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Development of SCAR primers for the detection of *Cadophora luteo-olivacea* on kiwifruit and pome fruit and of *Cadophora malorum* on pome fruit

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Summary. In recent years a postharvest disease of kiwifruit, characterized by skin pitting appearing after 3 or more months of storage, and caused by *Cadophora luteo-olivacea*, has been reported in most Italian packinghouses. *Cadophora malorum* - morphologically indistinguishable from *C. luteo-olivacea* - is a soilborne or wood-associated species that may also cause side rot on apples and pears. Forty-four isolates of *Cadophora* spp. from Italian kiwifruit harvested during the period 2001–2006 and nine reference strains of *C. luteo-olivacea* and *C. malorum* were tested for their postharvest pathogenicity on kiwifruit, apple and pear. The isolates were pathogenic on the three fruit types stored at 1°C for 120 days with variying degrees of virulence. A PCR-based method to identify both pathogen species on kiwifruit or pome fruits was developed. Sequencing of the ITS1, 5.8S gene and ITS2 region of the rDNA showed high similarity between both *Cadophora* species. Variation within the ITS was used to design one reverse primer common to both species and two species-specific forward primers to distinguish isolates of *C. luteo-olivacea* and *C. malorum*. Each sequence characterized amplified region (SCAR) primer pair was specific for either *Cadophora* species, when cross-tested, tested on other species of *Cadophora* or on species of other postharvest pathogens of kiwifruit, apple and pear.

Key words: *Actinidia deliciosa*, nuclear rDNA genes, side rot, skin pitting, virulence.

Introduction

Kiwifruit (*Actinidia deliciosa* cv. Hayward) can be stored for 3–6 months at 0±1°C and 90–95% RH (Feng *et al*., 2006). Controlled atmospheres (CA) of 1–2% O_2 and 3–5% CO_2 can further extend the storability of cold-stored kiwifruit (Kader, 1997). The most important postharvest decay of kiwifruit is grey mould caused by *Botrytis cinerea* (Snowdon, 1990). In recent years a new postharvest disease of kiwifruit, characterized by skin pitting appearing after 3 or more months of storage, and caused by *Cadophora luteo-olivacea* (van Beyma) Harrington & McNew, has been reported in most Italian packinghouses (Spadaro *et al.,* 2010). Initially, the disease was sporadic and economically insignificant, mostly affecting kiwifruit produced in Southern Italy (Gorini, 1991). Incidence of the disease increased in 1998 and 1999, reaching 20–30% in some kiwifruit batches in 2000 (Piano *et al.*, 2001) and 65% in 2006 (Spadaro *et al.,* 2010).

The first disease symptoms - often minor lesions - appear in packinghouses after 100–120 days storage, when relevant storage costs have already been sustained. Symptoms become economically significant when the kiwifruit reach distribution chains and consumers (Snowdon, 1990). Skin pitting on kiwifruit is of economic concern due to the lack of effective postharvest fungicides (Directive 2009/128 EC on sustainable use of pesticides).

Knowledge about the causative pathogen and disease epidemiology is limited. During 2001–2006, a survey was carried out in several kiwifruit packinghouses with the aim to identify possible

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agronomic factors and storage techniques predisposing fruit to development of the disease (Gilardi *et al.*, 2007). Batches with predisposing factors, such as low dry matter (DM) and high nitrogen content, should be carefully managed, reducing the storage life and avoiding excessively rapid CA establishment (Spadaro *et al.*, 2010). An unusual disease, named elephantiasis, has been recently observed in Italian orchards of kiwifruit cv. Hayward. *Phialophora*-like isolates obtained from necrotic woods were identified as *C. melinii* and *C. luteo-olivacea*, with the isolates of *C. melinii* giving greater colonization indices ex on kiwifruit tissues (Prodi *et al.*, 2008).

Another species of *Cadophora*, *C. malorum* (Kidd & Beaumont) Gams - morphologically indistinguishable from *C. luteo-olivacea* - is a soilborne or wood-associated fungus that may also rot apple fruit and asparagus (Frisullo *et al.*, 2002). *Cadophora malorum* has been recognized as the causal agent of wood discoloration and decay on the trunks of old kiwifruit 'Hayward' vines (Di Marco *et al.*, 2004). Symptoms of the disease also appear on the foliage in late summer as small, pale green spots irregularly distributed on upper leaf surfaces. The spots enlarge and coalesce on each leaf, developing into a series of irregularly shaped chlorotic areas that soon become necrotic and eventually cover most of the leaf surface. When the disease is severe, affected leaves tend to curl, wilt and drop prematurely. Fruits on diseased vines are stunted and do not reach full maturity. Symptoms normally appear every year on diseased plants, and the disease causes reduced productivity and longevity of kiwifruit orchards (Di Marco *et al.*, 2002).

Cadophora malorum is also a postharvest pathogen on apples and pears. It resides in orchard soil and wood, and may be transported into packinghouses on fruit skins or in soil adhering to harvest bins (Sugar and Spotts, 1993). This fungus is primarily a saprophyte living in the surface soil and upon the bark and in cankerous woody tissue of apple trees. Apple fruits become infected while on the trees, and under favourable conditions the fungus develops and causes serious and unpredictable losses in fruit storage. The fungus enters fruit through lenticels, insect injuries and mechanical punctures (McColloch, 1944).

The first objective of the present study was to ascertain the species of the isolates of *Cadophora* sp. coming from kiwifruit with skin pitting symptoms. Once it was confirmed that all the isolates belonged to *C. luteo-olivacea*, we decided to assess the degree of virulence of the fungus to kiwifruit, and if these isolates could also be pathogenic to apples and pears. In Italy, packinghouses dealing with kiwifruit generally also process apples, so pathogenic fungal species could potentially attack both fruit species. For this reason, pathogenicity tests were carried out on apples, pears, and kiwifruit stored at 1°C for 120 days. A further objective was to develop a rapid detection tool to specifically identify isolates of *C. malorum* and *C. luteo-olivacea*. Based on the sequence differences of the ITS1-5.8S-ITS2 region, one reverse primer common to both species and two forward primers specific for either species were designed. Both sequence characterized amplified region (SCAR) primer pairs were evaluated for specificity on the genomic DNA of other *Cadophora* species and other postharvest fungal pathogens of apples, pears and kiwifruit.

Materials and methods

Fungal isolates and culture conditions

A collection of 44 *Cadophora*-like isolates, obtained from diseased kiwifruit in Italy during the period 2001–2006, was maintained at AGROINNOVA - Centre of Competence for the Innovation in the Agro-environmental Sector, University of Torino (Italy), on potato dextrose agar (PDA, Merck, Darmstadt, Germany; 39 g L^{-1}) medium at 4°C. These isolates had been obtained from fruit, which were surface-disinfected by dipping in a solution of 0.5% NaClO and 0.05% Tween-20 for 5 min and by rinsing in sterile distilled water for 1 min. The outer tissues of the lesions were removed and small pieces, approximately 2 mm in diameter, were taken from the margins of the rotten tissues and placed in Petri dishes containing PDA amended with streptomycin (25 μg L^{-1}). The dishes were incubated at 20°C for 30 days. Sixteen representative isolates of other *Cadophora* species (*C. luteo-olivacea, C. malorum, C. melinii, C. fastigiata, C. lagerbergii, C. verrucosa, C. repens, C. americana* and *C. sessilis*) were included as reference strains. Table 1 outlines the isolates tested, their species, sources dates of isolation, geographical origin and GenBank accession numbers of the ITS regions se-

Table 1. Isolates, species, sources, areas of origin, year of isolation, collections and GenBank ITS sequence numbers for fungi investigated in this study.

Isolate	Species	Source of isolation	Origin	Year	Collection	ITS sequence (GenBank No.)
Phi K I	*C. luteo-olivacea*	*Actinidia deliciosa*, fruit	Lagnasco, Italy	2001	Univ. Torino	GU128545
Phi K1 II	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128546
Phi K2 III	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128551
Phi K2 IV	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128557
Phi K3 II	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	FJ486274
Phi K3 III	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128552
Phi K3 IV	*C. luteo-olivacea*	*A. deliciosa*, fruit	Borgo d'Ale, Italy	2001	Univ. Torino	GU128558
Phi K5 II	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GQ214536
Phi K6 II	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128548
Phi K6 III	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128553
Phi K7 III	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128554
Phi K8 III	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128555
Phi K9 II	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128584
Phi K9 III	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128556
Phi K10 II	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128549
Phi K11 II	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2001	Univ. Torino	GU128550
PAV Vittone	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2002	Univ. Torino	GU128566
PAV 40A	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2002	Univ. Torino	GU128562
PAV 40B	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2002	Univ. Torino	GU128563
PAV 40D	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2002	Univ. Torino	GU128564
PAV 83D	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2002	Univ. Torino	FJ486273
PAV 83E	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2002	Univ. Torino	GU128565
Phi 1/04	*C. luteo-olivacea*	*A. deliciosa*, fruit	Lagnasco, Italy	2004	Univ. Torino	GU128567
Phi 2/04	*C. luteo-olivacea*	*A. deliciosa*, fruit	Lagnasco, Italy	2004	Univ. Torino	GU128568
Phi 3/04	*C. luteo-olivacea*	*A. deliciosa*, fruit	Lagnasco, Italy	2004	Univ. Torino	GU128569
Phi 4/04	*C. luteo-olivacea*	*A. deliciosa*, fruit	Lagnasco, Italy	2004	Univ. Torino	GU128570
Phi 4 A I	*C. luteo-olivacea*	*A. deliciosa*, fruit	Lagnasco, Italy	2004	Univ. Torino	GQ214537
Phi C 2 I	*C. luteo-olivacea*	*A. deliciosa*, fruit	Italy	2004	Univ. Torino	GU128560
Phi E I	*C. luteo-olivacea*	*A. deliciosa*, fruit	Italy	2004	Univ. Torino	GU128561
Phi ACC 3A	*C. luteo-olivacea*	*A. deliciosa*, fruit	Cuneo, Italy	2004	Univ. Torino	GU128578
Phi mix reis	*C. luteo-olivacea*	*A. deliciosa*, fruit	Italy	2004	Univ. Torino	GU128582
PH 5-2	*C. luteo-olivacea*	*A. deliciosa*, fruit	Latina, Italy	2005	Univ. Torino	FJ486275
PH 10-3	*C. luteo-olivacea*	*A. deliciosa*, fruit	Latina, Italy	2005	Univ. Torino	GQ214538
PH 11-3	*C. luteo-olivacea*	*A. deliciosa*, fruit	Latina, Italy	2005	Univ. Torino	GU128572
PH 258/3	*C. luteo-olivacea*	*A. deliciosa*, fruit	Latina, Italy	2005	Univ. Torino	GU128574
PH 258-2-2	*C. luteo-olivacea*	*A. deliciosa*, fruit	Latina, Italy	2005	Univ. Torino	GU128575
PH 25813-3	*C. luteo-olivacea*	*A. deliciosa*, fruit	Latina, Italy	2005	Univ. Torino	GU128576
PH 263-3	*C. luteo-olivacea*	*A. deliciosa*, fruit	Latina, Italy	2005	Univ. Torino	GU128573

continues

Table 1. *continued*

Isolate	Species	Source of isolation	Origin	Year	Collection	ITS sequence (GenBank No.)
Kiwi Prova 30/2	*C. luteo-olivacea*	*A. deliciosa*, fruit	Italy	2006	Univ. Torino	GU128581
Kiwi Prova 30/3	*C. luteo-olivacea*	*A. deliciosa*, fruit	Italy	2006	Univ. Torino	GU128579
Kiwi Prova 30/4	*C. luteo-olivacea*	*A. deliciosa*, fruit	Italy	2006	Univ. Torino	GU128577
Kiwi Prova 30/5	*C. luteo-olivacea*	*A. deliciosa*, fruit	Italy	2006	Univ. Torino	GU128583
Kiwi Prova 30/6	*C. luteo-olivacea*	*A. deliciosa*, fruit	Italy	2006	Univ. Torino	GU128580
Kiwi Galliano 2006	*C. luteo-olivacea*	*A. deliciosa*, fruit	Latina, Italy	2006	Univ. Torino	FJ486276
Reference strains						
Pera Battaglio	*C. luteo-olivacea*	Pyrus communis cv. Williams, fruit	Italy		Univ. Torino	FJ486277
Phi Sugar	*C. malorum*	*P. communis* cv. Bosc, fruit	USA		David Sugar	GU128591
CBS 687.96	*C. malorum*	*Fagus sylvatica*, stem	Netherlands		CBS [b]	GU128592
CBS 352.87	*C. luteo-olivacea*	*Malus sylvestris*, fruit	Netherlands		CBS	GU128586
CBS 355.59	*C. luteo-olivacea*	*Malus sylvestris*, fruit	Netherlands		CBS	GU128587
CBS 851.69	*C. luteo-olivacea*	wheat-field soil	Germany		CBS	GU128585
CBS 141.41	*C. luteo-olivacea*	waste water	Sweden		CBS	GU128588
CBS 357.51	*C. luteo-olivacea*	*Malus sylvestris*, fruit	Italy		CBS	GU128589
CBS 268.33	*C. melinii*	Unknown	Sweden		CBS	AY249072
CBS 307.49	*C. fastigiata*	*Pinus*, blue stain	Sweden		CBS	AY249073
CBS 266.33	*C. lagerbergii*	*Pinus sylvestris*, wood	Sweden		CBS	AF083197
CBS 294.39	*C. repens*	*Pine lumber*	USA		CBS	AF083195
CBS 220.97	*C. americana*	*Linden* tree	USA		CBS	PAU31837
CBS 138.67	*C. verrucosa*	Unknown	France		CBS	GU128593
CBS 243.85	*C. sessilis*	*Picea abies*, resin	Netherlands		CBS	AY857542

[a] ATCC stands for American Type Culture Collection, Manassas, VA, United States.
[b] CBS stands for Centralbureau voor Schimmelcultures, Utrecht, The Netherlands.

quenced. Moreover, eleven postharvest pathogens common on pear, apple and kiwifruit (including *Botrytis cinerea, Alternaria alternata, Penicillium expansum, Mucor piriformis, Phoma pomorum, Phomopsis mali, Fusarium oxysporum, Sclerotinia sclerotiorum, Diaporthe actinidiae, Botryosphaeria obtusa* and *Cladosporium cladosporioides*) were used to test the specificity of the primers designed. All the fungal isolates were previously isolated from rotten fruits and then stored both at 4°C on PDA with streptomycin (25 μg L^{-1}) and at -80°C in 50% glycerol.

Pathogenicity tests and statistical analyses

Each isolate of *Cadophora* was grown on PDA in Petri dishes at 20°C. After 30 days, fungal cultures were aseptically filtered through four layers cheesecloth and conidia were brought to a final cell density of 10^5 CFU mL^{-1} in sterile distilled H_2O. The relative virulence of isolates of *C. luteo-olivacea* and *C. malorum* was assessed by inoculating artificial wounds in apples (*Malus domestica*, cv. Golden Delicious), in kiwifruit (*Actinidia deliciosa* cv. Hayward) and in pears (*Pyrus communis*, cv. Williams) with 10 μL of a conidial suspension con-

taining 10^5 conidia mL^{-1}. The fruit were each disinfected in sodium hypochlorite (NaClO, 1.0% chlorine) and rinsed under tap water, dried at room temperature and punctured with a sterile needle at the equatorial region (3 mm depth; 3–4 mm wide; three wounds per apple, two per pear, two per kiwifruit). After 4 months of storage at 1°C, the lesion diameters were measured. The experiments were performed twice. The identity of the causal agents (*C. luteo-olivacea* or *C. malorum*) was confirmed by plating diseased tissue from lesion margins on PDA in Petri dishes and by analysing the ribosomal region ITS1-5.8S-ITS2. The pathogenicity tests were repeated twice for each isolate of *C. luteo-olivacea* (50) and *C. malorum* (three). Analysis of variance (ANOVA) was performed for the data obtained, and a Tukey HSD all-pairwise comparisons test at $P<0.05$ level was used to compare the mean lesion diameters on pear, apple and kiwifruit (Table 2). Statistical analyses were performed by using the SPSS software (SPSS Inc., version 17.0, Chicago, IL, USA).

DNA extraction, ITS amplification and sequencing

DNA was extracted from the fresh mycelium of 60 isolates of *Cadophora* spp. grown on PDA for 30 days (Table 1). The DNA was extracted using NucleoMag 96 Plant Kits (Macherey Nagel, Oesingen, Switzerland) and a Kingfisher magnetic particle processor (Thermo Labsystems, Basingstoke, United Kingdom), following the manufacturers' protocols. About 100 mg of mycelium of each isolate was ground in liquid nitrogen with a mortar and pestle. The fine powder was used for DNA extraction. DNA was amplified by using universal primers ITS1 and ITS4 (White *et al.*, 1990). PCRs were performed in a TGradient thermal cycler (Biometra, Göttingen, Germany). Each 20 µL PCR mix contained 1 µL DNA template (50 ng), 200 mM each deoxynucleotide triphosphate, 2 µL 10× buffer (*Taq* DNA Polymerase, Qiagen, Chatsworth, CA, USA), 0.7 mM each primer, and 1.0 U *Taq* DNA Polymerase (Qiagen). The programme followed was: 95°C, 3 min; 34 cycles: 94°C, 15 s; 55°C, 45 s; 72°C, 55 s; 72°C, 7 min; 4°C. A 10 µL aliquot of products from each reaction was electrophoresed in 2.0% agarose gel and stained with SYBR SAFE (Invitrogen, Eugene, OR, USA). Gel images were acquired with a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were cloned into the PCR4 TOPO vector (Invitrogen) using the TOPO TA cloning kit following the manufacturer's protocol and later they were sequenced in both directions by BMR Genomics Center (Padova, Italy) using an ABI PRISM 3730XL DNA Sequencer. The sequences obtained were analysed using the software BLASTn for homology and ClustalW for alignment. The genus and the species of each isolate were determined.

Phylogenetic analyses of ITS sequencing data

Phylogenetic analyses, based on the sequence analysis of ITS ribosomal DNA, were performed on a dataset comprising newly generated sequences from the 44 *Cadophora*-like isolates and 16 reference strains of *C. luteo-olivacea*, *C. malorum*, and other *Cadophora* species. Data were analysed using MEGA4.1 software (Tamura *et al.*, 2007; Kumar *et al.*, 2008) to obtain a UPGMA tree constructed using 10,000 bootstrap replicates (Figure 1a).

Primer design and PCR assay

According to the differences indicated by sequence alignment, a pair of specific primers for *C. luteo-olivacea* and a pair of specific primers for *C. malorum* were designed; the primers were designated *Clu*-FOR (specific for *C. luteo-olivacea*), *Cma*-FOR (specific for *C. malorum*), and *Clm*-REV (specific for both *C. luteo-olivacea* and *C. malorum*). The primer pair *Clu-FOR* and *Clm-REV*, and *Cma-FOR* and *Clm-REV* were tested for specificity (Figure 1 b and c). The following amplification conditions were chosen: 20 µL of reaction mix containing: 1 µL DNA template (50 ng), 200 mM each deoxynucleotide triphosphate, 2 µL 10× buffer (*Taq* DNA Polymerase, Qiagen), 0.7 mM each primer, and 1.0 U *Taq* DNA Polymerase (Qiagen). Initial denaturation at 95°C for 2 min; 34 cycles of 15 s denaturation at 95°C, 15 s annealing at 58°C, 1 min extension at 72°C; and 7 min final extension at 72°C. Amplified products (10 µL) were analysed by agarose gel electrophoresis as described previously. Primer specificity was tested on the genomic DNAs of 60 isolates of *Cadophora* spp. (Figure 1 b and c) and of 11 postharvest pathogens common on pear, apple and kiwifruit, including *B. cinerea, A. alternata, P. expansum, M. piriformis, P. pomorum, P. mali, F. oxysporum, S. sclerotiorum, D. actinidiae, B. obtusa* and *C. cladospori-*

Table 2. Mean lesion diameters (mm) and degrees of virulence ([a]) for 44 isolates of *Cadophora* sp. from kiwifruit, six isolates of *C. luteo-olivacea* and three isolates of *C. malorum,* inoculated in apples cv. Golden Delicious, pears cv. Williams and kiwifruit cv. Hayward stored at 1°C for 120 days.

Isolate[b]	Mean lesion diameter (mm)			Isolate[b]	Mean lesion diameter (mm)		
	Apple	Pear	Kiwi		Apple	Pear	Kiwi
Phi K I	16 **	17 ***	20 **	Phi C 2 I	12 *	13 **	18 *
Phi K1 II	13 **	14 **	20 **	Phi E I	14 **	16 **	33 ***
Phi K2 III	16 **	12 **	21 **	Phi ACC3A	18 **	11 *	30 **
Phi K2 IV	17 **	16 **	23 **	Phi Mix Reis	17 **	13 **	22 **
Phi K3 II*	16 **	14 **	25 **	Phi 5-2	21 ***	18 ***	18 *
Phi K3 III	13 **	16 **	32 ***	PH 10-3	14 **	17 ***	23 **
Phi K3 IV	18 **	16 **	24 **	PH 11-3	14 **	13 **	23 **
Phi K5 II	16 **	17 ***	21 **	PH 258/3	12 *	12 **	15 *
Phi K6 II	15 **	16 **	28 **	PH 258-2-2	17 **	17 ***	19 *
Phi K6 III	21 ***	14 **	21 **	PH 25813-3	19 **	15 **	24 **
Phi K7 III	17 **	14 **	23 **	PH 263-3	16 **	15 **	20 **
Phi K8 III	13 **	13 **	22 **	Kiwi Prova 30/2	13 **	14 **	25 **
Phi K9 II	19 **	17 ***	26 **	Kiwi Prova 30/3	15 **	12 **	28 **
Phi K9 III	21 ***	17 ***	32 ***	Kiwi Prova 30/4	16 **	12 **	22 **
Phi K10 II	13 **	13 **	26 **	Kiwi Prova 30/5	16 **	11 *	30 **
Phi K11 II	15 **	15 **	20 **	Kiwi prova 30/6	22 ***	12 **	21 **
PAV Vittone	14 **	15 **	21 **	Kiwi Galliano 2006	17 **	12 **	27 **
PAV 40A	16 **	15 **	27 **	Reference strains			
PAV 40B	16 **	14 **	20 **	Pera Battaglio	18 **	17 ***	23 **
PAV 40D	17 **	10 *	15 *	ATCC 36274 (b)	17 **	12 **	24 **
PAV 83D	13 **	14 **	29 **	Phi Sugar (b)	12 *	15 **	18 *
PAV 83E	16 **	19 ***	25 **	CBS 687.96 (b)	17 **	12 **	28 **
Phi 1/04	12 *	13 **	27 **	CBS 352.87	13 **	12 **	28 **
Phi 2/04	17 **	15 **	21 **	CBS 355.59	16 **	11 *	30 **
Phi 3/04	16 **	16 **	24 **	CBS 851.69	16 **	12 **	36 ***
Phi 4/04	16 **	18 ***	25 **	CBS 141.41	16 **	14 **	27 **
Phi 4 A I	17 **	14 **	23 **	CBS 357.51	17 **	12 **	26 **

[a] Degree of virulence: * lowly virulent, ** virulent, *** highly virulent. Values followed by the same number of asterisks are not statistically different by Tukey's test ($P<0.05$).
[b] All the isolates tested were *C. luteo-olivacea* except for three *C. malorum* isolates.

oides. The fruit pathogens were placed on PDA medium amended with streptomycin (25 μg L^{-1}) in Petri dishes. Their DNA was extracted from fresh mycelium of isolates grown on PDA following the previously described procedure.

Results

Pathogenicity testing and statistical analyses

Forty-four isolates of *C. luteo-olivacea* obtained from naturally infected Italian kiwifruit, together with six reference strains of *C. luteo-olivacea* and

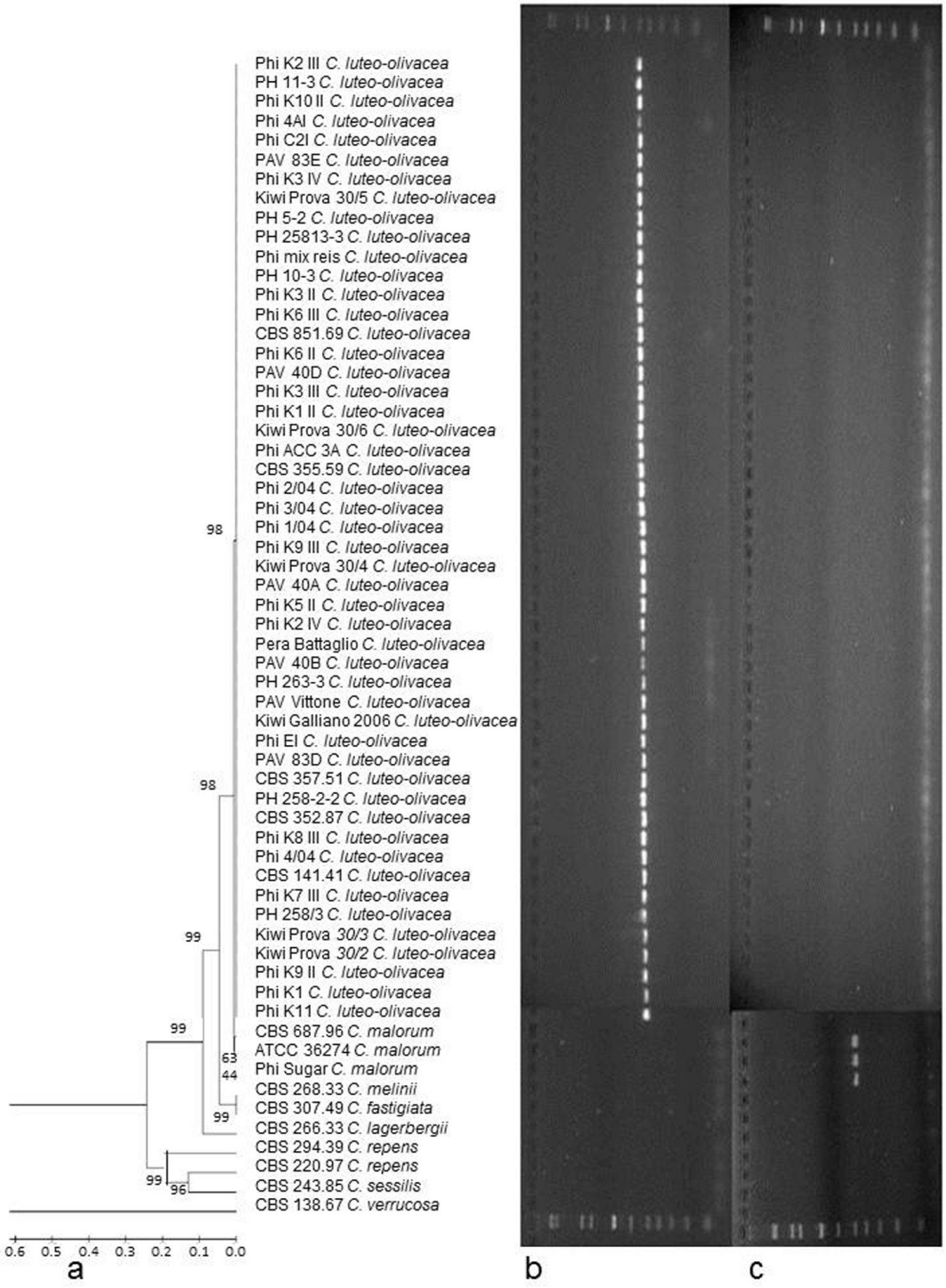

Figure 1. a) Dendrogram showing the relationship of *C. luteo-olivacea* isolates with other species of *Cadophora*, based on sequence of the ITS1-5.8S-ITS2 and the unweighted pair-group method using arithmetic averages (UPGMA). The sequence of *C. verrucosa* was used as an out-group and bootstrap values greater than 95% are shown adjacent to the appropriate branch points. b) Specificity of the primer pair *Clu*-FOR and *Clm-REV* for *C. luteo-olivacea*. c) Specificity of the primer pair *Cma-FOR* and *Clm-REV* for *C. malorum*.

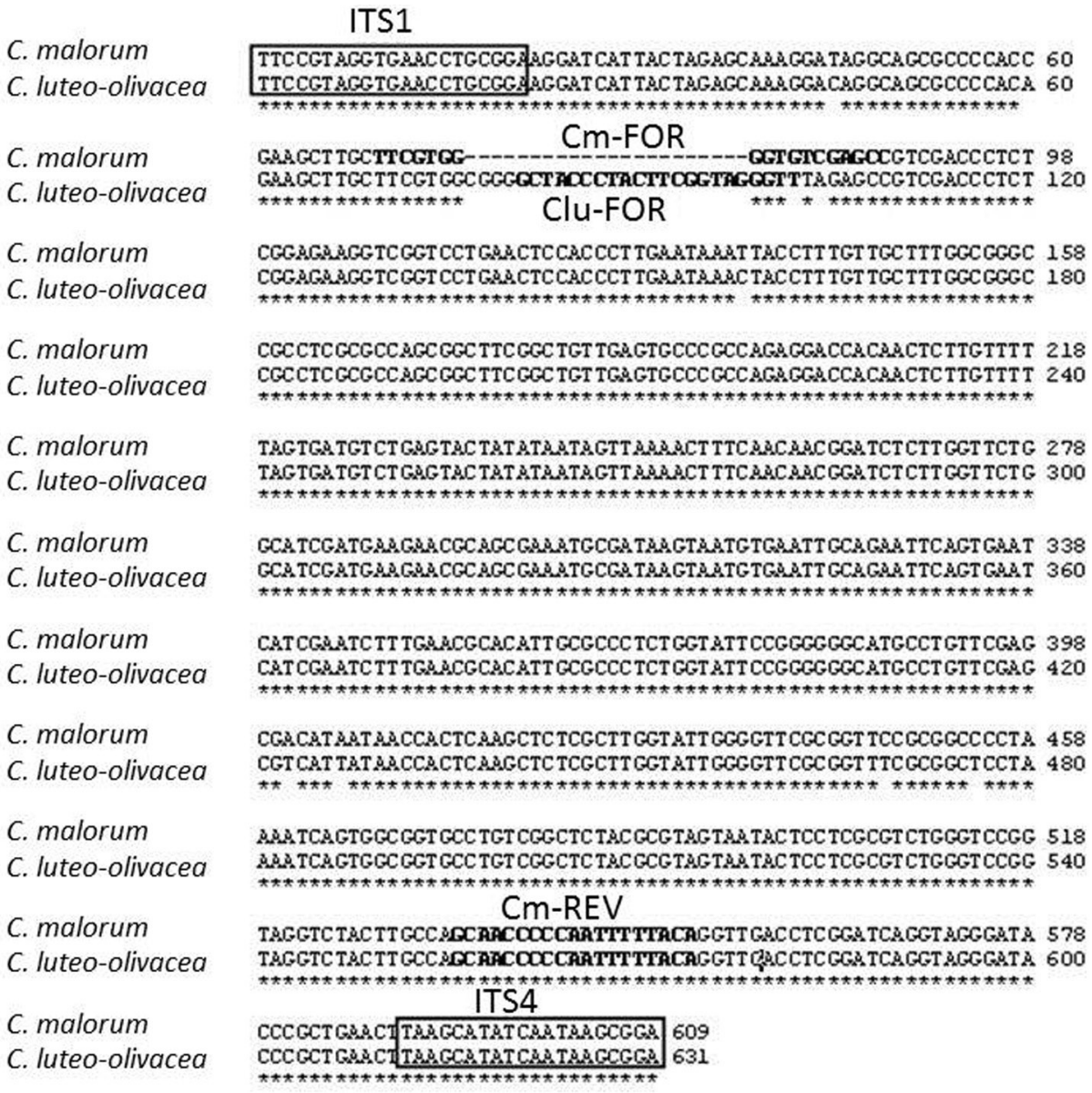

Figure 2. Alignment of the 5.8S RNA gene consensus sequences of *C. luteo-olivacea* and *C. malorum* isolates, showing positions of the primer pairs *Clu*-FOR, *Cma-FOR* and *Clm-REV* (bold font) with respect to the universal primer ITS1 and ITS4 (circumscribed).

three reference strains of *C. malorum*, were tested for their pathogenicity on apples cv. Golden Delicious, kiwifruit cv. Hayward and pears cv. Williams under controlled environmental conditions. Skin pitting symptoms appeared on kiwifruit after 3 or more months of storage, while side rot appeared on apples and pears after 45 d of storage. The *C. luteo-olivacea* and *C. malorum* isolates were grouped based on their degrees of virulence. The lesion diameter of each isolate was similar between the two trial repetitions. Tukey test (P=0.05) permitted three groups to be distinguished, according to their degree of virulence (lowly virulent, virulent, highly virulent) among the isolates tested for

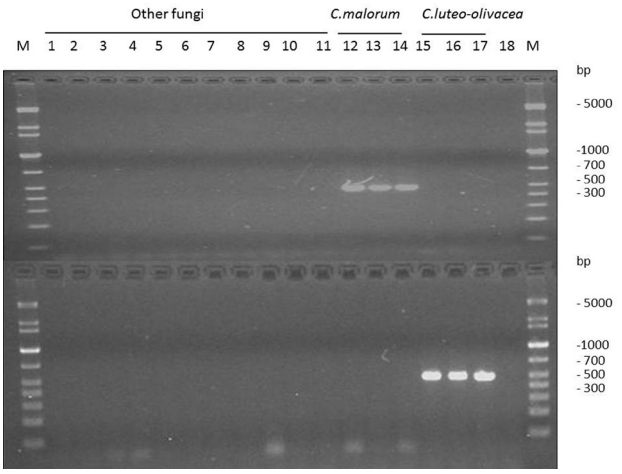

Figure 3. Specificity of the primer pairs *Cma-FOR* / *Clm-REV* for *C. malorum*, and *Clu-FOR* / *Clm-REV* for *C. luteo-olivacea*, tested on the genomic DNA of different postharvest pathogens. Lanes 1–11 (from left to right): *Botrytis cinerea, Alternaria alternata, Penicillium expansum, Mucor piriformis, Phoma pomorum, Phomopsis mali, Fusarium oxysporum, Sclerotinia sclerotiorum, Diaporthe actinidiae, Botryosphaeria obtusa* and *Cladosporium cladosporioides*; lanes 12–14: ATCC 36274, Phi Sugar, CBS 687.96 (*C. malorum*); lanes 15–17: CBS 141.41, CBS 357.51, Phi 1/04 (*C. luteo-olivacea*); Lane 18: water. M: molecular weight marker [GelPilot 1 kb Plus Ladder (100)].

pathogenicity (Table 2). Also the isolates belonging to the other seven species of *Cadophora* were tested for pathogenicity on apple, pear and kiwifruit, but none of them were pathogenic on the three fruit species (data not shown). On apples, four isolates were more virulent (lesion diam. ≥21mm) and four were less virulent (lesion diam. <13 mm). On pears, ten isolates were highly virulent (lesion diam. ≥17mm) and four were lowly virulent (lesion diam. <11mm). On kiwifruit, four isolates were highly virulent (lesion diam. 32–36mm) and six-were lowly virulent (lesion diam. <19mm). Most of the isolates showed a medium degrees of virulence on apples, pears and kiwifruit (Table 2). Greater pathogenicity was observed mainly on pears (isolates Phi KI, Phi K5 II, Phi K9 II, Phi K9 III, PAV 83E, Phi 4/04, PH 5-2, PH 10-3, PH 258-2-2 and Pera Battaglio), and sometimes on apples (isolates Phi K6 III, Phi K9 III, Phi 5-2, Kiwi Prova 30/6) or kiwifruit (isolates Phi K3 III, Phi K9 III, Phi E I, CBS 851.69). Skin pitting or side rot lesions were not detected on healthy control fruits. All the pathogenic isolates were successfully re-isolated from diseased hosts.

ITS sequence analyses and phylogenetic characterization of the isolates of *Cadophora* spp.

The GenBank accession numbers of the internal transcribed spacer (ITS1, 5.8S gene and ITS2) region of the rDNA (ITS) sequences of the 44 isolates and 16 reference strains of *Cadophora* spp. obtained in this study are indicated in Table 1. The ITS sequences of Italian isolates (44 from kiwifruit and one from pear), isolates from the American Type Culture Collection (ATCC, one isolate) and the Centraalbureau voor Schimmelcultures (CBS; 13 isolates), and the isolate of *C. malorum* isolated by David Sugar, Oregon State University (Sugar and Spotts, 1992) were aligned using ClustalW software. *Cadophora* spp. sequences showed high degrees of similarity. Multiple alignment of the ITS sequences revealed little intraspecific variation and low interspecific polymorphism. To represent the relationship between the different *Cadophora* species, the dataset was combined and analysed simultaneously by the unweighted pair-group method using arithmetic average (UPGMA analysis). The resulting dendrogram (Figure 1 a) confirmed the close genetic similarity between the species *C. malorum* and *C. luteo-olivacea*, which clustered separately from the other species of *Cadophora* tested: *C. melinii, C. fastigiata, C. lagerbergii, C. verrucosa, C. repens, C. americana* and *C. sessilis*. All the Italian isolates of *Cadophora* sp. from kiwifruit and pear clustered with the five *C. luteo-olivacea* isolates obtained from the CBS.

The UPGMA analysis (Figure 1a) provided a phylogenetic tree where three clades were observed. A first clade, supported by a 99% bootstrap value, comprised the 45 isolates coming from Piedmont (northern Italy) and Latium (central Italy) and five CBS isolates of *C. luteo-olivacea* coming from the Netherlands, Sweden, Germany and Italy. In this clade, three sequences of *C. malorum* were also included and they clustered together with the isolates of *C. luteo-olivacea* (63% bootstrap value). A second clade, supported by a 99% bootstrap value, included *C. melinii* and *C. fastigiata*. The sequence of *C. lagerbergii* clustered with the first and second clades (99% bootstrap value). A third clade (96% bootstrap value) – not containing any isolate of *C. malorum* or *C. luteo-olivacea*, included the three species *C. repens, C. americana* and *C. sessilis* (99% bootstrap value). The ITS sequence of *C. verrucosa* was the most distant from the isolates of the other *Cadophora* spp.isolate

SCAR primers for species specific PCR

The isolates of *C. luteo-olivacea* and *C. malorum* were in the same cluster, although there were a few differences. In particular, a sequence of 22 nucleotides present in *C. luteo-olivacea* but absent in *C. malorum* could be used to distinguish *C. luteo-olivacea* from *C. malorum* (Figure 3). A SCAR primer pair, the 22-bp-long forward primer *Clu*-FOR ($^{5'}$-GCTACCCTACTTCGGTAGGGTT-^{3}) and the 21-bp-long reverse primer *Clm-REV* ($^{5'}$-TGTAAAAATTGGGGGTTGCTG-^{3}) were designed for *C. luteo-olivacea*; the predicted size of the *C. luteo-olivacea* amplicon was 494 bp. Another SCAR primer pair, the 18-bp-long *Cma-FOR* ($^{5'}$-TTCGTGGGGTGTCGAGCC-^{3}) and the same reverse primer *Clm-REV*, were designed for *C. malorum* and the predicted size of the amplicon was 483 bp. Figure 2 shows the positions and the orientations of primers *Cma-FOR, Clu-FOR* and *Clm-REV* in the ITS region, with respect to the universal primers ITS1 and ITS4.

Specificity of both primer pairs

In specificity tests, the primer pair *Cma-FOR* and *Clm-REV* amplified a 483 bp DNA fragment from all *C. malorum* isolates tested. No product was amplified from other *Cadophora* species (Figure 1b and c) or from other postharvest pathogens of kiwifruit, apple and pear, including *B. cinerea, A. alternata, P. expansum, M. piriformis, P. pomorum, P. mali, F. oxysporum, S. sclerotiorum, D. actinidiae, B. obtusa* and *C. cladosporioides* (Figure 3). The same test was performed for the primers *Clu-FOR* and *Clm-REV* specific for *C. luteo-olivacea* (Figure 3). The primer pairs *Cma-FOR* / *Clm-REV* and *Clu*-FOR / *Clm-REV* were specific respectively for *C. malorum* and *C. luteo-olivacea*.

PCR assays using the primer pairs *Cma-FOR* / *Clm-REV* and *Clu*-FOR / *Clm-REV* were also used to detect *C. malorum* or *C. luteo-olivacea* on apple, pear and kiwifruit tissues. The results were consistent with the isolation of the pathogens on PDA. Specific amplicons were obtained by PCR assay from all the fruits infected with *C. malorum* or *C. luteo-olivacea*, and showing side rot on apple and pear or skin pitting symptoms on kiwifruit.

Discussion

All the *Cadophora*-like fungi isolated from kiwifruit in Italy belonged to *C. luteo-olivacea*, and no isolate was identified as *C. malorum*. The results obtained indicate that the populations of *Cadophora* analysed are genetically homogeneous, leading to identical sequences upon amplification of the highly conserved ITS1-5.8S-ITS2 region. This feature generated identical profiles among isolates of different geographical origin (Italy, the Netherlands, Germany and Sweden) or source of isolation (kiwifruit, pear, *Malus sylvestris*, wheat field soil and waste water), suggesting that the population of the fungal species is represented by a clonal lineage. The phylogenetic analysis performed in this study also suggests a close relationship between *C. malorum* and *C. luteo-olivacea*. According to morphology, biology and ITS sequence *C. luteo-olivacea* is close to *C. malorum*. Schol-Schwarz (1970) considered *Phialophora luteo-olivacea* and *P. goidanichii* synonyms for *P. malorum*, but the ITS sequence data separates the first two of these species from *C. malorum*. Using ITS and 28S (LSU) rDNA sequences, members of the genus *Cadophora* have been shown to be anamorphs of the Helothiales (Discomycetes) and distinct from the morphologically similar anamorph genus *Phialophora* (Harrington and McNew, 2003). *Phialophora atra* and *Cadophora heteroderae* have been synonymized with *C. malorum*, while *Phialophora goidanichii* has been synonymized

with *C. luteo-olivacea*. Previously, the same isolates coming from Italian kiwifruit used in this study were used for a RAPD analysis of genomic DNA using 14 decamers. The analysis of 352 polymorphic bands obtained showed that the fingerprinting of the kiwifruit isolates was similar to the *C. luteo-olivacea* isolates included in the study as references, and was phylogenetically distant from the *C. malorum* reference strains (Spadaro *et al.*, 2009). Another study, carried out on the ITS sequences of wood-destroying soft rot fungi isolated from the huts of an historic expedition in the Ross region of Antarctica, showed that the isolates of *C. malorum* and *C. luteo-olivacea* were closely related genetically and distant from other species of the genus *Cadophora* (Blanchette *et al.,* 2004).

This research confirmed that all the skin pitting symptoms occurring on kiwifruit cultivated in northern or central Italy from 2001 to 2006 were associated with *C. luteo-olivacea*. Moreover, the pathogenicity tests confirmed that *C. luteo-olivacea* can also be pathogenic on apple and pear fruit. The pathogenicity of *C. luteo-olivacea* on pome fruits was also confirmed by an isolate belonging to this species coming from a pear cv. Williams showing side rot, from Pera Battaglio, Italy. This result is in contrast with studies carried out by Sugar and Spotts (1992, 1993) in the United States, where side rot of pear was caused by *C. malorum*. To confirm the difference one isolate was provided by David Sugar and a second isolate was purchased from ATCC. The American isolates belonged to *C. malorum*. In contrast, three other isolates from *Malus sylvestris* in Italy (CBS 357.51) and the Netherlands (CBS 352.87 and CBS 355.59) belonged to *C. luteo-olivacea*. These results permit us to hypothesize that *C. luteo-olivacea* could be a typical postharvest pathogen on European kiwifruit, apples and pears, while *C. malorum* could be pathogenic typically on American pears. *Cadophora luteo-olivacea,* together with *C. melinii,* has also been recently isolated from kiwifruit plants with trunk hypertrophy, showing elephantiasis (Prodi *et al.,* 2008). Previously, Prodi *et al*. (2005) showed that *C. luteo-olivacea* was moderately pathogenic on kiwifruit while *C. melinii* had greater capacity for tissue colonization and deterioration. *Cadophora malorum* was also isolated from decayed trunks and cordons of Italian kiwifruit (Di Marco *et al*., 2004).

In Italy, packinghouses dealing with kiwifruit could also process apples and pears, so pathogenic fungicould potentially attack both fruit species. The pathogenicity tests carried out on apples, pears, and kiwifruit showed that inocula of *C. malorum* or *C. luteo-olivacea*, if present in packinghouses where kiwifruit and pome fruit are processed, can attack the different fruit species.

By analysing the ITS sequences of the isolates of *C. luteo-olivacea* and *C. malorum*, we have identified a sequence of 22 nucleotides present only on the isolates of *C. luteo-olivacea.* Therefore, though the two species are morphologically and phytopathogenetically indistinguishable, a simple and rapid molecular identification test could be developed. The PCR assay using the primer pairs *Clu*-FOR / *Clm-REV* and *Cma-FOR* / *Clm-REV* proved to be sensitive and specific. The test is a very promising tool which could be applied in orchards or during harvest, for early detection of pathogenic species, helping to predict the susceptibility to side rot on pome fruit or skin pitting on kiwifruit during fruit storage. The molecular assay developed in this study could also be used to distinguish *C. luteo-olivacea* or *C. malorum* from other *Cadophora* species, as well as from other postharvest pathogens frequently occurring on apple, pear and kiwifruit.

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