Fatal Exudative Dermatitis (FED) in Island Populations of Red Squirrels (Sciurus vulgaris): Spillover of a Virulent Staphylococcus aureus Clone (ST49) From Reservoir Hosts.

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Fatal exudative dermatitis in island populations of red squirrels (Sciurus vulgaris): spillover of a virulent Staphylococcus aureus clone (ST49) from reservoir hosts

Kay Fountain1,*, Tiffany Blackett2, Helen Butler3, Catherine Carchedi4, Anna-Katarina Schilling5, Anna Meredith5,6, Marjorie J. Gibbon1, David H. Lloyd4, Anette Loeffler4 and Edward J. Feil1

Abstract
Fatal exudative dermatitis (FED) is a significant cause of death of red squirrels (Sciurus vulgaris) on the island of Jersey in the Channel Islands where it is associated with a virulent clone of Staphylococcus aureus, ST49. S. aureus ST49 has been found in other hosts such as small mammals, pigs and humans, but the dynamics of carriage and disease of this clone, or any other lineage in red squirrels, is currently unknown. We used whole-genome sequencing to characterize 228 isolates from healthy red squirrels on Jersey, the Isle of Arran (Scotland) and Brownsea Island (England), from red squirrels showing signs of FED on Jersey and the Isle of Wight (England) and a small number of isolates from other hosts. S. aureus was frequently carried by red squirrels on the Isle of Arran with strains typically associated with small ruminants predominating. For the Brownsea carriage, S. aureus was less frequent and involved strains associated with birds, small ruminants and humans, while for the Jersey carriage S. aureus was rare but ST49 predominated in diseased squirrels. By combining our data with publicly available sequences, we show that the S. aureus carriage in red squirrels largely reflects frequent but facile acquisitions of strains carried by other hosts sharing their habitat (‘spillover’), possibly including, in the case of ST188, humans. Genome-wide association analysis of the ruminant lineage ST133 revealed variants in a small number of mostly bacterial-cell-membrane-associated genes that were statistically associated with squirrel isolates from the Isle of Arran, raising the possibility of specific adaptation to red squirrels in this lineage. In contrast there is little evidence that ST49 is a common carriage isolate of red squirrels and infection from reservoir hosts such as bank voles or rats, is likely to be driving the emergence of FED in red squirrels.

DATA SUMMARY
All genomes have been deposited with GenBank under Bioproject PRJNA679188 (accession numbers are given in Table S1, available in the online version of this article). Closed genome accession numbers: CP065353.1, CP065354.1 and CP065355.1.

INTRODUCTION
Red squirrels (Sciurus vulgaris) have been extirpated from most of the UK, remaining only in pockets of the north of England,
Impact Statement

Populations of endangered wildlife are threatened by many pressures such as loss of habitat, hunting and climate change. However, in some cases, such as Chytrid disease in frogs, or poxvirus disease in red squirrels, disease can be a significant cause of population decline, leading to local loss of a species or even global extinction. The investigation of wildlife disease is often given a low priority unless it directly affects human wellbeing. This study is an important contribution to our understanding of a disease that affects a population of threatened small mammals, and to our knowledge of how a common bacterium is transmitted amongst farmed animals, humans and wildlife. Red squirrels on the islands of Jersey (Channel Islands) and the Isle of Wight have been affected by a fatal skin disease associated with a particular clone of the bacterium, *Staphylococcus aureus*. In this study we sought to establish where squirrels carry this clone, and other *S. aureus* strains, and how these findings correlate with the incidence of the disease. Our data confirm a strong association between the clone and disease, and suggest that ST49 is rarely carried by healthy squirrels, pointing to a high level of virulence. In contrast, other island squirrel populations where the disease is not so common tend to acquire other *S. aureus* strains from co-residing host species.

METHODS

Sampling and isolation of staphylococci

A total of 228 red squirrels was sampled on the Isle of Arran, Brownsea Island, the IOW and Jersey (Channel Islands), between 2016 and 2019 as part of three projects, all approved by their relevant ethics oversight boards and licensing authorities [4, 15, 16]:

1. Squirrels trapped and anaesthetized for a separate leprosy study were swabbed opportunistically on the Isle of Arran (UK) (n=44) and Brownsea Island (n=44) [15].
2. On the IOW, three sick squirrels were sampled before treatment as part of the Wight Squirrel Project.
3. Sick, injured, orphaned and casualty red squirrels on Jersey (n=137) were sampled as part of the JSPCA squirrel surveillance project [4, 16].
4. For comparison purposes, on the Isle of Arran 20 domestic sheep from a flock grazing in the squirrel-trapping site, plus ten freshly culled wild red deer (*Cervus elaphus*) were also sampled.
For each squirrel, oral mucosae (where possible the oropharynx), the clean skin of the axilla or inguinal region and skin lesions were sampled using separate cotton tipped swabs. Swabs were rolled over each site for 2–5 s and placed into charcoal Amies medium (Technical Services Consultants, Heywood, UK) for transport to the laboratory.

Within 24 h of receipt, swabs were aseptically cut into tryptone soya broth (TSB) containing 10% salt (TSB+) (Oxoid, Thermo Fisher Scientific, Basingstoke, Hampshire, UK, and Sigma-Aldrich, Gillingham, Dorset, UK) and incubated at 37 °C for 24 h before streaking onto Columbia 5% sheep blood agar (Oxoid) and further incubation at 37 °C for 24 h. Presumptive S. aureus isolates were identified on the basis of colony morphology, haemolysis, and production of DNase (Oxoid) and up to five colonies were picked from different areas of the plate for each swab, yielding up to 20 isolates from a single animal. Isolates were stored at −20 °C and subcultures of 228 S. aureus isolates were dispatched on beads (Microbank, Prolab Diagnostics, Cheshire, UK) to MicrobesNG (Birmingham, UK) for sequencing.

**Sequencing**

Isolates (n=228) were sequenced on an Illumina platform by MicrobesNG (Birmingham) as follows:

Three isolates were also sequenced using long-read sequencing using Oxford Nanopore Technologies (ONT): for each squirrel, oral mucosae (where possible the oropharynx), the clean skin of the axilla or inguinal region and skin lesions were sampled using separate cotton tipped swabs. Swabs were rolled over each site for 2–5 s and placed into charcoal Amies medium (Technical Services Consultants, Heywood, UK) for transport to the laboratory.

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- Approximately 2×10^6 cells were used for high molecular weight DNA extraction using Nanobind CCB Big DNA Kit (Circulomics, Maryland, USA). DNA was quantified with the Qubit dsDNA HS assay in an Eppendorf AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol.

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**Bioinformatics**

Quality control, assembly and annotation of reads was undertaken using an in-house pipeline as follows: raw reads were trimmed using Trimmomatic [19], and quality confirmed with Fastqc [20]. Reads were assembled using Spades [21], and annotated with Prokka [22]. Hybrid assemblies of Illumina and Nanopore reads were built using Unicycler [23], and the assembly graphs visualized using Bandage [24]. MLSTs were determined using the software tool ‘MLST’ (profiles in Table S3) [17, 25]. Variants were called using Snippy against closed genome assemblies from this study as references and Snippy-Core used to generate full genome alignments [26]. SNP-dist was used to produce a distance matrix from an alignment [27].

Pangenes were built using Roary [28], and phylogenetic trees constructed from the core gene alignments using RAXML-NG [29]. Phylogenetic tree illustrations were produced in iTOL v5.7 [30]. Genomes were visually examined and manipulated using Artemis [31], further alignments were constructed using Clustal Omega [32], and viewed using Jalview [33]. BRIG was used to generate circular comparisons of genomes and Easyfig was used to create BLAST comparisons of phase genomes [34, 35].

Abricate was used to search for antimicrobial resistance and virulence genes, and plasmid replicons using the incorporated databases ARG-ANNOT, CARD, Resfinder, VFDB and PlasmidFinder [36–41]. A custom database was created from the Phaster [42] website database and BLAST was used to search for intact phages [43]. Potential plasmid sequences were confirmed using plasmidSPAdes and investigated using Mob-recon from Mob-suite to classify them against the database of known plasmids [44, 45].

A genome-wide association study was run using pySEER [46], using the phenotypes of ‘squirrel/not squirrel’ on a single MLST sequence type (ST133) to reduce the problem of lineage-related effects. A linear mixed model was used to generate a logistic model of GATK [47] and a phylogeny incorporated to control for population structure.

**RESULTS**

All 88 squirrels sampled on the Isle of Arran and Brownsea Island were deemed healthy on inspection; of the 137 squirrels sampled on Jersey, 122 were free of clinical signs of FED on inspection (classified as ‘healthy’), 14 had clinical signs or post-mortem evidence consistent with FED and one had other, non-FED-like skin lesions. The three diseased squirrels from IOW had skin lesions consistent with FED (Table 1). On Jersey, this is likely to underestimate the true carriage rate because posting delays might have resulted in false negative samples; the average time from sampling on Jersey to laboratory was 5 days with a
maximum of 41 days. *S. aureus* was also isolated from 13/20 (65%) sheep and from 8/10 deer (80%) (Table 1).

### Characterization of the *S. aureus* isolates

#### Genomes

A total of 228 genomes was sequenced using Illumina sequencing and genomes were assembled with a mean of 63 contigs (Table S1). Assemblies with N50 lower than 10,000 \( (n=4) \) were removed from core gene alignments constructed from the pangenome as the poor assemblies contained many apparent gene alleles, which distorted the branch lengths in the phylogenies.

In order to provide closed, accurate, closely related assemblies for mapping, and to enable examination of the context of mobile genetic elements, two red squirrel isolates from Jersey (ST49) and one from the Isle of Arran (ST133) were additionally sequenced on the Oxford Nanopore platform. Two ST49 isolates were chosen because they had different bacteriophage content, including the presence or absence of the phage carrying *lukM/F* genes (henceforth referred to as ‘the lukM phage’). Using a combination of long- and short-read data, complete assemblies consisting of a single closed chromosome with no plasmids were generated for all three isolates (Table S1). Genome data is publicly available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA679188

#### *S. aureus* lineages recovered

The *S. aureus* lineages recovered are reported in Table 1. Of the 44 healthy red squirrels from the Isle of Arran that were sampled, *S. aureus* was isolated from 14 of them. Three lineages of *S. aureus* were observed; seven individuals carried ST133, six individuals carried ST2328 and a single individual carried ST49. Healthy red squirrels on Brownsea Island (11/44) were found to carry four different lineages; four individuals carried ST692, four individuals carried ST133, two individuals carried ST188 and a single individual carried ST130.

Healthy sheep \( (n=13/20) \) from a single flock on the Isle of Arran, which grazed two sites close to the red squirrel sampling site carried ST1640, ST130 and ST8. Healthy free-ranging red deer from Arran \( (n=8/10) \) carried ST3237, 1640, 130, 1958 and 425. The deer were frequently (4/8) coinfected with more than one sequence type.

On Jersey, 14 squirrels, which had clinical signs or post-mortem evidence consistent with FED were found to carry ST49, and one of these was co-infected with ST2328. One squirrel, which had skin lesions not confirmed to be FED, also carried ST3237. A single healthy squirrel carried ST130.

On the IOW, of three red squirrels with skin lesions, one carried ST49 and one carried the single-locus variant of ST49,
ST1957, which was also reported by Simpson et al. (2013) on this island [6].

The lineages recovered from red squirrels have previously been recorded in other host species: ST133 and 2328 (ruminants), ST130 (small ruminants and small mammals), ST692 (avian), ST188 (livestock and human), ST49 (small mammals and pigs), ST1957 (red squirrels).

**Phylogenomic analysis suggests multiple host switch or spillover events**

Genome sequences corresponding to sequence types ST188, ST133 and ST49 were compared with publicly available
genomes for these lineages by constructing phylogenies using core gene alignments generated by pangenome analysis using Roary (Figs 1–3). The remaining sequence types did not have enough publicly available genomes to construct useful phylogenies.

On the ST188 tree, five ST188 strains isolated from two squirrels on Brownsea Island were resolved into two distinct sub-clusters corresponding to the individual squirrels from which they were isolated. They are positioned far apart on the tree (Fig. 1), suggesting independent introductions into the squirrel population. Although neither cluster is closely related to any of the publicly available genomes for this lineage, it is possible to infer that these isolates may have been acquired from humans based on phage content. All five squirrel isolates carried the β-haemolysin converting phage (Saphi3), which harbours genes associated with evasion of the human immune system such as sak, chp and scn, commonly termed the immune evasion complex (IEC). This phage has been reported to be more common in isolates recovered from human sources and is often absent from those recovered from animal sources [47]. Additionally, isolates from the two squirrels have a different combination of IEC genes (sak,
chp and scn, or sak and scn), which again is consistent with independent acquisitions from human sources.

From all geographic locations, 19 out of 26 healthy squirrels (73%) that carried S. aureus had strains that are typically associated with carriage by sheep or other small ruminants (ST133, 130 and 2328) [48].

From a phylogeny of a core-gene alignment of ST133 isolates from red squirrels and public databases we infer that squirrels on the Isle of Arran and Brownsea Island acquired ST133 independently, and that following acquisition there has been onward squirrel-to-squirrel transmission within each population (Fig. 2). However, while ST133 is a well-sampled lineage,

**Table 2.** Presence or absence of selected mobile genetic elements in the ST133 reference strain genome ED133 from a sheep, and ST133 and ST2328 isolates from red squirrels

|                  | Ovine-related phages | Hlb-converting phage | Ovine pathogenicity islands | Ovine-related virulence factors |
|------------------|----------------------|----------------------|-----------------------------|-------------------------------|
| ED133            | Phi SaOv1, 2, 3 (lukM) | No                   | SaovPI1, 2                  | sec, sell, tsst               |
| ST133 squirrel   | No                   | Yes                  | No                          | No                            |
| ST2328 squirrel  | No                   | Yes                  | SaovPI1, 2                  | sec, sell, tsst               |
| ST2328 squirrel  | No                   | No                   | No                          | No                            |

**Fig. 3.** Phylogeny of all publicly available ST49 isolates and isolates from red squirrels in this study constructed using a core-gene alignment from Roary. Leaf label text colours (host): dark blue=red squirrels, pink=bank vole, turquoise=human, green=pig, black=unknown. Background leaf labels (Island): red=Jersey, blue=IOW, green=Isle of Arran. Available on Microreact: https://microreact.org/project/8ZbXHY4VrD7vId7wXniQ.
we cannot completely rule out multiple transmissions from unsampled hosts, which do not appear in our phylogeny. For most of the publicly available genomes there is no information as to the host from which they were isolated, which limits the power of this analysis to identify possible reservoir hosts. To address this, we mapped those genomes for which host metadata was available against our closed ST133 assembly (from the Isle of Arran) and reconstructed a phylogeny based on genome-wide SNPs (Fig. S1). However, there is no clear correlation between phylogeny and host species, and we cannot infer the source of the isolates carried by red squirrels. However, this analysis does confirm that there are three distinct sub-clusters and greater overall diversity in the isolates from the Isle of Arran than those from Brownsea Island suggesting that these squirrels have been carrying these strains for longer.

Mobile genetic elements in CC133
Mobile genetic elements (MGE) such as phages and pathogenicity islands often carry genes that contribute to host adaptation and an examination of these elements could assist with identifying the host source of isolates found in red squirrels, and to assess the extent to which these isolates might have become adapted to squirrels. Carriage of the lukM phage has been reported to be common in the genomes of ruminant strains from multiple lineages, in which it helps S. aureus evade the bovine immune system and contributes to its virulence [12]. None of the ST133 isolates from squirrels contained this phage, however neither do other isolates in the same clade in the phylogeny (Fig. 2 marked with red bar) [13]. Phages and pathogenicity islands of ovine strains have previously been characterized primarily using the closed genome ED133 as an ST133 reference genome [49], and this and other sheep-derived isolates have been reported to carry specific ovine phages (including a lukM phage) and pathogenicity islands. We examined squirrel isolates in ST133 and ST2328 for the presence or absence of these elements and found them to be absent except in ST2328 isolates from the Isle of Arran (Table 2). However, as with the lukM phage we also found that other isolates within the clade lack these elements, it is therefore not clear if the isolates lost them before or after transmission to squirrels from another host (Fig. 2).

Other phages
Further examination of phages in isolates from red squirrels in ST133 and Arran ST2328 found that there is an intact phage inserting into the hlb gene (Table 2), but the phages from each lineage only share around 30% BLAST similarity with each other (Fig. S2). The hlb gene is the usual site of integration of the S. aureus phage 3, which carries the human IEC genes, and phage insertion will result in loss of function of this gene, but the hlb-converting phages in the red squirrel isolates do not carry any IEC-like genes in the expected site next to the amidase/holin module. ED133 does not carry this or any other phage at this site, and neither do the Jersey ST2328 isolates.

Genome wide association study in ST133
When bacteria switch to a new host from a host species to which they are adapted, a period of rapid adaptation may ensue. Previous studies have described an initial loss of genetic material following host switching, particularly of MGEs, but other adaptations including SNPs, insertions or recombinations have also been described [50]. Because the phylogeny of ST133 strains suggested that there is likely to
have been independent introductions of this lineage into the squirrel populations of the Isle of Arran and Brownsea Island, we carried out a GWAS to establish if there was genomic variation specific to both of these squirrel populations but absent from other ST133 genomes. We included 241 publicly available genomes, 15 squirrel isolate genomes from the Isle of Arran and nine from Brownsea Island. We used pySEER with a linear mixed model and k-mers [46]. Significant k-mers were filtered using a P-value of 2.80E-07 (determined from the number of unique k-mer patterns) and mapped against the closed genome in ST133 from this study (AR_39_merged_O1) (Fig. 4).

This highlighted significant hits in 11 genes spread across the length of the genome, of which six coded for cell-wall metabolism or transporter proteins, one coded for staphylocoagulase (coa), which interacts with host prothrombin, three coded for proteins in metabolic pathways, one was a urea transporter and one coded for a hypothetical protein, which was located between two membrane-associated operons (Table 3). This prominent representation of cell-wall protein genes may suggest host adaptation, as these proteins are likely to interact strongly with the host immune system. However, all except one of these 11 gene variants were found only in isolates from the Isle of Arran, not from Brownsea Island, and there were no common variants detected. Moreover, the single variant associated with the Brownsea Island population is synonymous, whereas 8/10 of the Isle of Arran SNPs are non-synonymous, which is consistent with a role in adaptation. The variants reported here were present in all the isolates from the Isle of Arran (or Brownsea), and in none of the other isolates in the analysis.

Table 3. Top hits from a GWAS of isolates in ST133 using pySEER plotted against the completed genome AR39_merged_O1. The protein product is as predicted by Prokka and the likely functional category of the protein is inferred from the available literature on these or similar proteins. Variant type is synonymous (s) or non-synonymous (ns) SNP. AR=Isle of Arran isolates only. BI=Brownsea Island isolates only.

| Gene          | Annotation                          | Predicted product                                      | Category               | Notes                       | Variant |
|---------------|-------------------------------------|--------------------------------------------------------|------------------------|-----------------------------|---------|
| AR39merged_00164 | coa_4                              | Staphylocoagulase precursor                            | Virulence              | Interaction with host prothrombin | AR nsSNP |
| AR39merged_00187 | tarK                               | Putative polyribitolphosphotransferase                 | Cell wall synthesis. Immune evasion. | AR sSNP |
| AR39merged_00191 | tarL                               | Putative polyribitolphosphotransferase                 | Cell wall synthesis. Immune evasion. | BI sSNP |
| AR39merged_00425 | marf                               | MOP superfamily PST family polysaccharide transporter | Carbohydrate metabolism | Membrane                  | AR sSNP |
| AR39merged_00634 | fruA                               | PTS system d-fructose-specific transporter             | Carbohydrate metabolism | Membrane                  | AR nsSNP |
| AR39merged_00809 | cdr                                | Coenzyme A disulfide reductase                         | Metabolism             | AR nsSNP                  |
| AR39merged_01042 | carB_1                             | Putative carbamoyl phosphate synthase                   | Amino acid metabolism  | AR nsSNP                  |
| AR39merged_02040 | htsC                               | Iron-binding ABC superfamily ATP-binding cassette      | Iron metabolism        | Transmembrane transport of staphyloferin | AR nsSNP |
| AR39merged_02117 | fnhb_1                             | Peptidoglycan pentaglycine interpeptide biosynthesis   | Metabolism             | Cell wall synthesis        | AR nsSNP |
| AR39merged_02141 | utp_1                              | Membrane-bound urea transporter                        | Metabolism             | Membrane                  | AR nsSNP |
| AR39merged_02211 |                                    | Hypothetical protein                                   |                       |                             | AR nsSNP |

Abbreviations: MOP; multidrug/oligosaccharidyl-lipid/polysaccharide, PST; polysaccharide transport, PTS; phosphotransferase system.

S. aureus ST49 was isolated from red squirrels on Jersey and IOW with clinical signs typical of FED. The single locus variant ST1957 was isolated from a squirrel on IOW with FED, but is not included in the phylogeny (Fig. 3). ST49 was also isolated from one apparently healthy red squirrel on the
Isle of Arran which was caught and released, so we cannot be sure that it did not subsequently develop disease.

The publicly available genomes, representing isolates from humans, pigs and other unknown sources, are distinct from the squirrel isolates on the ST49 phylogeny (Fig. 3), and none of these 26 isolates contain the lukM phage. In contrast, this phage is present in all but four of the 107 squirrel isolates plus the isolate from a bank vole (DIP), and the four exceptional isolates were obtained from one swab from each of two squirrels. The genome content of the lukM phage is nearly identical (99% identity) where it is present and will be fully described elsewhere. A further distinction is that the ST49 isolates from squirrels have no plasmids whereas the publicly available reference ST49 isolates have between 1 and 4, many of which are previously described (Table S5).

A phylogeny of the clade containing the squirrel and bank vole isolates was then constructed using a whole-genome alignment of isolates mapped to the closed assembly from a squirrel, which contained the lukM phage (JW31330_O1_dhc) (Fig. 5).

From Fig. 5 it is clear there have been limited introductions in each geographic site, and no migration between them. In order to explore transmission within Jersey between individual squirrels, we undertook a principal component analysis using SNP distances on each of the two separate clades of isolates from Jersey in the tree (Fig. S3). This showed four pairs of isolates, which clustered together, and the distances between each pair were between three and 23 SNPs (3–5, 5–6, 9–10 and 23), whereas the within-host diversity was less than three SNPs. Each of these pairs of isolates were recovered from squirrels found in similar geographical locations. Therefore, for three of these pairs it is not possible to rule out a squirrel-to-squirrel transmission, however we might also expect to see this pattern of SNP distances and topology of the phylogeny if squirrels were acquiring infection from an unsampled reservoir host in the same location.
The red squirrel isolates from Jersey cluster into two separate clades, which correspond to an east-west split of the location where the squirrels were found (Fig. 6). The SNP distances between isolates in these two clades are between 250–300 SNPs, and BLAST analysis using BRIG demonstrated that the SNPs are spread along the genome (Fig. S4). In an earlier study as part of the Jersey red squirrel surveillance project, 169 red squirrels had tissue samples taken for DNA and mitochondrial DNA sequencing revealing the existence of two mitochondrial control region haplotypes [52]. The geographical location of squirrels with these haplotypes shows a strong footprint, which is believed to result from the introduction of squirrels from either British or European stock (Fig. 7). While the two haplotypes are not completely separated, the east-west division strongly echoes the two clades of ST49 found in red squirrels (Fig. 6). The geography of Jersey clearly presents a barrier to mixing of animals from the east and west of the island. The barriers could be man-made, for example roads, or natural, such as breaks in woodland through which animals disperse.

**Within-host diversity in ST49 infected red squirrels**

Multiple sampling of several sites on a single host and of several isolates from each swab allows an estimation of the
within-host diversity. A host which has carried a strain for a longer time, has been infected with a more diverse inoculum or been infected on multiple occasions, would be expected to have a greater diversity of isolates [53]. We obtained multiple ST49 isolates from 14 red squirrels, so to estimate within-host diversity a SNP distance matrix was constructed from the mapping alignment using snp-dists and approximate duration of carriage calculated based on published S. aureus mutation rates (Table 4) [54]. In general, within-host diversity appears very low (maximum 2–3 SNPs), which suggests that ST49 is typically not carried for more than 2 months within each squirrel. This is consistent with the lack of evidence of

**Table 4.** Number of SNPs in a core-genome alignment of ST49 multiple isolates cultured from single hosts to assess within-host diversity. Divergence time (years)=[maximum pairwise SNP distance/(mutation rate x number of sites in alignment)]/2. Number of sites in alignment is 2.726 Mbp. A mutation rate of 2.7 SNPs per MB per year is used to estimate the length of time carried [54].

| ID          | No. of isolates | Max SNP | Min SNP | Estimated length of carriage (years) | Estimated length of carriage (days) |
|-------------|-----------------|---------|---------|--------------------------------------|-------------------------------------|
| JW_32635    | 16              | 3       | 0       | 0.20                                 | 74                                  |
| AR_056_18   | 3               | 0       | 0       | 0.00                                 | 0                                   |
| JW_32660    | 17              | 2       | 0       | 0.14                                 | 50                                  |
| JW_32979    | 5               | 2       | 0       | 0.14                                 | 50                                  |
| JW_32476    | 2               | 1       | 0       | 0.07                                 | 25                                  |
| JW_30866    | 6               | 2       | 0       | 0.14                                 | 50                                  |
| JW_31330    | 14              | 1       | 0       | 0.07                                 | 25                                  |
| JW_34223    | 8               | 1       | 0       | 0.07                                 | 25                                  |
| JW_34783    | 3               | 2       | 1       | 0.14                                 | 50                                  |
| JW_34662    | 5               | 0       | 0       | 0.00                                 | 0                                   |
| JW_34602    | 3               | 1       | 0       | 0.07                                 | 25                                  |
| JW_34680    | 4               | 2       | 0       | 0.14                                 | 50                                  |
| JW_34594    | 2               | 0       | 0       | 0.00                                 | 0                                   |
| IOW_HB733   | 13              | 0       | 0       | 0.00                                 | 0                                   |
squirrel-to-squirrel transmission in the phylogeny and also with the clinical time-frame of the disease in which death occurs in days once the squirrel has become sick enough to be captured.

DISCUSSION

The carriage rate of *S. aureus* in red squirrels in this study varies according to the island location (12–32%). However, in healthy squirrels on the Isle of Arran and Brownsea Island the rate was between 25–30%, which is comparable to that reported in humans [55]. The strains carried also showed considerable variation dependent on the island and the presence of sympatric species. This tendency to acquire strains from other hosts in the environment has also been reported in wild and captive rats in Europe, and in that case the clones also included CC49 and CC130 [8]. However, the evidence in red squirrels here points to both recent spillover events, and possible host switches involving a longer time scale and some genomic adaptation to the new host [50].

Healthy red squirrels on the Isle of Arran (14/44) were found to carry STs 133, 2328 and 49 (Table 1). ST133 is commonly isolated from small ruminants, especially sheep, but also other hosts including cattle and wildlife [56, 57]. ST2328 is a double-locus variant of ST133, which is a rarely reported isolate of sheep [17]. It is noteworthy that in our limited sampling of sheep and deer close to the red squirrel sampling site on the Isle of Arran we did not recover any ST133 isolates, which may point to a host switch occurring prior to the red squirrels’ introduction to the island, anecdotally reported in the 1930s and 1950s (P. Lurz. personal communication). The carriage rates of *S. aureus* in sheep (65%) and red deer (80%) appear to be high, but when the small sample sizes are accounted for, the rate in sheep is broadly consistent with reported rates of 30–45%, and a study in free-ranging red deer in Lombardy (Italy) also reported a nasal carriage rate of 91% [58][59].

Healthy red squirrels on Brownsea Island (11/44) were found to carry ST’s 692, 133, 188 and 130. ST692 has been isolated from farmed game birds in the UK, and poultry meat in South Korea, and is part of the avian specific clade CC385 [60, 61]. ST188 is geographically widespread and found in many different host species, including humans [62]. ST130 is also predominantly associated with ruminants, although it is also found in humans, and was the main lineage found in hedgehogs in Sweden and rats in central Europe [63, 64, 8].

On Jersey, despite sampling over 100 healthy red squirrels, ST49 was not recovered as a normal carriage isolate. However, in contrast with other islands, no other *S. aureus* carriage isolates were recovered, apart from one squirrel carrying ST130 and two with skin lesions carrying ST2328, which means that around 88% of squirrels were not colonized. Therefore, another explanation for the failure to find ST49 in red squirrels on other islands might be the possibly protective effect of carriage of less virulent strains such as ST133. This could be mediated through general immunity to *S. aureus* stimulated by the less virulent strains, or by direct inter-strain competition such as has been observed between *S. epidermidis* and *S. aureus* in humans [65].

On the Isle of Wight two out of three red squirrels with clinical signs of FED carried *S. aureus*, one ST49 and a second one the single locus variant ST1957, which was also reported in the 2013 study [6]. The significance of this apparent greater diversity of strains causing disease on IOW is unclear and merits further study.

Red squirrels on Brownsea Island are in close contact with other wild and farmed species, and are regularly fed by humans. Avian strains (ST692) found here are consistent with acquisition from feeders known to be shared with farmed game birds. The ST188 isolates recovered were possibly acquired from humans because strains isolated from humans usually contain a β-haemolysin converting phage carrying IEC genes, while similar strains isolated from animals do not [47, 54, 66]. However, the time scale of loss or gain of the phage is unclear, as experimentally it appears to be quite stable. A study transferring a human strain to gnotobiotic piglets found that while other genes were lost within hours of transfer, the β-haemolysin converting phage was still present after 16h [67], and in a separate experiment, which passaged human strains in sheep it was still present after more than a year [68] (R. Fitzgerald, unpublished information). Hence we cannot be sure if ST188 strains were acquired recently, or even directly from humans.

The predominant strains carried by healthy red squirrels in this study (72%) are typically associated with small ruminants (ST133, 2328 and 130)[48, 49]. This observation may reflect squirrels’ utilization of ruminant-grazed fields adjacent to woodland to bury caches of nuts during autumn. Alternatively, ruminant genome adaptations may also be conducive to the survival of *S. aureus* in red squirrels. Experimental evidence suggests that immediately following a host-switch, variation occurs in the MGE with loss or gain of phages and plasmids, however longer-term adaptation may involve fixation of mutations in the core genome [51, 67, 69, 68]. In a study of *S. aureus* strains infecting rabbits, a single non-synonymous SNP in the *dltB* gene appears to confer high infectivity for rabbits. The *dltB* gene encodes a cell-membrane-associated protein and alleles of this gene were found in other rabbit-infecting strains suggesting convergent evolution [69]. The GWAS on ST133 isolates carried by red squirrels in this study also highlighted variants in several genes encoding for cell-membrane-associated proteins, which suggests a degree of host-adaptation in the strains we sampled, however it is not clear if this is a permanent host-switch, and we did not find the same variants in isolates from both the Isle of Arran and Brownsea Island. GWAS analysis in *S. aureus* is hampered by strong lineage effects, and in previous studies a false positive rate of up to 70% was identified, so laboratory investigation to functionally verify the effect of the variants is needed [70].

The ST133 isolates recovered from red squirrels may have been acquired directly from small ruminants, or from an
unsampled secondary host. They cluster in the phylogeny with other isolates which have lost ovine-specific elements including the lukM phage. In contrast, in the phylogeny of ST49 isolates, those from squirrels all contain the phage, as does the isolate from a bank vole, which clusters closely (max SNP distance=~500) (Fig. 3), perhaps suggesting a role for the lukM phage in virulence in squirrels and in adaptation to bank voles. In a study of *S. aureus* in small mammals in Europe, ST49 was found in bank voles (*Myodes glareolus*) and yellow-necked mice (*Apodemus flavicollis*), however while the latter carried other strains, the bank vole carried ST49 exclusively [7].

There is considerable interest in methods to identify bacterial transmission using genome sequence data [53]. Methods that utilize SNP thresholds can be misleading if within-host diversity is not taken into account, however core SNP thresholds between 23 and 40 have been suggested, with pairs of isolates under the threshold regarded as transmission events [53]. For our ST49 data neither the topology of the phylogeny nor a principal component analysis using SNP distances was able to unequivocally demonstrate whether squirrels acquired infection from other squirrels or from an unsampled reservoir host in the same location.

Ongoing disease surveillance of red squirrels in Scotland has not recorded any cases of FED. Although bank voles live in woodland and are common throughout the UK, including IOW and on Jersey, which has its own subspecies [71], on the Isle of Arran they have been found only intermittently and in small numbers, and not at all on Brownsea Island (Dorset Wildlife Trust; personal communication) [71]. This pattern precisely mirrors our findings of ST49 in red squirrels, and supports the hypothesis that the bank vole is a reservoir host for ST49 infection in red squirrels, in which it is rapidly fatal. However, an unsampled host such as rats, which have been recorded carrying CC49 strains and are numerous on Jersey, is a plausible alternative [8]. Carriage sampling of small mammals at all locations is warranted to explore the significance of all these findings.

**CONCLUSION**

Red squirrels carry *S. aureus* strains usually associated with other species, primarily small ruminants, possibly as a result of an historic host-switch, however where squirrels are in close contact with birds or humans they will acquire strains from these species. We speculate that on Jersey and IOW red squirrels acquire *S. aureus* ST49 strains from a reservoir species, possibly the bank vole or rat, and rapidly develop fatal disease. There is little evidence of transmission of ST49 strains from squirrel to squirrel, and it was only recovered once from squirrels on other islands. The identification of the reservoir species on Jersey is a research priority for the development of mitigation measures to reduce the incidence of fatal exudative dermatitis in red squirrels on the island. The role of *lukM/F* genes in virulence in red squirrels and in adaptation to other small mammals remains to be elucidated.

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**Author contributions**

Conceptualization: D.H.L., A.M., A.L., K.F. and E.J.F. Data curation: K.F., M.J.G. Formal analysis; K.F., M.J.G. Investigation: K.F., A.S., A.L., H.B., T.B., M.J.G. Methodology: K.F., A.L., E.J.F. Project administration: K.F., A.L., A.M., E.J.F. Resources: K.F., A.S., A.L., T.B., H.B., A.M., M.J.G., D.H.L., A.L., E.J.F.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

The study was approved by the Royal Veterinary College Clinical Research Ethical Review Board (CRERB URN M2016 0093) and by the University of Bath Animal Welfare and Ethical Review Body. The Jersey Society for the Protection of Animals permitted the sampling of animals under their care and the Jersey States Vet permitted the export of swab samples to the UK. Red squirrels on Isle of Arran and Brownsea Island were captured and sampled under home office licence (PPL 70/9023), Natural England License 2016–24517-SCI-SCI and Scottish Natural Heritage Licence 90896 with ethical approval from the University of Edinburgh’s Animal Welfare and Ethical Review Body. The Forestry Commission Scotland gave permission to sample culled deer.

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