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Widespread distribution and high prevalence of an alpha-proteobacterial symbiont in the tick *Ixodes ricinus*

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Summary

The tick *Ixodes ricinus* is responsible for the transmission of a number of bacterial, protozoan and viral diseases to humans and animals in Europe and Northern Africa. Female *I. ricinus* from England, Switzerland and Italy have been found to harbour an intracellular α-proteobacterium, designated IricES1, within the cells of the ovary. IricES1 is the only prokaryote known to exist within the mitochondria of any animal or multicellular organism. To further examine the distribution, prevalence and mode of transmission of IricES1, we performed polymerase chain reaction screening of *I. ricinus* adults from 12 countries across its geographic distribution, including tick colonies that have been maintained in the laboratory for varying periods of time. IricES1 was detected in 100% of field-collected female ticks from all countries examined ($n = 128$), while 44% of males were found to be infected ($n = 108$). Those males that are infected appear to harbour fewer bacteria than females. Sequencing of fragments of the 16S rRNA and *gyrB* genes revealed very low nucleotide diversity among various populations of IricES1. Transmission of IricES1 from engorged adult females to eggs was found to be 100% ($n = 31$). In tick colonies that had been maintained in the laboratory for several years, a relatively low prevalence was found in females (32%; $n = 25$). To our knowledge, IricES1 is the most widespread and highly prevalent of any tick-associated symbiont.

Introduction

Ticks are regarded as important vectors of viral, bacterial and protozoan pathogens. In addition, many ticks carry intracellular bacteria that are apparently harmless to mammals (Cowdry, 1925; Mudrow, 1932; Suitor and Weiss, 1961; Burgdorfer *et al*., 1973). Such bacteria are generally known as endosymbionts, and are found primarily in the ovaries or malpighian tubules of ticks (Noda *et al*., 1997). This tissue specificity reduces the chances of endosymbionts being transferred to the tick's vertebrate host during a blood meal, thereby reducing the probability of horizontal transfer to other blood-sucking arthropods. They are more likely to spread via transmission to the eggs. Although several tick endosymbionts have been studied using classical microscopy (Roshdy, 1961; Aeschlimann and Hecker, 1970; Lewis, 1979; Sutakova and Rehacek, 1991), the biology of these bacteria is poorly understood compared with endosymbionts present

in other invertebrates (e.g. *Buchnera aphidicola*, *Wolbachia pipientis*).

The European sheep tick *Ixodes ricinus* (Acari: Ixodidae) has a distribution that stretches from Scandinavia down to Northern Africa, and across to Russia and Turkey (Estrada-Pena *et al*., 1998). It has an XX:XY sex-determination system (Kiszewski *et al*., 2001). During an electron microscopy (EM) study of *I. ricinus* from England, intracellular bacteria were discovered in the ovary (Lewis, 1979). Interestingly, the bacteria were found inside mitochondria as well as in the cytoplasm. It was suggested that the bacteria replicated in the mitochondria, which then burst and released bacteria into the cytoplasm. Later, female *I. ricinus* from Switzerland were found to harbour bacteria with the same characteristics (Zhu *et al*., 1992); bacteria were not seen in male specimens.

In a further EM study using engorged female ticks collected in Italy, bacteria were seen colonizing mitochondria between the inner and outer membranes, and consuming the mitochondrial contents (Sacchi *et al*., 2004). It was proposed that the bacteria had a life cycle similar to the predatory bacterium *Bdellovibrio bacteriovorus*. Molecular techniques used on *I. ricinus* collected in Italy showed that the bacteria seen in EM represent a single species within the α-proteobacteria. This species was given the temporary designation IricES1 (*I. ricinus* EndoSymbiont 1), pending further taxonomic studies. During two recent screening studies of bacteria present in *I. ricinus* from Germany (Schabereiter-Gurtner *et al*., 2003) and Italy (Sanogo *et al*., 2003), 16S rRNA sequences almost identical to that of IricES1 were recovered, although prevalence in the sampled populations was not reported. Polymerase chain reaction (PCR) screening of ticks collected in Italy ($n = 158$) and England ($n = 8$) showed that it was present in 96% of adult females, but not in males (Beninati *et al*., 2004). This result was in agreement with the high prevalence in females based on EM studies (Zhu *et al*., 1992). However, the sensitivity of the 16S rRNA PCR was not determined. The possibility that males contained the bacterium, although at relatively low concentrations, was not ruled out.

Whether IricES1 is a parasite, mutualist or commensalist is not yet understood. The prevalence of an endosymbiont within a host species may provide clues about the interaction that is occurring between host and symbiont. For example, in obligatory mutualistic interactions such as that between *B. aphidicola* and its aphid hosts, 100% prevalence is invariably seen (Baumann *et al*., 1997). To examine whether the very high prevalence of IricES1 in Italy, England and Switzerland extends to other parts of the distribution of *I. ricinus*, we performed a PCR-based screening of ticks collected from 12 countries. We also investigated the genetic diversity of IricES1 in different host populations, the transmission efficiency of the bacterium from the ovaries to the eggs, and its prevalence in long-term laboratory colonies.

Results and discussion

Phylogenetic analysis

Figure 1 shows the phylogenetic relationship of IricES1 to other members of the α-proteobacteria. The IricES1 16S rRNA sequence was most closely related to two sequences amplified from the tick *Haemaphysalis wellingtoni* (Parola *et al*., 2003). The cellular localization of these bacteria is not yet known. Other bacteria closely related to IricES1 include symbionts from *Acanthamoeba* spp. UWC8 and UWC36, two uncharacterized bacteria from microbial mats, and another bacterium from the tick *Ixodes persulcatus*. Together, IricES1 and the aforementioned bacteria formed a clade with 99% quartet puzzling support. This clade was the sister group of a separate clade containing the genera *Wolbachia*, *Anaplasma*, *Ehrlichia* and *Neorickettsia*.

Prevalence and genetic diversity of IricES1 in field-collected ticks

Ixodes ricinus adult ticks from 12 localities in 10 countries were initially screened by PCR of 16S rRNA, adding to previously published data from four localities in Italy and England (Table 1, Fig. 2). At each collection site, numbers of females and males collected were approximately equal, with no evidence for sex ratio bias (data not shown). This result was in agreement with the sex ratio of various populations of *I. ricinus* studied previously (de Meeus *et al*., 2002). Each of the localities was considered a 'population', and the prevalence of IricES1 in each population was measured. The PCR primers used are highly specific for IricES1, and do not amplify the 16S rRNA gene from closely related genera such as *Rickettsia*, *Ehrlichia* and *Wolbachia* (Beninati *et al*., 2004). The prevalence of IricES1 in females within each population was generally high, being near or at 100% in most populations. Prevalence in three populations from Algeria and Tunisia were lower, being 77% ($n = 9$), 79% ($n = 14$) and 75% ($n = 4$) (Table 1). The overall prevalence in female ticks, based on PCR of 16S rRNA in this study, was 95% (121/128), while that in males was 0% ($n = 64$). The viability of DNA from all negative samples was confirmed using the primers 12S-air and Met-20, specific for the mitochondrial AT-rich region (Simon *et al*., 1994). Overall, the results were in agreement with previous PCR examinations of ticks collected in Italy and England (Beninati *et al*., 2004), as well as EM examinations of ticks collected in Switzerland (Zhu *et al*., 1992), in which 96–100% of females were infected and males did not show evidence of harbouring the bac-

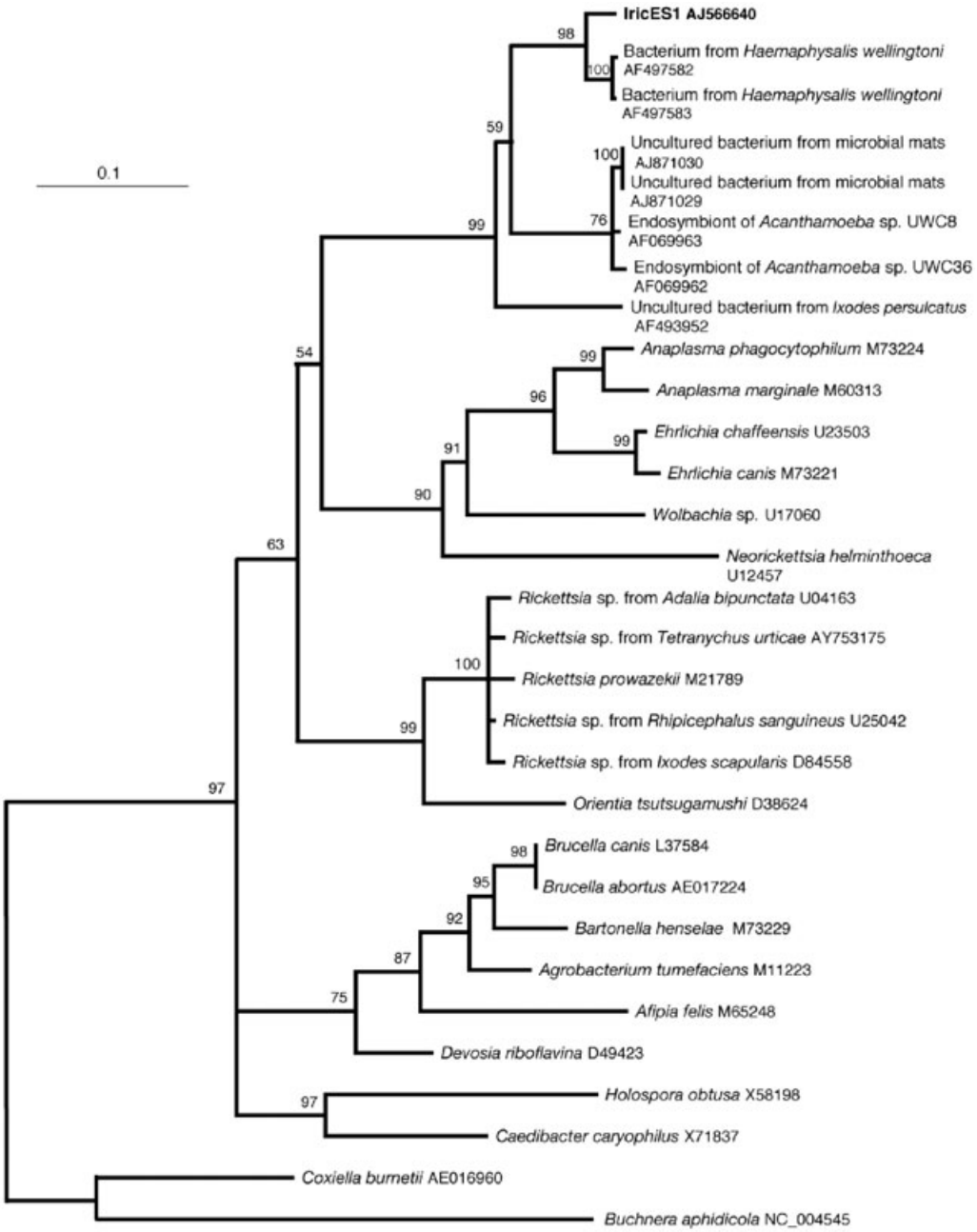

Fig. 1. Phylogenetic comparison of the 16S rRNA of IricES1, an intracellular bacterium from the tick *Ixodes ricinus*, with members of the α-proteobacteria. The tree was constructed using TREEPUZZLE 5.0, with the TN+G model of substitution. GenBank accession numbers for each sequence are shown adjacent to each strain. Numbers above each node represent quartet puzzling support values. The tree was rooted with representatives of the γ-proteobacteria (*Coxiella burnetii* and *B. aphidicola*). Scale bar represents the number of inferred substitutions per site.

terium (see Table 1). Serial dilution studies of purified plasmid DNA showed that the 16S rRNA PCR assay used in this study (as well as in Beninati *et al*., 2004) was effective in detecting as few as 200 bacterial equivalents per PCR tube (approximately equal to 20 000 bacterial equivalents in one tick).

A second PCR was developed in order to examine whether those samples negative in the 16S rRNA PCR were actually infected, although with fewer bacteria. This PCR involved amplification of a 145 bp fragment of *gyrB*, a gene encoding the protein DNA gyrase subunit B. In this new assay, 20 bacterial equivalents could be detected per PCR tube (approximately 400 bacterial equivalents in one tick). This increased level of sensitivity was most likely due

Table 1. IricES1 prevalence in field-collected ticks.

Collection details		IricES1 Prevalence			
		16S rRNA PCR assay		Including *gyrB* PCR assay[a]	
Country	Locality (number on map)	Female	Male	Female	Male
Sweden	Alsike (1)	5/5 (100%)	0/3	ne	2/3 (67%)
	Stockholm (2)	5/5 (100%)	0/2	ne	1/2 (50%)
Russia	Moscow (3)	9/9 (100%)	0/6	ne	2/6 (33%)
Ireland	Galway (4)	16/16 (100%)	0/12	ne	7/12 (58%)
England	Somerset (5)	4/4 (100%)[c]	0/4[c]	ne	1/4 (25%)
Germany	Berlin (6)	11/11 (100%)	ne	ne	ne
Czech Republic	Ceske Budejovice (7)	17/18 (94%)	0/8	18/18 (100%)	3/8 (37%)
Austria	Hohenhau (8)	17/17 (100%)	0/8	ne	6/8 (75%)
Switzerland[b]	Neuchâtel (9)	8/8 (100%)	ne	ne	ne
Italy	Trento (10)	53/55 (96%)[c]	0/37[c]	55/55 (100%)	17/37 (46%)
	Veneto (11)	3/3 (100%)[c]	0/3[c]	ne	2/3 (67%)
	Tuscany (12)	5/6 (83%)[c]	ne	6/6	ne
	Marche (13)	12/12 (100%)[c]	0/4[c]	ne	ne
Turkey	Zekeriyakoy (14)	12/12 (100%)	0/19	ne	5/19 (26%)
Algeria	Tizi Ouzou (15)	7/9 (77%)	0/2	9/9 (100%)	0/2 (0%)
Tunisia	El Jouza (16)	11/14 (79%)	0/2	14/14 (100%)	1/2 (50%)
	Col des Ruines (17)	3/4 (75%)	0/2	4/4 (100%)	1/2 (50%)

a. Numbers represent cumulative prevalences based on 16S rRNA and *gyrB* PCR results. In the case where 100% of females were found positive for 16S rRNA, PCR of *gyrB* was not performed.
b. In a previous study of ticks collected from the field in Neuchâtel and examined by electron microscopy, all females were found to contain a bacterium with the characteristics of IricES1, while the bacterium was not seen in males (Zhu *et al*., 1992).
c. Previously reported results from Beninati and colleagues (2004).
ne, not examined.

to the number of amplification cycles used (40 compared with 35 for the 16S rRNA PCR), as well as the short size of the fragment, and a larger aliquot of DNA (see *Experimental procedures*). Polymerase chain reaction of *gyrB* was performed on all samples negative for the 16S rRNA PCR, including negative females and males from a previous study (Beninati *et al*., 2004). All female samples were found to be positive, while 48/108 males were positive. Thus, the prevalence of females and males, taking into consideration all PCR results for both 16S rRNA and *gyrB*, rose to 100% ($n = 208$) and 44% ($n = 108$) respectively.

An examination of the genetic diversity of the symbiont was performed for two to three representatives of populations from 11 countries by sequencing two gene fragments: a 380 bp region of the 16S rRNA gene, and a 519 bp region of the *gyrB* gene. The region of 16S rRNA was selected for sequencing on account of it being relatively variable among close relatives of IricES1 (based on the alignment of Beninati *et al*., 2004). 16S rRNA sequences from all populations were found to be identical to that originally sequenced from *I. ricinus* collected in Italy, with the exception of those from Algeria and Tunisia, which were identical and shared one base-pair difference with the other sequences (EMBL-Align Accession No. ALIGN_000937). *gyrB* sequences were found to be only slightly more variable, with two synonymous substitutions being found among representatives of the 11 countries examined (ALIGN_000938). One of these substitutions was from one representative of the Algerian population, while the other was from both examined representatives of the Irish population.

Prevalence and transmission of IricES1 in laboratory-raised ticks

Included in this study were *I. ricinus* that had been maintained in two different laboratories (Insect Services, Berlin, Germany and The Institute of Zoology, University of Neuchâtel, Switzerland) for varying periods of time. The ticks used to start these laboratory colonies were collected locally. For ticks (F1) derived from females collected from vegetation but raised in the Berlin laboratory colony, female prevalence was 100% ($n = 6$) based on the 16S rRNA PCR assay, while one of the two males examined was positive in the *gyrB* PCR (Table 2). These results were in agreement with the 100% prevalence of IricES1 in wild female ticks collected in Berlin. All F2 females ($n = 4$) were also infected; however, only 18% of F5 females ($n = 11$) (from lines maintained in the laboratory for 7–8 years) were found to be positive based on the 16S rRNA PCR assay. This value did not change following screening using the more sensitive *gyrB* PCR, suggesting that several ticks were free of the bacterium.

In the case of the colony from Neuchâtel, 44% ($n = 14$) of female F7 ticks were infected based on PCR of 16S rRNA; this value did not change following screen-

Fig. 2. Collection localities of *Ixodes ricinus*. Numbers correspond to the names shown in Table 1.

ing using the *gyrB* PCR assay. The two F7 males examined were negative in both PCR assays. In the original study of Lewis (1979), ticks that had been maintained in the laboratory for several years were used for EM studies. Although the number of ticks examined was not given, a number of female ticks were reported not to contain the bacterium, in agreement with the results from our study.

The transmission efficiency of IricES1 from female adults to progeny was examined by allowing four F2 females from Berlin to engorge on separate guinea pigs. With one exception, all eggs from each female were found to be positive in the 16S rRNA PCR [transmission rates: 92% ($n = 12$), 100% ($n = 8$), 100% ($n = 6$), 100% ($n = 5$)]. The single negative egg was positive during the *gyrB* PCR assay.

Table 2. IricES1 prevalence in tick laboratory colonies.

			IricES1 Prevalence					
			16S rRNA			Including *gyrB* PCR assay[a]		
Country	Locality	Generation	Female	Male	Eggs	Female	Male	Eggs
Germany	Berlin	F1	6/6 (100%)	0/2 (0%)	ne	ne	1/2 (50%)	ne
		F2	4/4 (100%)	ne	30/31[b] (97%)	ne	ne	31/31[b] (100%)
		F5	2/11 (18%)	ne	ne	2/11 (18%)	ne	ne
Switzerland	Neuchâtel	F7	6/14 (43%)	0/2 (0%)	ne	6/14	0/2 (0%)	ne

a. Numbers represent cumulative prevalences based on 16S rRNA and *gyrB* PCR results.
b. pooled number of eggs from four different females.
ne, not examined.

Conclusions

We have shown that IricES1 is found in all females throughout the geographical range of its host, *I. ricinus*. This would make it the most widespread symbiont of any tick examined so far. Some females from populations in Tunisia and Algeria were found to be positive only with the more sensitive *gyrB* assay, indicating that they are infected by fewer bacteria than other females. IricES1 was detected in 44% of males across the distribution of its host, although only during the *gyrB* assay. Males, if they are infected, appear to harbour fewer bacteria than females. In larval and nymphal male *I. ricinus* collected in Neuchâtel, Switzerland, no bacteria were visible during EM examinations (Zhu *et al*., 1992). It is possible that these males were free of IricES1. Alternatively, some males may have been infected with very low numbers of the bacterium, which were difficult to visualize during EM.

IricES1 was detected in 100% of eggs, indicating that the major route of transmission for the bacterium is via maternal inheritance. A previous study found 100% of female larvae and nymphs derived from a single engorged female to be infected, while no males contained the bacterium (Zhu *et al*., 1992). Taken together, these results suggest that if an infected egg develops into a female, the bacteria continue to survive in primordial ovarian tissues. In contrast, if the egg develops into a male, most or all of the bacteria are lost.

Maintenance in the lab for several generations appears to lead to a loss of IricES1 in a majority of female hosts. Possible reasons for this include the fact that animals used to rear ticks are commonly fed antibiotics, and the fact that ticks are consistently exposed to room temperature conditions. In the wild, *I. ricinus* generally inhabits areas with cool climatic conditions, and it is exposed to subzero temperatures during winter. Arthropod symbionts are known to commonly be lost under laboratory conditions, and temperature is believed to play a role in some cases (Weeks *et al*., 2002). If IricES1 is important to the vitality of *I. ricinus*, the fact that it is frequently absent in laboratory-maintained ticks may have implications for the experimental use of these ticks, for example, in the study of the pathogens they vector.

The genetic diversity of IricES1 across its geographic distribution appears to be low, based on the few substitutions seen in the two gene fragments sequenced. This could indicate a relatively recent selective sweep of the bacterium across populations of *I. ricinus*. Alternatively, IricES1 may have been present in the last common ancestor of all *I. ricinus* populations, being inherited to all extant members of the species. The 100% prevalence of IricES1 may be important in limiting the extent of vertical transmission of pathogenic bacteria (e.g. *Rickettsia* spp.) by *I. ricinus*, as has been suggested for the symbiont *Rickettsia peacockii* in the American tick *Dermacentor andersoni* (Burgdorfer *et al*., 1981). As some long-term laboratory tick lines are apparently uninfected by IricES1, it should be possible to examine, via *in vitro* infection (Broadwater *et al*., 2002; Fingerle *et al*., 2002), whether other intracellular bacteria are more easily able to infect the ovaries when IricES1 is not present.

Experimental procedures

Tick collection and DNA extraction

Free-living *I. ricinus* adult ticks were collected by flagging vegetation in 12 localities in 10 countries (Fig. 2, Table 1), and identified and sexed using standard taxonomic keys. Owing to a previous study, which indicated that only females were infected with IricES1 (Beninati *et al*., 2004), the screening study was performed predominantly on females. DNA was extracted from either live ticks or specimens preserved in 100% ethanol, using a Bioneer Accuprep Genomic DNA Extraction Kit, following the manufacturer's instructions, into a final volume of 100 μl.

Phylogenetic analyses

A 16S rRNA sequence for IricES1 obtained previously (Beninati *et al*., 2004) was subjected to BLAST (http://www.ncbi.nlm.nih.gov/blast) analysis and aligned with close relatives as well as other proteobacterial sequences. Alignment of the corresponding 16S rRNAs was performed using software at the Ribosomal Database Project website (Cole *et al*., 2003), taking secondary structure into account. Phylogenetic analyses were performed under maximum likelihood (ML) criteria, using the program TREEPUZZLE 5.0 (Strimmer and von Haeseler, 1996). Default settings were used with the exception that the TN+G model of sequence evolution was selected.

Screening for IricES1 via PCR of 16S rRNA

Polymerase chain reaction of 16S rRNA was performed as described in Beninati and colleagues (2004), using the primers IricES1-F (5′-TGTAGCGATACAGAGTTCTGC-3′) and IricES1-R (5′-CACCCCAGTCGTCAACCTTAC-3′). Amplifications were performed in 30 μl of buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin] with 0.2 mM each deoxynucleoside triphosphate, 20 pmol each primer, 1 U of *Taq* polymerase (Perkin Elmer) and 1 μl of DNA sample. Cycling conditions were: 94°C for 1 min and 30 s; five cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 2 min; 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 2 min; 5 min at 72°C.

The effectiveness of the 16S rRNA PCR assay in detecting the symbiont was estimated as follows. The amplified fragment was cloned into a TA cloning vector (Promega pGEM-T Easy Vector Cloning Kit), and a plasmid miniprep was made (Sambrook *et al*., 1989) and purified (Macherey Nagel

NucleoSpin Plasmid Quick Pure). An aliquot of pure plasmid was then quantified using UV spectrophotometry at 260 and 280 nm, serially diluted and added to PCR tubes to give an equivalent number of gene copies from 2 to 2×10^9. Polymerase chain reaction was performed as described above, with the addition of 1 μl of an *I. ricinus* male DNA sample known to be uninfected by IricES1 (as determined using the *gyrB* assay described below). This was added to control for any effect of tick DNA sample on the PCR.

A 380 bp fragment of 16S rRNA was also sequenced for two to three ticks from each of the 10 countries examined in this study. The templates used for these sequencing reactions were from PCR amplifications using IricES-F and IricES-R (described above). Sequencing was performed using an ABI BigDye Terminator Kit on an ABI-Prism 310 Sequencing machine. The primers used for sequencing were IricES-F and IricES-R2 [5′-TCGTATTACCGCGGCTGCTG-3′; equivalent to positions 519–538 of the *Escherichia coli* K12 16S rRNA (GenBank Genome Accession No. NC_000913)].

Screening for IricES1 via PCR of gyrB

A second, species-specific PCR assay for the gene *gyrB* was developed. Initially, a 703 bp fragment of this gene was amplified from DNA extracted from the ovary of an engorged *I. ricinus* female known to be infected with IricES1 (Beninati *et al*., 2004). The primers used for this PCR were gyrB-consF [5′-TCNTT(T/C)TT(A/G)AA(T/C)TCNGGNGT-3′; equivalent to positions 583–602 of *E. coli gyrB*] and gyrB-consR [5′-CCNGCNGA(A/G)TCCCNTT(C/T)TA-3′; equivalent to positions 1267–1286 of *E. coli gyrB*], each of which was designed based on an alignment of various proteobacterial *gyrB* amino acid sequences. Conditions for this PCR were as follows: 94°C for 2 mins; 35 cycles of 94°C for 30 s, 52°C for 45 s and 72°C for 1 min; 10 min at 72°C. 5 μl of DNA sample were used in this PCR. Following cloning and sequencing of the fragment, the specific primers gyrB-Fa (5′-CTTGAGAGCA GAACCACCTA-3′; equivalent to positions 621–640 of *gyrB* from *E. coli*) and gyrB-Ra (5′-CAAGCTCTGCCGAAATA TCTT-3′ equivalent to positions 740–760 from *E. coli*) were then designed to amplify a 145 bp fragment. Conditions were as follows: 94°C for 2 mins; 40 cycles of 94°C for 30 s, 60°C for 1 min; 10 min at 72°C. Controls using bacteria found in *I. ricinus* (*Rickettsia* spp., *Ehrlichia* spp., *Borrelia* spp.; see Beninati *et al*., 2004) were included to check for non-specific amplification. Serial dilution experiments of cloned *gyrB*, similar to those described above for 16S rRNA, were also performed to determine the detection limit. Polymerase chain reaction of *gyrB* was performed on all samples negative for the 16S rRNA PCR. The three negative females from a previous study of ticks collected in Italy (Beninati *et al*., 2004), as well as negative males collected in Italy and England, were also screened using the *gyrB* PCR.

Polymerase chain reaction and sequencing of a 572 bp fragment was also performed on two to three tick representatives from each of 11 countries. The primers used for this PCR were gyrB-Fa and gyrB-Rb (5′-ATCACTTTTTTGCC TTTTGAG-3′; equivalent to positions 1171–1191 of the *E. coli* homologue). Conditions for the PCR reaction were as follows: 94°C for 2 mins; 35 cycles of 94°C for 30 s, 57°C for 45 s and 72°C for 1 min; 10 min at 72°C.

Laboratory ticks

Various generations (F1–F7) of adult ticks that had been maintained in the laboratory on pathogen-free New Zealand White Rabbits were screened for PCR as described above. Vertical transmission of IricES1 was examined by raising four F2 adult females from Berlin on pathogen-free guinea pigs. Following detachment, engorged females were incubated at 25°C and 70% relative humidity. Eggs began to appear 5–6 days later, and a subset of these were examined individually by DNA extraction and PCR.

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