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Phenotypic Variability during Selection and Phylogenetic Characteristics of Dermatophytes

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Dermatophytes; Spores; Dermomycete strain; Sporogenesis; Dissociation; Vaccine.

Abstract

This article presents the results of a comparative study of the phenotypic variability of isolates (strai2ns) of cultures of fungi of the genus Trichophyton, isolated from various animal species, the effect of the frequency of reseeding on their cultural and morphological characteristics, in particular, on the dynamics of sporogenesis by the method of mycelial reseeding (generations) of dermatophyte cultures, as well as their phylogenetic characteristics. Trichopyton strains with a high level of sporogenesis were selected for further study of their immunogenicity, virulence, and the possibility of using them in the design of new vaccines against animal dermatophytosis.

Discipline: Veterinary epidemiology.

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1 Introduction

Fungal populations are a set of genetically different strains (biotypes), within which there is a continuous process of the emergence of new forms. It is known that intraspecific variability is a necessary prerequisite for the survival and development of a species [2, 15].

The study of the phenotypic variability of fungi showed that the existence of strains as genetically pure lines is short. This phenomenon is based on mutational variability, which is one of the main factors in the evolution of fungi and provides material for natural selection [1, 14].

Intraspecific polymorphism is especially pronounced in dermatophytes, while the change in properties occurs both in the direction of strengthening and weakening of typical and atypical characters [3].

Certain variants crowd out others from the population, become predominant and even the only ones [4]. Dermatophytes with multiple transfers on nutrient media, during the production of vaccines, show high variability due to spontaneous mutations. This process occurs continuously and leads to the formation of variants that differ in their morphological and biological properties from subcultures isolated from a particular strain.

The high variability of dermatophytes suggests that this group of fungi has species instability, is in the process of speciation, and, possibly, is moving to obligate parasitism [5, 6].

It was revealed that dermatophytes with multiple subcultures on nutrient media, during the production of vaccines, show high variability due to spontaneous mutations. The main disadvantage of intraspecific polymorphism of dermatophyte variability is the change in biological properties, both in the direction of strengthening and weakening typical and atypical characters, which requires continuous phenotypic selection of dermatophyte properties on the basis of sporulation and immunogenic activity [7, 11].

Highly productive fungal strains used in the bioindustry, including those obtained by selection of the original strain, are subject to natural variability and gradually lose their useful properties, “degenerate”. In the process of natural variability, variants arise that lose their inherent useful feature, but nevertheless, maintaining the initial level of activity of the production strain is possible. This is achieved by carrying out continuous supporting culture selection by means of monospore seedings with careful culling of all variants that deviate from the main type (initial biotype), selection of conditions and environment favorable for the development of the main variant, strict adherence to each strain of reseeding regimes (number and frequency), excluding the possibility of genetic changes occurring during culture aging so that their activity does not decrease, but rather tends to increase [8, 9, 13].

Great success in the selection of highly active fungal strains has been achieved due to the use of various inducing agents that increase the range of variability and create conditions for selecting the most productive forms from the population [10, 12].

Domestic and farm animals are the main reservoir of dermatophyte fungi and the most important vector for the spread of dermatophytosis among humans. The incidence of ringworm in humans and animals is closely related, and an effective fight against these diseases is possible only with the creation of highly effective drugs that have not only preventive but also therapeutic properties.

The main means of combating animal dermatomycosis is the creation of associated vaccines that include antigens of several types of dermatophytes and provide immunity to several pathogens. Currently, live and inactivated vaccines are registered and used in veterinary practice in our country.

On the territory of the Russian Federation, in Germany, Turkey, Bulgaria and in the CIS countries - Belarus, Kazakhstan and Armenia, more than 10 vaccines against animal dermatophytosis are used (as of 01.10.2022).

It should be noted that specific immunotherapy and immunoprophylaxis have become the leading methods of combating animal dermatophytosis in the Russian Federation, while chemotherapeutic antifungal agents are more common in most foreign countries, which is obviously due to the insufficient effectiveness of vaccines on the market. The lack of efficiency is due to the lack of knowledge or experience in the field of biotechnology for the manufacture, selection and production of highly sporulating and immunogenic fungal strains used in vaccine design. Therefore, it is currently relevant to improve or create new highly immunogenic means for the prevention and treatment of animal dermatomycosis, taking into account modern data on monitoring the etiological structure of fungal diseases common to humans and animals.

The aim of the study is to select and study the phenotypic variability of isolates (strains) of cultures of fungi of the genus Trichophyton isolated from various animal species, the effect of the multiplicity of reseeding on their cultural and morphological characteristics, in particular on the dynamics of sporogenesis by the method of mycelial reseeding (generations) of dermatophyte cultures, and also presents their phylogenetic characteristics.

Solving this problem will improve the culture of production of vaccines against dermatophytosis in general, will allow the development of a methodology for selecting productive strains, taking into account

modern data on the etiological structure of pathogens of animal dermatophytosis, obtaining new productive strains with high sporogenesis, increasing the stability of the properties of manufactured drugs, creating prerequisites for the production of high-quality and competitive products.

2 Materials and Methods

In the work, we used cultures of fungal strains of the genus Trichophyton, which were isolated from pathological material selected from sick animals with signs of dermatophytosis in the Department of Mycology, as well as production strains of dermatophytes obtained from the All-Russian State Collection of Microorganism Strains Used in Veterinary Medicine and Animal Husbandry (FGBU " VGNKI).

Table 1: Sources of isolation of dermatophyte strains

Type of microscopic fungus	Strain number	Source of strain isolation
Trichophyton verrucosum Bodin, 1902.	153	A calf with trichophytosis
	291	A calf with trichophytosis
	168	A calf with trichophytosis
	130 L	A calf with trichophytosis
Trichophyton mentagrophytes (Robin) Blanchard , 1896.	1221	dog
	5421	dog
	18	cavy
	4121	silver-black fox
	9921	cavy
	27	white laboratory mouse
Trichophyton equinum (Matruchot et Dassonville) 1902	2021	horse
	6421	foal
	2251	horse

2.1 Nutrient Media

Cultivation of cultures and the study of their natural variability was carried out on a standardized wort agar of our own production.

To study the cultural and morphological properties of dermatophytes, we used Sabouraud medium (M063, Sabourand dextrose agar, HiMedia) with the addition of chloramphenicol (FD033, Chloramphenicol Selective Supplement, HiMedia), selective media Trichophyton agar No. 1-7 (Trichophyton media 1 - 5) (HiMedia Laboratories Pvt. Ltd.).

2.2 Cultivation Mode

The crops were cultivated at a temperature of +28°C for 14-21 days.

Obtaining pure cultures, their storage

Colonies of microscopic fungi obtained at the stage of primary isolation were assessed by morphology and microscopic structure, then they were seeded using the exhaustive stroke method on Sabouraud's medium with chloramphenicol. Next, 2–3 isolated colonies were selected, suspended in 0.9% NaCl solution, and 100 µl of the suspension were inoculated into tubes with slant wort agar or Sabouraud medium, using 2 tubes for each culture. as well as in the wells of a 12-well culture plate with Sabouraud's medium. The tubes were cultivated for 14 days at +28°C with cotton-gauze stoppers, then the stoppers were replaced with silicone ones and the tubes were placed in a refrigerator at +(2–8)°C.

Stepwise selection and study of the natural variability of dermatophytes

In the work, we used the method of selection of dermatophyte cultures, developed in the department of mycology of the Federal State Budgetary Institution "VGNKI". The method consists in seeding on wort-agar in Petri dishes a suspension of spores of a number of dilutions, ensuring the production of monospore colonies and

so that the number of grown colonies in one Petri dish does not exceed 30. After cultivation, I study the morphology of the grown colonies.

Selection by the level of sporulation

The selection of cultures was carried out according to the following scheme:

- determination of the level of sporulation of the initial strains;
- obtaining subcultures of strains with the highest level of sporulation;
- determination of the level of sporulation of the resulting subcultures;
- selection of subcultures with the highest level of sporulation;
- obtaining the next generation of subcultures and determining their level of sporulation.

Determining the level of sporulation

The level of sporulation was determined on the surface of the nutrient medium in the wells of a 6-well culture plate.

Suspension of microconidia with a density of 1.0E + 03 / ml was sown on the surface of the nutrient medium (wort-agar) in a volume of 100 µl (0.1 ml) and evenly distributed. The plates were cultured at +28°C for 7 days.

At the end of the cultivation period, microconidia were washed off the culture surface in each well with 3.0 ml of sterile distilled water with the addition of 0.5% (v/v) polysorbate-80 (Tween-80) and the number of microconidia in 1.0 ml of suspension was counted using the Goryaev camera. The spore yield (sporulation index) was calculated by the formula:

$$Bc = \frac{A*3}{9,6}, where: \quad (1)$$

Bc – spore yield from 1.0 cm^2 of culture (an indicator of sporulation);

A - the number of microconidia counted in 1.0 ml of suspension in the Goryaev chamber;

9.6 is the surface area of the culture plate well.

Then, the median value of the sporulation index was determined; for the next stage of selection, the crops with the highest level of sporulation were selected (the Bc index is higher than the median value).

Obtaining and analyzing subcultures

The suspension of microconidia obtained at the first stage was diluted to such an extent that 1.0 ml of the suspension contained no more than 100 microconidia, and 100 µl (0.1 ml) were sown on Petri dishes, evenly distributing the material over the surface of the nutrient medium. The crops were cultivated at a temperature of +28°C until isolated colonies were obtained.

Isolated colonies were cut out from the surface of the nutrient medium, suspended in sterile distilled water with the addition of 0.5% (v/v) polysorbate-80 (Tween-80), and the number of microconidia in 1.0 ml of the suspension was counted using a Goryaev chamber.

The resulting suspension was diluted to a density of 1.0E + 03 / ml, sown in 100 µl (0.1 ml) per 2 wells of a 6-well culture plate with a nutrient medium (wort-agar) and evenly distributed over the surface.

The plates were cultured at a temperature of +28°C for 7 days, after which the spore yield in each well was calculated as described earlier, and the average value was calculated over 2 wells.

Then, the median value of the sporulation index was determined; for the next stage of selection, the crops with the highest level of sporulation were selected (the Bc index is higher than the median value).

Comparative study of cultural and morphological properties of dermatophyte strains.

A comparative study of the cultural and morphological properties of dermatophyte strains was carried out, simultaneously making inoculations with an injection on the above media, after growing for 14-21 days, the morphology of the grown colonies was described.

Molecular genetic methods identification of dermatophyte strains, software

The isolation of DNA from a suspension of dermatophyte cultures was carried out using DNA-sorb-V reagent kits (Federal Budgetary Scientific Institution Central Research Institute, Russia) in accordance with the manufacturer's instructions.

Amplification of the genome fragments of micromycetes of the genus Trichophyton in the region of the internal transcribed spacer (ITS) was carried out by the «hot start» method using a modified DNA polymerase (HS Taq polymerase) on a Tertsik amplifier (DNA technology). Electrophoretic detection of PCR products was carried out in 1.8% agarose gel containing ethidium bromide in TAE buffer. The electrophoresis results were visualized using an ultraviolet transilluminator with an Infinity 1500/36M Xpress video gel-documentation system (Vilber Lourmat).

The Sanger DNA sequencing reaction with fluorescently labeled terminators was performed by the cycle sequence method on a 2720 Thermal Cycler according to the standard operating procedure. Capillary electrophoresis was performed on an ABI PRISM 3130/3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

The search for homologous DNA sequences was performed using BLAST using the NCBI database [Madden T. The BLAST sequence analysis tool. The NCBI Handbook [Internet]. 2nd edition. – National Center for Biotechnology Information (US), 2013.]. For phylogenetic analysis, the MEGA4 program was used [Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599].

3 Result and Discussion

3.1 Selection of Epizootic Strains of T. *verrucosum*. on the Basis of Spore Formation

As is known, the production of vaccines against animal dermatophytosis requires the presence of highly productive strains. Maintaining a high level of sporulation is possible with a continuous maintenance selection of strains for this trait. To study the variability of cultures on the basis of spore formation and the possibility of stabilizing their populations, 10 stages of selection of supporting selection were carried out (Table 2).

As can be seen from Table 2, the range of variability in terms of sporulation of T. verrucosum 153 strains was quite high: 65.9–88.9 million/cm^3 compared with the control strain T. verrucosum 130 L, but the highest sporulation rates were recorded in T. verrucosum 16 and 168. On the basis of spore formation, 453 lines (variants) of cultures were studied.

In the composition of the population of all strains, the presence of both low- and high-sporulating variants was observed.

When studying the stability of the population composition of the studied strains, it was found that the control production strain T. verrucosum 130 L and T. verrucosum 16 and 168 had the most stable genotype. The results are shown in Figure 1.

Table 2: Selection based on sporulation of T. verrucosum strains 153, 16, 168 130 L VGNKI

Name and number of strain	Selection stage	Number of options	Concentration of spores in 1 cm^3 in the Goryaev chamber, million			
			min	max	average	original version (isolate)
T. verrucosum 153	10	118	65.9	88.9	77.4	10.6
T. verrucosum 16		115	44.4	96.6	70.5	8.7
T. verrucosum 168		126	32.6	99.6	66.1	3.8
T. verrucosum 130 L control		94	55.6	78.8	67.2	64.2
Note: average data for 10 stages of selection are given.						

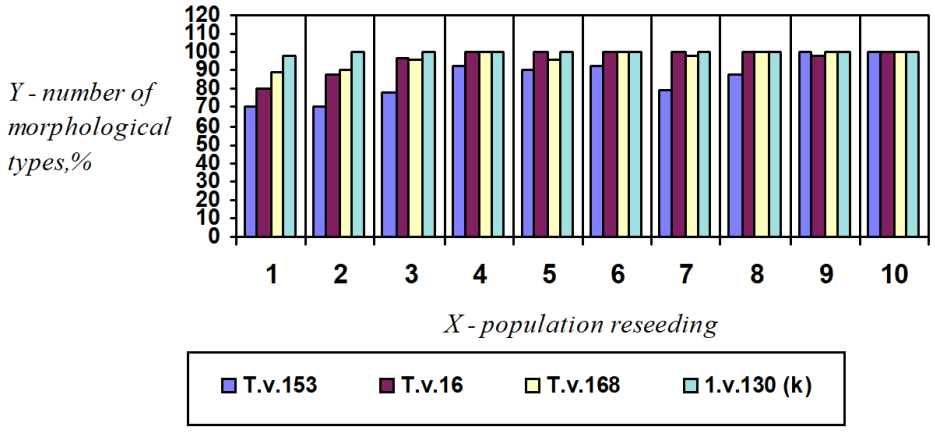

Figure 1: Variation in population composition of T. verrucosum strains. during the selection process.

Conducted 10 stages of selection according to cultural and morphological characteristics showed that the population of cultures of strains T. verrucosum Nos. 16, 168 and 130L VGNKI (control strain) at almost every stage consisted of 100% morphologically typical colonies for this strain.

In the process of selection, highly sporulating, population-stable lines of cultures of strains T. verrucosum 16 and 168 were selected, their molecular genetic identification was carried out, the strains were subjected to lyophilization and stored for further study of properties on the basis of immunogenicity and virulence.

3.2 Selection of Epizootic Strains of T. *mentagrophytes*. on the Basis of Spore Formation

To study the variability of cultures on the basis of sporulation and the possibility of stabilizing their populations, 10 stages of selection of the supporting selection of T. mentagrophytes strains 5097, 18, 7621, 1221 were carried out, the production strain 27 VGNKI was used as a control (Table 3). To study the variability of cultures on the basis of spore formation and the possibility of stabilizing their populations, 10 stages of selection of supporting selection were carried out.

As can be seen from Table 3, during the selection process, it was possible to significantly increase the level of sporogenesis of cultures of T. mentagrophytes 1221 and 18 strains despite the significant range of variability in this trait of sporulation: from 65.9 to 128.9 and 55.4.2 - 106.8 million/cm^3 spores, respectively.

According to the average indicators, the population of cultures of strains T. mentagrophytes 1221, 18, 5097 managed to be stabilized, which is confirmed by the concentration of spores in the control.

Table 3: Selection for sporulation and morphological features of T. mentagrophytes strains 1221, 5097, 7621, 18, 27 VGNKI.

Name and number of strain	Selection stage	Number of options	Concentration of spores in 1 cm^3 in the Goryaev chamber, million			
			min	max	average	original version
T. mentagrophytes 1221	10	150	65.9	128.9	97.4	7.2
T. mentagrophytes 5097		46	34.2	66.2	50.2	6.8
T. mentagrophytes 7621		122	12.6	30.6	21.6	5.2
T. mentagrophytes 18		38	55.4	106.8	81.1	9.1
T. mentagrophytes 27 VGNKI control		26	66.6	84.2	75.4	64.2
Note: average data for 10 stages of selection are given.						

According to cultural and morphological characteristics, 382 variants of colonies of cultures of T. mentagrophytes strains 1221, 18, 7621, 5097 and 27 VGNKI (control strain) were studied.

When studying the stability of the composition of the populations of the studied strains, it was found that the control production strain T. mentagrophytes 27 and T. mentagrophytes 1221 and 18 had the most stable genotype. The results are shown in Figure 2. Conducted 10 stages of selection for cultural and morphological characteristics showed that the population of cultures of strains of T. mentagrophytes Nos. 1221, 18 and 27 VGNKI (control strain) during the selection process, a relatively high phenotypic stability of the population composition of 100% was observed at the last stages of selection.

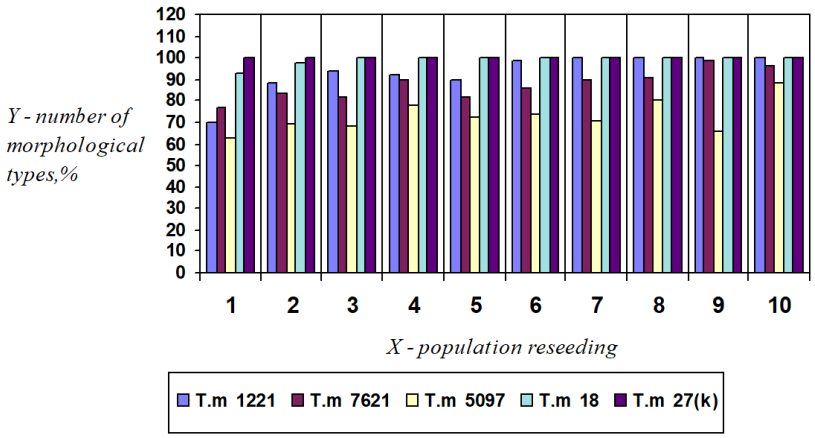

Figure 2: Variation in population composition of T. mentagrophytes strains during breeding selection.

Perhaps in the future, the use of the method of stepwise selection according to cultural and morphological characteristics will make it possible to stabilize these strains in terms of population composition.

The population of the culture strain line T. mentagrophytes 1221, 18 is of interest for further study of their biological properties.

3.3 Selection of Epizootic Strains of Trichophyton equinum on the Basis of Sporulation

According to cultural and morphological features and the sign of spore formation, 10 stages of selection of epizootic strains of Trichophyton equinum No. 6421, 2021 were carried out, the production strain of Trichophyton equinum 2251 VGNKI was used as a control.

On the basis of spore formation, 331 culture lines were studied (Table 4).

In the process of selection, the level of sporogenesis of cultures of T. equinum 6421 strains on average for the population was stabilized, despite the presence of low-sporulating variants in it: the minimum sporulation

was 31.6 million/cm^3, the maximum was 130.5 million/cm^3. During the selection process, it was not possible to increase the level of sporulation in the epizootic strain T. equinum 2021, the level of sporulation remained at the level of the original variant.

Table 4: Selection for sporulation and morphological features of Trichophyton equinum strains 2251, 6421, 2021.

Name and number of strain	Selection stage	Number of options	Concentration of spores in 1 cm^3 in the Goryaev chamber, million			
			min	max	average	original version
T. equinum 6421	10	94	31.6	130.5	81.0	17.6
T.equinum 2021		144	12.6	30.6	21.6	12.5
T.equinum 2251 (control)		48	86.4	114.8	100.6	78.8
Note: average data for 10 stages of selection are given.						

When studying the stability of the composition of the populations of the studied strains, it was found that the control production strain T. equinum 2251 and 6421 had the most stable genotype (Figure 3).

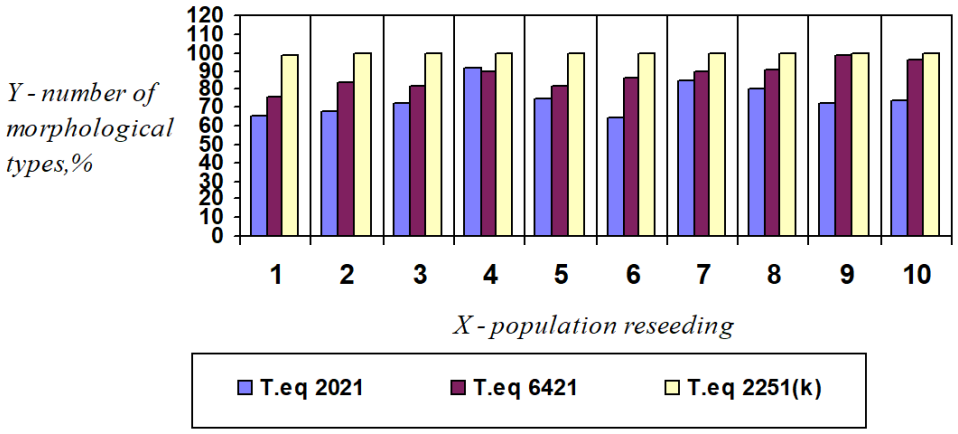

Figure 3: Variation in population composition of T. equinum strains during breeding selection.

According to cultural and morphological features, 286 variants of colonies were studied. The population of strains Trichophyton equinum 6421, 2021 at each stage of breeding at 60-80% consisted of colonies of the same morphological type, they were distinguished by high heterogeneity, characteristic of these strains, for the control strain Trichophyton equinum 2251 VGNKI, the population of a colony of one morphological type was 100%.

It is also possible that the use of the method of stepwise selection according to cultural and morphological characteristics will make it possible to stabilize the strains in terms of population composition.

The population of the Trichophyton equinum 6421 culture strain line is of interest for further study of their biological properties.

3.4 Phylogenetic Analysis of Micromycetes from the Collection of the Federal State Budgetary Institution "VGNKI"

Phylogenetic analysis was carried out for additional species confirmation of the epizootic and industrial strains of micromycetes of the genus Trichophyton from the collection of the VGNKI: Tr. mentagrophytes 5097, Tr. mentagrophytes 18, Tr. mentagrophytes 27, Tr. mentagrophytes M04, Tr. mentagrophytes 6401, Tr. verrucosum 130, Tr. verrucosum 168, Tr. verrucosum 16, Tr. equinum 6421. As a target for phylogenetic analysis of micromycetes, we chose a genome region in the region of the internal transcribed spacer (ITS), a

spacer sequence located between the genes of the small and large rRNA subunits in the chromosome. The ITS region is widely used by researchers to identify fungi at the genus and species level and has been recommended as a universal sequence for barcoding fungi [11]. The main advantages of this region of the genome are its multicopy nature, which ensures high sensitivity even with a small amount of sample for study, as well as the presence of highly conserved fungal-specific fragments directly adjacent to the variable regions [12].

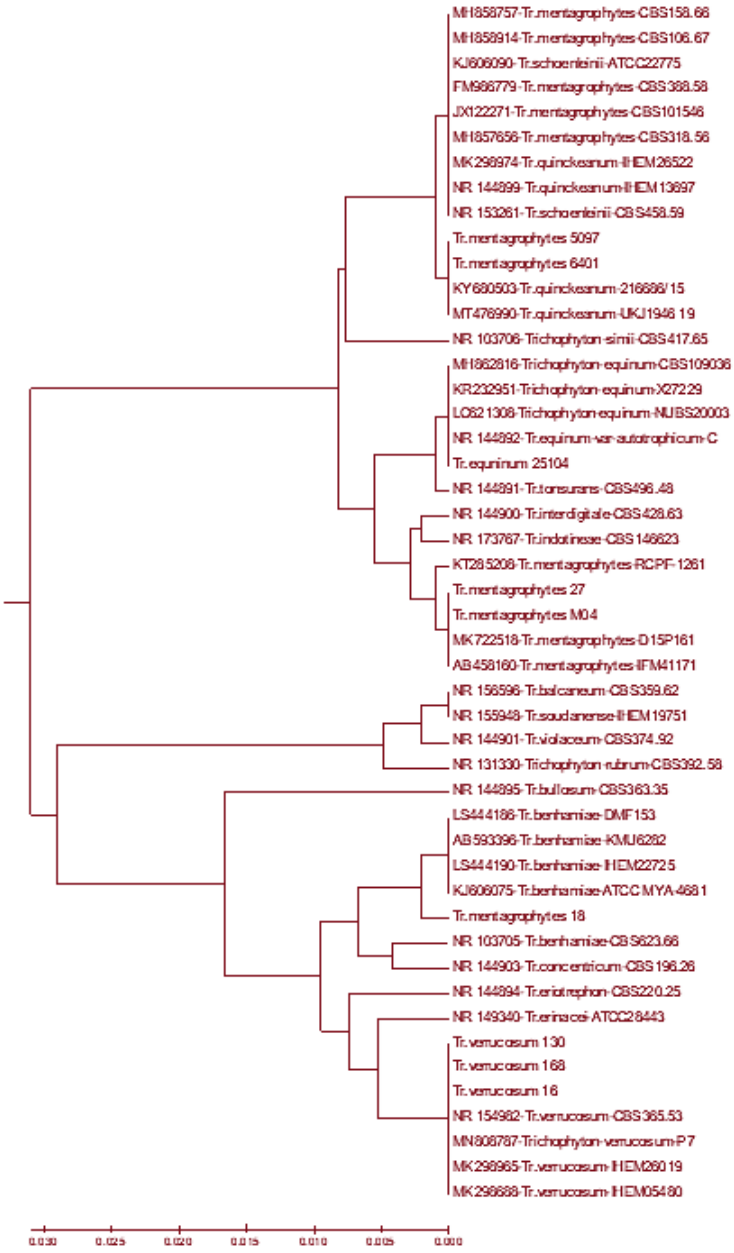

Figure 4: Dendrogram of differences in nucleotide sequences of a fragment of the ITS region of dermatophytes of the genus Trichophyton. MEGA4 program, UPDMA construction method.

In this work, ITS1 + ITS4 universal primers proposed by White T. et al. [13]. In the NCBI nucleotide sequence database, most reference fungal ITS sequences were obtained using these primers.

A phylogenetic analysis was carried out in order to establish the relationship between industrial strains of micromycetes of the genus Trichophyton from the collection of the Federal State Budgetary Institution "VGNKI" and micromycetes from databases, dendrograms of differences in nucleotide sequences were constructed. To construct a dendrogram, sequences of dermatophytes of the genus Trichophyton were selected from the NCBI database. The dendrogram of the differences in the nucleotide sequences of the fragment of the ITS region is shown in Figure 4.

Phylogenetic analysis by ITS confirmed the species affiliation of most industrial strains of micromycetes: collection strains were clustered in the corresponding branches of the phylogenetic tree. The strain Tr. mentagrophytes 18 was assigned to Trichophyton benhamiae based on the ITS fragment. Strains Tr. mentagrophytes 5097 and Tr. mentagrophytes 6401 are assigned to Trichophyton quinckeanum. Such results are most likely due to the problem of changing the species names of micromycetes. After the widespread use of molecular genetic methods of identification in the world, micromycetes, previously identified as Trichophyton mentagrophytes, were assigned to different species, but databases contain both new and old species nomenclature [14, 15]. The ITS region is widely used by researchers for fungal genotyping; databases contain a large number of sequences of various species, which allows for more accurate identification even taking into account the problem of changing the nomenclature of dermatophytes.

4 Conclusion

Selective selection contributes to the preservation of the uniformity of the composition of the population of dermatophytes according to cultural characteristics and provides an increase in the level of sporulation. Based on this, it can be concluded that the sign of sporulation in dermatophytes should be under polygenic control.

The results of the studies performed allow us to conclude that it is expedient to use in future work selected strains of fungi of the genus Trichophyton with a high level of sporogenesis, population composition, to conduct a comparative study of their immunogenic, virulent properties, stored in the collection of the institution of the most famous, relevant attenuated strains of Trichophyton verrucosum 130L, Trichophyton mentagrophytes 27 and Trichophyton equinum 2251, which will improve or design new vaccines using molecular genetic technologies.

5 Availability of Data and Material

Data can be made available by contacting the corresponding authors.

6 Acknowledgement

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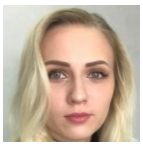

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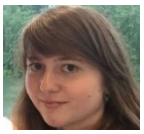

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