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Research Article

Isolation and Identification of Fungal Species from Pine Trees in the East of Iran

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ABSTRACT

In order to identify fungi associated with stem and leaf of pine trees in the city of Birjand, samples were collected from different regions of Birjand during 2013 -14. Sampling was carried out from discolored and decay tissues of pine trees. After transferring the samples to the laboratory, small fragments of infected tissues were isolated and surface sterilized and the fungi were isolated using general and specific media culture. Purification of isolates was performed using single spore or hyphal tip methods. Identification of isolates was carried out based on the morphological characteristics of isolates. ITS regions of fungi were amplified and sequenced using ITS1 and ITS4 primers. Calmodulin gene was amplified and sequenced using CMD5 and CMD6 primers in molecular studies. Initial setup was designed using Mega6 analysis software and phylogenetic trees. Strength of phylogenetic trees was evaluated with the application of Bootstrap (1000). Based on morphological and molecular data isolates of this research were determined to be *Aspergillus terreus*, *Embellisia* sp, *Epicoccum nigrum*, *Fusarium acuminatum* and *F. solani* species.

Key words: Mycoflora, *Aspergillus*, *Embellisia*, *Epicoccum*, *Fusarium*

INTRODUCTION

Many species of fungal pathogens have been isolated and reported from pine trees in the world. *Alternaria alternata* species were associated with have been introduced as a foliar and pine bark disease in many forest areas (Huang, 1990). *Aspergillus* species are the reason for decay disease of pine tree seed in America and Europe(Brown and Wylie, 1991). *Botrytis* is gray mold causative agent in most coniferous especially in fir trees, larch and Scots pine in forests of Europe and America(Munoz and Campos, 2013). *Colletotrichum gloeosporioides* species are agents of buds blight disease, browning of young leaves and branch, and death in pine trees around the world(Griffin *et al.*, 1987). *Cercospora sequoiae* and *C. pi-densiflora* species are needle blight agents in pine trees in the forests of the Japan and India (Reddy and Pandey, 1973, Suto, 1986). *Phytophthora*, *Fusarium*, *Rhizoctonia*, *Pythium* and *Cylindrocladium* species are reported from around the world as agents of seedling death disease in pine(Meier *et al.*, 2013). *Dothistroma septosporum* also known as red band needle blight (Bednarova *et al.*, 2007) and species of *Diplodia pinea* and *D. scrobiculata* cause tip blight of pine trees(Ong *et al.*, 2007).

Several species of *Fusarium* have been introduced as agents of dieback, seeds and root rot, and seedlings death and stunting and wilting of trees in different parts of the United States of America(Gordon, 2006).

Studies on fungi associated with pine trees are very limited in Iran. This study aimed to isolate some fungal species associated with pine trees in the city of Birjand in the East of Iran. Birjand, in the South Khorasan province, is known as an evergreen city. There are more than half a million pine trees in the streets of the city. Since, one of the factors that influencing growth of pine is fungal infections, identification of the fungi associated with pine is particularly important.

MATERIALS AND METHODS

The leaves and stems with specific or suspicious symptoms were selected from pine trees in the city of Birjand and transferred to the laboratory in order to isolation pathogenic agents. Small pieces of pine tissues were selected from the between healthy and diseased parts

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of leaves and stems. Infected tissues were surface-sterilized for 1min in 1% sodium hypochlorite solution and were cultured on the potato dextrose agar (PDA) medium (Gordon, 2006). Purification of fungal isolates was performed by single spore or hyphal tip methods using 2% water agar (WA) medium. Identification of isolates was done based on characteristics of colony and microscopic features.

For molecular studies, genomic DNA was extracted by the CTAB method and polymerase chain reaction (PCR) amplification was done using ITS1:5-TCCGTAGGTG AACCTGCGG-3 and ITS4:5-TCCTCCGCTTATTGA TATGC-3 primers (Brandfass and Karlovsky, 2008). PCR mixture contained 2.5 µl of 10X PCR buffer, 0.5 M Tris-HCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.04 Units/ul Taq DNA polymerase(Cinagene, Iran), 0.1µM from forward and reverse primers and 10pg of template DNA (White *et al.*, 1990). The PCR amplification protocol used follows: 1 cycle of 4 min at 95°C, 30 cycles of 30s at 95°C, 1min at 50°C, 1 min at 72 °C and finally 1 cycle of 5 min at 72°C (Darvishnia *et al.*, 2010, Martin and Rygiewicz, 2005). The PCR products were visualized and photographed under UV light.

PCR products were sequenced by the Macrogen Co (South Korea). DNA sequences were manually edited using Bioedit v7.1.3 and aligned using ClustalW software (Gräfenhan *et al.*, 2013). Phylogenetic analysis was conducted using MEGA 6.0 software (Tamura *et al.*, 2013) by neighbor-joining analysis methods and the degree of confidence in phylogenetic branching was assessed by using 1,000 bootstrap re samplings.

RESULTS AND DISCUSSION

In this study, a total of 31 fungal isolates were isolated from infected tissues of pine trees in the city of Birjand. This isolates were belonged to *Aspergillus terreus*, *Embellisia* sp., *Epicoccum nigrum*, *Fusarium acuminatum* and *F. solani* species based on morphological characteristics and molecular data.

Aspergillus terreus

Color of colony of this species was dark yellow-orange which changed to brown by aging (Figure 1a). Color of the underside of the colony was dark brown with yellow margins (Figure 1b). Growth rates of this fungus on PDA culture medium at 25 ° C were 3 cm in 7 days. Semi-spherical vesicles had transparent color and with both round and cylindrical spores (Figure 1c-f). Conidia were smooth, round and spherical as much as 1.5 to 2.5 micrometers (Figure 1g-h). Calmodulin gene sequences of this isolates had 100% similarity with the sequence of the above area in *Aspergillus* species of Gene bank. A total of 8 isolates from this species were isolated from pine trees in Birjand. This is the first report of *A. terreus* from pine trees in Birjand.

This saprotrophic species is prevalent in warmer climates such as tropical and subtropical regions. *A. terreus* has also been found in several habitats such as decomposing vegetation and dust. This spices is commonly used in industry to produce important organic acids, such as itaconic acid and cis-aconitic acid, as well as enzymes, like xylanase (Balajee, 2009).

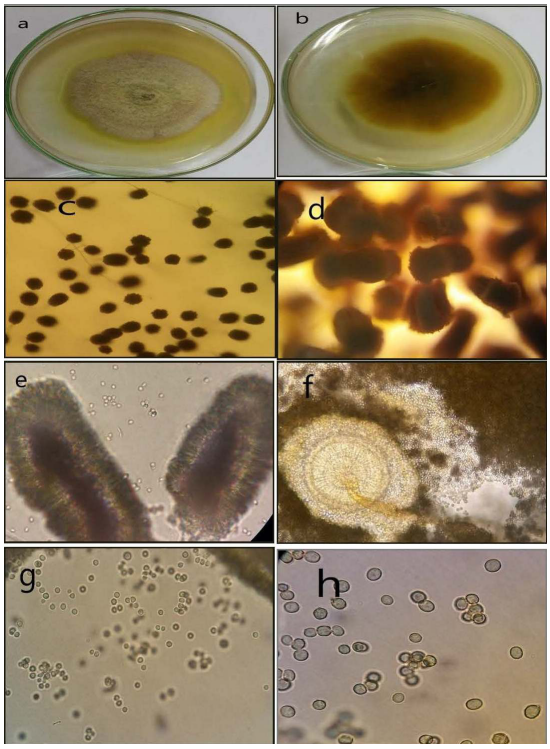

Fig. 1: *Aspergillus terreus*: colony on PDA medium (a-b), conidial head (c-f), conidia (g-h)

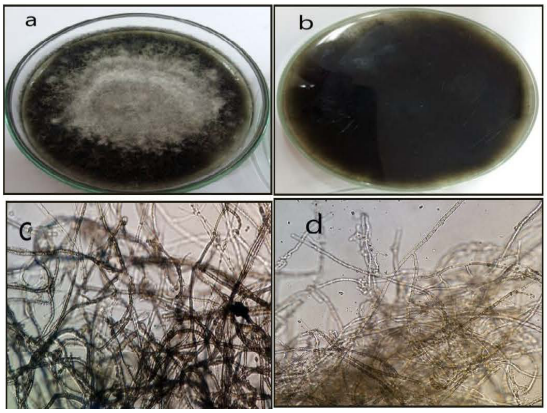

Fig. 2: *Embellisia* sp: colony on PDA medium (a-b), mycelium (c-d)

Embellisia sp.

Color of colony of this species was Gray-brown or Gray-dark olive green (Figure 2a) and the colony was Olive-brown (Figure 2b). Hyphae diameter was 5.3 micrometers (Figure 2c-d) and growth rate was 1.5 cm after 7 days on PDA culture medium at 25°C. These species did not produce spore but sequences of ITS region of these isolates had high similarity with *Embellisia* sp. isolates in GeneBank (Figure 6). A total of 4 isolates from this species were isolated from pine trees in Birjand.

Epicoccum nigrum

At first, the colony of this fungus was yellow to reddish orange which later became brown to black (Figure 3a-b). Mostly black and rare number of white large sporodochium was observed in the colony of this fungus.

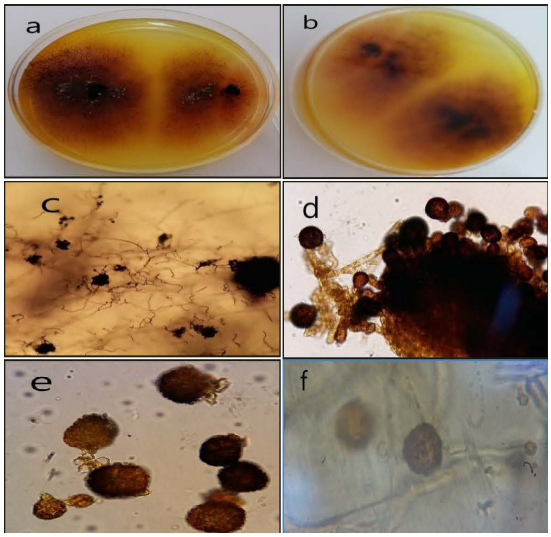

Fig. 3: *Epicoccum nigrum*: colony on PDA medium (a-b), conidia(c-f)

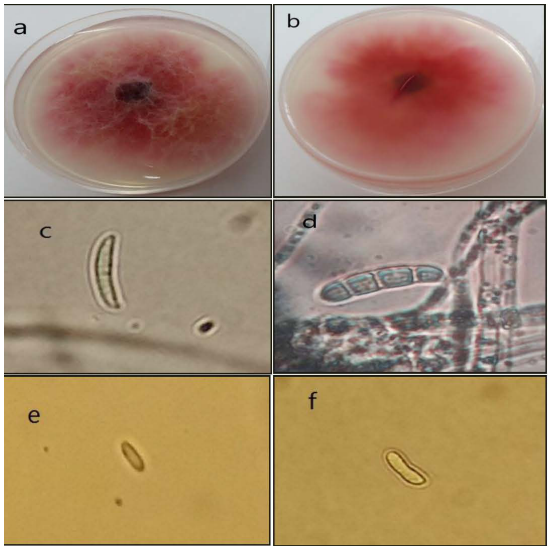

Fig. 4: *Fusarium acuminatum*: colony on PDA medium (a-b), conidia (c-f)

Spores were single or multi-celled, warty brown on the outside, spherical or pear shaped with a funnel-shaped base (Figure 3c-f). Mature spores were 10-15 micrometers in diameter. Sequences of ITS region of this isolates had 100% similarity with the sequence of *E. nigrum* isolates from gene bank (Figure 6). A total of 4 isolates from this species were isolated from pine trees in Birjand.

Fusarium acuminatum

The mean colony growth was 86 mm after 7 days; with white aerial mycelium, developing brownish pigmentation in the center on PDA (Figure 3a-b). The dorsal side of the colony had rose to burgundy pigmentation. Macroconidia were broadly falcate with 3-5 septa, and 3 to 8 × 39 to 64 µm. Microconidia were absent and chlamydospores formed in chains, 20 to 50 µm. ITS sequences of this isolates had one hundred percent similarity with the sequence of F1 isolates of gene bank (Figure 6).

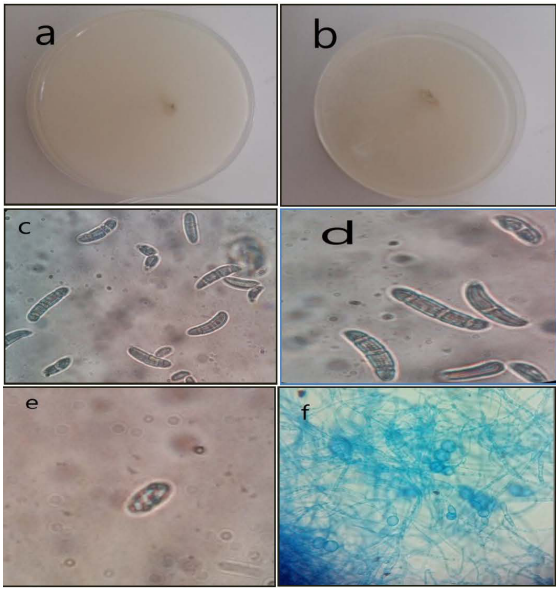

Fig. 5: *Fusarium solani*: colony on PDA medium (a-b), conidia(c-f)

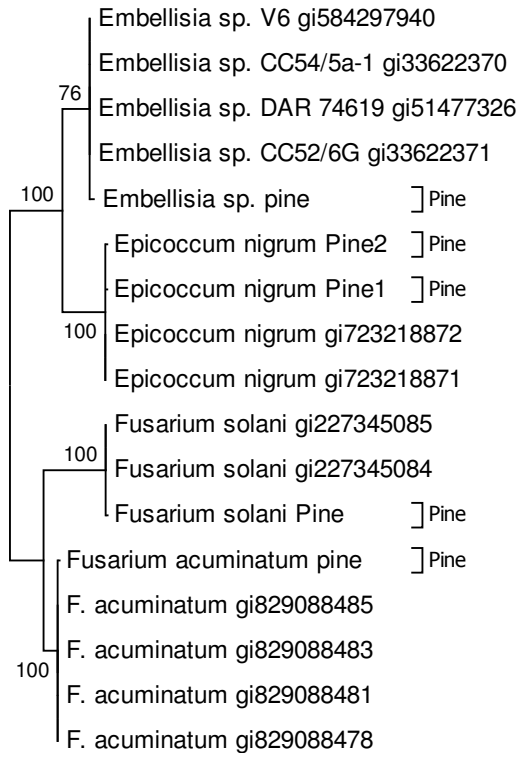

Fig. 6: Phylogenetic analysis of fungal isolates from pine based on ITS1 and 4 sequences using Neighbor-joining methods with 1000 bootstraps

Four isolates of *F. acuminatum* species were recovered from roots and 6 isolates from soil samples in this researc.

Fusarium solani

Growth rate on PDA culture medium at 25 °C was 5 to 7 cm in 7 days. They produced scattered white to cream mycelium and the colony was colorless to cream color

(Figure 5 a-b). Conidiophores were simple to branched. Microconidia were round, spherical and oval shaped and had one to two cells with dimensions of 18.5 × 4.5 mm(Figure 5e). Macroconidia were 4.5 × 43.5 mm ,slightly curved with large and round apical cell and 3 to 4 walls. Chlamydospores were spherical to oval, single or dual to triple chains with dimensions of 9.5 × 7.8 mm (Figure 5 c-f).

Sequences of ITS region of this isolates had one hundred percent similarity with the sequence of *F. solani* isolates of GeneBank. This isolate was in a same phylogenetic groups in phylogenetic tree with GeneBank isolates *F. solani* based on sequences of ITS region (Figure 6). From *F. solani* species, 5 isolates containing 2 isolates from roots and soil of pine and 3 isolates from different parts of pine were isolated.

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