Mycologia Iranica 11(1): 117–122, 2024 **Short Article** DOI:10.22092/mi.2024.367535.1291

Identification of *Rhizopus arrhizus* associated with some dried fruits and the traditional food Terkhêna in Mahabad, western Iran

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Abstract: In a study on the taxonomy of mycotoxigenic fungi associated with raisins and other dried fruits including apricots, apples, and white mulberries, and the traditional food Terkhêna in Mahabad, western Iran, 33 isolates resembling members of the genus *Rhizopus* were collected. Using ISSR technique, based on DNA fingerprinting patterns generated by primer $(GTG)_5$, all isolates were placed in two distinct clusters. A representative isolate from each cluster was selected for phylogenetic analysis. Based on ITS phylogeny, two representative isolates IRAN 5249C and CJA OGh31 were identified as *Rhizopus arrhizus* which has previously been reported from soil and clinical samples in Iran. To our knowledge, it is the first report of *R. arrhizus* on dried fruits apricot and white mulberry and the traditional food Terkhêna around the globe. To clarify the taxonomy of *R. arrhizus*, we recommend more investigation on the phylogeny of this species using multigene phylogenetic analyses.

Keywords: *Mucorales*, Mycotoxigenic fungi, Phylogeny, Taxonomy.

INTRODUCTION

Rhizopus species are of significant importance in industry, medicine, and agriculture (Zheng et al. 2007). Members of the genus are saprobic and ubiquitous fungi and have historically been used in industry to process fermented foods and produce organic acids. Medically, some species are producers of medicines and some are known as the causal agents of mucormycosis in humans and animals. In agriculture, *Rhizopus* species are known as plant pathogens causing decay and rot symptoms on host tissues and spoilage of plant products during storage (Schipper 1984, Schipper and Stalpers 1984, Inui et al. 1995, Schipper et al. 1996, Voigt et al. 1999, Abe et al. 2007, Zheng et al. 2007, Abe et al. 2010, Hoffmann et al. 2013). Post-harvest fungal spoilage of agricultural products causes qualitative and quantitative damages leading to significant economic losses and threatens consumer safety. Moreover, *Rhizopus* species pose risks of food and feed contamination by synthesizing mycotoxins and other biologically active compounds (Jennessen et al. 2005). The genus *Rhizopus* was established by Ehrenberg in 1820, based on the type species *Rhizopus nigricans*. Subsequently, *Rhizopus stolonifer* was introduced as the type species (Inui et al. 1965, Zheng et al. 2007). Few studies have focused on the taxonomy and phylogeny of *Rhizopus* from which some have discussed the history of *Rhizopus* taxonomy based on morphological and molecular data (Abe et al. 2007, Hoffmann et al. 2013, Walther et al. 2013, Li et al. 2016). So far, more than 140 species have been listed in Index Fungorum (April 2024; www.indexfungorum.org), but sequences data, mostly ITS sequence, are available for a few species. Dried fruits and a traditional food called "Terkhêna" are very popular foods in Zagros region as a part of Mesopotamia from prehistoric times. In a recent study on taxonomy of mycotoxigenic fungi associated with some dried fruits and Terkhêna from Mahabad, located in the west of Iran, 415 fungal isolates were collected of which 33 isolates resemble *Rhizopus* examined morphologically and phylogenetically based on ITS sequence data.

MATERIALS AND METHODS

Samples of dried fruits including apricots, apples, white mulberries and raisins, and a traditional food Terkhêna, were collected during 2021–2022 from Mahabad. Fungi were isolated on potato dextrose agar (PDA) and purified using the hyphal tip technique on water agar (WA). Total genomic DNA was extracted from freeze-dried mycelium of fungal isolates grown on potato dextrose broth (PDB) following the modified method of Raeder and Broda (1985), as detailed by Abdollahzadeh et al. (2009). To reduce the number of isolates for morphological and molecular studies, according to Alves et al. (2007) fungal isolates were clustered at the species level using the inter-simple sequence repeat (ISSR) technique based on DNA fingerprinting patterns generated by $(GTG)_5$. PCR conditions and electrophoresis of

Submitted 1 May 2024, accepted for publication 21 June 2024
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PCR products were adjusted following Alves et al. (2007). For clustering of the isolates, DNA fingerprinting profiles were visually analyzed. To generate sequence of the ITS region, representative isolates from each cluster were amplified and sequenced using primer pairs ITS5 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS4 (5′-TCCGTAGGTGAACCTGCGG-3′) (White et al.1990). PCR conditions were adjusted as described by Abe et al. (2006). PCR products were purified and sequenced by BGI (China) via BMG (Bio Magic Gene) Company. (Karaj, Iran). Consensus sequences extracted with BioEdit v. 7.0.0 (Hall, 2004) were submitted to GenBank. The generated sequences were subjected to BLAST analysis and aligned with the type or authentic strains of *Rhizopus* species using MAFFT online service (Katoh et al. 2019). The alignment was phylogenetically analyzed by Maximum Parsimony (MP) method through the online CIPRES Science Gateway (Miller et al. 2012) using PAUP v. 4.a168 (Swofford, 1991). The MP analysis was executed according to Bashiri et al. (2022). The resulting phylogenetic trees were visualized using FigTree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree) and the final trees were edited using Adobe Illustrator 2023 v. 27.1.0.189. Colony morphology was recorded on PDA in 90 mm petri plates at 25 °C after seven days. Fungal structures grown on PDA were observed and documented with an Olympus BX51 microscope equipped with an Olympus DP72 camera. Measurements of at least 30 fungal structures were generated using a Cell Sense Entry measurement module and analyzed to estimate each structure dimensions. The recorded images were used to make a plot using Adobe Photoshop 2021 v. 22.5.8. One of the two sequenced isolates was deposited in the culture collection of the Iranian Research Institute of Plant Protection (IRAN, Tehran, Iran).

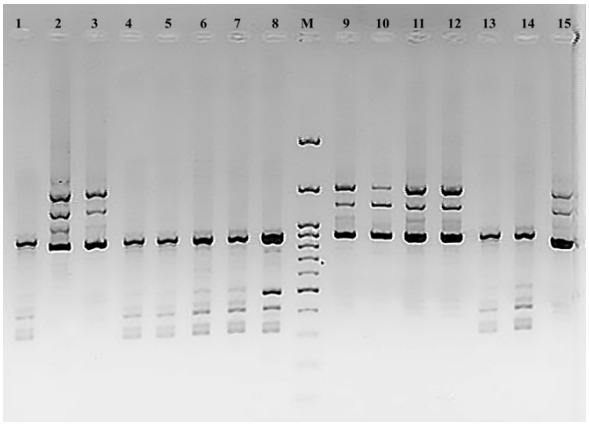

Fig. 1. DNA fingerprinting profile of some isolates of *Rhizopus* generated by primer $(GTG)_5$. M: GeneRuler DNA Ladder Mix (100bp).

RESULTS AND DISCUSSION

Phylogeny

In total, we collected 33 isolates resembling *Rhizopus* of which 10 were obtained from Terkhêna, 6 from raisins, and 17 from other dried fruits including 9 from apples, 3 from white mulberries, and 5 from apricots. Based on visual comparison of DNA fingerprinting patterns generated with primer $(GTG)_5$ using ISSR technique all 33 fungal isolates were placed in two distinct clusters each containing 8 and 25 isolates (Fig. 1). From each determined cluster one isolate was selected as representative for phylogenetic analysis based on ITS sequence data. BLAST search with ITS sequences of selected isolates IRAN 5249C (PQ533178) and CJA OGhD31 (PQ533179) showed that our isolates are close to *Rhizopus* species. Both isolates together with 10 isolates belonging to nine *Rhizopus* species and *Phycomyces blakesleeanus* CBS 284.35 as an outgroup retrieved from GenBank were aligned and subjected to phylogenetic analysis with MP method. The aligned ITS dataset consisted of 990 characters containing gaps of which 215 were constant, 233 were variable and parsimony-uninformative and 542 were variable and parsimony-informative. Analysis of the remaining 542 characters resulted in one most parsimonious tree (TL=2086, CI=0.74, RI=0.64, and HI=0.26) that is shown in Fig. 2, with MP bootstrap support values indicated at the nodes.

Our isolates with one difference with the neotype strain NRRL 1469 in ITS sequence data were phylogenetically placed in a highly supported clade and recognized as *Rhizopus arrhizus*. This species has been isolated as *R. oryzae*, a heterotypic synonym, from raisins (Lugauskas et al. 2005) and dried apple fruit (Bulut et al. 2022); however, no reports are available for Terkhêna, dried apricots and white mulberries. *Rhizopus arrhizus* has previously been reported from soil and clinical samples in Iran (Erami et al. 2013, Ziaee et al. 2016, Dolatabadi et al. 2017, Najafzadeh et al. 2017). To our knowledge, this species is reported here as a new record on Terkhêna, dried apricots, and white mulberries all over the world.

Taxonomy

Rhizopus arrhizus A. Fisch. in Rabenh., Kryptog. Fl. 1: 233 (1892)

Colonies on PDA first white with aerial hyphae developing both vertically and laterally, later becoming grey to black, reverse hyaline. *Sporangiophores* arising from aerial mycelia or stolons, straight to curved, smooth, with a thick and dark wall, 533–2083 × 8.2–13.8 µm. *Sporangia* hemispherical to spherical, smooth, transparent, brown, quickly deliquescing. *Rhizoids* if present, finger-like or branched, yellowish to brown, short.

Stolons poorly developed sub-hyaline to brown, sometimes septate. *Sporangiospores* quite irregular in shape, with striation on the surface, light brown to grey when solitary, grayish brown in mass, 2.6–9.2 × 4.5–7 (av. = 7.3 × 5.7 µm) (Fig. 3).

Notes: Morphologically, our isolates differ from the *R. arrhizus* in dimensions of sporangiophores and sporangiospores according to the description provided by Zheng et al. (2007). In addition, variation in fungal structures between various varieties or subspecies (var.: *arrhizus*, *delemar* and *tonkinensis*) can be detected in available descriptions (Zheng et al. 2007). Moreover, our isolates were also placed in two distinct clusters based on DNA fingerprinting profiles generated by $(GTG)_5$. Therefore, we recommend examining this clade in a multigene phylogeny using sequences of protein-coding genes (e.g. *rpb2*, *tef1*, *tub2*).

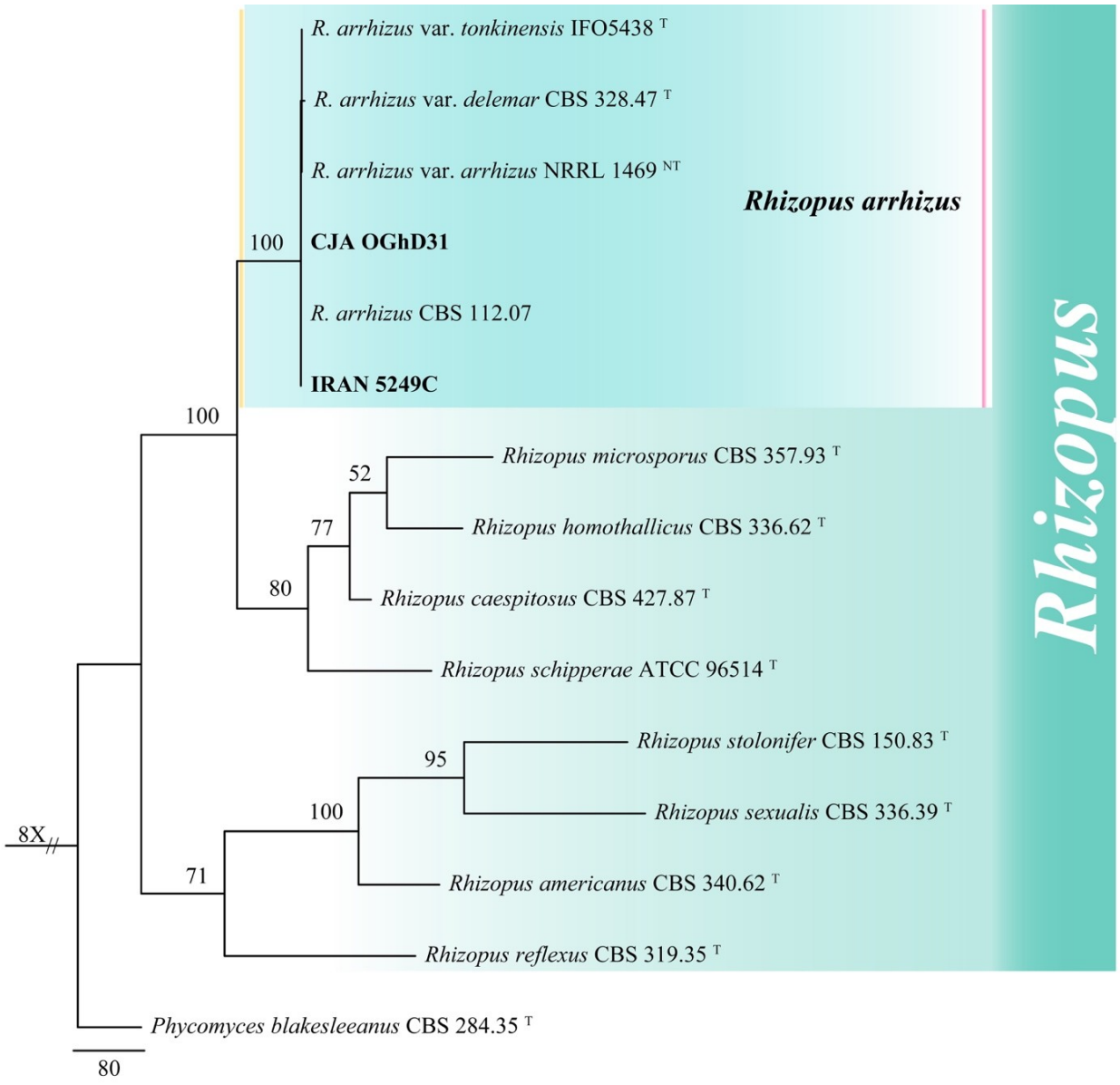

Fig. 2. The most parsimonious tree resulted based on ITS sequence alignment. Maximum parsimony bootstrap values >50% are shown at the nodes. The tree was rooted with *Phycomyces blakesleeanus* CBS 284.35. Scale bar = 80 nucleotide changes. Isolates sequenced in this study are in boldface. T indicates ex-type and NT neotype strains.

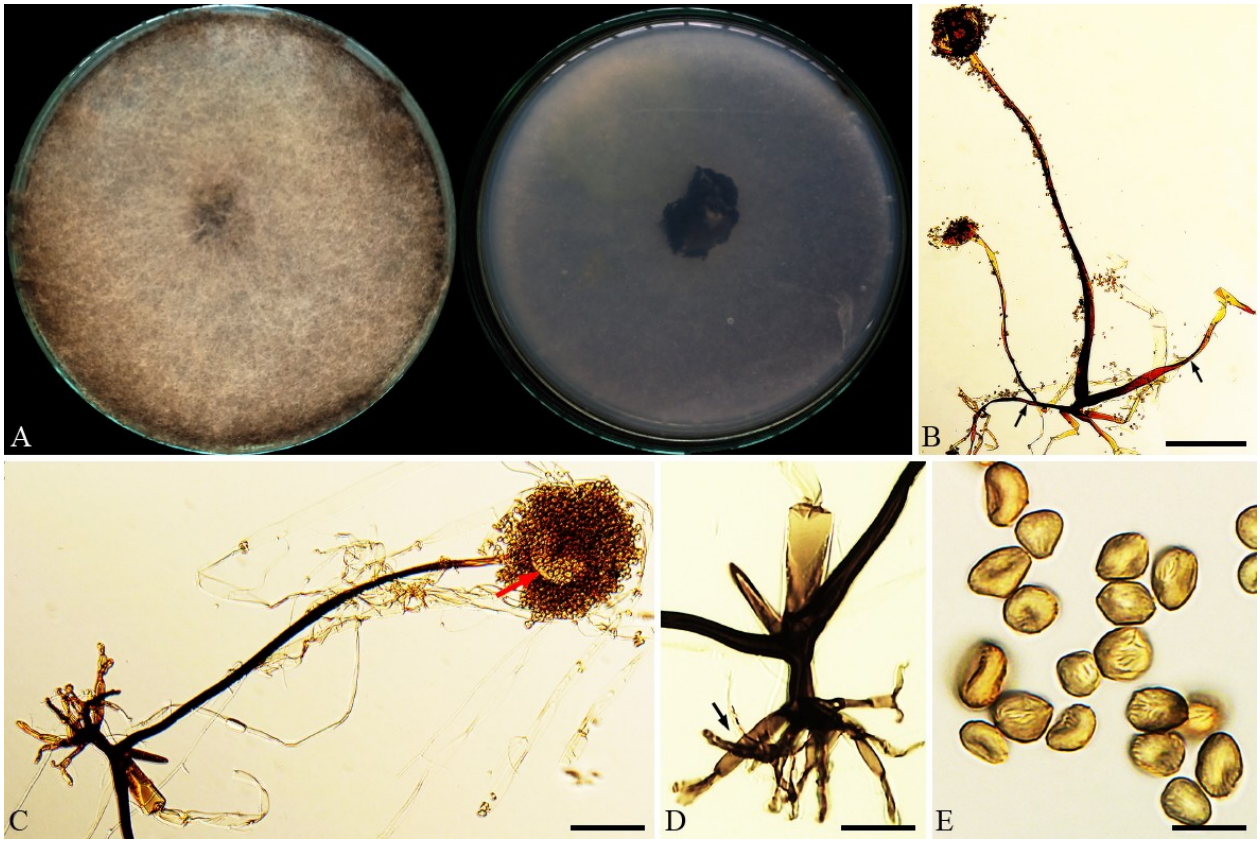

Fig. 3 *Rhizopus arrhizus* (IRAN 5249C). A. Colony morphology (surface and reverse) from left to right on PDA at 25 °C after 7 d. B. Sporangiophores, sporangia, and stolons (black arrows). C. Columella (red arrow). D. Rhizoid (black arrow). E. Sporangiospores. Scale bars: B = 300 µm, C = 100 µm, D = 50 µm, E = 10 µm.

ACKNOWLEDGMENTS

This study was financially supported by the University of Kurdistan. O. Ghaderi was financially supported by the ″Iranian Mycological Society″.

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شناسایی گونه *Rhizopus arrhizus* مرتبط با برخی میوه های خشک و غذای سنتی ترخینه از شهرستان مهاباد (غرب ایران)

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چکیده: طی یک مطالعه تاکسونومیکی روی قارچ‌های مولد توکسین مرتبط با کشمش و دیگر میوه‌های خشک از قبیل زردآلو، سیب و توت سفید و غذای سنتی ترخینه در شهرستان مهاباد واقع در غرب ایران، تعداد ۳۳ جدایه قارچی شبیه اعضای جنس *Rhizopus* جمع‌آوری شد. با استفاده از تکنیک ISSR، براساس الگوی باندی DNA تکثیر شده با آغازگر $(GTG)_5$ همه جدایه‌ها در دو گروه مجزا قرار گرفتند. از هر گروه یک جدایه به عنوان نماینده برای تجزیه و تحلیل‌های تبارزایی انتخاب شد. براساس تجزیه و تحلیل‌های تبارزایی ناحیه ITS، دو جدایه منتخب IRAN 5249C و CJA OGh31 به گونه *R. arrhizus* تعلق داشتند. بر اساس اطلاعات موجود این گونه برای اولین بار در جهان از میوه‌های خشک زردآلو، توت سفید و غذای سنتی ترخینه گزارش می‌شود. این گونه قبلا از خاک و نمونه‌های بالینی در ایران گزارش شده است. به منظور مشخص شدن وضعیت تاکسونومیکی گونه *R. arrhizus*، توصیه می شود تبارزایی چند ژنی این گونه بررسی شود.

کلمات کلیدی: *Mucorales*، قارچهای توکسین زا، فیلوژنی، تاکسونومی.

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تاریخ دریافت: ۱۴۰۳/۰۲/۱۲ تاریخ پذیرش: ۱۴۰۳/۰۴/۰۱