

Small islands and large biogeographic barriers have driven contrasting speciation patterns in Indo-Pacific sunbirds (Aves: Nectariniidae)

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Birds of the Indo-Pacific have provided biologists with many foundational insights. This study presents evidence for strong phylogeographic structure in two sunbird species from the heart of this region, the olive-backed sunbird, *Cinnyris jugularis*, and the black sunbird, *Leptocoma aspasia*. We assessed population divergence using morphological, plumage, bioacoustic and molecular data (mitochondrial *ND2/ND3*). Our findings indicate that the olive-backed sunbird should be recognized as multiple species, because birds from Sulawesi and the Sahul Shelf are closely related to each other, but widely separated from those in other regions. In addition, we provide evidence for an endemic species on the Wakatobi Islands, an archipelago of deep-sea islands off south-east Sulawesi. That a small bird could exhibit a range all the way from Sulawesi to Australia, while diverging on a small archipelago within this range, illustrates the complex interplay between dispersal and speciation. Our black sunbird genetic data also suggest unrecognized population structure, despite relatively weak plumage divergence. Black sunbirds in Sulawesi are likely to be a separate species from those in New Guinea, with a mean genetic distance of 9.1%. Current taxonomy suggests these sunbird species transcend classic biogeographic barriers, but our results suggest that these barriers are not easily bypassed.

ADDITIONAL KEYWORDS: birds – dispersal – evolution – island biogeography – Passeriformes – taxonomy – zoogeography.

INTRODUCTION

Wallacea (Fig. 1) is a central Indonesian region composed of islands separated by deep water, situated between the much shallower Sunda and Sahul continental shelves (Merrill, 1924; Dickerson *et al.*, 1928). Due to changes in sea level during glaciations (Voris, 2000), the boundaries between these contrasting water depths have acted as barriers to the dispersal of many organisms, leading to marked differences in animals found on either side (Lohman *et al.*, 2011). Wallacean islands played a vital role in the evolution of songbirds, providing avenues for dispersal and radiation after

the group originated in Australia (Moyle *et al.*, 2016). Wallacea's largest island, Sulawesi, has a complex geological history that has shaped its marked patterns of biological endemism (Michaux & Ung, 2021). The western boundary between Wallacea and the Sunda Shelf is known as Wallace's Line (Wallace, 1863; Huxley, 1868), although Wallace had difficulty deciding where to position his line relative to Sulawesi (Ali & Heaney, 2021) and considered this island 'anomalous' (Wallace, 1880). The eastern boundary between Wallacea and the Sahul Shelf was first described as a biogeographic barrier by Heilprin (1887), but is now best known as Lydekker's Line (Lydekker, 1896; Ali & Heaney, 2021). As a zone of transition between strikingly different biotas (Merrill, 1924; Dickerson *et al.*, 1928), Wallacea

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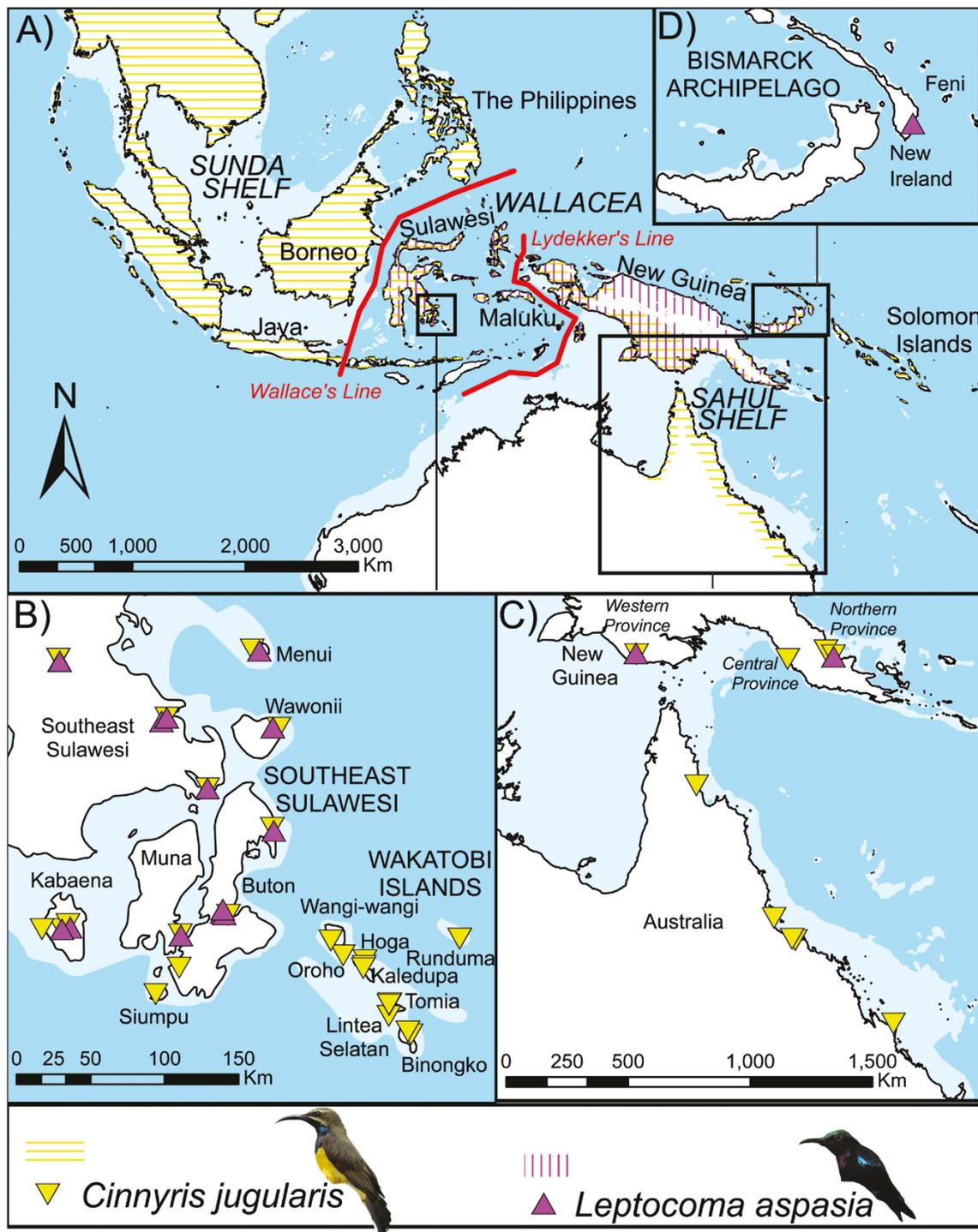


Figure 1. A, map of the Indo-Pacific region with study regions marked inside boxes. The range of the olive-backed sunbird is shaded horizontally in yellow, the range of the black sunbird vertically in purple, both according to BirdLife International. Seas deeper than 200 m are represented by a darker blue. Biogeographic barriers (Wallace, 1863; Lydekker, 1896) are represented with red lines. B, map of south-east Sulawesi and the Wakatobi Islands in Wallacea, with olive-backed sunbird sampling sites marked with yellow downward-pointing triangles, black sunbird sampling sites with purple upward-pointing triangles. C, map of Australia and New Guinea on the Sahul Shelf, with olive-backed sunbird sampling sites marked with yellow downward-pointing triangles, black sunbird sampling sites with purple upward-pointing triangles. D, map of the Bismarck Archipelago with the sampling site of the B10K black sunbird marked with a purple triangle.

has furnished the field of biogeography with many foundational insights (Wallace, 1860, 1863), and work in the region continues to improve our understanding of evolutionary theory in general as well as the evolutionary histories of many different organisms (Moyle *et al.*, 2016; Rowe *et al.*, 2019; Hardianto *et al.*, 2021; Purnomo *et al.*, 2021).

Wallacea is recognized as a hotspot of threatened biodiversity (Myers *et al.*, 2000). The importance of Wallacean biodiversity is becoming ever more apparent: the latest edition of the current reference work on the birds of the region (Eaton *et al.*, 2021) recognizes 27 additional endemic species compared to the first edition, published just over four years earlier. Eaton *et al.* (2021) sorted their taxonomic recommendations into two categories: splits and ‘limbo splits’, which are ‘possible splits that have either been mentioned in the literature but we feel that support is weak or insufficient, or they have generally not been mentioned in the previous literature and we feel that potential for splitting is considerable’ (Rheindt, 2021). The vast majority of new Wallacean taxa, including both splits and limbo splits, are restricted to particular islands (Eaton *et al.*, 2021) and are thus strictly allopatric. The consistent delimitation of allopatric taxa remains challenging, even when data are available (Tobias *et al.*, 2021). Therefore, specific and detailed investigation is still needed to clarify the diversity of birds across the many islands of Wallacea. One solution to the problem of allopatry (e.g. Cheke *et al.*, 2001; Mayr & Diamond, 2001) is to deal with ‘superspecies’, defined as monophyletic groups of allopatric populations that are inferred to be reproductively isolated, based on comparison with sympatric species (Amadon, 1966).

The islands of Wallacea are diverse in size and degree of isolation, making this region an ideal ‘natural laboratory’ (Whittaker *et al.*, 2017) for the study of biogeographic questions (e.g. Ó Marcaigh *et al.*, 2021a, b, 2022). For instance, in the south-east Sulawesi region there are continental land-bridge islands like Wawonii (or Wowoni), Kabaena, Muna and Buton (or Butung), which were connected by land to the much larger Sulawesi and to each other during geologically recent glaciations (Hall, 2013). On the other hand, the smaller Wakatobi Islands (also known as the Tukangbesi Islands) have not been attached to any larger landmasses since they first emerged (Nugraha & Hall, 2018). The Wakatobi Islands are recognized as an Important Bird Area (BirdLife International, 2021), but despite their importance they received little ornithological attention until recently (O’Connell *et al.*, 2020). Although the Wakatobi Islands are just 27 km from Buton, they harbour several endemic species (Kelly *et al.*, 2014; O’Connell *et al.*, 2019c), evidence of significant evolutionary independence from Sulawesi

and its land-bridge islands. An additional small island, Menui (or Manui), sits to the north of Wawonii. The channel between Menui and Sulawesi is particularly complex geologically, but does not appear to have formed a land bridge during Pleistocene glaciations (Nugraha & Hall, 2018).

The sunbirds (Nectariniidae) are a family of small passerines with a distribution extending from Africa in the west to Australia in the east. In a region where birds have provided the basis for much crucial evolutionary work, sunbirds have often drawn special attention (e.g. Jardine, 1843; Wallace, 1855; Shelley, 1876–1880). Many exhibit strikingly colourful plumages, which have informed taxonomists of their diversity (Cheke *et al.*, 2001). Indeed, the sunbirds as a group ‘deriv[e] their appellation from their brightly-tinted dress, appearing in higher splendour when played on by the sun-beams’ (Jardine, 1843). Much remains to be clarified in relation to their evolution as species continue to be divided on the basis of new sources of information like genetics and bioacoustics (Rheindt, 2021). Our understanding of biodiversity continues to develop as we continue to document and identify species-level lineages (Fišer *et al.*, 2018).

One challenge to sunbird taxonomy is that many species are widespread and varied in their plumage. The olive-backed sunbird, *Cinnyris jugularis* (Linnaeus, 1766), is one such member of the family. Under most taxonomic treatments [including Gill *et al.* (2022), followed here], this species ranges from China to north-east Australia, crossing both Wallace’s and Lydekker’s lines. Gill *et al.* (2022) recognize 21 subspecies across this range. However, it has been suggested that there may be more structure to these populations than is currently appreciated, and the olive-backed sunbird may warrant treatment as a superspecies. Some recent work (Eaton & Rheindt, 2017; Berryman & Eaton, 2020a, b; O’Connell *et al.*, 2020; Eaton *et al.*, 2021) has introduced the name ‘Sahul sunbird *Cinnyris clementiae*’ (elevated from the subspecies, *Cinnyris jugularis clementiae* Lesson, 1827) for birds occurring from Sulawesi to the Sahul Shelf. This new species-level taxon would include, among others, the subspecies *plateni* (Blasius, 1885) from Sulawesi and its land-bridge islands, the subspecies *frenatus* (Müller, 1843) from Australia and New Guinea and the subspecies *flavigastra* (Gould, 1843) from the Solomon Islands. This is part of a proposed three-way split based on differences in vocalization and mitochondrial DNA (mtDNA) (Eaton *et al.*, 2021), whereby birds on the Sunda Shelf are also elevated to a full species, ‘ornate sunbird *Cinnyris ornatus*’ (Lesson, 1827) and *Cinnyris jugularis* is retained for the Philippine population, which includes *obscurior* (Ogilvie-Grant, 1894) and *aurora* (Tweeddale, 1878) among its subspecies. Lohman *et al.* (2010) had previously

proposed that the Philippine population represented a distinct species based on mtDNA, also suggesting the same might be true for Sulawesi populations. [Eaton *et al.* \(2021\)](#) abolish the common name ‘olive-backed sunbird’, referring to the proposed Philippine species as the ‘garden sunbird’. Under the [Eaton *et al.* \(2021\)](#) treatment, Wallace’s Line acts as a boundary between *Cinnyris* Cuvier, 1816 sunbird species, but Lydekker’s Line does not. However, sampling is incomplete and [Eaton *et al.* \(2021\)](#) highlight that more work is needed to further clarify the taxonomy. Our work aims to contribute to this clarification.

The black sunbird, *Leptocoma aspasia* [Lesson & Garnot, 1828](#) ([Dickinson *et al.*, 2015](#)), overlaps with the olive-backed sunbird in Sulawesi and New Guinea, but it does not occur on the Sunda Shelf and is missing from Australia and the Wakatobi Islands ([Fig. 1](#)). Thus, the distribution of the black sunbird as currently recognized ([BirdLife International, 2022](#)) crosses Lydekker’s Line but is bounded by Wallace’s Line. Although there are 21 recognized subspecies of black sunbird ([Gill *et al.*, 2022](#)), including *porphyrolaema* ([Wallace, 1865](#)) on Sulawesi and its land-bridge islands and the nominate *aspasia* on New Guinea, [Eaton *et al.* \(2021\)](#) do not propose to elevate any of these as species-level splits or ‘limbo splits’.

The divergence of birds on islands is driven by traits of both the islands and the birds. Geographic context may present barriers, which will prevent gene flow in some species but not others ([Smith *et al.*, 2014](#)). Despite the use of the word ‘lines’, biogeographic barriers like Wallace’s Line have always been seen as at least partially permeable ([Huxley, 1868](#)), and the division of biotas by such barriers has been shown to be driven by differences in dispersal ability ([White *et al.*, 2021](#)). Sunbirds might be expected to be subject to more isolation (and thus evolutionary divergence) compared to other birds: despite their success as island colonists, [Wallace \(1856\)](#) noted that the wings of sunbirds are not suited to long-distance dispersal, being ‘short and round, quite incapable of any powerful flight’. The length and pointedness of the wing of a bird is an effective index of its dispersal ability, with important ecological consequences ([Sheard *et al.*, 2020](#)). Certain birds, known as ‘great speciators’, are adept at colonizing islands across relatively short distances, but still diverge readily across their range ([Diamond *et al.*, 1976](#)). Some sunbirds have displayed a propensity to diverge on the smallest of scales, indeed Philippine sunbirds of the genus *Aethopyga* [Cabanis, 1851](#) have diverged both between and within islands ([Hosner *et al.*, 2013](#)). On a larger scale, Wallace’s and Lydekker’s lines have been shown to play a role in the distribution of nectarivorous birds ([Carstensen & Olesen, 2009](#)).

The black and olive-backed sunbirds of Wallacea make for an interesting biogeographic comparison, as they are two of the only representatives of their family in this region. The sunbirds are believed to have originated in Asia ([del Hoyo *et al.*, 1992–2013](#)), with only six species occurring in Wallacea ([Carstensen & Olesen, 2009](#)). The black and olive-backed sunbirds are currently placed in different genera, but have been congeneric in the past, and most sunbird genera are thought to be closely related and ‘not well-marked’ in their differences ([Cheke *et al.*, 2001](#)). The two species share some ecological similarities, including their diet of nectar, fruit and insects ([Cheke *et al.*, 2001](#)), but they contrast in ways that are relevant to their dispersal. For example, the olive-backed sunbird occurs more commonly in mangroves than does the black sunbird ([Eaton *et al.*, 2021](#)). Mangrove species are often good island colonizers, either because they are susceptible to being dispersed by the elements or because these marginal habitats are easier to invade ([Wilson, 1959](#)).

As well as their position between Wallace’s and Lydekker’s lines, ‘anomalous’ Sulawesi and the Wakatobi Islands are of considerable biogeographic interest themselves. To date, a lack of genetic data has prevented full assessment of these sunbird populations. Prior to this study, no black sunbirds and only three olive-backed sunbirds from Sulawesi had been subjected to genetic analysis ([Lohman *et al.*, 2010](#)). Furthermore, no olive-backed sunbird sequences from the Wakatobi Islands have been published before this study. [Hartert \(1903\)](#) named the Wakatobi sunbird as an endemic species, *Cinnyris infrenata* [Hartert, 1903](#), noting that it lacked the yellow facial stripes of *Cinnyris jugularis* and had darker plumage on its ‘upper surface’, particularly the head. This taxon has since been treated as a subspecies of the olive-backed sunbird ([Hartert, 1920](#); [Gill *et al.*, 2022](#)), and its distribution was misrepresented in the literature until recently ([Kelly & Marples, 2011](#)). Two other Wakatobi endemic species named by [Hartert \(1903\)](#), which were later combined with other species, have recently been recognized as full species once more ([Kelly *et al.*, 2014](#); [O’Connell *et al.*, 2019c](#)). Previous work has noted the morphological and ecological distinctiveness of Wakatobi *infrenatus* sunbirds, as [O’Connell *et al.* \(2019a\)](#) found they exhibited increased sexual dimorphism and higher population density compared to *plateni* birds from Sulawesi and the land-bridge islands. [O’Reilly *et al.* \(2018\)](#) tested their automatic Bird Vocalisation Difference method on the olive-backed sunbird and found diagnosable bioacoustic differences between Wakatobi and Sulawesi populations. Unpublished genetic work based on the *COI* and *ND3* genes

(Kelly, 2014) identified the Wakatobi sunbird as an ‘incipient species’, which does not share haplotypes with the Sulawesi population, evidence that long-term gene flow has been limited by the barrier surrounding the Wakatobi Islands. However, Kelly (2014) did not find sufficient evidence to advise a species-level split. Eaton *et al.* (2021) highlighted the Wakatobi sunbird as a ‘limbo split’, recommending more acoustic and genetic work on them, which this study aimed to provide. Menui’s populations of black and olive-backed sunbirds, on the other hand, were unrecorded in the literature until recently (Monkhouse *et al.*, 2018) and so have not been officially assigned to any subspecies. Small, isolated islands like Menui and the Wakatobi hold a disproportionate share of Earth’s biodiversity (Cowie & Holland, 2006), so a full understanding of evolution requires sampling of these populations.

On a wide geographic scale, we sought to investigate the effect of Wallace’s and Lydekker’s lines on sunbirds by studying their population structure across Wallacea and the continental shelves. We hypothesized that this structure would follow one of three general patterns, offering varying levels of support for different taxonomic treatments:

1. A low level of genetic divergence across Wallacea and both continental shelves in the olive-backed sunbird, and across Wallacea and the Sahul Shelf in the black sunbird, in keeping with the Gill *et al.* (2022) taxonomy. This would suggest that the biogeographic barriers have had only a minor effect on sunbirds, as the olive-backed sunbird is subject to neither barrier while the black sunbird is subject to only one, Wallace’s Line.
2. Species-level divergence within the olive-backed sunbird, with the ‘Sahul sunbird’ of Wallacea and the Sahul Shelf strongly separated from Sunda Shelf populations, but no strong divergence in the black sunbird, supporting the Eaton *et al.* (2021) taxonomy. This would indicate that biogeographic barriers have the same moderate impact on both black and olive-backed sunbirds, both species being subject to Wallace’s Line while transcending Lydekker’s Line.
3. Additional diversity and population structure beyond what is indicated by either Eaton *et al.* (2021) or Gill *et al.* (2022). If either the black or olive-backed sunbird exhibits genetic divergence between Wallacea and the Sahul Shelf, for instance, this would indicate a major effect of biogeographic barriers, as both Wallace’s and Lydekker’s lines have the potential to halt gene flow in sunbirds. If a barrier affects one species but not the other, this raises further questions about how they have evolved and dispersed through the region.

MATERIAL AND METHODS

SAMPLING AND DNA SEQUENCING

We carried out mist-netting to sample birds on expeditions between 1999 and 2017 across south-east Sulawesi, the land-bridge islands off its coast, Menui and the Wakatobi Islands (Fig. 1). We followed the methodologies laid out in Redfern & Clark (2001) to measure the following morphological data: wing length (maximum chord), bill length (tip of bill to the base), body mass (in grams) and tarsus length (minimum). We also photographed birds (Supporting Information, Figs S1–S4) and wrote plumage descriptions in the field. We collected small numbers of contour feathers from the flank of each bird from which to extract DNA. The use of feather samples minimized the risk of injury and avoided disrupting their flight abilities and plumage-based visual signals (McDonald & Griffith, 2011). Additional tissue subsamples were provided from the Australian National Wildlife Collection (ANWC). Feather samples were stored in sealed paper envelopes until DNA was extracted from them. The ANWC tissue subsamples were processed immediately upon receipt. All extracted genetic samples were stored at -20° C. In total we produced DNA sequences from 84 olive-backed sunbirds: 26 of the *plateni* (Sulawesi) subspecies, 33 of the *infrenatus* subspecies (Wakatobi), five from Menui and 20 of the *frenatus* subspecies (15 from Queensland in Australia, five from New Guinea). We also produced sequences from 33 black sunbirds, including 21 from Sulawesi and its land-bridge islands (the *porphyrolaema* subspecies), five from Menui and seven from New Guinea (the nominate *aspasia* subspecies). The New Guinea birds came from three provinces of the nation of Papua New Guinea (PNG): the Western Province on the southern coast of the island and the Central and Northern provinces, both located in the south-east of the island. A complete list of samples used in our analyses is available in the Supporting Information (Supporting Information, Table S1), and all sampling sites are shown in Figure 1.

We used Qiagen DNeasy Blood and Tissue Kits to produce our genetic samples by extracting DNA from feathers and tissue. When working with feather samples we added 5 μ L of 1 M dithiothreitol (DTT) at the digest phase to release the genetic material shielded by keratin in the feather calamus. Otherwise, we followed the manufacturer’s instructions. Our genetic analyses were based on the mitochondrial genes NADH dehydrogenase subunit 2 (*ND2*) and NADH dehydrogenase subunit 3 (*ND3*). Evolutionary histories inferred from mtDNA and nuclear DNA sometimes differ (Rubinoff & Holland, 2005; Phillimore *et al.*, 2008); however, *ND2* has shown a particularly high level of agreement with nuclear markers (Campillo

et al., 2019). Although introgression and male-biased dispersal can obfuscate the patterns explored (Toews & Brelsford, 2012), *ND2* and *ND3* have been found to provide better resolution in younger radiations compared to single nuclear genes (Andersen *et al.*, 2015), and the sensitivity of mtDNA to population structure is useful in biogeographic studies (Linck *et al.*, 2016). As we aimed to study relatively recent divergences, we deemed *ND2* and *ND3* appropriate for this investigation. We targeted these two genes using touchdown Polymerase Chain Reactions (PCRs). This technique involved beginning the reaction with an annealing temperature 10° C above the melting temperature of the primers before reducing it gradually, to provide an increased yield compared to standard PCR protocols (Korbie & Mattick, 2008). The reagents and quantities we used in our PCRs are shown in the Supporting Information (Fig. S5). We amplified the *ND3* gene using the L10755-F and DOC-ND3-R1 primer pair (Chesser, 1999; O'Connell *et al.*, 2019b) and the *ND2* gene using internal and external primers of our own design, in order to target the gene in two halves (Supporting Information, Fig. S6; Table S2). We screened our PCR products on 2% electrophoresis gels stained with GelRed (Biotium) at a 1 × concentration, then had them Sanger sequenced at the GATC Eurofins Laboratory in Cologne, Germany. We used the ClustalW function in BIOEDIT v.7.2.5 (Hall, 1999) to align the resulting sequences and then concatenated *ND3* to *ND2* in MESQUITE v.3.51 (Maddison & Maddison, 2018). This gave a total of 1392 base pairs of DNA from each bird.

In order to compare genetic distances between our samples and those of other sunbird populations, we downloaded previously published sunbird sequences from GenBank (Supporting Information, Table S1). We obtained four full 1392 bp concatenated *ND2–ND3* sequences from our focal species and close relatives: *Cinnyris chalybeus* (Linnaeus, 1766) from Bowie *et al.* (2016), *Antheptes malacensis* (Scopoli, 1786) from Hosner *et al.* (2013), *Leptocomasperata* (Linnaeus, 1766) from Moyle *et al.* (2011) and a black sunbird sequenced by the B10K Project Consortium (GenBank accession number NC_051024). This bird (USNM-608697) was collected on New Ireland in the Bismarck Archipelago, and is listed as the *corinna* subspecies in the B10K Project Database (B10K Project Consortium, 2022). In addition, we downloaded the 20 olive-backed sunbird *ND2* sequences that were available. These came from the Sunda Shelf (*ornatus*), the Philippines (*obscurior*, *jugularis* and *aurora*) and the Solomon Islands (*flavigastra*), allowing us to examine genetic diversity across this range and assess which of our three hypotheses would be supported. These *ND2* sequences (from Smith & Filardi, 2007; Nyári *et al.*,

2009; Campbell, 2013; and Boyce *et al.*, 2019) could not be incorporated into the main, concatenated phylogeny as there was either no corresponding information on *ND3* or the *ND2* sequences were incomplete (see Supporting Information, Table S3 for details). Even so, these *ND2* sequences allowed us to use a 697 bp section of the gene to give an overview of the genetic patterns across a large part of the range of the olive-backed sunbird.

GENETIC AND PHYLOGENETIC ANALYSES

To create a general picture of population structure in black and olive-backed sunbirds, we calculated the pairwise p-distances between *ND2* haplotypes, as well as the average genetic distance between populations, using MEGA X v.10.1.8 (Kumar *et al.*, 2018) (Supporting Information, Tables S4, S5). A p-distance or 'proportion distance' represents the proportion of varying to matching nucleotide sites across two sequences. These distance measures used the *ND2* gene alone, to make them more widely comparable. We investigated the phylogenetics of sunbirds using both maximum likelihood (ML) and Bayesian approaches. We applied these methods, separately, to our concatenated 1392 bp *ND2–ND3* haplotypes and to the shorter 697 bp alignment that included birds from a larger part of the olive-backed sunbird range. Phylogenies included one representative of each concatenated *ND2/ND3* haplotype. We used the ModelFinder function (Kalyaanamoorthy *et al.*, 2017) in IQTREE v.1.6.12 (Nguyen *et al.*, 2015) to select the most appropriate nucleotide substitution model for our alignment. Choosing an appropriate nucleotide substitution model, which defines the probability of a given nucleotide base in the gene changing to another, is important in calculating the likelihood of a phylogenetic tree (Sullivan & Joyce, 2005).

We carried out separate ML analyses on our concatenated *ND2/ND3* sequences and the shorter olive-backed sunbird sequences in IQTREE using the GTR+F+I+G4 model. The confidence interval of the ML phylogeny was based on 1000 bootstraps (Felsenstein, 1985). We used MRBAYES v.3.2.7 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) to carry out Bayesian phylogenetic inference on our concatenated *ND2–ND3* sequences, a technique to construct phylogenies using the Markov chain Monte Carlo (MCMC) method (Mau *et al.*, 1999). We selected a GTR+G+I model, the closest equivalent to our ML model, with two MCMC runs and four chains per run, sampling every 1000 generations. As the MCMC algorithm converges on the data over time, with the time it spends on a given tree equivalent to the Bayesian posterior probability,

that is, the probability that said tree is correct (Huelsenbeck *et al.*, 2001), we discarded the first 25% of each MCMC run as ‘burn-in’ (Mau *et al.*, 1999). It is also important to confirm that the MCMC algorithm has ‘converged’ on a good approximation of the data and not just a local maximum (Besag & Green, 1993). We assessed convergence by reading the output parameters of the Bayesian model in TRACER v.1.7.1 (Rambaut *et al.*, 2018). Following O’Connell *et al.* (2019c), we deemed it acceptable once Average Standard Deviation in Split Frequencies (ASDSF) reached 0.01 and Effective Sample Size (ESS) of model parameters reached 200. Both requirements had been surpassed by the time our MCMC algorithm had iterated 4 million generations. We exported a 50% majority rule consensus tree from MRBAYES and imported both this and the ML tree into the R package ‘ggtree’ (Yu *et al.*, 2016), which we used to combine both into a single tree. The Supporting Information includes full, separate Bayesian (Fig. S7) and ML (Fig. S8) trees, with all outgroup and ingroup haplotypes represented.

We created Templeton–Crandall–Sing (TCS) haplotype networks using POPART v.1.7 (Leigh & Bryant, 2015) to visualize the genetic structure of our sunbird populations. Our haplotype networks were based on 1392 bp concatenated *ND2–ND3* sequences. The TCS method uses an agglomerative algorithm, working progressively to combine clusters sharing one or more connecting edge (Templeton *et al.*, 1992). This makes it ideal for the analysis of divergence at the population level, as ancestral haplotypes are likely to be common in the population and variation tends to be lower compared to analyses at higher taxonomic levels (Clement *et al.*, 2000).

MOLECULAR SPECIES DELIMITATION

We used Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.*, 2012) to perform distance-based molecular species delimitation on our sunbird sequences. This method divides sequences into putative ‘species’ with smaller genetic distances within than between them. ABGD calculates a minimum threshold interspecific distance, the ‘barcode gap’, which can be calibrated based on the expected prior intraspecific divergence of the gene or taxon. We ran our ABGD analysis on the web-server (<https://bioinfo.mnhn.fr/abi/public/abgd/>) using the default Kimura-2-Parameter (K2P) model and settings (Pmin = 0.001, Pmax = 0.1, Steps = 10, relative gap width = 1.5, Number of bins = 20). Our main ABGD analysis included all our focal *Cinnyris jugularis* and *Leptocoma aspasia* samples, as well as the congeneric *Cinnyris chalybeus* and *Leptocoma sperata* sequences downloaded from GenBank. As ABGD is a distance-based method, we restricted it to a single gene, *ND2*. We also ran a second

ABGD analysis using the partial olive-backed sunbird *ND2* sequences from GenBank, in a 697 bp alignment with our focal olive-backed sunbird sequences, to assess population structure across this wider range and see if it would support species status for the Philippine and Sunda Shelf populations (Eaton *et al.*, 2021).

BIOACOUSTIC AND MORPHOLOGICAL ANALYSES

Bioacoustics are an increasingly important aspect of species delimitation, and this approach has previously been recommended to help clarify the status of the Wakatobi olive-backed sunbird population (Eaton *et al.*, 2021). We recorded 68 olive-backed sunbirds in the Sulawesi and Wakatobi islands using a Zoom H2 Handy Recorder, Tokyo, Japan with a Sennheiser Me62 external microphone and a Telinga V2 parabolic reflector, Uppsala, Sweden. The analysis was carried out on calls rather than the more complex mating songs, as we had more recordings of these. Songs are commonly used in assessing pre-mating reproductive isolation, but a difference in calls might be stronger evidence of speciation, as calls tend to change more slowly (Marler, 2004). However, changes in calls are sometimes driven by changes in body mass (Potvin, 2013). We used RAVEN PRO v.1.6 (Center for Conservation Bioacoustics, 2019) to prepare spectrograms from these calls and extracted data on standard acoustic traits (Tobias *et al.*, 2010). We used both temporal features: namely duration, number of notes and pace (number of notes divided by duration); and spectral features: specifically maximum frequency, minimum frequency, bandwidth (maximum frequency minus minimum frequency) and peak frequency (frequency with highest amplitude). To account for the variation in vocalizations within individual birds, individual means were calculated for the calls in each recording. The mean data for each recording were then used as sample points. We analysed these acoustic data using R v.4.1.1 (R Core Team, 2021).

Only adult birds processed by the same author were included in morphological analyses, and males and females were treated separately due to sexual dimorphism. This provided a morphological sample size of 147 male olive-backed sunbirds (41 from Sulawesi and its land-bridge islands, 106 from the Wakatobi Islands) and 95 female olive-backed sunbirds (25 from Sulawesi and its land-bridge islands, 69 from the Wakatobi Islands, one from Menui). The one olive-backed sunbird from Menui was grouped with the Sulawesi birds based on genetic results. Our morphological analysis on the black sunbird used female birds because the sample was larger (27 from Sulawesi and its land-bridge islands, seven from Menui). We used Multivariate Analysis of Variance (MANOVA) on our morphological and bioacoustic data to investigate

whether these would support the clades suggested by our genetic work. We used histograms and Pearson's r to check that the assumptions of MANOVA were not violated, that is, that the residuals were normal and that the dependent variables were linearly related without being overly strongly correlated ($0.1 < r < 0.8$). We generated boxplots (Supporting Information, Figs S9–S11) to visualize morphological and acoustic differences between our populations of interest.

We also subjected our bioacoustic and morphological data to a points-based integrative species delimitation system (Tobias *et al.*, 2010). This system has been widely used by organizations such as BirdLife International, the global authority for birds for the IUCN Red List, and reference works such as the *Handbook of the Birds of the World* (del Hoyo *et al.*, 1992–2013). However, it has been criticized by some authors (Rheinhardt & Ng, 2021) and is best combined with multiple lines of evidence, including genetic data (Tobias *et al.*, 2021). The system allows a difference between populations to be scored on morphology, acoustics, plumage and some other sources of information that were not applicable to our study. A score of seven (or more) points indicates a species-level difference between the populations being compared. The Tobias *et al.* (2010) system uses effect sizes (Cohen's d) to build 'score' values. Effect sizes are considered to provide a more objective measure than P -values for statistical assessments in biology (Nakagawa & Cuthill, 2007), but we also ran Welch's two-sample t -tests to support our effect size calculations (Supporting Information, Figs S9–S11). We used the R package 'effsize' (Torchiano, 2020) to calculate Cohen's d for the morphological and acoustic differences between the Sulawesi (including the land-bridge islands) and Wakatobi populations of the olive-backed sunbird, and scored the plumage differences using our photographs and field descriptions. We used male plumage for the taxonomic scoring as this forms the basis of the current taxonomy of subspecies in the olive-backed sunbird. Our field descriptions covered ten males from Sulawesi and the land-bridge islands and 31 males from the Wakatobi Islands. We also examined photographs of each of these birds.

Finally, we estimated relative dispersal ability of focal olive-backed sunbirds by calculating a dispersal index following O'Connell *et al.* (2019c) and Ó Marcaigh *et al.* (2022), using the wing length and weight data. We ran a Welch's two-sample t -test to see if sunbirds on the Wakatobi Islands differed in dispersiveness from their relatives on the land-bridge islands, as loss of dispersiveness has been described in other birds on similarly isolated islands (Pedersen *et al.*, 2018; Le Pepke *et al.*, 2019). More information on this index is provided in the Supporting Information (Appendix S1), and it is plotted in Supporting Information, Figure S12.

RESULTS

GENETIC AND PHYLOGENETIC RESULTS

Our ModelFinder analysis found strong support for a General Time Reversible (GTR) model with a gamma shape parameter and a proportion of invariable sites (GTR+F+I+G4), indicating that mutations in our selected genes can occur but subsequently reverse as time passes (Lanave *et al.*, 1984; Tavaré, 1986). Biological constraints prevent change in certain parts of the gene, termed invariant sites (Waddell & Steel, 1997). Both Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) supported this model choice (Johnson & Omland, 2004).

Our haplotype networks and phylogenetic trees revealed substantial geographic population structure in both the olive-backed and black sunbirds. In the olive-backed sunbird (Figs 2, 3), the *plateni* birds from Sulawesi and its land-bridge islands were seen to be closely related to the *frenatus* birds from Australia and PNG. The *infrenatus* birds from the Wakatobi Islands formed their own cluster. In the black sunbird network and tree (Figs 2, 4), the black sunbirds from PNG formed a clade, while a second clade included the birds from Sulawesi, Menui and the land-bridge islands. The single *corinna* bird from New Ireland formed a sister clade to the PNG birds. Our tree and network showed separate Western Province (LA_P01 and P02) and Northern Province (LA_P03–06) clusters in New Guinea in the black sunbird. The black sunbirds from Menui were separated from the rest of the Sulawesi cluster by a similar number of mutations as the populations in mainland PNG were separated from each other.

ABGD found strong support for species-level splits within both black and olive-backed sunbirds. The most favoured partitioning of the full *ND2* sequences, compatible with the widest range of prior intraspecific divergences, produced seven groups with a barcode gap of 2.2%. The first group, CJ_S, contained all olive-backed sunbirds from Sulawesi, its land-bridge islands, Menui, PNG and Australia. The second, CJ_W, contained all olive-backed sunbirds from the Wakatobi Islands. The *Cinnyris chalybeus* outgroup sequence was grouped by itself. The fourth group, LA_S, contained all black sunbirds from Sulawesi, its land-bridge islands and Menui. The fifth group, LA_P, contained all black sunbirds from the mainland provinces of PNG. The single black sunbird sequence from the Bismarck Archipelago was grouped by itself as LA_B. The seventh 'group' contained the outgroup sequence of *Leptocoma sperata*.

We found a mean genetic distance of 9.1% between the LA_S group of black sunbirds and the LA_P group. Within these populations, there was a mean genetic distance of 1.4% between Menui and Sulawesi

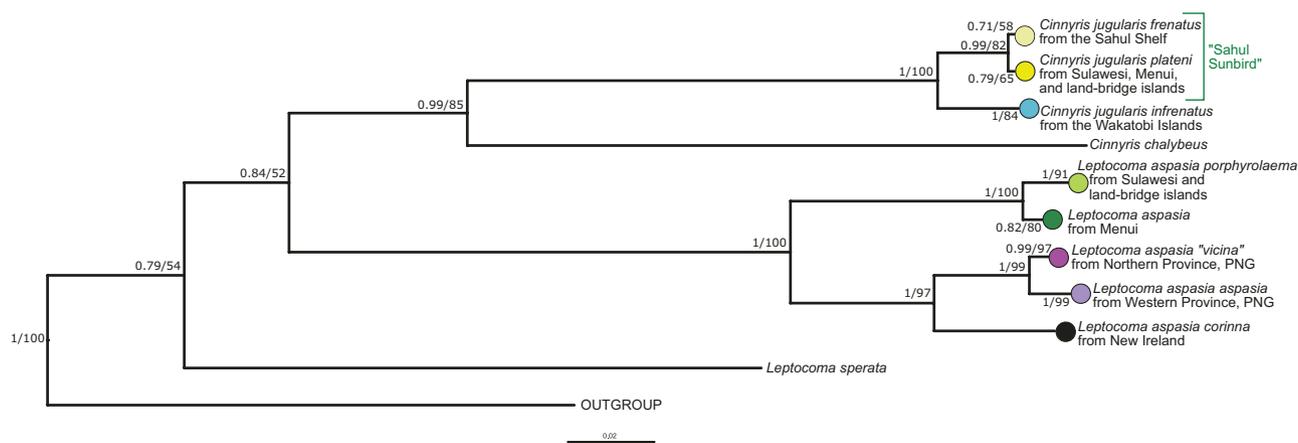


Figure 2. Simplified version of a combined maximum likelihood (ML) and Bayesian phylogenetic tree of *Cinnyrus* and *Leptocoma* species sampled in Wallacea and the Sahul Shelf. In this figure, the outgroup is omitted and each major clade in the data is collapsed into a single branch. Tips representing focal populations are marked with coloured circles. Nodes are labelled with Bayesian probability/ ML bootstraps. Full versions of the ML and Bayesian trees, including all outgroup taxa, are provided in the Supporting Information (Figs S7, S8).

(including the land-bridge islands) and a mean distance of 1.4% between the Northern and Western provinces of PNG. The bird from the Bismarck Islands (LA_B) had a mean genetic distance of 9.2% from the LA_S birds and 4.9% from the LA_P birds. Mean intrapopulation genetic distance was equal to 0.1% in the Menui population, 0.2% in Sulawesi and the land-bridge islands, 0.1% in the Northern Province of PNG and 0.3% in the Western Province of PNG.

Within Wallacea, the mean genetic distance (p-distance) between olive-backed sunbirds caught on the Wakatobi Islands and those from Sulawesi, Menui and the land-bridge islands was 3.3%. The mean genetic distance between the Wakatobi and Sahul Shelf populations was 3.1%. Meanwhile, the mean distance between the Sulawesi and Sahul Shelf populations was just 0.6%, leading us to group these together as the 'Sahul sunbird' in further analyses. The mean intra-group genetic distances were 0.2% for the Wakatobi, 0.4% for Sulawesi and the land-bridge islands, and 0.2% for the Sahul Shelf.

Our analyses using a portion of the *ND2* gene to assess a wider part of the olive-backed sunbird range also found strong evidence of species-level splits (Fig. 5). Using 697 bp of *ND2*, ABGD found a strong consensus for five species in the olive-backed sunbird superspecies, with a barcode gap of 2%. The first group contained all of the 'Sahul sunbird' (Eaton *et al.*, 2021) sequences, including our *plateni* samples from Sulawesi, its land-bridge islands and Menui, our *frenatus* samples from PNG and Australia and the partial sequences of *flavigastra* published by Smith & Filardi (2007) from the Solomon Islands. The second group ('garden sunbird') contained all sequences of the *jugularis* and *obscurior* subspecies from the Philippines. The two

birds of the *aurora* subspecies from Busuanga in the western Philippines formed a third group. The single *ornatus* sequence from Borneo was grouped by itself ('ornate sunbird'). All of our *infrenatus* sequences from the Wakatobi Islands made up the fifth and last group ('Wakatobi sunbird'). Each of the 'species' assigned by ABGD based on 697 bp of *ND2* formed its own well-supported clade in the partial *ND2* Bayesian and ML trees (Fig. 5C). In this 697 bp portion, there was a mean genetic distance of 4.5% between the *aurora* subspecies and the 'garden sunbird'. The 'garden sunbird' exhibited a mean genetic distance of 6.4% from the 'Wakatobi sunbird', a distance of 7.6% from the 'ornate sunbird' sequence and a 7.2% mean distance from the 'Sahul sunbird'. The *aurora* subspecies had a mean genetic distance of 6.3% from the 'Wakatobi sunbird' and a 6.1% mean distance from the 'Sahul sunbird'. The 'ornate sunbird' sequence exhibited a 7.7% mean genetic distance from the *aurora* sequences, a distance of 5.7% from the 'Wakatobi sunbird' and a 5.7% distance from the 'Sahul sunbird'.

RESULTS OF BIOACOUSTIC AND MORPHOLOGICAL ANALYSIS

We carried out an integrative taxonomic analysis (Tobias *et al.*, 2010) of the 'Wakatobi sunbird' by comparing the plumage, morphology and acoustics of this population to those of the neighbouring 'Sahul sunbird' populations of Sulawesi, its land-bridge islands and Menui. Eaton *et al.* (2021) highlight two plumage differences between the males of the Wakatobi Islands and the 'Sahul sunbird': they describe the back of Wakatobi birds as 'brown' rather than olive and they note that their face is plain where the 'Sahul sunbird'

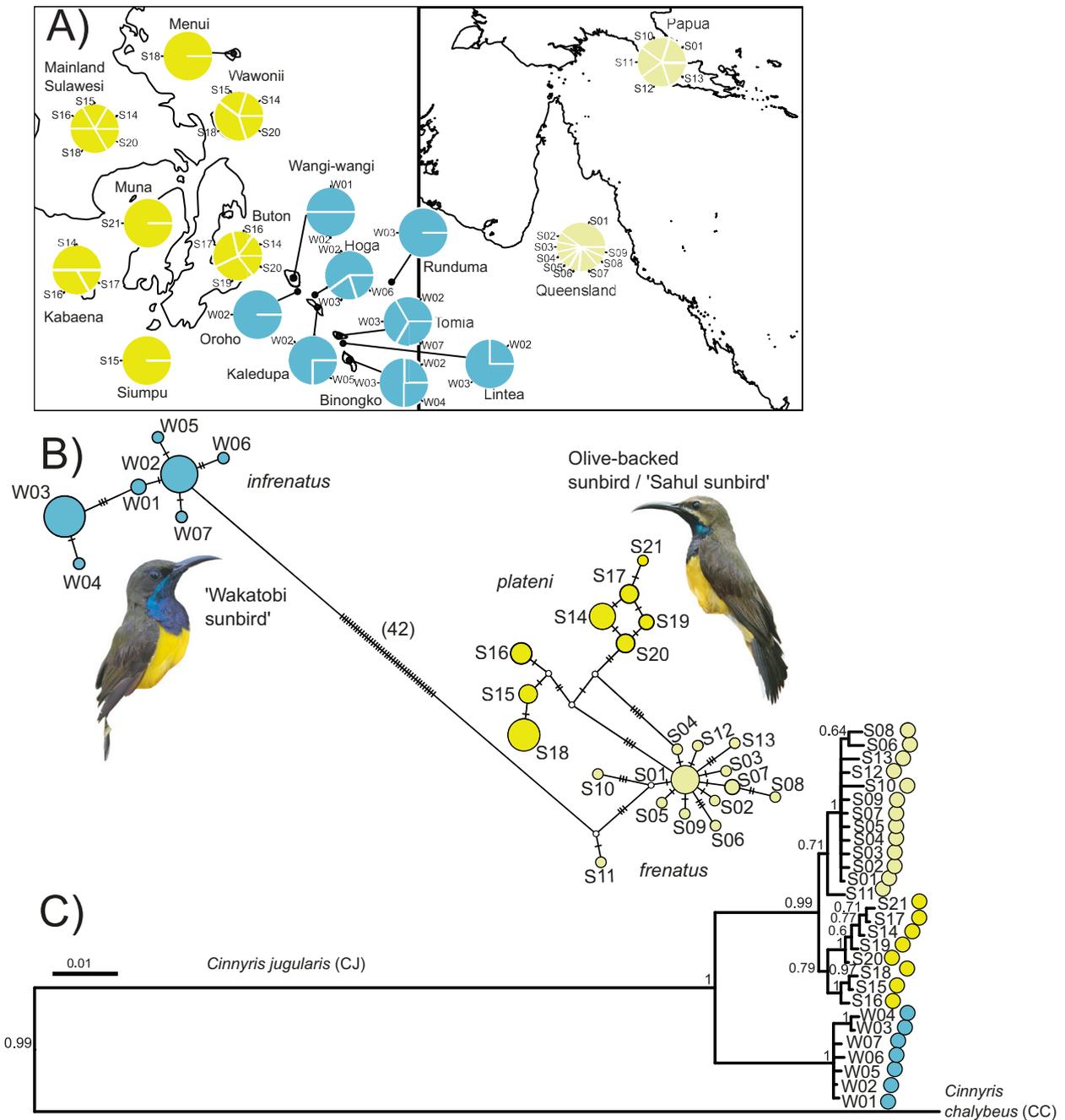


Figure 3. A, geographic distribution of *Cinnyrus jugularis* (sensu Gill *et al.*, 2022) haplotypes in Wallacea and the Sahul Shelf. Each circle represents an island and the fractions within the circle the haplotypes found on that island, proportioned to represent the frequency of each haplotype. The haplotypes are named according to the species-level divisions suggested by ABGD and coloured to represent the clades supported by our phylogenetic analyses. B, TCS haplotype network of *Cinnyrus* haplotypes. Each circle represents a unique *ND2-ND3* haplotype, sized to represent how many birds carried that haplotype. The hatch marks represent mutations between haplotypes, also given as numbers in brackets for the wider divergences. The unfilled, white nodes represent hypothetical ancestral states. C, Bayesian consensus tree of *Cinnyrus* haplotypes. Nodes are labelled with Bayesian probabilities.

has distinct yellow moustache stripes and supercilium. Our photographs (Supporting Information, Figs S1, S2) and field descriptions found the back colour to

be somewhat variable in both populations; however, the Wakatobi birds were generally darker. Under the Tobias *et al.* (2010) system we rank this as a minor

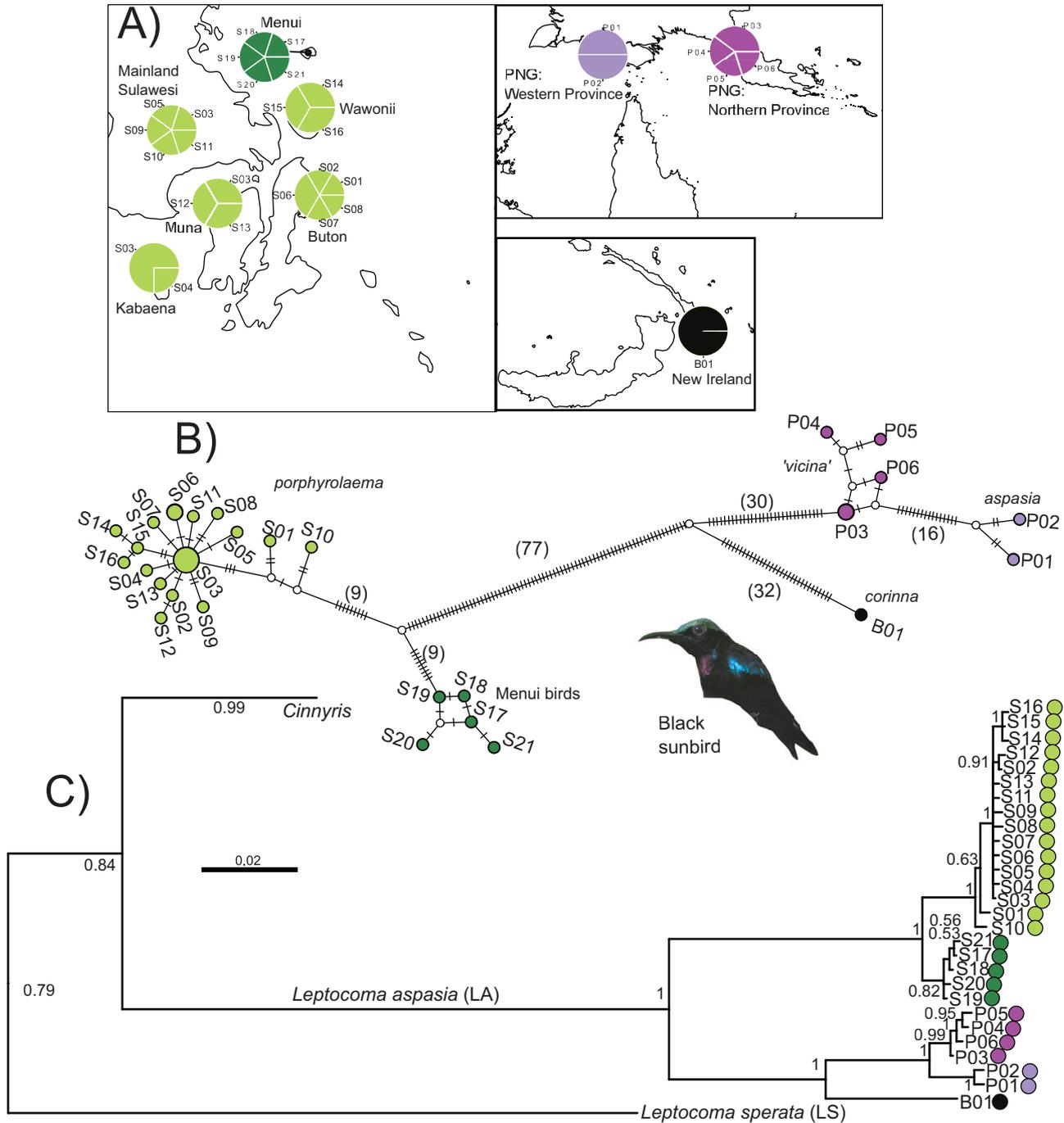


Figure 4. A, geographic distribution of *Leptocoma aspasia* haplotypes in Wallacea and the Sahul Shelf. Each circle represents an island and the fractions within the circle the haplotypes found on that island, proportioned to represent the frequency of each haplotype. The haplotypes are named according to the species-level divisions suggested by ABGD and coloured to represent the clades supported by our phylogenetic analyses. B, TCS haplotype network of *Leptocoma* haplotypes. Each circle represents a unique *ND2–ND3* haplotype, sized to represent how many birds carried that haplotype. The hatch marks represent mutations between haplotypes, also given as numbers in brackets for the wider divergences. The unfilled, white nodes represent hypothetical ancestral states. C, Bayesian consensus tree of *Leptocoma* haplotypes. Nodes are labelled with Bayesian probabilities.

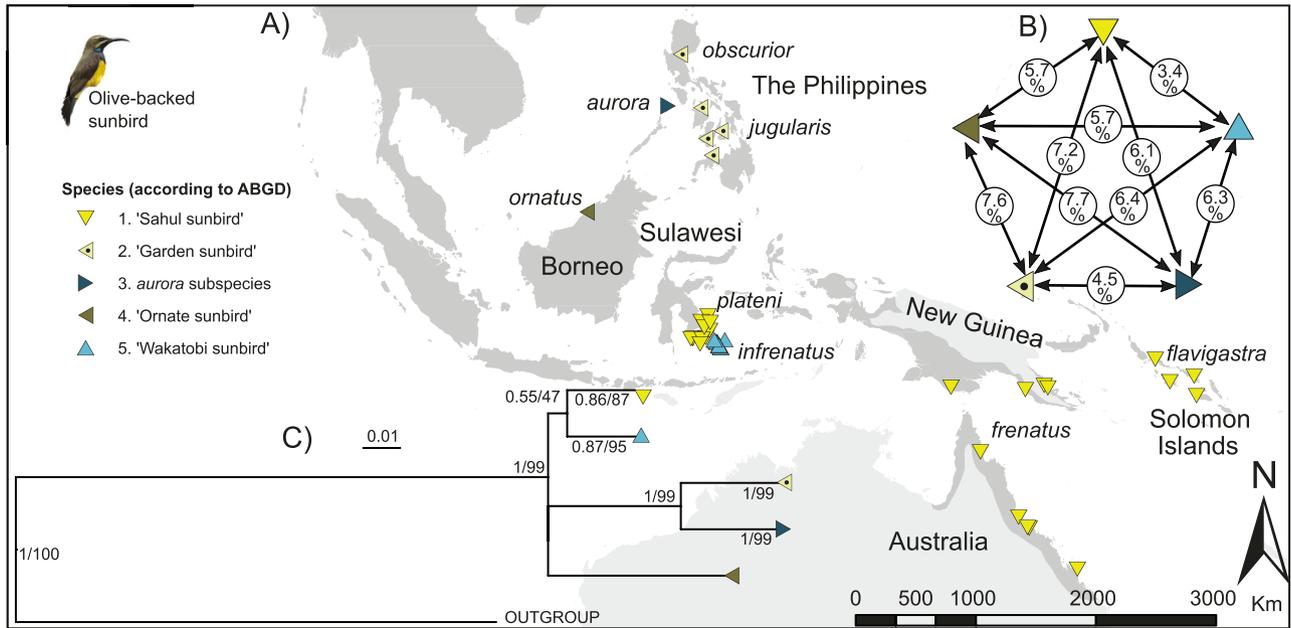


Figure 5. A, map of the Indo-Pacific with the range of the olive-backed sunbird shaded, as currently recognized by BirdLife International. Sampling sites of the birds included in our 697 bp partial *ND2* analysis are marked with different triangles, according to the species they were assigned to by ABGD. Currently recognized subspecies are labelled (Gill *et al.*, 2022). B, mean genetic distance (uncorrected p-distance) between each of the species recognized by ABGD, based on a 697 bp partial *ND2* alignment. C, simplified version of a combined maximum likelihood (ML) and Bayesian phylogenetic tree of 697 bp of olive-backed sunbird *ND2*. In this figure the outgroup is omitted and each of the ABGD species is collapsed into a single branch. Nodes are labelled with Bayesian probability/ ML bootstraps.

difference ('a slightly different wash or suffusion to all or part of any area'). We found that the faces of males from the Wakatobi Islands are distinctly darker, with the lack of markings presenting a marked contrast. We categorized this as a major difference ('contrastingly different hue/colour to all or part of a significant area').

The largest morphological decrease in the male olive-backed sunbird population of the Wakatobi Islands was in bill length (Cohen's *d* of -0.9 , $P < 0.01$). The largest morphological increase in the male Wakatobi birds was in tarsus length (Cohen's *d* of 2 , $P < 0.0001$). These traits are plotted in the Supporting Information (Fig. S9). In our bioacoustics analysis, we found the largest spectral effect size was in bandwidth (Cohen's *d* of 1.4 , $P < 0.0001$, with the 'Sahul sunbirds' singing with larger bandwidths) and the largest temporal effect size was in pace (Cohen's *d* of 0.5 , $P < 0.05$, with the 'Sahul sunbird' singing at faster paces). These traits are plotted in Supporting Information (Fig. S11). This gives a total 'Tobias score' across all characters of nine, exceeding the threshold of seven points, indicating a species-level difference between the 'Wakatobi sunbird' and the 'Sahul sunbird'. Using female birds instead of males for the morphological analysis would have given a total of eight, still surpassing the threshold.

The difference between the 'Wakatobi sunbird' and the 'Sahul sunbird' (specifically the olive-backed sunbird populations of south-east Sulawesi and its land-bridge islands) was reinforced by the bioacoustic and morphological MANOVA results. Only peak frequency, minimum frequency and bandwidth could be included in the bioacoustic MANOVA, as the other variables were either not linearly related or too strongly correlated (Pearson's $r > 0.8$ or < 0.1). This MANOVA revealed that the 'Wakatobi sunbird' is strongly differentiated in song from the 'Sahul sunbird' ($P < 0.001$, full output in Supporting Information, Table S6). The morphological MANOVA included the wing length, bill length, weight and tarsus length data, and found that the 'Wakatobi sunbird' is strongly differentiated from the 'Sahul sunbird' in these traits ($P < 0.001$, full output in Supporting Information, Tables S7, S8). This result was consistent, no matter whether male or female birds were used.

Our Welch's *t*-test found that the 'Wakatobi sunbird' has a considerably lower dispersal index than the 'Sahul sunbird' populations of south-east Sulawesi, its land-bridge islands and Menui ($P < 0.00005$ in males, $P < 0.005$ in females).

Because the Menui black sunbird population was suggested as a subspecies by our genetic work, we

used MANOVA to assess whether this population was distinct from Sulawesi and the land-bridge islands in wing length, bill length, weight and tarsus length (all of these variables being acceptably correlated). This analysis used female birds as only two adult male black sunbirds were caught on Menui (vs. seven adult females). We found that female black sunbirds are morphologically distinct on Menui compared to the land-bridge islands ($P < 0.001$, full MANOVA outputs in [Supporting Information, Table S9](#), plotted in [Fig. S10](#)). We did not carry out Tobias scoring on the black sunbird, because there were no clear plumage differences and less song data was available.

DISCUSSION

CINNYRIS TAXONOMY

Our results indicate that the olive-backed sunbird represents a superspecies and should be split into at least four species ([Supporting Information, Table S10](#)). We have found support for the three-way split suggested by [Eaton *et al.* \(2021\)](#) while also providing the first genetic, acoustic and morphological evidence that the ‘Wakatobi sunbird’ warrants recognition as a full species. The Wakatobi population exhibits a strikingly non-linear arrangement of population structure, with a range enveloped within that of the more widespread species, and had previously been suggested as a ‘limbo split’ ([Rheindt, 2021](#)) based on plumage differences alone ([Eaton *et al.*, 2021](#)). Our study has supported these differences with matching patterns in mtDNA and integrative species delimitation or ‘Tobias scoring’ ([Tobias *et al.*, 2010](#)). Due to the lower effective population size of mtDNA, along with other factors, differences in mtDNA should be integrated with other forms of evidence in this way ([Rubinoff & Holland, 2005](#)). In the light of this new integrative evidence, we recommend that the ‘Wakatobi sunbird *Cinnyris infrenatus*’, originally named by [Hartert \(1903\)](#), be reinstated as a separate species. In addition to its genetic divergence ([Fig. 3](#)), the ‘Wakatobi sunbird’ has shorter wings, a shorter bill and longer tarsi than the ‘Sahul sunbird’ ([Supporting Information, Fig. S9](#)), as well as exhibiting slower and higher pitched calls over a smaller bandwidth ([Supporting Information, Fig. S11](#)). This study is one of several to have remarked upon the distinctness of the Wakatobi avifauna ([Kelly *et al.*, 2014](#); [O’Connell *et al.*, 2019a, c](#)), and so we reiterate the recommendation of [O’Connell *et al.* \(2020\)](#) that the Wakatobi Islands should be protected as an Endemic Bird Area ([Stattersfield *et al.*, 1998](#)).

In addition to the distinctive nature of the Wakatobi lineage, our work supports the splits suggested by [Eaton *et al.* \(2021\)](#) and one possible additional split in the Philippines ([Fig. 5](#)). Under the [Eaton *et al.*](#)

(2021) treatment, populations from Sulawesi to the Sahul Shelf and the Solomon Islands were recognized as a species-level taxon, the ‘Sahul sunbird *Cinnyris clementiae*’, the Sunda Shelf populations become ‘ornate sunbird *Cinnyris ornatus*’ and the Philippine birds retain the *Cinnyris jugularis* name and take ‘garden sunbird’ as a common name. This is supported by deep genetic divergences (all greater than 5%) between these three putative species, with the Sahul Shelf represented by our new Sulawesi, Australian and PNG sequences along with Solomon Islands birds from [Smith & Filardi \(2007\)](#), the Sunda Shelf by a sequence from Borneo ([Boyce *et al.*, 2019](#)) and the Philippines by a number of previously published partial sequences ([Supporting Information, Table S3](#)). Although this split has been suggested previously based on limited sampling and incomplete sequences, our more comprehensive sampling of full sequences, taken from both ends of the new ‘Sahul sunbird’ species range, offers stronger support for the division. Our study has also suggested another potential split in this species complex ([Fig. 5](#)), outside the geographic range covered in detail by [Eaton *et al.* \(2021\)](#). ABGD considered the *aurora* sequences from Busuanga in the western Philippines to represent a distinct species, with a mean genetic distance of 4.5%, from the greater Philippine archipelago (subspecies *obscurior* and *jugularis*). Ornithologists ([Rand, 1951](#); [Billerman *et al.*, 2022](#)) have grouped the *aurora* subspecies separately from these other Philippine subspecies due to its orange breast plumage. The western chain of islands on which the *aurora* subspecies occurs is geologically and biogeographically distinct from the greater Philippine archipelago ([Diamond & Gilpin, 1983](#)). The *aurora* subspecies displayed a similar level of genetic divergence to that of the more thoroughly sampled ‘Wakatobi sunbird’, but was represented in our analysis by two partial *ND2* sequences from [Campbell \(2013\)](#), and so we recommend further sampling of this population.

The lack of divergence between the Menui population and the wider south-east Sulawesi population in the ‘Sahul sunbird’ confirms that the Menui population belongs to the *plateni* subspecies. On the Sahul Shelf itself, our ‘Sahul sunbirds’ exhibit a uniform population across PNG and Australia. This is in keeping with the current assignment of these populations to one subspecies, *Cinnyris jugularis frenatus*.

LEPTOCOMA TAXONOMY

The movement of species between the genera *Cinnyris*, *Leptocoma* and *Nectarinia* has previously caused confusion for taxonomists working with the black sunbird ([LeCroy, 2010](#)). Our analyses suggest that the *Leptocoma* genus is not monophyletic ([Fig. 2](#)). However,

the genus-level branches in our tree had lower support than the species- and subspecies-level branches due to our use of mtDNA. We strongly recommend further sequencing of all species within *Leptocoma* to clarify the genus taxonomy.

Previous research based on plumage differences has not proposed any splits or ‘limbo splits’ within the black sunbird (Eaton *et al.*, 2021). Nonetheless, we found that the black sunbird exhibited genetic divergence consistent with a species-level split between Wallacea and the Sahul Shelf, with a high genetic distance between Sulawesi and PNG (9.1%). This presents a marked contrast to the ‘Sahul sunbird’, which exhibited minimal divergence between those same populations. Further sequencing of birds from intervening areas (such as the Maluku Islands) would help to clarify the boundaries between these potential species. As several intervening populations are absent from our analysis, we cannot rule out the possibility of clinal variation between the Sulawesi and PNG populations (Brumfield, 2005; Cros & Rheindt, 2017). However, the genetic divergence we found was so strong (mean p-distance of 9.1%, higher than any observed in the olive-backed sunbird complex) that it seems more likely the two populations represent two species. The patterns we have observed suggest a division along Lydekker’s Line, and so we predict that sampling of the intervening populations would show a Wallacean species occurring from Sulawesi to the Maluku Islands and a separate species on New Guinea. This would involve the elevation of one of the Wallacean subspecies names to species level, as Lesson & Garnot (1828) named the black sunbird initially for a specimen from Manokwari (Doréry) on the New Guinea mainland. The discovery of cryptic species in the black sunbird highlights the importance of comprehensive sampling of species across their range, even in the absence of obvious plumage differences.

The structure within the black sunbird contrasted with the patterns shown by the olive-backed sunbird and ‘Sahul sunbird’ in several ways. The black sunbird exhibited a split between Sulawesi and PNG, where the ‘Sahul sunbird’ had a continuous population. The black sunbird also exhibited structure within both of these regions. Most taxonomic treatments (Cheke *et al.*, 2001; Billerman *et al.*, 2022) split the black sunbird into distinct subspecies in PNG’s Western Province (*L. a. aspasia*), Northern Province (*L. a. vicina* Mayr 1936) and the Bismarck Islands (*L. a. corinna*). However, Gill *et al.* (2022) merged *L. a. vicina* into *L. a. aspasia*, following Rand (1967). Our genetic work supports *L. a. aspasia* and *L. a. vicina* as distinct subspecies, whereas *L. a. corinna* appears to warrant species status based on ABGD. However, as this was based on a single sequence, we recommend that the Bismarck populations be examined further

before a judgement is made. The Menui black sunbird population was genetically distinct from Sulawesi and the land-bridge islands, with a mean genetic distance (1.72%), a distance greater than that between the two subspecies in PNG (1.48%). This population was also found to be distinct in wing length, bill length, weight and tarsus length (MANOVA, $P < 0.001$ in females). These findings suggest the Menui population of black sunbird may warrant recognition as an endemic subspecies.

IMPLICATIONS FOR BIOGEOGRAPHY AND EVOLUTION

The division between the ‘Sahul sunbird’ in Sulawesi and the ‘ornate sunbird’ in neighbouring Borneo (Eaton *et al.*, 2021) reflects one of the most iconic patterns in biogeography. Between these islands runs the boundary between the shallow Sunda Shelf and the deeper waters of Wallacea, which corresponds to the original and best-known version of ‘Wallace’s Line’ (Wallace, 1863; Fig. 1). However, Wallace’s Line, Wallacea and related biogeographic ideas have been treated in an entangled way in the literature, the treatment of Sulawesi and the Philippines being particularly contentious (Ali & Heaney, 2021). The three-way split of the olive-backed sunbird (Eaton *et al.*, 2021), which our work supports, reinforces the more widely used versions of Wallace’s Line (west of Sulawesi, south-east of the Philippines) and Wallacea (excluding the Philippines), as there are separate species in Wallacea (‘Sahul sunbird’), the Philippines (‘garden sunbird’) and the Sunda Shelf (‘ornate sunbird’).

Unlike Wallace’s Line, a similar barrier in the east has not prevented gene flow in this species, as the ‘Sahul sunbird’ appears to cross Lydekker’s Line with only shallow divergence between populations on either side. It is remarkable that the ‘Sahul sunbird’ appears to maintain a range all the way from Sulawesi to Australia, while the ‘Wakatobi sunbird’ seems to have diverged in a small archipelago nested in this range. Buton is only 27 km from the Wakatobi Islands, while the stretch of islands between Sulawesi and New Guinea contains deep-water barriers up to 100 km wide (this largest barrier is that between Misool and Seram). This striking pattern appears to conform with the ‘taxon cycle’ (Wilson, 1959, 1961; Ricklefs & Bermingham, 2002), whereby taxa become less dispersive as they age. In this framework, the ‘Sahul sunbird’ would occupy an early stage in the taxon cycle, leading to a dispersive lifestyle and a relatively recent colonization of the Sahul Shelf (or perhaps a back-colonization of Sulawesi) across Lydekker’s Line. This would be in keeping with previous hypotheses that the olive-backed sunbird colonized the Sahul Shelf from Wallacea quite recently (Schodde, 1977). The ‘Wakatobi sunbird’ would occupy a later stage of the taxon cycle,

maintaining a sedentary lifestyle in a small range. The ‘Wakatobi sunbird’ may thus represent a biological species, at it would have maintained its genetic and phenotypic divergence during this recent colonization wave. In other bird families that are widespread in this region, populations on small oceanic islands have been subject to selection for reduced dispersal ability, leading to a pattern of widespread continental species and isolated island relatives (Pedersen *et al.*, 2018; Le Pepke *et al.*, 2019). We have found evidence that this might also be the case in sunbirds, as the ‘Wakatobi sunbird’ exhibited a significantly lower dispersal index than the ‘Sahul sunbird’ populations of Sulawesi, its land-bridge islands and Menui. Since the earliest days of biogeography (Wallace, 1880), small, isolated ‘oceanic’ islands like the Wakatobi Islands have been known to harbour disproportionate numbers of endemic species. Birds on smaller islands are subject to different evolutionary processes than those on continents, exhibiting lower effective population sizes and losing fewer deleterious mutations to natural selection (Leroy *et al.*, 2021).

This study also demonstrates the somewhat stochastic nature of island colonization, as we have found that the sunbirds of Runduma colonized this island (located over 50 km east of the main Wakatobi Archipelago) from an entirely different direction than the white-eyes of the island. These are among the only two small passerines on Runduma that are not island specialists or human commensals. The ‘Wakatobi sunbird’ arrived on this tiny island from the Wakatobi Islands: CJ_W03 was the only haplotype found on Runduma, and is shared with Hoga, Tomia, Binongko and Lintea (Fig. 3). Runduma’s white-eye population, on the other hand, is most closely related to that of mainland south-east Sulawesi (O’Connell *et al.*, 2019c). Despite their evolutionary importance, many small islands in the Indo-Pacific have received little ornithological attention until recently (Monkhouse *et al.*, 2018; O’Connell *et al.*, 2020; Sin *et al.*, 2021).

The black sunbird was already known to be subject to Wallace’s Line, as Sulawesi represents the western extent of its range. Cheke *et al.* (2001) hypothesized that the black sunbird might be part of a superspecies, with its sister species on the other side of Wallace’s Line. Unlike the ‘Sahul sunbird’, our work indicates that *Leptocoma* sunbirds have speciated across Lydekker’s Line, based on the strength of divergence between populations in Sulawesi and PNG. Where the ‘Sahul sunbird’ exhibits a similar population throughout Australia and PNG, the black sunbird exhibits noticeable structure on this scale. That the Bismarck Islands hold an endemic taxon illustrates once again the importance of small and isolated islands in generating biodiversity.

CONCLUSION

We have found additional diversity and population structure in sunbirds beyond what is indicated by either Eaton *et al.* (2021) or Gill *et al.* (2022), i.e. the third of our hypothesized general patterns. Our findings support Eaton *et al.* (2021) in splitting the olive-backed sunbird, which is treated as one species by Gill *et al.* (2022), but where Eaton *et al.* (2021) split the species in three, our work supports the ‘Wakatobi sunbird’ as a fourth species. We have also identified cryptic populations in the black sunbird which were not suggested by either of these taxonomic treatments, with a stronger mitochondrial divergence between Wallacean and Sahul Shelf *Leptocoma* populations than between *Cinnyris* species. That Lydekker’s Line acts as a biogeographic barrier to the black sunbird but not the ‘Sahul sunbird’ is curious, perhaps reflecting a recent colonization or back-colonization across the Line by the ‘Sahul sunbird’. The olive-backed sunbird may be a stronger disperser than the black sunbird overall, as its tendency to occur in mangroves (Eaton *et al.*, 2021) would indicate. The cicadabird *Edolisoma tenuirostre* (Jardine, 1831) undertook a similarly recent colonization of this region, resulting in the entire Sahul Shelf being inhabited by an Australo-Papuan clade with strong dispersal abilities (Pedersen *et al.*, 2018). Perhaps, over time, species-level divergence could occur between the ‘Sahul sunbird’ populations separated by Lydekker’s Line.

Sunbirds have been admired by naturalists for centuries, and by artists for even longer: olive-backed sunbirds are depicted in reliefs on Java’s Borobudur Temple, dating to the 8th or 9th century CE, their search for nectar representing the Buddhist quest for enlightenment (Ashari *et al.*, 2021). However, improved sampling and modern integrative methods are only now revealing the true diversity within these species. As more island populations are sampled, such as those on the Wakatobi Islands, Menui and the Bismarck Archipelago, we learn more of their important role in evolutionary processes. It is interesting to contrast these effects with the large-scale barriers like Wallace’s and Lydekker’s lines. From tiny archipelagos to massive continental shelves, the threatened biodiversity of Wallacea and the Indo-Pacific needs to be examined at multiple scales if we are to be enlightened to its evolutionary history (Cicero *et al.*, 2021) and conservation (Myers *et al.*, 2000).

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ETHICS APPROVAL

RISTEKDIKTI provided the necessary permits and approvals, under permit numbers 0143/SIP/FRP/SM/VII/2010, 278/SIP/FRP/SM/VII/2012, 279/SIP/FRP/SM/VIII/2012, 174/SIP/FRP/E5/Dit. KI/V/2016, 159/SIP/FRP/E5/Fit. KIVII/2017 and 160/SIP/FRP/E5/Fit. KIVII/2017. Handling of animals was also approved by the Trinity College Dublin Animal Research Ethics Committee, under reference number 060417.

AUTHOR CONTRIBUTIONS

FÓM, DOC, DJK and NMM conceived this study. FÓM, DJK and NMM led the writing of the manuscript. DJK, NMM, DOC, FÓM, AK and KA carried out the fieldwork. FÓM, JM and NL carried out the laboratory work. ET analysed the acoustic data, supervised by FÓM. FÓM analysed the other data. All authors contributed to revising and improving the manuscript.

DATA AVAILABILITY

The new ND2 and ND3 DNA sequences produced during this study have been deposited in GenBank under accession numbers OM965843–OM966076.

Olive-backed sunbird vocalization recordings will be uploaded to xeno-canto. They are available here: <https://xeno-canto.org/set/7971>. The acoustic and morphological data are available in the [Supporting Information \(Files S2, S3\)](#). The R code underlying the analyses is provided in the [Supporting Information \(Appendix S2\)](#).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

- Figure S1.** Male olive-backed sunbirds from Sulawesi and its land-bridge islands, photographed by the authors.
- Figure S2.** Male olive-backed sunbirds from the Wakatobi Islands, photographed by the authors.
- Figure S3.** Female olive-backed sunbirds from Sulawesi and its land-bridge islands, photographed by the authors.
- Figure S4.** Female olive-backed sunbirds from the Wakatobi Islands, photographed by the authors.
- Figure S5.** Example PCR protocol used in this study, showing the reagents and quantities in the master mix along with the thermal cycling programme used.
- Figure S6.** Diagram of the mitochondrial *ND2* gene, labelled to demonstrate our sequencing technique.

Figure S7. Full Bayesian tree showing all haplotypes and outgroup taxa.

Figure S8. Full maximum likelihood (ML) tree showing all haplotypes and outgroup taxa.

Figure S9. Boxplots of (a) male and (b) female olive-backed sunbirds, comparing the ‘Sahul sunbird’ to the ‘Wakatobi sunbird’.

Figure S10. Boxplots of female black sunbird morphology, comparing the birds netted on Menui to those netted on Sulawesi and its land-bridge islands.

Figure S11. Boxplots of olive-backed sunbird bioacoustics, comparing the ‘Sahul sunbird’ to the ‘Wakatobi sunbird’.

Figure S12. Boxplots of (a) male and (b) female olive-backed sunbird dispersal index, comparing the ‘Sahul sunbird’ to the ‘Wakatobi sunbird’.

Table S1. DNA samples used for phylogenetic reconstruction.

Table S2. PCR primers used in this study.

Table S3. Additional olive-backed sunbird *ND2* sequences used to assess species status.

Table S4. P-distances between olive-backed sunbird concatenated *ND2–ND3* haplotypes.

Table S5. P-distances between black sunbird concatenated *ND2–ND3* haplotypes.

Table S6. Output of MANOVA on acoustics of olive-backed sunbirds from the Wakatobi Islands vs. Sulawesi, Menui and the land-bridge islands.

Table S7. Output of MANOVA on morphology of female olive-backed sunbirds from the Wakatobi Islands vs. Sulawesi, Menui and the land-bridge islands.

Table S8. Output of MANOVA on morphology of male olive-backed sunbirds from the Wakatobi Islands vs. Sulawesi, Menui and the land-bridge islands.

Table S9. Output of MANOVA on morphology of female black sunbirds from Menui vs. Sulawesi and the land-bridge islands.

Table S10. Potential taxonomic revisions supported by our work.

Appendix S1. Dispersal index methodology and rationale.

Appendix S2. R code used in analyses.

File S1. Supplementary Figures, Tables and Appendices.

File S2. Mean acoustic data extracted from olive-backed sunbird call recordings.

File S3. Raw morphological data taken from sunbirds.