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ORIGINAL PAPER

# *Blastobotrys nigripullensis*, a new yeast species isolated from a fungal outbreak on an ancient Roman shipwreck in the Netherlands

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**Abstract** A new species of the yeast genus *Blastobotrys* was discovered on ancient ship timbers in the Netherlands. The species had developed on the wood of a river barge dating to the Roman period. The growth occurred after the preservative polyethylene glycol (PEG 4000) was washed out of some of the timbers due to an undetected leak in the storage unit. Mycological analysis of various timber samples revealed the presence of *Microascus melanosporus* (predominant), *Microascus paisii*, a member of the *Acremonium chrysogenum*-clade, and a new *Blastrobotrys* species. The new species produced sporothrix-like conidiophores with clavate blastoconidia (3–7 × 1–3.5 µm) and was found to be osmotolerant, capable of growth on low water activity media like malt yeast 50% glucose agar (MY50G). In this article we formally describe and introduce *Blastrobotrys nigripullensis* (CBS 17879 $^{T}$) based on its morphology, physiology and phylogenetic placement.

**Keywords** Yeasts phylogeny · Buried river barge · Oak wood preservation · Polyethylene glycol · *Trichomonascaceae* · *Trichomonascus*

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10482-023-01898-x.

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## Introduction

In the early 1970s, three large wooden river barges and three smaller Roman-era vessels were discovered during construction work in the village of Zwammerdam in the Netherlands. At the time, the care facility 'Ipse de Bruggen' was being built in Zwammerdam on the Hooge Burch estate and by chance the workers came across the Zwammerdam vessels. The vessels were all made of oak and the river barges were exceptionally large, ranging from 20 to 34 m in length (De Weerd 1988).

In the years leading up to the discovery, the University of Amsterdam conducted archaeological research in the area and investigated a Roman fort that was marked on a Roman road map as the military point *Nigrum Pullum* (De Weerd 1988). This fort was part of the Roman *Limes*, a border where a series of forts along the river Rhine formed a defensive line in the Lower Germanic region (Fig. 1A). This defence

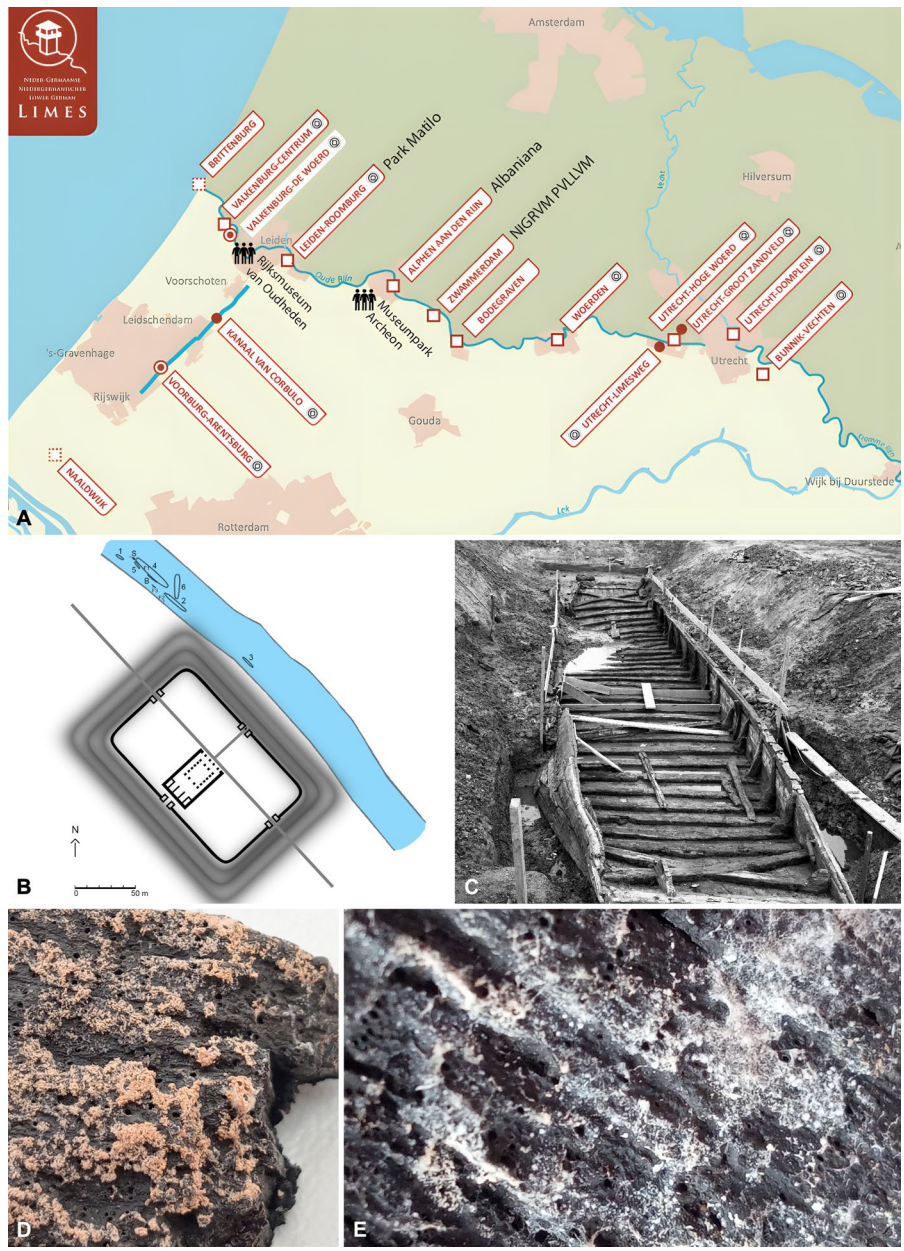


**Fig. 1** **A** The Roman *Limes* with the fort of *Nigrum Pullum* situated in today's Zwammerdam, the Netherlands (drawing by T. Hazenberg). **B** The position of the vessels of Zwammerdam, the Netherlands, in front of the fort of *Nigrum Pullum*. **C** The Zwammerdam 6 ship during the excavation (photo by G. Verkuyl, collection ACASA, University of Amsterdam, the Netherlands). **D** Fungal growth of *Microascus melanosporus* (dominant) and *Acremonium* species on wood from an L-shaped chine girder. E. White patches with *Blastobotrys nigripullensis* and *Microascus paisii* on wood from a portside wale

line also functioned as a transport corridor, a strictly controlled long-distance waterway from the interior regions inland towards the coast.

The vessels found near the site of *Nigrum Pullum* were scattered along a section of timber shoring in front of the fort, where the Rhine once flowed, and were located at various depths (Fig. 1B). They were essentially found in the riparian zone of a silted up river bed. To document and salvage the ships, construction work had to be halted and, under great time pressure, only the ships were properly examined. At that time, the relationship to the fort could not be further explored (De Weerd 1988). Why the ships sank or were scuttled in the vicinity of the fort thus remains unknown.

An early form of crowdfunding, supported by former Queen Juliana of the Netherlands, allowed the vessels to be salvaged and conserved to enable future display in a museum. The conservation of the wood with polyethylene glycol (PEG, 4000 g/mol), a water-soluble waxy solid, was carried out in batches starting in 1976 and took an average of two years per load of timbers. When heated, high-molecular weight PEG can penetrate wood tissues and replace water molecules. The PEG also bulks the deteriorated wood cells and thus reinforces the structure of the wood, improving dimensional stability. After PEG treatment, the wood was stored stacked in shipping containers. Decades later in 2017, a project started, organized by Hazenberg Archeologie and Museumpark Archeon in the Netherlands, that enabled the restoration of the vessels.

Unfortunately, an undetected leak in one of the shipping containers with remains of the sixth ship of Zwammerdam (Fig. 1C) had resulted in the preservative being washed out of some of the timbers. These timbers had started to dry out, crack and warp in some areas and had developed visible fungal growth (Fig. 1D, E). To seek advice for the removal of this growth, the project staff contacted the Westerdijk Fungal Biodiversity Institute (WI) in Utrecht and send in some samples to investigate further.

During the study of the fungal diversity present, a putative new *Blastobotrys* species was isolated. In the dual nomenclature era, *Blastobotrys* was considered the asexual genus (or anamorph) associated with the sexual genus (or teleomorph) *Trichomonascus*. This relationship was confirmed by Smith et al. (2011a, b). Following the abandonment of dual nomenclature for fungi (Hawksworth et al. 2011; McNeill et al. 2012), a decision on what generic name to apply to the *Blastobotrys*/*Trichomonascus* lineage remains to be made. This was discussed in a recent revision published for the lineage by Visagie et al. (2023) in which *B. davinci* was described, a species that has been detected from house dust and historically important artifacts including the self-portrait of Leonardo da Vinci which dates from ca 1512. The goal of the current study is to report on the fungi isolated from Zwammerdam vessel wood samples, to compare the putative new species with known *Blastobotrys* and *Trichomonascus* species and to formally introduce *B. nigripullensis* sp. nov. For this, we provide a phylogenetic analysis based on the internal transcribed spacer (ITS1-5.8S-ITS2) rDNA region (ITS) and D1/D2 regions of the large subunit (LSU), and describe the new species based on morphological and physiological characters.

## Materials and methods

### Sampling and isolations

A large oak knee detached from a floor timber with fungal growth as well as small pieces of a few centimetres of oak from hull planks with small white patches of suspected fungal growth on the surface were sent from Archeon (Alphen aan den Rijn, the Netherlands) to the Westerdijk Fungal Biodiversity Institute (WI, Utrecht, the Netherlands). Here, the suspected fungal material was plated directly on dichloran 18% glycerol agar (DG18), malt yeast 50% glucose agar (MY50G) and malt extract agar (MEA, Oxoid) supplemented with the antibiotics penicillin (6600 U per plate) and streptomycin (20,000 U per plate) to inhibit bacterial growth. The plates were incubated for 10–14 days at 25 °C in the dark. After incubation, colonies were transferred to fresh agar media. The strains were preserved in 30% glycerol, stored at − 80 °C and accessioned in the working culture collection of the Food and Indoor Mycology research group (DTO) housed at WI. Some of these strains were also preserved in the CBS culture collection and fungarium of WI.

### DNA extraction, sequencing and phylogenetic analysis

DNA was extracted from 7 d old colonies grown on MEA using the UltracleanTM Microbial DNA isolation Kit (MoBio Laboratories Inc., Solana Beach, USA). The ITS was amplified for all strains using the primer pair V9G and LS266 and used to make initial identifications to genus level. For species level identifications, additional regions were amplified as needed. These included LSU, translation elongation factor 1-$\alpha$ (*TEF*) and beta-tubulin (*BenA*) using primer pairs LR5 and LROR, EF1 and EF2 and Bt2a and Bt2b, respectively. All PCR amplifications were made following Samson et al. (2019). Amplification profiles were as follow: initial denaturing 94 °C for 5 min, 36 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, followed by a final elongation step of 72 °C for 10 min. Sequencing reactions were prepared using the BigDye Terminator Cycle Premix Kit (Applied Biosystems, Waltham, USA) and were run on an ABI PRISM 3730xl genetic analyser with the same primer pairs used for PCR amplification. Sequence contigs were assembled in SeqMan Pro v.15.0.0 (DNASTAR) and newly generated sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (accessions OQ876811–OQ876820, OQ876860, OQ876861 and OQ918213). Initial identifications were made by performing similarity searches using BLAST against the nucleotide sequence database. To compare the putative new *Blastobotrys* strains with known *Blastobotrys*/*Trichomonascus* species, the same DNA sequence reference dataset (Table 1) and phylogenetic approaches published by Visagie et al. (2023) were used. Alignments and treefiles were uploaded to the University of Pretoria's research data repository hosted on Figshare (https://doi.org/https://doi.org/10.25403/UPresearchdata.23815032).

### Morphological and physiological characterization of the yeast isolates

Two strains (CBS 17879 and CBS 17880) were morphologically and physiologically characterised as described by Visagie et al. (2023) using MEA, yeast morphology agar (YMA), potato dextrose agar (PDA) and glucose yeast peptone agar (GYPA), 5% glucose in yeast nitrogen broth and Dalmau plates on yeast morphology agar. The physiological growth profile of the strains was assessed using methods commonly used in yeast taxonomy (Kurtzman et al. 2011).

## Results

### Isolations and identifications

Three wood samples with visible fungal growth were studied and isolations made from these resulted in five strains representing four species. Light brown to beige fungal spots were present on the wood from an L-shaped chine girder sample (a combined bottom/side timber in the hull of a Roman river barge) on port side (timber code ZW6-K2 bb), and isolations revealed the presence of *Microascus melanosporus* (DTO 457-E5: ITS = OQ876812, LSU = OQ876817, *BenA* = OQ876861) and a member of the *Acremonium chrysogenum*-clade (DTO 457-E6: ITS = OQ876813, LSU = OQ876818). The other two samples (knee of a floor timber [timber code ZW6-S18] and part of a wale on port side [timber code ZW6-BE2 bb]) showed white spots (Fig. 1E) and direct microscopic analysis of these spots showed (pseudo)hyphae and yeast cells. Initial isolations only showed growth of yeast-like colonies on the MY50G plates; however, subculturing on MEA/PS and DG18 showed that these colonies could also grow on these media. A *Blastobotrys* species (CBS17879 = DTO 457-E3: ITS = OQ876814, LSU = OQ876819; CBS17880 = DTO 455-G9: ITS = OQ876815, LSU = OQ876820) was growing on the two investigated wood samples, and the new species *B. nigripullensis* is introduced below. Besides *B. nigripullensis*, also *Microascus paisii* (DTO 457-E4: ITS = OQ876811, LSU = OQ876816, *BenA* = OQ876860, *TEF* = OQ918213) was isolated from wood sample ZW6-BE2 bb, but no structures of this species were observed during the direct examination of the material.

*Microascus* species are both widespread and prefer substrates with cellulose, while *Acremonium* species occurs in soils and on decaying plant matter (Barron et al. 1961, Domsch et al. 1993; Hou et al. 2023). Considering the context of archaeological wood taken from the ground and then decomposed in parts, the occurrences of these species fit the picture. The surfaces of samples ZW6-K2 bb and ZW6-BE2 bb were steam cleaned and seven months later no regrowth was evident. However, the knee of the floor timber

**Table 1** Strains used for phylogenetic analyses

| Taxon name | Strains[a] | Location | Source | GenBank: ITS | GenBank: LSU |
|---|---|---|---|---|---|
| *Blastobotrys adenini-vorans* | CBS 8244 = NRRL Y-17692 IFO 10858 = IGC 4638 = PYCC 4638 (ex-type) | The Netherlands: Wageningen | Soil | KY101746 | DQ442697 |
| *Blastobotrys allociferrii* | CBS 5166 = NBRC 10194 = IFO 10194 (ex-type) | Germany | Human | LC158134 | LC158143 |
| *Blastobotrys americanus* | CBS 10337 = NRRL Y-6844 = LRB 70B3 (ex-type) | USA: Kansas | Unknown | KY101748 | DQ442699 |
| *Blastobotrys arbuscula* | CBS 227.83 = NRRL Y-17585 (ex-type) | Finland | Indoor air | OL772655 | DQ442689 |
| *Blastobotrys aristatus* | CBS 521.75 = NRRL Y-17579 = ATCC 34215 = CCM F-410 = HFM 2672 = UAMH 4665 (ex-type) | Czech Republic | Moldy plaster | OL772656 | DQ442686 |
| *Blastobotrys attinorum* | CBS 9734 = NRRL Y-27639 = UNESP-S156 (ex-type) | Brazil: Sao Paulo | Fungal garden of nests of the leaf-cutting ant (*Atta sexdens*) | KY101749 | AY442294 |
| *Blastobotrys baotianman-ensis* | CBS 16024 = CICC 33083 (ex-type) | China: Henan Province, Baotianman Nature Reserve | Gut of ground beetle (*Pterostichus gebleri*) | KU128710 | KU128725 |
| *Blastobotrys bombycis* | CBS 15274 (ex-type) | India: Dharwad | Silkworm (*Bombyx mori*) | OL772657 | OL772653 |
| *Blastobotrys buckinghamii* | CBS 13900 = NRRL Y-63727 = yHAB 196 (ex-type) | USA: Michigan, Tahquamenon Falls State Park | Mushroom associated with American beech (*Fagus grandfolia*) | OL772658 | OL772654 |
| *Blastobotrys capitulata* | CBS 287.82 = NRRL Y-17573 (ex-type) | South Africa | Flower, decaying tissue of candelabra tree (*Euphorbia ingens*) | OL772659 | DQ442688 |
| *Blastobotrys chiropterorum* | CBS 6064 = NRRL Y-17071 (ex-type) | Columbia | Liver of bat (*Mormoops megalophylla*) | KY101750 | DQ442682 |
| *Blastobotrys davincii* | CBS 16861 = DAOMC 251467 = CMW 56638 = CN 002G3 (ex-type) | Canada: Ontario, Stittsville | House dust | MW367648 | MW367634 |
| *Blastobotrys elegans* | CBS 530.83A = NRRL Y-17572 (ex-type) | Finland | Indoor air | OL772660 | DQ442687 |
| *Blastobotrys farinosus* | CBS 140.71 = NRRL Y-17593 = IGC 4592 = JCM 2935 (ex-type) | The Netherlands | *Hirneola auricula-judae* | OL772661 | DQ442685 |
| *Blastobotrys fungorum* | CBS 259.70 = CMW 17165 = UAMH 3678 (ex-type) | Germany | Old *Fomes fomentarius* basidiome | KX590837 | KX590883 |
| *Blastobotrys illinoisensis* | CBS 10339 = NRRL YB-1343 (ex-type) | USA: Illinois, Marion, Wohlwend farm | Tree | KY101751 | DQ442696 |
| *Blastobotrys indianensis* | CBS 9600 = NRRL YB-1950 (ex-type) | USA: Indiana, Spencer, McCormick's Creek State Park | White fungus associated with pine | KY101752 | DQ442692 |
| *Blastobotrys malaysiensis* | CBS 10336 = NRRL Y-6417 = EMMONS S3,539A (ex-type) | Malaysia | Cave soil | KY101753 | DQ442695 |
| *Blastobotrys meliponae* | CBS 14100 = URM7224 (ex-type) | Brazil: Pernambuco, Recife | Honey | KT448719 | KR779217 |
| *Blastobotrys mokoenaii* | CBS 8435 = NRRL Y-27120 (ex-type) | South Africa | Soil | KY101754 | DQ442694 |

**Table 1** (continued)

| Taxon name | Strains[a] | Location | Source | GenBank: ITS | GenBank: LSU |
|---|---|---|---|---|---|
| *Blastobotrys mucifer* | CBS 7409 = CCY 29–170-1 = IFO 10918 (ex-type) | Brazil: Manaus | Liver of toad (*Rhinella granulosa*) | KY102217 | KY106587 |
| *Blastobotrys muscicola* | CBS 10338 = NRRL Y-7993 (ex-type) | USA: Louisiana, near New Orleans | Moss on fallen log | KY101755 | DQ442680 |
| *Blastobotrys navarrensis* | CBS 139.77 = ATCC 36955 = IJFM 2642 = UAMH 4664 (ex-type) | Spain: Pampelona | Black pepper (*Piper nigrum*) | OK623478 | OK623486 |
| *Blastobotrys nigripullensis* | clone CMH114 | USA: Missouri, Kansas City | House dust and indoor air | KF800205 | – |
| *Blastobotrys nigripullensis* | clone S23T_59 | Unknown | Sea water | KU164259 | – |
| *Blastobotrys nigripullensis* | CBS 17880 = DTO 455-G9 | The Netherlands: Zwammerdam | Oak wood from ancient Roman ship | OQ876815 | OQ876820 |
| *Blastobotrys nigripullensis* | CBS 17879 = DTO 457-E3 (ex-type) | The Netherlands: Zwammerdam | Oak wood from ancient Roman ship | OQ876814 | OQ876819 |
| *Blastobotrys nivea* | CBS 163.67 = NRRL Y-17581 = ATCC 18420 = HFM 26 = UAMH 4663 = MUCL 6078 (ex-type) | Germany | Municipal compost | OL772662 | DQ442690 |
| *Blastobotrys parvus* | CBS 6147 = NRRL Y-10004 (ex-type) | Antarctic Ocean | Seawater | KY101757 | DQ442693 |
| *Blastobotrys peoriensis* | CBS 10340 = NRRL YB-2290 (ex-type) | USA: Illinois, Peoria | Unknown | KY101758 | DQ442700 |
| *Blastobotrys persicus* | CBS 14259 = IBRC-M30238 (ex-type) | Iran: Ilam | Soil | OL772663 | KU659141 |
| *Blastobotrys proliferans* | CBS 522.75 = NRRL Y-17577 = ATCC 34216 = CCM F-493 = HFM 2673 = UAMH 4666 (ex-type) | Brazil | Mite-infested nut (*Bertholletia excelsa*) | EU343812 | DQ442684 |
| *Blastobotrys raffinosifermentans* | CBS 6800 = NRRL Y-27150 (ex-type) | Unknown | Unknown | KY101759 | DQ442698 |
| *Blastobotrys robertii* | CBS 10106 = NRRL Y-27775 (ex-type) | The Netherlands: Wageningen forest | Rotten pine wood (*Pinus sylvestris*) | KY101760 | DQ839395 |
| *Blastobotrys serpentis* | CBS 10541 = NRRL Y-48249 = MTCC 8332 = W113A = YS W113A (ex-type) | India: Hyderabad City | Trinket snake gut | KY101761 | AM410667 |
| *Blastobotrys terrestris* | CBS 7376 = NRRL Y-17704 = CSIR Y914 = IFO 10859 = IGC 5133 = PYCC 5133 (ex-type) | South Africa: Barberton | Soil | KY101762 | DQ442683 |
| *Blastobotrys vanleeneni-anus* | CBS 14902 (ex-type) | The Netherlands | Soil | MG986487 | MG986492 |
| *Blastobotrys xishuangban-naensis* | CBS 16044 = CICC 33360 (ex-type) | China: Yunnan Province, Jinghong | Rotting wood | MK682811 | MK682809 |
| *Trichomonascus apis* | CBS 10922 = NRRL Y-48475 = NCAIM Y.01848 (ex-type) | Hungary | Mouldy honeycomb | KY105699 | EU790643 |

**Table 1** (continued)

| Taxon name | Strains[a] | Location | Source | GenBank: ITS | GenBank: LSU |
|---|---|---|---|---|---|
| *Trichomonascus ciferrii* | CBS 5295 = NRRL Y-10943 = ATCC 58443 = CCRC 21427 = Goto TH-26 = IFO 1854 = IGC 4164 = IMI 344641 = JCM 7621 (ex-type) | The Netherlands | Pig | AY493435 | DQ442681 |
| *Trichomonascus petasosporus* | CBS 9602 = NRRL Y-B2092 (ex-type) | USA: Missouri, Salem | White Oak (*Quercus sp*) | KY105704 | DQ442691 |
| *Wickerhamiella domercqiae* | CBS 4351 = NRRL Y-6692 (ex-type) | South Africa | Wine vat | DQ911463 | DQ438240 |

[a]Acronyms of culture collections in alphabetic order: *ATCC*, American Type Culture Collection, Manassas, Virginia; *CBS*, Westerdijk Fungal Biodiversity Institute (formerly Centraalbureau voor Schimmelcultures), Utrecht, the Netherlands; *CCM*, Czech Collection of Microorganisms, Brno, Czech Republic; *CCRC*, Bioresources Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan; *CCY*, Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia; *CICC*, China Center of Industrial Culture Collection, Beijing, China; *CMW*: (culture collection housed at the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa); *CSIR*, Council for Scientific and Industrial Research, Pretoria, South Africa; *DAOMC*, Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, Canada; *DTO*, Internal Culture Collection of the Department of Applied and Industrial Mycology of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; *IBRC*, Iranian Biological Resources Center, Academic Center for Education Culture and Research (ACECR), Tehran, Iran; *IFO*, Institute for Fermentation, Osaka, Yodogawa-ku, Osaka, Japan; *IGC*, Center of Biology, Gulbenkian Institute of Science, Oeiras, Portugal; *IJFM*, Instituto Jaime Ferrán de Microbiología Consejo Superior de Investigaciones Científicas, Madrid, Spain; *IMI*, CABI's collection of fungi and bacteria, Wallingford, UK; *JCM*, Japan Collection of Microorganisms, Tsukuba, Japan; *MTCC*, Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India; *MUCL*, Mycothèque de l´Université Catholique de Louvain, Louvain-la-Neuve, Belgium; *NBRC*, NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan; *NCAIM*, National Collection of Agricultural and Industrial Microorganisms, Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary; *NRRL*, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, US Department of Agriculture, Peoria, IL, USA; *PYCC*, Portuguese Yeast Culture Collection, C. R. M., New University of Lisbon, Lisbon, Portugal; *UAMH*, UAMH Centre for Global Microfungal Biodiversity (formerly University of Alberta Microfungus collection and Herbarium), Gage Research Institute, University of Toronto, Toronto, Canada; *URM*, culture collection at the Federal University of Pernambuco, Recife, Brazil; Others, personal collections and isolate numbers

(ZW6-S18) was steam cleaned twice after *Blastrobotrys* reappeared after the first cleaning attempt, but seven months later the fungus again formed visible growth on the wood surface. Next, a treatment with 70% alcohol is planned in the hope that this will be sufficient in the long term.

Species delineation and phylogenetic placement of B. nigripullensis sp. nov

Phylogenetic analyses based on ITS and LSU included all species currently accepted in *Blastobotrys/Trichomonascus* (Visagie et al. 2023). The ITS and LSU alignments were 669 and 576 bp long, respectively, with TVM+I+G being the best model for the ITS partition and GTR+G the best for the LSU partition. *Blastobotrys nigripullensis* resolved on a fully supported branch distinct of all other species (Fig. 2). The tree had a general lack of backbone support, and its closest relative(s) could not be determined as a result. Two DNA reads in Genbank, namely clones CMH114 (KF800205) and S23T-59 (KU164259), represent the same species. KF800205 originated from house dust and/or indoor air collected from Kansas City (USA) (Rittenour et al. 2014), and KU164259 originated from

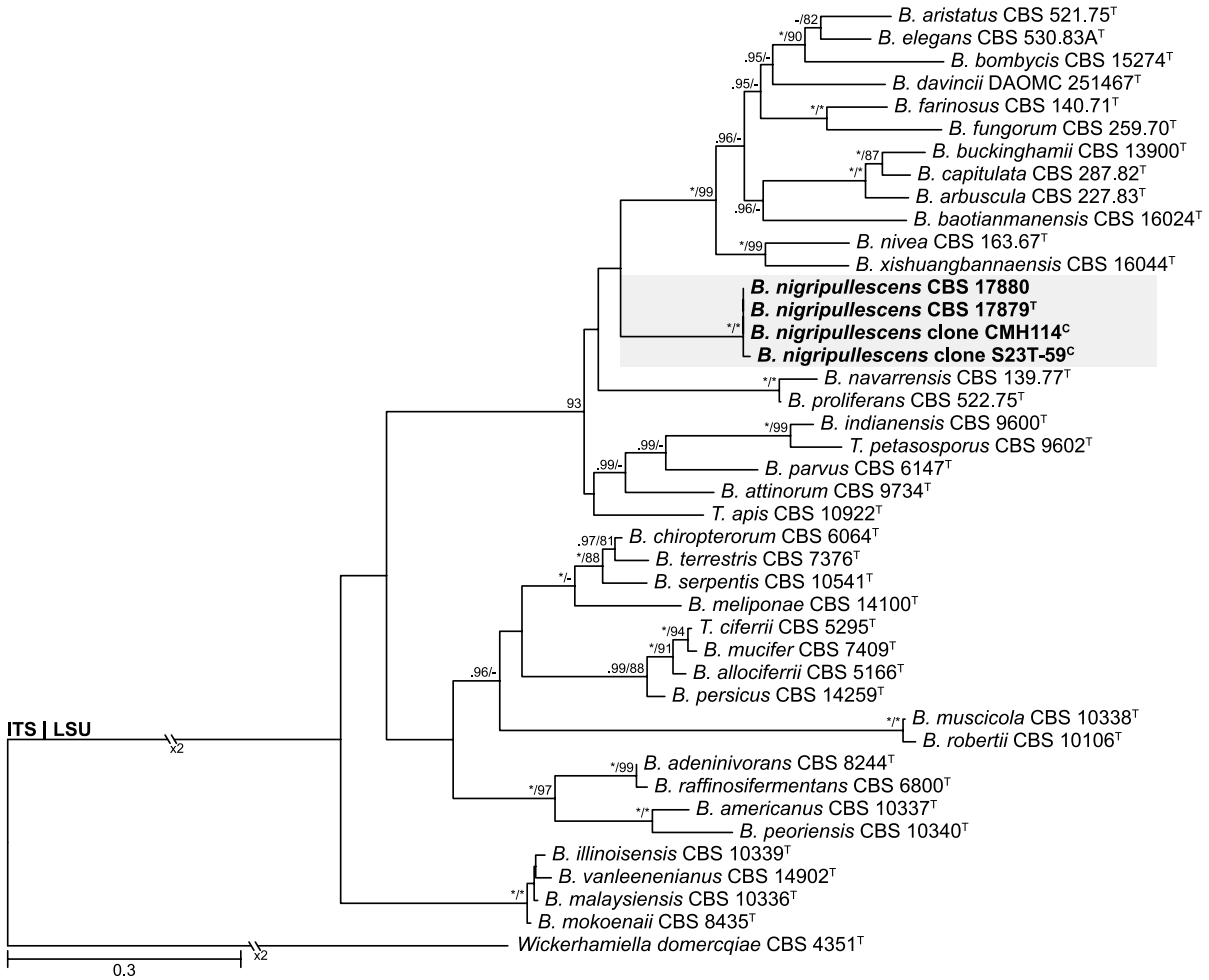


**Fig. 2** Combined phylogenetic tree of described *Blastobotrys* and *Trichomonascus* species based on ITS and LSU, indicating the phylogenetic placement of *Blastobotrys nigropullensis*. The tree is rooted to *Wickerhamiella domercqiae* CBS 3451 $^{T}$. Posterior probabilities (≥0.95) and Bootstrap support values (≥80%) are given above branches. The new species is indicated in bold text and grey box, $^{T}$=ex-type strain, $^{c}$=sequence from cloned DNA fragment

sea water collected from an unknown locality. Similarities between *B. nigripullensis* (CBS 17879 $^{T}$) and other species were low with LSU that differed by 42nt or more (based on alignment from 5'-ATTGCCCTA GTAACGGCGAGCG-3') and ITS by 130nt or more (based on alignment from 5'-AAGGATCATTAC CGAA-3') nucleotide substitutions, thus warranting recognition as a distinct species.

The new species lacks the typical morphology of most *Blastobotrys* as it did not form the branched conidiophores ending in conidiogenous cells producing blastoconidia as seen in e.g., *B. davincii* and other *Blastobotrys* (and those formerly classified in *Trichomonascus*) species (Smith et al. 2011a, b, Visagie et al. 2023). It does, however, resemble conidiophores produced by most *Sporothrix* species (Fig. 3). The nutritional growth requirements of strains CBS 17879 and CBS 17880 are presented in Table 2 and can be summarized as follows: the strains ferment glucose, but no other sugars tested; they utilize many of the

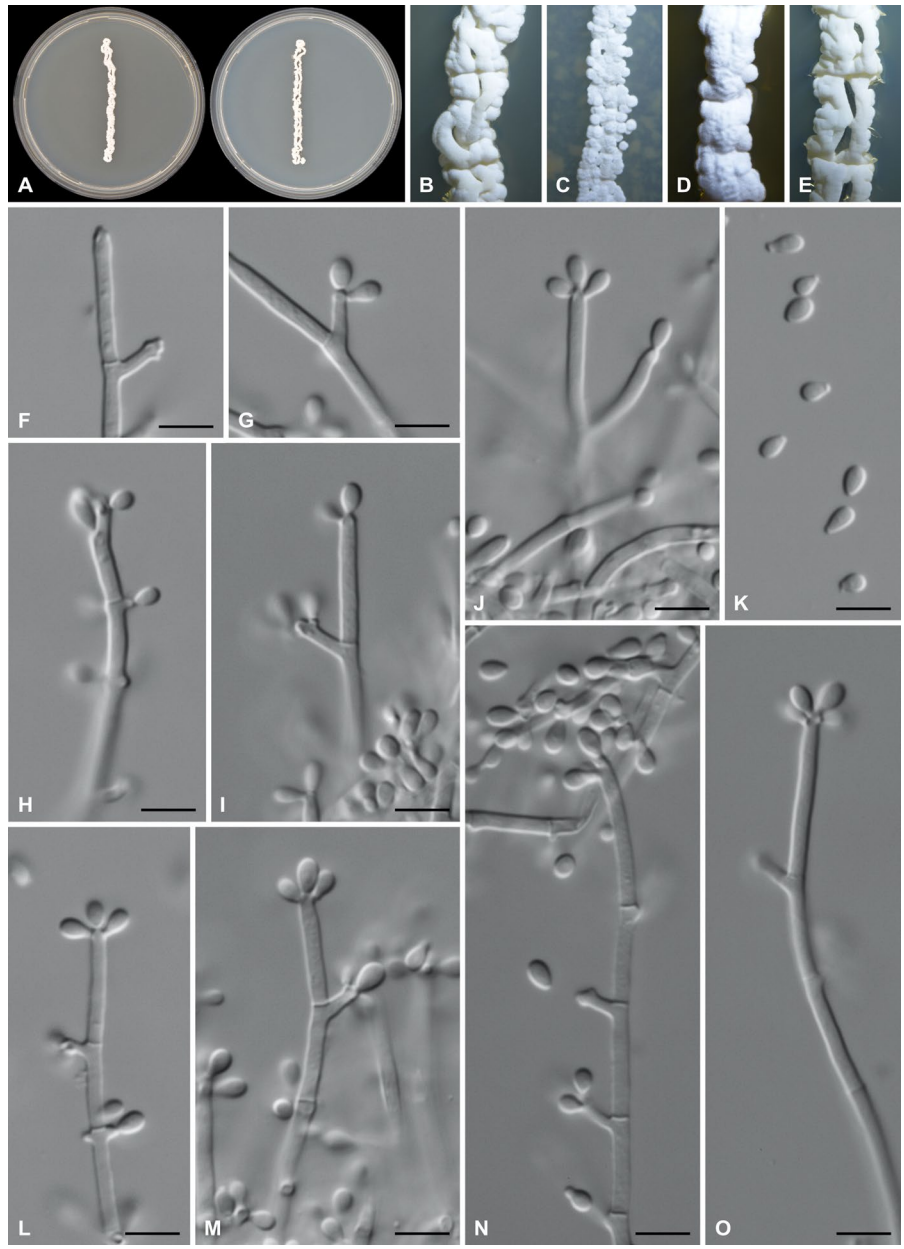


**Fig. 3** *Blastobotrys nigripullensis* (CBS 17879 $^{T}$). **A** Colonies on, from left to right, YMA and GYPA. Colony close-ups on MYA **B**, PDA **C**, MEA **D** and YMA **E**. **F**–**O**. Conidiophores and conidia from YMA observed under light microscope. Scale bars 10 µm

**Table 2** Growth characteristics of two isolates of *Blastobotrys nigripullensis*

| | CBS 17879 | CBS 17880 | | CBS 17879 | CBS 17880 |
|---|---|---|---|---|---|
| Growth on carbon compounds | | | Fermentation | | |
| D-Glucose | + | +` | D-Glucose | d | d |
| D-Galactose | + | + | Galactose | – | – |
| L-Sorbose | + | + | Maltose | – | – |
| D-Glucosamine | + | + | Sucrose | – | – |
| D-Ribose | + | + | Trehalose | – | – |
| D-Xylose | + | + | Lactose | – | – |
| L-Arabinose | + | + | Raffinose | | – |
| D-Arabinose | w,d | w | Xylose | – | – |
| L-Rhamnose | –,w,d | w,d | | | |
| Sucrose | + | + | Growth on nitrogen compounds | | |
| Maltose | – | – | Nitrate | – | – |
| α, α Trehalose | + | + | Nitrite | – | v [inhibition zone] |
| Methyl α-glucoside | – | – | Ethylamine | + | + |
| Cellobiose | + | + | L-Lysine | + | + |
| Salicin | + | + | Cadaverine | + | + |
| Arbutin | + | + | Creatine | – | – |
| Melibiose | –,w | – | Creatinine | – | – |
| Lactose | + | + | Glucosamine | – | – |
| Raffinose | – | – | Imidazole | – | – |
| Melezitose | – | – | Proline | – | – |
| Inuline | w | w | Tryptophane | – | – |
| Soluble starch | – | w | | | |
| Glycerol | + | + | Growth without vitamins: not done | | |
| meso Erythritol | + | + | | | |
| Ribitol | + | + | Other tests | | |
| Xylitol | – | – | Growth with 0,01% cycloheximide | + | + |
| L-Arabinitol | w | w | Growth with 0,1% cycloheximide | + | + |
| D-Glucitol | +,d | + | Growth on 50% glucose | + | + |
| D-Mannitol | + | + | Growth on 60% glucose | – | – |
| Galactitol | + | + | Starch production | – | – |
| myo-Inositol | + | + | Urea test | – | – |
| Glucono d-lactone | – | – | DBB reaction | – | – |
| 2-Keto-D-gluconate | – | w | MYA 30% glucose | + | +` |
| D-Gluconate | – | – | MYA 50% glucose | + | + |
| D-Glucuronate | d | d | MEA 20% sucrose | + | + |
| D-Galacturonate | – | – | MEA 40% sucrose | + | + |
| DL-Lactate | – | – | MEA 12,5% NaCl | + | + |
| Succinate | – | – | CMA 18% NaCl | + | + |
| Citrate | – | – | CMA 36% NaCl | + | + |
| Methanol | – | w | | | |
| Ethanol | d | d,w | Growth at various temperatures | | |
| Propane 1,2 diol | d,w | d,w | 4 °C | – | – |
| Butane 2,3 diol | w,- | d | 10 °C | w | w |
| Quinic acid | – | – | 18 °C | + | + |
| Saccharate | – | – | 30 °C | + | + |

**Table 2** (continued)

| | CBS 17879 | CBS 17880 | | CBS 17879 | CBS 17880 |
|---|---|---|---|---|---|
| Galactonic acid | – | – | 35 °C | – | – |

Abbreviations and symbols: +, growth; –, no growth; d, delayed growth; w, weak growth

tested carbon sources, but no maltose, and do not utilize nitrate and nitrite; the species is mesophilic and able to grow from 18 to 30 °C, but not at 35 °C; no growth in 0.1% cycloheximide, no production of starch or starch-like compounds; growth in substrates with elevated sugar (50%) and NaCl (36%) concentrations. Salient growth characteristics of the new species, and species that belong to the two most closely related clades are given in Supplemental Table 1. Like *B. baotianmenensis* the species does not utilize maltose, but can be separated from this species by growth on L-sorbose, D-xylose, L-arabinose, D-ribose, ethanol, galactitol, mannitol, glucitol, and myo-inositol.

Here we adopt a phylogenetic species concept to delineate our new species and formally describe it below as *B. nigripullensis*.

## Taxonomy

*Blastobotrys nigripullensis* Boekhout, M. Groenew., Meijer, and Visagie, sp. nov.—MycoBank MB 847655; Fig. 3.

*Etymology*: Latin, *nigripullensis,* after *Nigrum Pullum*, the Roman fort located at Zwammerdam, province Zuid-Holland, the Netherlands, along the Rhine River [now Old Rhine River].

*Type specimen*: The Netherlands, Zwammerdam, isolated from wood of Roman shipwreck, 15 September 2021, collected by Y. Vorst, (holotype CBS H-25194 (preserved in a metabolically inactive state in the fungarium), culture ex-type CBS 17879 = DTO 457-E3).

*DNA barcodes*: ITS = OQ876815, LSU = OQ876820.

*UNITE species hypothesis*: https://doi.org/10.15156/BIO/SH1135984.09FU.

*Description:* Sporothrix-like. After two weeks at 25 °C in 5% glucose in yeast nitrogen broth, white floating flocks and white sediment occur with short hyphal cells, 6–16 × 2.5–3.5 µm, with sympodial and lateral budding. After two weeks at 25 °C on yeast morphology agar, colonies ca. 7 mm wide, flat, dry, and tough, dull white to cream, under lens surface venose, with entire margin. Hyphae branched, hyaline, 1.5–3 µm wide, with lateral, clavate blastoconida, 3–7 × 1–3.5 µm, on short to longer denticles forming a sympodial rachis of blastoconidia; thicker, inflated regions, 7–130 × 7–20 µm, may be present with finger-like outgrowths. Dalmau plate on yeast morphology agar with short adhering filaments. Similar growth on GYPA, YMA, PDA and MEA. Fermentation, assimilation, results from other growth and biochemical tests, and growth at different temperatures are listed in Table 2.

*Additional strains examined*: The Netherlands, Zwammerdam, isolated from wood of Roman shipwreck, 15 September 2021, collected by Y. Vorst, CBS 17880 = DTO 455-G9.

## Discussion

The morphology of *B. nigripullensis* resembles that of the genus *Sporothrix*, more than that of *Blastobotrys/Trichomonascus* (Smith et al. 2011a, b, Visagie et al. 2023) as the typical inflated conidiogenous cells of the latter are absent. The molecular phylogeny, however, clearly shows that *B. nigripullensis* belongs to the core clade of *Blastobotrys/Trichomonascus* that include the generic type species, *Blastobotrys nivea*.

*Blastobotrys* species are common and previously found on straw, rotting/dead wood, mushrooms, nuts and black pepper; in soil compost, the indoor environment (dust and air), the Antarctic Ocean and open waters. Species of the genus are usually osmotolerant, xerotolerant and thermotolerant, and some are applied in biotechnology (Visagie et al. 2023). *Blastrobotrys nigripullensis* was isolated from wood of a Roman shipwreck that was preserved in alluvial clay deposits near the former Roman fort of *Nigrum Pullum*, located in the present-day village of Zwammerdam, province Zuid-Holland, the Netherlands. The oak of the shipwreck was preserved with PEG 4000, but in this case, due to an undetected leak, the preservative washed out and, consequently, the wood slowly

dried. This resulted in some visual growth of fungi that may be tolerant to high PEG concentrations. Two DNA clones that originated from house dust in the USA and seawater, had the same ITS sequences and might indicate the presence of *B. nigripullensis* in those samples as well. It indicates a rather broad ecological and, likely, also a broad geographical preference. The new *Blastobotrys* species was isolated together with *Microascus melanosporus* (predominant), *Microascus paisii* and a member of the *Acremonium chrysogenum*-clade. Both *Microascus* species are widespread and prefer substrates with cellulose, while *Acremonium* species occurs in soils and on decaying plant matter (Samson et al. 2019). It will therefore be interesting to investigate further whether the novel species is also able to assimilate substrates such as cellulose. Preliminary experiments did not observe growth of *B. nigripullensis* strains on agar media with lignin and ferulic acid (R.P. de Vries, unpubl. observations).

*Blastobotrys davincii* was recently described from house dust collected in Canada and has previously been detected in environmental DNA from several historically important artifacts, including a mummy (Palermo, Italy), Leonardo da Vinci's self-portrait and cave paintings (Maijishan Grottoes, China) (Visagie et al. 2023). Together with *B. nigripullensis* isolated from a Roman shipwreck, albeit treated with PEG, it seems that *Blastobotrys* species can occur on historical artefacts and may be more common than previously thought when dealing with such substrates. The discovery of *B. nigripullensis* raised the question of whether the yeast was already present when the wood was processed and is therefore a species from Roman times or whether it arrived via infiltration of groundwater into the archaeological site or after excavation, entering the conservation process. Since it is a species of a genus with representatives that has also been found on other historical and ancient objects, might a contemporary origin not be as likely as later contamination? Could *B. nigripullensis* have flourished again under the right circumstances, in this case pieces of PEG-washed out degrading wood? More research is needed to answer this question. The initial isolation of *B. nigripullensis* from the wooden remains was done on MY50G, an agar medium containing 50% glucose, which is only used to isolate extremely xerophilic fungi. However, this medium is not routinely used by most laboratories dealing with fungi on archaeological remains. Extreme xerophiles, such as *Aspergillus halophilicus* that grows on climate-controlled stored heritage artefacts, can only be detected and grown on media with low water activity (e.g. MY50G) (Bastholm et al. 2022), while *B. nigripullensis* can also grow on media with higher water activity. The species was able to grow on agar media with up to 36% NaCl, indicating salt tolerance. Further investigations are needed to understand why this yeast grew on PEG-contaminated remains of a Roman shipwreck.

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**Declarations**

**Conflict of interests** The authors declare no conflict of interests.

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