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Borrelia Lineages Adjacent to Zoonotic Clades in Black Flying Foxes (*Pteropus alecto*), Australia, 2018–2020

Appendix

Appendix Table 1. PCR primers and reaction conditions used for *Borrelia* sp. PCR testing*

Target gene	Primer name	Primer orientation	Sequence, 5′-3′	Annealing temperature, °C†	Cycles	Amplified fragment, bp	Reference
16S rRNA	1A	Forward	CTAACGCTGGCAGTGCGTCTTAAGC	70–61, 60	10 + 40	≈724	(*1*)
16S rRNA	1B	Reverse	AGCGTCAGTCTTGACCCAGAAGTTC				
16S rRNA	$Bf1_{24}$	Forward	GCTGGCAGTGCGTCTTAAGCATGC	72–63, 62	10 + 40	≈1,395	Modified from (*2*)
16S rRNA	$Br1_{24}$	Reverse	GCTTCGGGTATCCTCAACTCGGGT				
16S-23S rRNA IGS	F	Outer forward	GTATGTTTAGTGAGGGGGGTG	55	30	Variable	(*3*)
16S-23S rRNA IGS	R	Outer reverse	GGATCATAGCTCAGGTGGTTAG				
16S-23S rRNA IGS	Fn	Inner forward	AGGGGGGTGAAGTCGTAACAAG	55	30	Variable	
16S-23S rRNA IGS	Rn	Inner reverse	GTCTGATAAACCTGAGGTCGGA				
flaB	FlaLL	Outer forward	ACATATTCAGATGCAGACAGAGG	52	30	≈665	(*4*)
flab	FlaRL	Outer reverse	GCAATCATAGCCATTGCAGATTGT				
flab	442f	Inner forward	GCTGAAGAGCTTGGAATGCAACC	55	30	≈524	This paper
flaB	FlaRL	Inner reverse	GCAATCATAGCCATTGCAGATTGT				(*4*)

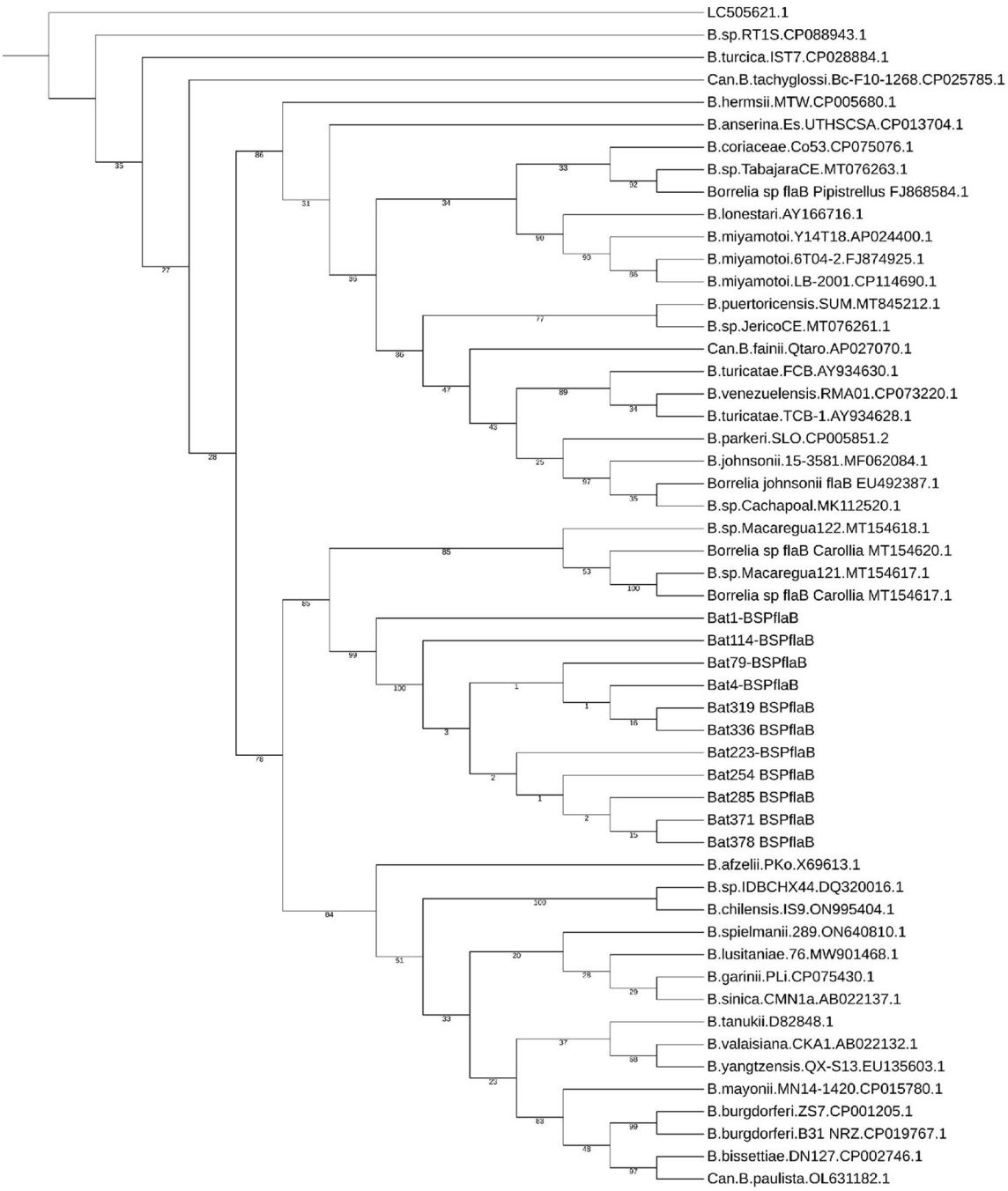
*PCR assays used Promega GoTaq Green master mix (Promega, Madison, WI, USA). Primers were used at 0.5 mM concentration. All PCRs began with an initial denaturation step at 94°C, 2 minutes. Thereafter, cycles consisted of 94°C, 30 seconds; annealing temperature as indicated in the table for 30 seconds; and extension at 72°C, 30 seconds for 16S 1A/1B and *flaB* primers or 72°C, 1 minutes for 16S $Bf1_{24}$/$Br1_{24}$ and IGS primers.
†The 16S rRNA screening primers (1A/1B) and the longer region 16S primers ($Bf1_{24}$/$Br1_{24}$) used a touchdown PCR approach. The annealing temperature was dropped 1°C in each of the first 10 cycles, followed by 40 additional cycles with annealing temperature as shown in the table.

Appendix Table 2. PCR positivity for *Borrelia* infections summarized across *Pteropus alecto* roosts and sampling sessions*

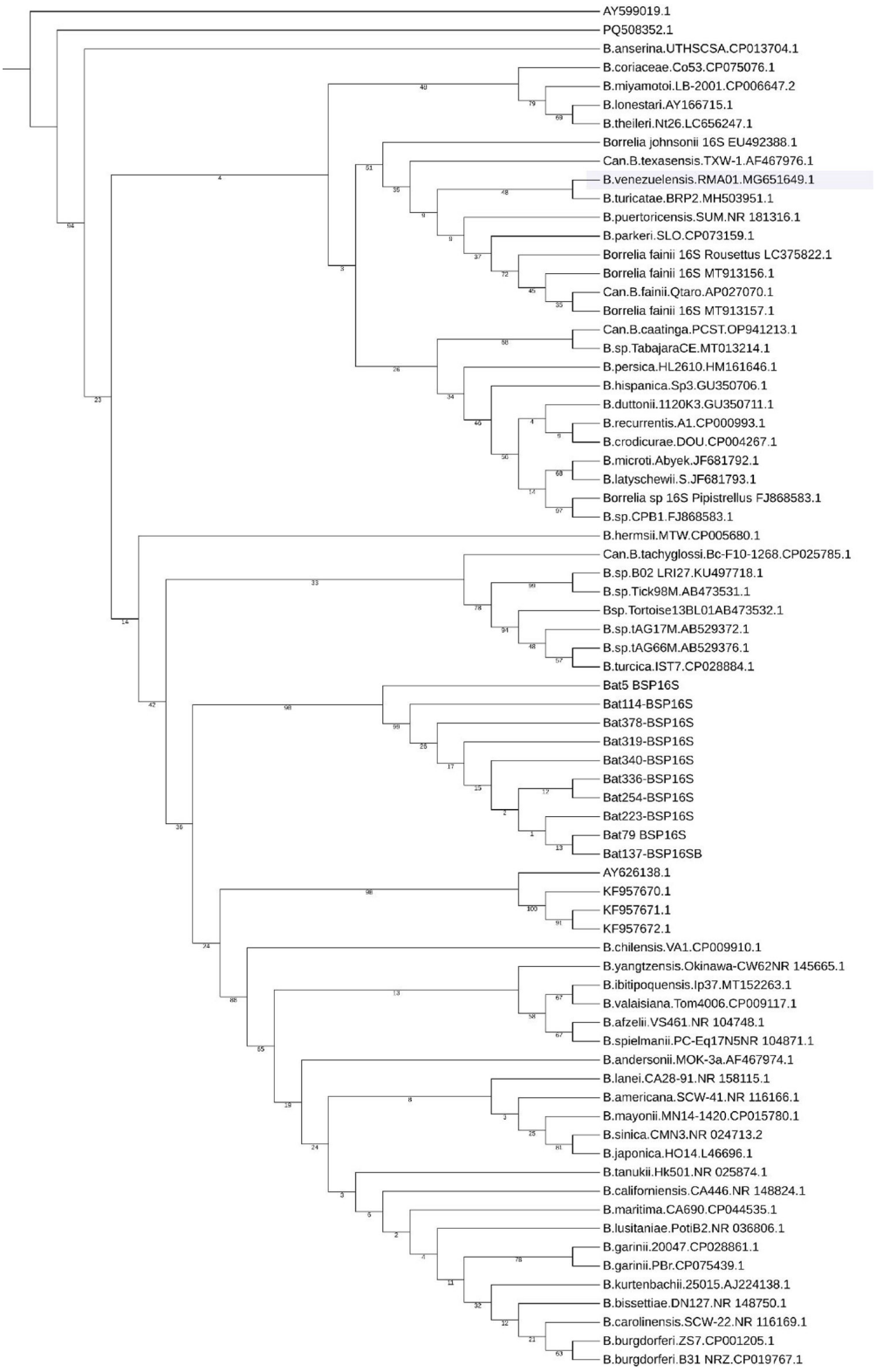
Site	Sampling session	Session prevalence	Total no. sampled	Site prevalence
Gympie	31 January 2019	0.20	20	0.20 (4/20)
Hervey Bay	15 July 2018	0	6	0
Hervey Bay	28 July 2020	0	30	
Maclean	9 July 2018	0	1	0
Mount Ommaney	17 January 2019	0	7	0
Redcliffe	25 May 2018	0.04	24	0.03 (10/375)
Redcliffe	27 July 2018	0	10	
Redcliffe	14 September 2018	0	19	
Redcliffe	14 December 2018	0.05	21	
Redcliffe	8 March 2019	0	59	
Redcliffe	28 May 2019	0.03	30	
Redcliffe	9 July 2019	0.03	32	
Redcliffe	10 September 2019	0.03	30	
Redcliffe	3 December 2019	0.03	32	
Redcliffe	3 March 2020	0.07	30	0.03 (10/375)
Redcliffe	11 May 2020	0.07	30	
Redcliffe	7 July 2020	0	30	

Site	Sampling session	Session prevalence	Total no. sampled	Site prevalence
Redcliffe	7 September 2020	0	28	
Toowoomba	3 June 2018	0.05	21	0.007 (3/402)
Toowoomba	21 July 2018	0.04	26	
Toowoomba	8 September 2018	0	21	
Toowoomba	8 December 2018	0	22	
Toowoomba	11 January 2019	0	9	
Toowoomba	15 March 2019	0.02	58	
Toowoomba	14 May 2019	0	30	
Toowoomba	2 July 2019	0	29	
Toowoomba	23 July 2019	0	6	
Toowoomba	3 September 2019	0	30	
Toowoomba	10 December 2019	0	30	
Toowoomba	10 March 2020	0	29	
Toowoomba	4 May 2020	0	30	
Toowoomba	14 July 2020	0	30	
Toowoomba	1 September 2020	0	30	

*Bats are considered infected if testing positive for at least 1 of our 3 markers (i.e., 16S rRNA gene, *flaB* gene, and 16S–23S rRNA ITS).



Appendix Figure 1. Maximum likelihood phylogenetic tree displaying evolutionary relationships between *Borrelia* spp. using the *flaB* gene. The tree was constructed using RAxML 8 (*5*) and a GTR+I+G nucleotide substitution model. Branch support was calculated with 1,000 rapid bootstrap replicates.



Appendix Figure 2. Maximum likelihood phylogenetic tree displaying evolutionary relationships between *Borrelia* spp. using the 16S gene. The tree was constructed using RAxML 8 (*5*) and a GTR+I+G nucleotide substitution model. Branch support was calculated with 1,000 rapid bootstrap replicates.

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