

RESEARCH ARTICLE

First metagenomic detection and partial genomic characterization of *Bossavirus delphinidgamma1* in captive common bottlenose dolphins in Southeast Asia



Augustine Natasha¹ , Annisa Wening Maharani Putri² , Nunak Nafiqoh³ , Sara Puspareni Prayitno¹ , Lila Gardenia³ , Hesty Novita² , Tanjung Penataseputro² , Agustin Indrawati⁴ , Desy Sugiani² , Uni Purwaningsih² , Tuti Sumiati² , Dewi Syahidah² , Rifky Rizkiantino² , Ji Eun Park¹, Harimurti Nuradji² , Angela Mariana Lusiastuti² and Won-Keun Kim^{1,5}

1. Department of Microbiology, Hallym University College of Medicine, Chuncheon 24252, Republic of Korea.

2. Research Center for Veterinary Science, National Research and Innovation Agency (BRIN), Bogor 16911, Indonesia.

3. Directorate of Scientific Collection Management, National Research and Innovation Agency (BRIN), Bogor 16911, Indonesia.

4. Division of Medical Microbiology, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor 16680, Indonesia.

5. Institute of Medical Science, College of Medicine, Hallym University, Chuncheon 24252, Republic of Korea.

ABSTRACT

Background and Aim: *Bossavirus delphinidgamma1* is a dolphin-associated gammaherpesvirus for which genomic information from tropical Asian marine mammal populations remains scarce. This study aimed to detect and partially characterize *Bossavirus delphinidgamma1* in captive common bottlenose dolphins (*Tursiops truncatus*) in Indonesia and evaluate an integrated Oxford Nanopore Flongle–Illumina sequencing workflow for viral surveillance using non-invasive, low-biomass samples.

Materials and Methods: Fifteen blowhole, anal, and reproductive tract swabs were collected from five clinically healthy captive dolphins between January 2023 and January 2025. RNA and DNA were extracted from individual or pooled samples. RNA underwent sequence-independent single-primer amplification followed by Oxford Nanopore Flongle sequencing, whereas DNA from Dolphin 4 was sequenced using Illumina NovaSeq X. Metagenomic reads were subjected to taxonomic identification, reference mapping, consensus generation, coding sequence annotation, protein similarity analysis, multiple-sequence alignment, and maximum-likelihood phylogenetic analysis.

Results: Oxford Nanopore sequencing detected *Bossavirus delphinidgamma1* sequences exclusively in the anal swab from Dolphin 4. The run generated 87,070 reads, of which 109 (0.12%) mapped to the viral reference genome, with a mean coverage depth of 0.28×. Subsequent DNA metagenomic sequencing of pooled Dolphin 4 swab extracts confirmed viral genetic material. Combined Oxford Nanopore and Illumina mapping covered 63.7% of the reference genome, with a median depth of 1×; 0.71% of the genome achieved ≥10× coverage. The recovered sequence contained a nearly complete DE1 gene and >50% coverage of several additional coding regions. Twelve predicted coding regions showed 68.9%–97.1% amino acid identity to *Bossavirus delphinidgamma1* or a closely related dolphin gammaherpesvirus. Phylogenetic analysis placed the Indonesian sequence with the Sarasota reference strain, with 100% bootstrap support.

Conclusion: This study provides the first metagenomic evidence and partial genomic characterization of *Bossavirus delphinidgamma1* in captive common bottlenose dolphins in Indonesia and Southeast Asia. The integrated sequencing approach enabled substantial partial-genome recovery from non-invasive samples and supports its application for exploratory marine mammal viral surveillance. Larger longitudinal studies incorporating targeted molecular confirmation, serology, and environmental sampling are warranted.

Keywords: *Bossavirus delphinidgamma1*, bottlenose dolphin, gammaherpesvirus, marine mammal, metagenomic sequencing, non-invasive sampling, Southeast Asia, viral surveillance.

INTRODUCTION

Marine mammal stranding and mortality investigations have contributed substantially to the detection and characterization of wildlife pathogens, including viruses of relevance to animal and public health [1, 2]. Viral host range and cross-species transmission are influenced by evolutionary relationships between viruses and their

Corresponding Author: Angela Mariana Lusiastuti and Won-Keun Kim

E-mail: ange003@brin.go.id; wkkim1061@hallym.ac.kr

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Co-authors: AN: B23007@hallym.ac.kr; AWMP: anni024@brin.go.id; NN: nuna001@brin.go.id; SPP: pusparenisara@gmail.com; LG: lila003@brin.go.id; HN: hess001@brin.go.id; TP: tanj002@brin.go.id; AI: indraseta@apps.ipb.ac.id; DS: desy008@brin.go.id; UP: unip001@brin.go.id; TS: tuti.sumiati@brin.go.id; DSy: dewi050@brin.go.id; RR: rifky.rizkiantino@brin.go.id; JEP: wlsttl313@gmail.com; HNu: hari056@brin.go.id

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hosts, and greater phylogenetic distance between hosts can affect patterns of viral transmissibility and virulence [3]. Interactions at the human–animal interface can also create opportunities for pathogens to cross-species barriers and establish infections in new hosts [4, 5]. Comparative phylogenetic analyses across virus families and their eukaryotic hosts have further demonstrated that both host codivergence and cross-species transmission contribute to viral evolutionary histories [6].

Compared with free-ranging populations, captive marine mammal facilities represent a distinct epidemiological setting characterized by repeated animal–caretaker interactions and managed aquatic environments [7]. Such facilities also provide opportunities for routine clinical observation and longitudinal non-invasive sampling [7, 8]. Consequently, captive facilities, particularly those in tropical regions where marine mammal viral surveillance remains limited, may serve as valuable sentinel settings for monitoring persistent or latent viral infections and changes occurring at the animal–human–environment interface [8, 9]. However, viral genomic surveillance of captive marine mammals remains poorly represented in Asia, particularly for cetacean herpesviruses, because many regional investigations have focused on stranded or clinically affected animals rather than longitudinal surveillance of clinically healthy captive populations [9, 10].

Marine mammals are increasingly recognized as valuable sentinels of pathogen circulation and changes in marine ecosystem health [9, 11]. Common bottlenose dolphins (*Tursiops truncatus*) are listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora, highlighting the importance of conservation-sensitive approaches to their health assessment [12]. Non-invasive, multisite sampling may therefore provide a practical approach for pathogen surveillance in living dolphins while minimizing disturbance associated with invasive sample collection.

Herpesviruses are large, double-stranded DNA viruses that infect a wide range of vertebrate hosts [13, 14]. Compared with several other viral groups, double-stranded DNA viruses exhibit relatively frequent patterns of host codivergence [15]. The subfamily *Gammaherpesvirinae* includes the genus *Bossavirus*, which comprises dolphin-associated gammaherpesviruses [16]. *Bossavirus delphinidgamma1*, previously referred to as common bottlenose dolphin gammaherpesvirus 1, was characterized from a common bottlenose dolphin in Sarasota Bay, Florida, USA [17, 18]. However, information regarding the geographic distribution and genomic diversity of this virus remains limited, particularly among dolphin populations in tropical Asian waters.

Marine mammal virology across Asia and Australasia remains geographically uneven, with much of the available regional evidence originating from stranded animals, mortality investigations, or targeted pathogen testing [10, 19, 20]. A global systematic review similarly demonstrated that published investigations of virus-associated mortality in marine mammals have been predominantly retrospective and geographically concentrated [9]. Longitudinal surveillance of clinically healthy animals using non-invasive metagenomic approaches remains particularly limited in Southeast Asia, restricting current understanding of viral occurrence, persistence, distribution, and genomic diversity in regional marine mammal populations.

Viral metagenomics provides a culture-independent approach for detecting known and previously uncharacterized viruses in aquatic organisms, including viral infections that may occur without overt clinical signs [21, 22]. Nevertheless, metagenomic analysis of non-invasive marine mammal swabs presents substantial technical challenges because these specimens may contain low concentrations of viral nucleic acids, abundant host-derived material, and fragmented viral genomes, thereby limiting viral detection and genome reconstruction. In addition, multiplexed Nanopore sequencing can be affected by barcode cross-talk, in which reads are incorrectly assigned among barcoded samples, potentially complicating the interpretation of low-abundance viral signals [23].

Oxford Nanopore sequencing provides rapid sequencing and long-read capability, whereas Illumina sequencing (Illumina, Inc., San Diego, CA, USA) offers high short-read accuracy and sequencing depth. Integration of these complementary platforms may therefore improve viral detection and partial-genome recovery from challenging biological specimens [24, 25]. Oxford Nanopore Flongle provides a relatively low-cost sequencing option for small-scale or single-sample viral investigations and, despite its lower throughput, can generate sufficient sequencing data for viral detection [26, 27]. These characteristics make Flongle potentially useful for pilot surveillance involving limited, low-biomass, or difficult-to-obtain specimens, including non-invasive swabs collected from marine mammals [26, 27].

Despite advances in marine mammal viral metagenomics, important geographic, epidemiological, and methodological knowledge gaps remain. Published information on *Bossavirus delphinidgamma1* is geographically restricted, and genomic evidence for this virus from Indonesia and the wider Southeast Asian region is lacking.

Furthermore, regional marine mammal virus investigations have largely relied on stranded, deceased, or clinically affected animals, whereas metagenomic surveillance of clinically healthy captive dolphins using repeated-access, non-invasive specimens remains limited [9, 10, 19, 20]. The performance of low-throughput Oxford Nanopore Flongle sequencing in combination with high-depth Illumina sequencing for recovering *Bossavirus delphinidgamma1* genomic material from low-biomass dolphin swabs has also not been sufficiently characterized. Consequently, it remains unclear whether an integrated sequencing strategy can provide useful viral genomic information from these challenging specimens. Addressing these gaps is important for establishing baseline information on cetacean viral diversity and distribution in Southeast Asia and for developing practical genomic surveillance approaches suitable for living marine mammals.

Therefore, this study aimed to detect and partially characterize *Bossavirus delphinidgamma1* sequences in clinically healthy captive common bottlenose dolphins in Indonesia using non-invasive, multisite swab sampling and to evaluate an integrated Oxford Nanopore Flongle–Illumina metagenomic sequencing workflow for viral detection and partial-genome recovery from low-biomass specimens. Specifically, the study sought to identify viral sequence reads, determine their genomic coverage and similarity to previously characterized *Bossavirus delphinidgamma1* sequences, characterize recovered coding regions, and assess the phylogenetic relationship of the detected viral sequence with previously reported dolphin gammaherpesvirus sequences. The findings are intended to provide baseline genomic evidence for *Bossavirus delphinidgamma1* in Southeast Asia and to assess the feasibility of combining complementary sequencing platforms for non-invasive viral surveillance of captive marine mammals.

MATERIALS AND METHODS

Ethical approval

The study protocol was reviewed and approved by the Ethics Committee for the Use and Care of Animals of the National Research and Innovation Agency (BRIN), Republic of Indonesia (approval no. 075/KE.03/SK/07/2023, 28-07-2023). All sampling procedures were performed using non-invasive swab collection from clinically healthy captive common bottlenose dolphins (*T. truncatus*). The sampling approach was designed to obtain biological material from living dolphins while minimizing animal disturbance and avoiding invasive tissue collection.

Study period and location

The study was conducted from January 2023 to January 2025 at Wersut Seguni Indonesia, a marine conservation facility located on the northern coast of Java Island, Indonesia. Five captive common bottlenose dolphins were included in the study. The dolphins were clinically healthy at the time of sampling and had no known history of illness (Table 1).

Table 1: Metadata of the five common bottlenose dolphins (*T. truncatus*).

Code	Sex	Age (years)	Reproductive status	Clinical status at sampling
Dolphin 1	Male	14	The dolphin has reach full reproductive maturity both physically and socially	Clinically healthy
Dolphin 2	Female	2	The dolphin is still in adolescent phase or transitioning away from complete dependence on nursing from its mother	Clinically healthy
Dolphin 3	Male	16	The dolphin has reached full reproductive maturity both physically and socially	Clinically healthy
Dolphin 4	Male	20	The dolphin has reach full reproductive maturity both physically and socially	Clinically healthy
Dolphin 5	Female	16	The dolphin is in its prime reproductive age, fully sexually mature, and highly fertile	Clinically healthy

Study design

This study employed an exploratory metagenomic surveillance design using non-invasive, multisite swab sampling followed by sequential Oxford Nanopore (Oxford Nanopore Technologies, Oxford, UK) and Illumina sequencing. Five dolphins were sampled from three anatomical sites: the blowhole, anus, and reproductive tract. Three separate swabs were collected from each anatomical site using a new sterile swab for each sampling replicate to minimize cross-contamination between samples and anatomical sites. Each swab was individually placed in a tube containing 10 mL of phosphate-buffered saline (PBS; pH 7.4; Bacto Laboratories Pty Ltd., Mount Pritchard, New South Wales, Australia) as the transport medium.

Site-specific nucleic acid preparations were initially subjected to Oxford Nanopore sequencing to screen for viral sequences and determine the anatomical origin of detectable viral signals. This analysis identified *B. delphinidgamma1* sequences in the anal swab from Dolphin 4. Because the DNA yield from this anal swab was insufficient for subsequent Illumina sequencing, DNA extracts obtained from the blowhole, anal canal, and reproductive tract swabs of Dolphin 4 were pooled before Illumina library preparation. Therefore, the Illumina-derived reads and subsequent partial viral genome recovery represented the pooled Dolphin 4 sample and could not be assigned to an individual anatomical site.

RNA and DNA extraction

Swabs immersed in PBS were vigorously vortexed to maximize nucleic acid recovery. For RNA extraction, a total of 1 mL of pooled PBS was divided among four extraction tubes. In each tube, 250 µL of sample was mixed with 750 µL of TRIzol™ LS reagent (Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. After drying, the RNA pellets from the four tubes were sequentially resuspended using the same 10 µL volume of RNase-free water (Thermo Fisher Scientific) to concentrate the recovered RNA into a single preparation. RNA quality was assessed using the 260/280 absorbance ratio, which ranged from 1.61 to 2.01.

For Dolphin 4, additional DNA extraction was performed from the remaining PBS associated with each swab using GENEzol™ reagent (Invitrogen (Thermo Fisher Scientific) according to the manufacturer's protocol and following the same extraction strategy. DNA extracts from the three anatomical sampling sites were subsequently pooled for Illumina sequencing because the DNA yield from the positive anal swab alone was insufficient for library preparation.

Integrated metagenomic next-generation sequencing workflow

For RNA samples, sequence-independent single-primer amplification was performed. Host ribosomal RNA was initially depleted using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) V2 (New England Biolabs, Ipswich, MA, USA). The depleted RNA was subsequently hybridized with the FR26RV-N primer (5'-GCC GGA GCT CTG CAG ATA TCN NNN NN-3'). A 12-µL RNA-primer mixture containing 2 µL of 10 pM FR26RV-N primer, 1 µL of 50% dimethyl sulfoxide, and 9 µL of RNA template was prepared. The mixture was incubated at 65°C for 5 min and subsequently held at 4°C.

First-strand complementary DNA (cDNA) was synthesized using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific). An 8-µL reverse-transcription master mix containing 4 µL of 5× SSIV Buffer, 1 µL of 100 mM dithiothreitol, 1 µL of RNaseOUT™ ribonuclease inhibitor, 1 µL of 10 mM deoxynucleotide triphosphate mixture, and 1 µL of SuperScript IV Reverse Transcriptase was added to the annealed RNA-primer mixture, resulting in a final reaction volume of 20 µL. Reverse-transcription was performed at 23°C for 10 min, 50°C for 10 min, and 80°C for 10 min, followed by a hold at 4°C.

Double-stranded (ds) cDNA was generated using Klenow 3'→5' exo DNA polymerase (New England Biolabs, Ipswich, MA, USA). The 20-µL reaction mixture contained 2 µL of 10× Klenow DNA Polymerase Buffer, 1 µL of Klenow DNA Polymerase (5 U/µL), 1 µL of RNase H, 0.5 µL of deoxynucleotide triphosphate mixture (0.5 mM each), and 15.5 µL of first-strand cDNA. The reaction was incubated at 37°C for 10 min and 75°C for 20 min. The resulting double-stranded complementary DNA (ds cDNA) was purified using Agencourt AMPure XP beads (Beckman Coulter Life Sciences, Brea, CA, USA).

Random-fragment amplification (RFA) of the purified ds cDNA was performed using MyTaq Red Mix (Bioline, Taunton, MA, USA) and the FR20RV primer (5'-GCC GGA GCT CTG CAG ATA TC-3'). Each 25-µL polymerase chain reaction (PCR) mixture contained 12.5 µL of 2× MyTaq Red Mix, 1 µL of 10 pM FR20RV primer, and 11.5 µL of ds cDNA template. The cycling conditions consisted of initial denaturation at 98°C for 30 s, followed by 38 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 20 s, and extension at 72°C for 45 s. Second amplification was performed using the same PCR mixture with 11.5 µL of the RFA product serving as the template. The thermal cycling conditions were maintained as described for the RFA, with the amplification step carried out for 25 cycles. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter Life Sciences).

The resulting amplicons were prepared for sequencing using a ligation sequencing kit compatible with Flongle flow-cell chemistry according to the manufacturer's protocol (Oxford Nanopore Technologies). Each library was sequenced as a singleplex assay using a MinION Mk1C system equipped with a Flongle flow cell (Oxford Nanopore Technologies). Following the detection of DNA viral sequences during the initial RNA-based metagenomic sequencing workflow, total DNA metagenomic sequencing was subsequently performed to confirm the viral signal. The pooled DNA sample from Dolphin 4 was prepared for metagenomic sequencing and sequenced

using the Illumina NovaSeq X platform according to the manufacturer's instructions (Illumina, Inc.).

Bioinformatic analysis

Raw reads generated using the Flongle flow cells were adapter-trimmed with Porechop and subjected to read-level taxonomic classification using Centrifuge, *de novo* assembly, and Basic Local Alignment Search Tool (BLAST)-based downstream sequence analysis implemented in VirPipe [25, 28]. VirPipe was operated using its built-in default configuration. To verify the viral sequences identified during the initial analysis, raw reads were mapped against the corresponding reference sequence using CLC Genomics Workbench version 22 (QIAGEN Digital Insights, Aarhus, Denmark) (Figure 1).

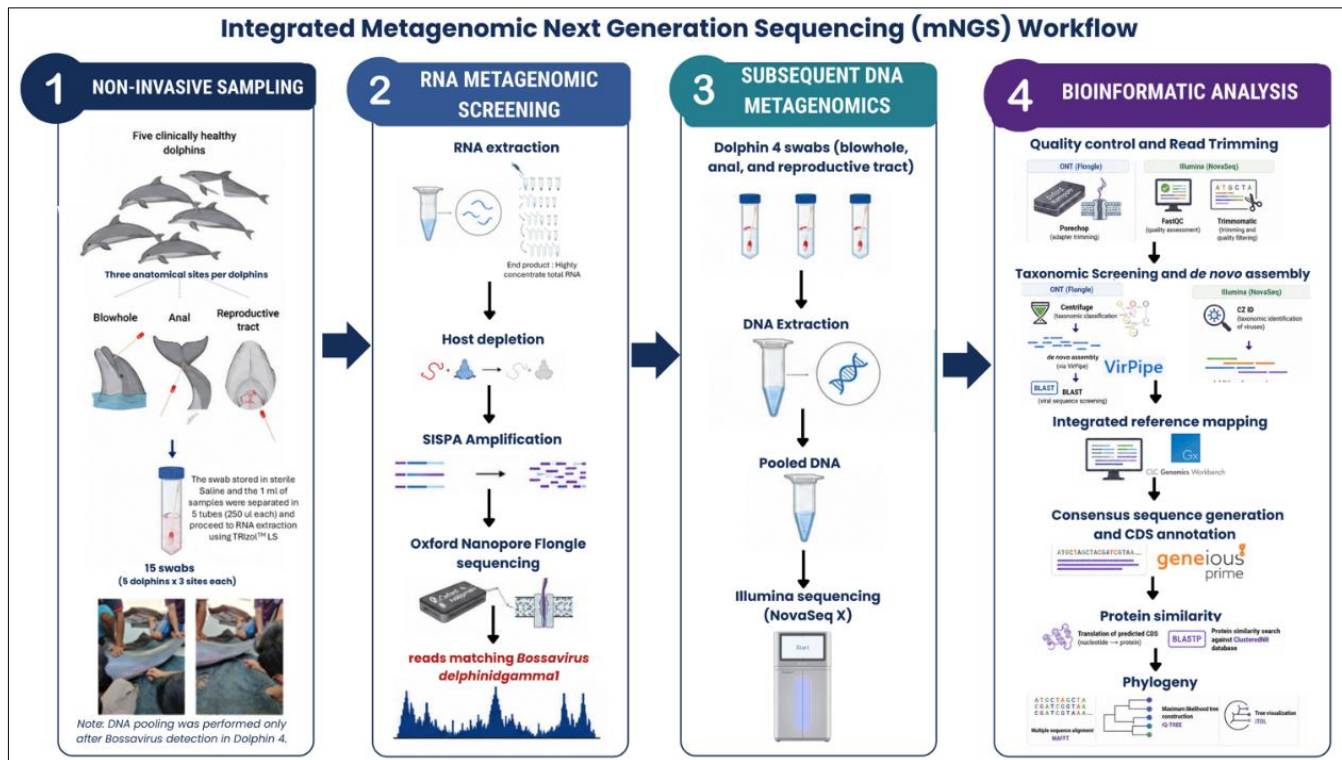


Figure 1: Integrated metagenomic next-generation sequencing workflow for the detection of *Bossavirus delphinidgamma1*. Fifteen non-invasive swabs were collected from five clinically healthy dolphins and processed individually for ribonucleic acid extraction, sequence-independent single-primer amplification, and Oxford Nanopore Flongle sequencing. Reads matching *B. delphinidgamma1* were detected in an anal swab from Dolphin 4. Because the DNA yield from the anal swab was insufficient for Illumina sequencing, DNA extracts from the blowhole, anal, and reproductive tract swabs of Dolphin 4 were pooled before Illumina NovaSeq X sequencing. Bioinformatic analysis included quality-control, taxonomic screening, *de novo* assembly, reference mapping, consensus-sequence generation, coding DNA sequence annotation, protein similarity analysis, and phylogenetic reconstruction. Flongle sequencing was conducted at the National Research and Innovation Agency laboratory under controlled conditions.

For Illumina metagenomic sequencing, raw paired-end reads were initially assessed using FastQC v0.12.1 (Babraham Institute, Babraham, UK) [29]. Quality-control reports were evaluated for per-base sequence quality, per-base sequence content, GC-content distribution, sequence duplication, overrepresented sequences, and adapter contamination. Reads were subsequently processed using Trimmomatic v0.39 (Usadel Lab, RWTH Aachen University / Forschungszentrum Jülich, Germany) in paired-end mode [30]. The first 25 nucleotides were removed using HEADCROP:25, and the remaining reads were cropped to 100 nucleotides using CROP:100, thereby excluding the final 25 nucleotides of the original reads. Quality trimming was performed using a four-base sliding window, with trimming initiated when the mean Phred quality score decreased below 20 (SLIDINGWINDOW:4:20). Reads shorter than 75 nucleotides after processing were discarded (MINLEN:75). Adapter clipping was not performed because the initial quality assessment identified no substantial adapter contamination. The processed paired-end reads were reassessed using FastQC v0.12.1 (Babraham Institute) before downstream metagenomic analysis [29]. The processed Illumina reads were subsequently analyzed using CZ ID (CZ ID; Chan Zuckerberg Initiative, Redwood City, CA, USA) [21].

Following identification of the viral sequences, raw reads generated by Illumina and Oxford Nanopore

sequencing were remapped to the *B. delphinidgamma1* reference genome using CLC Genomics Workbench. A consensus-sequence was extracted and annotated for coding DNA sequences (CDSs) using Geneious Prime. Predicted CDSs were translated, and protein identities were evaluated using Protein Basic Local Alignment Search Tool (BLASTP) against the ClusteredNR database [31]. The resulting consensus-sequence was aligned with related viral sequences using MAFFT v7.490 (Kazutaka Katoh, Osaka/Tokyo, Japan) [32]. The alignment was subsequently subjected to maximum-likelihood phylogenetic analysis using the IQ-TREE web server, and the resulting phylogenetic tree was visualized using Interactive Tree of Life (iTOL) (European Molecular Biology Laboratory (EMBL), Heidelberg, Germany) [33, 34]. Unless otherwise specified, bioinformatic tools were operated using their default settings.

RESULTS

B. delphinidgamma1 sequences were detected in samples from one captive common bottlenose dolphin in Indonesia. To our knowledge, this represents the first report of *B. delphinidgamma1* genetic material in Indonesia and Southeast Asia and the first detection in an Asian dolphin population, extending its published geographic distribution beyond the previously reported Sarasota Bay population in Florida, USA [17].

Sample characteristics and sequencing performance using the Flongle flow-cell

Five dolphins were sampled using blowhole, anal, and reproductive tract swabs. All dolphins were born and maintained at the Wersut Seguni Indonesia conservation facility, located on the northern coast of Java Island. The facility uses treated seawater obtained from the Java Sea (Figure 2). All sampled dolphins were clinically healthy at the time of sampling and remained clinically healthy during the observation period. According to the available facility records, none of the sampled dolphins had known contact with wild dolphins.



Figure 2: Geographic location of the Wersut Seguni Indonesia facility on the northern coast of Java Island, Indonesia. The red dot indicates the location of the facility, and the green area represents the Republic of Indonesia. The map was generated using MapChart.net (Minas Giannekas, Athens, Greece) and edited using Adobe Illustrator 2025 (Adobe Inc., San Jose, CA, USA).

RNA was recovered from the swab transport media. RNA measurements were available for Dolphins 3–5 only, with 260/280 absorbance ratios ranging from 1.61 to 2.01 (Table 2). This variation may have resulted from residual proteins or extraction reagents. Following bead-based purification and host ribosomal RNA depletion, the recovered RNA was used for library preparation and subsequent Flongle sequencing. Sequencing runs for Dolphins 3 and 4 showed low pore occupancy despite the availability of an adequate number of active pores (Figure 3).

Table 2: RNA concentration and quality of swab specimens after the RNA concentration procedure.

Specimen ID	RNA concentration (ng/μL)	260/280 ratio
Dolphin 3, blowhole swab	7.04	1.64
Dolphin 3, anal swab	7.806	1.63

Specimen ID	RNA concentration (ng/ μ L)	260/280 ratio
Dolphin 3, reproductive tract swab	11.710	1.82
Dolphin 4, blowhole swab	9.155	1.73
Dolphin 4, anal swab	5.778	1.63
Dolphin 4, reproductive tract swab	17.797	2.01
Dolphin 5, blowhole swab	7.636	1.61
Dolphin 5, anal swab	15.804	1.97
Dolphin 5, reproductive tract swab	8.949	1.73

Note: RNA concentration and A260/A280 ratio data were unavailable for Dolphins 1 and 2; therefore, only data for Dolphins 3–5 are presented.

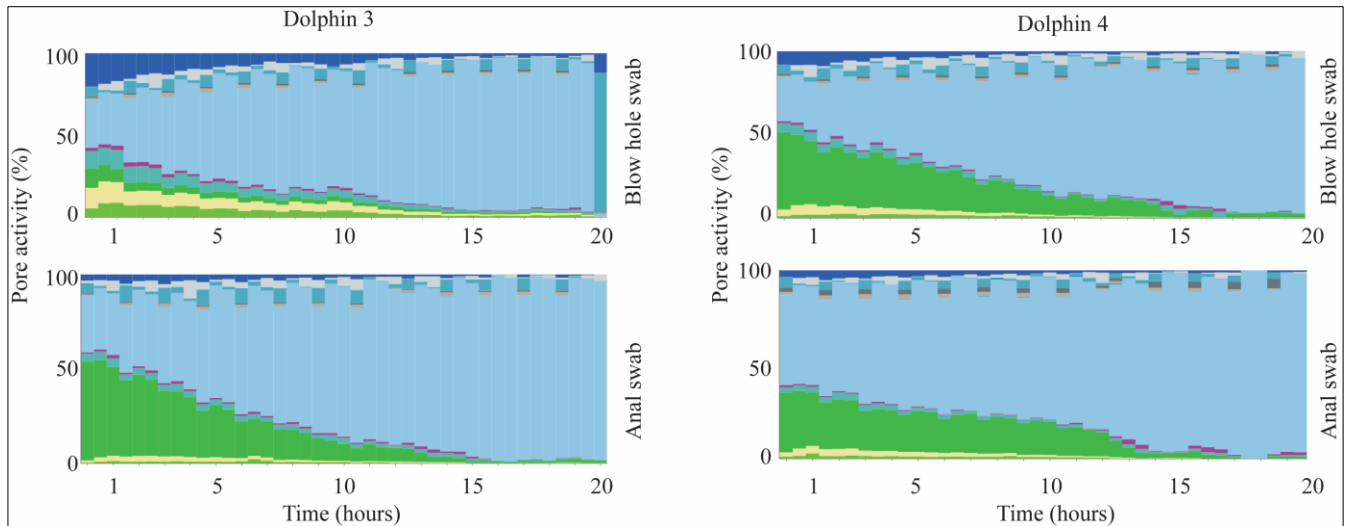


Figure 3: Pore occupancy during Oxford Nanopore Flongle sequencing of samples from Dolphins 3 and 4. Both sequencing runs showed low pore occupancy despite an adequate number of active pores. The observed low occupancy was associated with insufficient final library concentrations rather than an insufficient number of available active pores.

Initial detection of *B. delphinidgamma1* using metagenomic sequencing

RNA metagenomic sequencing did not yield meaningful RNA virus reads. However, partial sequences spanning multiple coding regions of *B. delphinidgamma1* were detected exclusively in the anal swab from Dolphin 4. Flongle sequencing generated 87,070 reads, of which 109 (0.12%) mapped to the *B. delphinidgamma1* reference genome, resulting in a mean coverage depth of 0.28 $\times$. No additional viruses were significantly detected among the remaining reads.

The Flongle sequencing run was performed as a single-sample assay, and comparable *B. delphinidgamma1* sequences were not detected in samples from the other dolphins. Because *B. delphinidgamma1* is a DNA virus, DNA metagenomic sequencing was subsequently performed to further evaluate the viral signal detected in Dolphin 4.

The DNA yield from the Dolphin 4 anal swab alone was insufficient for Illumina sequencing. Therefore, DNA extracts from the blowhole and reproductive tract swabs were pooled with the anal swab extract before Illumina library preparation. Consequently, the Illumina sequencing results represent the pooled Dolphin 4 sample and cannot be assigned to a specific anatomical sampling site. Initial detection was obtained using the Oxford Nanopore analysis workflow for Flongle reads, whereas CZ ID was used to analyze the subsequently generated Illumina reads from pooled Dolphin 4 DNA extracts. Samples collected from Dolphins 1, 2, 3, and 5 yielded either negative results or low total read counts (Supplementary Table 1). Sequencing statistics and *B. delphinidgamma1* detection results for each dolphin and anatomical sampling site are presented in Supplementary Table 1.

Partial genomic characterization of *B. delphinidgamma1*

DNA metagenomic sequencing provided broader coverage of the *B. delphinidgamma1* genome, although a complete-genome was not recovered. Combined mapping of Oxford Nanopore and Illumina reads using CLC Genomics Workbench covered 63.7% of the *B. delphinidgamma1* reference genome. Coverage was low and uneven, with a median depth of 1 $\times$. Only 0.71% of the reference genome achieved a sequencing depth of $\geq 10\times$, whereas several short genomic regions exhibited disproportionately high coverage (Figure 4).

The recovered sequence contained a nearly complete DE1 gene and >50% coverage of several additional coding regions (Table 3). Protein similarity analysis identified 12 predicted coding regions with similarities to *B.*

delphinidgamma1 or a related dolphin gammaherpesvirus. Amino acid identities ranged from 68.9% for open reading frame (ORF)7 to 97.1% for ORF3. The recovered regions included DE1, DE2, DE3, ORF3, ORF7, ORF8, ORF9, ORF11, ORF39, ORF49, ORF53, and ORF65 (Table 3).

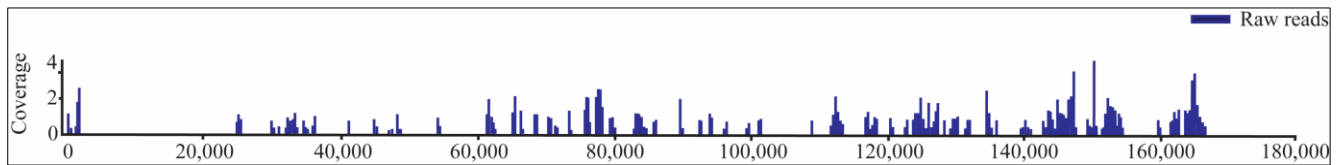


Figure 4: Reference-genome coverage of *Bossavirus delphinidgamma1* obtained from metagenomic sequencing reads. The mapped reads covered 63.7% of the *B. delphinidgamma1* reference genome (NC_035117.1), with low and uneven coverage and a median depth of 1×. Only 0.71% of the reference genome achieved a sequencing depth of $\geq 10\times$, whereas several short genomic regions exhibited disproportionately high coverage.

Table 3: Genomic features with high similarity to known viral proteins in public databases.

Coding region	Query coverage (%)	Length (bp)	BLASTP result	Organism	Amino acid identity (%)
De1	100	534	Apoptosis inhibitor FLIP De1	<i>B. delphinidgamma1</i>	90.4
De2	71.3	807	Membrane protein De2	<i>B. delphinidgamma1</i>	89.6
De3	54.5	750	Protein De3	<i>B. delphinidgamma1</i>	76.9
ORF3	67.3	3939	Protein G3	<i>B. delphinidgamma1</i>	97.1
ORF7	67.1	2148	DNA packaging terminase subunit 2	<i>B. delphinidgamma1</i>	68.9
ORF8	54.2	2667	Glycoprotein B	<i>T. truncatus</i> gammaherpesvirus	94.6
ORF9	81.3	3036	DNA polymerase catalytic subunit	<i>B. delphinidgamma1</i>	95.1
ORF11	79.8	1278	Virion protein G11	<i>B. delphinidgamma1</i>	92.16
ORF39	80.2	1122	Envelope glycoprotein M	<i>B. delphinidgamma1</i>	92.1
ORF49	63.4	922	Protein G49	<i>B. delphinidgamma1</i>	84.1
ORF53	90.3	321	Envelope glycoprotein N	<i>B. delphinidgamma1</i>	84.9
ORF65	92.9	420	Small capsid protein	<i>B. delphinidgamma1</i>	84.17

BLASTP = Basic Local Alignment Search Tool for Proteins; bp = Base pairs; CDS = Coding DNA sequence; DE = Dolphin-specific gene; FLIP = FLICE-like inhibitory protein; ORF = Open reading frame.

Phylogenetic analysis was performed to assess the relationship between the sequence recovered from Dolphin 4 and previously reported viral sequences. The Dolphin 4 sequence, designated Java-WSI/2023, and the Sarasota reference strain formed a sister clade with 100% bootstrap support and an estimated patristic distance of 0.054 substitutions/site (Figure 5).

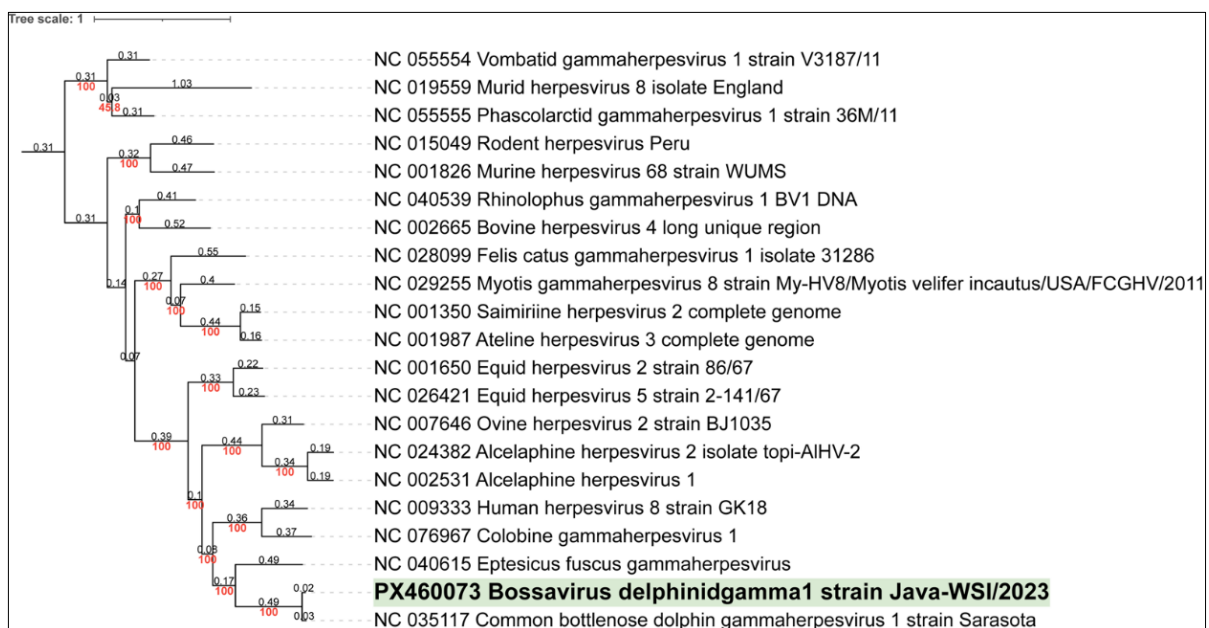


Figure 5: Maximum-likelihood phylogenetic analysis of *Bossavirus delphinidgamma1*-related sequence recovered from Dolphin 4. The phylogenetic tree was inferred using the IQ-TREE web server. The general time-reversible model with empirical base frequencies and FreeRate heterogeneity with 10 categories was selected according to the Bayesian information criterion, and branch support was assessed using 1,000 bootstrap replicates. The Java-WSI/2023 sequence and Sarasota reference strain formed a sister clade with 100% bootstrap support and an estimated patristic distance of 0.054 substitutions/site. Black numbers indicate branch lengths, and red numbers indicate bootstrap support values.

The annotated partial-genome sequence obtained from Dolphin 4 was deposited in GenBank under accession no. PX460073. The corresponding Oxford Nanopore raw reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under BioProject PRJNA1504244, BioSample SAMN62049142, and Sequence Read Archive (SRA) accession no. SRR39901519. The paired-end Illumina metagenomic reads were also submitted under the same BioProject and were undergoing BioSample taxonomy curation at the time of manuscript preparation; the corresponding accession number will be added when assigned.

DISCUSSION

Detection and geographic significance of *B. delphinidgamma1*

B. delphinidgamma1 was originally characterized from a rectal lesion biopsy collected from a common bottlenose dolphin in Sarasota Bay, Florida, USA [17]. The detection of *B. delphinidgamma1* genetic material in Indonesia extends the published geographic record of this virus to Southeast Asia and, to our knowledge, represents its first reported detection in Indonesia and the wider Southeast Asian region (Supplementary Table 2). Importantly, the sampled dolphins were clinically healthy and had no known history of illness. Detection of viral genetic material in a clinically healthy animal is compatible with subclinical viral presence; however, the present data are insufficient to determine whether the detected sequences represent latent infection, active viral replication, or viral shedding. Therefore, longitudinal molecular and serological surveillance is required to establish the biological and clinical significance of this finding.

The present study also demonstrates the potential value of non-invasive sampling for genomic surveillance of captive dolphins. Repeated sampling of captive animals can facilitate longitudinal monitoring while reducing reliance on invasive tissue collection [7, 35]. Captive populations additionally provide opportunities for standardized health assessments and repeated specimen collection that are considerably more difficult to achieve in free-ranging cetaceans [7, 35]. These characteristics may make captive marine mammal facilities useful settings for investigating the persistence and temporal dynamics of cetacean-associated viruses.

Performance of the integrated sequencing approach

Integration of the Oxford Nanopore and Illumina metagenomic datasets increased coverage of the *B. delphinidgamma1* reference genome. Although combining raw reads generated by these platforms requires careful interpretation because of their different sequencing characteristics, the N50 of the Flongle reads in the present study was <200 bp. Thus, the Nanopore dataset predominantly comprised short, fragmented reads of lengths approaching those of the 100–150-bp Illumina reads. Both datasets were mapped to the same reference genome using CLC Genomics Workbench, enabling their combined contribution to reference-genome coverage. Nevertheless, platform-specific sequencing errors remain an important consideration, particularly when interpreting individual nucleotide variants from regions with low sequencing depth.

The integrated analysis covered 63.7% of the *B. delphinidgamma1* reference genome; however, coverage was low and uneven, with a median depth of 1×, and only 0.71% of the genome achieved a depth of ≥10× (Figure 4). These characteristics precluded complete-genome reconstruction and limit confidence in nucleotide-level comparisons. Therefore, the principal value of the integrated workflow in the present study was the recovery and characterization of dispersed viral genomic regions rather than high-confidence reconstruction of a complete viral genome. The portability and real-time sequencing capability of Nanopore technology, complemented by the sequencing depth and accuracy of Illumina, may nevertheless provide a useful approach for exploratory viral surveillance involving limited, non-invasive marine mammal specimens [24].

Genomic and phylogenetic characterization

DNA metagenomic sequencing of the pooled Dolphin 4 sample provided broader genomic coverage than the initial RNA-based workflow. BLASTP analysis identified 12 predicted coding regions with their closest matches to *B. delphinidgamma1* or a closely related dolphin gammaherpesvirus (Table 3). Query coverage ranged from 54.2% to 100%, whereas amino acid identity ranged from 68.9% for ORF7 to 97.1% for ORF3. Several predicted proteins exhibited >90% amino acid identity, including ORF3/G3 (97.1%), ORF9/DNA polymerase catalytic subunit (95.1%), ORF8/glycoprotein B (94.6%), ORF11/virion protein G11 (92.16%), ORF39/envelope glycoprotein M (92.1%), and DE1/apoptosis inhibitor FLIP (90.4%) (Table 3). Additional similarities included DE2 (89.6%), ORF53/envelope glycoprotein N (84.9%), and ORF65/small capsid protein (84.17%).

The recovered coding regions represented multiple viral functional categories, including genome replication, DNA packaging, virion structure, membrane-associated functions, and envelope glycoproteins. The presence of

multiple independently recovered herpesvirus-associated regions, rather than a single isolated database match, together with reference mapping and phylogenetic placement, provides convergent sequence-based support for the presence of *B. delphinidgamma1*-related genomic material [17]. Nevertheless, the fragmented nature of the recovered sequences and low and uneven read depth require cautious interpretation. Sequence similarity alone cannot demonstrate functional expression of the predicted proteins or completely exclude environmental contamination, laboratory contamination, or mapping artifacts.

ORF8 produced its closest BLASTP match to an entry annotated as *Tursiops truncatus* gammaherpesvirus rather than *B. delphinidgamma1* (Table 3). This discrepancy may reflect differences in database nomenclature because the Sarasota virus was originally reported as common bottlenose dolphin gammaherpesvirus 1 and is currently classified by the International Committee on Taxonomy of Viruses within the species *Bosavirus delphinidgamma1* [17, 18]. The International Committee on Taxonomy of Viruses record associates delphinid gammaherpesvirus 1 and common bottlenose dolphin gammaherpesvirus 1 with the Sarasota reference genome [18]. Therefore, the ORF8 result is consistent with a closely related dolphin gammaherpesvirus within the *B. delphinidgamma1* lineage rather than providing evidence for a distinct viral species. Taxonomic interpretation should consequently consider sequence identity, query coverage, reference accession, and phylogenetic relationships rather than database organism labels alone.

Phylogenetic analysis further supported the relationship between the sequence recovered from Dolphin 4 and the Sarasota reference strain (Figure 5). However, phylogenetic inference based on incomplete herpesvirus sequences may be influenced by sequence length, limited taxon representation, and uneven availability of homologous genomic regions [36]. Therefore, the present findings support identification and partial genomic characterization of *B. delphinidgamma1*-related genetic material but are insufficient to establish a novel viral strain, reconstruct a complete-genome, or infer lineage-specific functional adaptation.

Epidemiological and surveillance implications

The detection of *B. delphinidgamma1* genetic material in a single dolphin cannot establish its prevalence within the study facility, other captive dolphin populations, or the wider Java Sea ecosystem (Supplementary Table 1). Furthermore, the absence of reported contact between the sampled dolphins and wild dolphins prevents determination of the source or timing of viral acquisition (Table 1). Comparative sampling across additional captive facilities, geographic regions, marine environments, and free-ranging dolphin populations is required to determine the distribution of the virus and investigate potential environmental and host-associated factors associated with its occurrence [37, 38].

The single-sample Flongle sequencing approach and absence of comparable sequences in the other sampled dolphins reduce concerns regarding barcode-mediated cross-sample contamination [23]. However, environmental or laboratory contamination cannot be completely excluded because extraction controls, environmental testing, and targeted molecular confirmation were not reported. Consequently, detection using metagenomic sequencing should be independently investigated using targeted molecular approaches in future studies.

Importantly, detection of *B. delphinidgamma1* sequences should not be interpreted as evidence of zoonotic transmission or human infectivity. The present study provides no direct evidence of transmission between dolphins and humans or between dolphins and other animal species. Further epidemiological, molecular, serological, and environmental investigations are required to determine the broader biological and health relevance of the detected viral sequences [11].

Limitations and future research directions

This study has several limitations. First, only five dolphins from a single captive facility were examined, and *B. delphinidgamma1* genetic material was detected in only one animal, precluding estimation of prevalence or broader epidemiological inference. Second, the low DNA yield from the positive anal swab required pooling of DNA extracts from the anal, blowhole, and reproductive tract samples before Illumina sequencing. Consequently, the anatomical source of the Illumina-derived viral reads cannot be determined, limiting conclusions regarding tissue tropism or routes of viral shedding. Third, only 63.7% of the reference genome was covered, with low and uneven sequencing depth, preventing complete-genome reconstruction and robust nucleotide-level characterization. Fourth, the absence of reported extraction controls, environmental testing, targeted PCR or quantitative PCR (qPCR) confirmation, and serological testing limits the ability to exclude contamination and determine infection status, viral activity, or previous exposure. Finally, the cross-sectional detection does not provide information regarding viral persistence, temporal shedding patterns, transmission routes, or clinical

consequences.

Future investigations should therefore include larger numbers of dolphins from multiple facilities and geographic regions and incorporate longitudinal, site-specific sampling to determine the persistence and temporal dynamics of viral detection. Parallel sampling of environmental water and facility-associated interfaces may help investigate potential environmental sources and transmission pathways. Targeted PCR or qPCR, together with serological testing, should be used to independently confirm metagenomic findings, with investigate infection and exposure status. Targeted enrichment or hybrid-capture sequencing could also improve sequencing depth and facilitate more complete-genome recovery, thereby enabling higher-resolution genomic and phylogenetic analyses.

Broader comparative analyses incorporating conserved viral genes and host phylogenies may further clarify the evolutionary history of cetacean gammaherpesviruses [38]. Further comparative analyses are needed to determine whether cetacean gammaherpesvirus diversification reflects host codivergence, historical host switching, or both.

CONCLUSION

This study provides the first metagenomic evidence and partial genomic characterization of *B. delphinidgamma1* in a captive common bottlenose dolphin in Indonesia and, to our knowledge, Southeast Asia. The integrated Oxford Nanopore Flongle–Illumina sequencing approach recovered reads covering 63.7% of the reference genome from non-invasive swab samples obtained from a clinically healthy dolphin. Although coverage was low and uneven, with a median depth of 1× and only 0.71% of the genome reaching a depth of ≥10×, multiple coding regions were recovered. Predicted proteins showed 68.9%–97.1% amino acid identity to *B. delphinidgamma1* or a closely related dolphin gammaherpesvirus. Phylogenetic analysis further demonstrated a close relationship between the Indonesian sequence and the Sarasota reference strain.

A major strength of this study was the integration of non-invasive sampling with Oxford Nanopore and Illumina sequencing, which enabled recovery of multiple viral genomic regions from low-biomass specimens.

The detection of *B. delphinidgamma1*-related genetic material in a clinically healthy captive dolphin extends the documented geographic occurrence of this virus to Indonesia and provides baseline genomic information for Southeast Asia. Although the findings require confirmation through larger longitudinal and targeted investigations, the study demonstrates the feasibility of integrating Oxford Nanopore Flongle and Illumina sequencing with non-invasive sampling for exploratory marine mammal viral surveillance.

DATA AVAILABILITY

The annotated partial-genome sequence of *B. delphinidgamma1* generated in this study has been deposited in GenBank under accession no. PX460073. The Oxford Nanopore metatranscriptomic raw sequencing reads are available in the NCBI SRA under BioProject PRJNA1504244, BioSample SAMN62049142, and SRA accession no. SRR39901519. The Illumina raw sequencing dataset has been submitted under the same BioProject, with SRA accession no. SRR39918380.

GENERATIVE AI DECLARATION

Generative artificial intelligence (AI) tools were used to assist with figure preparation and language editing, including grammar correction. Generative AI was not used for data analysis, interpretation of results, or generation of scientific conclusions.

AUTHORS' CONTRIBUTIONS

AN, AWMP, NN, HN, AML, and WKK: Conceptualization and study design. AN, AWMP, NN, SPP, LG, HN, TP, AI, DS, UP, HNu, AML, and WKK: Investigation, methodology, sample collection, formal analysis, data analysis, data curation, writing—original draft, and writing—review and editing. TS, DSy, RR, and JEP: Writing—review and editing. All authors have read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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