

# Study on genetic diversity of *Pseudomonas tolaasii* and *Pseudomonas reactans* bacteria associated with mushroom brown blotch disease employing ERIC and BOX-PCR techniques\*

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**ABSTRACT:** To distinguish the pathogenic strains of *Pseudomonas tolaasii* and saprophytic strains *P. reactans*, many mushroom cultivation centers in Iran were surveyed and samples were taken from compost, soil cover and button caps with or without visible symptoms preferably from flush two and three. Fifty five bacteria were isolated on the basis of colony morphology, pathogenicity test on excised tissue blocks of the fresh *Agaricus bisporus*, white line test, soft rot on potato tuber slices, erythrocytes hemolysis and certain biochemical and molecular characteristics. A unique PCR product with a molecular weight of 270bp was obtained using specific primer set, PTOF/PTOR of ITS1 region of *P. tolaasii* strains. To confirm the identification of *P. reactans* strains, two strains of this group (A2 and A6) were subjected to RNA polymerase beta-subunit gene (*rpoB*) amplification analysis using primer sets of long amplicon primers LAPS and LAPS27. The polymerase chain reaction products of these strains were sequenced and recorded in NCBI GenBank. In total, 16 isolates of *P. reactans* and 15 isolates of *P. tolaasii* were identified and compared with standard isolates. Subsequently, genetic diversity of the isolates of each species was determined separately with general primers ERIC 1R, ERIC2 and BOX AIR and clusters were drawn. This is for the first time the genetic diversity bacterial pathogen of brown blotch *P. tolaasii* and the saprophyte *P. reactans* was studied in Iran.

**Key words:** Brown blotch, Mushroom, *Pseudomonas tolaasii*, *P. reactans*, *Agaricus bisporus*, White button mushroom

\*This manuscript has been obtained from the M.Sc. thesis of the first author.

## INTRODUCTION

The button mushroom (*Agaricus bisporus*) is the most common cultivated mushroom around the world (Soler-Rivas et al. 1999). It has a very pleasant taste and culture easy. This pleasant edible food is threatened by *Pseudomonas tolaasii*, the causal agent of brown blotch disease in turn, led to serious losses in crop yield (Soler-Rivas et al. 1999). In fact, brown blotch disease or bacterial blotch is one of the most important cultivated mushrooms bacterial diseases in the world with significant annual crop loss.

The disease was identified by Tolaas in year 1915 and called *P. tolaasii* (Geels 1995). Later it was described by Paine (Paine 1919). Lelliott et al. 1966 on the other hand, showed that *P. tolaasii* was indistinguishable from some isolates of *P. fluorescens* and suggested that *P. tolaasii* could be regarded as a normal constituent of the microflora of mushroom beds (Royse and Wuest 1980). Since then on the basis of 16S rRNA sequences, *P. tolaasii* has been placed in the *P. fluorescens* group.

The symptoms of brown blotch begin on the cap of the mushroom then after a few days this causes the dark brown and pitting of level cap and even stripe, and will lead to wet and sunken lesions, which render the crop unmarketable (Lo Cantore and Iacobellis 2004; Wells et al. 1996; Wong and Preece 1982).

*P. reactans* is another saprophytic bacterium which exists in all most mushroom beds. To distinguish these two bacteria, few diagnostic tests were available. These include pathogenicity test, white line and hemolysis tests.

When two types of pathogenic forms of *P. tolaasii* and saprophytic bacterium *P. reactans* were streaked 1cm apart onto King's B medium (king et al. 1954), nutrient agar and pseudomonas agar F (PAF) and incubated at 24°C for 48 h, a white precipitate could be observed, as a line in between colonies both two strains (Soler-Rivas et al. 1999; Andolfi et al. 2008). The white line results from a specific interaction between a diffusible compound produced by *P. reactans* (called the white line inducing principle, WLIP) and tolaasin (Soler-Rivas et al. 1999). Several other studies also demonstrate specific interaction between the two types *Pseudomonas* strains (Munsch and Alatossava 2002).

Molecular methods were also frequently used to detect and identify the bacterial agents. PCR-based fingerprinting techniques currently being exploited in strain discrimination. Phylogenetic analysis of fluorescent pseudomonads on the basis of internal transcribed spacer I region (ITS I region) of ribosomal DNA was also well-documented (Kwon et al. 2000).

Initially the disease was first reported from cultivated button mushroom in northern and central parts of Iran (Rahimiyan et al. 1995). Later it became an endemic of different parts of the country. Phenotypic and genotypic characterization of *P. tolaasii* and its partial has been already reported (Khabbaz-Jolfaee and Rahimiyan 2002; Tajalipour et al. 2012).

The aim of this study is to identify the strains of both *P. tolaasii* and *P. reactans* species and to evaluate genetic diversity of these by employing ERIC and BOX PCRs.

## MATERIALS AND METHODS

### **Sampling and Isolation**

To screen the bacteria *P. tolaasii* and *P. reactans*, major mushroom cultivation centers of Tehran province of Iran were surveyed during 2010-2011 and enormous samples were collected from compost, soil cover and button caps with or without visible symptoms. Approximately 300g from each sample was placed in sterile disposable boxes and covered with selephon nylon. All samples were transferred to the plant pathology laboratory and were kept in the refrigerator at 4°C prior to isolation. Three caps were selected from each sample randomly and were washed with sterile distilled water. The appropriate suspensions were prepared and a loopful of sample suspension was streaked on nutrient agar (NA) and King's B (KB) media. Petri plates were incubated at 25°C for 48-72 hours. In order to obtain a pure culture, a single colony was selected and subcultured on fresh media.

### **Phenotypic characterization**

All bacterial isolates were examined for LOPAT tests (levan, oxidase, potato soft rot, arginine dihydrolyase and tobacco hypersensitive reaction) described by Lelliott and Stead 1987 and Lo Cantore and Iacobellis 2004. Additional phenotypic characteristics such as pathogenicity on the sporophores of *A. bisporus*, production of typical fluorescent pigment on KB medium white line test and hemolysis of erythrocytes were performed (Schaad et al. 2001 and Lelliott and Stead 1987).

### **Pathogenicity test on *A. bisporus***

The isolates were tested for pathogenicity assay on tissue blocks of *A. bisporus* (Ercolani 1970 and Nair and Fahy 1972). The caps were excised in cube-shaped pieces with 2x2.5cm<sup>3</sup> thickness. Each piece was inoculated with fresh culture of individual bacterium at a concentration 10<sup>7</sup>cfu/ml. The test was performed for all isolates each with four replications. Negative control blocks were inoculated with sterile distilled water. Blocks were incubated at temperature 25±1°C and relative humidity of 90% in a moist chamber (Obradovic et al. 2008). After 24 to 48 hours, the blocks were evaluated for disease symptoms appearance. To fulfill Koch's postulates and to determine disease severity the pathogenic strains were re-isolated from diseased blocks on King's B medium.

### **White line test**

This test was performed according to Wong and Preece (1979). Both authentic isolates of *P. tolaasii* NCPPB2192 and endemic strain *P. tolaasii* Pt6 were cultured on NA, KB and *Pseudomonas* Agar F media. The representative isolates suspected to *P. reactans* were streaked with several millimeters distance on either side of *P. tolaasii* NCPPB2192 and *P. tolaasii* Pt6. *P. fluorescens* was used as negative control. Likewise the same method was repeated for authentic isolates of *P. reactans* NCPPB1311 and endemic strain *P. reactans* (A2) against the suspected isolates of *P. tolaasii*. The formation of a precipitate in either tests during 48-72 hours at temperature 25°C was considered as a positive reaction (positive white line).

### ***Erythrocytes hemolysis assay***

*P. tolaasii* demonstrates hemolytic activity, causing lysis of erythrocytes. Hemolytic activity is specific diagnostic traits of *P. tolaasii* and *P. reactans*. Blood Agar Base (BAB) medium was prepared according to the supplier's indications. 5% of sterile defibrinated blood from sheep was aseptically added to the base medium (Munsch and Alatossava 2002; Andolfi et al. 2008). After thorough mixing, the medium was dispensed in several sterile Petri plates. All preliminary identified strains of *P. tolaasii* and *P. reactans* were spot inoculated with standard strain *P. tolaasii* NCPPB2192 and standard strain *P. reactans* NCPPB1311, respectively. *P. fluorescens* was used as a negative control. Plates were incubated at temperature 25°C for 48 h. The diameter of the hemolytic zones for each isolate was evaluated.

### ***PCR assay for detection of P. tolaasii***

Bacterial cells were subjected to the alkaline lysis method to release genomic DNA (Rademaker and De Bruijn 1997). A cell suspension was made in 10 µl of distilled water (about 10<sup>6</sup> cells/ml). After addition of 100 µl of 0.05 M NaOH, the tubes were incubated at 95°C in water bath for 15 min. The mixture was then centrifuged for 2 min at 14000 rpm and the supernatant was used as PCR template. The DNAs of 15 isolates were extracted for PCR analyses where internal transcribed spacer ITS1 sequences with specific primer pair of PTOF and PTOR (Kwon et al. 2000) was amplified. Sequences of the primers were as follows:

PTOF: 5'\_GAACACACAGAGCAGGAGTG\_3'  
PTOR: 5'\_CGTCGTTGATAGTTCGCCTGG\_3'

### ***PCR assay for detection of two representative isolates of P. reactans***

The genomic DNAs of two *P. reactans* isolates (A2 and A6) were also extracted by the alkaline lysis method. PCR amplification of RNA polymerase beta-subunit gene (*rpoB*) from bacterial genomic DNA was carried out using long amplicon primers LAPS and LAPS27 (Ait Tayeb et al. 2005). Sequences of the primers were as follows:

LAPS: 5'-TGGCCGAGAACCAGTTCGCGT-3'  
LAPS27: 5'-CGGCTTCGTCCAGCTTGTTTCAG-3'

### ***Determination the genetic diversity of isolates of P. tolaasii and P. reactans***

Genetic diversity of *P. tolaasii* and *P. reactans* isolates were analyzed by ERIC-PCR and BOX-PCR pattern based on nucleic acid fingerprint templates of isolates (Rademaker and De Bruijn 1997). The two standard strains of *P. reactans* NCPPB1311 and *P. tolaasii* NCPPB2192 were employed to determine the genetic diversity of target strains.

### ***PCR Protocol for DNA amplification***

The DNA sequences of the primers used for DNA fingerprinting were as follows:

ERIC 1R: 5'\_ATGTAAGCTCCTGGGGATTCAC\_3'  
ERIC2: 5'\_AAGTAAGTGAAGTGGGGTGGAGCG\_3'  
BOX AIR: 5'\_CTAGGGCAAGGCGACGCTGACG\_3'

Polymerase chain reactions were carried out in a total volume of 25 ml containing of 2.5 µl PCR Buffer (10x), 1.5 µl (50mM) MgCl<sub>2</sub>, 1.5 µl dNTP (25mM), 1µl of each forward and reverse primers (50 pmol), 0.3 µl Taq DNA Polymerase (5U/µl), 2µl chromosomal DNA and 15.2 µl DDH<sub>2</sub>O were prepared. The reaction mixtures were incubated in a thermocycler (BioRad) model MJ Research PTC. DNA thermal cycler used for PCR amplification was programmed as follows: (i) an initial extensive denaturation step at 92°C for 4 min; (ii) 35 cycles, with each cycle consisting of 92°C for 1 min: 10s, 48°C for 1 min: 10s, and 68°C for 2 min: 40s; and (iii) a final extension step at 68°C for 14 min. The reaction mixture for the primer BOX was similar to ERIC-PCR with slight (Rademaker and De Bruijn 1997).

### ***Electrophoresis of PCR products***

5µl of each amplified mixture was analyzed by agarose (1/2%, w/v) gel electrophoresis in Tris-acetate-EDTA (TAE) buffer containing 0.5 mg of ethidium bromide per ml. Each isolate with 2 ml of buffer loading (Loading Buffer), was completely mixed and the samples were added into the wells of the gel. The molecular size of an unknown piece of DNA can be estimated by adding 2 µl of a molecular marker (O'GeneRuler DNA Ladder Mix) alongside the samples. After 3-2h, the power supply was disconnected and the gel was stained in 0.5 mg/L ethidium bromide solution for 10min. The gel was then photographed with UV Transilluminator. The amplicons of some isolates were sequenced from forward and reverse primers (Sambrook et al. 1989).

### **DNA sequence analysis**

PCR products with 20µl of primers were sent to the Takapozist Company for direct DNA sequencing. Matching sequences was obtained with Seqman (DNASTAR Inc., Madison, Wis.). The resulting sequences were aligned with sequences in the GenBank using BLASTn program (Rademaker and De Bruijn 1997; Altschul et al. 1990). Using NCBI databases two bacterial isolates were sequenced and deposited accordingly. In order to evaluate genetic diversity among the pathovars of *P. tolaasii* and *P. reactans*, DNA primers corresponding to conserved motifs in bacterial repetitive ERIC and BOX elements were used. Two strains of *P. tolaasii* were deliberately deleted because of their similarities to other isolates. The PCR products were subjected to electrophoresis in a 1.2% agarose gel and stained with ethidium bromide. To estimate the size of DNA fragments, a DNA marker (O'GeneRuler DNA Ladder Mix) was used. Similarity analyses were done with the NTSYS-PC program, version 2.0. Similarities between the strains were determined on the basis of Jaccard similarity coefficient in the SAHN program of the NTSYS-pc software. For each coefficient, the similarity matrix was used to construct dendrograms with the help of the unweighted pair grouping by mathematical averaging (UPGMA) method, using the SAHN and TREE programes in NTSYS. Similarly, cluster analysis was also conducted using MEGA, version 5 (Tamura et al. 2007) software.

## **RESULTS**

### **Isolation and identification**

Fifty five strains were obtained belonging to the genus *Pseudomonas*. Of these, 15 isolates belonged to *P. tolaasii* and 16 isolates belonged to *P. reactans*.

### **Phenotypic characterization of bacterial isolates**

LOPAT tests (Levan, Oxidase, Potato soft rot, Arginine dihydrolyase and Tobacco hypersensitive reaction) were performed for all isolates. On KB culture medium, the pseudomonads produced diffusible fluorescent pigment. *P. tolaasii* colonies were produced bluish diffusible pigment and *P. reactans* colonies were produced greenish pigment. Other phenotypic characteristics of the *P. tolaasii* and *P. reactans* are shown in table 1.

### **Pathogenicity test on blocks of *A. bisporus* tissues**

All the 15 strains of *P. tolaasii* were produced variable browning (blotch) symptoms and associated with pitting on blocks of edible button mushroom. These were shown diverse severity among the different strains. Based on disease symptoms and disease severity on *A. bisporus*, the *P. tolaasii* isolates were categorized into 4 groups. Group I with single strain developing very severe symptoms. The group II contains 7 isolates with less severe symptoms. The group III with 5 isolates causing moderate symptoms and the forth group comprises of 2 isolates with ability to induce mild symptoms. *P. tolaasii* Pt6 was the representative of the first group with high level of disease severity. Some strains of *P. reactans* also caused mild brown spots *A. bisporus*. Yet few strains induced yellow lesions and majority did not cause any visible disease symptoms on its host.

### **White line test**

Atypical white line precipitate occurred between *P. tolaasii* and white line reacting bacterium *P. reactans*. The results were similar with both native and authentic cultures of *P. tolaasii* NCPPB2192 and also *P. tolaasii* Pt6 with *P. reactans* NCPPB1311 and *P. reactans* (A2) on three NA, KB and *Pseudomonas* agar F culture media.

### **Hemolytic activity or hemolysis of erythrocytes**

Most of the isolates capable of lysis red blood cells in Blood Agar Medium (BAB) within 24-48h by inducing colorless halo around the colonies. The variation in size indicates the degree of hemolytic activity among the strains. Generally, strains of *P. reactans* gave inhibition zones greater than the *P. tolaasii* strains. *P. fluorescens* as a negative control did not induce any hemolytic zones.

### **PCR assay for detection of *P. tolaasii***

The DNAs of *P. tolaasii* isolates were extracted and amplified with primer set of PTOF and PTOR. Unique PCR products with a molecular weight of 270bp were obtained (Fig. 1).

### **Genotype identification of two representative strains of *P. reactans***

Two strains of *P. reactans* from group 1 were selected for molecular diagnosis. This was done by amplification and sequencing of the *rpoB* gene. The DNAs were amplified with primer set of LAPS and LAPS27.

The PCR products with a molecular weight of 1250bp were obtained and subsequently were sequenced and deposited in NCBI GenBank. rpoB gene sequences of these two strains (ippbc pra2 isolate A2 and ippbc pra6 isolate A6) are available at GenBank under accession numbers of JX491482 and JX491485, respectively. BLAST (BLASTn) search against the NCBI sequence databases were indicated 99% similarity between strain A2 and P. reactans strain LMG 5329 (HMO70042.1) and 98% identity to P. reactans strain LMG 5329 (HMO70042.1) for strain ippbc pra6 isolate A6.

**Evaluation of genetic diversity of P. tolaasii and P. reactans strains**

Similarities and differences in DNA sequences were obtained by detecting the number and position of the bands per profile the weak and strong bands in which were generated. Approximate range size of the bands in ERIC-PCR and BOX-PCR methods were between 200bp-3000bp and 200bp-4000bp, respectively. Two dendrograms were constructed based on BOX-PCR and ERIC-PCR profiles (Figures 4 and 5).

As it was shown in Fig. 4, P. tolaasii strains were classified into two major groups based on amplification patterns obtained with set primers of ERIC2/ERIC 1R and the primer BOX AIR. Group I was included 11 strains encoded as Pt 1, Pt 2, Pt 3, Pt4, Pt6, Pt7, Pt8, Pt10, Pt11, Pt12, Pt13 and the group II comprised of two strains of Pt5 and Pt9.

After PCR analysis of strains P. reactans, amplification and electrophoresis PCR product using primer sets ERIC 1R/ ERIC2 and primer BOX AIR, P. reactans strains were indicated the presence of four distinct groups. Group I includes strains Pr 1, Pr 3, Pr 8, Pr 10, Pr 14 and Pr 16. Group II includes five strains Pr 2, Pr 5, Pr 11, Pr 12 and Pr 15. Group III includes three strains of Pr 4, Pr 6, Pr 13 and the group IV includes strains Pr 7 and Pr 9.

Table 1. Phenotypic features of P. tolaasii and P. reactans isolated from cap and bed mushroom.

Characteristic	P.	P.
	reactans	tolasii
Gram reaction	-	-
Fluorescent	+	+
Catalase	+	+
White line test(vice versa)	+	+
Levan	-	-
Oxidase	+	+
Potato soft rot	-	-
Tobacco hypersensitivity	-	-
Arginine dihydrolyase	+	+
Utilization of:		
Citrate	+	+
2-ketogluconate	+	+
Hydrolysis of:		
Gelatin	+	+
Casein	+	+
Starch	-	-
Lecithinase	+	+
Utilization of glucose	+	+
L-Tartrate	+	-
D-Xylose	+	-
Sucrose	-	-

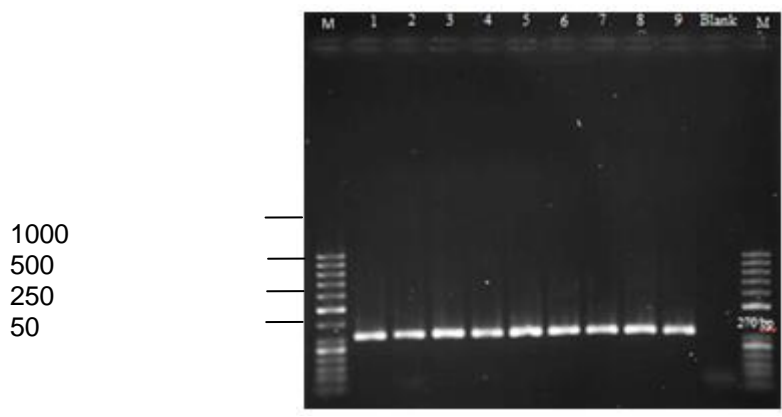


Figure 1. PCR amplification of ITS1 region of *P. tolaasii* strains using specific primer set, PTOF/PTOR. M, 50bp DNA marker on both side of the gel; Lane 1-9 *P. tolaasii* strains: Pt 1, Pt 2, Pt 3, Pt 4, Pt 5, Pt 6, Pt 7, Pt 8, *P. tolaasii* NCPPB2192, Blank.

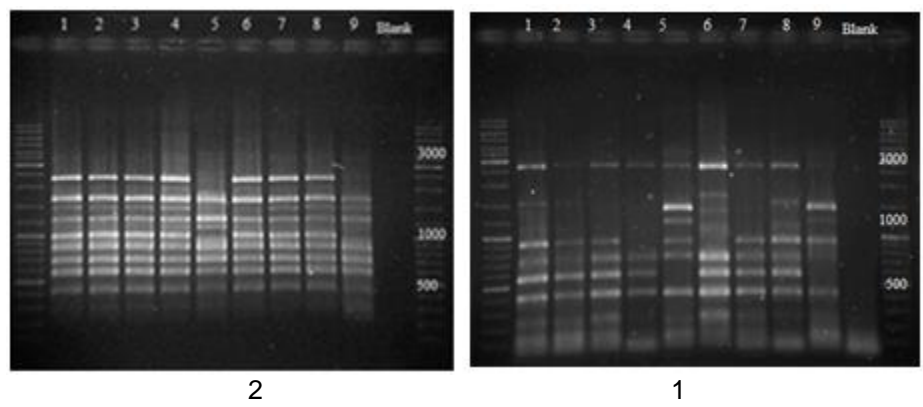


Figure 2. Genetic fingerprints of genomic DNA isolates obtained from of *P. tolaasii* strains using general primer set, ERIC-PCR(1) and BOX-PCR(2).

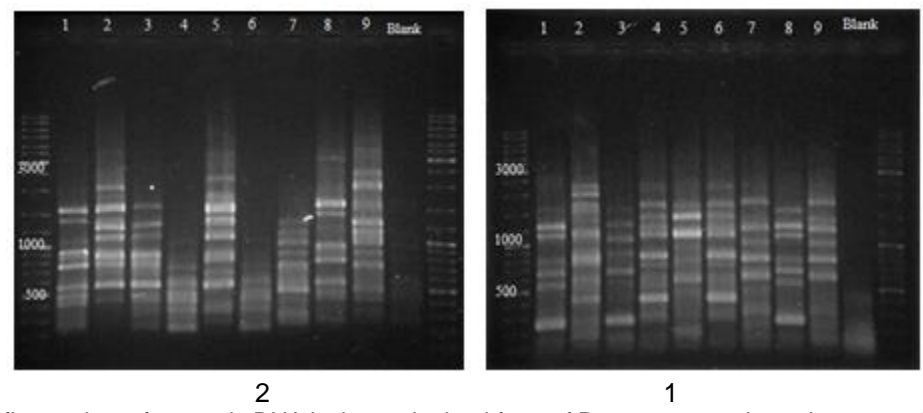


Figure 3. Genetic fingerprints of genomic DNA isolates obtained from of *P. reactans* strains using general primer set, ERIC-PCR(1) and BOX-PCR(2).

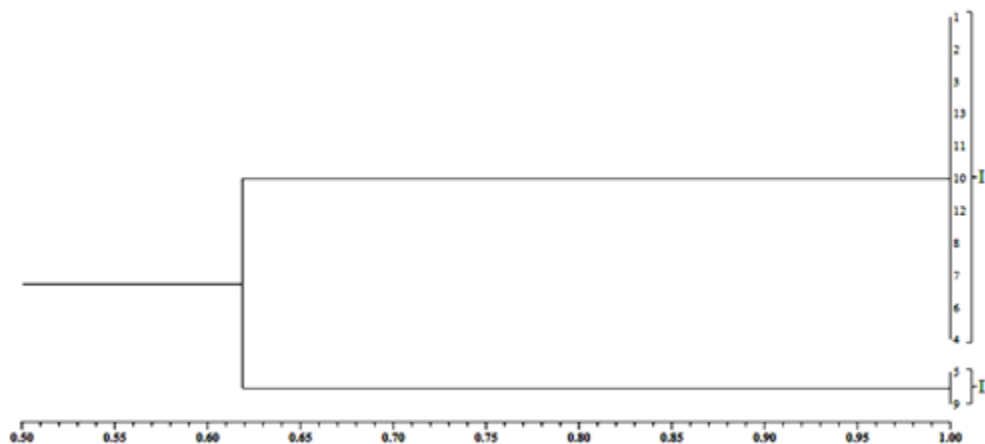


Figure 4. Dendrogram associated to electrophoresis PCR product ERIC-PCR and BOX-PCR of *P. tolaasii* strains.

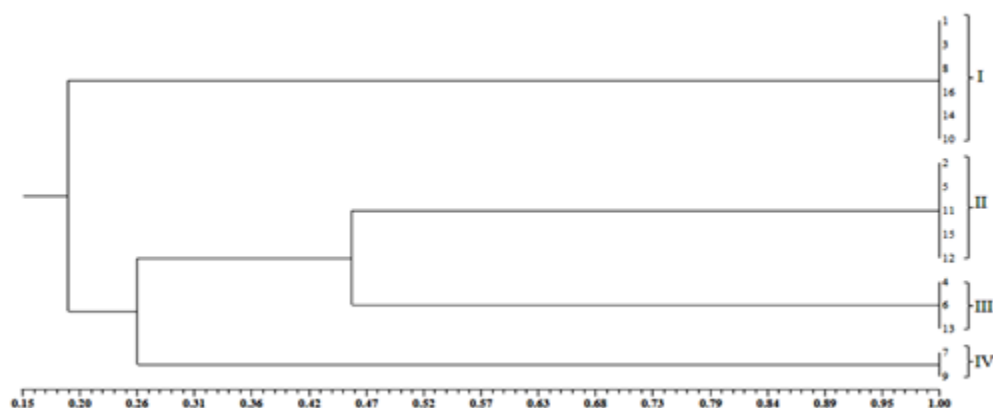


Figure 5. Dendrogram associated to electrophoresis PCR product ERIC-PCR and BOX-PCR of *P. reactans* strains.

## DISCUSSION

At present, bacterial blotch disease is the main problem in industrial units growing mushroom throughout the world. It also affects significantly the crop yield in Iran. It is well-documented that *P. tolaasii* is not an easy bacterium to eliminate because it can modify its biochemical pathways to survive in many adverse environments. (Nair and Fahy 1973).

The main characteristic of this bacterium is its capability to produce an extracellular toxin called as tolaasin (tolassin group).

From pathogenic points of view, we observed variable disease symptoms on edible button mushroom (*A. bisporus*) produced by different strains of *P. tolaasii*. Based on this trait, the strains were categorized into 4 major groups. Group I with a single strain caused severe symptoms.

No doubt this is due to the differences in genetic structure of pathogenicity, particular is in level production tolaasin. On the other hand, recent studies were shown that the WLIP, the lipodepsipeptide compound produced by *P. reactans* is a potential inhibitor of the symptoms in blotch disease which in turn, leads to inactivation tolaasin inactivation (Soler-Rivas et al. 1999).

In this study we also tried to investigate genetic diversity of pathogenic strains of *P. tolaasii* and associated bacterium *P. reactans* in Tehran province. Comparable results were obtained with PCR products of *P. tolaasii* strains by using specific primer set PTOF/PTOR (Kwon et al. 2000).

To identify the two strains of *P. reactans* at molecular level, these were subjected to RNA polymerase beta-subunit gene (*rpoB*) amplification using primer sets of long amplicon primers LAPS and LAPS27. PCR products of the strains A2 and A6 were then sequenced and recorded in NCBI GenBank.

Based on these results, strains of the two species *P. tolaasii* and *P. reactans* were distinguished on the basis of genetic structure. A thorough and complementary research work was needed to evaluate the genetic diversity among the well known bacterial pathogens of mushroom (Goor et al. 1986).

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