Ophiostoma and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile

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Bark beetles (Coleoptera: Scolytidae) are common vectors of *Ophiostoma* spp., which include primary tree pathogens as well as important agents of sapstain. In Chile, *Hylurgus ligniperda* and *Hylastes ater*, which are native to Europe, commonly occur on the exotic *Pinus radiata*. Little research has been done on *Ophiostoma* spp. associated with bark beetles in Chile and especially those carried by introduced pine-infesting insects. We recently collected specimens of these bark beetles and their galleries, and the aim of this study was to isolate and identify *Ophiostoma* spp. associated with the two bark beetle species. Identification was achieved using morphological characteristics and where appropriate, DNA sequencing. A total of five ophiostomatoid fungi (*Ceratocystiopsis minuta*, *Ophiostoma galeiformis*, *O. huntii*, *O. ips*, and *O. quercus*) were found associated with the bark beetles, all of which are recorded from Chile for the first time.

Keywords: *Ophiostoma*, *Leptographium*, *Pesotum*, *Ceratocystiopsis*, Ascomycetes, Scolytidae, *Hylurgus*, *Hylastes*

Introduction

Pinus spp. are native to the Northern Hemisphere, where species diversity is greatest in Central America and Southeast Asia (Richardson, 1998). Many pine species, however, have been introduced into Southern Hemisphere countries such as New Zealand, Australia, Chile and South Africa, where some species are grown in very large commercial plantations (Le Maitre, 1998; Richardson, 1998). In Chile, *Pinus radiata* is the most economically important exotic plantation species and covers an area of 1.5 million ha (INFOR, 2000).

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Many bark beetle species (Coleoptera: Scolytidae) infest *Pinus* spp. (Wood and Bright, 1992). Most of these bark beetles are not considered as pests in their native environment, but when introduced into new areas, and particularly where uniform stands of *Pinus* spp. are planted, they can become problematic (Wingfield and Swart, 1994; Wingfield *et al*., 2001). In Chile, *Hylurgus ligniperda* (Fabricius) and *Hylastes ater* (Paykull) are exotic pests of European origin infesting exotic *P. radiata* (Wood and Bright, 1992; Billings, 1993). Both of these insects can breed in fresh stumps and slash shortly after trees are felled (Ciesla, 1988).

Many bark beetles are vectors of ophiostomatoid fungi, which include a number of primary pathogens and agents of sapstain (Whitney, 1982; Harrington, 1988; Seifert, 1993; Brasier and Mehrotra, 1995; Paine *et al*., 1997). In Chile, eight ophiostomatoid fungi have been reported from different hosts thus far (Table 1). However, little research has been conducted on the fungal associates of pine-infesting bark beetles in this country.

Table 1. Ophiostomatoid fungi previously reported from Chile.

Fungal species	Host	Reference(s)
Ophiostoma nothofagi (Butin) Rulamort	*Nothofagus dombeyi*	Butin and Aguilar, 1984.
O. piceae (Münch) H. & P. Sydow	*Nothofagus* spp.; *Pinus* spp.; *Laurelia* spp.	Butin and Aguilar, 1984; Butin and Peredo, 1986; Billings, 1993; Harrington *et al*., 2001.
O. piliferum (Fr.) H. & P. Sydow	*Nothofagus pumilio*	Butin and Aguilar, 1984.
O. valdivianum (Butin) Rulamort	*N. alpina*; *N. dombeyi*	Butin and Aguilar, 1984.
Pesotum sp.	*Pinus radiata*	Peredo and Alonso, 1988.
Sporothrix curviconia de Hoog	*P. radiata*	Peredo and Alonso, 1988.
S. schenckii Hekt. & Perkins	Human	Travassos and Lloyd, 1980.
Sporothrix sp.	*P. radiata*	Peredo and Alonso, 1988.

In South Africa, as in Chile, exotic pine plantations constitute a significant section of the forestry industry. In recent years, much research has been conducted on fungi associated with bark beetles in exotic pine plantations of South Africa (Wingfield and Swart, 1989; Zhou *et al*., 2001, 2002). A comparison of the fungi associated with introduced bark beetles in Chile, with fungi from the same niche in South Africa, could provide insight into the spread of bark beetles and their fungi, south of the equator.

Recently, we had the opportunity to examine bark beetles and their breeding galleries from Chile, and to isolate *Ophiostoma* spp. occurring on the beetles and in their galleries. The aim of this study was to identify these fungi based on morphology and comparisons of ITS rDNA sequences.

Materials and Methods

Isolation of fungi

In the Valdivia area of Chile, 34 specimens of *H. ater* were collected from roots of dying *P. radiata* trees, and 80 specimens of *H. ligniperda* were collected from felled trees of the same species in log stacks. All bark beetle specimens were kept separately, and frozen at -70°C for twenty minutes before fungi were isolated from them. Four fungal isolates were also collected directly from galleries of *H. ater*. Isolation of fungi from bark beetles and their galleries were conducted within two weeks after samples had been collected.

Bark beetles from the same gallery were squashed directly onto the selective medium for *Ophiostoma* spp. (20g Biolab malt extract, 20g Biolab agar and 1000ml deionised water, amended with 0.05% cycloheximide and 0.04% streptomycin). Galleries were examined, and spore masses were transferred to the selective medium. Fungal isolation was conducted as described by Zhou *et al*. (2001).

All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphological studies

Both teleomorph and anamorph structures, when present, were mounted in lactophenol containing cotton blue on glass slides, examined microscopically, and characteristic structures measured. To induce the production of perithecia, isolates with only anamorphs present were grown on 2% WA medium (20g Biolab agar and 1000 ml distilled water), to which sterilised pine twigs had been added.

DNA sequencing and phylogenetic analysis

Isolates used

Two isolates (CMW9480 and CMW9481) produced only a *Pesotum*-like anamorph in culture, resembling that of *O. piceae* (Münch) H. & P. Sydow, *O. quercus* (Georgévitch) Nannfeldt, and *O. floccosum* Mathiesen. These isolates were difficult to identify based on morphology, and their single hyphal tip cultures were prepared for DNA sequence comparisons (Table 2).

DNA extraction

Each culture was grown in 50mL malt extract broth (20g Biolab malt extract, and 1000mL distilled water) at 25°C in the dark for 10 days. Mycelium was then harvested by filtration through Whatman no. 1 filter paper and freeze-dried.

DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985). Freeze-dried mycelium was ground to a fine powder in liquid nitrogen. Approximately 0.5mL of the powdered mycelium was suspended in 800µL of extraction buffer (200mM Tris-HCl pH 8.0, 150mM NaCl, 25mM EDTA pH 8.0, 0.5% SDS). Phenol (500µL) and chloroform (300µL) were added to the suspension, and the mixture was mixed, then centrifuged in a Beckman JA 25.50 rotor (12 000 rpm, 60 minutes, 4°C). The upper aqueous layer was transferred to sterilized Eppendorf tubes. 200µL of phenol and an equal volume of chloroform were added, mixed, and then centrifuged for 5 minutes. The aqueous phase was transferred again to new Eppendorf tubes, and chloroform extraction (400µL) was repeated once or twice until the interface was clear. Nucleic acid was then precipitated with 0.1 vol. of 3 M NaAc (pH 5.4) and 1 vol. of isopropanol. The nucleic acid was pelleted using centrifugation (12 000 rpm, 30 minutes, 4°C), and the salt removed by washing with 70% ethanol once. The vacuum-dried pellet was resuspended in 50µL of sterile water and 2µL of RNAse (10mg/mL, Roche Molecular Biochemicals) was added to digest any RNA. The reaction was incubated in a water bath overnight at 37°C. Agarose (Promega, Madison, CT, USA) gel electrophoresis (1%) was used to determine the presence of DNA. The DNA was visualized using ethidium bromide and UV light. DNA concentration was determined using UV spectroscopy (Beckman Du Series 7500 Spectrophotometer).

PCR amplification

The ITS1 and ITS2 (internal transcribed spacer) regions, including the 5.8S gene of the ribosomal RNA operon, were amplified using primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990). The template DNA was amplified in a 50 µL PCR reaction volume, consisting of 0.5 µL of DNA solution (100-200ng μL^{-1}), 0.5µL of Expand High Fidelity

Table 2. Fungi isolated from bark beetles and their galleries in Chile and isolates of selected species used as reference material in this study.

Species	Isolate No.	GenBank No.	Collector/supplier	Origin	Host	Insect	No. of isolates
Ceratocystiopsis minuta	[a]CMW10770		MJ Wingfield	Chile	*Pinus radiata*	*Hylurgus ligniperda*	1
Ophiostoma galeiformis	CMW9478; CMW9479		MJ Wingfield	Chile	*P. radiata*	*Hylastes ater*	4
	CMW9482; CMW9483		R Ahumada	Chile	*P. radiata*	*H. ligniperda*	2
O. huntii	CMW10768; CMW10769		R Ahumada	Chile	*P. radiata*	*H. ater*	9
O. ips	CMW5089; CMW6402		MJ Wingfield	Chile	*P. radiata*	*H. ligniperda*	27
O. quercus	[b]CMW9480	AY328519	MJ Wingfield	Chile	*P. radiata*	*H. ater*	2
	[b]CMW9481	AY328520					
O. floccosum	CMW7661	AF493253	ZW de Beer	South Africa	*P. elliottii*		
		AF198231	A Käärik	Sweden	*Picea* or *Pinus*		
O. piceae	CMW7649	AF081130	JN Gibbs	UK	*P. sylvestris*		
		AF081129	SH Kim *et al*.	Canada	*Picea mariana*		
O. quercus	CMW7650	AF198238	PT Scard, JF Webber	UK	*Quercus* sp.		
		AF081132	SH Kim *et al*.	Canada	*Tsuga*		
	CMW3119	AF493244	ZW de Beer	South Africa	*Pinus* chips		
	CMW7660	AF493252	ZW de Beer	New Zealand	*Pinus* chips		
	CMW7652		RA Blanchette	New Zealand	*P. radiata*		

[a] CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b] Isolates used in rDNA sequence analyses in the present study

PCR System enzyme mix (1.7 U) (Roche Molecular Biochemicals, Alameda, CA), 5µL of Expand HF buffer (10×) without $MgCl_2$, 3µL of $MgCl_2$ (25 mM), and 1.5µL of each primer (10 mM). PCR reactions were performed on an Eppendorf Mastercycler® Personal (PerkinElmer, Germany). The PCR conditions were as follows: 95°C for 2 minutes, followed by 40 cycles, where each cycle included 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C. A final elongation step was conducted for 8 minutes at 72°C. A negative control, using water without DNA, was included with each PCR. PCR products were visualized under UV illumination on a 1% agarose gel stained with ethidium bromide (10mg mL^{-1}). Amplification products were purified using the High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany).

DNA Sequencing

Sequencing reactions were carried out with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer Applied BioSystems) following the manufacturer's instructions. Sequencing was performed on an ABI PRISM 377 Autosequencer (PerkinElmer Applied BioSystems). PCR products were sequenced with the same primers used for PCR, as well as two additional internal primers, CS2 (5'-CAATGTGCGTTCAAAGATTCG-3') (Wingfield *et al*., 1996), and ITS3 (5'-GCATAGATGAAGAAGCAGC-3') (White *et al*., 1990).

Phylogenetic analysis

Sequences were aligned using Sequence Navigator version 1.01 (ABI PRISM, PerkinElmer). The alignment was checked manually and compared with data of related isolates from other studies obtained from GenBank (Table 2). Aligned data were analysed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1998). Uninformative characters were excluded from the analyses. The most parsimonious trees were produced using a heuristic search with TBR (Tree Bisection and Reconstruction) branch swapping. Bootstrap analysis (1000 replicates) was run to determine confidence intervals of the branching points.

Results

Isolation of fungi

In total, 45 fungal isolates were obtained from the specimens collected. Of these, 30 were isolated from *H. ligniperda*, and 15 from *H. ater*. Eleven isolates, representing all the morphological groups present, were selected for further investigation (Table 2).

Morphological studies

Morphological investigation showed that three ophiostomatoid fungi, *Ceratocystiopsis minuta* (Siemaszko) Upadhyay & Kendrick, *Ophiostoma galeiformis* (Bakshi) Mathiesen-Käärik, and *O. ips* (Rumbold) Nannfeldt, were commonly associated with *H. ligniperda*. Three ophiostomatoid fungi were found associated with *H. ater*: *O. galeiformis*, *O. huntii* (Robinson-Jeffrey) de Hoog & R. J. Scheffer, and a *Pesotum* sp. resembling the anamorphs of *O. piceae*, *O. quercus*, and *O. floccosum*.

Sequence analysis

DNA fragments (574 base pairs) were amplified for the isolates with *Pesotum* anamorphs. Manual alignment of these sequences resulted in a total of 579 characters. Of these, 45 were parsimony informative, 6 parsimony uninformative, and 528 were constant. Heuristic searches without using an outgroup taxon resulted in one most parsimonious tree (CI = 1.000, RI = 1.000, HI = 0.000), in which three main clades were well resolved (Fig. 1). The two isolates obtained in the present study (CMW 9480 and CMW 9481) resided in the first clade representing the *O. quercus* group. The second and third clades represent the *O. piceae* and *O. floccosum* groups, and have bootstrap supports of 90% and 100%, respectively.

Discussion

In this study, three ophiostomatoid species were found associated with each of the two bark beetle species from Chile. From *Hylurgus ligniperda*, *Ceratocystiopsis minuta*, *Ophiostoma galeiformis* and *O. ips* were isolated, while *O. galeiformis*, *O. huntii*, and *O. quercus* were found with *Hylastes ater*. *Ophiostoma galeiformis* was the only fungal species present on both bark beetle species. This study represents the first report of these five fungal species

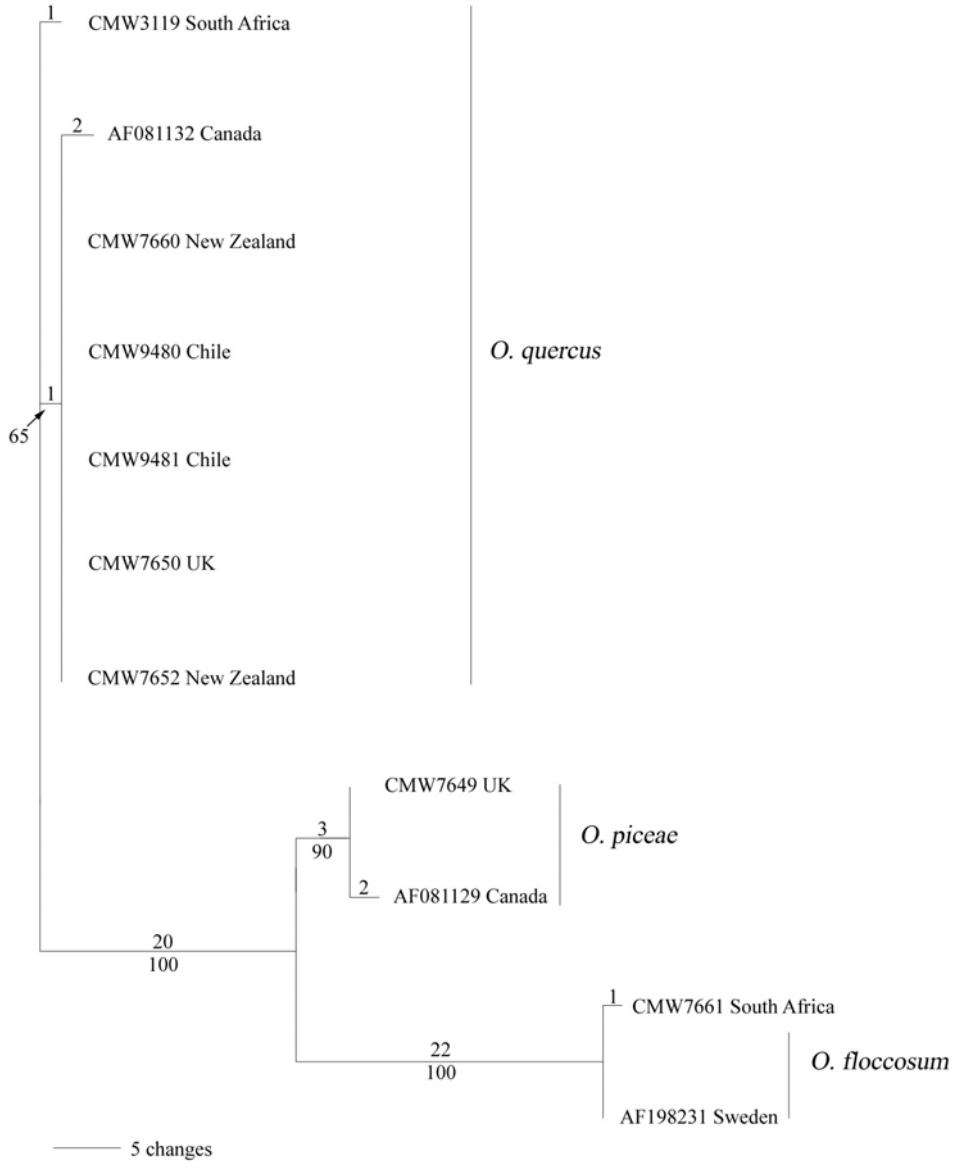

Fig. 1. Phylogram using ITS DNA sequences (ITS1 and ITS2 regions, including the 5.8S rRNA gene) from the group of isolates from Chile with a *Pesotum* stage. Base substitution numbers are indicated above the branches and bootstrap values (1000 bootstrap repeats) below the branches.

from Chile, and this list considerably increases the number of ophiostomatoid fungi known from the country.

Ceratocystiopsis minuta was first described by Siemaszko (1939) from *Picea abies* infested by *Ips typographus* (Linnaeus) in Poland. The fungus is associated with a wide variety of conifer-infesting bark beetles in many parts of the world (Davidson, 1942; Mathiesen-Käärik, 1953; Upadhyay, 1981; Solheim, 1986; Stone and Simpson, 1990; Yamaoka *et al*., 1998). In South Africa, *Cop*. *minuta* has been found on the exotic *Hylastes angustatus* (Herbst) and *Hylurgus ligniperda* (Zhou *et al*., 2001). The presence of *Cop*. *minuta* on *H*. *ligniperda* infesting *P*. *radiata* in Chile is not surprising, given its association with these European bark beetles in South Africa.

Ophiostoma galeiformis is associated with many different bark beetle species. The fungus was first described by Bakshi (1951) in Scotland, where it was isolated from *Larix kaempferi* infested by *Hylurgops palliatus* (Gyllenhal), *Dryocoetes autographus* (Ratzeburg), and *Trypodendron lineatum* (Olivier) (Bakshi, 1951). In Sweden, *O*. *galeiformis* has been isolated from *Picea abies* infested by *Hylastes cunicularius* (Errichson) (Mathiesen-Käärik, 1953), as well as from pine-infesting bark beetles (Hunt, 1956). The discovery of the fungus in Chile in the present study is not surprising, as it has also been found associated with the exotic pine-infesting *H*. *ligniperda* in South Africa (Zhou *et al*., 2001). The *Hylastes* vectors of these fungi, however, differ in the two areas, although both originate from Europe.

Ophiostoma huntii has been associated with several different bark beetle species on *Pinus* and *Picea* spp. (Jacobs and Wingfield, 2001). This fungus was originally isolated from pine infested with a *Dendroctonus* sp. in Canada (Robinson-Jeffrey and Grinchenko, 1964), and has been reported to be associated with *Dendroctonus ponderosae* (Hopkins), *Ips pini* (Say), and *Hylastes macer* (LeConte) in the USA, and *Tomicus piniperda* (Linnaeus) in Europe (Davidson and Robinson-Jeffrey, 1965; Harrington, 1988; Gibbs and Inman, 1991; Wingfield and Gibbs, 1991; Jacobs *et al*., 1998). *Ophiostoma huntii* also occurs in Australia and New Zealand, where it is associated with the European root-infesting bark beetle, *H*. *ater* (Jacobs *et al*., 1998). Studies on populations of fungi such as *O*. *huntii* in Chile, Australia and New Zealand, might provide useful information on how the bark beetles and fungi have been distributed throughout the Southern Hemisphere.

Ophiostoma ips was first described from *Ips calligraphus* (Germar) on *Pinus echinata*, *P*. *sylvestris*, and *P*. *rigida* in the USA (Rumbold, 1931), and has since been found associated with many conifer-infesting bark beetles in the Northern Hemisphere (Rumbold, 1931; Nisikado and Yamauti, 1933; Mathiesen-Käärik, 1953; Hunt, 1956; Mathre, 1964; Upadhyay, 1981; Rane

and Tattar, 1987; Lieutier *et al*., 1989; Perry, 1991; Masuya *et al*., 1999). In the Southern Hemisphere, it has been reported in Australia from galleries of *Ips grandicollis* (Eichhoff) on *Pinus taeda* (Vaartaja, 1966; Stone and Simpson, 1990), and from New Zealand on *P. elliottii* and *P. radiata* (Hutchison and Reid, 1988; Farrell *et al*., 1997). The fungus has also been reported from South Africa associated with *Orthotomicus erosus* (Wollaston), *H. angustatus* and *H. ligniperda* occurring on *P. radiata*, *P. patula* and *P. elliottii* (Wingfield and Marasas, 1980; Zhou *et al*., 2001). In this study, *O. ips* was isolated from *H. ligniperda* on *P. radiata* in Chile, which is similar to the situation in South Africa.

Ophiostoma quercus (Halmschlager *et al*., 1994; De Beer *et al*., 2003a) occurs primarily on hardwoods, but occasionally also on conifers, while *O. piceae* occurs on a wide range of hardwoods and conifers (Brasier and Kirk, 1993; Harrington *et al*., 2001; De Beer *et al*., 2003b). These two species are morphologically difficult to distinguish, but can be separated based on ITS rDNA sequence data (Harrington *et al*., 2001; De Beer *et al*., 2003b). DNA sequence comparisons in the present study have confirmed the association of *O. quercus* with *H. ater* on *P. radiata* in Chile. In South Africa, *O. quercus*, but not *O. piceae*, has been reported from various hardwoods and pine (De Beer *et al*., 2003b). The results of the present study raise suspicion that previous reports of *O. piceae* from *Nothofagus*, *Laurelia*, and *Pinus* spp. in Chile (Butin and Aguilar, 1984; Butin and Peredo, 1986; Billings, 1993; Harrington *et al*., 2001), might have represented *O. quercus*, and not *O. piceae*. Further surveys are necessary to clarify this issue.

In Chile, Australia and New Zealand, both *H. ligniperda* and *H. ater* occur (Swan, 1942; Anonymous, 1974). This is in contrast to South Africa where only *H. ligniperda* has been reported (Tribe, 1991). Both bark beetle species were accidentally introduced from Europe to these Southern Hemisphere countries, but the pine species planted originate from North America. Very little is known about the fungal associates of these bark beetle species in their native environments in the Northern Hemisphere. The only records are two old reports from Sweden where five ophiostomatoid species were recorded from *H. ater*. These include: *O. ips*, *O. penicillatum* (Grossman) Siemaszko, *O. piceae*, *L. lundbergii* Lagerberg & Melin, *O. piliferum* (Fries) H. & P. Sydow [= *O. coeruleum* (Münch) H. & P. Sydow], and *Graphium areum* Hedgcock (Mathiesen, 1950; Mathiesen-Käärik, 1953). None of these species have, however, been recorded in association with *H. ater* in the Southern Hemisphere.

As a result of this preliminary study, five ophiostomatoid fungi have been recorded associated with *Hylurgus ligniperda* and *Hylastes ater* in Chile.

Further surveys are required to extend the area that has been considered. In addition, pathogenicity tests with these fungi and assessments of their potential to cause sapstain on conifer lumber will be conducted.

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