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THE GENOTYPIC PROPERTIES OF *PSEUDOMONAS LUPINI* –BACTERIAL AGENT OF LUPIN SPOTTINESS

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The G+C content in genome DNA and nucleotide sequence of 16S RNA of Pseudomonas lupini Beltyukova, Korolyova strains have been determined for the first time. On the basis of these results and earlier obtained genotypic properties of Pseudomonas lupini the majority of the strains belongs to Pseudomonas syringae. .In our opinion the only exception is strain 8531, which according to its genotype belongs to Pseudomonas savastanoi pv. glycinea.

Key words: *Pseudomonas lupini, Pseudomonas syringae, Pseudomonas savastanoi, G+C content, nucleotide sequence of 16S RNA.*

Pseudomonas lupini is considered to be the bacterial agent of lupin, Beltyukova, Korolyova. The specific name of the pathogen is identified on the basis of some phenotypic features [1, 6, 10]. In modern determinative manuals the separate species of *Pseudomonas lupini* is absent. The majority of phytopathogenic *Pseudomonas* is represented by 45 pathovars that belong to two phenotype-like species *Pseudomonas syringae* and *Pseudomonas savastanoi* [11, 12]. Molecular and genetic research methods, sequencing of 16S rRNA gene, molar content of guanine and cytosine in total genomic DNA and DNA-DNA-hybridization, in particular, are widely used in bacterial systematization in the recent years [14, 16, 17, 18, 20]. At the same time, the polyphase approach which includes detailed bacteria research, *i.e.* the integration of analysis of phenotypic features with molecular and genetic research [7, 8, 18], is useful in questionable issues of bacteria systematization. We studied a wide complex of phenotypic features of *P.lupini* earlier [2, 3, 4, 5]. 22 strains of *Pseudomonas lupine* according to pathogenicity for 10 species of plants, morphologo-cultural, physiological and 49 biochemical properties, antigenic and fat acids content of total cell lipids are related to each other as well as to typical strains of *Pseudomonas syringae pv. Syringae 8511* and *Pseudomonas savastanoi pv. phaseolicola 9066* [3].

As the analysis of phenotypic features did not give the final answer concerning species belonging bacterial agent of lupin spottiness, therefore, the aim of our research was to study genotypic features of the collection and new strains of *P.lupini*, namely molar content of G+C in genomic DNA and nucleotide sequence of the 16S rRNA gene of these strains.

Materials and Methods. 5 collections, 2 new strains of *P.lupini*, and the typical strain of *Pseudomonas syringae pv. syringae 8511* were chosen for our research (Table 1).

The extraction and purification of genomic DNA to determine G+C content were conducted according to commonly accepted methods [9, 13]. The molar content of guanidine and cytosine sum in percent (%) was determined by the analysis of DNA denaturation curve using spectrophotometer DU-8 (Becman). DNA melting

Table 1.
Strains used in the research

Strain, No.	Origin	Source of strain obtaining
Pseudomonas lupini collection 8531-8535.	lupin	The collection of the department of phytopatogenic bacteria of the Institute of Microbiology and Visurology NAS of Ukraine
Pseudomonas lupini 6, 17	lupin	Extracted by us
Pseudomonas syringae pv. syringae 8511- UCM** B1027T ATCC-19310, NCPPB*-281	lilac	UCM**

Note: * - NCPPB – National Collection of Plant Pathogenic Bacteria, UK
** - UCM – Ukrainian Collection of Microorganisms

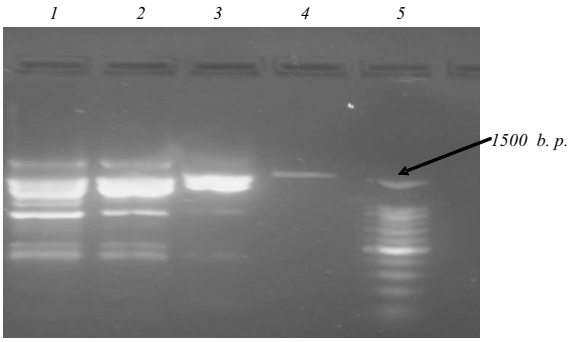

Fig.1 The search for optimal annealing temperature of primers at PCR: ***1***-50 C; ***2***-55 C; ***3***-60 C; ***4***-65 C; ***5***- marker DNA

temperature was determined as the midpoint on the function curve of relative absorption to temperature. G+C content in DNA was calculated as:

$\%G+C = (T_m\text{-}53.9?):0.41+ \%G+C$

T_m – melting temperature of DNA

%G+C – correction, which is calculated using DNA *E.coli* ATC 11775^T [9, 19].

To extract DNA for polymerase chain reaction (PCR) Silica Spin columns and the set of reagents "DNA-sorb-B" were used. The DNA copy of 16S rRNA gene was amplified using the universal primers pA - 5 - AGAGTTTGATCCTGGCTCAG-3 (8-27 in *E.coli* numbering system) and pH - 3 - AAGGAGGTGATCCAGCCGCA - 5 (1542-1523 in *E.coli* numbering system) at experimentally specified conditions, as follows, DNA denaturation – 94 C/15sec, annealing - 65 C/15sec, elongation - 72 C/5min, (Fig.1) [15].

The products of amplification were cloned using terminal transferase activity of Taq-polymerase into T-vector on the basis of pBLuescript SK(+) in EcoR**V** restriction site. 16S rDNA amplificate was sequenced by automatic sequencer 3130 Genetic Analyzer (Hitachi). The search for homologous nucleotide sequences of 16S rDNA, deposited in GenBank, was performed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Homologous sequences of nucleotides with likelihood were selected using AliBee program (http://www.genebee.msu.su/genebee.html). The topology of reconstructed maximum likelihood tree was created by the bootstraps method. The construction of maximum likelihood tree was performed on the basis of likelihood distance matrix using TreeTop program – Phylogenetic Tree Prediction (http://www.genebee.msu.su/services/phtree).

Results and Discussion. It is known that G+C content in genomic DNA is one of the most important features of a species. According to Yamamoto G+C content in genomic DNA for II cluster, inside *Pseudomonas* family, to which belong G^-, movable, rod-like microorganisms, capable of producing fluorescent pigment and growing at 4?C and not capable of growing at 41?C, varies in the range of 59.0-63.6 mol.%. The author also included so called ***syringae complex***, which consists of *Pseudomonas amygdali*, *Pseudomonas caripapayae*, *Pseudomonas ficuserctae*, the pathovars of *Pseudomonas syringae* and *Pseudomonas savastanoi* species [20] to this cluster. It was found that molar % of G+C varies from 58.5 up to 60.8 % (Fig.2), for the majority of the investigated strains of *P. lupini*, which, according to the literature data, corresponds to the values for *Pseudomonas syringae* and *Pseudomonas savastanoi* [16, 20].

In our research molar % of G+C in genomic DNA of typical strain 8511 values to 58.8%, which agrees with [16]. The only exception is *P.lupini* strain 8531, the molar % of G+C in genomic DNA of which valued to 62.5. It means that the G+C content in DNA of *P.lupini strain 8531* is 2.5mol.% higher than in other *P.lupini* strains and the typical strain, which according

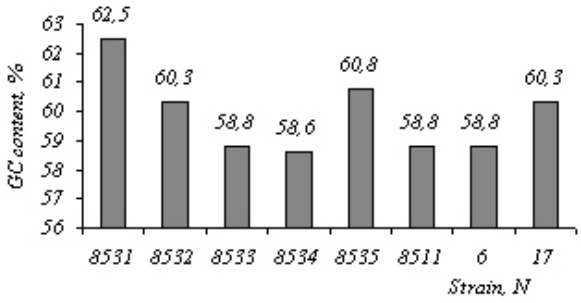

Fig 2. Molar % of G+C in DNA of *Pseudomonas lupini* and typical *Pseudomonas syringae pv. syringae 8511* strains.

Table 2.
The level of genotype likelihood of bacterial taxons determined by different methods.

Taxons, which are being compared	The level of likelihood		
	The difference in G+C content in DNA, mol. %	The level of DNA-DNA-hybridization, %	The level of likelihood of 16S rRNA genes, %
Families(or genus of one family)	*	* less than 15	* less than 90
The species of one genus	more than 10	not less than 20	91-98
Strains belonging to different species	from 1 to 10	less than 60-70	less than 97
The strains of one species	from 0 to 1	60-70	97.5-99.9

to the literature data [12] testifies to its belonging to another species (Table 2).

It was found that only *P.lupini* strain *8531* has 100% homology of 16S rRNA gene sequence with the referent strain *Pseudomonas savastanoi pv. glycinea* in the nucleotide database GenBank (Table 3).

As it is seen from the diagram, strain 8531 is the most genetically isolated in comparison with other investigated strains and typical *Pseudomonas syringae pv. syringae 8511* strain (Fig. 3).

It is worth mentioning that the higher value of G+C content in DNA, comparing to other strains by 2 mol.%, also has *P.lupini 8535*, and by 1.5 mol.% strains 8532 and 17. However, the strains *P.lupini 8535*, *8532* and *17* as well as the rest of strains have high likelihood of sequences of 16S rRNA gene with those deposited in GenBank nucleotide sequences of this gene of some pathovars, which according to modern scientific data belong to both species *Pseudomonas syringae* and *Pseudomonas savastanoi*.

Our results support completely the literature data about the high percentage of identity of nucleotide sequences of 16S rRNA gene in closely related species of *Pseudomonas syringae* and *Pseudomonas savastanoi* [20]. According to the data of Gardan *et al*., the pathovars of phytopathogenic pseudomonades *glycinea* and *phaseolicola*, which cause angular spottiness of soy and kidney beans are transferred from *P.syringae* to *P.savastanoi* species, exceptionally on

Table 3.
The identity of nucleotide sequences of 16S rRNA gene of P.lupini strains with nucleotide sequences of 16S rRNA gene of bacteria in the nucleotide database GenBank

Investigated strain number	Species name, pathovar (i) number of reference strain in the nucleotide database GenBank	The quantity of nucleotides in the fragment of 16S rRNA	The identity of sequences, %
8531	*P.savastanoi pv. glycinea* *P.syringae pv. syringae B 728a* *P.savastanoi pv. phaseolicola* *P.savastanoi pv. savastanoi ATCC 13522T* *P.syringae pv.pisi*	1478	100
8532, 8533, 8534, 8535, 6, 17	*P.savastanoi pv. glycinea* *P.syringae pv. syringae B 728a* *P.savastanoi pv. phaseolicola* *P.savastanoi pv. savastanoi ATCC 13522T* *P.syringae pv.pisi*	1476 1491	99

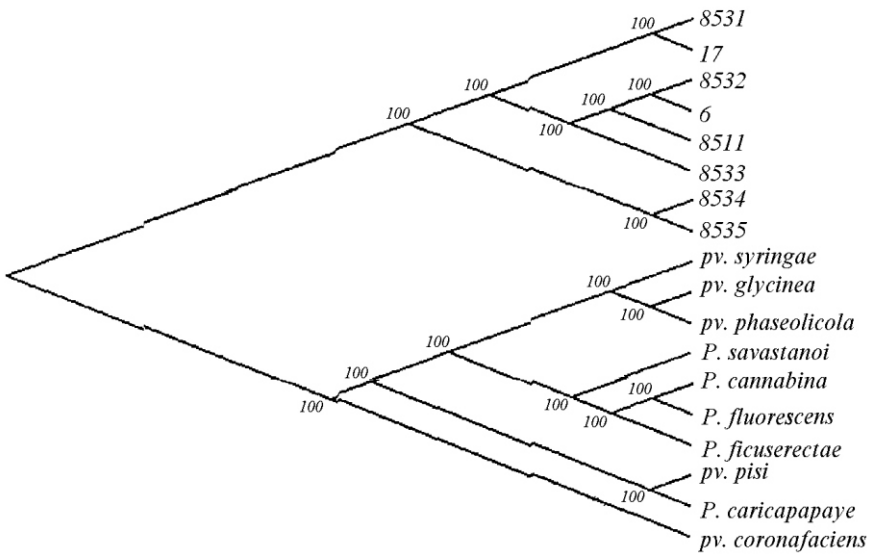

Fig. 3 Phylogenetic tree created on the basis of 16S rRNA gene sequence data, which reflects genetic relationship of strains.

the basis of the results of DNA-DNA hybridization [16, 20, 21]. Thus, the level of likelihood of DNA, found as the result of DNA-DNA hybridization, between typical strain *P.savastanoi 1392* and typical strains of pathovars *phaseolicola* and *glycinea* valued to 72-83%, and between these strains and typical strain *P.syringae pv. syringae 1670*^{T} – 50-56%, which evidences the author's opinion, belonging of pathovars *phaseolicola* and *glycinea* to *P.savastanoi* species [20]. This reclassification is still considered to be the questionable issue in phytopathogenic pseudomonades systematization [17, 18].

Therefore, the previously performed phenotype features analysis and the presented analysis of genotypic features in the current paper testify to the absence of *Pseudomonas lupini* species in nature. The majority of investigated strains belong to the *Pseudomonas syringae* species according to previously shown phenotypic likelihood and recently investigated G+C content in genomic DNA, nucleotide sequence of 16S rRNA gene with typical strain *Pseudomonas syringae pv. syringae* 8511. The exception is strain *8531* which was ascribed by us to *Pseudomonas savastanoi pv.glycinea* on the basis of G+C content of genomic DNA and the nucleotide sequence of 16S rRNA gene.

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Генотипические свойства возбудителя бурой бактериальной пятнистости люпина *Pseudomonas lupini*

Резюме

Определено содержание GC-пар в геномной ДНК и нуклеотидная последовательность гена 16S рРНК штаммов P. lupini Beltjukova, Koroljova. На основе этих и полученных ранее фенотипических признаков большинство штаммов P. lupini отнесено к Pseudomonas syringae. Исключение составил штамм P. lupini 8531, который из-за сродства GC-состава геномной ДНК и нуклеотидной последовательности гена 16S рРНК отнесен нами к Pseudomonas savastanoi pv. glycinea.

Ключевые слова: Pseudomonas lupini, Pseudomonas syringae, Pseudomonas savastanoi GC-состав ДНК, нуклеотидная последовательность гена 16S рРНК.

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