VILNIUS UNIVERSITY
NATURE RESEARCH CENTRE, INSTITUTE OF BOTANY

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PHYTOPLASMAS AND THEIR INSECT VECTORS IN LITHUANIA

Summary of doctoral dissertation
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ALGIRDAS IVANAUSKAS

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INTRODUCTION

Plant yellows diseases cause big damage to beneficial plants and inflict tremendous losses to agriculture.

For a long time it was thought that yellows was caused by viruses. In 1967, a group of Japanese researchers, using electron microscopy methods, found mycoplasma-like organisms (MLO) in ultrathin tissue sections of diseased plants. This discovery revealed new group of microorganisms and defined them as main agent of yellows diseases (Doi et al., 1967; Bertaccini, Duduk, 2009). In 1994, the term "phytoplasma" was officialy accepted instead of "MLO" (Bove, Garnier, 2002).

Phytoplasmas are wall-less, belonging to *Mollicutes* class, found in phloem and insect vector tissues, pathogenic, uncultivable *in vitro* bacteria. To date about several hundreds of plant species are known to be affected by these microorganisms. Major losses are brought by phytoplasmas to woody plant growers, who cultivate such plants as coconut palm trees, grapevines, stone fruit trees and apple trees (Bertaccini, Duduk, 2009). Antibiotics, thermotherapy, recovery from meristem are temporary, laborious and costly solutions to overcome these infections. Most effective ways to fight phytoplasmal infections still are diseased plant removal and preventive abundant use of pesticides in order to minimize and eliminate populations of vectors and weeds. It is important to collect as much as possible data about life cycles, ways of spreading in plant and environment, natural hosts and their relationships, influence of the anthropogenic factors on these bacteria. Elucidation of these aspects will lead to creation of strategies and models that would show how to effectively protect plants, stop the spreading of disease and how to change plant features to achieve these goals (McCoy, Williams, 1982; Staniulis, 1988; Bove, Davis, Lee, 2000; Garnier, 2002; Bertaccini, 2007).

Phytoplasmas have been ivestigated for thirty years in Lithuania (Staniulis, 1988). Advanced molecular biology methods during the last decades allowed to detect in various herbaceous and woody plants phytoplasmas that belong to five groups and seventeen subgroups (according to Lee et al., 1998 classification system) (Staniulis et al., 2000; Jomantienė et al., 2000; 2002a, b; Samuitienė et al., 2002; Urbanavičienė et al., 2005; Valiunas, 2003; Valiunas et al., 2000, 2001a, b, 2004; 2006, 2009a, 2010). In this study, we present results about new phytoplasmas and their position in classification scheme. We point out possible phytoplasma vectors and show results of performed transmission trials.

The aim of the research was to identify the phytoplasmas detected in insects that were found on various phytoplasma-infected plants, and to reveal phytoplasma insect-vectors as well as phytogenetical relationships of identified phytoplasmas.

Main tasks:

1. To define possible insect vectors, which were collected from plants with phytoplasmal symptoms.
2. To identify potential insect vectors of phytoplasmas and their plant hosts by using PCR, RFLP methods.
3. To identify and classify phytoplasmas detected in insect and symptomatic plant specimens by analysis of 16S rRNA, *rp* and *secY* gene sequences.

4. To compare data on phytoplasma vectors and plant hosts obtained during the research with the data presented in literature.
5. To perform insect transmission trials in order to define their ability to transmit phytoplasmas among plants from which insects where collected.
6. To carry out phylogenetical analysis of 16S rRNA and ribosomal protein genes, and to compare them with other phytoplasma genes deposited in internet databases of gene sequences.

Theme relevance

Phytoplamas are causing big economical losses to economies of agrarian countries by inducing epidemics of cultivated plants. In Lithuania, phytoplasmas impare important plants such as raspberries, oats, barley, carrots, rapes, onions and others, they were also detected in many of ornamental plants.

From previous research, we already know a few mostly widespread phytoplasma groups, subgroups, and many of their host plants in Lithuania. Phytoplasmas detected in our work cause damage and may induce epidemics of valuable plants such as strawberries, black salsifies, black currants, apple trees, sweat and sour cherries and industrial plants such as scots pines and Norway spruces. The data on potential vectors of these bacteria are very scarce in Lithuania. The identification and research of insect vectors will help to create more effective strategies and systems to fight with phytoplasmal infections. It will allow to earlier detect new and invasive phytoplasmas. Identification of phytoplasmas and their vectors will provide important data for research of ecology, distribution, origin, epidemiology, and ways of spreading of these pathogens. Such information is beneficial for plant protection (quarantine) institutions and plant growers in Lithuania and neighbouring countries. It will help to ascertain possible invasive insect species and phytoplasma strains which immigration to Lithuania caused the climatic changes.

Scientific novelty of the research

Comprehensive studies have been carried out on phytoplasma vectors in Southern, Western and Central Europe, but in Eastern and Northern Europe, the data on such research are scarce. The data collected in our study show high value in terms of investigations determined to identify the variety of phytoplasma insect vectors and the distribution of phytoplasmas in our region.

Phytoplasmas have been investigated in Lithuania for more than thirty years. Initially phytoplasma studies were based on the examination of ultrathin sections of plant or insect tissue through electron microscope. This method only allowed to confirm the infection and determine morphological features of bacteria. Molecular biology methods used in our and previous studies made possible to precisely and effectively detect and classify phytoplasmas, and define the variety and distribution of phytoplasmas in Lithuania. During this research for the first time in Lithuania, we determined possible phytoplasma insect vectors using molecular biology methods. Most of the detected phytoplasma subgroups were found in the identified insect species for the first time in Lithuania and worldwide. We found phytoplasmas in two insect species for the first time in Lithuania and abroad. Our data on new potential insect vector species extend the spectrum of phytoplasma vectors in our region. Phytoplasmas were detected for the first time in five plant species in Lithuania. We identified in this work one new phytoplasma

subgroup for Lithuania and world, and one new subgroup for Lithuania and their plant-hosts. New phytoplasma subgroups and their host plants found during this study contribute to biodiversity and distribution research of phytoplasma detected in Europe and worldwide. We revealed in our results not only the detected phytoplasmas and their host plants, but also for the first time in Lithuania we identified and confirmed insect vectors of identified phytoplasmas.

Defended statements:

1. Insect species from *Hemiptera* order are potential phytoplasma vectors in Lithuania. Eleven identified insect species represent possible vectors of phytoplasmas detected in Lithuania. Five plant species are new plant hosts for phytoplasmas in Lithuania.
2. Detected phytoplasmas belong to 16SrI-A, B, C, 16SrIII-B, P, T and 16SrXXI-A phytoplasma subgroups.
3. Detected possible insect vectors can have wide range of transmitted phytoplasmas.
4. *E. incisus* transmit phytoplasma attributed by classification scheme to 16SrI-C subgroup in Lithuania.
5. Detected phytoplasmas are closely related to other 16SrI-A, B, C, 16SrIII-B, P, T and 16SrXXI-A subgroup phytoplasmas detected in Lithuania and other countries.
6. 16SrIII-T and 16SrIII-P subgroup phytoplasmas could belong to the same species.
7. Xylem feeding leafhoppers as phloem feeding leafhoppers can acquire and maintain phytoplasmas.

Approbation of the results

The dissertation material was reported at two international conferences and one conference in Lithuania. The results of the researchwere presented in tree scientific papers and two abstracts of conferences.

Structure of dissertation

The dissertation consists of the following chapters: Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusions, References (216 sources), List of Publications. The dissertation covers 133 pages including 45 figures and 6 tables. The text of the dissertation is written in Lithuanian with the summary in English.

MATERIALS AND METHODS

Insect sample collection and processing

Suspected insect vectors of phytoplasmas were collected in July–September by net-sweeping and beating tray from a meadow and an orchard in Kaunas and Vilnius districts of Lithuania, where phytoplasma-infected plants had previously been found. Insects were preserved in 90% ethanol until DNA extraction.

Insect identification and classification

Adult male specimens were identified to the species level by morphological analysis under a stereomicroscope using entomological keys of common in Europe *Hemiptera* order insects.

Transmission trials

For insect transmission tests, healthy plants of Madagascar periwinkle (*Catharanthus roseus* (L.) G.Don) and white clover (*Trifolium repens* L.) were grown from seed in an isolated greenhouse section equipped with yellow sticky traps for surveillance for insects. Individuals of *E. incisus* for artificial breeding were collected from healthy-appearing plants in Lithuanian meadows and were placed in insect-proof cages containing white clover (*Trifolium repens* L.) plants. Insects in the artificially reared, caged colonies and plants on which they fed in the cages were analysed for phytoplasma infection; the results were negative. To obtain source plants for insect transmission tests, plants of white clover were collected in the Lithuanian orchard and tested for infection by the '*Ca.* Phytoplasma asteris' strain, CPh phytoplasma, the member of subgroup 16SrI-C. Phytoplasma-positive plants served as the original source of CPh phytoplasma for transmission of the phytoplasma to healthy plants of Madagascar periwinkle by the use of dodder (*Cuscuta* sp.). The CPh phytoplasma was then transmitted, using dodder, from the infected periwinkle to healthy white clover seedlings. This last set of clover plants was used as CPh source for insect acquisition feeding in the transmission tests. All plants were tested for phytoplasma-free or phytoplasma-infected status before and after the tests of CPh phytoplasma transmission from clover to clover.

The insect transmission tests were carried out in a greenhouse that was equipped with yellow sticky traps for insect surveillance. Groups of 20–30 artificially raised, phytoplasma-uninfected *E. incisus* 3rd to 4th stage nymphs were placed on caged plants (4 plants per cage) of CPh phytoplasma-infected white clover for an acquisition feeding period of 14 days. Surviving individuals (20–25–23, min-max-average) of these insects then were placed for an inoculation feeding period of 2–3 weeks on each of 20 white clover plants, and an equal number of healthy control white clover seedlings, without insects, were each held separately in insect-proof cages. After the inoculation feeding period, plants were treated with insecticide, and samples of plant tissue and insects were collected for phytoplasma detection and identification. The plants were held in the cages throughout the duration of the experiment until they died. All plants were tested for phytoplasma infection 2–3 weeks following the 2–3 week inoculation feeding period. Three identical trials were performed: one in 2011 (June–September) and two in 2012 (June–September).

Plant samples

To determine possible plant hosts, the samples of plants, showing symptoms of possible phytoplasma infection, were collected from the same locations, where insect samples were collected.

DNA extraction

DNA was extracted using Genomic DNA purification kit (MBI Fermentas, Vilnius, Lithuania) according to the kit protocol.

Nested PCR for phytoplasma detection

Extracted DNA from insects and plant samples was used as template in three separate experiments, each involving nested polymerase chain reactions (PCRs) primed by universal primer pairs P1A/16S-Sr (Lee et al., 2003; 2006) and R16F2n/R16R2n (Lee et al., 2006). PCRs were carried out and products were analysed as previously described (Lee et al., 1998).

RFLP analysis

Amplified DNA products of nested PCRs were analysed by single enzyme digestion, according to manufacturer's instructions, with *Alu*I, *Bfa*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Kpn*I, *Mse*I, *Taq*I and *Rsa*I (MBI Fermentas, Vilnius, Lithuania).

DNA electrophoresis

Restriction fragment length polymorphism (RFLP) patterns were analysed by electrophoresis through a 5% polyacrylamide gel. The DNA size marker was phiX174 DNA/*Bsu*RI (*Hae*III) (MBI Fermentas, Vilnius, Lithuania). RFLP patterns were compared with patterns previously published (Lee, 1998; Jomantiene, 2002).

Gene cloning

Products of PCRs primed by R16F2n/R16R2n were cloned in *E. coli* using the InsTAclone™ PCR cloning kit (MBI Fermentas, Vilnius, Lithuania).

Gene sequencing

Fragments of 16S rRNA, *rp* and *seY* genes sequenced using automated DNA sequencing (Institute of Biotechnology DNA Sequencing Centre, Macrogen Sequencing Centre). Sequence reads were assembled using SeqMan, and alignments of the sequences were accomplished with MegAlign, DNASTAR LaserGene software (DNASTAR, Madison, WI) and MEGA version 5 (Tamura et al., 2011).

Analysis of nucleotide sequences and construction of phylogenetic trees

Nucleotide sequences of the phytoplasma 16S rDNA amplicons were analysed through the use of BLAST searches at the National Centre for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/). The *i*PhyClassifier web tool (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi.) was used to obtain virtual RFLP gel images and to classify phytoplasma strains according to 16Sr groups and subgroups (Zhao et al., 2009). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al. 2011).

RESULTS AND DISCUSSION

Collected and identified insects and plants

We collected and identified insect samples of 35 species belonging to *Hemiptera* order. Eight species due to lack of male specimens were identified to genus level (Table 1).

Table 1. All collected insect species and genera

Insect species	**Locality**	**Date**
Suborder: *Auchenorrhyncha* Superfamily: *Cercopoidea* Family: *Aphrophoridae*		
Aphrophora alni	Dvarčionys, Vilnius d.	08 25 2012
Aphrophora alni	Mažieji Gulbinai, Vilnius d.	08 30 2012
Aphrophora alni	Muniškiai, Kaunas d.	08 07 2012
Aphrophora alni	Muniškiai, Kaunas d.	08 28 2010
Lepyronia coleoptrata	Muniškiai, Kaunas d.	08 12 2012
Neophilaenus sp.	Muniškiai, Kaunas d.	09 08 2010
Philaenus spumarius	Dvarčionys, Vilnius d.	08 24 2012
Philaenus spumarius	Mažieji Gulbinai, Vilnius d.	08 29 2012
Philaenus spumarius	Muniškiai, Kaunas d.	08 09 2012
Philaenus spumarius	Muniškiai, Kaunas d.	09 09 2010
Superfamily*: Membracoidea* Family: *Cicadellidae* Subfamily: *Agalliinae*		
Agallia sp.	Muniškiai, Kaunas d.	08 25 2010
Anaceratagallia ribauti	Muniškiai, Kaunas d.	08 26 2010
Subfamily*: Aphrodinae*		
Aphrodes sp.	Mažieji Gulbinai, Vilnius d.	08 31 2012
Aphrodes sp.	Muniškiai, Kaunas d.	08 08 2012
Aphrodes sp.	Muniškiai, Kaunas d.	08 27 2010
Subfamily: *Deltocephalinae*		
Arthaldeus pascuellus	Muniškiai, Kaunas d.	08 29 2010
Arthaldeus pascuellus	Muniškiai, Kaunas d.	08 02 2012
Arthaldeus striifrons	Muniškiai, Kaunas d.	08 30 2010
Arthaldeus striifrons	Muniškiai, Kaunas d.	08 02 2012
Athysanus sp.	Muniškiai, Kaunas d.	08 31 2010
Balclutha calamagrostis	Mažieji Gulbinai, Vilnius d.	08 23 2012
Balclutha punctata	Muniškiai, Kaunas d.	09 01 2010
Elymana sulphurella	Muniškiai, Kaunas d.	08 02 2012
Elymana sulphurella	Mažieji Gulbinai, Vilnius d.	08 23 2012
Errastunus ocellaris	Muniškiai, Kaunas d.	09 03 2010
Euscelis incisus	Muniškiai, Kaunas d.	08 11 2012

Euscelis incisus	Muniškiai, Kaunas d.	09 04 2010
Graphocraerus ventralis	Muniškiai, Kaunas d.	09 05 2010
Jassargus flori	Mažieji Gulbinai, Vilnius d.	08 23 2012
Macrosteles cristatus	Mažieji Gulbinai, Vilnius d.	08 23 2012
Macrosteles sexnotatus	Muniškiai, Kaunas d.	09 06 2010
Macrosteles sexnotatus	Dvarčionys, Vilnius d.	08 24 2012
Macrosteles sp.	Muniškiai, Kaunas d.	09 07 2010
Macrosteles sp.	Muniškiai, Kaunas d.	08 02 2012
Psammotettix alienus	Muniškiai, Kaunas d.	09 10 2010
Psammotettix confinis	Muniškiai, Kaunas d.	08 02 2012
Psammotettix confinis	Mažieji Gulbinai, Vilnius d.	08 23 2012
Psammotettix sp.	Mažieji Gulbinai, Vilnius d.	08 23 2012
Sagatus sp.	Muniškiai, Kaunas d.	09 11 2010
Verdanus abdominalis	Muniškiai, Kaunas d.	08 02 2012
Subfamily: *Cicadellinae*		
Cicadella viridis	Muniškiai, Kaunas d.	09 02 2010
Cicadella viridis	Mažieji Gulbinai, Vilnius d.	08 10 2012
Subfamily: *Typhlocibinae*		
Chlorita paolii	Muniškiai, Kaunas d.	08 02 2012
Chlorita paolii	Mažieji Gulbinai, Vilnius d.	08 23 2012
Edwardsiana sp.	Muniškiai, Kaunas d.	08 02 2012
Edwardsiana sp.	Žiežmariai, Kaišiadoriai d.	08 02 2012
Edwardsiana sp.	Dvarčionys, Vilnius d.	08 24 2012
Empoasca vitis	Dvarčionys, Vilnius d.	08 24 2012
Empoasca vitis	Mažieji Gulbinai, Vilnius d.	08 23 2012
Eupteryx atropunctata	Dvarčionys, Vilnius d.	08 24 2012
Notus flavipennis	Mažieji Gulbinai, Vilnius d.	08 23 2012
Zygina flammigera	Dvarčionys, Vilnius d.	08 24 2012
Superfamily: Fulgoroidea **Family: Delphacidae** **Subfamily: Delphacinae**		
Javesella pellucida	Muniškiai, Kaunas d.	08 02 2012
Javesella pellucida	Mažieji Gulbinai, Vilnius d.	08 23 2012
Xanthodelphax straminea	Muniškiai, Kaunas d.	08 02 2012
Subfamily: *Stenocraninae*		
Stenocranus minutus	Dvarčionys, Vilnius d.	08 24 2012
Stenocranus minutus	Mažieji Gulbinai, Vilnius d.	08 23 2012
Suborder: *Sternorrhyncha* **Superfamily*: Psylloidea*** **Family: *Psyllidae***		
Cacopsylla mali	Muniškiai, Kaunas d.	08 02 2012
Cacopsylla mali	Žiežmariai, Kaišiadoriai d.	08 02 2012

The collected plant samples belonged to 39 species. Three species were identified to genus level (Table 2).

Table 2. The list of collected and used for experiments plant species.

Plant species	**Locality**	**Symptoms**
Allium cepa L. – onion	Babtai, Kaunas d.	Phyllody
Alnus glutinosa (L.) Gaerth. – black alder	Tamošava, Trakai d.	Yellowing
Apium graveolens L. – celery	Tamošava, Trakai d.	Yellowing
Armoracia rusticana P.Gaertn., B. Mey et Scherb. – horseradish	Punia, Alytus d.	Yellow leaf borders and veins, declined bloom
Campanula sp. L. – bellflower.	Punia, Alytus d.	Phyllody
Cannabis sativa L. – hemp.	Tamošava, Trakai d.	Yellowing
Caragana arborescens Lam. – caragana	Merkinė, Varėna d.	Branching
Centaurium erythraea Rafn – common centaury	Muniškiai, Kaunas d.	Yellowing, branching, stunt
Cirsium arvense (L.) Scop. – creeping thistle	Muniškiai, Kaunas d.	Yellowing, branching, stunt
Cirsium arvense (L.) Scop. – creeping thistle	Balbieriškis, Prienai d.	White leafs
Convolvulus arvensis L. – field bindweed	Liškiava, Varėna d.	Yellowing
Dactylis glomerata L. – cock's-foot	Muniškiai, Kaunas d.	Yellowing, stunt
Echinops sphaerocephalus L. – great globe thistle	Merkinė, Varėna d.	Yellowing, branching
Epilobium sp. – willowherb	Muniškiai, Kaunas d.	Yellowing, branching
Euonymus europeatus L. – European spindle	Punia, Alytus d.	Yellow, bushy, wrinkled leafs on branch tips
Fragaria x ananassa Duchesne – strawberry	Dvarčionys, Vilnius d.	Phyllody, stunt
Malva sp. – mallow	Tamošava, Trakai d.	Yellowing
Melilotus officinalis (L.) Pall. – yellow sweet clover	Merkinė, Varėna d.	Yellowing
Oenotera rubricaulis Kleb. – dotted evening primrose	Liškiava, Varėna d.	Stem tip branching
Origanum vulgare L. – oregano	Babtai,	Phyllody

	Kaunas d.	
Picea abies (L.) Karst. – Norway spruce	Girionys, Kaunas d.	Yellowing, branching
Picris hieracioides L.–- hawkweed oxtongue	Muniškiai, Kaunas d.	Yellowing
Pinus sylvestris L. – Scots pine	Liškiava, Varėna d., Girionys, Kaunas d.	Witches‘ broom, small needles, decline
Populus tremula L. – European aspen	Tamošava, Trakai d.	Yellowing, witches‘ broom
Prunus avium (L.) L. – sweet cherry	Muniškiai, Kaunas d.	Yellows, decline
Prunus avium (L.) L. – sweet cherry	Babtai, Kaunas d.	Decline
Prunus cerasus L. – sour cherry	Babtai, Kaunas d.	Decline, late blossom, branching
Prunus domestica L. – plum	Muniškiai, Kaunas d.	Shoot proliferation, Yellowing
Quercus robur L. – oak	Liškiava, Varėna d.	Yellowing, small leafs
Ribes nigrum L. – black currant	Vilnius d.	Shoot malformation, and shortening
Rosa rugosa Thunb. – rugosa rose	Liškiava, Varėna d.	Branching
Rubus idaeus L. – raspberry	Vilnius d.	Yellowing
Scorzonera hispanica L. – black salsify	Tamošava, Trakai d.	Yellowing
Syringa vulgaris L. – common lilac	Tamošava, Trakai d.	Decline, yellows
Stellaria media (L.) Vill. – chickweed	Vilnius d.	Phyllody
Taraxacum officinale F. H. Wigg – common dandelion	Muniškiai, Kaunas d.	Yellowing, phyllody, small leafs
Thalictrum minus L. – meadow rue	Merkinė, Varėna d.	Yellowing
Trifolium pratense L. – red clover	Muniškiai, Kaunas d.	Phyllody
Trifolium repens L. – white clover	Babtai, Kaunas d.	Stunt, phyllody
Valeriana officinalis L. – garden valerian	Babtai, Kaunas d.	Yellowing
Vincetoxicum luteum Woffrugg. Et	Liškiava,	Yellowing

Link. – swallow-wort	Varėna d.	

Collected for the study, but not investigated insect species of possible vectors

We identified during this research 35 insect species (eight to genus level) from *Hemiptera* order (Table 1). Phytoplasmas detected commonly in Lithuania were identified in 10 insect species and 1 unidentified species from *Aphrodes* genus. Other insect samples of 24 insect species_were left for future research. Some of the insects already are known from literature as possible phytoplasma vectors.

Balclutha punctata Fabricius 1775 is known as a vector of mulberry dwarf (16SrI-B phytoplasma subgroup) (Han, 2012) and four other phytoplasma subgroups in Aliaska (Pantoja et al., 2009). Insects of *Balclutha* genus are possible vectors of almond witches' broom (16SrIX phytoplasma subgroup) (Dakhil et al., 2011) and stolbur (16SrXII-A phytoplasma subgroup) (Riedle-Bauer et al., 2006) disease agents.

Phytoplasmas of stolbur (Riedle-Bauer et al., 2006) and 16SrI-F subgroup (Orságová et al., 2011) were detected in samples of *Errastunus ocellaris* (Fallen 1806) species.

Javesella pellucida (Fabricius 1794) is possible vector of 16SrI-C and 16SrI-F phytoplasma subgroups (Orságová et al., 2011). Additionally, it can transmit OSDV (Oat Sterile Dwarf Virus), MRDV (Maize Rough Dwarf Virus) and EWSMV (European Wheat Striate Mosaic Virus) viruses (Kunz et al., 2010).

Macrosteles cristatus (Ribaut 1927) insects are mentioned as vectors of clover phyllody (16SrI-C phytoplasma subgroup), clover stunt (16SrIII-B phytoplasma subgroup) (Weintraub, Beanland, 2006) and stolbur (16SrXII-A phytoplasma subgroup) (Březíková et al., 2007).

Leafhopper species *P. alienus, P. confinis* from the *Psammotettix* genus, are known to transmit stolbur phytoplasmas in Europe (Fos et al., 1992). Phytoplasmas of 16SrI-A and 16SrI-C subgroups detected in *P. confinis* insects (Drobnjaković et al., 2011), therefore, we can assume that they are possible vectors of these phytoplasma subgroups in Lithuania. *P. alienus* species is detected to transmit BMWR (Band Mosaic of Wheat and Rye) and WDV (Wheat Dwarf Virus) viruses (Kunz et al., 2010).

16SrI phytoplasma group

Phytoplasmas of this group are most abundant in Lithuania. During this research in plant and insect samples, we detected phytoplasmas belonging to 16SrI-A, 16SrI-B and 16SrI-C subgroups.

16SrI-A phytoplasma subgroup

Symptomatic plant and insect samples were collected in Kaunas and Vilnius districts.

Extracted total DNA was used as template for nested PCR with universal primer pairs P1A/16S-Sr (Lee et al., 2003; Lee et al., 2006) and R16F2n/R16R2n (Lee et al., 2006). Obtained 1,2 kbp size rDNA fragment confirmed phytoplasmal infection in *Stenocranus minutus* insects, Norway spruce (*Picea abies* (L.) H.Karst.) and strawberry (*Fragaria* x *ananassa* Duchesne) plants. Phytoplasma after RFLP analysis was appointed to 16SrI-A subgroup (tomato big bud) according to classification scheme (Lee et al., 1998).

16S rRNA and ribosomal protein (*rpl22*/*rpls3*) gene fragments acquired from phytoplasma detected in spruce were cloned and sent for sequencing. Received sequences were compared using BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlBlastSear&LINK_LOC=blasthome) with sequences of known phytoplasmas given in NCBI database. Comparison showed that the gene sequences of detected phytoplasma are most similar to the sequences of phytoplasmas from 16SrI-A subgroup. Seqeunces also were analysed with *i*PhyClassifier (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) online tool. This tool confirmed relationship of given phytoplasma to 16SrI-A subgroup and created virtual RFLP (Fig. 1) profile, which was compared with profiles of classification schemes provided by Zhao et al. (2009) and Lee et al. (1998).

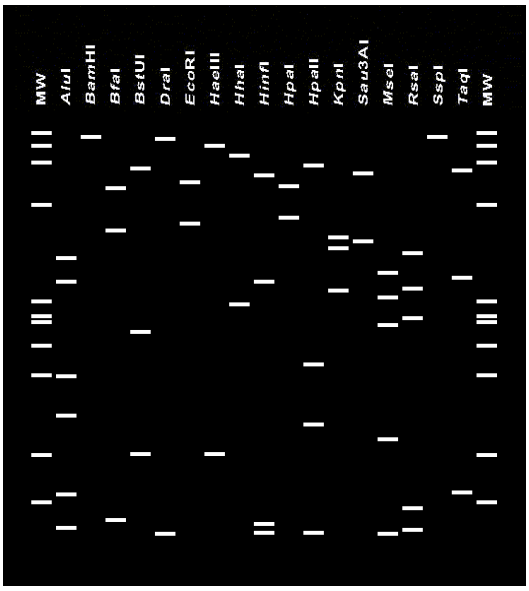


Fig. 1. RFLP analysis made by *i*PhyClassifier (Zhao et al., 2009) of 16S rRNA gene sequence from *S. minutus*, classified in subgroup 16SrI-A (MW- marker phiX174 DNA/*Bsu*RI (*Hae*III) fragment sizes: 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118, 72 bp)

Discussion: 16SrI-A phytoplasma subgroup belongs to the biggest and worldwide-detected aster yellows phytoplama group.

Other researchers found phytoplasmas in various plants in Lithuania. They found them in such cultivated plants as carrots (*Daucus sativus* Röhl.), oats (*Avena sativa* L.) and onions (*Allium cepa* L.) (Jomantiene et al., 2002b; Valiunas, 2003; Urbanaviciene et al., 2007; Jomantiene et al., 2010) and ornamental plants as monkshood (*Aconitum napellus* L.), wavyleaf sea-lavender (*Limonium sinuatum* (L.) Mill.), hyacinth (*Hyacinthus orientalis* L.), daisy (*Bellis perennis* L.), firewheel (*Gaillardia pulchella* Foug.), black-eyed Susan (*Rudbeckia hirta* L.), sneezeweed (*Helenium autumnale* L.), Japanese pachysandra (*Pachysandra terminalis* (Siebold & Zucc.)), bleeding heart (*Dicentra formosa* (Haw.) Walp.), gladiolus (*Gladiolus* sp. L.), doubtful knight's spur (*Consolida ajacis* (L.) Schur), Majorcan hellebore (*Helleborus lividus* Aiton), Chinese globeflower (*Trollius chinensis* Bunge), avens (*Geum coccineum* Lindl.) (Valiunas, 2003; Samuitienė et al., 2007).

We assume that *S. minutus* species is a possible vector of 16SrI-A subgroup phytoplasmas in strawberries in Lithuania, because these insects and the plants infected with these phytoplasmas were found in the same location. Acording to the literature this insect species feeds mainly on *Dactylis* genus plants. We suppose that this species can accidentally acquire these bacteria from strawberries and possibly transmit them.

We found no data on phytoplasmas detected or transmitted by these insects in our region and worldwide. Our data expand the range of natural plant hosts and possible vectors of 16SrI-A subgroup phytoplasmas in Lithuania.

16SrI-A subgroup phytoplasmas were found in spruce and strawberries for the first time in Lithuania. Phytoplasmas of this subgroup were also found in blue spruce in Poland (*Picea pungens* Engelm.) (Kaminska et al., 2011). In strawberries, additionally to

16SrI-A subgroup 16SrXII-E subgroup phytoplasmas were also found in Lithuania (Valiunas et al., 2006).

16SrI-B phytoplasma subgroup

Insect and plant samples infected with 16SrI-B subgroup phytoplasmas were collected in the orchards and meadows of Kaišiadoriai, Kaunas and Vilnius districts. Phytoplasmal infections were identified after amplification of 16S rDNA 1,2 kbp size fragment. Infections were discovered in *Aphrophora alni*, *Aphrodes* sp., *Cacopsylla mali*, *Cicadella viridis*, *Lepyronia coleoptrata*, *Philaenus spumarius* insect samples and in cherry plum (*Prunus cerasifera*), apple tree (*Malus domestica*), hawkweed oxtongue (*Picris hieracioides*), red clover (*Trifolium pratense*), plum (*Prunus domestica*), common centaury (*Centaurium erythraea*), cock's-foot (*Dactylis glomerata*), black currant (*Ribes nigrum*), black salsify (*Scorzonera hispanica*), chickweed (*Stellaria media*) plant samples. RFLP analysis of amplified 16S rDNA fragments confirmed relatedness to 16SrI group B phytoplasma subgroup.

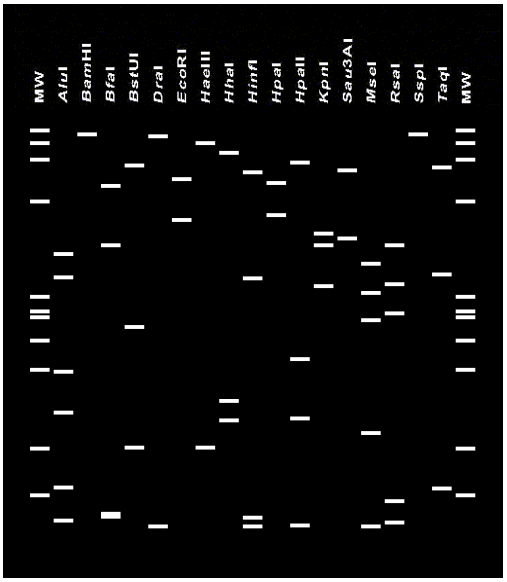


Fig. 2. RFLP analysis made by *i*PhyClassifier (Zhao et al., 2009) of 16S rRNA gene sequence from strain AY1A112, GenBank No. KC283215, classified in subgroup 16SrI-B (MW- marker phiX174 DNA/*Bsu*RI (*Hae*III) fragment sizes: 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118, 72 bp)

16S rDNA and ribosomal protein gene fragments were amplified from cherry plum and *C. viridis*, *Aphrodes* sp. insect samples were cloned to *E. coli*. After clone selection by PCR and RFLP methods, the selected clones were sent to sequencing companies. The obtained sequences were compared with the deposited in NCBI database sequences of other phytoplasmas. The derived data confirmed that the detected phytoplasma belongs to 16SrI-B/rpI-B phytoplasma subgroup. Analysis of sequence with *i*PhyClassifier tool and acquired virtual RFLP pattern (Fig. 2) supported our findings.

Discussion: Phytoplasmas of 16SrI-B subgroup form the biggest cluster of strains in 16SrI group. This subgroup is widely spread through the world in herbaceous and woody plants (Lee et al., 1998; Lee et al., 2004).

To date these phytoplasmas have been found by other researchers in Lithuania in alfalfa (*Medicago sativa* L.) (Jomantiene et al., 2000a), willow (*Salix* sp.), caragana (*Caragana arborescens* Lam.), sweet cherry (*Prunus avium*), sour cherry (*Prunus cerasus*), valerian (*Valeriana officinalis* L.), pear (*Pyrus communis* L.) (Valiunas, 2003; Valiunas et al., 2009). Urbanavičienė et al. (2005; 2006; 2007) identified 16SrI-B subgroup phytoplasmas in important for agriculture plants such as oat (*Avena sativa*) and barley (*Hordeum vulgare* L.). Samuitienė et al. (2007) detected bacteria of this subgroup in many of ornamental plants such as burning love (*Lychnis chalcedonica* L), moss

campion (*Silene orientalis* Hort.), showy stonecrop (*Sedum spectabile* Boreau), delphinium (*Delphinium cultorum*), butterfly flower (*Schizanthus pinnatus* Ruiz et Pav.).

A. alni species is considered as a possible vector of apple proliferation phytoplasma (Seemüller et al., 1990), although to date these data have not been confirmed.

Aphrodes bicincta and *A. makarovi* species are hard to distinguish. *A. bicincta* species is a confirmed vector of 16SrI-A, 16SrI-C, 16SrIII-B, 16SrIV and 16SrXII-A (stolbur) subgroup phytoplasmas (Nielson, 1968; Brčák, 1979; Lee et al., 1998; Weintraub, Beanland, 2006).

Apple proliferation phytoplasmas were detected in *C. mali* samples in Northern Italy (Baric et al., 2010). Species of the *Cacopsylla* genus: *C. melanoneura*, *C. picta*, *C. pruni*, *C. pyri*, *C. pyricola*, *C. pyrisuga* are main vectors of apple proliferation (16SrX-A subgroup phytoplasma), European stonefruit yellows (16SrX-B) and pear decline (16SrX-C) phytoplasmas (Weintraub, Beanland, 2006).

Mazzoni et al. (2001) mentions *C. viridis* as a possible vector of yellows diseases.

L. coleoptrata is a possible vector of apple proliferation (16SrX-A subgroup phytoplasma) phytoplasmas (Seemüller, 1990).

In *P. spumarius* insects other researchers found phytoplasmas of 16SrI-F (Orságová et al., 2011) and 16SrIII-A (Landi et al., 2007) subgroups, and 16SrV (Matteoni, Sinclair, 1988) and 16SrX groups (Seemüller, 1990).

According to our data, 16SrI-B subgroup phytoplasmas in *A. alni*, *Aphrodes* sp., *C. mali*, *C. viridis*, *P. spumarius*, *L. coleoptrata* insects were found for the first time in Lithuania and worldwide. These data widen the variety of phytoplasmas detected in these leafhoppers.

We suppose that *A. alni*, *Aphrodes* sp., *C. mali*, *C. viridis*, *P. spumarius*, *L. coleoptrata* species can possibly transmit 16SrI-B subgroup phytoplasmas in Lithuania and our region.

A. alni, *Aphrodes* sp., *C. viridis*, *P. spumarius*, *L. coleoptrata* species in literature are mentioned as polyphagous insects that can successfully feed on herbaceous and on woody plants. According to the obtained data, we assume that these species can transmit 16SrI-B subgroup phytoplasmas among plants in which they were found during our and other researches in Lithuania.

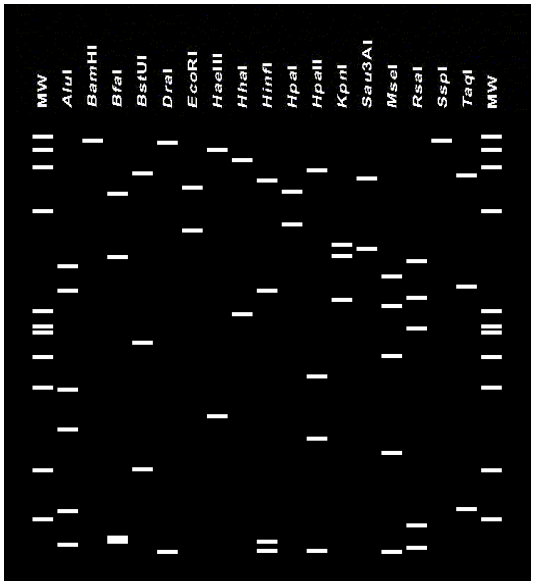
A. alni, *C. viridis*, *L. coleoptrata*, *P. spumarius* belong to the group of *Auchenorrhyncha* insects that feed exclusively on xylem (Purcell, 2008; Weintraub, Wilson, 2010). During our research, we found phytoplasmas in the samples of these species. Such results are in agreement with Crews et al. (1998) findings, that these insects by positioning their stylets can puncture phloem cells and ingest their contents. Rosa et al. (2014) also determined *Lepyronia quadrangularis* Say as phytoplasma vector. Because of these data, we should look at xylem feeding insects identified in this study not only as hosts, but also as possible vectors of phytoplasmas.

C. mali is monophagous species that feeds mainly on plants of *Malus* genus. We suppose that this species is a possible vector of 16SrI-B subgroup phytoplasmas among apple trees in Lithuania and neighbouring countries. This insect is known to transmit apple proliferation phytoplasmas in Europe. It is important to control and survey the distribution of this species in our country and the region.

Phytoplasmas of 16SrI-C subgroup were found for the first time during our research in common centaury, black salsify and chickweed plants in Lithuania.

16SrI-C phytoplasma subgroup

Symptomatic plant and insect samples were collected in the meadow and orchard of Kaunas district. 16S rDNA 1,2 kbp size gene fragments were amplified from *Anaceratagallia ribauti, Aphrodes* sp., *Arthaldeus striifrons, Euscelis incisus, L. coleoptrata*, *Macrosteles sexnotatus, P. spumarius*, cherry plum (*Prunus cerasifera*), white clover (*Trifolium repens*) and creeping thistle (*Cirsium arvense*) total DNA samples. Amplicons used in RFLP analysis and derived restriction profiles (Fig. 3) were compared with classification scheme profiles. Detected phytoplasmas were classified in 16SrI group C subgroup.



Note. The *i*PhyClassifier figure shows results from analysis of the nucleotide sequence from just one of two rRNA operons of a given phytoplasma.

Fig. 3. RFLP analysis made by *i*PhyClassifier (Zhao et al., 2009) of 16S rRNA gene sequence from strain CPhM15-7, operon *rrnA*, GenBank No. KC283214, classified in subgroup 16SrI-C (MW- marker phiX174 DNA/*Bsu*RI (*Hae*III) fragment sizes: 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118, 72 bp)

Performed transmission trials confirmed *E. incisus* capability to transmit 16SrI-C subgroup phytoplasmas in white clover (*T. repens*) plants in Lithuania.

Marker gene fragments acquired from *A. ribauti, Aphrodes* sp., *E. incisus, M. sexnotatus and P. spumarius* were cloned and sequenced. Analysis of sequences by BLAST and *i*Phyclassifier tools revealed that sequences of detected phytoplasmas are most similar to sequences of 16SrI-C subgroup phytoplasmas and showed their relationships to heterogenic ribosomal operons *rrnA* or *rrnB* of this subgroup.

Sequences are deposited in Genebank database under accession numbers: KC283213, KC283214, KC283218.

Discussion: Phytoplasmas of this subgroup distributed in the world were found in monocotyledonous and dicotyledonous plants (Lee et al., 2004).

16SrI-C subgroup phytoplasmas were found in white clover (*Trifolium repens* L.) (Staniulis et al., 2000), smooth meadow-grass (*Poa pratensis* L.), common gaillardia (*Gaillardia aristata* Pursh) (Valiunas et al., 2007), tall fescue (*Festuca arundinacea* Schreb.) (Urbanavičienė et al., 2006).

A. ribauti is confirmed as a vector of stolbur phytoplasma (16SrXII-A phytoplasma subgroup) (Riedle-Bauer et al., 2008; Drobnjaković et al., 2011).

M. sexnotatus species is a vector of hyacint „Lissers“ phytoplasma and 16SrI-A, 16SrI-B, 16SrXII subgroup phytoplasmas (Battle, 2000; Weintraub, Beanland, 2006; Duduk et al., 2008).

E. incisus insects transmit 16SrI-B, 16SrI-C, 16SrVI and 16SrXII-A subgroup phytoplasmas (Brčák, 1979; Weintraub, Beanland, 2006). Additionally, in these insects were detected phytoplasmas of 16SrI-F and 16SrIII-B subgoups (Orságová et al., 2011).

16SrI-C subgroup phytoplasmas were detected for the first time in *A. striifrons, L. coleoptrata, M. sexnotatus,* and *P. spumarius* insect samples in Lithuania and worldwide. Our data expand the knowledge about phytoplasmas found in these insects.

We found no data about phytoplasmas detected or transmitted by *A. striifrons* species in other regions. Our findings widen the circle of possible 16SrI-C subgroup phytoplasma vectors.

16SrI-C subgroup phytoplasmas in *Aphrodes* sp., *E. incisus* and *M. sexnotatus* insects have been found in other regions, but in Lithuania, this subgroup in these species was found for the first time.

L. coleoptrata, M. sexnotatus, P. spumarius are polyphagous and *E. incisus* is olygophagous species (Nickel, Remane, 2002). The variety of plant hosts of these species is very wide and overlapping. It is known that these insects prefer to feed on plants of *Poaceae* and *Fabaceae* families (Ossiannilsson, 1981; 1983; Nickel, Remane, 2002). We assume that they transmit phytoplasmas among plants of these families in Lithuania.

A. striifrons is a monophagous species and mainly feeds on plants of *Festuca* family (Nickel, Remane, 2002). We conclude that this species has a possibility to transmit phytoplasmas of 16SrI-C subgroup among tall fescue (*F. arundinacea*) plants in Lithuania.

M. sexnotatus is a confirmed vector of stolbur phytoplasmas in Europe. As potential vector, it poses a threat to Lithuanian agriculture and it is important to keep it under survey.

Phylogenetic trees of 16SrI group phytoplasmas

Phylogenetic trees were constructed using phytoplasmas 16S rRNA, *rpl22*/*rps3*, and *secY* gene sequences received during this research and sequences acquired from NCBI database.

Tree of 16SrI group (Fig. 4) was created using sequences of 16S rDNA of 46 phytoplasmas and one acholeplasma ((*Acholeplasma palmae*). It showed relationships among known phytoplasmas and phytoplasma strains of 16SrI-A, 16SrI-B and 16SrI-C subgroups detected in our research. The order of the branches confirmed classification status of the identified 16SrI group phytoplasmas.

16SrI-A subgroup phytoplasma detected in spruce was most related to phytoplasma strains of 16SrI-A subgroup detected earlier in Lithuania (oat proliferation, plantago virescence, gaillardia yellows) and in other countries (Texas potato purple top, carrot phytoplasma sp.).

Phytoplasma of 16SrI-B subgroup was detected in *Aphrodes* sp. most related to strain of 16SrI-B subgroup phytoplasma detected in *Scorzonera hispanica* in Lithuania, and to strain of 16SrI-O subgroup phytoplasma detected in the United States of America.

Arrangement of 16SrI-C subgroup phytoplama strains, detected in *Aphrodes* sp., *E. incisus*, *M. sexnotatus*, and *P spumarius*, on phylogenetic tree showed that they are nearly related to earlier in Lithuania detected gaillardia yellows, poa stunt and cirsium yellows phytoplasmas. The tree also showed ralationship of detected strain 16S rRNA gene sequences to *rrnA* and *rrnB* ribosomal operons.

Phylogenetic analysis and phylogenetic trees were constructed using helper marker genes: ribosomal protein (*rpl22*/*rps3*) and secretion system (*secY*) gene sequences.

Phylogenetic tree of phytoplasma ribosomal protein gene sequences was constructed using *rpl22*/*rps3* gene sequences of 28 rpI group phytoplasmas and one acholeplasma (*Acholeplasma laidlawii*). We included in our analysis also *rp* gene of phytoplasma detected in larch (*Larix* sp.) samples from the Ukraine. Derived tree was in consent with 16S tree and confirmed relationship of the detected phytoplasmas to 16SrI-A, -B, -C subgroups. Phytoplasma of rpI-B subgroup identified in the samples from the Ukraine is less similar to Lithuanian phytoplasmas, and formed separate branch.

Phylogenetic tree based on secretion system genes was constructed using sequences of *secY* gene of 21 phytoplasmas from secY-I group, and one acholeplazma (*Acholeplasma laidlawii*). Tree branching pattern showed that sequences of *secY* gene acquired from *Aphrodes* sp., *E. incisus*, *A. ribauti* insect samples have the highest resemblance with aster yellows phytoplasmas KVG, CPh, and KVE strains, which belong to secY-I-C subgroup.

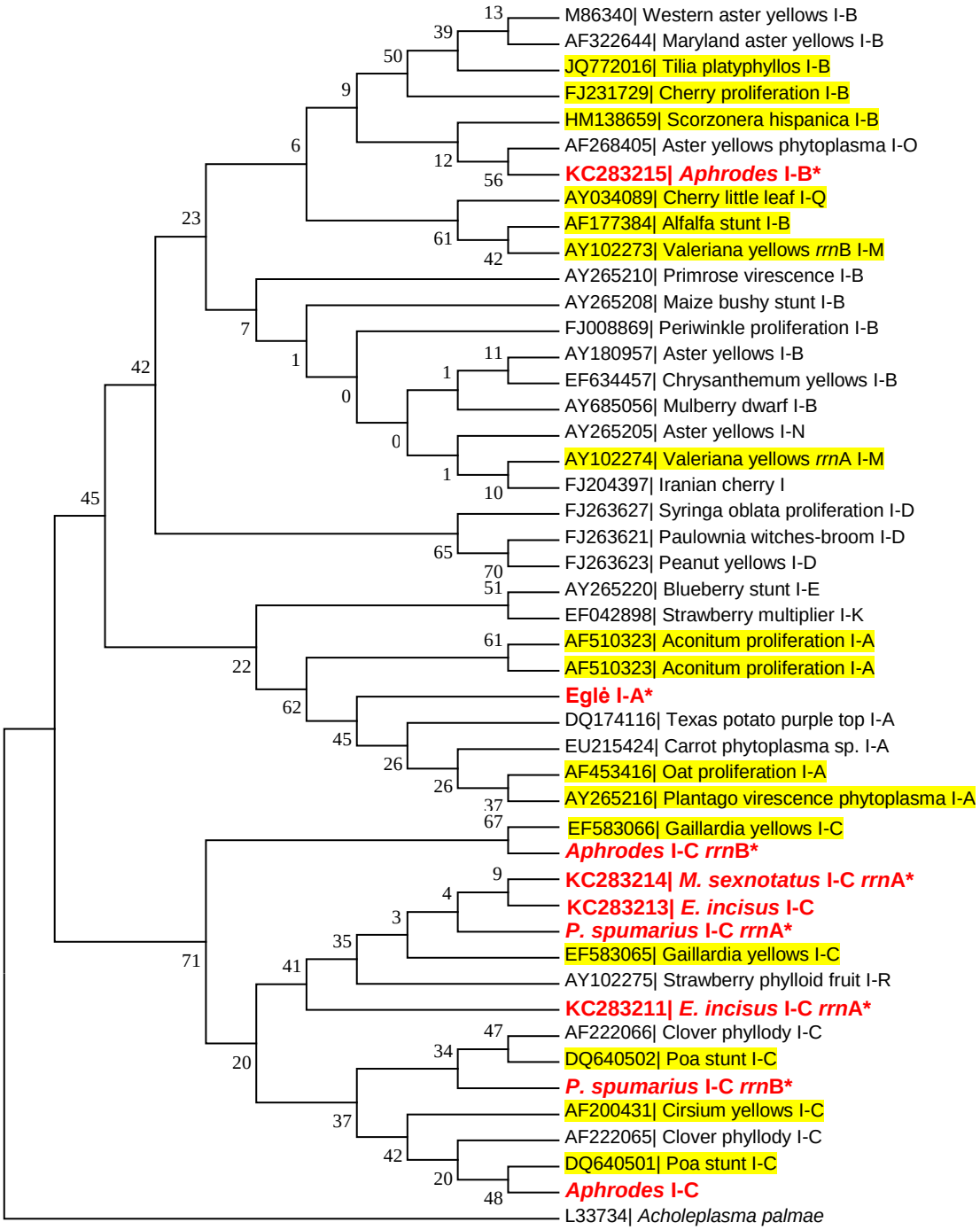


Fig. 4. Phylogenetic tree, comparing the 16S rRNA sequences of detected 16SrI-A, 16Sr-B and 16Sr-C subgroup phytoplasmas with 43 phytoplasmas of 16SrI group and 1 acholeplasma (*Acholeplasma palmae*) as an out-group constructed by the Neighbor-Joining method with Mega 5 software. Number in front – Gene bank accession number. Roman numbers refer to phytoplasma 16Sr RFIP groups, capital letters – subgroups. Numbers near branches show Bootstrap values. * – phytoplasma strain detected during research. *rrn*A, *rrn*B names of the heterogenic 16S rDNR phytoplasma operons. Highlighted – phytoplasma strains detected in Lithuania

16SrIII phytoplasma group

Phytoplasmas of this group are found less frequent in Lithuania. During our research, we detected phytoplasmas of 16SrIII-B, -P subgroups, and new 16SrIII-T subgroup. 16SrIII-B subgroup phytoplasma was identified in *A. ribauti* and red clover (*Trifolium pratense*); 16SrIII-P – in *Aphrodes* sp., *E. incisus* and dandelion; 16SrIII-T – in sour cherry.

16SrIII-B phytoplasma subgroup

Samples of insects and red clover plants with phylody, yellowing, small leave symptoms were collected in the meadows of Kaunas district.

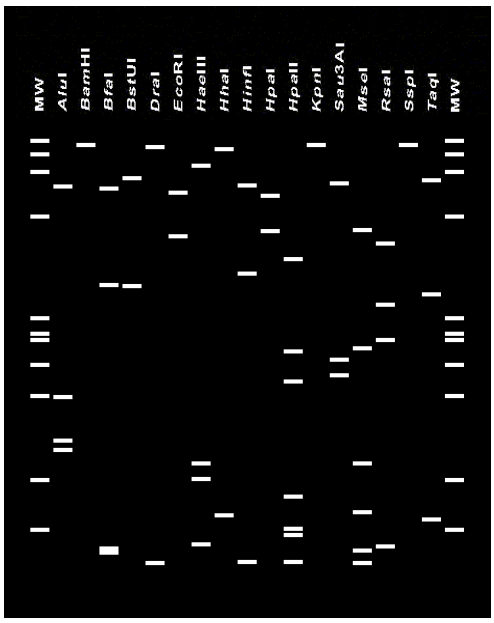


Fig. 5. RFLP analysis made by *i*PhyClassifier (Zhao et al., 2009) of 16S rRNA gene sequence from strain CYEA1, GenBank No. KC283217 classified in subgroup 16SrIII-B (MW- marker phiX174 DNA/*Bsu*RI (*Hae*III) fragment sizes: 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118, 72 bp)

Extracted total DNA was used as template in PCR reactions with universal phytoplasma primers. PCR's yielded 1,2 kbp size fragments, which were used in RFLP analysis. Comparison of the restriction profiles to classification scheme profiles allowed to identify the detected phytoplasma as a member of 16SrIII-B subgroup. Cloned to *E. coli* fragment was sequenced. Sequence is deposited in Gene bank under a.n.: KC283217. BLAST comparison of this sequence confirmed relationship to 16SrIII-B subgroup. *i*PhyClassifier group appointment and derived virtual RFLP profile (Fig. 5) affirmed the data.

Discussion: Phytoplasmas of 16SrIII-B subgroup have been detected in a variety of plants in various world regions (Jomantienė et al., 1998b; Lee et al., 1998; Seemuller et al., 1998; Postman et al., 2001; Montano et al., 2011).

Thus far these bacteria have been found in red clover (*Trifolium pratense* L.) (Staniulis et al., 2000), soybean (*Glycine max* (L.) Merr.) and firewheel (*Gaillardia pulchella* Foug.) (Jomantiene et al., 2000a, 2002a) in Lithuania.

We found out that 16SrIII-B subgroup phytoplasmas in *A. ribauti* insects were detected for the first time in Lithuania and worldwide. Our data expand the knowledge about these insects' abilities to transmit phytoplasmas.

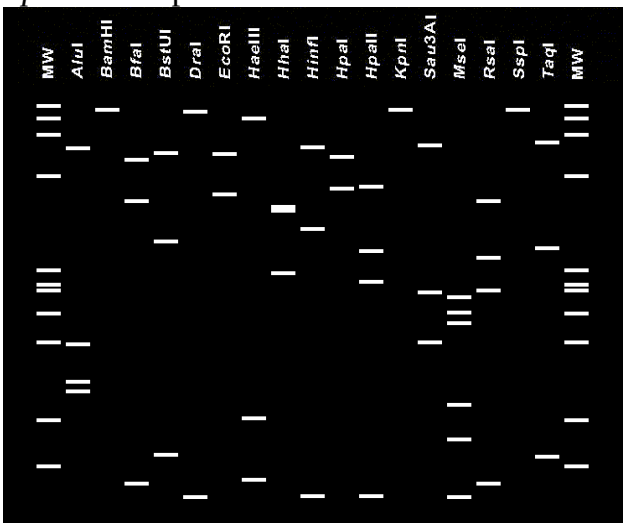
We suspect that this species is a possible vector of 16SrIII-B subgroup phytoplasmas among red clover plants.

16SrIII-P phytoplasma subgroup

Phytoplasma specific 1,2 kbp size 16S rDNA fragment was acquired from *Aphrodes* sp., *E. incisus* insects and dandelion (*T. officinale*) plant samples. The samples were collected in the meadow in Muniškiai (Kaunas district). Analysis of RFLP profiles appointed the detected phytoplasmas to 16SrIII-P subgroup. This subgroup is distinct

from other 16SrIII group phytoplasmas by profiles of *Hha*I and *Hae*III restriction endonucleases.

We cloned and sent for sequencing the fragment of 16S rRNA gene amplified from *Aphrodes* sp. and *E. incisus*. The received sequences we compared to known phytoplasma sequences in NCBI database. We used *i*PhyClassifier online tool to classify phytoplasmas and create virtual RFLP profiles (Fig. 6). Both methods revealed that cloned fragment is most similar to one of heterogenic ribosomal operons – to *rrnA* operon.



Note. The *i*PhyClassifier figure shows results from analysis of the nucleotide sequence from just one of two rRNA operons of a given phytoplasma.

Fig. 6. RFLP analysis made by *i*PhyClassifier (Zhao et al., 2009) of 16S rRNA gene sequence from strain DanVirA213, operon *rrnA*, GenBank No. KC283216, classified in subgroup 16SrIII-P (MW- marker phiX174 DNA/*Bsu*RI (*Hae*III) fragment sizes: 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118, 72 bp)

Discussion: Phytoplasma of this subgroup was found for the first time by Jomantiene and colleagues. According to symptoms caused in inflorescence, this phytoplasma received DanVir (dandelion virescence) name (Jomantienė et al., 2002).

We detected the phytoplasma of this subgroup in *Aphrodes* sp. and *E. incisus* insect samples. To date there were no data on vectors of these microorganisms. We suppose that these insects are potential vectors of 16SrIII-P subgroup phytoplasmas.

Unidentified species of the genus *Aphrodes* was found to harbour phytoplasmas of 16SrI-B, 16SrI-C and 16SrIII-P subgroups. These insects are polyphagous and have much wider spectre of plant hosts and transmitted phytoplasmas than monophagous species. In order to minimize phytoplasma infection chances and effects, it is important to control distribution and quantity of these insects in cultivated fields.

E. incisus species insects are polyphagous and transmit stolbur phytoplasmas in other regions. These insect qualities and gradual shift of the geographical pathogen distribution areas due to climatic changes pose a danger for Lithuanian agriculture.

16SrIII-T phytoplasma subgroup

Phytoplasmas of this subgroup were detected in sour cherry (*Prunus cerasus*) plant samples collected in the orchard of Kaunas district. We did not found phytoplasmas of this subgroup in the collected insect samples.

Total DNA extracted from sour cherry was used in nested PCR with P1A/16S-Sr (Lee et al., 2003; Lee et al., 2006) and R16F2n/R16R2n (Lee et al., 2006) primer pairs. Comparison of amplified 1200 bp size 16S rDNA fragments RFLP profiles with classification scheme profiles (Lee et al., 1998; Marcone et al., 200l; Jomantienė et al., 2002a) revealed that the detected phytoplasma belongs to a completely new 16SrIII-T subgroup (named as ChD, cherry decline). This strain had different *Hinf*I restriction

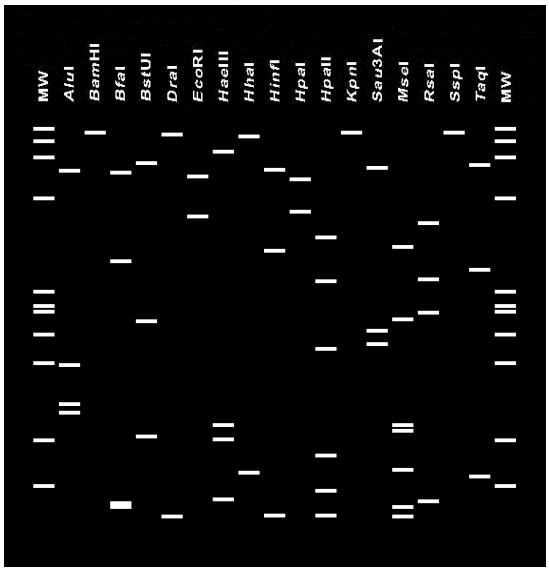


Fig. 7. RFLP analysis made by *i*PhyClassifier (Zhao et al., 2009) of 16S rRNA gene sequence from strain ChD (GenBank no. FJ231728) classified in subgroup 16SrIII-T (MW-marker phiX174 DNA/*Bsu*RI (*Hae*III) fragment sizes: 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118, 72 bp)

profile, which differentiated it from other 16SrIII group phytoplasmas. 16S rDNA fragment of this phytoplasma was cloned and sequenced. The received sequence was compared to sequences of known phytoplasmas deposited in NCBI database and was used to create virtual RFLP image (Fig. 7) with *i*PhyClassifier tool. The obtained data confirmed that detected phytoplasma should be separated in to new subgroup. The research data were published in the paper (Valiunas et al., 2009).

Discussion: Phytoplasma of new 16SrIII-T subgroup was detected for the first time in Lithuania. Our finding broadens variety of detected 16SrIII group phytoplasmas in Lithuania and worldwide.

Phytoplasmas of 16SrI-Q subgroup have been found in sour cherries in Lithuania (Valiunas et al., 2009a). Phytoplasmas in sour cherries have also been found in Hungary (16SrX-B; 16SrI-B) and other countries (Varga et al., 2001; Valiunas et al., 2009).

It is not known who could transmit phytoplasmas of this subgroup in Lithuania. We suppose that it could be the same insects that transmit 16SrIII-P subgroup phytoplasmas (*E. incisus*, *Aphrodes* sp.), because these insects are polyphagous and possibly transmit 16SrIII-P subgroup phytoplasmas that are related to 16SrIII-T subgroup phytoplasmas.

Phylogenetic trees of 16SrIII group phytoplasmas

16S rRNA gene phylogenetic tree (Fig. 8) was constructed using 16S rDNA sequences of 34 phytoplasmas from 16SrIII group and one acholeplasma (*Acholeplasma palmae*). The tree shows phylogenetic relatedness of the detected 16SrIII-B, -P and -T subgroup phytoplasmas in Lithuania to other 16SrIII group phytoplasmas. It confirms that detected in sour cherry phytoplasma belongs to the new 16SrIII-T subgroup. The high similarity of the named as ChD (cherry decline) 16S rRNA gene sequence to that of DanVir *rrnB* suggests the possibility that ChD and DanVir may belong to a single phytoplasma species and that dandelion is possibly an alternate host of ChD phytoplasma.

Phytoplasma of 16SrIII-B subgroup detected in *A. ribauti* is most related to gaillardia phyllody phytoplasma (Jomantienė et al., 2002) found in Lithuania, and branches out from 16SrIII-B phytoplasmas found in other regions.

16S rDNA sequences of phytoplasmas found in *E. incisus* and *Aphrodes* sp. are similar to the sequences of *rrnA* operon of dandelion virescence phytoplasmas (16SrIII-P subgroup), but are different from 16SrIII-P subgroup phytoplasmas with *rrnB* operon.

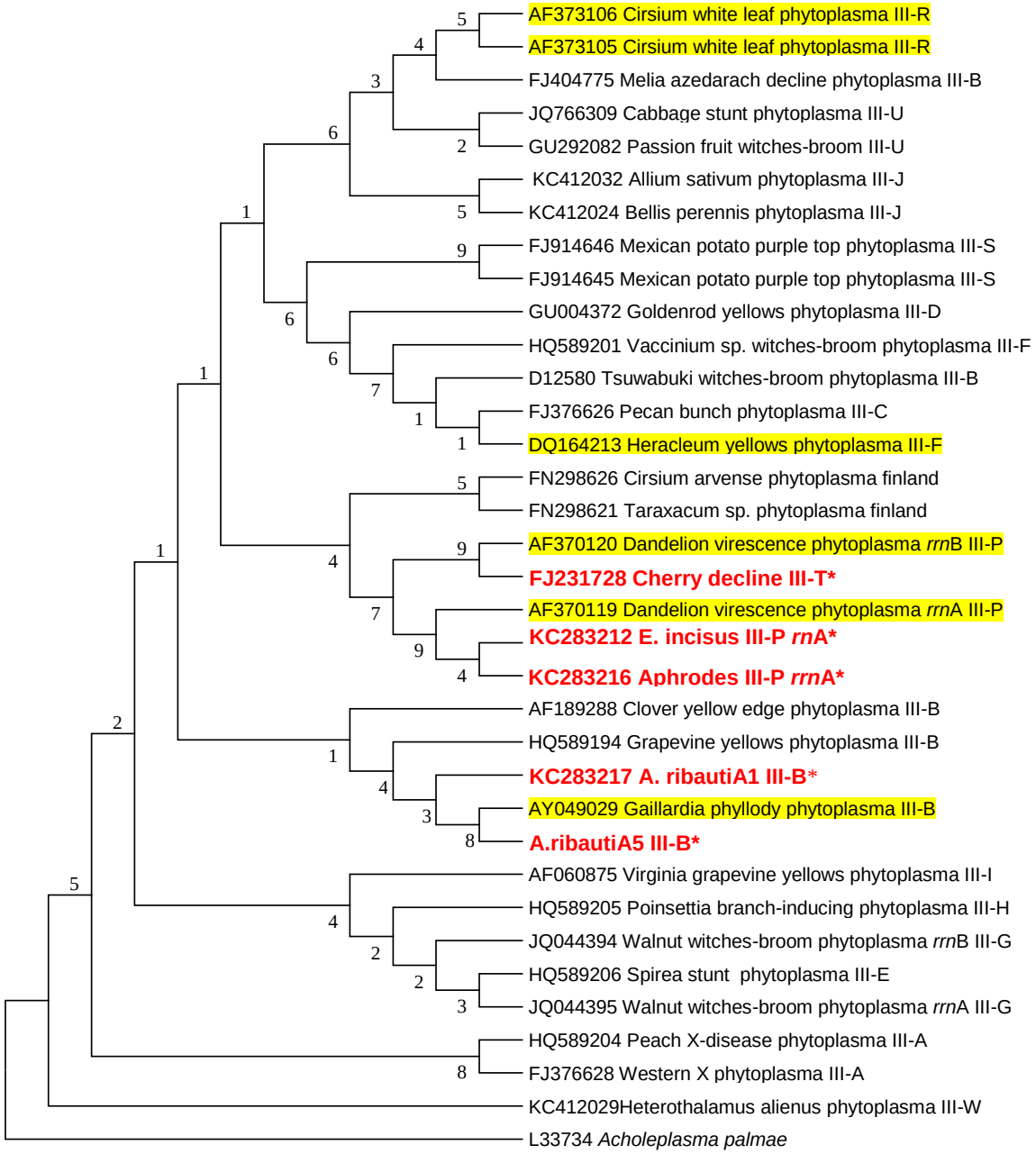


Fig. 8. Phylogenetic tree, comparing the 16S rRNA sequences of detected 16SrIII-B, 16SrIII-P, 16SrIII-T subgroup phytoplasmas with 31 phytoplasmas of 16SrIII group and 1 acholeplasma (*Acholeplasma palmae*) as an out-group constructed by the Neighbor-Joining method with Mega 5 software. Number in front – Gene bank accession number. Roman numbers refer to phytoplasma 16Sr RFIP groups, capital letters – subgroups. Numbers near branches show Bootstrap values. * – phytoplasma strain detected during research. *rrn*A, *rrn*B names of the heterogenic 16S rDNR phytoplasma operons. Highlighted – phytoplasma strains detected in Lithuania

16SrXXI-A phytoplasma subgroup

Samples of needles and shoots collected from pines with witche's broom, short needle, needle yellowing, dwarfism symptoms from Liškiava, Girionys, Neringa localities in Lithuania. We also collected samples of insects, though we did not detect phytoplasmas of this subgroup in them.

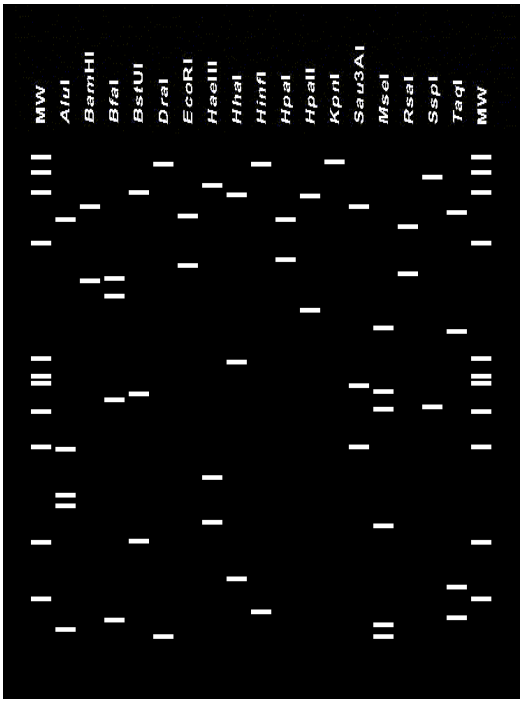


Fig. 9. RFLP analysis made by *i*PhyClassifier (Zhao et al., 2009) of 16S rRNA gene sequence from strain PineBT (GenBank no. GU289676) classified in subgroup 16SrXXI-A (MW- marker phiX174 DNA/*Bsu*RI (*Hae*III) fragment sizes: 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118, 72 bp)

Extracted total DNA was used in nested PCR reaction with universal phytoplasma primers. Amplified 16S rDNA fragments were used in RFLP analysis. Comparison of RFLP the profiles with classification scheme appointed detected phytoplasma to 16SrXXI-A subgroup. We cloned acquired fragments to *E. coli* and sent the selected clones for sequencing. The received sequence we compared with sequences of phytoplasmas deposited in NCBI database and used for creation of virtual RFLP image (Fig. 9) with *i*PhyClassifier tool. Strain of the detected phytoplasma received the name of pine bunchy top phytoplasma (PineBT). Tentative results were presented at the conference (Valiunas et al., 2010).

Discussion: 16SrXXI-A subgroup phytoplasmas were detected only in gymnosperm plants from *Pinaceae* family: *Pinus halepensis*, *Pinus sylvestris*, *Abies procera*, *Picea pungens*, *Pinus banksiana*, *P. mugo*, *P. nigra*, *P. tabuliformis*, *Taxodium distichum* var. *imbricarium*, *Tsuga canadensis* (Schneider et al., 2005; Sliwa et al., 2008; Huang et al., 2011; Kaminska, Berniak, 2011; Kaminska et al., 2011).

These phytoplasmas can be transmitted by grafting. Natural vectors of these phytoplasmas are still unknown.

Timber industry and export are important branches of Lithuanian economy. It is crucial to ascertain the ways and degree of distribution, and the impact of these pathogens on wood ecosystems.

Phylogenetic tree of 16SrXXI group phytoplasmas

Phylogenetic tree (Fig. 10) was created using 16S rDNA sequences of four 16SrXXI-A subgroup phytoplasmas and one acholeplasma (*Acholeplasma palmae*). Pattern of the branches shows that Lithuanian strain is more related to phytoplasma strains detected in Germany and Poland and divergent from Spanish strain.

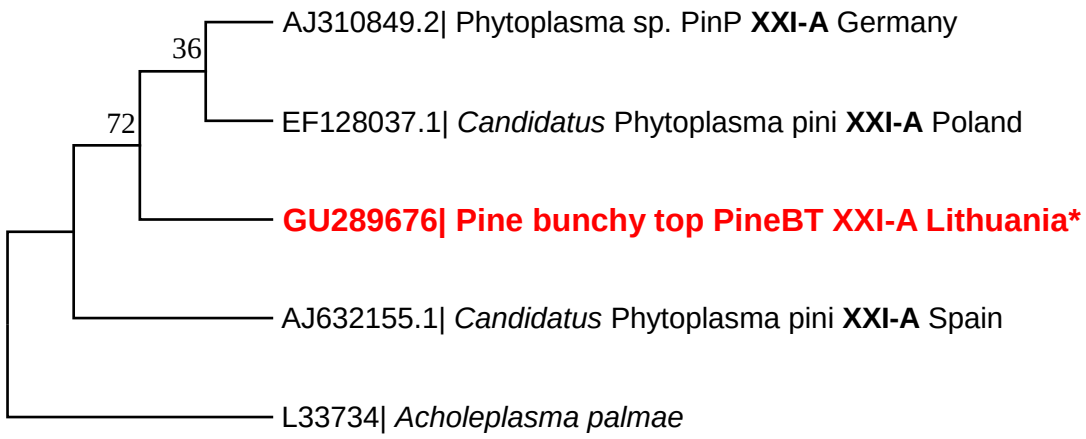


Fig. 10. Phylogenetic tree of 16SrXXI-A subgroup phytoplasmas. Number in front – Gene bank accession number. Roman numbers refer to phytoplasma 16Sr RFIP groups, capital letters – subgroups. Numbers near branches show Bootstrap values. * – phytoplasma strain detected during the research.

Summary of the results

During this research, phytoplasmas of 16SrI-A, B, C, 16SrIII-B, P, T and 16SrXXI-A subgroups were detected in collected samples. 16SrXXI-A phytoplasma subgroup was detected for the first time in Lithuania, and 16SrIII-T phytoplasma subgroup was detected for the first time in Lithuania and worldwide.

We detected phytoplasmas in insect samples belonging to 10 species and 1 unidentified species from the genus *Aphrodes* (Table 3). Two new possible insect vector species (*A. striifrons*, *S. minutus*) were identified during the research.

Table 3. Possible phytoplasma vectors and phytoplasmas detected in field-collected insects, and classified in 16S rDNA RFLP groups and subgroups.

Insect species/genus	**Total/infected insects**	**16Sr subgroup classification of detected phytoplasma strain**	**GenBank acc. no. of 16S rRNA gene sequences from detected phytoplasmas**	**Strain[a]/GenBank number of reference strain**
Anaceratagallia ribauti	6/4	16SrIII-B	KC283217	CYE /AF173558
		16SrI-C	NA	CPh/L33762

Aphrodes sp.	6/6	16SrIII-P	KC283216	DanVir/AF370119
		16SrI-B	KC283215	AY1/L33767
				CPh/L33762
		16SrI-C	NA	
Aphrophora alni	1/1	16SrI-B	NA	AY1/L33767
Arthaldeus striifrons	4/4	16SrI-C	NA	CPh/L33762
Cacopsylla mali	10/8	16SrI-B	NA	AY1/L33767
Cicadella viridis	3/3	16SrI-B	NA	AY1/L33767
Euscelis incisus	10/10	16SrI-C	KC283211KC283213	CPh/L33762
		16SrIII-P	KC283212	DanVir/AF370119
Lepyronia coleoptrata	5/5	16SrI-B	NA	AY1/L33767
				CPh/L33762
		16SrI-C	NA	
Macrosteles sexnotatus	10/3	16SrI-C	KC283214	CPh/L33762
Philaenus spumarius	5/4	16SrI-B	NA	AY1/L33767
				CPh/L33762
		16SrI-C	KC283218	
Stenocranus minutus	5/3	16SrI-A	NA	BB/AF222064

Strain – reference strain of the indicated phytoplasma 16Sr subgroup**.**

Bolded – confirmed phytoplasma vector identified in this study.

Default – possible phytoplasma host/vector identified in this study.

CYE-clover yellow edge, AY1-Maryland aster yellows, DanVir-dandelion virescence, CPh-clover phyllody.

NA – Not available.

For the first time in our region 16SrI-A, -B, -C; 16SrIII-B, -P subgroup phytoplasmas were detected in *A. alni*, *A. ribauti*, *Aphrodes* sp., *A. striifrons*, *C. mali*, *C. viridis*, *L. coleoptrata*, *M. sexnotatus*, *P. spumarius* and *S. minutus* insect species. These data complement knowledge about vector and host variety of these phytoplasmas.

Phytoplasmas detected in plant samples belong to 18 plant species (Table 4). In five plant species (black salsify, chickweed, common centaury, Norway spruce, Scots pine) phytoplasmas were detected for the first time in Lithuania.

Table 4. Plant species and detected phytoplasma 16Sr subgroups.

Nr.	**Plant species**	**16Sr subgroup classification of detected phytoplasma strain**	**Collection place**
1.	*Centaurium erythraea*	16SrI-B	Muniškiai, Kaunas d.
2.	*Cirsium arvense*	16SrI-C	Muniškiai, Kaunas d.
3.	*Dactylis glomerata*	16SrI-B	Muniškiai, Kaunas d.
4.	*Fragaria x anannassa*	16SrI-A	Dvarčionys, Vilnius d.
5.	*Malus domestica*	16SrI-B	Muniškiai, Kaunas d., Žiežmariai, Kiašiadoriai d.
6.	*Picea abies*	16SrI-A	Girionys, Kaunas d.
7.	*Picris hieracioides*	16SrI-B	Muniškiai, Kaunas d.
8.	*Pinus sylvestris*	16SrXXI-A	Girionys, Liškiava
9.	*Prunus avium*	16SrI-B	Muniškiai, Kaunas d.
10.	*Prunus domestica*	16SrI-B	Muniškiai, Kaunas d.
11.	*Prunus cerasifera*	16SrI-B; 16SrI-C	Muniškiai, Žiežmariai

12.	*Prunus cerasus*	16SrI-B; 16SrIII-T	Kaunas d.
13.	*Ribes nigrum*	16SrI-B	Vilnius d.
14.	*Scorzonera hispanica*	16SrI-B	Tamošava, Trakai d.
15.	*Stellaria media*	16SrI-B	Vilnius d.
16.	*Taraxacum officinale*	16SrIII-P	Muniškiai, Kaunas d.
17.	*Trifolium pratense*	16SrI-B	Muniškiai, Kaunas d.
18.	*Trifolium repens*	16SrI-C	Muniškiai, Kaunas d.

Phylogenetic analysis and constructed phylogenetic trees confirmed the appointment of the detected phytoplasmas to 16SrI-A, B, C; 16SrIII-B, P, T and 16SrXXI-A subgroups, and showed their relationship to other phytoplasmas detected in Lithuania and other countries.

Eight 16S rRNA nucleotide sequences of detected phytoplasma strains identified in insect and diseased plant samples are deposited in the International Gene Bank database.

CONCLUSIONS

1. During the research, we identified 35 insect species of the *Hemiptera* order. Eleven insect species were infected with phytoplasmas that belong to seven 16Sr subgroups. The identified insect species are potential vectors of the detected phytoplasmas. The data also revealed five new phytoplasma plant hosts in Lithuania.
2. Analysis of 16S rRNA, *rp* and *secY* gene sequences of phytoplasmas detected in insect and plant samples showed that the detected phytoplasmas belong to 16SrI-A, B, C, 16SrIII-B, P, T and 16SrXXI-A phytoplasma subgroups. 16SrIII-T phytoplasma subgroup was found in cherries for the first time in Lithuania and worldwide.
3. Two detected insect species (*Arthaldeus striifrons*, *Stenocranus minutus*) are new potential phytoplasma vectors in Lithuania and worldwide. The data revealed that the detected 11 insect species have wider range of vectored phytoplasmas. These data complement the knowledge about the vector and host variety of the identified phytoplasmas.
4. Transmission trials confirmed ability of *E. incisus* to transmit 16SrI-C subgroup phytoplasmas to white clover (*Trifolium repens*).
5. Phylogenetic analysis of gene sequences revealed that the investigated phytoplasmas are closely related to earlier in Lithuania detected phytoplasmas.
6. Phylogenetic analysis showed that sequence fragment of 16S rRNA gene of 16SrIII-T subgroup phytoplasma is similar to *rrnB* operon sequence of 16SrIII-P subgroup phytoplasma. This could mean that both phytoplasmas belong to the same species.
7. The research confirmed that xylem-feeding insects acquire and maintain phytoplasmas. We assume that probably they can be not only hosts, but also vectors of the detected phytoplasmas.

LIST OF PUBLICATIONS

1. Valiunas D., Jomantiene R., **Ivanauskas A.**, Abraitis R., Staniene G., Zhao Y., Davis R.E., 2009: First report of a new phytoplasma subgroup, 16SrIII-T, associated with decline disease affecting sweet and sour cherry trees in Lithuania. – Plant Disease, 93(5): 550.
2. Jomantiene R., Valiunas D., **Ivanauskas A.**, Urbanaviciene L., Staniulis J., Davis R..E., 2011: Larch is a new host for a group 16SrI, subgroup B phytoplasma in Ukraine. – Bulletin of Insectology, 64(S): S101–S102.
3. **Ivanauskas A.**, Valiunas D., Jomantiene R., Staniulis J., Alma A., Picciau L., Davis R.E., 2011: First report of potential phytoplasma vectors: *Euscelis incisus* and *Macrosteles sexnotatus* in Lithuania. **–** Bulletin of Insectology, 64(S): S131–S132.
4. **Ivanauskas A.**, Valiunas D., Jomantiene R., Picciau L., Davis R.E., 2014: Possible insect vectors of 'Candidatus Phytoplasma asteris' and 'Candidatus Phytoplasma pruni'-related strains in Lithuania. – Zemdirbyste-Agriculture, 101(3). Accepted for publication.

Conference reports

1. Valiunas D., Jomantiene R., **Ivanauskas A.**, Sneideris D., Staniulis J., Davis R.E, 2010: A possible threat to the timber industry: '*Candidatus* Phytoplasma pini' in Scots pine (*Pinus sylvestris* L.*)* in Lithuania. – Abstract book of the combined meeting of Work Groups 1-4, COST Action FA0807, Editors A. Bertaccini, A. Lavifia, E. Torres. Current status and perspectives of phytoplasma disease research and management, Sitges, Spain, 1–2 February 2010.
2. Jomantiene R., Valiunas D., **Ivanauskas A.**, Urbanaviciene L., Staniulis J., Davis R.E., 2011: Larch is a new host for a group 16SrI, subgroup B phytoplasma in Ukraine. – *Second International Phytoplasmologist Working Group Meeting*. Neustadt/Weinstrasse, Germany, 12–16 September 2011.
3. **Ivanauskas A.**, Valiunas D., Jomantiene R., Staniulis J., Alma A., Picciau L., Davis R.E., 2011: First report of potential phytoplasma vectors: *Euscelis incisus* and *Macrosteles sexnotatus* in Lithuania. **–** *Second International Phytoplasmologist Working Group Meeting*. Neustadt/Weinstrasse, Germany, 12–16 September 2011. http://www.ipwgnet.org/index.php?option=com_content&view=article&id=38&Itemid=33
4. **Ivanauskas A.,** 2013: Phytoplasmas and their insect vectors in Lithuania. – Conference for young researchers „BIOFUTURE: perspectives of nature and life sciences. Vilnius, Lithuania, 11 December 2013.

Fitoplazmos ir jų vabzdžiai platintojai Lietuvoje

Santrauka

Geltligės yra vienos iš žalingiausių naudingųjų augalų ligų, sukeliančios nemažus ekonominius nuostolius. Pagrindiniai šių ligų sukėlėjai yra fitoplazmos – sienelės neturinčios ląstelės, aptinkamos augalų karnienos ląstelėse ir vabzdžių šeimininkų audiniuose, neaugančios dirbtinėse terpėse, *Mollicutes* klasės bakterijos. Šių mikroorganizmų sukeliamų ligų mechanizmas yra dar pilnai neištirtas. Manoma, kad augalų pažeidimus jos sukelia sutrikdydamos augalų hormonų arba jų pirmtakų apytaką. Būdingiausi šių bakterijų sukelti simptomai yra augalo organų pageltimas, žiedų pažaliavimas, augalo žemaūgė, vegetatyvinių augalo dalių formavimasis vietoj generatyvinių, „raganų šluotos“. Efektyviausi būdai kovoti su fitoplazminėmis infekcijomis yra sergančių augalų naikinimas bei tetraciklino, termo terapijos, tačiau paskutinieji metodai yra brangūs ir mažai efektyvūs. Siekiant apsisaugoti nuo šių patogenų arba sumažinti nuostolius, svarbu vykdyti griežtą karantino politiką bei vabzdžių pernešėjų ir piktžolių kontrolę.

Fitoplazmos tyrinėjamos pasitelkiant molekulinės biologijos ir imunologinius metodus. Jų aptikimui ir klasifikacijai naudojami 16S rRNR ir eilės kitų genų sekų analizė.

Šio darbo metu, nuo augalų su išreikštais fitoplazminiais simptomais, buvo surinkti vabzdžių pavyzdžiai. Taip pat surinkti simptomatinių augalų pavyzdžiai. Vabzdžiai buvo klasifikuoti pagal entomologinius raktus, naudojamus Europoje paplitusių *Hemiptera* būrio atstovų apibūdinimui. Iš viso identifikuotos cikadelių aštuonios gentys ir 27 rūšys.

Fitoplazmų aptikimui buvo panaudotas lizdinės PGR metodas su universaliais fitoplazmų pradmenimis. Šios bakterijos buvo aptiktos vabzdžių pavyzdžiuose priklausančiuose *Hemiptera* būrio vienai genčiai (rūšis nenustatyta) ir dešimčiai rūšių. Fitoplazmos aptiktos 17 rūšių ir genties augalų pavyzdžiuose.

Pagal pagausintų 1,2 kbp dydžio,16S rDNR sekų realių ir virtualių restrikcijos ilgio polimorfizmo analizės profilių palyginimus su klasifikacijos schemų profiliais (Lee et al., 1998; Wei et al, 2007), fitoplazmos buvo priskirtos 16SrI-A, -B, -C; 16SrIII-B, -P, -T; 16SrXXI-A pogrupiams. Šių mikroorganizmų 16Sr rRNR, *rp*, *secY* genų sekos buvo klonuotos ir nuskaitytos. Gautos sekos palygintos su duomenų bazėse (NCBI) esančiomis žinomų fitoplazmų genų sekomis bei atlikta jų filogenetinė analizė (MEGA5). Palyginimai bei filogenetinių medžių šakojimosi tvarka patvirtino aptiktų fitoplazmų priskyrimą atitinkamiems pogrupiams.

Anaceratagallia ribauti - 16SrIII-B, 16SrI-C, *Aphrophora alni* - 16SrI-B, *Aphrodes* sp. - 16SrIII-P, 16SrI-B, 16SrI-C, *Arthaldeus striifrons* - 16SrI-C, *Cacopsylla mali* - 16SrI-B, *Cicadella viridis* - 16SrI-B, *Euscelis incisus* - 16SrI-C, 16SrIII-P, *Lepyronia coleoptrata* - 16SrI-B, 16SrI-C, *Macrosteles sexnotatus* - 16SrI-C, *Philaenus spumarius* - 16SrI-B, 16SrI-C, *Stenocranus minutus* - 16SrI-A – šių vabzdžių pavyzdžiuose atitinkamų fitoplazmų pogrupių kamienų aptikimas įrodo, kad jie yra šių kamienų šeimininkai bei galimi pernešėjai. Gauti rezultatai taip pat parodo, jog polifaginės cikadelės *A. ribauti*, *Aphrodes* sp., *E. incisus*, *L. coleoptrata*, *P. spumarius* gali įgyti daugiau nei vieną skirtingų fitoplazmų grupių/pogrupių kamieną. Pirmą kartą mūsų regione 16SrI-A, -B, -C; 16SrIII-B, -P pogrupių fitoplazmos buvo identifikuotos *A. alni*,

A. ribauti, Aphrodes sp., *A. striifrons, C. mali, C. viridis, L. coleoptrata, M. sexnotatus, P. spumarius* ir *S. minutus* vabzdžiuose. Taip pat nustatyti du nauji Lietuvai ir pasauliui galimi fitoplazmų vektoriai – *A. striifrons* ir *S. minutus*.

Tyrimo metu išaiškinti vienas naujas Lietuvai 16SrXXI-A ir naujas pasauliui 16SrIII-T fitoplazmų pogrupiai. Pirmą kartą Lietuvoje 16SrI-A pogrupio fitoplazma aptikta braškėje. Mūsų gauti duomenys praplečia Lietuvoje bei pasaulyje aptinkamų fitoplazmų, jų vabzdžių platintojų ir augalų šeiminikų ratus.

Tyrimo duomenys parodė, kad medienos ląstelių sultimis mintančios cikadelės *A. alni, C. viridis, L. coleopterata* ir *P. spumarius* gali įgyti fitoplazmas, todėl yra galimybė, kad jos gali būti ne tik jų šeimininkais, bet ir pernešėjais.

Atlikti fitoplazmų pernešimo *E. incisus* vabzdžiais bandymai tarp baltųjų dobilų patvirtino šios rūšies gebėjimą jas platinti tarp šių augalų. Taip pat nustatyti galimi tik Lietuvoje aptinkamų 16SrIII-P pogrupio fitoplazmų platintojai tarp kiaulpienių – *E. incisus* ir *Aphrodes* sp. vabzdžiai.

Mūsų atlikta filogenetinė analizė patvirtino Lietuvoje identifikuotų fitoplazmų klasifikavimo į atitinkamas 16Sr RFLP grupes ir pogrupius rezultatus bei padėjo išaiškinti naujus jų kamienus. Be to, išaiškinta, jog 16SrIII-T pogrupio fitoplazmos 16S rDNR seka beveik identiška 16SrIII-P pogrupio fitoplazmų vieno iš heterogeniškųjų *rrnB* operono sekai, tai galėtų reikšti, kad abi fitoplazmos priklauso tai pačiai rūšiai. Šio darbo metu aptiktų fitoplazmų genų sekos didžiausią gimingumą parodė su anksčiau Lietuvoje aptiktų fitoplazmų genų sekom ir atsiskyrė nuo kituose regionuose aptinkamų kamienų.

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Nov. 2013– present	Biologist. Nature Research Centre, Institute of Botany, Laboratory of Plant Viruses, Vilnius, Lithuania

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