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EXPERIMENTAL ARTICLES

Change in the Temperature Preferences of *Beauveria bassiana sensu lato* isolates in the Latitude Gradient of Siberia and Kazakhstan

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Abstract—The radial growth of twenty isolates of the entomopathogenic fungus *Beauveria bassiana sensu lato* from different natural zones of Western Siberia and Kazakhstan (from 65 to 43°N) was tested under different temperatures (5–35°C). It was shown that the thermotolerance of the fungal isolates increased significantly from the north to south. The cold activity of the cultures did not significantly correlate with the latitude of origin and the sum positive temperatures of the regions. A distinct group of the steppe thermotolerance isolates was shown by the analysis of genomic polymorphism using seven intermicrosatellite DNA markers (ISSR). The steppe isolates had high levels of virulence to the wax moth *Galleria mellonella* and the Colorado potato beetle *Leptinotarsa decemlineata* at high temperatures (>30°C) compared to that of the forest-steppe isolates. The obtained data indicate that the use of isolates from the steppe zone will be most promising for the insect pest control under the conditions of continental and arid climate.

Keywords: *Beauveria*, climatic zones, temperature, radial growth, virulence, genomic polymorphism, adaptation, *Leptinotarsa*

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The anamorphic ascomycete *Beauveria bassiana sensu lato* is one of the world's most widespread entomopathogenic fungi infecting insects of various orders, actively using as an agent of biological control. [1–3]. The tolerance to the limiting abiotic environmental factors and, in particular, to high or low temperatures is different between strains of *B. bassiana* [4–6]. The temperature limits of mycelial growth for this fungus are within 5–37°C with the optimum between 20 and 28°C [5–8]. It has been shown on the cultures from different geographic regions of the world that thermotolerance of the populations of entomopathogenic fungi was increased with a decrease in the locality latitude [9–11] or, on the contrary, their cold activity was increased in higher latitudes [6, 11]. However, no such patterns were revealed in other studies [5–6, 12]. The question of the genetic characteristics of the strains with certain temperature preferences remains to be studied in greater detail [13].

The plains territories of Western Siberia and Kazakhstan are a unique region for studying the temperature preferences of microorganisms in the north–south gradient, since latitude zoning is ideal in this territory [14]. Moreover, this region has a high rate of preservation of natural biocenoses [15].

The search for entomopathogenic fungi with different temperature preferences is also topical from the applied point of view. High temperatures are one of the main factors limiting the use of entomopathogenic fungi under the conditions of continental and arid climate [13]. It should be noted that a number of authors have shown a relationship between the fungal tolerance to high or low temperatures determined in vitro and their capacity for infecting the host insects at the temperatures which are suboptimal for micromycetes [11, 16, 17]. Other researchers, however, did not observe such patterns [18].

In this connection, the tasks of the present work were set: (1) to assess radial growth in the 5–35°C temperature range for *Beauveria bassiana* isolates from different natural climatic zones of Western Siberia and Kazakhstan (65–43°N), (2) to analyze their genetic differences, and (3) to study the virulence of the cultures under different temperature conditions.

MATERIALS AND METHODS

Fungal isolates. Twenty *Beauveria bassiana* isolates from the collection of entomopathogenic microorgan-

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The *Beauveria bassiana* isolates studied

no.	Isolate	Natural zone	Geographic origin	Latitude	Longitude
1	N-2	F-T	town Nadym	65°29′	72°31′
2	As-584	F-T	100 km S from town Salekhard	65°23′	64°42′
3	As-588	F-T	100 km S from town Salekhard	65°23′	64°42′
4	As-589	F-T	100 km S from town Salekhard	65°23′	64°42′
5	Bol-1	F-S	Novosibirsk oblast, town Bolotnoe	55°41′	84°22′
6	Bol-2	F-S	Novosibirsk oblast, town Bolotnoe	55°41′	84°22′
7	Bol-4	F-S	Novosibirsk oblast, town Bolotnoe	55°41′	84°22′
8	K-18	F-S	Novosibirsk oblast, village Rep'evo	55°04′	83°31′
9	Zh-17	F-S	Novosibirsk oblast, village Zherebtsovo	55°06′	83°18′
10	Bs-4	F-S	Novosibirsk oblast, village Novososedovo	54°37′	83°57′
11	I-2	F-S	Novosibirsk oblast, village Burmistrovo	54°38′	82°47′
12	Uns-3	N-S	Novosibirsk oblast, town Karasuk	53°45′	77°42′
13	BBK-1	N-S	Novosibirsk oblast, town Karasuk	53°42′	78°10′
14	Sar-31	N-S	Novosibirsk oblast, town Karasuk	53°41′	78°02′
15	Sem-2	N-S	N-E Kazakhstan, city Semei	50°22′	80°22′
16	Sem-3	N-S	N-E Kazakhstan, city Semei	50°22′	80°22′
17	Uk-2	S-S	S-E Kazakhstan, 70 km W form Almaty	43°25′	75°50′
18	Uk-4	S-S	S-E Kazakhstan, 70 km W form Almaty	43°25′	75°50′
19	Uk-5	S-S	S-E Kazakhstan, 70 km W form Almaty	43°25′	75°50′
20	Uk-6	S-S	S-E Kazakhstan, 70 km W form Almaty	43°25′	75°50′

Note: F-T, forest-tundra; F-S, forest-steppe; N-S, northern (the herb–bunchgrass) steppe; S-S, southern (desert) steppe.

isms of the Institute of Systematics and Ecology of Animals, Siberian Branch, Russian Academy of Sciences, and the All-Russian Institute of Plant Protection, Russian Academy of Agricultural Sciences, isolated on the territory of Western Siberia and Kazakhstan were used in the research (table). The cultures were isolated from the insects of different taxonomic groups or the soil collected in the plain and low-altitude (up to 700 m above sea level) regions in the biocenoses typical for the natural zones. Depending on the regions of isolation, the isolates were divided into four groups: (1) forest-tundra, (2) forest-steppe, (3) northern steppe (from the zone of herb–bunchgrass steppe), and (4) southern steppe (from the desert steppes of the TienShan foothills). The strains were stored on Czapek and Waksman [19] artificial nutrient media (ANM) at 4°C with yearly transfers.

Radial growth studies. Mycelial growth of the cultures was tested on the Waksman agarized medium. Discs 8 mm in diameter were cut out from the four-day old cultures and placed on the medium at the center of 90-mm petri dishes. The cultures were incubated in thermostats at 5, 10, 15, 20, 25, 30, and 35°C. In the 10–35°C range of temperatures, 14 days were taken to register the linear growth of the colonies (the time when the fastest-growing isolates reached the edges of the petri dishes at optimal temperatures). The measurement of the colonies was made crosswise with an accuracy of up to 1 mm. To level out the individual differences in the growth rates, we used relative parameter of growth (relative growth): = a / b × 100%, where a is the colony diameter at a certain temperature, and b is the colony diameter at the temperature optimum (25°C for most cultures). When the radial growth of the cultures at 5°C was analyzed, 60 days was taken as the registration point, and only the absolute values (the colony diameter, mm) were used. All the experiments were carried out in three replicates. The data were analyzed using Factorial Anova (STATISTICA 6). Fisher's test was used to assess the significance of differences.

Analysis of genetic differences. Seven ISSR primers proposed by Estrada et al. [20] for the study of biodiversity of *B. bassiana* strains and their phylogenetic relations were used for analyzing the genetic differences: 808—$(AG)_8C$; 809— $(AG)_8G$; 818—$(CA)_8G$; 842—$(GA)_8YG$; 885—BHB$(GA)_7$; 889—DBD$(AC)_7$; 891—HVH$(GT)_7$.

Fungi were cultivated in Czapek liquid medium with peptone (0.4%) for five days in a rotary shaker at 110 rpm and 26°C. The obtained biomass was separated from the liquid culture by centrifugation. DNA was isolated from 100 mg of precipitated mycelium using a DNeasy Plant Mini Kit (QIAGEN).

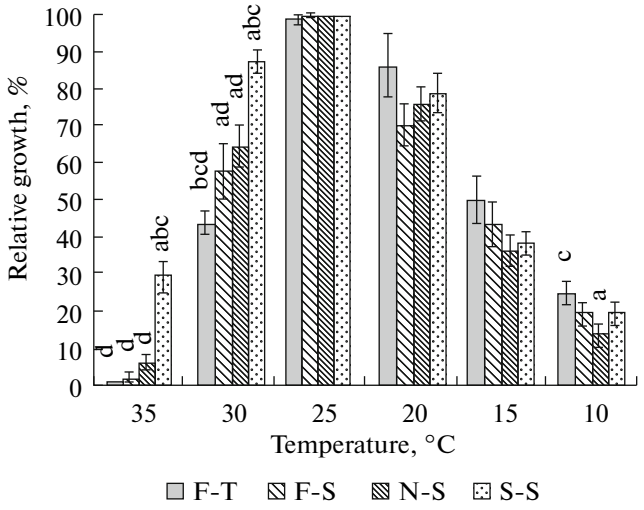

Fig. 1. Relative radial growth of 20 *B. bassiana* isolates from different latitude zones of Western Siberia and Kazakhstan. Designation of the zones is according to the table. Significant ($p < 0.05$) differences: from F-T (a); from F-S (b); from N-S (c); and from S-S (d). The vertical lines are the standard errors.

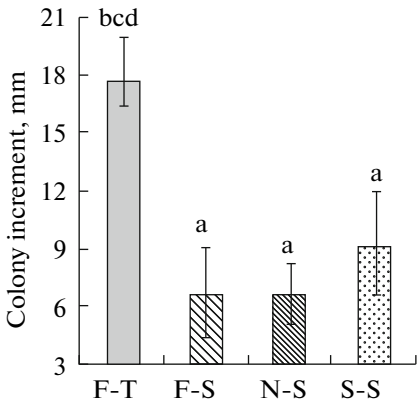

Fig. 2. Radial growth of 20 *B. bassiana* isolates from different latitude zones of Western Siberia and Kazakhstan at 5°C within 60 days. See Fig. 1 for the designations.

The PCR was performed in 25 μL of the mixture containing 2.5 μL of 10× PCR buffer (100 mM KCl; 200 mM Tris-HCl, pH 8.8; 0.1% Triton X-100), 2.5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.2 mM of each dNTP, 5 pmol of primer, 50 ng of the DNA template, and 1.25 U. of *Taq*-polymerase (SibEnzim, Russia).

The samples were amplified on a BIS-110 amplifier. Conditions of the PCR were: denaturation, 94°C, 5 min; annealing, 94°C, 30 s; 52°C, 40 s; 72°C, 40 s, 35 cycles; elongation 72°C, 15 min. Analysis of the PCR DNA fragments was carried out using electrophoresis in 1.5% agarose gel with ethidium bromide.

In order to determine the level of genetic similarity between the isolates, we compiled the total binary matrix where the presence or absence of a band on the electrophoregram was marked. The method of complete linkage using the STATISTICA 6 software was used for the construction of the dendrogram.

Bioassay. The caterpillars of second and third instars of the greater wax moth *Galleria mellonella* L. and the fourth-instar larvae of the Colorado potato beetle *Leptinotarsa decemlineata* (Say) were used. Biological testing was carried out according to the standard techniques described earlier [21, 22]. After infection, the insects were maintained for 10–15 days under the temperature conditions optimal for the mycoses (21 or 25°C) and unfavorable for their development (30 or 33°C). The temperature was controlled every 60 min with autonomous temperature recorders (Relsib) put into the containers with insects. The dead insects were put on moist filter paper in petri dishes and maintained under different temperature conditions in order to establish the possibility of the formation of conidia. In order to assess the significance of the differences in mortality rate of the insects and the formation of conidia on the cadavers, Student's *t*-test was used.

RESULTS

Radial growth studies. The average optimum of mycelial growth in all groups of isolates was 25°C (Fig. 1). The isolates from the southern latitudes were more tolerant to high temperatures than those from the northern latitudes. All southern steppe cultures, as well as some northern steppe cultures (Sem-3 and Sar-31), were able to grow at the high temperature (35°C). Significant differences in the rate of radial growth of the isolates were noted at 30°C, where the pattern of more active growth of the southern versus northern cultures was distinctly observed. At lower temperatures (15–10°C), no significant differences were noted between the northern and southern isolates in most cases; however, the tendency for the forest-tundra cultures to grow more quickly was observed. With a more strong temperature decrease (5°C), the differences between the forest-tundra isolates and more southern isolates proved to be significant (Fig. 2). A significant negative correlation was revealed between the relative colony growth at high temperatures and the locality latitude: $r = -0.70$, $p = 0.0005$ (growth at 30°C) and $r = -0.76$, $p = 0.0001$ (growth at 35°C), as well as a direct correlation between the sum of positive temperatures above 10°C in the regions and the growth of colonies at 30 and 35°C: $r = 0.71$, $p = 0.0005$ and $r = 0.81$, $p = 0.00001$, respectively. The correlation analysis did not reveal significant relationships between the growth of colonies at low temperatures and the locality latitude or the sum of positive temperatures in the regions ($r < 0.43$, $p > 0.05$).

Analysis of genetic differences. Analysis of the ISSR profiles revealed polymorphism in the group of the studied isolates. The number of unique (not repeated)

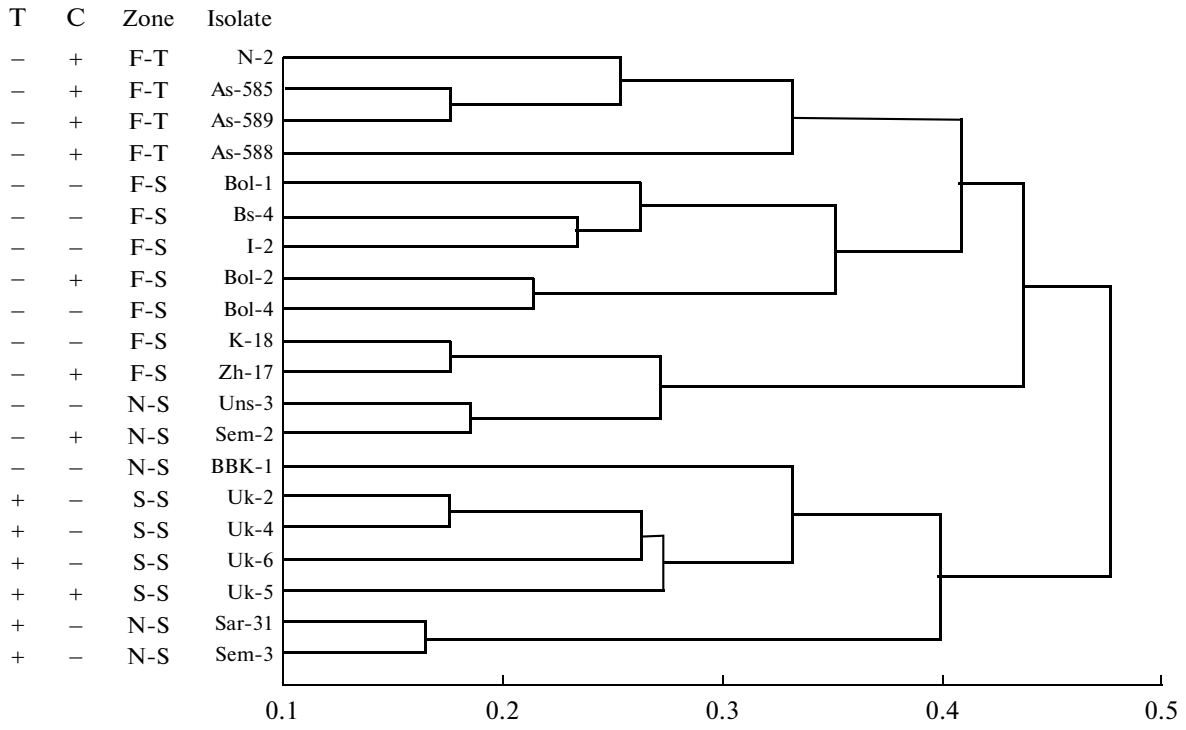

Fig. 3. Dendrogram of genetic relationships between the isolates from different natural climatic zones of western Siberia and Kazakhstan based on the comparison of the profiles of seven ISSR primers. T, tolerance to increased temperatures (capacity for growth at 35°C); C, cold activity (growth by more than 1 cm at 5°C for 60 days). The designations of the natural zones are in accordance with the table.

profiles constituted, depending on the primer used, from 50 (primer 885) to 100% (primers 818, 842). In the dendrogram (Fig. 3), the group of southern and northern steppe isolates growing at high (35°C) temperatures was distinctly clustered. Only one isolate (BBK-1) that did not grow at this temperature was found in this group. The other dendrogram block included the cultures intolerant to high temperatures, which were grouped into three large clusters: forest-tundra, forest-steppe, and mixed (forest-steppe and northern steppe). The fact that some of ISSR profiles (Fig. 4) are characterized by the presence of unique bands inherent in the cultures capable for growth at 35°C is noteworthy. On the whole, this electrophoresis was marked by considerable similarity between the ISSR spectra of the thermotolerant isolates (Sar-31, Sem-3, Uk-2, Uk-4, UK-5, Uk-6), despite the geographic remoteness of their origin (Fig. 4, profiles *14, 16–20*). This suggests the possibility of using of the ISSR profiles for identification of the cultures tolerant to high temperatures.

Bioassay. In order to study the virulence of the isolates at different temperatures regimes, five steppe cultures capable of mycelial growth at 35°C (Sem-3, Uk-2, Uk-4, Uk-5, Uk-6) and five forest-steppe isolates that did not grow at this temperature (K-18, Zh-17, Bs-4, Bol-2, I-2) were chosen. The experiments with *G. mellonella* showed an absence of differences in virulence between the steppe and forest-steppe groups of isolates at the temperature optimum for the fungi (26°C) (Fig. 5). Although at higher temperatures (30°C) the steppe isolates showed higher virulence compared with the forest-steppe isolates, only the steppe cultures were able to cause mycoses at still higher temperature (33°C). Similar results were obtained when larvae of the *L. decemlineata* infected with fungi were placed in different temperature conditions (Fig. 6). Moreover, only the steppe isolates of the fungus were able to complete the life cycle and produce conidia on the dead insects at increased temperatures (33°C). Thus, at 25°C, the share of the mycelium-overgrown cadavers of *L. decemlineata* was 79 ± 8.2 and 74 ± 7.1% for the forest-steppe and steppe cultures, respectively ($p > 0.05$). However, at 33°C, the level of this parameter was 5 ± 1.9% for the forest-steppe isolates and 66 ± 14.7% for the steppe cultures ($p < 0.05$).

DISCUSSION

Our study has shown more active growth of the southern isolates compared with the northern ones at high temperatures (30–35°C), whereas changes in the cold activity in the studied group of isolates were less noticeable. Earlier, Fargues et al. [5] did not find relationships between the growth of *B. bassiana s. l.* colonies in the 8–37°C range and the origin of the isolates

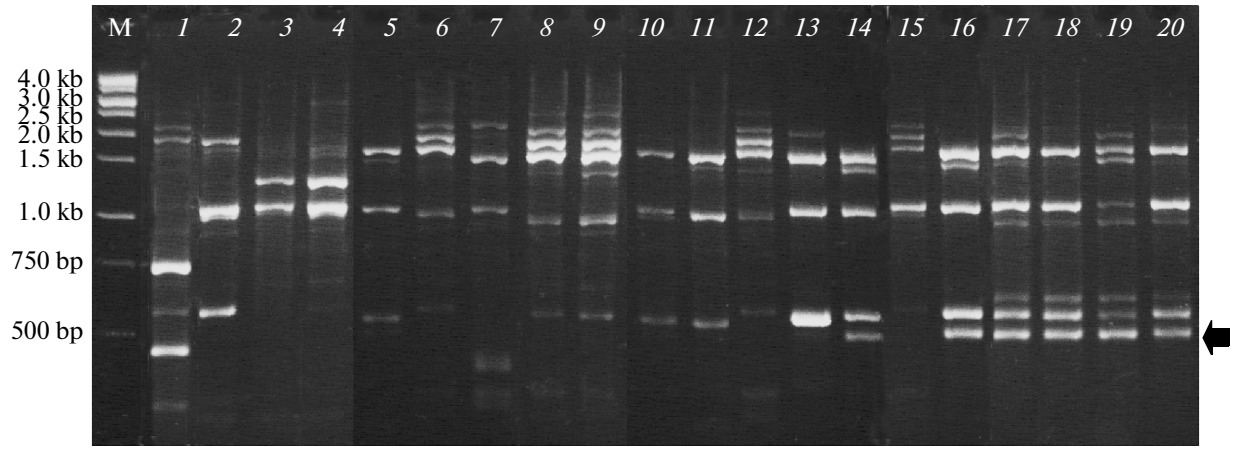

Fig. 4. Electrophoregram of primer 891 for *B. bassiana* isolates. The isolate numbers correspond to the table. The arrow indicates a band which is unique for the cultures tolerant to 35°C.

from different climatic zones of North and South Americas, Europe, Africa, China, etc. Fernandes et al. [6] studied the tolerance to high temperatures and cold activity in 53 *B. bassiana* isolates from Brazil and the United States. The authors showed that the isolates from higher latitudes (25–46°C) had a higher cold activity than the isolates from low latitudes (0–22°C). However, they did not find correlation between the latitude of origin and the tolerance of the fungus to high temperatures. The authors assumed that *B. bassiana* populations were adapted not only to the climatic zones, but to specific habitats inside them. It is also possible that the absence of this correlation was connected with the fact that the locality altitude and the heat supply to the regions were not considered.

Our study agrees with the data of Bidochka et al. [11] who examined *B. bassiana* strains from the territory of Canada and the United States. Based on the allozyme analysis, the researchers showed distinct isolation of the genetic groups of the strains isolated from the arctic, forest, and agricultural habitats. The isolates from the agricultural habitats were more adapted to high temperatures (37°C), whereas the forest and arctic isolates were more adapted to low temperatures (8°C). Moreover, the capacity for growth on ANM was correlated with virulence to the test insects (*G. mellonella* and *Tenebrio molitor* L.) at different temperatures. In their earlier work [23], these authors showed, on the basis of the allozyme analysis and the RAPD and RFLP PCR, that Canadian *Metarhizium anisopliae s. l.* strains clearly divide on two groups: the

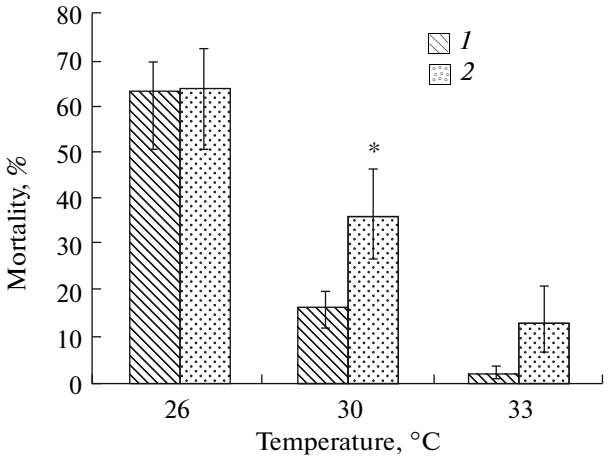

Fig. 5. Virulence of the forest-steppe (*1*) and steppe (*2*) isolates of *B. bassiana* to *Galleria mellonella* larvae under different temperature conditions. The suspension titer was 2.5×10^7 conidia/mL, the duration of the experiment was 15 days, * $p < 0.05$.

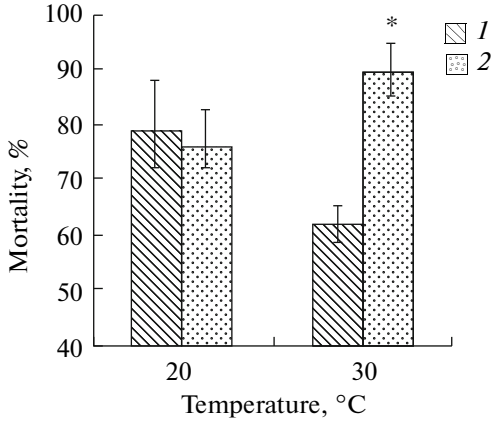

Fig. 6. Virulence of the forest-steppe (*1*) and steppe (*2*) isolates of *B. bassiana* to *Leptinotarsa decemlineata* larvae at different temperatures. The suspension titer was 2.5×10^7 conidia/mL, the duration of the experiment was 10 days, * $p < 0.05$.

forest strains showing a higher cold activity, and those isolated in agricultural habitats characterized by tolerance to high temperatures.

Our results also agree with the data of Vidal et al. [9] who showed that *Isaria fumosorosea* Wize strains isolated from the territory of India had a higher tolerance to high temperature compare to the cultures from western Asia or in the south of North America. The latter isolates were more thermotolerant than the European ones. Rangel et al. [10] showed that the high-latitude (36°–61°) *Metarhizium* isolates were more sensitive to heat stress than those isolated from the near-equator regions.

Earlier, it was shown by the example of a group of isolates from a restricted territory (Novosibirsk oblast) that the specialization of *B. bassiana s. l.* cultures was not associated with their origin from the hosts belonging to one or another taxonomic group [21]. Similar results were obtained by other authors with various molecular genetic methods [11, 24]. However, the specialization of entomopathogenic fungi may be connected with the environmental factors acting on both links of the insect–entomopathogen system [13, 25, 26]. For example, it was shown that the strains of *Beauveria* isolated from locusts (*Acrididae*) were more thermotolerant than the cultures isolated from the insects of other systematic groups [5], which is likely to be connected with the habitat of locusts and their ability to increase the body temperature during mycoses [27]. Accordingly, the strains adapted to high temperatures are able to infect the locusts and to complete their life cycle on these hosts more successfully. In all probability, what plays a leading role in the differentiation of the *Beauveria* populations is the conditions of the host habitat and, in particular, the abiotic environmental factors.

The data obtained allow us to suggest that the using of isolates from the steppe biocoenosis would be the most promising for the development of biological preparations, which were highly efficacious under the continental climate conditions. Our experiments indicate that the ability of mycelial growth at 35°C, as well as the unique ISSR profiles, may be the markers of the strain's tolerance to the high temperatures. These tests may be used for choice of the cultures of potential producers of biological preparations.

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