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Prevalence and burden of two rickettsial phylotypes (G021 and G022) in *Ixodes pacificus* from California by real-time quantitative PCR

Du Cheng^{a,1}, Katie Vigil^{a,1}, Paula Schanes^{a,1}, Richard N. Brown^b, and Jianmin Zhong^a

^aDepartment of Biological Sciences, Humboldt State University, Arcata, CA, 95521, USA

^bDepartment of Wildlife, Humboldt State University, Arcata, CA, 95521, USA

Abstract

The western black-legged tick, *Ixodes pacificus* Cooley and Kohls, commonly bites humans in the far western U.S. In addition to transmitting Lyme borreliosis and anaplasmosis, it is a host of nonpathogenic bacteria as well as some of unknown pathogenicity. In this study, we report the detection, prevalence, and burden of 2 rickettsial phylotypes with unknown pathogenicity in *I. pacificus* ticks from 6 California counties using real-time quantitative PCR with phylotype-specific primers and probes. Prevalence of rickettsial phylotypes G021 and G022 from 247 *I. pacificus* ticks was 100% and 2.0%, respectively. The median burden of phylotype G021 was 7.3 per tick cell, whereas the burden of phylotype G022 was 0.8 per tick cell. The burden of phylotype G021 significantly differed between collection sites and between vegetation habitats. Ticks collected from the coastal sage scrub habitat of southern California had a lower burden of phylotype G021 when compared to central California oak woodland, northern California oak woodland, and mixed evergreen and ponderosa pine-oak habitats of northern California. No significant correlation was found between the burden of the phylotype G021 in the presence and absence of the phylotype G022 in *I. pacificus*, suggesting that the presence of these *Rickettsia* species do not interfere with each other in *I. pacificus*.

Keywords

Rickettsia; *Ixodes pacificus*; Prevalence; Burden; Microhabitat

Introduction

Ticks are important vectors of diseases in humans and other animals (Jongejan and Uilenberg, 2004). In the U.S., *Ixodes pacificus* Cooley and Kohls is distributed throughout California, Oregon, Washington, and sporadically in parts of Arizona, Nevada, and Utah (Furman and Loomis, 1984). In addition to transmitting *Borrelia burgdorferi* Johnson et al.

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*Corresponding author. Department of Biological Sciences, Humboldt State University, 1 Harpst Street, Arcata, CA 95521, USA. Tel.: +1 707 826-5554; fax: +1 707 826-3201. jz15@humboldt.edu (Jianmin Zhong).

¹These authors contributed equally.

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and *Anaplasma phagocytophilum* (Foggie) Dumler et al. (Naversen and Gardner, 1978; Burgdorfer et al., 1985; Barlough et al., 1996; Richter et al., 1996), *I. pacificus* harbors nonpathogenic spotted fever group rickettsiae, the Tillamook agent, and '*Rickettsia midichlorii*', for which no pathogenicity has been reported in vertebrates (Gottlieb et al., 2006; Hughes et al., 1976; Philip et al., 1978, 1981; Troughton and Levin, 2007). Nonpathogenic rickettsiae are antigenically closely related to, but distinct from, pathogenic rickettsiae (Hughes et al., 1976; Philip et al., 1978, 1981). The prevalence of apparently nonpathogenic rickettsiae varies widely among different tick species, ranging from 0% in *Amblyomma naponense* Packard, to 100% in *Amblyomma rotundatum* Koch (Labruna et al., 2004; Perlman et al., 2006). Although little is known about roles of the nonpathogenic rickettsiae, it has been hypothesized that some nonpathogenic rickettsiae interfere with the transovarial transmission of pathogenic *Rickettsia rickettsii* (Wolbach) Brumpt in ticks (Price, 1953; Macaluso et al., 2002; Niebylski et al., 1997). Some nonpathogenic rickettsiae are endosymbiotic and have been shown to play essential roles in host fitness (Giorgini et al., 2010; Himler et al., 2011), including reproductive manipulation, such as male-killing (Werren et al., 1994; von der Schulenburg et al., 2001), parthenogenesis (Giorgini et al., 2010), and fecundity (Sakurai et al., 2005; Perotti et al., 2006). The effects of rickettsial presence on tick reproduction remain unknown.

The putative symbionts have only occasionally been cultivated in conventional cell culture, and often require tick cell lines for cultivation (Wolbach et al., 1922; Raoult and Roux, 1997). Conventional PCR is reproducible and capable of verifying the size of a specific PCR amplicon. Drawbacks of this method include lack of rapid quantification of the amplicon and the requirement of post-PCR gel electrophoresis step. Quantitative real-time polymerase chain reaction (qPCR) is a frequently used and practical tool for quantification of particular bacterial species in ticks (Jasinskas et al., 2007; Zhong et al., 2007). While cell culture remains a vital and important means of bacterial detection, qPCR is rapid, highly sensitive, reproducible, and has an advantage in applying a linear amplification over a wide, dynamic range of targeted bacterial species.

Recently, our laboratory has identified 2 rickettsial phylotypes (G021 and G022), or bacterial phylogenetic types, in *I. pacificus* from California's Napa Valley (Phan et al., 2011). However, the ecology and biology of the rickettsial phylotypes in *I. pacificus* remain incompletely characterized. Here, we report the prevalence, burden, and broad habitat associations of 2 recently identified rickettsial phylotypes, G021 and G022, in *I. pacificus* from 7 collection sites in California, assessed by qPCR.

Materials and methods

Tick collections

I. pacificus ticks were collected by dragging a 1-m² white flannel cloth over grass and shrubs. The dragging at each collection site was conducted by 2–5 persons for one hour. The cloth was examined every 2 min, and ticks were manually removed from the cloth with a forceps. A total of 247 adult *I. pacificus* was collected from 7 collection sites in 6 California counties in 2009 and 2010, including 30 adults from Contra Costa County [standard universal transverse mercator grid system (UTM) coordinates: 10N 585409 4183882], 63 adults from Humboldt County (UTM: 10N 429550 4530570), 50 adults from Mendocino County (UTM: 10N 485625 4345185), 33 adults from Orange County (UTM: 11N 439605 3705363), 31 adults from Middle Creek in Shasta County (UTM: 10N 547409 4493951), 15 adults from Whiskeytown in Shasta County (UTM: 10N 531122 4507650), and 25 adults from Annadel State Park in Sonoma County (UTM: 10N 531979 4256013) (Fig. 1).

Four discrete habitat types were assigned after combining information on latitude, terrain, precipitation, vegetation, and climate (Fig. 1). Dominant plants in coastal sage scrub habitat include California sagebrush (*Artemisia californica* Less.), buckwheat (*Eriogonum fasciculatum* Benth.), California lilac (*Ceanothus* spp.), manzanita (*Arctostaphylos* spp.), sage (*Salvia* spp.), coyote brush (*Baccharis* spp.), lemonade berry [*Rhus integrifolia* (Nutt.) Engl.], laural sumac (*Malosma laurina* Engl.), and scrub oak (*Quercus dumosa* Nutt.). The central California oak woodland habitat is comprised of interior live oak (*Quercus wislizeni* A. DC.), valley oak (*Quercus lobata* Née), Oregon white oak (*Quercus garryana* Douglas ex Hook.), gray pine (*Pinus sabiniana* Douglas), Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], California bay [*Umbellularia californica* (Hook. & Arn.) Nutt.], manzanita, coffeeberry, and redberry (*Rhamnus* spp.), toyon [*Heteromeles arbutifolia* (Lindl.) M. Roem.], and various grasses and forbs. Common plants in northern California oak woodland habitat include interior live oak, black oak (*Quercus kelloggii* Newb.), gray pine, California buckeye (*Aesculus californica* Nutt.), buckbrush (*Ceanothus cuneatus* A. Gray), coffeeberry (*Rhamnus californica* Eschsch.), manzanita, poison oak (*Toxicodendron diversilobum* Greene), and various grasses and forbs. The habitat in the mixed evergreen and ponderosa pine-oak has common plants including ponderosa pine (*Pinus ponderosa* Douglas ex Loudon), canyon live oak (*Quercus chrysolepis* Liebm.), Oregon white oak, tanoak (*Lithocarpus densiflorus* Rehder), chamise (*Adenostoma fasciculatum* Hook. & Arn.), manzanita, and various grasses and forbs (Mayer and Laudenslayer, 1988). Central California oak woodland was the most common habitat compared to the southern coastal sage scrub, northern oak woodland, and mixed evergreen and ponderosa pine-oak habitats. Among 4 types of habitat, 105 ticks were sampled from central California oak woodland habitat in Contra Costa, Mendocino, and Sonoma Counties; 33 from coastal sage scrub habitat in Orange County; 78 from mixed evergreen and ponderosa pine-oak habitat in Humboldt County, and Whiskeytown in Shasta County; and 31 from northern California oak woodland near Middle Creek of Shasta County (Fig. 1).

Of 247 *I. pacificus*, 134 ticks were collected from Contra Costa, Orange, Shasta, and Sonoma Counties in late winter, whereas 50 ticks from Mendocino County and 63 ticks from Humboldt County were collected in late fall. The collected ticks were identified under a dissecting microscope using a standard key for morphological identification (Furman and Loomis, 1984). All ticks were stored in 70% ethanol until use.

DNA extraction

I. pacificus adults were rinsed 3 times in 70% ethanol to reduce surface bacteria. Tick samples were individually placed in microcentrifuge tubes and were frozen in liquid nitrogen for exoskeleton and tissue pulverization with DNase-free plastic pestles. Total genomic DNA was individually extracted using DNeasy Blood & Tissue Kits (QIAGEN, Valencia, CA), as described previously (Zhong et al., 2007). A negative control mock extraction (without tick specimens) was prepared for every 10 samples extracted using the same DNA extraction procedure as stated above.

Cloning and sequencing

The genes encoding outer membrane protein A (*ompA*) from rickettsial phylotypes G021 and G022 and actin from *I. pacificus* were amplified by GoTaq® Green Master Mix (Promega, Madison, WI). PCR reactions were carried out using *I. pacificus* genomic DNA pooled from 96 field-collected adult ticks in Humboldt County as a DNA template and a final concentration of 0.2 µM of each primer (Table 1). G021 and G022 *ompA* genes were cloned, as well as the *I. pacificus* actin gene, using StrataClone™ PCR Cloning Kit, as described by the manufacturer (Agilent Technologies, Santa Clara, CA). Plasmids were purified using the PureYield™ Plasmid Miniprep System (Promega, Madison, WI). A

Thermo Scientific NanoDrop™ 1000 Spectrophotometer was used to determine the concentration of DNA. All constructs were confirmed by nucleotide sequencing (Elim Biopharm, Inc., Hayward, CA).

Real-time quantitative PCR (qPCR)

To design primers and probes for qPCR, 2 alignments were carried out using ClustalX Software (Version 1.83). One alignment contained sequences of the *ompA* gene from phylotypes G021 (GenBank accession number: GQ375161) and G022 (GQ375162) and homologous regions of all *Rickettsia* species in GenBank. The second alignment contained sequences of the actin gene of *I. pacificus* (GU556973) and homologous regions of related tick species. Regions with unique nucleotides were used to design primers and probes by Primer Express Software (Version 2.0, Life Technologies Corporation, Carlsbad, California). The sequences of the primers and probes are listed in Table 1.

A Taqman® assay was used to quantify amplicons of rickettsiae and tick cells using Applied Biosystems 7300 Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA). Briefly, the qPCR conditions included a denaturation step of 10 min at 94°C and 40 cycles consisting of denaturation for 30 s at 94°C and annealing and elongation at 60°C for 1 min. qPCR was performed using qPCR MasterMix containing ROX passive reference and 1.5 mM MgCl₂ (AnaSpec, Fremont, CA) and 0.25 μM of each primer and probe (Table 1). A final concentration of 0.04% bovine serum albumin was added in the qPCR reactions in order to overcome unknown inhibitors. The qPCR amplified a 76-bp G021 *ompA* gene fragment, a 98-bp G022 *ompA* gene fragment, and a 108-bp *I. pacificus* actin gene fragment, respectively. DNA from each tick was individually analyzed to quantify the burden and prevalence of rickettsiae. In our study, bacterial burden is defined as the number of outer membrane protein A *ompA* gene copies of the phylotype relative to the number of *I. pacificus* actin gene copies (Jasinskas et al., 2007; Zhong et al., 2007). The number of rickettsiae per tick cell was determined from a standard curve generated using 10-fold serially diluted known copies of phylotype G021 and G022 *ompA* gene clones, and an *I. pacificus* actin clone. qPCR results were confirmed by gel electrophoresis. Every tick sample was measured in duplicate for *I. pacificus* actin and G021 *ompA* genes. The *ompA* gene from G022 was measured 4 times to reduce the error of detecting a low quantity of rickettsial DNA in some samples. The mock extracts were quantified concurrently with tick samples in order to confirm reliability of the DNA extractions.

Sensitivity, specificity, and efficiency were determined in the qPCR assay. A 10-fold dilution series of clone DNA containing either phylotype *ompA* gene or *I. pacificus* actin gene was prepared to determine qPCR sensitivity and linear dynamic range. The dilution series copy number ranged from 3.1×10^{-1} – 3.1×10^9 , 3.6×10^{-1} – 3.6×10^9 , and from 2.8×10^{-1} – 2.8×10^9 for G021 phylotype, G022 phylotype, and the actin gene, respectively. qPCR sensitivity was determined by testing the lowest detectable DNA copy numbers in the assay. A sample was considered positive if its threshold cycle (Ct) value was less than the lowest limit of detection. All possible combinations of primers and probes for the phylotypes were tested to determine specificity of qPCR. Isolated DNA from *Ixodes scapularis* Say ISE6, a *Rickettsia*-free cell line, was also used to determine the specificity of qPCR (Munderloh and Kurtti, 1995). Probes and primers were considered specific if they amplified only phylotype-specific clones. The efficiency of qPCR was calculated with the formula $E = 10^{(-1/\text{slope})} - 1$, where slope of the standard curve was calculated by the ABI 7300 System SDS Software (Cikos and Koppel, 2009).

Statistical analysis

Linear regression was carried out using the SPSS statistical software package (version 17) (IBM SPSS North American Headquarters, Chicago, IL). All other statistical analyses were carried out using the R environment software package (version 2.13.2) (Chambers, 1998). A generalized linear model was used to analyze the effects of tick collection sites and habitats on the burdens of rickettsiae in *I. pacificus*. ANOVA and Tukey's post hoc tests were used to compare the log-transformed burdens of rickettsiae among tick samples. The influence of the presence of phylotype G022 on the burden of phylotype G021 was evaluated using one-way ANOVA. Linear regression was tested to evaluate the relationship between the burdens of the 2 rickettsial phylotypes in ticks. Results of statistical comparisons with *P* values less than 0.05 were considered significant.

Results

Sensitivity, specificity, efficiency, and linear dynamic range of the qPCR assay

The primer set specificity for G021 and G022 phylotypes was confirmed by NCBI BLAST analysis; the nucleotide sequences for phylotypes G021 and G022 primers and probes were not significantly homologous between 2 phylotypes. The G021 primer set and probe showed an 8.7% (2 of 23 bases) and 4.3% (1 of 23 bases) mismatch to the most homologous *ompA* DNA sequence of *R. aeschlimannii* Beati et al. (GenBank accession number HQ335160). G022 primers and probe showed a 14.8% (4 of 27 bases) and 18.8% (3 of 16 bases) mismatch to most homologous *ompA* DNA sequence of '*R. monacensis*' (accession number FJ919651). Probe and primer sets generated phylotype-specific qPCR products with no PCR amplification of another cloned phylotype; qPCR products were not obtained with any other combinations of primers and probes of the 2 phylotypes. qPCR results with the DNA template of *I. scapularis* ISE6 were negative (Fig. 2B).

The lowest detectable copy number of phylotypes G021 and G022 *ompA* genes, and *I. pacificus* actin gene was determined as 3.1, 3.6, and 2.8 copies, respectively. Gel electrophoresis confirmed the sensitivity of the qPCR and the sizes of the 3 amplicons (Fig. 2A). The average efficiency of qPCR amplification for rickettsial phylotypes G021, G022, and actin were calculated to be 93.4% ($\sigma=4.6\%$), 94.3% ($\sigma=6.5\%$), and 99.1% ($\sigma=5.6\%$), respectively. Cloned phylotype DNA had similar qPCR efficiency when diluted with either water or elution buffer from the DNeasy Blood & Tissue Kits (QIAGEN). The linear dynamic range of phylotypes G021 and G022 *ompA* genes, and *I. pacificus* actin gene was determined to be $3.1\text{--}3.1\times 10^7$ ($R^2=0.998$), $3.6\text{--}3.6\times 10^7$ ($R^2=0.996$), and $2.8\text{--}2.8\times 10^7$ ($R^2=0.997$), respectively (Fig. 2C). Within this range, the copy number of the samples was accurately quantified.

Prevalence and burden of rickettsial phylotypes

A total of 247 (100% prevalence) *I. pacificus* ticks was qPCR-positive for the rickettsial phylotype G021. In contrast, only 5 [2.0% prevalence; 95% confidence interval (CI): 0.3–3.7%] of 247 ticks tested positive for phylotype G022. Ticks from Contra Costa and Humboldt County tested 10% and 3.2% positive for phylotype G022, respectively (Table 2). Although 2 collection sites tested positive for phylotype G022, the prevalence was not significantly different between Contra Costa and Humboldt County (Fisher's exact test; *P* > 0.05). The median burden of phylotype G021 was 7.3 per tick cell (95% CI: 1.3–13.4) and differed between collection sites (one-way ANOVA; *P* < 0.05).

The median burden of G021 was 9.8 per tick cell in Contra Costa County, 4.8 in Humboldt County, 6.0 in Mendocino County, 0.9 in Orange County, 25.7 in Whiskeytown of Shasta County, 8.1 in Middle Creek of Shasta County, and 12.8 in Annadel State Park of Sonoma

County (Fig. 3). Based on Tukey's post-hoc comparison, the burden of phylotype G021 between some collection sites was significantly different ($P < 0.001$), whereas other sites were not significantly different (Table 3). For example, ticks collected from Whiskeytown in Shasta carried a higher burden of phylotype G021 than did all collection sites other than Annadel State Park in Sonoma County ($P < 0.05$), but the burden at Annadel State Park was not shown to be different from burdens in ticks from Mendocino County and Whiskeytown and Middle Creek in Shasta County ($P > 0.05$). Additionally, a *t*-test analysis showed that the burden of phylotype G021 in *I. pacificus* was not significantly different between ticks collected in late fall (median burden of 4.9 per tick cell) and late winter (median burden of 9.1 per tick cell) ($P > 0.05$).

Ecological variables were evaluated to investigate rickettsial burden between collection sites. One-way ANOVA was used to analyze the rickettsial burden grouped by broad habitat types. The burden of phylotype G021 was different among habitats ($P < 0.01$). Tukey's post-hoc analysis showed a significant difference of burden of phylotype G021 in *I. pacificus* ticks collected from coastal sage scrub habitat than in 3 other habitat types combined; we found a median burden of 0.9 rickettsiae per tick cell from coastal sage scrub habitat (95% CI: 0–6.1) which was lower than 8.6 (95% CI: 0–19.5) from central California oak woodland ($P < 0.05$), 6.8 (95% CI: 0–18.6) from mixed evergreen ponderosa pine-oak habitat ($P < 0.01$), and 8.1 (95% CI: 3.9–12.3) from northern California oak woodland ($P < 0.01$) (Fig. 4).

The median burden of phylotype G022 was 0.8 per tick cell (95% CI: 0–2.9); ranging from 3.3 per tick cell in ticks from Humboldt County and 2.1×10^{-4} for Contra Costa County to zero for Mendocino, Orange, Shasta, and Sonoma County. No significant differences of the burdens of the phylotype G022 were found among collection sites ($P > 0.05$), habitat types ($P > 0.05$), and time of collections ($P > 0.05$). There was no significant difference between the mean burden of G021 alone and that of G021 and G022 co-infections ($F = 0.12$; $df = 263$; $P > 0.05$). Also there was no correlation observed between the burdens of the phylotype G021 and G022 at the 2 collection sites where both phylotypes were found ($R^2 = 0.288$; residual mean-square error = 0.024; $P > 0.05$).

Discussion

In this study, we developed a qPCR assay with optimal sensitivity, specificity, and efficiency to demonstrate the presence of 2 rickettsial phylotypes in *I. pacificus* in 6 counties within the state of California. By accessing the geographic information of these collection sites, we report that the burden of rickettsiae may be associated with the habitat in which its host resides.

100% of ticks surveyed tested positive for rickettsial phylotype G021, whereas the prevalence of phylotype G022 was only 2.0% from 7 tick collection sites (Table 2). The high prevalence of G021 is similar to *Rickettsia* sp. IXLII reported in *I. lividus* Koch (Graham et al., 2010) and *Rickettsia helvetica* Beati et al. reported in *I. ricinus* Linnaeus (Nielsen et al., 2004). A widespread and prevalent distribution of *Rickettsia* species has been observed in other arthropods, in which *Rickettsia* was found in up to 100% of cat fleas, *Ctenocephalides felis* Bouché, and whiteflies, *Bemisia tabaci* Gennadius (Gottlieb et al., 2006; Reif et al., 2008). The high prevalence of phylotype G021 suggests that the bacterium is an endosymbiont of *I. pacificus* (Chen et al., 1996; Azad and Beard, 1998; Perlman et al., 2006; Weinert et al., 2009). Previous phylogenetic analysis demonstrated that the rickettsial phylotype G021 has high homology with the endosymbiotic *Rickettsia* species in *I. scapularis* (Phan et al., 2011). Additionally, preliminary data indicate a transovarial

transmission route for the phylotype G021, which further supports the endosymbiont hypothesis (Zhong, unpublished data).

Our previous phylogenetic analysis supports phylotype G022 as a novel spotted fever group *Rickettsia* species (Phan et al., 2011). In contrast to phylotype G021, the prevalence of phylotype G022 was comparatively low in *I. pacificus*. Interestingly, the low prevalence of phylotype G022 was similar to the prevalence (4.35%) of the Tillamook agent, a previously reported SPF *Rickettsia* species in *I. pacificus* (Hughes et al., 1976; Lane et al., 1981). This disparity of rickettsial prevalence in *I. pacificus* may be the result of different transmission routes for the 2 phylotypes. Little is known about the maintenance of these 2 rickettsial phylotypes in *I. pacificus*. The low prevalence of phylotype G022 implies that it might rely on other tick or vertebrate hosts to ensure its survival in nature and that it may cause disease in vertebrate hosts (Munderloh and Kurti, 1995; Raoult and Roux, 1997; Azad and Beard, 1998; Paddock et al., 2004). Previously, the Tillamook agent was reported as a mildly virulent strain since it causes mild fever in guinea pigs (Hughes et al., 1976).

The burdens of phylotype G021 varied among tick collection sites in California. Although time of tick collections does not affect the burden of the phylotype in sampled ticks, ticks collected from the coastal sage scrub habitat had the lowest burden of phylotype G021 when compared to Central California oak woodland, Northern California oak woodland, and mixed evergreen and ponderosa pine-oak habitats (Table 2). Despite small sample sizes, our data suggest that this phylotype may exhibit higher vitality in specific microhabitats that include predominantly wooded areas. As suggested recently, this observation could be due to a variety of biotic and abiotic microhabitat factors (Lane et al., 2007; Swei et al., 2011). In contrast to other habitats sampled in this study, coastal sage scrub is characterized by dry shrubs and a relative lack of densely wooded areas; it is a drought-resistant deciduous plant community that thrives in Mediterranean-like, drier locations at lower elevations, similar to southern California's coastal habitats (Kirkpatrick and Hutchinson, 1980; Westman, 1981). The sensitivity to desiccation of *I. pacificus* has been shown repeatedly and this likely affects their abundance (Clover and Lane, 1995; Bertrand and Wilson, 1996; Peavey and Lane, 1996) and questing behavior (Burri et al., 2011; Randolph and Storey, 1999) in arid environments. It may be that host health and questing behavior, as determined by the humidity of different microclimate conditions, are determining factors in ticks for the maintenance of robust bacterial infections (Stafford, 1994; Bertrand and Wilson, 1996; Fielden and Rechav, 1996; Peavey and Lane, 1996; Padgett and Lane, 2001). Due to low rainfall and lack of a canopy, sensitivity to desiccation is necessarily aggravated in the southern California coastal sage scrub climate, and this may account for the depressed *Rickettsia* bacteria counts found in our study (Harrison et al., 1971). Habitat-type may be a key variable that supports bacterial burden in distinct regions, and this relationship should be evaluated in future ecological investigations.

Many research groups have failed to detect co-infections of *Rickettsia* species in ticks (Fernandez-Soto et al., 2004; Marquez et al., 2006; Labruna et al., 2007). Conversely, many studies have successfully displayed co-infections in various tick hosts (Carmichael and Fuerst, 2006; Rydkina et al., 1999; Halos et al., 2010; Berrada et al., 2011). *Ixodes ricinus* has been shown to harbor *R. helvetica* and a rickettsial symbiont (Halos et al., 2010). *Rickettsia sibirica* Zdrodovskii and *Rickettsia conorii* Brumpt have been shown to co-infect *Rhipicephalus pumilio* Schulze, *Rh. sanguineus* Latreille, and *Dermacentor nuttalli* Olenov (Rydkina et al., 1999). Naturally occurring tri-infections of *Rickettsia bellii* Philip, *Rickettsia montanensis* (Lackman) Weiss and Moulder, and *R. rickettsii* in *D. variabilis* Say have also been detected (Carmichael and Fuerst, 2006). Infection of *I. pacificus* with 2 rickettsial phylotypes raises questions of a possible relationship between G021 and G022 burdens in co-infected ticks because of reported interference among different rickettsiae in

ticks (Burgdorfer et al., 1981; Telford, 2009). However, burdens of infection by the 2 phylotypes in *I. pacificus* collected for this study were not correlated suggesting that the presence of one rickettsial phylotype does not influence infections of the other in *I. pacificus*, although low statistical power associated with the low prevalence and burden of phylotype G022 make it difficult to make a meaningful statement concerning this relationship.

Together, our results indicate the presence of 2 newly detected rickettsial phylotypes in *I. pacificus*, although it is likely that phylotype G0722 is identical, or closely related to, the Tillamook agent (Hughes et al., 1976; Lane et al., 1981) and '*R. midichlorii*' (Troughton and Levin, 2007) in *I. pacificus*. High prevalence and burden of G021 suggest an established, symbiotic relationship between the bacteria and host as previously demonstrated for other rickettsial species (Chen et al., 1996; Azad and Beard, 1998; Perlman et al., 2006; Weinert et al., 2009). Future studies on transovarial and transstadial transmissions of the rickettsial phylotypes in *I. pacificus* and strain isolations using cell culture techniques are needed to confirm symbiotic status. Additionally, our findings support a significant influence of habitat on bacterial load within their tick hosts. Although data of ecological parameters in all habitats are not comprehensive, our findings provide baseline data for future, large-scale research on the prevalence of rickettsial phylotypes in *I. pacificus* from different habitats. Understanding the biology and ecological requirements of tick endosymbionts can contribute toward developing novel control strategies. Experimental manipulation of the bacterial symbiont may lead to suppression of tick populations and reduction of tick-borne diseases in the future.

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Fig. 1. Geographic distributions of the collected tick samples. The California County Map was provided with permission of use by the California State Association of Counties.

B. Specificity

Primers	Probe	DNA template	Amplification
G021	G021	<i>Ixodes scapularis</i> ISE6	no
G022	G022	<i>Ixodes scapularis</i> ISE6	no
G021	G022	G021 clone	no
G021	G022	G022 clone	no
G022	G021	G021 clone	no
G022	G021	G022 clone	no
G021	G021	G021 clone	yes
G022	G022	G022 clone	yes

C. Linear Dynamic Range and Efficiency

Fig. 2.

The sensitivity, specificity, linear dynamic range, and efficiency of qPCR. (A) Sensitivity. Fragments of the phylotype G021, G022, and actin gene of *Ixodes pacificus* were amplified by qPCR. The lowest detectable copy numbers of the 3 amplicons by qPCR were confirmed by running on a 3% agarose gel. (B) Specificity. Different combinations of the primers and probes of the 2 rickettsial phylotypes were used to amplify either clone DNA or *Ixodes scapularis* tick cell line ISE6. The positive result indicated the detection of the PCR amplicon by qPCR. (C) Linear dynamic range and efficiency. qPCR amplification plots and corresponding standard curves of the genes (phylotype G021, G022, and actin). The linear dynamic range of the standard curves of the genes was determined to be from 3 to 3×10^7

copies. K represents the slope of the standard curve that was used to calculate the qPCR efficiency. R^2 represents the coefficient of determination of the standard curve.

Fig. 3.

Box-whisker plots of Log_{10} ratios of copies of *Rickettsia* species phylotype G021 *ompA* gene to copies of *Ixodes pacificus* actin gene from 7 collection sites. Real-time qPCR assays with gene-specific primers and Taqman® probes were used to estimate *ompA* gene copies per tick cell.

Fig. 4. Box-whisker plots of Log_{10} ratios of copies of *Rickettsia* species phylotype G021 *ompA* gene to copies of *Ixodes pacificus* actin gene from 4 habitats. Gene copies per tick were estimated by real-time quantitative polymerase chain reaction assay with gene-specific primers and Taqman® probes.

Table 1

Primers and probes used in the study.

Gene name	Primer/probe	Sequence (5' to 3')
<i>ompA</i> (phylotype G021)	21	CTGCAGATATAGCCGGTCGTATT
<i>ompA</i> (phylotype G021)	21	TAATCGAACCAACGACGGTATTT
<i>ompA</i> (phylotype G021)	21	VIC-CTCCCGTAGGTCTAAA-MGB
<i>ompA</i> (phylotype G022)	22	ACGGCTGGAGGAGTAGCTAATG
<i>ompA</i> (phylotype G022)	22	GATTACCACCGTAAGTAAATGCCTTAT
<i>ompA</i> (phylotype G022)	22	6FAM-TCCTGTTGACGGTCCT-MGB
actin	actin-F	TTGTCCGCGACATCAAGGA
actin	actin-R	CGGGAAGCTCGTAGGACTTCT
actin	actin-probe	6FAM-CCGCCGCCTCTTCCTCTTCCT

Table 2

Prevalence and burden of 2 rickettsial phylotypes in *Ixodes pacificus* collected from 6 counties in California.

Collection sites	Ticks Examined (total number)	Phylotype G021		Phylotype G022
		Prevalence	Burden (95%CI)	Prevalence (95%CI)
Contra Costa	30	100%	9.8(0.4–21.5)	10% (0–20.7%)
Humboldt	63	100%	4.8(3.0–7.9)	3.2% (0–7.6%)
Mendocino	50	100%	6.0(4.1–9.4)	0%
Orange	33	100%	0.9(0.6–8.9)	0%
Shasta Whiskeytown	15	100%	25.7(18.8–40.5)	0%
Shasta Middle Creek	31	100%	8.1(6.4–10.8)	0%
Sonoma Annadel State Park	25	100%	12.8(10.0–17.8)	0%
Total	247	100%	7.3(1.3–13.4)	2.0% (0.3–3.8%)

CI: confidence interval.

Table 3

Significance of post-hoc comparisons of the burden of phylotype G021 in *Ixodes pacificus* from 7 collection sites in California.

Collection sites	1	2	3	4	5	6	7
1	n/a	—*	—	—	<0.01	<0.05	<0.01
2		n/a	—	—	<0.01	—	<0.05
3			n/a	—	<0.01	—	—
4				n/a	<0.01	<0.05	<0.01
5					n/a	<0.01	—
6						n/a	—
7							n/a

1, Contra Costa County; 2, Humboldt County; 3, Mendocino County; 4, Orange County; 5, Whiskeytown in Shasta County; 6, Middle Creek in Shasta County; 7, Annadel State Park in Sonoma County.

* $P > 0.05$; n/a: not applicable.