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SHORT COMMUNICATION

Coronavirus shedding in New Zealand bats: insights and future perspectives

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Abstract: The current COVID-19 pandemic emphasises the dramatic consequences of emerging zoonotic pathogens and stimulates the need for an assessment of the evolution and natural cycle of such microbes in a "One Health" framework. A number of recent studies have revealed an astonishing diversity of bat-borne coronaviruses, including in insular environments, which can be considered as simplified biological systems suited for the exploration of the transmission cycles of these viruses in nature. In this work, we present two new lineages of *Alphacoronaviruses* detected by screening the only two extant New Zealand bat species: the lesser short-tailed bat (*Mystacina tuberculata*) and the long-tailed bat (*Chalinolobus tuberculatus*). Infection prevalence reaching 60% in long-tailed bats makes this host-pathogen model relevant for the investigation of maintenance mechanisms in a bat reservoir with peculiar physiological adaptations to temperate climates. A phylogenetic analysis shows that these viral lineages do cluster with coronaviruses hosted by bat sister species from Australia, supporting co-diversification processes. These patterns provide an interesting framework for further research aiming at elucidating the natural history and biological cycles of bat-borne coronaviruses.

Introduction

The current COVID-19 pandemic has brought to the fore front investigations on zoonoses and further illustrated the importance of understanding both the evolutionary history and the natural cycles of infectious agents with zoonotic potential. While an animal origin for SARS- (Severe Acute Respiratory Syndrome) and MERS- (Middle East Respiratory Syndrome) coronaviruses is reaching consensus (Chan et al. 2013; Cui et al. 2019), the actual identification of the species acting as reservoirs for such emergent viruses is still far from complete. Further, the natural cycles of these viruses in their animal reservoirs remain essentially a black box, stressing the need for a comprehensive characterisation of biotic and abiotic parameters influencing viral transmission in nature with the aim of highlighting putative emergence drivers.

Coronaviruses (CoVs) are classified into four genera. Members of *Alphacoronavirus* and *Betacoronavirus* are commonly detected in bats but are also responsible for diseases in humans and other mammalian hosts (Cui et al. 2019), while members of *Gammacoronavirus* and *Deltacoronavirus* have been mostly described in birds (Wille & Holmes 2020). The diversity and classification of CoVs can be assessed based on partial RNA-dependent RNA polymerase (RdRp) gene sequences (Wilkinson et al. 2020). This allows the identification of a large diversity of CoVs sub-genera associated with their host species, in particular in *Alphacoronavirus* and *Betacoronavirus*.

Considering the high diversity of bat species worldwide, current knowledge on bat-borne CoVs remains limited and strongly biased by oversampled taxa or regions (Anthony et al. 2017). For example, some tropical regions such as Southeast Asia (Lacroix et al. 2017), Western Africa (Maganga et al. 2020), or the islands of the Southwestern Indian Ocean (Joffrin et al. 2020) have been extensively explored while several resource-limited countries have been hardly investigated (Anthony et al. 2017). Given that Chiroptera is the second most diversified mammalian order following Rodentia, the actual diversity of bat-borne CoVs is certainly underestimated, especially considering that each bat species may host 2–5 unique CoVs (Peel et al. 2020). The diversity of bat-borne CoVs and the apparent complexity of their evolutionary history, including co-diversification and host switching events (Anthony et al. 2017), make this host-pathogen system a relevant model for the investigation of the evolution of zoonotic viruses. Recent investigations have reported strong co-phylogenetic signals between bats and hosted CoVs, which further strengthens the role of bats as natural reservoirs for these emerging pathogens (Joffrin et al. 2020; Latinne et al. 2020). Insular ecosystems do facilitate the investigation of host-pathogen interactions as they exhibit some peculiar properties such as geographical isolation that limits migration of animal reservoirs and associated

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viruses, a limited number of species facilitating the screening of putative reservoirs, and the age of island formation that can be considered as time boundaries for divergence/co-divergence processes (Tortosa et al. 2012).

In the present report, we explore the diversity of bat-borne CoVs in the relatively simple insular system of New Zealand. Indeed, the country hosts only two extant bat species, *Mystacina tuberculata* (Mystacinidae) and *Chalinolobus tuberculatus* (Vespertilionidae), known as the lesser short tailed- and long tailed-bat, respectively. Both are temperate rain-forest species, which primarily roost in colonies in tree cavities and rarely caves (O'Donnell & Sedgeley 2006). Both species were common throughout the country, which, up until c. 800 years ago was fully forested. Over 75% of forests have been cleared since the arrival of humans in New Zealand, and both species are now threatened (O'Donnell et al. 2010, 2018). However, both species are still widely distributed throughout the country, albeit in relatively low numbers and largely confined to forest remnants (Fig. 1). Social organisation of the two species differs, with long-tailed bats forming adjacent small colonies, usually < 200 bats (O'Donnell & Sedgeley 2006). In comparison, lesser short-tailed bats form colonies of > 6000 individuals (Lloyd 2001). Neither extant species are known to share colonial roosts and today, there are few locations where both species occur in the same forests.

A previous study reported the full genome sequence of an alphacoronavirus obtained through next generation sequencing of five bat scats from lesser short-tailed bats (Wang et al 2015). We screened scats from 181 bat specimens belonging to both species and report molecular sequences revealing two new distinct CoVs hosted by New Zealand bats. The prevalence of CoV-positive bats together with a phylogenetic analysis including CoVs hosted by Australian bats pave the way for future studies aiming at inferring the evolution of these viruses as well as their natural cycle in a non-tropical insular environment.

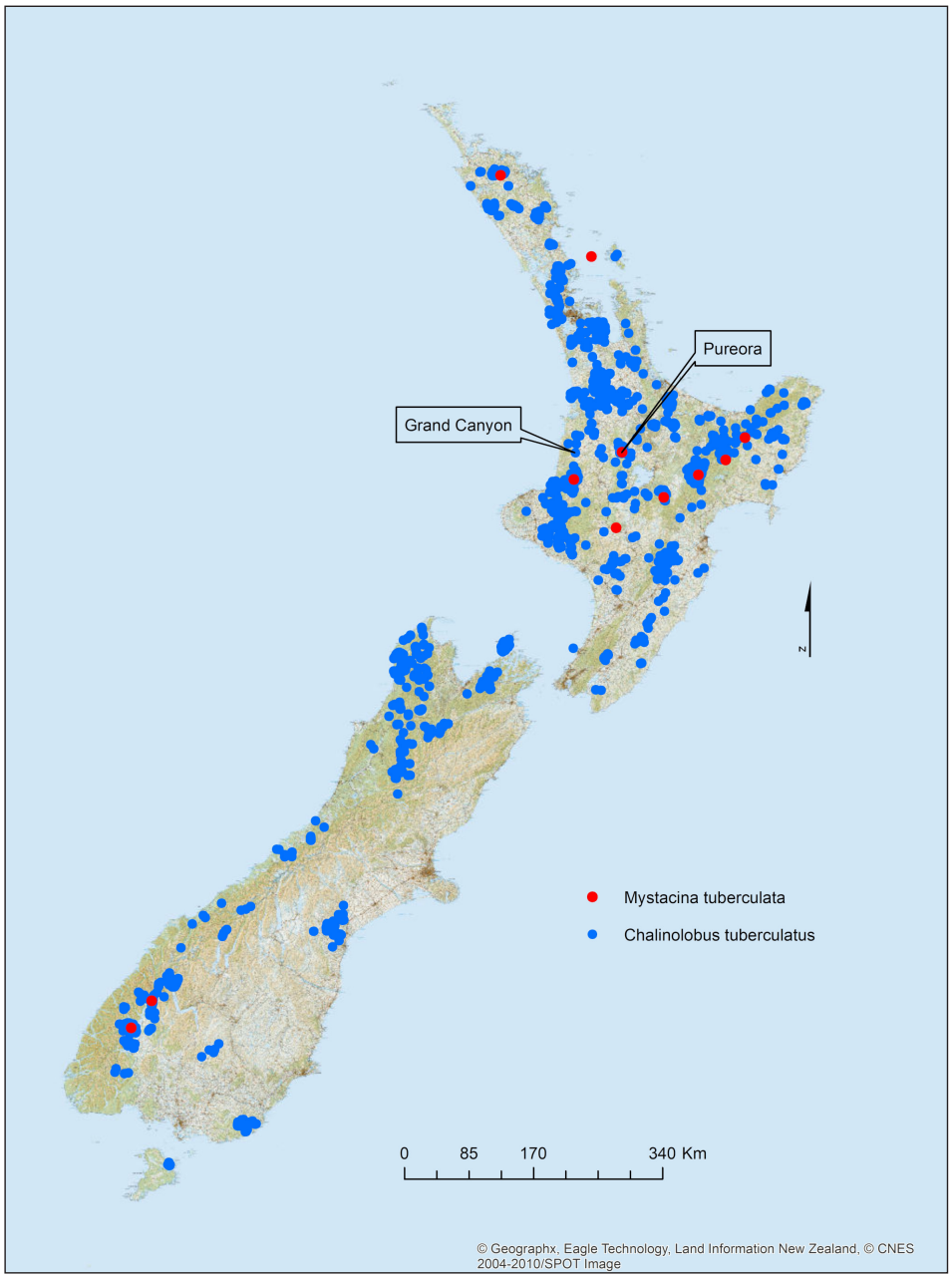

Figure 1. Occurrence of *C. tuberculatus* and *M. tuberculata*, and localisation of the two sampling sites. Bat records include surveys done by DOC, councils, contractors, private groups, and individuals since 2002.

Methods

Bats sampling

Lesser short-tailed bats (*M. tuberculata*) were sampled by collecting scats from clean tarpaulins that were spread at the bottom of tree holes used as day roosts near Pureroa, North Island (Fig. 1). Scats were sampled the next morning using sterile toothpicks and stored individually in 500 μL RNAlater (Qiagen, Valencia, USA). Long-tailed bat (*C. tuberculatus*) specimens were trapped using harp traps in Grand Canyon cave (Fig. 1) and maintained individually in cotton bags from which one single scat was sampled and preserved in a tube containing 500 μL RNAlater. Scats were then stored at room temperature and placed within a week following sampling in −70°C freezers until molecular analyses. All samplings were carried out from 21–27 February 2020.

CoV screening

Nucleic acids were obtained using trizol (Invitrogen, Carlsbad, USA) extraction. Briefly, roughly half of each bat scat was taken out of RNA later using a sterile tip and crushed into 1mL of cold trizol using a sterile pestle. Then RNA was extracted following the manufacturer's protocol and eventually resuspended in 40 μL RNAse-free water. cDNAs were obtained using Takara Primescript Reverse Transcriptase, oligo (dT) primer and random 6 mers (Takara, Kusatsu, Japan) following the manufacturer's protocol. The detection of CoV cDNA was assessed using a previously published semi-nested PCR targeting a portion of the RNA-dependent RNA Polymerase (RdRp) gene (Poon et al 2005) except that the reverse primer was replaced by IN7* primer (5'-TAR CAM ACA ACA CCR TCA TCA GA-3'), which was designed using additional CoV genomes made available since the original publication. Twenty amplicons randomly selected among the 50 CoV-PCR positive samples (including the only two positive samples from short-tailed bats) reported herein were Sanger sequenced on both strands at the Genetic Analyses Services from the University of Otago.

Phylogenetic analyses

We constructed a first Bayesian phylogeny essentially by using RdRp sequences included in previously published phylogenies presenting the diversity of Australasian bat-borne CoVs (Smith et al. 2016; Prada et al. 2019; Peel et al. 2020). JModelTest v.2.1.4 (Darriba et al. 2012) was used to determinate the best sequence evolution model. Then a Bayesian inference analyze was realised using Mr. Bayes v.3.2.3 (Ronquist et al. 2012) and GTR+I+G as substitution model with two independent runs of four incrementally heated Metropolis Coupled Markov Chain Monte Carlo starting from a random tree. The Metropolis Coupled Markov Chain Monte Carlo was run for 2 million generations with trees sampled every 100 generations. The convergence level of each phylogeny was verified by an average standard deviation of split frequencies inferior to 0.05. The initial 10% of trees for each run were discarded as burn-ins, and the consensus phylogeny with posterior probabilities were obtained from the remaining trees. The resulting phylogeny was visualised with FigTree v.1.4.2 (Rambaut 2014).

We then extended the dataset by aggregating data presented in three published papers (Lacroix et al. 2017; Joffrin et al 2020; Latinne et al. 2020), leading to a dataset of 1150 sequences. Duplicated sequences as well as sequences that did not correspond to the RdRp locus were manually discarded from the dataset, leading to a final dataset composed of 812 sequences. An unrooted ML phylogeny was constructed using FastTree/One click on NGPhylogeny (Lemoine et al. 2019), which can be visualised at https://itol.embl.de/tree/9213012 8120274381648222266.

Results and Discussion

The study revealed high CoV infection prevalence (> 60%) in long-tailed bats and limited infection in lesser short-tailed bats (< 5%; Table 1). Such figures suggest an important role of long-tailed bats in the maintenance of the detected CoV, but the limitations of the sampling scheme call for cautious interpretations. Strong temporal dynamics have been previously reported in CoVs transmission within bat colonies and the present data represent a single time point (Drexler et al. 2011; Wacharapluesadee et al. 2018; Joffrin et al. 2022). For instance, a longitudinal survey carried out on a tropical insectivorous bat species has revealed a marked infection peak in March, reaching 80% prevalence, while parturition occurs synchronously toward the end of December in that species (Joffrin et al. 2022). This peak has been attributed to juveniles losing the maternal protection provided by lactation. The samples used in the present study were all collected in the last week of February and might correspond to a period with high infection prevalence 2–3 months after parturition, which is also highly synchronous, occurring mostly in the last week of November in the central North Island (O'Donnell 2002a; O'Donnell & Borkin 2021). It is also important to note that long-tailed bats were individually processed by hand, identified at the species level and scats rapidly stored in RNA later. By contrast, scats from lesser short-tailed bats were obtained through a non-invasive method, and the absence of handling of each individual bat precludes definitive identification of these bat samples.

Table 1. Summary of bat roosts, sampled species, and number of tested and CoV positive bats.

Roost Reference Code	Nature of site	Bat species	Sample size	Number of CoV-positive samples (%)
Pureroa CR6	Tree hole-day roosts	*M. tuberculata*/ Lesser short-tailed bat	43	2 (4.7%)
Pureroa CR8			5	0 (0.0%)
Pureroa CR13			32	0 (0.0%)
Grand Canyon cave	Cave-night roost	*C. tuberculatus*/ Long-tailed bat	81	50 (61.7%)

The phylogenetic analysis (Fig. 2) revealed two distinct lineages with lineage two overwhelmingly dominant (19 out of the 20 obtained sequences, including the two sequences obtained from *M. tuberculata*). In addition, there was limited diversity within lineage two, in keeping with previous studies showing limited intra-species diversity at the RNA-dependent RNA polymerase encoding gene (Joffrin et al. 2020). Although only Grand Canyon cave was sampled for long tailed bats, opportunities for these bats to mix with individuals from unrelated colonies are likely high compared to lesser short-tailed bats (O'Donnell 2000; O'Donnell et al. 2016): long-tailed bats form temporary social groupings at night roosts, including at this sampling site where over 350 animals can appear at night, with the majority gone by morning (O'Donnell 2002b).

Importantly, the phylogenetic analysis (Fig. 2) showed that both lineages one and two are embedded within clades containing sequences from CoV shed by *Chalinolobus gouldii*, the Australian sister species of *C. tuberculatus* (long-tailed bat). We further constructed a comprehensive phylogeny including 812 sequences covering a substantial portion of the currently known bat-borne CoV diversity at a global scale. In this phylogeny (https://itol.embl.de/tree/9213012812027 4381648222266), lineage one was embedded within a clade containing viral sequences obtained from several bat species, including two distinct Australian *Chalinolobus* species, hence supporting a co-diversification of these CoV lineages and their *Chalinolobus* bat hosts from Australasia.

Of note, lesser short-tailed bats have been previously reported as hosting CoV (Hall et al. 2014) genetically distant from those detected in the present study. A detailed inspection of the RdRp sequence shows that the semi-nested PCR used herein is likely not adapted to the detection of this previously

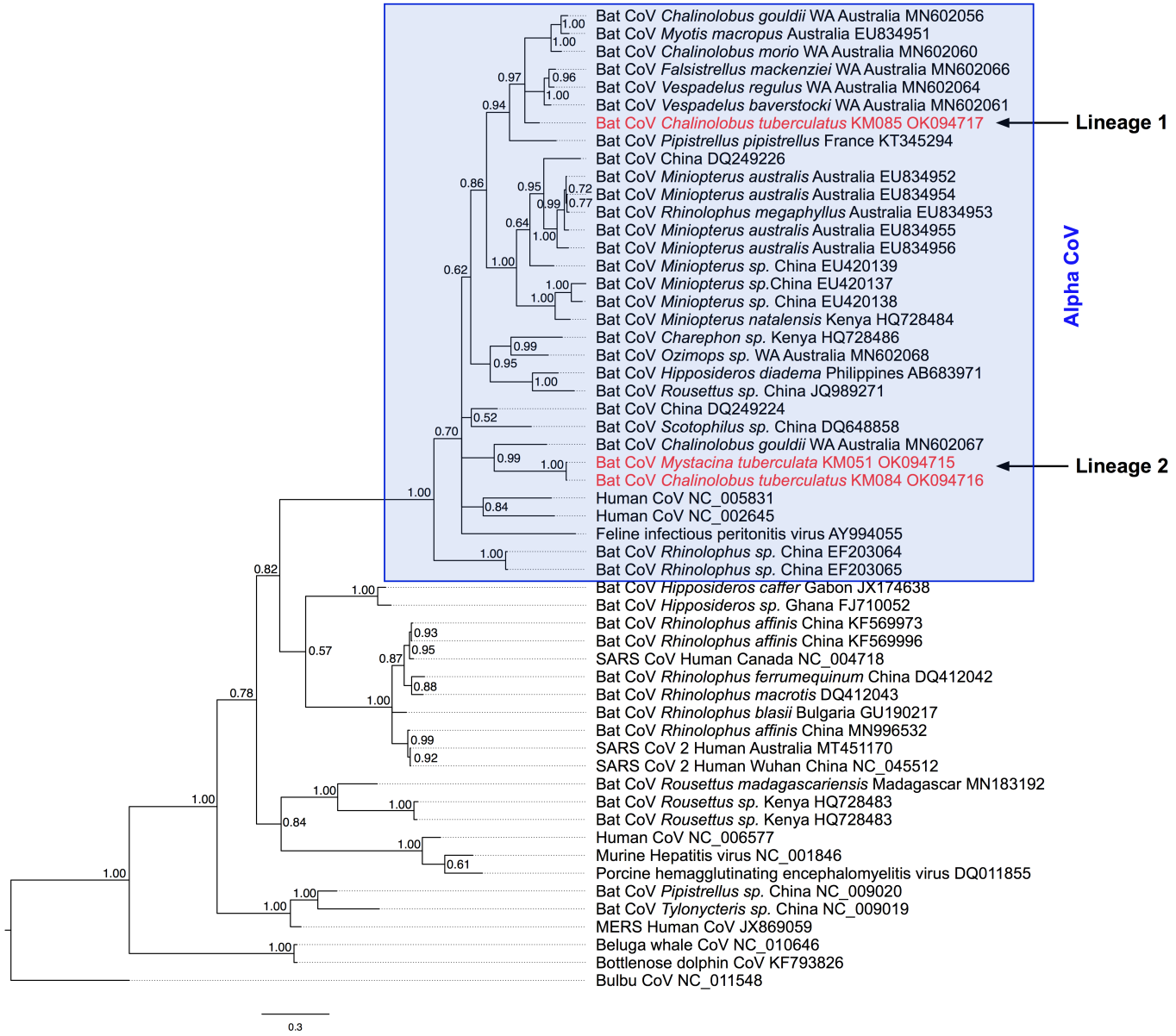

Figure 2. Bayesian phylogenetic reconstruction of bat-borne CoVs using 55 amplicons of the RdRp sequence (406 nt). Gene accession number is provided for each sequence. The tree is rooted on Bulbul CoV. Alphacoronaviruses are boxed in blue, lineages reported herein are highlighted in red. The analysis was conducted with Mr. Bayes v.3.2.3 under the GTR+I+G substitution model. Node values correspond to posterior probabilities and the scale bar to the number of nucleotide substitutions per site. Adapted from Peel et al (2020).

described viral lineage, because of numerous mismatches in one of the used primers (CoV-Fwd2, not shown).

From an epidemiological perspective, it is important to note that both reported lineages are embedded within *Alphacoronavirus* b clade, while all three CoV that have emerged in human populations in the last decades are betacoronaviruses, more specifically belonging to *Sarbecovirus* and *Merbecovirus* subgenera. Therefore, the viruses investigated and reported herein cannot be classified as of highest risk of emergence in human populations.

From an evolutionary standpoint, the topologies of the two presented phylogenies are coherent with a *Chalinolobus* ancestral population(s) colonising New Zealand from Australia while infected with CoV. Although we did not provide evidence that the topologies of bats and associated CoVs are congruent, these viral sequences appear as good candidates to infer evolutionary rates of these viruses using molecular information from bat hosts, including mitochondrial sequences and microsatellite data (Dool et al. 2016). It is worth noting that *Chalinolobus* is a relatively recent colonist of New Zealand (< 1 Myr), compared to lesser short-tailed bats (> 20 Myr) (Lloyd 2003; O'Donnell & Borkin 2021), which is coherent with the limited diversity of associated CoV reported herein. Anyway, the acquisition of full viral genome sequences is a necessary step towards a robust exploration of the evolutionary history of these coronaviruses.

The high infection prevalence (reaching 61.7% in long-tailed bats), the dispersal capacities and the ability of New Zealand bats to enter into torpor make this biological model relevant to explore the mechanisms of maintenance of bat-borne CoVs in the wild. It has been proposed that torpor or hibernation are physiological adaptations that may facilitate viral maintenance in bats (Calisher et al. 2006). It is thus relevant to implement a longitudinal survey in order to address the temporal dynamics of these infections through one or two successive seasons. Such a non-invasive survey could allow identifying infection peaks within colonies throughout the reproductive season (Dietrich et al. 2015; Joffrin et al. 2022) during which bats should be protected from any human disturbance/contact.

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Additional information and declarations

Author contributions: PT, CL, and RP conceived and designed the analyses. KM and CFJO'D provided their knowledge on the biology of New Zealand bats and implemented the sampling of all biological material investigated herein. PT produced molecular data. YG carried out the phylogenetic analyses. MP gathered all available published and unpublished information and prepared the map presenting bat species occurrence throughout New Zealand. All authors participated in writing and editing the manuscript.

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Data and code availability: The novel CoV sequences (387 and 400 nucleotides from the RdRp gene) from New Zealand bats were deposited in GenBank under accession numbers OK094715, OK094716 and OK094717. The dataset composed of 812 (387nt long) sequences is provided in supplemental fasta file.

Ethics: Sampling was conducted by the New Zealand Department of Conservation staff, in conformity with the Conservation Act 1987.

Conflict of interest: The authors declare no conflict of interest.

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