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NEW OR UNUSUAL DISEASE REPORTS

Neofusicoccum luteum as a pathogen on Tejocote (*Crataegus mexicana*)

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Summary. Tejocote (*Crataegus mexicana*), a small pome crab-apple-like fruit, is becoming economically important in California with increasing production, so consideration of diseases that hinder the yield is important. Diseased trees of tejocote were observed in four orchards of Riverside and San Diego Counties of California. Ten symptomatic/asymptomatic samples were studied from each of the orchards. Five most frequently isolated fungi were identified on the basis of morphological characters and sequence data of the internal transcribed spacer ITS1-5.8S-ITS2 and partial β-tubulin gene. Three isolates were identified as *Neofusicoccum luteum* and two as *Phomopsis* sp. Pathogenicity tests were conducted by inoculating detached shoots of healthy tejocote trees. Significant lesions were observed on all shoots inoculated with the three *N. luteum* isolates (designated UCR1190, UCR1191, and UCR1192), but not on the shoots inoculated with other isolates or the non-inoculated controls. Results indicated that all three *N. luteum* isolates are aggressive pathogens on tejocote. This pathosystem should be further studied with a goal of designing appropriate disease management strategies.

Key words: Botryosphaeriaceae; branch canker; dieback; Mexican hawthorn.

Introduction

Tejocote, also known as Mexican hawthorn (*Crataegus mexicana*, Family Rosaceae), (www.plants.usda.gov; Phipps *et al*., 1990) was recorded as introduced to California in 1868 (Riedel, 1957), but was not cultivated for fruit production. In the 1990s, the United States Department of Agriculture Smuggling, Interdiction and Trade Compliance Program confiscated many tejocote fruits at the point of entry during frequent attempts to get them into the United States from overseas (www.ers.usda.gov). The fruits of *C. mexicana* are each small (1–3 cm in diameter), pome, and crab-apple-like, with three to five brown seeds and a hard skin covering the fleshy pulp that becomes soft when ripe. Fruit ripens from October to December in California when the colour is mostly greenish-yellow with russet or black spots or occasionally red to dark purple. Fruits may be eaten raw, cooked, or used as raw material in the processing of concentrated pulp, jam, jellies, and marmalades (Vivar-Vera *et al*., 2007). Tejocote production in California is becoming economically important; thereby generating attention about the possible pathogens that are hindering its yield.

Many fungal genera have been associated with dieback, and trunk and branch cankers in many woody hosts, including *Phomopsis* sp. and members of the Botryosphaeriaceae. Specifically, *Neofusicoccum luteum* has been reported as a pathogen associated with dieback and canker in many woody hosts, including kiwifruit (Pennycook and Samuels, 1985), grapevine (Philips *et al*., 2002), avocado, (McDonald *et al*., 2009), olives (Sergeeva *et al*., 2009), and citrus (Adesemoye *et al*., 2011). Usually, openings and wounds in host plants make invasion easier for species in the Botryosphaeriaceae, including *N. luteum*, and specifically, pruning wounds are important points of entry (Rolshausen *et al*., 2010; Amponsah *et al*. 2012).

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In 2010, canker and dieback (Figure 1A) of tejocote trees were observed in Riverside and San Diego Counties of California. Symptoms included grayish to brown discoloration on the rind, bark cracking (Figure 1B), and when a cut was made through the tree, canker was seen covering xylem advancing regions (Figure 1C). The objective of the present study was to identify the pathogens associated with branch canker and dieback of tejocote in the two counties.

Materials and methods

Sample collection, isolation, and morphology of fungal isolates

In 2010, symptomatic branches of dying tejocote trees, most of which showed severe canker and dieback, were sampled from four orchards in Riverside and San Diego Counties, California. Ten branch samples were collected from each orchard and transported in a cooler to the laboratory at the University of California, Riverside (UCR). Samples were washed with deionized water and cankers were surface-disinfested with 95% ethanol and flamed. Pieces of symptomatic tissues, 1–2 mm^2 in size, were plated onto nutrient agar (NA) and incubated for about 36 h at 27°C to isolate bacteria. Similar tissues were plated onto potato dextrose agar amended with 0.01% tetracycline (PDA-tet) and incubated in the dark for 4 d at 25°C to isolate fungi. Pure cultures of fungal isolates were obtained by transferring hyphal tips to fresh PDA-tet plates while single colonies of bacterial isolates were streaked on fresh NA.

Preliminary identification of fungal isolates was based on morphological characters (Alexopoulos *et al*., 1996; Farr *et al*., 2002; Luque *et al*., 2005). Isolates suspected to belong to the Botryosphaeriaceae were grown on oatmeal agar, slightly modified from Gooding and Lucas (1959) or 2% water agar on which autoclaved pine needles were placed and incubated at 25°C under a UV light with 12 h photoperiod for 2–4 weeks (McDonald and Eskalen, 2011) until fertile pycnidia were formed. Digital images of 20 conidia per isolate were taken with a Leica DFC420 camera mounted on a compound microscope (Olympus BX40). The length and width of the conidia were determined using the SPOT Imaging Software v4.7.0.35 (Diagnostic Instruments, Inc., MI, USA). Conidial characteristics were compared with those in previous reports (Phillips *et al*., 2002; Luque *et al*., 2005; Úrbez-Torres *et al*., 2006).

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from fungal isolates by shaking in FastPrep24 (MP Biomedicals LLC, Solon, OH, USA) at a speed of 4.0 for 20 s, and using the methods of Cenis (1992). Polymerase chain reaction (PCR) was performed in a MyCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using primers Bt2a/2b for β-tubulin (BT) and/or ITS4/5 for 18S rRNA, internal transcribed spacer ITS1-5.8S-ITS2, and 28S rRNA regions (White *et al*., 1990; Farr *et al*. 2002; Slippers *et al*., 2004). Each PCR reaction contained 12.5 µL of GoTaq Green Master Mix (Prome-

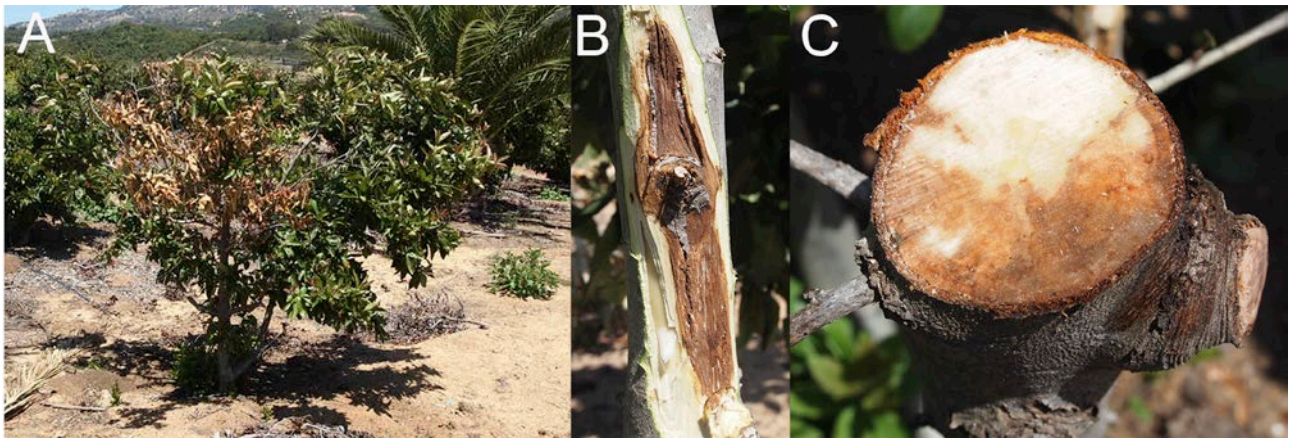

Figure 1. (a) A tejocote tree showing dieback, (b) canker on the trunk, and (c) cross section of a branch canker.

ga, Madison, WI), 9.3 μL of PCR-grade water, 0.6 μL of 10 μM of each primer, and 2 μL of DNA template. The reaction protocol included an initial preheat at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 45 s, and a final extension at 72°C for 5 min.

The PCR products were verified in 1% agarose gel and the gels were documented with Dark Reader DR88X transilluminator (Clare Chemical Research, Dolores, CO, USA). The PCR products were purified using Isopure PCR purification kit (catalog # CM-0100-100, Denville Scientific Inc, Metuchen, NJ). The quality of the PCR products was estimated with a NanoDrop Spectrophotometer ND-1000 (Thermal Scientific, Wilmington, DE). Sequencing was done at the Institute for Integrative Genome Biology of the University of California, Riverside. Sequences were edited and aligned with Sequencher software 4.6 (Gene Codes, Corporation, Ann Arbor, MI, USA). The sequences have been deposited in GenBank under Accession numbers JF921867 to JF921871 for ITS and JF921872 to JF921874 for β-tubulin.

Phylogenetic analysis

ITS and β-tubulin sequences were assembled with 38 others retrieved from GenBank, representing 15 species of *Botryosphaeria* with one outgroup species (*Guignardia philoprina*). Sequences were aligned with Clustal X (Thompson *et al*., 1997), and analysis of data sets was performed using PAUP* version 4.0 b10 (Sinauer Associates) (Swofford, 2002). Separate analyses were run for the ITS and β-tubulin data sets and a partition homogeneity test was performed in PAUP to determine if the two data sets could be combined in a single analysis. Maximum parsimony analysis was performed using the heuristic search option. Bootstrap support values were evaluated from 100 replicates to test branch strength. Tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated.

Pathogenicity test

Pathogenicity was tested on detached shoots of healthy tejocote trees (*Crataegus mexicana*), the same variety from which the isolates were originally obtained. One wound per shoot was made on 1–2 y old detached shoots using a 3 mm diam. cork borer, and the freshly wounded surfaces were inoculated with 3 mm diam. mycelial plugs of 5 d old cultures of each isolate growing on PDA-tet (Adesemoye and Eskalen, 2011). Inoculated wounds and shoot ends were covered with petroleum jelly and wrapped with Parafilm to prevent desiccation. Control shoots were inoculated with sterile agar plugs and incubated similarly. There were ten replicate shoots for each isolate and the control treatment. Shoots were incubated at 25°C in moist chambers for 4 weeks after which time lesion lengths were recorded. The experiment was repeated. Re-isolation and identification of fungi from the inoculated and control shoots were carried out using similar methods as described above.

Data analyses

Lesion lengths were analyzed using GLM procedure of Statistical Analysis System 9.3 (SAS Institute, Cary, NC), and Fisher's protected LSD was used to separate means with P=0.05.

Results

Characteristics of isolates

Isolates UCR1190 to UCR1192 growing on PDA were white with fluffy rope-like hyphae reaching the lid of the Petri dish within 3 d. Yellowing of the growth medium was obvious after 2 d, matching previous descriptions of *N. luteum*, which also produces a yellow pigment that diffuses into the agar (Pennycook and Samuels, 1985; Phillips *et al*., 2002; Sergeeva *et al*., 2009). The yellow colour (Figure 2a) gradually turned grey to violaceous/black after 8–10 d of incubation (Figure 2b). After 16 d, conidia could be seen oozing out of the pycnidia formed on pine needle agar. Conidia were ellipsoidal to fusiform with rounded ends, unicellular, hyaline, and some developed one or two septa. The mean conidial size was 19.8 μm × 6.5 μm for UCR1191, 20.5 μm × 6.5 μm for UCR1190, and 21.4 μm × 6.9 μm for UCR1192. Two isolates of another frequently isolated fungus were identified morphologically as *Phomopsis* sp. (Farr *et al*., 2002).

Molecular identification and phylogenetic analysis

The BLAST search showed that three of the isolates matched 100% to *N. luteum* in GenBank. For the

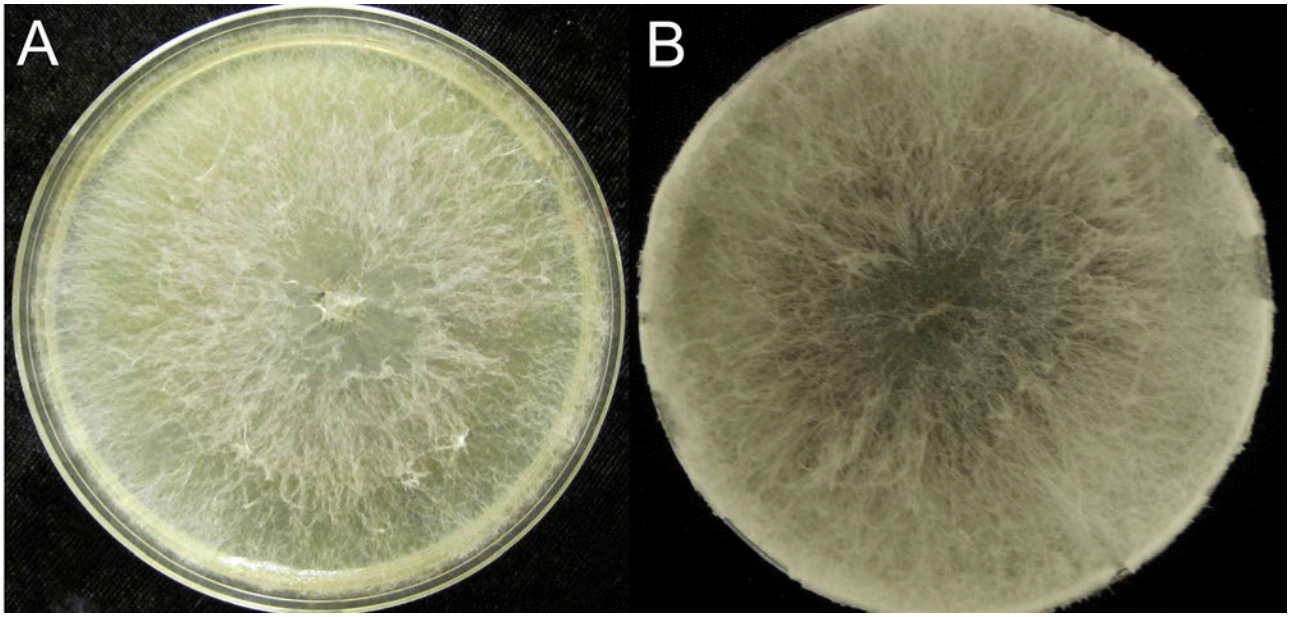

Figure 2. Neofusicoccum luteum strain UCR1190 (a) yellow pigment on a 3 d old Petri plate culture, (b) darkened 12 d old culture.

two isolates earlier identified through morphology as *Phomopsis* sp., strain UCR1193 matched 98% to *Phomopsis* sp. and strain UCR1197 matched 99% to *Phomopsis theicola*. *Erwinia* sp. was isolated from one of the samples.

Of the 564 characters analyzed for the ITS data set; 434 characters were constant and 87 were parsimony uninformative. Maximum parsimony analysis of the remaining 43 parsimony informative characters resulted in 210 equally parsimonious trees with TL = 144, CI = 0.965278, RI = 0.947917, RC = 0.915003, and HI = 0.034722. Of the 400 characters analyzed for the β-tubulin data set 281 characters were constant, 81 were parsimony uninformative. Maximum parsimony analysis of the remaining 38 parsimony informative characters resulted in 139 equally parsimonious trees with TL = 135, CI = 0.962963, RI = 0.939024, RC = 0.904246, and HI = 0.037037. The most parsimonious trees for ITS and β-tubulin regions recovered similar topology with a high degree of clustering, showing clustering of isolates UCR1190, UCR1191, and UCR1192 with isolates of other *N. luteum*, but with low bootstrap support (63%). The clade for *N. luteum* was well separated from the other clades of sequences obtained from GeneBank for *Botryosphaeria dothidea*, *B. parva*, *B. australe*, and strain CBS44768 (outgroup).

A partition homogeneity test indicated that the ITS and β-tubulin data sets could be combined (P=0.01). Of the 963 characters analyzed for the combined data set, 714 were constant, 168 characters were parsimony uninformative and 81 parsimony informative. One hundred trees were retained in the analysis and a strict consensus tree was generated. The consensus tree (Figure 3) recovered a topology with strong bootstrap support (94%) for the clade of *N. luteum* isolates including UCR1190, UCR1190, and UCR1192.

Pathogenicity test

After 4 weeks, all detached shoots inoculated with the three isolates of *N. luteum* (UCR1190, UCR1191, and UCR1192) had brown lesions that spread from the inoculation points to both ends of the shoots, and pycnidia of *N. luteum* were observed on all the infected shoots. The mean lesion lengths were not significantly (P=0.05) different among isolates but were different from the control (Table 1). All three isolates were recovered from all inoculated shoots but not from the control shoots. The two isolates of *Phomopsis* sp., which were included in the pathogenicity test, did not show any lesion different from the control.

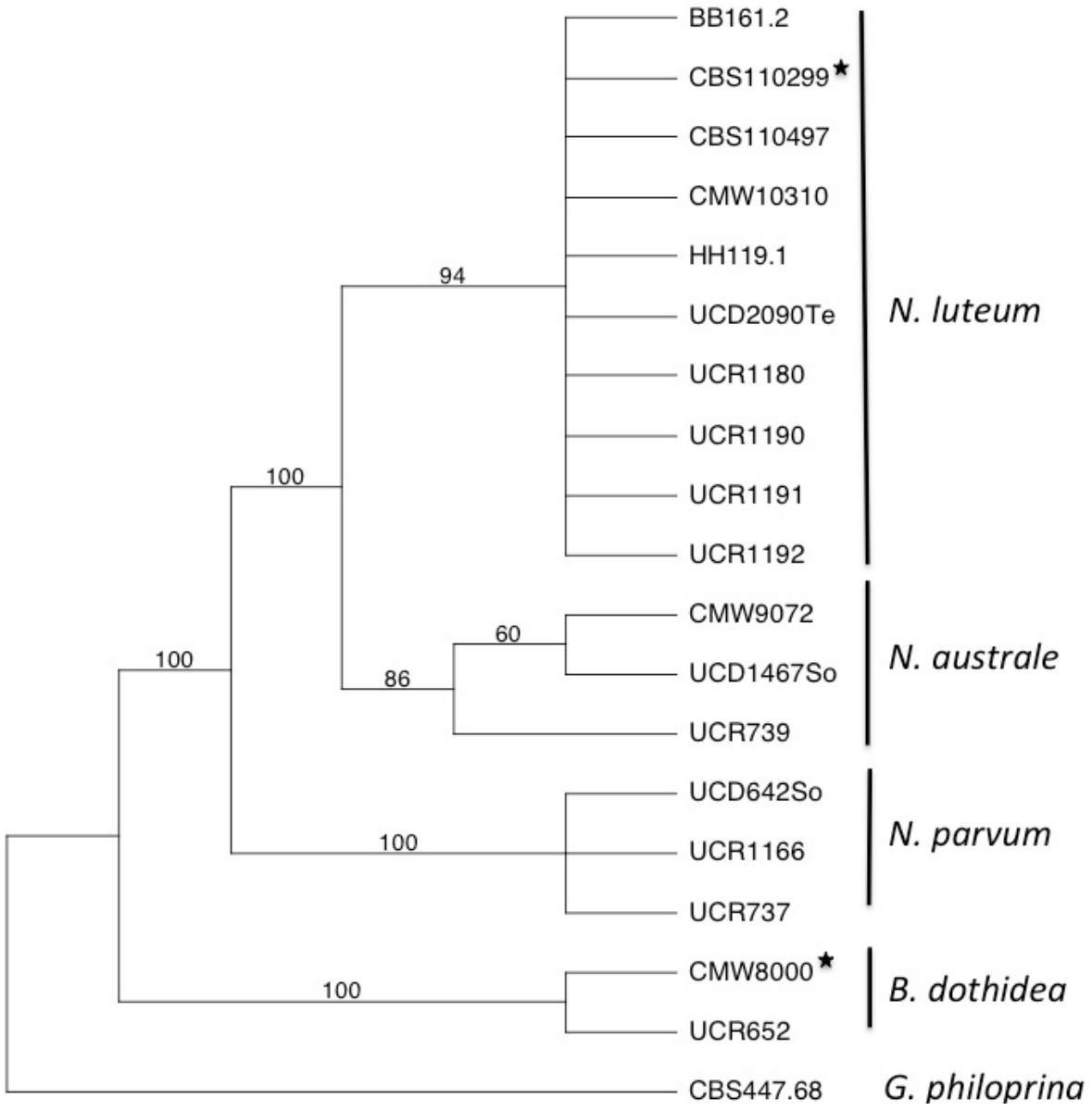

Figure 3. Consensus tree obtained from combined ITS and b-tubulin sequence data sets. (TL=279 steps, CI= 0.964158, RI= 0.943820, RC= 0.909992). Bootstrap support values from 1000 replicates higher than 50% are reported at the nodes. (*) ex-type sequences from GenBank.

Discussion

This study confirmed the identity of the isolates UCR1190, UCR1191, and UCR1192 as *N. luteum*, and established the association of the isolates with branch and shoot canker of Tejocote. There is evidence that *Phomopsis* sp. were not involved in the cause of tejocote canker, although the genus *Phomopsis* has been associated with cankers of many plant hosts, including members of the Rosaceae in which tejocote belongs (Farr *et al*., 2002). Although one isolate of *Erwinia* sp., (an important pathogen in dieback, canker, and blight in apples), was obtained during this study, *Erwinia* sp. did not play any role

Table 1. Mean lesion lengths induced on shoots by three *N. luteum* and two *Phomopsis* sp. strains.

Strain	Identity	Lesion length [a] (cm)
UCR1190	*N. luteum*	14.3 a
UCR1191	*N. luteum*	15.83 a
UCR1192	*N. luteum*	14.17 a
UCR1193	*Phomopsis* sp.	0.82 b
UCR1197	*Phomopsis* sp.	1.0 b
Non-inoculated control		0.83 b
LSD [b]		1.68

[a] Means followed by different letters are significantly different (P =0.05).
[b] Least significant difference.

in tejocote canker, as comparable levels of dieback were observed on trees where only *N. luteum* but no *Erwinia* sp. was isolated.

Neofusicoccum luteum is one species in the Botryosphaeriaceae that may be widespread on more hosts than anticipated. In a recent survey of Botryosphaeriaceae on avocado in California, *N. luteum* was the most frequently isolated, accounting for about 40% of isolates obtained, while 60% of the isolates were five other species (McDonald and Eskalen, 2011). In a study on citrus, *N. luteum* isolates were obtained from citrus samples collected in San Diego and San Luis Obisco counties of California and were very aggressive during pathogenicity tests on citrus (Adesemoye *et al*., 2011).

The aggressiveness of *N. luteum* on tejocote in tests conducted raises concern, although the extent to which the pathogen could hinder plant productivity is yet to be determined. A susceptible tree could possibly act as a source of inoculum or alternative host for infection of nearby crops. For example, an isolate of *N. luteum* was obtained from a Sycamore tree, which was near to one of the tejocote orchard sample locations in Riverside County.

There is need for more attention on cankers caused by *N. luteum* on tejocote. Earlier, Sergeeva *et al*. (2009) called for more attention following their report of *N. luteum* on olive in Australia. Further studies should include the possible impacts of other pathogens or their interactions with *N. luteum* on tejocote trees and the fruits. For example, it has been reported that the shelf-life of tejocote fruits is 4–8 d after harvesting before excessive softening occurs (Vivar-Vera *et al*., 2007), but the cause of this has not yet been determined. The present study, to our knowledge, is the first association of *N. luteum* with tejocote in California. It is hoped that this new knowledge will form the basis for future studies that are needed on this plant as its production continues to expand.

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