

# Reducing the number of accepted species in *Aspergillus* series *Nigri*

C. Bian<sup>1</sup>, Y. Kusuya<sup>2,3</sup>, F. Sklenář<sup>4,5</sup>, E. D'hooge<sup>6</sup>, T. Yaguchi<sup>2</sup>, S. Ban<sup>2</sup>, C.M. Visagie<sup>7</sup>, J. Houbraken<sup>8</sup>, H. Takahashi<sup>2,9,10\*</sup>, V. Hubka<sup>2,4,5\*</sup>

<sup>1</sup>Graduate School of Medical and Pharmaceutical Sciences, Chiba University, Chiba, Japan; <sup>2</sup>Medical Mycology Research Center, Chiba University, Chiba, Japan; <sup>3</sup>Biological Resource Center, National Institute of Technology and Evaluation, Kisarazu, Japan; <sup>4</sup>Department of Botany, Faculty of Science, Charles University, Prague, Czech Republic; <sup>5</sup>Laboratory of Fungal Genetics and Metabolism, Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic; <sup>6</sup>BCCM/IHEM collection, Mycology and Aerobiology, Sciensano, Bruxelles, Belgium; <sup>7</sup>Department of Biochemistry, Genetics, and Microbiology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa; <sup>8</sup>Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; <sup>9</sup>Molecular Chirality Research Center, Chiba University, Chiba, Japan; <sup>10</sup>Plant Molecular Science Center, Chiba University, Chiba, Japan

\*Corresponding authors: H. Takahashi, [hiroki.takahashi@chiba-u.jp](mailto:hiroki.takahashi@chiba-u.jp); V. Hubka, [vit.hubka@gmail.com](mailto:vit.hubka@gmail.com)

**Abstract:** The *Aspergillus* series *Nigri* contains biotechnologically and medically important species. They can produce hazardous mycotoxins, which is relevant due to the frequent occurrence of these species on foodstuffs and in the indoor environment. The taxonomy of the series has undergone numerous rearrangements, and currently, there are 14 species accepted in the series, most of which are considered cryptic. Species-level identifications are, however, problematic or impossible for many isolates even when using DNA sequencing or MALDI-TOF mass spectrometry, indicating a possible problem in the definition of species limits or the presence of undescribed species diversity. To re-examine the species boundaries, we collected DNA sequences from three phylogenetic markers (*benA*, *CaM* and *RPB2*) for 276 strains from series *Nigri* and generated 18 new whole-genome sequences. With the three-gene dataset, we employed phylogenetic methods based on the multispecies coalescence model, including four single-locus methods (GMYC, bGMYC, PTP and bPTP) and one multilocus method (STACEY). From a total of 15 methods and their various settings, 11 supported the recognition of only three species corresponding to the three main phylogenetic lineages: *A. niger*, *A. tubingensis* and *A. brasiliensis*. Similarly, recognition of these three species was supported by the GCPSR approach (Genealogical Concordance Phylogenetic Species Recognition) and analysis in DELINEATE software. We also showed that the phylogeny based on *benA*, *CaM* and *RPB2* is suboptimal and displays significant differences from a phylogeny constructed using 5 752 single-copy orthologous proteins; therefore, the results of the delimitation methods may be subject to a higher than usual level of uncertainty. To overcome this, we randomly selected 200 genes from these genomes and performed ten independent STACEY analyses, each with 20 genes. All analyses supported the recognition of only one species in the *A. niger* and *A. brasiliensis* lineages, while one to four species were inconsistently delimited in the *A. tubingensis* lineage. After considering all of these results and their practical implications, we propose that the revised series *Nigri* includes six species: *A. brasiliensis*, *A. eucalypticola*, *A. luchuensis* (syn. *A. piperis*), *A. niger* (syn. *A. vinaceus* and *A. welwitschiae*), *A. tubingensis* (syn. *A. chiangmaiensis*, *A. costaricensis*, *A. neoniger* and *A. pseudopiperis*) and *A. vadenis*. We also showed that the intraspecific genetic variability in the redefined *A. niger* and *A. tubingensis* does not deviate from that commonly found in other aspergilli. We supplemented the study with a list of accepted species, synonyms and unresolved names, some of which may threaten the stability of the current taxonomy.

**Key words:** *Aspergillus luchuensis*, *Aspergillus niger*, *Aspergillus tubingensis*, clinical fungi, indoor fungi, infraspecific variability, multigene phylogeny, multispecies coalescence model, ochratoxin A, species delimitation.

**Citation:** Bian C, Kusuya Y, Sklenář F, D'hooge E, Yaguchi T, Ban S, Visagie CM, Houbraken J, Takahashi H, Hubka V (2022). Reducing the number of accepted species in *Aspergillus* series *Nigri*. *Studies in Mycology* 102: 95–132. doi: 10.3114/sim.2022.102.03

**Received:** 6 October 2022; **Accepted:** 6 December 2022; **Effectively published online:** 19 December 2022

**Corresponding editor:** Robert A. Samson

## INTRODUCTION

*Aspergillus niger* and its relatives play important roles in food mycology (Taniwaki *et al.* 2018), biotechnology (Schuster *et al.* 2002, Yang *et al.* 2017), the fermentation industry (Hong *et al.* 2014) and medical mycology (Howard *et al.* 2011). Consequently, *A. niger* is the most frequently cited species name in the genus *Aspergillus* (Samson *et al.* 2017). Since, on most occasions, species in section *Nigri* have been classified as GRAS (generally regarded as safe) by the Food and Drug Administration of the US government, they are suitable organisms for genetic manipulation and thus are used by biotechnology industries to produce hydrolytic enzymes (such as amylases and lipases) or organic acids (such as citric acid and gluconic acid) (Ward 1989, Bennett & Klich 1992, Andersen *et al.* 2011). On the other hand, *Aspergillus* section *Nigri* species (including some *A. niger* strains) cause food spoilage or contaminate a wide variety of food products (Samson

*et al.* 2019) and may produce mycotoxins such as ochratoxin A, fumonisins and oxalic acid (Frisvad *et al.* 2011, 2018). *Aspergillus niger*, *A. tubingensis* and their lesser known cryptic species (phylogenetically supported but phenotypically nearly identical or undistinguishable) can also act as opportunistic pathogens that cause invasive mycoses in immunocompromised patients and non-invasive mycoses (aspergilloma, allergic aspergillosis, fungal otitis externa and keratomycosis) in otherwise healthy patients (Hubka *et al.* 2012, D'hooge *et al.* 2019, Hashimoto *et al.* 2017, Salah *et al.* 2019, Vidal-Acuña *et al.* 2019, Gits-Muselli *et al.* 2021, Nargesi *et al.* 2022). Some previous studies also demonstrated that different antifungal susceptibility patterns to azole derivatives are present between *A. niger* and *A. tubingensis* complexes (Alcazar-Fuoli *et al.* 2009, Hendrickx *et al.* 2012, Szigeti *et al.* 2012).

The infrageneric classification of *Aspergillus* has a long history with Thom & Church (1926), Thom & Raper (1945) and Raper & Fennell (1965) recognizing that its species could be classified

into “groups” based on their morphological similarities. This infrageneric classification was formalised when Gams *et al.* (1986) introduced names as subgenera and sections in *Aspergillus*, e.g., all black aspergilli were classified in section *Nigri* in the subgenus *Circumdati*. Recently, Houbraken *et al.* (2020) reviewed this infrageneric classification with the help of multigene phylogenies, and showed that it mostly agreed with the infrageneric classification proposed by Gams *et al.* (1986). They introduced series rank and subdivided section *Nigri* into five series. The most economically important section *Nigri* species, including *A. niger*, *A. tubingensis* and *A. luchuensis* belong to the series *Nigri*.

The taxonomy of section *Nigri* at the species level has been turbulent over time. Originally, Mosseray (1934) proposed 35 species. This number was reduced by Raper & Fennell (1965), who recognized 12 species and two varieties, and provided detailed morphological characteristics to distinguish among their accepted species. Al-Musallam (1980) revised the species limits using cluster analysis based on morphological and cultural parameters, suggesting at least seven species, including *A. carbonarius*, *A. ellipticus*, *A. helicothrix*, *A. heteromorphus*, *A. japonicus*, *A. foetidus* and *A. niger*. In this classification, *A. niger* was subdivided into six varieties and two forms. Kozakiewicz (1989), who drew her taxonomic conclusions from conidial ornamentation under scanning electron microscopy, distinguished 16 taxa, among which three belong to the current series *Nigri*, namely, *A. acidus*, *A. niger* with six varieties and *A. citricus* with two varieties. Early molecular genetic studies indicated that *A. niger* consisted of at least two cryptic species, *A. niger* and *A. tubingensis* (Kusters-van Someren *et al.* 1991, Varga *et al.* 1994, Peterson 2000), which are morphologically undistinguishable (Pitt & Hocking 2009, Crous *et al.* 2009). Using a polyphasic approach combining morphological features, extrolite profiling and  $\beta$ -tubulin DNA sequences, Samson *et al.* (2004) accepted 15 species in the section, comprising eight species in the clade currently known as series *Nigri*, i.e., *A. brasiliensis*, *A. costaricensis*, *A. foetidus*, *A. lacticoffeatus*, *A. niger*, *A. piperis*, *A. tubingensis* and *A. vadensis*. Subsequent phylogenetic studies supported the recognition of several cryptic phylogenetic species in the series *Nigri*, such as *A. awamori* (Perrone *et al.* 2011), later synonymized with another cryptic species, *A. welwitschiae* (Hong *et al.* 2013). Similarly, Varga *et al.* (2011) recognized *A. acidus*, which was later synonymized with the resurrected species *A. luchuensis* (Hong *et al.* 2013). Another two cryptic species related to *A. tubingensis*, i.e., *A. eucalypticola* and *A. neoniger*, were described by Varga *et al.* (2011), who also synonymized *A. foetidus* and *A. lacticoffeatus* with *A. niger*. As a result, the last overview of accepted *Aspergillus* species (Houbraken *et al.* 2020) assigned ten species to the series *Nigri*: *A. brasiliensis*, *A. costaricensis*, *A. eucalypticola*, *A. luchuensis*, *A. neoniger*, *A. niger*, *A. piperis*, *A. tubingensis*, *A. vadensis* and *A. welwitschiae*. Four novel cryptic species have been proposed since. Silva *et al.* (2020) introduced *A. vinaceus* as a close relative of *A. niger* and Khuna *et al.* (2021) introduced *A. chiangmaiensis*, *A. pseudopiperis* and *A. pseudotubingensis* as close relatives of *A. tubingensis*.

Vesth *et al.* (2018) and de Vries *et al.* (2017) *de novo* sequenced the genomes of the majority of accepted section *Nigri* species and performed phenotypic and genomic comparative analyses. The whole genomes of the ex-neotype strain of *A. niger* (CBS 554.65) and 24 *A. niger sensu stricto* strains were subsequently sequenced, and the mating-type distribution was analysed (Ellena *et al.* 2021, Seekles *et al.* 2022). Isolates with both *MAT1-2-1* and *MAT1-1-1* mating-type gene idiomorphs were found among

genome-sequenced strains of *A. niger*, in agreement with previous PCR-based detection studies (Varga *et al.* 2014, Mageswari *et al.* 2016). This fact indicates that the species might have a cryptic sexual cycle, as previously observed in *A. tubingensis* (Horn *et al.* 2013). Additionally, recent phylogenomic studies indicated that section *Nigri* might belong to the subgenus *Nidulantes* (de Vries *et al.* 2017, Steenwyk *et al.* 2019) and not subgenus *Circumdati* (Jurjević *et al.* 2015, Kocsubé *et al.* 2016).

The reliable identification of section *Nigri* species is of great importance, as evidenced by their diverse positive and negative significances for humans. However, there is an increasing number of isolates that cannot be satisfactorily classified into the currently recognized species despite using multilocus sequence data (Howard *et al.* 2011, Negri *et al.* 2014, D'hooge *et al.* 2019). This fact also resulted in the recent description of several cryptic species related to *A. niger* and *A. tubingensis* (Silva *et al.* 2020, Khuna *et al.* 2021). The narrow species definition in the series *Nigri* is also associated with unsatisfactory identification results of MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry), a widely used identification tool in diagnostic laboratories and applied spheres (Gautier *et al.* 2016, D'hooge *et al.* 2019, Ban *et al.* 2021). All of the abovementioned problems may indicate that the species limits are not defined correctly and that the species definitions applied in the series are too narrow. This situation prompted the initiation of this study, where we are concerned with the verification of species boundaries at the molecular level based on a large number of strains.

A common requirement of species delimitation methods is proper sampling (a high number of strains of all studied species ideally isolated from a wide range of substrates and localities) and the presence of intraspecific variability (Carstens *et al.* 2013). This condition is, however, frequently difficult to fulfil in fungal taxonomy (Ahrens *et al.* 2016). From this point of view, series *Nigri* represents a perfect model group for studying species limits on a large scale due to the frequent occurrence of its species in many habitats and a high representation of molecular data in public databases. To do so, we have gathered extensive sequence data and applied a wide range of phylogenetic methods. The synthesis of the resulting data and the consideration of its practical taxonomic implications have led to a significant reduction in the number of species, as detailed below.

## METHODS

### Molecular studies

The DNA sequences of three loci,  $\beta$ -tubulin (*benA*), calmodulin (*CaM*) and the RNA polymerase II second largest subunit (*RPB2*), were gathered for a total of 276 strains from series *Nigri*. For this process, we removed short sequences or those that contained obvious errors. Additional species from other series of section *Nigri* were selected as outgroups or reference species depending on the analysis. The sequences were either downloaded from the GenBank database and mostly originated from previous studies, or they were taken from genes amplified and sequenced in this study. Sequences downloaded from the GenBank database were mostly from studies by D'hooge *et al.* (2019), Peterson (2008), Jurjević *et al.* (2012), Fungaro *et al.* (2017) and Hashimoto *et al.* (2017). Sequence alignments from the Silva *et al.* (2020) study were kindly provided by the authors. Information about the provenance of all strains is listed in Table 1.

**Table 1.** List of *Aspergillus* series *Nigri* strains included in the phylogenetic analyses.

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DBJ accession Nos. <sup>2</sup>			
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS
<i>A. brasiliensis</i>	CBS 101740 = IMI 381727 = ATCC MYA 4553 = IBT 21946 <sup>T</sup>	Brazil	soil	genome <sup>2</sup>	genome	EF661063	MH862749
	PPRI 26017 = CMV 007D1	South Africa	onion	MK451119	MK451325	MK450774	MK450631
	PPRI 3388 = CMV 010B8	South Africa	garlic ( <i>Allium sativum</i> )	MK451152	MK451326	MK450775	MK450632
	PPRI 3328 = CMV 004I8	South Africa	mangrove leaves	MK451015	MK451324	MK450773	MK450630
	NRRL 26651	USA	unknown	EF661094	EF661160	EF661064	KC796389
	NRRL 26650	USA	unknown	EF661079	EF661159	EF661062	EF661196
	NRRL 35542	USA	peanut seed	EF661096	EF661162	EU021641	EF661199
	NRRL 26652	USA	unknown	EF661095	EF661161	EF661063	EF661198
	CBS 121619 = DTO 24-D5 = ITEM 6139	Portugal	grapes	AM295185	AM295176	OP081976	AM295181
	CBS 121618 = DTO 24-D2 = ITEM 4539	Portugal	grapes ( <i>Tinta barroca</i> )	AM295182	AM295179	OP081975	—
	NBRC 9455 = ATCC 16404 = CBS 733.88 = DSM 1387 = DSM 1988 = KCTC 6317 = MUCL 29039 = MUCL 30113 = CECT 2574 = IFO 9455 = NCPF 2275 = IHEM 3766	USA	blueberry ( <i>Vaccinium</i> sp.)	genome	genome	genome	—
	NBRC 105650 = ATCC 9642 = CBS 246.65 = JCM 16265 = NRRL 3536 = ATHUM 2856 = CECT 2700 = DSM 63263 = IFO 6342 = IMI 091855 = MUCL 19001 = IHEM 3797	Australia	wireless set	genome	genome	genome	—
	IFM 66950 = CCF 3991	Spain	cave air	genome	genome	genome	—
	IFM 66951 = CCF 4962	Romania	microbial mat in cave	genome	genome	genome	—
	IHEM 23046	Belgium	unknown	MH614413	MH644886	OP081977	MH613107
	IHEM 23047	Belgium	unknown	MH614444	MH644887	OP081978	MH613108
	IHEM 5185 = NRRL 2276	Unknown	unknown	MH614565	MH644892	OP081982	MH613109
	IHEM 23049	Belgium	unknown	MH614414	MH644889	OP081979	MH613081
	IHEM 3766	USA	fruit, <i>Vaccinium</i> subg. <i>Cyanococcus</i>	MH614415	MH644891	OP081980	MH613079
	IHEM 3797	Australia	radio set	MH614416	MH644890	OP081981	MH613110
<i>A. niger</i>	CBS 554.65 = ATCC 16888 = IFO 33023 = IHEM 3415 = JCM 10254 = NRRL 326 = CCFC 222006 = IMI 050566 = NBRC 33023 <sup>T</sup>	USA	tannin-gallic acid fermentation	EF661089	EF661154	EF661058	FJ629337
	CBS 101883 = CECT 20581 = IBT 22031 <sup>T1</sup>	Indonesia	coffee bean	genome	genome	genome	—
	PPRI 8682 = CMV 005B2	South Africa	mopane debris	MK451027	MK451462	MK450792	—
	PPRI 8734 = CMV 005B7	South Africa	mopane debris	MK451032	MK451464	MK450793	—
	PPRI 26011 = CMV 005G9	South Africa	soil	MK451060	MK451468	MK450794	—
	PPRI 18719 = CMV 004I3	South Africa	frass of <i>Busseola fusca</i> feeding inside maize stems	MK451010	MK451458	MK450789	—
	PPRI 3640 = CMV 004I6	South Africa	old photo paper	MK451013	MK451459	MK450790	—
	IFM 63326	Japan	human sputum	MK854742	OP081973	genome	—
	IFM 63604	Japan	human sputum	MK854748	OP081974	genome	—
	IFM 59636	Japan	human abdominal drain	OP081896	OP081969	genome	—
	104	Brazil	coffee bean	OP081838	OP081913	OP082051	—

Table 1. (Continued).

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DBJ accession Nos. <sup>2</sup>			
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS
<i>A. niger</i>	1400	Brazil	brazil nuts	OP081842	OP081917	OP082055	—
	1551	Brazil	brazil nuts	OP081848	OP081923	OP082061	—
	2334	Brazil	brazil nuts	OP081860	OP081935	OP082073	—
	2504	Brazil	brazil nuts	OP081861	OP081936	OP082074	—
	4.17	Brazil	grapes	OP081863	OP081938	OP082076	—
	4.18	Brazil	grapes	OP081864	OP081939	OP082077	—
	643	Brazil	coffee bean	OP081878	OP081953	OP082091	—
	6504	Brazil	brazil nuts	OP081879	OP081954	OP082092	—
	7061	Brazil	brazil nuts	OP081884	OP081959	OP082097	—
	8219	Brazil	coffee bean	OP081888	OP081963	OP082101	—
	902	Brazil	brazil nuts	OP081890	OP081965	OP082103	—
	1.2	Brazil	grapes	OP081836	OP081911	OP082049	—
	1357	Brazil	brazil nuts	OP081841	OP081916	OP082054	—
	23.115	Brazil	onions	OP081859	OP081934	OP082072	—
	CBS 117.80	Unknown	unknown	OP081891	GU195632	OP082104	—
	IHEM 18069	French Guiana, France	soil under palm tree	MH614489	MH645000	OP082110	MH613155
	IHEM 2312	Belgium	cosmetics	MH614521	MH645010	OP082127	MH613218
	IHEM 17892	Belgium	chronic sinusitis	MH614520	MH644877	OP082108	KP131598
	IHEM 23979	Belgium	otitis	MH614500	MH645005	OP082139	MH613182
	IHEM 25482	France	human bronchoalveolar lavage	MH614474	MH644860	OP082150	MH613113
	IHEM 23907	Belgium	human ear	MH614492	MH645003	OP082136	MH613168
	IHEM 24459	India	otitis	MH614502	MH645006	OP082144	MH613185
	IHEM 5788	Unknown	Chinese gall	MH614437	MH645007	OP082169	MH613213
	IHEM 21604	Cuba	outdoor air	MH614478	MH644996	OP082117	MH613124
	IHEM 5844	Unknown	unknown	MH614434	MH645002	OP082170	MH613157
	IHEM 5296	Unknown	leather	MH614519	MH645009	OP082167	MH613217
	IHEM 6126	Belgium	human sputum	MH614453	MH644998	OP082171	MH613135
	IHEM 5622	Unknown	unknown	MH614451	MH645001	OP082168	MH613156
	IHEM 4023	Belgium	mycotic otitis externa	MH614496	MH645004	OP082162	KP131606
	IHEM 9673	France	dwelling environment dust	MH614523	MH645012	OP082176	MH613219
	IHEM 25481	France	human bronchoalveolar lavage	MH614473	MH644859	OP082149	MH613112
	IHEM 21605	Cuba	nickel deposit	MH614479	MH644997	OP082118	MH613125
	IHEM 6147	Belgium	human nose	MH614439	MH645008	OP082172	MH613215
	IHEM 9709	France	cement	MH614522	MH645011	OP082177	MH613084
	IFM 56816	Japan	human ear	OP081895	OP081968	genome	—
	2.6	Brazil	grapes	OP081856	OP081931	OP082069	—
	2.8	Brazil	grapes	OP081858	OP081933	OP082071	—
	102.2706	Brazil	grapes	OP081837	OP081912	OP082050	—
	13.09	Brazil	coffee bean	OP081840	OP081915	OP082053	—
	148.727	Brazil	onions	OP081846	OP081921	OP082059	—
ITAL 47.456 = IBT 35556 <sup>12</sup>	Brazil	surface of grape berries	MN583579	MN583580	MN583581	MN575692	
ITAL 49.577	Brazil	grapes	OP081869	OP081944	OP082082	—	
ITAL 57.1243	Brazil	grapes	OP081875	OP081950	OP082088	—	

Table 1. (Continued).

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DDBJ accession Nos. <sup>2</sup>			
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS
<i>A. niger</i>	ITAL 62.1583	Brazil	grapes	OP081877	OP081952	OP082090	—
	ITAL 66.1929	Brazil	grapes	OP081881	OP081956	OP082094	—
	ITAL 48.545	Brazil	grapes	OP081867	OP081942	OP082080	—
	47.514	Brazil	grapes	OP081865	OP081940	OP082078	—
	48.544	Brazil	grapes	OP081866	OP081941	OP082079	—
	49.580	Brazil	grapes	OP081870	OP081945	OP082083	—
	55.1175	Brazil	grapes	OP081874	OP081949	OP082087	—
	58.1292	Brazil	grapes	OP081876	OP081951	OP082089	—
	CBS 139.54 <sup>T3</sup>	Namibia	<i>Welwitschia mirabilis</i>	MN969369	genome	MN969100	MH857271
	PPRI 23385 = CMV 00415	South Africa	animal feed	MK451012	MK451548	MK450816	MK450663
	PPRI 4966 = CMV 00411	South Africa	<i>Chrysomelidae</i> beetle	MK451008	MK451546	MK450814	MK450661
	PPRI 13255 = CMV 00412	South Africa	chicken house bedding, mostly sunflower husks	MK451009	MK451547	MK450815	MK450662
	PPRI 26014 = CMV 006G2	South Africa	soil	MK451097	MK451562	MK450819	MK450669
	PPRI 9362 = CMV 005D1	South Africa	twigs and leaves from <i>Colophospermum mopane</i>	MK451039	MK451556	MK450818	MK450666
	IFM 50267	Japan	human sputum	OP081894	OP081967	genome	—
	IFM 63309	Japan	human, bronchoalveolar lavage fluid	MK854741	OP081972	genome	—
	IFM 49718	Japan	human sputum	OP081893	OP081966	genome	—
	IFM 62618	Japan	human, bronchoalveolar lavage fluid	MK854725	OP081971	genome	—
	IFM 60653	Japan	human sputum	OP081897	OP081970	genome	—
	15477	Brazil	onions	OP081847	OP081922	OP082060	—
	15580	Brazil	onions	OP081849	OP081924	OP082062	—
	15646	Brazil	onions	OP081850	OP081925	OP082063	—
	16158	Brazil	onions	OP081851	OP081926	OP082064	—
	16285	Brazil	onions	OP081852	OP081927	OP082065	—
	171804	Brazil	onions	OP081854	OP081929	OP082067	—
	181832	Brazil	onions	OP081855	OP081930	OP082068	—
	2.7	Brazil	grapes	OP081857	OP081932	OP082070	—
	48282	Brazil	onions	OP081868	OP081943	OP082081	—
	53295	Brazil	onions	OP081871	OP081946	OP082084	—
	53a	Brazil	yerba mate	OP081872	OP081947	OP082085	—
	54298	Brazil	onions	OP081873	OP081948	OP082086	—
	6573	Brazil	brazil nuts	OP081880	OP081955	OP082093	—
	69363	Brazil	onions	OP081883	OP081958	OP082096	—
	714	Brazil	brazil nuts	OP081885	OP081960	OP082098	—
	77390	Brazil	onions	OP081887	OP081962	OP082100	—
	88430	Brazil	onions	OP081889	OP081964	OP082102	—
	127a	Brazil	yerba mate	OP081839	OP081914	OP082052	—
	145723	Brazil	onions	OP081843	OP081918	OP082056	—
	146.724	Brazil	onions	OP081844	OP081919	OP082057	—
	146725	Brazil	onions	OP081845	OP081920	OP082058	—
	361	Brazil	brazil nuts	OP081862	OP081937	OP082075	—
	670	Brazil	brazil nuts	OP081882	OP081957	OP082095	—
716	Brazil	brazil nuts	OP081886	OP081961	OP082099	—	

Table 1. (Continued).

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DBJ accession Nos. <sup>2</sup>			
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS
<i>A. niger</i>	17.179	Brazil	grapes	OP081853	OP081928	OP082066	—
	DTO 267-13	Thailand	house dust	OP081892	KP330149	OP082105	—
	IHEM 22373	India	otomycosis	MH614468	MH644935	OP082121	KP131600
	IHEM 17902	Belgium	chronic sinusitis	MH614508	MH644965	OP082109	KP131599
	IHEM 25483	France	human sputum	MH614447	MH644929	OP082151	MH613114
	IHEM 3095	Mauritius	soil	MH614488	MH644942	OP082160	MH613151
	IHEM 3090	Mauritius	soil	MH614487	MH644884	OP082159	MH613150
	IHEM 20620	Cuba	soil	MH614516	MH644973	OP082115	MH613208
	IHEM 23644	India	otitis	MH614435	MH644948	OP082129	MH613171
	IHEM 6727	France	bronchopulmonary cancer	MH614450	MH644972	OP082174	KP131609
	IHEM 21970	Mauritius	soil	MH614484	MH644938	OP082120	MH613111
	IHEM 24434	India	otitis	MH614498	MH644952	OP082141	MH613179
	IHEM 18351	French Guiana, France	bark kapok tree	MH614481	MH644934	OP082112	MH613128
	IHEM 23822	India	otitis	MH614493	MH644947	OP082132	MH613169
	IHEM 23651	India	otitis	MH614491	MH644946	OP082131	MH613167
	IHEM 24707	Belgium	otitis	MH614503	MH644957	OP082145	MH613189
	IHEM 25101	Belgium	Unknown	MH614441	MH644961	OP082147	MH613194
	IHEM 21697	Belgium	onion	MH614448	MH644939	OP082119	MH613137
	IHEM 23892	Belgium	human	MH614570	MH644944	OP082134	MH613161
	IHEM 23965	France	otitis	MH614497	MH644951	OP082138	MH613178
	IHEM 15980	Belgium	ball from Pakistan	MH614518	MH644976	OP082107	MH613210
	IHEM 20621	Cuba	soil	MH614480	MH644933	OP082116	MH613126
	IHEM 18080	French Guiana, France	soil of riverside	MH614513	MH644969	OP082111	MH613077
	IHEM 25485	France	human bronchoaspiration	MH614477	MH644932	OP082153	MH613116
	IHEM 5077	Belgium	human ear	MH614515	MH644971	OP082166	MH613078
	IHEM 25340	Belgium	human toenail	MH614506	MH644962	OP082148	MH613195
	IHEM 18676	Peru	guano	MH614446	MH644928	OP082113	MH613082
	IHEM 24328	Unknown	horse (crust) - clinical sample	MH614499	MH644953	OP082140	MH613180
	IHEM 2951	Belgium	mycotic otitis externa	MH614569	MH644963	OP082156	KP131605
	IHEM 2969	India	soil	MH614449	MH644941	OP082157	MH613149
	IHEM 22432	Somalia	otitis	MH614509	MH644966	OP082126	KP131604
	IHEM 26623	Belgium	human ear	MH614486	MH644940	OP082155	MH613146
	IHEM 26214	Belgium	otitis	MH614505	MH644960	OP082154	MH613192
	IHEM 23648	India	otitis	MH614495	MH644950	OP082130	MH613174
	IHEM 24450	India	otitis	MH614469	MH644955	OP082142	MH613184
	IHEM 25484	France	human bronchoaspiration	MH614476	MH644931	OP082152	MH613115
	IHEM 22377	Belgium	conjunctivitis - human eye	MH614483	MH644937	OP082123	KP131602
	IHEM 22378	Somalia	human auditory canal	MH614510	MH644967	OP082124	MH613076
	IHEM 23913	Belgium	human ear	MH614501	MH644954	OP082137	MH613183
	IHEM 4781	Belgium	mycotic otitis externa	MH614454	MH644974	OP082165	KP131608
	IHEM 22374	India	otomycosis	MH614482	MH644936	OP082122	KP131601
	IHEM 23642	India	otitis	MH614452	MH644861	OP082128	MH613170

Table 1. (Continued).

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DBJ accession Nos. <sup>2</sup>				
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS	
<i>A. niger</i>	IHEM 4063	Bahamas	sea water	MH614440	MH644995	OP082163	MH613152	
	IHEM 23895	Belgium	human ear secretions	MH614584	MH644945	OP082135	MH613163	
	IHEM 14389	Belgium	chronic obstructive bronchopneumopathy (bronchoalveolar lavage)	MH614517	MH644975	OP082106	KP131597	
	IHEM 18678	Peru	guano	MH614475	MH644930	OP082114	MH613074	
	IHEM 3710	Unknown	unknown	MH614490	MH644943	OP082161	MH613158	
	IHEM 24767	India	otitis	MH614507	MH644964	OP082146	MH613198	
	IHEM 9388	France	air	MH614438	MH644979	OP082175	MH613220	
	IHEM 3019	Zaire	soil from palm grove ( <i>Elaeis guineensis</i> )	MH614514	MH644970	OP082158	MH613206	
	IHEM 24454	India	otitis	MH614470	MH644956	OP082143	MH613186	
	IHEM 22379	India	otomycosis	MH614511	MH644968	OP082125	KP131603	
	IHEM 4461	Belgium	mycotic otitis externa	MH614585	MH644978	OP082164	KP131607	
	IHEM 23888	Belgium	otitis	MH614494	MH644949	OP082133	MH613173	
	IHEM 6350	Belgium	dried food for fish	MH614471	MH644977	OP082173	MH613214	
	<i>A. eucalypticola</i>	CBS 122712 = DTO 53-A2 = IBT 29274 <sup>T</sup>	Australia	leaves of <i>Eucalyptus</i> sp.	genome	genome	genome	OL711732
	<i>A. luchuensis</i>	CBS 205.80 = IFM 47726 = NBRC 4281 = IFO 428 = KACC 46772 = RIB 2642 <sup>T</sup>	Unknown	awamori-koji	JX500062	JX500071	MN969081	JX500081
NRRL 4750 = CBS 128.52 = KACC 47005 <sup>T4</sup>		USA	unknown	EF661087	EF661152	EF661052	MH856956	
RIB 2601 = IFO 4033 = NBRC 111188 = ATCC 38854 = IFM 46994		Unknown	unknown	genome	genome	genome	LC573600	
IFO 4308 = NBRC 4308		Unknown	unknown	genome	genome	genome	AB573884	
IHEM 26285		France	human broncho-aspiration	MH614561	MH644869	OP082038	MH613141	
IHEM 25486		France	human trimming liquid	MH614563	MH644862	OP082030	MH613118	
CBS 112811 = CECT 20582 = IBT 26239 <sup>T5</sup>		Denmark	black pepper	genome	genome	genome	OL711715	
IHEM 23904		India	otitis	MH614551	MH644990	OP082022	MH613164	
PPRI 13091 = CMV 010I3		South Africa	soil	MK451175	MK451492	MK450797	—	
IHEM 5316		Belgium	human ear	MH614562	MH644893	OP082046	MH613216	
PPRI 9197 = CMV 005C9		South Africa	soil	MK451038	MK451491	MK450796	MK450641	
PPRI 13230 = CMV 011A9		South Africa	maize roots	MK451187	MK451493	MK450798	—	
PPRI 8983 = CMV 005C2		South Africa	twigs and leaves from <i>Colophospermum mopane</i>	MK451035	MK451490	MK450795	MK450640	
<i>A. pseudotubingensis</i>	SDBR CMUO2 <sup>T</sup>	Thailand	rhizosphere soil	MK457206	MK457205	MK457208	—	
	SDBR CMUO8	Thailand	rhizosphere soil	MW602908	MW602907	MW602909	—	
	SDBR CMU20	Thailand	rhizosphere soil	MW602913	MW602912	MW602914	—	
<i>A. tubingensis</i>	CBS 133056 = DTO 213-F6 = NRRL 4875 <sup>T</sup>	Unknown	unknown	EF661086	EF661151	EF661055	EF661193	
	PPRI 8683 = CMV 005B3	South Africa	soil	MK451028	MK451452	MK450786	—	
	PPRI 8733 = CMV 005B6	South Africa	plant debris from <i>Colophospermum mopane</i>	MK451031	MK451453	MK450787	—	
	PPRI 21344 = CMV 004I4	South Africa	seed of soybean ( <i>Glycine max</i> )	MK451011	MK451448	MK450782	—	
IFM 57258	Nagasaki, Japan	soil	OP081902	OP081909	genome	—		

Table 1. (Continued).

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DBJ accession Nos. <sup>2</sup>			
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS
<i>A. tubingensis</i>	IHEM 18329	French Guiana, France	rotten mango	MH614467	MH644992	OP082002	MH613202
	IHEM 18205	French Guiana, France	soil	MH614419	MH644865	OP082001	MH613127
	IHEM 21599	Cuba	rail feather	MH614458	MH644985	OP082008	MH613073
	IHEM 18097	French Guiana, France	rotten mango	MH614417	MH644981	OP081998	MH613119
	IHEM 21607	Cuba	indoor environment	MH614464	MH644988	OP082012	MH613133
	IHEM 18042	French Guiana, France	soil under mango tree	MH614462	MH644980	OP081997	MH613117
	IHEM 21597	Cuba	indoor air	MH614457	MH644984	OP082007	MH613122
	IHEM 2463	Belgium	nutmeg ( <i>Myristica fragrans</i> )	MH614465	MH644989	OP082028	MH613147
	IHEM 21593	Cuba	cotton cloth	MH614460	MH644987	OP082006	MH613131
	IHEM 18136	French Guiana, France	soil under palm tree	MH614456	MH644983	OP082000	MH613121
	IHEM 21592	Cuba	human ear	MH614420	MH644866	OP082005	MH613130
	IHEM 21606	Cuba	indoor environment	MH614466	MH644991	OP082011	MH613199
	IHEM 21590	Cuba	indoor air	MH614418	MH644986	OP082004	MH613129
	IHEM 22375	India	otomycosis	MH614461	MH644867	OP082015	KP131633
	IHEM 22376	India	otomycosis	MH614463	MH644868	OP082016	KP131634
	IHEM 18106	French Guiana, France	leaf of mango tree	MH614455	MH644982	OP081999	MH613083
	IHEM 21602	Argentina	clinical material	MH614459	MH644864	OP082010	MH613123
	PPRI 21345 = CMV 005A8	South Africa	seed of soybean ( <i>Glycine max</i> )	MK451024	MK451450	MK450784	—
	PPRI 21347 = CMV 005A9	South Africa	seed of soybean ( <i>Glycine max</i> )	MK451025	MK451451	MK450785	—
	IHEM 21971	Mauritius	soil	MH614546	MH644993	OP082013	MH613134
	IHEM 25327	Belgium	laboratory contaminant	MH614421	MH644994	OP082029	MH613139
	IHEM 21601	Argentina	clinical material	MH614564	MH644917	OP082009	—
	PPRI 7393 = CMV 005A5	South Africa	unknown	MK451021	MK451542	MK450813	MK450660
	NRRL 4851 = ATCC 16879 = CBS 115.48 = CBS 558.65	Wisconsin, USA	unknown	EF661085	EF661150	EF661054	—
	CBS 134.48 = ITEM 7040	Unknown	unknown	AY820007	AJ964876	EF661055	FJ629354
	IHEM 5802	Unknown	unknown	MH614558	MH644919	OP082048	MH613204
	IHEM 22772	Belgium	human ear	MH614545	MH644871	OP082018	MH613132
	PPRI 25991 = CMV 001B7	South Africa	walnut kernels ( <i>Juglans regia</i> )	MK450891	MK451540	MK450811	MK450658
	PW3161	Hong Kong	human nail	LC000547	LC000560	LC000573	AB987902
	PPRI 6720 = CMV 005A3	South Africa	beetle ( <i>Aspidimorpha areata</i> )	MK451019	MK451541	MK450812	MK450659
	IFM 54640	Japan	human sputum	OP081898	OP081905	genome	—
	IFM 55763	Japan	vineyard	OP081899	OP081906	genome	—
	IFM 57143	Japan	human	OP081901	OP081908	genome	—
	IFM 56815	Japan	human ear	OP081900	OP081907	genome	—



Table 1. (Continued).

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DBJ accession Nos. <sup>2</sup>			
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS
<i>A. tubingensis</i>	IFM 61612	Japan	human, bronchoalveolar lavage fluid	OP081903	OP081910	genome	—
	IHEM 22370	Belgium	otitis	MH614525	MH644894	OP082014	KP131632
	IHEM 1941	Belgium	dust from mattress	MH614550	MH644900	OP082003	MH613159
	IHEM 17170	Belgium	chronic sinusitis	MH614537	MH644923	OP081989	KP131625
	IHEM 25487	France	environment	MH614524	MH644879	OP082031	MH613120
	IHEM 23971	Belgium	mycotic otitis externa	MH614555	MH645013	OP082025	MH613181
	IHEM 17440	Belgium	chronic sinusitis	MH614540	MH644924	OP081991	KP131627
	IHEM 23890	Belgium	human ear	MH614530	MH644905	OP082020	MH613172
	IHEM 16879	Morocco	otitis human ear	MH614559	MH644921	OP081986	MH613211
	IHEM 26291	Belgium	unknown	MH614552	MH644903	OP082039	MH613165
	IHEM 17439	Belgium	chronic sinusitis	MH614539	MH644876	OP081990	KP131626
	IHEM 13662	Belgium	hospital environment	MH614553	MH644898	OP081985	MH613153
	IHEM 17033	Belgium	mycotic otomycosis externa	MH614536	MH644922	OP081987	KP131623
	IHEM 23911	Belgium	otitis	MH614566	MH644908	OP082024	MH613177
	IHEM 17904	Belgium	chronic sinusitis	MH614538	MH644875	OP081996	MH613212
	IHEM 17893	Belgium	chronic sinusitis	MH614542	MH644926	OP081994	KP131630
	IHEM 25949	France	human sputum	MH614528	MH644873	OP082034	MH613144
	IHEM 26200	Belgium	otitis	MH614568	MH644901	OP082036	MH613160
	IHEM 26645	Belgium	human ear secretions	MH614549	MH644897	OP082043	MH613148
	IHEM 26163	Belgium	otitis externa	MH614527	MH644872	OP082035	MH613140
	IHEM 23900	Belgium	otitis	MH614529	MH644904	OP082021	MH613166
	IHEM 26661	Belgium	onychomycosis human toe nail	MH614556	MH644911	OP082044	MH613193
	IHEM 17903	Belgium	chronic sinusitis	MH614543	MH644927	OP081995	KP131631
	IHEM 17441	Belgium	chronic sinusitis	MH614541	MH644925	OP081992	KP131628
	IHEM 26350	France	air	MH614548	MH644883	OP082040	MH613143
	IHEM 17168	Belgium	chronic sinusitis	MH614534	MH644920	OP081988	KP131624
	IHEM 26622	Belgium	human ear	MH614567	MH644896	OP082042	MH613145
	IHEM 25777	Belgium	human wound	MH614423	MH644913	OP082033	MH613197
	IHEM 25773	Belgium	otitis externa	MH614472	MH644912	OP082032	MH613196
	IHEM 4379	unknown	unknown	MH614554	MH644899	OP082045	MH613154
	IHEM 23985	Belgium	otitis	MH614531	MH644907	OP082026	MH613176
	IHEM 26215	Belgium	otomycosis	MH614433	MH644914	OP082037	MH613200
	IHEM 17442	Belgium	chronic sinusitis	MH614533	MH644915	OP081993	KP131629
	IHEM 10349	China	grains	MH614535	MH644918	OP081984	MH613207
	IHEM 23886	Belgium	otitis	MH614424	MH644910	OP082019	MH613188
	IHEM 5615	Unknown	unknown	MH614425	MH644916	OP082047	MH613201
	IHEM 24447	India	otitis	MH614532	MH644909	OP082027	MH613187
	CBS 115574 = IBT 23401 = ITEM 7555 = CECT 20579 <sup>16</sup>	Costa Rica	soil	genome	genome	HE984361	MH862988
	CBS 553.65 = NRRL 5121 = ATCC 16880 = IMI 23599	Costa Rica	soil	FJ629278	OP081904	OP081983	FJ629327
	CBS 115656 = IBT 20973 = NRRL 62634 <sup>17</sup>	Venezuela	mangrove water	KC796361	KC796377	genome	OL711719
	IHEM 23909	India	otitis	MH614422	MH644906	OP082023	MH613175
	IHEM 22394	India	conjunctivitis - human eye	MH614544	MH644870	OP082017	KP131635

Table 1. (Continued).

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DDBJ accession Nos. <sup>2</sup>			
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS
<i>A. tubingensis</i>	SDBR CMUI4 <sup>18</sup>	Thailand	rhizosphere soil	MK457200	MK457199	MK457202	—
	SDBR CMUI5	Thailand	rhizosphere soil	MW602898	MW602897	MW602899	—
	SDBR CMUI1 <sup>19</sup>	Thailand	rhizosphere soil	MK457194	MK457193	MK457196	—
	SDBR CMUI7	Thailand	rhizosphere soil	MW602903	MW602902	MW602904	—
<i>A. vadensis</i>	CBS 113365 = IMI 142717 = CECT 20584 = IBT 24658 <sup>T</sup>	Egypt	air	AY585531	genome	HE984371	—
	IHEM 26351	France	human sputum	MH614547	MH644878	OP082041	MH613142
<i>A. carbonarius</i>	CBS 111.26 = ITEM 4503 = NRRL 369 = ATCC 1025 = ATHUM 2854 = CBS 556.65 = IMI 016136 = MUCL 13583 = NCTC 1325 = NRRL 1987 = NRRL 369 = ITEM 5010 <sup>T</sup>	Unknown	paper	EF661099	EF661167	EF661068	EF661204
	NRRL 67 = NBRC 5864 = ATCC 8740 = CBS 420.64 = DSM 872 = IFO 5864 = IMI 41875 = MUCL 30479 = NRRL 1737 = NRRL 605	Unknown	unknown	EF661097	EF661165	EF661066	EF661202
	NRRL 4849 = CBS 114.29	USA	unknown	EF661100	EF661168	EF661069	EF661205
	NRRL 346 = ATCC 6277 = CBS 146284	Honduras	unknown	EF661098	EF661166	EF661067	EF661203
<b>Additional strains used only in DELINEATE and STACEY analyses</b>							
<i>A. carbonarius</i>	IHEM 661	France	indoor air in bakery	MH614442	MH645014	—	—
	IHEM 1931	Belgium	dust from mattress	MH61458	MH644880	—	—
	IHEM 25902	France	human sputum	MH614575	MH645015	—	—
	DTO 179-F4	South Africa	house dust	KP329846	KJ775280	—	—
	DTO 179-C6	South Africa	house dust	KP329845	KJ775278	—	—
	CCF 3388	Czech Republic	toenails, human clinical material	HE577803	HE649500	—	—
	144-3-K2	Unknown	unknown	KJ599604	KJ599576	—	—
	A-1759	Israel	grape	KC520549	KC520552	—	—
	G187	Slovakia	dried vine fruits	MT166308	MK046876	—	—
	A-2160	Spain	grape	KC520550	KC520553	—	—
<i>A. ibericus</i>	NRRL 35644 = CBS 121593 = IMI 391429 = DTO 24-F1 = ITEM 4776 = IHEM 23498 <sup>T</sup>	Portugal	wine grapes	EF661102	EF661163	XM_025715438	—
	NRRL 35645 = ITEM 6601 = IMI 391430	Portugal	wine grapes	EF661101	EF661164	EF661065	—
<i>A. ellipticus</i>	CBS 707.79 = IMI 172283 <sup>T</sup>	Unknown	unknown	AY585530	EF661170	EF661051	—
	CBS 677.79 = IMI 278383 = ITEM 4499 <sup>T10</sup>	Costa Rica	unknown	AY819993	AM117810	HE984363	—
<i>A. heteromorphus</i>	NRRL 4747 = CBS 117.55 = ATCC 12064 = IMI 172288 = IHEM 5801 = ITEM 7045 <sup>T</sup>	Brazil	culture contaminant	EF661103	EF661169	EF661050	—
	IHEM 18645	France, Martinique	indoor wall	MH614573	MH645030	—	—
<i>A. sclerotii carbonarius</i>	CBS 121057 = IBT 121057 <sup>T</sup>	Unknown	culture contaminant	EU159229	EU159235	MN969091	—
<i>A. sclerotioniger</i>	CBS 115572 = IBT 22905 = ITEM 7560 <sup>T</sup>	Unknown	unknown	FJ629304	FN594557	HE984369	—
<i>A. japonicus</i>	CBS 114.51 = ITEM 7034 <sup>T</sup>	Unknown	unknown	HE577804	AJ964875	MN969079	—
	PPRI 4286 = CMV 005H7	South Africa	soil	MK451062	MK451430	MK450779	—
	ITEM 14787	USA	indoor air	HE984413	HE984430	HE984377	—

Table 1. (Continued).

Species	Strain No. <sup>1</sup>	Country	Substrate	GenBank/ENA/DDBJ accession Nos. <sup>2</sup>				
				<i>benA</i>	<i>CaM</i>	<i>RPB2</i>	ITS	
<i>A. japonicus</i>	ITEM 14805	USA	indoor air	HE984416	—	HE984378	—	
	CBS 123.27 = NRRL 360 = ATCC 1042 = IFO 4106 = IMI 358697	Puerto Rico	soil	HE577805	EF661141	EF661047	—	
	NRRL 1782 = ATCC 16873 = CBS 568.65 = IMI 211387	Panama	soil	—	EF661144	EF661048	—	
	NRRL 35494 = ITEM 15926	Unknown	unknown	—	EU021690	EU021639	—	
	NRRL 35541	USA	peanut field soil	EF661104	EF661143	EU021640	—	
	NRRL 4839 = IHEM 5627 = NCTC 3792 = MUCL 13578 = IMI 312983 = CBS 113.48	Unknown	unknown	MH614586	EF661142	EF661049	—	
	IHEM 26043	France	human sputum	MH614587	MH645026	—	—	
	CBS 115571	Bahamas	marine environment	EU482434	EU482432	—	—	
	AJP01	Unknown	unknown	JX103558	JX103559	—	—	
	CCF 4079	Unknown	unknown	HE577811	FR751423	—	—	
	ITEM 14789	USA	indoor air	HE9844174	HE984432	—	—	
	NBRC 4408	Unknown	soil	LC573650	LC573708	—	—	
	NBRC 32856	Japan	living leaf	LC573651	LC573709	—	—	
	<i>A. aculeatus</i>	CBS 172.66 = IHEM 5796 = NRRL 5094 = IMI 211388 = CCRC 32190 = ATCC 16872 = ITEM 7046 <sup>T</sup>	Unknown	tropical soil	HE577806	EF661148	XM020198603	—
		PPRI 7513 = CMV 005A6	Unknown	unknown	MK451022	MK451291	MK450752	—
PPRI 26016 = CMV 007C9		South Africa	soil	MK451118	MK451296	MK450756	—	
F-719		Unknown	unknown	HE577810	HE578093	—	—	
PPRI 4070 = CMV 005F1		South Africa	soil	MK451044	MK451292	MK450753	—	
ITEM 14807		USA	indoor air	HE984409	HE984424	HE984372	—	

<sup>1</sup>Acronyms of culture collections in alphabetic order: ATCC, American Type Culture Collection, Manassas, Virginia; ATHUM, Athens Collection of Fungi, University of Athens, Athens, Greece; CBS, Westerdijk Fungal Biodiversity Institute (formerly Centraalbureau voor Schimmelcultures), Utrecht, the Netherlands; CCF, Culture Collection of Fungi, Department of Botany, Charles University, Prague, Czech Republic; CCF, Canadian Collection of Fungal Cultures, living fungal collection associated with the DAOM herbarium (Agriculture and Agri-Food Canada), Ottawa, Canada; CCM (F-), Czech Collection of Microorganisms, Brno, Czech Republic; CCRC (=BCRC), Bioresources Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan; CECT, Colección Española de Cultivos Tipo, Universidad de València, Edificio de Investigación, Burjassot, Spain; CMV, working and formal culture collections housed at FABI (Forestry and Agricultural Biotechnology), Innovation Africa, University of Pretoria, South Africa; DSM, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; DTO, internal culture collection of the Department Applied and Industrial Mycology of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; IBT, Culture Collection at the Department of Biotechnology and Biomedicine, Lyngby, Denmark; IFM, Culture Collections for Pathogenic Fungi and Actinomycetes, Medical Mycology Research Center, Chiba University, Chiba, Japan; IFO, Institute for Fermentation, Osaka, Japan (IFO strains were transferred to the NBRC NITE collection); IHEM (BCCM/IHEM), Belgian Coordinated Collections of Micro-organisms, Fungi Collection: Human and Animal Health, Sciensano, Brussels, Belgium; IMI, CABI's collection of fungi and bacteria, Wallingford, UK; JCM, Japan Collection of Microorganisms, Tsukuba, Japan; KACC, Korean Agricultural Culture Collection, Wanju, South Korea; KCTC, Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Jeongseup, South Korea; MUCL (BCCM/MUCL), Agro-food & Environmental Fungal Collection, Louvain-la-Neuve, Belgium; NBRC (NITE), National Institute of Technology and Evaluation, Biological Resource Center, Department of Biotechnology, Kisarazu, Chiba, Japan; NCTC, National Collection of Type Cultures, Central Public Laboratory Service, London, UK; ITEM, Agri-Food Toxigenic Fungi Culture Collection, Institute of Sciences of Food Production, Bari, Italy; NRRL, Agricultural Research Service Culture Collection, Peoria, Illinois, USA; PPRI, culture collection of the National Collections of Fungi, housed at the Agricultural Research Council - Plant Health and Protection, Roodeplaat, South Africa; RIB, National Research Institute of Brewing, Tax Administration Agency, Higashihiroshima, Hiroshima, Japan; SDBR, Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University, Chiang Mai Province, Thailand. Other acronyms represent personal strain numbers (without permanent preservation).

<sup>2</sup>Accession numbers to genomic sequences analysed in this study are listed in Supplementary Table S3.

<sup>T</sup>ex-type strain. <sup>T1</sup> = *A. lacticoffeatus*; <sup>T2</sup> = *A. vinaceus*; <sup>T3</sup> = *A. welwitschiae*; <sup>T4</sup> = *A. nakazawae*; <sup>T5</sup> = *A. piperis*; <sup>T6</sup> = *A. costaricensis*; <sup>T7</sup> = *A. neoniger*; <sup>T8</sup> = *A. chiangmaiensis*; <sup>T9</sup> = *A. pseudopiperis*; <sup>T10</sup> = *A. helicothrix*.

Total genomic DNA was isolated from 5-d-old cultures using the Zymo Research Fungal/Bacterial DNA Kit™ (Zymo Research, Irvin, CA, USA) or the Invisorb Spin Plant Mini Kit (Invitex, Berlin, Germany). PCR amplification of *CaM* was performed using the primer pairs cmd5 and cmd6 (Hong *et al.* 2006) or CF1L and CF4 (Peterson 2008); *benA* with Bt2a and Bt2b (Donaldson *et al.* 1995) or Ben2f (Hubka & Kolarik 2012) and Bt2b; and *RPB2* with fRPB2-5F and fRPB2-7CR (Liu *et al.* 1999). Samples were amplified using the following cycling parameters: one initial step of 10 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C and a single extension step of 10 min at 72 °C. The PCRs were performed in 25 µL volume containing 2 µL of DNA (2 pM), 2 µL of forward primer, 2 µL of reverse primer, 19 µL of H<sub>2</sub>O, and PuReTaq Ready-To-Go PCR beads (GE Healthcare UK, Little Chalfont, UK). The PCR products were sequenced using the BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) using the same primers used for PCR and analysed on an ABI Prism 3130 genetic analyser (Applied Biosystems) according to the manufacturer's instructions.

Newly obtained DNA sequences were inspected and assembled in Geneious Prime v. 2020.2.4 (Biomatters Ltd.). Sequences were deposited into GenBank with the accession numbers listed in Table 1.

## Phylogenetic analyses and species delimitation

Alignments of *benA*, *CaM* and *RPB2* were performed using the FFT-NS-i option implemented in the MAFFT online service (Kato *et al.* 2019). The alignments were trimmed, concatenated, and then analysed using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) methods. The final alignments are available from the DRYAD digital repository (<https://doi.org/10.5061/dryad.866t1g1td>).

Suitable partitioning schemes and substitution models for the analyses were selected based on the Bayesian information criterion using a greedy strategy implemented in PartitionFinder 2 (Lanfear *et al.* 2017) with settings allowing introns, exons and codon positions to be independent datasets (Supplementary Table S1). ML trees were constructed with IQ-TREE v. 1.4.4 (Nguyen *et al.* 2015) with nodal support determined by ultrafast bootstrapping (BS) with 10<sup>5</sup> replicates. Bayesian posterior probabilities were calculated using MrBayes v. 3.2.6 (Ronquist *et al.* 2012). The analysis ran for 10<sup>7</sup> generations, two parallel runs with four chains each were used, every 1 000<sup>th</sup> tree was retained, and the first 25 % of trees were discarded as burn-in. The convergence of the runs and effective sample sizes were checked in Tracer v. 1.6 (<http://tree.bio.ed.ac.uk/software/tracer>). Maximum parsimony (MP) trees were calculated using PAUP\* v. 4.0b10 (Swofford 2003). Analyses were performed using the heuristic search option with 100 random taxon additions; tree bisection-reconnection (TBR); maxtrees were set to 1 000. Branch support was assessed by bootstrapping with 500 replications.

The rules for the application of the GCPSR approach were adopted from Dettman *et al.* (2003a, b) and slightly modified for the different design of this study (different numbers of loci and methods used). To recognize a clade as an “evolutionary lineage”, it had to satisfy one of two criteria: (a) genealogical concordance - the clade was present in the majority (2/3) of the single-locus genealogies; (b) genealogical nondiscordance - the clade was well supported in at least one single-locus genealogy, as judged by ML ultrafast bootstrap proportions ≥95 %, MP bootstrap proportions ≥70 % and BI posterior probabilities ≥95 %, and was not contradicted in any other single-locus genealogy at the same level of support. When

deciding which evolutionary lineages represent “phylogenetic species”, two additional criteria were applied and evaluated according to the combined phylogeny of three genes: (a) genetic differentiation - species had to be relatively distinct and well differentiated from other species to prevent minor tip clades from being recognized as a separate species; (b) all individuals had to be placed within a phylogenetic species, and no individuals were to be left unclassified.

Species delimitation analyses based on the multispecies coalescent model were performed as described previously (Sklenář *et al.* 2021). In brief, the haplotype function from R v. 4.0.2 (R Core Team 2016) package PEGAS (Paradis 2010) was used to retain only unique sequences in the alignments. The best fitting models were obtained in jModelTest v. 2.1.7. Parameters and the other settings of the STACEY, GMYC, bGMYC, PTP, and bPTP methods were consistent with previous studies (Sklenář *et al.* 2021, Glässnerová *et al.* 2022). Two different settings were used for the GMYC method. The ultrametric input trees for this method were calculated in BEAST v. 2.6.6 (Bouckaert *et al.* 2014) with a chain length of 10<sup>7</sup> generations. As a model for creating input trees, we set up the coalescent constant population prior and performed two tree reconstruction methods: one with the common ancestor height (CAh) setting and a second with the median height (Mh) setting. We only show the delimitation results of both settings when they were different. The results of all analyses are graphically summarised in iTOL v. 6 (Interactive Tree of Life) (Letunic & Bork 2021).

The multilocus species delimitation method STACEY was performed in BEAST (Bouckaert *et al.* 2014) using the STACEY v. 1.2.5 add-on (Jones 2017). For this and the DELINEATE analysis (see below), we added several additional species from other series of section *Nigri* (Table 1). We set up the length of MCMC chain to 10<sup>9</sup> generations, the species tree prior was set to the Yule model, the molecular clock model was set to a strict clock, the growth rate prior was set to a lognormal distribution (M = 5, S = 2), the clock rate priors for all loci were set to a lognormal distribution (M = 0, S = 1), the PopPriorScale prior was set to a lognormal distribution (M = -7, S = 2) and the relative DeathRate prior was set to a beta distribution ( $\alpha = 1$ ,  $\beta = 1\ 000$ ). The substitution models were as follows: *benA* - K80+I; *CaM* - TrNef+G; *RPB2* - TrNef+G. The output was processed with SpeciesDelimitationAnalyzer (Jones *et al.* 2015). For the presentation of the STACEY results, we first created a plot showing how the number of delimited species and the probability of the most likely scenarios changed in relation to the value of the *collapseheight* parameter, and then we created similarity matrices using code from Jones *et al.* (2015) with different values of *collapseheight* chosen from the plot (see the Results section).

Independent testing of various species boundary hypotheses was performed in the DELINEATE software (Sukumaran *et al.* 2021). After splitting the dataset into hypothetical populations (Supplementary Table S2) using the “A10” analysis in BPP v. 4.4 (Yang 2015), the species tree for these populations was estimated in starBEAST (Heled & Drummond 2010) implemented in BEAST v. 2.6.7 (Bouckaert *et al.* 2014). The analysis was run in the Python (van Rossum & Drake Jr FL 2014) package DELINEATE (Sukumaran *et al.* 2021). In every simulated model, some populations delimited by BPP were assigned to tentative (fixedly defined) species based on the species delimitation results from previous analyses or were based on the common species definitions from previous studies. Other populations were left free to be delimited (unassigned to any species). As a result, the algorithm implemented in DELINEATE can either recognize the unassigned population as a separate

species, define a wider monophyletic species composed of several unassigned populations, or merge unassigned populations with some of the predefined species.

## Whole genome sequencing and assembly

Whole-genome 100 bp paired-end (PE) sequencing was performed in 18 strains with the aid of HiSeq 1500 (Illumina, San Diego, CA) using the HiSeq reagent kit v. 1, according to the manufacturer's instructions. A 300 bp PE sequencing was performed on a MiSeq (Illumina) using the MiSeq Reagent Kit v. 3, according to the manufacturer's instructions. Long read sequencing of IFM 61612 was performed on the MinION platform (ONT; Oxford Nanopore Technologies, Cambridge, UK). A DNA library for ONT sequencing was prepared using a short-read eliminator kit (Nippon Genetics, Tokyo, Japan) and a ligation sequencing kit (SQK-LSK109), and sequencing was performed with a FLO-MIN106D flow cell (R9.4.1) for 48 h, according to the manufacturer's instructions. A total of 83 443 ONT reads were generated with a mean length of 12 011 bp using ONT Guppy v. 2.3.5.

Adapters and low-quality bases of Illumina reads were trimmed by Trim Galore v. 0.6.4 (Krueger 2015) with the default settings selected ([http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore)). The mitochondrial genomes were assembled using GetOrganelle v. 1.6.4 (Jin *et al.* 2020) with trimmed reads. To filter the mitochondrial reads, the trimmed reads were aligned against the mitochondrial genomes by BWA v. 0.7.17-r1188 (Li & Durbin 2009), and the mapped reads were filtered by SAMtools v. 1.9 (Li *et al.* 2009) and SeqKit (Shen *et al.* 2016). For eight isolates sequenced by MiSeq, the filtered reads were used to assemble the nuclear genomes using SPAdes v. 3.14.0 (Bankevich *et al.* 2012) with the options '--cov-cutoff auto' and '--careful'. For six isolates sequenced by HiSeq 1500, the assembly of nuclear genomes was carried out as follows: (1) the filtered reads were used to assemble the contigs using VelvetOptimiser v. 2.2.6 (Zerbino & Birney 2008); (2) the contigs were used to generate *in silico* long mate-pair reads with inserts of  $3\ 000 \pm 300$  bp using Wgsim v. 0.3.1-r13 (<https://github.com/lh3/wgsim>) with the options '-e 0 -1 100 -2 100 -r 0 -R 0 -X 0 -d 3000 -s 300 -N 4000000'; (3) Illumina PE and simulated mate-pair (MP) reads were assembled by ALLPATHS-LG v. 52488 (Gnerre *et al.* 2011). The genome of IFM 61612 was assembled using NECAT v. 0.0.1\_update20200803 (Chen *et al.* 2021), and polished with the ONT reads by using Racon v. 1.4.20 (Vaser *et al.* 2017) and Medaka v. 1.4.4 (<https://github.com/nanoporetech/medaka>) and with Illumina PE reads by using HyPo v. 1 (Kundu *et al.* 2019). Most tools were obtained through Bioconda (Grüning *et al.* 2018).

## Genome annotation

The annotation of assembled genomes was performed using the Funannotate pipeline v. 1.7.4 (<https://funannotate.readthedocs.io/en/latest/>). Following the identification of repeat sequences by RepeatModeler v. 1.0.11 (<http://www.repeatmasker.org/RepeatModeler.html>) and RepeatMasker v. 4.0.7 (<https://www.repeatmasker.org>), Funannotate *ab initio* prediction was performed with the option '--busco\_seed\_species=aspergillus\_oryzae' by Augustus v. 3.3.3 (Stanke *et al.* 2006), GeneMark-ES v. 4.38 (Ter-Hovhannisyan *et al.* 2008), GlimmerHMM v. 3.0.4 (Majoros *et al.* 2004), and SNAP v. 2006-07-28 (Korf 2004) using exon hints from the proteins of *A. niger* CBS 513.88 and *A. oryzae* RIB40 downloaded from the Aspergillus Genome Database (Bairoch & Apweiler 2000, Cerqueira *et al.* 2014). The functional annotations

of predicted genes were performed using the Swiss-Prot, InterPro v. 5.42-78.0 (Jones *et al.* 2014), eggNOG v. 4.5.1 (Buchfink *et al.* 2015, Huerta-Cepas *et al.* 2016), MEROPS v. 12.0 (Rawlings *et al.* 2018, Mitchell *et al.* 2019), and the dbCAN v. 8.0 (Yin *et al.* 2012) databases. Genome annotations of the reference strains were obtained from the Joint Genome Institute (<http://genome.jgi.doe.gov/>) (Supplementary Table S3). The completeness of the draft genomes and predicted proteins were evaluated using BUSCO v. 4.0.6 (Seppey *et al.* 2019) with the database "euotiales\_odb10".

## Phylogenetic analyses based on the genomic sequences

Orthologous genes among the 31 strains (18 newly sequenced and 13 available from previous studies) were identified by OrthoFinder v. 2.3.12 (Emms & Kelly 2019). Then, the amino acid sequences of 5 752 single-copy orthologous proteins were aligned using the G-INS-i option in MAFFT v. 7.471 and concatenated into one long protein sequence. A phylogenetic tree was constructed using multithreaded RAxML (Stamatakis 2014), selecting the PROTGAMMAWAG model and using 100 bootstrap replicates to determine node support.

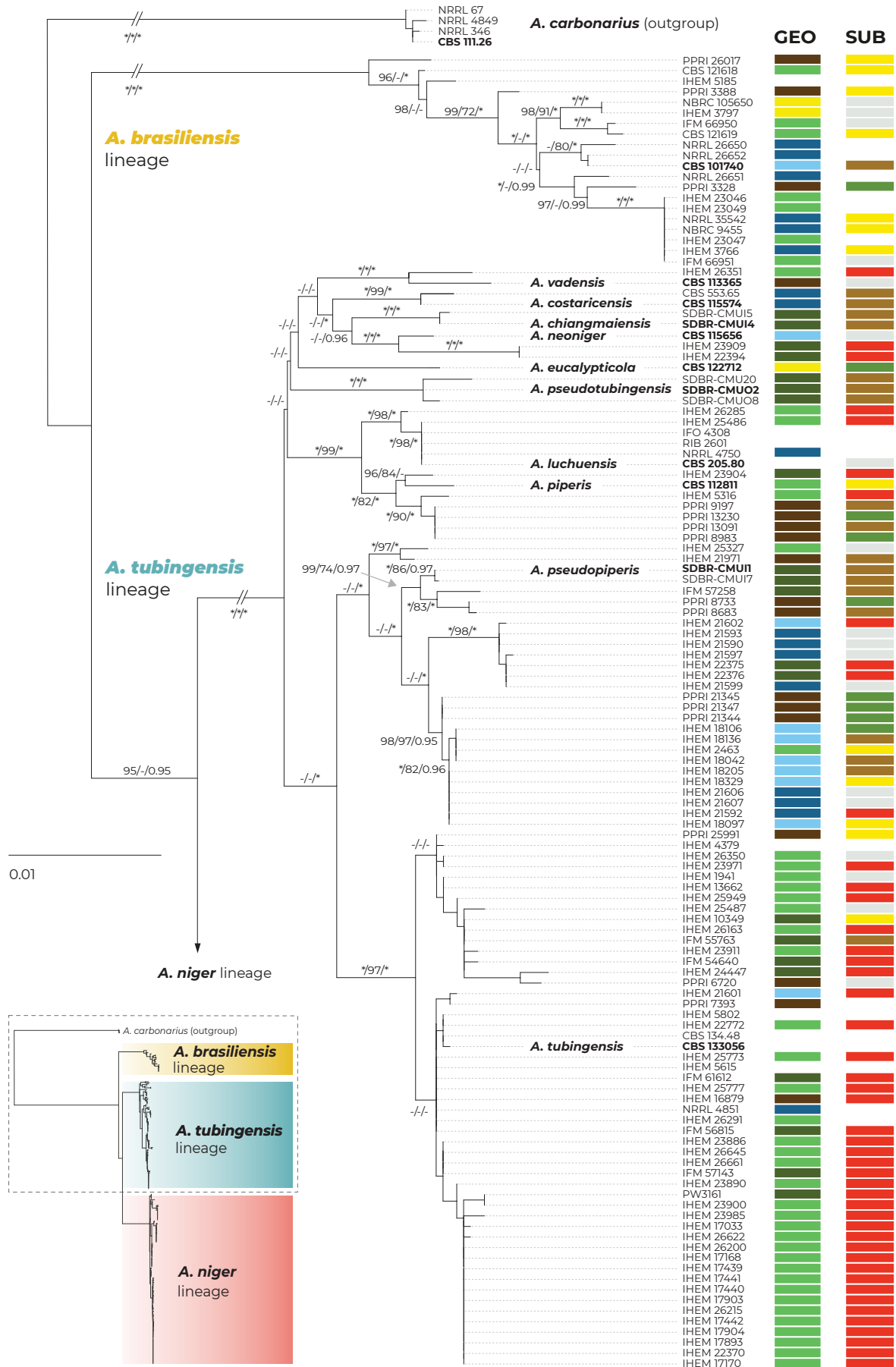
The predicted protein sets of 31 strains were evaluated with BUSCO protein mode. A total of 2 754 genes present at a single copy were identified among all strains. From these, 200 genes were randomly selected for analyses using the STACEY method and randomly distributed into ten independent analyses, each containing 20 genes (Supplementary Table S4). Coding regions (exons) of the corresponding 200 nucleotide sequences were extracted for each strain from the *de novo* assemblies. Each sequence matrix was aligned using MAFFT, and well-aligned regions were extracted using Gblocks v. 0.91b (Talavera & Castresana 2007) with settings allowing no gap positions.

## RESULTS

### Combined phylogeny based on three loci

A maximum likelihood (ML) phylogenetic tree based on three loci in 280 strains (276 belonging to the series *Nigri* and four *A. carbonarius* strains used as outgroups) is presented in Fig. 1, with the geographic origin and substrate plotted on the tree in the form of colour strips. The topology of the trees inferred by the maximum parsimony (MP) and Bayesian inference (BI) methods was similar to ML and the bootstrap support values and posterior probabilities, respectively, were appended to the nodes.

There are three main lineages in the tree and for practical reasons, we named them based on the priority rules. The *A. brasiliensis* lineage (ABL) only contains the ex-type isolate of *A. brasiliensis*, while the *A. niger* lineage (ANL) contains the ex-type isolates of *A. lacticoffeatus*, *A. niger*, *A. vinaceus* and *A. welwitschiae* (the old species names, which are usually considered synonyms, are omitted). The *Aspergillus tubingensis* lineage (ATL) contains ex-types of ten species: *A. chiangmaiensis*, *A. costaricensis*, *A. eucalypticola*, *A. luchuensis*, *A. neoniger*, *A. piperis*, *A. pseudopiperis*, *A. pseudotubingensis*, *A. tubingensis* and *A. vadensis*. The sequences of the three recently described species, *A. chiangmaiensis*, *A. pseudopiperis* and *A. pseudotubingensis* (Khuna *et al.* 2021), were of poor quality and contained a large number of errors (indels) in the coding regions and probably also in the noncoding regions of all three genes. Due to this fact and the



**Fig. 1.** Multilocus phylogeny of *Aspergillus* series *Nigri* based on three loci (*benA*, *CaM*, *RPB2*) and 276 isolates (and four *A. carbonarius* isolates as an outgroup). The best-scoring maximum likelihood (ML) tree inferred in the IQ-TREE is shown; ultrafast bootstrap support values (ML bs) are appended to nodes along with maximum parsimony bootstrap support values (MP bs) and Bayesian inference posterior probabilities (BI pp); only support values  $\geq 95\%$ ,  $\geq 70\%$  and  $\geq 0.95$ , respectively, are shown; a dash indicates lower statistical support for a specific node or the absence of a node in the phylogeny while an asterisk indicates full support; the ex-type strains are designated with a bold print; the information on geographic origin and isolation source is plotted on the tree (see legend). Alignment characteristics, partitioning schemes and substitution models are listed in Supplementary Table S1.



absence of genomic sequences, we excluded these species from most analyses. However, we manually corrected some apparent errors in the exonic regions (partially revised sequences can be found in the alignments deposited in the Dryad digital repository: <https://doi.org/10.5061/dryad.866t1g1td>) and added these species to the classical phylogenetic trees based on the ML, MP and BI methods. In these phylogenies, the positions of *A. chiangmaiensis* and *A. pseudotubingensis* differ significantly from the tree presented by Khuna *et al.* (2021). The combined phylogeny without these species is shown in Supplementary Fig. S1.

The bootstrap support values in the combined trees (Fig. 1 and Supplementary Fig. S1) are high on many terminal branches in ATL, and the majority of the currently accepted species form their own well-supported terminal clades. On the other hand, some deeper nodes are poorly supported and the relationships among the species in ATL are mostly unresolved. If only the monophyly and statistical support of branches in these combined phylogenetic trees were considered, all currently recognized species in ATL could be accepted. Bootstrap values in the trees based on concatenated datasets are, however, often falsely high and are not a suitable criterion for deciding about species limits (Kubatko & Degnan 2007, Seo 2008). The boundaries between currently accepted species in ANL are much less clear, as there are several poorly supported clades in the proximity of *A. vinaceus*, *A. niger* and *A. welwitschiae* whose species identification is unclear.

The geography and substrate of isolation showed no clear patterns that could be associated with particular species, although clinical isolates from European countries (mostly strains from the study of D'hooge *et al.* (2019)) were overrepresented in the clade containing the *A. tubingensis* ex-type strain CBS 133056, while isolates from food and South America were more common throughout ANL (strains predominantly from Silva *et al.* (2020)).

### Incongruences between single gene trees: consequences for the GCPSR approach and BLAST similarity searches

Single-gene trees based on *benA*, *CaM* and *RPB2* loci are shown in Fig. 2 and Supplementary Fig. S2 (only one isolate per unique multilocus haplotype was included). The coloured connecting lines show changes in the positions of isolates between single-gene trees (Fig. 2); the branches were rotated so that the trees maximally correspond to each other; the trees without connecting lines are shown in Supplementary Fig. S2. There was no conflict in topology among the three main lineages, *i.e.*, there was no isolate changing position between ABL, ANL and ATL in different single-gene trees. When applying the rules of the GCPSR approach in ABL and ANL, only one phylogenetic species (PS) can be recognized in each lineage because the composition of their subclades is variable in terms of the isolates resolved in them, while the statistical support of the subclades is low or unstable between phylogenies. A more complicated situation is present in the ATL, where some clades can be recognized as evolutionary lineages, as they are well supported in at least two phylogenetic trees. For example, the clade containing the two strains of *A. vadensis* (CBS 113365 and IHEM 26351) was present and supported in all phylogenies, while the clade containing seven strains attributed to *A. luchuensis*/*A. piperis* was present and supported in the *benA* and *CaM* trees. The singleton lineage containing the ex-type of *A. eucalypticola* (CBS 122712) was also present in all trees, but it cannot be evaluated by the GCPSR criteria as there are no isolates clustering with this strain. Although

the abovementioned evolutionary lineages were recognized by the grouping criteria, they cannot be recognized as PS because doing so would have resulted in a situation where other strains belonging to the ATL and forming several nonmonophyletic clades in the combined tree (Fig. 1) could not be assigned to any phylogenetic species and were left unclassified. As a result, applying GCPSR criteria in the ATL would also lead to the recognition of only one phylogenetic species.

To show the practical consequences of the incongruences between single-gene datasets, we created a local BLAST database containing sequences of *benA*, *CaM* and *RPB2* of the ex-type strains belonging to the series *Nigri*. Then, we performed BLAST searches for all sequences of these genes against this database (only one isolate per unique multilocus haplotype was used) in Geneious PRIME v. 2020.2.4 (<https://www.geneious.com>). The results of the BLAST searches and the closest similarities to the ex-type strains are presented in Fig. 3. It is apparent that sequences of different genes from the same strain frequently resulted in the closest sequence similarities with ex-type strains of different species and thus resulted in variable species identifications. This phenomenon does not involve *A. brasiliensis*.

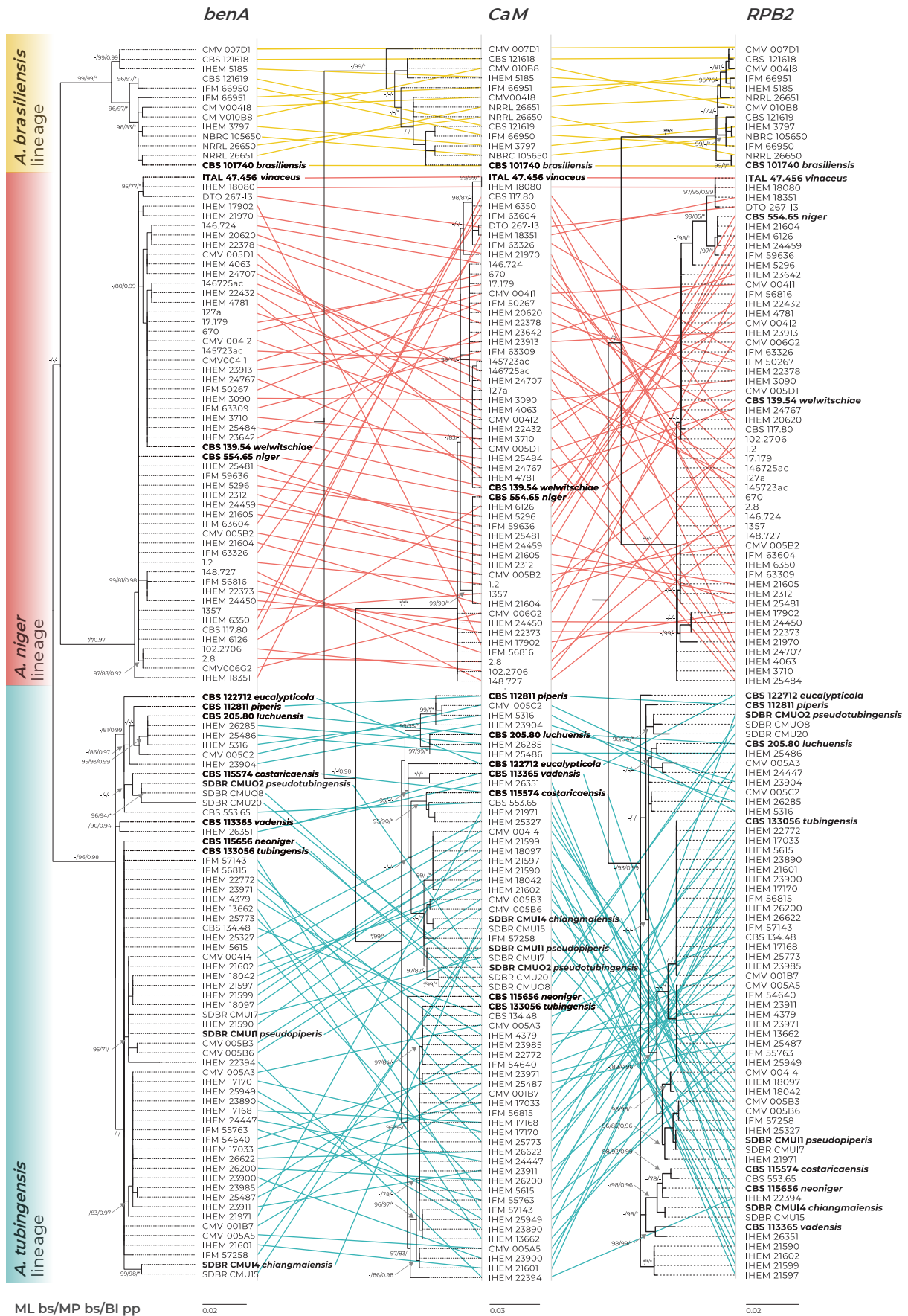
In the ANL, different species identifications commonly resulted for different loci, which was observed in 21 out of 53 haplotypes (39.6 %). In seven haplotypes (13 %; CBS 117.80, IFM 63326, IFM 63604, IHEM 6350, 148.727, 2.8 and 102.2706), the three BLAST searches resulted in three different identifications, *i.e.*, *A. niger*, *A. vinaceus* and *A. welwitschiae*. This demonstrates that there are phylogenetic conflicts throughout the entire clade and that there is no barcode gap for these species at any of these loci. These incongruences are also visible in the single-gene phylogenetic trees shown in (Fig. 2, Supplementary Fig. S2).

In ATL, unstable identification based on different genes was less prevalent than in ANL, but was still present in 14 out of 54 haplotypes (24 %). It occurred mostly between *A. tubingensis*, *A. costaricensis*, *A. neoniger*, *A. pseudopiperis*, *A. luchuensis* and *A. piperis*. Four haplotypes (7 %; IHEM 23904, IHEM 25327, IHEM 21971 and IFM 57258) were identified as a different species for each gene region. This phenomenon also has its basis in the single-gene tree incongruences (Fig. 2, Supplementary Fig. S2).

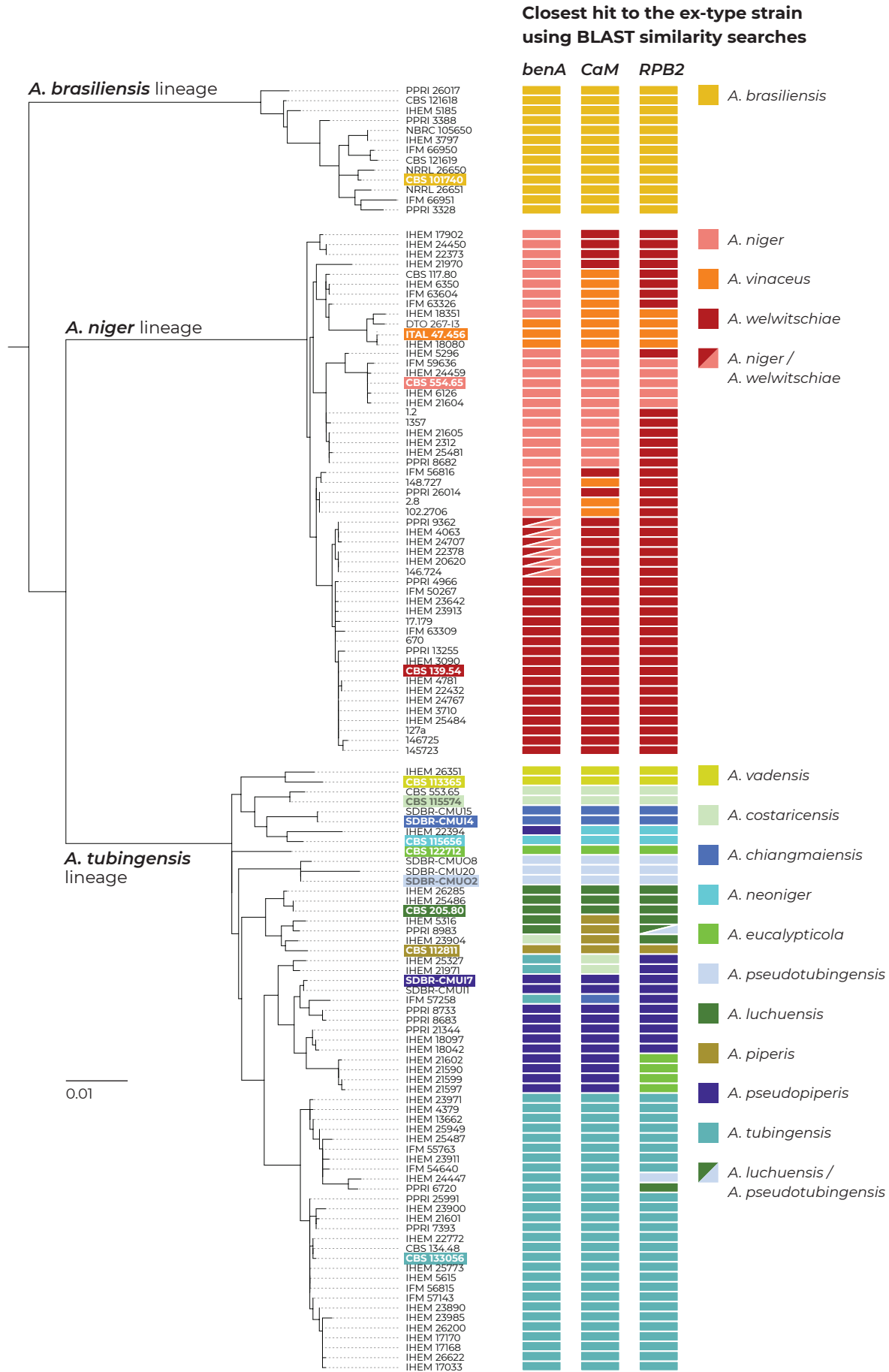
### Phylogenomic tree

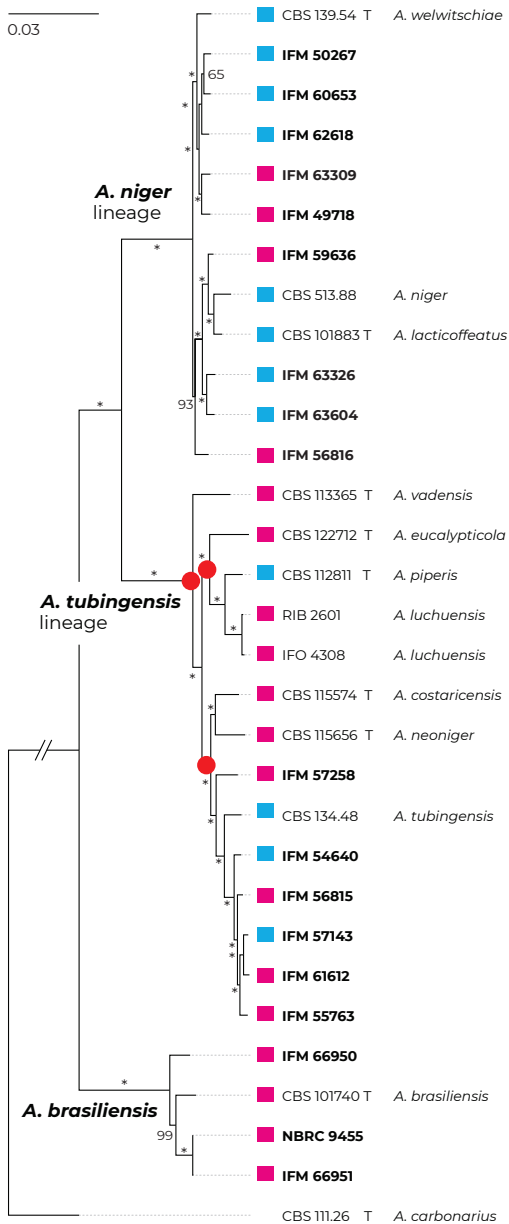
In Fig. 4, the topology of the phylogenomic tree was compared to the topology of the three-locus ML tree (alignment reduced to unique multilocus haplotypes; *A. chiangmaiensis*, *A. pseudopiperis* and *A. pseudotubingensis* were excluded; list of haplotypes in Supplementary Table S5) and to the topology of the species tree constructed in starBEAST. The phylogenomic tree was based on 5 752 protein orthologues extracted from 30 whole-genome sequences of series *Nigri* and one sequence of *A. carbonarius* as an outgroup. Nine out of 18 newly sequenced genomes (Supplementary Table S3; designated by bold print in Fig. 4) belonged to ANL, six to ATL and three to ABL. Almost all clades in the phylogenomic tree gained full statistical support, in contrast to the trees based on the *benA*, *CaM* and *RPB2* loci. The topologies among the different analyses were mostly congruent, but several incongruences were observed (most of them were present at unresolved nodes with low statistical support). For example, *A. vadensis* was resolved as a separate branch sister to all other species in the ATL in the phylogenomic and species tree but clustered with *A. costaricensis* and *A. neoniger* with a high support of 95 % in the ML tree. In the phylogenomic and species tree, *A. neoniger* and *A. costaricensis*





**Fig. 2.** Comparison of single-gene genealogies based on the *benA*, *CaM* and *RPB2* loci and created by three different phylogenetic methods (only one isolate per unique multilocus haplotype is included in each phylogeny). The coloured connecting lines show changes in the positions of isolates between single-gene trees (the branches were rotated so that the trees maximally correspond to each other). Best-scoring single-locus maximum likelihood (ML) trees are shown; ML ultrafast bootstrap support values (ML bs), maximum parsimony bootstrap support values (MP bs) and Bayesian inference posterior probabilities (BI pp) are appended to nodes. Only support values  $\geq 95\%$ ,  $\geq 70\%$  and  $\geq 0.95$ , respectively, are shown. A dash indicates lower statistical support for a specific node, or the absence of a node in the phylogeny, while an asterisk indicates full support. The x-type strains are designated with a bold print. Alignment characteristics, partitioning schemes and substitution models are listed in Supplementary Table S1.

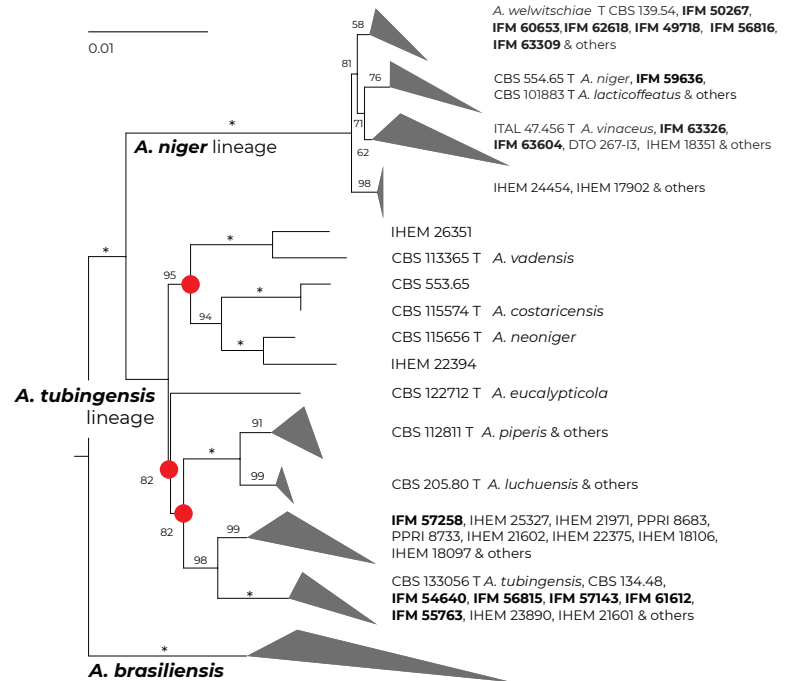
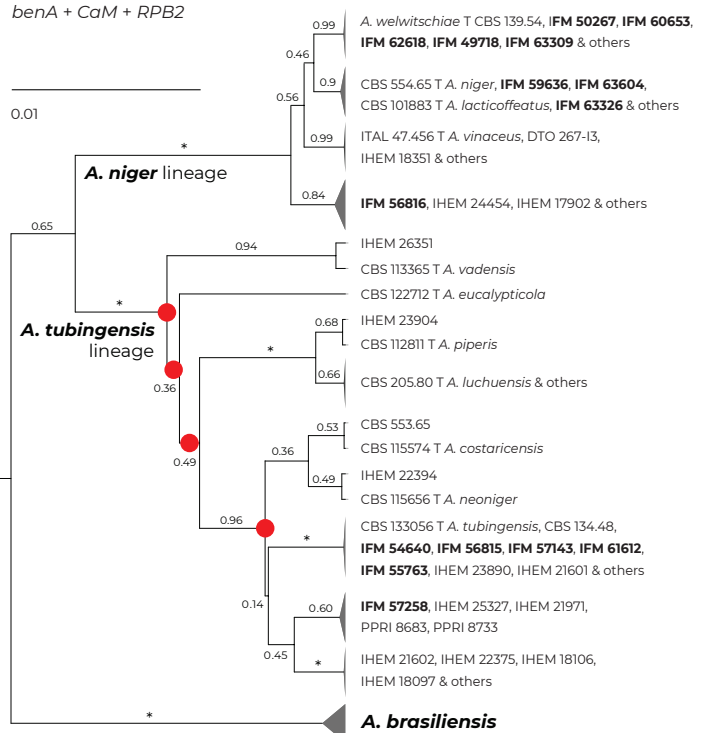


**Maximum likelihood**5 752 single copy  
orthologous proteins

● Main topological incongruences  
between different phylogenies

**MAT gene idiomorph**

■ *MAT1-1*  
■ *MAT1-2-1*

**Maximum likelihood***benA* + *CaM* + *RPB2***Species tree***benA* + *CaM* + *RPB2*

**Fig. 4.** Comparison of topologies of phylogenetic trees constructed on the basis of different methods and data. Incongruences between phylogenies are designated with red circles. Maximum likelihood (ML) tree constructed based on the 5 752 orthologous proteins extracted from the 31 whole genome sequences (WGS; Supplementary Table S3) (left side); ML tree based on *benA*, *CaM* and *RPB2* loci estimated in IQ-TREE (top right); species tree based on *benA*, *CaM* and *RPB2* loci estimated in starBEAST (bottom right); the last two mentioned trees were constructed from combined three-gene alignments reduced to unique multilocus haplotypes (Table S5), and *A. chiangmaiensis*, *A. pseudopiperis* and *A. pseudotubingensis* were excluded. The most significant differences can be observed in the positions of *A. neoniger*, *A. costaricensis* and *A. eucalypticola*. Bootstrap support values or posterior probabilities are appended to the nodes, the support values equal to 100 % and 1.00, respectively, are designated with an asterisk; the ex-type strains are designated with the letter "T". Isolates for which whole genomic sequences were generated in this study are designated with a bold print; mating-type gene idiomorphs are plotted on the tree based on the WGS data.

clustered with *A. tubingensis* isolates, in contrast to the ML tree, where they clustered distantly from the latter. Last, *A. eucalypticola* clusters with *A. luchuensis* and *A. piperis* in the phylogenomic tree, while in ML and species trees, it formed a long separate branch.

Both *MAT1-2-1* and *MAT1-1-1* mating-type gene idiomorphs were found in the genomes of strains belonging to the ANL and ATL, while only the *MAT1-2-1* idiomorph was found in the *A. brasiliensis* strains (Fig. 4).

## Multispecies coalescent model-based (MSC) methods

The results of the species delimitation using four single-locus species delimitation methods (GMYC, bGMYC, PTP and bPTP) and one multilocus method, STACEY, are summarised in Fig. 5. For the GMYC and STACEY methods, we showed the results of two different settings, as they differed in the number of delimited species.

All methods and their settings delimited the entire ANL as a single species without any exception. The method STACEY and all single-locus methods except for three delimited only one species in the *A. brasiliensis* lineage. Three methods (GMYC with a common ancestor height (CAh) setting; bPTP based on *benA*, and bPTP based on *CaM*) recognized isolate PPRI 26017 as a singleton species separated from *A. brasiliensis*. All single-locus methods except for two unequivocally proposed that ATL should be considered a single broad species. The first exception was the GMYC method based on *benA* with a CAh setting that divided the ATL into three species: *A. vadensis* and another two species that are not monophyletic in any multigene phylogeny that are shown in Figs 1 and 3. The second was the bPTP method based on *CaM*, which divided ATL into eight species corresponding to *A. vadensis*, *A. eucalypticola*, *A. tubingensis*, a species comprising both *A. piperis* and *A. luchuensis*, and four additional species whose delimitation has no support in the multigene phylogeny as they are not monophyletic (Figs 1 and 3).

The multilocus method STACEY was performed using two different datasets. The first dataset contained unique multilocus haplotypes from the *Nigri* series and the second dataset was supplemented with eight additional species outside the *Nigri* series. The results of both analyses were identical and supported the delimitation of one or four species in the ATL depending on the value of the *collapseheight* parameter. Detailed results of STACEY based on the second dataset are presented in Fig. 6, where subfigure A illustrates the effect of the *collapseheight* parameter value on the number of delimited species. On the y-axis on the left side, there is the number of delimited species with the given *collapseheight* value (black line). The support for the most likely scenario (yellow line), and the support for the second most likely scenario (blue line) are shown; other less supported scenarios were omitted. The vertical dashed lines in subfigure A represent the scenarios illustrated in detail in Fig. 6B and Fig. 6C in the form of similarity matrices showing posterior probabilities of each pair of isolates being included in the same species. There are three main scenarios that gained reasonable support and which delimited 15 (*collapseheight* = ~0.004–0.006), 14 (*collapseheight* = ~0.006–0.008) and 11 species (*collapseheight* = ~0.009 and higher) (Fig. 6), corresponding to 3 or 7 species in the series *Nigri*. In all three scenarios, the ANL and ABL were always resolved as single species. At *collapseheight* of ~0.005, four species were delimited in the ATL: (1) *A. costaricensis* + *A. neoniger* + *A. tubingensis*; (2) *A. luchuensis* + *A. piperis*; (3) *A. eucalypticola*; and (4) *A. vadensis*. When the *collapseheight* increases and reaches values in the

range 0.006–0.008, the support for the delimitation of multiple species in the ATL decreases, but four species are still supported and not lumped together. The only difference in the scenario with 14 species compared to that with 15 species is outside series *Nigri*, namely, *A. aculeatus* is delimited as one or two species, respectively. In the scenario with 11 species and a *collapseheight* of approximately 0.009 and higher, the whole *A. tubingensis* lineage is delimited as a single species.

## Species delimitation using DELINEATE

The species hypotheses were independently tested in DELINEATE software, where we set up 10 different models. These models were divided into three categories based on the broadness of the fixed species predefined within the ANL and ATL: narrow (Models 1–4), medium (Models 5–7) and broad (Models 8–10). The results are summarised in Fig. 7, where every column represents one model with individual populations either assigned into species (grey bars = predefined species), or left unassigned and free to be delimited (brown bars). The red frames show the resulting solution proposed by DELINEATE for every model, *i.e.*, an unassigned population either recognized as a separate species, merged with other unassigned population(s), or merged with some predefined species.

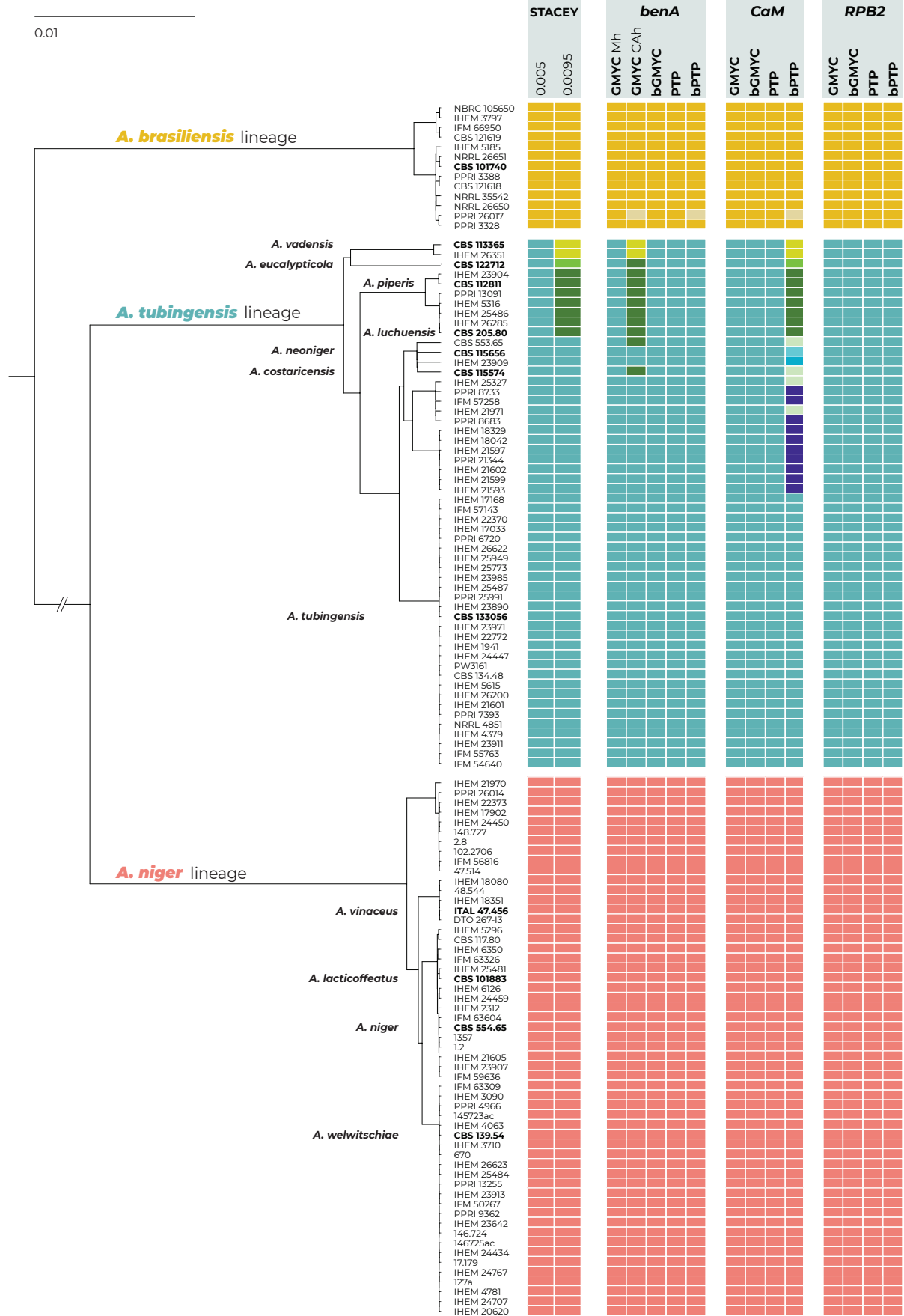
Models 1, 5 and 8 focused on delimitation within the ABL, where four populations were recognized by BPP software. In all three models, these populations were left unassigned, and they were always lumped to form a single broad species regardless of whether the species in the ANL and ATL were predefined as narrow or broad.

Models 2, 6 and 9 focused on the ANL, in which all populations were left free to be delimited, while the populations of the ATL were segregated into eight narrow species (Model 2), four broader species (Model 6) and one broad species (Model 10). In all of these settings, the ANL was always recognized as a single broad species regardless of the species number and species limits in the ATL.

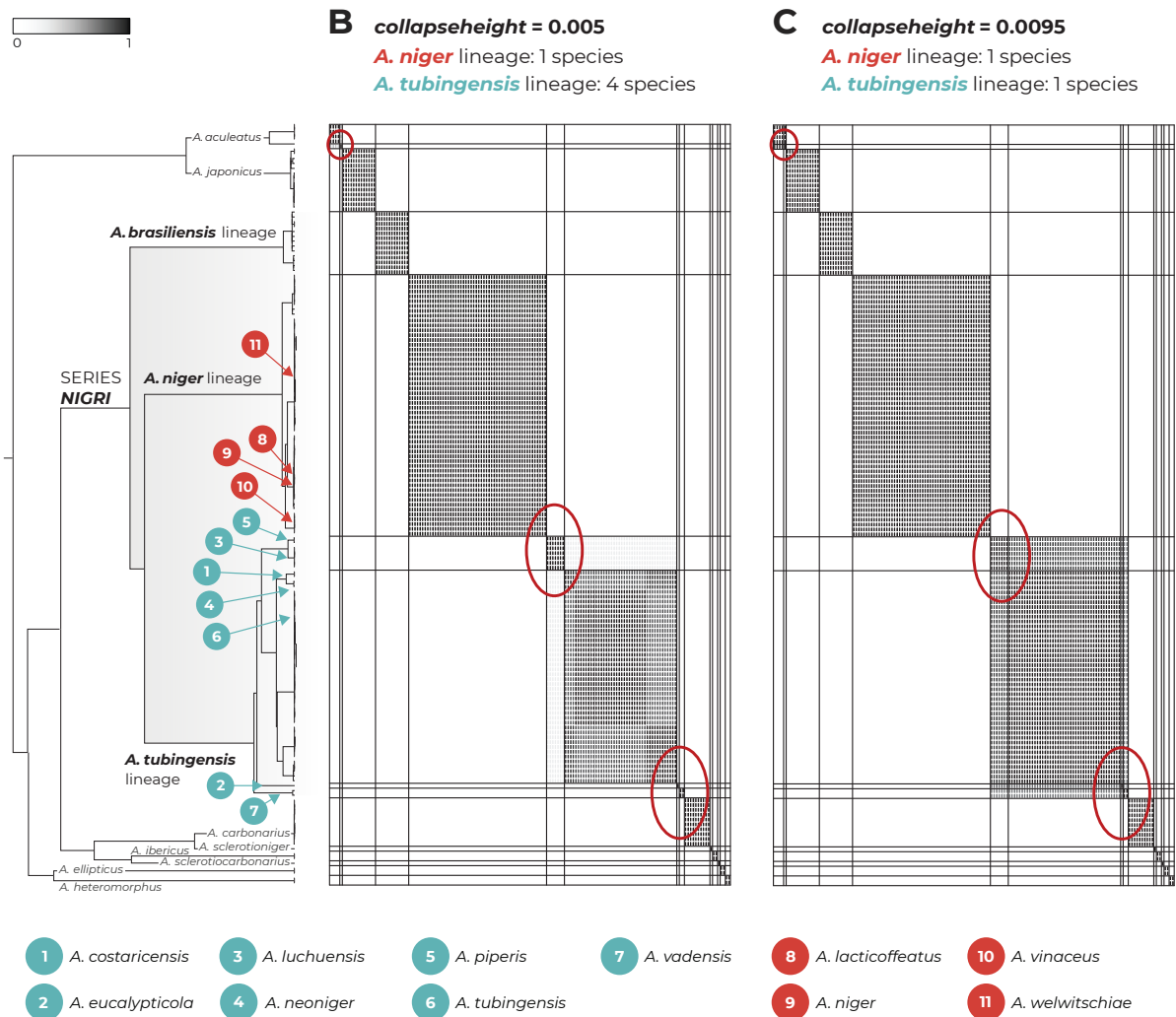
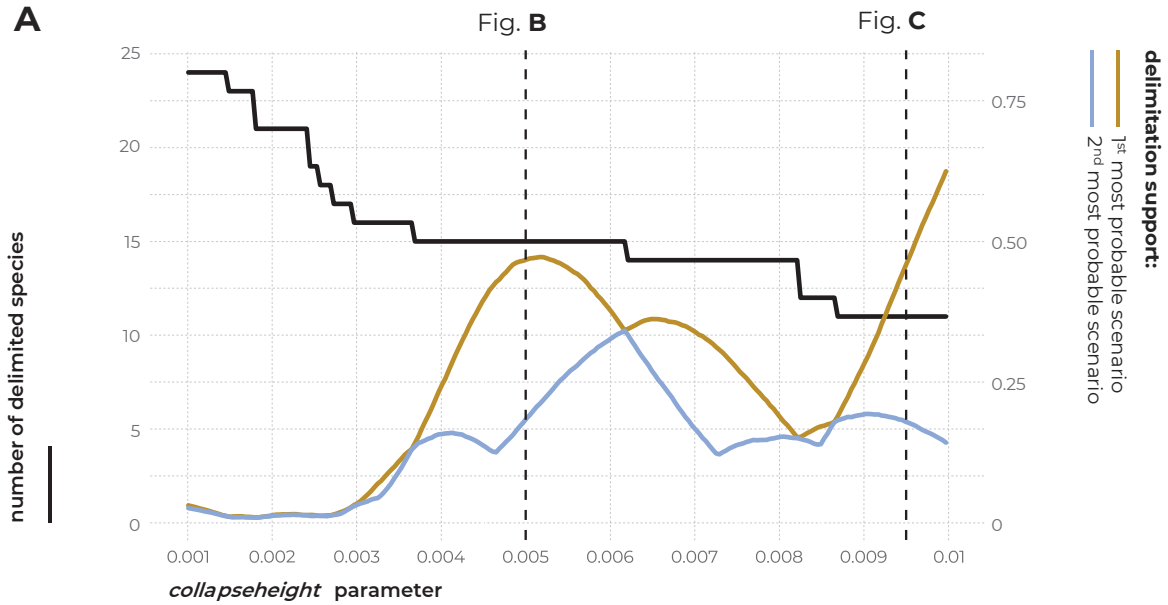
More variable delimitation results were obtained in the ATL. Models 3 and 4 simulated situations where *A. niger* populations (pop1 to pop8) were segregated into four and six species, respectively. In these cases, the ATL populations (pop1 to pop13) were arranged into four species as follows: (1) *A. piperis* + *A. luchuensis*; (2) *A. tubingensis* + *A. costaricensis* + *A. neoniger*; (3) *A. eucalypticola*; and (4) *A. vadensis*. In Models 7 and 10, the populations of the ANL were predefined into two and single species, respectively. In both cases, the ATL was delimited as a single species.

## STACEY analysis using genomic data

To analyse species limits using MSC methods on the genome scale, we randomly selected 200 single-copy orthologous genes from the genomes. The alignments of these genes were randomly distributed into ten datasets each containing 20 genes that were analysed separately (Supplementary Table S4). The results are summarized in Fig. 8. The upper part of the figure shows the course of each analysis using different colours and the dependence of the delimitation results on the changing value of the *collapseheight* parameter. For every analysis, there are two curves, the first representing the number of delimited species (left y-axis) and the second representing the probability of the most likely species delimitation scenario at a specific *collapseheight* value (ranging from 0 to 1, right y-axis).

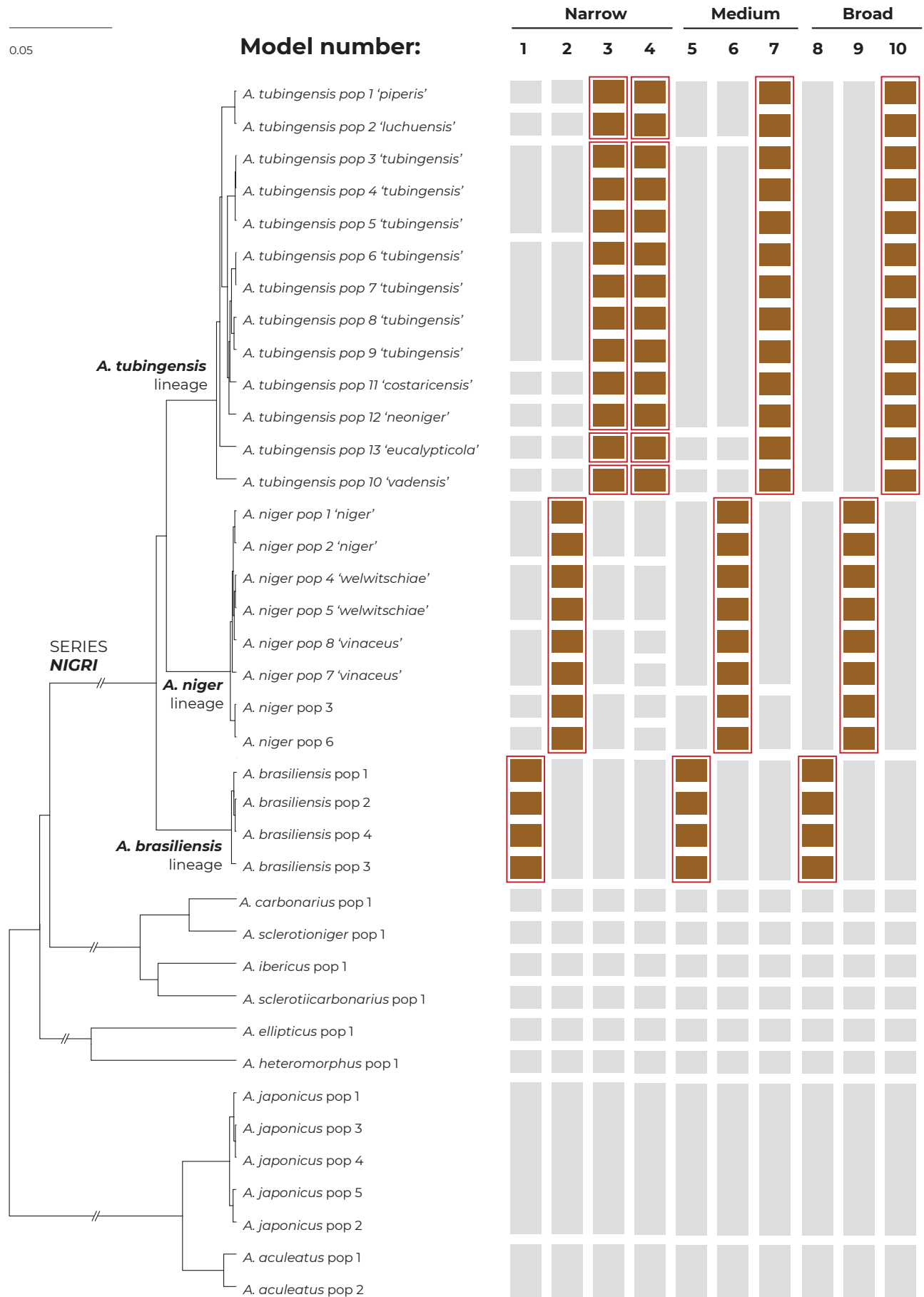


**Fig. 5.** Schematic representation of the results of species delimitation methods in the series *Nigri*. One multilocus method (STACEY) and four single-locus methods (GMYC, bGMYC, PTP, bPTP) were applied to a dataset of three loci (*benA*, *CaM*, *RPB2*). The results are depicted by coloured bars with different colours or shades indicating species delimited by specific methods and settings. Ex-type isolates are highlighted with a bold print. The STACEY results with two *collapseheight* parameter values, 0.005 and 0.0095, are shown. For the GMYC method, the coalescent constant population tree model was used as an input with both common ancestor heights (CAh) and median height (Mh) settings (both settings are only shown when they produced different delimitation results). The phylogenetic tree was calculated by STACEY analysis and is used solely for the comprehensive presentation of the results from different methods.



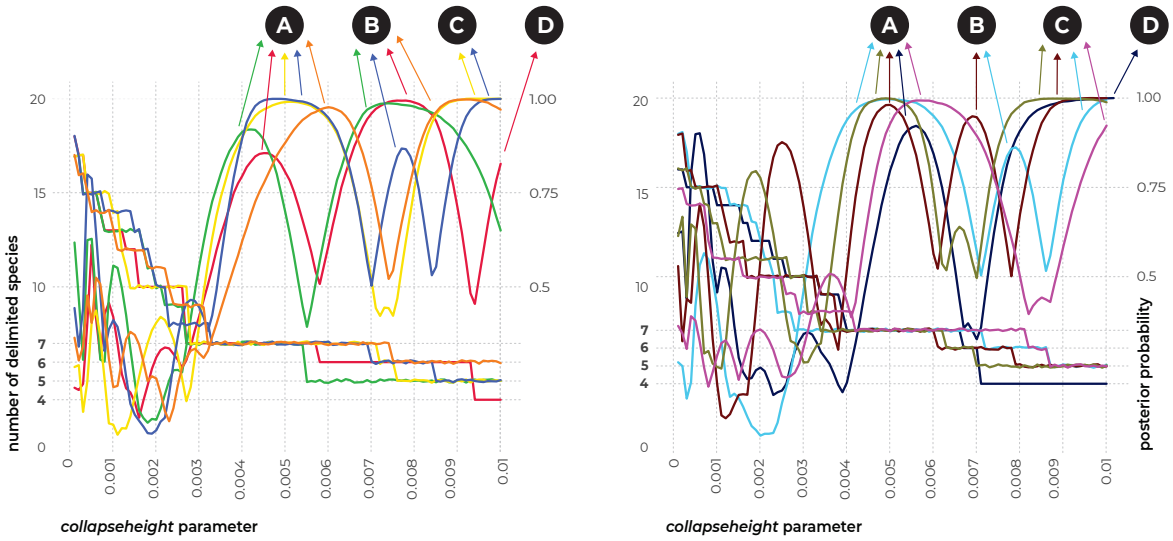
**Fig. 6.** The results of species delimitation by using the STACEY method based on the three loci: *benA*, *CaM* and *RPB2*. (A) Dependence of the delimitation results on the *collapseheight* parameter. The black solid line represents the number of delimited species (left y-axis) depending on the changing value of the *collapseheight* parameter (x-axis). The yellow line represents the probability (range from 0 to 1, right y-axis) of the most likely scenario at specific *collapseheight* value. The blue line represents the probability of the second most probable scenario at a specific *collapseheight* value; curves representing other less probable scenarios are omitted. Dashed vertical lines mark two values of the *collapseheight* parameter (0.005 and 0.0095) whose results are shown in detail by similarity matrices in subfigures B and C. The similarity matrices give the posterior probability of every two isolates belonging to the same multispecies coalescent cluster (tentative species). The darkest black shade corresponds to a posterior probability of 1, while the white colour is equal to 0. Thicker horizontal and vertical lines in the similarity matrices delimit species or their populations that gained delimitation support in some scenarios.

0.05

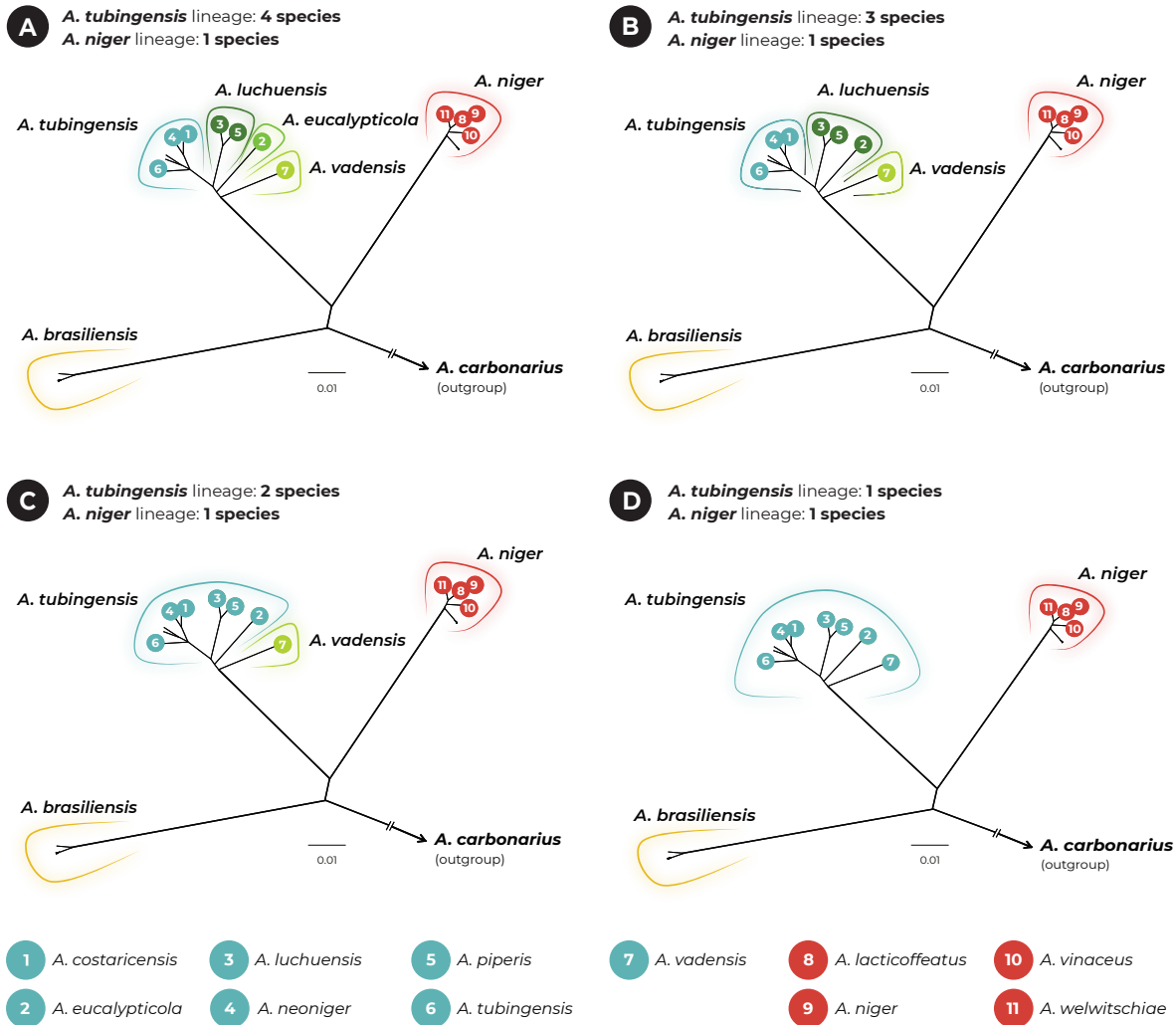


**Fig. 7.** Species delimitation using DELINEATE software. In total, ten models of species boundaries were set up and tested. The brown bars represent unassigned populations left free to be delimited, while the grey bars represent the predefined species. The resulting solutions suggested by DELINEATE are depicted by red frames around the bars. The populations were delimited by using BPP software; isolates belonging to each population are listed in Supplementary Table S2. The displayed tree was calculated in starBEAST based on the *benA*, *CaM* and *RPB2* loci and is only used for the comprehensive presentation of the results from different models.

STACEY BASED ON 20 GENES: analysis No. 1 2 3 4 5 6 7 8 9 10



TAXONOMIC SOLUTIONS PROPOSED BY STACEY ANALYSES



**Fig. 8.** Species delimitation results from ten independent STACEY analyses, each based on 20 genes randomly selected from genomes of series *Nigri* species ( $n = 30$ ; plus one genome of *A. carbonarius* as the outgroup). The upper part of the figure shows the dependence of the delimitation results on the changing value of the *collapseheight* parameter; each analysis is designated with a different colour. There are two curves for each analysis, the first representing the number of delimited species (left y-axis) and the second representing the probability of the most likely species delimitation scenario at a specific *collapseheight* value (ranging from 0 to 1, right y-axis). Every analysis proposed two to three taxonomic solutions having similar probabilities. In total, there were four possible scenarios (A, B, C, D) differing in the number of delimited species within the *A. tubigenis* lineage (one to four species). These delimitation results are schematically shown in the lower part of the figure. The list of genes used in every analysis can be found in Supplementary Table S4.

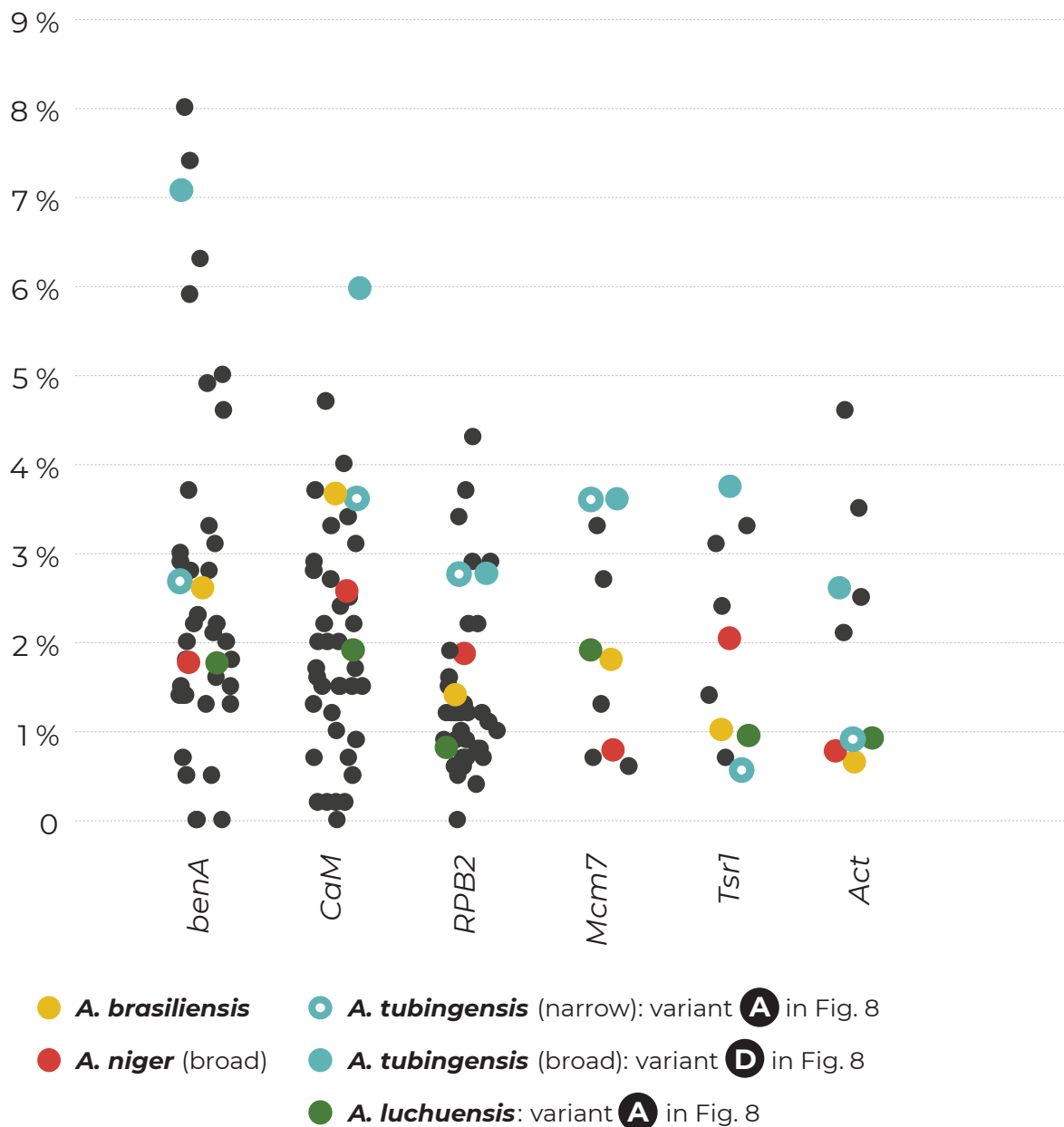


Surprisingly, every analysis proposed two to three taxonomic solutions having high probabilities (two to three major peaks). There were four possible scenarios (A, B, C, D) resulting in 3–6 delimited species in the *Nigri* series. However, none of these analyses supported the division of the ANL or ABL into more species. Thus, all of these scenarios only differed in the number of delimited species in the ATL, ranging from one to four delimited species. These delimitation results are schematically shown in the lower part of Fig. 8. All ten analyses supported the scenario with four species: (1) *A. costaricensis* + *A. neoniger* + *A. tubingensis*; (2) *A. luchuensis* + *A. piperis*; (3) *A. eucalyptica*; and (4) *A. vadensis*. In addition, six analyses supported scenarios with three or two species in the ATL, and only two analyses lumped all species into one at high *collapseheight* values (Fig. 8).

### Intraspecific genetic diversity in *Aspergillus* and series *Nigri*

To determine whether the intraspecific genetic variability in the redefined species in the series *Nigri* falls within the range commonly found in *Aspergillus*, we made an intraspecific diversity comparison across *Aspergillus* (basic data in Supplementary Table S6). We downloaded sequences of the *benA*, *CaM* and *RPB2* genes from 34 species outside section *Nigri* (fewer common markers, such as *Mcm7*, *Tsr1* and *Act*, were available for only some species). The criteria for inclusion were as follows: (1) species boundaries were re-examined using MSC methods, and (2) the strains of the species were obtained from at least three countries (sufficient sampling).

The results of the comparison (Fig. 9) showed that dissimilarities in the common taxonomic markers were usually lower than 4 % except in *benA*, where a higher degree of dissimilarity was found occasionally.



**Fig. 9.** Jitter plot showing maximum sequence dissimilarity between isolates of the same *Aspergillus* species whose species limits have been delimited using methods based on a multispecies coalescent model. Only species represented by isolates from at least three countries were included to ensure relevant sampling and thus representative intraspecific genetic variability. In total, 34 species were included that were classified outside section *Nigri*. The data from *benA*, *CaM* and *RPB2* were collected from all 34 species, while less common markers (minichromosome maintenance factor 7 - *Mcm7*, ribosome biogenesis protein - *Tsr1* and actin - *Act*) were available for only a limited number of species. Species belonging to the series *Nigri* are designated with coloured circles. The basic data used for construction of the jitter plot are listed in the Supplementary Table S6.

This was mainly caused by long intronic regions in *benA*. However, the two highest dissimilarity values in *benA* belong to *A. vitricola* (sect. *Restricti*) and *A. subalbidus* (sect. *Candidi*), which could each be a complex of two species because the species delimitation results were ambiguous (Sklenář *et al.* 2017, Glässnerová *et al.* 2022). Thus, the real maximum sequence dissimilarity in *benA* may be lower than that displayed in Fig. 9.

The intraspecific variability in the broadly defined *A. niger* (corresponding to the whole ANL) and in *A. brasiliensis* falls comfortably within the range frequently found in *Aspergillus*. The intraspecific genetic diversity of the broadly defined *A. tubingensis* in *benA* and *CaM* is higher than that commonly found in *Aspergillus*, while the variability within the narrowly defined *A. tubingensis* (excluding *A. luchuensis*/*A. piperis*, *A. eucalypticola* and *A. vadensis*) is within the range common for *Aspergillus* (Fig. 9).

## TAXONOMY

The following list contains the names of accepted species in the series *Nigri* and their synonyms. Illegitimate and invalid names are also included as some of them are still used in the applied sphere. This list builds on previously published lists of accepted species and their synonyms in section *Nigri* (Thom & Church 1926, Thom & Raper 1945, Raper & Fennell 1965, Al-Musallam 1980, Kozakiewicz 1989). Compared to previous lists, this list is primarily based on available molecular data. If this data is missing, the names are included in the sub-list of unresolved or doubtful names.

One of the main goals of this list is to point out that the taxonomy of series *Nigri* is still unresolved and to stimulate further research to resolve names for which original material is available in the form of cultures or herbarium specimens. Some of these names may still threaten the stability of the taxonomy of series *Nigri*. Most importantly, some of the synonyms or unresolved names compete for priority with economically significant species such as *A. tubingensis*, *A. luchuensis* or even *A. niger*. Although *A. niger* Tiegh. 1867 was conserved against *Ustilago ficuum* Reichard 1867 and *Ustilago phoenicis* Corda 1840 (Kozakiewicz *et al.* 1992), it can still be predated by *A. phaeocephalus* Durieu & Mont. 1848 (no specimens available), *A. nigrescens* C.P. Robin 1853 (no specimens available) and *A. nanus* Mont. 1856 whose type is preserved in the Muséum National d'Histoire Naturelle in France but no DNA data are available. The situation is even more complicated with *A. tubingensis* Mosseray 1934. This species is conspecific with several older species names, such as *A. pulverulentus* (basonym *Sterigmatocystis pulverulenta* McAlpine 1896), *A. schiemaninae* Thom 1916, *A. elatior* Mosseray 1934, *A. pseudoniger* Mosseray 1934 and possibly some other species listed among unresolved species. *Aspergillus tubingensis* is the most frequently used name compared to other competing names and members of the International Commission of *Penicillium* and *Aspergillus* (ICPA) are thus currently in discussions about its possible conservation.

### Accepted species and their synonyms in *Aspergillus* section *Nigri* series *Nigri*

**1. *Aspergillus brasiliensis*** Varga, Frisvad & Samson, *Int. J. Syst. Evol. Microbiol.* 57: 1929. 2007. MycoBank MB 510581. — Type: CBS 101740. Ex-type: CBS 101740 = IMI 381727 = IBT 101740. DNA barcodes: ITS = FJ629321; *benA* = FJ629272; *CaM* = FN594543; *RPB2* = EF661063.

**2. *Aspergillus eucalypticola*** Varga, Frisvad & Samson, *Stud. Mycol.* 69: 9. 2011. MycoBank MB 560387. — Type: CBS H-20627. Ex-type: CBS 122712 = IBT 29274. DNA barcodes: ITS = EU482439; *benA* = EU482435; *CaM* = EU482433; *RPB2* = MN969070.

**3. *Aspergillus luchuensis*** Inui, *J. Coll. Sci. Imp. Univ. Tokyo* 15: 469. 1901. MycoBank MB 151291. — Type: CBS H-24280. Ex-type: CBS 205.80 = NBRC 4281 = KACC 46772 = IFM 47726 = RIB 2642. DNA barcodes: ITS = JX500081; *benA* = JX500062; *CaM* = JX500071; *RPB2* = MN969081.

*Synonym:* *Aspergillus perniciosus* Inui, *J. Coll. Sci. Imp. Univ. Tokyo* 15: 473. 1901. [no MycoBank record]. Synonymized by Hong *et al.* (2013). No living culture available.

*Synonym:* *Aspergillus awamori* Nakaz., *Rep. Govt. Res. Inst. Formosa* 1. 1907. [MycoBank MB 119955]. Synonymized by Hong *et al.* (2014). Culture ex-type: JCM 2261 = IAM 2112 = KACC 47234.

*Synonym:* *Aspergillus velutinus* Mosseray, *La Cellule* 43: 252. 1934. [MycoBank MB 255342]. The *benA* sequence (FJ629283) derived from the ex-type strain CBS 139.48 (= NRRL 4877 = CCM F-286 = VKM F-2084 = WB 4877) is identical to the ex-type of *A. luchuensis*.

*Synonym:* *Aspergillus aureus* var. *acidus* Nakaz., Simo & A. Watan., *Bull. Agric. Chem. Soc. Japan* 12: 960. 1936. [MycoBank MB 493809]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2013). Culture ex-type: NBRC 4121 = NRRL 4796 = CBS 564.65 = KACC 45131.

*Synonym:* *Aspergillus aureus* var. *pallidus* Nakaz., Simo & A. Watan., *Bull. Agric. Chem. Soc. Japan* 12: 961. 1936. [MycoBank MB 372388]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2014). Culture ex-type: NBRC 4123 = NRRL 4797 = CBS 565.65 = KACC 47004.

*Synonym:* *Aspergillus awamori* var. *piceus* Nakaz., Simo & A. Watan., *Bull. Agric. Chem. Soc. Japan* 12: 956. 1936. [MycoBank MB 250923]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2013). Representative strain: CBS 119.52 = NRRL 4846.

*Synonym:* *Aspergillus kawachii* Kitahara & Yoshida, *J. Ferment. Technol.* 27. 1949. [No MycoBank record]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2013). Culture ex-type: NBRC 4308 = CBS 117.80 = NRRL 4886 = KACC 46771. Hong *et al.* (2013) and Ban *et al.* (2021) examined phylogenetic position of *A. kawachii* based on strains KACC 46771 and NBRC 4308, respectively. Their sequences are identical with the ex-type of *A. kawachii* in contrast to the sequences from strain CBS 117.90 that belong to the ANL (Table 1). Our conclusion is that the strain CBS 117.90 is contaminated with *A. niger*.

*Synonym:* *Aspergillus citricus* var. *pallidus* (Nakaz., Simo & A. Watan.) Kozak., *Mycol. Pap.* 161: 114. 1989. [MycoBank MB 127748]. Not validly published – basonym *A. aureus* var. *pallidus* Nakaz., Simo & A. Watan. is not a validly published [Art. 41.5; Turland *et al.* (2018)].

*Synonym:* *Aspergillus inuii* Sakag., Iizuka & M. Yamaz., *J. Agric. Chem. Soc. Japan* 3: 97. 1950. [Mycobank MB 309227]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2014). Culture ex-type: JCM 22302 = IAM 2255 = CBS 125.52 = KACC 47235.

*Synonym:* *Aspergillus nakazawae* [as *nakazawai*] Sakag., Iizuka & M. Yamaz., *J. Agric. Chem. Soc. Japan* 4: 3.

1950. [Mycobank MB 309232]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2014). Culture ex-type: NRRL 4750 = CBS 128.52 = KACC 47005.

*Synonym: Aspergillus foetidus* var. *acidus* (Nakaz., Simo & A. Watan.) Raper & Fennell, *The Genus Aspergillus*: 326. 1965. [Mycobank MB 353273]. Synonymized by Hong *et al.* (2013). Not validly published – basionym *A. aureus* var. *acidus* Nakaz., Simo & A. Watan. is not a validly published name [Art. 41.5; Turland *et al.* (2018)].

*Synonym: Aspergillus foetidus* var. *pallidus* (Nakaz., Simo & A. Watan.) Raper & Fennell, *The Genus Aspergillus*: 325. 1965. [Mycobank MB 353274]. Synonymized by Hong *et al.* (2014). Not validly published – basionym *A. aureus* var. *pallidus* Nakaz., Simo & A. Watan. is not a validly published name [Art. 41.5; Turland *et al.* (2018)].

*Synonym: Aspergillus niger* var. *awamori* (Nakaz.) Al-Musallam, *Revision of the black Aspergillus species*: 60. 1980. Doctoral diss., Rijksuniversiteit Utrecht, Netherlands. [Mycobank MB 118405]. Hong *et al.* (2014) showed that the ex-type strain NRRL 4948 (= CBS 557.65 = KACC 46996 = ATCC 16877) is closely related to the ex-type of *A. welwitschiae* (ANL) and is synonymized here with *A. niger*.

*Synonym: Aspergillus acidus* Kozak., *Mycol. Pap.* 161: 110. 1989. [Mycobank MB 127765]. Synonymized by Hong *et al.* (2013). Culture ex-type: KACC 45131 = CBS 564.65 = ATCC 16874 = NBRC 4121 = IMI 104688 = NRRL 4796.

*Synonym: Aspergillus coreanus* Yu T.S. *et al.*, *J. Microbiol. Biotechnol.* 14:182. 2004. [No Mycobank record]. Illegitimate name [Art. 54.1; Turland *et al.* (2018)] and *nomen nudum*. Synonymized by Hong *et al.* (2013). Authentic strain: KACC 41731 = CBS 119384.

*Synonym: Aspergillus piperis* Samson & Frisvad, *Stud. Mycol.* 50: 57. 2004. [Mycobank MB 500009]. Synonymized in this study.

*Synonym: Aspergillus luchuensis* mut. *kawachii* (Kitahara & Yoshida) S.B. Hong, O. Yamada & R.A. Samson, *Appl. Microbiol. Biotechnol.* 98: 560. 2014. [No Mycobank record]. Not validly published: basionym was not validly published [Art. 41.5; Turland *et al.* (2018)] and protologue does not include citation of the identifier issued for the name by a recognized electronic repository, e.g. Mycobank [Art. F.5.1; Turland *et al.* (2018)].

**4. *Aspergillus niger*** Tiegh., *Ann. Sci. Nat., Bot. ser.* 5, 8: 240. 1867. MycoBank MB 284309. *Nomen conservandum* taking precedence over *Ustilago phoenicis* Corda and *Ustilago ficuum* Reichard which were published earlier (Kozakiewicz *et al.* (1992)). — Type: CBS 554.65. Ex-type: CBS 554.65 = NRRL 326 = ATCC 16888 = IFO 33023 = IHEM 3415 = IMI 050566ii = IMI 50566 = JCM 10254 = QM 9270 = QM 9946 = Thom 2766 = WB 326. DNA barcodes: ITS = EF661186; *BenA* = EF661089; *CaM* = EF661154; *RPB2* = EF661058.

*Synonym: Ustilago ficuum* Reichardt, *Verh. Zool.-Bot. Ges. Wien* 17: 335. 1867. [Mycobank MB 187740]. *Nomen rejiciendum*, rejected name in favour of *A. niger* Tiegh. – see Kozakiewicz *et al.* (1992). The ex-type strain IHEM 3710 (= WB 364 = NRRL 364 = IMI 91881 = CBS 555.65 = ATCC 16882) derived from the type IMI 91881 (Kozakiewicz *et al.* 1992) is located in the ANL (Fig. 1).

*Synonym: Eurotium nigrum* (Tiegh.) de Bary, *Beiträge zur Morphologie und Physiologie der Pilze*: 21. 1870. [No Mycobank record]. *Basionym: A. niger* Tiegh.

*Synonym: Sterigmatocystis nigra* (Tiegh.) Tiegh., *Bull. Soc. Bot. France* 24: 102. 1877. [Mycobank MB 231646]. *Basionym: A. niger* Tiegh.

*Synonym: Ustilago welwitschiae* Bres. in *Sacc, Bol. Soc. Brot.* 11: 68. 1893. [Mycobank MB 176748]. Synonymized in this study.

*Sterigmatocystis ficuum* (Reichardt) Henn., *Hedwigia* 34: 86. 1895. [Mycobank MB 100573]. See *Ustilago ficuum* Reichardt (basionym).

*Synonym: Sterigmatocystis welwitschiae* (Bres.) Henn. in H. Baum, *Kunene-Zambesi Expedit.*, Berlin: 168. 1903. [Mycobank MB 233714]. See *Ustilago welwitschiae* Bres. in *Sacc.* (basionym).

*Synonym: Aspergillus batatas* [as *batatae*] Saito, *Zentralbl. Bakteriol. Parasitenkn. Abt. II*, 18: 34. 1907. [Mycobank MB 214829]. Synonymized by Hong *et al.* (2014). Culture ex-type: NRRL 4785 = CBS 115.34 = NBRC 4034 = KACC 46995.

*Synonym: Aspergillus welwitschiae* (Bres.) Henn. in Wehmer, *Zentralbl. Bakteriol. Parasitk. Abt. II*, 18: 294. 1907. [Mycobank MB 490584]. See *Ustilago welwitschiae* Bres. in *Sacc.* (basionym).

*Synonym: Aspergillopsis nigra* (Tiegh.) Speg., *Anales Mus. Nac. Hist. Nat. Buenos Aires, ser. 3*, 13: 435. 1911. [Mycobank MB 210007]. *Basionym: A. niger* Tiegh.

*Synonym: Rhopalocystis nigra* (Tiegh.) Grove, *J. Econ. Biol.* 6: 41. 1911. [Mycobank MB 432029]. *Basionym: A. niger* Tiegh.

*Synonym: Aspergillus niger* var. *altipes* E. Schiemann, *Z. Indukt. Abstamm. Vererbungsl.* 8: 1–35. 1912. [Mycobank MB 493817]. This variety belongs to the ANL based on the ITS sequence (MH854603) derived from the ex-type strain CBS 102.12 (= ATCC 10549 = IBT 19348 = IBT 26343 = IFO 4067 = MUCL 13608 = NRRL 4863 = WB 4863).

*Synonym: Sterigmatocystis batatas* [as *batatae*] (Saito) *Sacc. Syll. Fung.* 22: 1261. 1913. [Mycobank MB 209678]. See *A. batatas* Saito (basionym).

*Synonym: Aspergillus aureus* Nakazawa, *Rep. Govt. Res. Inst. Formosa* 4: 215–222. 1915. [No Mycobank record]. Synonymized by Hong *et al.* (2014). Culture ex-type: CBS 121.28 = NRRL 4784 = NBRC 4031 = RIB 2014 = KACC 47003 = IFO 4031 = IMI 104687 = WB 4784. Illegitimate name [Art. 54.1; Turland *et al.* (2018)]. Not *A. aureus* Berk. 1836 [Mycobank MB 214770].

*Synonym: Aspergillus ficuum* (Reichardt) Thom & Currie, *J. Agric. Res.* 7: 12. 1916. [Mycobank MB 101909]. See *Ustilago ficuum* Reichardt (basionym).

*Synonym: Aspergillus citricus* Mosseray, *Ann. Univ. Sci. Budap. Rolando Eotvos Nominatae Biol.* 43: 262. 1934. [Mycobank MB 251457]. Synonymized by Frisvad *et al.* (2011) and Houburaken *et al.* (2014). The genotype of the ex-type strain IHEM 5622 (= CBS 126.48 = WB 337 = NRRL 337 = NCTC 1692 = MUCL 28130 = IMI 15954 = IFO 6428 = DSM 734 = CCRC 30206 = ATCC 10254) is identical to the ex-type strains of *A. niger* (Fig. 1).

*Synonym: Aspergillus bainieri* Mosseray, *Ann. Soc. Sci. Brux.* 54: 79. 1934. [No Mycobank record]. The description is based on the same strain like *A. longobasidia* Bainier ex Mosseray (CBS 121.48).

*Synonym: Aspergillus longobasidia* Bainier ex Mosseray, *La Cellule* 43: 227. 1934. [Mycobank MB 253086]. The ex-

- type strain CBS 121.48 (= NRRL 4857) belongs to the ANL (Hong *et al.* 2013).
- Synonym: Aspergillus pseudocitricus* Mosseray, La Cellule 43: 228. 1934. [Mycobank MB 254148]. The ex-type strain CBS 127.48 (= NRRL 4869) can be identified as *A. niger* based on *benA* (Hong *et al.* 2013).
- Synonym: Aspergillus niger* mut. *fusca* Blochwitz, Ann. Mycol. 32: 87. [No MycoBank record]. The ex-type strain CBS 113.33 (= NRRL 4864 = ATCC 10548 = WB 4864) can be identified as *A. niger* based on *benA* (Hong *et al.* 2013).
- Synonym: Aspergillus hennebergii* Blochwitz, Ann. Mycol. 33: 238. 1935. [MycoBank MB 266969]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. The ex-type strain CBS 118.35 (= NRRL 4883 = CECT 2801 = QM 9704 = WB 4883 = VKM F-4392) can be identified as *A. niger* based on *benA* (Hong *et al.* 2013).
- Synonym: Aspergillus aureus* var. *brevior* [as *brevius*] Nakaz., Simo & A. Watan. Bull. Agric. Chem. Soc. Japan 12: 962. 1936. [MycoBank MB 493810]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. The representative strain CBS 113.52 (= WB 4841) can be identified as *A. niger* based on *benA* (Hong *et al.* 2013).
- Synonym: Aspergillus awamori* var. *fuscus* Nakaz., Simo & A. Watan., Bull. Agric. Chem. Soc. Japan 12: 959. 1936. [MycoBank MB 250921]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2013, 2014). Representative strain: CBS 117.52 = NRRL 4844 = WB 4844.
- Synonym: Aspergillus awamori* var. *minus* Nakaz., Simo & A. Watan., Bull. Agric. Chem. Soc. Japan 12: 955. 1936. [MycoBank MB 250922]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2013). Representative strain: CBS 118.52 [the whole-genome sequence was generated by Seekles *et al.* (2022)].
- Synonym: Aspergillus miyakoensis* Nakaz., Simo & A. Watan., Bull. Agric. Chem. Soc. Japan 12: 963. 1936. [MycoBank MB 253392]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Synonymized by Hong *et al.* (2014). Culture ex-type: NRRL 4859 = CBS 117.51 = KACC 46998.
- Synonym: Aspergillus pyri* W.H. English, Res. Stud. State Coll. Wash. 8: 127. 1940. [MycoBank MB 453796]. *Nomen nudum*. Subsequent work by English led to recognition of the name as a synonym of *A. niger* and withdrawal of the name (Thom & Raper 1945, Raper & Fennell 1945).
- Synonym: Aspergillus foetidus* Thom & Raper, A manual of the Aspergilli: 219. 1945. [Mycobank MB 284300]. Synonymized by Varga *et al.* (2011). Culture ex-type: CBS 121.28 = NRRL 4784 = NBRC 4031 = RIB 2014 = KACC 47003 = IFO 4031 = IMI 104687 = WB 4784.
- Synonym: Aspergillus usamii* Sakag., Iizuka & M. Yamaz., J. Appl. Mycol. Japan 4: 1. 1950. [No MycoBank record]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Validly published later – see *A. usamii* Sakag., Iizuka & M. Yamaz. ex Iizuka & K. Sugiy.
- Synonym: Aspergillus usamii* mut. *shirousamii* Iizuka & Yamaguchi. J. Gen. Appl. Microbiol. 1: 194–200. 1954. [No MycoBank record]. Not validly described. Synonymized by Hong *et al.* (2014). Culture ex-type: NRRL 4889 = KACC 47001.
- Synonym: Aspergillus usamii* Sakag., Iizuka & M. Yamaz. ex Iizuka & K. Sugiy., J. Jap. Bot.: 232. 1965. [MycoBank MB 326664]. Synonymized by Yamada *et al.* (2011) and Hong *et al.* (2014). Culture ex-type: CBS 139.52 = NRRL 4760 = ATCC 11364 = ATCC 14331 = NBRC 4388 = RIB 2602 = IAM 2185 = KACC 47000 = IFO 4388 = QM 8164 = WB 4760.
- Synonym: Aspergillus niger* f. *hennebergii* Blochwitz ex Al-Musallam, Revision of the black *Aspergillus* species: 68. 1980. Doctoral diss., Rijksuniversiteit Utrecht, Netherlands. [MycoBank MB 118404]. See *A. hennebergii* Blochwitz (basionym).
- Synonym: Aspergillus niger* var. *usamii* (Sakag., Iizuka & M. Yamaz. ex Iizuka & K. Sugiy.) Al-Musallam, Revision of the black *Aspergillus* species: 64. 1980. Doctoral diss., Rijksuniversiteit Utrecht, Netherlands. [MycoBank MB 118400]. See *A. usamii* Sakag., Iizuka & M. Yamaz. ex Iizuka & K. Sugiy. (basionym).
- Synonym: Aspergillus niger* var. *ficuum* (Reichardt) Kozak., Mycol. Pap. 161: 112. 1989. [MycoBank MB 127745]. See *Ustilago ficuum* Reichardt (basionym).
- Synonym: Aspergillus lacticoffeatus* Frisvad & Samson, Stud. Mycol. 50: 52. 2004. [Mycobank MB 500008]. Synonymized by Varga *et al.* (2011) and confirmed in this study.
- Synonym: Aspergillus vinaceus* Ferranti *et al.*, J. Fungi 6: 371 - p14. 2020. [MycoBank MB 833399]. Synonymized in this study.
- 5. *Aspergillus tubingensis*** Mosseray, La Cellule 43: 245. 1934. [MycoBank MB 255209]. — Type: CBS H-24288. Ex-type: NRRL 4875 = CBS 133056 = QM 8904 = WB 4875. DNA barcodes: ITS = EF661193; *benA* = EF661086; *CaM* = EF661151; *RPB2* = EF661055.
- Synonym: Sterigmatocystis pulverulenta* McAlpine, Agric. Gaz. N.S.W., Sydney 7: 302. 1896 (1897). [MycoBank MB 194998]. Synonymized by Houbraeken *et al.* (2014). The ex-type strain IHEM 5615 (= CBS 558.65 = CBS 115.48 = MUCL 13592 = MUCL 15630 = NRRL 4851 = WB 4851 = IMI 211396 = ATCC 16879) is close to the ex-type of *A. tubingensis* (Fig. 1).
- Synonym: Aspergillus pulverulentus* (McAlpine) Wehmer, Zentralbl. Bakteriologie Parasitenkd., Abt. II, 18: 394. 1907. [MycoBank MB 121243]. See *Sterigmatocystis pulverulenta* McAlpine (basionym).
- Synonym: Aspergillus cinnamomeus* E. Schiemann, Z. Indukt. Abstamm. Vererbungslehre. 8: 1–35. 1912. [MycoBank MB 122195]. The *benA* sequence (FJ629307) derived from the ex-type strain CBS 103.12 = ATCC 1027 = IBT 16906 = IBT 28139 = IBT 29892 = IFO 4043 = IMI 016148 = LSBH Ac42 = MZKI A-76 = NCTC 3774 = NRRL 348 = QM 326 = Thom 3534B = WB 348) is identical with the ex-type of *A. tubingensis*.
- ? *Synonym: Aspergillus fuscus* E. Schiem., Z. Indukt. Abstamm. Vererbungslehre. 8: 1–35. 1912. [MycoBank MB 450741]. Illegitimate name [Art. 54.1; Turland *et al.* (2018)]. Not *A. fuscus* Bonord. 1861 [MycoBank MB 206602]. Illegitimate name *A. fuscus* was replaced by *nomen novum* *A. schiemanniae* Thom 1916 (see *A. schiemanniae*).
- ? *Synonym: Aspergillus proteus* E. Schiem., Z. Indukt. Abstamm. Vererbungslehre. 8: 1–35. 1912. [No MycoBank record]. Illegitimate name – not accepted by the author itself in the original publication [Art. 36.1; Turland *et al.* (2018)].
- Synonym: Aspergillus pulverulentus* (McAlpine) Thom, J. Agric. Res. 7: 11. 1916. [No MycoBank record]. Illegitimate name [Art. 54.1; Turland *et al.* (2018)] – a later homonym of *A. pulverulentus* (McAlpine) Wehmer 1907.

- ? *Synonym: Aspergillus schiemanniae* Thom [as *schiemanni*], J. Agric. Res. 7: 11. 1916. [Mycobank MB 102113]. The ITS sequence (MH854950) derived from the ex-type strain CBS 122.28 (= ATCC 1040 = NRRL 361 = IFO 4091 = IMI 091895 = IMI 16269 = LSHB AC.37 = NCTC 3781 = QM 327 = Thom 3534C = WB 361) belongs to the ATL.
- Synonym: Aspergillus elatior* Mosseray, Ann. Univ. Sci. Budap. Rolando Eotvos Nominatae Biol. 43: 253. 1934. [Mycobank MB 251950]. The ex-type strain IHEM 5615 (= CBS 558.65 = CBS 115.48 = MUCL 13592 = MUCL 15630 = NRRL 4851 = WB 4851 = IMI 211396 = ATCC 16879) is similar to the ex-type of *A. tubingensis* (Fig. 1).
- Synonym: Aspergillus pseudoniger* Mosseray, La Cellule 43: 256. 1934. [Mycobank MB 472168]. The ex-type strain IHEM 4379 (= CBS 128.48 = IMI 313491 = WB 4870 = MUCL 31312) is similar to *A. tubingensis* (Fig. 1).
- Synonym: Aspergillus niger* mut. *cinnamomeus* (E. Schiemann) Thom & Raper, A manual of the Aspergilli: 223. 1945. [Mycobank MB 440825]. See *A. cinnamomeus* E. Schiemann (basonym).
- ? *Synonym: Aspergillus niger* mut. *schiemanniae* (Thom) Thom & Raper [as *schiemanni*], A manual of the Aspergilli: 224. 1945. [Mycobank MB 123940]. See *A. schiemanniae* Thom (basonym).
- Synonym: Aspergillus saitoi* Sakag., Iizuka & M. Yamaz., J. Appl. Mycol. Japan 3: 68. 1950. [No MycoBank record]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Validly published later – see *A. saitoi* Sakag., Iizuka & M. Yamaz. ex Iizuka & K. Sugiy.
- Synonym: Aspergillus saitoi* var. *kagoshimaensis* Sakag., Iizuka & M. Yamaz., J. Appl. Mycol. Japan 4: 3. 1950. [No MycoBank record]. Not validly published [Art. 39.1; Turland *et al.* (2018)]. Validly published later – see *A. saitoi* var. *kagoshimaensis* Sakag., Iizuka & M. Yamaz. ex Iizuka & K. Sugiy.
- Synonym: Aspergillus awamori* var. *hominis* Bat. & Maia, An. Soc. Biol. Pernambuco 15: 186. 1957. [Mycobank MB 351895]. The *benA* sequence (FJ629318) derived from the ex-type strain CBS 107.55 (= NRRL 4740 = ATCC 12074 = WB 4740) is almost identical (419/420 bp) to the ex-type of *A. tubingensis*.
- Synonym: Aspergillus saitoi* Sakag., Iizuka & M. Yamaz. ex Iizuka & K. Sugiy., J. Jap. Bot. 40: 230. 1965. [Mycobank MB 326655]. Synonymized by Hong *et al.* (2014). Culture ex-type: CBS 136.52 = CBS 552.65 = NRRL 4757 = IAM 2209 = ATCC 11362 = IMI 211395 = KACC 46993 = WB 4757.
- Synonym: Aspergillus saitoi* var. *kagoshimaensis* Sakag., Iizuka & M. Yamaz. ex Iizuka & K. Sugiy., J. Jap. Bot. 40: 231. 1965. [Mycobank MB 349043]. Synonymized by Hong *et al.* (2014). Culture ex-type: CBS 137.52 = NRRL 4758 = IMI 214827 = ATCC 11363 = KACC 46994 = QM 8162 = IAM 2190 = WB 4758.
- Synonym: Aspergillus niger* f. *pulverulentus* (McAlpine) Al-Musallam, Revision of the black *Aspergillus* species: 58. 1980. Doctoral diss., Rijksuniversiteit Utrecht, Netherlands. [Mycobank MB 118403]. See *Sterigmatocystis pulverulenta* McAlpine (basonym).
- Synonym: Aspergillus niger* var. *pulverulentus* (McAlpine) Kozak., Mycol. Pap. 161: 113. 1989. [Mycobank MB 127747]. See *Sterigmatocystis pulverulenta* McAlpine (basonym).
- Synonym: Aspergillus niger* var. *tubingensis* (Mosseray) Kozak., Mycol. Pap. 161: 112. 1989. [Mycobank MB 127746]. *Basionym: A. tubingensis* Mosseray.
- Synonym: Aspergillus costaricensis* [as *costaricaensis*] Samson & Frisvad, Stud. Mycol. 50: 52. 2004. [Mycobank MB 369151]. Synonymized in this study.
- Synonym: Aspergillus neoniger* Varga, Frisvad & Samson, Stud. Mycol. 69: 16. 2011. [Mycobank MB 560390]. Synonymized in this study.
- Synonym: Aspergillus Chiangmaiensis* S. Khuna, N. Suwannarach & S. Lumyong, Front. Microbiol. 12: 705896 - p6. 2021. [Mycobank MB 830887]. Synonymized in this study.
- Synonym: Aspergillus pseudopiperis* S. Khuna, N. Suwannarach & S. Lumyong, Front. Microbiol. 12: 705896 - p6. 2021. [Mycobank MB 830888]. Synonymized in this study.
- 6. *Aspergillus vadensis*** Samson, de Vries, Frisvad & Visser, Antonie van Leeuwenhoek 87: 201. 2005. [Mycobank MB 340234]. — Type: CBS 113365. Ex-type: CBS 113365 = CECT 20584 = IMI 313493 = IBT 24658. DNA barcodes: ITS = AY585549; *benA* = AY585531; *CaM* = FN594560; *RPB2* = HE984371.
- Synonym: Aspergillus vadensis* de Vries *et al.*, Appl. Environ. Microbiol. 70: 3954. 2004. [Mycobank MB 560390]. Not validly described (*nomen nudum*).

### Unresolved or doubtful names most probably belonging to the series *Nigri*

*Aspergillus phaeocephalus* Durieu & Mont., Exploration scientifique de l'Algérie 1: 342. 1848. [Mycobank MB 179669]. No specimens are available.

*Synonym: Sterigmatocystis phaeocephala* (Durieu & Mont.) Sacc., Syll. Fung. 4: 76. 1886. [Mycobank MB 233071].

*Aspergillus nigrescens* C.P. Robin, Histoire naturelle des végétaux parasites: 518. 1853. [Mycobank MB 182212]. Considered either *A. fumigatus* or *A. niger* by various authors (Wilhelm 1877, Wehmer 1901, Raper & Fennell 1965), no material is available (species described from pathological specimens only).

*Aspergillus nanus* Mont., Syll. Gen. Sp. Crypt. (Paris): 300. 1856. [Mycobank MB 193617]. Typus in Muséum National d'Histoire Naturelle, France, Paris (PC); no DNA sequence available. The strains of *A. niger* var. *nanus* examined by Al-Musallam (1980) belong either to the ANL or ATL, e.g., CBS 105.47 (MH856174; ATL), CBS 106.47 (FJ629281; ATL), CBS 110.30 (MH855092; ANL), CBS 115.50 (MH856565; ANL), CBS 131.52 (the whole-genome sequence was generated by Seekles *et al.* (2022); ANL).

*Synonym: Aspergillus niger* var. *nanus* (Mont.) Al-Musallam, Revision of the black *Aspergillus* species: 62. 1980. Doctoral diss., Rijksuniversiteit Utrecht, Netherlands. [Mycobank MB 118406].

*Sterigmatocystis antacustica* C.E. Cramer, Vierteljahrsschr. Naturforsch. Ges. Zürich 4: 325. 1859. [Mycobank MB 209963].

*Synonym: Rhopalocystis antacustica* (C.E. Cramer) Grove, J. Econ. Biol.: 41. 1911. [Mycobank MB 503610].

*Aspergillus nigricans* Wreden, C. R. Hebd. Seances Acad. Sci. 65: 368. 1867. [No MycoBank record]. No specimen is available.

*Aspergillus fuliginosus* Peck, Bull. Buffalo Soc. Nat. Sci. 1: 69. 1873. [Mycobank MB 208679]. Typus in New York State Museum, USA (NYSf 1265); no DNA sequence available.

*Synonym: Sterigmatocystis fuliginosa* Bainer, Bull. Soc. Bot. France 28: 78. 1881. [No MycoBank record].

*Aspergillus nigricans* Cooke, Grevillea 6: 127. 1878. [Mycobank

- MB 182128]. Illegitimate name. Not *A. nigricans* Wreden 1867.  
*Alliospora sapucaya* [as *sapucayae*] Pim, J. Bot. London 21: 234. 1883. [Mycobank MB 215122].  
*Aspergillus cookei* Sacc., Syll. Fung. 4: 71. 1886. [Mycobank MB 206435]. A replaced synonym for illegitimate name *A. mucoroides* Cooke.  
*Basionym*: *Aspergillus mucoroideus* Cooke, Grevillea 12: 9. 1883. [Mycobank MB 187861]. Illegitimate name [Art. 54.1; Turland et al. (2018)]. Not *A. mucoroides* Corda 1838 [Mycobank MB 187771].  
*Aspergillus subfuscus* Johan-Olsen, Nordiskt Med. Ark. 18: 14. 1886. [Mycobank MB 184212].  
*Basionym*: *Sterigmatocystis subfusca* (Johan-Olsen) Sacc., Syll. Fung. 10: 526. 1892. [Mycobank MB 226258].  
*Aspergillus nigriceps* Berk. & Curt., Grevillea 17: 21. 1888. [No MycoBank record]. A slide from material in the Harvard Collection (Curtis collection, Wright no. 927) showed species inseparable from *A. niger* (Raper & Fennell 1965).  
*Aspergillus ustilago* Beck in H. Wawra, Itinera Principum S. Coburgi 2: 148. 1888. [Mycobank MB 161832].  
*Synonym*: *Sterigmatocystis ustilago* (Beck) Sacc., Syll. Fung. 10: 526. 1892. [Mycobank MB 198044].  
*Sterigmatocystis castanea* F. Patt., Bull. Torrey Bot. Club 27: 284. 1900. [Mycobank MB 195664]. Typus in BPI Herbarium (BPI 410863).  
*Aspergillus gallomyces* Calmette, Germ. Pat. Abstr. 129: 164. 1902. [No MycoBank record].  
*Aspergillus strychni* Lindau, Hedwigia 43: 306. 1904. [Mycobank MB 183848].  
*Synonym*: *Sterigmatocystis strychni* (Lindau) Sacc. & D. Sacc., Syll. Fung. 18: 516. 1906. [Mycobank MB 225824].  
*Sterigmatocystis pseudonigra* Costantin & Lucet, Bull. Soc. Mycol. France 19: 33. 1903. [Mycobank MB 195028]. No specimen available.  
*Synonym*: *Aspergillus pseudoniger* (Costantin & Lucet) Saincl., Centralbl. Gesammte Forstwesen: 103. 1949. [Mycobank MB 292856]. Illegitimate name. Not *A. pseudoniger* Mosseray 1934. [Mycobank MB 472168].  
*Sterigmatocystis luteonigra* M.L. Lutz, Bull. Soc. Bot. France 53: 50. 1907. [Mycobank MB 228634].  
*Synonym*: *Aspergillus luteoniger* (M.L. Lutz) Thom & Church, The Aspergilli: 166. 1926. [Mycobank MB 269972].  
*Aspergillopsis intermedia* Speg., Anales Mus. Nac. Hist. Nat. Buenos Aires, ser. 3, 13: 435. 1911. [Mycobank MB 209760]. Typus deposited in the Herbarium of Museo de La Plata, Argentina (LPS 12691); no DNA sequence is available. The strains considered *A. niger* var. *intermedius* by Al-Musallam (1980) belong either to the ANL or ATL, e.g., CBS 117.32 (MH855233; ATL), CBS 115.29 (MH855018; ATL), CBS 117.52 (MH856951; ANL), CBS 130.52 (FJ629359, FJ629310; ATL), CBS 107.55 (FJ629367, FJ629318; ATL).  
*Synonym*: *Aspergillus niger* var. *intermedius* (Speg.) Al-Musallam, Revision of the black *Aspergillus* species: 66. 1980. Doctoral diss., Rijksuniversiteit Utrecht, Netherlands. [Mycobank MB 118402].  
*Aspergillus phoenicis* (Corda) Thom, J. Agric. Res. 7: 14. 1916. [Mycobank MB 101952].  
*Basionym*: *Ustilago phoenicis* Corda, Icones fungorum hucusque cognitorum 4: 9. 1840. [Mycobank MB 169060].  
*Nomen rejiciendum*, rejected name in favour of *A. niger* Tiegh. (Kozakoewict et al. 1992). Type located in Corda's herbarium (PRM, Czech Republic) but no sequence derived from the original material is available. Representative strains NRRL 363, NRRL 365 and NRRL 1956 do either belong to ANL or ATL (Houbraken et al. 2014).  
*Synonym*: *Sterigmatocystis phoenicis* (Corda) Pat. & Delacr., Bull. Soc. Mycol. France 7: 119. 1891. [Mycobank MB 232882].  
*Synonym*: *Aspergillus niger* var. *phoenicis* (Corda) Al-Musallam, Revision of the black *Aspergillus* species: 56. 1980. Doctoral diss., Rijksuniversiteit Utrecht, Netherlands. [Mycobank MB 118401].  
*Aspergillopsis tropicalis* Speg., Bol. Acad. Nac. Cienc. Córdoba 23: 588. 1918. [Mycobank MB 209817].  
*Aspergillus fumaricus* Wehmer ex Thom & Church, The Aspergilli: 181. 1926. [Mycobank MB 122538].  
*Sterigmatocystis gigantea* Mattlet, Ann. Soc. Belge Méd. Trop. 6: 31. 1926. [Mycobank MB 252349].  
*Synonym*: *Aspergillus giganteus* (Mattlet) C.W. Dodge, Medical Mycology: 629. 1935. [No MycoBank record]. Illegitimate name. Not *A. giganteus* Wehmer 1901 [Mycobank MB 206765].  
*Synonym*: *Aspergillus mattletii* Hendr., Publ. Inst. Nat. Étude Agron. Congo Belge 35: 7. 1948. [Mycobank MB 284307].  
*Aspergillus atropurpureus* Blochwitz, Ann. Mycol. 32: 86. 1934. [No MycoBank record]. Illegitimate name. Not *A. atropurpureus* Zimm. 1902 [Mycobank MB 214593].  
*Aspergillus biourgei* Mosseray, La Cellule 43: 241. 1934. [Mycobank MB 251009].  
*Aspergillus buntingii* Mosseray, La Cellule 43: 236. 1934. [Mycobank MB 251145].  
*Aspergillus churchii* Mosseray, La Cellule 43: 242. 1934. [Mycobank MB 251413].  
*Aspergillus densus* Mosseray, La Cellule 43: 232. 1934. [Mycobank MB 251792].  
*Aspergillus granulatus* Mosseray, La Cellule 43: 249. 1934. [Mycobank MB 252446]. Culture ex-type: CBS 120.48 = NRRL 4855 = WB 4855 (no DNA sequence available).  
*Aspergillus guttifer* Mosseray, La Cellule 43: 235. 1934. [Mycobank MB 252493].  
*Aspergillus microcephalus* Mosseray, La Cellule 43: 225. 1934. [Mycobank MB 253336]. The strain CBS 122.48 is considered the ex-type strain (Al-Musallam 1980) but no DNA sequence is available.  
*Aspergillus niger* var. *fermentarius* Nakaz., Simo & A. Watan., J. Agric. Chem. Soc. Japan 10: 171. 1934. [No MycoBank record].  
*Aspergillus olivaceofuscus* Mosseray, La Cellule 43: 258. 1934. [Mycobank MB 253675].  
*Aspergillus praecox* Mosseray, Ann. Soc. Sci. Brux. 54: 79. 1934. [No MycoBank record].  
*Aspergillus pseudoelator* Mosseray, La Cellule 43: 255. 1934. [Mycobank MB 254152].  
*Aspergillus rutilans* Mosseray La Cellule 43: 234. 1934. [Mycobank MB 254522].  
*Aspergillus sclerotifer* Mosseray, Ann. Soc. Sci. Brux. 54: 79. 1934. [No MycoBank record].  
*Aspergillus variegatus* Mosseray La Cellule 43: 238. 1934. [Mycobank MB 255332].  
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## DISCUSSION

Taxonomic studies on *A. niger*, *A. tubingensis* and their related species have been the subject of many taxonomic controversies, rearrangements, name resurrections and repeated synonymizations. Currently, the species number in series *Nigri* is at one of its historical peaks and involves 14 accepted species (Houbraken *et al.* 2020, Silva *et al.* 2020, Khuna *et al.* 2021). Distinguishing among these species using morphology is impossible, and DNA sequencing is the gold standard for reliable classification and species identification (Howard *et al.* 2011, Varga *et al.* 2011). Therefore, these species can be considered cryptic in the truest sense. However, even when using molecular methods, some isolates cannot be identified satisfactorily at the species level (Howard *et al.* 2011, Negri *et al.* 2014, D'hooge *et al.* 2019). Contradictory species identifications in significant part of isolates using BLAST similarity searches with different genes (Fig. 3) can serve as evidence of taxonomic issues and may indicate the need for a taxonomic reclassification. To verify this assumption, we gathered a large dataset of a series *Nigri* sequences from three common phylogenetic markers and *de novo* sequenced 18 genomes and employed various species delimitation methods. All of these methods unequivocally suggested species number reduction.

### Phylogenetic support of species reduction

We demonstrated that MSC delimitation methods using three genes broadly agreed on the distinction of only three species in the series *Nigri* (*A. brasiliensis*, *A. niger* and *A. tubingensis*). Only four analyses from among the 15 depicted in Fig. 5 proposed the delimitation of more species. These conclusive results, especially in the *A. niger* lineage (ANL) and *A. brasiliensis* lineage (ABL),

are supported by the collection of a large number of strains well representing the intraspecific genetic variability.

In the *A. tubingensis* lineage (ATL), the multilocus method STACEY gave similar support to the delimitation of one or four species. This dilemma has not been satisfactorily resolved even at the genome level because ten STACEY analyses based on 20 genes each proposed several solutions ranging from one to four species in the ATL (Fig. 8), with four species being most preferred. The datasets containing 20 genes (and not higher) were selected due to the relatively high computational requirements of the method and because 20 genes are usually enough to resolve the phylogeny with a similar level of accuracy to the genome-wide data (Rokas *et al.* 2003, Yang & Rannala 2010, 2014). The uncertainty regarding the number of delimited species can probably be attributed to the underrepresentation of isolates related to *A. vadensis* and *A. eucalypticola* and, to a lesser extent, other species in the ATL, with the exception of *A. tubingensis* itself. Generally, it is thought that when a dataset contains a few isolates with large genetic distances, the delimitation methods may be prone to over delimitation, but with a large set of isolates representing genetic variability of every species, the probability of methods finding real species boundaries increases (Pante *et al.* 2015, Chambers & Hillis 2020). Another reason for the conflicting results can be the widely present incongruences between single-gene genealogies as typically present in recently speciating species or in populations that have not completed their speciation process. In recently diverged species, it is typically caused by retaining ancestral polymorphisms (incomplete lineage sorting), or it can be caused by past hybridization events (Hubka *et al.* 2018, Steenkamp *et al.* 2018, Matute & Sepúlveda 2019).

At this point, it is appropriate to mention that the single-gene phylogenies based on *benA*, *CaM* and *RPB2* loci were highly incongruent (Fig. 2, Supplementary Fig. S2) and that the topology of the resulting multigene phylogeny shows several conflicts or poorly resolved clades compared to the whole-genome phylogeny, as we demonstrated in Fig. 4. Suboptimal phylogenetic signals contained in these three loci can further contribute to the discrepancy between the results of the MSC methods (support of a single species in the ATL) and those of the STACEY analyses based on 20 loci randomly selected from genomes (predominant support of four species in the ATL) (Fig. 8). When species limits are evaluated using the GCPSR approach (Taylor *et al.* 2000, Dettman *et al.* 2003a) using the *benA*, *CaM* and *RPB2* loci only, the obvious conclusion is the recognition of only three species in the series *Nigri*: *A. brasiliensis*, *A. niger* and *A. tubingensis*. The recently proposed *A. vinaceus* (Silva *et al.* 2020) from the ANL was also described based on the GCPSR approach, but the phylogeny lacked some isolates with intermediate genotypes included here. The presence of these strains in the phylogenies contradicts the delimitation of *A. vinaceus* as a separate phylogenetic species.

Another independent testing of species hypotheses was performed in the recently released software DELINEATE (Sukumaran *et al.* 2021). The software needs some a priori defined species that are correctly delimited in the dataset. This requirement was fulfilled by the inclusion of some species belonging to the other series of section *Nigri*. In addition, *A. brasiliensis* is well defined and can serve as a "reference species" when dealing with delimitation in *A. niger* and *A. tubingensis* lineages. The results of the various models were relatively stable, always supporting the broad concept of *A. niger* (comprising *A. niger*, *A. welwitschiae* and *A. vinaceus*) and *A. brasiliensis* (Fig. 7). In the ATL a broad species definition (comprising all species in the lineage) gained support in Models

7 and 10 when *A. niger* was defined as a broad species as well. In models where the ANL was divided into four and six species, the unassigned ATL populations were segregated into four tentative species. This scenario is, however, improbable because the ANL was never divided into several species in Models 2, 6 and 9. Model 6 shows the situation where populations of the ATL are predefined into four species identical to those delimited by Models 3 and 4, while populations of the ANL are unassigned. In this situation, *A. niger* was also delimited as a single broad species. In summary, DELINEATE analysis convincingly supported that the series *Nigri* contains only three species based on *benA*, *CaM* and *RPB2*.

## Phenotype and sexual reproduction in series *Nigri*

Species in the series *Nigri* are all biserial and present colonies with brown, dark brown to black sporulating areas and white to yellowish white mycelial areas. Generally, the species exhibit largely overlapping characteristics in terms of both macromorphology (colony diameter and colour) and micromorphology (size, shape and ornamentation of conidia, diameter and shape of vesicle, and length and width of stipes) (Table 2). Not only particular species but also the main lineages of *A. niger* and *A. tubingensis* are indistinguishable from each other using morphology. Additionally, species identification is sometimes complicated by the occurrence of atypical strains exhibiting unusual morphological characteristics. For example, *A. lacticoffeatus* was described based on its different colony colour and a specific extrolite pattern (Samson *et al.* 2004). However, it was later synonymized with *A. niger*, because it was phylogenetically inseparable (Varga *et al.* 2011). Within the ATL, *A. vadensis* stands out among the others for its slow growth and short stipes (Table 2), but the original description is based on only one isolate (Vries *et al.* 2005).

Series *Nigri* species are known for producing a wide range of extrolites, and their spectra were summarised by Samson *et al.* (2007), Nielsen *et al.* (2009) and Frisvad *et al.* (2018). It is often asserted that species in series *Nigri* could be identified by different secondary metabolite patterns. However, the extrolite profiles can vary among strains, which makes its use to distinguish between species complicated. Additionally, the usability of secondary metabolite production for taxonomy and evolutionary biology in general might be limited due to the potential for horizontal gene transfer of secondary metabolism gene clusters between species of the same genus or even between fungal genera (Richards 2011, Slot & Rokas 2011, Szöllösi *et al.* 2015). The high frequency of this phenomenon is also suggested from the genomes of series *Nigri* species (Vesth *et al.* 2018). Considering our proposed reclassification of series *Nigri*, the extrolite profiles of the six species we accepted need to be reassessed to determine if they support the delineation proposed here based on a phylogenetic species concept.

Sexual reproduction in heterothallic fungi is governed by mating-type (*MAT*) genes (Dyer & O’Gorman 2011). In section *Nigri*, the development of asci and ascospores occurs in the stroma of sclerotia (Horn *et al.* 2013). Therefore, sclerotia formation is considered a prerequisite for sexual reproduction. In the ATL, sclerotia were reported in most species except for *A. eucalypticola*, *A. pseudotubingensis* and *A. vadensis*. In the ANL, sclerotia were formed in some strains of *A. vinaceus* and *A. niger* (Frisvad *et al.* 2014, Silva *et al.* 2020). Sclerotia in the ATL were mostly pink to yellow, while those in the ANL were white to cream (Table 2). Despite the heterogeneity in sclerotia production among series *Nigri* species, most of them were proven to be heterothallic or protoheterothallic,

which means that mating-type gene idiomorph(s) associated with heterothallism have been detected among the examined strains of these species (Houbraken *et al.* 2020). Currently, *A. tubingensis* is the only species in series *Nigri* with an experimentally proven sexual cycle (Horn *et al.* 2013). In this study, we detected both *MAT1-1-1* and *MAT1-2-1* idiomorphs among isolates of ANL and ATL (Fig. 4), in agreement with previous studies (Horn *et al.* 2013, Varga *et al.* 2014, Mageswari *et al.* 2016, Seekles *et al.* 2022). An interesting observation was made by Horn *et al.* (2013), who sequenced five genes in the parental isolates of *A. tubingensis* and their progeny resulting from sexual reproduction. The authors found that *A. neoniger* cannot be separated from *A. tubingensis* in the *benA* or *tsr1* phylogenies, while in the other phylogenies (*CaM*, *Mcm7* and *RPB2*), it is resolved outside the least inclusive clade containing all *A. tubingensis* strains and its ex-type. In our opinion, this fact demonstrates gene flow between *A. tubingensis* and *A. neoniger* and supports the synonymization made in this study. *Aspergillus costaricensis* was not included by Horn *et al.* (2013), but it is a close relative of *A. neoniger* and must be synonymized together to retain the monophyly of the redefined *A. tubingensis* (Fig. 5).

## Updated taxonomy of series *Nigri* and species identification

We showed based on various independent species delimitation analyses that the number of accepted species in the *Nigri* series is too high and needs to be reduced. The methods broadly agreed that the ABL and ANL contain only one species each. The only uncertainty was about the species number in the ATL, where single-gene MSC methods and the GCPSR approach based on the commonly used phylogenetic markers *benA*, *CaM* and *RPB2* supported only one species, while STACEY analyses supported up to four species: *A. tubingensis*, *A. luchuensis*, *A. eucalypticola* and *A. vadensis*. After considering all results and practical consequences, we decided on a conservative solution by retaining four species in the ATL. This newly proposed taxonomic treatment of series *Nigri* with six accepted species overall is shown in Fig. 10.

The following findings played a major role in our decision-making process. *Aspergillus luchuensis* is industrially extremely important (Hong *et al.* 2013, 2014), and its imprudent synonymization without further confirmation could cause unnecessary taxonomic instability. The phylogenetic signal from the dataset containing only *benA*, *CaM* and *RPB2* loci may be suboptimal when compared to the predominant signal obtained from whole genome data (Fig. 4); thus, the results should be considered with caution before data from more genes/genomes are evaluated. We also showed that the intraspecific genetic diversity of the broadly defined *A. tubingensis* would be higher than that commonly found for *Aspergillus* species, while the variability within *A. tubingensis*, excluding *A. luchuensis*, *A. eucalypticola* and *A. vadensis* comfortably falls within this range (Fig. 9).

We believe that the user community will benefit from the simplified taxonomy of series *Nigri* with a lower number of species. This new classification will facilitate species identification that is currently complicated by inconsistent identification results when using sequence data of different genes (Fig. 3) and the impossibility of finding species-specific mass spectra for some narrow species when using the MALDI-TOF method (Gautier *et al.* 2016, D’hooge *et al.* 2019, Ban *et al.* 2021). DNA sequence identification of these newly defined species is possible by using *benA*, *CaM* and *RPB2* markers, although in a minority of cases, there can be conflicting identification in the ATL (Fig. 3). Because of this, we recommend that if identification to a species level is required in the ATL, it should be



Table 2. Overview of macro- and micromorphological features of species belonging to the series *Nigri*.

Species	CYA, 7 d (mm)		MEA, 7 d, 25 °C (mm)	Prevailing colony colour(s) on CYA	Vesicle diam (µm), shape	Stipe (µm)		Conidia: diam (µm), shape, surface	Sclerotia (mm), colour	References <sup>1</sup>
	25 °C	37 °C				Length	Width			
<i>A. brasiliensis</i>	71–76	71–76	52–70	cream-coloured to light brown	30–45, nearly globose	700–1 700	8–13	3.5–4.8, subglobose, echinulate	1–1.5, white	Varga <i>et al.</i> (2007)
<i>A. niger</i>	70–85	70–85	n/a	black	45–80, globose	1 500–3 000	15–20	3.5–5.5, globose, finely to distinctly roughened	0.5–0.7, cream	Crous <i>et al.</i> (2009), Samson <i>et al.</i> (2010), Frisvad <i>et al.</i> (2014)
<i>A. lacticoffeatus</i>	71–76	59–75	52–70	cream to light brown	40–65, nearly globose	(200–)300–1 200	(7–)10–15(–18)	3.5–4.1 × 3.4–3.9, subglobose, usually smooth to very finely roughened	not observed	Samson <i>et al.</i> (2004)
<i>A. vinaceus</i>	64–66	60–63.5	35–39	black	63–75, globose to subglobose	1 300–1 800	16–21	3–5.5, globose to subglobose, finely roughened to echinulate	0.9–1.5, white to cream	Silva <i>et al.</i> (2020)
<i>A. welwitschiae</i>	similar to <i>A. niger</i>	similar to <i>A. niger</i>	similar to <i>A. niger</i>	black	45–85, globose	similar to <i>A. niger</i>	n/a	3.5–5.5, globose, finely to distinctly roughened	not observed	Hong <i>et al.</i> (2013), Silva <i>et al.</i> (2020)
<i>A. chiangmaiensis</i>	62–63	57–66	68–70	dark brown to black	35–68, globose to ellipsoidal	370–1 430	10–15	3–4.5, globose to subglobose, echinulate	0.4–1.4, white	Khuna <i>et al.</i> (2021)
<i>A. costaricensis</i>	63–78	58–62	26–62	white with sparse black-sporulating areas	40–90, globose	(800–)1 000–1 700(–1 900)	(12–)13–20(–22)	3.1–4.5, globose to subglobose, smooth to distinctly roughened	1.2–1.8, pink to yellow	Samson <i>et al.</i> (2004)
<i>A. eucalypticola</i>	68–72	30–50	46–51	beige to cream yellow	30–55, globose	n/a	8–14	2.5–3.5, globose, smooth to coarsely roughened	not observed	Varga <i>et al.</i> (2011)
<i>A. luchuensis</i> <sup>2</sup>	37–80	30–67	43–68	white, gray, brown, black	15–90, subglobose to globose	1 500	10–22	3–4.5, globose, smooth	0.8–1.3, cream	Samson <i>et al.</i> (2010), Varga <i>et al.</i> (2011), Hong <i>et al.</i> (2013), Frisvad <i>et al.</i> (2014)
<i>A. neoniger</i>	72–80	37–67	54–61	gray to black	30–50, globose	n/a	8–12	3.5–5, globose, coarsely roughened to echinulate	observed (without description)	Varga <i>et al.</i> (2011), Frisvad <i>et al.</i> (2014)
<i>A. piperis</i>	60–75	64–82	59–78	cream-coloured with sparse black-sporulating areas	40–55, subglobose	(300–)400–3 000	(7–)12–15(–20)	2.8–3.6, subglobose to broadly ellipsoidal, distinctly roughened	1–1.7, yellow to pink brown	Samson <i>et al.</i> (2004)
<i>A. pseudopiperis</i>	63–65	61–64	55–60	greenish brown to dark brown	15–39, globose to ellipsoidal	200–2 125	10–18	3–5, globose to subglobose, roughened to spinulose	0.2–1 × 0.2–0.8, light yellow to pinkish orange	Khuna <i>et al.</i> (2021)
<i>A. pseudotubingensis</i>	51–58	55–68	62–63	grayish brown to dark brown	20–70, globose to ellipsoidal	740–3 060	10–18	3–6, globose to subglobose, fine spiny surface	not observed	Khuna <i>et al.</i> (2021)
<i>A. tubingensis</i>	n/a	n/a	40–65	beige, grayish brown, brown to black	27–80, globose	550–2 300(–6 000)	10–35	3–5, globose, coarsely roughened	0.4–1.8, cream to pinkish tan	Samson <i>et al.</i> (2007), Varga <i>et al.</i> (2007), Hong <i>et al.</i> (2013), Horn <i>et al.</i> (2013), Ismail (2017)
<i>A. vadensis</i>	25	n/a	30	light brown to olive green brown	25–35, globose	up to 150	6–15	3–4, globose, rough-walled to finely echinulate	not observed	de Vries <i>et al.</i> (2005)

n/a - exact data not available.

<sup>1</sup>if there are multiple studies in the column "references", the description and dimensions are shown as a summary from all these sources.<sup>2</sup>the dimensions shown here represent summary from description of *A. luchuensis* and its synonym *A. acidus* as they appeared in the publications of Varga *et al.* (2011) and Hong *et al.* (2013).

***A. tubingensis***

- 1 *A. costaricensis*
- 4 *A. neoniger*
- 6 *A. tubingensis*

OTHER SYNONYMS:

- *A. chiangmaiensis*
- *A. pseudopiperis*

*A. cinnamomeus*, *A. elatior*,  
*A. hennenbergii*, *A. pseudoniger*,  
*A. pulverulentus*, etc.  
(see section Taxonomy)

***A. luchuensis***

- 3 *A. luchuensis*
- 5 *A. piperis*

OTHER SYNONYMS:

*A. acidus*, *A. awamori*, *A. inuii*,  
*A. kawachii*, *A. nakazawae*,  
*A. perniciosus*, etc.  
(see section Taxonomy)

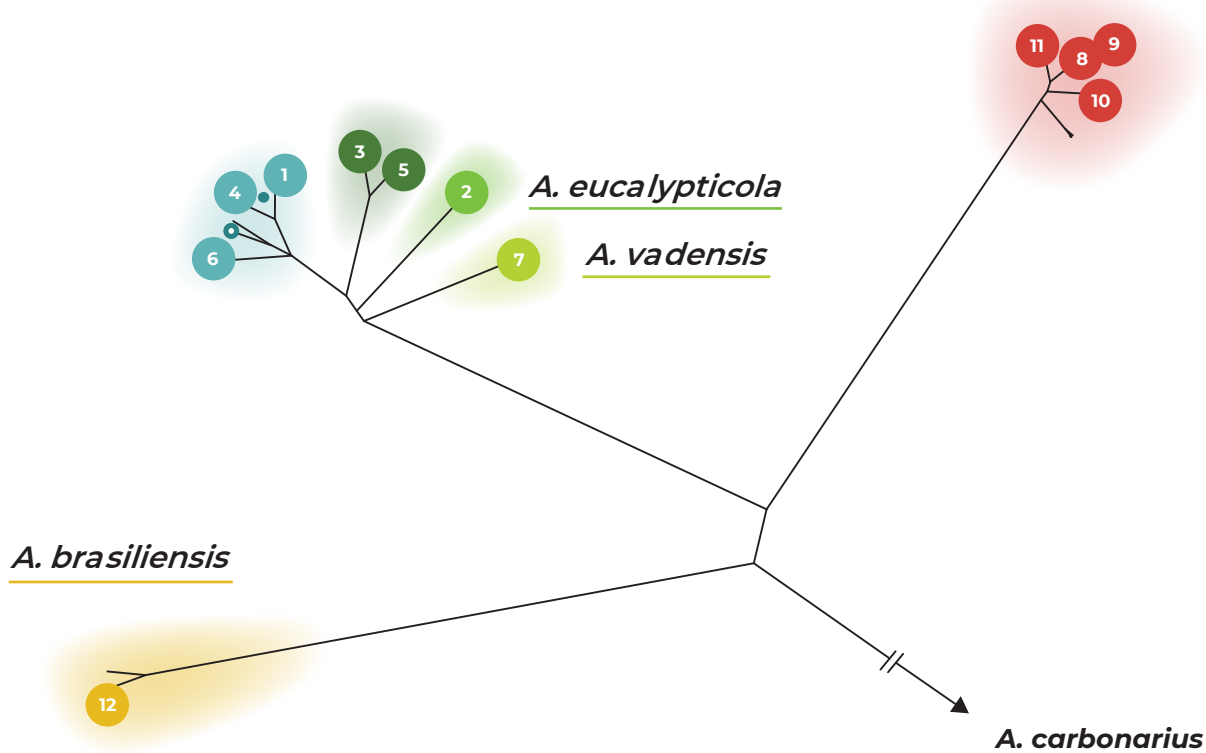
***A. niger***

- 8 *A. lacticoffeatus*
- 9 *A. niger*
- 10 *A. vinaceus*
- 11 *A. welwitschiae*

OTHER SYNONYMS:

*A. batatas*, *A. ficuum*, *A. citricus*,  
*A. foetidus*, *A. longobasidia*,  
*A. pseudocitricus*, *A. usamii*, etc.  
(see Taxonomy section)

0.01



**Fig. 10.** Taxonomic rearrangement of series *Nigri* into six species with marked synonymizations. The proposed taxonomy is schematically shown in the form of a radial tree (species tree based on *benA*, *CaM* and *RPB2* calculated in starBEAST). The positions of *A. chiangmaiensis* and *A. pseudopiperis* are approximated based on the tree shown in Figs 1, 3 as they were not included in this phylogenetic analysis.

done through DNA sequencing of all three markers and performing phylogenetic analysis. The ITS barcoding sequence can only distinguish among ABL, ANL and ATL (Supplementary Fig. S3).

As mentioned above, we excluded three newly described species, *A. chiangmaiensis*, *A. pseudopiperis* and *A. pseudotubingensis* (Khuna *et al.* 2021), from the analyses based on the MSC model because of the poor quality of their available sequences. However, we propose that *A. chiangmaiensis* and *A. pseudopiperis* are synonyms of *A. tubingensis*, as their phylogenetic position in the combined ML, MP and BI trees is within the redefined *A. tubingensis*: the *A. pseudopiperis* ex-type strain (SDBR-CMU1) is located in a clade sister to the clade containing the *A. tubingensis* ex-type (CBS 133056), and *A. chiangmaiensis* is closely related to *A. costaricensis* and *A. neoniger* (Figs 1, 3), which are also synonymized with *A. tubingensis*. The status of *A. pseudotubingensis* remains uncertain and needs to be revised in the future as it forms a relatively separate lineage in the combined phylogeny based on three loci similar to *A. eucaalypticola* (Figs 1, 3). The phylogeny presented by Khuna *et al.* (2021) is affected by

numerous errors in the DNA sequences of the newly described species and it shows clear signs of the long branch attraction phenomenon (Kück *et al.* 2012). We believe that a more reliable position is shown in our phylogenies constructed based on partially corrected sequences (Figs 1, 3).

The species reduction proposed in this study is in line with some other studies using MSC model-based methods, genomic data or more complex approaches for species delimitation in fungi. For example, species number was significantly reduced in the plant pathogens from the *Diaporthe eres* complex (Hilário *et al.* 2021) and *Alternaria* section *Alternaria* (Woudenberg *et al.* 2015), the edible mushroom *Flammulina* (Wang *et al.* 2018), the lichen-forming fungus *Bryoria* (Boluda *et al.* 2019), the indoor fungi from *Aspergillus* series *Versicolores* (Sklenář *et al.* 2022) and the opportunistic human and animal pathogens from *Aspergillus* series *Viridinutantes* (Hubka *et al.* 2018). Numerous examples can also be found outside the *Fungi* kingdom (Li *et al.* 2019, Feng *et al.* 2021, Parker *et al.* 2021). These studies demonstrate that species over splitting similar to that observed in series *Nigri* is relatively common

in extensively studied groups and species number reduction can become an increasingly common trend in taxonomy.

## Future prospects

Further reduction of species number in the series *Nigri* cannot be ruled out in the future after collecting more strains and more whole genome sequences from underrepresented taxa such as *A. eucalypticola* and *A. vadensis*. Inclusion of more variability from these rare taxa should resolve persistent ambiguities in the definition of species limits and increase the probability of methods to delimit species more conclusively. As mentioned above, the position of *A. pseudotubingensis* should be re-examined, and higher quality DNA sequences should be obtained, ideally whole genome sequences. For now, we recommend avoiding using this name until the abovementioned issues are resolved.

Advanced phylogenetic methods spanning the whole genome will allow for a systematic and comparable metric of species differentiation. For example, reciprocal monophyly and a high level of concordance between thousands of markers on the genome scale should allow better differentiation between processes such as recombination, incomplete lineage sorting and speciation in most cases (Kobmoo *et al.* 2019, Matute & Sepúlveda 2019, Mavengere *et al.* 2020). A dense population-level representation in genome-scale phylogenetic studies is important for accurate identification of species boundaries and for resolving evolutionary relationships between species and higher-level taxonomic ranks in *Aspergillus* (Steenwyck *et al.* 2022). Our efforts in the following years will be directed towards achieving this goal.

## DECLARATION ON CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ACKNOWLEDGMENTS

Vit Hubka was supported by the Czech Ministry of Health (grant NU21-05-00681), the Charles University Research Centre program no. 204069 and the Czech Academy of Sciences Long-term Research Development Project (RVO: 61388971). František Sklenář was supported by the project of Charles University Grant Agency (GAUK 380821). This work has been partly supported by JST SPRING awarded to Cai Bian (JPMJSP2109). CMV acknowledges the Plant Health and Protection - Agricultural Research Council (ARC-PHP), where research on the PPRI strains included in this study was initiated and financially supported by a grant (nr 110441; reference FBIS170406226088) received from the Foundational Biodiversity Information Programme (FBIP) of the National Research Foundation of South Africa. VH is grateful for the support from the Japan Society for the Promotion of Science - Postdoctoral Fellowships for Research in Japan (Standard) and Grant-in-aid for a JSPS research fellow (No. 20F20772). We are grateful to Jan Karhan for the help with graphical adjustments of analysis outputs. We thank Kateřina Glässnerová for her assistance in the laboratory and Osamu Yamada for providing copies of some old taxonomic studies.

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**Supplementary Material:** <https://studiesinmycology.org/>

**Fig. S1.** Multilocus phylogeny of *Aspergillus* series *Nigri* based on three loci (*benA*, *CaM* and *RPB2*) without species *A. chiangmaiensis*, *A. pseudopiperis* and *A. pseudotubingensis*. Best-scoring Maximum Likelihood (ML) tree inferred in the IQ-TREE is shown; ultrafast bootstrap support values (ML bs) are appended to nodes; only support values  $\geq 95\%$  are shown; the ex-type strains are designated with a bold print; the information on geographic origin and isolation source is plotted on the tree (see the legend).

**Fig. S2.** Comparison of single-gene genealogies based on the *benA*, *CaM* and *RPB2* loci and created by three different phylogenetic methods (only one isolate per unique multilocus haplotype is included in each phylogeny). Best-scoring single-locus maximum likelihood (ML) trees are shown; ML ultrafast bootstrap support values (ML bs), maximum parsimony bootstrap support values (MP bs) and Bayesian inference posterior probabilities (BI pp) are appended to nodes. Only support values  $\geq 95\%$ ,  $\geq 70\%$  and  $\geq 0.95$ , respectively, are shown. A dash indicates lower statistical support for a specific node, or the absence of a node in the phylogeny, while an asterisk indicates full support. The ex-type strains are designated with a bold print. Alignment characteristics, partitioning schemes and substitution models are listed in Supplementary Table S1.

**Fig. S3.** Best scoring maximum likelihood tree inferred in IQ-TREE based on ITS rDNA region sequences of series *Nigri* members (Table 1). It is apparent from the tree that a limited variability present in this locus can only differentiate the three main lineages, and more detailed differentiation is not possible. The ITS sequence of the *A. vadensis* ex-type was excluded from the analysis because it contains numerous errors and unresolved positions (accession number AY585549). Alignment characteristics, partitioning schemes and substitution models are listed in Supplementary Table S1.

**Table S1.** Characteristics of alignments, partition-merging results and best substitution model for each partition according to the Bayesian information criterion.

**Table S2.** Assignment of strains into populations for analysis in DELINEATE based on BPP v. 4.3.

**Table S3.** *Aspergillus* series *Nigri* genomes analyzed in this study and their basic characteristics.

**Table S4.** Distribution of 200 orthologous genes into ten separate STACEY analyses.

**Table S5.** List of unique multilocus haplotypes.

**Table S6.** Maximum sequence dissimilarity between isolates of the same *Aspergillus* species whose species limits have been delimited using methods based on a multispecies coalescent model; only species represented by isolates from at least three countries were included.