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Characterization of *Eutypa lata* and *Cytospora pistaciae* causing dieback and canker of pistachio in Italy

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Summary. During the winter of 2017, dieback and canker symptoms were observed on pistachio (*Pistacia vera*) in two orchards in the Bronte area, Catania Province, Sicily, Southern Italy. Two different fungi were consistently isolated from infected tissues. Morphological observations and multi-locus phylogenies using five genomic loci (ITS, *act*, *rpb2*, *tef1* and *tub2*) identified these fungi as *Cytospora pistaciae* and *Eutypa lata*. Pathogenicity tests on 5-y-old potted plants of *P. vera* grafted on terebinth (*P. terebinthus*) reproduced similar symptoms as those observed in nature, and Koch's postulates were fulfilled for these two pathogens. This study is the first to report dieback and canker diseases of pistachio caused by *C. pistaciae* and *E. lata* in Italy.

Keywords. Pathogenicity, molecular analysis, disease symptoms, *Pistacia vera*.

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INTRODUCTION

Pistachio is cultivated in the southern regions of Italy, of which Sicily is the main production area. The province of Catania (with 430 ha of pistachio), followed by the provinces of Caltanissetta (with 220 ha) and Agrigento (with 145 ha) are the largest pistachio-producing areas, with a total production of 3,878 tons (AGRISTAT, 2017). Currently, the commune of Bronte in Catania Province represents the most important area of Sicily for pistachio production, and pistachio is an important economic resource for this territory (Barone and Marra, 2004). In this area, different pistachio cultivars are grafted on terebinth plants which are grown on volcanic soils (Barone *et al*., 1985). Few studies have been conducted to investigate pistachio diseases occurring in Italy, and only a few diseases have been reported to date. These include branch dieback (caused by *Botryodiplodia* sp.), leaf spot (*Alternaria alternata*), anthracnose, branch and twig cankers (*Botryosphaeria dothidea*)

and phylloptosis and leaf spots (mainly caused by *Septoria pistaciae*) (Casalicchio, 1963; Schilirò and Privitera, 1988; Frisullo *et al*., 1996; Vitale *et al*., 2007). In eastern Sicily, cankers and decline caused by *Liberomyces pistaciae* Voglmayr, Vitale, Aiello, Guarnaccia, Luongo & Belisario are the most important pistachio diseases (Vitale *et al*., 2018). Blight caused by *Arthrinium xenocordella* Crous was also recently reported on pistachio fruit in the Agrigento Province (Aiello *et al*., 2018).

During the winter of 2017, pistachio trees with dieback, canker and gummosis symptoms were observed in the area of Bronte. Following culturing from necrotic tissues, two fungal species were consistently isolated. Cankers from one orchard generated colonies of *Cytospora* while cankers from a second orchard generated *Eutypa* colonies.

The aim of the present study was to investigate the etiology of pistachio canker diseases, which could represent new threats for the pistachio production of Sicily.

MATERIALS AND METHODS

Isolation and morphology of fungi

Surveys were conducted in ten pistachio orchards with histories of branch canker and dieback in eastern Sicily (Catania Province). Approximately 20 symptomatic pistachio branches with canker were collected from each orchard for analyses. Sub-cortical and wood fragments (about 5 × 5 mm) were cut from the margins between affected and healthy branch tissues. Tissue pieces were disinfected in 1.2% sodium hypochlorite for 60 s, rinsed in sterile water and dried on sterile filter paper. The fragments were then placed into Petri plates containing potato dextrose agar (PDA, Oxoid) amended with 100 mg L^{-1} of streptomycin sulfate (Sigma-Aldrich), and incubated at room temperature (25 ± 5°C). Fungal colonies consistently growing from symptomatic tissues were cultured into new PDA plates. To obtain pure cultures, single-conidium or hyphal-tip isolations were performed after 1 month incubation at room temperature under natural light conditions. Isolates for each putative fungal pathogen (four isolates of *Eutypa* and three of *Cytospora*) were characterized by morphological, molecular and phylogenetic analyses (Table 1). These cultures were deposited in the working collection of Dr Pedro Crous (CPC), at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (Table 1). Size and shape of conidia were recorded for each fungal isolate grown on PDA for 2 weeks at 25 ± 1°C.

DNA extraction, PCR amplification and sequencing

Extractions of genomic DNA were performed from pure cultures, as reported elsewhere (Guarnaccia and Crous, 2017), using the Wizard Genomic DNA Purification Kit (Promega Corporation). Partial regions of five loci were amplified. The primers ITS5 and ITS4 (White *et al*., 1990) were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the 18S rRNA, the first internal transcribed spacer region, the 5.8S rRNA gene; the second internal transcribed spacer region and the 5' end of the 28S rRNA gene. The primers ACT-512F and ACT-783R (Carbone and Kohn, 1999) were used to amplify part of the actin gene (*act*). The partial beta-tubulin (*tub2*) gene was amplified with primers Bt-2a and Bt-2b (Glass and Donaldson, 1995). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1-α gene (*tef1*). The primers 5f2/7cr were used to amplify part of *rpb2* (O'Donnell *et al*., 2010). The regions ITS, *act*, *tef1* and *rpb2* were amplified for the species of *Cytospora* using the PCR programmes adopted by Lawrence *et al*. (2018) and Jami *et al*. (2018). The regions ITS and *tub2* were amplified for the species of *Eutypa* following the PCR programmes used by Moyo *et al*. (2018a). The PCR products were sequenced in both directions using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies), after which amplicons were purified through Sephadex G-50 Fine columns (GE Healthcare) in MultiScreen HV plates (Millipore). Purified sequence reactions were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies). The DNA sequences generated were analyzed and consensus sequences were computed using the program SeqMan Pro (DNASTAR).

Phylogenetic analyses

Novel sequences generated in this study were blasted against the NCBIs GenBank nucleotide database, to determine the closest relatives to be included in the phylogenetic analyses. Blast analyses indicated that three isolates belonged to *Cytospora* and the remaining four to *Eutypa*. Sequence alignments of the different gene regions, including sequences obtained from this study and sequences from GenBank, were initially performed using the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index. html) (Katoh and Standley, 2013), and then manually adjusted in MEGA v. 7 (Kumar *et al*., 2016). To establish the identity of the fungal isolates, phylogenetic analyses were conducted using

one locus (data not shown) as well as concatenated analyses of four loci (ITS, *act*, *tef1* and *rpb2*) for *Cytospora* spp. and two loci (ITS and *tub2*) for *Eutypa* spp., as indicated by blast analysis. Additional reference sequences were selected based on recent studies on *Cytospora* and *Eutypa* species (Lawrence *et al*., 2018, Moyo *et al*., 2018a, b). Phylogenetic analyses were based on Maximum Parsimony (MP) for all the individual loci and for the multi-locus analyses. The MP analyses were carried out using PAUP (Swofford, 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on 'best trees' only, with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony and the bootstrap analyses (Hillis and Bull 1993) were based on 1,000 replicates. Sequences generated in this study were deposited in GenBank (Table 1).

Pathogenicity of representative isolates

Pathogenicity tests with one representative isolate of *C. pistaciae* (CPC34208) and one of *E. lata* (CPC34213; Table 1) were carried out to satisfy Koch's postulates. These tests were carried out in a growth chamber maintained at 25 ± 1°C. Potted 5-y-old plants of *P. vera* grafted onto *P. terebinthus* were used for artificial inoculations. Three plants were inoculated with each isolate. Six wounds were made on individual plant stems approx. 8-10 cm apart from each other.

Inoculations were made on stems after removing of bark discs with a cork borer, placing a 5 mm plug from a 14-d-old PDA culture of test isolate into the wound and covering with Parafilm® (Pechney Plastic Packaging Inc.) to prevent desiccation. An equivalent number of plants and inoculation sites were inoculated with sterile PDA plugs to serve as controls. The inoculated plants were observed once each month for symptoms development, and a final assessment was conducted 5 months after inoculation. To fulfil Koch's postulates, re-isolations were carried out following the procedure described above, where tissue fragments were plated onto PDA. Each re-isolated fungus was identified through morphological characteristics.

RESULTS AND DISCUSSION

Symptomatic plants showed cankers with cracking and gum exudation, and often branches or shoots showed dieback. Under the bark of affected branches, cankers were characterized by discolouration and necrosis, and in some cases discolouration extended to the vascular tissue (xylem) and pith. Two different fungal colony types were consistently obtained from isolations from symptomatic tissues (Figure 1) taken from the two orchards. Cankers from one orchard generated *Cytospora* colonies while cankers from the other orchard generated *Eutypa* colonies. The same symptoms in the remaining orchards investigated in the Bronte area produced colonies of *L. pistaciae* (Vitale *et al*., 2018).

Conidia of three representative isolates of *Cytospora* were in accordance with the description by Lawrence *et al*. (2018) of *C. pistaciae* Lawr., Holland & Trouillas. The four MP trees derived from the single gene sequence alignments (ITS, *act*, *tef1* and *rpb2*) were topologically similar, confirming that the three isolates used for the molecular analyses were *Cytospora*. The combined phylogeny of *Cytospora* species consisted of 35 sequences, including the outgroup sequences of *Diaporthe limonicola* (culture CBS 142549; Guarnaccia and Crous, 2017). A total of 2,056 characters (ITS: 1–574, *act*: 581–890, *tef1*: 897–1289, *rpb2*: 1296–2056) were included in the phylogenetic analysis of *Cytospora* spp. For the phylogeny of *Cytospora* species, 489 characters were parsimony-informative, 336 were variable and parsimony-uninformative and 1,213 characters were constant. A maximum of 1,000 equally most parsimonious trees were saved (Tree length = 1 552, CI = 0.743, RI = 0.782 and RC = 0.581). Bootstrap support values from the parsimony analysis were plotted on the phylogenetic trees presented in Figure 2. In the combined analyses, the three representative isolates clustered with four reference strains of *C. pistaciae*. The individual alignments and trees of the single loci used in the analyses were compared with respect to their performance in species recognition. *Cytospora pistaciae* was differentiated and identified in all single-gene analyses.

Cytospora terebinthi Bres. has been reported in Italy as the causal agent of cankers and gummosis of pistachio (Corazza *et al*., 1990; Furnitto, 1984), while other *Cytospora* species have been reported in other crops, including peach (Hampson and Sinclair., 1973; Banko and Helton, 1974). The taxonomy of *Cytospora* species associated with fruit and nut crops was recently revised, and *C. pistaciae* was described as a new species on pistachio in California, but the pathogenicity of this species was not investigated (Lawrence *et al*. 2018).

Conidia of four isolates of *Eutypa* were in accordance with the description of *E. lata* by Moyo *et al*. (2018b). The two MP trees derived from the single gene sequence alignments (ITS and *tub2*) were topologi-

cally similar, and this confirmed that the four isolates used in this study were *Eutypa*. All the species belonging to *Eutypa* and other Xylariales used in the multilocus phylogeny consisted of 29 sequences with the outgroup sequences of *L. pistaciae* (CBS 144255; Vitale *et al*., 2018). A total of 1,076 characters (ITS: 1–582, *tub2*: 589–1,076) were used for the Xylariales analysis, and 453 characters were parsimony-informative, 166 were variable and parsimony-uninformative and 451 characters were constant. A maximum of 1,000 equally most parsimonious trees were saved (Tree length = 1 669, CI = 0.648, RI = 0.786 and RC = 0.509). Bootstrap support values from the parsimony analysis were plotted on the phylogenetic trees presented in Figure 3. In the combined analyses, the four isolates were related to reference isolates of *E. lata*. The individual alignments and trees of the single loci used in the analyses were compared with respect to their performance in species recognition. *Eutypa lata* was differentiated and identified in all single-gene analyses.

Eutypa lata is a pathogen with a wide host range, occurring in more than 160 hosts (Farr and Rossman, 2017). In Italy, *E. lata* has been reported on *Acer* sp. in Sicily (Greuter *et al*., 1991), *Ribes rubrum* (Prodorutti *et al*., 2008), olive trees (Tosi and Natalini, 2009) and *Vitis vinifera* (Acero *et al*., 2004). Eutypa dieback and gummosis of pistachio caused by *E. lata* has been reported only in Greece (Rumbos, 1986).

Five months after artificial inoculation, symptoms produced from each fungus in trees were similar to those present on trees in the field. These consisted of external cankers and gumosis produced around the inoculation sites, with small cracks present in each sunken lesion. After removing the bark, a dark discolouration and necrotic tissues were visible (Figure 1). The respective inoculated pathogens were re-isolated from symptomatic tissues, thus fulfilling Koch's postulates. No symptoms were observed on control (uninoculated) plants.

This is the first report of *E. lata* and *C. pistaciae* associated with cankers on pistachio in Europe. Further

Figure 1. Symptoms reproduced from mycelial plug inoculation with *Cytospora pistaciae* (a) and *Eutypa lata* (b) on 5-y-old potted plants of *Pistacia vera* 5 months after inoculation with respective fungi. Cultural characteristics of *Cytospora pistaciae* (c) and *Eutypa lata* (d) colonies grown on PDA are also illustrated.

Table 1. Collection details and GenBank accession numbers for isolates included in this study.

Species	Culture No.	Host	Locality	GenBank Number[a]				
				ITS	*act*	*tef1*	*rpb2*	*tub2*
Cytospora austromontana	**CMW 6735**	*Eucalyptus pauciflora*	Australia	NR137542	-	-	-	-
C. berkeleyi	**StanfordT3T**	*Eucalyptus globulus*	California, USA	AY347350	-	-	-	-
C. californica	**9c–24 = CBS 144234**	*Juglans regia*	California, USA	MG971935	MG972083	MG971645	-	-
	KARE264	*Pistacia vera*	California, USA	MG971920	MG972069	MG971630	-	-
C. cincta	CFCC 89956	*Prunus cerasifera*	China	KR045624	-	-	KU710953	-
C. cinereostroma	**CMW 5700**	*Eucalyptus globulus*	Chile	AY347377	-	-	-	-
C. diatrypelloidea	**CMW 8549**	*Eucalyptus globulus*	Australia	AY347368	-	-	-	-
C. disciformis	**CMW 6509**	*Eucalyptus grandis*	Uruguay	AY347374	-	-	-	-
C. eriobotryae	**IMI136523**	*Eriobotrya japonica*	India	AY347327	-	-	-	-
C. eucalypticola	**ATCC 96150**	*Eucalyptus nitens*	Tasmania, Australia	AY347358	-	-	-	-
	CMW 5309	*Eucalyptus grandis*	Entebbe, Uganda	AF260266	-	-	-	-
	CMW 40051	*Eucalyptus camaldulensis*	Zimbabwe	KF923249	-	-	-	-
	CMW 40048	*Eucalyptus camaldulensis*	Zimbabwe	KF923248	-	-	-	-
C. gigaspora	**CFCC 89634**	*Salix psammophila*	China	KF765671	KU711000	-	KU710960	-
C. granati	**CBS 144237**	*Punica granatum*	USA	MG971799	MG971949	MG971514	-	-
C. joaquinensis	**CBS 144235**	*Populus deltoides*	USA	MG971895	MG972044	MG971605	-	-
C. leucostoma	CFCC 50015	*Sorbus pohuashanensis*	China	KR045634	-	-	-	-
C. nivea	MFLUCC 15–0860	*Salix acutifolia*	Russia	KY417737	KU711006	-	KY417805	-
C. parapistaciae	KARE232	*Pistacia vera*	California, USA	MG971807	MG971957	MG971522	-	-
	KARE268	*Pistacia vera*	California, USA	MG971806	MG971956	MG971521	-	-
	KARE269	*Pistacia vera*	California, USA	MG971805	MG971955	MG971520	-	-
	KARE270 = CBS 144506	*Pistacia vera*	California, USA	MG971804	MG971954	MG971519	-	-
C. parasitica	**MFLUCC 15–0507**	*Malus domestica*	Russia	KY417740	-	-	KY417808	-
C. pistaciae	KARE441	*Pistacia vera*	California, USA	MG971800	MG971950	MG971515	-	-
	KARE442	*Pistacia vera*	California, USA	MG971803	MG971953	MG971518	-	-
	KARE443 = CBS 144238	*Pistacia vera*	California, USA	MG971802	MG971952	MG971517	-	-
	KARE444	*Pistacia vera*	California, USA	MG971801	MG971951	MG971516	-	-
	CPC 34208 = CBS 144226	*Pistacia vera*	Italy	*MN078066*	*MN078063*	*MN078077*	*MN078080*	-
	CPC 34209	*Pistacia vera*	Italy	*MN078067*	*MN078064*	*MN078078*	*MN078081*	-
	CPC 34211	*Pistacia vera*	Italy	*MN078068*	*MN078065*	*MN078079*	*MN078082*	-
C. punicae	5A-80 = CBS 144244	*Punica granatum*	USA	MG971943	MG972091	MG971654	-	-
C. sacculus	CFCC 89624	*Juglans regia*	China	KR045645	-	-	KU710976	-

(Continued)

Table 1. (Continued).

Species	Culture No.	Host	Locality	GenBank Number[a]				
				ITS	*act*	*tef1*	*rpb2*	*tub2*
C. salicacearum	**MFLUCC 16–0509**	*Salix alba*	Russia	KY417746	-	-	KY417814	-
C. salicicola	**MFLUCC 14–1052**	*Salix alba*	Russia	KU982636	-	-	-	-
Cryptosphaeria subcutanea	CBS 240.87	*Salix borealis*	Norway	KT425232	-	-	-	KT425167
Diaporthe limonicola	**CBS 142549**	*Citrus limon*	Malta	MF418422	-	MF418501	MH797629	-
Diatrypella atlantica	**HUEFS 194228**	unknown	Brazil	KM396615	-	-	-	KR363998
Eutypa cremea	CBS 120837	*Prunus salicina*	South Africa	KY752762	-	-	-	KY752791
Eutypa lata	CBS 121430	*Prunus armeniaca*	South Africa	KY752766	-	-	-	KY752794
	ADSC300	*Schinus molle*	Australia	HQ692610	-	-	-	HQ692493
	SACEA01	*Ceanothus* sp.	Australia	HQ692615	-	-	-	HQ692499
	EP18	*Vitis vinifera*	Australia	HQ692611	-	-	-	HQ692501
	CPC 34213	*Pistacia vera*	Italy	*MN078069*	-	-	-	*MN078073*
	CPC 34214	*Pistacia vera*	Italy	*MN078070*	-	-	-	*MN078074*
	CPC 34215	*Pistacia vera*	Italy	*MN078071*	-	-	-	*MN078075*
	CPC 34216	*Pistacia vera*	Italy	*MN078072*	-	-	-	*MN078076*
Eutypa leptoplaca	ADFIC100	*Ficus macrophylla*	Australia	HQ692608	-	-	-	HQ692485
Eutypa maura	CBS 219.87	*Acer pseudoplatanus*	Switzerland	AY684224	-	-	-	DQ006967
Eutypa tetragona	CBS 284.87	*Sarothamnus scoparius*	France	DQ006923	-	-	-	DQ006960
Eutypella citricola	STEU 8098	*Vitis vinifera*	South Africa	KY111634	-	-	-	KY111588
Eutypella microtheca	STEU 8107	*Vitis vinifera*	South Africa	KY111629	-	-	-	KY111608
Eutypella vitis	MSUELM13	*Vitis vinifera*	USA	DQ006943	-	-	-	DQ006999
Liberomyces pistaciae	**CBS 144225**	*Pistacia vera*	Italy	MH797562	-	-	-	MH797697
Peroneutypa alsophila	CBS 250.87	*Arthrocnemum fruticosum*	France	AJ302467	-	-	-	-
Peroneutypa curvispora	HUEFS 136877	unknown	Brazil	KM396646	-	-	-	-
Peroneutypa diminutiasca	**MFLUCC 17–2144**	unknown	Thailand	MG873479	-	-	-	MH316765
Peroneutypa diminutispora	**HUEFS 192196**	unknown	Brazil	KM396647	-	-	-	-
Peroneutypa kochiana	F-092,373	*Atriplex halimus*	Spain	AJ302462	-	-	-	-
Peroneutypa longiasca	**MFLUCC 17–0371**	*Hevea brasiliensis*	Thailand	MF959502	-	-	-	-
Peroneutypa rubiformis	**MFLUCC 17–2142**	unknown	Thailand	MG873477	-	-	-	MH316763
Peroneutypa scoparia	DFMAL100	*Robinia pseudoacacia*	France	GQ293962	-	-	-	GQ294029
	CBS 242.87	*Robinia pseudoacacia*	France	AJ302465	-	-	-	-
	MFLUCC 17–2143	unknown	Thailand	MG873478	-	-	-	MH316764

[a] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; act: actin; tef1: translation elongation factor 1-α gene; rpb2: RNA polymerase second largest subunit; tub2: beta-tubulin. Sequences generated in this study indicated in italics. Ex-type and ex-epitype cultures are indicated in bold.

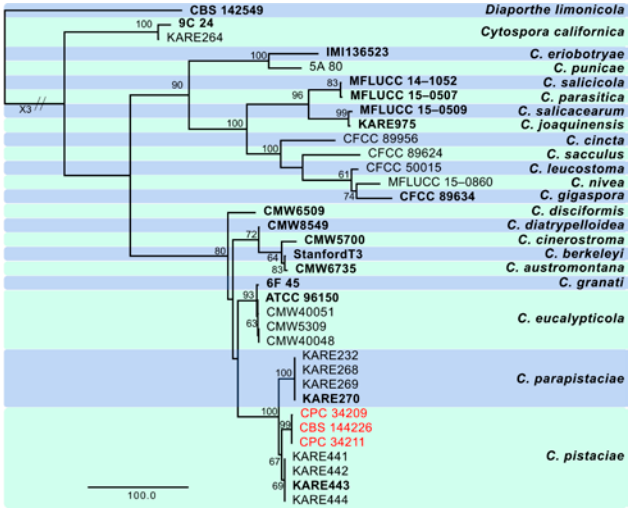

Figure 2. The first of two equally most parsimonious trees obtained from a heuristic search of the combined ITS, *act*, *tef1* and *rpb2* sequence alignments of *Cytospora* spp. Bootstrap support values are shown at the nodes. The strains isolated in this study are shown in red and the scale bar represents the number of changes. The tree was rooted to *Diaporthe limonicola* (CBS 142549).

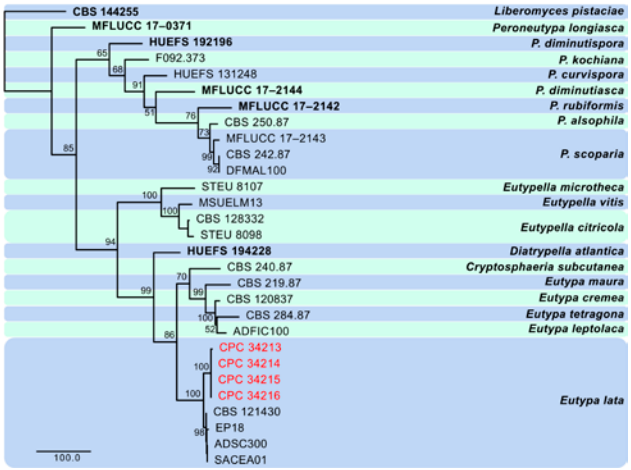

Figure 3. The first of four equally most parsimonious trees obtained from a heuristic search of the combined ITS, and *tub2* sequence alignments of species belonging to *Eutypa* and other genera of Diatrypaceae. Bootstrap support values are shown at the nodes. The strains isolated in this study are shown in red and the scale bar represents the number of changes. The tree was rooted to *Liberomyces pistaciae* (CBS 144255).

studies should investigate the role of propagation material, mechanical injuries and pruning wounds in disease transmission and spread.

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