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Aspergillus species in retail samples of pistachio, walnut and hazelnut in Kerman, Iran

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Abstract: Nuts are among Iran's most important crops consumed by many people due to their nutritional and nutraceutical properties. Fungi from the genus *Aspergillus* contaminate them during pre- and post-harvest stages. *Aspergillus* species are responsible for various agricultural products' secondary spoilage, and they can produce mycotoxins harmful to humans and animals. The present study evaluated the fungal contamination of nuts marketed in local stores in Kerman. Samples of pistachio, walnut, and hazelnut were collected throughout Kerman province, Iran, to characterize *Aspergillus* species contaminating nuts marketed in retail shops. *Aspergillus* species were examined by morphological and molecular criteria to explore the diversity of this genus. The phylogenetic relationships of these species were determined using sequences from partial *β*-tubulin and calmodulin sequences. *Aspergillus* species were identified as *A. flavus*, *A. parasiticus*, *A. arachidicola*, *A. tamarii*, *A. caelatus*, *A. nomius*, *A. leporis*, *A. quadrilineatus*, *A. unguis*, *A. spelunceus*, *A.ochraceus*, *A. auricomus*, *A. westerdijkiae*, *A. montevidensis*, *A. pseudoglaucus*, *A. subalbidus* and *A. taichungensis*. Populations of *Aspergillus* species on nuts, how these populations vary among different types of nuts, and their mycotoxin production potential are discussed.

Keywords: Calmodulin, retail shop, mycotoxin, Sequencing, phylogeny

INTRODUCTION

The genus *Aspergillus* is one of the most common and important filamentous fungal genera that frequently contaminate crops resulting in substantial economic loss worldwide (Bayman et al. 2002). Species of this genus are important since they are used in the fermentation industry, produce toxic metabolites harmful to humans and animals, act as opportunistic. Pathogens to humans, and infect and decay plant products (Perrone et al. 2007; Gholami-Shabani et al. 2017). They produce various mycotoxins in foods and feeds. The most important toxins are aflatoxins and ochratoxin A (Varga et al. 2004). Aflatoxins are genotoxic, carcinogenic, and teratogenic to both humans and animals. They are produced by *Aspergillus* section *Flavi* including *A. flavus* and *A. parasiticus* (Horn & Dorner 2002). Ochratoxin A is a potent nephrotoxin and a possible human carcinogen produced by *Aspergillus* section *Circumdati* including *A. ochraceus* and *A. westerdijkiae* (Visconti et al. 2008). *Aspergillus* species can contaminate nuts at different stages, including pre-harvest, harvest, processing, handling, and storage (Perrone et al. 2007). Pistachio, walnut, and hazelnut are among Iran's most important crops consumed throughout the country by many people due to the nutritional and nutraceutical properties of the nuts. Nuts are consumed as raw kernels and processed kernels or used in the confectionery industry. Mycotoxins at different levels have been reported in nuts by several authors (Amiri et al. 2013; Palumbo et al. 2015; Ait Mimoune et al. 2018). Trade concerns for exporting nut cargos have stimulated many authors to study mycotoxigenic flora and try eliminating mycotoxins and *Aspergillus* species from nut kernels. However, less is known about populations of *Aspergillus* species on nuts or how these populations vary among different types of nuts when marketed in local stores. The precise identification and characterization of *Aspergillus* spp. that could survive and proliferate on the nuts in the local market are less studied than on nuts in the field and the exporting cargos. This study aimed to examine the occurrence of *Aspergillus* species in three nuts marketed in local stores in Kerman County, Iran. We collected samples of conventionally produced pistachio, walnut, and hazelnut, and used morphological and molecular methods to analyze the biodiversity and the phylogenetic relationships within the obtained isolates.

MATERIALS AND METHODS

Fungal isolation

A total of 120 samples of pistachio, hazelnut, and walnut, each of approximately 500 g, were collected

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from retail shops in Kerman County during 2019-2021. The direct plating technique (Pitt & Hocking 2009) was used to isolate *Aspergillus* species from samples. From each sample, 50 g of nuts were surface disinfected by immersion in 1% NaOCl for 1 min, rinsed in sterile distilled water, and placed on sterile filter paper to dry. Pieces of each nut were plated on dichloran Rose Bengal chloramphenicol (DRBC) agar according to the methodology of King et al. (1979). Plates were incubated in the dark for 5-7 days at 25 °C. All isolates with the appearance of belonging to *Aspergillus* species were grown on Czapek yeast autolysate agar (CYA) and incubated at 25 °C for seven days (Pitt & Hocking, 2009) to see colony characteristics. The Putative isolates of *Aspergillus* spp. were sub-cultured onto CYA (Czapek yeast extract agar) for subsequent identification of species using morphological and molecular methods. Isolates were deposited in the KGUT Fungal Culture Collection at the Kerman Graduate University of Advanced Technology, Kerman, Iran, stored in 10% glycerol at – 20 °C for short term and at -80 °C for long-term preservation.

Morphological characterization

For macro morphological observations, spore suspensions (in 0.2% agar) were inoculated at three points onto 9 cm plates of MEA (Malt Extract Agar), CYA, CY20S (Czapek Agar with 20% sucrose), and CZ (Czapek's agar) as described by Samson et al. (2014). Plates were incubated for seven days in the dark at 25°C. One additional CYA plate for each isolate was inoculated and incubated at 37°C. Plates were placed upside down. Colony texture, diameter, color, pigmentations, and exudates were observed after seven days. The production of sclerotia was recorded. Plates of MEA and CYA were incubated for more extended periods (up to 2 months) for the possible production of a sexual state.

For micro-morphological observations, mounts were prepared from colonies grown on MEA medium in cotton blue and 50% lactic acid. For removing air bubbles and spreading conidia, a drop of alcohol was added. Digital images were made using a Dino-eye microscope camera USB lens (The Microscope Store, LLC, USA). All isolates were preliminarily attempted to be identified based on their morphological features according to available keys (Klich 2002; Klich and Pitt 1998) and papers (Houbraken et al. 2020). The identifications were further confirmed by molecular analysis.

DNA extraction, amplification, and sequencing

The cultures used for the molecular studies were grown in PD broth at room temperature for seven days on a rotary shaker; the mycelium was harvested, washed in sterile water, and freeze-dried. The cells were lysed using CTAB solution, and the DNA extraction was performed using DNG^{TM}-Plus solution (Sinaclon, Iran) following the manufacturer's instructions. The quality of the genomic DNA was checked on 1% agarose gel and visualized by staining with ethidium bromide solution.

Partial calmodulin gene (580 bp) was amplified from *Aspergillus* isolates using the primers cmd5 (5′ CCG AGT ACA AGG AGG CCT TC 3′) and cmd6 (5′ CCG ATA GAG GTC ATA ACG TGG 3′) (Hong et al. 2006). Amplification of part of (550bp) the β-tubulin gene was performed using Bt2a (5′ GGT AAC CAA ATC GGT GCT TTC 3′) and Bt2b (5′ACC CTC AGT GTA GTG ACC CTT GGC 3′) (Glass & Donaldson 1995). Twenty-five µL PCR reactions contained 1X reaction buffer, 0.4 mM of each primer (Metabion, Germany), 200 mM dNTPs, 2.5 mM MgCl2, 20 ng of DNA, and 1 unit of Taq polymerase. A Biometra TAdvanced Thermal Cycler (Biometra, Göttingen, Germany) was used to perform PCRs with the cycling conditions consisting of 95 °C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and then 5 min at 72°C.

Molecular identification and Phylogenetic analysis

The obtained sequences were edited by Geneious (Biomatters Inc., USA) when needed and compared to the sequences in the NCBI (GenBank) database using BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) to find the most likely taxonomic designation of each isolate.

All the sequences generated in this study were deposited in GenBank, and the accession numbers were obtained. Furthermore, a number of DNA sequences were retrieved from GenBank and included in the phylogenetic analyses to determine the taxonomic status of *Aspergillus* isolates (Table 1). Maximum parsimony phylogenies were applied using heuristic searches in PAUP v. 4.0a133 (Swofford 2002) with bootstrap analysis of 1,000 replicates to test the support of the branches.

RESULTS

Fungal Species Identification

In this study, *Aspergillus* species were isolated from 120 samples of pistachio, hazelnut, and walnut collected from retail shops in Kerman County, Iran. In total, 260 *Aspergillus* isolates were obtained. The morphological and molecular investigations led to the identification of 17 species that are, according to Houbraken et al. 2020, resided in 11 Series, namely series Flavi, Kitamyces, Nomiarum, and Leporum from section *Flavi*; series Nidulantes, Unguium. and Speluncei from section *Nidulantes*; ser. Circumdati from section *Circumdati*; ser. Candidi from section *Candidi*; and series Chevalierorum, Aspergillus and Rubri from section *Aspergillus* (Fig 1). The most prevalent genera belonged to section *Flavi* (42% of all isolates).

The identified species included *A. flavus*, *A. parasiticus*, and *A. arachidicola*, from ser. Flavi; *A. tamari* and *A. caelatus* from ser. Kitamyces; *A. nomius* from ser. Nomiarum; *A. leporis* from ser. Leporum (section *Flavi*, Figs 2 to 8); *A. quadrilineatus* from ser. Nidulantes; *A. unguis* from ser. Unguium; *A. spelunceus* from ser. Speluncei (section *Nidulantes*, Figs 9 to 11); *A.ochraceus*, *A. auricomus*, and *A.*

westerdijkiae from ser. Circumdati (section *Circumdati*, Figs 12 to 14); *A. montevidensis* from ser.

Table 1. Fungal species included in phylogenetic analysis of this study.

species	Strain No	Source	GenBank accession numbers	
			calmodulin	β-tubulin
A.Subalbidus	C14	pistachio	MN986426 [a]	OP265126 [a]
A.Subalbidus	DTO:129-E3	house dust	KJ775249	KJ775068
A.Subalbidus	DN68	soil	MW480770	LT908034
A. taichungensis	MD17	Hazelnut	MN986427 [a]	OP265127 [a]
A. taichungensis	IBT 19404	holotype	EU076310	EU076297
A. taichungensis	DTO:270-C9	house dust	KJ775253	KJ866981
A. campestris	CBS 348.81	type material	EU076311	EU076296
A. dobrogensis		bat	OU641290	OU641294
A. tritici	DTO 438-H6	Rice	MZ028007	KX495180
A. flavus	W15	walnut	MN986404 [a]	MT009226 [a]
A. flavus	P42	pistachio	MN986406 [a]	MT009228 [a]
A. tamarii	MD5	pistachio	MN986414 [a]	MN993903 [a]
A. tamarii	CBS 129.49		KJ175555	KJ790260
A. pseudotamarii	CBS 765.97	Peanut Argentina	EF202031	EF203126
A. caelatus	MD7	hazelnut	MN986416 [a]	MN993914 [a]
A. caelatus	NRRL 26100	-	EF661523	EF661471
A. pseudocaelatus	CCDCA 11425	*Phaseolus vulgaris*	MG746452	MG746539
A. leporis	MD8	pistachio	MN986417 [a]	MN993905 [a]
A. leporis	CBS 349.81		FJ491486	FJ491475
A. parasiticus	MD9	pistachio	MN986418 [a]	MN993906 [a]
A. parasiticus	MD10	hazelnut	MN986419 [a]	MN993907 [a]
A. parasiticus	CBS 117618		EF202039	FJ491485
A. arachidicola	MD12	pistachio	MN986421 [a]	MN993909 [a]
A. arachidicola	CBS 117615		EF202050	EF203161
A. arachidicola	DTO 010-H5	*Arachis glabrata*	MG517999	MG517627
A. flavus	NRRL 447		EF661506	EF661483
A. nomius	MD15	pistachio	MN986424 [a]	MN993912 [a]
A. nomius	MD16	hazelnut	MN986425 [a]	MN993913 [a]
A. nomius	ITAL 1230		KJ816328	KJ789984
A. pseudonomius	NRRL 3353	field soil	LC314726	EF661495
A. aflatoxiformans	DTO 215-F5	peanut cake	MG518053	MG517682
A. austwickii	DTO 228-G8	sesame kernels	MG518082	MG517712
A. minisclerotigenes	DTO 009-F5	*Arachis hypogaea* seed	MG518007	EF203153
A. bertholletius	CCT 7615	Brazil nut	JX198674	KY924666
A. luteovirescens	NRRL 26010	type material	EF661533	EF661498
A. coremiiformis	CBS 553.77	type material	FJ491488	FJ491482
A. alliaceus	DTO 034-B2	*Allium cepa*	MG518004	MG517632
A. neoalliaceus	CBS 134375	soil	MG518158	MG517613
A. aspearensis	DTO 203-D4	soil	MG518038	MG517667
A. avenaceus	CBS 102.45		FJ491495	FJ491480
A. pipericola	DTO 228-H4	black pepper	MG518087	MG517717
A. texensis	NRRL 66855	holotype	MN987070	MK119752
A. montevidensis	CBS 119376		LT671235	KX463375
A. montevidensis	KAS5878	dust	KX463324	KX463375
A. montevidensis	P13	Pistachio	MN986429 [a]	OP265128 [a]
A. montevidensis	EMSL No. 2730	filter	LT671254	MZ826418
A. pseudoglaucus	H33	Pistachio	ON855051 [a]	OP265129 [a]
A. pseudoglaucus	EMSL No. 2844	swab	LT671298	MW488413
A. pseudoglaucus	DUCC6003	furniture	MN619779	MW357166
A. ruber	S48	rice	MZ826368	MZ826402
A. teporis	CBS 141768	corn kernels	LT671195	LT671194
A. tamarindosoli	CBS 141775	soil	LT671192	LT671191
A. proliferans	KAS6292	dust	KX463333	KX463381
A. chevalieri	PRK9a	groundnuts	KU872184	KU872182
A. sloanii	DTO:245-A9	dust	KJ775314	KJ775077
A. xerophilus	NRRL 6132		EF651984	EF651924

A. appendiculatus	CBS 101746		HE801319	HE801334
A. costiformis	CCF 4097	nail	FR837974	FR837970
A. intermedius	NRRL 4817		EF652014	EF651894
A. tonophilus	NRRL 5124	lectotype	EF652000	EF651919
A. glaucus	NRRL 117		EF651990	EF651888
A. restrictus	DTO:237-A2	dust	KJ775295	KY117749
A. ochraceus	N5	Hazelnut	MT079833 a	OP265130 a
A. ochraceus	FV54	fruit	MW259084	MG254195
A. ochraceus	CMV006D9	wheat	MK451474	EF150882
Aneobridgeri	PLR9	Pistacia lentiscus	MK680532	MK680531
Asclerotiorum	CMV007B4	food	MK451507	MZ062566
Apallidofulvus	CMV012D2	soil	MK451477	MT084116
A. auricomus	MD24	Walnut	ON855055 a	OP265131 a
A. auricomus	NRRL 391	neotype	EF661379	EF661320
A. westerdijkiae	MD23	Hazelnut	ON855054 a	OP265132 a
A. westerdijkiae	UTHSC:DI15-5	Clinical Sample	LT574773	LT574738
A. westerdijkiae	DTO:246-B5	dust	KP330165	KP329876
A. steynii	DTO:245-I5	dust	KJ775324	EF661347
A. ostianus	CBS:311.80	pulses	KJ775240	KJ775052
A. sesamicola	DTO:148-B4	sesami seed	KJ775233	KJ775063
A. westlandensis	DTO:231-B1	air	KJ775231	KJ775067
A. affinis	418	leaf litter	JF805764	GU721092
A. fresenii	CBS 550.65		FJ491561	
A. muricatus	NRRL 35071		EF661365	EF661355
A. tanneri	NRRL 62425	type material	JN896583	JN896582
A. spelunceus	MD21	Walnut	ON855052 a	OP265133 a
A. spelunceus	S59	cave sediment	LN873971	LN873954
A. spelunceus	CCF 4000	cave air	LN873969	FR775321
A. unguis	DTO:270-D7	dust	KJ775396	KP329863
A. unguis	NRRL 6328		EF652421	EF652333
A. quadrilineatus	APR22	Walnut	MN993915 a	OP265135 a
A. quadrilineatus	NRRL 4904		EF652396	EF652308
A. sydowii	DTO 438-E4	Rice	MZ028003	MZ027942
A. sublatus	DTO 433-A8	soil	MW671044	KP329853
A. aureolatus	DN61	soil	MW480763	LR593495
A. askiburgiensis	CCF 4085	*Myotis myotis*	LN873966	FR775376
A. foveolatus	CBS 279.81	type material	MN969229	KX423622
A. creber	CMV011F9	seed	MK451356	OU641287
A. croceus	CCF 4721	cave air	LN873964	LN873951
A. tennesseensis	KAS5657	house dust	KX894570	LS423457
A. israelensis	CBS 140628	evaporation pond	KU866798	KU866916
A. oleicola	DTO 322-A9	indoor	KU866770	KU866904
A. qinqixianii	DTO 098-H7		KU866723	KU866894
A. varians	DTO 063-I1	cork	KU866720	KX423620
A. dromiae	DTO 061-B3	*Dronica crythropus*	KU866713	KU866893
A. pepii	AV11051B_IX	holotype	KU613365	KU613371
A. versicolor	DTO:270-D1	dust	KJ775395	KP329933
A. fructus	DTO:267-G8	dust	KJ775367	KP329905
A. nidulans	DTO:244-I3		KJ775305	MK749975
A. undulatus	CBS 261.88		EU443989	KU866902
A. venezuelensis	CBS 868.97	type material	EU443977	AY339998
A. purpureus	NRRL 6133	holotype	EF652418	AB248315
A. asperescens	NRRL 5036		EF652407	EF652319
A. stellatus	NRRL 4793		EF652391	EF652303
A. aurantiobrunneus	NRRL 4545		EF652377	EF652289
A. aeneus	NRRL 4769		EF652386	EF652298

[a] Sequences generated for this study.

Chevalierorum; *A. pseudoglaucus* from ser. Rubri (section *Aspergillus*, Figs 15 to 16); *A. subalbidus* and *A. taichungensis* from ser. Candidi (section *Candidi*, Figs 17 to 18). The species with the frequency of at least five isolates are reported in this study. Approximately 11% of the obtained isolates were from section *Nigri* that were excluded from this study because they are common in plant products and have been isolated from various sources.

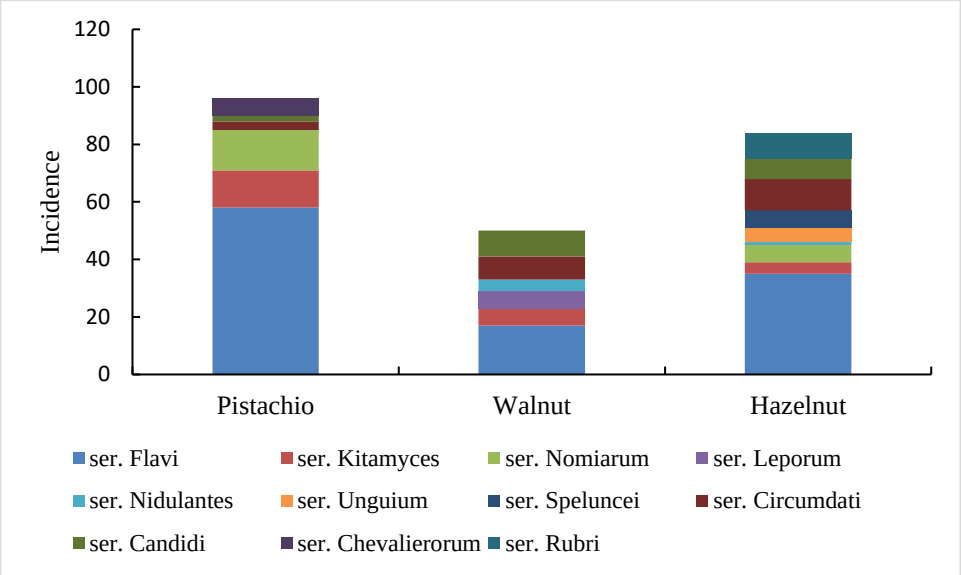

Fig. 1. Frequencies of *Aspergillus* series obtained from pistachio, hazelnut, and walnut collected from retail shops in various areas of Kerman County (n = 260).

Species in section *Flavi* include species with colonies in shades of yellow-green to brown with usually biseriate conidial heads, which produce dark sclerotia. These species are important mycotoxin producers, including aflatoxins, among which aflatoxin B_1 is the most toxic secondary metabolite produced by fungi. Seven species from this section were obtained and identified based on morphology and sequence data, as *A. flavus*, *A. parasiticus*, *A. tamari*, *A. nomius*, *A. arachidicola*, *A. caelatus*, and *A. leporis* (Figs 2 to 4).

Aspergillus section *Nidulantes* includes species with brown-pigmented stipes, usually biseriate conidial heads and ascomata when present embedded in masses of Hülle cells. Vesicles are usually globose, subglobose, or subclavate. Conidia are globose and echinulate, green in mass. The species in this section produce the carcinogenic mycotoxin sterigmatocystin and aflatoxins. Three species from this section were obtained and identified as *A. quadrilineatus*, *A. unguis*, and *A. spelunceus* (Fig 5).

Aspergillus section *Circumdati* includes species with rough walled stipes and usually biseriate conidial heads. They are also called the *Aspergillus ochraceus* group. These species produce yellow to ochre conidia. The species in this section produce ochratoxins. Species such as *Aspergillus ochraceus* are identified as human and animal pathogens. Many species are reported from agricultural and stored foods. The most important species regarding potential ochratoxin A contamination in agricultural products are *A. ochraceus* and *A. westerdijkiae*. Three species from this section were obtained and identified as *A.ochraceus*, *A. auricomus*, and *A. westerdijkiae* (Fig 6).

Aspergillus section *Aspergillus* includes species with globose to subglobose vesicles and uniseriate conidiophores. These species produce green conidia. These species are common in indoor air, cereals, and food products. Species in this section are xerophilic and can grow at minimum moisture levels. Species are reported from cases of superficial infections and sporadic invasive infections; however, they are not important pathogens. Two species from this section were obtained and identified as *A. montevidensis* and *A. pseudoglaucus* (Fig 7). They are reported to produce potentially toxic echinulin.

Aspergillus section *Candidi* includes species with slow-growing colonies having white to yellowish conidia. Conidial heads are globose; conidiophores are smooth and usually small, metulae in most species cover the entire vesicle. Species in this section produce species-specific extrolites that, they have not been found in any other *Aspergillus* species. Two species from this section were obtained and identified as *A. subalbidus* and *A. taichungensis* (Figs 17 to 18).

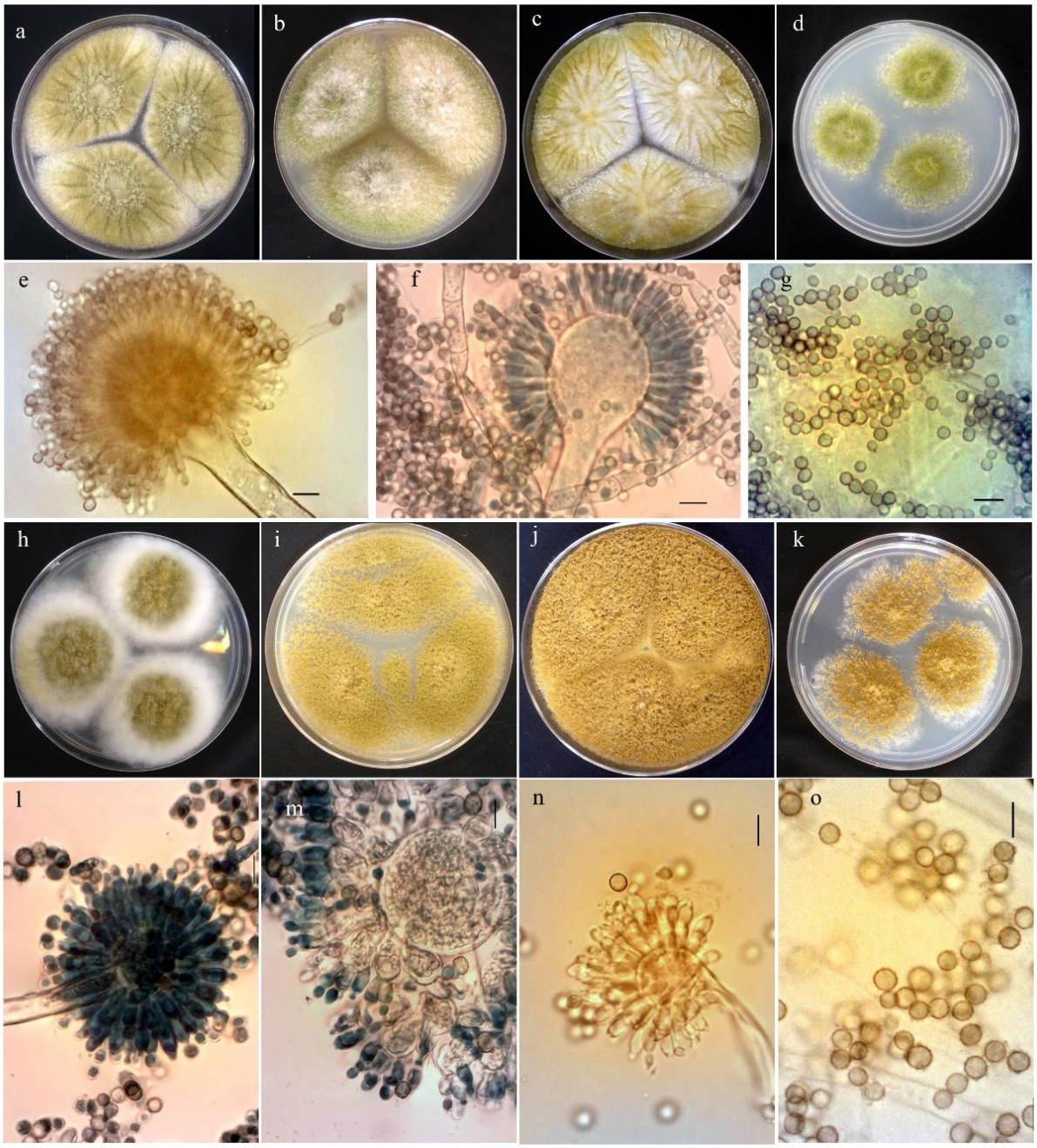

Fig. 2. *Aspergillus flavus*, a-d) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, e-f) Conidiophores, g) Conidia; *Aspergillus tamarii*, h-k) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, l-n) Conidiophores, o) Conidia; Scale bars: 10 µm.

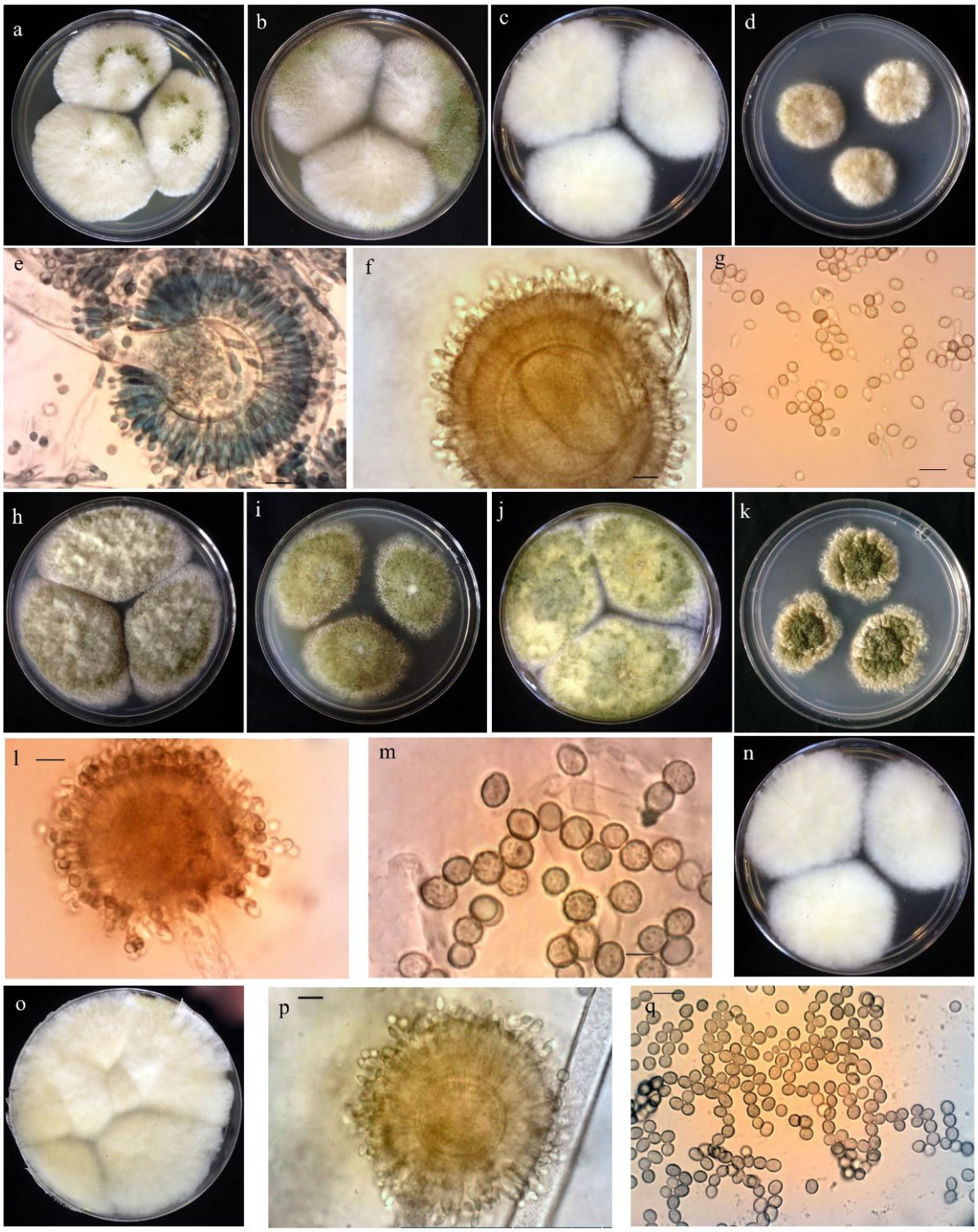

Fig. 3. *Aspergillus nomius*, a-d) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, e-f) Conidiophores, g) Conidia, *Aspergillus parasiticus*, h-k) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, l) Conidiophore, m) Conidia; *Aspergillus leporis*, n-o) Colonies after 7 d at 25 °C, left to right, CYA, MEA, p) Conidiophore, q) Conidia, Scale bars: 10 µm, e: 5 µm.

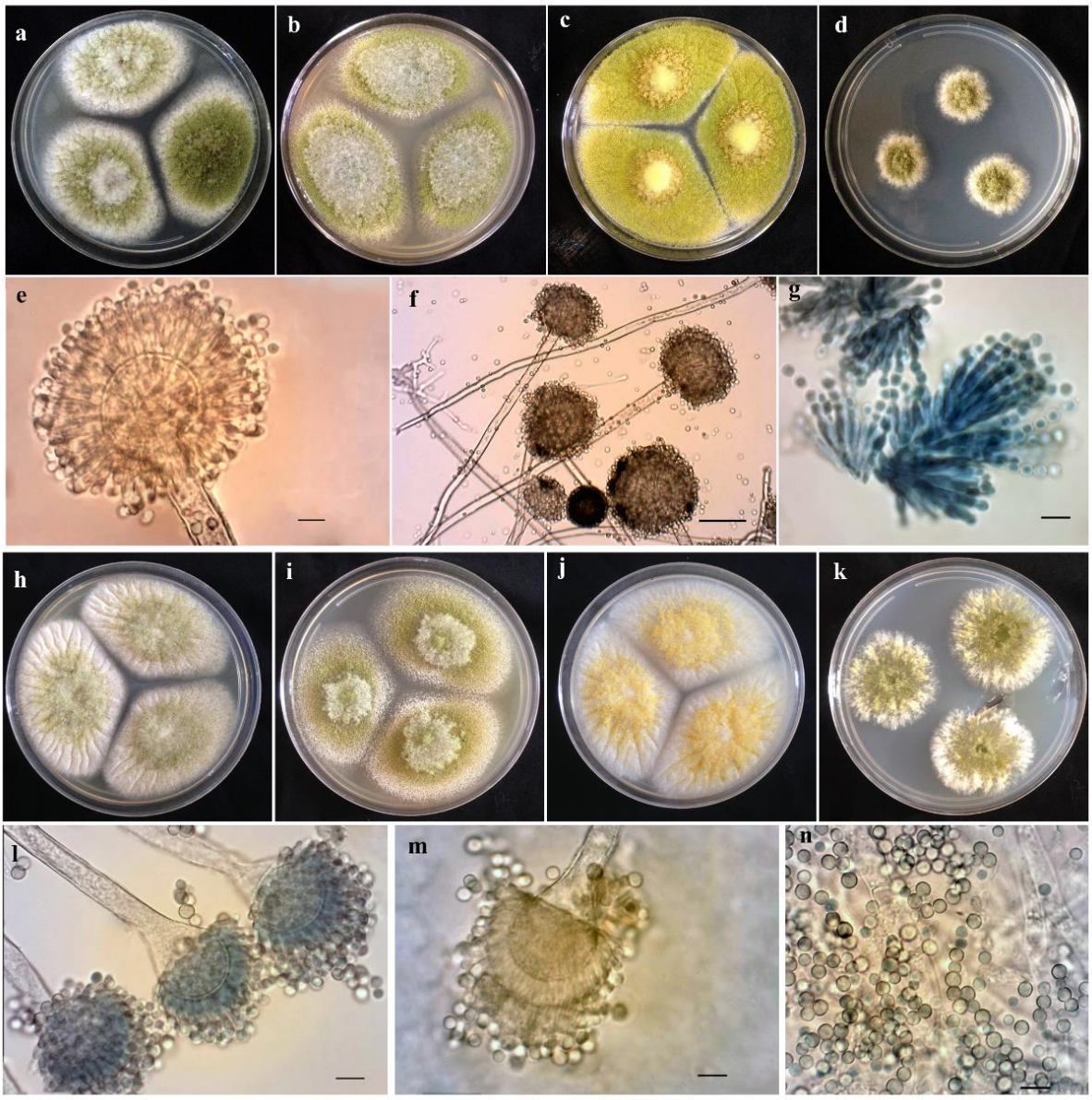

Fig. 4. *Aspergillus caelatus*, a-d) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, e-f) Conidiophores, g) Conidia; *Aspergillus arachidicola*, h-k) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, i-m) Conidiophores, n) Conidia, Scale bars: 10 µm.

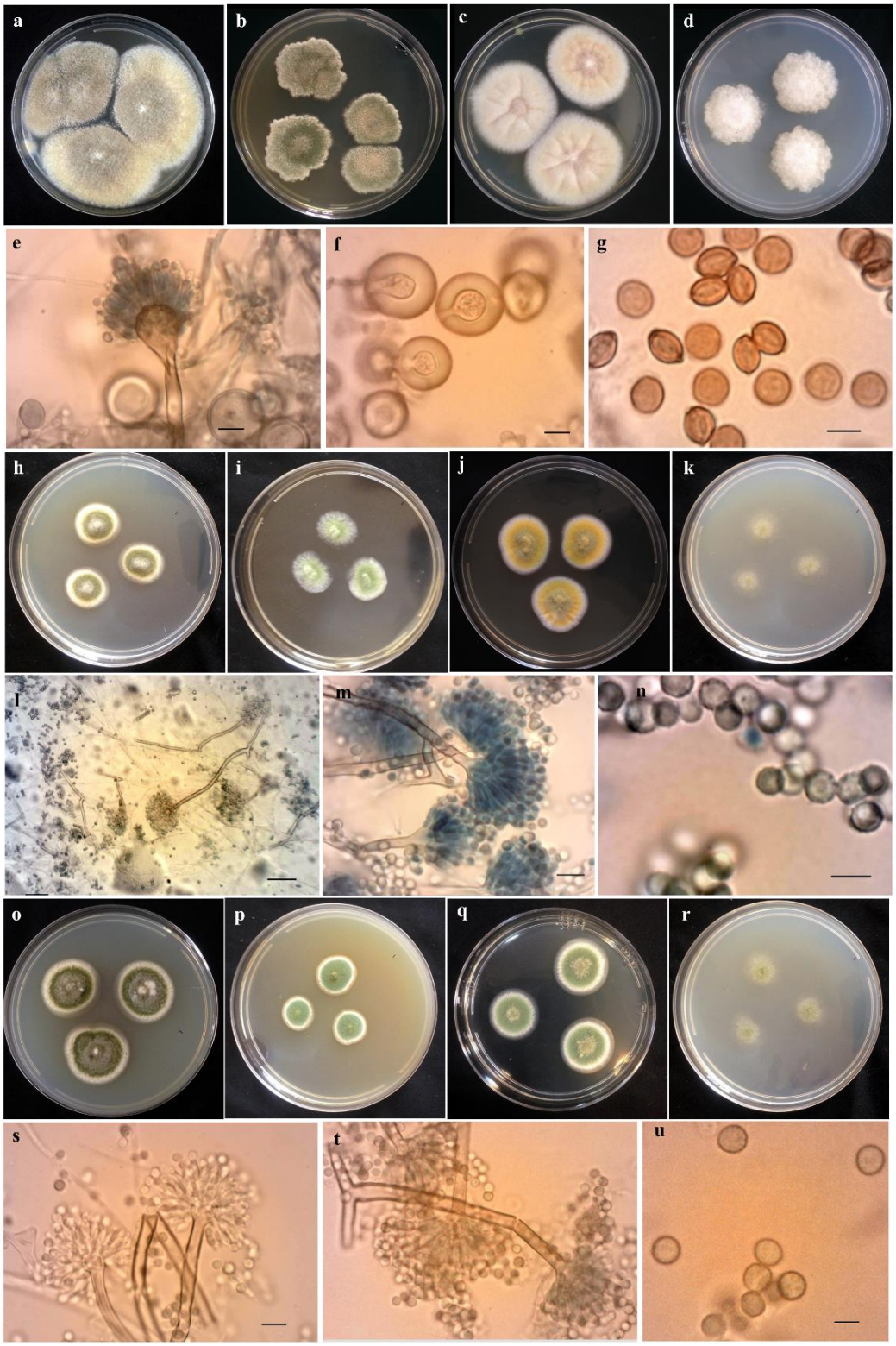

Fig. 5. *Aspergillus quadrilineatus*, a-d) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, e) Conidiophores, f) Hull cells, g) Ascospores; *Aspergillus spelunceus*, h-k) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, l-m) Conidiophores and Conidia, n) Conidia; *Aspergillus unguis*, o-r) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, s-t) Conidiophores, u) Conidia, Scale bars: e-f: 10 µm, g: 5 µm. l: 50 µm, m: 5 µm, n: 5 µm, s-t: 10 µm and u: 5 µm.

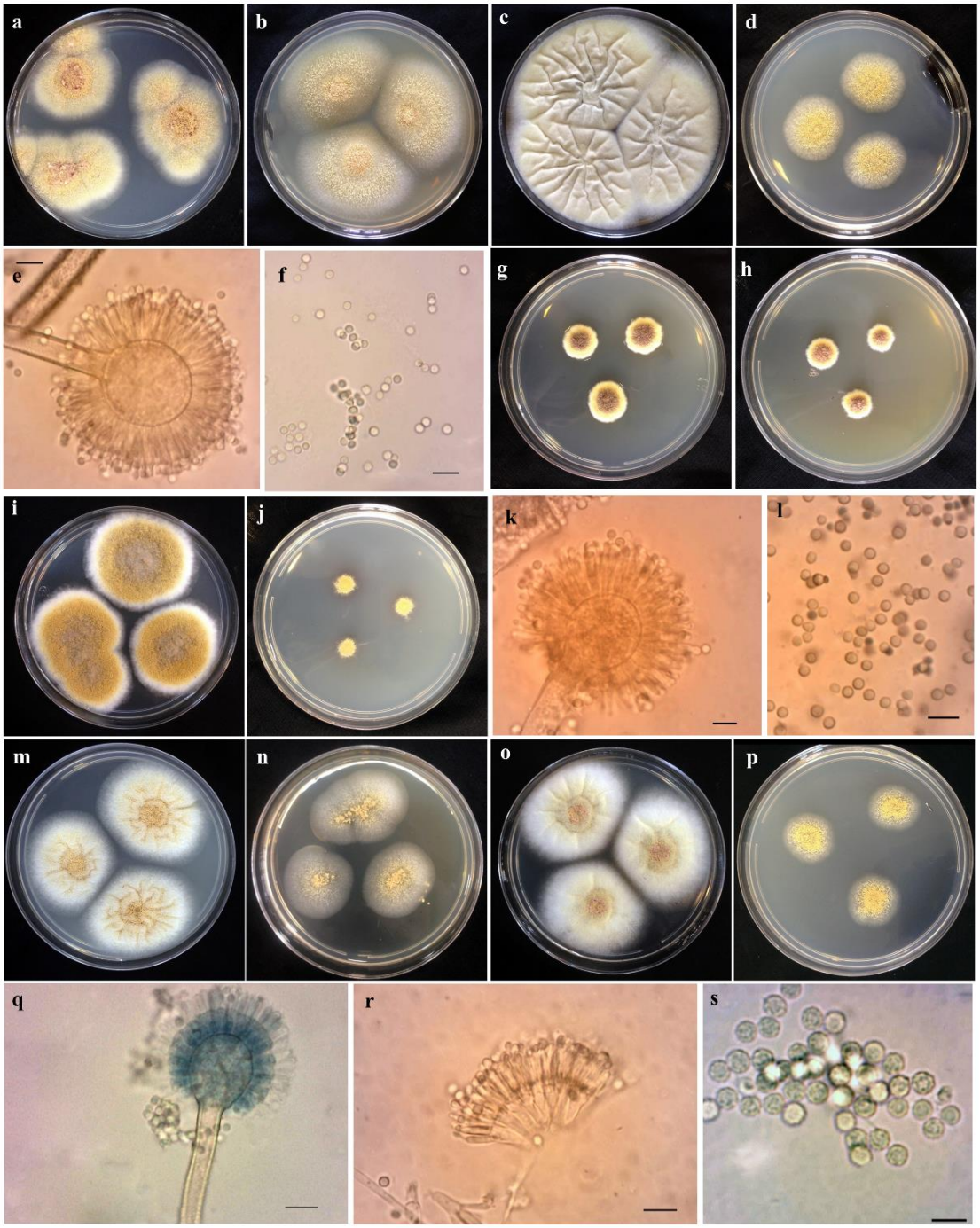

Fig. 6. *Aspergillus ochraceus*, a-d) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, e-g) Conidiophores, h) Conidia; *Aspergillus auricomus*, g-j) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, k) Conidiophore, l) Conidia; *Aspergillus westerdijkiae*, m-p) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, q) Conidiophore, r) Phyalids, s) Conidia, Scale bars: e-r: 10 µm s: 5 µm.

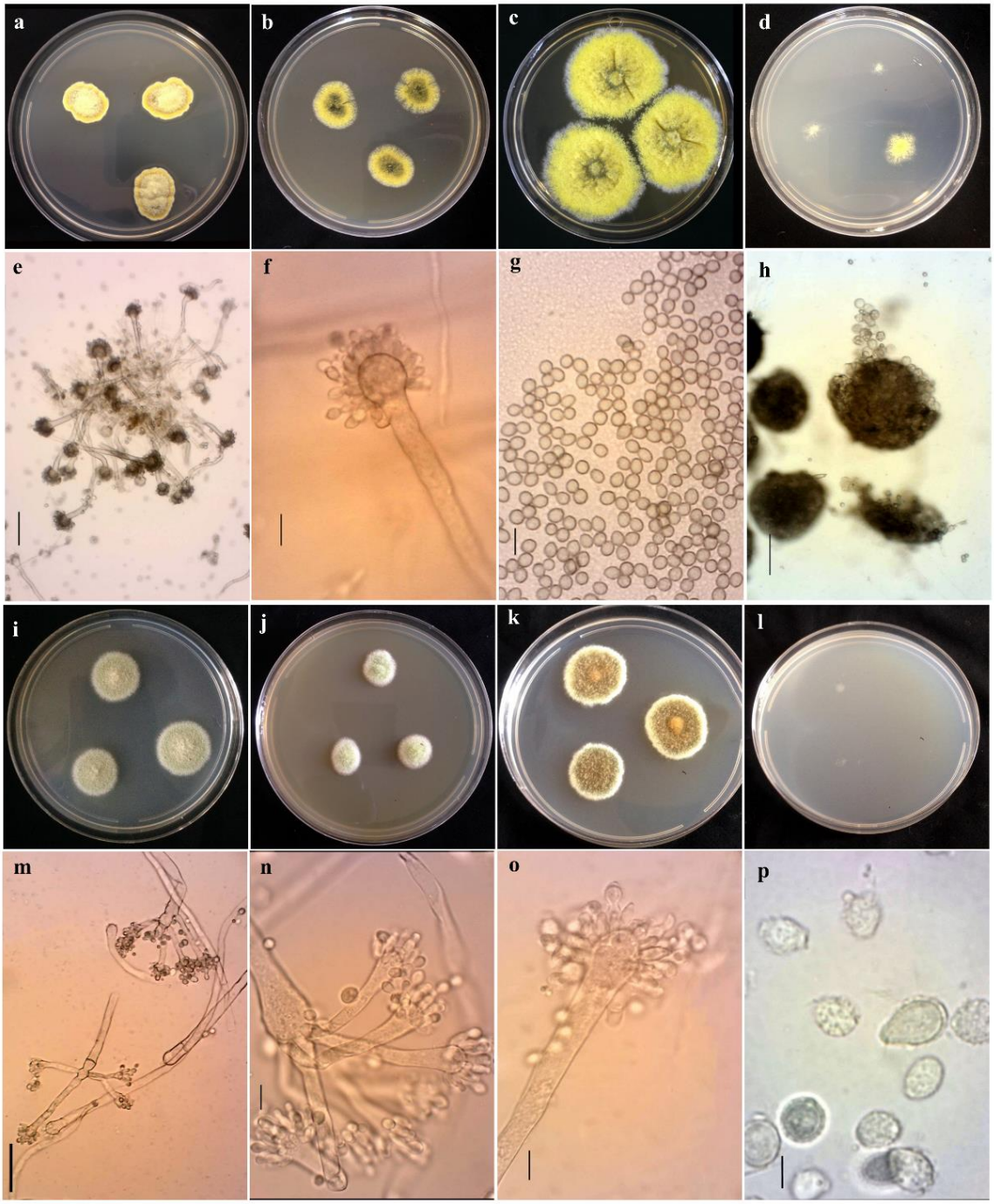

Fig. 7. *Aspergillus montevidensis*, a-d) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, e-f) Conidiophores, g) Conidia, h) Ascomata; *Aspergillus pseudoglaucus* , i-l) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, m-o) Conidiophores, p) Ascospores; Scale bars: e: 50 µm and f-h: 10 µm, m-o: 10 µm and p: 5 µm.

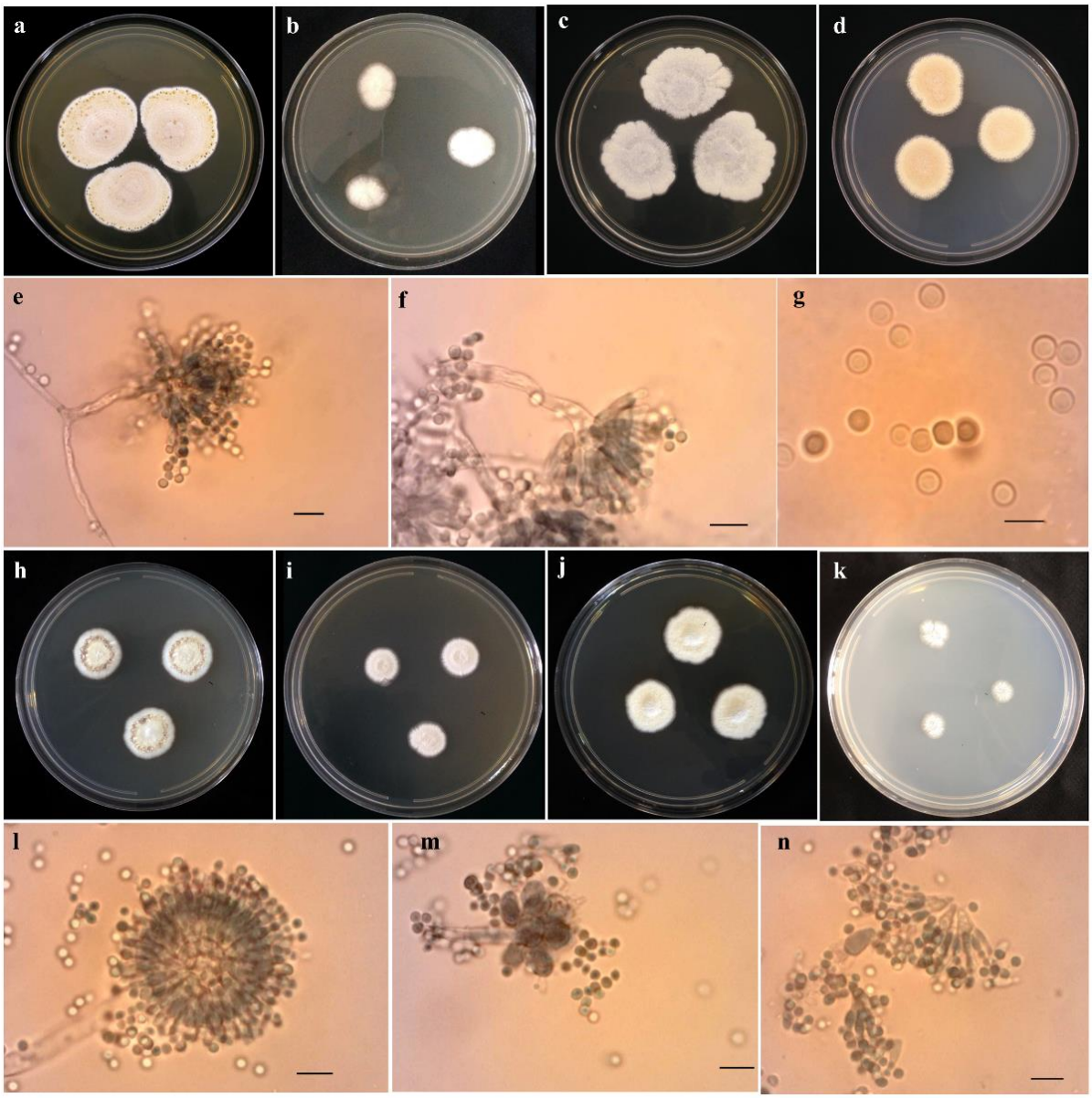

Fig. 8. *Aspergillus subalbidus*, a-d) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, e-f) Conidiophores, g) Conidia; *Aspergillus taichungensis*, h-k) Colonies after 7 d at 25 °C, left to right, CYA, MEA, CY20S, CZ, l) Conidiophore, m-n) Phyalids and Conidia, Scale bars: e-f: 10 µm g: 5 µm, l-n: 10 µm.

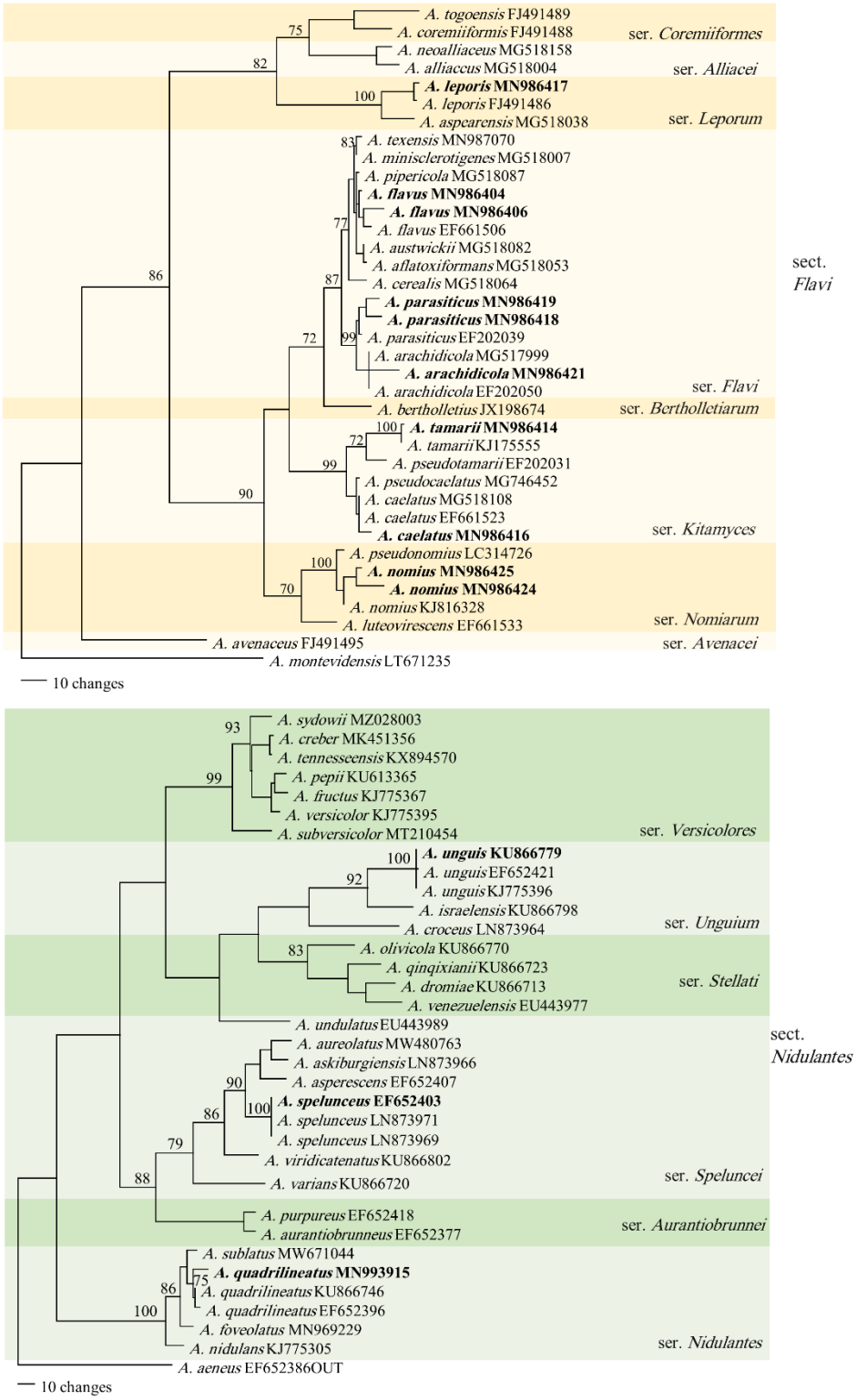

Fig 9. Phylogram derived from maximum parsimony inference analysis of partial β-tubulin and calmodulin nucleotide data set of sections *Flavi* and *Nidulantes*. Bootstrap values are presented at the nodes above the branches. The bar indicates the number of substitutions per site.

DISCUSSION

The present study was conducted to explore the biodiversity of the genus *Aspergillus* in pistachio, hazelnut, and walnut collected from retail shops in Kerman County, Iran. Although many research has been done on the determination of mycotoxins in nuts (Amiri et al. 2013; Ostadrahimi et al. 2014; Mimoune et al. 2018), the occurrence of *Aspergillus* spp. is less studied in these products as compared to that in nuts in the field and the exporting cargos. Species-level identification of nuts contamination to toxigenic fungi is the key step in developing an efficient plan to monitor and protect agricultural products.

Sequences of calmodulin and β-tubulin are used in this study to confirm morphological identifications and determine the genetic relatedness of the isolates obtained in this study. The overall topologies of our phylogenetic trees were in agreement with the latest phylogenies of the *Aspergillus* genus (Houbraken et al. 2020). The obtained isolates resided in sections *Flavi*, *Nidulantes*, *Circumdati*, *Aspergillus*, and *Candidi*. Molecular phylogenetic analyses combined with morphological characteristics were used to identify *Aspergillus* species. The identified species included *A. flavus*, *A. parasiticus*, *A. arachidicola*, *A. tamarii*, *A. caelatus*, *A. nomius*, *A. leporis*, *A. quadrilineatus*, *A. unguis*, *A. spelunceus*, *A.ochraceus*, *A. auricomus*, *A. westerdijkiae*, *A. montevidensis*, *A. pseudoglaucus*, *A. subalbidus*, and *A. taichungensis*. Literature reported that the genus *Aspergillus* is predominant in nuts (Bayman et al. 2002). One reason can be due to their ability to resist adverse environmental conditions. *A. flavus* was the most common species found on pistachio (23.5%) and hazelnut (14.6%), in agreement with studies of many authors (Doster & Michailides 1994; Rahimi et al. 2007; Saffari et al. 2021) while only 0.7% of the *A. flavus* isolates were isolated from walnut.

Our results showed the presence of species with aflatoxin-producing ability in hazelnut samples. Contamination of hazelnuts to aflatoxins is reported (Ozay 2007; Baltaci et al. 2012; Houshyarfard & Javadi 2018). According to these findings, a concerning situation can be perceived about hazelnuts marketed in retail shops in Iran. Other species of importance with mycotoxigenic risks that we isolated from hazelnut are *A. ochraceus* and *A. westerdijkiae*, that; both can produce large amounts of ochratoxin A (Morello et al. 2007; Ghitakou et al., 2006). Some researchers have as well found *Aspergillus* species other than aflatoxigenic ones to be predominant in hazelnuts. Lombardi et al. (2022) evaluated the fungal contamination of ready-to-eat hazelnuts collected from different boreal hemisphere areas, and they isolated *A. niger*, *A. ochraceus*, and *A. welwitschiae*, which are potential ochratoxin producers. Another species that we isolated from hazelnut is *A. pseudoglaucus* which can produce potentially toxic echinulin (Chen et al. 2017). Fungal contamination of hazelnut kernels is affected by the cultivation area climate, harvest time, and storage condition (Pscheidt et al., 2019; Lombardi et al. 2022). Drying the kernels is suggested to remove the fungal contamination of hazelnuts (Turan 2018). However, *Aspergillus* species can survive high temperatures and extreme stress (Dijksterhuis 2007). Therefore, the drying process cannot remove mycotoxin risk. Researchers (Mao et al. 2016; Lombardi et al. 2022) have suggested strategies such as irradiation with gamma rays, plasma technology, chemical treatments, and biological control using antagonistic microorganisms.

Pistachios were higher than other nuts in the frequency of colonization by aflatoxin-producing species of section *Flavi*. This might be due to the nutrient constitution of this substrate, processing techniques, and storage conditions of this product that have been favorable for aflatoxigenic species growth. This agrees with previous observations that have reported members of the *Aspergillus* section *Falvi* to occur most frequently on this nut product (Mojtahedi et al., 1979; Doster & Michailides 1994, Vaamonde et al. 2003; Jamali et al. 2012; Houshyarfard et al. 2014; Habibi & Afzali 2021). Many researchers have reported the presence of aflatoxins in pistachio samples in Iran. Cheraghali et al. (2007), analyzed Iranian pistachio nuts for aflatoxins and observed that 36.7% of the total samples were contaminated and the AFB_1 level was above the maximum tolerated level in 11.8% of samples. Shakeri et al. (2019), studied the level of aflatoxins in nuts in Isfahan in 2016, and reported that the concentration of aflatoxins in 37.5% of positive samples was higher than the approved limit of the Iranian National Standard. Our results are in agreement to previous studies show that contamination of pistachios in retail shops in Kerman to aflatoxins and can adversely affect the consumer's health. Another species of importance that was isolated from pistachio samples was *A.ochraceus*, which can produce ochratoxin A which needs the attention of consumers.

In this study, walnut samples were less contaminated with *Aspergillus* section *Flavi* species than pistachio and hazelnuts. *A. flavus* and *A. tamarii* were isolated from walnut samples. Research has been done on the determination of aflatoxins in walnut, and some authors have reported the occurrence of aflatoxin in walnut (Fani et al. 2013; Shakeri, et al. 2019). Rezaei et al. (2014) studied aflatoxin contamination in nuts, including walnut samples, and reported that samples had aflatoxin contamination but met the legal limits of the National Standard of Iran and were not at a hazardous level. Taghizadeh et al. (2020), evaluated walnut samples for aflatoxin B_1 and observed that Aflatoxin B1 was found in more than half of the samples; However their results showed that is no carcinogenic risk for Iranians posed by walnut consumption. On the other hand, some researchers have reported high levels of aflatoxin in walnut samples. Imani Nejad and Farahani (2012) detected

aflatoxin in 35 samples of raw walnuts from Iranian supermarkets in Tehran and reported that in total seven (20.0%) samples were above the limit set by Codex Alimentarius (2001). Ostadrahimi et al .2014 walnut samples were contaminated to aflatoxin, which was more than the maximum tolerated level in Iran. According to our results in this study, due to low contamination of the walnuts to *Aspergillus* section *Flavi*, the risk posed by walnut consumption was not hazardous. Another *Aspergillus* species of importance that we isolated from walnuts was *A. quadrilineatus*. This species is reported to be the causative agent of Invasive aspergillosis in immunodeficiencies and cancer patients (Verweij et al. 2008). The clinical significance of the presence of this species on walnuts requires further investigations.

Contamination of nuts to *Aspergillus* spp. can occur at any stage before marketing, *i.e*. production, drying, processing, storage, and transportation (Norlia et al. 2019). Fluctuations in climate can affect the extent of contamination to *Aspergillus* species, alternate their community structure, and influence the predisposition of hosts to contamination via altering crop development (Cotty & Jaime-Garcia 2007). It has been shown that storage duration in warm, humid conditions affects fungal growth and toxin-production in nuts. Prolonged storage significantly increases the rate of contamination by *Aspergillus* species and potentially toxin-producing isolates (Saleemullah et al. 2006). The production of inhibiting compounds by other fungal or bacterial species might be influencing the contamination of kernels to *Aspergillus* species as well. Palumbo et al. (2006) showed that some bacteria isolated from almonds had antifungal activity against aflatoxigenic *A. flavus* via production of diffusible metabolites.

CONCLUSION

In conclusion, this study reports the diversity of *Aspergillus* spp. in nuts marketed in Kerman County, Iran. The results demonstrated that nuts are frequently contaminated with *Aspergillus* species with the potential to produce mycotoxins and should probably be regularly monitored and specific safety control actions be taken. The data obtained in this study, combined with the information available in the literature, suggests that the *Aspergillus* species isolated from the nuts impose a potential health hazard for the consumer. The species-level identification of the commonly occurring *Aspergillus* species in nut kernels is a key starting point for subsequent choosing of protective technologies and further investigations.

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جداسازی و شناسایی گونه‌های Aspergillus از پسته، گردو و فندق جمع آوری شده از خرده فروشی‌ها در کرمان، ایران

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چکیده: مغزیجات در ایران از اهمیت خاصی برخوردار هستند و به دلیل خواص تغذیه‌ای توسط بسیاری از مردم مصرف می‌شوند. قارچ‌های جنس آسپرجیلوس در طی مراحل پیش از برداشت تا پس از برداشت می‌توانند این محصولات را آلوده کنند. گونه‌های آسپرجیلوس بسیاری از محصولات کشاورزی را به صورت ثانویه آلوده کرده و قادر به تولید انواع مایکوتوکسین‌های مضر برای انسان و دام هستند. در این پژوهش آلودگی قارچی مغزیجات موجود در فروشگاه‌های محلی در سطح استان کرمان ارزیابی شد. نمونه‌های پسته، گردو و فندق از سرتاسر استان کرمان جمع‌آوری شد و آلودگی آن‌ها به گونه‌های آسپرجیلوس بررسی گردید. گونه‌های این جنس با استفاده از روش‌های ریخت‌شناختی و داده‌های مولکولی شناسایی شدند. ارتباط فیلوژنتیک بین گونه‌ها با استفاده از توالی‌های بتاتوبولین و کالمودولین تعیین شد. گونه‌هایی که شناسایی شد عبارتند از *A. flavus* ،*A. parasiticus* ،*A. arachidicola* ،*A. tamarii* ،*A. caelatus* *A. westerdijkiae* ،*A. nomius* ،*A. leporis* ،*A. quadrilineatus* ،*A. unguis* ،*A. spelunceus* ،*A.ochraceus* ،*A. auricomus* ،*A. taichungensis* ،*A. subalbidus* ،*A. pseudoglaucus* ،*A. montevidensis* جمعیت گونه‌های آسپرجیلوس خصوصاً گونه‌های با پتانسیل تولید مایکوتوکسین روی مغزیجات و پراکنش آن‌ها روی انواع مختلف مغز در این پژوهش مورد بحث قرار گرفته است.

کلمات کلیدی: مایکوتوکسین، خرده فروشی، کالمودولین، فیلوژنی، توالی‌یابی

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