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Comparative Analysis of Quan and Watanabe Pan-Coronavirus Assays for Bat Coronavirus Diversity in Sarawak, East Malaysia

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ABSTRACT

Bats are natural reservoirs for a diverse range of coronaviruses (CoVs), including those closely related to SARS-CoV and SARS-CoV-2, making them crucial for understanding CoV genetics and zoonotic transmission. The exceptional bat diversity in Sarawak, Malaysian Borneo, provides an ideal setting to investigate CoV diversity and potential transmission pathways. This study examined CoV prevalence and diversity in 346 fecal samples from bats across 29 species in northern and western Sarawak, employing two pan-CoV PCR assays: Quan (Q-assay) and Watanabe (W-assay). The Q-assay and W-assay estimated the CoV prevalence to be 14.45% and 12.72%, respectively. The overall true prevalence based on both assays was 22.83%. There was a fair agreement between both assays ($\kappa = 0.286$) with comparable performance in detecting the virus (McNemar $p > 0.05$). Phylogenetic analyses identified six distinct clades within alphacoronaviruses (α-CoVs) and betacoronaviruses (β-CoVs), comprising two unclassified Borneo-Alpha CoVs and four from the subgenera *Minunacovirus*, *Rhinacovirus*, *Nobecovirus*, and *Sarbecovirus*. This study represents the first report of Sarawak bat CoVs derived from rectal and fecal samples, addressing a significant knowledge gap. The findings highlight the need for complementary molecular assays to enhance CoV surveillance and deepen understanding of viral ecology in regions of high biodiversity, with implications for zoonotic disease prevention.

1 | Introduction

Bats are recognized as hosts for thousands of viruses from at least 28 different families [1], some of which cause severe and often fatal diseases in humans and animals. Many of these viruses are only identified after causing significant disease outbreaks in humans or livestock [2]. Studies have shown that bats harbor a significantly higher number of viruses compared to other mammalian orders, emphasizing their role as critical viral reservoirs [3]. The emergence of SARS-CoV-1 and the discovery of two other human coronaviruses (CoVs) (HCoV-NL63 and HCoV-HKU1) further emphasize the importance of bats as reservoirs for zoonotic viruses. Notably, the closest known relatives to SARS-CoV-2, such as BANAL-52 (96.8% similarity) from *Rhinolophus malayanus* and RaTG13 (96.1% similarity) from *Rhinolophus affinis*, highlight the risks posed by

bat-borne CoVs. Intriguingly, *R. affinis* has also been found in Sarawak, Malaysian Borneo, reinforcing the need for focused research in this region.

Sarawak, with its rich bat diversity, acts as a natural laboratory for studying the genetic diversity of CoVs and potential pathways of transmission to humans and animals. To date, 80 species of bats from nine families (Pteropodidae, Emballonuridae, Megadermatidae, Nycteridae, Rhinolophidae, Hipposideridae, Vespertilionidae, Miniopteridae, and Molossidae) have been documented in Sarawak [4]. Despite this biodiversity, studies on bat virology, especially in relation to their role as CoV reservoirs, remain limited. Previous research in the region have demonstrated the presence of α-CoVs and β-CoVs in local bat species [5, 6], underscoring the importance of continued surveillance to understand the risks posed by these viruses.

Accurate detection of bat CoVs is critical for the early identification of potential zoonotic threats. Reverse transcription-polymerase chain reaction (RT-PCR) assays are the gold standard for detecting viral RNA due to their high sensitivity and specificity. Molecular detection of CoVs typically involves degenerate primers targeting conserved regions of the RdRp gene, minimizing false negatives [7–10]. Among the widely used pan-CoV PCR assays for bat CoV detection, the Quan (Q-assay) [11] and Watanabe (W-assay) [12] protocols stand out. The Q-assay provides broad detection across multiple bat species, while the W-assay targets specific viral lineages. Although these assays have been utilized in diverse ecological settings [13–16], their application in Sarawak remains underexplored.

This study compares the sensitivity and specificity of the Q-assay and W-assay for detecting CoVs in insectivorous and frugivorous bats sampled from northern and western Sarawak. By evaluating their performance across different bat species, this research seeks to deepen our understanding of CoV diversity, transmission dynamics, and spillover risks in this underexplored region. The findings could contribute to global surveillance efforts and enhance pandemic preparedness strategies.

2 | Materials and Methods

2.1 | Bat Sampling

Between 2021 and 2023, bats were captured from their natural environments, including orchards and protected areas such as national parks, in Sarawak's western and northern regions. Sampling was conducted from 1800 to 0600 h using harp traps and mist nets. Bats were identified following Payne et al. [17]. Rectal swabs or fecal samples were collected from live frugivorous and insectivorous bats, then placed in viral transport medium (VTM). During fieldwork, these samples were temporarily preserved in portable coolers or freezers at −20°C. Samples were processed using the protocols approved by Universiti Malaysia Sarawak Animal Ethics Committee (UNIMAS-AEC). Upon reaching our laboratory at Universiti Malaysia Sarawak (UNIMAS), the samples were stored in a −80°C freezer until further analysis.

2.2 | RNA Extraction and cDNA Synthesis

Viral RNA from bat samples was extracted with High Pure Viral Nucleic Acid Extraction Kit (Roche, Manheim, Germany) according to the manufacturer's protocol, and was eluted with 50 μL of Roche elution buffer. The RNA extract was kept at −80°C for further analysis. The cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) with specific primers from two established protocols, Q-assay [11] and W-assay [12]. Briefly, 4 μL 5X RT buffer, 2 μL dNTPs (10 mM each), 1.0 μL of specific primer, 0.5 μL of Ribolock (40 U/μL), and 1.0 μL of RevertAid Reverse Transcriptase (200 U/μL) were mixed 5 μL of extracted RNA. Reverse transcription was performed via incubation for 10 min at 42°C, 60 min at 50°C, and held at 20°C.

2.3 | Quan-Assay vs. Watanabe-Assay for PCR Detection

The Q and W assays PCR protocols targeted the RNA-dependent polymerases (RdRp) region RNA polymerase, involving nested and hemi-nested RT-PCR. The details of primers used in this study are shown in Table 1, and the illustration of the primer-targeted regions in Figure 1. For the Q-assay and W-assay, the PCR protocols were performed by following the published protocols [11, 12]. The PCR reaction mixture contained 5 μL of cDNA was mixed with 10 X of Taq Buffer, 3.0 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 0.025 U/μL of Taq DNA Polymerase and 33.50 μL of water nuclease-free giving a final volume of 50 μL. Each assay run included a confirmed bat CoV positive control (GenBank Accession number OP328800) and a negative control (water) to validate the test. PCR products were visualized using a 2% agarose gel with a 100 bp DNA ladder at 120 V for 30 min. Amplified products of the expected band size were for Sanger sequencing (Apical Scientific, Malaysia).

2.4 | Phylogenetic Analysis

The identities and similarities of the sequenced isolated were analyzed using the Basic Local Alignment Search Tool (BLASTn) of the National Center for Biotechnology Information (NCBI). The positive CoV sequences from the Q and W assays were aligned with reference sequences available from GenBank using MUSCLE [18]. For both alignments, the general time reversible (GTR) model with a gamma distribution of rate heterogeneity was the best-fitting model. The maximum-likelihood (ML) trees were built with 1000 bootstrap replicates for both datasets using MEGA version 11.0.10 [19]. The trees were annotated using Interactive Tree of Life (iTOL) by adding labels, species, and other relevant information.

For this study, 45 positive bat CoV sequences from the Q-assay and 31 from the W-assay were successfully sequenced. However, for phylogenetic analysis, only 20 sequences from Q-assay and 19 from the W-assay were selected as representative sequences for each lineage. The accession numbers for these sequences are as follows: LC855489, LC858020, LC858043, LC858046-LC858062 [Q-assay], and LC863021-LC863039 [W-

TABLE 1 | List of CoV primers used in this study.

Type of PCR assay	PCR fomat	Primer's name	Primer sequence (5′ to 3′)	Amplicon size (bp)	Targeted region[a]	References
(Q)-assay	Two-step RT-PCR	CoV-FWD1	CGTTGGIACWAAYBTVCCWYTICARBTRGG	520	17 480–17 820	Quan et al. [11]
		CoV-RVS1	GGTCATKATAGCRTCAVMASWWGCNACATG			
	Nested PCR	CoV-FWD2	GGCWCCWCCHGGNGARCAATT	328		
		CoV-RVS2	GGWAWCCCCAYTGYTGWAYRTC			
(W)-assay	Two-step RT-PCR	CoV-FWD3	GGTTGGGAYTAYCCHAARTGTGA	440	14 370–14 750	Modified from Watanabe et al. [12]
		CoV-RVS3	CCATCATCASWYRAATCATCATA			
	Hemi-nested	CoV-FWD4	GAYTAYCCHAARTGTGAYAGAGC			

[a]The position of PCR product was aligned and compared with the whole genome of HCoV-229E (GenBank accession No.: NC_002645.1) [15].

assay]. Additionally, reference sequences for comparative analysis were retrieved from the GenBank database (Supporting Information S1: Table S1 and Supporting Information S2: Table S2).

2.5 | Statistical Analysis

Statistical analyses were conducted using IBM SPSS Statistics version 29.0.2.0 (20) software. The results from the Q-assay and W-assay were evaluated using Cohen's Kappa (κ) statistics to assess the level of agreement between the two tests. Kappa values were interpreted according to the guidelines by [20], where values between 0.00 and 0.20 indicate slight agreement, 0.21–0.40 reflect fair agreement, 0.41–0.60 indicate moderate agreement, 0.61–0.80 suggest substantial agreement, and values greater than 0.80 represent almost perfect agreement.

3 | Result

3.1 | Bat Sample Collection

Between 2021 and 2023, a total of 346 bat individuals were captured from the northern and western Sarawak. Rectal swabs and fecal samples were collected from these bats. The detailed sampling locations are shown in Figure 2. The sampled bats represented 29 species, distributed across 13 genera and 6 families. Bats from family Pteropodidae ($n = 154$) and family Rhinolophidae ($n = 103$), accounted for 74.28% of the total samples. The species that were most sampled were *Cynopterus brachyotis* ($n = 62$) and *Rhinolophus creaghi* ($n = 55$). There were also several single samples collected and these were *Kerivoula minuta* ($n = 1$), *Kerivoula pellucida* ($n = 1$), *Rhinolophus luctus* ($n = 1$), and *Rhinolophus trifoliatus* ($n = 1$). This is the first documentation in Sarawak for the bat CoV detection using live bat samples.

3.2 | Bat CoV Detection

The overall prevalence of bat CoVs in this study was 22.83% ($n = 79/346$), based on detection using the Q-assay and W-assay (Table 2). In the western region, the bat CoVs positivity was 28.38% ($n = 42/142$). Meanwhile, in the northern region which includes Niah National Park and Mulu National Park, the bat CoVs positivity were 4.0% ($n = 2/50$) and 22.78% ($n = 35/154$), respectively. It was noted that CoVs were detected in 4 families (Pteropodidae, Hipposideridae, Miniopteridae and Rhinolophidae) and 13 bat species across the western and northern regions of Sarawak, demonstrating a high diversity of bat species serving as carriers of CoVs (Figure 3). Among the family Pteropodidae (frugivorous bats), *C. brachyotis* had the highest CoV positivity rate, 37.10% ($n = 23/62$). Other notable species included *P. lucasi* and *E. spelaea* with similar CoV positivity of 33.33% ($n = 12/36$; $n = 3/9$). For the three other families (insectivorous bats), the species *R. creaghi* showed the highest positivity of CoVs, with 43.63% ($n = 24/55$). It was observed that *R. borneensis* and *R. philippinensis* also have CoV positivity of 20.0% ($n = 4/20$) and 33.33% ($n = 4/12$), respectively. The sole species from the family Miniopteridae, *M. magnater* had one individual tested positive for CoV, 25% (one out of four samples collected).

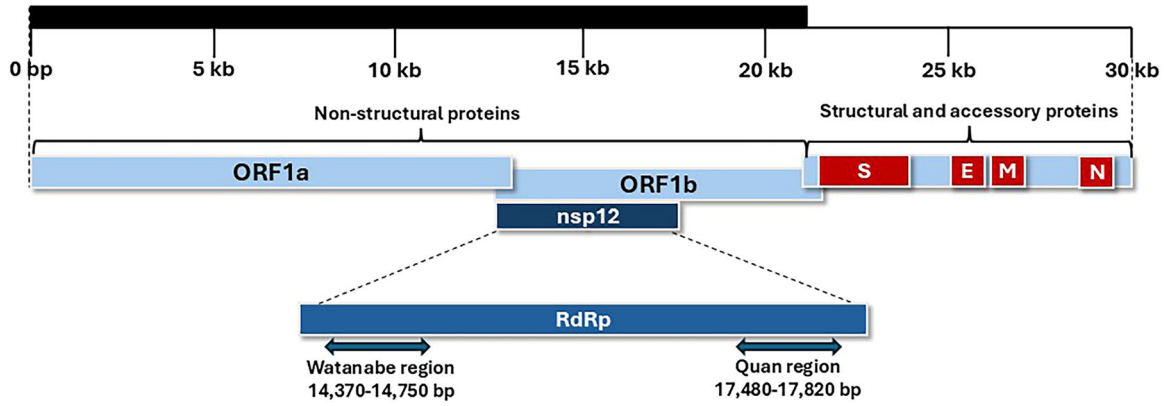

FIGURE 1 | Genomic organization of a CoV highlighting the RdRp region targeted by Quan and Watanabe primers.

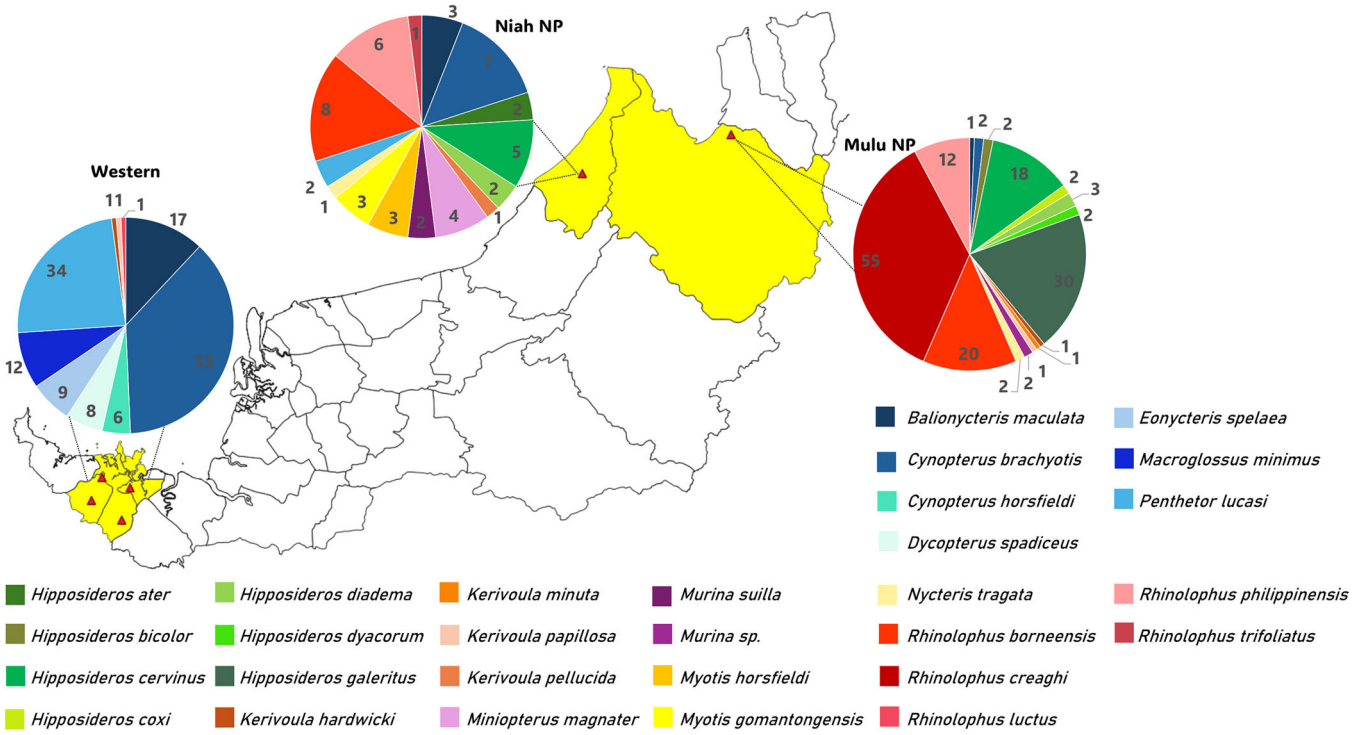

FIGURE 2 | Geographic distribution and bat species diversity in northern and western Sarawak, Malaysian Borneo. Pie charts represent the relative abundance of bat species sampled from three regions: Western Sarawak, Niah National Park (NP), and Mulu National Park (NP). The chart segments are color-coded to indicate different bat species, with species names listed in the legend. Sampling sites are marked on the map with red triangles.

3.3 | Phylogenetic Classification of Bat CoVs

The phylogenetic trees which depict the evolutionary relationships among the bat CoVs identified in the study were constructed using 20 RdRp sequences for the Q-assay and 19 RdRp sequences for the W-assay (Supporting Information S3: Table S3). These trees revealed six subgenera, including two unclassified Borneo-Alpha CoVs (*Clade A2 and Clade A3*), as well as several unclassified CoVs in subgenera *Minunacovirus* (*Clade A1*), *Rhinocovirus* (*Clade A4*), *Nobecovirus* (*Clade B1*), and *Sarbecovirus* (*Clade B2*). However, due to the use of only small genetic fragments in this study, they could not be classified based on the CoV classification criteria (Figure 4). *Nobecovirus*-related CoVs (*Nobecovirus-rCoVs*) were detected in bats from the Pteropodidae family at a significantly higher rate compared to bats from other families. Additionally, *Rhinocovirus*-related CoVs (*Rhinocovirus-rCoVs*) were detected in bats of the family Rhinolophidae and *Minunacovirus*-related CoVs (*Minunacovirus-rCoVs*) were found in bats of the family Miniopteridae family.

The *Minunacovirus-rCoVs* (*Clade A1*) was identified in *Miniopterus* species, *Miniopterus magnater* (NNP-064) from Niah National Park serving as a key representative. This clade showed perfect congruence in both assays, grouping together with BatCoV/MpGD17/*M. pusillus*/China/2017 (OQ175069), BatCoV/AFCD307/*Miniopterus*/Hong Kong (EU420137), and BatCoV/HKU8/AFCD77/*Miniopterus*/Hong Kong (EU420139), with sequence similarities between 91.99% and 93.20%. Basic congruence was observed in unclassified Borneo-Alpha CoVs

TABLE 2 | Summary of the bat species and overall prevalence of bat CoVs in this study.

Bat species	Western region			Northern region						Total no. of bats [Total no. of positives] n [+ve]
				Niah National Park			Mulu National Park			
	No. bats	Q/W	Q & W	No. bats	Q/W	Q & W	No. bats	Q/W	Q & W	
Frugivorous bats										
Family Pteropodidae										
Balionycteris maculata	17	1	0	3	0	0	1	0	0	**21 [1]**
Cynopterus brachyotis	53	13	9	7	1	0	2	0	0	**62 [23]**
Cynopterus horsfieldi	6	0	0	0	0	0	0	0	0	**6**
Dycopterus spadiceus	8	2	0	0	0	0	0	0	0	**8 [2]**
Eonycteris spelaea	9	3	0	0	0	0	0	0	0	**9 [3]**
Macroglossus minimus	12	1	0	0	0	0	0	0	0	**12 [1]**
Penthetor lucasi	34	12	0	2	0	0	0	0	0	**36 [12]**
Insectivorous bats										
Family Hipposideridae										
Hipposideros ater	0	0	0	2	0	0	0	0	0	**2**
Hipposideros bicolor	0	0	0	0	0	0	2	0	0	**2**
Hipposideros cervinus	0	0	0	5	0	0	18	2	0	**23 [2]**
Hipposideros coxi	0	0	0	0	0	0	2	0	0	**2**
Hipposideros diadema	0	0	0	2	0	0	3	0	0	**5**
Hipposideros dyacorum	0	0	0	0	0	0	2	0	0	**2**
Hipposideros galeritus	0	0	0	0	0	0	30	1	0	**30 [1]**
Family Vespertilionidae										
Kerivoula hardwicki	1	0	0	0	0	0	1	0	0	**2**
Kerivoula minuta	0	0	0	0	0	0	1	0	0	**1**
Kerivoula papillosa	1	0	0	0	0	0	1	0	0	**2**
Kerivoula pellucida	0	0	0	1	0	0	0	0	0	**1**
Murina suilla	0	0	0	2	0	0	0	0	0	**2**
Murina sp.	0	0	0	0	0	0	2	0	0	**2**
Myotis horsfieldi	0	0	0	3	0	0	0	0	0	**3**
Myotis gomantongensis	0	0	0	3	0	0	0	0	0	**3**
Family Miniopteridae										

(Continues)

TABLE 2 | (Continued)

Bat species	Western region			Northern region						Total no. of bats [Total no. of positives] n [+ve]
				Niah National Park			Mulu National Park			
	No. bats	Q/W	Q & W	No. bats	Q/W	Q & W	No. bats	Q/W	Q & W	
Miniopterus magnater	0	0	0	4	0	1	0	0	0	**4 [1]**
Family Nycteridae										
Nycteris tragata	0	0	0	1	0	0	2	0	0	**3**
Family Rhinolophidae										
Rhinolophus borneensis	0	0	0	8	0	0	20	4	0	**28 [4]**
Rhinolophus creaghi	0	0	0	0	0	0	55	17	7	**55 [24]**
Rhinolophus luctus	1	1	0	0	0	0	0	0	0	**1 [1]**
Rhinolophus philippinensis	0	0	0	6	0	0	12	4	0	**18 [4]**
Rhinolophus trifoliatus	0	0	0	1	0	0	0	0	0	**1**
Total	**142 [42]**			**50 [2]**			**154 [35]**			**346 [79]**

Note: No. bats represent the number of bat individuals captured in each locality. Q/W represents the number of bats CoV positive in Q or W assay. Q&W represents the number of bats CoV positive in both Q and W assays.

(*Clade A2* and *Clade A3*) for both assays. The sequences from *Rhinolophus* species from Mulu National Park, *R. borneensis*, *R. creaghi* and *R. philippinensis* prominently grouped in *Clade A2* together with other *Rhinolophus* and *Hipposideros* species from China, Jordan, and Malaysia. Moreover, this clade is only observed in Q-assay and a sister clade to the *Pedacovirus* (AF353511). Whereas *Clade A3* is more divergent than *Clade A2*, with a broader spread of sequences from both frugivorous and insectivorous bat species, including previously described BorneoAlpha-1 (MZ574065) and BorneoAlpha-2 (OP328796) (bolded in blue in Figure 4) indicating higher genetic variability within this group. Similarly, *Clade A3* was only observed in W-assay with no overlapping sequences with *Clade A2*. Additionally, there were unclassified Borneo-Alpha CoVs from *Clade A2* and *Clade A3*, indicating the presence of novel strains yet to be fully characterized. Interestingly, 2 concordance sequences from this study, *R. creaghi* from Mulu National Park (MNP002 and MNP023) were observed in both assays. However, these sequences group in Borneo-Alpha CoVs (*Clade A2*) in Q-assay and *Rhinocovirus-rCoVs* (*Clade A4*) in W-assay. The *Rhinocovirus-rCoVs* (*Clade A4*) was observed to cluster together with SADS-rCoV/141378/*R. pusillus*/China/2014 (MF769476) and BatCoV/HKU2/*R. affinis*/China (MN312347) with sequence similarity of 83.70%, highlighting their genetic proximity.

In the case of β-CoVs, a distinct clade, *Nobecovirus-rCoVs* (*Clade B1*) was consistently identified across both assays. This clade was predominantly associated with *C. brachyotis* and other frugivorous bats, including *P. lucasi*, *M. minumus*, and *E. spelaea*. Notably, in the Q-assay, bat CoVs from *Hipposideros* and *Rhinolophus* species were observed clustering together with those from other frugivorous bats. In this clade, six bat CoV sequences from *C. brachyotis* in this study were concordance in both assays (KPG008, KBP1002, MG023, MG055, MG065, and MG066). Overall, our bat CoV sequences in *Clade B1* were grouped together with *Nobecovirus* sequences, BatCoV/GCCDC1 356/*R. leschenaulti*/China/2014 (KU762338) and BatCoV/HKU9-4/China (EF065516) as well as with other *Pteropus* species bat CoV sequences from Thailand, Cambodia and India and previously described BorneoBeta-3 (OP328793). The *Sarbecovirus-rCoVs*, *Clade B2* was derived from genus *Rhinolophus*, specifically the *R. creaghi* species from Mulu National Park. One sequence identified in our study, MNP063 exhibited discordant grouping across the two assays. In the Q-assay, this sequence grouped within the α-CoV genus (*Clade A2*), clustering with BatCoV/PREDICT CoV-78/PSW01471/*R.cf.arcuatus*/Malaysia/2015 (MT064634) with sequence similarity of 92.51%.

Conversely, in the W-assay, MNP063 was grouped within the β-CoV genus (*Clade B2*), alongside BatCoV/PREDICT CoV-84/PSW01983/*R. creaghi*/Malaysia/2016 (MT083289) with a higher sequence similarity of 97.83%. Furthermore, it formed a sister grouping with other *Sarbecovirus-rCoVs*, including BANAL-20-52 and RaTG13.

3.4 | Concordance Between the Q-Assay and W-Assay

The contingency (Table 3) shows the detection of CoVs in frugivorous and insectivorous bats using two assays (Q and W). Of the total 346 samples, 18 samples were positive for both

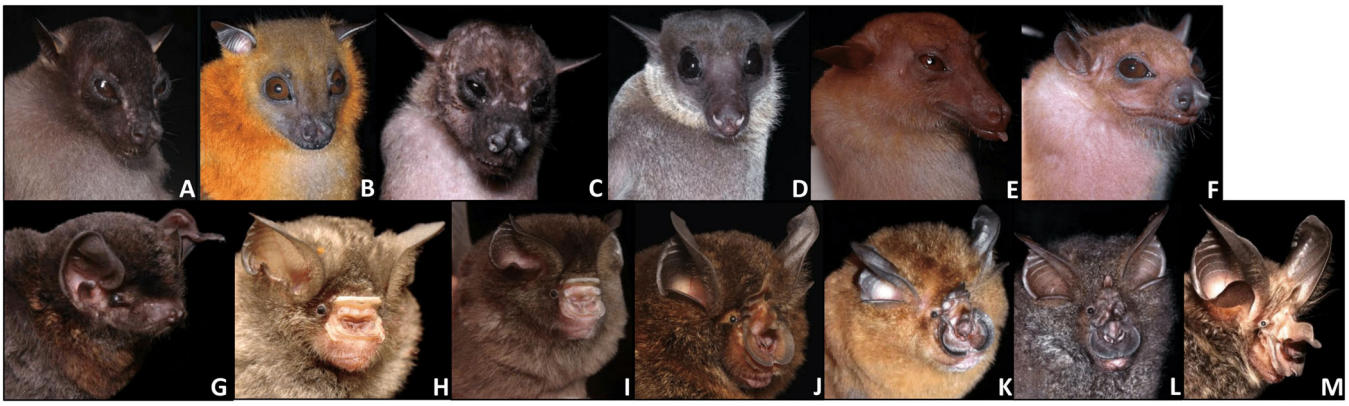

FIGURE 3 | The photographs of the 13 CoV positive bat species are shown here. Family Pteropodidae: A—*Balionycteris maculata*; B—*Cynopterus brachyotis*; C—*Dycopterus spadiceus*; D—*Eonycteris spelaea*; E—*Magroglossus minimus*; F—*Penthetor lucasi*. Family Miniopteridae: G—*Miniopterus magnater*. Family Hipposideridae: H—*Hipposideros cervinus*; I—*Hipposideros galeritus*. Family Rhinolophidae: J—*Rhinolophus borneensis*; K—*Rhinolophus creaghi*; L—*Rhinolophus luctus*; M—*Rhinolophus philippinensis*.

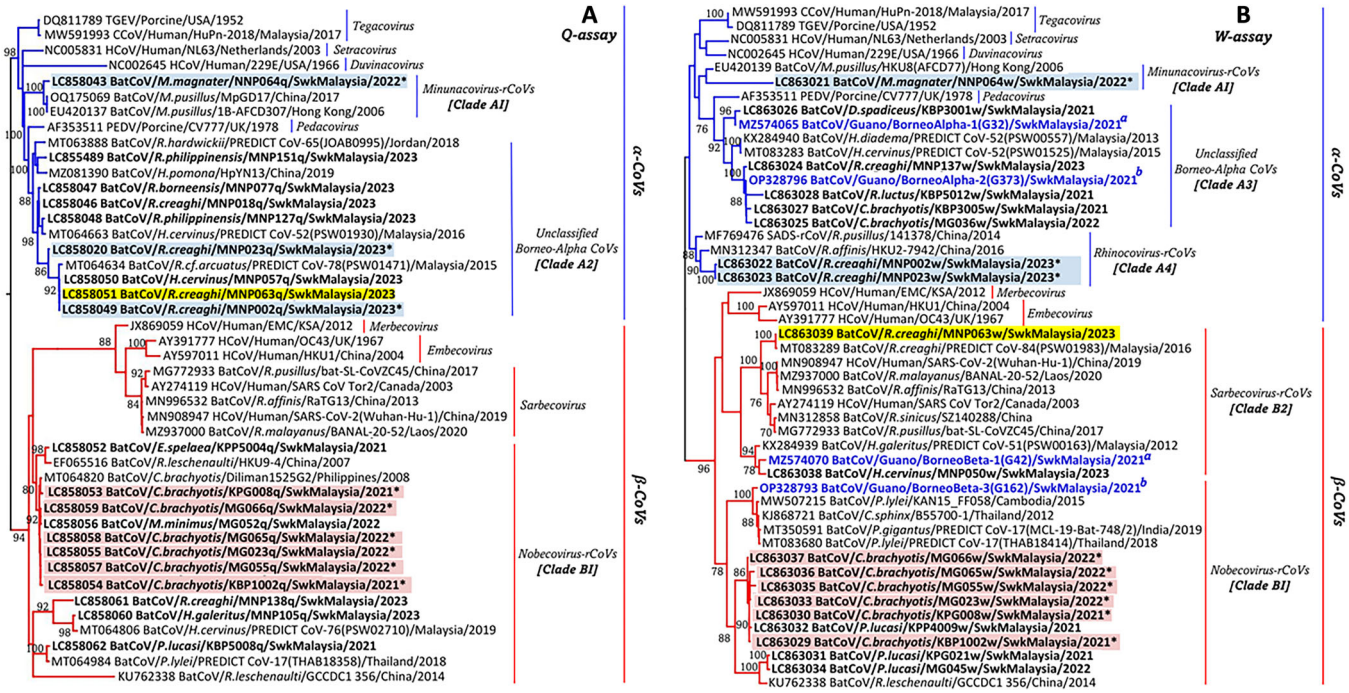

FIGURE 4 | Maximum likelihood phylogenetic tree for representative partial bat CoV RdRp sequences. (A) Phylogenetic tree generated using the Quan-assay. (B) Phylogenetic tree generated using the Watanabe-assay. The trees represent the evolutionary relationships of α-CoVs (blue branches) and β-CoVs (red branches), with bootstrap values indicated at branch nodes (values ≥ 70%). Black bolded sequences indicate those detected in this study. Blue bolded sequences with superscripts represent representative sequences from previous studies: *a*: [6], and *b*: [5]. Sequences highlighted in blue indicate concordance within α-CoVs, while sequences highlighted in red indicate concordance within β-CoVs. Sequences highlighted in yellow represent discordant sequence detected by the Quan and Watanabe assays.

assays (W+Q+) and 270 samples were negative for both assays (W−Q−). The Kappa statistic indicates a fair agreement between the two assays (Quan and Watanabe), with a Kappa value of 0.286, which is statistically significant ($p < 0.001$). This suggests that while there is some level of agreement, the classification between the two assays is not perfect. The McNemar test result showed a *p*-value of 0.512, suggesting no significant difference between the two assays (Quan and Watanabe) in terms of their performance in detecting bat CoVs (as $p > 0.05$) (Table 4).

4 | Discussion

The emergence of CoVs such as SARS-CoV, MERS-CoV, SADS-CoV, and SARS-CoV-2 underscores the substantial public health risks associated with zoonotic transmission since the beginning of the 21st century [21–23]. Bats are often identified as the primary reservoirs and are commonly labeled as the culprits for these viruses due to their role in harboring closely related strains. The increasing discoveries of diverse α-CoVs and β-CoVs within bat populations underscore the need for a deeper

TABLE 3 | Contingency table of Q-assay vs. W-assay.

Test A Frugivorous & insectivorous bats	W+	W−	Total
Q+	18	32	50
Q−	26	270	296
Total	44	302	346
Test B Frugivorus bats	**W+**	**W−**	**Total**
Q+	9	8	17
Q−	22	115	137
Total	31	123	154
Test C Insectivorous bats	**W+**	**W−**	**Total**
Q+	9	24	33
Q−	4	155	159
Total	13	179	192

TABLE 4 | Cohen's Kappa (κ) and McNemar test values for Q-assay and W-assays.

Metric	Result
Cohen's Kappa (κ)	0.2860
McNemar test	0.512, $p > 0.05$

understanding to improve surveillance and implement effective spillover prevention strategies. However, there has been some ambiguity in the selection of appropriate assays for CoV detection, with the Quan and Watanabe assays being the most widely utilized. Despite their effectiveness, differences in sensitivity and specificity present challenges in accurately identifying these viruses.

Our study investigates the prevalence and diversity of bat CoVs in northern and western Sarawak, underscoring the critical role of bats as natural reservoirs for these viruses in this region. The diversity of bat species and their associated CoVs in this region mirrors findings from other Southeast Asian countries, where bats have been identified as key hosts for a wide variety of CoVs, including those with zoonotic potential such as SARS-CoV and MERS-CoV [13, 14]. Notably, this research fills a gap in existing literature, as limited studies have been conducted in Sarawak, and we present the first data from the region using bat fecal samples. Previous studies [5, 6] primarily relied on guano samples (non-live fecal sampling) making this approach distinct and potentially more comprehensive for detecting CoVs in local bat populations. Additionally, our work further refines existing classifications of bat α-CoVs and β-CoVs in Sarawak, as studies on bat CoVs suggest that bats are primarily hosts to approximately 16 CoV subgenera [24].

A novel finding in this study is the detection of a *Minunacovirus-rCoVs* (*Clade A1*) in *M. magnater* species captured in Niah NP. In both Q-assay and W-assay, our strain clustered together with the *Miniopterus* strains from other studies that includes the one from China and Hong Kong with genetic similarities between 91.99% and 93.20% [25–27]. Previous studies have demonstrated that the predominant hosts associated with *Minunacovirus* subgenus are often from genus *Miniopterus*, which aligns with our study here [25, 28]. Of particular concern is the identification of the putative precursor virus of SARS-CoV in *M. magnater*, underscoring the potential of new zoonotic CoVs [29]. Considering the diverse range of habitats occupied by this species, including human settlements, plantations, and open land, it is essential to focus research efforts on *Miniopterus* genus in Sarawak. Such focus is essential for evaluating their potential risks and deepening our understanding of their role in potential zoonotic transmission, especially considering their well-documented association with a wide array of recombinant strains.

Our α-CoV clade analysis for our samples in both Q and W assays phylogenetic trees exhibited interesting clading among the rhinolophids or horseshoe bats. Rhinolophids bats serve as key reservoirs for CoVs [24, 30]. In the Q-assay tree, the CoVs found in the three *Rhinolophus* species, which are *R. borneensis, R. creaghi*, and *R. philippinensis* were clustered in the unclassified Borneo-Alpha CoVs (*Clade A2*). This is significant because *Rhinolophus* species have been identified as reservoirs for SARS-related CoVs. These CoVs formed a distinct clade with representative sequences from Sabah, Malaysia Borneo, i.e. BtCoV/PREDICT CoV-78/*R.cf.arcuatus* and BtCoV/PREDICT CoV-52/*H. cervinus*, suggesting possible ecological similarities or overlapping behaviors between populations. These bats have communal roosting and such traits, including food-sharing/feeding habits, may facilitate viral circulation. Furthermore, dense forest habitats support stable *Rhinolophus* populations and viral persistence [31]. This genetic similarity across regions may also imply limited CoVs divergence within these bat populations.

In contrast, the W-assay revealed a distinct clade, the unclassified Borneo-Alpha CoVs (*Clade A3*) linking frugivorous and insectivorous bat CoVs including the previously identified BorneoAlpha-1 and BorneoAlpha-2 [5, 6]. This pattern points to shared viral reservoirs among bat populations across western Sarawak, suggesting frequent interspecies interactions in habitats with ecological features like limestone caves and dense vegetation, with such connectivity may foster CoV persistence and circulation. Another important clade that caught our attention here is the *Rhinocovirus-rCoVs* (*Clade A4*) in W-assay. *R. creaghi* formed a sister clade with the *Rhinocovirus* strains, BtCoV/HKU2 and swine acute diarrhea syndrome related CoV (SADS-rCoV), BtCoV/SADS-rCoV/141378 found in *R. affinis* and *R. pusillus* that were identified in China. Based on Latinne and colleagues, they reported that subgenus *Rhinocovirus* is host specific within the rhinolophid bats, including the sequences related to HKU2-CoV and SADS-CoV [24]. The HKU2 bat CoV is significant due to its close relation to viruses that have demonstrated zoonotic potential, particularly the SADS-CoV in pigs. Discovered in *Rhinolophus* bat species, HKU2 highlighted the capacity of bat to jump between species and emphasized the potential for CoVs to affect agricultural animals and possibly humans [32–34]. The co-detection of α- and β-CoVs in *R.*

creaghi highlights this species as a significant reservoir of diverse CoVs, emphasizing the importance of ongoing surveillance in these regions.

Our study detected CoVs in bats of the family Pteropodidae, which are the frugivorous bats such as *C. brachyotis*, *D. spadiceus*, *E. spelaea, M. minimus*, and *P. lucasi*. Our findings indicated that frugivorous bats, *C. brachyotis* and *P. lucasi* generally have a higher positivity rate for CoVs compared to insectivorous bats when tested using the W-assay. This result aligns with Watanabe (2010), where four out of six tested bat species in their study were frugivorous. The higher CoVs detection rate may suggest that the primer is particularly suited for frugivorous bats or in our case, more frugivorous bats were sampled. If these potential sampling biases are discounted, other factors that may contribute to this higher detection rate may also include dietary exposure to virus-laden fruits and social behaviors that facilitate viral transmission. Another study reported that the sensitivity of Watanabe primers for detecting CoVs was reduced when the primer sequences contained more than four mismatches [10].

Interestingly, our CoVs from the Pteropodidae family exhibit a diversity of α-CoV and β-CoV. For the W-assay, we noticed that our fruit bats CoVs clustered together with insectivorous bats in the unclassified Borneo-Alpha CoVs subgenus (*Clade A3*). For both Q-assay and W-assay, most of the CoVs found in frugivorous bats clustered in the *Nobecovirus-rCoVs* (*Clade B1*), which this subgenus is known to be very host-specific to this Pteropodidae family. In our previous study [5], we described the BorneoBeta-3 CoV, which in this study was seen clustered together with Rousettus bat GCCDC1 related CoVs (RoGCCDC1r-CoVs) and *Pteropus* sp. found from Southeast Asia and India. It was reported that after the initial detection of *Rousettus* bat CoV GCCDC1 (RoBat-CoV GCCDC1) in *Rousettus leschenaultii* in Yunnan province in 2016, this virus was subsequently identified in *E. spelaea* populations, which are known to co-roost with *R. leschenaultii* [35]. Of one interest, the CoV found in *E. spelaea* was clading together with the BtCoV/HKU9 strain (EF065516) from China. *R. leschenaultii* species have not been documented in Sarawak, however this species is in Peninsular Malaysia [4]. While the zoonotic potential of the subgenus *Nobecovirus* remains unclear, viruses like RoBat-CoV GCCDC1 and HKU9 have uncertain risks. Previous research has reported a tendency for recombination in this subgenus, particularly in the widely distributed GCCDC1 lineage in Asia, which carries a *p10* gene insertion derived from an orthoreovirus [36] This recombination potential greatly increases the chance of new viral strains emerging within host populations, raising the risk of spillover events and complicating control efforts for emerging CoV outbreaks.

In the *Sarbecovirus-rCoVs*, *Clade B2* observed in W-assay, two noteworthy clustering patterns emerged for β-CoVs. First, the previously described BorneoBeta-1 sequence grouped with Hipposideros spp. CoVs from Mulu NP and Sabah (BtCoV/PREDICT CoV-51/PSW00163). The pooled guano samples from Wind Cave's roosting site indicate a complex co-roosting dynamic among *Hipposideros* bat species, which may explain the shared CoV sequences detected in these samples. Second, bat CoV from *R. creaghi* clustered with BtCoV/PREDICTCoV-84/PSW01983, formed a sister clade to the *Sarbecovirus* subgenus. This observation underscores the importance of pursuing full-genome sequencing for these sequences to better understand their genomic characteristics and facilitate accurate classification. A particularly significant finding from our study is the absence of *Sarbecovirus*-like CoVs in the sampled bat populations, suggesting no current evidence of reverse zoonotic transmission from human CoVs to bats in Sarawak. This supports the hypothesis that the detected non-human CoVs likely originated from endemic bat species, notably within the Rhinolophidae family represented in this analysis. These results expand our understanding of CoV ecology in the region and highlight potential transmission dynamics, emphasizing the value of continued surveillance to evaluate spillover risks between bat populations and humans.

In this study, we observed a discordant result for the sequence MNP063, where the Q-assay and W-assay classified the sequence differently. In the Q-assay, MNP063 grouped within the unclassified α-CoV subgenus (*Clade A2*), showing a 92.51% sequence similarity to another α-CoV from Malaysia. Meanwhile, in the W-assay, MNP063 was classified in the *Sarbecovirus-rCoVs* subgenus (*Clade B2*), with a higher sequence similarity of 97.83%. This discordance may be due to differences in how the assays target viral genetic regions, leading to different results. The Q-assay may be more sensitive to certain α-CoVs, while the W-assay may better detect β-CoVs, particularly those related to *Sarbecoviruses* (like RaTG13 and BANAL-20-52), to which MNP063 is closely related. Interestingly, two other sequences from the same species, *R. creaghi* (MNP002 and MNP023), grouped consistently in both assays, though they were classified in slightly different subgroups (unclassified α-CoVs in Q-assay and *Rhinocovirus* in W-assay). This suggests that while the assays agree on some sequences, there are still biases in how they categorize certain viruses. The discordance between the assays for MNP063 highlights the genetic diversity of bat CoVs, especially in regions like Borneo, where viruses may not fit neatly into existing categories. These differences emphasize the need for more accurate methods to classify viruses, such as next-generation sequencing (NGS), which can provide a clearer picture of viral diversity.

Our study suggests that the Quan and Watanabe primers are not true pan-CoV primers. Consequently, previous prevalence studies [11, 12, 14, 15, 24, 37–39] using these primers may have underestimated the actual prevalence of coronaviruses. While unbiased NGS appears to be a promising tool for pathogen discovery, its high cost remains a significant barrier for laboratories in many low- and middle-income countries (LMICs).

To address this limitation, alternative amplicon sequencing strategies need to be developed to support LMIC researchers in their efforts to identify novel pathogens. For example, in human papillomavirus (HPV) detection, the degenerate MY09/11 primer set was found to have lower sensitivity for detecting HPV in primary specimens [40]. An improved version, PGMY09/11, which uses a cocktail of specific primers, demonstrated significantly higher sensitivity [41]. Similarly, pan-CoV or family-wide PCR approaches may benefit from a multiplexed cocktail of specific primers targeting the same binding sites rather than relying solely on a single pair of highly degenerate primers.

5 | Conclusion

This study provides valuable insights into the prevalence and diversity of bat CoVs in northern and western Sarawak, highlighting bats as key reservoirs for zoonotic CoVs. We found significant viral diversity in species like *M. magnater* and *R. creaghi*, which are closely related to zoonotic strains, including those related to SARS-CoV. While both the Quan and Watanabe assays are effective, their differences in sensitivity and specificity can lead to discordant results, underscoring the importance of using both assays for comprehensive surveillance. Our findings emphasize the need for ongoing monitoring of bat populations and the refinement of diagnostic methods to improve CoV detection. Future research should continue to investigate the role of bats in zoonotic transmission, especially in remote regions in Sarawak, to better assess spillover risks and improve public health preparedness.

Sarawak Emerging Pathogen Surveillance Study Group

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Ethics Statement

This study was approved by the UNIMAS Animal Ethics Committee (UNIMAS/AEC/R/F07/074).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data supporting the findings of this study are included in the article and its Supporting Information Materials.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.