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A report of *Rhizopus oryzae* causing postharvest soft rot of apple fruit in China

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Abstract
Postharvest soft rot was found on apples in a local market in Beijing, China. Based on the morphological characteristics and molecular analyses of the ITS rDNA regions and Elongation factor alfa-1 (*EFl-α*) gene, the causal fungus was identified as *Rhizopus oryzae*. This is the first report of *Rhizopus*-associated with soft rot on apple fruit in China.

Keywords *Rhizopus oryzae* soft rot · Apple · Postharvest rot · ITS sequence · Ef-α1gene

In 2016, China produced 50% (89.3 million tones) of the world production of apples (*Malus pumila*). The USA, Poland, Turkey, India and Iran each produce 3–5% of the world's apples (FAOSTAT 2017). Vegetables, fruits, and ornamental plants are often infected by postharvest pathogens that cause diseases like crown rot, fruit core rot, blue/grey/green mould, anthracnose and soft rot. Fungal infection of fruit and apples can result in significant postharvest losses if it is not stored at suitable environmental conditions or if it is damaged during handling or storage (Agrios 2005). In August 2018, soft rot symptoms suspected of being caused by *Rhizopus* were detected on apple fruit at local markets in the Haidian district of Beijing, China (39°57′55.984" N, 116°20'8.124" E). The earliest symptom on the surface of infected fruit were small, water soaked lesions which soon turned brown and rapidly expanded, resulting in a sunken, soft rot of the entire fruit (Fig. 1a). White hyphae developed on the rotten tissue and within three days grey sporangiophores bearing sporangia were produced from the hyphae. The infected fruit were collected from local markets and the fungus associated with the symptoms was isolated on potato dextrose agar (PDA). To isolate the causal agent, small pieces (2 mm) from rotten lesions were cut from infected fruit samples, sterilised with 75% ethanol for 20 s followed by 0.5% NaOCl for 3 min, rinsed three times with sterile distilled water, placed on PDA, and incubated at 25 °C for 2 days.

Purified fungal colonies from infected tissues were initially white and cottony, then became grey to blackish-grey due to mature sporangia on PDA within three days (Fig. 1b). Sporangiophores were mostly erect, unbranched, smooth walled, aseptae, subhyaline to brown, singly or in groups and came up from stolons in opposite direction to rhizoids, in sets of 3–5. Sporangia were globose to subglobose, mostly 35-210 μm in diameter, white at first then becoming black due to the production of conidia (Fig. 1e). Columella were mostly globose to sub-globose and 30-90 μm × 50–110 μm. Sporangiospores were abundant, pale greyish to brown, sub-globose or ovoid, angular, having striations, and 3.8- 10 μm × 3-5 μm (Fig. 1f).

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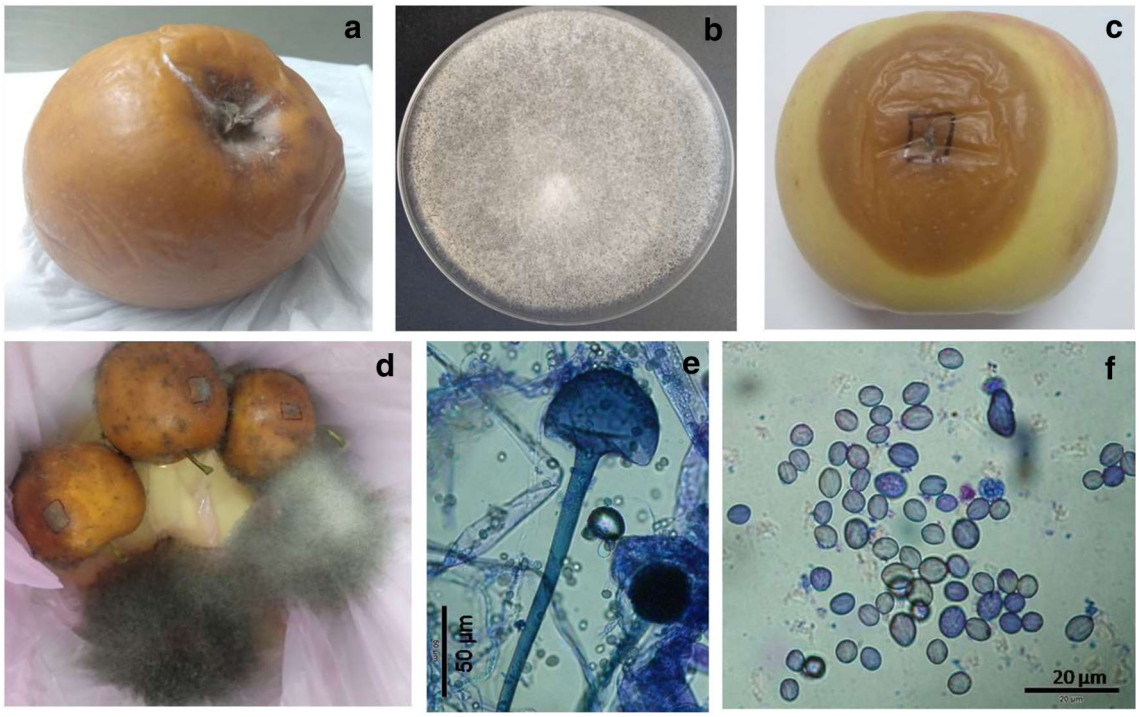

Fig. 1 *Rhizopus oryzae* on apple – (**a**) soft rot symptoms on fruit collected from local markets, (**b**) isolate growing on PDA for 3 days at 25 °C, (**c**) soft rot symptoms on incubated fruit after 2 days incubation, (**d**) soft rot symptoms on incubated fruit after 7 days incubation, (**e**) sporangiophore bearing a sporangium, (**f**) sporangiospores

The in vitro growth response to temperature is important to distinguish between *Rhizopus stolonifer* (syn. *R. nigricans*) which does not grow above 37 °C and *R. oryzae* which does grows at 40 °C (Kwon et al. 2014; Schipper and Stalpers 2003). In this study, growth rate was also determined by measuring the diameters of fungal colonies on PDA plates incubated at 40 °C. *Rhizopus* isolates from affected apples grew at 30 mm/day on PDA at 40 °C, which together with their morphological features is characteristic of *R. oryzae*. The fungus was identified as *Rhizopus oryzae*, based on the morphological characteristics and growth temperature (Lunn 1977; Liou et al. 2007; Kwon et al. 2014; Schipper and Stalpers 2003).

Nine cultures of the fungus tentatively identified as *R. oryzae* were grown on PDA at 25 ± 2 °C for 4 days for DNA extraction using fungal genomic DNA isolation kit (TIANGEN, China). The internal transcribed spacer (ITS) regions of rDNA and the partial of Elongation factor-alfa1 (Ef-α1) gene were amplified with primers ITS1/ITS4 (White et al. 1990) and EF1-728F/EF1-986R (Carbon and Kohn 1999) respectively, and sequenced. DNA sequences of the nine isolates were identical, therefore, ITS (MH973158) and Ef-α1 (MK310275) sequences of a representative isolate (YC-IK4) were deposited in Genbank. The resulting ITS and Ef-α1sequences showed 99% similarity with *R. oryzae* accessions (HQ897687, MH156644, MF470368, AB097334, AB181304, AB281527, AB281530, AB281531, AB281534, AB281540). The fungal isolate YC-IK4 is deposited (CGMCC Accession No.16961) in the China General Microbiological Culture Collection Center, Bejing, China (Fig. 2).

To prove Koch's postulates, nine apples were artificially inoculated with isolate YC-IK4 using a wound infection method. A spores suspension (0.1 mL; 10^6 conidia/mL of sterile distilled water) was injected under the surface of each apple at one point using a sterile needle under aseptic conditions in a laminar flow cabinet. Three apples were inoculated with sterile distilled water as a control treatment. All inoculated apples were placed in a closed chamber at 80% RH and 30 °C. After 48 h of incubation, fungal rot symptoms, similar to those on affected fruit collected from the local markets, developed on

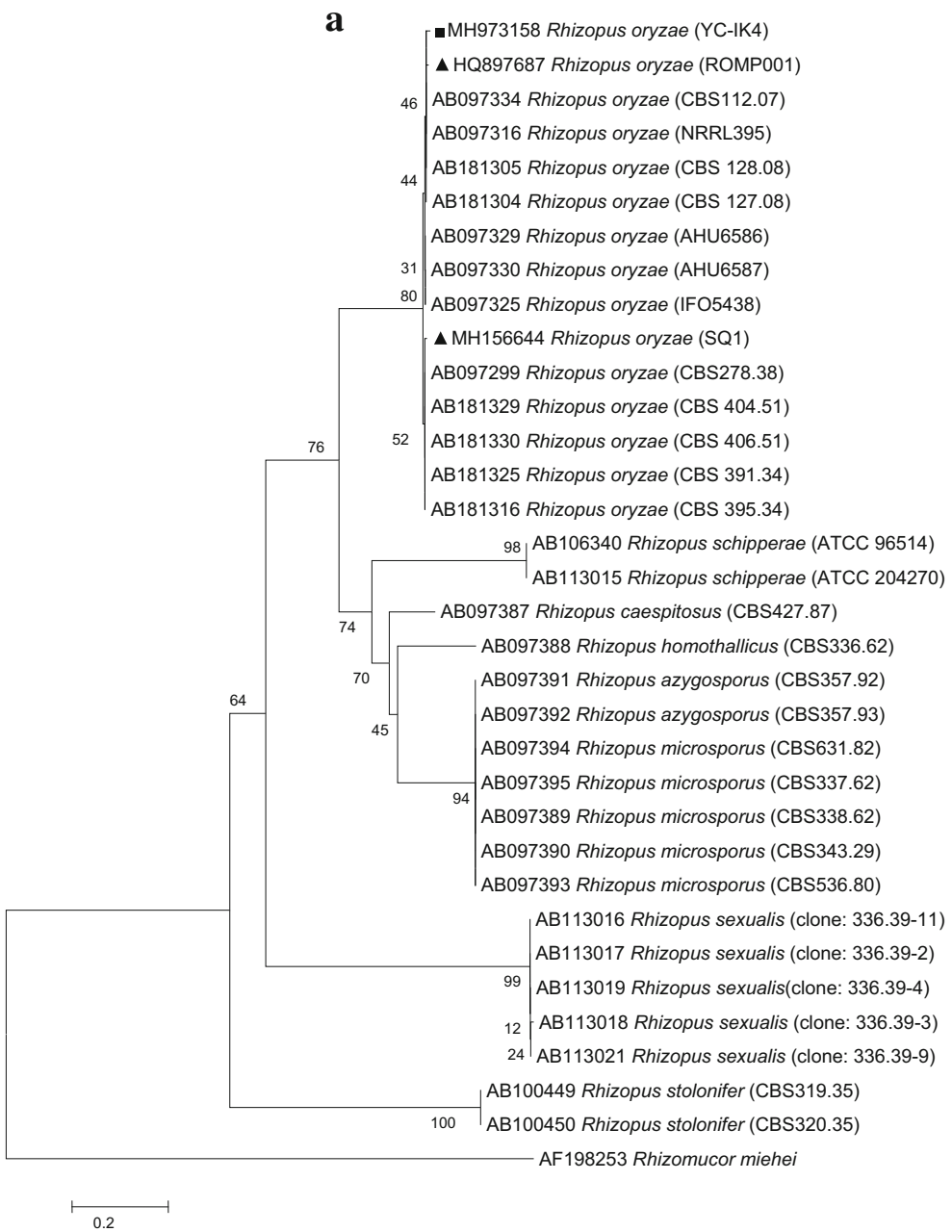

Fig. 2 Neighbor-Joining (NJ) phylogenetic trees of genus *Rhizopus*, Based on (**a**) the rDNA ITS sequence and (**b**) Elongation factor-α1 (EF-α1) gene. ■MH973158 *Rhizopus oryzae* (YC-IK4) is isolated from apple from china while *R. oryzae* sequences ▲HQ897687 (ROMP001) and ▲MH156644 (SQ1) indicate isolates reported on apple from Korea and Saudi Arabia respectively

the inoculated apples. Control apples remained asymptomatic. The causal fungus was reisolated from the artificially infected apples and was identified as *R. oryzae*.

Postharvest soft rot of apple, sweet potato, and banana fruit caused by *R. oryzae* (syn; *R. arrhizus*) have been reported in Korea and Saudi Arabia (Kwon et al. 2011, 2012a, b; Al-Dhabaan 2018). The pathogen has also been reported to be associated with postharvest root rot of *Codonopsis lanceolata* in Korea (Park et al. 2014). However, to our knowledge, this is a new record of

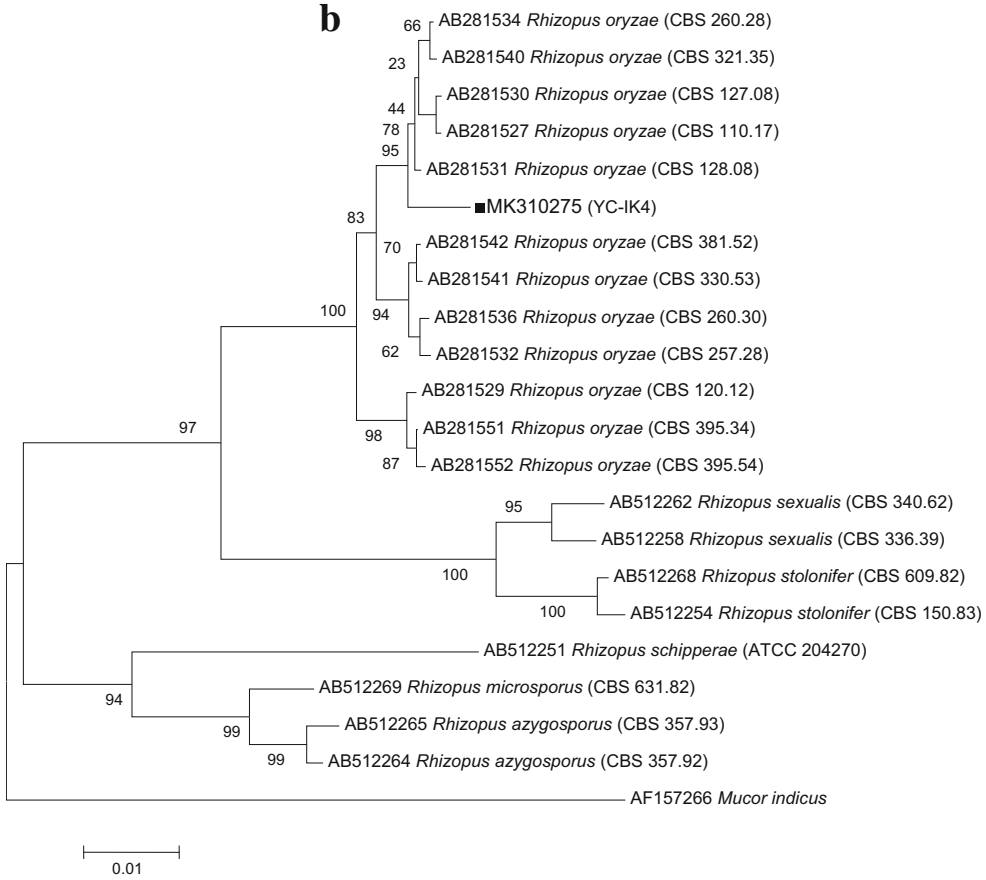

Fig. 2 continued

R. oryzae as a postharvest pathogen causing soft rot on apple in China.

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