Molecular identification of *Oidium neolycopersici* on greenhouse tomato in Kazakhstan

Alexandr Pozharskiy[1,2*], *Aruzhan* Mendybayeva[1], *Gulnaz* Nizamdinova[1], *Valeriya* Kostyukova[1], *Dilyara* Gritsenko[1]

[1]Institute of Plant Biology and Biotechnology, Almaty, 050040, Kazakhstan
[2]al-Farabi Kazakh National University, Almaty, 050040, Kazakhstan

Abstract. Powdery mildew caused by *Oidium neolycopersici* is a harmful diseases of tomato widespread all over the world, particularly affecting tomato plants in greenhouses. Despite the occurrence of the disease in Kazakhstan, no studies on molecular identification of the pathogen were conducted to date. In the present work, third generation sequencing of partial rRNA gene sequence was used to identify the isolate of the powdery mildew fungus from a local greenhouse, in addition to traditional identification based on symptoms and micromorphology. As a result, 5,693 reads specific to the target genomic region allowed to obtain a partial consensus sequence rRNA gene of 499 bp length. This sequence was used to identify the fungus. Although the specificity of this sequences was not certain within genera *Erysiphe* and *Pseudoidium,* in addition to morphological and pathological traits, it provided sufficient data to identify the found fungal isolate as *Oidium [Erysiphe] neolycopersici.* More precise identification and characterization require additional studies using purified genetic material of the fungus.

1 Introduction

Powdery mildew is a group of infectious diseases of plants caused by ascomycetes of Erysiphaceae family, class Leocyomycetes. With more than 800 species capable of infecting about 10,000 host plant species [1,2], powdery mildews pose a significant threat virtually for all economically important plants. Erysiphaceae are obligate biotrophic parasites colo-nizing plant epidermis [3]; except for hemiendophytic genera of tribe Phyllactinieae, most species have epiphytic localization [4]. General symptoms of powdery mildews include typical white powdery spots on leaves, shoots, or fruits, spreading over the plant and caus-ing necrotic damage and wilting [2]. Most species have dual classification due to their complex life cycle with asexual (anamorph) and sexual (teleomorph) stages with differing morphology [5].

For tomato, three powdery mildew fungi are known: Oidium neolycopersici L. Kiss synonyms Pseudoidium neolycopersici, Erysiphe neolycopersici) [6], Leveilulla taurica (Lev.) G. Arnaud (syn. Erysiphe taurica Lev.) [7], and Oidium lycopersici, which is an endemic species

* Corresponding author: aspozharsky@gmail.com

of Australia [6]. Leveilulla taurica, assigned to Phyllactinieae, demonstrates signif-icant differences in symptoms, whereas two Oidium species are similar and were previously classified as one species [6]. Since 1986, when a first significant outbreak was observed in Netherlands, Oidium neolcypersici became an important problem worldwide, as the usual greenhouse conditions are very favorable for the growth of the fungus [8].

Despite the wide occurrence of powdery mildew on tomato plants in open ground farms and greenhouses, to date no studies on formal identification of tomato powdery mildew using molecular methods were conducted in Kazakhstan. In the present work, we have used third generation DNA sequencing tecnhoology by Oxford Nanopore to obtain sequences of rRNA genes with internal transcribed spacers (ITS) and identify powdery mil-dew species, in addition to traditional phytopathology and microscopic observations. The obtained results will be useful for further development of plant pathology and protection on the contemporary molecular genetic foundations in our country.

2 Materials and Methods

2.1 Collection and maintenance of infected plant material

The tomato leaves displaying characteristic symptoms of powdery mildew were collected in the private greenhouse in Almaty region, Kazakhstan. The leaf fragments with white powdery spots were washed with standard phosphate buffer saline (PBS) solution (137 mM sodium chloride, 2 mM potassium chloride, 10 mM phosphate puffer pH 7.4; Amres-co, USA) to suspend conidia. Tomato plants were grown from the mixture of hybrid seeds on universal turf substrate, in the following conditions: 20°C, relative humidity 50%, 12 h light day period. One month old seedlings were inoculated by spraying with PBS suspension of powdery mildew conidia. The condition of the inoculated plants was monitored daily, and the passage of infection to the fresh healthy plants was conducted when needed to maintain a living culture of the fungus.

2.2 Light microscopy

The isolated fungal mycelium and conidia were prepared by pressing a tomato leaf frag-ment with white powdery spots to a glass slide covered with 10% glycerol solution in PBS. After drying on the air, the slide was fixed by quick heating with an alcohol burner, the leaf fragment was removed, and the fungus was fixed on the glass was stained for an hour with 0.05% methylene blue water solution, rinsed with distilled water, and dried on the air. Fungal mycelia were prepared by fixing fresh infected leaves in 96% ethanol overnight, followed by staining with Giemsa solution, washing in distilled water, and air drying. All fixed preparations were mounted for permanent storage using epoxy resin. Additionally, freshly obtained wet conidia preparations were observed using dark field and phase contrast microscopy.

2.3 DNA sequencing and data analysis

A suspension of conidia for PCR was prepared by shaking infected leaf fragments (about 2 cm^2) in 150 µL of the standard phosphate buffer for 30 min at room temperature.

Primers AITS (5'-CGATTGAATGGCTAAGTGAGG-3') and TW14 (5'-GCTATCCTGAGGGAAACTTC-3') were used for amplification of rRNA gene region, according to Bradshaw and Tobin [9]. PCR reaction mix was prepared in the final volume 50 µL and contained 1U Phusion® dDNA-polymerase (Thermo Fisher, USA) with 10 µL of supplied reaction buffer (five-fold stock solution), 1 μL of 10 mM dNTP mix, 2 μL of each primer (10 μM), and 5 μL of conidia suspension prepared immediately before use. Results of PCR were checked by electrophoresis in 1.5% agarose gel in tris-acetate electrophoretic buffer. The amplification product was quantified using Qubit with dsDNA Broad Range reagent kit.

Sequencing of the PCR product was carried out using Oxford Nanopore MinION device with FLO-MIN114 (v. R10) flowcell and SQK-RBK114-96 Rapid Barcoding library preparation kit, following manufacturer's protocols. Obtained reads were mapped using BWA-MEM tool with parameters recommended for Nanopore long reads [10]. The scaffold level genome assembly of Erysiphe [Oidium] neolycopersici ASM361085v1 (https://www.ncbi.nlm.nih.gov/genome/; accession number GCA_003610855.1) [11] was used as a reference for read mapping. UGENE software [12] was used to obtained consensus sequence covered by the reads. NCBI Blast tool [13] was used to identify the obtained consensus sequences.

3 Results and Discussion

The collected tomato leaf material with typical symptoms of powdery mildew was used to obtain the pathogen (Fig. 1, A). The symptoms were reproduced on tomato plants grown in the laboratory conditions (Fig. 1, B). In the used growth conditions, first signs of white powdery spots appeared about 14-16 days post inoculation; the appearing symptoms were rapidly spreading on nearby leaves and shoots, leading to their necrosis. Further inoculations with the secondary obtained infectious material demonstrated the similar diseases development.

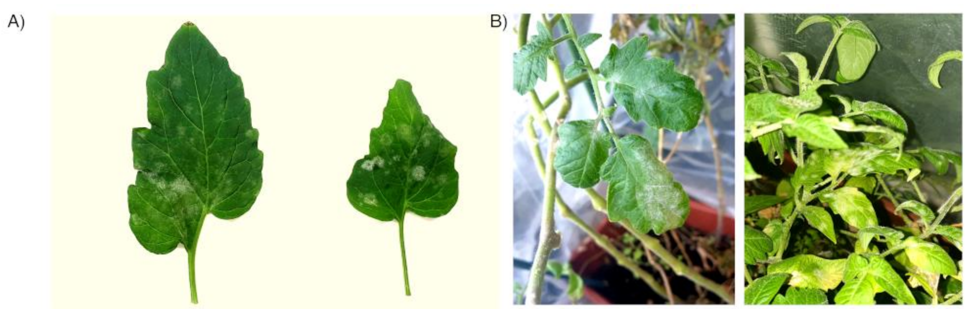

Fig. 1. Symptoms of powdery mildew of tomato: A) white powdery spots on tomato leaves collected in a greenhouse; B) white powdery spots on tomato plants after first and following inoculations

Microscopic examination was conducted throughout the in vivo cultivation of powdery mildew fungus. Several methods was used to observe micromorphology of the pathogen (Fig. 2). The morphology of conidia and conidiophores was in agreement with the published data on *Oidium neolycopersici* [6, 14]. Due to the relatively low humidity of growing environment, conidiophores were observed to form separate conidia rather than long pseudochains [15]. The morphological properties were stable across a series of inoculations, confirming the identity of the infectious agent.

The known specificity of powdery mildew species growing on tomato plants indicate two species, *Oidium neolycopersici* and *Leveilulla taurica* as the main candidates for the found pathogenic isolate. Along with the visual symptoms on the plants, the morphology of the fungus allowed to initially attribute it as *O. neolycopersici* rather than *L. taurica*.

The sequencing of partial rRNA gene was performed using long read Oxford Nanopore technology. Unlike the traditional Sanger sequencing, this method is useful for identification of sequences in case of mixed DNA, as it allows to sequence individual DNA molecules. It is important in case of rRNA genes and ITS, which are ubiquitous among all cellular organisms, so the amplification of sequences from a particular species is difficult. Indeed, only 9,044 of all 74,990 obtained reads were succesfully mapped against the reference genome of *Oidium neolycopersici*, and 5,693 reads were specific to the target region (rRNA gene located in scaffold MCFK01000579). After mapping and filtering poorly mapped reads out, a high quality partial consensus sequence of rRNA gene with length 499 bp was obtained. BLAST search results revealed high similarity with species of genera Erysiphe and Pseudoidium (Table 1). For all considered hits (100 best BLAST hits) the query coverage was 88%, identity from 89.6 to 90.06%, E-value near zero (not exceeding 10-147). *Erysiphe [Oidium] neolycopersici* and *E. aquilegiae*, phylogenetically close species [16], had the highest numbers of hits among identified sequences.

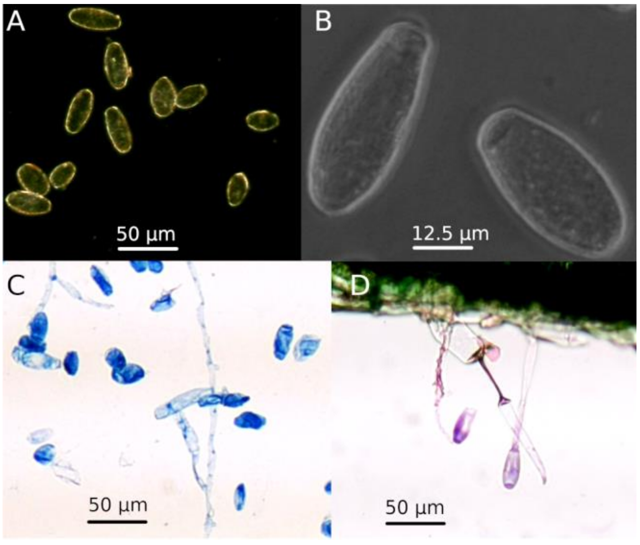

Fig. 2. Micromorphology of *Oidium neolycopersici.* Note: A) dark field microscopic image of conidia; B) phase contrast microscopic image of conidia; C) isolated hyphae, conidiophores, and conidia fixed by heat and stained with methylene blue; D) conidiophore on the leaf surface fixed with ethanol and stained with Giemsa solution.

Although the specific identification of the found powdery mildew fungus remains not finally conclusive for the obtained DNA sequence, in combination with morphological data, symptoms, and the host plant observation, it allows to conclude that it should be classified as *Oidium [Erysiphe] neolycopersici* with high degree of confidence. The results demonstrate that Nanopore Sequencing technology is suitable for targeted identification of plant pathogens; however, although this method can be used for mixed DNA samples, it still requires higher concentration of target DNA comparing to any side components, e.g. pathogen's vs host's DNA. For instance, more precise identification and characterization of the discovered isolate would require more accurate isolation of pure conidia and their DNA.

Table 1. Summary of BLAST search results for obtained partial consensus sequence of rRNA gene of candidate Oidium [Erysiphe] neolycopersicum isolate. Only species with more than one hit are shown

Species	**BLAST score**	**Number of hits**
Erysiphe aquilegiae	545	11
Erysiphe (unidentified species)	545	10
Erysiphe neolycopersici	545	8
Erysiphe chloranthi	545	5
Erysiphe sedi	545	5
Erysiphe ruyongzhengiana	545	5
Erysiphe begoniae	545	5
Erysiphe viburni	540	5
Erysiphe lonicerae	540	5
Erysiphe caricae-papayae	545	4
Erysiphe pseudoviburni	540	4
Erysiphe macleayae	545	3
Erysiphe takamatsui	545	3
Erysiphe cf aquilegiae HAL 3134 F	545	2
Erysiphe parnassiae	545	2
Erysiphe aquilegiae var ranunculi	545	2
Erysiphe lentaginis	540	2
Pseudoidium pedaliacearum	545	8
Pseudoidium hortensiae	545	4

4 Conclusion

The present work demonstrates the first results of use of third generation of sequencing technologies for targeted identification of tomato powdery mildew pathogen, *Oidium neolycopersici,* in Kazakhstan. Despite high economical importance of tomato production in Kazakhstan, and potential harm imposed by locally distributed and imported powdery mildew isolates, only limited efforts have been made to date to implement modern DNA based methods of pathogen identification and monitoring. The results of this work show that, although having shortcomings related to purity and specificity of analyzed samples, modern DNA sequencing methods are important tool to achieve this goal. In addition to traditional visual and microscopic methods, the retrieved fragment of rRNA gene sequence allowed to attribute species of the found fungal isolate. The further work aimed to improve purity and analyzed samples, increase data output and accuracy, will be important step in studies of powdery mildews spreading in our country, not only *Oidium neolycopersici* on tomato, but also harmful pathogens of other important crops.

Acknowledgment

The work was performed within the framework of a targeted funding program BR18574149 ‘‘Development of highly productive cultivars and lines of agricultural crops using innovative technologies’’ (Ministry of Science and Higher Education of the Republic of Kazakhstan).

References

1. U. Braun, The current systematics and taxonomy of the powdery mildews (Erysiphales): an overview. Mycoscience **52,** 210–212 (2011). https://doi.org/10.1007/S10267-010-0092-1
2. D.A. Glawe, The powdery mildews: a review of the world's most familiar (yet poorly known) plant pathogens. Annual Review of Phytopathology **46,** 27–51 (2008). https://doi.org/10.1146/annurev.phyto.46.081407.104740
3. R. Hückelhoven, Powdery mildew susceptibility and biotrophic infection strategies. FEMS Microbiology Letters **245,** 9–17 (2005). https://doi.org/10.1016/j.femsle.2005.03.001
4. Y. Mori, Y. Sato, S. Takamatsu, Evolutionary analysis of the powdery mildew fungi using nucleotide sequences of the nuclear ribosomal DNA. Mycologia **92,** 74–93 (2000). https://doi.org/10.1080/00275514.2000.12061132
5. U. Braun, The impacts of the discontinuation of dual nomenclature of pleomorphic fungi: the trivial facts, problems, and strategies. IMA Fungus **3,** 81–86 (2012). https://doi.org/10.5598/imafungus.2012.03.01.08
6. L. Kiss, R.T.A. Cook, G.S. Saenz, J.H. Cunnington, S. Takamatsu, I. Pascoe, M. Bardin, P.C. Nicot, Y. Sato, A.Y. Rossman, Identification of two powdery mildew fungi, Oidium neolycopersici sp. nov. and O. lycopersici, infecting tomato in different parts of the world. Mycological Research **105,** 684–697 (2001). https://doi.org/10.1017/S0953756201004105
7. J.C. Correll, T.R. Gordon, V.J. Elliott, Host range, specificity, and biometrical measurements of *Leveillula taurica* in California. Plant Disease **71,** 248–251 (1987)
8. A. Lebeda, B. Mieslerová, M. Petřivalský, L. Luhová, M. Špundová, M. Sedlářová, V. Nožková-Hlaváčková, D.A.C. Pink, Review of tomato powdery mildew - A challenging problem for researchers, breeders and growers. Acta Horticulturae **1159**, 107–115 (2017). https://doi.org/10.17660/ActaHortic.2017.1159.17
9. M. Bradshaw, P.C. Tobin, Sequencing herbarium specimens of a common detrimental plant disease (powdery mildew). Phytopathology **110,** 1248–1254 (2020). https://doi.org/10.1094/PHYTO-04-20-0139-PER
10. H. Li, R. Durbin, Fast and accurate long-read alignment with Burrows–Wheeler transform. Bioinformatics **26,** 589–595 (2010). https://doi.org/10.1093/bioinformatics/btp698
11. Y. Wu, X. Ma, Z. Pan, S.D. Kale, Y. Song, H. King, Q. Zhang, C. Presley, X. Deng, C.-I. Wei, S. Xiao, Comparative genome analyses reveal sequence features reflecting distinct modes of host-adaptation between dicot and monocot powdery mildew. BMC Genomics **19,** 705 (2018). https://doi.org/10.1186/s12864-018-5069-z
12. K. Okonechnikov, O. Golosova, M. Fursov, A. Varlamov, Y. Vaskin, I. Efremov, O.G. German Grehov, D. Kandrov, K. Rasputin, M. Syabro, T. Tleukenov, Unipro UGENE: A unified bioinformatics toolkit. Bioinformatics **28,** 1166–1167 (2012). https://doi.org/10.1093/bioinformatics/bts091
13. G.M. Boratyn, C. Camacho, P.S. Cooper, G. Coulouris, A. Fong, N. Ma, T.L. Madden, W.T. Matten, S.D. McGinnis, Y. Merezhuk, Y. Raytselis, E.W. Sayers, T. Tao, J. Ye, I. Zaretskaya. BLAST: a more efficient report with usability improvements, Nucleic Acids Research **41,** W29–W33 (2013). https://doi.org/10.1093/nar/gkt282
14. H.-Y. Hsiao, H.A. Ariyawansa, C.-C. Hsu, C.-J. Wang, Y.-M. Shen, New Records of Powdery Mildews from Taiwan: Erysiphe ipomoeae comb. nov., E. aff. betae on Buckwheat, and E. neolycopersici comb. nov. on *Cardiospermum halicacabum.* Diversity **14,** 204 (2022). https://doi.org/10.3390/d14030204
15. W. Oichi, Y. Matsuda, T. Nonomura, H. Toyoda, L. Xu, S. Kusakari, Formation of

conidial pseudochains by tomato powdery mildew *Oidium neolycopersici*. Plant Disease **90,** 915-919 (2006). https://doi.org/10.1094/PD-90-0915

16. A. Lebeda, B. Mieslerová, M. Petřivalský, L. Luhová, M. Špundová, M. Sedlářová, V. Nožková-Hlaváčková, D.A.C. Pink, Resistance mechanisms of wild tomato germplasm to infection of *Oidium neolycopersici*. Eur J Plant Pathol **138.** 569–596 (2014). https://doi.org/10.1007/s10658-013-0307-3