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RESEARCH PAPERS

Biological and molecular characterisation of *Pilidium lythri*, an emerging strawberry pathogen in Iran

Kaivan KARIMI[1,2], Mahdi ARZANLOU[1], Asadollah BABAI-AHARI[1] and Ilaria PERTOT[2]

[1] Plant Protection Department, Faculty of Agriculture, University of Tabriz, Tabriz, P O Box: 5166614766, Iran
[2] Department of Sustainable Agro-Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach (FEM), San Michele all'Adige, Italy

Summary. Strawberry production is hampered by numerous biotic factors including fungal pathogens. Symptoms of dark brown necrotic lesions on fruits, stems and leaves were observed in a survey of strawberry fields in the Kurdistan province of Iran. Symptomatic plant tissues were collected and several fungal isolates were recovered from these tissues. Based on a combination of morphological characteristics and sequence data for ITS and LSU ribosomal DNA, the isolates were identified as *Pilidium lythri* (previously named *P. concavum* or *Hainesia lythri*). A pathogenicity assay confirmed that all new isolates induced symptoms resembling those observed in the field, as well as two *P. lythri* isolates from olive included in the assay; no significant disease incidence or severity differences were detected between isolates. This is the first report of *P. lythri* on strawberry in Iran. Genetic diversity between *Pilidium lythri* isolates from strawberry (18 isolates) and olive (two isolates) was evaluated using RAPD and M13 markers. No polymorphism was detected within and among the isolates, indicating limited genetic variability probably due to lack of recombination events and/or recent introduction. Given recent outbreaks and the presence of the pathogen in most strawberry growing areas in the Kurdistan province, quickly applied and appropriate management programmes are necessary to prevent spread of the disease.

Key words: tan-brown spot, morphological description, molecular identification, Kurdistan, *Pilidium concavum*.

Introduction

Strawberry (*Fragaria* × *ananassa* Duchesne) is one of the most valuable crops grown worldwide, and also in Iran (FAO, 2013), where it is grown both in open fields and in greenhouses (Banaeian *et al*., 2011). The Kurdistan province accounts for around 53% of strawberry production in Iran. Strawberry plants and fruits are attacked by numerous fungal pathogens, *e.g. Botrytis cinerea* Pers.: Fr., *Colletotrichum* spp. and *Verticillium dahliae* Kleb. (Maas, 1998). In recent years, a new fungal disease caused by *Pilidium lythri* (Desm.) Rossman [previously named *P. concavum* (Desm.) Höhn. and *Hainesia lythri* (Desm.) Höhn.] has occasionally been isolated from strawberry worldwide. The disease was first reported in Brazil and since then in several other countries, including Belgium, China and the USA (Lopes *et al*., 2010; Debode *et al*., 2011; Geng *et al*., 2012; Fernández-Ortuño *et al*., 2014). In strawberry, *P. lythri* causes tan-brown fruit rot, resembling the symptoms of anthracnose, although the pathogen can also infect leaves, stems and roots (Debode *et al*., 2011; Geng *et al*., 2012; Fernández-Ortuño *et al*., 2014). *Pilidium lythri* was recently isolated from healthy strawberries as an endophyte (Hipol *et al*., 2014). This pathogen had already been isolated worldwide from other hosts, *e.g. Rosa* sp., *Hieracium caespitosum* Dumort., *Olea europaea* L., *Paeonia suffruticosa* Andrews., *Bergenia crassifolia* (L.) Fritsch., *Eucalyptus* sp., *Prunus domestica* L., and *Fallopia japonica* (Houtt.) Ronse Decr. (Table 1).

The genus *Pilidium* was first described with *P. acerinum* (Alb. & Schwein.) Kunze from the leaves of

Corresponding author: M. Arzanlou
E-mail: Arzanlou@tabrizu.ac.ir

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Table 1. *Pilidium lythri* strains used for phylogenetic analysis with GenBank accessions.

Species	GenBank No. ITS (LSU[a])	Host	Country	Reference
Pilidium lythri	JQ790789.1	*Fallopia japonica*	USA	Bruckart *et al*., 2013
P. lythri	JX047867.1	*Hieracium caespitosum*	France	Caesar *et al*., 2012
P. lythri	KF911079.1	*Fragaria* × *ananassa*	USA	Fernández-Ortuño *et al*., 2014
P. lythri	KC845228.1	*Olea europaea*	Iran	Arzanlou *et al*., 2013
P. lythri	JQ995228.1	*Fragaria* × *ananassa*	China	Geng *et al*., 2012
P. lythri	GU126750.1	*Paeonia suffruticosa*	China	Duan *et al*., 2010
P. lythri	FM211810.1	*Bergenia crassifolia*	France	Cardin *et al*., 2009
P. lythri	KJ908840.1	*Fragaria* × *ananassa*	Philippines	Hipol *et al*., 2014
P. lythri	KF060281.1	*Eucalyptus* sp.	Mozambique	Maússe-Sitoe *et al*., 2016
P. lythri	KF646103.1	*Rosa rugosa*	Lithuania	Menkis *et al*., 2014
P. lythri	KC614564.1	*Prunus domestica*	Iran	Sayari *et al*., 2013
P. lythri	AY487094.1 (AY487095)	*Rosa* sp.	USA	Rossman *et al*., 2004
P. lythri	AY487097.1 (AY487098)	*Paeonia suffruticosa*	Japan	Rossman *et al*., 2004
P. lythri CCTU[b] FCh12	KX639606	*Fragaria* × *ananassa*, fruit	Iran	This study
P. lythri CCTU PN6	KX639607 (KX639613)	*Fragaria* × *ananassa*, petiole	Iran	This study
P. lythri CCTU FT7	KX639608	*Fragaria* × *ananassa*, fruit	Iran	This study
P. lythri CCTU FP2	KX639609	*Fragaria* × *ananassa*, fruit	Iran	This study
P. lythri CCTU FT1	KX639610	*Fragaria* × *ananassa*, fruit	Iran	This study
P. lythri CCTU SCh23	KX639611	*Fragaria* × *ananassa*, stem	Iran	This study
P. lythri CCTU FS6	KX639612	*Fragaria* × *ananassa*, fruit	Iran	This study
P. pseudoconcavum	KF777184.1 (KF777236.1)	*Greyia radlkoferi*	South Africa	Crous *et al*., 2013
P. acerinum	AY487088 (AY487089)	*Aesculus hippocastanum*, ex dead leaf	Netherlands	Rossman *et al*., 2004
P. acerinum	AY487091.1 (AY487092)	garden soil	Netherlands	Rossman *et al*., 2004
P. eucalyptorum	KT950854.1 (KT950868.1)	*Eucalyptus robusta*	France	Crous *et al*., 2015
Chaetomella raphigera	AY487076.1 (AY487077)	*Vaccinium corymbosum*	USA	Rossman *et al*., 2004

[a] GenBank accession numbers for LSU sequences are shown in brackets.
[b] CCTU: Culture Collection of Tabriz University.

deciduous trees (Rossman *et al*., 2004). Four species are currently accepted in *Pilidium*, including *P. acerinum*, *P. lythri*, *P. pseudoconcavum* and *P. eucalyptorum* (Rossman *et al*., 2004; Kirk *et al*., 2008; Crous *et al*., 2013; 2015). Members of this genus usually produce two types of conidiomata, black pycnidia and pale sporodochia. Black pycnidia are most commonly encountered in nature, while pale sporodochia are found in pure culture (Palm, 1991; Rossman *et al*., 2004). The sporodochial form, known as *Hainesia lythri*, was previously reported on strawberry as causal agent of Hainesia leaf spot and tan-brown

rot (Opgenorth and White, 1991; Golebniak and Jarosz, 2004). To avoid considering two conidiomatal types as distinct species, Palm (1991) stated that the enclosed, black sclerotium-like pycnidia of *P. lythri* and the light-coloured, fleshy, discoid, stalked sporodochia of *Hainesia lythri* were synanamorphs (Rossman *et al*., 2004). The genus is phylogenetically located in the Leotiomycetes and the sexual morph of *P. lythri* has been described as *Discohainesia oenotherae* (Cooke & Ellis) Nannf. (Rossman *et al*., 2004; Kirk *et al*., 2008; Arzanlou *et al*., 2013). To comply with recent ''one name – one fungus'' changes in fungal nomenclature (Art. 59 of the new ICN, McNeill *et al*., 2012), among the three generic names available for this fungus, *Pilidium* Kunze 1823 has been preferred. A new combination was proposed to replace *P. concavum* (Desm. 1847) Höhn. with *P. lythri* (Desm. 1846) Rossman (Johnston *et al*., 2014).

Because of the importance of the strawberry industry and the frequent occurrence of tan-brown rot symptoms in Iran; our initial aim was to assess the extent of occurrence of *P. lythri* in strawberry fields. Extensive sampling carried out in Kurdistan province in 2014 led to the isolation of several *P. lythri* isolates. We characterised isolates using morphological, molecular and pathogenicity traits, and further deduced how they may have been introduced to Iran.

Materials and methods

Sampling and fungal isolation

Extensive sampling was carried out in strawberry fields in the Kurdistan province of Iran, and specifically in Sanandaj, Kamyaran and Sarvabad, the counties with the main strawberry production. Sampling was designed in "M" figure transect across each field to insure consistency. Symptomatic plant parts showing leaf spot, stem necrosis and/or fruit rot were collected and transferred to the laboratory in single sterile boxes. Conidial masses developing on fruit surfaces were transferred to acidified potato dextrose agar (PDA; 200 g potatoes, 20 g dextrose, 15 g agar, 1,000 mL distilled water) in Petri plates. For other plant parts, infected segments were obtained from the edges of lesions, disinfected in sodium hypochlorite (1%) for 30 s, followed by 70% ethanol for 60 s, and rinsed in sterile distilled water three times for 10 s each. The segments were allowed to dry on sterile paper under a laminar flow hood and placed on PDA in Petri plates. Petri plates were incubated at 25°C for 7 d in darkness. Single spore cultures were then prepared (Arzanlou *et al*., 2007) and preserved on potato carrot agar slants (PCA; extract from 20 g potatoes and 20 g carrots, 15 g agar, 1,000 mL distilled water) at 4°C in the Culture Collection of Tabriz University (CCTU).

Morphological description

Cultural features of isolates including colony colour, shape and growth rates were assessed on PDA, malt extract agar (MEA, 3%) and oat meal agar (OA; all Sigma Aldrich) after 7 d of incubation at 25°C in darkness. For microscopic characteristics, the Riddell slide culture technique (Riddell *et al*., 1950), was used. Thirty measurements of each microscopic structure were made and photographs of relevant traits were taken using an Olympus digital camera (DM-21) mounted on a light microscope (Olympus BX41, Olympus Optical Co).

Pathogenicity assay

To fulfill Koch's postulates and evaluate the aggressiveness of *P. lythri* isolates, the pathogenicity of 20 *P. lythri* isolates [eighteen isolates from strawberry and two from olive (Table 2, Figure 1)] was assessed on detached untreated fruits of *Fragaria* × *ananassa* cv. Elsanta. The fruits were surface-disinfected by dipping in 1% sodium hypochlorite, followed by 70% ethanol for 30 s, and rinsed three times in sterile distilled water. They were then allowed to dry on sterile paper under a laminar flow hood. Five fruits were inoculated with each isolate by spraying 300 µL of an aqueous conidial suspension (1×10^6 conidia mL^{-1}) containing Tween 20 (0.1%; v/v). Sterile distilled water with 0.1% Tween 20 was used for the untreated controls. After 7 d, disease incidence was calculated as the percentage of infected fruits and disease severity was assessed based on the index recommended by Haung *et al*. (2011) with some modifications. A 0 to 8 scale was used, where 0 = healthy fruit and infection rates were classified as 1 = 12%; 2 = 25%; 3 = 37%, 4 = 50%; 5 = 62%; 6 = 75%; 7 = 87%; 8 = 100%).

DNA extraction, amplification and sequencing

Single-spore cultures developed on PDA were used for DNA extraction, using the protocol of Möller

Table 2. Sampling areas and their distances (km), with sample codes of isolates obtained from symptomatic strawberry in Iran.

Area (county)	Chenareh (San[a])	Noshur (K[b])	Paygelan (Sar[c])	Sou (San)	Tazabad (San)	Tarom (Zan[d])	Isolate (CCTU[e])
Chenareh	-	17.28	33.47	6.44	15.82	263.18	F[f]Ch8[g]
							FCh12
							FCh13
							FCh14
							FCh18
							SCh19
							SCh20
							SCh22
							SCh23
							LCh29
							LCh30
Noshur		-	34.03	14.88	1.88	283.87	PN6
							SN6
Paygelan			-	27.81	33.06	291.22	FP2
Sou				-	13.07	268.76	FS6
Tazabad					-	277.88	FT1
							FT2
							FT7
Tarom (Zan)						-	1200
							OLV2

[a] San: Sanandaj; [b] K: Kamyaran; [c] Sar: Sarvabad; [d] Zan: Zanjan; [e] CCTU: Culture Collection of Tabriz University. [f] The first letter in the isolate code following CCTU refers to plant parts as F, fruit; C, crown; P, petiole; S, stem; L, leaf; [g] The second/third letters indicate the sampling areas.

et al. (1992). The DNA concentration of each isolate was measured using a Nano-Drop nd-1000 spectrophotometer (Nano Drop Technologies) in three replications, and suitable dilutions were then prepared. In the polymerase chain reaction (PCR), the partial regions of two loci including ITS-rDNA and LSU-rDNA genes were amplified using, respectively, primers of ITS1+ITS4 (White *et al*., 1990) and LR0R + LR7 (Vilgalys and Hester, 1990; Rehner and Samuels, 1994). All PCR reactions were each carried out in a total volume of 25 µL, consisting of 50 ng genomic DNA, 1 × Dream Taq Green PCR Mastermix (Fer-mentas) and 0.2 µM of each primer. The LSU and ITS PCR conditions were as described, respectively, by de Gruyter *et al*. (2009) and Woudenberg *et al*. (2009). Amplicons were separated on 1% agarose gel stained with ethidium bromide and their sizes were determined in comparison to the GeneRuler molecular marker (GeneRuler™, #SM0333). For sequencing, PCR products were purified with Exo-Sap enzyme (Euroclone S.p.a.), according to the manufacturer's instructions. DNA amplicons were then sequenced using a BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Phylogenetic analysis

Assembled sequences were generated using both forward and reverse ABI raw trace files with the

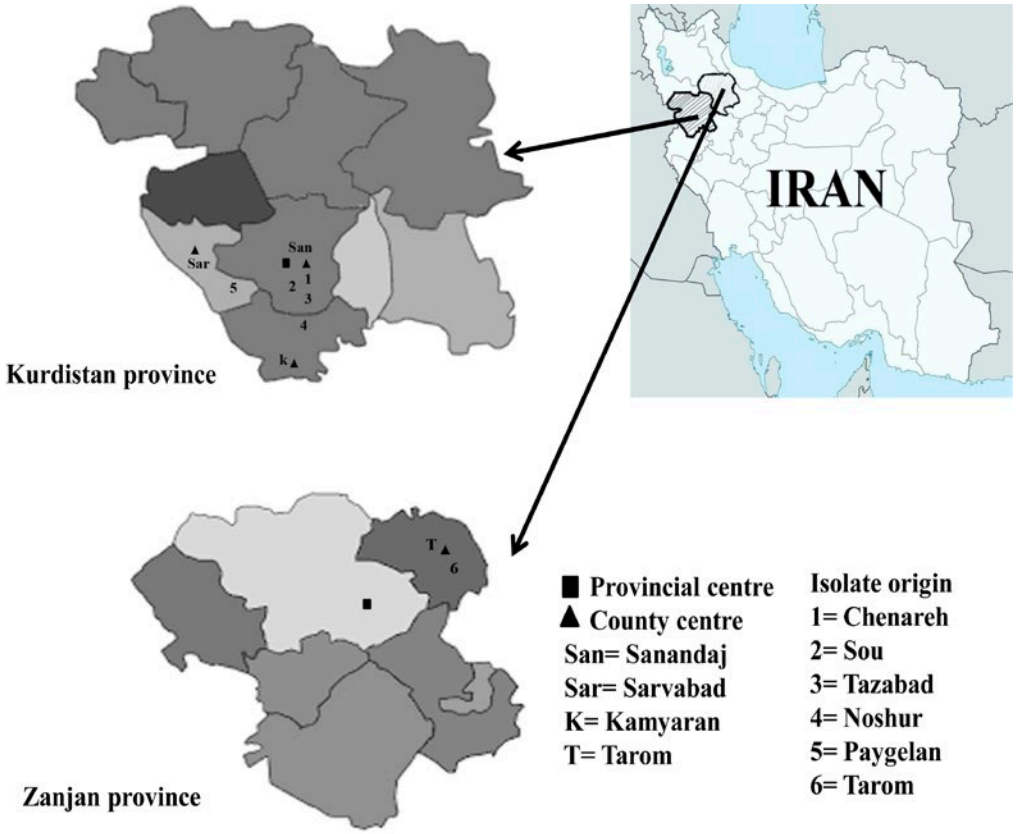

Figure 1. Sampling sites in Kurdistan and Zanjan provinces of Iran for *Pilidium lythri*.

Staden program, version 2.0.0b9-src.tar.gz (Staden, 1996). Assembled sequences were used as a query for BLAST (Mega BLAST from NCBI) in GenBank. Subjects with a high similarity and ex-type and ex-epitype strains were then downloaded from GenBank (Table 1). All downloaded sequences, along with the sequences obtained in this study, were individually and separately aligned for each ITS and LSU gene, using Muscle implemented in Mega5 (Tamura *et al*., 2011) and checked manually. The best-fit evolutionary model for each aligned gene file was selected using MrModelTest software, v. 2.3 (Nylander, 2004). For a limited dataset, ITS and LSU datasets were then concatenated using Mesquite software (Maddison and Maddison, 2011). Bayesian inference (BI) was used to build phylogenetic trees with MrBayes v. 3.2.1 (Ronquist and Huelsenbeck, 2003). We ran two distinct BIs, for ITS datasets individually and concatenated datasets including ITS and LSU. For both BIs, the heating parameter was set at 0.15 and four Markov Chain Monte Carlo (MCMC) chains were run, starting from random trees for 1 million generations, with trees sampled every 1,000 generations. The first 25% of saved trees were discarded as burn-in. Consensus tree and posterior probabilities (PP) were determined from the remaining trees, and inspected and printed using FigTree v1.4.2. For both analyses, gaps were treated as missing data. Trees obtained from both ITS and multi-gene analyses were rooted with *Chaetomella raphigera* voucher BPI 843541 (Arzanlou *et al*., 2013).

Random amplified polymorphic DNA (RAPD) and M13 analyses

In order to assess infra-species diversity between the *P. lythri* isolates obtained from strawberry in this study and to compare them with two strains originating from olive (Table 2, Figure 1), RAPD and M13 fingerprinting were used to reveal patterns of variation.

With both methods, PCR reactions were performed in volumes of 15 μL, consisting of 50 ng genomic DNA, 2 × Dream Taq Green PCR Mastermix (Fermentas) and 0.2 μM of each primer. For RAPD and M13 analyses the primers were as follows; OPA 01 (5′- CAGGCCCTTC-3′); OPA 13 (5′-CAGCACCCAC- 3′); OPB 01 (5′-GTTTCGCTCC-3′); OPB 10 (5′-CTGCTGGGAC-3′) and M13 minisatellite (5′-GAGGGTGGCGGTGGTTCT-3′) as a representative primer in M13 analysis. The thermal cycles in RAPD were adjusted for initial denaturation at 94°C for 5 min, followed by 45 cycles at 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 5 min (Trkulja and Hristov, 2012). For M13 minisatellite markers, an amplification cycle was designed as described by Queiroz *et al*. (2013). The multiple bands amplified were separated by electrophoresis on 1.5% agarose gel in 1 × Tris-acetate-EDTA buffer. Gels were stained with ethidium bromide and pictured under UV light. Amplicon sizes were determined by comparison with a 1 kb DNA ladder (Cat. No. PR901645, CinnaGen). All amplifications were repeated at least twice to ensure reproducibility of results. Distinct and reproducible bands were considered for analysis of the DNA profiles.

Statistical analyses

Parametric data in the pathogenicity test were analysed using a one way ANOVA with SAS software (SAS Institute, Inc., 2003). Least Significant Difference (LSD) was used for mean comparison. Microsoft Office Excel 2007 was used to draw the graphs.

Results

Fungal isolates

During the sampling process a total of 113 fungal isolates were isolated in association with the tan-brown and black spot symptoms on strawberry. Of these, based on shape and growth rate of the colonies, shape and size of conidia and conidiomata, 18 *P. lythri* isolates were initially grouped together on morphological grounds. One isolate was selected for each group. The selected isolates originated from the three counties of Sanandaj, Sarvabad and Kamayaran and specifically from the Chenareh (eleven isolates), Noshur (two isolates), Paygelan (one isolate), Sou (one isolate) and Tazabad areas (three isolates) (Figure 1). The majority of the isolates (ten) originated from fruits, with five from stems, two from a leaf and one from a petiole (Table 2, Figure 1). *Pilidium* isolates comprised 18 of the total 113 isolates obtained in this study (i.e. 16% of all isolates). For sampling, 27 strawberry fields were inspected (Chenareh (four fields), Noshur (seven fields), Paygalan (eight fields), Sou (five fields) and Tazabad (three fields).

Morphological descriptions

The symptoms in the field associated with the isolation of *P. lythri* were sunken tan-brown spots on fruits, similar to those with anthracnose infection. However, unlike anthracnose fruit rot with salmon-orange conidial masses, pink conidial masses were produced on the lesion surfaces (Figure 1c). Visible symptoms on stems were necrotic lesions, and on leaves were tan-brown spots.

Colonies on all media tested, were flat, circular, with sparse aerial mycelium, regular edges, white to cinnamon colour on PDA and white to buff on MEA or OA. Occasionally, a creamy to brown circle appeared around the centre of each colony (Figure 2d, e and f). Profuse stalked sporodochia but no pycnidia were produced on all growing areas in buff (PDA) to creamy colour (MEA and OA), with slimy conidial masses (Figure 2g). Sporodochia were discoid, cupulate to hemispherical (Figure 2g). Conidiophores were hyaline, smooth-walled, septate and 30 μm long. Conidiogenous cells (phialides) were 8–12 × 2.0–2.5 μm in size, hyaline, smooth-walled and cylindrical to ampulliform (Figure 2h). Conidia were unicellular, allantoid, slightly curved, pointed on both sides, hyaline and smooth (Figure 2i). The sexual stage was not observed. The growth rates of colonies and conidial size of the isolates are presented in Table 3.

Pathogenicity assay

The pathogenicity assay (disease incidence on inoculated fruits) showed that all isolates, including strains obtained from olive, caused tan-brown spots on all of the inoculated strawberry fruits (Figure 2a and b). No statistically significant differences were observed between the isolates treated in terms of disease severity ($df = 19$; $F = 1.23$; $P=0.253$), including the isolates from olive, and mean disease severity was ranged from 46 to 69% for the isolates examined (Figure 3).

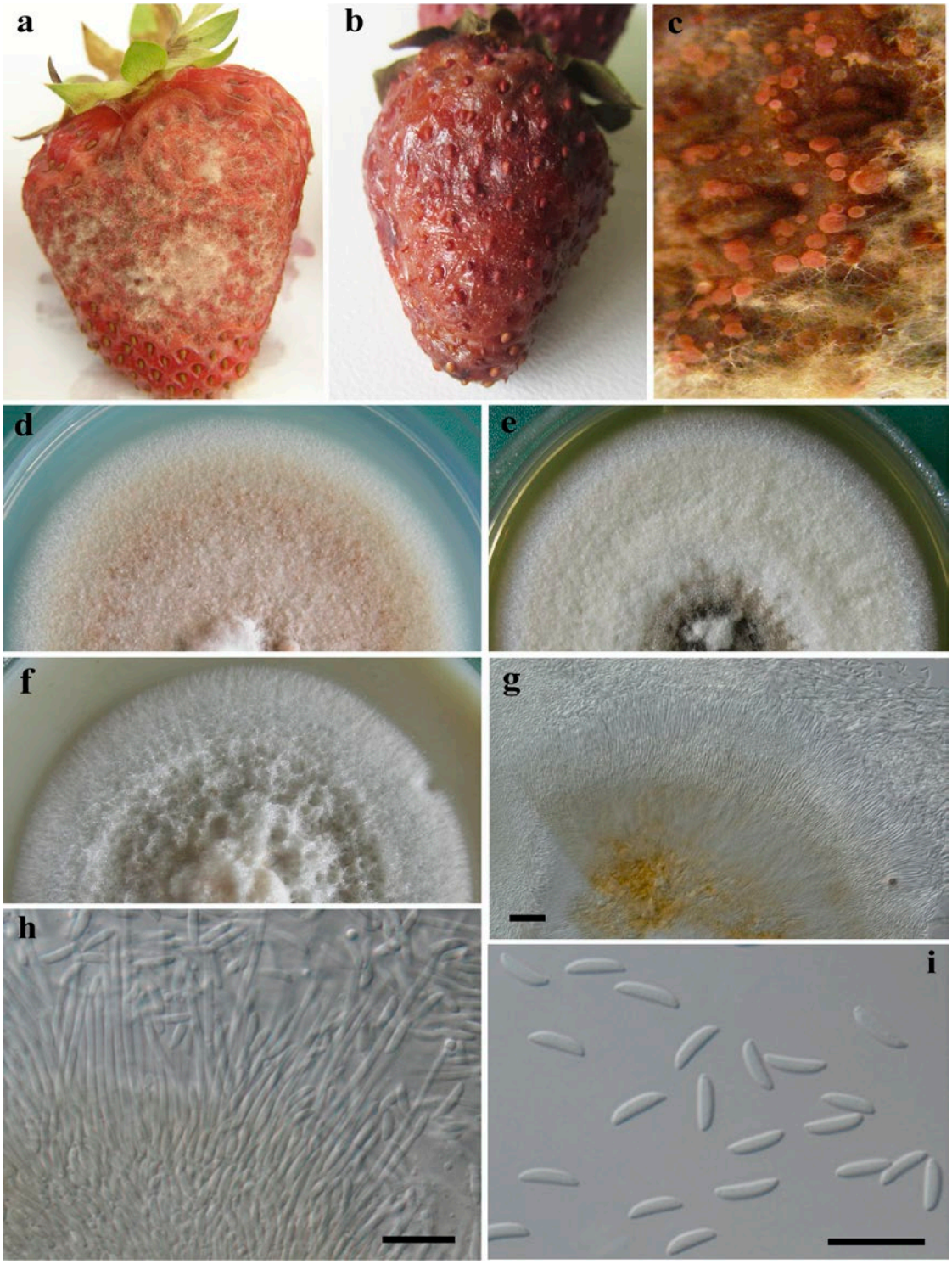

Figure 2. Morphological characterisation of the *Pilidium lythri* strain CCTU PN6. a and b, Tan-brown fruit rot induced in pathogenicity test by CCTU PN6 and CCTU 1200. c, Sporodochia formed on fruit tissue. d to f, Colony shape and colour on PDA, MEA and OA. g, Sporodochia on PDA. h, Conidiophores and conidiogenous cells. i, Conidia. Scale bars = 20 μm for g and 10 μm for h and i.

Table 3. Colony growth rates (mm diameter after 7 d) of *Pilidium lythri* strains on potato dextrose (PDA), oatmeal (OA) and malt extract agar (MEA), and conidium dimensions.

Isolate (CCTU)	PDA (mm)	MEA (mm)	OA (mm)	Conidial size on PDA (µm)
CCTU FCh13	50-51	49-50	48-49	(6.5–)7.3–8(–8.5) × (1.5–)1.8–2.0(–2.2)
CCTU FS6	51-55	50-53	46-48	(6–) 6.7 – 7.2 (–8) × (1.3–) 1.7 – 2.0 (–2.3)
CCTU LCh29	51-55	50-52	47-49	(5.5–) 6.5 – 7.0 (–8.1) × (1.5–) 1.6 – 1.7 (–2.0)
CCTU SCh19	47-50	46-49	44-47	(6–) 6.7 – 7.0 (–7.9) × (1.2–) 1.7 – 1.8 (–2.0)
CCTU FCh8	44-46	44-45	45-44	(7.7–) 7.2 – 7.6 (–8.3) × (1.5–) 1.7 – 1.9 (–2.1)
CCTU LCh30	52-53	52-54	46-48	(6.9–) 7.4 – 7.8 (–8.3) × (1.4–) 1.75 – 1.9 (–2.1)
CCTU SCh20	50-51	50-52	45-46	(6.7–) 6.9 – 7.27 (–8.3) × (1.6–) 1.9 – 2.2 (–2.5)
CCTU SCh23	47-49	46-48	42-43	(5.2–) 6.4 – 6.8 (–7.4) × (1.1–) 1.7 – 1.9 (–2.5)
CCTU FP2	45-46	44-45	42-43	(7–) 7.75 – 8.11 (–8.5) × (1.8–) 2.1 – 2.2 (–2.5)
CCTU PN6	45-46	44-47	42-43	(6.8–) 7.28 – 7.5 (–7.3) × (1.4–) 1.8 – 1.9 (–2.0)
CCTU FCh12	40-41	42-44	41-42	(6.7–) 7.2 – 7.5 (–8) × (1.6–) 1.9 – 2 (–2.3)
CCTU FCh14	49-49	48-49	44-45	(5.1–) 6.2 – 6.8 (–7.8) × (1.3–) 1.6 – 1.8 (–2.1)
CCTU FT1	45-47	44-46	40-42	(5.5–) 7 – 7.5 (–8.3) × (1.6–) 1.8 – 1.9 (–2.3)
CCTU SCh22	45-45	46-47	42-43	(5.6–) 6.3 – 6.8 (–7.6) × (1.3–) 1.5 – 1.7 (–1.9)
CCTU FT2	45-46	45-47	44-46	(6.6–) 6.9 – 7.21 (–7.9) × (1.5–) 1.8 – 1.9 (–2.1)
CCTU FT7	47-48	45-46	43-44	(6.5–) 7 – 7.3 (–7.9) × (1.6–) 1.9 – 2.1 (–2.5)
CCTU FCh18	50-55	52-53	43-46	(6.8–) 7.2 – 7.6 (–8.2) × (1.5–) 1.8 – 2.0 (–2.4)
CCTU SN6	45-46	44-46	41-42	(6.4–) 6.9 – 7.2 (–7.6) × (1.7–) 1.9 – 2.0 (–2.2)
CCTU FN6	46-47	45-46	41-43	(6.8–) 7.5 – 7.9 (–8.3) × (1.5–) 1.9 – 2.1 (–2.4)

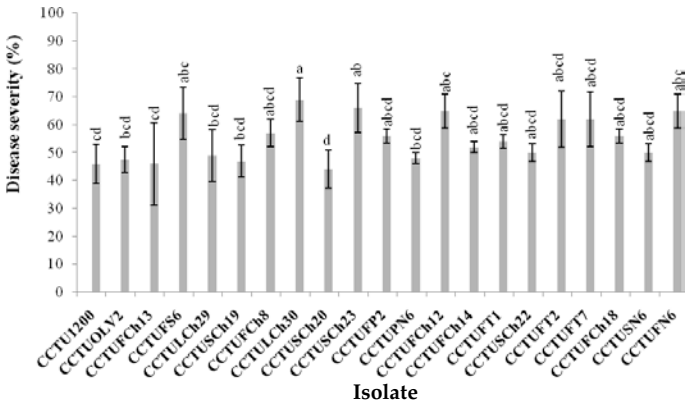

Figure 3. Mean severity of tan-brown rot caused by *Pilidium lythri* isolates from Iran on *Fragaria* × *ananassa* cv. Elsanta fruits in pathogenicity assays. CCTU 1200 and OLV2, strains obtained from olive, the other strains are from strawberry. NC, negative control. Data analysed using one-way ANOVA (df = 19; $F = 1.23$; P=0.253); error bars indicate the standard errors of means. Different letters show least significant difference at P>0.05.

Phylogenetic analysis

For phylogenetic analysis of ITS, seven strawberry isolates were used, and for concatenated ITS and LSU sequences one strawberry isolate was used. The final aligned ITS dataset contained 23 in-group taxa, with a total of 410 characteristics containing 63 unique site patterns. Concatenated ITS and LSU datasets contained only seven in-group taxa (because of missing LSU sequences for the majority of lodged species in GenBank), with a total of 1,709 characteristics (gene boundaries, ITS: 1–411 and LSU: 412–1,709), including 104 unique site patterns (ITS: 53 and LSU: 51). Using MrModeltest v. 2.3, we found the models of JC+G as the most fitting replacement model for ITS and HKY+G for LSU. ITS phylogeny located all Iranian isolates examined in the *P. lythri* clade with high posterior probability, including different strains of *P. lythri* originating from various hosts and regions (Figure 4). Analysis of concatenated sequences revealed again two distinct monophyletic groups with highest posterior probability. One contained the ex-epitype strain of *P. lythri* and ex-type strain of *P. pseudoconcavum,* separated from another group containing the ex-epitype strain of *P. acerinum* and ex-type strain of *P. eucalyptorum,* as a sister group (Figure 5). Sequences derived from this study were deposited at NCBI's GenBank nucleotide database (http://www. ncbi.nlm.nih.gov; Table 1).

RAPD and M13 fingerprinting

In RAPD analyses, DNA patterns made by the OPA01 primer had to be excluded because of failure of amplification of some isolates. For the rest of the primers, including three RAPD primers (OPA13, OPB01, OPB10) and the M13 minisatellite, no infraspecies variation was revealed among the 20 *P. lythri* isolates originating from strawberry and olive. For all primers, similar DNA patterns at each locus were recorded for all isolates examined from strawberry

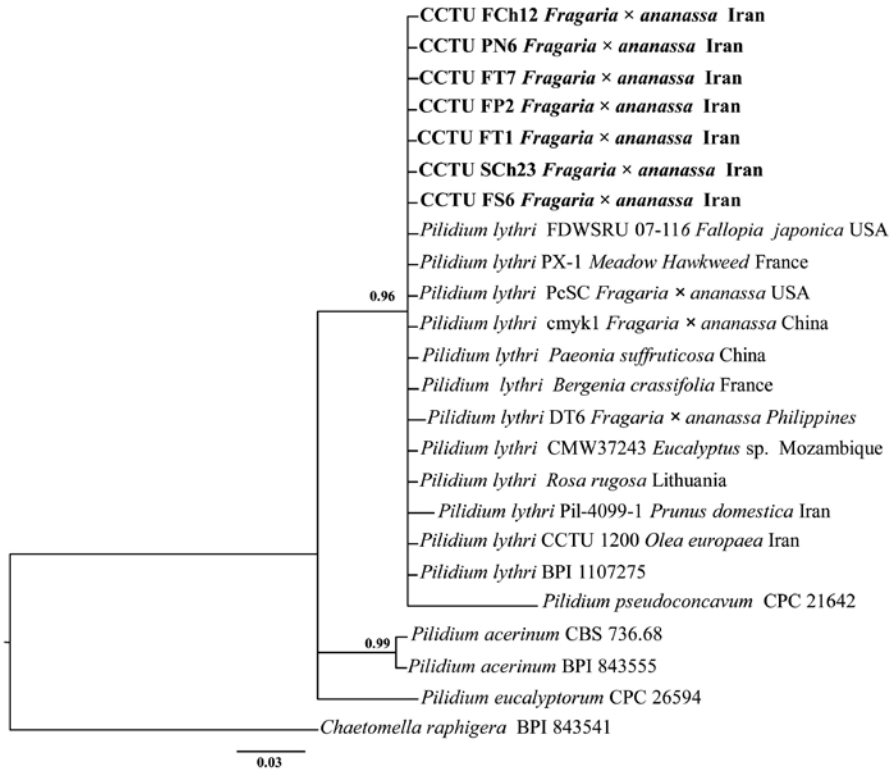

Figure 4. ITS phylogeny of different strains of *Pilidium lythri,* including the isolates obtained in this study, using Bayesian inference based on the JC+G model. The tree was rooted with *Chaetomella raphigera* voucher BPI 843541. The scale bar shows 0.03 expected changes per site. The strawberry isolates used for phylogenetic analysis in this study are in bold type.

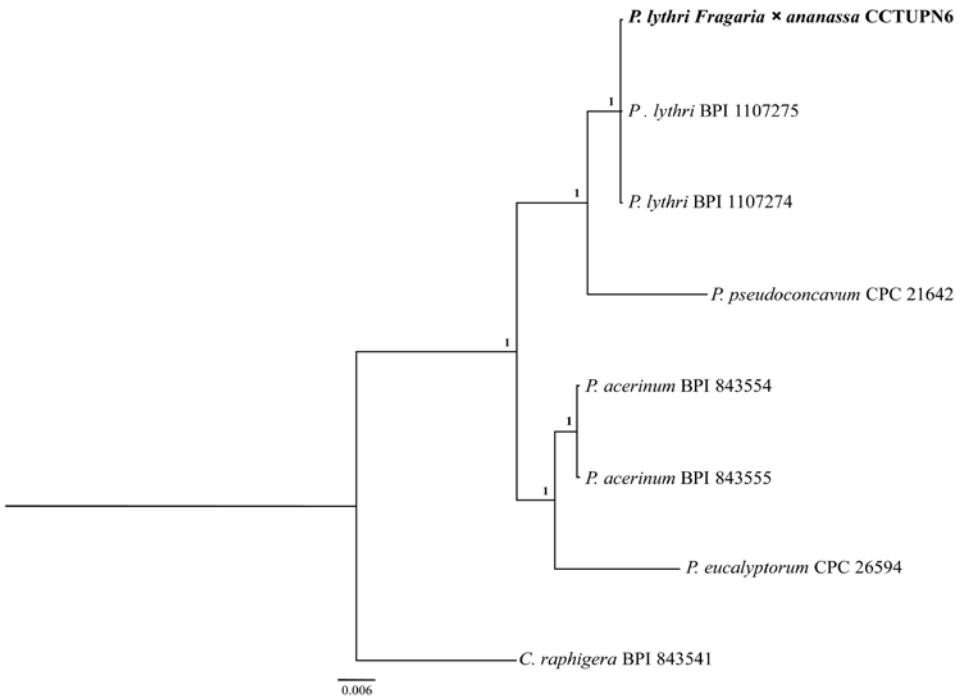

Figure 5. Bayesian tree generated for concatenated sequences of ITS and LSU including ex-epitype and ex-type strains of *Pilidium* species based on the models of JC+G and HKY+G respectively. Our isolate (CCTU PN6) is shown in bold font. The scale bar shows 0.006 expected changes per site. The tree was rooted by *Chaetomella raphigera* voucher BPI843541. The figures on the nodes indicate posterior probability.

and olive. Amplicon sizes ranged from 600 to 2,000 bp for OPA13, 600 to 2,750 bp for OPB01, 400 to 2,500 bp for OPB10 and 600 to 1,500 bp for the M13 minisatellite (Figure 6a to d).

Discussion

The occurrence of *P. lythri* in strawberry is reported for the first time in Iran, and 18 representative isolates are characterised. The morphological characteristics of all the isolates were in full agreement with the descriptions of *P. lythri* (synanamorph *H. lythri*) (Palm, 1991; Rossman *et al*., 2004). Only sporodochia were formed on all media tested and pycnidia were absent, in agreement with previous reports of Pilidium rot on strawberry and other hosts (Cardin *et al*., 2009; Lopes *et al*., 2010; Bruckart *et al*., 2013; Fernández-Ortuño *et al*., 2014). In a sequence analyses for the ITS and LSU regions, LSU BLAST of our representative isolate (CCTU PN6) showed complete similarity (100%) with the ex-epitype strain of *P. lythri* (BPI 1107275 and BPI 1107274) and *P. lythri* (CCTU1200), while ITS BLAST analysis showed complete similarity with various *P. lythri* sequences lodged in GenBank. In addition, the identity of our isolates was further corroborated in phylogenetic analysis using ITS-rDNA, individually and with concatenated combined datasets of ITS and LSU rDNA: all isolates were placed in the *P. lythri* clade (Figures 4 and 5). As in the ITS phylogeny, concatenation with LSU sequences segregated each species. Two species are each represented by only one isolate, *P. pseudoconcavum* grouping basal to *P. lythri*, and *P. eucalyptorum* basal to *P. acerinum* (Figure 5). In accordance with the morphological features, the two species *P. lythri* and *P. pseudoconcavum,* which are known to produce sporodochia in culture (Rossman *et al*., 2004; Crous *et al*., 2013), were in a single clade as a sister group of two other species, *P. acerinum* and *P. eucalyptorum*, which both produce pycnidia in culture (Rossman *et al*., 2004; Crous *et al*., 2015). The ITS tree confirmed the placement of the isolates

examined, together with strains isolated from various hosts and of different geographical origins (sequences deposited in GenBank) in the *P. lythri* clade (Table 1, Figure 4). Since there are more reports associated with diseases caused by *P. lythri* than of other *Pilidium* spp., *P. lythri* has a wider host range, is more widespread, and is not a specific parasite of a single plant species. This reflects its flexibility (host range and geographical distribution). The genus is in the Helotiales, which are one of the largest non lichen-forming Ascomycete groups. This includes a wide spectrum of fungi such as plant pathogens, endophytes, mycorrhizae, ectomycorrhizal parasites, fungal parasites, terrestrial saprobes, aquatic saprobes, root symbionts and wood rot agents, which develop in various ecosystems and occupy a wide range of niches (Wang *et al*., 2006).

Pilidium was previously only known to have two species, namely *P. lythri* and *P. acerinum* (Rossman *et al*., 2004; Kirk *et al*., 2008), but in recent years *P. pseudoconcavum* and *P. eucalyptorum* have been found on *Greyia radlkoferi* and *Eucalyptus robusta* (Crous *et al*., 2013, 2015). Among the hosts reported for *P. lythri*, strawberry and olive (Lopes *et al*., 2010; Arzanlou *et al*., 2013) are of economic significance worldwide. *Pilidium lythri* has been found to cause tan-brown leaf spot on strawberry in some countries (Table 1), and also fruit rot of *P. domestica* and *O. europaea* in northern and north-west Iran (Sayari *et al*., 2013; Arzanlou *et al*., 2013). To the best of our knowledge, this is the first report of *P. lythri* as the causal agent of tan-brown spot on strawberry plants in Iran.

In the pathogenicity assay, no significant differences between *P. lythri* isolates were detected for disease incidence or severity (Figure 3). This indicates that *P. lythri* can easily infect strawberries and represents a serious additional potential problem for the strawberry industry in Iran. *Pilidium lythri* isolates obtained in this study represented 16% of the all isolates obtained. Arzanlou *et al*. (2013) reported a 2.4% occurrence of olive fruit rot caused by *P. lythri* in seven orchards inspected in Iran. These percentages show that the disease is progressing, probably through infected plant material, where the fungus acts as an opportunistic non-specific pathogen (Table 1).

The molecular tools used in this study, including RAPD (Figure 6a, b and c) and M13 (Figure 6d) analyses, did not detect any polymorphism among the isolates. Furthermore, homogeneity was confirmed for isolates from olive with those from strawberry, although the sampling sites were more than 250 km apart in the provinces of Kurdistan (strawberry) and Zanjan (olive) (Figure 1). The primers used in RAPD fingerprinting have previously shown high rates of polymorphism in other fungal populations such as *Cercospora beticola*, *Macrophomina phaseolina* and *Botrytis cinerea* (Álvaro *et al*., 2003; Moyano *et al*., 2003; Trkulja and Hristov, 2012). The most differentiating M13 minisatellite marker has been successfully used to study populations of *Cryptococcus gattii, C. neoformans, Lactobacillus* spp., *Mycosphaerella fijiensis* and *Togninia minima* (Ulrich *et al*., 2009; Liaw *et al*., 2010; Mercanti *et al*., 2011; Queiroz *et al*., 2013; Arzanlou and Narmani, 2016), confirming similar RAPD results. The results for molecular markers in this study further corroborate the homogeneity observed in the phylogenetic analysis and in pathogenicity testing. Given the high homogeneity detected in isolates examined in this study, two hypotheses can be drawn: i) either *P. lythri* populations propagate clonally, and mechanisms creating variations such as sexual, parasexual and mitotic recombination are not significant among individuals, or ii) with reference to recent reports of this pathogen on several crops in Iran (Arzanlou *et al*., 2013; Sayari *et al*., 2013), the pathogen inoculum has only recently been introduced, having a single origin and a wide host range. In any case, the large-scale monoculture of a susceptible host plant is bound to attract more new diseases.

In conclusion, Iranian strawberry production will have to face the new challenge posed by the introduction of this new pathogen. Although genetic diversity and disease incidence (16%) are still low, the possible appearance of new more aggressive pathotypes adapted to local climatic conditions cannot be excluded because of the polyphagous habit of this species. Therefore, the adoption of quarantine programmes in *P. lythri*-free areas, selecting the most effective fungicides and utilization of cultivars with resistance/tolerance are critical measures to prevent spread of the disease.

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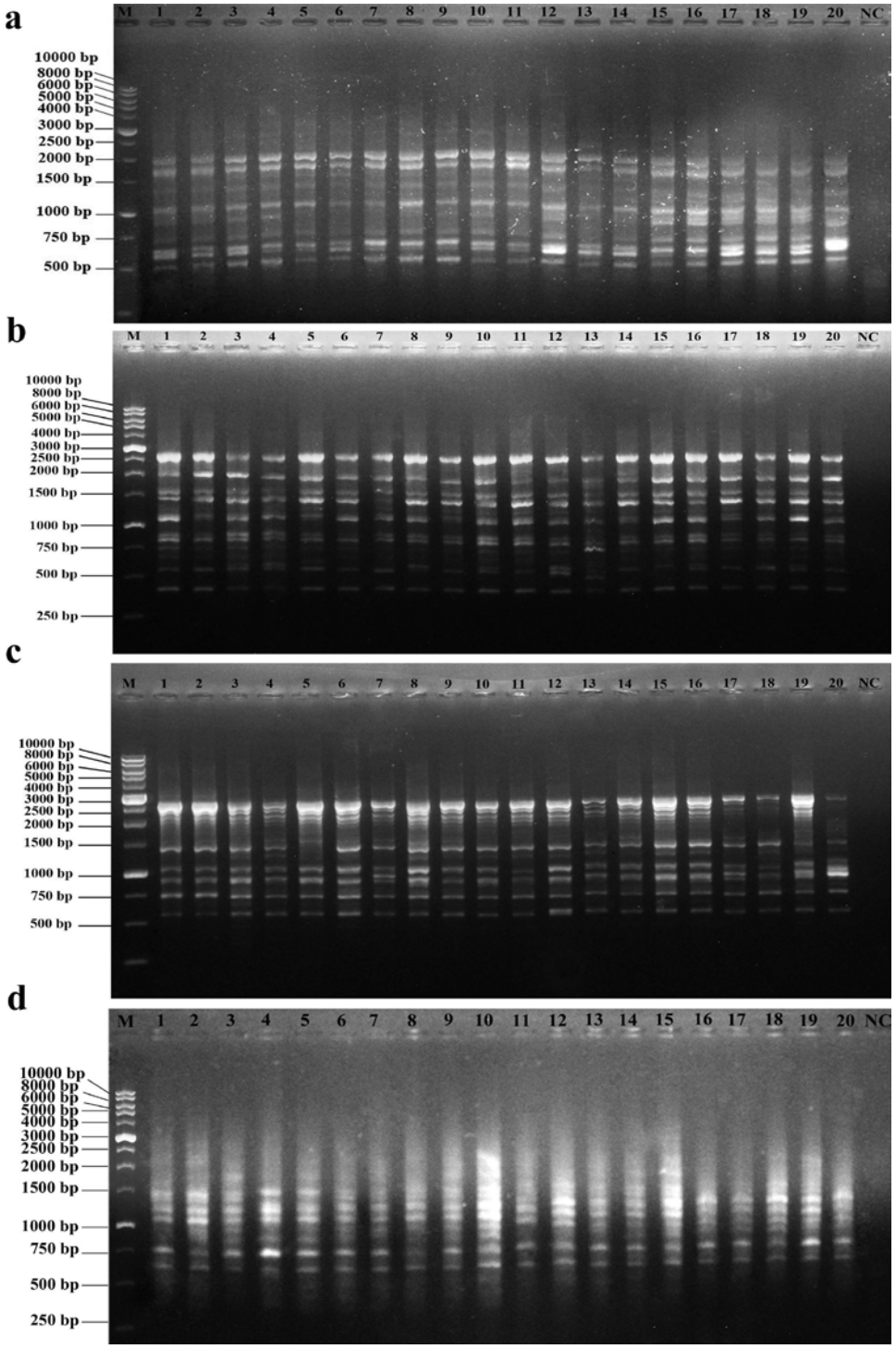

Figure 6. DNA fingerprinting of *Pilidium lythri* isolates by four primers of OPA13 (a), OPB10 (b), OPB01 (c), M13 minisatellite (d), in RAPD and M13 analyses. Lane M, 1Kb DNA ladder represents 13 fragments ranging from 250 bp to 10,000 bp. Lanes 1–2, isolates obtained from olive CCTU 1200 and CCTU OLV2. Lanes 3–20, isolates from strawberry (CCTU FCh13, CCTU FS6, CCTU LCh29, CCTU SCh19, CCTU FCh8, CCTU LCh30, CCTU SCh20, CCTU SCh23, CCTU FP2, CCTU PN6, CCTU FCh12, CCTU FCh14, CCTU FT1, CCTU SCh22, CCTU FT2, CCTU FT7, CCTU FCh18, CCTU SN6). NC, negative control.

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