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Mammaliicoccus sciuri's Pan-Immune System and the Dynamics of Horizontal Gene Transfer Among *Staphylococcaceae*: a One-Health CRISPR Tale

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Abstract
Recently emancipated from the *Staphylococcus* genus due to genomic differences, *Mammaliicoccus sciuri*, previously classified as an occasional pathogen, emerges as a significant player in the landscape of resistance gene dissemination among *Staphylococcaceae*. Despite its classification, its role remained enigmatic. In this study, we delved into the genomic repertoire of *M. sciuri* to unravel its contribution to resistance and virulence gene transfer in the context of One Health. Through comprehensive analysis of publicly available genomes, we unveiled a diverse pan-immune system adept at defending against exogenous genetic elements, yet concurrently fostering horizontal gene transfer (HGT). Specifically, exploration of CRISPR-Cas systems, with spacer sequences as molecular signatures, elucidated a global dissemination pattern spanning environmental, animal, and human hosts. Notably, we identified the integration of CRISPR-Cas systems within SCC*mec*s (Staphylococcal Cassette Chromosome *mec*), harboring key genes associated with pathogenicity and resistance, especially the methicillin resistance gene *mecA*, suggesting a strategic adaptation to outcompete other mobile genetic elements. Our findings underscored *M. sciuri*'s active engagement in HGT dynamics and evolutionary trajectories within *Staphylococcaceae*, emphasizing its central role in shaping microbial communities and highlighting the significance of understanding its implications in the One Health framework, an interdisciplinary approach that recognizes the interconnectedness of human, animal, and environmental health to address global health challenges.

Keywords CRISPR-Cas · One health · *Staphylococcaceae* · *mecA* · SCC*mec* · Antimicrobial resistance

Introduction

The *Staphylococcaceae* family encompasses over one hundred bacterial species, including notable opportunistic pathogens implicated in various superficial and systemic diseases in both humans and animals (Parte et al., 2020). Among these, *Staphylococcus aureus* stands as the family's protagonist, while other members like *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* are recognized as relevant nosocomial pathogens, often associated with infections linked to invasive medical devices (Becker et al., 2014; Rossi et al., 2024). Additionally, emerging veterinary pathogens such as *Staphylococcus pseudintermedius*, *Staphylococcus coagulans*, and *Staphylococcus schleiferi* contribute to skin infections in domestic animals, otitis externa, and pyoderma in dogs (Souza-Silva et al., 2022).

As novel strains are isolated and genomic data becomes increasingly available in public repositories, the taxonomic landscape evolves through reclassifications and reassignments. Such is the case with *Staphylococcus sciuri* and four other species, which have undergone reclassification into the newly proposed genus *Mammaliicoccus* following meticulous phylogenomic analyses (Madhaiyan et al., 2020).

Originally described in 1976 as gram-positive cocci, nonmotile, and nonsporeforming bacteria, *Mammaliicoccus sciuri* derives its name from gray squirrels (*Sciurus carolinensis*), where it is commonly found on their skin. However, it has also been identified within the microbiota of various other animals, including opossums, dogs, sheep, and humans (Kloos et al., 1976). Presently, *M. sciuri* emerges as

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a ubiquitous species, inhabiting diverse environments ranging from animal-derived foods to dairy farms and swine manure treatment plants (Adkins et al., 2022; Martins-Silva et al., 2023; Yang et al., 2022).

Despite typically being considered a commensal and innocuous species, *M. sciuri* has been implicated in severe and occasionally fatal veterinary infectious processes (de Carvalho et al., 2022; Sacramento et al., 2022). Although human infections caused by *M. sciuri* are infrequent, its zoonotic potential, coupled with its ubiquity, underscores the need for vigilance. The presence of various virulence and antimicrobial resistance genes within *M. sciuri* raises concerns regarding the potential transfer of these genes to other members of the *Staphylococcaceae* family, potentially augmenting the pathogenicity of other species with greater health risks to humans (Nemeghaire et al., 2014).

The concept of gene reservoirs is well-established among less pathogenic members of the *Staphylococcaceae* family (Rossi et al., 2020), with the dynamics of horizontal gene transfer encompassing diverse processes such as transduction, conjugation, transformation, and vesiduction (Rodríguez-Beltrán et al., 2021). These processes are regulated by an array of bacterial defense systems against mobile genetic elements, with the CRISPR-Cas system representing a crucial component. Comprising a locus of short direct repeat sequences interspersed with exogenous origin sequences known as spacers, the CRISPR system not only functions as an adaptive bacterial immune system but also serves as a molecular marker for horizontal gene transfer in specific lineages (Bernheim & Sorek, 2020; Rossi et al., 2019).

In light of the foregoing, this study aims to undertake a comprehensive analysis of publicly available *M. sciuri* genomes to elucidate its role in the dynamics of genetic material exchange among its relatives. Specifically, our objectives include investigating the involvement of CRISPR systems and other bacterial defense mechanisms, such as restriction-modification and abortive infection systems, in horizontal gene transfer processes and correlating this data with the presence and characteristics of key mobile genetic elements, thereby elucidating their exchange among *Staphylococcaceae* facilitated by *M. sciuri*.

Materials and Methods

Mammaliicoccus sciuri Genome Sequences Retrieval

A comprehensive collection of *M. sciuri* genomic sequences was obtained from the National Center for Biotechnology Information (NCBI) GenBank database. A search query using the species name was conducted in January 2023, yielding a total of 183 genomic sequences (Table S1). Subsequently, all relevant files were systematically retrieved and downloaded for subsequent computational analyses. These sequences were sourced from diverse origins, encompassing environmental samples as well as isolates from animal and human hosts.

Search and Analysis of CRISPR-Cas Systems

All collected genomes underwent a thorough examination to identify the presence and types of CRISPR systems using CRISPRCasFinder version 1.1.2 (Couvin et al., 2018), employing default settings as defined by the developers. Subsequently, direct repeat and spacer sequences from strains identified as CRISPR-positive were extracted into separate files for further scrutiny regarding their origin and conservation. Direct repeat sequences were aligned using the Clustal Omega algorithm within MEGA version 11.0.13 (Tamura et al., 2021), and the resulting alignment was utilized to generate a sequence logo using WebLogo version 3 (Crooks et al., 2004). The origin of each spacer sequence was determined using BLASTn with default parameters against the Genbank database. Moreover, complete sequences of CRISPR-Cas loci were subjected to phylogenetic analysis to assess correlation among *M. sciuri* strains containing these loci. Alignment was performed using MEGA, followed by Bayesian inference analysis using MrBayes program version 3.2.7., adhering to suggestions provided by the developers (Ronquist et al., 2012).

Search for *M. sciuri* Defense Systems and Mobile Elements

In addition to CRISPR systems, other defense mechanisms against exogenous genetic elements present in the *M. sciuri* genomes were explored. Anti-CRISPR operons were investigated using AcrFinder (Yi et al., 2020), while DefenseFinder version 1.0.9 (Tesson et al., 2022) was utilized to analyze additional defense systems. Default settings provided by the developers were applied for consistency and comparability across the datasets. Furthermore, the presence of prophages within the *M. sciuri* genomes was assessed using PHASTER (Arndt et al., 2016).

Analysis of the Genetic Context of CRISPR-Cas Systems in *M. sciuri* Genomes

To investigate the potential correlation between CRISPR-Cas systems and mobile genetic elements harboring resistance genes, our methodology followed a systematic approach. Initially, we utilized ISFinder (Siguier et al., 2006) to identify insertion sequences (IS) proximal to the CRISPR-Cas systems. Subsequently, potential genomic islands were predicted using IslandViewer 4.0 (Bertelli et al., 2017). The sequences spanning the regions between the flanking IS of

the CRISPR loci were then extracted and analyzed using ResFinder version 4.3.3 and VirulenceFinder 2.0 (Bortolaia et al., 2020) to detect any antimicrobial resistance and virulence genes, respectively. Furthermore, we investigated the presence of the staphylococcal cassette chromosome *mec* (SCC*mec*), which carries the methicillin resistance *mecA* or *mecC* gene, utilizing SCC*mec*Finder (Kaya et al., 2018). All analyses were conducted following the default parameters provided by the respective software developers. Finally, the remaining genes within the collected sequences were annotated through a search in the UniProt database (The UniProt Consortium, 2019).

Analysis of the Dissemination of *M. sciuri* Mobile Genetic Elements Among Other *Staphylococcaceae*

To investigate the dissemination of mobile genetic elements of *M. sciuri* among other *Staphylococcaceae* species, we examined the conservation of the mobile region surrounding the identified CRISPR systems in other genomes of the *Staphylococcaceae* family. This comparative analysis was conducted using BLASTn with standard parameters, against all sequences of the NCBI's Genbank public database. Subsequently, conserved sequences found in other species were converted into GenBank format using Prokka version 1.14.5 (Seemann, 2014). Gene plotting and synteny analysis were performed using the Clinker program version 0.0.28 (Gilchrist & Chooi, 2021), following the recommendations of the developers. Additionally, the Mauve software version 2.4.0 (Darling et al., 2004) was employed to align homologous sequences from different species, enabling a comprehensive comparison of genetic elements across the *Staphylococcaceae* family.

Results

Defense Systems are Diverse and Varied Across the *M. sciuri* Population

Our analysis of 183 publicly available genomes of *M. sciuri* from diverse geographical locations revealed a rich array of defense systems, comprising 24 different types (Fig. 1). These defense mechanisms primarily encompass abortive infection (Abi), restriction-modification (RM), and CRISPR-Cas systems. Interestingly, the distribution of defense

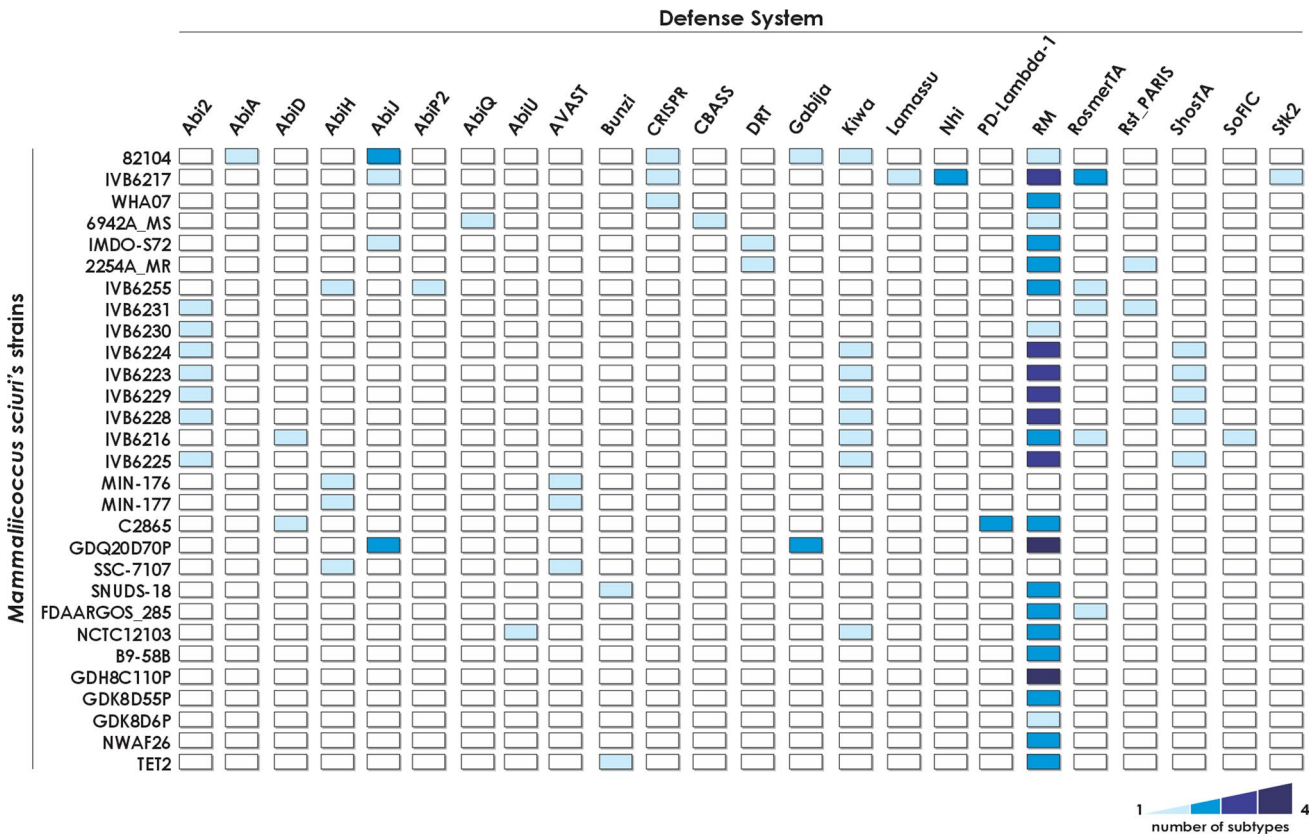

Fig. 1 The pan-immune system of *Mammaliicoccus sciuri*. Distribution of known defense systems across publicly available genomes of *Mammaliicoccus sciuri*: the varying shades of blue, from lighter to darker, indicate the presence of 1 to 4 subtypes of the same system. White rectangles indicate the absence of a respective defense system. In the missing strains, no defense systems were detected

systems varied among populations of *M. sciuri*, with different strains exhibiting a diverse combination of defense mechanisms against bacteriophages or other mobile genetic elements. Upon examination, genomes positive in the DefenseFinder search were found to harbor between 1 and 7 different defense systems, highlighting the variability within the *M. sciuri* population. Notably, restriction-modification systems emerged as a prevalent defense mechanism, being present in 85% of the positive strains, with some strains containing up to 4 subtypes. However, despite this diversity, only a minority of strains (13%; 29 strains) harbored known defense systems.

CRISPR-Cas Systems Highlight the Dissemination of *M. sciuri* Across Different Hosts and Distant Geographical Regions

Out of the 183 sequences analyzed, only 3 (less than 1.5%) were found to possess CRISPR-Cas systems. Remarkably, these systems were identified in *M. sciuri* strains isolated from vastly different environments and distant locations. The first strain, 82,104, was isolated from a human urine culture in China. The second, WHA07, was obtained from the bioaerosol of a chicken farm in a city over 900 km away from the 82,104 strain location, within the same country. The third strain, IVB6217, originated from nasal swabs of a camel in Kenya, situated over 6,000 km apart from the other two locations. This information was obtained from their GenBank data, as no publications featuring these genomes are currently available. Despite their disparate origins, the CRISPR-Cas systems exhibited striking similarities. All three strains harbored the type IIIA CRISPR-Cas system, with Cas10 serving as the principal effector protein in the interference process (Fig. 2A). Phylogenetic analysis of the CRISPR-Cas locus revealed a closer evolutionary relationship between strains 82,104 and WHA07 in comparison to strain IVB6217. Additionally, the direct repeats within the CRISPR arrays exhibited a high level of conservation among the three strains, sharing 92% identity across their 38 nucleotides (Fig. 2B), indicative of a common ancestral origin.

CRISPR Spacers of *M. sciuri* Witness the Multi-Host Transmission of this Bacterium

To gain insights into the probable dissemination dynamics of *M. sciuri* strains, we examined the spacer sequences of the three identified CRISPR elements and their order of acquisition. Remarkably, the three strains shared two identical spacers, and strains 82,104 and WHA07 shared additional 13 spacers with the same sequence and organization. Each distinct spacer was sequentially numbered from 1 to 47 (Fig. 3). The chronological incorporation of spacers, coupled with the prevalence of identical spacers, suggests a common ancestral strain possessing spacers 1 and 2, which subsequently diverged through various evolutionary pathways: (i) one evolutionary pathway led to strain IVB6217, acquiring spacers

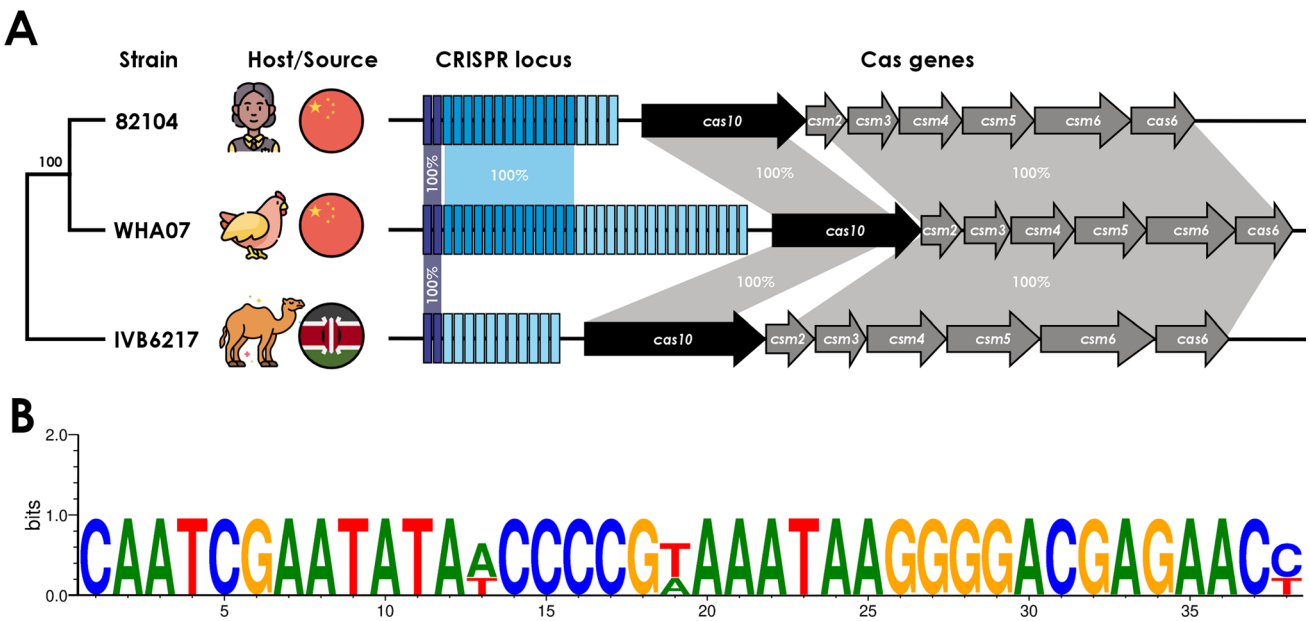

Fig. 2 CRISPR-Cas systems in *Mammaliicoccus sciuri*. (**A**) *Mammaliicoccus sciuri* strains positive for CRISPR loci, with information on their host and country of isolation. Darker blue rectangles represent spacer sequences identical in all strains, medium-shaded blue rectangles represent spacer sequences shared by two strains, and light-shaded blue rectangles represent strain-specific spacers. (**B**) Conservation of the direct repeat sequences from the CRISPR of the three strains

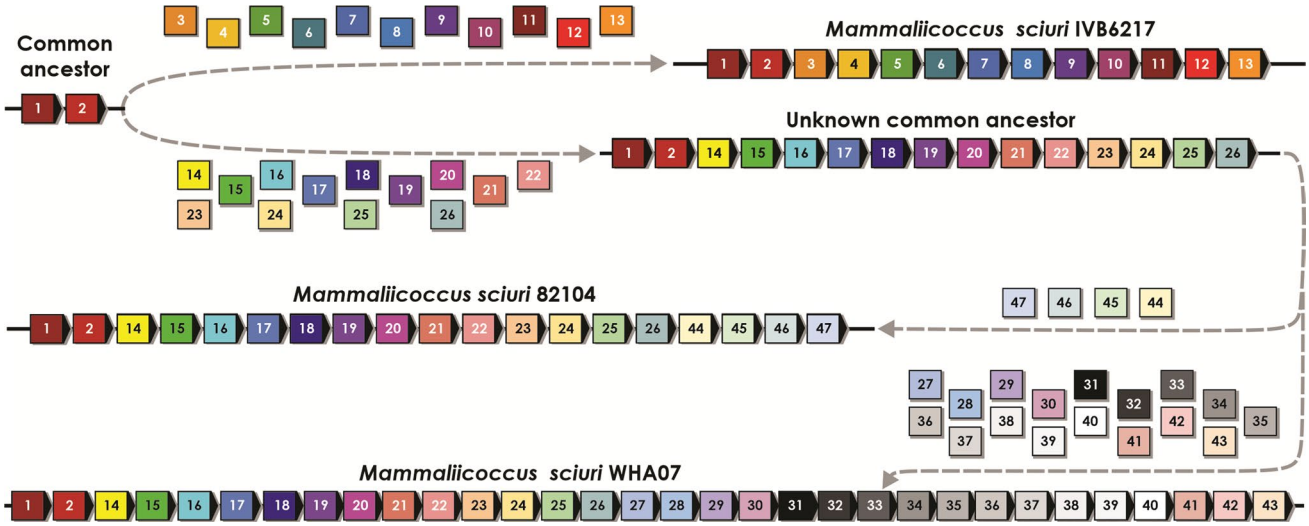

Fig. 3 Proposal of the global and multi-host dispersion of the CRISPR-bearing *Mammaliicoccus sciuri* strains, based on the chronological order of acquisition of spacers over time. Each distinct spacer sequence was assigned a unique color and number

2 to 13; (ii) another pathway gave rise to a new ancestor, acquiring spacers 14 to 26; (iii) this subsequent ancestor further diverged, resulting in strains WHA07—acquiring spacers 27 to 35—and 82,104—acquiring spacers 44 to 47 (Fig. 3).

Notably, none of the 47 unique spacers exhibited homologs in public sequence databases, posing challenges in determining their origin—whether viral, plasmid or other mobile genetic element. Additionally, all these spacers are exclusive to *M. sciuri* and have not been identified in other *Staphylococcaceae* species.

Mammaliicoccus sciuri CRISPR Systems Reside Within an SCC*mec* and Between Insertion Sequences

In our investigation of the mobilome within *M. sciuri* strains containing CRISPR-Cas systems, we made an intriguing observation: none of these strains harbored prophages in their genomes. However, what we did find was that all CRISPR loci were flanked by insertion sequences, specifically IS*257* on the left and IS*Sau3* on the right (Fig. 4A). The *M. sciuri* genome is rich in IS elements, with strain IVB6217, for instance, containing a total of 71 non-redundant IS elements in its complete genome (data not shown). Upon closer examination, the sequences of the CRISPR loci flanked by IS*257* and IS*Sau3* in Fig. 4A were identified as SCC*mec* elements in strains WHA07 and 82,104, approximately 112 kb in size, with remnants of it found in strain IVB6217, approximately 84 kb in size. Interestingly, in addition to the presence of the methicillin resistance gene *mecA*, the CRISPR loci were found to be adjacent to the beta-lactam resistance gene *blaZ*. The loci of the three strains were found to be located within genomic islands (Fig. 4B). Further analysis revealed intriguing evolutionary insights. Strain IVB6217, as suggested by phylogeny and spacer acquisition history, appears to have diverged earlier. An alignment of the SCC*mec* elements from strains WHA07 and 82,104 with the genome of IVB6217 unveiled that the cassette of the latter strain had undergone various rearrangements, with its fragments dispersed throughout its genome (Fig. 4C). However, despite these rearrangements, both the *mecA* and *blaZ* genes remain present, with the latter positioned immediately after the *cas* genes.

The SCC*mecs* of *M. sciuri* Carry a Range of Resistance-Related Genes Shared with Other *Staphylococcaceae*

The SCC*mec*s of *M. sciuri* emerge as reservoirs of various resistance-related genes, echoing the adaptability of *Staphylococcaceae* in challenging environments. Beyond the concerning presence of *mecA* and *blaZ*, these elements house an array of genes crucial for *M. sciuri*'s survival and persistence (Table S2). Noteworthy among these are heavy metal pump systems encoded by the *cad* and *ars* operons, a lysostaphin encoded by the *lytM* gene, and restriction-modification systems encoded by the *hsd* genes. What's particularly striking is the shared genetic landscape between *M. sciuri* and other *Staphylococcaceae* species, whose sequences are available in Genbank (Fig. 5). Approximately 30% of the *M. sciuri* SCC*mec* exhibits striking similarity with strains of *Staphylococcus aureus* OC3, isolated from a fatal human pediatric patient in Russia (Khokhlova et al., 2015); *Staphylococcus epidermidis* HD99-4, retrieved from the nasal cavity of a human in Germany (Both et al., 2021); and *Staphylococcus pseudintermedius* KM241, obtained from a terrier dog with

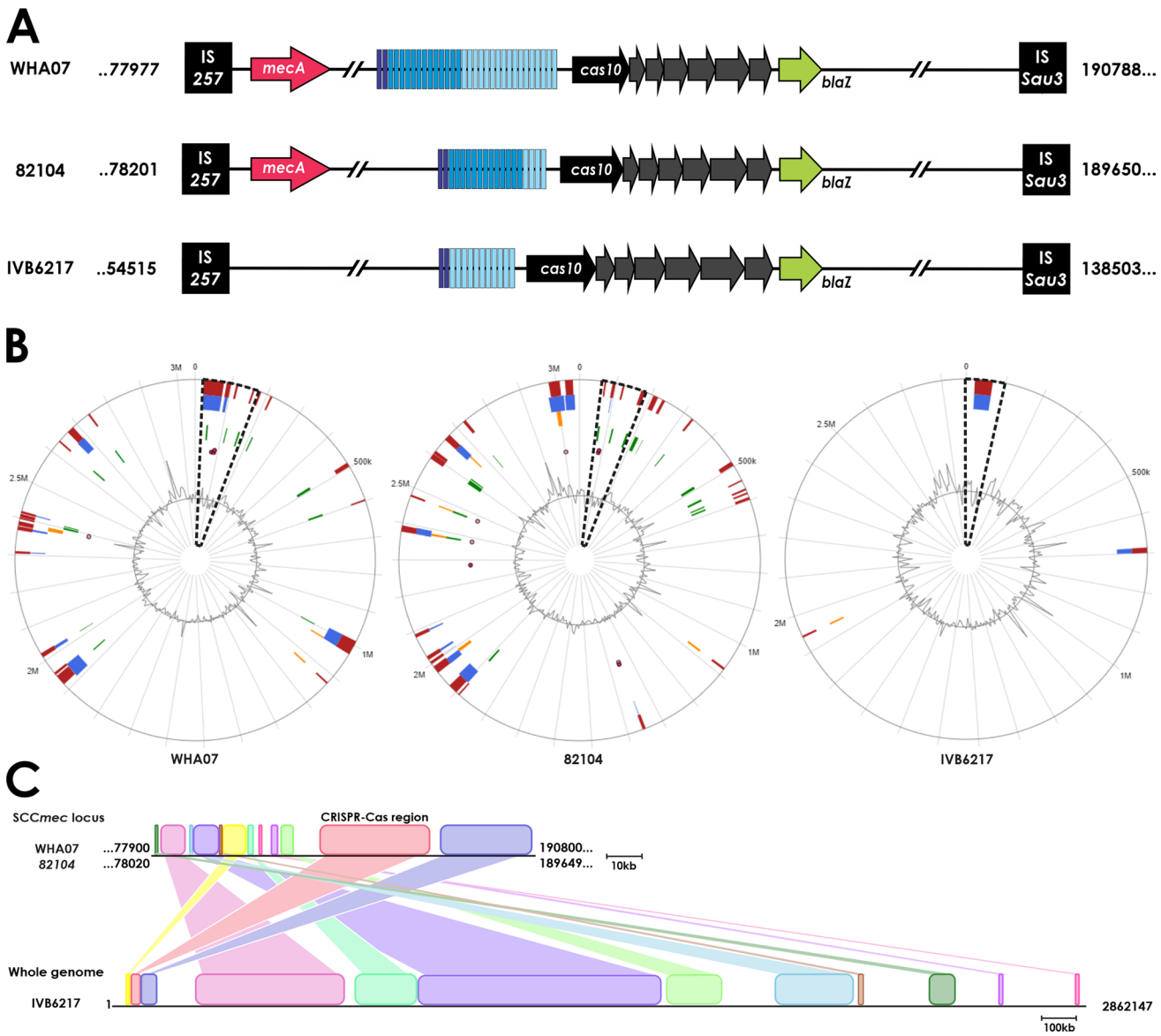

Fig. 4 *Mammalicoccus sciuri*'s CRISPR location within SCC*mec*s. (**A**) Flanking insertion sequences of the *Mammaliicoccus sciuri* CRISPR-Cas systems and colocalization with the beta-lactam resistance genes *mecA* and *blaZ*. (**B**) Localization of the CRISPR flanking region within genomic islands, highlighted in *Mammaliicoccus sciuri*'s genome by dotted lines. (**C**) Genetic synteny between the SCC*mec* from strains WHA07 and 82,104, and that of the strain IVB6217

otitis in Switzerland (Descloux et al., 2008). Additionally, a similar genomic segment is shared with *Staphylococcus capitis* TW2795, a strain isolated from humans in Japan.

Discussion

While the recent reclassification of *Mammaliicoccus sciuri* into a distinct genus, separate from *Staphylococcus* (Madhaiyan et al., 2020), suggests an evolutionary divergence, the clinical significance of this species within the *Staphylococcaceae* family remains nuanced. This is especially important when considered within the framework of a One Health context, which recognizes the need of studying bacterial dispersion, horizontal gene transfer, and antimicrobial resistance emergence as interconnected aspects of animal health, human health, and the environment (Hernando-Amado et al., 2019). Despite generally being considered a commensal with low pathogenic potential, its documented involvement in infections (de Carvalho et al., 2022; Sacramento et al., 2022), particularly in veterinary contexts, underscores the need to comprehend its biology, genomic characteristics, and host interactions.

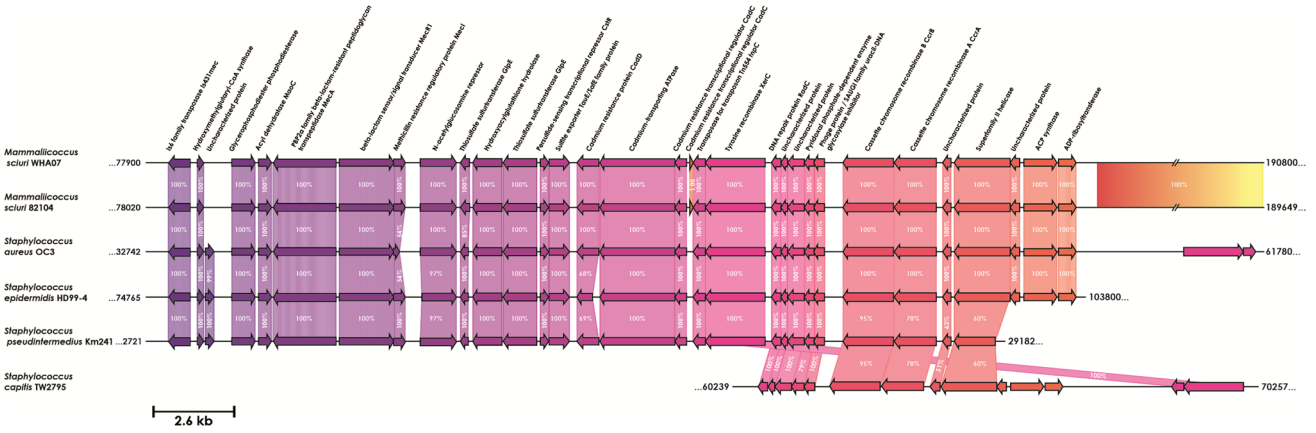

Fig. 5 Genetic synteny between the SCC*mec* from *Mammaliicoccus sciuri* strains and several species of *Staphylococcus* of human and animal origins, with emphasis on the presence of various antimicrobial resistance genes. Numbers between the alignments indicate the homology between the sequences

In previous studies, we emphasized that some underestimated *Staphylococcaceae* species could pose a significant threat to public health due to their potential to act as reservoirs of resistance genes for more pathogenic species, such as *S. aureus*, thereby enhancing their ability to resist available antimicrobial therapies (Rossi et al., 2020). These findings align with the scarcity of restrictive systems for mobile elements in these bacteria, which appears to favor inter-species horizontal gene transfer. For instance, CRISPR-Cas systems, found in 50% of bacterial genomes (Makarova et al., 2020), are present in only 3 to 4% of *Staphylococcaceae* (Rossi et al., 2017a, 2017b).

This low proportion of CRISPR-Cas systems, as observed in *Staphylococcus* spp., persists in *M. sciuri*. Although the pan-immune system of *M. sciuri* described here comprises at least 24 different types of defense systems, their overall proportion is low. However, the diversity of systems suggests that this array ensures versatility in combating various invasive agents, thereby safeguarding bacterial survival or fitness at a population level rather than an individual one, as proposed for other species (Bernheim & Sorek, 2020). Conversely, while these systems ensure species perpetuity, their scarcity increases susceptibility to the entry of exogenous genetic material, especially plasmids and bacteriophages, likely facilitating horizontal gene transfer.

The majority of systems found in *M. sciuri* genomes are described as active against bacteriophages primarily. Abortive infection systems (Abi), for example, encompass diverse mechanisms but share the common function of guiding the infected cell to self-sacrifice during phage infection, preventing viral spread and ensuring population survival (Lopatina et al., 2020). On the other hand, restriction-modification (RM) systems involve at least two enzymes: one responsible for cleaving specific sequences in the target DNA, and another for modifying the same sequence in the bacterial DNA, mostly through methylation, thereby preventing it from being cleaved as well (Sneppen et al., 2015).

CRISPR-Cas systems, besides targeting invasive bacteriophages, provide adaptive immunity against plasmids in *Staphylococcaceae* (Hatoum-Aslan et al., 2014), which likely explains their scarcity among family members, given the importance of horizontal gene transfer for the evolution of these organisms. Here, the presence of identical spacers in the same organization order, a consequence of their known chronological incorporation, reinforced the zoonotic nature of *M. sciuri*. It not only witnessed the multi-host and multi-regional dispersal of its strains but also of the SCC*mec* carrying this element.

SCC*mec* elements are mobile genetic elements found in *Staphylococcaceae*, responsible for methicillin resistance. They consist of at least the *mec* gene complex, which includes the *mecA* gene encoding penicillin-binding protein 2a (PBP2a)—or the emerging *mecB* and *mecC* genes, conferring resistance to beta-lactam antibiotics, and the *ccr* gene complex, which includes recombinase genes responsible for the mobility of these elements. SCC*mec* elements vary in size and genetic composition, resulting in different SCC*mec* types (I to XV). These variations contribute to the diversity of methicillin-resistant staphylococcal strains (Wolska-Gębarzewska et al., 2023). The primary function of SCC*mec* elements is to provide antibiotic resistance, posing a significant challenge in treating staphylococcal infections and major public health concern. In the case of *M. sciuri* shown here, SCC*mec* elements also carry additional genes related to resistance and virulence, enhancing the adaptability of this species.

As new SCC*mec* elements carrying CRISPR-Cas systems are described, previously found in *S. pseudintermedius*, *S. schleiferi*, *S. capitis*, and *S. aureus* (Rossi et al., 2019), the data highlight the one health nature of horizontal transfer

among *Staphylococcaceae*. They also raise questions about the significance of the association between these two genetic elements. Here, we propose that, considering the fitness cost that an SCC*mec* may represent for the hosting cell, given its size of several tens of thousands of base pairs, including a CRISPR-Cas system inside it could function as protection against the acquisition of new elements, thereby ensuring the maintenance of bacterial fitness.

Although all CRISPR-Cas systems carried by SCC*mec*s are of type III—type II systems are also found in *Staphylococcaceae* (Rossi et al., 2017b)—those of *M. sciuri* seem to have diverged long ago from those found in *Staphylococcus* spp., as they do not share common spacers. Besides, the fact that none of these spacers have homologs in other sequences in the GenBank databases highlights an underrepresentation of *M. sciuri* mobile element sequences in the databases. This gap in knowledge about the genomics of this bacterium is noteworthy, given that mobile genetic elements are among the major players in the genetic plasticity of *Staphylococcaceae*. Insertion sequences, in particular, present in dozens of copies throughout the genome of these bacteria, are involved in transposition events responsible for a wide alteration of microbial genomic organization, serving as a strategy for rapid adaptation and evolution of *Staphylococcaceae* pathogenicity (Bouchami et al., 2016). The same seems to occur in *M. sciuri*, as, although the same SCC*mec* sequences are present in all three strains studied here, in one of them, the distribution of some genes has diversified throughout its genome.

Studies characterizing SCC*mec* elements of *M. sciuri* on livestock farms have emerged, drawing attention to the ability of these bacteria to carry not only the *mecA* gene—the primary public health concern regarding *Staphylococcus* currently—but also another methicillin resistance gene, *mecC* (de Moura et al., 2023). Single nucleotide polymorphism analyses of strains obtained from both humans and animals further suggest cross-contamination (Dhaouadi et al., 2022).

Here, we demonstrate that the SCC*mec*s of *M. sciuri* strains share a significant portion of their sequences that exhibits a high degree of homology with various potentially pathogenic *Staphylococcus* species isolated from both humans and animals. The fact that these shared sequences not only carry the *mecA* gene but also various other genes associated with stress resistance, antimicrobial resistance, and pathogenicity factors underscores that *M. sciuri* plays an equally important role, alongside its relatives, in the dynamics of genetic sharing within the family.

Because of this, it is crucial to dedicate more studies to this ubiquitous species and exercise caution regarding the selective pressure imposed on *M. sciuri* strains, especially in veterinary practice. This becomes even more important as evidence points to the irrefutable interconnectivity and inseparability between the environment, animal health, and human health.

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Author contributions ACA and CCR: Conceptualization, data curation, investigation, writing – original draft. ACA: Methodology. ACA, MGM and CCR: Writing – review & editing. MGM and CCR: Funding acquisition and visualization. CCR: Project administration, Supervision.

Data availability Data available on request from the authors.

Declarations

Conflict of Interest None to declare.

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