, one employed solely mitochondrial data (Christidis et al., 2014) and the other, while also incorporating nuclear microsatellite data, only sampled five African and Malagasy species (Miller-Butterworth et al., 2005). Ours is the first study to independently estimate phylogenetic relationships and species limits using coalescent analyses; we use data from multiple independent nuclear loci to test hypotheses of relationships inferred from mitochondrial and distributional data. Given the subtle morphological distinctions drawn among *Miniopterus* species, the genus is a particularly fitting subject for species delimitation analyses. Our goals are therefore to (a) determine how many evolutionarily distinct lineages are distinguished using multilocus coalescent delimitation methods; (b) explore patterns of diversification within the entirety of the Afrotropical Realm, including Madagascar, Comoros, Zanzibar and São Tomé, using gene tree and species-tree approaches; and (c) assess the geographic scale of endemism among sub-Saharan African *Miniopterus*.

2 | MATERIAL AND METHODS

2.1 | Selection of taxa and sampling

The genetic data set is based on 352 *Miniopterus* individuals. We generated original genetic data from 263 individuals collected at 77 georeferenced localities, and complemented them with 99 mitochondrial sequences from 58 localities downloaded from GenBank (we obtained new sequence data for 10 individuals with prior GenBank records; see Figure S1 and Appendix S1). All individuals were sequenced for cyt-*b* in order to maximize assessment of genetic diversity; however, redundant haplotypes were removed for subsequent phylogenetic analyses (see Appendix S1 for complete list of individuals sequenced). As in recent phylogenetic studies of the Afrotropical bats *Otomops* (Patterson et al., 2018) and *Scotophilus* (Demos, Webala, Bartonjo, & Patterson, 2018). This work has been greatly facilitated by newly available samples from East and Central Africa. Voucher specimens for the bats newly sequenced for this study ($n = 253$) were collected during small mammal surveys across sub-Saharan Africa, with relatively dense sampling in East Africa. Initial assignment of individuals to species for East African specimens was determined using meristic, mensural and qualitative characters published in the bat keys of Thorn and colleagues (Thorn, Kerbis Peterhans, & Baranga, 2009) and Patterson and Webala (2012). Collection methods followed mammal guidelines for the use of wild mammals in research (Sikes & Animal Care and Use Committee of the American Society of Mammalogists, 2016) and were approved under Field Museum of Natural History IACUC #2012-003. See Appendix S1 for voucher numbers and institutions, locality data and GenBank accession numbers. The use of museum voucher specimens in these analyses permits the genetic patterns we resolve in our analysis to be evaluated alongside subsequent dental, cranial and skeletal studies of the same specimens. To avoid adding to current taxonomic confusion in *Miniopterus*, we purposefully took a conservative approach in assigning names to clades in our analyses. Where a clade's taxonomic identity was ambiguous or unknown, we referred to it simply as a numbered clade (clades 1–10 in this study). Integrative taxonomic diagnoses of the various clades supported by our analyses will be necessary to determine which (if any) existing names may apply to them.

2.2 | DNA extraction, amplification and sequencing

Genomic DNA from preserved tissue samples was extracted using the Wizard SV 96 Genomic DNA Purification System (Promega Corporation). Fresh specimens were sequenced for mitochondrial cyt-*b*, using the primer pair LGL 765F and LGL 766R (Bickham, Patton, Schlitter, Rautenbach, & Honeycutt, 2004; Bickham, Wood, & Patton, 1995), and five unlinked autosomal nuclear introns: ACOX2 intron 3, ACPT intron 4, COPS7A intron 4, ROGDI intron 7 (Salicini, Ibáñez, & Juste, 2011) and STAT5A (Matthee, Burzlaff, Taylor, & Davis, 2001) for specimens of *Miniopterus* and the close vespertilionid outgroup *Myotis tricolor* (see Table S1 for primer information). PCR amplification, thermocycler conditions and sequencing were identical to Demos et al. (2018) and Patterson et al. (2018). Sequences were assembled and edited using GENEIOUS PRO v.11.1.5 (Biomatters Ltd.). Sequence alignments were made using MUSCLE (Edgar, 2004) with default settings in GENEIOUS. Protein coding data from cyt-*b* were translated into amino acids to determine codon positions and confirm the absence of premature stop codons, deletions and insertions. Several gaps were incorporated in the nuclear intron alignments, but their positions were unambiguous.

We resolved nuclear DNA to haplotypes with software PHASE (Stephens, Smith, & Donnelly, 2001) and set the probability threshold to 0.70 following Garrick, Sunnucks, and Dyer (2010). PHASE files were formatted and assembled using the SeqPhase online platform (Flot, 2010).

Sequence alignments used in this study have been deposited on the FIGSHARE data repository (10.6084/m9.figsh

are.9927032). All newly generated sequences were deposited in GenBank with accession numbers (MN503247–MN503252 and MN503882–MN504408); (see also Appendix S1).

2.3 | Phylogenetic analyses

jMODELTEST2 (Darriba, Taboada, Doallo, & Posada, 2012) on CIPRES Science Gateway v.3.1 (Miller, Pfeiffer, & Schwartz, 2010) was used to determine the sequence substitution models that best fit the data using the Bayesian Information Criterion for cyt-*b* and the five nuclear introns. Uncorrected sequence divergences (*p*-distances) between and within species/clades were calculated for cyt-*b* using a 327 sequence alignment that excluded all Asian taxa with the exception of *Miniopterus paululus* in MEGA X 10.0.5 (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). Maximum-likelihood (ML) analyses were performed using the program IQ-TREE version 1.6.0 (Chernomor, von Haeseler, & Minh, 2016; Nguyen, Schmidt, von Haeseler, & Minh, 2015) on the CIPRES portal for separate gene trees (cyt-*b*, ACOX2, ACPT, COPS7A, ROGDI, and STAT5A) and a concatenated alignment, partitioned by gene, using the five nuclear introns. Following Hillis and Bull (1993), nodes supported by bootstrap values (BP) ≥70% were considered strongly supported. Gene tree analyses under a Bayesian Inference (BI) framework were inferred in MRBAYES v.3.2.6 (Ronquist et al., 2012) on the CIPRES portal for the same set of genes as the ML analyses. Two independent runs were conducted in MrBayes and nucleotide substitution models were unlinked across partitions for each nuclear locus in the concatenated alignment. Four Markov chains were run for 1×10^7 generations for individual gene trees, and 2×10^7 generations for the concatenated analysis, using default heating values and sampled every 1,000th generation. A conservative 25% burn-in was applied and stationarity of the MRBAYES results was assessed in Tracer v.1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). Majority-rule consensus trees were constructed for each Bayesian analysis. Following Erixon, Svennblad, Britton, and Oxelman (2003), nodes supported by posterior probability (PP) ≥0.95 were considered strongly supported.

Miniopterus taxa for inclusion in species-tree analyses were assigned to either species or numbered clades based on clade support in the ML and BI gene tree analyses of the cyt-*b* data set. Thus, results from gene tree analyses were used to define populations to be used as "candidate species" (as in Demos et al., 2015) in a coalescent-based species-tree approach implemented in StarBEAST2 (Ogilvie, Bouckaert, & Drummond, 2017), an extension of BEAST v.2.5.1 (Bouckaert et al., 2014; Drummond, Suchard, Xie, & Rambaut, 2012). Species-tree analysis was conducted using the five nuclear intron alignments. Substitution, clock, and tree models were unlinked across all loci. A lognormal relaxed-clock model was applied to each locus under a Yule tree prior and a linear with constant root population size model. Four independent replicates were run with random starting seeds and chain lengths of 2×10^8 generations and parameters were sampled every 20,000 steps. For the StarBEAST2 analyses, evidence of convergence and stationarity of model parameter posterior distributions was assessed based on ESS values >200 and examination of trace files in Tracer v.1.7. The burn-in was set at 20% and separate runs were assembled using LOGCOMBINER v.2.5.1 and TREEANNOTATOR v.2.5.1 (Rambaut et al., 2018).

2.4 | Coalescent lineage delimitation

On the basis of well-supported monophyletic clades obtained by the cyt-*b* gene tree analyses, a lineage delimitation scenario with 11 candidate species (clades 1–9, *mossambicus*, and *natalensis*) was tested. We inferred the evolutionary isolation of their gene pools using the phased nuclear DNA data set (ACOX2, ACPT, COPS7A, ROGDI and STAT5A; 66 individuals) for joint independent lineage delimitation and species-tree estimation evaluated under the multi-species coalescent model using the program BPP v.3.3 (Rannala & Yang, 2017; Yang & Rannala, 2014). This analysis was carried out to guide future investigations of the species status of evolutionarily isolated lineages inferred here, using an integrative species taxonomic approach to include morphological, morphometric and acoustic characters, as well as, ectoparasitic associations and distributional data. Species/clade memberships for BPP were identical to individuals assigned to lineages in the species-tree analyses. The validity of our assignment of individuals to populations was tested using the guide-tree-free algorithm (A11) in BPP. Two independent runs for each of four different combinations of divergence depth and effective population sizes priors (τ and θ, respectively; Table S2) were tested, as the probability of delimitation by BPP is sensitive to these two parameters (Leaché & Fujita, 2010; Rannala & Yang, 2017; Yang & Rannala, 2014). Two independent MCMC chains were run for 5×10^4 generations. The burn-in was 20% and samples drawn every 50th generation. In total, eight BPP runs were carried out using five-phased nuclear intron alignments. Lineages were considered to be statistically well supported when the delimitation posterior probabilities generated were ≥0.95 under all four prior combinations.

3 | RESULTS

The 273 cyt-*b* sequences used in the ML and BI gene tree analyses ranged from 525 to 1,133 base pairs (bp) in length (88% coverage). To aid in visualizing the phylogenies inferred from this matrix, we reduced a matrix of 352 individuals to a set of mostly unique haplotypes, resulting in the final alignment of 273 sequences (see Appendix S1 for information

on all 352 sequences). The number of base pairs for the sequence alignments (not including outgroup) used in individual ML and BI gene tree and Bayesian species-tree analyses were: ACOX2 ($n = 68$), 402–436 bp; ACPT ($n = 66$), 351–430 bp; COPS7A ($n = 67$), 735–740 bp; ROGDI ($n = 67$), 337–382 bp; STAT5A ($n = 68$), 466–533 bp; and 5 intron concatenated alignment ($n = 68$), 1,733–2,520 bp. The best-supported substitution models for each gene estimated by jMODELTEST2 are listed in Table S1. Uncorrected cyt-*b* *p*-distances for African and Malagasy *Miniopterus* species and numbered clades (removing Eurasian and Australasian sequences except for *M. paululus*) averaged 0.093 and ranged from 0.017 to 0.139 between species/clades, while within species/clade distances averaged 0.009 and ranged from 0.000 to 0.026 (Table S3).

3.1 | Mitochondrial gene trees

Phylogenetic trees based on the 273 sequence cyt-*b* mitochondrial data set were well resolved for a sub-Saharan African clade that includes *Miniopterus fraterculus*, *M. mossambicus*, *M. newtoni*, and numbered clades 1 to 10 but were poorly resolved at most basal nodes (Figure 2). The ML and BI analyses had similar topologies and the combined analysis is presented. The majority of Malagasy *Miniopterus* species cluster together in a poorly supported group (9 of 12 species) that contains two well-supported sub-clades consisting of (*gleni* + *griffithsi* + *majori*) and (*aelleni* + *ambohitrensis* + *brachytragos* + *egeri* + *manavi* + *petersoni*). The positions of *Miniopterus griveaudi* and *M. soroculus* from Madagascar and East African clade 10 are poorly resolved. *Miniopterus natalensis* is well supported as sister to all African + Malagasy species and clades. Of the Palearctic, Indomalayan and Australasian clades represented, western Palearctic *M.* sp. (Israel) and (*M. pallidus* [*M. schreibersii*, *M. maghrebensis*]), appear as successive sisters to *M. natalensis* + the remaining African and Malagasy species/clades. The monophyly of each of the four African and 12 Malagasy species in the analyses are strongly supported by cyt-*b* sequences, with the exception of *Miniopterus manavi* which is recovered as paraphyletic (*M. manavi* 1 *and M. manavi* 2 in Figure 2). The numbered clades 1 to 10 all are strongly supported as monophyletic with the exception of clade 1, which contains two well-supported subclades. Multiple long branches are inferred for clades 1 to 10, and individuals from several of these cyt-*b* lineages are sympatric in East Africa (i.e., clades 1, 4, 7 and 8 are all present in Nakuru County, Kenya).

3.2 | Multilocus nuclear gene trees

The BI gene tree inferred from the concatenated nuclear genes ACOX2, ACPT, COPS7A, ROGDI, and STAT5A (68 individuals; matrix 98% complete) is shown in Figure 3. This tree resembled the ML tree in having mostly poor support for relationships among species and numbered clades (see Figure S2 for individual ML and BI intron gene trees). Nuclear analyses failed to recover *M. mossambicus* or clades 1, 2 and 9 as monophyletic and these are labelled as merged in Figure 3. All species and numbered clades are strongly supported as monophyletic with the exceptions of clade 4 (PP < 0.95 and BS < 70) and *M. mossambicus* + clade 9 (PP = <0.95 and BS > 70). As in the mitochondrial gene tree (Figure 2), the same grouping of sub-Saharan African species and numbered clades is well supported (although nuclear introns were unavailable from *M. fraterculus* and *M. newtoni*). *Miniopterus natalensis* is securely recovered well inside the sub-Saharan African lineage; clade 10 appears as the basal-most/earliest diverging African lineage outside Madagascar, although its position is poorly supported. Eleven of 12 Malagasy species cluster together (excluding *Miniopterus griffithsi*) although none of their interspecific relationships are well supported. *Miniopterus australis* (Indomalayan) + *M. tristis* (Australasian) are well supported as sister to *M. schreibersii* (Palearctic) + all African and Malagasy clades.

3.3 | Nuclear species tree

Samples from all posterior parameter values of the four independent StarBEAST analyses using the five intron nuclear data set had ESS values >200. We discarded the first 10% of each run, leaving 18,000 species trees in the posterior distributions that were merged in LogCombiner. The topology of the maximum clade credibility tree (Figure 4; also see Figure S2 for individual ML and BI intron trees) was nearly identical across all four independent runs. Species-tree analysis using StarBEAST resulted in a topology that was generally poorly supported, with only six of 25 nodes having PP ≥ 0.95. There was strong support for Palearctic *Miniopterus schreibersii* as sister to all 24 Afrotropical species and numbered clades included in the analyses. As in the multilocus nuclear gene tree analyses (Figure 3), the species tree strongly supported *M. australis* + *M. tristis* as sister to *M. schreibersii* and the African + Malagasy clade, which was poorly resolved. In both the cyt-*b* and intron gene trees, there is poor support for the monophyly of either Malagasy or sub-Saharan African lineages to the exclusion of the other (Figures 2 and 3).

3.4 | Lineage delimitation

Results from the replicated BPP analyses show that choice of priors had minimal effect on delimitation probabilities for most (nine of 11) tested species and numbered clades (Table 1). *Miniopterus mossambicus* and clade 9 had poor delimitation support as evolutionarily isolated lineages in Prior

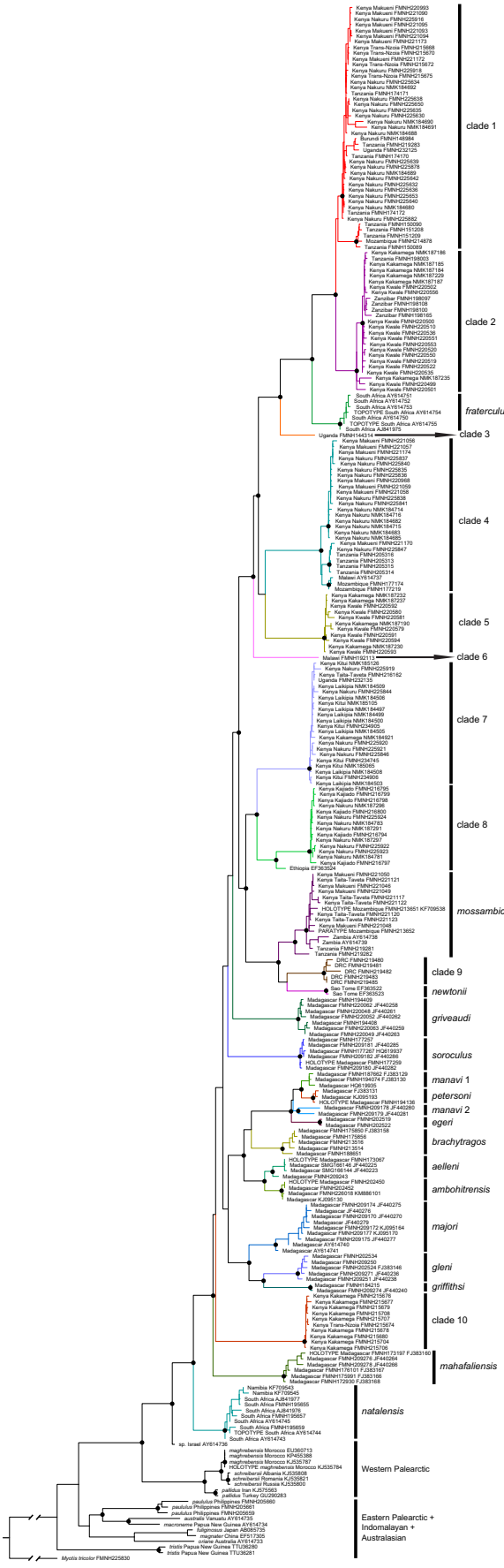

FIGURE 2 Maximum-likelihood phylogeny of mitochondrial cytochrome-*b* sequences of *Miniopterus*. The phylogeny was inferred in IQ-TREE and its topology was very similar to the Bayesian phylogeny calculated in MRBAYES. Filled black circles on nodes denote bootstrap values (BS) ≥ 70% and Bayesian posterior probabilities (PP) ≥0.95. Branch colours indicate individual clade membership [Colour figure can be viewed at wileyonlinelibrary.com]

Schemes 1 and 2 (defined in Table S2). These unsupported lineages had short branch lengths in the species tree (Figure 4) and were paraphyletic in multilocus nuclear gene tree analyses (Figure 3). All of the remaining numbered clades (1–8) and *M. natalensis* had strong delimitation support (PP ≥ 0.98) and are distinguished as robustly defined lineages across all prior schemes. Contrary to multilocus gene tree inference (Figure 3), but in accord with mitochondrial gene tree inference (Figure 2), clade 1 and clade 2 are strongly inferred as genetically isolated lineages (Table 1). The eight strongly delimited clades that could not be confidently named (i.e., clades 1–8 and clade 9 + *M. mossambicus*) are candidates to be evaluated as potentially valid species using independent data in an integrative taxonomic framework.

4 | DISCUSSION

4.1 | Multiple new divergent lineages

Miniopteridae is thought to have diverged from Vespertilionidae + Cistugidae ~48 Mya, and the crown age for the family—marking the split between the Afrotropical and the Indomalayan + Australasian clades—was dated at 13 Mya (Amador et al., 2018). Relative to other bat families, Miniopteridae is therefore relatively young and its main diversification occurred in the middle Miocene (Amador et al., 2018). In the most comprehensive phylogenetic study of Afrotropical Miniopteridae, we recovered relatively deep lineage divergence among species and the 10 numbered clades (clades 1–10). Four of the latter likely represent populations reported as the species *Miniopterus africanus*, *M. arenarius*, *M. inflatus*, and *M. minor*, as we analysed samples taken within the currently recognized ranges of each of them. Yet we refrained from assigning scientific names where species identity was uncertain due to taxonomic uncertainty (e.g., members of the *M. inflatus* species complex; Simmons, 2005), confirmatory type material was unavailable, or morphology was ambiguous. Assuming that all four were included among our ten clades (Figure 2), at least five and perhaps six other species-ranked lineages are currently unrecognized and thus either new to science and undescribed, or else are currently regarded as synonyms. The overall uncorrected mean pairwise genetic distance among species and species ranked clades of the cyt-*b* data

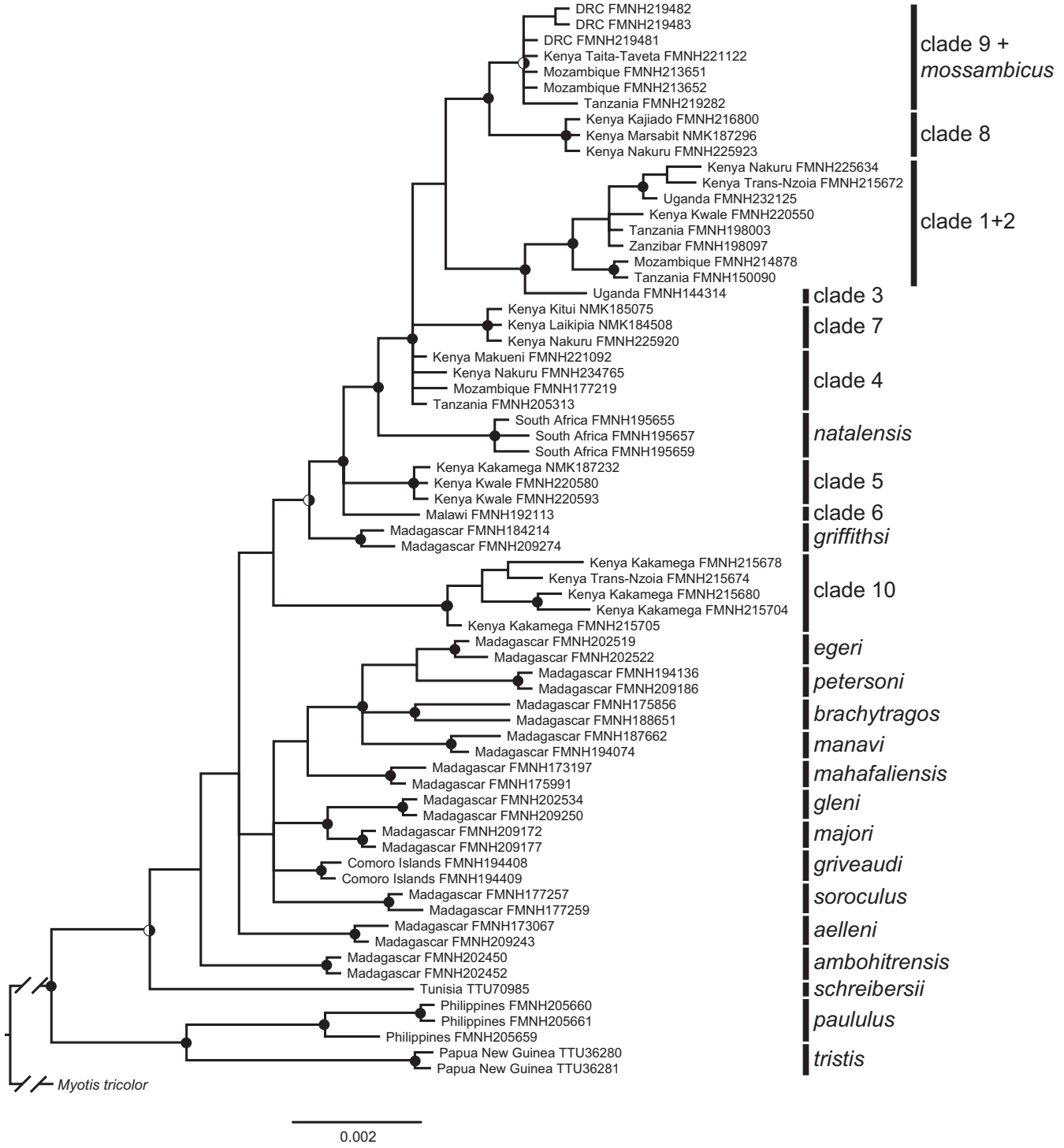

FIGURE 3 Bayesian phylogeny of *Miniopterus* based on five nuclear introns. The phylogeny was inferred in MRBAYES and its topology closely resembled the maximum-likelihood phylogeny calculated in IQ-TREE. Filled black circles on nodes denote bootstrap values (BS) ≥ 70% and Bayesian posterior probabilities (PP) ≥ 0.95, right-half-filled black circles indicate BS ≥ 70% and PP < 0.95, and unmarked nodes indicate BS < 70% and PP < 0.95

set was 0.093. In other Afrotropical bat surveys using the same mitochondrial marker, overall cyt-*b* distances averaged 0.10 among *Scotophilus* (Demos et al., 2018), 0.14 among *Myotis* (Patterson et al., 2019), 0.10 among *Rhinolophus* (Demos, Webala, Goodman, et al., 2019), and 0.17 among *Nycteris* (Demos, Webala, Kerbis Peterhans, et al., 2019). *Miniopterus* genetic distances for cyt-*b* were comparable among all sub-Saharan African species-ranked clades (0.094) and all those from the Madagascar region (0.083). Branch lengths for clades 1–10 in the nuclear gene tree

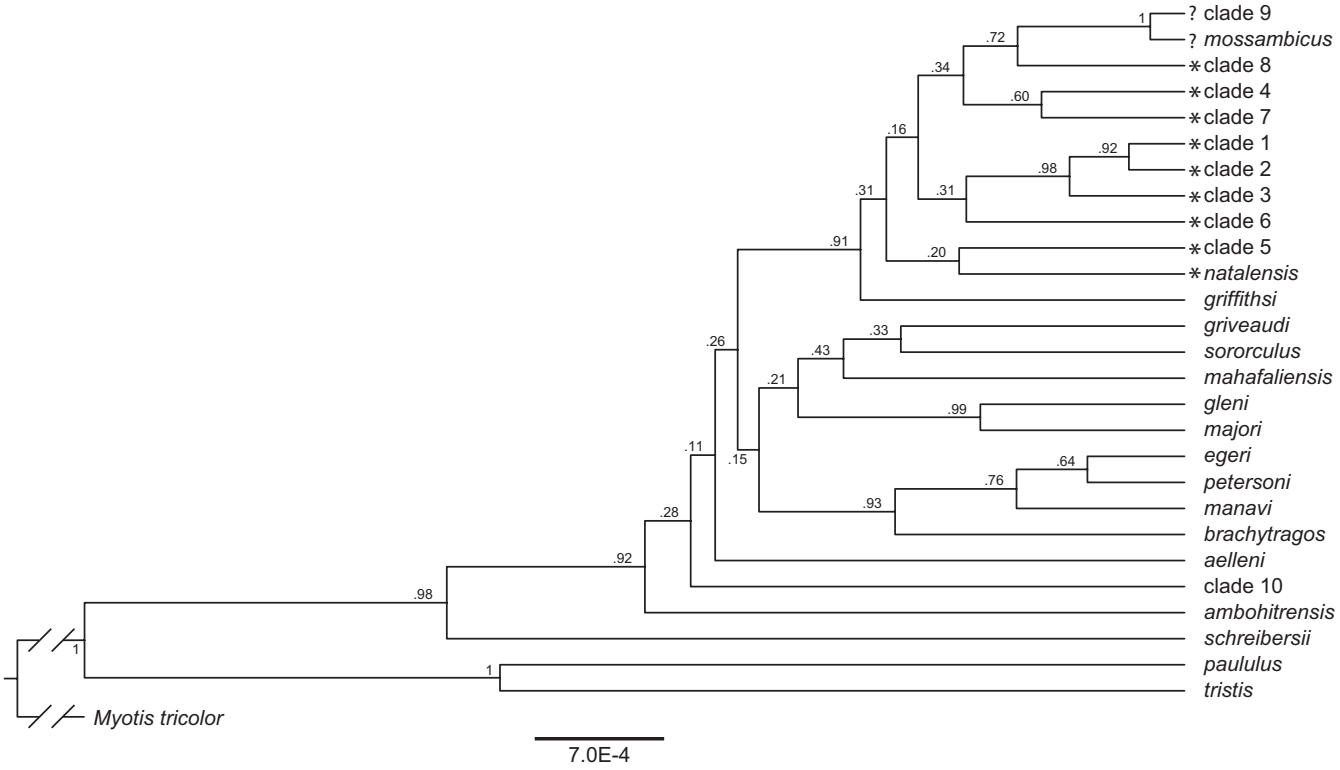

FIGURE 4 Species tree of *Miniopterus* estimated in StarBEAST2 using the five nuclear intron data set. Numbers adjacent to nodes indicate posterior probabilities. Terminal tips in the tree that are statistically well supported (PP ≥ 0.95) from BPP are indicated by '*' preceding the clade name, and terminal tips that had PP < 0.95 are indicated by '?' preceding the clade name

(Figure 3) and nuclear species-tree (Figure 4) analyses were also comparable to those of recognized *Miniopterus* species.

4.2 | Phylogenetic relationships of *Miniopterus*

Previous phylogenetic analyses of Afrotropical *Miniopterus* have been limited by poor geographic and population-level sampling of sub-Saharan African taxa (Amador et al., 2018; Miller-Butterworth et al., 2005; Shi & Rabosky, 2015). However, phylogenetic studies of *Miniopterus* all strongly recover an Afrotropical clade. More extensive taxon and gene sampling resolves this as sister to a Palearctic + Indomalay an + Oceanian clade (Amador et al., 2018; Shi & Rabosky, 2015). Although few members of the second clade were represented in our multilocus nuclear analysis, there was strong

TABLE 1 Lineage delimitation results based on the five intron data set for the Afrotropical clade of *Miniopterus* under four different parameter sets

Putative species	BPP PS1	BPP PS2	BPP PS3	BPP PS4
Clade 1	0.98	0.98	0.99	0.99
Clade 2	0.98	0.98	0.99	0.99
Clade 3	0.99	0.99	1.0	1.0
Clade 4	1.0	0.99	1.0	1.0
Clade 5	0.99	0.99	1.0	1.0
Clade 6	0.99	0.99	1.0	1.0
Clade 7	1.0	1.0	1.0	1.0
Clade 8	1.0	1.0	1.0	1.0
Clade 9	0.32	0.38	0.98	0.98
mossambicus	0.32	0.38	0.98	0.98
natalensis	1.0	1.0	1.0	1.0

Note: See section 2 for parameter details. Values for BPP PSs are average posterior probabilities (PP) of delimitation from three replicated BPP runs under each of four different Prior Schemes for two data sets (PS; Table S2).

support for monophyly of the Afrotropical taxa (Figures 2 and 3).

Within the Afrotropical clade, *M. natalensis* from southern Africa was well supported as sister to the remaining African + Madagascar clades in the mitochondrial tree (Figure 2), but this was strongly contradicted by the nuclear gene tree and species tree where basal nodes within the Afrotropical clade were poorly supported (Figures 3 and 4). None of the phylogenetic analyses inferred support for the monophyly of either the Madagascar taxa or the sub-Saharan clades. The placement of clade 10 varied widely among phylogenetic analyses: it was poorly supported as nested within Madagascar lineages in the mitochondrial gene tree (Figure 2) and nuclear species tree (Figure 4). Phylogenetic uncertainty associated with long branches is a recurrent systematic theme. Christidis et al. (2014) analysed relationships among Malagasy *Miniopterus* using cyt-*b*, recovering 18 clades that they combined into five primary lineages. Only one of their primary lineages has even partial support in our intron and species trees: the sister relationship between *Miniopterus gleni* and *M. majori,* although *M. griffithsi* is not recovered with them and in both trees appears more closely related to sub-Saharan species than to any on Madagascar. A general pattern of long terminal taxon branches subtending short backbone internodes accounts for the poor support for deeper nodes in both the mitochondrial and nuclear phylogenetic analyses. This pattern also suggests a rapid radiation model of diversification. In applying the term radiation to the diversification of Afrotropical *Miniopterus* we purposefully refrain from characterizing this process as an adaptive radiation. Although consensus is lacking on all of the attributes required to accurately characterize a radiation as adaptive (Givnish, 2015; Losos & Miles, 2002), most definitions include the evolution of a diversity of ecological roles on the basis of adaptations within a lineage either through the evolution of key innovations (e.g., flight) or exploitation of resources that are underutilized by other species (e.g., colonization of isolated islands or landmasses). However, data are unavailable as to how Miniopteridae species have ecologically diverged into various adaptive zones or niches. Although Christidis et al. (2014) characterized the rapid radiation of *Miniopterus* species on Madagascar as an adaptive radiation, evidence for the role of phenotypic disparity and ecological divergence in the rapid diversification of Malagasy taxa was lacking. Because the role of morphological disparity in facilitating ecological divergence is unknown in *Miniopterus*, this process might equally be described as a non-adaptive radiation.

Whether the diversification of *Miniopterus* species on Madagascar was facilitated by either adaptive or non-adaptive processes, the accelerated tempo of speciation is suggested by rough divergence dates presented by Christidis et al. (2014), applying a 2% substitution rate per million years to a mitochondrial gene tree analysis of 11 Malagasy and five sub-Saharan African *Miniopterus* species and the fossil-calibrated mitochondrial gene tree of Amador et al. (2018). Christidis et al. (2014) estimated a crown age of 7.24 Ma for an Afrotropical *Miniopterus* clade and 10.16 Ma for Miniopteridae, whereas Amador et al. (2018) inferred a crown age of 8.66 Ma for Afrotropical *Miniopterus* and 12.83 Ma for the genus as a whole. Employing a phylogenetic species concept, Christidis et al. (2014) estimated as many as 18 *Miniopterus* species may occur on Madagascar; they opined that as many as 100 species may occur across the Old World range of the genus (where 35 are currently recognized). Genetic evidence suggests that as many as five evolutionarily isolated populations in our study may represent additional species—integrative taxonomic assessments of their rank are needed. Geographic sampling in our data set is very sparse for the species-rich rainforests of West and Central Africa, so continent-wide Miniopteridae diversity is probably grossly underestimated.

Recent studies of Afrotropical mammal diversification date the earliest divergence of many genus-level clades or species complexes to the Plio-Pleistocene boundary; subsequent diversification is thus temporally concordant with and perhaps driven by the initiation of Pleistocene climatic oscillations. Corresponding expansions and contractions of forest species into Pleistocene refugia has been well documented in several Afrotropical small mammal groups including rodents (Demos, Peterhans, Agwanda, & Hickerson, 2014; Mizerovská et al., 2019; Nicolas et al., 2011), shrews (Demos et al., 2015; Jacquet et al., 2015), and golden moles (Mynhardt et al., 2015). The role of expanding and contracting Pleistocene habitat in isolating African bat populations remains to be tested.

4.3 | Lineage delimitation and taxonomic reappraisal

Our data set is the largest yet assembled for Afrotropical *Miniopterus*. It infers support for at least eight independent evolutionary lineages among the numbered clades 1 to 10. Clade 10 was not analysed in delimitation analyses because of its unstable phylogenetic position and deep divergence from other *Miniopterus* clades. Coalescent delimitation analyses strongly support clades 1–8 as evolutionarily independent lineages while the independence of clade 9 is ambiguous, potentially conspecific with *M. mossambicus* (Table1, Figure 4). Four of the eight recognized sub-Saharan African species are confidently named in this study (*M. fraterculus*, *M. mossambicus*, *M. natalensis*, *M. newtoni*), and four of those not named (*M. africanus*, *M. arenarius*, *M. inflatus*, *M. minor*) may well be represented among the numbered clades. But four or five additional lineages are supported in the nuclear coalescent delimitation and species-tree analyses as candidate species for future assessment with corroborative data.

As discussed by Monadjem et al. (2013), numerous problems remain to be resolved in the systematics of Afrotropical *Miniopterus*, including the probable paraphyly of recognized taxa (e.g., *M. inflatus*, *M. minor*, and *M. natalensis*), all of which exhibit very broad distributions as currently understood.

It is worth noting that we did not attempt to incorporate phenotypic characters in the current study of species differentiation. Recent studies describing and delimiting *Miniopterus* species on Madagascar have relied on a system of molecular sequence analysis to assign individual specimens to clades and then assessment of their morphological attributes for differences. Recognized clades commonly show broad overlap in mensural characters, such as forearm or maxillary toothrow length, and hence these are of little use in species diagnosis. However, most Malagasy *Miniopterus* clades show discrete morphological character variation that is diagnostic of individual clades, most notably in the tragus, a feature of the external ear that is directly associated with echolocation (Gannon, Sherwin, deCarvalho, & O'Farrell, 2001; Lawrence & Simmons, 1982). Further, echolocation calls associated with these same samples could be used with tragus morphology to provide largely consistent separation of individuals belonging to the different clades (Ramasindrazana, Goodman, Schoeman, & Appleton, 2012). Analysing the numbered clades reported in the current study using their qualitative and quantitative morphology, bioacoustics, internal and external parasites, and distribution is a logical next step. It will permit descriptions of their biological differentiation, assessment of their diagnostic characteristics, and application of scientific names.

4.4 | Distribution patterns

Sampling of Afrotropical taxa in this study was focused on Eastern and Southern Africa and Madagascar. Most sub-Saharan samples were taken in Kenya, one of 54 African countries (Figure S1). The number of *Miniopterus* clades with Kenyan members may be useful to indicate the extent of *Miniopterus* diversity in lesser sampled African regions. Six species of *Miniopterus* are reported to occur in Kenya, namely *M. africanus*, *M. fraterculus, M. inflatus*, *M. minor*, *M. mossambicus*, and *M. natalensis* (Musila et al., 2019). But the divergent monophyletic clades in the cyt-*b* gene tree (Figure 2) and coalescent delimitation results (Table 1, Figure 4) strongly support at least eight *Miniopterus* lineages in Kenya (clades 1, 2, 4, 5, 7, 8, and 10, and *M. mossambicus*). Four of these species/clades have geographic ranges that include Nakuru County, and four species are also sympatric in the Kakamega Forest in western Kenya. Thus, the number of *Miniopterus* species-ranked lineages we have documented in Kenya exceeds by one the number of currently recognized species for all of continental sub-Saharan Africa. We expect that many additional *Miniopterus* species will come to light with additional sampling in undersampled regions of Africa.

The distribution and endemism patterns of Afrotropical *Miniopterus* are clouded by several factors, including the very subtle cranial and external characters that distinguish named taxa within *Miniopterus* (Monadjem et al., 2013; Ramasindrazana et al., 2012), the lack of support for deeper nodes in our mitochondrial and nuclear phylogenies, and wholly inadequate sampling in western and central Africa. Nonetheless, some patterns are clearly discernable. First, the sympatric occurrence in at least two different regions of Kenya of individuals from four species-level clades is remarkable, and this diversity was found to characterize both savannah and lowland rainforest habitats. Elsewhere in the range of Miniopteridae, two to three body-size classes have been observed to co-occur (e.g., small, medium, and large; Monadjem et al., 2013). Local co-occurrence of multiple *Miniopterus* species is probably fostered by their typical reliance on caves for roosting, and this influence could be especially marked in more arid regions. Second, based on our sampling, the only two species-level clades that appear restricted to lowland rainforest are clade 9 from the Congo Basin, Democratic Republic of Congo, and *M. newtoni* from São Tomé Island in the Gulf of Guinea, West Africa. These two taxa are well supported as sisters and in turn are sister to *M. mossambicus* whose distribution is restricted to eastern and southeastern Africa (Figures 1 and 2). Finally, both the mitochondrial (Figure 2) and nuclear (Figures 3 and 4) phylogenies recovered two deeply diverged lineages represented by single individuals (clade 3 and clade 6) from mountains in the African Rift. Clade 3 is known only from the Ruwenzori Mts. in the Albertine Rift, and clade 6 is known only from the Southern Rift in Malawi. Both of these montane regions house multiple endemic mammal species (Brooks et al., 2004; Kahindo, Bowie, & Bates, 2007). Whether these two lineages are actually restricted to African Rift habitats, and therefore constitute Rift endemics, will require further investigation.

An unexpected result of species delimitation analyses was ambiguous support for clade 9 from Democratic Republic of Congo as being either included with or genetically isolated from *M. mossambicus*, distributed in far eastern Africa (Kenya through Zimbabwe, possibly including Zambia (López-Baucells et al., 2017; Monadjem et al., 2013). However, association with *M. mossambicus* may be an artefact of uninformative nuclear markers for this lineage: clade 9 is relatively deeply diverged from *M. mossambicus* in the cyt-*b* gene tree, where it is well supported as monophyletic and sister to *M. newtoni* (Figure 2). Our samples of *M. mossambicus* include individuals from both Kenya and Tanzania in a well-supported monophyletic clade with the *M. mossambicus* holotype.

4.5 | Broader significance

Recently, part of the deadly Ebola virus genome was recovered from a bat identified as *M. inflatus* in Liberia, close to the deadly outbreak of the disease in Guinea and neighbouring regions between 2013 and 2016 (Kupferschmidt, 2019). Although it is important to note that researchers did not isolate the virus itself, and there is no evidence that the bats serve as reservoirs or vectors of the disease, the broad distribution of *M. inflatus* as it is currently understood raises disturbing questions for public health and safety. This species has its type locality in Cameroon, but *Miniopterus* from 16 different African nations—from Guinea in West Africa to Ethiopia in northeast Africa to Zimbabwe in southern Africa—are currently allocated to this species and known by this name (IUCN, 2019; Monadjem & Schlitter, 2017). Yet the conservative morphology of *Miniopterus* and the cryptic diversity uncovered in genetic analyses of the genus suggest much finer-scale differentiation. Five of the eight evolutionarily independent lineages we documented in Kenya are at present either restricted to that country or extend only into a neighbouring country; a similar localized extent and density of *Miniopterus* distributions also characterizes Madagascar. Additional surveys of *Miniopterus* in tropical western and central Africa are needed to determine the extent of miniopterid diversity and the true extent of their geographic ranges.

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ORCID

Terrence C. Demos https://orcid.org/0000-0003-0522-3708
Bruce D. Patterson https://orcid.org/0000-0002-2249-7260

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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