Systematics of *Aspergillus* species of subgenus *Nidulantes* in Iran

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Abstract

Aspergillus subgenus *Nidulantes* accommodates species of industrial and medical importance. Thirty isolates morphologically assigned to subgenus *Nidulantes* were studied, 25 isolated from cereals in the north and northwestern provinces and the rest from other substrates in Iran. Based on morphological and molecular data (sequences of the ITS rDNA and β-tubulin), nine species were identified belonging to the sections *Nidulantes*, *Usti*, *Terrei* and *Flavipedes*. Phylogenetic analyses based on the β-tubulin gene resolved the relationship among the examined *Aspergillus* species largely concordant with morphological characters. Among the identified species, *Aspergillus aurantiobrunneus*, *A. calidoustus*, *A. flavipes*, *A. iizukae*, *A. insuetus*, *A. kassunensis*, *A. quadrilineatus* and are new records to the Iranian mycobiota.

Keywords: *Aspergillaceae*, biodiversity, phylogeny, taxonomy

بررسی سیستماتیک گونه‌های *Aspergillus* متعلق به زیرجنس *Nidulantes* در ایران*

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خلاصه

این زیر جنس که اغلب از اندام‌های هوایی غلات در مناطق شمال و شمال‌غرب و تعدادی هم از سایر بسترها در ایران به دست آمده بودند، مورد مطالعه قرار گرفتند. براساس داده‌های ریخت‌شناسی و مولکولی (توالی‌های مناطق ژنی ITS rDNA و β-tubulin)، تعداد نه گونه *Aspergillus* متعلق به زیرجنس *Nidulantes* شناسایی گردید که در بخش‌های *Nidulantes*، *Usti*، *Terrei* و *Flavipedes* گروه‌بندی شدند. آنالیزهای فیلوژنتیکی انجام گرفته براساس توالی‌های ژن β-tubulin در مقایسه با توالی‌های ITS rDNA روابط میان گونه‌های *Aspergillus* مطالعه شده را به خوبی منعکس کرد که در حد زیادی منطبق با ویژگی‌های ریخت‌شناسی نسبت داده شده به این گونه‌ها بود. از میان گونه‌های شناسایی شده *Aspergillus aurantiobrunneus*، *A. calidoustus*، *A. flavipes*، *A. iizukae*، *A. insuetus*، *A. kassunensis* و *A. quadrilineatus* برای نخستین بار از ایران گزارش می‌شوند.

واژه‌های کلیدی: آسپرژیلاسه، تاکسونومی، تنوع زیستی، فیلوژنی

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Introduction

Aspergillus P. Micheli ex Link is one of the economically important fungal genera in fermentation industry, food microbiology, biodeterioration and human health. Aspergilli have been reported from almost all kinds of soils (Raper & Fennell 1965, Christensen & Tuthill 1985, Bennett & Klich 1992, Domsch *et al.* 2007) and agricultural products as the predominant contaminants (Kozakiewicz 1989, Pitt & Hocking 1997, Domsch *et al.* 2007). The number of *Aspergillus* species was estimated around 250 up to 2007 by Geiser *et al.* (2007). Since then almost 52 new species have been described (Houbraken *et al.* 2007, Samson *et al.* 2007a, Hong *et al.* 2008, Mares *et al.* 2008, Noonim *et al.* 2008, Perrone *et al.* 2008, Peterson 2008, Pildain *et al.* 2008, Varga *et al.* 2008, Zalar *et al.* 2008, Balajee *et al.* 2009, Varga *et al.* 2010a, b, Samson *et al.* 2011a, b, Varga *et al.* 2011a, b, Davolos *et al.* 2012, Jurjevic *et al.* 2012). Therefore, the total number of *Aspergillus* species so far described would be ~300.

To deal with numerous species in the genus, early researchers divided them into groups and series based on conidial and conidiohphore colour, vesicle size and shape, seriation, presence of a teleomorph and hülle cells (Thom & Church 1926, Thom & Raper 1945, Raper & Fennell 1965). The groups were formally classified as subgenera and sections by Gams *et al.* (1985) to comply with the International Code of Botanical Nomenclature. Placement of some species and existence of some of these groups have been questioned by Kozakiewicz (1989), Samson & Frisvad (1991) and Peterson (2000). In a revision of the genus *Aspergillus* based on rDNA sequences, Peterson (2000) proposed eliminating three of the six subgenera established by Gams *et al.* (1985), retaining 12 of the 18 sections, modifying three of the sections and deleting the other three. Frisvad *et al.* (2005) proposed sect. *Ochraceorosei* to accommodate the species *A. ochraceoroseus* Bartoli & Maggi and *A. rambellii* Frisvad & Samson. The relationship among Aspergilli was further studied by Peterson (2008) who accepted five subgenera (*Aspergillus*, *Circumdati*, *Fumigati*, *Nidulantes* and *Ornati*) and 16 sections based on a multiple phylogeny (RPB2, calmodulin and ITS-LSU nrDNA). To some extent, this infrageneric taxonomy was supported by Houbraken & Samson (2011) which conducted the phylognetic study of the family *Trichocomaceae* using four combined loci (RPB1, RPB2, Tsr1 and Cct8).

The taxon *Trichocomaceae* was introduced by Fischer (1897) based on a teleomorph genus, *Trichocoma* Jungh. The classification of this family was studied extensively using phenotypic characters (Malloch & Cain 1972a, Subramanian 1972, Malloch 1985a, b, von Arx 1986). Malloch & Cain (1972b) classified the anamorph genera with phialidic structures including *Aspergillus* under the *Trichocomaceae*. However, in the last revision of the family *Trichocomaceae* (Houbraken & Samson 2011), the oldest family name, *Aspergillaceae* was re-instated to include *Aspergillus* and its associated teleomorph genera.

The taxonomy of the genus *Aspergillus* has evolved from a simple morphological species concept (Raper & Fennell 1965, Samson 1979, Klich & Pitt 1988) into a polyphasic approach including macro- and micro-morphology, growth temperature regimes, profiles of secondary metabolites (extrolites) and molecular data, mainly ITS rDNA, β-tubulin and calmodulin genes. This approach has been successfully applied for most *Aspergillus* sections, including *Candidi* (Varga *et al.* 2007b), *Nigri* (Samson *et al.* 2007b, Varga *et al.* 2011a), *Usti* (Houbraken *et al.* 2007, Samson *et al.* 2011b), *Clavati* (Varga *et al.* 2007a), *Fumigati* (Samson *et al.* 2007a, Hong *et al.* 2008), *Terrei* (Samson *et al.* 2011a) and *Flavi* (Varga *et al.* 2011b).

Aspergillus is a good example of a genus where dual nomenclature has been applied. The concept of "Dual Nomenclature" which simply means the use of more than one name for a single taxon was established in the International Code of Botanical Nomenclatue (ICBN) in 1910, to resolve the problem of naming fungi that exhibit pleomorphic life cycle (Cline 2005). Article 59 of

ICBN governs the naming of these fungi. Recently, the proposal to revise article 59 was accepted at the IBC Nomenclature section at Melborne, 2011 and the principle of "One Fungus = One Name" was established (Norvell 2011) which imply on using only one name for a single taxon. As an extension of this session, a symposium entitled "One Fungus = Which Name" was held on 12–13 April, 2012 in Amsterdam, the Netherlands to address which name of pleomorphic fungi should be used in future. Several criteria such as priority, taxonomic clarity, prevalence in nature, usage of names in various industries and stability and relevance were discussed. As an example, the name *Aspergillus* was suggested to be used for all species even those having teleomorphs, due to its priority (an older name than teleomorph genera) and prevalent usage in pathology, industry and quarantine issues.

In an investigation of *Aspergillus* species associated with barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) in the northern and northwestern provinces of Iran 2005–08, 30 isolates, morphologically assigned to the subgenus *Nidulantes*, were selected and studied. Based on morphological and molecular data, seven species new to the Iranian mycobiota were identified. In addition, the association between morphological and molecular data is discussed.

Materials and Methods

- Strains, media and morphological observations

Fungal strains were mainly obtained from cereals (wheat, barley and maize) collected from the northern and northwestern provinces (Ardabil, E and W Azerbaijan) of Iran. A few additional strains were obtained from Iranian Fungal Culture Collection (IRAN …C). Our strains were isolated using the modified method of Raper & Fennell (1965) based on direct isolation of fungi from plant materials (see Asgari & Zare 2011).

For macro-morphological observations, Czapek Yeast Autolysate (CYA) and 2% Malt Extract Agar (MEA) were used (Samson *et al.* 2010). The isolates were inoculated at three points on the agar plates and incubated at 25º C in the dark for 7 d. Colony texture and colour are described on CYA, growth rate and reverse on CYA and MEA. For micro-morphological observations, microscopic mounts were made in lactic acid from MEA colonies and a drop of alcohol was added to remove air bubbles and excess conidia. Average and standard deviations were calculated with BioloMICS 1.0.2 software (provided by Dr. V. Robert, BioAware, S.A., 2003). Photographs were taken using an Olympus (DP25) digital camera.

Subcultures of the strains obtained in this study are preserved at the Iranian Fungal Culture Collection (IRAN …C) at the Iranian Research Institute of Plant Protection, Tehran, Iran.

- DNA extraction and screening

DNA was extracted with a slightly modified method of Cenis (1992). To select representative strains for sequencing, the amplified parts of the β-tubulin gene of all strains were subjected to PCR-RFLPs. Partial β-tubulin gene was amplified using primers Bt2a [5′-GGT AAC CAA ATC GGT GCT GCT TTC] and Bt2b [5′-ACC CTC AGT GTA GTG ACC CTT GGC] (Glass & Donaldson 1995). The PCR reaction (25 µl) contained 50 ng of genomic DNA, 12.5 pmol of each primer, 0.3 mM dNTPs (CinnaGen, Iran) and 1× PCR buffer containing 2 mM $MgCl_2$, 1.5 U *Taq* DNA polymerase (CinnaGen, Iran). PCR amplification was carried out on an MWG (AG Biotech, Ebersberg, Germany) thermocycler. The PCR program for amplification of parts of β-tubulin gene was 94º C/3 min (initial denaturation), 94º C/1 min, 60º C/1 min, 72º C/2 min (35×) and 72º C/10 min (final extension).

Ten restriction enzymes (REs) including *Bam*HI, *Msp*I, *Hin*6I, *Pst*I, *Alu*I, *Kpn*I, *Hin*fI, *Sal*I, *Bsu*RI (*Hae*III) (Fermentas, Germany) and *Taq*I (Vivantis, Malaysia) were tested to select those that revealed maximum polymorphisms. Three of them, *Bsu*RI (*Hae*III) (GG:CC), *Hin*fI (G:ANTG) and *Taq*I (T:CGA) produced the highest numbers of polymorphic bands and were subsequently used to digest amplicons from all isolates examined in this study. The restriction fragments were

separated by horizontal electrophoresis in 1.8% agarose gel in 1× TBE buffer. The electrophoresis was carried out for about 4 h at 100 Volt (Appelex PS 1006P). Three µl of 100-bp size marker (0.1 µg/µl; Fermentas, Germany) was loaded into two wells on each side of the gels.

The banding patterns were analysed using Jaccard's similarity coefficient with UPGMA in MVSP (multivariate statistical package, v. 3.11a: Kovack Computing, Anglesey, UK). Binary codes were used to score the bands for presence (1) or absence (0). Separate and combined analyses were performed using different combinations of RFLP data (dendrograms not shown).

- PCR amplification and DNA sequencing

DNAs showing polymorphism in PCR-RFLPs were amplified for the ITS region of ribosomal DNA and partial β-tubulin gene. The ITS region (ITS1-5.8S-ITS2) was amplified using primers ITS1 [5′-TCC GTA GGT GAA CCT GCG G] and ITS4 [5′-TCC TCC GCT TAT TGA TAT GC] (White *et al.* 1990) or ITS1-F [5′-CTT GGT CAT TTA GAG GAA GTA A] (Gardes & Bruns 1993) and ITS4. The PCR program for the ITS region amplification was 94º C/3 min (initial denaturation), 94º C/45 s, 50º C/50 s, 72º C/2 min (40×) and 72º C/5 min (final extension). PCR reaction and conditions for amplification of partial β-tubulin gene was as described above.

The PCR products were purified using a Core-One™ DNA cleaning kit (Cat No. PP 200, S. Korea) or AccuPrep® DNA cleaning kit (Cat. No. K-3034-1, Bioneer, Inc., USA). The purified DNA samples were then submitted to a capillary sequencing machine (ABI 3730 Capillary Electrophoresis Genetic Analyzer, University of California, Davis) for sequencing.

- Phylogenetic analysis

The programs EditSeq and SeqMan, parts of the DNA*Lasergene (DNAstar, Madison, WI) software package, were used to assemble and edit the sequence files. The alignments were initially obtained using the Pairwise Alignment option in GeneDoc (Nicholas & Nicholas 1997). Sequences of the ITS region (592 positions) and partial β-tubulin gene (432 positions) were analysed separately using MEGA5 (Tamura *et al*. 2011). The evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing alignment gaps, and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). The MP tree was obtained using the Close-Neighbor-Interchange algorithm of Nei & Kumar (2000) with search level 1 (Felsenstein 1985, Nei & Kumar 2000) in which the initial trees were obtained with the random addition of sequences (100 replicates). The tree branches were drawn to scale, with lengths calculated using the average pathway method (Nei & Kumar 2000), in the units of the number of changes over the whole sequence. The codon positions included were 1st+2nd+3rd+Non-coding. All alignment gaps were treated as missing data.

Results and Discussion

Out of 30 *Aspergillus* isolates examined in this study, 25 were obtained from barley, wheat and maize in the northern and northwestern provinces of Iran and the other five were obtained from other substrates in Iran, including soil, *Arachis hypogaea* L., *Pistacia vera* L. and *Musa sapientum* L. All examined isolates had the morphological traits of subgenus *Nidulantes*, having biseriate conidiogenous cells and conidia of variable colour (Gams *et al.* 1985).

Subgenus *Nidulantes* originally contained the sections *Nidulantes*, *Flavipedes*, *Terrei*, *Usti* and *Versicolores* (Gams *et al.* 1985). The sect. *Nidulantes* is characterized by short, brown stipes, green conidia, abundant globose hülle cells and *Emericella* teleomorph. *Aspergillus* species with brown stipes and dull red, brown or olivaceous conidia and elongated hülle cells are placed in sect. *Usti*, those with hyaline stipes and buff to orange-brown conidia in sect. *Terrei*. Section *Versicolores* includes species with hyaline to pale-brown stipes and green, grey-green or blue-green conidia. The species included in sect. *Flavipedes* also have hyaline to pale-brown stipes like sect. *Versicolores*, but have white to buff conidia. In the last revisions of infrageneric taxonomy of *Aspergillus* based on molecular data

(Peterson 2008, Houbraken & Samson 2011), subgenus *Nidulantes* phylogenetically only contains the sections *Nidulantes*, *Ochraceorosei*, *Usti* and *Sparsi*, whilst sections *Terrei* and *Flavipedes*, are in subgen. *Circumdati*. Here we still include these sections because of their distinct morphology.

Nine species of *Aspergillus* obtained during this study found belonging to the sections *Nidulantes*, *Usti*, *Terrei* and *Flavipedes*. Among them *A. aurantiobrunneus* (G.A. Atkins, Hindson & A.B. Russell) Malloch, *A. quadrilineatus* Thom & Raper (from sect. *Nidulantes*), *A. kassunensis* Baghd., *A. insuetus* (Bainier) Thom & Church, *A. calidoustus* Varga, Houbraken & Samson (from sect. *Usti*), *A. flavipes* (Bainier & R. Sartory) Thom & Church and *A. iizukae* Sugiy. (from sect. *Flavipedes*) are new records to the Iranian mycobiota. *Aspergillus nidulans* var. *nidulans* (Eidam) G. Winte from sect. *Nidulantes*, and *A. terreus* Thom from sect. *Terrei* had already been reported from Iran (Ershad 2009).

To clarify the relationship among the identified species we conducted phylogenetic analyses using sequences of ITS ribosomal DNA (Fig. 1) and β-tubulin gene (Fig. 2). The sequences of the identified species of *Aspergillus* were aligned against those available in GenBank (Table 1) through BLAST search (Altschul *et al.* 1990). Compared with the ITS region, the sequences of β-tubulin gene (Fig. 2) proved to elucidate the relationships of examined *Aspergillus* species better, mostly in concordance with morphological characters. The association between morphological and molecular data is discussed below.

Aspergillus aurantiobrunneus (G.A. Atkins, Hindson & A.B. Russell) Raper & Fennell, The Genus *Aspergillus*: 511 (1965) (Fig. 3)

≡ *Emericella nidulans* var. *aurantiobrunnea* G.A. Atkins, Hindson & A.B. Russell, Trans. Br. Mycol. Soc. 41: 501 (1958).

Teleomorph also had known as *Emericella aurantiobrunnea* (G.A. Atkins, Hindson & A.B. Russell) Malloch, Can. J. Bot. 50: 61(1972).

Colonies reaching 6–8 mm diam on MEA and 5–10 mm on CYA in 7 d at 25º C, dense, plane, forming pale bluish-white crusts of stromata surrounding the ascomata; reverse dull orange-brown on MEA and dark brown on CYA. Ascomata ovate to globose, purple-black, 250–450 µm diam; hülle cells forming the structure of stromata, globose to ellipsoidal, 18–28 µm diam; asci 8-spored, globose to ellipsoidal, 9–11.5 µm diam; ascospores bright purple-red, lenticular, smooth-walled, 5–6.5 × 3.5–4.5 µm, with two pleated sinuous and entire equatorial rings, 1–1.3 µm wide.

Anamorph: Reduced conidiophores were only formed on SNA (Gams *et al.* 1998). Conidial heads radiating to nearly globose, dull buff; stipes pale brown, smooth-walled, (55–)75–100(–130) × 3–4.5 µm; vesicles globose to subglobose, 9–11(–15) µm diam; biseriate; metulae covering almost the entire surface of vesicles, 3.5–4.5 × 2.5–3 µm; phialides 5–6 × 2–2.5 µm; conidia globose to subglobose, smooth-walled, 3–3.5 µm.

Specimens examined: E Azerbaijan province, Ahar, on *Hordeum vulgare* seed, 14-I-2008 (IRAN 2042C and IRAN 2043C). Both collected and isolated by B. Asgari.

Emericella nidulans var. *aurantiobrunnea* was originally described by Atkins *et al.* (1958) based on a strain isolated from canvas respirator bag in Australia. After closely examining the ex-type strain (WB 4545), Raper & Fennell (1965) raised the variety to species level. The ex-type strain examined by Raper & Fennell (1965) had smaller vesicles, metulae, phialides and conidia, mainly smooth-walled, compared with the original description provided by Atkins *et al.* (1958).

Table 1. Isolates used to draw the phylogenetic trees in this study

Taxon	Strain	Source	GenBank accession numbers	
			ITS	β-tubulin
A. allahabadii	NRRL 4101, IMI 362227, WB 4101	Soil, San Salvador	--	EF669533
A. amylovorus	CBS 600.67 (T), ATCC 18351, IMI 129961, MUCL 15648	Wheat starch, Kharkiv, Ukraine	--	FJ531190
A. aurantiobrunneus	NRRL 4545 (T), IFM 42008, ATCC 16821, CBS 465.65, DSL48, IFO 30837, IMI 139281, IMI 74897, CBS H-12381, IMI 074897	Canvas haversack for respirator, Australia	EF652465	AB248306
A. aurantiobrunneus	IRAN 2043C, A302	Seed of *Hordeum vulgare*, Ahar, Iran	KC473927	KC473911
A. aurantiobrunneus	IRAN 2042C, A471	Seed of *Hordeum vulgare*, Ahar, Iran	KC473928	KC473912
A. aureofulgens	NRRL 6326 (T), CBS 653.74	Natural truffle soil, France	--	EU014079
A. calidoustus	CBS 121601 (T)	Bronchoalveolar lavage, Netherlands	--	FJ624456
A. calidoustus	IRAN 227C, A573	Seed of *Hordeum vulgare*, Karaj, Iran	KC473932	KC473909
A. carneus	CBS 111.49	Air	--	FJ491721
A. cleistominutus	IFM 48170	--	AB248989	--
A. corrugatus	IFM 54741	--	AB264792	--
A. flavipes	CBS 587.65, NRRL 4578, ATCC 16795, IMI 135424, QM 1994	Soil, Haiti	--	EU014082
A. flavipes	IRAN 2062C, A180	Straw of *Triticum aestivum*, Moghan, Iran	KC473934	KC473913
A. flavipes	IRAN 939C, A568	*Musa sapientum*, Sistan-o-Baluchestan province, Iran	--	KC473914
A. flavipes	ATCC 1030, NRRL 286	--	AY373849	--
A. iizukae	NRRL 3750 (T), CBS 541.69, IMI 141552	Stratigraphic core sample, Japan	--	EU014086
A. iizukae	IRAN 2063C, A351	Seed of *Hordeum vulgare*, Kaleybar, Iran	--	KC473915
A. iizukae	NRRL 35046	Soil, Benton County, Oregon, USA	EU021601	--
A. insuetus	CBS 119.27, NRRL 4876	Soil, Iowa, USA	EF652481	EF652305
A. insuetus	IRAN 2055C, A420	Straw of *Hordeum vulgare*, Sarab, Iran	KC473933	KC473908
A. insuetus	CCF 3995	Bronchoalveolar lavage, Czech Republic	FR733861	--
A. kassunensis	CBS 419.69 (T), NRRL 3752, IMI 334938	Soil, Berza, Damascus, Syria	--	FJ531181
A. kassunensis	IRAN 1280C, A554	Soil, Shiraz, Iran	--	KC473910
A. keveii	CCF 2596	Arable soil, Czech Republic	--	FR775324
A. nidulans var. *nidulans*	CBS 589.65 (NT), ATCC 10074, IHEM 3563, IMI 086806, IMI 126691, NRRL 187	Froidchapelle, Belgium	--	AY573547
A. nidulans var. *nidulans*	IRAN 2044C, A155	Straw of *Triticum aestivum*, Moghan (Bilesavar), Iran	KC473930	KC473905
A. nidulans var. *nidulans*	IRAN 2045C, A181	Straw of *Triticum aestivum*, Moghan (Bilesavar), Iran	KC473931	KC473906
A. nidulans var. *acristatus*	IFM 42016 (NT), ATCC 16839, CBS 119.55, IFM 54231, IMI 061453, LCP 84.2558, NRRL 2394	Exposed fabric, USA	--	AB248304
A. nidulans var. *dentatus*	IFM 42024	--	--	AB248342
A. nidulans var. *dentatus*	IFM 42021	Herbal drug	AB249000	--
A. nidulans var. *echinulatus*	IFM 54201	--	AB248966	--
A. nidulans var. *latus*	IFM 42011 (Isotype), ATCC 16848, CBS 492.65, IFM 49660, IFO 30847, IMI 074181, NRRL 200	--	AB248992	AB248334
A. nidulans	UOA/HCPF 10647	Bronchial secretions from a patient with cystic fibrosis	GQ461904	--
A. nidulans	RGT-S3	*Rumex gmelinii*	HQ674655	--
A. pseudodeflectus	F02Z2172	Wuyishan Nature Reserve, Fujian province, China	--	HM060542

Table 1 (contd.)

A. pseudodeflectus	IBL 03111	*Coffea arabica*, USA	DQ778908	--
A. purpureus	IFM 42012	--	AB248973	AB248315
A. quadrilineatus	IFM 42006 (NT), ATCC 16816, CBS 591.65, IFO 30850, IMI 89351, NRRL 201	Soil, USA	--	AB248335
A. quadrilineatus	784501	Cystic fibrosis patients, France	GU594759	--
A. quadrilineatus	IRAN 235C, A569	Seed of *Arachis hypogaea*, Jiroft, Iran	KC473929	KC473907
A. raperi	NRRL 2641 (T), CBS 123.56, ATCC 16917, IFO 6416, IMI 070949, Herb. K	Grassland soil, Yangambi, Zaire	--	EF652278
A. rugulosus	IFM 54242	--	AB249002	--
A. subsessilis	NRRL 4905 (T), CBS 502.65, ATCC 16808, IMI 135820, CBS H-6766, IMI 135820	Desert soil, California, USA	--	EF652309
A. terreus	UOA/HCPF 12626A	Pre-cooked pasta meal, Greece	--	JF509461
A. terreus	IRAN 2056C, A24	Leaf of *Hordeum vulgare*, Shabestar, Iran	KC473935	KC473916
A. terreus	IRAN 1097C, A558	Seed of *Pistacia vera*, Isfahan, Iran	KC473936	KC473917
A. terreus	CCF 3315	Outdoor air, Prague, Czech Republic	FR837967	--
A. ustus	UOA/HCPF 10218-1	Bronchial secretions, Greece	--	GQ376126
A. ustus	7-07	--	HQ594530	--
A. variecolor	RGT-S7	*Rumex gmelinii*	HQ674656	--
F. nivea	NRRL 6134 (T), CBS 444.75, IMI 334935, ATCC 1575	Soil, Maharashtra, India	--	EF669532

Underlined sequence numbers are generated in this study, others are from GenBank. *A.* = *Aspergillus*, *F.* = *Fennelia*, (T) = ex-type strain, (NT) = ex-neotype strain.

ATCC, American Type Culture collection, Manassas, Virginia, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IFM, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japanese Federation of Culture Collections; IFO, Institute for Fermentation, Osaka, Japan; IMI, CABI Bioscience, Egham, UK; IRAN, Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Tehran, Iran; LCP, Laboratoire de Cryptogamie, Paris, France; MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA; QM, Quartermaster Collection of Filamentous Fungi, USA; other abbreviations are not registered.

The isolates examined in this study had the same morphological characters of *A. aurantiobrunneus* described by Raper & Fennell (1965), except that their conidia had the same dimensions as the ex-type strain described by Atkins *et al.* (1958). The sequence data of ITS rDNA (Fig. 1) grouped our strains with *A. aurantiobrunneus* and *A. purpureus* Samson & Mouch. with 99% bootstrap support; the sequences of β-tubulin (Fig. 2), however, revealed a closer relationship with *A. aurantiobrunneus* (86% bootstrap support) than with *A. purpureus*. The latter species is finely distinguished by possessing red-purple ascospores (6–7 × 4.5–5.2 µm) with low crests, and hyaline conidiophores producing hyaline, smooth-walled, cylindrical conidia, measuring 3.5–5.5 × 1.5–2 µm (Samson & Mouchacca 1975). *Aspergillus aurantiobrunneus* is a new record to the Iranian mycobiota.

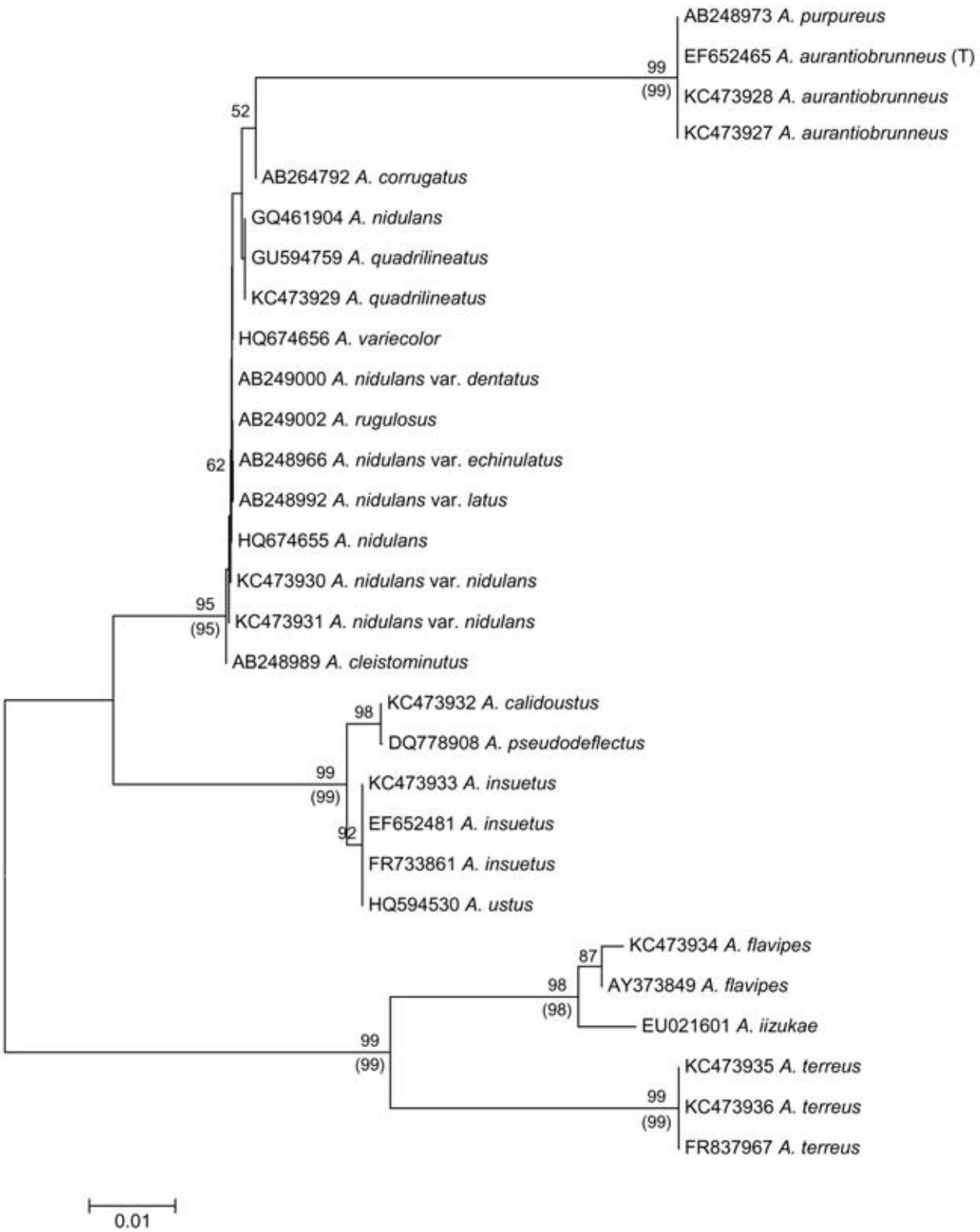

Fig. 1. Unrooted neighbor-joining tree based on ITS region sequences. Bootstrap values > 50% (1000 replicates) of NJ analysis is shown above the branches and those of parsimony below the branches in brackets. The scale bar indicates the nucleotide substitution in NJ analysis. *A.* = *Aspergillus*, (NT) = ex-neotype strain, (T) = ex-type strain.

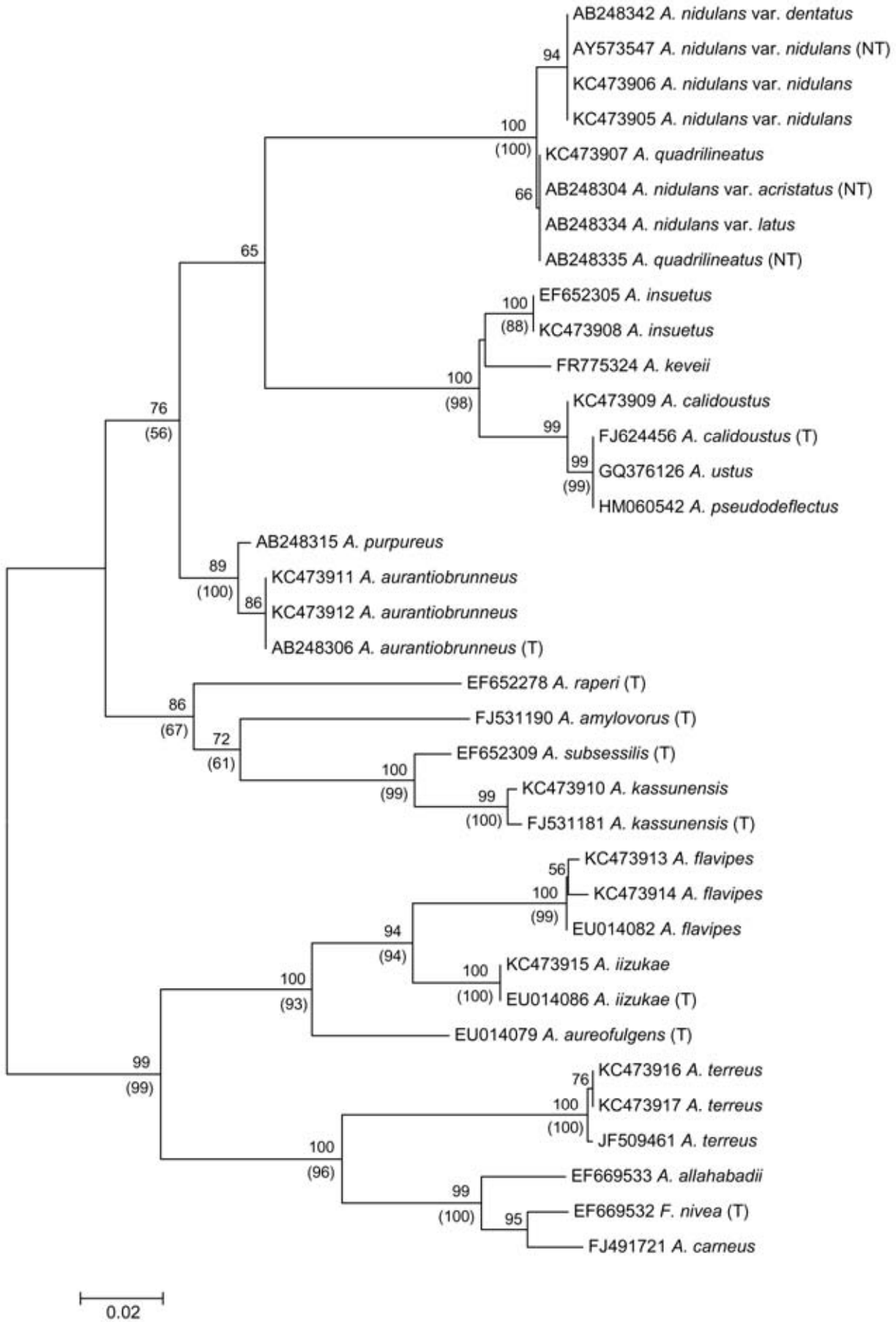

Fig. 2. Unrooted neighbor-joining tree based on β-tubulin gene sequences (details as in Fig. 1).

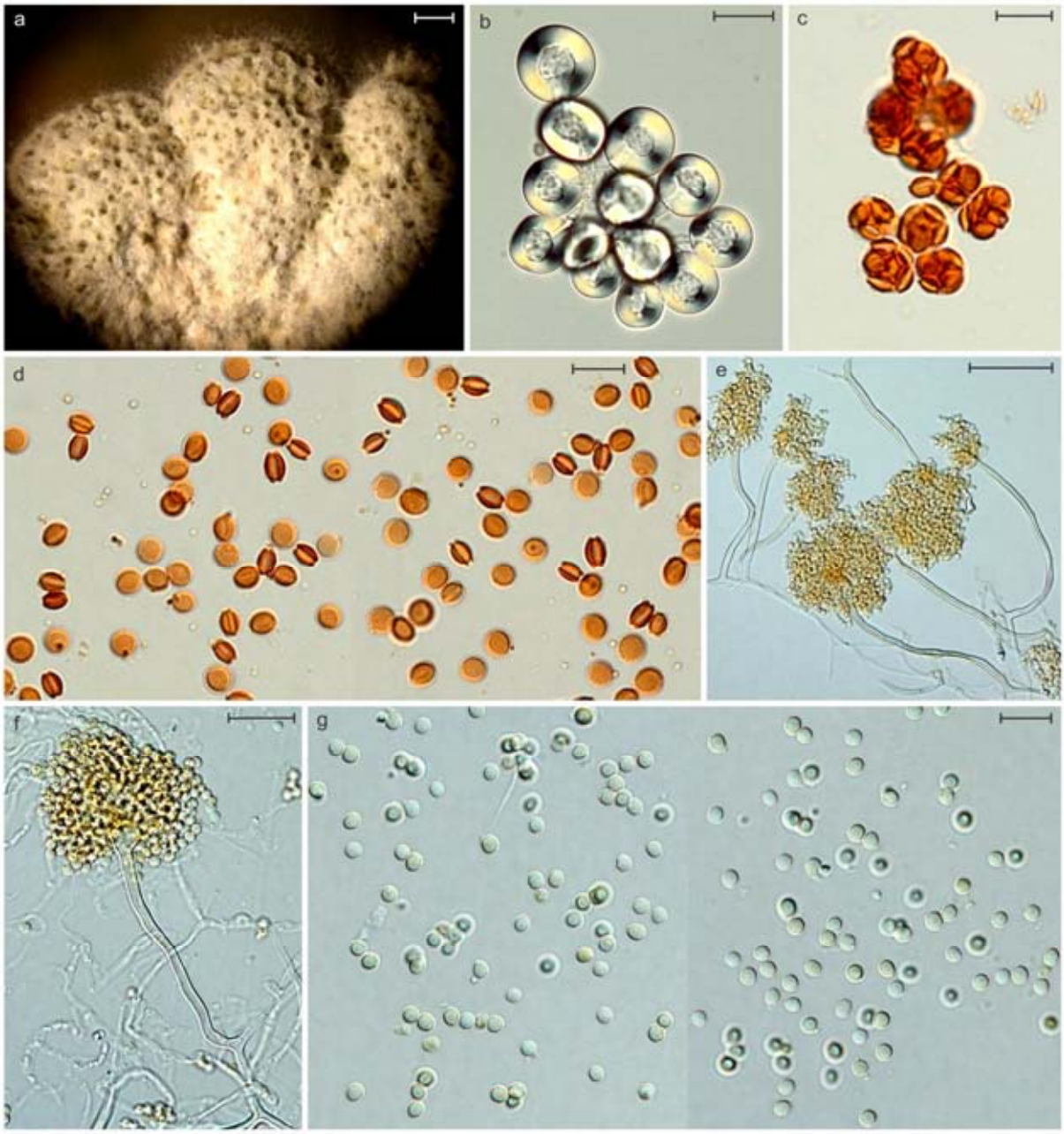

Fig. 3. *Aspergillus aurantiobrunneus*: a–d. Teleomorph and e–g. Anamorph. a. Stromata containing ascomata, b. Hülle cells, c. Asci, d. Ascospores, e, f. Conidiophores, g. Conidia (Bars: a = 1000 µm; e = 50 µm; b, f = 20 µm; c, d, g = 10 µm).

Aspergillus nidulans var. ***nidulans*** (Eidam) G. Winter, Rabenh. Krypt.-Fl., Edn 2 (Leipzig) 1.2: 62 (1884) (Fig. 4)

Teleomorph known as *Emericella nidulans* (Eidam) Vuill., Compt. Rend. Hebd. Séances Acad. Sci. Paris 184: 137 (1927)

Specimens examined: Ardebil province, Moghan, Bilesavar, on *Triticum aestivum* straw, 25-VIII-2006 (IRAN 2044C and IRAN 2045C), 6-IX-2006 (IRAN 2052C, IRAN 2048C and IRAN 2049C); Moghan (Parsabad), on *Triticum aestivum* seed, 14-I-2008 (IRAN 2046C and IRAN 2051C); E Azerbaijan province, Sarab, on *Hordeum vulgare* seed, 14-I-2008 (IRAN 2047C); Kaleybar, on *Hordeum vulgare* seed, 27-XI-2007 (IRAN 2053C); Bostanabad, on *Hordeum vulgare* seed, 14-I-2008 (IRAN 2050C and IRAN 2054C). All collected and isolated by B. Asgari.

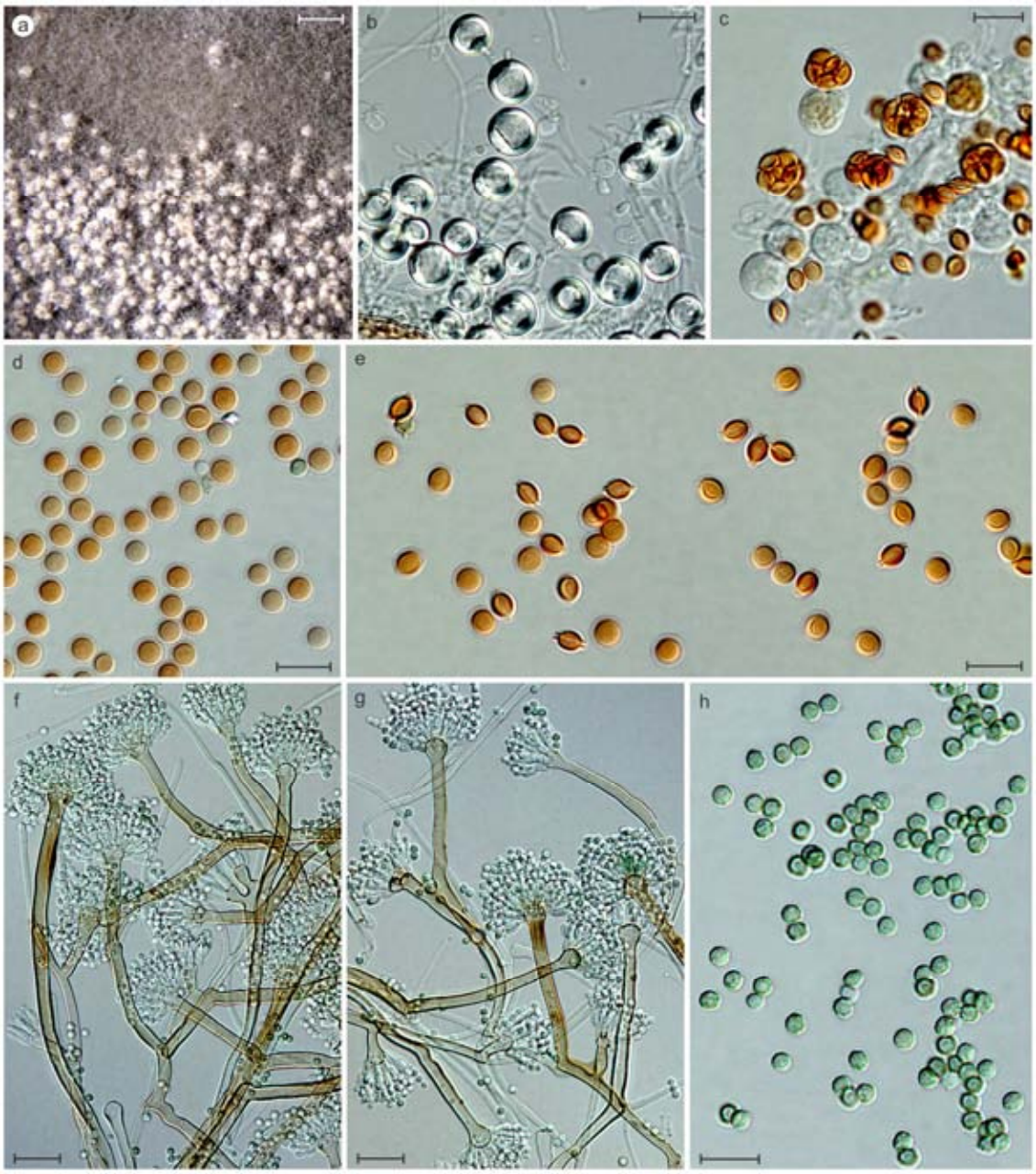

Fig. 4. *Aspergillus nidulans* var. *nidulans*: a–e. Teleomorph and f–h. Anamorph. a. Ascomata surrounded by hülle cells and conidial heads, b. Hülle cells, c. Asci, d, e. Ascospores, f, g. Conidiophores, h. Conidia (Bars: a = 1000 µm; b, f, g = 20 µm; c–e; h = 10 µm).

Aspergillus quadrilineatus and *A. rugulosus* are the closest species to *A. nidulans*, possessing red-purple, lenticular, smooth-walled ascospores with two equatorial crests. However, *A. quadrilineatus* (Benjamin 1955) has ascospores with two additional equatorial flanges which are narrower and less distinct. *Aspergillus rugulosus* (Benjamin 1955) has also slowly-growing colonies (reaching to 2–3 mm diam in 7 d at 25º C) and ascospores with conspicuously rugulose walls compared with *A. nidulans*.

Four varieties of *A. nidulans* had been described based on ascospore ornamentation and flanges; var. *acristatus* Fennell & Raper has ascospores without longitudinal crests; var. *dentatus* D.K. Sandhu & R.S. Sandhu has ascospores with narrow, conspicuously dentate equatorial crests; var. *echinulatus* Fennell & Raper has ascospores with convex surface uniformly echinulate and var. *latus* Thom & Raper is characterized by ascospores with broad equatorial crests up to 1.5–1.8 µm.

Our phylogenetic analyses based on the β-tubulin gene (Fig. 2) revealed the close relationship between molecularly examined strains (IRAN 2044C and IRAN 2045C) with the ex-neotype strain of *A. nidulans* var. *nidulans* (CBS 589.65) and another strain labeled *A. nidulans* var. *dentatus* from unknown origin (AB248342, submitted to GenBank by Matsuzawa *et al.* 2006, unpubl.). However, the morphological characters of the Iranian strains fit well *A. nidulans* var. *nidulans* (Thom & Raper 1939, Raper & Fennell 1965). The examined strains are clearly distinguished from *A. nidulans* var. *dentatus* by possessing larger ascospores (vs 3.5 × 2.5–2.8 µm) without dentate equatorial crests. *Aspergillus nidulans* has been previously reported from Iran on *Arachis hypogaea* (Pourabdollah & Ershad 1997) and *Pistacia vera* (Mojtahedi *et al.* 1979).

Aspergillus quadrilineatus Thom & Raper, Mycologia 31: 660 (1939) (Fig. 5)

Teleomorph known as *Emericella quadrilineata* (Thom & Raper) C.R. Benj., Mycologia 47: 680 (1955)

Colonies reaching 45 mm diam on MEA and 50 mm on CYA in 7 d at 25º C, dull green, plane, spreading, slightly wrinkled, velutinous with fimbriate margins; mycelium white, inconspicuous; reverse dull brownish-yellow on MEA and orange-brown on CYA. Ascomata developing separately throughout the colony, globose to subglobose, partially embedded in the mycelial felt, appearing pale brown due to surrounding hülle cells, 200–300 µm diam; hülle cells globose, 15–18.5 µm diam; asci 8-spored, globose to ellipsoidal, 9.5–12 µm diam; ascospores red, lenticular, regularly ornamented with colourful projections, 4.5–5.5 × 3.5–4 µm, with four equatorial crests (less than 1 µm wide), two of these very obvious and the other two quite indistinct.

Anamorph: Conidia formed too sparse on both media to affect the colony appearance. Conidial heads radiating to columnar; stipes dull brown, sinuous, smooth-walled, mostly covered with colourful projections, (25–)60–100 × 3–4 µm; vesicles pyriform, 8–11.5 µm diam; biseriate; metulae covering only the upper third to half of the vesicles, 5–6.5 × 2–3 µm; phialides 4–6 × 2–3 µm; conidia globose, finely roughened (2.5–)3–3.5 µm.

Specimen examined: Kerman province, Jiroft, on *Arachis hypogaea* seed, 1995, coll. & isol. Sh. Pourabdollah (IRAN 235C).

Phylogenetic analyses based on partial β-tubulin (Fig. 2) placed the examined strain in this study with *A. nidulans* var. *acristatus*, *A. nidulans* var. *latus* and *A. quadrilineatus* in a monophyletic clade with 66% bootstrap support. However, a morphological examination demonstrated its close relationship with *A. quadrilineatus* rather than the varieties of *A. nidulans*. Nevertheless, the examined strain slightly deviated from *A. quadrilineatus* (Benjamin 1955) by possessing ascospores mostly ornamented with colourful projections compared with smooth ascospore valves of *A. quadrilineatus*. This species is a new record to Iran.

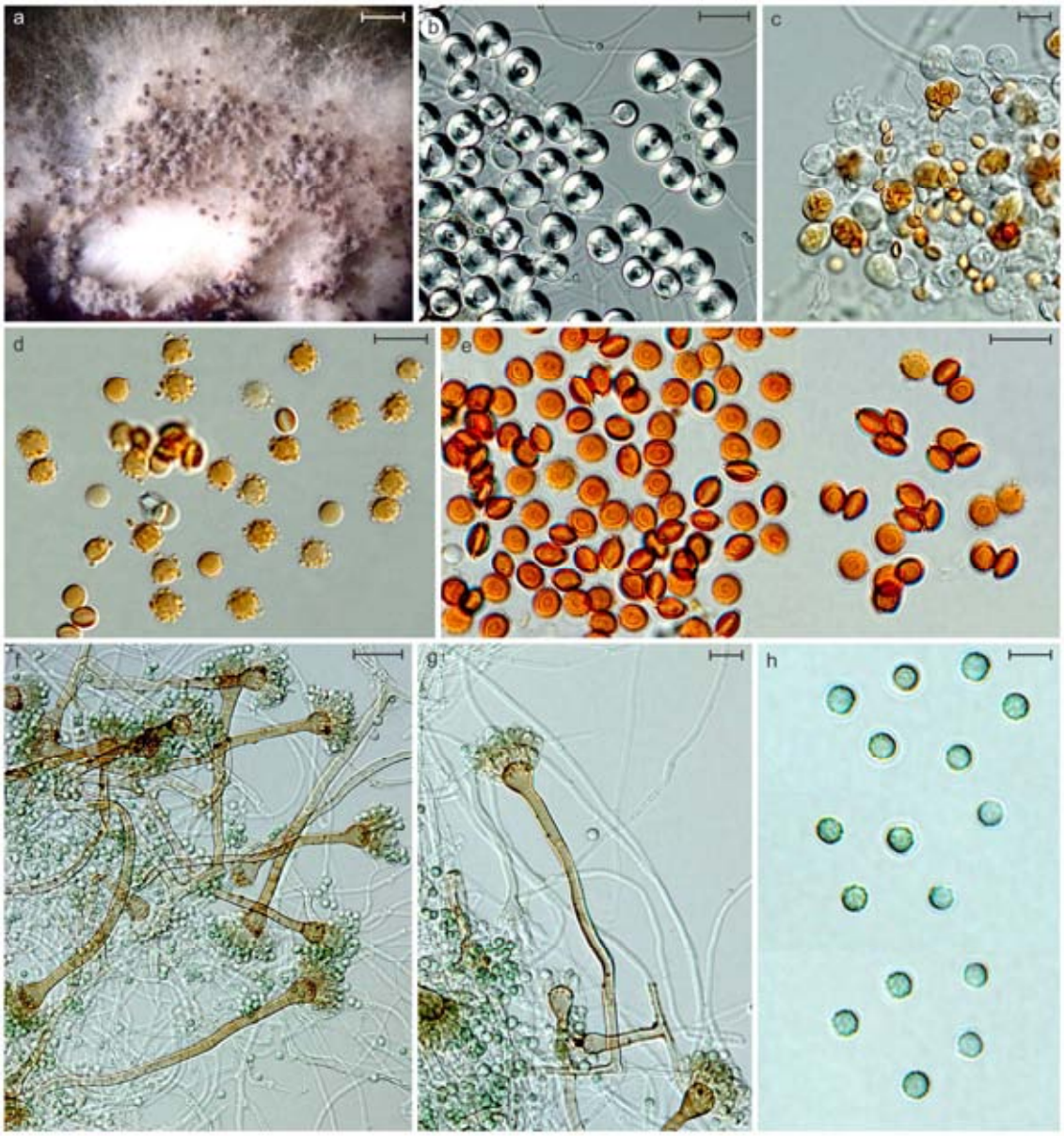

Fig. 5. *Aspergillus quadrilineatus*: a–e. Teleomorph and f–h. Anamorph. a. Ascomata surrounded by hülle cells, b. Hülle cells, c. Asci, d, e. Ascospores, f, g. Conidiophores, h. Conidia (Bars: a = 1000 µm; b, f = 20 µm; c–e; g = 10 µm; h = 5 µm).

Aspergillus kassunensis Baghd., Nov. Sist. Niz. Rast. 5: 113 (1968) (Fig. 6)

Colonies reaching 20 mm diam on MEA and 10 mm on CYA in 7 d at 25º C, floccose, consisting of tough basal felt, raised and irregularly wrinkled in central area, white at first, soon becoming pale grey with the development of a limited number of inconspicuous conidial heads on aerial mycelium; reverse pale grey-brown on MEA and orange-brown on CYA. Conidial heads poorly developed, small, loosely radiating, formed on short stalks from aerial hyphae; stipes uncoloured, smooth-walled, 15–35 × 2.5–4 µm; vesicles variable in shape, ranging from globose to flattened or dome-shaped, 3–6 µm diam; biseriate; metulae covering only the upper third to half of the vesicles, 4–6 × 2–3 µm; phialides 4–6 × 2–2.5 µm; conidia globose to subglobose, smooth-walled, 2–3 µm; hülle cells abundant, scattered through the mycelial felt, globose, 27–34 µm diam.

Specimen examined: Fars province, Shiraz, soil, 2008, coll. & isol. S. Jamali (IRAN 1280C).

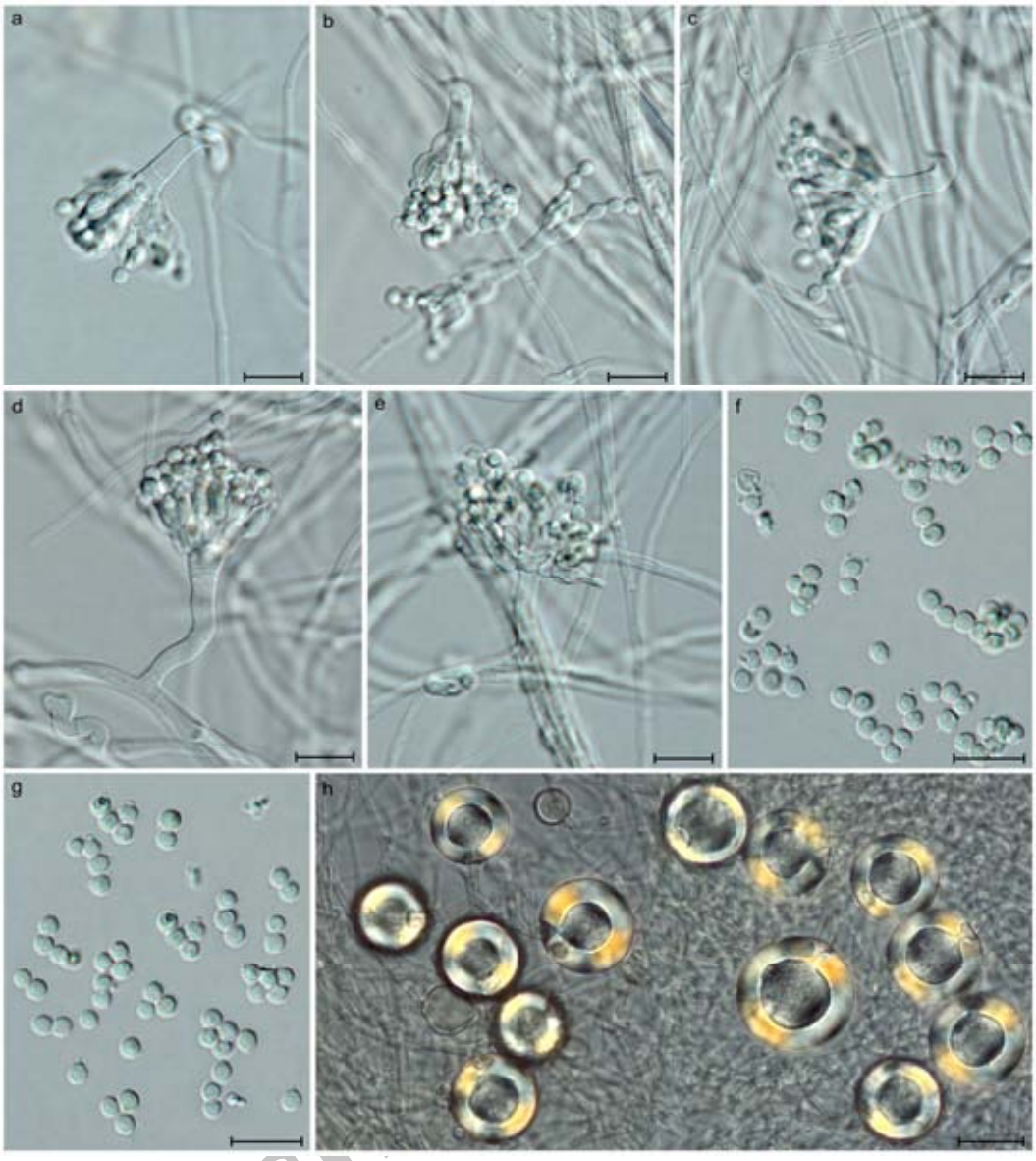

Fig. 6. *Aspergillus kassunensis*: a–e. Conidiophores, f, g. Conidia, h. Hülle cells (Bars: h = 20 µm, a–g = 10 µm).

Aspergillus kassunensis (Baghdadi 1968) was treated as a synonym of *A. subsessilis* Raper & Fennell by Samson & Mouchacca (1975) and Samson (1979). However, Peterson (2008) and Samson *et al.* (2011b) considered *A. kassunensis* as a separate species based on molecular examination of the ex-type strains. No morphological characters were determined as the discriminating criteria between these two species. We also included the ex-type strains of *A. kassunensis* (CBS 419.69) and *A. subsessilis* (NRRL 4905) in β-tubulin gene sequence analysis to resolve the taxonomic identity of the examined strain, IRAN 1280C. As a result, our strain was molecularly assigned to *A. kassunensis* rather than *A. subsessilis*. The morphological examination of this strain revealed its close relationship with *A. subsessilis* described by Raper & Fennell (1965); nevertheless, it had more distinct, longer and wider stipes (vs 5–6 × 2.2–2.5 µm) and slightly smaller (vs 3–3.5 µm) mainly smooth-walled conidia than the description of *A. subsessilis*.

Aspergillus insuetus (Bainier) Thom & Church, Manual of the Aspergilli: 153 (1929) (Fig. 7)

Colonies reaching 40 mm diam on MEA and 30 mm on CYA in 7 d at 25º C, dark grey at the centre, shading through white, with sterile, floccose marginal area; reverse orange-brown on MEA and yellow-olivaceous on CYA. Conidial heads small, radiating to hemispherical; stipes brown, smooth-walled, (160–)180–300 × 5–7 µm; vesicles subglobose to hemispherical, (11.5–)13–22 µm diam; biseriate; metulae covering half of the vesicles, 4.5–6 × 2.5–3.5 µm; phialides 6–7 × 2.5–3 µm; conidia globose, distinctly roughened, tuberculate, 3–3.7 µm; hülle cells in scattered groups, straight or slightly curved, 25–60(–80) × 13.5–18(–20) µm.

Specimen examined: E Azerbaijan province, Sarab, on *Hordeum vulgare* straw, 5-III-2007, coll. & isol. B. Asgari (IRAN 2055C)

Aspergillus ustus was described by Thom & Church (1926) based on *Sterigmatocystis usta* Bainier. These authors also accepted *A. insuetus* based on *S. insueta* Bainier, but later this species was abandoned (Thom & Raper 1945) and included in the broad description of *A. ustus* by Raper & Fennell (1965). Houbraken *et al.* (2007) clarified that *A. insuetus* is a valid species which can be distinguished from *A. ustus* and other species assigned to *Aspergillus* sect. *Usti* based on molecular grounds (ITS nrDNA, calmodulin, β-tubulin), metabolite profiles and phenotypic characters. The molecular data provided by Houbraken *et al.* (2007) showed that, *A. insuetus* is more closely related to *A. calidoustus* and *A. pseudodeflectus* Samson & Mouch. than to *A. ustus*. They mentioned the production of a pergillin-like compound by *A. insuetus* as a major difference between this species and the others.

These findings were additionally supported by Peterson (2008) who performed the phylogenetic analyses of *Aspergillus* strains assigned to sect. *Usti*. In this study, two authentic strains of *A. minutus* E.V. Abbott (NRRL 4876 and NRRL 279) were grouped with an authentic strain of *A. insuetus* (NRRL 1974) in a clade with 100% bootstrap support. This clade was clearly separated from the other clade including the ex-type strains of *A. puniceus* Kwon-Chung & Fennell (NRRL 5077), *A. ustus* (NRRL 275) and *A. granulosus* Raper & Thom (NRRL 1932).

In this study, the examined strain was placed in a clade with *A. insuetus* and *A. ustus* with 92% bootstrap support based on ITS rDNA (Fig. 1). However, the β-tubulin analysis (Fig. 2) grouped this strain with *A. insuetus* (100% bootstrap support). The morphological characters of the examined strain were in concordance with *A. insuetus* as illustrated by Houbraken *et al.* (2007) based on the ex-type strain, CBS 107.25. This strain was also differentiated from *A. ustus* (Houbraken *et al.* 2007) by producing dark grey colonies on MEA (vs green to olive-brown), shorter and wider stipes, larger vesicles and smaller conidia (vs 3.2–4.5 µm) with more roughened and tuberculate walls. This species is a new record for Iran.

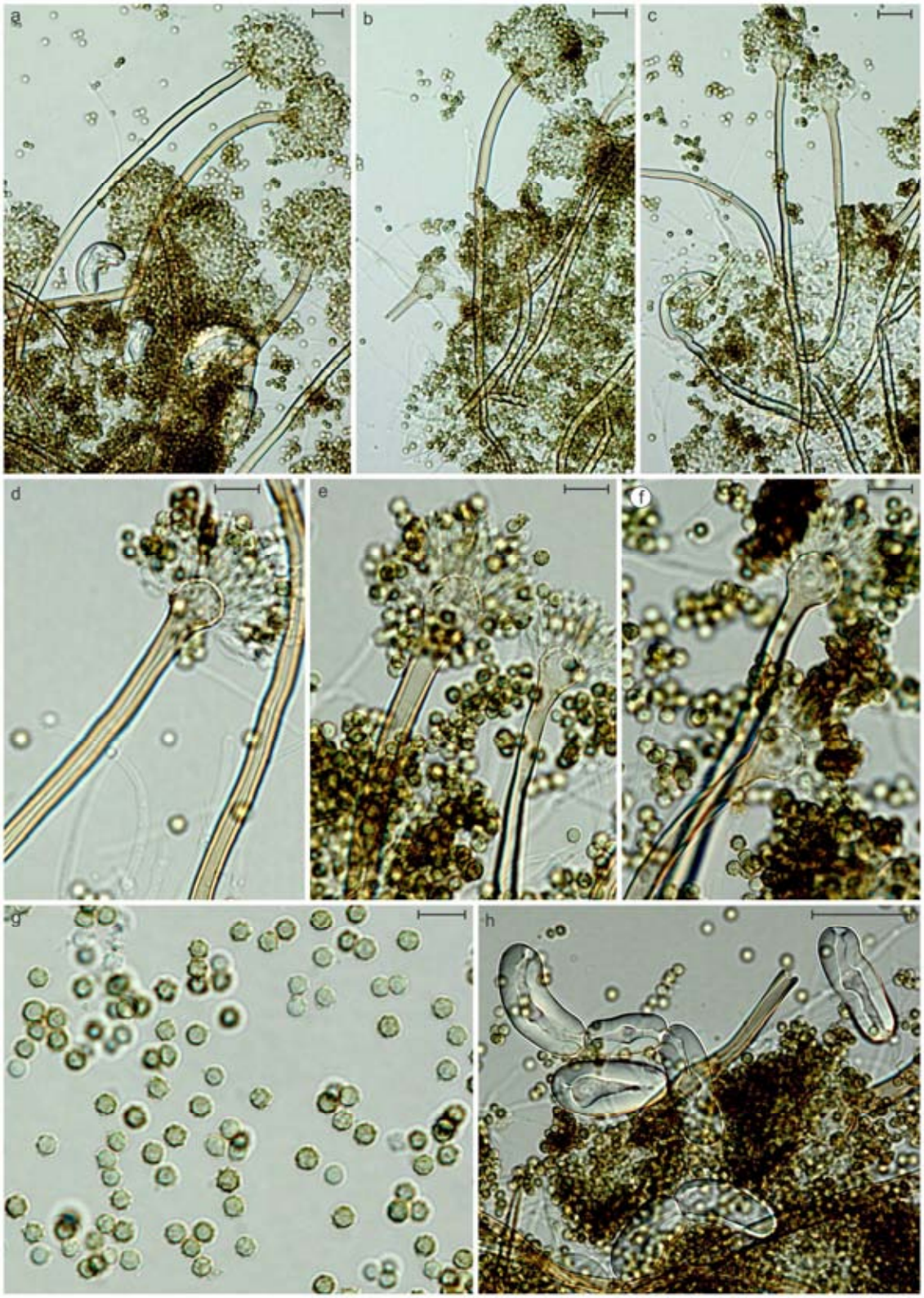

Fig. 7. *Aspergillus insuetus*: a–f. Conidiophores, g. Conidia, h. Hülle cells (Bars: a–c, h = 20 µm; d–f = 10 µm; g = 5 µm).

Aspergillus calidoustus Varga, Houbraken & Samson, Eukaryot. Cell 7(4): 636 (2008) (Fig. 8)

Colonies reaching 45 mm diam on MEA and 25 mm on CYA in 7 d at 25º C, brownish-grey, low, plane, floccose; reverse yellow with olive-brown centre on both media. Conidial heads small, loosely columnar; stipes brown, smooth-walled, (40–)70–160(–190) × 3–4.5 µm; vesicles pyriform or broadly spatulate, 7.5–12 µm diam; biseriate; metulae covering the upper half of the vesicles, 5–7 × 2.5–3.5 µm; phialides 5–7 × 2.3–3 µm; conidia globose, coarsely roughened to echinulate, 3.3–4 µm diam. Hülle cells were not formed on the tested media.

Specimen examined: Alborz province, Karaj, on *Hordeum vulgare* seed, 1992, coll. & isol. A. Nejat Salari (IRAN 227C).

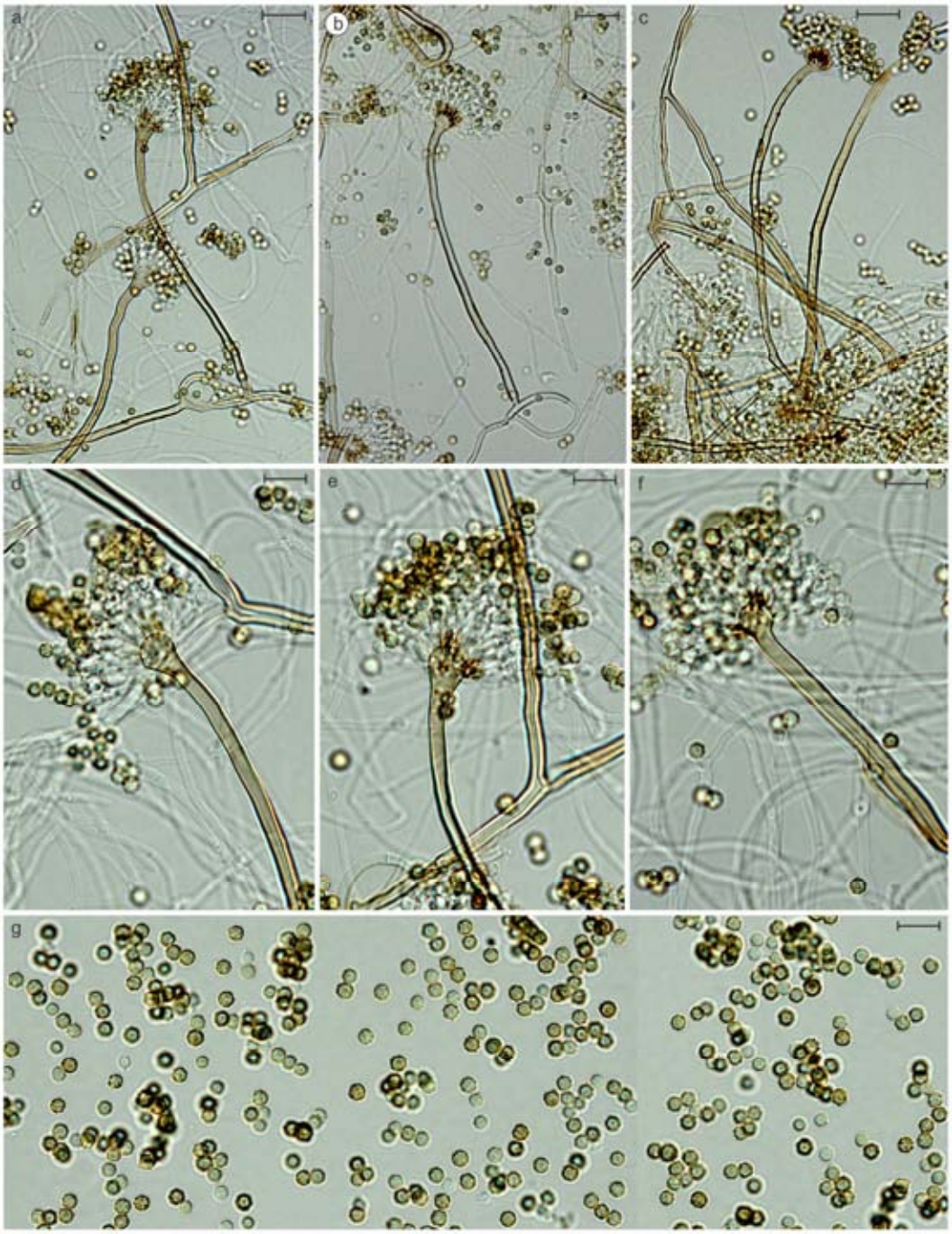

Fig. 8. *Aspergillus calidoustus*: a–f. Conidiophores, g. Conidia (Bars: a–c = 20 µm, d–g = 10 µm).

Phylogenetic analyses based on β-tubulin gene sequences (Fig. 2) grouped the examined strain with *A. ustus*, *A. calidoustus* and *A. pseudodeflectus* (99% bootstrap support). This strain had similar morphology to *A. calidoustus* (Varga *et al.* 2008). However, it slightly deviated from it by possessing narrower stipes (vs 4–7 µm) and slightly larger conidia (vs 2.7–3.5 µm). *Aspergillus pseudodeflectus* Samson & Mouchacca (1975), the closest species to *A. calidoustus*, is characterized by white, velvety and non-sporulating colonies on both MEA and CYA, curved and narrow stipes (2.5–3.5 µm wide), and larger conidia (3.5–5 µm diam) mainly ornamented with small warts. Hülle cells which are sparsely formed by *A. calidoustus* strains, are absent in *A. pseudodeflectus*. *Aspergillus ustus* is also differentiated from the above species mainly by different colony morphology and regular production of hülle cells that are typically scattered or formed in irregular masses, not associated with pigmented mycelium in any case.

Aspergillus terreus Thom, Am. J. Bot. 5: 85–86 (1918) (Fig. 9)

Specimens examined: E Azerbaijan province, Shabestar, on *Hordeum vulgare* leaf, 16–X-2006 (IRAN 2056C); Marand, on *Triticum aestivum* leaf, 27-IX-2005 (IRAN 2061C); Kaleybar, on *Hordeum vulgare* seed, 27-XI-2007 (IRAN 2059C and IRAN 2060C); Ardebil province, Moghan, on *Triticum aestivum* seed, 14-I-2008 (IRAN 2057C and IRAN 2058C), all collected and isolated by B. Asgari; Moghan, on *Zea mays* seed, 1991, coll. & isol. Gh.A. S. Karavar (IRAN 137C and IRAN 138C); Isfahan province, Isfahan, on *Pistacia vera* seed, 2004, coll. & isol. P. Rahimi (IRAN 1097C); Kerman province, Jiroft, on *Arachis hypogaea* seed, 17-V-1995, coll. & isol. Sh. Pourabdollah (IRAN 245C).

Aspergillus sect. *Terrei* includes *Aspergillus* species with columnar conidial heads in shades of buff to brown. The most important species of this section is *A. terreus* which is a ubiquitous fungus. Strains of *A. terreus* have been frequently isolated from desert and grassland soils and as contaminant of plant products like stored corn, barley and peanut (Kozakiewicz 1989).

Aspergillus terreus was the only species assigned to the *A. terreus* species group by Raper & Fennell (1965) with two additional varieties, *A. terreus* var. *africanus* Fennell & Raper ≡ *Aspergillus neoafricanus* Samson, S.W. Peterson, Frisvad & Varga, and *A. terreus* var. *aureus* Thom & Raper ≡ *Aspergillus aureoterreus* Samson, S.W. Peterson, Frisvad & Varga. Since then, several molecular studies have indicated that, this section should be expanded to include a number of other species (Peterson 2000, Varga *et al.* 2005). In the most recent study conducted by Samson *et al.* (2011a), the taxonomic status of sect. *Terrei* was clarified using a polyphasic approach. Three varieties of *A. terreus* including var. *aureus*, var. *floccosus* Y.K. Shih and var. *africanus* were raised to species level. Furthermore, some species formerly placed in sections *Flavipedes* and *Versicolores* were transferred to sect. *Terrei* and two additional new species were described.

Aspergillus terreus is mainly characterized by compactly columnar, pale grey to brownish-orange conidial heads, with tightly packed metulae, very small (2–2.5 µm diam), smooth-walled conidia and the presence of lateral conidia on submerged hyphae. In this study, the affinity of the molecularly examined strains (IRAN 2056C and IRAN 1097C) to *A. terreus* was firmly supported by both sequence data of ITS rDNA (Fig. 1) and β-tubulin gene (Fig. 2) with 99% and 100% bootstrap support, respectively. This fungus has been previously reported from Iran on *Arachis hypogaea*, *Ficus carica* L., *Hordeum vulgare*, *Pistacia vera*, *Sesamum indicum* L., *Vitis vinifera* L. and *Zea mays* (Ershad 2009).

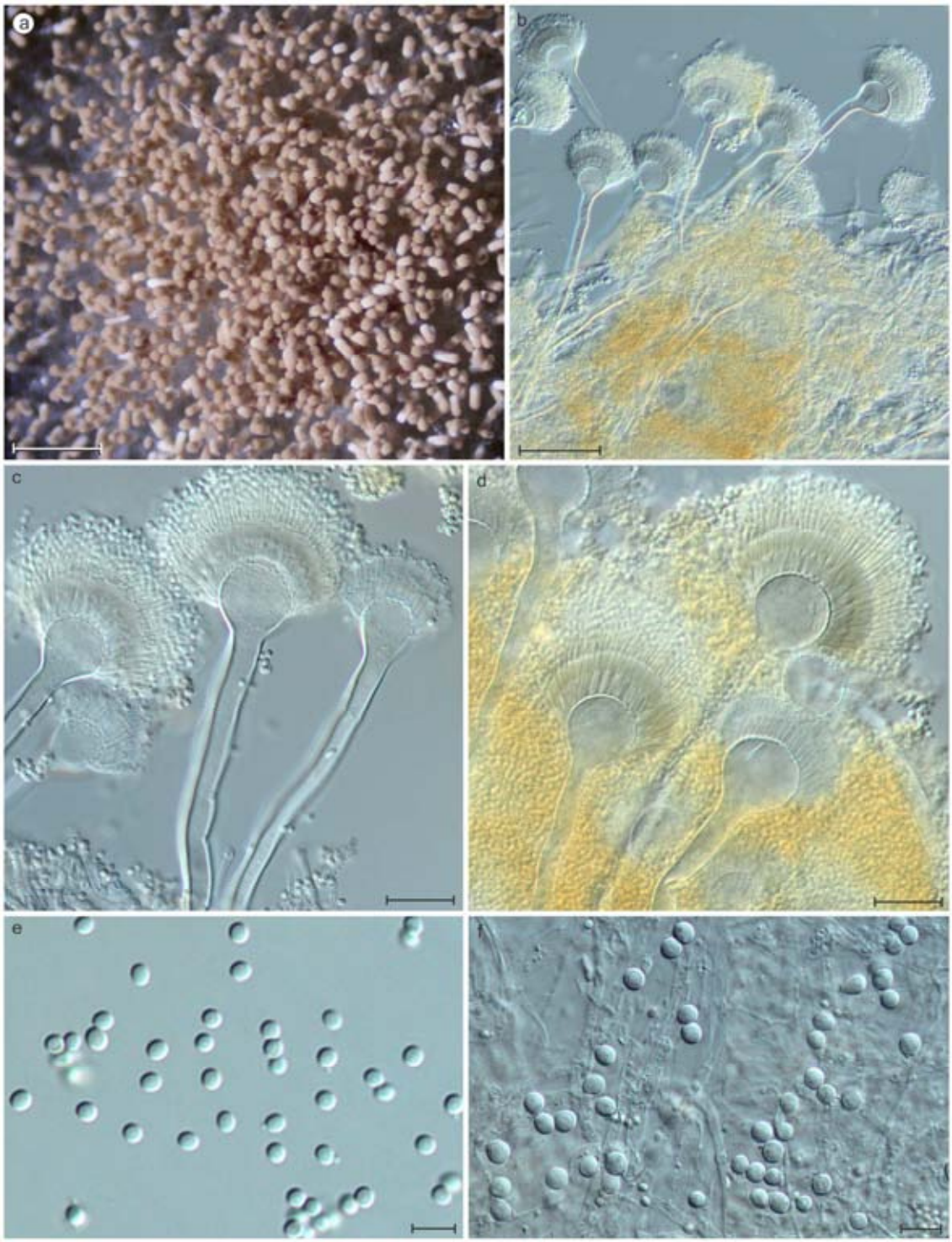

Fig. 9. *Aspergillus terreus*: a. Conidial heads, b–d. Conidiophores, e. Conidia, f. Aleuroconidia (Bars: a = 500 µm; b = 50 µm; c, d = 20 µm; f = 10 µm; e = 5 µm).

Aspergillus flavipes (Bainier & R. Sartory) Thom & Church, Manual of the Aspergilli: 179 (1926) (Fig. 10)

Colonies reaching 25–30 mm diam on MEA and 20–25 mm on CYA in 7 d at 25º C, white to very pale buff on CYA, but dull pinkish-buff on MEA, velutinous to slightly granular, occasionally forming sectors of sterile, floccose mycelia; exudate yellow to brown when present; reverse golden-brown on MEA and uncoloured to yellow-brown on CYA. Conidial heads radiating to loosely columnar; stipes yellow-brown, thick-walled, smooth-walled to finely roughened, (650–)1000–1300 (–1500) × 5–7.5 µm; vesicles subglobose to spatulate, 14–20 × (11.5–)13–18 µm; biseriate; metulae covering the upper one-third of the vesicle in small heads, but in large heads the whole vesicle, 5–8 × 2–3 µm; phialides 6–8 × 1.5–2.5 µm; conidia globose to subglobose, smooth-walled, 2–3 µm diam.

Specimens examined: Ardebil province, Moghan, on *Triticum aestivum* straw, 13-VIII-2006, coll. & isol. B. Asgari (IRAN 2062C); Sistan-o-Baluchestan province, on *Musa sapientum* L., 2004, coll. & isol. M. Amani (IRAN 939C).

The morphological characters of the strains examined in this study fit well the descriptions by Raper & Fennell (1965) and Klich (2002). The identification was additionally supported by phylogenetic analyses based on partial β-tubulin sequences (100% bootstrap support) (Fig. 2). It is distinct from its purported teleomorph *Fennellia flavipes* B.J. Wiley & E.G. Simmons 1973 as shown by Peterson (2008). This species is a new record for Iran.

Aspergillus iizukae Sugiy., J. Fac. Sci. Tokyo Univ., Section 3, 9(11): 390 (1967) (Fig. 11)

Colonies reaching 25 mm diam on MEA and 20 mm on CYA in 7 d at 25º C, pale buff, velutinous to slightly granular; soluble pigment orange-brown; exudate yellow; reverse dark reddish-brown on MEA and pale brown on CYA. Conidial heads radiating to columnar; stipes yellow-brown, thick-walled, smooth-walled, (500–)600–1000(–1300) × 7–9 µm; vesicles spatulate, 22–25 × 17–21 µm; biseriate; metulae mostly covering the whole vesicles, 4–6 × 1.7–2.5 µm; phialides 6–7 × 1.5–2 µm; conidia globose, smooth-walled, 2.3–3 µm diam.

Specimen examined: E Azerbaijan province, Kaleybar, on *Hordeum vulgare* seed, 27-XI-2007, coll. & isol. B. Asgari (IRAN 2063C).

The phylogenetic tree drawn on partial β-tubulin (Fig. 2) revealed the close relationship between the strain examined here with the ex-type strain of *A. iizukae*, NRRL 3750 (100% bootstrap support) as well as its separation from *A. flavipes*. This is in concordance with Peterson (2008) who showed *A. iizukae* to be molecularly distinct from *A. flavipes* based on sequences of RPB2, calmodulin and ITS-LSU rDNA.

In our morphological examination of IRAN 2063C, it slightly deviated from *A. flavipes* by having wider conidiophores terminating in larger vesicles, narrower metulae mainly covering the entire vesicles, narrower phialides and slightly larger conidia. Examination of metabolite profiles and growth-temperature relationships of the ex-type strains of *A. iizukae* and *A. flavipes* is required to establish criteria that reliably distinguish these close species.

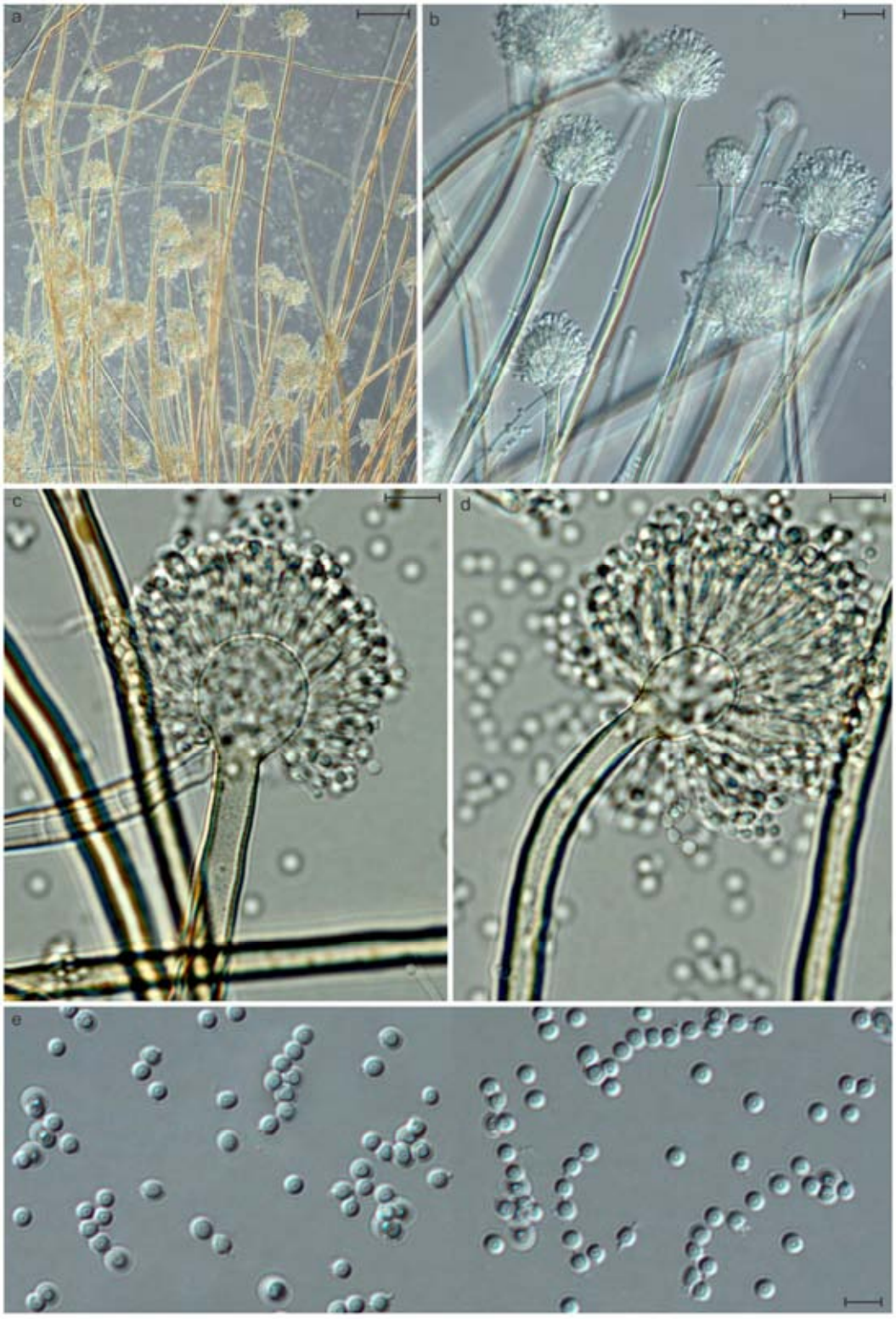

Fig. 10. *Aspergillus flavipes*: a–d. Conidiophores, e. Conidia (Bars: a = 100 µm, b = 20 µm, c–d= 10 µm, e = 5 µm).

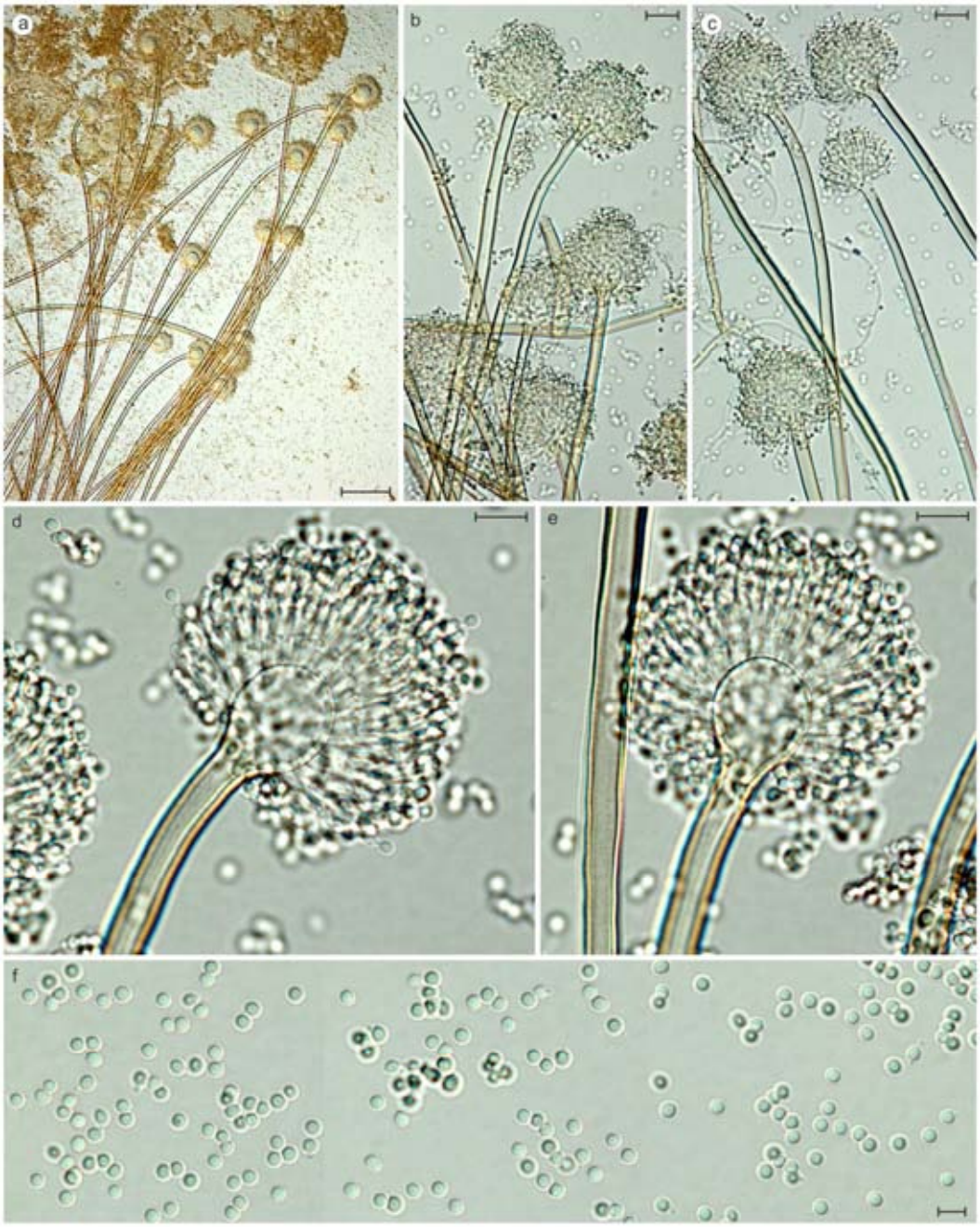

Fig. 11. *Aspergillus iizukae*: a–e.Conidiophores, f. Conidia (Bars: a = 100 µm; b, c = 20 µm; d, e = 10 µm; f = 5 µm).

Acknowledgment

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