Widespread occurrence of *Candidatus* Phytoplasma ulmi infecting elm species in Germany

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Abstract

Background: *Candidatus* Phytoplasma ulmi is the causative agent of elm yellows and has been categorised in the European Union as a quarantine pathogen in the past. For central and northern European countries, information on the occurrence and distribution of the pathogen and its impact on elms is scarce, so a survey of native elm trees has been conducted in Germany. Results: About 6,500 samples in total, from *Ulmus minor*, *Ulmus laevis* and *Ulmus glabra*, were collected nationwide. Phytoplasma detection was performed by applying a universal 16S rDNA-based real-time PCR assay and a novel *Ca. P. ulmi* species-specific real-time PCR assay targeting the 16S-23S spacer region. Both assays revealed that 28% of the samples were infected by *Ca. P. ulmi*, but infection rates of the elm species and regional incidences differed. The infection of trees is not correlated to disease-specific symptoms. The survey identified a regional disparity of infection which was high in east, south and central Germany, whereas only a few infected sites were found in the western and northern parts of the country. No correlation was apparent between altitude level and the prevalence of infection. First insights into the monitoring of the seasonal titre of *Ca. P. ulmi* in an infected tree by real-time PCR revealed high colonisation in all parts of the tree throughout the year. Conclusions: *Ca. P. ulmi*-infection is widely present in elms in Germany. The rare occurrence of symptoms indicates either a high degree of tolerance in elm populations or a low virulence of pathogen strains enabling high infection rates in a long-living host.

Background

Phytoplasmas are characterised as obligate parasites from the bacterial class Mollicutes, where they form the monophyletic taxon '*Candidatus* Phytoplasma'. They colonise the nutrient-rich phloem of their plant host and rely for transmission on phloem-feeding hemipteran insect vectors.

Phytoplasmas are associated with diseases of more than 1,000 plant species, including many important crops [1]. Several phytoplasma groups comprise the description 'yellows' or 'yellowing' in their name, due to an observed phytoplasma-associated leaf chlorosis. This is also the case for *Candidatus* Phytoplasma ulmi infecting elm trees [2]. *Ca. P. ulmi* is phylogenetically closely related to economically important plant pathogenic phytoplasmas such as *flavescence dorée* phytoplasma, causing grapevine yellows [3], *Candidatus* Phytoplasma rubi, causing rubus stunt [4], *Candidatus* Phytoplasma ziziphi causing jujube witches' broom [5], and alder yellows phytoplasma, causing alder yellows [6]. These phytoplasmas belong to the elm yellows group and are classified as 16SrV group members based on their restriction fragment length polymorphism pattern in PCR-amplified 16S rDNA [2].

Elm yellows was first described in 1938 in North America [7], but historic reports indicate earlier sightings [8, 9]. Most North American elm species are highly susceptible to an infection by the bacterium and show a dramatic course of disease progression [10–12]. The trees usually die within two years post-infection, displaying a number of characteristic symptoms such as leaf yellowing, witches' broom formation and phloem necrosis. In the US, the disease spread gradually from the mid-western states to the east and to

the south, causing a considerable loss of native elm trees [12]. In Europe, the disease was first reported in Italy and then later on in France, Bulgaria, Serbia and Croatia [13–15]. The disease symptoms displayed by the European elm species resembled those of the North American elm species, but phloem necrosis did not occur. Therefore, the European elm species were considered less susceptible than their American relatives [16].

Since 1975, *Ca. P. ulmi* has been regarded in the EU as a harmful organism and regulated by the Council Directive 2000/29/EC [17]. A comprehensive analysis on the re-categorisation of *Ca. P. ulmi* was conducted by the European Food and Safety Authority in 2014 [18], but due to limited information on its distribution, strain virulence, potential insect vectors and effects on European elm species, the report remained inconclusive. Ensuing reports of elm yellows findings from the UK, the Czech Republic, Poland and Belgium, however, demonstrated that *Ca. P. ulmi* is more widespread in the EU than previously thought [19–23]. In response to the new situation the European Plant Protection Organization moved *Ca. P. ulmi* in 2017 from Annex I list A1, as a pathogen absent from the EU, to list A2, in recognition of its regional presence [24]. In December 2019, the new Council Directive 2016/2031 will deprive the quarantine status of *Ca. P. ulmi* for continental Europe, but the status for Great Britain will remain in place.

In Germany, *Ca. P. ulmi* was first reported in 1992 from a single Scots elm (*Ulmus glabra*) displaying witches' broom symptoms in south-western Germany [25]. DNA-DNA hybridisation studies with elm yellows-specific probes resulted in identical hybridisation profiles between German, French, Italian and American accessions, thereby providing evidence that the European elm yellows strains were closely related – or actually identical to – the American strains [14]. In a more recent study, 59 European white elm (*Ulmus laevis*) trees, four of which showed stunted growth and leaf chlorosis, were examined in the states of Brandenburg and Berlin [26]. Based on 16Sr DNA nested PCR assays, half of the tree samples were identified as phytoplasma-positive, and sequence analyses confirmed the presence of *Ca. P. ulmi*. However, all symptomatic elm trees tested phytoplasma-negative. The unexpected high presence of *Ca. P. ulmi*, and the absence of visual disease symptoms found in this study, prompted a nationwide survey of the pathogen's distribution and occurrence in the native elm species *U. glabra*, *U. laevis* and *U. minor*. The results of this survey, comprising a distribution map with incidence levels at sampling sites and a newly designed elm yellows-specific real-time PCR assay, are presented herein.

Results

Wide absence of disease symptoms of elms in Germany

A total of 6,486 elm accessions from 339 sites were collected. The accessions comprised 2,630 Scots elm, 2,049 European white elm and 1,807 field elm samples (Table 1, Fig. 3A). The individuals' ages ranged from one-year-old seedlings to trees of more than 400 years. The trunk diameter of the trees ranged from 0.5 cm to 3.5 m.

Table 1
Overview on number and elm samples collected during the survey in the federal states of Germany.

Federal State/elm species and no. of samples	U. glabra	U. laevis	U. minor	No.
Baden-Württemberg	371	90	265	726
Bavaria	374	90	124	588
Berlin	0	34	46	80
Brandenburg	204	412	304	920
Hamburg	0	0	29	29
Hesse	70	15	25	110
Lower Saxony	113	59	102	274
Mecklenburg West-Pomerania	467	494	40	1001
Rhineland-Palatinate	160	91	129	380
North Rhine-Westphalia	223	228	231	682
Saarland	29	0	31	60
Saxony	270	180	150	600
Saxony-Anhalt	46	138	170	354
Schleswig-Holstein	215	142	78	435
Thuringia	88	75	84	247
Number of species and total number	2630	2048	1808	6486

Ca. *P. ulmi*-specific symptoms were rarely observed, considering the number of trees, the infection rate at some sites, their different ages and environment. However, in 2017, 2018 and 2019, symptomatic Scots elms, approximately 2 to 3 m in height, were observed along roadsides in Müncheberg, Brandenburg (Fig. 1A, B). The trees showed numerous witches' brooms clearly visible in winter time and during new shoot development in July/August. These plants showed an early bud break at the witches' broom sites in March compared to non-symptomatic parts of the trees. Otherwise, the trees looked inconspicuous. One tree, about the same height and in the same area, showed a different symptomatology, in that all branches were severely stunted, and the leaves were small and brittle. In 2018, field elms displaying little leaf, yellowing and stunting symptoms were observed in Ingelheim am Rhein, Rhineland-Palatinate (Fig. 1C, D, E) and near Haßfurt, Bavaria. In many natural habitats, Scots elms and field elms were severely affected by the Dutch elm disease clearly showing xylem discolouration. Fungal-induced wilting and dieback symptoms were aggravated during the 2018 summer drought in Germany.

Characteristics of the Ca P. ulmi-specific TaqMan assay

A universal real-time assay for the detection of phytoplasma infection and for control of the template quality (18Sr DNA plant) was routinely used [27]. A specific-assay for the detection of Ca. P. ulmi was not available and was developed in this work. The universal and the species-specific assay have been applied within this study enabling the detection of phytoplasma infections in general and of Ca. P. ulmi in particular, respectively. The selected forward and reverse primers and the TaqMan probe of the specific assay are located in the spacer region at positions 1,677 to 1,698, 1,700 to 1,721 and 1,754 to 1,772, respectively, relative to the first nucleotide of the Ca. P. ulmi sequence deposited under acc. no AF122911 and amplified a fragment of 96 bp in length. The Ca. P. ulmi sequences showed most differences in relation to the sequences of alder witches' broom, flavescence dorée, rubus stunt and Ca. P. balanitae in the region between the forward primer (fEY_spacer-rt) and the TaqMan probe (qEY_spacer-rt) annealing site. The reverse primer (rEY_spacer-rt) differs only by a T at the 3' end relative to the sequences of alder witches' broom, flavescence dorée and rubus stunt, or by an internal nucleotide difference relative to Ca. P. balanitae (Fig. 2). Temperature gradient assays revealed an optimum temperature of 56 °C, when considering product yield and assay specificity. At this temperature, only the Ca. P. ulmi strains ULW and EYC maintained in C. roseus and the Ca. P. ulmi-infected field samples were amplified (data not shown).

To assess the number of phytoplasmas in the phloem tissue of infected elm samples, DNA standards with copy numbers from 10⁰⁸ to 10⁰¹ per µl were PCR-amplified with the universal phytoplasma and Ca. P. ulmi-specific assays. One µl of DNA extract from a healthy elm tree was added to each reaction, to simulate the assay conditions with unknown samples. Both assays showed a dynamic range of amplification with Ct values of 18.2 to 37.6 for the lowest and highest dilution, respectively, with serial dilution steps differing by Ct values of 2 to 3 (Table 2).

Table 2
Ct values of DNA standards after real-time PCR with the universal phytoplasma- and EY-specific spacer real-time PCR assay.

Copy number/assay	Universal phytoplasma assay ^a	EY-specific spacer assay ^a
10 ⁰⁸	19.7	21.6
10 ⁰⁷	21.4	23.2
10 ⁰⁶	22.5	24.3
10 ⁰⁵	25.7	27.1
10 ⁰⁴	30.0	30.4
10 ⁰³	31.3	32.0
10 ⁰²	34.5	35.4
10 ⁰¹	37.2	35.1
^a , Mean of four technical replicates including DNA from a non-infected elm tree.		

Real-time PCR results with the universal phytoplasma assay highlights established infection in elm stands

The internal 18Sr DNA amplification control showed strong amplification (Ct values 9 to 22), due to the high content of plant DNA and also confirmed the template quality. Samples with a Ct value > 22 were re-assayed, or the DNA extraction was repeated. With the universal phytoplasma assay, 1,803 of the 6,486 elm samples were rated phytoplasma-positive, thus representing an infection rate of 27.8% based on the total number of samples (Table 3).

Table 3

Categorization of sample Ct values obtained with universal phytoplasma- and spacer-specific real-time PCR assays.

Assay and elm species/Ct range	≤ 34	≤ 28	≤ 22	≤ 18
Universal phytoplasma assay	27.79 ^{a, b} (1803)	24.0 (1558)	9.0 (584)	1.8 (119)
U. glabra	11.6 (751)	10.6 (685)	5.7 (371)	1.5 (100)
U. laevis	10.3 (665)	8.7 (562)	1.5 (97)	0.1 (7)
U. minor	6.0 (387)	4.8 (313)	1.8 (117)	0.2 (14)
EY-specific spacer assay	27.75 ^b (1801)	20.4 (1302)	2.5 (164)	0.1 (5)
U. glabra	11.6 (750)	9.9 (645)	2.0 (131)	0.1 (4)
U. laevis	10.2 (664)	6.5 (423)	0.2 (12)	- (1)
U. minor	6.0 (387)	4.0 (257)	0.3 (22)	- (-)

^a, Percentage of phytoplasma-positive samples based on the total number of examined samples. ^b, Percentage values with two decimals reflecting the difference of two additional positive results for the universal phytoplasma assay. Rounding differences cause percentage sums deviations.

The elm species were infected to various degrees. European white elm samples showed the highest infection rate with 32.5% (665 of 2,048), followed by Scots elm and field elm samples with 28.6% (751 of 2,630) and 21.4% (387 of 1,808), respectively. The phytoplasma number represented by the Ct value was generally high amongst the samples. In total, 1,558 of 1,803 positive samples showed Ct values ≤ 28, and of these 584 and 119 samples showed Ct values ≤ 22 and ≤ 18, respectively. Scots elm trees showed a higher phytoplasma titre compared to the other elm species. However, the correlation of

phytoplasma titre and *Ca. P. ulmi*-symptoms was not apparent. The amplification curves in assays with the DNA of a non-infected elm tree and a no-template control always ranged below the threshold line.

Real-time PCR results with *Ca. P. ulmi*-specific spacer assay indicates rare occurrence of other phytoplasmas

The *Ca. P. ulmi*-specific assay provided almost the same number of positive samples (1,801) compared to the universal phytoplasma assay (1,803; Table 3). The difference in the two cases was due to trees infected by other phytoplasma strains. Partial sequence analysis of two P1/P7 PCR fragments revealed that one phytoplasma sequence (1,631 bp) was identical to the sequence of a *flavescence dorée* phytoplasma strain (acc. no. AF176319), whilst the other sequence (1,631 bp) was identical to phytoplasmas found in *Artemisia vulgaris* (acc. no. MK440304) and *Alnus glutinosa* (acc. no. MK440303). However, both phytoplasmas were members of the elm yellows group. The sequences have been deposited in GenBank under the accession numbers MN394841 and MN394842. Whereas the overall number of positively rated samples from both assays was nearly identical, the assay performance in samples with a higher phytoplasma number was lower compared to the universal phytoplasma assay. This was most apparent when comparing the samples with Ct values ≤ 22 (Table 3).

Distribution map of *Ca. P. ulmi* in Germany highlights hot spots

Elm samples were collected at 339 sites in Germany. The elm species were not homogeneously distributed across the territory (Fig. 3A). The approximate species frequency in the federal states is reflected by the numbers given in Table 1. The presence of *Ca. P. ulmi* was likewise not homogeneous (Fig. 3B). Regions with sites showing an infection rate of more than 66.7% (red dot) were clustered in Saxony, Saxony-Anhalt and Brandenburg. Other hotspots occurred along the upper Rhine valley, and some were present in Bavaria and Hesse. Sites showing a lower infection rate (yellow and orange dots) were mostly found in the vicinity of these hotspots. The infection rate decreased towards the west and north, and only five sites with *Ca. P. ulmi* infection were found above a virtual line drawn from Trier to Rostock (Fig. 3B). At one of these sites in North Rhine-Westphalia, *U. laevis* showed an infection rate of 30% (six out of 20 samples). The four sites in Schleswig-Holstein showed infection rates of 2.5% (one out of 40 samples) and 20% (four out of 20 samples) for *U. glabra*, and 5% (one out of 20 samples) and 15% (six out of 40 samples) for *U. laevis* and *U. minor*, respectively.

Infection rate correlated to altitude or tree age

The majority of sampling sites were located in the German lowlands at altitudes ≤ 100 m above the average mean sea level (AMSL). However, quite a few sites were also located in the low mountain range, from 300 to 1,100 m AMSL. About the same number of sites ranged in between (Fig. 4). The proportion of sites free of phytoplasmas, and those with a low (up to 1/3 of individuals), high (up to 2/3 of individuals)

and extreme infection rate (up to 100%), were almost identical between the zones, thereby indicating a broad habitat for potential insect vectors.

Except for monumental trees and seedlings, the ages of trees were unknown and calculated on the basis of the trunk diameter. For trees up to 5 cm, 10 cm, 20 cm and 50 cm in diameter, age was calculated at 10, 20, 39 and 98 years, respectively. The oldest tree was a European white elm in Gültz (Brandenburg), estimated to be 400 to 700 years old and with a diameter of 3.5 m. The number of infected individuals was determined in relation to trunk diameter (Fig. 5). The graph revealed different disease progressions for the three species. While *U. glabra* showed a steady increase in infection with age, *U. laevis* showed a strong increase in infected individuals in trees up to 20 years of age, reaching a plateau thereafter. A different situation was observed for *U. minor*. An infection rate of 20% was determined in the youngest age group, and this number did not change much in the other age categories. The graph also shows the diminishing number of older trees for Scots elms and field elms, due to the mortal effects of Dutch elm disease on their population. Nevertheless, the survey included 111 trees with diameters over 100 cm (> 195 years), of which 38 individuals were infected by *Ca. P. ulmi*, comprising 29 *U. laevis*-, eight *U. glabra*- and one *U. minor* tree.

All-season colonization of elm

To assess the seasonal fluctuation of pathogen numbers, a monthly screening of different plant parts from an infected Scots elm tree was performed with the universal real-time PCR assay. The mean monthly Ct values of all samples within the examined period ranged from 24.2 to 28.6. The June to December Ct means were slightly lower than the January to May means. The Ct means between trunk samples and root samples of the same month never differed by more than 3. The Ct values of the individual monthly trunk samples were close and never apart by more than four cycles. The lowest phytoplasma titre was recorded from January to March in buds, with Ct means ranging from 28.4 to 31.5. Considering the lowest average Ct (24.2) value, a phytoplasma number of 10^{08} per gram of phloem tissue was calculated. The highest Ct average (Ct 31.5) found in bud material represents a phytoplasma number two orders of magnitude lower.

Discussion

From poor information to a representative survey for the German territory

A recent survey in the states of Brandenburg and Berlin identified an unexpected high infection rate of *U. laevis* by *Ca. P. ulmi* [26]. This small survey reflected overall poor knowledge on the distribution of the pathogen in Germany and prompted a nationwide survey in which ca. 6,500 samples were collected at 339 sites. The results of this survey can be regarded as representative for the German territory, as most of the natural habitats of the three elm species were covered. Preliminary results of this survey have been published as a contribution for the IPWG conference in 2019 [35].

At the beginning of the survey, no information was available on the colonisation of *Ca. P. ulmi* in infected elm trees. To obtain meaningful real-time PCR results for a large number of samples, the most suited 'plant-part-to-collect' had to be determined. In the well-studied phytoplasma diseases of pome fruit trees, the phytoplasma titre displays strong seasonal fluctuations, reaching a low in late winter to early summer [36, 37]. The examination of an *U. glabra* tree, however, quickly revealed that *Ca. P. ulmi* colonises roots and shoots all year round in a constantly high titre. Therefore, branches or trunk material were sampled throughout the survey. Even though petioles or leaf midribs would have been easier to collect, the choice fell on branches or trunk material, as the sampling period could be extended with this material.

Spacer-specific real-time assay enables reliable detection of *Ca. P. ulmi* infection

The real-time PCR assays corroborated the results of the seasonal course study, as in most trees a fairly high number of *Ca. P. ulmi* was identified. More than 86% and 72% of the positive samples showed Ct values lower than 28, with the universal phytoplasma or specific *Ca. P. ulmi* spacer-based real-time PCR assay, respectively, representing an organism titre of 10^{06} per gram of phloem tissue and higher. Both real-time PCR assays worked reliably, although the species-specific assay showed a slightly lower performance in respect to the Ct values. This was most likely caused by the lower G+C content of the primers and probe and the reduced binding strength compared to the oligonucleotides of the universal phytoplasma assay. The substitution of adenine bases with 2,6-diaminopurin, to increase the melting temperature of the TaqMan spacer probe, did not change performance significantly compared to the assay with the non-modified probe. Nevertheless, the spacer assay proved to be a reliable diagnostic tool for the species-specific DNA amplification of *Ca. P. ulmi* strains in this study.

Occurrence of *Ca. P. ulmi* in Germany in comparison to the situation in other European countries

It is evident that *Ca. P. ulmi* was present in almost 28% of the elm samples, thus demonstrating the high number of infected trees in German elm populations. A higher overall *Ca. P. ulmi* infection rate of 46% was found in Croatian elm populations [38]. However, if the German survey would have focused only on phytoplasma hotspots, an even higher infection rate would have resulted, as 77 of the 339 sites examined showed infection rates over 66.7%. Therefore, the high overall incidence rate in Croatia might simply be a matter of sample size. In other studies, a preferential collection of material from symptomatic trees has been executed and infection rates of 85% and higher have been found, which also might not reflect the real infection rate [39]. The three elm species showed different disease rates. While almost one-third of the *U. laevis* samples tested *Ca. P. ulmi*-positive, *U. glabra* and *U. minor* were infected to a lesser degree. This difference is not due to sample size, as the number of tested accessions from each species was about similar. In Croatia, different elm species infection rates were also found, with almost 75% of infected *U. laevis*, followed by *U. minor* (10.8%) and *U. glabra* (4%) [38]. However, in this work, the latter

two species were also infected by other phytoplasmas, and therefore the infection rates are not directly comparable.

Occasional detection of other phytoplasmas in elm

In all, except for two infected elm samples, *Ca. P. ulmi* was identified. These phytoplasmas were only identified by the different specificity of the two real-time PCR assays employed. Sequence analyses of ribosomal fragments, however, revealed that both phytoplasmas were related to *Ca. P. ulmi* and belong to the elm yellows or 16SrV group. Phytoplasmas with other taxonomic affiliations, such as *Candidatus* Phytoplasma solani and *Candidatus* Phytoplasma asteris, have been described in *U. glabra* and *U. minor* displaying rather unspecific symptoms of leaf yellowing and drying [38]. These symptoms could not be observed in the two phytoplasma-infected trees. The presence of other phytoplasmas in elms is most likely caused by an occasional feeding of infected phytoplasma insect vectors. However, the fact that only 16SrV-group phytoplasmas were found in elm trees might indicate a certain host-pathogen group specificity.

Rare symptom formation indicates tolerance in a long-living host

Despite the high number of phytoplasmas in the sieve tubes, German elms seem to react in a tolerant way upon infection, which stands in striking contrast to the reactions of American and Asian elm species [12, 16]. The few symptomatic *U. glabra* and *U. minor* trees that were found, however, displayed typical *Ca. P. ulmi* symptoms with witches' brooms, stunting and leaf chlorosis. Mittempergher (2000) also concluded after extended observations in Italian elm breeding stations that European elm species tolerate a *Ca. P. ulmi* infection quite well, although the number of symptom sightings from southern Europe [38-40] for *U. minor* and *U. laevis* seem to be more frequent compared to reports from central and northern regions of the continent. However, this could also be caused by a stronger awareness of plant pathologists or breeders, regional differences of *Ca. P. ulmi* strain virulence, the genetic background of elm populations or other undetermined stress factors.

The different infection rates of the elm species and within the age categories are difficult to explain with our present knowledge. The progressive infection in aging *U. glabra* populations is easily comprehensible, but the plateau phase for *U. laevis*, and the constant infection rate of *U. minor* age classes, is not as easy to deduce. Of particular interest is the situation of old and monumental trees of more than 100 cm in trunk diameter. This category was represented by 124 European white elms, 29 of which were phytoplasma-infected, and five field elms, with one phytoplasma-infected individual. How some of these old trees escaped phytoplasma infection remains unclear, as they were well located in regions of high infection pressure. Therefore, it seems that a certain degree of resistance is present in the populations.

However, other factors might be involved too, like the protective role of the plants' microbiome [41], albeit insufficient information is available to assess its influence so far.

Unequal distribution of *Ca. P. ulmi* might be linked to vectors

Infection hotspots were located in the eastern, central and south-western parts of Germany, whereas infected sites became rare towards the north and north-western regions. The most plausible explanation is that an insect vector migrated from the southern-to-eastern side into the territory moving towards the north and west. Hence, the distribution map given in **Fig. 3B** might simply reflect a snapshot. Beside the verified *Ca. P. ulmi* vector *Macropsis mendax* for Italy [42], the phytoplasma has only been identified by PCR in *Hyalesthes luteipes* in Serbia, but no transmission experiment was performed [39]. For Germany, no data are available. The fact that infected trees were found from sea level up to an altitude of 750 m might indicate a vector different to *M. mendax*, as this insect is only known to occur up to an altitude of 400 m [43]. A spread of the bacterium through the exchange or trade of infected elms for planting purposes can be excluded, as elm timber has little forest use. In addition, root bridges can be discounted, as this would only explain spread in a small area.

Ca. P. ulmi infection is common in European elm stands

This survey demonstrated a nationwide distribution of *Ca. P. ulmi* in all three native elm species. The number of infected individuals is such that the eradication of this quarantine pathogen is impossible. In 2017, the EPPO realised the more general occurrence of *Ca. P. ulmi* in its member states and moved the pathogen from Annex I/A 1 to Annex I/A2, recognising its regional presence. However, even this categorisation does not seem to be justified in light of the general distribution demonstrated in this survey. The recent finding of *Ca. P. ulmi* in Belgium [23] demonstrates its presence also west of Germany, although the confirmed numbers of infected elms are still low. The new EU Council Directive 2016/2031, effective from mid-December 2019, will deprive *Ca. P. ulmi* of its quarantine status in member states of continental Europe and implement a legal change supported by the findings of this survey and also from other recent surveys.

Regardless of the legal status of the disease, this work has established a sound basis for future research with respect to transmission and phytoplasma-host interaction. Examples of tolerance are often overlooked by phytopathologists and are rarely reported. It is remarkable that the closely related elm yellows and alder yellows phytoplasmas share this feature [44], which might be the result of a long-term co-evolution of these phytoplasmas with their hosts. A deeper understanding of such a particular phytoplasma-host interaction may provide the key to developing new strategies to cope with phytoplasmosis in agriculture.

Conclusions

This work presents the first nationwide survey of *Ca. P. ulmi* infection of native elm species in Germany providing representative figures of infection incidence in the federal states. Almost 30% of all elm accessions tested EY-positive. Elm species were infected to a different degree with regional disparity of infection. Hot-spots of infection were identified in East-, Southeast and Central Germany while infection rates in West- and North Germany were low. Despite the high infection rate disease symptoms were rarely found indicating a high degree of tolerance of native elm species to infection. An infection of elm trees by other phytoplasmas was only identified in two cases. A species-specific real time TaqMan assay based on 16S-23S spacer sequence motives has been developed providing sensitive and reliable diagnosis of the pathogen. The occurrence of infected elm trees in regions beyond 400 m of altitude suggests insect vectors different to the verified EY-vector *Macropsis mendax*. This work broadens the knowledge of *Ca. P. ulmi* occurrence in Germany and provides a sound basis for future work regarding species tolerance and pathogen virulence.

Methods

This study aims to clarify the infection status of elms in Germany with respect to *Ca. P. ulmi*. Therefore, the survey comprises the sampling of plant material from elms in Germany, followed by DNA extraction providing the templates for screening by real-time PCR assays. Diagnostic real-time PCR assays were performed by applying universal phytoplasma primers [27] and a new specific primer set for *Ca. P. ulmi* (this study).

Sampling of plant material from elms

Elm samples were collected at 339 sites, based on a survey published in 2007 on the genetic resources of elm species in Germany [28]. The monumental elm tree sites were taken from the web resource on monumental trees (<https://www.monumentaltrees.com>). In addition, one- to two-year-old seedlings were obtained from nurseries in Riedlingen (Baden-Württemberg), Müncheberg and Waldsiedersdorf (both Brandenburg). Additional elm trees were randomly sampled during the surveys at roadsides or in public parks. The diameter of each trunk was measured at a height of 1.3 m, and the approximate age was estimated by software provided on the website www.baumportal.de [29]. Elm shoot samples were collected from October 2017 until May 2019. Where possible, two shoot samples, about 25 cm in length and 0.5 to 4 cm in diameter, were collected from 20 randomly selected trees per site. The phytoplasma strains ULW (elm yellows phytoplasma) and ALY (alder yellows phytoplasma) maintained in *Catharanthus roseus* were used as reference strains. The coordinates of all collected elm accessions were recorded in WGS84 format, using a portable GPS device. In a first attempt, the seasonal colonisation of *Ca. P. ulmi* was monitored for one year in an infected Scots elm 3 m in height and 6 cm in diameter. Samples were taken on a monthly basis from the roots, at trunk ground level and then at distances of 25 cm up to a height of 1.75 m. Phloem tissue at these sites was extracted from a square of 0.5 cm². Midrib or bud samples from the top and bottom of the tree were also examined.

DNA extraction

DNA from all elm shoot samples was extracted from phloem tissue, using a CTAB procedure [30]. DNA was extracted from 125 mg of phloem tissue, using 3 ml of CTAB buffer. The tissue was homogenised with a steel ball homogeniser in plastic extraction bags (Bioreba, Grenzach). The nucleic acids pellet was resuspended in 200 µl of sterile water and stored at -20 °C until use. Small-scale phloem DNA extractions (15 mg) were performed with a Beadruptor (Biolab, Beverly, MA) in microfuge tubes containing ceramic beads and 250 µl of CTAB buffer. The nucleic acids pellet was resuspended in 50 µl of sterile water and stored as described above. DNA from other phytoplasma strains (AAY, American aster yellows; AT, apple proliferation strain AT; BGWL, Brachiaria grass white leaf; EYC, elm yellows strain from US; GVX, green valley strain of Western X disease; LWB, Candidatus Phytoplasma omanense; PA, Candidatus Phytoplasma australiense; RS, rubus stunt; FD90, flavescence dorée isolate FD90) was obtained from Kerstin Zikeli and Michael Maixner (both Julius Kuehn-Institute, Institute for Plant Protection in Fruit Crops and Viticulture) and used as positive or negative controls.

Real-time PCR standard

The 16S-23S DNA of the *Ca. P. ulmi* strain ULW was amplified with P1/P7 primers [31, 32] as described previously [33]. The 1.8 kb PCR fragment was ligated into the cloning vector pGEM-T (Promega, Madison), and the insert was verified by sequencing with vector primers (M13, M13rev) and the universal phytoplasma primers fU5 and rU3 [30]. The recombinant plasmid was bulk-extracted, its quantity determined by Qubit fluorometric quantification (Invitrogen, Carlsbad) and serially diluted with sterile water to obtain plasmid concentrations ranging from 10^8 to 10^{01} copies per µl (referred to as 'DNA standard'). P1/P7 PCR fragments of phytoplasma field isolates were sequenced as described above. Sequences differing from *Ca. P. ulmi* database entries were deposited at GenBank. The standard was only used to roughly estimate the plant phytoplasma titre and was not included in routine screenings.

Calculation of phytoplasma titres

The phytoplasma titre was determined by the Ct values of the above mentioned serially diluted DNA standard relative to the Ct value of the elm samples. The calculation considered the amount of plant tissue represented in the nucleic acids pellet, the volume of the reconstituted nucleic acids pellet and the copy number of the target gene. The calculated figures are approximate values.

Design of *Ca. P. ulmi*-specific primers

16S-23S spacer sequences from *Ca. P. ulmi* database entries with acc. nos. AF122911, AF189214 and EU184021 and spacer regions of related phytoplasmas (flavescence dorée phytoplasma, acc. no. AF176319; rubus stunt phytoplasma, acc. no. Y16395; *Ca. P. balanitae*, acc. no. AB689678 and alder witches' broom phytoplasma, acc. no. MK440303) were aligned using ClustalX [34]. The primers and probe were selected based on regions of complete homology to *Ca. P. ulmi* sequences and the maximal number of base differences or gaps to the related phytoplasmas. The derived primers and probe were as

follows (5' – 3'): Forward primer fEY_spacer-rt, ATATCAGGAAAATATTTACTAC; reverse primer rEY_spacer-rt, CGCCCTTACTTTCTTCAAT; TaqMan probe pEY_spacer-rt, FAM-TTGAAGAAAGTTCTTTGAAAAG-BHQ1. Double underlined nucleotides were replaced by 2,6-diaminopurin to increase the melting temperature.

Diagnostic real-time assays

A TaqMan real-time PCR assay for universal phytoplasma detection was performed [27] with the following modifications. The assay was performed in 10 µl reactions containing 1 µl of nucleic acids extract, 10 pmol of each forward and reverse primer and a probe (5'-FAM/BHQ1-3') for phytoplasma detection, 3.3 pmol of each forward and reverse primer and a probe (5'-Cy5/BHQ3-3') for plant 18Sr DNA amplification and 5 µl of 2 x primaQUANT mastermix (Steinbrenner, Wiesenbach). The reactions were cycled as described previously [27] in a qTower (Analytik Jena AG, Jena), except that the initial 50 °C and 95 °C steps were replaced by a three-minute denaturation step at 95 °C. The plant 18Sr DNA assay served as an internal amplification control to exclude the presence of inhibitory co-extracted compounds. The *Ca. P. ulmi*-specific spacer assay was performed in 10 µl reactions containing 10 pmol of each forward and reverse primer and probe, albeit without plant-specific primers and probe. The cycling parameters were as follows: One cycle for 3 min at 95 °C, 40 cycles at 95 °C for 15 sec and 56 °C for 25 sec. Data evaluation was done via the cycler software provided by the manufacturer. Due to the high number of samples, verification of real-time PCR results was performed on a step-by-step basis. Samples were re-examined if the Ct value between universal and species-specific assays differed by more than three, or if one of the assays was rated negative. In each run, a phytoplasma-positive field sample with a Ct value of 29, ULW DNA (positive control), DNA of a non-infected healthy elm and a water control (negative controls) were included, to verify run consistency. The Ct values were assessed stringently, and samples with Ct values > 34 were considered negative.

Abbreviations

EU, European Union

Ct, cycle threshold

AMSL, average mean sea level

IPWG, International Phytoplasma Working Group

EPPO, European Plant Protection Organisation

CTAB, Cetyltrimethylammoniumbromide

Declarations

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article. Specific datasets on coordinates of elm stands analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BS, RK and MK designed the sampling. BS carried out the surveys, performed all experiments and drafted the manuscript. MK designed the study and contributed to manuscript preparation. All authors have read and approved the manuscript.

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Figures

Figure 1

Ca. P. ulmi-specific disease symptoms. (A) Branches of a healthy (left) and diseased (right) U. glabra tree in winter time. (B) Rare witches' broom formation of U. glabra in summer time. (C) Twig of a healthy U. minor with normal leaf size and leaf colouration. (D) U. minor branch with stunted growth. (E) U. minor with little leaf symptom and leaf chlorosis.

Ca. P. ulmi (AF122911)	TATATCAGGAAATATTACTACTTTGAAGAAAGTTCTTTGAAAG~TTCAATAAAATTGAAGAAAGTAAGGGCGT
Flavescence dorée (AF176319)	-.-.-C-.....~t.
Rubus stunt (Y16395)	-.-.-C-.....~t.
Ca. P. balanitae (AB689678)	C.....-.-.-C-.....~t.
Alder WB (MK440303)	-.-.-C-.....~t.

Fig. 2

Figure 2

Alignment of 16S-23S spacer regions (5' – 3') of *Ca. P. ulmi* and closely related phytoplasma strains. Nucleotide differences of the other sequences to the *Ca. P. ulmi* sequence are indicated by lowercase letters. The positions of the forward primer, the TaqMan probe and the reverse primer are underlined and given from left to right, respectively. -, indicates gaps in alignment. ≈, indicates bases not depicted.

Fig. 3

Figure 3

A, sampling sites with predominant elm species. Blue square, *U. glabra*; Green square, *U. laevis*; Orange square, *U. minor*. B, presence of *Ca. P. ulmi* at the sampling sites based on real-time PCR results. Green dot, no phytoplasmas found; Yellow dot, up to one-third of trees infected; Orange dot, more than one-third and up to two-thirds of trees infected; Red dot, more than one-third and up to 100% of trees infected.

Fig. 4

Figure 4

Overall infection rate of elm species at different altitude levels. Altitude, number of sites and number of individuals examined indicated. Left (light grey), percentage of sites with non-infected trees. In a clockwise direction, percentage of sites with up to one-third, more than one-third and up to two-thirds and more than two-thirds of individuals infected by *Ca. P. ulmi*.

Fig. 5

Figure 5

Infection rate of *U. minor*, *U. laevis* and *U. glabra* in relation to trunk diameter. Grey bars represent non-infected individuals. White bars represent phytoplasma-infected individuals. The number of individuals considered is given in the bars. The infection percentage in an age group is given on the right.