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An investigation of the presence of *Xylella fastidiosa* in Cicadomorpha specimens collected in different habitats in Poland

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Abstract: A quarantine organism, the bacterium *Xylella fastidiosa* (Xf), is a xylem-inhabiting, vector-transmitted, Gram-negative, and very slow-growing bacterium in the *Lysobacteraceae* (earlier *Xanthomonadaceae*) family. The spreading of *X. fastidiosa* over long distances occurs mainly via import/export human-mediated transportation of mainly latently or symptomatically infected plant material. Short-distance distribution is usually by xylem sap-feeding insects. Until now, the presence of *X. fastidiosa* has not been reported or studied in Poland. During our study, over 500 individuals from the four families: Cicadellidae, Aphrophoridae, Delphacidae and Membracidae were collected in different geographical regions of Poland. Real-time PCR with primers for *rimM* gene and nested PCR to detect *X. fastidiosa*, using DNA extracted directly from selected insects known as potential vectors of *X. fastidiosa*, did not confirm the bacterium's presence in these insects.

Keywords: xylem-limited bacterium; insects; detection; real-time PCR; nested PCR

The bacterium *Xylella fastidiosa* is a polyphagous plant pathogen with a very broad host range, including monocotyledonous and dicotyledonous species, herbaceous and woody plants, cultivated crops and weeds, native flora, and riparian and landscape shade trees, with the list of potential hosts increasing every year. Currently, according to the European Food Safety Authority (EFSA), the updated list of *X. fastidiosa* hosts comprises 679 plant species from 88 plant families, excluding detection methods (EFSA et al. 2023). Due to the large number of infected plant species, *X. fastidiosa* can cause a variety of symptoms. In addition to the commonly known Pierce's disease (PD) of grapevine (*Vitis* spp.), citrus variegated chlorosis (CVC) and the so-called leaf scorch diseases in *Prunus* spp. [including almond leaf scorch (ALS) in *Prunus amygdalus*] and other hosts, the best known and most serious diseases are the phoney peach disease (PPD) in peach and different kinds of leaf scorch diseases, e.g. oleander leaf scorch (OLS), coffee leaf scorch (CLS), and plum (*Prunus domestica*) leaf scald (PLS) (Davis et al. 1978; Wells 1983, 1987; Chang et al. 1993; Newman et al. 2003; Schuenzel et al. 2005; Janse & Obradovic 2010; Nunney et al. 2014).

Several pathogenic varieties of the bacterium have been described and subdivided into subspecies based on phylogenetic studies and sequencing (Schaad et al. 2004; Schuenzel et al. 2005; Hernandez-Martinez et al. 2007; Randall et al. 2009; Nunney et al. 2014). Out of the subspecies proposed, at present only *fas-*

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tidiosa, multiplex and *pauca* are officially considered as valid names by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB) (Bull et al. 2012; Denancé et al. 2019). Recently, the subspecies *sandyi* and *morus* have been proposed for inclusion in the subspecies *fastidiosa* (Marcelletti & Scortichini 2016; Denancé et al. 2019).

To date, the presence of *X. fastidiosa* and most of the diseases caused by this bacterium have been reported from North and South America – the recent reports come from Costa Rica and Mexico (Legendre et al. 2014; Nunney et al. 2014), Argentina (Haelterman et al. 2019), Brazil (Coletta-Filho et al. 2016), and others (Hilton et al. 2017). According to the EPPO Global Database (https://gd.eppo.int/taxon/XYLEFA/distribution), only a few cases have been reported outside of this area in Europe and the Mediterranean basin. The presence of *Xylella* has been reported in territories formerly part of Yugoslavia (Berisha et al. 1998), Switzerland (EPPO 2023a), France and Italy (Elbeaino et al. 2014; Loconsole et al. 2014; Marcelletti & Scortichini 2016; Martelli et al. 2016; Denancé et al. 2017; EPPO 2023b), Iran (Amanifar et al. 2016, 2019), Taiwan (Leu & Su 1993), Spain (Olmo et al. 2017; EPPO 2023c), Switzerland (EPPO 2023a), and recently in Portugal (EPPO 2023d).

The spreading of *X. fastidiosa* over long distances occurs mainly via import/export human-mediated transportation of mainly latently or asymptomatically infected plant material, while short-distance distribution is by xylem sap-feeding insects (Redak et al. 2004; Chatterjee et al. 2008). Although there have been outbreaks of *X. fastidiosa* in Italy, France, Spain and the UK, the bacterium has not yet been reported in Poland. Since *X. fastidiosa* can infect a large number of plant species, and many of them, including *Vitis* planting material, were and are still imported, the risk of introduction (especially in latent form) must not be underestimated. The absence of disease caused by this pathogen is mainly due to the failure to recognise infected plant material or potential vectors, or both. However, so far, there have been no systematic inspections or extensive research in Poland to detect and accurately identify *X. fastidiosa* in insect bodies, which would help determine the prevalence of this bacterium's vectors. Only sporadic surveys have been conducted by the Main Inspectorate of Plant Health and Seed Protection (GIORiN).

According to a scientific opinion of the European Food Safety Authority (EFSA), the putative vectors of *X. fastidiosa* that occur in Poland include the following species: Sharpshooter Cicadellidae *Cicadella viridis* (L., 1758), *Graphocephala fennahi* Young 1977; *Evacanthus interruptus* (L., 1758); *Errhomenus brachypterus* Fieber, 1866; Spittlebugs Aphrophoridae: *Aphrophora alni* (Fallen 1805), *A. corticea* Germar 1821, *A. major* Uhler 1896, *A. pectoralis* Matsumura 1903, *A. salicina* (Goeze 1778), *A. similis* (Lethierry 1888), *Lepyronia coleoptrata* (L., 1758), *Neophilaenus albipennis* (Fabricius 1798), *N. campestris* (Fallen 1805), *N. exclamationis* (Thunberg 1784), *N. infumatus* (Haupt 1917), *N. lineatus* (L., 1758), *N. minor* (Kirschbaum 1868), *Peuceptyelus coriaceus* (Fallen 1826), *Philaenus spumarius* (L., 1758); Spittlebugs Cercopidae: *Cercopis sanguinolenta* (Scopoli 1763), *C. vulnerata* Rossi 1807 (Malumphy 2017). However, the same report emphasised that all xylem-sap-feeding insects in Europe were considered potential vectors of *X. fastidiosa*.

In Poland, 552 species of planthoppers (Fulgoromorpha Evans) or leafhoppers (Cicadomorpha Evans) have been recorded to date (Walczak et al. 2016). These insects feed on a wide variety of vascular plants, including woody and herbaceous plants, grasses, sedges, and conifers, in almost all terrestrial ecosystems. Many of them are monophagous or oligophagous, but they are also polyphagous. Currently, the threat of *X. fastidiosa* spreading to Eastern Europe appears low (Godefroid et al. 2019). However, given climate change, the large number of host plants, and the potential for *X. fastidiosa* to spread through additional vectors, the probability of this invasive pathogen spreading in the future cannot be ruled out. It is therefore important to recognise the threat, taking into account, in the first place, the potential vectors of this bacterium in selected terrestrial ecosystems, including some major and minor crops and their surroundings.

The detection and identification of *X. fastidiosa* are not as straightforward as for other phytopathogens. It is a slow-growing (fastidious) bacterium, and there are two points to consider before working with it. First, 2–3 weeks are needed to obtain colonies on different agar media; second, and most importantly, the bacterium does not grow on many common culture media. Generally, some good semi-selective/selective media have been developed, such as PD2, PW, CS20 and BCYE (Schaad et al. 2001; Almeida et al. 2004). Among the serological methods, the enzyme-linked immunosorbent assay (ELISA) (Sherald 1991; Chang et al. 1993; Leu et al. 1998) is the most commonly used (Carbajal et al. 2004; Buzkan et al. 2005; Schuenzel et al. 2005; Loconsole et al. 2014). The most common and specific methods for identifying and detecting *X. fastidiosa* are those that rely on analysis of bacterial DNA. Extraction of bacterial DNA from host tissues has been achieved using standard commercial kits and basic CTAB buffer (De Souza et al. 2003; Rodrigues et al. 2003; Loconsole et al. 2014). Subsequently, extensive studies have been undertaken, and many techniques employing specific primers that recognise target sequences in genomic DNA have been developed. To date, PCR-based assays (Minsavage et al. 1994; Rodrigues et al. 2003; Huang et al. 2006; Huang 2009) and PCR derivatives, including RFLP (restriction fragment length polymorphism) and RAPD (random-amplified polymorphic DNA) analyses (Pooler & Hartung 1995; Mehta et al. 2001), as well as real-time and LAMP PCR, and others (Francis et al. 2006; Harper et al. 2010; Guan et al. 2013; Li et al. 2013; Mang et al. 2016; Martelli et al. 2016; Amoia et al. 2023; Cesbron et al. 2023), have been successfully used for the detection and differentiation of the *X. fastidiosa* bacterium in different hosts.

The application of detection methods directly to insects enables the prediction of the potential threat of bacterial transmission in Poland. As the bacterium had not been reported nor studied in Poland before, the aim of our study was: *(i)* to collect and identify insects from different plant species in different geographical regions of Poland, and *(ii)* to check for the presence of *X. fastidiosa* bacteria in the bodies of those insects. The presented study fits into European surveillance efforts for *Xylella fastidiosa*, realised in the framework of i.a. PROMODE, CURE-XF, XF-ACTORS and POnTE.

MATERIAL AND METHODS

Methods of sampling insects

Sample collection site. The study on the occurrence of Auchenorrhyncha was conducted between 2018 and 2021 across 16 fruit orchards in several regions of Poland (Figure 1). Besides apple trees, the monitoring included peach, sweet cherry in orchards, grapevines in vineyards, and the collection of plum, pear, and apple tree cultivars in the Botanical Garden of the Polish Academy of Sciences in Powsin. Additionally, samples were taken from ornamental trees (beech, hornbeam, oak) in the Arboretum in Rogów, rhododendron in the Botanical Garden,

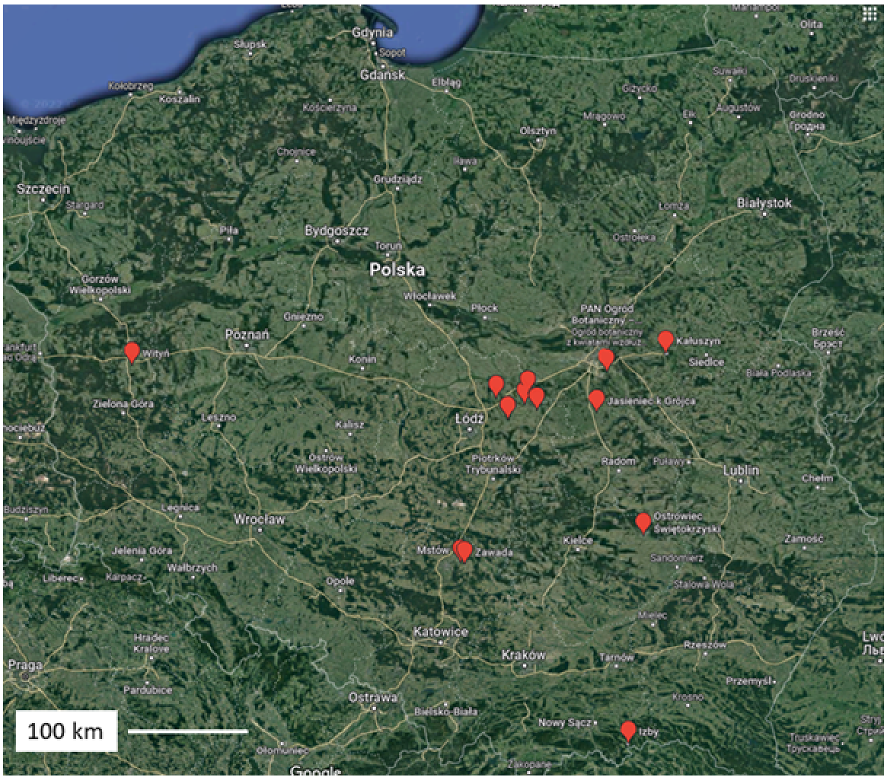

Figure 1. Locations surveyed to determine the presence of Auchenorhyncha specimens

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rose plantation, juniper plantings in ornamental nurseries, meadows, and boundary strips adjacent to the examined objects (Table 1). A total of 62 samples were collected during the survey period. Insect samples were collected from June to October. Most of the orchards and vineyards included in the study were protected with insecticides in accordance with integrated pest management guidelines.

Sampling of insects. Samples of Auchenorrhyncha were collected from both the inter-row strips and the canopies of fruit trees in orchards, fruit cultivar collections and vines in vineyards, from herbaceous plants growing under the trees, as well as from ornamentals, meadow and herbaceous plants growing near the surveyed orchards: *(i)* Samples from tree canopies were taken using the beat-tray method. A sample collection unit was defined as the number of individual specimens obtained from 100 randomly selected branches from 25 randomly selected trees in the tested area of approximately 0.5 ha at each test location. *(ii)* Samples of Auchenorrhyncha from herbaceous plants growing under the trees and other uncultivated vegetation were collected with a sweep net (38 cm diameter). For every stand, four samples were taken. Each sample consisted of 25 sweeps. *(iii)* Samples of insects from the rhododendron were collected using an aspirator.

The contents of the sweep net were emptied into a plastic bag, properly labelled and sealed. All samples were frozen at –20 °C, and the insects were later separated in the laboratory and preserved in 98% ethanol (EtOH) until identification. Samples were examined under a microscope for species identification, based on reference to appropriate keys and illustrations (Ossiannilsson et al. 1981; Holzinger et al. 2003).

Molecular detection of *Xylella fastidiosa*

DNA isolation from insects. For DNA extraction from insects, the CTAB method, as recommended by EPPO (EPPO 2019), was chosen. Briefly, the insects stored in ethanol at –20 °C were, after identification, dried on sterile filter paper, placed on the lid of a Petri dish, and their heads were cut off with a scalpel. The heads were then transferred to a 2 mL Eppendorf tube (Axygen, USA). They were homogenised with one 5-mm tungsten carbide bead for 20 s at 24/s in a Tissue Lyser II homogeniser (Qiagen, Hilden, Germany). After that, 500 mL of CTAB buffer was added, and the contents of each tube were mixed well by shaking. Next, the samples were heated at 65 °C for 30 min, after which 500 μL of 24 : 1 chloroform : isoamyl alcohol was added. Each sample was mixed again by shaking and centrifuged at 16 000 g for 10 min. Subsequently, 400 μL of the supernatant was transferred to a 1.5 mL microcentrifuge tube (Axygen, USA), and 280 μL of cold 2-propanol was added. After mixing by inverting a few times, the tube was incubated at –20 °C for 1 h. Next, the samples were centrifuged at 16 000 g for 20 min; the pellet was then washed with 1 mL of 70% ethanol, centrifuged again at 16 000 g for 10 min, and decanted with 70% ethanol. The sample was finally air-dried, and the pellet was suspended in 30 μL of RNase- and DNase-free water. DNA concentrations were measured using a NanoDrop™ 2000/2000c spectrophotometer (ThermoFisher Scientific, Waltham, USA).

Quantitative PCR (qPCR) assay. After DNA isolation, the presence of *X. fastidiosa* in insects was detected using real-time PCR with a TaqMan probe following the Harper protocol (Harper et al. 2010) with primers designed for *rimM* gene: XF-F 5'-CACGGCTGGTAACGGAAGA-3', XF-R 5'-GGGTTGCGTGGTGAAATCAAG-3', XF-P 5'-6-FAM-TCGCATCCCGTGGCTCAGTCC-BHQ-1-3'. The reaction was conducted in a Bio-Rad CFX96 thermocycler using TaqMan™ Fast Universal PCR Master Mix (Applied Biosystems, USA). The PCR reaction mixture (20 μL in total volume) contained: 1 μL of the originally isolated DNA (at different concentrations) and, additionally, 1 μL of diluted DNA at a conc. of 10 ng/μL, 1 × TaqMan™ Fast Universal PCR Master Mix (Applied Biosystems, USA), and 0.3 μM of primers XF-F and XF-R and 0.3 μM of XF-P. For the positive control, the DNA from the reference strain *X. fastidiosa* subsp. *fastidiosa* CFBP 7970^T was used. Nuclease-free Milli-Q water was used as the negative control. The limits of detection (LoD) of the real-time PCR assay were determined using a 10-fold dilution series prepared in TE buffer, with bacterial genomic DNA isolated from a pure culture of CFBP 7970^T. The dilution series ranged from ~70 ng/μL to 0.7 fg/μL. Initial DNA concentrations were determined with a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The PCR conditions used were as previously described in the original paper (Harper et al. 2010).

Nested PCR assay. Additionally, the presence of *X. fastidiosa* in insects was checked using nested PCR (Cruaud et al. 2018). For this purpose, two

Table 1. Auchenorhyncha collected during surveys carried out in 2018–2020 in different regions of Poland

No.	Family/species	No. of individuals			Type of habitat	Trophic relations	Hosts	Vector status of X.F. in Europe	No. genera- tion	Over- vintering stage	References
		collected	tested	infected							
	Aphrophoridae										
1	*Philaenus spumarius* (L., 1758)	4	4	0	Meadow adjacent to a home garden, which includes a pedunculate oak, maples, and fruit trees	polyphagous	Herbaceous dicotyledonous plants	confirmed	1	egg stage	Elbeaino et al. (2014); Saponari et al. (2014a); Cornara et al. 2019; Avosani et al. (2022); Nickel & Remane (2002)
2	*Neophilaenus lineatus* (L., 1758)	3	3	0		polyphagous	*Poaceae, Cyperaceae, Juncaceae*	potential	1	egg stage	Malumphy (2017)
3	*Neophilaenus campestris* (Fallen, 1805)	4	4	0		olyphagous1	*Poaceae*	confirmed	1	egg stage	Elbeaino et al. (2014); Cavalieriet al. (2018, 2019); Lago et al. (2021); Morente et al. (2018) Cornara et al. (2019); Nickel & Remane (2002)
	Cicadellidae										
4	*Cicadella viridis* (L., 1758)	9	9	0	Inter-row strips of herbaceous vegetation in an apple orchard	polyphagous	*Carex, Juncus,* other	potential	1	egg stage	Bodino et al. (2022); Malumphy (2017); Nickel & Remane (2002)
		8	8	0	Inter-row strips of herbaceous vegetation in a plum cultivar collection						
		2	2	0	Inter-row herbaceous vegetation in a vineyard						
		10	10	0	Herbaceous plants under alder, hornbeam, and beech trees in the dendrological collection of the Arboretum						
		4	4	0	Roses plantation						

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Table 1. To be continued...

No.	Family/species	No. of individuals			Type of habitat	Trophic relations	Hosts	Vector status of X.F. in Europe	No. generation	Over-vintering stage	References
		collected	tested	infected							
5	*Graphocephala fennahi* Young, 1977	13	13	0	*Rhododendron* collection in the botanical garden	monophagous	*Rhododendron* sp.	potential	1–2	egg stage	Nickel & Remane (2002); Di Serio et al. (2019); Malumphy (2017)
6	*Cicadella lasiocarpae* Ossiannilsson, 1981	42	42	0	Meadow adjacent to the home garden, which includes a pedunculate oak, maples, and fruit trees	polyphagous	*Carex, Juncus*, other	potential	1	egg stage	
7	*Euscelis incisus* (Kirschbaum, 1858)	1	1	0	Inter-row strips of herbaceous vegetation on the rose plantation	olygophagous	*Poaceae, fabaceae*	potential	2	Nymphal/ egg stage	Malumphy (2017); Nickel & Remane (2002); Junkiert & Gorczyca (2025)
		1	1	0	Inter-row strips of herbaceous vegetation in an apple orchard						
		1	1	0	Inter-row strips of herbaceous plants in a vineyard						
8	*Athysanus quadrum* Boheman, 1845	5	0	0	Inter-row strips of herbaceous plants on a blueberry plantation	monophagous1	*Filipendula, Inula, Lathyrus*	unconfirmed role as a vector	1	egg stage	
9	*Arthaldeus striifrons* (Kirschbaum, 1868)	1	0	0	Meadow adjacent to a home garden, which includes a pedunculate oak, maples, and fruit trees	Olygophagous1	*Festuca arundinacea, F. pratensis, Lolium perenne*?	unconfirmed role as a vector	2	egg stage	
10	*Cicadula (Cicadula) persimilis* (Edwards, 1920)	4	0	0		monophagous1	*Dactylis glomerata* 2	unconfirmed role as a vector	2	egg stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
11	*Balclutha punctata* (Fabricius, 1775)	1	0	0	Herbaceous plants under trees in the pear collection	olygophagous 1s	*Poaceae*	unconfirmed role as a vector	1	adult stage	
12	*Elymana sulphurella* (Zetterstedt, 1828)	1	0	0	Inter-row strips of herbaceous plants in a pear orchard	olygophagous	*Poaceae*	unconfirmed role as a vector	1	egg stage	

Table 1. To be continued...

No.	Family/species	No. of individuals			Type of habitat	Trophic relations	Hosts	Vector status of X.F. in Europe	No. genera-tion	Over-vintering stage	References
		collected	tested	infected							
13	*Macrosteles laevis* (Ribaut, 1927)	2	0	0	Inter-row strips of herbaceous plants in a pear orchard	polyphagous	*Poaceae*	unconfirmed role as a vector	2	egg stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
		47	5	0	Inter-row strips of herbaceous plants in vineyards						
		62	0	0	Inter-row strips of herbaceous plants in a sweet cherry orchard						
		13	0	0	Inter-row strips of herbaceous plants in an apple cultivar collection						
		5	0	0	Boundary strip between apple tree orchards						
		4	0	0	Rose plantation						
14	*Macrosteles* spp	1	0	0	Semi-natural area adjacent to the forest	–	–	–	–	–	–
15	*Mocydiopsis attenuata* (Germar, 1821)	7	0	0	Inter-row strips of herbaceous plants under trees in the apple cultivars collection	monophagous2	*Festuca ovina*, *F. rubra*, *F. heterophyla*	unconfirmed role as a vector	1	adult stage	Nickel & Remane (2002), Junkiert & Gorczyca (2025)

Table 1. To be continued...

No.	Family/species	No. of individuals			Type of habitat	Trophic relations	Hosts	Vector status of X.F. in Europe	No. generation	Overvintering stage	References
		collected	tested	infected							
16	*Psammotettix alienus* (Dahlbom, 1850)	2	0	0	Inter-row strips of herbaceous plants in a peach orchard	olygophagous1	*Poaceae*	unconfirmed role as a vector	2	egg stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
		56	56	0	Inter-row strips of herbaceous plants in vineyards						
		23	0	0	Inter-row strips of herbaceous plants in the apple cultivars collection						
		10	0	0	Herbaceous plants on the Roses plantation in the ornamental nursery						
		9	0	0	Herbaceous plants on *Juniperus* planting in the ornamental nursery						
		23	0	0	Boundary strip between apple tree orchards						
17	*Streptanus sordidus* (Zetterstedt, 1828)	1	0	0	Inter-row strips of herbaceous plants in the apple cultivar collection	olygophagous1	*Agrostis stolonifera., A. capillaris*	unconfirmed role as a vector	2	egg stage	
		1	0	0	Inter-row strips of herbaceous plants in a peach orchard						
18	*Psammotettix confinis* (Dahlbom, 1850)	12	0	0	Meadow adjacent to a home garden, which includes a pedunculate oak, maples, and fruit trees	olygophagous1	*Poaceae*	unconfirmed role as a vector	2	egg stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
19	*Psammotettix dubius* Ossiannilsson, 1974	14	0	0		olygophagous1	*Poaceae*	unconfirmed role as a vector	1	egg stage	
20	*Oncopsis flavicollis* (Linnaeus, 1761)	1	0	0	Inter-row strips of herbaceous plants in the Pear cultivar collection	monophagous2	*Betula pendula, B. pubescens*	unconfirmed role as a vector	1	egg stage	

Table 1. To be continued...

No.	Family/species	No. of individuals			Type of habitat	Trophic relations	Hosts	Vector status of X.F. in Europe	No. generation	Over-vintering stage	References
		collected	tested	infected							
21	*Empoasca pteridis* (Dahlbom, 1850)	23	0	0	Inter-row strips of herbaceous plants in a peach orchard	polyphagous	Herbaceous plants	unconfirmed role as a vector		egg stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
		2	0	0	Semi-natural area adjacent to the forest						
22	*Eupteryx* spp.	1	0	0	Inter-row strips of herbaceous plants in vineyards	–	–	–	–	–	–
23	*Zyginidia* (*Zyginidia*) *pullula* (Boheman, 1845)	11	0	0		olygophagous1	*Poaceae*	unconfirmed role as a vector	2	adult stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
Membracidae											
24	*Stictocephala bisonia* Kopp & Yonke, 1977	11	0	0		polyphagous	*Fabaceae, Rosaceae*	unconfirmed role as a vector	1	eggs	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
Delphacidae											
25	*Dicranotropis* (*Dicranotropis*) *hamata* (Boheman, 1847)	1	0	0	Inter-row strips of herbaceous plants in the apple cultivar collection	olygophagous1	*Poaceae*	unconfirmed role as a vector	2	nymphal stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
26	*Javesella* (*Javesella*) *dubia* (Kirschbaum, 1868)	10	0	0		olygophagous1	*Agrostis cappillaris, A.stolonifera*	unconfirmed role as a vector	2	nymphal stage	
27	*Javesella* (*Javesella*) *pellucida* (Fabricius, 1794)	2	0	0	Inter-row strips of herbaceous plants in a vineyard	polyphagous	*Poaceae, Ceperaceae, Juncaceae*	unconfirmed role as a vector	2	nymphal stage	
		4	0	0	Inter-row strips of herbaceous plants in an apple orchard						
		1	0	0	Inter-row strips of herbaceous plants in a peach orchard,						

Table 1. To be continued...

No.	Family/species	No. of individuals			Type of habitat	Trophic relations	Hosts	Vector status of X.F. in Europe	No. genera-tion	Over-vintering stage	References
		collected	tested	infected							
27	*Javesella* (*Javesella*) *pellucida* (Fabricius, 1794)	5	0	0	Inter-row strips of herbaceous plants on a blueberry plantation	polyphagous	*Poaceae, Cyperaceae, Juncaceae*	unconfirmed role as a vector	2	nymphal stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
28	*Javesella* spp.	12	0	0	Inter-row strips of herbaceous plants in the apple cultivar collection	–	–	–	–	–	–
29	*Laodelphax striatellus* (Fallen, 1826)	1	0	0	Inter-row strips of herbaceous plants in a peach orchard	polyphagous	*Poaceae, Cyperaceae, Juncaceae*	unconfirmed role as a vector	2	nymphal stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
		2	0	0	Inter-row strips of herbaceous plants in the apple cultivar collection						
		2	0	0	Inter-row strips of herbaceous plants in an apple orchard						
		3	0	0	Inter-row strips of herbaceous plants on a blueberry plantation						
		3	0	0							
30	*Nothodelphax albocarinatus* (Stal, 1858)	1	1	0	Inter-row strips of herbaceous plants in the Vineyard	monophagous1	*Carex* spp.	unconfirmed role as a vector	2	nymphal stage	Nickel & Remane (2002)
31	*Struebingianella lugubrina* (Boheman, 1847)	4	0	0	Inter-row strips of herbaceous plants in an apple cultivar collection	monophagous2	*Glyceria maxima, G. fluitans*	unconfirmed role as a vector	2	nymphal stage	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
		3	0	0	Inter-row strips of herbaceous plants in a pear cultivar collection						
32	*Xanthodelphax flaveolus* (Flor, 1861)	4	0	0	Blueberry plantation	monophagous1	*Poa pratensis*	unconfirmed role as a vector	1?	nymphal stage	

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Table 1. To be continued...

No.	Family/species	No. of individuals			Type of habitat	Trophic relations	Hosts	Vector status of X.F. in Europe	No. generation	Overvintering stage	References
		collected	tested	infected							
33	*Stenocranus major* (Kirschbaum, 1868)	1	0	0	Inter-row strips of herbaceous plants in the Apple cultivar collection	monophagous1	*Phalaris arundinacea*	unconfirmed role as a vector	1	adult staghe	Nickel & Remane (2002); Junkiert & Gorczyca (2025)
		4	0	0	Inter-row strips of herbaceous plants in the Pear cultivar collection						
	RAZEM	518	164	0							

X.F. – *Xylella fastidiosa;* Monophagous1 – 1st degree monophagous, 1 plant species; monophagous2 – 2nd degree monophagous, 1 plant genus; olygophagous1 – 1st degree oligophagous, 1plant family; olygophagous2 – 2nd degree; olygophagous – 2 plant families or less than 5 species of less than 5 families primer pairs targeting the *holC* gene were adopted, i.e. external ones holC-for 5'-ATGGCACGCGCCGACTTCT-3' / holC-rev 5'-ATGTCGTGTTTGTTCATGTGCAGG-3' (Yuan et al. 2010) and internal HolC-NesF1 5'-ATTTGATTKCCAAACCGCGCT-3'/ HolC-NesR378 5'-CGCGTGYCTTGTAGTGTTTC-3' (Cruaud et al. 2018). Both PCR reactions were conducted according to the protocol adopted from the original papers, with a few modifications. The PCRs were performed in a 50 μL reaction mixture using 1 × DreamTaq Buffer (ThermoFisher Scientific, Waltham, USA), 0.2 mM each dNTP, 0.4 μM of each primer, 1 U of DreamTaq Polymerase (ThermoFisher Scientific, Waltham, USA) and template DNA (PCR 1–20% of the purified insect DNA in the first PCR, and PCR 2–10 and 50 times diluted mixture from PCR 1). The DNA from the reference strain *Xylella fastidiosa* subsp. *fastidiosa* CFBP 7970 was used as the positive control. Nuclease-free Milli-Q water was used as the negative control. The limits of detection (LoD) of the nested PCR assay were tested using a 10-fold dilution series prepared in TE buffer, with bacterial genomic DNA isolated from a pure culture of CFBP 7970^{T} (for PCR1). The dilution series ranged from ~70 ng/μL to 0.7 fg/μL. For the second PCR, 10- and 50-fold diluted mixtures from PCR 1 were used as template DNA. Initial DNA concentrations were determined with a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The PCR reactions were conducted in a T3000 thermocycler (Biometra, Germany). Amplification was confirmed on a 1.5 % agarose gel in 0.5× TBE buffer (0.045 M trisboric acid, 0.001 M EDTA, pH 8.0). The reactions were performed three times.

RESULTS

Occurrence of Auchenorrhyncha. During this study, a total of 518 individuals belonging to four families: Cicadellidae, Aphrophoridae, Delphacidae and Membracidae were collected in different regions of Poland (Figure 1, Table 1). The most abundant families were Cicadellidae and Delphacidae, whereas individuals belonging to the families Aphrophoridae and Membracidae were collected in low numbers.

Twenty-three species (352 specimens) of Auchenorrhyncha were collected with a sweep net from inter-row strips of herbaceous plants in orchards, vineyards and fruit tree cultivar collections [Electronic Supplementary Material (ESM) Table S1]. Three of those species were the most numerous: *Macrosteles laevis* Ribaut, followed by *Psammotettis alienus* Dahlbom, and *Cicadella viridis* L. Among the species found, *Cicadella viridis* (ESM Figure S1) requires special attention as a potential vector of the bacterium *Xylella fastidiosa*.

In the samples collected from herbaceous plants, eleven species (115 specimens) were identified. As in the orchards, the family Cicadellidae was the most abundant, comprising 104 specimens belonging to 9 species. Among those, *Cicadella lasiocarpae* Ossiannilsson was particularly important as a potential vector of *X. fastidiosa*, while only 11 specimens were identified as Aphrophoridae. Among those, three species were identified: *Philaenus spumarius* (ESM Figures S2–4), *Neophilaenus campestris* and *N. lineatus,* all of which have been known in Europe as vectors of *X. fastidiosa* (ESM Table S2).

The fewest Auchenorrhyncha were caught from herbaceous plants growing under ornamental trees and shrubs. Of the identified species, only *C. viridis* and *G. fennahi* (ESM Figure S5) may be considered as potential vectors of *X. fastidiosa* (ESM Table S3). By comparison, we found no Auchenorrhyncha in the samples collected from tree canopies using the beat-tray method.

Detection of *Xylella fastidiosa* in Cicadomorpha and Fulgoromorpha (Auchenorrhyncha). DNA analysis for *X. fastidiosa* was performed for 164 specimens in total, belonging to *Cicadella viridis* (33 specimens), *Cicadella lasiocarpae* (42 specimens), *Philaenus spumarius* (4 specimens), *Neophilaenus lineatus* (3 specimens), and *Neophilaenus campestris* (4 specimens) and *Graphocephala fennahi* (13), all known to be xylem feeders, and *Euscelis incisus* (3 specimens), *Macrosteles laevis* (5 specimens), *Psammmotettix alienus* (56 specimens), *Nothodelphax albocarinatus* (1 specimen), all recognised as phloem feeders. The results of this study showed that *Xylella fastidiosa* was absent in all tested spittlebug species, regardless of the methodology used (ESM Figure S6). The LoD of the different assays was satisfactory. In the analyses, we detected 0.7 ng/reaction of genomic DNA in real-time PCR and 0.7 fg/reaction in nested PCR.

DISCUSSION

Studies on the early detection and monitoring of *X. fastidiosa* in insects enable the prediction of the bacterium's possible distribution (Elbeaino et al. 2014; Cruaud et al. 2018; Cunty et al. 2020). According to a study by Gargani et al. (2021), a survey of putative vectors is a necessary first step in understanding the epidemiology of infection. As suggested, this is even more justified in buffer zones or areas where no symptoms of *X. fastidiosa* have yet been detected/recorded.

Of the specimens classified as potential vectors of *X. fastidiosa*, only the representatives of two species have so far been confirmed as vectors of *X. fastidiosa* in Europe, i.e. *Philaenus spumarius* (Hemiptera: Aphrophoridae) and *Neophilaenus campestris* (Hemiptera: Aphrophoridae) (Cornara et al. 2017; Cavalieri et al. 2019). Although *N. campestris* has been shown to effectively transmit *X. fastidiosa* by feeding on xylem sap, its role in the epidemiology of this disease is still unknown. Since this species is also common in Poland and may contribute to the spread of *X. fastidiosa*, it needs to be monitored. *Cicadella viridis* L. (Hemiptera: Cicadellidae) is similarly common in Europe, including Poland, and is also considered a vector of *X. fastidiosa.*

Other Auchenorrhyncha species that stood out most in terms of abundance were *Macrosteles laevis* and *Psammotettix alienus*. Both species are polyphagous feeders on a wide range of host plants, particularly on crops, vegetables, herbs and some ornamentals (Klejdysz & Walkowski 2008; Klejdysz 2013). Both species are considered vectors of (Soika & Kamińska 2000; Kamińska & Soika 2001).

Buffalo treehopper (*Stictocephala bisonia*) is an important pest of vines. Symptoms of damage to vines are visible as reddening of leaves or whole branches (Arzone & Vidano 1987). There are no reports in the available literature on its role in pathogen transmission between host plants.

The absence of leafhoppers in the canopies of the examined fruit tree species can be attributed to several factors. Most Auchenorrhyncha species identified in this study were classified as first- or second-degree monophages or oligophages, with their host plants mainly grasses. Only a few were polyphagous, including *Philaenus spumarius*, *Cicadella viridis*, and *C. lasiocarpae*a. It should be emphasised that most of these species occur on ground vegetation, mainly grasses, where they feed, mate, and lay eggs. Of the Auchenorhyncha species listed, only adult *P. spumarius* individuals are capable of migrating to the tree crowns, where they stay and feed, which occurs in Poland between June and September (unpublished data). In Italy, it has been observed that adult *P. spumarius* exhibit distinct vertical migration during the dry summer period, moving from the herbaceous ground layer to canopies and other trees and shrubs (Bodino et al. 2019). It is suspected that time of day is an important factor influencing the distribution of canopies and inter-row vegetation; however, no observations have confirmed this. Additionally, it is possible that the beating trap methods used to collect samples from tree canopies were insufficient to detect leafhoppers. Perhaps using yellow sticky traps to collect Auchenorhyncha flying in orchards would be more effective. However, collecting individuals from sticky traps to assess the presence of *X. fastidiosa* is difficult and, in many cases, impossible.

Our results revealed no presence of *X. fastidiosa* in xylem sap-feeding insects that could play a role as its potential source and dissemination agent, even though the insects were collected in different geographical regions and from different plant material/crops, and in the periods considered by other authors as the most suitable for detecting the bacterium in insects (Cruaud et al. 2018; Bodino et al. 2019). Of the methods developed so far for *X. fastidiosa* detection (Baldi & Porta 2017), we selected real-time PCR with a TaqMan probe following the Harper protocol (Harper et al. 2010), as recommended by EPPO (EPPO Bulletin 2019) for the detection of bacteria in insects and considered by other authors as the most reliable, sensitive, relevant and highly specific test for detecting *X. fastidiosa* (Modesti et al. 2017; EPPO Bulletin 2019; Cunty et al. 2020; Gargani et al. 2021). The second method adopted, i.e. the nested PCR (Cruaud et al. 2018), was applied because in the studies by scientists from Corsica, real-time PCR with TaqMan as described by Harper et al. (Harper et al. 2010) had revealed some limitations in the detection of *X. fastidiosa* in insect vectors and thus underestimated the real prevalence of the bacterium. In that study, a nested PCR targeting the holC gene revealed that *X. fastidiosa* was present at higher prevalence across all populations. The LAMP, although enabling direct detection of the bacterium in the field, was less sensitive and could yield false-negative results (Harper et al. 2010). Based on this, the results from southern Italy (Elbeaino et al. 2014) should be interpreted with caution. On another site, although Cruaud et al. (Cruaud et al. 2018) had reported lower sensitivity of Harper real-time PCR, Cunty et al. (Cunty et al. 2020) found the method to be specific, reproducible, sensitive, and relevant for the detection of *X. fastidiosa* in *P. spumarius* collected in infected areas in France. The robustness of real-time PCR (Harper et al. 2010) had also been confirmed at the inter-laboratory level by tests performed within the framework of the Euphresco project 2015-F-146. Therefore, all these studies justify the appropriateness of our use of the two highly sensitive surveillance methods for detecting *X. fastidiosa.* The other thing that we should always take into account is the threshold limit. In our study, we obtained a quite high limit of detection for bacteria. Based on the nested PCR results, the sensitivity was much higher. This is strictly connected to the methodology, which uses two successive rounds, significantly enhancing sensitivity and specificity by targeting an internal sequence and reducing non-specific amplification, thereby allowing the detection of low-abundance targets. Thanks to its use, we could increase sensitivity, which is key for detecting DNA Xf in insects. Cruaud et al. (2018) obtained similar results in studies using nested PCR; the sensitivity was several dozen times higher than with real-time PCR. Cunty et al. (Cunty et al. 2020) had reported that the ddPCR originally developed to detect *X. fastidiosa* in plants (Dupas et al. 2019) allowed them to determine 10^3 cells/head or insect, and the authors stated that this was the technical limit of this protocol. Cruaud et al. (2018) suggested that the low prevalence of *X. fastidiosa* in *P. spumarius* (even in the late season, recommended as the most appropriate) may still underestimate the prevalence of *X. fastidiosa* and may be related to the relatively poor ability of the detection methods. Although the present study on the detection of *X. fastidiosa* showed no presence of this quarantine bacterium, the probability of its introduction into Poland through trade in nursery materials for ornamental and fruit plants remains high. There are currently no chemicals to control *X. fastidiosa*. The only way is to remove and destroy infected plants and control the vectors. Since it is difficult to prevent the entry of bacteria and vectors, further inspections to monitor the presence of *X. fastidiosa* in different hosts and its vectors should be conducted, especially given the worldwide exchange of plant material and imports from areas where the bacterium has already been detected.

CONCLUSION

The results of this study showed that the *X. fastidiosa* bacterium was absent in all tested spittlebug individuals of the tested species, regardless of the methodology used. To our knowledge, this study constitutes the first survey of insect vectors for *X. fastidiosa* in Poland.

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

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