A New Bat Species from Southwestern Western Australia, Previously Assigned to Gould’s Long-eared Bat

Nyctophilus gouldi Tomes, 1858

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Abstract. A distributional isolate in southwestern Western Australia previously assigned to Gould’s Long-eared Bat Nyctophilus gouldi Tomes, 1858 is demonstrated to be a distinct and previously unnamed cryptic species, based on a lack of monophyly with eastern populations and substantial DNA sequence divergence (5.0 %) at the mitochondrial gene COI. Morphologically both species are alike and overlap in all measured characters but differ in braincase shape. The new species has one of the most restricted geographic ranges of any Australian Vespertilionidae and aspects of its ecology make it vulnerable to human impacts.

Introduction

Long-eared bats of the genus Nyctophilus are small to medium-sized species of the cosmopolitan family Vespertilionidae. The genus is centred on mainland Australia and the island of New Guinea (Burgin, 2019). Nine species are recognized from Australia, all of which roost in cavities and crevices or foliage of trees, and buildings (Churchill, 2008), with occasional suspected opportunistic cave use (e.g., Kutt, 2003).

Gould’s Long-eared Bat Nyctophilus gouldi Tomes, 1858, as currently understood, is found on mainland southeastern Australia extending from far southeastern South Australia, through Victoria and NSW to eastern Queensland as far north as the Atherton Tableland (Pennay et al., 2008). An isolated occurrence in far southwestern Western Australia (WA) was first tentatively recognized by Kitchener & Vicker (1981), following the realization by Hall & Richards (1979) that N. gouldi was a species distinct from the larger Greater Long-eared Bat N. timoriensis (Geoffroy, 1806). Throughout most of the 20th century N. gouldi had been treated as the southeastern Australian subspecies of N. timoriensis and the presence there of a larger species had been overlooked prior to Hall & Richards (1979). Consequently, although specimens of N. gouldi from WA existed in research collections including the Australian Museum (AM) in the early 20th century, they remained unrecognized and were assigned to N. timoriensis.

Tomes (1858) based his description of N. gouldi on two specimens from Moreton Bay (Brisbane, Qld) and one from Bathurst, NSW. Thomas (1915) designated a female from Moreton Bay as lectotype and provided a re-diagnosis that supported its distinction from N. geoffroyi Leach, 1821 and N. timoriensis (using the name N. major Gray, 1844). Unfortunately for most of the remaining 20th century N. gouldi was confused with N. timoriensis and all authors prior to Hall & Richards (1979) adopted the view of Iredale & Troughton (1934) who treated N. gouldi as the southeastern Australian subspecies of N. timoriensis.

The unresolved status of different morphological forms within N. daedalus Thomas, 1915 from northern Western Australian and the Northern Territory might also be relevant to an evaluation of the taxonomic status of N. gouldi. Parnaby (2009) suggested that two or more broadly sympatric species might be contained within N. daedalus. A smaller-bodied form of N. daedalus with relatively much longer ears and a more gracile skull are features shared with N. gouldi but its...
status remains unresolved.

Unresolved taxonomic issues affect at least half of the nine species of Nyctophilus currently recognized from mainland Australia. The need to assess the taxonomic status of southwestern WA N. gouldi was identified 20 years ago (Reardon, 1999) but until now, remained unresolved. There is considerable variation in body size and morphology in eastern Australian N. gouldi. Populations from lower rainfall, inland regions are on average smaller as noted by Parnaby (1987) and Lumsden & Bennett (1995). Churchill et al. (1984) considered that smaller animals from north Queensland might represent a separate species.

This paper arose from efforts to use a DNA bar-coding approach to confirm species identifications of microbat specimens sampled during Australian Museum fieldwork (Eldridge et al., 2020). The successful identification of some samples required extensive comparisons with bat reference samples held by the Australian Museum and vouchered specimen data from online databases (NCBI GenBank and BOLDSystems). During this work phylogenetic analysis of samples of N. gouldi from WA and eastern Australia were found not to be monophyletic and differed by a level of DNA sequence divergence greater than that found between some currently recognized microbat species (Eldridge et al., 2020).

**Methods**

**DNA sequencing**

Total genomic DNA was extracted from 10 mg of tissue using the Isolate II Genomic DNA Kit (Bioline Australia) following manufactures protocol. Fragments of two mitochondrial DNA genes, cytochrome b (CytB) and cytochrome oxidase 1 (COI), were PCR amplified using the following primers: CytB - L14841 (F), AAAAACCTTCCATCAACATCTC AGCATGATGAAA, H15149 (R) AAACCTGCAGCCCCCTC AGAATGTATTTGTCTCTCA (Kocher et al., 1989); COI-BAK1490 (F) CTCCAAAACACAAAGACGATCGG, BAK2198 (R) TAGACCTTCGTTGTCGCGAAGAATCA (Neaves et al., 2018). PCRs were performed in 25 μl reactions using 10-20 ng of genomic DNA, 1 × MyTaq Red Reagent Buffer (Bioline Australia), 2 pmol primers and 0.5 Units MyTaq Red DNA polymerase (Bioline Australia). Thermocycling was performed on an Eppendorf Mastercycler Nexus Gradient (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation 94°C (3 min), 38 cycles of denaturation at 94°C (20 s), annealing at 54°C (40 s) and extension at 72°C (40 s) with a final extension step of 72°C for 5 min. The partial COI fragment for Eastern Australian N. Geoffroyi was amplified using alternative primers LCO1490 (F) GGTCACAACATCATAAGAATTTGG and HCO2198 (R) TAAACTTCAGGGTGACCAAAAATCA (Folmer et al., 1994) under the PCR conditions outlined above except that the annealing temperature was 52°C. PCR products were visualized on a 2% E-gel (Life Technologies Corp. #G5018-02), then purified using ExoSAP-IT reagent (ThermoFisher Scientific #78201.1.ML) and sequenced at the Australian Genome Research Facility (AGRF, Sydney). Sequences were edited using Sequencher v5.4 (Gene Codes Corporation, Ann Arbor, USA) and aligned using ClustalW in Mega 7.0.21 (Kumar et al., 2016).

The evolutionary history of haplotypes was inferred using Maximum Likelihood based on the HKY model and 500 bootstraps. The most appropriate evolutionary model was determined using the “Find Best Model Fit” in Mega 7.0.21 (Kumar et al., 2016). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

**Morphological comparisons**

Measurements were taken with Vernier callipers to the nearest 0.05 mm as illustrated by Parnaby (2009). Abbreviations for measurements are: GSL, Greatest length of skull: from the most anterior extension of the premaxilla to the posterior of the lambdoidal crest; CON, posterior of occipital condyles to anterior most point of premaxilla; CM3, Length of maxillary tooth row: from anterior cingulum of canine to posterior cingulum of M3; C1-C1, Outer breadth across upper canines from cingula; PAL, Palatal-sinual length, from the most posterior margin of the anterior palatine imbrication to the most anterior margin of the interterygoid fossa; ZYG, Zygomatic breadth, maximum breadth across zygomatic arches; INT, Least inter-temporal breadth; M33, Maximum breadth from left M3 to right M3, from labial cingula; BRH, Braincase height: calliper blade positioned along basioccipital-basiphenoid bones and along the sagittal crest; MASB, maximum breadth across mastoids; BAS, Basicranial length, from anterior most margin of foramen magnum to most anterior margin of interterygoid fossa; BTB, Least inter-bulla distance, least distance between each bulla; BUL, Bulla length, from base of eustachian tube when present. Ear Length, taken from the junction of outer ear margin near the jaw; FA, Forearm length, from the posterior tuberile of the radius; D3p1, third metacarpal length and D5p1, first metacarpal length, both taken from the anterior of the radius to the middle of the metacarpal-phalanx joint; HL, Lower hind leg length, taken with the lower leg with the ankle and knee joints bent. Note that HL is not equivalent to tibia length.

Female N. gouldi average larger for most external and cranial measurements (Parnaby, 1987; Young & Ford, 2000) and each sex was grouped separately in statistical analyses. Summary statistics and Principal Components analyses (PCA) were run using the Palaentological Statistics (PAST) software package (Hammer et al., 2001), version 4.0 (January, 2020). PCAs were run using both correlation and variance-covariance matrices, using only specimens with complete measurements. Standardized character coefficients where used to explore the possible contribution of individual characters to each PC axis.

Institutional abbreviations used throughout the text are: AM, Australian Museum, Sydney; AMNH, American Museum of Natural History, New York; ANWC, Australian National Wildlife Collection, CSIRO, Canberra; MV, Museum of Victoria, Melbourne; QM, Queensland Museum, Brisbane; WAM, Western Australian Museum, Perth. Specimens used in the DNA and morphological analyses are listed in the Appendix.
Results

DNA sequencing

For the two partial mtDNA genes amplified no premature STOP codons or indels were detected which would suggest the amplification of NU.MTs. To investigate species boundaries within the genus a 584 base pair (bp) fragment of COI was aligned from 28 vouched Nyctophilus specimens from six species groups (GenBank Accession numbers MT246209–MT246234, MW965505, MW965506). The maximum likelihood tree with the highest log likelihood (Fig. 1a) shows eight well supported monophyletic lineages within sampled Nyctophilus. Two divergent non-monophyletic clades were present in both N. gouldi and N. geoffroyi. Analysis of a 284 bp fragment of CytB from 21 Nyctophilus produced a tree (Fig. 1b) with similar topology but with reduced support at some nodes (GenBank Accession numbers MT246593–MT246605, MW976994). The two lineages identified within N. gouldi separated specimens from eastern and western Australia and were 5.0% divergent for COI (Table 1) (5.7% divergent for CytB). Within each N. gouldi clade the average divergence for COI was 0.4–1.0%.

The two identified N. geoffroyi clades separated individuals sampled from western and eastern Australia and showed 10.4% average sequence divergence (Table 1) (10.6% divergence for CytB). Currently recognized Nyctophilus species showed average sequence divergences ranges from 1.4–12% for COI (Table 1) (2.0–11.9 for CytB).

Morphological comparisons

Measurement ranges of all 13 cranial and five external characters overlap between eastern and western N. gouldi for each sex (Table 2). A PCA based on nine cranial characters failed to achieve separation of western from eastern N. gouldi (Fig. 2), nor was separation achieved in separate PCAs (not shown) based on a reduced sample size, that enabled inclusion of the nine cranial characters along with BUL, BTB and BAS, and a separate analysis using the initial nine characters and FA.

In a PCA based on a correlation matrix using nine cranial characters, western N. gouldi formed a group with partial separation from eastern N. gouldi on the first two PCA axes, although most specimens fell within the range of PC scores of eastern N. gouldi (Fig. 2a). Specimen scores on the first PC axis reflect overall size as indicated by positive character loadings of approximately similar magnitude on that axis (Table 3). The second PC axis, which influenced the peripheral position of western N. gouldi specimens, were mainly driven by an inverse trend in the magnitude of INT compared to other characters as indicated by character loadings on that axis (Table 3). The relatively broader INT of western N. gouldi shown in a bivariate plot of CON vs. INT (Fig. 3a) indicates that the loadings on PC 2 reflect a proportionately broader INT in western compared to the majority of eastern N. gouldi. Western N. gouldi fell completely within the scatter of scores for eastern N. gouldi on all subsequent PC axes (not shown).

Patterns of variation of eastern and western N. gouldi amongst specimens used in the PCA were examined further by consideration of the smaller average size of inland N. gouldi compared with those from higher rainfall regions. Specimens of eastern N. gouldi (n = 79) were divided into four geographic regions based on a combination of broad differences in rainfall and landform and patterns of between-locality variation evident in individual characters. These groups are: (a) the higher rainfall areas of eastern NSW and far southeastern Qld from which subcoastal and montane regions (n = 35); (b) the higher rainfall areas of southern and eastern Victoria south of the northern slopes of the Dividing Range (n = 19); (c) southern inland through to northern Qld from largely inland districts (n = 11) and (d) animals from inland northern Victorian lowlands and inland NSW, west of the western slopes (n = 14). An example of mean differences in size between these regions (GSL for adult males, Table 4) typifies that measurement ranges overlap between the four regions but means differ. As sample size is small and we are interested only in broad trends in the data, we have not applied analyses of statistical significance. The trend for larger mean GSL (which is also apparent for other characters including FA, not shown) for the groups a and b above from mesic regions is apparent (Table 4).

The PC scores on the first two PC axes of the initial PCA using 9 cranial characters was re-examined by assigning specimens according to the four geographic groups of N. gouldi and western N. gouldi. When specimens are labelled according to geographic group, a plot of PC scores on the first two axes (Fig. 2b) reveals that PC scores of western N. gouldi extensively overlap those from the lower rainfall group of inland Victoria and NSW but to a far lesser extent, those of the inland and north Qld group, with minimal overlap with specimens from the two groups from mesic Victoria and NSW. The PCA. This PCA was based on a single matrix treating all specimens as one group. Two multi-group PCAs were run using the same nine characters and specimens, one using between-group, the other within-group comparisons. The relationships of specimens on the first two PC axes of both analyses were essentially the same as the initial one-group PCA, i.e. PC scores for western N. gouldi were

| Table 1. Average sequence divergence among (below diagonal) and within (along diagonal) sampled Nyctophilus taxa for COI. |
|----------------------------------------------------------|
|                           | holtorum sp. nov. | gouldi E | geoffroyi E | geoffroyi W | corbeni | bifax | daedalus | arnhemensis |
|---------------------------|------------------|----------|-------------|-------------|---------|-------|----------|------------|
| Nyctophilus holtorum sp. nov. | 0.5              | 5.0      | 10.5        | 12.1        | 11.4    | 7.8   | 7.4      | 16.3       |
| Nyctophilus gouldi E       | 5.0              | 0.4      | 9.5         | 9.8         | 8.7     | 7.5   | 7.5      | 13.9       |
| Nyctophilus geoffroyi E    | 10.5             | 9.5      | 0.4         | 10.4        | 10.2    | 9.9   | 11.1     | 14.9       |
| Nyctophilus geoffroyi W    | 12.1             | 9.8      | 10.4        | 0.5         | 10.6    | 11.9  | 11.1     | 14.2       |
| Nyctophilus corbeni        | 11.4             | 8.7      | 10.2        | 7.1         | 0       | 11.6  | 11.1     | 14.6       |
| Nyctophilus bifax          | 7.8              | 7.5      | 11.9        | 10.6        | 7.1     | 10.4  | 1.4      | 7.9        |
| Nyctophilus daedalus       | 3.9              | 5.2      | 9.9         | 11.6        | 11.1    | 11.4  | 7.9      | 0          |
| Nyctophilus arnhemensis    | 7.4              | 7.5      | 11.1        | 10.2        | 11.1    | 1.4   | 7.9      | 0          |
| Vespadelus darlingtoni     | 16.3             | 13.9     | 14.9        | 14.2        | 14.9    | 14.6  | 15.6     | 14.4       |
Figure 1. Highest maximum likelihood tree of relationships amongst *Nyctophilus* using: (a) 28 COI haplotypes, and (b) 21 CytB haplotypes. Bootstrap support (%) is shown at branch nodes. All M numbers refer to AM specimens except where indicated.
Figure 2. Specimen scores on the first two axes of a PCA based on a correlation matrix of nine cranial characters of adult male *N. holtorum* sp. nov. (squares, *n* = 6) and *N. gouldi* sensu stricto (circles, *n* = 79): (a) PCA scores grouped by species, and (b) same plot with *N. gouldi* coded by four geographic localities: montane and subcoastal NSW and far southeastern Qld (closed circles); southern and eastern Victorian montane regions (closed diamonds); inland and northern Qld (open circles) and inland NSW and northern Victoria (triangles).
Table 2. Summary statistics for adults of each sex of *Nyctophilus holtorum* sp. nov. and *N. gouldi* sensu stricto: SE, standard error; SD, standard deviation; and CV, coefficient of variation.

|                   | *Nyctophilus holtorum* sp. nov. | *Nyctophilus gouldi* sensu stricto |
|-------------------|----------------------------------|-----------------------------------|
|                   | males                           | males                            |
|                   | mean  | SE   | SD   | CV   | mean  | SE   | SD   | CV   |
| CON               | 15.177| 0.163| 0.398| 2.62 | 14.55 | 15.5 | 6    |
| GSL               | 16.602| 0.161| 0.395| 2.38 | 16.0  | 16.9 | 6    |
| CM3               | 5.867 | 0.082| 0.341| 5.51 | 6.1   | 6.1  | 6    |
| CI-C1             | 4.484 | 0.053| 0.307| 4.3  | 4.6   | 7    |
| ZYG               | 9.955 | 0.096| 2.353| 9.63 | 10.17 | 9.63 |
| INT               | 3.753 | 0.059| 1.676| 4.44 | 4.37  | 4.03 |
| M33               | 6.282 | 0.325| 0.796| 12.67| 4.68  | 8.66 |
| BRH               | 5.983 | 0.092| 0.224| 3.75 | 5.73  | 6.31 |
| MASB              | 8.892 | 0.138| 0.256| 2.98 | 8.53  | 9.16 |
| BTB               | 1.655 | 0.059| 0.159| 9.5  | 1.5   | 1.8  |
| BUL               | 3.835 | 0.072| 0.159| 3.97 | 3.69  | 4.05 |
| BAS               | 5.472 | 0.153| 0.303| 5.53 | 5.09  | 5.8  |
| PAL               | 6.273 | 0.05 | 0.152| 2.42 | 6.4   | 6.4  |
| EAR               | 22.45 | 0.475| 0.112| 4.25 | 24.5  | 27.7 |
| FA                | 39.885| 0.255| 0.865| 22.22| 35.8  | 41.1 |
| D3p1              | 39.241| 0.278| 0.878| 23.6 | 36.3  | 38.5 |
| D5p1              | 36.455| 0.291| 0.919| 2.52 | 35.3  | 37.7 |
| HL                | 18.711| 0.216| 0.647| 3.46 | 17.8  | 19.9 |

|                   | females                        | females                        |
|-------------------|--------------------------------|--------------------------------|
|                   | mean  | SE   | SD   | CV   | mean  | SE   | SD   | CV   |
| CON               | 15.61 | 0.11 | 0.216| 1.38 | 15.37 | 15.83| 4    |
| GSL               | 17.028| 0.069| 0.139| 0.82 | 16.87 | 17.18| 4    |
| CM3               | 6.096 | 0.047| 0.106| 1.73 | 9.56  | 6.21 | 4    |
| CI-C1             | 4.697 | 0.034| 0.082| 1.75 | 4.6   | 4.8  | 6    |
| ZYG               | 10.448| 0.087| 0.173| 1.66 | 10.27 | 10.66| 4    |
| INT               | 3.696 | 0.061| 0.134| 3.61 | 3.47  | 3.8  | 5    |
| M33               | 6.792 | 0.061| 0.136| 2.0  | 6.61  | 6.94 | 4    |
| BRH               | 6.13  | 0.098| 0.177| 2.89 | 5.96  | 6.32 | 4    |
| MASB              | 9.205 | 0.076| 0.151| 1.64 | 9.06  | 9.33 | 4    |
| BTB               | 1.725 | 0.014| 0.029| 1.87 | 1.7   | 1.75 | 4    |
| BUL               | 3.865 | 0.055| 0.111| 2.87 | 3.75  | 4.0  | 4    |
| BAS               | 5.65  | 0.126| 0.252| 4.45 | 5.3   | 5.9  | 4    |
| PAL               | 6.29  | 0.076| 0.169| 2.69 | 6.0   | 6.4  | 5    |
| EAR               | 26.4  | 0.432| 0.967| 3.66 | 25    | 27.6 | 5    |
| FA                | 41.378| 0.427| 1.282| 3.1  | 39.5  | 43.7 | 9    |
| D3p1              | 38.925| 0.416| 1.176| 3.02 | 37.4  | 40.8 | 8    |
| D5p1              | 38.213| 0.394| 0.794| 3.69 | 37.9  | 41.2 | 8    |
| HL                | 19.6  | 0.273| 0.771| 3.93 | 17.9  | 20.3 | 8    |

Table 3. Character loadings, eigenvalues, and % variance on each axis of a PCA using a correlation matrix of nine cranial characters of male *Nyctophilus holtorum* sp. nov. (n = 6) and *N. gouldi* sensu stricto (n = 79).

|                  | PC 1  | PC 2  | PC 3  | PC 4  | PC 5  | PC 6  | PC 7  | PC 8  | PC 9  |
|------------------|------|------|------|------|------|------|------|------|------|
| CON               | 0.368| -0.152| -0.013| -0.346| -0.198| 0.068| -0.176| -0.374| 0.712|
| GSL               | 0.369| -0.148| 0.076| -0.243| -0.246| 0.056| -0.291| -0.391| -0.691|
| CM3               | 0.332| -0.386| -0.320| -0.161| 0.100| 0.489| -0.019| 0.600| -0.047|
| CI-C1             | 0.336| -0.032| -0.259| -0.118| 0.766| -0.436| 0.063| -0.150| -0.051|
| ZYG               | 0.352| 0.068| 0.035| 0.366| -0.242| -0.500| -0.507| 0.405| 0.079|
| INT               | 0.235| 0.871| -0.039| -0.130| 0.124| 0.346| -0.166| 0.067| -0.004|
| M33               | 0.334| 0.009| -0.355| 0.709| -0.118| 0.224| 0.294| -0.331| 0.012|
| BRH               | 0.303| -0.124| 0.828| 0.232| 0.304| 0.218| 0.094| 0.035| 0.049|
| MASB              | 0.350| 0.159| 0.096| -0.273| -0.352| -0.308| 0.707| 0.214| -0.059|
| Eigenvalue        | 6.651| 0.779| 0.466| 0.319| 0.288| 0.226| 0.148| 0.100| 0.023|
| % variance        | 73.90| 8.652| 5.173| 3.545| 3.197| 2.515| 1.649| 1.108| 0.251|
Table 4. Summary statistics for GSL of 79 adult male *Nyctophilus holtorum* sp. nov. and *N. gouldi* sensu stricto, used in the PCA, assigned to four geographic groups.

| Group                                      | n  | min | max  | mean  | SE  | SD  | CV  |
|--------------------------------------------|----|-----|------|-------|-----|-----|-----|
| Montane and coastal NSW and far southeastern Qld | 35 | 16.6| 18.5 | 17.551| 0.089| 0.528|3.01 |
| Inland and northern Queensland              | 11 | 15.6| 17.0 | 16.436| 0.127| 0.420|2.56 |
| Inland New South Wales and northern Victoria| 14 | 16.2| 17.3 | 16.683| 0.092| 0.344|2.06 |
| Southern and montane Victoria               | 19 | 16.3| 18.4 | 17.458| 0.113| 0.492|2.83 |
| *Nyctophilus holtorum* sp. nov.             | 6  | 16.0| 16.9 | 16.602| 0.161| 0.395|2.38 |

Figure 3. Bivariate plots (mm) showing overlap in measurements of adult *N. holtorum* sp. nov. (squares, polygons) and adult *N. gouldi* sensu stricto (circles). Solid symbols are females, open symbols male: (a), condylo-basal skull length (CON) vs. least inter-temporal breadth (INT); (b) CON vs. mastoid breadth (MASB); (c), CON vs. braincase height (BRH); (d) forearm length (FA) vs. greatest skull length (GSL), and (e) FA vs. length from canine to upper rear molar (CM3).
Differences in skull proportions and braincase shape between western and eastern *N. gouldi* were apparent from skull comparisons. The braincase of western *N. gouldi* tends to be more inflated anteriorly, and relatively wider; the palate tends to extend further posteriorly, enclosing a much higher proportion of the interpterygoid fossa (Fig. 4). Skulls of eastern *N. gouldi* of equivalent CON, tend to have smaller INT (Fig. 3a) and narrower MASB (Fig. 3b) but overlap extensively in BRH (Fig. 3c). For example, male eastern *N. gouldi* of equivalent CON (≤ 15.5 mm) have smaller mean MASB (mean = 8.56 mm, s.e. 0.033, n = 24 vs. 8.89 mm s.e. 0.11, n = 6). Western *N. gouldi* also tend to have smaller skulls relative to eastern *N. gouldi* of equivalent FA (Fig. 3d) and shorter tooth rows (FA vs. CM3, Fig 3e). Although these differences are evident as trends in bivariate plots, the differences are far more apparent from direct skull comparisons and are poorly captured in the linear measurements used in this study. Differences evident from visual inspection of skulls appear to be based on shape factors not fully represented by these measurements. Bivariate plots of ratios of various measurements were also examined (e.g., MASB/CON vs. CM3/FA, not shown) but these showed no further separation than plots of simple measurements.

Apart from braincase shape, no consistent cranial or dental differences were found between eastern and western *N. gouldi*. Baculum morphology of eastern *N. gouldi* is variable (see illustrations of Hill & Harrison, 1987; Parnaby, 2009) and broadly resembles that of western *N. gouldi* in general shape, based on the few baculi examined (WAM M.4845, WAM M.10036).

Despite the overall morphological similarity (which is typical of taxa within *Nyctophilus*), the genetic divergence, lack of monophyly between eastern and western *N. gouldi* sensu lato and the identification of differences in braincase shape leads us to conclude that the southwest WA isolate previously assigned to *N. gouldi* represents an undescribed cryptic species.

**Systematics**

**Family Vespertilionidae**

**Genus Nyctophilus** Leach, 1821

**Type species.** *Nyctophilus geoffroyi* Leach, 1821.

**Nyctophilus holtorum** sp. nov.

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Figs 1–6

**Holotype:** WAM M.64188 (previously registered AM M.39799), field number 7HP43, adult male, body in alcohol, skull extracted, captured in a harp trap (bat trap) set on a forest road on the evening of 27 November, 2007 by H. Parnaby and T. Reardon. Field measurements (mm) of the holotype are: FA, 40.9; snout-vent length, 50; vent-tail tip length, 46; ear length (from notch), 26.2; hindleg length (with knee and ankle bent), 19.9; body weight, 9 g. Frozen tissue samples (liver) stored at the AM.

**Paratypes:** (total 8 adults, all bodies in alcohol). Northcliffe-Windy Harbour Road, 200 m north of road to Mt Chudalup, D’Entrecasteau National Park, 34°45′37″S 116°05′06″E, WA, collected by H. Parnaby, T. Reardon and S. Ingleby on 27 November 2007: AM M.39806 (7HP29) and AM M.39807 (7HP30) both male. Northcliffe-Windy Harbour Road, 3.2 km south of road to Mt Chudalup, 34°47′17″S 116°04′30″E D’Entrecasteau National Park, WA, collected by H. Parnaby and T. Reardon on 27 November 2007: AM M.39809 (7HP33) female, AM M.39810 (7HP34) male; AM M.39811 (7HP38) male; AM M.39812 (7HP40) male; c. 10 km northeast of Waroona, 32°47′54″S 116°00′53″E, WA, collected H. Parnaby and T. Reardon on 27 November 2007: AM M.39813 (7HP41) female. Manjimup Post Office, 34°15′00″S 116°32′00″E: WAM M.19164, female, body in alcohol, skull extracted, collected by M. Sawle 1980. Frozen tissue samples (liver) stored at the AM and SAM for all paratypes except WAM M.19164.

**Specimens examined.** See Appendix. The type series consists of 9 specimens, others are referred specimens.

**Type locality:** State Forest c. 10 km northeast of Waroona, 32°47′54″S 116°00′53″E [WGS84 ±20 m], Western Australia.

**Diagnosis:** A species of medium body size for the genus, closely resembling *N. gouldi* sensu stricto in external appearance, cranial and dental morphology but differs by an average sequence divergence of 5.0 % at the mitochondrial gene COI. It differs further in that the braincase tends to be broader for *N. gouldi* sensu stricto of equivalent GSL (Figs 3 and 4), as reflected by greater MASB (Fig. 3a); the anterior of the braincase tends to be more inflated laterally, and the skull tends to be relatively shorter e.g., FA vs. GSL (Fig. 3c) and FA vs. CM3 (Fig. 3d).

Diffs from *N. daedalus* sensu stricto, which has a relatively broader, larger skull (GSL: males greater than 17.3 mm, females greater than 17.6 mm); relatively much smaller auditory bullae that are set further apart, and more reduced M3, i.e. the second and third “commissures” of M3 are much shorter relative to the first commissure. Further differs in typically having a more developed (higher) post-nasal mound and relatively longer ears than *N. daedalus* sensu stricto.

Diffs from sympatric *N. geoffroyi* in shape and relative development of the post-narial snout elevation, which is divided by a vertical median groove (Fig. 5) compared to the distinct median Y-shaped groove in *N. geoffroyi* and the latter species averages smaller in general size, e.g., FA typically less than 38 mm; smaller mean body weight (e.g., combined sexes mean 6.3 vs. 10.0, Fullard et al., 1991).

Distinguished from sympatric *N. major* which has a low post-nasal snout mound and is a distinctly larger species, e.g., FA typically greater than 42 mm; GSL greater than 18.8 mm vs. less than 17.3; C1-C1 greater than 5.7 mm vs. less than 4.9 mm; CM3 greater than 7.0 mm vs. less than 6.2 mm.

Diffs from *N. major* tor which has a low post-nasal snout mound; has a more reduced M3; has a longer and more elongate baculum shaft, and averages larger for body and skull dimensions (see Parnaby, 2009).

Diffs from *N. arnhemensis* Johnson, 1959 which has relatively shorter ears (less than 24 mm), a relatively smaller postnasal snout mound. If further differs from that species in relatively much larger auditory bullae, distal tip of the baculum forms a simple point compared to a bifid tip in *N. arnhemensis*, and the latter species has relatively much smaller urethral lappets.
Figure 4. Relatively greater inflation of the braincase of left, the holotype of *N. holtorum* sp. nov. (WAM M.64188), compared with *N. gouldii* (AM M.51228). Both are adult males with equal GSL.
Etymology. Named in honour of the late Dr John Holt and Mrs Mary Holt in recognition of their generous long-term support of Australian biodiversity research and conservation.

Distribution. Restricted to four IBRA regions in far southwestern Western Australia (Fig. 6). We are aware of only one voucher-based locality record from the southern Avon Wheatbelt (from the Tambellup district), a region that has been extensively cleared of native vegetation. The specimen (WAM M.593) was collected by F. R. Bradshaw and registered in 1923 (probably Frederick Robert Bradshaw of Tambellup, Whittell, 1954). The species is primarily found in taller marri and jarrah forests with a dense shrubby understory. Two other Nyctophilus species are sympatric with Nyctophilus sp. nov., N. major and N. geoffroyi.

Common name. Holt’s Long-eared Bat.

Discussion

Here we have elevated Western Australian populations previously considered to be N. gouldi sensu lato to species status (N. holtorum sp. nov.) based primarily on a lack of monophyly and substantial DNA sequence divergence. We believe this arrangement more accurately reflects the long isolation of the southwest WA populations from those in eastern Australia through the presence of the arid Nullarbor Barrier and is consistent with the recognition of eastern and western species of Falsistrelles (Kitchener et al., 1986), and a range of other mesic-adapted taxa including potoroos (Frankham et al., 2012), honeymeanes (Toon et al., 2010) and black cockatoos (White et al., 2011).

Our two samples of N. daedalus and those of N. holtorum sp. nov. form a separate clade from N. gouldi sensu stricto in the maximum likelihood trees. Several possibly sympatric species are thought to be included within N. daedalus and specimens currently assigned to that species exhibit extensive morphological variability (see Parnaby, 2009). Both of our genetic samples of N. daedalus are from the Pilbara but whether they represent N. daedalus sensu stricto, type locality Daly River, Northern Territory, remains to be determined. The relationships of N. holtorum sp. nov. to the N. daedalus “complex” cannot be resolved here and the morphological criteria given in our diagnoses between these two species will need to be refined once more extensive sampling across northern Australia and analysis has been conducted. However, it appears that N. holtorum sp. nov. is closer genetically to Pilbara populations of “N. daedalus” than to N. gouldi from eastern Australia.

We have not established a single diagnostic morphological criterion to distinguish N. holtorum sp. nov. from N. gouldi sensu stricto using the traditional morphological comparisons used in this study but suspect that a more comprehensive comparative analysis or statistical analysis (e.g., discriminant function analysis) using larger samples will do so. Although numerous differences are apparent from our skull illustrations of the two species, occasional examples of N. gouldi sensu stricto display morphology more typical of N. holtorum sp. nov. Differences in skull shape, particularly the more inflated braincase of N. holtorum sp. nov. that were apparent from direct skull comparisons, were not adequately reflected in our data set and geometric morphometric techniques might better explore cranial differences between these species. We also noticed a trend for a relatively shorter mesopterygoid fossa in N. holtorum sp. nov. that should be assessed using larger samples. A more refined definition of morphological differences between N. holtorum sp. nov. and N. gouldi sensu stricto might also emerge from a clearer understanding of the substantial variation apparent in eastern Australian N. gouldi. Our results support previous assessments that populations of N. gouldi from inland Victoria (Lumsden & Bennett, 1995) and NSW and Qld (Churchill et al., 1984; Parnaby, 1987) have smaller average body size than those from higher rainfall areas of coastal and montane regions. Churchill et al. (1984) believed that inland and northern Queensland N. gouldi were a separate species, although the limited analysis by Parnaby (1987) failed to find consistent morphological differences between inland and mesic regions. A previous assessment of morphological variation in N. gouldi and similar taxa (Parnaby, unpublished) concluded that variation between western and eastern Australian N. gouldi sensu lato equated at most to subspecific differences. A comprehensive examination of the status of the small eastern form of N. gouldi is therefore warranted. Our maximum likelihood trees (Fig. 1) included samples of two small N. gouldi from eastern and central Pilliga Forests of NSW, an interzone between faunal elements from inland and eastern faunas. The two Pilliga individuals nested within remaining samples of N. gouldi from montane and coastal areas of NSW. This suggests that, at least in NSW, the inland form represents a reduction in average body size in populations of N. gouldi, presumably in response to environmental variables but this needs to be corroborated by adequate sampling across the inland range of the species.

Our maximum likelihood trees also reveal significant new insights into relationships of three other species of Nyctophilus. Lineages of N. geoffroyi from western and eastern Australia were paraphyletic and showed an average divergence of 10.4% in the COI data, indicating that two species also exist within N. geoffroyi. Nomenclatural revision is therefore required but the allocation of names requires taxonomic decisions that are best left to a comprehensive taxonomic assessment and improved genetic sampling across the transcontinental distribution of this taxon. Specifically, type localities will need to be assigned to the earliest available names but such decisions are beyond the scope of the current study. Of the five available names that are currently synonyms of mainland N. geoffroyi Leach, 1821, type localities cannot be determined for the two earliest names, beyond “Australia” for geoffroyi and “Islands of the Pacific” for pacificus Gray, 1831 (see Mahoney & Walton, 1988). The type locality of the next
available name *australis* Peters, 1861, was given as Western Australia in the original description but Iredale & Troughton (1934) have suggested that this is an error for Sydney, but without giving reasons. More extensive sampling would also be required of *N. geoffroyi* from central and northern Australia.

Our exemplars of *N. bifax* and *N. arnhemensis* from opposite sides of the continent, had an average divergence of 1.4% in our COI data (2.5% for CytB). This level of divergence is at the lower end of interspecific variation (Bradley & Baker, 2001), some bat taxa that are recognized as full species on morphological data have similarly low average divergence. For example, levels of DNA divergences between the recognized species of Malagasy *Miniopterus* (family Miniopteridae) ranged from 2.5 to 12.9% (Christidis et al., 2014) using CytB which typically has a higher mutation rate than COI. The specific distinction of *N. bifax* and *N. arnhemensis* has not been questioned in the past but requires further investigation. Both can be difficult to distinguish morphologically in the Gulf of Carpentaria, Queensland and a preliminary morphological assessment suggests that both might be present on Cape York Peninsula where *N. bifax* appears to have a consistently longer skull (H. Parnaby, unpublished). Again, a more detailed morphological and genetic analysis with expanded sampling is required to address these issues.

In comparison to *N. gouldi* sensu stricto, comparatively little is known of the biology of *N. holtorum* sp. nov. Similar to several other species of *Nyctophilus*, *N. holtorum* sp. nov. has low intensity, high frequency echolocation calls and slow, highly manoeuvrable flight, suited to its typical foraging habitat inside stands of densely cluttered vegetation (Bullen & McKenzie, 2001; 2002). McKenzie (cited in Pennay et al., 2008) suggested that a shrubby understorey is an essential habitat element for *N. holtorum* sp. nov. and hollows in large old trees are a critical roost resource for the species (Webala et al., 2010; Burgar et al., 2015). There are two published studies of diet (Fullard et al., 1991; Burgar et al., 2014) but little is known of its reproductive biology.

Of the 36 extant species of Vespertilionidae recognized from Australia (Jackson & Groves, 2015), two of the three species with the most limited geographic range are restricted to the forest region of far southwestern WA. A third species, the Flute-nosed bat *Murina florium* Thomas, 1908 is localized in the rainforests of northern Queensland (Woinarski et al., 2014). In addition to *N. holtorum* sp. nov., the Western Falsistrelle *Falsistrellus mackenziei* Kitchener, Caputi and Jones, 1986 is also restricted to the southwestern forest region. A further taxon, *N. major major*, is possibly specifically distinct from the parapatric *N. major tor* Parnaby,
2009 from more arid environments, and is also largely restricted to the region.

Long-eared bats with low intensity echolocation calls that are “gleaners” in cluttered vegetation have been identified globally as a vulnerable group (Safi & Kerth, 2004) and this will apply to species of Nyctophilus. Several studies of N. gouldi sensu stricto have identified aspects of its biology that suggest the species is vulnerable to population decline and populations are believed to be declining (Pennay et al., 2008). Populations in South Australia and adjacent eastern Victoria were shown to be adversely affected by habitat fragmentation and it is possible that N. holtorum sp. nov. might be similarly sensitive. Law et al. (1999) found that the species is likely to be affected by habitat fragmentation on the southwestern slopes of NSW. Fuller (2013) showed decreased genetic diversity and elevated measures of inbreeding in population isolates in South Australia and adjoining southeastern Victoria, in contrast to N. geoffroyi from the same area, which showed limited impacts of fragmentation. Threlfall et al. (2012) found that N. gouldi avoided urban environments and concluded that it was an urban sensitive species. Lunney et al. (1988) found that hollows in large, old eucalypts were an essential habitat component for maternity colonies of the species and Rueegg et al. (2018) suggest a need for high densities of hollows large enough for colony formation. Corben’s Long-eared Bat (Nyctophilus corbeni) is listed as Vulnerable under Australian Federal legislation. That species is sensitive to habitat fragmentation, with remaining strongholds in NSW being centred on the largest remaining forest blocks (Turbill & Ellis, 2006). The species is also sensitive to adverse impacts to foraging and roosting habitat from fire (Law et al., 2016).

In concert with a relatively restricted geographic distribution, populations of N. holtorum sp. nov. face threats from loss of hollow trees, increased fire frequency and intensity and increased aridity arising from trends of gradual drying that have been documented during the past four decades (Climate Commission, 2014; CSIRO and BOM, 2020). Bullen (2008) suggested that the Western Falsistrelle Falsistrellus mackenziei, a species with a similar distribution to N. holtorum sp. nov., has contracted from western areas of its former geographic range during the past few decades, which he attributed to a trend in increasing drying in southwestern WA.

Gould’s long-eared bat sensu lato is currently listed as Least Concern on the IUCN Red List (Pennay et al., 2008). It is listed as Endangered under South Australian legislation, where the species has a limited distribution, but is not listed under any threat category in the other States in which it occurs, including Western Australia. The Extent of Occurrence of N. holtorum sp. nov. based on the polygon method using localities given in Fig. 6, exceeds the upper threshold for Vulnerable of 20 K km² under Red List criterion B (IUCN, 2012). This estimate assumes that the species is still present at those localities. Only six of the 26 locality records post-date 1999, four from the Warren bioregion and two from the Jarrah Forest bioregion. We do not know if this reflects a distributional decline or a decline in the retention of vouchers. A comprehensive survey of the species present distribution is an essential step towards effective conservation management of the species.

We recommend that a detailed review of the conservation status of N. holtorum sp. nov. is required because the species could be vulnerable to significant decline due to its restricted geographic occurrence, dependence on tree hollows that take centuries to form, and by implication, the known vulnerabilities of other Nyctophilus species to increased fire frequency and intensity, and habitat fragmentation, all of which are predicted to increase. Investigations of the biology and conservation management of this species should be a priority. Given that the species cannot be reliably identified from other Nyctophilus species based on call characteristics (Wentzel et al., 2019), surveys should be based on live capture using mist nets or harp traps. This would also provide an opportunity to take wing biopsies from the captured individuals to genetically confirm their identification and facilitate investigation of the presence of genetic structuring within their fragmented habitat in southwest WA.
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Appendix

**Specimens from which DNA samples were used in genetic analyses**

*Nyctophilus arnhemensis*: WAM M.60490, WAM M.60491. *N. bifax*: AM M.23448. *N. daedalus*: AM M.22127, AM M.22128. *N. corbeni*: AM M.25355, AM M.25356. *N. geoffroyi (NSW)*: AM M.47969, AM M.49337, AM M.49338, AM M.49342, AM M.49360. *N. gouldi*: AM M.23966, AM M.45568, AM M.47801, AM M.47891, AM M.49333, AM M.49334, AM M.49363, AM M.51196. *N. holtorum* sp. nov.: WAM M.64188*, AM M.39807*, AM M.39811*, AM M.39812*.

**Specimens included in statistical analyses**

*Nyctophilus gouldi*: Males (total, 79): AM: M.3041, M.3912, M.5514, M.8471, M.11217, M.11521, M.11656, M.13171, M.13227, M.13389, M.13393, M.13403, M.13406, M.13577, M.14104, M.14108, M.14110, M.16007, M.16008, M.16009, M.16010, M.16011, M.16016, M.16017, M.16019, M.16020, M.16021, M.16029, M.16036, M.25789, M.26490, M.26495, M.34151, M.35510, M.35511, M.36756, M.37647, M.51128, AM M.51308, ANNH: 66147. ANWC: CM1895, CM2033, CM2062, CM2077, CM2109, CM2334, CM2345, CM2382, CM2387, CM4029, CM573, CM591, MV: C25355, C25356, C25360, C25366, C25367, C25374, C25643, C26953, C26954, C26956, C26957, C26961, C26966, C26967, C26968, C26975, C26976, C26977, C26978. QM: J20378, J6184, J6185, J6221, J6303, JM06, JM5248, JM5360, JM5361, JM5364. Females (total, 53): AM: M.12967, M.13228, M.13235, M.13380, M.13410, M.14114, M.14116, M.14117, M.14181, M.15985, M.15986, M.15987, M.15988, M.15989, M.16023, M.16024, M.16030, M.16750, M.27221, M.27224, M.3414, M.34875, M.3545, M.36882, M.37664, M.37718, M.51216, M.5450, M.5956, M.7025. ANWC: CM1575, CM2323, CM574, CM612. MV: C25343, C26951, C26952, C26956, C26958, C26959, C26960, C26964, C26965, C26970, C26973, C26974. QM: J6302, JM1113, JM5359, JM5365, JM5366, JM5381.

*Nyctophilus holtorum* sp. nov.: Males (total, 12): AM M.4209, AM M.39809*, AM M.39813*, WAM M.5273, WAM M.5274, WAM M.5285, WAM M.5286, WAM M.16854, WAM M.16856, WAM M.16858, WAM M.19164*, WAM M.24546.

* Specimen is part of type series