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Research Note

The most informative loci to identify trunk disease pathogens associated with grapevine and perennial fruit and nut crops

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Summary. Trunk disease (TD) fungi are taxonomically diverse, and accurate species delimitation relies on multilocus phylogenetic analyses. However, the loci commonly employed vary among fungal groups, leading to inconsistencies in species recognition. This paper provides a comparative overview of the most informative genetic loci for species identification within the main families associated with TDs, including *Botryosphaeriaceae*, *Cytosporaceae*, *Diaporthaceae*, *Diatrypaceae*, *Phaeomoniellaceae*, *Togniniaceae*, *Nectriaceae* (*Ascomycota*), and *Hymenochaetales* (*Basidiomycota*). The internal transcribed spacer region (*ITS*) remains the universal primary barcode, but its discriminatory power is often limited. The most informative loci [translation elongation factor 1-α (*tef1*), β-tubulin (*tub2*), actin (*act1*), calmodulin (*cal*), histone (*his3*), and the RNA polymerase II second largest subunit (*rpb2*)] are identified, and optimal locus combinations for each fungal group are identified. This synthesis will aid selection of the most appropriate loci for robust phylogenetic inference and accurate pathogen identification, thereby improving epidemiological and management studies of TDs.

Keywords. Fungal taxonomy, multi-locus phylogeny, molecular identification, pathogen diagnosis.

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Trunk diseases (TDs) are among the most economically destructive disorders of woody plants, affecting forest trees and perennial fruit crops across temperate and tropical regions (Slippers and Wingfield, 2007; Gramaje *et al*., 2018; Guarnaccia *et al*., 2022; Martino *et al*., 2025). The pathogens involved are a highly diverse assemblage of *Ascomycota* and *Basidiomycota*, including members of the *Botryosphaeriaceae*, *Cytosporaceae*, *Diaporthaceae*, *Dia-*

trypaceae, *Nectriaceae*, *Phaeomoniellaceae*, *Togniniaceae*, and the *Hymenochaetales*. These fungi colonize woody tissues of their hosts primarily via wounds and/or natural openings, and cause vascular dysfunction, dieback, progressive yield decline, and eventual plant death. Accurate species-level identification is essential to underpin epidemiological studies, and to develop evidence-based, durable management strategies. The present paper specifically relates to fungal trunk pathogens associated with grapevine and perennial fruit and nut crops. Forest pathogens (e.g. *Cryphonectriaceae*), although relevant to canker diseases in woody plants, fall outside the scope of this review.

Identification of TD pathogens has relied heavily on morphological and cultural characteristics, which are often insufficient due to phenotypic plasticity and overlapping traits (Figure 1). The advent of molecular phylogenetics has revolutionized the taxonomy of these fungi, allowing for the use of multiple gene regions to resolve species boundaries. However, the loci selected for phylogenetic analyses have not been standardized, leading to discrepancies among studies. To address this, the present paper reviews current knowledge to determine which loci or locus combinations provide the best resolution for each TD-related fungus group.

The most complete taxonomic and phylogenetic studies describing TD-associated fungi were reviewed. These sources included revisions and monographs for each family, including Phillips *et al*. (2013) for *Botryosphaeriaceae*, Santos *et al*. (2017) and Manawasin-

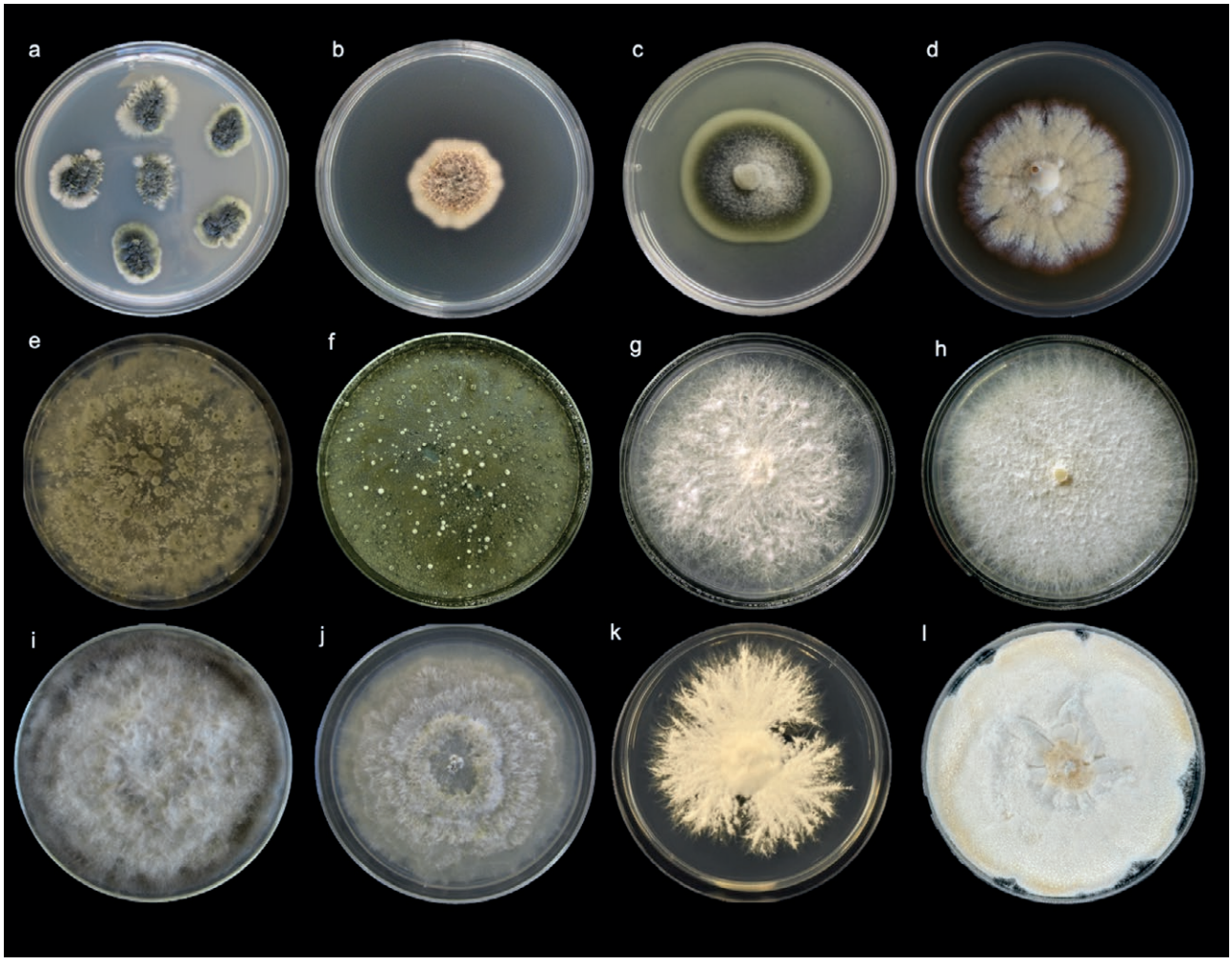

Figure 1. Colony morphologies of wood-inhabiting fungi grown on potato dextrose agar at 25°C: (a) *Phaeomoniella chlamydospora*, (b) *Phaeoacremonium parasiticum*, (c) *Cadophora luteo-olivacea*, (d) *Dactylonectria torresensis*, (e) *Cytospora pistaciae*, (f) *Cytospora sorbicola*, (g) *Diatrype stigma*, (h) *Eutypa leptoplaca*, (i) *Neofusicoccum parvum*, (j) *Diaporthe ampelina,* (k) *Schizophyllum commune*, and (l) *Ganoderma adspersum*.

ghe *et al*. (2019) for *Diaporthe*, Trouillas *et al*. (2010) for *Diatrypaceae*, Tegli *et al*. (2000) and Chen *et al*. (2022) for *Phaeomoniellaceae*, Cabral *et al*. (2012) for *Cylindrocarpon*-like species, Mostert *et al*. (2006) and Marin-Felix *et al*. (2019) for *Togniniaceae*, Travadon *et al*. (2015) and Chen *et al*. (2022) for *Cadophora* spp., Lin *et al*. (2024) for *Cytospora* spp., and Amalfi *et al*. (2012) for *Hymenochaetales*.

Each gene region reported in these studies was evaluated for its phylogenetic informativeness and resolution power. The analysis focused on loci commonly used in fungal taxonomy, including the internal transcribed spacer (*ITS*), translation elongation factor 1-α (*tef1*), β-tubulin (*tub2*), actin (*act1*), calmodulin (*cal*), histone (*his3*), and the RNA polymerase II second largest subunit (*rpb2*). Table 1 lists the respective primer pairs and amplification conditions for these gene regions. The loci were chosen based on their abilities to resolve closely related species, their reproducibility, and their representation in publicly available databases.

The comparative analysis revealed substantial variation in the informativeness of loci among fungal families associated with TDs. While ITS remains a universal barcode, it often lacks discriminatory power for closely related taxa. Figure 2 provides an overview of the optimal loci combinations for each group, with the most informative markers highlighted. Loci shown in green represent the most phylogenetically informative gene for each family, while loci in orange indicate additional markers necessary to resolve species complexes or describe new taxa. Table 1 presents the recommended primer pairs for each locus.

Table 1. Loci and respective primer pairs used to identify trunk disease fungi.

Loci	Primers	Sequence 5′-3′	References	Target Fungi
act1	ACT-512F	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn (1999)	ACT-512F & ACT-728R: *Cytosporaceae*
	ACT-783R	TACGAGTCCTTCTGGCCCAT	Carbone and Kohn (1999)	ACT-512F & ACT-783R: *Diaporthaceae* and *Togniniaceae*
	ACT-728R	TGGAGGGAGAAGAGCTACGA	Carbone and Kohn (1999)	
cal	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	Carbone and Kohn (1999)	CAL-228F & CAL-737R: *Diaporthaceae*
	CAL-737R	CATCTTCTGGCCATCATGG	Carbone and Kohn (1999)	
his3	CYLH3F	AGGTCCACTGGTGGCAAG	Crous *et al.* (2004)	CYLH3F & CYLH3R: *Nectriaceae*
	CYLH3R	AGCTGGATGTCCTTGGACTG	Crous *et al.* (2004)	CYLH3F & H3-1b: *Diaporthaceae*
	H3-1b	GCGGGCGAGCTGGATGTCCTT	Glass and Donaldson (1995)	
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White *et al.* (1990)	ITS1-F & ITS4: *Botryosphaeriaceae*, *Nectriaceae*, and *Hymenochaetales*
	ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)	ITS4 & ITS5: *Cytosporaceae*
	ITS4	TCCTCCGCTTATTGATATGC	White *et al.* (1990)	ITS1 & ITS4: *Diaporthaceae*, *Diatrypaceae*, and the genus *Cadophora*
	ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al.* (1990)	PCH1 & PCH2: *Phaemoniellaceae*
	PCH1	CTCCAACCCTTTGTTTATC	Tegli *et al.* (2000)	
	PCH2	TGAAAGTTGATATGGACCC	Tegli *et al.* (2000)	
rpb2	RPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu *et al.* (1999)	RPB2-5F & RPB2-7cR: *Cytosporaceae*
	RPB2-7cR	CCCATRGCTTGYTTRCCCAT	Liu *et al.* (1999)	
tef1	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn (1999)	EF1-688F & EF1-986R: *Botryosphaeriaceae*
	EF1-986R	TACTTGAAGGAACCCTTACC	Carbone and Kohn (1999)	EF1F & EF2R: *Cytosporaceae*
	EF1-688F	CGGTCACTTGATTTGTTGG	Alves *et al.* (2008)	EF1-728F & EF1-986R: *Diaporthaceae*
	EF1-1251R	CCTCGAACTCACCAGTACGA	Alves *et al.* (2008)	CylEF-1 & CylEF-R2: *Nectriaceae*
	EFIF	ATGGGTAAGGARGACAAGAC	O'Donnell and Cigelnik (1997)	EF1-688F & EF1-1251R: genus *Cadophora*
	EF2R	GGARGTACCAGTSATCATGTT	O'Donnell and Cigelnik (1997)	
	CylEF-1	ATGGGTAAGGAVGAVAAGAC	J.Z. Groenewald, unpublished	
	CylEF-R2	GCCATCCTTGGAGATACCAGC	Crous *et al.* (2004)	
tub2	BT2a	GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson (1995)	BT2a & Bt2b: *Botryosphaeriaceae*, *Cytosporaceae*, and *Diaporthaceae*
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson (1995)	T1 & Bt2b: *Nectriaceae*, *Togniniaceae*, and *Diatrypaceae*
	T1	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik (1997)	
	BTCadF	MATGCGTGAAATYGTAAGT	Travadon *et al.* (2015)	BTCadF & BTCadR: genus *Cadophora*
	BTCadR	TCAGCACCCTCAGTGTAATG	Travadon *et al.* (2015)	

Taxonomic group	ITS	*TEF1*	*TUB2*	*ACT1*	*RPB2*	*HIS3*	*CAL*	DNA lyase
Ascomycota								
Botryosphaeriaceae	X	X	X	X	X		X	
Cytosporaceae	X	X	X	X	X			
Diaporthaceae	X	X	X	X		X	X	X
Diatrypaceae	X		X		X			
Nectriaceae	X	X	X	X	X	X	X	
Phaeomoniellaceae	X	X		X				
Togniniaceae	X	X	X	X				
Cadophora	X	X	X					
Basidiomycota								
Hymenochaetales	X	X			X			

Excluding SSU and LSU.
The most informative locus to identify and analyse species in each group is highlighted in green. Sequencing additional loci, highlighted in orange in each group, is required for species separation for new species descriptions.

Figure 2. Genes for phylogenetic analyses and species identifications of fungi causing trunk diseases.

For the *Botryosphaeriaceae*, *tef1* and *tub2* consistently provide the greatest resolution for species delimitation, with *ITS* alone being insufficient. Additional markers such as *act1*, *rpb2*, and *cal* may further improve phylogenetic robustness (Inderbitzin *et al*., 2010; Phillips *et al*., 2013). Within *Diaporthaceae*, species delimitation requires a multilocus dataset combining *ITS*, *tef1*, *tub2*, *cal*, and *his3*, with *tef1* offering the best resolution (Santos *et al*., 2017; Manawasinghe *et al*., 2019). For *Diatrypaceae*, *ITS* and *tub2* consistently provide the highest resolution for species delimitation, with *ITS* being the most informative (Trouillas *et al*., 2010). Members of the *Phaeomoniellaceae*, including *Phaeomoniella*, are best resolved using *ITS* (Tegli *et al*., 2000; Chen *et al*., 2022). *Togniniaceae*, encompassing *Phaeoacremonium* spp., benefit from the combined use of *tub2* and *act1*. For *Nectriaceae*, encompassing '*Cylindrocarpon*'-like fungi, *his3* is the most informative locus, complemented by *ITS*, *tef1*, and *tub2* (Cabral *et al*., 2012; Lawrence *et al*., 2019). In *Cadophora* species, *ITS*, *tef1*, and *tub2* are typically used, with *tub2* being the most informative locus (Travadon *et al*., 2015). Within *Cytospora*, species delimitation requires a multilocus dataset combining *ITS*, *tef1*, *tub2*, *act1*, and *rpb2*, with *TEF1* offering the best resolution (Lawrence *et al*., 2018; Chen *et al*., 2022; Lin *et al*. 2024). Finally, within the Basidiomycota (*Hymenochaetales*), *ITS*, *tef1*, and *rpb2* suffice for accurate identification, with *ITS* being the most informative locus (Amalfi *et al*., 2012).

Advances in molecular taxonomy have considerably improved understanding of TD fungi. Despite significant progress, however, several challenges remain. A major obstacle is the heterogeneity of loci that have been used across studies, which complicates cross-comparison and meta-analyses of phylogenetic data. While *ITS* remains the official fungal barcode, it is often inadequate for species-level resolution among TD pathogens, due to low interspecific variability. Therefore, multi-locus sequencing analyses (MLSA) using *tef1*, *tub2*, and *his3*, as applicable to each fungal group (Figure 2), and these analyses have become the standard for accurate species delimitation, although this approach requires more laboratory resources and expertise than for single locus analyses.

The continuing discovery of cryptic and newly described species highlights the need for a unified molecular framework. Integration of genomic approaches, such as whole-genome sequencing (WGS) and MLSA, offers unprecedented opportunities. Genomic data provide increased resolution for population-level analyses, which enables increased understanding of evolutionary relationships, host adaptation, and pathogenicity. Nevertheless, these approaches are costly and require robust bioinformatic resources and capability.

To standardize taxonomy of TD pathogens, a community-based reference database should be established, including curated multi-locus datasets and metadata. This would facilitate consistent identification across laboratories, promote reproducibility, and reduce misidentifications that can hinder epidemiological interpretations. Furthermore, advances in high-throughput sequencing (Nilsson *et al*., 2019; Lofgren and Stajich, 2021), field-

deployable (portable) DNA barcoding using nanopore devices (Srivathsan *et al*., 2021), and machine-learning-based taxonomic classifiers (Wang and Cole, 2024), are likely to play important roles in the near future.

This summary highlights that there is no universal multilocus scheme suitable for all TD pathogens. Instead, optimal locus selection must be tailored to each fungus family to achieve accurate and reproducible results. The comparative framework presented here will support harmonization among laboratories, improve species delimitation, and facilitate further research into the biology and management of TD pathogens.

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