

# First report of *Nigrospora humicola* (Apiosporaceae, Xylariales) on *Phragmites* plant from North Asia

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## Abstract

In this study, one *Nigrospora* sp. strain was isolated from a stem of *Phragmites australis* collected in North Asia and identified as *N. humicola* based on the results of multilocus phylogeny of the internal transcribed spacer (ITS),  $\beta$ -tubulin (*tub*), translation elongation factor EF-1a (*tef*) genes, and a detailed analysis of the cultural and morphological characters of the fungus. This is the first detection of *N. humicola* in Russia and its first occurrence on a plant, so the substrate range of this species may be wider than currently known. Morphological analysis of the strain revealed the additional characteristics that are considered important for the identification of *N. humicola*: sterile cells; formation of stained clusters of conidiophores and conidiogenous cells; equatorial slits in some young conidia. Thus, further analysis of morphology would be useful in refining our understanding of the *Nigrospora* species limits.

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## Introduction

*Nigrospora* Zimm. (Ascomycota, Sordariomycetes, Xylariales) are ubiquitous fungi revealed on plants<sup>[1–4]</sup>, lichens<sup>[5]</sup>, insects<sup>[6,7]</sup>, marine organisms<sup>[8]</sup>, humans<sup>[9]</sup>, and in the soil<sup>[10]</sup>. These fungi are associated with different plants as endophytes, saprobes, parasites, and pathogens<sup>[2,4,11–13]</sup>.

Nowadays, with the emerging possibilities of accurate species identification of fungi, studying the patterns of their occurrence and abundance, as well as various characteristics is an urgent task. In recent years the revision of *Nigrospora* genus was carried out and new species were described on the basis of the molecular and morphological evidence<sup>[1,4,10,13–17]</sup>. The identified genetic diversity of *Nigrospora* indicates that many species of this genus have not yet been discovered. Moreover, the information on the distribution and host plants of known *Nigrospora* species remains often fragmentary. At least 30 *Nigrospora* species were detected in China on various substrates<sup>[10,11,13,14,16,17]</sup>, among them, 14 *Nigrospora* species were associated with *Poaceae* plants<sup>[11,13,14]</sup>.

In Russia, the *N. gorlenkoana* Novobr. occurrence on wheat, barley, oats, maize, and rapeseed were previously confirmed by molecular genetic methods<sup>[18]</sup>, while there is no information on the distribution of other *Nigrospora* species. In our previous study, we suggested that one strain of *Nigrospora* sp. MFG 70052, isolated from *Phragmites australis* stem can be described as a potential new species based on morphology and phylogenetic analysis data, which represented the distinct genetic line, sister to *N. chinensis* Mei Wang & L. Cai<sup>[18]</sup>.

The aim of study was to identify the *Nigrospora* sp. strain isolated from *Phragmites australis* collected in North Asia.

## Materials and methods

### Fungal strain

The *Nigrospora* sp. strain was isolated from the *Phragmites australis* collected in Primorsky Krai of Russia in 2010. The fragments

of stems with necrosis were carefully washed with tap water, and were surface sterilized by soaking in 5% NaOCl (3 min), and then rinsing with sterile water. Then, the plant tissues were placed on potato sucrose agar (PSA) containing 1 mL/L mixture of antibiotics (HyClone, Austria), and 0.4  $\mu$ L/L Triton X-100 (Panreac, Spain). Following incubation at 25 °C, the pure *Nigrospora* culture was sub-cultures onto fresh PSA. Suspension 20  $\mu$ L consisting of 20–50 conidia and sterilized distilled water was transferred to a Petri dish on PSA containing Triton X-100 and spread with a sterile spatula over the surface of the medium prepared the day before. Incubation continued for 48 h at 25 °C, after one germinated spore was selected and transferred to a new dish with PSA. The fungal growth was recorded after 3 d of incubation. The single-spored *Nigrospora* strain MFG 70052 was deposited in the culture collection of the All-Russian Institute of Plant Protection (VIZR, St. Petersburg, Russia).

### Molecular genetic analysis

Genomic DNA was extracted from fungal mycelium grown on PSA (7 d, 25 °C, in the dark) using the CTAB protocol<sup>[19]</sup>. The fragments of the internal transcribed spacer (ITS),  $\beta$ -tubulin gene (*tub*), and translation elongation factor EF-1a (*tef*) were amplified with ITS1/ITS4<sup>[20]</sup>, T1/T2<sup>[19]</sup>, and EF1-728F/EF1-986R<sup>[21]</sup> primer pairs and according to the authors' protocols, respectively. The sequencing of the fragments was carried out on an ABI Prism 3500 sequencer (Applied Biosystems, Japan) using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA).

The obtained combined sequences of three loci (ITS, *tub*, and *tef*) as well as the sequences of representative 88 *Nigrospora* spp. strains (Table 1) were aligned using the MEGA X 10.1 program. The best-fitting substitution model was determined using the IQ-TREE 2 v.2.1.3 program. The T92+G model was chosen for the multilocus analysis. The phylogenetic analysis was performed using maximum likelihood (ML) also in IQ-TREE 2 v.2.1.3 program. Bootstrap supports values were calculated with 1,000 replicates. To further infer phylogenetic relationships among taxa, a Bayesian analysis was conducted with MrBayes 3.2.1 using 2,000,000 generations of Markov chain Monte Carlo (MCMC), and trees were sampled at every 1000<sup>th</sup> generation.

**Table 1.** The information on the strains used for phylogenetic analysis in this study.

Species	Strain ID	Substrate	Origin	GenBank accession numbers		
				ITS	tub	tef
<i>Nigrospora anhuiensis</i>	QY-2	Rice	China	OP677969	PP103614	PP103590
<i>N. aurantiaca</i>	CGMCC 3.18130 T	<i>Nelumbo</i> sp., leaves	China	KX986064	KY019465	KY019295
<i>N. aurantiaca</i>	LC 7034	<i>Musa paradisiaca</i>	China	KX986093	KY019598	KY019394
<i>N. aurantiaca</i>	LC12063	<i>Saccharum officinarum</i> , leaves	China	MN215769	MN329933	MN264008
<i>N. bambusae</i>	CGMCC 3.18327 T	Bamboo, leaves	China	KY385307	KY385319	KY385313
<i>N. bambusae</i>	LC 7244	Bamboo, leaves	China	KY385306	KY385320	KY385314
<i>N. brasiliensis</i>	CMM1214 T	<i>Nopalea cochenillifera</i>	Brazil	KY569629	MK720816	MK753271
<i>N. brasiliensis</i>	CMM 1217	<i>Nopalea cochenillifera</i>	Brazil	KY569630	MK720817	MK753272
<i>N. camelliae-sinensis</i>	CGMCC 3.18125 T	<i>Camellia sinensis</i>	China	KX985986	KY019460	KY019293
<i>N. camelliae-sinensis</i>	LC12070	<i>Saccharum officinarum</i> , leaves	China	MN215773	MN329937	MN264012
<i>N. chinensis</i>	CGMCC 3.18127 T	<i>Machilus breviflora</i>	China	KX986023	KY019462	KY019422
<i>N. chinensis</i>	LC4433	<i>Castanopsis</i> sp.	China	KX986013	KY019536	KY019436
<i>N. chinensis</i>	LC 4660	<i>Quercus</i> sp.	China	KX986026	KY019548	KY019445
<i>N. chinensis</i>	QY	Rice	China	OP677966	PP103612	PP103588
<i>N. coryli</i>	W18	<i>Corylus heterophylla</i>	China	PP218065	PP320372	PP461302
<i>N. cooperae</i>	SFC20230324-M03	<i>Ishige</i> sp.	South Korea	OQ726361	OQ735179	OQ735196
<i>N. cooperae</i>	BRIP 72408b	<i>Themeda arguens</i>	Australia	OP035047	OP039537	OP039538
<i>N. covidalis</i>	CGMCC 3.20538 T	<i>Lithocarpus</i> sp.	China	OK335209	OK431479	OK431485
<i>N. covidalis</i>	LC15837	<i>Lithocarpus</i> sp.	China	OK335210	OK431480	OK431486
<i>N. endophytica</i>	URM8462 T	<i>Manihot esculenta</i>	Brazil	OM265233	OP572420	OP572416
<i>N. endophytica</i>	ARM687	<i>Manihot esculenta</i>	Brazil	OM265226	OP572418	OP572415
<i>N. falsivesicularis</i>	CGMCC 3.19678 T	<i>Saccharum officinarum</i> , leaves	China	MN215778	MN329942	MN264017
<i>N. falsivesicularis</i>	LC13553	<i>Saccharum officinarum</i> , roots	China	MN215779	MN329943	MN264018
<i>N. ficuum</i>	ZHKUCC 22-0143	<i>Ficus</i> sp.	China	OR164911	OR166318	na
<i>N. ficuum</i>	ZHKUCC 22-0125	<i>Ficus</i> sp.	China	OR164910	OR166317	na
<i>N. globosa</i>	CGMCC3.19633 T	Soil	China	MK329121	MK336134	MK336056
<i>N. globosa</i>	LC 12441	Soil	China	MK329122	MK336135	MK336057
<i>N. globospora</i>	CGMCC 3.20539 T	<i>Petasites hybridus</i>	China	OK335211	OK431481	OK431487
<i>N. globospora</i>	LC 15839	<i>Petasites hybridus</i>	China	OK335212	OK431482	OK431488
<i>N. gorlenkoana</i>	CBS 480.73	<i>Vitis vinifera</i>	Kazakhstan	KX986048	KY019456	KY019420
<i>N. gorlenkoana</i>	MFG 70030	Wheat, grain	Russia	OK563236	OK626358	OK626374
<i>N. gorlenkoana</i>	MFG 70036	Wheat, grain	Russia	OK563241	OK626363	OK626379
<i>N. gorlenkoana</i>	MFG 70038	Barley, grain	Russia	OK563242	OK626364	OK626380
<i>N. gorlenkoana</i>	MFG 70051	Oats, grain	Russia	OK563250	OK626372	OK626388
<i>N. guangdongensis</i>	CFCC 53917 T	<i>Cunninghamia lanceolata</i>	China	MT017509	MT024495	MT024493
<i>N. guangdongensis</i>	Tly068	<i>Cunninghamia lanceolata</i>	China	MT017510	MT024496	MT024494
<i>N. guilinensis</i>	CGMCC 3.18124 T	<i>Camellia sinensis</i>	China	KX985983	KY019459	KY019292
<i>N. guilinensis</i>	LC 7301	<i>Nelumbo</i> sp., stem	China	KX986063	KY019608	KY019404
<i>N. hainanensis</i>	CGMCC 3.18129 T	<i>Musa paradisiaca</i> , leaves	China	KX986091	KY019464	KY019415
<i>N. hainanensis</i>	LC13514	<i>Saccharum officinarum</i> , leaves	China	MN215780	MN329944	MN264019
<i>N. humicola</i>	CFCC 56884 T	Soil	China	ON555686	ON557392	ON557394
<i>N. humicola</i>	CFCC 56885	Soil	China	ON555687	ON557393	ON557395
<b><i>N. humicola</i></b>	<b>MFG 70052</b>	<b><i>Phragmites australis</i></b>	<b>Russia</b>	<b>OK563251</b>	<b>OK626373</b>	<b>OK626389</b>
<i>N. lacticolonia</i>	CGMCC 3.18123 T	<i>Camellia sinensis</i>	China	KX985978	KY019458	KY019291
<i>N. lacticolonia</i>	LC 7009	<i>Musa paradisiaca</i> , leaves	China	KX986087	KY019594	KY019454
<i>N. lacticolonia</i>	LC12059	<i>Saccharum officinarum</i> , leaves	China	MN215783	MN329947	MN264022
<i>N. macarangae</i>	MFLUCC 19-0141 T	<i>Macaranga tanarius</i> , dead leaves	Taiwan, China	MW114318	na	na
<i>N. macarangae</i>	NCYUCC 19-0177	<i>Macaranga tanarius</i> , dead leaves	Taiwan, China	MW114319	na	na
<i>N. macarangae</i>	NCYUCC 19-0312	<i>Macaranga tanarius</i> , dead leaves	Taiwan, China	MW114320	na	na
<i>N. magnoliae</i>	MFLUCC 19-0112 T	<i>Magnolia liliifera</i> , leaves	China	MW285092	MW438334	na
<i>N. magnoliae</i>	LC 6704	<i>Camellia sinensis</i>	China	KX986047	KY019571	KY019373
<i>N. manihoticola</i>	URM8461 T	<i>Manihot esculenta</i>	Brazil	OM265224	OM869479	OM914791
<i>N. marylouiseclawsiae</i>	BRIP 74865b T	<i>Spinifex sericeus</i>	Australia	PP125567	PP209362	PP209361
<i>N. mercuriadeae</i>	BRIP 75764a T	<i>Chromolaena odorata</i>	Australia	PP707904	PP712794	PP712793
<i>N. musae</i>	CBS 319.34 T	<i>Musa paradisiaca</i> , fruit	Australia	KX986076	KY019455	KY019419
<i>N. musae</i>	LC 6385	<i>Camellia sinensis</i>	China	KX986042	KY019567	KY019371
<i>N. oryzae</i>	LC 6759	<i>Oryza sativa</i>	China	KX986054	KY019572	KY019374
<i>N. oryzae</i>	LC 2707	<i>Rhododendron simiarum</i>	China	KX985954	KY019481	KY019307
<i>N. osmanthi</i>	CGMCC 3.18126 T	<i>Osmanthus</i> sp.	China	KX986010	KY019461	KY019421
<i>N. osmanthi</i>	LC 4487	<i>Hedera nepalensis</i>	China	KX986017	KY019540	KY019438
<i>N. pernambucoensis</i>	SCUA-Saf-N16	<i>Arthrocnemum macrostachyum</i>	Iran	PP256498	PP263820	PP263806
<i>N. pernambucoensis</i>	URM8463 T	<i>Manihot esculenta</i>	Brazil	OM265234	OM869481	OM914793
<i>N. philosophiae-doctoris</i>	CGMCC 3.20540 T	<i>Disporum sessile</i>	China	OK335213	OK431483	OK431489

(to be continued)

Table 1. (continued)

Species	Strain ID	Substrate	Origin	GenBank accession numbers		
				ITS	tub	tef
<i>N. philosophiae-doctoris</i>	LC 15838	<i>Disporum sessile</i>	China	OK335214	OK431484	OK431490
<i>N. pyriformis</i>	CGMCC 3.18122 T	<i>Citrus sinensis</i>	China	KX985940	KY019457	KY019290
<i>N. pyriformis</i>	LC 2694	<i>Rubus reflexus</i>	China	KX985945	KY019472	KY019300
<i>N. rubi</i>	CGMCC 3.18326 T	<i>Rubus</i> sp.	China	KX985948	KY019475	KY019302
<i>N. saccharicola</i>	CGMCC3.19362 T	<i>Saccharum officinarum</i> , leaves	China	MN215788	MN329951	MN264027
<i>N. saccharicola</i>	LC 12057	<i>Saccharum officinarum</i> , leaves	China	MN215789	MN329952	MN264028
<i>N. sacchari-officinarum</i>	CGMCC3.19335 T	<i>Saccharum officinarum</i> , roots	China	MN215791	MN329954	MN264030
<i>N. sacchari-officinarum</i>	LC 13531	<i>Saccharum officinarum</i> , roots	China	MN215792	MN329955	MN264031
<i>N. singularis</i>	CGMCC3.19334 T	<i>Saccharum officinarum</i> , roots	China	MN215793	MN329956	MN264032
<i>N. singularis</i>	LC 12068	<i>Saccharum officinarum</i> , roots	China	MN215794	MN329957	MN264033
<i>N. shadeganensis</i>	IRAN 4958C T	<i>Halocnemum strobilaceum</i>	Iran	PP256499	PP263821	PP263812
<i>N. shadeganensis</i>	SCUA-Saf-N28	<i>Aeluropus lagopoides</i>	Iran	PP256500	PP263822	PP263813
<i>N. sphaerica</i>	LC 7298	<i>Nelumbo</i> sp., leaves	China	KX985937	KY019606	KY019401
<i>N. sphaerica</i>	LC13530	<i>Saccharum officinarum</i> , roots	China	MN215795	MN329958	MN264034
<i>N. stoneae</i>	BRIP 75022a	<i>Cyperus aromaticus</i>	Australia	OR608744	OR604067	OR604065
<i>N. stoneae</i>	BRIP 75019a T	<i>Cyperus aromaticus</i>	Australia	OR608743	OR604066	OR604064
<i>N. vesicularifera</i>	CGMCC3.19333 T	<i>Saccharum officinarum</i> , leaves	China	MN215812	MN329975	MN264051
<i>N. vesicularifera</i>	LC 12055	<i>Saccharum officinarum</i> , leaves	China	MN215814	MN329977	MN264053
<i>N. vesicularis</i>	CGMCC 3.18128 T	<i>Musa paradisiaca</i> , leaves	China	KX986088	KY019463	KY019294
<i>N. vesicularis</i>	LC 0322	unknown host plant	Thailand	KX985939	KY019467	KY019296
<i>N. yunnanensis</i>	GUCC24-0008 T	<i>Juglans regia</i>	China	PP915796	PP947937	PP947933
<i>N. yunnanensis</i>	GUCC24-0009	<i>Juglans regia</i>	China	PP915797	PP947938	PP947934
<i>N. zimmermanii</i>	CBS 290.62	<i>Saccharum officinarum</i>	Ecuador	KY385309	KY385317	KY385311
<i>N. zimmermanii</i>	LC 13534	<i>Saccharum officinarum</i>	Pakistan	MN215824	MN329987	MN264063
<i>Nigrospora</i> sp.	LC 2725	<i>Symplocos zizyphoides</i>	China	KX985960	KY019487	KY019313
<i>Nigrospora</i> sp.	LC 4566	<i>Lithocarpus</i> sp.	China	KX986022	KY019545	KY019354
<i>Apiospora arundinis</i>	CBS 114316	<i>Hordeum vulgare</i> , leaves	Iran	KF144884	KF144974	KF145016

The analyzed *Nigrospora* strain is indicated in bold. T – the ex-type strain; na – the sequence is unavailable.

## Morphological study

The morphological characteristics of *Nigrospora* sp. strain MFG 70052 were observed during its growth on PSA in the dark at 25 °C for 7–30 d, while the diameter of the colony was measured on the third day of cultivation in two perpendicular directions and the average diameter of strain colony was calculated. The micromorphological structures were investigated and documented using Olympus BX53 microscope (Olympus America, USA) connected to a SUBRA camera (Jenoptik, Germany).

## Results

### Molecular identification

Phylogenetic analysis of the adjusted and aligned sequences of *Nigrospora* sp. MFG 70052, as well as reference strains, included the following lengths: ITS – 459 bp, *tub* – 588 bp, *tef* – 566 bp. The number of parsimony-informative sites per genome locus was 58 (12.6%), 184 (31.3%), and 305 (53.9%), respectively. The topology of the phylogenetic tree was concordant with that reconstructed previously<sup>[1,2,10]</sup>. The analyzed strain MFG 70052 formed a clade with the reference strains *N. humicola* Qin Yang & Ning Jiang CFCC 56884 and CFCC 56885 with high bootstrap support ML/BP 100/1.0, which confirms the belonging of the strain MFG 70052 to the *N. humicola* species (Fig. 1).

### Description morphology

The colony of *Nigrospora humicola* MFG 70052 reaching 80 mm on the third day of cultivation on PSA at 25 °C. Mycelium is flat, floccose, edge entire, initially white, later becoming gray when sporulation is abundant, and the edge is smooth/filamentous (Fig. 2). The reverse is smoke-gray, with age a noticeable pattern of dark hyphae and sporulation is noted.

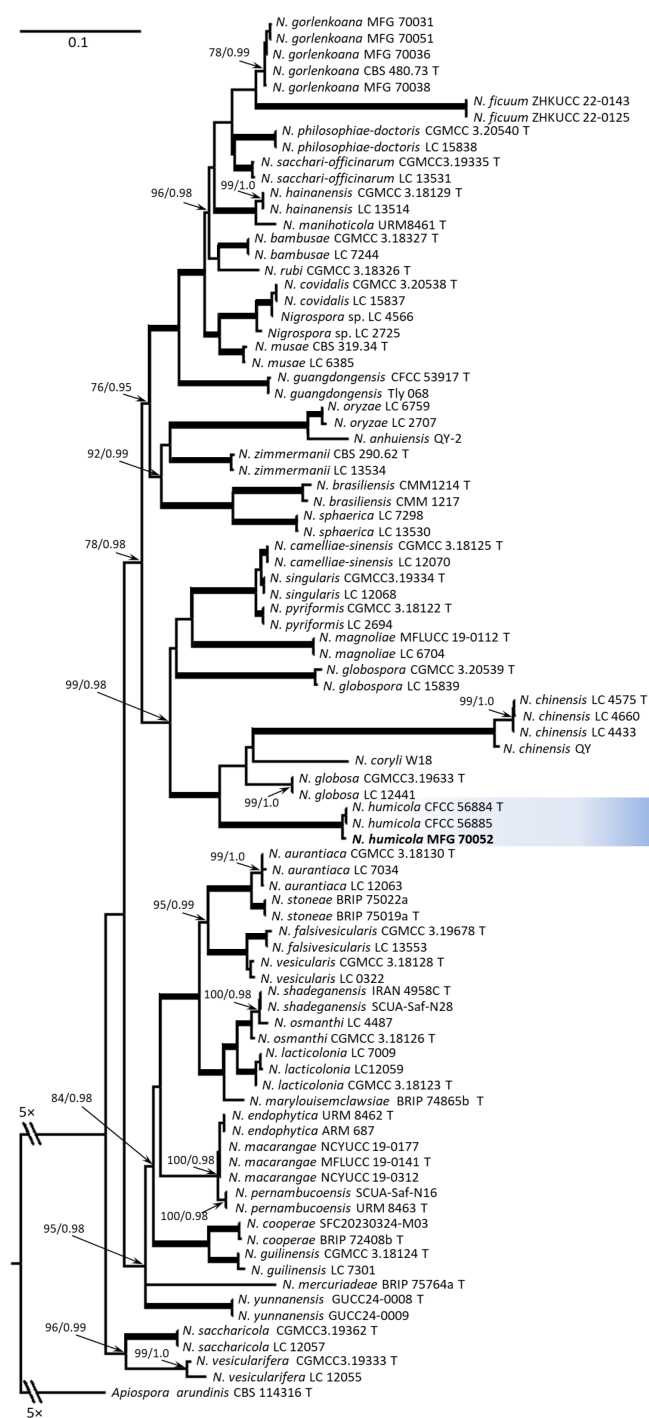
Hyphae 2.5–6 µm, smooth, branched, septate, hyaline, some becoming brownish with age. Setae not observed. Conidiophores are mostly reduced to conidiogenous cells, macronematous, aggregated in clusters on hyphae. Conidiogenous cells 4–10 × 4–8 µm, hyaline to pale brown, their coloration is especially noticeable when they are aggregated, ampulliform, spherical, or subspherical.

Conidia 10.8–16.7 × 11.2–18.7 µm (13.7 × 15.6 µm), solitary, acrogenous, spherical or broadly ellipsoidal, egg-shaped, aseptate, pale brown at the beginning of formation, later black, shiny, smooth, aseptate. In some light-colored young conidia of *N. humicola* the presence of equatorial slits is noticeable. Sterile cells 27.0–40.8 × 11.2–21.4 µm (33.6 × 16.7 µm), terminally formed on hyphae, dark brown to black, elongated-ellipsoid or club-shaped, sometimes curved or angular.

## Discussion

At present, investigations of fungal diversity rely almost invariably on sequencing several informative genome loci and the development of multilocus phylogeny, for this approach provides greater resolution than the sum of morphological features. In this study, using the phylogenetic analysis of the ITS, *tub*, and *tef* loci *Nigrospora* strain MFG 70052 isolated from reed in 2019 from the Primorsky region of Russia bordered with China, was accurately identified as *N. humicola*. This is the first finding of this species on the plant (*Poaceae*) and the first detection in Russia. *Nigrospora humicola* was described recently when two strains isolated from soil in 2021 in the Hebei Province of China were studied in detail<sup>[10]</sup>.

Some *Nigrospora* species are associated with different plants, e.g. *N. oryzae* (Berk. & Broome) Petch is the most ubiquitous species of this genus and has been reported from 40 different plant host genera including *Poaceae* family plants<sup>[11]</sup>, and largely distributed



**Fig. 1** Maximum likelihood (ML) phylogenetic tree based on DNA sequence data from three loci (ITS, *tub*, and *tef*) of *Nigrospora* fungi. ML bootstrap support values > 70 %, followed by Bayesian posterior probability (BP) scores > 0.95 are shown at the nodes. Thickened lines indicate ML/BP of 100/1.0. The tree was rooted on sequences of *Apiospora arundinis* CBS 114316. The analyzed strain is indicated in bold. T, ex-type strain.

in the territory of Russia, *N. gorlenkoana* was found in mycobiota of cereal crops and rapeseed plants<sup>[18]</sup>. Overall, *Nigrospora* species lacks host specificity<sup>[14]</sup>, so the host range of *N. humicola* may also be wide.

Morphologically, MFG 70052 strain similar to *N. humicola* strains described by Zhang et al.<sup>[10]</sup> in the cultural features, sizes and shapes of conidiogenous, and conidial cells. However, there are some

morphological differences that we consider important for identification of this species. One of them was a formation of sterile cells that looks like conidia due to dense dark pigmentation, but they are much larger and have an irregular shape. The sterile cells are formed directly on hyphae, not on conidiogenous cells and frequently observed in some *Nigrospora* species<sup>[11]</sup>. These morphological structures were described in the phylogenetically closest to *N. humicola* species *N. chinensis*, representing the sister clade<sup>[11]</sup>. In our opinion, they are an additional diagnostic feature. Moreover, in the culture of *N. humicola*, stained clusters of conidiophores and conidiogenous cells are formed, the same as described in *N. oryzae* and *N. lacticola* Mei Wang & L. Cai<sup>[11]</sup>. Equatorial slits are present in some young conidia of *N. humicola*, as in *N. gorlenkoana*<sup>[11]</sup>, but not mentioned in the description of the species<sup>[10]</sup>.

The results obtained in this study indicate that analysis of morphology is likely to be useful in refining our understanding of the *Nigrospora* species limits. The accurate identification of *Nigrospora* species is possible only by combined analysis of genetic, morphological, ecological, and physiological data<sup>[2,11,22]</sup>. The diversity of *Nigrospora* species in nature as well as their characteristics require further investigation.

## Author contributions

The authors confirm contribution to the paper as follows: study conception and design, data collection: Orina AS, Gagkaeva TYu, Gavrilova OP; analysis and interpretation of results: Orina AS, Gagkaeva TYu; draft manuscript preparation: Orina AS, Gagkaeva TYu. manuscript review and editing: Gavrilova OP. All authors reviewed the results and approved the final version of the manuscript.

## Data availability

The data generated and analyzed during this study are available in this article. DNA sequence data are available in the GenBank database, and the accession numbers are provided in Table 1. The *Nigrospora humicola* MFG 70052 strain has been deposited in the fungal culture collection of the Laboratory of Mycology and Phytopathology (VIZR, St. Petersburg, Russia).

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## Conflict of interest

The authors declare that they have no conflict of interest.

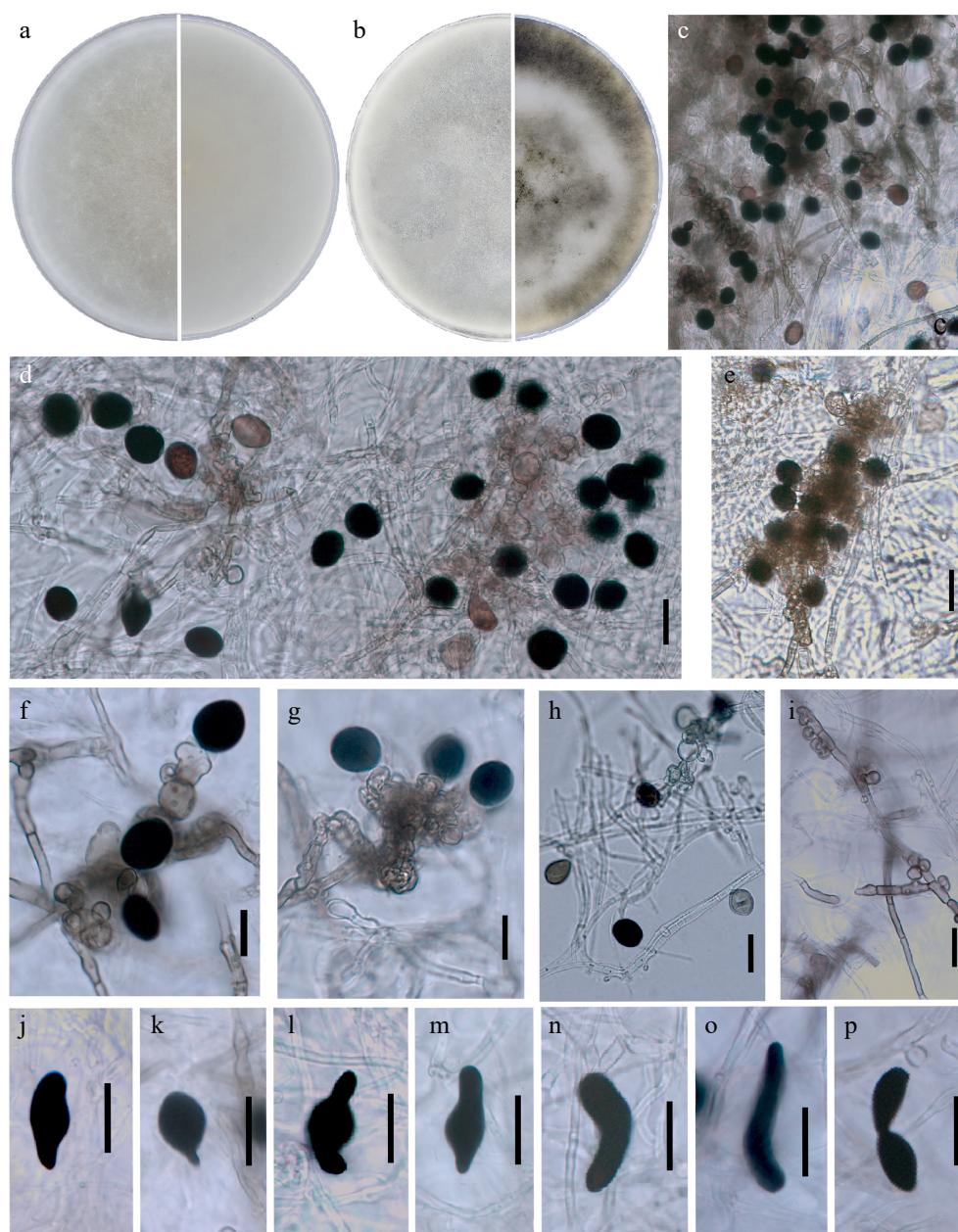
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**Fig. 2** *Nigrospora humicola* MFG 70052 (PSA, 25 °C in the dark). Upper surface and reverse overview after growth for (a) 7 d, and (b) 30 days. (c)–(h) Conidiophores and conidiogenous cells giving rise to brown-black conidia. (i) Septate hyphae and spherical vesicles aggregating in conidiophores. (j)–(p) Sterile cells. Scale bars = 20 µm.

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