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A novel dataset to identify the endemic herpetofauna of the New Caledonia biodiversity hotspot with DNA barcodes

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Abstract. New Caledonia is the smallest global biodiversity hotspot, yet has one of the highest levels of endemism for an insular region of its size. Lizards are the dominant vertebrate fauna, and, while ecologically important, can be difficult to identify and many are in decline due to anthropogenic threats. As an aid to facilitate identification, we generated a near-complete DNA barcode dataset for New Caledonian lizards, consisting of 601 mitochondrial CO1 sequences of 100 of the 107 described lizards, and a number of yet undescribed species. We use this dataset to assess the performance of CO1 in delimiting species recognised by other, more extensive data and in recovering phylogenetic signal. Most species had intraspecific genetic distances ≤3.7%. Most comparisons between described species were at least ~5% divergent, with the exception of three pairwise species comparisons showing interspecific distances > 2.5%. Maximum likelihood CO1 trees of the six most speciose genera recovered each as monophyletic and, although discordant with previously published ND2 trees using quantitative topology tests, showed similar patterns of intraspecific and interspecific divergence, supporting the utility of CO1 in taxonomic identification and species delimitation. Some species showed overlap between intra- and interspecific pairwise distances, suggesting cryptic taxa, a finding also supported by species delimitation analyses using GMYC and mPTP. This dataset not only provides the basis for economical and reliable identification of New Caledonian lizards encountered during biodiversity assessments, but also provides a potential tool for investigating the identity of native lizards and their ecosystem interactions, even from partial remains.

Keywords: conservation, cytochrome *c* oxidase subunit I, DNA barcode, geckos, lizards, NADH dehydrogenase subunit 2, New Caledonia, phylogeny, skinks, systematics, threatened species.

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Introduction

Many islands in the Pacific Ocean are regarded as being part of internationally recognised biodiversity hotspots with exceptionally high levels of floral and faunal endemism and vegetation loss, and have become a research focus to identify the effects of global climate change and habitat alteration on native taxa (Myers *et al.* 2000; Mittermeier *et al.* 2004; Bellard *et al.* 2014). The distribution of this diversity has been shaped by the complex geological history of the Pacific Ocean's landmasses (Aitchison *et al.* 1995; Hall 1996, 2002; Neall and Trewick 2008; Lohman *et al.* 2011), involving interlandmass exchanges, vicariance events, and *in situ* speciation and radiations (Lohman *et al.* 2011; Blackburn *et al.* 2013; Grismer *et al.* 2016; O'Connell *et al.* 2018; Tallowin *et al.* 2020). Although advances in molecular techniques and computational power have provided a powerful mechanism to more comprehensively identify the Pacific regional biodiversity, there is still a need to quickly and efficiently identify these species, especially given the global rise in extinction rates and the sensitivity of insular populations to extirpation and extinction (Keppel *et al.* 2014; Pimm *et al.* 2014). The conservation and maintenance of insular biodiversity has been concentrated on a number of biodiversity hotspots (e.g. Wulff *et al.* 2013; Françoso *et al.* 2015; Struebig *et al.* 2015). In this study, we perform a DNA barcoding assessment of the lizards of the New Caledonia biodiversity hotspot in the Pacific, the smallest listed in terms of area, but with one of the most species-rich and endemic insular lizard faunas in both relative and absolute terms.

New Caledonia is located in the south-west Pacific Ocean and comprises the New Caledonian mainland, or Grande Terre

(area = 16 664 km^2), numerous small satellite islands, and the larger Belep Islands, Isle of Pines, and Loyalty Islands (Fig. 1). The Grande Terre is dominated by a series of mountain chains with elevations of some peaks reaching 1600 m above sea level. The island's subtropical climate supports a highly endemic and heterogeneous flora with metalliferous soils in ultramafic regions (Jaffré 1992; Isnard *et al.* 2016). The diverse New Caledonian biota was once thought to be a product of the islands' ancient Gondwanan heritage or from long-distance dispersal from neighboring regions (Morat *et al.* 1986; Pole 1994), but recent geological evidence indicates a marine transgression event, with reemergence from inundation in the Oligocene (~37 mya) (Grandcolas *et al.* 2008; Cluzel *et al.* 2012; Nattier *et al.* 2017), followed by subsequent colonisations, *in situ* radiations, and adaptations of terrestrial flora and fauna (Nattier *et al.* 2013; Pillon *et al.* 2014; Paun *et al.* 2016; Skipwith *et al.* 2016). With no native non-volant land mammals on the islands, the native herpetofauna, comprising primarily diplodactylid geckos and eugongyline skinks (Bauer and Sadlier 2000), are the dominant land vertebrates. Of the 107 described lizard species present in the region (Uetz *et al.* 2020), 101 are endemic, or nearly so; the skink *Caledoniscincus atropunctatus* and gekkonid gecko *Gehyra georgpotthasti* have also been found on some of the adjacent islands of Vanuatu. Six other species of gekkonid geckos also occur in New Caledonia, but these are all widespread in the Pacific and are known or considered likely to have been introduced.

Invasive species and anthropogenic activities have all caused declines in gecko and skink populations in New Caledonia, and in combination with the impacts associated with climate change, are likely to lead to further declines. As a consequence, many taxa are threatened under International Union for Conservation of Nature (IUCN) criteria; a majority (64%) are listed as vulnerable (VU; IUCN threat status) and 20% of described species have had an increase in threat status or were evaluated as threatened since 2011 (IUCN 2018). Agricultural activities, forest fires, and nickel mining operations have resulted in habitat loss and fragmentation (McCoy *et al.* 1999; Pascal *et al.* 2008; Jaffré *et al.* 2010; Schroers and Tron 2013; Pouteau and Birnbaum 2016; Ibanez *et al.* 2017), as have introduced deer, pigs, and cattle (Bouchet *et al.* 1995; Gargominy *et al.* 1996; Robinet *et al.* 1998; Rouys and Theuerkauf 2003). Directly affecting the herpetofaunal populations, invasive ants and rodents attack and prey on the native lizards and their eggs and eliminate lizard dietary sources, leading to a decrease in local diversity and abundance (Jourdan *et al.* 2001; Palmas *et al.* 2017; Thibault *et al.* 2017). Among the most significant of threats are the feral cats that use geckos and skinks as food sources and significantly endanger populations (Rouys and Theuerkauf 2003; Palmas *et al.* 2017).

The systematics and taxonomy of the New Caledonian lizard fauna are still dynamic, with 45 species being described or undergoing taxonomic change in the past two decades, almost doubling the endemic lizard fauna (Bauer *et al.* 2006; Smith *et al.* 2007; Skipwith *et al.* 2014; Sadlier *et al.* 2009, 2014*a*, 2014*b*, 2014*c*, 2015). The molecular phylogenies generated by these studies have been based primarily on the mitochondrial gene NADH dehydrogenase subunit 2 (ND2). However, although phylogenetically informative, its long length, need for taxon-specific primers, and sensitivity to PCR conditions makes it difficult to amplify and sequence. As such, the use of shorter, yet phylogenetically informative loci with universal primers is an attractive alternative. DNA barcoding (sequencing of mitochondrial cytochrome *c* oxidase subunit I [CO1]) of animals has been used for the identification and delimitation of various taxonomic groups (Hebert *et al.* 2003; Clare *et al.* 2007), providing an alternative to traditional taxonomic approaches to accurately identify morphologically conserved taxa (Vences *et al.* 2012). Utilising CO1, individuals from populations can be lumped together as a single taxon or split into barcode 'species' if the genetic distance between CO1 sequences is below or above, respectively, a defined threshold value. Provided that there is more variation between species than within populations (i.e. the 'barcoding gap': Meyer and Paulay 2005), CO1 barcoding has proven to be a reliable species-specific marker and barcode gaps have been proven useful in inferring species boundaries (Nagy *et al.* 2012; Jeong *et al.* 2013; Čandek and Kuntner 2014; Shen *et al.* 2016; Vasconcelos *et al.* 2016). Although there may be a continuum of intra- and interspecific distances in certain scenarios, CO1 barcoding has been empirically shown to be a reliable species-specific marker and barcode gaps have been proven useful in inferring species boundaries and shedding light on regional biodiversity.

Reptile and amphibian DNA sequences are underrepresented in barcoding initiatives when compared with other vertebrate groups (Trivedi *et al.* 2016). Project Cold Code at the Southern China DNA Barcoding Center (Kunming Institute of Zoology) aims to barcode the world's amphibians and non-avian reptiles and increase the representation of these barcodes for a variety of uses (Murphy *et al.* 2013). We have generated the largest and most comprehensive DNA barcoding assessment to date of an insular vertebrate fauna, and the first DNA barcoding initiative for the rich, New Caledonian lizard fauna. We present and analyse this dataset here with the aims of (1) providing the most comprehensive barcode database as possible for the New Caledonian herpetofauna, (2) evaluating the performance of CO1 as a barcode for the New Caledonian herpetofauna by calculating pairwise genetic distances within and between species to determine whether barcode gaps potentially representing cryptic taxa were evident, (3) assessing the phylogenetic performance (informativeness) of CO1 in comparison to the widely employed and highly informative ND2 mitochondrial marker, and (4) determining whether the CO1 sequence data could also be meaningfully used to infer relationships between taxa.

Methods and materials

Sampling

Our sampling consists of 601 lizard specimens from New Caledonia, representing 100 of the 107 currently described species, 95 of which are native. Tissue samples from widespread and systematic collection efforts over 35 years used extensively in a diversity of published studies (see Bauer and Jackman 2006; Bauer *et al.* 2006, 2009, 2012; Sadlier *et al.* 2009, 2014*a*, 2014*b*, 2014*c*; Skipwith *et al.* 2014 and papers cited therein) were used to obtain genomic DNA. All but seven species of the native New Caledonian lizards were barcoded in this study. The gekkonid gecko *Gehyra georgpotthasti* and skinks *Kuniesaurus albiauris*,

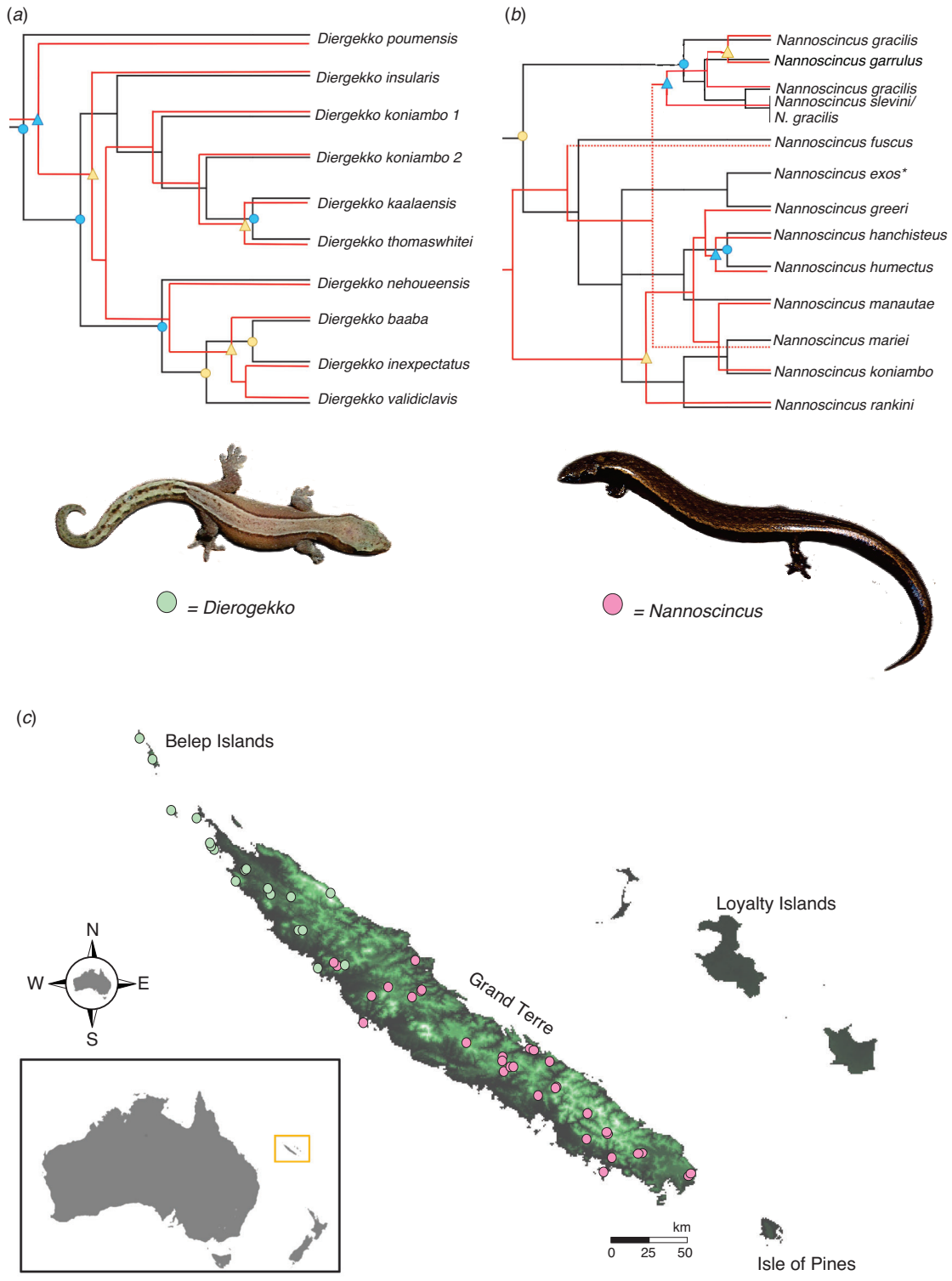

Fig. 1. Simplified CO1 (red) and ND2 (black) ML gene tree topology discordance for (*a*) *Dierogekko* and (*b*) *Nannoscincus*. Triangles and circles indicate bootstrap support values for CO1 and ND2 tree respectively; blue and yellow symbols indicate strong and moderate support respectively (no symbol = low support). Dashed branches have no phylogenetic meaning and are shown only for ease of viewing tree topology. Branch lengths have been edited for viewing purposes. (*c*) Map of New Caledonia and the surrounding Loyalty Islands, Belep Islands, and Isle of Pines. Map of the position of New Caledonia relative to Australia inset. Points represent sampling distribution of *Dierogekko* and *Nannoscincus* in the gene trees. Other generic trees, full locality data, and voucher specimen information are available in Supplementary Table S1. Note: *Nannoscincus exos* was not sequenced for CO1 but is represented in the ND2 tree. Specimen photo credits: *Dierogekko*: Aaron M. Bauer; *Nannoscincus*: Ross A. Sadlier.

Geoscincus haraldmeieri, *Phoboscincus bocourti*, *Phaeoscincus ouinensis*, *Epibator greeri*, and *Nannoscincus exos* were not sequenced as these taxa are known from one or only a few specimens (Bauer and Sadlier 2000) and tissue samples for most are not available. Of the 601 lizard samples selected, 211 of these represent specimens of the diplodactylid gecko genus *Bavayia*, which comprises 12 described and 27 putatively new species, identified on the basis of ND2 sequence data and morphological examinations from current research separate from this study (here referred to as the 'integrative taxonomy' approach: Bauer and Jackman 2006; Bauer *et al.*, unpubl. data). Although not endemic to New Caledonia, two species of snakes, the introduced typhlopid snake *Virgotyphlops braminus* (formerly *Indotyphlops braminus*) and the native sea krait *Laticauda laticaudata*, as well as the single (introduced) species of amphibian in New Caledonia, *Litorea aurea*, were also barcoded.

DNA extraction and sequencing

DNA was extracted from liver tissue and tail tips of wild-caught specimens using salt extraction protocols or Qiagen® DNeasy tissue extraction kits. Mitochondrial CO1 was amplified by polymerase chain reaction (PCR) with different combinations of primers: RepCO1-F/RepCO1-R, Chmf4/Chmr4, and CO1_C01(seq.)/CO1_C02/CO1_C03(seq.)/CO1_C04 (Che *et al.* 2012; Nagy *et al.* 2012), and verified using gel electrophoresis. Included in the reaction were 2.5 µL genomic DNA, 2.5 µL light strand primer, 2.5 µL heavy strand primer, 2.5 µL dinucleotide pairs, 2.5 µL 5X buffer, MgCl 10X buffer, 0.18 µL Taq polymerase, and 9.82 µL H_2O, using the primers listed above. PCR reactions were executed on an Eppendorf Mastercycler gradient theromocycler under the following conditions: initial denaturation at 94°C for 5 min, followed by a second denaturation at 95°C for 55 s, annealing at 49°C for 1 s, followed by a cycle extension at 72°C for 1:10 s, for 35 cycles, and a final extension step at 72°C for 10 min. All PCR products were visualised via 1.5% agarose gel electrophoresis. We purified PCR and sequencing products using SpeedBead magnetic carboxylate modified particles, and sent purified products to GENEWIZ facilities (South Plainfield, NJ, USA) and the Kunming Institute of Zoology, Chinese Academy of Sciences, to be sequenced on ABI 3730xl DNA analysers.

Phylogenetic analyses and species delimitation

While all barcode sequences contribute to our goal of providing a resource for future use in conservation, we only use genera (discussed below) with large sample sizes and multiple species for phylogenetic methods. Raw sequence data were aligned by eye and edited in Geneious® v7.1.7 with the MUSCLE alignment function with default parameters. Using currently accepted species boundaries established through morphology and (other) molecular data, we calculated inter- and intraspecific uncorrected pairwise distances for all lizard genera using the pairwise distance function in MEGA v7 (Kumar *et al.* 2016), to determine whether a barcoding gap exists.

To assess the phylogenetic utility of CO1 trees, maximum likelihood (ML) genealogies of the six most speciose lizard genera were reconstructed to ensure barcoded specimens formed clades that have also been recovered in published, well-supported ND2 and/or multilocus trees using the same specimens (Bauer and Jackman 2006; Skipwith *et al.* 2014; Sadlier *et al.* 2009, 2014*a*, 2014*b*, 2014*c*). ML methods were used (1) to obtain a point estimate of evolutionary relations, incorporating evolutionary models of nucleotide substitution and evolutionary rate heterogeneity parameters, and (2) draw comparisons to previously published analyses using different markers on the same specimens. We created CO1 gene trees for two genera of geckos (*Bavayia* and *Dierogekko*) and four genera of skinks (*Caledoniscincus*, *Nannoscincus*, *Marmorosphax* and *Sigaloseps*) with RAxML v8.2.12 (Stamatakis 2014) for 1000 bootstrap iterations under a GTRGAMMA molecular model of evolution, which accounts for rate heterogeneity amongst lineages in a phylogeny. Bootstrap values of 70–100 were interpreted as providing moderate to strong branch support (Hillis and Bull 1993). Outgroups and sample sizes for each tree are available in Supplementary Table S1.

To statistically compare the generic CO1 topologies to the respective ND2 topologies, we ran Shimodaira–Hasegawa (SH) (Shimodaira and Hasegawa 1999) and approximately unbiased (AU) (Shimodaira 2002) tests in PAUP* v4.0 (Swofford 2003) and CONSEL (Shimodaira and Hasegawa 2001). The SH, AU, and CONSEL topology tests evaluate if significantly different topologies exist by comparing a phylogenetic tree with its own and another DNA alignment matrix (in this case, assessing a CO1 tree with an ND2 dataset and an ND2 tree with a CO1 dataset). We utilise these methods on all trees to evaluate the degree of phylogenetic signal and informativeness above the species level in CO1, as has been done with reptiles before (e.g. *Cyrtodactylus* geckos: Brennan *et al.* 2017).

Additionally, to assess the efficacy of CO1 divergences (the barcode gap) for delimiting morphologically similar, cryptic taxa in particular, we ran multiple species delimitation analyses on *Bavayia*. This genus has the highest number of species, many of them as yet undescribed. Thus, we use *Bavayia* as a model genus to see if both DNA barcoding and species delimitation analyses suggest higher levels of diversity already identified by integrative methods, but not yet described. A general mixed Yule coalescent approach (GMYC) was implemented in the R package SPLITS in R v3.6.1 (Ezard *et al.* 2009; R Core Team 2019); due to its greater accuracy, the single-threshold approach was used instead of the multiple-threshold (Pons *et al.* 2006; Fontaneto *et al.* 2007; Monaghan *et al.* 2009; Fujisawa and Barraclough 2013). Because analyses such as GMYC use distances and thresholds, which ignore evolutionary relationships, we also ran a multirate Poisson tree process (mPTP) (Kapli *et al.* 2017) to compare species delimitation results between mPTP, GMYC, and integrative taxonomic approaches. The mPTP analysis was run using the 'multi' threshold option and a Markov Chain Monte Carlo (MCMC) approach for 100 million steps and a burn-in of 2 million steps. Both GMYC and mPTP were run on ultrametric CO1 trees of *Bavayia* under a Yule process and a relaxed lognormal clock in BEAST2 (Bouckaert *et al.* 2014). Sequences of CO1 for specimens in this study have been published on both GenBank and the Barcode of Life Database (BOLD) (see Supplementary Table S1 for accession numbers and barcode index numbers [BINs]).

Table 1. Ranges of interspecific and intraspecific uncorrected pairwise CO1 distances for specimens of each genus barcoded in this study

The number of species in this study for each genus are shown in parentheses. Sample sizes for individual species can be obtained from Supplementary Table S1. Intraspecific distances are provided in Supplementary Table S2. Intraspecific distance range does not apply for species with only one representative specimen

Genus (no. of species)	Interspecific distance range (%)	Intraspecific distance range (%)	No. of specimens (*n*)
Bavayia (12 + 27)	3.2–23.2[A]	0–9.3	211
Correlophus (3)	10.2–13.3	0	4
Dierogekko (9)	5.9–15.2	0–10.8	43
Eurydactylodes (4)	5.3–15.7	0.1	6
Hemidactylus (2)	23.0	0	3
Hemiphyllodactylus (1)	–	–	1
Lepidodactylus (1)	–	0	2
Mniarogekko (2)	7.1–7.3	0.2	3
Nactus (1)	–	0	2
Oedodera (1)	–	0–0.5	3
Paniegekko (1)	–	–	1
Rhacodactylus (4)	10.4–17.6	0–0.5	9
Caesoris (1)	–	–	1
Caledoniscincus (15)	1.2–20.0[A]	0.5–11.1	93
Celatiscincus (2)	11.1–12.4	0.2–1.8	4
Cryptoblepharus (1)	–	0.2	2
Emoia (2)	22.1–23.2	0–5.7	5
Epibator (2)	3.7–4.1	0.5–2.1	5
Graciliscincus (1)	–	0.4–1.3	3
Kanakysaurus (2)	10.1–10.3	0–0.3	4
Lacertoides (1)	–	0–0.3	4
Lioscincus (2)	17.3	0–3.8	5
Marmorosphax (5)	2.5–19.0	0–7.9	59
Nannoscincus (11)	0–18.4[A]	0–11.7	53
Phaeoscincus (1)	–	–	1
Phasmasaurus (2)	19.5–20.6	0.3–3.4	5
Phoboscincus (1)	–	1.4–3.0	3
Sigaloseps (6)	1.1 –18.7[A]	0–8.5	56
Simiscincus (1)	–	0	2
Tropidoscincus (3)	3.2–16.3	0.2–1.5	8

[A]Scenarios in which one or more species pairs do not show a barcode gap that is seen in the other members of the genus.

Results

Species delimitation

A total of 601 unique lizard CO1 sequences were generated, representing 95 described, native lizard species and five introduced gekkonid geckos (and an additional five sequences representing two snakes (*Laticauda laticaudata*, $n = 2$; *Virgotyphlops braminus*, $n = 1$) and one amphibian (*Litorea aurea*, $n = 2$)). The CO1 sequence length with the highest frequency was 669 base pairs (bp). The minimum and maximum bp counts in this study were 291 and 699 bp respectively. We obtained barcodes for all described species in our sampling and for 27 putatively new species of *Bavayia*. PCR amplification yielded moderate to high quality sequence of at least 500 bp, the minimum required for barcode compliance by BOLD (www.boldsystems.org), for 99% of the specimens in this study. About 87% (529 CO1 sequences) were barcode compliant (linked to BOLD accession numbers); all sequences from this study were deposited in BOLD. For all lizard specimens in this study, all 100 described species were assigned to BINs (595 of 601 sequences). Although a small percentage of specimens were amplified and sequenced using primers Chmf4/Chmr4, and CO1_C01(seq.)/CO1_C02/CO1_C03(seq.)/CO1_C04 (3.1% and 1% respectively), a majority of specimens (94.5%) were amplified and sequenced to ≥2X coverage using the primer pair RepCO1-F/RepCO1-R. Additionally, 1.4% of specimens were amplified by using both RepCO1 and Chm primers and RepCO1/CO1_C01/C02 primers.

Across the 127 described and putative lizard species barcoded in this study, 102 species (including putative species) were represented by multiple samples. Of these species, ~80% (82 species) had intraspecific distances no greater than 3.7% (Supplementary Table S2) and ~95% had barcoding gaps that distinguished them from their congeners (Table 1). Intraspecific CO1 distances for taxa within skink genera ranged from 0 to 11.7%, but most species fell below or near 3%. Seven skink species distributed across four genera showed large intraspecific distances, exceeding 7%, and may reflect the existence of cryptic species in *Caledoniscincus*, *Nannoscincus*, *Marmorsphax*, and *Sigaloseps* (See Discussion, Table 1, Supplementary Table S2).

For geckos, the intraspecific distances ranged from 0 to 10.8%, most of which were below 1%, with the exception of

several taxa in *Bavayia* and *Dierogekko* (Table 1). Two species in *Bavayia* and one *Dierogekko* had high intraspecific distances, which, similar to skinks, may be suggestive of undescribed species (Supplementary Table S2). The greatest interspecific CO1 distance range amongst all lizards was seen in geckos, between described species of *Bavayia* (3.2–23.2%) (Table 1), some of which have long been regarded as composite. All other geckos had smaller interspecific range values (Table 1), except *Dierogekko koniambo*, which had a maximum intraspecific distance of 10.8% (Table 1; Supplementary Table S2) and was polyphyletic in the CO1 phylogeny (Fig. 2), providing evidence that it may represent multiple species. For the other diplodactylids (*Rhacodactylus*, *Correlophus*, *Eurydactylodes*, and *Mniarogekko*) there was no overlap between maximum intraspecific distances and minimum interspecific CO1 distances (Table 1).

Only three species pairs, one in each of three genera of skinks, had interspecific distances less than 2.5% and lacked a barcoding gap. Low levels of interspecific variation were found between the sister species pairs *Nannoscincus gracilis* and *N. slevini* (0–1.2%), *Sigaloseps ruficauda* and *S. balios* (1.1%), and *Caledoniscincus aquilonius* and *C. terma s.s.* (1.2–5.5%).

Phylogenetic signal

The ML CO1 trees recovered *Bavayia*, *Dierogekko*, *Caledoniscincus*, *Nannoscincus*, *Sigaloseps*, and *Marmorosphax* as monophyletic (Figs 1, 2; see Supplementary Figs S1–S6). Nodes close to the root of each tree were poorly to moderately supported, while most intermediate and distal nodes were well supported. No bootstrap values in any of the CO1 trees were higher than those in respective branches in the ND2 trees. The SH/AU topology tests revealed that tree topologies between ND2 and CO1 datasets were significantly different from each other ($P < 0.05$), with the exceptions of *Dierogekko* and *Caledoniscincus* (Table 2). In all CO1 trees, sister group relationships consistent with the more robust ND2 data set were frequently recovered (Fig. 1, Supplementary Fig. S7), and, in the case of the *Bavayia* tree, so were the two major clades representing the *Bavayia cyclura* group and the *Bavayia sauvagii* group (similar to the ND2 dataset; see Supplementary Fig. S7). Reciprocally monophyletic groups within the skinks *M. boulinda* and *M. taom* were recovered with strong support. These clades represent populations from localities that are geographically distinct from their conspecifics, and had pairwise distances of 7–8% and 3–4%, respectively, to their sister clades (see Supplementary Fig. S4 for gene tree). *Nannoscincus mariei* also recovered geographically separate clades that were ~4–10% divergent from each other (see Supplementary Fig. S5). All species were recovered as monophyletic, with the exceptions of *N. gracilis*, *N. slevini*, *C. aquilonius*, *C. terma*, and *D. koniambo* (see Supplementary Figs S2, S3, S5). The case of *D. koniambo* includes specimens from two different (but proximate) localities having pairwise distances of 9.8–10.2%. In *Bavayia*, 12 clades representing formally described species and 27 clades that represent putative species were recovered in both the CO1 tree and ND2 trees (Bauer and Jackman 2006). The GMYC analysis recovered 56 species of *Bavayia* from the CO1 data, with a significant likelihood ratio of 20.68 ($P = 3.22 \times 10^{-5}$), while the mPTP supported 44 species. The GMYC analysis split 13 and lumped none of the *Bavayia* taxa, while mPTP split 7 and lumped 2 *Bavayia* taxa (Fig. 2). The undescribed lineages of *Bavayia* are taxa that have also been recognised on the basis of independent ND2 and morphological data (Bauer and Jackman 2006; Bauer *et al.*, unpubl. data).

Discussion

All of the endemic New Caledonian gecko and skink genera with multiple constituent species had well defined barcode gaps. Considering only polytypic genera, only three species pairs of the 52 scincids had interspecific distances below 3%; these instances were not unexpected based on prior studies of the respective taxa (discussed below). Due to the range in interspecific distances among geckos and skinks, it would be difficult to extrapolate any species delimitation threshold for taxa in these groups as a whole. However, there are cases within *Bavayia*, *Dierogekko*, *Caledoniscincus*, *Nannoscincus*, and *Marmorosphax*, where high levels of intraspecific divergence indicate the possible presence of putative cryptic species within currently recognised taxa. In the case of the paraphyletic *Dierogekko koniambo* and the divergent clades of *C. orestes*, *M. taom*, *M. boulinda*, and *N. mariei*, the monophyletic groups that were recovered are each from discreet geographic areas, and represent lineages that have evolved in allopatry or parapatry (see below). The greatest extent of cryptic diversity is within *Bavayia*. In addition to the 12 already-described taxa, a further 27 lineages have been identified as putative species using an integrative taxonomy approach (total = 39). The use of GMYC and mPTP further corroborates this; although the number of taxa differs for each approach, there is quantitative support of cryptic diversity in the CO1 tree that is significantly greater than currently described diversity (12 species). Analyses such as GMYC and mPTP provide a statistical framework for testing hypotheses of diversity across a group, and may even perform better than studies using 2% or 3% thresholds (Avise and Walker 1999; Hebert *et al.* 2003; Tang *et al.* 2012; Fujisawa and Barraclough 2013; Vasconcelos *et al.* 2016). For *Bavayia* this was not unexpected given each of the currently recognised taxa within the genus have long been recognised as containing a number of undescribed species (Bauer and Jackman 2006). While our GMYC and mPTP results indicate that there is undescribed diversity within *Bavayia*, subsequent morphological examination of voucher specimens and in some instances increased sampling is needed before it can be established that observed divergences represent distinct species; thus, we consider them as operational taxonomic units. It is worth noting that while BOLD algorithmically distributes BINs to cluster DNA sequences together, the number of lizard BINs in this study (212: see Supplementary Table S1) greatly exceeds the number of described and potentially undescribed species we recognise in our sampling and results (100 described species, several putative species or undescribed lineages). Because our analyses incorporate evolutionary models and do not solely rely on genetic distances, we do not consider BINs to be representative of species or operational taxonomic units.

Our analyses found a few cases of low levels of interspecific divergence between taxa, and where the barcode data would suggest the lumping of these described species. These represent

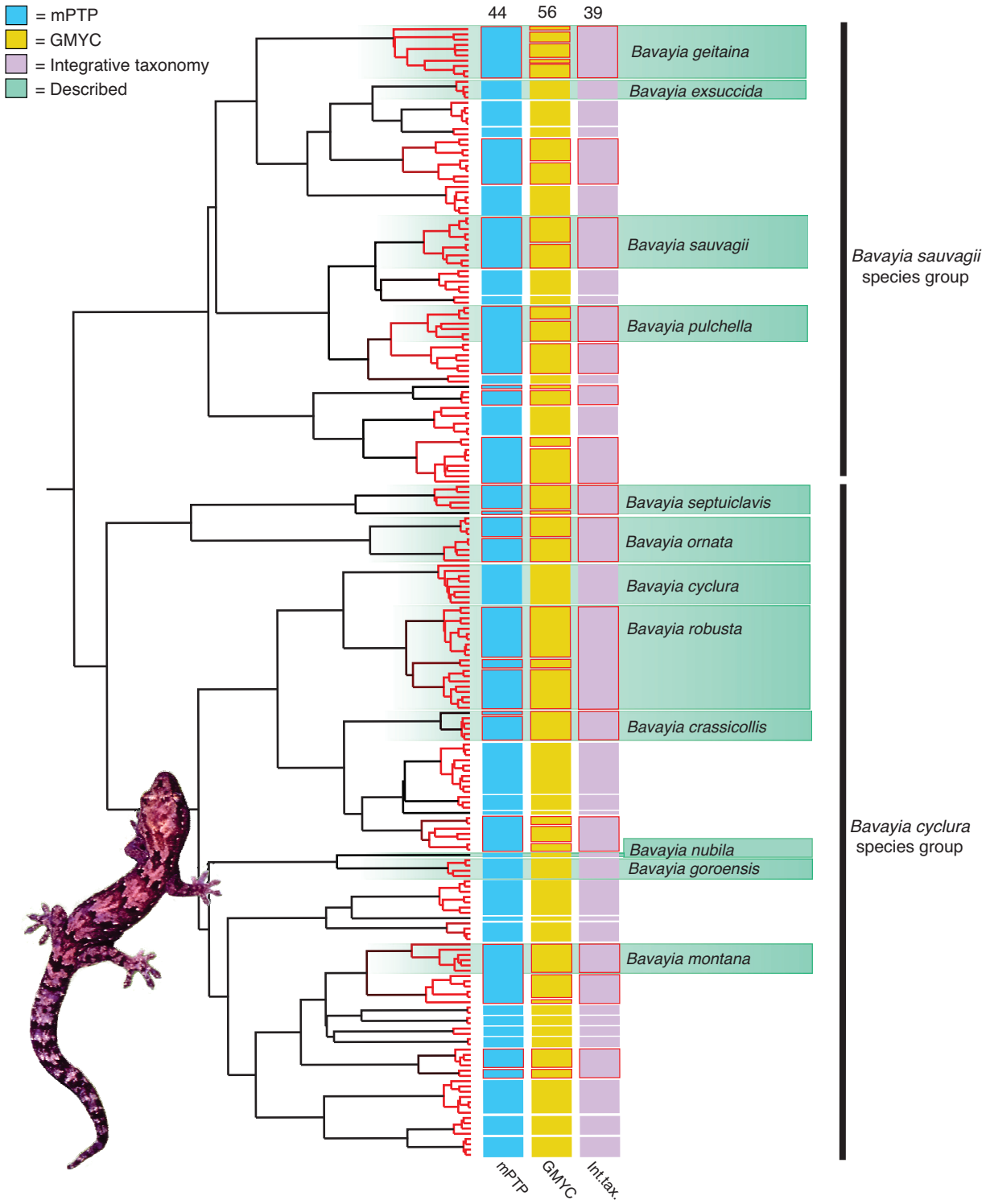

Fig. 2. Ultrametric ML CO1 tree of *Bavayia*, with red clades and single terminal black branches representing species from the mPTP analysis. Species delimitation results of mPTP (blue), GMYC (yellow), and integrative taxonomy (purple) methods are shown, with the respective suggested number of entities/species above each column. Species delimitation results with red-bordered boxes represent incongruency between at least two species delimitation methods. Clades highlighted in green represent the formally described species (*sensu stricto*); unhighlighted groups represent putative species (*Bavayia cyclura* spp. 1–16 or *Bavayia sauvagii* spp. 1–11) based on a minimum branch length between formally described forms. Specimen photo credit: Ross A. Sadlier.

Table 2. *P*-value of the SH and AU tests from PAUP* and CONSEL
Significant differences of CO1 trees using the ND2 dataset ($CO1_{ND2}$) and of ND2 trees using the CO1 dataset ($ND2_{CO1}$) are represented by *P*-values ≤0.05 and an asterisk (*)

Taxon	PAUP*/CONSEL SH/AU $CO1_{ND2}$	PAUP*/CONSEL | SH/AU $ND2_{CO1}$
Bavayia	0*/0*	0*/0*
	0*/$2e^{-43}$*	0*/$2e^{-156}$*
Caledoniscincus	0.1401/.1332	0.0004/∼0*
	0.077/0.068	0*/$8e^{-52}$*
Marmorosphax	0*/0*	0.0001*/0*
	0*/$8e^{-7}$*	0*/$2e^{-23}$*
Nannoscincus	0.0002*/0*	0*/0*
	$2e^{-4}$*/$2e^{-4}$*	0*/$8e^{-8}$*
Sigaloseps	0*/0*	0*/0*
	0*/$3e^{-5}$*	0*/$2e^{-10}$*
Dierogekko	0.0947/0.0567	0.5039/0.5134
	0.066/0.036*	0.133/0.107

instances in which the literature and other data have identified a similarly low level of differentiation. The single specimen of *S. balios* included in our dataset was only 1.1% divergent from *S. ruficauda*. This result was not surprising, given previous studies that have used ND2 found that while these two taxa differ from each other in scalation and colour pattern, they were only 0.9–2.7% divergent from one another, possibly as the result of a recent and rapid divergence event (Sadlier *et al.* 2014*a*).

Within *Caledoniscincus* the absence of a barcoding gap between intra- and interspecific CO1 distances lies in the current concept of the taxonomy of the species *C. terma* and *C. aquilonius*. Differentiation of these species on morphological criteria is difficult, but this barcoding study has provided an alternative assessment of the taxonomic position of these taxa (non-monophyly of *C. aquilonius* in this study) that is parsimonious with the results observed. The description of *C. terma* (Sadlier *et al.* 1999) was based on a population from a single locality (Mt. Mandjelia) on the Grand Terre, and that of *C. aquilonius* on several populations in adjacent areas. Since then, further sampling from intervening areas has been made and a strict CO1 pairwise comparison between *C. terma s.s.* (the type population on Mt. Mandjelia) with samples assigned to *C. aquilonius* from Tiebaghi and Riviere Nehoué based on morphological criteria showed a level of sequence divergence of only 1.1%. Rather than considering *C. terma* and *C. aquilonius* conspecific, the relationship between the two taxa in the CO1 phylogeny suggests that *C. terma* is not restricted to Mt. Mandjelia and has populations in Tiebaghi and Riviere Nehoué. This also suggests a geographically revised *C. aquilonius*, populations of which were 3.6–5.4% divergent in CO1 from *C. terma*; this also warrants a review of the morphological differences between the taxa. Alternatively, mitochondrial introgression of *C. aquilonius* and *C. terma* or small sample sizes may produce this result, and multilocus sampling would help clarify the history of these species.

Within *Nannoscincus* the clade represented by *N. gracilis* + *N. slevini* + *N. garrulus* (Fig. 1) is problematic. Both the ND2 tree and CO1 tree identify *N. gracilis*, as currently conceived, as paraphyletic by inclusion of *N. garrulus* within *N. gracilis*, and both also fail to distinguish between the parapatric populations of *N. gracilis* and *N. slevini* (Sadlier *et al.* 2014*b*). *Nannoscincus slevini* is diagnosed from *N. gracilis* on a single morphological character: a loss of the fifth digit of the manus, which is unique within eugongyline skinks. However, the lack of genetic differentiation between these individuals suggests that this morphological trait may simply represent a recently evolved but geographically discreet polymorphism within a single lineage.

The results of our study clearly show that CO1 has been able to identify ('barcode') existing recognised taxa, and the discreet highly differentiated lineages identified in previous studies utilising ND2, and confirms the problematic status of the few taxa that are not supported as distinct species by CO1 data. With respect to phylogenetic signal, many deep and intermediate nodes were poorly supported, and, as evidenced quantitatively in our study, the CO1 and ND2 topologies are significantly different. Although deeper nodes in the trees were poorly supported, the overall performance allows conspecifics and sometimes sister taxa/species groups to be recovered with strong support, even in extremely speciose groups, as has been found in previous studies on *Cyrtodactylus* geckos (Brennan *et al.* 2017). Although not optimal for phylogenetic reconstruction, we are confident that the use of CO1 can help delimit the New Caledonian herpetofauna and identify unknown samples at the level of species, genus, or other higher order groups.

DNA barcoding has been used increasingly for the identification of animals in conservation projects (Li *et al.* 2017). Although herpetofaunal barcoding studies exist (e.g. Hawlitschek *et al.* 2013; Jeong *et al.* 2013; Vasconcelos *et al.* 2016), reptiles still have the lowest representation on BOLD (http://www.boldsystems.org/; accessed May 2020), and have a number of records that are orders of magnitude less than those of amphibians, mammals, birds, and actinopterygiians (which total to ∼95% of all entries on BOLD listed under 'Chordata'). This study adds 100 (95 newly added) described lizard species, 27 putatively new species of *Bavayia* currently being described (Bauer *et al.*, unpubl. data), and potentially undescribed taxa of *Caledoniscincus*, *Dierogekko*, *Nannoscincus*, and *Marmorosphax* to BOLD, greatly increasing the herpetofaunal representation of this database and Project Cold Code. Our dataset includes 94% of the terrestrial herpetofauna of New Caledonia and represents a large, insular fauna that is nearly entirely endemic and a majority of which are threatened. Only 54% of protected areas are covered by strict limitations on nickel mining (Jaffré *et al.* 1998; Wulff *et al.* 2013), a major threat to the native herpetofauna, and many reptile species are not found within the areas that are protected (IUCN 2018). The dataset generated in this study provides a tool for the identification of evolutionarily significant units (ESUs) at both the species and intraspecific lineage level to which conservation measures can be applied. In identifying these ESUs, it also provides the means to more comprehensively assess the biodiversity and history of the islands' geographic entities, in particular the isolated massifs and mountains that already exhibit high levels of endemism.

Based solely on habitat loss, New Caledonia has been ranked as one of the top 10 regions, globally, to most likely face extinction of plants and terrestrial vertebrates (Brooks *et al.* 2002). Although the integration of genomic data into conservation efforts has been recognised and advocated (Garner *et al.*

2016), not all facilities have the financial resources to afford high-throughput sequencing technologies; additionally, many of the individuals who work with the conservation of the New Caledonian biota also lack the taxonomic expertise to delimit morphologically conserved species. Thus, the quick and efficient identification of this fauna is critical for conservation and establishment of ESUs, especially using cost- and time-efficient methods that do not sacrifice accuracy. The utility of DNA barcoding can extend beyond the identification of whole animal samples, and is now proving to be particularly valuable in the identification of partial remains of dead specimens, and even from loose DNA in the scats of invasive feral cats (Deagle *et al.* 2005). With lizards as the dominant vertebrates of New Caledonia, feral cats consume lizards as a major prey source when other animals, such as introduced rodents and rabbits, are scarce (Nogales *et al.* 2013). Studies in Australia have estimated that, per day, more than one million reptiles are preyed on by cats (Woinarski *et al.* 2018), which have been identified as one of the primary threats to hundreds of vertebrate species in different island systems (Bonnaud *et al.* 2011; Medina *et al.* 2011; Bellard *et al.* 2017). The utility of DNA barcoding from partial remains lends itself not just as an identifier, but also sheds light on the prey composition and dynamics of mammalian species (Shehzad *et al.* 2012). As time has progressed, more of the New Caledonia herpetofauna has been identified as threatened (IUCN 2018); with the decline of populations on the rise, quick and efficient creation of DNA datasets in public repositories to aid in identification are essential to integrate different data types into effective conservation measures.

Conclusion

The data provided by our study represent the largest barcoding study for an insular fauna to date, and a near-complete DNA barcoding assessment for the herpetofauna of the world's smallest, but one of the most endemic-rich, biodiversity hotspots. We present molecular sequence data for 93.4% of described, terrestrial species and a number of putatively undescribed new species. CO1 molecular barcodes are informative enough when used with model-based phylogenetic methods to identify and delimit species, and determine sister species and species group relationships, though not deeper evolutionary relationships. The CO1 sequence data provide evidence that several lizard genera show high levels of intraspecific variation, potentially representing unrecognised taxa, which has been shown in previous studies but using the more difficult (and thus more expensive) to sequence mitochondrial ND2. Using CO1 as a barcode provides a time- and cost-efficient method for identifying cryptic biodiversity in the herpetofauna of New Caledonia, a threatened region from which new genera and species are still being discovered (e.g. Sadlier *et al.* 2019*a*, 2019*b*). Although some of the taxa in our study are represented by a few samples, many of these species are threatened and known from few specimens with limited distributions (IUCN 2018). Our dataset will be useful in the identification of unknown specimens of this endemic and threatened fauna, in which many of the species are conservative in morphology and are difficult to unequivocally identify, even by expert taxonomists. The methods used here show that DNA barcodes have the ability to detect extensive unrecognised lineage diversity in geckos and skinks, and identify contentious taxa that warrant further taxonomic work. These methods and results are transferrable to other island systems that are understudied and in need of quick biodiversity assessments at relatively low costs.

Conflicts of interest

The authors declare no conflicts of interest.

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