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MOLECULAR IDENTIFICATION OF WOOD-DECAYING FUNGI OF *ARMILLARIA* GENUS WIDESPREAD IN EASTERN SIBERIA AND THE FAR EAST OF RUSSIA USING ITS, IGS-1-1 AND *TEF-1*A GENETIC MARKERS

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The genus *Armillaria* is an essential component of forest ecosystems playing very important ecological role in dead wood decomposition, but it often becomes a serious pathogen causing white root rot in trees. It is also known that *Armillaria* species significantly differ in the level of pathogenicity. Thus, accurate identification of *Armillaria* is critical for assessing the risk of tree disease. In this study we analyzed 28 *Armillaria* isolates from Siberia and the Far East using nucleotide sequences of ITS, IGS-1-1 and *TEF-1α* gene regions and generated phylogenetic trees based on maximum likelihood method. In total, four *Armillaria* species were identified: *A borealis*, *A. cepistipes*, *A. ostoyae* and *A. gallica*. *A. borealis* was the most frequent among collected isolates (18 out of 28 isolates). *A. gallica*, *A. cepistipes* and *A. ostoyae* were much less frequent with two, five and three isolates out of 28, respectively. Thedistribution of *Armillaria* species in Siberia and the Far East was described for the first time. It is concluded that further studies are necessary to determine the role of *Armillaria* in trees pathological dieback, and *A. borealis* should be a key focus.

Ключевые слова: Armillaria, Basidiomycota, Far East, IGS, ITS, phylogeny, Siberia, taxonomy, *TEF-1α*, white root rot

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INTRODUCTION

Armillaria (Fr.) Staude is widespread in both hemispheres with more than 40 well-described species recorded worldwide that play a significant role in the dynamics of forest ecosystems (Shaw, Kile, 1991; He et al., 2019). Species diversity and wide geographical distribution of this genus are likely promoted by a combination of several features such as high environmental plasticity (Guillaumin et al., 1993), use of both saprotrophic and parasitic ecological strategies (Cruickshank et al., 2011) and effective

spreading through both rhizomorphs and root contacts (Rishbeth, 1985).

Armillaria can infect a wide range of plant hosts and causes loss of crops, urban areas and forests. Moreover, the level of virulence, rhizomorph production and host preferences also varies among species (Morrison, 2004; Prospero et al., 2004). For example, *A. ostoyae* is known as aggressive pathogen and produces abundant rhizomorphs, but thinner than those of *A. gallica* and *A. cepistipes* and mainly inhabits conifer trees (Wahlström, Johansson, 1992; Guillaumin et al., 1993). *A. borealis* parasites on both coniferous and deciduous trees, and produces rhizomorphs faster than *A. ostoyae,* but its virulence level varies (Guillaumin et al., 1993; Lushaj et al., 2010; Heinzelmann et al., 2016). *A. gallica* and *A. cepistipes* have weaker pathogenicity than *A. ostoyae* and *A. borealis* and act mostly as saprotrophs on deciduous trees, their main hosts (Rishbeth, 1982; Guillaumin et al., 1993; Baumgartner et al., 2011; Lushaj et al., 2010). *A. cepistipes* is capable of producing abundant rhizomorph networks (Rishbeth, 1985), which are helpful for new substrates exploration.

It has been shown that several *Armillaria* species can coexist in the same forest stand, predominantly saprotrophic together with parasitic species. For example, *A. cepistipes* or *A. sinapina* often occur with *A. ostoyae*, and *A. mellea* with *A. gallica*, reflecting the development of different ecological strategies due to competition for the same substrates (Cruickshank et al., 2011; Dettman, Kamp, 2011; Mesanza et al., 2017).

In recent years, DNA sequence data have been increasingly used for fungal species identification (Cai et al., 2011). The first studies were carried out with the use of nucleotide sequences of the intergenic spacer 1 (IGS-1-1) located between the 28S and 5S rRNA genes and internal transcribed spacer (ITS) located between the 18S and 28S rRNA genes (Harrington, Wingfield, 1995; Kim et al., 2000). Then, the *TEF-1α* gene was used for phylogenetic analysis of *Armillaria* and gave a better separation of closely related species (Shaw, Kile, 1991; Wahlström, Johansson, 1992; Maphosa et al., 2006). Today, multilocus phylogenies replace monogenic ones since they describe the evolutionary history of the species more precisely (Tsykun et al., 2013).

According to phylogenetic studies the genus *Armillaria* belongs to the *Agaricales* order of the *Basidiomycota* division, in which it is placed in the *Physalacriaceae* family together with other genera (Collins et al., 2013; He et al., 2019). Recent phylogenomic analysis based on 835 conserved genes showed that *Guyanagaster* and *Cylindrobasidium* are the most phylogenetically close genera to *Armillaria* (Sipos et al., 2017), and it was discovered that the separation of the *Armillaria* and *Guyanagaster* occurred about 40–50 million years ago (Koch et al., 2017; Sipos et al., 2017).

In this study, we collected samples of *Armillaria* in Eastern Siberia and Far East (Russia) and analyzed them using sequences of three DNA regions – ITS, IGS-1-1 and *TEF-1α*. The distribution of species helped us better understand the diversity of the phylogenetic lineages of *Armillaria* associated with forest dieback in Russia.

MATERIALS AND METHODS

Sampling sites and symptoms of infection. In total, 28 pure culture samples isolated from wood, fruitbodies (basidioma, basidiocarp) and basidiospores of *Armillaria* with different levels of pathogenicity and growing conditions were collected from both coniferous and deciduous trees in Siberia and Far East. The sampling sites included forest dieback of the southern taiga and Siberian forest steppe, mountain taiga forests of the Western and Eastern Sayan Mountains, Kuznetsk Alatau and Sikhote-Alin Mountains and were visited in the period of 2000–2020 once in every 1–3 years.

The forest area of pathological dieback varied from 0.1 to 30 ha. Trees of all sizes and ages including 50–260-year-old trees were affected by the dieback. *Pinus sibirica* Du Tour, *Abies sibirica* Ledeb. and *Abies nephrolepis* (Trautv. ex Maxim.) Maxim. were more susceptible to dieback than other species. Unlike *Heterobasidion annosum* s.l., dieback of conifers due to *Armillaria* often happened very quickly without any prior noticeable weakening (Fig. 1).

Death of trees with well-developed crown that did not express any signs of oppression happened very quickly. Lifespan of their needles, tree size, and growth of shoots did not differ from the average values for the forest stand. The hypothesis is that the fungus can parasitize on the roots for a long time (as evidenced by numerous excavations of root systems). However, with a decrease in the defensive ability of the tree and the penetration of the mycelium into

Fig. 1. Dieback of conifer trees due to *Armillaria*.

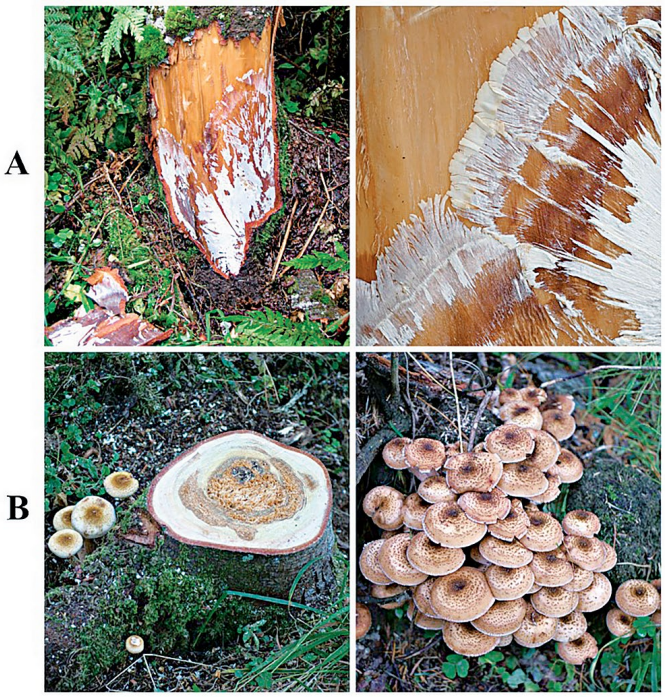

Fig. 2. Typical signs of the pathogenic action of *Armillaria borealis*: A – spread of fungal mycelium under the bark of *Abies sibirica*; B – fruiting bodies and the decay caused by *Armillaria borealis*.

the zone of the root collar, a rapid ringing of the trunk and death occurs (Fig. 2).

Colony morphology and microstructure. The morphology of the fungi was investigated in a culture on carrot agar (CA) and Norkrans medium (NM) (Norkrans, 1953) at 23±1 °C for 28 days without illumination. Key indicators were pigmentation of agar nutrient medium, radial growth rate (mm per day), presence of rhizomorphs, their total number, pigmentation and length (for 14 days). Microscopic observations were made using a Nikon Eclipse Ci microscope with a photo-documentation system (Nikon, Japan) and a scanning electron microscope Hitachi SU3500 (Hitachi, Japan). Samples were prepared according to the standard protocol for fungi cultured on solid media (Alves et al., 2013).

Pathogenicity *in situ*. Pathogenic effect of *Armillaria in situ* was assessed using a three-point scale: 1 point – low pathogenicity: dying trees in the lower layer, death of individual trees without the formation of groups and rare thin mycelium on roots; 2 points – average pathogenicity: dying single trees or small groups (up to 7 trees), dominating in the upper canopy, over several years with a pronounced preliminary weakening and gradual falling of needles, well-defined mycelium on the roots, rarely rising above the root collar; 3 points – high pathogenicity: dying trees prevailing in the upper canopy, without signs of preliminary weakening; a well-defined group of dying trees (more than 7 trees); fast drying of trees (within 1 year); well-developed *Armillaria* mycelium on the roots; the fan of the mycelium rises significantly above the root collar.

Pathogenicity *in vitro*. The inoculum of *Armillaria* strains was introduced into sterile aspen chips (10×10 mm) moistened with NM. Sterile chips were inoculated with agar blocks with pre-grown strains at the age of 21 days. The incubation period was 2 months at 23 °C in the dark, after which the chips were used for inoculation of plants. The plant test objects were 45 cm long and 38–40 mm in diameter cuttings of *Populus tremula* L. with its own roots. For each fungal strain, the number of rooted cuttings was 10. Cuttings were inoculated with colonized and sterile (control) chips at a height of 5 and 10 cm above the soil surface. The chips were inserted into the bark incisions and secured with parafilm tape. The cuttings were placed in plastic containers with pre-calcined sand and incubated for 3 months in a climatic chamber at 25 °C with bottom heating and periodic moistening of the sand with illuminance level of 5000 lx. Estimated indicators at the end of the experiment were the presence of a fan of mycelium, the presence of necrosis of the cambial zone, and the intensity of the spread of necrosis. Pathogenic effect *in vitro* was assessed on a three-point scale: 1 point – low pathogenicity: uneven spread of necrosis from the point of inoculation at a distance of up to 15 mm, mycelium is rare and poorly developed; 2 points – medium pathogenicity: uniform spread of necrosis from the point of inoculation at a distance of up to 30 mm, mycelium is dense and well developed; 3 points – high pathogenicity: uniform spread of necrosis from the point of inoculation at a distance of 30 mm or more, the presence of fusion of necrotic zones along the ring at the point of inoculation, mycelium is dense with a well-developed fan.

Isolation methods. Pure cultures of *Armillaria* spp. were isolated from fruitbodies, basidiospores and diseased wood of *Abies nephrolepis, Abies sibirica*, *Betula pendula* Roth, *Picea jezoensis* (Siebold et Zucc.) Carrière, *Picea obovata* Ledeb., *Pinus sibirica, Pinus sylvestris* L., *Populus tremula* L.) on 2% malt extract agar with 0.5% tannin (MEA + T). The pure cultures were maintained on slants of 2% (w/v) malt extract agar (MEA) medium at 6 °C. The list of Siberian and Far Eastern strains isolated in a pure culture, their hosts, geographical location and pathogenicity are presented in Supplementary Table S1 (Kolesnikova et al., 2023).

Isolation from wood. For isolation, wood with signs of necrosis was used. The wood surface was briefly sterilized in the flame of an alcohol burner, then a depression was made with a sterile scalpel on the border of healthy and necrotic tissue, and thin chips several cm long were cut out. They were placed in a sterile Petri dish, pressing the sharp edge into the MEA + T using sterile forceps. Several pieces of wood were placed in a Petri dish at a distance from each other. The dishes were incubated at 22 °C in the dark and regularly checked for fast growing microscopic fungi.

These fast growing colonies were removed together with agar medium using a sterile scalpel, while slow-growing *Armillaria* colonies were transferred from the surface of the agar medium to slants of MEA in test tubes.

Isolation from fruitbodies. The fruitbodies were carefully cleaned of plant debris and soil. The surface of the fruitbody was wiped with 96% ethanol and dried on filter paper. Then, they were cut with a sterile scalpel, the inner sterile part excised, and the fragments of the fruitbody transferred to MEA+T and incubated at 22 °C in the dark. Young growing mycelium, which appeared on the pieces of fruitbodies, was transferred to the slants of MEA in test tubes.

Isolation from basidiospores. The fruitbodies were carefully cleaned as described above and placed on sterile parchment paper with the spore-bearing layer down to obtain a spore print for 24–48 hours. Spores from a spore print were spread on the surface of 1% malt-extract agar medium and incubated 1–3 days at 24 °C. Germinated single spores were isolated using the method of Korhonen and Hintikka (1980).

DNA extraction, PCR and sequencing. DNA was isolated from pure isolate cultures using the DNeasy Plant Mini Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions. DNA was stored at −20 °C until required. The IGS-1–1 region was amplified using primers 0–1 and LR12R (Harrington, Wingfield, 1995). Amplicons from the ITS region (including the ITS-1, 5.8S gene and ITS-2 regions) were obtained using primer pair ITS-1/ITS-4 (White et al., 1990). The *TEF-1α* gene was amplified using primers EF595F and EF1160R (Kauserud, Schumacher, 2001).

PCR reactions were conducted using a HotStarTaq® DNA Polymerase kit (Qiagen Inc., Manchester, UK). PCR mixtures comprised 10× PCR buffer, $MgCl_2$, dNTPs, primers (100 μM each), 0.2 μl HotStarTaq DNA Polymerase, 2 μl template DNA (20–100 ng) and 6.8 μl sterile MilliQ water. PCR was performed using a T100 Thermal Cycler (Bio-Rad, Foster City, California, USA). The following PCR amplification protocol was used: 15 min of initial denaturation at 95 °C, 35 cycles of 1 min denaturation at 95 °C, primer annealing for 1 min at 50 °C and extension for 1 min at 72 °C, a final extension for 20 min at 72 °C was included to complete the reaction followed by the hold at 4 °C. PCR products were visualized after electrophoresis in a 1.5% agarose (Sigma, Saint Louis, Missoury, USA) gel stained with Roti – GelStain (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). PCR products of the expected size were purified using innuPREP Gel Extraction Kit (AJ Innuscreen GmbH, Jena, Germany), and the amplicons of the ITS, *TEF-1α* and IGS-1 DNA loci were sequenced in both directions in 28 fungal samples using the ABI Genetic Analyzer 3130xL (Applied Biosystems, Foster City, USA) at the Department of Forest Genetics and Forest Tree Breeding, University of Göttingen (Germany).

Sequence analysis. In total, 264 DNA sequences of the ITS, *TEF-1α* and IGS-1 DNA loci (88 sequences per each locus) for *Armillaria* species were used including 84 sequences representing 28 isolates sequenced by the authors and 180 sequences downloaded from NCBI GenBank (Supplementary Table S2 in Kolesnikova et al., 2023) including *Guyanagaster necrorhizus* (*TEF-1α*: KU289108.1, ITS: KU170948.1, IGS: KU254225.1), which was used as an outgroup species to root the phylogenetic trees.

Multiple sequence alignment for each separate marker was generated using the UGENE v. 34.0 (Okonechnikov et al., 2012) with MUSCLE alignment algorithm (Edgar, 2004). Further, all three alignments were concatenated in the SeaView v. 3.2 (Gouy et al., 2010). Three individual marker multisequence alignments and an alignment concatenated for all three markers in the PHYLIP and MEGA formats are presented in Supplementary Data S1 in Kolesnikova et al. (2023).

The search for the best evolutionary models of nucleotide substitutions in alignment consisting of three markers was done using PartitionFinder2 v. 2.1.1 (Lanfear et al., 2017) based on AIC (Akaike Information Criterion) (Burnham, Anderson, 2004). Best models for individual marker alignments were determined using ModelFinder (Kalyaanamoorthy et al., 2017). PartitionFinder2 divided the alignment into 3 parts: 1) TEF1a_1, TEF1a_3, 2) ITS, TEF1a_2 and 3) IGS-1. The TRNEF + I + G model was identified as the best one for the first set (TEF1a_1, TEF1a_3), model K81UF + I + G for the second set (ITS, TEF1_2) and TRN + G for IGS-1. For individual marker alignments the following best models have been identified: TNe + G4 for *TEF-1α* and HKY + F + G4 for ITS and IGS-1. The markers were checked for congruence using Concaterpillar v. 1.8a (Leigh et al., 2008). The trees were built using the IQ-TREE program v. 1.6.12 based on the maximum likelihood method (Nguyen et al., 2015). Node support was determined using ultrafast bootstrap analyses with 1000 replicates.

To search for groups of strains that likely represent the same individual was done via calculating the matrix of pairwise genetic distance using the MEGA program v. 11.0.13 (Tamura et al. 2021) and the Maximum Composite Likelihood model (Tamura et al. 2004), which is presented in Supplementary Data S2 in Kolesnikova et al. (2023).

RESULTS

Morphology

All strains of *Armillaria* were able to colonize agar culture media *in vitro*. On carrot-agar at 23 °C, most strains formed colonies with white-cream-brown color aerial

mycelium, flat, from fluffy to leathery-velvety texture. All strains of *A. ostoyae*, *A. gallica*, 80% of *A. cepistipes* strains, and 56% of *A. borealis* strains formed well-developed, highly branching white rhizomorphs, which were darkened during the aging of the culture (Fig. 3, Supplementary Table S3 in Kolesnikova et al., 2023). The number and total length of rhizomorphs (per Petri dish) varied from 10 to 45 pcs and 93–785 mm, respectively. The proportion of pigmented rhizomorphs on the 21st day of cultivation ranged from 10 to 70%. Several types of color reactions were noted near the inoculation block: brown and dark brown pigmentation of the medium (all rhizomorph-forming strains of *A. cepistipes* and *A. gallica*), discoloration (all rhizomorph-forming strains of *A. borealis*) and lack of pigmentation (all *A. ostoyae* strains; non-rhizomorphic *A. borealis* and *A. cepistipes* strains). The radial growth rate varied within 0.2–0.6 mm per day.

On NM at 23 °C, most of the studied strains formed squat colonies with a meager, poorly developed velvety-leathery aerial mycelium or white non-pigmented mycelium. Rhizomorphosis was observed in the same strains as on Carrot agar, however, the morphology of rhizomorphs was significantly different: short, sharp, needle-like, slightly branching, darkening already on the seventh day of cultivation. The number and total length of rhizomorphs varied from 1 to 10 pcs and from 5 to 785 mm, respectively. Brown and light brown agar pigmentation was observed in all rhizomorph-forming strains *A. cepistipes*, *A. gallica*, and *A. borealis*. The radial growth rate was in the range of 0.1–0.5 mm/day, which is comparable to that on carrot agar. In single strains of *A. borealis*, the growth rate on NM was twice as lower than in carrot agar, in *A. cepistipes* strains – twice higher.

Microstructures

Observations did not reveal significant differences in micromorphology in the studied *Armillaria* isolates. Most of the strains grown on NM formed a well-developed septate mycelium. Generative hyphae were light brown, translucent, branching at an acute angle, less often at right angles, simple, moderate to medium branching. The hyphae width varied from 2 to 3.5 μm.

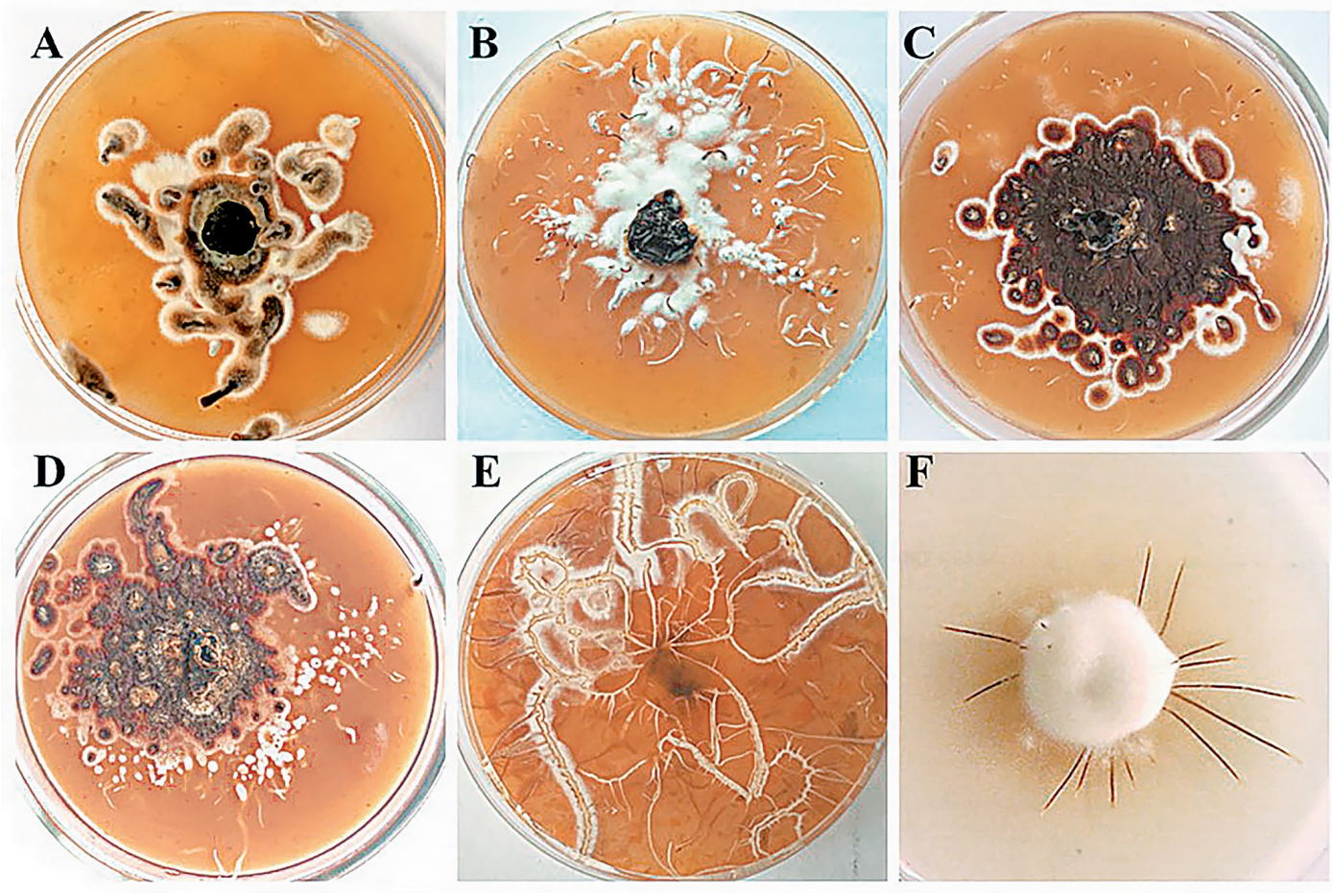

Fig. 3. Colony morphology of *Armillaria borealis* (A), *A. cepistipes* (B, E, F), *A. gallica* (C), and *A. ostoyae* (D) on carrot agar after 21 days of cultivation: colonies are squat with white-creamy-brown aerial mycelium; colony texture from fluffy to leathery velvety; on carrot agar (E) and Norkrans medium (F) *A. cepistipes* forms well-developed rhizomorphs.

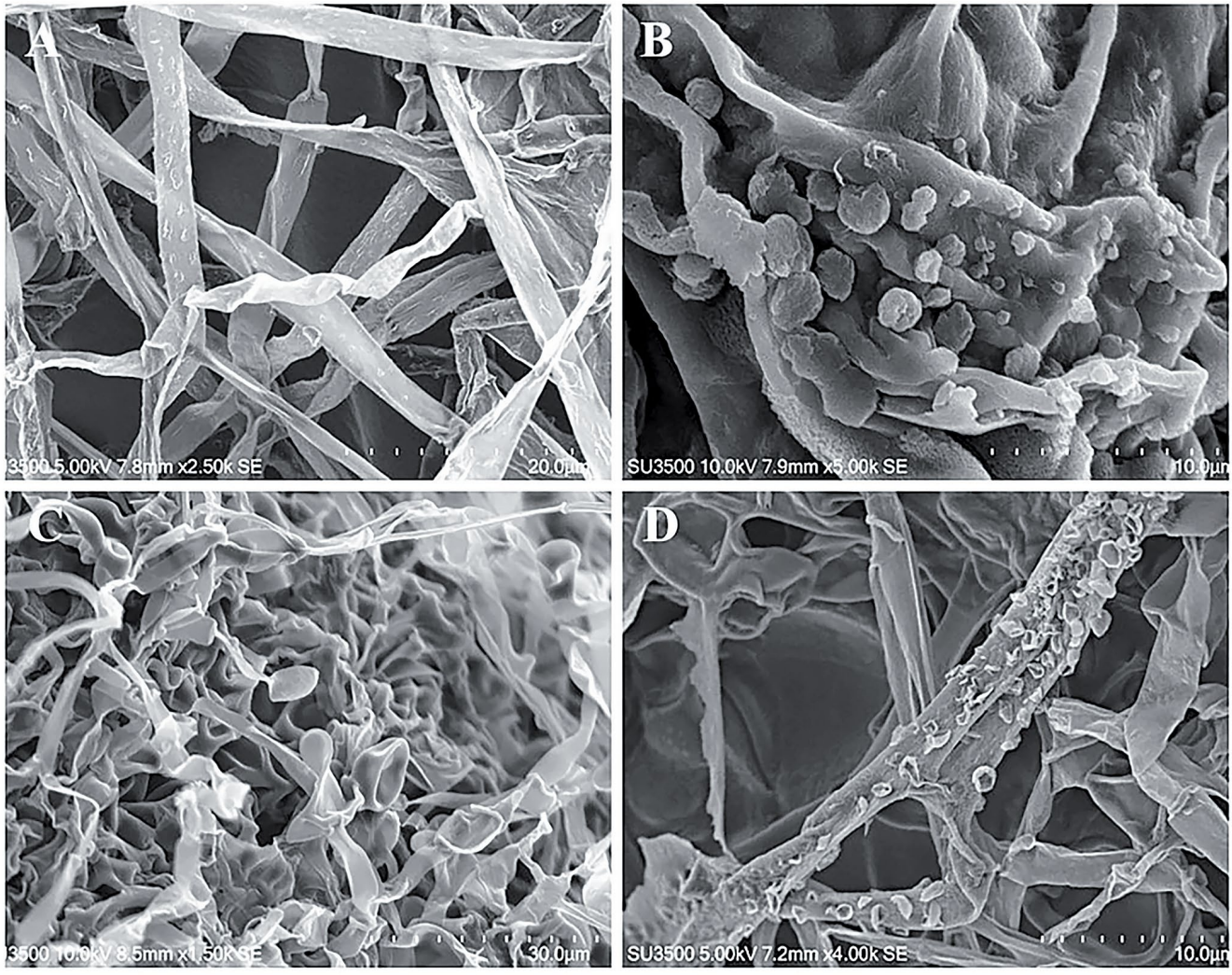

Fig. 4. Scanning electron microscopy of *Armillaria* strains (×1500–5000): A – vegetative hyphae of *A. borealis*; B – spherical structures on the hyphae of *A. cepistipes*; C – accumulation of thick-walled spherical cells in the old part of the *A. gallica* colony; D – encrusted hyphae of *A. ostoyae*.

On individual hyphae, spherical structures up to 3–5 µm in diameter were formed; hyphae acquired a rough surface (Fig. 4). In the old part of the mycelium, spherical swellings with a diameter from 4 to 7.5 µm were formed; voids appeared inside the hyphae and the number of anastomoses was moderate. Skeletal hyphae were formed when the culture has reached the age of 30–35 days; the color ranged from light to dark brown, without any partitions, 1.5–3.0 µm wide. In the old parts of the colony, massive clusters of spherical thick-walled hollow cells (less often ellipsoidal) with a diameter from 5 to 16 µm were formed. The cells were localized on unstained hyphae or in pigmented mycelial plexuses (rounded cells also become yellow, rusty and light brown). Swollen, colorless, thick-walled cells, not necessarily spherical, were noted in rhizomorphs. They were under the top layer of pigmented hyphae. The sizes of swollen cells varied within the range of 9.7–22 µm.

Phylogenetic identification of isolates

In total, 28 isolates of *Armillaria* collected from dying coniferous and deciduous trees with different levels of pathogenicity in Siberia (Krasnoyarsk Territory and the Republic of Khakassia) and the Far East (Sikhote-Alin), were analyzed. The analysis of the three markers in our study for congruence showed that they were not fully congruent. However, lack of congruence is common when comparing phylogenetic trees based on different individual genes (Leigh et al., 2011). It is mainly due to their incomplete gene sorting, ancient admixture, gene introgression, different evolutionary history and divergence rate (Kartavtsev, Redin, 2019). There is no a single best approach to deal with this problem, but use of several genes at the same time allows to correct this problem, and the more genes, the better (Leigh et al., 2011; Matute, Sepúlveda, 2019; Bryant, Hahn, 2020). Therefore, we used the supermatrix approach, although we are aware that this approach can

cause sometimes inconsistency of phylogenetic estimates (see references in McGowen et al., 2008 for discussion). This approach uses concatenated gene alignments, which are used then to generate a single species tree using standard phylogenetic tree reconstruction methods. This approach with the same set of marker genes that were used in our study has been and continues to be used for a very large number of species in a large number of publications, including identification of *Armillaria* species by other researchers (e.g., Tsykun et al., 2013; Denman et al., 2013).

In our study, the phylogenetic trees were based on concatenated multiple nucleotide alignments of three markers ITS, IGS-1, and *TEF1-α*. Only a few genetically identical sequences were found (highlighted in Supplementary Table S2 and Data S2 in Kolesnikova et al., 2023), and variation within species was relatively high. The isolates clustered into five major groups that corresponded to known species with high level of statistic support (Fig. 5). Five isolates formed the *A. cepistipes* group, two the *A. gallica* group, and three the *A. ostoyae* one. *A. borealis* formed two groups or clades: one large, which included 17 isolates, and a small one, which included a single isolate.

Phylogeny based only on the *TEF1-α* marker also revealed five clades (Supplementary Fig. S1 in Kolesnikova et al., 2023). Five isolates were grouped into the *A. cepistipes* clade, one – in the *A. gallica* clade, and three – in *A. ostoyae*. The species *A. borealis* again formed two or three clades: one large, which included 19 isolates (with 17 our samples), and a small one, which included three isolates (A618 from Switzerland, A1 from Finland, and our sample 399/17 collected in Southern Siberia, Krasnoyarsk Territory). The A618 from Switzerland likely represents a different lineage or clade. In addition, a mixed group was formed, which included *A. gallica* and *A. mellea* strains from the NCBI GenBank database.

Phylogeny based only on the ITS marker revealed three mixed clades (Supplementary Fig. S2 in Kolesnikova et al., 2023) with six isolates clustered in the clade *A. cepistipes/A. sinapina*, two – in *A. cepistipes/A. sinapina/A. gallica*, and 20 – in *A. ostoyae/A. borealis*.

Phylogeny based only on the IGS-1 marker revealed two mixed clades (Supplementary Fig. S3 in Kolesnikova et al., 2023): 21 isolates clustered in the mixed clade *A. ostoyae/A. borealis*, and seven – in *A. cepistipes/A. gallica/A. sinapina/A. nabsnona*.

Prevalence and distribution of isolates and species

Based on phylogenetic analysis, four species were identified in total: *A. borealis*, *A. gallica*, *A. ostoyae* and *A. cepistipes*. *A. borealis* and *A. cepistipes* occurred on the Krasnoyarsk Territory, *A. borealis*, *A. cepistipes* and *A. gallica* – on the territory of the Republic of Khakassia (Fig. 6), while *A. ostoyae*, *A. gallica* and *A. cepistipes* – on the territory of the Far East (Fig. 7).

The groups of isolates that likely represent the same strain or possibly individuals for samples collected in the same place were also identified for two samples of *A. tabescense* collected in Japan: 2006-20-01 (Kanagawa, Japan) and 96_3_3 (Tokyo, Japan), two samples of *A. mellea* also collected in Japan: 94-10-1 (Gunma, Japan) and A_10 (Tokyo, Japan), three samples of *A. borealis*: one collected in Germany, A5 and two in Southern Siberia, Khakassia, 73/8 (20) and 111 (18), three samples of *A. borealis*: one collected in Far East, Sikhote-Alin, 27D (21) and two in Central Siberia, Krasnoyarsk Territory, D48 (04) and S16 (06), and four samples of *A. borealis*: three collected in Far East, Sikhote-Alin 19D (26), 26D (27), 40D-2 (23) and one in Southern Siberia, the Republic of Khakassia, 22/12 (25) (see Supplementary Table S2 and absolutely identical samples with zero genetic distance between each other based on all three markers in Supplementary Data S2).

Pathogenicity

The pathogenicity of the studied *Armillaria* strains *in situ* varied from low to high. The maximum level was noted for the Far Eastern *A. ostoyae* strains (3 points; drying area from 4.8 to 16 ha) and Siberian strains of *A. borealis* (2–3 points; area of decline from 0.1 to 31.8 ha; in rare cases decline of single trees was noted). The pathogenicity of the *A. cepistipes* strains did not exceed 1 point; large-scale foci of forest decline were not found.

The pathogenicity of *Armillaria* strains *in vitro* was also heterogeneous. The data obtained were consistent with *in situ* observations. The strains of *A. ostoyae* and *A. borealis* showed the maximum level of pathogenicity (2–3 points). A significant advance of the mycelium fan along the *Popolus tremula* wood from the point of inoculation (12–45 mm) and a high degree of cambium necrosis (35–75 mm) were noted. Cases of merging of necrotic zones that created large areas of damage along the growing ring were noted on the cuttings (Fig. 8).

Armillaria ostoyae was found only in the area of trees with aerial dieback dominating in the upper canopy with pronounced groups of drying trees and without any signs of preliminary weakening. A rapid dying trees (within a year) and the presence of a high-rising fan of mycelium were observed. *A. cepistipes* was found only in area of dying trees in the lower layer; the death of individual trees did not form groups; a rare and thin mycelium was found on the roots. Similar to *A. cepistipes*, *A. gallica* was isolated from trees with aerial dieback in the lower layer with a rare thin mycelium on the roots, or in area with the single dying trees with preliminary weakening and pronounced mycelium on the roots. *A. borealis* was found both on trees with mycelium on the roots, rarely rising above the root

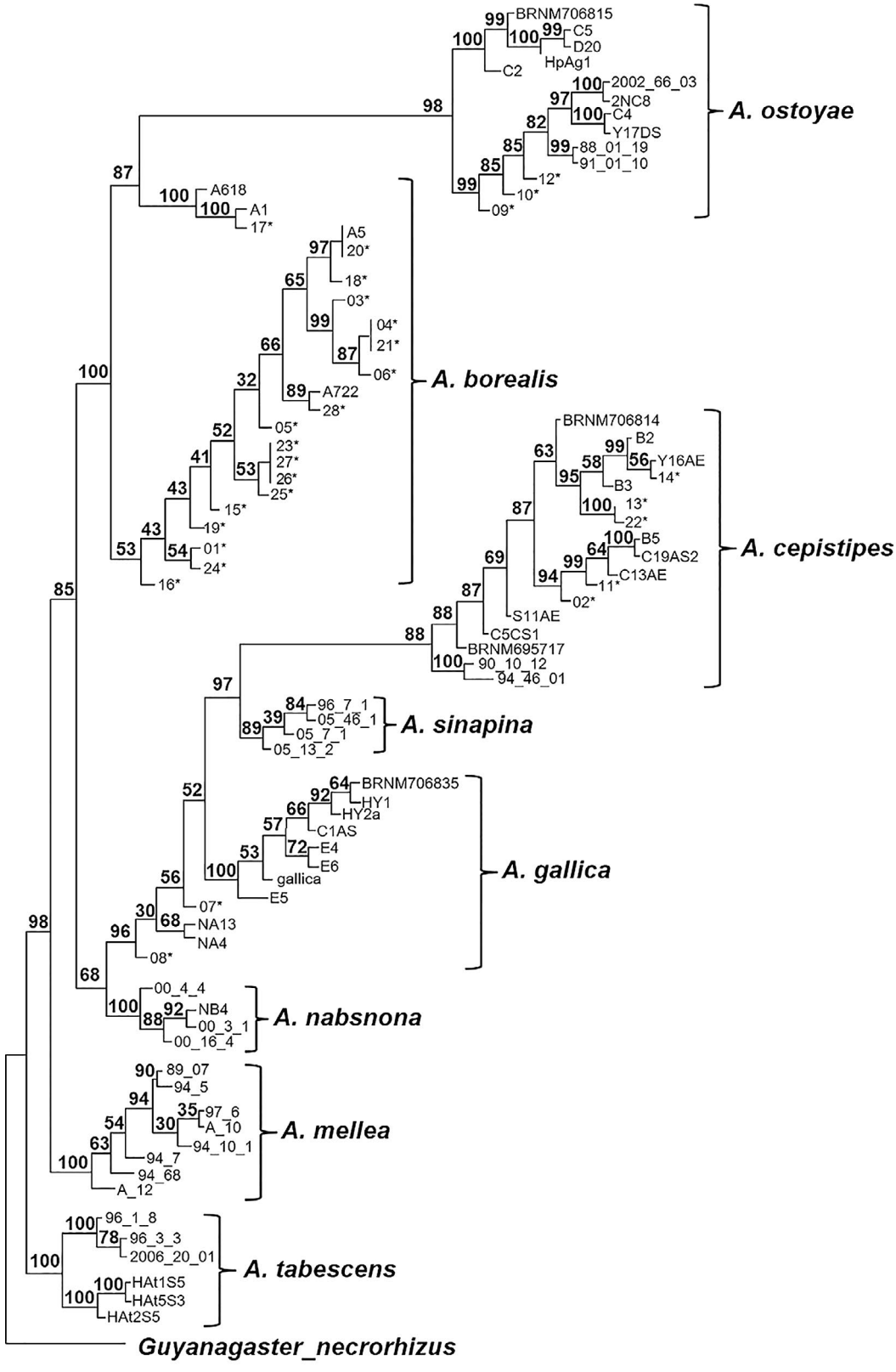

Fig. 5. Maximum likelihood (ML) phylogenetic tree of 28 new *Armillaria* isolates and sequences of *Armillaria* species from NCBI GenBank based on the 88 concatenated *TEF-1α*, ITS and IGS-1-1 nucleotide sequence alignments (264 sequences in total). Bootstrap values are indicated above the branches next to the clusters. *Guyanagaster necrorhizus* was used as an outgroup. Isolates numbered 01–28 were sequenced by the authors and marked with asterisk (see also Supplementary Tables S1 and S2 – Kolesnikova et al., 2023).

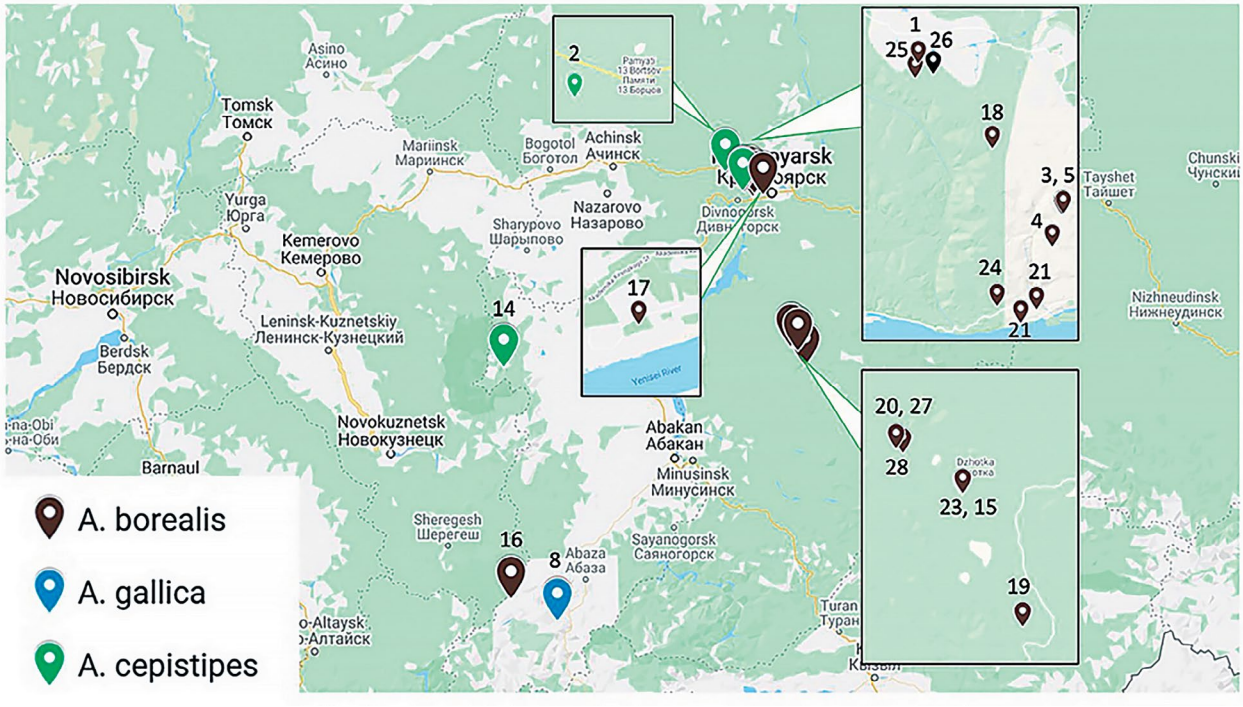

Fig. 6. Distribution of the *Armillaria* species in East Siberia.

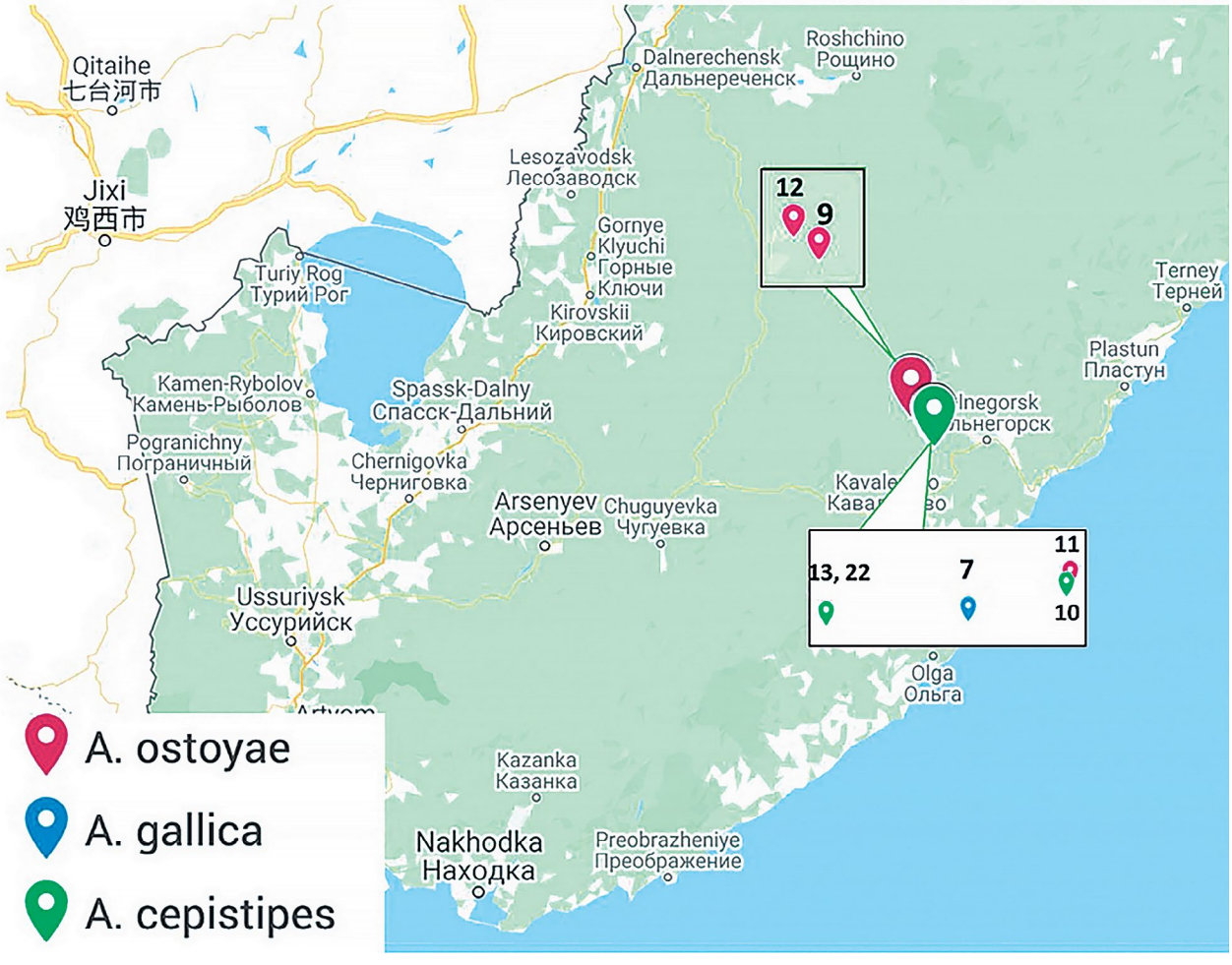

Fig. 7. Distribution of the *Armillaria* species on the Far East territory.

Fig. 8. *In vitro* spread of *Armillaria borealis* mycelium fan (strain D48) and necrosis of *Populus tremula* cambium. The movement of the mycelium fan and the presence of necrosis are shown right at the top.

collar, with a pronounced preliminary weakening as well as on trees without signs of preliminary weakening with a high-rising mycelium.

In vitro pathogenicity of *A. borealis*, depending on the strain, was 2 and 3 points, *A. cepistipes* – 1 point, *A. gallica* – 1 and 2 points, and *A. ostoyae* – 3 points.

An increase in the temperature of the near-ground air layer was characteristic for all regions of mass forest drying in Siberia (data available at https://climexp.knmi.nl; Pavlov, 2015). At the south of Siberia (53.70N, 91.70E, WMO station 29866, MINUSINSK), the warming trend was observed for the period 1890–2019: annual temperature of the near-ground air layer was increased on average by 2.6 °C in the period of the last 100 years, and April temperature – by 3.5 °C for the same period). A warmer winter (from 1916 to 2018 the winter temperature was increased by 4.9 °C) creates better conditions for fungus overwintering. The most intense increase in temperature was observed for the period 1970–2001 with May temperature increased by 3.6 °C on average reaching 15.5 °C in some years and promoting earlier and active growth of the mycelium of root pathogens at temperatures between 10 and 31 °C, with an optimum between 20 and 22 °C (Rishbeth, 1978). A simultaneous increase of precipitation also creates more favorable conditions for *Armillaria*.

DISCUSSION

The goal of identifying *Armillaria* isolates was achieved using a three-marker-based phylogenetic analysis. The isolates represented the species *A. borealis*, *A. gallica*, *A. ostoyae*, and *A. cepistipes*, which were previously identified in Siberia only by morphological characteristics (Pavlov, 2015). The validity and informativeness of the markers for identification of the *Armillaria* species that we used in our study is also confirmed by the review Coetzee et al. (2018).

Phylogenetic trees based on three marker sequences well-differentiated all samples. In contrast, trees based on single marker ITS or IGS-1 produced mixed clades such as *A. ostoyae*/*A. borealis* and *A. gallica*/*A. cepistipes*/*A. sinapina*. This can be explained by the fact that *A. ostoyae* and *A. borealis* are indeed closely related species. Their similarity could be due to sharing ancestral polymorphism and hybridization between them (Anderson, Stasovski, 1992). *A. gallica*, *A. cepistipes* and *A. sinapina* are also phylogenetically closely related species and are similar morphologically (Antonín et al., 2009). The filamentous fungal taxon delimitation power of the ITS markers is comprehensively reviewed in Vu et al. (2019). Among individual markers *TEF1-α* was the most discriminatory. Previous studies have also shown that *TEF-1α* has higher variation than ITS and IGS-1 (Antonín et al., 2009; Ross-Davis et al., 2012; Tsykun et al., 2013).

It is interesting that both phylogenetic trees based on either *TEF-1α* or all three markers demonstrated two or three clades or lineages consisting of the *A. borealis* isolates. A similar phenomenon has been observed in other studies where these two clades did not have any geographical connections (Guo et al., 2016; Klopfenstein et al., 2017). This is also in agreement with Antonín et al. (2009), according to which *A. borealis* splits into two clades based on the sequences of the same *TEF-1α* marker (see Fig. 5 there). However, they used only three samples of supposedly *A. borealis* – one from the Czech Republic formed a clade together with *A. ostoyae* and *A. gemina*, and the other two from Finland and Germany, respectively, formed another clade. Moreover, a phylogenetic tree based on a single marker is probably not sufficient for species identification, unlike our tree based on three markers.

We additionally blasted the ITS sequence of the type specimen of *A. borealis* Marxm. et Korhonen collected in Finland (the NCBI GenBank accession NR_159624, which is identical to the NCBI GenBank accession MH861561.1 and is listed in Supplementary_Table_S2.xlsx; Kolesnikova et al., 2023) with our samples, and the best matching was with samples of a large clade, starting with S16/06 (MW418555) from Central Siberia, Krasnoyarsk Territory, and then 111/18 (MW418547) from Southern Siberia, Khakassia, 396d/16 (MW418559) and 108d/15 (MW418558) from Southern Siberia, Krasnoyarsk Territory, R1-2/10 (MW418552), R1-1/9 (MW418537), 36/8 (MW418536), 206g/11 (MW418548) and D47/3 (MW418556) from Central Siberia, Krasnoyarsk Territory.

In our study, both clades of *A. borealis* also have mixed geography and contain both European and Asian strains.

This suggests that *A. borealis* can be represented by two different phylogenetic lineages, or that *A. borealis* has two lineages of the *TEF-1α* gene, which appear as distinct clusters in phylogenetic trees (Antonín et al. 2009; Mulholland et al., 2012).

A. borealis was found to be the most common species. It was found both on trees with mycelium on the roots, rarely rising above the root collar, with a pronounced preliminary weakening, and on trees without signs of preliminary weakening, with the presence of a high-rising fan of mycelium. Thus, this species appears to be the dominant in Siberia.

The second most common species, *A. cepistipes*, was isolated only from the places of dying trees in the lower layer; but the dead trees did not form groups. A rare and thin mycelium was found on the roots. The data on the dominance of *A. borealis* and *A. cepistipes* are consistent with the results of the work carried out earlier in Siberia using the method of species identification based on sexual compatibility tests (mating test) (Pavlov, 2015).

Isolates of the *A. ostoyae*, which has long been recognized as an aggressive primary pathogen, were found only in places of dying trees dominating in the upper canopy, without signs of preliminary weakening and with pronounced groups of dying trees. Rapid drying of trees (within a year) and the presence of a high-rising fan of mycelium were also observed, which also corresponds to several *A. borealis* isolates.

Due to the high degree of pathogenicity and wide spread of *A. ostoyae*, numerous studies of this species have been carried out (Omdal et al., 1995; Legrand et al., 1996; Morrison, Pellow, 2002; Prospero et al., 2008; Heinzelmann et al., 2020). Unfortunately, knowledge about the ecology of *A. borealis* is still insufficient. Field studies have shown that *A. borealis* is often less pathogenic than *A. ostoyae*, and that *A. borealis* is less likely to participate in primary attacks on living trees (Gregory, Watling, 1985; Bendel et al., 2006) and can act as a secondary pathogen, infecting trees that were previously weakened by other abiotic factors (Bendel et al., 2006). However, current studies in Finland, Siberia, and Far East demonstrated that *A. borealis* is much more pathogenic than *A. ostoyae* causing vast dry-outs (Pavlov, 2015). While experimental data on pathogenicity and *A. borealis* are limited, the data obtained suggest that *A. borealis* is similar or more virulent than *A. ostoyae* for conifer seedlings (Morrison, 2004), which is consistent with experimental data of Siberian isolates of *A. borealis* in our study.

A. gallica was isolated in places of dying trees in the lower layer with rare thin mycelium on the roots, or in places with sporadic dying trees with preliminary weakening and pronounced mycelium on the roots. This species is considered a saprotroph, like its closely related species *A. cepistipes*. Previously, it was shown that species producing monopodially branched rhizomorphs, such as *A. gallica* and *A. cepistipes,* are less aggressive than species with dichotomously branched rhizomorphs (*A. mellea*, *A. borealis*, and *A. ostoyae*) (Morrison, 2004).

Moreover, *Pinus sibirica* Du Tour, *Abies sibirica* Ledeb. and *Abies nephrolepis* (Trautv. ex Maxim.) Maxim. were more susceptible to dieback than other species. Unlike *Heterobasidion annosum* s.l., dieback of conifers due to *Armillaria* often happened very quickly without any prior noticeable weakening (Fig. 1). Death of trees with well-developed crown that did not express any signs of oppression happened very quickly. Lifespan of their needles, tree size, and growth of shoots did not differ from the average values for the forest stand. The hypothesis is that the fungus can parasitize on the roots for a long time (as evidenced by numerous excavations of root systems). However, with a decrease in the defensive ability of the tree and the penetration of the mycelium into the zone of the root collar, a rapid ringing of the trunk and death occurs.

In our study, strains 10 and 11 (*Armillaria cepistipes* and *A. ostoyae*, respectively) were isolated from neighboring trees. The coexistence of several *Armillaria* species in closely spaced territories was previously reported mainly for *A. cepistipes* and *A. ostoyae* (Prospero et al., 2006). The rest of the strains collected from neighboring trees turned out to be the same organisms.

CONCLUSION

For the first time, genetic identification of Eastern Siberian and Far Eastern isolates of *Armillaria* was carried out. Phylogenetic analysis based on three markers made it possible to determine with high accuracy the species identity of the studied isolates. However, *TEF-1α* also showed good differentiation and potentially can be used as a single molecular marker for species identification with some reservations. The isolates represented four different species, mostly *A. borealis* (18 out of 28 isolates). It is hard to conclude how many different clades or lineages have been identified in our study. We did not set the task of fully resolving all lineages; it can be solved only by using a larger number of markers or whole-genome sequencing. Our task was rather modest – to determine species identity among collected samples, and we believe that three highly polymorphic diagnostic markers, which are widely used in barcoding to identify species, are mostly sufficient, and we successfully completed this task.

The pathogenicity level of all isolates was consistent with the known data. Phylogenetic trees based on three markers, as well as based on a single marker *TEF-1α*, revealed two or possibly three clades of *A. borealis*. Additional taxonomic and ecological studies are needed to verify whether strains in these clades represent separate

phylogenetic lineages and to find out whether they have different level of pathogenicity.

The maximum level of phytopathogenicity *in situ* and *in vitro* was noted in the Far Eastern strains of *A. ostoyae* (3 points; pathological drying area is from 4.8 to 16 ha) and the Siberian strains of *A. borealis* (2–3 points; drying area is from 0.1 to 31.8 ha; in rare cases, the death of single trees is noted).

The dominant species on the territory of Southern Siberia is *A. borealis*, which is characterized by morphological, phytopathogenic and genetic heterogeneity and demonstrates two behavioral strategies: 1) moderate (secondary) pathogen, infecting trees previously weakened by other abiotic factors – the fungus colonizes trees with a pronounced preliminary weakening; the mycelium fan develops mainly on the roots and rarely rises above the root collar, 2) an aggressive (primary) pathogen capable of causing epiphytoties and occurring on trees without signs of preliminary weakening; the mycelium fan rises high along the trunk; the death of a tree can occur very quickly, within a year.

Supplemental information for this article and NCBI GenBank accession numbers of all nucleotide sequences of three gene regions (*TEF1*, ITS and IGS-1) used in the study are available in the Supplemental Files at https://doi.org/10.6084/m9.figshare.21644732.v6 (Kolesnikova et al., 2023).

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Молекулярная идентификация дереворазрушающих грибов рода *Armillaria*, распространенных в Восточной Сибири и на Дальнем Востоке России с использованием генетических маркеров ITS, IGS-1-1 и *TEF-1α*

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Род *Armillaria* является важным компонентом лесных экосистем, играя очень важную экологическую роль в разложении валежной древесины, но часто становится серьезным патогеном, вызывающим белую корневую гниль деревьев. Также известно, что виды *Armillaria* значительно различаются по уровню патогенности. Таким образом, точная идентификация *Armillaria* имеет решающее значение для оценки риска заболевания деревьев. В данном исследовании мы проанализировали 28 изолятов *Armillaria* из Сибири и Дальнего Востока с использованием нуклеотидных последовательностей участков генов ITS, IGS-1-1 и *TEF-1α* и построили филогенетические деревья на основе метода максимального правдоподобия. Всего было идентифицировано четыре вида *Armillaria*: *A. borealis*, *A. cepistipes*, *A. ostoyae* и *A. gallica*. Среди собранных изолятов наиболее часто встречался *A. borealis* (18 из 28 изолятов). *A. gallica*, *A. cepistipes* и *A. ostoyae* встречались значительно реже: два, пять и три изолята из 28 соответственно. Впервые описано распространение видов *Armillaria* в Сибири и на Дальнем Востоке. Сделан вывод о том, что необходимы дальнейшие исследования для определения роли *Armillaria* в патологическом отмирании деревьев, и *A. borealis* должен быть в центре внимания.

Ключевые слова: Дальний Восток, белая корневая гниль, Сибирь, таксономия, филогения, *Armillaria*, *Basidiomycota*, IGS, ITS, *TEF-1α*