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PHYTOPLASMA DISEASES: A REVIEW OF 50 YEAR HISTORY AND CURRENT ADVANCES

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Abstract

Phytoplasma diseases were known long before the discovery of their agent. Since the early 1930s in the former Soviet Union the infectious nature of the disease known under the name of "stolbur of tomato" has been recognized. Attempts were made to find vectors of the disease (I.K. Korachewski, 1934; V.L. Ryzhkov et al., 1934). In 1945 it was found that planthopper *Hyalesthes obsoletus* can be the disease vector (K. Sukhov et al., 1946). Searching for an infectious agent of plant diseases with symptoms of dwarfism, yellowing, damaged leaves and generative organs, which was unable to grow on artificial nutrient media was unsuccessfully conducted for several decades in our country and abroad. The discovery was made only in 1967 by Japanese researchers (Y. Doi et al., 1967). The causative agent of the disease, the unknown earlier phytopathogen from *Mollicutes* class, was similar to mycoplasma pathogens of animals. Later it was found that the life cycle of phytoplasmas is associated with the phloem cells of the plants, in which they multiply, and with vector insects of *Hemiptera* order which are feeding plant juice and able not only to support the phytoplasma reproduction in their body, but even transmit phytoplasma to the offspring. Phytoplasmas, like other *Mollicutes*, have no cell wall; they have a minimal genome known to cellular organisms, which causes their obligate parasitism. In the ex-USSR, phytoplasma disease was extensively studied by electron microscopy, immunological methods, and phytoplasma were successfully cultured on artificial nutrient media. Three decades later Italian researchers managed to culture the infective agent on artificial media and confirmed its belonging to phytoplasma by DNA sequencing (A. Bertaccini et al., 2010; N. Contaldo et al., 2012, 2013). In 1990s, a great step forward was made due to molecular methods of phytoplasma diagnosis and study. Phytoplasmas' taxonomy was developed based on the conservative 16S ribosomal RNA gene and further elaborated with the involvement of other genes having both highly and less conserved sequences (I.-M. Lee et al., 1993; B. Schneider et al., 1993, 1997; I.-M. Lee et al. 1998, 2010; M. Martini et al., 2007). The next important step was a discovery of virulence factors of phytoplasma affecting host plants and making them more attractive to insect vectors naturally involved in the spread of phytoplasmas. In recent years, a lot of genomic data has been obtained for various phytoplasmas; attention is paid to elucidate phytoplasma metabolism which is important to understand the host—pathogen—vector interactions (K. Oshima et al., 2004; X. Bai et al., 2006; A. Hoshi et al., 2009; A. Sugio et al., 2011; A. MacLean et al., 2011; K. Sugawara et al., 2013; Z. Orlovskis et al., 2016). In Russia, molecular methods have allowed the researchers to reveal the phytoplasma nature of a group of diseases with unclear etiology that gives the key to control of these widespread and harmful diseases. Prevention is the primary means of controlling phytoplasma diseases, including the use of healthy planting material, resistant varieties, methods aimed at spatial isolation from sources of infection, weed eradication, and the use of biopreparation and bioagents capable of producing tetracycline antibiotics.

Keywords: phytoplasma, phytoplasma diseases, yellows, witches'-broom, stolbur, phytoplasma vectors, phytoplasma taxonomy, phytoplasma marker genes

In 2017, there were several anniversaries in phytopathology: the 125th

anniversary of the discovery of viruses by D.I. Ivanovsky, the 110th anniversary of the organization of the Phytopathological Laboratory in Russia by A.A. Yachevsky and 50 years from the discovery of phytoplasmas and viroids; what is more, the diseases caused by both these pathogens were known and were considered viral long before their agents were detected. Phytopathogens, which were named phytoplasma in 1994, were discovered in 1967 by Japanese virologists. This discovery could have happened 10 years earlier in the USA, but for unfortunate reasons, it did not happen. In the phloem of plants affected by yellows-like diseases (witches' broom, dwarfism), bacterial polymorphic bodies resembling mycoplasma, which is the pathogen of bovine pleuropneumonia (pleuropneumonia like-organisms, PPLO = PLT) were detected, not viruses [1]. They were also detected in vectors of the disease — leafhoppers (family *Cicadellidae*), and later in jumping plant lices or psyllids (family *Psyllidae*).

In the former USSR, long before the discovery of phytoplasmas, plant diseases such as stolbur, yellows, alfalfa witches' broom, potato and wood witches' broom, grain dwarfism were known. Most often, due to the similarity of symptoms, they were referred to as a virus, but sometimes they were considered non-infectious. The revolutionary discovery by D.I. Ivanovsky in 1892 of the microorganism that causes a mosaic disease of tobacco was followed by a series of discoveries of the so-called filterable viruses, the pathogens of humans and animals, but in phytopathology, virological studies were continued only in the 1920s after A.A. Yachevsky visited the USA. He described a number of viral diseases of potato (*Solanum tuberosum* L.), among which in 1926 was witches' broom [2]. Until the 1930s, studies of viral diseases were limited to describing virus-like symptoms in plants of different species in different regions and comparing them with the diseases described in foreign reviews. In the 1930s and 1940s, the first virology laboratories were organized and data were obtained on the infectivity of a number of diseases considered viral, including the stolbur, and cereal pupation, which were classified as yellows.

For the first time, a disease called Stolbur (from Ukrainian “stovbur” — a “trunk” or “stem”) was observed on tomatoes with symptoms of fruit lignification in the late 1920s. This word later became an international term for phytoplasmas belonging to the 16SrXII group. In 1934, I.K. Korachevsky described the characteristic stolbur symptoms on tomatoes [3]. The infectious nature of the disease was proven by grafting a tomato with stolbur on healthy plant [4]. The disease was not transmitted by seeds or by inoculation of the juice in the tissue of a healthy plant. At the same time, there were cases of the rapid spread of stolbur, leading to a massive infection of plantations. I.K. Korachevsky tried to find a vector of the disease among insects, but the test of aphids, thrips, bugs, and some species of leafhoppers did not give results; therefore, the cause was attributed to the effects of various abiotic factors on the physiology of tomatoes. This was a significant step back in understanding the nature of stolbur [5].

In 1945, K.S. Sukhov and A.M. Vovk, realizing that the environmental hypotheses of the disease causes lead the wrong way, began to persistently look for a vector. They identified the entire species composition of the insects of the *Hemiptera* order, the *Auchenorrhyncha* (or *Cicadinea*) suborder, which visited tomatoes. These were insects from the families *Cixiidae* (2 species), *Delphacidae* (3 species), *Aphrophoridae* (spittlebugs, 1 species), *Cicadellidae* (leafhoppers, 11 species). In total, 17 species of *Cicadinea* and several rarely encountered species were tested (without determining the species). It was possible to identify only one vector species, the *Hyalesthes obsoletus* (Sign.) from the *Cixiidae* family (planthoppers) [6]. In subsequent years, these data were confirmed [7, 8]. The discovery of Soviet scientists was an important step in explaining the epidemiol-

ogy of widespread and harmful diseases of economically significant crops in Eurasia, caused by the stolbur phytoplasmas.

For the first time, infection of potatoes presumably with stolbur was recorded in the Crimea in 1935 [9]. In 1940, it was shown that potatoes can be infected by inoculation [10]. The first outbreaks of stolbur on potatoes were recorded in 1943 in Kyrgyzstan and in 1944 in the Moscow Province [11]. Since that time, the term "stolbur" has been widely used to describe diseases with characteristic symptoms of yellowing and redness of the lamina and growth retardation [12]. In 1945, from 40 to 70% of the potato plantations in the Krasnodar Territory suffered from stolbur. The disease caused a serious decrease in crop yields and led to catastrophic economic losses [12]. The disease had a serious economic impact in other regions of the Russian Federation, especially in the Volga Region [7], as well as in the Union republics of the former USSR: in Crimea [4, 5, 13, 14], in Ukraine [15, 16], in Moldova [17], Armenia [18], Georgia [19, 20], Azerbaijan [21, 22] and in the Central Asian republics [23-25].

In Georgia, a new species of tomato stolbur vector was discovered, the planthopper *Hyalesthes mlokosiewiczi* Sign. (*Cixiidae* family) [26]. Its larvae, nymphs, and adults captured on stolbur-infected corn bindweed plants (*Convolvulus arvensis* L.) were transferred under isolators to healthy tomatoes, where these insects fed for some time, and 21-23 days after feeding, the first symptoms of infection appeared [20, 26].

For a long time in the domestic phytopathological literature, there has been a discussion about the ecological or fungal nature of the stolbur wilting of potatoes Even at present, for the first time encountering the stolbur wilting of potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.) or eggplant (*Solanum melongena* L.), phytopathologists associate it with the damage made exclusively by the fungi of the genus *Fusarium, Rhizoctonia, Verticillium or Colletotrichum* [27]. This is explained by the fact that phytoplasma infected plants of vegetable and grain crops are more susceptible to damage root rot and vascular wilting pathogens, as well as to fungal leaf spots caused, for example, by Alternaria and Cladosporium [28-30].

In 1955, A.M. Vovk and G.S. Nikiforova made the first attempt in the USSR to determine the size and shape of the stolbur pathogen in the juice of a diseased tomato using electron microscopy [31].

In 1890-1900, the main cause of peach tree decline and dieback in the USA, Delaware, was the yellows. In the early 1930s, L.O. Kunkel found the disease vector, leafhopper *Macropsis trimaculata* (Fitch) [32]. Studying the ecology of the vector, during electron microscopic examination of *M. trimaculata* and fixed vessel sections of peach, which showed symptoms of the yellows, A. Hartzell found plasma-like bodies and inclusions. However, because of their lability, specific morphology and differences from the phytoviruses, he failed to identify the pathogen [33, 34].

The American phytovirologist K. Maramorosch injected leafhoppers *Macrosteles fascifrons* Stal. with juice of the aster plant diseased by aster yellows, as well as an extract from leafhopper-vector, and showed the possibility to reproduce pathogen in both the plant and the vector [35, 36]. In one experiment, antibiotics (penicillin, streptomycin, and tetracycline) were added to the extracts. The experimenter was convinced that they should not affect infection, since it was thought that the infectious agent was a virus. However, in the case of tetracycline use, infection did not occur. Finding none explanation for this fact, the author attributed it to the effect of high temperatures in the greenhouse. The experiment was not repeated.

In 1966, K. Maramorosch visited the laboratory of the famous virologist

and immunologist W. Henle in Philadelphia. He brought with him electron microscopic photographs of the salivary glands of leafhoppers. The electron microscopy expert T. Hummeler, who worked at the same institute, after looking at these photographs, drew the author's attention to the presence of structures similar to mycoplasmas. However, K. Maramorosch did not attach any importance to this, because he was not familiar with the work on the successful cultivation of *Mycoplasma pneumonia*, did not know what the word "mycoplasma" means, and did not even take an interest in this [37, 38].

In 1967, during the annual meeting of the Japanese Society of Phytopathologists, E. Shikata from the University of Hokkaido, a former employee of K. Maramorosch, studied the abstract of the article by Y. Doi and colleagues, which considered mycoplasma-like organisms found in the mulberry (genus *Morus*) with symptoms of dwarfism. E. Shikata suspected that such microorganisms were in electron microscopic photographs of the aster yellows pathogen made by him in 1954, when he worked in the laboratory of K. Maramorosch, and asked Maramorosch to send him the photoplates. Photographic negatives were found, but because of the absence of viruses on them, they were not sent on the request [37].

A key role in the recognition of phytoplasmas was played by a veterinarian from the University of Tokyo K. Koshimizu. After studying electron micrographs taken by Y. Doi in 1967, he discovered the similarity of the structures visible on them to mycoplasmas and suggested testing the effect of tetracycline on trees. Y. Doi did not leave this information unaddressed. At the suggestion of Y. Doi, his manager H. Asuyama instructed his other employee T. Ishii to conduct an experiment on healing a diseased young plant of mulberry with tetracycline, which was done with a positive result. As a result, three reports were presented at the annual meeting in Sapporo (Japan), which marked the discovery of mycoplasma-like organisms in plants, later known as "phytoplasmas" [39-41].

In May 1968, the first paper by J. Giannotti et al. [42] on determining the mycoplasma-like organisms in forest apple tree with signs of proliferation appeared in France. The researchers did not make references to the publications of Japanese scientists, presenting their work as a pioneer paper. Later J. Giannotti published data on the cultivation of mycoplasma-like organisms on artificial nutrient media. However, other scientists, in particular, J. Bovй and R. Davis, failed to repeat the cultivation of mycoplasma-like organisms. An attempt to cultivate phytoplasmas in the laboratory of K. Maramorosch was also unsuccessful due to the formation of pseudo-colonies formed with an excess of horse serum [43]. Several unsuccessful attempts made in different laboratories led to the adoption by the international committee of mycoplasmologists of the postulate that it is impossible to cultivate mycoplasmas on artificial nutrient media. However, later K. Maramorosch expressed the hope that the cooperation of phytoplasmologists with other microbiologists will eventually lead to the possibility of cultivating these microorganisms [37].

After the discovery of phytoplasmas, active electron microscopic studies of plant yellows pathogens and their tetracycline therapy began worldwide. The number of papers focused on plant mycoplasmas began to increase progressively: in 1967, four papers were published, in 1968 — 29, in 1969 — 61, in 1970 — 90, and by 1974, the number of detected cases of phytoplasma diseases reached 50. In the USSR, the study of mycoplasma-like diseases was conducted at the Institute of Microbiology of the USSR Academy of Sciences, at the Zabolotny Ukrainian Institute of Microbiology and Virology, at the All-Union Scientific Research Institute for Plant Protection (VIZR), where the laboratory of viral and mycoplasmal diseases was headed by Professor Yu. I. Vlasov, the follower of

Professor K.S. Sukhov, and at some other research institutes.

The purpose of the research carried out at VIZR was to study the patterns of phytoplasma diseases spreading in biocenoses and the development of methods to control them. The classical scheme of the circulation of the stolbur pathogen in nature was described earlier by K S. Sukhov and A. M. Vovk (1949) in the Krasnodar Territory. They noted the natural-focal nature of the disease. Subsequently, corrections and additions were made to this scheme, mainly relating to the species composition of vectors and infection reservoirs [44, 45]. In addition to the planthoppers *Hyalesthes obsoletus*, the disease is spread by meadow froghoppers *Phylaenus spumarius* L. (family *Aphrophoridae*, spittlebugs), leafhoppers *Aphrodes bicinctus* Schrank and *Cicadella viridis* L. (family Cicadellidae), as well as planthopper *Pentastiridius leporinus* L. [46]. Leafhoppers get an infection, feeding on infected perennial plants, i.e. bindweed *Convolvulus arvensis* L., *Goebelia alopecuroides* (L.) Bunge, Canada thistle *Cirsium arvense* (L.) Scop., etc. The migration of leafhoppers from weed and wild plants to crops occurs when the nutritional conditions of insects in a natural focus become unfavorable, for example, when wild plants dry out under the conditions of dry hot weather.

In the 1970s-1990s, the prevalence of stolbur on tomatoes in some seasons reached 50-60%.in the Astrakhan and Volgograd Regions, in the North Caucasus, as well as in Armenia and Uzbekistan. In addition, witches' broom was common on potatoes (*Solanum tuberosum* L.) and alfalfa (*Medicбgo satнva* L.), and phyllody on clovers (*Trifolium* L.). Alfalfa witches' broom was often found in Kazakhstan, Kyrgyzstan, Uzbekistan, and the Volga Region. As a result of the studies of these diseases, carried out by VIZR personnel, republican institutes for plant protection and other institutions, the list of vectors and reservoirs of infection was expanded, the properties of the pathogen were studied, measures to prevent and control diseases were substantiated, taking into account their natural focal nature, practical guidelines were published [47, 48].

In the 1970s-1980s, the laboratory of viral and mycoplasma diseases of VIZR actively cooperated with many institutes in the USSR and other countries. In the joint work, experts from Armenia, Georgia, and Ukraine were engaged. In 1981-1985 the productive cooperation was with the expert from Institut national de la recherche agronomique (INRA, Paris, France) Dr. J. Giannotti. French scientists were interested in a rich collection of plant samples infected by phytoplasmas collected in different regions of the USSR (Astrakhan, Volgograd, Armenia, Uzbekistan) from tomatoes, potatoes, eggplants, and other crops. Electron microscopy revealed the presence of an infectious agent belonging to the *Mollicutes* class. French researchers suggested using artificial nutrient media to study the microbiological properties of pathogens.

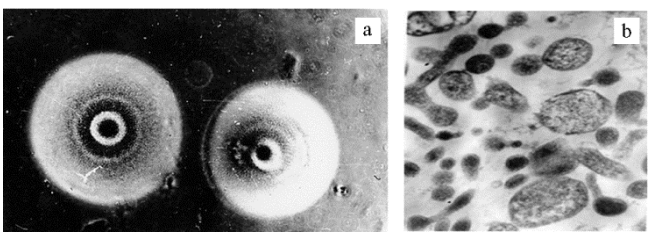

Fig. 1. Growth of stolbur pathogen on an artificial nutrient medium as a "fried egg" type colonies (×480) (a)**; the pathogen of tomato stolbur on a section** (electron microscopy, ×20,000) (b). Photo by L.N. Samsonova [44].

It has been already indicated that after several unsuccessful attempts to culture phytoplasmas on artificial nutrient media, their non-culturability began to be considered as an irrefutable fact. However, in the 1980s, VIZR actively engaged in the culture of microorganisms of the *Mollicutes* class (Fig. 1). Microorganisms isolated on media were serologically related to *Acholeplasma laidlawii* and *Spiroplasma citri*. Now it is difficult to say whether they belonged to phyto-

plasmas, but at that time researchers were sure of this and developed complex nutrient media for their culture [49, 50]. These were the first works of Soviet scientists on the possibility of phytoplasma culturing confirmed in the 21st century by Italian phytoplasmologists by molecular genetic methods.

Beginning from 2010, papers began to appear in Italy that showed the possibility of achieving the growth of phytoplasmas from various 16Sr groups on special commercial media using fragments of shoots of periwincle *Catharanthus roseus* (L.) G. Don. The growth of phytoplasma colonies on agar usually occurs within 2-5 days, although a relatively long pre-incubation in a liquid medium is required. Under equal conditions, phytoplasmas and mycoplasmas form morphologically similar colonies of 0.1-0.2 mm in size. However, quantitative indicators cannot be a differential characteristic, since they can vary widely and depend on the species, the strain of mycoplasma, the medium composition, the temperature and time of incubation, etc. Polymerase chain reaction (PCR) revealed the presence of phytoplasma DNA in cultured microorganisms used as a source of DNA matrix. Identification using restriction fragment length polymorphism analysis (RFLP) and direct amplicon sequencing also confirmed that it is a phytoplasma [51-53].

Until the 1990s, a precise definition of the taxonomic identity of a pathogen causing the stolbur or similar diseases was not possible. In the early 1990s, molecular identification methods appeared. Using 16S rRNA gene, specific oligonucleotide primers were developed, which allowed PCR amplification of the phytoplasmas' 16S rDNA fragments in a wide range of host plants infected by phytoplasmas [54, 55]. It has become possible to determine, differentiate and classify phytoplasmas by RFLP analysis. PCR-amplified phytoplasma 16S rDNA fragments are exposed to restriction endonucleases (each separately), resulting in DNA fragments of different length, which are then separated by electrophoresis in polyacrylamide gel and compared with published restriction maps. The length of the fragments and their number depend on the number of restriction sites in the amplicon molecule for the endonuclease used. Recently, amplicons are more often exposed to direct sequencing and further virtual in silico (computer) cleavage and separation of DNA fragments. A new phytoplasma classification system was developed based on differences in the primary structure of DNA encoding the 16S rRNA gene [54-56].

During the next two decades, phytoplasmas have been found in many plants under diseases of unknown etiology with characteristic yellowing symptoms. The development of virtual RFLP analysis (computer simulated RFLP) allows analyzing a large number of 16S rRNA gene sequences of phytoplasmas deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and greatly facilitates the ability to update their list [57]. Currently, this list contains about 50 groups and over 100 subgroups of phytoplasmas 16S rRNA (16Sr) [58]. It was shown that 16Sr groups correspond to phylogenetic clades established by the method of phylogenetic analysis of the 16S rRNA gene full-length sequence, which indicates the validity of the classification based on RFLP analysis (Fig. 2).

Since 2006 (first within the framework of the Russian-American cooperation on the project of the International Science and Technology Center — ISTC, now —Astana, Republic of Kazakhstan, and then on the Russian programs), the All-Russian Research Institute of Phytopathology has continued to identify phytoplasmas using PCR/RFLP analysis. Phytoplasmic diseases affecting potatoes were monitored in eight economic regions of the Russian Federation: North, North-West, Central, Central Black Earth, North Caucasus, Ural, and West-Siberian. During 7 years of research, more than 1,200 samples with phytoplasma infection symptoms have been tested. Phytoplasmas belonging to five

16Sr groups and eight subgroups have been identified: 16SrI-B, 16SrI-C, 16SrI-P, 16SrII-A, 16SrIII-B, 16SrVI-A, 16SrVI-C, and 16SrXII-A. It is shown that symptoms like stolbur on potatoes can cause both phytoplasma of the stolbur group (16SrXII-A) and phytoplasmas belonging to other groups (16SrI, 16SrIII, 16SrVI) [60].

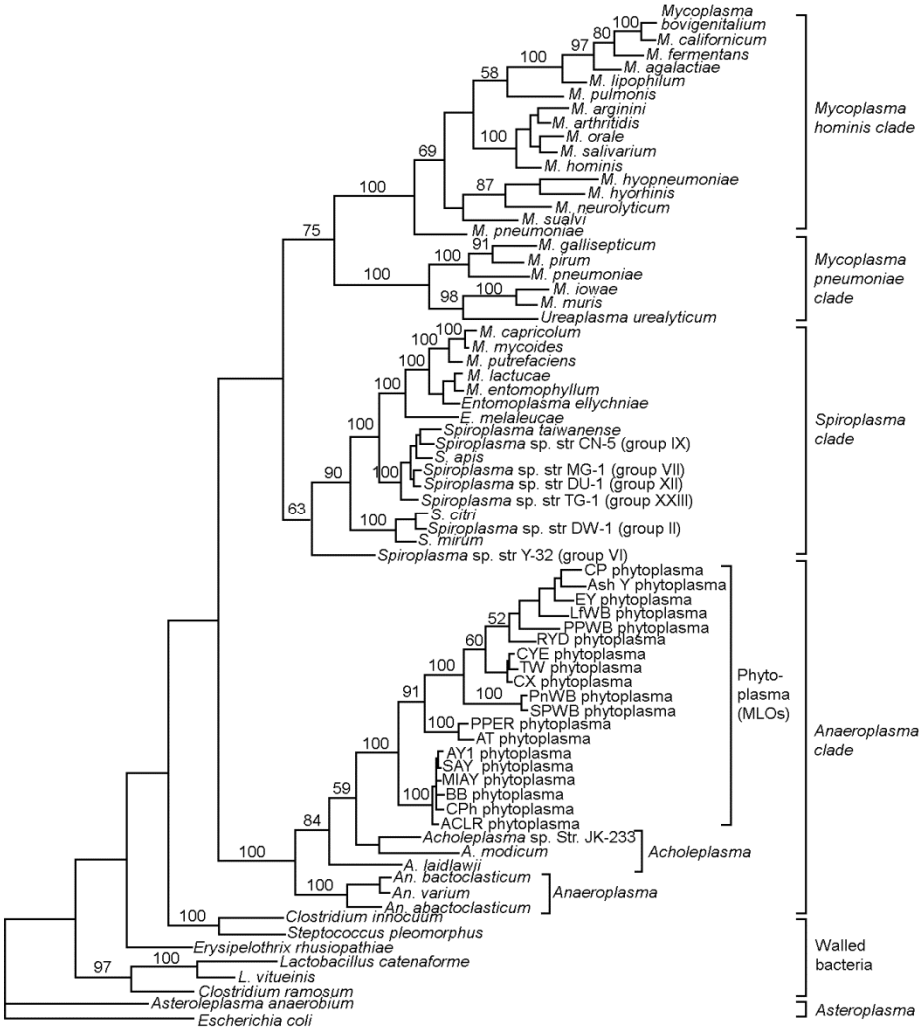

Fig. 2. Phylogenetic tree built using the parsimony analysis based on the full-lenght 16S rRNA gene sequence for 46 members of the *Mollicutes* class and a few representatives of bacteria with cell wall. *Escherichia coli* is used as an external group. The branches length is proportional to the number of changes in the sequence. The bootstrap analysis values for the internal node are indicated on the branches of the tree [59].

Monitoring of phytoplasmic legume diseases in four Russian regions (Northern, Central, Volga and West Siberian) showed that the majority of infected clover plants had typical symptoms of Clover phyllody (CPh), Clover yellow edge (CYE), and Clover proliferation (CP) diseases. These same diseases occurred on plants of other genera and species, but their symptoms could vary significantly. Infected alfalfa showed the typical symptoms of witches' broom. In total, phytoplasmas belonging to four groups and six subgroups were identified on legumes, with the phytoplasmas of Clover yellow edge (16SrIII-B) and Clover phyllody (16SrI-C) being more common; stolbur subgroups (16SrXII-A) and clover proliferation (16SrVI-A) were less often; in a few cases phytoplasmas of

the 16SrI-B and 16SrIII-F subgroups were identified [61]. In addition to potatoes and legumes, phytoplasma was isolated from cultivated and wild plants of more than 30 families, among which, along with grass, were shrubs and woody species. The most rarely encountered groups of phytoplasmas include phytoplasma of the Peanut witches' broom (PnWB) (16SrII), isolated from potatoes and wormwood (*Artemisia vulgaris* L.) in 2009; phytoplasma of the Elm yellows (EY) group (16SrV) isolated from large-leaved elm tree (*Ulmus laevis* Pall), and phytoplasma of the Apple proliferation (AP) 'group (16SrX) isolated from pears (*Pyrus communis* L.) [62]. More than 20 species of insects from the order *Hemiptera*, collected in the Moscow region, have been tested for several years for the phytoplasma carrier state. In eight species of leafhoppers and three species of spittlebugs (*Aphrophoridae*), phytoplasmas of 16SrI (16SrI-B, 16SrI-C, and 16SrI-P subgroups), 16SrIII (16SrIII-O subgroup), 16SrVI and 16SrXII-A groups were found. *Euscelis incisus* Krs., *Macrosteles laevis* Rib. and *Aphrodes bicinctus* Schrk. leafhoppers prevailed [60, 61]. In the Volga Region, *Dictyophara europaea* L. was identified as a carrier of phytoplasma of the 16SrIX group, planthoppers *Hyalesthes obsoletus* Sign. and *Pentastiridius leporinus* L. as carriers of phytoplasma of the 16SrXII-A subgroup and *Psammotettix striatus* L. was infected with the phytoplasma of the group 16SrIII. All the listed insect species of the *Hemiptera* can be potential vectors of phytoplasmas in the central region of Russia and in the Volga Region [62].

Only the phloem-feeding species, mainly from the suborder *Cicadinea*, families *Aphrophoridae* (froghopper), *Cicadellidae* (leafhoppers), *Membracidae* (treehoppers), *Cixiidae*, *Delphacidae* and *Dictyopharidae* (planthoppers), and suborder *Sternorrhyncha*, family *Psillidae* (jumping plant lice) are capable of phytoplasma transfer. It is assumed that the specificity of the vector and phytoplasma connection is determined by the interaction of the main antigenic protein (Amp) of the phytoplasma membrane and the insect microfilament complex, which determines the transition of phytoplasma through the stylet to the intestine, and then to the hemolymph and salivary gland in which phytoplasma multiplies and reaches an infectious titer. This period is called latent. Thus, the majority of *Cicadinea* from the *Cixiidae* family *(Hyalesthes obsoletus* Signoret*, Pentastiridius leporinus* Linnaeus*, Cixius wagneri* China*, Reptalus panzeri* Lцw*,* etc.) have a specific connection with the stolbur group phytoplasmas (16SrXII), and the incubation period can be 20 days.

Fourteen sequences of Russian isolates of potato phytoplasma (EU333397, EU333398, EU333400, EU344884, KP864663-KP864669, KP864672-KP864675) and 42 sequences of legume phytoplasma (KX773491-KX773530, KY587524, and KY587525) were deposited in the GenBank database (Fig. 3) [60, 61].

IRPCM Phytoplasma/Spiroplasma Working Team — Phytoplasma Taxonomy Group (2004) recommended the term *Candidatus* Phytoplasma for naming new types of 16S rRNA gene that has less than 97.5% similarity to the previously described *Ca*. Phytoplasma. Due to the high conservation of 16S rDNA, many biologically and ecologically different strains of phytoplasma, which could be considered as new taxa, are not considered them given this criterion. In this case, to determine the species, additional unique biological properties should be taken into account, such as the specificity of the antibodies, the range of host plants, specific vectors, and molecular criteria.

Another household gene *sec*Y, encoding the translocation of a ribosomal subunit, is also successfully used as a marker to identify more distinctions within groups and subgroups of phytoplasmas. It can be used to differentiate genetically close, but ecologically different strains that cannot be distinguished by analyzing the 16S rRNA gene [67]. RFLP analysis of amplified fragments containing the

sequence of the phytoplasma *groEL* gene, encoding the heat shock proteins from the HSP60 family, made it possible to differentiate eight different strains previously attributed by the ribosomal classification to one 16SrI-B subgroup [68]. The sequence of the 16S-23S rRNA intergenic region has also been used to differentiate phytoplasmas [69, 70]. The indicator of the genetic diversity of phytoplasmas is significantly increased by analyzing *vmp1* and *stamp* genes encoding phytoplasma membrane proteins [71, 72].

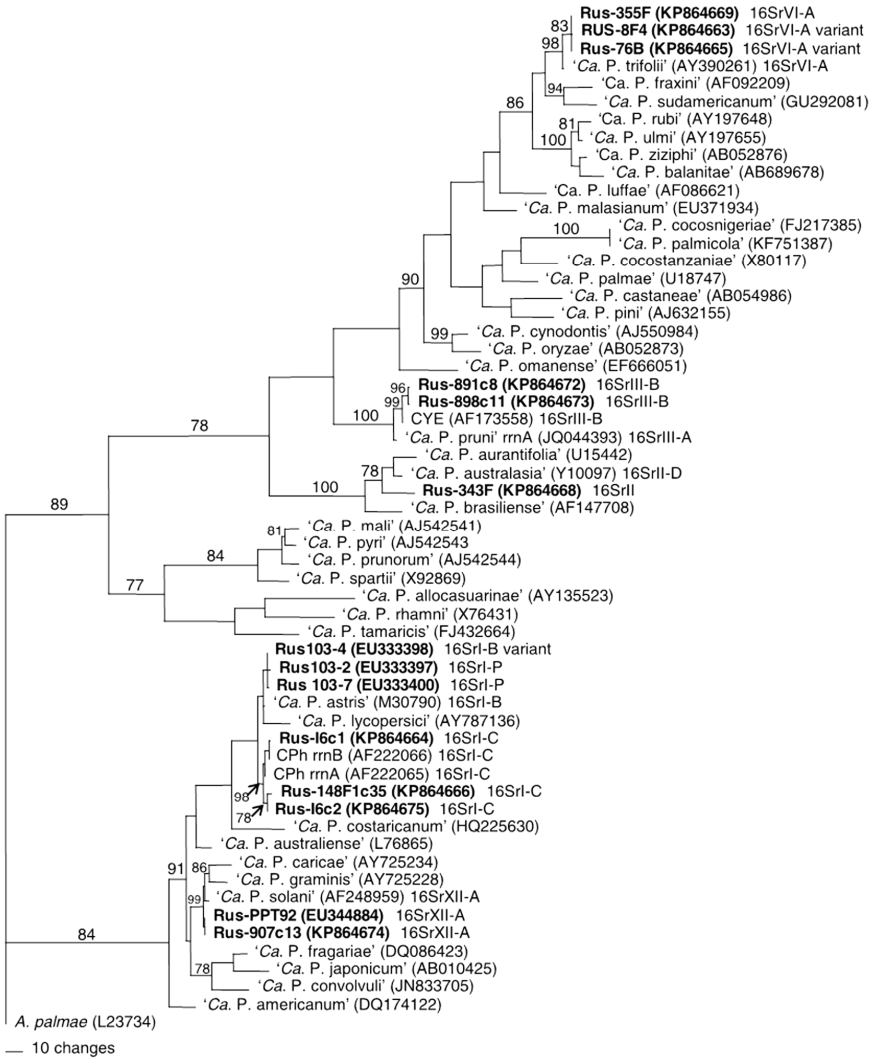

Fig. 3. Phylogenetic tree constructed using the parsimony analysis based on a partial sequence of the 16S rRNA gene for Russian potato phytoplasma strains of the *Candidatus* Phytoplasma species. *Acholeplasma palmae* was used as an external group for the tree rooting. The branches length is proportional to the number of changes in the sequence. The bootstrap analysis values are shown on the main branches of the tree. In the brackets, there are numbers under which sequences are deposited in GenBank. Numbers of Russian isolates are in bold [60].

In 2004, the full genome was sequenced in the *Candidatus* Phytoplasma asteris strain OY-M. Its size was 800 thousand bps. This was a minimum set of genes necessary for the existence of an obligate intracellular parasite. As it turned out, even less metabolic functions are encoded in the phytoplasma genome than in mycoplasmas. In addition, the pentose phosphate cycle and ATP synthase subunits, which were thought to be necessary for life, were not found in the studied phytoplasma. This may be the result of intracellular parasitism in a nutri-

ent-rich environment [73]. *Acholeplasmas* and phytoplasmas differ from mycoplasmas and spiroplasmas in that the UGA triplet serves as stop codon, whereas in the rest of prokaryotes, including most *Mollicutes*, this triplet encodes the amino acid tryptophan.

Like many plant pathogens, phytoplasmas produce virulence factors (i.e. effectors) that interfere with the host's normal life processes, changing them in favor of the pathogen. The first such effector protein described, the "tengu-su inducer" (TENGU), was isolated from onions (*Allium* sp.) infected with phytoplasma which caused yellowing [74]. This protein is transported via the phloem into other cells, including cells of the apical and axillary meristem, and causes characteristic symptoms, the witches' broom and dwarfism. The N-terminus of TENGU contains an 11 amino acid signal peptide which is cleaved *in vivo* during proteolysis by plant serine protease. It is assumed that this fragment at the N-terminus of the protein directly induces the development of the observed symptoms [75].

After 2 years, a report appeared on deciphering the genome of another phytoplasma strain which is the pathogen of the witches' broom of lettuce (*Lactuca sativa* L.). While the first strain belonged to the 16SrI-B subgroup, the second belonged to the 16SrI-A subgroup. This is the largest subgroup causing more than 100 economically significant diseases [76]. It was shown that strain AY-WB uses at least two protein effectors (SAP54 and SAP11) to affect the host plant, making the plant more suitable for colonization by insect vectors. The spread of phytoplasmas in nature [77-79] depends entirely on them.

A genome sequence was constructed for four phytoplasmas belonging to the 16SrIII group (X-disease) — the MA strain that causes the witches' broom of cranberries *Vaccinium* subgen. *Oxycoccus* (Hill) A. Gray, the JR1 strain that causes the phyllody of clover (*Trifolium* L.) in Italy, phytoplasmas that induce branching of poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch), and phytoplasma of euphorbia yellows (*Euphorbia antiquorum* L.). All four strains, despite their belonging to different subgroups of the 16SrIII group, had similar genomes and included a highly conservative portion (the DNA sequence identity was 92-98% for 500 bps) and small strain-specific regions. The genes encoding functional proteins that provided interaction with the host plant (membrane transport, proteases, DNA methylases, effectors, etc.) differed from each other and from strains of other species [80].

German researcher M. Kube compared four phytoplasmas, the OY and AY-WB strains of *Candidatus* Phytoplasma *asteris*, as well as *Ca.* P. *australiense* and *Ca.* P. *mali*. A complete set of genes required for glycolysis was found in all phytoplasmas except for *Ca.* P. *mali* [81]; therefore, the issue of the alternative way of obtaining ATP arose. Phytoplasmas do not have a set of genes for sterol biosynthesis, tricarboxylic acid cycle, phosphotransferase, *de novo* nucleotide synthesis, and amino acid synthesis. This explains the need for localization of the pathogen in sieve tubes, since it is their juice that contains the necessary metabolites. Although its composition varies depending on the type of plant, it always has a large amount of carbohydrates. Despite the difference in genome size, the set of functional proteins is the same for all phytoplasmas [82].

In recent years, methods for diagnostic of phytoplasma diseases have been developed, the phytoplasma taxonomy continues to be improved, a database has been developed, which expands the possibilities of studying the alleged virulence factors, and a lot of information has been obtained about the organization of the genomes of various phytoplasmas [83-86]. The basis for controlling phytoplasmic diseases are prevention methods: obtaining healthy planting material, the use of resistant varieties and agricultural methods that determine resistance to both the

pathogen and its vectors, spatial isolation from sources of infection, the destruction of infectious reservoirs and vectors, the use of biopreparations and bio-control agents capable of producing antibiotic substances of the tetracycline group.

Thus, over the 50 years that have passed since the discovery of the causative agents of phytoplasma diseases, significant progress has been made in studying these pathogens: the list of hosts-plants and vectors of diseases has been extended, the genetic aspects of harmfulness have been studied and continue to be studied, the papers considering the metabolism of phytoplasmas have appeared, which is important for understanding the host—pathogen—vector interaction. The study of phytoplasmic diseases has confirmed their wide distribution and catastrophic harmfulness. Monitoring studies conducted in Russia in the last decade indicate that phytoplasmas affect various cultures, which requires the deeper experimental researches and joint efforts of virologists and experts related to agriculture and forestry.

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