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Research Article

Characterization of *Mycothermus thermophilus* engaged in mushroom composting in Iran

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Abstract: The thermophilic fungus *Mycothermus thermophilus* is one of the most important thermophilic fungi in mushroom composting process. Thirty nine isolates of *M. thermophilus* were collected from nine provinces of Iran and were identified as *M. thermophilus* based on morphological features and ITS regions. The studied isolates significantly increased the growth of *Agaricus bisporus* hyphae compared to control when used *in vitro* situation. Also the colony morphology of the mushroom changed when it grew on the colony of *M. thermophilus.* While the studied thermophilic isolates were morphologically different, no diversity was observed in terms of Random Amplified Polymorphic DNA (RAPD) finger-printing. The genetically clonal population of *M. thermophilus* collected from Iranian mushroom composting farms was attributed to lack of sexual reproduction, similar raw materials used in compost formulations, compost temperature, and concentration of ammonia during pasteurization as selection pressures.

Keywords: *Agaricus bisporus*, cultivated mushroom, genetic diversity, RAPD, thermophilic fungi. *Mycothermus thermophilus*

Introduction

The foremost prerequisite for the successful cultivation of *Agaricus bisporus* (Lange) Imbach (the white button mushroom) is the selective nutritional substrate called compost (Parati *et al.*, 2011). Composting process is composed of two main step called phase I and phase II. In phase I, the raw materials such as pre-wetted wheat straw, broiler litter, gypsum and urea or ammonium sulphate are completely mixed together and then transferred to bunkers or indoor tunnels. The first phase of composting takes 9-12 days based on the nature of the raw materials. Due to the microbial activities temperature of the compost increases and nitrogen-rich lignocellulose complex is formed. The temperature of the compost reaches 75-80 °C in this step. Phase II is performed in specialized pasteurization tunnels and takes 5-7 days. In this step, the harmful pests and moulds are killed and ammonia disappears (Noble and Gaze, 1996;Souza *et al.*, 2014).

Mycothermus thermophilus (Cooney & R. Emerson 1964) Natvig, Taylor, Tsang, Hutchinson and Powell, gen. et comb. nov. (Natvig *et al.*, 2015) has the main role in removal of ammonia and selectivity of the compost for the growth of *A. bisporus* (Ross

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and Harris, 1983a, b). *M. thermophilus* rapidly colonizes all parts of the compost in conditioning stage of phase II, then its biomass is used as food by *A. bisporus* in the next stage of mushroom cultivation. There are several studies confirming stimulation of mushroom mycelium growth by *M. thermophilus* and significant correlation between the population of this thermophilic fungus and mushroom yield has been demonstrated (Coello-Castillo *et al.*, 2009; Salar and Aneja, 2007; Straatsma *et al.*,1991; Straatsma *et al.*, 1989; Straatsma and Samson, 1993).

Mycothermus thermophilus was known before as *Scytalidium thermophilum* (Natvig*et al.*, 2015). This fungus has two types of growth. Type I has very dark single spores produced on short lateral hyphal branches. In dual culture of this type with *A. bisporus*, at first bilateral inhibition is seen, then the mushroom grows over the thermophilic fungus and uses it as food by lysis mechanism. In type II, spores are intercalary, produced in chains and slightly pigmented. In this type, the mushroom mycelia can immediately colonize the colony of *M. thermophilus* (Cooney and Emerson, 1964; Lyons *et al.*, 2000;Mouchacca, 1997;Straatsma and Samson, 1993).

Lyons *et al.*, (2000) proved that the ITS1-5.8S-ITS2 sequences show high identity between the isolates of these two types and also was not able to differentiate these two types but RAPD grouping clearly separated the two types from each other. The results of RAPD were correlated morphologically and thermo-gravimetrically.

Studies on genetic population of *M. thermophilus*, contribute on special strains of *M. thermophilus* having higher ability to colonize the substrate, produce more enzyme, consume ammonia and easy-available carbohydrates during conditioning period; thereby increasing the growth of *A. bisporus* mycelia and mushroom yield. Despite the importance of this thermophilic fungus in the mushroom composting process, no considerable investigation has been done regarding its population genetics in Iran. Hence this study was performed in order to determine the situation of *M. thermophilus* isolates from various composting farms of the country.

Materials and Methods

Collection and isolation of *Mycothermus thermophilus*

Mycothermus thermophilus isolates were collected from different mushroom composting sites of Iran (nine provinces) during 2014. From each composting farm, at least four parts of the second phase compost were sampled. The codes, date of collection, geographic origin of the 39 isolates studied in this investigation are presented in Table 1.

Isolation was done using serial dilution. Briefly, five gram of each sample was added to 95 ml sterilized distilled water containing 0.01% tween 20. After agitating for ten minutes, 100µL of diluted homogenate was inoculated on Yeast Dextrose Agar (YDA) plates containing Streptomycin sulphate (50 mgl^{-1}) and Penicillin G ($50mgl^{-1}$). The inoculated plates were incubated at 48 ± 1 °C for three days in the dark. During the incubation period, these plates were screened for the presence of fungal colonies. Single spore cultures were obtained for genetic purification. The isolates were maintained on YDA slant tubes at 4 °C.

Identification of the isolated fungi was followed using taxonomic criteria presented by Barnett and Hunter (2006), Straatsma and Samson (1993) and Natvig *et al.* (2015).

Morphological observation and mycelial growth rate

Colony characteristics and growth measurements of all isolates were studied on YDA medium. Five mm inoculum plugs from the growing edge of *M. thermophilus* colonies were transferred and placed at the center of nine cm Petri dishes in five replicates. The cultures were incubated at 48 ± 1 °C in the dark. Mycelial growth of the colonies was monitored and recorded every day until the colony reached the edge of Petri dishes.

Table 1 Characteristics of *Mycothermus thermophilus* isolates collected from various composting farms in Iran.

Isolate	Collection Site	Composting farm	Date of collection	Exact location (Latitude and Longitude)	Colony diameter (mm) at 48 °C	Growth of *Agaricus* in dual culture test compared to the control (%)
Ar1	Ardabil, Divlaq	Chatrak	Jul 03, 2014	N38.256330 E48.14445	72.0 a–i	29.4 a–j
Ar2	Ardabil, Divlaq	Chatrak	Jul 03, 2014	N38.256330 E48.14445	75.3 a–g	28.2 b–j
Es2a	Isfahan, Mohammadabad	Yekta	Aug 21, 2014	N32.353188 E52.15105	75.7 a–f	16.5 i–k
Es4b	Isfahan, Mohammadabad	Yekta	Aug 21, 2014	N32.353188 E52.15105	65.0 g–l	44.7 a
Es4	Isfahan, Mohammadabad	Yekta	Aug 21, 2014	N32.353188 E52.15105	60.0 j–n	36.7 a–f
Po1	Hashtgerd, Tankaman	Yekta	Aug 21, 2014	N35.894126 E50.633044	70.3 a–i	40.0 a–d
Po2	Hashtgerd, Tankaman	Yekta	Aug 21, 2014	N35.894126 E50.633044	73.3 a–h	20.0 f–k
TMU1	Tehran, Peikanshahr	TMU	Jul 24, 2014	N35.744076 E51.162741	68.3 c–j	9.4 k
TMU2	Tehran, Peikanshahr	TMU	Jul 24, 2014	N35.744076 E51.162741	52.3 n	23.5 d–k
De1	*Dezful,* Siahmansour	Golgeh Dez	Dec 02, 2014	N32.338570 E48.500767	71.0 a–i	23.5 d–k
De2	*Dezful,* Siahmansour	Golgeh Dez	Dec 02, 2014	N32.338570 E48.500767	67.3 e–k	18.8 g–k
De3	*Dezful,* Siahmansour	Golgeh Dez	Dec 02, 2014	N32.338570 E48.500767	68.7 c–j	17.6 h–k
De5	Dezful, Siahmansour	Golgeh Dez	Dec 02, 2014	N32.338570 E48.500767	63.0 h–m	31.8 a–j
De6	Dezful, Siahmansour	Golgeh Dez	Dec 02, 2014	N32.338570 E48.500767	80.7 ab	22.4 e–k
De8	Dezful, Siahmansour	Golgeh Dez	Dec 02, 2014	N32.338570 E48.500767	75.7 a–f	37.6 a–e
De10	Dezful, Siahmansour	Golgeh Dez	Dec 02, 2014	N32.338570 E48.500767	79.3 ab	29.4 a–j
Ja	Abyek, Zargar	Javaneh	Jun 29, 2014	N36.071261 E50.396072	69.3 b–j	28.2 b–j
Ja8	Abyek, Zargar	Javaneh	Jun 29, 2014	N36.071261 E50.396072	78.7a–c	15.3 jk
Ja1	Abyek, Zargar	Javaneh	Jun 29, 2014	N36.071261 E50.396072	84.0 a	30.6 a–j
Ker1	Kermanshah	Niloufar	Sep 09, 2014	N34.388292 E46.955291	72.7 a–i	29.4 a–j
Ker2	Kermanshah	Niloufar	Sep 09, 2014	N34.388292 E46.955291	62.3 i–m	35.3 a–g
Ker3	Kermanshah	Niloufar	Sep 09, 2014	N34.388292 E46.955291	67.7 e–k	37.6 a–e
Ker4	Kermanshah	Niloufar	Sep 09, 2014	N34.388292 E46.955291	74.7 a–f	30.6 a–j
Ma1	Mashhad	Hamisheh Bahar	Sep 25, 2014	N36.497121 E59.364517	79.9 ab	23.5 d–k
Ma2	Mashhad	Hamisheh Bahar	Sep 25, 2014	N36.497121 E59.364517	65.3 f–l	15.3 jk
Ma3	Mashhad	Hamisheh Bahar	Sep 25, 2014	N36.497121 E59.364517	77.3 a–e	27.1 c–j
Ma4	Mashhad	Hamisheh Bahar	Sep 25, 2014	N36.497121 E59.364517	64.7 h–l	30.6 a–j
Ma5	Mashhad	Hamisheh Bahar	Sep 25, 2014	N36.497121 E59.364517	75.3 a–g	30.6 a–j
Sa1	Abyek, Gazersang	Sadaf	Jun 27, 2014	N36.012824 E50.524558	72.7 a–i	34.1 a–h
Sa2	Abyek, Gazersang	Sadaf	Jun 27, 2014	N36.012824 E50.524558	80.3 ab	15.3 jk
Sa3	Abyek, Gazersang	Sadaf	Jun 27, 2014	N36.012824 E50.524558	78.0 a–d	30.6 a–j
Sa4	Abyek, Gazersang	Sadaf	Jun 27, 2014	N36.012824 E50.524558	78.7 a–c	42.4 a–c
Se1	Shahrud, Bastam, Dehkheyr	–	Sep 27, 2014	N36.550437 E54.956910	79.3 ab	22.4 e–k
Se2	Shahrud, Bastam, Dehkheyr	–	Sep 27, 2014	N36.550437 E54.956910	80.0 ab	45.9 a
Se3	Shahrud, Bastam, Dehkheyr	–	Sep 27, 2014	N36.550437 E54.956910	66.0 f–l	40.0 a–d
Gha1	Ghazvin	Chatre sepid	Jun 22, 2014	N36.264514 E50.128712	58.0 k–n	43.5 a–c
Gha2	Ghazvin	Chatre sepid	Jun 22, 2014	N36.264514 E50.128712	69.3 b–j	32.9 a–i
Gha4	Ghazvin	Chatre sepid	Jun 22, 2014	N36.264514 E50.128712	54.7 mn	16.5 i–k
Gha5	Ghazvin	Chatre sepid	Jun 22, 2014	N36.264514 E50.128712	57.3 l–n	32.9 a–i

Values within a column followed by the same letters do not differ significantly by Duncan test (P = 0.05). TMU: Tarbiat Modarres University.

All micro-morphological features such as hyphae, conidiogenesis structures and spores measurement were examined. A thin layer of YDA was poured on sterilized slide placed in the Petri dishes. Inoculum plugs of each isolate was placed on the sterilized slide in the Petri dishes. Differential interference contrast microscope (Olympus BX51) equipped with Olympus DP72 camera was used for measurement and observation. For each isolate at least 40 samples were measured using MycoCam V4. software. No stain was used for slide preparation.

Culture of *Agaricus bisporus*

Agaricus bisporus (Sylvan A15, a white mid-hybrid variety) was used in the dual culture test. This commercial strain was maintained on compost extract agar media in slant tubes at 5 °C.

Dual culture

Dual culture of *A. bisporus* and *M. thermophilus* was performed based on den Camp *et al.* (1990) procedure with some modification. Inoculum plugs (10 mm diam.) from the active growing edge of 3 days old cultures of *M. thermophilus* were transferred to the edge of the nine cm Petri dishes (with 10 mm distance from the edge of Plates) containing Compost Extract Agar (CEA). The inoculated Petri dishes were incubated at 48 ± 1 °C for four days, when much of each culture medium was colonized by *M. thermophilus*. Then temperature of the plates was reduced to 24-25 °Cand mycelial plugs of *A. bisporus* from active growing edge of 20 days old cultures were transferred to CEA plates previously colonized with the thermophilic fungal isolates. The plugs were placed across from the M. *thermophilus* colony at a distance of 10 mm from its border. The controls consisted of the plates in which only the plugs of *A. bisporus* were placed. The growth of the mushroom mycelia was monitored daily and compared with the control. This experiment was repeated twice, each with four replications.

DNA extraction

Single spore cultures of *M. thermophilus* isolates were grown in 250 mL Erlenmeyer flasks containing yeast dextrose broth and incubated in shaker-incubator (LabTech, LSI-3016R) at 110 r/min for 3 d at 48 ± 1 °C. The produced mycelial biomass of the thermophilic isolates were harvested using vacuum filtration and washed at least twice with deionized distilled water and the dried biomass was stored at -70 °C until use.

The mycelial biomass was ground to a fine powder using liquid nitrogen. The genomic DNA was extracted using cetyl-trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980) with some modification. The ground biomasses were transferred to polypropylene tubes containing 600 µLDNA extraction buffer (100mM Tris HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB and 1% β-mercapto ethanol). The tubes were incubated at 65 °C for 30 min with gentle swirling at intervals. Two thirds volume of chloroform: isoamyl alcohl (24: 1, v/v) was added to ingredients in the tubes and mixed. The tubes were maintained for 15-25 min at -20 °C and then centrifuged (Bechman, 64R) at 13000 r/min for 10 min at 4 °C. DNA pellets were dried overnight at room temperature. The dried pellets were dissolved in deionized distilled water. For purification of the extracted DNA, *RNase* treatment was performed as Murray and Thompson (1980) method. The purified DNA was qualified and quantified by Eppendorf bio-photometer at A280/260 nm absorption spectrum and final concentration was adjusted to 20 ng/µL for use in PCR analysis.

RAPD analysis

Nine RAPD primers (Lyons*et al.*, 2000;Singh *et al.*, 2005) were chosen in this study. Sequences of primers and their characteristics are presented in Table 2. RAPD amplification was performed in 25µL reaction mixture containing 12.5µL of *Taq* PCR Master Mix (Amplicon Taq 2 × master mix red), 40ng genomic DNA, 2µM decamer primer and 8.5 µL deionized distilled water. Thermal cycler (Eppendorf, master cycler, epgradient, Germany) was programmed as follow: Initial denaturation for 3 min at 94 °C, 35 cycles of 94 °C for 1min, 36.2-43.7 °C for 1 min (annealing temperature for each decamer primer was optimized as indicated in Table 2), 72 °C for 1 min with a final extension step at 72 °C for 10 min.

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The amplified products were visualized on 1.4 agarose containing 0.1µL mL^{-1} safe fluorescent stain (NS1000, SMOBIO) gel in 1 × TBE buffer and numeric pictures of agarose gel was recorded using transilluminator (GelDoc, Vilber Lourmat, T-5 x 20-2A). The similarity matrix of RAPD product fingerprints were subjected to cluster analysis using NTSYSpc 2.1a software with the method of UPGMA and jaccard's coefficient and the appropriate dendrogram was drawn. 1kb DNA ladder (DM 3100, SMOBIO) was used as size marker.

Table 2 Characteristics of RAPD primers and polymorphism obtained by RAPD analysis in *Mycothermus thermophilus* isolates.

Primer	Source	Sequence (5′–3′)	Annealing temperature °C	No. of polymorphic loci	No. of monomorphic loci	Total no. of loci	Size range (bp)	Polymorphism (%)
OPP14	(Singh *et al.* 2005)	CCAGCCG AAC	43.7	0	11	11	300–2500	0.0
OPP6	(Singh *et al.* 2005)	GTGGGCT GAC	40.0	0	13	13	100–3000	0.0
OPN9	(Singh *et al.* 2005)	TGCCGGC TTG	38.6	0	13	13	200–2000	0.0
OPA1	(Singh *et al.* 2005)	CAGGCCC TTC	39.0	0	9	9	300–2000	0.0
OPA4	(Singh *et al.* 2005)	AATCGGG CTG	40.6	0	16	16	100–2000	0.0
A13	(Lyons *et al.* 2000)	CAGCACC CAC	36.2	0	10	10	300–2000	0.0
A3	(Lyons *et al.* 2000)	AGTCAGC CAC	41.4	0	9	9	200–1500	0.0
A1	(Lyons *et al.* 2000)	GTGCAAT GAG	43.5	0	7	7	200–3000	0.0
A11	(Lyons *et al.* 2000)	CAATCGC CGT	41.4	0	11	12	250–2000	8.3
Total				1	99	100		1.0

Amplification of ITS1-5.8S-ITS2 regions

The ITS region of genomic DNA was amplified using ITS1 (5′ GGAAGTAAAAGTCGTAACA AGG 3′) and ITS4 (5′ TCCTCCGCTTATT GATATGC 3′) primers (White *et al.* 1990). The amplification was performed in a total volume of 50µL reaction mixture containing 25µL of *Taq* PCR Master Mix (Amplicon Taq 2 × master mix red), 80ng genomic DNA, 2µM of each forward and reverse primers and 17µL deionized distilled water. Reaction was performed in 0.2mL thin wall polymerase chain reaction (PCR) tubes using Eppendorf epgradient thermocycler with the following program: one cycle of three min at 94 °C, 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and a final extension period for 7 min.

The PCR products were analyzed by electrophoresis on 1.5% agarose gel. The gel was stained with safe fluorescent Gel Stain (NS1000, SMOBIO) (0.50µgmL^{-1}) and visualized under UV to confirm DNA amplification. The molecular weight of amplified DNA was estimated by 1kb DNA ladder (DM 3100, SMOBIO). The amplified fragment was extracted from the gel and purified (Wizard; Promega) according to the manufacturer's protocol. Amplified PCR products were sequenced by Macrogen Co. (Seoul, Korea) with ABI PRISM, 3730XL Analyzer.

DNA sequence analysis

The results of ITS1, ITS2, and 5.8S rDNA sequences were edited with BioEdit program and then compared with the GenBank database using BLAST (Basic Local Alignment Search Tool) algorithm to query all the nucleotides databases presented at NCBI (National Center for Biotechnology Information). Sequences matched for the rDNA gene sequences were selected and the sequences of those with valid records were obtained for more analysis. Sequences were manually aligned by CLUSTAL W (Higgins *et al.*, 1992) and phylogenetic rooted tree was constructed using maximum likelihood analysis (ML) with MEGA5.05 program (Kumar *et al.*, 2001). For examination of reliability of the constructed tree, 1000 bootstrap replicates were employed (Felsenstein, 1985).

Statistical analysis

Data were analyzed by the SAS system (SAS Institute) using the general linear model. Analyses of variance (ANOVA) were performed and the means were compared by Duncan Multiple Range Test at $p \leq 0.05$.

Results

Sampling and identification

A total of 39 isolates of *M. thermophilus* were collected from nine provinces (Fig. 1). All isolates were identified as *M. thermophilus* based on the taxonomic criteria presented by Barnett and Hunter (2006), Straatsma and Samson (1993) and Natvig*et al*. (2015).

The produced conidia varied from globose to ellipsoidal and in most cases were sphaerical (Fig. 2 A-C). Dimensions of spores were 4.5-18.7 × 6.3-18.7 µm, No differentiated reproductive organs like phialides were observed and conidia were of arthrospore type; the mycelia were separated by transverse septa and arthrospres were formed (Fig. 2 D, E). Spores got swollen and changed from ellipsoidal to globose in shape (Fig. 2 F, G), then the cell wall of the young spores were thickened and conidia were observed as dark brown under the microscope (Fig. 2 J, K). The colonies of *M. thermophilus* in the early stages of growth were light colored then gradually turned black from the center of the Petri dishes with aging. The aerial mycelia and aerial chains of conidia were observed in the Petri dishes (Fig. 2L). The width of vegetative hyphae was 3.3-5.9 µm.

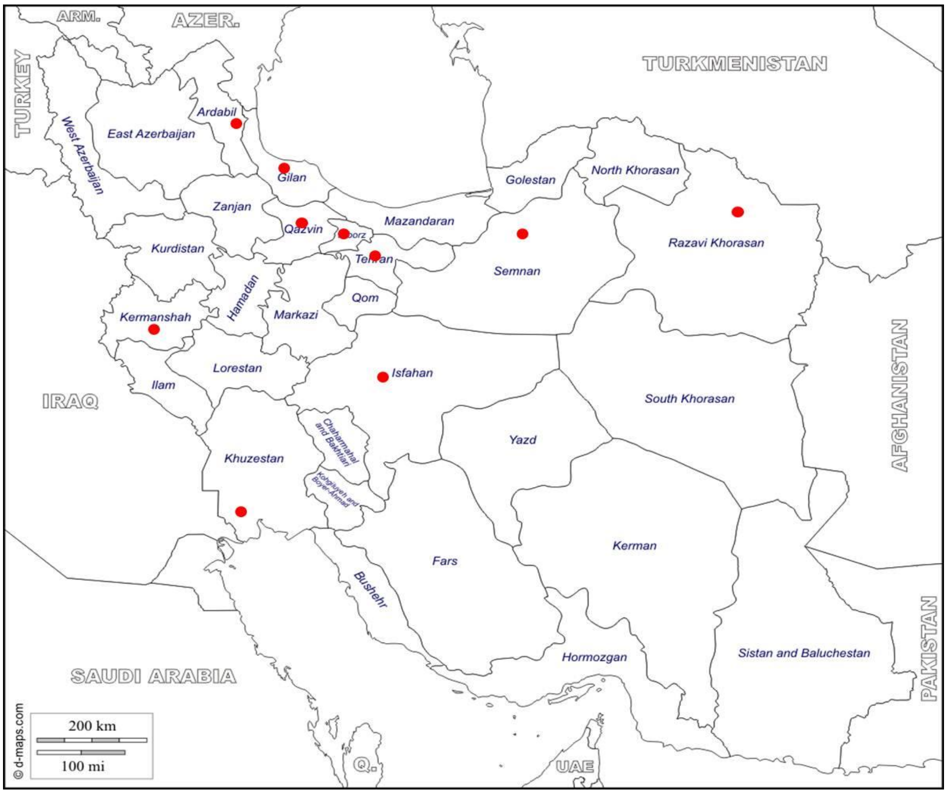

Figure 1 The isolation sites of *Mycothermus thermophilus* are shown by red spots on the map of Iran.

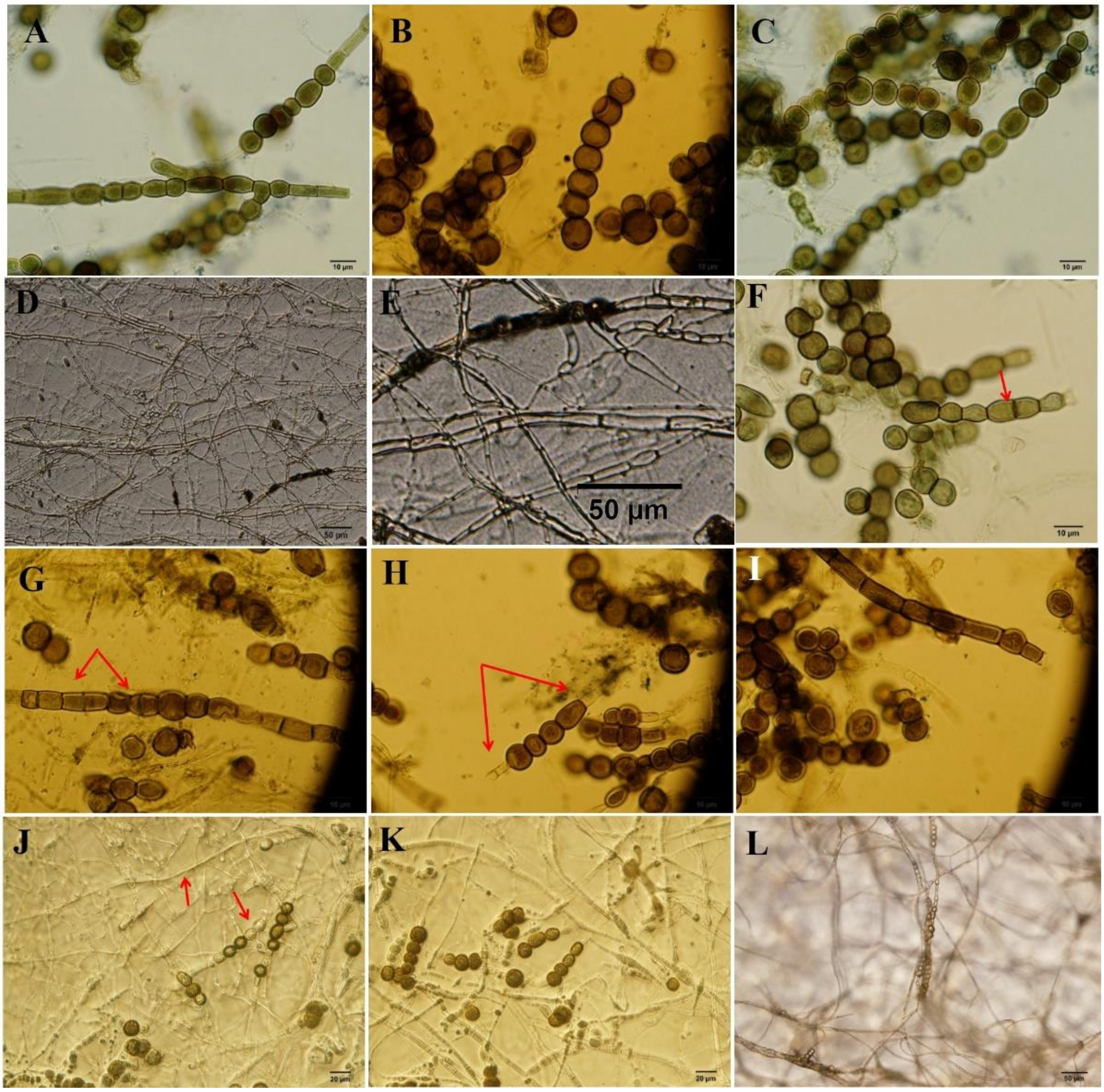

Figure 2 *Mycothermus thermophilus*. A-C: conidial chains. D, E: septa re are formed in the mycelium and cell wall is thickened. F, G: the septation between two cells that are swelling. H: the mycelia on the both sides of the chain of spores are fading. I: cylindrical spores produced by forming of septa. J, K: vegetative mycelia not yet differentiated into conidia are seen with the differentiated spores. L: aerial mycelia with spores produced on them. Bars: A-C 10 µm; D, E 50 µm; F-I 10 µm; J-K 20µm; L 50 µm. No staining was used in slide preparation and visualization.

The conidia were produced in chains (like the chain of beads) and were not disturbed or separated easily from each other by air flow or hits. For this reason, the procedure for single spore culture was modified.

Aleuriospores (single dark spores produced on short lateral hyphae) were not observed in any of the studied isolates indicating that all the *M. thermophilus* isolates belonged to the type II of the thermophilic fungus.

The morphology of colonies was different in the replicates of each isolate, but there were similarities in colony morphology among all isolates. At first the colonies were off-white to grey, then because of sporulation and with aging their color gradually turned black from

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the center of colony. The margin of colonies differed in shape from regular to lobed and filled the 10 cm Petri dishes (Fig. 3) at 48 °C during 80 h. Grouping of the isolates based on morphology of colonies was not possible, because of variations among the replications of each isolate and the sub-cultures. There were, however, significant differences at 5% probability level among isolates based on mycelial growth rate (Table 1). Isolate Ja1 with 84 mm in 72 h had the most rapid growth and TMU2 with 52.3 mm had the slowest mycelial growth on YDA Petri dishes.

Mycelial growth rate of *Agaricus bisporus* in dual culture

All the studied isolates significantly increased the growth of *A. bisporus* compared with the control. Also colony morphology of the mushroom changed when growing on the colony of *M. thermophilus* (Fig. 4). In this investigation the mushroom mycelia had the highest and lowest growth on the colonies of Se2 (45.9%) and TMU1 (9.4%) compared with the control, respectively. The mean comparison between isolates are shown in Table 1.

DNA polymorphism

DNA polymorphism detected by RAPD pattern was the same in all of the isolates for the two replicates. The RAPD products ranged in size from 200 to 3000 bp. The characteristics of the amplification products and DNA polymorphism observed for each RAPD primers are given in Table 2. Nine decamer primers produced a total 100 RAPD reproducible markers (99 monomorphic and 1 polymorphic). Among the primers, polymorphism was only observed in A11. Dendrograms generated by A11 divided isolates into two groups at 91.5 percent similarity level (Figs. 5, 6).

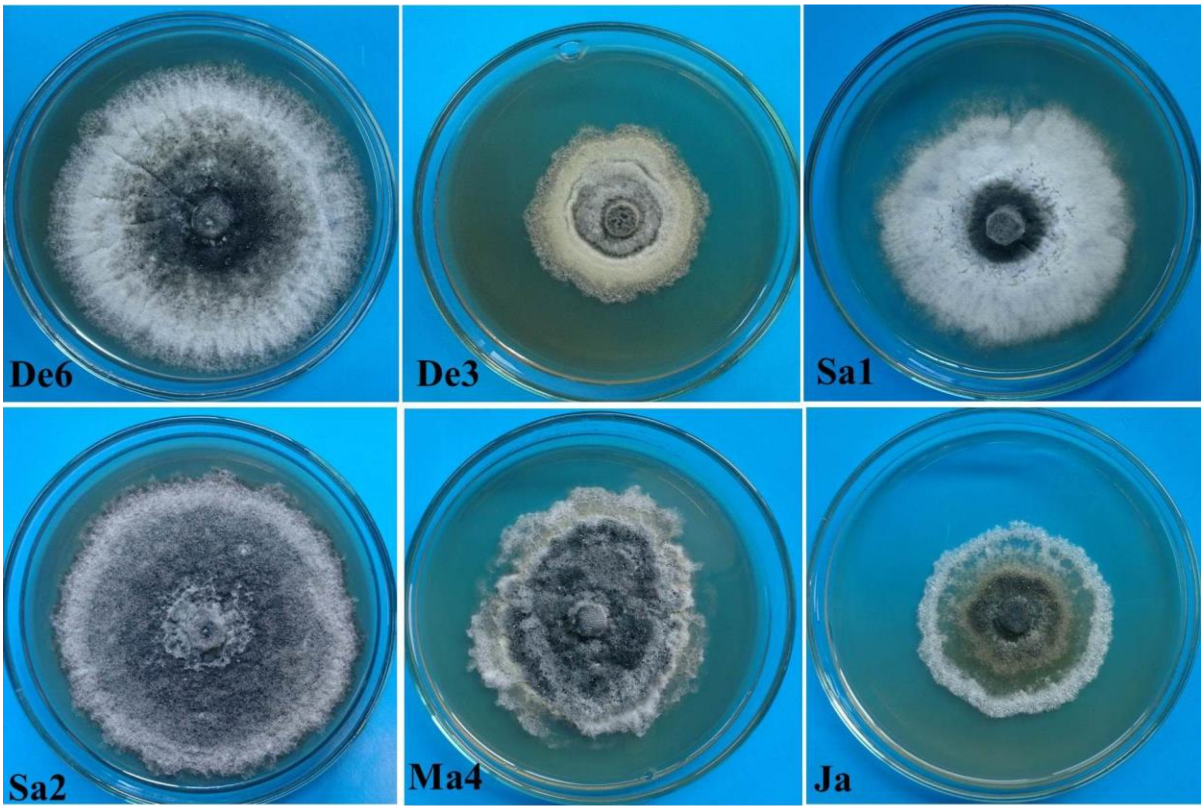

Figure 3 Colony appearance of some isolates of *Mycothermus thermophilus* on yeast dextrose agar (YDA) medium at 48 °C.

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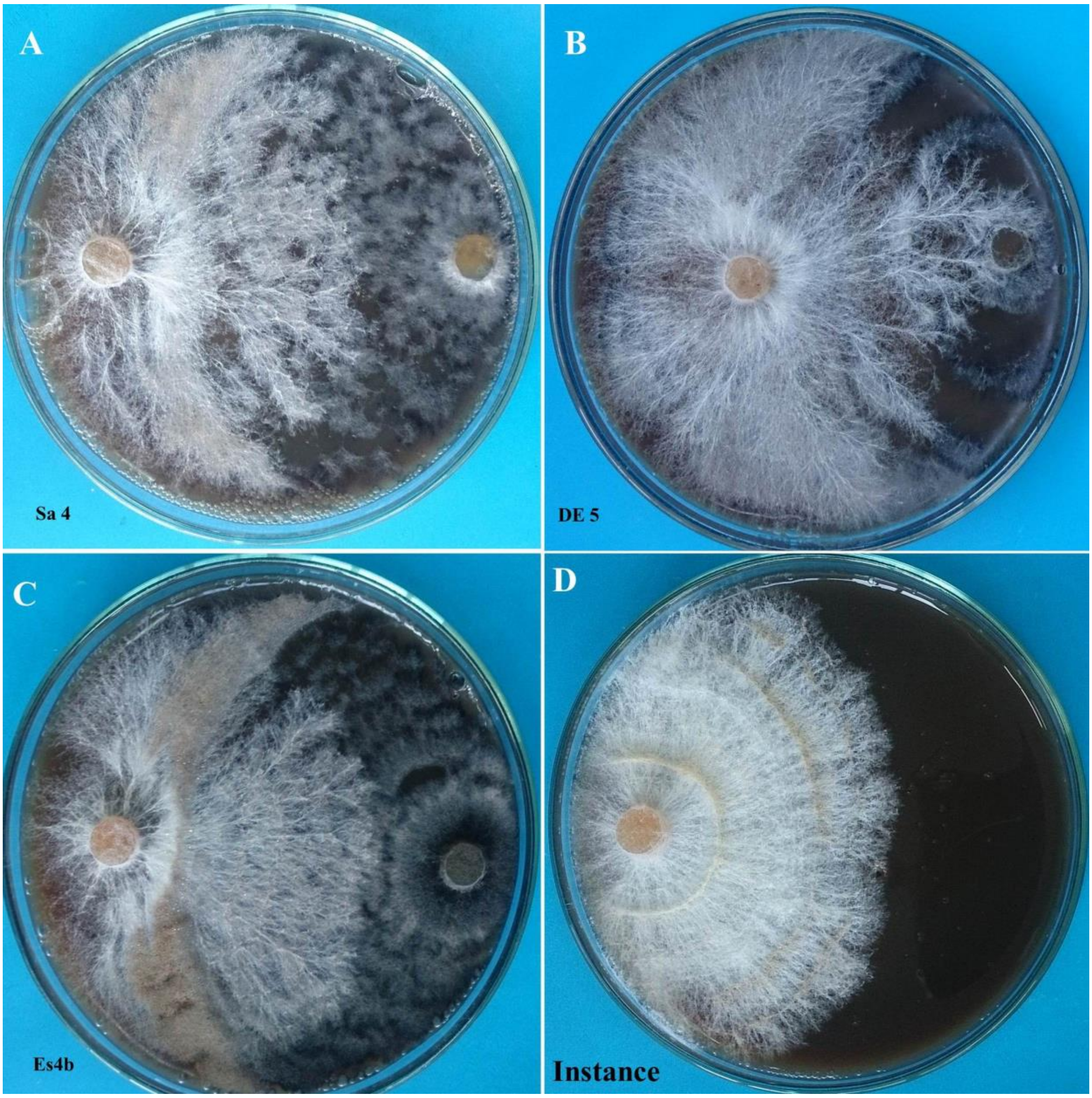

Figure 4 Dual cultures of *Agaricus bisporus* and *Mycothermus thermophilus* on compost extract agar (CEA) medium at 25 °C. A-C: increasing in the growth of *A. bisporus* and shape changes of the mycelia into more linear and thicker ones as a result of growing on the colonies of *M. thermophilus*. D: the control treatment that only the mushroom was cultured on CEA.

ITS sequence analysis

Due to the lack of genetic diversity among the isolates of *M. thermophilus*, only ten isolates belonging to both RAPD groupings were selected for sequencing. The ITS1 and ITS4 primer pairs amplified a 550-600 bps fragment containing ITS1, 5.8S and ITS2 regions (Fig. 7). The ITS sequences of the representative isolates were submitted in GenBank and the sequenced *M. thermophilus* isolates were deposited in Iranian fungal culture collection(WDCM 939).The NCBI accession numbers and Iranian fungal culture collection numbers are presented in Table 3.

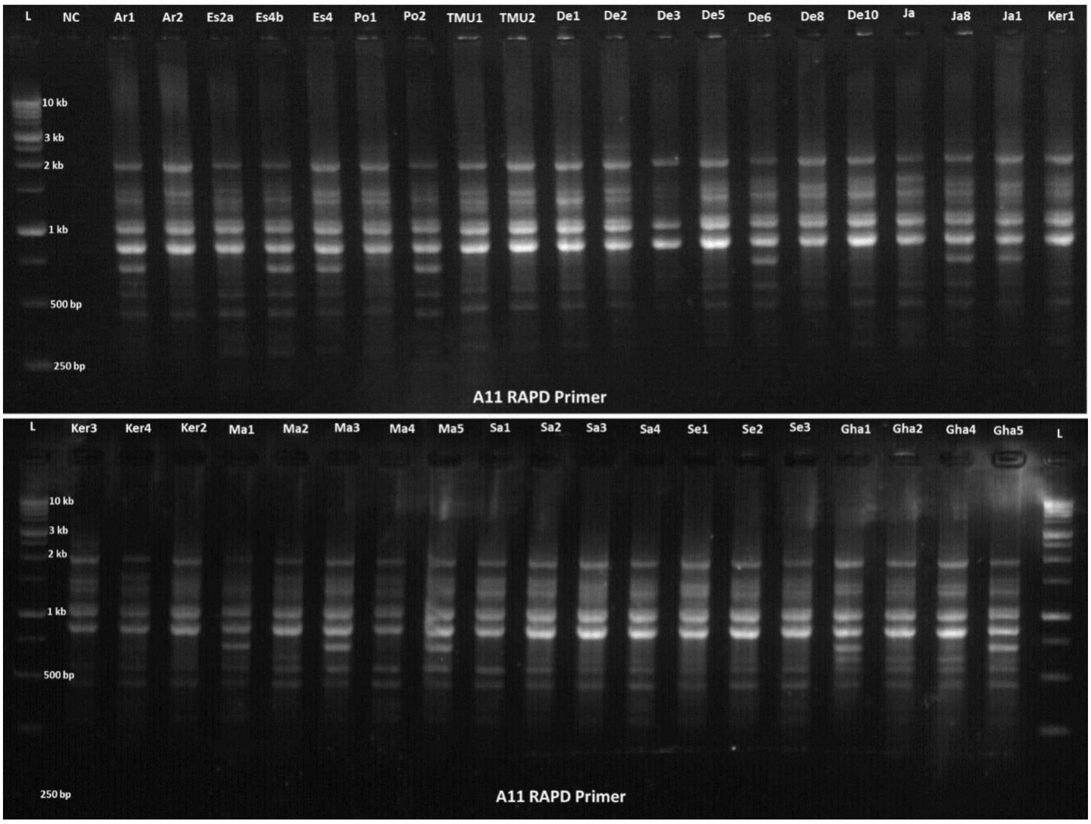

Figure 5 RAPD pattern in *Mycothermus thermophilus* isolates obtained with A11 primer, NC: negative control, L: 1kbp size marker ladder.

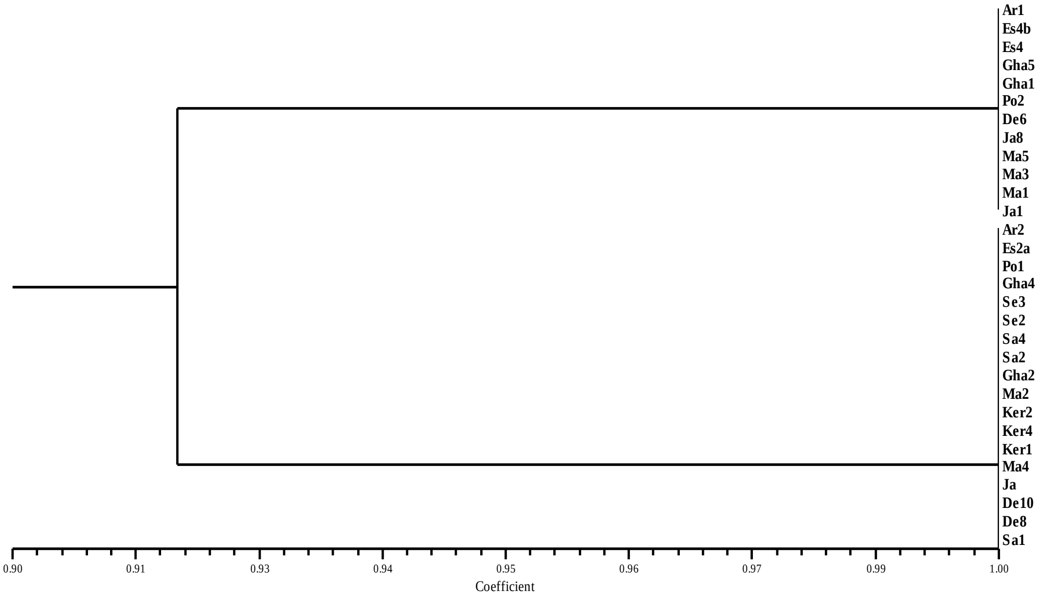

Figure 6 Dendrogram showing genetic relationship among the 39 Isolates of *Mycothermus thermophilus* collected from different places of Iran based on UPGMA clustering method and Jaccard's coefficient.

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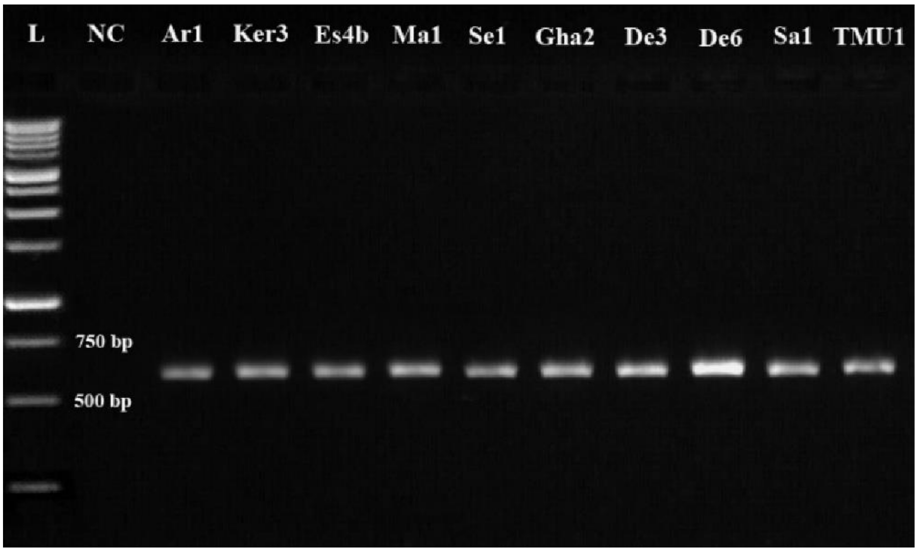

Figure 7 The amplified fragment with 550–600 bps containing ITS1, 5.8S and ITS2 of *Mycothermus thermophilus* isolates. L: molecular weight marker (1kb DNA ladder), NC: negative control.

Table 3 Accession numbers of the ITS sequences of *Mycothermus thermophilus* isolates deposited in GenBank and Iranian fungal culture collection.

Isolate	Accession number	Culture collection accession number	Isolate	Accession number	Culture collection accession number
Ar1	KX898140	IRAN 28012C	Gha2	KX898145	IRAN 2810C
Es4b	KX898141	IRAN 2805C	De6	KX898146	IRAN 2809C
Ker3	KX898142	IRAN 2811C	TMU1	KX898147	IRAN 2807C
Ma1	KX898143	IRAN 2813C	Sa1	KX898148	IRAN 2808C
Se1	KX898144	IRAN 2814C	De3	KX898149	IRAN 2806C

Comparison of ITS sequences of the thermophilic isolates with the valid records in NCBI showed that these isolates belong to *Scytalidium thermophilum* which in recent nomenclature is changed to *M. thermophilus*.

The phylogenetic tree based on ITS sequence analysis showed that the Iranian *M. thermophilus* isolates were completely identical with the ex-type isolate and the other valid records (Fig. 8). *Remersonia thermophila* and *Humicola fuscoatra* var. *fuscoatra* were used as out-group.

Discussion

The features related to the vegetative hyphae, conidiogenesis process, the shape and size of spores and the morphology of colony of *M. thermophilus* is in accordance with the results of investigations by Straatsma and Samson (1993) and Natvig *et al.* (2015).

In this study, the colony morphology of the *M. thermophilus* isolates were very different from each other in the same replications and in the subsequent sub-culturing, returning to the original morphological characteristics was seen. Straatsma and Samson (1993) also stated that the morphology of colony of *M. thermophilus* is variable and in each sub-culturing a different morphology of colony is seen. In the present study, all the isolates collected from different regions of the country were able to grow well at 48 °C and increased the mycelial growth rate of *A. bisporus* compared to the control. Also the morphology of the mushroom colony changed during its growth on the biomass of *M. thermophilus* isolates. Regardless of the fact that the growth of the mycelia of *A. bisporus* was significantly different on the isolates of *M. thermophilus*, no reduction or inhibition in growth of the mushroom mycelium was observed.

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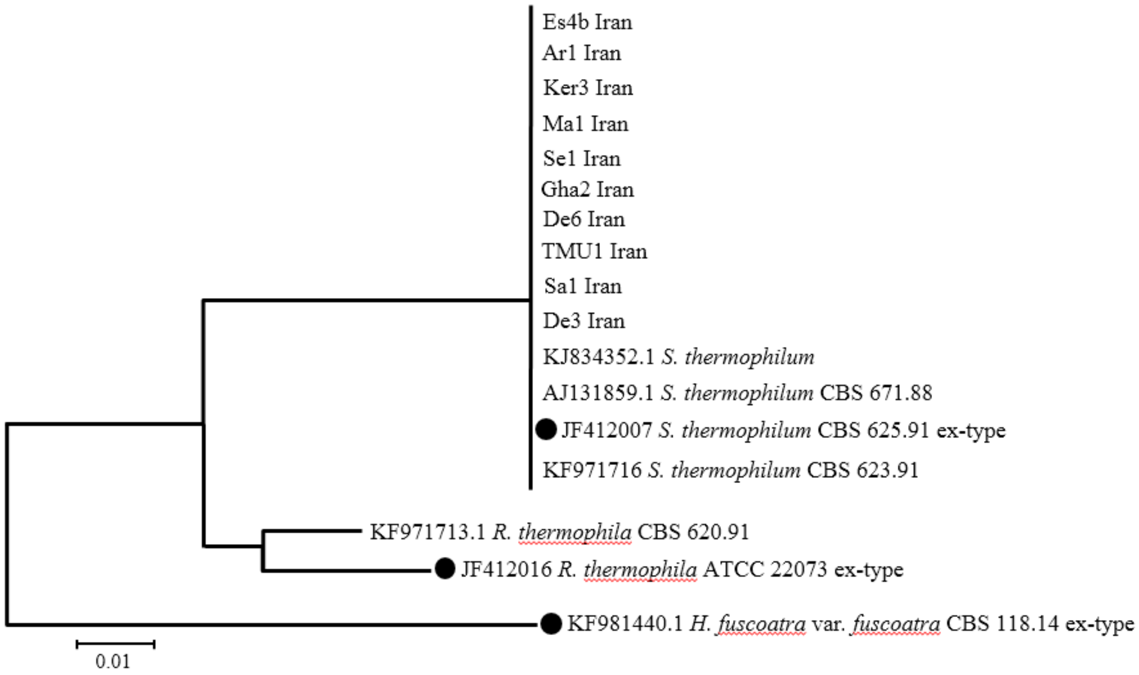

Figure 8 Maximum likelihood tree of *Mycothermus thermophilus* based on ITS1-5.8S-ITS2 sequences constructed using MEGA5.05 program. *Remersonia thermophila* and *Humicola fuscoatra* var. *fuscoatra* were used as out-group. The bootstrap values (1000 replicates) are shown for each node as percent. The black circles are the ex-type isolates.

Lyons *et al.* (1999) demonstrated that depending on the type of *M. thermophilus,* there are two kinds of interaction between *M. thermophilus* and *A. bisporus.* In type I of *M. thermophilus*, both *M. thermophilus* and *A. bisporus*, inhibit the growth of each other for a while when reaching each other (in dual culture) and finally the mushroom will colonize the colony of the thermophilic fungus and use it as source of food. In type II of the thermophilic fungus, *A. bisporus* immediately colonizes the colony of *M. thermophilus* (Lyons *et al.*, 1999). Comparing the results of the present study and those of Lyons*et al.* (1999) showed that all of the *M. thermophilus* isolates belong to the type II of *M. thermophilus.* There are numerous reports that show type II of *M. thermophilus* have been isolated from mushroom compost (Cailleux, 1973;Eicker, 1977; Olivier and Guillaumes, 1976;Straatsma*et al.*, 1989).

There is a positive correlation between the density of *M. thermophilus* in the compost and mushroom yield (Straatsma*et al.*, 1989). The thermophilic fungus produces special metabolites, stimulating the growth of *A. bisporus* (Straatsma and Samson, 1993). This could be the reason of enhancing the growth of the mushroom mycelia in the dual cultures and also in the compost. In one study the biomass of *M. thermophilus* accounted for about two percent of the total dry weight of the compost (Sparling *et al.*, 1982). This microbial biomass is considered as an important nutrient source for the mycelia of *A. bisporus.*

Of the nine primers used to study the genetic diversity in the population of *M. thermophilus,* only one primer (A11) showed a low degree of polymorphism. We showed that there is no genetic diversity in the population of *M. thermophilus* collected from mushroom composting farms in Iran. All of the studied isolates were genetically clonal. The high level of genetically clonal fungal population also has been reported by the other researchers (Bonnen and Hopkins, 1997; Collopy *et al.*, 2001;Largeteau *et al.*, 2004, 2006, 2008;Mehrparvar *et al.*, 2012).

The two types of *M. thermophilus* were clearly differentiated from each other using RAPD marker (A1, A3, A11 and A13 primers) (Lyons*et al.*, 2000). In the present investigation, all the isolates belonged to the second type of *M. thermophilus* and the cluster created by A11 primer was related to the genetic diversity within the second type. It is suggested that the other molecular markers (such as AFLP, URP, ISSR and etc.) could be used and also, in addition to the mushroom composting farms, samples be taken from the other sources such as poultry farms and croplands to better clarify the genetic situation of this thermophilic fungus in Iran.

There are several factors influencing the genetic structure of *M. thermophilus*. It was proposed that wheat straw can be regarded as a primary source of *M. thermophilus* propagules (Straatsma *et al.*, 1994). In Iran the raw ingredients, like wheat straw and broiler litter, for mushroom composting farms are supplied by certain regions (Ardebil and Golestan provinces in the north and Khuzestan province in the south, Iran) and transported to the composting farms situated in other provinces. This is the main reason of spreading the fungus all over the country. Sexual reproduction is considered as the main source of genetic diversity in the fungal populations but no teleomorph has been observed for *M. thermophilus* in the nature or laboratory so far (Straatsma and Samson, 1993). The raw materials used in compost formulation, temperature of compost and concentration of ammonia during pasteurization are similar in the different batches of composts. All of these factors can act as selective pressures and shift the population of *M. thermophilus* to a genetically uniform state.

Comparing the ITS regions of the representative isolates in this study revealed that they were identical to each other and also with the ex-type *M. thermophilus* strain. These results are in agreement with the results of Lyons*et al.* (2000) that reported the sequences of ITS regions of *M. thermophilus* isolates had high level of homology together and can't differentiate them from each other.

However, RAPD marker failed to separate *M. thermophilus* isolates from each other based on their mycelial growth rate and their geographical origin. All the *M. thermophilus* were significantly different with each other at five percent probability level, also no correlation was found between the mycelial growth rate of *M. thermophilus* isolates at 48 °C and the morphology of colonies with RAPD patterns.

Conflict of interest disclosure

The authors declare that there are no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed. Also all named authors have agreed to publication of the work; and the manuscript does not infringe any personal or other copyright or property rights.

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خصوصیات قارچ گرمادوست *Mycothermus thermophilus* مربوط به کمپوست قارچ خوراکی در ایران

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چکیده: قارچ گرمادوست *Mycothermus thermophilus* از مهم‌ترین قارچ‌های گرمادوست در فرایند تولید کمپوست به‌شمار می‌رود. نمونه‌برداری از نه استان ایران انجام و ۳۹ جدایه قارچ گرمادوست براساس ویژگی‌های ریخت‌شناختی و ناحیه ITS ریبوزومی شناسایی شدند. تمام این جدایه‌ها از نظر خصوصیات مولکولی و ریخت‌شناختی با یک‌دیگر مقایسه شدند. تمامی جدایه‌های قارچ گرمادوست مورد مطالعه، موجب افزایش رشد میسلیوم قارچ خورکی دکمه‌ای در مقایسه با شاهد شدند. هم‌چنین شکل پرگنه قارچ خوراکی به محض رشد روی پرگنه قارچ گرمادوست تغییر کرد. تمامی جدایه‌های قارچ گرمادوست از نظر خصوصات ریخت‌شناختی با یک‌دیگر تفاوت معنی‌داری داشتند اما هیچ تنوع ژنتیکی با استفاده از نشانگر RAPD بین آنها مشاهده نشد. جمعیت کاملاً یک‌دست از نظر ژنتیکی قارچ گرمادوست *M. thermophilus* به عدم وجود تنوع ژنتیکی، یکسان بودن مواد خام مورد استفاده در ساخت کمپوست، دمای کمپوست در هنگام ساخت و غلظت آمونیاک در زمان پاستوریزه به‌عنوان عوامل فشار انتخابی نسبت داده شد.

واژگان کلیدی: *Agaricus bisporus*، قارچ خوراکی، تنوع ژنتیکی، RAPD، قارچ گرمادوست *Mycothermus thermophilus*