Pol. J. Environ. Stud. Vol. 31, No. 5 (2022), 4549-4556
DOI: *10.15244/pjoes/146217* ONLINE PUBLICATION DATE: 2022-08-30

Original Research

Detection of Phytopathogenic Bacteria Damaging Weeping Birch (Betula Pendula) by Molecular Identification Method

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Received: 20 October 2021
Accepted: 28 January 2022

Abstract

The article discusses the morphological changes of birch trees due to bacterial dropsy growing in the "Green Belt" artificial forest in the surrounding of the Nur-Sultan city. Samples of kernel were taken from diseased tree trunks, and, afterward, isolated bacterial cultures were grown in a nutrient medium. For molecular genetic studies, one strain (designated as DDdd-1), the most typical of morphological characteristics, was selected, the colonies of which showed the greatest growth on nutrient media. The nucleotide sequences 8F and 806R of 16SrRNA were determined on the basis of sequencing. The resulting sequences were compared in GenBank from the NCBI database showing related species and their similarities. Extraction of cores from infected birch trunks, isolation of pure strains of the pathogen, molecular identification of ribosomal RNA 16S nucleotide chains were performed using modern methods in the field of bacteriology. Then, the results obtained were compared with relevant data from the International Genbank database. Molecular characteristics of isolated bacterial DDdd-1 strain have been found to correspond to typical Dickeya dadantii by 96.72%. According to the International Genbank, the DDdd-1 strain is defined as pathovar D. dadantii pv. betulae. The data obtained demonstrate close relation of DDdd-1 strain Dickeya dadantii pv. betulae to some strains of Dickeya chrysanthemi. The species identified in International Genbank as Erwinia chrysanthemi with index numbers KJ541470.1 and AF373175.1 are assumed to be identical to D. dadantii pv. betulae. Dickeya is a complex taxon that still requires a lot of research efforts on species composition, close family bonds, the size and structure of host plants.

Keywords: bacterial dropsy, Betula pendula 16SrRNA, Dickeya dadantii

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Introduction

Bacteriosis affects woody plant species in forests around the world. Bacterial dropsy, also known as Slime flux, Alcoholic flux, or Wetwood, deserves special attention. It harms many types of tree species, including birch, cedar, fir, oak, beech, pine, spruce, linden, ash, and poplar. Birch (Betula pendula) is especially vulnerable to bacterial dropsy. The areas of affected birch forests are very significant [1-3]. Dropsy affects all parts of trees and manifests itself in the form of spots, cracks, and ulcers where methane-containing sap starts oozing out [3-5]. Consequently, this bacterial infection makes the valuable tree species dry out en masse [6].

Nowadays, bacterial dropsy is widely spread in the world in temperate and tropical latitudes. On the Eurasian continent, the disease is observed in Great Britain and from Eastern Europe to Japan, including Lithuania, Belarus, Ukraine, the European part of Russia, the Caucasus, Iran, the Southern Ural, the south of Western Siberia, southern Central Siberia, Primorsky Krai, Kazakhstan, and Kyrgyzstan [7, 8]. The disease covers the entire range of the genus Betula in Russia. In Kazakhstan, only small foci were previously noted, but after 2010, the disease acquired a massive character. Currently, in Kazakhstan, this disease is widespread in the Kostanay, North Kazakhstan, and Pavlodar regions [9].

Human economic activity, lack of due understanding, and ignorance of the specifics of bacterial diseases are principal factors in the incidence of the phenomenon. Felling, care activities on plantations, and preparation of seeds and cuttings foster the spread of the pathology [10]. Contaminated sowing and planting material are often replicated in forestry operations. In the natural environment, pathogenic dropsy-causing bacteria expand with atmospheric moisture, soil waters and are distributed by animals. Pathogens penetrate plants through mechanical damage to the dermal tissues system (wounds, cracks) and natural holes (lenticels, stomata). High humidity and temperatures advance the spread of pathogenic bacteria. Besides, the infection can be transmitted to offspring through seeds.

Bacterial dropsy of birch trees is a very harmful parenchymal disease of this tree family. The main symptoms begin with the appearance of orange-pink spots on various parts of the tree trunk, sometimes on branches or the surface of the periderm. Gradually, the place of such spots becomes larger and eventually cracks. A light brown malodorous liquid begins leaking out of the cracks. The liquid that flows on the surface of the tree bark dries quickly and turns into a large orange-brown stain that can be seen from afar. In places of damage, tissues begin to die off, forming wounds with torn edges. Over time, such wound increases, and a dead dark brown plots and living wet wood, emitting a sour smell, emerge under the bark of the affected areas. The disease expands deep into the tree trunk and covers the core, the conductive tissue is destroyed, clogged, and the leaves begin to fade and fall. In the beginning, some branches of the tree wither, and, eventually, the whole tree dries up.

The forestry aspects of the disease, such as the prevalence of the disease, the phytosanitary state of plants in certain areas, forestry measures to limit the spread of the disease, and the economic damage, are currently being studied more frequently. However, phytobacteriological studies that allow determining the pathogenic mechanism and establishing the true causative agent of the disease remain insufficient.

Opinions on the causative agents of bacterial dropsy differ. According to previous sources, two types of causative agents are indicated [11]. Particularly for the birch bacterial dropsy, Erwinia multivora is considered as the pathogen of the disease [2, 3, 7]. Until recently, the study of bacterial dropsy of birches in Kazakhstan has not received enough attention. However, another study classified the gram-negative facultative anaerobes bacteria isolated from affected birch trees in Northern Kazakhstan as Erwinia multivora [12]. Yet, they were considered as bacteria of other species, such as Pseudomonas sp., Pectobacterium caratovorum, Dickeya dadantii [13]. Also, in this study, strains of phytopathogenic bacteria belonging to the Enterobacteriaceae family were isolated. The authors assumed that the disease of birches on the territory of Kazakhstan, diagnosed as bacterial dropsy belongs to another type of bacteriosis.

In the last published system of Enterobacterales order, the only Enterobacteriaceae family was divided into eight independent families [14]. The genus Erwinia on the one hand, and the genera Pectobacterium, Brenneria, Dickeya, on the other hand, was included in families Erwiniaceae and Pectobacteriaceae, accordingly [15, 16].

Hence, since the Republic of Kazakhstan belongs to the countries with a small area of forest plantations and at the same time, there is a degradation of forest plantations caused by worsening ecological situation, the necessity of enhanced phytosanitary control over forest plantations is undoubted. Bacterioses have a significant impact on the weakening of the forest stands. Therefore, establishing authentic pathogens of this disease in Kazakhstan is crucial for further phytosanitary control of the plantations. The purpose of this study was to identify the bacterial phytopathogen that provokes bacterial dropsy in the birch trees around the Nur-Sultan area through modern molecular genetic methods.

Experimental

Institution and Region of Study

The study was conducted in the laboratory of the Department of Biology and Genomics, L.N. Gumilyov

Eurasian National University, and based on the National Center for Biotechnology of the Republic of Kazakhstan. The materials for research were obtained from the 'Green Belt' artificial forests (Kyzylzhar, Vyacheslavka, and Arshaly) planted in summer and autumn months around the city of Nur-Sultan. The reason for choosing this forest is the large number of birches and their different ages (15-30 years). Also, on the territory of these forestry enterprises, birch forests planted in the 70s of the last century and natural birches are growing. It has been established, that numerous diseases on plantations of the artificial green belt, including bacterial dropsy, can spread from such old forests.

Characteristics of Plant's Condition

An approach used herein to describe the state of plants was taken from other studies [12]. Test sites with at least 200 Betula pendula trees were identified in the forest area. A total of 1000 trees were examined, and the age and morphometric parameters of plants were evaluated. The intensity of the disease was assessed by applying the following classification: class 1 – healthy plants, no signs of degradation; class 2 – slight weakening, rare bark bloating, no signs of degradation; class 3 – medium impact degree, the crown is partly defoliated, bark bloating all over the trunk, rare red-brown leaks; class 4 – severe weakening, the crown is very defoliated, numerous leaks and bloating of the bark; class 5 – recent deadwood, no living foliage in the crown and partially preserved only on the water sprouts; class 6 – old deadwood, fruiting bodies of the birch trumpet throughout the trunk (Piptoporus betulinus, and birch sapwood holes (Scolytus ratzeburgi).

Collection of Samples

Cores were removed from the birch trunks with signs of the disease using a special Haglof drill in the horizontal direction (length 100-500 mm, dm 4.3 mm) (Fig. 1a). The cores obtained (3-12 cm) were wiped externally with 90% ethanol, placed in sterile tubes, and stored in a refrigerator at 3-4ºC until further use (Fig. 1b). A total of 40 cores were taken, 10 from each location.

a) b)

Fig. 1. a) core removal from diseased birch (Betula pendula) using the Haglof drill; b) core samples in a sterile tube.

Bacterial Culture

Potato agar was used to isolate microorganisms from the cores and obtain pure strains, and standard meat-peptone was were used to enrich the cultures. Potato agar was prepared in the following way: 200 grams of raw peeled potatoes were poured into 1 liter of water and boiled for 30 minutes, the broth was put aside and filtered in a cold state. Then, the volume of the filtrate was brought to the initial, pH was adjusted to 7.1±0.1, 5 g of peptone with 25 g of agar were added, heated until complete dissolution of the latter, and filtered. Eventually, 1 g of chalk was added, and the mixture was sterilized for 30 minutes at a temperature of 120ºC. Standard meat-peptone broth had the following composition: peptic digest of animal tissue - 10 g/l, beef serum - 500 g/l, and sodium chloride - 5 g/l. The final pH value (at 25ºC) was 7.5±0.2.

Under sterile conditions, slices of 0.8-1 mm in size were cut out from the inside of each radial core in the laminar box chamber (20-30 slices from each core) and placed on nutrient medium in Petri dishes. The inoculated nutrient medium was placed in an incubator at 26.5ºC. Emerging colonies were monitored daily and characterized subsequently (colony formation and shape, density, color, and growth rate). Re-plating was used to isolate bacterial colonies from other microorganisms in the nutrient medium. Isolation of pure culture was carried out following the Drygalsky method.

Isolation of DNA from Bacterial Cultures

Of all the obtained isolates, one the most typical strain by its morphological characteristics was selected for molecular genetic studies, the colonies of which showed the greatest growth on nutrient media.

Following Wilson's method [20], the culture in a glass tube was first transferred to a plastic tube to isolate DNA from bacterial cultures for genetic typing, and 1 ml of culture was then used. The sample was centrifuged at 12.000 rpm for 10 minutes. The precipitate was mixed (suspended) with 500 µl of TE buffer, and 20 µl of lysozyme was added and incubated at 37ºC for 1 hour. Then, 30 µl of 10% SDS and 3 µl of proteinase K (20 mg/ml) was added. To remove fragments of the cell membrane, polysaccharides, and protein residues, 100 µl of 5 M NaCl was added. Afterward, it was stirred in a vortex solution, including 80 µl of CTAB mixture (07 M NaCl in 10% CTAB). After repeated stirring in the vortex, the mixture was incubated at 65ºC for 10 min, and the isolated DNA was purified by phenol/chloroform method.

For this purpose, 700 µl of chloroform/isoamyl alcohol (24/1) was added to the resulting suspension,

shaken, and centrifuged for 10 minutes at 12.000 rpm. The separated portion of liquid was poured into a new test tube with the addition of another phenol/chloroform (1:1) mixture and centrifuged again. At that, unwanted proteins and cell membrane residues from the culture were removed. Afterward, the top layer of the liquid (1.5 ml) was poured into another test tube, and 600 µl of isopropyl alcohol was added with the subsequent DNA precipitation. The DNA deposited at the bottom of the tube was washed with 70% ethanol. Subsequently, 100 µl of TE buffer was added to purified DNA samples and placed in a freezer at -20ºC. The concentration of isolated DNA samples was determined using a NanoDrop spectrometer at a wavelength of 260 nm.

Amplification of the 16SrRNA Gene Fragment

PCR was performed with the following universal primers: 8f 5' – agagtttgatcctggctcag-3 and 806R -5'ggactaccagggtatctaat with a total reaction volume of 20 µl. The PCR mixture contained 150 ng of DNA, 1 unit of MaximaHotStartTaqDNAPolymerase (Fermentas), 0.2 mM dNUF (deoxynucleoside triphosphate) each, one-time PCR buffer (Fermentas), 2.5 mM MgCl2, each with 10 p/mol primer. According to the PCR amplification program, denaturation was performed first at 95ºC, and then in 30 alternating cycles: 30 seconds at 95ºC, 40 seconds at 55ºC, 1 minute at 72ºC, and the final extension lasted 7 minutes at 72ºC.

Determination of the Nucleotide Chain

The enzymatic method of exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas) was used to purify PCR products from unbound primers. The sequencing reaction was performed using the BigDye® Terminator v3.1 loop sequence kit (Applide Biosystems) in the DNA Analyzer (Applide Biosystems) 3730x, automated genetic analyzer via sequencer that arranged the nucleotide chain into fragments. The PCR analysis was performed using a BioRad T100 amplifier (BioRad).

As described above, the nucleotide sequences 8F and 806R 16SrRNA were determined based on the sequencing of pure culture strains isolated from diseased tree trunks (cores). Afterward, the end fragments (low-quality primers and nucleotide sequences of the fragments) were removed. The resulting sequences were compared in the Genbank from NCBI database using BLAST (Basic Local Alignment Search Tool) and phylogenetic genealogy, showing the associated species and their relationships (similarities). The results were calculated for 650 bp of the sequential 16SrRNA chain. Phylogenetic genealogy was created in a maximum close way to the reality of a computer software package Clustal Omega <Multiple Sequence Alignment <EMBL-EBI.

Results

Analysis of Phytopathological Condition of Birch

Analysis of the phytopathological condition of birch (Fig. 2) on trial planting areas with the trees aged 15-30 years showed that 32% of trees were affected by bacterial dropsy in Kyzylzhar, 27% in Vyacheslavka, and 28% in Arshaly. At that, plants with light and medium impact degrees prevailed. In an area of birch forests planted in the 70s with the inclusion of natural birches, bacterial dropsy was detected in 46% of plants, with a medium and severe degree of damage prevailing (Fig. 3a). However, separate groups of dead plants were detected in all forest stands (Fig. 3b).

Characteristics of 5-day-old Bacterial Colonies

After five days, 12 similar colonies of bacteria were obtained. The characteristics of 5-day-old bacterial colonies that began to grow in a nutrient medium (Fig. 4) were as follows: colonies were located along the edge of the slices, the edges are unbranched, uneven, wavy, white, and are disposed at a distance of 0.2-0.4 cm from kernel disks. The purified 5-day inoculated colonies had a round shape, glossy, white, pale, and

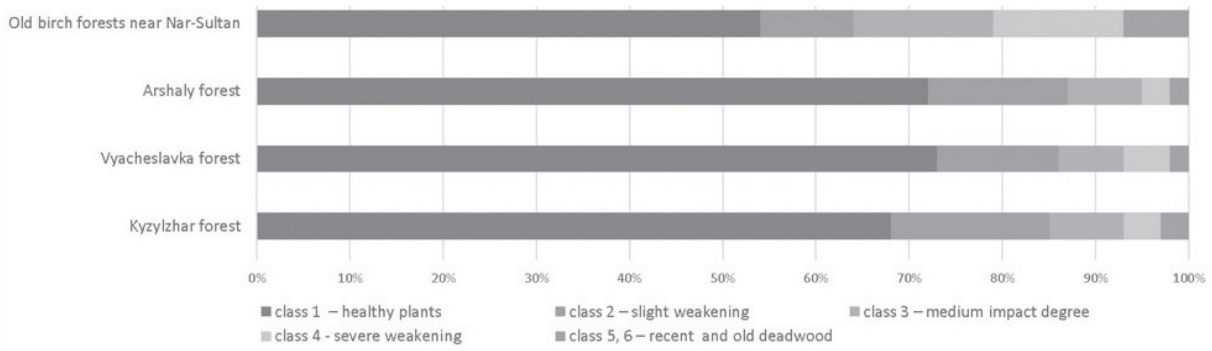

Fig. 2. Phytopathological condition of birch in different areas studied. Kyzylzhar forest, Vyacheslavsky forest and Arshalin forest - the trees are 15-30 years old, the old forest near Nur-Sultan is about 50 years old.

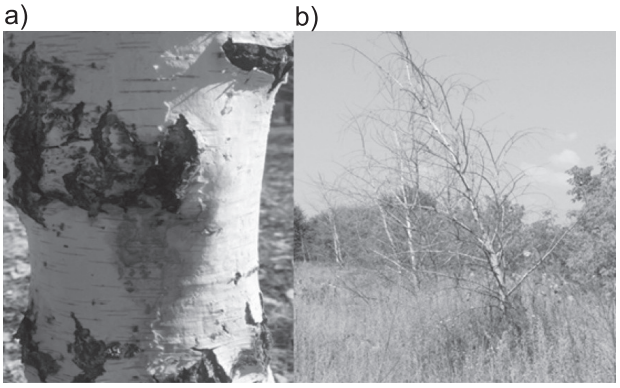

Fig. 3. Birch with bacterial dropsy: a) foci of dropsy located in the trunk; b) mass dying.

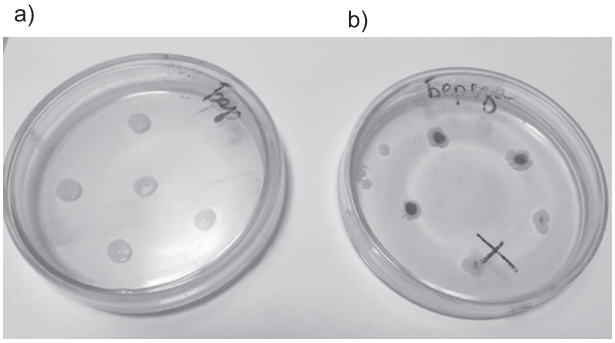

Fig. 4. a) the first 5-day vaccination; b) 5-day purified vaccine.

smooth edges, dense texture, were thick with the sizes of 9x10 mm, 4x7 mm, 3x6 mm, 4x6 mm, and 5x9 mm.

The Nucleotide Chain Sequence of the Studied Strain

Bacterial strain isolated from the birches with bacterial dropsy and cultivated in a laboratory nutrient medium was labeled as DDdd-1 strain. The nucleotide chain sequence of the studied DDdd-1 strain showed that its 16SrRNA gene fragment in Genbank from NCBI is similar (homologous) to Dickeya dadantii by 96.72%. The phylogenetic relationship with other species was determined based on the nucleotide sequence of this strain (Fig. 5). In the phylogenetic tree of the test sample, the strain DDdd-1 -1 16SrRNA showed a close relationship with the bacteria NR041921.1 Dickeya dadantii, KJ541470.1 Erwinia chrysanthemi, and AF373175.1 Erwinia chrysanthemi in Genbank from NCBI on nucleotide chains. In the dendrogram, the DDdd-1 strain of Dickeya dadantii (strain examined in this study) corresponded to the group of Erwinia chrysanthemi strains selected from the Genbank from NCBI. Therefore, the DDdd-1 strain corresponded to a cluster uniting two closely related species – Dickeya dadantii and Dickeya chrysanthemi (syn. Erwinia chrysanthemi, syn. Pectobacterium chrysanthemi).

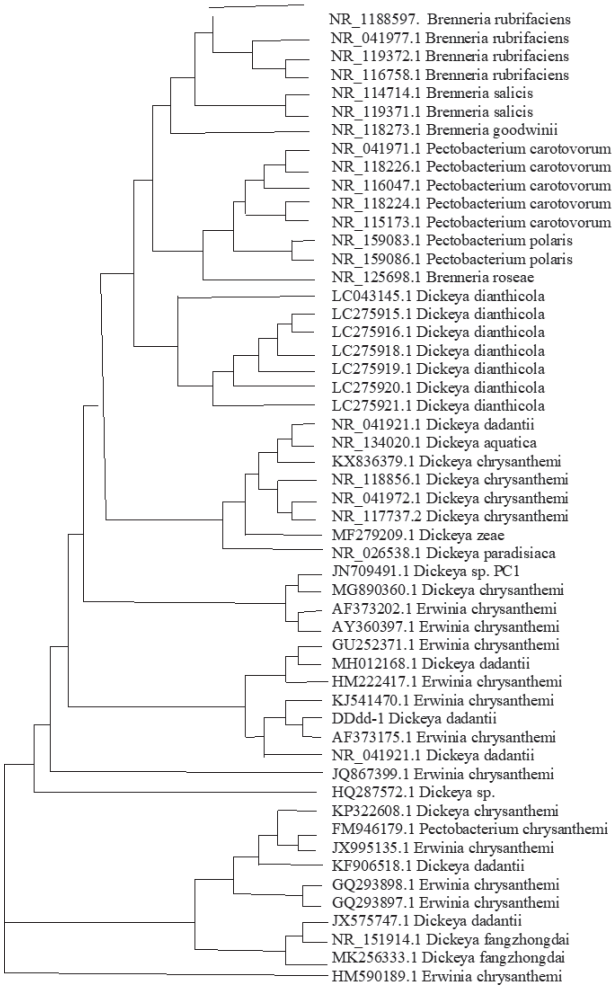

Fig. 5. Phylogenetic family tree of the test sample.

However, considering that only one locus was analyzed and a relatively small homology of 96.7% was established, the belonging of the strain to Dickeya dadantii cannot be stated with absolute certainty. Perhaps, the studied strain belonged to another species, sequences of which are not available in the database.

Discussion

The question of identifying authentic pathogen of bacterial dropsy. Currently, eight independent families are distinguished within the order of Enterobacterales, and the Erwinia genus is included in the Erwiniaceae family [14]. Erwinia nimipressuralis was indicated as a causative agent of hardwood bacterial dropsy in Ukraine [17] and coniferous species in Central Siberia [10]. The species Erwinia nimipressuralis was believed to affect beech and fir, while Erwinia multivora was considered to influence only birch.

Nevertheless, in European literary sources, the rank of independent species was not indicated, and it was cited as an atypical bacterium for the genus Erwinia with ‘questionable pathogenic properties’ [18-21].

According to available data, E. nimipressuralis cannot produce pectolytic enzymes and cause tissue maceration. Besides, it does not affect roots and seeds, which contradicts the phytopathological picture of bacterial dropsy. Based on 16S rRNA analysis, this species of bacteria was established to be closely related to the bacteria of the genus Enterobacter. Thus, the species was reclassified as Enterobacter nimipressuralis, [21], which confirms the inability of bacteria of this genus to produce pectolytic enzymes.

On the other hand, Erwinia multivora is capable of producing pectolytic enzymes that cause tissue maceration and affect roots and seeds [1, 5]. Undoubtfully, E. nimipressuralis and E. multivora are different species. Therefore, for a better understanding of the problem, a brief description of the taxonomic transformations of bacteria from the genus Erwinia is quite relevant. In the beginning, the name Erwinia was used. Then, several species were renamed to emphasize their belonging to the genus Pectobacterium [22]. However, the species name Pectobacterium stopped to be used by the 90s, and species provoking tissue maceration were singled out within the genus Erwinia into the carotovora group. Based on the biochemical characteristics, the genus Erwinia was divided into four groups, one of which was Erwinia carotavora affecting the surface and underground (like tubers) parts of various plant families and causing symptoms, such as tissues darkening and rotting and leaves curling and yellowing [23].

Based on the analysis of 16S rDNA, divided the species of the genus Erwinia into three phylogenetic groups: the first group is the genus Erwinia itself, the second group is the genus Pectobacterium, to which species E. carotovora and E. chrysanthemi were transferred among others, and the third group is the genus Brenneria [15]. Using phenotypic testing, DNA-DNA hybridization, serology, and 16S rDNA, Pectobacterium chrysanthemi may be classified as belonging to the new genus Dickeya [12]. Besides, some new species have been described in this genus, including D. dadantii. According to the last system of Enterobacterales order, the bacteria of Brenneria, Pectobacterium, and Dickeya genera are included in the Pectobacteriaceae family. All these bacteria are capable of producing pectolytic enzymes and inciting tissue maceration. In terms of determining the authentic pathogen of bacterial dropsy, bacteria Pectobacterium carotovorum and Dickeya chrysanthemi deserve special attention.

Pectobacterium carotovorum has been determined to be similar to E. multivora by physiological and biochemical properties. Also, this bacterium is capable of producing pectolytic enzymes and inciting tissue maceration. However, for a long time, P. carotovorum was believed to cause soft rot of vegetable crops, unlike the last species that affect woody plants. Nevertheless, some specific variants of this species were confirmed to provoke 'Slime flux' on many woody plants [24]. Some authors suggest considering the identity of P. carotovorum and referring the first species as a pathovar of the second one on the woody vegetation [1]. At the very minimum, E. multivora requires reclassification as P. multivorum. In previous studies of the bacterial birch dropsy performed in Kazakhstan, the pathogen causing this bacteriosis was identified as E. multivora [2]. However, the following examination did not reveal a presence of E. multivora in any of the samples, although, in 30% of the samples, bacteria similar to Pectobacterium carotovorum and Dickeya dadantii in genetic structure was detected [9].

The authors concluded that for a long time, the disease of birch forests in Kazakhstan was incorrectly diagnosed as bacterial dropsy, although it referred to the generalized bacterial diseases of forest species. Performed research confirmed the presence of Dickeya dadantii bacteria in the birch forests near Nur-Sultan and its probable involvement in the pathogenesis of this pathology in woody plants of Kazakhstan. Besides, the data obtained showed a close relationship of the strain DDdd-1 Dickeya dadantii to some strains of Dickeya chrysanthemi.

The species identified in International "Genbank" from NCBI as Erwinia chrysanthemi with index numbers KJ541470.1 and AF373175.1 are assumed to be identical to D. dadantii. Its difference from other bacteria implies maximum aggressiveness at high temperatures, possibility to be transferred from plant to plant by insects, rapid spread through the plant's conduction system to the plant's body, and delay at low temperatures.

The analysis of multilocus chains of recA, dnaX, rpoD, gyrB, and 16S rDNA was performed. Based on the results of PCR genomic fingerprinting and biochemical tests, Japanese scientists isolated the genus Dickeya from 24 different plant species and classified them into six groups: 1st group is D. chrysanthemi, 2nd group is D. dadantii, 3rd group is D. dianthicola, 4th group is D. zeae, and 5th and 6th groups included newly identified species [25]. Dickeya chrysanthemi is recognized as one of the most pathogenic bacteria. Subsequent studies have shown that it causes bacteriosis in sixteen dicotyledonous and ten monocotyledonous plants. This species is also known to cause damages in plants of many tropical and subtropical regions by initiating a light rot. D. dadantii incites necrosis, biodegradation, and 'soft rot' of plants, usually affecting potato tubers, vegetable bulbs, and ornamental crops. The species of the genus Dickeya has not yet been distinguished from woody plants. Based on the results of this study, the strain DDdd-1 is considered as pathovar D. dadantii pv. betulae. Nevertheless, the role of Dickeya chrysanthemi and D. dadantii in the pathogenesis of bacterial birch dropsy requires further research. However, considering that only one locus was analyzed and a relatively small homology of 96.7% was established, the belonging of the strain to Dickeya dadantii cannot be stated with absolute certainty. Perhaps, the studied strain belonged

to another species, sequences of which are not available in the Genbank from NCBI.

The limitation of this molecular genetic study is that only the 16S RNA amplification method was used and one of the most typical morphological characteristics of the strain was selected, the colonies of which showed the greatest growth on nutrient media. Therefore, this study should be considered as preliminary. Dickeya is a complex taxon that still requires extensive research into species composition, close family relationships, and host size and structure. The role of D. chrysanthemi and D. dadantii in the pathogenesis of bacterial dropsy birch requires further study using more extensive factual material, taking into account Koch postulates.

Conclusions

Thus, cultural, morphological, and molecular genetic studies of DDdd-1 bacterial strains, which were isolated from bacterial dropsy found in birches of the Green Belt around Nur-Sultan, and using a comparative analysis with the corresponding species of the International Genbank from NCBI, the isolated D. dadantii pv. betulae belongs to the genus Dickeya. Evidence of this can be seen in the phylogenetic genealogy of closely related species shown in Fig. 4. There is a reason to believe that species of the same genus Dickeya (noted in International Genbank as Erwinia chrysanthemi with index numbers KJ541470.1 and AF373175.1) as the species considered in this study (DDdd-1 Dickeya dadantii) are similar to Dickeya dadantii. It is because each researcher has named this pathogen (Dickeya chrysanthemi) in different ways, initiating, thus, a very wide range of adaptations to host plants. In general, Dickeya is a complex taxon that still requires a lot of research efforts on species composition, close family ties, and differences and structure of host plants.

Conflict of Interest

The authors declare no conflict of interest.

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