Exp Appl Acarol (2017) 73:461–475
https://doi.org/10.1007/s10493-017-0186-y

Assessment of bacterial diversity in *Hyalomma aegyptium*, *H. marginatum* and *H. excavatum* ticks through tag-encoded pyrosequencing

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Received: 19 June 2017 / Accepted: 1 November 2017 / Published online: 9 November 2017

Abstract Ticks are among the most significant human-biting ectoparasites and they play a major role in transmission of many pathogenic agents to humans. In the present study, three species of *Hyalomma* ticks, *Hyalomma aegyptium*, *H. marginatum* and *H. excavatum*, were examined for the presence of zoonotic bacteria, both male and female ticks alike. Examination of microbial diversity with tag-encoded pyrosequencing indicates that *H. marginatum* and *H. excavatum* were more diversity rich than *H. aegyptium*. Although numerous pathogenic and non-pathogenic bacterial genera were detected, including *Acidovorax*, *Bacillus*, *Bacteroides*, *Bdellovibrio*, *Clostridium*, *Curvibacter*, *Escherichia*, *Flavobacterium*, *Limnohabitans*, *Paenibacillus*, *Ralstonia*, *Sarcina*, *Sediminibacterium*, *Segetibacter Stenotrophomonas* and *Variovorax*, the predominant zoonotic bacteria represented in these ticks were genera *Borrelia, Francisella,* and *Rickettsia.* To the authors' knowledge, this work represents the first detection of *Yersinia enterocolitica* in the tick *H. excavatum*, raising questions regarding the vector competency of this tick, as well as associations of different disease representations perhaps through previously unforeseen routes of pathogen introduction. Likewise, similar questions are related to the presence of *Legionella pneumophila* in one *H. excavatum* sample.

Keywords *Hyalomma* · Humans · Pyrosequencing · Turkey · Zoonotic bacteria

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Introduction

Disease-causing organisms that can be transferred between animal and human hosts are called zoonotic pathogens which have global importance (Chomel et al. 2007; Magnarelli 2009). Many zoonotic pathogens are transferred by way of an arthropod vectors; such as, mosquitoes, biting flies, lice, fleas, and ticks (Parola and Raoult 2001; Beugnet and Marié 2009; Badiaga and Brouqui 2012). Ticks are the most significant human-biting arthropods and they play an important role in transmission of many zoonotic pathogens (Parola and Raoult 2001; Jongejan and Uilenberg 2004). Within these ticks, zoonotic disease causing organisms co-exist with other non-pathogenic organisms in a very diverse bacterial community in the same way that all living things harbor unique microbial communities (Jones et al. 2010; Andreotti et al. 2011). It is thought that the composition and interactions of organisms within these microbial communities may affect the ability of pathogenic bacteria to colonize and persist within an individual vector, invade the vector community, or ultimately affect the transfer of these organisms to vertebrate hosts (Jones et al. 2010). Therefore, investigating the bacterial diversity of tick parasites may ultimately shed light on the competency of these vectors to transmit disease to vertebrate hosts.

In Turkey, recent studies indicate a significant variety of tick genera and species biting humans as well as other vertebrate hosts, implicating these ticks as potential vectors of zoonotic pathogens. Many of these studies reported that *Hyalomma* species are the most prevalent human biting ticks in Turkey (Gargili et al. 2011; Bursali et al. 2013; Keskin et al. 2015). There are several studies about detection of microbial community in *Amblyomma*, *Dermacentor*, *Ixodes*, and *Rhipicephalus* ticks (Andreotti et al. 2011; Carpi et al. 2011; Lalzar et al. 2012; Williams-Newkirk et al. 2014); however, very little is known about the complete microbial community within the *Hyalomma* ticks.

Molecular approaches to study the microbiota of ticks have been successfully employed, and while effectively avoiding the growth bias and other issues surrounding cultural techniques (Shi et al. 2010; Andreotti et al. 2011). The focus of this paper is to examine the microbial communities in 12 ticks removed from human and tortoise hosts. We will identify the prevalence of zoonotic bacteria, detect the presence of opportunistic pathogens, and elucidate what appears to be common and commensal flora of ticks according to species from Turkey. This work, to the authors' knowledge, represents the first time tag-encoded pyrosequencing has been used to describe the microbiome of *Hyalomma aegyptium, H. marginatum,* and *H. excavatum* ticks.

Materials and methods

Tick samples

Two adult males and two adult females of *H. aegyptium* (from tortoises crushed by cars in Tokat, 2010), *H. marginatum* (from human in Tokat, 2008) and *H. excavatum* (from human in Amasya, 2008) samples from Turkish Tick Collection (TTC) at Gaziosmanpasa University, Department of Biology were randomly selected and used for the present study (Table 1). *Hyalomma marginatum* and *H. excavatum* ticks removed from humans were donated to TTC by Directorate of Primary Health Care, Ministry of Health. All samples were stored in 70% ethanol. Ticks were identified to species using the morphological keys (Merdivenci 1969). At the time of sample preparation, ticks were washed firstly in 70%

Table 1 Metadata for tick samples in Turkey. Samples are numbered 13–24. Following each sample number is a code providing metadata for each sample. The first two letters indicate the tick species. The third letter indicates the sex of the tick. The forth letter indicates the host from which it was removed

Location of collection	Year of collection	Sample #	Tick species	Sex	Host	Sample ID
Tokat	2010	13	*H. aegyptium*	m	Tortoise	13-HaMT
		14	*H. aegyptium*	f	Tortoise	14-HaFT
		15	*H. aegyptium*	m	Tortoise	15-HaMT
		16	*H. aegyptium*	f	Tortoise	16-HaFT
		17	*H. marginatum*	m	Human	17-HmMH
		18	*H. marginatum*	f	Human	18-HmFH
		19	*H. marginatum*	m	Human	19-HmMH
		20	*H. marginatum*	f	Human	20-HmFH
Amasya	2008	21	*H. excavatum*	m	Human	21-HeMH
		22	*H. excavatum*	f	Human	22-HeFH
		23	*H. excavatum*	m	Human	23-HeMH
		24	*H. excavatum*	f	Human	24-HeFH

ethanol then, in molecular grade water for 5 min and allowed to air dry in a sterilized hood. DNA was extracted as described by Dowd et al. (2008b).

Ethical statement

According to Turkish legislation no specific permits were required for the use of samples in invertebrate animal collections. However, approval for the field collection of ticks was obtained from the Ministry of Food, Agriculture and Livestock (Agreement # B.12.0.TAG.0.05.03-604.02-993). The described studies did not involve any live animals, endangered or protected animal species and use of private land, wild life, national parks and protected areas. All necessary oral permits were obtained for the use of ticks collected from human hosts, including permission of Directorate of Primary Health Care, Ministry of Health.

Molecular techniques

Recent studies have shown that the hypervariable region targeted by the primer plays a critical role in determining the profile of a microbial community generated by pyrosequencing. In order to avoid experimental bias, two primer sets were used to target the V1–V3 and V6–V8 variable regions of the 16S rRNA gene in order to compare the relative percent abundance as suggested (Kumar et al. 2011).

Template DNA, primers, and Quiagen HotStar Master Mix PCR kit reagents were combined and subjected to a single 35 cycle PCR step with the addition of 0.5U of HotStar HiFidelity Polymerase in each reaction (Qiagen, Valencia, CA, USA). The finished PCR products were sequenced using the bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) as described previously, utilizing the Titanium sequencing platform rather than the FLX platform (Roche Applied Science, Indianapolis, IN, USA) in order to attain longer average read lengths generated by the Titanium methodology (Dowd et al. 2008a, c; Andreotti et al. 2011). The B27 primer sequence extends from the 27F 5′ AGRGTTT-GATCMTGGCTCAG 3′ to 519r 5′ GTNTTACNGCGGCKGCTG 3′ in relation to *E. coli*

16S extending across V1 and into the V3 ribosomal region. The B939 primer sequence extends from the 939F 5′ TTGACGGGGGCCCGCAC 3′ to 1394r 5′ ACGGGCGGTGTGTRC 3′ in relation to *E. coli* 16S extending across V6 and into the V8 ribosomal region. Amplicon sequencing was performed based upon the manufacturer's protocols for Titanium sequencing on the FLX-titanium platform.

Data analysis

The Q25 sequence data derived from the pyrosequencing process was generated using a proprietary analysis pipeline (www.mrdnalab.com). Sequences are first depleted of barcodes and primer sequences, followed by the removal of short sequences (sequences < 200 base pairs), sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 base pairs. Sequences are then de-noised and chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity) (Dowd et al. 2008a, c; Capone et al. 2011). Operational taxonomic units were then taxonomically classified using BLASTn against a curetted GreenGenes database (DeSantis et al. 2006) and compiled into each taxonomic level as both "counts" and "percentage" files. Counts files contain the actual number of sequences while the percent files contain the relative percentage, based on proportion, of sequences within each sample that map to the designated taxonomic classification; such that, if there are 1000 sequences total and 100 of the sequences are classified as *Staphylococcus*, then we represent this as *Staphylococcus* being 10%. Data generated by the proprietary analysis pipeline were used to calculate the Shannon Diversity indices.

Results

For the B939 primer set, a total of 121,509 sequences were utilized for analysis of the 12 tick samples resulting in 647 OTUs averaging 414 ($\sigma = 9.85$) bp in length. From these OTUs, a total of 161 genera and 202 bacterial species were identified in the 12 samples. For the B27 primer set, a total of 88,635 sequences were utilized for analysis of the 12 tick samples resulting in 482 OTUs averaging 386 ($\sigma = 7.89$) bp in length. From these OTUs, a total of 116 genera and 203 bacterial species were identified in the 12 samples.

The relative percent abundance (RPA) of bacterial genera according to each tick species and primer set are summarized in Fig. 1. The *H. aegyptium* samples are by far the least rich in diversity. The genera *Francisella* and *Borrelia* make up the vast majority of the RPA, with between 85.3% (B27) and 92.1% (B939) *Francisella*, and 7.8% (B939) and 14.6% (B27) *Borrelia.* The average number of unique genera and species in *H. aegyptium* samples for the B939 primer are six genera and eight species, and three genera and four species for the B27 primer.

The *H. marginatum* and *H. excavatum* tick samples have a considerably more diverse microbiome. Organisms from the genera *Flavobacterium*, and *Rickettsia* represent between 50 and 75% RPA in the *H. marginatum* tick samples. The average number of unique genera and species in *H. marginatum* samples for the B939 primer are 48 genera and 57 species, and 30 genera and 35 species for the B27 primer. Other organisms present in the *H. marginatum* samples identified by B27 are *Curvibacter, Segetibacter* and *Bacteroides*; and *Escherichia, Sarcina,* and *Limnohabitans* by B939.

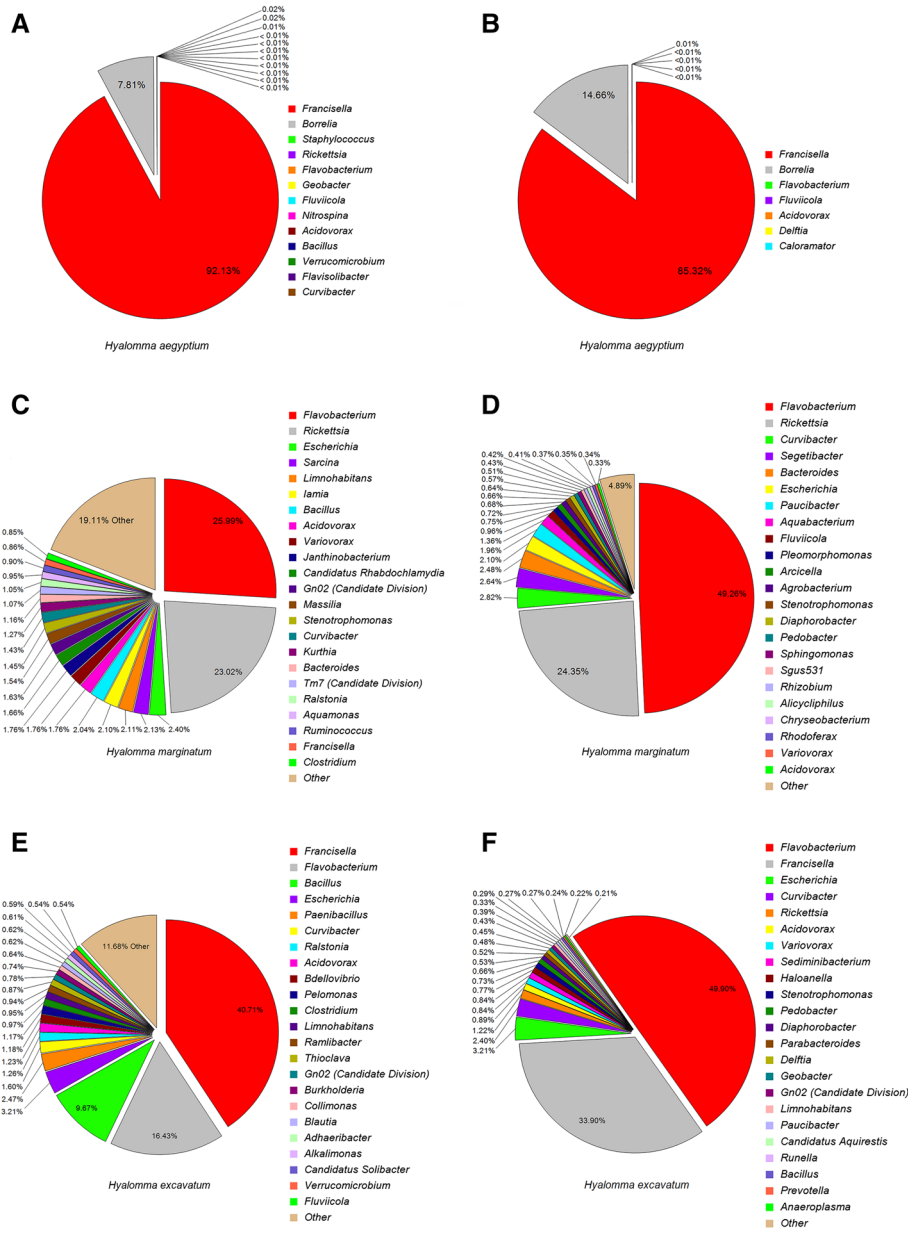

Fig. 1 Relative abundance (%) of bacterial genera (complete microbiome) according to each tick species. **a**, **c** and **e** were performed with primer B939; **b**, **d** and **f** were performed with primer B27. The organisms listed in the legends appear from top to bottom in order of highest relative abundance (RPA), and pair with the percentage decreasing in a clockwise rotation around the pie chart

Organisms from the genera *Francisella, Flavobacterium*, *Escherichia,* and *Curvibacter* predominate the microbiome of the *H. excavatum* tick samples. The average number of unique genera and species in *H. excavatum* samples for the B939 primer are 42 genera and

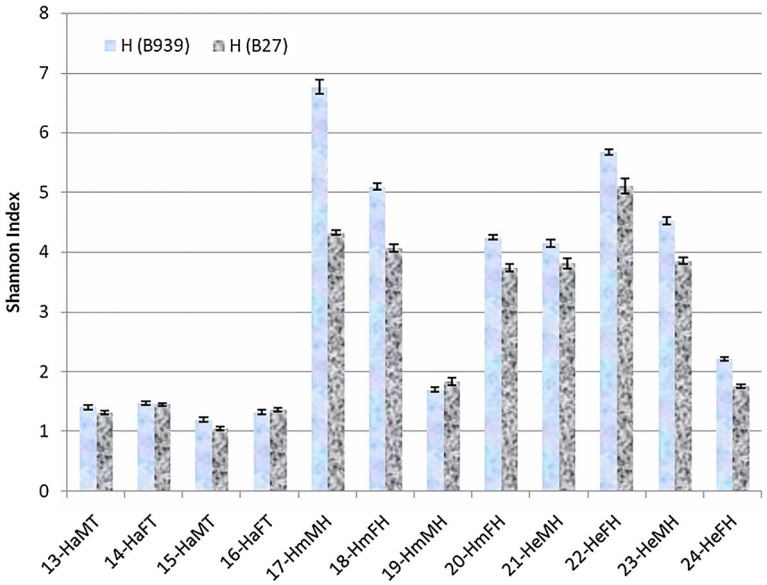

Fig. 2 Shannon diversity index for each sample as generated for each primer set. The Shannon Index was calculated by averaging Qiime generated values over 110 random sampling iterations at various sequences per sample values. Error bars represent the median absolute deviation. See Table 1 for explanation of the sample codes

52 species, and 33 genera and 39 species for the B27 primer. The B939 primers indicate *Bacillus, Paenibacillus, Ralstonia, Acidovorax*, and *Bdellovibrio* as highly prevalent genera, while the B27 primers indicate *Rickettsia, Acidovorax, Variovorax,* and *Sediminibacterium*.

The primer sets differ considerably in the RPA values for the various organisms representing the major components of the microbiome. The minor organism component RPA values are more similar. In order to compare the data of these two primer sets, the Shannon diversity and equitability indices are calculated.

The Shannon Diversity Index accounts for both richness and evenness of OTUs. Figure 2 compares the median Shannon Diversity Index values (± median absolute deviation) for each tick sample. The median absolute deviation is a measure of statistical dispersion, and is less influenced by outlying data points within a set than the standard deviation (Grice et al. 2009). The Shannon Equitability Index, which assumes a value between 0 and 1, can be calculated from the data in Fig. 3. The Shannon Equitability indicates the taxonomic evenness, with 1 representing complete evenness. The taxonomic evenness may more readily be described as the relative distribution of sequences among the OTUs. These values appear to follow the same trends when comparing results of both primer sets for a given sample to both primer sets of other samples. However, it becomes apparent in sample 17-HmMH that, though the hyper variable regions for the two primer sets both indicate a higher degree of diversity than samples 13–16, there is perhaps some discrepancy in the estimates of species richness. This trend is what led Kumar et al. (2011) to suggest using concatenated data from multiple primer sets in order to reduce the influence targeting only one rather than multiple hypervariable regions may have on the composition of the microbial community.

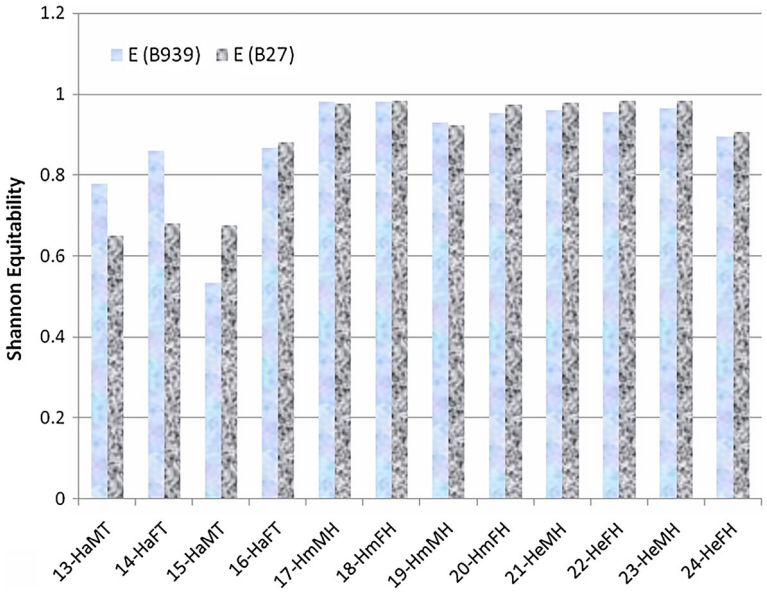

Fig. 3 Shannon Equitability for each sample as a function of the primer set. The Equitability value was calculated using the median Shannon Index value generated in Fig. 2 and the average number of observed species as generated by Qiime based on estimates of unique operational taxonomic units

Table 2 summarizes the relative percent abundance of zoonotic bacteria identified according to the respective primer sets, B939 and B27, based on the proportion of sequences within each sample that map to the designated taxonomic classification. In several instances both of the primer sets generate OTUs that map back to identify the same organisms; such as, *Borrelia turcica, Francisella* endosymbiont of *Dermacentor variabilis, Francisella novicida,* and *Rickettsia conorii* at the species level. OTUs from both primer sets mapped back to the genus level for *Francisella* and *Rickettsia*, and to the family *Legionellaceae*. The B939 primer set identified *Francisella noatunensis* and *Yersinia enterocolitica*. The B27 primer set identified an unclassified *Borrelia* species, *Legoinella pneumophila,* and *Rickettsia sibirica.* Table 2 underscores the need for scientist to concatenate data generated from multiple hypervariable regions in order to differentiate potentially pathogenic organisms at the species level.

Discussion

Most studies involving hard ticks focus on the tick directly as an ectoparasite to humans and vertebrate hosts, and indirectly as a vector of a variety of pathogens; including bacteria, viruses, protozoa, and nematodes. This research is the first effort in elucidating the bacterial community within the *Hyalomma* tick using the titanium pyrosequencing platform. An assortment of pathogenic bacteria undergo at least a portion of their life cycle harbored in ticks, which include the genera *Anaplasma, Babesia, Borrelia, Coxiella, Cowdria, Ehrlichia, Francisella,* and *Rickettsia* (Scoles 2004; Scoles et al. 2007). The γ-proteobacteria genera *Coxiella* and *Franscisella*, and the α-proteobacteria *Rickettsia* also contain endosymbionts of ticks that are closely related to pathogenic bacteria (Scoles 2004;

Table 2 Relative abundance (%) of zoonotic bacterial species identified according to the respective primer sets: B939 and B27

Organism ID	13-HaMT	14-HaFT	15-HaMT	16-HaFT	17-HmMH	18-HmFH	19-HmMH	20-HmFH	21-HeMH	22-HeFH	23-HeMH	24-HeFH	Primer
Borrelia sp.	0.378	0.399	0.135	0.132	0	0	0	0	0	0	0	0	B27
Borrelia turcica	8873	13,784	3756	5619	0.337	0	0	0	0	0	0	0	B939
	16,722	22,867	7453	11,188	0	0	0	0	0	0	0	0	B27
Francisella sp.	21,276	16,687	22,969	22,140	0	0.114	0.883	0	16,552	1116	0.774	28,379	B939
	16,567	14,973	2594	2922	0	0	0	0	13,215	0	0	35,199	B27
Francisella endosymbiont of *Dermacentor variabilis*	12,552	16,460	13,296	12,652	0	0.569	0.353	0	4978	0.587	0.155	7475	B939
	64,429	58,757	67,458	64,320	0	0	0.653	0	26,684	0	0.317	51,958	B27
Francisella noatunensis	4112	3790	4144	4697	0	0	0.589	0	3755	0.176	0.516	5238	B939
Francisella novicida	51,994	47,998	52,884	51,578	0	0.176	1617	0	34,541	1321	1446	54,656	B939
	0.675	0.639	0.966	0.116	0	0	0	0	0.128	0	0	0.346	B27
Legionella pneumophila	0	0	0	0	0	0	0	0	0	0.152	0	0	B27
Legionellaceae	0	0	0	0	0	0	0	2324	0	0	0.361	0	B939
	0	0	0	0	0	0	0	0.197	0	0	0	0	B27
Rickettsia sp.	0	0	0	0	0.793	0	2882	0	0	0.528	0	0	B939
	0	0	0	0	0	0	24,995	0.632	0	0.634	0	0	B27
Rickettsia conorii	0.636	0	0	0	0	0	88,239	0	0	0	0.516	0	B939
	0	0	0	0	0	0	23,245	0	0	0	0	0	B27
Rickettsia sibirica	0	0	0	0	0	0	47,723	0	0	0.634	0	0	B27
Yersinia enterocolitica	0	0	0	0	0	0	0	0	0	0	0	0.284	B939

Andreotti et al. 2011). These endosymbionts can have commensal, mutualistic, or parasitic relationships with the tick host (Noda et al. 1997; Scoles 2004; Andreotti et al. 2011). The next generation pyrosequencing approach for the study of microbial diversity has been used effectively to differentiate between pathogenic strains and endosymbionts within insects (Shi et al. 2010; Andreotti et al. 2011). The bacterial community of *Hyalomma* presented in this work revealed the presence of several zoonotic bacteria in the samples, and an organism from the genus *Borrelia* with unknown pathogenicity.

Francisella spp.: *Francisella* is the only genus in the family *Francisellaceae*, and includes three species: the etiological agent of tularemia *Francisella tularensis*, *F. philomiragia*, and *F. novicida*. The latter two differ in both geographical distribution and virulence, yet are still implicated in human disease. Recent articles suggests *F. philomiragia* and *F. novicida* are only associated with disease in immune-compromised patients (Hollis et al. 1989; Clarridge et al. 1996; Escudero et al. 2010). There have also been *Francisella* strains implicated in human disease whose taxonomic position has not been established (Whipp et al. 2003; Escudero et al. 2010). In this study, 10 of the 12 samples collected contained a *Francisella* endosymbiont of tick species *Dermacentor variabilis*, where the organism was first isolated. Only samples 17-HmMH and 20-HmFH did not contain this organism. In 9 of the samples containing the endosymbiont, *Francisella novicida* and *F. noatunensis* were also identified by the B939 primers, demonstrating the specificity of the next generation pyrosequencing technique. Sample 18-HmFH did not contain *F. noatunensis. F. noatunensis* was not differentiated from other *Francicella* species by the B27 primer set. Current literature demonstrates that the clinical representation of disease and innate immune response to *Francisella* strains are highly dependent on the route of infection. Intra-dermal infection in murine models indicate early production of important inflammatory cytokines, where as infection via the respiratory route does not result in the production of these cytokines until later stages of infection. This delay has been shown to contribute significantly to the septicemia indicative of respiratory tularemia, which is the deadliest form of the disease in humans resulting in a 30–40% mortality rate (Sharma et al. 2009). The incidence of *F. novicida* in Turkey is currently not reported, though new regions of Turkey are recognizing periodic incidences of tularemia (Ozsurekci et al. 2011).

Rickettsia conorii: Mansueto et al. (2012) reviewed the immunity and immunopathology of rickettsial diseases emphasizing the global importance of these pathogens as emerging zoonoses. In all, 6 of the 12 samples spanning all three tick species contained organisms in the genus *Rickettsia*. Other species of *Rickettsia* besides *Rickettsia rickettsii,* including *Rickettsia conorii*, have been implicated in human disease as indicated by serologic screening in the Black Sea region (Tekin et al. 2010), and in the Trakya regions of Turkey (Kuloglu et al. 2012). *Rickettsia conorii* is well known as the agent of Mediterranean spotted fever, and was identified in three samples, 13-HaMT, 19-HmMH, and 23-HeMH, as indicated in Table 2. Ticks, both male and female alike, are carriers of *R. conorii*.

Rickettsia sibirica: Several strains of *R. sibirica* have been detected in ticks and humans in Europe (De Sousa et al. 2006). Recently, *Rickettsia sibirica mongolitimonae*, was detected in *Hyalomma marginatum* ticks collected from humans (Keskin and Bursali 2016), but there is no report to human infection with *R. sibirica* in Turkey.

Borrelia turcica: The ecology, transmission, epidemiology, and clinical symptoms of Lyme borreliosis in Europe and the 18 genospecies (at least) that currently make up the *Borrelia burgdorferi* sensu lato complex have recently been reviewed (Rizzoli et al. 2011). In Europe, species of *Borrelia* that are pathogenic, non-pathogenic, and those with still an

unknown pathogenicity have all been identified. The ecology of borreliosis is known to be a function of the interactions between the microbe, the vector, and the vertebrate host (Rizzoli et al. 2011). In 2003, a new spirochete was isolated from the hard tick *H. aegyptium* using BSK-II medium in Turkey, and was further characterized as *Borrelia turcica*, and shown to be phylogenetically distinct from both relapsing fever associated *Borrelia* and from Lyme-disease related *Borrelia* (Guner et al. 2004). The original work mentioned that normal ddY mice and cyclophophamide-treated mice did not show any symptoms of disease after inoculation with *B. turcica*, and spirochetes could not be re-isolated from the spleen, kidney, ear, blood, bladder, or heart. *B. turcica* was identified in all four *H. aegyptium* ticks removed from tortoises, and sample 17-HmMH removed from a human. However, the organism was not present in any of the *H. excavatum* ticks removed from human hosts.

Yersinia enterocolitica: Sample 24 was a *H. excavatum* female tick removed from a human host, and contained a small amount of the zoonotic organism *Yersinia enterocolitica*. Though typically less than 0.1% relative abundance in the sample is considered a trace amount, this OTU has a read length of 461 bp, percent homology of %99.33, and an e value of 0, and thus is an excellent match. To the authors' knowledge, this is the first detection of this pathogen in *H. excavatum,* and represents an intriguing find. The genus *Yersinia* contains three species that are known to be pathogenic to immune competent humans; *Yersinia pestis* (causative agent of Plague typically carried by fleas), *Y. pseudotuberculosis,* and *Y. enterocolitica* (causative agent of yersiniosis in humans and animals) (Perry and Fetherston 1997). In addition to acute gastroenteritis, the infection may lead to various post-infectious complications such as reactive arthritis (Fredriksson-Ahomaa et al. 2006). This organism is a common food borne pathogen primarily found in porcine tongue, liver, heart, and kidneys, though pathogenic strains are seldom isolated through conventional culture methods (Fredriksson-Ahomaa et al. 2006). There have been no previous investigations as to the vector competency of ticks for this organism to humans or animals.

Legionella pneumophila: The Legionellales are an order of proteobacteria with two families, Legionellaceae and Coxiellaceae. Legionellaceae are gram-negative proteobacteria made up of three genera; *Flouribacter, Legionella,* and *Sarcobium.* Legionnaires' disease is caused by *Legionella pneumophila*, and was first identified as a human pathogen in 1976 (Fraser et al. 1977). Cases of community acquired Legionnaires' disease have been epidemiologically linked to residential water supplies through consumption, or aerosolized inhalation (Stout et al. 1992). A recent article has pointed out that some cases of pneumonia have also presented with acute kidney injury, which interestingly can develop days before clinical demonstration of pneumonia (Haines and Calhoon 1987; Clement et al. 2007). Though closely related to *Coxiella burnetii*, the eteological agent of Q-fever known to be vectored by insects, *L. pneumophila* is not considered a zoonotic pathogen. Previous studies identifying *L. pneumophila* in *Hyalomma* ticks, or the potential for these ticks to be competent vectors of *L. pneumophila* are lacking, though clearly the organism multiplies within human monocytes (Horwitz and Silverstein 1980). It is also unknown if non-respiratory presentations of Legionnaires' disease might be related to alternate routes of infection; such as transfer through an insect vector. Samples 20-HmFH and 23-HeMH both contained organisms traced back to the family Legionellaceae. Sample 22-HeFH contained *L. pneumophila* as identified by the B27 Primer set.

Previous bacterial diversity studies in *Rhipicephalus annulatus* and *Dermacentor marginatus* ticks also collected in Turkey have supported community ecology theory that diversity rich microbiomes are more resistant to bacterial invasion than diversity deprived

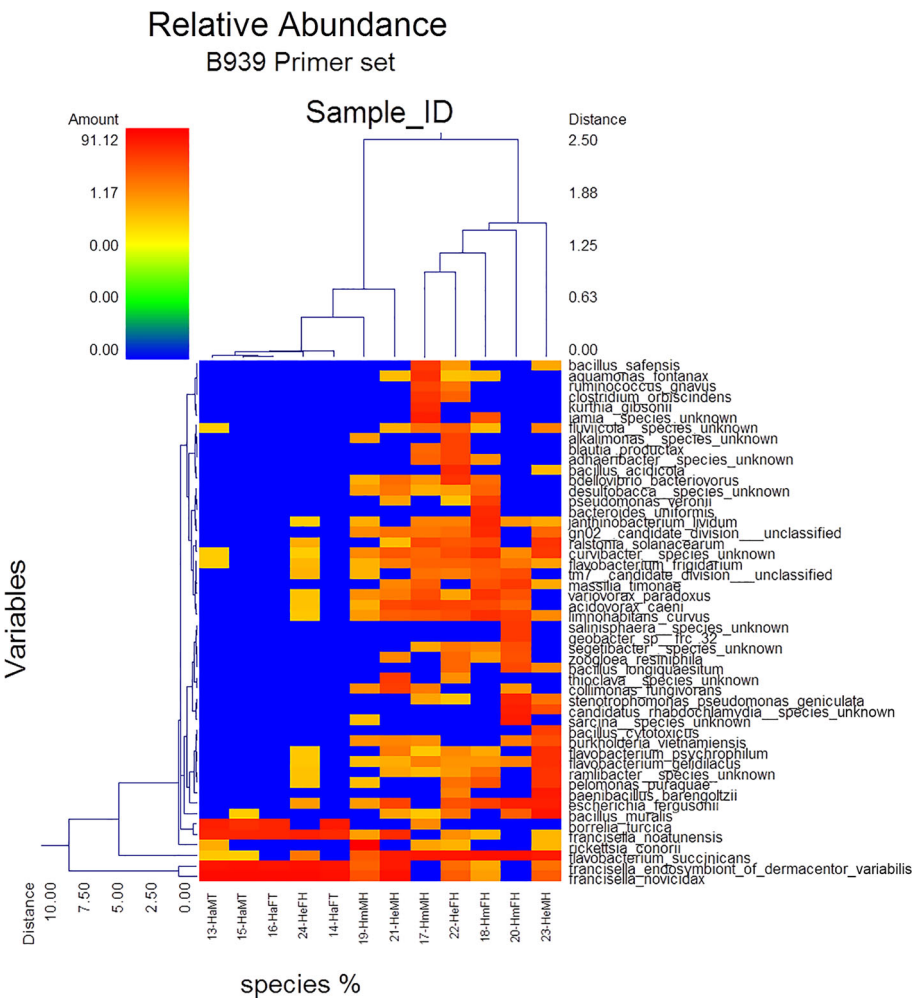

Fig. 4 Heat map depicting the bacterial communities and relative abundance of ten tick samples from 3 *Hyalomma* ticks labeled with species and tick sex for primer B939

ones (Tekin et al. 2017). Figure 4 (primer B939) and 5 (primer B27) depicts the 50 organisms of highest percent relative abundance as identified according to each primer set in the form of heat maps. Both primer sets indicate that the microbiome of *H. aegyptium* has very poor diversity. Moreover, certain species of the organisms present, *Borrelia* and *Francisella,* are will associated with zoonosis which supports the community ecological theory. The majority of species diversity is seen in *H. marginatum* and *H. excavatum.* In these eight samples, a much higher percentage of known non-pathogenic tick endosymbionts such as *Flavobacterium* are present. This seems to reduce the over-all per sample RPA of pathogenic organisms in most of the samples. Revisiting Table 2 for a closer look reveals the theory is still supported, particularly in samples 19-HmMH where the majority of pathogens are from the genus *Rickettsia*, and 21-HeMH where *Francisella* predominates. Sample 17-HmMH likewise supports the theory as it shows significant microbial

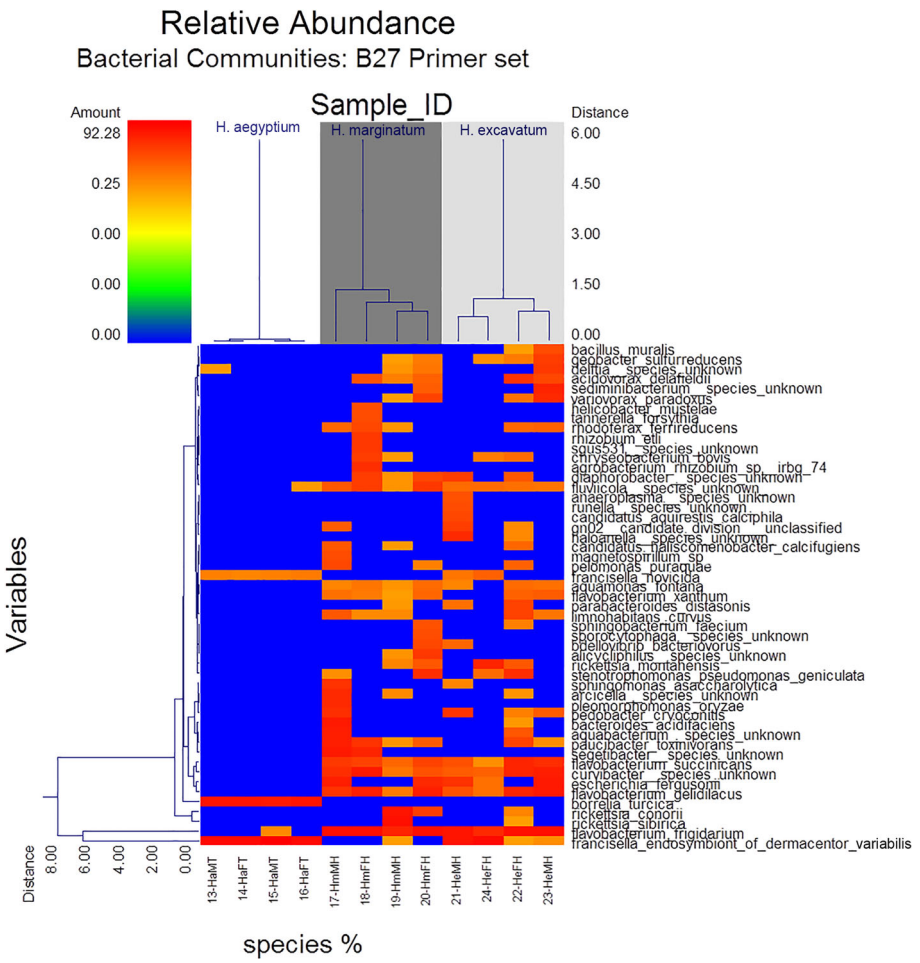

Fig. 5 Heat map depicting the bacterial communities and relative abundance of ten tick samples from 3 *Hyalomma* ticks labeled with species and tick sex for primer B27

diversity of endosymbionts in Figs. 4 and 5, yet only small amounts of *Borrelia turcica* and *Rickettsia* spp. representing the only pathogenic organisms present in that sample. The challenge remains that the vector competence of ticks for many of these organisms is well established, as well is relatively high virulence for these pathogens. Therefore, the presence of any zoonotic pathogen within a tick that could potentially be transferred to humans and animals must be considered significant, regardless of quantity.

Examination of the microbial diversity within these three species indicates that *H. marginatum* and *H. excavatum* are more diversity rich than *H. aegyptium*. This work has shown that all 3 species of *Hyalomma* ticks examined harbor zoonotic bacteria, both male and female ticks alike, and could potentially act as vectors for know zoonoses in Turkey. The predominant zoonotic bacteria represented in these ticks are genera *Borrelia, Francisella,* and *Rickettsia.* To the authors' knowledge, this work represents the first detection of *Yersinia enterocolitica* in the tick *H. excavatum*, raising questions regarding the vector competency of this tick, as well as associations of different disease representations perhaps

through previously unforeseen routes of pathogen introduction. Likewise, similar questions are related to the presence of *Legionella pneumophila* in one *H. excavatum* sample. Ultimately, more light has been shed on the perceived "normal" microbiota of *Hyalomma* ticks and their associations with pathogenic bacteria.

Acknowledgements Authors thank to Directorate of Primary Health Care, Ministry of Health donation of tick samples to the Turkish Ticks Collection. We also thank to Eray Simsek (Gaziosmanpasa University, Department of Biology) for the designing of figures. This study is partially supported by The Council of Higher Education and a Grant (TBAG 105T457) from The Scientific & Technological Research Council (TUBITAK) of Turkey.

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