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# Morphology, phylogeny and cultural characteristics of *Aspergillus rosettanus*, a novel species in section Circumdati isolated from Wadi-El-Natron, Egypt

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**ABSTRACT**

*Aspergillus* species can colonize a wide range of substrates, and they are frequently found in a wide range of situations. More than 340 species make up the genus *Aspergillus* (family *Aspergillaceae*); some of these species are toxic to humans, animals, or plants and produce aflatoxins and ochratoxins. This research discovered a novel *Aspergillus* strain from the *Aspergillus*: section Circumdati in a soil sample taken from a region near Rosetta Lake at Wadi-El-Natron region, Egypt. It was originally identified as *A. insulicola* and deposited with the entry number MF075156 into the NCBI nucleotide database. Based on molecular analyses of the internal transcribed spacer (ITS) region and comparisons between the strain's macroscopic and microscopic characteristics with those of other species in section Circumdati, the strain was suggested as a novel species in the current research and given the name *Aspergillus rosettanus*. This novel species can be distinguished from the existing *Aspergillus* species in section Circumdati by having smaller conidial heads (45-65 µm), metulae (4-6 µm), and phialides (5-7 µm).

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## Introduction

*Aspergillus* species are often found in a wide range of environments because they can colonize a wide range of substrates (Moubasher & Mazen 1972; Moubasher et al. 2016; Moubasher et al. 1985; Moubasher et al. 2018; Abdel-Hafez et al. 1988; Abdel-Hafez et al. 1978; Abdel-Hafez et al. 1983; Abdel-Hafez 1981; Mohamed et al. 2020; Al-Bedak et al. 2021; Al-Bedak et al. 2020; Al-Bedak & Moubasher 2020; Ismail et al. 2002; Ismail & Abdullah 1977; Ismail et al. 2017). Genus *Aspergillus* (Family: *Aspergillaceae*) has more than 340 species, some are harmful to plants, animals or people and/or generate toxins such as, aflatoxins and ochratoxins (Abdel-Azeem et al. 2020). Since the revision of the section Circumdati by Christensen (Christensen 1982), only three new species have been described: *A. sepultus* (Tuthill & Christensen 1986), *Neopetromyces muricatus* (Udagawa et al. 1994; Frisvad & Samson 2000), and *A. persii* (Zotti & Montemarteni 2002). Peterson (2000) determined that *Petromyces alliaceus*, *P. albertensis*, and *A. lanosus* are members of section Flavi based on rDNA sequence data, which has been verified by phenotypic traits (Frisvad & Samson 2000). *A. campestris* was another species that was initially classified as Circumdati. Rahbaek et al. (1999), Peterson (2000), and Rahbæk et al. (2000) classified this species as Candidi. Finally, *A. dimorphicus* and *A. sepultus* were transferred to section Wentii (Peterson 1995; Peterson 2000; Frisvad & Samson 2000), leaving section

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Circumdati with a homogeneous series of very closely related fungi, with only *A. robustus* differing from the remaining species (Peterson 2000). Peterson (2008) updated the molecular taxonomy of the section Circumdati and discovered an undescribed species while essentially corroborating the findings of a prior single locus analysis in which seven novel species were described (Frisvad et al. 2004). *Aspergillus affinis* was described by (Davolos et al. 2012). Al-Bedak et al. (2020) and Al-Bedak & Moubasher (2020) added two new species, *A. curvatus* and *A. gaarensis*, respectively. The teleomorphic species *Neopetromyces muricatus* and more than 25 identified *Aspergillus* species, which are phenotypically and phylogenetically distinct despite being closely related, are included in this section up to now. Species of section Circumdati are essential sources of mycotoxins, including ochratoxin A, penicillic acid, xanthomegnin, viomellein and vioxanthin (Van der Merwe et al. 1965; Hesseltine et al. 1972; Ciegler 1972; Stack & Mislivec 1978; Robbers et al. 1978). This section has subsequently introduced in a molecular study on genetic variation, and identifying many new species were introduced during the last few decades (Varga et al. 1998; Tuthill & Christensen 1986; Udagawa et al. 1994; Frisvad & Samson 2000; Frisvad et al. 2004; Davolos et al. 2012; Gil-Serna et al. 2015; Al-Bedak & Moubasher 2020; Zotti & Montemarteni 2002; Al-Bedak et al. 2020). Consequently, this investigation gives insight into the morphological and phylogenetic characteristics of a new member within the genus *Aspergillus*: section Circumdati isolated from a soil sample close to Rosetta Lake, Wadi-El-Natron, Egypt.

## Materials and Methods

### *The study site*

Wadi-El-Natron is located at 30°17' and 30°38"N, 30°2' and 30°30' E in the Western desert parallel to Egypt's Nile Delta. It lies 23 meters downstream and 38 meters below the Rosetta branch of the Nile River (Ismail et al. 2017). Rosetta Lake is a 1.05 $Km^2$ area. It dries up in the summer and contains little sodium carbonates (Figure 1).

### *Fungal isolation and preservation*

The novel *Aspergillus* species was isolated from a soil sample close to Rosetta Lake in Wadi-El-Natron, Egypt. The isolation was carried out using the dilution plate method described by Warcup (1950) on Czapek's agar (Ismail et al. 2017). One mL of the soil suspension with appropriate dilution, was transferred to Petri plates containing the isolation medium. The plates were then incubated at 25 °C for 15 days. The developed colonies were purified on Cz agar (Samson 2010), and the pure culture of the fungus was preserved in the culture collection of Assiut University Mycological Centre as AUMC 11042, and Herbarium specimen as AUMC H-11042.

**Fig 1.** Lake Rosetta from which the new species, *A. rosettanus*, was isolated.

### *Morphological studies*

Growth rates of the new species were studied on Czapek's agar (CZ; (Raper & Fennell 1965), malt extract agar (MEA; (Samson et al. 2010), Czapek's yeast Autolysate agar (CYA; (Pitt 1979), yeast extract sucrose agar (YES; (Frisvad 1981), Blakeslee's malt extract agar (MEAbl; (Blakeslee 1915), malt extract (20%) with sucrose agar (M20S; (Samson et al. 2010), M40S, CYA with 20% sucrose agar (CY20S; (Klich 2002), Creatine sucrose agar (CREA; (Frisvad 1981), urease enzyme (Paterson & Bridge 1994), mannitol agar (Brayford & Bridge 1989) and tannin sucrose agar (TAN; (Thrane 1986). Inoculations were made from spore suspensions prepared in a 0.2% agar and 0.05% Tween 80 solution (Samson et al. 2014). Plates were inoculated in a three-point pattern using a micropipette and inoculum size of 1.0 µL/spot, and the plates were then incubated in the dark at 25°C with additional CYA plates incubated at 37°C for 7 days. Colony colors were identified according to Kornerup & Wanscher (1978), and the microscopic characteristics were examined from the MEA culture.

### *DNA extraction, PCR, and sequencing*

DNA was extracted using the protocol described by Moubasher et al. (2019), which involved grounding and transferring a 0.2 g of fungal mycelia that were 7 days old and cultivated on MEA to a 1.5 mL microfuge tube. The CTAB method (800 µL CTAB buffer containing 3% CTAB, 1.4 M NaCl, 0.2% Mercaptoethanol, 20 mM EDTA, 100 mM TRIS-HCl pH 8.0, and 1% PVP-40) was used to isolate the DNA. To amplify the ITS region, a PCR reaction was conducted utilizing SolGent EF-Taq

and the universal primers ITS1 and ITS4 (White et al., 1990).

### *Phylogenetic analysis*

Using the DNASTAR computer program (version 5.05), a contiguous sequence of *A. rosettanus* AUMC 11042 was created. The 40 strains in the ITS dataset were 38 for the closely related strains from the genus *Aspergillus*: section Circumdati, which contained the type species that could be obtained through downloads from GenBank, one sequence for the newly discovered species, and one sequence for *A. oryzae* AUMC 10329, which was used as an outgroup. For this research, MAFFT (Katoh & Standley 2013) was used to align all sequences using its default settings. BMGE (Criscuolo & Gribaldo 2010) was applied to maximize the parsimony uninformative characters and alignment gaps. MEGA X version 10.2.6 was used to carry out maximum-likelihood (ML) and maximum-parsimony (MP) phylogenetic analyses (Kumar et al. 2018). By using 1000 bootstrap replications, the most parsimonious trees' resilience was assessed (Felsenstein 1985). With Modeltest 3.7, the Akaike information criterion (AIC) was used to identify the most optimal nucleotide replacement model for data set analyses (Posada & Crandall 1998). The phylogenetic tree was drawn and visualized using MEGA X (Kumar et al. 2018). The resulting tree was saved as a TIF file (Al-Bedak & Moubasher 2020). The GenBank deposition for the ITS sequence is MF075156. The new species was identified and registered as MB856184 at MycoBank.

## Results

### *Taxonomy*

***Aspergillus rosettanus*** O.A.M. Al-Bedak, sp. nov. (Fig. 2).

GenBank No: MF075156

MycoBank No: MB856184

### *Etymology*

Named after Rosetta Lake in Wadi-El-Natron, Egypt, where the fungus was isolated.

### *Macroscopic and microscopic characteristics*

Colonies on MEA attaining a diameter of 42–50 mm after 7 days at 25 °C, floccose, centrally white, raised, orange-white (6A2–3), reddish-white (7A2/8A2) with moderate to heavy sporulation. Margin entire, greyish-orange (5B3). Soluble pigments and exudates lacking. Reverse coffee to raw umber (5F7–8) in the center, camel to sunburn towards the margin (6D–E4–5). Soluble pigments and exudates absent. Colonies on CYA attaining a diameter of 38–46 mm after 7 days at 25 °C, floccose, centrally white, raised, orange-white (6A2–3), reddish-white (7A2/8A2) with abundant sporulation. Margin entire. Reverse brownish-beige, greyish-brown to brown (6D–F3–4), pale orange, light orange to greyish-orange (5A–B3–4). Soluble pigments and exudates absent. Conidiophores pale brown to brown, rough-walled, straight, sinuous, slightly curved at the upper part, commonly 200–400 (–600) µm × 7–10 µm ($\bar{x}$ = 300 µ × 8.5 µm, n = 50). Conidial heads radiate, biseriate, 45–65 µm ($\bar{x}$ = 55 µm, n = 50). Vesicles globose, commonly (10–) 20–25 (–30) µm ($\bar{x}$ = 22.5 µm, n = 50). Metulae 4–6 µm ($\bar{x}$ = 5 µm, n = 50). Phialides 5–7 µm ($\bar{x}$ = 6 µm, n = 50). Conidia globose 1.5–3.0 µm ($\bar{x}$ = 2.25 µm, n = 50). Sclerotia up to 500-700 µm ($\bar{x}$ = 600 µm, n = 50). Hülle cells and teleomorph not observed (Figure 2). Colony diameters of *A. rosettanus* on different media are shown in Table 1.

**Table 1.** The colony diameter of *A. rosettanus* on the remaining media after 7 days at 25 °C.

| Medium | Diameter (mm) | Medium | Diameter (mm) |
|---|---|---|---|
| Cz | 35–40 | MEAbl | 36–50 |
| Yes | 40–60 | CREA (No acid) | 30–35 |
| $CY_{20}S$ | 42–60 | Urea (-ve) | 30–35 |
| CYA 37°C | 10–13 | Mannitol (-ve) | 35–50 |
| $M_{20}S$ | 40–75 | Tannic acid | 12–13 |
| $M_{40}S$ | 42–62 | | |

### *Known distribution*

Soil sample close to Rosetta Lake, Wadi-El-Natron, Egypt.

### *Typification*

Egypt, Wadi-El-Natron, a soil sample close to Rosetta Lake, 2012, isolated by Osama A. M. Al-Bedak (Holotype AUMC 11042; ex-type AUMC H-11042).

### *Distinguishing characteristics*

Small conidial heads (45–65 µm), metulae (4–6 µm), and phialides (5–7 µm) can differentiate this species from other species in section Circumdati.

### *Molecular studies*

The most comparable species in the NCBI nucleotide database using a mega blast search with the ITS sequence of the novel species compared to type species in GenBank are *A. curvatus* AUMC 11038 (=EMCCN 2213) [(GenBank MN006961; Identities = 554/579 (95.68%); Gaps = 6 (1%)] and *A. ochraceopetaliformis* CAF 073 (=ATCC 12066 = CBS 123.55 = IMI 211804 = NRRL

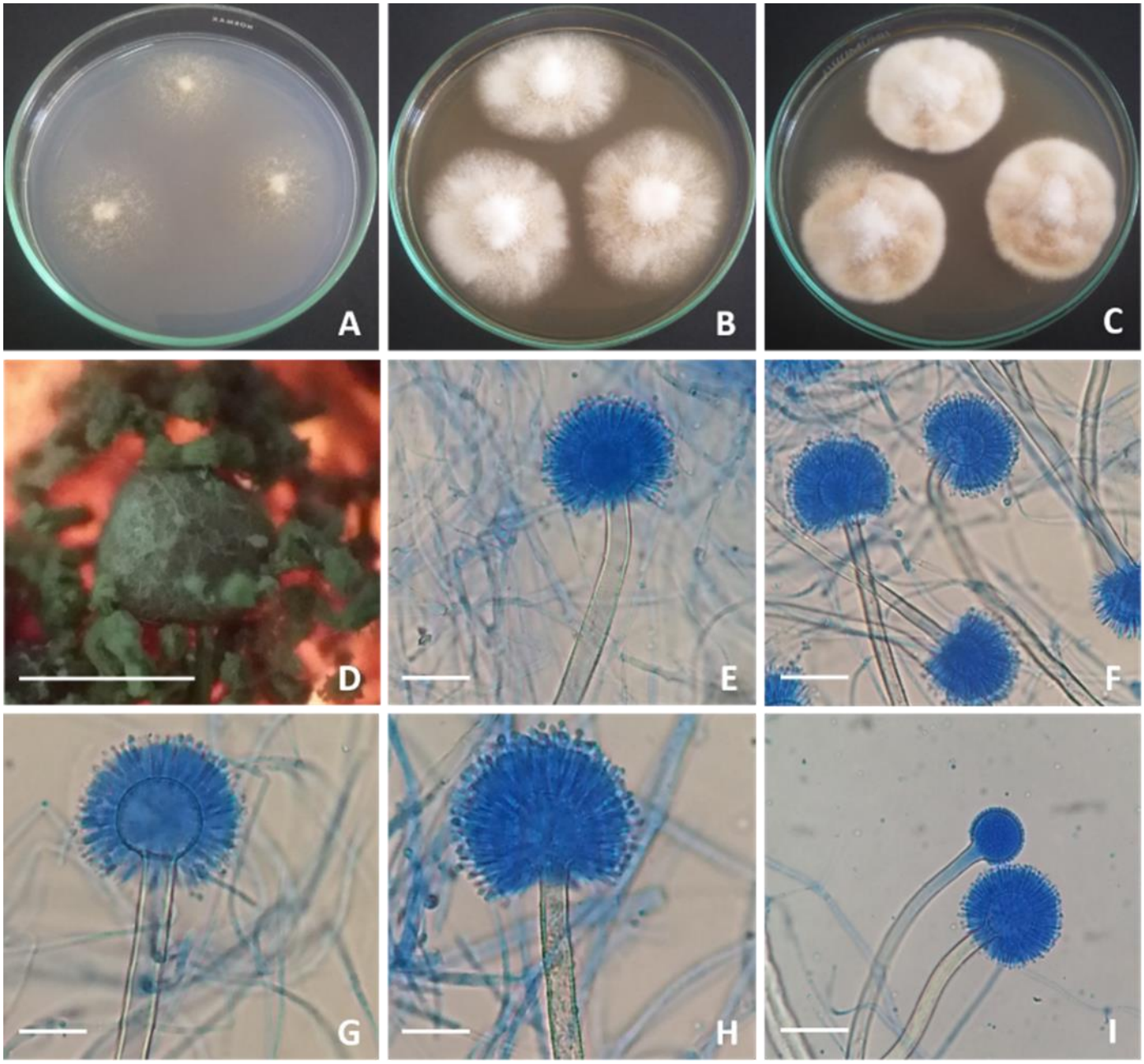


**Fig 2.** *Aspergillus rosettanus* AUMC 11042. A–C. Seven–day–old colonies on Cz, MEA and CYA at 25 °C. D. Sclerotia. E-I. Conidiophores and conidial heads. (Scale bars: D = 500 µm. E–I = 20 µm).

4752) [(GenBank KU 821470; Identities = 513/556 (92.27%); Gaps = 15 (2%)]. Phylogenetic analysis of ITS dataset was employed to determine the taxonomic status of our strain relative to other members of *A. ochraceus* group.

***Phylogenetic analysis***

The entire ITS dataset comprised 40 strains. The maximum parsimony dataset consisted of 576 characters with 372 constant characters (no gaps, no N), 110 variable characters that were parsimony-uninformative (29.6% of constant characters), and 57 characters were counted as parsimony informative (15.3% of constant). Kimura 2-parameter model by using a discrete Gamma distribution (K2+G) was the perfect model for substitution of nucleotides. The most parsimonious tree generated from MP analysis with a tree length of 388 steps, final ML optimization likelihood value of -2888.47, tree size of 0.85515, consistency index of 0.627517, retention index of 0.821830, and composite index of 0.515712, was selected to represent and discuss the phylogenetic relationships among taxa (Figure 3). The phylogenetic result depicts the relationships of the new species with other *A. ochraceus* group. The calculated ML tree

revealed strong bootstrap support for most terminal clades and the tree backbone. In the phylogenetic tree, our strain formed a distinct lineage in which it can be recognized as a new species. The novel species was placed in a single branch as a part of *A. ochraceopetaliformis* CAF 073 and *A. curvatus* AUMC 11038 clade (100% ML/100% MP) (Figure 3). The interspecific differences in the ITS sequences were 25 nucleotides between *A. rosettanus* AUMC 11042 and *A. curvatus* AUMC 11038 and 43 nucleotides between *A. rosettanus* and *A. ochraceopetaliformis* CAF 073.

## Discussion

Hypersaline lakes can be identified by their high NaCl concentrations (up to 30%), frequent big concentrations of other ions, and substantial pH concentrations (Ismail et al. 2017). Soils with a greater pH level (> 8) are created after soda evaporates, showing extreme environments (Ismail et al. 2017). For the emergence of new species and the development of various ecological interactions between individuals which account for such environmental extremes, the study of these extremes is crucial. Recently, some novel fungi from Egypt's Wadi-El-Natron hypersaline lakes have been added to the mycobiota, including *Ramophialophora chlamydospora* A.H. Moubasher, M.A. Ismail, O.A. Al-Bedak, & R.A. Mohamed, from the water of the Fasida lake (Moubasher et al. 2019), *Paracremonium moubasheri* Al-Bedak & M.A. Ismail, from sediment of Hamra lake (Al-Bedak et al. 2019), *Aspergillus gaarensis* O.A. Al-Bedak & A.H. Moubasher, from soil of El-Gaar lake (Al-Bedak & Moubasher 2020) and *Aspergillus curvatus* O.A. Al-Bedak & A.H. Moubasher, from water of Khadra lake (Al-Bedak et al. 2020).

In this research, a novel *Aspergillus* strain from the *Aspergillus*: section Circumdati was discovered in a soil sample taken from a region near Rosetta Lake at Wadi-El-Natron region, Egypt. It was originally recognized as *A. insulicola* and entered with the entry number MF075156 into the NCBI nucleotide database (Ismail et al. 2017). Based on molecular analyses of the internal transcribed spacer (ITS) region and comparisons between macroscopic and microscopic characteristics of the strain with those of other species in section Circumdati, the strain was suggested as a novel species in the current research and given the name *Aspergillus rosettanus*. The new species *A. rosettanus* shared traits with the Circumdati group, including biseriate and radiate conidial heads, rough-walled conidiophores, and small conidia. Sequencing of the ITS region revealed a closest relationship of the new species to other Circumdati members.

Due to its zones of high continuity and variability, which have been used to classify *Aspergillus* species, the ITS zone between the *18S rRNA* genes and the *28S rRNA* genes has a great interest in the differentiation of closely related organisms. (Henry et al. 2000; Al-Bedak & Moubasher 2020; Accensi et al. 1999; Moubasher & Soliman 2011; Al-Bedak et al. 2020). Morphological characteristics of new species compared with other species in section Circumdati are summarized in Table 2.

The unique strain *A. rosettanus* in this investigation differed micro- and macromorphologically from the two strains *A. curvatus* and *A. ochraceopetaliformis*, as well as from other closely related species in the section Circumdati, suggesting the presence of a distinct species. By having sclerotia, which in *A. ochraceopetaliformis* evolved as reddish-brown sclerotia-like structures, *A. rosettanus* may be distinguished from this species. In turn, the *A. rosettanus* forms orange-white to reddish-white colonies on MEA and CYA; a colony color which can differentiate this new species from both *A. ochraceopetaliformis* that usually occupied by white mycelia, covering dark yellow to light brown to gray colonies, and *A. curvatus* that forms orange gray to dark blond to yellowish-brown colonies on MEA and brown fur, bronze to brown mustard on CYA. As a result, the conidiophores of *A. rosettanus* in the top parts have a slight curvature as opposed to the firmly bent conidiophores of *A. curvatus*. *A. rosettanus* also differs from the other species in this group by having relatively small conidial heads (45–65 µm), metulae (4–6 µm), and short phialides (5–7 µm).

## Consent for publication

All authors agree to participate and publish.

## Competing interests

The authors declare no conflict of interest.

## Authors' contributions

All authors participated equally to data analysis, authoring, and revising the article. The final version of the manuscript has been reviewed and approved by the authors.

## Accessibility of data

Pure culture of the new strain was preserved as frozen and lyophilized samples in the culture collection of Assiut University Mycological Centre as AUMC 11042. ITS sequence was deposited in GenBank as MF075156. The description of the new species was uploaded to MycoBank as MB856184. Sequence alignments have been submitted for all data sets to TreeBASE http://purl.org/phylo/treebase/phylows/study/TB2:S31743 (study no. 31743).

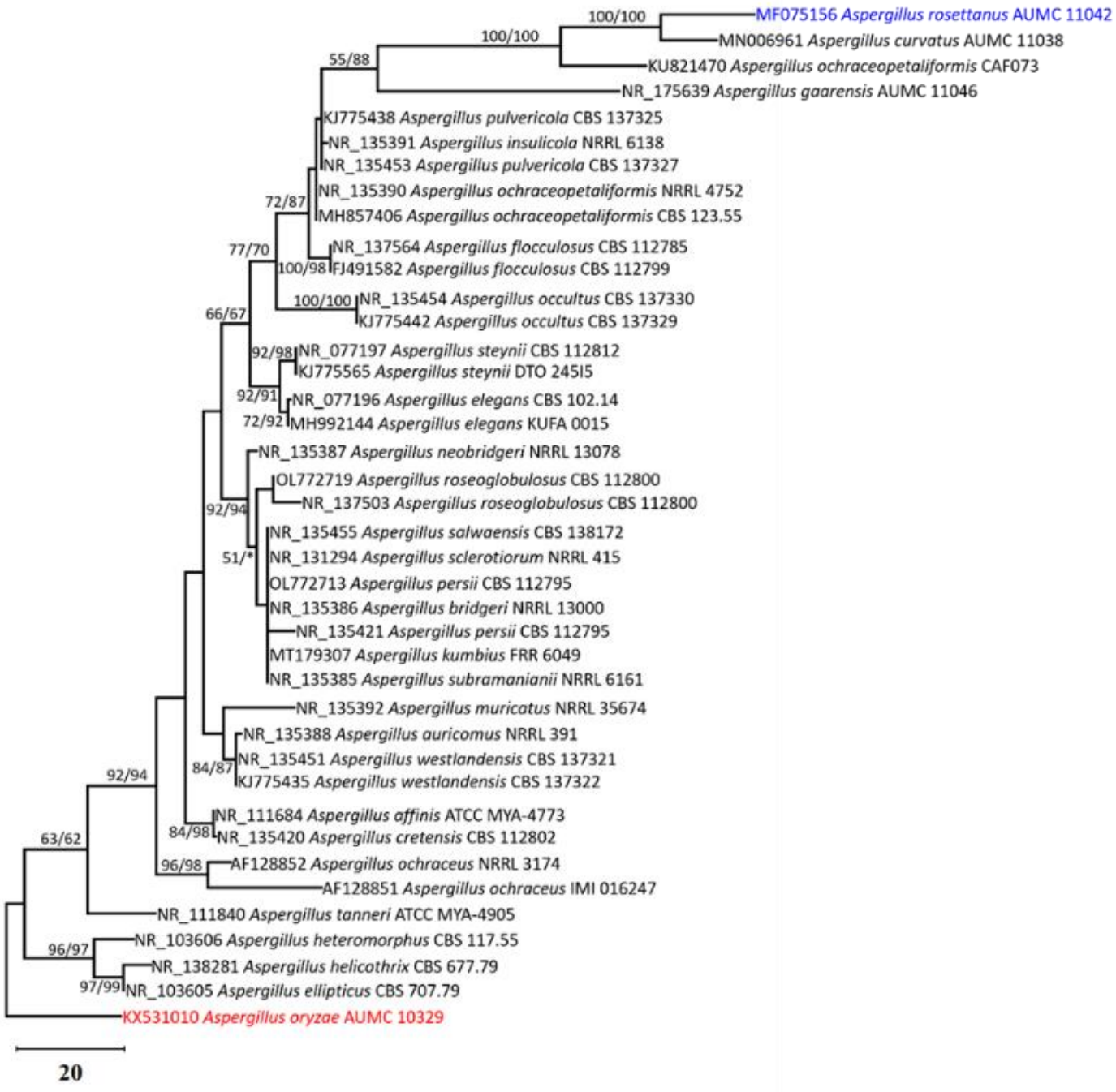


**Fig 3.** The most parsimonious phylogenetic tree obtained from a heuristic search (1000 replications) of *A. rosettanus* AUMC 11042 based on the ITS sequence (in blue) compared to other closely similar species belonging to the genus *Aspergillus*: section Circumdati in GenBank. Bootstrap support values for ML/MP ≥50% are indicated near the respective nodes. The tree is rooted to *A. oryzae* AUMC 10329 as an outgroup (in red).

**Table 2.** Morphological characteristics of *Aspergillus rosettanus* AUMC 11042 in comparison to other species in *Aspergillus*: section *Circumdati* on MEA after 7 days at 25 °C.

| Morphological features | Sporulation | Sclerotia | Conidiophores | Vesicles | Metulae | Conidia |
|---|---|---|---|---|---|---|
| ***A. rosettanus*** **This study** | Orange–white to reddish–white | 500–700 µm | Pale brown to brown, rough–walled, straight, sinuous, slightly curved at the upper part, 200–400 (–600) µm × 7–10 µm | Vesicles globose, (10–) 20–25 (–30) µm | 4–6 µm | Globose 1.5–3.0 µm |
| *A. auricomus* (Christensen 1982) (Visagie et al. 2014) | Golden yellow to orange yellow | Abundant on Czapek's agar | Hyaline to dark brown, rough, 190–1360 × 5–11 µm | 13–53 µm | 6.5–14.5 × 4–6 µm | Smooth, 3–4 × 2.5–3 µm |
| *A. curvatus* (Al-Bedak et al. 2020) | Orange grey | 600–1000 µm long and up to 800 µm wide | Brown, rough-walled, sinuous, strongly curved, 200–300 (–500) µm × 5–10 µm | Globose to subglobose, (10–) 20–25 (–30) µm | 10-17 µm | Globose to subglobose, smooth, 2–3 µm × 2–3 |
| *A. flocculosus* (Frisvad et al. 2004) | Dull yellow to greyish yellow | (360–)400–590(–650) µm | Light brown, rough, 1000–1500 µm | Globose to pyriform, (16–) 20–44 (–46) µm | (7–)8–26(–28) µm | Globose, (1.9–)2–2.5(–2.7) µm |
| *A. gaarensis* (Al-Bedak and Moubasher 2020) | Yellowish-white to pale yellow | Absent | Pale brown to brown, rough, 200 – 600 × 5–8 µm | Globose, 20–40 µm | 8–13 µm | 2.5–3.0 × 2.5–3.0 µm |
| *A. insulicola* (Christensen 1982) (Visagie et al. 2014) | Light orange to greyish orange | Absent | Hyaline to yellow to brown, rough walled, 250–600 × 6–8.5 µm | Globose, 15–30 µm | 7–12 µm | Globose to subglobose, smooth, 2–3 × 2–3 µm |
| *A. ochraceopetaliformis* (Visagie et al. 2014) | Dull yellow to olive brown to brown | Sclerotia-like structures reddish brown, 350–650 µm | Hyaline to yellow to brown, rough walled, 260–1300 × 8–10 µm | Globose to pyriform, 25–45 µm | 10–20 (–28) µm | Globose, smooth, 2–3 × 2–3 µm |
| *A. ochraceus* (Christensen 1982) | Warm buff to cinnamon buff | In some strains, 1000–2000 µm | 700–1500 x 10–14 µm | Globose to somewhat 35–50 µm | 15–20 µm | Globose to sub-globose, finely roughened, 2–3.5 µm |
| *A. pulvericola* (Visagie et al. 2014) | Yellowish white | Sometimes present, white to cream, 250–510 µm | Hyaline to brown, rough, 190–1000 × 5–9 µm | Globose, 15–53 µm | 5.5–16.5 µm | Globose, smooth, 2–3 × 2–3 µm |

**Table 2.** (contd.)

| Morphological features | Sporulation | Sclerotia | Conidiophores | Vesicles | Metulae | Conidia |
|---|---|---|---|---|---|---|
| *A. sclerotiorum* (Christensen 1982) | Pale yellow | In some strains, 1000–1500 µm | Up to 800–1200 µm | Less than 40 µm | 6.5–12 µm | Globose, smooth or delicately roughened,2–3 µm |
| *A. westerdijkiae* (Frisvad et al. 2004) | Velvety, pale to light or dull yellow | Sparsely formed, (460–)480–760(–840) × (430–)480–660 (–720) µm on CYA and (440–)450–720(–750) × (430–)430–650(–700) µm on OA | Up to 1800 µm | Globose to spathulate, (16–)20–35(–42) × (3)3.5–5.7(–7.1) µm | (10.5–)11 × 19(–23) µm | Globose, finely roughened, (2.3–)2.5–3(–3.1) × (2.2–)2.3–2.8(–3.1) µm |

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