



Characterisation of typical enteropathogenic *Escherichia coli* (tEPEC) lineages and novel *bfpA* variants detected in Australian fruit bats (*Pteropus poliocephalus*)

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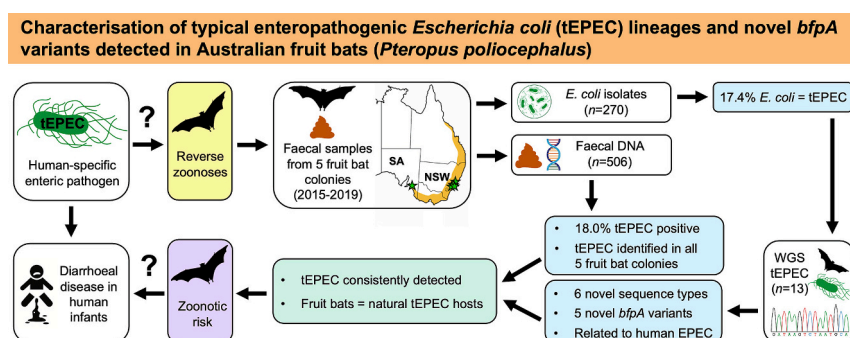
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HIGHLIGHTS

- tEPEC comprised 17.4 % of *E. coli* cultured from wild fruit bat faeces.
- Novel tEPEC sequence types and *bfpA* variants indicate bat-specific tEPEC lineages.
- Fruit bat tEPEC are related to human EPEC and show zoonotic potential in vitro.
- tEPEC consistently detected in urban fruit bats and may pose a public health risk.
- Fruit bats appear to be natural tEPEC hosts.

GRAPHICAL ABSTRACT



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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) is an important cause of diarrhoeal disease in human infants. EPEC strains are defined by the presence of specific virulence factors including intimin (encoded by the *eae* gene) and bundle forming pili (Bfp). Bfp is encoded by the *bfp* operon and includes the *bfpA* gene for the major pilus subunit. By definition, Bfp are only present in typical EPEC (tEPEC), for which, humans are considered to be the only known natural host. This study detected tEPEC in faecal samples from a wild Australian fruit bat species, the grey-headed flying-fox (*Pteropus poliocephalus*). Whole genome sequencing of 61 *E. coli* isolates from flying-foxes revealed that 21.3 % (95%CI: 13 %–33 %) were tEPEC. Phylogenetic analyses showed flying-fox tEPEC shared evolutionary lineages with human EPEC, but were predominantly novel sequence types (9 of 13) and typically harboured novel *bfpA* variants (11 of 13). HEp-2 cell adhesion assays showed adherence to human-derived

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epithelial cells by all 13 flying-fox tEPEC, indicating that they all carried functional Bfp. Using an EPEC-specific duplex PCR, it was determined that tEPEC comprised 17.4 % (95%CI: 13 %–22 %) of 270 flying-fox *E. coli* isolates. Furthermore, a tEPEC-specific multiplex PCR detected the *eae* and *bfpA* virulence genes in 18.0 % (95% CI: 8.0 %–33.7 %) of 506 flying-fox faecal DNA samples, with occurrences ranging from 1.3 % to 87.0 % across five geographic areas sampled over a four-year period. The identification of six novel tEPEC sequence types and five novel *bfpA* variants suggests flying-foxes carry bat-specific tEPEC lineages. However, their close relationship with human EPEC and functional Bfp, indicates that flying-fox tEPEC have zoonotic potential and that dissemination of flying-fox tEPEC into urban environments may pose a public health risk. The consistent detection of tEPEC in flying-foxes over extensive geographical and temporal scales indicates that both wild grey-headed flying-foxes and humans should be regarded as natural tEPEC hosts.

1. Introduction

Enteropathogenic *Escherichia coli* (EPEC) is an important cause of diarrhoea and mortality in infants in low- and middle-income countries (Kaper et al., 2004; Levine et al., 2020), and prolonged diarrhoea in children in high-income countries (Afset et al., 2004; Nguyen et al., 2006). EPEC are characterised by their ability to cause attaching and effacing lesions of the intestinal epithelium, resulting in microvilli destruction and diarrhoea (Nataro and Kaper, 1998; Trabulsi et al., 2002). All EPEC carry specific virulence factors, most notably a ~ 35 kb chromosomal locus for enterocyte effacement (LEE) pathogenicity island which encodes an outer membrane protein, intimin (encoded by the *eae* gene), a type 3 secretion system, and several type-3 secreted effectors, including a translocated intimin receptor (*tir* gene), EspA, EspB, EspH and Map (Elliott et al., 1998; Wong et al., 2011). In addition to the LEE-encoded genes, there are numerous non-LEE encoded (Nle) type 3 secreted effectors (Wong et al., 2011) and adhesins (Bardiau et al., 2010b) which may be associated with increased pathogenicity (Wong et al., 2011).

EPEC strains which carry the EPEC adherence factor plasmid (pEAF) are classified as typical EPEC (tEPEC) and those lacking this plasmid are known as atypical EPEC (aEPEC) (Trabulsi et al., 2002). pEAF carries the bundle forming pilus (*bfp*) operon and plasmid encoded regulators (*per*), all of which are required for tEPEC pathogenicity in humans (Levine et al., 1985). Bundle-forming pili facilitate the formation of stable microcolonies on epithelial cell monolayers in vitro, giving rise to a pattern known as localised adherence, which is characteristic of tEPEC (Nataro and Kaper, 1998).

LEE has multiple evolutionary variants, with distinct EPEC lineages being associated with specific LEE insertion sites and *eae* subtypes (Ingle et al., 2016; Müller et al., 2009). Multiple variants of the EAF plasmid and *bfpA* allele also exist, but unlike LEE, they are not highly conserved within tEPEC lineages (Lacher et al., 2007). EPEC lineages have historically been characterised by O and H antigens (O:H serotype), with 12 classical EPEC O-serogroups recognised in 1987 (World Health Organisation, 1987) and multiple non-classical O-serogroups identified subsequently (Lacher et al., 2007; Ori et al., 2019). The continuing emergence of new EPEC O-serogroups indicates that the LEE (and pEAF in tEPEC) can be acquired independently by diverse *E. coli* lineages (Ingle et al., 2016; Lacher et al., 2007).

Humans, especially children, are the primary reservoir of EPEC and humans are considered the only natural host for tEPEC (Chandran and Mazumder, 2013; Nataro and Kaper, 1998). Only rarely have tEPEC been identified in non-human hosts, with sporadic detections reported in domestic animals; dogs, cats, pigs, lambs and cattle (Begum et al., 2014; Blanco et al., 2005; Chandran and Mazumder, 2013; Goffaux et al., 2000; Nakazato et al., 2004; Stephan et al., 2004; Wani et al., 2009), and in wildlife; birds, bats, boar, coyote and deer (Alonso et al., 2017; Borges et al., 2017; Chandran and Mazumder, 2013; Gioia-Di Chiacchio et al., 2018; Ishii et al., 2007; Nowak et al., 2017; Saldenberg et al., 2012; Sanches et al., 2017). In contrast, numerous studies have reported the presence of aEPEC in companion animals and livestock (Chandran and Mazumder, 2013; Goffaux et al., 2000; Ishii et al., 2007; Kobayashi et al., 2003; Nakazato et al., 2004; Xu et al., 2017) and in diverse wild

mammal and bird species (Alonso et al., 2017; Bardiau et al., 2010a; Borges et al., 2017; Carvalho et al., 2003; Chandran and Mazumder, 2013; Kobayashi et al., 2009; Mora et al., 2018; Nowak et al., 2017; Sacristán et al., 2014; Saldenberg et al., 2012; Sanches et al., 2017; Xu et al., 2017).

Although reports of the carriage of enteric bacterial pathogens by bats are sparse (Agustin et al., 2019; Ajayi, 2020; Brygoo et al., 1971; Cabal et al., 2015; Heard et al., 1997; Henry et al., 2018; Höhne et al., 1975; Iovine et al., 2015; Islam et al., 2013; Nowak et al., 2017), tEPEC have been isolated from two bat species (Nowak et al., 2017), and key tEPEC virulence genes, *eae* and *bfpA*, have been identified in faecal DNA from a further eight bat species (Cabal et al., 2015), suggesting bats may be an important wildlife group associated with tEPEC carriage. However, genetic characterisation of tEPEC from bats is very limited, with sequence typing performed on only four bat tEPEC isolates (Nowak et al., 2017), and no whole genome sequencing has been undertaken to examine phylogenetic relationships between bat and human tEPEC strains. Additionally, the prevalence of tEPEC carriage in bat populations has not been determined over geographical or temporal scales. Thus, it is currently unknown if tEPEC detections in bats are sporadic zoonanthroponotic transmission, or if bats are tEPEC reservoir hosts and pose a zoonotic risk.

Bats belong to the order Chiroptera which is divided into two sub-orders, Yinpterochiroptera (predominantly fruit bats/flying-foxes but includes some microbats) and Yangochiroptera (microbats) (Teeling et al., 2005). On mainland Australia, there are four native species of fruit bats (*Pteropus* spp.), each with different geographical distributions. The only endemic species, the grey-headed flying-fox (*Pteropus poliocephalus*), is widely distributed across eastern Australia, and is the predominant fruit bat species in New South Wales, Victoria and South Australia (Australian Government, 2021). Grey-headed flying-foxes, occupy tree roosts and form large colonies that can number in the tens of thousands (Australian Government, 2021). Movement of individuals between colonies occurs frequently, with an average daily colony turnover of 17.5 %, making them an extremely mobile metapopulation (Welbergen et al., 2020). Grey-headed flying-foxes are highly urban-adapted and are renowned for forming large colonies in parklands located within cities, placing them, and their copious faecal deposits, in close proximity to humans (Parry-Jones and Augee, 2001; Williams et al., 2006). This close proximity provides increased opportunity for zoonotic pathogen transmission to occur between humans and grey-headed flying-foxes (Gibb et al., 2020). Exposure to faecal pathogens may be via direct contact between humans and grey-headed flying-foxes, or via indirect contact with their faeces or contaminated food and water in the environment (Davy et al., 2023; Kruse et al., 2004). To date, research examining zoonotic pathogens in grey-headed flying-foxes has largely focused on viral pathogens (Boardman et al., 2020), with only two reports of zoonotic enteric bacterial pathogens, both being *Salmonella enterica* (McDougall and Power, 2021; Van Brussel et al., 2023).

This study investigated the occurrence of tEPEC in wild grey-headed flying-foxes, hereafter referred to as flying-foxes, and examined the genetic characteristics and evolutionary relationships between flying-fox tEPEC strains and human EPEC strains using whole genome sequence (WGS) data and analysis of key virulence genes.

2. Materials and methods

2.1. Grey-headed flying-fox faecal sampling

Faecal samples were collected from five wild flying-fox colonies in two Australian states; New South Wales (NSW, four colonies) and South Australia (SA, one colony) (Fig. 1).

Faecal samples were collected at 10 sampling timepoints over a four-year period, between March 2015 and April 2019 (Table S1). Adelaide Botanic Park (ABP) was sampled on four occasions, Blackalls Park (BP) on three occasions and Camellia Gardens (CG), Centennial Park (CP) and Ku-ring-gai Flying-Fox Reserve (KFFR) were each sampled once (Table S1). A total of 600 faecal samples were collected, of which, 468 were cultured for *E. coli* and 506 underwent DNA extraction (Table S1).

For NSW sites, faecal samples were collected opportunistically by placing between six and 16 plastic drop sheets measuring 2.6 m × 3.6 m, under multiple roost trees throughout the flying-fox colony. Between five and 10 discrete faecal samples were selected from each sheet, avoiding samples positioned close together to minimise the risk of repeated sampling from the same individual. For sampling in SA, individual flying-foxes were trapped using mist nets and then placed in clean a cotton holding bag and transferred to the Adelaide Zoo veterinary hospital for processing. Trapped flying-foxes were anaesthetised, and rectal swabs taken from individuals or faeces collected from the clean holding bags. Faecal samples for culture were transferred using a sterile swab into a FecalSwab™ tube (COPAN, Brescia, Italy) or into sterile faecal jars for those being used for DNA only analysis. All faecal samples

were stored at 4 °C and cultured within 72 h of collection.

All sample collections were conducted under approval from Macquarie University Animal Ethics Committee (No. 2017/013), the University of Adelaide Animal Ethics Committee (No. S-2015-028), and under NSW Government Scientific Licence (No. SL101898) and SA Department of Environment and Water Wildlife Scientific Permit (M-23671-1, -2 and -3).

2.2. Isolation and phylotyping of *E. coli* from grey-headed flying-fox faecal samples

The *E. coli* strains were isolated by inoculating FecalSwab media directly onto Chromocult Coliform Agar (Merck Millipore, Massachusetts, USA) followed by incubation overnight at 37 °C. *E. coli* colonies were identified by their dark blue to violet appearance. Where morphologically different *E. coli* colonies (based on colony and halo colour) were present on the same plate, a representative colony of each type was selected. Otherwise, a single colony was randomly selected.

All *E. coli* isolates were assigned to phylogroups A, B1, B2, C, D, E, F, or cryptic clade I using the Clermont phylotyping method (Clermont et al., 2013), as previously described (McDougall et al., 2022), using cell lysate as the DNA template. *Escherichia coli* lysates were obtained by culturing isolates in 10 mL Luria-Bertani (LB) broth, Miller (Difco Laboratories, Detroit, USA) at 37 °C overnight, centrifugation (12,000 g, 10 min), resuspending *E. coli* pellet in 0.1 mL sterile PCR water, heating (95 °C, 10 min) and centrifugation (12,000 g, 10 min).

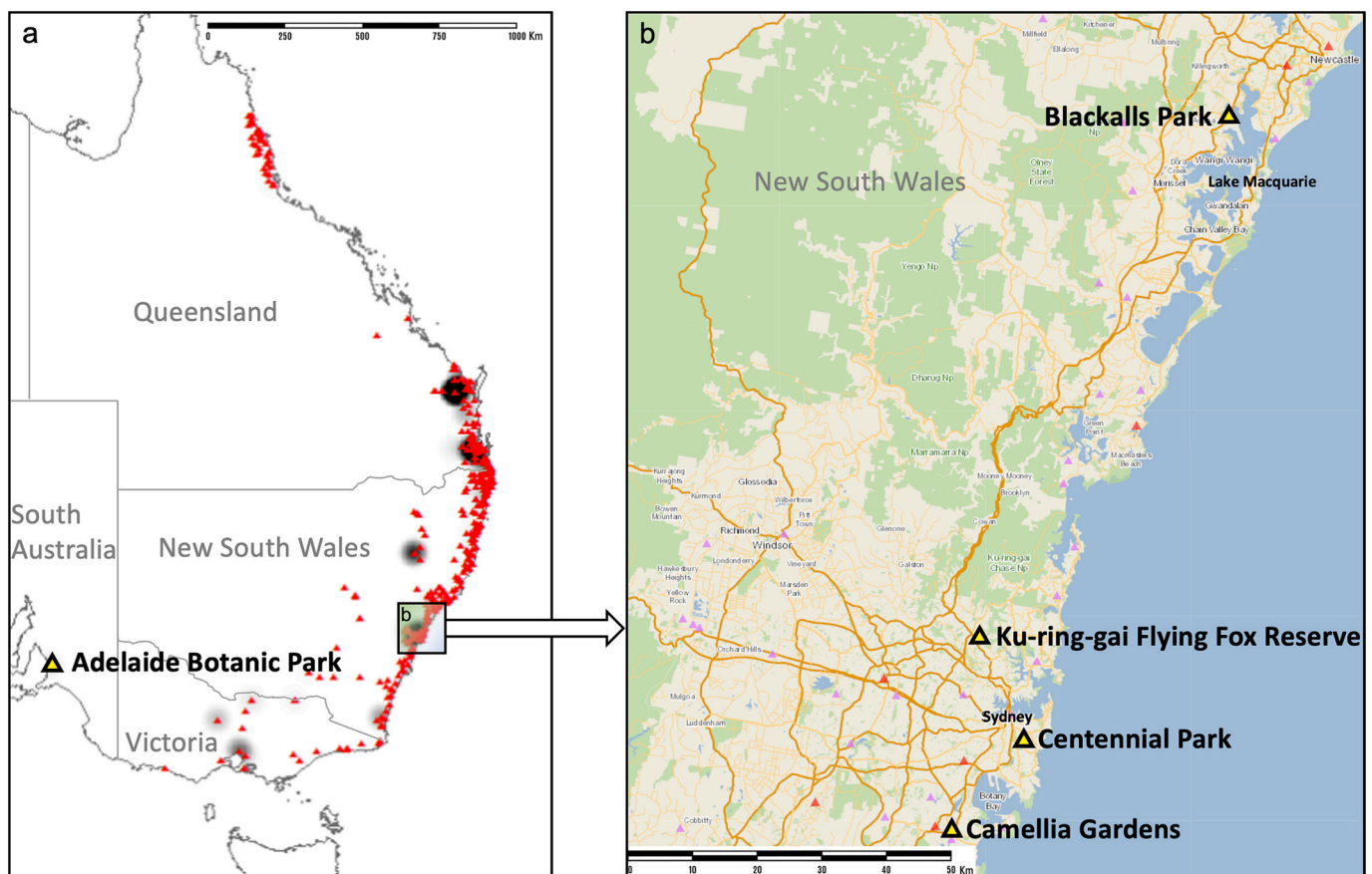


Fig. 1. Distribution of grey-headed flying-fox colonies across eastern Australia and the locations of sampled colonies. a. Red triangles indicate the geographical distribution of flying-fox colonies in eastern Australia. The yellow triangle indicates the location of the sampled Adelaide Botanic Park colony in South Australia. The map also shows the New South Wales (NSW) sampling area at low magnification. b. Map showing the NSW sampling sites at higher magnification. Yellow triangles indicate the four sampled colonies in NSW. Red triangles indicate permanently occupied colonies and pink triangles indicate seasonally occupied colonies. Both images were constructed using maps and May 2018 survey data sourced from the National Flying-Fox Monitoring Program (Australian Government) (<http://www.environment.gov.au/biodiversity/threatened/species/flying-fox-monitoring>).

2.3. *E. coli* whole genome sequencing

Phylotyping data was used to select a representative subset of *E. coli* isolates ($n = 61$) for WGS (Table S2). Selected isolates were grown in 5 mL LB broth at 35 °C overnight with shaking at 150 r.p.m., after which DNA was extracted using the ISOLATE II Genomic DNA Kit (Bioline, London, UK). The concentration of gDNA was determined using a Qubit dsDNA BR assay kit (Invitrogen, Waltham, USA). Libraries were prepared for WGS using a Nextera XT DNA sample preparation kit (Illumina, San Diego, USA) and the Nextera XT index kit (Illumina) with 0.5 ng of input DNA. WGS was performed on an Illumina MiSeq platform using a 600-cycle MiSeq Nextera XT reagent kit v3 (2×300 -bp paired-end reads). Raw sequence reads were assembled as de novo genome sequences using CLC Genomics Workbench 8 (CLC Bio, Aarhus, Denmark). Raw sequence reads for all flying-fox *E. coli* isolates were uploaded to NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA613581 and BioProject ID PRJNA857315. Assembled sequences are available in Enterobase (Supplementary data 1).

2.4. Analysis of assembled DNA sequences

Assembled DNA sequences were screened for virulence factors (VFs) using VirulenceFinder 2.0 (Joensen et al., 2014) (available at <http://www.genomicepidemiology.org/services/>) and Abricate VFDB (<https://github.com/tseemann/abricate>) (Chen et al., 2016) in Galaxy Australia (available at <https://usegalaxy.org.au/>), including VFs associated with EPEC; *eae*, *esp* genes, *map*, *tir*, *bfpA*, *perA* and Nle-effectors (*cif*, *nleB*, *nleE*) and adhesins (*efa1*, *iha*, *lpfA*, *paa*, *toxB*). Additional screening for VFs which may be associated with EPEC included toxins (*astA*, *cdtAB*, *stx*, *vat*), invasion protein *ibeA*, yersiniabactin (*fyuA*, *irp1*, *irp2*) and miscellaneous VFs (*iss*, *malX*). Plasmid replicons were identified in paired raw sequence reads using PlasmidFinder 2.0 (Carattoli et al., 2014) (available at <http://www.genomicepidemiology.org/services/>).

The *eae* and *bfpA* sequences were extracted from assembled DNA sequences using Geneious Prime 2022.0.2 (Biomatters Limited, Auckland, New Zealand) and BLASTn searches performed (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) against *eae* allele reference sequences (listed in Table S3) (Lacher et al., 2006; Wasilenko et al., 2012) and human *bfpA* allele reference sequences (listed in Table S4) (Blank et al., 2003; Blank et al., 2000; Lacher et al., 2007). ResFinder 3.2 (Zankari et al., 2012) and PointFinder 3.1.0 (Zankari et al., 2017) (both available at <http://www.genomicepidemiology.org/services/>) were used to screen sequences for antimicrobial resistance. Serotypes (O:H) were assigned using SeroTypeFinder 2.0 (Joensen et al., 2015). Sequence type (ST) was determined using Multi-Locus Sequence Typing (MLST) 2.0 (Larsen et al., 2012) and *fimH* type using FimTyper 1.0 (available at <http://www.genomicepidemiology.org/services/>). Novel strains were assigned a ST according to the Achtman seven gene MLST scheme in Enterobase (<http://enterobase.warwick.ac.uk/species/index/ecoli>).

Full-length *bfp* operon sequences of flying-fox tEPEC were obtained by mapping raw sequence reads to an EAF plasmid reference sequence from *E. coli* O127:H6 E2348/69 (plasmid pMAR2, GenBank FM180569) using Geneious Prime 2022.0.2 (Biomatters Limited, Auckland, New Zealand). BLASTn searches of flying-fox *bfp* operons were performed (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the closest matches in GenBank. Full-length flying-fox *bfp* operon sequences are available in GenBank and accession numbers are provided in Table S5.

2.5. Phylogenetic analysis and comparison of flying-fox, human and animal EPEC

Phylogenetic relationships between all flying-fox phylogroup B2 *E. coli* isolates selected for WGS and additional Australian sourced phylogroup B2 *E. coli* isolates (collection supplied by David Gordon, Supplementary data 2) were inferred by core-genome alignment and

identification of single nucleotide polymorphisms (SNPs) using the Harvest suite of tools, including Parsnp (<https://www.cbcb.umd.edu/software/harvest>), to construct a maximum likelihood phylogeny (Treangen et al., 2014).

Based on the distribution of flying-fox EPEC in the phylogeny of Australian sourced phylogroup B2 isolates, additional EPEC isolates closely related to the flying-fox EPEC were identified in Enterobase by ST searches using Achtman seven gene MLST with up to two allele mismatches (<http://enterobase.warwick.ac.uk/species/index/ecoli>) (Supplementary data 3). Closely related EPEC which also carried the shiga toxin virulence gene (*stx*), referred to as enterohaemorrhagic *E. coli* (EHEC), were also included. Four phylogroup B2 EPEC clade trees were then constructed using all flying-fox EPEC, Australian EPEC and the additional EPEC isolates identified in Enterobase. The EPEC clade tree phylogenies were inferred using core-genome alignment and the Harvest suite of tools as described above (<https://www.cbcb.umd.edu/software/harvest>) (Treangen et al., 2014).

The *eae* and *bfpA* phylogenetic trees were constructed using *eae* and *bfpA* sequences extracted from B2 EPEC in all four clades also using core-genome alignment and the Harvest suite of tools (<https://www.cbcb.umd.edu/software/harvest>) (Treangen et al., 2014). Reference sequences and GenBank accession numbers for *eae* subtypes and *bfpA* allele variants are described in Tables S3 and S4. All trees were annotated using Interactive Tree of Life v5 (iTOL) (<https://itol.embl.de>) (Letunic and Bork, 2019).

The LEE sequence was obtained for all *bpf* positive isolates (tEPEC and EHEC) by mapping raw sequence reads to a LEE reference sequence from *E. coli* strain RHB10-C14 (GenBank CP055967) using Geneious Prime 2022.0.2 (Biomatters Limited, Auckland, New Zealand) and BLASTn searches performed (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the LEE chromosomal insertion sites.

Whole genome single nucleotide polymorphism (SNP) analysis of all flying-fox tEPEC and two flying-fox aEPEC isolates was performed using CSI Phylogeny 1.4 (available at <https://cge.cbs.dtu.dk/services/>) (Kaaas et al., 2014). Paired raw sequencing reads were uploaded and SNP analysis performed using default settings against the reference genome EPEC E2348/69 variety 2 (GenBank LT827011). A SNP matrix was constructed using SNP counts between all genomes.

2.6. HEP-2 adhesion assays with flying-fox tEPEC isolates

In vitro patterns of adherence by flying-fox tEPEC isolates to HEP-2 cells were determined using a modified Center for Vaccine Development (CVD) method (Vial et al., 1990). Three human clinical *E. coli* isolate controls were included; E2348/69 (tEPEC control), W1056 (aEPEC control) and HS (non-pathogenic commensal control). Two flying-fox aEPEC isolates that lacked the *bfp* operon, but otherwise shared almost identical genomes to two flying-fox tEPEC isolates, were also included as controls in these assays. All bacterial isolates were grown in Antibiotic Medium No. 3 Assay Broth (Oxoid, Basingstoke, UK) for 16 h, without shaking at 37 °C. Approximately 10^7 bacteria were incubated with HEP-2 cell monolayers grown to ~70 % confluence in the presence of 0.5 % D-mannose at 37 °C in 5 % CO₂. After 3 h, the monolayers were washed three times with PBS to remove non-adherent bacteria and cells were then fixed with 3 % formalin in PBS before staining with 10 % Giemsa stain. Assays were performed in triplicate and adherent bacteria were observed by brightfield microscopy using a Zeiss Axio Imager Z2 microscope. Nine representative images (x400 magnification) for each isolate were captured using a Zeiss Axiocam 506 digital camera and examined blind for adherence patterns; localised adherence (LA), aggregative adherence (AA), diffuse adherence (DA), indeterminate adherence (IA) and no adherence (NA), where non-adherence was defined as <10 of 200 HEP-2 cells having 5 or more adherent bacteria.

2.7. EPEC-specific duplex PCR of *E. coli* isolates

To enable rapid screening for EPEC virulence factors in *E. coli* isolates, two primer sets targeting *eae* and *bfpA* were designed using sequences obtained from the WGS of flying-fox EPEC. The primers were *eaeF* 5'-GGCTGGCATGAGTCATACAATA-3' and *eaeR* 5'-CGTAACGGCTGCCTGATAAT-3' which give a product size of 373 bp and *bfpAF* 5'-CTGTATATTGGGCAGACCAGTT-3' and *bfpAR* 5'-CGTTGCAAGACTAACACATGC-3' with a product size of 219 bp. A duplex PCR for both genes was performed using GoTaq® Green Master Mix (Promega, Madison, WI, USA), *eae* and *bfpA* primers at 0.5 µM each and 2 µL *E. coli* isolate DNA (using cell lysate as described for the phylotyping PCRs). The cycling conditions were 94 °C 3 min; 35 cycles of 94 °C 30 s, 60 °C 30 s, 72 °C 1 min 30 s; 72 °C 5 min. Primer pairs were validated by correlating PCR results from 20 flying-fox *E. coli* isolates with WGS results (Table S6).

2.8. tEPEC-specific multiplex PCR of faecal DNA

To determine the occurrence of tEPEC in all flying-fox faecal samples, the tEPEC-specific duplex PCR was adapted with the addition of *chuA* phylotyping primers (*ChuA.1b* and *ChuA.2*) from the Clermont quadruplex PCR method (Clermont et al., 2013), as an internal control to confirm the presence of *E. coli* from phylogroups B2, D, E and/or F. All primers were 0.4 µM each and the PCR performed using the *E. coli* phylotyping PCR conditions as previously described. The faecal multiplex PCR was validated by correlating faecal DNA PCR results ($n = 20$) to the corresponding *E. coli* PCR and WGS results (Table S6). The tEPEC multiplex PCR was used to screen faecal DNA extracted using the Biotec ISOLATE Faecal DNA Kit following the manufacturer's instructions. Faecal DNA samples that failed to amplify in the multiplex tEPEC PCR underwent a 16S rRNA PCR to confirm PCR competency, using universal eubacterial primers *f27* and *r1492* (Lane, 1991) with GoTaq® Green Master Mix (Promega, Madison, WI, USA) and cycling conditions of 94 °C 3 min; 35 cycles of 94 °C 30 s, 58 °C 30 s, 72 °C 1 min 30 s; 72 °C 5 min.

2.9. Statistical analyses

Counts of EPEC isolates (as determined by the presence of *eae* +/- *bfpA*) in cultured *E. coli* or tEPEC virulence genes (*eae* and *bfpA*) in faecal DNA samples were summarised as total count with percent and 95 % confidence interval (CI) using the Wilson method (Wilson, 1927). To estimate the overall prevalence of tEPEC positive faecal DNA samples, a mixed-effects binomial regression was used whereby a random effect was used to control for repeated measures from the same location. Results are expressed as estimated percentage with 95%CI.

3. Results

3.1. Detection of tEPEC in wild grey-headed flying-fox faecal samples

A total of 270 *E. coli* isolates were obtained from 468 flying-fox faecal samples (Table S2). A subset of these isolates (61 of 270) underwent WGS (Table S2), which revealed that 33 were EPEC, with 13 being tEPEC (*eae* and *bfpA* positive) and 20 being aEPEC (*eae* positive and *bfpA* negative). The remaining 28 isolates were negative for *eae* and *bfpA*, and designated 'Non-EPEC'.

The 13 flying-fox tEPEC isolates belonged to eight different sequence types (STs). Additionally, nine of the 13 flying-fox tEPEC belonged to one of six novel STs. The largest number of flying-fox tEPEC isolates (7 of 13) were serogroup O non-typable (ONT), with O48, O71, O91, O109, O180 and O118/O151 each occurring once. The most frequent H-antigens in the 13 tEPEC were H1 ($n = 4$), H45 ($n = 3$), H34 ($n = 2$) and H51 ($n = 2$), with H6 and H40 both occurring once. EPEC status, phylotype, ST and serotype for all 61 flying-fox *E. coli* that underwent WGS are

provided in Table S7.

3.2. Phylogeny of Australian phylogroup B2 *E. coli* from flying-foxes, humans and animals

Of the 61 flying-fox *E. coli* that underwent WGS, 47 were assigned to phylogroup B2, which included the 13 tEPEC, 18 aEPEC, and 16 Non-EPEC isolates (Supplementary data 2). The additional 109 Australian-sourced phylogroup B2 *E. coli* sequences from humans and other animals included 18 EPEC and 91 Non-EPEC sequences (Supplementary data 2). Phylogenetic analysis of these 47 flying-fox phylogroup B2 *E. coli* and 109 Australian-sourced phylogroup B2 *E. coli*, placed all EPEC strains from flying-foxes, humans and other animals, into one of four broad clades, designated Clades A, B, C and D (Fig. 2). The flying-fox tEPEC were distributed within three clades, with most clustered in Clade A (10 of 13), two in Clade C and one in Clade D (Fig. 2). Clade B contained flying-fox aEPEC only.

3.3. Phylogeny of flying-fox tEPEC and globally sourced phylogroup B2 EPEC isolates

Six of 10 flying-fox tEPEC in Clade A belonged to a broad cluster designated ST7799 ONT:H51, which also contained one human aEPEC sourced from the United Kingdom (Fig. 3a). Of the four remaining flying-fox tEPEC in Clade A, three ST2346 isolates were clustered with an aEPEC of Australian mammal of unknown species (Fig. 3a). The tenth flying-fox tEPEC in Clade A (ST8437) belonged to a cluster containing predominantly human sourced aEPEC and one human sourced tEPEC of serotype O33:H34 (Fig. 3a). Clade A contained tEPEC of the classical serotype, O142:H34, however, all 10 Clade A flying-fox tEPEC were placed in other lineages (Fig. 3a).

The two flying-fox tEPEC in Clade C belonged to distant clusters. One isolate exhibited a novel ST (ST7796) and was clustered with a flying-fox aEPEC of the same ST and serotype. The other isolate was clustered with predominantly human sourced aEPEC (Fig. 3b). The single flying-fox tEPEC isolate in Clade D exhibited a novel ST (ST9731) and was also clustered with a flying-fox aEPEC of the same ST and serotype (Fig. 3c). Overall, only three of 13 of flying-fox sourced tEPEC isolates shared STs, serotypes and/or phylogenetic clusters with human associated EPEC (Fig. 3).

Single nucleotide polymorphism (SNP) whole genome analysis of all GHFF tEPEC and the two closely related aEPEC revealed little variation between closely clustered isolates (0–5 SNP counts between genomes) and large variations between tEPEC from different clades (9506–12,191 SNP counts between genomes) (Fig. S1). All flying-fox tEPEC had SNP counts between 8443 and 10,006 against the reference genome tEPEC E2348/69 (Fig. S1).

3.4. Virulence factors of phylogroup B2 tEPEC clades

The LEE-encoded genes, *eae*, *espABDH*, *map* and *tir*, were present in almost all phylogroup B2 EPEC isolates in Clades A to D, whereas Nle-effectors and adhesins were variable (Fig. 3, Fig. S2 and Table S8). Of the 13 tEPEC isolates from flying-foxes, *cif* was present in six, followed by *nleB2* in three and *nleB2* plus *paa* in one (Fig. 3 and Table S8). *perA* was detected in all 13 flying-fox tEPEC, whereas *efa1*, *iha*, *lpfA*, *nleB1*, *nleE* and *toxT* were absent from these isolates (Fig. 3 and Table S8).

Other virulence factors (*astA*, *cdtAB*, *vat*, *ibeA*, *fyuA/irp*, *malX*) showed variable distributions in flying-fox and human EPEC but were somewhat conserved within closely related clusters (Fig. 3). Of the 13 flying-fox tEPEC, *astA* was present ten, *ibeA* and *malX* were both present in two, and *cdtAB* and *vat* were present in one isolate each (Fig. 3). None of the 13 flying-fox tEPEC isolates carried shiga toxin *stx* genes or yersinabactin (*fyuA/irp*) (Fig. 3).

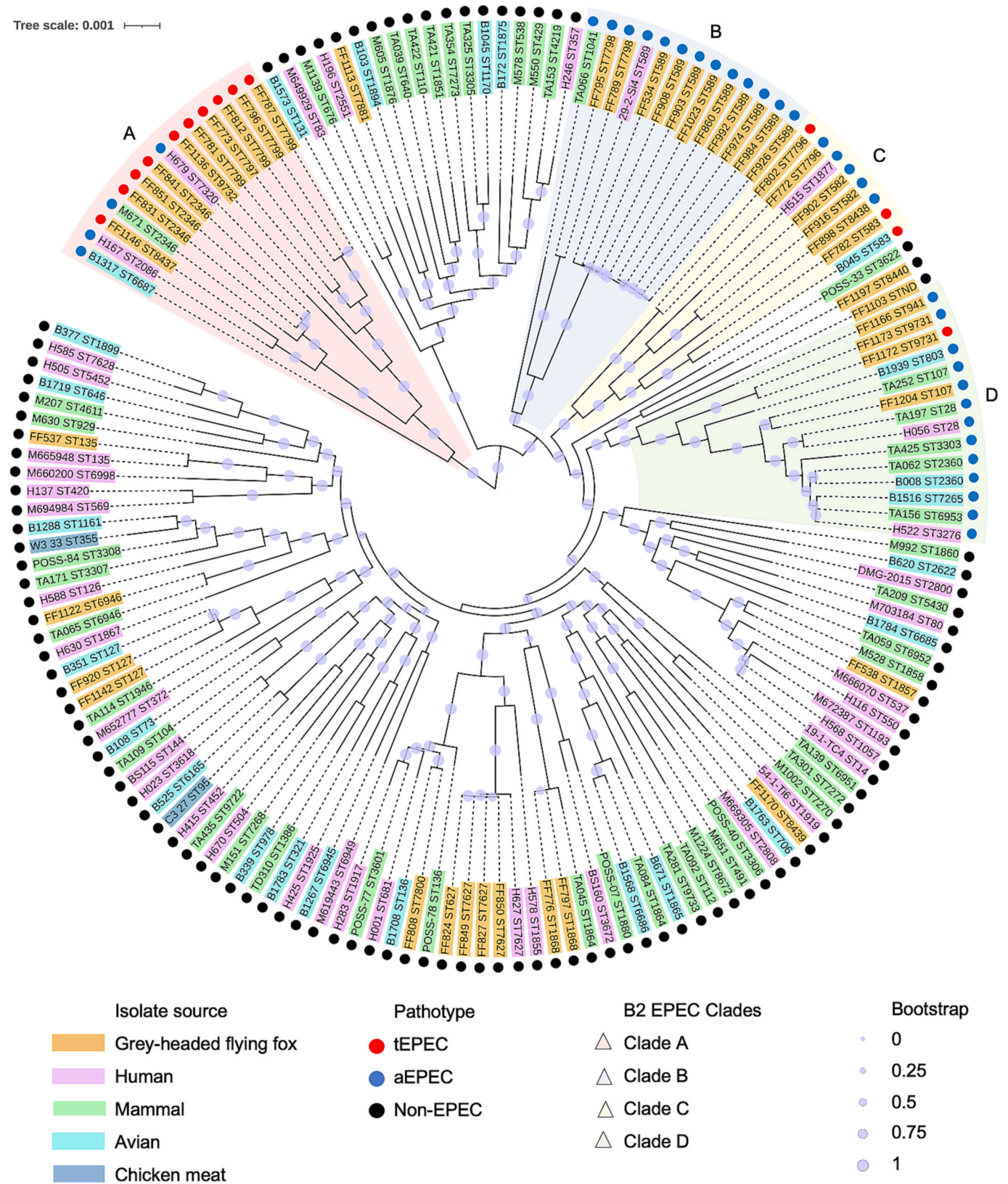


Fig. 2. Phylogenetic tree of 47 phylogroup B2 *E. coli* isolates from grey-headed flying-foxes and 109 phylogroup B2 strains isolated from humans and animals in Australia. Labels indicate isolate name and sequence type (ST). Pathotypes: tEPEC, typical enteropathogenic *E. coli*; aEPEC, atypical enteropathogenic *E. coli*. STND, sequence type not determined. Isolate source: 'Mammal' refers to mammals other than GHFF and humans. WGS data sourced by David Gordon. B2 isolate metadata is presented in Supplementary data 2.

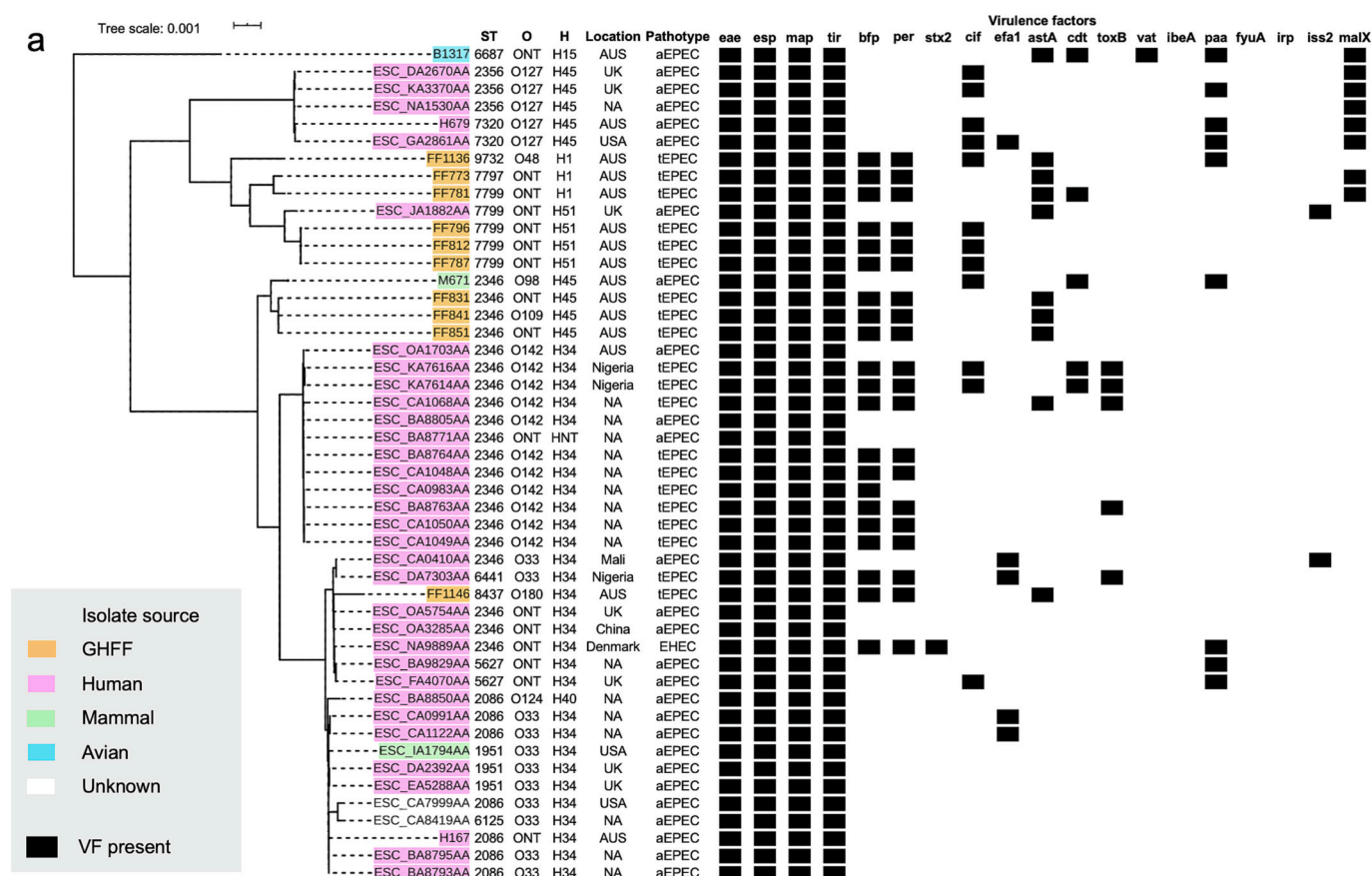


Fig. 3. Comparative phylogenetics of phylogroup B2 enteropathogenic *Escherichia coli* of Clades A, C and D isolated from grey-headed flying-foxes and other sources. Phylogenetic trees were constructed using Australian and closely related strains identified in EnteroBase and are presented with associated metadata and virulence factors) a. Phylogroup B2 Clade A. b. Phylogroup B2 Clade C. c. Phylogroup B2 Clade D. Metadata abbreviations: GHFF, grey-headed flying-fox; ST, sequence type; O, O-serogroup; H, H-antigen; NT, non-typable; esp., *espABDH*; bfp, *bfp* operon; per, *perA*; cdt, *cdtAB*; ibe, *ibeABC*; irp, *irp1* and *irp2*. Location abbreviations: AUS, Australia; NA, not available; NTL, Netherlands; UK, United Kingdom; USA, United States of America. Pathotype abbreviations: tEPEC, typical enteropathogenic *E. coli*; aEPEC, atypical enteropathogenic *E. coli*; EHEC, enterohaemorrhagic *E. coli*. Isolate source: 'Mammal' refers to mammals other than GHFF and humans. Clade trees isolate metadata is presented in Supplementary data 3.

3.5. *Eae* phylogenetic comparison of phylogroup B2 EPEC clades

Twelve *eae* subtypes were identified across the four phylogroup B2 EPEC clades, with each subtype largely conserved within clades and closely related clusters (Fig. S3). Nine of the 12 *eae* subtypes were detected in the 13 flying-fox tEPEC isolates, with the most frequent *eae* subtypes being $\epsilon 8$ ($n = 3$) and μ ($n = 3$) (Fig. 4). Of the 13 flying-fox tEPEC *eae* sequences, 11 showed between 99.65 % to 100 % identity to reference sequences (Table S9). One sequence showed 97.45 % identity while another sequence exhibited <94 % identity and was designated a novel *eae* subtype (Table S9). The chromosomal insertion site of *eae* and the associated LEE pathogenicity island was *trnA-Sec* (*selC*) for all 13 flying-fox tEPEC (Fig. 4).

3.6. *Bfp* phylogenetic comparison of flying-fox tEPEC

In contrast to the *eae* gene, *bfpA* allele type was not conserved within clades or clusters (Fig. 4). Twelve of 13 flying-fox *bfpA* allele sequences formed genetically distinct clusters (Fig. 4). Only two flying-fox *bfpA* alleles were close matches to human sourced alleles. FF802 showed 99.15 % identity with the *bfpA* $\beta 1$ reference allele (GenBank accession No. AF304471) and FF1172 was a 98.63 % match to a non-reference human *bfpA* variant designated $\beta 14$ (GenBank accession No. AB247933) (Table S10). All other flying-fox *bfpA* sequences ($n = 11$) were ≤ 94.84 % matches to reference *bfpA* alleles and considered to be

five novel *bfpA* variants, hereby designated FF-1 to FF-5 (Fig. 4). An identical novel *bfpA* variant (FF-1) was detected in four flying-fox tEPEC isolates from two different phylogenetic lineages that were collected at two flying-fox colonies located approximately 1200 Km apart (Fig. 4). Isolates FF781 and FF1136 shared an almost identical (99.49 %) novel *bfpA* variant (FF-4), that also belonged to different lineages and were collected at different sampling locations (Fig. 4). All three ST2346 flying-fox isolates shared the Novel-5 *bfpA* variant (Fig. 4).

3.7. Sequence variations in flying-fox *bfpA* alleles and *bfp* operons

Alignment of all flying-fox *bfpA* nucleotide sequences with seven of the most closely related human *bfpA* alleles identified 135 positions of nucleotide variation across the 20 *bfpA* sequences (Fig. 5a). The translated flying-fox *bfpA* amino acid sequences showed highly conserved regions, particularly in the first third (N-terminal) and highly variable regions in the last two-thirds (C-terminal) (Fig. 5b). These conserved and variable regions in flying-fox *bfpA* alleles correlated with amino acid polymorphisms in the human-associated *bfpA* reference alleles (Fig. 5b).

All 13 flying-fox tEPEC carried a full-length *bfp* operon and sequence comparison showed that *bfp* operon type correlated with *bfpA* allele type (Fig. 4). For flying-fox tEPEC with the same *bfpA* variant, little variation (1–5 SNPs) was observed in the full-length *bfp* operon sequences (Table S5). In BlastN searches, all full-length flying-fox *bfp* operon sequences showed 96.96 % to 97.78 % identity to *bfp* operons from human

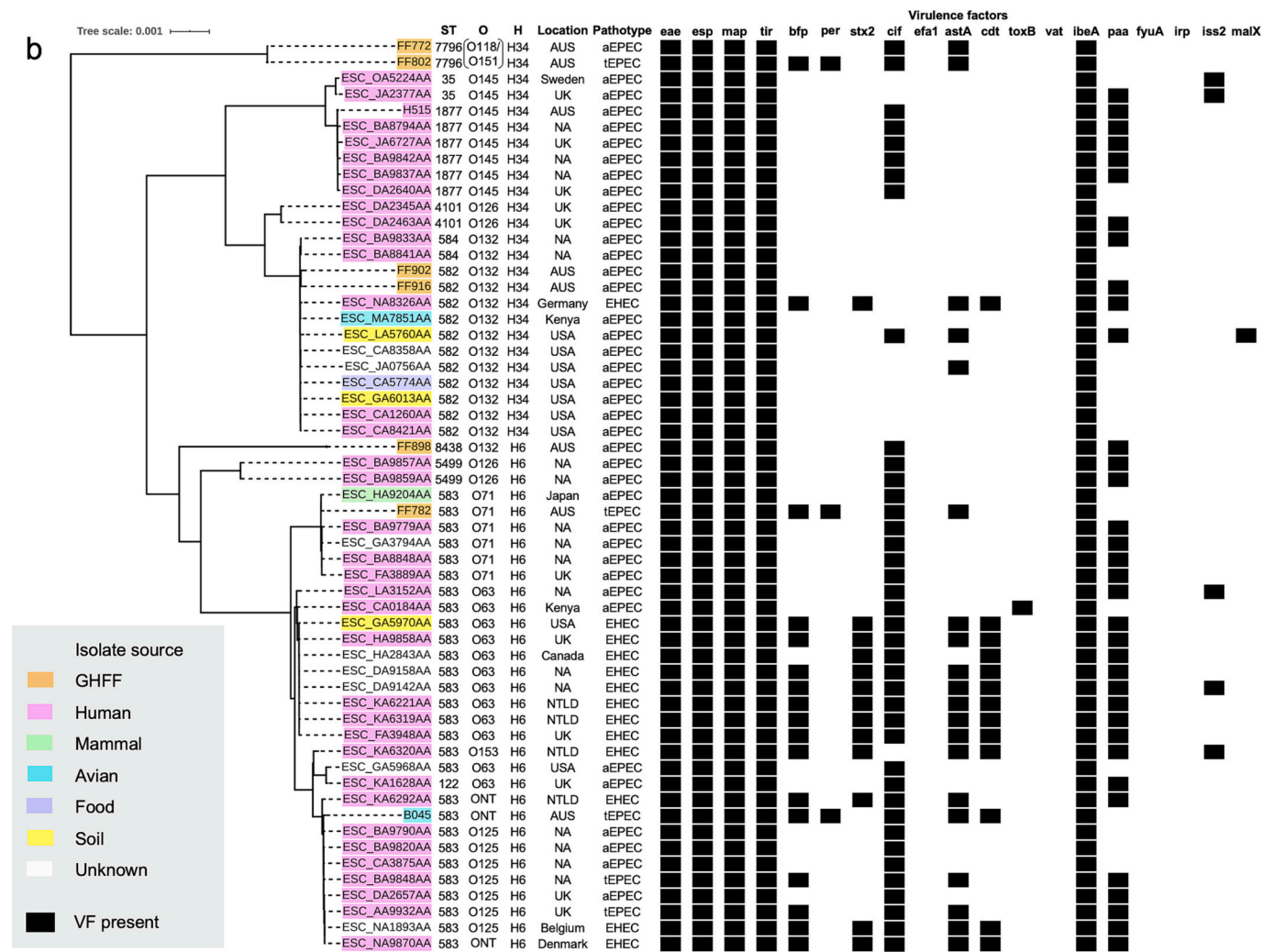


Fig. 3. (continued).

associated tEPEC isolates in GenBank (Table S5).

3.8. Plasmid replicons in flying-fox tEPEC

Plasmid replicons were identified in all 13 flying-fox tEPEC (mean 3.9, range 2–6), with all 13 tEPEC carrying at least one IncFII replicon, 11 of 13 tEPEC carried an IncFIB replicon and 9 of 13 carried at least one IncI replicon (Table S11). The two paired aEPEC isolates (FF772 and FF1173) belonging to the same ST and serotype as two tEPEC isolates (FF802 and FF1172 respectively), notably lacked two and three IncF replicons compared to their respective tEPEC pair (Table S11).

3.9. HEP-2 adhesion assays

All 13 flying-fox tEPEC and two flying-fox aEPEC isolates were investigated in vitro adhesion assays with HEP-2 cells. The two aEPEC isolates (FF772 and FF1173) were also examined because they shared almost identical genomes to two tEPEC isolates (five and one SNPs with FF802 and FF1172 respectively), but lacked the *bfp* operon (Fig. S1). All 13 flying-fox tEPEC isolates showed the characteristic localised adherence (LA) pattern associated with tEPEC after 3 h incubation with HEP-2 cells (Fig. 6a). Three isolates (FF773, FF812 and FF1136) showed very strong LA that did not correlate to a specific *bfpA* variant or tEPEC lineage (Fig. 6b). Both paired flying-fox aEPEC (FF772 and FF1173) that lacked the *bfp* operon, were non-adherent after 3 h incubation (Fig. 6c).

3.10. PCR determination of EPEC occurrence in all cultured *E. coli* isolates

PCR screening for the defining EPEC virulence factors, *eae* and *bfpA*, was performed with DNA from all 270 *E. coli* isolates cultured from 468 flying-fox faecal samples (Fig. 7c). EPEC-specific duplex PCR of *E. coli* isolates determined that tEPEC comprised 17.4 % (47 of 270, 95%CI: 13.4 %–22.4 %) *E. coli* isolates, with occurrence ranging from 0.0 % to 65.6 % across the five flying-fox colonies (Fig. 7c). Additionally, 11.1 % (30 of 270, 95%CI: 7.9 %–15.4 %) isolates were aEPEC, with occurrence ranging from 0.0 % to 25.0 % across the five colonies (Fig. 7c). Individual sample data for all *E. coli* isolates is provided in Supplementary data 4 and all prevalence data is tabulated in Table S12).

3.11. PCR determination of tEPEC virulence genes in flying-fox faecal DNA

Flying-fox faecal DNA samples ($n = 506$) from all five flying-fox colonies, were screened directly for the presence of tEPEC using an EPEC-specific multiplex PCR targeting the *eae* and *bfpA* virulence genes, and an internal control gene, *chuA* (Fig. 7d). Overall, 18.0 % (95%CI: 8 %–33.7 %) of all flying-fox faecal DNA samples were positive for tEPEC virulence genes (*eae*, *bfpA* and *chuA*) (Fig. 7d). For faecal samples obtained from individual flying-foxes in the ABP colony ($n = 95$), the highest occurrence of tEPEC positive samples was 87.0 % in February 2017 (40 of 46) and the lowest occurrence was 6.1 % in August 2017 (3

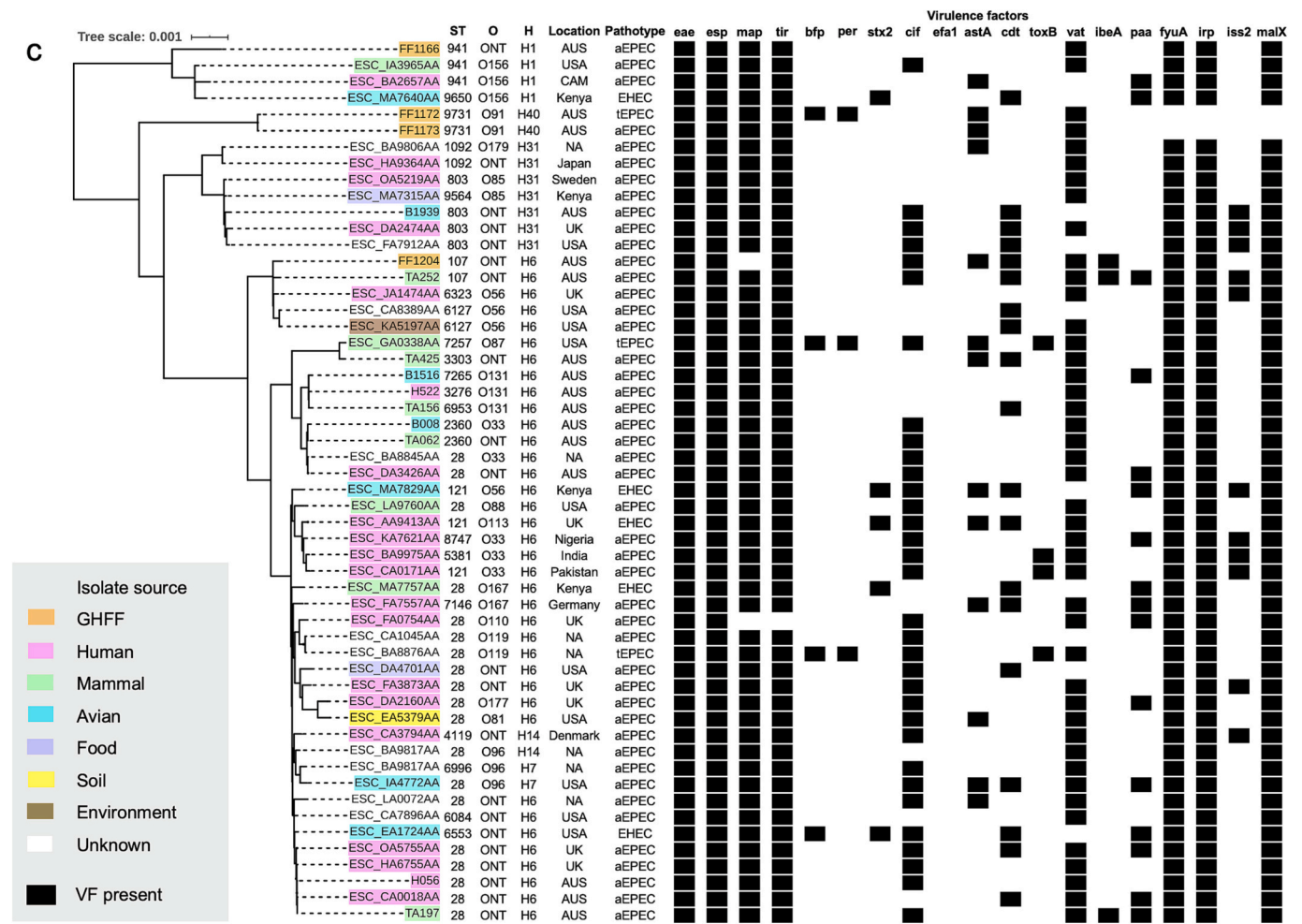


Fig. 3. (continued).

of 49) (Fig. 7d). For faecal samples obtained from under flying-fox roosts in NSW colonies ($n = 411$), the highest occurrence of tEPEC positive samples was 22.0 % from the CG colony in January 2018 (11 of 50) and the lowest occurrence was 1.3 % from the BP colony in December 2017 (1 of 75) (Fig. 7d). Individual sample data for all faecal DNA samples is provided in Supplementary data 5 and PCR data in Table S13.

4. Discussion

In this study, we found that wild grey-headed flying-foxes across eastern Australia carry diverse tEPEC lineages, including six novel sequence types and five novel *bfpA* allele variants. Finding these diverse and novel tEPEC lineages and *bfpA* variants was unexpected given that tEPEC have been almost exclusively associated with human hosts (Chandran and Mazumder, 2013; Nataro and Kaper, 1998). The inferred phylogeny of flying-fox associated tEPEC and human-associated EPEC indicates shared evolutionary lineages, with the largely conserved nature of *eae* subtypes within EPEC clusters, and a common *selC* LEE insertion site (Ingle et al., 2016) providing further support for shared lineages. Subtyping of *eae* alleles indicated that the 10 Clade A flying-fox tEPEC shared phylogenetic lineages with several human aEPEC lineages, but not the classical tEPEC O142:H34 (WHO, 1987) which also belongs to Clade A. The presence of multiple novel *bfpA* allele variants in Clade A flying-fox tEPEC suggests that they acquired *bfp* operons or EAF plasmids independently (Hazen et al., 2016), and then evolved as flying-fox specific lineages. Additionally, the presence of almost identical novel *bfp* operon variants in flying-fox tEPEC from different phylogenetic lineages

and different flying-fox colonies suggests that these *bfp* operons, and probably EAF plasmids, are circulating within flying-fox microbiomes. The two wild flying-fox colonies sharing novel *bfpA* allele and *bfp* operon variants in phylogenetically distinct tEPEC are located over 1200 Km apart and were sampled 13 months apart. Flying-foxes exhibit a high level of movement between colony sites and are considered to be one metapopulation (Welbergen et al., 2020). Over time, individual flying-foxes can move distances >1200 Km, but they will rarely travel 1200 Km directly between NSW and SA (Boardman et al., 2021; Tidemann and Nelson, 2004; Welbergen et al., 2020). The constant influx and outflux of individuals between colonies (Welbergen et al., 2020) establishes transmission pathways for tEPEC and *bfp* operons between flying-foxes in different colonies (inter-colony transmission), as previously documented with *Klebsiella pneumoniae* transmission between flying-fox colonies (Vezina et al., 2022). These findings indicate that these novel *bfpA* variants may be host specific to flying-foxes and have been circulating within the flying-fox metapopulation for a long time.

Phylogenetic analysis and *eae* subtyping of the flying-fox tEPEC isolates in Clades C and D also suggested that they share lineages with human associated aEPEC rather than tEPEC. Two of these flying-fox tEPEC belonged to divergent clusters void of human associated EPEC, but harboured *bfpA* alleles that were closely related to human *bfpA* variants. Interestingly, each of these divergent flying-fox clusters contained paired isolates with almost identical genomes, but one was aEPEC and the other tEPEC, indicating that either the tEPEC strains had recently acquired the *bfp* operon or EAF plasmid, or more likely that the aEPEC had lost the *bfp* operon (Hazen et al., 2016). Loss of pEAF by the

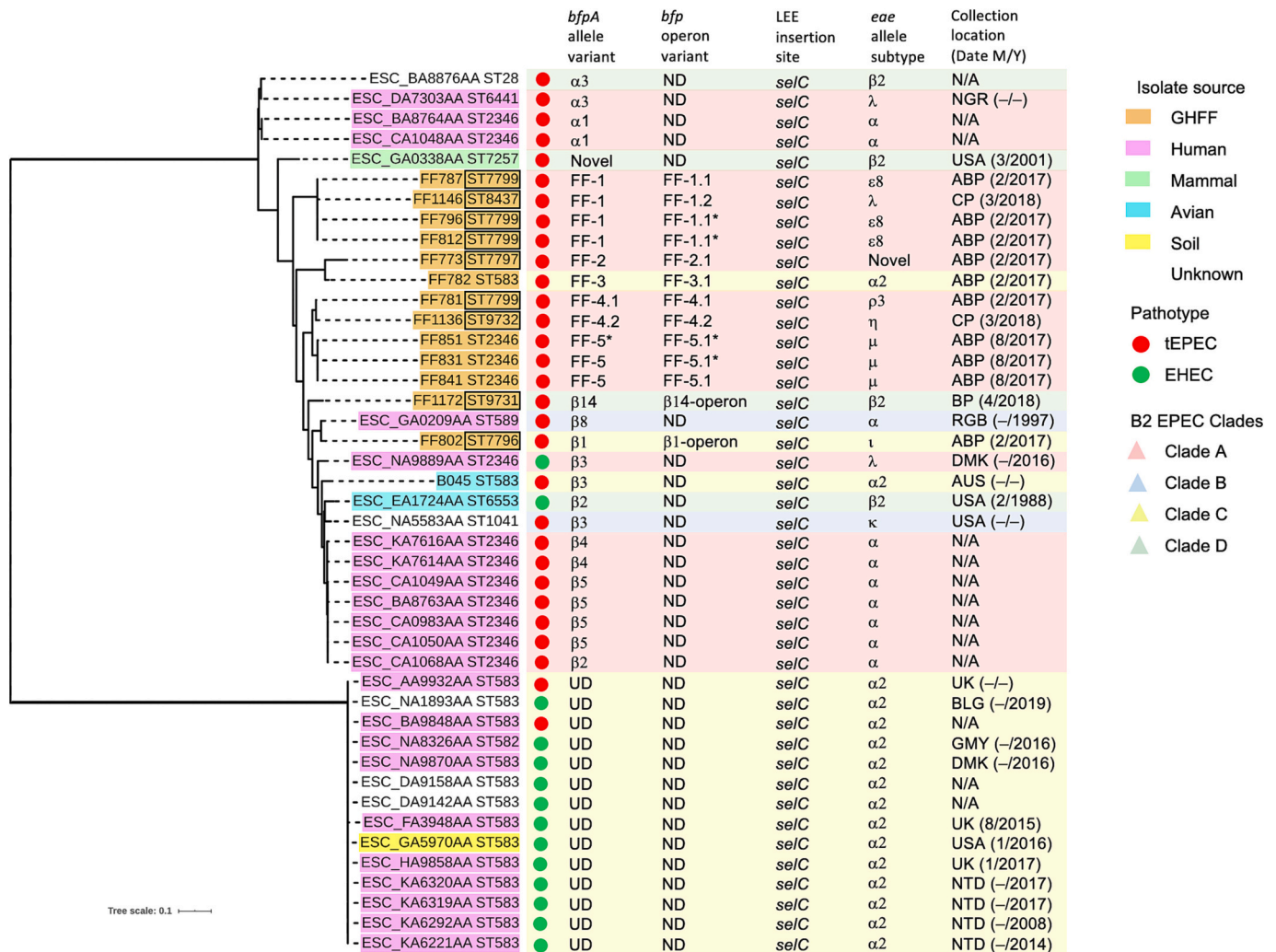


Fig. 4. Phylogeny of *bfpA* alleles from *Escherichia coli* in Clades A, B, C and D

The phylogenetic tree was constructed using *bfpA* sequences from tEPEC isolated from grey-headed flying-foxes (GHFF) and all *bfpA* positive isolates identified in clade trees. Isolate labels indicate name and sequence type (ST). Black boxes denote novel STs. Pathotype abbreviations: tEPEC, typical enteropathogenic *E. coli*; EHEC, enterohaemorrhagic *E. coli*. Columns indicate closest *bfpA* allele and *eae* subtype match to reference and GenBank sequences (provided in Tables S12 and S13). *bfpA* alleles: novel, *bfpA* allele sequence <94 % identity with reference and GenBank sequences; UD, undefined, *bfpA* allele sequence was a 100 % match to an EHEC associated *bfpA* allele (GenBank accessions CP039405, CP027551 and CP070230); ND, not determined. Asterisk (*) indicates partial sequences obtained: FF851 *bfpA*, 94.4 % coverage; FF796, FF812, FF831, FF851 *bfp* operons, 98.0 % to 98.5 % coverage. Isolate source: 'Mammal' refers to mammals other than GHFF and humans. Collection location: ABP, Adelaide Botanic Park flying-fox colony; BP, Blackalls Park flying-fox colony; CP, Centennial Park flying-fox colony; BLG, Belgium; DMK, Denmark; GMY, Germany; NGR, Nigeria; RGB, Republic of Guinea-Bissau; USA, United States of America. Date M/Y, Collection date month/year. N/A, Not available. Accession numbers for flying-fox *bfp* operon sequence variants are provided in Table S5.

prototypical tEPEC strain, E2348/69, during the course of infection in humans has been reported previously (Levine et al., 1985).

The conserved regions of the flying-fox BfpA proteins are consistent with conserved regions of human BfpA, which are predicted to be domains with critical roles in the structural integrity of Bfp (Blank et al., 2000). The variable regions in flying-fox BfpA proteins also correlate with amino acid polymorphisms observed in human BfpA and are predicted to present diverse surface regions to the host immune system (Blank et al., 2000). The characteristic LA to HEp-2 cells by all 13 flying-fox tEPEC, including those with novel *bfpA* variants, indicates that flying-fox tEPEC harbour functional *bfp* operons, as predicted by sequence analysis and presence of other key virulence factors. The absence of a *bfp* operon in the paired aEPEC isolates was correlated with their inability to adhere to HEp-2 cells in vitro, in contrast to the LA observed with their respective, closely related *bfp* positive tEPEC strains, further indicating that *bfp* operons are responsible for the characteristic LA. Although these novel tEPEC may be host specific to flying-foxes,

their adherence to HEp-2 cells in vitro suggests they may be able to adhere to human intestinal epithelial cells and should be considered potential zoonotic pathogens.

The high occurrence of tEPEC in *E. coli* isolates from flying-foxes (17.4 %) and consistent detection of tEPEC virulence genes in faecal DNA samples from five wild flying-fox colonies over a four-year period (18.0 %, 95%CI: 8 %–33.7 %) was unexpected given that tEPEC have been almost exclusively associated with human hosts (Chandran and Mazumder, 2013; Nataro and Kaper, 1998). Such high occurrences of tEPEC have not been previously reported for any wildlife or domestic animal species, with sporadic reported occurrences ranging from 1.1 % to 8.0 %, including; dogs 1.1 % to 2.1 % (Chandran and Mazumder, 2013; Nakazato et al., 2004), lambs 0.9 % to 1.9 % (Wani et al., 2009), cattle 2.1 % (Blanco et al., 2005), captive and wild birds in Brazil 1.2 % to 6.5 % (Borges et al., 2017; Gioia-Di Chiacchio et al., 2018; Saidenberg et al., 2012; Sanches et al., 2017), wild boar 1.1 % (Alonso et al., 2017), wild coyotes 5.8 % (Chandran and Mazumder, 2013) and fruit bats in

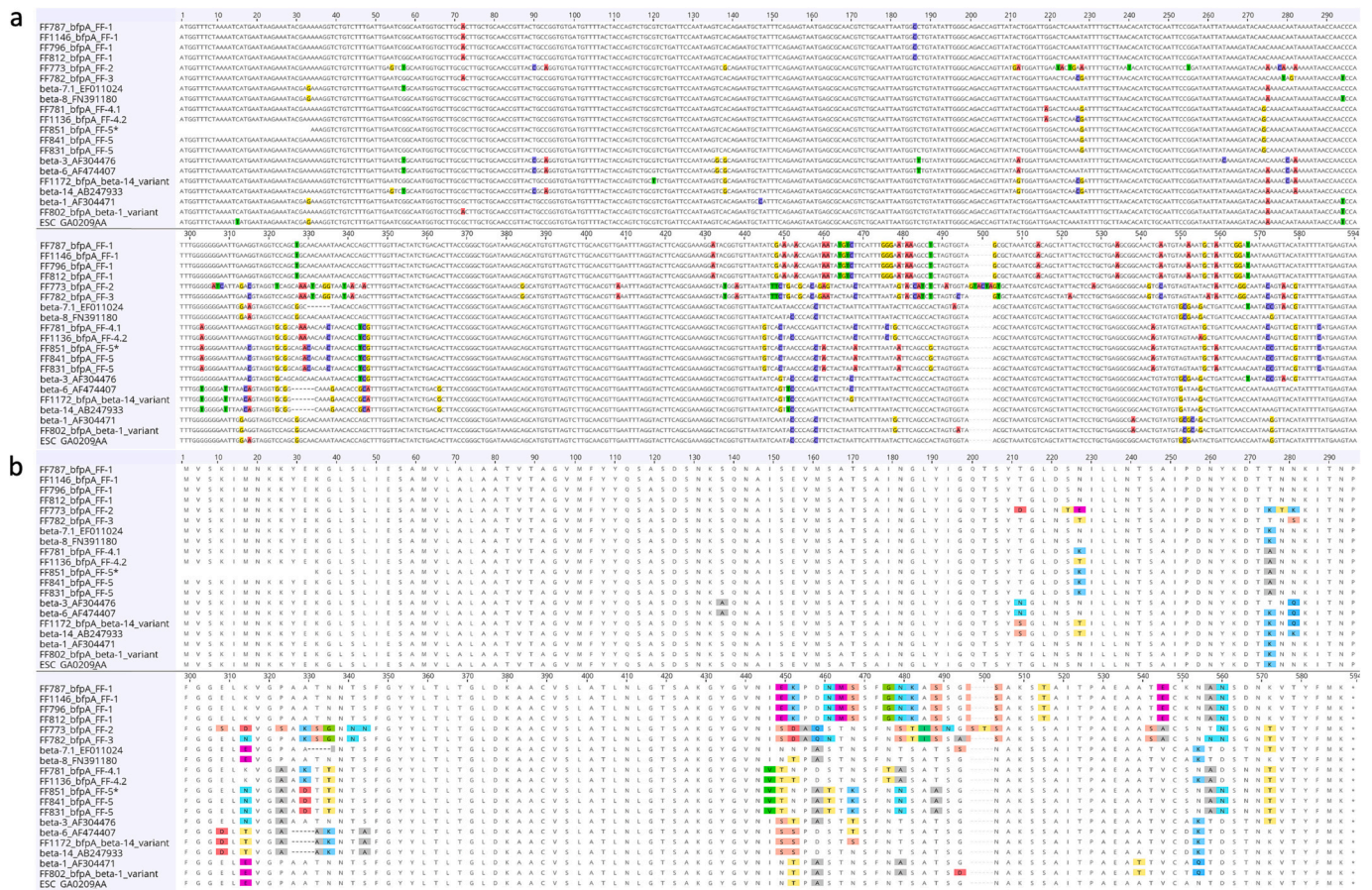


Fig. 5. Comparison of grey-headed flying-fox *bfpA* nucleotide and translated amino acid sequences with reference alleles. Alignment of all grey-headed flying-fox *bfpA* sequences (denoted by names commencing with 'FF') with the closest reference *bfpA* alleles (beta-1, beta-3, beta-4, beta-7.1) and close non-reference sequence matches identified in the *bfpA* phylogenetic trees (ESC_GA0209AA) and GenBank BLASTn searches (AB247933 and FN391180). Asterisk (*) indicates a partial sequence was obtained for FF851 *bfpA* allele (94.4 % coverage). **a.** Nucleotide sequence alignment. **b.** Translated amino acid sequence alignment. Variations from the consensus sequence are highlighted and colour coded according to nucleotide or amino acid type.

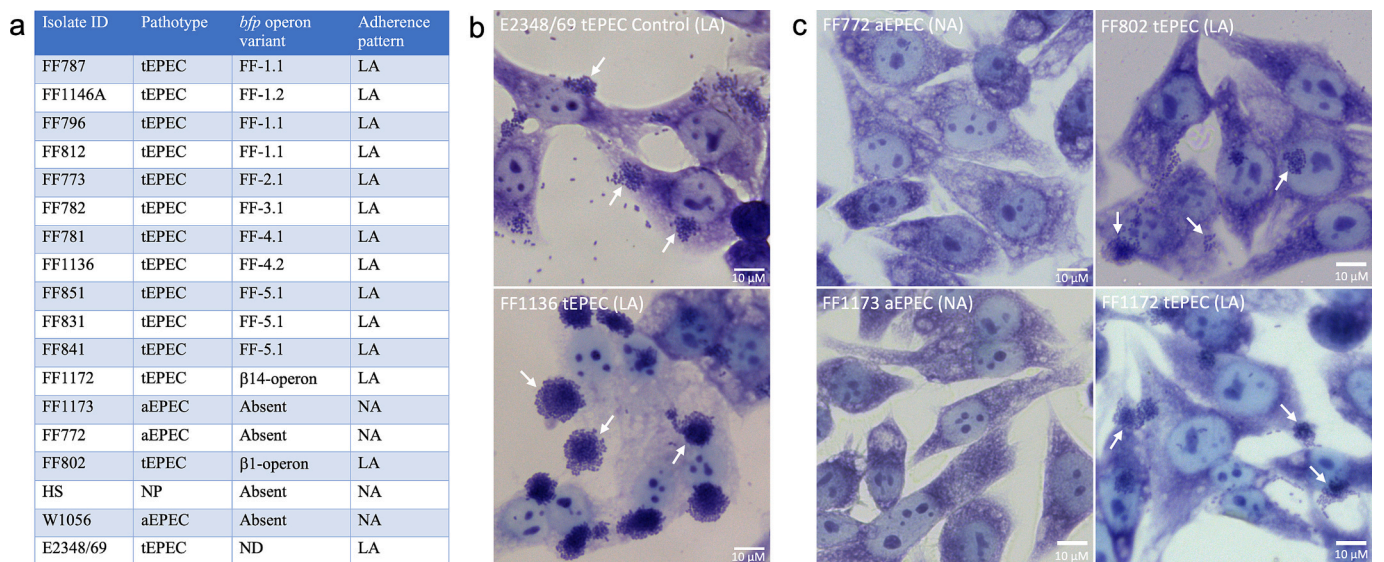


Fig. 6. Patterns of adherence of flying-fox EPEC to Hep-2 cells. **a.** Adherence patterns of flying-fox-associated tEPEC, paired aEPEC and control *E. coli* strains to Hep-2 cells by after co-incubation for 3 h. Hep-2 adherence pattern: LA, localised adherence; NA, non-adherent. **b.** Light microscopic images showing LA patterns for the prototypical tEPEC control strain E2348/69 and strong LA by tEPEC FF1136. **c.** Adherence patterns of the paired aEPEC/tEPEC isolates FF772/FF802 and FF1173/FF1172. Arrows indicate localised adherence pattern.

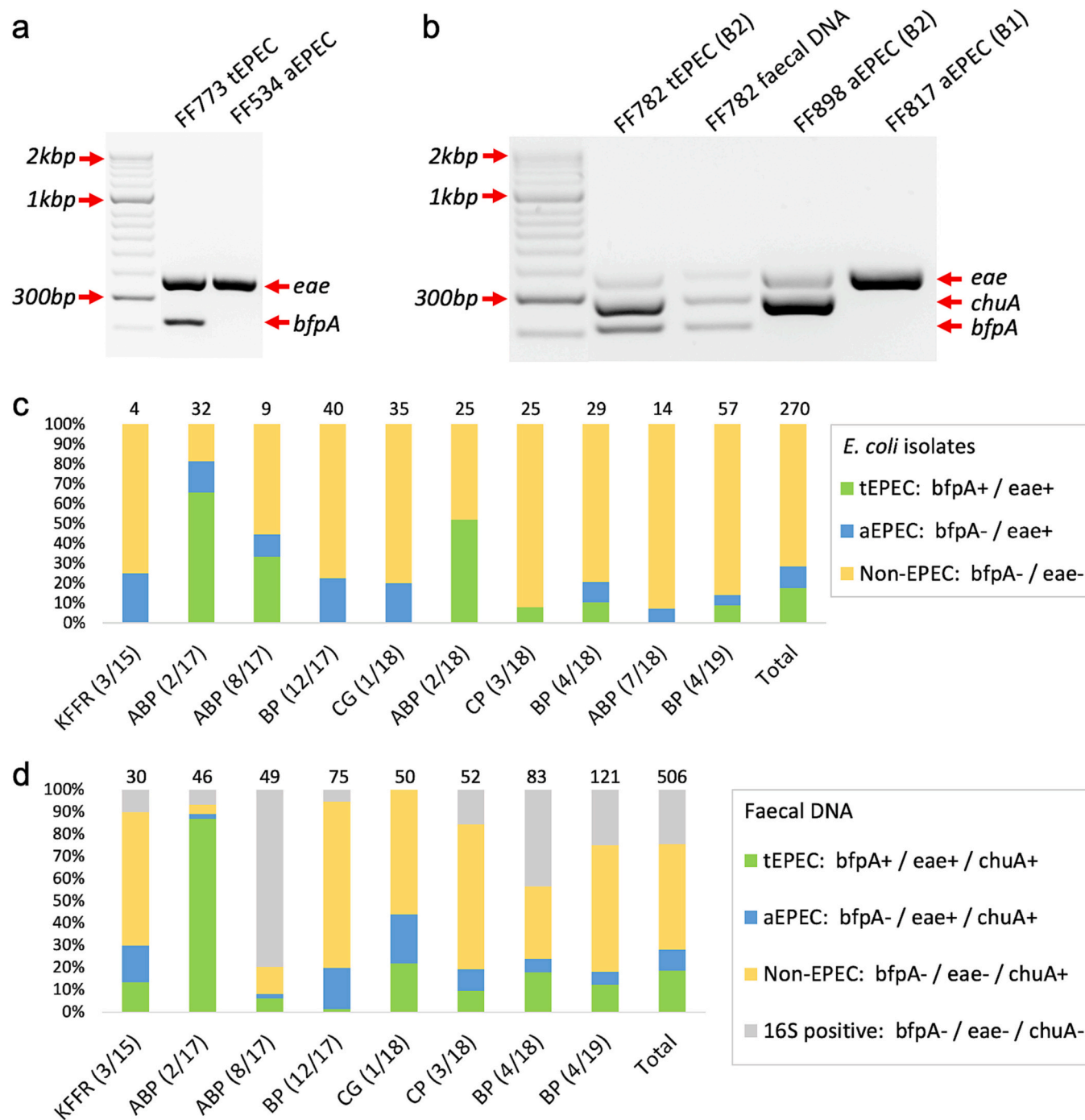


Fig. 7. Prevalence of enteropathogenic *Escherichia coli* (EPEC) in wild grey-headed flying-foxes in five colonies. a. Representative image of an EPEC PCR gel targeting *eae* and *bfpA* with isolate DNA. b. Representative image of an EPEC PCR gel targeting *eae*, *bfpA* and *chuA* with faecal DNA. B1, phylogroup B1; B2, phylogroup B2. c. Occurrence of typical (tEPEC) and atypical aEPEC in *E. coli* isolates from grey-headed flying-foxes. The number of *E. coli* isolates tested is shown above the bars. Sampling dates (month/year) are indicated underneath the locations. KFFR, Ku-ring-gai Flying-Fox Reserve; ABP, Adelaide Botanic Park; BP, Blackalls Park; CG, Camellia Gardens; CP, Centennial Park. Individual sample data is provided in Supplementary data 4. d. Occurrence of typical (tEPEC) virulence genes in faecal DNA from grey-headed flying-foxes. The number of faecal DNA samples tested is shown above the bars. Sampling dates (month/year) are indicated underneath the locations. KFFR, Ku-ring-gai Flying-Fox Reserve; ABP, Adelaide Botanic Park; BP, Blackalls Park; CG, Camellia Gardens; CP, Centennial Park. Individual sample data is provided in Supplementary data 5.

the Republic of Congo 8.0 % (Nowak et al., 2017). In studies which determined the serotype and/or sequence type of tEPEC from other animal species, the vast majority were tEPEC strain types associated with human infection, indicating zoonanthroponotic transmission to non-human hosts (Alonso et al., 2017; Borges et al., 2017; Carvalho et al., 2007; Carvalho et al., 2003; Gioia-Di Chiacchio et al., 2018; Nakazato

et al., 2004). A notable exception is the detection of tEPEC with novel sequence types ($n = 3$) in two fruit bat species (*Myonycteris torquata* and *Epomops franqueti*) in the Republic of Congo (Nowak et al., 2017). The identification of six novel tEPEC sequence types and five novel *bfpA* alleles in grey-headed flying-foxes, combined with the consistent detection of tEPEC over a four-year period and across a large

geographical distance (>1200 Km), indicates that tEPEC carriage is not the result of zoonanthroponotic transmission, but that grey-headed flying-foxes are natural hosts of flying-fox-specific tEPEC lineages.

Flying-foxes have a short intestinal tract relative to body length, a simple hindgut with no microbial fermentation chamber and a rapid gastrointestinal transit time of 18–32 min (Tedman and Hall, 1985). A study of Australian mammals suggested that in the absence of a modified hindgut, *E. coli* can only establish intestinal populations in large bodied species (Gordon and Cowling, 2003). Additionally, *E. coli* densities in the intestinal microbiome decrease as the gastrointestinal transit time of ingested food increases (O'Brien and Gordon, 2011). Laboratory models using bio-reactors also indicate that bacterial populations are less likely to establish in the gut when there is a fast transit time (Ballyk and Smith, 1999; Ballyk et al., 2001; Jones et al., 2002). However, bacterial strains which can adhere to the intestinal mucosa are more likely to establish, regardless of gut length or transit time (Ballyk and Smith, 1999; Ballyk et al., 2001; Jones et al., 2002). Thus, the presence of adhesion factors, such as Bfp in tEPEC, may allow these bacteria to persist in the flying-fox intestinal tract, despite the rapid gastrointestinal transit time and simple hindgut (Nowak et al., 2017). In addition, the attaching and effacing capacity of all varieties of EPEC may allow them to remain in the intestinal tract for extended periods, giving EPEC an evolutionary advantage over other enteric bacteria.

Carriage of aEPEC in flying-foxes was not an unexpected finding, as diverse aEPEC have previously been associated with diverse mammalian wildlife species (Alonso et al., 2017; Bardiau et al., 2010a; Chandran and Mazumder, 2013; Mora et al., 2018; Xu et al., 2017) including bats (Cabal et al., 2015; Iovine et al., 2015; Nowak et al., 2017). The flying-fox sourced aEPEC isolates reported here typically shared STs, serotypes and/or phylogenetic clusters with human associated aEPEC and were detected in four wild flying-fox colonies geographically located up to 1200 Km apart over a four-year period. These findings suggest widespread acquisition of diverse human-associated aEPEC by flying-foxes, indicating they may also be reservoirs for potentially zoonotic aEPEC.

The occurrence and distribution of tEPEC and aEPEC varied considerably in colonies from different locations and at different sampling timepoints, which may relate to variables including exposure levels to sources of EPEC in the environment (Sidhu et al., 2013; von Holy et al., 2006), horizontal transmission rates between individuals within a colony (Mühldorfer, 2013), climate (Gordon and FitzGibbon, 1999) or diet (Gordon and Cowling, 2003).

We do not know if EPEC are pathogenic for flying-foxes but the high occurrence of tEPEC observed in apparently healthy adults sampled in the ABP colony suggests that this is not the case. A study of EPEC in flying-fox pups may be warranted given that EPEC are pathogenic for human infants but infected adults are typically asymptomatic (Nataro and Kaper, 1998).

5. Conclusions

Strains of tEPEC obtained from grey-headed flying-foxes do not belong to the classical O:H serotypes associated with human infections (Lacher et al., 2007), but instead predominantly belong to novel tEPEC lineages and/or carry novel *bfpA* alleles, suggesting that flying-fox tEPEC have evolved in a host-specific manner. HEP-2 adhesion assays indicated that these novel flying-fox tEPEC isolates harbour functional *bfp* operons. This study is the first to report the consistent detection of tEPEC in a non-human host, over extensive geographical and temporal scales. The high occurrences of tEPEC in large flying-fox colonies located in urban environments has the potential for significant zoonotic and public health risks. Future studies are warranted to examine the potential infectivity of flying-fox tEPEC for humans, mechanisms of adherence, and the microbiological role and dynamics of tEPEC in grey-headed flying-foxes and other fruit bat species.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.166336>.

CRedit authorship contribution statement

Fiona McDougall: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. David Gordon: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data Curation, Writing - Review & Editing. Roy Robins-Browne: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Review & Editing, Visualization. Vicki Bennett-Wood: Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Review & Editing, Visualization. Wayne Boardman: Investigation, Resources, Data Curation, Writing - Review & Editing. Petra Graham: Statistical analysis, Writing - Review & Editing. Michelle Power: Conceptualization, Methodology, Investigation, Resources, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Assembled sequences for all flying-fox *E. coli* isolates are available in Enterobase (Supplementary data 1). Raw sequence reads for all flying-fox *E. coli* isolates were uploaded to NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA613581 (EPEC isolates) and BioProject ID PRJNA857315 (Non-EPEC isolates). Enterobase and BioSample accession numbers for all flying-fox EPEC isolates and associated metadata are provided in a supplementary excel file (Supplementary data 1). Annotated sequences for novel flying-fox *bfp* operon variants have been uploaded to GenBank (accession numbers are listed in Table S5).

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