Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2021.012075

ARTICLE

A New Record of *Aspergillus vadensis* (Ascomycota) Isolated from Soil in Yunnan Province, China

Sadia Nadir[1,2,3], Sehroon Khan[1], Dhanushka N. Wanasinghe[1,2], Saowaluck Tibpromma[1,2], Shahid Iqbal[1,2], Jianchu Xu[1,2,*] and Peter Mortimer[1,2,*]

[1]CAS Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Science, Kunming, 650201, China

[2]Centre for Mountain Futures, Kunming Institute of Botany, Kunming, 654400, China

[3]Department of Chemistry, University of Science and Technology, Bannu, 28100, Pakistan

*Corresponding Authors: Jianchu Xu. jxu@mail.kib.ac.cn; Peter Mortimer. peter@mail.kib.ac.cn

Received: 13 June 2020 Accepted: 12 October 2020

ABSTRACT

During a survey of soil fungi in Yunnan Province, several isolates of *Aspergillus* were obtained. Based on morphology and molecular analyses of internal transcribed spacer regions and intervening 5.8S nrRNA gene (ITS), β-tubulin and calmodulin (CaM) genes sequences, two isolates were identified as *Aspergillus vadensis* (section *Nigri*). A phylogenetic tree, detailed descriptions, illustrations and scanned electron microscopy morphology are provided for the new isolates. This is the first record of *A. vadensis* from China.

KEYWORDS

Morphology; phylogeny; section *Nigri;* soil fungi

1 Introduction

Aspergillus is a large and diverse fungal genus in the family *Aspergillaceae*, with over 400 recognized species [1] classified into six subgenera and 25 sections [2]. The genus *Aspergillus* is one of the most studied fungal genera due to its application in human and animal health, the food industry, enzyme technology, pharmacology, and as genetic model organisms [3–6].

Aspergillus sect. *Nigiri*, known as 'black aspergilli' [7], includes 27 accepted species [8] occurring predominantly in soil, but they have also been isolated from other sources [2,9–11]. Species in this section are versatile and are of industrial and biotechnological importance [12–14]. *Aspergillus niger* is used in the fermentation industry for the production of enzymes and citric acid and has been granted status as a GRAS (generally recognized as safe) organism [12,14]. Other members are exploited for the production of biopharmaceuticals, mycotoxins and a variety of extracellular enzymes [12,13]. *Aspergillus tubingenesis* was reported to be a degrader of polyurethane plastics, a promising discovery in the field of mycoremediation [15]. Some species in this section are reported to be pathogenic to both humans and animals [16,17] and are also known to produce ochratoxin, a mycotoxin that is toxic and carcinogenic [18].

Members of the section *Nigri* have been divided into two separate clades based on their conidiophore morphology: Biseriate species (e.g., *A. carbonarius* and *A. niger* complex) and uniseriate species

(e.g., *A. aculeatus* and *A. japonicas*), which have been shown to exhibit differences in their sexual morphs, sclerotium formation and extrolite profiles [19].

Aspergillus vadensis belongs to the section *Nigri* and was first identified in 2004 [20]. This species displays several unique characteristics and differs from other members of sect. *Nigri* in its restriction fragment length polymorphism (RFLP) and production of secondary metabolites. Unlike other fungi of this section that predominantly produce black colonies, *Aspergillus vadensis* was found to produce olive-green to brown colonies when grown on Czapek Yeast Agar (CYA) and malt extract agar (MEA) media.

During a survey of soil microfungi in Yunnan Province, China, several axenic cultures of *Aspergillus* were obtained. The objectives of this study were (1) To sequence one non-translated loci and two protein-coding genes of the new isolates; (2) Identify phylogenetic relationships of the new isolates, with close affinities in *Aspergillus* by using ML, MP and BI analyses; and (3) Provide comprehensive morphological descriptions, micro-photographic illustration and discussions.

2 Materials and Methods

2.1 Soil Sampling and Fungal Isolation

The study was carried out in February 2018, utilizing abandoned (over 12 months) agricultural land in Kunming, Yunnan Province, China. First, surface litter was scraped away to obtain a uniformly thick slice of soil from the surface to the plough depth at each spot. Next, a V-shaped cut was made with a sterilized spade (using 70% ethanol) to remove a 1–2 cm slice of soil and placed into a clear plastic bag. After that, the soil was cleared of large objects such as stones, litter, wood pieces and plant roots by removing them with gloved hands. Using this method, samples were collected from five randomly selected spots within a 20 m × 20 m area. Later, these samples were pooled into a single sample. *Aspergillus* species were obtained from this pooled soil sample by using the soil plate method [21] on malt extract agar (MEA) media in petri dishes, with the incubation temperature at 28°C for seven days.

2.2 Molecular Study

DNA was isolated from pure cultures (seven days old) grown on MEA. DNA was extracted using Biospin Fungal Genomic DNA isolation Kit–BSC14S1 (BioFlux, China). Polymerase chain reaction (PCR) was used to amplify partial gene regions of internal transcribed spacer regions and intervening 5.8S nrRNA gene (ITS), β-tubulin and calmodulin (CaM) gene regions as described by Tanikiwa et al. [22]. Primers used are shown in Tab. 1. PCR amplicons were separated on 1% agarose gel and sequenced by Sangon Biotech, Shanghai, China.

Table 1: List of genes and primers used in this study

Genes	Primer	References
ITS	ITS1/ITS4	[23]
β-tubulin	BT2a/BT2b	[24]
Calmodulin	cmd5/cmd6	[25]

2.3 Phylogenetic Analyses

BLASTn searches were made using the newly generated sequences to determine the closest relatives for the taxonomic framework. Sequence data used for phylogenetic analyses in this study were downloaded from the NCBI database based on recently published data [7] (Tab. 2). Each locus was automatically aligned with MAFFT v. 7 [26] after which alignments were manually checked and improved using BioEdit v. 7.0.5.2 [27]. Phylogenetic methods used in this study included maximum-likelihood (ML), maximum parsimony (MP)

and Bayesian criteria (BI). Generated phylograms were visualized using the FigTree v1.4.0 program [28], reorganized in Microsoft PowerPoint (2016) and deposited in TreeBASE, submission ID: 26171.

Table 2: The species and strains used in the phylogenetic analyses

Species name	Strain	GenBank accession numbers		
		ITS	β-tubulin	CaM
Aspergillus aculeatinus	CBS 121060^T	EU159211	EU159220	EU159241
Aspergillus aculeatus	CBS 172.66^T	NR_111412	HE577806	EF661148
Aspergillus brasiliensis	CBS 101740^T	FJ629321	FJ629272	FN594543
Aspergillus brunneoviolaceus	CBS 621.78^T	AJ280003	EF661105	EF661147
Aspergillus carbonarius	CBS 111.26^T	EF661204	EF661099	EF661167
Aspergillus costaricaensis	CBS 115574^T	DQ900602	FJ629277	FN594545
Aspergillus costaricaensis	IHEM 21971	MH613134	MH614546	MH644993
Aspergillus costaricaensis	IHEM 25327	MH613139	MH614421	MH644994
Aspergillus ellipticus	CBS 482.65^T	EF661221	EF661083	EF661148
Aspergillus eucalypticola	CBS 122712^T	EU482439	EU482435	EU482433
Aspergillus fijiensis	CBS 313.89^T	FJ491680	FJ491688	FJ491695
Aspergillus flavus	CBS 569.65^T	AF027863	EF661485	EF661508
Aspergillus floridensis	NRRL 62479^T	–	HE984412	HE984429
Aspergillus heteromorphus	CBS 117.55^T	EU821305	EF661103	EF661169
Aspergillus homomorphus	CBS 101889^T	EF166063	AY820015	FN594549
Aspergillus ibericus	NRRL 35644^T	EF661200	EF661102	EF661163
Aspergillus indologenus	CBS 114.80^T	AJ280005	AY585539	AM419750
Aspergillus japonicas	CBS 114.51^T	AJ279985	HE577804	FN594551
Aspergillus labruscus	ITAL 22.223^T	KU708544	KT986014	KT986008
Aspergillus luchuensis	CBS 205.80^T	JX500081	JX500062	JX500071
Aspergillus luchuensis	IHEM 25486	MH613118	MH614563	MH644862
Aspergillus luchuensis	IHEM 26285	MH613141	MH614561	MH644869
Aspergillus neoniger	CBS 115656^T	FJ491682	FJ491691	FJ491700
Aspergillus neoniger	IHEM 18136	MH613121	MH614456	MH644983
Aspergillus neoniger	IHEM 18329	MH613202	MH614467	MH644992
Aspergillus neoniger	NRRL 62634	KC796401	KC796361	KC796377
Aspergillus niger	CBS 554.65^T	EF661186	EF661089	EF661154
Aspergillus niger	IHEM 2312	MH613218	MH614521	MH645010
Aspergillus niger	IHEM 5296	MH613217	MH614519	MH645009
Aspergillus niger	IHEM 9673	MH613219	MH614523	MH645012
Aspergillus piperis	CBS 112811^T	EU821316	FJ629303	EU163267
Aspergillus piperis	IHEM 23904	MH613164	MH614551	MH644990

(Continued)

Table 2 (continued).

Species name	Strain	GenBank accession numbers		
		ITS	β-tubulin	CaM
Aspergillus piperis	IHEM 5316	MH613216	MH614562	MH644893
Aspergillus saccharolyticus	CBS 127449^T	NR_135441	HM853553	HM853554
Aspergillus sclerotiicarbonarius	CBS 121057^T	EU159216	EU159229	EU159235
Aspergillus sclerotioniger	CBS 115572^T	DQ900606	FJ629304	FN594557
Aspergillus trinidadensis	NRRL 62479^T	–	HE984420	HE984434
Aspergillus tubingensis	IHEM 17441	KP131628	MH614541	MH644925
Aspergillus tubingensis	IHEM 17893	KP131630	MH614542	MH644926
Aspergillus tubingensis	IHEM 17903	KP131631	MH614543	MH644927
Aspergillus tubingensis	NRRL 4875^T	EF661193	EF661086	EF661151
Aspergillus uvarum	CBS 121591^T	AM745757	AM745751	AM745755
Aspergillus vadensis	CBS 113365^T	AY585549	AY585531	FN594560
Aspergillus vadensis	IHEM 26351	MH613142	MH614547	MH644878
Aspergillus vadensis	**KUMCC 19-0219**	**MN698977**	**MN752699**	**MT547194**
Aspergillus vadensis	**KUMCC 19-0220**	**MN698977**	**MT394617**	**MT547195**
Aspergillus welwitschiae	CBS 139.54^T	FJ629340	FJ629291	KC480196
Aspergillus welwitschiae	IHEM 15980	MH613210	MH614518	MH644976
Aspergillus welwitschiae	IHEM 6350	MH613214	MH614471	MH644977
Aspergillus welwitschiae	IHEM 9388	MH613220	MH614438	MH644979

* T = ex-type strains; Bold = new sequences.

2.4 Morphological Analysis

For macromorphological observations, colonies were grown on MEA and Czapek yeast extract agar (CYA) media. The cultures were inoculated in the center of each plate and incubated at 28°C for seven days. The culture plates were observed for colony colour, size, texture and production of exudates. Microscopic mounts prepared in lactic acid were used to study the microscopic features. Scanning electron microscopy (SEM, Hitachi S570) was performed as described previously by Khan [15].

3 Results and Discussion

3.1 Phylogenetic Analysis

The combined alignment (ITS, β- tubulin and CaM) contained 51 strains with 2094 characters representing section *Nigri*, the new strains proposed in this study, and the outgroup taxon (*Aspergillus flavus*). The topology of the multi-locus ML, MP phylogenetic trees and Bayesian inference is largely concordant, and the same clades are supported. The overall topology of these yielded trees was similar to previous work [7,14]. The best scoring tree with RAxML analysis gave a final optimization value of −13115.709914 (Fig. 1). The ML model of the concatenated data showed the following parameters: Estimated base frequencies: A = 0.214563, C = 0.284009, G = 0.262361, T = 0.239066; substitution rates: AC = 0.894395, AG = 2.869504, AT = 1.314187, CG = 0.567345, CT = 4.215652, GT = 1.000; proportion of invariable sites: I = 0.397753; gamma distribution shape parameter α = 1.204389. The MP analyses generated the maximum of 26 equally most parsimonious trees, and the most parsimonious is

shown in Fig. 1 with the following parameters: Length = 2244, CI = 0.638, RI = 0.869, RC = 0.555 and HI = 0.362. From the analysed characters, 1192 were constant, 180 were variable and parsimony-uninformative and 722 were parsimony-informative. For Bayesian analysis, dirichlet base frequencies and the GTR+I+G model were recommended by MrModelTest. The dataset comprised 1101 unique patterns. Neglecting the initial 20% of trees, 1601 trees remained and were used to calculate Bayesian PP (Fig. 1; third value: PP > 0.95 shown).

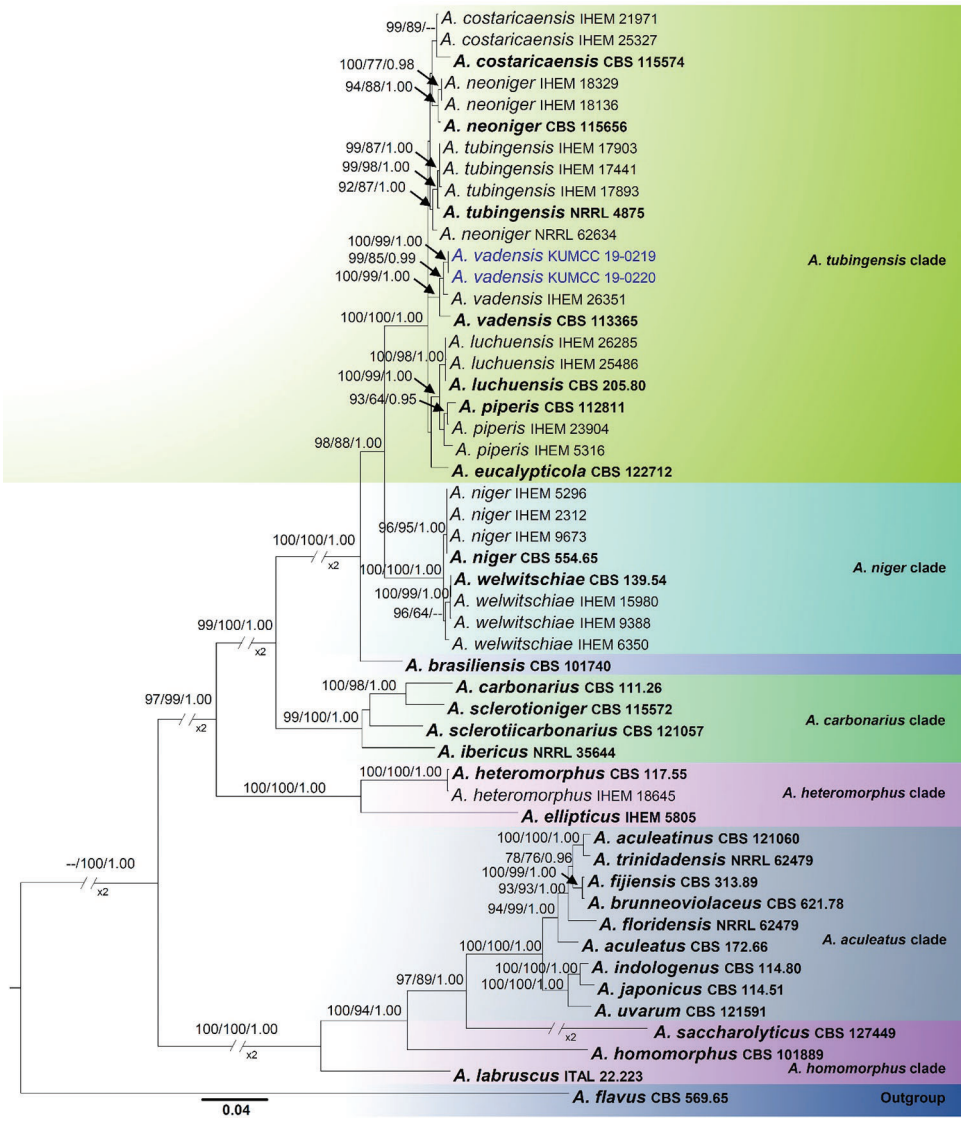

Figure 1: Phylogenetic tree based on analysis of the combined sequence data of ITS, beta tubulin and calmodulin gene region for *Aspergillus* section *Nigri*. Bootstrap support values >60% for ML and MP, and Bayesian PP values higher than 0.95% are defined above the internal branches, respectively. The tree is rooted to *Aspergillus flavus* (CBS 569.65) in the section *flavi*. Bold = ex-type strains; Blue = new isolate

In the concatenated sequence data analysis, *A. aculeatus*, *A. carbonarius*, *A. homomorphus*, *A. niger* and *A. tubingensis* clades were supported by high bootstrap support values (Fig. 1). But the species in the *A. heteromorphus* clade are not monophyletic and constitute a basal lineage sister to the *A. aculeatus*

clade. Two of our new isolates, KUMCC 19-0219 and KUMCC 19-0220, clustered in the *A. tubingensis* clade (Fig. 1). Within the *A. tubingensis* clade, our new isolates were monophyletic with *A. vadensis* (CBS 113365 and IHEM 26351), and this relationship was statistically well-supported (ML = 100%, MP = 99% and PP = 1.00, Fig. 1)

3.2 Taxonomy

Aspergillus vadensis Samson, de Vries, Frisvad & Visser, Antonie van Leeuwenhoek 87 (3): 201 (2005)

IndexFungorum number: IF340234

Colony dimensions after 2 days at 25°C were as follows: MEA: 24 mm–25 mm, CYA: 21 mm–22 mm. Colony colours and texture on MEA for 7 days showed seriate margins, upper surface granular and black, flat mycelium white in beginning become black after 10 days, sporulate within 24 hours, densely covered with black conidial heads, conspicuous exudates like big black droplets, and creamy white wrinkled reverse that becomes dull yellow with age. Colony colours and texture on CYA for 7 days showed circular colony with curled edge, mycelium white, flossy, velvety with sporulate after 24 h, densely covered with black conidial heads. *Mycelium* composed of hyaline, branched, septate, smooth-walled hyphae. *Conidial heads* radiating. *Stipes* 4 µm–7.5 µm (n = 15) µm diam., hyaline, smooth to finely rough-walled, variable in length, just below vesicles 5 mm–8 mm. *Vesicles* 25–35 × 30–35 µm ($\bar{x}$ = 31 × 34 µm, n = 15), globose to subglobose, hyaline, entirely covered by phialides, *Conidiophores* 4–6 × 3–4 µm ($\bar{x}$ = 5 × 3.5 µm, n = 15) uniseriate or biseriate, hyaline, cylindrical to ampulliform, smooth-walled, wide at the top, non-septate or with occasional septum, reduced short conidiophores or solitary phialides frequently present on the aerial mycelium. *Conidia* 3–5 × 3–5 µm ($\bar{x}$ = 3.87 × 3.89 µm, n = 30), arranged in long chains, globose to subglobose, echinulate, hyaline to greenish and coarsely roughened.

Material examined: CHINA, Yunnan Province, Kunming, Panlong, 25.135016° N, 102.747458° E, from soil, 22 February 2018, Sadia Nadir, G3, MFLU 20–0141, living culture KUMCC 19–0219; from soil, G7, MFLU 20–0142, living culture KUMCC 19–0220.

Notes: de Vries et al. [20] introduced *Aspergillus vadensis* from air in Egypt as a taxon in *Aspergillus* section *Nigri*. During our investigation on the diversity of soil microfungi in Yunnan Province, two isolates were recovered from soil samples in Kunming. Morphological characters such as conidial heads, vesicles, conidiophores and conidia fit well within the type description of *Aspergillus vadensis*, which was derived from culture. In the phylogenetic study, our new strains cluster with *Aspergillus vadensis* as a monophyletic clade with 100% ML, 99% MP and 1.00 PP support (Fig. 1). Comparison of ITS, beta tubulin and calmodulin sequence data revealed there is no significant difference (no differences in ITS, 3 pb differences in beta tubulin and 6 bp differences in calmodulin) between our new isolate and *Aspergillus vadensis*. Therefore, we would like to keep our new isolates as new records of *Aspergillus vadensis* from China.

Species within *Aspergillus* section *Nigri* require both morphological and molecular analyses for accurate species delamination. For the phylogenetic analyses, ITS alone is not sufficient. Additional gene regions (β-tubulin and calmodulin) are recommended [11,12,29], and our analyses were conducted accordingly. Despite the fact that the new isolates bear close morphological and phylogenetic resemblances to the type species of *Aspergillus vadensis*, there are some observed differences worth mention. The ex-type culture has brown to olive-green colony on CYA and MEA while we observed white mycelium that became black upon maturation on these media. We observed that the colonies grown on MEA (Fig. 2a) and colonies grown on CYA (Fig. 2b) differed from each other in their appearance (top view), and this was not reported from the ex-type culture. Phialides of the new isolates were uniseriate at the immature stage (3 days) and became biseriate after 7 days. This was also not mentioned in de Vries et al. [20]. Furthermore, the new isolates were found to produce exudates on MEA appearing as big black droplets, which was also not reported from the type description.

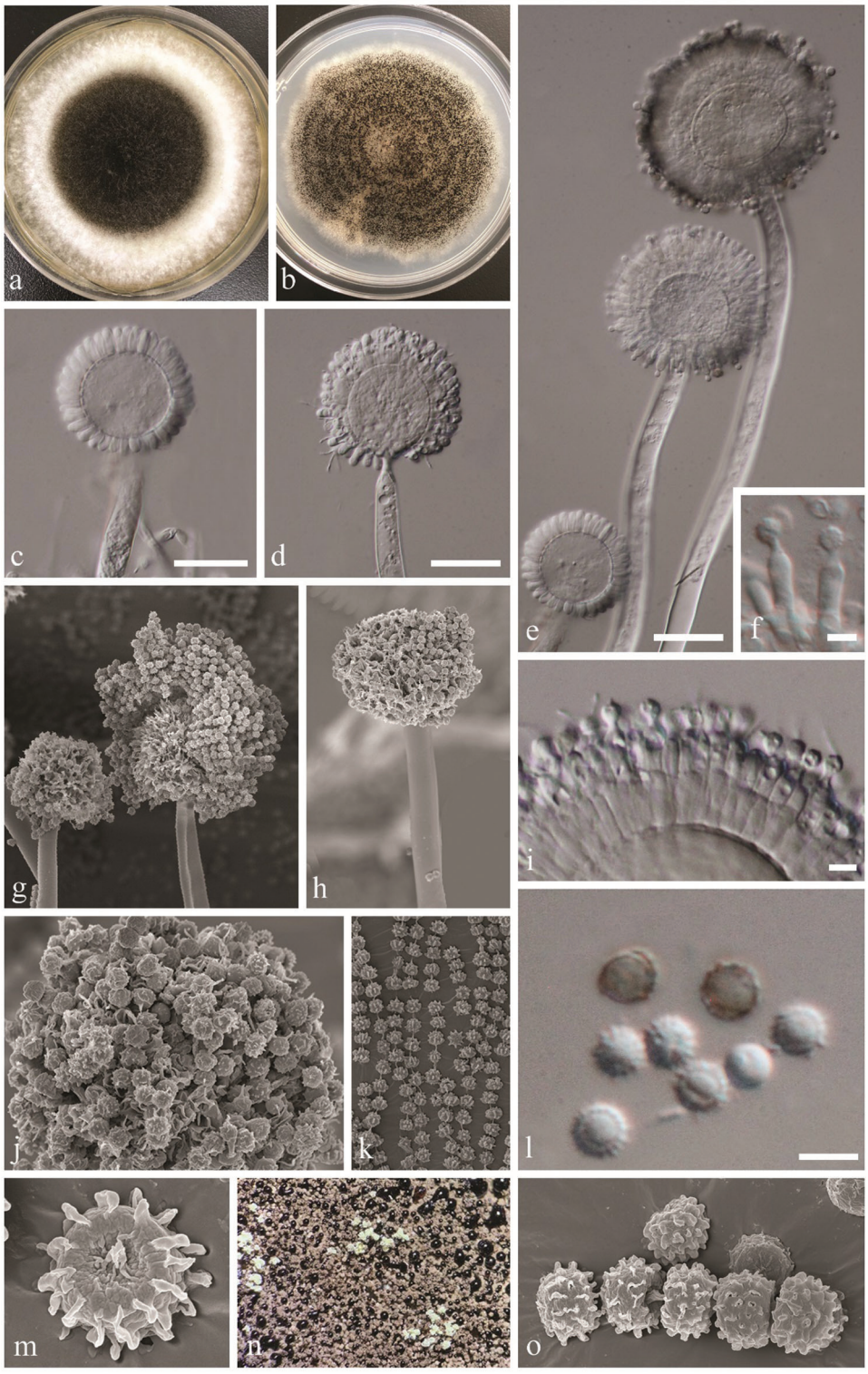

Figure 2: *Aspergillus vadensis* (KUMCC 19-0219, new record). a: Culture on MEA at 28°C. b: Culture on CYA at 28°C. c, d, e: Conidiophores. f, i: Close up conidia head and conidia (note: phialides attached to metulae in i). g, h: Conidiophores and conidia under SEM. j, k, m, o: Conidia under SEM. l: Close up conidia. n: Culture surface and exudate production. Scale bars: b–e: = 20 µm, f, l = 5 µm, i = 10 µm

Funding Statement: This research was funded by Key Research Project "Agroforestry Systems for Restoration and Bio-industry Technology Development", Grant No. 2017YFC0505101. S. Khan, S. Tibpromma and D. Wanasinghe are thankful to the CAS President's International Fellowship Initiative (PIFI Grant Nos. 2019PC0011, 2020PC0009 and 2019PC0008) and S. Tibpromma is thankful to the International Postdoctoral Exchange Fellowship Program (No. Y9180822S1), China Postdoctoral Science Foundation, Yunnan Human Resources and Social Security Department Foundation. D. Wanasinghe is thankful to the 64th batch of China Postdoctoral Science Foundation (Grant No. Y913083271). P. Mortimer is thankful to the National Science Foundation of China (NSFC), Project Codes 41761144055 and 41771063.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

1. Wijayawardene, N. N., Hyde, K. D., Al-Ani, L. K. T. (2020). Outline of Fungi and fungus-like taxa. *Mycosphere, 11(1),* 1060–1456. DOI 10.5943/mycosphere/11/1/8.
2. Samson, R. A., Visagie, C. M., Houbraken, J. J., Hong, S. B., Frisvad, J. C. (2014). Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Studies in Mycology, 78,* 141–173. DOI 10.1016/j.simyco.2014.07.004.
3. Punt, P. J., van Biezen, N., Conesa, A., Albers, A., Mangnus, J. et al. (2002). Filamentous fungi as cell factories for heterologous protein production. *Trends in Biotechnology, 20(5),* 200–206. DOI 10.1016/S0167-7799(02)01933-9.
4. Wösten, H., Scoltmeijer, K., de Vries, R. (2007). Dijksterhuis, J., Samson, R. (eds.), *Food mycology: A multifaceted approach to fungi and food*. pp. 183–196. Boca Raton, FL, USA: CRC Press.
5. Nierman, W. C., Pain, A., Anderson, M. J., Wortman, J. R., Kim, H. S. et al. (2005). Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature, 438(7071),* 1151–1156. DOI 10.1038/nature04332.
6. Meijer, M., Houbraken, J. A. M. P., Dalhuijsen, S., Samson, R. A., de Vries, R. P. (2011). Growth and hydrolase profiles can be used as characteristics to distinguish *Aspergillus niger* and other black aspergilli. *Studies in Mycology., 69(Suppl 1),* 19–30. DOI 10.3114/sim.2011.69.02.
7. D'hooge, E., Becker, P., Stubbe, D., Normand, A., Piarroux, R. et al. (2019). Black aspergilli: A remaining challenge in fungal taxonomy. *Medical Mycology, 57(6),* 773–780. DOI 10.1093/mmy/myy124.
8. Vesth, T. C., Nybo, J. L., Theobald, S., Frisvad, J. C., Larsen, T. O. et al. (2018). Investigation of inter- and intraspecies variation through genome sequencing of *Aspergillus* section *Nigri*. *Nature Genetics, 50(12),* 1688–1695. DOI 10.1038/s41588-018-0246-1.
9. Abrunhosa, L., Calado, T., Venancio, A. (2011). Incidence of fumonisin B2 production by *Aspergillus niger* in Portuguese wine regions. *Journal of Agricultural and Food Chemistry, 59(13),* 7514–7518. DOI 10.1021/jf202123q.
10. Palumbo, J. D., O'Keefe, T. L., Vasquez, S. J., Mahoney, N. E. (2011). Isolation and identification of ochratoxin A-producing *Aspergillus s*ection *Nigri* strains from *California raisins*. *Letters in Applied Microbiology, 52(4),* 330–336. DOI 10.1111/j.1472-765X.2011.03004.x.
11. Perrone, G., de Girolamo, A., Sarigiannis, Y., Haidukowski, M. E., Visconti, A. (2013). Occurrence of ochratoxin A, fumonisin B2 and black aspergilli in raisins from Western Greece regions in relation to environmental and geographical factors. *Food Additives & Contaminants Part A Chemistry Analysis, Control, Exposure and Risk Assessment, 30(7),* 1339–1347. DOI 10.1080/19440049.2013.796594.
12. Varga, J., Frisvad, J. C., Kocsubé, S., Brankovics, B., Tóth, B. et al. (2011). New and revisited species in *Aspergillus* section *Nigri*. *Studies in Mycology, 69,* 1–17. DOI 10.3114/sim.2011.69.01.
13. Pel, H. J., de Winde, J. H., Archer, D., Dyer, P. S., Hofmann, G. et al. (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology, 25(2),* 221–231. DOI 10.1038/nbt1282.
14. Fungaro, M. H. P., Ferranti, L. S., Massi, F. P., de Silva, J. J., Sartori, D. et al. (2017). *Aspergillus labruscus* sp. nov., a new species of *Aspergillus* section *Nigri* discovered in Brazil. *Scientific Reports, 7(1),* 5736. DOI 10.1038/s41598-017-06589-y.

15. Khan, S. (2017). Biodegradation of polyester polyurethane by *Aspergillus tubingensis*. *Environmental Pollution., 225(8),* 469–480. DOI 10.1016/j.envpol.2017.03.012.
16. Serra, R., Abrunhosa, L., Kozakiewicz, Z., Venâncio, A. (2003). Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes. *International Journal of Food Microbiology, 88(1),* 63–68. DOI 10.1016/S0168-1605(03)00085-0.
17. Bui-Klimke, T. R., Wu, F. (2013). Ochratoxin A and human health risk: A review of the evidence. *Critical Reviews in Food Science and Nutrition, 55(13),* 1860–1869. DOI 10.1080/10408398.2012.724480.
18. Abarca, M. L., Accensi, F., Bragulat, M. R., Cabañes, F. J. (2001). Current Importance of Ochratoxin A–Producing *Aspergillus* spp. *Journal of Food Protection, 64(6),* 903–906. DOI 10.4315/0362-028X-64.6.903.
19. Serra, R., Cabanes, F. J., Perrone, G., Castellá, G., Venâncio, A. et al. (2017). *Aspergillus ibericus*: A new species of section *Nigri* isolated from grapes. *Mycologia, 98(2),* 295–306. DOI 10.1080/15572536.2006.11832702.
20. de Vries, R. P., Frisvad, J. C., van de Vondervoort, P. J., Burgers, K. et al. (2005). *Aspergillus vadensis*, a new species of the group of black Aspergilli. *Antonie van Leeuwenhoek, 87(3),* 195–203. DOI 10.1007/s10482-004-3194-y.
21. Warcup, J. H. (1950). The soil-plate method for isolation of fungi from soil. *Nature, 166(4211),* 117–118. DOI 10.1038/166117b0.
22. Taniwaki, M. H., Pitt, J. I., Iamanaka, B. T., Sartori, D., Copetti, M. V. et al. (2012). *Aspergillus bertholletius* sp. nov. from Brazil Nuts. *PLoS One, 7(8),* e42480. DOI 10.1371/journal.pone.0042480.
23. White, T. J., Burns, T., Lee, S., Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J. (eds.), *PCR Protocols: A guide to methods and applications*. pp. 315–322. San Diego: Academic Press. 10.1016/b978-0-12-372180-8.50042-1.
24. Glass, N. L., Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology, 61(4),* 1323–1330. DOI 10.1128/AEM.61.4.1323-1330.1995.
25. Hong, S. B., Cho, H. S., Shin, H. D., Frisvad, J. C., Samson, R. A. (2006). Novel *Neosartorya* species isolated from soil in Korea. *International Journal of Systematic and Evolutionary Microbiology, 56(2),* 477–486. DOI 10.1099/ijs.0.63980-0.
26. Katoh, K., Standley, D. M. (2016). A simple method to control over-alignment in the MAFFT multiple sequence alignment program. *Bioinformatics, 32(13),* 1933–1942. DOI 10.1093/bioinformatics/btw108.
27. Hall, T. (2004). Bioedit version 6.0.7. http://www.mbio.-ncsu.edu/bioedit/bioedit.html.
28. Rambaut, A., Drummond, A. (2008). *FigureTree: Tree figures drawing tool, version 1.2.2*. University of Edinburgh, Institute of Evolutionary Biology.
29. Samson, R. A., Houbraken, J. A. M. P., Kuijpers, A. F. A., Frank, J. M., Frisvad, J. C. (2004). New ochratoxin A or sclerotium producing species in *Aspergillus* section. *Studies in Mycology, 50(1),* 45–61.