A genetic analysis reveals low prevalence of phytoplasma infection in *Hyalesthes obsoletus* Signoret, vector of 'bois noir', in SW-Germany

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Abstract:

Bois Noir is a grapevine disease responsible for severe economic losses in wine production. Bois Noir is caused by *Candidatus* Phytoplasma solani, cell wall-less bacteria belonging to the taxonomic group 16Sr-XII-A. In Germany, they are known to be vectored from plant to plant by the cixiid *Hyalesthes obsoletus*, but so far the prevalence of the disease in the vector population, as well as its spatio-temporal distribution is poorly understood. We therefore analyzed infections of *H. obsoletus* collected in different vineyards in Baden (South-Western Germany) with quantitative real-time PCR. From 125 analyzed individuals, only five were infected with *Ca*. Phytoplasma solani. All infected individuals were colonized by *Ca*. Phytoplasma solani type I which is associated with the host plant *Urtica dioica* (stinging nettle). More research is needed to understand the reasons of this surprisingly low prevalence of Bois Noir in the population of *H. obsoletus* in South-West Germany.

Introduction:

Phytoplasma are cell-wall less prokaryotes. Lacking common metabolic pathways, they need host tissues for their survival and are therefore obligate parasites (Christensen et al., 2005). Phytoplasma have developed complex life cycles that involve replications in both insects and plants. In plants, they are usually found in phloem tissues, while in insects they need to cross the gut cells, replicate within internal tissues, and then reach salivary glands for transmission to new plants (Hogenhout et al., 2008). This peculiar life cycle allows them to easily reach new host plants taking advantage of the mobility provided by the insect hosts. In cultivated crops, they can also be transmitted through agricultural practices such as pruning and grafting (Hodgetts et al., 2013).

While the number of known insect vectors for phytoplasma is limited (so far, phytoplasma were only found among phloem feeders of the order Hemiptera (Hogenhout et al 2008)), there is a broad range of host plants. Phytoplasma infections have been found in over 700 plant species (Hoshi et al., 2009). Some of these infections cause severe damage to agricultural crops with serious economic impact. Some examples are: lethal yellowing of palms, (Harrison et al. 2008), peach X-disease, apple proliferation (Bertaccini et al., 2010), and Bois Noir (BN), a grapevine phytoplasmosis. The latter is the focus of this study. Typical BN disease symptoms on *Vitis vinifera* include necrotic leaves with downward rolled margins, unlignified branches and shriveled berries. At the end of the veraison the shoots do not lignify and turn black, giving the disease its name of Bois Noir. The infection usually leads to a significant decrease in yield (Garau et al., 2007). Due to the growing economic impact of the disease in European grapevine production (Arnaud et al., 2007, Garau at al., 2007), a better understanding of the transmission and dynamics of this phytoplasma is therefore of great importance.

According to current literature, the main vector of BN in Western Europe is the ciixid plant hopper *Hyalesthes obsoletus* Signoret 1865 (Maixer, 2006). Although *H. obsoletus* feeds on a wide range of herbaceous plants, its life cycle can only be completed on few hosts. The two most relevant host plants in Germany are stinging nettle (*Urtica dioica*) and field bindweed (*Convolvulus arvensis*) *(*Maixner et al., 2005). These two hosts are related to distinct strains of BN causing phytoplasma: *Candidatus* Phytoplasma solani type I is related to *U. dioica,* while *Ca*. Phytoplasma solani type II is associated to *C. arvensis* (Quaglino et at, 2013). Both strains have grapevine as a "dead end host", which means that insects can infect a grape plant, but cannot acquire phytoplasma from infected grape plants (Kaul et al., 2009). Before the year 2000, *H. obsoletus* was considered a rare species in Germany, and *C. arvensis* was the major host plant for this epidemic cycle (Sergel, 1986). In the last 20 years, however, *H. obsoletus* was found more frequently on *U. dioica*. It has been speculated that the increasing mean temperatures could be connected to this host plant shift (Boudon-Padieu et al., 2007). The new host allowed the insect to colonize new areas (that is where *U. dioica* is present and *C. arvesis* absent); but also increase its population density in areas where it was already present (Maxiner et al., 2007). For these reasons, BN has become an increasing concern in Germany.

Our study took place in the region of Baden. Located in South-West Germany at the borders of France and Switzerland, Baden is one of the most important areas of Germany for wine production. Since BN is a relatively new challenge, only few studies have examined this

epidemiological system in the area object of our study. Darimont et al. (2001) conducted an analysis of insect infestation in the year 1999 and 2000. Breuer et al. (2008) conducted a monitoring of the occurrence of *H. obsoletus* proving the insect's presence in all wine-growing districts, and Panassiti et al., (2013) reported the presence of the insect all over the region. From these studies, we have a basic understanding of the ecology of the disease, but additional data is needed on disease prevalence and spatial distribution of the disease in the insect population in the area. In addition, it would be desirable to better understand whether the phytoplasma infection has any consequences for the vector in terms of body size and thus implicates consequences for the vector fitness (e.g. on fecundity (Honěk, 1993)).

To address this problem, we analyzed infection incidence (via genetic analysis) and morphological features of *H. obsoletus* individuals, collected in 45 vineyards across the region of Baden.

We want to investigate what is the prevalence of the disease in the vector population, distinguishing between the two different types of phytoplasma (type I or type II). We analyze the spatial distribution of the infected insects in order to have an overview of the infestation distribution in the area. We check also the weight and length of the specimen for a comparison between infected and non-infected individuals.

Materials and Methods:

Sampling of *H. obsoletus* individuals was performed as described in Panassiti et al. (2013). In brief, 125 individuals were collected at 45 locations spread over the Baden region (Figure 1) between June and August in 2012 and 2013. The locations were selected randomly. Yellow sticky traps and the viticulture prediction tool "vitimeteo" (www.vitimeteo.de) were used to monitor and predict the flight activity of the insects, in order to guarantee optimal sampling conditions. In each of the locations, potential host plants were identified. If *U. dioica* was found, the sampling consisted of sweeping a sweep net (30 cm diameter) over the selected plants. If *C. arvensis* was found, suction sampling was performed. Stinging nettle patches were swept 5 times per square meter. Suction sampling was applied for 3 minutes for every square meter of the patch.

The collected insects were transported in a cooling box and freeze dried in the laboratory with a freeze dryer "Christ Alpha 1-2 LDplus", (Martin Christ Freeze Dryers, Germany) and then further analyzed. We determined species and gender following the identification keys of Biedermann and Niedringhaus (2004) with a microscope "Zeiss Stereo LUMAR 1.2", (Carl Zeiss, Germany) (figure 2). We measured body length with the software "Axiovision Rel 4.8". The length of an individual was defined as the distance from head to the bottom of the fore-wings (Figure 2). The samples were then frozen in liquid nitrogen and conserved at -80 °C for further analyses.

DNA extraction and quantitative real-time PCR (qPCR) was carried out with the same procedure for extraction and the same primers, probes and cycle settings for qPCR as in Fahrentrapp et al. (2013). This method utilizes hydrolysis probes specific for selective DNA fragments of both, phytoplasma types I and II, as well as for insect DNA. The method allows to detect an infection and to distinguish the phytoplasma type. Based on the obtained results, we calculated the amount of phytoplasma DNA relative to the insect DNA, using the method $2^{-\Delta\Delta Ct}$ presented by Livak et al., (2001) for relative quantification of gene expression.

Results

Of the 125 collected *Hyalesthes obsoletus* individuals, 52 were identified as males, 70 as females and 3 were not identifiable due to the lack of the final abdominal segments. The observed female ratio of 56% is not significantly different from an even sex ratio (p=0.18 with a binomial test against H_0 = 50%; the 95% confidence interval spans 47% - 65%). Of all 125 individuals, five (four females and one male) were infected with *Ca*. Phytoplasma solani, type I. This can be translated into a disease prevalence of 4%, with the 95% confidence interval from a binomial model ranging from 1.3% to 9.2%. The five infected individuals were caught in different locations spread all over Baden (Figure 1). The amount of phytoplasma DNA detected in the samples varied substantially. The sample with the highest amount of phytoplasma DNA has roughly 17 times more phytoplasma-DNA than the sample with the lowest amount (Table 1).

We observed a difference in weight and length between males and females. Females were in general longer and heavier than males. The average length and dry weight for females and males was 4.95 (+/-0.31), 4.01 (+/-0.28) mm as well as 1.7 (+/-0.57) and 0.67 (+/-0.31) mg, respectively. Our results for sex ratio, weight and length are also displayed table 2 and in figures 3 and 4. Due to the low number of infected insects, it was not possible to draw any conclusions about the influence of infections on insect length and body mass. The five infected individuals show values that are well within the range found for non-infected individuals.

Discussion

The prevalence of *Ca.* Phytoplasma solani infection in *H. obsoletus* determined in this study was surprisingly low. We only found five infected individuals in 125 samples analyzed (4%). Studies in different areas showed higher infection rates. Langer (2004) observed an infection rate of 26.5%. In a study conducted in the north of Italy, Lessio et al. (2007) reported a variable infection rate during different timeframes, reaching up to 80%. Darimont et al. (2001), who also sampled in Baden, but over different years, reported an average infection rate of 23% over several years. We have no clear explanation for the low disease prevalence in the present study. The sample size of this study is relatively low, but as the 95% confidence interval extended only up to 9.2%, random variation is unlikely to be an explanation for this data if the true disease prevalence were around 20%. A plausible explanation is that the sampling methods used in our study were different from previous studies. In our study, sampling locations were chosen randomly. In the other studies mentioned, collection site selection was not clearly described and it could be that sampling locations were chosen close to vineyards in which the disease has already been observed. In this case a higher prevalence would be logically expected. Therefore there is a need for further studies to resolve this open question.

The length measurements of *H. obsoletus* are in agreement with values from the literature. Alma (2002) described body length of 3.7-4 and around 5 mm for males and females, respectively. To our knowledge no previous studies reported on the dry body weight of the insects that could be compared to our results. No significant bias of the sex-ratio was observed in this study as expected for our sampling method. Other studies that utilized traps for sampling, observed a male-biased sex ratio (Lessio et al., 2007). It is in fact known that in

many Homoptera species, males have a greater flight activity and a higher dispersal rate compared to females (Lessio et al., 2004).

In conclusion, our study showed an unexpectedly low prevalence of BN-causing phytoplasma in individuals of the insect vector *H. obsoletus* caught in Baden. We were not able to draw any conclusion on differences in size of the individuals due to the low number of infected individuals.

Further surveys with a higher number of specimen, sampling locations and an analysis of plant material for *U. dioica*, *C. arvensis* and *V. vinifera*, will help to understand if this low prevalence reflects a true decline of the disease in the vector population, or whether it can be explained with systematic differences with previous studies.

Acknowledgments

Phytoplasma infected *H. obsoletus*-derivedDNA samples used as positive controls in qPCR were kindly provided by M. Maixner.

We thank Dr. Breuer and the Staatliches Weinbauinstitut Freiburg, for providing the possibility to conduct this study and the financial support.

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268 **Table 1:**

Table 1: Overview of qPCR results and morphological measurements for the insects in which an infection was detected, including length, weight, and folds of DNA.

Sample	Sex	Length (mm)	Dry weight (mg)	DNA Folds*	St. Dev.
1114	Female	4.74	1.7	2.6	0.3
1109	Female	4.03	1.7	17.2	0.5
1123	Female	5.19	2.3	3	0.7
1041	Female	4.9	2.1	13.3	0.7
1134	Male	3.48	1.1	1	1

*Relative titers of DNA normalized to sample 1134.

269
270 We here present the results of the morphological observations and qPCR measurements. The
271 DNA folds are calculated using the ratio phytoplasma DNA/insect DNA. We then expressed
272 the results in folds relatively to the sample with the lowest amount of DNA (1134). This
273 means that, as an example, sample 1114 was found having 2.6 times more phytoplasma DNA
274 present in his body compared to sample 1134.
275
276

277 **Table 2:**
278

Table 2: Maximum, minimum and average values of morphological observations grouped by sex of all specimens collected in this study.

Measurement	Average value (Std. Dev.)	Max.	Min
Sex ratio (%)	56 (9)		
Male length (mm)	4,01 (0.28)	4.49	3.18
Female length (mm)	4.95 (0.42)	5.7	3.73
Male body mass (mg)	0.67	1.4	0.1
Female body mass (mg)	1.7 (0.57)	2.7	0.2

279
280 In table 2 the results of morphological observations are presented. Females clearly show
281 bigger values for length and weight compared to males, usually shorted and lighter.

282 **Figures:**

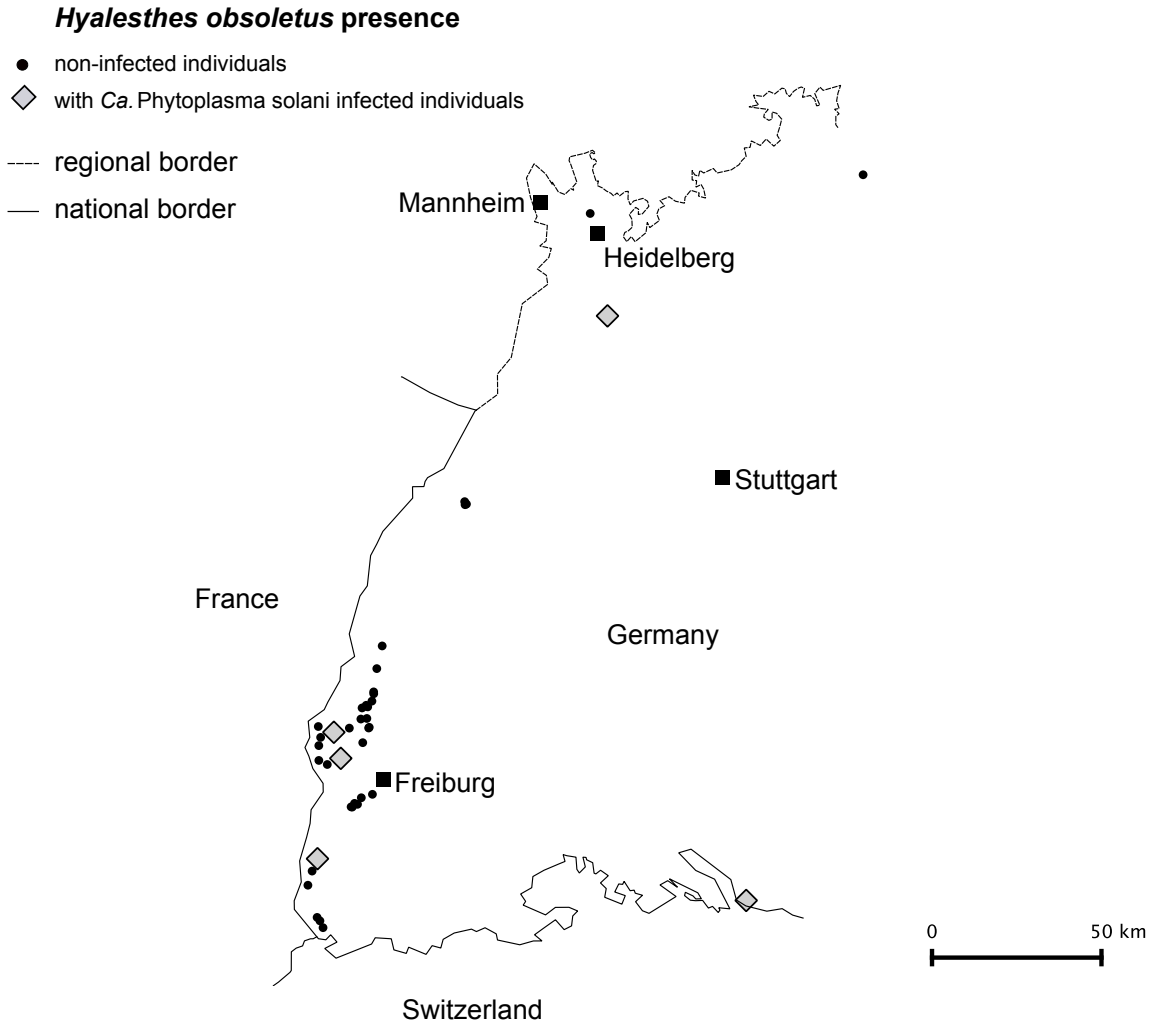

Figure 1: Map representig the collection sites in the region of Baden. Black dots are the sampling locations while grey squares represent the sampling locations where infected H. obsoletus were found.

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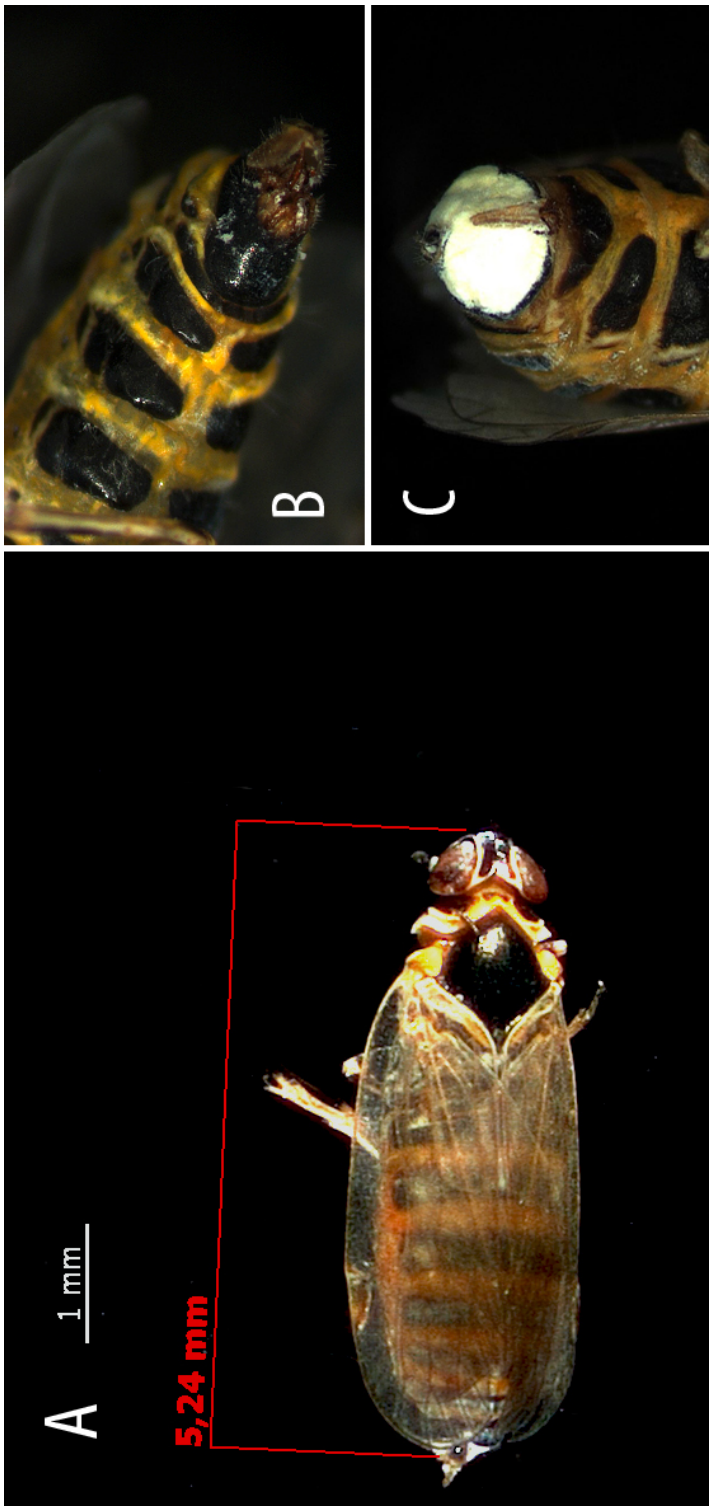

Figure 2: Magnified photographs of (A) H. obsoletus, (B) male genital capsule, and it's (c) female ovipositor.

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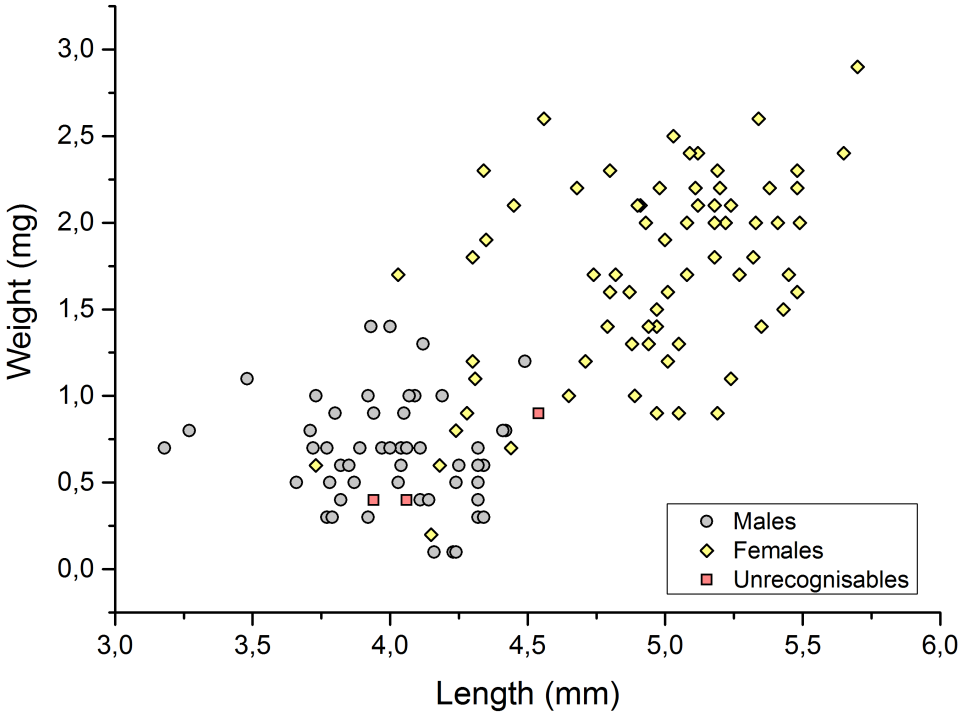

Figure 3: Distribution of length and weight for the individuals analyzed. Females are colored in yellow diamonds, males are represented in red dots and the samples with the abdomen missing (unrecognisable) are shown in blue squares.

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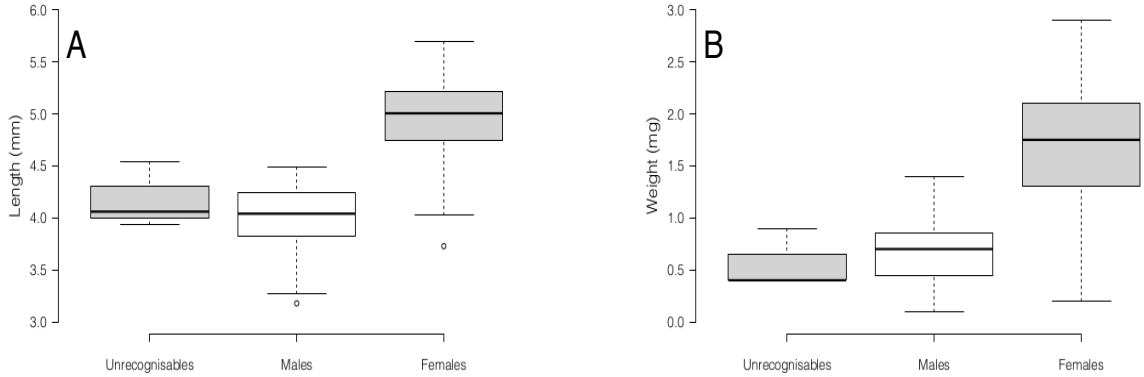

Figure 4, A: Box plots of distribution of length (A) and Weight (B) of specimen collected in this study; red indicates males, yellow females and blue the unrecognisable samples.

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