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ORIGINAL PAPER

Morpho-molecular study of entomopathogenic fungi associated with citrus orchard pests in Northern Iran

Alireza Armand[2,3] · Seyed Akbar Khodaparast[1] · Saeed Nazari[1] · Arash Zibaee[1]

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Abstract

Entomopathogenic fungi play a significant role in regulating insect populations in nature and have potential applications in pest management strategies in different regions. *Citrus* spp. are among the important horticultural products in northern Iran, and the orchards are affected by different insect pests, especially mealybugs. This study aimed to isolate and identify entomopathogenic fungi associated with citrus orchard pests in northern Iran, focusing on *Akanthomyces* and *Lecanicillium* species on mealybugs. Through the samples collected from different regions within Guilan province, 12 fungal isolates were collected and identified based on the combination of morphological characteristics and molecular data. *Akanthomyces lecanii*, *A. muscarius*, *Engyodontium rectidentatum*, *Lecanicillium aphanocladii* and *Lecanicillium rasoulzarei* sp. nov. were identified. Of these, *A. muscarius* on *Lepidosaphes* sp., *E. rectidentatum* on Coccidae, and *L. aphanocladii* on *Tetranychus urticae* are reported as new fungal-host records from Iran. Moreover, a new species, *Lecanicillium rasoulzarei*, is illustrated, described, and compared with closely related species.

Keywords Biocontrol · Insect pathogens · Diversity · Mealybugs · Phylogeny · Taxonomy

Introduction

Iran ranks as the sixth most significant producer of citrus globally, boasting a production yield of 4.1 million tons in the year 2016. The cultivation landscape encompasses a total expanse of 276,000 hectares dedicated to various citrus species within Iranian agricultural domains (Valedsaravi et al. 2021). Presently, the northern region stands out as the foremost hub for citrus production in Iran, marking a transformative evolution in the citrus industry (Alipour et al. 2013).

Insect pests represent a significant menace to global food security, inflicting substantial economic losses amounting to 18% of the world's agricultural production (Savary et al. 2012). Citrus trees face a barrage of pests including mealybugs, scales, aphids, spider mites, leaf miners, thrips, and whiteflies (Mahmood et al. 2014; Nath and Sikha 2019), resulting in diminished citrus yields both in terms of quantity and quality on a worldwide scale. Nonetheless, the citrus mealybug, scientifically known as *Planococcus citri* Risso (Hemiptera: Pseudococcidae), stands out as the most pernicious threat to *Citrus* spp., inflicting both direct and indirect damages upon citrus orchards (Demirci et al. 2011; Martelli 2014).

Citrus agroecosystem has been noticed as a rich environment containing natural enemies (Urbaneja et al. 2015). The long-term application of insecticides, particularly non-selective pesticides, has had detrimental effects on the natural enemies of pests, resulting in their resurgence and the subsequent outbreak of pests (Pourian et al. 2019). Indeed, entomopathogenic fungi have garnered significant attention in global research due to their extensive biological activity and their potential functions within agroecosystems (Wood et al. 2015; Nicoletti and Becchimanzi 2020). Among entomopathogenic fungi, hypocrealean fungi are prevalent

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✉ Seyed Akbar Khodaparast
blumeria2015@gmail.com

[1] Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

[2] Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand

[3] School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand

entomopathogens renowned for their efficacy against a wide array of pest species (Hajek and Delalibera 2010).

Molecular investigations carried out by Zare et al. (2000) and Sung et al. (2001) have elucidated the existence of a distinct cluster within *Verticillium* sec. *Prostrata*. Subsequently, Gams and Zare (2001) proposed the establishment of the genus *Lecanicillium* to accommodate clavicipitaceous species resembling *Verticillium* and previously categorized within the section *Prostrata*. This reclassification was based on a combination of morphological characteristics and the analysis of combined internal transcribed spacer (ITS), small subunit rDNA (SSU), and large subunit rDNA (LSU) sequence data. Phylogenetic revisions of the section *Prostrata* were conducted by Zare and Gams (2001, 2008), leading to the recognition of various *Lecanicillium* species. *Lecanicillium* has been placed in the *Cordycipitaceae* along with *Akanthomyces* Lebert., *Beauveria* Vuill. (Imoulan et al. 2017; Khonsanit et al. 2020), *Engyodontium* de Hoog., *Gibellula* Cavara., *Isaria* Pers., *Microhilum* H.Y. Yip & A.C. Rath., *Parengyodontium*, and *Simplicillium* W. Gams & Zare. (Sung et al. 2007; Johnson et al. 2009; Vega et al. 2012; Tsang et al. 2016; Wijayawardene et al. 2022). The utilization of multi-gene phylogenetic analysis method has increased over the last decade, leading to improvements in the taxonomy and nomenclature systems of genera and species in the Cordycipitaceae family (Bischoff et al. 2009; Imoulan et al. 2017; Kepler et al. 2017; Mitina et al. 2017; Chen et al. 2018; Mongkolsamrit et al. 2018; Zhou et al. 2018; Bustamante et al. 2019; Cabaleiro et al. 2019; Khonsanit et al. 2020; Wang et al. 2020; Zhang et al. 2021; Rizal et al. 2024). Following a comprehensive reassessment of the Cordycipitaceae by Kepler et al. (2017), numerous taxa, notably *L. lecanii*, the prototypical representative of *Lecanicillium*, underwent reclassification into the genus *Akanthomyces*. Despite this taxonomic revision, the appellation *Lecanicillium* persists in scholarly discourse, and recent taxonomic investigations have unveiled several novel species (Crous et al. 2018; Huang et al. 2018; Su et al. 2019; Zhou et al. 2018, 2022; Chen et al. 2020b).

The genus *Akanthomyces* is primarily taken into account as a pathogen for lepidopteran insects (Aini et al. 2020). Some species of the genus that are Torrubiella-like can infect spiders (e.g., *A. novoguineensis*). Moreover, *Akanthomyces* was listed as an asexual state for some species of *Cordyceps* (e.g., *C. tuberculata* and *C. confragosa*), which are characterised as pathogens of scale insects and have a wide host range (Sung et al. 2007; Kepler et al. 2017; Wijayawardene et al. 2017).

Mitina et al. (2017) isolated and identified *Lecanicillium muscarium, L. longisporum, L. psalliotae*, and *L. pissodis*, predominantly on the insects from the order Hemiptera. *Lecanicillium psalliotae* has been reported from India, infecting cardamom thrips (*Sciothrips cardamomi*) (Senthil Kumar et al. 2015). *Akanthomyces attenuatus* (=*Lecanicillium attenuatum*) has been isolated from pea aphid (*Acyrthosiphon pisum*) in China (Wang et al. 2017). Whereas, Du et al. (2019) showed its virulence against *Megalurothrips usitatus* (Thysanoptera: Thripidae). Broumandnia et al. (2021) identified *Akanthomyces lecanii* (=*Lecanicillium lecanii*) and *A. muscarius* (=*Lecanicillium muscarium*) from *Bemisia tabaci* populations in northern Iran, highlighting the scarcity of information regarding indigenous Iranian isolates. Furthermore, *Akanthomyces muscarius* (Naeim Amini et al. 2010), *Akanthomyces lecanii* (Naeim Amini et al. 2010), *Metarhizium anisopliae* (Pereira et al. 2011; Chartier FitzGerald et al. 2016), *Isaria farinosa* (Demirci et al. 2011), and *Beauveria bassiana* (Amnuaykanjanasin et al. 2013; Chartier FitzGerald et al. 2016) were reported as effective fungal species against mealybugs. Additionally, Ghaffari et al. (2017) showed the efficiency of *Akanthomyces lecanii* and *A. dipterigenus* (=*Lecanicillium longisporum*) against *Planococcus citri* (Risso).

Despite its paramount significance, the isolation and identification of entomopathogenic fungi that infect pests in citrus orchards, have been inadequately investigated in northern Iran (Naeim Amini et al. 2010; Karimi and Kamali 2021). Moreover, the efficacy of a regionally developed fungal biopesticide may vary in different countries and locations due to differences in fungal races, environmental conditions, and ecological characteristics (Lockwood 1993; Goble et al. 2010). Therefore, it is crucial to isolate and identify indigenous entomogenous fungi to enhance understanding of the natural biodiversity in specific areas and to establish a valuable source of biological control agents for future pest management purposes (Quesada-Moraga 2007; Jacas and Urbaneja 2010; Dreistadt 2012; Bouvet et al. 2019).

Materials and methods

Sample collection

Arthropod cadavers infected by fungi were collected from the citrus orchards in different municipal places of Guilan province, Iran from October 2018 to October 2019 (Fig. 1). Samples were transferred to the laboratory, and the arthropod hosts were identified at species, genus or family level.

Fungal isolation and morphological identification

The collected cadavers were examined under a stereomicroscope and cadavers showing signs of infection, were

Fig. 1 Different pests associated with the leaves of *Citrus sinensis*, infested by entomopathogenic fungi

directly taken with a fine sterile needle and plated onto potato dextrose agar (PDA; Merck) culture medium. In the cases of mature mealybug cadavers, the cadavers were first surface disinfested in 1% sodium hypochlorite solution for 3 min, then rinsed three times with sterile distilled water and transferred again onto PDA culture medium (Quesada-Moraga et al. 2007). The petri plates were incubated at 25 ± 1 °C for 2–4 days for fungal growth. The growing fungal isolates were purified using the hyphal tip method (Vinit et al. 2018). Morphological attributes, encompassing conidial features (such as shape, size, and color), conidial arrangement on the phialides, phialide characteristics (including shape, size, and color), the quantity and arrangement of phialides in whorls, as well as the presence or absence of pigmentation and octahedral crystals, were meticulously observed and documented from cultures cultivated on the PDA medium, kept at 25 ± 1 °C for 10 days using a Leica DM1000 microscope, equipped with a Canon 600D camera. The permanent slides were prepared using distilled water and Congo Red reagent (1%) as a mountant, and the coverslips were fixed on the slides using transparent nail polish (Senanayake et al. 2020). Identification of the isolates was done according to Humber (2012), Zare and Gams (2001), and related publications (Gams et al. 1984; Samson et al. 1988; Luangsa-ard et al. 2007). Living cultures were deposited in the culture collection of the Iranian Ministry of Agriculture (Iranian Research Institute of Plant Protection, Tehran, Iran).

Table 1 Primers and their respective sequences used in this study

Primer	Sequence 5`→3`
ITS5	GGA AGT AAA AGT CGT AAC AAG G
ITS4	TCC TCC GCT TAT TGA TAT GC
EF1-983 F	GCY CCY GGH CAY CGT GAY TTY AT
EF1-2218R	ATG ACA CCR ACR GCR ACR GTY TG

DNA extraction and PCR

DNA extraction was performed using the HotSHOT protocol outlined by Montero-Pau et al. (2008). A small piece of fungal mycelium was harvested using an inoculating needle from a 10-day-old culture on the PDA medium and transferred into 1.5 mL tubes containing 100 µL of alkaline lysis buffer (0.2 mM disodium ethylene diamine tetraacetic acid, 25 mM NaOH, pH 8.0, Merck) and centrifuged for 30 min at 2000× g. Then, the tubes were incubated at 95 °C for 30 min and immediately cooled on ice for five min. Finally, 100 µL of Tris-HCl solution (Sigma-Aldrich, Vienna, Austria; 40 mM, pH 5.0) was added to the tubes, vortexed, and stored at -20 °C as a template for PCR. The ITS region and partial *EF1α* gene were amplified and sequenced using the primer pairs ITS5/ITS4 (White et al. 1990) and 983 F/2218R (Rehner and Buckley 2005), respectively. The sequences of primers are shown in Table 1.

The amplifications were performed in a total volume of 25 µL. PCR mixtures contained 12.5 µL of master mix (CinnaGen, Iran) (including 10×PCR buffer, MgCl2, dNTPs, Taq DNA Polymerase), 7.5 µL of double-distilled water, 1 µL of each primer, and 3 µL of DNA solution. PCR was run on an Eppendorf Thermal Cycler (Eppendorf Personal, Darmstadt, Germany) under the following conditions: an initial denaturation cycle at 94 ºC for 2 min, followed by ten cycles of 94 ºC for 30 s, 68 ºC for 30 s, and 72 ºC for 90 s; 35 cycles of 94 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 90 s; and a final extension at 72 ºC for 10 min, for *EF1α*. For the ITS region, an initial denaturation cycle of 2 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 52 °C, 30 s at 72 °C, and a final extension of 5 min at 72 °C. Positive amplicons were visualized on a 1% agarose gel under UV light using a Gel DocTM XR + Molecular Imager (BIO-RAD, USA). Finally, the sequencing was carried out by Royan Zistagen Company, Tehran, Iran.

Phylogenetic analyses

The obtained sequences were checked using BioEdit v.7.0.9.0 and were compared against the NCBI GenBank nucleotide database using BLASTn search. Reliable sequences were selected from GenBank according to BLASTn search results and previous studies (Kepler et al. 2017; Aini et al. 2020; Chen et al. 2022) (Table 2).

Table 2 The GenBank accession numbers of taxa used in the phylogenetic analyses

Species	Strain	GenBank accession numbers	
		ITS	EF1α
Akanthomyces aculeatus	HUA 186,145^{T}	–	MF416465
A. aculeatus	TS772	KC519371	KC519366
A. araneosus	KY11341^{T}	ON502826	ON525443
A. araneosus	KY11342	ON502844	ON525445
A. attenuatus	CBS 402.78^{T}	AJ292434	EF468782
A. coccidioperitheciata	NHJ 6709	JN049865	EU369025
A. dipterigenus	CBS 126.27^{T}	AJ292385	–
A. farinosa	CBS 541.81	AY624180	JQ425686
A. kanyawimiae	TBRC 7244^{T}	MF140752	MF140836
A. lecanii	CBS 101,247^{T}	JN049836	DQ522359
A. lecanii	IMI 304,807	AJ292382	–
A. lecanii	IMI 304,817	AJ292383	–
A. lecanii	**L828-2, IRAN-3703 C**	**OR304363**	**OR352910**
A. lecanii	**Le817-2, IRAN-3685 C**	**OR304364**	**–**
A. lecanii	**Le817-7, IRAN-3686 C**	**OR304365**	**OR352911**
A. lecanii	**Sh901-5, IRAN-3695 C**	**OR304366**	**–**
A. lecanii	**T901-2, IRAN-3699 C**	**OR304367**	**OR352912**
A. lepidopterorum	SD05151^{T}	MT705971	–
A. lepidopterorum	SD05152	MT705972	–
A. muscarius	CBS 143.62^{T}	NR_111096	KR064305
A. muscarius	IMI 179,173	AJ292387	–
A. muscarius	IMI 282,532	AJ292435	–
A. muscarius	**F901-1, IRAN-3700 C**	**OR327055**	**OR352913**
A. muscarius	**Gh1009-1, IRAN-3687 C**	**OR327056**	**OR352914**
A. muscarius	**O1103-1, IRAN_3688C**	**OR327057**	**OR352915**
A. muscarius	**R128-3, IRAN_3704C**	**OR327058**	**OR352916**
A. neoaraneogenus	GZUIFDX2^{T}	KU893153	MH978187
A. neoaraneogenus	GZUIFDX1	KU893152	–
A. neoaraneogenus	GZUIFSN1	MH978177	MH978188
A. neocoleopterorum	GY11241^{T}	MN093295	MN097813
A. neocoleopterorum	GY11242	MN093297	MN097815
A. noctuidarum	BCC 36,265^{T}	MT356072	MT477978
A. pissodis	CBS 118,231^{T}	–	KM283822
A. pyralidarum	BCC28816^{T}	MT356080	MT477982
A. sabanensis	ANDES-F 1024^{T}	KC633232	KC633266
A. sulphureus	TBRC 7248^{T}	MF140758	MF140843
A. thailandicus	TBRC 7245^{T}	MF140754	MF140839
A. tiankengensis	KY11571^{T}	ON502848	ON525447
A. tiankengensis	KY11572	ON502821	ON525449
A. tortricidarum	BCC72638^{T}	MT356076	MT478004
A. tuberculatus	OSC 111,002	JN049830	DQ522338
A. uredinophilus	KACC 44,082^{T}	–	KM283806
A. uredinophilus	KACC 44,066	–	KM283808
A. waltergamsii	TBRC 7252^{T}	MF140748	MF140834
Engyodontium aranearum	CBS 309.85	AJ292391	DQ522341
E. parvisporum	IHEM 22,910	LC092896	–
E. rectidentatum	CBS 206.74	LC092893	LC425540
E. rectidentatum	CBS 547.82	LC092894	–
E. rectidentatum	CBS 641.74	LC092895	–
E. rectidentatum	**R820-1, IRAN-3690 C**	**OR327477**	**OR352918**
Gamszarea kalimantanensis	BTCC-F23^{T}	AB360356	–
G. testudinea	CGMCC3.18986^{T}	MH177616	MH184587
G. testudinea	CGMCC3.18987	MH177615	MH184586
G. wallacei	CBS 101,237^{T}	NR_111267	EF469073

Table 2 (continued)

Species	Strain	GenBank accession numbers	
		ITS	EF1α
Lecanicillium acerosum	CBS 418.81^T	NR_111268	KM283810
L. antillanum	CBS 350.85^T	AJ292392	DQ522350
L. aphanocladii	GZUIFR.SP477	KX021371	–
L. aphanocladii	**R801-2, IRAN-3692 C**	**OR327068**	**–**
L. aranearum	CBS 726.73^T	AJ292464	EF468781
L. araneicola	BTCC-F35^T	AB378506	–
L. cauligalbarum	GZUIFRZHJ01^T	MH730663	MH801920
L. dimorphum	CBS 363.86^T	AJ292429	EF468784
L. flavidum	CBS 300.70^T	EF641877	KM283813
L. fungicola var. aleophilum	CBS 357.80^T	NR_111064	KM283815
L. fungicola var. fungicola	CBS 992.69^T	NR_119653	KM283816
L. fusisporum	CBS 164.70^T	AJ292428	EF468783
L. primulinum	JCM 18,525^T	NR_119418	–
L. psalliotae	CBS 101,270	AJ292389	EF469066
L. rasoulzarei	**R613-1, IRAN-3689C^T**	**OR339890**	**OR352917**
L. saksenae	CBS 532.81^T	AJ292432	EF469067
L. subprimulinum	HKAS99548^T	MG585314	MG585317
L. subprimulinum	HKAS99549	MG585318	MG585321
L. sp.	CBS 639.85	AJ292386	KM283824
L. sp.	KACC 43,873	–	KM283809
Simplicillium lamellicola	CBS 116.25	AJ292393	DQ522356
S. lanosoniveum	CBS 101,267	AJ292395	DQ522357

Type strains are indicated by "^{T}", and the newly sequenced strains are in bold

The sequences of each locus were aligned with MAFFT v.7 (http://mafft.cbrc.jp/alignment/server) (Katoh et al. 2019). BioEdit v.7.0.9.0 was used to manually further adjust the alignment where necessary (Hall 1999). Aligned sequences were automatically trimmed using TrimAl software with the gappyout method. The alignment was converted to PHYLIP and NEXUS formats using the online tool ALTER (Glez-Peña et al. 2010). Maximum likelihood (ML) analysis was done using RAxML-HPC2 on XSEDE with 1000 bootstrap replicates and the GTR + GAMMA model of nucleotide evolution. The best-fit evolutionary model for each dataset was evaluated using the Akaike Information Criterion (AIC) using jModeltest 2.1.10 on the CIPRES online platform (Nylander 2004). The best-fit evolutionary model for ITS and *EF1α* was GTR + I + G. The Bayesian posterior probability (BYPP) analysis was done using a Markov Chain Monte Carlo (MCMC) algorithm with Bayesian posterior probabilities in MrBayes on XSEDE (Ronquist et al. 2012). Four MCMC chains were run from random trees for 1,000,000 generations and sampled every 100th generation. The first 25% of the generated trees were discarded as burn-in, and the remaining trees were used for calculating posterior probabilities. All these analyses were performed on the CIPRES Science Gateway (https://www.phylo.org/portal2) (Miller et al. 2011). The resulting phylograms were visualized in FigTree v. 1.4.0 and edited in Adobe Illustrator CC 22.0.0 (Adobe Systems, San Jose, CA, USA).

Results

During this study, 12 isolates were obtained, purified, and identified. The details regarding the isolates and hosts are presented in Table 3. Five species belonging to three genera, including *Akanthomyces*, *Engyodontium*, and *Lecanicillium* were identified based on combination of morphological characteristics and phylogenetic analyses of ITS and *EF1α* sequence data. Of these, *A. lecanii* and *A. muscarius* had the highest frequency with five and four isolates, respectively. *Lecanicillium aphanocladii*, *L. rasoulzarei* sp. nov. and *Engyodontium rectidentatum*, each with one isolate, had the lowest frequency in this study.

Phylogenetic analysis

Seventy-six strains are included in the combined analyses. The best RAxML tree with a final likelihood value of -10643.894192 is shown (Fig. 2). The matrix had 687 distinct alignment patterns with 22.24% undetermined characters or gaps. The evolutionary model applied for ITS and *EF1α* was GTR + I + G.

Phylogenetic analyses indicate that *L. rasoulzarei* sp. nov. shares a close relationship with *L. aranearum* (CBS 726.73) and *L. araneicola* (BTCC-F35) within a clade, supported by robust bootstrap values of 98/1.0 (ML/BYPP), albeit as a distinct lineage. The five strains of *Akanthomyces lecanii*

Table 3 Information regarding to the fungal isolates obtained in this study

Species	Isolate	Insect host	City	Location
Akanthomyces lecanii	L828-2	*Planococcus citri*	Lahijan	La: 37°13'02"N Lo: 49°58'19E A: -5 m
Akanthomyces lecanii	Le817-2	*Planococcus citri*	Langeroud	La: 37°10'09"N Lo: 50°07'40"E A: 45 m
Akanthomyces lecanii	Le817-7	*Planococcus citri*	Langeroud	La: 37°10'06"N Lo: 50°07'44"E A: 56 m
Akanthomyces lecanii	Sh901-5	Coccidae	Shaft	La: 37°09'59"N Lo: 49°24'14"E A: 45 m
Akanthomyces lecanii	T901-2	*Planococcus citri*	Talesh	La: 37°48'16"N Lo: 48°54'47"E A: 34 m
Akanthomyces muscarius	F901-1	*Planococcus citri*	Fouman	La: 37°13'13"N Lo: 49°19'42"E A: 27 m
Akanthomyces muscarius	Gh1009-1	Coccidae	Ghaleroudkhan	La: 37°06'03"N Lo: 49°15'45"E A: 168 m
Akanthomyces muscarius	O1103-1	*Lepidosaphes* sp.	Langeroud	La: 37°05'35"N Lo: 50°07'41"E A: 96 m
Akanthomyces muscarius	R128-3	*Planococcus citri*	Rasht	La: 37°11'55"N Lo: 49°39'00"E A: 26 m
Engyodontium rectidentatum	R820-1	Coccidae	Rasht	La: 37°11'44"N Lo: 49°38'31"E A: 29 m
Lecanicillium aphanocladii	R801-2	*Tetranychus urticae*	Rasht	La: 37°15'41"N Lo: 49°34'10"E A: 5 m
Lecanicillium rasoulzarei	R613-1	Aphididae	Rasht	La: 37°15'24"N Lo: 49°35'51"E A:7 m

La = Latitude; Lo = Longitude; A = Altitude

identified in this study formed a cluster with the ex-type of *A. lecanii*, *A. lecanii* (IMI 304,807), and *A. lecanii* (IMI 304,817), exhibiting a ML bootstrap value of 94%. Likewise, all strains of *Akanthomyces muscarius* were grouped with the ex-type, showing a bootstrap value of 92/0.99 (ML/BYPP). A single strain of *Lecanicillium aphanocladii* occupied a clade alongside *L. aphanocladii* (GZUIFR.SP477) with robust bootstrap values of 97/1.0 (ML/BYPP). *Engyodontium rectidentatum* (IRAN-3690 C) clustered with other strains of *E. rectidentatum*, supported by bootstrap values of 100/1.0 (ML/BYPP) (Fig. 2).

Taxonomy

Akanthomyces lecanii (Zimm.) Spatafora, Kepler & B. Shrestha 2017 Fig. 3.

Sexual morph not observed. **Asexual morph** *Hyphae* hyaline, septate, smooth-walled, 1–3 µm ($\overline{x}$ = 2 µm) wide. *Phialides* hyaline, aseptate, short, relatively thick, aculeate and strongly tapered toward the apex, (6–)11–20(–23) × 1–2(–2.2) µm ($\overline{x}$ = 15.5 × 1.5 µm, n = 30) µm, produced singly or in whorls, 3–6 phialides in a whorl, produced directly on prostrate hyphae, on conidiophores or secondarily on previous phialides. Frequently forming *short secondary necks* projecting from the apical part of the phialide. *Conidia* aggregate mostly in ellipsoidal heads at the top of the phialides, hyaline, short-ellipsoidal, 1-celled, (2.5–)3–5 × 1–1.5 µm ($\overline{x}$ = 3 × 1 µm, n = 30). *Octahedral crystals* present.

Culture characteristics Colonies slow growing on the PDA (Merck, Germany), reaching (10–)14–20 mm in diam. after 10 days at 25 °C, fluffy, circular, entire in margins. White on the upper side and light yellow on the reverse.

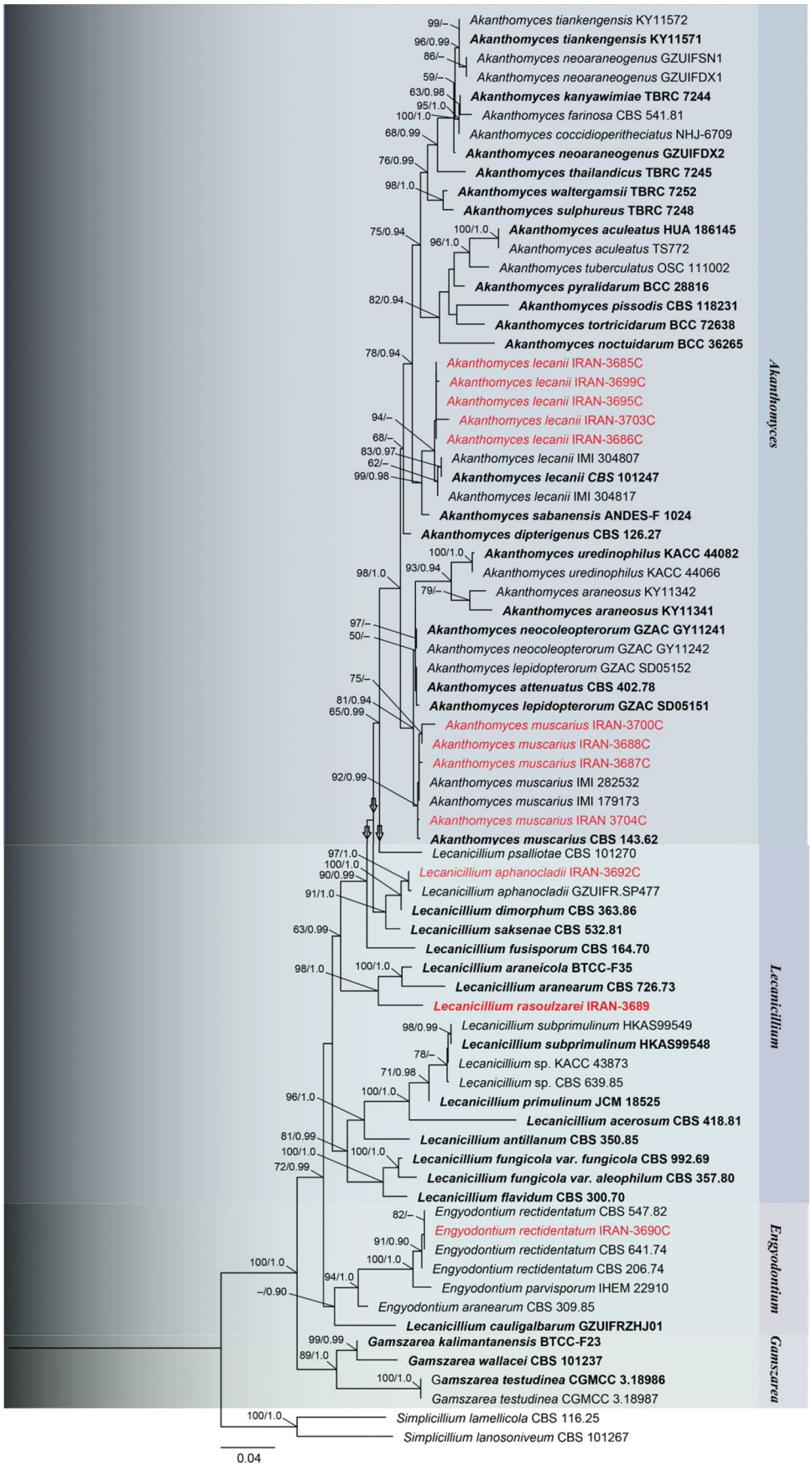

Fig. 2 Phylogenetic tree generated by maximum likelihood analysis based on the combined ITS and *EF1α* sequence data of the *Akanthomyces*, *Engyodontium*, and *Lecanicillium* species. ML bootstrap support values ≥ 50% and Bayesian posterior probabilities ≥ 0.90 are given at the nodes, respectively. The tree is rooted with *Simplicillium lamellicola* (CBS 116.25) and *Simplicillium lanosoniveum* (CBS 101,267). Ex-type strains are in bold, and the identified species are in red

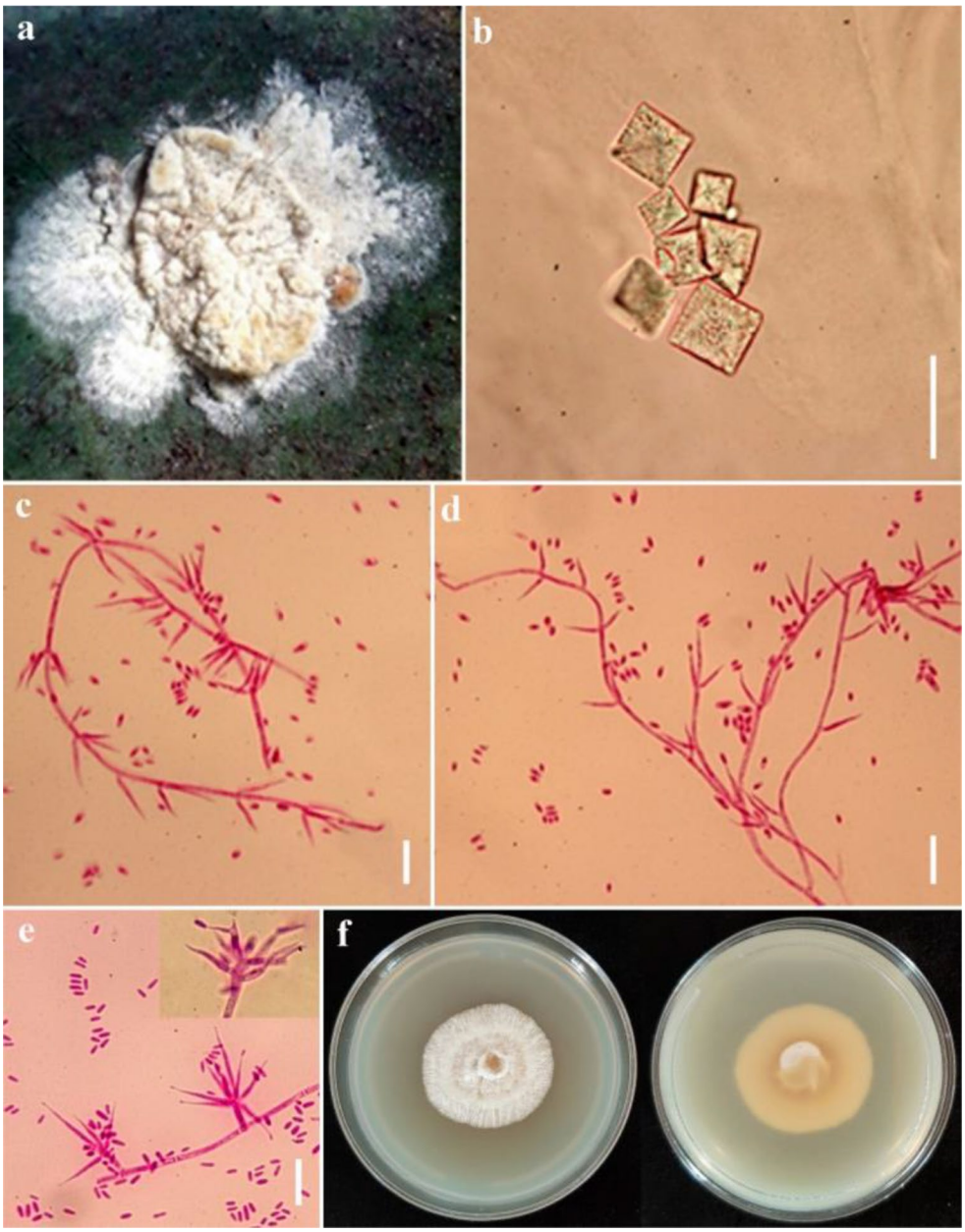

Fig. 3 *Akanthomyces lecanii*; **a** Insect host infected by the fungus **b** Octahedral crystals **c, d** Phialides and conidia (IRAN-3703 C) **e** Phialides and short secondary necks (IRAN-3686 C) **f** Obverse and reverse sides of culture on PDA. Scale bars: **b** = 20 µm, **c–e** = 10 µm

Materials examined Iran, Guilan province, Lahijan; isolated from dead *Planococcus citri* (Pseudococcidae); 19 November 2018, Alireza Armand, L828-2 (IRAN-3703 C). Iran, Guilan, Langeroud, Leylakooh; isolated from dead *Planococcus citri* (Pseudococcidae); 08 November 2018, Alireza Armand, Le817-7 (IRAN-3686 C).

Akanthomyces muscarius (Petch) Spatafora, Kepler & B. Shrestha 2017 Fig. 4.

Sexual morph not observed. **Asexual morph** *Hyphae* hyaline, septate, smooth-walled, 1–2.5 µm ($\overline{x}$ = 1.5 µm) wide. *Phialides* hyaline, aseptate, long, relatively thin, relatively aculeate and slightly tapered toward the apex, (12.5–)20–35(–38) × 1–1.8(–2) µm ($\overline{x}$ = 27 × 1.2 µm, n = 30) µm, produced singly or in whorls, 3–5 phialides in whorls, produced directly on prostrate hyphae, on conidiophores or secondarily on previous phialides. *Short secondary necks* very uncommon. *Conidia* aggregate in subglobose to ellipsoidal heads at the apex of the phialids, hyaline, cylindrical, 1-celled, (2.5–)3–6.5(–8) × 1–2 µm ($\overline{x}$ = 5 × 1.3 µm, n = 30). *Octahedral crystals* present.

Culture characteristics Colonies fast growing on PDA (Merck, Germany), reaching (22–)25–28 mm in diam. after 10 days at 25 °C, fluffy, circular, entire in margins. White on the upper side and light yellow on the reverse.

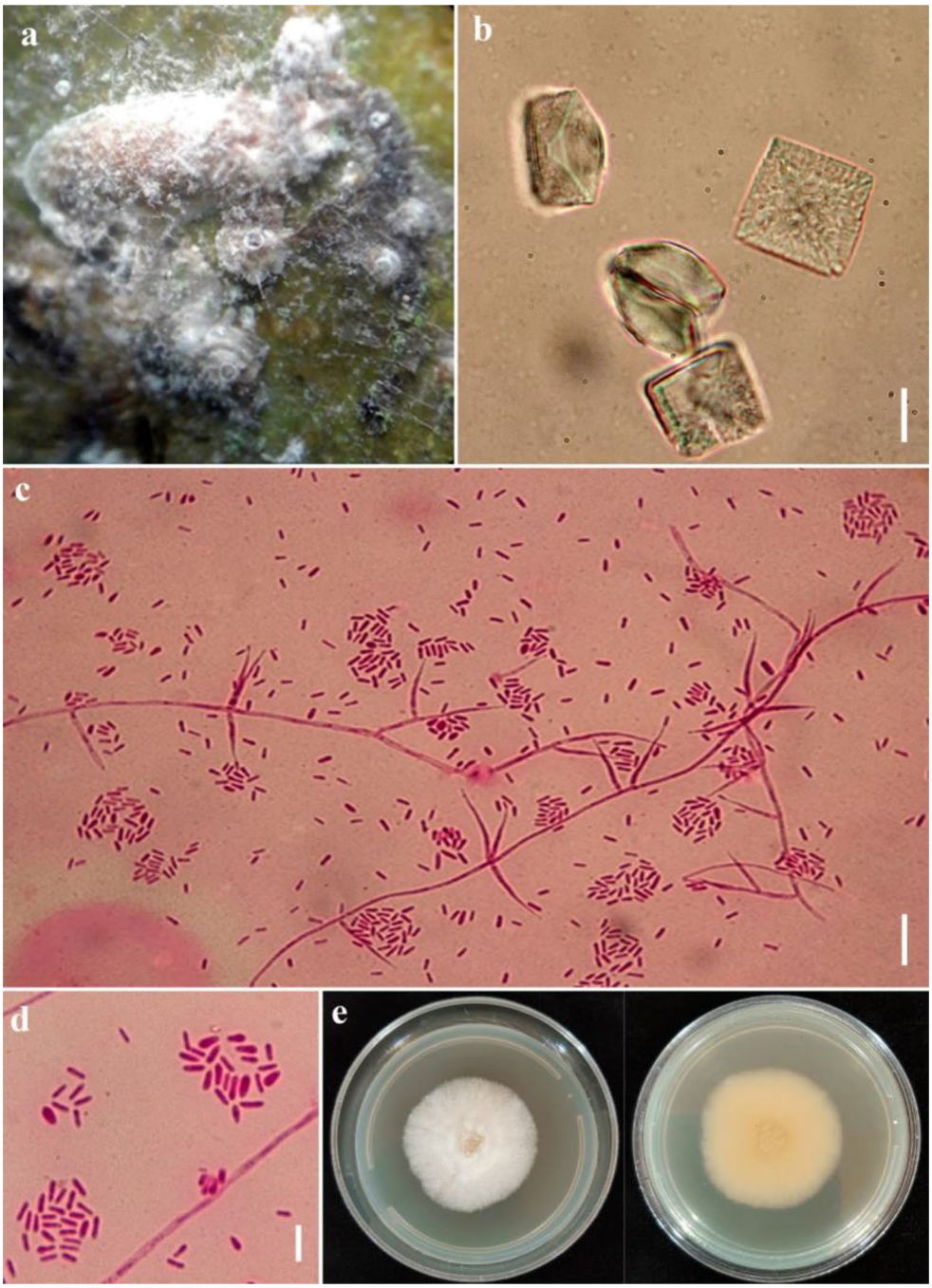

Fig. 4 *Akanthomyces muscarius* (IRAN-3688 C); **a** Insect host infected by the fungus **b** Octahedral crystals **c, d** Phialides and conidia **e** Obverse and reverse sides of culture on PDA. Scale bars: **b** = 20 µm, **c, d** = 10 µm

Material examined Iran, Guilan province, Langeroud, Otaghvar; isolated from dead *Lepidosaphes* sp. (Diaspididae); 23 January 2019, Alireza Armand, O1103-1 (IRAN-3688 C).

Engyodontium rectidentatum (Matsush.) W. Gams, de Hoog, Samson & H.C. Evans 1984 Fig. 5.

Sexual morph not observed. **Asexual morph** *Hyphae* hyaline, septate, smooth-walled, 1.5–2.5 µm ($\overline{x}$ = 2.3 µm) wide. *Conidiogenous cells* arising in whorls from prostrate hyphae, polyblastic, bearing thin, perpendicular denticles, scattered along the upper half, (15–)20–29(–34) × 1–1.5 µm ($\overline{x}$ = 25 × 1.2 µm, *n* = 30) µm. Conidia formed on straight, cylindrical, narrow and short denticles scattered along the upper half of the conidiogenous cells, measuring 1–2 × 0.2 µm. *Conidia* hyaline, smooth-walled, ovoid to fusiform, apiculate at the base, 1-celled, 3–5 × 1.2–2 ($\overline{x}$ = 4.5 × 1.5 µm, *n* = 30) µm. *Terminal conidia* solitary, cylindrical, straight, 6–10 × 1.5–2 ($\overline{x}$ = 7.5 × 1.8 µm, *n* = 30).

Culture characteristics Colonies on the MEA (Merck, Germany) reaching 12–13 mm in diam. after 6 days at 20 °C,

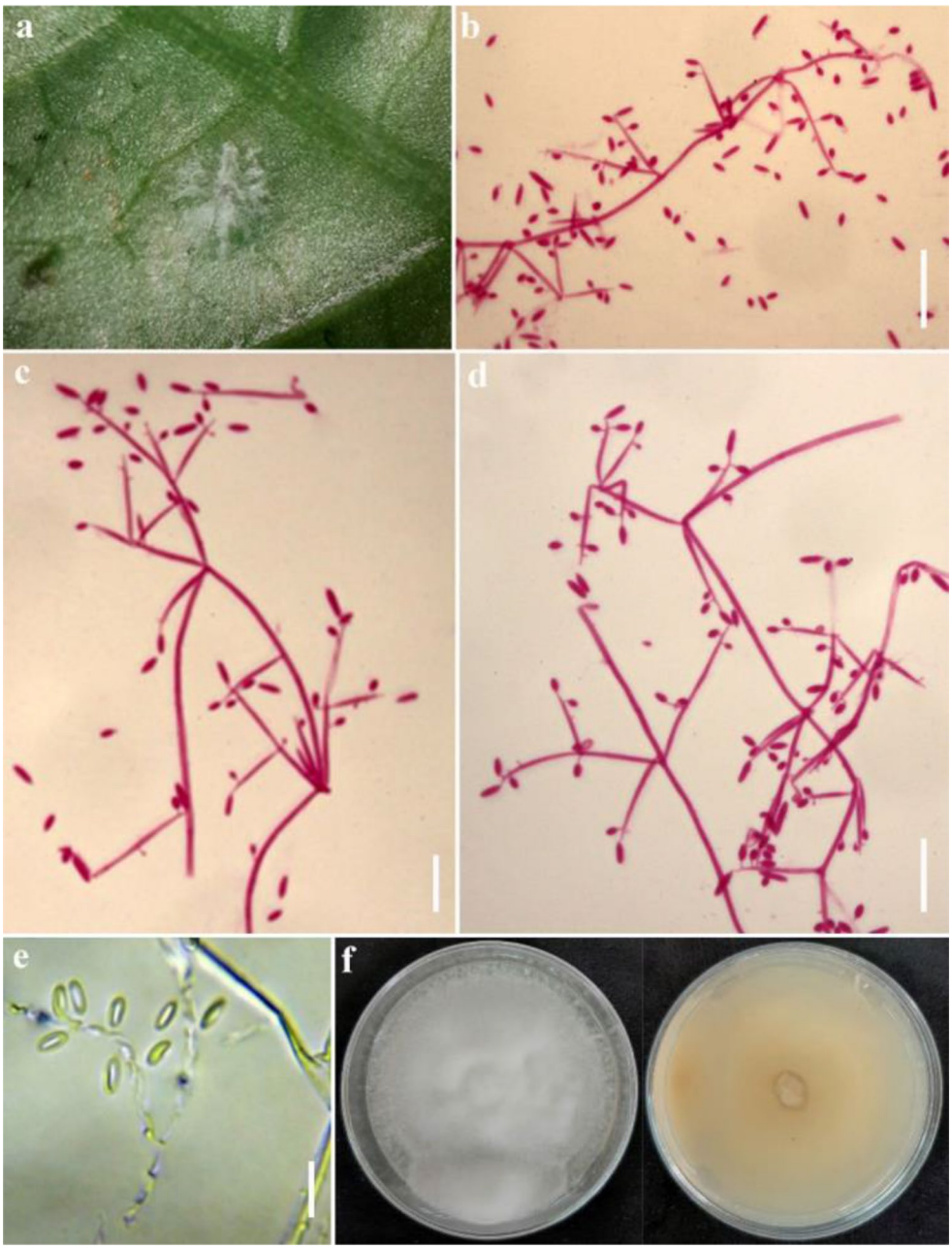

Fig. 5 *Engyodontium rectidentatum* (IRAN-3690 C); **a** Insect host infected by the fungus **b–d** Polyblastic conidiogenous cells, and conidia **e** Conidiogenous denticles and conidia on the rachids **f** Obverse and reverse sides of culture on the MEA. Scale bars: **b–e** = 20 µm

cottony, circular, entire in margins. White on the upper side and light yellow on the reverse.

Material examined Iran, Guilan province, Rasht; isolated from dead *Tetranychus urticae*; 11 November 2018, Alireza Armand, R820-1 (IRAN-3690 C).

Lecanicillium aphanocladii Zare & W. Gams 2001 Fig. 6.

Sexual morph not observed. **Asexual morph** *Hyphae* hyaline, septate, smooth-walled, 1–3 µm ($\overline{x}$ = 1.9 µm) wide. *Aphanophialides* hyaline, aseptate, short, flask-shaped at first, swollen at base, tapering toward the thread-like neck, soon collapsing and becoming reduced to very fine denticles, varying in size from small denticles to longer phialides, 4–11 × 1–2 µm ($\overline{x}$ = 5.5 × 1.5 µm, *n* = 30) µm, produced singly, in pairs, verticillate or in dense irregular clusters on prostrate hyphae. *Conidia* hyaline, solitary, oval to subglobose, 1-celled, 2.7–4.5 × 1.7–3 µm ($\overline{x}$ = 3.5 × 1.8 µm, *n* = 30). *Octahedral crystals* not observed.

Culture characteristics Colonies fast growing on PDA, reaching 40–41 mm in diam. after 10 days at 25 °C, cottony, circular, entire in margins. White on the upper side and red on the reverse, with red pigments diffusing into the media.

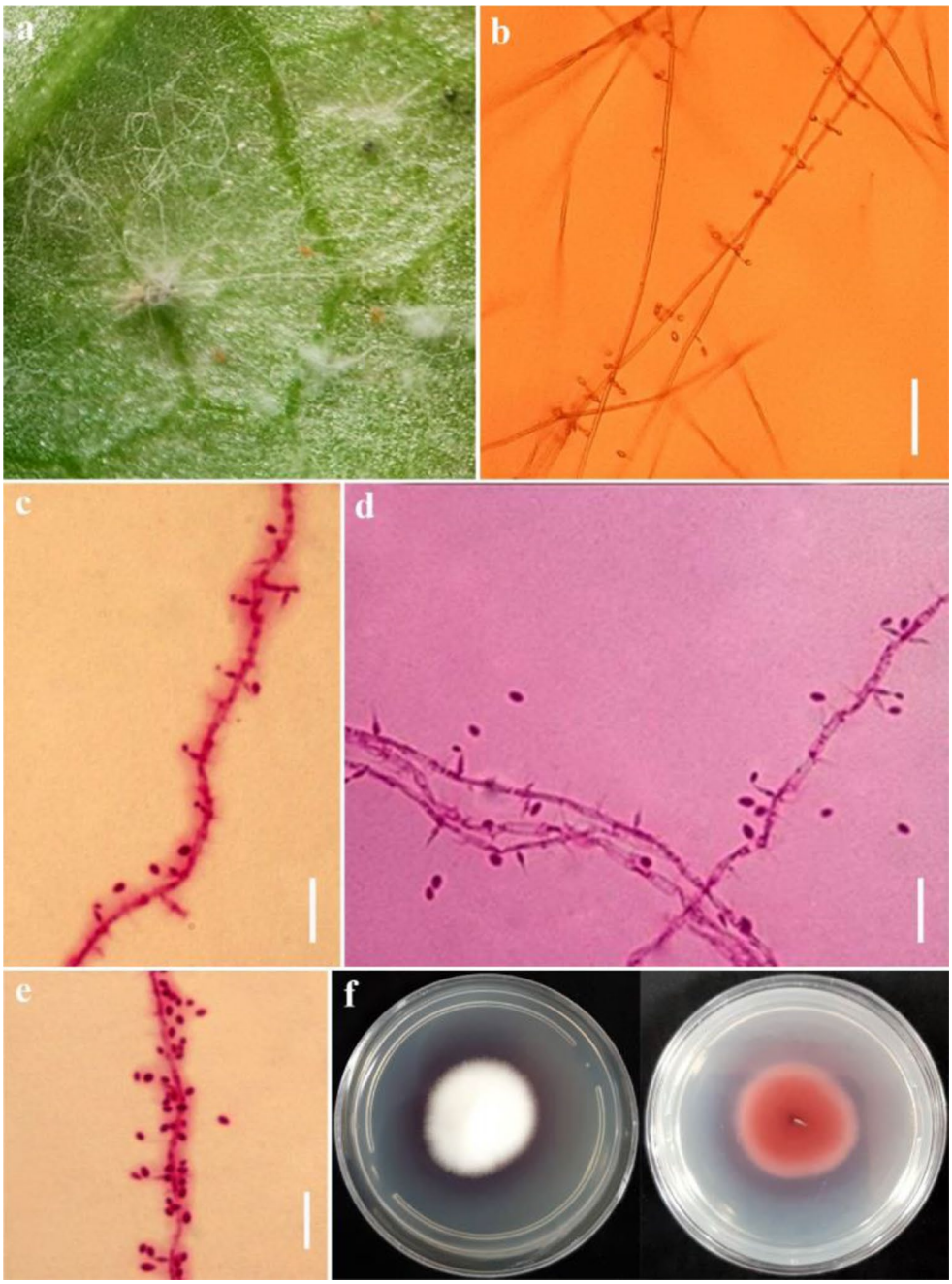

Fig. 6 *Lecanicillium aphanocladii* (IRAN-3692 C); **a** Insect host infected by the fungus **b–e** Aphanophialides and conidia f. Obverse and reverse sides of culture on the PDA. Scale bars: **b–e** = 10 µm

Material examined Iran, Guilan province, Rasht; isolated from dead *Tetranychus urticae*; 23 October 2019, Alireza Armand, R801-2 (IRAN-3692 C).

Lecanicillium rasoulzarei Armand A. & Khodap. **sp. nov.** Figure 7.

Index Fungorum number: IF 900873.

Etymology: In honor of the Iranian mycologist, Rasoul Zare, who is a pioneer in the molecular taxonomy of *Lecanicillium* and related genera.

Holotype: Iran, Guilan province, Rasht, on dead Aphididae, 04 September 2018, Alireza Armand, GUM 1199 (consisting of dried sheets of PDA culture). Ex-type culture IRAN-3689 C. GenBank Acc. No: ITS: OR339890, *EF1a*: OR352917.

Sexual morph not observed. **Asexual morph** *Hyphae* hyaline, septate, smooth-walled, 1–2 µm ($\bar{x}$ = 1.2 µm) wide. *Phialides* hyaline, aseptate, narrow, long, slightly aculeate and tapered toward the apex, straight or slightly curved, (13–)17–22(–26) × 1–1.2 µm ($\overline{x}$ = 20 × 1 µm, n = 30), produced solitary, in pairs or in whorls of 3–5 phialides directly on prostrate hyphae; *secondary necks* not observed. *Conidia* aggregated in subglobose to globose heads and of two types; *macroconidia* hyaline, falcate, straight or slightly

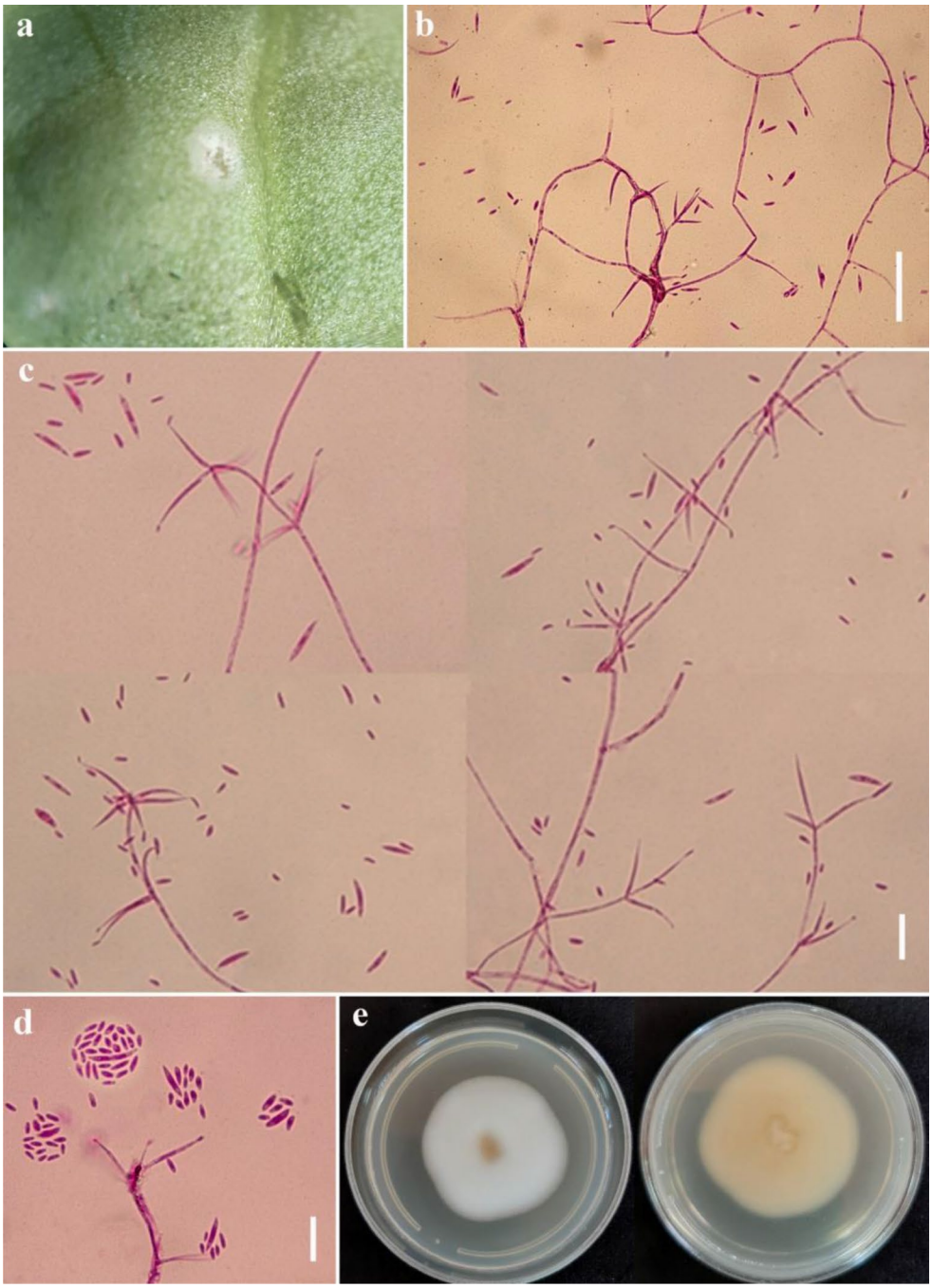

Fig. 7 *Lecanicillium rasoulzarei* (IRAN-3689 C); **a** Insect host infected by the fungus **b, c** Phialides and conidia **d** Conidial heads **e** Obverse and reverse sides of culture on the PDA. Scale bars: **b** = 40 µm, **c** = 20 µm, **d** = 10 µm

curved, asymmetrically narrowed, subacute or pointed at the ends, 1-celled, (6–)8–10(–12) × 1.2–1.5(–2) µm ($\overline{x}$ = 9.5 × 1.3 µm, n = 30); *microconidia* hyaline, short-cylindrical or ovoid, pointed at on end or both ends, 1-celled, (2.5–)3–5 × (0.7–)1–1.2 µm ($\overline{x}$ = 3.7 × 1 µm, n = 30). *Octahedral crystals* not observed.

Culture characteristics Colonies fast growing on PDA, reaching 36–37 mm in diam. after 10 days at 25 °C, cottony, circular, entire in margins. White on the upper side and light yellow on the reverse.

Notes: Lecanicillium aranearum was first described on Araneida from Ghana, India (Zare and Gams2001), while *L. araneicola* was isolated from Araneae in Indonesia (Sukarno et al. 2009). A pairwise comparison between *L. rasoulzarei* and *L. aranearum* revealed 92.7% identity in ITS (39/537 bp differences) and 94.5% identity in the *EF1α* sequence (53/964 bp differences). Despite the unavailability of the *EF1α* sequence for *L. araneicola*, a pairwise comparison of ITS region between *L. rasoulzarei* and *L. araneicola* showed 5% (23/515 bp) differences. *Lecanicillium rasoulzarei* differs from *L. aranearum* by producing two types of

conidia that can be easily distinguished from each other. In *L. aranearum* (CBS 726.73), conidia are homogenous and smaller than the macroconidia of *L. rasoulzarei* (Zare and Gams2001). *Lecanicillium rasoulzarei* is distinguished from *L. araneicola* by producing smaller macroconidia and more numbers of phialides in each whorl (up to 5) than those of *L. araneicola*. Forthermore, *L. araneicola* produces allantoid or ellipsoidal microconidia with rounded ends and slightly curved, while *Lecanicillium rasoulzarei* produces straight short-cylindrical or ovoid microconidia (Sukarno et al. 2009). *Lecanicillium rasoulzarei* was isolated from Aphididae and is distinct from the two species that occupied the clade (Fig. 2).

Discussion

The integration of molecular analyses alongside morphological observations has refined our understanding of the taxonomy and relationships within Cordycipitacaea. However, recent investigations have demonstrated that the genus *Lecanicillium* is paraphyletic (Sung et al. 2007). This reason, along with the abandonment of the dual nomenclature system for pleomorphic fungi, has led to the reclassification of *Lecanicillium* (Kepler et al. 2017), with a significant number being transferred to the overlooked genus *Akanthomyces* Lebert 1858, known for its entomogenous species. Later on, various studies described novel taxa within both *Akanthomyces* and *Lecanicillium* genera (Mongkolsamrit et al. 2018; Su et al. 2019; Aini et al. 2020; Chen et al. 2020a; Zhang et al. 2021). Nevertheless, some recently identified species of *Lecanicillium*, including *L. uredinophilum* (Manfrino et al. 2022) and *L. pissodis* (Chen et al. 2020b), have been transferred to the genus *Akanthomyces* using multi-gene phylogenetic studies. The aforementioned results underscore that, despite advancements in the phylogenetic understanding of this fungal group, the precise taxonomic placement of some *Lecanicillium* and *Akanthomyces* species remains a subject of active investigation.

Distinguishing morphological characteristics of *Lecanicillium* species include the production of aculeate phialides, forming either singly or in terminal and intercalary whorls, ellipsoidal-cylindrical or fusiform-falcate conidia, adhering in fascicles at the tip of the phialides or solitary on denticles (e.g. *L. aphanocladii*) (Zare and Gams 2008). These morphological characteristics are not sufficient to set *Lecanicillium* apart from other closely related genera such as *Akanthomyces*. These two genera share indistinguishable morphological characteristics, which led to misidentifying the species within these genera (Zare and Gams2001; Kepler et al. 2017). Therefore, in this study, morphological and molecular characteristics were used to identify the species precisely.

Various phylogenetic studies have employed distinct molecular markers to reassess the phylogeny of the investigated group and to identify novel taxa. For instance, Sukarno et al. (2009) identified two new species, *L. araneicola* and *L. kalimantanense*, and introduced *L. saksenae* , using ITS sequence data. Kaifuchi et al. (2013) used LSU and ITS sequences to introduce *L. primulinum*. Kepler et al. (2017) reassessed *Clavicipitaceae* using SSU, LSU, *TEF*, *RPB1*, and *RPB2*. Mongkolsamrit et al. (2018) described four new species, *A. kanyawimiae*, *A. sulphureus, A. thailandicus*, and *A. waltergamsii* utilizing a multi-gene phylogenetic study of combined ITS, LSU, *TEF1*, *RPB1*, and *RPB2* dataset. The same markers were used to introduce *L. araneogenous* as a new species in China (Chen et al. 2018). To discriminate between species, the phylogenetic study performed in this study using combined ITS and *TEF* sequence data, was consistent with the above studies.

The phylogenetic trees generated through maximum likelihood and Bayesian analyses exhibited similar topologies, with well-supported branches in both trees. In light of a comprehensive review of existing literature and a thorough evaluation of various gene regions employed in previous studies on the phylogeny of the targeted fungal group, the Internal Transcribed Spacer (ITS) and Elongation Factor 1-alpha (*EF1α*) gene regions have been selected with the assurance that they can effectively delineate all the studies species within the investigated fungal group. The phylogenetic results indicated that the combined ITS and *EF1α* sequence dataset successfully delineated *Engyodontium*, *Gamszarea*, *Akanthomyces* and *Lecanicillium* species into distinct clades (Fig. 2). In the recent study conducted by Zhou et al. (2018), a novel species named *L. cauligalbarum* was described based on a comprehensive dataset comprising concatenated sequences of ITS, nc SSU and LSU rDNA, *TEF*, *RPB1*, and *RPB2*. In their phylogenetic analysis two strains of this species clustered with the *Blackwellomyces* clade, contrasting our phylogenetic analyses, which placed the ex-type of *L. cauligalbarum* with the *Engyodontium* clade (Fig. 2). Notably, the original study related to the new species did not encompass *Engyodontium* species in the phylogenetic analyses. In a separate study, Zhang et al. (2021) established a new genus, *Gamszarea*, to accommodate the species previously classified in *Lecanicillium*, employing ITS, SSU and LSU rDNA, *TEF*, *RPB1*, and *RPB2* sequence data. Similarly, their phylogenetic analysis excluded *Engyodontium* species and revealed that *L. cauligalbarum* strains occupied an intermediate position between the *Blackwellomyces* and *Ascopolyporus* clades. These results collectively indicate that *L. cauligalbarum* lacks a fixed position within the *Lecanicillium* clade, necessitating the proposal of a new

combination for this species. However, the phylogenetic results of the present study were consistent with those of Zhang et al. (2021), supporting the segregation of *Gamszarea* species into a distinct clade separate from other genera.

A single isolate of *L. aphanocladii* (IRAN-3692 C) was morphologically similar to the strains described by Zare and Gams (2001), and the phylogenetic analysis indicated that the newly isolated strain belongs to the species *L. aphanocladii* (Fig. 2). *Lecanicillium aphanocladii* has mainly been isolated from *Agaricus* spp., but it has also been reported from *Trialeurodes vaporariorum*, the leaf litter of *Acacia karroo*, *Abelmoschus esculentus*, and *Sphaerotheca fuliginea*. However, the fungus has been isolated and illustrated for the first time from *Tetranychus urticae* (Tetranychidae).

Morphologically, *Akanthomyces lecanii* is similar to *A. sabanensis*. However, it differs from *A. sabanensis* by producing ellipsoidal conidia that are relatively longer than those of *A. sabanensis* (Chiriví-Salomón et al. 2015). Phylogenetic analysis showed that the studied *A. lecanii* isolates grouped in a clade with the type (CBS 101,247) and representative strains with a 94% ML bootstrap value, and *A. sabanensis* formed a sister clade with 99/0.98 (ML/BYPP) bootstrap values (Fig. 2).

Akanthomyces muscarius isolated in this study shared the same morphological characteristics as *A. lecanii*; however, it can be distinguished from *A. lecanii* by producing longer phialides and conidia and the absence of secondary necks. Moreover, *Akanthomyces muscarius* grows considerably faster than *A. lecanii* on the PDA. The phylogenetic tree showed that our isolates clustered well with the ex-type strain (CBS 143.62) in a clade with 92/0.99 (ML/BYPP) bootstrap values (Fig. 2). *Akanthomyces muscarius* and *A. lecanii* were the dominant species associated with *Planococcus citri*; however, *Akanthomyces muscarius* (O1103-1) was found to infect *Lepidosaphes* sp. This is the first record of *Akanthomyces muscarius* occurrence in Diaspididae.

The *Engyodontium* strain is similar to the type strain described by Gams et al. (1984). Based on phylogenetic analysis, the strain was placed within the *E. rectidentatum* clade with 91/0.90 (ML/BYPP) bootstrap values (Fig. 2). *Engyodontium* species were mostly reported from Diaspididae, Coccidae, and spiders (de Hoog 1978; Gams et al. 1984). This is the first report of the occurrence of *Engyodontium* in *Tetranychus urticae*.

Conclusions

This study aimed to explore indigenous entomopathogenic fungi associated with the pests infecting different citrus orchards in Guilan province, Northern Iran. This research reported three new insect hosts — previously undocumented specific insect hosts infected by specific fungi — and described a new *Lecanicillium* species from Iran. The research contributed to the understanding of the natural biodiversity of entomopathogenic fungi in specific geographical areas and established a valuable source of biological control agents for future research. The findings underscore the importance of further studies on the role of entomopathogenic fungi in pest control in citrus-cultivating regions and their potential applications in sustainable agricultural practices.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-024-03944-2.

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Author contributions Armand A. contributed to conceptualization, performing the research, and writing the paper; Khodaparast S.A. contributed to conceptualization, providing laboratory facilities, and editing the paper; Zibaee A. did insect host identification and advisory service during the research; Nazari S. collaborated with sample collection, finding the target geographical regions, and getting permission. The first draft of the manuscript was written by Armand A. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets used for the phylogenetic analyses in the present study are available from the corresponding author by request and as supplementary files.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests in this research.

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