Biology of *Pentastiridius leporinus* and approaches to control the main vector of the syndrome ‘basses richesses’ in sugar beet
Biology of *Pentastiridius leporinus* and approaches to control the main vector of the syndrome ‘basses richesses’ in sugar beet

Dissertation

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List of abbreviations

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List of abbreviations

A  Alanine
AAP  Acquisition access period
AIC  Akaike information criterion
ANOVA  Analysis of variance
ATP  Adenosine triphosphate
BBCH  Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und chemische Industrie
BLO  Bacterium-like organism
BN  ‘Bois noir’
BOLD  Barcode of Life Datasystem
bp  Base pairs
C  Cytosine
C. arvensis  Convolvulus arvensis
C. placida  Cicadula placida
C. riparius  Chironomus riparius
C. roseus  Catharanthus roseus
C. wagneri  Cixius wagneri
Ca.  Candidatus
COI  Cytochrome oxidase I gene
conv.  Convariety
D. citri  Diaphorina citri
D. europaea  Dictyophara europaea
D. idonea  Delphacodes idonea
DAPI  4′,6-diamidino-2-phenylindole
DLB  Dyella-like bacterium
dmPCR  Direct multiplex PCR
DNA  Deoxyribonucleic acid
dsDNA  Double-stranded DNA
E. affinis  Empoasca affinis
E. decipiens  Empoasca decipiens
E. pteridis  Empoasca pteridis
EDTA  Ethylenediaminetetraacetic acid
F. florii  Fieberiella florii
FDP  ‘Flavescence dorée phytoplasma’
List of abbreviations

FISH          Fluorescence in situ hybridization
fw            Forward
G             Guanine
H. crudus     Haplaxius crudus
H. luteipes   Hyalesthes luteipes
H. obsoletus  Hyalesthes obsoletus
H. scotti     Hyalesthes scotti
H. sellatiformis Hishimonoides sellatiformis
HR            Hazard ratio
hsp20         Heat shock protein 20
IAP           Inoculation access period
ITS           Internal transcribed spacer
J. obscurella Javesella obscurella
J. pellucida  Javesella pellucida
L. angustifolia Lavandula angustifolia
L. cervi      Lipoptena cervi
LAMP          Loop-mediated isothermal amplification
LP            Latency period
M. hiroglyphicus Matsumuratettix hiroglyphicus
manA          Mannose 6-phosphate isomerase
MLO           Mycoplasma-like organism
MLSA          Multilocus sequence analyses
MR            Maize redness
N. campestris Neophilaenus campestris
N. lugens     Nilaparvata lugens
O. ishidae    Orientus ishidae
O. polyphemus Oliarus polyphemus
P. alienus    Psammotettix alienus
P. australis  Phragmites australis
P. beieri     Pentastiridius beieri
P. leporinus  Pentastiridius leporinus
P. maximum    Panicum maximum
PBS           Phosphate-buffered saline
PCR           Polymerase chain reaction
qPCR          Quantitative PCR
R. cuspidatus Reptalus cuspidatus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>R. melanochaetus</td>
<td>Reptalus melanochaetus</td>
</tr>
<tr>
<td>R. panzeri</td>
<td>Reptalus panzeri</td>
</tr>
<tr>
<td>R. quinquecostatus</td>
<td>Reptalus quinquecostatus</td>
</tr>
<tr>
<td>R. sativus</td>
<td>Raphanus sativus</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RISA</td>
<td>rDNA intergenic spacer analysis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>rp</td>
<td>Ribosomal protein</td>
</tr>
<tr>
<td>RPA</td>
<td>Recombinase polymerase amplification</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RTD</td>
<td>Rubbery taproot disease</td>
</tr>
<tr>
<td>rv</td>
<td>Reverse</td>
</tr>
<tr>
<td>S. alba</td>
<td>Sinapis alba</td>
</tr>
<tr>
<td>S. aureofaciens</td>
<td>Streptomyces aureofaciens</td>
</tr>
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<td>S. bisonia</td>
<td>Stictocephala bisonia</td>
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<td>Sorghum halepense</td>
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<tr>
<td>S. kolophon</td>
<td>Sogatella kolophon</td>
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<td>Spiroplasma melliferum</td>
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<td>Salvia sclarea</td>
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<tr>
<td>S. tithonius</td>
<td>Scaphoideus titanus</td>
</tr>
<tr>
<td>S. tuberosum</td>
<td>Solanum tuberosum</td>
</tr>
<tr>
<td>SBR</td>
<td>Syndrome ‘basses richesses’</td>
</tr>
<tr>
<td>SMC</td>
<td>Strawberry marginal chlorosis</td>
</tr>
<tr>
<td>sp.</td>
<td>Species</td>
</tr>
<tr>
<td>ssp.</td>
<td>Subspecies</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>T. absoluta</td>
<td>Tuta absoluta</td>
</tr>
<tr>
<td>T. confusum</td>
<td>Tribolium confusum</td>
</tr>
<tr>
<td>T. japonicus</td>
<td>Trissolcus japonicus</td>
</tr>
<tr>
<td>TDTA</td>
<td>(3E,8Z,11Z)-3,8,11-tetradecatrienyl acetate</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>U. dioica</td>
<td>Urtica dioica</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>V. agnus-castus</td>
<td>Vitex agnus-castus</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume percent</td>
</tr>
<tr>
<td>var.</td>
<td>Variety</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile organic compounds</td>
</tr>
<tr>
<td>wg</td>
<td>Wingless gene</td>
</tr>
</tbody>
</table>
1. Introduction

Planthoppers in the family Cixiidae are well known vectors for several plant diseases caused by phloem-limited bacteria (e.g. Jović et al. 2009; Jović et al. 2019; Šafářová et al. 2018). Cixiids can adapt to new plant species, thus they can transmit plant pathogens to new hosts (e.g. Salar et al. 2010a; Sémétey et al. 2018). In sugar beet (Beta vulgaris) Cixiidae together with Delphacidae and Cicadellidae are members of Auchenorrhyncha (Pfitzer et al. 2020; Sémétey et al. 2007a). In Cixiidae, Pentastiridius leporinus, Hyalesthes obsoletus, and Cixius wagneri (Hemiptera: Cixiidae) are vectors for the syndrome ‘basses richesses’ (SBR) which is a fast spreading sugar beet disease in Central Europe that can seriously threaten economy of sugar beet production (Behrmann et al. 2021; Bressan et al. 2008; Gatineau et al. 2002; Mahillon et al. 2022; Peter 2020, 2022; Sémétey et al. 2007b). SBR disease is caused by two phloem-limited bacterial pathogens i) ‘Candidatus Arsenophonus phytopathogenicus’ (here called: SBR-proteobacterium) and ii) ‘Candidatus Phytoplasma solani’ (here called: SBR-phytoplasma) (Bressan et al. 2012; Firrao et al. 2005; Gatineau et al. 2002). Both pathogens are transmitted by cixiid planthoppers and the main economic vector, P. leporinus, is a less studied species (Bressan et al. 2008; Pfitzer et al. 2020; Sémétey et al. 2007a). P. leporinus adapted from its natural host plants (e.g. Phragmites australis) to sugar beet crop rotations what is probably the reason for the spread of SBR disease (Bressan et al. 2009a; Bressan 2009, 2014).

SBR is an important emerging disease in sugar beet which can lead to strong sugar content and yield losses (Bressan et al. 2008; Sémétey et al. 2007b). Therefore, the development of control strategies is urgently needed. Investigating the life cycle, molecular identification, and the establishment of a permanent rearing of P. leporinus, are essential to understand the interaction of vector and pathogens aiming to control the vector and disease. Furthermore, analysis of agricultural measures and their potential to reduce the emergence of adult P. leporinus by interrupting the life cycle of this planthopper are important to develop sustainable approaches for vector control.

1.1 Syndrome ‘basses richesses’ of sugar beet

1.1.1 History and geographical distribution

The first observation of SBR symptoms occurred in the French region Burgundy in 1991, which led to large income losses of 50% over ca. 1,000 ha sugar beet cultivation area in 1992 (Gatineau et al. 2002; Richard-Molard et al. 1995). In the following years, the severity of SBR symptoms fluctuated and already 1,800 ha were affected in France (regions Burgundy and Jura) in 2004 (Sémétey et al. 2007b). In Germany, SBR symptoms were
observed for the first time in 2008 on less than 150 ha and the first detection of SBR-proteobacterium in sugar beet was reported in 2009 (Schröder et al. 2012). Similar to France, SBR appeared in fluctuating severity in Germany and already ca. 16,400 ha were affected in 2018 (Pfitzer et al. 2020; Sémétey et al. 2007b). Two major infected areas are Southwest Germany and in the valley of the Elbe river (federal states Brandenburg Saxony, and Saxony-Anhalt) (Behrmann et al. 2021; Pfitzer et al. 2020). West Switzerland is another heavily SBR infested area, where the disease was firstly observed in 2017 and affected ca. 3,000 and 5,000 ha in 2019 and 2021, respectively (Mahillon et al. 2022; Peter 2020, 2022). Peter (2022) suggested that SBR disease yearly propagates ca. 15 km. Thus, SBR disease is fast spreading and threatening sugar beet cultivation in Central Europe.

1.1.2 Symptoms and yield decline

![Figure 1](image)

**Figure 1** Comparison of field grown SBR free (A, C) sugar beets and plants showing typical SBR symptoms on the leaves such as chlorotic or necrotic older leaves and asymmetric younger leaves (B) and necrosis in the vascular bundles of the taproots (D) in Baden-Württemberg.

Gatineau et al. (2002) described SBR symptoms in sugar beet in detail, which can be observed in late summer. SBR-proteobacterium and SBR-phytoplasma infections can lead to cell wall lignification and to cell necrosis and infections with SBR-proteobacterium can also
lead to deposition of phenols in the phloem cells’ lumen (Gatineau et al. 2002). Thus, the vascular bundles of the taproots display necrosis. Further, the older leaves are chlorotic or necrotic and the younger leaves are asymmetric (Figure 1) (Gatineau et al. 2002). Therefore, sugar beet fields can show large-scale yellowing (Figure 2) (Gatineau et al. 2002).

**Figure 2** Large scale yellowing of a SBR affected sugar beet field in Baden-Württemberg (Germany) in 2020.

SBR infections can cause up to 5 % reductions in absolute sugar content (Bressan et al. 2008; Sémétey et al. 2007b). Bressan et al. (2008) artificially inoculated potted sugar beets (plant age: 35-50 days old) with SBR-proteobacterium using 4-5 field-collected *P. leporinus* adults per sugar beet plant and *Ca. Phytoplasma solani* using 8-10 *H. obsoletus* adults. Generally, the plants’ reaction to SBR infection was comparable between both pathogens (after five months incubation time at 26 ± 5 °C). According to Bressan et al. (2008), both SBR pathogens can lead to a strong reduction of total plant biomass, however, the phytoplasma has a higher reduction effect on the taproot mass and can lead to a stronger leaf proliferation compared to SBR-proteobacterium under controlled conditions. Thus, taproot fresh mass of infected sugar beets can be reduced more than 25 % (Bressan et al. 2008). Mixed infections with both SBR pathogens can also occur in sugar beets (see Introduction section 1.1.6). Therefore, SBR disease can result in significant losses of income for farmers and industries (Gatineau et al. 2002).
1. Introduction

1.1.3 SBR pathogens: Taxonomy and transmission

Phloem-limited phytopathogens such as bacteria and viruses are well known to induce severe plant diseases that can significantly harm plants and play a growing and important role for various crops (reviewed in Bendix and Lewis 2018; reviewed in Gross et al. 2022). The phloem contains high sugar and nutrient concentrations, thus it is a suitable niche for numerous plant pathogens (reviewed in Bendix and Lewis 2018; reviewed in Gross et al. 2022). Both SBR-proteobacterium and SBR-phytoplasma are phloem-limited pathogens that cannot be cultivated in vitro and are exclusively transmitted by insect vectors (Sémétéy et al. 2007b). Generally, most phloem-limited bacteria cannot be cultivated in vitro, leading to the fact that the postulates of Koch were not fulfilled, so they were named ‘Candidatus’ (reviewed in Bendix and Lewis 2018; reviewed in Bressan 2014; reviewed in Gross et al. 2022). Bové and Garnier (2002) assumed that these bacteria cannot be cultivated in vitro because some nutrients in the phloem remained unknown which are crucial components for the bacterial development. However, Ha et al. (2019) recently established and maintained a host-free biofilm culture of ‘Ca. Liberibacter asiaticus’ (α-proteobacteria) for more than two years. In contrast, the xylem is less nutrient-rich than the phloem; thus, in vitro cultivation of most xylem-limited bacteria succeeded (reviewed in Bové and Garnier 2002).

The phloem-limited intracellular bacteria can be separated into walled and wall-less bacteria (reviewed in Bendix and Lewis 2018; reviewed in Gross et al. 2022; reviewed in Musetti and Pagliari 2019). Within wall-less bacteria (class: Mollicutes), two groups are known: Spiroplasmas (e.g. Spiroplasma kunkelli, Spiroplasma citri) and phytoplasmas. Numerous phytoplasmas, e.g. ‘Ca. Phytoplasma asteris’ (subgroup 16SrI-A), ‘Ca. Phytoplasma solani’ (stolbur subgroup 16SrXII-A), or ‘Ca. Phytoplasma mali’ (16SrX-A) can cause more than thousand different plant diseases (reviewed in Bendix and Lewis 2018; reviewed in Musetti and Pagliari 2019). More, according to their phylogeny, phytoplasmas can be categorized into several classes (16SrI to XXXIII) which consist of different subclasses according to the sequences of 16S ribosomal RNA (rRNA) gene (reviewed in Gonella et al. 2019). Examples for gram-negative walled bacteria are α-proteobacteria such as Ca. Liberibacter asiaticus, ‘Ca. Liberibacter africanus’, and ‘Ca. Liberibacter americanus’ that are causal agents of Huanglongbing or Citrus Greening disease, and ‘Ca. Liberibacter solanacearum’ that is the causal agent of Zebra chip disease in Solanaceae, respectively, and γ-proteobacteria, e.g. ‘Ca. Phlomobacter fragariae’ that is the causal agent of strawberry marginal chlorosis (SMC) disease and SBR-proteobacterium (reviewed in Bendix and Lewis 2018; reviewed in Gross et al. 2022). ‘Ca. Liberibacter’ is the sole genus within α-proteobacteria (Gross et al. 2022).
1. Introduction

Different routes are known to mediate transmission of phytopathogenic bacteria to the phloem tissue of plants, i) agronomical methods (e.g. grafting of diseased plants), ii) vascular transmission by parasitic plant species from infected to healthy plants, iii) transmission by seeds, and iv) transmission by insect vectors which is the most important pathway (reviewed in Gross et al. 2022). Thus, this study focuses on an insect vector transmitted bacterial disease. Generally, the transmission cycle of bacteria (here: Phytoplasma) by vector insects comprises three steps. Firstly, insects passively uptake the pathogen with their mouth parts and infect themselves while exploiting phloem sap (reviewed in Alma et al. 2019; reviewed in Weintraub and Beanland 2006). The acquisition access period (AAP) of bacteria can range from less than one hour to several days and longer AAPs are connected to a higher probability that the insect acquires the bacterial pathogen (Purcell 1982). Secondly, the pathogens multiply and propagate within the insects body (e.g. salivary glands) during the latency period (LP) that can range from a few days to more than two months, before planthoppers can transmit it to plants (reviewed in Alma et al. 1997; reviewed in Weintraub and Beanland 2006). Thirdly, the infective insect vectors transmit the pathogen to the host plants, what is defined as inoculation access period (IAP). Vector insects transmit phytoplasmas to plants in a persistent-propagative mode, thus, AAPs can be short (a couple of days), LPs need to be longer (weeks), and IAPs require a medium-short amount of time (reviewed in Alma et al. 2019).

Several transmission experiments under controlled climatic conditions have shown that *P. leporinus* can transmit SBR-phytoplasma or SBR-proteobacterium to sugar beet and periwinkle (*Catharanthus roseus*) (Gatineau et al. 2001; Mahillon et al. 2022). However, transmission efficiency of SBR-phytoplasma to sugar beet was rather low for *P. leporinus* (18.3 %, sugar beet plants were inoculated with each 10–50 specimens) (Gatineau et al. 2001), compared to transmission efficiency of *H. obsOLEtUS* (68.4 %) (see Introduction section 1.1.2) (Bressan et al. 2008). According to Sémétey et al. (2007b), field collected adult *P. leporinus* can transmit SBR-proteobacterium within a 24 h IAP and induce SBR symptoms as well as remarkable sugar content losses. However, stronger SBR symptoms and sugar content losses (5 % maximum reduction of absolute sugar content) were observed from single adults after longer IAPs (16 days maximum) or after inoculation of sugar beets with higher inoculation densities (up to 20 *P. leporinus* per sugar beet with a 24 h IAP) (Sémétey et al. 2007b). Additionally, the ability to persistently (up to 33 days after field collection of *P. leporinus* adults) transmit SBR-proteobacterium to sugar beets was shown under controlled climatic conditions (Bressan et al. 2009b). In these experiments, *P. leporinus* females displayed a higher transmission efficiency (64.7 %, n=51) of SBR-proteobacterium to sugar beets and a higher longevity (maximum 33 days) compared to males (24.1 %, n=29;
12 days maximum longevity) (Bressan et al. 2009b). Generally, adults are the important vectors of bacterial diseases and they are more mobile than nymphs due to their ability to fly (reviewed in Alma et al. 2019). However, in addition to adults, *P. leporinus* nymphs can also transmit SBR-proteobacterium to sugar beets (Bressan et al. 2009b).

### 1.1.4 Plant-pathogen interactions

#### 1.1.4.1 SBR-proteobacterium: The bacterial clade *Arsenophonus*

In general, little information is available on the interaction between γ-proteobacteria and plants (reviewed in Gross et al. 2022). The size of the SBR-proteobacterium is 2.0–2.5 μm in length and 0.28–0.32 μm in width (Gatineau et al. 2002). In sugar beets, the phloem can be systemically colonized by SBR-proteobacterium (reviewed in Gross et al. 2022). Thus, Mahillon et al. (2022) found high titers of SBR-proteobacterium in rootlets, taproots, and petioles from old leaves. Generally, plants can deposit callose into sieve plates and companion cell plasmodesmata as a response to damages (wounds) and infection with pathogens such as SBR-proteobacterium, to interrupt the pathogen spread within the plant (reviewed in Bendix and Lewis 2018; reviewed in Gross et al. 2022). However, the function of the phloem can be negatively affected, thus, sugar contents can be reduced in sugar beets when infected with SBR-proteobacterium (see Introduction section 1.1.2) (reviewed in Gross et al. 2022).

SBR-proteobacterium is phylogenetically closely related to other bacterial endosymbionts of insects from the order Hemiptera such as *Ca. Phlomobacter fragariae* (Salar et al. 2010b; Sémétey et al. 2007c) that was originally identified as bacterium-like organism (BLO) (Nourrisseau et al. 1993). Later, this BLO was classified into γ-3 proteobacterium group (Zreik et al. 1998). Bressan (2014) proposed to classify *Phlomobacter* bacteria into *Arsenophonus* group.

Genome analysis of insect endosymbionts such as analysis of genome size, GC content, and phylogenetic analysis can provide information about their interaction with hosts (reviewed in Fan et al. 2016). In general, numerous plant-pathogenic bacteria that are limited to the plants’ phloem have smaller genomes, because they miss numerous genes that are necessary for metabolic mechanisms (reviewed in Bendix and Lewis 2018). Instead, these bacteria frequently have mechanisms which support acquisition of components that were produced by their plant host (reviewed in Bendix and Lewis 2018).

It is assumed that phloem-limited α-proteobacteria manipulate the environment in the plants to promote their own development, by changing plant metabolite contents (reviewed in Gross et al. 2022). Thus, α-proteobacteria can modify the contents of nutrients or primary plant
metabolites in different plant parts (tubers, stems, or leaves) (reviewed in Gross et al. 2022). Further, plant starch, sugar and carbohydrate levels, as well as non-volatile secondary metabolites (e.g. phenols) can be changed due to infections with Ca. Liberibacter (reviewed in Gross et al. 2022). Finally, infections with Ca. Liberibacter asiaticus can modify the source-sink relationship in citrus plants, thus, starch contents were increased in leaf material (source) and lower in the roots (sink) (reviewed in Gross et al. 2022). Furthermore, plant resistance against α-proteobacteria is also influenced by the composition of plant metabolites (reviewed in Gross et al. 2022). For example, resistance against Ca. Liberibacter asiaticus was associated with high phenol and flavonoid contents in potato (Solanum tuberosum) plants (reviewed in Gross et al. 2022).

1.1.4.2 SBR-phytoplasma

Phytoplasmas were initially named mycoplasma-like organisms (MLOs), they are gram-positive procaryotes in plants (Garnier et al. 2001; reviewed in Orlovskis et al. 2015; reviewed in Weintraub and Beanland 2006) and their mean size is 400 nm (80-900 nm) (reviewed in Marcone 2014). Generally, phytoplasmas such as SBR-phytoplasma are insect parasites and obligate plant parasites (Bai et al. 2004; reviewed in Kumari et al. 2019; Oshima et al. 2011) that are hard to control (Garnier et al. 2001). Further, phytoplasma infections can modify composition (e.g. nutrients, amino acids, carbohydrates, sugars) of different plant parts including leaves, stems, or tubers (reviewed in Gross et al. 2022). Thus, it was suggested that the transport pathways within the plants can be interrupted and starch or carbohydrate contents can be increased in leaves (source) and reduced in roots (sink) tissues (reviewed in Gross et al. 2022). The effect on the concentrations of plant metabolites can vary in different plant species or genotypes (reviewed in Gross et al. 2022). This can be also affected by the insect vector attacks or additional biotic stresses (reviewed in Gross et al. 2022). It was suggested that the contents of non-volatile secondary metabolites such as phenolic compounds and flavonoids may change during plant defense response to phytoplasma infections (reviewed in Gross et al. 2022).

In mycoplasmas (class: Mollicutes) and phytoplasmas, genome analysis can also help to better understand their underlying pathogenesis mechanisms (reviewed in Namba 2019). For example, it was shown that these bacteria have small genomes and lack significant parts of their genome (metabolic genes) (reviewed in Namba 2019). One explanation for this phenomenon is that symbiotic or parasitic bacteria live in habitats with a high nutrient level provided by their hosts, thus, many genes are not needed (reviewed in Namba 2019). More, mycoplasmas miss the genome parts that are necessary for transport of electrons or oxidative phosphorylation, tricarboxylic acid cycle, and biosynthesis of fatty and amino acids.
Introduction

In addition, phytoplasmas miss vitally important genes for adenosine triphosphate (ATP) synthase, transport system of phosphotransferase, and the pathway of pentose phosphate (reviewed in Marcone 2014; reviewed in Namba 2019). Additionally, phytoplasmas are not capable to synthesize nutrients or life-essential components (reviewed in Marcone 2014; reviewed in Namba 2019). Instead, they acquire nutrients and metabolic substances from cells of their host (plants or insects) (reviewed in Marcone 2014; reviewed in Namba 2019). The host ranges of phytoplasmas are generally limited for insect hosts but can be large for plant hosts (reviewed in Marcone 2014; reviewed in Namba 2019). The molecular mechanisms of phytoplasma pathogenicity and symptom development has been reviewed (reviewed in Namba 2019). For example, conserved effector proteins for phytoplasmas such as 'TENGU' can induce witches' boom symptoms. Within the infected plants, this effector protein can cause plant symptoms by hindering auxin signalling after being divided into a peptide and moved to the apical meristem of the plant shoot. Phytoplasma infections can also lead to phyllody in plants which is a consequence of floral organ manipulation. Witches' boom and phyllody symptoms promote proliferation of young and small leaves that are more attractive for insects and these modified leaves stay green longer compared to flowers that decline after a short time. This contributes to the higher attractivity for insects. Therefore, these effects of phytoplasmas led to maintain their viability and further spread by their insect vectors (reviewed in Namba 2019). Similarly, Bressan et al. (2008) also observed stronger proliferation of young and small leaves in sugar beets artificially inoculated with Ca. Phytoplasma solani.

Besides SBR, Rubbery taproot disease (RTD) is another sugar beet disease that is caused by phytoplasma (Ćurčić et al. 2021a; Ćurčić et al. 2021b). This disease was firstly observed in Serbia in the 1960s. In the following time, RTD appeared in lower severity. Recently, another outbreak was observed in 2018 (Ćurčić et al. 2021a; Ćurčić et al. 2021b). Recent studies showed that Ca. Phytoplasma solani is the exclusive causal agent and according to transmission experiments H. obsoletus, Reptalus quinquecostatus, and Reptalus cuspidatus (all Hemiptera: Cixiidae) are the vectors of RTD disease (Ćurčić et al. 2021a; Ćurčić et al. 2021b; Duduk et al. 2023; Kosovac et al. 2023). RTD symptoms include wilting, chlorosis, and necrosis of the leaves and are firstly observed in late July. In some cases, all leaves can be necrotic what leads to plant death and rotting of the taproots. In contrast to SBR, SBR-proteobacterium is not involved and the taproots of RTD diseased sugar beets have no visible symptoms compared to healthy plants, however, they turn rubbery and are not sliceable anymore (Ćurčić et al. 2021a; Ćurčić et al. 2021b). In the sugar factory, sugar beets are sliced to increase recovery of sugar. Further, heated water is added to the slices to extract the sugar according to diffusion processes (reviewed in Draycott 2006). Thus,
infected plants do not fulfill the quality demands and are unusable for sugar production. Sugar beet varieties differ in susceptibility or prevalence of RTD, respectively (Čurčić et al. 2021a; Ćurčić et al. 2021b).

1.5 Insect vector-pathogen interactions

1.5.1 SBR-proteobacterium: The bacterial clade *Arsenophonus*

Generally, transovarially transmitted endosymbionts such as *Arsenophonus* bacteria can be divided into i) obligate or primary symbionts, ii) facultative or secondary symbionts, iii) parasites, and iv) phytopathogens transmitted by vectors (reviewed in Bressan 2014). More, obligate symbionts can provide nutrients for their insect hosts (reviewed in Bressan 2014) and facultative symbionts can protect insects from enemies such as entomopathogens or parasitic insects (Łukasik et al. 2013; Oliver et al. 2003) or improve heat-stress tolerance of insects (Montllor et al. 2002). Indeed, *P. leporinus* and *C. wagneri* host and transmit SBR-proteobacterium and *Ca. Phlomobacter fragariae* obligatorily to plants (reviewed in Bressan 2014). Further, walled bacteria in the clade *Arsenophonus* developed from endosymbionts of their insect vectors to plant pathogens and can change their insect host’s life history (reviewed in Bendix and Lewis 2018; Bressan et al. 2012). Indeed, most of the development of SBR-proteobacterium takes place in the planthopper (year round along the complete life cycle) and it remains unclear how SBR-proteobacterium affects *P. leporinus* (Bressan 2014; Bressan et al. 2009b). SBR-proteobacterium is localized in the ovaries, gonads, fat tissue, guts, and salivary glands of *P. leporinus* (Bressan et al. 2009b; Bressan et al. 2012). More specifically, the highest titers were shown in male and female gonads and female salivary glands (Bressan et al. 2009b). However, the titers of SBR-proteobacterium in the salivary glands were significantly lower in males compared to females. This could be one of the reasons for the lower transmission efficiency by males (Bressan et al. 2009b). Bressan et al. (2009b) further reported that infected female *P. leporinus* can vertically transmit SBR-proteobacterium to their offspring (30 % transmission rate under controlled climatic conditions). Thus, SBR-proteobacterium has the potential to be retained in the insect vector without being transmitted to sugar beets (Bressan et al. 2009b). According to Bressan et al. (2009b), the infection rates of field-collected nymphs increased over the sampling time or developmental stages (57 % in second to third instar nymphs collected in autumn and nearly 100 % in fourth to fifth instar nymphs collected in spring), respectively. Furthermore, the infection rates of late instar nymphs and emerging adults were comparable (Bressan et al. 2009b). However, due to the low vertical transmission efficiency, it was concluded that horizontal transmission plays a primary role in the spread of SBR disease and that SBR-proteobacterium could not survive for a long-term (several generations) exclusively in *P. leporinus* (Bressan 2014). In the study of Salar et al. (2010a), all developmental stages of...
C. wagneri carried Ca. Phlomobacter fragariae. The detection rates in third to fifth instar nymphs (70–75 %) were higher than in adults (19 %).

In contrast to α-proteobacteria, little information is available for γ-proteobacteria regarding chemical processes in insect-pathogen interactions (reviewed in Gross et al. 2022). Infection with α-proteobacteria or feeding on infected plants showed positive, negative, or neutral effects on the life history traits of insect vectors (reviewed in Gross et al. 2022). Diaphorina citri (Hemiptera: Livididae) were more fecund and population growth rates were increased after feeding on Ca. Liberibacter asiaticus infected plants, however, Bactericera cockerelli (Hemiptera: Triozidae) were less fecund and had a higher mortality following feeding on plants that were infected with Ca. Liberibacter solanacearum (reviewed in Gross et al. 2022). D. citri nymphal survival was negatively, but development time and egg number were positively affected after feeding on Ca. Liberibacter asiaticus infected citrus plants what led to increased population growth rates and it was suggested that the host plant infection had a higher impact on D. citri life history than the infection with the bacterium (reviewed in Gross et al. 2022). Finally, in addition to the interaction between plant pathogenic bacteria with host plants or insect vectors, they interact with other bacteria. This interaction can be positive, harmful, or competitive for bacteria (reviewed in Gonella et al. 2019).

1.1.5.2 SBR-phytoplasma

The phloem composition can differ between insect resistant and insect susceptible cultivars and affect insect vector fitness, feeding behaviour, and mortality (reviewed in Gross et al. 2022). Further, phytoplasmas can have positive, neutral, or negative effects on health, survival, and morphometric parameters of their insect vectors (reviewed in Gross et al. 2022; reviewed in Krüger and Fiore 2019). It is generally proposed that the positive effects on insect vectors promote transmission and spread of plant pathogens (reviewed in Gross et al. 2022). Macrosteles quadrilineatus (Hemiptera: Cicadellidae) had a higher fecundity (increased egg deposition and nymph production) on phytoplasma infected Arabidopsis thaliana (Sugio et al. 2011), and females had a higher longevity and increased nymph production on phytoplasma infected aster plants compared to non-infected plants (Beanland et al. 2000). However, ‘Flavescence dorée phytoplasma’ (FDP) exposed Scaphoideus titanus (Hemiptera: Cicadellidae) were less fecund, thus, the number of hatching nymphs was reduced and eggs from infected females hatched later (Bressan et al. 2005). In contrast, phytoplasma infection had no negative effect on fertility and longevity of Paraphlepsius irratus (Hemiptera: Cicadellidae) (Chiykowski 1991).

In contrast to Arsenophonus bacteria, only few studies suggested transovarial transmission of phytoplasmas in Auchenorrhyncha or psyllids. Alma et al. (1997) detected phytoplasma in
different life stages of *S. titanus* including eggs, newly hatched first instar nymphs, older nymphal instars, and adults that were kept on healthy *Vicia faba* plants. Thus, the authors assumed that transovarial phytoplasma transmission could be possible, however, further studies are required to prove this assumption and to quantify vertical transmission rates (Alma et al. 1997). Kawakita et al. (2000) found mulberry dwarf phytoplasmas (16SrI-B, aster yellows group) in several tissues from *Hishimonoides sellatiformis* (Hemiptera: Cicadellidae), e.g. genitals (ovaries, seminal receptacles, testes), salivary glands, brain or intestines using electron microscopy and polymerase chain reaction (PCR). Thus, Kawakita et al. (2000) suggested vertical phytoplasma transmission in *H. sellatiformis*. Furthermore, Hanboonsong et al. (2002) detected the phytoplasma that causes sugarcane white leaf disease by PCR in all life stages (eggs, nymphs and adults) from two consecutive generations of *Matsumuratettix hiroglyphicus* (Hemiptera: Cicadellidae). Additionally, in situ PCR confirmed the presence of the phytoplasma in various tissues, e.g. reproductive organs of females, digestive tracts, and salivary glands. More, phytoplasma was less detected in males compared to females. Thus, Hanboonsong et al. (2002) summarized that *M. hiroglyphicus* can be a reservoir for the phytoplasma. Additionally, transovarial transmission of ‘Ca. Phytoplasma prunorum’ was shown in *Cacopsylla pruni* (Hemiptera: Psyllidae) (Tedeschi et al. 2006). More, the phytoplasma was detected in eggs, nymphs and recently hatched adults that were able to infect healthy plum plants (Tedeschi et al. 2006). However, information about possible effects and interactions between SBR-phytoplasma and plants or insect vectors are completely missing.

Further, volatile organic compounds (VOCs) can have a significant effect on insect behaviour and attractiveness of plants for insects and can alter between phytoplasma infected and phytoplasma free plants (reviewed in Gross et al. 2022). Thus, they can manipulate the behaviour of insect species (e.g. psyllids). *Cacopsylla picta* (Hemiptera: Psyllidae) preferred *Ca. Phytoplasma mali* infected over non infected apple trees according to VOC release (β-caryophyllene) of the trees. Further, emigrant specimens were also more attracted to infected trees, however, remigrant *C. picta* preferred to oviposit at pathogen free apple trees (reviewed in Gross et al. 2022). Chuche et al. (2016) compared host preferences of *S. titanus*. Both nymphs and adults preferred FDP infected grapevine over healthy grapevine. However, it was not clearly stated whether these preferences were more caused by the leaf colour or by VOCs (Chuche et al. 2016).

### 1.1.6 Pathogen detection and mixed infection

Classical phytoplasma detection focused on diagnosis procedures such as symptom observation, transmission of phytoplasmas by insect vectors, dodder or grafting and
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4′,6-diamidino-2-phenylindole (DAPI) staining that was combined with analysis of the plant’s phloem using electron microscopy (reviewed in Bertaccini et al. 2019). Thus, SBR diseased plants can be identified according to the symptoms described by Gatineau et al. (2002). However, plant diseases which are caused by MLOs can be hardly differentiated due to comparable symptom expression (reviewed in Namba 2019). Finally, molecular methods are required for unequivocal identification of sugar beets infected with SBR pathogens (Pfitzer et al. 2020). An optimum detection of pathogens (here: Phytoplasma) requires three steps i) DNA extraction/preparation, ii) choice of DNA target sequence and iii) diagnostic technique (reviewed in Bertaccini et al. 2019). Serological methods such as immunofluorescence or Enzyme-linked Immunosorbent Assay (ELISA) were also used for detection of phytoplasma-infected plants. Nowadays, phytoplasma detection in plant material is done by PCR amplification and sequencing of 16S rRNA gene fragments. Other molecular techniques for detection of phytoplasmas include nested PCR, quantitative (q)PCR or Real-Time PCR, restriction fragment length polymorphism (RFLP) analyses, microarrays, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA) (reviewed in Bertaccini et al. 2019).

SBR-phytoplasma can be detected in sugar beets or planthoppers by nested PCR using P1/P7 and fU5/rU3 primer pairs (Gatineau et al. 2001). In general, nested PCR can be beneficial when phytoplasma concentrations are relatively low or when inhibitory substances are present, thus, this method can improve sensitivity and specificity of molecular detection (reviewed in Bertaccini et al. 2019). The amplified DNA can be used for grouping the isolates of stolbur phytoplasma in RFLP after digesting the 16S amplicon with Tru9I restriction enzyme (Gatineau et al. 2001). P1/P7 primer combination is suitable for amplification of a long fragment that includes nearly the complete 16S rRNA gene, 16S–23S rRNA internal transcribed spacer (ITS), the entire transfer RNA (tRNA)-Ile gene, and a short 23S rRNA gene fragment (reviewed in Bertaccini et al. 2019). Further, fU5/rU3 primer combination was originally designed to amplify a part of 16S ribosomal DNA (rDNA) of various phytoplasma strains (Lorenz et al. 1995). Later, Bressan et al. (2008) used STOL11f3/STOL11r2 primer combination in direct PCR to specifically detect stolbur phytoplasma in cixiid planthoppers. Further, STOL11f2/STOL11r1 and STOL11f3/STOL11r2 primer combinations were used in nested PCR to specifically detect stolbur phytoplasma in sugar beets (Bressan et al. 2008) which originated from the study of Clair et al. (2003).

Different protocols were used in various studies for PCR detection of SBR-proteobacterium. Sémétey et al. (2007a) used different primer combinations (Fra5/rP1, Fra5/L1r, and Alb1/Oliv1) for detection of SBR-proteobacterium. Fra5 was originally designed by Zreik et al. (1998) to match 16S rDNA from Ca. Phlomobacter fragariae, rP1 was originally designed by
Weisburg et al. (1991) to match 16S rDNA from bacteria, and L1r was designed by Borneman and Triplett (1997) to match 23S rDNA from bacteria, respectively. Sémétey et al. (2007a) used Fra5/L1r primer combination for rDNA intergenic spacer analysis (RISA) of SBR-proteobacterium and Alb1/Oliv1 primer combination for specific amplification of a 16S-ITS region of SBR-proteobacterium. Sémétey et al. (2007b) used fD1/rP1 and Fra4/Fra5 primer combinations to amplify 16S rDNA of SBR-proteobacterium, where fD1 was originally designed to match 16S rDNA of various eubacteria (Weisburg et al. 1991) and Fra4/Fra5 were originally used to match 16S rDNA of Ca. Phlomobacter fragariae (Zreik et al. 1998). Later, Alb1/Oliv1 primer combination was also used for specific detection of SBR-proteobacterium in various studies (Bressan et al. 2009b; Bressan et al. 2007; Bressan et al. 2008; Bressan et al. 2011; Bressan et al. 2012). Additionally, Pfitzer et al. (2020) used Fra5/L1r and Alb1/Oliv1 primer combinations in nested PCR. Further, SBR-proteobacterium can also be detected by fluorescence in situ hybridization (FISH) (Bressan et al. 2012). Recently, Real-Time PCR protocols were established to detect SBR-proteobacterium based on spoT gene sequences (Mahillon et al. 2022), or mannose 6-phosphate isomerase (manA) and heat shock protein 20 (hsp20) gene (Zübert and Kube 2021). The sequence of these genes has been obtained using metagenomic shotgun sequencing on DNA from infected sugar beet plant (Zübert and Kube 2021). Amplification and sequencing of 16S rRNA has been used for detection of both SBR pathogens (Behrmann et al. 2022).

Infection rates of adult *P. leporinus* varied between planthopper collection times and sites. SBR-proteobacterium and SBR-phytoplasma were found in 7-100 % and 0-13.3 % of adults in France, respectively (Bressan et al. 2011; Gatineau et al. 2001; Sémétey et al. 2007a). In Germany, infection rates with SBR-proteobacterium (28-65 %) after nested PCR (Pfitzer et al. 2020; Schröder et al. 2012) or ca. 15-85 % after TaqMan Real-Time PCR (Behrmann et al. 2022) and SBR-phytoplasma were comparable to French studies (0-5 %, nested PCR, Pfitzer et al. 2020). In Switzerland, infection rates of *P. leporinus* adults in sugar beet fields were 64.4 % with SBR-proteobacterium in 2020 using Alb1/Oliv1 primer combination (n=45) (Mahillon et al. 2022). Recently, SBR-phytoplasma infection rates of field collected adult *P. leporinus* ranged from 10-94.3 % in Germany in 2021 using TaqMan Real-Time PCR assay (Behrmann et al. 2022). However, SBR-phytoplasma infection rates (0-25 %) were lower in 2020 (Behrmann et al. 2022). Gatineau et al. (2002) assumed that the flight distance to sugar beet fields and presence of SBR-phytoplasma reservoir plants during migration may affect SBR-phytoplasma infection rate of *P. leporinus*. Double infected *P. leporinus* have rarely been reported. Pfitzer et al. (2020) reported a 5 % (n=59) maximum double infection rate from one collection site in 2018. However, Behrmann et al. (2022) recently reported double infections in planthoppers between 0 up to 50 % in 2020 and 2021 using TaqMan.
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Real-Time PCR assay. Besides planthoppers, sugar beets were also mainly infected with SBR-proteobacterium and double infections occurred in rare cases (Bressan et al. 2011; Pfitzer et al. 2020; Sémétey et al. 2007b). A total of 525 sugar beet samples from eleven different field sites and five different years in total were analysed in the study of Sémétey et al. (2007b). SBR-proteobacterium (Fra5/rP1 primer combination), SBR-phytoplasma (nested PCR using P1/P7 and fU5/rU3 primer pairs and grouping the isolates in RFLP after digesting with Tru9I restriction enzyme), and double infections were observed from 55, 2 and 0.6 % of the samples, respectively (Sémétey et al. 2007b). According to Pfitzer et al. (2020), SBR-proteobacterium but not SBR-phytoplasma was detected in 32 mixed samples which originated from at least 96 sugar beets displaying SBR symptoms. Bressan et al. (2011) described a positive correlation between planthopper abundance and their infection rates with SBR-proteobacterium. Furthermore, a positive correlation was described between P. leporinus abundance and the infection rate of sugar beets (Bressan et al. 2011). Finally, to the current state, SBR-proteobacterium is the causal agent of SBR disease, however the role of SBR-phytoplasma should be reinvestigated according to recent results.

1.1.7 Pentastiridius leporinus and other potential SBR vectors

P. leporinus is the most important SBR vector (Bressan et al. 2008; Mahillon et al. 2022; Pfitzer et al. 2020; Sémétey et al. 2007a). This species has a palearctic distribution and was collected up to 750 m above the sea level (EPPO 2022; Holzinger et al. 2003). Initially, this species was named Cicada leporina, Flata pallens or Flata pallidus (Holzinger et al. 2003). Additionally, due to misidentification, this species was accidentally named Oliarus pallens (now: Pentastiridius suzensis) or Pentastiridius beieri (Gatineau et al. 2002; Holzinger et al. 2003). P. leporinus belongs to Cixiidae that is one of the biggest Fulgoromorpha families and approximately 30 species within the family Cixiidae are distributed in Central Europe (Holzinger et al. 2002; Holzinger et al. 2003; Mühlethaler et al. 2019).

In addition to P. leporinus, many Auchenorrhyncha species belonging to the Cixiidae, Delphacidae, Cicadellidae, Membracidae, Tettigometridae, Flatidae, and Aphrophoridae families were reported from sugar beet fields (Pfitzer et al. 2020; Sémétey et al. 2007a). According to Sémétey et al. (2007a), at least 22 Auchenorrhyncha species were collected within and around French sugar beet fields between 1998 and 2003. Additionally, 42 Auchenorrhyncha species were collected in Southwest German sugar beet fields in 2018 (Pfitzer et al. 2020). In both studies, P. leporinus was the predominant species, however other cixiids such as R. quinquecostatus and H. obsoletus were also collected that are phylogenetically closely related based on morphological traits (Ceotto and Bourgoin 2008; Pfitzer et al. 2020; Sémétey et al. 2007a). Sémétey et al. (2007a) exclusively detected
SBR-proteobacterium in *Pentastiridius* sp. by PCR using Alb1/Oliv1 primers but not in any other Auchenorrhyncha species. However, in the study of Bressan et al. (2008), SBR-proteobacterium was detected in 82.7% of *P. leporinus* and 43.4% of *C. wagneri* (n=83) specimens with PCR using Alb1/Oliv1 primers. Further, stolbur phytoplasma was detected in 62.3% of *H. obsoletus* (n=61) from *Convolvulus arvensis* and in 76% of *H. obsoletus* (n=25) specimens from *Urtica dioica* in direct PCR using STOL11f2/STOL11r1 primers (Bressan et al. 2008). Additionally, stolbur phytoplasma was detected in 1.6% *Pentastiridius* sp. (n=471) and 4.8% *H. obsoletus* (nested PCR using P1/P7 and fU5/rU3 primer pairs and grouping the isolates in RFLP after digesting with Tru9I restriction enzyme, n=63) (Sémétey et al. 2007a). According to Bressan et al. (2008), *C. wagneri* can transmit SBR-proteobacterium and from *C. arvensis* collected *H. obsoletus* can transmit SBR-phytoplasma to sugar beet. *C. wagneri* is the dominant vector of *Ca. Phlomobacter fragariae*, the causal agent of SMC disease (Bressan et al. 2008; Danet et al. 2003; Zreik et al. 1998).

### 1.1.8 Host range, host shift, reservoir plants, and life cycle of insect vectors and phloem-limited bacteria

*P. leporinus* has a univoltine life cycle in Central Europe as generally described for cixiids (Biedermann and Niedringhaus 2004; Holzinger et al. 2002; Holzinger et al. 2003). In general, Auchenorrhyncha species produce five nymphal and one adult stage, however the nymphal stages of *P. leporinus* were not be described before the start of this thesis (Biedermann and Niedringhaus 2004). Female Cixiidae oviposit hypogean and some species use soil clefts to get into the soil (Holzinger et al. 2002; Holzinger et al. 2003). In general, Cixiidae such as *P. leporinus* cover their eggs with wax filaments, presumably to regulate humidity (Holzinger et al. 2002; Holzinger et al. 2003) and to protect immature stages from enemies (Mühlethaler et al. 2019). Generally, Auchenorrhyncha are hemimetabolic and polyphagous insects, feed from plant sap and cixiid planthoppers are phloem feeders (Biedermann and Niedringhaus 2004; Holzinger et al. 2002). In natural habitats, adult *P. leporinus* were observed to feed on the aboveground parts of reed grass (*P. australis*), *Scirpus*, *Carex* and *Eriophorum* grasses (Holzinger et al. 2003; Nickel 2003). Generally, cixiid nymphs develop in the soil and feed from roots of their host plants (Holzinger et al. 2002). However, the host plants of *P. leporinus* nymphs are unknown (Biedermann and Niedringhaus 2004; Holzinger et al. 2002; Holzinger et al. 2003). Nickel (2003) suggested that the nymphs may feed on other host plants than reed grass. According to Bourgoin (2022), Pfitzer et al. (2020), and Rizwan et al. (2020), adult *P. leporinus* were collected in *Carex*, cottongrass (*Eriophorum*), rice (*Oryza sativa*), *P. australis*, *Phragmites communis*, pedunculate oak (*Quercus robur*), *Scirpus*, *Triticum*, Bermuda grass (*Cynodon dactylon*),
Egyptian clover (*Trifolium alexandrinum*), cucumber (*Cucumis sativus*), sweetcorn (*Zea mays* conv. saccharata var. rugosa), chives (*Allium schoenoprasum*), millet, and celery (*Apium graveolens*) fields. However, it is unknown whether *P. leporinus* reproduces on these plants. Recently, *P. leporinus* has adapted to sugar beet and cereals [winter wheat (*Triticum aestivum*) or barley (*Hordeum vulgare*)] crop rotations (Figure 3) (Bressan et al. 2009a; Bressan 2009; Bressan et al. 2011).

Figure 3 Life cycle of *Pentastiridius leporinus* in sugar beet-winter wheat crop rotations. Dashed arrows mark the research objectives of this thesis, if variations in soil tillage and succession crops following SBR-infested sugar beet are potential options to control *P. leporinus*.

This host shift enabled the spread of this insect vector and SBR disease (Bressan 2014). Thus, adult *P. leporinus* migrate to sugar beet fields during summer (June-July) (Bressan et al. 2011). According to Bressan et al. (2009a), the females carry an average of 46.6 ± 1.9 eggs and oviposit close to sugar beet taproots into the soil. Afterwards, the nymphs hatch and feed subterranean on taproot sap and in September, the nymphs (second to third instar) largely stay in 10-25 cm depth before sugar beet harvest (Bressan et al. 2009a). Recently, Behrmann et al. (2022) assumed that the nymphal distribution depends on soil temperature, indicating that at colder temperatures (below 5.6 °C, measured from 5-10 cm soil depth), nymphs are less frequently found in the 0-10 cm soil layer and concentrate in deeper soil layers (10-20 cm and 20-30 cm). Moreover, at temperatures above 8.6 °C, the nymphs concentrate in the topsoil layer (0-10 cm) (Behrmann et al. 2022). Bressan (2009) speculated that lower temperatures induce a diapause for the nymphs in autumn, when the nymphs are in the second and third instar (Bressan et al. 2011). Recently, Behrmann et al. (2022)
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reported that *P. leporinus* nymphs were still active when soil temperatures were below 0 °C. Bressan et al. (2009a) collected nymphs (third to fifth instar) from 0-10 cm depth in winter wheat in May. More, the nymphs developed from fourth and fifth instar to adults feeding on cereal roots (Bressan et al. 2011). It is currently unknown, whether *P. leporinus* independently adapted to sugar beet crop rotations in the different SBR infested areas.

The flight activity of *P. leporinus* is variable in sugar beet-winter wheat crop rotations. So far, the shortest presence in sugar beet fields, from late June to late July 1999 was reported by Bressan et al. (2009a), however, the longest flight activity from mid of May to late September 2020 was reported by Behrmann et al. (2022). In 1999, the flight activity of *P. leporinus* lasted from early June to early July and in 2000, it lasted from late May to late June in winter wheat fields in France (Bressan et al. 2009a). In addition, Bressan et al. (2010) analysed the migration of adult *P. leporinus* from winter wheat (preceded by sugar beet) to different field crops including sugar beet, corn (*Zea mays*), soybean (*Glycine max*) and winter wheat (without the pre crop sugar beet). Adults were captured on sticky traps in all analysed crops, but the highest flight activity was reported from sugar beet fields. After the nymphs had left winter wheat (preceded by sugar beet), adults were exclusively captured in sugar beet (Bressan et al. 2010). Therefore, Bressan et al. (2010) assumed that colonization of adult *P. leporinus* only occurred in sugar beet. Additionally, nymphs were exclusively found in the winter wheat field preceded by sugar beet, before adult collection was done with sticky traps (Bressan et al. 2010).

Generally, reservoir plants (usually weeds) are necessary for pathogen uptake by the insect vectors (Jović et al. 2009; Jović et al. 2019) and cixiid planthoppers are important vectors of several bacterial plant diseases. Besides *P. leporinus*, other cixiids shifted host and transmit pathogens that cause severe plant diseases which share interesting parallels with the SBR disease. Initially, *H. obsoletus* exclusively reproduces on perennial herbs (Lessio et al. 2007), e.g. *U. dioica, Artemisia vulgaris, Amaranthus retroflexus,* and *Senecio erucifolius* (reviewed in Mori et al. 2013; Nickel 2003) and the principal natural host is *C. arvensis* (Holzinger et al. 2003). Further nymphal hosts e.g. *Onosma armenum, Crambe orientalis,* and *Geranium tuberosum* were also reviewed in Riolo et al. (2012). *H. obsoletus* is the primary vector of the economically most important cixiid-transmitted phytoplasma, *Ca. Phytoplasma solani* (Jović et al. 2019), thus, causing several plant diseases such as ‘bois noir’ (BN) disease in grapevine (Maixner et al. 1995; Šafářová et al. 2018; Sforza et al. 1998), maize redness (MR) disease (Mori et al. 2013), potato stolbur disease (Mitrović et al. 2016), and of *Ca. Phytoplasma solani* induced diseases in *Vitex agnus-castus, Salvia sclarea* (reviewed in Jović et al. 2019), celery and tomato (Carraro et al. 2008; Popović et al. 2021). It is known or at least assumed that *H. obsoletus* reproduces and acquires *Ca. Phytoplasma solani* from
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weeds and then transmits it to crop species, e.g. Mitrović et al. (2016) or Bressan et al. (2008) showed that *H. obsoletus* can acquire *Ca.* Phytoplasma solani from the reservoir *C. arvensis* and transmits it to potato or sugar beet (see Introduction sections 1.2 and 1.3). According to Kosovac et al. (2016), *V. agnus-castus* and *S. sclarea* are reservoirs for *Ca.* Phytoplasma solani where *H. obsoletus* can acquire the pathogen and transmit it to crops. However, *H. obsoletus* cannot reproduce in the above mentioned crops (reviewed in Jović et al. 2019). Danet et al. (2003) assumed that *H. obsoletus* may be the main vector of stolbur phytoplasma in SMC disease. According to Sémétey et al. (2018), *H. obsoletus* is also a vector of lavender decline disease. Interestingly, lavender (*Lavandula angustifolia*) is a reproductive host of *H. obsoletus*. Thus, *H. obsoletus* nymphs acquire *Ca.* Phytoplasma solani while feeding on lavender or *C. arvensis* roots, which is also the most important reservoir of the phytoplasma. This finding is in contrast to other phytoplasma induced plant diseases where the pathogen reservoirs usually are weeds (described above). Later, emerged and infected adults transmit *Ca.* Phytoplasma solani to new lavender plants (Sémétey et al. 2018). Thus, *H. obsoletus* also adapted its life cycle to this crop, as it was also described for *P. leporinus* in SBR disease (described above).

*Reptalus panzeri* (Hemiptera: Cixiidae) was originally found on shrubs, herbaceous plants, bushes, or woods (Biedermann and Niedringhaus 2004; Holzinger et al. 2003; Nickel 2003). Jović et al. (2007) showed that *R. panzeri* is an important vector species of MR disease due to transmission of *Ca.* Phytoplasma solani. According to Jović et al. (2009), *R. panzeri* adopted its life cycle to maize-winter wheat crop rotations. Thus, adult females oviposit into maize fields and the hatching nymphs acquire *Ca.* Phytoplasma solani during feeding on the roots of infected maize plants. Vertical transmission of the pathogen from females to their offspring has not been observed. Comparable to the life cycle of *P. leporinus* in sugar beet-winter wheat rotations, *R. panzeri* nymphs reach second or third instars in autumn and complete their life cycle in the following summer on wheat roots. In this pathosystem, maize, winter wheat, and *Sorghum halepense* were identified to host *Ca.* Phytoplasma solani and therefore they act as reservoirs (Jović et al. 2009). Additionally, *R. panzeri* vectors potato stolbur disease and it can transmit *Ca.* Phytoplasma solani acquired from *S. halepense* to potato (Mitrović et al. 2016).

*C. wagneri* was originally found on trees, shrubs or herbaceous plants (Holzinger et al. 2003). However, *C. wagneri* is the dominant vector of *Ca.* Phlomobacter fragariae, the causal agent of SMC disease and infection rates of insects can range between 19-87 % (Bressan et al. 2008; Danet et al. 2003; Salar et al. 2010a; Zreik et al. 1998). According to Nourrisseau et al. (1993), SMC disease was firstly observed in France in 1988. Comparable to SBR disease, SMC disease is also additionally caused by a stolbur phytoplasma and the induced
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Symptoms in strawberries are comparable between both pathogens (Danet et al. 2003). Symptoms of SMC disease are red discolorations in leaflets, reduced size of younger leaves, fruit deformations, and root necrosis in strawberries (Nourrisseau et al. 1993). Stolbur phytoplasma was primarily found in strawberry nursery plants, however, Ca. Phlomobacter fragariae was mainly detected in strawberry crops (Danet et al. 2003). C. wagneri adopted to strawberry cultures (Salar et al. 2010a), thus, adult females oviposit into the soil, the nymphs overwinter in strawberry crops, develop on strawberry roots, and emerge adult in the following summer (June). Thus, SMC disease and its vector have interesting similarities to SBR disease. All nymphal stages successfully transmit Ca. Phlomobacter fragariae to strawberry plants (Salar et al. 2010a). Danet et al. (2004) successfully transmitted Ca. Phlomobacter fragariae to C. roseus by C. wagneri. Recently, it was shown that Ca. Phlomobacter fragariae can also be transmitted from infected plants to daughter plants through stolons (Dittmer et al. 2021).

1.1.9 Management of insect vectors

Control of bacterial (here: Phytoplasma) induced diseases focuses on the elimination of reservoir plants, cultivation of resistant/tolerant cultivars, and insecticide treatments to control the vector (reviewed in Bianco et al. 2019). According to Salar et al. (2010a), no C. wagneri specimens were found in a strawberry plot in June, after a single insecticide (organochlorine) treatment in March, thus, the authors assumed that the insecticide treatment successfully controlled C. wagneri nymphs in the soil. In contrast, a high number of insecticide treatments was necessary to show effects against adult P. leporinus in a field experiment with special permission (Pfitzer et al. 2020). Possible reasons are the limited persistence of insecticides (Bressan 2009), long distance flight activity of adults, and high numbers of adults in the field (Pfitzer et al. 2020). Further, it is completely unknown, which insecticides are effective against P. leporinus and no insecticides are currently authorized against this planthopper. Accordingly, H. obsoletus adult control with insecticides was not practicable in lavender crops, due to potential side effects on pollinators and subsoil development of nymphs (reviewed in Jović et al. 2019). However, cultivation of tolerant cultivars helped to control lavender decline (reviewed in Jović et al. 2019).

Indeed, only one study about P. leporinus control was reported by Bressan (2009) that targeted control of nymphs in the soil since the immature stages take the longest time within P. leporinus development. Firstly, changes in soil tillage after sugar beet harvest and before sowing winter wheat led to a 28 % reduction in emerged adults. Secondly, growing spring barley instead of winter wheat after sugar beet harvest led to 81 % and 80 % reductions in nymphs and emerging adults, respectively. However, these results have not been replicated,
since the field experiments were each conducted on one field trial site only (Bressan 2009). Growing of non-host plants for the insect vector is another method for disease management. For example, a high mortality on non-host plants was observed for *Nilaparvata lugens* (Hemiptera: Delphacidae) which causes dramatic damage in rice by virus transmission (Oka 1979). Further, this strategy was used for controlling nymphs of the planthopper *Haplaxius crudus* (Hemiptera: Cixiidae, previously called *Myndus crudus*) which vectors ‘Ca. Phytoplasma palmae’, causing lethal yellowing disease in coconut palms (Beltran-Aldana et al. 2020; Howard 1990). However, other measures such as mulching had a disadvantageous effect for control of *H. crudus* (Howard and Oropeza 1998) and *H. crudus* adult emergence was higher in some mulched plot treatments compared to unmulched plots. In the study of (Lamp et al. 1984), herbicide treatments successfully reduced abundances of most Auchenorrhyncha species (families Cicadellidae and Delphacidae) in alfalfa, probably due to removal of grasses that were potential hosts (Lamp et al. 1984). Further, few reports are known about parasitism in cixiid planthoppers. One report was given by Sforza et al. (1999), where one adult *H. obsoletus* was parasitized by a parasitoid wasp belonging to the family Dryinidae (Hymenoptera). Similarly, parasitoid wasps (*Embolemus* sp. and *Ampulicomorpha* sp.) can harm cixiid nymphs (reviewed in Guglielmino and Bückle 2013). Generally, dryinid parasitic wasps are promising insects for Auchenorrhyncha control (Guglielmino 2002). However, no reports are available where *P. leporinus* was parasitized by biological antagonists.

### 1.1.10 Rearing and life history data of Auchenorrhyncha

The development of a laboratory rearing represents an important tool to ensure a constant supply of insects (here: Psylloidea) that is independent from environmental conditions and the availability in their natural habitat (reviewed in Jarausch et al. 2019). Additionally, a laboratory rearing is important to study the life history and biology of Auchenorrhyncha species and the disease which they transmit to develop control strategies (Beltran-Aldana et al. 2020). However, due to the complexity of planthopper vector life cycles, it remains difficult to reproduce them under controlled conditions (Bressan 2014). Generally, only few rearing protocols of cixiids are available. Further laboratory rearing protocols of cixiids are only described for *H. crudus* (Beltran-Aldana et al. 2020; Tsai et al. 1976), *H. obsoletus* (Chuche et al. 2018; Kessler et al. 2011; Sforza et al. 1999) and *Oliarius polyphemus* (Hemiptera: Cixiidae) (Hoch and Howarth 1993). Sforza et al. (1999) established a laboratory rearing and provided life history traits such as development time, sizes and mortality in different nymphal stages of *H. obsoletus*. However, a mass rearing failed due to high insect mortality (Sforza et al. 1999). The establishment of a *P. leporinus* rearing is necessary, however, to keep this insect species under controlled conditions was considered as a challenge (Bressan 2009,
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2014), due to high mortalities of *P. leporinus* adults under controlled conditions after collection in the field (reviewed in Bressan et al. 2009a). Bressan (2014) assumed that it is hard to reproduce the life cycles of planthopper vectors that carry *Arsenophonus* bacteria under controlled conditions. However, as SBR-proteobacterium cannot be cultivated in vitro, a rearing of infected planthopper is necessary to study these bacteria (Bressan 2014).

1.1.11 Monitoring and detection of insect vectors

Auchenorrhyncha collection can be carried out with quantitative, semi-quantitative, and qualitative methods (Holzinger et al. 2003). Further, insect collection methods are divided into active or passive methods (reviewed in Krüger and Fiore 2019). Usually, several methods are combined to acquire sufficient data and the method selection depends on the aim of the study, species and development stage of the insect (reviewed in Krüger and Fiore 2019). Examples for quantitative methods are suction or vacuum sampling using D-Vac or G-Vac (reviewed in Holzinger et al. 2003; reviewed in Krüger and Fiore 2019). In these methods, nymphs from unknown species can be reared under controlled conditions until adult emergence and before species identification. Further, Auchenorrhyncha flight activity can be studied with colour bowl attraction traps (mostly yellow coloured), suction traps, light or sticky traps. Emergence traps, e.g. ground photo-eclectors are useful to analyse the densities of Auchenorrhyncha species with subsoil nymphal development (e.g. cixiids) by quantifying adult emergence from soil. Semi-quantitative collection methods (e.g. sweep netting, pitfall, Malaise or sticky traps) can provide standardised results which are hardly comparable. Qualitative methods can be used to analyse the host plants of Auchenorrhyncha with sweep netting, suction sampling, visual inspection e.g. searching or selective sampling of insects on the potential host plants (reviewed in Holzinger et al. 2003; reviewed in Krüger and Fiore 2019).

According to Holzinger et al. (2003), traditionally, species identification of Auchenorrhyncha is based on the observation of external morphological traits or dissection. Relevant traits can be found on different parts of the head, legs, wings, and genital structures, e.g. Cixiidae (such as *R. quinquecostatus*, *H. obsoletus*, and *P. leporinus*) are characterized by a roof-shaped resting position of the wings, male genitalia are completely sclerotinized, and the absence of i) apical processes on the lateral keels of the frons and ii) lateral processes on the head (Holzinger et al. 2003). Within Cixiidae, different characteristics can be used for species identification, e.g. the number of keels on the mesonotum, number of spines on the first segment of the hind tarsus, as well as the characteristics of scutellum, vertex, and pronotum. Finally, the observation of male genitalia is necessary for the unequivocal identification of many cixiids, e.g. *Pentastiridius* species (Biedermann and Niedringerhaus
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2004; Holzinger et al. 2003). *P. leporinus* has five keels on the mesonotum, at least 12 spines on the first segment of the hind tarsus, and the spine of the aedeagus has a 90° angle to its tip (Biedermann and Niedringhaus 2004; Holzinger et al. 2003). *R. quinquecostatus* and *H. obsoletus* are morphologically closely related to *P. leporinus* (Ceotto and Bourgoin 2008) and they also have five keels on the mesonotum (Holzinger et al. 2003). However, for many cixiids such as *P. leporinus*, no morphological keys are available for females and immature stages (Bertin et al. 2010a). Morphological species identification of cixiid planthoppers can only be done by few experienced entomologists, and it may lead to misidentification (Bertin et al. 2010a, 2010b; Liu et al. 2018). Furthermore, morphological identification requires intact insect samples and the number of samples that can be processed is limited due to labor and time requirements (Liu et al. 2018). Hence, adequate handling and storage of the collected insects are required before morphological species identification can be done (Biedermann and Niedringhaus 2004; Holzinger et al. 2003).

Later, molecular methods were established to support insect identification. DNA barcoding based on mitochondrial cytochrome oxidase I gene (COI) sequences was established by Hebert et al. (2003). In this method, template DNA from an unknown specimen is amplified with universal primers using PCR (reviewed in Bertaccini et al. 2019). Afterwards, the obtained COI fragment is sequenced and compared to a database (e.g. Barcode of Life Datasystem (BOLD) or GenBank database) where other DNA barcodes with known origin of species are published (Bressan et al. 2009a, Bressan et al. 2009c; Wilson 2012). Thus, DNA barcoding allows global animal species identification even for non-experts (Hebert and Gregory 2005) or identification of plant-pathogenic bacteria (reviewed in Bertaccini et al. 2019). However, DNA barcoding is hampered when insects are misidentified, no further DNA barcodes from these species are available in database, or when samples are contaminated (reviewed in Bertaccini et al. 2019; Virgilio et al. 2010).

Further, COI sequence analysis was used to identify various planthopper species, e.g., *P. leporinus, R. quinquecostatus, and H. obsoletus* (Bertin et al. 2010a, 2010b; Bressan et al. 2009a). Additionally, numerous species-specific PCR assays, PCR-RFLP or direct multiplex PCR (dmPCR) assays were developed for detection of insect species such as *Trissolcus japonicus* (Hymenoptera: Scelionidae), *Reptalus* ssp., *Hyalesthes* ssp., *Hishimonus* ssp. (Hemiptera: Cicadellidae), or *Nilaparvata* ssp. (Hemiptera: Delphacidae) (Bertin et al. 2010a, 2010b; Chen et al. 2021; Hao et al. 2015; Liu et al. 2018). In these methods, sequences of the analysed species were multiple aligned and species-specific primers were designed on highly conserved intra-specific parts of the genome with inter-specific mismatches to the excluded species. Insect species were identified according to characteristic fragments obtained from PCR amplification and separation of the amplicons on agarose-gels and
sequencing of PCR products were not required (Bertin et al. 2010a, 2010b; Chen et al. 2021; Hao et al. 2015; Liu et al. 2018). However, there is a risk of incorrect results in PCR assays (Hebert et al. 2003) and sequence analysis of COI gene allows reliable species identification (reviewed in Weissensteiner and Lanchbury 1996). Additionally, these methods enable to identify cixiid nymphs and to analyse their host plants from which they have been collected (Bertin et al. 2010a, 2010b). Generally, molecular methods for species identification are simple, time- and cost-saving (Chen et al. 2021; Hao et al. 2015; Liu et al. 2018). In contrast to traditional morphological identification, molecular methods can also be used to identify damaged specimens (Liu et al. 2018). Finally, molecular methods are a helpful tool to support monitoring of insect vectors and to identify suitable approaches for vector control (Bertin et al. 2010a, 2010b).

Numerous methods are used for template DNA preparation of insects before PCR amplification. However, several ingredients may interfere PCR amplification of DNA, e.g., secondary plant metabolites (polyphenols and polysaccharides), thus, diluting DNA templates can help in some cases, or these inhibitors must be removed at all (reviewed in Bertaccini et al. 2019). Some methods include a purification step of DNA, e.g., 'DNeasy Blood & Tissue Kit’ (QIAGEN GmbH, Hilden, Germany). In contrast, other time- and cost-saving methods are described, where insects are simply crushed in distilled water, ethylenediaminetetraacetic acid (EDTA), or phosphate-buffered saline (PBS), incubated in boiling water (100 °C) and the resulting crude insect tissue fluid was used for PCR amplification (Priti et al. 2021). Various methods for isothermal amplification of nucleic acid are described that have the potential to be used on-site, such as RPA or LAMP (reviewed in Bertaccini et al. 2019).
2. Research objectives

SBR is a fast spreading and threatening sugar beet disease and the high losses of sugar content and yield can significantly harm profitability of sugar beet cultivation (Bressan et al. 2008; Gatineau et al. 2002; Sémétey et al. 2007b). Consequently, control strategies against the economic vector *P. leporinus* are urgently required. To this aim, studying the life cycle, interaction with host plants, host range, rearing, and the establishment of simple methods for detection and monitoring of the main insect vector are essential.

Before the beginning of this thesis, no *P. leporinus* rearing protocol under laboratory conditions was available that allows the year-round availability and fast-tracking research on vector control measures. Further, suitable host plants, pots, substrates, and abiotic conditions that allow a mass production of *P. leporinus* were unknown. Additionally, basic life history traits of the different developmental stages such as mortality, development time, body size or head capsule width were completely missing. However, this information would contribute to a better understanding of the vector and disease. To overcome this problem, this study aimed to characterize *P. leporinus* developmental stages and to provide life history traits that allow to distinguish the nymphal instars. Further, a method for *P. leporinus* mass rearing was established (manuscript I).

There was evidence that agronomic practices such as variation of succession crops or soil tillage after sugar beet harvest could reduce numbers of *P. leporinus* that emerge adult from the soil (Bressan 2009). However, these preliminary findings were not reproduced and no information about host suitability of different plant species for *P. leporinus* is available. Thus, a further objective of this thesis was to systematically test various succession crops and soil tillage treatments in field trials under different environments to identify potential agricultural practices for vector control. Additionally, survival and development of *P. leporinus* nymphs on alternative host plants was analysed under laboratory conditions to confirm the field trial results (manuscript II).

Currently, monitoring of male *P. leporinus* adults is based on species identification by observation of external morphological traits (Biedermann and Niedringhaus 2004; Holzinger et al. 2003). However, this method is labor and time intensive and may lead to misidentification (Liu et al. 2018). Further, identification on species level is only possible for male *P. leporinus*, thus, it cannot be used for female and immature specimens (Biedermann and Niedringhaus 2004; Holzinger et al. 2003). This thesis aimed to provide a molecular method/tool that allows reliable large-scale monitoring and species identification of
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*P. leporinus*, which is also a crucial step for the detailed analysis of this fast spreading insect vector and the development of targeted vector control measures (manuscript III).

Finally, the results reported in the different manuscripts are related and the significance of the findings is discussed.
3. Manuscript I

Life history traits and a method for continuous mass rearing of the planthopper *Pentastiridius leporinus*, a vector of the causal agent of syndrome ‘basses richesses’ in sugar beet

Short title: Life history traits and mass rearing of *P. leporinus*

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Abstract:

BACKGROUND: The planthopper *Pentastiridius leporinus* (Hemiptera: Cixiidae) is the main vector of the γ-3 proteobacterium 'Candidatus Arsenophonus phytopathogenicus' which causes the syndrome “basses richesses” (SBR) in sugar beet. SBR is a new and fast spreading disease in Central Europe that leads to high yield losses. To date the development of management strategies is hampered by insufficient knowledge about general life history traits of the planthopper and, most importantly, the year round availability of insects reared under controlled conditions. Rearing of *P. leporinus* has been considered challenging and to date no protocol exists.

RESULTS: Here we describe a method for mass rearing *P. leporinus* on sugar beet from egg to adult, which has produced five generations and >20,000 individuals between June 2020 and March 2022. An alternative host such as wheat is not necessary for completing the life cycle. No-choice experiments showed that *P. leporinus* lays 139.1 ± 132.9 eggs on sugar beet, whereas no oviposition was observed on its nymphal host wheat. Head capsule width was identified as a trait that unequivocally distinguished the five nymphal instars. Developmental time from first instar to adult was 193.6 ± 35.8 days for males and 193.5 ± 59.2 days for females. Infection rates of adults were tested with nested polymerase chain reaction (PCR). The results demonstrated that 70-80% of reared planthoppers across all generations carried the SBR proteobacterium.

CONCLUSION: The mass rearing protocol and life history data will help overcome an important bottleneck in SBR research and enhance efforts in developing integrated pest management tools.

Keywords:

Cixiidae, *Candidatus Arsenophonus phytopathogenicus*, proteobacterium, development time, insect culture, mass rearing
1 INTRODUCTION

The planthopper *Pentastiridius leporinus* (Linnaeus 1761) is a member of the Cixiidae (Hemiptera, Fulgoromorpha) family, which comprises almost 2,000 species worldwide.\(^1\) The insect has a palearctic distribution but does not occur in the northern regions.\(^2\) In natural ecosystems, adult *P. leporinus* are known to feed on aboveground parts of reed grass (*Phragmites australis*) but no information is available on host plants used by the immature stages that ingest phloem sap from roots.\(^1\)-\(^3\) In agricultural systems, *P. leporinus* feeds on sugar beet (*Beta vulgaris*) leaves, thereby transmitting the phloem-restricted \(\gamma\)-3 proteobacterium ‘*Candidatus Arsenophonus phytopathogenicus*’ that causes a disease known as syndrome “basses richesses” (SBR).\(^4\)-\(^6\) In recent years, the planthopper has become a major economic pest in several Central European sugar beet growing regions, including eastern France, Germany, and Switzerland.\(^7\)-\(^9\) During the summer, adults migrate to sugar beet fields, where the females oviposit into the soil. The hatching nymphs start feeding on sugar beet roots but usually complete their development on winter wheat, which frequently follows in crop rotation. In the subsequent year, adult planthoppers emerge from the soil, climb up on wheat plants and then migrate into a nearby sugar beet field.\(^4\),\(^10\),\(^11\)

Interestingly, *P. leporinus*, is not very common in natural ecosystems and in Germany it is even red-listed as an endangered species.\(^12\) A recent host plant shift to sugar beet and cereals such as winter wheat (*Triticum aestivum*) or barley (*Hordeum vulgare*) has probably led to the tremendous increase in population sizes of *P. leporinus* and thus to the fast spread of SBR.\(^4\),\(^10\),\(^11\) The sugar beet disease SBR leads to severe economic losses due to low sugar contents and yield reductions of up to 25%.\(^6\),\(^8\),\(^13\) Typical symptoms are chlorosis and necrosis of the older leaves, asymmetric young leaves and necrosis of the vascular bundles of the tap roots.\(^6\) SBR was first recorded in eastern France in 1991 and detected in Germany in 2009.\(^9\),\(^14\) More than a decade later, the area of affected sugar beet has accumulated to 3,000 ha (2019) in Switzerland and 16,400 ha (2018) in Germany.\(^7\),\(^8\) In eastern France, SBR is currently not a problem because the sugar factory was closed and sugar beet cultivation ceased (Laudinat V, 2022, pers. comm.).

Apart from the \(\gamma\)-proteobacterium *Ca. Arsenophonus phytopathogenicus*, the phytoplasmal ‘*Ca. Phytoplasma solani*’, belonging to the stolbur group (16SrXII), has also been identified as causal agent of SBR symptoms.\(^4\),\(^6\),\(^15\)-\(^17\) However, so far only the proteobacterium plays a major etiological role in France and Germany.\(^6\),\(^8\),\(^9\) Adult *P. leporinus* and nymphs can transmit the SBR proteobacterium persistently to sugar beets.\(^18\) Furthermore, adult *P. leporinus* can also vertically transmit the proteobacterium to their offspring.\(^18\) Whether these microbes benefit the insect and its spread, is still an open question.
So far, no management strategies are available to farmers for controlling SBR and its insect vector. Due to the high mobility and the extended migration period of the adult planthopper, chemical control with available insecticides has not been practicable.\textsuperscript{8} While crop rotation and changes in soil tillage practices may show some promise and could be part of a future control strategy, further research is urgently needed.\textsuperscript{10}

An important prerequisite for accelerated research on the biology, life history and management of \textit{P. leporinus}, is a laboratory rearing that can provide a constant and sufficient supply of all developmental stages of the insect. Until now, continuous rearing of \textit{P. leporinus} has been considered challenging and no protocol has been available.\textsuperscript{18,19} Here, we provide a method for the year round mass production of \textit{P. leporinus} from egg to adult on sugar beet. In addition, data on various life history traits are presented. The information will enhance efforts in studying this economically important vector and its bacterial symbionts.

2 MATERIALS AND METHODS

2.1 Plants

Seeds of sugar beet, \textit{Beta vulgaris} cv. Vasco (SESVanderHave Deutschland GmbH, Eisingen, Germany) without seed coating were grown in different pots and substrates, depending on the experiments. Plants were cultivated in a greenhouse with a temperature range of 20-35 °C. Natural daylight was supplemented with artificial light at 85 µmol (s m\textsuperscript{2})\textsuperscript{-1} (‘Elektrox SUPER BLOOM HPS 400 Watt’, Grow In AG, Berlin, Germany) when needed to maintain a 16:8 h light/dark photoperiod. If not stated otherwise, a 3:1 mixture of peat (‘Fruhstorfer Erde Typ P 25’, HAWITA Gruppe GmbH, Vechta, Germany) and sand (0-2 mm diameter) was used as substrate. Plants were fertilized with a solution of 1 g l\textsuperscript{-1} ‘Hakaphos® Blau 15-10-15(+2)’ when necessary (COMPO EXPERT GmbH, Münster, Germany).

2.2 Insects

Adult \textit{P. leporinus} were collected with a sweep net from a sugar beet field close to Neckarsulm in Germany (49°12'17.2"N 9°10'48.8"E) on 19 - 20 June 2020. Insects were identified according to their scutellum, vertex, pronotum, hind tarsus and male genital structures.\textsuperscript{2}

If not stated otherwise, all experiments were carried out in a controlled environment room at 20.9 ± 1 °C, 48 ± 12.2% r.h. and a photoperiod of 16:8 h light/dark. Light intensity was 80 µmol (s m\textsuperscript{2})\textsuperscript{-1}, provided by full-spectrum LED lights (‘Bioledex GoLeaf E2 LED Pflanzenleuchte Vollspektrum 120cm 50W IP44’, DEL-KO GmbH, Germany). Host plants and insects were cultivated in 60 x 60 x 60 cm cages (mesh size: 150 µm, ‘BugDorm-2120F Insect Rearing Tent’, MegaView Science Co., Ltd., Taiwan). Plants were only watered when
showing very first signs of wilting to prevent planthoppers from drowning. Water was filled into the saucers, thus leaving the top part of the soil dry.

2.3 Role of plant container, substrate and environmental conditions for oviposition

As a first step in establishing a continuous rearing, the role of two different plant containers and three substrates for oviposition success was investigated in two environments. Transparent polystyrene cylinders (‘small pots’, 170 ml volume, 4.8 cm diameter, 10.4 cm height, ‘Zuchtgläschen’, K-TK e.K., Retzstadt, Germany) with a hole (1 cm diameter) in the bottom and 2.4 l polypropylene plant pots (‘large pots’, 16 cm top diameter, 15 cm height) were used. Both container types were filled with either field soil (loamy clay), a mix of peat and sand (3:1) or peat mixed with cracked expanded clay (‘Original LamstedtDan’, 4–8 mm, Fibo ExClay Deutschland GmbH, Lamstedt, Germany) (3:1). Field soil was collected from the sugar beet field where planthoppers were caught. The soil was heat-treated (45°C) for 48 h before use, in a ‘Kempson’ apparatus. A single sugar beet plant (small pots: growth stage according to BBCH 12-14, large pots: BBCH 16) was grown in each pot. Sugar beets in small pots received six female and three male adults, while plants in large pots received ten female and five male adults. The number of total planthoppers was adapted to reflect the difference in pot sizes, while maintaining a 2:1 ratio of females to males. We assumed that one male would mate with more than one female and that an excess of males could stress ovipositing females. A transparent, perforated polypropylene bag (small pots: 25 cm length x 15 cm width, large pots: 38 cm length x 25 cm width) (‘CPP-Brötchenbeutel genadelt’, www.der-verpackungs-profi.de GmbH, Göttingen, Germany) was placed over the plant and was secured with a rubber band around the pot. Inoculated plants in small and large pots were kept in two different environments: (A) 14:10 h light/dark photoperiod, 24:18 °C, 60-80% r.h., 200 µmol (s m⁻¹)⁻¹ (Valoya 03-155-230 R150 AP673 LED Oberlicht’, Valoya Oy, Helsinki, Finland) and (B) 16:8 h light/dark photoperiod, 20.9 ± 1 °C, 48 ± 12.2% r.h. and 80 µmol (s m⁻¹)⁻¹ of full-spectrum light (described above). Egg batches were removed and quantified 16-18 days after inoculation. Each treatment was tested with six replicates.

2.4 Role of host plant species for adult survival and oviposition

The role of sugar beet and wheat plants for survival and oviposition in *P. leporinus* was assessed. Sugar beet (BBCH 12-14, n = 17) and two growth stages of wheat plants *Triticum aestivum* cv. Dekan (KWS SAAT SE & Co. KGaA, Einbeck, Germany) were compared in a no-choice experiment (‘small wheat plants’: BBCH 10-14, n = 15 and ‘large wheat plants’: BBCH 19, n = 9). Plants were cultivated in small pots with a single sugar beet or 2-3 wheat plants per pot. Sugar beet and small wheat plants were grown under controlled climatic conditions (see 2.2). Large wheat plants were grown in a greenhouse before the experiment.
(see 2.1). All plants were supplied with a single, newly emerged (max. 24 h old) female and one male adult planthoppers and covered with a transparent, perforated polypropylene bag (25 cm length x 15 cm width). Plants were checked regularly and a second male was added in a few cases where the male planthopper could not be retrieved or had died. Plants were arranged in a complete randomized design in the controlled environment room. Oviposition of adults was evaluated daily and survival of adults was evaluated every third day. Egg batches were removed from the substrate and the number of eggs was counted under a stereomicroscope (model ‘M3Z’, Wild Heerbrugg AG, Heerbrugg, Switzerland).

2.5 Continuous rearing of Pentastiridius leporinus

For oviposition, five female and three male adult planthoppers were kept on a single potted sugar beet plant (BBCH 12-14). Plants were grown in small pots and covered with a perforated bag (Fig. 4A). Over a 4-6 week period, egg batches were removed after seven days (Fig.4B) and groups of 4-5 egg batches were transferred to a plastic container (18 cm length x 13.5 cm width x 6 cm height) (Salatschale NP eckig - Becher - Polystyrol weiß - 1000 g, Papier Brinkmann GmbH, Münster, Germany), which contained a 1 cm layer of 3:1 peat-sand mix (Fig. 4C) and was kept in darkness by placing eight containers in two inverted plastic trays (60 cm length x 40 cm width x 7.5 cm height) (Newbox 15, beku Lagertechnik GmbH, Klagenfurt, Austria). The container was closed with a transparent lid that contained holes for gas exchange. The substrate surrounding the egg batches was kept moist by adding a few drops of water once per week until nymphs hatched to prevent eggs from desiccating, whereas the rest of the substrate inside the box was kept dry. This measure was taken as several nymphs had died and expressed mycosis when humidity in the container was too high. Bisected or quartered taproots from sugar beet (BBCH 19: 10 to 20 unfolded leaves), grown in polypropylene pots (9 cm length x 9 cm width x 9.5 cm height) in a greenhouse, were added as a food source for the hatching nymphs (Fig. 4D). Taproots were replaced after 14 days or earlier when showing signs of deterioration. Every second week, rearing containers were checked for fifth instar nymphs (first appearance after ca. eight weeks). All fifth instars were gently removed with a laboratory spoon (Fig. 4E), transferred to another container and released into an insect rearing tent with potted sugar beet plants (large pots with a single plant in BBCH 16-19: 6 to 14 unfolded leaves) to complete metamorphosis on an intact plant (Fig. 4F). Large pots were inoculated with 100 fifth instar nymphs. The substrate consisted of two layers with a 3:1 peat-sand mix as the lower part and a top layer of approx. 5 cm expanded clay (‘Original LamstedtTon 8–16 mm’, Fibo ExClay Deutschland GmbH). Cages were checked once per week for emerged adult planthoppers. Adults were gently removed with an aspirator (Fig. 4G), sexed and used for oviposition as described above or for further experiments.
2.6 Characterisation of developmental stages

In Central Europe, winter wheat is the most common crop grown after sugar beet harvest. Accordingly, *P. leporinus* nymphs spend most of their development on wheat roots until reaching the imago stage. To measure the developmental time of immature planthoppers, a single nymph was placed in a plastic container (11 cm length x 8 cm width x 5 cm height) (‘Saladboxx 250 cc’, Pro-Pac Ostendorf Plastic Thermoformfolien und Verpackungen GmbH & Co. KG, Vechta, Germany) with small holes in the lid for gas exchange. The container was filled with a layer of 3:1 peat-sand mix (25 g), which was kept moist and replaced after ca. 4 weeks. Two 4-10 days old wheat seedlings were added as a food source and replaced after eight days or earlier, if needed. The entire experiment was carried out twice. In the first replicate, the offspring of at least seven different parental females from ten different egg batches were analysed (4-14 nymphs per egg batch) and recordings started with second instar nymphs (n = 78) and ended with the fifth instar. Until reaching the second stage, neonates were kept on sugar beet. In the second replicate, the entire life cycle for the offspring of 12 different parental females (8-12 nymphs per female) was assessed. This experiment commenced by taking egg batches with known oviposition dates and transferring these to Petri dishes. At the day of hatching, first instar nymphs (n = 105) were transferred to the described experimental setup using a small paint brush. Survival and development of the nymphs were recorded every day under a stereomicroscope.

Morphometric measurements of each nymphal instar and of eggs from three different generations were taken to determine if body size can be used to differentiate between juvenile stages. Maximum length and width of eggs was recorded with a micrometer under a stereomicroscope (40x magnification). Body length of nymphal instars was measured (10-40x magnification) from the tip of the vertex to the tip of the abdomen. Head capsule width was determined as indicated in Fig. 1.

![Figure 1](image)

*Figure 1* Anterior part of *Pentastiridius leporinus* nymph. Lines and arrow indicate how head capsule width was determined.

Photos of the different developmental stages were taken with focus stacking under a stereomicroscope model ‘MSV266’ to which a camera model ‘DMC5400’ (both Leica Microsystems GmbH, Wetzlar, Germany) was fitted.
2.7 Infection rate of planthoppers

To examine the infection rate with SBR pathogens in the rearing, randomly selected adult *P. leporinus* (*n* = 10 per generation) from three consecutive generations were analysed with nested polymerase chain reaction (PCR). Insects were individually stored in 96% (v/v) ethanol at -20 °C until use. DNA was extracted with DNeasy® Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. Presence of *Ca. Arsenophonus phytopathogenicus* was verified with the primers Fra5/L1r and Alb1/Oliv1 as described in Sémétey et al. (2007), infection with phytoplasma was tested with the primers P1/P7 and U5/U3 as described in Gatineau et al. (2001).16,23

2.8 Statistical analyses

All statistical analyses were carried out with SAS 9.4 (SAS Institute Inc., Cary, NC, USA). In the oviposition experiment, data were analysed separately for both environments. Two-way analysis of variance (ANOVA) was carried out after testing for homogeneity of variances and normal distribution. Accordingly, oviposition data in the environment (b) were square-root transformed to meet the condition of variance homogeneity. Least square means were calculated to analyse significant differences at *p*<0.05 using ‘lsmeans’ in the PROC GLIMMIX procedure.24

Survival analysis of the no-choice oviposition experiment was carried out with Cox proportional hazard models and PROC PHREG in SAS 9.4. Hazard ratios (HR) >1 or <1 describe higher or lower probabilities of mortality compared to the referred treatment, respectively.24

One-way or two-way ANOVA were carried out in the PROC MIXED procedure for data of development time, body length and head capsule width. Residuals were tested for normal distribution with the Kolmogorov-Smirnov test at *p*<0.05. Data were log-transformed to meet normality. The GROUP syntax was used to estimate individual covariances for each effect under the assumption of variance heterogeneity. Akaike information criterion (AIC) was used to identify the best model. A Tukey-Kramer test was conducted to analyze significant differences between the treatments at *p*<0.05 using the macro PDMIX800.24,25

3 RESULTS

3.1 Role of plant container size, substrate and environmental conditions for oviposition success

Egg batches were produced in all provided plant containers, substrates, and environments but significant differences were found among treatments (Fig. 2). In both environments, the
The highest number of egg batches (≥ 1.5) per female was found in large pots with field soil. Significantly fewer egg batches were found in pots with other substrates, although no clear pattern could be discerned with regard to the role of pot size and substrate. In environment A, only small pots with field soil contained a median of more than 1 egg batch per female (Fig. 2A). No significant differences were found between large pots filled with either peat-sand or peat-expanded clay substrate. The same was true for small pots with both peat substrates. In environment B, no differences in the number of egg batches (median 0.81) were detected between different substrates in small pots (Fig. 2B). Interestingly, large pots with either peat substrate contained fewer or similar numbers of egg batches than small containers.

**Figure 2** Oviposition of *Pentastiridius leporinus* in potted sugar beet in dependence of pot type, substrate and two environments: (A) 14:10 h light/dark photoperiod, 24:18 °C, 60-80% r.h. and 200 µmol (s m⁻²)⁻¹ and (B) 16:8 h light/dark photoperiod, 20.9 ± 1 °C, 48 ± 12.2% r.h. and 80 µmol (s m⁻²)⁻¹. Small pots (S) with 4.8 cm diameter and large pots (L) with 16 cm diameter were compared (n = 6). Boxes show 25th, 50th and 75th percentiles, whiskers show 10th and 90th percentiles. Treatments with the same letter within a graph are not significantly different according to two-way ANOVA (α = 0.05).

### 3.2 Role of host plant species for adult survival and oviposition

The mortality rates of female adults kept on small (HR: 13.6, Cox proportional hazard model, p < 0.001) and large (HR: 13.2, Cox proportional hazard model, p < 0.001) wheat plants were significantly higher compared to female adults kept on sugar beet (Fig. 3). No statistical differences in mortality were observed on small compared to large wheat plants (HR: 1.04, Cox proportional hazard model, p = 0.93). Median survival times were 6 days on small and large wheat plants and 24 days on sugar beet.
Neither the production of wax filaments on the tip of the female’s abdomen, a characteristic trait of ovipositing *P. leporinus*, nor any egg batches were found in the wheat treatments. In contrast, a maximum of eight oviposition events per planthopper was observed in sugar beet (Table 1). In total, 13 of 17 female adults laid eggs and more than six oviposition events were observed in a single female. Females were observed to lay eggs up to 42 days after eclosion. The mean number of eggs per egg batch was 49.6 ± 31.3 with most eggs per egg batch being laid in the first oviposition event. The largest batch consisted of 186 eggs. A mean of 139.1 ± 132.9 and a maximum of 406 eggs per female adult were observed from 17 females.

**Table 1** Temporal oviposition pattern of *Pentastiridius leporinus* in a no-choice experiment. Data show time after eclosion when egg batches were laid and number of eggs per egg batch for each oviposition event of a single female. Oviposition occurred only in sugar beet plants (*n* = 17), no eggs were laid in wheat.

<table>
<thead>
<tr>
<th>Oviposition event</th>
<th>Days Range</th>
<th>Median</th>
<th>Mean±SD</th>
<th>Min-Max</th>
<th>Median</th>
<th>Mean±SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6-17</td>
<td>11</td>
<td>10.6±3.5</td>
<td>33-186</td>
<td>51</td>
<td>51±44.9</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>10-25</td>
<td>16.5</td>
<td>16.9±4.3</td>
<td>31-77</td>
<td>43.5</td>
<td>46.6±15.4</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>14-24</td>
<td>19</td>
<td>19.4±3.6</td>
<td>37-77</td>
<td>47</td>
<td>49.8±15.9</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>18-32</td>
<td>29</td>
<td>26.6±5.6</td>
<td>18-32</td>
<td>37</td>
<td>40.2±17.5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>29-36</td>
<td>30</td>
<td>31.7±3.8</td>
<td>10-17</td>
<td>13.5</td>
<td>13.5±4.9</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>33-42</td>
<td>37.5</td>
<td>37.5±6.4</td>
<td>8-38</td>
<td>23</td>
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<td>39</td>
<td>-</td>
<td>39</td>
<td>39</td>
<td>1</td>
</tr>
</tbody>
</table>
A continuous mass rearing of *Pentastiridius leporinus* was successfully established. The rearing started with field collected planthoppers in June 2020 and until March 2022 a maximum of five generations and >20,000 *P. leporinus* individuals were produced under controlled climatic conditions. An overlap between generations was observed due to variations in development times, and third to fifth generation insects were reared in March 2022. Crucial points were the transfer of fifth instar nymphs to potted sugar beets with a top layer of expanded clay for metamorphosis (Fig. 4F) to avoid the development of adults inside the containers (Fig. 4C-E). This step prevented losing adult planthoppers due to starvation as they are
probably unable to feed on roots. The nymphs immediately hid between the expanded clay particles after being transferred. Without a top layer of expanded clay metamorphosis failed, as most transferred nymphs left the pot and died within a few days (data not shown). Adult emergence started approximately 4 weeks after the transfer (Fig. 5) and 140 days later 68.7% of the nymphs had developed into adults. The majority of emerging adults were male (59.2%). After 140 days, the recording was stopped since adult emergence had nearly ceased.

![Figure 5](image-url) Accumulated percentage of emerged adults from fifth instar nymphs on potted sugar beet plants which were grown in large pots with a top layer of expanded clay (n = 3). Symbols show mean values, whiskers show standard deviation.

### 3.4 Characterisation of developmental stages

The highest mortality (25.7%) was observed in first instar nymphs (Table 2). Lowest mortality (3.0-3.1%), on the other hand, was found in third and fourth nymphal instars. In total, 56.2% of the analysed specimens from the second generation completed their life cycle on wheat seedlings. With older instars, the average developmental time increased significantly, however no differences were observed between first and second instar. Additionally, the range of developmental time increased with older instars. When comparing both generations, we found that in third instars, development time was significantly longer for insects from the first compared to the second generation (p = 0.011). In all other instars, no statistical differences were observed in development times between the different generations (data not shown). First female and male adults emerged 104 and 145 days after hatching. Body length and head capsule width increased significantly with older nymphal instars (Table 3). In contrast to body length, head capsule width did not overlap between nymphal instars. All nymphal instars produced wax filaments. All developmental stages are depicted in Fig. 6.
Table 2 Larval mortality and duration of developmental stages of *Pentastiridius leporinus* reared on wheat plants at 20.9 ± 1 °C. Insects from two generations were analysed. *n* = number of individuals with known beginning and ending of a developmental stage. SD = Standard deviation. Range indicates minimum and maximum numbers of days per stage. Treatments with the same letter are not significantly different in duration of developmental stages according to one-way ANOVA (α = 0.05).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Mortality [%]</th>
<th>Days</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>Egg batch</td>
<td>-</td>
<td>17-25</td>
<td>22</td>
</tr>
<tr>
<td>First instar</td>
<td>25.7</td>
<td>13-21</td>
<td>16</td>
</tr>
<tr>
<td>Second instar</td>
<td>12.9</td>
<td>13-27</td>
<td>17</td>
</tr>
<tr>
<td>Third instar</td>
<td>3.0</td>
<td>14-49</td>
<td>20</td>
</tr>
<tr>
<td>Fourth instar</td>
<td>3.1</td>
<td>17-203</td>
<td>38.5</td>
</tr>
<tr>
<td>Fifth instar</td>
<td>13.4</td>
<td>17-211</td>
<td>90</td>
</tr>
<tr>
<td>First instar-male adult</td>
<td>-</td>
<td>145-264</td>
<td>185</td>
</tr>
<tr>
<td>First instar-female adult</td>
<td>-</td>
<td>104-354</td>
<td>181</td>
</tr>
</tbody>
</table>

Table 3 Morphometric measurements showing length and width of egg and nymphal stages of *Pentastiridius leporinus*. In nymphs, width was measured from head capsules. Treatments with the same letter are not significantly different according to one-way ANOVA (α = 0.05).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Length [mm]</th>
<th>Width [mm]</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Egg</td>
<td>0.57-0.74</td>
<td>0.65±0.05</td>
<td>0.28-0.36</td>
</tr>
<tr>
<td>First instar</td>
<td>0.74-1.24</td>
<td>0.92±0.13</td>
<td>0.26-0.29</td>
</tr>
<tr>
<td>Second instar</td>
<td>1.11-1.76</td>
<td>1.43±0.18</td>
<td>0.31-0.40</td>
</tr>
<tr>
<td>Third instar</td>
<td>1.60-2.41</td>
<td>2.03±0.22</td>
<td>0.43-0.57</td>
</tr>
<tr>
<td>Fourth instar</td>
<td>1.80-4.20</td>
<td>2.83±0.48</td>
<td>0.60-0.78</td>
</tr>
<tr>
<td>Fifth instar</td>
<td>2.76-5.93</td>
<td>4.26±0.67</td>
<td>0.85-1.22</td>
</tr>
</tbody>
</table>
3. Manuscript I

Figure 6 Development stages of *Pentastiridius leporinus*. (A) Egg batch covered with waxy filaments, (B) single egg, (C) first instar nymph, (D) second instar nymph, (E) third instar nymph, (F) fourth instar nymph, (G) fifth instar nymph, (H) female adult and (I) copulation.

3.5 Infection rate of adult planthoppers

Adults infected with the proteobacterium were detected in the first three generations of the rearing (Table 4). The infection rates (70-80%) were comparable between the different generations. In contrast, no phytoplasma was detected in any of the analysed adults.

Table 4 Infection rate of adult *Pentastiridius leporinus* with the γ-3 proteobacterium (Proteo) *Ca. Arsenophonus phytopathogenicus* and phytoplasma (Phyto) *Ca. Phytoplasma solani* in different generations of the rearing. The ratio of positive to total tested specimens is shown.

<table>
<thead>
<tr>
<th></th>
<th>1&lt;sup&gt;st&lt;/sup&gt; generation</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; generation</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; generation</th>
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<tbody>
<tr>
<td>Proteo</td>
<td>7/10</td>
<td>8/10</td>
<td>8/10</td>
</tr>
<tr>
<td>Phyto</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>
4 DISCUSSION

Here we describe a new protocol for the continuous mass rearing of the planthopper *P. leporinus*. Our study also provides basic life history data on this little-known insect species and a simple method for distinguishing nymphal stages.

As a first step towards developing a suitable rearing method and to obtain an idea which plant container size, substrate or combination of environmental conditions would yield a high number of egg batches per female, an oviposition experiment was performed with field-collected adult planthoppers. We found that it was possible to produce *P. leporinus* eggs in two different environmental settings that represented climate chamber conditions common for rearing insects of the temperate zone. The combination of small pots with six female planthoppers versus large pots with ten female planthoppers did not yield a clear picture, with one exception: the highest number of egg batches was always found in large pots containing field soil. In these pots, egg batches were mostly detected in the gap between substrate and pot wall. Field soil contained many cracks and cavities, while both alternative substrates were rather compacted. Field soil therefore allowed *P. leporinus* females to walk into the cracks for oviposition, which is relevant as *P. leporinus* has not been observed to dig. Although large pots filled with field soil turned out to be superior compared to all other combinations, the important information gained in this trial was that *P. leporinus* also accepted small transparent polypropylene pots with standardized, commercially available substrates for oviposition. The small size of the containers allowed easy handling, while the transparent polystyrene made it possible to regularly monitor the presence of egg batches. Hence, these options were chosen for further mass rearing.

In a second trial, we studied the oviposition pattern and longevity of *P. leporinus* on sugar beet and wheat plants. The results were unequivocal as no eggs were laid at all in pots with wheat and planthoppers feeding on this host plant lived only for around 18 days. Also, no abdominal wax filaments were produced by females caged onto wheat. Such wax filaments were generally found in mature females about to lay eggs in the sugar beet treatment and are a characteristic feature in several cixiid planthoppers.\(^1\)\(^2\)\(^6\)\(^27\) Females offered sugar beet, on the other hand, produced several egg batches with an average of 139 eggs during their entire life span of 51 days. These egg batches contained a mean of 50 eggs. The results are interesting because wheat is a very suitable host for nymphal development and hence the question of why *P. leporinus* females reject this species as an oviposition substrate awaits further exploration. A falsifiable hypothesis could be that wheat phloem sap cannot be exploited by adult *P. leporinus* as an appropriate food source that supports egg production.
In the sugar beet – winter wheat system, nymphs spend most of their life on wheat roots.\(^4\) Hence, the developmental time and mortality of nymphal stages was recorded on this host. We found that highest mortality occurred in first instars at a rate of 25.7\% and declined strongly in instars three and four. However, fifth instar nymphs were more vulnerable again, accounting for 13.4\% in mortality. Remarkably, not only developmental time increased consistently with each nymphal instar but also the time range that nymphs spent in the respective stages. While the first instar stage lasted 13-21 days, fourth and fifth instars varied considerably between 17 and 203 and between 17 and 211 days, respectively. No statistical differences were observed between both generations of fourth and fifth instars, therefore other reasons need to be considered for the large differences in development time of individual nymphs. Since all nymphs were kept under the same environmental conditions, we assume that this trait is genetically determined. Whether it is an adaptive trait, remains to be studied. Overall, male and female planthoppers had the same lifespan and the whole life cycle was completed in approximately seven months under laboratory conditions.

Additionally, morphometric measures of all immature stages were taken. Head capsule width was thereby identified as a trait that lends itself well to unequivocally differentiate between nymphal instars, as no overlap was found for head capsule widths.

With the gained insights, we were able to establish a continuous mass rearing as described above and shown in Fig. 4. We exclusively used sugar beet as a host, which furthermore demonstrates that \(P.\ leporinus\) can complete its life cycle entirely on this plant without the need for alternative hosts such as wheat. From June 2020 until March 2022, we were able to produce a maximum of five generations and over 20,000 nymphs under controlled climatic conditions at constant 20.9 ± 1°C. Obviously, \(P.\ leporinus\) does not require an obligatory diapause, although the species is known to have a univoltine life cycle in Central Europe.\(^2,3\) As a crucial step in our rearing, we found that it was necessary to provide fifth instars with potted sugar beet plants that had a 5 cm layer of expanded clay on top of the peat-sand substrate. This allowed the insects to gain access to the roots and complete their metamorphosis. Attempts to produce adults on sugar beet plants without this top layer of expanded clay had failed. In our experiments, 68.7\% of fifth instar nymphs emerged as adults within 140 days of which the majority (59.2\%) were male. So far, we are unaware of any other protocol for mass rearing of Cixiidae with belowground nymphal stages. Sforza et al. (1999) reported the rearing of epigean nymphs of the related species \(H.\ obsoletus\). However, mass rearing was not possible due to high mortality of the insects.\(^22\)

Adult planthoppers from three consecutive generations were taken from the rearing and tested for the presence of \(Ca.\ Arsenophonus\) phytopathogenicus and \(Ca.\ Phytoplasma\) solani by nested PCR. While the sample size was comparatively low, the emerging picture
was clear, showing that 70-80% of insects from three generations were infected with the proteobacterium. The phytoplasma was absent in all 30 specimens tested. Bressan et al. showed that vertical transmission of the SBR proteobacterium to the next generation is possible. This finding could be confirmed in our studies because the three generations of *P. leporinus* were held separately. In summary, our study provides relevant information on several life history traits of *P. leporinus* and introduces a method for its continuous mass rearing. The presented data will facilitate research on the biology and ecology of this economically important vector insect and can thus critically contribute to the development of integrated pest management strategies.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest. The Kuratorium für Versuchswesen und Beratung im Zuckerrübenanbau had no role in the design of the study, in the collection, analyses or interpretation of data, in the writing of the manuscript or in the decision to publish the results.

Data availability statement

Research data are not shared.

References


4 Bressan A, Moral García FJ and Boudon-Padieu E, The prevalence of *Candidatus Arsenophonus phytopathogenicus* infecting the planthopper *Pentastiridius leporinus*


3. Manuscript I


4. Manuscript II

Effects of succession crops and soil tillage on suppressing the syndrome “basses richesses” vector Pentastiridius leporinus in sugar beet

Short title: Effects of succession crops and soil tillage on Pentastiridius leporinus

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Abstract:

BACKGROUND: *Pentastiridius leporinus* (Hemiptera: Cixiidae) is the most important vector of syndrome "basses richesses" (SBR), a new disease that leads to severe economic losses in sugar beet. In this study, different soil tillage methods (ploughing and cultivator) and crops (winter wheat, spring wheat, spring barley, maize and bare soil) following SBR-infested sugar beet were tested as potential management options in field trials. In the laboratory, the survival and development of first and third instar nymphs on wheat, maize and barley (first instar only) was studied to further assess their suitability as host plants.

RESULTS: In five out of seven field sites, reduced soil tillage had no effect on adult planthopper emergence compared to ploughing. In two sites, reduced tillage resulted in higher emergence rates. In nearly all field sites, up to 98.9% fewer emerging adults were detected in bare soil and maize, when compared to winter wheat. Under laboratory conditions, the lowest survival rate was found in first instar nymphs feeding on maize seedlings (4.2%), while 29.2% survived on barley, and 66.7% on wheat, over a period of 300 days. In contrast, 73.3% and 70% of third instar nymphs survived on wheat and maize over a period of 150 days.

CONCLUSION: Soil tillage had little effect against *P. leporinus*. Maize is a poor host for first instars but a suitable resource for third instar nymphs, the stage which encounters maize under field conditions. Hence, reductions in planthopper emergence in the field were likely caused by starvation due to the long host-free period between sugar beet harvest and the sowing of maize.

Keywords:
Cixiidae, life cycle, host plant, vector control, maize, field trials
1 INTRODUCTION

Syndrome “basses richesses” (SBR) is a sugar beet (*Beta vulgaris*) disease, which leads to severe reductions of up to 5% in absolute sugar content and sugar yield losses up to 25%.\(^1\)\(^-\)\(^3\) Symptoms are chlorosis and necrosis of older leaves, asymmetrically formed younger leaves and necrosis in tap root vascular bundles.\(^1\) Two prokaryotic bacterial pathogens, the γ-3-proteobacterium, ‘*Candidatus Arsenophonus phytopathogenicus*’, and phytoplasma ‘*Candidatus Phytoplasma solani*’ of the stolbur group (16SrXII), are known as causal agents of SBR.\(^1\)\(^-\)\(^7\) The phloem feeding planthopper *Pentastiridius leporinus* L. (1761) (Hemiptera: Cixiidae) is the most important SBR vector in France, Southwest Germany and Switzerland.\(^3\)\(^,\)\(^8\)\(^,\)\(^9\) According to Holzinger et al., the only reported natural host of adult *P. leporinus* is reed (*Phragmites australis*), while the host plant species of the nymphs are still unknown.\(^10\) The host shift towards sugar beet and cereals, winter wheat (*Triticum aestivum*) or spring barley (*Hordeum vulgare*), has probably occurred only recently, as *P. leporinus* had not been reported as a sugar beet pest before its first appearance in eastern France, where it had caused significant economic damage.\(^7\)\(^,\)\(^11\)\(^,\)\(^12\) In early summer, adults migrate into sugar beet fields where females oviposit into the soil. The hatching nymphs first develop on sugar beet roots and, after harvest in early autumn, continue their development on roots of winter wheat, which most frequently follows sugar beet in the crop rotation.\(^7\) Overall, five immature stages have been described for *P. leporinus*. Second to third instar nymphs were found in autumn (September) and third to fifth instar nymphs in spring (May).\(^12\)\(^,\)\(^13\) Bressan assumed that nymphs probably diapause in late autumn and winter due to low temperatures.\(^11\) From the end of May until the beginning of July, the insects complete their development into adults in cereal crops and migrate back into sugar beet fields.\(^7\)\(^,\)\(^12\) So far, *P. leporinus* is known to produce only one generation per year in Central Europe.\(^10\)\(^,\)\(^14\)

The first observation of SBR symptoms in sugar beet was recorded in 1991 in the eastern French regions of Burgundy and Jura and, by 2004, 1,800 ha were infected.\(^2\) According to Schröder et al., the first detection of the γ-proteobacterium in German sugar beet fields occurred in 2009.\(^15\) The estimated area of infestation in 2018 was already 16,400 ha and further spread of this disease was reported by Behrmann et al.\(^8\)\(^,\)\(^16\) In Switzerland, the first SBR disease detection was in 2017 and its fast spread from 3,000 ha to 5,000 ha was reported between 2019 and 2021.\(^9\)\(^,\)\(^17\)\(^,\)\(^18\) Unlike many new invasive pests, *P. leporinus* was not introduced. Instead, the species is native and, with the exception of the northern regions, has a widespread Palaearctic distribution.\(^10\) It is therefore likely that SBR has the potential to affect at least all sugar beet growing areas where *P. leporinus* occurs.
Clearly, there is a strong need to identify vector control measures for this fast-spreading disease as it poses a major threat to sugar beet production. Hence, different soil tillage methods and crops following SBR-infested sugar beet were explored in this study to assess their effects on the vector. Various authors have described agronomic measures, including the cultivation of poor host plants, to successfully control different planthopper and leafhopper species.\textsuperscript{19-21} Indeed, the first preliminary indication that soil tillage and crop rotation may negatively affect \textit{P. leporinus} populations was provided by Bressan who showed that growing spring barley instead of winter wheat after sugar beet led to 81% and 80% reductions in nymphs and emerging adults, respectively.\textsuperscript{11} Furthermore, a 28% reduction in emerging adults in winter wheat was observed following reduced tillage compared to ploughing after sugar beet harvest.\textsuperscript{11} However, data were obtained from one field in a single year only. Hence, we carried out two years of field experiments on a total of nine different locations to obtain further data on the potential of agronomic control measures against \textit{P. leporinus}. Our hypotheses were: i) changes in soil structure due to soil tillage should reduce the number of emerging adults; ii) \textit{P. leporinus} adult emergence can be reduced by planting an appropriate succession crop after sugar beet. iii) the sowing date of the succession crop plays a crucial role in reducing adult numbers.

2 MATERIALS AND METHODS

2.1 Effect of soil tillage and crop rotation on \textit{Pentastiridius leporinus} adult emergence

Field trials were set up in different regions of Southwest Germany (Kirchardt, Neckarsulm, Ilvesheim, Dettenheim, Ladenburg, and Untereisesheim) and in the valley of the river Elbe (Arzberg) after sugar beet harvests in autumn 2018 and 2019. Trial sites were selected according to the following criteria: i) high presence of \textit{P. leporinus} nymphs in the soil, ii) strong and uniform SBR symptoms and iii) uniform field topography. \textit{Pentastiridius leporinus} adult emergence was assessed following different soil tillage treatments (ploughing at depths of at least 20 cm and cultivator treatment at depths up to 20 cm) in combination with various crops cultivated after the sugar beet harvest. In 2019, we compared winter wheat with spring wheat and spring barley, while in 2020, winter wheat was compared with maize and bare soil (Table 1). Thus, we evaluated the effects of two early spring crops (barley and spring wheat) that are sown between end of February to mid/end of March and a late spring crop (maize), sown in mid-April. Bare soil was included as a control to assess the absence of new roots that could be used as a nymphal food source.

In one location (Kirchardt 2019), winter durum (\textit{Triticum durum}) instead of winter wheat was cultivated. In our study, reduced tillage was carried out using a rigid tine cultivator instead of a disc plough applied in the study of Bressan, because this is more commonly used by
In Ladenburg 2019 and Untereisesheim 2019, winter wheat was compared to spring barley; both after reduced tillage. However, in Ladenburg 2019, using a cultivator for soil tillage was impossible due to the dry soil conditions, so a disc harrow was used instead. Agronomic measurements (time of soil tillage, tilling depths) and plant protection methods (use of herbicides and fungicides) were performed according to good agronomic practice in the wheat, barley, and maize plots. Emerging weeds in bare soil treatment plots were controlled with the herbicides Adengo (Bayer CropScience Deutschland GmbH, Monheim, Germany), Glyphosate or with a combination of Laudis (Bayer CropScience Deutschland GmbH), Spectrum and Bo 235 (both BASF SE, Ludwigshafen, Germany) when necessary. Detailed information is given in Tables 1 and 2.

### Table 1 Details of experimental field sites.

<table>
<thead>
<tr>
<th>Field trial site</th>
<th>Coordinates</th>
<th>Sugar beet harvest</th>
<th>Depths and dates of soil tillage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2018/19</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untereisesheim</td>
<td>49°12'54.1&quot;N 9°11'27.0&quot;E</td>
<td>18/9/18</td>
<td>20 cm, 15/10/18</td>
</tr>
<tr>
<td>Ladenburg</td>
<td>49°27'11.5&quot;N 8°37'55.1&quot;E</td>
<td>17/10/18</td>
<td>*10 cm, 17/10/18</td>
</tr>
<tr>
<td>Arzberg</td>
<td>51°30'31.3&quot;N 13°09'46.0&quot;E</td>
<td>2/11/18</td>
<td>22 cm, 8/11/18</td>
</tr>
<tr>
<td>Dettenheim</td>
<td>49°10'54.9&quot;N 8°23'22.5&quot;E</td>
<td>13/10/18</td>
<td>15 cm, 19/10/18</td>
</tr>
<tr>
<td>Kirchardt</td>
<td>49°12'33.0&quot;N 8°58'20.1&quot;E</td>
<td>1/10/18</td>
<td>10 cm, 5/10/18</td>
</tr>
<tr>
<td><strong>2019/20</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arzberg</td>
<td>51°31'07.6&quot;N 13°07'24.9&quot;E</td>
<td>27/9/19</td>
<td>20 cm, 4/10/19</td>
</tr>
<tr>
<td>Neckarsulm</td>
<td>49°12'19.3&quot;N 9°10'35.1&quot;E</td>
<td>25/10/19</td>
<td>15-20 cm, 30/10/19</td>
</tr>
<tr>
<td>Ilvesheim</td>
<td>49°27'38.3&quot;N 8°34'33.7&quot;E</td>
<td>24/10/19</td>
<td>15 cm, 25/10/19</td>
</tr>
<tr>
<td>Dettenheim</td>
<td>49°10'51.3&quot;N 8°23'31.1&quot;E</td>
<td>25/10/19</td>
<td>10-12 cm, 26/10/19</td>
</tr>
</tbody>
</table>

*Disc harrow was used instead of the rigid tine cultivator in the Ladenburg testing site

### Table 2 Dates and depths of seedbed preparation in the different field trial sites. Seedbed preparation was carried out with a rotary harrow at each testing site.

<table>
<thead>
<tr>
<th>Field trial site</th>
<th>Winter wheat</th>
<th>Spring wheat</th>
<th>Spring barley</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2018/19</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untereisesheim</td>
<td>4-5 cm, 16/10/18</td>
<td>4-5 cm, 28/2/19</td>
<td></td>
</tr>
<tr>
<td>Ladenburg</td>
<td>2-3 cm, 16/10/18</td>
<td>2-3 cm, 28/2/19</td>
<td></td>
</tr>
<tr>
<td>Arzberg</td>
<td>4 cm, 8/11/18</td>
<td>4 cm, 4/3/19</td>
<td></td>
</tr>
<tr>
<td>Neckarsulm</td>
<td>2 cm, 8/11/18</td>
<td>2 cm, 4/3/19</td>
<td></td>
</tr>
<tr>
<td>Ilvesheim</td>
<td>3-5 cm, 4/11/18</td>
<td>8 cm, 26/2/19</td>
<td></td>
</tr>
<tr>
<td>Dettenheim</td>
<td>2 cm, 4/11/18</td>
<td>2 cm, 26/2/19</td>
<td></td>
</tr>
<tr>
<td>Kirchardt</td>
<td>5 cm, 8/10/18</td>
<td>5 cm, 13/3/19</td>
<td></td>
</tr>
<tr>
<td><strong>2019/20</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arzberg</td>
<td>4 cm, 26/10/19</td>
<td>8 cm, 14/4/20</td>
<td></td>
</tr>
<tr>
<td>Neckarsulm</td>
<td>4-5 cm, 31/10/19</td>
<td>10 cm, 11/4/20</td>
<td>8 cm, 11/4/20</td>
</tr>
<tr>
<td>Ilvesheim</td>
<td>8 cm, 26/10/19</td>
<td>8 cm: 1/4/20, 8/4/20</td>
<td>8 cm: 1/4/20, 8/4/20</td>
</tr>
<tr>
<td>Dettenheim</td>
<td>3-5 cm, 27/10/19</td>
<td>8-10 cm, 9/4/20</td>
<td>-</td>
</tr>
</tbody>
</table>
The field trial layout used a completely randomized block design. A plan of the layout is provided in Supplementary Fig. S1. In 2019, the trials were designed with three blocks. This was increased to four blocks in 2020, due to the nonhomogeneous nature of *P. leporinus* adult emergence observed in 2019. Plot width was 6 m except at the sites Untereisesheim 2019 (3 m), Ladenburg 2019 (3 m) and Arzberg 2020 (12 m).

### 2.2 Quantification of field-emerged *Pentastiridius leporinus* adults

Three cages (base area approx. 3.6 m² with a 1.5 m maximum height, Supplementary Fig. S2) were set up in each plot to catch *P. leporinus* adults emerging from soil. The cages were placed at 10 m distance. An additional cage per plot was erected in Arzberg in 2020. All cages were covered with a 1.35 mm mesh gauze (FA.BIO 01 Kulturschutznetz Rettichnetz, Hartmann-Brockhaus, Pfaffenhofen-Wagenhofen, Germany) with gauze ends buried in the ground. Inside each cage, a 10 x 25 cm yellow sticky trap (Gelbe Insekten-Leimtafeln, Aeroxon Insect Control GmbH, Waiblingen, Germany) was attached at the top to catch emerging adults. Each sticky trap was replaced once per week. Counting periods of adult *P. leporinus* were 13/5 to 15/7/19 in Arzberg and 14/5 to 8/7/19 in all other field trial sites in 2019, and 29/4 to 19/7/20 in Arzberg and 22/4 to 20/7/20 in all other field trial sites in 2020. Thus, the sampling periods ended when only very low numbers of adults were caught. On 29/6/20 in Neckarsulm, Ilvesheim and Dettenheim and on 30/6/20 in Arzberg, maize plants were cut to a maximum height of 1 m, to avoid the plants reaching the top of the cages and to ensure that emerging adult *P. leporinus* were still attracted to the sticky traps while the maize plants stayed alive.

### 2.3 Identification of *Pentastiridius leporinus*

All field collected planthopper adults were identified to genus level according to their scutellum, vertex, pronotum and hind tarsus, using a stereomicroscope. From each field trial site, a representative sample of 25 male *Pentastiridius* sp. were then identified to species level according to their genital morphology, using the key of Biedermann and Niedringhaus. All analysed insects in the genus *Pentastiridius* sp. were identified as *P. leporinus*, therefore, all *Pentastiridius* sp. were counted as *P. leporinus*.

### 2.4 Performance of planthopper nymphs on different crop plants

The performance (survival and ability to reach the adult stage) of first instar *P. leporinus* nymphs on seedlings of wheat (cv. Dekan, KWS SAAT SE & Co. KGaA, Einbeck, Germany), maize (cv. Ronaldino, KWS SAAT SE & Co. KGaA) and barley (cv. Orbit, KWS SAAT SE & Co. KGaA) was analysed under controlled environmental conditions (21 ± 1°C, 49 ± 11.9% relative humidity, 24 h darkness). Wheat was considered as a control, since it is the crop that
generally follows sugar beet harvest. First instars were chosen as this is the most sensitive stage. In a second experiment, we also compared the performance of third instar nymphs on maize against wheat. The rationale behind this was to find out, whether the suitability of maize as a host plant is dependent on the nymphal age. The planthopper nymphs would encounter maize seedlings in spring, when they are in the third instar or later.¹²

The experiments were carried out in 11 x 8 x 5 cm plastic containers with perforated lids. Containers were filled with 25 g of substrate [3:1 parts mixture of Fruhstorfer Erde Typ P 25 (HAWITA Gruppe GmbH, Vechta, Germany) and sand (diameter 0-2 mm)]. Seedlings were grown in conical 600 mL plastic pots (11 cm upper diameter; 9 cm height) filled with sand (diameter 0-2 mm) under controlled environmental conditions as described above but with a 16:8 h light/dark photoperiod and 80 µmol (s m⁻²)⁻¹ light intensity. Light was provided by full-spectrum LED lights (‘Bioledex GoLeaf E2 LED Pflanzenleuchte Vollspektrum 120 cm 50W IP44’, DEL-KO GmbH, Germany). Seeds were not treated with insecticides or fungicides. The seedlings (4-10 d) were carefully removed from the sand and cleaned with tap water. Seedling roots were placed on top of the substrate to allow the nymphs easy access for feeding. Each container received two seedlings of either wheat or barley or only one seedling of maize due to its larger biomass. A single nymph from our laboratory rearing (described in Pfitzer et al.¹³) was added to each container. Third instar nymphs had been fed on pieces of sugar beet tap root before the experiment. A total of 24 first and 30 third instar nymphs were tested per plant species. Seedlings were replaced when displaying first signs of deterioration or after eight days at the latest. The substrate was kept moist and was replaced after four weeks or earlier, if necessary. The survival and development of nymphs was evaluated at least twice per week for a period of 160 days (first instars) and 150 days (third instars), respectively. Nymphal development was assessed by checking moulting events and head capsule width measurements.¹³

2.5 Statistical analysis

Statistical analysis was carried out using SAS 9.4 (SAS Institute Inc., Cary, USA). The total number of field-emerged *P. leporinus* adults per m² was analysed. The mean values of the emerged adults were calculated from three or four cages within each plot. Those means from three or four plots or blocks per treatment and testing site were then used for a two-way analysis of variance (ANOVA) and tested for their homogeneities of variance and normal distribution. Least square means were determined with LSMEANS in the PROC GLIMMIX procedure separately for each testing site to analyse for significant differences at the p < 0.05 significance level. Block*treatment interactions were used as a random factor. The assumption of the homogeneity of the variances was not fulfilled in the field trials, where the
role of soil tillage and cultivation of winter wheat was compared to spring wheat or to maize and bare soil. To fulfil this assumption, the data were square root transformed to analyse for significant differences. The data were then back transformed to calculate the estimated mean values and standard errors in each treatment and for each testing site. Cox proportional hazard models and PROC PHREG were used in SAS 9.4 for survival analysis of the no-choice performance experiments. Hazard ratios (HR) >1 or <1 describe the higher or lower probabilities of mortality compared to the referred treatment.

3 RESULTS

3.1 Effect of succession crops and soil tillage on adult emergence of *Pentastiridius leporinus*

3.1.1 Role of cultivation of winter wheat compared to barley

On the two testing sites, the emergence of adults from barley was compared to winter wheat (Fig. 1). There were no differences on the first dates of adult emergence (3/6/19) and peaks (24/6/19) on both trial sites (Figs. 1A, 1B). For Untereisesheim 2019, a significantly lower total adult emergence rate of 36.3% ($p = 0.038$) was observed in barley, compared to winter wheat (Fig. 1C). In contrast, a 23.9% increase of *P. leporinus* adult emergence was observed in the barley treatment in Ladenburg 2019 after soil tillage with disc harrows. However, this was not significantly different ($p = 0.488$) from the wheat treatment (Fig. 1D). Details for the statistical analyses of field trials is given in Supplementary Table S1.
3.1.2 Role of soil tillage and cultivation of winter wheat compared to spring wheat

On the three field trial sites, cultivation of spring wheat was compared to winter wheat after soil tillage treatments with plough or cultivator (Fig. 2). The first adults were caught 3/6/19 in Dettenheim, 11/6/19 in Arzberg and 17/6/19 in Kirchardt (Figs. 2A-C). At Arzberg and Dettenheim, the highest numbers of emerging adults were observed 17/6/19 in all treatments, except for 24/6/19 in Dettenheim in the plough and spring wheat treatment. In Kirchardt, emerging adults peaked 24/6/19 in winter wheat and spring wheat after ploughing, and then 1/7/19 in winter wheat and spring wheat after soil tillage by cultivator.

In general, spring wheat cultivation did not lead to significant reductions in *P. leporinus* adult emergence when compared to winter wheat (*p* > 0.05). Only in Arzberg 2019, a significant reduction of 60.7% (*p* = 0.039) was observed in spring wheat after soil tillage with cultivator, when compared to winter wheat (Fig. 2D). In Dettenheim 2019, no differences between crops were found, but ploughing led to statistically significant lower emergence rates than cultivator treatment (*p* = 0.044; Fig. 2E). Very low adult emergence rates were observed in Kirchardt 2019 that did not differ between treatments (Fig. 2F).

**Figure 1** Temporal patterns of *Pentastiridius leporinus* adult emergence at the trials sites: (A) Untereisesheim; and (B) Ladenburg in 2019. Treatments were CW = cultivator + winter wheat, CB = cultivator + spring barley, DW = disc harrow + winter wheat, DB = disc harrow + spring barley. Figures (C+D) show total mean numbers of emerged *Pentastiridius leporinus* depending on trial site and treatment. Bars indicate standard errors. Mean values with the same letter within a site are not significantly different at *p* < 0.05, based on two-way ANOVA.
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Figure 2 Temporal patterns of *Pentastiridius leporinus* adult emergence in the trials sites in 2019: (A) Arzberg; (B) Dettenheim; and (C) Kirchardt. Treatments were PW = ploughing + winter wheat, CW = cultivator + winter wheat, PS = ploughing + spring wheat and CS = cultivator + spring wheat. Figures (D-F) show the estimated total mean numbers of emerged *Pentastiridius leporinus* depending on trial site and treatment. Bars indicate standard errors. Mean values with the same letter within a site are not significantly different at p < 0.05 based on two-way ANOVA. The y-axis scales in the graphs of Kirchardt in 2019 (C, F) differ from the graphs of the other trial sites due to lower numbers of that adults emerged.

3.1.3 Role of soil tillage and cultivation of winter wheat compared to maize and bare soil

*Pentastiridius leporinus* adult emergence in maize was analysed on four field trial sites and on three of these sites additionally in bare soil with no roots of newly developing seedlings. The treatments were analysed after soil tillage treatment from ploughing or cultivator use (Fig. 3). On all field trials sites, the number of adults emerging from maize and bare soil were lower compared to winter wheat. The initial *P. leporinus* adult emergence varied between the different field trial sites (Fig. 3A-D): 4/5/20 in Neckarsulm, 12/5/20 in Dettenheim and Ilvesheim, and 31/5/20 in Arzberg. In general, the peaks of adult emergence in maize and bare soil treatments occurred earlier compared to wheat treatment, followed by a strong decline of emergence.
Figure 3 Temporal patterns of Pentastiridius leporinus adult emergence in the trial sites: (A) Arzberg; (B) Neckarsulm; (C) Ilvesheim; and (d) Dettenheim in 2020. Treatments were PW = ploughing + winter wheat, CW = cultivator + winter wheat, PM = ploughing + maize, CM = cultivator + maize, P- = ploughing + bare soil and C- = cultivator + bare soil. Figures (E-H) show estimated total mean numbers of emerged Pentastiridius leporinus depending on the trial site and treatment. Bars indicate standard errors. Mean values with the same letter within a field trial site are not significantly different at p < 0.05 based on two-way ANOVA. The y-axis scales in the graphs of Dettenheim 2020 (D, H) differ from the graphs of the other trial sites due to lower numbers of adults emerged.

Consistent and significant reductions (p < 0.05) were observed on all field trial sites in the maize and bare soil treatments, compared to winter wheat. The strongest effects were
observed in Neckarsulm 2020 with reductions of 98.3% and 98.9% adult emergences from maize and bare soil, respectively, compared to winter wheat (p < .001), both after soil tillage by cultivator. Exceptions were observed in Arzberg 2020, where the plough-winter wheat treatment was not significantly different from all other treatments and in Dettenheim 2020, where no significant differences were observed between the ploughed winter wheat and cultivator-bare soil treatments compared to all other treatments. In Arzberg 2020, Ilvesheim 2020 and Dettenheim 2020, the reduced emergence in maize compared to wheat were 83.8%, 88.3% and 79%, respectively, after ploughing and 88.5%, 92.4% and 86.2%, respectively, after soil tillage with a cultivator. In Ilvesheim 2020, the mean number of emerged adults was significantly higher in the plough-bare soil treatment, compared to the cultivator-maize treatment (p < 0.033). In Neckarsulm 2020, the number of emerging adults was significantly higher in the plough-bare soil treatment, compared to the plough-maize (p = 0.045) and cultivator-maize (p = 0.032) treatments. In two out of seven field trial sites, a significant reduction in adult emergence was observed in winter wheat after tilling the soil with a plough compared with a cultivator (Dettenheim 2019: -87.9%, p = 0.044 and Neckarsulm 2020: -42%, p = 0.004; Figs. 2E, 3F). On the other sites, no significant differences were found between the soil tillage methods, although the ploughing effect (-39.3% in Arzberg 2019 (Fig. 2D), -44.4% in Arzberg 2020 (Fig. 3E) and -58.4% in Dettenheim 2020 (Fig. 3H)) and differences in the temporal pattern between the soil tillage treatments were generally quite high. Ilvesheim 2020 was the only testing site where the number of emerging adults in winter wheat was increased after ploughing (+18.5%).

3.2 Performance of nymphs on different plant species

3.2.1 Performance of first instar nymphs

The performance of *P. leporinus* first instar nymphs was assessed on three plant species (wheat, barley, and maize). After 300 days, the survival rate was 66.7% in wheat, 29.2% in barley and 4.2% in maize (Fig. 4). Significantly higher mortality rates were found in nymphs kept on barley (Cox proportional hazard model, HR: 2.6, p = 0.025) and maize (HR: 10, p < 0.001) when compared with nymphs kept on wheat. The mortality rates of nymphs feeding on maize were also significantly higher than on barley (HR: 3.7, p < 0.001). Most of the nymphs feeding on maize died at the beginning of the experiment (12.5% survival rate after eight days).
The development of the *P. leporinus* life stages on the different plant species was analysed in detail (Fig. 5). After 169, 121 and 158 days, first adults were observed in wheat, barley, and maize, respectively. Despite the survival rate of nymphs being lower in barley compared to wheat, the development in barley was slightly faster. On maize seedlings, only 8.3% of the specimens reached the second or higher nymphal instars. In addition, most deaths in nymphs on barley occurred in the first and second nymphal instars, whereas instars three to five were less vulnerable.
3.2.2 Performance of third instar nymphs

The performance of *P. leporinus* third instar nymphs feeding on wheat and maize plants was assessed. After 150 days, the survival rate was 73.3% in wheat and 70% in maize (Fig. 6). The mortality rates of nymphs kept on maize seedlings were not significantly different from nymphs kept on wheat (Cox proportional hazard model, HR: 1.113, p = 0.83).
Figure 6 Survival of *Pentastiridius leporinus* third instar nymphs on seedlings of wheat and maize (n = 30) in a no-choice experiment.

The development of third instar nymphs on the plant species was analysed in detail (Fig. 7). After 70 and 84 days first adults were observed in wheat and maize, respectively. Like the survival rate of nymphs, the development was also comparable in both treatments. Thus, 56.7% and 50% of the specimens reached the adult stage on maize and wheat seedlings, respectively.
4 DISCUSSION

SBR is a fast-spreading bacterial disease that poses a serious threat to sugar beet cultivation and the sugar industry in Central Europe. Hence, there is a strong need for developing sustainable vector control measures. In the present study, we analysed if disturbances of the nymphal habitat due to different soil tillage methods or different crop plants succeeding sugar beet affected the emergence of adult *P. leporinus*. No conclusive results were obtained with respect to soil tillage or the planting of various cereals as follow-up crops after sugar beet. However, planting maize or keeping the field fallow led to strong reductions in planthopper populations. Since third instar nymphs developed well on maize, we presume that starvation rather than the unsuitability of maize as a host plant was the cause.

In the present study field experiments were carried out in several locations in southern Germany and in the valley of the river Elbe for two years. We anticipated that changes in the soil structure due to different soil tillage methods would result in changes in the number of
emerging adults. Ploughing, in particular, can transfer sugar beet harvest residuals into deeper soil layers or speed up microbial degradation of harvest residuals, thus depriving the nymphs from food. Yet, we were unable to identify a soil tillage method that gave consistent results on all sites. Mouldboard ploughing led to significant reductions in the number of emerging planthoppers in only two out of seven field sites where wheat was grown when compared to reduced tilling. Nevertheless, a general trend for lower insect numbers was discernible in other sites too. Why ploughing did not lead to equally strong effects in all sites remains unknown. We hypothesize that the effects of soil tillage may depend on the individual field and its soil conditions (e.g., pores or cavities in the soil where nymphs reside) but further research is necessary to assess the role of soil structure and correlated factors. Interestingly, our findings are in contrast to the report of Bressan, in which reduced tillage rather than mouldboard ploughing (25 cm depth) lowered planthopper numbers. However, this experiment was performed only once on a particular field site. Furthermore, reduced tillage was carried out using a rigid tine cultivator with 20 cm maximum tillage depth in our experiments, instead of a disc plough with 10-15 cm depth. Only in Ladenburg 2019, a disc harrow was used instead of rigid tine cultivator before winter wheat and spring barley cultivation, however, this was not related to any other tillage practice. Therefore, both reduced studies on reduced tillage are not entirely comparable.

Our findings confirm previous observations that wheat as a follow-up crop is a very suitable host plant for *P. leporinus.* The highest number of emerging adults in field trials and the best survival and development rates in the laboratory study were found in wheat. Spring wheat cultivation did not result in consistently reduced numbers of emerging adults. Therefore, we conclude that the delayed sowing of spring wheat (end of February to mid-March) did not lead to nymph starvation and a reduction in emerging adults compared to winter wheat cultivation (sown October to November). The reduction in adult emergence observed on one of the two trial sites in spring barley and the lower performance of first instar *P. leporinus* nymphs on barley seedlings may indicate that barley seedlings are a less suitable host compared with wheat. These findings confirm a previous experiment by Bressan, since lower numbers of adult *P. leporinus* were observed in barley as well. However, the effects in the present study were less pronounced.

In contrast to all other treatments, a clear reduction in adult emergence was found in fields where maize was grown after sugar beet or where fields were left fallow. Remarkably, maize was the last sown (mid-April) spring crop in the field trials and no clear differences were observed compared to bare fallow. These findings led to the question whether the late sowing date reduced *P. leporinus* populations due to nymphal starvation or whether maize had to be considered a non-host. Two no-choice tests were therefore carried out to assess
the suitability of maize seedlings in supporting *P. leporinus* development. In the first experiment, neonate nymphs were used to test the most vulnerable stage, while in the second experiment, third instars were assessed. In the latter, neonate nymphs were initially fed on sugar beet until they reached the third instar to simulate conditions in the field. Our results clearly showed that the host suitability of maize depended on the instar to which the seedlings were offered: while nearly all planthoppers died before reaching the adult stage when maize was fed to first instars, the contrary was the case in third instars. Here, almost all individuals emerged as adults, demonstrating that maize is a suitable host for older nymphs. These results also suggest that the low adult numbers found in our field trials were caused by the fact that *P. leporinus* nymphs were deprived of host plants for too long (> 5 months) and had starved to death.

Some individuals of *P. leporinus* also emerged from the bare soil treatment, albeit in very low numbers. Sugar beet harvest residues might have served as food sources for the nymphs here. When the cages were set up in our trials (mid-April 2020), nymphs were found next to sugar beet harvest residues in some spots (data not shown). Moreover, we had demonstrated previously that *P. leporinus* can finish their development exclusively on pieces of beet tap root. Weeds, on the other hand, can be excluded as a potential food source as plots had been treated with herbicides. Further studies should evaluate the role of sugar beet harvest residues in the field to assess their contribution to nymphal survival.

In summary, this study provides information on *P. leporinus* management options in sugar beet cropping rotations and the host suitability of different crop species for *P. leporinus* nymphs. We conclude, that *P. leporinus* is able to survive on all succession crops but some control could be achieved by the cultivation of late sown spring crop species after sugar beet cultivation. However, to avoid bare soil after sugar beet harvest and the associated risk of soil erosion, break crops should be cultivated before late sown crop species. Further studies need to identify break crops that can be integrated into sugar beet crop rotations but at the same time serve as non-host plants for *P. leporinus* control.

**Acknowledgements**

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Conflict of Interest

The authors declare no conflict of interest.

Data availability statement

Research data are not shared.

References


### Supplementary information

**Table S1** Statistical analysis of experimental field sites based on two-way ANOVA. DF= Degrees of freedom.

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Figure S1 Schematic representation of the experimental setup for the field trials; (a) in 2019; and (b) in 2020. Rectangles show the position of the cages.
Figure S2 Representation of the cages to catch *Pentastiridius leporinus* adults emerging from the soil.
5. Manuscript III

Molecular detection of *Pentastiridius leporinus*, the main vector of the syndrome ‘basses richesses’ in sugar beet

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Simple Summary: *Pentastiridius leporinus* is the main vector of a new and fast spreading disease, the syndrome ‘basses richesses’ (SBR) in sugar beet. SBR causes high sugar content and yield losses in Central Europe. Monitoring of this insect vector based on morphological identification is challenging as two other cixiid species *Reptalus quinquecostatus* and *Hyalesthes obsoletus* with similar external characters are known to additionally appear in sugar beet fields. In this study, a PCR-based method is provided for simple and reliable detection of *P. leporinus* collected via sweep nets and sticky traps. This method also detects eggs and all nymphal stages and differentiates this vector from the most common Auchenorrhyncha species occurring in sugar beet fields. Furthermore, the phylogenetic relationship of these morphologically close cixiid species was investigated based on the mitochondrial cytochrome oxidase I gene (*COI*).

Abstract: Monitoring of *Pentastiridius leporinus* (Hemiptera: Auchenorrhyncha: Cixiidae), representing the main vector of the syndrome ‘basses richesses’ (SBR) disease in sugar beet is based on morphological identification. However, two other cixiid species, *Reptalus quinquecostatus* and *Hyalesthes obsoletus* with similar external characters are known to appear in sugar beet fields and are challenging to be distinguished from *P. leporinus*. We present a PCR-based method for species-specific detection of both male and female *P. leporinus*, directly after sweep net collection or after up to 18 months long term storage on sticky traps. Two methods of DNA template preparation, based on a commercial extraction kit or on simple grinding in phosphate-buffered saline (PBS) were compared. The latter method was also established for eggs and all five nymphal instars of *P. leporinus* from a rearing. Furthermore, *in silico* primer analysis showed that all Auchenorrhyncha species including far related species reported from sugar beet fields can be differentiated from *P. leporinus*. This was PCR-confirmed for the most common Auchenorrhyncha species from different German sugar beet fields. Sequence analysis of the *P. leporinus* mitochondrial cytochrome oxidase I gene (*COI*) amplicon showed a close relationship to *COI* from *P. beieri* but separated from the *Reptalus* and *Hyalesthes* species which are grouped into the same family Cixiidae. We present a sensitive, cost- and time-saving PCR-based method for reliable and specific detection of eggs and all nymphal instars, as well as male and female *P. leporinus*, after different methods of planthopper collection and template DNA template preparation that can be used in large scale monitoring assays.

Keywords: Cixiidae; mitochondrial cytochrome oxidase I; phylogeny; simple DNA preparation; species-specific primers
1. Introduction

The syndrome ‘basses richesses’ (SBR) is a fast-spreading sugar beet (*Beta vulgaris*) disease leading to up to 5% absolute sugar content loss and severe yield reduction of the taproot [1–3]. Since the first report in 1991, a fast spread of SBR occurred in eastern France, and 1800 ha were infected in 2004 [4]. The first detection of SBR in German sugar beet fields was in 2009, whereas the estimated area of infestation was more than 16,000 ha in 2018 [5]. A further spread of the disease into additional sugar beet growing regions of Germany was reported by Behrmann et al. [6]. Additionally, SBR appeared in sugar beet fields (5000 ha) in Switzerland in 2021 [7]. Two SBR causal agents were described, the γ3-proteobacterium ‘*Candidatus Arsenophonus phytopathogenicus*’ (here called: ‘SBR proteobacterium’) and the stolbur phytoplasma (16SrXII group) ‘*Candidatus Phytoplasma solani*’ [2,4,8–10]. Both pathogens are exclusively transmitted by planthoppers [1,3].

The most important SBR vector in sugar beet fields is *Pentastiridius leporinus* (Hemiptera: Auchenorrhyncha: Cixiidae), due to its high population densities, infection rates and the ability to transmit both pathogens to sugar beet plants [1,3,5,10,11]. Additionally, female adult *P. leporinus* can vertically transmit the SBR proteobacterium to their offspring [12]. This allows maintenance of the pathogen over various generations in a rearing under lab conditions [13]. The only known natural host plant of *P. leporinus* is reed (*Phragmites australis*) [14]. Recently, *P. leporinus* host-shifted to sugar beet and winter wheat (*Triticum aestivum*) or barley (*Hordeum vulgare*) crop rotations. This phenomenon together with increasing *P. leporinus* populations led to a significant SBR spread [8,15,16].

Several Auchenorrhyncha species from various taxonomic families and subfamilies have been collected in sugar beet fields [5,11,17]. Among them, there are two closely related cixiid planthoppers. Sémétey et al. [11] reported, that adult *Reptalus* sp. were present in sugar beet fields in the French regions, Burgundy, and Franche-Comté. In German field studies in Baden-Württemberg in 2018, *R. quinquecostatus* was the most common cixiid planthopper species after *P. leporinus* [5,17]. No other species besides *R. quinquecostatus* were found in sugar beet within the genus *Reptalus*. These observations were confirmed by sampling from different sugar beet fields in Baden-Württemberg in 2019 and 2020 [17,18].

The second species, *Hyalesthes obsoletus* (Hemiptera: Cixiidae) is an important vector for several plant diseases e.g., “bois noir” [19–21], potato stolbur disease [22], lavender decline [23] and the maize redness disease [24] by transmission of Ca. P. solani. Sémétey et al. [11] and Bressan et al. [1] reported this planthopper species from French sugar beet fields and demonstrated that *H. obsoletus* is a potential vector of the SBR disease under controlled environmental conditions. *H. obsoletus* was collected from different sugar beet fields in
Germany (Baden-Württemberg) between 2018 and 2020 [5,17,18]. *R. quinquecostatus* and *H. obsoletus* can be hardly distinguished from *P. leporinus* by morphological traits (Figure 1). These three species have hyaline or transparent wings, the fore wings are characterised by a roof-shaped resting position, the mesonotum has five keels and the absence of a post-tibial calcar at the hind legs [14]. These species are also closely related in a phylogenetic analysis based on morphological traits [25]. Due to the fast spread of the SBR disease, *P. leporinus* monitoring is much needed. Usually, adult Auchenorrhyncha collection is carried out with sweep netting or sticky traps [26]. *P. leporinus* eggs or nymphs can be directly collected from soil [12]. Sticky traps represent an important tool to monitor the vector spread, but glue removal and species classification of planthoppers by morphological traits are time-consuming and error prone [27,28]. Further problems of traditional species identification are the need for highly skilled and experienced personnel [28,29]. This clearly limits the throughput in practical monitoring [28]. More, morphological keys for *P. leporinus* identification at the species level are exclusively described for male adults [14,30]. To our knowledge, a morphological method is lacking to discriminate female adults or immature *P. leporinus* including eggs from other cixiid species. Molecular methods can be used to support or substitute morphological species identification [27,28,31,32]. Hebert et al. [33] established the use of DNA barcoding based on mitochondrial cytochrome oxidase I gene (*COI*) sequences for taxonomic insect identification. The *COI* gene was used for identification of planthoppers in the genera *Reptalus* or *Hyalesthes* using species-specific primers [27,29], for sequence analysis of several cixiid species including *P. leporinus*, *R. quinquecostatus*, and *H. obsoletus* [16,27,29] or for phylogenetic analysis of cixiid and delphacid planthoppers including *P. leporinus*, *Reptalus cuspidatus*, and *H. obsoletus* [34].

Figure 1 Morphology of three close cixiid planthoppers showing hyaline or transparent wings, the fore wings are characterised by a roof-shaped resting position, and the mesonotum has five keels. (A) *Pentastiridius leporinus*, (B) *Reptalus quinquecostatus*, (C) *Hyalesthes obsoletus*. Scale bar represents 1 mm.
The aim of this study was to establish a species-specific, inexpensive and time-saving PCR detection for *P. leporinus* eggs, immature stages and both male and female adults allowing differentiation from two other closely related species (*R. quinquecostatus* and *H. obsoletus*). In addition, sequence analysis showed that the designed primers enable differentiation of *P. leporinus* from all other Auchenorrhyncha species that have been described from sugar beet fields, including morphologically and taxonomically close as well as distantly related species. Furthermore, two common sources of insect collections (sweep netting with direct preservation or sticky trap collection) and two methods of template DNA preparation were evaluated. The evolutionary relationships based on the *P. leporinus* partial sequence of the *COI* gene confirmed the relationship between closely and distantly related Auchenorrhyncha species.

2. Materials and Methods

2.1. Planthopper Collection and Morphological Identification

Closely related cixiids (adult *P. leporinus*, *R. quinquecostatus*, and *H. obsoletus*) were field collected with sweep nets or yellow sticky traps during summer 2020 from several locations in Germany (Baden-Württemberg, Rhineland-Palatinate, and Saxony). Morphological identification of the sweep net collected insects was carried out within 24 h after collection. Sticky traps 10 cm × 25 cm (‘Gelbe Insekten-Leimtafeln’, Aeroxon Insect Control GmbH, Waiblingen, Germany) were collected after seven days and transferred into polypropylene cards (‘office discount Sichthüllen DIN A4 glasklar glatt 0,12 mm’, office discount GmbH, Neufahrn bei München, Germany). Sticky trap collected specimens were stored on the traps for 14–18 months (long term) at room temperature (15–25 °C) before morphological identification was carried out.

The most common Auchenorrhyncha species reported from German sugar beet fields (species are provided in Section 2.4) were collected during summer 2020 and stored on sticky traps for 1–2 weeks (short term) before morphological identification was performed [17].

Morphological identification of planthoppers was carried out with a stereomicroscope according to the taxonomic key of Biedermann & Niedringhaus [30]. Family and genus of individual female adult specimens were identified by observation of wings, pronotum, mesonotum, postnotum, and tarsus. Furthermore, the genital structures of male adults were evaluated to allow morphological identification at the species level. Hereafter, sweep net collected specimens were preserved in 96% ethanol and at ~20 °C and sticky trap collected specimens with glue attached were preserved in 60% or 70% ethanol at room temperature until further use. Additionally, *P. leporinus* eggs and all five nymphal instars were obtained.
from a rearing on sugar beet [13]. Developmental stages of nymphs were determined under a stereomicroscope according to the key of Pfitzer et al. [13], before specimens were preserved in 96% ethanol at −20 °C until further use.

2.2. Template DNA Preparation

Detailed information about experimental samples is provided in Supplementary Table S1. Insect DNA templates were obtained either by using ‘DNeasy Blood & Tissue Kit’ (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions or simply by crushing the insects in phosphate-buffered saline (PBS) as described by Priti et al. [35] with slight modifications. Individual insects were transferred into 1.5 mL microcentrifuge tubes with 60 μL (Stictocepha la bisonia adults: 120 μL) or 30 μL (eggs and nymphs) PBS (pH 7.4), then crushed with a sterile micropestle and incubated at 100 °C for 10 min. Additionally, the tubes were centrifuged for 10 min with 13,500×g at room temperature. The supernatant (template DNA concentrations are provided in Section 2.3) was used as a PCR template. DNA quality and quantity were analyzed with a spectrophotometer (‘DeNovix DS-11’, DeNovix Inc., Wilmington, DE, USA). To avoid DNA contamination between samples, we used a single undamaged insect for DNA preparation. Furthermore, to avoid DNA degradation, DNA extracts by means of DNeasy Blood & Tissue Kit were diluted in AE buffer and PBS extracted DNA were used in a short time, within a week.

2.3. Primer Design and PCR Conditions

COI sequences of P. leporinus, R. quinquecostatus, and H. obsoletus were obtained from the NCBI database (National Center for Biotechnology Information, U.S. National Library of Medicine, Rockville Pike, MD, USA) and multiple-aligned with the software BioEdit 7.2 [36] for primer design. COI sequences of P. leporinus were also compared to each two additional representative taxonomically close Reptalus and Hyalesthes species (R. melanochaetus, R. panzeri, H. luteipes, and H. scotti) for species-specific primer design. The specific P. leporinus fw1 and rv1 primers were designed to have no miss-match with the COI gene of P. leporinus but show miss-match with the COI gene of the closely related species.

Furthermore, the specificity of the designed primers was tested in silico on all Auchenorrhyncha species reported to occur in sugar beet fields [5,11,17] for which COI sequences were available at the NCBI database. A list of primers (Table 1) is provided.
Table 1 List of primers used for partial amplification of mitochondrial cytochrome oxidase I gene (COI) from *Pentastiridius leporinus*, *Reptalus quinquecostatus* and *Hyalesthes obsoletus*.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>PCR-Product Size [bp]</th>
<th>Reference</th>
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<tr>
<td>Ron (fw)</td>
<td>GGATCACCTGATATAGCATTCCC</td>
<td>~1000</td>
<td>Argüello Caro [37]</td>
</tr>
<tr>
<td>Calvin (rv)</td>
<td>GGRAARAAWGTAAARTTWACTCC</td>
<td>~1000</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. leporinus</em> fw1 TATTGCAGTACCAACAGGT</td>
<td>341</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. leporinus</em> rv1 TGTTGAATTTACTCCGTAATATAGTAAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UEA3 (fw)</td>
<td>TATAGCATTCCACGAATAAATAA</td>
<td>~1000</td>
<td>Lunt et al. [38]</td>
</tr>
<tr>
<td>UEA8 (rv)</td>
<td>AAAATGTTGAGGAAAAATGTTA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Species-specific PCR reactions with *P. leporinus* fw1 and rv1 primers were carried out in a final volume of 20 μL, consisting of 10 μL ‘DreamTaq PCR Master Mix (2X)’ (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 μM of each primer and 13–27 ng (eggs), 80–270 ng (nymphs), 25–150 or 2–8 ng (sweep net or sticky trap collected adults after kit extraction), and 7–73 ng (adults after preparation in PBS) template DNA. PCR conditions were 98 °C for 2 min, 30 cycles at 95 °C for 30 s, 56 °C for 25 s and 72 °C for 25 s and a final step at 72 °C for 10 min.

A ~1000 bp fragment of the COI gene was amplified with primers Ron and Calvin [37] and used as a control for DNA quality. Another PCR protocol was used for amplification of a ~1000 bp fragment of the COI region from *R. quinquecostatus* and *H. obsoletus* with primers UEA3 and UEA8 according to Lunt et al. [38]. PCR reactions were carried out in a mixture with a final volume of 20 μL, consisting of 10 μL DreamTaq PCR Master Mix (2X), 0.5 μM of each primer and the same (UEA3 and UEA8) or double (Ron and Calvin) template DNA concentrations compared to species-specific PCR (described above). Thermocycling conditions consisted of 95 °C for 2 min, 35 cycles at 95 °C for 30 s, 51 °C (Ron and Calvin) or 54 °C (UEA3 and UEA8) for 30 s and 72 °C for 75 s and a final 72 °C step for 10 min.

PCR products were separated on 1 % agarose gels and stained with ‘Gelred’ (Biotium, Landing Pkwy, CA, USA) next to a ‘GeneRuler 1 kb DNA ladder’ (Thermo Fisher Scientific, Waltham, MA, USA). PCR products were sequenced (Microsynth Seqlab GmbH, Göttingen, Germany) and the data were used in phylogenetic analysis. Furthermore, COI sequences were aligned to sequences from the NCBI database to support morphological determination (see section 2.1).

2.4. Application to Adult and Immature Specimens

The specificity of *P. leporinus* fw1 and rv1 primers was tested on both male and female adults of *P. leporinus*, *R. quinquecostatus*, and *H. obsoletus* using the two template preparation methods. Furthermore, these primers were also tested for detection of eggs and all nymphal instars of *P. leporinus* after PBS template preparation. For these assays, we had
no access to *R. quinquecostatus* and *H. obsoletus* immature specimens, so only adults were used as the negative control.

Additionally, PCR specificity tested for the most common Auchenorrhyncha species reported from German sugar beet fields [17] including morphologically and taxonomically close and distant species from various families (Cixiidae, Delphacidae, Membracidae, and Cicadellidae). These species included: *P. leporinus*, *Empoasca pteridis*, *Empoasca affinis*, *Cicadula placida*, *Orientus ishidae*, *R. quinquecostatus* (closely related), *Psammotettix alienus*, *Empoasca decipiens*, *Fieberiella florii*, *Javesella pellucida*, *S. bisonia*, and *Javesella obscurella*. *J. obscurella* (family: Delphacidae, 20th most common Auchenorrhyncha species from sugar beet) was added, due to absence of *COI* mismatches on the 3′ end with *P. leporinus* fw1 primer (see below).

### 2.5. Evolutionary Relationships

The amplified part of the *COI* (341 bp in size) of *P. leporinus* was sequenced and applied for BLAST search. Ten representative entries from *Pentastiridius* spp., *Reptalus* spp., and *Hyalesthes* spp. were selected to test their phylogenetic relationship using the neighbour-joining method [39]. *Catonia carolina* (family: Achilidae) and *Tettigometra virescens* (family: Tettigometridae) were used as outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [40]. Furthermore, evolutionary divergence between sequences was estimated and the number of base substitutions per site from between the sequences is shown. The evolutionary distances were computed using the Maximum composite likelihood method [41] and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analysis was conducted using MEGA X [42]. Similarly, the part of the *COI* gene (ca. 1000 bp depending on the species) amplified from *P. leporinus*, *R quinquecostatus*, and *H. obsoletus* using universal primers, was sequenced, and used in a BLAST search. Each one representative *COI* sequence from the NCBI database per Auchenorrhyncha family and subfamily reported from sugar beet fields [17] was aligned and used for phylogenetic analysis, including another taxonomically close family Delphacidae [43]. This was to show whether the amplified *COI* sequence is helpful to group these closely and far related species.
3. Results

3.1. Species-Specific Primer Design

*In silico* analysis was conducted to test the specificity of the newly designed *P. leporinus* fw1 and rv1 primers towards the COI gene of various species within the genera *Pentastiridius*, *Reptalus*, and *Hyalesthes* available from the NCBI database. No mismatches to the primers were observed for the different *P. leporinus* sequences (FN179289, FN179288, Figure 2A). However, one to four mismatches to the forward and three to nine mismatches to the reverse primer were observed in the sequences of each three *Reptalus* and *Hyalesthes* species, respectively. Each sequence displayed at least one mismatch at the 3′ ends of both primers and the mismatches accumulated at the 3′ ends (Figure 2A). The primer positions on *P. leporinus* COI are displayed in Figure 2B. *In silico* a 341 bp PCR product was obtained.

![Figure 2 Alignment of the specific primers (P. leporinus fw1 and rv1) to the COI gene of Pentastiridius leporinus and different members of Reptalus spp. and Hyalesthes spp. (A) Dots mark identical nucleotides in the specific primers and the analyzed sequences. Asterisks mark the positions of conserved nucleotides within primer sequences. Nucleotide mismatches between primers and analyzed sequences are highlighted with letters and numbers indicated for each sequence. (B) The schematic map represents the location of specific primers on the partial COI gene. Arrows represent the locations of the primers which amplify a 341 bp fragment (light blue) on *P. leporinus* COI sequence available from the NCBI database (dark blue).](image)

Alignment of the specific primers to the COI gene of various Auchenorrhyncha genera or species, reported from sugar beet fields, showed 2 to 14 mismatches to the fw1 primer and 3 to 23 mismatches to the rv1 primer (Supplementary Figure S1). Most of the mismatches occurred at the primers 3′ end. Two exceptions (*J. obscurella* and *N. campestris*), where the mismatches to *P. leporinus* fw1 primer were not located at the 3′ ends, were observed.
However, seven and ten mismatches, respectively, were observed for these two species to P. leporinus rv1 primer and at least two of the mismatches were located at the 3’ ends. Therefore, distantly related Auchenorrhyncha species from sugar beet fields may not be detected with these specific primers.

**Figure 3** Alignment of the universal primers (Ron/Calvin and UEA3/UEA8) to P. leporinus, R. quinquecostatus, and H. obsoletus COI sequences and primer location within the COI gene. Alignment of (A) Ron/Calvin and (B) UEA3/UEA8 primers to the COI gene of P. leporinus, R. quinquecostatus, and H. obsoletus. Dots mark identical nucleotides in the primers and the analyzed sequences. Asterisks mark the positions of conserved nucleotides within primer sequences. Nucleotide mismatches between primers and analyzed sequences including numbers are indicated for each sequence. (C) P. leporinus, R. quinquecostatus, and H. obsoletus COI schematic maps. Arrows represent the locations of the primers on the COI gene and show that Ron and UEA3 as well as Calvin and UEA8 partly overlapped. The green color shows the fragment that was amplified and sequenced in this study for each species. The blue color shows the available sequence from the NCBI database.

Furthermore, the universal COI primer pairs Ron/Calvin and UEA3/UEA8 were aligned to the P. leporinus, R. quinquecostatus, and H. obsoletus COI sequences. Ron and Calvin primers were used for molecular detection of cixiids according to Urban et al. [44] and UEA3 and UEA8 primers were designed for general COI amplification of hemipteran insects [38]. The numbers and positions of mismatches are shown in Figure 3A,B. Primer positions on the COI
sequences are represented in Figure 3C. Ron and Calvin primers each had a maximum of one mismatch with *P. leporinus*, *R. quinquecostatus*, and *H. obsoletus* COI. UEA8 primer had three mismatches with *P. leporinus* COI (one mismatch on the next-to-last nucleotide at the 3’ end, Figure 3B) which is expected to interfere with PCR amplification (Figure 3C).

### 3.2. PCR Validation on Adult Planthoppers

The specificity of *P. leporinus* fw1 and rv1 primers was tested on DNA templates, prepared with a DNeasy Blood & Tissue Kit, from male and female adult *P. leporinus*, *R. quinquecostatus*, and *H. obsoletus*. In the specific *P. leporinus* PCR, 100% of the *P. leporinus* specimens and no unspecific sample were detected (Figure 4). However, in the general PCR using universal primers (Ron and Calvin), for both sweep net and sticky trap collected specimens, all samples were detected. Notably, 25% of sticky trap collected insects produced only weak bands. Furthermore, in the general COI PCR using UEA3 and UEA8 primers, no DNA amplification was observed for *P. leporinus* specimens but 100% of the *R. quinquecostatus* and *H. obsoletus* specimens produced amplicons. However, 50% of the PCR products obtained from sticky trap collected insects were rather weak (Supplementary Figure S2).

![Figure 4](image-url)

**Figure 4** Electrophoretic patterns of PCR products show specific *P. leporinus* detection. Total DNA was extracted from adult specimens collected from either sweep nets (A1, B1, C1) or sticky traps (A2, B2, C2) using a blood and tissue kit. In panels (A1 and A2), universal Ron and Calvin primers, in panels (B1 and B2), specific *P. leporinus* primers and in panels (C1 and C2), universal UEA3 and UEA8 primers were used for PCR. Lanes 1, 2 represent *P. leporinus* male adult samples; 3, 4 *P. leporinus* female adult; 5, 6 *R. quinquecostatus* male adult; 7, 8 *R. quinquecostatus* female adult; 9, 10 *H. obsoletus* male adult; 11, 12 *H. obsoletus* female adult; C: Negative control (water). The sizes of amplicons are shown on the left side and compared with 1 kb ladder (L).
Amplification of COI fragments from PBS extracts is shown in Figure 5. PBS extracts had a lower quality, compared with DNeasy Blood & Tissue Kit DNA extracts (data not shown). A part of the COI was amplified from 75% of the sweep net and 100% of the sticky trap collected specimens in the general COI PCR with Ron and Calvin primers, however 25% of the sticky trap collected samples produced weak bands. In specific P. leporinus PCR, 100% of P. leporinus specimens and none of the other samples were detected. In the general COI PCR with UEA3 and UEA8 primers, DNA from none of P. leporinus and 75% (sweep net collected) or 100% (sticky trap collected) of R. quinquecostatus and H. obsoletus samples were amplified. However, most of the sticky trap collected samples produced rather weak bands. The obtained COI sequences in this study from P. leporinus, R. quinquecostatus, and H. obsoletus using universal primers were aligned and the consensus sequences were submitted to the NCBI database (accession numbers ON094072, ON094073, and ON210854).

**Figure 5** Electrophoretic patterns of PCR products show specific P. leporinus detection from PBS extracts. Adult specimens were collected from either sweep nets (A1, B1, C1) or sticky traps (A2, B2, C2). In panels (A1 and A2), universal Ron and Calvin primers, in panels (B1 and B2), specific P. leporinus primers and in panels (C1 and C2), universal UEA3 and UEA8 primers were used for PCR assay. Lanes 1, 2 represent P. leporinus male adult samples; 3, 4 P. leporinus female adult; 5, 6 R. quinquecostatus male adult; 7, 8 R. quinquecostatus female adult; 9, 10 H. obsoletus male adult; 11, 12 H. obsoletus female adult; C: Negative control (water). The sizes of amplicons are shown on the left side and compared with 1 kb ladder (L).

### 3.3. Detection of Immature Life Stages of P. leporinus

The COI was amplified from all immature P. leporinus specimens, including eggs and all five nymphal stages, using the universal Ron and Calvin primers and specific P. leporinus primers (Figure 6). No DNA was amplified from immature specimens using UEA3 and UEA8
primers. In general, single, and clear bands with the expected product size were obtained for all specimens with specific primers.

![Figure 6 Electrophoretic patterns of PCR products showing detection of P. leporinus eggs and nymphs from PBS extracts.](image)

(A) L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 C

(B) L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 C

(C) L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 C

3.4. Detection of Distantly Related Species from Sugar Beet Fields

The specificity of *P. leporinus* primers was tested on the most common Auchenorrhyncha species from German sugar beet fields including closely and distantly related species. No DNA was amplified from other species besides *P. leporinus* with specific *P. leporinus* PCR (Supplementary Figures S2 and S3). In general COI PCR with Ron and Calvin primers, a part of the COI gene was amplified from *P. leporinus*, *R. quinquecostatus*, *H. obsoletus*, *F. florii*, *J. pellucida*, and *J. obscurella* specimens. The obtained COI sequences in this study of *F. florii* and *Javesella* sp. were aligned, and the consensus sequences were submitted to the NCBI database with the accession numbers OP090544, OP068197, and OP103664. In the general COI PCR with UEA3 and UEA8 primers, DNA from *R. quinquecostatus*, *H. obsoletus* and one *P. alienus* specimen was amplified.

3.5. Evolutionary Relationships

The phylogenetic relationship of morphologically closely related planthoppers was analyzed based on partial *P. leporinus* COI sequence amplified with specific primers and NCBI COI sequences of various species from the genera *Pentastiridius*, *Reptalus*, and *Hyalesthes*. 
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(Figure 7). The aim was to test whether the specifically amplified COI fragment is sufficient to differentiate those closely related species. Members of the three species clearly separated to different main branches of the phylogenetic tree, confirming morphological differences. Based on this analysis, two *P. leporinus* specimens from Russia (FN179288) and France (FN179289) were phylogenetically closest to the German collections and *P. beieri* was the closest species to *P. leporinus* in this study. Thus, intraspecific genetic distance to *P. leporinus* from Russia (0.0) and France (0.6) was lower than interspecific distance to *P. beieri* (5.1) (Supplementary Table S2). Therefore, the specifically amplified COI fragment was variable enough to differentiate *Pentastiridius* spp., *Reptalus* spp., and *Hyalesthes* spp. from each other.

![Phylogenetic tree](image)

**Figure 7** Evolutionary relationships of selected members of Cixiidae based on the partial COI sequence amplified from *P. leporinus* using specific primers in this study and COI sequences from the NCBI database of closely related species from genus *Pentastiridius* and each three species from two taxonomically close genera *Reptalus* and *Hyalesthes*. The sequence obtained in this study is shown in bold. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The specifically amplified COI fragment is differentiating the closely related species. *Catonia carolina* from the Achilidae family and *Tettigometra virescens* from the Tettigometridae family were used as outgroups.

The phylogenetic relationship of closely and distantly related Auchenorrhyncha species reported from sugar beet fields based on the COI sequence amplified with universal primer pairs showed that *P. leporinus*, *R. quinquecostatus*, and *H. obsoletus* are closely related and grouped into Cixiidae (Supplementary Figure S4). This confirms the close morphological features for these species. Additionally, these COI sequences were useful to clearly differentiate Cixiidae members from Delphacidae and all other representatives from different Auchenorrhyncha families and subfamilies reported in sugar beet fields.
4. Discussion

DNA barcoding is a well-established method for insect species identification [31]. It is based on the COI sequence comparison with database sequences [31,32]. In addition, insect sequences from internal transcribed spacers (ITS) or 5.8S-ITS2 rDNA are used for species-specific detection [27,28,45]. Species-specific molecular detection methods are rapid and cost-saving compared to analysis of morphological traits and reduce the risk of misidentification [31,46]. In the presented study, species-specific primers were designed on highly conserved parts of the COI gene of the target species as the COI gene was variable enough to distinguish P. leporinus from all other Auchenorrhyncha species reported from sugar beet fields. Supporting our approach, several studies demonstrated that the COI gene was exclusively and successfully used for species-specific insect detection. For example, the COI gene was used for species-specific detection of Reptalus spp. [27], Hyalesthes spp. [29], Trissolcus japonicus [46], and Hishimonus spp. [47].

In this study, a specific PCR assay was established to detect the main vector of the SBR disease in sugar beet. The method can be applied to detect P. leporinus and discriminate this insect from other morphologically closely related cixiids including R. quinquecostatus and H. obsoletus [25]. Additionally, the in silico analysis demonstrated that other more distantly related Auchenorrhyncha species, reported from sugar beet fields, will not be detected due to missing target sequence similarity. Supporting the in silico analysis, P. leporinus was differentiated from the most common Auchenorrhyncha species reported from German sugar beet fields, including taxonomically distantly related species such as Empoasca spp., F. florii or C. placida.

Immature stages represent the longest time-period of the P. leporinus life cycle [8] and morphological description as well as taxonomic keys are missing to precisely discriminate P. leporinus immature stages from other cixiids. Molecular methods have been used to identify the immature stages of insects which also expands the monitoring period of insect vectors [29,31]. Similarly, Figure 6 shows that the developed protocol allows detection of all P. leporinus immature stages.

We provide a PCR method that reliably (100% detection rate of P. leporinus specimens) detects both male and female P. leporinus, either from sweep net or sticky trap collection, even if the insects were preserved in 96% ethanol at −20 °C within 24 h after sweep net collection or stored for a short (1–2 weeks) or long time (up to 18 months) on the sticky traps at room temperature before they were preserved in 60 or 70% ethanol. Sticky trap collected specimens were successfully detected without removing sticky trap glue from the insect bodies. Additionally, we established this method with a simple and time saving DNA
preparation by grinding specimens in PBS. PBS extracts were successfully used for specific detection of all insect life stages including eggs, nymphs, and adults. Thus, this simple and cheap method is suitable for large scale monitoring assays. Furthermore, sequencing of PCR products is not required due to the species-specificity of this protocol.

The published universal primers (Ron and Calvin) allow the detection of *P. leporinus* only after sequencing the PCR products which is time consuming. In addition, the amplicons for some samples are low in concentration possibly due to the degeneracy of primers. With the lower quality of template DNA in PBS extracts, this degeneracy resulted in weaker signals. Due to the 100% amplification rate of the analyzed *P. leporinus* samples with specific primers, the provided specific primers are more efficient and precise, compared to universal PCR with Ron and Calvin primers. The Ron primer was originally designed for general amplification of lepidopterans, dipterans, coleopterans, thysanopterans, hemipterans, and homopterans [48] and the Calvin primer was originally used to analyze species from the genera Enchenopa and Campylrenchia within the family Membracidae [49]. Later, the primer pair Ron and Calvin was used for molecular detection of planthoppers from the infraorder Fulgoromorpha and the families Cixiidae and Delphacidae [44]. Amplification of delphacid DNA with Ron and Calvin primers was also demonstrated in the study of Argüello Caro et al. [37]. In our experiments, cixiid (*P. leporinus, R. quinquecostatus, H. obsoletus*) and delphacid (*J. pellucida, J. obscurella*) DNA was amplified. However, specimens of the families Cicadellidae (exception: *F. florii*) and Membracidae (*S. bisonia*), which belong to the infraorder Cicadomorpha were not detected. Therefore, the Ron and Calvin primer combination was no perfect choice to generally detect all Auchenorrhyncha species by sequencing.

Although UEA3 and UEA8 primers were designed for general *COI* amplification of hemipteran insects [38], due to mismatches, they never amplified *P. leporinus* in this study. This primer pair therefore may only be of use as a negative control for *P. leporinus* detection. Additionally, only one of three *P. alienus* specimens was amplified besides *R. quinquecostatus* and *H. obsoletus* and no other distantly related species, suggesting that this primer pair is not suitable for general *COI* amplification of Auchenorrhyncha species from sugar beet fields.

The evolutionary relationships of numerous cixiid species including *Pentastiridius* sp., *R. quinquecostatus*, and *H. scotti* have been extensively analyzed based on a large fragment (3652 bp in size) of *COI*, Cytochrome b, nuclear 18S rDNA and 28S rDNA genes [43]. Similarly, the *COI* gene (800 bp in size) was used for phylogenetic analysis of cixiids and delphacids including *P. leporinus, R. cuspidatus*, and *H. obsoletus* [16,34]. Therefore, the
COI gene is a suitable gene for differentiation of these species. In our study, we confirmed that a partial COI fragment (341 bp) that was specifically amplified from *P. leporinus* in comparison to sequences from *R. quinquecostatus* and *H. obsoletus* can be sufficient to differentiate these morphologically close species.

In addition, phylogenetic analysis for these species based on the generally amplified COI fragments (~1000 bp) in comparison to representative species of all Auchenorrhyncha families and subfamilies reported from sugar beet fields confirmed the close morphological features for these three species and that the two close families Cixiidae and Delphacidae can be clearly separated (Figure S4). In several studies, the close relationship between Cixiidae and Delphacidae has been reported [25,43,44] which supports the presented phylogenetic analysis based on the COI gene.

In conclusion, we provide here a sensitive, cost- and time-saving molecular method for reliable and specific detection of all immature stages as well as male and female *P. leporinus*, after different methods of planthopper collection and template DNA preparation. This technique has the potential to be used in large scale monitoring assays.

**Author Contributions:** Conceptualization, R.P., M.V. and O.E.; planthopper collection and species determination, R.P.; primer design: R.P., O.E.; lab research: G.H., R.P.; phylogenetic analyses: O.E., R.P.; writing—original draft preparation, R.P.; writing—review and editing, M.V., O.E.; visualization, R.P., O.E.; supervision, M.V.; project administration, M.V.; funding acquisition, M.V. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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11. Sémétey, O.; Bressan, A.; Gatineau, F.; Boudon-Padieu, E. Development of a specific assay using RISA for detection of the bacterial agent of ‘basses richesses’ syndrome of
sugar beet and confirmation of a *Pentastiridius* sp. (Fulgoromophia, Cixiidae) as the economic vector. *Plant. Pathol. 2007*, 56, 797–804.


### Supplementary information

#### Table S1 Table of experimental samples.

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Table S2  Evolutionary divergence between sequences of selected members of Cixiidae based on the partial COI sequence amplified from *P. leporinus* using specific primers in this study and COI sequences from the NCBI database of closely related species from the genus *Pentastiridius* and each three species from two taxonomically close genera *Reptalus* and *Hyalesthes*. The sequence obtained in this study is shown in bold. The specifically amplified COI fragment shows that intraspecific genetic distance was lower than the interspecific distance. *Catonia carolina* from the Achilidae family and *Tettigometra virescens* from the Tettigometridae family were used as outgroups.

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Figure S1 Alignment of the specific primers (P. leporinus fw1 and P. leporinus rv1) to the COI of all Auchenorrhyncha species, which were reported from sugar beet fields. Identical nucleotides in the specific primers and the target sequences are marked with dots. Letters highlight nucleotide dissimilarities between primers and target sequences. The number of nucleotide mismatches is summarized in the column at the right side of each alignment. Asterisks mark the positions of conserved nucleotides within primer sequences.
Figure S2 Electrophoretic patterns of PCR products show the specific detection of *P. leporinus*. Male adult insects were grinded in PBS for DNA template preparation. The insects were collected from sticky traps and stored on sticky traps for 1–2 weeks before use. In panel A, universal Ron and Calvin primers, in panel B, specific *P. leporinus* primers and in panel C, universal UEA3 and UEA8 primers were used for PCR assay. Lanes 1–3 represent *P. leporinus*; 4–6 *E. pteridis*; 7–9 *E. affinis*; 10–12 *C. placida*; 13–15 *O. ishidae*; 16–18 *R. quinquecostatus*. 19 *H. obsoletus* (after sweep net collection, control); C: negative control (water). The sizes of amplicons are shown on the left side and compared with 1 kb ladder (L).

Figure S3 Electrophoretic patterns of PCR products show the specific detection of *P. leporinus*. Male adult insects were grinded in PBS for DNA template preparation. The insects were collected from sticky traps and stored on sticky traps for 1–2 weeks before use. In panel A, universal Ron and Calvin primers, in panel B, specific *P. leporinus* primers and in panel C, universal UEA3 and UEA8 primers were used for PCR assay. Lanes 1–3 represent *P. alienus*; 4–6 *E. decipiens*; 7–9 *F. florii*; 10–12 *J. pellucida*; 13–15 *S. bisonia*; 16–17 *J. obscurella*; 18 *P. leporinus* (control); 19 *R. quinquecostatus* (control); 20 *H. obsoletus* (after sweep net collection, control); C: negative control (water). The sizes of amplicons are shown on the left side and compared with 1 kb ladder (L).
Figure S4  Evolutionary relationships of selected members of Cixiidae (P. leporinus, R. quinquecostatus, and H. obsoletus) using the sequences of COI gene that were PCR amplified with universal primers (Ron and Calvin; UEA3 and UEA8) in this study compared to the available sequences from NCBI database of representative members of all Auchenorrhyncha families and subfamilies reported from sugar beet fields. The sequences from this study are shown in bold. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The obtained COI fragments were useful to clearly differentiate P. leporinus, R. quinquecostatus, and H. obsoletus from other taxonomically close and far species.
6. Discussion

6.1 Life cycle of *Pentastiridius leporinus* under controlled and field conditions and characterization of development stages

The first aim of this thesis was to establish a *P. leporinus* rearing under laboratory conditions that allows detailed studies about the planthopper’s life cycle, vector control measures and the etiology of SBR disease. In literature, only a small number of rearing protocols for cixiid planthoppers, such as *H. obsoletus*, *H. crudus*, and *O. polyphemus* are available (Beltran-Aldana et al. 2020; Chuche et al. 2018; Hoch and Howarth 1993; Kessler et al. 2011; Tsai et al. 1976). Generally, the nymphs of cixiids have a subterranean development, what differs from other Auchenorrhyncha such as delphacids (Holzinger et al. 2003). In this thesis, a *P. leporinus* mass rearing was successfully established at 20.9 ± 1 °C, 48 % ± 12.2 % RH, a 16:8 h light/dark photoperiod, and 80μmol (s m⁻²)⁻¹ of full-spectrum light (manuscript I). The *H. crudus* mass rearing described by Beltran-Aldana et al. (2020) shares interesting parallels. Under natural conditions, *H. crudus* adults feed on the leaves of coconut palms and transmit *Ca. Phytoplasma palmae* (see Introduction section 1.1.9; Beltran-Aldana et al. 2020; Howard 1990). After copulation, the females oviposit in the underneath grasses and the nymphs develop by feeding on the grass roots (Beltran-Aldana et al. 2020). Initially, Tsai et al. (1976) developed a small scaled laboratory rearing of *H. crudus* on St. Augustine grass (*Stenotaphrum secundatum*). Beltran-Aldana et al. (2020) aimed to simulate *H. crudus* life cycle to establish a *H. crudus* mass rearing under semi-controlled conditions. To this aim, when *Panicum maximum* young plants were transplanted into larger plastic pots, only 2/3 of the pots were then filled with substrate. Therefore, the top part (1/3) of the plant roots was grown above the substrate level and the pots were covered with aluminum insulator to promote growth of fine roots under dark conditions. Further, this measure was taken to ensure that *H. crudus* nymphs can access the roots for feeding and to develop subterranean until reaching the adult stage. For oviposition, the plants were transferred into mesh cages and inoculated with *H. crudus* adults and the leaves of potted coconut palms were also added into the mesh cages as a food source for the adults. In contrast to the study of Beltran-Aldana et al. (2020), *P. leporinus* nymphs could not be reared in masses exclusively on the same (sugar beet) plants, due to strong expression of SBR symptoms and partial plant decline during ca. seven months development time from egg to adult (data not shown). Thus, the nymphs were reared in plastic containers until reaching fifth instar and could be collected easily for various experiments. In this study, fifth instar nymphs were transferred to potted sugar beets and in the top layer of expanded clay, the nymphs developed under dark conditions and access sugar beet roots until metamorphosis what is comparable to the
system used by Beltran-Aldana et al. (2020). The high rate of *P. leporinus* that emerged adult (68.7 % within 140 days) is comparable to the study of Beltran-Aldana et al. (2020) (71 %) and demonstrates that the system using potted sugar beets with a top layer of expanded clay was suitable to produce adults from fifth instar nymphs (Figure 5 in manuscript I). In this study, 59.2 % of *P. leporinus* which had emerged adult from fifth instar nymphs were male in controlled environments (manuscript I). Bressan et al. (2009b) also reported an equal sex ratio of *P. leporinus*, however no details were given.

In the protocol of Sforza et al. (1999), *H. obsoletus* nymphs showed an unexpected epigean behaviour as they were observed at the base and basal shoots of *L. angustifolia* and mass rearing failed. This contrasted to field observations where *H. obsoletus* nymphs develop in the soil (Sforza et al. 1999). Furthermore, Sforza et al. (1999) used a relatively compacted substrate (1:1 mixture of small gravel (0.5 mm diameter) and peat) where the nymphs presumably had no chance to enter the soil to feed from *L. angustifolia* roots. Chuche et al. (2018) reared *H. obsoletus* on *S. sclarea*, however, under controlled conditions, adult production rate from field collected nymphs was low (ca. 23 %, n=240) and no information about substrates used were provided. Moreover, Kessler et al. (2011) cultivated *H. obsoletus* on *U. dioica* grown in peat substrate, however, survival and reproduction rate were low. Further, only ca. 8 % of eggs and nymphs reached the adult stage and mass rearing failed (Kessler et al. 2011).

In this study, *P. leporinus* nymphs have also been found epigean on sugar beet taproots, but only when a 3:1 peat-sand substrate was provided without a top layer of expanded clay. However, due to low reproduction rate of planthoppers, this system was not further used (data not shown). Thus, it may be concluded that a suitable habitat for the nymphs which allows natural (subterraneous) development (e.g. using expanded clay) is a key factor for the establishment of a mass rearing for cixiids.

In this study, one generation was completed within ca. seven months (median) on wheat seedlings under controlled conditions, and maximum five generations were produced in the rearing on sugar beet within 21 months, much shorter periods compared to insects appearing in the field (manuscript II; see Introduction section 1.1.8; Biedermann and Niedringhaus 2004; Holzinger et al. 2002; Holzinger et al. 2003). The transition from one generation to the next was thus faster under controlled conditions, producing more than one generation per year compared to the field-living *P. leporinus* that in general only produces one generation (per year) (Biedermann and Niedringhaus 2004; Holzinger et al. 2002; Holzinger et al. 2003). However, Pfitzer et al. (2020) assumed that “extreme” weather conditions can lead to more than one generation and that above-average temperatures forced the expression of a second
P. leporinus generation in late summer which was the case in 2019. Indeed, photoperiod and temperature are critical factors for Auchenorrhyncha development and possible dormancy (Witsack 2002). In the laboratory rearing, the environmental conditions were kept constant and potential harmful environmental factors for the planthoppers were avoided. According to the literature, mostly 16:8 light/dark photoperiods were used for rearing of cixiids (Chuche et al. 2018; Sforza et al. 1999), except the 18:6 light/dark photoperiod used by Kessler et al. (2011). Beltran-Aldana et al. (2020) reared H. crudus at 25.7 ± 3.4 °C and 85 % ± 13 % RH. Sforza et al. (1999) also reported that development time of H. obsoletus nymphs was reduced (50 %) under controlled (23 ± 1 °C) compared to field conditions (one generation per year). In the study of Chuche et al. (2018), H. obsoletus produced over ten generations on S. sclarea within three years (23-25 °C). Sforza et al. (1999) assumed that the absence of consecutive dormancy led to accelerated H. obsoletus development under controlled conditions (Sforza et al. 1999). Indeed, consecutive dormancy of Central European Auchenorrhyncha can be initiated by unsuitable conditions and can be stopped or avoided by optimum environmental or external conditions such as photoperiod and temperature (Witsack 2002). Thus, it may be assumed that a diapause is not mandatory for P. leporinus development (see Discussion in manuscript I) and the controlled conditions were favourable for the year-round continuous and accelerated production and supply of planthoppers of P. leporinus under absence of dormancy what is a key factor to conduct various studies.

Further objectives of this thesis were to characterize P. leporinus developmental stages and to provide a possibility to distinguish the five nymphal instars. Life history traits of only few cixiid species are available in literature. In this study, the mean number of eggs per egg batch was 49.6 ± 31.3. Bressan et al. (2009a) found 46.6 ± 1.9 eggs per field collected female adults (n=30) after dissection. In the study of Bressan et al. (2009a), P. leporinus egg batches consisted of 20-35 eggs under controlled environments.

On wheat seedlings, 56.2 % of P. leporinus first instar nymphs reached the adult stage (manuscript I). More, only the first (25.7 % mortality) nymphal instar was relatively vulnerable and the mortalities in the second (12.9 %), third (3 %), fourth (3.1 %), and fifth (13.4 %) instar were lower. In the H. obsoletus rearing protocol of Sforza et al. (1999), first (54.5 %, n=33), second (93.3 %, n=15), third (77.8 %, n=9), and fourth (50 %, n=6) instar mortalities were remarkably higher compared to the P. leporinus rearing in this thesis. Data on nymphal mortality are not available for other cixiid species. For example, Ballou et al. (1987) analysed nymphal mortality of Sogatella kolophon and Delphacodes idonea (both Hemiptera: Delphacidae). In S. kolophon, first instar (27.3 %, n=22) was also more vulnerable compared to second to fifth instars (0-9.7 %, n=26-31) (Ballou et al. 1987) as also reported by Calvert and Wilson (1986), where the first instar of Stenocranus lautus (Hemiptera: Delphacidae)
was also more vulnerable (68.2 %, n=22) compared to the second (42.9 %, n=7) and third (50 %, n=4) instars. In contrast, *D. idonea* mortality was 0-6.7 % in first and second instars and 16.7-19.4 % in third to fifth instars (n=29-36) (Ballou et al. 1987).

Various morphological traits such as body or wing pad size as well as numbers of teeth on the spur, pit-like sensoria, tarsomeres, metatarsal segments, and metatibial spines were partly used in literature to distinguish the five nymphaul instars of numerous Auchenorrhyncha species (*Lacertinella australis*, *Pentagramma longistylata*, *Prokelisia crocea* (all Hemiptera: Delphacidae), *Phylloscelis pallescens* (Hemiptera: Dictyopharidae), *H. obsoletus*, and *H. crudus*) (Cargnus et al. 2012; Holder and Wilson 1992; McPherson and Wilson 1995; Rossi Batiz and Lenicov 2014; Wilson and Tsai 1982; Wilson and Wheeler 1986). In this study, a possibility to distinguish *P. leporinus* nymphaul instars is provided, by measuring head capsule width (manuscript I). This allows detailed studies on the different nymphaul instars of *P. leporinus* from the rearing after unequivocal identification.

6.2 Improvement and limitations of the *Pentastiridius leporinus* mass rearing

By using the established protocol for mass rearing, more than 20,000 specimens were produced between June 2020 and March 2022 (manuscript I), and it is strongly assumed that even higher numbers of planthoppers can be produced with this protocol. In order to ensure such high numbers, it is necessary to optimize and improve the used procedures to scale up the rearing and make it more time effective. Firstly, egg production can be optimized. According to the method described in manuscript I, transparent polystyrene cylinders were inoculated with each five females and three males and covered with perforated polypropylene bags. However, egg batch collection from this system can be difficult and time consuming as the work has to be done carefully to avoid that planthoppers escape from the perforated polypropylene bags when opened for egg batch collection. Instead, groups of three or four potted sugar beet plants (e.g. polypropylene pots: 9 cm length × 9 cm width × 9.5 cm height) can be placed into mesh cages (60 × 60 × 60 cm) and inoculated with 300 or more adult planthoppers. In this improved system, egg batches were mostly found on the bottom of the pots and could easily be collected once per week or each second week (data not shown). Secondly, adult production from fifth instar nymphs can be scaled-up. Currently, large pots (2.4 L polypropylene plant pots, 16 cm top diameter, 15 cm height) were inoculated with 100 fifth instar nymphs each. However, bigger polypropylene pots can also be used (e.g. 38 cm length × 14 cm width × 14 cm height, three sugar beet plants per pot) and inoculated with up to 1,000 fifth instar nymphs. More, these pots were also successfully used in groups of two pots per mesh cage (data not shown). Thirdly, the rearing can be scaled-up in general. This work was faced with time and space limitations, thus, only a
fraction of emerged adults from each generation were used for oviposition and production of the next generation. In addition, a significant number of *P. leporinus* (several thousand nymphs and adults) was taken from the rearing for various experiments. Larger time and space capacities would also allow to use more adults for oviposition and to produce larger numbers of *P. leporinus*. The fact that *P. leporinus* mass rearing was established despite these circumstances, represents the potential of the protocol presented. Furthermore, it could be tested if higher temperatures, such as 23-25 °C as reported by Chuche et al. (2018), can speed up *P. leporinus* development in controlled environments and lead to a higher planthopper production in the rearing as Witsack (2002) reported that temperature has an impact on the development of Auchenorrhyncha.

Rearing of insects can lead to alterations in their biology compared to natural conditions due to adaption to the specific conditions in the laboratory (Tsakas and Zouros 1980). In addition, genetic impoverishment can be a consequence when insect populations are too small (Nowak et al. 2007a). In the study of Nowak et al. (2007a), different levels of inbreeding and inbreeding coefficients of 0 (natural population), 0.125 (crossbreed of eleven laboratory strains), and 0.375 (full siblings were crossed over two generations) were compared in *Chironomus riparius* (Diptera: Chironomidae). Indeed, life history traits such as mortality, mean emergence time, egg batch size, sex ratio, and growth rates of populations were negatively affected by inbreeding (Nowak et al. 2007a). In addition, *C. riparius* strains with a low level of genetic variation from laboratory rearing had diminished fitness parameters and were significantly affected under environmental stress (Cadmium exposure) compared to strains with higher genetic variation (Nowak et al. 2008). In the study of Nowak et al. (2007b), heterozygosity decreased in a *C. riparius* rearing over 23 generations (population size: 350-500 larvae, crossbreed of eleven *C. riparius* strains). Further, field collected *C. riparius* had a higher heterozygosity when compared to a crossbreed of eleven different *C. riparius* strains from laboratory rearings (each strain was reared for at least two generations under controlled conditions before crossbreeding and each 13 males and females were used per laboratory strain for crossbreeding) and the authors assumed that field collected insects are favourable to refresh genetic diversity in laboratory rearing (Nowak et al. 2007b). Since the initial population of the rearing (ca. 2,000 *P. leporinus* adults, data not shown) and generations (ca. 5,000-6,000 specimens per generation, data not shown) were relatively large and generation number was rather low (three to five generations until March 2022), it is assumed that the risk of inbreeding was relatively low at this stage. However, no empirical comparisons of life history traits or inbreeding coefficients have been conducted between the different laboratory generations. In this study, development time was compared between the first and second generation and no significant differences were found except in third nympha...
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instars (manuscript I). Further life history traits cannot be compared under this aspect because each time only one generation was analysed for oviposition (Table 1, Figure 2 in manuscript I) and adult emergence from the fifth-instar nymphs (Figure 5 in manuscript I). Indeed, based on the findings of Nowak et al. (2007a) and Nowak et al. (2007b), it may help to regularly crossbreed field collected specimens with planthoppers from the rearing to maintain fitness of *P. leporinus* what also supports reproducibility of experiments under controlled conditions (Nowak et al. 2008).

6.2.1 Interaction of *Pentastiridius leporinus* with SBR-proteobacterium and SBR-phytoplasma

SBR-proteobacterium and SBR-phytoplasma cannot be cultivated *in vitro* (Sémétey et al. 2007b). Thus, i) cultivation and ii) transmission of the pathogens to sugar beet plants rely on *P. leporinus*. In the newly developed approach for mass rearing, SBR-proteobacterium was maintained and transmitted over multiple *P. leporinus* generations and infection rates were 70-80 % in the first three generations of the rearing (*n*=10) (manuscript I). Since the different planthopper generations were kept separately, transovarial transmission of SBR-proteobacterium at a high rate that was initially described by Bressan et al. (2009b), was confirmed in this study (see Discussion manuscript I), however, no conclusions can be drawn about the vertical transmission rate. In the study of Bressan et al. (2009b), the vertical transmission rate of SBR-proteobacterium was 30 %. To ensure that the rearing method is suitable for long-term maintenance of SBR-proteobacterium, subsequent *P. leporinus* generations should also be tested for presence of this pathogen. Generally, transovarial transmission of phytoplasmas is not the rule in Auchenorrhyncha or psyllids (see Introduction section 1.1.5.2). Thus, SBR-phytoplasma was not detected by PCR in *P. leporinus* during the first three generations of the rearing. Since SBR-phytoplasma infection rate was not analysed in the initial (parental) generation of the rearing, no conclusions can be drawn in this study regarding possible transovarial transmission of SBR-phytoplasma in *P. leporinus* and this should be evaluated in further studies.

Genome sequence analysis of *Arsenophonus* bacteria can provide information about their lifestyle (Fan et al. 2016) and several genome sequences of *Arsenophonus* and endosymbiotic bacteria were published. Thus, Fan et al. (2016) analysed the draft genome sequence of ‘*Ca. Arsenophonus nilaparvatae*’. More, genome size as well as GC-content and phylogenetic analysis showed that *Ca. Arsenophonus nilaparvatae* is a facultative endosymbiont of *N. lugens* (Fan et al. 2016). Nováková et al. (2016) and Zeng et al. (2018) provided the complete genome sequences of ‘*Ca. Arsenophonus lipopteni*’ (γ-proteobacterium) that is an obligate symbiont for the hematophagous insect *Lipoptena*
cervi (Diptera: Hippoboscidae) and Cardinium cSfur which is a bacterial endosymbiont of Sogatella furcifera (Hemiptera: Delphacidae), respectively. Furthermore, the genome of Ca. Arsenophonus lipopteni contains genes which are capable to synthesize several vitamins such as B2 (riboflavin), B6 (pyridoxine), and B7 (biotin) for L. cervi, probably to compensate low contents of these vitamins in the deer blood (Nováková et al. 2016). The interaction between P. leporinus and the SBR-proteobacterium is poorly characterized so far. In future studies, genome analysis could be the base to better unravel the role of the proteobacterium for the insect. Bressan (2014) already raised the question whether P. leporinus benefits from infection with SBR-proteobacterium and thus might be capable to propagate in sugar beet-winter wheat crop rotations. To this aim, life history needs to be compared between SBR-proteobacterium infected and non-infected P. leporinus on sugar beet and wheat plants. The rearing method presented can provide SBR-proteobacterium infected planthoppers to deeper study this question. To further analyse the role of the SBR pathogens for P. leporinus, life history, longevity and fecundity (egg deposition, number of hatching nymphs) should be compared between infected and non-infected P. leporinus as already described for other insects species (reviewed in Gross et al. 2022, see Introduction section 1.1.5).

Since SBR-phytoplasma was not present so far, it is a key question, how this pathogen can be introduced into and maintained in a P. leporinus rearing. Further, a rearing of SBR-phytoplasma infected P. leporinus would also allow detailed studies about insect-pathogen interactions. However, it is completely unknown, if P. leporinus nymphs can acquire SBR-phytoplasma from infected sugar beet plants and transmit it to healthy plants, since, no information is available about AAPs, LPs, and IAPs of SBR-phytoplasma for P. leporinus. Further, only information about IAPs of SBR-proteobacterium is available for P. leporinus (Sémétey et al. 2007b), although such information would be crucial to understand SBR pathogen acquisition, propagation and transmission by P. leporinus. Comparable to P. leporinus, information was also lacking for H. obsoletus. Thus, Sforza et al. (1999) suggested to establish a rearing of pathogen-free H. obsoletus that would allow detailed studies on transmission of Ca. Phytoplasma solani.

Basic research in acquisition and transmission of SBR pathogens is urgently required regarding the different developmental stages of P. leporinus. According to Jović et al. (2009), R. panzeri nymphs can acquire Ca. Phytoplasma solani from maize roots in fields infested with MR disease (see Introduction section 1.1.8), however, infection rates of field collected nymphs were rather low and depended on nymphal instar (second instar: 0 %, n=66; third instar: 4.3 %, n=70; fifth instar: 5.1 %, n=39) (Jović et al. 2009). Under controlled conditions, healthy R. panzeri third instar nymphs were able to acquire Ca. Phytoplasma solani from
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*C. roseus* (AAP: 8 days) and transmit it (LP+AP: 45 days) to maize (89.4 % transmission efficiency, n=19) and wheat plants (7.1 % transmission efficiency, n=28) (Jović et al. 2009). Subsequently, surviving nymphs were reared on wheat plants for another two months and *Ca. Phytoplasma solani* was found in 47.4 % (n=59) of planthoppers using nested PCR (Jović et al. 2009). Galetto et al. (2016) reported that healthy *S. titanus* nymphs (third to fifth instar) successfully acquired FDP from an infected grapevine branch (AAP: 7 days, LP: 3 weeks).

Under the assumption that *P. leporinus* nymphs can acquire SBR-phytoplasma, *P. leporinus* adults should be collected which carry the pathogen and need to feed and potentially transmit it to sugar beet plants. Further, the resulting nymphs also need to feed on these plants to acquire SBR-phytoplasma. Thus, the sugar beets for oviposition should be cultivated with a top layer of expanded clay (see manuscript I) or according to the previously described aluminum insulator system of Beltran-Aldana et al. (2020) by growing sugar beet instead of *P. maximum*. Consequently, the females oviposit and the resulting nymphs develop in the expanded clay layer or under aluminum insulator, respectively. Another critical aspect is the risk of sugar beet plant decline after infection with SBR pathogens during the in average seven months development time of planthoppers from egg to adult (described above). To avoid this problem, the nymphs should regularly be transferred to new plants until reaching the adult stage. Finally, the emerging adults could be used to produce the next generation. However, the first attempts in *P. leporinus* nymphs to acquire SBR-phytoplasma from infected sugar beets and to transmit it to healthy sugar beets failed (Mark Varrelmann, pers. comm.). Based on the studies of Galetto et al. (2016) and Jović et al. (2009), it may also be tested if healthy *P. leporinus* can acquire SBR-phytoplasma from infected plants and transmit it to sugar beet to keep this pathogen in the rearing.

When aiming to produce *P. leporinus* that are exclusively infected with SBR-phytoplasma, one can be faced with another problem, as only one study (Behrmann et al. 2022) reported that *P. leporinus* can exclusively be infected with this pathogen. Indeed, planthoppers were mostly infected with SBR-proteobacterium and co-infections with both SBR pathogens were only found in rare cases (Behrmann et al. 2022; Pfitzer et al. 2020) (see Introduction section 1.1.6). Thus, collection of planthoppers that are exclusively infected with SBR-phytoplasma may be difficult.

Therefore, a *P. leporinus* rearing with SBR pathogen free insects should be established to further compare the life history with infected *P. leporinus* following Sforza et al. (1999) regarding *H. obsoletus*. To this aim, one approach would be to collect SBR pathogen free *P. leporinus* from the field. It was already shown that in most populations, a part of the
insects remains without SBR pathogen infection (Behrmann et al. 2022; Pfitzer et al. 2020; Sémétey et al. 2007a; Sémétey et al. 2007b) (see Introduction section 1.1.6). However, there is a risk of false negative results in molecular detection methods (reviewed in Weissensteiner and Lanchbury 1996). Recently, SBR pathogen free insects were successfully collected from natural habitats in Germany (Mark Varrelmann, pers. comm.), what enables to study the insect-pathogen interaction and the role of SBR-proteobacterium in detail, potentially unravelling the host shift of P. leporinus. However, the phylogenetic relationships between P. leporinus from natural habitats and sugar beet are unsolved and need to be studied in future to better understand the adaption of P. leporinus to sugar beet crop rotations in the different areas infested with SBR disease.

If pathogen-free insects are not available, there are (other) options to cultivate pathogen-free planthoppers, for instance by curing P. leporinus from the SBR-proteobacterium, as has been described for α-proteobacteria in different insect species. Thus, in Nasonia vitripennis (Hymenoptera: Pteromalidae), which is a parasitoid wasp, experimentally extended diapauses (two years at 4 °C) of larvae which were infected with two Wolbachia strains led to partial losses of the strains (Perrot-Minnot et al. 1996). Cytoplasmic incompatibility in Tribolium confusum (Coleoptera: Tenebrionidae) is caused by Wolbachia bacteria (Gharabigloozare and Bleidorn 2022). In the study of Stevens and Wicklow (1992), T. confusum fed from wheat seeds that were artificially inoculated with Streptomyces aureofaciens (Actinomycetales: Streptomycetaceae), a bacterium that is known to produce tetracycline (Stevens and Wicklow 1992). Finally, 85 % of specimens (n=20) were cured from cytoplasmic incompatibility, presumably by tetracycline production of S. aureofaciens (Stevens and Wicklow 1992). In addition, 90 % of T. confusum larvae (n=20) were cured from cytoplasmic incompatibility after cultivation at 37 °C for 25 days (Stevens 1989). Gharabigloozare and Bleidorn (2022) recently reported that Wolbachia density in T. confusum was the lowest after cultivation of adults at 34 °C, compared to lower temperatures (30-33 °C), but reproduction was negatively affected by the high temperatures.

In Introduction section 1.1.5, it was already described how symbiotic or parasitic bacteria can alter the life history of their respective insect vector. In addition, the bacteria can also interact, thus, these interactions can be positive, harmful, or competitive for bacteria (reviewed in Gonella et al. 2019). For SBR-proteobacterium and SBR-phytoplasma, the interactions in P. leporinus are completely unknown and need to be analysed in future studies. In this context, the facts that mixed infections are rarely reported in P. leporinus (see Introduction section 1.1.6; Behrmann et al. 2022; Pfitzer et al. 2020) and that transovarial transmission of SBR-proteobacterium is possible (Bressan et al. 2009b and manuscript I), can be hints for interaction amongst the bacteria. Several bacteria such as Wolbachia are
widely distributed in insect vectors (reviewed in Gonella et al. 2019). These bacteria have the potential to manipulate reproduction of insects, however their exact role is mostly unknown (reviewed in Gonella et al. 2019). According to Bressan et al. (2009c), different bacterial symbionts were found in *P. leporinus* such as ‘*Ca. Sulcia muelleri*’, ‘*Ca. Purcelliella pentastirinorum*’ (γ-proteobacterium), and *Wolbachia* bacteria that were also found in *H. obsoletus* (reviewed in Gonella et al. 2019). According to phylogenetic studies, *Ca. Sulcia muelleri* and *Ca. Purcelliella pentastirinorum* also occur in *R. cuspidatus* and various *Oliarus* spp. (Bressan et al. 2009c). Further, *Ca. Sulcia muelleri* is the most important obligate symbiont in Auchenorrhyncha (reviewed in Gonella et al. 2019). In *H. obsoletus*, Iasur-Kruh et al. (2017) detected *Ca. Sulcia* by deep sequencing of 16S rRNA gene and a *Dyella*-like bacterium (DLB) belonging to Xanthomonadaceae family using FISH analysis. In an *in vitro* test, the DLB harmed *Spiroplasma melliferum* growth. Since *S. melliferum* is closely related to phytoplasmas, the authors assumed that the DLB could be used against *Ca. Phytoplasma solani* to control BN disease in grapevine (Iasur-Kruh et al. 2017). Later, the DLB was introduced in phytoplasma infected grapevine and reduced symptom expression, presumably more caused by inhibition than competition of bacteria based on genome analysis of the DLB (Iasur-Kruh et al. 2018).

*Dictyophara europaea* (Hemiptera: Dictyopharidae) vectors FDP in grapevine (Krstić et al. 2018). In *D. europaea*, *Wolbachia* and phytoplasma infections were negatively correlated suggesting that the *Wolbachia* strain was an antagonist to FDP or both bacteria had a competitive relationship (reviewed in Gonella et al. 2019; Krstić et al. 2018). In contrast, concentrations of *Ca. Liberibacter asiaticus* and *Wolbachia* were positively correlated in *D. citri* (Fagen et al. 2012). Further, when different phytoplasma strains were sequentially offered to leafhoppers, they primarily transmitted the firstly acquired strain, what may be explained by competition between the strains and that the firstly acquired strain propagates in the insect’s body and can be transmitted to plants after reaching salivary glands (reviewed in Gonella et al. 2019). Another remarkable aspect is that *C. wagneri* can carry and transmit SBR-proteobacterium and *Ca. Phlomobacter fragariae*, however, both pathogens were not detected in *C. wagneri* at the same time (reviewed in Gonella et al. 2019). However, no conclusions can be drawn about interaction of these bacteria in *C. wagneri* (reviewed in Gonella et al. 2019).

**6.2.2 Identity of SBR-proteobacterium and SBR-phytoplasma**

Identity, origin, and role of SBR-phytoplasma are still unsolved. Under the assumption that SBR-phytoplasma is not transmitted transovarially, the origin/reservoir of this pathogen remains unclear and should be analyzed in further studies. According to Bressan et al.
(2008), *H. obsoletus* adults that were collected from *C. arvensis* and no *U. dioica* collected *H. obsoletus* transmitted *Ca.* Phytoplasma solani to sugar beet and the authors assumed that these phytoplasmas have different plant host ranges (Bressan et al. 2008; see Introduction section 1.1.2). Due to the fact that *H. obsoletus* was found in sugar beet fields (Pfitzer et al. 2020; Sémétey et al. 2007a), one possibility may be that *H. obsoletus* adults play a crucial role in transmission of SBR-phytoplasma to sugar beet. Further, Gatineau et al. (2002) assumed that *P. leporinus* may acquire SBR-phytoplasma from weed species during migration to sugar beet fields (see Introduction section 1.1.6). Finally, the origin of SBR-phytoplasma in sugar beets should be investigated in further studies.

Generally, 16S rRNA gene is widely used for detection and characterization of phytoplasmas, however, due to high conservation of rRNA sequences, their usage can have limitations when different phytoplasma strains are compared (reviewed in Martini et al. 2019). Consequently, genes with less conservation were used in addition (reviewed in Martini et al. 2019). Finally, multilocus sequence analyses (MLSA) is a helpful approach to analyse identity of phytoplasmas which induce plant diseases such as RTD in sugar beet (Čurčić et al. 2021a; reviewed in Martini et al. 2019). Number and selection of target genes can vary in MLSA (reviewed in Glaeser and Kämpfer 2015). Thus, Čurčić et al. (2021a) analysed the sequences of *stamp*, *tuf*, and *vmp1* genes of *Ca.* Phytoplasma solani what is useful to differentiate different strains of this phytoplasma (reviewed in Rossi et al. 2019). According to MLSA results, Čurčić et al. (2021a) assumed that *H. obsoletus* from *U. dioica* play a secondary or insignificant role in transmission of RTD. Thus, the authors found parallels with the study of Bressan et al. (2008), where from *U. dioica* collected *H. obsoletus* also played no role in transmission of *Ca.* Phytoplasma solani to sugar beet (Čurčić et al. 2021a).

Furthermore, Čurčić et al. (2021a) analysed genetic variability of *Ca.* Phytoplasma solani strains using MLSA. To better understand origin and vector role of SBR-phytoplasma in sugar beet, MLSA needs to be applied in future studies. Additionally, sequencing of housekeeping genes (such as the genes *secY, secA, rpoB, tuf*, and *groEL* as well as ribosomal protein (*rp*)) can contribute to detailed characterization of phytoplasmas that are closely related (reviewed in Martini et al. 2019; reviewed in Namba 2019). In another study, alternatively, the housekeeping genes *gyrB, rpoB, atpD, and infB* were used to classify and to analyse the identity, and phylogeny different *Pantoea* sp. (γ-proteobacteria) strains in MLSA (Brady et al. 2008).

No further information on genetic variability of SBR-proteobacterium is available that can also contribute to clarify identity and potential origin. Thus, further studies should compare genome sequences of SBR-proteobacterium to evaluate genetic variability from different SBR infested areas such as France, Southwest Germany, region of the river Elbe in
Germany, and Switzerland. However, providing the sequences of bacterial insect symbionts can be difficult when these insects also carry other bacteria (e.g. endosymbionts or parasites) (Vancaester and Baxter 2023) and it was described previously that *P. leporinus* can carry different bacterial species at the same time (Bressan et al. 2009c).

Further, analysis of molecular mechanisms that induce SBR symptoms in sugar beet (as reviewed in Namba 2019) is required to better understand the etiology of the SBR disease. Various high-throughput methods have been established to study phytoplasma-host plant or insect host interactions (reviewed in Dermastia et al. 2019). Thus, for a better understanding of the interactions between SBR-phytoplasma and sugar beets and the involvement of proteome and RNAs, such techniques may be a helpful tool and need to be carried out in further studies (reviewed in Dermastia et al. 2019). Additionally, phytoplasma genomes can be entirely or partially sequenced (reviewed in Dermastia et al. 2019). Next generation sequencing techniques focus on micro RNAs, small RNAs, and long noncoding RNAs of plants that are infected with phytoplasmas and contributed to extensive characterization of the gene expression in the genome (reviewed in Dermastia et al. 2019). Micro-RNAs that are small noncoding and endogenous play a significant role for the comprehension of host plant-phytoplasma interactions, since they are involved in post-transcriptional regulation of genes when plants respond to biotic stresses (reviewed in Dermastia et al. 2019). Long noncoding RNA expression is also connected to plant infections with phytoplasmas (reviewed in Dermastia et al. 2019). Phytoplasmas can have effects on symptoms expression by modifying gene expression and protein levels in plants and alter the pathways for hormones, stress signalling and nutrition (reviewed in Dermastia et al. 2019). Studies on the transcriptome and proteome have shown the down-regulation of numerous genes involved in photosynthesis of different phytoplasma-infected plants such as grapevine (reviewed in Dermastia et al. 2019). More, maize plants that were artificially inoculated with maize bushy stunt phytoplasma (*Ca. Phytoplasma asteris*, Ramos et al. 2020) had higher levels of phenols, protein, and reducing sugars compared to healthy plants (Junqueira et al. 2004). In contrast, the chlorophyll level was reduced, thus, the authors assumed that the phytoplasma disturbs photosynthesis (Junqueira et al. 2004). Such mechanisms were also observed in other phytoplasma-induced diseases (e.g. in *C. roseus*) and it was assumed that this causes chlorosis in leaves (reviewed in Dermastia et al. 2019). Generally, it is assumed that the phytoplasmas manipulate infected cells to acquire hexoses due to reducing carbohydrate level to improve the conditions for their own development (reviewed in Dermastia et al. 2019). Finally, such studies are also required to better understand the proteomic and transcriptomic mechanisms in sugar beets caused by infections with SBR pathogens.
6.3 Control measures and non-hosts for Pentastiridius leporinus

6.3.1 Agronomic measures

This thesis further aimed to identify agricultural measures for *P. leporinus* control. To this aim, it was analysed, whether soil tillage (ploughing or reduced tillage using cultivator) reduces *P. leporinus* adult emergence. However, only in two field trial sites, significant effects were observed in the winter wheat plots after ploughing (manuscript II). Behrmann et al. (2022) analysed *P. leporinus* nymphal distribution in the soil from two consecutive years. The nymphal distribution depended on the temperatures in the top soil layer (0-10 cm). Thus, at colder temperatures, the nymphs preferred deeper soil layers (Behrmann et al. 2022; see Introduction section 1.1.8). In this thesis, nymphal distribution in the soil was not analysed before soil tillage. Further, tilling depth was not standardized between the different field trial sites and adapted to the individual field conditions. Thus, it cannot be concluded whether the effects observed from ploughing on two of seven field trial sites were caused by soil tillage or different tilling depths of plough and cultivator tillage and it was assumed that the effects were more likely caused by individual soil conditions in the fields (see Discussion in manuscript II). In future studies, ploughing and tillage by cultivator should be done in the same tilling depths on different field trial sites to address this question. In contrast, studying the nymphal distribution in the soil before soil tillage is not considered practicable since it is time- and labor-intensive and *P. leporinus* nymphs can be distributed unevenly in the soil (data not shown).

In general, only few reports are known, focusing on soil tillage for the control of cixiid planthoppers. According to Maixner (2007), ploughing in winter time transferred *H. obsoletus* nymphs to the soil surface and frost killed them, leading to reduced numbers of *H. obsoletus* that emerged adult in the following summer. Mori et al. (2020) further assumed that a late frost event (end of April, minimum -4.4 °C) significantly reduced *H. obsoletus* nymphal population. In contrast, no information is available on *P. leporinus* nymphs regarding possible effects of (cold) temperatures. Further studies should firstly evaluate the effect of different temperatures and durations of frost events under controlled conditions. Secondly, if frost has a harmful effect on *P. leporinus* survival, it can be analysed if ploughing before frost events (see Maixner 2007) also transfers *P. leporinus* nymphs to the soil surface, being subsequently harmed by frost. However, during sugar beet cultivation this would only be possible if frost events occur after harvest and before sowing of winter wheat, however, in this study, the time gap was short (maximum four weeks, see manuscript II). Consequently, more time would be available for ploughing when spring crops are sown after sugar beet harvest and soil remains bare until then.
The reduction effects observed from maize (late sown spring crop, sown in April) and bare soil treatments compared to winter wheat were clear (79-99%) in all three and four field sites, respectively. Thus, *P. leporinus* nymphs were probably starved by the long host-free period after sugar beet harvest, by planting maize or keeping the field fallow which turned out as potential agricultural practices for *P. leporinus* control (manuscript II). Planthopper control of species with aboveground or sub-soil nymphal stages by cultivation of non-host plants or maintenance of bare soil has been previously described. In the study of Oka (1979), *N. lugens* was reared on 34 different weed and grass species; on most species nymphs were produced but nearly all died. However, no experiments were conducted to confirm these results under field conditions (Oka 1979). Further, it was assumed that cultivation of non-host plant species after rice harvest or bare fallow land were measures for vector control (Oka 1979). Howard (1990) identified that certain grass species reduced the number of *H. crudus* that emerged as adults in container trials, in comparison to St. Augustine grass that was used as the control. This was also confirmed in field trials under coconut palms (Howard 1990).

So far, no other study evaluated if other (late sown) spring crops, e.g. *Glycine max*, *Helianthus annuus*, *S. tuberosum*, *Pisum sativum*, *Vicia faba*, or *Lupinus* sp. also have the potential to reduce *P. leporinus* adult emergence when grown after sugar beet. Due to the relatively long host-free period between sugar beet harvest and late sown spring crops, future studies should analyse, if cover crops (e.g. *Sinapis alba*, *Guizotia abyssinica*, *Raphanus sativus*) can be integrated (see Discussion in manuscript II). Due to the fact that cover crops shorten the period in which the soil is kept fallow after sugar beet harvest, the plant species should not allow *P. leporinus* nymphs to develop. Different studies have shown that biomass accumulation of cover crops such as *S. alba* or *R. sativus* was negatively affected by late sowing dates (Cottney et al. 2022; Toom et al. 2019). Thus, a potential caveat is that the harvest of sugar beets in autumn (this study: From mid-September to beginning of November) can interfere with the establishment of cover crops. Consequently, sugar beets would need to be harvested sooner, but early harvest dates can lead to decreased sugar yields (Lauer 1997). Another option could be to test further winter crops such as winter barley, *Secale cereale*, or *Triticale* sp. Comparable to cover crops, growing winter crops only makes sense when they cause mortality in *P. leporinus* nymphs. Practicality, economy, as well as political and phytosanitary aspects may be potential caveats that also need to be evaluated. Although possible, maize cultivation after sugar beet is connected to further disadvantages. For example, sugar beet late root rot with strong yield losses caused by *Rhizoctonia solani* can be increased when maize is integrated into sugar
beet crop rotations (Buhre et al. 2007). Finally, any changes in succession crops after sugar beet harvest need to be accepted and implemented by the farmers.

The newly developed experimental setup allows to test the performance (survival and ability to reach the adult stage) of *P. leporinus* nymphs in controlled environment and this thesis contributes to the knowledge about the host range of *P. leporinus*. In future studies, firstly, performance of *P. leporinus* nymphs should be analysed on potential (non) host plant species in controlled environment since the field tests are extensive and complex. However, nymphal performance was only tested on plants in the seedling stage (4-10 days old) in this study. In order to improve this system and to adapt it more closely to field conditions, the planthopper performance should be further tested on potted plants that grow under light conditions. Since a 16:8 h light/dark photoperiod and ca. 21 °C were suitable for *P. leporinus* mass rearing, this may be used. To simulate autumn and winter conditions, performance of *P. leporinus* nymphs may also be tested in shorter photoperiods and/or colder temperatures under controlled conditions. To this aim, it is assumed that the system with a 5 cm top layer of expanded clay which was used for adult production from fifth instar nymphs on sugar beet, may also be useful to test other plant species with some adaptions. Furthermore, adapting the system of Beltran-Aldana et al. (2020) (described above) may also be another option to test nymphal performance. Secondly, selected promising crops should be tested in field trials since the most important aim is to control *P. leporinus* under field conditions.

Although the effects observed from the maize and bare soil treatments were clear, it cannot be concluded how such measures can contribute to sustainable vector management in SBR diseased areas and reduce yield losses in sugar beet. Bressan et al. (2011) used transparent sticky traps (30 cm length, 21 cm width) to quantify *P. leporinus* adults from a total of 29 sugar beet fields in 2006 and 2007. It was clearly shown that the larger the *P. leporinus* populations (2-509 *P. leporinus* / sticky trap), the higher were the probabilities that the adults were infected with SBR-proteobacterium (7-100 %) and sugar beets expressed SBR symptoms (0-90 % diseased plants) (Bressan et al. 2011). However, no conclusions can be drawn from adult numbers on sticky traps to exact *P. leporinus* populations (*P. leporinus* / hectare). Further, the connection between population size and significant yield damage is completely unknown and should be further investigated to evaluate potential reduction effects from bare soil or maize. In field trials, sugar beets covered with mesh gauze were successfully inoculated artificially using *P. leporinus* adults under field conditions (data not shown). Thus, in future studies, field grown sugar beets should be inoculated artificially with different *P. leporinus* population densities. Finally, sugar yield should be analysed to study the relationship between planthopper population density and yield reduction.
Even though clear differences in survival and development were observed between host plant species for *P. leporinus* nymphs, the factors determining host suitability for different nymphal instars remain completely unknown. Thus, future studies should analyse phloem components (e.g. nutrient, metabolites, sugar or amino acid content) of plant species and dietary requirements of *P. leporinus* since these factors define host suitability for insects (reviewed in Gross et al. 2022).

Behrmann et al. (2022) recently conducted host choice experiments with *P. leporinus* nymphs under controlled conditions. *P. leporinus* nymphs preferred sugar beet and maize roots over wheat roots and no statistical differences were observed between sugar beet and maize. However, the authors did not state which nymphal instar(s) was/were used, thus, no clear comparisons can be drawn to the findings in this thesis. Bressan et al. (2010) assumed that olfactory and visual factors may influence *P. leporinus* adult behaviour. To obtain further information about the reasons for host plant selection, basic research on olfactory or visual factors that determine attraction of *P. leporinus* are crucial. Riolo et al. (2012) conducted Y-tube olfactometer bioassays to compare attraction of male and female *H. obsoletus* adults to different plant species. In addition, the authors used gas chromatography and identified VOCs that were mirrored in electrophysiological reactions of the antennae (Riolo et al. 2012). Finally, such studies are also required to identify VOCs that are attractants or repellents for *P. leporinus* and how VOC release of the plants is affected by SBR-proteobacterium and SBR-phytoplasma (reviewed in Gross et al. 2022).

### 6.3.2 Chemical control

Mori et al. (2014) conducted detailed field studies for chemical control of *H. obsoletus* in *U. dioica*. Glyphosate in combination with Flazasulfuron and additionally Imidacloprid treatments of *U. dioica* in the end of April significantly reduced the numbers of adult *H. obsoletus* that were distributed within vineyards and significantly reduced the numbers of emerging adults during summer, respectively. In another experiment, both neonicotinoids (Imidacloprid and Thiamethoxam) that were sprayed in the beginning of June (immediately before initial adult emergence) significantly reduced *H. obsoletus* adult emergence compared to the untreated control. However, a Glyphosate treatment in late April was more effective (Mori et al. 2014). Significant reductions in adult planthopper and leafhopper (Auchenorrhyncha) specimens per m² in alfalfa were shown by Lamp et al. (1984), when grass herbicides where applied which reduced the biomass of grass weeds as host plants. According to Maixner (2007), application of a systemic insecticide in spring significantly reduced the number of emerging *H. obsoletus* from *U. dioica* during summer. However,
detailed information about the timing and insecticide used in this study are not available (Maixner 2007).

Currently, it is completely unknown which insecticides are effective against *P. leporinus* under practical conditions and no insecticides are currently authorized (see Introduction section 1.1.9), this would require basic research. To this aim, under controlled conditions, performance of *P. leporinus* nymphs from the laboratory rearing can be tested on plants that were treated with insecticides. The insecticides would need to be distributed systematically in the plants phloem to be potentially up taken by the nymphs as it was previously described that Cixiidae such as *P. leporinus* feed from the plant phloem cells (Biedermann and Niedringhaus 2004; Holzinger et al. 2002) (see Introduction section 1.1.8). Obviously, use of insecticides for insect control is not considered sustainable (reviewed in Gross et al. 2022; reviewed in Witzgall et al. 2010). Thus, alternative methods should be taken into account.

### 6.3.3 Alternative/biological control

Gross et al. (2022) reviewed innovative insect vector management strategies, which can be summarized as non-chemical measures that include attract-and-kill or push-and-pull measures, mass collections, repellents, antifeedants, deterrents, or interrupting in copulation (reviewed in Gross et al. 2022). In insects with subterranean larval development, application of pheromones targets the adult stage and may be advantageous compared to using insecticides (reviewed in Witzgall et al. 2010). Generally, use of pheromones for insect control is useful for sustainable insect pest control and techniques can be divided into i) disturbance of copulation and ii) mass destruction of insects (reviewed in Witzgall et al. 2010). Techniques for mass destruction of insects are cost-effective and can further be divided into mass trapping or attract-and-kill methods (reviewed in Witzgall et al. 2010). Indeed, sex pheromones can be used for monitoring and mass trapping of various insect pests such as *Tuta absoluta* (Lepidoptera: Gelechiidae) (reviewed in Witzgall et al. 2010). In *T. absoluta*, the sex pheromone release by females was analysed using gas chromatography. The most important sex pheromone was (3E,8Z,11Z)-3,8,11-tetradecatrienyl acetate (TDTA). Afterwards, attraction of male adults to different TDTA concentrations was tested in wind tunnel bioassays and field experiments. More, in the field experiments, attractiveness of males to TDTA depended on the time of day and trap designs (Attygalle et al. 1995; Attygalle et al. 1996; Ferrara et al. 2001). In the study of Minuz et al. (2020), adult *H. obsoletus* were repelled from grapevine that were treated with benzothiadiazole. Additionally, a combination of attractants and repellents can be used to further develop push-and-pull strategies (Eben and Gross 2013). However, none of these measures have been used for sustainable *P. leporinus* management so far and no
information about pheromone release is available in Auchenorrhyncha (Mühlethaler et al. 2019). Thus, basic research is required to test if these measures based on pheromones can also be applied against *P. leporinus*. As already discussed, the rearing protocol presented can provide *P. leporinus* to carry out various tests under controlled conditions to identify sex pheromones of *P. leporinus* and use them for their control.

Dryinid wasps are promising for the use in biological control of Auchenorrhyncha (Guglielmino 2002) (see Introduction section 1.1.9). Furthermore, cixiid nymphs were found to be parasitized by *Embolemus* sp. and *Ampulicomorpha* sp. (reviewed in Guglielmino and Bückle 2013) (see Introduction section 1.1.9). In general, low information is available on *P. leporinus* being parasitized. Furthermore, in the study of Moussa et al. (2021), entomopathogenic nematodes (e.g. *Steinernema carpocapsae*) and fungi (e.g. *Isaria fumosorosea*) effectively controlled *H. obsoletus* under controlled environmental conditions. In the study of Langer et al. (2005), *Metarhizium anisopliae* was successful in controlling *H. obsoletus* adults under controlled conditions. Thus, field-collected *P. leporinus* nymphs and adults (alive or dead) should be examined closely for parasitation and infections with entomopathogenic fungi or nematodes. Furthermore, possible harmful effects of Embolemidae or Dryinidae against *P. leporinus* can be tested under controlled conditions by using insects from the mass rearing. If successful, reproducibility under field conditions is mandatory. Indeed, entomopathogenic fungi were isolated from field collected *P. leporinus* nymphs. However, these isolates were not effective against *P. leporinus* nymphs in virulence tests under controlled conditions. In addition, *Metarhizium* sp. isolates were only partly effective against *P. leporinus* (data not shown). Thus, biological control of the planthopper remains difficult.

Furthermore, RNA interference (RNAi) can be used for gene silencing in insects (reviewed in Gross et al. 2022). RNAi can help to understand the role of genes in molecular processes and to establish vector control measures (reviewed in Jain et al. 2020). Furthermore, it is highly specific to the targeted organisms and it is a sustainable method in pathogen and insect pest control (reviewed in Menezes et al. 2022). This method targets the organisms molecular processes and aims to silence specific genes, which was already applied against numerous planthoppers (reviewed in Menezes et al. 2022; Yu et al. 2014). Yu et al. (2014) tested RNAi on *S. furcifera*, which has macropterous (long wings) and brachypterous (short or reduced wings) lineages. More, the wingless gene (*wg*) has a crucial role in the wing dimorphism of the planthopper. *S. furcifera* nymphs (second instar) were artificially fed with double-stranded RNA of *wg*. Consequently, RNAi reduced the *wg* expression in nymphs as well as body weight and wing length in adults. Furthermore, the wings displayed deformations (Yu et al. 2014). Finally, RNAi should be tested for *P. leporinus* control.
6.3.4 Resistance breeding

According to Bianco et al. (2019), breeding for resistant varieties is another effective method to control bacterial-derived (here: Phytoplasma-derived) plant diseases. Regarding SBR disease, no resistant varieties are currently available. Other important steps are the establishment of a biotest system that allows rapid research on potential tolerance or resistance mechanisms in sugar beet. For this purpose, suitable climatic controlled conditions (temperature, humidity, photoperiod, light intensity and spectrum), materials and experimental setups (plant pot size, substrates, plant age, planthopper numbers, IAPs) are required. Several groups successfully reproduced SBR symptoms in sugar beets under controlled conditions (Bressan et al. 2008; Mahillon et al. 2022; Sémétéy et al. 2007b). However, it took at least ca. seven weeks until the first SBR symptoms were observed and even longer until emergence of strong symptoms such as chlorosis in old leaves (Bressan et al. 2008; Mahillon et al. 2022; Sémétéy et al. 2007b). This was also confirmed in own studies (data not shown). Obviously, such systems are not suitable for large scale and rapid testing of genotypes. Thus, alternative protocols should be proposed. The presented mass rearing protocol also contributes to accelerated research of this question under controlled conditions since SBR-proteobacterium infected P. leporinus were produced. As previously described, the establishment of a rearing with SBR-phytoplasma infected P. leporinus may be a key factor to test the effects of SBR-phytoplasma and SBR-proteobacterium in detail. Alternatively, grafting can be used to transmit phytoplasmas to plants (reviewed in Namba 2019). Recently, Mahillon et al. (2022) artificially transmitted SBR-proteobacterium to C. roseus using P. leporinus adults (see Introduction section 1.1.3) and grafting. However, transmission of both SBR pathogens by grafting still has to be established for sugar beets. If possible, this technique would not require planthoppers, thus tests could be performed independently from the insect vector.

6.3.5 Curative applications

In contrast, curing of plant diseases caused by phytoplasmas concentrates more on (perennial) crops than on insect vectors, however, no efficacious methods are available (reviewed in Laimer and Bertaccini 2019). In older studies, chemotherapeutic treatments by tetracycline injection into phytoplasma infected plants temporarily reduced plant symptoms in different perennial crops (reviewed in Laimer and Bertaccini 2019). However, the symptoms generally reappeared after some time or regular treatments were necessary and antibiotic treatments of crops are not permitted in European countries (reviewed in Laimer and Bertaccini 2019). In addition, thermoderapeutic methods (e.g. treating graft material with hot water or hot air) were effective, however, only tested for perennial crops such as grapevines that were infected with FDP (reviewed in Laimer and Bertaccini 2019). Obviously,
6. Discussion

thermotherapeutic treatments of sugar beets are not possible under field conditions, thus, future research should focus on other control methods (described above).

6.4 Monitoring of Pentastiridius leporinus, host plants, and SBR

Detailed knowledge about the host range and spread of *P. leporinus* are essential for targeted vector control and the development of integrated pest management strategies. In this thesis, for the first time a method is presented that allows reliable identification of *P. leporinus*, including eggs, all five nymal instars, male and female adults (manuscript III). A key advantage is that this method makes *P. leporinus* monitoring available for non-experts in Auchenorrhyncha morphology and taxonomy. Especially regions where *P. leporinus* newly immigrated lack the experience in *P. leporinus* identification based on morphological traits. However, in this study, species selection was based on Auchenorrhyncha reported from sugar beet fields (Pfitzer et al. 2020; Sémétey et al. 2007a) and species lists should be updated in future regarding appearance and possible host plants.

To further evaluate the potential and significance of vector control measures as reported in manuscript II, exact knowledge about the host plant range is essential. Currently it is assumed or suggested that *P. leporinus* only reproduces in sugar beet crop rotations or on reed grass (see Introduction section 1.1.8). Thus, targeted *P. leporinus* control would be more difficult, if significant numbers develop on additional plant species as suggested by Nickel (2003). Consequently, these specimens would not be controlled by the agricultural measures suggested in manuscript II. Thus, the newly established DNA based species-specific detection method can be used in studies on the oviposition hosts and the host plant range of the nymphs or adults after selective sampling on the potential host plants (reviewed in Holzinger et al. 2003; reviewed in Krüger and Fiore 2019). Further, rearing of collected nymphs to the adult stage, as reviewed in Holzinger et al. (2003), is not required any more before species identity of immature specimens can be determined. In this thesis, a morphological key is provided to unequivocally distinguish *P. leporinus* nymphal instars (manuscript I). However, use of morphological traits for identification of nymphs can be limited, especially if species are mixed in a sample of several nymphs, e.g. *Hyalesthes* sp. and *Pentastiridius* sp. nymphs (Sforza et al. 1999). Thus, according to the morphological key provided in manuscript I, only *P. leporinus* nymphal instars can be distinguished and species identification is not possible in mixed populations. Finally, molecular methods can help to overcome this problem (Sforza et al. 1999). Therefore, a combination of head capsule width measurement (manuscript I) and molecular detection (manuscript III) can confirm nymphal instar and species identity in practical monitoring assays. Finally, the molecular method reported in manuscript III can contribute to a better understanding of the vector’s host range.
In this study, a PCR based molecular method was developed that enables monitoring *P. leporinus* and detecting all life stages of the insect using crude DNA templates. However, this method still requires a thermocycler and a gel electrophoresis step which are not practical for the on-site detection of the vector. Further studies are required to develop on-site detection of *P. leporinus* based on isothermal methods such as LAMP or RPA (reviewed in Dickinson and Hodgetts 2019). Generally, RPA is a fast method that allows species-specific detection of insects or pathogens using specific primers. In this assay, primer binding is supported by enzymes which divide double-stranded DNA (dsDNA). More, in RPA assays, enzymes and proteins (strand-displacing polymerase and recombinase single-stranded binding protein) are combined to isothermally amplify DNA. RPA works under a moderate temperature (37-42 °C) and in a short time (5-30 minutes) (reviewed in Bertaccini et al. 2019; reviewed in Dickinson and Hodgetts 2019; Priti et al. 2021) which allows rapid on-site species-specific detection of target genes (reviewed in Bertaccini et al. 2019; reviewed in Dickinson and Hodgetts 2019; Priti et al. 2021). The designed primers in this study for the specific detection of *P. leporinus* can be tested for detection by RPA. However, specific detection of *P. leporinus* using LAMP assay needs to develop new primers and experimental set up.

Molecular detection of phloem-limited bacteria is challenging (reviewed in Bendix and Lewis 2018). In phytoplasma induced plant diseases, detection of infected plants already before symptom expression can help to remove these plants to avoid the spread of plant diseases (reviewed in Dickinson and Hodgetts 2019). Since RPA and LAMP can be used for detection of pathogens (reviewed in Bertaccini et al. 2019), a further aim could be to establish rapid methods for early detection of both SBR-phytoplasma as well as SBR-proteobacterium in sugar beets contributing to monitoring of the SBR disease.
7. Summary

*Pentastiridius leporinus* (Hemiptera: Cixiidae) is the main vector of Syndrome ‘basses richesses’ (SBR), a fast-spreading sugar beet (*Beta vulgaris*) disease in Central Europe. The disease is caused by two procaryotic phloem-limited bacterial pathogens, the γ-3 proteobacterium ‘*Candidatus Arsenophorus phytopathogenicus*’ (here: SBR-proteobacterium), and the stolbur phytoplasma (16SrXII-A subgroup) ‘*Candidatus Phytoplasma solani*’ (here: SBR-phytoplasma). SBR infections in sugar beet can lead to maximum 5% losses in absolute sugar content and more than 25% yield reduction in the taproots. *P. leporinus* has adapted from its natural host reed (*Phragmites australis*) to sugar beet in crop rotation with cereals such as winter wheat (*Triticum aestivum*) or spring barley (*Hordeum vulgare*). However, the developmental stages of *P. leporinus* have not been characterized and no rearing protocol for this planthopper is available. In addition, agronomical measures for sustainable *P. leporinus* control are lacking. Further, other species of Auchenorrhyncha were reported from sugar beet fields and species such as *Hyalesthes obsoletus* and *Reptalus quinquecostatus* (Hemiptera: Cixiidae) are morphologically closely related to *P. leporinus*, can be hardly distinguished and only male adults can be identified to the species-level using morphological keys. Thus, a reliable molecular method is required for species identification of all *P. leporinus* developmental stages.

To establish a *P. leporinus* rearing, suitable conditions such as temperature, humidity and light were optimized. The established rearing allowed continuous production of ca. 20,000 specimens and up to five generations exclusively on sugar beet in 21 months without dormancy. In a no-choice experiment, median survival time for adults was 24 days and females laid 139.1 ± 132.9 eggs on sugar beet. In contrast, the females rapidly died (6 days median survival time) and did not oviposit on wheat plants. Development time of second to fifth instars increased with the age and first instar was most vulnerable (25.7% mortality), compared to the other instars (3-13.4% mortality). Finally, development time from first instar to adult was remarkably faster under controlled (males: 193.6 ± 35.8 days, females: 193.5 ± 59.2 days) compared to field conditions (ca. one year). The fifth-instar nymphs were successfully (68.7%) emerged as adults within 140 days after being transferred to potted sugar beets with a top layer of expanded clay. Further, in the first three generations of the rearing, 70-80% of adult planthoppers (n=10) carried SBR-proteobacterium according to polymerase chain reaction (PCR). In contrast, SBR-phytoplasma was not detected. Thus, SBR-proteobacterium infected *P. leporinus* can be cultivated using this protocol.

To study agronomical measures for *P. leporinus* control, adult *P. leporinus* emergence was quantified after variations in soil tillage (tillage by plough or cultivator) and succession crops
(spring wheat, spring barley, maize, and bare soil were compared to winter wheat) following SBR-infested sugar beet in field experiments. In five of seven field trial sites, adult numbers from winter wheat did not differ between tillage by plough or cultivator and in two sites, adult *P. leporinus* emergence was significantly (p<0.05) lower after ploughing. Further, adult numbers were significantly reduced (up to 98.9 %) in bare soil and in the latest sown spring crop maize, in all three and four analysed field trial sites, respectively.

Under controlled environmental conditions, survival and development of *P. leporinus* first and third instar nymphs were tested on wheat, maize, and barley (only tested for first instar) seedlings. The survival rates in first instar were 4.2 % on maize, 29.2 % on barley seedlings and 66.7 % on wheat over 300 days. Furthermore, 4.2 %, 29.2 %, and 62.5 % of first-instar nymphs developed into adults on maize, barley, and wheat, respectively. In third instar nymphs, similar survival rates were observed for maize (70 %) and wheat (73.3 %) over 150 days. More, 56.7 % and 50 % of third instar nymphs developed into adults on maize and wheat, respectively. It is assumed that the effects observed in maize were caused by starvation of nymphs due the long host-free period after sugar beet harvest.

To establish a PCR-based molecular method for reliable, time- and cost-saving identification of all *P. leporinus* development stages, species-specific primers were designed on highly conserved parts of mitochondrial cytochrome oxidase I gene (*COI*) which had mismatches on the 3'-ends when compared to sequences of *R. quinquecostatus* and *H. obsoletus*. PCR method was established using two methods of template DNA preparation including a commercial kit or a simple, time- and cost-saving insect crushing in phosphate-buffered saline (PBS). The established PCR-based method allowed species-specific detection of *P. leporinus* male and female adults and differentiation from *R. quinquecostatus* and *H. obsoletus*, and also other common Auchenorrhyncha species that were collected from German sugar beet fields. Further, all immature stages of *P. leporinus* (eggs and all five nymphal instars) were specifically detected. In addition, phylogenetic analysis based on the specifically amplified *COI* fragment showed that *P. leporinus* differs from the other close planthopper species including *R. quinquecostatus* and *H. obsoletus*.

In conclusion, this study provides basic knowledge on the biology of *P. leporinus* and the mass rearing protocol enables year-round availability and accelerated research on the planthopper. Furthermore, agronomic measures for vector control were analysed on multiple field trial sites and potential further steps for research are discussed. The established molecular method can be used for monitoring of all life stages of *P. leporinus* in sugar beet and other host plants without additional sequencing steps.
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8. References


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Eidesstattliche Erklärung

1. Hiermit erkläre ich, dass diese Arbeit weder in gleicher noch in ähnlicher Form bereits anderen Prüfungsbehörden vorgelegen hat.

Weiter erkläre ich, dass ich mich an keiner anderen Hochschule um einen Doktorgrad beworben habe.

Bühlertann, den 31.01.2023

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Bühlertann, den 31.01.2023

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