



Published in final edited form as:

Zoonoses Public Health. 2020 June ; 67(4): 435–442. doi:10.1111/zph.12703.

An unprecedented cluster of Australian bat lyssavirus in *Pteropus conspicillatus* indicates pre-flight flying fox pups are at risk of mass infection

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Abstract

In November 2017, two groups of *P. conspicillatus* pups from separate locations in Far North Queensland presented with neurological signs consistent with Australian bat lyssavirus (ABLV) infection. These pups ($n = 11$) died over an 11-day period and were submitted to a government laboratory for testing where ABLV infection was confirmed. Over the next several weeks, additional ABLV cases in flying foxes in Queensland were also detected. Brain tissue from ABLV-infected flying foxes during this period, as well as archived brain tissue, was selected for next-generation sequencing. Phylogenetic analysis suggests that the two groups of pups were each infected from single sources. They were likely exposed while in crèche at night as their dams foraged. This study identifies crèche-age pups at a potentially heightened risk for mass ABLV infection.

Keywords

lyssavirus; public health; whole genome sequencing; zoonoses

1 | INTRODUCTION

Australian bat lyssavirus (ABLV) is the only lyssavirus endemic to Australia and causes both human and animal fatalities in the state of Queensland (Annand & Reid, 2014; Francis et al., 2014; Hanna et al., 2000). There are two genetic variants of ABLV: a flying fox variant that has been detected in the four most common Australian flying fox

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CONFLICT OF INTEREST

None declared.

species (*Pteropus* sp.), and an additional variant, to date detected only in the yellow-bellied sheath-tail bat (*Saccolaimus flaviventris*). The prevalence of ABLV antigen—indicative of current infection—in wild bats is less than 1%; however, prevalence is higher in sick or injured bats which are more likely to be found grounded or ‘hanging low’ (Barrett, 2004; Field, 2018). Between 2013 and 2016, ABLV was detected in 8 to 14 bats per year in Queensland, Australia (Wildlife Health Australia, 2017). Because of its zoonotic risk and high case fatality rate, ABLV in bats is of concern to public health authorities and to wildlife carers who oversee the welfare, treatment and rehabilitation of sick and injured bats in Australia.

There is an extensive network of volunteer wildlife carers in Australia that rescue sick, injured and orphaned native animals for rehabilitation and release. In Queensland, wildlife, including bats, is protected under the *Nature Conservation Act 1992*. The state Department of Environment and Science licenses people to rescue and keep wildlife in accordance with the conditions of their licence and legislation regarding the care of sick, injured or orphaned protected animals (QLD DES, 2013). In addition to being responsible for the rescue, acute care and rehabilitation of bat species, wildlife carers also provide a crucial service to their communities by responding to incidents where bats are found in public spaces and private homes. Due to the low likelihood but potentially fatal consequences of exposure to ABLV, members of the public are urged to avoid contact with bats whenever possible. Health and biosecurity authorities recommend contacting a vaccinated, licensed wildlife carer if a bat is found in need of help (<https://www.business.qld.gov.au/industries/farms-fishing-forestry/agriculture/livestock/animal-welfare/pests-diseases-disorders/australian-bat-lyssavirus>).

On 31 October 2017, a group of spectacled flying fox pups (*Pteropus conspicillatus*; $n = 9$; Group 1) were found hanging from a fence near an established flying fox colony in Far North Queensland (FNQ). The pups ranged in age between 4 and 7 weeks of age based on forearm measurements taken by wildlife carers. One pup had bite wounds to the head severe enough to necessitate euthanasia. Three of the eight remaining rescued pups had wounds consistent with recent bites from another bat. Within a day of rescue, the pups had been distributed to several wildlife carers to be hand-raised. As early as 13 days after the rescue, rescued pups began presenting with clinical signs consistent with ABLV infection; over a 9-day period, 6 of the 8 pups found together had died or were euthanized. The pups presented with clinical signs including but not limited to paresis, aggression and cranial nerve defects (Table 1). Cranial nerve defects impacting tongue and mouth movements can be observed in one of the Group 1 pups at <https://animalhealthaustralia.com.au/incidence-of-australian-bat-lyssavirus-in-queensland>. The carers reported their suspicion of ABLV infection to Biosecurity Queensland.

On 6 November 2017, a large group of *P. conspicillatus* pups ($n = 18$; Group 2) were found on the ground under a tree near an established flying fox colony approximately 80 kilometres south-west from where the first group of *P. conspicillatus* pups were rescued. Forearm measurements indicated that these pups ranged from 4 to 9 weeks of age. Two weeks later, the carer reported to Biosecurity Queensland that several pups ($n = 5$) had developed clinical signs consistent with ABLV. Clinical signs specific to individual bats were not recorded. Within two days, all 5 affected pups died or were euthanized. These

pups were each isolated as soon as abnormal clinical signs were recognized, minimizing the opportunity for further transmission to other bats in care.

Whole carcasses from the two clusters of affected pups ($n = 11$) were submitted to the Queensland Government Biosecurity Sciences Laboratory, Coopers Plains, QLD. Real-time PCR testing of fresh brain detected the flying fox variant of ABLV in all 11 pups. Concurrent real-time PCR tests for the *S. flaviventris* ABLV variant were negative. Nine of the 11 pups were also tested by fluorescent antibody test, corroborating the diagnosis of ABLV infection. These cases were initially reported by wildlife and animal health authorities in Wildlife Health Australia's 'ABLV Bat Stats' and in Animal Health Australia's Animal Health Surveillance Quarterly (AHA, 2017; WHA, 2017).

In the weeks following this temporal cluster of ABLV infections in FNQ, ABLV was detected in a further 3 *Pteropus alecto* from South East and Central Queensland, and an additional *P. conspicillatus* pup from FNQ (Figure 1). Due to the high number of ABLV infections detected during this 8-week period, as well as the unprecedented clustering and suspected mass infection amongst rescued pups (where mass infection is defined as more than 4 individuals infected as part of an incident), the molecular epidemiology of these cases was investigated using next-generation sequencing (NGS).

2 | MATERIALS AND METHODS

2.1 | Samples

We sequenced brain tissue from 15 flying foxes from Queensland infected with ABLV during the 8 weeks of November 2017 and January 2018. These were compared with samples from 16 ABLV-infected Queensland bats submitted previously over a much longer time course between January 2013 and May 2017 that had been stored at -80°C at the Biosecurity Sciences Laboratory (Table 2).

2.2 | RNA extraction and next-generation sequencing

Total nucleic acid was extracted from 31 flying fox brain samples using the RNeasy Mini Kit (Qiagen) following manufacturer's instructions but without the addition of RNase free DNase to Buffer RLT. Final elution volume was in 30 μl of Buffer EB. RNA concentration was measured using the Qubit Fluorometer and the Qubit RNA HS Assay Kit (Thermo Fisher Scientific). The sequence library was prepared using the TruSeq Stranded mRNA Library Preparation Kit substituting the Oligo-dT capture beads with those from the RiboZeroTM rRNA Removal Kit (Human/Mouse/Rat, Illumina). cDNA was prepared using SuperscriptTM II Reverse Transcriptase (ThermoFisher Scientific), and all purification steps utilized AMPure XP kit paramagnetic beads (Beckman Coulter). The size and purity of the pooled sequence library were quantified using the 2200 TapeStation (Agilent), with the final equimolar concentration quantified using the Qubit DNA dsDNA HS Assay Kit (ThermoFisher Scientific). The library was sequenced on a NextSeq 500 Sequencing Platform using a NextSeqMid Output Kit v2 300 (Illumina). Indexing quality control and FASTQ file generation were performed using the online server BaseSpace Sequence Hub (Illumina), with additional trimming performed using the Geneious[®] 11.0.3 (Biomatters)

plugin BBDuk (Brian Bushnell). Continuing to use Geneious®, individual reads were paired and mapped to a reference (GenBank accession [AF418014](#)).

Consensus sequences were submitted to NCBI GenBank (accession numbers: [MK492309–MK492319](#), [MK944082–MK944095](#)).

2.3 | Phylogenetic and selection analysis

Phylogenetic analysis was performed using whole genome sequence data derived from the NGS and additional whole genome ABLV sequences from flying foxes available on GenBank. Full-length was analysed using the GTR nucleotide substitution model with gamma distributed rate variation as identified using IQtree ModelFinder (Kalyaanamoorthy, Minh, Wong, Haeseler, & Jermin, 2017). Maximum-likelihood trees were constructed using PhyML with 1000x bootstrap support (Guindon et al., 2010). Phylogenetic tree was formatted using FigTree (version 1.4.4, <http://tree.bio.ed.ac.uk/software/figtree/>).

3 | RESULTS

Average coverage across sequences was 4,345. Sequences from the majority of individuals covered 100% of the genome, while remaining sequences covered 99.6%–99.9% of the genome; areas not covered were limited to genomic termini, known to be highly conserved amongst lyssaviruses (Marston et al., 2007) and unlikely to impact downstream analyses.

ABLV whole genome sequences from pups in Group 1 ($n = 6$) and Group 2 ($n = 5$) were identical or highly similar. A single nucleotide synonymous substitution within the RNA polymerase coding region differentiates Group 1 from Group 2 (Figure 2). Within Group 1, nucleotide sequences were 100% homologous, whereas slight variations (1–2 nucleotide substitutions) exist in 3 of 6 sequences within Group 1. Whole genome sequences from pups in Groups 1 and 2 (QA0018–QA028) were distinct from sequences from all other ABLV cases submitted to Biosecurity Queensland between November 2017 and January 2018 (QA029–QA032). Comparison with sequences derived from archived ABLV-infected flying fox brain tissue (QA001–QA017) shows that ABLV sequences from Groups 1 and 2 pups are most similar to QA010, extracted from a *Pteropus scapulatus* in South East Queensland in 2014. The additional ABLV-infected *P. conspicillatus* pup from Far North Queensland (QA031) was most similar to QA016, a sequence obtained from a *P. alecto* found in a Brisbane suburb in 2016. The other sequences obtained from ABLV-infected *P. alecto* between November 2017 and January 2018 show similar diversity and are more homologous to sequences derived from archived flying foxes than to one another (Figure 3).

Apart from the highly similar sequences of Groups 1 and 2, two sequences from archived tissue (QA003, QA004) within a small spatiotemporal window of 7 weeks and less than 5 km appeared to be closely related, with only three nucleotide substitutions. This is in contrast to all other sequences derived from archived flying fox samples which, despite a substantial sampling bias towards bats from South East Queensland, do not indicate any pattern of transmission specific to location or a single species based on the similarity of viral sequences, consistent with previous molecular work on the flying fox ABLV variant (Barrett, 2004; Foord et al., 2006; Guyatt et al., 2003).

4 | DISCUSSION

With the exception of the *P. conspicillatus* pup clusters, all other ABLV cases between November 2017 and January 2018 were sufficiently genetically distinct to eliminate the possibility of a propagating epidemic along the Queensland coast in the summer of 2017 (Figure 3). Despite close geographic proximity, an additional ABLV case in a *P. conspicillatus* pup (QA031), which occurred approximately two months after the ABLV pup clusters, was not closely related. The endangered *P. conspicillatus* is largely confined to the northern Wet Tropics region of FNQ (Department of the Environment, 2019), yet ABLV sequences from infected *P. conspicillatus* (groups 1 and 2, QA031) cluster with sequences from archived brain tissue collected from *P. scapulatus* (QA010) and *P. alecto* (QA016) submitted from South East Queensland. These more nomadic flying fox species presumably play a role in the geographic dissemination of ABLV (Field, 2004).

There appear to be a number of distinct ABLV lineages that circulate across temporal, geographic and *Pteropus* species in the state of Queensland. Notably, sequences available on GenBank from infected bats from Western Australia are embedded within these lineages despite enormous geographic distances (Figure 3). This is consistent with previous molecular studies into ABLV epidemiology which indicates that flying fox species form one continuous host population when it comes to ABLV infection, including the geographically limited *P. conspicillatus* (Barrett, 2004; Foord et al., 2006). ABLV is, to our knowledge, unique in this respect considering that most bat lyssaviruses are markedly species-specific (Marston et al., 2018).

Next-generation sequencing of ABLV from infected bats in Queensland between November 2017 and January 2018 has demonstrated that while viral sequences from each pup within Groups 1 and 2 were significantly different to all other ABLV sequences from other bats during this time period (Figure 3), the sequences within each group were highly similar.

In addition, the degree of homology between Groups 1 and 2 is much higher than anticipated, with only a single nucleotide synonymous substitution within the RNA polymerase coding region clearly delineating Group 1 from 2 (Figure 2). One possibility is that a single bat infected both groups of pups, and a within-host mutation occurred in the period between attacks, differentiating Group 1 and Group 2. However, while point mutations during passage have been documented in lyssaviruses, within-host mutations to our knowledge have not (Bonnaud et al., 2019). It is unlikely that a flying fox, already sufficiently progressed in its disease course to infect a group of pups, could survive long enough to fly approximately 80 kilometres and then infect another group of pups 6 days later. Clinical progression to death in flying foxes rescued with clinical signs due to ABLV is rapid, usually 1–3 days (Barrett, 2004). One clinically ill adult bat that was observed for 8 days prior to death was unable to fly when first observed, suggesting the complex function of flight is lost early in the clinical course (Barrett, 2004). In an experimental study, no ABLV-infected flying foxes exhibiting clinical signs survived >4 days (Barrett, 2004). We speculate that the two clusters were infected by two different bats, but that these two bats had been infected by a single bat (Figure 4). These two bats would have then dispersed during their respective incubation periods prior to infecting pups in Groups 1 and 2.

The short period during which observed clinical onset occurred within pups of Group 1 (within nine days) and Group 2 (within 2 days) means it is very unlikely any pups in a group had subsequently infected the others (Table 1). Previous experimental studies of ABLV and RABV in bats have indicated that virus in saliva is only detectable at onset or immediately before onset (<24 hr) prior to recognizable clinical signs (Barrett, 2004; Jackson et al., 2008). While it is possible that pup QA018 (clinical onset Day 10) infected pup QA023 (clinical onset Day 19), the observation that QA023 had bite marks at the time of rescue supports the notion that this pup and the others found on the ground were all exposed prior to coming into care. The onset of clinical signs of ABLV in all 5 pups in Group 2 occurred over only 2 days, precluding the possibility that one infected bat in that group had infected the others.

At 4 to 9 weeks of age flying fox pups cannot fly, but are sufficiently independent to be left in roosting trees in a crèche overnight while dams forage for food (Markus & Blackshaw, 2002). Pups in groups 1 and 2 were found close to the ground on the edge of established colonies; Group 2 was found beneath a tree that may have been used for crèche (J. Mclean personal communication, November 24 2017). Clusters of ABLV infection in pups which have not been reported in adults or neonatal pups suggest pups in crèche may be uniquely susceptible to mass infection due to the combination of their nocturnal communal roosting and their relative inability to avoid bites and scratches from an infected adult. This represents a brief but highly vulnerable period in their development in which they are left alone overnight without the protection of their dams and are too young to fly away.

ABLV infections in Groups 1 and 2 were identified because wildlife carers routinely checked on the welfare of the flying foxes within their local roosting colonies. Similar mass infections of crèche-age flying foxes may have gone undetected. Two additional accounts from Queensland wildlife carers describe attacks towards flying foxes of this age group: a carer observed a group of pre-flight flying foxes being attacked by a highly aggressive, dysphonic adult flying fox who was euthanized shortly thereafter. The pups who were unable to fly away or fend off attack came into care and subsequently declined in condition and died in care from what is suspected ABLV (wildlife carer personal communication, June 25 2018). A previously documented case describes a dam, later confirmed to be infected with ABLV, observed attacking juvenile bats as well as her own pup so severely its skull was punctured (Skerratt, Speare, Berger, & Winsor, 1998). Whether this elevated risk factor extends to other bat species that use crèches is unknown.

That the pups were found together on the ground, some with fresh bite wounds, supports that Groups 1 and 2 were exposed to ABLV just prior to being found. The apparent incubation periods ($n = 11$, ranging from 10 to 19 days) in these clusters are shorter than those previously observed in naturally infected flying foxes (30 days and 36–57 days; Field, McCall, & Barrett, 1999; Warrilow et al., 2003). However, the incubation periods are consistent with those observed following the experimental inoculation of flying foxes with a particularly virulent ABLV isolate (Barrett, 2004). Groups 1 and 2 feature 2 amino acid substitutions on the phosphoprotein coding gene and 3 on RNA polymerase coding gene. Although most studies have focused on the role of the glycoprotein for pathogenicity, substitutions on the phosphoprotein and RNA polymerase may impact neuroinvasiveness

and the rate of transcription and replication, thereby influencing the incubation period and potentially viral load (Streicker, Altizer, Velasco-Villa, & Rupprecht, 2012; Yamaoka et al., 2013).

This unprecedented cluster of ABLV amongst *P. conspicillatus* pups, likely exposed while vulnerable in crèche, exhibited a wide array of clinical signs and a brief and unexpectedly consistent incubation period. Wildlife carers and other people who may come into contact with bats in Australia need to be alert for bats showing a wide spectrum of potentially subtle neurological signs as more overt clinical signs (e.g., aggression) are not always present. Wildlife carers in Australia should be particularly wary of ABLV clinical signs in the pre-flight age group and can help limit further transmission to other bats in their care by adhering to the Australian Bat Lyssavirus General Biosecurity Obligation guidelines (Queensland Government, 2019).

Investigations into the large number of ABLV-infected bats in Queensland between November 2017 and January 2018 revealed the diversity of ABLV circulating amongst flying fox populations in the state and identified the mass infection of two groups of *P. conspicillatus* pups. Next-generation sequencing allowed for differentiation of closely related viruses, including nearly identical sequences, which would have been indistinguishable based on N or G gene sequencing alone. This study demonstrates the value of whole genome sequencing for investigations into the transmission of lyssaviruses and other zoonotic pathogens.

ACKNOWLEDGEMENTS

We thank Gavin S. Wilkie for his sequencing expertise and assistance, and the staff at Biosecurity Queensland's Biosecurity Sciences Laboratory. We also want to thank the volunteer wildlife carers of Tolga Bat Hospital, Connie Kerr, Dave Pinson, Jesse Jansen and Alicia Knudsen. This study was funded by Biosecurity Queensland, University of Queensland School of Veterinary Science, and a Wet Tropics Management Authority student grant. Author H. Field was supported in part by the United States Agency for International Development (USAID) Emerging Pandemic Threats PREDICT (Cooperative Agreement No. AID-OAA-A-14-00102) and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (Award No. R01AI110964).

Funding information

University of Queensland; Biosecurity Queensland; Wet Tropics Management Authority

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Impacts

- Whole genome sequences of Australian bat lyssavirus contribute to an understanding of the diversity of the virus in *Pteropus* species in Queensland.
- A wide range of Australian bat lyssavirus clinical signs in *Pteropus conspicillatus* pups are documented.
- We identify a heightened risk of mass Australian bat lys-savirus infection in crèche-age *Pteropus* pups.

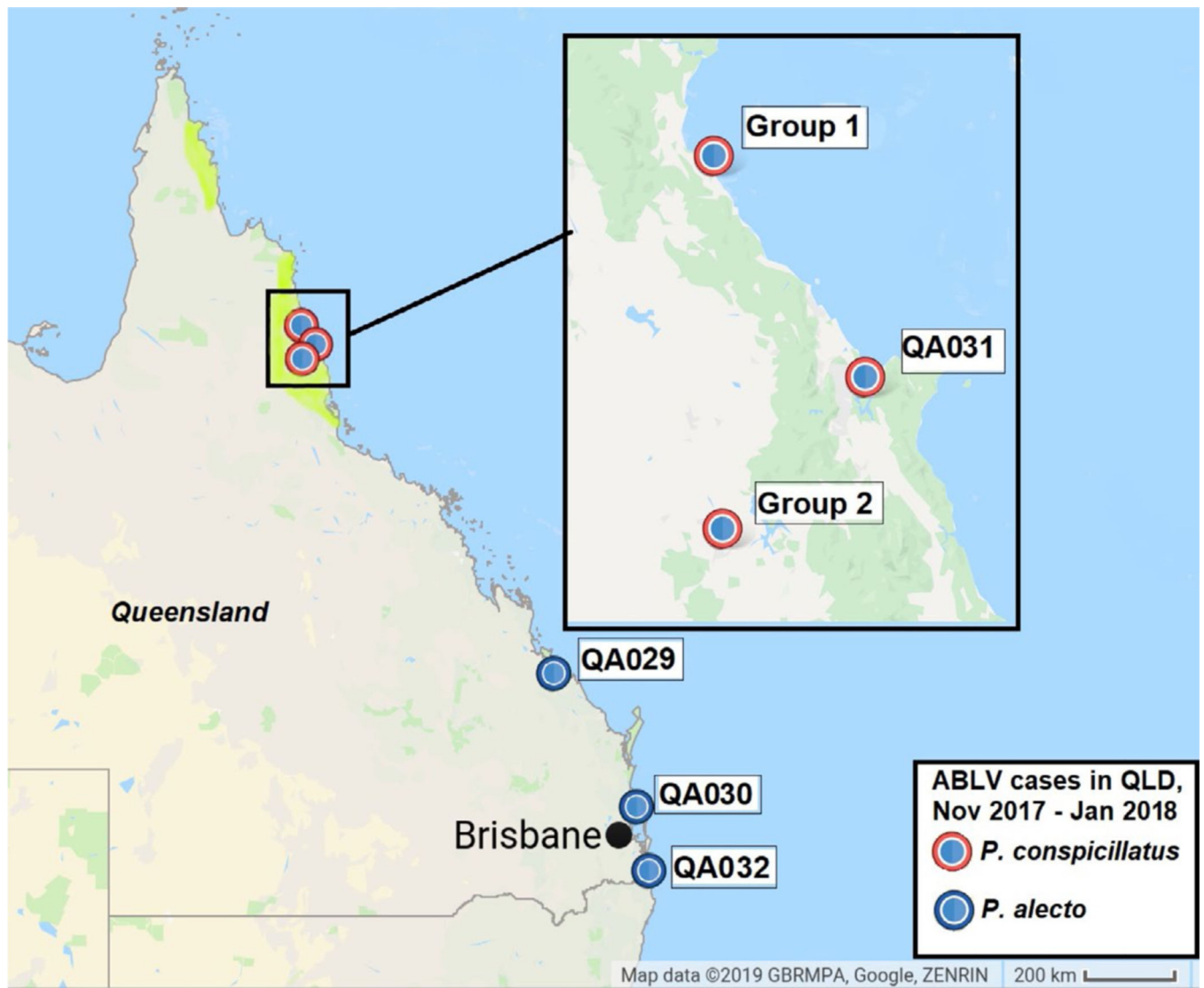


FIGURE 1.

ABLV sequences derived from infected *Pteropus conspicillatus* and *Pteropus alecto* bats submitted to Biosecurity Queensland between November 2017 and January 2018. The distribution of *P. conspicillatus*, restricted to the northern part of the state, is marked in yellow

Sequence	Group	*	*	*	*	*	*	*	*	*	*	*		
QA018	Group 1	T	C	T	C	A	T	G	C	G	T	C	T	G
QA019	Group 1	T	C	T	C	A	T	G	C	G	T	C	T	G
QA020	Group 1	T	C	T	C	A	T	G	C	G	T	C	T	G
QA021	Group 1	T	C	T	C	A	T	G	C	G	T	C	T	G
QA022	Group 1	T	C	T	C	A	T	G	C	G	T	C	T	G
QA023	Group 1	T	C	T	C	A	T	G	C	G	T	C	T	G
QA024	Group 2	T	C	T	C	T	T	G	C	G	T	C	T	G
QA025	Group 2	T	C	T	C	T	T	G	C	G	T	C	T	G
QA026	Group 2	T	C	T	C	T	T	G	C	G	T	C	T	G
QA027	Group 2	T	C	T	C	T	T	G	C	G	T	C	T	G
QA028	Group 2	T	C	T	C	T	T	G	C	G	T	C	T	G

FIGURE 2.

A single nucleotide substitution (T --> A) on the RNA polymerase coding region differentiates Group 1 and Group 2

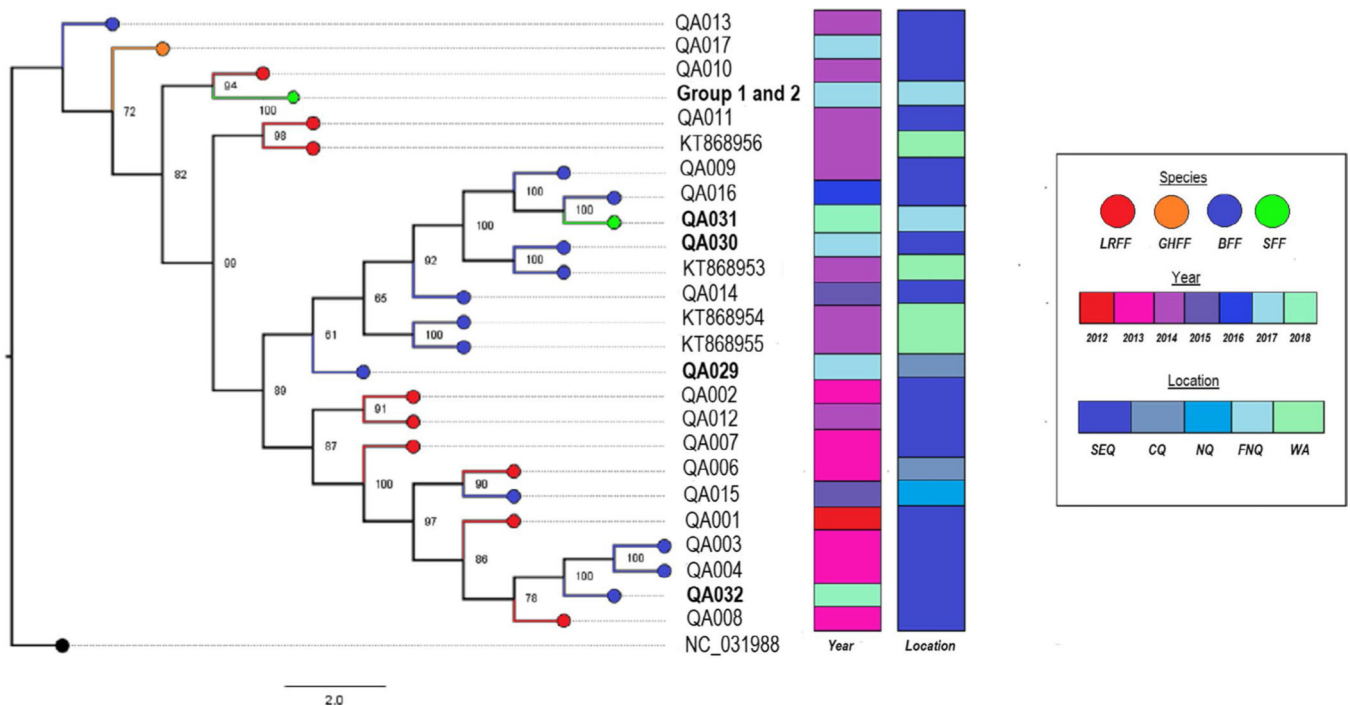


FIGURE 3.

Maximum-likelihood tree of whole genome ABLV sequences with 1,000× bootstrap support. Four ABLV whole genomes from bats in Western Australia are included for comparison, and NC_031998.1 (Gannoruwa bat lyssavirus) is used as an outgroup. The 11 sequences comprising Group 1 and Group 2 are clustered together, while the additional sequence from a *P. conspicillatus* pup in FNQ during the same time period as the pup cluster (QA031) is notably distant. Sequences from flying foxes submitted between November 2017 and January 2018 are in bold. ^aSpecies names are condensed to LRFF (little red flying fox, *P. scapulatus*), GHFF (grey-headed flying fox, *P. poliocephalus*), BFF (black flying fox, *P. alecto*) and SFF (spectacled flying fox, *P. conspicillatus*). ^bLocations are condensed as SEQ (South East Queensland), CQ (Central Queensland), NQ (North Queensland), FNQ (Far North Queensland) and WA (state of Western Australia)

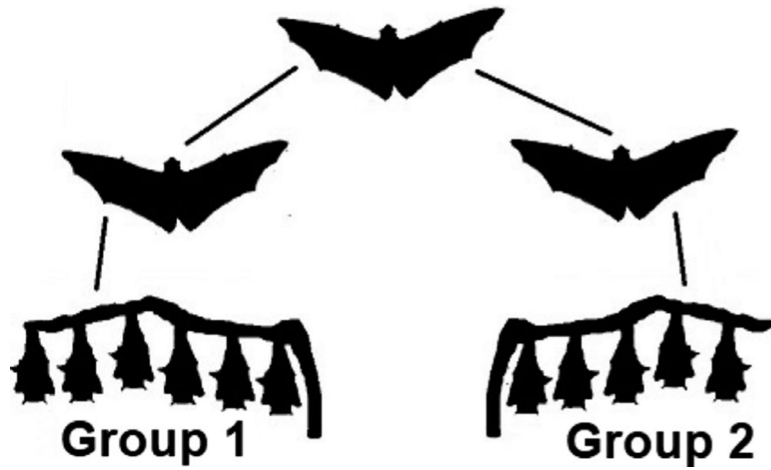


FIGURE 4.

Hypothesized transmission pathway: pups in Group 1 and Group 2 each infected by a single bat. Based on the nucleotide similarity between ABLV sequences in Group 1 and Group 2, we speculate that two flying foxes were infected by the same source before dispersing at least 80 kilometres to infect the two groups of *P. conspicillatus* pups

TABLE 1

Clinical signs of ABLV in rescued pups (Group 1)

Sample ID number	Bat carer	Weight loss/weight gain	'Wobbly', uncoordinated	Paresis	Aggression	Laboured breathing	Dysphonic	Cranial nerve defects	'Irritable/distressed/agitated'	Days post-rescue
QA018	1	X	nr	nr	nr	nr	nr	nr	nr	10–13
QA019	1	nr	X	X	X	X	nr	nr	X	13–15
QA020	2	X	nr	X	nr	nr	nr	X	nr	13–19
QA021	3	nr	nr	X	X	X	X	nr	X	16–17
QA022	3	X	X	nr	X	nr	X	nr	X	19–21
QA023	1	X	nr	X	X	nr	nr	X	X	19–21

Note: X, clinical sign reported; nr, not reported. Paresis (of varying severity), aggression, 'agitation' and weight loss/failure to gain weight were the most common clinical signs exhibited amongst pups in Group 1. In each instance, clinical signs were limited to duration of 2–3 days until euthanasia or natural death. Clinical signs specific to individual bats were not recorded for Group 2.

TABLE 2
 ABLV sequences from selected flying foxes submitted to the Biosecurity Sciences Laboratory, Biosecurity Queensland

Sample ID number	Species	Date of submission (month/year)	Location (within QLD)	GenBank accession No.	Notes
QA001	<i>P. scapulatus</i>	12/2012	South East	MK9444082	
QA002	<i>P. scapulatus</i>	01/2013	South East	MK9444083	
QA003	<i>P. alecto</i>	03/2013	South East	MK9444084	
QA004	<i>P. alecto</i>	04/2013	South East	MK9444085	
QA006	<i>P. scapulatus</i>	09/2013	Central	MK9444086	
QA007	<i>P. scapulatus</i>	10/2013	South East	MK9444087	
QA008	<i>P. scapulatus</i>	12/2013	South East	MK9444088	
QA009	<i>P. alecto</i>	02/2014	South East	MK9444089	
QA010	<i>P. scapulatus</i>	04/2014	South East	MK492318	
QA011	<i>P. scapulatus</i>	04/2014	South East	MK9444090	
QA012	<i>P. scapulatus</i>	05/2014	South East	MK9444091	
QA013	<i>P. alecto</i>	05/2014	South East	MK9444092	
QA014	<i>P. alecto</i>	01/2015	South East	MK9444093	
QA015	<i>P. alecto</i>	05/2015	North	MK9444094	
QA016	<i>P. alecto</i>	11/2016	South East	MK492319	
QA017	<i>P. poliocephalus</i>	05/2017	South East	MK9444095	Group 1
QA018	<i>P. conspicillatus</i>	11/2017	Far North	MK492309	Group 1
QA019	<i>P. conspicillatus</i>	11/2017	Far North	MK92310	Group 1
QA020	<i>P. conspicillatus</i>	11/2017	Far North	MK492311	Group 1
QA021	<i>P. conspicillatus</i>	11/2017	Far North	MK492312	Group 1
QA022	<i>P. conspicillatus</i>	11/2017	Far North	-	Group 1
QA023	<i>P. conspicillatus</i>	11/2017	Far North	-	Group 1
QA024	<i>P. conspicillatus</i>	11/2017	Far North	MK492313	Group 2
QA025	<i>P. conspicillatus</i>	11/2017	Far North	-	Group 2
QA026	<i>P. conspicillatus</i>	11/2017	Far North	-	Group 2
QA027	<i>P. conspicillatus</i>	11/2017	Far North	-	Group 2
QA028	<i>P. conspicillatus</i>	11/2017	Far North	-	Group 2
QA029	<i>P. alecto</i>	12/2017	Central	MK492314	
QA030	<i>P. alecto</i>	12/2017	South East	MK492315	

Sample ID number	Species	Date of submission (month/year)	Location (within QLD)	GenBank accession No.	Notes
QA031	<i>P. conspicillatus</i>	01/2018	Far North	MK492316	
QA032	<i>P. alecto</i>	01/2018	South East	MK492317	

Note: ABLV sequences from 15 flying foxes submitted between November 2017 and January 2018 and archived brain tissue from 16 flying foxes submitted between January 2013 and May 2017. Two sequences from Group 1 and all but one from Group 2 were not submitted to GenBank as they were identical.