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Nanotools for molecular identification two novels *Cladosporium cladosporioide*s species (Cladosporiaceae) collected from tomato phyloplane

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Magnetic nanoparticles (MNPs) consisting of magnetite (Fe_3O_4) are promising as nanodiagnostic tools in plant pathology. Magnetic nanoparticles (MNPs) were produced using hydrothermal protocols. MNPs size diameter and size distribution were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Fe_3O_4 nanoparticles are monodispersible and spherical with an average diameter of 82 nm. Dynamic light scattering analysis of the same samples revealed that the synthesized MNPs were highly monodispersed and had a hydrodynamic diameter ranging from 100 to 201. Fungal DNA was extracted using MNPs in comparison with the conventional sodium dodecyl sulfate (SDS) method in the context of quality, quantity and timing process. The quality and yields of the isolated DNA from all *Cladosporium* strains using magnetic nanoparticles were higher compared to the DNA isolation method via sodium dodecyl sulfate (SDS). PCR using specific primers targeting ITS and Actin genes were amplified 100% of varying sized gene fragments, verifying the high quality of the isolated DNA. Forty seven fungal isolates belonging to the *Cladosporium cladosporioides* complex were observed and phylogenetically evaluated on the basis of DNA sequences of the internal transcribed spacer regions ITS1 and ITS2, in addition to partial actin and translation elongation factor 1-α gene sequences. Maximum likelihood phylogenetic analyses were performed for the combined data set (ITS + ACT + TEF) using RAxML. The three *Cladosporium* strains were isolated from tomato in Saudi and Egypt was identified very similar to *Cladosporium asperulatum* and *Cladosporium myrtacearum* based on their molecular phylogenetic characteristics. DNA isolation using magnetic nanoparticles will expectedly be used commonly both in plant pathology laboratories and in the nanobiotechnology industry.

Key words: *Cladosporium*, magnetic nanoparticle, DNA recovery.

INTRODUCTION

Cladosporium is one of the largest and most heterogeneous genera of hyphomycetes which comprise 189 species (Bensch et al., 2015; Crous et al., 2014). *Cladosporium* species are also known to be general as

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endophytic fungi (Riesen and Sieber, 1985; El-Morsy, 2000) as well as phylloplane fungi (Islam and Hasin, 2000; Levetin and Dorseys, 2006). The genus including certain species are important as potential biocontrol agents for plant diseases (Köhl et al., 2015) or, in the food industry, as fruit contaminants causing spoilage in low temperature storage or on cereals such as barley, oat, rye and wheat (Kulik et al., 2014; Frasz and Miller, 2015). Concomitant with other plant pathogenic fungi (Agrios, 2005; Bensch et al., 2012). In this genus, *C. cladosporioides* is a very common, cosmopolitan, saprobic species and has been applied to several taxa that have been demonstrated as distinct in recent decades (Bensch et al., 2012). The phylloplane, or leaf surface, represents an important terrestrial habitat that harbours a wide range of microorganisms (Lindow and Brandl, 2003). Filamentous fungi from the phylloplane may be either parasites, saprophytes, endophytes or epiphytes*. Alternaria alternata, A. citrifolia, Aspergillus niger, A. flavus, A. candidus, A. nidulans, Penicillium expansum, Curvlaria lunata, Cladosporium cladosporioides, C. herbarum, Fusarium oxysporum, Trichoderma harzianum and Rhizoctonia solani* was isolated from tomato leafs (Beenish and Paul, 2016). Accurate identification of plant pathogenic fungi by morphological methods may take quite a few days and expert researchers therefore need rapid detection tools that can provide results within a few hours. To achieve these detection methods, plant pathologists are working with nanotechnologists to develop quick and better detection systems (Sharon et al., 2010). DNA has also become a preferred material for nanotechnologists because of its unique properties of structural stability, programmability of sequences, and predictable self-assembly (Zahid et al., 2013). Magnetic nanoparticles (MNPs) consisting of magnetite (Fe3O4) are promising as nanodiagnostic tools in plant pathology (Alghuthaymi et al., 2016; Khiyami et al., 2014)**.** In previous study, an attempt was made to develop a method of DNA extraction protocol from *R. solanacearum* and infested potato tubers based on the use of synthesized magnetite nanoparticles, using few microliters of bacterial ooze and cell lysate as the starting material in the presence of polyethylene glycol (PEG)/NaCl and RNAse A (Alghuthaymi et al., 2016). The magnetic nanoparticle offers a valuable technique for the detection of *R. solanacearum* in potato tubers and pure culture which gives good yields of amplifiable DNA. MNPs can diffuse quickly in a reaction mixture and can then be easily collected by an external magnetic field (Deng et al., 2013). A comprehensive review of the literature regarding magnetic nanoparticle binding of DNA was explained by Pershina et al. (2014). In the current research, the MNPs were applied to adsorb DNA template after *Cladosporium* mycelium was lysed. The DNA binding MNPs were directly subjected to polymerase chain reaction (PCR) to amplify ITS and Actin specific sequence of *Cladosporium* strains, which can improve both the utilization rate of the DNA template and detection sensitivity. Analysis of data confirmed two novels *Cladosporium cladosporioide*s was identified in comparison with references isolates.

MATERIALS AND METHODS

Synthesis and characterize of magnetic nanoparticles

MNPs were produced by chemical co-precipitation of Fe^{2} + and Fe^{3} + ions in an alkaline solution, followed by treatment under hydrothermal conditions. The Fe_3O_4 nanoparticles were dried in a vacuum oven at 60°C for 24 h (Bandyopadhyay et al., 2011). The particles were characterized for size using transmission electron microscopy (TEM). Also, the average nanoparticle size and size distribution were verified by dynamic light scattering (Zetasizer, Malvern Instruments Ltd, Malvern, UK).

Isolation of fungal DNA using magnetic nanoparticles

Single-conidial isolates were obtained from tomato phyloplane and cultured as detailed in Crous (1998). Fungal isolates were grown on PDA for 3 to 4 weeks at 16°C in the dark and total genomic DNA was isolated from 50 to 100 mg of axenic mycelium scraped from the edges of the growing, mycelium was ground to a fine powder with liquid nitrogen culture using magnetic nanoparticles. One milliliter (1 mL) binding buffer (1.25 mol L^{-1} NaCl, 10% PEG-6000), also 4 µL RNAse A (10 mg mL^{-1}), were added and microtube was incubated for 10 min at 65°C. Thirty microliters (30 µL) of MNPs dissolved in 1% (w/v) sodium dodecyl sulfate solution was added. The nanoparticles should be freshly prepared, water dispersed and sonicated at 60 MHz for at least 20 min before use. Sodium acetate (140 µL) was added; the tube was mixed by gentle inversion three times and incubated at deep freezer for 5 min. Themagnetic pellets were immobilized using externalmagnetic (from outside the microtube to collect magnetic nanoparticles), and the supernatant discarded.

The magnetic nanoparticles were washed twice with 750 µL cold 70% (v/v) ethanol and dried at room temperature for 10 min. Finally, the magnetic particles were resuspended in 50 µL of TE buffer (50 mM Tris-HCl and 1 mM EDTA, pH 8.0) and the bound DNA was eluted at 65°C for 5 min with moderate agitation (Alghuthaymi et al., 2016). To compare the efficiency of DNA isolation method for MNPs, DNA was isolated from Cladosporium mycelium according to the protocol published by Moslem et al. (2010). The quality and concentration of DNA were assessed by agarose electrophoresis. The DNA solution was transferred into a new microcentrifuge tube and kept at -20°C until use. The Gel Doc 1000 system (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) was used for image capturing under UVillumination and the graphic files were exported as 8-bit TIFF images.

PCR amplification and DNA sequencing

PCR amplification was performed using universal fungal ITS rRNA genespecific oligonucleotide primers ITS1 (5′-TCCG TAGGTGAACCTGCGG-3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC-3′) (White et al., 1990), the actin gene (ACT) using the primers ACT-512F and ACT-783R (Carbone and Kohn, 1999). Each reaction contained 2 µL of template DNA (10 ng/µL), 0.2 µM from each primer, 1.5 mM MgCl2, 0.5 mM dNTPs and 0.5 µL of Taq DNA polymerase (Jena Bioscience, Germany) in 5 µL of the manufacturer's reaction buffer with a total of 50 µL reaction volume. The PCR reactions were run for 35 cycles

(each cycle is 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s) in a thermocycler (Techne TC-312, Techne, Stone, UK), with an initial hot start (94°C for 15 min) and a final extension (72°C for 10 min). PCR amplification was confirmed on 1.5% agarose electrophoresis gels stained with ethidium bromide. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacurer's instruction. The purified PCR products were directly sequence in both directions using the respective forward and reverse primers. The amplified PCR fragments were sent to a commercial sequencing provider (Shanghai Sangon Biological Engineering Technology and Services Co., Shanghai, P.R. China).

Phylogenetic analysis

Sequencing and sequence alignment

The combined TEF, Actin and ITS sequence data set using 50 *Cladosporium* strains with *Cladosporium xylophilum* as the outgroup taxon. The other sequences used in the analyses (Table 1) were obtained from GenBank based on blast searches and recently published data (Bensch et al., 2010). The multiple alignments were automatically done by MAFFT v. 7.036 (http://mafft.cbrc.jp/alignment/server/index.html; Katoh and Standley, 2013) using the default settings and latter refined where necessary, using BioEdit v. 7.0.5.2 (Hall, 2004).

Phylogenetic analyses

Maximum-likelihood (ML) analysis was performed in RAxML (Stamatakis, 2006) implemented in raxmlGUI v.0.9b2 (Silvestro and Michalak, 2012), employing mixed models of evolution settings of the program and Bootstrap support obtained by running 1000 pseudo replicates. The online tool Findmodel was used to determine the best nucleotide substitution (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) model for each partition. Maximum Likelihood bootstrap values (ML) equal or greater than 60% are given above each node in red (Figure 1). Phylograms were visualized with FigTree v1.4.0 program (Rambaut, 2012) and reorganized in Microsoft power point (2007) and Photoshop CS3 Extended 10.0 (Adobe Systems Inc, USA).

RESULTS

Properties of magnetic nanoparticles

Figure 1A shows the TEM image of Fe_3O_4 nanoparticles are monodispersible and spherical. The average diameter of magnetic nanoparticles is 82 nm. Dynamic light scattering analysis of the same samples revealed that the synthesized MNPs were highly monodispersed and had a hydrodynamic diameter ranging from 100 to 201 nm (Figure 2). The size distribution was recorded automatically by the software of these repeated measurements. When the external magnetic field was applied to the edge of the microtube for a few seconds, the magnetic nanoparticles were magnetized and were aggregated together. When gentle stirring without external magnetic field was applied, the aggregations were quickly redispersed again. The particles thus obtained exhibited a strong magnetic response.

DNA quality

The DNA yield was estimated by assessment of intensity of DNA bands in ethidium bromide stained agarose gel. The molecular weight of the isolated DNA was 3200 bp. It shows that our protocol yields high-quality DNA, which is transparent, non-viscous and no RNA was present. No smearing of DNA occurred on the gel, and the 100-bp DNA marker fragment was clearly visible, indicating that DNA degradation had not occurred (Figure 3). The quality and yields of the isolated DNA from all the samples using magnetic nanoparticles were higher to the DNA isolation method via sodium dodecyl sulfate (SDS). The quality of extracted fungal DNA was assessed by PCR amplification and sequencing of ITS and Actin genes.

Phylogenetic analysis

The combined gene data set of ITS, Actin and Tef consists of 47 strains of 34 taxa with the out group taxon. The phylogenetic trees (Figure 4) show that the *C. cladosporiodes* complex in this study segregates into 6 distinct clades. The phylogenetic tree also suggested that this KSA and Egypt strains might be close to *C. asperulatum* and *C. myrtacearum* as they formed a subclade, although without support. The two strains of *Cladosporium* species formed a sister clade with *C. asperulatum*.

DISCUSSION

Molecular data play a pivotal role in modern mycological taxonomy, but have some constraints in application. In recent years, the survey of unexplored habitats and sources by using molecular techniques has expanded our knowledge of fungal diversity (Sandoval-Denis et al., 2016). Although methods involving their magnetic separation have been extensively studied, there is currently a need for an efficient technique to isolate DNA for highly sensitive diagnostic applications. MNP protocol involves less steps, microcentrifuge tubes and time therefore is more cost-effective when compared with traditional techniques (Maeda et al., 2016). In the current research, the produced Fe_3O_4 nanoparticles are monodispersible and spherical with an average diameter of 82 nm. Dynamic light scattering analysis of the same samples revealed that the synthesized MNPs were highly monodispersed and had a hydrodynamic diameter ranging from 100 to 201. MNPs with diameters of 100 to 500 nm were prepared and carboxyl group, hydroxyl group and amino group modifications were achieved by appropriate chemical reactions (Zhao et al., 2012). The fungal DNA extracted by the traditional SDS extraction process displayed similar electrophoretic pattern of DNA fragments, but with lower yields, which is in agreement with a previous study (Basu et al., 2013; Zhou et al.,

Table 1. Reference sources of *Cladosporium* species isolates used in this study (46 isolates, 34 taxa).

Cladosporium species	Accession number	host	Country	Collector	GeneBank Numbers		
					ITS gene	TEF gene	ACT gene
C. acalyphae	CBS 125982*; CPC 11625	Acalypha australis	South Korea	H.D. Shin	HM147994,	HM148235,	HM148481
C. angustisporum	CBS 125983*; CPC	Alloxylon wickhamii	Australia	B.A. Summerell	HM147995,	HM148236,	HM148482
C. asperulatum	CBS 113744	Grape bud	U.S.A.: Washington	F.M. Dugan	HM147996,	HM148237,	HM148483
C. asperulatum	CBS 126339; CPC 11158	Eucalyptus leaf litter	India	W. Gams	HM147997,	HM148238,	HM148484
C. chubutense	CBS 124457*; CPC	Pinus ponderosa	Argentina	A. Greslebin	FJ936158,	FJ936161,	FJ936165
C. cladosporioides	CBS 113738	Grape bud	U.S.A.: Washington	F.M. Dugan	HM148004,	HM148245,	HM148491
C. cladosporioides	CBS 101367	Soil	Brazil	-	HM148002,	HM148243,	HM148489
C. cladosporioides	CBS 122130; ATCC	Bamboo slats	Japan	-	HM148008,	HM148249,	HM148495
C. cladosporioides	CPC 13734	Areca sp.	Thailand	I. Hidayat	HM148036,	HM148277,	HM148523
C. cladosporioides	CPC 14009; MRC 10150	Wheat	South Africa	-	HM148037,	HM148278,	HM148524
C. cladosporioides s. lat. lineage 1	CBS 116744	Leaves of Acer	Germany	L. Pehl	HM148053,	HM148294,	HM148540
C. cladosporioides s. lat. lineage 1	CPC 14296; BA1695	Indoor building	Denmark	B. Andersen	HM148056,	HM148298,	HM148543
C. cladosporioides s. lat. lineage 2	CPC 11664; Hill 1076-2	Oncoba spinosa	New Zealand	C.F. Hill	HM148058,	HM148300,	HM148545
C. cladosporioides s. lat. lineage 2	CBS 306.84	Urediniospores of Puccina alli	U.K.	G.S. Taylor	HM148057,	HM148299,	HM148544
C. cladosporioides s. lat. lineage 3	CBS 109082	Silene maritima	U.K.	A. Aptroot	EF679354,	EF679429,	EF679506
C. cladosporioides s. lat. lineage 4	CBS 113746	Bing cherry fruits	U.S.A.: Washington	R.G. Roberts	HM148061,	HM148303,	HM148548
C. cladosporioides s. lat. lineage 4	CPC 13978	Needles of Pinus	Argentina	A. Greslebin	HM148064,	HM148306,	HM148551
Cladosporium sp1	KSA-13	Tomato leafs	Egypt	Alghuthaymi	Submitted	-	Submitted
Cladosporium sp1	KSA-37	Tomato bract	Saudi	Alghuthaymi	Submitted	-	Submitted
Cladosporium sp2	KSA-85	Tomato leafs	Saudi	Alghuthaymi	Submitted	-	Submitted
C. colocasiae	CBS 119542; CPC 12726;	Colocasia esculenta	Japan	-	HM148066,	HM148309,	HM148554
C. colocasiae	CBS 386.64*; ATCC	Colocasia esculenta	Taiwan	K. Sawada	HM148067,	HM148310,	HM148555
C. delicatulum	CBS 126342; CPC 14287;	Indoor air	Denmark	B. Andersen	HM148079,	HM148323,	HM148568
C. delicatulum	CBS 126343; CPC 14299;	Building material	Denmark	B. Andersen	HM148080,	HM148324,	HM148569
C. exile	CBS 125987*; CPC 11828	leaves of Corylus avellana	U.S.A.: Washington	D. Glawe	HM148091,	HM148335,	HM148580
C. flabelliforme	CBS 126345*; CPC14523	Melaleuca cajuputi	Australia	B.A. Summerell	HM148092,	HM148336,	HM148581
C. funiculosum	CBS 122128; ATCC16160; IFO 6536; JCM	Ficus carica	Japan	-	HM148093,	HM148337,	HM148582
C. gamsianum	CBS 125989*; CPC 11807	Strelitzia sp.	South Africa	W. Gams	HM148095,	HM148339,	HM148584
C. globisporum	CBS 812.96*	Meat stamp	Sweden	M. Olsen	HM148096,	HM148340,	HM148585
C. hillianum	CBS 125988*; CPC	Leaf mold of Typha	New Zealand	R. Beever	HM148097,	HM148341,	HM148586
C. inversicolor	CBS 131.29; ATCC	Triticum aestivum	—	F.T. Bennett	HM148099,	HM148343,	HM148588
C. myrtacearum	CBS 126349; CPC 13689;	Eucalyptus placita	Australia	B.A. Summerell	HM148116,	HM148360,	HM148605
C. oxysporum	CBS 125991; CPC 14371;	Soil, near the terracotta	China: Xi'an,	B. Andersen	HM148118,	HM148362,	HM148607
C. perangustum	CBS 125996*; CPC	Cussonia sp.	South Africa	P.W. Crous	HM148121,	HM148365,	HM148610
C. phyllophilum	CBS 125992*; CPC 11333	Taphrina sp. on Prunus	Germany	K. Schubert	HM148154,	HM148398,	HM148643

Table 1. Contd.

C. pini-ponderosae	CBS 124456*; CPC	Pinus ponderosa	Argentina	A. Greslebin	FJ936160,	FJ936164,	FJ936167
C. pseudocladosporioides	CBS 117134	Cloud water	—	M. Sancelme	HM148156,	HM148400,	HM148645
C. rectoides	CBS 125994*; CPC 11624	Vitis flexuosa	South Korea	H.D. Shin	HM148193,	HM148438,	HM148683
C. scabrellum	CBS 126358*; CPC	Ruscus hypoglossum	Slovenia	H.J. Schroers	HM148195,	HM148440,	HM148685
C. subuliforme	CBS 126500*; CPC 13735	Chamaedorea metallica	Thailand	I. Hidayat and J.	HM148196,	HM148441,	HM148686
C. tenuissimum	CBS 125995*; CPC 14253	Lagerstroemia sp.	U.S.A.: Louisiana	P.W. Crous	HM148197,	HM148442,	HM148687
C. uredinicola	CPC 5390; ATCC 46649	Hyperparasite on Cronartium fusiforme	U.S.A.: Alabama	-	AY251071,	HM148467,	HM148712
C. varians	CBS 126360; CPC 11327	Ulmus sp.	Germany	K. Schubert	HM148222,	HM148468,	HM148713
C. verrucocladosporioides	CBS 126363*; CPC12300	Rhus chinensis	South Korea	H.D. Shin	HM148226,	HM148472,	HM148717
C. vignae	CBS 121.25; ATCC200933; MUCL 10110	Vigna unguiculata	U.S.A.	M.W. Gardner	HM148227,	HM148473,	HM148718
C. xylophilum	CBS 113749	Bing cherry fruits	U.S.A.	F.M. Dugan	HM148228,	HM148474,	HM148719

ATCC: American Type Culture Collection, Virginia, U.S.A.; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; MUCL: Mycotheque de l'Universite catholique de Louvain.

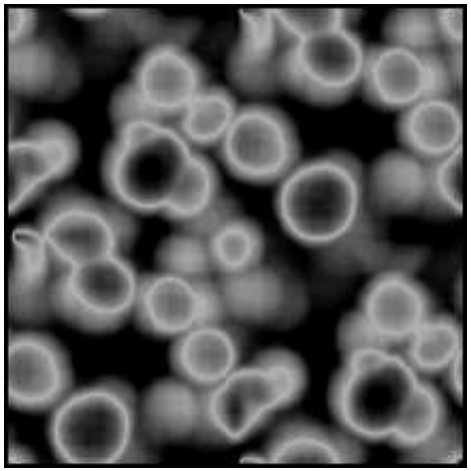

Figure 1. Transmission electron microscopy (TEM) image of iron oxide nanoparticle revealing the size of the particle to be 20 nm.

2013). The higher yield with the current method is probably attributed to the nanosize of the magnetic particles and optimum conditions for DNA binding (Saiyed et al., 2006). DNA recovery from a sample containing a single bacterial genome was performed by means of imidazole-modified magnetic nanoparticles (Imi-MNPs) and subsequent successful PCR detection were demonstrated on *Bacillus subtilis* as a model bacterium (Maeda et al., 2016). The current method is quick and results in pure DNA ready for sequencing. The DNA binding MNPs were directly subjected to polymerase chain reaction (PCR) to amplify the internal transcribed spacer (ITS) region, as well as fragments of the actin (Act) genes of three *Cladosporium* strains. A multilocus DNA sequence typing approach, employing three loci (the internal transcribed spacers of the rDNA genes (ITS), and partial actin and translation elongation factor 1-α gene sequences), as well as was used for the identification and delimitation of two new *Cladosporium* species. Several authors have demonstrated the usefulness of EF-1α and Act loci to allow a good species delimitation in *Cladosporium* (Bensch et al., 2012; Schubert et al., 2007). *Cladosporium asperulatum* is phylogenetically close to *C. myrtacearum* forming a distinct sister clade (Bensch et al., 2012; Bensch et al., 2010). Morphologically this species is comparable with *C. subtilissimum*, but differs in having 0 to 12-septate, somewhat longer conidiophores and narrower conidia. An isolate from North America (CBS 113744) is morphologically and phylogenetically slightly distinct from the other two isolates and only tentatively assigned to the present species (Bensch et al., 2012; Bensch et al., 2010). The current placement of two *Cladosporium* species with *C. asperulatum* at the family level, however, cannot be confirmed without morphological chracteriztion. *Cladosporium pini-ponderosae* is genetically close to *C. chubutense*, which has also been described from dead and living needles of *Pinus ponderosa* collected in pine plantations in Argentina (Patagonia). ITS data of the two species are almost identical, but ACT and TEF sequence are distinct. Our phylogenetic studies agree with previous revisions of the genus

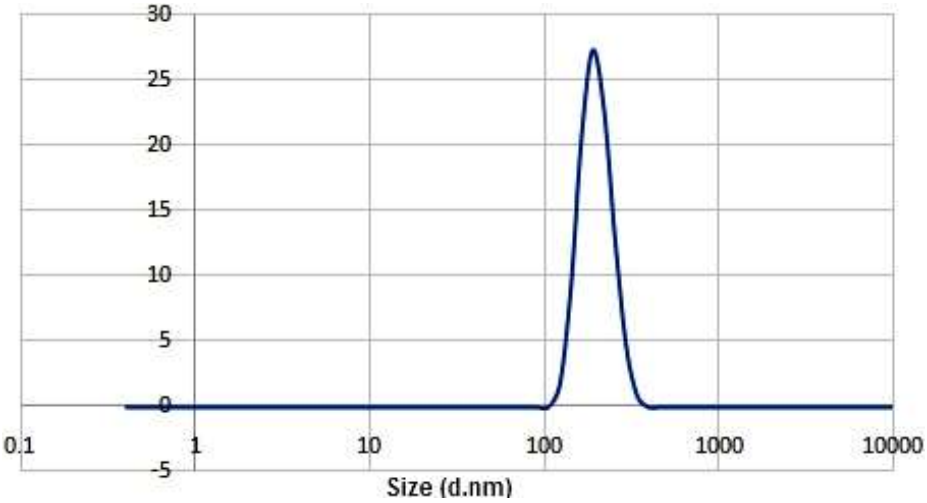

Figure 2. Dynamic light scattering (DLS) data of magnetic nanoparticle, showing the size distribution by number of the nanoparticles.

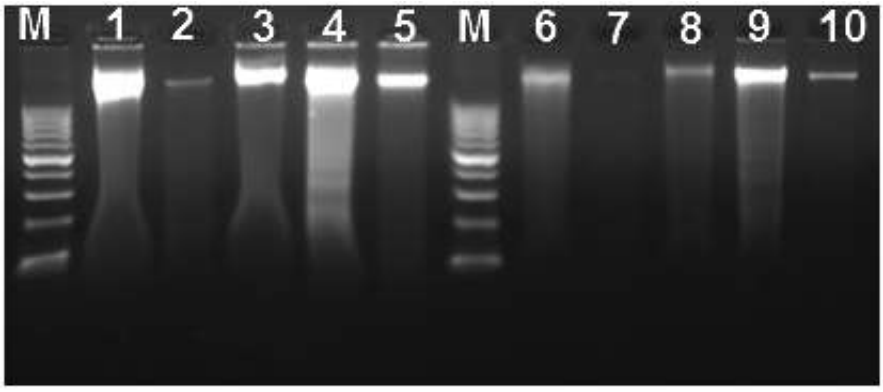

Figure 3. Agarose gel electrophoresis of genomic DNA isolated using magnetic nanoparticles (MNPs). Lanes 1-5: DNA isolated from Cladosporium isolates using MNPs; lanes 6-10: DNA isolated from Cladosporium isolates using SDS methods according to Moslem et al. (2010). Lane M = 100 bp DNA Ladder (Jena Bioscience, Germany) was loaded in the first and middle lanes.

(Bensch et al., 2012; Sandoval-Denis et al., 2016; Schubert et al., 2007; Schubert et al., 2009; Zalar et al., 2007). The most phylogenetic informative markers were *actA* and *tef1*, while ITS sequences were usually identical for species of the same complex as previously reported by Bensch et al. (2010). Future prospects in plant disease diagnostic will continue in miniaturization of biochip technology to the nanoscale range. DNA nanodevices could enable accurate tracking, detection and diagnosis of plant pathogens in the early stages of plant (Khiyami et al., 2014).

Conclusion

The quality and yields of the isolated DNA from all the samples using magnetic nanoparticles were higher to the DNA isolation method via SDS. Three *Cladosporium* strains were isolated from tomato in Saudi and Egypt was identified very similar to *C. asperulatum* and *C. myrtacearum* based on their molecular phylogenetic characteristics. It requires further examinations and morphological data are urgently needed to establish the identity and clarify the taxonomic status of two identified species. Also, more experiments on nucleic acid isolation of other fungal plant pathogens using the magnetic nanoparticles are in progress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

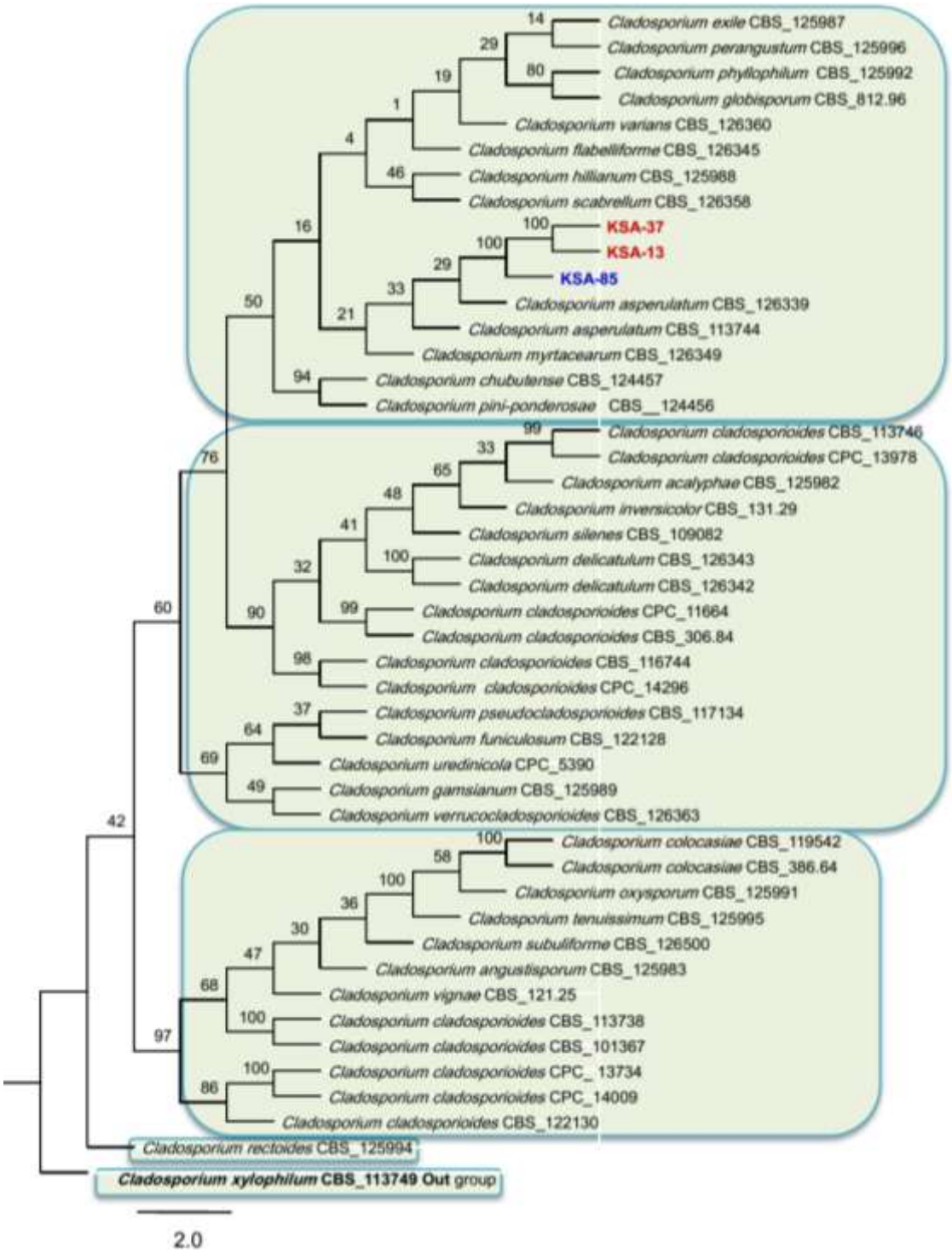

Figure 4. RAxML tree generated by the analysis of combined data set of ITS, Actin and Tef sequences. Bootstrap support values greater than 50% for maximum likelihood (ML) analyses are given above the nodes. The original isolate numbers are noted after the species names. *C. xylophilum* (CBS 113749) is the out group taxon.

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