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**New genetically distinct phytoplasmas and insect carriers associated with
pine tree disease revealed by a survey in Curonian Spit, Lithuania**

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Abstract: Our previous studies reported that phytoplasma was the causative agent of the pine disease in Curonian spit, Lithuania. In this study, insects from diseased pine trees and their adjacent areas were collected from 2016 to 2019 to further identify potential insect vectors that spread phytoplasmas. A total of 1018 phloem-feeding insects (order Hemiptera) were identified, 98.62% of which were aphids (Aphididae), and no known phytoplasma vectors were found. Results from semi-nested PCR using phytoplasma-universal primers revealed that phytoplasmas were detected in scots pine aphids (*Cinara pini*), waxy grey pine needle aphids (*Cinara pineti*), and species-unknown aphids. Further sequence analysis and virtual RFLP analysis of aphid-harbored phytoplasma strains indicated that they were closely related to ‘*Candidatus Phytoplasma pini*’ (16SrXXI-A), but mainly 16SrXXI-A variants, which were also main strains identified in diseased pine trees. In addition, three new phytoplasma subgroups were delineated in the present study. Subgroups 16SrXXI-C and 16SrXXI-D were unveiled from previously identified (but classification was overlooked) Lithuanian pine phytoplasma strains. Subgroup 16SrXXI-E was discovered from the newly identified aphid-harbored phytoplasmas. Further transmission trial study on these aphids will provide insights into the epidemiology, and pathosystem of pine phytoplasma diseases, as well as the disease management.

Key words: genetic diversity, insect transmissible plant pathogens, aphid

1. Introduction

Phytoplasmas, previously known as mycoplasma-like organisms (MLOs), are minute cell wall-less, and phloem-restricted intracellular bacteria that infect a wide variety of plant species worldwide, including agronomically important crops and ornamental plants (Lee et al. 2000). Phytoplasmas can be transmitted from plant to plant by insects, dodder (*Cuscuta cephalanthi* Engelm.), and grafting. All three modes of transmission are widely used in experimental plants, but in nature, phytoplasmas are transmitted in a persistent-propagative manner by phloem sap feeding insects in the order Hemiptera. They are mainly leafhoppers (Cicadelloidea), planthoppers (Fulgoroidea) and psyllids (Psylloidea) (Weintraub and Beanland 2006; Hogenhout et al. 2008). During the process of the persistent-propagative transmission, phytoplasmas must overcome several barriers such as intestinal and salivary gland barriers in the vector body (Lee et al. 2000; Weintraub and Beanland, 2006; Hogenhout et al. 2008). The epidemiology of phytoplasmas is closely related to the capacity of the insect vectors in survival, acquisition, transmission, and host range (Alma et al. 2019).

Due to phage-mediated genetic recombination and fusion, phytoplasma genomes are highly plastic, ranging in length from 530-1350 Kb (Oshima et al. 2004; Bai et al. 2006; Wei et al. 2008a). In addition, the phytoplasma genomes have undergone reductive evolution and have lost many genes involved in essential metabolic pathways, such as pentose phosphate pathway, tricarboxylic acid cycle, fatty acid biosynthesis, sterol biosynthesis, *de novo* nucleotide synthesis, and biosynthesis of most amino acids (Tan et al. 2021). This determines that phytoplasma is highly dependent on nutrients imported from host cells, and therefore, despite many attempts, it has not been possible to achieve *in vitro* culture of phytoplasmas. The traditional phenotype and biological properties-based classification approaches for culturable bacteria are not applicable to unculturable phytoplasmas, and phytoplasma taxonomy and classification depend largely on the molecular biology approaches. Currently, phytoplasmas are assigned in the provisional genus '*Candidatus* Phytoplasma' (IRPCM 2004), and 46 '*Candidatus* Phytoplasma' species have been named on the basis of 16S rRNA gene sequences, or distinct ecological features, such as unique vectorship and host specificity (Kirdat et al. 2020; Rodrigues et al. 2020; Jones et al. 2021; Zhao et al. 2021). In addition, based on distinct 16S

rRNA gene restriction fragment length polymorphism (RFLP) patterns, phytoplasmas have been classified into 36 ribosomal groups, and more than 150 subgroups (Lee et al. 1998; Seemüller et al. 1998; Wei et al. 2007; Zhao et al. 2009; Rodrigues et al. 2020).

The Curonian Spit is an elongated peninsula (98 km long and 0.4-4 km wide) in the Baltic Sea that surrounds the Curonian Lagoon. As a trans-boundary UNESCO World Heritage Site, southern and northern portions of the Curonian Spit are located in Russia and Lithuania, respectively. The Curonian Spit possesses the largest travelling sand dunes in Europe, and it is therefore called “Lithuanian Sahara”. The survival of the spit is the result of continual efforts in stabilization and reforestation against natural wind and sea erosion (United Nations Educational, Scientific, and Cultural Organization 2000). Appropriately 70% of the spit land is currently covered with forests, mainly conifers, such as native scots pine (*Pinus sylvestris* L.) and alien mountain pine (*Pinus mugo* Turra) trees (Kutorga et al. 2012). Pine trees play a key role in sand dune stabilization and afforestation, especially, *P. mugo*. *P. mugo*, native to the highlands of central Europe, was introduced to the Lithuanian seacoast spit in 1825 to stabilize dunes (Danusevičius et al. 2012). In addition, pine trees in the Curonian Spit also attract a lot of tourists and photography enthusiasts because of their unique “dancing or drunken” appearance (Korneevets et al. 2018). Since 2008, however, both scots pine and mountain pine trees have displayed symptoms such as excessive branching, and needle dwarfing. Phytoplasmas have been identified as the etiological agents of the disease (Valiunas et al. 2015). Interestingly, phytoplasma strains in two distinct 16S ribosomal groups (16SrI and 16SrXXI) are associated with pine tree diseases in Lithuania (Valiunas et al. 2015).

In order to better understand the epidemiology of Lithuanian pine phytoplasma disease and to develop effective disease management strategies, the purpose of this study was to investigate potential insect vectors responsible for the spread of the pine disease. Prior to the present study, there were scattered reports of leafhoppers, planthoppers and psyllids that feed and overwinter on pine trees (Waloff 1973; Jarausch and Jarausch 2014). However, no insects had been confirmed to transmit any pine-infecting phytoplasmas. In this study, the insects fed on and live in the vicinity of diseased pines

90 in the Curonian Spit, Lithuania were collected. In our insect collection, aphids were dominant and no
91 phytoplasma insect vectors as previously reported were found. Such results indicated the possibility
92 that the pine-infecting phytoplasmas might be transmitted by other phloem feeding insects. The semi-
93 nested PCR results showed that phytoplasmas were detected in scots pine aphids (*Cinara pini*), waxy
94 grey pine needle aphids (*Cinara pineti*, previously known as *Schizolachnus pineti*) and other aphids
95 (*Cinara spp.*, unknown species). The sequence analysis and *in silico* RFLP analysis revealed that
96 aphid derived phytoplasma strains were closely related to ‘*Candidatus* Phytoplasma pini’ and
97 belonged to group 16SrXXI. The findings raise an interesting question of whether these aphids play
98 a role in spreading pine-infecting phytoplasmas in the region. Further transmission trial study will
99 provide more information on the epidemiology of pine phytoplasma diseases.

Draft

2. Materials and Methods

To investigate the potential insect vectors of phytoplasmas associated with pine diseases, insect collection was conducted in Curonian spit, Lithuania for three consecutive years from 2016-2019. The symptomatic scots pine and mountain pine trees mainly distribute in four locations: Preila (55°22'43.5"N/21°03'47.5"E), Pervalka (55°25'16.5"N/21°05'10.7"E), Juodkrante (55°34'30.0"N/21°07'16.4"E; 55°36'19.3"N/21°07'23.0"E). Insects were collected from symptomatic pine trees and adjacent area in these four locations by a net-sweeping and beating tray methods. The insects were stored in a -20 °C freezer with 90% ethanol until morphological identification and DNA extraction were performed. The collected insect specimens were subjected to morphological analysis under stereoscopic microscope by using entomological keys (Ribaut 1952; Kerzhner and Jachevskij 1964; Ossiannilsson, 1978, 1981, 1983; Freude et al. 1965–1989; Blackman and Eastop 2000, 2020) and identified to the genus and species levels.

Because the body size of aphid is small, 20-30 insect specimens from the same species were pooled together to one sample. The total DNA was extracted using Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Lithuania). The extracted DNA from insects and plant samples was used as template in three separate experiments, each involving polymerase chain reactions (PCRs) primed by universal primer pairs: P1/16S-SR (Lee et al. 2006) followed by P1A/16S-SR (Lee et al. 2004). The DNAs extracted from the symptomatic pine trees, and water were used as positive and negative control for PCR examination, respectively. The PCR was carried out in 25 µl reaction mixtures containing 1 µl of undiluted total DNA, 200 µM of each dNTP, and 0.4 µM of each primer, 15 mM MgCl₂, 2.5 units of LATaq DNA-proofreading polymerase (Takara Bio USA, Madison, WI). The following thermocycling conditions were used: denaturation at 94 °C for 1 min, annealing for 2 min at 55 °C, and primer extension for 3 min at 72 °C for 35 cycles (10 minutes for the final extension). One microliter of diluted (1:20) PCR product from the first amplification was used as the template in the reaction mixtures for semi-nested PCR. The PCR products were analysed by 1% agarose gel electrophoresis, with added SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), and visualized with a UV transilluminator. The semi-nested PCR amplicons (1.5 kb) of 16S rDNA

gene were cloned in *Escherichia coli* using the Invitrogen TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Recombinant plasmids were purified using Bio-Rad Quantum Prep Plasmid Miniprep kit (Bio-Rad, USA). Sequencing was conducted using Macrogen next generation sequencing services (Macrogen Inc., Rockville, MD USA). At least three clones were selected for each amplicon to ensure >6X coverage for each base position. The consensus sequences were assembled using DNASTAR LaserGene software package Seqman application (DNASTAR, Madison, WI). The obtained nucleotide sequences were deposited into GenBank.

The sequence similarity analysis was conducted against *iPhyClassifier* (Zhao et al. 2009). The virtual RFLP profiles were created, and similarity coefficients were calculated using *iPhyClassifier* (Zhao et al. 2009). With MEGA6 software, the phylogenetic analysis was conducted by using Maximum Likelihood method (Tamura et al. 2013). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The taxa used in the phylogenetic tree construction included previously reported 16SrXXI group of phytoplasma strains; *Acholeplasma palmae* served as an out-group during the phylogenetic tree construction.

3. Results

3.1. Insect identification and phytoplasma detection

A total of 1348 insects were collected from the diseased pine trees and their adjacent area in Curonian Spit of Lithuania (Fig. 1a and b). Morphological identification using a stereomicroscope revealed that these insects belonged to six families and ten genera. Among them, 1018 insect specimens were identified in the order Hemiptera, belonging to three families Aphididae, Myridae, and Lygaeidae (Table 1). Aphids (Aphididae) accounted for 98.62% including scots pine aphids (*Cinara pini*), and waxy grey pine needle aphids (*Cinara pineti*, Table 1, Fig. 1c and d). The presence of phytoplasmas in 1018 insects was examined by semi-nested PCR with phytoplasma 16S rRNA gene specific primers. Amplicons of around 1.5kb were amplified from insect samples

belonging to two known aphid species, *C. pini*, and *C. pineti* (Table 1; Fig. 1). *C. pini* and *C. pineti* are common pine aphids, known as scots pine aphids and waxy grey pine needle aphids, respectively. In addition, phytoplasmas were also amplified from some unknown aphid species (*Cinara spp.*). However, no phytoplasmas were detected in the insect samples within Families Myridae and Lygaeidae, or other aphid species *Cinara pinea* and *Anoecia corni* (Table 1).

3.2. The sequence similarity analysis

PCR amplicons of six aphid samples (Cpini-1, Cpini-2, Cpineti, Cinara.sp-1, Cinara.sp-2, and Cinara.sp-3) were cloned and sequenced. The obtained nucleotide sequences, covering a nearly full-length of the 16S rRNA gene and a partial 16S-23S RNA intergenic region, were deposited to the GenBank (Accession numbers: MW695531-MW695536, Table 2). Aphid-harbored phytoplasma strains were designated as Cpini-1, Cpini-2, Cpineti, Cinara.sp-1, Cinara.sp-2, and Cinara.sp-3, respectively. Six strains were nearly identical and most closely related to the reference strain of ‘*Candidatus Phytoplasma pini*’ (AJ632155). They shared 99.5-99.9% sequence similarity in 16S rRNA gene. No 16SrI-A related phytoplasma strains were found in aphid samples.

3.3. Virtual RFLP analysis

The purpose of this study was to investigate the potential insect vectors for the transmission of pine disease phytoplasmas. Therefore, a comparative virtual RFLP analysis was performed between phytoplasmas detected in aphids (this study) and diseased pine trees (previously reported) by employing *iPhyClassifier* (Zhao et al. 2019). A total of thirteen 16S rRNA sequences (>1250bps) of ‘*Candidatus Phytoplasma pini*’-related strains were retrieved from NCBI database (Table 2, Schneider et al. 2005; Costanzo et al. 2016; Valiunas et al. 2015, 2019). The reference strain of ‘*Candidatus Phytoplasma pini*’ (AJ632155, Schneider et al. 2005) was found from *Pinus halepensis* in Spain and identified belonging to 16SrXXI-A (Fig. 2a and Table 2). The RFLP profiles of most pine phytoplasma strains including German strain (AJ310849, detected in *Pinus sylvestris*, Schneider et al. 2005), and eight Lithuanian strains were identical (Fig. 2b). These eight Lithuanian strains were

detected in *Pinus sylvestris* (GU289676, KR051471, MK089817), and the remaining five were found in *Pinus mugo* (KF801674, KR051475, KR051470, KR051472, KR051473, Valiunas et al. 2015, 2019). The underscored strains were shown in Table 2 as the representatives. The similarity coefficient among the above mentioned nine strains and the 16SrXXI-A reference strain is 0.98, the threshold for a variant delineation (Wei et al. 2008). Therefore, these strains were designated as 16SrXXI-A variants (Fig. 2b and Table 2). It is obvious that 16SrXXI-A variant strains are the majority of Lithuanian pine phytoplasma strains.

Previously reported '*Candidatus Phytoplasma pini*' (KU242428) was identified in USA and classified into 16SrXXI-B (Costanzo et al. 2016). This strain possesses a unique RFLP pattern, and its similarity coefficient against all known phytoplasma strains is less than 0.85 (the threshold to designate a new group, Wei et al. 2007, Table 2), thus, it should be redesignated to a new group. Virtual RFLP analysis also showed that previously reported two strains '*Candidatus Phytoplasma pini*' PineBLD (MK089819) and '*Candidatus Phytoplasma pini*' PineDYN27 (KR051474) belonged to two new 16SrXXI subgroups. They had distinct RFLP profiles (Fig. 2c and d) and the similarity coefficients against other 16SrXXI group of phytoplasmas were less than 0.97, the threshold for a new subgroup delineation (Wei et al. 2008). Hereby, '*Candidatus Phytoplasma pini*' PineBLD and PineDYN27 were designated as 16SrXXI-C, and 16SrXXI-D, respectively (Table 2).

Virtual RFLP analysis of phytoplasma strains detected in the aphid samples showed the same trend as pine-derived strains. Among a total of six aphid-harbored phytoplasma strains, four strains Cpini-1, Cpini-2, Cinara.sp-1, and Cinara.sp-2 had the same RFLP pattern (Fig. 2b and Table 2), that was identical to that of the 16SrXXI-A variant shown in Fig. 2b. In addition, the other two aphid phytoplasma strains Cpineti and Cinara.sp-3 shared the same RFLP profile (Fig. 2e), which, however, was distinct from other strains. The similarity coefficient score <0.97 compared with other strains in 16SrXXI group indicated that Cpineti and Cinara.sp-3 is a new subgroup. Therefore, we designated it as 16SrXXI-E, and aphid strain phytoplasma Cpineti (MW695533) is the reference strain (Table 2). Three new subgroups, 16SrXXI-C, 16SrXXI-D, and 16SrXXI-E could be differentiated from the

known 16SrXXI-A by *Hae*III enzyme digestion alone, and three new subgroups could be distinguished by further *Dra*I and *Taq*I enzyme digestions (Fig. 2f).

3.4. Phylogenetic analysis

The phylogenetic analysis of 16S rRNA gene sequences (1250 positions) using Maximum Likelihood method was carried out to study the evolutionary relationship between aphid-harbored pini phytoplasma strains and other known 16SrXXI group/subgroup reference strains. The tree topology demonstrated that all the 16SrXXI phytoplasma strains fell in the monophyletic phytoplasma clade but apparently represented two different phylogenetically subclades. The newly identified aphid-derived phytoplasma strains belonged to the same subclade as all previously reported European pine phytoplasma strains in the 16SrXXI group, while '*Candidatus* Phytoplasma pini' USA strain fell into the other subclade (16SrXXI-B, Fig. 3). This result was consistent with that of virtual RFLP analysis.

4. Discussion

Among the insects collected during the survey, not previously known phytoplasma insect vectors such as leafhoppers, planthoppers, and psyllids were found. This may be due to the special smell of pine trees and the sticky resin/wax they secreted that facilitated the repellent behavior of these insects (Roark, 1939). In addition, due to strong winds and waves and seawater erosion, Curonian Spit may not be an ideal habitat for the afore-mentioned recognized phytoplasma insect vectors (Møller, 2013; Ivinskis and Rimšaitė, 2005). We suspected that pine phytoplasmas may be transmitted by other phloem feeding insects. In our entire insect collection, aphids make up the majority. It has been reported that the different phytoplasmas exist in different aphids (Cainelli et al. 2007; Galetto et al. 2011; Kong et al., 1997; López and Pantoja, 2014; Mehrotra, 2013; Soufi, 2012; Wang and Hughes, 1976). For examples, apple proliferation phytoplasmas were detected in three species of apple aphids (*Aphis pomi*, *Dysaphis plantaginea*, and *Eriosoma lanigerum*) (Cainelli et al. 2007). Chrysanthemum yellows phytoplasmas were also found in two aphid species (*Aphis craccivora* and *Myzus persicae*) (Galetto et al. 2011). To date, there have been no confirmed cases of successful transmission of

phytoplasma by aphids (D'Arcy and Nault, 1982; Cainelli et al. 2007; Galetto et al. 2011). But it is noteworthy that phytoplasmas (MLOs) have been observed in the salivary glands and mid-gut tissues of pine needle aphids (*Eulachnus rileyi*) under light and electron microscopy (Gopo et al. 2002). These pine needle aphids fed on pines and cypress trees infected with phytoplasmas (Gopo et al. 2002). Phytoplasmas must overcome the intestinal barrier and salivary gland barrier of the insect vector in order to complete vector-borne transmission. Phytoplasmas were found in salivary glands and midgut tissues of pine needle aphids, suggesting the potential of aphids as vectors for the transmission of phytoplasmas associated with pine and cypress phytoplasma diseases. The nested PCR results from the present study indicated the aphids were pine phytoplasma carriers. Further transmission trials are needed to determine if the phytoplasma-carrying aphids are vectors. New subgroup lineages (16SrXXI-C, 16SrXXI-D, and 16SrXXI-E) identified in diseased pine trees and aphid carriers broadened our understanding of genetic diversity of phytoplasmas associated with pine diseases.

5. Conclusions

Our study confirmed that most of phytoplasma strains identified in surveyed aphids were consistent with the major strains found in previously reported diseased scots pine and mountain pine trees. The results suggest that scots pine aphids (*Cinara pini*), waxy grey pine needle aphids (*Cinara pineti*), and species-unknown aphids are phytoplasma insect carriers. In addition, the findings from this study also revealed new genetically distinct phytoplasmas strains, representing new subgroup lineages. Further transmission trials will contribute to a better understanding of the epidemiology of pine phytoplasma diseases.

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Fig. 1. Sampling and identification of insects from and around the symptomatic pine trees in Curonian Spit, Lithuania. (a) insects were collected from four different sampling sites (gray solid ellipses) in Curonian Spit area where symptomatic pine trees (b) were found. The map is under the Creative Commons Attribution-Share Alike 3.0 Unported license. Attribution: Knutux at the Lithuanian language Wikipedia (<https://upload.wikimedia.org/wikipedia/commons/0/08/KursiuNerija.png>). The map was modified with Adobe Photoshop. (c-d) stereo dissecting microscopic images of two species of insects that have detected phytoplasmas associated with pine diseases. (c) *Cinara pini* (scots pine aphid). (d) *Cinara pineti* (also known as *Schizolachnus pineti*, waxy grey pine needle aphid). Scale bar = 1mm.

Fig. 2. *In silico* virtual RFLP profiles of 16S rRNA gene F2nR2 fragments from phytoplasma strains detected in aphid samples (this study) and previously reported pine phytoplasma strains (a-e) and differentiation of 16SrXXI subgroups by key restriction enzyme digestions (f). RFLP profiles of (a) 16SrXXI-A, the reference strain of ‘*Candidatus* Phytoplasma pini’ (AJ632155). (b) 16SrXXI-A variant, phytoplasma strains detected in aphid samples (MW695531, MW695532, and MW695534) and previously reported pine phytoplasma strains (AJ310849, KF801674, and MK089817). (c) 16SrXXI-C, ‘*Candidatus* Phytoplasma pini’ PineBLD (MK089819). (d) 16SrXXI-D, ‘*Candidatus* Phytoplasma pini’ PineDYN27 (KR051474). (e) 16SrXXI-E, aphid strain Phytoplasma Cpineti (MW695533) and aphid strain Phytoplasma Cinara.sp-3 (MW695536). Recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *AluI*, *BamHI*, *BfaI*, *BstUI* (*ThaI*), *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI* (*MboI*), *MseI*, *RsaI*, *SspI*, and *TaqI*. (f) The new subgroups 16SrXXI-C, 16SrXXI-D, and 16SrXXI-E can be differentiated from 16rXXI-A subgroup by *HaeIII* enzyme digestion, and 16SrXXI-C, 16SrXXI-D and 16SrXXI-E can be further distinguished by *DraI* and *TaqI* digestions. MW, ϕ X174 DNA-*HaeIII* digestion.

Fig. 3. Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search

449 were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of
450 pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then
451 selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch
452 lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in
453 MEGA6 (Tamura et al., 2013). The taxa used in the phylogenetic tree construction included 16S
454 rRNA gene F2nR2 fragments (1250 positions) of all previously reported 16SrXXI group of
455 phytoplasma strains associated with pine diseases, and aphid phytoplasma strains identified in the
456 current study (highlighted in bold). Subgroups of 16SrXXI-A, 16SrXXI-B, 16SrXXI-C, 16SrXXI-D,
457 and 16SrXXI-E were highlighted in gray boxes. *Acholeplasma palmae* served as an out-group during
458 the phylogenetic tree construction. The scale bar represents evolutionary distance in nucleotide
459 substitutions per base position.

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Table 1. Species/genus identification of insects collected from the Curonian Spit in Lithuania and detection of phytoplasmas in insect samples by semi-nested PCR.

Insect identification		Total number of insect specimens collected	Number of phytoplasma-positive samples/number of samples tested
Order Hemiptera	Family Myridae		
	<i>Lygus rugulipennis</i> (Poppius, 1911)	2	0/2
	Family Lygaeidae		
	<i>Gastrodes grossipes</i> (DeGeer, 1773)	12	0/12
	Family Aphididae		
	<i>Cinara pini</i> (Linnaeus, 1758)	240	6/8**
	Scots pine aphid		
	<i>Cinara pineti</i> (Fabricius, 1781)	85	2/4**
	waxy grey pine needle aphid		
	<i>Cinara</i> spp.	633*	17/21**
	<i>Cinara pinihabitans</i> (Mordvilko, 1894)	20	0/1**
	<i>Cinara pinea</i> (Mordvilko, 1894)	25	0/1**
	<i>Anoecia corni</i> (Fabricius, 1775)	1	0/1

* 633 aphids have not yet been identified at the species level, but there are apparently more than three different new species. ** 20-30 insect specimens were pooled in one sample for DNA extraction and PCR detection.

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Table 2. Similarity coefficients derived from virtual RFLP analysis of 16S rRNA genes of phytoplasma strains identified in aphid samples (new subgroup 16SrXXI-E and 16SrXXI-A variant) and other 16SrXXI phytoplasmas

	Phytoplasma strain (Accession number)	Host (Country)	Subgroup classification	Reference	1	2	3	4	5	6	7	8	9	10	11	12	13
1	'Candidatus Phytoplasma pini' (AJ632155)	<i>Pinus halepensis</i> (Spain)	16SrXXI-A	Schneider et al., 2005	1.00												
2	'Candidatus Phytoplasma pini' (AJ310849)	<i>Pinus sylvestris</i> (Germany)	16SrXXI-A variant	Schneider et al., 2005	0.98	1.00											
3	'Candidatus Phytoplasma pini' Smiltyne1 (KF801674)	<i>Pinus mugo</i> (Lithuania)	16SrXXI-A variant	Valiunas et al., 2015	0.98	1.00	1.00										
4	'Candidatus Phytoplasma pini' PineBL2 (MK089817)	<i>Pinus sylvestris</i> (Lithuania)	16SrXXI-A variant	Valiunas et al., 2019	0.98	1.00	1.00	1.00									
5	'Candidatus Phytoplasma pini' (KU242428)	<i>Pinus virginiana</i> (USA)	16SrXXI-B ^a	Costanzo et al., 2016	<u>0.79</u>	<u>0.81</u>	<u>0.81</u>	<u>0.81</u>	1.00								
6	'Candidatus Phytoplasma pini' PineBLD (MK089819)	<i>Pinus mugo</i> (Lithuania)	16SrXXI-C	Valiunas et al., 2019; this study	0.93	0.95	0.95	0.95	<u>0.76</u>	1.00							
7	'Candidatus Phytoplasma pini' PineDYN27 (KR051474)	<i>Pinus mugo</i> (Lithuania)	16SrXXI-D	Valiunas et al., 2015; this study	0.95	0.97	0.97	0.97	<u>0.78</u>	0.93	1.00						
8	Aphid strain Phytoplasma Cpini-1 (MW695531)	<i>Cinara pini</i> (Lithuania)	16SrXXI-A variant	this study	0.98	1.00	1.00	1.00	<u>0.81</u>	0.95	0.97	1.00					
9	Aphid strain Phytoplasma Cpini-2 (MW695532)	<i>Cinara pini</i> (Lithuania)	16SrXXI-A variant	this study	0.98	1.00	1.00	1.00	<u>0.81</u>	0.95	0.97	1.00	1.00				
10	Aphid strain Phytoplasma Cpineti (MW695533)	<i>Cinara pineti</i> (Lithuania)	16SrXXI-E	this study	0.96	0.98	0.98	0.98	<u>0.79</u>	0.93	0.97	0.98	0.98	1.00			
11	Aphid strain Phytoplasma Cinara.sp-1 (MW695534)	<i>Cinara sp.</i> (Lithuania)	16SrXXI-A variant	this study	0.98	1.00	1.00	1.00	<u>0.81</u>	0.95	0.97	1.00	1.00	0.98	1.00		
12	Aphid strain Phytoplasma Cinara.sp-2 (MW695535)	<i>Cinara sp.</i> (Lithuania)	16SrXXI-A variant	this study	0.98	1.00	1.00	1.00	<u>0.81</u>	0.95	0.97	1.00	1.00	0.98	1.00	1.00	
13	Aphid strain Phytoplasma Cinara.sp-3 (MW695536)	<i>Cinara sp.</i> (Lithuania)	16SrXXI-E	this study	0.96	0.98	0.98	0.98	<u>0.79</u>	0.93	0.97	0.98	0.98	1.00	0.98	0.98	1.00

468 ^a the subgroup 16SrXXI-B (KU242428) should be redesignated to a new group because the similarity coefficients (underscored) are less than 0.85, a threshold for a new
469 group assignment.

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Fig. 1.

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Fig. 2.

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Fig. 3.

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