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Pathogenic Micromycete *Neocatenulostroma germanicum* in Pine Plantations of the Republic of Armenia

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ABSTRACT

From the needles of the Scots pine (*Pinus sylvestris* L.) collected in 2022 and 2023 in Northeastern and Central regions of the Republic of Armenia the causative agent of pine needles browning *Neocatenulostroma germanicum* (Crous & U. Braun) Quaedvlieg & Crous was identified. Both morphological and molecular approaches along with phylogenetic analysis were applied. This is the first report of *N. germanicum* identified in the territory of Armenia.

Keywords: ascomycetous fungus; pine needles browning; *Pinus sylvestris*; morphological examination; internally transcribed spacer (ITS) region; phylogenetic analysis

INTRODUCTION

Armenia has relatively limited forest resources. The total forest area in the country is approximately 11% of the total land area (Mkrtchyan and Grigoryan 2014, Bondarev 2020). Among these, pine plantations constitute the smallest share among the main forest-forming species, accounting for 5.3% of Armenia's total forest area (Alaverdyan 2002).

Despite the relatively small area of pine forests in Armenia, they play an important economic and nature conservation role. Pine planting (*Pinus* spp.) in Armenia started in the mid-20th century primarily to replenish the forest fund in areas initially treeless or bare as a result of logging, in areas where pine was the most acceptable species for the existing soil and climatic conditions and where reforestation using other species was considered inappropriate. Currently, pine forests play an anti-erosion role on mountain slopes. They also have a certain protective value for agricultural lands, and contribute to the creation of favorable microclimatic conditions, particularly in areas of traditional recreation.

Drying of pine plantations, mainly artificially planted *Pinus sylvestris*, in Armenia was first observed in 2018 in the northern provinces. In the following years, this phenomenon spread widely to the central and southern regions of the country as well. To elucidate the underlying causes of this phenomenon, comprehensive surveys were carried out, including studies of insect pests (Karagyan et al. 2024) and nematodes (Arbuzova et al. 2025). Preliminary data on the fungal community were also obtained (Shchukovskaya et al. 2024).

In the frame of the reconnaissance surveys of fungal communities' symptoms of lesion (browning and dying of the ends of needles), symptoms similar in appearance to those caused by *Neocatenulostroma germanicum* were found on needles of *Pinus sylvestris* L.

The genus *Neocatenulostroma* includes both plant-pathogenic species and those occurring on various substrates (Quaedvlieg et al. 2014). Within the genus, a distinct complex has formed, consisting of three morphologically similar and phylogenetically closely related species: *N. abietis* (Butin & Pehl) Quaedvlieg & Crous, *N.*

germanicum (Crous & U. Braun) Quaedvlieg & Crous, and *N. microsporum* (Joanne E. Taylor & Crous) Quaedvlieg & Crous (Quaedvlieg et al. 2014, Markovskaja et al. 2016).

Recently, the identification of pathogenic micromycetes has increasingly relied on a combination of morphological and molecular diagnostic methods, which were also used in our study. This is the first record of this pathogenic species in the mycoflora of the country.

MATERIALS AND METHODS

Sample Collection

During 2022 and 2023, the surveys of drying pine plantations were carried out in some provinces of Armenia (Kotayk, Gegharkunik, Tavush, Lori and Aragatsotn) (Figure 1, Table 1). Samples were collected from 5 to 10 trees in each location, with the number of pine needles analyzed from each tree being approximately 15. Needles showing obvious signs of damage (such as discoloration, formation of spore-bearing structures, necrosis, and wilting) were collected. Wood fragments were collected for further mycological analyses as well.

Fungi Isolation, Microscopy and Morphometry

Needle fragments showing signs of necrosis were sterilized in 70% ethanol for 10 seconds, then rinsed with sterile water 3–5 times, dried, placed in a moist chamber, and incubated for 1–2 weeks at 20–22°C (Stamets and Chilton 1983). The culture of the fungus was obtained by subculturing well-developed sporodochia that had formed on infected needles onto a nutrient medium potato dextrose agar (PDA). PDA medium was prepared from dry powder using the commercial "HiMedia Laboratories Pvt. Limited" (India) according to the manufacturer's instruction. Fragments of mycelium for subculturing were taken from the periphery of actively growing colonies to

Table 1. Sample collection data in Armenia.

Locality	Dates of collection	GPS	Habitats
env. Tsilkar	28.06.2022 07.07.2023	N40.73039 E44.19521 El. 2168 m	artificial pine forest
env. Gyulagarak, "Sochut" dendropark	28.06.2022 06.07.2023	N40.93709 E44.48176 El. 1481 m	mix of artificially grown trees
env. Dilijan	29.06.2022 04.07.2023	N40.75897 E44.89333 El. 1180 m	artificial pine forest
env. Semyonovka	29.06.2022 04.07.2023	N40.67862 E44.88021 El. 1898 m	artificial pine forest mixed with deciduous trees
env. Hankavan	30.06.2022 08.07.2023	N40.63037 E44.47231 El. 2016 m	artificial pine forest
env. Tsaghkadzor	30.06.2022 08.07.2023	N40.53125 E44.73662 El. 1796 m	artificial pine forest
env. Tsovagyugh	01.07.2022 04.07.2023	N40.61208 E44.95750 El. 1929 m	artificial pine forest on the former bottom of the lake Sevan
env. Tsapatagh	01.07.2022	N40.40041 E45.47052 El. 1905 m	artificial pine forest on the former bottom of the lake Sevan
env. Jil	01.07.2022	N40.47126 E45.40908 El. 1914 m	artificial pine forest on the former bottom of the lake Sevan
env. Yeranos	03.07.2023	N40.25537 E45.21249 El. 1916 m	artificial pine forest on the former bottom of the lake Sevan
env. Nerkin Getashen	03.07.2023	N40.16468 E45.26379 El. 1918 m	artificial pine forest on the former bottom of the lake Sevan
env. Tsovak	03.07.2023	N40.20168 E45.64730 El. 1923 m	artificial pine forest on the former bottom of the lake Sevan
env. Koghb, "Zikatar" Environmental Center	05.07.2023	N41.12210 E44.92322 El. 1309 m	beech forest with artificially grown plots of pine trees
env. Jrvezh, Forest Park	10.07.2023	N40.17968 E44.61874 El. 1559 m	artificial mixed forest with plots of pine trees

avoid contamination by bacteria and other fungi. Isolation was also carried out on a growth medium (PDA), incubated for 3 to 6 weeks at the temperature of 20–22°C. For molecular analysis, a pure fungal culture was obtained through multiple subcultures on PDA.

The formed mycelium was examined under a light microscope Olympus BX53 Evident equipped with Olympus DP74 digital camera for revealing morphological structures. The sporodochia formed on the surface of the needles were photographed using a Zeiss SteReo Discovery V12 stereomicroscope equipped with a Canon EOS 5D MkIII camera. Initial species identification was carried out by morphological features using reference literature (Crous et al. 2007, Quaedvlieg et al. 2014, Markovskaja et al. 2016).

DNA Extraction, Polymerase Chain Reaction (PCR) Amplification and Sequencing

Genomic DNA was isolated from sporulating cultures using the commercial “PhytoSorb” kit (Syntol, Russia), based on purification with magnetic particles, in accordance with the manufacturer’s instructions. In the two studied isolates, the amplification of the nuclear genome region internal transcribed spacer (ITS) was performed using the following pair of universal primers (White et al. 1990):

- ITS-5- 5’- GGAAGTAAAAGTCGTAACAAG G -3’,
- ITS-4 - 5’- TCCTCCGCTTATTGATATGC -3’.

The PCR mixture (25 µl) contained 5 µl 5× PCR buffer Mas^{OR} TaqMix-2025 (“Dialat Ltd”, Russia), 0.5 µl of each primer, 2 µl of the respective genomic DNA extract and 17 µl sterile water. PCR conditions were as follows: initial denaturation for 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 52°C and 30 s at 72°C, with final extension for 7 min at 72°C.

Visualization of PCR products was performed by gel electrophoresis in 1% agarose gel stained with ethidium bromide. PCR products intended for Sanger sequencing were purified using the GeneJET PCR Purification Kit (Thermo Fisher, USA) according to the manufacturer’s instructions.

The sequencing reaction was performed using BigDye Terminator v3.1 Cycle Sequencing Kit reagents (Applied Biosystems, USA) according to the manufacturer’s instructions, followed by separation of fragments on a 3500 genetic analyzer (Applied Biosystems, USA).

Two original sequences of *N. germanicum* specimens from Hankavan village (accession number PX219661) and Dilijan town (PX219657) obtained for the 572 bp fragment, and one sequence of *Diplodia sapinea* from Tsilkar village (PX219648) obtained for the 600 bp fragment of the ITS region were submitted to GenBank.

Samples and Sequence Alignment, Phylogenetic Tree Construction

Nucleotide sequences obtained in this study were edited and aligned using the Clustal W algorithm in the BioEdit software (Hall 1999). Comparative analysis of the obtained nucleotide sequences with the sequences deposited in GenBank was carried out using the BLAST program on the NCBI website (https://www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed in MEGA 11.0 program (MEGA software development team, USA) using Maximum Likelihood (ML) method and Kimura 2-parameter model. The standard bootstrap (1000 replicates) was used to evaluate the statistical nodal support of the tree (Kimura 1980, Kumar et al. 2016).

For phylogenetic analysis the ITS region sequences of 27 samples, both original and those obtained from GenBank were used, namely: 12 samples of *Neocatenulostroma germanicum*, 3 samples of *N. microsporum,* 4 samples of *N. abietis*, 1 sample of *N. castaneae* Phukhams., Bhunjun & K.D. Hyde, 6 species belonging to the other genera of order Mycosphaerellales and *Diplodia sapinea* (Fr.) Fuckel (Botryosphaeriales, as out group) (Table 2).

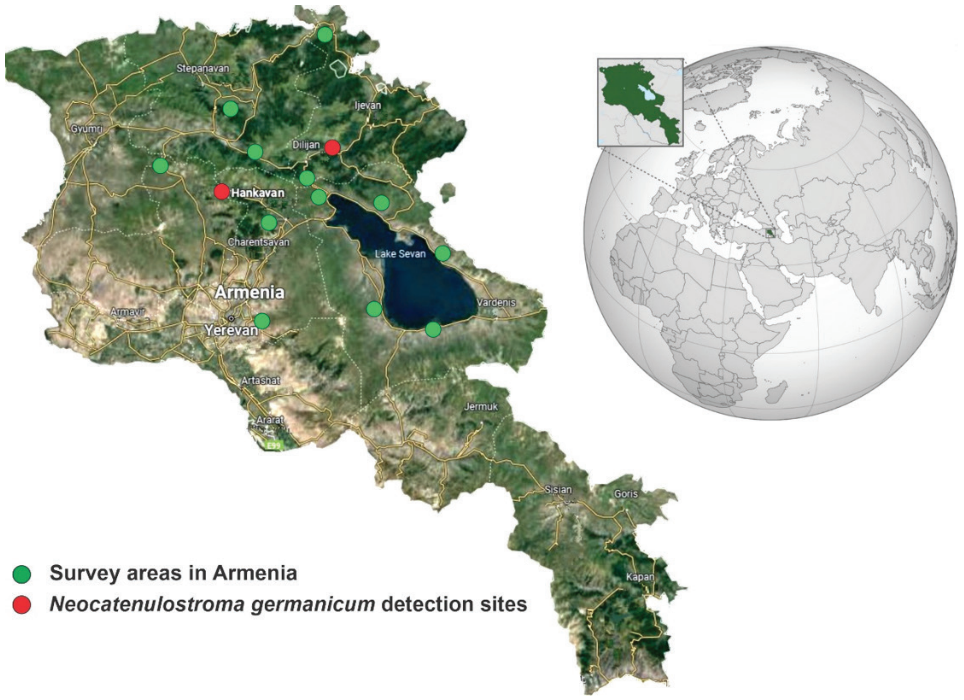

Figure 1. Map with sampling sites and localities of the record of *Neocatenulostroma germanicum* in Armenia.

Table 2. Species of Mycosphaerellales and *Diplodia sapinea* (Botryosphaeriales) taken as out group used for phylogenetic analysis (ITS region).

Species	Country	Isolation source	GenBank Accession No	Reference
Neocatenulostroma germanicum	Armenia	*Pinus sylvestris*	PX219661 PX219657	This study
N. germanicum	Lithuania	*P. mugo*	KR995100 KR995103	Markovskaja et al. 2016
		P. sylvestris	KR995101 KR995102	
N. germanicum	Ukraine	*P. nigra* subsp. *pallasiana*	KR995104 KR995105 KR995106	
N. germanicum	Germany	Stone (CBS 539.88)	EU019253.2	Crous et al. 2007
N. germanicum	Germany	Holotype of *Catenulostroma germanicum* Crous & U. Braun (CBS 539.88)	MH862143.1	Vu et al. 2019
N. germanicum	Belarus	*Pinus nigra*	MK622897.1	Golovchenko et al. 2021
N. microsporum	South Africa	*Protea cynaroides*	KF901499.1	Quaedvlieg et al. 2014
N. microsporum	South Africa	Culture from holotype of *Trimmastostroma microsporum* (CBS 101951)	EU167572.1	Simon et al. 2009
N. microsporum	South Africa	*Protea cynaroides*	NR_145114.1	Quaedvlieg et al. 2014
N. abietis	USA	Soil	MW471927.1	Bazzicalupo et al. 2022
N. abietis	Germany	Type of *Trimmatostroma abietis* Butin & Pehl (CBS 459.93)	NR_145103.1	Crous and Groenewald 2005
N. abietis	Mallorca	Limestone	AY559362.1	Ruibal et al. 2005
N. abietis	Germany	Type of *Trimmatostroma abietis* Butin & Pehl (CBS 459.93)	MH862431.1	Vu et al. 2019
N. castaneae	Italy	*Castanea sativa*	MZ519072.1	Phukhamsakda et al. 2022
Teratosphaeria verrucosa	South Africa	*Eucalyptus cladocalyx*	KF901645.1	Quaedvlieg et al. 2014
T. cryptica	Australia	*E. globulus*	KF901573.1	Quaedvlieg et al. 2014
Catenulostroma protearum	South Africa	*Hakea sericea*	GU214628.1	Crous et al. 2009
C. hermanusense	South Africa	*Phaenocoma prolifera*	JF499833.1	Crous and Groenewald 2011
Mycosphaerella parva	Australia	*Austroafricana parva*	AY509780.1	Maxwell et al. 2005
Neophaeothecoidea proteae	South Africa	*Protea repens*	NR_157417	Crous et al. 2008
Diplodia sapinea	Armenia	*P. sylvestris*	PX219648	This study

RESULTS AND DISCUSSION

Cultural and Morphological Characteristics of *Neocatenulostroma Germanicum*

Laboratory studies on the surface of the necrotized part of the needles collected in the vicinity of Dilijan town (Tavush Province) and Hankavan village (Kotayk Province) revealed the formed conidial sporulation of the fungus – black stromatic beds, as well as sporodochia exiting through the stomata (Figures 2 a, b).

Conidia are thick-walled, dark brown, ellipsoid, with one septum, combined into short chains, 9.4–25.2 × 4.9–6.4 µm (Figure 2 c).

Colonies are slow-growing, on the 25th day at 20°C the average diameter was 11–12 mm, their shape is rounded, with smooth edges (Figure 2 d). At the initial stage of growth, the colony appears gray with an olive tinge; as it grows, it takes on a rich dark gray (steel-like) color with a faint olive-green tint. The colony reverse is uniformly black in color.

According to morphological characteristics, the isolated fungus was identified as *Neocatenulostroma germanicum* (Crous et al. 2007, Markovskaja et al. 2016).

Comparison of the Sequences and Phylogenetic Analysis

According to Maximum Likelihood (ML) phylogenetic analysis of the original sequences obtained in this study and deposited in GenBank, some sequences showed that they belong to the *Neocatenulostroma abietis/germanicum/microsporum* species complex (Figure 3). As expected, these species form a single clade with high support (99%) of the bootstrap test, whereas the newly described species *N. castaneae* from Italy (MZ519072.1) is not included in this clade. It should be noted that the target isolates from Armenia are 99.6–99.8% identical to *N. germanicum* isolates from Germany (EU019253.2), Latvia (KR995101), and Ukraine (KR995104, KR995105, KR995106).

Among morphologically similar species of the group, which include *Neocatenulostroma abietis*, *N. germanicum* and *N. microsporum*, one of the most potentially dangerous and poorly studied pathogenic micromycetes is *N. germanicum*, the causal agent of needle browning. The species is distributed in several European countries, including Germany, Poland, Lithuania, Montenegro, Belarus, and some regions of Russia (Markovskaja et al. 2016, Lasarević and Menkis 2020, Golovchenko et al. 2021, Bulgakov 2022). *N. germanicum* can behave not only as a saprotroph developing on various substrates, but also as a serious pathogen, massively affecting the needles of young pines in spring and summer, sometimes together with fungi of the genus *Dothiostroma* sp. (Markovskaja et al. 2016). Therefore, the application of both microscopic and morphometric methods, as well as molecular analysis (using a part of ITS region) confirmed the presence of *N. germanicum* in two localities of the artificial pine plantations in the vicinity of the Dilijan town and the Hankavan village, in the Republic of Armenia. In both localities, artificially grown pine forests planted on fairly steep, south-facing slopes are present. Despite different altitudes, the habitats are characterized by similar ecological conditions.

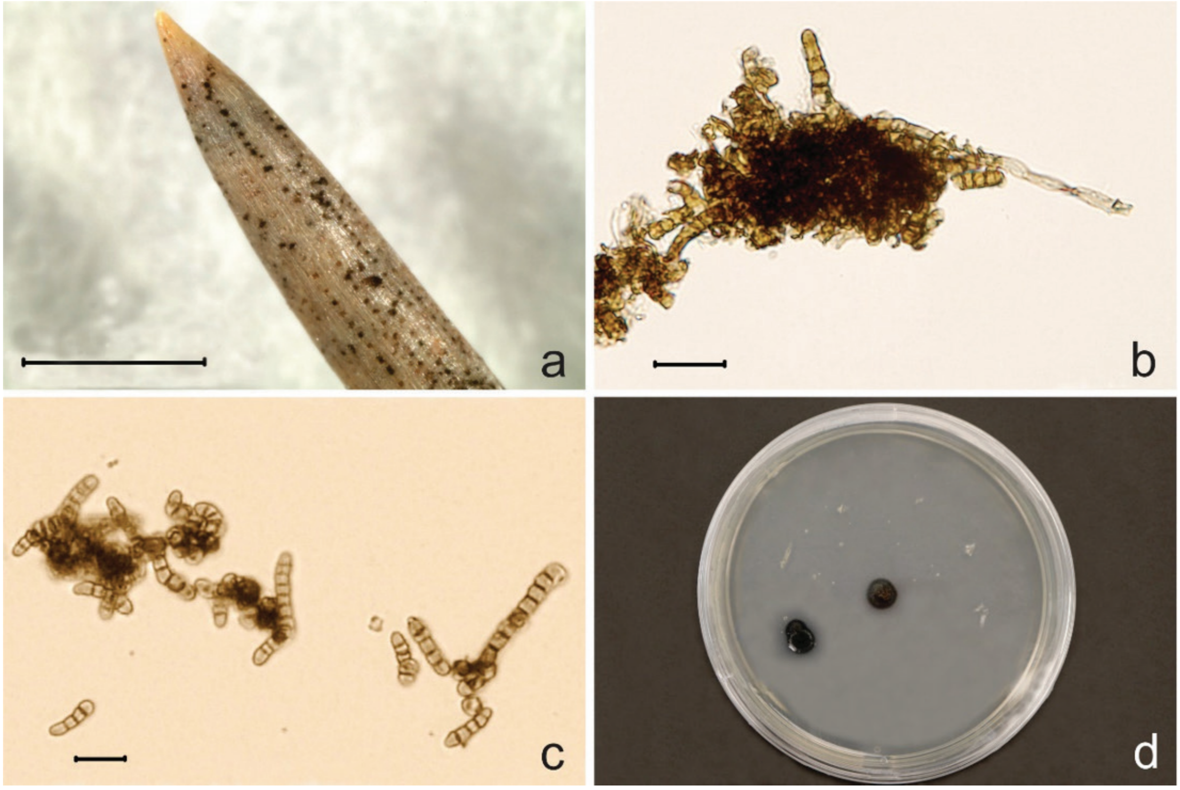

Figure 2. *Neocatenulostroma germanicum.* **(a)** Dark sporodochia on the necrotic part of pine needles. Scale bar = 3.2 mm; **(b)** Sporodochia with conidia chains. Scale bar = 100 µm; **(c)** Chains of conidia. Scale bar = 200 µm; **(d)** Colonies on PDA after 25 days.

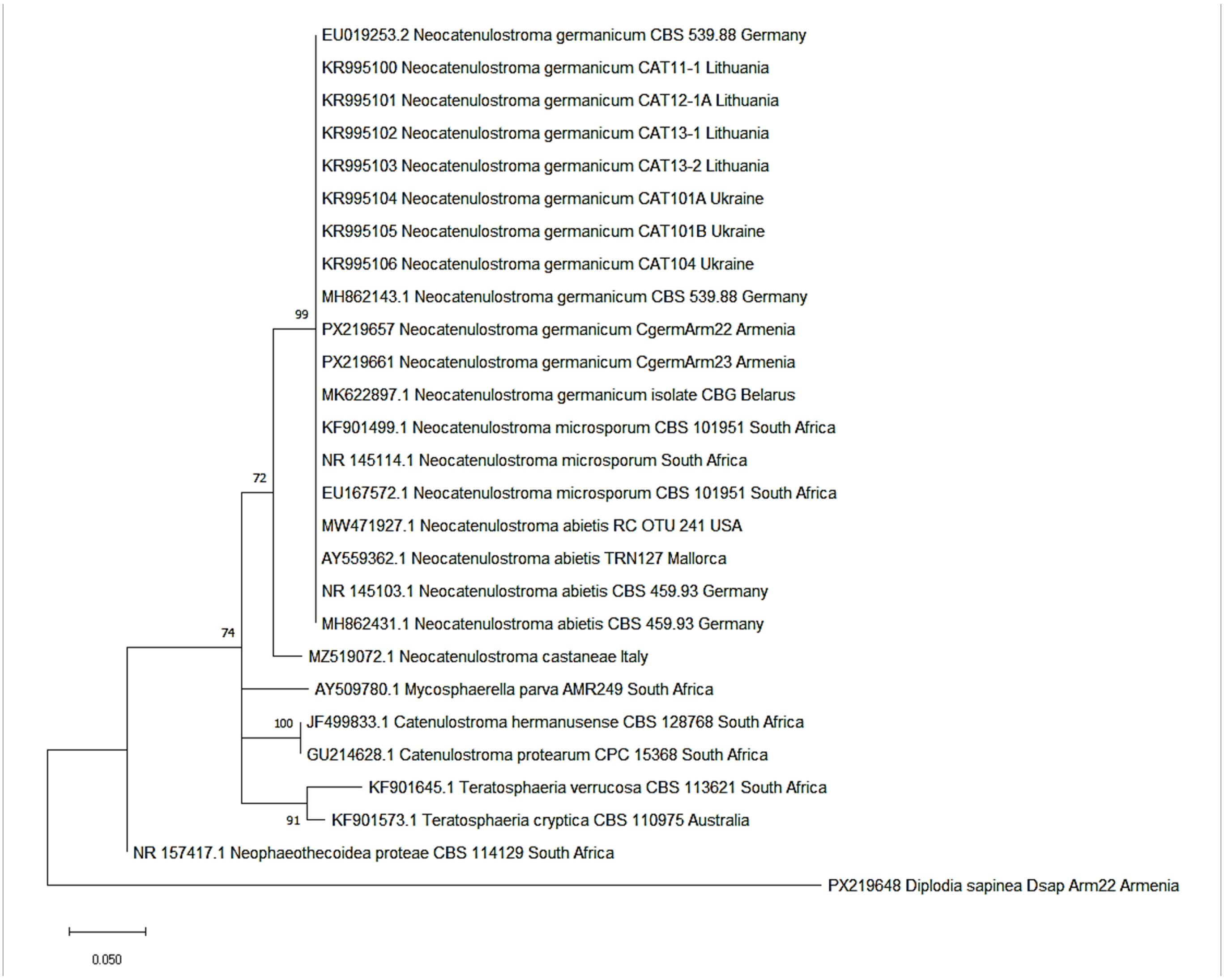

Figure 3. Maximum Likelihood (ML) phylogenetic tree based on sequences of ITS genome fragment of 12 specimens of *Neocatenulostroma germanicum*, some representatives of the order Mycosphaerellales and *Diplodia sapinea* as out group. The robustness determined by bootstrap analysis (1000 alternative dendrograms) is indicated as percentage at nodes. The GenBank accession number and country of origin are listed for every sequence.

CONCLUSION

The results of our research allow us to draw a definite conclusion that in the north-eastern and central regions of the Republic of Armenia (Tavush and Kotayk provinces) on the needles of Scots pine (*Pinus sylvestris* L.) a pathogenic micromycete *Neocatenulostroma germanicum* has been revealed, whose species identity is confirmed by the results of studies based on morphological, microscopic and molecular diagnostic methods. This is the first report of the discovery of the species on the territory of the Republic of Armenia.

It is important to note that further molecular genetic studies are planned, during which more variable and informative regions of the nuclear genome will be tested to enable more precise species differentiation within the genus *Neocatenulostroma*.

Author Contributions

KG, GT – conceived and designed the research, KG, AK, GT, SA, AE, EI, SI – conducted field research and collected material, SA, KN, – performed laboratory studies, SA, KN, KG – processed the data, KG - secured the field research funding; KG, KM – assisted with the preparation of the drafted manuscript, SA – wrote the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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