AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 4, pp. 745-756
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.934.55:591.2

doi: 10.15389/agrobiology.2023.4.745eng
doi: 10.15389/agrobiology.2023.4.745rus

THE ROLE OF FUNGI IN THE ETIOLOGY OF MASS SKIN LESIONS IN SABLES *Martes zibellina* L. 1758 IN TOMSK REGION

R.S. OVCHINNIKOV[1] ✉, A.V. ZHIGALIN[2, 3], A.G. GAYNULLINA[1], A.G. YUZHAKOV[1], O.Yu. TUTENKOV[2], V.A. SAVINOV[1], N.S. MOSKVITINA[2], A.V. KAPUSTIN[1], A.I. LAISHEVTCEV[1], A.M. GULYKIN[1]

[1]*Federal Scientific Centre Skryabin and Kovalenko All-Russian Research Institute of Experimental Veterinary RAS*, 24/1, Ryazanskii Prosp., Moscow, 109428 Russia, e-mail rsovchinnikov@mail.ru (✉ corresponding author), gainullinaalla@gmail.com, Anton_oskol@mail.ru, visik06@mail.ru, kapustin_andrei@mail.ru, a-laishevtsev@bk.ru, admin@viev.ru;
[2]*National Research Tomsk State University,* 36, Prosp. Lenina, Tomsk, 634056 Russia, e-mail alex-zhigalin@mail.ru, tutenkov@mail.ru, mns_k@mail.ru;
[3]*Dagestan State University, Institute of Ecology and Sustainable Development,* 43a, ul. Gadzhieva, Makhachkala, Republic of Dagestan, 367000 Russia
ORCID:
Ovchinnikov R.S. orcid.org/0000-0001-6634-9879
Zhigalin A.V. orcid.org/0000-0003-4661-0560
Gaynullina A.G. orcid.org/0000-0002-0545-0559
Yuzhakov A.G. orcid.org/0000-0002-0426-9678
Tutenkov O.Yu. orcid.org/0009-0003-2844-9635
Savinov V.A. orcid.org/0000-0003-1891-0005
Moskvitina N.S. orcid.org/0000-0002-3425-7723
Kapustin A.V. orcid.org/0000-0003-0136-2487
Laishevtcev A.I. orcid.org/0000-0002-5050-2274
Gulykin A.M. orcid.org/0000-0003-2160-4770
The authors declare no conflict of interests
Acknowledgements:
The study was carried out within the framework of the state assignments of the Ministry of Science and Higher Education of the Russian Federation (projects No. 0721-2020-00190 and No. FGUG-2022-0009), and with the support of the Russian Foundation for Basic Research (RFBR) grant No 20-04-60010 "Study of the diversity, circulation and pathogenic potential of coronaviruses in natural reservoirs on the territory of Western and Eastern Siberia"
Final revision received July 23, 2022
Accepted February 2, 2023

Abstract

Mass skin lesions in sables *Martes zibellina* in Siberia have been known since the 18th century, but their etiology is still not well understood. The disease affects up to 62 % of hunted sables, causing damage to the skin and causing serious economic loss. One of the versions suggests the participation of microscopic fungi in the occurrence of this dermatosis. In the present work, it was established for the first time that keratinophilic dermatophyte fungi of various species are involved in the etiology of skin disease in sables, some of which were discovered in the territory of the Russian Federation for the first time. The aim of the work was to reveal and identify clinically significant fungi in sables with clinical manifestations of skin diseases. Pathological material (hair, crusts) was taken from the affected areas of the skins of wild sables (*Martes zibellina* L. 1758), hunted during the 2018-2019 hunting season in various areas of the Tomsk region. A total of 28 samples of pathological material were studied. Mycological examination included a Wood's lamp test, direct microscopy of the pathological material, inoculation on mycological media, followed by identification of isolated fungal cultures. Inoculation was performed on DTM-Expert, a differential diagnostic medium for dermatophytes (FNTs VIEV RAS, Russia) and on Sabouraud medium with chloramphenicol (HiMedia Laboratories Pvt. Ltd., India). The incubation was carried out under aerobic conditions at 26-28 °C, the incubation period was up to 21 days. To study the cultural and morphological features, the cultures were re-inoculated on Sabouraud agar in Petri dishes, incubated for 10-14 days. For molecular genetic identification, isolated colonies of fungi grown on Sabouraud's medium were selected for 10 days at 26-28 °C. DNA was isolated using the GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The resulting DNA was used to carry out the polymerase chain reaction (PCR). Regions of the internal transcribed spacer (ITS) of the ribosomal RNA gene were sequenced. Phylogenetic analysis of the obtained nucleotide sequences was performed using the SeqMan application (DNASTAR Lasergene v.7.1.0, https://www.dnastar.com/software/lasergene/). Sequence alignment with those available in the GenBank database was performed using the Standard Nucleotide BLAST software package (http://www.ncbi.nlm.nih.gov/BLAST/). The resulting nucleotide sequences of particular interest were deposited to the GenBank NCBI database. When examining the affected sables, skin lesions were found, which were localized mainly in the back, waist,

and sides. They were characterized by loss of guard hairs, alopecia, formation of crusts and scabs. Dark spots were often observed in the area of lesions from the side of the skin. Lesions were observed both in males and females, mainly in young animals. During visual examination, samples of pathological material were sticky bundles of hair (downy, less often guard hair) with dried crusts and scales at the base. The result of the fluorescent test with a Wood's lamp in all samples was negative. Microscopy revealed bundles of downy hairs stuck together and a large amount of purulent debris, which made it difficult to detect fungal elements. As a result of cultural mycological analysis, 51 cultures were isolated, 18 taxa (species and genera) of fungi were identified. At the same time, keratinophilic dermatophyte fungi (*Arthroderma cuniculi*, *Chrysosporium carmichaelii*, *Chrysosporium* spp.) were isolated from 12 % of the samples, probably acting as etiological agents of dermatosis. Growth of dermatophytes was observed only on the DTM-Expert selective differential diagnostic medium; fast-growing non-dermatophyte fungi grew on ordinary media. Non-dermatophyte fungi with keratinolytic properties were also isolated — *Scopulariopsis brevicaulis* (16 %), *Acremonium* spp. (14 %), *Aspergillus* spp. (36 %), which can act as secondary opportunistic pathogens.

Keywords: pathogenic fungi, animal mycoses, dermatomycoses, dermatophytes, *Arthroderma*, *Chrysosporium*, *Martes zibellina*, sable

In recent decades, a sharp increase in the incidence of opportunistic mycoses has been noted among domestic and wild animals. Some mycoses are highly contagious, capable of causing death, and affecting large populations in nature [1]. Typical examples are amphibian chytridiomycosis, white nose syndrome (WNS) in bats, and snake fungal disease (SFD) [2-4]. Mycogenic infections cause significant damage to the biodiversity of natural ecosystems. When commercial animals are affected, mycoses also lead to significant economic losses.

Therefor, a detailed study of the mycobiota of wild animals which can be both contaminants and saprobionts, and potentially pathogenic species of fungi (pathobionts), becomes important. Research on the human and animal microbiome has focused on prokaryotes, while fungi have been studied to a much lesser extent [5]. The species composition of the mycobiota of wild animals in the Russian Federation and abroad has not been sufficiently studied [6].

Some representatives of animal mycobiota can be dangerous to humans, that is, they have epidemiological significance. Thus, pathogens of adiaspiromycosis are capable of causing respiratory mycoses [7]. In 2015, a new species of *Emmonsia* spp. was discovered, causing not only respiratory but also disseminated mycoses, and a sharp increase in incidence was noted (8). In 2018, one of the first ecological-epidemiological studies to detect fungal pathogens in wild animals was undertaken in Brazil. Fungi were found in 102 of 1063 samples, including pathogenic species in 89 samples [9].

In Russia, the *Mustelidae* species with valuable fur, including sable (*Martes zibellina* L. 1758), are of great commercial importance.

The first mention of a skin disease in free-ranging sables in Siberia was found back in the 18th century, but its etiology remained unclear for many years. The disease is recorded throughout the species' range. The only Russian work by N.D. Stepanenko written in the late 1960s, and devoted to mycogenic infections of wild sables was closed to readers for a long time and republished only in 2007 [10]. It describes a massive fungal infection of sables, which occurs in the form of chronic skin lesions and causes enormous economic damage to the fur trade. N.D. Stepanenko and his colleagues were the first to conduct a mycological study of affected skins. In most samples, fungi of the genus *Cephalosporium* (syn. *Acremonium*) were isolated which were indicated as the causative agents of the disease.

In the 1960s, skin defects known as "pockmarks" affected up to 70% of wild Siberian sables [10]. Currently, skin lesions are still widespread. O.Yu. Tyutenkov et al. [11] inspected more than 2 thousand sable skins in procurement organizations and coududcted a questionnaire survey of hunters. In the bulk sample, a significant proportion of affected skins was identified, 53.5±1.1% [11]. According to the authors' report, massive skin lesions of sables are caused by fungi, but

proper mycological diagnostic studies have not yet been carried out.

This work for the first time finds out that the etiology of skin disease in sables involves keratinophilic dermatophyte fungi of various species some of which were discovered for the first time in the Russian Federation.

The goal of the work was detection and identification of clinically significant microscopic fungi in sables with signs of skin diseases.

Materials and methods. Pathological material (hair, crusts) was taken from the affected areas of the skins of wild sables (*Martes zibellina*) during hunting season of 2018-2019 in various areas of the Tomsk region, 3 samples from Kolpashevo District, 19 samples from Bakcharsky District, 6 samples from southern regions (Tomsk, Kozhevnikovsky and Shegarsky). A total of 28 samples of pathological material were studied.

Mycological examination included a Wood's lamp test, direct microscopy of pathological material, inoculation on mycological media, followed by identification of isolated fungal cultures. Inoculation was carried out on the differential diagnostic medium for dermatophytes DTM-Expert (FSC VIEV RAS, Russia) and on Sabouraud's medium with chloramphenicol (HiMedia Laboratories Pvt. Ltd., India).

The cultures were incubated under aerobic conditions at 26-28 °C up to 21 days. To study cultural and morphological traits, cultures were reseeded by stab-inoculation into Sabourau's agar in Petri dishes and incubated for 10-14 days. Crushed drop preparations of fungal cultures were microscoped in a dry Micro-optix MX 100 microscope system (West Medica, Austria) at a magnification of ×100 and ×400.

Species phenotypic identification of fungi was carried out with the key [12].

For direct microscopy, pathological material were prepared in a 15% solution of potassium hydroxide (KOH) and microscoped at ×100 and ×400.

For molecular genetic identification, individual colonies of fungi grown on Sabouraud medium for 10 days at 26-28 °C were selected. DNA was isolated using the GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The resulting DNA was used for polymerase chain reaction (PCR).

The internal transcribed spacer of ribosomal RNA (ITS) were amplified with primers (5′→3′) ITS1Fwfun TTGGTCATTTAGAGGAAGTAAAAGTC, ITS1Rvfun CTGCGTTCTTCATCGATGC. Amplification (an amplifier DTprime 5, DNA-Technologies LLC, Russia) was run as follows: 5 min at 94 °C; 15 min at 94 °C, 20 s at 50 °C, 20 s at 72 °C (35 cycles); 5 min at 72 °C.

Regions of the internal transcribed spacer of the ribosomal RNA gene (ITS) were sequenced with the same primers using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

For double-strand sequencing, the same primers were used as for PCR. The nucleotide sequence was determined (an ABI Prism 3100 automatic sequencer, Applied Biosystems, USA) according to the manufacturer's instructions.

Phylogenetic analysis of the obtained nucleotide sequences was performed with the SeqMan application (DNASTAR Lasergene v.7.1.0, https://www.dnastar.com/software/lasergene/). The Standard Nucleotide BLAST software package (http://www.ncbi.nlm.nih.gov/BLAST/) was used to align sequences with those available in the GenBank database. Nucleotide sequences of particular interest have been deposited in the NCBI GenBank database https://www.ncbi.nlm.nih.gov/genbank/).

Results. When examining the sable skins, lesions were discovered, which were localized mainly in the back, lower back, and sides. They were characterized by loss of guard hairs, alopecia, crusts and scabs formation. Hairline was of uneven length, hair pulled out in clumps. Dark spots were often observed in the area of

lesions on the mesternal side. Some of the lesions were hidden by the undercoat and were revealed by palpation (tubercles, crusts). Lesions were observed in both males and females, mainly in young animals.

Upon visual examination, samples of pathological material were sticky tufts of hair (downy, less often guard hair) with dried crusts and scales at the base. The result of the Wood's lamp fluorescent test was negative in all samples.

During microscopy, tufts of sticky downy hair and a large amount of purulent debris were observed, making it difficult to detect fungal elements, which is why spores and fungal mycelium could not be detected in the samples.

By cultural mycological analysis of 28 samples of pathological material, fungi were isolated from 25 samples (89.2%). In total, 51 cultures were isolated, 18 taxa (species and genera) of fungi were identified (Fig. 1).

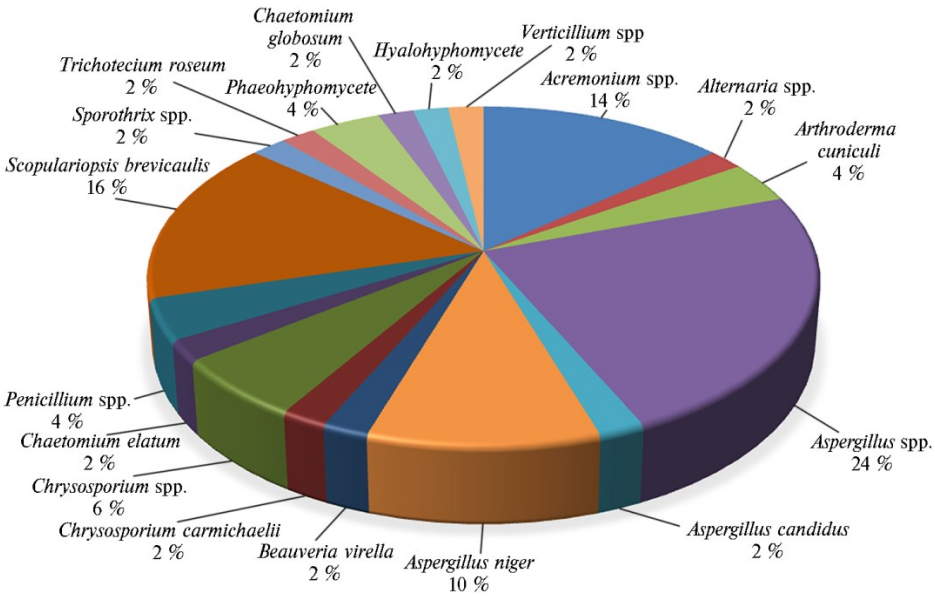

Fig. 1. Taxonomic composition and percentage of fungi isolated from samples of pathological material from affected areas of wild sable skins (*Martes zibellina* L. 1758) (Tomsk Province, 2018-2019)**.**

The mycobiota of skin lesions was dominated by fungi of the genus *Aspergillus* (36%, *Aspergillus* spp. 24%, *A. niger* 10%, *A. candidus* 2%). The most common species also were *Scopulariopsis brevicaulis* (16%) and *Acremonium* spp. (14%). In addition, several species of keratinophilic dermatophyte fungi have been isolated, e.g., *Arthroderma cuniculi* (4%), *Chrysosporium carmichaelii* (2%), and *Chrysosporium* spp. (6%). In total, the proportion of keratinophilic fungi was 12%. The share of other fungal taxa is 2-4% (*Alternaria* spp., *Beauveria virella*, *Chaetomium elatum*, *Chaetomium globosum*, *Penicillum* spp., *Sporothrix* spp., *Trichotecium roseum*, *Verticillium* spp., *Phaehyphomycete*, *Hyalohyphomycete*).

The occurrence of fungi ranged from 0 to 4 species per sample. From most samples, two species were identified. Of particular interest are two isolates of dermatophyte fungi (isolates No. 1.1-19 and No. 3.11-19), initially identified by morphological characteristics as *Trichophyton* spp. Both cultures were isolated from sables of the Bakchar region. On days 7-9 of growth, both isolates caused reddening of the DTM-Expert medium and formed white velvety-powdery colonies characteristic of dermatophyte fungi (Fig. 2).

When subcultured by stab-inoculation into Sabouraud's agar, white velvety-woolly colonies, slightly convex, with ciliated, hyaline edges, were observed. The reverse side of the colonies was light brown. The diameter of the colonies was

30-40 mm on day 14 of growth. Microscopy revealed hyaline branching mycelium and numerous oval and drop-shaped unicellular microconidia. Multicellular spores (macroconidia) were not observed. During long-term incubation of cultures, the formation of spirally convoluted hyphae was observed.

Fig. 2. Growth of the dermatophyte *Trichophyton* spp. No. 3.11-19, isolated from samples of pathological material from the affected areas of the skins of wild sables (*Martes zibellina* L. 1758), on the selective medium DTM-Expert.

To clarify the species identity, molecular genetic identification of *Trichophyton* spp. isolates No. 1.1-19 and No. 3.11-19 was carried out by sequencing the ITS region (Table).

According to ITS sequencing, both isolates of *Trichophyton* spp. (No. 1.1-19 and No. 3.11-19) showed the greatest homology (100 and 97.7%, respectively) with the strain *Trichophyton* spp. IFM 41172, isolated from a badger. The isolates showed significant similarity with the strain *Trichophyton* spp. NWHC 44736-43-02- 01B, isolated from a gopher snake. The third closest homologue was *Arthroderma cuniculi* CBS 492.71, isolated from a human (see Table). Thus, the closest homolog identified to species for both isolates is *Arthroderma cuniculi.*

As can be seen from the dendrogram based on sequenced ITS regions of *A. cuniculi* isolates No. 1.1-19 and *A. cuniculi* No. 3.11-19 (Fig. 3), they form a separate cluster together with the strain *Trichophyton* spp. IFM 41172, adjacent to clusters of three *A. cuniculi* and two *A. tuberculatum* strains.

Another isolate (No. 3.5-19), presumably a dermatophyte, was phenotypically identified as *Chrysosporium* spp. When growing on Sabouraud's agar, white, uniformly colored colonies, velvety fluffy, convex, with radial folding, were observed. The edges were smooth. The reverse side of the colonies was yellow to light brown, with radial furrows. The diameter of the colonies on day 14 of growth was 30-40 mm. The culture produced numerous oval and round microconidia characteristic of the genus *Chrysosporium*. When sequencing the ITS region, an isolate of *Chrysosporium* spp. No. 3.5-19 showed 100% homology with the strain *Chrysosporium carmichaelii* E00083342, isolated from a human nail (GenBank: KC923439.1, https://www.ncbi.nlm.nih.gov/nucleotide/KC923439.1).

In addition to the three mentioned cultures, three more were identified by morphological features as *Chrysosporium* spp., however, their species identity requires further molecular clarification.

The ITS sequences of three dermatophyte isolates identified by sequencing were deposited in the GenBank NCBI under numbers MN534766.1 (*A. cuniculi* 1.1-19), MN653980.1 (*A. cuniculi* 3.11-19), MT556012.1 (*C. carmichaelii* 3.5-19).

The clinical signs of skin lesions in sables in the present study is in many ways similar to the description previously made by other authors. The lesions were characterized as "pockmarks," "bald patches", "scabs", "cut and matted hair", and hair loss in tufts [10]. Such descriptions are characteristic of a chronic inflammatory process. Direct microscopy of the pathological material was uninformative due to the large amount of purulent debris. The cultural method of mycological analysis showed a significant species diversity of fungi that make up the mycobiota of skin lesions of wild sables. The vast majority of the studied samples (89.2%) contained from 1 to 4 species of fungi. Moreover, 88% of the isolated fungi turned out to be non-dermatophytic molds. Obviously, they play the role of contaminants for the skin of sables. Scopulariopsis brevicaulis was a common species (16%). This species, although not a dermatophyte, has keratinolytic activity [13] and is capable of causing superficial mycoses in humans [14] and animals 15]. Isolation may indicate its certain etiological significance as an opportunistic pathogen.

Results of BLAST analysis of ITS sequences of *Trichophyton* spp. isolates No. 1.1-19 and No. 3.11-19 from affected areas of wild sable skins (*Martes zibellina* L. 1758) (Tomsk Province, 2018-2019)

Closest strain	Homology, %		Accession number in GenBank	Isolated from
	isolate No. 1.1-19	isolate No.3.11-19		
Trichophyton sp. IFM 41172.	100.00	97.73	AB458161.1 https://www.ncbi.nlm.nih.gov/nuccore/AB458161.1	Badger (*Meles meles*)
Trichophyton sp. NWHC 44736-43-02-01B	93.24	92.56	KX148667.1 https://www.ncbi.nlm.nih.gov/nuccore/KX148667.1	Bullsnake (*Pituophis catenifer*)
Arthroderma cuniculi CBS 492.71	90.59	90.94	NR_077138.1 https://www.ncbi.nlm.nih.gov/nuccore/NR_077138.1	Human, skin lesions

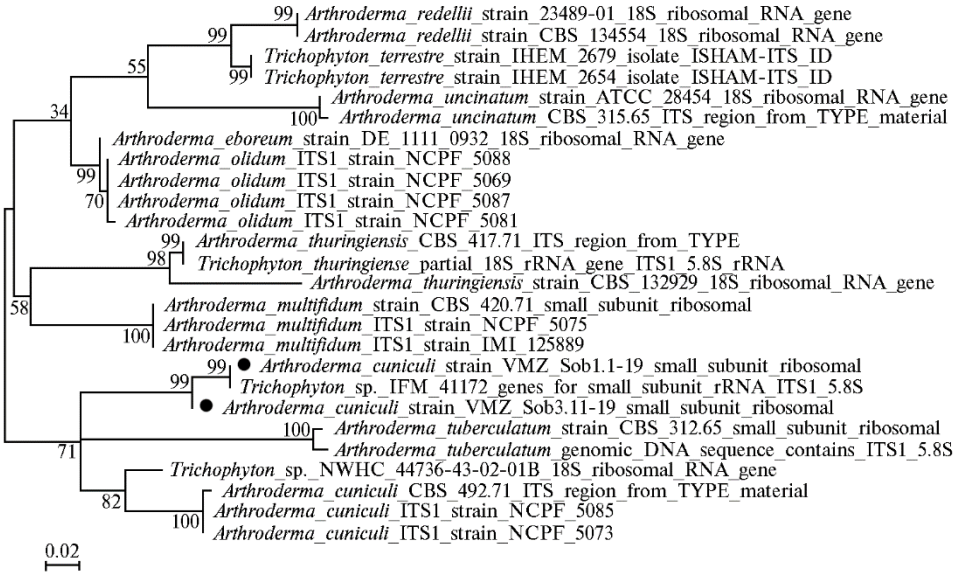

Fig. 3. Phylogenetic dendrogram based on ITS region sequencing of isolates *Arthroderma cuniculi* No. 1.1-19 and *A. cuniculi* No. 3.11-19 (marked with circles) **from affected areas of wild sable skins (*Martes zibellina* L. 1758)** (Tomsk Province, 2018-2019)

Representatives of the genus *Acremonium* (14% of pool) deserve special attention. Samples from some animals contained two morphotypes of *Acremonium* spp. (white and yellow morphotypes). Their species identity requires clarification. Fungi of the genus *Acremonium* also have keratinolytic activity [16] and cause diseases in humans [17] and animals [15].

In the study by N.D. Stepanenko [10], it was the fungi of the genus *Acremonium* (according to the old nomenclature *Verticillium*) that were isolated from most samples and recognized as the main etiological factor of the disease. In our opinion, this taxon may play a certain role in the pathogenesis of the disease, but does not serve as the main (primary) causative agent.

The most likely pathogens leading to skin lesions are keratinophilic dermatophyte fungi (genus *Onygenales*) with 12% share in the mycobiota. Two detected isolates of *Arthroderma cuniculi* form a separate cluster along with the strain *Trichophyton* sp. IFM 41172 from a badger. The molecular genetic characteristics of the *A. cuniculi* strains we isolated and their taxonomic position require further study.

The genus *Arthroderma* (family *Arthrodermataceae*) is the largest genus of dermatophyte fungi, which includes 27 species [18]. The species *A. cuniculi* is poorly studied, and its ecological niche and clinical significance are not yet entirely clear. It was isolated and first described in 1963 [19]. It was isolated in several cases both from wool and from soil in the habitats of animals, in particular hares (https://www.ncbi.nlm.nih.gov/nuccore/KT155576.1). Phylogenetical relatives have been isolated from snake skin lesions (https://www.ncbi.nlm.nih.gov/nuccore/KX148667.1) and also from human in case of dermatophytosis lesions (https://www.ncbi.nlm.nih.gov/nucleotide/NR_077138.1), indicating the pathogenic potential of *A. cuniculi*. In our opinion, this species may be the main etiological factor in skin infections in sables. We discovered *A. cuniculi* for the first time in Russia, and for the first time it was isolated from sables. It is possible that *A. cuniculi* is zooanthropophilic and poses a threat to humans, like many other dermatophyte species that infect animals [20].

In addition, keratinophilic fungi of the genus *Chrysosporium* were isolated from sables. One isolate, the *Chrysosporium carmichaelii* was identified to species

by sequencing. The species identity of three other isolates of *Chrysosporium* spp. requires clarification. The ecology of the species *C. carmichaelii* has not been sufficiently studied. It has been isolated from soil and dust [21], from bats, and from a human fingernail (https://www.ncbi.nlm.nih.gov/nucleotide/KC923439.1), indicating its pathogenic potential. Fungi of the genus *Chrysosporium* are known as causative agents of superficial and deep mycoses in humans and animals [22] and can be considered as possible causative agents of skin infections in sables. According to available publications, here we submit the first report of the detection of *C. carmichaelii* isolation in the Russian Federation.

Importantly, keratinophilic dermatophyte fungi, such as *A. cuniculi* and *Chrysosporium* spp., are fastidious and slow growing in culture; their growth is easily suppressed by fast-growing molds. Cultures of *A. cuniculi* were obtained only on the selective medium for dermatophytes DTM-Expert, recently developed at the Federal Scientific Center All-Russian Institute of Experimental Veterinaty RAS (Moscow) [23]. Most cultures of *Chrysosporium* spp. were also isolated on the DTM-Expert medium, while on the standard Sabouraud's medium their growth was apparently inhibited by fast-growing molds.

We hypothesize that due to the lack of selective media for dermatophytes N.D. Stepanenko [10] failed to isolate dermatophyte fungi from sables, but found only non-dermatophyte fungi with a predominance of the genus *Acremonium* which were taken as infectious agents. Although in our work the proportion of dermatophyte fungi was 12%, in reality their prevalence in sables may be higher, but not in all cases such fungi can be isolated from clinical material. The true distribution of pathogenic fungi can be further studied using modern highly sensitive diagnostic techniques, in particular metagenomic sequencing [24].

There are only a few publications devoted to the study of the mycobiota of mustelids in terms of various diseases. In Czechoslovakia, a high prevalence (from 30 to 73%) of adiaspiromycosis, a respiratory disease caused by species of the genus *Emmonsia* (formerly classified as genus *Chrysosporium*), was diagnosed among mustelids. Main pathogen *Emmonsia parva* is a typical saprotroph that lives in soil and on plant debris [25]. In the UK, adiaspiromycosis in the wild was diagnosed in almost a third (28%) of animals of different species examined; the main pathogen detected was *Emmonsia crescens*, which can also infect humans [26]. It cannot be excluded that species of the genus Emmonsia, morphologically close to the genus *Chrysosporium*, also circulate in the population of Siberian sables, which may be revealed in further studies.

It should be noted that in world literature, infectious diseases of sables are covered very poorly, since in nature this species lives only in Russia, Kazakhstan, Mongolia, China, Korea and Japan.

Thus, from sables with skin lesions caught in the Tomsk region, representatives of 18 different taxa of fungi were isolated, including keratinophilic dermatophyte fungi (*Arthroderma cuniculi*, *Chrysosporium carmichaelii*, *Chrysosporium* spp.), probably acting as etiological agents of mass dermatosis. The species *A. cuniculi* and *C. carmichaelii* have not been previously diagnosed in the Russian Federation, and this is the first report of their occurrence in sables. Non-dermatophytic fungi with keratinolytic properties (*Scopulariopsis brevicaulis*, *Acremonium* spp., *Aspergillus* spp.) may play the role of secondary opportunistic pathogens in this disease. Studies have demonstrated the presence of dermatophyte fungi in the wild, indicating the need for further investigation of the prevalence of clinically significant fungi in wild animals. It is advisable to develop a set of measures to combat fungal infections of wild animal that cause significant economic damage.

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