International Journal for Parasitology: Drugs and Drug Resistance 14 (2020) 274–283

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Detection of target-site and metabolic resistance to pyrethroids in the bed bug *Cimex lectularius* in Berlin, Germany[1]

Arlette Vander Pan [a,*], Carola Kuhn [a], Erik Schmolz [a], Georg von Samson-Himmelstjerna [b], Jürgen Krücken [b]

[a] German Environment Agency, Boetticher Str. 2 Haus 23, 14195 Berlin, Germany
[b] Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany

ARTICLE INFO

Keywords:
Cimex lectularius
Pyrosequencing
Target-site resistance
Metabolic resistance
Germany

ABSTRACT

Knockdown-resistance (*kdr*) against pyrethroids in bed bugs (*Cimex lectularis*) is associated with the presence of several point mutations in the voltage-sensitive sodium channel α-subunit gene and/or an increased metabolic detoxification by cytochrome P450 monooxygenases (CYPs). In the present study, pyrosequencing assays were developed to quantify the presence of the *kdr* substitutions (V419L or L925I substitution) in bed bugs in Berlin, Germany. In 14 of 17 bed bug field strains, pyrosequencing revealed the presence of the substitution L925I with allele frequencies between 30% and 100%. One field strain additionally carried the substitution V419L with allele frequencies of 40% in males and 96% in females. In seven of the 17 field strains, mRNA levels of four CYP genes were examined using RT-qPCR. Relative to a susceptible laboratory reference strain, five field strains showed significantly higher mRNA levels of *cyp397a1* with 7.1 to 56-fold increases. One of these strains additionally showed a 4.9-fold higher mRNA level of *cyp398a1* compared to the reference strain, while *cyp4cm1* and *cyp6dn1* showed no significant differences. Our findings indicate that multiple resistance mechanisms are present in German *C. lectularius* populations simultaneously.

1. Introduction

Over the last 20 years, there has been an increase in reports of bed bug (*Cimex lectularius*) infestations in hotels, public buildings, and private houses worldwide (Ter Poorten and Prose, 2005; Harlan, 2006; Masetti and Bruschi, 2007; Dang et al., 2017; Cambronero-Heinrichs et al., 2020). Globalization, international travel and migration (Davis et al., 2009; Kolb et al., 2009), trade of second-hand articles, and the development of resistance against insecticides, especially against pyrethroids (Davies et al., 2012; Dang et al., 2017) are considered to be responsible for the expansion of *C. lectularius*. Also, the limited number of effective insecticidal active substances has an influence on the development of resistance in bed bugs and their dispersal (Kilpinen et al., 2011; Romero, 2011). In the EU, most of the authorized products contain pyrethroids, which are primarily used for bed bug control (Kilpinen et al., 2011; Davies et al., 2012; Dang et al., 2017; Gonzalez-Morales and Romero, 2019). Pyrethroid resistance in bed bugs has been described in many countries (Booth et al., 2015; Palenchar et al., 2015; Dang et al., 2017; Balvin and Booth, 2018; Caceres et al., 2019; Holleman et al., 2019; Vander Pan et al., 2019). German pest control companies have also observed increasing difficulties in controlling bed bugs with pyrethroids (personal communications with pest controllers).

For determination of resistance levels, filter paper contact bioassays or topical application assays are easy to perform and thus widely used (Romero et al., 2007; Lilly et al., 2009; Seong et al., 2010; Durand et al., 2012; Dang et al., 2014a). However, large numbers of bed bugs are required. In most cases, even heavy infestations do not provide enough specimens to perform a robust bioassay. Therefore, it is necessary to rear the collected bed bugs, which might lead to a decrease in resistance levels over time (Zhu et al., 2013; Gordon et al., 2015). Molecular assays have the potential to complement the bioassay-based resistance monitoring using relatively small numbers of bed bugs collected in the field. Several types of molecular assays have been proven to be useful for the detection of insecticide resistance in bed bugs (Yoon et al., 2008; Seong et al., 2010; Zhu et al., 2010; Mamidala et al., 2012; Dang et al., 2014b). Regarding parasitic gastrointestinal nematodes, quantification of alleles

* Corresponding author. ArthroScience GmbH, Lönsstraße 9, 13125 Berlin, Germany.
E-mail address: a.vanderpan@arthroscience.de (A. Vander Pan).
[1] Note: Supplementary data associated with this article.

https://doi.org/10.1016/j.ijpddr.2020.11.003
Received 22 July 2020; Received in revised form 16 November 2020; Accepted 16 November 2020
Available online 20 November 2020

conferring resistance to benzimidazole anthelmintics by pyrosequencing assays has been widely used (von Samson-Himmelstjerna et al., 2009; Skuce et al., 2010; Barrère et al., 2013; Demeler et al., 2013; Ramünke et al., 2016).

In different bed bug populations, four mechanisms responsible for insecticide resistance have been described: 1. Reduced penetration of insecticides, 2. Enhanced sequestration and excretion of insecticides, 3. Enhanced metabolic detoxification of insecticides, and 4. Reduced effect of insecticides at their target site (Bai et al., 2011; Mamidala et al., 2011; Zhu et al., 2013). These mechanisms can occur individually or in combination (Adelman et al., 2011; Mamidala et al., 2011, 2012; Zhu et al., 2013).

Previous molecular analyses of pyrethroid-resistant *C. lectularius* populations indicated that the presence of two single nucleotide polymorphisms (SNPs) in the *Clvssc* gene encoding the pyrethroid receptor – the voltage-sensitive sodium channel α-subunit – is associated with knockdown-resistance (*kdr* or *kdr*-type resistant mechanisms) to pyrethroids (Yoon et al., 2008; Seong et al., 2010; Dang et al., 2014b, 2017; Holleman et al., 2019). A SNP at position 1255 (GTC→CTC) leads to the substitution of valine by leucine (V419L). A second substitution at position 2773 (CTT→ATT) leads to the replacement of leucine with isoleucine (L925I). Increased metabolic detoxification by esterases, glutathione S-transferases, and especially by cytochrome P450 (CYP) monooxygenases have been described as additional mechanisms causing pyrethroid resistance in bed bugs (Adelman et al., 2011; Bai et al., 2011; Mamidala et al., 2011, 2012; Zhu et al., 2013). Zhu et al. (2013) showed, in comparison to a susceptible laboratory strain, significantly increased mRNA expression levels of four CYP genes (*cyp397a1*, *cyp398a1*, *cyp4cm1*, *cyp6dn1*) in bed bug field populations.

The objectives of this study were to obtain data about the occurrence of these pyrethroid resistance mechanisms in bed bugs collected from 23 infested locations in Berlin, Germany. For this purpose, pyrosequencing assays using genomic PCR fragments of individual and pooled bed bugs were established to analyze the presence of the two polymorphisms (V419L, L925I) and to quantify the different genotypes. Additionally, relative mRNA expression levels of the four CYP genes *cyp397a1*, *cyp398a1*, *cyp4cm1,* and *cyp6dn1* were determined using reverse transcription quantitative PCR (RT-qPCR). In a first step, the molecular assays were validated using five *C. lectularius* field strains of which the resistance ratios for deltamethrin had been determined in a previous study (Vander Pan et al., 2019). In a second step, the validated molecular assays were used to examine 15 additional field-collected strains.

Very few studies regarding the polymorphisms in the *Clvssc* gene associated with *kdr* in bed bug strains collected in Germany are available (Booth et al., 2015; Balvin and Booth, 2018). Our findings should supplement these few results and provide an initial overview of local variation in the frequency of the resistance-associated alleles and relative mRNA expression levels of the four CYP genes.

2. Materials and methods

2.1. Bed bugs

Between 2008 and 2013, bed bugs were collected from 23 infested locations in Berlin, Germany (Table 1 and Supplementary Information Fig. S1). Except for the SK strain (collected from four separate infested sites (Vander Pan et al., 2019)) all strains originated from individual infested locations. Bed bugs from the different collection sites were identified as *C. lectularius* using the taxonomic key in , specifically the pronotum width-to-length ratio. Sequences from the vssc cDNA obtained for all strains that were successfully bred showed >99.5% identity to *C. lectularius* and <97% identity to *C. hemipterus*, which confirmed the morphological identification. The *C. lectularius* strain of the German Environment Agency (UBA) was used as an insecticide susceptible reference strain (Vander Pan et al., 2019).

Laboratory rearing of five field strains (named after the location where they were collected: LB, HO, SK, OB, and AS) was successful. In a previous study (Vander Pan et al., 2019), the phenotypic status of pyrethroid resistance in these strains was determined in a filter paper contact bioassay using deltamethrin (EC_{50} (RR): UBA: 0.08 μg/cm^2; LB: 0.35 μg/cm^2 (4.3), HO: 0.4 μg/cm^2 (4.9), SK: 0.59 μg/cm^2 (7.2), OB: 0.89 μg/cm^2 (11.0), AS: 1.68 μg/cm^2 (20.7)). Sufficient numbers of bed bugs for these bioassays were obtained approximately one year (about six generations) after the initial introduction in the laboratory. Thus laboratory-reared descendants were also used for the molecular assays (Table 1).

Table 1
Rearing procedures and experiments conducted with bed bug strains and populations collected from 23 infested locations in Berlin, Germany. Except for the SK strain (collected from four separate infested sites (Vander Pan et al., 2019)), all strains originated from individual infested locations. The UBA strain was used as an insecticide susceptible reference strain (Vander Pan et al., 2019).

Strain/ population	Laboratory rearing	Pyrosequencing 5 technical replicates		RT-qPCR 2 technical replicates
		pooled bed bugs	individual bed bugs	individual bed bugs
UBA	since 1947	100 M[a]/ 100 F[b]	10 M/10 F	10 M
SK OB LB AS HO	6 generations	100 M/ 100 F	10 M/10 F	10 M
GW	2 generations	75 M/75 F	n.a.	10 M
T		200 mix (M, F, I[c])	n.a.	10 M
RS		5 M/5 F	n.a.	n.a.
LBII		5 M	n.a.	n.a.
LBIII		5 M	n.a.	n.a.
	n.a.	n.a.	1 M	n.a.
B2		4 M/4 F	n.a.	n.a.
B3		n.a.	1 M	n.a.
B4		4 M/3 F	n.a.	n.a.
B5		3 M	1 F	n.a.
B6		n.a.	1 M and 4th I	n.a.
B7		2 3rd I	n.a.	n.a.
B8		2 5th I	n.a.	n.a.
B9		5 M/5 F	n.a.	n.a.
B10		5 M/5 F	n.a.	n.a.

[a] Male.
[b] Female.
[c] Instar.

All bed bug strains were reared without insecticide selection pressure, as previously described by Vander Pan et al. (2019). After seven feedings (6 wks), most of the bed bugs were adult, and individuals had a maximum difference in age of 7 d. The molecular assays with reared bed bugs were performed with adults 8 d after their last blood meal. Previous studies have shown that sex-specific differences in bed bugs can influence test results (Siljander et al., 2008; Pfiester et al., 2009; Aak et al., 2014; Vander Pan et al., 2019). Therefore, allele frequencies of *kdr* substitutions were examined in both sexes (Table 1).

Despite initially high numbers of bed bugs, rearing of five other strains (named by the location where they were collected: GW, RS, LB2, LB3, and T) was commenced as described before but was not successful due to high mortality and low reproduction rates. Consequently, laboratory-reared descendants from the second generation were used for the molecular assays (Table 1). Except for the T strain, only adults of the same age and feeding status as described above were used. Bed bugs from the ten remaining collection sites (populations: B1–B10) consisted of less than 20 bed bugs. Therefore, laboratory rearing was not possible within an adequate investigation period, and field-caught bed bugs were used for the experiments instead of laboratory-reared descendants (Table 1).

Bed bugs from the 20 field strains and populations as well as bed bugs from the susceptible UBA strain were frozen at −20 °C and −80 °C until further use in the pyrosequencing experiments and RT-qPCRs, respectively.

2.2. Pyrosequencing of genomic DNA

The substitutions V419L or L925I were quantified in ten field strains (SK, OB, LB, AS, HO, GW, RS, LBII, LBIII, and T) and in seven bed bug populations (B2, B4, B5, B7, B8, B9, and B10) performing pyrosequencing as a rapid quantitative sequencing method. In order to analyze allele frequencies of the two polymorphisms, *C. lectularius* from each strain and population were pooled as presented in Table 1.

Furthermore, pyrosequencing of genomic DNA of individual male and female bed bugs was performed in order to evaluate the genotypes (Table 1). Pyrosequencing assays for the quantitative analysis of pyrethroid resistance-associated SNPs at position 1255 (V419L) and 2773 (L925I) of the *Clvssc* gene were developed using Pyromark Assay Design software 2.0 (Qiagen, Hilden, Germany).

Genomic DNA of the pooled and individual bed bugs was isolated with the NucleoSpin® 8 Tissue Kit (Macherey-Nagel, Düren, Germany) and the corresponding NucleoVac 96 vacuum manifold. Bed bugs were crushed with a pestle in a 1.5 ml reaction tube, adding 50 µl 1 × PBS for better homogenization. Then 180 µl lysis buffer (T1) and 25 µl proteinase K were added, and tubes were incubated at 56 °C and 900 rpm overnight. The following DNA extraction was performed according to the manufacturer's instructions, and DNA was collected in 50 µl elution buffer. DNA concentrations were measured by determining the optical density of samples using a Take 3 plate in the Epoch plate reader (Bio-Tek, Winooski, VT, USA) at 260, 280, and 320 nm using the Gen5™ software. DNA samples were stored at −20 °C until further use. DNA fragments with the potential SNPs at position 1255 (V419L) and 2773 (L925I) were amplified by PCR (Phusion Hot Start II High-Fidelity DNA Polymerase, Thermo Fisher Scientific Inc., Schwerte, Germany) using sequence specific primer pairs (Table 2). PCR was carried out in 50 µl using 12.5 ng template DNA with 1 × Phusion HF buffer, 10 mM dNTP-mix, 250 nM of each primer, and 1 U Phusion Hot Start II DNA-Polymerase (Thermo Fisher Scientific Inc., Schwerte, Germany). Thermocycling was conducted in a Bio-Rad C1000 or S1000 cycler (Bio-Rad Laboratories, Feldkirchen, Germany) with initial denaturation at 98 °C for 30 s followed by 45 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min as final elongation. PCR products were analyzed by agarose gel electrophoresis and were ready to be used as templates for pyrosequencing.

The accuracy of the pyrosequencing assay was evaluated with a standard curve for the respective SNPs (Supplementary Information Fig. S2). DNA containing the wild-type or resistance-associated allele was isolated as plasmid DNA. For this purpose, PCR products were purified from agarose gels using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Europe, Freiburg, Germany) according to the manufacturer's specifications. Afterwards, they were cloned into the pCR™4Blunt-TOPO® Vector (Thermo Fisher Scientific Inc., Schwerte, Germany) and transformed into One Shot® TOP10 Chemically Competent *E. coli*-cells (Thermo Fisher Scientific Inc., Schwerte, Germany) using the Zero Blunt® TOPO® PCR Cloning Kit (Thermo Fisher Scientific Inc., Schwerte, Germany) according to the instruction manual. Plasmid DNAs were prepared using the EasyPrep® Pro Plasmid Miniprep Kit (Biozym Scientific, Hessisch Oldendorf, Germany) according to the manufacturer's specifications. Positive clones were identified by sequence analysis. To obtain a standard curve for each SNP with different percentages of each polymorphism, 0.5 ng plasmid DNA containing the resistance-associated and wild-type allele were mixed in twelve different ratios (Proportion of the resistance-associated allele: 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%). Thermocycling was conducted as described above. Linear regression analysis for both pyrosequencing assays with GraphPad Prism 8.4.2 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used to determine the correlation between the calculated and observed allele frequencies.

Table 2
Primers used for PCR, pyrosequencing, and quantitative real-time PCR experiments.

Primer name	Sequence (5'→ 3')	Substitution/ Gene
PCR		
Clkdr1up[a]	GTG GCA CAT GTT GTT CTT CAT AGT	*kdr* V419L
Clkdr1lo[b] (HPLC)	CGC CTT CTT TTG CAG TTC A	
Clkdr1lo-biotin (HPLC)	Biotin -CGC CTT CTT TTG CAG TTC A	
Clkdr2up	CCC ATC ACA GCA AAG ATG AAA AT	*kdr* L925I
Clkdr2lo (HPLC)	ATT ATG GGC AGA ACA GTG GGT	
Clkdr2lo-biotin (HPLC)	Biotin -ATT ATG GGC AGA ACA GTG GGT	
Pyrosequencing		
Clkdr1-seq (HPLC)	CCT GGG ATC ATT CTA CC	*kdr* V419L
Clkdr2-seq (HPLC)	ACA CAA AAG TTA AAT TAC CA	*kdr* L925I
Quantitative real-time PCR reference genes		
ClRpl8F[a]	AAA GGC ACG GTT ACA TCA AAG GTG	*rpl8*
ClRpl8R[b]	TAG TCT TGA ACC TAT AGG GGT CCC	
ClRpl11F	GAA GAA TGT CAT GCG AGA TGT CAG G	*rpl11*
ClRpl11R	CCT TCG AGA AGA CTG GCT GCT G	
ClRps16F	ACA GCA GTC GCC TAC TGC AAA C	*rps16*
ClRps16R	TCC TGG AGT TTG TAC TGG AGG ACC	
Quantitative real-time PCR CYP genes		
cyp4cm1forward	AGG AGA ATG TCA GAA AGC GTC ACC	*cyp4cm1*
cyp4cm1reverse	GCA TTA ATT TTT CCC AAC TTT CTT	
cyp6dn1forward	AGC CCA CAG CTC GGA AAC AG	*cyp6dn1*
cyp6dn1reverse	CTT ATG CCT TGG TGG GAG AGT	
cyp397a1forward	CTC GGG CTC ACC ACT CTC AAC A	*cyp397a1*
cyp397a1reverse	ACC GTC ATG GCT CCC GTC AG	
cyp398a1forward	AAT CGC CCA CAG GAA AAA CAA CAA	*cyp398a1*
cyp398a1reverse	CCG GGT GGG AAG CGA GTA GG	

[a] Up and F: forward primer.
[b] Lo and R: reverse primer.

For quantitative analysis of both SNPs, preparation of all PCR products with the PyroMark Q24 MDx Vacuum Workstation and pyrosequencing assay (PyroMark® Q24 System, Software, Workstation, and PyroMark Gold Q24 reagents; Qiagen, Hilden, Germany) was performed as described in the PyroMark® Q24 User Manual using self-designed sequencing primer pairs (Table 2). For each sample, five technical replicates in at least two different runs were analyzed. Differences between the susceptible UBA strain and the field strains were tested for statistical significance with a one-way ANOVA, and p-values were adjusted with Dunnett's post-hoc test. Differences between males and females of the respective strain were tested for statistical significance with a one-way ANOVA, and p-values were adjusted with Sidak's post-hoc test. Statistics were performed in GraphPad Prism 8.4.2 for Windows (GraphPad Prism Software, San Diego, CA, USA, www.graphpad.com).

In order to examine any adverse effects of the L925I substitution, particularly of the homozygous genotype, on the field strains LB, HO, SK, OB, and AS reared without pyrethroid selection pressure, allele distribution was examined concerning the existence of a Hardy–Weinberg equilibrium (package "Hardy-Weinberg" for R, version 3.1.3). Due to the small sample size, the "likelihood ratio" test was particularly used as recommended by Engels (2009).

2.3. Reverse-transcription quantitative PCR

Relative mRNA expression levels of the four CYP genes *cyp397a1*, *cyp398a1*, *cyp4cm1,* and *cyp6dn1* of the insecticide susceptible UBA strain and the field strains LB, HO, SK, OB, AS, GW, and T were compared. Ten single male bed bugs of each strain were examined in two technical replicates. For normalization, mRNA expression of the three reference genes *rpl8*, *rpl11,* and *rps16* was used, which were previously evaluated for stable expression in the context of pyrethroid resistance by Zhu et al. (2012). The BestKeeper tool was applied to re-evaluate the stability of these reference genes (Supplementary Information Table S2). Bed bugs were crushed on ice with a pestle in 1 ml peqGOLD Trifast™. RNA was extracted according to the provided manual. RNA precipitates were dissolved in DEPC-treated water and stored at −80 °C until use. RNA concentrations were measured by determining the optical density as described for DNA before. The integrity of all RNA samples was evaluated using Lab-on-Chip-Technology with the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Measurements were performed according to the manufacturer's Quick Start Guide. The study was carried out prior to the detection of the "hidden break" in the 28S rRNA of *C. lectularius* by Natsidis et al. (2019). Thus, RNA integrity was determined by manually inspecting the peak patterns of the electropherograms and gel-like images. Contaminating genomic DNA was removed by digestion with DNaseI (Rnase-free; Thermo Fisher Scientific Inc., Schwerte, Germany) according to the manufacturer's specifications. According to the manufacturer's instructions, reverse transcription was performed with 50 ng total RNA by using the DyNAmo cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Schwerte, Germany). No template controls (NTC) and no-RT-controls (-RT) were included in each run to detect possible contaminations. To evaluate the gene specific PCR efficiencies, plasmid standards containing the PCR fragment as insert were prepared, and dilution series were analyzed with every RT-qPCR run. Sequence specific primer pairs used for the PCR and RT-qPCR were those described by Zhu et al. (2012) for the three reference genes and by Adelman et al. (2011) for the four CYP genes (Table 2). Plasmid concentration was measured, and a stock dilution for all samples with 4×10^8 copies was stored at −20 °C. Final dilutions were 4×10^7 to 40 for *rpl8*, *rps16* and *cyp6dn1*, 1×10^7 to 10 for *rpl11*, *cyp398a1* and *cyp4cm1* and 4×10^6 to 4 for *cyp397a1*. Dilutions were freshly prepared prior to the RT-qPCR. To compare the different mRNA expression levels between runs, a calibrator was amplified on each plate as a reference point. The calibrator consisted of cDNA from ten bed bugs belonging to the UBA strain mixed in equal quantities. Amplifications were performed in a Bio-Rad CFX96 real-time PCR detection system using the 2 × GoTaq® qPCR Master Mix (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. In each reaction, 200 nM forward and reverse primer and 4 µl cDNA were used. The included GoTaq® Hot Start Polymerase was activated by an initial denaturation at 95 °C for 2 min. The following 49 cycles consisted of two steps of 95 °C for 15 s and 60 °C for 60 s. At the end of every extension phase, the amount of double stranded PCR product was measured as BRYT Green® fluorescence, and the quantification cycle (Cq) was determined according to the MIQE guidelines (Bustin et al., 2009). The absence of non-specific PCR products was confirmed by melting curve analysis. Therefore, the temperature was raised from an initial 60 °C–95 °C by 0.5 °C every 5 s. Relative quantitative analysis of amplification data was performed using the CFX Manager 2.0 Software (Bio-Rad Laboratories, Feldkirchen, Germany). mRNA expression of the four genes of interest was compared to the three reference genes. Statistical differences ($\alpha = 0.05$) between the resulting mRNA expression levels of the four CYP genes from the seven field strains and those from the susceptible UBA strain were calculated with a Kruskal-Wallis test and Dunn's post-hoc test.

3. Results

3.1. Allele frequencies encoding the two substitutions V419L and L925I

Pyrosequencing assay evaluation resulted in an excellent correlation of calculated and observed frequencies for the substitutions V419L ($r = 0.98$) and L925I ($r = 0.99$) (Supplementary Information Fig. S2). However, at low frequencies, there was some overestimation of the frequency of the resistance-associated alleles. Based on this analysis, relative resistance-allele frequencies below 15% were considered to be technical background of the pyrosequencing assay while the technical background for the susceptible wild-type alleles was slightly smaller with 10%.

The polymorphism V419L was not detected in the UBA strain and any of the five field strains successfully reared in the laboratory (Fig. 1A). Pyrosequencing further revealed the absence of the L925I polymorphism in the susceptible UBA strain and the HO field strain. In contrast, the L925I allele was identified in the SK, AS, OB, and LB field strains (Fig. 1B). Female bed bugs from the AS strain (67%) and male bed bugs from the OB strain (59%) showed the highest frequency of L925I, whereas male (30%) and female (31%) bed bugs from the LB strain showed the lowest frequency (Fig. 1B). Male bed bugs from the SK (44%) and AS (51%) strains as well as female bed bugs from the SK (39%), and OB (60%) strains showed intermediate allele frequencies (Fig. 1B). Differences in allele frequencies of male and female bed bugs between the four field strains SK, OB, LB, and AS and the susceptible UBA strain were found to be statistically highly significant ($p < 0.0001$, for all strains).

Pyrosequencing indicated that both substitutions were absent in the *Clvssc* gene of the T strain (Fig. 1C and D). The V419L substitution was identified in female (96%) and male (40%) bed bugs from the RS strain (Fig. 1D). Both allele frequencies were significantly higher ($p < 0.0001$) than those of the susceptible UBA strain. Male bed bugs from the GW strain showed an average of 99% resistance-associated alleles encoding L925I (Fig. 1C). The frequency of the L925I substitution from the remaining strains GW (female), RS, LBII, and LBIII was 100% (Fig. 1C). Statistically highly significant differences in allele frequencies were found between the susceptible UBA strain and the strains GW, RS, LBII, and LBIII ($p < 0.0001$, for all strains).

Pyrosequencing results for L925I showed no resistance-associated alleles in males and females from B10. Female bed bugs of B4 showed the lowest L925I frequency (82%). Frequencies of resistance-associated alleles in male bed bugs of B4 and females of B2 were 94% and 99%, as well as 96% in the tested juveniles from B7. A 100% frequency of L925I was found in B2 (males), B5, B8, and B9 (Fig. 1E). Compared to the susceptible UBA strain, statistically significantly higher allele frequencies were observed for all tested populations - except for B10 males and females - with $p < 0.001$. None of the tested populations had the V419L variant (Fig. 1F).

Differences in allele frequencies of L925I between male and female bed bugs of the AS strain (females: 67%; males: 51%) and B4 population (females: 82%; males: 94%) were found to be statistically highly significant ($p < 0.0001$, for both). In the RS strain (females: 96%; males: 40%), differences in allele frequencies of V419L between males and females were also found to be statistically highly significant ($p < 0.0001$).

3.2. Genotyping of individual Cimex lectularius

None of the published studies has given comprehensive information about the impact of homo- and heterozygosity of V419L and L925I substitution on the resistance status of bed bug populations so far. Therefore, DNA of ten single male and female *C. lectularius* from the UBA, SK, OB, LB, AS, and HO strain, as well as few isolated bed bugs from the populations B1, B3, B5, B6 and B8 was sequenced. Pyrosequencing revealed that both substitutions were not present in any of the

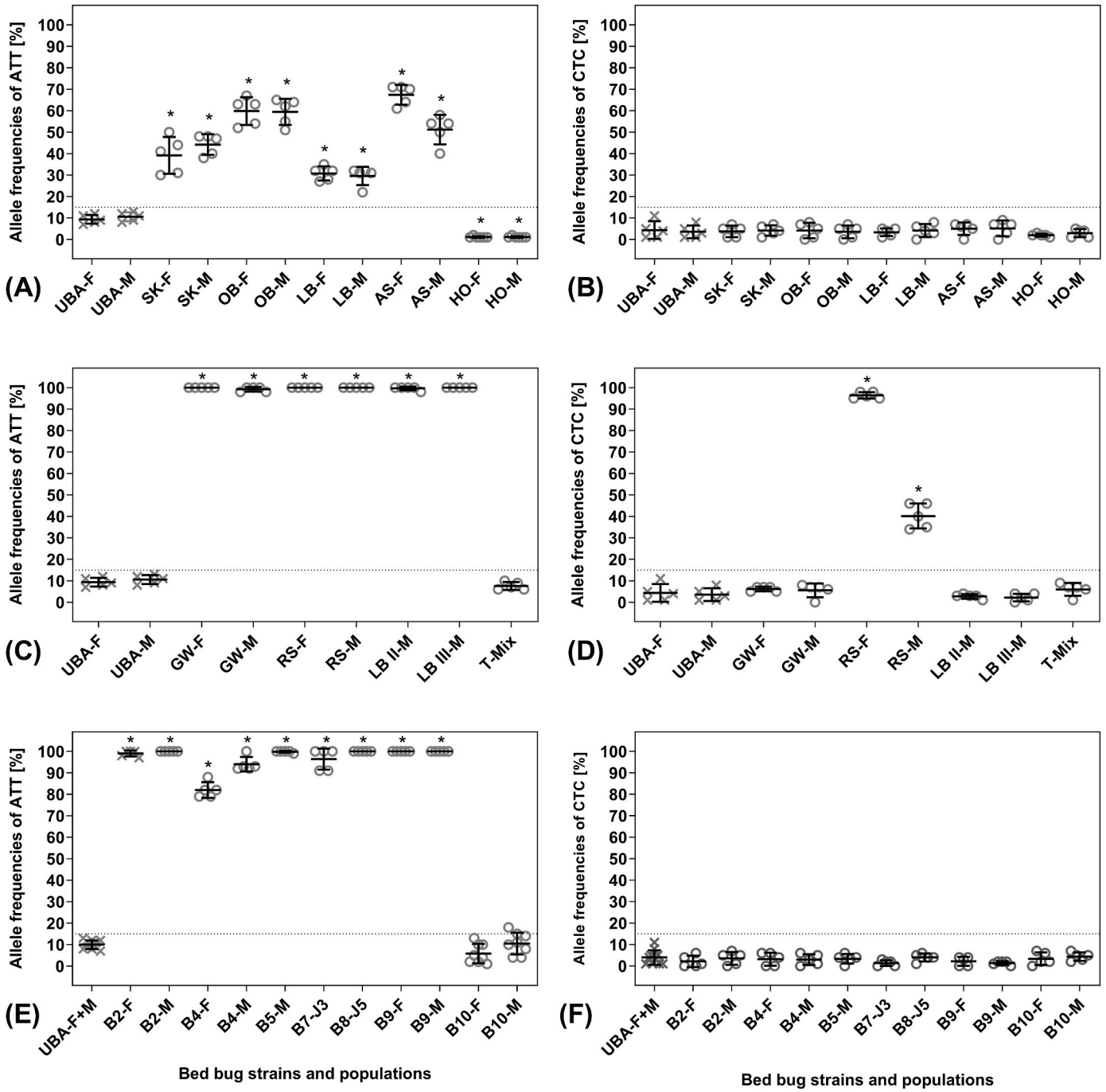

Fig. 1. Allele frequencies for the polymorphisms V419L (GTC→CTC) and L925I (CTT→ATT) in the voltage-sensitive sodium channel α-subunit gene of *Cimex lectularius* from the susceptible UBA strain and (**A**), (**B**) the five field strains that were reared for about one year (**C**), (**D**) the five field strains that failed to breed and (**E**), (**F**) the field-collected bed bug populations where rearing was not possible (examination of single bed bugs is not shown). Differences within the same sex between the susceptible UBA strain and the field strains were tested for statistical significance (*, $p < 0.0001$) with a one-way ANOVA followed by Dunnett's post-hoc test. The crosses and circles show the five technical replicates of the pyrosequencing of each strain, the horizontal lines show the means, and the error bars represent the standard deviation.

specimens from the susceptible UBA strain, which was therefore considered to be homozygous wild-type. In contrast, in all five field strains reared in the laboratory, homozygous wild-type, heterozygous, and homozygous resistance-associated genotypes of bed bugs regarding the substitution L925I were present (Table 3).

In all tested bed bug populations, for which rearing could not be accomplished, only bed bugs homozygous for the resistance-associated L925I exchange were found. No strains and populations showed alleles with the substitution V419L and thus were homozygous wild-type (Table 3).

In order to address if these alleles, particularly the homozygous resistance-associated genotype, might cause disadvantages under laboratory rearing conditions in the absence of insecticide selection pressure, allele distribution was examined concerning the presence of a Hardy–Weinberg equilibrium. Due to the small sample size, the likelihood-ratio test included in the Hardy-Weinberg-package in R was favored, but the other tests in the package were conducted as well. Although p-values differed between the individual tests, the actual results remained

Table 3
Different genotypes (homozygous wild-type, heterozygous and homozygous resistance-associated) of the V419L and L925I substitutions in ten single male and female bed bugs of the susceptible UBA strain and the five successfully reared field strains. For the characterization of the DNA from individual bed bugs from populations B1, B3, B5, B6, and B8, the table shows the sex or developmental stages.

Strains/ Populations	Homozygous wild-type [F[a]/M[b]]		Heterozygous [F/M]		Homozygous resistance-associated [F[a]/M[b]]	
	V419L	L925I	V419L	L925I	V419L	L925I
UBA	10/10	10/10	0/0	0/0	0/0	0/0
LB	10/10	7/9	0/0	2/1	0/0	1/0
HO	10/10	0/1	0/0	8/9	0/0	2/0
SK	10/10	0/4	0/0	10/4	0/0	0/2
OB	10/10	4/4	0/0	5/5	0/0	1/1
AS	10/10	4/5	0/0	4/5	0/0	2/0
B1	M[b]	0	0	0	0	M
B3	1/1	0/0	0/0	0/0	0/0	1/1
B5	F[a]	0	0	0	0	F
B6	M/4th I[c]	0	0	0	0	M/4th I
B8	F	0	0	0	0	F

[a] Female.
[b] Male.
[c] Instar.

unchanged regardless of the test used. The statistical power of the analysis remained low due to the small number of examined bed bugs. However, the results show a principal tendency. The distribution of alleles encoding L925I was not significantly different from Hardy-Weinberg equilibrium in the LB ($p = 0.226$), SK ($p = 0.059$), OB ($p = 0.656$), and AS ($p = 0.908$) strain. In contrast, the genotypes present in bed bugs of the HO strain were statistically significantly different from the Hardy–Weinberg equilibrium ($p = 0.001$), with heterozygous individuals being much more frequently observed than expected. For the SK strain, the same tendency was observed, but the difference was not significant. Thus, homozygous resistance-associated genotypes were significantly underrepresented in the HO strain.

3.3. mRNA expression levels of the CYP genes cyp397a1, cyp398a1, cyp4cm1 and cyp6dn1

The suitability of the three reference genes *rpl8*, *rpl11,* and *rps16* used for normalization of target gene expression was verified with the Excel-based BestKeeper software tool. Descriptive statistics of all Cq values were computed for each reference gene and compared over the entire study. For the three reference genes ($n = 3$), a total of 95 Cq values (one sample of each duplicate tested per gene and bed bug strain) were examined (Supplementary Information Table S2). The fold over- or under-expression of individual samples compared to the geometric mean Cq for the same reference gene were ±1.73-fold for *rpl8*, ±2.11-fold for *rpl11,* and ±1.93-fold for *rps16*. Thus, *rpl8* showed with 0.82 cycles the lowest and *rpl11* with 1.12 cycles, the highest variation of the Cq values (SD [± Cq]). Variation in the expression of *rps16* was intermediate, with an SD [± Cq] of 0.99 cycles. The BestKeeper index was calculated as the geometric mean of the Cq values of the three reference genes and showed an SD [± Cq] of 0.92 cycles. According to the BestKeeper tool, any studied reference gene with an SD [± Cq] higher than one can be considered inconsistent. For that reason, a second BestKeeper index was calculated after the removal of *rpl11* ($n = 2$) (Supplementary Information Table S1). This resulted in a decrease of the BestKeeper index with an SD [± Cq] of 0.87 cycles.

Multiple pair-wise correlation analysis was performed to estimate the inter-gene relations among the three reference genes, and the BestKeeper index ($n = 3$). Relations were described by the Pearson correlation coefficient (r). The correlation analysis showed a significant correlation between *rpl8* and *rpl11* ($r = 0.869$; $p = 0.001$), *rpl8,* and *rps16* ($r = 0.870$; $p = 0.001$) as well as *rpl11* and *rps16* ($r = 0.819$; $p = 0.001$). Furthermore, a significant correlation existed when comparing the reference genes with the BestKeeper index (*rpl8*: $r = 0.962$, *rpl11*: $r = 0.942$ and *rps16*: $r = 0.945$ with $p = 0.001$ for all data sets). Additional regression analysis and the resulting coefficients of determination showed that 92.5%, 89.3%, and 88.7% of the variability were explained by *rpl8*, *rps16,* and *rpl11*, respectively. Therefore, variation was adequately captured by the model, including all three reference genes.

Correlation analysis without *rpl11* ($n = 2$) showed the same significant correlation ($p = 0.001$), with $r = 0.870$ between *rpl8* and *rps16* as before. In comparison to the correlation analysis with all three reference genes, the correlation between the index and the reference genes *rpl8* and *rps16* increased ($r = 0.966$ and $r = 0.968$), whereas the significance level remained the same ($p = 0.001$). The coefficient of determination increased within this model by 0.8% (*rpl8*: 93.3% of variability explained) and 4.4% (*rps16*: 93.7%). Since differences between both models were only small, all three reference genes were included in the mRNA expression analysis.

Gene specific PCR efficiencies (E) were evaluated with plasmid standard dilution series for all genes, which were analyzed with every RT-qPCR run. The highest efficiency of amplification of the reference cDNA showed *rpl8* with $E = 97.7\%$, followed by *rps16* with $E = 80.3\%$ and *rpl11* with the lowest efficiency of $E = 64.7\%$. The highest amplification of CYP cDNAs was detected for *cyp397a1* with $E = 96.9\%$ and lowest for *cyp4cm1* with $E = 84.4\%$. Amplification of *cyp398a1* and *cyp6dn1* was in between with $E = 96.4\%$ and $E = 90.6\%$. There was also an excellent positive linear correlation between the Cq values and the logarithmically transformed copy numbers of the dilution series (*rpl8*: $r = 0.9993$, *rps16* and *rpl11*: $r = 0.9991$, *cyp4cm1*: $r = 0.9996$, *cyp398a1*: $r = 0.9987$, *cyp6dn1*: $r = 0.9979$ and *cyp397a1*: $r = 0.9940$).

The mRNA expression analysis was always performed with the pyrethroid susceptible UBA strain as a control, and expression levels are given as fold differences relative to the UBA expression level (Fig. 2). The analysis revealed that the average mRNA expression level of *cyp397a1* was significantly higher for the strains HO, with 7.1- ($p = 0.0421$), SK with 17.3- ($p = 0.0009$), AS with 17.5- ($p = 0.0011$), GW with 56- ($p < 0.0001$) and T with 44.9-fold ($p < 0.0001$) increased expression, respectively, in comparison with UBA. No statistically significant differences between mRNA expression levels of the UBA strain and the field strains LB (5.1, $p = 0.6115$) and OB (5.3, $p = 0.3131$) were detected. The average expression levels of *cyp398a1* were 4.9-fold higher in the T field strain ($p = 0.0042$) than in the UBA strain. No increased mRNA expression levels of *cyp398a1* were found in the other field strains (Fig. 2). Furthermore, no significant increases in mRNA expression levels of the CYP genes *cyp6dn1* and *cyp4cm1* were found between the analyzed strains (Fig. 2).

4. Discussion

The present study provides the first evidence of the occurrence of a significant increase in mRNA expression levels of the CYP genes *cyp397a1* and *cyp398a1* in bed bugs collected in Germany. Moreover, the substitution L925I in the voltage-sensitive sodium channel α-subunit gene was detected in almost all examined bed bug strains. The presence of the substitution V419L, in combination with the substitution L925I, was demonstrated in one strain. The screening of 20 field strains and populations revealed for the first time that in German bed bug populations, target-site resistance and metabolic resistance mechanisms appear simultaneously, which has also been shown for other countries (Adelman et al., 2011; Zhu et al., 2013). An overview of all detected potential resistance mechanisms of the field strains and populations, as well as the previously determined resistance ratios (RR) for deltamethrin (Vander Pan et al., 2019), is shown in Table 4.

The pyrosequencing method is frequently used to determine alleles

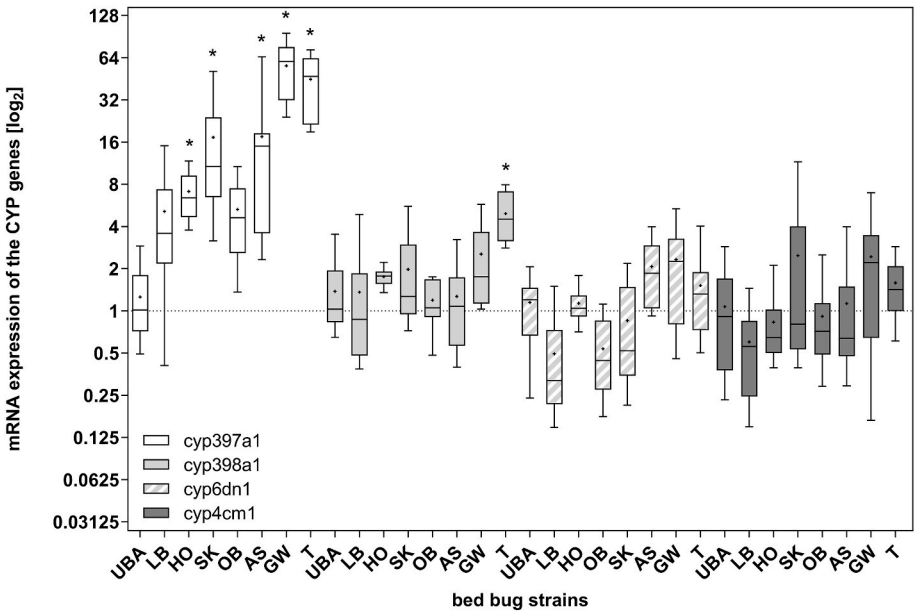

Fig. 2. mRNA expression levels of the four CYP genes *cyp397a1*, *cyp398a1*, *cyp6dn1,* and *cyp4cm1* relative to the reference genes *rpl8*, *rpl11,* and *rps16* normalized to the susceptible UBA strain. Differences between the susceptible UBA strain and the other strains were tested for statistical significance with a Kruskal-Wallis test and Dunn's post-hoc test. Boxes extend from the 25th to 75th percentiles, and whiskers represent min to max. The line in the middle of the box is plotted at the median and the cross at the mean. *, $p < 0.05$.

Table 4
Overview of the examined resistance mechanisms that were detected in each of the bed bug field strains, populations, and the susceptible UBA strain. Allele frequencies were determined among n = 100 males and females each for both SNPs causing V419L and L925I substitutions and the different genotypes of the L925I exchange in n = 10 single male and female bed bugs, respectively. Only mRNA expression levels of n = 10 single male bed bugs with statistically significant differences between the UBA strain and the tested field strains are shown. Resistance ratios were determined as fold difference in the EC50 value in a 24-well filter contact bioassay adapted from Romero et al. (2007) in a previous study (Vander Pan et al., 2019).

FS/P[a]	Allele frequencies [%]				Genotypes L925I	mRNA expression levels of the CYP genes				RR[d]
	V419L		L925I			*cyp397a1*	*cyp398a1*	*cyp4cm1*	*cyp6dn1*	
	F[b]	M[c]	F	M						
UBA	–		–	–	0 H[e] 0 HM[f]	–	–	–	–	–
LB	–	–	31	30	3 H, 1 HM	–	–	–	–	4.34
HO	–	–	–	–	17 H, 2 HM[j]	7.1	–	–	–	4.92
SK	–	–	39	44	14 H, 2 HM	17.3	–	–	–	7.24
OB	–	–	60	59	10 H, 2 HM	–	–	–	–	11.00
AS	–	–	67	51	9 H, 2 HM	17.5	–	–	–	20.73
GW	–	–	100	99	n.a.	56	–	–	–	n.a.
T	-[g]		-[g]		n.a.	44.9	4.9	–	–	n.a.
RS	96	40	100	100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
LBII	n.a.	–	n.a.	100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
LBIII	n.a.	–	n.a.	100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
B1	n.a.	n.a.	n.a.	n.a.	1 HM	n.a.	n.a.	n.a.	n.a.	n.a.
B2	–	–	99	100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
B3	n.a.	n.a.	n.a.	n.a.	2 HM	n.a.	n.a.	n.a.	n.a.	n.a.
B4	–	–	82	94	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
B5	n.a.	–	n.a.	100	1 HM	n.a.	n.a.	n.a.	n.a.	n.a.
B6	n.a.	n.a.	n.a.	n.a.	2 HM	n.a.	n.a.	n.a.	n.a.	n.a.
B7	-[h]		96[h]		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
B8	-[i]		100[i]		1 HM	n.a.	n.a.	n.a.	n.a.	n.a.
B9	–	–	100	100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
B10	–	–	–	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

[a] Field strains and populations.
[b] Females.
[c] Males.
[d] Resistance ratios.
[e] Heterozygous.
[f] Homozygous.
[g] Pool of 200 mixed bed bug stages.
[h] Third instar.
[i] Fifth instar.
[j] Bed bug strains in which the potential *kdr* substitutions were only detected during the single bed bug study (genotyping) and not during the investigation of the allele frequencies.

associated with benzimidazole-resistance in parasitic nematodes and thus fairly accessible in the community of parasitologists interested in resistance research. However, it is clearly not as accessible as simple qualitative methods such as restriction-fragment length polymorphism analysis of PCR products, allele-specific PCR, or simple Sanger sequencing of PCR products. In comparison to the latter, costs are comparable, while turn-around time is slightly in favor of pyrosequencing since this is a real-time method while Sanger sequencing typically runs overnight. The most important advantage of pyrosequencing is its ability to provide quantitative data on allele frequencies. This is not possible at all with Sanger sequencing, where even identical adjacent bases might differ considerably in peak height. Qualitative methods such as allele specific PCR have a much lower background than the 15% technical background applied in the present study and can detect resistance alleles already at very low frequencies. However, they do not offer any option of allele quantification. Real time PCRs using, e.g., allele-specific probes would also have fewer problems to detect very low allele frequencies but would allow less precise measurements if both alleles have similar frequencies. Typically, differences smaller than twofold are hard to quantify by real time PCR. Therefore, pyrosequencing adds an additional method that can be used for quantification of *kdr* alleles in bed bug populations to rapidly evaluate if resistance alleles are present in a population.

Pyrosequencing revealed that the substitution L925I was present in 17 of the 20 German bed bug field strains and populations. Bed bugs from the RS field strain additionally carried the V419L substitution. The latter was only found in one strain (RS) combined with L925I and thus occurred considerably less frequently than L925I. The rarer occurrence of V419L alone or in combination with L925I has also been described in other European studies (Durand et al., 2012; Booth et al., 2015; Balvin and Booth, 2018). Studies from the USA and Australia also showed that V419L rarely occurs alone but often in combination with L925I (Zhu et al., 2010, 2013; Adelman et al., 2011; Dang et al., 2014b, 2017; Holleman et al., 2019).

Unlike the other field strains, pyrosequencing data of the HO strain were contradictory (Table 4). Neither the substitution V419L nor L925I was found in the allele frequency study with 100 pooled males and females. However, genotyping of ten single male and female bed bugs each revealed that from 20 examined bed bugs 17 were heterozygous and two homozygous for the resistance-associated L925I substitution. Since all examined bed bugs came from the same stock, a mix-up is hard to imagine. Both experiments were repeated with new batches of bed bugs. However, the results were very similar to the initial round for both experiments (data not shown), and it was impossible to find a convincing explanation. Independently generated RT-PCR products from the HO strain that were analyzed by Sanger sequencing confirmed the presence of L925I in the pool that was used for RNA isolation (data not shown). We therefore consider the results for pyrosequencing of the pooled HO strain bed bugs as incorrect, although we have no explanation of why this error occurred. We do not consider a problem in pyrosequencing because quantification of other samples in the same runs worked properly.

Genotyping of individual bed bugs of ten field strains revealed heterozygous as well as homozygous individuals for the substitution L925I. In contrast to other studies (reviewed by Holleman et al. (2019)), many individuals of the five laboratory reared strains exhibit the heterozygous genotype. In contrast, no heterozygotes were detected in these strains that were not reared in the laboratory (Table 3), which corresponds to the other studies. Furthermore, four of the field strains without rearing success (GW, RS, LBII, and LBIII) and four bed bugs from the populations B2, B5, B8, B9 showed allele frequencies of about 100% for L925I (Table 4). This means that these strains had a high frequency of homozygous individuals with a resistance-associated genotype. However, rearing and thus the determination of RR by bioassay was only possible for the LB, HO, SK, OB, and AS strains due to the above-mentioned breeding problems with the other strains. As expected, from the five field strains which had been reared successfully, the strains with the highest RR AS, OB, and SK also showed the highest allele frequencies for L925I (Table 4). These results coincide with the results of Seong et al. (2010), who found that an increase in the frequency of the resistance alleles is correlated to an increase of deltamethrin resistance levels in *C. lectularius*. However, allele frequencies for L925I in field strains reared for about one year under laboratory conditions were considerably lower in comparison to bed bug strains without rearing success or where no rearing was possible. This might be due to resistance loss as a consequence of rearing over several generations without insecticide selection pressure (Zhu et al., 2013; Gordon et al., 2015), suggesting that the presence of resistance alleles results in lower fitness under laboratory conditions without exposure to pyrethroids (Polanco et al., 2011; Zhu et al., 2013). It is probable that the RRs of the five laboratory reared field strains were appreciably higher in the original population and more homozygous resistance-associated genotypes were present.

Significant differences in allele frequencies of male and female bed bugs were only found in two strains and one population. In the AS and RS strain, female bed bugs showed higher allele frequencies, and in the B4 population, those of the males were higher. These results suggest that allele frequencies of *kdr* substitutions are not systematically skewed towards higher frequency in one of the sexes. Differences in susceptibility to insecticides between both sexes may also involve other sex-specific metabolic detoxification mechanisms known to occur in other insects (Abd-Elghafar et al., 1990; Pruett et al., 2001; Le Goff et al., 2006). We were not able to test this within the timeframe of our study. For that reason, further studies are required where both sexes are used in bioassays and molecular assays.

Although the frequency of bed bugs homozygous for the allele encoding L925I was low (10%), the AS field strain showed the highest RR (Table 4) in a filter paper contact bioassay. Furthermore, it was tested positive for pyrethroid resistance in an insecticide bioassay simulating practical conditions (Vander Pan et al. (2019)). This could indicate that the heterozygous genotype (45%) results in a decreased pyrethroid susceptibility. Our findings are supported by the assumption of Romero et al. (2007) that genes responsible for pyrethroid resistance are inherited incompletely dominant. Holleman et al. (2019) suggested that compared with the southern cattle tick and the sub-Saharan mosquito, bed bugs with the heterozygous genotype are less susceptible to insecticides than those homozygous susceptible but more than bed bugs with homozygous resistance-associated genotypes. However, it has not been clearly shown whether the heterozygous occurrence of the substitution results in an increase of the RR or whether the genotype must be homozygous to confer a resistant phenotype.

Besides *kdr* genotypes, increased mRNA expression levels of the four CYP genes *cyp397a1*, *cyp398a1*, *cyp4cm1,* and *cyp6dn1* have previously been implicated in pyrethroid resistance in bed bugs (Zhu et al., 2013). RT-qPCR was used to compare the mRNA expression between the insecticide susceptible UBA strain and seven field strains. In five of these field strains, the average mRNA expression levels of *cyp397a1* were significantly higher compared to the susceptible UBA strain (Table 4).

Differences in RR (Table 4) to the UBA strain found in our previous study (Vander Pan et al., 2019) can well be explained by the resistance mechanisms detected. Allele frequencies encoding the substitution L925I were nearly 60% for the AS and OB field strains in both sexes. The RR of the AS strain was twice as high as that of the OB strain, which can be associated with the 17-fold increase of the mRNA expression level of *cyp397a1* in the AS field strain. The lower RR in the SK strain might be explained by the very similar increase in *cyp397a1* but a frequency of alleles encoding L925I of approximately 40% in comparison to the AS strain. None of the tested CYP genes was upregulated in the LB field strain. The frequency of alleles with the substitution L925I in male bed bugs was approximately 30%, which can also explain the considerably lower RR of the LB strain in comparison to the other field strains. These findings support those of previous studies, which demonstrated that decreased susceptibility and resistance to pyrethroids in bed bugs could

be associated with the presence of the substitutions V419L or L925I (Zhu et al., 2010; Dang et al., 2017) and a simultaneously increased metabolic detoxification by CYPs (Mamidala et al., 2011; Zhu et al., 2013; Dang et al., 2017).

The GW and T strains showed the highest mRNA expression levels of *cyp397a1*. In addition, the mRNA expression level of *cyp398a1* was slightly increased in the T strain. Determination of the RR with the 24-well filter contact bioassay (Vander Pan et al., 2019) was not possible since breeding of both strains was not successful. Thus, it is not possible to draw conclusions on the influence of highly increased expression levels of *cyp397a1* on resistance phenotypes of these strains. However, previous studies (Adelman et al., 2011; Zhu et al., 2013), e.g., by Adelman et al. (2011), also showed a significantly increased (>36-fold) transcript-level of *cyp397a1* in a pyrethroid resistant bed bug strain. Therefore, we assume that both strains would be resistant to pyrethroids in the field. Since the GW strain additionally showed allele frequencies of about 100% for L925I, it is very likely that bed bugs would be highly resistant.

For long-lasting control strategies and successful resistance management of bed bug infestations, especially in huge apartment buildings, it is necessary to provide evidence of pyrethroid resistance in field populations within a few days (Stanley, 2014). These and previous studies with other insects (Zhu et al., 2013; Knolhoff and Onstad, 2014) demonstrated that pyrosequencing and RT-qPCR are appropriate qualitative and quantitative methods for monitoring the occurrence of resistance mechanisms in bed bug field populations. With the help of such molecular methods, it is conceivable that bed bugs suspected to be resistant by pest controllers could be examined for resistance mechanisms in the laboratory in a short period of time. Based on the results of such monitoring studies, control options should be adjusted, e.g., by rotating insecticides with different active ingredients or even insecticide free methods. For the pyrethroids, pyrosequencing assays even provide the option to conduct a molecular diagnosis of a resistance genotype to predict if this pesticide class is still likely to work.

Several of the field-collected bed bugs could not be successfully reared under laboratory conditions. A possible reason could be the presence of fitness disadvantages due to insecticide resistance, as already shown in bed bugs and other insects (Roush and Plapp, 1982; Carriere et al., 1994; Roy et al., 2010; Brito et al., 2013; Diniz et al., 2014; Gordon et al., 2015). In contrast, Polanco et al. (2011) even reported fitness advantages in resistant bed bug strains. However, the examination of the allele distribution concerning the presence of a Hardy-Weinberg equilibrium of L925I in the LB, SK, OB, and AS field strain showed that the resistance-associated genotype had no significant adverse effect on the frequency of individuals carrying a homozygous resistant genotype or frequency of heterozygous bugs. In particular, low levels of bed bugs homozygous for L925I would have been expected to be observed if this genotype would be associated with fitness costs in the absence of selection by pyrethroids. However, due to the small number of tested bugs, the power of the tests was low, and thus the effects of genotypes should not be excluded using the present data. Also, other cytochrome P450 enzymes or other xenobiotic metabolizing enzymes such as esterases or glutathione S-transferases, other mutations in the *Clvssc* gene, e.g., I936F (Dang et al., 2014b; Palenchar et al., 2015) and penetration resistance can have an influence on the susceptibility or the fitness of the bed bug field strains examined here. Further studies are needed to examine other pyrethroid resistance mechanisms and the contribution of each in pyrethroid resistance in the collected field strains.

In conclusion, our study indicates for the first time that multiple resistance mechanisms are present in “German” *C. lectularius* populations simultaneously. Pyrethroid resistance in bed bugs collected in Berlin is based on the L925I *kdr* genotype and upregulation of various cytochrome P450 enzymes. It is likely that additional mechanisms not investigated here might have an influence on the susceptibility of “German” bed bugs to pyrethroids. Like in other countries, these results may explain why German pest control companies increasingly report about problems or failures regarding the control of bed bug infestations using only pyrethroids. To prevent the development and further spread of resistant bed bugs, integrated control approaches, taking advantage of rapid (molecular) resistance diagnosis is necessary. The study gives the first detailed description of the pyrosequencing method that can be used in the future to screen for pyrethroid resistant bed bug populations. Pyrosequencing assays provide the option to quantify *kdr* genotypes in bed bug populations to rapidly identify pyrethroid resistant genotypes. Using this method, the establishment of a breeding colony is not necessary, and the actual resistance status can be determined directly after bed bug collection.

Acknowledgments

The authors are grateful to pest controllers and bed bug dog detection teams for helping to identify collection sites. We thank Sabrina Ramünke for the technical support concerning pyrosequencing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2020.11.003.

Funding

This work was supported by the German Environment Agency.

Declarations of interest

None.

References

Aak, A., Rukke, B.A., Soleng, A., Rosnes, M.K., 2014. Questing activity in bed bug populations: male and female responses to host signals. Physiol. Entomol. 39, 199–207.

Abd-Elghafar, S.F., Appel, A.G., Mack, T.P., 1990. Toxicity of several insecticide formulations against adult German cockroaches (dictyoptera: blattellidae). J. Econ. Entomol. 83, 2290–2294.

Adelman, Z.N., Kilcullen, K.A., Koganemaru, R., Anderson, M.A., Anderson, T.D., Miller, D.M., 2011. Deep sequencing of pyrethroid-resistant bed bugs reveals multiple mechanisms of resistance within a single population. PloS One 6, 1–9.

Bai, X., Mamidala, P., Rajarapu, S.P., Jones, S.C., Mittapalli, O., 2011. Transcriptomics of the bed bug (*Cimex lectularius*). PloS One 6, e16336.

Balvin, O., Booth, W., 2018. Distribution and frequency of pyrethroid resistance-associated mutations in host lineages of the bed bug (Hemiptera: cimicidae) across Europe. J. Med. Entomol. 55, 923–928.

Barrère, V., Keller, K., von Samson-Himmelstjerna, G., Prichard, R.K., 2013. Efficiency of a genetic test to detect benzimidazole resistant *Haemonchus contortus* nematodes in sheep farms in Quebec, Canada. Parasitol. Int. 62, 464–470.

Booth, W., Balvin, O., Vargo, E.L., Vilimova, J., Schal, C., 2015. Host association drives genetic divergence in the bed bug, *Cimex lectularius*. Mol. Ecol. 24, 980–992.

Brito, L.P., Linss, J.G., Lima-Camara, T.N., Belinato, T.A., Peixoto, A.A., Lima, J.B., Valle, D., Martins, A.J., 2013. Assessing the effects of *Aedes aegypti kdr* mutations on pyrethroid resistance and its fitness cost. PloS One 8, e60878.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.

Caceres, M., Santo-Orihuela, P.L., Vassena, C.V., 2019. Evaluation of resistance to different insecticides and metabolic detoxification mechanism by use of synergist in the common bed bug (heteroptera: cimicidae). J. Med. Entomol. 56, 1324–1330.

Cambronero-Heinrichs, J.C., Sanchez-Portilla, L.S., Calderon-Arguedas, O., Troyo, A., 2020. *Cimex lectularius* linnaeus, 1758 (Hemiptera: cimicidae) in Costa Rica: first case report confirmed by molecular methods in Central America. J. Med. Entomol. 57, 969–973.

Carriere, Y., Deland, J.-P., Roff, D.A., Vincent, C., 1994. Life-history costs associated with the evolution of insecticide resistance. Proc. Royal Soc. B 258, 35–40.

Dang, K., Doggett, S.L., Veera Singham, G., Lee, C.Y., 2017. Insecticide resistance and resistance mechanisms in bed bugs, *Cimex* spp. (Hemiptera: cimicidae). Parasites Vectors 10, 318.

Dang, K., Lilly, D.G., Bu, W., Doggett, S.L., 2014a. Simple, rapid and cost-effective technique for the detection of pyrethroid resistance in bed bugs,*Cimex* spp. (Hemiptera: cimicidae). Aust. Entomol. 54, 191–196.

Dang, K., Toi, C.S., Lilly, D.G., Bu, W., Doggett, S.L., 2014b. Detection of knockdown resistance mutations in the common bed bug, *Cimex lectularius* (Hemiptera: cimicidae), in Australia. Pest Manag. Sci. 71, 914–922.
Davies, T.G., Field, L.M., Williamson, M.S., 2012. The re-emergence of the bed bug as a nuisance pest: implications of resistance to the pyrethroid insecticides. Med. Vet. Entomol. 26, 241–254.
Davis, R.F., Johnston, G.A., Sladden, M.J., 2009. Recognition and management of common ectoparasitic diseases in travelers. Am. J. Clin. Dermatol. 10, 1–8.
Demeler, J., Krüger, N., Krücken, J., von der Heyden, V.C., Ramünke, S., Küttler, U., Miltsch, S., López Cepeda, M., Knox, M., Vercruysse, J., Geldhof, P., Harder, A., von Samson-Himmelstjerna, G., 2013. Phylogenetic characterization of β-tubulins and development of pyrosequencing assays for benzimidazole resistance in cattle nematodes. PloS One 8, e70212.
Diniz, M.M.C.d.S.L., Henriques, A.D.d.S., Leandro, R.d.S., Aguiar, D.L., Beserra, E.B., 2014. Resistance of *Aedes aegypti* to temephos and adaptive disadvantages. Rev. Saude Publica 48, 775–782.
Durand, R., Cannet, A., Berdjane, Z., Bruel, C., Haouchine, D., Delaunay, P., Izri, A., 2012. Infestation by pyrethroids resistant bed bugs in the suburb of Paris, France. Parasite 19, 381–387.
Engels, W.R., 2009. Exact tests for hardy–weinberg proportions. Genetics 183, 1431–1441.
Gonzalez-Morales, M.A., Romero, A., 2019. Effect of synergists on deltamethrin resistance in the common bed bug (Hemiptera: cimicidae). J. Econ. Entomol. 112, 786–791.
Gordon, J.R., Potter, M.F., Haynes, K.F., 2015. Insecticide resistance in the bed bug comes with a cost. Sci. Rep. 5, 10807.
Harlan, H.J., 2006. Bed bugs 101: the basics of *Cimex lectularius*. Am. Entomol. 52, 99–101.
Holleman, J.G., Robison, G.A., Bellovich, I.J., Booth, W., 2019. Knockdown resistance-associated mutations dominate populations of the common bed bug (Hemiptera: cimicidae) across the south Central United States. J. Med. Entomol. 56, 1678–1683.
Kilpinen, O., Kristensen, M., Vagn Jensen, K.-M., 2011. Resistance differences between chlorpyrifos and synthetic pyrethroids in *Cimex lectularius* population from Denmark. Parasitol. Res. 109, 1461–1464.
Knolhoff, L.M., Onstad, D.W., 2014. Resistance by ectoparasites. In: Onstad, D.W. (Ed.), Insect Resistance Management Biology, Economics, and Prediction, 2 ed. Academic Press, London, UK, pp. 185–231.
Kolb, A., Needham, G.R., Neyman, K.M., High, W.A., 2009. Bedbugs. Dermatology Ther 22, 347–352.
Le Goff, G., Hilliou, F., Siegfried, B.D., Boundy, S., Wajnberg, E., Sofer, L., Audant, P., ffrench-Constant, R.H., Feyereisen, R., 2006. Xenobiotic response in Drosophila melanogaster: sex dependence of P450 and GST gene induction. Insect Biochem. Mol. Biol. 36, 674–682.
Lilly, D.G., Doggett, S.L., Zalucki, M.P., Orton, C.J., Russel, R.C., 2009. Bed Bugs that Bite Back. Confirmation of Insecticide Resistance in Australia in the Common Bed Bug, *Cimex Lectularius*. Professional Pest Manager, pp. 22–24.
Mamidala, P., Jones, S.C., Mittapalli, O., 2011. Metabolic resistance in bed bugs. Insects 2, 36–48.
Mamidala, P., Wijeratne, A.J., Wijeratne, S., Kornacker, K., Sudhamalla, B., Rivera-Vega, L.J., Hoelmer, A., Meulia, T., Jones, S.C., Mittapalli, O., 2012. RNA-Seq and molecular docking reveal multi-level pesticide resistance in the bed bug. BMC Genom. 13, 6.
Masetti, M., Bruschi, F., 2007. Bedbug infestations recorded in Central Italy. Parasitol. Int. 56, 81–83.
Natsidis, P., Schiffer, P.H., Salvador-Martinez, I., Telford, M.J., 2019. Computational discovery of hidden breaks in 28S ribosomal RNAs across eukaryotes and consequences for RNA Integrity Numbers. Sci. Rep. 9, 19477.
Palenchar, D.J., Gellatly, K.J., Yoon, K.S., Mumcuoglu, K.Y., Shalom, U., Clark, J.M., 2015. Quantitative sequencing for the determination of kdr-type resistance allele (V419L, L925I, I936F) frequencies in common bed bug (Hemiptera: cimicidae) populations collected from Israel. J. Med. Entomol. 52, 1018–1027.
Pfiester, M., Koehler, P.G., Pereira, M.H., 2009. Effect of population structure and size on aggregation behavior of *Cimex lectularius* (Hemiptera: cimicidae). J. Med. Entomol. 46, 1015–1020.
Polanco, A.M., Brewster, C.C., Miller, D.M., 2011. Population growth potential of the bed bug, *Cimex lectularius* L.: a life table Analysis. Insects 2, 173–185.
Pruett, J.H., Kammlah, D.M., Guerrero, F.D., 2001. Variation in general esterase activity within a population of *Haematobia irritans* (Diptera: muscidae). J. Econ. Entomol. 94, 714–718.
Ramünke, S., Melville, L., Rinaldi, L., Hertzberg, H., de Waal, T., von Samson-Himmelstjerna, G., Cringoli, G., Mavrot, F., Skuce, P., Krücken, J., Demeler, J., 2016. Benzimidazole resistance survey for *Haemonchus, Teladorsagia* and *Trichostrongylus* in three European countries using pyrosequencing including the development of new assays for *Trichostrongylus*. Int J Parasitol Drugs Drug Resist 6, 230–240.
Romero, A., 2011. Moving from the old to the new: insecticide research on bed bugs since the resurgence. Insects 2, 210–217.
Romero, A., Potter, M.F., Potter, D.A., Haynes, K.F., 2007. Insecticide resistance in the bed bug: a factor in the pest's sudden resurgence? J. Med. Entomol. 44, 175–178.
Roush, R.T., Plapp, F.W.J., 1982. Effects of insecticide resistance on biotic potential of the house fly (Diptera: muscidae). J. Econ. Entomol. 75, 708–713.
Roy, S., Mukhopadhyay, A., Gurusubramanian, G., 2010. Fitness traits of insecticide resistant and susceptible strains of tea mosquito bug *Helopeltis theivora* Waterhouse (Heteroptera: Miridae). Entomol. Res. 40, 229–232.
Seong, K.M., Lee, D.-Y., Yoon, K.S., Kwon, D.H., Kim, H.C., Klein, T.A., Clark, J.M., Lee, S.H., 2010. Establishment of quantitative sequencing and filter contact vial bioassay for monitoring pyrethroid resistance in the common bed bug, *Cimex lectularius*. J. Med. Entomol. 47, 592–599.
Siljander, E., Gries, R., Khaskin, G., Gries, G., 2008. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. J. Chem. Ecol. 34, 708–718.
Skuce, P., Stenhouse, L., Jackson, F., Hypsa, V., Gilleard, J., 2010. Benzimidazole resistance allele haplotype diversity in United Kingdom isolates of *Teladorsagia circumcincta* supports a hypothesis of multiple origins of resistance by recurrent mutation. Int. J. Parasitol. 40, 1247–1255.
Stanley, B.H., 2014. Monitoring resistance. In: Onstad, D.W. (Ed.), Insect Resistance Management Biology, Economics, and Prediction, 2 ed. Academic Press, London, UK, pp. 485–513.
Ter Poorten, M.C., Prose, N.S., 2005. The return of the common bedbug. Pediatr. Dermatol. 22, 183–187.
Vander Pan, A., Schmolz, E., Krücken, J., Kuhn, C., 2019. A novel simulated-use test for determining the efficacy of insecticides against bed bugs (Hemiptera: cimicidae). J. Econ. Entomol. 112, 2345–2353.
von Samson-Himmelstjerna, G., Walsh, T.K., Donnan, A.A., Carriere, S., Jackson, F., Skuce, P.J., Rohn, K., Wolstenholme, A.J., 2009. Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing. Parasitology 136, 349–358.
Yoon, K.S., Kwon, D.H., Strycharz, J.P., Hollingsworth, C.S., Lee, S.H., Clark, J.M., 2008. Biochemical and molecular analysis of deltamethrin resistance in the common bed bug (Hemiptera: cimicidae). J. Med. Entomol. 45, 1092–1101.
Zhu, F., Gujar, H., Gordon, J.R., Haynes, K.F., Potter, M.F., Palli, S.R., 2013. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. Sci. Rep. 3, 1456.
Zhu, F., Sams, S., Moural, T., Haynes, K.F., Potter, M.F., Palli, S.R., 2012. RNA interference of NADPH-cytochrome P450 reductase results in reduced insecticide resistance in the bed bug, *Cimex lectularius*. PloS One 7, e31037.
Zhu, F., Wigginton, J., Romero, A., Moore, A., Ferguson, K., Palli, R., Potter, M.F., Haynes, K.F., Palli, S.R., 2010. Widespread distribution of knockdown resistance mutations in the bed bug, *Cimex lectularius* (Hemiptera: cimicidae), populations in the United States. Arch. Insect Biochem. Physiol. 73, 245–257.