Records of the Australian Museum (2021)
vol. 73, issue no. 1, pp. 53–66
https://doi.org/10.3853/j.2201-4349.73.2021.1766

Records of the Australian Museum
a peer-reviewed open-access journal
published by the Australian Museum, Sydney
communicating knowledge derived from our collections
ISSN 0067-1975 (print), 2201-4349 (online)

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

Keywords: cryptic species; new species; *Nyctophilus arnhemensis*; *Nyctophilus bifax*; *Nyctophilus daedalus*; *Nyctophilus geoffroyi*
Taxonomic registration: urn:lsid:zoobank.org:pub:BA8640CF-1E05-468D-8633-980DADE98BB6
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Received: 1 March 2020 **Accepted**: 15 February 2021 **Published**: 19 May 2021 (in print and online simultaneously)
Publisher: The Australian Museum, Sydney, Australia (a statutory authority of, and principally funded by, the NSW State Government)
Citation: Parnaby, Harry E., Andrew G. King, and Mark D. B. Eldridge. 2021. A new bat species from southwestern Western Australia, previously assigned to Gould's Long-eared Bat *Nyctophilus gouldi* Tomes, 1858. *Records of the Australian Museum* 73(1): 53–66.
https://doi.org/10.3853/j.2201-4349.73.2021.1766
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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), AAAAAGCTTCCATCCAACATCTC AGCATGATGAAA, H15149 (R) AAACTGCAGCCCCTC AGAATGATATTTGTCCTCA (Kocher *et al*., 1989); COI-BAK1490 (F) CTCAACCAACCACAAAGACATCGG, BAK2198 (R) TAGACTTCTGGGTGGCCGAAGAATCA (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). Thermo-cycling 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 LCOI490 (F) GGTCAACAAATCATAAAGATATTGG and HCO2198 (R) TAAACTTCAGGGTGACCAAAAAATCA (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 M^3; **C1-C1**, Outer breadth across upper canines from cingula; **PAL**, Palatal-sinual length, from the most posterior margin of the anterior palatial imargination to the most anterior margin of the interpterygoid fossa; **ZYG**, Zygomatic breadth, maximum breadth across zygomatic arches; **INT**, Least inter-temporal breadth; **M33**, Maximum breadth from left M^3 to right M^3, from labial cingula; **BRH**, Braincase height: calliper blade positioned along basioccipital-basisphenoid 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 interpterygoid 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 tubercle of the radius; **D3p1**, third metacarpal length and **D5p1**, firth 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 Palaeontological 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 vouchered *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							
Nyctophilus gouldi E	5.0	0.4						
Nyctophilus geoffroyi E	10.5	9.5	0.4					
Nyctophilus geoffroyi W	12.1	9.8	10.4	0.5				
Nyctophilus corbeni	11.4	8.7	10.2	7.1	0			
Nyctophilus bifax	7.8	7.5	11.9	10.6	11.5	—		
Nyctophilus daedalus	3.9	5.2	9.9	11.6	10.4	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

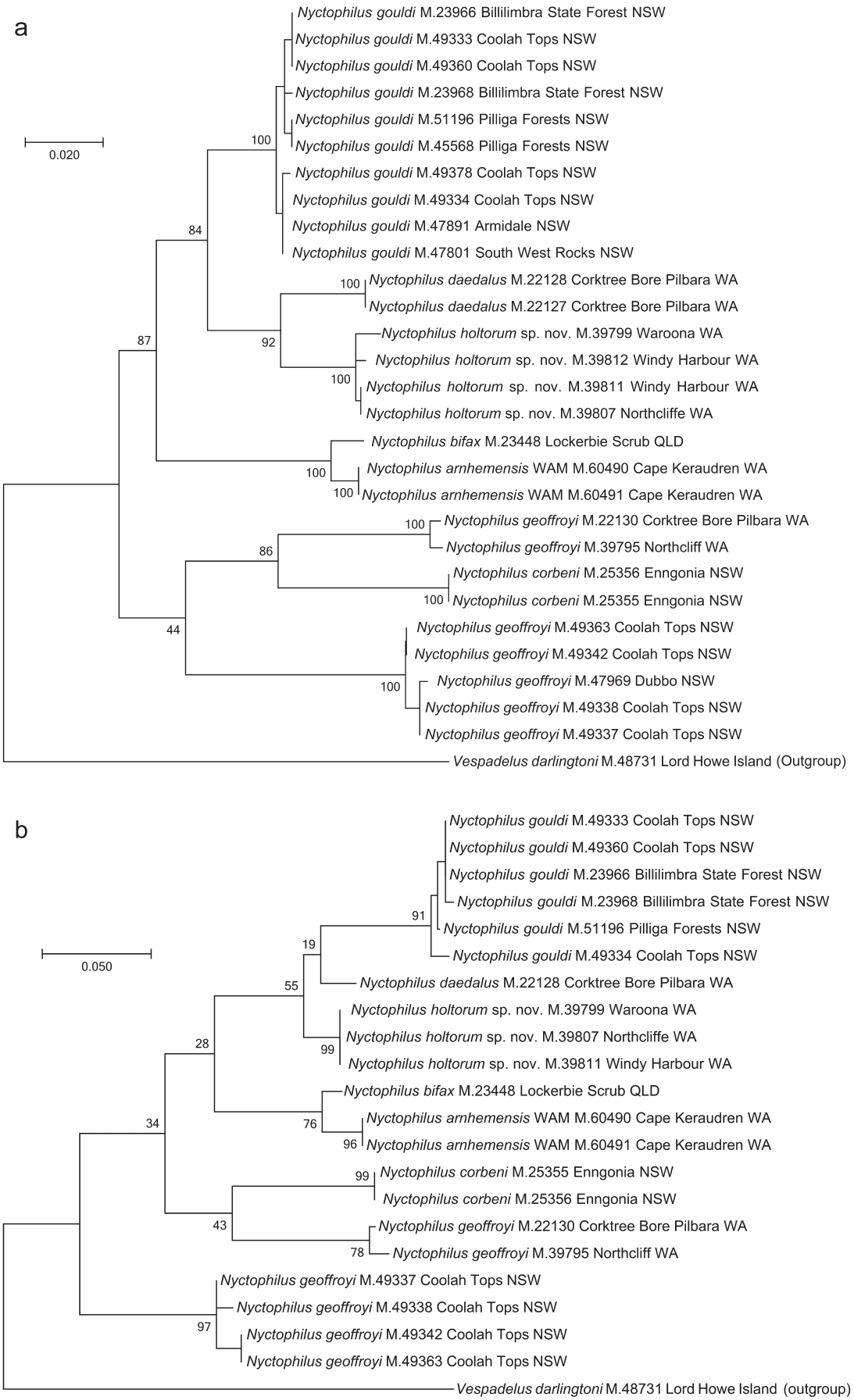

Figure 1. Highest maximum likelihood tree of relationships amongst *Nyctophilus* using: (*a*) 28 COI haplotypes, and (*b*) 21 CytB haplotypes. Bootstrap support (%) is shown at branch nodes. All M numbers refer to AM specimens except where indicated.

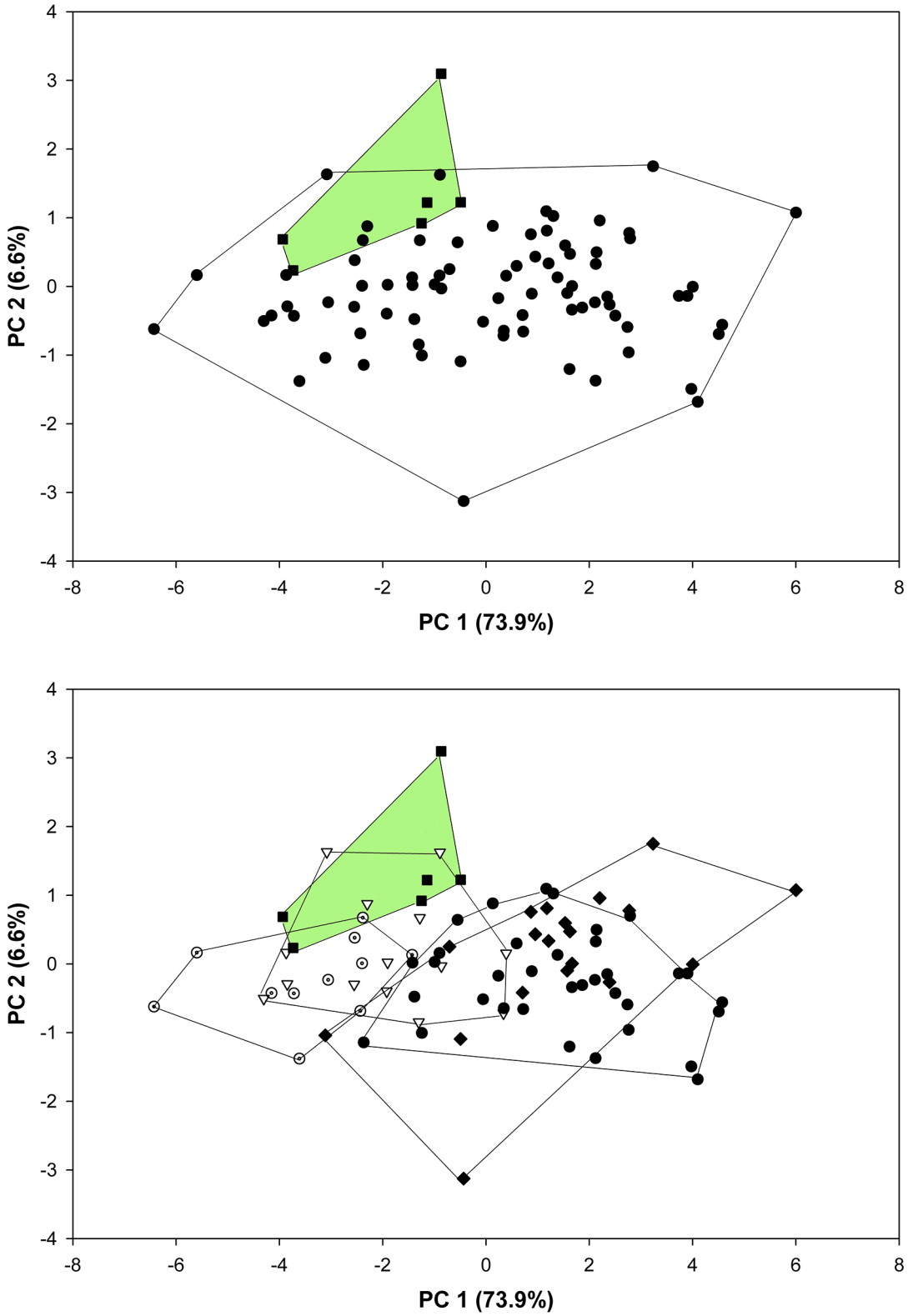

Figure 2. Specimen scores on the first two axes of a PCA based on a correlation matrix of nine cranial characters of adult male *N. holtorum* sp. nov. (squares, n = 6) and *N. gouldi* sensu stricto (circles, n = 79): (*a*) PCA scores grouped by species, and (*b*) same plot with *N. gouldi* coded by four 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*						
	males							males						
	mean	SE	SD	CV	min	max	n	mean	SE	SD	CV	min	max	n
CON	15.177	0.163	0.398	2.62	14.55	15.5	6	15.835	0.065	0.61	3.85	14.2	17.2	88
GSL	16.602	0.161	0.395	2.38	16.0	16.9	6	17.229	0.071	0.649	3.76	15.6	18.5	83
CM3	5.867	0.082	0.2	3.41	5.51	6.1	6	6.298	0.026	0.242	3.85	5.8	7.0	88
C1-C1	4.486	0.052	0.138	3.07	4.3	4.68	7	4.72	0.024	0.219	4.65	4.2	5.3	87
ZYG	9.955	0.096	0.235	2.36	9.63	10.17	6	10.153	0.041	0.384	3.78	8.9	11.1	87
INT	3.753	0.068	0.167	4.44	3.57	4.03	6	3.686	0.017	0.152	4.11	3.2	4.1	82
M33	6.282	0.325	0.796	12.67	4.68	6.86	6	6.755	0.031	0.275	4.07	6.0	7.5	81
BRH	5.983	0.092	0.224	3.75	5.73	6.31	6	6.096	0.027	0.251	4.11	5.4	6.7	84
MASB	8.892	0.108	0.265	2.98	8.53	9.16	6	8.93	0.036	0.333	3.73	8.2	9.7	86
BTB	1.665	0.079	0.159	9.5	1.5	1.8	4	1.559	0.018	0.147	9.41	1.23	1.97	66
BUL	3.835	0.076	0.152	3.97	3.69	4.05	4	4.001	0.017	0.142	3.56	3.69	4.26	67
BAS	5.472	0.151	0.303	5.53	5.09	5.8	4	5.838	0.038	0.311	5.32	5.17	6.56	67
PAL	6.273	0.062	0.152	2.42	6.0	6.4	6	6.25	0.154	0.378	6.05	5.9	6.9	6
EAR	25.95	0.348	1.102	4.25	24.5	27.7	10	27.165	0.178	1.94	7.14	23.5	32.1	119
FA	39.858	0.255	0.885	2.22	38.5	41.1	12	39.709	0.181	1.969	4.96	34.8	42.6	119
D3p1	37.236	0.278	0.878	2.36	36.3	38.5	10	38.519	0.191	2.038	5.29	33.7	42.9	114
D5p1	36.455	0.291	0.919	2.52	35.3	37.7	10	38.082	0.195	2.056	5.4	33.5	42.6	111
HL	18.711	0.216	0.647	3.46	17.8	19.9	9	19.809	0.115	1.207	6.09	17.0	22.6	110
	females							females						
	mean	SE	SD	CV	min	max	n	mean	SE	SD	CV	min	max	n
CON	15.61	0.108	0.216	1.38	15.37	15.83	4	16.225	0.059	0.444	2.74	15.4	17.1	57
GSL	17.028	0.069	0.139	0.82	16.87	17.18	4	17.639	0.069	0.508	2.88	16.5	18.7	54
CM3	6.096	0.047	0.106	1.73	5.96	6.21	5	6.454	0.026	0.199	3.09	6.1	7.0	57
C1-C1	4.697	0.034	0.082	1.75	4.6	4.8	6	4.895	0.029	0.22	4.5	4.5	5.4	57
ZYG	10.448	0.087	0.173	1.66	10.27	10.66	4	10.481	0.038	0.282	2.69	9.8	11.1	56
INT	3.696	0.06	0.134	3.61	3.47	3.8	5	3.735	0.022	0.161	4.3	3.3	4.12	55
M33	6.792	0.061	0.136	2.0	6.61	6.94	5	6.95	0.035	0.257	3.69	6.3	7.5	54
BRH	6.13	0.089	0.177	2.89	5.96	6.32	4	6.126	0.027	0.206	3.36	5.6	6.6	56
MASB	9.205	0.076	0.151	1.64	9.06	9.35	4	9.084	0.035	0.266	2.92	8.5	9.75	56
BTB	1.725	0.014	0.029	1.67	1.7	1.75	4	1.68	0.018	0.12	7.17	1.39	1.97	44
BUL	3.863	0.055	0.111	2.87	3.75	4.0	4	4.039	0.019	0.125	3.09	3.77	4.26	44
BAS	5.65	0.126	0.252	4.45	5.3	5.9	4	5.945	0.034	0.225	3.78	5.49	6.4	44
PAL	6.29	0.076	0.169	2.69	6.0	6.4	5	6.76	0.157	0.351	5.19	6.4	7.3	5
EAR	26.4	0.432	0.967	3.66	25	27.6	5	27.944	0.182	1.788	6.4	24.2	32.3	96
FA	41.378	0.427	1.282	3.1	39.5	43.7	9	42.13	0.194	1.934	4.59	36.7	45.3	99
D3p1	38.925	0.416	1.176	3.02	37.4	40.8	8	40.248	0.213	2.012	5.0	34.3	46.2	89
D5p1	38.213	0.281	0.794	2.08	36.9	39.2	8	39.761	0.2	1.868	4.7	34.9	43.5	87
HL	19.6	0.273	0.771	3.93	17.9	20.3	8	20.293	0.13	1.213	5.98	17.8	23.2	87

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	–0.690
CM3	0.332	–0.386	–0.320	–0.161	0.100	0.489	–0.019	0.600	–0.047
C1-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	8.659	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

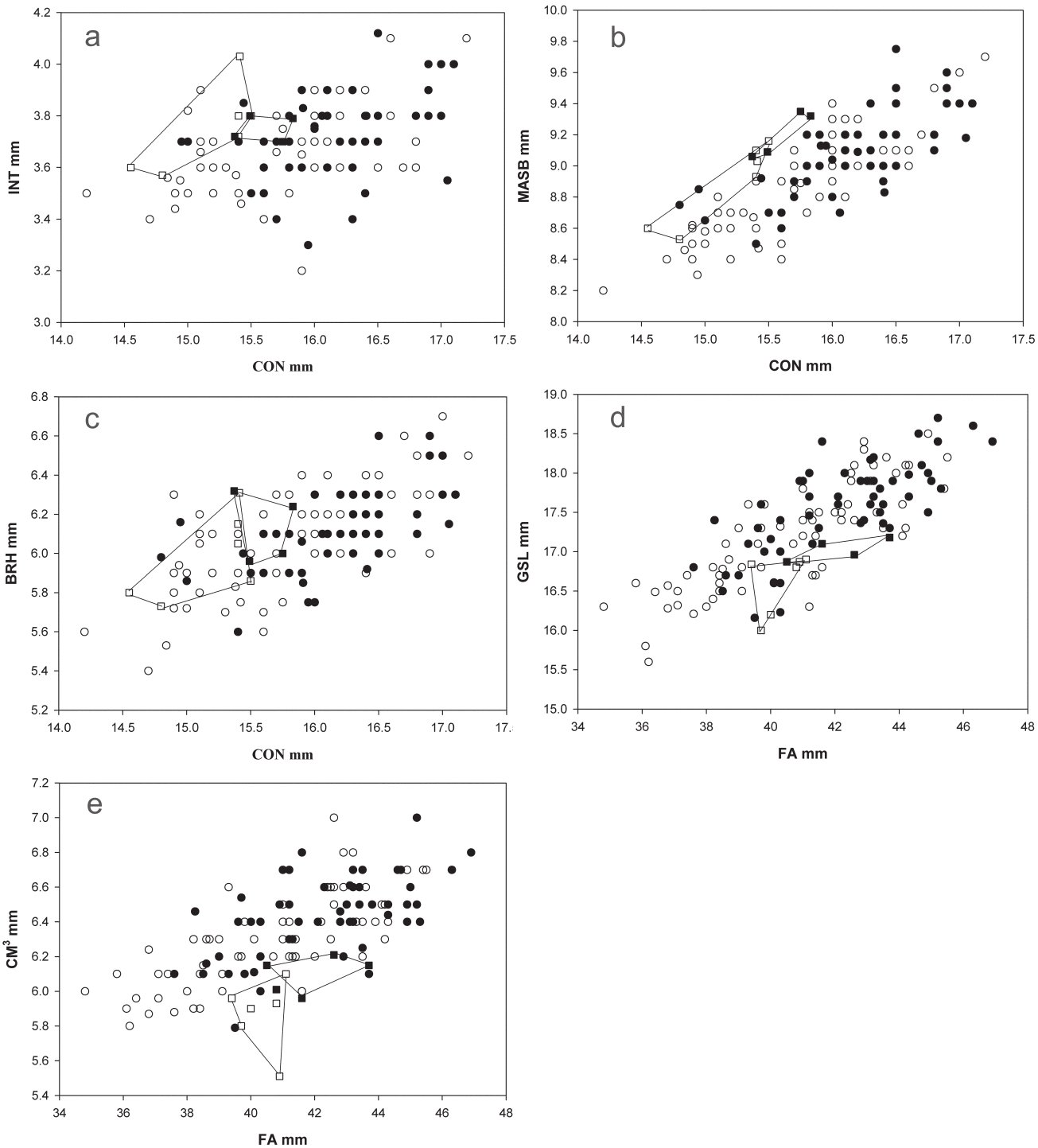

Figure 3. Bivariate plots (mm) showing overlap in measurements of adult *N. holtorum* sp. nov. (squares, polygons) and adult *N. gouldi* sensu stricto (circles). Solid symbols are females, open symbols male: (*a*), condylo-basal skull length (CON) vs. least inter-temporal breadth (INT); (*b*) CON vs. 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).

peripheral though extensively overlapped those of inland *N. gouldi* (not shown).

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 monophylly 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.

urn:lsid:zoobank.org:act:39AD0974-589C-4E9C-B075-E1D9A499AA7E

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).

Differs 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 M^3, i.e. the second and third "commissures" of M^3 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.

Differs 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.

Differs from *N. major tor* which has a low post-nasal snout mound; has a more reduced M^3; has a longer and more elongate baculum shaft, and averages larger for body and skull dimensions (see Parnaby, 2009).

Differs 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. gouldi* (AM M.51228). Both are adult males with equal GSL.

Figure 5. Frontal view of AM M.39811, male, paratype of *N. holtorum* sp. nov. showing enlarged dorsal snout mound posterior to the noseleaf. Scale 5 mm.

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 *N. holtorum* 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), honeyeaters (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

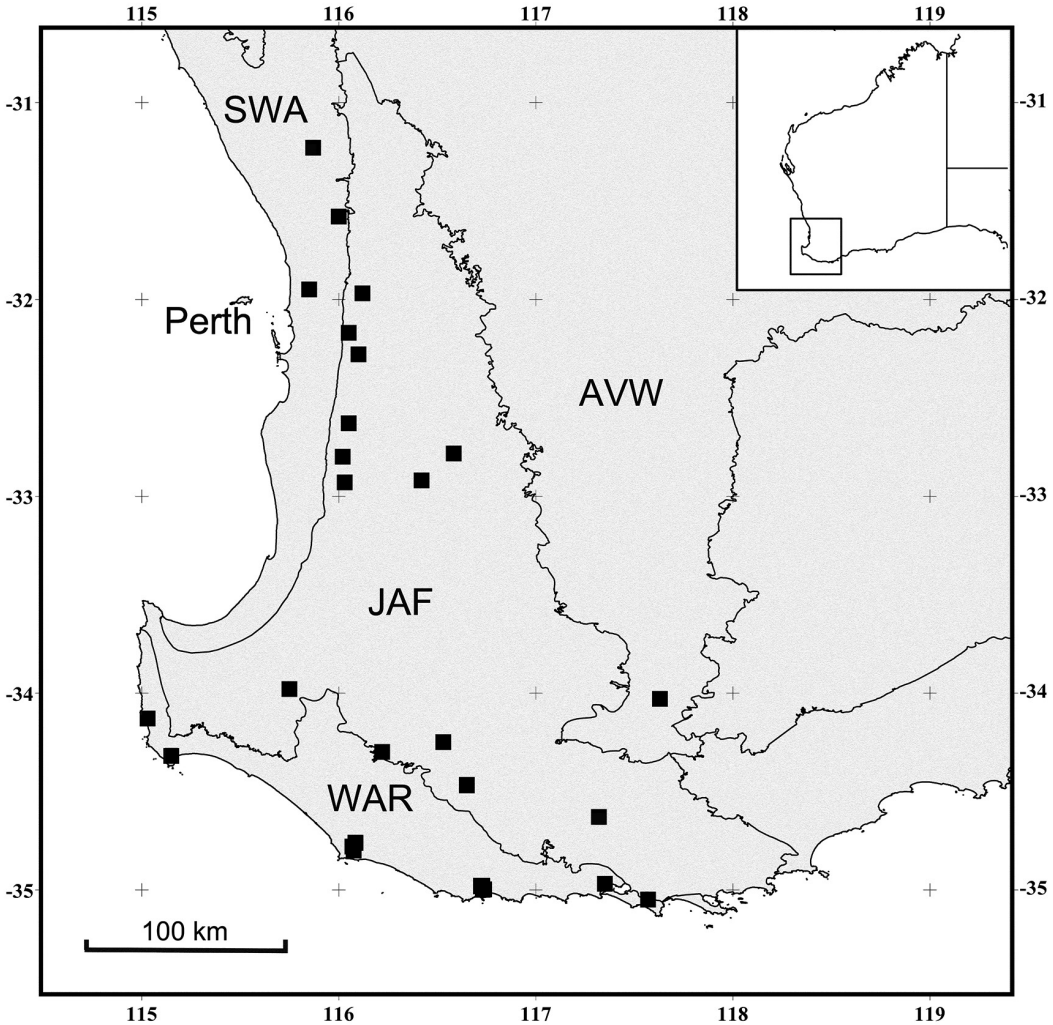

Figure 6. Distributional records of *N. holtorum* sp. nov. based on AM and WAM voucher specimens examined (n = 26 localities) and bioregional boundaries of the Interim Biological Regionalization Scheme (IBRS version 7; DSEWPAC, 2012). Regions are: AVW, Avon Wheatbelt; JAF, Jarrah Forests; SWA, Swan Coastal Plain and WAR, Warren.

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 Rueegger *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 km^2 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.

Acknowledgements. We thank Sandy Ingleby, Anja Divljan and Scott Ginn (Australian Museum); Kenny Travouillon and Melissa Taylor (Western Australian Museum); Heather Janetski (Queensland Museum); Leo Joseph (Australian National Wildlife Collection, Canberra) and staff of Museum Victoria for access to specimens and tissue samples in their care. Thanks to Dr Norm MacKenzie (then of CALM, Perth) whose guidance largely facilitated the success of field work for this study. Stacked imaging kindly provided by Ron Lovatt, Digivol volunteer (Australian Museum) with permission from Leonie Prater (Digivol Lab Co-ordinator). We applaud the constructive input from reviewers Jessica Worthington Wilmer (Queensland Museum) and Andrew Baker (Queensland University of Technology), and editor Shane McEvey. We thank Rebecca Johnson, Cameron Slatyer and David Alquezar for their support. Thanks to John Whittall (Area Manager, Baradine Office of Environment & Heritage) for expediting our field permits.

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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* (WA): AM M.22130, AM M.39795. *N. geoffroyi* (NSW): AM M.47969, AM M.49337, AM M.49338, AM M.49342, AM M.49363. *N. gouldi*: AM M.23966, AM M.23968, AM M.45568, AM M.47801, AM M.47891, AM M.49333, AM M.49334, AM M.49360, AM M.49378, 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.14111, M.16007, M.16008, M.16009, M.16010, M.16011, M.16016, M.16017, M.16019, M.16020, M.16021, M.16029, M.25789, M.26490, M.26495, M.34151, M.35510, M.36756, M.37647, M.51228, AM M.51308. AMNH: 66147. ANWC: CM1895, CM2033, CM2062, CM2077, CM2109, CM2334, CM2345, CM2382, CM2387, CM4029, CM573, CM591. MV: C25355, C25360, C25366, C25367, C25374, C25643, C26953, C26954, C26955, C26957, C26961, C26966, C26967, C26968, C26975, C26976, C26977, C26978. QM: J20378, J6184, J6185, J6221, J6303, JM506, JM5248, JM5360, JM5361, JM5364. **Females** (total, 53): AM: M.12967, M.13228, M.13235, M.13380, M.14106, M.14114, M.14116, M.14117, M.14118, M.14184, M.15985, M.15986, M.15987, M.15988, M.15989, M.16023, M.16024, M.16030, M.16750, M.27221, M.27248, M.3414, M.34875, M.3545, M.36882, M.37646, 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.4249, AM M.39806*, AM M.39807*, AM M.39810*, AM M.39811*, AM M.39812*, AM M.33382. AMNH 256866. WAM M.10036, WAM M.16861, WAM M.19079, WAM M.64188*. **Females** (total, 9): AM M.4209, AM M.39809*, AM M.39813*; WAM: WAM M.5273, WAM M.16854, WAM M.16856, WAM M.16858, WAM M.19164*, WAM M.24546.

* Specimen is part of type series