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Original article

Phylogenetics of the *Spiroplasma ixodetis* endosymbiont reveals past transfers between ticks and other arthropods

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ABSTRACT

The bacterium *Spiroplasma ixodetis* is a maternally inherited endosymbiont primarily described from ticks but also found widespread across other arthropods. While it has been identified as a male-killing agent in some insect species, the consequences of infection with *S. ixodetis* in ticks are entirely unknown, and it is unclear how this endosymbiont spreads across tick species. Here, we have investigated this aspect through the examination of the diversity and evolutionary history of *S. ixodetis* infections in 12 tick species and 12 other arthropod species. Using a multi-locus typing approach, we identified that ticks harbor a substantial diversity of divergent *S. ixodetis* strains. Phylogenetic investigations revealed that these *S. ixodetis* strains do not cluster within a tick-specific subclade but rather exhibit distinct evolutionary origins. In their past, these strains have undergone repeated horizontal transfers between ticks and other arthropods, including aphids and flies. This diversity pattern strongly suggests that maternal inheritance and horizontal transfers are key drivers of *S. ixodetis* spread, dictating global incidence of infections across tick communities. We do not, however, detect evidence of *S. ixodetis*-based male-killing since we observed that infections were widely present in both males and females across populations of the African blue tick *Rhipicephalus decoloratus*.

1. Introduction

Ticks commonly harbor maternally inherited bacterial endosymbionts (Bonnet et al., 2017; Duron et al., 2017). Some of these endosymbionts, such as *Coxiella* and *Francisella*, are essential for the life cycle of ticks: they are obligate nutritional mutualists able to synthesize B vitamins that are deficient in the blood meal of ticks (Duron et al., 2018; Gerhart et al., 2016; Gottlieb et al., 2015; Smith et al., 2015). The experimental elimination of these symbionts results in decreased tick survival, fecundity and egg viability, as well as in some physical abnormalities (Duron et al., 2018; Guizzo et al., 2017; Li et al., 2018; Zhang et al., 2017; Zhong et al., 2007), which can be fully restored with an oral supplement of B vitamins (Duron et al., 2018). These obligate symbionts are, by definition, present in most individuals of a given tick species. However, many other maternally inherited bacteria, for instance *Arsenophonus* or *Rickettsiella*, are facultative endosymbionts: they are not essential for the life cycle of ticks and exhibit variable infection frequencies across tick populations (Cafiso et al., 2016; Clay et al., 2008; Duron et al., 2017, 2016; Lalzar et al., 2012). Facultative endosymbionts are expected to play various roles in tick fitness, reproduction or immunity. Their effects remain largely unstudied yet (Bonnet et al., 2017; Narasimhan and Fikrig, 2015).

Spiroplasma ixodetis (Mollicutes: Entomoplasmatales: Spiroplasmataceae) is one of the most intriguing facultative endosymbionts of ticks. The *Spiroplasma* genus includes a variety of commensal, pathogenic and mutualistic species of arthropods and plants (Gasparich, 2002). In 1981, a new *Spiroplasma* was isolated from extracts of Western black-legged ticks, *Ixodes pacificus* (Tully et al., 1981). This bacterium was morphologically and genetically distinct from known *Spiroplasma* species (Tully et al., 1983), and was formally described as *S. ixodetis* (Tully et al., 1995). Since its initial discovery, membership of the species *S. ixodetis* has expanded, and it is now known to be widespread in ticks: in addition to *I. pacificus*, it was found in *I. ricinus*, *I. arboricola*, *I. frontalis*, *I. uriae*, *I. ovatus*, *I. persulcatus*,

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several other undetermined *Ixodes* species, *Dermacentor marginatus*, *Rhipicephalus annulatus*, *R. decoloratus*, *R. pusillus* and *R. geigyi* (Bell-Sakyi et al., 2015; Duron et al., 2017; Henning et al., 2006; Hornok et al., 2010; Qiu et al., 2014; Taroura et al., 2005; Van Oosten et al., 2018).

Surveys of microbial diversity have further uncovered *S. ixodetis* in other arthropod groups, including spiders, mites, butterflies, leafhoppers, phasmids, aphids, various flies and coleopterans (Duron et al., 2008; Fukatsu et al., 2001; Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Martin et al., 2012; Martin et al., 2013; Sanada-Morimura et al., 2013; Simon et al., 2011; Tinsley and Majerus, 2006; DiBlasi et al., 2011; Pérez-Ruiz et al., 2015; Weinert et al., 2007). In all examined cases, *S. ixodetis* showed stable maternal (transovarial) transmission through successive host generations (Fukatsu et al., 2001; Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Tinsley and Majerus, 2006). In most insects, such as the African queen butterfly *Danaus chrysippus* and a variety of ladybird species, *S. ixodetis* acts as a male-killer: the viability and fertility of infected females is usually either not, or only mildly, affected, but *S. ixodetis* can be strongly virulent in males, often killing them (Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Sanada-Morimura et al., 2013; Tinsley and Majerus, 2006). The bacterium typically induces the death of male hosts during embryonic development and results in (almost) all-female broods from infected mothers (Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Sanada-Morimura et al., 2013; Tinsley and Majerus, 2006). Insect species harboring *S. ixodetis* thus show a sex bias in infection prevalence, with some females found infected while infection is never (or rarely) observed in males (Hurst et al., 1999; Jiggins et al., 2000; Majerus et al., 2000, 1999; Sanada-Morimura et al., 2013; Tinsley and Majerus, 2006). Male hosts being "dead ends" for transmission, male-killing benefits *S. ixodetis* transmission by favoring reproduction of infected female hosts through the reallocation of resources from males to females (e.g. by reducing sibling competition for food), or through the reduction of inbreeding depression (Engelstadter and Hurst, 2009). However, not all *S. ixodetis* members are male-killers since there is no evidence of sex bias in infection in spiders, leafhoppers or crane flies (Duron et al., 2008). The tree-hole tick *I. arboricola* is the single tick species examined for sex bias in *S. ixodetis* infection but no evidence of male-killing was found (Van Oosten et al., 2018).

How *S. ixodetis* infections have spread across tick species is still unclear. Phylogeny can be used to this aim by estimating proximity of current infections and retracing their evolutionary histories. To date, the 16S rRNA gene sequence has been used as an exclusive marker for the *S. ixodetis* description, with all known strains represented only by this gene (e.g. Bell-Sakyi et al., 2015; Duron et al., 2008; Fukatsu et al., 2001; Jiggins et al., 2000). However, this marker is inadequate for inferring a reliable intraspecific phylogeny since the inner topology is poorly resolved because of insufficient 16S rRNA sequence polymorphism. Therefore, in the present study, we characterized *S. ixodetis* variation in ticks and other arthropods at five genes (16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG*). We further attempted to characterize overall *S. ixodetis* diversity and to infer the evolutionary processes shaping this diversity. We additionally tested the African blue tick, *Rhipicephalus decoloratus* for possible evidence of male-killing activity, by assessing sex bias in *S. ixodetis* infection.

2. Material and methods

2.1. Tick collection

The multi-locus typing of *S. ixodetis* was conducted using a collection of DNA templates from 12 tick infected species (Table 1). Of the 12 tick species examined, seven belong to the *Ixodes* genus, four to *Rhipicephalus* and one to *Dermacentor*. Most specimens were collected on taxonomically diverse vertebrate hosts or their environment (Table 1). In addition, another collection of DNA templates from 12 arthropod species infected by *S. ixodetis* was used: it includes a parasitic mite species and 11 insect species (five species of Hemiptera and six of Diptera) (Table 1). For each DNA template, *S. ixodetis* infection had been previously confirmed through sequencing of the 16S rRNA gene (Duron et al., 2017, 2008), or of intergenic ribosomal spacer and adjacent regions (Martin et al., 2012, 2013). Within each examined species, there were one to four *S. ixodetis*-infected individuals separately investigated for the multi-locus typing analysis (Table 1).

The consequences of *S. ixodetis* infection for tick reproduction was examined using field-caught adult specimens of the African blue tick *R. decoloratus* sampled from three localities in South Africa and one in Benin (Table 2). All specimens were sexed on the basis of their morphological characters and further examined for a sex bias in *S. ixodetis* infection prevalence.

2.2. Molecular screening and typing

Each DNA template was obtained from individual extraction of tick whole body using the DNeasy Blood and Tissue Kit (QIAGEN) following manufacturer instructions. To design specific PCR primers for multi-locus typing, we used the draft genome of *S. ixodetis* (*S. ixodetis* Y32 type strain, early isolated from *I. pacificus* extracts by Tully et al., 1981, and further sequenced by X. Bailly, unpublished data) as a reference. Along with the 16S rRNA gene, we selected four other genes (*rpoB*, *dnaK*, *gyrA* and *EpsG*) present in a single copy within the *S. ixodetis* Y32 draft genome. Gene features, primers and PCR conditions are detailed in Table 3. Fragments of these five genes were amplified using nested or semi-nested PCR assays which were efficient at increasing sensitivity and specificity for *S. ixodetis* detection. The efficiency of our typing method was ascertained through positive PCR amplification and clear sequences for the five loci from DNA samples of *I. ricinus* infected by *S. ixodetis* as we further detailed in the Results section. The nested PCR assay targeting the 16S rRNA gene was further used to examine the specimens of *R. decoloratus* for sex bias in *S. ixodetis* infection prevalence.

Nested and semi-nested PCR amplifications were performed as follows: the first PCR run with the external primers was performed in a 10 μL volume containing ca. 20 ng of genomic DNA, 3 mM of each dNTP (Thermo Scientific), 8 mM of $MgCl_2$ (Roche Diagnostics), 3 μM of each primer, 1 μL of 10 × PCR buffer (Roche Diagnostics), and 0.5 U of Taq DNA polymerase (Roche Diagnostics). A 1 μL aliquot of the PCR product from the first reaction was then used as a template for the second round of amplification. The second PCR was performed in a total volume of 25 μL and contained 8 mM of each dNTP (Thermo Scientific), 10 mM of $MgCl_2$ (ThermoScientific), 7.5 μM of each of the internal primers, 2.5 μL of 10 × PCR buffer (Thermo Scientific), and 1.25 U of Taq DNA polymerase (Thermo Scientific). All PCR amplifications were performed under the following conditions: initial denaturation at 93 °C for 3 min, 35 cycles of denaturation (93 °C, 30 s), annealing (Tm = 49–56 °C, depending on primers, 30 s), extension (72 °C, 1 min), and a final extension at 72 °C for 5 min. To prevent possible contamination, first and second PCR runs have been physically separated from one another, in entirely separate rooms. Known positive and negative individuals were used as controls in each PCR assay. All PCR products were visualized through electrophoresis in a 1.5% agarose gel. All amplicons from nested and semi-nested PCR assays were purified and sequenced in both directions (EUROFINS). The chromatograms were manually inspected and cleaned with CHROMAS LITE (http://www.technelysium.com.au/chromas_lite.html) and sequence alignments were done using CLUSTALW (Thompson et al., 2002), both implemented in MEGA (Tamura et al., 2007). Alleles were determined on the basis of sequence identity (i.e. the number of characters which match exactly between two different sequences). If two sequences differed by one or more bases, they were described as two different alleles. Nucleotide sequences of *S. ixodetis* have been deposited in the GenBank nucleotide database (Accession numbers: 16S rRNA: MK248055-MK248068, MK241976,

Table 1
List and origin of tick and other arthropod species examined for multi-locus typing of *Spiroplasma ixodetis*.

Arthropod species	Origin	Number of DNA templates	Reference for DNA templates positive for *Spiroplasma ixodetis*
Arachnida: Ixodida: Ixodidae (hard ticks)			
Ixodes pacificus Cooley and Kohls, 1943	Hopland, Mendocino Co., California, 1985, on vegetation	1	(Duron et al., 2017)
Ixodes arboricola Schulze and Schlottke, 1930	Middelheim Park, Belgium, 2012, in nests of passerine birds	2	(Duron et al., 2017)
Ixodes frontalis (Panzer, 1798)	Belgium, specimens derived from engorged females collected in nests of passerine birds	2	(Duron et al., 2017)
Ixodes ricinus (Linnaeus, 1758)	Sénart, France, 2010, on European roe deer (*Capreolus capreolus*)	2	(Duron et al., 2017)
Ixodes uriae White, 1852	Bering Island, Russia, 2008, on common guillemot (*Uria aalge*)	1	(Duron et al., 2017)
Ixodes sp.1	Guiglo, Ivory Coast, 1994, on leopard (*Panthera pardus*)	1	(Duron et al., 2017)
Ixodes sp.3	Ariy Kamen Islet, Kamchatka, Russia, 2008, on red-legged kittiwake (*Rissa brevirostris*)	1	(Duron et al., 2017)
Dermacentor marginatus (Sulzer, 1776)	France, 2011 and 2013, on vegetation	2	(Duron et al., 2017)
Rhipicephalus annulatus (Say, 1821)	Gogonou, Benin, 2012, on zebu (*Bos indicus*)	1	(Duron et al., 2017)
Rhipicephalus decoloratus Koch, 1844	Sandvelt, South Africa, 2011, on southern eland (*Taurotragus oryx*)	2	(Duron et al., 2017)
Rhipicephalus geigyi (Aeschlimann and Morel, 1965)	Burkina-Faso, 2013, on zebu (*Bos indicus*)	1	(Duron et al., 2017)
Rhipicephalus pusillus Gil Collado, 1936	Montmarin, France, 2011, on European rabbit (*Oryctolagus cuniculus*)	1	(Duron et al., 2017)
Arachnida: Mesostigmata: Dermanyssidae (parasitic mites)			
Dermanyssus gallinae (De Geer 1778)	France, 2007-2008	4	This study
Insecta: Hemiptera: Aphididae (aphids)			
Acyrthosiphon pisum (Harris, 1776)	Tende, France, 2009	1	This study
Cinara confinis (Koch, 1856)	Montpellier, France, 2010	1	This study
Cinara maritimae Eastop & Hille Ris Lambers, 1976	Ondres, France, 2010	1	This study
Insecta: Hemiptera: Cicadellidae (leafhoppers)			
Cicadella viridis (Linnaeus, 1758)	L'Olme, France, 2006	1	(Duron et al., 2008)
Insecta: Hemiptera: Miridae (mirid bugs)			
Notostira elongata (Geoffroy, 1785)	L'Olme, France, 2006	1	(Duron et al., 2008)
Insecta: Diptera: Scathophagidae (dung flies)			
Ceratinostoma ostiorum (Curtis, 1832)	West Lothian, Scotland, United Kingdom	1	(Martin et al., 2012)
Scathophaga stercoraria (Linnaeus, 1758)	Lethbridge, Canada	1	(Martin et al., 2012)
Insecta: Diptera: Empididae (dance flies)			
Clinocera sp.	Lompret, Belgium	1	(Martin et al., 2013)
Insecta: Diptera: Dolichopodidae (long-legged flies)			
Ethiromyia chalybea (Wiedemann, 1817)	Denderleeuw, Belgium	1	(Martin et al., 2013)
Poecilobothrus chrysozygos (Wiedemann, 1817)	La Gué de la Chaine, France	1	(Martin et al., 2013)
Thrypticus tarsalis Parent, 1932	Meilegem, Kaaimeersen, Belgium	1	(Martin et al., 2013)

KY674400-KY674420, EU727097, EU727098; *rpoB*: MK267069-MK267097; *dnaK*: MK266994-MK267018; *gyrA*: MK267043-MK267068; *EpsG*: MK267019-MK267042).

2.3. Molecular phylogenetics

The GBLOCKS program (Castresana, 2000) with default parameters was used to remove poorly aligned positions and to obtain unambiguous sequence alignments. All sequence alignments were also checked for putative recombinant regions using the RDP3 computer analysis package which combines several non-parametric recombination detection methods (Martin et al., 2010). Phylogenetic relationships were evaluated between *S. ixodetis* strains using 16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* gene sequences from ticks and other arthropods. In addition to sequences produced in this study, additional *S. ixodetis* sequences, as well as a subset of sequences from other *Spiroplasma* species, available in GenBank were also used. The evolutionary models most closely fitting the sequence data were determined using Akaike information criterion with the MEGA program (Tamura et al., 2007). Phylogenetic analyses were based on maximum likelihood (ML) analyses. A ML heuristic search, using a starting tree obtained by neighbor-joining, was conducted and clade robustness was further assessed by bootstrap analysis using 1000 replicates in MEGA (Tamura et al., 2007).

2.4. Statistics

Statistical analyses (Fisher's exact test) were carried out using the R statistical package (http://www.r-project.org/).

3. Results

3.1. Multi-locus typing of *Spiroplasma ixodetis*

The diversity of *S. ixodetis* was examined using 16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* gene sequences. The *S. ixodetis* 16S rRNA gene was amplified from all DNA templates ($n = 32$), while the *rpoB*, *dnaK*, *gyrA* and *EpsG* genes were amplified from part of them ($n = 28$, 24, 25 and 23, respectively as shown in Table 4). The five genes were amplified from *S. ixodetis* of seven tick species ($n = 10$ specimens) and six other arthropod species ($n = 9$). Only one to four genes were amplified from the five remaining tick species ($n = 7$) and the six other arthropod species ($n = 6$). The failure of *rpoB*, *dnaK*, *gyrA* and *EpsG* PCR amplifications in some cases may be due to a consequence of mutations in the priming regions. All sequences were easily readable without double

Table 2
Populations of *Rhipicephalus decoloratus* examined for sex bias in infection prevalence of *Spiroplasma ixodetis*.

Population	Vertebrate host	*n*			Number of infected specimens		
		males	females	males and females	males	females	males and females
Vaalwater, South Africa, 2010	Zebu (*Bos indicus*)	10	9	19	2 (0.20%)	0 (0%)	2 (0.11%)
Sandvelt, South Africa, 2011	Blue Wildebeest (*Connochaetes taurinus*), Greater Kudu (*Tragelaphus strepsiceros*) and Southern Eland (*Taurotragus oryx*)	10	16	26	4 (0.40%)	9 (0.56%)	13 (0.50%)
Hopestatt, South Africa, 2012	Zebu (*Bos indicus*)	10	13	23	0 (0%)	0 (0%)	0 (0%)
Gogonou, Benin, 2012	Zebu (*Bos indicus*)	0	6	6	0 (0%)	0 (0%)	0 (0%)
All populations	-	30	44	74	6 (0.20%)	9 (20.5%)	15 (20.3%)

Table 3
List of genes and primers used for *Spiroplasma ixodetis* typing.

Gene	Hypothetical product	Primers (5'-3')	Tm and Fragment size	References
16S rDNA	Small ribosomal subunit	Spixo_F2- TTAGGGGCTCAACCCCTAACCGC Spixo_R2- CCCTGCCTTTGGCAGCTCCCTCC Spixo_F2- TTAGGGGCTCAACCCCTAACCGC Spixo_R1- TCTGGCATTGCCAACTCTCGTGG	First PCR run: 56 °C, Spixo_F2/Spixo_R2: 865 bp Second PCR run: 56 °C, Spixo_F2/Spixo_R1: 822bp	This study (modified from Duron et al., 2008)
rpoB	DNA-directed RNA polymerase β chain	Spixo_rpoB_F1- TTGTTGGTACGGGTGTTGAG Spixo_rpoB_R2- CATCAAAAACAGGRGTTGCT Spixo_rpoB_F2- GGACCTTCRATGAAAGATGG Spixo_rpoB_R1- CAACCGGTGTTCCATCTTCT	First PCR run: 52 °C, Spixo_rpoB_F1/Spixo_rpoB_R2: 984 bp Second PCR run: 54 °C,Spixo_rpoB_F2/Spixo_rpoB_R1: 628bp	This study
dnaK	Chaperone protein DNAK	Spixo_dnaK_F1- GCTGCCATTGCTTATGGTTT Spixo_dnaK_R2- ACACCTCTTGGTGCTGGTTC Spixo_dnaK_F2- TGCTGCTGAAAAAGCAAAAA Spixo_dnaK_R1- ACAGCGGGTTGATTGTCTTC	First PCR run: 52 °C, Spixo_dnaK_F1/Spixo_dnaK_R2: 871bp Second PCR run: 49 °C, Spixo_dnaK_F2/Spixo_dnaK_R1: 515bp	This study
gyrA	DNA gyrase subunit A	Spixo_gyrA_F1- TCAAGAGCTTTACCAGATGCAA Spixo_gyrA_R- TGCACCTGTAGGAAAGTCAGG Spixo_gyrA_F2- CCAGATGCAAGAGATGGATTG	First PCR run: 54 °C, Spixo_gyrA_F1/Spixo_gyrA_R: 573bp Second PCR run: 54 °C, Spixo_gyrA_F2/Spixo_gyrA_R: 561bp	This study
EpsG	Transmembrane protein EPSG	Spixo_EpsG_F1- CAACCCAACGATCAAAATCA Spixo_EpsG_R2- ACGCAATGCTGATGTCATTC Spixo_EpsG_F2- CATCAGCAAATTGTGCGTAAA	First PCR run: 51 °C, Spixo_EpsG_F1/Spixo_EpsG_R2: 676bp Second PCR run: 54 °C, Spixo_EpsG_F2/Spixo_EpsG_R2: 505bp	This study

Table 4

Allelic profile of the five polymorphic genes in *Spiroplasma ixodetis* from the 12 tick species and 12 other arthropod species examined in this study. Letters a–q represent the different alleles at each *S. ixodetis* locus. Dashes indicate an absence of gene PCR amplification. Letters A–V represent the seven *S. ixodetis* haplotypes identified on the basis of 16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* allelic profiles.

Host species of *Spiroplasma ixodetis*	*Spiroplasma ixodetis* genes					*Spiroplasma ixodetis* haplotypes
	16S rDNA	*rpoB*	*dnaK*	*gyrA*	*EpsG*	
Ixodes pacificus	a	a	a	a	a	A (*S. ixodetis* Y32 type strain)
Ixodes arboricola	b	b	_	_	_	B
Ixodes frontalis	c	c	b	b	b	C
Ixodes ricinus	d	c	b	b	b	D
Ixodes uriae	d	c	b	b	_	E
Ixodes sp. (#1)	e	d	c	c	c	F
Ixodes sp. (#3)	d	c	b	b	_	E
Dermacentor marginatus	f	_	_	_	d	G
Rhipicephalus annulatus	g	e	d	d	b	H
Rhipicephalus decoloratus	g	e	d	d	b	H
Rhipicephalus geigyi	g	e	d	d	b	H
Rhipicephalus pusillus	h	_	_	_	_	I
Dermanyssus gallinae (1)	i	f	e	e	e	J
Dermanyssus gallinae (2)	i	f	f	f	e	K
Acyrthosiphon pisum	a	g	_	g	f	L
Cinara confinis	j	_	_	_	_	M
Cinara maritimae	g	h	_	h	b	N
Cicadella viridis	k	i	g	i	g	O
Notostira elongata	l	j	h	j	h	P
Ceratinostoma ostiorum	m	k	i	k	_	Q
Scathophaga stercoraria	n	l	j	_	_	R
Clinocera sp.	o	m	k	l	_	S
Ethiromyia chalybea	a	n	l	m	b	T
Poecilobothrus chrysozygos	p	o	m	m	b	U
Thrypticus tarsalis	q	c	b	n	i	V

peaks, indicating a confident degree of primer specificity for *S. ixodetis* nested and semi-nested PCR amplifications. All these sequences belong unambiguously to the *S. ixodetis* species as described below. Further characterizations were thus based on 1 to 5 *S. ixodetis* gene markers per DNA template (Table 4).

On the basis of DNA sequencing, we characterized 9 to 17 distinct alleles depending on *S. ixodetis* gene (Table 4), including 17 16S rRNA alleles (97.4–99.9% pairwise nucleotide identity), 16 *rpoB* alleles (86.6–99.8%), 13 *dnaK* alleles (85.3–99.8%), 14 *gyrA* alleles (94.2–99.8%) and 9 *EpsG* alleles (94.5–99.8%). The 16S rRNA alleles we observed here showed a 98.3–100% pairwise nucleotide identity with the *S. ixodetis* Y32 type strain, suggesting that all these bacteria belong to the *S. ixodetis* species. Overall, the allelic variation at the five gene markers led to the identification of 22 genetically different *S. ixodetis* haplotypes (A–V hereafter, cf. Table 4) allied to 12 tick species (9 haplotypes) or to the 12 other arthropod species (13 haplotypes). In the six arthropod species for which more than one individual was examined, no sequence variation at any *S. ixodetis* gene was observed between DNA templates from the same arthropod species, excepted for the poultry mite *Dermanyssus gallinae* for which two *S. ixodetis* haplotypes were found (Table 4).

The *S. ixodetis* A haplotype from our *I. pacificus* sample showed 100% nucleotide identity at the five gene markers with the *S. ixodetis* Y32 type strain. All the other tick and arthropod species were infected by *S. ixodetis* haplotypes differing from the *S. ixodetis* Y32 type strain (Table 4). Most of the arthropod species harbored a different *S. ixodetis* haplotype with the exception of a few tick species, which were each infected by a genetically identical *S. ixodetis* haplotype on the basis of 16 rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* typing. These tick species are *I. uriae/Ixodes* sp. 3 (*S. ixodetis* haplotype E) and *R. annulatus/R. decoloratus/R. geigyi* (*S. ixodetis* haplotype H) (Table 4).

3.2. Phylogenetics of Spiroplasma ixodetis

We further examined the evolutionary relationships between the different *S. ixodetis* haplotypes, including the *S. ixodetis* Y32 type strain. A comparison with other *S. ixodetis* strains reported from previous studies was however restricted to the 16S rRNA gene sequences since no *S. ixodetis rpoB*, *dnaK*, *gyrA* and *EpsG* gene sequences were available in GenBank. A maximum-likelihood (ML) analysis based on 16S rRNA gene sequences was first used to examine the phylogenetic relationship of our 32 *S. ixodetis* 16S rRNA gene sequences with 16 other *S. ixodetis* sequences (including male-killer strains of the butterfly *Danaus chrysippus*, the planthopper *Laodelphax striatellus* and the ladybirds *Anisosticta novemdecimpunctata*, *Harmonia axyridis*, *Adalia bipunctata*) available in GenBank, as well as sequences of 16 other *Spiroplasma* species (Fig. 1). We recovered the clustering of all *S. ixodetis* 16S rRNA gene sequences within a robust monophyletic clade, and the *S. ixodetis* characterized in this study are phylogenetically closely related to the *S. ixodetis* found in other arthropod species. All other *Spiroplasma* species (including other endosymbionts of insects such as *S. poulsonii*) cluster outside the *S. ixodetis* clade (Fig. 1).

No *S. ixodetis* subclade specific to ticks are evident along 16S rRNA gene tree: the *S. ixodetis* found in ticks are rather scattered among *S. ixodetis* of parasitic mites, spiders and insects (Fig. 1). Indeed, the *S. ixodetis* of *I. ricinus* and *I. uriae* are closely related to the *S. ixodetis* male-killers of the ladybird *Anisosticta novemdecimpunctata* and of the planthopper *Laodelphax striatellus*. Another example includes the *S. ixodetis* of *R. pusillus*, *R. geigy*, *R. annulatus* and *R. decoloratus* which all cluster together in the same clade with the *S. ixodetis* of the aphid *Cinara maritimae*. However, the inner topology of the *S. ixodetis* clade based on 16S rRNA gene sequences remains too poorly resolved in many other cases (as shown by low support values of inner branches) for inferring the exact relatedness between all *S. ixodetis* from different arthropod species (Fig. 1).

Novel ML analyses based on *rpoB*, *dnaK*, *gyrA* and *EpsG* gene sequences were conducted to refine the *S. ixodetis* intrageneric phylogeny using our *S. ixodetis*-positive DNA templates, the *S. ixodetis* Y32 type strain genome sequences and other *Spiroplama* genomes available in GenBank. When the sequences were examined separately for each gene, ML analyses confirmed that all *S. ixodetis* described in this study unambiguously belong to the *S. ixodetis* species (Fig. 2A–D). We observed no sign of recombination in the data set: the analysis of concatenated sequences did not detect significant recombination events between *S. ixodetis* strains (all $P > 0.05$ for the GENECONV and RDP recombination-detection tests). An additional ML analysis was conducted using 16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG S. ixodetis* concatenated dataset from 18 of our 32 DNA templates (at least one of the genes was not amplified from the 14 other DNA templates, see Table 4). It also confirms that all the strains characterized in this study belong to the *S. ixodetis* species (Fig. 3).

The single *rpoB*, *dnaK*, *gyrA* and *EpsG* gene phylogenies and concatenated phylogeny consistently showed that ticks harbor divergent *S. ixodetis* strains (Figs. 2A–D and 3). The *S. ixodetis* strain of *Ixodes pacificus* (including the Y32 type strain) was markedly different to other *S. ixodetis* strains on the basis of the *dnaK* and *EpsG* gene phylogeny (Fig. 2B and D). Similar observations were also obvious for the *S. ixodetis* strains of *Ixodes arboricola* for the *rpoB* gene (Fig. 2A), *Ixodes* sp.1 (*rpoB*, *dnaK*, *gyrA* and *EpsG*, Figs. 2A–D and 3) and *D. marginatus* (*EpsG*, Fig. 2D). Closely related *S. ixodetis* strains can also be found in phylogenetically divergent arthropod taxa: the *S. ixodetis* strains of *I. ricinus*, *I. uriae*, *Ixodes* sp. 3, *R. annulatus*, *R. decoloratus* and *R. geigyi* all

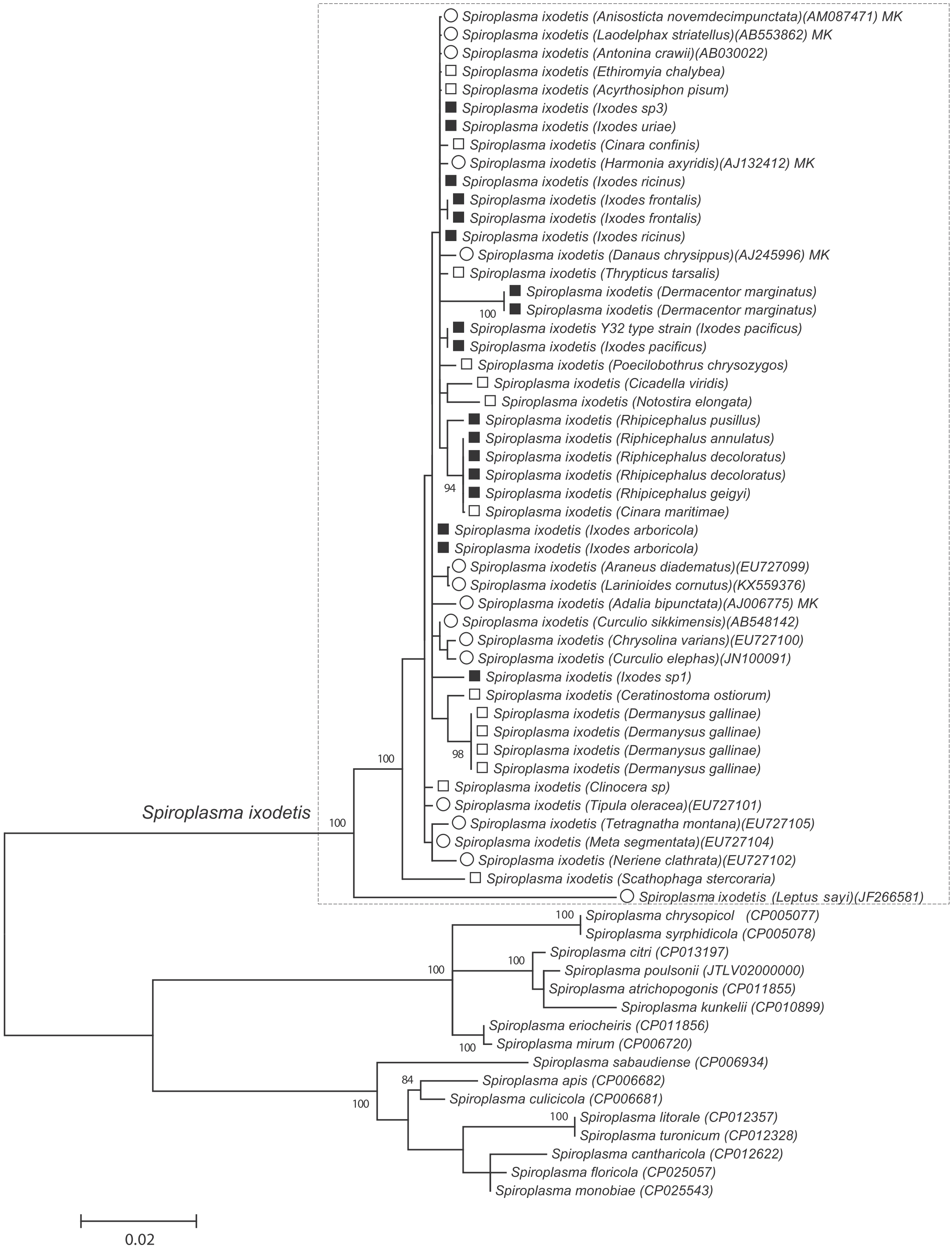

Fig. 1. Phylogeny of *Spiroplasma ixodetis* constructed using maximum-likelihood (ML) estimations based on 16S rRNA gene sequences (763 unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR + G + I). Sequences from other *S. ixodetis* and *Spiroplasma* species available in GenBank were added to the analysis. The box delineates the *S. ixodetis* species. Black and white symbols indicate *S. ixodetis* strains found in ticks and other arthropods, respectively. Square symbols indicate *S. ixodetis* strains characterized in this study while circle symbols show other *S. ixodetis* strains already available in GenBank. MK indicates known *S. ixodetis* male-killer strains of insects. Bacterial name, host species (if applicable) and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values > 70% are shown). The scale bar is in units of substitution/site.

clustered together with the *S. ixodetis* strains of insects, including flies (e.g. the long-legged fly *Poecilobothrus chrysozygos*), aphids (the pea aphid *Acyrtosiphon pisum*), leafhoppers (*Cicadella viridis*) or mirid bugs (*Notostira elongata*) (Figs. 2A–D and 3). Overall, these phylogenies revealed that *S. ixodetis* strains that were present in ticks did not cluster together within the same *S. ixodetis* subclade. However, it is noteworthy that the *S. ixodetis* strains of *Rhipicephalus* species clustered together (Figs. 1, 2A–D and 3), showing that related tick species may also harbor

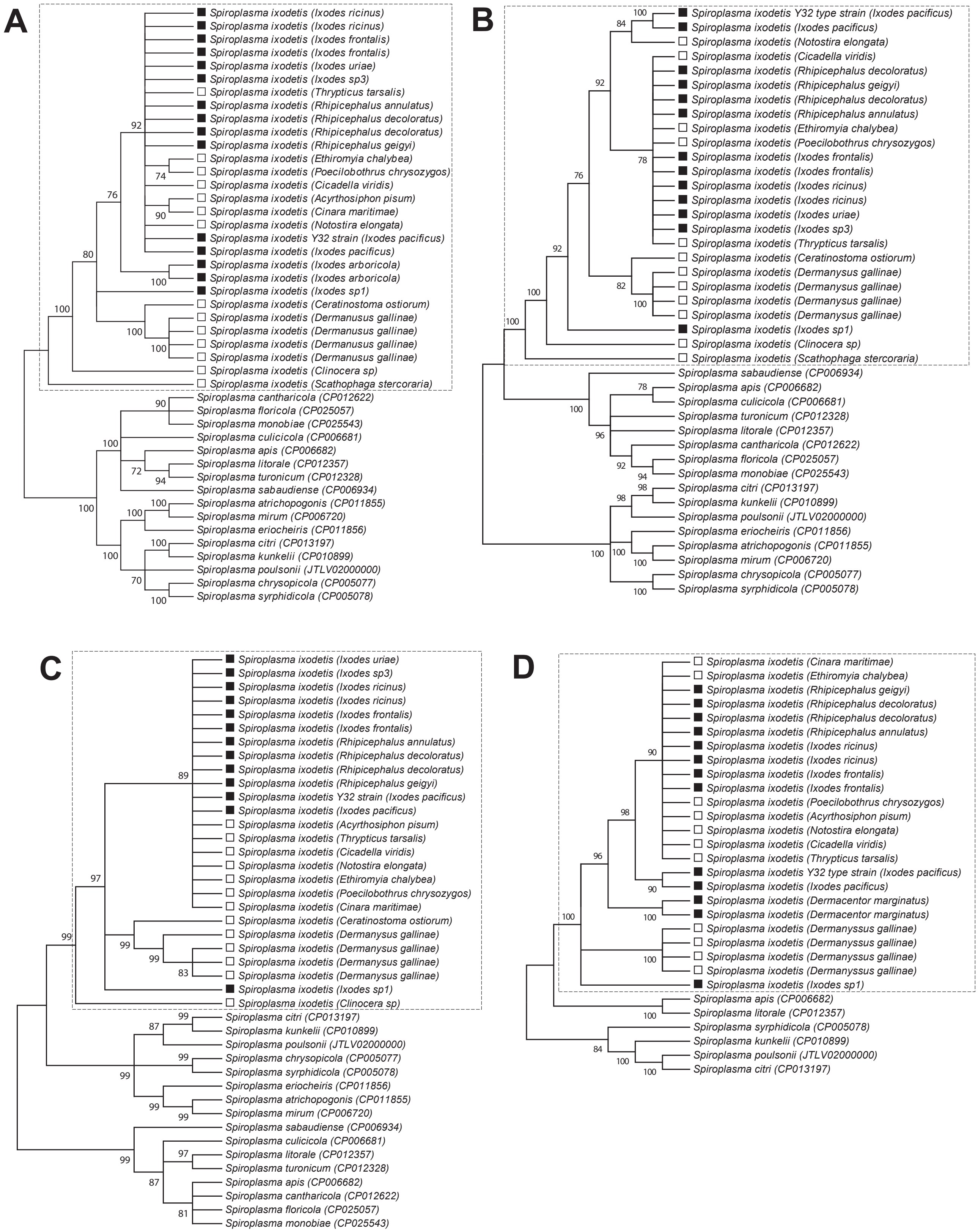

Fig. 2. Cladograms depicting the 70% majority-rule consensus of *Spiroplasma ixodetis* phylogenetic trees constructed using maximum-likelihood (ML) estimations based on (**A**) *rpoB* (585 unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR + G + I), (**B**) *dnaK* (476 bp; GTR + G + I), (**C**) *gyrA* (519 bp; GTR + G) and (**D**) *EpsG* (462 bp; GTR + G + I) sequences. The box delineates the *S. ixodetis* species. Black and white squares indicate *S. ixodetis* strains found in ticks and other arthropods, respectively. Square symbols indicate *S. ixodetis* strains characterized in this study. Bacterial name, host species (if applicable) and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values > 70% are shown).

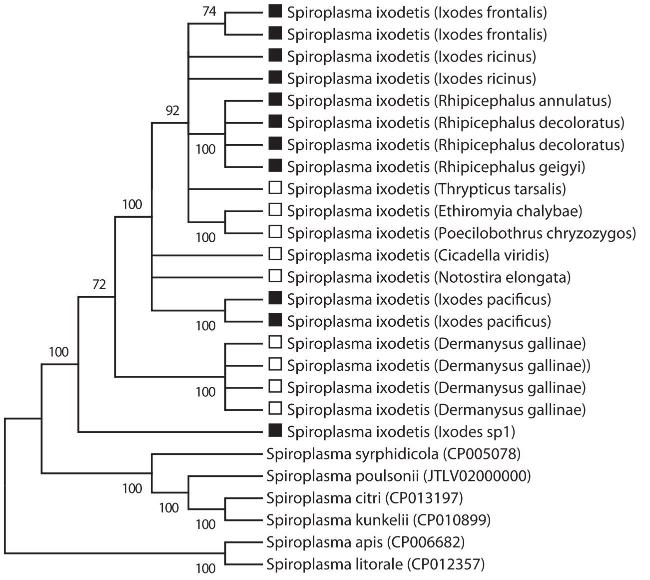

Fig. 3. Cladograms depicting the 70% majority-rule consensus of *Spiroplasma ixodetis* phylogenetic trees constructed using maximum-likelihood (ML) estimations based on concatenated 16S rRNA, *rpoB*, *dnaK*, *gyrA* and *EpsG* sequences (2805 unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR + G + I). The box delineates the *S. ixodetis* species. Black and white squares indicate *S. ixodetis* strains found in ticks and other arthropods, respectively. Square symbols indicate *S. ixodetis* strains characterized in this study. Bacterial name, host species (if applicable) and GenBank accession numbers are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values > 70% are shown).

related *S. ixodetis* strains.

3.3. Sex bias in infection prevalence in Rhipicephalus decoloratus

We examined 74 *R. decoloratus* specimens (30 males and 44 females) from four populations for sex bias in *S. ixodetis* infection prevalence, as an indication of potential male-killing activity. Through the 16S rRNA nested PCR assay, we detected *S. ixodetis* infection in 15 specimens (20.3%) (Table 2). Infected specimens were found in two of the four populations, i.e. the Vaalwater ($n = 2$ infected specimens of 19) and the Sandvelt ($n = 13$ infected specimens of 26) populations. However, *S. ixodetis* showed no evidence of sex-biased prevalence in adult *R. decoloratus*: 6 of 30 males (20%) and 9 of 44 females (20.5%) were infected showing that there was no evidence to reject the null hypothesis of equal prevalence of *S. ixodetis* in male and females (Fisher's exact test, $p = 0.76$).

4. Discussion

We examined 12 tick species and 12 other arthropod species for the diversity of *S. ixodetis*, a maternally inherited endosymbiont primarily described from the Western black-legged tick, *I. pacificus* (Tully et al., 1981). Our multi-locus typing scheme confirmed the presence of *S. ixodetis* infections in diverse tick species, as shown by recent studies using the 16S rRNA gene sequence as an exclusive marker (Bell-Sakyi et al., 2015; Duron et al., 2017; Henning et al., 2006; Hornok et al., 2010; Qiu et al., 2014; Taroura et al., 2005; Van Oosten et al., 2018). Importantly, the examination of *rpoB*, *dnaK*, *gyrA* and *EpsG* gene sequences revealed a wider diversity of *S. ixodetis* than previously known from 16S rRNA gene sequences.

Phylogenetic analyses based on the multi-locus data set consistently showed that ticks harbor divergent *S. ixodetis* strains. The structure revealed was complex, showing that evolution of *S. ixodetis* has been shaped by both maternal inheritance and horizontal transfers (HT) with frequent host turnover. Because of maternal inheritance, persistence of infection in a tick species over long periods of time should result in diversification of *S. ixodetis* alongside its tick host, that is to say in cocladogenesis. This process may explain why the four *Rhipicephalus* species examined in this study harbor closely related *S. ixodetis* strains. Nevertheless, in most cases, our results rather revealed extensive HT since the *S. ixodetis* strains found in ticks do not form a tick-specific subclade. The *S. ixodetis* strains of ticks have different evolutionary origins as shown by their phylogenetic proximity with strains found in insects. The best examples are found in the *Ixodes* genus: the *S. ixodetis* strains found in *I. ricinus*, *I. uriae* and *Ixodes* sp. 3 are more closely related to strains found in aphids, leafhoppers and flies than the *S. ixodetis* strains found in *I. pacificus* (including the *S. ixodetis* Y32 type strain), *I. arboricola* and *Ixodes* sp. 1. The rarity of cocladogenesis suggests that the long term maintenance of *S. ixodetis* infections is mainly possible through repeated interspecies transfers between ticks and other arthropods. A few case studies have demonstrated the key importance of ectoparasitic mites, which feed on arthropod haemolymph, for HT. Indeed, mites can serve as interspecific vectors of *Spiroplasma* spp. by picking up the endosymbiont from infected arthropods, and subsequently transferring the infection to recipient arthropods (Jaenike et al., 2007; DiBlasi et al., 2011). Specific adaptations facilitating HT are also likely present since *S. ixodetis* is one of the rare endosymbionts of arthropods able to grow outside the host cells (Tully et al., 1995). This ability may enhance the likelihood of successful HT between arthropod species, as empirically shown for another endosymbiont, *Arsenophonus* (Duron et al., 2010; Skinner, 1985).

Spiroplasma ixodetis infections in ticks showed the typical features of secondary endosymbionts: the patchy distribution within Ixodidae suggests that ticks are not dependent upon infection for survival or reproduction (Bell-Sakyi et al., 2015; Duron et al., 2017; Henning et al., 2006; Hornok et al., 2010; Qiu et al., 2014; Taroura et al., 2005; Van Oosten et al., 2018). The maternal transmission of *S. ixodetis* however implies that its transmission success should broadly depend on its effect on arthropod fitness (Engelstadter, Hurst, 2009). While the *S. ixodetis* strains found in ticks are related to the male-killer strains found in insects, we did not observe evidence for this in *R. decoloratus*, a result also previously observed in *I. arboricola* (Van Oosten et al., 2018). In this context, the effects of *S. ixodetis* infection on ticks may thus be more complex than previously considered in other arthropods. One can assume that *S. ixodetis* may confer multiple benefits to ticks with diverse roles in development, reproduction, defense against environmental stress or immunity. It is worthy of interest that some *Spiroplasma* spp. are defensive symbionts that can protect their insect hosts from their natural enemies, strongly reducing individual mortality, e.g. in pea aphids *versus* pathogenic fungi, or in *Drosophila versus* parasitic nematodes (Lukasik et al., 2013; Jaenike et al., 2010). Another possibility is that detection of *S. ixodetis* in ticks results from cross-contamination due to the presence of infected parasitic wasp eggs: although *S. ixodetis* has not been found in parasitic wasps of ticks so far, the presence of the endosymbiont *Wolbachia* in parasitic wasp eggs contained in the bodies of tick samples caused incidental detection of symbiont DNA (Tijsse-Klasen et al., 2011; Plantard et al., 2012). Anyway, the precise nature of how *S. ixodetis* affects tick biology remains to be characterized, suggesting a need for further investigations.

In conclusion, the *S. ixodetis* endosymbiosis represents an important but insufficiently studied component of tick biology. An important diversity of *S. ixodetis* is certainly present in ticks, yet what remains unclear is what possible role they may play in shaping tick biology, or the ecological processes that govern their distribution and abundance. Interesting in this regard is the intriguing pattern of *S. ixodetis* distribution within ticks: while infection is common in hard ticks (Ixodidae), the symbiont has never been detected in soft ticks (Argasidae) despite intensive screening efforts (Duron et al., 2017). Why soft ticks do not seem to harbour *S. ixodetis* is not currently understood, but suggests that some so far unknown degree of host specificity may exist.

Competing interests

The authors declare that they have no competing interests.

Ethic statements

Some of the tick specimens were removed from wild and domestic animals, in accordance with the recommendations on the French National charter on the ethics of animal experimentation. The capture of animals was also carried out in strict accordance with good animal care practices recommended by the European guidelines. All captures were performed by competent people without causing avoidable pain, suffering, distress or lasting harm to animals.

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References

Bell-Sakyi, L., Palomar, A.M., Kazimirova, M., 2015. Isolation and propagation of a *Spiroplasma* sp. from Slovakian *Ixodes ricinus* ticks in *Ixodes* spp. cell lines. Ticks Tick. Dis. 6, 601–606.

Bonnet, S.I., Binetruy, F., Hernandez-Jarguin, A.M., Duron, O., 2017. The Tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. Front. Cell. Infect Microbiol. 7, 236.

Cafiso, A., Bazzocchi, C., De Marco, L., Opara, M.N., Sassera, D., Plantard, O., 2016. Molecular screening for *Midichloria* in hard and soft ticks reveals variable prevalence levels and bacterial loads in different tick species. Ticks Tick. Dis. 7, 1186–1192.

Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.

Clay, K., Klyachko, O., Grindle, N., Civitello, D., Oleske, D., Fuqua, C., 2008. Microbial communities and interactions in the lone star tick, *Amblyomma americanum*. Mol. Ecol. 17, 4371–4381.

DiBlasi, E., Morse, S., Mayberry, J.R., Avila, L.J., Morando, M., Dittmar, K., 2011. New *Spiroplasma* in parasitic *Leptus* mites and their *Agathemera* walking stick hosts from Argentina. J. Invertebr. Pathol. 107, 225–228.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J., Hurst, G.D., 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol. 6, 27.

Duron, O., Wilkes, T.E., Hurst, G.D., 2010. Interspecific transmission of a male-killing bacterium on an ecological timescale. Ecol. Lett. 13, 1139–1148.

Duron, O., Cremaschi, J., McCoy, K.D., 2016. The high diversity and global distribution of the intracellular bacterium *Rickettsiella* in the polar seabird tick *Ixodes uriae*. Microb. Ecol. 71, 761–770.

Duron, O., Binetruy, F., Noël, V., Cremaschi, J., McCoy, K.D., Arnathau, C., Plantard, O., Goolsby, J., Pérez de León, A.A., Heylen, D.J.A., Van Oosten, A.R., Gottlieb, Y., Baneth, G., Guglielmone, A.A., Estrada-Peña, A., Opara, M.N., Zenner, L., Vavre, F., Chevillon, C., 2017. Evolutionary changes in symbiont community structure in ticks. Mol. Ecol. 26, 2905–2921.

Duron, O., Morel, O., Noel, V., Buysse, M., Binetruy, F., Lancelot, R., Loire, E., Ménard, C., Bouchez, O., Vavre, F., Vial, L., 2018. Tick-bacteria mutualism depends on B vitamin synthesis pathways. Curr. Biol. 28, 1896–1902.

Engelstadter, J., Hurst, G.D.D., 2009. The ecology and evolution of microbes that manipulate host reproduction. Annu. Rev. Ecol. Evol. Syst. 40, 127–149.

Fukatsu, T., Tsuchida, T., Nikoh, N., Koga, R., 2001. *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). Appl. Environ. Microbiol. 67, 1284–1291.

Gasparich, G.E., 2002. *Spiroplasmas*: evolution, adaptation and diversity. Front. Biosci. 7, 619–640.

Gerhart, J.G., Moses, A.S., Raghavan, R., 2016. A *Francisella*-like endosymbiont in the Gulf Coast tick evolved from a mammalian pathogen. Sci. Rep. 6, 33670.

Gottlieb, Y., Lalzar, I., Klasson, L., 2015. Distinctive genome reduction rates revealed by genomic analyses of two *Coxiella*-like endosymbionts in ticks. Genome Biol. Evol. 7, 1779–1796.

Guizzo, M.G., Parizi, L.F., Nunes, R.D., et al., 2017. A *Coxiella* mutualist symbiont is essential to the development of *Rhipicephalus microplus*. Sci. Rep. 7, 17554.

Henning, K., Greiner-Fischer, S., Hotzel, H., Ebsen, M., Theegarten, D., 2006. Isolation of*Spiroplasma* sp. from an *Ixodes* tick. Int. J. Med. Microbiol. 296 (Suppl. 40), 157–161.

Hornok, S., Meli, M.L., Perreten, A., Farkas, R., Willi, B., Beugnet, F., Lutz, H., Hofmann-Lehmann, R., 2010. Molecular investigation of hard ticks (Acari: Ixodidae) and fleas (Siphonaptera: Pulicidae) as potential vectors of rickettsial and mycoplasmal agents. Vet. Microbiol. 140, 98–104.

Hurst, G.D.D., von der Schulenburg, J.H., Majerus, T.M.O., Bertrand, D., Zakharov, I.A., Baungaard, J., Völkl, W., Stouthamer, R., Majerus, M.E., 1999. Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. Insect Mol. Biol. 8, 133–139.

Jaenike, J., Polak, M., Fiskin, A., Helou, M., Minhas, M., 2007. Interspecific transmission of endosymbiotic *Spiroplasma* by mites. Biol. Lett. 3, 23–25.

Jaenike, J., Unckless, R., Cockburn, S.N., Boelio, L.M., Perlman, S.J., 2010. Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. Science 329, 212–215.

Jiggins, F.M., Hurst, G.D.D., Jiggins, C.D., Von Der Schulenburg, J.H., Majerus, M.E.N., 2000. The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. Parasitology 120, 439–446.

Lalzar, I., Harrus, S., Mumcuoglu, K.Y., Gottlieb, Y., 2012. Composition and seasonal variation of *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* bacterial communities. Appl. Environ. Microbiol. 78, 4110–4116.

Li, L.H., Zhang, Y., Zhu, D., 2018. Effects of antibiotic treatment on the fecundity of *Rhipicephalus haemaphysaloides* ticks. Parasit. Vectors 11, 242.

Lukasik, P., Guo, H., van Asch, M., Ferrari, J., Godfray, H.C., 2013. Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. J. Evol. Biol. 26, 2654–2661.

Majerus, T.M., Graf von der Schulenburg, J.H., Majerus, M.E., Hurst, G.D., 1999. Molecular identification of a male-killing agent in the ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). Insect Mol. Biol. 8, 551–555.

Majerus, M.E., Hinrich, J., Schulenburg, G.V., Zakharov, I.A., 2000. Multiple causes of male-killing in a single sample of the two-spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae) from Moscow. Heredity 84, 605–609.

Martin, D., Lemey, P., Lott, M., et al., 2010. RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics 26, 2462–2463.

Martin, O.Y., Gubler, A., Wimmer, C., Germann, C., Bernasconi, M.V., 2012. Infections with *Wolbachia* and *Spiroplasma* in the Scathophagidae and other Muscoidea. Infect. Genet. Evol. 12, 315–323.

Martin, O.Y., Puniamoorthy, N., Gubler, A., Wimmer, C., Bernasconi, M.V., 2013. Infections with *Wolbachia*, *Spiroplasma*, and *Rickettsia* in the Dolichopodidae and other Empidoidea. Infect. Genet. Evol. 13, 317–330.

Narasimhan, S., Fikrig, E., 2015. Tick microbiome: the force within. Trends Parasitol. 31, 315–323.

Pérez-Ruiz, M., Martìnez-Rodriguez, P., Herranz, J., Bella, J.L., 2015. A survey of *Wolbachia*, *Spiroplasma* and other bacteria in parthenogenetic and non-parthenogenetic phasmid (Phasmatodea) species. Eur. J. Entomol. 112, 409–418.

Plantard, O., Bouju-Albert, A., Malard, M.A., Hermouet, A., Capron, G., Verheyden, H., 2012. Detection of *Wolbachia* in the tick *Ixodes ricinus* is due to the presence of the hymenoptera endoparasitoid *Ixodiphagus hookeri*. PLoS One 7, e30692.

Qiu, Y., Nakao, R., Ohnuma, A., Kawamori, F., Sugimoto, C., 2014. Microbial population analysis of the salivary glands of ticks: a possible strategy for the surveillance of bacterial pathogens. PLoS One 9, e103961.

Sanada-Morimura, S., Matsumura, M., Noda, H., 2013. Male killing caused by a *Spiroplasma* symbiont in the small brown planthopper, *Laodelphax striatellus*. J. Hered. 104, 821–829.

Simon, J.C., Boutin, S., Tsuchida, T., Koga, R., Le Gallic, J.F., Frantz, A., Outreman, Y., Fukatsu, T., 2011. Facultative symbiont infections affect aphid reproduction. PLoS One 6, e21831.

Skinner, S.W., 1985. Son-killer: a third extrachromosomal factor affecting the sex ratio in the parasitoid wasp, *Nasonia* (= *Mormoniella*) *vitripennis*. Genetics 109, 745–759.

Smith, T.A., Driscoll, T., J.J, G., Raghavan, R., 2015. A *Coxiella*-like endosymbiont is a potential vitamin source for the Lone Star Tick. Genome Biol. Evol. 7, 831–838.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Taroura, S., Shimada, Y., Sakata, Y., Miyama, T., Hiraoka, H., Watanabe, M., Itamoto, K., Okuda, M., Inokuma, H., 2005. Detection of DNA of' *Candidatus* Mycoplasma haemominutum' and *Spiroplasma* sp. in unfed ticks collected from vegetation in Japan. J. Vet. Med. Sci. 67, 1277–1279.

Thompson, J.D., Gibson, T.J., Higgins, D.G., 2002. Multiple sequence alignment using ClustalW and ClustalX. In: Baxevanis, A.D., Petsko, G.A., Stein, L.D., Stormo, G.D. (Eds.), Curr. Protoc. Bioinformatics. John Wiley & Sons Inc., Hoboken pp. 2.3.1–2.3.22.

Tijsse-Klasen, E., Braks, M., Scholte, E.J., Sprong, H., 2011. Parasites of vectors - *Ixodiphagus hookeri* and its *Wolbachia* symbionts in ticks in the Netherlands. Parasit. Vectors 4, 228.

Tinsley, M.C., Majerus, M.E., 2006. A new male-killing parasitism: *spiroplasma* bacteria infect the ladybird beetle *Anisosticta novemdecimpunctata* (Coleoptera: Coccinellidae). Parasitology 132, 757–765.

Tully, J.G., Rose, D.L., Yunker, C.E., Cory, J., Whitcomb, R.F., Williamson, D.L., 1981. Helical mycoplasmas (spiroplasmas) from *Ixodes* ticks. Science 212, 1043–1045.

Tully, J.G., Whitcomb, R.F., Rose, D.L., Williamson, D.L., Bove, J.M., 1983. Characterization and taxonomic status of tick spiroplasmas: a review. Yale J. Biol. Med. 56, 599–603.

Tully, J.G., Rose, D.L., Yunker, C.E., Carle, P., Bové, J.M., Williamson, D.L., Whitcomb, R.F., 1995. *Spiroplasma ixodetis* sp. nov., a new species from *Ixodes pacificus* ticks collected in Oregon. Int. J. Syst. Evol. Microbiol. 45 (23), -28.

Van Oosten, A.R., Duron, O., Heylen, D.J.A., 2018. Sex ratios of the tick *Ixodes arboricola* are strongly female-biased, but there are no indications of sex-distorting bacteria. Ticks Tick. Dis. 9, 307–313.

Weinert, L.A., Tinsley, M.C., Temperley, M., Jiggins, F.M., 2007. Are we underestimating the diversity and incidence of insect bacterial symbionts? A case study in ladybird beetles. Biol. Lett. 3, 678–681.

Zhang, C.M., Li, N.X., Zhang, T.T., Qiu, Z.X., Li, Y., Li, L.W., Liu, J.Z., 2017. Endosymbiont CLS-HI plays a role in reproduction and development of *Haemaphysalis longicornis*. Exp. Appl. Acarol. 73, 429–438.

Zhong, J., Jasinskas, A., Barbour, A.G., 2007. Antibiotic treatment of the tick vector *Amblyomma americanum* reduced reproductive fitness. PLoS One 2, e405.