Next generation sequencing of cannabis flower samples and their respective quantitative PCR assays reveals false positive detection of off-target *Aspergillus* species

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Abstract
Cannabis has a complex microbiome that can, in rare circumstances, contain four *Aspergillus* species of clinical and regulatory concern. *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus terreus* are regulated pathogens in many legal cannabis jurisdictions in the U.S. (21 states) and Canada. Molecular methods that interrogate the DNA of these pathogenic species have been adopted due to the challenges in resolving these species from other benign and ubiquitous *Aspergillus* species with culture or plating methods. Despite the rapid and competitive adoption of qPCR in this field, discordances still arise and require resolution. We describe the use of two different next generation sequencing methods to better characterize the targets of *Aspergillus* qPCR assays used in the cannabis field.

Introduction
In order to resolve discordant samples that provided inconsistent qPCR results across replicate samples with the same assay and also discordant results between competitive qPCR kits, qPCR amplicons were subjected to Illumina sequencing. In addition to sequencing the qPCR products, longer confirmatory ITS amplicons (450 bp and 750 bp) were generated from these pre-PCR samples and sequenced on Oxford Nanopore Sequencers. These longer ITS amplicons provide a more comprehensive picture of the microbiome before qPCR.

We discovered that certain qPCR kits in the cannabis testing space are targeting highly conserved beta tubulin genes described by Nasri *et al.*(Figure 1).[1] The primers described by Nasri *et al.* are not unique for *A. flavus*, *A. fumigatus*, *A. niger* and *A. terreus* sequence amplification, because they also amplify sequences in *Aspergillus brasiliensis*, *Aspergillus nidulans*, *Aspergillus tamarii* and even other fungal genera like *Fusarium oxysporum, Botrytis cinerea,* and *Penicillium citrinum* (Figure 2A and 2B). As described by Nasri *et al.*, these amplicons require restriction digests to properly speciate.

While this restriction digest coupled with gel electrophoresis may resolve closely related species, any contaminant or restriction digest inhibitor that slows the digestion reaction or induces star activity may lead to a lack of specificity and higher false positive or false negative rates.
NOTE: Star activity is the relaxation or alteration of the specificity of restriction enzyme mediated cleavage of DNA that can occur under reaction conditions that differ significantly from those optimal for the enzyme.

Many of the *Aspergillus* qPCR kits available in the cannabis field use a simple boil preparation that does not purify the DNA before digestion or PCR amplification. This failure to purify DNA will expose the digestion reaction to inhibitors (terpenes, cannabinoids, and flavonoids), and may result in increased digestion failure rates or alteration of the melting temperature of the PCR primers (Figure 3A and 3B)[2]. Failure to digest the DNA may result in a lack of specificity of the amplification primers. Failure to purify and concentrate the DNA leads to higher dilutions and sub-sampling. Single CFU tests need to compensate for extreme sub-samplings. You need 13 cell doublings (2^13 = 8,192) to compensate for 7,500X sub-sampling. *Aspergillus* doubling rate is 2-3 hours and thus requires 26-39 hours to compensate for 7,500 fold sub-samplings. Crude sample preps usually require extreme dilutions to dilute the inhibitors and this comes at the direct expense of long enrichment times which may enable one off-target *Aspergillus* to out-grow the target.

To address off-target PCR, Nasri *et al.* used agarose gel electrophoresis and post PCR restriction digests known as PCR-RFLP (Figure 1). The implementation of Nasri *et al.* into the cannabis field likely modified the qPCR protocol to perform the restriction digestion before qPCR as opposed to after it (Figure 3). It is unclear if the restriction enzyme or recognition site was modified to adjust for this change in workflow. Failure to fully digest the gDNA will leave off-target but closely related *Aspergillus* species gDNA amplification competent. These primers also amplify members of other genera like *F. oxysporum, B. cinerea* and *P. citrinum*, which are commonly found on cannabis. While the exact qPCR probe sequence cannot be ascertained with this analysis, it is harder for probes to deliver specificity if their respective PCR primers amplify indiscriminately.

Results

Another hypothesis supported by this data is that Nasri *et al*. was utilized by Vendor B as a template to design qPCR primers for the cannabis field but due to the large size of the Nasri amplicon (over 500 bp), a smaller and more qPCR compatible set of amplicons were designed targeting sequence internal to the Nasri primers. Further examination of the Illumina sequencing data of the qPCR amplicons show that the reads don't map well to *A. niger, A. terreus* or *A. fumigatus*, but instead do map to *A. tamarii* and *A. flavus* albeit with the identical variants present in each amplicon (Figure 4A-4E). The sequence this amplicon maps to in the *A. tamarii* genome is 153 bp in length and is 100% identical between *A. flavus* and *A. tamarii* underscoring this amplicon's inability to differentiate these related species. A multiple sequence alignment of the consensus sequence does identify conserved regions on the ends of the amplicons that are not specific to the *Aspergillus* genus (Figure 4E).

Likewise, any restriction digests performed on the gDNA may render assays targeting other loci in the genome impossible to amplify thus making any direct comparison of kits using post digested gDNA meaningless. For example, Nasri *et al*. describe the use of the restriction enzyme *Alw*l (GGATC) to help discern amplicons derived from these non-specific primers. This restriction site also exists in the *A. flavus* amplicon of another qPCR kit provider and as a result, no amplification can be achieved with the *A. flavus* assay applied to gDNA that has been digested with this enzyme. When comparing different qPCR assays, it is important to be aware of these digestion steps and how they may render one kit to generate false positives while rendering others to produce false negative results that would not be observable if the assays were compared directly from undigested gDNA.

To confirm the Illumina sequencing of the qPCR amplicon, two different alternative ITS amplicons were generated (450 bp and 750 bp) and sequenced with an Oxford Nanopore Technology Mk1C sequencer. These broader microbiome surveys provide a picture of the complexity of the background the qPCR primers are attempting to discern. Multiple different fungi are identified including some of the highest E-values and read counts for *A. tamarii* (Figure 5). A survey of a different sample on the same qPCR plate, highlights many species native to cannabis that the Nasri primers or putative qPCR primers (as inferred from the Illumina sequence) are likely to amplify based on conserved sequence homology (Figure 6).

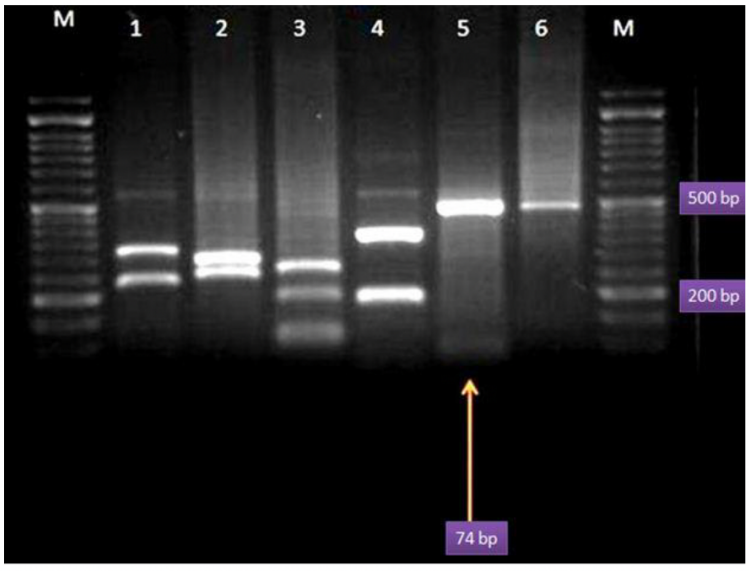


Fig. 1. Restriction profiles of BT2 gene by *Alw1* for the common pathogenic *Aspergillus*. Lane 1: *A. niger* (IFRC 407), lane 2: *A. flavus* (CBS 204301), lane 3: *A. clavatus* (CBS 514.65), lane 4: *A. terreus* (IFRC 1002), lane 5: *A. fumigatus* (CBS 113.26) and lane 6: *A. nidulans* (CBS 879). M, 50-bp molecular size marker.

Figure 1. Reproduced from Nasri *et al.* All *Aspergillus* species and some *Fusarium*, *Botrytis*, and *Penicillium* species amplify with Nasri *et al*. and restriction digests are required to speciate. Failure to digest samples may result in false positive qPCR signals for off-target *Aspergillus*.

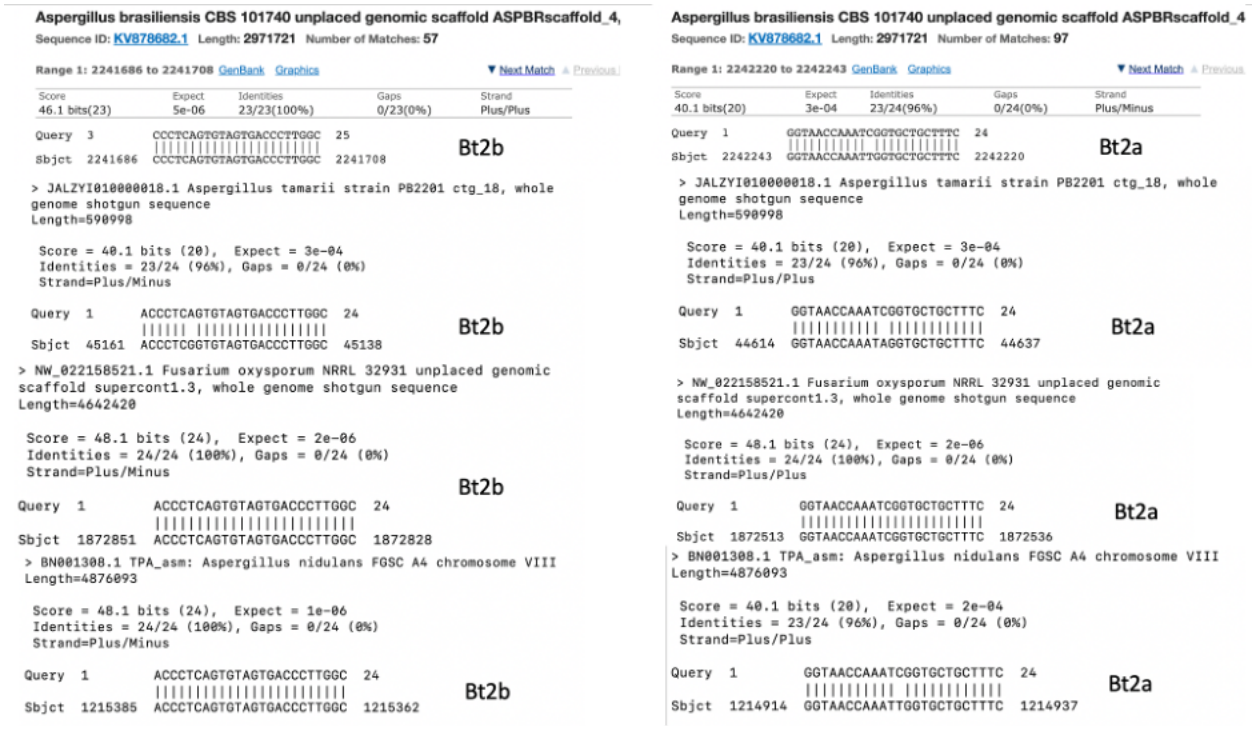


Figure 2A. BLAST of Nasri *et al.* primers demonstrate perfect homology to fungi outside of the *Aspergillus* genus (*F. oxysporum*) and single nucleotide polymorphisms on the 5 prime end of many *Aspergillus* off target species.

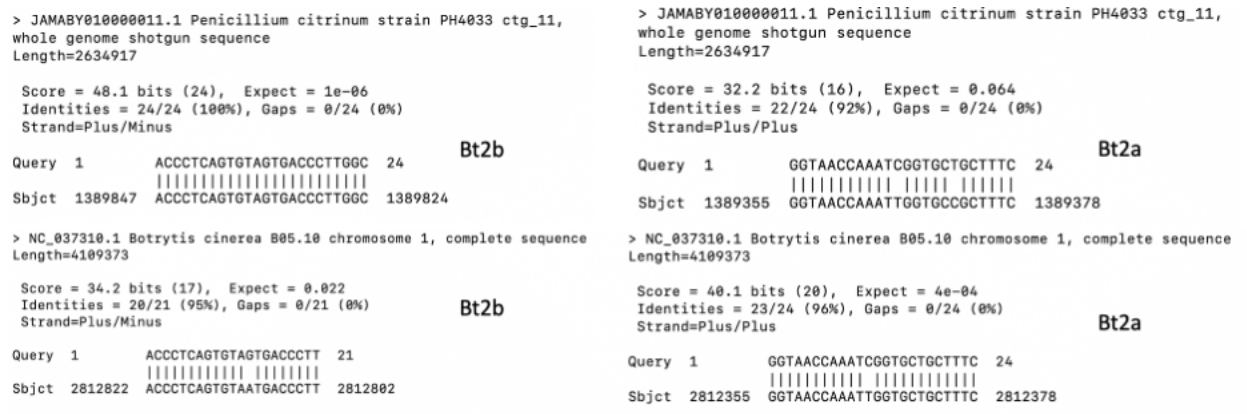


Figure 2B. BLAST of Nasri *et al.* primers demonstrate homology to fungi outside of the *Aspergillus* genus (*P. citrinum, B. cinerea*) and single nucleotide polymorphisms on the 5 prime end of many of these amplicons.

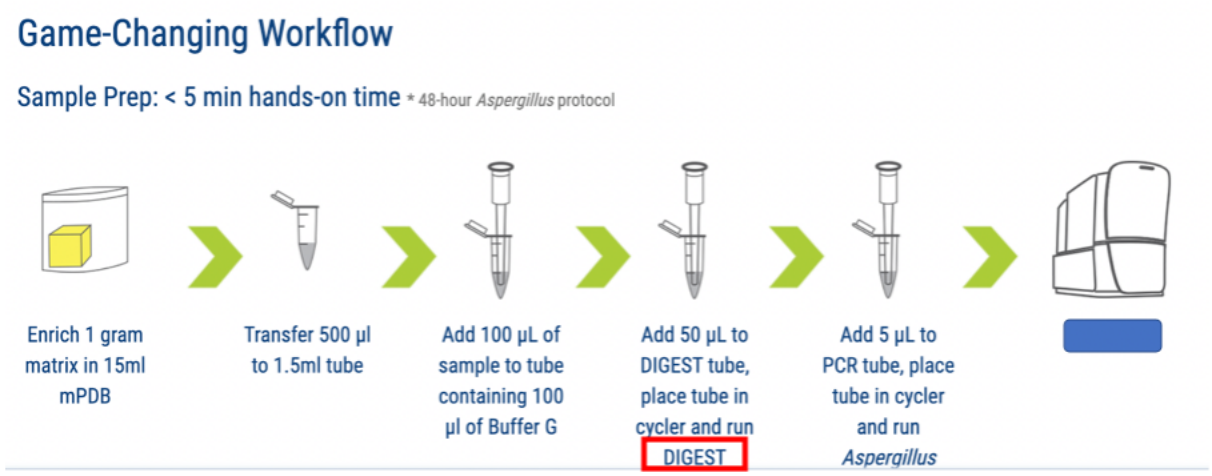


Figure 3A. qPCR from Vendor B performs a 7,500-fold sub-sampling (500 µL/15 mL *100 µL/500 µL *5 µL/250 µL =1/30*1/5*1/50) with a restriction enzyme digestion (red) prior to qPCR. Assuming *Aspergillus* has a doubling time of 3 hours, 24-hour enrichment would only provide 256-fold compensation for this dilution and fail to sample single CFU samples. 48-hour enrichment is necessary to compensate for such extreme sub-samplings. DNA purification can also compensate for extreme sub-samplings by concentrating the DNA into smaller volumes if the DNA elution volume is less than the starting purification volume. Image source (https://invisiblesentinel.com/products/cannabis-hemp-tests/gene-up-aspergillus-pro/)

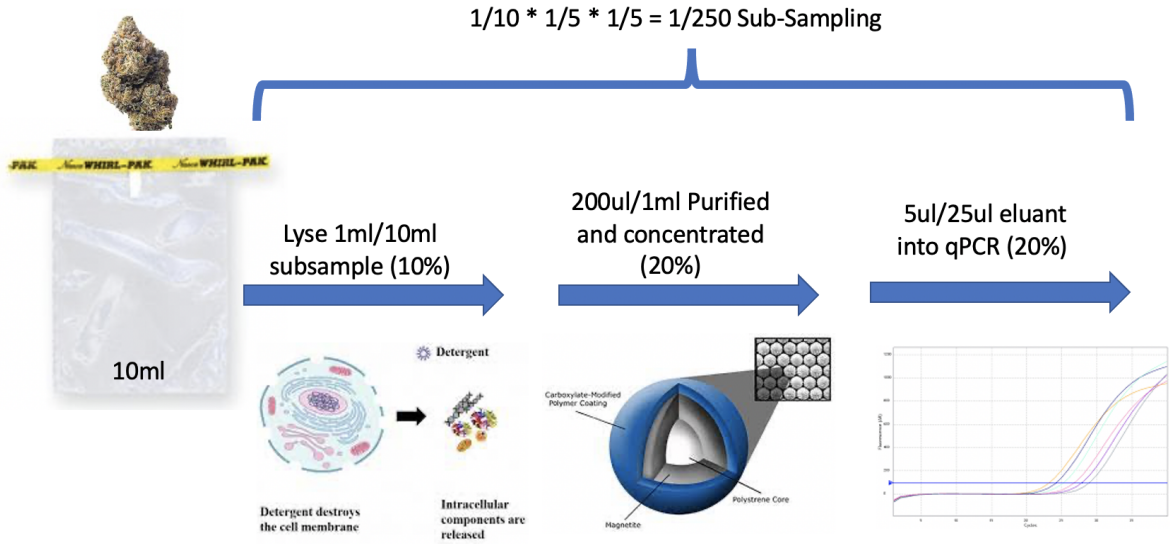


Figure 3B. A qPCR workflow from an assay that utilizes a DNA purification step to eliminate plant inhibitors and concentrate the sample. This concentration step affords lower sub-sampling and less enrichment time. Extended enrichment times are vulnerable to excessive growth of off-target species.

Figure 4A. Illumina sequence coverage of Vendor B qPCR amplicon mapped to *A. tamarii* identifies the Beta Tubulin gene described by Nasri *et al*. *A. tamarii* is cited as a biocontrol organism. https://www.nature.com/articles/s41522-022-00321-z.pdf. Of note, the Illumina library generation process uses a nuclease that hydrolyses the ends of the amplicons to generate phosphates and 3' hydroxyl groups required for sequencing adaptor ligation. As a result, there is no Illumina sequence coverage over the Nasri *et al*. primer sequences but there is sufficient coverage over the ends of the qPCR amplicons to assess the specificity of any internally designed qPCR primers.

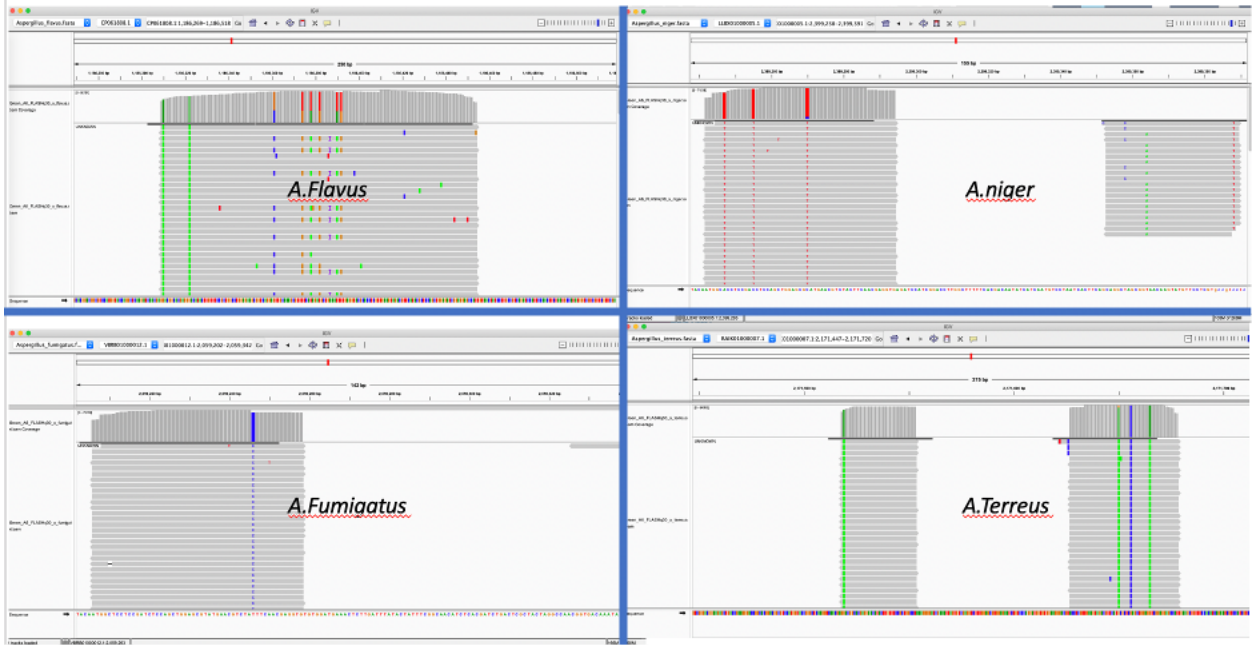


Figure 4B. Illumina sequence of the Vendor B amplicon mapped back to respective references demonstrates contiguous coverage across a putative amplicon only in *A. flavus* and *A. tamarii.*

>JALZYI010000018.1:44879-45031
CAATGGCTCCTCCGATCTCCAGCTGGAGCGTATGAACGTCTACTTCAACGAGGTGCGT
ACCTCACATTTTTCAGCCTCTTTGACAACGCTTTGCAAGTCCTGACCGCTTCTCCAGG
CCAGCGGAAACAAGTATGTCCCTCGTGCCGTCCTTGT

Figure 4C. Beta Tubulin sequence with over 80,000X coverage in the *A. tamarii* genome. This sequence is also 100% identical to the beta tubulin sequence in *A. flavus* (GenBank: MW217749.1) suggesting this amplicon cannot speciate these two organisms with any restriction digest or selective probe sequence.

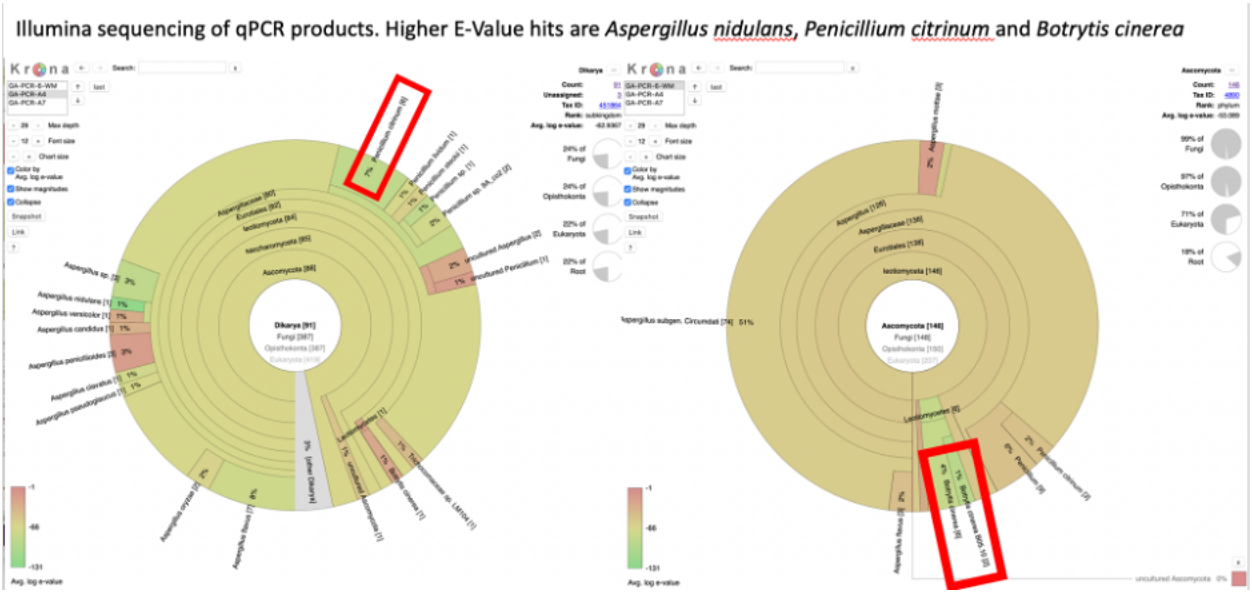


Figure 4D. Krona Plot of Illumina sequencing coverage of qPCR amplicons. Highest E-Values are for *A. nidulans, P. citrinum*, and *B. cinerea*. While these primers may amplify many off target sequences, the probe sequence used may not detect them.

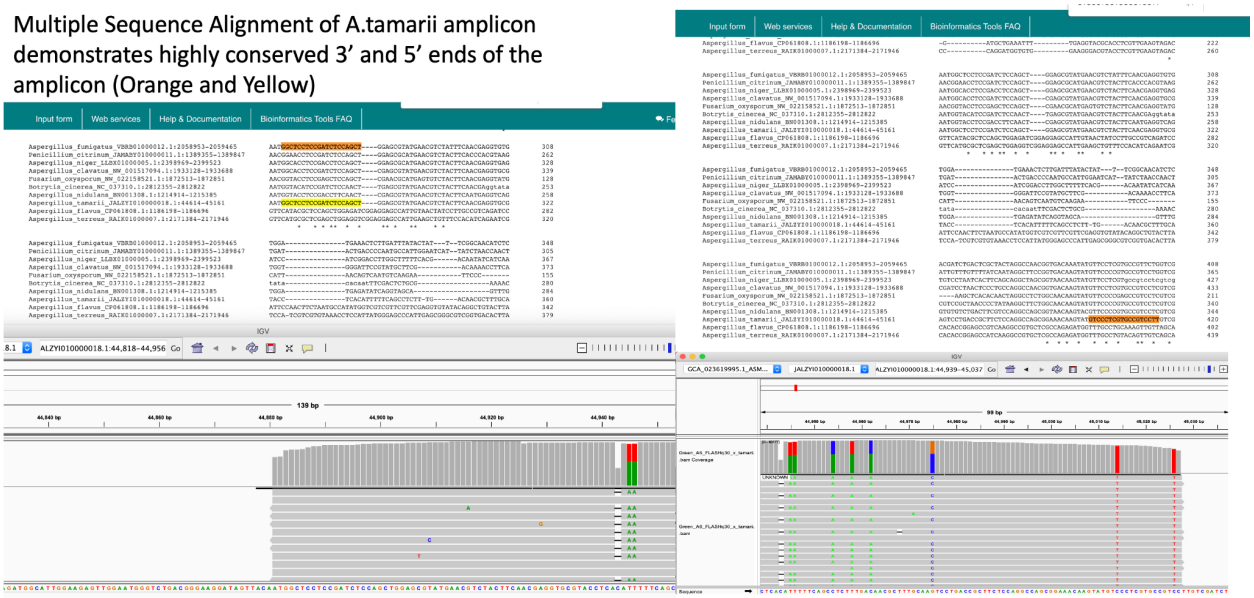


Figure 4E. Multiple Sequence Alignment (MSA) of the qPCR generated and Illumina sequenced *A. tamarii* amplicon across hypothetical off-target genomes demonstrates highly conserved terminal amplicon sequences (highlighted in orange and yellow). These are the most likely candidates for the PCR primer sequences. The abrupt change in read coverage marks the 5' end of the amplification primers. The 3' end of the qPCR primer sites cannot be determined with this sequencing method, but the 4 base pair deletion is a likely candidate as it discerns *A. niger* and *A. fumigatus* from *A. flavus*, *A.terreus*, and *A. tamarii.*

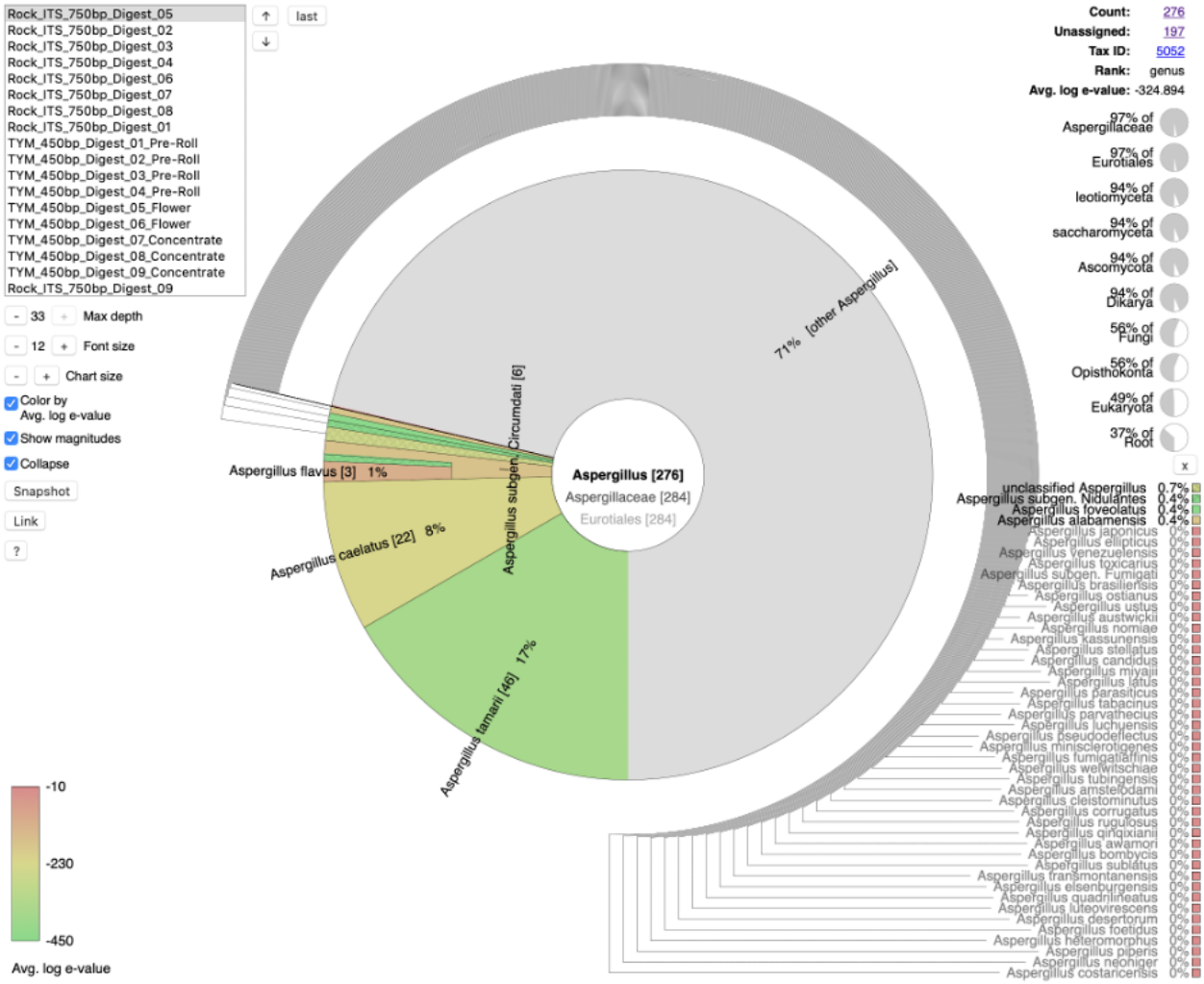


Figure 5. Oxford Nanopore sequencing of 750 bp ITS amplicons derived from the Cheng *et al.* primers applied to a '*Flavus* positive' sample identified with Vendor B qPCR primers. These data demonstrate 10-fold (3 vs 46) more reads with higher E-value alignments to *A. tamarii* than *A. flavus*. These data further confirm that *A. tamarii* is the dominant species present and the Vendor B amplicon cannot discern the difference.

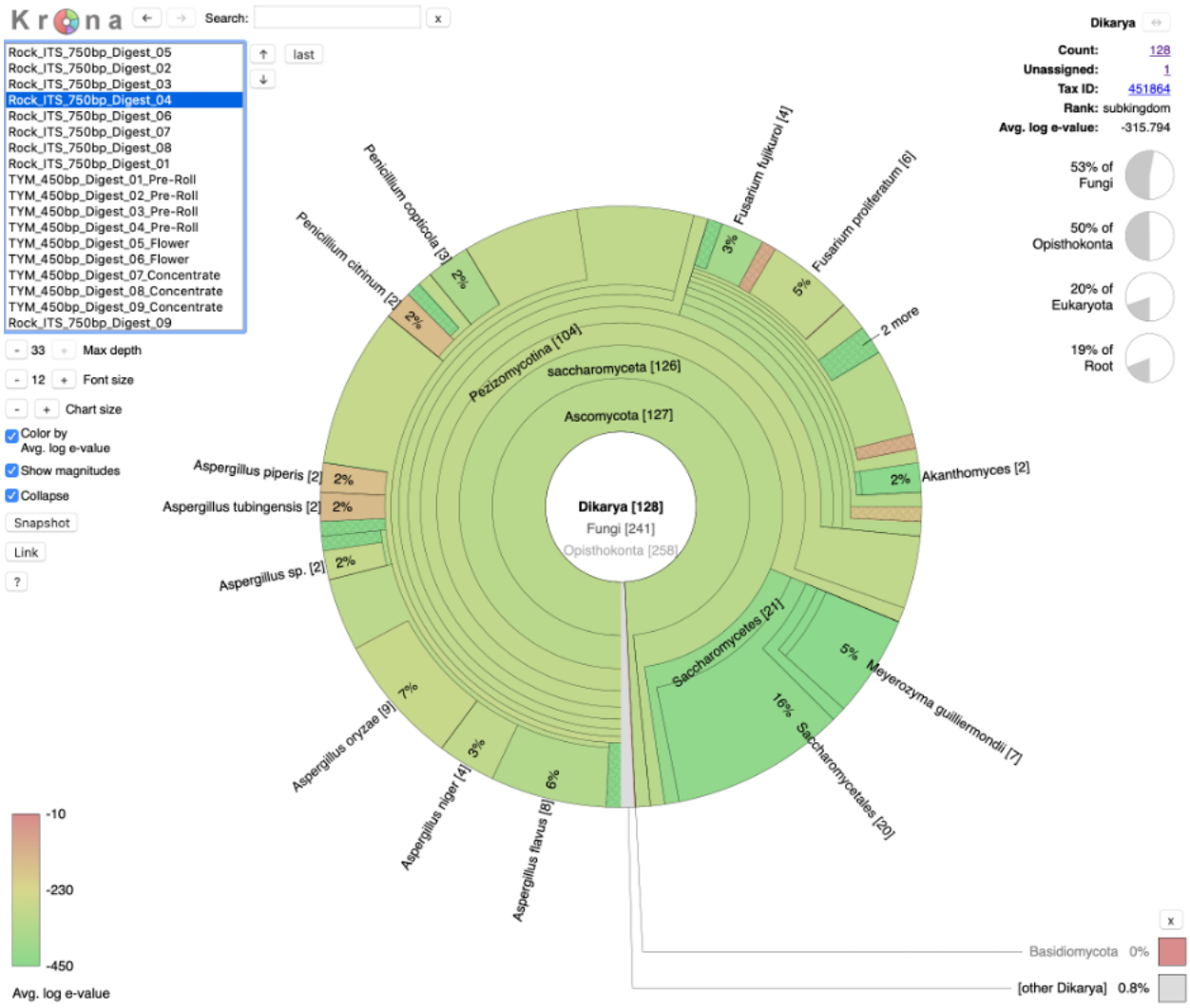


Figure 6. Oxford Nanopore sequencing of 750 bp ITS amplicons derived from Cheng *et al.* primers applied to Pre-Roll sample 04 demonstrates many *Aspergillus* species in addition to many *Fusarium* and *Penicillium* species are present in a given pre roll sample.

Confirmation of *A. tamarii* in qPCR

To further confirm off-target *A. tamarii* amplification, qPCR assays were obtained from Vendor B and Vendor C and tested. *A. tamarii* was sourced from ATCC (ATCC #1005), purified and run on qPCR with these two vendor's kits according to their respective manufacturers instructions. Both kits amplified *A. tamarii* and Vendor C failed to amplify *A.terreus* ATCC# 20542D-2 (Figure 7). After review of Vendor C's scientific poster, it appears *A. tamarii*, *A. paraciticus*, *A. orzyae*, *A. penicillioides*, and *A. vadensis* are known off-target hits that were previously published (https://www.bio-rad.com/webroot/web/pdf/fsd/literature/CSC_West_tech_poster.pdf), but then later omitted from their marketing material and AOAC exclusion testing (https://www.bio-rad.com/sites/default/files/webroot/web/pdf/fsd/literature/Bulletin_3212.pdf). This is 5/46 *Aspergillus* exclusion organisms that trigger positive tests for samples they should

exclude. A 10 fold lower LOD for *A. fumigatus* was also reported despite the use of 50 cycles of PCR and 58°C annealing temperatures.

Sample	Target (FAM)	Ct (FAM)	Target (TexasRed)	Ct (TexasRed)	Target (HEX)	Ct (HEX)
Aspergillus tamarii ATCC#1005 in cannabis background	A. flavus, A. fumigatus, A.niger	19.27	A. terreus	ND	Internal control	30.32
Aspergillus tamarii pure culture ATCC#1005	A. flavus, A. fumigatus, A.niger	22.28	A. terreus	ND	Internal control	30.12
10,000 copies Aspergillus flavus DNA	A. flavus, A. fumigatus, A.niger	18.19	A. terreus	ND	Internal control	30.06
10,000 copies Aspergillus fumigatus DNA	A. flavus, A. fumigatus, A.niger	24.33	A. terreus	ND	Internal control	27.78
10,000 copies Aspergillus niger DNA	A. flavus, A. fumigatus, A.niger	23.72	A. terreus	ND	Internal control	28.16
10,000 copies Aspergillus terreus DNA	A. flavus, A. fumigatus, A.niger	ND	A. terreus	ND	Internal control	27.70
Positive Control		28.43		27.4		27.53
NTC		ND		ND		27.55

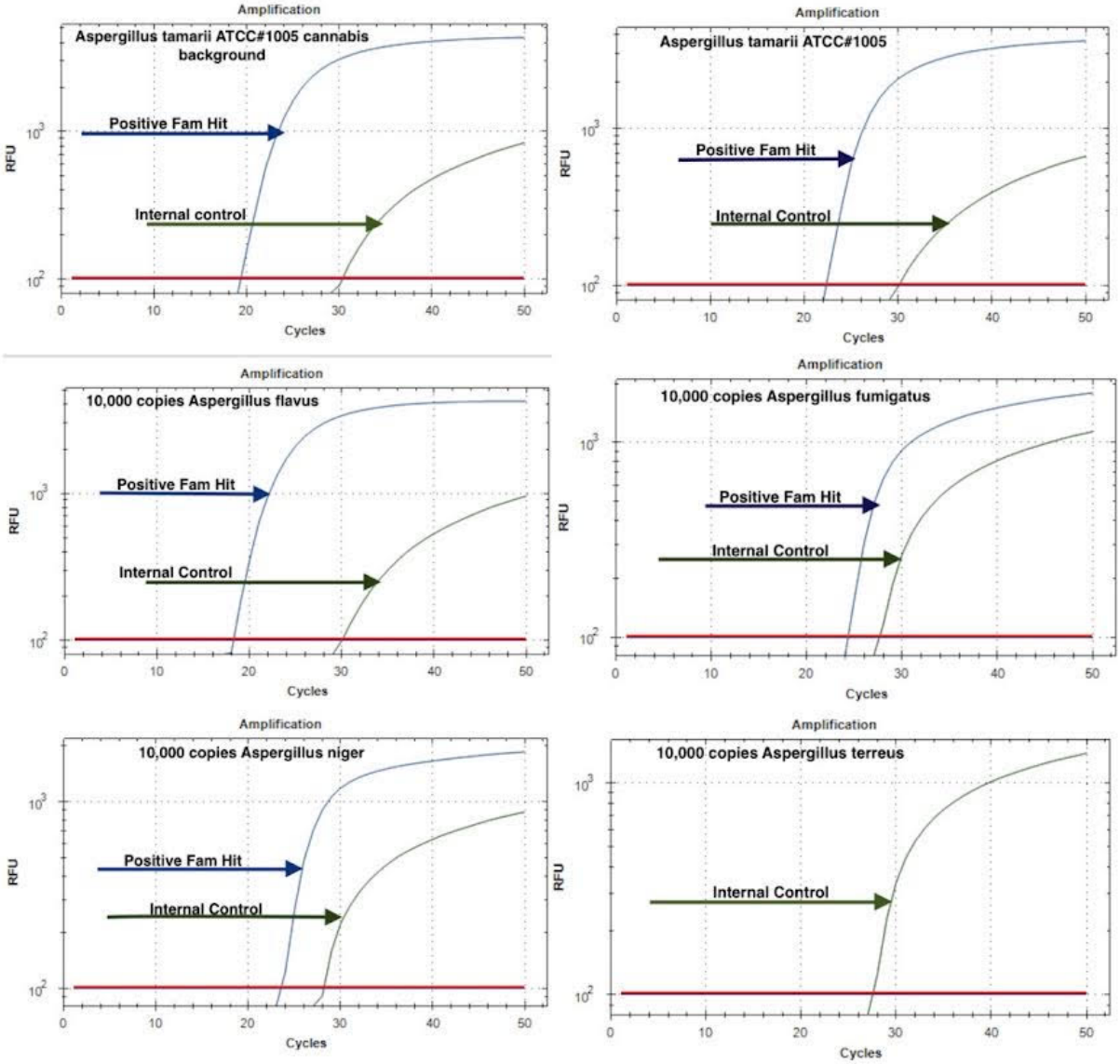


Figure 7. qPCR with *A. tamarii* using Vendor C's *Aspergillus* qPCR kit. *A. tamarii* shows positive amplification while *A. terreus* fails to detect 10,000 copies of ATCC sourced *A. terreus*.

Methods

Aspergillus qPCR products were provided by Green Scientific Laboratory and amplified according to the manufacturer's protocol. Illumina sequencing libraries were generated using the Watchmaker Genomics DNA library prep kit. Libraries were barcoded, amplified and sequenced with Azenta genomics on an Illumina 4000 with paired 150 base reads. Reads were trimmed with Trimgalore and merged with FLASH[3,4] and mapped to 5 respective *Aspergillus* species using bwa mem[5].

A.niger NCBI Accession number: LLBX01000001.1
A.flavus NCBI Accession number: CP061804.1
A.terreus NCBI Accession number: RAIK01000001.1
A.fumigatus NCBI Accession number: VBRB01000022.1
A.tamarii NCBI Accession number: JALZYI010000001.1

Primer Name	Primer Sequence 5->3	Expected Amplicon Size
Nasri: Bt2a	GGTAACCAAATCGGTGCTGCTTTC	500bp
Nasri: Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
McKernan: MGC-ITS3F	TACACGACGTTGTAAAACGACGCATC GATGAAGAACGCAGC	450bp
McKernan: MGC-ITS3R	AGGATAACAATTTCACACAGGATTTGA GCTCTTGCCGCTTCA	
Cheng: ITS-u1	GGAAGKARAAGTCGTAACAAGG	750bp
Cheng: ITS-u4	RGTTTCTTTTCCTCCGCTTA	

500 bp Beta Tubulin amplicons described by Nasri *et al.*
450 bp ITS amplicons were generated with primers described by McKernan *et al.*[6]
750 bp ITS amplicons were generated with primers described by Cheng et al[7].

Briefly, gDNA samples were purified with 1.8X Ampure (Beckman Genomics) and eluted in 100 µL of ddH20. 5 µL of a 1:10 dilution of this eluent is amplified with NEB 2X LongAmp (New England Biolabs) in a 25 µL reaction.

Long Amp Mastermix creation.

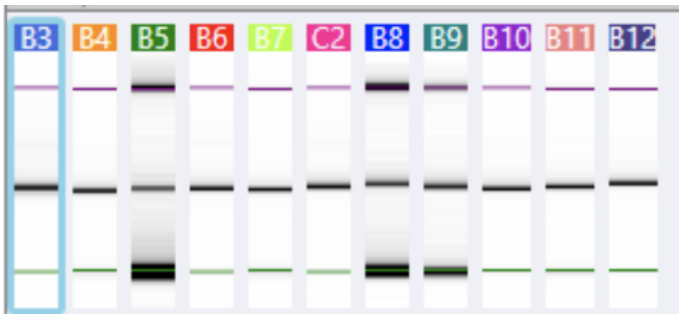
1) 16.5 µL of NEB LongAmp Mastermix
2) 1 µL of 12.5 µM ITS Primers
3) 6.5 µL of ddH20

4) 5 µL of 1:10 dilution of Lysed DNA

PCR protocol

1) 94°C for 30 sec
2) 94°C for 30 sec
3) 61.4°C/65°C for 90 sec (750 bp/450 bp amplicon)
4) Repeat steps 2 & 3 (above) 32 more times
5) Maintain at 4°C

Purification (1.8X Ampure) of these PCR products leads to clean peaks on an Agilent D5000 high sensitivity tape station.



Oxford Nanopore Sequencing

ITS amplicons were converted into sequence ready barcoded libraries using the Oxford Nanopore Rapid Barcoding Kit (SQK-RBK004). Purified libraries were quantitated on an Agilent Tape station and sequenced on an ONT Flongle (FLO-FLG001). Thousands of reads per barcode were obtained. Reads were base called with the SUP model and quality filtered for reads >Q14 reads at least 200 bp in length and less than 800 bp in length. Quality filtered reads were matched against NCBI NR with BLAST and plotted in Krona, color coded according to the E-Value.

Discussion

We demonstrate that certain AOAC certified *Aspergillus* qPCR kits utilized in the cannabis field have the potential to confuse various *Aspergillus* species due to the non-unique beta tubulin sequences used as targets. We further confirm this Illumina sequencing with Oxford nanopore sequencing of two alternative ITS amplicons and demonstrate higher read counts for the off-target *A. tamarii* species. *A.tamarii* was not listed in the manufacturers exclusion list and various exclusion samples were derived from samples from their own private biobank.

AOAC International validation and certification of microbial assays in the cannabis industry is a welcomed improvement in harmonizing assay performance and robustness. Despite this recent improvement and adoption of AOAC approved methods, the AOAC process does not require inclusion and exclusion testing to be performed in the cannabis matrix, nor does it require that

the organisms used in inclusion and exclusion testing to be widely available. Many manufacturers resort to using organisms from privately held internal biobanks which cannot be verified by outside parties. Likewise, no DNA sequencing confirmation of the assay's performance nor measurements of the limit of detection (LOD) are currently required. This can lead to many AOAC approved assays that demonstrate different sensitivity and specificity once utilized in a real matrix. The AOAC SMPR process does include a matrix study, but the cannabis chemotype diversity in the matrix study can be limited to a single cannabis chemotype.

Methods that fail to purify the gDNA prior to analysis are exposed to the wide chemotype diversity found in cannabis delivering a broad and variable portfolio of potential inhibiting compounds. Cannabis flowers can contain up to 20% (w/w) cannabinoids and 4% (w/w) terpenoids. The 20% cannabinoids can vary from CBD, to THC, CBG, CBDA, THCA, CBGA *etc*. and DNA purification eliminates these potential inhibitors with thorough ethanol washing of the DNA. Methods deploying simple boil preparations that are validated on a single cannabis matrix may be exposed to inhibitors that can alter the specificity of restriction digests or polymerase performance. It is of note that the Vendor B qPCR cycling conditions have a very low annealing temperature (57°C) and are run for 45 cycles of PCR. This can lead to off target amplification with dirtier boil preparations. Vendor C's cited poster shows 50 cycles of PCR.

Assays that employ digestion steps cannot be readily compared to other assays with gDNA that is collected after digestion. These data may help to inform the field as to the source of potential discordances found in the various cannabis testing assays.

Mass spectrometry (MALDI-TOF) is often suggested as an alternative speciation validation method but as one can see with this case, many closely related *Aspergillus* species have conserved amino acid sequences, while intronic or synonymous DNA variation can readily separate them. Tam *et al*. reported the inability to speciate *A. tamarii* from *A. flavus* and *A. nominus* with MALDI-TOF and recommended ITS, beta tubulin or calmodulin PCR as preferable methods[8]. Despite the progress regarding MALDI-TOF, current literature implies it is inadequate to reliably speciate *Aspergillus* and ITS sequencing remains the gold standard[9].

A. tamarii has several industrial fermentation applications for enzyme production and biocontrol. It is used in the production of soy sauce[10]. Genome sequencing of 23 *Aspergillus* genomes has discovered a complete lack of the Aflatoxin gene cluster in *A. tamarii* and places *A. tamarii* in a separate clade as *A. flavus*[11]. While certain mammalian culture conditions can induce *A.tamarii* to synthesize cyclopiazonic acid (CPA), its concentration was very dependent on the type of growth media used. Most human infections require ocular injury and keratitis. It is unknown if CPA is produced *in-planta*[12].

This study has several limitations in that the qPCR probe sequence for Vendor B and Vendor C are proprietary and while the DNA sequencing is demonstrating many off-target amplicons, it cannot be assumed that these amplicons are producing probe hydrolysis and additional qPCR signals. The sequencing methods utilized did not capture single stranded oligos or probes, so the primer sequences discussed within are inferred from prior publications describing methods targeting the same regions for similar speciation requirements. The Illumina sequencing utilized a nuclease-based fragmentation procedure that did not provide coverage over the Nasri primer sequence but the regions penultimate to the Nasri primers are deeply covered and suggestive of alternative PCR primer sites. Nevertheless, the sequencing does expose potential primer regions, the lack of specificity associated with these PCR targets and the inability of these sequences to discern *A. flavus* from *A. tamarii*. False positive and false negative rates can escalate with any assay that resorts to very crude boil preparations and the current AOAC certifications do not adequately assess inclusion and exclusion criteria in a diverse cannabis matrix. Inclusion and exclusion organisms should only be sourced from independent biobanks that anyone can replicate in the field. DNA sequencing of the amplicons generated and the background microbiome being amplified is a very valuable tool in characterizing the specificity of a given assay. Further sequencing is underway to understand the frequency of *A.tamarii* in the cannabis market.

Data availability

Illumina and ONT sequencing data-
https://figshare.com/articles/dataset/Nanopore_sequencing_of_quantitative_PCR_assays_reveals_off_target_Aspergillus_species_commonly_found_in_Cannabis_flowers/20776714

Krona Plots - https://figshare.com/account/articles/20769646

Acknowledgements

Kristofer Marsh and Ahmed Munshi Green Scientific Laboratories for supplying discordant DNA samples and encouraging the resolution of the discordancies.

Authors contributions

KJM: Study design in conjunction with Green Scientific, generated ONT libraries and sequencing, performed confirmatory read mapping and BLAST analysis, manuscript preparation.
SM: Read Mapping, Krona plot generation, Figure generation, analysis
NH: PCR and ONT Sequencing.
LTK: MSA analysis, read mapping and figure generation
YH: Amplicon analysis and manuscript preparation.
LZ: Illumina sequencing.
SH: Manuscript preparation and review

Conflicts of Interest- Authors are employed by Medicinal Genomics Corporation which manufactures *Aspergillus* qPCR kits for the Cannabis industry.

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