t-RFLP identification of grapevine pathogens and endophytes

Landcare Research
Manaaki Whenua

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Introduction

Grapevine fungal pathogens can cause significant economic losses in viticulture. Common fungal truck diseases include: *Phaeoacremonium* sp. and *Phaeomoniella chlamydospora* (Petri disease), *Botryosphaeria* sp. (canker), *Eutypa lata* (dieback), and *Cylindrocarpon* sp. (blackfoot).

However, many of the fungi isolated from grapevines are non-pathogenic or even beneficial endophytes. Healthy vines may have greater diversity of fungal species inside them than those vines that are of poor health. Pathogenic fungi can also be found in healthy, productive vines (Halleen et al., 2003). Some pathogenic fungi may be suppressed by endophytes, or an absence of stress event to trigger disease. Some diseases such as esca require a combination of at least two pathogenic species before disease symptoms appear (Mugnai et al., 1999).

Current diagnostic tests using PCR can detect the presence or absence of disease causing-fungi (usually one species at a time), and is more sensitive than culturing from symptomatic tissue. However, this provides only a partial view of the complexities of grapevine fungal communities. Here a relatively new method, called 'database t-RFLP' is used to examine the entire fungal population inside a vine, including pathogens and endophytes. This method can screen vines for the most common fungal pathogens, and also has potential as a research tool to monitor population changes before and after certain treatments.

Fig 1. Cross-section through the trunk of a 25 year old grapevine. A multiple infection by various fungal pathogens has caused necrosis, discolouration, and black gooey sap.

Methods

Tissue samples were taken from young grapevines for fungal isolation onto MEA amended with streptomycin, and DNA was extracted from sub-cultures of isolates using the REDextract™ kit (Sigma). Isolates were identified to genus by sequencing the ITS region, and for *Phaeoacremonium* and *Botryosphaeria* to species level by β-tubulin sequences. The DNA extracts from up to three isolates of each identified genus or species was used to obtain standard t-RFLP profiles.

The extracts were PCR amplified with terminally labelled ITS1F and ITS4 primers. The ITS fragments were digested separately with *Hae*III and *Cfo*I and run on an ABI 3130xl Genetic analyser, with a 1000-bp standard (Fig 2.). The electropherogram was analysed with GeneMapper 4.0. Data including peak size, height, and area, was exported from GeneMapper into an R statistical package TRAMPR (Fitzjohn et. al., 2007), where peaks were analysed and compared against database standards.

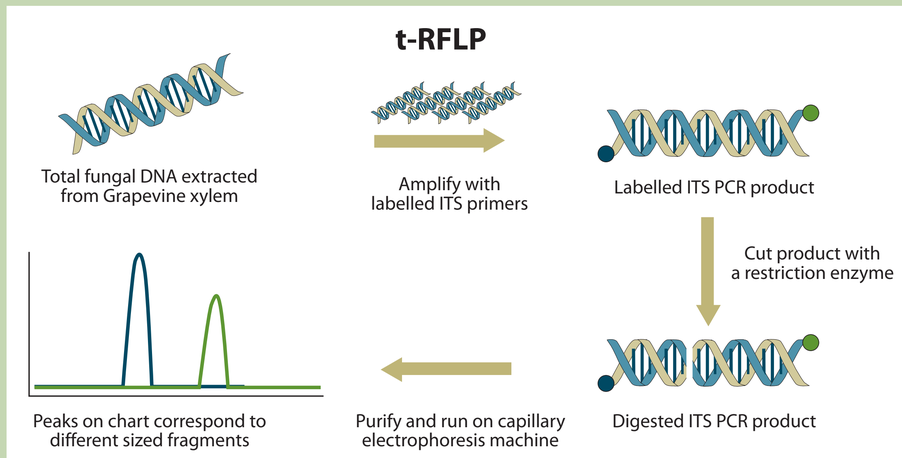


Fig 2. Schematic overview of the method for producing a t-RFLP profile. Different fungal species will have different peak profiles, due to sequence and length variation in the amplified PCR product.

Results

Fig 3. The peak-profile of a pure culture of the pathogen *Cylindrocarpon liriodendri*. The ITS PCR product amplified from this strain was fluorescently labelled at both ends (ITS1F, ITS4). Then cut (independently) with *Cfo*I and *Hae*III. When analysed this results in four peaks, these are stored as a standard profile for this species and can be recalled later for identification in a mixed sample.

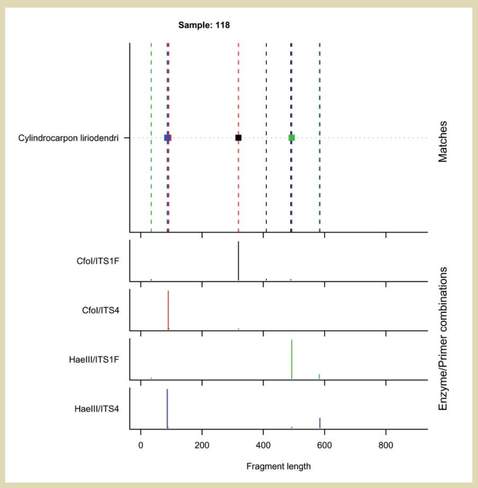
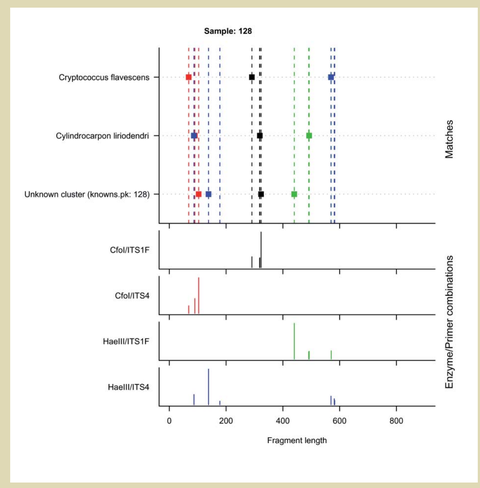


Fig 4. This plot shows the identified fungi present in a small sample of grapevine xylem. The bottom section of the image is the peak profile obtained from cutting the ITS DNA fragment. The identified species are *Cylindrocarpon liriodendri* (a pathogen), and *Cryptococcus flavescens* (an endophytic yeast). This sample also contains another as-yet-unidentified fungi that regularly occurs in peak profiles.



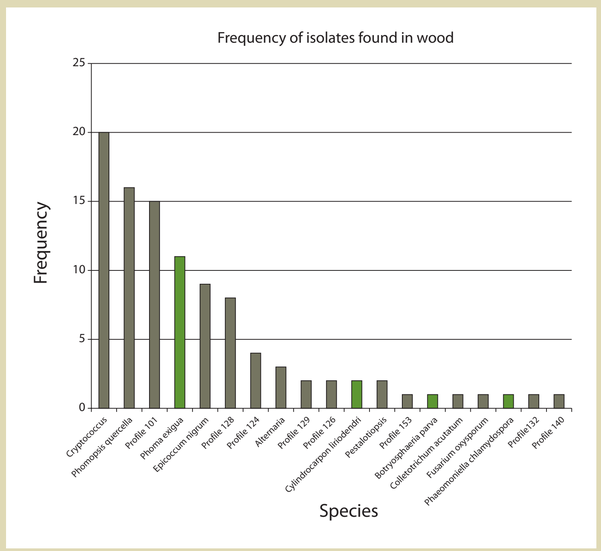


Fig 5. Frequency of fungal profiles found in a survey of grapevine xylem. Green bars are known pathogens.

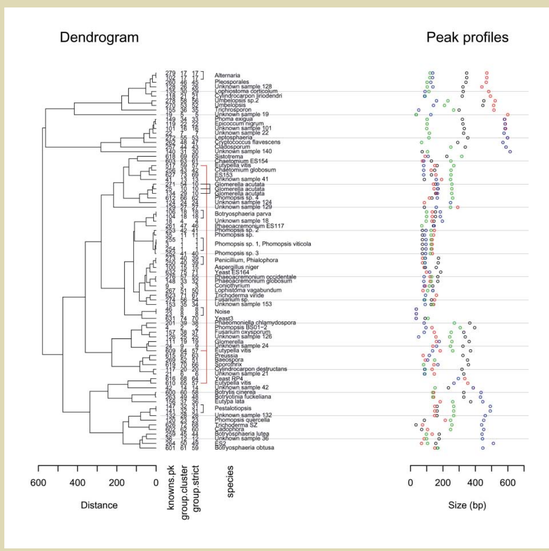


Fig 6. The current database of known t-RFLP profiles. Note the dendrogram does not necessary represent phylogenetic grouping.

Conclusions

t-RFLP shows promise both as a diagnostic technique for identifying grapevine pathogens, and a research tool for investigating the efficacy of control methods. We have also examined the succession of fungi following the hot water treatment of grapevines, which has allowed us to monitor for how long this treatment is effective. Furthermore t-RFLP has been used to track changes in fungal populations in vines after exposure to a pathogen. With the current importance of sustainable viticulture, the t-RFLP database is being further developed to include bio-control agents and beneficial organisms such as Mycorrhizae. This will allow tracking of these species in both plants and soil to monitor their effectiveness at colonising vines with beneficials and preventing or suppressing pathogen infections. In the future, t-RFLP could also be used to monitor the effect of environmental stresses on fungal populations in the vines. Similar methods may be used for a range of other horticultural crops.

References

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