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Occurrence of *Borrelia burgdorferi* s.l. in different genera of mosquitoes (Culicidae) in Central Europe

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ABSTRACT

Lyme disease or Lyme borreliosis is a vector-borne infectious disease caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex. Some stages of the borrelial transmission cycle in ticks (transstadial, feeding and co-feeding) can potentially occur also in insects, particularly in mosquitoes. In the present study, adult as well as larval mosquitoes were collected at 42 different geographical locations throughout Germany. This is the first study, in which German mosquitoes were analyzed for the presence of *Borrelia* spp. Targeting two specific borrelial genes, *flaB* and *ospA* encoding for the subunit B of flagellin and the outer surface protein A, the results show that DNA of *Borrelia afzelii*, *Borrelia bavariensis* and *Borrelia garinii* could be detected in ten Culicidae species comprising four distinct genera (*Aedes*, *Culiseta*, *Culex*, and *Ochlerotatus*). Positive samples also include adult specimens raised in the laboratory from wild-caught larvae indicating that transstadial and/or transovarial transmission might occur within a given mosquito population.

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1. Introduction

In the second half of the 20th century, infectious diseases became increasingly important. About 15 million (>25%) of 57 million annual deaths worldwide are estimated to be directly related to emerging and re-emerging infectious diseases (Morens et al., 2004). Of these, an accelerating number is transmitted by obligate haematophagous arthropods with mosquitoes and ticks as the most crucial vectors.

Lyme borreliosis is a multisystemic infectious disease caused by distinct spirochetes belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex, which are transmitted by hard ticks (Ixodidae). Early signs of the disease often include unspecific symptoms such as headache, fever and fatigue. After 3–30 days, 70–80% of infected individuals developed a ring-like skin rash at the ticks bite site (erythema migrans). If left untreated, the pathogens can disseminate, leading to more severe symptoms involving diverse organs, e.g. the heart, the joints, and the central nervous system. The *B. burgdorferi* s.l. complex comprises a group of at least 20 genospecies worldwide (Casjens et al., 2011; Skuballa et al., 2012; Ivanova et al., 2014). Regarding their potential to cause the disease, the 20 genospecies can be divided into two groups of which the first group consists of five genospecies frequently isolated from patient samples while the second group comprises genospecies with either uncertain pathogenic potential or that have never been detected in human samples (Rudenko et al., 2011). Among human pathogenic genospecies, *Borrelia garinii*, *B. burgdorferi*, and *Borrelia afzelii*, the three main European ones are maintained by birds or small mammals. *Borrelia bavariensis*, a serotype formerly regarded as *B. garinii* OspA type 4 is also adapted to rodents (Skuballa et al., 2012; Margos et al., 2013). Of these, different genospecies can be present simultaneously in a single tick (Rauter and Hartung, 2005; Wodecka et al., 2010). This is of particular interest as genospecies can be associated with different clinical manifestations, e.g. *B. burgdorferi* sensu stricto (s.s.) is often associated with arthritis, *B. garinii* with neurological manifestation, *B. afzelii* almost always induces acrodermatitis chronica atrophicans (Skotarczak, 2014) and *B. bavariensis* has been linked to neuroborreliosis (Margos et al., 2013). Therefore, an infection with various genospecies may result in the manifestation of multiple disease symptoms. Although ticks are the prominent transmission vectors of these particular pathogens, borreliae have also been detected in other arthropods, especially

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in mosquitoes. Mosquitoes have been known since the late 19th century to be the vectors for a number of several human diseases, such as malaria, dengue, chikungunya or yellow fever. Recently, it has been demonstrated that human cases of tularemia caused by *Francisella tularensis holarctica* are transmitted by mosquitoes (Lundström et al., 2011; Thelaus et al., 2014).

Especially in Germany, there is a lack of knowledge which pathogens can be successfully transmitted by diverse mosquito species. In mosquitoes, Spirochetes were found for the first time, in a *Culex* species in 1907 (Jaffé, 1907). Concerning spirochetes of the *B. burgdorferi* s.l. complex, different genospecies could be identified in mosquitoes already (Halouzka et al., 1999; Žákovska et al., 2004). So far the maximal periods of survival for borreliae in naturally infected mosquitoes are still unknown (Magnarelli and Anderson, 1988). In experimentally fed mosquitoes borreliae could be detected up to 14 days after a blood meal in two species (*Aedes aegypti* and *Aedes triseriatus*) (Magnarelli et al., 1987). Additionally, there is one report of a patient who developed erythema migrans after a mosquito bite (Hård, 1966).

Owing to the very different feeding time and frequency of mosquitoes and ticks, Matuschka and Richter (2002) postulated that transmission of *Borrelia* species by mosquitoes appears to be impossible, with the result that research on this topic stopped nearly completely for over a decade. This is astonishing, as it is still unknown whether these insects may be able to play a small role influencing the ecology and epidemiology of Lyme borreliosis.

Aim of this study is to check whether borreliae can be found in different species or even genera of mosquitoes with different feeding habits. Therefore, anthropophilic, mammalophilic as well as ornithophilic species have been screened for the presence of borreliae. In addition, we sought to examine, whether borreliae can also be detected directly after the metamorphosis in mosquitoes, as transstadial transmission is known in ticks, which is a crucial step for a vector-borne pathogen to be transmitted later on.

2. Material and methods

2.1. Collection of samples

Mosquitoes were sampled from April to October 2013 at 42 sampling sites in Germany during a mosquito monitoring program, which included the detection of possible pathogens. Most of these sites were in Saxony ($n = 9$) followed by Brandenburg ($n = 6$) and Hesse ($n = 15$) (SI 1). Individuals were collected using EVS-traps with dry ice, BG-Sentinel™ traps (Biogents AG, Regensburg, Germany) with CO_2 as an attractant, hand nets and by human bait (Supplemental Table 1). Larvae were collected in natural (e.g. puddles, ponds, tree holes) as well as in artificial (e.g. vases, rain barrels) water pools using hand nets. After capture, larvae were kept alive and raised to adults. For this study, no specific permissions were required and no endangered or protected species are involved.

Supplementary Table 1 related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.10.018.

2.2. Morphological identification

Morphological identification of mosquito species was carried out with a stereomicroscope. The morphological characters of each specimen were analyzed using two identification keys (Mohrig, 1969; Becker et al., 2010).

2.3. DNA-extraction

For DNA-extraction, morphologically identified mosquitoes were pooled by species, collection date and collection site, each pool contained up to ten individuals. Samples were homogenized individually with a tissue mill (MM400, Retsch GmbH, Germany) and 2 stainless steel beads (3 mm, VWR, Germany) for 2 min at 25 Hz. Subsequent DNA-extraction was carried out with glass fiber plates (Pall GmBH, Dreieich, Germany) following the protocol of Ivanova et al. (2006).

2.4. Detection of borrelial DNA by nested PCR

Nested PCR was applied to detect two different borrelial genes, *flaB* and *ospA* in the collected samples. The *flaB* fragments were amplified using primers FL3/5 and FL6/7and primer pairs N1/C1 and N2/C2 were used to identify the *ospA* sequences (Table 1) (Picken, 1992; Rijpkema et al., 1997; Žákovska et al., 2004). The reaction mixtures for primers FL3/5 as well as N1/C1 (both 25 μl) consisted of 12.5 μl Master Mix (Peqlab Biotechnology GmbH, Erlangen, Germany) containing 0.4 mM dNTP, 4 mM MgCl2, 40 mM Tris–HCl, 32 mM $(NH_4)_2SO_4$, 0.02% Tween 20 and 1.25 U Taq-Polymerase, 1 μl of each primer (10 pmol μl^{-1}) 9 μl ddH_2O and 1.5 μl genomic DNA. The reactions for the FL6/7 nested PCR contained 12.5 μl Master Mix, 1 μl of each primer (10 pmol μl^{-1}) 5.5 μl ddH_2O and 5 μl PCR-product of FL3/5. The amplification of N2/C2 was performed with 12.5 μl Master Mix, 1 μl of each primer (10 pmol μl^{-1}) 9 μl ddH_2O and 1.5 μl PCR-product of N1/C1. For FL6/7 the cycle parameters followed the protocol of Picken et al. (1996): FL3/5 = 1 cycle of 94 °C, 12 min; 30 cycles of 94 °C, 1 min; 70 °C, 2 min and 72 °C, 3 min followed by terminal extension of 72 °C, 7 min and a final ramping to 8 °C; FL6/7 = 1 cycle of 94 °C, 12 min; 30 cycles of 94 °C, 1 min; 54 °C, 2 min and 72 °C, 3 min, final extension at 72 °C, 7 min and a final ramping to 8 °C; N1/C1 = 1 cycle of 37 °C, 5 min and 94 °C for 10 min; 30 cycles of 94 °C, 1 min; 45 °C, 1 min and 72 °C (extended for 5 s with each cycle), 1 min, final extension at 72 °C, 5 min and a final ramping to 8 °C; N2/C2 = 1 cycle of 94 °C, 10 min; 25 cycles of 94 °C, 1 min; 43 °C, 1 min and 72 °C, 1 min, and a final extension at 72 °C for 5 min and a final ramping to 8 °C (slightly modified after Rijpkema et al. (1997)). In each PCR attempt, a positive as well a negative control were performed to rule out the possibility of laboratory contamination. Quality and yield of PCR products was analyzed by Midori Green (Nippon Genetic EUROPE GmbH) staining and agarose gel-electrophoresis. For subsequent Sanger-sequencing reactions, with product specific forward primers, positive samples were purified using the peqGOLD Cycle-Pure Kit (Peqlab Biotechnology GmbH, Erlangen, Germany). Each obtained sequence was edited using BioEdit (Hall, 1999) and compared with sequences deposit in GenBank using the BLAST algorithm (Altschul et al., 1997).

The sequences obtained were deposited in EMBL Nucleotide Sequence Database under accession numbers LN650604–LN650631.

3. Results

In total 3615 adult mosquitoes were morphologically identified (Supplemental Table 2) and analyzed, including 74 adults reared from field-caught larvae in the laboratory. Specimens which could not be identified morphologically to species level because of their poor state of preservation were analyzed individually.

Supplementary Table 2 related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.10.018.

For all analyses, no distinction was made between *Culex pipiens* and *Culex torrentium* and specimens were treated as pools of "*Cx. pipiens*/*torrentium*" as well as between *Ochlerotatus annulipes* and *Ochlerotatus cantans*, which were treated as pools of "*Oc. annulipes*/*cantans*". The analyzed adult mosquitoes belong to the genera *Aedes* ($n_{pools} = 265$), *Anopheles* ($n_{pools} = 12$), *Coquillettidia*

Table 1
Primers used for the detection of *Borrelia* species.

Primer	Target gene	Primer sequence (5′ → 3′)	Fragment length [bp]
FL3	flaB	GAGCTTCTGATGATGCTGCTGGYATGGGRG	874
FL5	flaB	GRGGAACTTGATTAGCYTGYGCAATCATTGCC	
FL6	flaB	TTCAGGRTCTCAAGCKTCTTGGAC	276
FL7	flaB	GCATTTTCWATTTTAGCAAGWGATG	
N1	ospA	GAGCTTAAAGGAACTTCTGATAA	560
C1	ospA	GTATTGTTGTACTGAATTGT	
N2	ospA	ATGGATCTGGAGTACTTGAA	351
C2	ospA	CTTAAAGTAACAGTTCCTTCT	

(n_{pools} = 12), *Culex* (n_{pools} = 116), *Culiseta* (n_{pools} = 7) and *Ochlerotatus* (n_{pools} = 270).

All pools were analyzed for the presence of borrelial DNA targeting the *flaB* and *ospA* genes. Of the 682 tested pools, seventeen pools were positive for *flaB* and eleven for *ospA*. Sequence analysis and comparison with those deposited in GenBank revealed positive results for *B. afzelii*, *B. bavariensis* as well as *B. garinii*. borreliae were detected in eight out of 265 *Aedes* spp. pools with a total of 1861 specimens of wild-caught adults as well as in eighteen of 270 *Ochlerotatus* spp. pools with a total of 865 individuals. We also found borrelial DNA in one of 116 *Culex* spp. pools consisting of 796 specimens, and in one of twelve specimens of *Culiseta* spp. In total, detection of borrelial DNA was successful in 25 of 682 adult field-caught mosquito pools and three pools from laboratory-raised specimens, which evaluated the nested PCR method positively for the detection of borreliae in mosquitoes. Out of the laboratory-raised pools, three samples of *Ochlerotatus* (two single individuals and one two specimen pool) were positive for *B. burgdorferi* s.l.

The most frequent species was *B. garinii*, which was found in twelve mosquito pools; *B. afzelii* could be detected in six mosquito pools consisting of four species and in all three positive laboratory-raised pools. *B. bavariensis* DNA could be detected in seven pools. Table 2 shows the occurrence of the different *Borrelia* species in the respective mosquito species.

The distribution pattern of the detected *Borrelia* species (*B. afzelii*, *B. bavariensis*, and *B. garinii*) is summarized in Fig. 1. *B. afzelii* was more frequently found in the southern and western collection sites of Germany, while *B. bavariensis* appears to be the dominant species at the eastern sampling sites. A diverse distribution could be suggested for *B. garinii* which was found in southwestern, southern as well as in eastern localities. The hosts of the identified *Borrelia* and mosquito species are shown in Fig. 2.

4. Discussion

About 51 mosquito species are known in Germany of which 24 were detected during the present study. Differences in the number of species identified at the distinct collection sites may be attributed to differences in mosquito abundance. The observed differences can most likely be connected to sampling at altered time points, variation of species activity or various collection methods used.

Previously published epidemiological data indicate that haematophagous insects could be involved in the transmission of borreliae in some areas (Magnarelli et al., 1986; Kosik-Bogacka et al., 2002). But so far the role of arthropods other than ticks in the transmission cycle of borreliae remains unclear, although a few reports have been published on cases of Lyme borreliosis following an insect bite (Doby et al., 1986; Hård, 1966; Luger, 1990). So, borreliae have already been detected in previous studies on mosquitoes in the United States, where *Oc. stimulans* females from Norwich contained DNA of *B. burgdorferi* (Magnarelli and Anderson, 1988). In the Czech Republic, motile borreliae have been isolated from *Aedes cinereus*, *Ae. vexans*, *Oc. cantans*, *Ochlerotatus communis* and *Ochlerotatus sticticus* as well as the *Cx. pipiens* complex (Halouzka, 1998; Halouzka et al., 1998; Žákovska et al., 2006). The detection of numerous spirochaete species in salivary glands of *Oc. cantans* (Zeman, 1998) resembles previous results, which showed spirochetes in British anophelines (Sinton and Shute, 1939). We found borrelial DNA in ten mosquito species comprising four genera. In total in 25 of 682 adult field-caught mosquito pools and three pools from laboratory-raised specimens. Out of the laboratory-raised pools, three samples of *Ochlerotatus* (two single individuals and one two specimen pool) were positive for *Borrelia* spp. In total borreliae were detected in ten mosquito species of which most show mainly mammalo- or anthropophilic feeding habits. The identification of spirochetes in the salivary glands suggests that mosquitoes may potentially transmit on occasion bacteria. In ixodid ticks, borreliae can be transmitted by different pathways: transstadial, feeding and co-feeding (Vennestrøm et al., 2008). At least feeding and transstadial (although not a sort of transmission as just one individual is involved) could be possible pathways along with insects, in particular mosquitoes. In ticks, the outer surface protein OspA appears to be responsible for the attachment of the spirochetes to the midgut of the ticks by the TROPSA receptor (Li et al., 2007). When the tick begins to feed and the spirochetes in the midgut begin to multiply, most spirochetes cease expressing OspA on their surfaces while beginning simultaneously expressing OspC and migrate to the salivary gland. This upregulation of OspC begins during the first day of feeding and peaks 48 h after attachment (Schwan and Piesman, 2000). OspC, which interacts with the salivary protein Salp15 (that way the spirochete gets protected from complement-mediated killing [Schuijt et al., 2008]) plays an essential role during the very early stage of mammalian infection (Tilly et al., 2006) and may also be necessary to allow *B. burgdorferi* to invade and attach to the salivary gland after leaving the midgut of the tick (Pal et al., 2004a; Grimm et al., 2004). However, neither Salp15 nor the tick gut epithelial cell protein TROSPA (Pal et al., 2004b), is known to be present in *Culicidae*, as well as no homologous proteins have been described so far in *Culicidae*. Thus, it is tempting to speculate that other, unrelated proteins might act as potential ligands for OspA and OspC.

Most studies on the presence of *Borrelia* spp. in *Culicidae* have been done in the Czech Republic. The infection rate in these previous studies was between 0.7% and 7.6% for adults. Nejedlá et al. (2009) reported that 3.3% of the analyzed mosquitoes (*Aedes* spp., *Culex* spp., *Ochlerotatus* spp.) were positive for spirochetes using dark field microscopy (DFM) of which 0.7% being positive for borreliae in a subsequent PCR. In other studies, the rate ranged from 0.7 to 7.6% (Sanogo et al., 2000). For north-eastern Poland, the rates were 1.1% for *Aedes* spp. and 0.3% for *Culex* spp. (Nejedlá et al., 2009) and according to another study, in 1.25% of the *Aedes* spp. and *Culex* spp. in Poland borrelial DNA was detected (Kosik-Bogacka et al., 2002). In later studies 0.8% of the tested mosquitoes were positive for borreliae (Kosik-Bogacka et al., 2004, 2006, 2007). This is similar to our findings; however, assuming the minimum of one infected specimen per pool would result in the total percentage within the same range of previous studies. Nevertheless, the

Table 2
Distribution of the identified *Borrelia* species in the respective mosquitoes. The table summarizes the sampling point, the accession numbers as well as the strain with the highest similarity found in GenBank and their respective accession number. *Borrelia* species have been identified by nested-PCR targeting the *flaB* and *ospA* genes.

Gene	*Borrelia* species	Mosquito species	Number of pooled individuals	Sampling point	Length of sequence	Accession number comparison	Strain/isolate	Accession number	Similarity
FL	*B. afzelii*	*Ae. cinereus*	2	Wielenbach	151	KJ810661.1	Not stated	LN650604	100
		Oc. communis	10	Wielenbach	191	KJ810661.1	Not stated	LN650605	99
		Oc. sticticus	1	Wielenbach	162	CP009212.1	Tom3107	LN650606	99
		Oc. sp	2	Rhön	201	CP009212.1	Tom3107	LN650607	99
		Oc. sp	1	Nussloch	168	KJ810661.1	Not stated	LN650608	100
		Oc. sp	1	Nussloch	136	KJ810661.1	Not stated	LN650609	99
	B. garinii	*Ae. cinereus*	10	Görlitz 3	149	KF894055.1	iso: 66-S-12	LN650612	100
		Ae. vexans	10	Neißewiesen Hagen-werder	215	KF894054.1	iso: 66-S-12	LN650613	99
		Ae. vexans	10	Neißewiesen Hagen-werder	115	KF894055.1	iso: 66-S-12	LN650614	100
		Ae. vexans	10	Berlin	165	KF894055.1	iso: 66-S-12	LN650617	100
		Ae. vexans	10	Berlin	165	KF894055.1	iso: 66-S-12	LN650616	100
		Ae./Oc. sp	1	Neißewiesen Hagen-werder	183	KF894055.1	iso: 66-S-12	LN650619	100
		Ae./Oc. sp	1	Berlin	155	KF894055.1	iso: 66-S-12	LN650610	100
		Cs. subochrea	1	Darmstadt	124	KF894055.1	iso: 66-S-12	LN650611	100
		Cx. pipiens/torrentium	1	Berlin	210	D63368.1	Not stated	LN650620	98
		Oc. communis	10	Neißewiesen Hagen-werder	144	KF894055.1	iso: 66-S-12	LN650615	100
		Oc. communis	2	Neißewiesen Hagen-werder	180	KF894055.1	iso: 66-S-12	LN650618	100
OspA	*B. afzelii*	*Ae. cinereus*	2	Wielenbach	276	GU826936.1	iso: 991	LN650621	99
		Oc. caspius	10	Berlin	134	FJ546608.1	Strain IPT179	LN650624	100
		Oc. sticticus	10	Haarsee	184	FJ546608.1	Strain IPT179	LN650622	99
	B. bavariensis	*Ae. vexans*	10	Berlin	225	JX889267.1	Strain mutant 20	LN650625	99
		Oc. cataphylla	1	Berlin	128	JX889267.1	Strain mutant 20	LN650628	98
		Oc. communis	2	Niederspree (Wiese)	204	JX889267.1	Strain mutant 20	LN650630	97
		Oc. communis	6	Ullersdorf	209	JX889266.1	Strain mutant 19	LN650629	100
		Oc. dorsalis	1	Breesen	168	JX889267.1	Strain mutant 20	LN650627	98
		Oc. geniculatus	2	Ullersdorf	274	JX889267.1	Strain mutant 20	LN650631	100
		Oc. sticticus	7	Görlitz 3	239	JX889267.1	Strain mutant 20	LN650626	95
	B. garinii	*Oc. sticticus*	10	Wielenbach	284	KM397123.1	Strain IO-TP-TW	LN650623	99

number of infected specimens in the recent study might be much higher as we can give only information about the possible range of infected specimens. Therefore a direct comparison of the values is not possible owing to the design of the present study to analyze pooled samples in contrast to investigate individual mosquitoes. In our investigation no big variation has been shown in the infection rate between the distinct mosquito species.

The calculated infection rates of borreliae seem to largely depend on the method of choice. There are some studies in which the presence of spirochetes was determined using DFM in comparison with the identification of borrelial DNA in the respective sample by PCR (Kosik-Bogacka et al., 2007). While the percentage of spirochetes was relatively high when analyzed by DFM, the numbers are lower when analyzed by PCR, because of the relatively low sensitivity of the single step PCR approach and by the fact that with DFM borreliae cannot be distinguished from other spirochetes. Limitation in the detection of *Borrelia* infections in ticks has already been reported (Kahl et al., 1998; Cisak et al., 2006). The same reason may have influenced the results of the present study, especially the difficulties in amplifying parts of both gene fragments. The fact that only low numbers of borreliae can be present (34% of positive ticks harbor less than 10 borreliae [Hubalek and Halouzka, 1998]), can therefore lead to false-negative results. Previously there has to be a minimum of about 130 borreliae in a given sample in order to obtain positive PCR results (Nejedlá et al., 2009). Another problem is the lack of standardization of PCR for the identification of *Borrelia* spirochetes. This lack makes it impossible to compare the reports on this subject matter. PCR detection of *B. burgdorferi* s.l. uses various molecular markers, which show varying results (Wodecka et al., 2010). These already in the literature mentioned varying results are a matter that explains also, why the PCR results for both gene fragments vary in this study, as the sensitivity for both gene fragments varies greatly.

In the present study, the distribution of the detected species (*B. afzelii*, *B. bavariensis*, *B. garinii*) is in line with previously published data where *B. afzelii* has been reported to be the most frequent species in ticks in the Bonn area (Maetzel et al., 2005), which is situated north-west of our sampling area. In another study, the

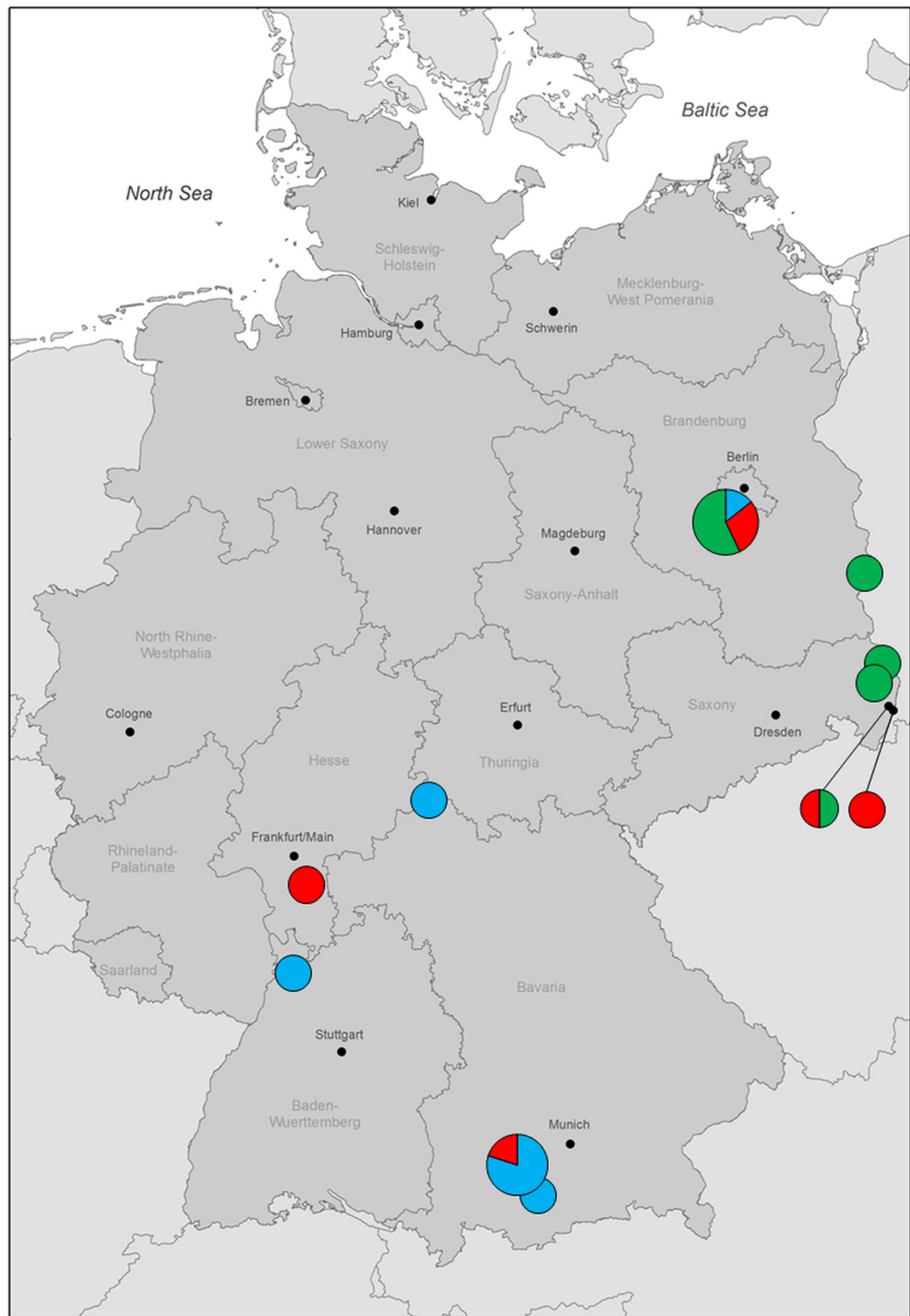

Fig. 1. Distribution of the detected *Borrelia* species. *Borrelia afzelii*: blue circles, *B. bavariensis*: green circles, *B. garinii*: red circles. The species detection was based on the detection of fragments of the *flaB* and *ospA* genes. The circle size varies for better illustration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

prevalence of borreliae in ticks in Thuringia has been analyzed where *B. garinii* was the predominant species (Hildebrandt et al., 2003). Thuringia is situated between the different sampling sites in the present study and the results of the actual study show a tendency that the abundance of *B. garinii*/*B. bavariensis* increases from southern to central/northern Germany and vice versa for *B. afzelii*, which was previously described for *B. garinii* and *B. afzelii* (Rauter and Hartung, 2005). However, the distribution pattern of the two species does not reflect data reported for Saxony (Bigl et al., 1999), Bonn (Kurtenbach et al., 2001; Maetzel et al., 2005) and the Rhine-Main area (Bingsohn et al., 2013). In those studies, *B. afzelii* was detected more frequently than *B. garinii*. The distribution of the various species shows spatial and temporal differences, which depend mainly on the activity of the ticks and the presence of adequate reservoir hosts (Gern, 2009; Rizzoli et al., 2011). Therefore in a certain area where *B. garinii* predominates, *B. afzelii* temporarily become the dominant species (Gern, 2009). In any case, the distribution and prevalence of various borrelial genospecies varies on a local and regional scale (Rizzoli et al., 2011), what influences to some extent the appearance of certain symptoms of Lyme borreliosis in a given region. The regional distribution is also of interest because the different *Borrelia* species are associated with

Mosquito species	anthropophil	mammalophil	ornithophil	*Borrelia* species	reservoir hosts
Ae. cinereus	+	+		*B. afzelii*	?
				B. garinii	
Ae. vexans	+	+		*B. garinii*	
				B. bavariensis	
Ae./Oc. sp.				*B. garinii*	
				B. afzelii	
Cs. subochrea		+		*B. garinii*	
Cx. pipiens/ Cx. torrentium	+	+	+	*B. garinii*	
Oc. caspius	+	+		*B. garinii*	
Oc. cataphylla	+	+		*B. bavariensis*	
Oc. communis	+	+		*B. afzelii*	
				B. garinii	
				B. bavariensis	
Oc. dorsalis	+			*B. bavariensis*	
Oc. geniculatus	+	+		*B. bavariensis*	
Oc. sticticus	+	+		*B. afzelii*	?
				B. garinii	

Fig. 2. Positive tested mosquito species and detected *Borrelia* species. The corresponding (reservoir-) hosts of borreliae are shown as shadows of the vertebrate class; feeding habits of the mosquito species, as far as known, are shown behind each species as crosses.

particular reservoir hosts, e.g. *B. garinii* with birds while *B. afzelii* and *B. bavariensis* tend to be associated with rodents (Rizzoli et al., 2011; Rudenko et al., 2011). One of these two species is in every sampled area of the present study the predominant species (*B. afzelii* in the west, *B. bavariensis* in the east). These localities were mainly of rural character where certain mice species are common, what can explain the distribution. Nevertheless, it has to be taken into consideration that since *B. bavariensis*, formerly *B. garinii* OspA type 4, has only recently been classified as a distinct genospecies (Rudenko et al., 2011), it cannot be excluded that sequences deposited in Genbank which are attributed to *B. garinii*, may actually belong to *B. bavariensis*.

Culicidae are not considered as possible vectors for *borreliae*. Anyway, we could detect borrelial DNA in laboratory-raised adults, which have never fed on blood and have been collected as larvae. This finding suggests, although it gives no information about possible vector abilities, at least that there is a mode how *borreliae* can persist in *Culicidae*, even from the larval to the adult stage. The capacity of ticks to successfully transmit *borreliae* depends on several factors. The blood-feeding act of ticks takes much longer than the one of mosquitoes. Mosquitoes are vessel feeders and generally cannulate dermal capillaries, drawing blood directly from the vessel lumen and rarely from the interstitial tissue. Spirochetemia caused by *B. burgdorferi* s.l. is a transient, process of short duration whereas spirochetes can persist in the skin for a very long time over years as shown in patients with Acrodermatitis chronica atrophicans. Thus, the destruction of skin tissue by tick feeding facilitates the uptake of *borreliae* to a much greater degree than does the cannulation of capillaries by mosquitoes. For these reasons, it has been previously suggested that mosquitoes do not play a role as competent vectors (Matuschka and Richter, 2002). These works did not take into account that also various biotic and abiotic factors, such as climatic conditions, vegetation type and human management of the area (by direct influence on the behavior/activity of the vector), as well as host behavior, abundance and susceptibility influence the transmission (Gern, 2009). While it has been clearly established, that ticks are the primary vectors of Lyme disease spirochetes, it may be possible that mosquitoes play a role as an occasional mechanical vector.

Borreliae have already been found in overwintering female mosquitoes (Halouzka et al., 1998; Sanogo et al., 2000) and larvae (Žákovska et al., 2004) supporting the results of the present study in which laboratory-reared adults were tested positive for borreliae although they lacked any opportunity to get infected via a blood meal. This may also indicate a transstadial and/or transovarial transmission within mosquito populations.

In summary, here we show that different mosquito species and even genera harbor borrelial DNA from *B. afzelli*, *B. garinii*, and *B. bavariensis*. The questions whether mosquitoes can serve as a secondary mechanical vector remains unclear. Thus further studies are required to confirm natural transmission of spirochetes via mosquitoes to a competent host. Our results also raise the questions, if mosquitoes have functional-related proteins enabling at least the attachment and survival of borreliae. With the detection of *borreliae* in laboratory hatched specimens, we could show for the first time that *borreliae* endure the metamorphosis from larvae to pupae and finally to adults in mosquitoes, as the adult specimens were analyzed without having a prior blood meal. Nevertheless, the question of how mosquito larvae acquire *borreliae* remains unclear and is a focus of ongoing studies.

Conflict of interest

No one of the authors has any conflicts of interest.

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