ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2019, 57, 246–255
doi: 10.1093/mmy/myy007
Advance Access Publication Date: 8 March 2018
Original Article

Original Article

Direct detection of *Coccidioides* from Arizona soils using CocciENV, a highly sensitive and specific real-time PCR assay

J. R. Bowers[1,†], K. L. Parise[2,†], E. J. Kelley[1], D. Lemmer[1], J. M. Schupp[1], E. M. Driebe[1], D. M. Engelthaler[1], P. Keim[1,2] and B. M. Barker[1,2,*]

[1]Pathogen Genomics Division, Translational Genomics Research Institute North, Flagstaff, Arizona and [2]Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, Arizona

*To whom correspondence should be addressed. Bridget M. Barker, PhD. Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ 86011, USA. Tel: +928-523-6074; E-mail: bridget.barker@nau.edu

†JRB and KLP contributed equally to this work.

Received 19 July 2017; Revised 11 January 2018; Accepted 6 February 2018; Editorial Decision 17 January 2018

Abstract

Coccidioides immitis and *Coccidioides posadasii* are soil fungi endemic to desert regions of the southwestern United States, and the causative agents of valley fever, or coccidioidomycosis. Studies have shown that the distribution of *Coccidioides* in soils is sporadic and cannot be explained by soil characteristics alone, suggesting that biotic and other abiotic factors should be examined. However, tools to reliably and robustly screen the large number of soils needed to investigate these potential associations have not been available. Thus, we developed a real-time polymerase chain reaction (PCR) assay for testing environmental samples by modifying CocciDx, an assay validated for testing clinical specimens to facilitate coccidioidomycosis diagnosis. For this study, we collected soil samples from previously established locations of *C. posadasii* in Arizona and new locations in fall 2013 and spring 2014, and screened the extracted DNA with the new assay known as CocciEnv. To verify the presence of *Coccidioides* in soil using an alternate method, we employed next generation amplicon sequencing targeting the ITS2 region. Results show our modified assay, CocciEnv, is a rapid and robust method for detecting *Coccidioides* DNA in complex environmental samples. The ability to test a large number of soils for the presence of *Coccidioides* is a much-needed tool in the understanding of the ecology of the organism and epidemiology of the disease and will greatly improve our understanding of this human pathogen.

Key words: Coccidioides, valley fever, soil sampling, molecular detection, real-time PCR.

Introduction

Coccidioides posadasii and *Coccidioides immitis* are species of soil fungi endemic to arid regions of the Americas, and both cause valley fever, or coccidioidomycosis, a potential threat to the health of residents of the arid West.[1–4] Our current knowledge suggests that in Arizona, Mexico, Texas,

© The Author(s) 2018. Published by Oxford University Press on behalf of The International Society for Human and Animal Mycology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024

and Central and South America, this disease is caused by *C. posadasii*, whereas in the Central Valley of California and as far north as eastern Washington State the disease is caused by *C. immitis*. [5,6] Valley fever starts with inhalation of *Coccidioides* conidia from the environment, yet very little is known about the prevalence of the organism and factors associated with high levels of *Coccidioides* in the environment. Previously, cultures of *C. posadasii*, obtained from soils in Tucson, Arizona, showed the distribution of *Coccidioides* is sporadic and not explained by soil characteristics alone, suggesting a potential role of biotic or other factors in the distribution of the organism in the environment. [7] As soil disturbance is highly correlated with coccidioidomycosis,[8] our knowledge of *Coccidioides* ecology must grow in order to protect public health.

Current culture-based methods of environmental *Coccidioides* detection rely on standard media plate methods to grow the fungus directly from soils, or passage of soil solutions in mice susceptible to coccidioidomycosis and retrieval of viable culture from infected tissue.[5,7,9–13] Both methods have limitations and are cost and labor intensive. Direct culture requires a large number of plates, which is a complicated task in the confines of a biosafety level 3 (BSL3) laboratory, and generally results in low yields of *Coccidioides*.[14] The rapid overgrowth of other fungi that outcompetes *Coccidioides* is frequently stated as the main drawback to this method.[7,14] Mouse passage requires the presence of infectious arthroconidia, which only form at certain times of the *Coccidioides* life cycle, resulting in low success rates. Additionally, this method detects only strains pathogenic to the mice. If nonpathogenic strains exist, mouse passage will not indicate the true distribution of the organism in the environment.

Molecular based assays have been proposed as useful methods to screen soils for the presence of *Coccidioides*.[5,7] Several groups have developed nested PCR applications, targeting the multi-copy internal transcribed spacer (ITS) region common to many fungal species.[14–16] The methodology employs amplification of a conserved region flanking the variable ITS and uses the PCR product as the template for a second, more stringent PCR targeting a *Coccidioides*-specific region, followed by sequencing of the final product. However, the resulting sequence is frequently found to have low or no homology to *Coccidioides*.[15] Additionally, PCR involving the manipulation and further amplification of amplicon DNA is prone to contamination and false positives.[17,18] To improve this process, we developed a TaqMan PCR assay that is highly sensitive and specific to *Coccidioides*. The assay, CocciEnv, is based on the CocciDx assay, which has been validated[19] and recently received FDA clearance (https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpmn/denovo.cfm?ID=DEN170041) as a coccidioidomycosis diagnostic assay.[19] CocciDx has been used for limited soil and air analyses[5,20]; however, additional investigation of the assay and the target for environmental application was needed, and we therefore conducted this study. The assay targets a repetitive region of DNA known only in the *Coccidioides* genus. This method employs a sensitive and specific amplification that can provide results in a few hours after extraction of DNA. In order to further increase assay sensitivity for soil microbe detection, we increased the number of the target alleles captured by the assay, based on newly sequenced isolates of *Coccidioides*, and validated its use for environmental screening. We propose this as a robust method to detect *Coccidioides* DNA in environmental samples and as an indispensable tool for understanding the ecology of this understudied pathogen.

Methods

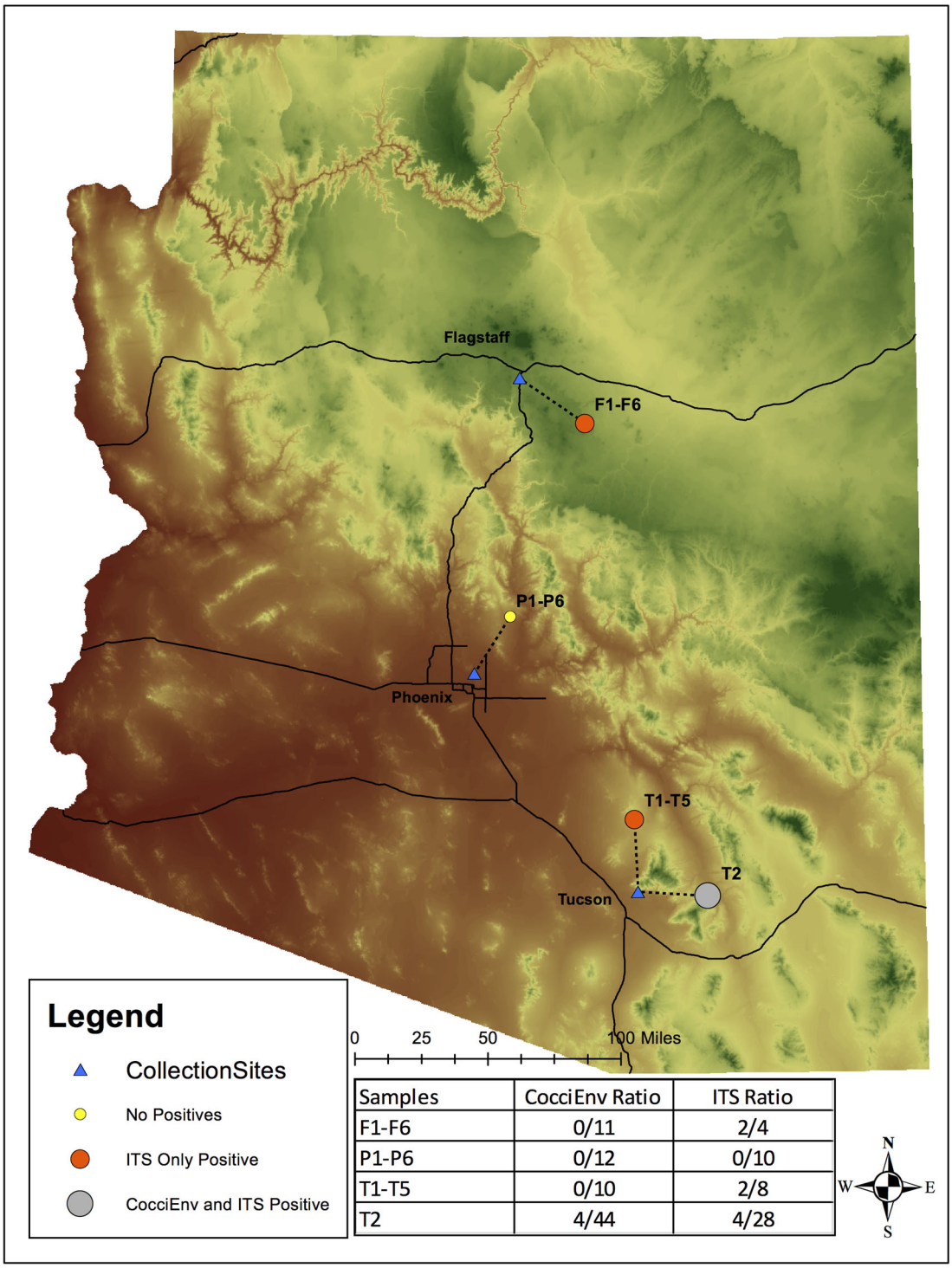
Site description and soil sampling

Soil sampling occurred in September–October, and the following April when the fungus is thought to be actively growing in the soil.[21–23] Several areas in Tucson that were previously identified as culture-positive for *Coccidioides*[7] were sampled as potential positive controls. Additional soil samples in the fall of 2013 were collected from rodent burrows in Phoenix and Flagstaff areas for comparison (Fig. 1, Table S1). Samples were collected from each site as a composite by removing the surface soil and collecting and combining 2 cm to 10 cm depth layers in sample collection bags or sterile 50 ml conical tubes. Implements were decontaminated with 10% bleach and rinsed with distilled water between collections, and samples placed in Ziploc gallon bags and surface-decontaminated with 10% bleach for transport on dry ice and storage at 4°C.

DNA extraction and preparation

For assay validation, genomic DNA from pure cultures of 562 *Coccidioides* collected from various clinical specimen types from several endemic regions was assayed (Table S2). All DNA samples were whole genome-amplified (WGA) using the REPLI-g Mini Kit (Qiagen, Boston, MA, USA) or illustra Single Cell GenomiPhi DNA Amplification Kit (GE Healthcare, Addison, IL, USA). WGA DNA was diluted 1:1000 before real-time PCR. Genomic DNA from four other Onygenales species, *Amauroascus mutatus* ATCC® 90275, *Amauroascus niger* ATCC® 22339, *Byssoonygena ceratinophila* ATCC® 64724, and *Chrysosporium queenslandicum* ATCC® 4404 was included in a set of genomic DNA from various fungal and bacterial species for

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024



Samples	CocciEnv Ratio	ITS Ratio
F1-F6	0/11	2/4
P1-P6	0/12	0/10
T1-T5	0/10	2/8
T2	4/44	4/28

Figure 1. Map of Arizona sampling locations. Triangles represent sampling locations. Small yellow circles indicate samples collected from the site were negative for the presence of *Coccidioides* DNA. Medium red circles indicate that sites were positive using amplicon sequencing. Large gray circles indicate samples were positive using both methods.

specificity screening (Table S3). DNA was extracted from the latter isolates using the DNeasy Blood and Tissue Kit (Qiagen) with lytic enzymes appropriate for the species.

For soil samples, cell lysis and DNA extraction was conducted using the PowerSoil® DNA Isolation Kit (MO BIO). For each soil sample, DNA was separately extracted from 1 g of soil taken from two to four different sections of the collection bag to test reproducibility. (These replicates are labeled A, B, C, or D in Table S1.). Extractions were carried out according to manufacturer's instructions, with

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024

TABLE 1. CocciDx and CocciEnv real-time PCR assays.

Assay component	Name	Sequence	Final concentration in PCR (μM)
CocciDx Assay			
Forward primer	CocciDx_F1	GTGTTAGGTAGTCCAACTAGCACCT	0.6
Forward primer	CocciDx_F2	GTGTTAGGTAATCCAACCAGCACCT	0.6
Forward primer	CocciDx_F3	GTGTTAGGTAATCCAACTAGCACCT	0.6
Reverse primer	CocciDx_R1	CTGATGGAGGACTCGTATGCTTGT	0.6
Reverse primer	CocciDx_R2	CTGATGGAGGACTTGTACACTTGT	0.6
Reverse primer	CocciDx_R3	CTGATGGAGGAATTGTATGCTTGT	0.6
Reverse primer	CocciDx_R4	CTGATGGAGGACTTGTATGCTTGT	0.6
Taqman probe	CDxQ_FAM-MGB	6FAM-ACCCACATAGATTAGC-MGBNFQ	0.25
CocciEnv Assay			
Forward primer	CocciEnv_F1d1	CGTTGCACRGGGAGCACCT	0.375
Forward primer	CocciEnv_F2	AAGCTTTGGATCTTTGTGGCTCT	0.375
Forward primer	CocciEnv_F3	AATTGATCCATTGCAAGCACCT	0.25
Forward primer	CocciEnv_F4	AATCCAACCTTTGGAACTACACCT	0.25
Forward primer	CocciEnv_F5	TTTTCCGGTATGGACTAGCACCT	0.375
Forward primer	CocciEnv_F6d2	TGTTAGGTAATCYAACYAGCACCT	0.125
Forward primer	CocciEnv_F7d2	TRTTAGGTAATYCAACTAGCACCT	0.125
Forward primer	CocciEnv_F8d1	TGTTAGATAATCCAACYAGCACCT	0.125
Forward primer	CocciEnv_F9d2	GKTARGTAATCCAACTAGCACCT	0.125
Forward primer	CocciEnv_F10d2	TGTTAGGTARTCCAACTAGCAYCT	0.125
Forward primer	CocciEnv_F11d2	TGTTAGGTAATCCAACTMGCACYT	0.125
Reverse primer	CocciEnv_R1	GATGGAGGACTCTATATGCTTGT	0.375
Reverse primer	CocciEnv_R2	ATGGAGGACTCGTTATGCCTGT	0.375
Reverse primer	CocciEnv_R3	GGAGGACCCGTATGCTTGTGT	0.375
Reverse primer	CocciEnv_R4	TGCTAAATGATGGAGGGCTTGT	0.375
Reverse primer	CocciEnv_R5	GATGGAGGCTCGTATGCTTGT	0.375
Reverse primer	CocciEnv_R6	AAGGGGTTTGTGGTGAATCCTTA	0.375
Reverse primer	CocciEnv_R7	CAGAAAAATAGCCGTATGCTTGT	0.375
Reverse primer	CocciEnv_R8d2	TRATGGAGRACTTGTATGCTTGT	0.125
Reverse primer	CocciEnv_R9d1	TGATGGAGGACTCGTATGCYTGT	0.125
Reverse primer	CocciEnv_R10d2	TGATGGARRACTCATATGCTTGT	0.125
Reverse primer	CocciEnv_R11d2	TGATAGAGAACTTGTATRCTTRT	0.125
Reverse primer	CocciEnv_R12d2	TGATGAAGAACTTRTATRCTTGT	0.125
Reverse primer	CocciEnv_R13d2	TGATRRAGGACTTGTATGCTTGT	0.125
Reverse primer	CocciEnv_R14	TGATGGAAAACTTGTATGCTTGT	0.125
Reverse primer	CocciEnv_R15d2	TGATGGAGGACTTGTAYAYTTGT	0.125
Reverse primer	CocciEnv_R16d2	TGATGGAGGACTTGTAYGCTTRT	0.125
Reverse primer	CocciEnv_R17d2	TGATGGAGGACTYATATGCTTRT	0.125
Reverse primer	CocciEnv_R18d2	GATGGAGGACTCGTWYGCTTGT	0.125
Taqman probe	CocciEnv_FMGB	6FAM-ACCCACATAGATTAGC-MGBNFQ	0.25

one exception: the FastPrep-24 Instrument at 6.5 m/s for 60 s (MP Biomedicals, Santa Ana, CA, USA) was used to bead-beat the sample. DNA was quantified using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and diluted to a standard concentration of 100 ng/μl for PCR assays.

Real-time PCR assay development and screening

The real-time PCR assay, CocciDx (Table 1), was developed by the Translational Genomics Research Institute (TGen).[5] The CocciDx target was identified by surveying for repeat regions among *Coccidioides* genomes (http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/coccidioides-genomes, sequence data now available as Genbank BioProject PRJNA46299. Repeat regions were first identified in the *C. immitis* RS genome by a pairwise BLAST of the genome against itself, using a word size of 50 and a minimum aligned length of 50 bp with 90% sequence identity, then determining which queries hit at multiple loci. These candidate repeat sequences were checked *in silico* for

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024

ubiquity among *Coccidioides* genomes and for specificity to *Coccidioides* by BLAST of the NCBI nucleotide database. One candidate sequence was selected based on its high number of repeats, sensitivity, and specificity. In the NCBI database, the sequence is annotated as a copia-like retrotransposon. Alleles of the repeated region were aligned using SeqMan (DNAStar) and an assay was designed to conserved regions using Primer Express® 3.0 (ThermoFisher Scientific).

After *in silico* development, sensitivity, specificity, and limits of detection of the CocciDx assay were characterized.[19] The assay was optimized on the 7900HT Real-Time PCR System (ThermoFisher Scientific). Each 10 μl reaction mixture contained 1X PerfeCTa qPCR FastMix II (Quanta Biosciences, Beverly, MA, USA), assay concentrations outlined in Table 1, and 200 ng DNA template. Thermocycling conditions were initial denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

To validate sensitivity, the CocciDx assay was screened across WGA DNA of 562 unique isolates of *Coccidioides*, including 40 *C. immitis*, 436 *C. posadasii*, and 86 *Coccidioides* species unknown (Table S2). To validate specificity, the assay was screened across a panel of DNA from various species including human, other fungal pathogens, one genetic neighbor, and several bacterial pathogens that may cause similar clinical presentation to coccidioidomycosis (Table S3). For limit of detection experiments a synthesized plasmid control containing one copy of the CocciDx target (Blue Heron Biotech, LLC, Tobermory, ON) was used. In order to precisely quantify copy numbers of the plasmid, serial dilutions of the plasmid, including dilutions down to extinction, were run on a real-time PCR assay that targets the β-lactamase gene present in the plasmids (Fig. S1). Using the Poisson distribution, plasmid copy number was calculated based on the observed number of amplification events of the lowest dilutions of the plasmid. To determine the limit of detection of CocciDx, 20 replicates of serial dilutions of the quantified plasmid control were screened to determine the lowest number of target copies that resulted in 95% positive results. The process was repeated in 60 replicates for confirmation.

With the recent deposition of new *Coccidioides* genome sequences in public databases,[24] we hypothesized that we could improve the analytical sensitivity of the assay by adding primers to capture more variants of the CocciDx target. Using a local BLAST database of the available *Coccidioides* genomes, we queried for hits with 100% identity to and 100% coverage of the CocciDx Taqman probe sequence. For each hit, we extracted the probe region and flanking sequence and aligned them. We designed 29 new primers to increase the number of alleles of the target captured by the assay (Table 1), and refer to the new environmental sample assay as CocciEnv. The total number of different alleles and copy numbers of the CocciEnv target in *Coccidioides* genomes were estimated bioinformatically (Fig. 2). The new assay was run using the same conditions as for CocciDx, with modifications only to primer concentrations (Table 1).

CocciEnv was subject to a more concise validation than for CocciDx given the extensive validation of CocciDx but included sensitivity and specificity screening across a subset of the DNAs mentioned above, along with DNA from four additional Onygenales species: *Amauroascus mutatus* ATCC® 90275, *Amauroascus niger* ATCC® 22339, *Byssoonygena ceratinophila* ATCC® 64724, and *Chrysosporium queenslandicum* ATCC® 4404 (Table S3). Additionally, CocciDx and CocciEnv were tested by using genomic DNA (not whole genome-amplified) from 23 *Coccidioides* isolates.

CocciEnv was run on soil DNA using the 7900HT Real-Time PCR System (ThermoFisher Scientific). Each 20 μl reaction contained 1 × PerfeCTa qPCR ToughMix (Quanta Biosciences) with 100 ng total DNA template and assay concentrations outlined in Table 1, with the following

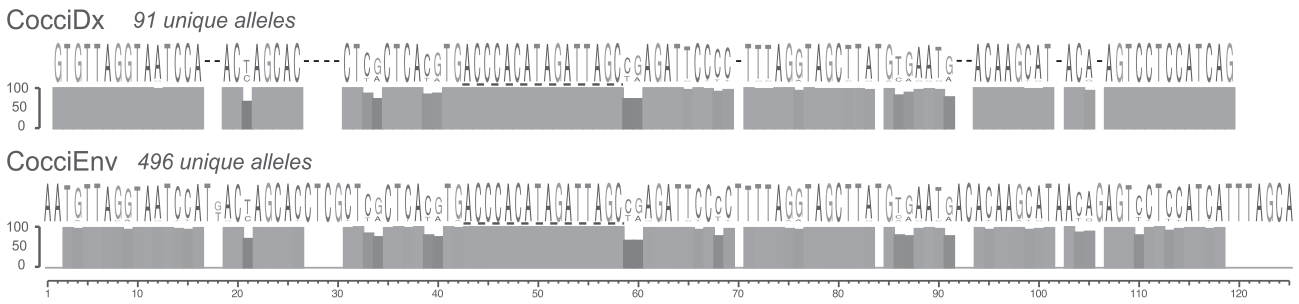


Figure 2. Consensus sequence comparison illustrating the allele diversity in Coccidioides genomes captured by CocciDx and CocciEnv. One representative sequence of each allele was included in the consensus (i.e., identical alleles were removed). The height of each nucleotide is proportional to its frequency in that position among the alleles. Gaps in the CocciDx consensus correspond to insertions in alleles captured by CocciEnv that are not captured by CocciDx. The histograms illustrate the percent frequency of each position in all alleles captured by each assay. The Taqman probe sequence is underlined with the dashed line. Figure was created using MegAlign Pro (DNAStar, Inc).

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024

thermocycling conditions: initial denaturation for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All reactions were set up in a PCR cabinet to prevent contamination, and three technical replicates were run for each DNA sample. A reaction was considered positive if it showed logarithmic amplification, produced a C_T value of <40, and all controls performed as expected.

PCR validation of fungal genomic targets in soil

To confirm that soil DNA samples contained fungal DNA and were amenable to PCR, each was screened for fungal DNA using primers ITS1 and ITS4 targeting the ribosomal RNA operon.[25] Each 50 μl reaction contained 1 × MyFi™ Mix (Bioline) with 21 μl DNA template and 10 μM each forward and reverse primers, with the following thermocycling conditions: initial denaturation for 1 min at 95°C followed by 40 cycles of 15 s at 95°C, 20 s at 55°C, and 45 s at 72°C, and a final extension of 10 min at 72°C. PCR products were visualized via agarose gel electrophoresis. If bands were not present for a sample, it was not processed further, and DNA was re-extracted from that soil sample. Samples positive for fungal DNA were screened with CocciEnv, as well as by ITS2 amplicon sequencing (see below).

Validation of target amplification in soil DNA samples

As soil is a complex sample, Sanger sequencing was employed to confirm the presence of the assay target when detected in a soil sample. PCR was run in 20 μl reactions that included 100 nM of each primer CocciDx_F3 and CocciDx_R4 (Table 1), 2 ng DNA template, and *Taq* DNA polymerase (ThermoFisher Scientific). Thermocycling conditions consisted of an initial denaturation of 10 min at 95°C followed by 40 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C, and a final extension of 10 min at 72°C. PCR products were cleaned using ExoSAP-IT™ (Affymetrix, Santa Clara, CA, USA), and sequenced using the above PCR primers with BigDye® Terminator v3.1 chemistry (ThermoFisher Scientific). Reaction products were analyzed on a 3130xl automated genetic analyzer (ThermoFisher Scientific). Sequencing reaction results were assembled in Seqman (DNAStar).

ITS2 amplicon sequencing

The ITS2 region in fungal PCR-positive soil samples was amplified in triplicate using published primers.[26] PCR was performed in 8 μl reactions containing 0.1 U/μl Phusion Hot Start II DNA polymerase (ThermoFisher Scientific), 1 μM each primer, 200 μM dNTPs, and 6% glycerol (v/v). Thermocycling was as follows: 95°C for 2 min, and 25 cycles of 95°C for 30 s, 55°C for 30 s, 60°C for 4 min. Replicate reactions were pooled for indexing. Index PCR conditions and reagents were the same as above except for the indexing primers and 15 PCR cycles were performed. Indexed products were bead-purified,[27] quantified with PicoGreen® fluorescence (ThermoFisher Scientific), and pooled to equimolar concentrations. The final pool was bead-purified and quantified by qPCR using Library Quantification Kit, Illumina/ABI Prism (KAPA Biosystems) and sequenced in 2 × 250 mode on the MiSeq platform (Illumina).

We analyzed amplicon sequencing results using the TGen-developed bioinformatic tool, ASAP.[28,29] ASAP links together several bioinformatic programs with parameters set for customized sequencing analysis and results generation. In this case, ASAP first merged sequence read pairs with PEAR.[30] The reads were then trimmed of Illumina adapter (ligated during the sample library preparation process) and further trimmed based on sequence quality with Trimmomatic.[31] Specifically, a 5 bp sliding window across the read checked for average Phred scores below 20. Any windows that fit that criterion were removed. The full ITS2 *C. posadasii* reference sequence was obtained from the NCBI database (Genbank accession number KF539879) and trimmed to the expected amplicon size (334 bp) to serve as the reference sequence for the first round of ASAP. Trimmed, merged reads were then mapped to the reference sequence with the bowtie2 aligner.[32] Binary alignment map (BAM) files, generated by the aligner (one generated for each sample), were analyzed to determine the breadth and depth of coverage of the reference and identity to the reference. Thresholds to identify whether a sample was positive or negative were set at 100% breadth at 1 × depth of coverage at ≥97% identity (i.e., the full length of the 334 bp ITS2 reference sequence had to have a pair-merged read align with 10 or fewer single-nucleotide polymorphisms [SNPs]). This identity threshold was set according to the lowest identity of all known *Coccidioides* ITS2 sequences in the NCBI nucleotide database. Tablet[33] was used to verify results.

Upon analysis with ASAP, several samples were found for which reads aligned to the *C. posadasii* ITS2 amplicon reference that did not pass the 97% identity filter. To determine what other organisms might be the source of these sequences, any reads that aligned to the ITS2 reference that didn't meet the 97% identity, 100% breadth criteria were binned for analysis. A BLAST analysis of these reads showed hits to several other fungal species. These sequences were added as references for ASAP to determine if CocciEnv could be cross-reacting with other fungal species.

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024

Results

CocciDx and CocciEnv assay validation and comparison

The WGA DNA samples from all 562 unique isolates of *Coccidioides* (Table S2) were positive on the CocciDx assay (real-time PCR Ct values were all < 35.0) and all DNA samples from various other species (Table S3) were negative (Ct values were all > 40.0), illustrating 100% sensitivity and 100% specificity. These data reflect the recently published CocciDx clinical validation data, in which sensitivity was 100% and specificity between 93.8% and 100% for DNA extracted from clinical specimens run on the GeneSTAT instrument (DxNA, LLC).[19] Using a serial dilution of a precisely quantified synthetic plasmid standard (Blue Heron Biotech, LLC), the CocciDx assay limit of detection was determined to be 15 target copies/reaction and the linear range was between 10^8 and 10^1 copies/reaction (Fig. S1).

On the CocciEnv assay, 45 out of the 45 *Coccidioides* WGA DNA samples tested were positive (Ct values < 35.0), and all 28 of the nontarget DNA samples, which included the Onygenales family members (Table S3), were negative (Ct value > 40.0). A comparison of CocciEnv and CocciDx showed that the CocciEnv assay resulted in an average of 1.8 (range of 1.6 to 2.1) Ct values earlier than those from the CocciDx assay when screened on the same genomic DNA, inferring a limit of detection three to over fourfold lower than that of CocciDx.

Genomes from 84 *Coccidioides* isolates were bioinformatically screened to determine the number of perfect matches to each assay that would result in the expected PCR product. Collectively in all 84 genomes, target alleles that were a perfect match to the CocciEnv assay were found a total of 4,614 times, which makes an average of ~55 copies/genome, while the alleles that were a perfect match to CocciDx were found 471 times, an average of ~6 copies/genome. Although the actual assays would likely capture additional alleles that are close, but not perfect, matches to the primer or probe sequences, thereby exhibiting sensitivity beyond what is described here, this was not further explored. The matches were dereplicated to determine the number of unique alleles that would be captured with a perfect match by each assay. CocciEnv captures 496 different alleles of the target, while CocciDx captures 91 different alleles (Fig. 2).

CocciEnv soil screening

Soil DNA was tested with CocciEnv in triplicate technical replicates. Results were considered positive if two of the three replicates had Ct values <40. Four samples tested positive out of 73 screened. These four samples were biological replicates of one soil sample collected near an apparently unoccupied large rodent burrow (Table S1), illustrating the reproducibility of both the DNA extraction and the CocciEnv assay.

ITS2 amplicon sequencing

Presumably, the vast majority of fungal species are not known, and soils have highly complex microorganism composition. We therefore set stringent parameters for determining the presence of *Coccidioides* in soil by ITS2 amplicon sequencing. The number of pair-merged reads that matched the *Coccidioides* ITS2 region reference sequence at ≥97% identity is shown in Table S1. Of the 50 soil samples tested, eight had one or more reads positive for *Coccidioides* (≥97% identity, Table S1, Table 2). Four of these are the four that tested positive by CocciEnv. Unfortunately, we did not have enough material to screen CocciEnv on three of the four other ITS2-positive samples (Table 2). The last sample had one read align and tested negative on CocciEnv.

We also identified by BLAST the closest species match to each pair-merged read that aligned to the *C. posadasii* ITS2 reference that did not pass the 97% identity threshold. The top BLAST hit for each was one of the following: *Chrysosporium keratinophilum*, *Chrysosporium tropicum*, *Aphanoascus verrucosus*, *Aphanoascus canadensis*, *Uncinocarpus reesi*, *Uncinocarpus queenslandicus*, *Arthroderma multifidum*, *Castanedomycs australiensis*, or *C. posadasii* (at <97% identity). The ITS2 sequences from these species were added to ASAP as references and results from this analysis are shown in Table S1. In 12 cases, the best hit of the reads was *C. posadasii*, at <97% identity, which could be indicative of an unknown *Coccidioides* ITS2 sequence or an unknown species. Seven of these 12 samples also had reads pass the 97% identity filter for *C. posadasii*, so were considered positive, suggesting the presence of unknown *Coccidioides* ITS2 sequences. Four of these seven tested positive on CocciEnv, while the other three were not tested. The five samples that did not have additional reads pass the identity filter tested negative on CocciEnv, suggesting the presence of a yet unknown fungal species (Table S1).

Discussion

Characterizing the natural reservoirs of *Coccidioides* is necessary for coccidioidomycosis epidemiology and public health protection. Unfortunately, a paucity of data exists to address this.[34] It is understood that *Coccidioides* has a sporadic, unpredictable distribution in the environment.[35] Because exposure of a susceptible host to arthroconidia often leads to infection, understanding the environmental

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024

TABLE 2. Comparison of CocciEnv and ITS2 sequencing on a subset of soil samples. Data for all soil samples are in Table S1. 97% sequence identity is a common cutoff for species assignment for fungal metagenomics.

Sample ID	Location	CocciEnv mean C_t value	ITS2 Read counts ≥97% sequence identity	ITS2 Read counts <97% sequence identity
F2A	Flagstaff	Not performed	4	13
F2B	Flagstaff	Negative	0	0
F3A	Flagstaff	Negative	1	0
P2A	Phoenix	Negative	0	2
P2B	Phoenix	Not performed	0	0
P3B	Phoenix	Negative	0	0
P4A	Phoenix	Not performed	0	0
T2A	Tucson	Negative	0	11
T2B	Tucson	Negative	0	1
T3A	Tucson	Negative	0	10
T3B	Tucson	Negative	0	6
T4A	Tucson	Not performed	2	3
T4B	Tucson	Not performed	2	3
T5A	Tucson	Negative	0	4
T2-1a	Tucson	Negative	0	2
T2-1c	Tucson	Negative	0	2
T2-2a	Tucson	32.6	7	15
T2-2b	Tucson	31.3	14	22
T2-2c	Tucson	32.0	8	39
T2-2d	Tucson	32.0	22	43
T2-4a	Tucson	Negative	0	31
T2-4b	Tucson	Negative	0	12
T2-4c	Tucson	Negative	0	36
T2-4d	Tucson	Negative	0	7
T2-5a	Tucson	Negative	0	17
T2-5b	Tucson	Negative	0	10
T2-5c	Tucson	Negative	0	6
T2-5b	Tucson	Negative	0	13
T2-6a	Tucson	Negative	0	1
T2-10a	Tucson	Negative	0	12
T2-10b	Tucson	Negative	0	12
T2-10c	Tucson	Negative	0	11
T2-10d	Tucson	Negative	0	10

reservoir is critical to quantifying the risk of exposure. In fact, a recent study linked rising coccidioidomycosis cases with land use-induced soil disturbances in Antelope Valley in California.[8] With the development of a rapid, inexpensive, and high-performance screening tool, many ecological questions become answerable regarding favorable and unfavorable biotic and abiotic factors, mechanisms of dispersal, seasonality, and locations and persistence of *Coccidioides* foci in the environment.

Coccidioidomycosis is on the rise, and there are several nonexclusive phenomena that might be responsible; including population growth in endemic areas, increase of susceptible populations, heightened awareness of coccidioidomycosis, and increasing rates of exposure to arthroconidia through landscape disturbance.[8,36] Our understanding of the contributions of each of these factors is lacking.[36] A sensitive and specific soil-screening tool would enable studies to elucidate the role that landscape disturbance plays in the incidence of coccidioidomycosis. Additionally, such a screening tool would inform regulatory agencies in endemic regions (e.g., environmental, occupational health, corrections, and public health agencies) of risk of exposure to workers and communities within the vicinity of any proposed project where there is the potential for soil disturbance and dust, and inform remediation efforts. A recent epidemiological investigation of coccidioidomycosis outbreaks in prisons in California's Central Valley did not identify an association of coccidioidomycosis with outdoor activities.[37] Comprehensive soil surveys could pinpoint hotspots of *Coccidioides*, and be highly informative

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024

for investigations such as this, and direct effective mitigation practices.

CocciEnv and its clinical diagnostic counterpart CocciDx are rapid, straightforward, highly sensitive, and inexpensive assays to detect *Coccidioides* DNA in environmental and clinical samples, respectively.[38] CocciDx recently received FDA clearance as a coccidioidomycosis diagnostic test (https://www.tgen.org/news/2017/december/06/tgen-technology-results-in-new-fast-accurate-valley-fever-test/). CocciEnv, with slight differences from CocciDx in primer number and sequence, is designed to be more specific and sensitive than CocciDx making it especially suited for testing environmental samples.

Of note, there were seven samples that contained several reads that matched the known *Coccidioides* ITS2 at ≥97% identity that also contained ITS2 sequences that did not pass the identity filter but whose top BLAST hits were *C. posadasii*. This may be evidence of a more genetically diverse population of *Coccidioides* than is currently described, or an as yet unknown *Coccidioides*-like fungus that cohabits with *Coccidioides*. The majority of sequences deposited in the NCBI database are clinical isolates, thus in-depth studies of *Coccidioides* soil isolates are necessary to determine if sequences detected might represent a non-pathogenic form of *Coccidioides*.

The identification of our highly-repeated assay target as a copia-like retrotransposon is not surprising. Retrotransposons replicate via RNA intermediates, which interact with a self-encoded integrase to integrate into the host genome, leaving the original template intact. In this way retrotransposons continuously increase in number. Eukaryotic genomes have numerous copies of some retrotransposons; the human genome contains more than a million copies of the *alu* retrotransposable element.[39] Thus, targeting a retrotransposon makes for a highly sensitive detection assay. We have targeted a portion of a retrotransposon in the Ty1/Copia superfamily, a superfamily originally defined in *Saccharomyces cerevisiae* and abundant throughout eukaryotic genomes.[40,41] The copia-like element we target with CocciEnv is specific to *Coccidioides*, making an ideal assay for applications requiring maximum sensitivity while maintaining specificity. Heterogeneity among copies of a given copia-like retrotransposon can be significant,[40] as we show here in *Coccidioides*, and is the reason behind the numerous primers in the CocciEnv assay.

The assay presented here addresses many of the limitations that have previously prevented needed fine-scale modeling of *Coccidioides* in the environment: the lack of a high throughput system to screen a large number of soils, the lack of sensitivity of microbiological methods, and the lack of reliable molecular tools. Additionally, CocciEnv obviates culture of *Coccidioides*, a BSL3 organism, and does not preclude the discovery of non-pathogenic strains, as mouse passage does. Our CocciEnv assay is sensitive and specific on the soils we tested, despite the fact that we did not find many positive soils. As the assay was designed using known fungal DNA sequence and validated with DNA from available fungal isolates, it is possible that we could detect unknown fungi resulting in false positive hits. Despite this, we promote the assay as a rapid and cost-effective screening tool to identify soils to more thoroughly investigate. For example, in the current study a majority of samples were negative for the two screening approaches, significantly reducing the number of samples that would be further processed using culture-based methods. Thus, the successful detection of *Coccidioides* with CocciEnv will be used as our screening method for future soil sample collections to identify novel sites for *Coccidioides* ecology research, recovery of viable organisms, and epidemiological information.

Supplementary material

Supplementary data are available at *MMYCOL* online.

Acknowledgments

Thanks to Adina Doyle, Remy Hilsabeck, Lela Andrews, Stephanie Rivas, Jason Travis, Dr. Eric Lewis, and Kevin Dickinson for technical support. John Taylor kindly provided select DNA samples. This work was supported by an internal Northern Arizona University TRIF grant (1002378) and Arizona Biomedical Research New Investigator Grant (ADHS16-162415) to BMB and Centers for Disease Control and Prevention (contract no. 200201461029) to DME.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Flynn NM, Hoeprich PD, Kawachi MM et al. An unusual outbreak of windborne coccidioidomycosis. *N Engl J Med*. 1979; 301: 358–361.
2. Pappagianis D, Einstein H. Tempest from Tehachapi takes toll or Coccidioides conveyed aloft and afar. *West J Med*. 1978;129: 527–530.
3. Schneider E, Hajjeh RA, Spiegel RA et al. A coccidioidomycosis outbreak following the Northridge, Calif, earthquake. *JAMA* 1997; 277: 904–908.
4. Talamantes J, Behseta S, Zender CS. Fluctuations in climate and incidence of coccidioidomycosis in Kern County, California: a review. *AnnN Y Acad Sci*. 2007;1111: 73–82.
5. Litvintseva AP, Marsden-Haug N, Hurst S et al. Valley fever: Finding new places for an old disease. *Coccidioides immitis* found in Washington State soil associated with recent human infection. *Clin Infect Dis*. 2015; 60: E1–E3.
6. Lewis ER, Bowers JR, Barker BM. Dust devil: the life and times of the fungus that causes valley fever. *PLoS Pathogens*. 2015; 11: e1004762.
7. Barker BM, Tabor JA, Shubitz LF, Perrill R, Orbach MJ. Detection and phylogenetic analysis of *Coccidioides posadasii* in Arizona soil samples. *Fungal Ecol*. 2012; 5: 163–176.

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024

8. Colson AJ, Vredenburgh L, Guevara RE, Rangel NP, Kloock CT, Lauer A. Large-scale land development, fugitive dust, and increased coccidioidomycosis incidence in the Antelope Valley of California, 1999–2014. *Mycopathologia*. 2017; 182: 439–458.
9. Baptista-Rosas RC, Hinojosa A, Riquelme M. Ecological niche modeling of Coccidioides spp. in western North American deserts. *Ann N Y Acad Sci*. 2007;1111: 35–46.
10. Brillhante RS, Moreira Filho RE, Rocha MF et al. Coccidioidomycosis in armadillo hunters from the state of Ceara, Brazil. *Mem Inst Oswaldo Cruz*. 2012; 107: 813–815.
11. Lacy GH, Swatek FE. Soil ecology of *Coccidioides immitis* at Amerindian middens in California. *Applied Microbiol*. 1974; 27: 379–388.
12. Swatek FE, Omieczynski DT. Isolation and identification of *Coccidioides immitis* from natural sources. *Mycopathologia*. 1970; 41: 155–166.
13. Wanke B, Lazera M, Monteiro PC et al. Investigation of an outbreak of endemic coccidioidomycosis in Brazil's northeastern state of Piaui with a review of the occurrence and distribution of *Coccidioides immitis* in three other Brazilian states. *Mycopathologia*. 1999; 148: 57–67.
14. Greene DR, Koenig G, Fisher MC, Taylor JW. Soil isolation and molecular identification of *Coccidioides immitis*. *Mycologia*. 2000; 92: 406–410.
15. Baptista-rosas RC, Catalán-dibene J, Romero-olivares AL, Hinojosa A, Cavazos T, Riquelme M. Molecular detection of *Coccidioides* spp. from environmental samples in Baja California: linking valley fever to soil and climate conditions. *Fungal Ecol*. 2012; 5:177–190.
16. Lauer A, Baal JD, Baal JC, Verma M, Chen JM. Detection of *Coccidioides immitis* in Kern County, California, by multiplex PCR. *Mycologia*. 2012; 104: 62–69.
17. Aslanzadeh J. Preventing PCR amplification carryover contamination in a clinical laboratory. *Ann Clin Lab Sci*. 2004; 34: 389–396.
18. Zhang T, Fang HH. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl Microbiol Biotechnol*. 2006; 70: 281–289.
19. Saubolle MA, Wojack BR, Wertheimer AM, Fuayagem AZ, Young S, Koeneman BA. Multicenter clinical validation of a cartridge-based real-time PCR system for detection of *Coccidioides* spp. in lower respiratory specimens. *J Clin Microbiol*. 2018; 56: e01277–17.
20. Chow NA, Griffin DW, Barker BM, Loparev VN, Litvintseva AP. Molecular detection of airborne *Coccidioides* in Tucson, Arizona. *Med Mycol*. 2016; 54: 584–592.
21. Comrie AC. Climate factors influencing coccidioidomycosis seasonality and outbreaks. *Environ Health Perspect*. 2005; 113: 688–692.
22. Comrie AC, Glueck MF. Assessment of climate-coccidioidomycosis model: model sensitivity for assessing climatologic effects on the risk of acquiring coccidioidomycosis. *Ann N Y Acad Sci*. 2007; 1111: 83–95.
23. Tamerius JD, Comrie AC. Coccidioidomycosis incidence in Arizona predicted by seasonal precipitation. *PloS One*. 2011; 6: e21009.
24. Engelthaler DM, Roe CC, Hepp CM et al. Local population structure and patterns of western hemisphere dispersal for *Coccidioides* spp., the fungal cause of valley fever. *MBio*. 2016; 7: e00550–16.
25. White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A guide to methods and applications*. Cambridge, MA, USA: Academic Press; 1990: 315–322.
26. Taylor DL, Walters WA, Lennon NJ et al. Accurate estimation of fungal diversity and abundance through improved lineage-specific primers optimized for illumina amplicon sequencing. *Appl Environ Microbiol*. 2016; 82: 7217–7226.
27. Rohland N, Reich D. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Res*. 2012; 22: 939–946.
28. Bowers JR, Lemmer D, Sahl JW et al. KlebSeq, a diagnostic tool for surveillance, detection, and monitoring of *Klebsiella pneumoniae*. *J Clin Microbiol*. 2016; 54: 2582–2596.
29. Colman RE, Schupp JM, Hicks ND et al. Detection of low-level mixed-population drug resistance in *Mycobacterium tuberculosis* using high fidelity amplicon sequencing. *PLoS One*. 2015; 10: e0126626.
30. Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics*. 2014; 30: 614–620.
31. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014; 30: 2114–2120.
32. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 2012; 9: 357–359.
33. Milne I, Stephen G, Bayer M et al. Using Tablet for visual exploration of second-generation sequencing data. *Brief Bioinform*. 2013; 14: 193–202.
34. Li Z, Chaturvedi V. The powers and perils of PCR in the search for the natural reservoirs of *Coccidioides* species. *Mycopathologia*. 2017; 182: 435–438.
35. Nguyen C, Barker BM, Hoover S et al. Recent advances in our understanding of the environmental, epidemiological, immunological, and clinical dimensions of coccidioidomycosis. *Clinical Microbiol Rev*. 2013; 26: 505–525.
36. CDC. Increase in reported coccidioidomycosis—United States, 1998–2011. *MMWR Morb Mortal Wkly Rep*. 2013; 62: 217–221.
37. Benedict K, Purfield AE, Mohle-Boetani J, Wheeler C, Park BJ. Awareness and environmental exposures related to coccidioidomycosis among inmates at two California prisons, 2013. *J Correct Health Care*. 2016; 22: 157–163.
38. Marsden-Haug N, Hill H, Litvintseva AP et al. *Coccidioides immitis* identified in soil outside of its known range—Washington, 2013. *MMWR Morb Mortal Wkly Rep*. 2014; 63: 450.
39. Daniel C, Behm M, Ohman M. The role of Alu elements in the cis-regulation of RNA processing. *Cell Mol Life Sci*. 2015; 72: 4063–4076.
40. Flavell AJ, Pearce SR, Heslop-Harrison P, Kumar A. The evolution of Ty1-copia group retrotransposons in eukaryote genomes. *Genetica*. 1997; 100: 185–195.
41. Llorens C, Munoz-Pomer A, Bernad L, Botella H, Moya A. Network dynamics of eukaryotic LTR retroelements beyond phylogenetic trees. *Biol Direct*. 2009; 4: 41.

Downloaded from https://academic.oup.com/mmy/article/57/2/246/4925226 by guest on 23 April 2024