

Molecular basis of cycloheximide resistance in the Ophiostomatales revealed

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Abstract

Resistance to the antibiotic Cycloheximide has been reported for a number of fungal taxa. In particular, some yeasts are known to be highly resistant to this antibiotic. Early research showed that this resulted from a transition mutation in one of the 60S ribosomal protein genes. In addition to the yeasts, most genera and species in the Ophiostomatales are highly resistant to this antibiotic, which is widely used in to selectively isolate these fungi. Whole genome sequences are now available for numerous members of the Ophiostomatales providing an opportunity to determine whether the mechanism of resistance in these fungi is the same as that reported for yeast genera such as *Kluyveromyces*. We examined all the available genomes for the Ophiostomatales and discovered that a transition mutation in the gene coding for ribosomal protein eL42, which results in the substitution of the amino acid Proline to Glutamine, likely confers resistance to this antibiotic. This change across all genera in the Ophiostomatales suggests that the mutation arose early in the evolution of these fungi.

Keywords: Cycloheximide resistance · Ophiostoma · Ophiostomatoid · Ribosomal protein eL42

Introduction

The Ophiostomatales (Ascomycetes) are best known as arthropod associated fungi that include important pathogens of trees such as the Dutch elm disease fungi *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (Brasier1991; Gibbs 1978), human and animal pathogens in the genus *Sporothrix* (de Beer et al. 2003; Rodrigues et al. 2016)

and agents of sap-stain in lumber (Seifert 1993). An unusual characteristic of species in the Ophiostomatales is that they are consistently highly tolerant to the antibiotic cycloheximide. This biochemical characteristic was initially recognized by Fergus (1956) who showed that some wood staining species of *Ophiostoma* shared this feature.

Fungi in the Ophiostomatales have had a long and complex taxonomic history. This has more specifically concerned to the separation of the genera *Ophiostoma* and *Ceratocystis* and their relatives (de Hoog and Scheffer 1984; Wingfield et al. 1993; Seifert et al. 2015). Confusion regarding the generic boundaries of these fungi dates back to a time when their taxonomy relied almost exclusively on morphology (Uphadhyay 1991; Wingfield et al. 1993). Specifically, their various shared morphological characteristics, arising from convergent evolution that facilitates associations with arthropod vectors resulted in confusion regarding the appropriate taxonomic boundaries between the genera *Ophiostoma* and *Ceratocystis*, which were collectively referred to as the Ophiostomatoid fungi (Wingfield et al. 1993; Seifert et al. 2015).

For many years, cycloheximide tolerance provided a useful non-morphological characteristic that clearly separated species related to *Ceratocystis* from those related to *Ophiostoma* (Harrington 1981). The more recent emergence of DNA sequence based phylogenies has strongly supported the fact that these two groups of fungi are unrelated and reside respectively in unrelated Orders (Hausner et al. 1993a, b; Spatafora and Blackwell 1994). These are the Ophiostomatales defined by *Ophiostoma sensu lato* (de Beer et al. 2013) and the Microascales including genera in the Ceratocystidaceae (de Beer et al. 2014) and the Gondwanamycetaceae including species of *Knoxdaviesia* (Reblova et al. 2011). A recent revision of the Ophiostomatales based on multiple gene genealogies as well as whole genome data (de Beer et al. 2022) has defined 16 genera including all those species that have, in various studies, been shown to tolerate high levels of cycloheximide in culture.

Cycloheximide is a powerful antibiotic that is not generally applied for medical purposes. It is, however, commonly used in research experiments to inhibit translation of messenger RNA and thus protein synthesis in eukaryotic cells. For example, Rao and Grollman (1967) showed that its mechanism of action was associated with the 60S ribosomal subunit in *Saccharomyces cerevisiae*. Studies using *S. cerevisiae* and *Tetrahymena thermophila* mutants with low levels of resistance to cycloheximide showed that this was the result of an amino acid substitution in the ribosomal protein L29 (Kaufers et al. 1983; Yao and Yao 1991).

Most Eukaryotes are sensitive cycloheximide. There are however various exceptions, other than in the Ophiostomatales mentioned above, such as in some ascomycetous yeasts (Saccharomycetaceae). For example, resistance to the antibiotic in species of *Kluyveromyces*, *Candida* and *Schwanniomyces* has been shown to result from the substitution of a Glutamine (Gln) in the place of a Proline (Pro) at position 56 in the ribosomal protein L41 (Dehoux et al. 1993; Sasnauskas et al. 1992). Using genetic transformants of *Saccharomyces cerevisiae*, Kawai et al. (1992) showed that a Pro to Gln change in the ribosomal protein L41 results in resistance to cycloheximide at concentrations of 100 µg/ml. Likewise in the green alga *Chlamydomonas reinhardtii*, mutants with point mutations in the ribosomal protein gene L41 (RPL41) where a

Proline at position 56 has been replaced with either Leucine or Serine are also resistant to cycloheximide (Stevens et al. 2001). The Leucine mutation in this case results in higher levels of resistance.

There is a reasonably robust literature showing that cycloheximide resistance arises from amino acid substitutions in specific ribosomal proteins. A complication in understanding this trait arises from the fact that the ribosomal proteins have been named variously for the prokaryotes and eukaryotes in the past (Wittmann et al. 1971, Kruiswijk and Planta 1974; Wool et al. 1995). Thus, to compare the names of these proteins in different publications, it is necessary to be aware of their variable nomenclature. Specifically, and pertinent to this study, ribosomal protein L41 was renamed L42 (Planta and Mager 1998) and is now referred to as eL42 (Ban et al. 2014). Thus references to substitutions in ribosomal protein L41 are most correctly referred to as being in ribosomal protein eL42.

In the recent revision of the Ophiostomatales, de Beer et al. (2022) included Genome sequences for 31 species representing 11 of 14 currently recognized genera (excluding *Afroraffaelea*, *Aureovirgo* and *Paleoambrosia*). The availability of these genome sequences has provided an opportunity to determine the basis of their resistance to cycloheximide and whether this might be similar to that described in many yeasts. The aim of this study was thus to use the available Ophiostomatales genome sequences to identify the amino acid sequence of the ribosomal protein eL42. Consequently to determine whether the predicted amino acid Proline at position 56 has been substituted by Glutamine or some other amino acid.

Materials and Methods

Taxon sampling and genome collection

To provide a phylogenomic framework for this study, a genome data set was assembled and analysed including 69 genomes for species in the Sordariomycetes and the Saccharomycetes. This dataset included all currently available genome sequences for genera in the Ophiostomatales (*Ceratocystiopsis*, *Chrysosphaeria*, *Esteya*, *Fragosphaeria*, *Graphilbum*, *Grosmannia*, *Hawksworthiomyces*, *Intubia*, *Leptographium*, *Ophiostoma*, *Sporothrix* and *Raffaelea*) and thus fungi known or expected to be tolerant to cycloheximide. For comparative purposes, genomes for representative genera in the Microascales including the Ceratocystidaceae (*Ambrosiella*, *Bretziella*, *Catunica*, *Ceratocystis*, *Davidsoniella*, *Endoconidiophora*, *Huntiella* and *Thielaviopsis*), Gondwanamycetaceae (*Knoxdaviesia*) and Microascaceae (*Microascus*) were included. With the exception of *Microascus*, these are known to be sensitive to the antibiotic. In addition, genomes for a selection of other Sordariomycetes genera reported to be cycloheximide sensitive (*Colleototrichum*, *Cryphonectria*, *Diaporthe*, *Fusarium*, *Geosmithia*, *Magnaporthe*, *Neurospora*, *Phaeoacremonium*, *Thielavia* and *Trichoderma*) were also included. In order to accommodate yeasts (Saccharomycetes) 16 species in 12 genera (*Ascoidea*, *Brettanomyces*, *Candida*, *Eremothecium*, *Komagataella*, *Kluyveromyces*, *Lachancea*, *Ogataea*, *Pachysolen*, *Pichia*, *Saccharomyces* and *Saccharomycopsis*), some of which are known to be either sensitive or tolerant to cycloheximide, were included (Table 1). All genome sequences were downloaded from JGI Genome Portal or NCBI genome databases with accession numbers and references provided in Table 1.

Table 1. Species of Saccharomycetes and Sordariomycetes included in the analyses, their genome accession numbers and corresponding reference for the genome sequences

Species	Isolate number	Cycloheximide resistance	Accession number	Reference
<i>Ascoidea asiatica</i>	JCM 7603	No	GCA_001600695.1	Shen et al. (2018)
<i>Ascoidea rubescens</i>	DSM 1968	No	GCF_001661345.1	Riley et al. (2016)
<i>Komagataella phaffii</i>	GS115	Yes	GCA_900235035.1	De Schutter et al. (2009)
<i>Brettanomyces bruxellensis</i>	UCD 2041	Yes	GCF_011074885.1	Roach and Borneman (2020)
<i>Candida arabinofermentans</i>	NRRL YB-2248	Yes	GCA_001661425.1	Riley et al. (2016)
<i>Ogataea polymorpha</i>	NCYC 495	Yes	GCF_001664045.1	Riley et al. (2016)
<i>Pichia membranifaciens</i>	CBS 107	No	GCA_001661235.1	Riley et al. (2016)
<i>Eremothecium gossypii</i>	ATCC 10895	Yes	GCF_000091025.4	Dietrich et al. (2004)
<i>Kluyveromyces lactis</i>	NRRL Y-1140	Yes	GCF_000002515.2	Dujon et al. (2004)
<i>Lachancea meyersii</i>	CBS 8951	No	GCA_900074715.1	Vakirlis et al. (2016)
<i>Lachancea thermotolerans</i>	CBS 6340	No	GCF_000142805.1	Souciet et al. (2009)
<i>Pachysolen tannophilus</i>	NRRL Y-2460	No	GCA_001661245.1	Riley et al. (2016)
<i>Saccharomyces kluyveri</i>	NRRL Y-12651	No	GCA_000149225.2	Cliften et al. (2003)
<i>Saccharomycopsis capsularis</i>	NRRL Y-17639	Yes	GCA_003705375.1	Shen et al. (2018)
<i>Saccharomycopsis fibuligera</i>	KPH12	Yes	GCA_001936155.1	Choo et al. (2016)
<i>Saccharomycopsis malanga</i>	KCN26	Yes	GCA_001599215.1	Shen et al. (2018)
<i>Cryphonectria parasitica</i>	EP155	No	GCA_011745365.1	Crouch et al. (2020)
<i>Diaporthe ampelina</i>	DA912	No	GCA_001006365.1	Morales-Cruz et al. (2015)
<i>Colleotrichum graminicola</i>	M1.001	No	GCF_000149035.1	O'Connell et al. (2012)
<i>Geosmithia morbida</i>	1262	No	GCF_012550715.1	Schuelke et al. (2017)
<i>Trichoderma reesei</i>	QM6a	No	GCF_000167675.1	Martinez et al. (2008)
<i>Fusarium graminearum</i>	NRRL 31084	No	GCF_000240135.3	Cuomo et al. (2007)
<i>Fusarium oxysporum</i>	4287	No	GCF_000149955.1	Ma et al. (2010)

<i>Magnaporthe grisea</i>	70-15	No	GCF_000002495.2	Dean et al. (2005)
<i>Magnaporthe poae</i>	ATCC 64411	No	GCA_000193285.1	Okagaki et al. (2015)
<i>Ambrosiella xylebori</i>	CBS 110.61	No	GCA_002778035.1	Vanderpool et al. (2018)
<i>Bretziella fagacearum</i>	CMW 2656	No	GCA_002018255.1	Wingfield et al. (2016a)
<i>Catunica adiposa</i>	CBS136.34	No	GCA_001640685.1	Wingfield et al. (2016b)
<i>Ceratocystis fimbriata</i>	CBS 114723	No	GCA_000389695.3	Wilken et al. (2013)
<i>Davidsoniella virescens</i>	CMW17339	No	GCA_001513805.1	Wingfield et al. (2015ay)
<i>Endoconidiophora polonica</i>	CBS100205	No	GCA_001856765.1	Wingfield et al. (2016b)
<i>Huntiaella moniliformis</i>	CBS 118127	No	GCA_000712465.1	Van der Nest et al. (2014a)
<i>Thielaviopsis musarum</i>	CMW1546	No	GCA_001513885.1	Wingfield et al. (2015a)
<i>Knoxdaviesia proteae</i>	CMW40885	No	GCA_001510565.1	Aylward et al. (2016)
<i>Microascus trigonosporus</i>	CBS 218.31	Yes	NA	JGI
<i>Ceratocystiopsis brevicomis</i>	CBS 137839	Yes	GCA_002778105.1	Vanderpool et al. (2018)
<i>Ceratocystiopsis minuta</i>	CBS 138717	Yes	GCA_001676865.1	Wingfield et al. (2016b)
<i>Chrysosphaeria jan-nelii</i>	CMW47058	Yes	GCA_020002325.1	Nel et al. 2021
<i>Esteya vermicola</i>	CBS 115803	Yes	GCA_002778215.1	Vanderpool et al. (2018)
<i>Fragosphaeria purpurea</i>	CBS 133.34	Yes	GCA_002778095.1	Vanderpool et al. (2018)
<i>Graphilbum fragrans</i>	CBS 138720	Yes	GCA_001513895.1	Wingfield et al. (2015a)
<i>Grosmannia clavigera</i>	kw1407	Yes	GCF_000143105.1	DiGuistini et al. (2011)
<i>Grosmannia galeiformis</i>	CBS 115711	Yes	GCA_004028395.1	Wingfield et al. (2018)
<i>Grosmannia penicillata</i>	CBS 116008	Yes	GCA_001938055.1	Wingfield et al. (x)
<i>Hawksworthiomyces lignivorus</i>	CBS 119148	Yes	GCA_002917075.1	Wingfield et al. (2017a)
<i>Intubia macrotermitinae</i>	CMW47056	Yes	GCA_020002355.1	Nel et al. 2021
<i>Leptographium lundbergii</i>	CBS 138716	Yes	GCA_001455505.1	Wingfield et al. (2015b)
<i>Leptographium procerum</i>	CMW34542	Yes	GCA_000806385.1	Van der Nest et al. (2014b)

<i>Ophiostoma bicolor</i>	ZLVG358	Yes	NA	Lah et al. (2017)
<i>Ophiostoma ips</i>	CBS 138721	Yes	GCA_002917055.1	Wingfield et al. (2017a)
<i>Ophiostoma novo-ulmi</i>	H327	Yes	GCA_000317715.1	Forgetta et al. (2013)
<i>Ophiostoma piceae</i>	UAMH 11346	Yes	GCA_000410735.1	Haridas et al. (2013)
<i>Ophiostoma ulmi</i>	W9	Yes	NA	Khoshraftar et al. (2013)
<i>Raffaelea aguacate</i>	RL272	Yes	GCA_002777955.1	Vanderpool et al. (2018)
<i>Raffaelea albimanens</i>	CBS 271.70	Yes	GCA_002778245.1	Vanderpool et al. (2018)
<i>Raffaelea ambrosiae</i>	CBS 185.64	Yes	GCA_002778195.1	Vanderpool et al. (2018)
<i>Raffaelea arxii</i>	CBS 273.70	Yes	GCA_002778165.1	Vanderpool et al. (2018)
<i>Raffaelea lauricola</i>	RL570	Yes	GCA_002778145.1	Vanderpool et al. (2018)
<i>Raffaelea quercivora</i>	JCM 11526	Yes	GCA_001662465.1	Masuya et al. (2016)
<i>Raffaelea quercus-mongolicae</i>	KACC44405	Yes	GCA_002215975.1	Jeon et al. (2017)
<i>Raffaelea sulphurea</i>	CBS 380.68	Yes	GCA_002778055.1	Vanderpool et al. (2018)
<i>Sporothrix brasiliensis</i>	5110	Yes	GCF_000820605.1	Teixeira et al. (2014)
<i>Sporothrix globosa</i>	CBS 120340	Yes	GCA_001630435.1	Huang et al. (2016)
<i>Sporothrix pallida</i>	SPA8	Yes	GCA_000710705.2	D'Alessandro et al. (2016)
<i>Sporothrix phasma</i>	CBS 119721	Yes	GCA_011037845.1	Liu et al. (2019)
<i>Sporothrix schenckii</i>	1099-18	Yes	GCF_000961545.1	Teixeira et al. (2014)
<i>Thielavia terrestris</i>	NRRL 8126		GCF_000226115.1	Berka et al. (2011).
<i>Neurospora crassa</i>	OR74A	No	GCA_000182925.2	Galagan et al. (2003)
<i>Phaeoacremonium aleophilum</i>	UCRPA7		GCF_000392275.1	Blanco-Ulate et al. (2013)

Phylogenomic analyses

All genome sequences were subjected to BUSCO v4.0.5 analysis using the ascomycota_odb10 dataset (Seppey et al. 2019). Single copy BUSCO genes that were shared across all 69 species were identified and these were used to construct a species tree utilizing a coalescence approach. The amino acid sequences for each BUSCO gene were aligned with PRANK v.170427 (Löytynoja 2014) using the default parameters and trimmed with Trimal v1.4 (Capella-Gutiérrez et al. 2009) with the “automated1” option. After trimming, an additional filtering step was carried out to remove datasets with less than 100 sites in alignment length or less than 50 parsimony-informative characters. Datasets that did not include all taxa after the aligning and trimming steps were also excluded from further analyses.

Maximum likelihood trees were constructed on the remaining datasets using IQTREE v1 with automatic model selection and 1000 ultrafast bootstrap replicates (Hoang et al. 2018, Minh et al. 2020). After collapsing the branches having less than 10 % bootstrap support from individual gene trees using Newick Utilities (Junier & Zdobnov 2010), the species phylogeny was inferred from the resulting gene trees in ASTRAL v5.7.7 (Mirarab et al. 2014). Finally, RaxML v 8.2.11 (Stamatakis 2014) was applied to estimate branch length for the species phylogeny with the concatenated alignment of all BUSCO genes used for species tree constructions.

Ribosomal protein eL42 annotation and comparison

Protein coding genes present in all genomes were predicted with Augustus v3.2.3 (Stanke et al. 2006) using the species models for *Neurospora crassa* and *Kluyveromyces lactis* as the representatives for taxa in the Sordariomycetes and the Saccharomycetes respectively. Genes encoding the ribosomal protein eL42 were identified by carrying out a BLASTP analysis with the *Kluyveromyces lactis* ribosomal protein eL42 (GenBank accession M94988.1) as query against a protein database consisting of all amino acid sequences obtained with Augustus prediction of all 69 genomes. The genome sequences and DNA sequences of eL42 gene were extracted from all species and these were aligned in MAFFT v7 with the E-INS-i option (Katoh & Standley 2013). The resulting alignment was then used to verify and manually curate (where necessary) the protein coding sequences of the eL42 genes from all species. Finally, the eL42 amino acid sequences all species were aligned in MAFFT v7 (Katoh & Standley 2013) and the alignment was visualized on the phylogenomic tree with iTOL v4 (Letunic & Bork 2019).

Results

Phylogenomic tree construction

A total of 312 shared single copy BUSCO genes were identified across 69 species, 248 of which were retained for the construction of the species phylogeny. The phylogenomic tree inferred with ASTRAL showed two major lineages represented by species of the Saccharomycetes and Sordariomycetes respectively (Figure 1). The evolutionary relationships of species residing in the Saccharomycetes included in this study were consistent with those in the phylogeny produced by Krassowski et al. 2018. The Sordariomycete and Saccharomycete lineages grouped together, species in the Ophiostomatales formed a monophyletic clade and species in Microascales

including the Ceratocystidaceae, Gondwanamycetaceae and *Microascus* grouped together.

Ribosomal protein eL42 annotation and comparison

A single gene encoding for the eL42 protein was predicted from each of the 69 genomes included in this study. The total length of the predicted protein was 100 amino acids in all species investigated. The amino acid alignment of the protein sequence displayed a high level of conservation (supplementary Figure 1) across species in the Saccharomycetes and those in the Sordariomycetes. There were, however, a range of introns present in the predicted gene sequences, from one (in species of the Saccharomycetes) to six in *Knoxdaviesia protea* (Sordariomycetes).

All species of the Ophiostomatales had a Glutamine (Q) at position 56 in the eL42 protein (Figure 1). In contrast, all species in the Sordariomycetes known to be cycloheximide sensitive including those in the Ceratocystidaceae and Gondwanamycetaceae had a Proline (P) at position 56. In the case of *Microascus trigonosporus*, which is known to be cycloheximide resistant, there was a Proline (P) at position 56. All the other species in the Sordariomycetes included in this study have a Proline (P) at position 56 in the eL42 protein and are known to be cycloheximide sensitive. All the species in the Saccharomycetes with known resistance to cycloheximide had a Glutamine at position 56, in contrast to a Proline at this position for species that are susceptible to the antibiotic.

There were three additional amino acid substitutions in the predicted eL42 protein that are shared between the Ophiostomatales, but not present in the close relatives utilized as outgroups in this study. These were Threonine at positions 30 and 88 and Lysine at position 81. These amino acid differences are not shared with yeasts known to be highly resistant to cycloheximide and that have the Glutamine substitution in position 56 of eL42. These additional amino acid differences are thus unlikely to be linked to cycloheximide resistance in the Ophiostomatales.

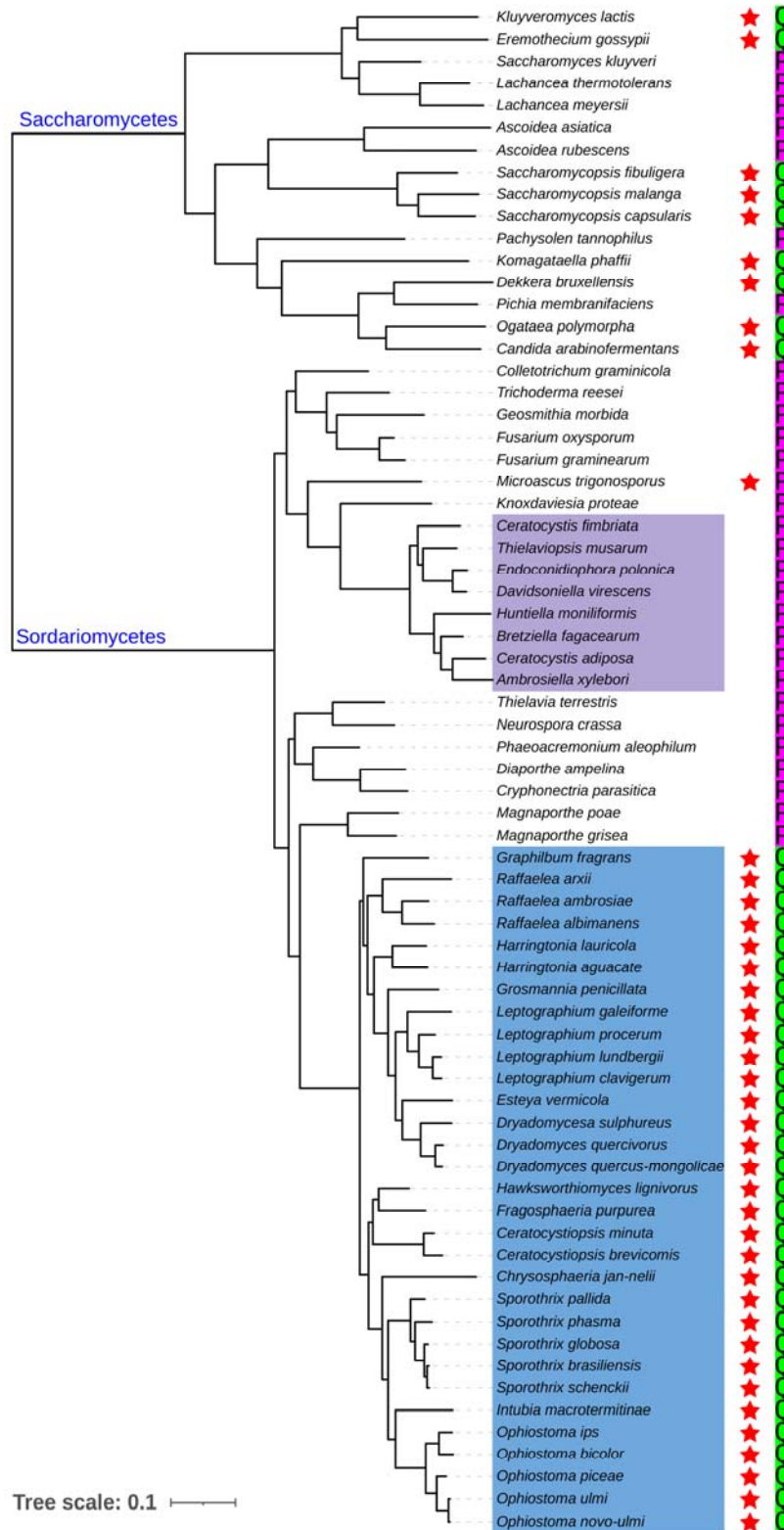


Figure 1 Phylogenomic tree of all the species in this study. The red stars indicate species that are known to be resistant to cycloheximide. The red arrows indicate position 56 in the protein where a Glutamine (Q) is present only in species that are resistant to cycloheximide whereas a Proline (P) is present in species that are known to be susceptible to this antibiotic (except for *Microascus trigonosporus*).

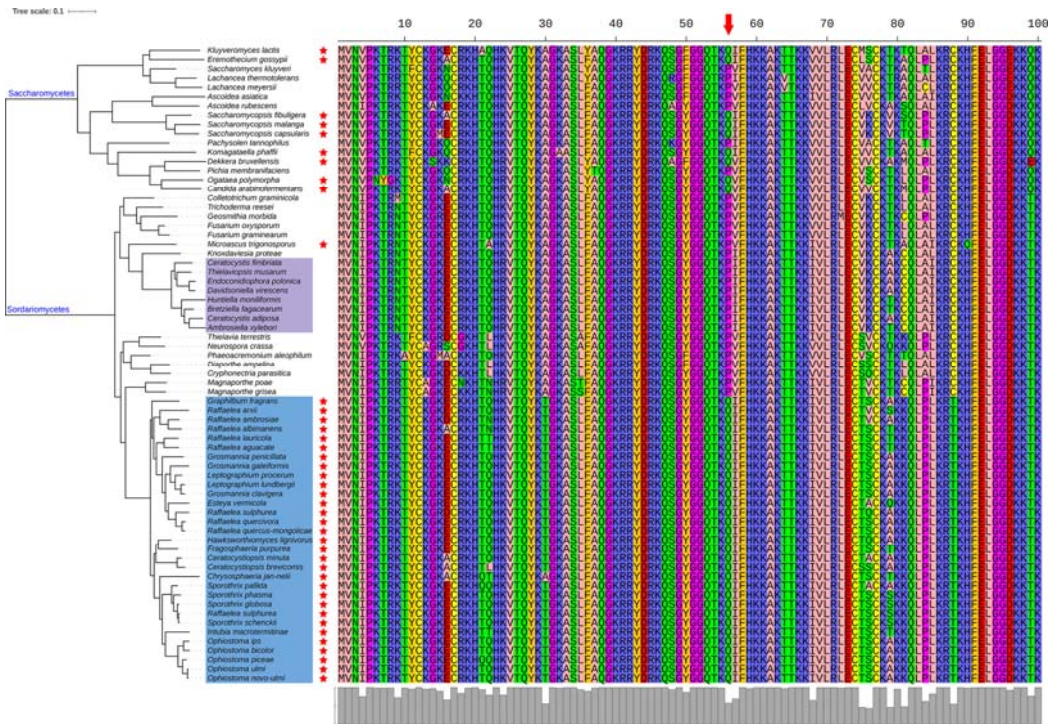


Figure 1 SUPPLEMENTARY. Phylogenomic tree (left) and alignment of the ribosomal protein eL42 sequences (right) in 69 fungal species residing in the Saccharomycetes and the Sordariomycetes. The red stars indicate species that are known to be resistant to cycloheximide. The red arrows indicate position 56 in the protein where a Glutamine (Q) is present only in species that are resistant to cycloheximide whereas a Proline (P) is present in species that are known to be susceptible to this antibiotic (except for *Microascus trigonosporus*).

Discussion

Cycloheximide resistance has been well known in species of the Ophiostomatales for many years. However, the molecular basis of this characteristic has never been considered. In this study, we were able to show that cycloheximide tolerance in these fungi is due to a substitution of the amino acid Proline in the ribosomal protein eL42 at position 56 with a Glutamine. This is the same as has been shown in various species of yeasts where the Proline at position 56 in eL42 is replaced with a Glutamine (deHoux et al. 1993; Sasnauskas et al. 1992)

We included in this study an analysis of the ribosomal protein eL42 in species of the Microascales, more specifically the Ceratocystidaceae and Gondwanamycetaceae. This was due to the long-standing confusion between members of these Families and the Ophiostomatales in the past. Unsurprisingly, none of these species had the Glutamine substitution in position 56 of eL42. This confirms the molecular basis of cycloheximide sensitivity in these fungi, which has been well-known for those species and for which the trait has previously been tested (Harrington 1981).

Microascus trigonosporus was included in this study due to its placement in the Microscales and thus its relationship with the Ceratocystidaceae and Gondwanamycetaceae. This fungus is a dermatophyte and has been established as cycloheximide resistant in previous studies (Brasch et al. 2019). The fact that *M.*

trigonosporus has a Proline at position 56 in the predicted protein eL42 suggests that the resistance of this fungus to cycloheximide is not as a consequence of a change in the protein eL42, but rather due to a different mechanism. Given that its close relatives in the Ceratocystidaceae and Gondwanamycetaceae are sensitive to cycloheximide, a different molecular basis for the trait is perhaps not surprising. This could for example be due to over expression of the ATP-binding cassette (ABC) transporters, (Moran et al. 1998), the presence of the multi - drug resistance *MDR 1* gene (Gupta et al. 1998) or the ability to convert cycloheximide to a less toxic derivative (Shearer and Sypherd 1988). Interestingly, this last form of resistance is not limited to fungi but has also been reported in carrot cell culture (Sung et al. 1981). Additionally, Shen et al. (2021) report a number of amino acid substitutions in ribosomal proteins that result in cycloheximide resistance in *Neurospora crassa*. In the case of eL42 these were P56L and F58L and for uL15 they reported two different mutations, Q38K and Q38L. None of these mutations are found in the genome of *M. trigonosporus*. Further research to determine the molecular basis of cycloheximide tolerance in *M. trigonosporus* is likely to yield interesting and useful findings.

Numerous yeasts, relatively widely distributed across the Saccharomycetaceae are known to be highly resistant to cycloheximide and the results of the present study are consistent with that fact. In the case of *Candida* and *Kluyveromyces*, cycloheximide resistance is the result of a single amino acid substitution in eL42 (Dehoux et al. 1993; Sasnauskas et al. 1992). This is the ribosomal protein to which cycloheximide binds and that underpins its mode of action. It is, therefore, not surprising that species in the Ophiostomatales, known to be highly resistant to this antibiotic have a substitution in the same ribosomal protein. What was perhaps unexpected is that the substitution is exactly the same as that found in various yeast taxa. In this regard, it suggests that the mutation allows for a functional protein but that also provides cycloheximide resistance. What is also interesting is that the amino acid (Glutamine) which is substituted in the Ophiostomatales, is also the same as that observed in yeasts. It seems likely that other cycloheximide resistant eukaryotes would have this same mutation and that this would have then arisen separately in different lineages.

It is particularly relevant that all species in the Ophiostomatales are tolerant to high levels of cycloheximide. This is a relatively large Order of the fungi and there are no known exceptions. The situation in the yeasts is different where this biological characteristic is present variously across the Saccharomycetales without any clear pattern of occurrence. This suggests that there has been a selection for cycloheximide tolerance early in the evolution of the Ophiostomatales and that this selective pressure has been maintained over a long evolutionary history. In contrast, the occurrence of this trait across the ascomycetous yeasts suggests that it has thus either arisen independently in different lineages or been lost across evolutionary time in lineages, where there is no selective pressure to maintain it.

The results of this and previous studies provide robust evidence that all species in the Ophiostomatales are highly tolerant to cycloheximide. This implies that there has been strong evolutionary pressure across a relatively large assemblage of fungi to maintain this unique characteristic. The Ophiostomatales are well-known associates of arthropods including various groups of insects and mites (Wingfield et al 2017b). and it is reasonable to speculate that cycloheximide tolerance has contributed to the

establishment of this niche. Some evidence supporting this view emerges from the close association of between some wood boring beetles and *Streptomyces* (Actinomycetes) that produce cycloheximide (Grubbs et al. 2020). While this might only be a limited example, the fact that most if not all Ophiostomatales likely have some association with arthropods, including those such as mites that occur in soils, suggests that they have evolved in an environment rich in cycloheximide or together with organisms that produce this antibiotic. Further understanding this relationship is likely to be lucrative in new scientific discovery.

References

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