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A systematic survey of environmental DNA in Palau's lakes and waterfalls reveals an increase in *Leptospira* levels after flooding

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A B S T R A C T

Objective: Leptospirosis is an important bacterial zoonosis which is widespread in tropical and subtropical islands and influences human and animal health which has secondary economic effects. Although leptospirosis is endemic in Palau, an Oceanian Pacific Island country, few systematic surveys of potential risk factors for *Leptospira* infection, such as weather and host animals, have been conducted in the natural environment. We used environmental DNA metabarcoding to assess the distribution, species diversity, and abundance of pathogenic *Leptospira* in this endemic region to investigate the potential environmental risks.
Methods: Forty-two paired water samples, representing fine and rainy weather conditions, were collected from four representative waterfalls and lakes on Babeldaob Island, the largest island in Palau. High-throughput sequencing analysis was conducted for polymerase chain reaction products of leptospiral 16S rRNA and vertebrate animal mitochondrial 12S rRNA genes.
Results: We revealed greater *Leptospira* diversity and abundance in samples collected after continuous rain, particularly in the presence of flooding, compared with samples collected under typhoon, monsoon, or fine weather conditions. From same samples, six mammalian species including cats (*Felis catus*), mice (*Mus musculus*), Yap flying fox (*Pteropus yapensis*), rats (*Rattus spp.*), and pigs (*Sus scrofa*) were repeatedly detected. These may be candidates of host animals of *Leptospira* in Palau; however, their detection was not clearly correlated with that of *Leptospira*.
Conclusion: We repeatedly detected several species of pathogenic *Leptospira* from water samples of a wide region of Babeldaob Island. We confirmed that *Leptospira* contamination in freshwater environments increased under rainy conditions, particularly in the presence of flooding. This information could be used to improve public health control measures in this region.

1. Introduction

Leptospirosis is a zoonotic disease caused by the bacterial genus *Leptospira,* which occurs mainly in tropical and subtropical regions. The genus *Leptospira* comprises at least 69 species which can be classified into 2 subclades (S1, S2) of non-pathogenic species and 2 subclades (P1, P2) composed of species with variable pathogenic potential [1–4]. Members of the P1 subclade are pathogenic species that have been reported to cause infection in both humans and animals [4]. From the urine of chronically infected reservoirs, pathogenic *Leptospira* spreads into environmental substrates, such as soil and river water, finally leading to human infection by direct exposure to contaminated soil or water [3–5]. Infection is often acquired during water-related outdoor activities such as swimming, kayaking, and working in paddy lands, and by handling livestock and pet animals [6]. The estimated burden of leptospirosis is more than 1 million cases, with almost 60,000 fatalities worldwide each year [7]. The importance of leptospirosis has been emphasized given the wide variety of host animals and recent effects of climate change [3,7–9]. In particular, heavy rain and flooding are thought to increase the risk of leptospirosis by elevating the concentrations of pathogenic *Leptospira* in fresh water [5,10–16]. Consequently, the risk of leptospirosis coupled with meteorological events such as typhoons, monsoons, and flooding should be considered in countries located in temperate, subtropical, and tropical regions.

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Leptospirosis has been reported in the Republic of Palau, an Oceanian Pacific Island country (reviewed in Guernier et al. 2018) [17]. Although limited literature is available before 2000, 81 cases of leptospirosis were reported between 2000 and 2006 after the establishment of the national disease surveillance system in Palau, with a relatively high estimated mean annual incidence rate of 65.2/100,000 [18]. In 2014, two cases of leptospirosis were reported in Japanese travelers who were thought to have become infected while swimming in the Ngardmau Waterfall on Babeldaob Island, Palau, 2 days after a typhoon (Figure 1A, B); *Leptospira* species was unidentified [19]. The main host animals of *Leptospira* in Palau are thought to be rats, stray dogs, and pigs [18]. Domestic cattle, goats, and pigs were subjected to serological assays using the microscopic agglutination test (MAT), and 40–45 % were found to be positive for *Leptospira* [17,20]. The presumptive serogroups in animals were Icterohaemorrhagiae, Panama, Pyrogenes, Sejroe, and Canicola; and the putative serogroups in humans were Icterohaemorrhagiae and Javanica. Several investigations of *Leptospira* have been reported in Palau; however, DNA-based species-level studies are lacking. Therefore, an understanding of *Leptospira* diversity, potential host animals, and the associated environmental factors in Palau is limited.

In this study, we aimed to address the eco-epidemiological aspects of leptospirosis in Palau, particularly its distribution, possible association with weather and rain, and potential host animals of *Leptospira*. We used an environmental DNA (eDNA) metabarcoding method, which provides a detailed analysis from the bacterial to the animal level directly from water samples [9,11,21]. We focused on Babeldaob Island, the largest island of the Republic of Palau (Fig. 1A), and chose four representative tourism and water leisure spots: Ngardmau Waterfall, Ngardok Lake, Ngatpang Waterfall, and Ngchesar Waterfall, as research sites (Fig. 1). Outdoor activities in these areas may increase the risk of exposure to *Leptospira*. Using water samples from multiple sites in these locations, we addressed the relationship between *Leptospira* detection and weather by comparing samples collected on fine and rainy days. In addition, the possible correlation between *Leptospira* and the detection of terrestrial vertebrates was assessed using eDNA metabarcoding data by massive DNA sequencing.

The motivation for this study was an increase in the number of cases of leptospirosis in Palau since 2018, particularly in 2021 and 2022 (Fig. 2). Although *Leptospira* are analyzed by culturing specimens from human patients, livestock, and other animals, some pathogenic species of *Leptospira* has been difficult to be cultured [1]. The culturing methods of *Leptospira* have been improved currently using rich media containing

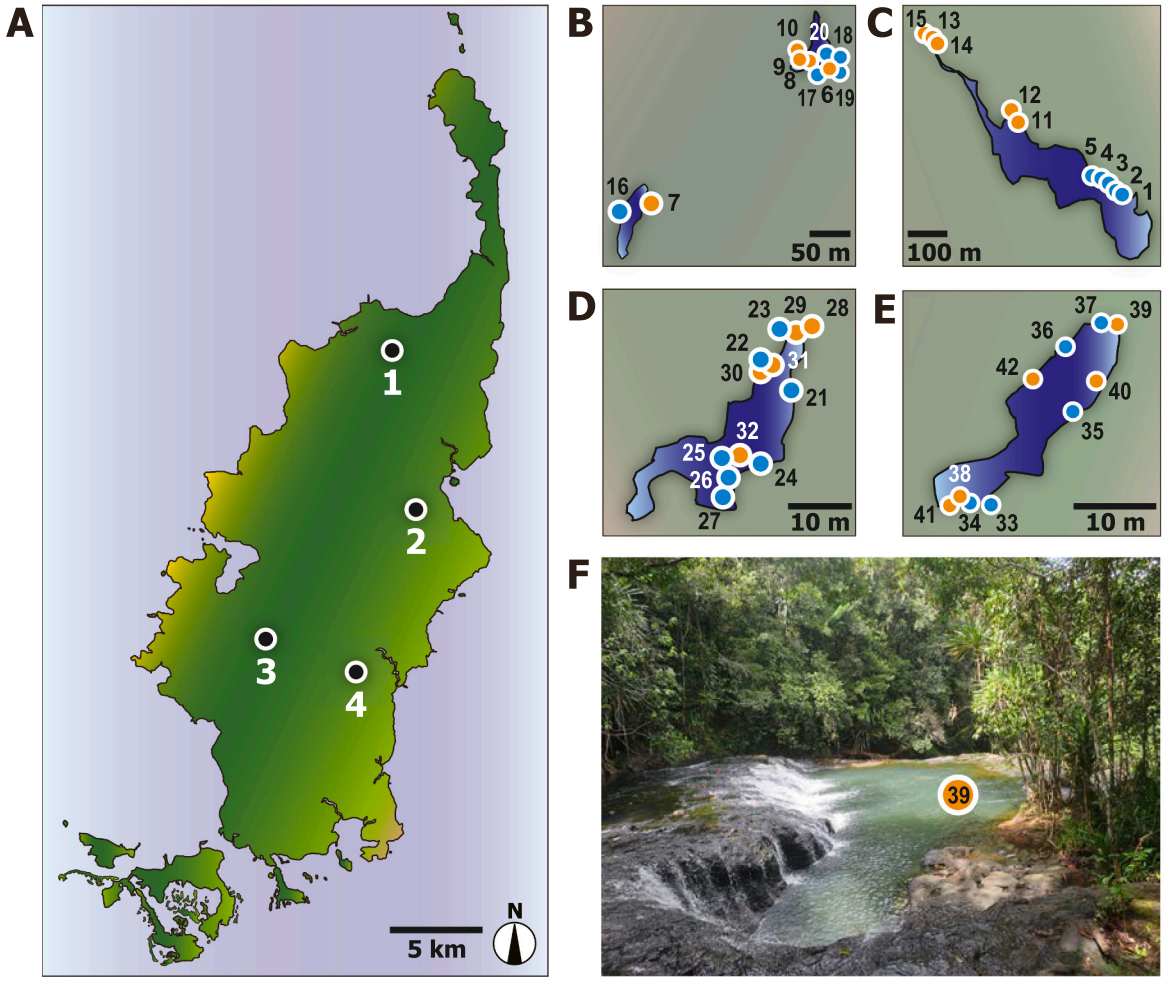

Fig. 1. Babeldaob Island of the Republic of Palau and locations of water sampling sites. (A) Babeldaob and Koror Islands. The numbers 1, 2, 3, and 4 indicate the location of the Ngardmau Waterfall, Ngardok Lake, Ngatpang Waterfall, and Ngchesar Waterfall, respectively. (B) Ngardmau Waterfall and sampling locations alongside of the waterfall. (C) Ngardok Lake and sampling locations alongside of the lake. (D) Ngatpang Waterfall and sampling locations alongside of the waterfall. (E) Ngchesar Waterfall and sampling locations alongside of the waterfall. Water sampling locations on fine and rainy days are shown as orange and blue circles, respectively. (F) Picture of a representative sampling site next to the Ngchesar Waterfall (sample number 39 in the panel E, fine day). The map imagery was obtained from OpenStreetMap (https://www.openstreetmap.org/) licensed under the Open Data Commons Open Database License by the OpenStreetMap Foundation and the Creative Commons Attribution-ShareAlike 2.0 license (CC BY-SA 2.0). Adobe Illustrator 2021 v25.2.1 was used to draw the Figure with map imagery. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

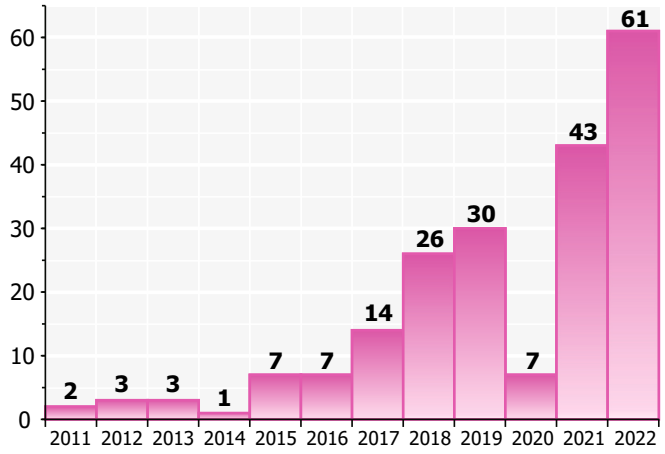

Fig. 2. Number of cases of leptospirosis recorded from 2011 to 2022 in the Belau National Hospital in Koror, Republic of Palau. Data are provided from this hospital and Ministry of Health and Human Services, Republic of Palau, on cases diagnosed using an IgM- and IgG-antibody assay kit. The data do not cover all cases of leptospirosis in Palau because the hospital is only one institution that diagnose leptospirosis, and private clinics are not required to report cases of leptospirosis. The information of locality (Koror or Babeldaob Island) and ethnicity of patients is provided in Supplementary material 2.

rabbit sera and bovine serum albumin. However, the total diversity of pathogenic *Leptospira* and their distribution in the wild environment would be more easily assessed using an eDNA-based approach [9,11,21]. We conducted a semi-quantitative analysis of *Leptospira* diversity and their relative abundance in the eDNA of freshwater waterfalls and a lake. Simultaneously, animals co-occurring with *Leptospira* and related environmental factors such as weather were analyzed using an eco-epidemiological approach. By focusing on whether the type of rain was episodic (typhoon and monsoon) or continuous (flooding) [12,13,22–25], we applied an integrative approach to evaluate potential risk factors for leptospirosis in Palau.

2. Materials and methods

2.1. Environmental water sampling

In total, 42 environmental water samples (listed in Supplementary material 3) were collected from four localities on Babeldaob Island in the Republic of Palau from October 2021 to May 2022: Ngardmau Waterfall, Ngardok Lake, Ngatpang Waterfall, and Ngchesar Waterfall (Fig. 1). The temperature of Palau is stable throughout the year (with daily highs and lows of approximately 31 °C and 24 °C, respectively). The four sampling sites are tourist spots in Palau used as hiking destinations and for water leisure activities and cover the northern to southern parts of the largest island in Palau. The water sampling was designed to collect paired water samples from each location, with one sample collected on a fine day and the other sample collected on a day when a meteorological event associated with an increased the risk of infection (rain, monsoon, flooding, or typhoon) was forecast. The latter samples were collected as soon as possible after the meteorological event with a maximum delay of 3 days (Supplementary material 3). Detailed observation and record of the meteorological events at the sampling of present study were described in the caption of the table of Supplementary material 3.

Surface water was sampled at the edge of the lake or water pocket, where the researchers had access (Fig. 1B–F) using a clean plastic bucket. A 0.2 L to 1.5 L volume of environmental water was filtered by a Sterivex unit with a 0.45 μm pore size membrane (Merck Millipore, Milan, Italy) at each sampling site using a 50 mL disposable syringe SS-50LZ (Terumo, Tokyo, Japan). The filtration pore size has been confirmed to capture *Leptospira* bacteria in a previous study [11]. Ideally, the water sampling may be standardized with regard to the amount (mL), however, water filtration at the same volume was practically difficult because waters became muddy when rainy weather compared to fine days. Accordingly, the water filtration was conducted until the filters became clogged with filtrate to standardize the sampling by an approximate amount of the filtrated materials. The volume of the water (mL) filtered was recorded for each sample. Following the on-site water filtration, the Sterivex unit was filled by 2.0 mL of the DNAiso Reagent (Takara Bio, Shiga, Japan) for sample fixation and DNA preservation, and tightly sealed with a polypropylene Luer-fitting cap (ISIS, Osaka, Japan). The samples were stored at −30 °C in the laboratory in Palau Island and kept at room temperature for several days during their transfer to Japan, followed by storage at freezing temperature in the University of the Ryukyus until DNA extraction. The weather at the sampling site and the date of sample collection were recorded by the author (R.S.). Data on the amount of rainfall (mm) (Supplementary material 3) were provided by the National Oceanic and Atmospheric Administration, National Weather Service Office, Republic of Palau.

2.2. DNA extraction

Total eDNA was extracted from the water filtrates in the Sterivex unit using a DNeasy PowerWater Sterivex Kit (Qiagen, Hilden, Germany). The manufacturer's standard protocol was used with minor modifications. The Sterivex units were placed at room temperature for 15 min to thaw the DNAiso reagent. Subsequently, the reagent was removed and discarded using a 10 mL disposable syringe (Becton, Dickinson and Company). After adding the 65 °C MBL solution, filter units were incubated at the same temperature on a heat block for 10 min to lyse the cells and other biomaterials in the filtered residues. For the final eDNA elution, 30 μL of microbial DNA-free water (Qiagen) was used, obtaining a total of 60 μL eDNA solution from each filter unit by the elution step twice. The eDNA was stored at −30 °C after quantification of its concentration and quality using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. PCR amplification and sequencing from water eDNA for metabarcoding analysis

From each eDNA sample extracted above, we amplified partial fragments of the 16S rRNA gene of *Leptospira*, which is more specific than *lipL32* gene when using environmental samples [9]. Partial fragments of the mitochondrial (mt) 12S rRNA genes of various vertebrates were amplified separately to analyze the fauna and potential host species of *Leptospira* [9,11,21]. The known PCR primer sequences targeting leptospiral 16S rRNA [26], broad bacterial 16S rRNA V4 partial region [27], and vertebrate mt-12S rRNA (named MiFish primers) [28] were used with adding the priming sequences for second-round indexing PCR and DNA sequencing, and random hexamer or dimer nucleotides for effective sequencing using the MiSeq platform (Illumina, San Diego, CA, USA) [11,28]. The oligo-nucleotide sequences used are described in a previous report [21]. The typical target length (base pairs, bp) and the final concentration of primers of the PCR were 330 and 169 bp, and 0.38 and 0.29 μM for leptospiral 16S rRNA and vertebrate mt-12S rRNA, respectively.

The Multiplex PCR Assay Kit version 2 (Takara Bio) was used for PCR determination of *Leptospira* and broad bacteria, with 2.0 μL of template eDNA in a total reaction volume of 10.0 μL through 37 cycles of amplification at an annealing temperature of 50 °C. A quarter concentration of additional broad bacterial 16S rRNA V4 primers was used for positive control amplification, with a typical target length of 259 bp. PCR was performed twice for each sample as a replicate. Negative control PCR was performed using 2.0 μL of microbial DNA-free water (Qiagen) as a template. For the PCR of vertebrates, the HiFi HotStart ReadyMix (Kapa Biosystems) was used with 1.5 μL of templates in a total

reaction volume of 12.0 μL through 35 amplification cycles with two patterns of annealing temperatures of 60 °C and 65 °C. PCR was performed at different annealing temperatures separately for all samples. Applying two annealing temperatures with different fidelity enable amplification of relatively more diverse DNA types than is possible using a single temperature because of their different susceptibilities to DNA sequences, particularly to secondary structures. The other PCR conditions were as described previously [11]. Negative control PCR was performed using 1.5 μL of microbial DNA-free water (Qiagen) as a template. The 1st-round PCR products were diluted 30- and 20-fold in leptospiral 16S rRNA and vertebrate mt-12S rRNA, respectively, in RNase-free water (Thermo Fisher Scientific), and subjected to 2nd-round PCR to add the dual-index tags (D5, D7, A5, and A7 series) and MiSeq flow cell-binding sites (Illumina) using Ex Taq Hot Start Version (Takara) as described previously [11,27].

DNA sequences of the tag-indexed 2nd-round PCR products were determined using MiSeq (Illumina). PCR products with unique combinations of dual indices for each sample were pooled in equal amounts for semi-quantitative purposes. The pooled samples were purified using a 1.0 % L03 agarose gel (Takara Bio) and a MinElute Gel Extraction Kit (Qiagen) according to a standard protocol. The eluted DNA solution was further purified and concentrated by removing short DNA fragments (<100 bp) using AMPure XP magnetic beads (Beckman Coulter) according to a standard purification protocol. The obtained sequencing library was quantified using the Qubit 3.0 with the dsDNA HS Assay Kit (Thermo Fisher Scientific), and the 4 nM library was obtained by dilution using microbial DNA-free water (Qiagen). The volume molarity of the libraries was calculated based on the average molecular weight of a DNA nucleotide (660 g/mol), DNA concentration, and mean length (bp) of the second-round PCR products of the library, which were 526, 454, and 365 bp in the leptospiral 16S rRNA, bacterial 16S rRNA V4, and vertebrate mt-12S rRNA genes, respectively. DNA sequencing was performed using the MiSeq Reagent Kit v2 500 or 300 cycles (Illumina) for 251 or 151 bp paired-end sequencing of leptospiral and bacterial 16S rRNA or vertebrate mt-12S rRNA genes, respectively.

2.4. Metabarcoding data analysis of Leptospira and potential host animals

The sequence data obtained using MiSeq were subjected to quality-based primary data processing. The 3′-tail nucleotides of each sequence with an error rate $> 10^{-1}$ were removed using the program DynamicTrim [29]. The tail-trimmed paired-end sequences were connected using FLASH software [30] and processed using custom Perl scripts (available at Dryad data repository: https://doi.org/10.5061/dryad.54v2q) [28] to exclude sequences containing base call failures (N bases) and having atypical lengths compared with the expected PCR target sizes described above. The filter-pass ranges of the sequence length before primer removal were 280–450 bp for leptospiral 16S rRNA and bacterial 16S rRNA V4, and 204–254 bp for vertebrate mt-12S rRNA. Primer sequences with a maximum five-base mismatch were removed using TagCleaner [31]. Sequences lacking primers at either end were discarded. The identical sequences within each sample were merged into a dereplicated sequence while keeping its count information using UCLUST (derep_fulllength command) [32]. For vertebrate mt-12S rRNA sequences, the singleton sequences in each sample were aligned with ≥2 counts effective sequences at ≥99 % sequence similarity to remove sequencing error and/or intra-species variation (denoising). The number of aligned singletons was added to the count information of the matched effective sequence and unmapped singletons were discarded to exclude unexpected experimental contamination by various genomes. For leptospiral 16S rRNA sequences, the environmental sequence data, including singletons, were analyzed using molecular phylogeny, as described in the subsection below on “Molecular phylogenetic analysis,” because the PCR primers used were confirmed to be specific to pathogenic *Leptospira* [9,11,21,26].

These quality-filtered effective sequences were analyzed to estimate their taxonomic origin based on sequence similarity to known reference sequences using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) Plus program [33]. The NCBI nucleotide collection database (nt) [34] was used as the reference database to analyze the leptospiral 16S rRNA gene. MitoFish [35–37] and NCBI nt were used to analyze the vertebrate mt-12S rRNA gene. For the leptospiral 16S rRNA, the BLASTN analysis was performed at sequence similarity and an *e*-value threshold of 85 % and 10^{-3}, respectively. This relatively moderate parameter setting was aimed at avoiding false negatives (type II errors) in the preliminary sequence annotation. According to the BLAST top-hit results, these provisional annotations were confirmed or corrected based on the molecular phylogenetic analysis with known representative leptospiral 16S rRNA gene sequences as described in the subsection on “Molecular phylogenetic analysis” below. The sequence counts for each species from the two PCR replicates were summed for each sample.

For the vertebrate mt-12S rRNA gene, the BLASTN-based species annotation was performed at the sequence similarity and *e*-value thresholds of 90 % and 10^{-5}, respectively. This relatively strict parameter setting was because of the completeness of the vertebrate mtDNA database, which is generally higher than that of bacteria or *Leptospira*. The sequence counts of the species from two separate PCR amplifications with different annealing temperatures (60 °C and 65 °C) were summed for each sample.

2.5. Molecular phylogenetic analysis

Representative 16S rRNA gene sequences of the genus *Leptospira* were collected based on the phylogenetic trees constructed by Guglielmini et al. [1] and Vincent et al. [2]. The obtained reference sequences and the partial 16S rRNA sequences determined in our eDNA analysis were aligned using MAFFT version 7.310 [38]. A maximum-likelihood (ML) phylogenetic tree was constructed using MEGA X version 10.2.5 [39]. A general time-reversible model of nucleotide substitution [40] was applied with auto-adjustment of invariable sites and gamma correction parameters. The support values for the tree nodes were estimated using 100 replications of bootstrapping analysis. We considered that a bootstrap value ≥50 % was reliable and indicated it on the nodes of the phylogenetic tree. The estimated phylogenetic tree was examined to confirm whether it was largely compatible with the reference leptospiral phylogenetic trees of Guglielmini et al. [1] and Vincent et al. [2].

2.6. Statistical analysis

The significance of the difference in average values was examined using Welch’s *t*-test based on the mean and variance scores. This test was applied for the analysis of the amount of water samples filtered from each location (mL), and concentration (ng/μL) and quality values ($OD_{260/280}$) of the extracted eDNA. To examine the effects of weather conditions (fine weather or rain; Supplementary material 3) on the eDNA detection of *Leptospira*, we constructed two generalized linear mixed models (GLMMs) with a Poisson error structure and a log link. This analysis assumed the Poisson distribution to model count data consisting of nonnegative integers without an upper limit, such as DNA sequence numbers. The GLMM analyses with log link function estimated parameters such as β_1, β_2 using the maximum likelihood method to predict response variable by fitting data to an exponential function such as $\exp.(\beta_1 + \beta_2 x_1)$. The response variables for each model were the total number of *Leptospira* sequences and number of *Leptospira* operational taxonomic units (OTUs) detected in each sample. These models included weather conditions (fine or rainy) as a fixed effect, sampling location as a random effect, and amount of water samples filtered and total number of raw sequences from each sample as offsets. The offsets were set to evaluate the detection of *Leptospira* per unit amount of water samples and number of raw sequences.

To test whether the detection pattern (presence/absence) of *Leptospira* differed between fine and rainy weather conditions, the numbers of *Leptospira*-positive and -negative samples from the four localities (Ngardmau Waterfall, Ngardok Lake, Ngatpang Waterfall, and Ngchesar Waterfall) were pooled by weather and analyzed using Fisher's exact test. We also compared the total number of *Leptospira* sequences among four different types of rain conditions (rain, monsoon, flooding, and typhoon; Supplementary material 3) by performing a two-group permutated Brunner-Munzel test, which is a nonparametric approach based on the null hypothesis of the appearance of equal sequence numbers among all pairwise groups of rain. The significance levels of the multiple comparisons were corrected using the sequential Bonferroni method. A possible relationship between the number of eDNA sequences detected from *Leptospira* and vertebrates was tested using Pearson's product-moment correlation coefficient (r). The false discovery rate (FDR) of r was evaluated using the Benjamini–Hochberg method. The statistical analyses were performed using *R* version 4.3.3 (https://www.R-project.org) [41].

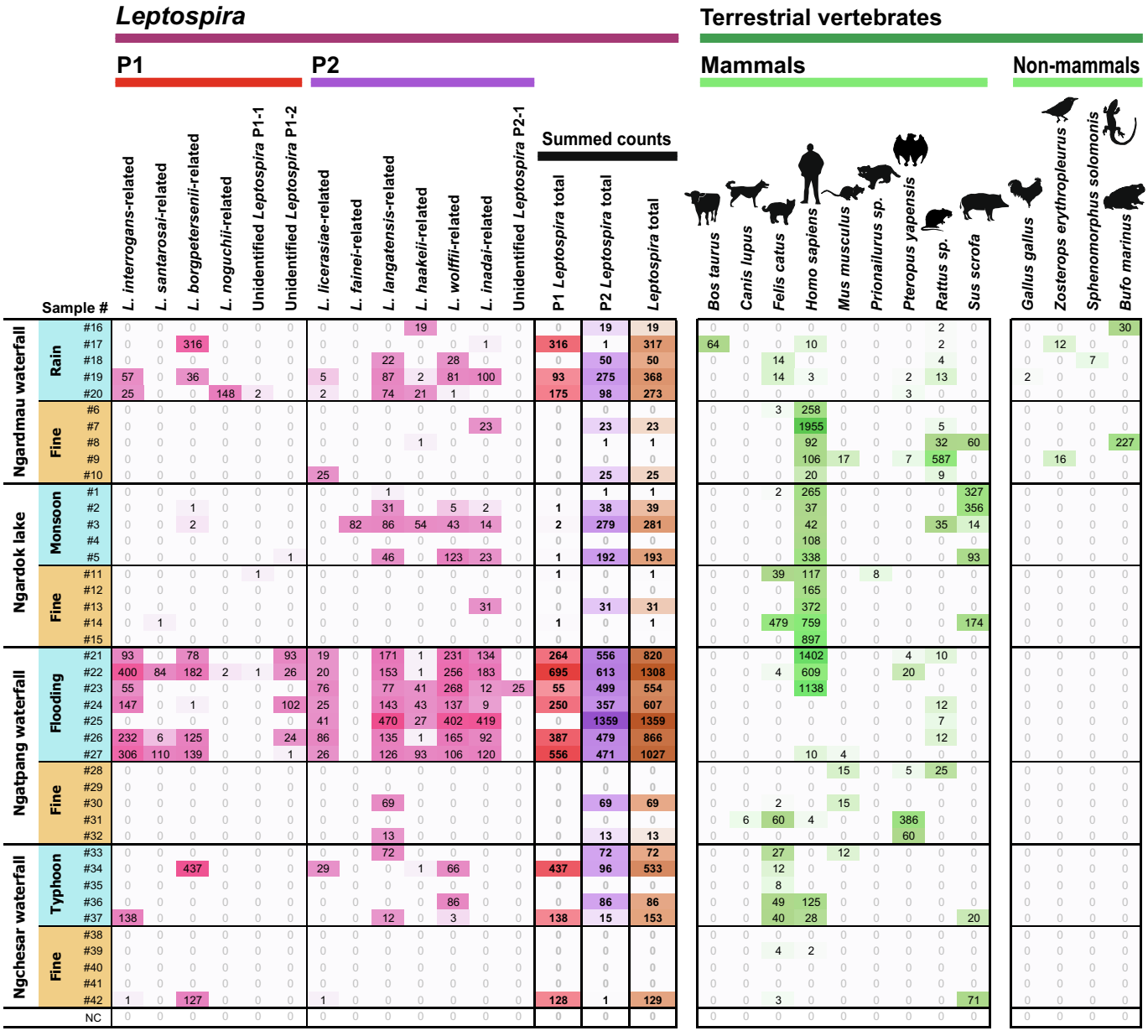

Fig. 3. Environmental DNA detection of partial leptospiral 16S rRNA and tetrapod mitochondrial 12S rRNA genes. Columns denote the operational taxonomic unit (OTU) of *Leptospira* and species of vertebrate detected by environmental DNA (eDNA) metabarcoding. Rows indicate sample names and number of eDNA sequences detected in each sample. The sequence numbers are shown with colored matrixes in magenta and green shading for *Leptospira* and vertebrates, respectively, with the colour intensity relative to the sequence numbers. P1 and P2 denote phylogenetic subclades of pathogenic *Leptospira* based on Vincent et al. [2] and present molecular phylogenetic analysis (Supplementary material 1). The "P1 *Leptospira* total, " "P2 *Leptospira* total," and "*Leptospira* total" columns indicate the summed sequence numbers across P1, P2, and all *Leptospira* OTUs, respectively. These categories are shown with colored matrixes in red, purple, and brown shading, respectively. NC indicates a negative control sample (RNase-free water). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Environmental DNA metabarcoding sequencing for detecting Leptospira

To detect *Leptospira* species distributed on Babeldaob Island in the Republic of Palau, we collected 42 water samples from four localities of the inland lake and waterfall (Fig. 1). Five to seven water samples were collected on fine and rainy days at each locality to examine the effect of weather on leptospiral detection (Fig. 1B–E). The mean (± S.E.) volume of filtered water was 593.1 ± 50.2 mL (Supplementary material 3). From each sample, a mean of 3.16 ± 0.23 ng/μL of total eDNA was extracted with mean $OD_{260/280}$ quality values of 1.96 ± 0.13 (Supplementary material 3). The mean volume of filtered water was significantly lower on rainy days than on fine days (Welch's *t*-test, $p < 0.001$), and the average concentration and amount of extracted eDNA were significantly higher on rainy days than on fine days (Welch's *t*-test, $p < 0.001$). The $OD_{260/280}$ quality values of the extracted eDNA did not differ significantly between rainy and fine days (Welch's *t*-test, $p = 0.178$).

From each eDNA sample, partial fragments of the leptospiral 16S rRNA gene and a broadly conserved bacterial 16S rRNA gene V4 region were amplified in a multiplex manner and sequenced using MiSeq. In total 2,132,648 pairs of raw sequences were obtained with an average of 25,389 ± 346 reads per one PCR replicate of each sample. After primary data processing, 2,074,006 reads remained as quality-filtered sequences, with 24,691 ± 340 reads per PCR replicate. Both primer ends of the amplified bacterial 16S rRNA V4 region were found in 2,064,703 reads (24,580 ± 344 reads per PCR replicate), and those of leptospiral 16S rRNA were found in 9303 reads (111 ± 24 reads per PCR replicate).

From the 9303 putative leptospiral 16S rRNA gene sequences, we identified 9219 leptospiral sequences (pink, red, purple, and brown shading in Fig. 3). Among the 9303 sequences, 11 exhibited BLAST-hit to bacteria other than *Leptospira*, and 72 had no BLAST hits against the NCBI nt database [34]. Leptospiral annotation of the remaining 9220 sequences, which was primarily achieved from the BLAST top-hit against the NCBI nt, was carefully corrected based on the results of the molecular phylogeny, as analyzed with the reference 16S rRNA gene sequences of known *Leptospira* species (Supplementary material 1). One eDNA-derived sequence was estimated to be phylogenetically unclear with regards to its position among the other leptospiral clades (denoted as "P20 r1 eDNA-sequence-219" in Supplementary material 1). We identified 9219 phylogenetically-defined environmental *Leptospira* sequences.

3.2. Leptospira detection and distribution, and their association with weather conditions

The *Leptospira* species detected using eDNA metabarcoding, and the number of sequences detected from each location under each weather condition are shown in Fig. 3. Through a careful species annotation based on the molecular phylogenetic analysis described above, we identified 13 OTUs of *Leptospira*. According to the molecular phylogeny (Supplementary material 1), the leptospiral eDNA included sequences genetically related to L. *interrogans*, *L. santarosai*, *L. borgpetersenii*, and L. *noguchii* from the P1 subclade, and L. *licerasiae*, *L. fainei*, *L. langatensis*, *L. haakeii*, *L. wolffii*, and L. *inadai* from the P2 subclade [1,2]. We also found three *Leptospira* clades that did not include known closely related *Leptospira* sequences: two and one from the P1 and P2 subclades, respectively. We provisionally named these OTUs as Unidentified *Leptospira* P1–1, P1–2, and P2–1 (Fig. 3; Supplementary material 1). In general, the obtained species annotation was relatively obscure and limited in terms of resolution because of the shorter eDNA-derived sequences (Supplementary material 1), although the classification of the P1 and P2 clades and their respective total number of sequences was not affected.

The total number of *Leptospira* sequences per unit amount of water samples and number of raw sequences from each sample was significantly higher in rainy weather than in fine weather (GLMM; estimated fixed effects: weather [rain to fine], exponential coefficient (e^{Coef}) = 66.780, exponential standard error (e^{SE}) = 1.061, $z = 55.170$, $d.f. = 1$, $p < 0.001$). The number of *Leptospira* OTUs detected per unit amount of water samples and number of raw sequences was also significantly higher in samples collected on rainy days than in those collected on fine days (GLMM; estimated fixed effects: weather [rain to fine]: e^{Coef} = 18.390, e^{SE} = 1.375, $z = 6.550$, $d.f. = 1$, $p < 0.001$). Regarding the presence/absence of the *Leptospira* in each sample, *Leptospira* OTUs were detected in 20 of the 22 samples collected in rainy conditions, and 9 of the 20 samples collected during fine weather conditions (Fig. 3). The number of samples in which any *Leptospira* OTUs were detected was significantly higher in samples collected in rainy weather than in those collected in fine weather (two-sided Fisher's exact test, $p = 0.002$). Furthermore, multiple comparisons of the total number of *Leptospira* sequences detected under four different types of rain conditions (rain, monsoon, flooding, and typhoon; Fig. 3) showed that the number of *Leptospira* sequences was significantly higher in the presence of flooding than under the other three types of rain conditions (permutated Brunner-Munzel test, sequential Bonferroni method-corrected $p < 0.05$; Table 1). The total number of *Leptospira* sequences did not differ significantly among the other three rain conditions (rain, monsoon, and typhoon; permutated Brunner-Munzel test, sequential Bonferroni method-corrected $p > 0.05$; Table 1). Regarding the eDNA PCR products of the broad bacterial 16S rRNA V4 gene, the number of sequences obtained during flooding did not differ significantly from those in samples collected in other types of rain (typhoon, monsoon, or rain) or on fine days. The mean number of broad bacterial 16S rRNA V4 sequences per a PCR replicate was 23,303 ± 538, 24,391 ± 978, 27,508 ± 345, 23,026 ± 677, and 24,730 ± 568 in flooding, rain, monsoon, typhoon, and fine weather conditions, respectively.

3.3. Environmental DNA metabarcoding sequencing of vertebrates potentially correlated with Leptospira

From each eDNA sample, partial fragments of vertebrate mt-12S rRNA gene were amplified and sequenced by the MiSeq, obtaining a total 344,577 pairs of raw sequences with an average of 4102 ± 443 reads per one of the two PCRs with different annealing temperatures (60 °C and 65 °C) from each sample. After primary data processing, a total of 189,480 reads remained as quality-filtered sequences with 2256 ± 450 reads per a PCR with both primer ends of the amplified vertebrate mt-12S rRNA identified.

From these putative vertebrate sequences, we found a total of 173,196 true vertebrate sequences (green shading in Fig. 3). From the filtered 189,480 sequences, a total of 176,072 non-singleton (≥2 counts) effective sequences in a replicate were conferred species annotation after re-mapping analysis of singleton sequences and BLAST-based analysis using a customized version of the MiFish pipeline [35–37] with tetrapod data from the NCBI nt database [34]. Among these, 12,120 and 294 sequences were annotated as mammalian and non-

Table 1
Pairwise comparisons of total sequence counts of *Leptospira* operational taxonomic units (OTUs) in samples collected in four types of rain conditions (rain, monsoon, flooding, typhoon).

	Rain	Monsoon	Flooding	Typhoon
Rain		*n.s.*	*	*n.s.*
Monsoon			*	*n.s.*
Flooding				*
Typhoon				

*$p < 0.05$ after sequential Bonferroni correction.
n.s., no significant difference.

mammalian (avian, reptile, and amphibian) vertebrates, respectively. The other 160,782 sequences were annotated as teleost fish. The remaining 2876 sequences showed no BLAST hits. The average number of annotated vertebrate eDNA sequences was 4124 ± 828 per sample, indicating 47 vertebrate species, including nine mammalian, two avian, one reptilian and amphibian, and 34 teleost fish species. The right-hand panel of Fig. 3 shows the detected number of sequences of terrestrial vertebrates, including mammals, which are potential hosts for the *Leptospira*, from each sample. A full list of vertebrate species and their sequence counts is provided in Supplementary material 4.

Although several mammalian species were detected multiple times, including cats (*Felis catus*), mice (*Mus musculus*), Yap flying fox (*Pteropus yapensis*), rats (*Rattus sp.*), and pigs (*Sus scrofa*), the co-occurrence relationship between these mammals and *Leptospira* was not statistically clear. The correlation between the number of sequences of mammalian species and *Leptospira* OTUs that were detected from more than two samples (Fig. 3) was not significant when correlation was estimated across all 42 samples ($r = -0.154$ to 0.259, $d.f. = 40$, $p = 0.332$ to 0.098; Benjamini–Hochberg-corrected FDR $q > 0.01$). When the correlation was analyzed according to the four localities (Fig. 1B–E), cats (*Felis catus*) were significantly correlated with detection of L. *wolffii*-related OTU in the Ngardmau Waterfall ($r = 0.866$, $d.f. = 8$, $p = 0.001$; Benjamini–Hochberg-corrected FDR $q < 0.01$), although the sample size was small. No other significant correlations between mammals and *Leptospira* OTUs that were detected more than twice were found in any locality. When the more conservative Fisher's 2 × 2 exact test for the presence or absence of *Leptospira* or mammals was applied, no significant association was detected for any mammalian species, including cats, in all localities (two-sided Fisher's exact test, $p = 0.167$ to >0.999).

4. Discussion

Using eDNA metabarcoding analysis of the leptospiral 16S rRNA partial gene from 42 freshwater samples from four localities of Babeldaob Island in Palau, we found that leptospiral eDNA detection was associated with rainy weather conditions, particularly continuous flooding. Molecular phylogenetic analysis of leptospiral sequences amplified from eDNA supports the finding that more diverse OTUs were detected in samples collected on rainy days than in samples collected on fine days, and the number of *Leptospira* sequences reflecting the biomass of *Leptospira* semi-quantitatively was also significantly greater on rainy days. This finding was also validated by a more rigorous analysis based on the presence or absence of *Leptospira*. In addition, we observed that the total amount of filtered water on rainy days was less than that on fine days, suggesting an increase in suspended material in the water. The eDNA concentration obtained on rainy days was consistently higher than that obtained on fine days. Taken together, these findings suggest that more soil exudate is suspended in freshwater environments during rainy weather, leading to increased contamination by *Leptospira* [5]. The relationship between rainy weather, decreased amounts of filtrated waters, and increased *Leptospira* detection suggests that *Leptospira* may be able to propagate in waterlogged soil, in addition to reservoir animals [14].

Regarding the types of rain, including ordinary rain, typhoon, monsoon, and flooding, flooding was associated with a marked increase in the detection of *Leptospira* from eDNA. The seven samples of flooding water from Ngatpang Waterfall covered most of the leptospiral OTUs found in this study, along with a significantly higher abundance of detected eDNA sequences compared with other localities and weather conditions. This finding emphasizes that continuous rainy weather, such as flooding, affects the exudation of *Leptospira* into freshwater environments [16,42,43]. The mechanism of the exudation of *Leptospira* into water may be explained by the model of leptospiral migration in soil proposed by Davignon et al. [3]. According to this hypothesis, continuous rain causes oxygen in the soil to rise toward the surface, leading to the movement of aerobic *Leptospira* into shallow beds of the soil. This causes leptospiral exudation into rivers and ponds if the rainfall continues for a certain period [3]. In addition, *Leptospira* may be able to propagate in waterlogged soil after flooding [14]. In Palau, cases of leptospirosis have been reported that were thought to have occurred after flooding of Ngardmau Waterfall [19]. Collectively, the relationship between leptospirosis, flooding, and increased leptospiral eDNA detection after flooding confirms that flooding and/or exposure to waterlogged soil are significant environmental risk factors for leptospirosis [12–16,22–25].

We did not find a clear association between *Leptospira* and vertebrate detection in this eDNA analysis of potential host animals. Previous serological studies in Palau have reported *Leptospira* positivity among rats, stray dogs, pigs, cattle, and goats [17,18,20]. Except for goats, all of these animals were detected in our eDNA analysis. However, the correlation between these animals and *Leptospira* was generally not significant. The only significant correlation was between cats and L. *wolffii*-related OTU in samples from the Ngardmau Waterfall, although it is unclear whether these cats were domestic or feral, or a wild species closely related to domestic cats. The correlation between cats and L. *wolffii*-related OTU warrants further investigation. Recently, serological and DNA analyses suggested that dogs living in the peridomestic environment of the Ecuadorian Amazon Basin and interacting with humans are hosts of pathogenic *Leptospira* [44]. According to our previous studies, the correlation between *Leptospira* and animal eDNA detection tends to be clear in stagnant water, such as irrigation water and paddy fields [9], whereas the correlation is sometimes unclear in moving water, such as rivers [21]. Because this study analyzed aquatic localities with relatively fast flows, such as waterfalls, information on the co-occurrence of *Leptospira* and animals may have been lost. The lack of correlation between *Leptospira* and animals supports the possibility that *Leptospira* may propagate in waterlogged soils and not only in reservoir animals [14]. Nevertheless, the animals repeatedly detected in this study, including cats, mice, fruit bats, rats, and pigs, should be considered as potential hosts of *Leptospira* in Palau. To confirm the host-pathogen relationship between these animals and *Leptospira*, further investigation is needed based on the direct serological assays and bacterial isolation from kidneys. Additionally, the detection of human eDNA in multiple localities may provide information for hygiene and sewage management in these areas.

This eDNA analysis has several limitations. First, the taxonomic resolution of leptospiral eDNA sequences was insufficient for complete species-level analysis of *Leptospira* because of the shorter length of the 16S rRNA gene amplified from the eDNA samples. Although the major phylogenetic clusters of *Leptospira* P1 and P2 [1,2] were accurately distinguished, the relationships between closely related *Leptospira* species were not fully resolved. To improve the phylogenetic resolution, we attempted to amplify the leptospiral *secY* gene [45] in our preliminary experiment; however, we could not obtain PCR products and sequences from eDNA samples. In our previous study, the leptospiral *flaB* gene [42] was also analyzed; however, PCR was successful only in a sample with high concentrations of *Leptospira* [9]. Our environmental DNA metabarcoding of *Leptospira* needs to be further improved by incorporating additional marker genes such as *lfb1* [46] to increase the molecular phylogenetic information. In addition, these results of quantitative analysis based on the number of eDNA sequences should be interpreted with caution because the genomic copy number of the 16S rRNA gene varies among bacteria, ranging from one to 15, depending on the bacterial species [47]. The mitochondrial DNA, which is used as a taxonomic marker for vertebrate eDNA analysis, varies in copy number among species, tissues, and cells [48]. The variable amounts of filtered water during the sampling (Supplementary material 3) may also affected the *Leptospira* and animal species detection sensitivity and their diversity. Accordingly, our results, based on the number of eDNA sequences, have technical limitations, although the number is thought to be approximately proportional to the biomass. As mentioned above, the eDNA-detection correlation between the *Leptospira* and animals only

imply the possible pathogen-host relationships. In addition, an environmental detection of *Leptospira* itself only reinforce the presence of them. The substantial and final evidence would be provided by direct isolation and culturing of *Leptospira* from the environment and animals.

In summary, we demonstrated that rainy weather, particularly continuous rain accompanied by flooding, increases the detection of *Leptospira* in the freshwater environments of Palau, based on eDNA metabarcoding analysis. Based on the detected leptospiral eDNA intensity, the risk of infection appears to be higher under conditions of continuous or episodic rain than in fine weather conditions. A relationship between rainy weather and *Leptospira* infection has been reported previously in Palau and other regions. This study documented the leptospiral distribution and relative abundance in natural environments of Palau through direct detection of leptospiral eDNA and comparison between rainy and fine weather conditions. Similarly, eDNA analysis of vertebrates identified possible host animals for *Leptospira* in Palau, including cats, mice, fruit bats, rats, and pigs. In addition, our results do not exclude the possibility that the detected *Leptospira* propagated in waterlogged soils. The methodology and results of this study provide important insights into potential risk factors for *Leptospira* infection in Palau. It also contains useful information for the management of livestock and other pathogens, and for public health.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2024.100898.

Ethics statement

Field work in Palau was conducted under the Bureau of Agriculture, Ministry of Agriculture, Fisheries and the Environment, Republic of Palau. Sample transfer and DNA experiment was conducted under collaboration research and material transfer agreement between the Bureau of Agriculture, Ministry of Agriculture, Fisheries and the Environment, Republic of Palau and Faculty of Medicine, University of the Ryukyus, Japan.

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CRediT authorship contribution statement

Yukuto Sato: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kaori Tsurui-Sato:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Yoichiro Uchima:** Visualization, Validation, Investigation, Formal analysis, Data curation. **Cheryl-Ann Udui:** Writing – review & editing, Resources, Conceptualization. **Osiro Lorin:** Resources, Conceptualization. **Kashgar Rengulbai:** Resources, Conceptualization. **Claudia Toma:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Ryo Suzuki:** Writing – review & editing, Validation, Resources, Project administration, Investigation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that authors did not use any generative AI and AI-assisted technologies in the writing process.

Declaration of competing interest

On behalf of all authors, the corresponding author states that there are no competing interests to declare.

Data availability

The raw sequence reads generated by MiSeq in the current study are available in the DDBJ Sequence Read Archive (DRA) for leptospiral and vertebrate metabarcoding data, respectively. Accession number: DRA018593 and DRA018594.

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