Profiling grapevine trunk pathogens *in planta*: A case for community-targeted DNA metabarcoding

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ABSTRACT

DNA metabarcoding, commonly used in exploratory microbial ecology studies, is a promising method for the simultaneous *in planta*-detection of multiple pathogens associated with disease complexes, such as the grapevine trunk diseases. Their detection is particularly challenging, due to the presence within an individual wood lesion of multiple co-infecting trunk pathogens and other wood-colonizing fungi, which span a broad range of taxa in the Fungal Kingdom. As such, we designed metabarcoding primers, using as template the ribosomal internal transcribed spacer of grapevine trunk-associated Ascomycete fungi (GTAA) and compared them to two universal primer widely used in microbial ecology. We first performed *in silico* simulations and then tested the primers by high-throughput amplicon sequencing of (i) multiple combinations of mock communities, (ii) time-course experiments with controlled inoculations, and (iii) diseased field samples from vineyards under natural levels of infection. All analyses showed that GTAA had greater affinity and sensitivity, compared to those of the universal primers. Importantly, with GTAA, profiling of mock communities and comparisons with shotgun-sequencing metagenomics of field samples gave an accurate representation of genera of important trunk pathogens, namely *Phaeomoniella*, *Phaeoacremonium*, and *Eutypa*, the abundances of which were greatly over- or under-estimated with universal primers. Overall, our findings not only demonstrate that DNA metabarcoding gives qualitatively and quantitatively accurate results when applied to grapevine trunk diseases, but also that primer customization and testing are crucial to ensure the validity of DNA metabarcoding results.

INTRODUCTION

Grapevine trunk diseases affect the longevity and productivity of grapevines (*Vitis vinifera*) in all major growing regions of the world [1-4]. They are caused by numerous species of fungi that infect and damage the wood, causing chronic infections [5-7]. Among the most common grapevine trunk diseases are Eutypa dieback (primarily caused by *Eutypa lata*), Esca (primarily caused by *Phaeoacremonium minimum*, *Phaeomoniella chlamydospora*, and *Fomitiporia* spp.), Botryosphaeria dieback (primarily caused by *Neofusicoccum parvum*, *Diplodia seriata*, among other fungi in the Botryosphaeriaceae family), Phomopsis dieback (primarily caused by *Diaporthe ampelina*), and Black foot (caused by *Cylindrocarpon*, *Campylocarpon*, and *Ilyonectria* spp.) [4, 8-11]. Because of the characteristic mixed infections, trunk diseases represent a disease complex [12, 13]. In addition to infections of pruning wounds by airborne and splash-dispersed spores, trunk pathogens may be introduced to a healthy vineyard by asymptomatic propagation material. Fungi associated with grapevine trunk diseases have been found in rootstock mother-plants, rooted rootstock cuttings, bench-grafts, and young grafted vines [14-16]. The presence of multiple species in the same vine complicates disease diagnosis and, consequently, proper timing of practices to limit infection in the vineyard and to propagate clean nursery stock.

Taxonomic identification of fungi associated with grapevine wood is currently done by the following steps: (i) plating grapevine woody tissue on nutrient-rich agar plates, (ii) hyphal-tip colony isolation to pure cultures, (iii) DNA extraction from fungal mycelium, (iv) PCR amplification of taxonomically informative loci, such as the nuclear ribosomal internal transcribed spacer (ITS), elongation factor, and β-tubulin, and (v) comparisons of amplicon sequences with sequence databases [17-19]. PCR-based diagnostics represent a significant improvement compared to traditional approaches that depend on morphological features for species

identification and, thus, require skilled expertise in mycology [20]. However, these approaches still require an initial culturing step, which may limit the detection of slow-growing fungi. Alternatively, with species or genus-specific markers, PCR could be used to determine *in planta* the presence of certain species, thereby skipping the culturing step [21, 22]. One limitation of this approach, however, is that it may not detect all trunk pathogens in a given sample [23, 24]. Indeed, certain combinations of fungi may be important in the severity of symptom expression [25].

Because trunk pathogens cause mixed infections, attempts have been made to characterize the composition of the trunk-pathogen community. For example, finger-printing techniques like Automated Ribosomal Intergenic Spacer Analysis (ARISA) [26] and Single-Strand Conformation Polymorphism (SSCP) [27, 28] have been used to compare fungal communities among different samples of grapevine wood, although these do not identify trunk pathogens to the species level. A DNA macroarray system, based on reverse dot-blot hybridization containing oligonucleotides complementary to portions of the β-tubulin locus, was developed for species-level identification, specifically for detection of trunk pathogens that cause Young vine decline [23]. We previously described a strategy, based on untargeted shotgun sequencing of metagenomic DNA and RNA, to detect and quantify trunk pathogens *in planta* simultaneously [13]. Despite clear advantages over other approaches, this method still has its limitations, such as relying on assembled genomes, as well as costly library preparation and computationally intensive analyses.

DNA metabarcoding, which has been used extensively for the analysis of microbial communities [29-32], may provide a cheaper and more scalable method for the characterization of trunk-pathogen communities. This approach has already been applied to other pathosystems to address a variety of research objectives. For example, DNA metabarcoding has been used to identify candidate pathogens [33, 34] and potential biocontrol agents [35], to profile putative plant

pathogens associated with insects [36], and to diagnose quarantine pathogens as part of national plant-protection programs [37-39]. DNA metabarcoding infers taxonomic composition of complex biological samples by amplifying, sequencing, and analyzing target genomic regions [40, 41]. The ribosomal ITS, which is under low evolutionary pressure and, thus, presents high levels of variation between closely related species, has been commonly used as a barcode for the analysis of fungal biodiversity [42, 43]. ITS is typically amplified by universal primers that anneal to the conserved flanking sequences. The "universality" of the primers, which derives from their ability to amplify a broad range of taxonomically unrelated species across the Fungal Kingdom [44], is exploited in studies that aim to profile fungal communities, typically in exploratory analyses of environmental samples. We hypothesized that although universal primers may capture broad biodiversity in exploratory analyses, they may provide less accurate representation of microbial pathogen communities than primers that are designed and optimized to amplify species known to be associated with those communities, based on prior knowledge of disease etiology. After all, grapevine-trunk diseases are one of the most widely studied disease complexes, in terms of species composition (Lamichane and Venturi, 2015). In this work, we designed and evaluated metabarcoding primers that were optimized to amplify the ITS regions of grapevine trunk pathogens. By a combination of *in silico* simulations, and analyses of 'mock' communities, samples from controlled inoculations, and samples from symptomatic vineyards, we demonstrated that community-customized metabarcoding provides greater more qualitatively and quantitevely accurate representation of trunk-pathogen communities than common universal primers.

RESULTS

Primer design, selection, and validation with target species

We designed multiple degenerate primers that target the internal transcribed spacer (ITS) of grapevine trunk-associated ascomycetes (GTAA) using the TrunkDiseaseID as reference database [20]. Primer potential was determined *in silico*, considering the amplicon size and estimating the number of sequence hits to the database, their alignment mismatches, and gap scores. From a total of twenty forward and three reverse degenerate primers, primers GTAA182f and GTAA526r (GTAA, hereinafter) performed the best and were selected for further testing. The GTAA primers target the entire ITS2 region with the forward and reverse primers aligning to the 5.8S ribosomal RNA and the large subunit ribosomal ribonucleic acid (LSU), respectively (**Table 1** and **Figure 1A**). The primers produced amplicons of approximately 350 bp from isolates of seven trunk pathogens, as expected based on the amplicon size predicted from the 213 ITS sequences of ascomycetes in the TrunkDiseaseID database (301.72 ± 7.53 bp; **Figure 1B**). We obtained a similar amplicon size when the GTAA primers were used to amplify total DNA extracted from naturally infected grapevines with trunk-disease symptoms (**Figure 1C**). Failure to amplify DNA of two negative controls, grape leaves, and of the bacterium *Agrobacterium tumefaciens*, supports their specificity to Fungi (**Figure 1B**). Amplicon sequences matched the correct species, when aligned to the NCBI non-redundant nucleotide database using BLASTn, thereby confirming the ITS region amplified by the GTAA primers is informative for taxonomic assignments (**Additional File 1**).

GTAA primer precision, sensitivity, efficiency, and usefulness for metabarcoding of grapevine trunk pathogens were compared to those of the BITS [45] and SP primers [46]. The BITS primers are widely used for fungal metabarcoding analysis in vineyards and grape must (e.g.: [47-51]), whereas the SP primers [46] were recently used by [52] for fungal microbial ecology, and implemented in the Earth Microbiome Project (http://www.earthmicrobiome.org; **Table 1 and**

Figure 1A). Samples were from DNA extracted from potted grapevines either inoculated with *N. parvum* or from non-inoculated controls. By sampling PCR reactions every five cycles, the GTAA amplicon was visible on an agarose gel starting at 20 cycles, whereas those of SP and BITS were visible at 25 and 30 cycles, respectively (**Figure 1D**). Furthermore, SP produced multiple bands, which may be due to non-specific binding and/or chimeric amplicons. Based on qPCR with the same samples, the average Ct values for GTAA were approximately nine cycles lower than those of BITS ($P < 1.85e^{-04}$) and SP ($P < 3.50e^{-04}$) (**Figure 1E**). Overall, our findings suggest a higher affinity of the GTAA primers, when amplifying samples containing grapevine trunk pathogens.

In silico simulation of amplification and taxonomic identification

We then carried out an *in silico* simulation that compared the potential amplification bias and taxonomic usefulness of GTAA, BITS, and SP primers using a comprehensive dataset of fungi associated with trunk diseases. We compiled a custom database of 521 full-length ITS sequences across 17 genera (**Figure 2A, Additional File 2**). We included only full-length ITS sequences to be able to compare primers that amplify different regions of the ITS (**Figure 1A**). *In silico* amplification of each sequence in the custom database was carried out considering all alternative sequences of degenerated primers and allowing a series of mismatches between primer and template sequences. *In silico* amplification was carried out testing all possible combinations of allowed mismatches, from 0 to 5 mismatches in the first five nucleotides of the primer (head) and 0 to 2 mismatches in the last two nucleotides of the primer (tail). GTAA primers amplified a higher number of sequences than BITS and SP primers, for every parameter tested (**Figure 2B**). When no mismatches between primer and target were allowed, GTAA primers amplified 85.80%, SP primers amplified 13.63%, and BITS primers were predicted to amplify none of the sequences in the database. When at least two mismatches were allowed in the tail of the primer, BITS and SP

158 primers amplified only 16.70% and 30.33% of target sequences, respectively, whereas GTAA
159 primers amplified 86.75%. With the most permissive parameters, GTAA primers amplified
160 98.08% of the sequences, and BITS and SP primers amplified 97.89% and 25.91%, respectively.
161 The requirement of multiple mismatches for BITS primers to achieve a similar number of
162 sequences as GTAA primers is consistent with the cycle-sampling results (**Figure 1D**), and
163 suggests that GTAA primers are more efficient than BITS at amplifying the ITS of grapevine trunk
164 pathogens.
165 To determine if amplicons generated by GTAA primers are informative for taxonomic assignment,
166 we analyzed with Mothur [53] the amplicons that were generated by the simulation. By comparing
167 the assigned genera (observed) with the expected genera for each primer set we assessed false
168 positive (FP; i.e, erroneously assigned), false negative (FN; i.e, not amplified or not assigned), and
169 true positive (TP; i.e, correctly assigned, **Figure 2C**) rates. GTAA primers had the highest
170 sensitivity (TP/(TP/FN)*100 = 89.50 ± 6.45%), followed by BITS (54.25 ± 47.86%), and SP
171 (20.50 ± 2.53%). SP and GTAA primers displayed similar precision (SP: TP/(TP+FP)*100 = 97.50
172 ± 1.00%; GTAA: 97.00 ± 0.00%), which was higher than that of BITS primers (72.25 ± 48.18%).
173 The different performance of the three primer sets in the simulation appeared to be mostly due to
174 amplification bias against certain genera (**Figure 2D**). GTAA primers amplified and correctly
175 assigned to the proper genera a larger fraction of sequences than the other two primer sets for 14
176 out of 17 genera tested. This was the case for the following widely distributed trunk pathogens:
177 *Eutypa* (GTAA: 98.0 ± 4.0%, BITS: 53.8 ± 53.8%, and SP: 44.0±4.0%), *Diaporthe* (GTAA: 96.5
178 ± 1.3%, BITS: 51.3 ± 53.6%, and SP: 18.7 ± 9.3%), and *Phaeoacremonium* (GTAA: 95.5 ± 3.0%,
179 BITS: 51.5 ± 56.1%, and SP: 18.7 ± 9.0%). BITS primers correctly assigned more sequences for
180 *Lasiodiplodia* (GTAA: 71.0 ± 8.0%, BITS: 75.0 ± 50.0%, and SP: 0.0 ± 0.0%) and *Cylindrocarpon*

(GTAA: 35.5 ± 41.0%, BITS: 46.3 ± 33.54%, and SP: 0.0±0.0%). SP primers correctly assigned more sequences for *Campylocarpon* (GTAA: 50.0 ± 57.7%, BITS: 50.0 ± 57.7%, and SP: 75.0 ± 50.0%). Overall, this simulation predicted that, unlike the two universal primer sets, GTAA primers amplify ITS of more trunk pathogens and allow taxonomic assignment with greater sensitivity (i.e., higher true positive rate) and specificity (i.e., lower false negative rate). SP primers were not included in further experiments, due to their poor performance in these early stages.

Analysis of mock communities and infection time course

To evaluate the primers for characterizing the species composition of mixed infections, we first started by sequencing with an Illumina MiSeq and analyzing mock communities (**Figure 3A**). Although DNA was extracted from stems with no symptoms of trunk disease to be used as a pure source of grape DNA, both primer sets detected fungi, mostly belonging to the genera *Campylocarpon* and *Phaeoacremonium* (**Figure 3A**). When grape DNA was mixed with DNAs of *Pheaoa. minimum* and *Phaeom. chlamydospora* both primer sets identified the correct taxa, with small relative deviation from expected values (GTAA δ = 11.02 ± 7.0 %; BITS δ = 16.68 ± 11.39%). For mock communities including *Eutypa*, GTAA primers detected this trunk pathogen in similar amounts to the expected abundance (δ = 9.74 ± 1.10%), whereas BITS primers greatly underestimated its abundance (δ = 88.87 ± 1.27%). In mock communities with equal concentrations of DNA from *E. lata*, *Phaeoa. minimum*, *Phaeom. chlamydospora*, *N. parvum, D. seriata*, and *D. ampelina*, there was underrepresentation of *Eutypa* (δ = 16.70 ± 0.12%), and *Phaeoacremonium* (δ = 13.10 ± 0.63%), and overrepresentation of *Phaeomoniella* (δ = 24.34 ± 1.56%) by BITS primers (**Figure 3A**). The correlation of expected and observed abundances in these mock communities was greater for GTAA (R = 0.92) than BITS (R = 0.67; **Figure 3B**). Because DNA was mixed in equal amounts, the expected relative abundance of each genus was

16.6%. GTAA primers detected *Eutypa* at 16.42 ± 2.41%, whereas BITS primers detected this trunk pathogen at 0.05 ± 0.03%. In the case of *Diplodia*, GTAA primers estimated the abundance of the genus at 3.13 ± 0.48% and BITS primers at 28.9 ± 0.8%. Interestingly, neither primer set was able to detect properly *Diaporthe*, reporting only 0.58 ± 0.23% and 0.87 ± 0.15% for GTAA and BITS primers, respectively. Nonetheless, GTAA primers provide a better qualitative and quantitative representation of important trunk pathogens.

We then tested the two primers using grape samples collected at different time points after controlled inoculation with a trunk pathogen. The objective of this analysis was to determine if the metabarcoding approach could detect quantitative differences between samples at early and late stages of infection. Vines were inoculated with *N. parvum* and stem samples were collected at 24 hours, 2 weeks, and 6 weeks post-inoculation. Plants non-inoculated wounded (NIW) and non-inoculated non-wounded (NINW) were included as controls. As expected, *Neofusicoccum* was predominant in the inoculated wounded (IW) samples, but absent from the controls (**Figure 4**), except for a single NIW sample, possibly due to cross-contamination during wounding or from contamination of the propagation material. Both primer sets revealed a five-fold increase in the average percentage of *Neofusicocum* between 24 hours and 6 weeks post-inoculation.

Analysis of field samples and comparison with reference-based shotgun metagenome sequencing

We then tested the primers on naturally infected grapevines. We used the same 28 field samples described in [13], which allowed us to compare the metabarcoding approach with the quantitative taxonomic profiles obtained by a reference-based shotgun metagenome sequencing. The samples were grouped according to symptoms into Eutypa dieback (ED), Esca (ES), wood canker without foliar symptoms (WC), and apparently-healthy (AH). All 28 field samples were amplified with

227 both GTAA and BITS primers, with SP primers used for a subset. Taxonomy assignment based
228 on amplicon metabarcoding detected 14 genera, in addition to those with genomes in the
229 multispecies reference, with abundances > 0.05% in one or more samples (**Additional File 3**).
230 Both GTAA and BITS primer sets identified *Alternaria*, *Cyphellophora*, and *Penicillium*, whereas
231 *Cladosporium*, *Aureobasidium*, *Gibberella*, and *Cryptovalsa* were only identified by GTAA
232 primers, and *Angustimassarina, Exophiala, Erysiphe, Meyerozyma, Acremonium*, and
233 *Vishniacozyma* by BITS primers. GTAA primers revealed species abundances at very similar
234 levels to those obtained by metagenomics analysis (**Figure 5A**), with a strong linear correlation
235 between the two approaches ($R = 0.95$; **Figure 5B**), which was higher than those of both BITS (R
236 $= 0.63$) and SP primers ($R = 0.27$). In agreement with the other results described above, BITS
237 primers underestimated *Eutypa* in Eutypa-dieback samples and overrepresented *Phaeomoniella* in
238 Esca samples. The even weaker correlation obtained with SP primers was due to the strong bias
239 against *Eutypa* and *Diaporthe*. GTAA primers showed stronger correlations across all genera of
240 trunk pathogens ($0.89 < R < 0.99$, **Figure 5C**) compared to those of BITS ($0.58 < R < 0.75$). Both
241 primer sets showed a low correlation for *Neofusicoccum*, likely due to the low abundance of this
242 genus in the samples assayed. Overall, our findings confirm the universal primers have a
243 significant bias against important taxa and were outperformed by our GTAA primers for trunk
244 pathogens.

245 **DISCUSSION**

246 In this study, we tested the application of DNA metabarcoding to profile the fungal taxa associated
247 with grapevine trunk diseases. We show that DNA metabarcoding of ribosomal ITS amplified with
248 commonly-adopted universal primers consistently misrepresented the abundance of important
249 trunk pathogen species, such as *Eutypa* and *Phaeomoniella*. The customization of primer design

using trunk pathogen sequences as template led to improved the results with greater sensitivity. This was likely due to greater homology between the GTAA primers and the ITS of the grapevine trunk pathogens they target. On average the sequence identity of the grapevine trunk pathogen targets was significantly greater with the GTAA primers (97.4 ± 5.5%; $P < 2e\text{-}16$) than with the other universal primers (BITS: 90.2 ± 7.1%; SP: 83.3 ± 0.2.2%) used in the study. Amplification bias of universal ITS primers due to higher levels of mismatches for certain taxonomic group were observed previously using *in silico* PCR [54, 55]. Importantly, we also showed that the GTAA primers had higher sensitivity while maintaining a precision threshold for taxonomic assignment of 97%, suggesting that the customization of the target region also played a role in improving the DNA metabarcoding for these organisms. We should stress out that BITS and SP primers are not the only available universal primers and the goal of this study was not to provide a comprehensive survey of all universal ITS metabarcoding primers. BITS and SP were selected, because they are both widely used DNA barcoding primers, including in studies conducted on vineyard and wine must samples [46-51]. We cannot rule out that other universal primers that were not tested in this study may have performed differently. However, the results presented in this study show that universal primers may not be always appropriate to study a fungal community and, when fungal community composition is available, researchers should consider customizing their DNA metabarcoding primers. In addition, we illustrate the value of assessing both the amplification and taxonomy usefulness of the metabarcoding primers *in silico* prior to downstream wet lab evaluations.

In addition to customization of primers, the inclusion of other DNA barcodes should help overcome some of the limitations associated with the ITS region, such as copy number variation between and within species and low resolution in separating some phylogenetically closely related

fungal species [42, 56]. For example, the ITS region does not accurately identify species of plant-pathogenic fungi like *Alternaria*, *Botryosphaeria,* and *Diaporthe* [57]. The genera for which the GTAA primers consistently underestimated abundances like *Lasiodiplodia, Botryosphaeria, Diplodia,* and *Diaporthe* are known to be difficult to be resolved with the ITS region alone [57]. The high correlation between metagenomics and metabarcoding results using the GTAA primers suggest that copy number variation of the ITS region is not an overwhelming issue for the grapevine trunk pathogens present in the field samples. Nonetheless, we expect that the inclusion of additional barcodes, such as β-tubulin and elongation factor 1-α, will help increase accuracy of taxonomic identification at the species level and help measure those genera for which the ITS is known not to be effective [20, 23, 58, 59].

CONCLUSIONS

As trunk diseases are complex diseases caused by mixed infections, DNA metabarcoding should provide a rapid and effective method for high-throughput multispecies identification overcoming the limitations of currently applied diagnostic methods. Universal primers are advantageous in exploratory analysis where *a priori* knowledge on the taxonomic composition of the samples is limited or not available. However, a more targeted approach should be used when the objective is to study a more defined group of microorganisms, like the grapevine trunk pathogens which symptoms have been consistently associated with certain fungal species [4, 20, 60]. Overall, the results presented here demonstrated that DNA metabarcoding can be applied to grapevine trunk diseases. With further improvement of taxonomic identification by combining multiple barcoding loci and of quantification by measurement of direct correlation between fungal biomass and PCR amplification cycles, we envision DNA metabarcoding to be routinely applied in trunk pathogen research and diagnostics. DNA metabarcoding provides multiple advantages to methods employed

296 in the past. Namely, there is no need of fungal isolation, it allows high number of samples to be
297 analyzed at the same time given the multiplexing potential of the technology, and takes advantage
298 of the constantly improving high-throughput sequencing technologies. Since wood pathogens may
299 remain asymptomatic in young, non-stressed vines, propagation material may contain latent fungal
300 infections and may become symptomatic after planting and serve as a source of inoculum for
301 further infections of potentially clean plants. Methods of virus detection and eradication have been
302 crucial in ensuring that the material in germplasm repositories and clean plant programs is free of
303 known viruses. By allowing the rapid testing of large number of wood samples from mother plants
304 in foundation blocks and propagation material in nurseries, we expect that the applications of
305 metabarcoding to trunk pathogen diagnostics will help reduce the amount of trunk pathogens
306 introduced into vineyards at planting as well as the incidence of young vine decline. Our results
307 also demonstrated that primer customization and testing are crucial to ensure the validity of DNA
308 metabarcoding results.

309

310 **METHODS**

311 **Metabarcoding primers targeting grapevine trunk-associated Ascomycetes (GTAA)**

312 Ribosomal Internal transcribed spacer (ITS) sequences of trunk pathogens and other wood-
313 colonizing fungi of grape, specifically in the Division Ascomycota, were retrieved from the
314 TrunkDiseaseID.org database [20]. Sequences were aligned using ClustalW2 (v2.1; [61]) to
315 identify conserved regions. Sequence alignment was used as input for the metabarcoding primer
316 design software Primer Prospector v1.0.1 [62], using a sensitivity threshold of 80% and an initial
317 primer seed size of 5 bp. The ITS sequence of *E. lata* (GeneBank KU721859.1) was used as a
318 'reference'. Primers were selected based on median amplicon size, and mismatches, gaps, and

numbers of matches to the sequences in the database. The base pairs 'AG' were used as a linker between the primer and an eight-nucleotide barcodes on the 5' region of the forward primer sequence. Barcode sequences were as described in [63]. A list of barcoded forward GTAA primers is listed in **Additional File 4**.

A custom database was compiled with full length ITS sequences of species in the following genera: *Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia, Neofusicoccum, Phaeomoniella, Diaporthe, Phaeoacremonium, Diatrype, Diatrypella, Eutypa, Xylaria, Cylindrocarpon, Campylocarpon, Dactylonectria, Ilyonectria*, and *Neonectria*. Sequences were retrieved from the NCBI GenBank repository. Completeness of the ITS sequences was validated using the hidden Markov models-based software ITSx [64]. Only sequences spanning the entire ITS region (ITS1, 5.8S, and ITS2) were kept for downstream analysis. Species and GenBank accessions of the complete ITS sequences included in the custom database are listed in **Additional File 2**. To reduce redundancy and identify outliers, the complete ITS sequences were clustered using the UCLUST algorithm [65] integrated in Qiime (v1.9.1; [66]) with 97% identity. The longest representative sequence of each cluster was selected, using the Qiime 'pick_rep_set.py' function. All representative sequences were aligned using Mafft v7.271 [67]) with the '--auto' argument and 1,000 iterations. Sequences clustering outside the expected family were removed from the final custom database.

The program Degenerate In-Silico PCR (dispr, https://github.com/douglasgscofield/dispr) was used to predict and evaluate the amplification of sequences of the custom ITS database, using our GTAA primers, and universal BITS [45], and SP [46] primers. Dispr allowed an amplicon size of 100 to 400 bp, all combinations up to five mismatches in the head of the primer ('H' or 5'-most region), and all combinations up to two mismatches in the tail of the primer ('T' or the remaining

342 3'-portion of the primer). The resulting amplicons produced *in silico* were then used for taxonomy
343 assignment with 80% confidence, using Mothur (v1.39.5; [53]), as it is integrated in Qiime
344 (v1.9.1). The UNITE database v7.2 [68] was used as taxonomic reference. True positives were
345 defined as sequences that were assigned to the expected genus, false positives were sequences
346 assigned to a different genus, and false negatives were sequences not assigned to any genus or
347 were not amplified by dispr.
348 To generate mock communities, we combined (i) DNA from a healthy grapevine with DNA from
349 pure cultures of three trunk pathogens at different concentrations, or (ii) equal concentrations of
350 DNA from pure cultures of six trunk pathogens. For the former, grape and fungal DNA were
351 combined as follows: 90% grape with 10% *E. lata* isolate Napa209 [69], *Phaeoa. minimum* isolate
352 1119 [70], or *Phaeom. chlamydospora* isolate C42 [71]; 80% grape with 10% of each of two fungal
353 isolates (in all three pair-wise combinations of the three isolates); and 70% grape DNA with 10%
354 of each of the three fungal isolates. For the latter, equal concentrations of DNA were combined
355 from the same three trunk pathogens and three additional species: *N. parvum* isolate UCD646So
356 [11], *Dia. ampelina* isolate Wolf911 [72], and *Diplodia seriata* isolate SBen831 [73]. Grape DNA
357 was extracted from the leaves of a non-inoculated, non-wounded plant; this DNA template came
358 from a previous experiment [74]. The mock communities containing grape DNA were amplified
359 and sequenced independently three times, whereas the mock community of six fungal DNAs was
360 amplified and sequenced independently five times. Prior to DNA extraction on Potato Dextrose
361 Agar (PDA; Difco laboratories, Detroit, MI). DNA was extracted as described in [73] and
362 measured by Qubit (Life technologies).
363 To test *in planta* detection of a trunk pathogen at variable levels of infection (i.e., from low to high
364 concentrations of fungal biomass over time), DNAs for the infection time course of *N. parvum*

were extracted from the same samples described in [74]. Briefly, 1-year-old potted *V. vinifera* 'Cabernet Sauvignon' FPS 19 plants were inoculated with isolate UCD646So mycelia. Woody stems were collected at seven time points: 0 hpi, 3 hpi, 24 hpi, 2 wpi, 6 wpi, 8 wpi, and 12 wpi. Wood samples from 1 cm below the inoculation site were collected using flame-sterilized forceps and immediately placed in liquid nitrogen for nucleic acids extraction. Infections were confirmed by positive recovery of the pathogen after 5-day growth on PDA.

To test *in planta* detection of multiple trunk pathogens in mixed infection (i.e., to characterize the species composition of a naturally established trunk-pathogen community), DNA from the same 28 field samples described in [13] was used to make cross-technology comparisons. These field samples were collected from mature vines (> 8 years-old) showing a variety of the most common symptoms associated with trunk diseases. Wood samples were collected from distinct plants with the following combinations of symptoms: Eutypa dieback foliar and wood symptoms, Esca foliar and wood symptoms, wood symptoms and no foliar symptoms, and apparently healthy plants with no foliar or wood symptoms.

High throughput sequencing libraries

Each sample was amplified using the unique 8-nt barcode forward primer sets for GTAA and BITS, to enable sample multiplexing. The 25-µl PCR reaction mix contained 2 ng of DNA template, 1X Colorless GoTaq flexi buffer (Promega Corporation, Madison, WI), 1.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 mM dNTPs, 0.4 µM of each primer, and 1.25 units of GoTaq Flexi DNA polymerase (Promega Corporation, Madison, WI). PCR program (Veriti thermal cycler, Applied Biosystems) was as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 45 seconds, 55°C for 1 min., and 72°C for 1 min., and a final extension at 72°C for 10 min. In the experiments to assess primer affinity, reactions were stopped after 5, 10, 15, 20, 25, 30, and 35

388 cycles. Following PCR, amplicon size and uniqueness were verified using gel electrophoresis, and
389 bands were cleaned using Ampure XP magnetic beads (Agencourt, Beckman Coulter). DNA
390 concentration was determined for each purified amplicon using Qubit (Life technologies). For the
391 single isolate validation, amplicons were sequenced with Sanger (DNA Sequencing Facility,
392 University of California, Davis).
393 For high-throughput sequencing, equimolar amounts of all barcoded amplicons were pooled into
394 a single sample, the total concentration of which was determined by Qubit. Five hundred
395 nanograms of pooled DNA were then end-repaired, A-tailed and single-index adapter ligated
396 (Kapa LTP library prep kit, Kapa Biosystems). After adapter ligation, the sample was size-selected
397 with two consecutive 1X bead-based cleanups; concentration and size distribution were
398 determined with Qubit and Bioanalyzer (Agilent Technologies), respectively. DNA libraries were
399 submitted for sequencing in 250-bp paired-end mode on an Illumina MiSeq (UCDavis Genome
400 Center DNA technologies Core). All FASTQ files with the amplicon sequences separated by
401 barcode were deposited in the NCBI Sequence Read Archive (BioProject: PRJNA485180; SRA
402 accession: SRP156804).
403 **Amplicon sequencing community analysis**
404 Adapter-trimming was carried out using BBDuk (BBMap v.35.82; http://jgi.doe.gov/data-and-
405 tools/bb-tools/) in paired-end mode with sequence "AGATCGGAAG" and the following
406 parameters: ktrim=r, k=10, mink=6, edist=2, ordered=t, qtrim=f and minlen=150. Adapter-
407 trimmed FASTQ files were then quality-filtered using Trimmomatic v0.36 [75] with paired-end
408 mode, phred33, a sliding windows of 4:19, and a minimum length of 150 bp. Sequencing data
409 were then processed in the Qiime environment v1.9.1 [66]. Barcodes were extracted from the
410 FASTQ files using the "extract_barcodes.py" function with the "-a" argument that attempts read

orientation and a barcode length of eight base pairs. The resulting sequences and barcodes were used to tag the reads with "split_libraries_fastq.py", a threshold quality score of 20, and a barcode size of eight basepairs. Operational taxonomic units (OTUs) were identified with a 99% similarity threshold using the UCLUST algorithm [65] with the reverse strand match enabled ("-z"), and the longest sequence of each OUT was chosen as representative sequence. Taxonomy assignment was carried out using Mothur (v1.39.5; [53] with the UNITE database v7.2 [68] as reference and a 80% confidence threshold. For each sample, sequences were randomly sampled with the function "single_rarefaction.py" from the OTU tables to obtained a total number of sequences per sample equal to the lowest number of reads across GTAA, BITS, and SP datasets. Taxonomy tables at the genus level were then created using "summarize_taxa.py".

DECLARATIONS

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All FASTQ files with the amplicon sequences separated by barcode were deposited in the NCBI Sequence Read Archive (BioProject: PRJNA485180; SRA accession: SRP156804).

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Conceived and designed the experiments: AMC and DC. Performed the lab experiments: RFB, AMC, ET, and JD. Performed field sampling: PER. Performed bioinformatic analysis: AMC. Wrote the manuscript: AMC, KB, and DC. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Primer design and testing. (A) Schematic representation of the annealing sites of forward and reverse GTAA, BITS, and SP primers in the fungal ribosal ITS. Reported amplicon sizes were calculated based on the ITS sequence of *Eutypa lata* (KU320617.1). (A) & (B): Bioanalyzer electropherograms showing amplicons generated using GTAA primers and (A) DNA from purified organisms as template and (C) field samples with different trunk disease symptoms.

683 *Agrobacterium tumefaciens*, *V. vinifera* and nuclease-free water were included as controls. AH:
684 apparently healthy, ED: Eutypa Dieback, ES: Esca and WC: wood cankers (no leaf symptoms).
685 (D) PCR products from grapevines inoculated with *N. parvum* at six weeks post-inoculation, which
686 were visible on agarose gels at five-cycle intervals, when amplified with primers GTAA, BITS, or
687 SP. L: 100 bp Ladder. (E) Cycle thresholds (Ct) measured by qPCR of the same reactions shown
688 in (D).

689 **Figure 2**. ***In silico*** **simulation of amplification and taxonomic assignment.** (A) Neighbor-
690 joining tree of the full-length ITS sequences included in the custom database used in the
691 simulation. (B) Barplots showing the number of sequences predicted to be amplified by each
692 primer set at different combinations of mismatches. H: primer head. T: primer tail. Numbers
693 correspond to the number of mismatches either in H or T. (C) Barplots showing the number of
694 false negative (FN), false positive (FP), and true positive (TP) sequences with each primer set. (D)
695 Percentage of sequences per genus correctly assigned with each primer set at the different
696 mismatch combinations.

697 **Figure 3. Results of DNA metabarcoding of mock communities.** (A) Stacked barplots showing
698 the relative abundance of genera in the mock communities identified using the GTAA and BITS
699 primers. Eu: *Eutypa*, Pa: *Phaeoacremonium*, Pm: *Phaeomoniella*, Di: *Diaporthe*, Dp: *Diplodia*,
700 Np: *Neofusicoccum,* and Vv: *Vitis vinifera.* (B) Linear correlations between observed and expected
701 abundances for each genus contained in the mock community.

702 **Figure 4**. **Results of DNA metabarcoding of an infection time-course.** Stacked barplots show
703 the relative abundance of the genera detected in a time course experiment after inoculation with
704 *N. parvum*. IW: wound-inoculated with *N. parvum*; NIW: non-inoculated non-wounded; NINW:
705 non-inoculated non-wounded controls.

706 **Figure 5. Results of DNA metabarcoding of field samples and comparisons with shotgun**
707 **whole-genome metagenomics (WGS).** (A) Stacked barplots showing the relative abundance of
708 the genera detected by WGS and DNA metabarcoding with GTAA, BITS, and SP primers. (B)
709 Scatter plots showing the correlations of the relative abundance obtained by DNA metabarcoding
710 and WGS. (C) Scatter plots showing the correlations of the relative abundance separately for each
711 genus obtained by DNA metabarcoding using the GTAA primers and WGS.

712 **TABLES**

713 **Table 1. Metabarcoding primer sequences targeting the ITS region used in this study**

Primer name	Direction	Primer sequence 5' to 3'	Citation
GTAA	Forward	AAAACTTTCAACAACGGATC	this study
GTAA	Reverse	TYCCTACCTGATCCGAGGTC	this study
BITS	Forward	CTACCTGCGGARGGATCA	[45]
BITS	Reverse	GAGATCCRTTGYTRAAAGTT	[45]
SP	Forward	CTTGGTCATTTAGAGGAAGTAA	[46]
SP	Reverse	GCTGCGTTCTTCATCGATGC	[46]

714

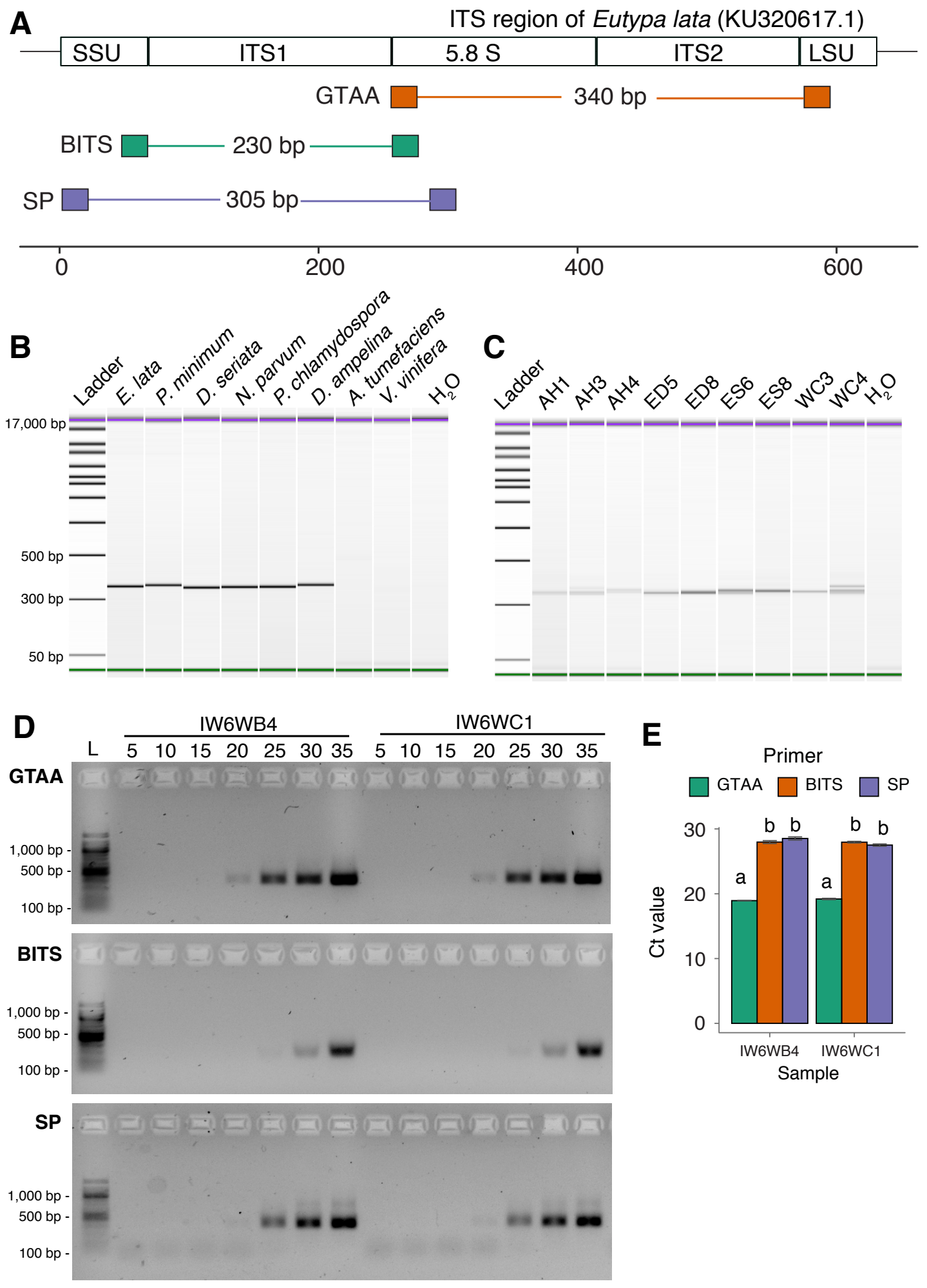
715 **ADDITIONAL FILES**

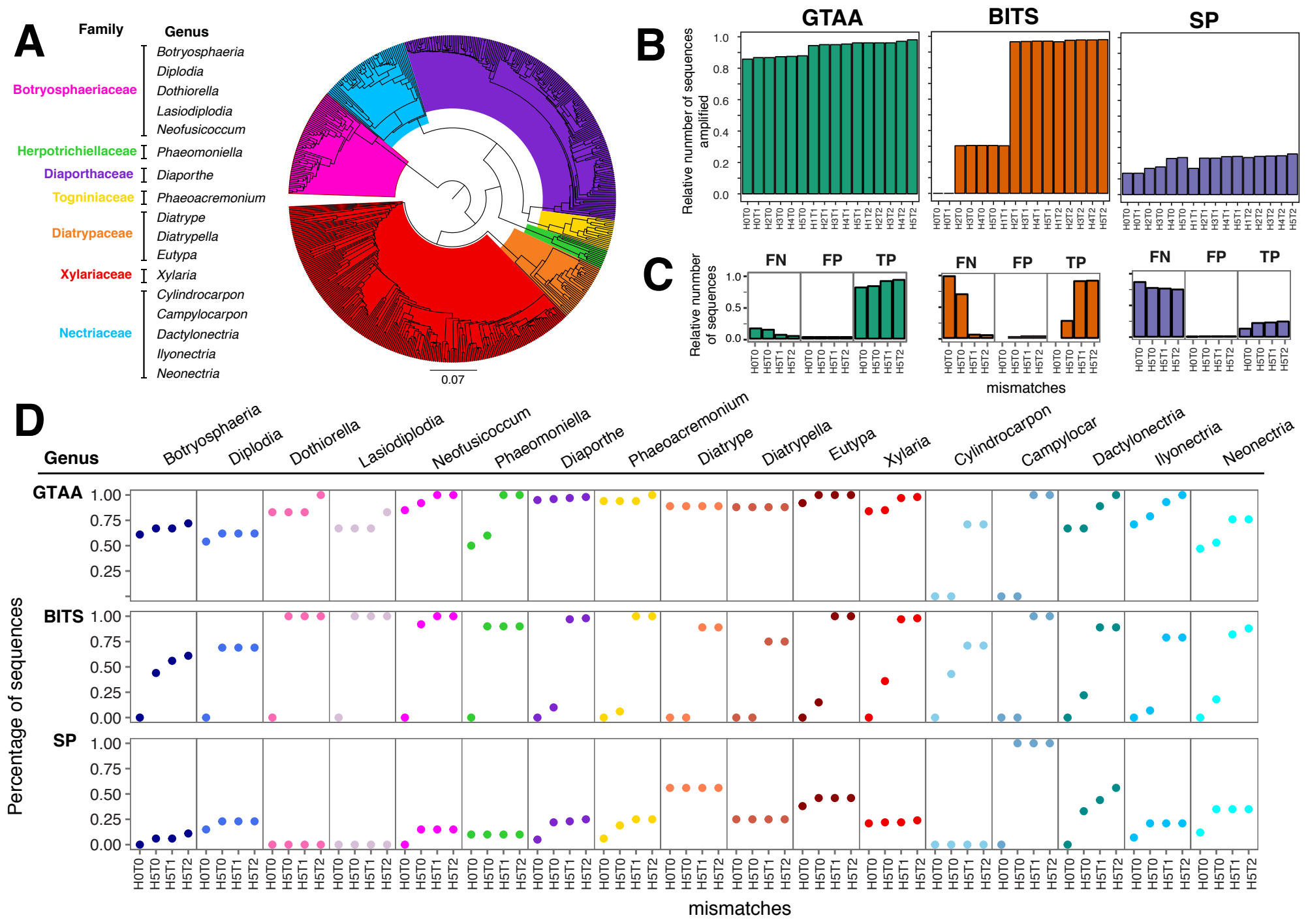
716 **Additional File 1: Text S1 (FASTA).** Assembled amplicon sequences produced by the GTAA
717 primers and sequenced with Sanger.

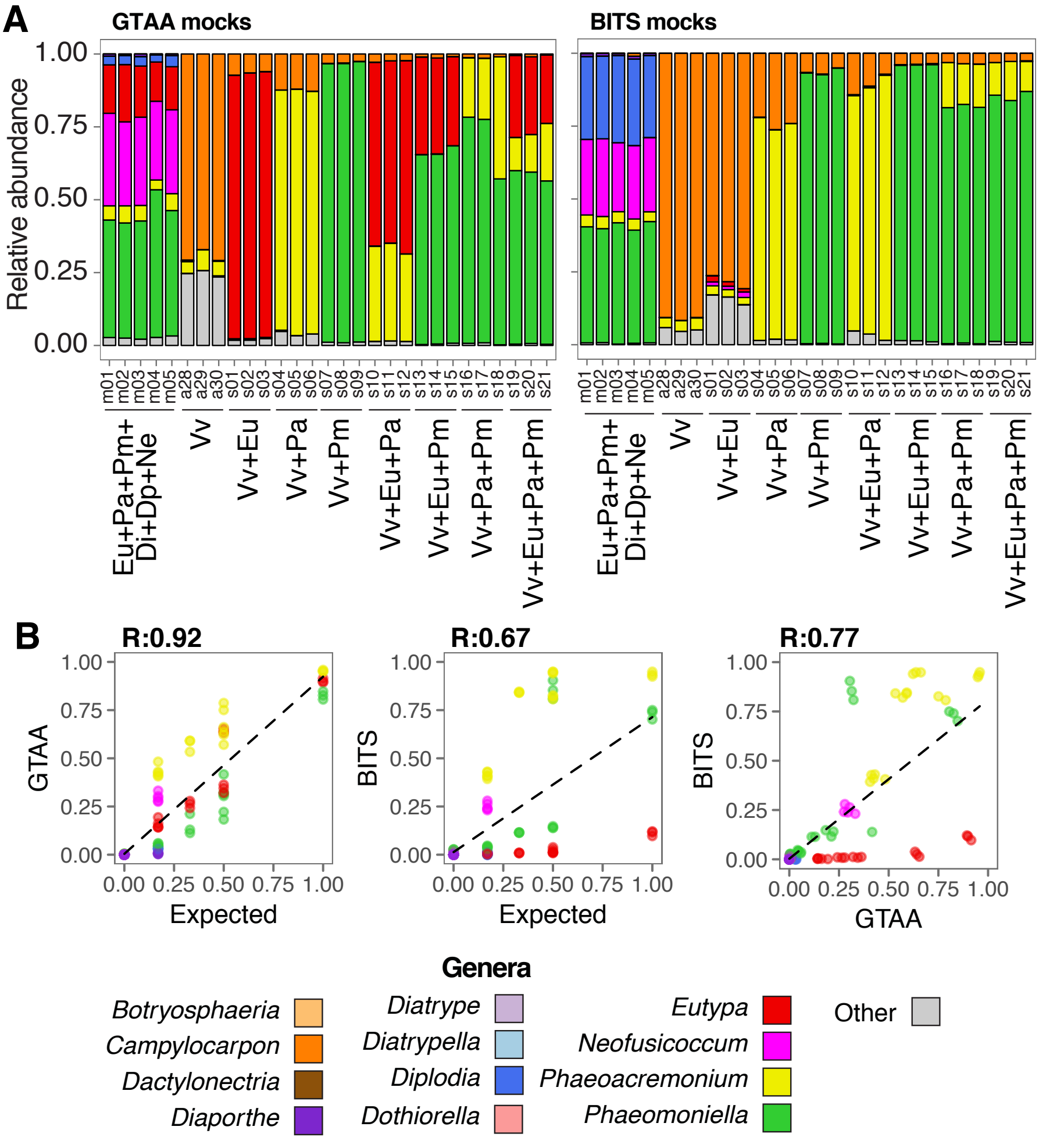
718 **Additional File 2: Table S1 (XLSX).** List of filtered sequences retrieved from the NCBI used as
719 a database for primer testing and test results per primer. TP: True Positive, FP: False Positive and
720 FN: False Negative.

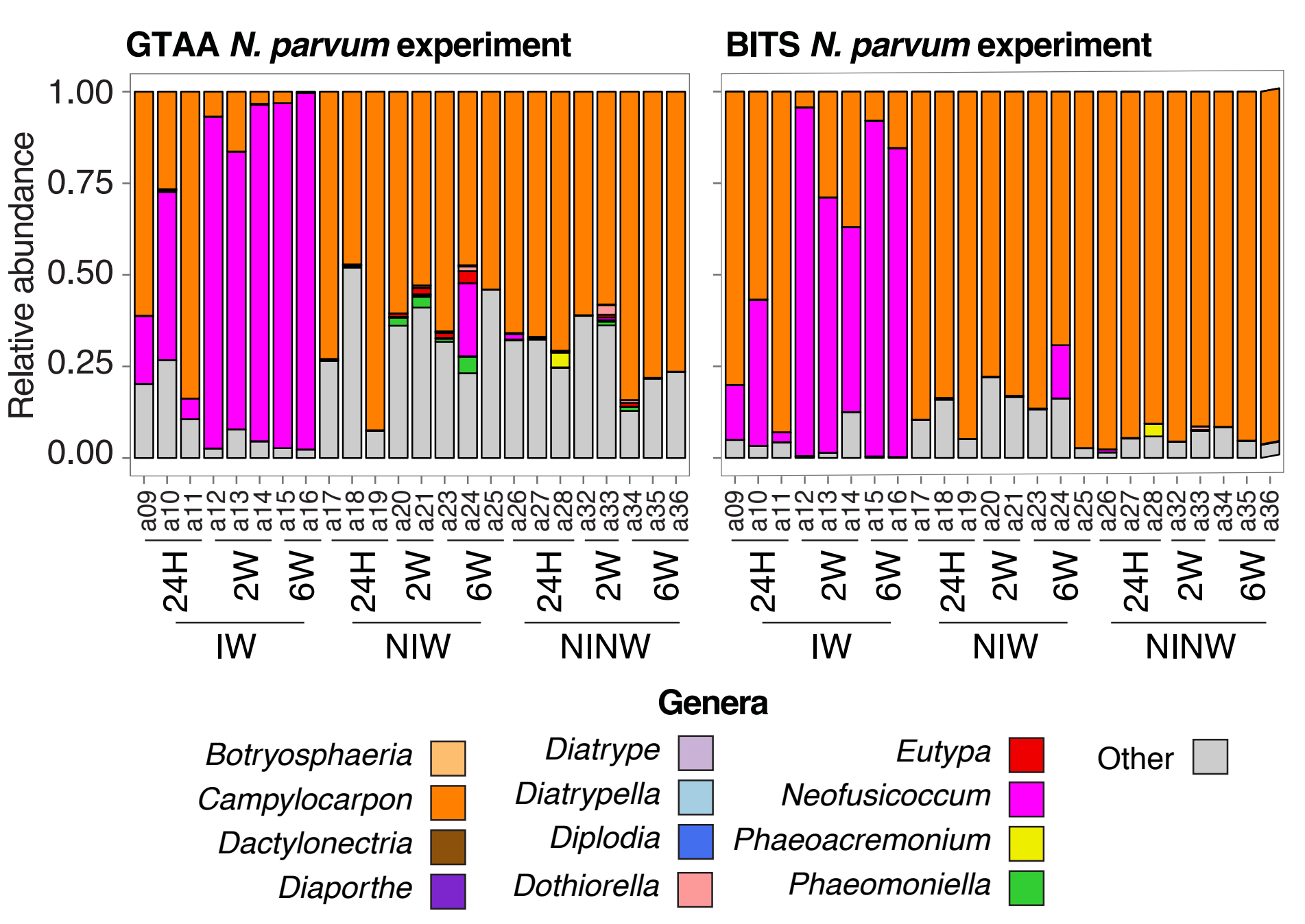
721 **Additional File 3: Figure S1 (PDF).** Venn diagram of genera detected by the GTAA and BITS
722 primers from the field samples detected with more than 0.05% abundance per sample.

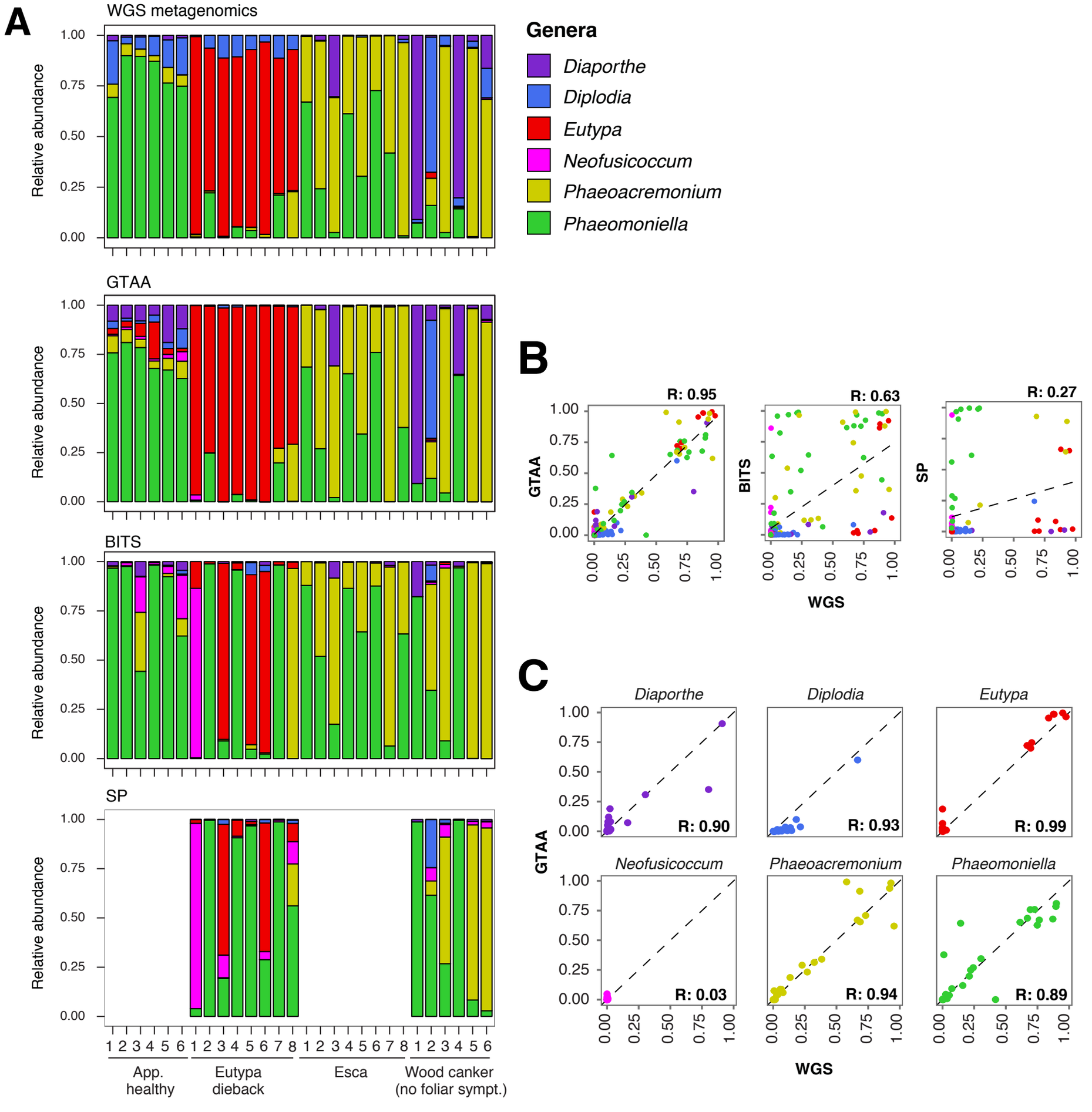
723 **Additional File 4: Table S2 (XLSX).** List of forward GTAA primers with linker and barcodes


A
ITS region of Eutypa lata (KU320617.1)
SSU
ITS1
5.8 S
ITS2
LSU
GTAA
340 bp
BITS
230 bp
SP
305 bp
0
200
400
600
B
Ladder
E. lata
P. minimum
D. seriata
N. parvum
P. chlamydospora
D. ampelina
A. tumefaciens
V. vinifera
H2O
17,000 bp
500 bp
300 bp
50 bp
C
Ladder
AH1
AH3
AH4
ED5
ED8
ES6
ES8
WC3
WC4
H2O
D
IW6WB4
IW6WC1
L
5 10 15 20 25 30 35
5 10 15 20 25 30 35
GTAA
BITS
SP
1,000 bp
500 bp
100 bp
E
Primer
GTAA
BITS
SP
Ct value
30
20
10
0
a
b
b
a
b
b
IW6WB4
IW6WC1
Sample


A
Family
Genus
Botryosphaeriaceae
Botryosphaeria
Diplodia
Dothiorella
Lasiodiplodia
Neofusicoccum
Herpotrichiellaceae
Phaeomoniella
Diaporthaceae
Diaporthe
Togniniaceae
Phaeoacremonium
Diatrypaceae
Diatrype
Diatrypella
Eutypa
Xylariaceae
Xylaria
Nectriaceae
Cylindrocarpon
Campylocarpon
Dactylonectria
Ilyonectria
Neonectria
0.07
B
GTAA
BITS
SP
Relative nunmber of sequences amplified
1.0
0.8
0.6
0.4
0.2
0.0
H0T0 H0T1 H2T0 H3T0 H4T0 H5T0 H1T1 H2T1 H3T1 H4T1 H5T1 H1T2 H2T2 H3T2 H4T2 H5T2
C
FN
FP
TP
Relative nunmber of sequences
1.0
0.5
0.0
H0T0 H5T0 H5T1 H5T2
mismatches
D
Genus
Botryosphaeria
Diplodia
Dothiorella
Lasiodiplodia
Neofusicoccum
Phaeomoniella
Diaporthe
Phaeoacremonium
Diatrype
Diatrypella
Eutypa
Xylaria
Cylindrocarpon
Campylocar
Dactylonectria
Ilyonectria
Neonectria
GTAA
BITS
SP
1.00
0.75
0.50
0.25
0.00
Percentage of sequences
H0T0 H5T0 H5T1 H5T2
mismatches


A
GTAA mocks
BITS mocks
Relative abundance
1.00
0.75
0.50
0.25
0.00
m01 m02 m03 m04 m05 a28 a29 a30 s01 s02 s03 s04 s05 s06 s07 s08 s09 s10 s11 s12 s13 s14 s15 s16 s17 s18 s19 s20 s21
Eu+Pa+Pm+ Di+Dp+Ne
Vv
Vv+Eu
Vv+Pa
Vv+Pm
Vv+Eu+Pa
Vv+Eu+Pm
Vv+Pa+Pm
Vv+Eu+Pa+Pm
B
R:0.92
R:0.67
R:0.77
GTAA
BITS
BITS
Expected
Expected
GTAA
0.00 0.25 0.50 0.75 1.00
Genera
Botryosphaeria
Campylocarpon
Dactylonectria
Diaporthe
Diatrype
Diatrypella
Diplodia
Dothiorella
Eutypa
Neofusicoccum
Phaeoacremonium
Phaeomoniella
Other


GTAA N. parvum experiment
BITS N. parvum experiment
Relative abundance
1.00
0.75
0.50
0.25
0.00
a09 a10 a11 a12 a13 a14 a15 a16 a17 a18 a19 a20 a21 a23 a24 a25 a26 a27 a28 a32 a33 a34 a35 a36
24H 2W 6W 24H 2W 6W 24H 2W 6W
IW NIW NINW
a09 a10 a11 a12 a13 a14 a15 a16 a17 a18 a19 a20 a21 a23 a24 a25 a26 a27 a28 a32 a33 a34 a35 a36
24H 2W 6W 24H 2W 6W 24H 2W 6W
IW NIW NINW
Genera
Botryosphaeria
Campylocarpon
Dactylonectria
Diaporthe
Diatrype
Diatrypella
Diplodia
Dothiorella
Eutypa
Neofusicoccum
Phaeoacremonium
Phaeomoniella
Other


A
WGS metagenomics
GTAA
BITS
SP
Relative abundance
1.00
0.75
0.50
0.25
0.00
Genera
Diaporthe
Diplodia
Eutypa
Neofusicoccum
Phaeoacremonium
Phaeomoniella
1 2 3 4 5 6 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6
App. healthy
Eutypa dieback
Esca
Wood canker (no foliar sympt.)
B
R: 0.95
R: 0.63
R: 0.27
GTAA
BITS
SP
WGS
C
Diaporthe
Diplodia
Eutypa
Neofusicoccum
Phaeoacremonium
Phaeomoniella
R: 0.90
R: 0.93
R: 0.99
R: 0.03
R: 0.94
R: 0.89
GTAA
WGS