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ORIGINAL RESEARCH

DNA barcoding reveals cryptic diversification and taxonomic discordance among bats and birds within Sub-Saharan Africa

Jake Mulvaney[1] · **Monika Moir**[1,2] · **Michael I. Cherry**[1,3]

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Abstract

Cryptic species present a challenge for conservation, as species diversity may remain undetected. In zoological research, DNA barcoding of the mitochondrial cytochrome c oxidase subunit I (COI) has become a useful heuristic tool for aiding species resolution and informing species discovery. Despite concerted efforts to genetically barcode bats and birds, comprehensive assessments have yet to be undertaken across the Afrotropics. We retrieved available DNA barcodes of native breeding Afrotropical bat and bird species. Using Bayesian phylogenetic modelling, we assessed DNA barcode performance at species identification, and sought to detect notable intraspecific clade partitioning hinting at cryptic speciation. Available DNA barcodes represent only 42.3% and 23.6% of the relevant bat and bird species diversity, respectively, with only 18.7% of bat species and 7.2% of bird species having geographically spread records. DNA barcodes afforded greater taxonomic resolution of Afrotropical bird species than of bats (96.8% vs. 84.0%), with bats having a higher proportion of species non-monophyly (25.5% vs. 4.8%). Well-supported (≥95% posterior probability) clade partitioning was inferable from twenty-one bat species and fifteen bird species, and a further single under-sampled bat species and fifteen such bird species showed deep (>2.0%) intraspecific divergences. These phylogenetic signatures allude to cryptic speciation within these volant taxa, and serve to prompt more comprehensive assessments of Afrotropical fauna. These findings also indirectly affirm the importance of paleoclimatic refugia to endemic vertebrate diversity. The current taxonomic status of birds is better supported by this molecular evidence than that of bats.

Keywords Afrotropics · DNA barcoding · COI · Chiroptera · Aves · Evolutionarily significant unit · Diversity

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✉ Jake Mulvaney
jakemulvaney@gmail.com

[1] Department of Botany and Zoology, Stellenbosch University, Stellenbosch 7600, South Africa

[2] Centre for Epidemic Response and Innovation, School for Data Science and Computational Thinking, Stellenbosch University, Stellenbosch 7600, South Africa

[3] Department of Zoology, Edward Grey Institute, South Parks Road, Oxford OX1 3PS, England

Introduction

COP15 has once again focused attention on the plight of the world's biodiversity. Yet documenting diversity faces many impediments, including limited finance and taxonomic skill. Denoting full species status rather than subspecies or similar can influence conservation prioritization including red listing (May 1990). It has been 20 years since DNA barcoding was proposed as a heuristic tool for aiding species resolution and delineation (Hebert et al. 2003a,b), thereby helping to document as many species as possible before they go extinct. This practice is informed principally by the phylogenetic species concept – that species should comprise genetically distinct evolutionarily groups (Nixon and Wheeler 1990). In animals, standard DNA barcoding uses 648 bp of mitochondrial cytochrome c oxidase I (COI) (Hebert et al. 2003a). This partial gene sequence typically shows high interspecific-, yet low intraspecific diversity, and so is a useful preliminary tool for assessing species boundaries - notwithstanding rate variation across taxa (Sigwart and Garbett 2018), intraspecific taxonomic complexity (Phillips et al. 2019), and parapatric hybridisation (Aliabadian et al. 2009).

DNA barcoding has proven reliable for identifying recognised species, and informing species-level systematics. Thus, it can contribute to conservation both by expediting biodiversity assessments, such as through environmental DNA metabarcoding (Beng and Corlett 2020); and by providing information about evolutionary histories and species phylogenetic diversity (Krishnamurthy and Francis 2012). Use of this standardised genetic tool has been especially valuable in the case of bat species, which can be challenging to differentiate based on morphological and acoustic criteria (Gager et al. 2016; Mota et al. 2022; Rydell et al. 2017). Even among birds, a group which comprises more readily phenotypically discernible species, there may be twice as many evolutionarily distinct species than reflected by traditional morphometrics (Barrowclough et al. 2016). Globally, DNA barcoding studies have provided insights useful for refining species boundaries across both bats (Benítez et al. 2021; Clare et al. 2007, 2011; Kruskop et al. 2012) and birds (Chaves et al. 2015; Kerr et al. 2009a,b; Saitoh et al. 2015), although these studies have been skewed predominantly towards the eastern Palaearctic, Nearctic, and especially the Neotropics. But despite several studies demonstrating the efficacy of this molecular tool, few have employed it on a broad geographic scale. Additionally, no large-scale DNA barcoding assessments have been undertaken for bats and birds within the Afrotropical realm – defined here as encompassing sub-Saharan Africa and the southern Arabian Peninsula (Olson et al. 2001), excluding the southwest Indian Ocean islands (Holt et al. 2013). The Afrotropics support approximately 20% of global bat diversity (Wilson and Mittermeier 2019), and 21% of terrestrial bird species (BirdLife International 2013), yet this area is projected to suffer the most extreme temperature increases from climate change from 2040 to 2059, as well as increased aridity, (UNDP Climate Impact Lab, 2022 https://impactlab.org/ accessed 1 December 2022). South Africa's avifauna has been identified as being particularly vulnerable to climate change, while for bats logging of forests in West Africa and in the Congo Basin presents an even greater threat (Harfoot et al. 2021). Currently, 33 bat species and 240 bird species which naturally breed within the sub-Saharan Africa are globally threatened or near threatened (IUCN 2022), with a further 85 bat species and 16 bird species listed as 'Data Deficient'.

In this context, it is essential that Africa's biota is adequately described as a matter of urgency, and DNA barcoding is a useful tool to guide this process. It has been argued that as

mammologists are more eager to embrace the phylogenetic species concept, the molecular revolution in taxonomy has resulted in more mammal species having been split than bird species, as many avian taxonomists remain wed to the biological species concept (Garnett and Christidis 2017). This seems apparent in the case of Africa: of the 328 bat species currently recognized from sub-Saharan Africa and the southwest Indian Ocean islands, 18% have been described over the last three decades (Taylor et al. 2019); over this same period, the number of formally recognised bird species breeding within the Afrotropics has increased by 13% (Dowsett et al. 1993; HBW and BirdLife International 2022). Birds are on current evidence taxonomically more speciose and more functionally diverse than bats, as they have a wider range of both diet and foraging guilds in addition to being both diurnal and nocturnal. Bats are the second most diverse order of mammals and, like birds, occupy a similarly wide range of habitats. Despite bats (an order) and birds (a class) occupying different taxonomic ranks, both are volant endothermic vertebrate taxa of generally small body size, high metabolic rates (when not dormant) and have considerably longer lifespans than non-volant mammals of comparable body size (Munshi-South and Wilkinson 2010). Both groups demonstrate relatively well-resolved taxonomies, and have similar DNA barcode representation within the Afrotropics (see below).

In this study, we have used available DNA barcodes to assess genetic relationships, and to attempt to understand current and potential species diversity among native breeding Afrotropical bat and bird species. Using both Bayesian phylogenetic modelling and genetic distances, we compare the reliability of DNA barcodes in informing species identity and relationships within these two vertebrate groups, with instances of non-monophyly taken as an indication of potential cryptic speciation. We hypothesised that DNA barcodes would more accurately reflect avian than bat species taxonomy, as we presume the former to be better resolved due to greater phenotypic species differentiation. By extension, we expected to uncover greater cryptic diversity among bats than in birds.

Methods

DNA barcoding dataset compilation

We compiled DNA barcode datasets representing all native breeding Afrotropical bat and bird species represented in the Barcode Of Life Data System v4 (BOLD) repository (Hebert and Ratnasingham 2007): https://www.boldsystems.org/index.php/). We derived comprehensive species lists from the total list of African species, as per the 'African Chiroptera Report 2022' (van Cakenberghe and Seamark 2022) for bats, and the 'Handbook of the Birds of the World and BirdLife International Digital Checklist of the Birds of the World, Version7' (HBW and BirdLife International, 2022) for birds. From these sources, we excluded introduced species, non-breeding Palearctic migrants, species confined to northern Africa (Palearctic), and island endemics of the western Indian Ocean and Southern Ocean. Using these filtered species lists, we retrieved DNA barcodes (mtDNA COI-5P) from the BOLD repository by searching either 'Aves' or 'Chiroptera' AND '[sub-Saharan African country]', subsequently searching '[genus name]' for the relevant bat and bird genera. This twofold search afforded collection of both nameless, and non-georeferenced records; missing spatial

data were sought from original studies to improve the phylogeographic insights therein. Extralimital records of native breeding Afrotropical species were retained.

To balance sample size and species representation alongside adequate sequence data, we considered DNA barcodes only from the 5' end, and omitted specimens < 541 bp in bats and < 513 bp in birds. Bat and bird sequences were aligned respectively using CLUSTAL X (Larkin et al. 2007) in GENEIOUS 7.1.4 (©Biomatters).

Our final DNA barcode datasets comprised 1844 specimens of at least 106 Afrotropical bat species (Appendix 1a), and 1440 specimens of 441 Afrotropical bird species (Appendix 1b). Included in these datasets are our own contributions of 89 records of 15 bat species and 197 records of 72 bird species collected from south-eastern South African forests, representing 7 and 40 novel bat and bird species records respectively (see Appendix 2 for field sampling and DNA barcoding procedures).

Phylogenetic analyses

Bayesian phylogenetic trees based on the above DNA barcode alignments were reconstructed in MrBayes v3.2.7 (Ronquist et al. 2012) at the Cipres Science Gateway v3.3. (Miller et al. 2010) on XSEDE. Bat and bird datasets were analysed separately, under the best nucleotide substitution model (GTR + I + G for both groups), as selected according to Akaike information criterion (AIC) (Posada and Buckley 2004) derived from PAUP* (Swofford 1998) likelihood scores in MRMODELTEST v2.3 (Nylander 2004). Tree reconstructions comprised two runs of four chains for 10 million generations, sampling every 10,000 generations using default parameters, and with a burn-in of 25%. Consensus trees were viewed in FigTree v1.4.4 (Rambaut 2020) (see Appendix 3 for phylogenetic trees). Coalescenc of species lineages were visually inspected, with ≥ 95% nodal posterior probabilities (*p.P.*) denoting well-supported clades. We identified instances of intraspecific reciprocal monophyly, polyphyly, and paraphyly following Meyer and Paulay (2005). To simplify phylogenetic interpretations, DNA barcodes which were not assigned to the appropriate genera, or related genera, were flagged as database errors (misidentification, low quality/contaminated sequences, or nuclear-mitochondrial inserts).

Finally, we estimated intraspecific and interspecific (within genus) genetic distances under the Kimura 2-parameter model (K2P) (Kimura 1980) with 1,000 bootstrap validations using MEGA X (Kumar et al. 2018). Although genetic distance thresholds are a controversial means of delimiting species (DeSalle and Goldstein 2019; Meyer and Paulay 2005; Sigwart and Garbett 2018), deep intraspecific divergences thresholds exceeding > 2.0% in bats (Clare et al. 2011) and > 1.6% in birds (Kerr et al. 2009a) were used to gauge cryptic diversification, especially among poorly sampled species.

Results

Current DNA barcodes spanning > 500 bp of the standard 648 bp COI-5P mtDNA partial gene have been collected for approximately 40.3% of the 263 native breeding Afrotropical bat species (Appendix 4a), and 23.6% of the 1863 native breeding Afrotropical bird species (Appendix 4b); of these, only 50 bat species (18.7%, Appendix 4c) and 135 bird species (7.2%, Appendix 4d) have geographically dispersed DNA barcodes. Available DNA

barcodes represent only 8/33 (24.2%, Appendix 4c) of native breeding Afrotropical bat species and 37/240 (15.4%, Appendix 4d) of native breeding Afrotropical bird species listed as threatened on the IUCN Red List, alongside 17/85 (20.0%) of bat species and 0/16 (0.0%) of bird species deemed 'Data Deficient'.

For both taxa, the DNA barcode sampling density of the species considered is highest in southern Kenya and eastern South Africa (Fig. 1). Avian barcodes appear more uniformly dispersed across the Afrotropics, but where bat barcoding has been undertaken, more intense sampling is evident. Overall, bat showed a substantially higher barcode count per species (mean: 13.2, median: 6, range: 1-143; Appendix 4c) than did birds (mean: 3.2, median: 2, range: 1–43; Appendix 4d), and a greater proportion of bat species were represented by multiple barcodes than were birds (81.1% vs. 68.5%; Appendix 4c-4d).

Despite overall better DNA barcode coverage for bats, phylogenetic tree reconstructions revealed that only 89/106 (84.0%) of bat species evaluated had unique DNA barcodes (Appendix 4c), compared to 427/441 (96.8%) of bird species evaluated (Appendix 4d). However, DNA barcode species resolution among bats may be hampered by inadequate phenotypic species identification, as 45 well-supported monophyletic clusters/independent lineages emerged among barcodes curated only to genus-level (Appendix 5). These generic

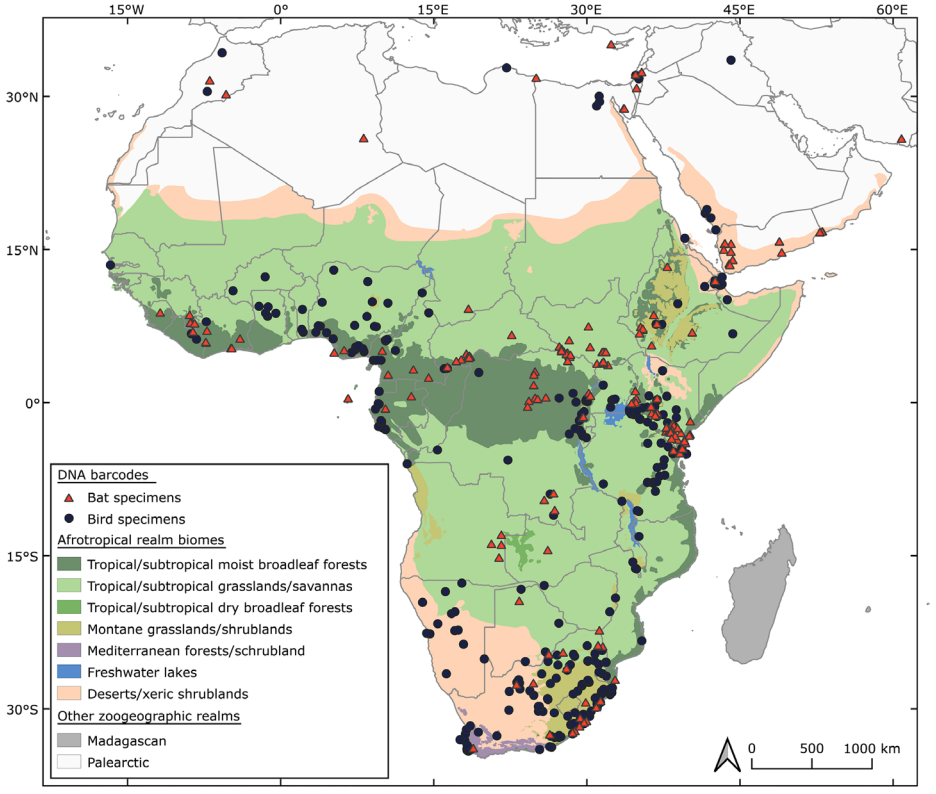

Fig. 1 Sampling localities of the phylogenetically informative DNA barcodes (>500 bp) from bat and bird species which naturally breed in the Afrotropics, with the major biomes of this zoological realm delineated according to Olson et al. (2001)

barcode cohorts likely represent many known, and possibly unknown, bat species, though these could not be identified. No generic avian analogues were observed.

In total, we detected 28 bat barcodes (1.5% of total, Appendix 1a) and 34 bird barcodes (2.4% of total, Appendix 1b) to be erroneous; however, these were mostly correctable species misidentifications, and only one bat and one bird barcode were omitted from analyses due to gross out-grouping. We further revised 282 bat barcodes (15.3% of total) of 18 species bearing outdated nomenclature (Appendix 1a), alongside 94 bird barcodes (6.5% of total) of 23 species (Appendix 1b). Additionally, 448 bat barcodes (24.3% of total) and 21 bird barcodes (1.5% of total) were originally curated to genus-level only (Appendices 1, 4 and 6). These barcodes were retained in the phylogenetic analyses and have been partially curated in Appendix 5.

Intraspecific distances were markedly higher among bats (mean: 2.0%, median: 0.8%, range: 0.0-15.7%; Appendix 4c) than among birds (mean: 0.6%, median: 0.2%, range: 0.0-8.1%; Appendix 4d). In both taxa, most species divergence were below the median values (Appendix 4), with mean values having been raised by the comparatively few highly divergent species (see below). Among genera with several barcoded species (including unspecified barcodes), interspecific distances were similarly higher among bats (n = 20, mean: 9.4%, median: 9.5%, range: 1.6–18.6%; Appendix 6a) than birds (n = 89, mean: 6.3%, median: 6.4%, range: 0.0-12.1%; Appendix 6b). Figure 2 shows that intraspecific distances were mainly lower than interspecific distances, yet neither bats nor birds showed an apparent 'barcode gap' between these two critical levels of divergence. For both faunal groups, genetic distances from DNA barcodes yield ambiguous species distinctions, with no obvious threshold values to identify overly divergent species.

Phylogenetic modelling showed that, of the identifiable species possessing multiple barcodes, 63/86 (73.3%) bat species and 286/302 (94.7%) bird species were monophyletic (Appendix 4c and 4d, respectively). Additionally, 16/20 (80.0%) bat species, and 134/139 (96.4%) bird species represented by only a single DNA barcode branched independently, and so could be differentiated from related species (Appendix 4c and d).

Well-supported intraspecific clade partitioning was evident in 21 bat species (Figs. 3) and 15 bird species (Fig. 4). Among bats, eight monophyletic species possessed reciprocally monophyletic intraspecific clades, while three were paraphyletic, and ten were polyphyletic

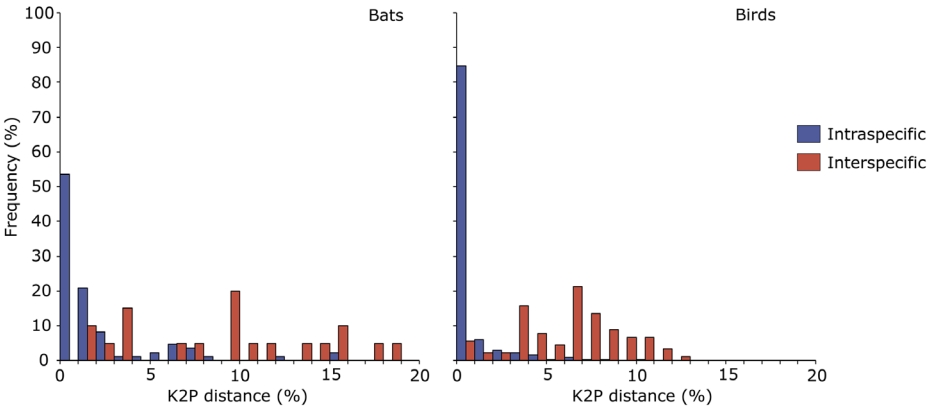

Fig. 2 Kimura-2-parameter COI genetic distances observed within recognised native breeding Afrotropical bat (left) and bird (right) species (purple) and genera (red)

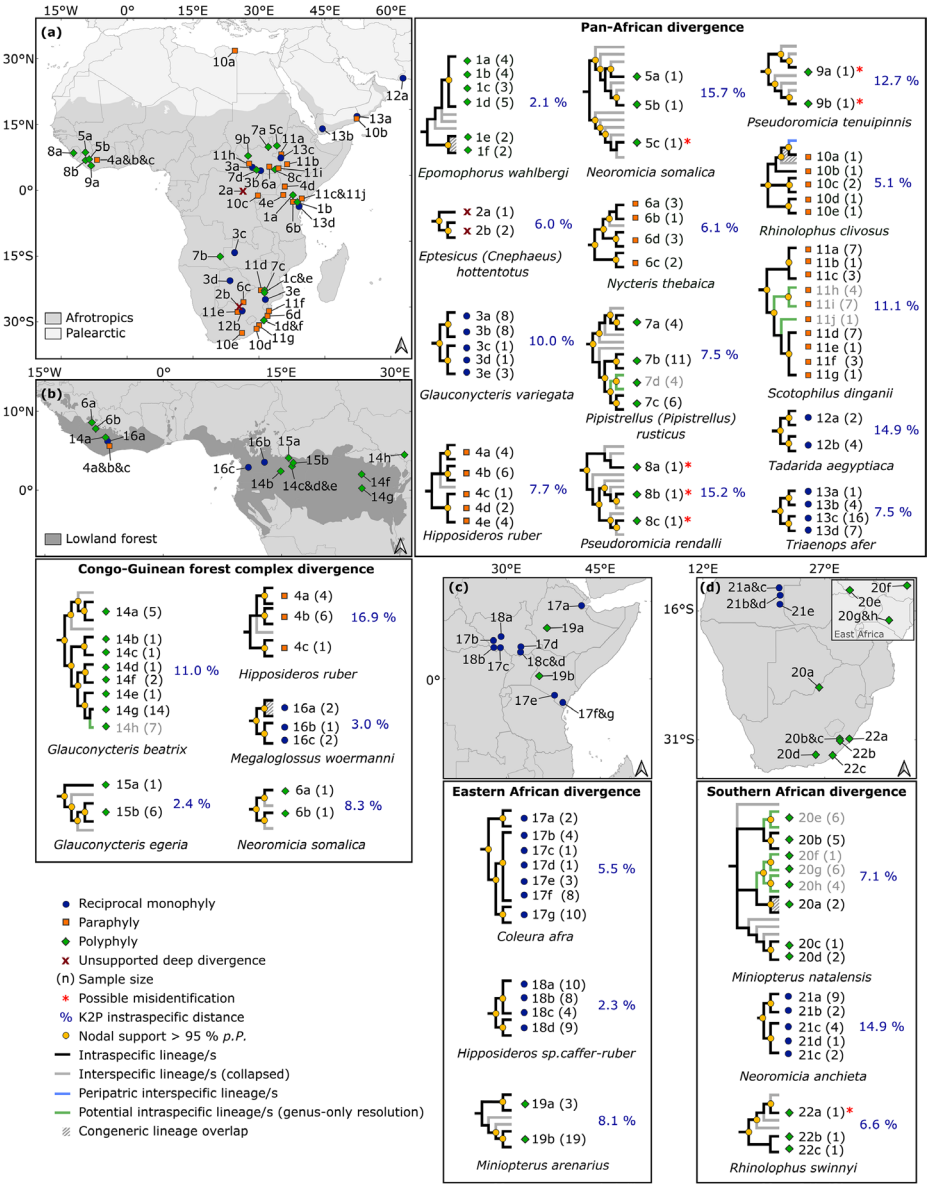

Fig. 3 Bayesian clustering of available DNA barcodes from native breeding Afrotropical bats revealed 21 species exhibiting well-supported (>95% *p.P.*) intraspecific clade partitioning and/or deep divergence (>2.0%) across (**a**) the Africa continent, (**b**) the Congo-Guinean lowland forest complex, (**c**) east Africa, (**d**) and southern Africa. Shown here are the sample localities of species demonstrating reciprocally monophyletic (blue circles), paraphyletic (orange squares), or polyphyletic (green diamond) clades, alongside those with deeply divergent lineages only (red crosses)

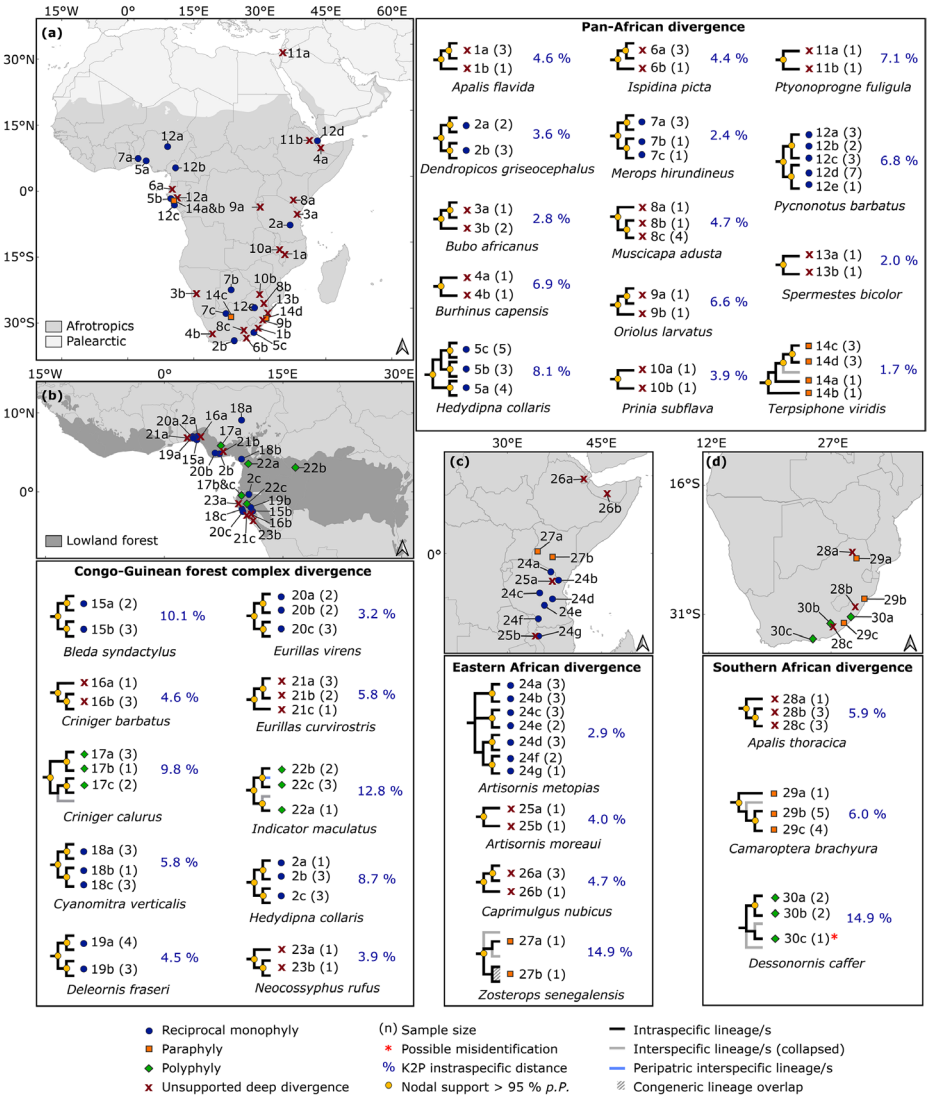

Fig. 4 Bayesian clustering of available DNA barcodes from native breeding Afrotropical birds revealed 30 species exhibiting well-supported (>95% *p.P.*) intraspecific clade partitioning and/or deep divergence (>2.0%) across (**a**) the Africa continent, (**b**) the Congo-Guinean lowland forest complex, (**c**) east Africa, (**d**) and southern Africa. Shown here are the sample localities of species demonstrating reciprocally monophyletic (blue circles), paraphyletic (orange squares), or polyphyletic (green diamond) clades, alongside those with deeply divergent lineages only (red crosses)

(Fig. 3). By comparison, eight monophyletic bird species had intraspecific reciprocal monophylies, four were paraphyletic, two were polyphyletic, and one was both para- and polyphyletic (Fig. 4). Additionally, one poorly sampled bat species (Fig. 3) and fifteen poorly sampled bird species (Fig. 4) showed deep intraspecific genetic divergences exceeding predetermined species-threshold values. Among the species showing potential cryptic diver-

gence were three bat species classified as 'Data Deficient' or 'Not Evaluated' by the IUCN Red List: *Glauconycteris egeria* (Fig. 3a), *Hipposideros sp. caffer-ruber* (Fig. 3c), and *Miniopterus arenarius* (Fig. 3c). One IUCN Red List bird species, *Artisornis moreaui* (Critically Endangered), also showed deep genetic divergence (Fig. 4c), despite poor sampling.

Seventeen bat species (Appendix 4c) and fourteen bird species (Appendix 4d) had overlapping lineages among congeners, and so were non-resolvable to species level. Notably, DNA barcode ambiguity was detected among four IUCN Red List species, namely the bat species *Laephotis angolensis* (Data Deficient), and the bird species *Agapornis fischeri* (Near Threatened), *Balearica regulorum* (Endangered), *Balearica pavonine* (Vulnerable), and *Poicephalus robustus* (Vulnerable). Additionally, four bat species (Fig. 3a, b and d) and one bird species (Fig. 4c) with deep divergences shared mitochondrial lineages with sister-species (see also Appendix 4c-4d), although misidentification is possible.

Unexpectedly, phylogenetic analysis of DNA barcodes revealed potential genus-level paraphyly, wherein certain bat and bird species demonstrated well-supported coalescence among non-congeners (Fig. 5). In bats, the three paraphyletic genera (Fig. 5a and c) were localised to the family Vespertilionidae (suborder Yangochiroptera/Vespertilioniformes). By comparison, six instances of avian genera paraphyly were observed across two avian families of two orders: Nectariniidae (order Passeriformes, Fig. 5d g), and Accipitridae (order Accipiteriformes, Fig. 5 h-5i).

DNA barcode comparisons between the avian orders and chiropteran sub-orders, respectively, showed uneven distributions of species-level divergences across the higher taxonomic ranks of both faunal groups. Between the two bat suborders, Yangochiroptera (Vespertilioniformes) had a higher proportion of species exhibiting intraspecific clade partitioning compared to Yinpterochiroptera (Pteropodiformes) (25.4% vs. 22.6%; Appendix 4c). Average genetic divergences were also deeper among species within Yangochiroptera compared to those within Yinpterochiroptera, both according to intraspecific (2.4% vs. 1.4%) and interspecific (10.0% vs. 7.4%) assessments (Appendix 4c). The higher genetic variability apparent among species within Yangochiroptera is despite these species having fewer barcodes on average than those within Yinpterochiroptera (9.8 vs. 19.8; Appendix 4c), and suggests faster mitochondrial evolution within the former suborder. This explanation would also con-

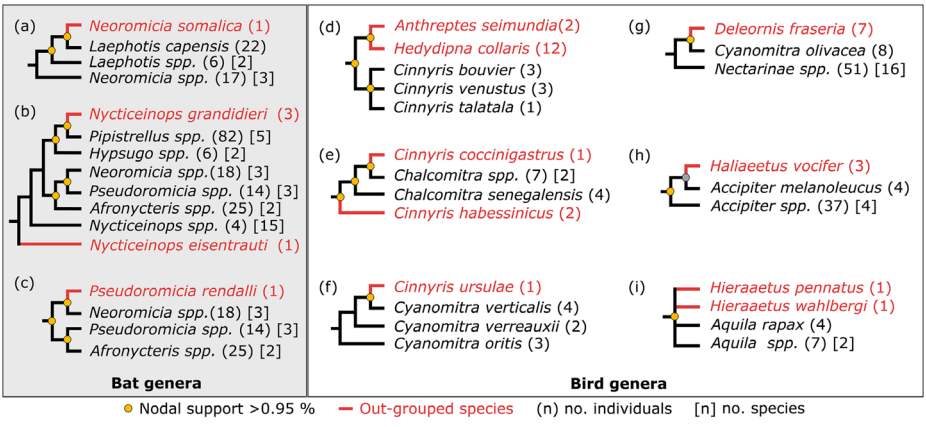

Fig. 5 Paraphyletic genera of Afrotropical bats (**a-c**) and birds (**d-i**) as revealed by Bayesian clustering of available DNA barcodes. Red branching highlights the out-grouped species nested within another genus

form to Yinpterochiroptera species having greater degree of DNA barcode ambiguity among congeners (22.2%) – possibly implying limited mitochondrial gene divergence between sister species – compared to those within Yangochiroptera (12.9%) (Appendix 4c). Similarly in birds, species within the Passeriformes (passerines) – the most speciose avian order, both globally and in the Afrotropics – showed higher DNA barcode variability than those from the 26 non-passerine orders assessed. A higher proportion of passerine species displayed intraspecific clade partitioning than non-passerines (6.8% vs. 2.3%), while relatively more non-passerine species had congeneric lineage overlap than did passerines (5.3% vs. 1.8%) (Appendix 4d). Passerines also showed deeper intraspecific (0.8% vs. 0.4%), and interspecific (6.6% vs. 5.9%) divergences compared to non-passerines (Appendix 4d), despite the former group averaging slightly fewer barcodes per species than the latter (3.4 vs. 3.1).

Discussion

Available DNA barcodes better depict known species diversity among native breeding Afrotropical birds compared to their chiropteran counterparts, with birds affording not only greater species resolution than bats (96.8% vs. 84.0%), but also far lower non-monophyly (4.8% vs. 25.5%). The higher rate of detection of species non-monophyly in bats than in birds may be influenced by bats averaging more barcodes per species (13.2 vs. 3.2), thus potentially encompassing broader intraspecific haplotype breadths. However, the assumption that higher chiropteran species non-monophyly may be an artefact of greater per species sampling is undermined by single-barcode bat species demonstrating a higher degree of non-independent branching compared birds (20% vs. 3.6%), despite relatively fewer single-barcode species among bats (18.9%) than birds (31.5%). The lower proportion of avian phylogenetic incongruencies may be attributed to there being 4.2 times more barcoded bird species than bats considered in this study, given that increased species representation reduces the rate of phylogenetic errors (Pollock et al. 2002; Zwickl and Hillis 2002). This counterbalances the limited sampling of individual bird species, which otherwise may have impaired correct species-level coalescence (Nielsen an Matz, 2006; Phillips et al. 2019). Additionally, discrepancies between avian and chiropteran mitochondrial evolution, and possibly sex-biased dispersal (detailed below) may contribute to the differing species-level divergence trends observed. On balance, the greater avian monophyly we report most likely reflects more accurate species phenotypic identification and taxonomic resolution of Afrotropical birds compared to bats.

A corollary to this explanation is that the utility of DNA barcodes hinges upon meticulous metadata curation of archived records. The elusive field identification of bats (Chornelia et al. 2022; Foley et al. 2017; Solari et al. 2019) impedes accurate barcode labelling more so than in birds, weakening the inferential power of chiropteran barcodes and potentially artificially inflating the degree of observed non-monophyly (Mutanen et al. 2016). Not only was there a higher degree of generic-only barcodes for bats than birds (24.3% vs. 1.5%; Appendix 1a-1b), but a further 185 omitted bat records had order/family-only curation (Appendix 1a). Among the unspecified bat barcodes, phylogenetic modelling revealed 45 well-supported, yet indeterminate ‘species’ clades, and these have been categorised accordingly in Appendix 5.

Because of ongoing taxonomic revisions, we observed an appreciable accumulation of DNA barcodes with outdated nomenclature, obfuscating concise species-barcode correspondence. Antiquated curation appears more prevalent among bat DNA barcodes compared to those of birds (15.3% vs. 7.0% of total records assessed, respectively), despite the greater avian species- and phylogenetic diversity considered in this study. In this regard, the recent state of flux in African bat systematics (Monadjem et al. 2021a; Rossoni et al. 2021; Taylor et al. 2022) exemplifies the overarching challenge for molecular databases to continuously update the curation of archived records. Bat biologists call on a suite of diagnostic tools (morphometrics, acoustics, and genetic markers) for species identification, but with frequent overlap between morphological and acoustics parameters emphasis often rests on molecular identification from reference databases. Accordingly, poor record curation within these databases can impede correct species assignment of new records, thereby perpetuating these problems. Appendix 1 amends individual barcode metadata where possible. The under-performance of assessed bat barcodes reinforces the importance of voucher specimen availability, and suggests that diagnostic data beyond photographs and georeferencing may be necessary accompaniments to DNA barcodes where species are morphologically indistinct. We suggest acoustic recordings of flight-cage flown bats should accompany DNA barcode uploads to facilitate accurate species identification with a further chiropteran taxonomic tool.

Beyond highlighting the need for improved DNA barcode curation, our phylogenetic modelling reveals Afrotropical bat and bird species cryptic diversification warranting further attention. Deep intraspecific divergences were present within 20.8% of assessed bat species (Figs. 3) and 6.8% of assessed bird species (Fig. 4), with 19.8% and 3.4% respectively displaying intraspecific clade partitioning. Deeper intraspecific divergences were broadly correlated to sampling intensity (sample size and geographic coverage), with most species having below median genetic distances (0.8% in bats and 0.2% in birds). Only 47.2% of bat species and 31.5% of bird species assessed had geographically dispersed barcode records (Appendix 1 and Appendix 4c-4d), of which most species retained shallow divergences (Appendix 4c-4d). The deeper intraspecific mitochondrial divergences detected could be inflated by female philopatry; regional selection sweeps; chance; or undetected database errors (Baker et al. 2009; Meyer and Paulay 2005; Moritz and Cicero 2004; Rubinoff et al. 2006), and so more comprehensive genomic and morphological substantiation of these observations is needed. Nevertheless, our findings highlight the cryptic diversification potential of both volant taxa throughout the Afrotropics, especially among bats.

The geographic distribution of available barcodes depicts a paucity of bat and bird barcodes from many countries along the northern bounds of the Afrotropical realm, as well as from most of Angola and Somalia (Fig. 1). There is also notable under-representation of DNA barcodes from the most extensive Afrotropical biome, tropical and subtropical grasslands and savanna (Fig. 1), despite increasing biodiversity surveying of these ecosystems (Bond and Parr 2010; Huntley et al. 2019a; Petermann and Buzhdygan 2021). Sampling is highly clustered in the montane grassland biome of South Africa, and discontinuously within the moist broadleaf forests from Kenya through Central Africa to Sierra Leone. These geographical patterns of sampling and sequencing are echoed in the number of whole animal genomes from Africa available only from South Africa and Nigeria (Hotaling et al. 2021) which may be attributed to resource and funding scarcity. New species discovery in the Afrotropics (with the exceptions of South Africa and Madagascar), lags behind

that in the Neotropical, Indo-Malayan, and Australasian regions (Grieneisen et al. 2014), at least partly due to lower research capacity within sub-Saharan Africa. Here, we simultaneously draw attention to the extent of cryptic diversification evident from currently available barcodes within the Afrotropics, and to the considerable unsampled diversity that persists within the realm. Hopefully, this will be addressed to some extent by additional funding to developing countries following COP15.

In the Afrotropics, appreciably deep intraspecific divergences of both birds and bats largely manifested at the inter-regional scale, suggesting greater regionalisation of presumably widespread species. Eight bat species (Fig. 3a) and thirteen bird species (Fig. 4a) possessed distinct southern African lineages, and a further six bat species and two bird species showed longitudinal isolation between western/eastern Africa/western Asia. Regional-scale deep intraspecific divergences skewed towards the Congo-Guinean lowland forest complex for both bats (five species; Fig. 3b), and especially birds (ten species; Fig. 4b), despite limited DNA barcoding efforts within these forests. This concentration of species divergence corroborates evidence of cryptic diversification within the Congo-Guinean forests for both bats (Hassanin et al. 2018; Huntley et al. 2019b; Monadjem et al. 2020; Nesi et al. 2013) and birds (Huntley et al. 2019a; Huntley and Voelker 2016; Voelker et al. 2013). Most other noteworthy regional divergences occurred within biodiversity hotspots over eastern Africa (three bat species, Fig. 3c; four bird species, Fig. 4c) and south-eastern Africa (three bat species, Fig. 3d; three bird species, Fig. 4d). Sample bias and taxonomic/database errors notwithstanding, this suggests heightened cryptic bat and bird diversification in these regions, as already observed in small mammals (Demos et al. 2014; Matamba et al. 2020, 2021). Altogether, cryptic divergences among the bats and birds assessed frequently mapped onto Afrotropical paleoclimatic mammalian and avian refugia (Levinsky et al. 2013; Mizerovská et al. 2019); some of the avian refugia having persisted since the Eocene (Fjeldså and Bowie 2008).

While most significant intraspecific clade partitioning emerged across spatially discrete sample sets, a few noteworthy divergences were also evident between overlapping/partially overlapping sample sets within seven (potentially eight) bat species (Fig. 3a and c) and two bird species (Fig. 4a and b). True reproductive isolation between these respective populations, however, requires further substantiation. Other cases of non-monophyly arose from incomplete mitochondrial lineage sorting between congeners, reflecting either introgressive hybridisation, limited gene fragment evolution between sister-species, or sample misidentification (Funk and Omland 2003). We detected seventeen instances of DNA barcode lineage ambiguity across seven bat genera (16.0% of species; Appendix 4c) – including *Epomophorus*, for which mitochondrial introgression is suspected (Hassanin et al. 2020; Nesi et al. 2011) – as well as fourteen instances across seven bird genera (3.2% of species; Appendix 4d).

Interestingly, certain partial DNA barcode ambiguity resulted from one monophyletic species nesting within the clade of a sister-species, as was observed once in bats (Appendix 4c) and three times in birds (Appendix 4d). This one-sided phylogenetic distinction may reflect recent speciation events arising through peripatry, wherein a smaller subpopulation isolated from a main ancestral population undergoes accelerated evolution which leads to an asymmetric divergence between respective populations (Colvin 2018; Losos and Glor 2003).

The observed DNA barcode ambiguity concerning four globally threatened IUCN Red List species (Appendix 4d) undermines the use of this genetic tool in wildlife forensic approaches which may be employed to protect these species (Galimberti et al. 2015). This is concerning given the potential vulnerability of the two parrot species *Agapornis fischeri* and *Poicephalus robustus*, which both have ambiguous DNA barcodes, to illegal poaching for the international pet trade (Martin 2018). Further limiting the use of conservation applications of DNA barcoding is that 75.8% and 84.6% of globally threatened naive breeding Afrotropical bat and bird species, respectively, remain unbarcoded. The lack of genetic assessment within many of these species risks overlooking even more precarious species populations (Krishnamurthy and Francis 2012). This is highlighted by the critically endangered bird species *Artisornis moreaui* which has previously been shown to exhibit deep genetic divergences between isolated populations (Bowie et al. 2018).

Beyond species-level assessments, our DNA barcode phylogenies suggest apparent genus-level paraphyly within one chiropteran family and two avian families (Fig. 5). Although DNA barcoding typically does not provide insights into genus-level classifications (Rubinoff et al. 2006; Zink and Barrowclough 2008), high statistical support for unexpected species clustering may convey presently inaccurate taxonomy. These anomalous findings represent the minority of congeneric relationships inferred from DNA barcodes examined, many of which could not be resolved reliably from this genetic tool alone. This dearth of inferable congeneric relationships, however, may afford credibility to those with high statistical support – including those between species of supposedly different genera. Additionally, these paraphyletic genera have partial literature support, and belong to families which have either not been comprehensively assessed, or are in a state of taxonomic flux. We therefore intend the inter-species relationships depicted in Fig. 5 to prompt more in-depth taxonomic re-assessments. The chiropteran family Vespertilionidae, to which the three paraphyletic bat genera were localised (Fig. 5a and c), is well-known for taxonomic incongruencies between both species and genera (van Cakenberghe and Seamark 2022), with *Laephotis-Neoromicia* (Fig. 5a) boundaries remaining unascertained (Monadjem et al. 2021b). Despite possessing six non-monophyletic genera (Fig. 5d g), the passerine family Nectariniidae lacks phylogenetic assessment in Africa, although spermatological insights support genus non-monophyly (Omotoriogun et al. 2016). Finally, in the family Accipitridae, the *Haliaeetus-Accipiter* paraphyly (Fig. 5h) seems peculiar, though plausible according to incomplete assessments of both genera (Breman et al. 2013; Kunz et al. 2019; Schreiber and Weitzel 1995; Seibold and Helbiga 1996), whereas the apparent *Hieraaetus-Aquila* synonymity (Accipitridae, Fig. 5i) affirms the classification of (Lerner and Mindell 2005).

In addition to greater rates of intraspecific clade partitioning, Afrotropical bats also displayed deeper genetic divergences within species and among congeners compared to Afrotropical birds. Deeper intraspecific divergences detected in bats may partly reflect higher female philopatry in mammals compared to birds (Greenwood 1980), although such mitochondrial haplotype variation is not necessarily the product of spatial isolation (Teske et al. 2018). More generally, however, this discrepancy in divergence values supports slower mitochondrial gene evolution in birds compared to mammals, controlling for body size (Nabholz et al. 2009, 2013; Pentinsaari et al. 2016). While combined nuclear and mitochondrial genetic diversification across Chiroptera appears fairly uniform (Shi and Rabosky 2015), the mitochondrial genes of Yangochiroptera bats may be subjected to stronger selection (Meganathan et al. 2012), contributing to the deeper species divergences observed

within this sub-order. Avian mitochondrial evolution itself appears quite variable (Lavinia et al. 2016; Nabholz et al. 2009), evolving fastest in passerines. However, passerines display largely homogenous rate variation in COI evolution (Nguyen and Ho 2016). Although Afrotropical passerines here exhibited correspondingly deeper genetic divergences compared to non-passerines, in the Neotropics the opposite was apparent (Chaves et al. 2015). Despite these variations in COI evolution, DNA barcodes still capture most Afrotropical bat and bird species diversity. The few incongruencies observed arise from DNA barcode curation errors, low DNA barcode resolution among certain congeners, or potential undetected cryptic diversification.

The extent of deep divergences observed within native breeding Afrotropical bats and birds, though suppressed by incomplete species population representation, highlights the under-appreciated potential for much greater diversity among sedentary African taxa. For example, ground-based and arboreal Afrotropical mammals show similar or deeper intraspecific mitochondrial divergences when compared to both bats and birds (Gaubert et al. 2015; Huntley et al. 2019b). Ultimately, the reconciliation of genetic and morphometric and other phenotypic data remains necessary to define species more coherently as evolutionary species units, both for systematics and for conservation (Taylor et al. 2019; Wells et al. 2021; Zachos 2018).

Conclusion

Phylogenetic analysis of available DNA barcodes for Afrotropical bats and birds shows that this genetic tool better represents avian than chiropteran diversity. This is less likely to reflect the inherent properties of the DNA barcodes, but rather the more reliable phenotypic classification of bird species diversity compared to bats. Consequently, archived bird barcodes are better curated to reflect current systematics, which in turn affords these records greater credibility when used to identify and delineate species boundaries. Currently, DNA barcodes are available for significantly more native breeding Afrotropical bat species (40.3%) compared to bird species (23.6%), and more Afrotropical bats species (18.7%) have geographically dispersed barcodes than do birds (7.2%). Despite this incomplete coverage of native breeding Afrotropical species, 21 bat species (8.0%) and 15 bird species (0.8%) exhibited well-supported intraspecific clade partitioning (polyphyly, paraphyly, or reciprocally monophyletic clades), with numerous under-sampled species having genetic divergences exceeding predetermined species threshold values. Observed species non-monophyly and deep divergences corresponded with previously identified Afrotropical paleoclimatic refugia for birds and mammals, suggesting that future sampling of these areas will likely uncover more unknown diversity. Alternatively, this trend may simply reflect greater research focus within these refugia. The hidden diversity uncovered within these two volant taxa suggests greater unrecognised cryptic speciation among sedentary Afrotropical taxa. Perhaps the most significant contribution that DNA barcoding affords conservation is species identification *in lieu* of sufficient phenotypic data – as seems particularly relevant for bats. Concerning species discovery, DNA barcoding remains an effective preliminary tool requiring more robust phylogenetic substantiation, which in turn should complement morphological and ecological studies to investigate the conservation status of taxa. Furthermore, DNA barcoding can aid in resolving species-level phylogenetic diversity for guiding

biodiversity conservation strategies which emphasize preserving deep branching and unique lineages within species (Krishnamurthy and Francis 2012), as described here for both taxa. Together these could contribute significantly to conservation efforts to mitigate further loss of biodiversity in the face of multiple human threats to biodiversity in the Anthropocene.

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Data Availability The sequences generated from the current study were deposited in Barcode of Life Data (BOLD) under the 'Foundational Biodiversity Eastern Cape' project on BOLD (https://www.boldsystems.org/index.php/) under the accession numbers ECBAT001-19 to ECBAT089-19 and BATEC001-23 to BATEC007-23 for bats, and ECFPB001-21 to ECFPB197-21 for birds. Accession numbers of the publicly available DNA barcodes analysed in this study are provided in Appendix 1.

Declarations

Competing interests The authors declare no competing interests.

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