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LUTSENKO V. A.[1], POLISHCHUK L. V.[1], HONG J.[2], FOMINA M. A.[1]✉
[1] D.K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine, Ukraine, 03143, Kyiv, Acad. Zabolotnoho str., 154, e-mail: M.Fomina@ukr.net
[2] Marine Biodiversity Institute of Korea, Korea, 33662, Chungcheongnam-do
✉ *M.Fomina@ukr.net*

XEROTOLERANT STRAIN OF *PENICILLIUM CHRYSOGENUM* MF18_10 ISOLATED FROM THE DAMAGED WALLS OF SAINT SOPHIA'S CATHEDRAL, KYIV

Aim. The aim of this work is to determine the taxonomic position of the fungal strain MF18_10 isolated from the damaged wall with medieval painting of St. Sophia's Cathedral (Kyiv, Ukraine). ***Methods.*** The fungus was isolated on selective medium for xerophilic fungi (70% sucrose Czapek agar). Macro- and micro-morphological phenotypic characterization was carried out using light and scanning electron microscopy. Molecular-biological identification was performed using nucleotide sequences of the ITS-fragment. ***Results.*** On the basis of phenotypical characteristics of the fungal isolate MF18_10, it was established its affiliation to the genus *Penicillium*. ITS analysis revealed that the isolate belongs to the species *P. chrysogenum* demonstrating 100% identity with other 78 *P. chrysogenum* strains in GenBank database including the type strains NR_077145 and AY373902, also sharing several distinct differences in substitutions, deletions and insertions within this group. ***Conclusions.*** The isolated xerotolerant fungus was identified as *P. chrysogenum*, the typical representative of the indoor environments and dust, and also common for mycobiota of damaged historic-cultural artifacts. The differences in the analyzed *P. chrysogenum* ITS primary structures did not correlate with the source of isolation.

Keywords: *Penicillium chrysogenum*, xerotolerant fungi, ITS, scanning electron microscopy.

Nowadays, the problem of the deterioration of cultural and historical monuments by microorganisms is becoming more and more relevant. Many historically valuable buildings all over the world undergo biodegradation, especially through microscopic fungi – micromycetes. Recently, the dark-stained deterioration has been discovered in some parts of the unique medieval murals of Saint Sophia's Cathedral in Kyiv, Ukraine, which could be caused by micromycetes. The knowledge of the nature of deterioration and the microorganisms involved is essential for fighting microbial invasion and saving cultural and historical heritage [1–5].

Among the areas of the ribosomal cistron, the region of the internal transcribed spacer (ITS) has the highest probability of successful identification for a wide range of fungi, with the most clearly distinguished barcode gap between interspecific and intraspecific variations, and therefore ITS was proposed as a standard barcode for fungi. ITS region combines the highest resolution to distinguish similar species with the high PCR and sequencing success in a wide range of fungi [6].

This work was aimed at the phenotypic and ITS-based molecular-biological identification of the culturable fungus isolated by us from the deteriorated walls with medieval painting in St. Sophia's Cathedral (Kyiv, Ukraine).

Materials and methods

The culture of the microscopic fungus MF18_10 was isolated in 2018 from the dark-stained deterioration on the walls of St Michael Altar in St. Sophia's Cathedral in Kyiv, Ukraine. Considering the interior conditions in the Cathedral, the selective medium for xerophilic fungi 70% sucrose Czapek agar (CZA70S) was used for isolation. The phenotypic characteristics of colonies were also observed on malt extract agar supplemented with 10 % NaCl (MEA + 10% NaCl), Czapek yeast extract agar supplemented with 20% sucrose (CY20S), malt yeast extract agar supplemented with 40% sucrose (MY40S), potato dextrose agar supplemented with 15% NaCl (PDA + 15% NaCl), and malt extract agar (MEA). The isolate was incubated for 14 days at 25°C in darkness [1, 4, 7–9]. Light microscopy of the mycelium was performed using Primo Star Zeizz light microscope equipped with a Canon PowerShot A640 camera. Tescan Mi-

ra 3 LMU microscope was used for scanning electron microscopy (SEM).

In order to detail the taxonomic position of the fungal culture MF18_10, we analyzed the nucleotide sequence of the isolate's ITS-fragment containing the 5'-end of the 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, the 3'-end of the 26S rRNA gene [10–12]. Universal fungi primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' were used to amplify the ITS chromosome fragment [10]. The nucleotide sequence of the ITS of the isolate MF18_10 (585 bp) was placed in GenBank under the accession number MK367422 and its primary structure was used in our study as a reference sequence [10–12].

Results and discussion

The phenotypical features of the fungal isolate MF18_10 demonstrated its affiliation to the genus *Penicillium* (family *Aspergillaceae*, order Eurotiales, phylum Ascomycota) (Fig. 1A). It was shown that the isolate formed distinctive for the genus dense brush-like spores-bearing structures conidiophores terminated by the clusters of flask-shaped phialides (length around 6–9 μm) with the chains of smooth conidiospores (size 2–4 μm). Phialides were supported by secondary branches of a conidiophore metulae (length around 6–9 μm) arising from a smooth stipe (width around 2–4 μm). The ability of the isolate MF18_10 to grow on various media simulating the low water activity (Table 1) as well as on conventional malt extract agar indicated the xerotolerance of this fungus with the potential ability to grow and cause damage on the walls under conditions of low humidity in the Cathedral.

BLAST nucleotide analysis carried out, using the ITS primary structure of studied *P. chrysogenum* MF18_10 (MK367422, 585 bp), revealed more than 12,000 sequences of rRNA clusters of fungi with varying homology to the reference one.

The molecular size of the ITS sequences homologous to the reference MK367422 varied from 585 bp. (e.g. JX136719 for *P. chrysogenum* XF5) to 1258 bp (e.g. LT558875 for *P. chrysogenum* DI16-53). BLASTN analysis showed 898 hits of the fungal rRNA clusters for *P. chrysogenum* species with identities to the primary reference varying from 100% to 81% and query cover ranging from 100% to 9%. It should be noted, that some of the sequences have smaller molecular sizes than the reference ITS sequence MK367422. For example, the ITS sequence of *P. chrysogenum* SDSP (MG214658, 543 bp) includes ITS1, 5.8S rRNA and ITS2; whereas ITS sequence NCPF2881 (241 bp) consisted of only ITS1 and 5.8S rRNA; as well as the *P. chrysogenum* DPL-8 ITS (MF000932, 256 bp) contained only 5.8S rRNA and ITS2. Our study was focused only on the database ITS sequences with the query cover 100% compared to our reference. The ITS of 105 strains of the genus *Penicillium* and uncultured penicillia were found to be fully identical (100%) to the MK367422 primary structure with 100% query cover and e value = 0.0 (Fig. 1B). Most of them (74%) were the ITS sequences of *P. chrysogenum* strains, including the fragment of the rRNA cluster of the type strain *P. chrysogenum* CBS 306.48 (NR_077145, 585 bp) (Fig. 2).

As known, the *P. chrysogenum* strains are the most common among the penicillia and are found on all continents. The strains, chosen through ITS BLAST analysis for their 100% identity to the reference one, were reported to be isolated from the samples collected in Europe (Ukraine, Bulgaria, Slovakia, Italy), North America (Canada, USA), South America (Brazil, Costa Rica), Asia (Russia, China, Japan), and North Africa. A number of *P. chrysogenum* strains (DTO 103E7, DTO 149C1, DTO 149C2, DTO 149C3) were isolated from soil samples taken in the Dry Valley of Antarctica. Considered representatives of *P. chrysogenum* species were found both in the outdoor environment (strawberry fields, groves of olive trees, cave walls), and in the indoors (various buildings, houses, graves, museums). These fungi are also common on plants (grapes, hyssop, olive trees, kiwi, barley), processed foods (cheese, ham, bread products, yoghurt), and also reported in cosmetics and on documents. Some representatives of *P. chrysogenum* species might also be pathogenic for humans, insects and plants as well as the contaminant microorganisms of laboratory cultures or production processes.

Table 1. The colony diameter (mm) after *P. chrysogenum* MF18_10 growth on different agar media for 14 days at 25°C

MEA	MEA+10%NaCl	PDA+15 % NaCl	MY40S	CY20S	C70S
25-35	28-35	1-4	19-27	21-30	15-21

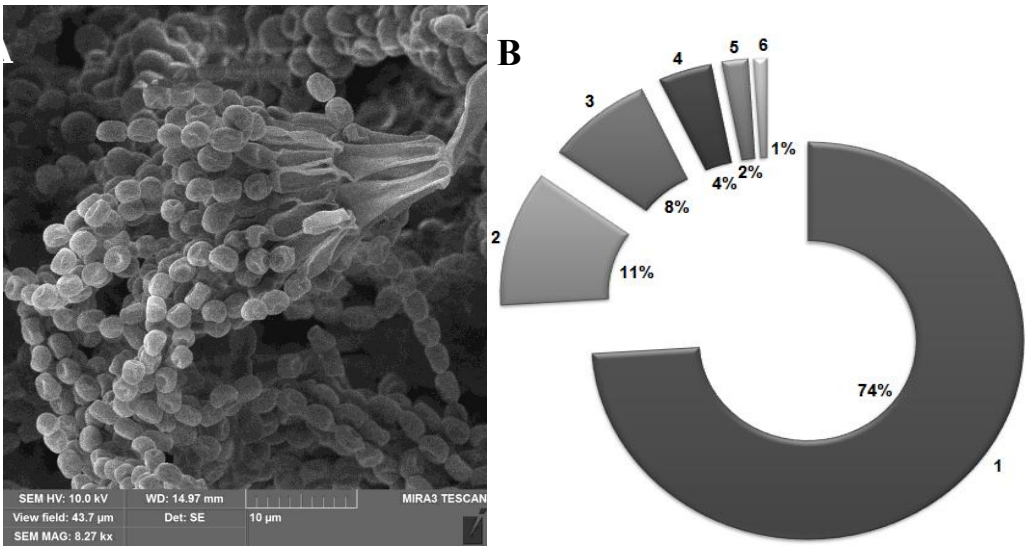

Fig. 1. (A) SEM of the conidiophore of *Penicillium* sp. isolate MF18_10; (B) ITS sequences for 105 representatives of *Penicillium* spp. with the primary structure identical (100%) to the isolate MF18_10: (1) *P. chrysogenum*, (2) *P. allii-sativi*, (3) *Penicillium* sp., (4) uncultured *Penicillium* fungi, (5) *P. tardochrysogenum*, (6) *P. desertorum*.

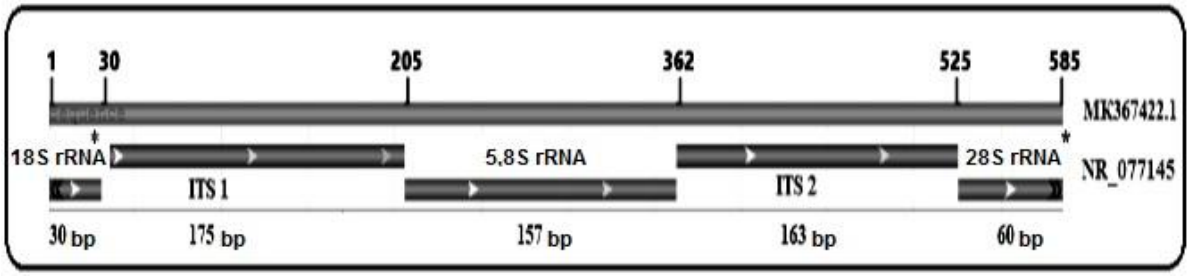

Fig. 2. ITS structure of *P. chrysogenum* strain MF18_10 (MK367422) compared to the type strain *P. chrysogenum* CBS 306.48 (NR_077145). * – a partial sequence of rRNA genes.

Considering that the *P. chrysogenum* MF18_10 strain was isolated by us from the medieval wall paintings, we were interested in other originated from similar environments representatives of this species with identical (100%) or highly homologous (99.9–96%) primary ITS structure. Among data for fully identical to the reference ITS sequences, several strains of this species were isolated from the surface of the art works: *P. chrysogenum* FX5 (JX136719) and *P. chrysogenum* FX43 (KJ780803) from wall paintings; *P. chrysogenum* DTO 149 B5 (JX997027) from oil painting; *P. chrysogenum* H09-022 (KC009774.1) and *P. chrysogenum* WL5-2B (MF422150) from 19th century collection items. Many of these strains were isolated from both walls and air samples taken in various parts of buildings, museums, tombs, industrial premises, and termitaries (Tables 2, 3) [9, 13, 14].

As a result of verified alignments, there were found ITS fragments of 93 *P. chrysogenum* strains with high homology values (identities = 99.8% – 96%; e value = 0.0; query cover = 100%). We determined the mismatches and gaps localization in their ITS structures (Fig. 3). It was found that 17.1% of the *P. chrysogenum* strains contained the inserts of 1 or 2 nucleotide residues corresponding to 6 nucleotide residues of the reference ITS. In addition, in 77% of the strains, the thymine residue corresponds to the 362nd cytosine residue of the reference ITS. In 14.5% of the strains, there are substitutions and gaps corresponding to 513rd and 516th residues. Inserts of 1 or 2 residues corresponding to the 577th nucleotide residue of the reference ITS were found in 3.1% of the strains (Fig. 3). Thus, both the regions of the spacers (ITS1 and ITS2) and the rRNA genes (18S rRNA and 28S rRNA) manifested the differences (substitutions and breaks) in the rRNA operons primary structure for the *P. chrysogenum* strains.

Table 2. Information about selected *P. chrysogenum* strains with ITS primary structures completely identical* to the isolated from St. Sophia's Cathedral *P. chrysogenum* MF18_10 (MK367422.1)

Strains	GenBank Accession N	Isolation sources
CBS 306.48	NR_077145	TYPE material, CBS collection, USA
FRR 807	AY373902	TYPE material, USA
JCM 22826	AB479305	the stone chambers in the Takamatsuzuka and Kitora Tumuli, Japan
F15	HQ380769	Mogao Grotoes caves, China
5	HQ380786	Mogao Grotoes caves, China
XF5	JX136719	surface of murals in Xu XianXiu's tomb, China
XF4	JX136718	surface of murals in Xu XianXiu's tomb, China
XF43	KJ780803	surface of murals in Xu XianXiu's tomb, China
DTO 149B5	JX997027	damaged oil painting, Ukraine: Kharkov
H09-022	KC009774	19-th century art collection, Costa Rica
WL5-2B	MF422150	19-th century art lamina, Costa Rica
DTO 102B2	JX996996	indoor environment, Canada
DTO 102B3	JX996997	indoor environment, Canada
DTO 102B5	JX996999	indoor environment, Canada
DTO 102B6	JX997000	indoor environment, Canada
DTO 102B9	JX997002	indoor environment, Canada
DTO 68C3	JX997046	indoor environment, Canada
DTO 68C5	JX997048	indoor environment, Canada
DTO313-A5	MF803947	house dust, USA
DTO312-I4	MF803946	house dust, USA
F4-02	JN561259	workshop on the production of rice wine, China
MT-12	MF765611	specimen voucher, China

Notes: * – identity grades of all strains were – Identities = 100%, E value = 0.0, Query cover = 100%.

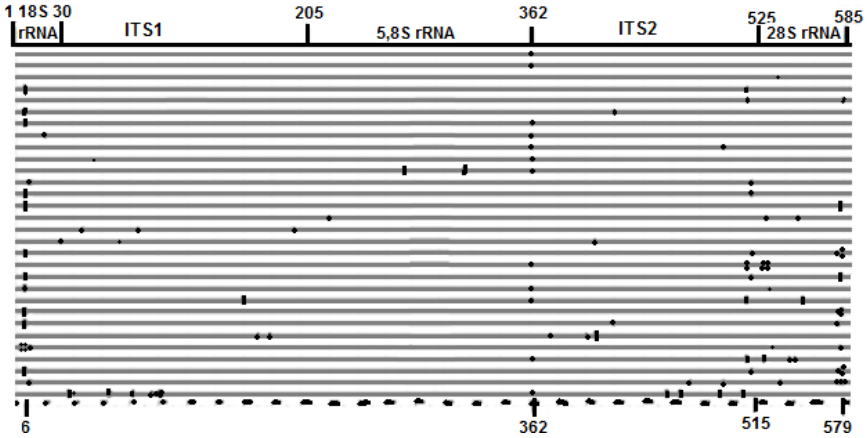

Fig. 3. The localization of nucleotide bases mismatches in the ITS region of the strain *P. chrysogenum* MF18_10 (MK367422) and the selected *P. chrysogenum* strains from GenBank.

It was found that nucleotide transitions take place more often (12 substitutions) than transversions (4 substitutions) (Table 3). The similar substitutions may happen for the strains originated from the diverse sources. There are the same substitutions in the nucleotide sequences of the ITS of *P. chrysogenum* strains JX997051 and JX996998 (transitions of 205 n.); EU833212 and KJ780802 (transitions of 259 n.); JX997044 and JX997045 (transitions of 514 n.). Some strains originated from geographically remote areas (e.g. EU833212 from Mexico and KJ780802 from China) as well as from the same locations (e.g. sequences of *P. chrysogenum* strains isolated from the surface of murals in Xu Xian Xiu's tomb, China KJ780802, JX136726, JX136729). Also, it was observed a certain trend in the substitutions in the ITS sequences originated from the Mogao Grottoes cave, China (HQ380775 and HQ380757): the ITS of these strains had the largest number of substitutions (Table 3).

Table 3. The differences in the primary structures of ITS region of selected *P. chrysogenum* strains (with identity 99.1–99.8%)[Ig] in comparison to the reference ITS of isolated from St. Sophia's Cathedral *P. chrysogenum* MF18_10 (MK367422)

Strains	GenBank Accession N	Identity (%) and mismatches in ITS primary structures (substitutions **R: X→Y**)	Isolation sources
DTO 87I2	JX997051	I=99.8% **R:** T→C(204 n.)* **Ts**	archive, Netherlands
102B4	JX996998	I=99.8% **R:** T→C (204 n.) **Ts**	house dust, USA
DTO 102B7	JX997001	I=99.8% **R:** A→G (514 n.) **Ts**	house dust, USA
DTO 64E8	JX997044	I=99.8% **R:** A→G (514 n.) **Ts**	industrial premises (vaccine production), Netherlands
DTO 68B8	JX997045	I=99.8% **R:** A→G (514 n.) **Ts**	industrial premises, Germany
P11.7	EU833212	I=99.8% **R:** A→G (259 n.) **Ts**	Los Riscos cave soil, Mexico
XF42	KJ780802	I=99.8% **R:** A→G (259 n.) **Ts**	Xu Xian Xiu's tomb murals, China
XF12	JX136726	I=99,8% **R:** T→G (69 n.) **Tv**	
F23	HQ380775	I=99.7% **R:** C→T (32 n.) **Ts** C→T (406 n.) **Ts** **Del.** 73 n.	Mogao Grottoes cave, China
XF15	JX136729	I=99.5% **R:** A→C (21 n.) **Tv** C→T (362 n.) **Ts**	Xu Xian Xiu's tomb murals, China
F2	HQ380757	I=99.1% **R**: G→A (170 n.) **Ts** T→A (178 n.) **Tv** A→T (375 n.) **Tv** T→C (400 n.) **Ts Ins.** T (406 – 407 n.)	Mogao Grottoes cave, China

Notes: [Ig] – **I**dentity **g**rades of selected strains: Query cover = 100%, E value = 0.0; **R** – Amino acid replacement, **X** – the nucleotide residue in reference ITS MK367422, **Y** – the nucleotide residue in the homologous ITS fragments, **Ins** – insertion, **Del** – deletion, **Tv** – transversion of nucleotide residue, **Ts** – transition of nucleotide residue. * – localization of substitutions, deletions and insertions.

Conclusions

Thus, on the basis of the phenotypical characteristics of the isolate MF18_10 from the deteriorated walls with medieval murals in St. Sophia's Cathedral in Kyiv, its affiliation to the genus *Penicillium* was established. Its taxonomic position as *P. chrysogenum* strain MF18_10 was confirmed by computerized analysis of the ITS-fragment nucleotide sequence (MK367422), showing its complete identity to the ITS-fragments of type strains NR_077145 and AY373902, and also other 78 *P. chrysogenum* strains in GenBank database. Our study showed that the isolated strain MF18_10 belongs to the common for indoor environments, dust, aerosols penicillia, which are also often isolated from historical-cultural artifacts. This *P. chrysogenum* isolate was found to be xerotolerant and might contribute to the destruction of the ancient wall paintings. However, considering that the deterioration of the valuable heritage objects is often caused by xerophilic fungi which are very difficult to cultivate under laboratory conditions, there will be needed further multidisciplinary studies on mycobiota of the dark stains on the St. Sophia's Cathedral murals.

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ЛУЦЕНКО В. О.[1], ПОЛІЩУК Л. В.[1], ХОНГ Дж.[2], ФОМІНА М. О.[1]
[1] Інститут мікробіології та вірусології ім. Д.К. Заболотного НАН України,
Україна, 03143, м. Київ, вул. Академіка Заболотного, 154, e-mail: M.Fomina@ukr.net
[2] Корейський Інститут Морського Біорізноманіття,
Південна Корея, 33662, Чунхчеонхем-до

КСЕРОТОЛЕРАНТНИЙ ШТАМ *PENICILLIUM CHRYSOGENUM* MF18_10, ІЗОЛЬОВАНИЙ ІЗ ПОШКОДЖЕНИХ СТІН СОФІЇВСЬКОГО СОБОРУ, КИЇВ

Мета. Метою роботи було визначення таксономічного положення штаму гриба MF18_10, ізольованого із пошкоджених стін з середньовічними фресками Софійського собору (Київ, Україна). ***Методи.*** Гриб було виділено в чисту культуру на селективному для ксерофільних грибів середовищі (агар Чапека з 70 % сахарози). Для макрота мікроморфологічної характеристики застосовували світлову та скануючу електронну мікроскопію. Молекулярно-біологічну ідентифікацію проводили з використанням нуклеотидної послідовності фрагменту ITS. ***Результати.*** На основі фенотипової характеристики грибного ізоляту MF18_10 було встановлено його приналежність до роду *Penicillium*. Аналіз ITS виявив, що ізолят належить до виду *P. chrysogenum*, демонструючи його 100 % ідентичність з іншими 78 штамами *P. chrysogenum* в базі даних GenBank, включаючи типові штами NR_077145 і AY373902, а також поділяючи характерні відмінності в заміщеннях, делеціях і вставках в межах цієї групи. ***Висновки.*** Ізольований ксеротолерантний гриб було ідентифіковано як *P. chrysogenum*, типовий представник внутрішніх приміщень і пилу, а також поширений в мікобіоті пошкоджених історично-культурних артефактів вид. Відмінності в проаналізованих первинних структурах ITS *P. chrysogenum* не корелювали з джерелом виділення.

Ключові слова: *Penicillium chrysogenum*, ксеротолерантні гриби, ITS, скануюча електронна мікроскопія.