Rotstop nanopore detection - testing for live *Phlebiopsis*

Report for Anna & Nils Håkanssons foundation/Partnerskap Alnarp (PA1277)

Abstract

Heterobasidion root rots are a highly damaging and costly disease of conifers that require proper control procedures to limit their damage. RotStop is a commercial biocontrol product that can be applied to cut stems to reduce stump infection by Heterobasidion species by instead promoting colonization by *Phlebiopsis gigantea*, a non-pathogenic saprotrophic wood decay fungi. The efficacy of RotStop treatment depends on its proper handling and application, and there are concerns that non-viable product is being applied. This study examined if RNA from RotStop could be detected from the sawblade-discharges of harvesting equipment used in the field to treat stumps using nanopore sequencing, in order to determine if the biocontrol agent was alive at the time of treatment. RNA matching *P. gigantea* was present in the field-collected samples, indicating that contractors had properly maintained the control agent. However, the RNA matches were not robust mostly due to short read lengths. The quality of the RNA extracted from field samples and of RotStop gel were poor, regardless of the methods used. However, RNA extracted from *P. gigantea* cultures was excellent, suggesting that the tested methods were functional for the organism, but unsuitable for the commercial product and field samples. This may be because of interfering components in the product, or because the P. gigantea spores in the product are in a stasis with little RNA present. Future will need to improve the RNA quality before nanopore sequencing can be implemented in a practical sense.

Introduction

In the face of worsening climate change, countries must strive towards a more sustainable bioeconomy, with forestry providing a fundamental contribution to a sustainable future. In Sweden, forestry has long been an integrated and essential part of the national economy; however, there are threats to sustainable wood production. Norway spruce (*Picea abies*) is the most common tree species in Sweden, and approximately 25% of all mature trees are infected by root rot. Root and butt rot in Norway spruce, mainly caused by *Heterobasidion* spp., leads to downgrading of timber, increased risk of wind throw and growth loss, resulting in severe financial losses to the Scandinavian forestry sector (Hodges 1999). The spores of *Heterobasidion* enter conifer stands primarily through the stumps created during thinning operations during the growing season (Brandtberg, Johansson & Seeger 1996; Rishbeth 1951a). The fungus spreads subsequently by mycelial growth to adjacent trees via root grafts and contacts (Rishbeth 1951b). The spread of *Heterobasidion* spp. can be reduced if a control treatment is applied to surfaces of freshly cut stumps (Rishbeth 1952, 1957). The saprophytic fungus *Phlebiopsis gigantea* and chemicals such as urea or borates are efficient stump treatment agents (Kallio 1971; Korhonen et al. 1993; Rishbeth 1963). In Sweden, *P. gigantea* has been the most frequently used agent for stump treatment (Thor 2003) and from 2017 is the only proven agent that can be legally used.

Several studies have demonstrated the long- and short-term efficacy of treating stumps with *P. gigantea*, using different spore concentrations of *P. gigantea*, the need to have a certain coverage of *P. gigantea* on the stump surface, and the survival of *P. gigantea* spores due to high pressure during application(Berglund & Rönnberg 2004; Korhonen et al. 1993; Oliva, Bendz-Hellgren & Stenlid 2011;

Thor, Bendz-Hellgren & Stenlid 1997). Preliminary results from a recent study investigating the efficacy from practical stump treatments indicate though that treatment is not perfect in real operations and the consequent result is that the effect on root rot might be negatively affected. There are several potential reasons for this, but because *P. gigantea* is a living biological agent, it requires certain handling procedures to survive application and be effective. Unofficial sources report a mishandling of the product, meaning there is a risk that not all stumps being treated are treated with live material. There is, therefore, great need for a method to reliably test for the presence of living *P. gigantea* in what is sprayed on the stumps in the forest.

Although the presence of *P. gigantea* in the treatment spray can be ascertained easily by looking for its DNA, this method would not determine whether the organism is alive, because DNA is relatively stable and can persist well after cell death. RNA can only be produced and maintained by living cells, making it the better nucleic acid to target for determining the viability of the *P. gigantea* spores in the treatment sprays. RNA is considerably less stable than DNA, degrades easily and is traditionally more expensive and complicated to sequence. However, recent advances in nanopore sequencing technology (i.e., the MinION sequencer from Oxford Nanopore Technology, ONT) allows for direct, rapid and portable sequencing of DNA and RNA and gives real-time results. The MinION sequencer is extremely promising as a plant disease diagnostic tool, and it has already been successfully used in a limited number of pathosystems, but these examples are largely under controlled laboratory settings and concerning agricultural systems and have almost exclusively focused on DNA detection rather than RNA. More robust tests of MinION's efficacy in forest systems and under in-field conditions are needed. Further development of this tool would be useful for foresters who need to get an answer within a short time at a low cost so that they can adjust their operating procedures if something is wrong with the treatment.

This project utilized ONT's MinION sequencer to determine if tree logging machinery in the field is actually applying living biocontrol agent *P. gigantea* as determined the presence of *P. gigantea* RNA in samples collected directly from cutting machines. RNA from the commercial product was also obtained and sequenced under laboratory conditions to test and develop RNA-extraction methods, sequencing protocols, and bioinformatical approaches for data analysis.

Materials and Methods

Sample Preparation

Rotstop S gel (Interagro Skog AB), stored at -20 °C, was thawed to room temperature and thoroughly mixed under sterile conditions prior to use. The gel was used to create the following samples types for RNA extraction: (1) undiluted gel (henceforth RSg), (2) gel diluted to manufacturer's recommended field strength of 1 g gel ddH_2O L^{-1} (1xRS), (3) gel diluted to field strength then frozen with liquid N (1xRS-F), and (4) *P. gigantea* mycelia (Pg). After diluting the gel, the 1xRS and 1xRS-F solutions were left at room temperature for 1 hour before proceeding to mimic sitting in a harvester tank; both solutions were then aliquoted into 50 mL tubes. 1xRS tubes were centrifuged at 4000 rcf to form a pellet that was used for subsequent RNA extractions. 1xRS-F tubes were immediately frozen in liquid N and stored at -80 °C overnight to mimic field samples (explained below). The 1xRS-F samples were then thawed in a 25 °C water bath before centrifugating like with the 1xRS samples. *Phlebiopsis gigantea* mycelia was grown from the gel by adding 0.1 mL of the gel to 40 mL of malt extract broth. Cultures were grown for 14 days in the dark under constant, gentle shaking (200 rpm). Mycelia was collected and pulverized to a fine powder in a mortar using liquid N and a pestle.

Field samples were collected from three different single grip harvesters at three locations, Virestad, Ljungby, and Vänneböke in Sweden on October 15, 2020. Drippings from the harvesting sawblade were collected in plastic bag placed under the blade while operators engaged the pump to expel the RotStop suspension from the tank. The box was stirred and the liquid put into multiple 50 mL vials using a 50 mL syringe. Vials were immediately frozen in liquid N and placed on dry ice until they could be stored in -80 °C freezer. Field samples were then thawed in a 25 °C water bath and centrifuged like above.

RNA from the RotStop gel was extracted using four separate methods, three intended for total RNA extractions and one for messenger RNA. The first method was a hot acid phenol method (henceforth TES method) derived from Collart and Oliviero (1993). For this method, tissue pellets were resuspended in 400 µl of ice cold TES solution (10 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0, and 0.5 % SDS) before adding 400 µl of acid-phenol:chloroform, pH 4.5 with isoamylalcohol (125:24:1) and vortexting vigorously. The tissue was incubated at 65 °C for 60 min with occasional, brief vortexing. Tubes were placed on ice for 5 min before centrifugation at 4 °C at 16k rcf. The top layer was transferred to a new tube, 400 µl of acid phenol was added and tubes vortexed. Tubes were placed on ice, centrifuged and the top layer transferred to a new tube like before. One tenth volume of 3 M sodium acetate pH 5.3, 2.5 volumes of ice cold absolute ethanol, and 1 µl of RNA grade glycogen (20 mg/mL stock) were added to each tube which were then vortexted and stored at -20 °C overnight. Tubes were centrifuged at 4 °C for 30 min at 12,000 rcf and the supernatant removed. Pellets were washed twice in 70% ethanol in nuclease free water by centrifuging at 4 °C for 10 min at 12k rcf. Pellets were resuspended in nuclease free water. The second extraction method used Sigma's RNAzol® RT following the manufacturer's protocol, except that 1 µl of RNA grade glycogen was added to each tube during the isopropanol precipitation step. The third method used OMEGA Bio-Tek's E.Z.N.A.® Plant RNA Kit and the fourth method used Invitrogen's Dynabeads mRNA DIRECT kit, but no RNA was ever recovered using either of these methods and they were not considered viable for these sample types. RNA from the field samples was extracted using only the RNAzol® RT method described above. RNA extracts were treated with Turbo DNAse following manufacturer's instructions and reactions cleaned up using AMPure XP beads.

RNA extracts were quantified using Qubit High Sensitivity RNA assays and purities determined by examining the 260/280 nm and 260/230 nm absorbance ratios on a Denovix DS-11 FX spectrophotometer. RNA quality was assessed on an Agilent Bioanalyzer 2100 using Agilent RNA 6000 Pico Kit.

Nanopore sequencing

RNA from the RotStop gel samples were run RNA was prepared for nanopore sequencing following the PCR-cDNA Barcoding protocol (SQK-PCB109). PCR amplification was done for 14 cycles with a 4 min extension step, and reactions were cleaned up with an AMPure XP bead to sample ratio of 1.5. For each sample, 1.4 ng of cDNA was pooled together to make a 100 fmol amplified cDNA barcoded pool; this assumed a 200 bp product. Sequencing runs were performed on a MinION MK1C using R9.4.1 flow cells running MinKNOW 21.11.7. Sequencing reactions were run for 48 hr using default settings on the MK1C device for voltage and using fast basecalling.

Data Analysis

A reference database was generated in EPI2ME using Fasta Reference Upload (ver.2022.05.20-14092) with the *P. gigantea* genome (Hori et al. 2014), *Heterobasidion annosum* (Olson et al. 2012), and *Botrytis cinerea* (Amselem et al. 2011) available on JGI Genome Portal. Fastq custom alignments to the reference

databases were done using EPI2ME (using ver.2021.11.26) using a minimum Q-score of 10 and minimum read length of 500 bp. To assess non-*P. gigantea* alignment matches, alignment of the data sets to a custom mixed species reference database was done using the Fastq WIMP analysis (ver.2021.11.26) in EPI2ME with the same Q score and read length requirements as above. To identify what our sequence data was aligning to, the relevant aligned reference sequence data was queried against taxa Fungi (taxid:4751) using NCBI BLASTn to find the best identified match to other fungi.

Results

RotStop gel samples

RNA quality of the Rotstop gel samples varied by sample, but overall was low quality. Electropherograms from the Bioanalyzer showed copious degradation of RNA from all but the two Pg samples. Pg samples have distinct 18S and 28S peaks and high quality. The other samples from the gel have a large number of peaks or humps between 20 and 30 sec and no clear 18S or 28S peaks. Marker peaks and ladder runs appear as expected.

The sequencing run for the RotStop gel samples generated 11.43 million reads of 2.5 Gbases with an average Q score of 12.2 and an average sequence length of 216 bases. Results for each sample are provided in Table 1. No stark differences are apparent between the two RNA extraction methods for any sample pair when examining the average read length and Q scores, except for the 1xRS samples, where the TES method gave RNA fragments more than twice as long on average compared to the RNAzol method. For the 1xRS samples, the TES method also nearly twice as many reads, resulting in a four times greater Mbase yield. All of the gel samples have mostly similar metrics (the aforementioned 1xRS TES sample notwithstanding). However, the Pg samples had higher average read lengths compared to the other samples, but the two RNA extraction methods were comparable in terms of number of reads, average Q scores. Unclassified reads, *e.g.*, those not assigned a barcode, were less abundant, had lower Q scores but were longer than most samples, except the Pg samples.

Table 1 Quality control data from RotStop gel samples

Sample	Total Reads	Total Yield (Mbases)	Average Q score	Average Length (nt)
RSg Zol[1]	2 029 920	206.3	13.0	101
RSg TES[2]	1 068 199	154.5	12.0	144
1xRS Zol	764 444	111.7	12.6	146
1xRS TES	1 519 436	474.5	11.9	312
1xRS-F Zol	1 067 411	117.0	12.5	109
1xRS-F TES	2 138 369	305.0	12.8	142
Pg Zol	909 016	403.8	11.5	444
Pg TES	1 213 309	463.4	11.6	381
unclassified	509 527	202.7	9.4	397

[1]Zol = RNA extacted with RNAzol reagent

[2]TES = RNA extracted using modified Collart and Oliviero (1993) method

Of the 10.71 mil reads (not including unclassified reads), 941 853 met the criteria for alignment, with most excluded reads being below the 500 bp threshold. Of the successful reads, 764 347 reads (81%) were aligned to the *P. gigantea* genome (Table 2). The TES extracted samples tended to have more

successful reads than the RNAzol extracted samples in all sample types, except Pg samples which were nearly equivalent. Both Pg samples and the 1Xrs TES samples had much higher total and successful reads compared to the other samples. All samples had similar average identities. There are only minor differences between the 1xRS and 1xRS-F samples of a given extraction method. All samples either aligned most frequently to KN840936.1 or KN840476.1, which correspond to mitochondrial DNA and an unknown genetic component, respectively. The average accuracy of the alignments to KN840936.1 was higher than to KN840476.1.

Table 2 Alignment results for RotStop gel samples to *Phlebiopsis gigantea* genome

Sample	Total Reads Analyzed	Successful Reads	Alignment Seq ID[1]	Reads Aligned to Seq ID	Average Alignment Length	Average Identity	Average Accuracy	Best Matching Accession	BLASTn match[2]
RSg Zol[3]	20 006	8120	KN840936.1	3704	542	96.7	89.5	LC707859.1	Mitochondrion
RSg TES[4]	31 002	14 650	KN840936.1	2469	548	96.7	89.7	LC707859.1	Mitochondrion
1xRS Zol	15 775	2581	KN840936.1	762	537	96.7	89.5	LC707859.1	Mitochondrion
1xRS TES	259 475	218 160	KN840476.1	19 407	533	96.5	77.3	-	No match
1xRS-F Zol	12 087	5716	KN840936.1	2340	545	96.7	89.5	LC707859.1	Mitochondrion
1xRS-F TES	68 991	33 268	KN840936.1	5554	545	96.7	89.8	LC707859.1	Mitochondrion
Pg Zol	257 010	242 360	KN840476.1	20 304	536	96.5	77.3	-	No match
Pg TES	277 507	239 492	KN840476.1	16 568	537	96.5	77.2	-	No match
Total[5]	941 853	764 347	-	-	-	-	-	-	-

[1]only the most frequently aligned match is show

[2]based on E-score of reference genome to all fungi using standard nucelotide basic local alignment search tool

[3]Zol = RNA extacted with RNAzol reagent

[4]TES = RNA extracted using modified Collart and Oliviero (1993)

[5]not including unclassified reads

When aligning the RotStop gel data to non-target fungal genomes, 59 067 of the 941 853 successful reads were aligned (6%), considerably lower than the amount that aligned to the *P. gigantea* genome. Most of these successful reads belonged to the two Pg samples and to 1xRS TES. The majority of the samples aligned most frequently to a small ribosomal gene of scaffold_09 of the *H. annosum* reference genome, while Pg Zol and 1xRS TES aligned most frequently to a hypothetical protein in scaffold_01. The alignment lengths for the hypothetical proteins are longer but less accurate than the alignments for the ribosomal gene.

Table 3 Alignment results for RotStop gel samples to non-target fungal genomes

Sample	Total Reads Analyzed	Successful Reads	Alignment Seq ID[1]	Reads Aligned to Seq ID	Average Alignment Length	Average Identity	Average Accuracy	Best Matching Accession	BLASTn match[2]
RSg Zol[3]	20 006	406	scaffold_09	287	510	93.4	87.4	MK500942.1	Small ribosome
RSg TES[4]	31 002	878	scaffold_09	437	578	93.4	87.5	MK500942.1	Small ribosome
1xRS Zol	15 775	356	scaffold_09	240	572	93.6	87.3	MK500942.1	Small ribosome
1xRS TES	259 475	17 512	scaffold_01	3789	839	85.7	69.1	XM_009542554.1	Partial hypothetical protein
1xRS-F Zol	12 087	546	scaffold_09	374	530	93.2	87.1	MK500942.1	Small ribosome
1xRS-F TES	68 991	1338	scaffold_09	642	555	93.7	87.8	MK500942.1	Small ribosome
Pg Zol	257 010	17 186	scaffold_01	4251	861	85.6	68.7	XM_009542554.1	Partial hypothetical protein
Pg TES	277 507	20 845	scaffold_09	4895	725	93.7	87.6	MK500942.1	Small ribosome
Total[5]	941 853	59 067	-	-	-	-	-	-	-

[1]only the most frequently aligned match is show

[2]based on E-score of reference genome to all fungi using standard nucelotide basic local alignment search tool

[3]Zol = RNA extacted with RNAzol reagent

[4]TES = RNA extracted using modified Collart and Oliviero

[5]not including unclassified reads

Field samples

As with the RotStop gel samples, RNA quality from the field samples was poor. Electropherograms from the Bioanalyzer show strong degradation of RNA, particularly of the samples from Skär, as evidenced by the large number of peaks and elevated baseline between 20 and 30 sec and the peak corresponding to the 28S is very small or missing from the samples. Marker peaks and ladder runs appear as expected.

The sequencing run for the field samples generated 5.28 million reads of 796 Mbases with an average Q score of 12.6 and an average sequence length of 151 bases. Sequencing metrics are generally similar between replicates for a site and between the sites (Table 3). All sites had an average Q score greater than 13 and a read length of approximately 100 nt, except Skär samples which were a bit shorter. Unclassified reads comprised about 30% of the total reads and had much lower Q scores on average, but were over two times longer than the reads assigned a barcode.

Table 4 Quality control data from field samples

Sample	Total Reads	Total Yield (Mbases)	Average Q score	Average Length (nt)
Skär 1	546,942	50.4	13.5	92
Skär 2	531,238	41.9	13.8	78
Skär 3	431,006	36.3	13.9	84
Ljun 1	426,818	45.3	14.1	106
Ljun 2	374,769	38.4	13.9	102
Ljun 3	279,139	30.9	13.5	110
Vänneböke 1	272,231	26.6	14.2	97
Vänneböke 2	476,762	51.3	14.0	107
Vänneböke 3	362,008	30.9	14.2	85
unclassified	1,579,046	444.4	9.6	281

Only 37 739 of the 3.70 million total reads met the criteria for alignment (1.0%), with most failing because of inadequate length (Table 5). Of these reads, 1402 (3%) aligned to the *P. gigantea* genome. The successful field samples overwhelmingly aligned to scaffold 529 (Genbank ID KN840966.1) and scaffold 92 (Genbank ID KN840531.1) of the reference *P. gigantea* genome, with 82% of all successful reads aligning to these two regions. Both scaffold regions correspond to 18S ribosomal genes of the

same Genbank accession. Accuracies of the alignments were moderately better for KN840966.1 than KN840531.1, but reads to KN840966.1 were shorter and less abundant.

Table 5 Alignment results of field samples to *Phlebiopsis gigantea* genome

Sample	Total Reads Analyzed	Successful Reads	Alignment Seq ID[1]	Reads Aligned to Seq ID	Average Alignment Length	Average Identity	Average Accuracy	Best Matching Accession	BLASTn match[2]
Skär 1	3280	82	KN840966.1	61	388	90.8	85.5	AB809163.1	18S ribosome
Skär 2	2740	100	KN840966.1	68	397	90.6	85.9	AB809163.1	18S ribosome
Skär 3	2821	51	KN840966.1	31	351	91.2	85.9	AB809163.1	18S ribosome
Ljun 1	4566	324	KN840531.1	259	593	84.7	78.8	AB809163.1	18S ribosome
Ljun 2	4457	239	KN840531.1	175	586	84.8	78.6	AB809163.1	18S ribosome
Ljun 3	3705	190	KN840531.1	139	599	84.6	78.8	AB809163.1	18S ribosome
Vänneböke 1	4340	119	KN840966.1	44	377	90.4	84.7	AB809163.1	18S ribosome
Vänneböke 2	8528	190	KN840966.1	66	380	90.8	85.2	AB809163.1	18S ribosome
Vänneböke 3	3302	107	KN840966.1	44	369	90.2	84.3	AB809163.1	18S ribosome
Total[4]	37 739	1402	-	-	-	-	-	-	-

[1]only the most frequently aligned match is show

[2]based on E-score of reference genome to all fungi using standard nucelotide basic local alignment search tool

[3]unclassified reads could not be assigned a barcode

[4]not including unclassified reads

Field samples had 1924 reads of the 37 739 total reads (5%) successfully align to the non-target fungal genomes which was higher than the amount aligned to the *P. gigantea* genome. Supercontig 1.189 of the *B. cinerea* reference genome, which corresponds to a 5S ribosomal gene, was the most commonly aligned to region overall and for each sample specifically (Table 6). Approximately 32% of all successful reads aligned there. Average identities and accuracies of the alignments were moderately higher for the Skär and Vänneböke samples than the Ljun samples. Average alignment lengths were longer for alignments to the *B. cinerea* genome than the *P. gigantea* genome.

Table 6 Alignment results for field samples to non-target fungal genomes

Sample	Total Reads Analyzed	Successful Reads	Alignment Seq ID[1]	Reads Aligned to Seq ID	Average Alignment Length	Average Identity	Average Accuracy	Best Matching Accession	BLASTn match[2]
Skär 1	3280	112	Supercontig_1.189	41	712	90.1	84.1	MK757685.1	5S ribosome
Skär 2	2740	130	Supercontig_1.189	42	645	90.7	85.5	MK757685.1	5S ribosome
Skär 3	2821	69	Supercontig_1.189	18	708	89.6	83.2	MK757685.1	5S ribosome
Ljun 1	4566	465	Supercontig_1.189	191	715	85.8	78.9	MK757685.1	5S ribosome
Ljun 2	4457	361	Supercontig_1.189	137	711	85.6	78.4	MK757685.1	5S ribosome
Ljun 3	3705	270	Supercontig_1.189	105	792	85.3	77.9	MK757685.1	5S ribosome
Vänneböke 1	4340	134	Supercontig_1.189	35	653	88.0	81.3	MK757685.1	5S ribosome
Vänneböke 2	8528	225	Supercontig_1.189	57	548	88.3	81.8	MK757685.1	5S ribosome
Vänneböke 3	3302	158	Supercontig_1.189	43	644	87.7	82.1	MK757685.1	5S ribosome
Total[4]	37 739	1924	-	-	-	-	-	-	-

[1]only the most frequently aligned match is show

[2]based on E-score of reference genome to all fungi using standard nucelotide basic local alignment search tool

[3]unclassified reads could not be assigned a barcode

[4]not including unclassified reads

Discussion

This study was the first attempt at developing a tool for detecting living biocontrol agent *P. gigantea* from treatment tanks on harvesting equipment. The results presented here are inconclusive as to whether field collected samples contained living *P. gigantea*. Interpretations of the results are hindered by the overall short read lengths, a poorly annotated reference genome, and low alignment accuracies that matched best to genomics regions that are not particularly informative for identification purposes. These issues mostly result from the overall poor RNA quality used for sequencing. Despite trying several different methods, only two methods were capable of yielding any amount of RNA, but neither were very successful in terms of yield or quality, for the field samples or the majority of the gel samples.

There were only roughly half as many total reads for the field samples as the gel samples. Starting inputs for the library preparation were the same, so the reason for this is not entirely clear but could be a result of difference in the quality of the flow cells. The proportion of reads aligning to the *P. gigantea* genome were also different between the gel and field samples. Only 3% of the acceptable field sample reads aligned, while 81% of the gel sample reads aligned. This difference could be because the field samples are not pure and contain many contaminating microbes. Indeed, after centrifugation before adding RNAzol, it was apparent that the field samples were feculent, particularly the Skär samples. This sediment almost certainly contained other microbes and microscopic organisms that diluted the presence of RotStop-derived *P. gigantea* RNA. This is supported by the result that more of the field reads aligned to the non-target fungal genomes. However, 6% of the RotStop gel reads aligned to the non-target genomes as well, so some non-specificity of the genomic regions is expected. It is also possible that no living RotStop gel was added to the harvester water tanks (or that it was obliterated by the pressurized pumping system), so no or very few *P. gigantea* reads would be present. We did not have a no RotStop negative control for field samples, so this cannot be conclusively determined. It is also possible that natural-occurring *P. gigantea* could be in the field samples, as it is a common fungal species in northern hemisphere temperate conifer forests (Käärik & Rennerfelt 1958; Menkis et al. 2012; Petäistö 1978). The water sources for the tanks on the different harvester are unknown, but it is possible, yet not likely, they are filled with nearby contaminated pond water.

The issue of unspecific regions of the genome is difficult to overcome with metagenomic datasets like this. When looked at in isolation, many parts of an organism's genome are not helpful for identification or taxonomic purposes because the genetic sequences are not distinct enough to make robust demarcations. Nanopore sequencing mitigates this complication by obtaining long length reads that span large portions of the genome or transcriptome. Unfortunately, this advantage was lost for this project because of the short average bp length of the RNA obtained.

Ideally, the sequencing would have been performed using messenger RNA (mRNA) rather than total RNA, but because mRNA constitutes only a small percentage of total RNA, it is more difficult to extract. mRNA is used for active protein translation and is readily synthesized and degraded by living cells, while rRNA is a major structural component of ribosomes and persists longer (Alberts et al. 2002). Therefore, mRNA can be considered a better target molecule for determining whether something is alive or dead. We intended to use mRNA for sequencing, but only total RNA extraction protocols could isolate RNA from the RotStop gel. RNA yields were too low and of too low quality to attempt mRNA enrichment. Future efforts should consider using a metabarcoding approach to enrich for genetic regions useful for identification purposes like the internal transcribed spacer (ITS), which is a DNA fragment situated the

small and large ribosomal subunits. The ITS is commonly used for identifying fungi (Schoch et al. 2012). While many of the reads from this study matched to ribosomal genes, it is not known if they contained the ITS region or not. During rRNA maturation, the ITS region is excised and rapidly degraded, so it may not have survived the RNA extraction protocol.

Although a comprehensive comparison of the TES method and RNAzol methods was beyond the intent of this study, the two methods appear to perform similarly in regards to RNA quality and sequencing output. The RNAzol method is considerably faster, easier, and requires less equipment, so it is preferable to the TES method when considering use in a field setting. However, neither method yielded high quality RNA for any of the gel or field samples, except for the *P. gigantea* mycelia, which were excellent. This indicates that while these two methods were conducted properly and are suitable for more common tissue samples (like mycelia), they are not sufficient for more difficult samples types like the RotStop gel and water-collected samples. Further testing of other methods is necessary to improve the quality of the RNA from the gel and field samples. For the sequencing output, the biggest differences between the two methods were the number of reads. However, since the cDNA of each sample is pooled into one library based on fmol amounts, which is estimated using the same average 200 bp length for all samples, some differences in read counts is not unexpected. Since the RNA was degraded for most samples, it is difficult to gauge how much of the differences in reads is due to differences in fragment lengths or poor library construction. Freezing and thawing of the field samples may have partially degraded the RNA by causing cell lysis before RNA can be protected by the extraction buffers, but this effect is likely minimal since there were no clear differences between the sequencing statistics of the 1xRS and 1xRS-F gel samples. Nonetheless, future efforts should consider filtering the liquid field samples and putting the filtrate into RNA storage solutions directly before returning to the lab.

The RNA from the gel samples most regularly aligned to an unknown genetic region and to a mitochondrial gene. These alignment regions were not reflected in the field samples, where the majority of field reads aligned to regions corresponding to ribosomal genes. Matches to ribosomal genes are to be expected because total RNA extracts were used for cDNA synthesis, and approximately 80-90% of RNA present in a cell is rRNA (Alberts et al. 2002). Field samples did have a very small number of reads align to the same unknown region and mitochondrial gene, and the gel samples also had reads align to the two rRNA genes from the field samples. It is not clear why the gel samples had more hits to non-rRNA. Perhaps the RNA species present in the gel are more indicative a resting or stasis state and field samples have RNA species more reflective of an active status. However, this seems doubtful since the RNA from Pg samples was from taken actively growing *P. gigantea* cells and yet the RNA of these samples aligned best to the mitochondria as well. Another possibility is that the field samples contain little *P. gigantea* and the sequencing results are dominated by the RNA of other species.

Overall, this study has taken the first steps to being able to determine if living Rotstop agent is actually being applied to tree stumps. However, obtaining high quality RNA proved exceedingly difficult and improving these efforts is paramount before further sequencing protocols can be tested. This would be especially critical before any portable, point-of-need based tools can be developed.

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