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**Short Communication**

# NEW REPORT OF *ASPERGILLUS AWAMORI* FRUIT ROT OF GUAVA IN PAKISTAN

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## ABSTRACT

Rotting of guava fruits, selling in local fruit markets of three districts of Punjab, Pakistan i.e. Lahore, Shiekhupura and Kasur was observed in September 2014. Isolation and identification of causal organism was carried out from the diseased fruits. *Aspergillus awamori* was identified based on morphological characters. Identification was further verified by nucleotide sequence analysis of ITS region of rDNA and partial calmodulin gene. Phyylogenetic analysis of amplified ITS and partial calmodulin gene nucleotide sequences clearly showed that *A. awamori* is a distinct species within black Aspergilli group and present in the *A. niger* clade. Finally, after confirmation of pathogenicity, *A. awamori* was reported for the first time as a guava fruit rot pathogen from Pakistan.

**Key words:** *Aspergillus,* guava, phylogenetic analysis, rot.

Guava (*Psidium guajava*) is a nutrient rich and unique flavored fiber fruit which is consumed fresh as well as in processed form (Jams, beverages, ice cream etc). This fruit contains vitamins; A, $B_6$, C, thiamine and riboflavin as well as essential minerals for example calcium, magnesium, potassium, iron and phosphorus. Being naturally rich in vitamin C, guava has antioxidant properties which are helpful in prevention and cure of many diseases (Chen and Yen, 2007; Joseph *et al.,* 2011). In September 2014, during a survey conducted to local fruit market of Lahore, Pakistan, guava fruits covered with white mycelium and abundant dark brown conidia were observed (Fig. 1A). Guava fruits showing the same symptoms were also collected from Shiekhupura and Kasur. Infected fruits from all the three sampling sites were brought to the laboratory for pathogen(s) identification. Pathogen was cultured and purified on Malt Extract Agar (Dhingra and Sinclair, 1995) and Czapek Dox Agar medium (Raper and Fennell, 1965). Seven days old pure fungus culture grown at 25 °C was used for the identification of pathogen. Initially purified fungal pathogen was identified on the basis of morphological and cultural characters (colony color, size, zonation, presence of exudates and sclerotia etc). Complete description of macroscopic (type of conidial heads, aerial and submerged mycelium) and microscopic (conidiophores, phialides, metulae, vesicles and conidia) characters was made using stereoscope and calibrated compound microscope respectively (Raper and Fennell, 1965 Pildain *et al.,* 2008).

Identification of pathogen based on morphology was confirmed by nucleotide sequence analysis of Internal Transcribe Spacer Sequence (ITS) and partial calmodulin gene (CAL). Total genomic DNA of fungal pathogen was extracted following the method of Akhtar *et al.* (2014) and used for the amplification of selected genes (White *et al.,* 1990). The PCR reaction was carried out as described by Nayab and Akhtar (2016). Detail of primers used in present study is provided in Table 1. Good quality amplified DNA fragments were sent for nucleotide sequencing. Resulting DNA sequences were analysed by nucleotide BLAST.

Phylogenetic analysis of nucleotide sequence data of amplified ITS region of the rDNA and partial calmodulin gene was carried out (Varga *et al.,* 2007; Noonim *et al.,* 2008; Varga *et al.,* 2011).

For the completion of Koch's postulates, healthy guava fruit were first surface sterilized and then inoculated with the spores of isolated pathogen. Under aseptic conditions, five healthy fruits were first wounded at three different points with the help of a sterilized needle and then $10^5$ spores were injected in each wound. Sterilized water was also injected to fruits to be served as control. All fruits were kept at 25 ± 2 °C in separate covered plastic boxes containing moistened blotting paper bedding. Fruits were observed regularly for fungal growth. Experiment was repeated three times. After 48 hours of spore injection, all inoculated fruits exhibited the same disease symptoms of decay with powdery black spores and white mycelium as found on the fruits collected initially for pathogen identification. However control fruits remained asymptomatic. Consistently re-isolation of *A. awamori* from artificially inoculated fruits fulfilled Koch's pathogenicity postulates.

Cultural studies were conducted on 7 days old pure culture. Three isolates were selected for detailed morphological studies. Colonies on MEA growing rapidly reaching 4.5 - 5.0 cm in 7 days at 25 ± 2 °C without zonation, colony texture was velutinous to floccose white from center, dark brown to black in color and creamy yellow in reserve. Sclerotia were absent while colonies on CZ had clear zones in culture and relatively slow growing attained a diameter of 3 - 4 cm. Abundant radial conidial heads were present.

Sterigmata were in two series (biseriate). Conidiophores were hyaline, smooth walled ranging in length from 300 – 700 mm while 6 µm in diameter becoming darker at the apex and terminating in sub-globose to globose vesicle that range 20 - 40 µm in diameter. Metulae covering 1/2 of the whole surface of the vesicle, phialides 4.8 - 17.5 × 2.4 - 6.7 µm in size. Conidia globose to subglobose in shape, 2.8 - 4.6 µm in size, smooth to conspicuously rough walled and have irregular ridges.

When morphological description was compared with the published literature, the pathogen was recognized as *Aspergillus awamori* (Klich, 2002; Varga *et al.,* 2011). Identified pathogen was submitted to First Fungal Culture Bank of Pakistan (FCBP), University of the Punjab, Lahore, Pakistan and assigned the accession number FCBP1500.

Pathogen identity was confirmed by amplification and nucleotide sequencing of rDNA ITS1-5.8S-ITS2 (ITS) and partial calmodulin gene (CAL) (Fig. 2). rDNA nucleotide sequence of *A. awamori* showed 99 % similarity with many GenBank strains *A. awamori* including WAIR120 (LC032125.1), CBS 113.3 (DQ196199.1), NRRL 4951 (KC796388.1) and that of calmodulin gene exhibited 100 % identity with its the respective gene of other strains of same species including LEMI993 (KJ777809.1), NRRL 4948 (KF288119.1) ITEM 4777 (FN394671.1). Amplified ITS nucleotide and partial calmodulin gene sequences were deposited to GenBank under the accession numbers KT026311 and LN874032 respectively.

The evolutionary history of all selected black *Aspergillus* strains, based on ITS and partial calmodulin gene sequences is inferred by the Tamura-Nei model (Tamura and Nei, 1993) based Maximum Likelihood method using the program MEGA6 (Tamura *et al.,* 2013). The trees with the highest log likelihood are shown (Fig. 3 and Fig. 4). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.

**Table 1. Primers used in present study.**

| Sr. No | Primer | Primer name | Sequence (3' - 5') |
|---|---|---|---|
| 1 | ITS (Forward) | ITS1 | TCCGTAGGTGAACCTGCGG |
| 2 | (Reverse) | ITS4 | TCCTCCGCTTATTGATATGC |
| 3 | Calmodulin (Forward) | cmd5 | CCGAGTACAAGGAGGCCTTC |
| 4 | (Reverse) | cmd6 | CCGATAGAGGTCATAACGTGG |

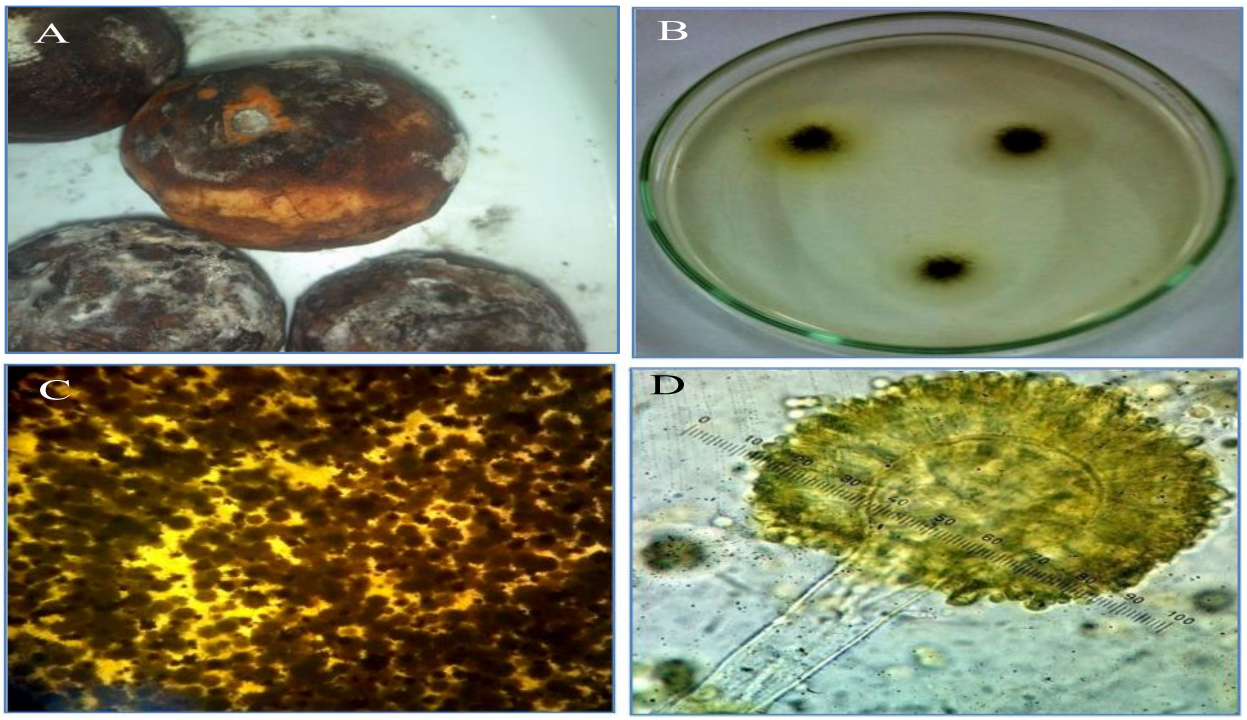


**Fig. 1. Guava rot caused by *A. awamori.* (A): Rotted fruits collected from fruit market; (B): Purified fungal colonies grown on Czapek Dox agar medium for 7 days at 25 ± 2 °C; (C) Radiate conidial heads of *A. awamori* under stereoscope; (D): A biseriate conidial head showing vesicle and attachment of phialides and metullae to vesicle.**

Morphology based identification of *A. awamori* was confirmed by sequence-based identification (Varga *et al.,* 2011). Phylogenetic tree of 23 species from *Aspergillus* section *Nigri* along with the *A. awamori* (FCBP1500) that was generated using ITS data (Figure 3) clearly depicted that *A. awamori* is a distinct species of black Aspergilli group. *A. awamori* could also be distinguished from the currently accepted species of black *Aspergilli* (Varga *et al.,* 2011) based on calmodulin sequence data as this species exhibited unique calmodulin sequence and present in the *A. niger* clade (Fig. 4).

Based on the results of present study, to our knowledge, this is the first report of guava fruit rot from Pakistan caused by *A. awamori*. Guava is an economically important fruit of Pakistan, therefore knowledge about the prevalence and geographical distribution of this new pathogen will facilitate the selection of control measures to limit the economic loss by this pathogen as well as possible health hazards to humans upon consumption of infected fruits.

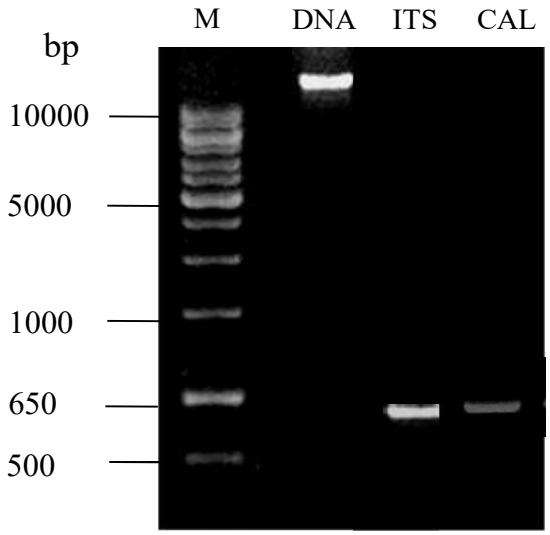


**Fig. 2: Gel electrophoresis of genomic DNA, amplified ITS region of rDNA (ITS) and partially amplified calmodulin gene (CAL) of *A. awamori* (FCBP1500). M=1 Kb DNA marker.**

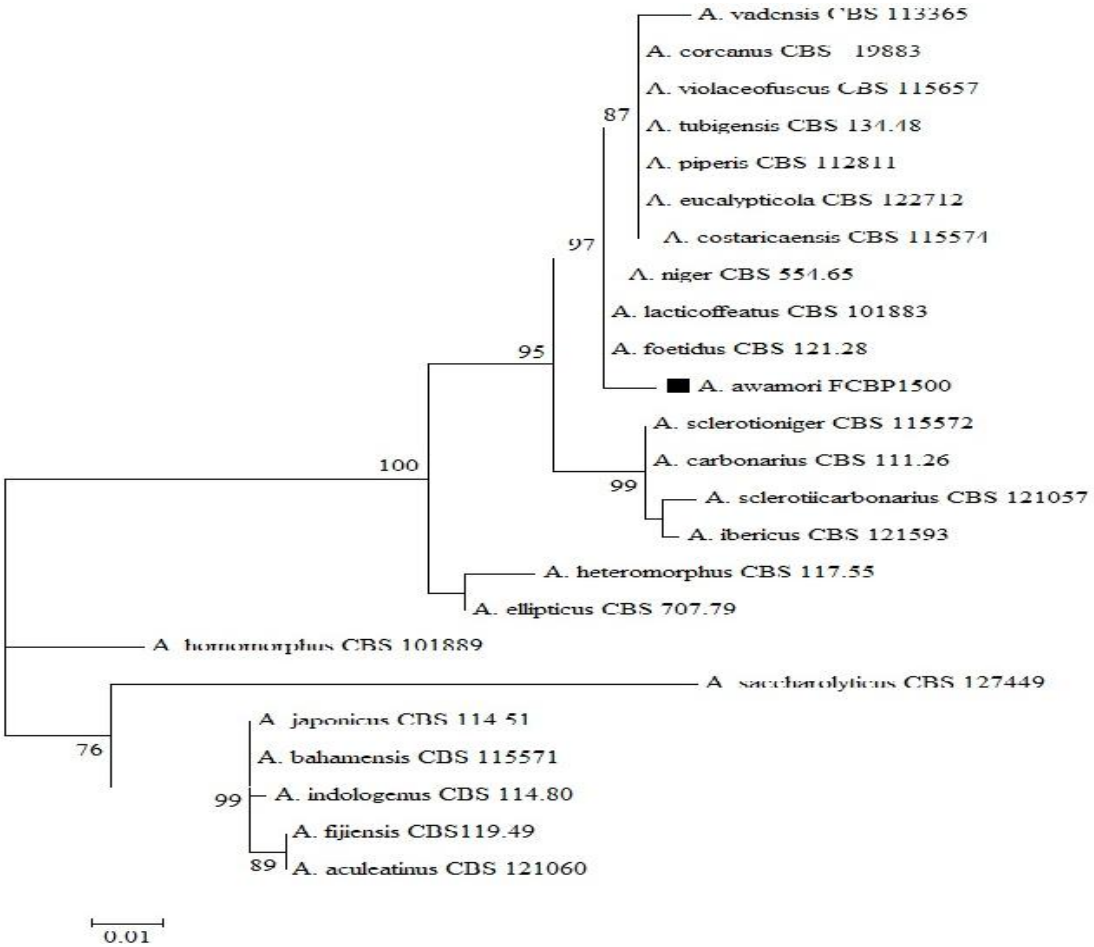


**Fig. 3: Molecular Phylogenetic analysis of ITS nucleotide sequences of members of *Aspergillus* Section *Nigri* by Maximum Likelihood method. Numbers mentioned above branch are values of bootstrap values and values above 70 % are indicated.**

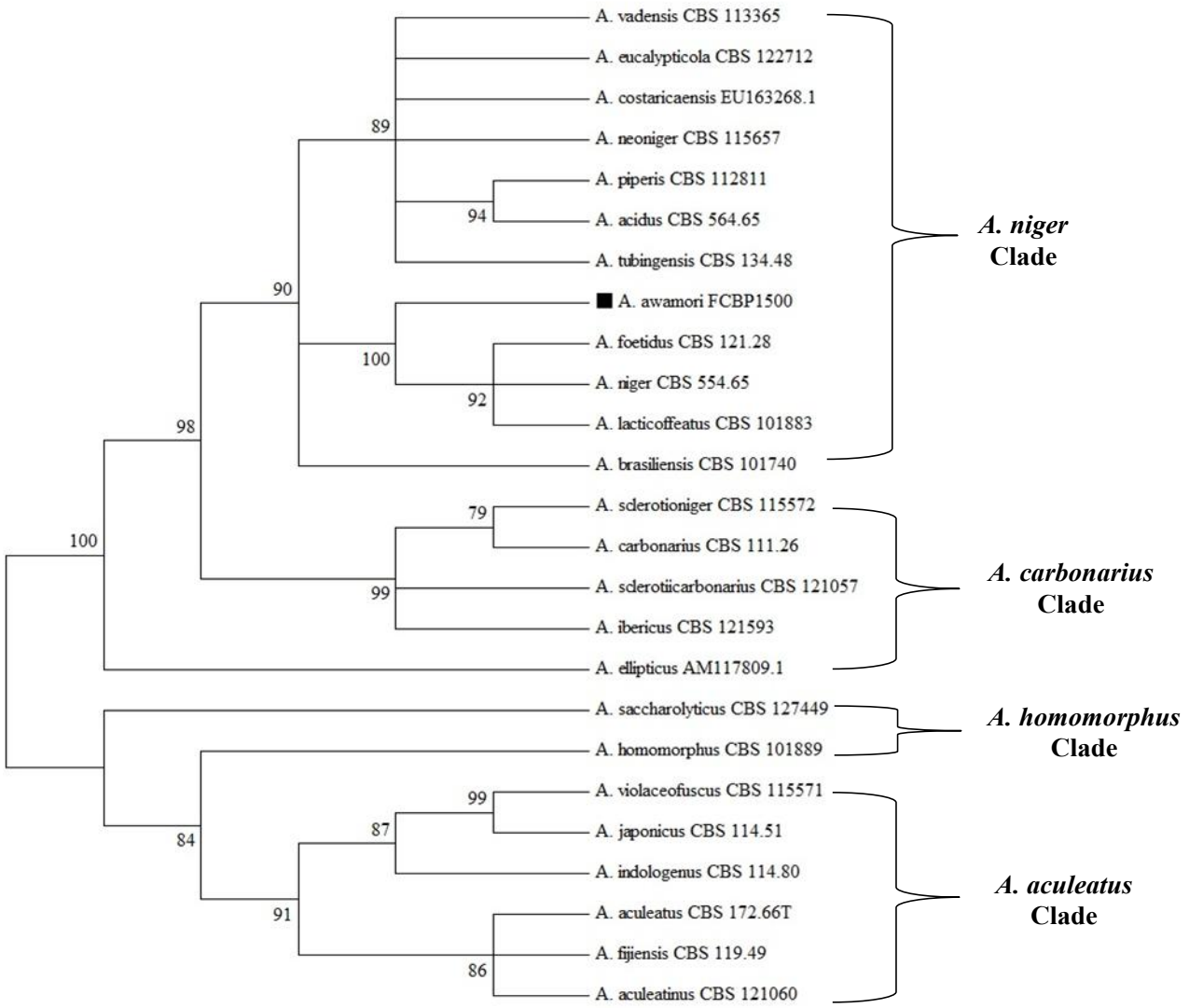


**Fig. 4: Molecular Phylogenetic analysis of partial Calmodulin nucleotide sequences of members of *Aspergillus* Section *Nigri* by Maximum Likelihood method. Numbers mentioned above branch are values of bootstrap values and values above 70 % are indicated.**

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