***Cadophora luteo-olivacea* isolated from apple (*Malus domestica*) fruit with post-harvest side rot symptoms in northern Italy**

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Accepted: 18 September 2021 / Published online: 17 October 2021
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Abstract Recently, *Cadophora luteo-olivacea* has been reported as a post-harvest pathogen of side rot of apple (*Malus domestica*) in Latvia, and of pear (*Pyrus communis*) in Italy and the Netherlands. In 2018 and 2019, in South Tyrol (northern Italy) 11 fungal cultures, identified as *C. luteo-olivacea* based on ITS (internal transcribed spacer region) sequence data, were obtained from apple fruit displaying post-harvest side rot symptoms. The fruit were sampled in packinghouses representing different apple growing areas of South Tyrol. For a more accurate taxonomic assignment, two additional molecular genetic loci, β -tubulin (TUB2) and translation elongation factor 1 α (TEF-1 α), of seven isolates were amplified and sequenced. Phylogenetic analysis based on multi-locus DNA sequence data confirmed that isolates from apple fruit with side rot symptoms indeed represented *C. luteo-olivacea*. A pathogenicity test performed on apple fruit of ‘Golden Delicious’ confirmed the virulence of a representative isolate. So far, *C. luteo-olivacea* represents a minor post-harvest pathogen of apple but considering the losses that this

fungus can cause to other crops, the awareness about this potential apple disease should be raised.

Keyword Fruit rot; fungal plant pathogens; emerging plant diseases; multi-locus sequence typing; molecular genetic identification

Modern apple cultivars generally show exceptional storage properties as fruit can be stored for a period of up to twelve months at low temperatures and under controlled atmosphere conditions (Sutton et al., 2014). During this process, fruit may deteriorate, which is caused by post-harvest physiological disorders or by diseases (Snowdon, 1990). The main post-harvest diseases of pome fruits are caused by various fungal pathogens and in terms of economic losses, blue mold (*Penicillium* spp.), gray mold (*Botrytis cinerea*), bull’s eye rot (*Phlyctema vagabunda*) and brown rot (*Monilinia* spp.) are regarded as the most important (Spadaro et al., 2019). Side rot of apple and pear has been considered a minor post-harvest disease, although it occasionally causes considerable economic damages on pears (*Pyrus communis*) in the Pacific Northwest USA and in Europe (Sugar, 2014). The principal pathogen associated with this disease is *Cadophora malorum* (Kidd and Beaumont) W. Gams (syns. *Phialophora malorum* [Kidd and Beaumont] McColloch, *Sporotrichum malorum* Kidd and Beaumont, and *Sporotrichum carpogenum* Rühle) (Spadaro et al., 2019). Bertrand et al. (1977)

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10658-021-02388-4>.

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described an outbreak of lateral rot on ‘Bosc’ pears in Oregon (USA), suggesting that *C. malorum* probably entered the host through lenticels or micro-wounds in the cuticle. Sugar and Spotts (1993a) postulated that spores of the fungus may be brought into commercial packinghouses on the surface of fruit or on harvest bins, and that the inoculum is transmitted to unaffected fruit through immersion dump tanks. Recently, *Cadophora luteo-olivacea* (J.F.H. Beyma) T.C. Harr. and McNew, has been revealed as a post-harvest pathogen of side rot on apple in Latvia, and on pear in Italy and the Netherlands (Grantina-Ievina, 2015; Spadaro et al., 2011; Wenneker et al., 2016). On pear fruit, *C. luteo-olivacea* and *C. malorum* were described to induce similar symptoms, which are manifested as dark brown circular to oval lesions that appear after about three, six or more months of cold storage (Spadaro et al., 2019; Sugar, 2014; Wenneker et al., 2016). Lesion diameters described on pear fruit caused by *C. luteo-olivacea* ranged from 0.5 to 1.0 cm (Wenneker et al., 2016), and those caused by *C. malorum* were less than 2 cm (Sugar, 2014).

South Tyrol is situated in the northern part of Italy and contributes to approximately 10% of the European apple production (Autonomous Province of Bozen-Bolzano, 2020). A survey on post-harvest

pathogens was performed in 2018 and 2019 in packinghouses located in different parts of the apple production area of South Tyrol (Amaral Carneiro & Baric, 2021a). Apple fruit with symptoms resembling side rot were found in 1% of the decayed fruit samples. In fact, these fruit that were stored for five to eight months under controlled atmosphere conditions showed symptoms that could be assigned to two categories (Fig. 1). On some apple fruit, the lesions were circular and slightly sunken, with a black center or black spots scattered over the lesion and resembled those of black rot caused by *Alternaria* spp. (Spadaro et al., 2019). In other cases, the symptoms were manifested as circular, flat to slightly sunken lesions, and the rotten tissue remained rather firm. The color of such lesions ranged from brown to dark brown and could be confused with bull’s eye rot as described by Sugar (2014) (Fig. 1B). In most of the cases, the lesion diameter was larger than 1 cm and reached up to approximately 4 cm. In general, the diseased tissue was spongy (Fig. 1A and Supplementary Figure S1).

From all explants of ten out of eleven apple fruit showing the above-described symptoms, the fungus *Cadophora* spp. was obtained on Potato Dextrose Agar (PDA; VWR International, Milan, Italy) in pure culture (Table 1, Fig. 1 and Supplementary

Fig. 1 The external and internal symptoms associated with side rot of apple after cold storage under controlled atmosphere conditions and the corresponding fungal isolates obtained on

PDA. **A:** ‘Golden Delicious’ (19-DSS-KA-4-060) after eight months of storage and **B:** ‘Roho 3615’/Evelina® (18-DSS-BS-EL-1-014) after five months of storage

Table 1 *Cadophora luteo-olivacea* isolates obtained from decayed apple fruit that were sampled in different packinghouses in 2018 and 2019 in South Tyrol (northern Italy)

No	Isolate code	Fungal species	Apple cultivar	Year of sampling	Cultivation method	Growing area	GenBank accession number		
							ITS	TUB2	TEF-1 α
1	18-DSS-BS-EL-1-014	<i>C. luteo-olivacea</i>	‘Roho 3615’/Evelina®	2018	Organic	Terlan/Andrian-Terlano/Andriano	MZ144208	-	-
2	18-DSS-CAFA-2-001	<i>C. luteo-olivacea</i>	‘Granny Smith’	2018	Integrated	Meran-Merano	MZ144207	MZ269721	MZ269727
3	18-DSS-KA-1-020	<i>C. luteo-olivacea</i>	‘Braeburn’	2018	Integrated	Unterland-Bassa Atesina	MZ144210	MZ269717	MZ269730
4	18-DSS-KA-2-057	<i>C. luteo-olivacea</i>	‘Braeburn’	2018	Integrated	Unterland-Bassa Atesina	MZ144206	-	-
5	18-DSS-MX-1-130	<i>C. luteo-olivacea</i>	‘Red Delicious’	2018	Integrated	Eisacktal-Valle Isarco	MZ144205	-	-
6	18-DSS-NTC-1-004	<i>C. luteo-olivacea</i>	‘SQ 159’/Natyra®	2018	Organic	Unterland-Bassa Atesina	MZ144211	-	-
7	19-DSS-BS-3-012	<i>C. luteo-olivacea</i>	‘Roho 3615’/Evelina®	2019	Organic	Überetsch-Oltradige	MZ144204	MZ269719	MZ269728
8	19-DSS-BS-3-014	<i>C. luteo-olivacea</i>	‘Roho 3615’/Evelina®	2019	Organic	Überetsch-Oltradige	MZ144209	MZ269723	MZ269729
9	19-DSS-KA-4-044	<i>C. luteo-olivacea</i>	‘Golden Delicious’	2019	Integrated	Unterland-Bassa Atesina	MZ144203	MZ269718	MZ269724
10	19-DSS-KA-4-060	<i>C. luteo-olivacea</i>	‘Golden Delicious’	2019	Integrated	Unterland-Bassa Atesina	MZ144202	MZ269722	MZ269726
11	19-DSS-MX-2-015	<i>C. luteo-olivacea</i>	‘Granny Smith’	2019	Integrated	Eisacktal-Valle Isarco	MZ144201	MZ269720	MZ269725

Figure S1). In one case, sample 19-DSS-MX-2-015, *Cadophora* spp. was isolated in conjunction with *Phlyctema vagabunda* (syn. *Neofabraea alba*) but could be separated in pure culture in a subsequent step. The fungal isolates on PDA were flat, felty and cottony in the center, with smooth margins, an even edge, and they varied in color from white to gray or black-olivaceous (see Fig. 1 and Supplementary Figure S1).

Pure single-spore cultures were obtained (Amaral Carneiro & Baric, 2021b) and DNA was extracted from approximately 100 mg of fresh mycelium of

each fungal isolate following the protocol of Cas-sago et al. (2002). The first molecular genetic identification was based on DNA sequencing of the internal transcribed spacer (ITS) region with the primer pairs UN-UP18S42/UN-LO28S22 (Lévesque & De Cock, 2004). Further PCR reactions with the primer pairs BT-CadF/BT-CadR (Travadon et al., 2015), EF1-728F/EF1-986R (Carbone & Kohn, 1999) and EF1-688F/EF1-1251R (Alves et al., 2008) were performed to amplify segments of β -tubulin (TUB2) and translation elongation factor 1 α (TEF-1 α), respectively. Each PCR reaction contained 7.5

μL of the 2x PCRBIO Taq Mix (PCR Biosystems Ltd., Oxford, UK; containing a premix of Taq DNA polymerase, dNTPs and the appropriate buffer system), 0.75 μL of each forward and reverse primer (10 μM) and 5 μL of sterile nuclease-free-water. The final volume of the PCR reaction of 15 μL was reached by the addition of 1 μL of template DNA (initial concentration of 10 ng μL^{-1}). The PCR conditions for ITS, TUB2 and TEF-1 α were followed as described by Amaral Carneiro and Baric (2021a), Travadon et al. (2015) and Glass and Donaldson (1995), respectively. While the ITS region of all the fungal isolates could be successfully amplified, specific PCR products with primers BT-CadF/BT-CadR designed by Travadon et al. (2015) were obtained only for seven of the eleven fungal isolates. Furthermore, despite intensive efforts to optimize the PCR reaction, no amplicons were obtained with primer pair EF1-728F/EF1-986R. Instead, amplicons could be generated with primers EF1-688F/EF1-1251R for seven isolates, but primers EF1-728F and EF1-986R were used as internal sequencing primers

for the PCR products of the TEF-1 α region. PCR products purified with the A'SAP™ PCR clean-up reagents (ArticZymes, Tromsø, Norway) were sent for Sanger sequencing to the company Microsynth SeqLab GmbH (Göttingen, Germany).

The quality of DNA sequences of the ITS region was checked with the software Geneious Prime version 2019.2 (Biomatters Ltd., Auckland, New Zealand), and each sequence was individually trimmed at the same position at either end, obtaining a final sequence length of 446 bp. The same procedure was applied to the seven TUB2 and TEF-1 α sequences, resulting in final sequence lengths of 413 bp and 355 to 363 bp, respectively. Subsequent BLAST analysis revealed that all the DNA sequences obtained in the present study showed the highest degree of similarity with sequences of the genus *Cadophora* contained in the GenBank database.

Pairwise distance matrices obtained separately for each genomic region and comprising DNA sequences of each isolate from South Tyrol revealed the presence of three different ITS haplotypes (ITS-a: 8

Fig. 2 Maximum-likelihood tree constructed with MEGA X software (Kumar et al., 2018) based on a multi-locus alignment (ITS, TUB2 and TEF-1 α) with 1,247 characters. *C. luteo-olivacea* isolates associated with side rot of apple in South Tyrol (highlighted in bold) and 18 reference isolates representing different species of the genus from the study of Travadon et al. (2015) were included in the analysis. *C. finlandia* was used as an outgroup. The numbers at the branches of the tree (> 95%) represent bootstrap values obtained after 1,000 replications

isolates, ITS-b: 1 isolate and ITS-c: 2 isolates). The haplotypes ITS-a and ITS-b differed in a single point mutation, while ITS-c differed from the others by two point mutations. The TUB2 sequences showed four different haplotypes (TUB2-a: 3 isolates, TUB2-b: 2 isolates, TUB2-c: 1 isolate and TUB2-d: 1 isolate). TUB2-a differed from TUB2-b in a single point mutation, and from TUB2-c and TUB2-d by two point mutations. Regarding the DNA sequences of the TEF-1 α gene, three haplotypes were found (TEF-1 α -a: 3 isolates, TEF-1 α -b: 3 isolates and TEF-1 α -c: 1 isolate). The TEF-1 α segment showed the highest degree of variability among the investigated isolates compared to the sequences of the other two loci. TEF-1 α -a differed from TEF-1 α -b by five point mutations, while TEF-1 α -c differed from TEF-1 α -a in 13 point mutations and from TEF-1 α -b in 14 point mutations.

Sequences of the same set of genomic regions as analyzed in this study of 18 reference isolates representing different species of the genus *Cadophora* were selected from Travadon et al. (2015) to perform a phylogenetic analysis (Supplementary Table S1).

The phylogenetic analysis was based on an alignment comprising 1,247 positions of concatenated multi-locus sequences and was accomplished using the maximum likelihood (ML) algorithm as implemented in the software Molecular Evolutionary Genetic Analysis (MEGA X) (Kumar et al., 2018). The resulting phylogenetic tree clustered all the isolates obtained in the present study in the monophyletic *C. luteo-olivacea* group (Fig. 2).

Phenotypic assessment based on in vitro growth rates and colony morphology, as well as conidia characteristics was conducted with pure single-spore cultures of representative isolates 18-DSS-CAFA-2-001, 19-DSS-BS-3-014 and 19-DSS-MX-2-015. The mycelium growth of these isolates after ten days at 20 °C showed an area of 5.2 ± 0.5 , 6.1 ± 0.3 and 5.1 ± 1.2 cm², respectively. The shape of the conidia was cylindrical with round ends, with average length and width of $8.6 \pm 2.3 \times 2.6 \pm 0.8$, $9.4 \pm 1.4 \times 2.1 \pm 1.2$ μ m and $8.4 \pm 2.5 \times 1.9 \pm 0.7$ μ m, respectively (Fig. 3).

Fig. 3 Colony appearance on the upper side (A) and reverse side (B) of isolate 18-DSS-CAFA-2-001 grown on PDA medium at 20 °C for 10 days, and conidia at a magnification of 400 $\times$ (C) and 1,000 $\times$ (D). Scale Bar = 50 μ m

Koch's postulates were proven with isolate 18-DSS-CAFA-2-001 on twelve apple fruit of 'Golden Delicious'. Preparation of the conidia suspension adjusted to 10^4 conidia mL^{-1} and inoculation of apple fruit were performed as described in Amaral Carneiro and Baric (2021a). Twelve control fruit were inoculated with sterile distilled water. Inoculated fruit were incubated for two weeks in moist chambers at 20 °C in the dark. The external symptoms were assessed, and the size of the lesions was measured. Isolate 18-DSS-CAFA-2-001 successfully infected 97% of the inoculated wounds ($N=36$). Lesions began to become evident at 5 days post-inoculation (dpi). At 21 dpi, the circular lesions had a dark-brown to black color and a diameter of 1.1 ± 0.5 cm (Supplementary Figure S2). Re-isolates from the lesions showed identical phenotypic characteristics as those previously described, and DNA sequences of the ITS region confirmed their identity. No lesions or rot developed on the negative control fruit.

C. luteo-olivacea is a pathogen frequently associated with wood-decay of grapevine (*Vitis vinifera*), reported in Canada, South Africa, Spain, Uruguay, and USA (Gramaje et al., 2011; Halleen et al., 2007; Navarrete et al., 2011; Travadon et al., 2015; Úrbez-Torres et al., 2014). In 2011, Spadaro et al. detected and characterized *C. luteo-olivacea* as a causative agent of the post-harvest disease skin pitting of kiwifruit (*Actinidia* spp.) in several Italian packing-houses. Additionally, this species was reported as a post-harvest pathogen of grape and kiwifruit in cold climates such as Ibaraki prefecture (Japan) and central Chile, respectively (Nakaune et al., 2016; Auger et al., 2018). On pome fruits, *C. luteo-olivacea* was isolated from rotten apple fruit at storage sites in Latvia (Grantina-Ievina, 2015), and from a pear sample in Italy (Spadaro et al., 2011), while in the Netherlands, *C. luteo-olivacea* was found to be an important post-harvest pathogen of pear (Wenneker et al., 2016), but less abundant on apple (Köhl et al., 2018). A closely related species, *C. malorum* is the main casual organism associated with side rot of apple in the United States (McColloch, 1944; Sugar, 2014; Sugar & Spotts, 1992, 1993a, 1993b).

Spadaro et al. (2011) suggested that *C. luteo-olivacea* could be a post-harvest pathogen of kiwi and pome fruit in Europe, while side rot of pome fruit in America is caused by *C. malorum*. However, this assumption is based on the analysis of a very small

number of isolates (two of *C. malorum*; three of *C. luteo-olivacea*) and by considering ITS as the only molecular marker region, DNA sequences of which were not even available for all the isolates. The information available about *C. malorum* on pears in the United States mainly derives from the publications of David Sugar and co-workers (Sugar, 2014; Sugar & Powers, 1986; Sugar & Spotts, 1992, 1993a, 1993b), while only one study describing *C. malorum* as a post-harvest pathogen of apple was retrieved (McColloch, 1944). These works primarily focused on the description of symptoms caused by the pathogen and on morphological characteristics of the fungus in vitro. However, many studies have affirmed that the biological characteristics and morphological features of *C. malorum* and *C. luteo-olivacea* are very similar (Harrington & McNew, 2003; Spadaro et al., 2011). Moreover, ITS sequence analysis does not permit to distinguish *C. malorum* and *C. luteo-olivacea* as distinct monophyletic groups. Therefore, it would be of interest to perform a multi-locus sequence typing analysis to ascertain whether all the isolates obtained in the past from apple and pear fruit with side rot symptoms in the USA would indeed cluster in the *C. malorum* group.

Blanchette et al. (2010), Travadon et al. (2015), and Eichmeier et al. (2020) have suggested that there is a relationship between environmental conditions and the distribution of *Cadophora* spp. Some species of the genus may be better adapted to cooler climates, as *C. malorum* was the main isolate obtained from degraded wood huts in Antarctica (Blanchette et al., 2010) or *C. novi-eboraci* was isolated from wood decay of grapevine in New York (Travadon et al., 2015), Germany (Gierl & Fischer, 2017), and from walnut trees with cankers and dieback symptoms in the Czech Republic (Eichmeier et al., 2020). *C. luteo-olivacea*, in contrast, was suggested to have an apparent preference for warmer, Mediterranean climate, since it has been isolated primarily in California (Travadon et al., 2015), South Africa (Halleen et al., 2007), and Spain (Gramaje et al., 2011). However, this assumption contradicts the study of Köhl et al. (2018), which showed that *C. luteo-olivacea* commonly occurs in pear and apple orchards in the Netherlands. The genus *Cadophora* has received little attention in taxonomic studies, which raises questions about the accurate classification of some specimens in fungal culture collections and DNA sequence

databases (Maciá-Vicente et al., 2020; Travadon et al., 2015). Therefore, an association of the distribution of different *Cadophora* species and environmental conditions seems to be premature.

There is little evidence available about *C. luteo-olivacea* as a post-harvest pathogen causing side rot of domesticated apple (*Malus domestica*) in Italy. The only indication of the presence of *C. luteo-olivacea* on European crab apple in Italy dates to 1950, when the strain CBS 357.51 was isolated from *Malus sylvestris* in Bologna (Emilia-Romagna) and was deposited in the fungal culture collection CBS-KNAW of the Fungal Biodiversity Centre (Utrecht, The Netherlands). So far, *C. luteo-olivacea* accounted for 1% of the isolates obtained from rotten apple fruit in our survey (see also Amaral Carneiro & Baric, 2021a). Nevertheless, epidemiological investigations at different apple-growing sites in this region would be recommended to identify possible sources of inoculum, as the fungus can be found on different plant residues in the orchard (Köhl et al., 2018). Such studies would represent a precautionary measure to avoid a possible scenario observed on kiwifruit in northern and central Italy. After its emergence on this crop, *C. luteo-olivacea* was initially considered irrelevant, but developed into one of the most important post-harvest pathogens of kiwifruit within a decade (Gorini, 1991; Piano et al., 2001; Spadaro et al., 2011). Furthermore, Sugar and Powers (1986) confirmed, by examining wound colonization, that *C. malorum* was a weak pathogen, and was able to invade the fruit only when its competitor, *Cladosporium herbarum*, was controlled in the orchard with benomyl sprays to which *C. malorum* was shown to be resistant (Bertrand & MacSwan, 1977). No such data are available for *C. luteo-olivacea*. Since there are no specific strategies implemented to control side rot of apple in this geographic area, it would be important to conduct fungicide sensitivity assays with products authorized in integrated pest management (Workgroup for Integrated Fruit Production in South Tyrol, 2021) and organic production, to determine whether the existing fungicide programs could also help preventing the development of *C. luteo-olivacea*.

Acknowledgements We are grateful to the Free University of Bozen-Bolzano for funding the research project. Furthermore,

we would like to thank the South Tyrolean Fruit Growers Cooperatives for providing samples of apple fruit with post-harvest diseases. The authors thank the Department of Innovation, Research, University and Museums of the Autonomous Province of Bozen/Bolzano for covering the Open Access publication costs.

Funding The research project “Development of a decision support system for the determination of post-harvest disorders and diseases of apples – DSSApple” was funded by the Free University of Bozen-Bolzano (Project Code IN2067—ID Call 2017).

Data availability All DNA sequences obtained in the present study were deposited in GenBank, whereas the multi-locus DNA sequence alignment was submitted to TreeBASE (study accession number S28299; <http://purl.org/phylo/treebase/phyloWS/study/TB2:S28299>).

Consent for publication Publication has been approved by all co-authors.

Conflicts of interest/Competing interests The authors declare that they have no conflict of interest.

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