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Massive Infection of Seabird Ticks with Bacterial Species Related to *Coxiella burnetii*

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Seabird ticks are known reservoirs of bacterial pathogens of medical importance; however, ticks parasitizing tropical seabirds have received less attention than their counterparts from temperate and subpolar regions. Recently, *Rickettsia africae* was described to infect seabird ticks of the western Indian Ocean and New Caledonia, constituting the only available data on bacterial pathogens associated with tropical seabird tick species. Here, we combined a pyrosequencing-based approach with a classical molecular analysis targeting bacteria of potential medical importance in order to describe the bacterial community in two tropical seabird ticks, *Amblyomma loculosum* and *Carios* (*Ornithodoros*) *capensis*. We also investigated the patterns of prevalence and host specificity within the biogeographical context of the western Indian Ocean islands. The bacterial community of the two tick species was characterized by a strong dominance of *Coxiella* and *Rickettsia*. Our data support a strict *Coxiella*-host tick specificity, a pattern resembling the one found for *Rickettsia* spp. in the same two seabird tick species. Both the high prevalence and stringent host tick specificity suggest that these bacteria may be tick symbionts with probable vertical transmission. Detailed studies of the pathogenicity of these bacteria will now be required to determine whether horizontal transmission can occur and to clarify their status as potential human pathogens. More generally, our results show that the combination of next generation sequencing with targeted detection/genotyping approaches proves to be efficient in poorly investigated fields where research can be considered to be starting from scratch.

Ticks are considered vectors of major medical and veterinary importance. Their peculiar ecology, including their extended life spans and ability to transfer pathogens horizontally via cofeeding (1), gives these arthropod vectors the additional and important role of pathogen reservoirs (2). Among ticks, those parasitizing wild birds have been proposed to play an additional epidemiological role, given the potential of some bird species to play a part in the long-distance dispersal of ticks and tick-borne pathogens (2, 3).

Seabird ticks have received little attention, despite the fact that ticks in sometimes uninhabited and geographically remote areas have been shown to harbor human pathogens (4–6). The high mobility of seabirds and their wide geographical distribution, together with their gregarious breeding behaviors, may result in these hosts and their ticks playing an important epidemiological role (5). Many attempts to characterize pathogen circulation in seabird ticks have been restricted to viruses (5, 7), while bacteria of epidemiological importance, such as the Lyme disease bacterium *Borrelia burgdorferi sensu lato*, have been described in the seabird tick *Ixodes uriae* worldwide (4, 8–11). Despite these data on some important bacterial pathogens, to date, little is known about the broader array of bacterial communities in seabird ticks under natural conditions.

Seabirds represent the most abundant and widespread avifauna of the western Indian Ocean (WIO) (12). Located at the intersection of major intercontinental and transoceanic migratory routes between Europe, Africa, Asia, Oceania, and the subantarctic islands (13–15), the WIO, which encompasses distant and mostly uninhabited islands, is prone to pathogen introduction and emergence (16). Recent disease outbreaks related to birds (e.g., West Nile virus in Madagascar, Sindbis virus in Mauritius [17]) support this idea. Two distinct tick species have been documented from seabirds breeding on islands of the WIO: a hard tick species (*Amblyomma loculosum*) and a soft tick species (*Carios* [*Ornithodoros*] *capensis*). These species exhibit significant ecological differences, which may entail differences in infection patterns: *C. capensis* ticks are typically nest-associated ticks with a strong specificity for seabirds, while *A. loculosum* ticks are generalist ectoparasites able to feed on reptiles, birds, and mammals (18, 19). While a recent study showed the circulation of *Rickettsia* pathogens in these two tick species in the WIO (20), information pertaining to the epidemiological role of tropical seabird ticks in broader bacterial circulation is scarce.

In this study, we evaluated the potential of a metagenomic approach to describe the bacterial community in seabird ticks from islands of the western Indian Ocean and to detect potential tick-borne bacterial pathogens of epidemiological importance in this region. We evaluated the role of the tick species and distribution in structuring bacterial communities by comparing bacterial diversity between *A. loculosum* and *C. capensis* ticks within the same island and in *C. capensis* ticks collected from different is-

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lands, respectively, and investigated the prevalence of *Coxiella* bacteria in soft and hard ticks of the region.

MATERIALS AND METHODS

Tick collection. Ticks were collected from different seabird species in 2011 and 2012 on six islands of the WIO: La Réunion Island, Bird and Aride Islands (the Seychelles), and Europa, Juan de Nova, and Tromelin Islands (the Eparses Islands). Ticks were collected either directly from adults and chicks or in the nest environment, as follows: soft ticks were sampled on red-tailed tropicbirds (*Phaeton rubricauda*) on Europa Island, on red-footed booby (*Sula sula*) specimens and nests on Tromelin Island, within reproductive colony boundaries of sooty terns (*Onychoprion fuscatus*) on Juan de Nova Island, and in wedge-tailed shearwater (*Puffinus pacificus*) nests on Petite Ile (La Réunion Island); hard ticks were collected on the ground of Petite Ile (La Réunion Island). Both soft and hard ticks were collected from a sooty tern colony at Bird Island. On Aride Island, ticks were collected from sooty terns (*Onychoprion fuscata*), tropical shearwaters (*Pufinus bailloni*), brown noddies (*Anous stolidus*), lesser noddies (*Anous tenuirostris*), white-tailed tropicbirds (*Phaeton lepturus*), and wedge-tailed shearwaters (*Pufinus pacificus*). All ticks were morphologically identified as *A. loculosum* or *C. capensis* using standard taxonomic keys (21, 22). Depending on the access to liquid nitrogen tanks, ticks were either conserved by dry flash freezing or by placing them in 70% ethanol until the samples were brought back to the laboratory and frozen at −80°C before analyses.

Sampling in La Réunion Island and in the Eparses Islands (Europa, Juan de Nova, and Tromelin Islands) was conducted under the approval of the Direction de l'Environnement, de l'Aménagement et du Logement and the Terres Australes and Antarctiques Françaises. Sample collection at Aride and Bird Islands and sample export to La Réunion Island were performed with the approval of the Seychelles Bureau of Standards and the Ministry of Environment.

Bacterial metabarcoding design. Ticks analyzed through metabarcoding were randomly selected from samples of 3 distinct and geographically distant sites, i.e., Juan de Nova, Tromelin, and La Réunion Islands. Pooled tick samples consisted of 5 adult ticks for both *C. capensis* and *A. loculosum*. For each pool, ticks were placed in a single 1.5-ml Eppendorf tube and crushed for 2 min at 25 Hz using two 3-mm tungsten beads in a TissueLyser apparatus (Qiagen, Valencia, CA). Total nucleic acids were extracted using a V2 minikit (Qiagen, Valencia, CA), and each pool was prepared for bacterial 16S rRNA metabarcoding (23) with the use of the GsFLX technology (Genoscreen, Lille, France). Briefly, 16S rRNA V3 and V4 variable regions were amplified via specific PCR primers targeting the upstream and downstream regions of the V3–V4 segment: the 3′ ends of forward (TACGGRAGGCAGCAG) and reverse (GGACTACCAGGGTATCTAAT) bacterium-specific primers were associated at the 5′ end with multiplex identifier (MID) tags, a GsFLX key, and GsFLX adapters. Each pool was independently amplified twice with distinct MID tags, allowing the individual identification of each pool, as well as each of the two PCR duplicates. The quantity of each PCR product was then determined with a PicoGreen kit, and all products were mixed together in equimolar concentrations prior to 454 GsFLX sequencing.

Sequence analyses and species identification. All 454 GsFLX sequences were separated into their respective pools by MID identification, performed using the Geneious software package (24). Those sequences that were less than 250 bp in length or that contained sequence ambiguities were discarded. Grouped sequences were exported in the FASTA format, and a locally executed nucleotide BLAST search was performed using the BLAST+ program, downloaded from the NCBI website (25). The reference 16S rRNA database used was also obtained from the NCBI file listings. One hundred alignments and 10 descriptions were exported for each BLAST result; all other search parameters were retained in their default settings. The MEGAN (v5) program was used to assign the BLAST results to bacterial taxa and to visualize the data (26). Normalized data sets were compared in MEGAN (v5), and unassigned reads were excluded from the analyses. The new minimum taxon cover functionality of MEGAN (v5) was exploited to provide accurate BLAST hit assignments within taxonomic trees, and the new core biome and shared biome functions were used to generate representations of the major bacterial groups present in each pooled sample. Grouped sequence similarities were compared using the principle component analysis (PCoA) methodology, and Shannon and Simpson indices were calculated from the leaves of a fully expanded taxonomic tree, irrespective of the taxonomic level of classification.

***Coxiella* PCR detection and genotyping.** Due to the high prevalence of *Coxiella* detection in all tick DNA pools (see Results), the 16S rRNA gene was amplified using endpoint PCR, as previously described (27). Positivity was assessed on agarose gels, and randomly selected PCR products were sequenced for each tick species and for samples from each island. Sequences were aligned with those of known *Coxiella* symbionts as well as with the sequence of *Coxiella burnetii* using the ClustalW program and subsequently trimmed, and the alignment was eventually refined over 100 iterations using the MUSCLE algorithm implemented in the Geneious Pro (v5.6.0) software package (24). A maximum likelihood phylogenetic tree with 1,000 bootstrap iterations was generated using the PhyML plugin from Geneious Pro (v5.6.0) and the optimal substitution model (GTR+I+G), as selected using the jModelTest (v2.1.2) tool (28). The appearance of the tree was edited in the FigTree (v1.4) program.

Nucleotide sequence accession numbers. Partial 16S rRNA gene sequences have been submitted to GenBank and may be found under accession numbers KF913893 to KF913928.

RESULTS

Bacterial identification by pyrosequencing. A total of 125,880 sequences were obtained from the four tick pools. The lengths of the sequences ranged from 30 to 568 bp, and 12,851 (10%) were shorter than 250 bp. The average and median read lengths were 401 and 442 bp, respectively, and sequence reads were evenly distributed across each multiplexed pool. Sequences were filtered using quality criteria to exclude reads containing ambiguities and/or sequences with length of less than 250 bp.

Independent sequencing repeats generated highly similar taxonomic representations, whereas samples from different islands or tick species generated characteristic diversity profiles (Fig. 1A). The overall levels of bacterial diversity measured in samples from *A. loculosum* from La Réunion Island and *C. capensis* from La Réunion and Tromelin Islands were comparable, whereas a greater level of diversity was obtained from *C. capensis* ticks from Juan de Nova Island (Fig. 1B and Table 1). However, rarefaction curves based on the leaves of a fully expanded taxonomic tree in MEGAN (v5) plateaued for each of the obtained samples, suggesting that the biodiversity of each sample had been exhaustively categorized.

Of the sequence reads assigned with MEGAN to a minimum taxonomic level of order, the bacterial orders *Legionales*, *Rickettsiales*, *Bacillales*, *Actinomycetales*, and *Rhodobacterales* were the most prominently represented across the studied tick samples (Fig. 1C).

Core and shared biomes and genera of possible medical importance. Characterization of the core bacterial biome constituted by combining data from all pooled samples identified *Coxiella*, *Bacillus*, and *Rickettsia* as the three most prominent bacterial genera present in the tested ticks of the region (Fig. 2D).

Interestingly, two of the most abundant genera identified in the tested ticks included known human pathogens: *Rickettsia*, the causative agent of rickettsioses such as African tick bite fever, has previously been identified to be abundant in ticks of the region

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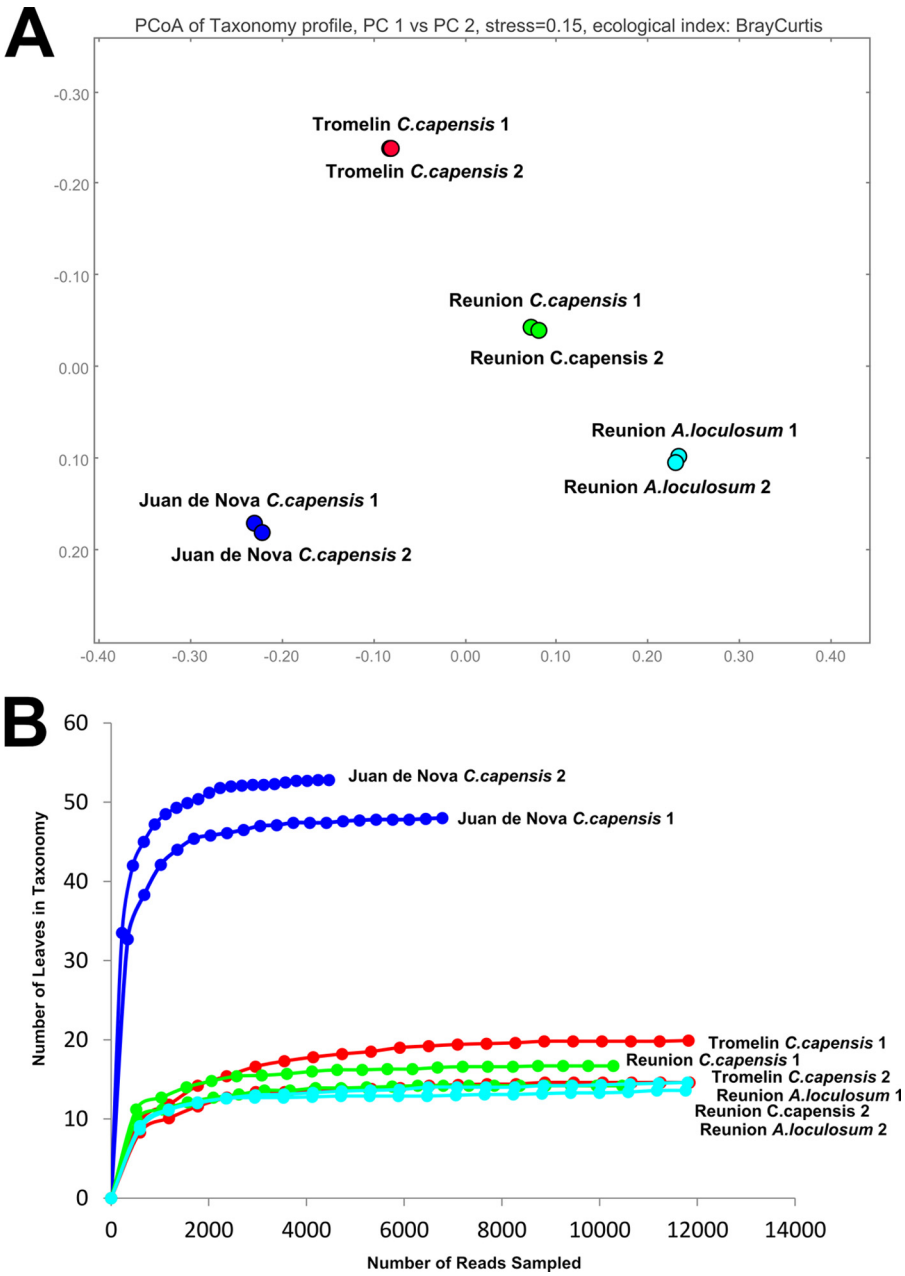

FIG 1 Pyrosequencing data. (A) Similarity testing by principle component analysis on the basis of the Bray-Curtis ecological index of fully expanded comparative taxonomic trees in the MEGAN (v5) program. PC1 is presented on the horizontal axis, and PC2 is presented on the vertical axis. (B) Rarefaction curves generated from data assigned to the leaves of fully expanded comparative taxonomic trees.

(20) and was observed to be prevalent within samples of *A. loculosum* from La Réunion Island. *Rickettsia* bacteria were also present but were less well represented in some samples of *C. capensis*. In contrast, *Coxiella* bacteria were present in large quantities in samples of *C. capensis* but at low levels in *A. loculosum*. *Coxiella* sequences exhibited between 98.4 and 98.7% genetic identities with *Coxiella burnetii*, the causative agent of Q fever (29). Interestingly *Borrelia* spp., the causative agents of Lyme disease and relapsing fever, were also identified in *C. capensis* from Tromelin Island; however, they were present only in trace quantities (13 of 24,797 reads). Additionally, *Kocuria* bacteria, which are increasingly associated with human pathologies (30), were identified in *A. loculosum*.

***Coxiella* prevalence in seabird ticks.** Although *Coxiella* spp. were found to be ubiquitous from 454 pyrosequencing, precise species identification and measures of population prevalence were impossible to obtain from these data. Thus, we set out to characterize the regional *Coxiella* prevalence by targeted PCR and classical Sanger sequencing using *Coxiella*-specific primer pairs that target the 16S rRNA gene (31).

Targeted PCR was performed on the full bank of available individual tick samples, which consisted of *C. capensis* and *A. locu-*

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TABLE 1 Summary of pyrosequencing data

Sample origin	Tick species	No. of ticks	Replicate no.	MID used	No. of raw reads	No. of reads after quality control	Shannon index	Simpson index
Tromelin Island	*Carios capensis*	5	1	MID1	14,743	13,652	1.63	2.48
			2	MID2	15,738	14,243	1.61	2.46
Juan de Nova Island	*Carios capensis*	5	1	MID3	15,115	13,423	3.71	6.88
			2	MID7	17,259	15,292	4.23	11.07
La Réunion Island	*Carios capensis*	5	1	MID9	13,691	12,417	1.79	2.02
			2	MID10	16,395	14,838	1.63	1.88
La Réunion Island	*Amblyomma loculosum*	5	1	MID11	17,336	15,746	1.47	2.27
			2	MID12	15,065	13,418	1.54	2.35

losum specimens collected from seabirds and their nests in Juan de Nova, Tromelin, Europa, Bird, Aride, and La Réunion Islands (Fig. 3). The *Coxiella* detection prevalence ranged from 46% to 100% (Table 2). Of all *C. capensis* ticks tested, 65.7% were positive for *Coxiella*, whereas this figure was 71.2% for *A. loculosum*. The prevalence of *Coxiella* bacteria was significantly lower in *C. capensis* ticks from La Réunion Island than in *C. capensis* ticks from the other locations ($P < 0.001$ by Fisher's exact test in all pairwise comparisons).

***Coxiella* diversity shows a strict tick-host specificity.** Phylogenetic analyses demonstrated that all *Coxiella* strains originating from the WIO were closely related to *Coxiella burnetii* and demonstrated nearly perfect species specificity for their tick hosts (Fig. 4). Two well-supported, separate bacterial clades were identified in hard ticks (e.g., *A. loculosum*, *Amblyomma americanum*, *Amblyomma cajennense*, and *Haemaphysalis longicornis*) and soft ticks (*C. capensis* and *Ornithodoros moubata*). Additionally, distinct *Coxiella* clades were identifiable for *C. capensis* and *A. loculosum* hosts. Furthermore, two distinct *Coxiella* haplotypes were identified in *C. capensis* ticks: one in a single *P. pacificus* nest in Petite Ile and the other in all other sequenced samples from the WIO. Genetic distances revealed that *Coxiella* strains from *C. capensis* were the most closely related to *Coxiella burnetii*. The mean ± standard deviation genetic identities to the *C. burnetii* group of the phylogenetic groups presented in Fig. 4 were as follows: 98.7% ± 0.19% for *C. capensis* symbiont group 1, 98.9% ± 0.31% for *C. capensis* symbiont group 2, 96.4% ± 0.21% for the *Amblyomma* species symbiont group, and 96.2% ± 0.20% for the *Hemaphysalis longicornis* symbiont group.

DISCUSSION

The present investigation shows that a combination of nontargeted 454 pyrosequencing with a subsequent specific investigation of a taxon of interest quickly provides a powerful set of data for

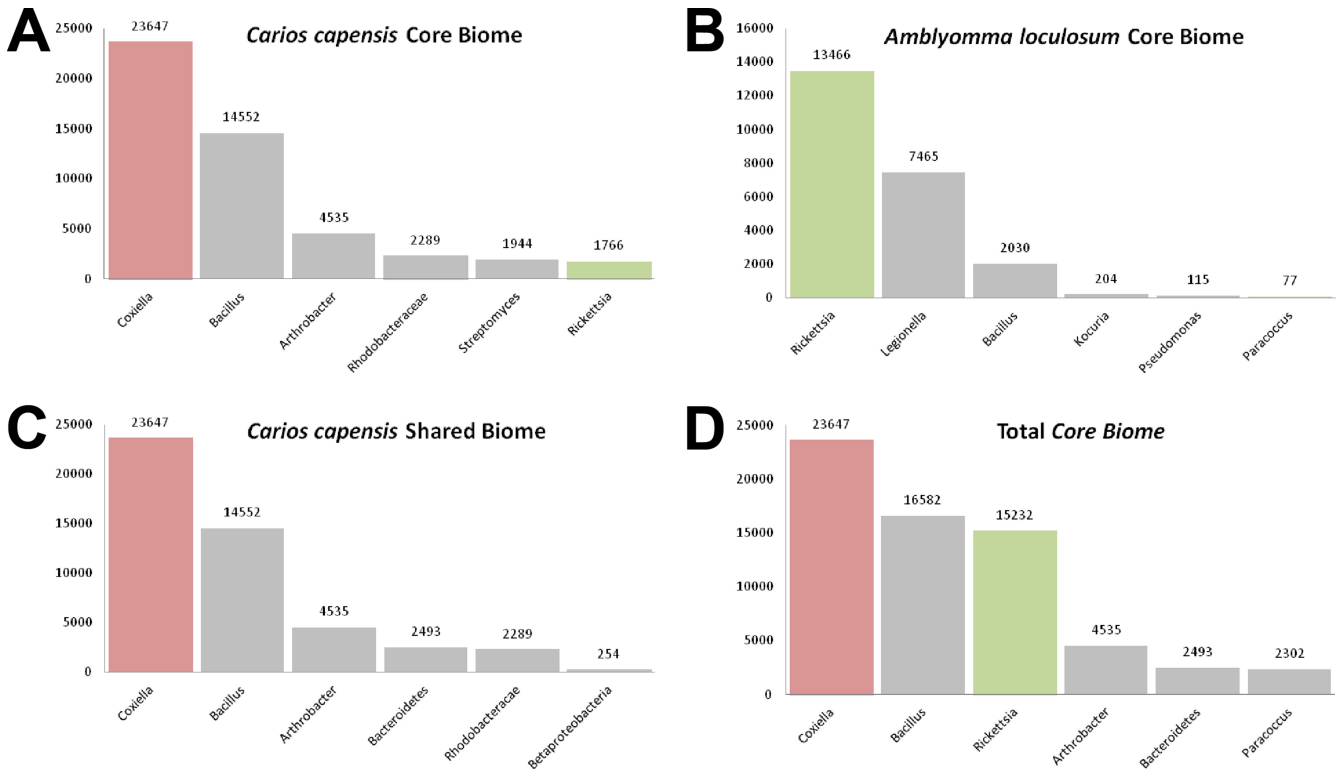

FIG 2 Bacteriome data from ticks of the WIO. Bars indicate the number of raw sequence reads assigned to named bacterial taxa. The numbers of sequences assigned to the named taxa are indicated above the bars. Bars corresponding to *Coxiella* spp. and *Rickettsia* spp. are colored red and green, respectively.

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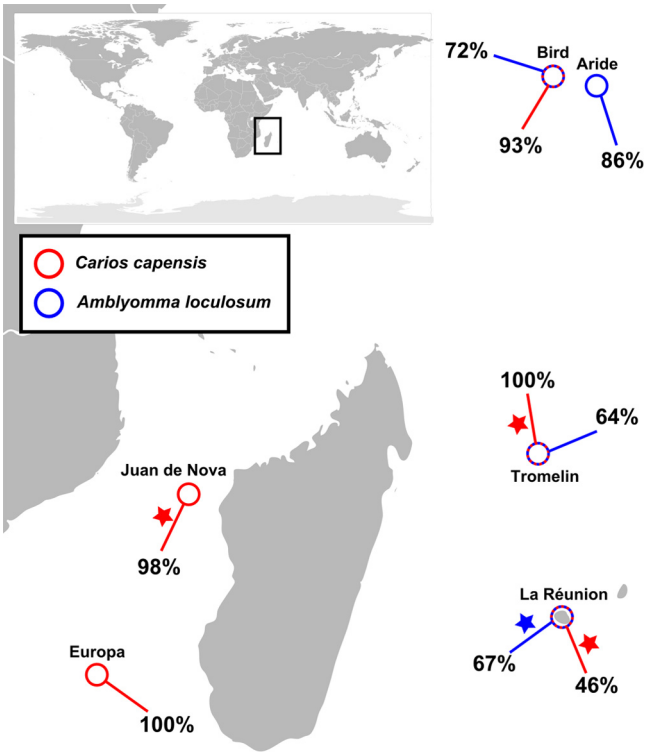

FIG 3 *Coxiella* in ticks of the southwestern Indian Ocean (SWIO) islands. The prevalence of *Coxiella* spp. detected in tick samples from different locations of the SWIO is shown. Stars, samples that were included in the 16S rRNA pyrosequencing analyses.

poorly investigated biological material, such as tropical seabird ticks. Products from two independent PCRs carried out on DNA from four independent pooled tick samples were sequenced, and sequencing indicated little intrasample variability, which may have occurred due to the experimental variability in deep sequencing and/or PCRs. This suggests that increasing the number of samples may be more relevant than duplicating PCRs and sequencing when designing such approaches.

The depth of the analysis provided by this next generation sequencing methodology was sufficient to identify bacterial species of *Kocuria* (an emerging human pathogen) and other genera that were present in minute trace quantities, such as *Borrelia* spp., which are known to be transmitted by seabird ticks (5) and which are the causative agents of Lyme disease and relapsing fever. Furthermore, high read numbers of *Rickettsia* and *Coxiella* spp. were obtained from the majority of tested tick samples, suggesting that ticks are a frequent and possibly amplifying reservoir host for these bacterial species. Our results corroborate recent work that has shown that these ticks are infected with the human pathogen *Rickettsia africae* (20). The investigation presented herein reveals massive infection of both hard and soft ticks with *Coxiella*-like organisms, with the bacteria infecting soft ticks presenting up to 99% identity with *C. burnetii*. The strict host specificity reported herein supports the vertical transmission of this bacterial species and the previously proposed long coevolution of both *Coxiella* lineages following an ancient infection in the ancestor of both main families of ticks: the Argasidae (soft ticks) and the Ixodidae (hard ticks) (31). We did not observe any bird species-associated specificity or any spatial structure, which suggests that the presented *Coxiella* spp. are likely transmitted among islands and bird species. However, the resolution of a 16S rRNA gene marker is not sufficient to completely address these points (O. Duron, personal communication), and the use of additional markers will be necessary to resolve the regional structure of this bacterial population.

Another important point raised by the 454 approach is that the results provide information not only on bacterial diversity but also on bacterial abundance, as previously discussed (32). Indeed, 29.5% of all bacterial 16S rRNA gene haplotypes were related to *Coxiella*, and another 19.0% were related to *Rickettsia*. Thus, although a quantitative PCR study of individual ticks will be required to determine bacterial copy numbers per host, our data show that these two bacterial genera are clearly prominent in *C. capensis* and *A. loculosum*.

The absence of some symbionts in the sample is also of interest. For instance, the absence of *Wolbachia* in these seabird ticks is coherent with the proposition of Plantard et al. that *Wolbachia* isolates that were previously detected in ticks had a parasitoid origin (33).

Two *C. capensis* ticks from the same nest of a wedge-tailed shearwater (*P. pacificus*) were infected with a clearly distinct *Coxiella* haplotype. Interestingly, the same two tick specimens were shown to be infected with two *Rickettsia* spp. substantially different from the other *Rickettsia* spp. infecting the WIO sample and were strongly related to *Rickettsia belli*, considered the earliest diverging *Rickettsia* species (34, 35). These two specimens may descend from a distinct tick population or lineage, as recently described for *C. capensis* (19), and thus be infected by distinct *Rickettsia* and *Coxiella* strains. Such a pattern would suggest that "a single bird with reservoir ticks may serve to seed a new geographic area," as previously suggested (36). A population genetics investigation of these ticks at a global scale may allow testing of this tempting but still speculative hypothesis.

Coxiella burnetii is the causative agent of Q fever, which can cause illness in many domesticated mammals, birds, and humans (37). *Coxiella*-like bacteria have now been identified in a number of distinct tick species (31, 38–42) and are considered symbionts with probable vertical transmission. Whether *Rickettsia* may be primarily considered an invertebrate symbiont or a vertebrate pathogen is still an open question (34, 43) and can be subjectively related to the main scientific interest of each researcher. Given the growing number of *Coxiella* species described in several distinct tick species and the prominent prevalence of this taxon in the

TABLE 2 Rate of detection of *Coxiella* spp. in ticks from WIO region

Tick species	Origin	No. of ticks:		Prevalence (%) of *Coxiella* detection
		Positive	Tested	
C. capensis	Tromelin Island	15	15	100
	Juan de Nova Island	42	43	98
	Europa Island	41	41	100
	La Réunion Island	87	189	46
	Bird Island	14	15	93
A. loculosum	Aride Island	25	29	86
	Tromelin Island	9	14	64
	La Réunion Island	37	55	67
	Bird Island	28	39	72

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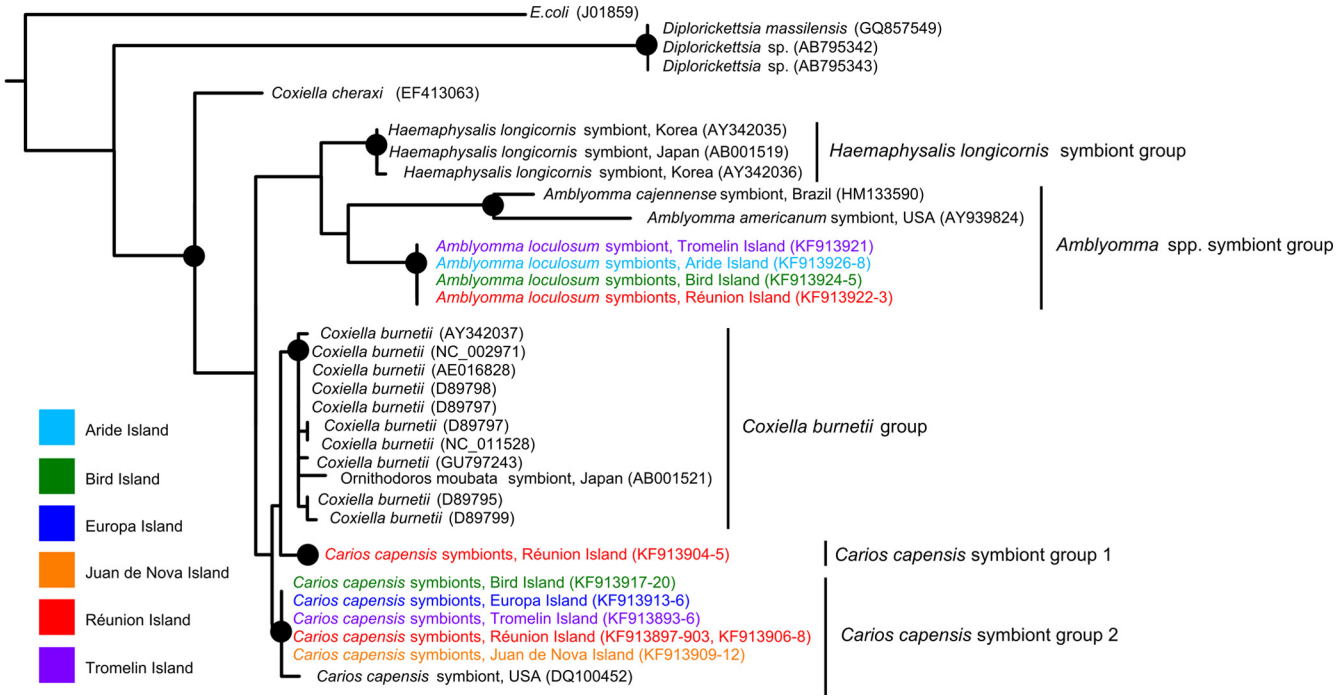

FIG 4 Phylogenetic relationships between the strains of *Coxiella* detected. The maximum likelihood phylogenetic tree was calculated with 1,000 bootstrap replicates of *Coxiella* MUSCLE-aligned partial 16S rRNA sequences from ticks, using selected sequences from GenBank (accession numbers are indicated in parentheses) as reference strains for *C. burnetii* and *C. cheraxi*. *Escherichia coli* (outgroup) and *Diplorickettsia* spp. (family *Coxiellacae*) were also included in the analysis. Dots indicate nodes with bootstrap support greater than 90%. The major phylogenetic clades are indicated on the right. Sequences generated as part of this study are indicated with a color code corresponding to the sampled island.

seabird ticks investigated herein, it seems that a proper assessment of the pathogenicity of these *Coxiella* species should today be considered an important issue.

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