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A new *Xylaria* species from Iran

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Abstract: Three species of the genus *Xylaria* (Xylariaceae) are presented based on collections from Guilan Province, Iran. Both morphological and molecular characteristics were used in order to identify these species. *Xylaria longissima* sp. nov. is described and illustrated as a new *Xylaria* species, and *X.* cf. *striata* and *X. xylarioides* are reported as new records fro Iranian mycobiota. *Xylaria longissima* and *X. xylarioides* were collected from wood of unknown dicotyledonous plants and *X.* cf. *striata* was collected from soil. Differences between these species and other closely related taxa are discussed. The result of this study indicates that sequences of internal transcribed spacer (ITS) region have sufficient resolution to distinguish between major species groups.

Key words: Ascomycetes, Xylariaceae, taxonomy, Guilan, Iran.

INTRODUCTION

Species of the genus *Xylaria* Hill ex Schrank usually grow on the rotten wood, but can be found in soil or on various substrates such as fallen leaves, petioles, herbaceous stems, dung, grasses, seeds or fruits and wood (Rogers 1986). The genus has been studied in various parts of the world such as America, Europe, Africa and Asia (Ellis & Everhart 1887a,b, Dennis 1956, 1957, 1958, 1961, 1964, Martin 1970, Ju & Tzean 1985, Rogers 1986, Rogers & Samuels 1986, Rogers et al. 1988, San Martin Gonzalez & Rogers 1989, Ju & Rogers 1999, San Martín et al. 2001, Rogers et al. 2008, Kshirsagar et al. 2009, Ma et al. 2011, 2012, 2013, Ju et al. 2012, Rogers & Ju 2012, Whalley et al. 2012), but there are only a few reports on *Xylaria* species from Iran (Soleimani 1976, Riedl & Ershad 1977, Daneshpazhuh 1980, Arefipour et al. 2004, Zare & Morid 2006, Zare & Asef 2008, Ershad 2009, Hashemi et al. 2013, 2014). More *Xylaria* species are reported from Guilan province of Iran in this paper.

MATERIALS AND METHODS

Isolates, media and morphological observation

Fungal materials were collected from Guilan Province, Iran. Cultures were obtained from ascospores as previously explained by Ju and Rogers (1996) and Ho and Ko (1997). Colony characteristics were recorded on fresh oatmeal agar (OA) [30 g rolled oats, 20 g agar and 1000 ml distilled water] (Stevens 1981) at 20 ± 0.5°C, 12/12 h light/dark regime. Growth rates were measured in duplicates after 10 days. Observations and measurements were made from specimens examined in distilled water for ascospores, in 10% Lactic acid for asci, and in Melzer's reagent for ascus apical rings. At least 30, 10 and 5 measurements were determined for ascospores, asci and ascus apical rings, respectively. A VANOX AHBS3 Olympus light-microscope was used to examine fungal structures and a M1000 Leica light-microscope equipped with a Canon EOS 600D digital camera was applied for photography. Fungal species were determined morphologically according to relevant literature (Dennis 1956, 1957, 1958, 1961, Rogers 1979, 1986, Rogers & Samuels 1986, Rogers et al. 1988, San Martin Gonzalez & Rogers 1989, Rogers & Ju 1998, Ju & Rogers 1999, Rogers et al. 2008, Hladki & Romero 2010, Rogers & Ju 2012). Specimens were deposited in the Fungal Reference Collection of the Ministry of Jihad-e Agriculture (IRAN) located at Iranian Research Institute of Plant Protection, Tehran and the fungal collection of the Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Iran (GUM).

Molecular studies

Total DNA was extracted from the scooped content of perithecia according to Chelex method (Walsh et al. 1991). Internal Transcribed Spacer (ITS) region was amplified using ITS5 [5′–gga agt aaa agt cgt aac aag g] and ITS4 [5′–tcc tcc gct tat tga tat gc] primers for the first PCR, and ITS1 [5′–tcc gta ggt gaa cct gcg g] and ITS4 for semi-nested PCR (White et al. 1990). The PCR reaction mixture (25 µl) contained 1X PCR buffer, 2 mM $MgCl_2$, 5 pmol of each primer, 0.2 mM dNTPs (Bioflux biotech), 0.75 U *Taq* DNA polymerase (Bioflux biotech) and 5 µl of template DNA for the first PCR or 0.5 µl of the first PCR

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product for semi-nested PCR. The PCR amplification conditions were as follows: initial denaturation of 90 sec at 95°C followed by 35 cycles of 95°C/30 sec, 52°C/ 30 sec, 72°C/30 sec and a final extension of 6.5 min at 72°C. PCR amplification was carried out using a thermal cycler MyGenie® (Bioneer, South Korea). The PCR products were electrophoresed in 1% agarose gel containing 1 µl CinnaGen DNA safe stain (CinnaGen, Tehran)/30 ml agarose gel and visualized by a gel documentation tool (UVITEC®, Cambridge). Amplified products were purified and sequenced by an ABI 3730XL capillary DNA sequencer (Sequetech, California).

Phylogenetic analysis

Sequence files were evaluated by Chromas 2.4 (Technelysium Pty Ltd., South Brisbane, Australia). Alignments were initially obtained using the Pairwise Alignment option in GeneDoc 2.7.000 (Nicholas et al. 1997). Phylogeny was inferred using Neighbor-Joining (p-distance) method with Pairwise Deletion option and the bootstrap test by 1000 replicates (Felsenstein 1985, Saitou & Nei 1987, Nei & Kumar 2000). Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). Relevant sequences representing nearly related taxa to our isolates were obtained from GenBank using a Blast search and incorporated to the analyses (Table 1). *Hypoxylon fragiforme* (JN979420) was used as outgroup.

RESULTS AND DISCUSSION

Three *Xylaria* species, *X. longissima* sp. nov., *X.* cf. *striata* and *X. xylarioides* are identified and illustrated. *Xylaria* cf. *striata* and *X. xylarioides* are reported as new records to Iranian mycobiota.

Xylaria longissima Hashemi, Khodaparast, Zare & Elahinia, *sp. nov.* — MycoBank MB 810915; Fig. 1

Etymology– Referring to long acute sterile apices of stromata.

Stromata caespitose, 55–71 mm long, fertile portion 6–8 × 1–1.5 mm diam., with very long (30–45 mm long) setiform and branched sterile apices; stipe pubescent, 17–20 × 1 mm diam.; stromatal surface

Table 1: Isolates and ITS sequences of *Xylaria* species used in this study. Underlined sequences are newly generated.

Taxon	Substrate/Origin	Herb./ Culture accession no.	GenBank accession no.
X. longissimi	wood of unknown plant/Iran	IRAN 16581 F	KP218905
X. longissimi	wood of unknown plant/Iran	IRAN 16582 F	KP218906
X. striata	soil/Iran	GUM 1150	KP218908
X. xylarioides	wood of unknown plant/Iran	GUM 1151	KP218909
X. filiformis	herbaceous stem of unknown plant/Iran	GUM 1052	KP218907
X. acuta	-/USA	ATCC 56487	AF163026
X. adscendense	wood/ French West Indies	570 (HAST, JF)	GU300101
X. apiculata	-/Colombia	CBS 365.81	AF163027
X. arbuscula	bark/Taiwan	89041211 (HAST)	GU300090
X. arbuscula var. *plenofissura*	wood/Taiwan	93082814 (HAST)	GU339495
X. bambusicola	Bamboo culm/Thailand	162 (JDR)	GU300088
X. bambusicola	*Bambusa oldhamii* culm/Taiwan	205 (WSP HOLOTYPE)	EF026123
X. bambusicola	Bamboo culm/China	HMJAU 22227	JX256820
X. cornu-damae	-/Canada	CBS 724.69	AF163031
X. digitata	wood/Ukraine	919 (HAST)	GU322456
X. digitata	-/-	CBS 161.22	AY909006
X. fioriana	-/South Africa	CBS 486.61	AF163034
X. fusispora	trunk/China	HMJAU 23625	JX256825
X. grammica	wood/Taiwan	479 (HAST)	GU300097
X. grammica	-/Thailand	ST2363	DQ322146
X. hypoxylon	wood/-	152 (HAST)	GU300096
X. hypoxylon	*Sorbus aucuparia*/Sweden (ex-epitype)	CBS122620	AM993141
X. ianthino-velutina	fruit of *Swietenia macrophylla*/Martinique	553 (HAST, JF)	GU322441
X. ianthino-velutina	-/Thailand	SUT123	DQ322147
X. juruensis	-/Taiwan	92042501 (HAST)	GU322439
X. leavis	wood/Martinique	419 (HAST, JF)	GU324746
X. leavis	bark/Taiwan	95072910 (HAST)	GU324747
X. liquidambaris[†]	*Liquidambar styraciflua*/Georgia	ATCC 42766	AY909021
X. longipes	*Fagus sylvatica*/Germany	CBS 148.73	AY909013
X. longipes	-/Netherlands	CBS 148.73 (KCTC 6575)	AF163038
X. mali	-/-	CBS 385.35	AF163040
X. multiplex	-/Thailand	ST2298	DQ322155
X. multiplex	wood of *Hibiscus tiliaceus*/USA	259 (JDR)	GU300099
X. oxyacanthae	fallen seeds/USA	859 (JDR)	GU322434
X. oxyacanthae	fruit of *Crataegus monogyna*/Germany	2010-502 (LZ)	HQ414587
X. polymorpha	wood/USA	JDR1012	GU322460
X. polymorpha	stump of *Fagus sylvatica*/Germany	M:M-0125909	FM164944
X. schweinitzii	bark/Taiwan	92092023 (HAST)	GU322463
X. schweinitzii	-/-	ST2349	DQ322161
X. scruposa	dead wood/Martinique	497 (HAST, JF	GU322458
X. striata	branch/China	304 (HAST)	GU300089
X. venosula	twigs/USA	94080508 (HAST)	EF026149
X. venustula	bark/Taiwan	88113002 (HAST)	GU300091
Hypoxylon fragiforme	bark/France	YMJ 383 (HAST)	JN979420

[†]This sequence appears in GenBank as *X. persicaria* but it has been renamed as *X. liquidambar* (*=X. liquidambaris* in Index Fungorum and Mycobank) by Rogers *et al*. (2000).

roughened from evidently to slightly perithecial elevations, with light brown peeling outer layer splitting in longitudinal bands; ostioles slightly papillate. Asci cylindrical, 8-spored, 165–180 × 6–7 µm, the spore bearing part 90–100 µm, with apical ring bluing in Melzer's iodine reagent, rectangular, (3–)3.5–4(–4.5) × 2.5 µm; ascospores (15–)16–18(–20) × 5–6 µm, inequilateral to navicular, with straight spore-length germ-slit.

Cultural characteristics — Colony growth on OA was 32 ± 6.9, 53.2 ± 10.1 and 76.8±9.1 mm after 10, 16 and 25 days at 20 ± 0.5°C and 12/12 h light/dark regime, respectively. Colony was at first white with gray center, and then turned to vinaceous buff with dark concentric rings which produced dark, branched and setiform stromata with white tip (Fig. 1H–I).

Fig. 1. *Xylaria longissima* holotype (IRAN 16581 F). **a.** Stromata on wood in natural conditions, **b.** Stromata with very long, setiform and branched sterile apices, **c.** Close-up of surface of stromata with brown peeling outer layer, **d.** Pubescent stipe, **e.** Ascospores and rectangular ascus apical ring bluing in Melzer's reagent, **f.** Close-up of ascospores with straight spore-length to nearly spore-length germ-slit, **g.** Colony on OA after 21 days at 20±0.5°C in 12/12 h D/L regime, **h.** Close-up of stromata produced on OA. Scale bar: E, F= 10 µm.

Specimens examined. IRAN, Guilan Province, Rasht, Saravan Forest Park, on the wood of unknown dicotyledonous plant, 30 Nov. 2012, *S.A. Hashemi*, holotype Herb. IRAN 16581 F, culture ex-type IRAN 2268 C; Guilan Province, Shaft, Dorud Khan, on the wood of unknown dicotyledonous plant, 4 Oct. 2012, *S.A. Hashemi*, Herb. IRAN 16582 F, culture ex-type IRAN 2269 C).

Notes — Phylogenetic analysis showed that *X. longissima* formed a distinct lineage (Fig. 4). Both morphology and rDNA sequences confirmed that our material represents a new species in *Xylaria*. *Xylaria longissima* is characterized by very long setiform sterile apices, more or less conspicuous perithecia, light brown peeling outer layer and 16–18 × 5–6 µm ascospores with straight spore-length germ-slit. Stromata were more robust (2 mm diam.) and longer (98 mm long), with longer (63 mm long) and unbranched sterile setiform apices in the second specimen collected from Shaft (IRAN 16582 F). However, it generally fits *X. longissima*. Comparison of its ITS sequence also shows 100% homology with the holotype of *X. longissima* (Fig. 4).

Morphologically, *X. longissima* is somewhat close to *X. filiformis*, *X. theissenii* var. *macrospora*, *X. juruensis* and *X. arbuscula*. *Xylaria filiformis* has individual naked perithecia dispread along the filiform stromata without peeling outer layer, wider ascospores 14–18 × 8–8.5 µm, and occurs on leaves and herbaceous debris (Ellis & Everhart 1887a, Rogers & Samuels 1986). *Xylaria theissenii* var. *macrospora* possesses very long (34–40 µm) ascospores (Rogers & Samuels 1986). *Xylaria juruensis* has naked individual perithecia dispread along the stromata, more or less pubescent stromata and 12–17 × 4–5 µm ascospores (Hennings 1904). *X. arbuscula* differs from *X. longissima* mainly based on its completely immersed perithecia, lack of very long acute sterile apices, inverted hat shape ascus apical ring and length of germ-slit (San Martin Gonzalez & Rogers 1989). Even though no sequences of the *X. filiformis* and *X. theissenii* are deposited at GenBank, *X. longissima* isolates formed a distinct lineage from our previously reported *X. filiformis* (Hashemi et al. 2014). Moreover, the ITS sequence of *X. longissima* differs from *Xylaria juruensis* and *X. arbuscula* sequences (Fig. 4).

Based on a BLAST search using ITS sequences, the closest taxa to *X. longissima* were *X. bambusicola* (KF381074, JX256820, JX256819 and JX256818) and *X. grammica* (HM752504) with 95% homology. Among them, *X. bambusicola* (KF381074) and *X. grammica* (HM752504) specimens have been reported as endophytes. *Xylaria longissima* differs from *X. bambusicola* mainly in its host plant, surface and apices of stromata and size of ascospore. *Xylaria bambusicola* which occurs on monocotyledonous material, has completely immersed perithecia, short acute sterile apices, with 9.5–11(–12.5) × 4–5 µm ascospore, and occasionally a tiny hyaline cellular appendage on one end (Ju & Rogers 1999). *Xylaria longissima* differs from *X. grammica* described by San Martin & Rogers (1989) mainly in ascospore size, diameter and tip of stromata and the color of peeling outer layer. *Xylaria grammica* has stromata with 5–8 mm diameter, rounded and fertile or acute and sterile apices, with whitish areas that appears as vertical strips, and (9.5–)10–12(–12.5) × (4.5–)5–5.5 µm ascospore.

Xylaria* cf. *striata Pat., Journ. Bot. (Morot) 1: 247. 1887. Fig. 2

Stromata gregarious on soil, several arising from common base, 44–120 mm long, fertile portion cylindrical, 9–18(–22) × (2–)2.5–3(–4) mm diam., with acute sterile apices; stipe root-like, 70–100 mm long, stromatal surface more or less roughened from whitish to pale peeling outer layer and minute wrinkles; perithecia almost immersed, 0.38–0.56 mm diam.; ostioles papillate to slightly papillate. Asci degenerated and not seen; apical ring bluing in Melzer's iodine reagent, rectangular, 3–3.5(–4) × 2(–3) µm; ascospores (13–)15–19(–22.5) × 5–7(–9) µm, fusiform to ellipsoid, often apiculate at one end when produced on stromata under natural conditions (Fig. 2E) but not apiculate when produced on stromata under laboratory conditions (Fig. 2F), with straight about 1/3 of spore-length.

Cultural characteristics — Colony growth on fresh OA 12±3.2 mm after 10 days at 20±0.5 °C in 12 h dark/12 h fluorescent light regime. Colonies covering 9 cm diam. Petri plates in 6–7 wk, at first white then becoming blackish brown at center, margins regular, zonate, with blackish brown furrows with dark and unbranched mature stromata with buff tip. Reverse side with dark furrows and zones. Anamorph not produced (Fig. 2H–I).

Specimen examined. IRAN, Guilan Province, Lahijan, Koshal village, on soil, 7 Feb. 2014, *S.A. Hashemi*, Herb. GUM 1150.

Notes — Morphology of Iranian specimen fits *X. striata* except for the shape of ascospore. Ascospores of *X. striata* are reported 16–18(–19) × 4.5–5 µm (San Martín et al. 1999) or 15–20 × 5–8 µm (Saccardo 1891, Lloyd 1917). Dennis (1957) described ascospores of *X. deserticola* (a species that San Martín *et al.* (1999) believed to be synonymous with *X. striata*) as 16–18 × 5–7 µm. Ascospores of *X. striata* have always been described as lacking apiculate ends, while in this study spores of the Iranian specimen are often apiculate at one or rarely two ends when produced on stromata under natural conditions (Fig. 2E). They are however not apiculated when produced on stromata in culture (Fig. 2F). Colony of Iranian material differs from San Martin et al. (1999) description by slower growth rate, blackish brown furrows and dark furrows and zones at the reverse side (Fig. 2H–I). Phylogenetic analysis of ITS sequences showed that our isolate was close to *X. striata* (GU300089) with high bootstrap support value (Fig. 4). Although the deeply buried stipe in the soil

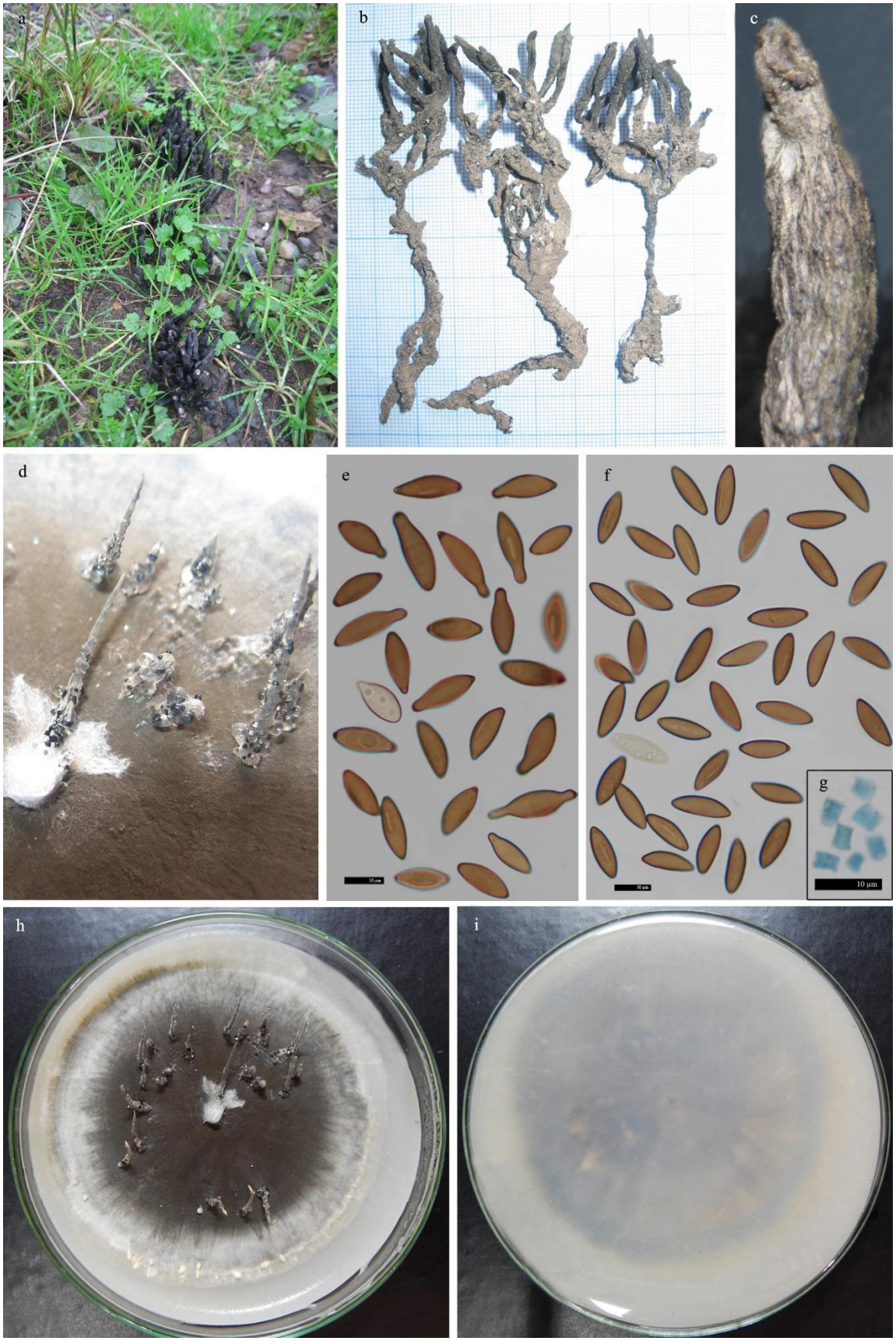

Fig. 2. *Xylaria* cf. *striata*. **a.** Gregarious stromata on soil, **b.** Stromata with long rooting base, **c.** Close-up of stromatal surface with whitish to buff color peeling outer layer, **d.** Stromata produced on OA with drops of ascospores logged from mature perithecia, **e.** Mostly papillate ascospores produced on stromata from natural conditions, **f.** Not apiculated ascospores produced on stromata in OA, **g.** Rectangular apical ring bluing in Melzer's iodine reagent, **h.** and **i.** Front and reverse sides of colony on OA after 44 days at 20±0.5°C in 12/12 h D/L regime, respectively. Scale bars = 10 µm.

suggests that the stromata comes from the immersed plant material (San Martín *et al.* 1999), we did not find any plant material at least in 20 cm depth of the soil at sampling site. *Xylaria* cf. *striata* is reported as a new record from Iran which is also the first Iranian *Xylaria* species reported on soil.

Xylaria xylarioides (Speg.) Hladki & A.I. Romero, Fungal Diversity 42: 80. 2010. Fig. 3

Stromata solitary or in small groups, 2–8.3 × (0.7–)1.1–1.5(–1.8) mm, fertile region cylindrical, subglobose to conical, 1.4–2.9(–3.2) × (0.7–)1.1–1.5(–1.8) mm, with acute sterile apices, stromatal surface, dark, roughened with perithecial contours, with brown peeling outer layer. Stipe short (0.1–)0.4–1.7(–5.4) ×0.2–0.7 mm. Perithecia 0.56–0.67 mm diam. Ostioles inconspicuous to minutely papillate. Asci 8-spored, 173–183(–203) × 7.5 µm, the spore-bearing part 117–127 µm, with apical ring bluing in Melzer's iodine reagent, rectangular, (5–)6 × (2.5–)2.8–3 µm; ascospores (16.5–)18–21(–21.5) × 6.5–8(–9.5) µm, brown, ellipsoid inequilateral, with straight nearly spore-length germ-slit.

Specimen examined. IRAN, Bibi Yanlo village, Astara, Guilan Province, on wood of unknown dicotyledonous plant, 10 July 2012, *S.A. Hashemi* (Herb. GUM 1151).

Notes — *X. xylarioides* has been recently segregated from *X. apiculata* (Hladki & Romero 2010). Based on BLAST search and the phylogenetic tree inferred from ITS sequences (Fig. 4), the closest sequence to our fungus is *X. apiculata* (AF163027), however, the authors used this name in wide sense without description in their paper (Lee et al. 2000), because *X. xylarioides* has been accepted and described later (Hladki & Romero 2010). Hence we do not clearly know this sequence belongs to which organism, i.e *X. apiculata* sensu stricto or *X. xylarioides* sensu Hladki & Romero. *Xylaria xylarioides* differs from *X. apiculata* mainly in stromatal surface, ascospore size and shape. *Xylaria apiculata* has smooth stromatal surface with completely immersed perithecia, larger ascospores, (16.0–)20–24.5(–30) × (5.0–)6.7–8.0(–9.0) µm with occasionally an inconspicuous cellular appendage on one end (Rogers & Samuels 1986) while *X. xylarioides* has roughened stromatal surface with conspicuous perithecial outlines, 17–21 × 6.5–9 µm and not appendiculate ascospores (Hladki & Romero 2010). This is the first report of this species from Iran.

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Fig. 3. *Xylaria xylarioides*. **a.** Stromata on wood, **b**. and **c.** Close-up of stromatal surface with perithecial counters and with short to very short stipe, **d.** Ascospores, **e.** Ascus apical ring bluing in Melzer's iodine reagent. Scale bars: D and E = 10 µm.

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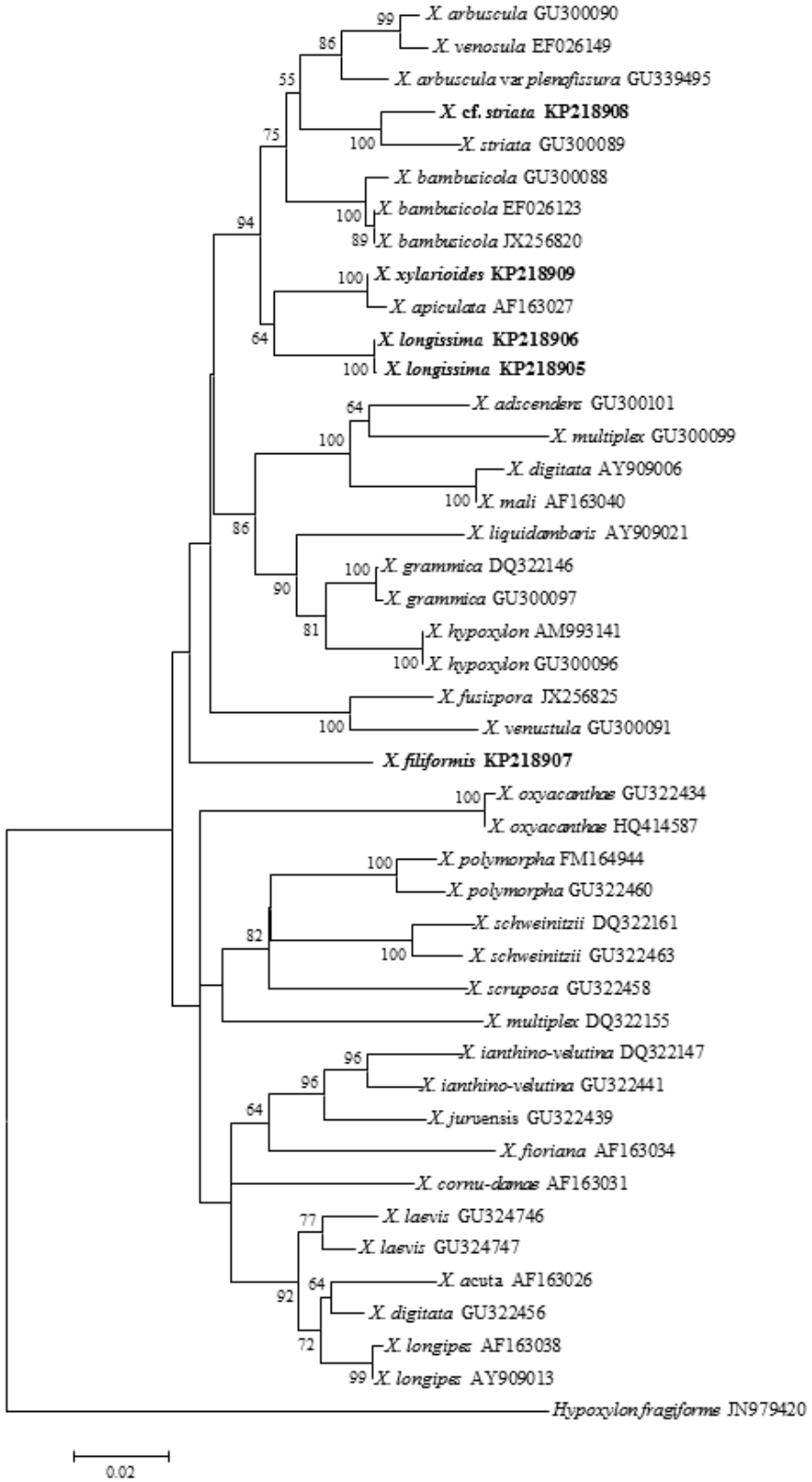

Fig. 4. Bootstrap consensus phylogenetic tree of *Xylaria* inferred from ITS (ITS1–5.8S–ITS2) sequences using Neighbor-Joining method in MEGA5 with 1000 bootstrap replications. The tree was rooted to *Hypoxylon fragiforme*. The bootstrap values (>50%) are shown above branches. Accession numbers of type strains are underlined.

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معرفی گونه ای جدید از جنس *Xylaria* از ایران

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چکیده: در این مطالعه بر اساس نمونه های جمع آوری شده از استان گیلان و بررسی ویژگی های ریخت شناسی و مولکولی، سه گونه از جنس *Xylaria* (Xylariaceae) معرفی شده است. *X. longissima* sp. nov. به عنوان یک گونه جدید برای جنس *Xylaria* و *X.* cf. *striata* و *X. xylarioides* به عنوان آرایه های جدید برای مایکوبیوتای ایران گزارش می شوند. *X. longissima* و *X. xylarioides* از چوب میزبان نامشخص دولپه ای و *X.* cf. *striata* از خاک جمع آوری شده اند. تفاوت این سه گونه با آرایه های نزدیک به آنها نیز مورد بحث قرار گرفته است.

کلمات کلیدی: Ascomycetes, Xylariaceae، تاکسونومی، گیلان، ایران

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