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Detection and phylogenetic analysis of Coccidioides posadasii in Arizona soil samples

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\textbf{A B S T R A C T}

Reports of coccidioidomycosis are on the rise in the southwestern US. However, the ecology of the pathogen, Coccidioides, remains obscure and there is limited knowledge of the environmental antecedents of disease outbreaks. Detection of the fungus in the environment remains a critical challenge to modeling the source of disease. Using BALB/c mice as a biosensor, 8.9 % of soils analyzed from the Tucson area (Pima County, Arizona) were found to contain the pathogen. The genotypes of 66 Coccidioides strains, recovered from 11 soils, were determined with diagnostic microsatellite loci. Comparison of these genotypes to clinical isolates revealed all were \textit{Coccidioides posadasii} and they grouped with Arizona isolates. Among sites where multiple strains were recovered, two indicated a clonal population, while others yielded a diversity of genotypes. A secondary goal of this research was to assess applicability of PCR, with its potential for high-throughput screening, as a method for identifying Coccidioides-containing soils.

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\textbf{Introduction}

\textit{Coccidioides immitis} and \textit{Coccidioides posadasii} are dimorphic fungi that change from a saprobic mycelial phase in soil into a parasitic spherule phase when arthroconidia are inhaled by a mammalian host (\textit{Cole & Sun 1985}). Coccidioidomycosis, a disease that can be severe in humans and other animals, can result from this exposure. Approximately 60 % of human infections are asymptomatic. Thirty percent result in a range of symptoms that can persist for months but are ultimately self-limiting. Fewer than 10 % of patients develop complications that require medical intervention. However, these cases may require lifelong therapies, or result in death. \textit{C. immitis} is proposed to be endemic to the semiarid desert areas of the central valley and southern deserts of California, and possibly into Baja California, whereas \textit{C. posadasii} is proposed to be endemic to southern Arizona, New Mexico, western Texas, northern Mexico and parts of Central and South America (\textit{Fisher et al. 2002}).
Based on human infection and positive skin test rates in the US, the highest exposure to Coccidioides is in the southern portion of the central valley of California (Kern and Tulare counties) and the Sonoran desert areas of southern and central Arizona (Pima, Pinal and Maricopa counties) (Fig 1; Ajello 1971; Kirkland & Fierer 1996; Pappagianis 1988). There has been a steady increase in reported cases of coccidioidomycosis with 1551 cases reported to the Arizona Department of Health Services in 1998, 5535 reported in 2006 and over 10000 reported in 2009 (Komatsu et al. 2003; Sunenshine et al. 2007; Hector et al. 2011). Despite this increase, and the associated interest due to the economic and human costs of coccidioidomycosis, little is known about the environmental source of the inoculum. On a regional scale, the major predictors of disease are climate, soil disturbance, and dust/wind events (Pappagianis 1994; Pappagianis et al. 1994; Comrie 2005; Comrie & Glueck 2007). However, at finer scales, the ecology of the fungus remains obscure and environmental antecedents of the disease are largely unstudied (Cox & Magee 2004). Reports have suggested it may be associated with alkaline soil that has a high salt content, rodent burrows and Amerindian middens at archeological sites; however, little is known about the specific niche of Coccidioides in the soil or how environmental factors impact risk of human infection (Galgiani 1999).

Previous studies of the ecology of Coccidioides, conducted primarily between the 1930s and the mid 1970s, demonstrated that it was difficult to isolate the fungus from soil. Overall reported recovery rates from soil are low, from 0% to 15% (Elconin et al. 1957; Maddy 1958; Swatek et al. 1967; Swatek &
Detection of \textit{C. posadasii} in soil

Omiecinski 1970; Greene et al. 2000. Historically, isolation of \textit{Coccidioides} from environmental samples entailed plating soil suspensions directly on media or inoculation of susceptible laboratory animals with soil suspensions (Stewart & Meyer 1932; Elconin et al. 1964; Maddy 1959; Greene et al. 2000). General conclusions from these previous studies are that \textit{Coccidioides} occurs in disjunct distributions in the soil, and it is isolated sporadically over time from the same site, usually associated with periods of drought after a rainy season. However, most of these studies were done prior to the availability of genetic markers that allow comparison of the strains repeatedly isolated from the same sites or with patient strains.

The goal of the current research was to isolate environmental strains of \textit{Coccidioides} from a limited geographic region in order to use modern methods of strain genotyping to compare them to clinical strains from the same region. This will improve our understanding of the population biology of this fungus by adding the genotypes of strains from the soil. We assessed three methods for identifying soil sites that contain \textit{Coccidioides}. We determined the genotypes of 66 isolates of \textit{Coccidioides} obtained from soil samples in the Tucson basin and compared these to the genotypes of previously characterized isolates (Fisher et al. 2002), to assign species and population to each isolate.

\section*{Methods}

\subsection*{Soil sampling, physical and chemical analyses}

More than 700 soil samples were collected in and around Tucson, Arizona. Samples were collected (1) from public/nonresidential property in proximity to addresses of seropositive dogs; (2) in the yards at residences of participants in a valley fever epidemiology study; (3) based on different soil types in the Tucson area; (4) from locations where patients suspected to have been exposed to the agent and acquired infection; or (5) from sites previously identified as \textit{Coccidioides}-positive (Converse & Reed (1966) and samples 572–583). These sites represent variation in soil type, physiographic position on the landscape, sample depth, proximity to plant species, and season of the year. Detailed descriptions of the soil and vegetation, along with GPS readings were recorded for each site. Most sites represented discrete locations, although in some cases multiple samples were collected at a single location. For example one set of samples, numbers 572–583, were collected based on proximity to a previous soil sample that tested positive for \textit{Coccidioides}. For this location, seven samples were collected from a 3 m long, 0.7 m deep trench and five were from the flood plain within 8 m of the trench along a medium-sized wash, with storm flows less than 56 m$^3$s$^{-1}$.

Soils were collected using a 7 cm diameter bucket auger or a small hand shovel sterilized in a 10 % bleach solution before sample collection. Soil samples were air-dried before storage at room temperature. Subsamples from a small number of sites were autoclaved before physical and chemical analysis ($n = 44$) by the University of Arizona Soil, Water and Plant Analysis Laboratory. Eleven of these 44 samples were identified as \textit{Coccidioides}-positive. The 33 \textit{Coccidioides} negative samples were selected to provide a representative range in soil physical and chemical characteristics for the study area. Thirty-one of these were selected by proximity to \textit{Coccidioides}-positive sites and six were selected to represent common soil types typical of Tucson, Arizona. All soils were analyzed for: electrical conductivity using a conductivity meter with 1:1 water extract; pH by ion electrode with 1:1 water extract; Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$ soluble cations by atomic absorption spectrometer using 1:1 water extract; cation exchange capacity by Mg(NO$_3$)$_2$ extraction; total N and total organic carbon by Walkley–Black high temperature combustion using a NCS analyzer; available B by hot water extraction; CaCO$_3$ equivalent by rapid titration; percent sand, silt and clay (USDA texture classification) by hydrometer method; water soluble F$^-$, Cl$^-$, NO$_3^-$, Br$^-$, NO$_4^-$, PO$_4^{3-}$ and SO$_4^{2-}$ by ion chromatography using 1:1 water extract; and water soluble KH$_2$O by inductively coupled plasma spectrometry using 1:1 water extract (Klute & Page 1982; Sparks 1996). Association of \textit{Coccidioides}-positive samples with soil characteristics was evaluated using logistic regression analysis.

\subsection*{Extraction of viable arthroconidia from soil for mouse injection and plating}

A total of 124 soil samples from 108 locations were prepared for injection into mice. Soil suspensions were prepared using a modification of the salt flotation method of Stewart & Meyer (1932). Five grams of soil were hydrated with 25 ml of a 30 % NaCl/0.01 % Tween 80 solution in a 50 ml centrifuge tube (BD-Falcon). Tubes were shaken thoroughly to break up soil particles and ensure complete suspension, and then aliquots of the supernatant and of the pellets on agar media were allowed to settle for 30 min. Twenty milliliters of the supernatant were pipetted off and 10 ml placed in each of two 50 ml centrifuge tubes. Forty milliliters of sterile distilled water (dH$_2$O) were added and the diluted saline suspensions were centrifuged at 3 200 g for 40 min at 4 °C. The pellets were resuspended in 25 ml sterile dH$_2$O and centrifuged as before. To determine the effect of settling time and exposure of arthroconidia to the saline solution, 10$^5$ arthroconidia in the pellet and that variation in settling time and exposure of arthroconidia to the saline solution, 10$^5$ arthroconidia were inoculated into 20 ml of the 30 % NaCl/0.01 % Tween 80 solution, shaken, and allowed to settle for 15, 30, 45 or 60 min. Arthroconidia were harvested according to Kellner et al. (2005) and counted using a hemacytometer; viable counts were determined by plating. Spiked saline suspensions were centrifuged as above and the supernatants decanted, followed by resuspension of the pellets in sterile dH$_2$O. Incubation of aliquots of the supernatant and of the pellets on agar media determined that the procedure resulted in recovery of most arthroconidia in the pellet and that variation in settling time in the 30 % NaCl solution had no effect on viability.

Suspensions for mouse inoculation were prepared as described above, except 20 000 U ml$^{-1}$ penicillin G (Sigma–Aldrich) and 300 μg ml$^{-1}$ streptomycin (Sigma–Aldrich) were added to each 10 ml quantity of supernatant drawn off from the 30 % saline hydration step and allowed to incubate for 30 min at room temperature prior to dilution with 40 ml distilled water and centrifugation. The two pellets from the original soil inoculation were combined into a single tube and washed again in 25 ml sterile distilled water. The final pellet
was resuspended in 1 ml sterile 0.9 % saline with 5 000 U ml$^{-1}$ penicillin G and 30 μg ml$^{-1}$ streptomycin for injection into two mice (0.5 ml/mouse).

For plating the soil extracts, a modified double-pour protocol from Swatek & Omieczynski (1970) was used. The final pelleted was suspended in 1 ml dH$_2$O and 0.25 ml was placed in each of four 100 mm culture plates (BD-Falcon 1050). Yeast extract top agar (30 ml of 0.5 % yeast extract, 1.5 % agar) was cooled to 50 °C and then cycloheximide (to 1 000 μg ml$^{-1}$ (AG Scientifics)), streptomycin (to 200 μg ml$^{-1}$) and chloramphenicol (to 100 μg ml$^{-1}$) were added and the mixture was poured over the soil extract. Two plates were incubated at ambient temperature ($\sim$24 °C) and two plates were incubated at 37 °C. Putative Coccidioides colonies were subcultured to fresh yeast extract media, and incubated at ambient conditions. All manipulations to soil samples and to viable cultures were performed in a Select Agent biosafety level 3 (BSL3) laboratory, either at the Southern Arizona Veterans Administration Health Care System, or in the University of Arizona Keating Building, using standard operating procedures developed for working with this Select Agent and approved by the Centers for Disease Control (CDC).

**Mouse inoculation and analysis**

Eight week old, female BALB/c mice were obtained from Harlan-Sprague-Dawley (Indianapolis, IN) and maintained in pans under microisolator hoods in a BSL3 laboratory according to NIH guidelines for care and use of laboratory animals. The University of Arizona Institutional Animal Care and Use Committee approved all procedures. BALB/c mice were used because they are reported to be the most sensitive mouse to coccidioidal infection, with approximately 50 arthroconidia injected intraperitoneally being a lethal dose for 90 % of the inoculated animals (Kirkland & Fierer 1983; Abuodeh et al. 1999). The mice were injected intraperitoneally with 0.5 ml of soil suspension extracted as described above. For each soil sample, two mice were inoculated and housed together. After injection, mice were observed once or twice daily for the first 3–4 d, then daily thereafter until euthanasia. In initial experiments, some mice died within 6 d of inoculation due to a toxic inflammation resulting from the injection. No microbial growth was recovered from tissues of these mice. These post-inoculation deaths were prevented in subsequent tests by treatment with the anti-toxic, anti-inflammatory drug flunixin meglumine (2.5 mg kg$^{-1}$).

If mice looked ill or had documented weight loss of 3 g, they were sacrificed and the lungs and spleens harvested aseptically and individually into Whirl-Pak sterile specimen bags (Nasco, Ft. Atkinson, WI) prior to culturing. Select tissue specimens — portions of lungs, spleens, or abdominal abscesses at injection sites — from ill mice with grossly apparent lung lesions, were fixed in 10 % formalin and examined histopathologically. At 21 d post-inoculation, remaining mice were euthanized and any evidence of disease was recorded. Lungs and spleens were collected for culture even if animals looked grossly normal.

To test whether our extraction and inoculation protocol could result in mouse infection, a sterile autoclaved soil sample (491A) was inoculated with 10$^6$ arthroconidia in 10 g of soil and arthroconidia extracted as described above, except that the final pellet was resuspended in 2.5 ml saline with antibiotics. Part of the resulting soil extract was injected into two mice in each of three experiments as controls (0.5 ml/mouse). The remaining soil suspension was plated using the double-pour method described. Five of the six inoculated mice were euthanized due to illness and weight loss prior to 21 d post-inoculation. Granulomatous and abscess-like lesions consistent with coccidioidal infection were identified in these five mice, fungal colonies grew from culture plates, and histopathology confirmed coccidioidomycosis. For the extract that was plated, approximately 100 colonies grew from 300 μl of soil suspension.

To screen murine tissues for fungus after euthanasia, 1 ml of 0.9 % saline was added to each Whirl-Pak bag containing an organ and the tissue was crushed using the flat end of a syringe plunger, resulting in a homogeneous suspension. The macerated tissue was plated on 100 mm Petri plates containing 1 % Difco$^\text{®}$ yeast extract (BD, USA), 2 % glucose, 1.5 % Difco Agar$^\text{®}$, 1 000 μg ml$^{-1}$ cycloheximide (AG Scientifics, San Francisco) and 200 μg ml$^{-1}$ of chloramphenicol (Sigma, USA). Any fungal colony that appeared was subcultured and incubated at 28 °C. Only one isolate per organ, or inoculation site, was retained. Microscopic analysis and DNA extractions were performed after growth for 2 weeks on all cultures. The first putative positive strain was analyzed using the Accuprobe Coccidioides immittis Culture Identification Test (Gen-Probe, San Diego), and arthroconidia were harvested and injected into mice to demonstrate pathogenicity. Other strains were verified as Coccidioides by molecular methods, which confirmed they were different from standard lab strains.

The sensitivity of detecting Coccidioides by inoculation of BALB/c mice with soil extracts was calculated using a binomial probability distribution. Sensitivity means the proportion of actual positive soils that are correctly identified. Calculations were done following additional testing of the soils determined to be Coccidioides-positive in the initial screenings. Four more mice were inoculated with extracts from two additional 5 g aliquots of each positive soil, prepared as described above. The results from four or six mice per sample (see Table 1) were pooled to calculate the probability of detecting Coccidioides in an arthroconidium-containing soil depending on the number of mice inoculated.

In addition to the 11 positive soils that were tested with six mice for the sensitivity estimate, 18 other soils that were negative when screened with two mice were subjected to further testing with an additional four mice. These were selected based on either their location near positive soils or because they were PCR-positive for coccidioidal DNA.

**DNA isolation, PCR and population structure analysis of fungal strains**

For each Coccidioides isolate, cultures were streaked to obtain single colonies. A single colony was transferred to a second plate and grown for 2 weeks. DNA was isolated by scraping mycelium from the culture as described in Kellner et al. (2005). The DNA concentration was checked using a spectrophotometer (Nanodrop, Wilmington, DE) and diluted to a final concentration of 10 ng μl$^{-1}$. To determine whether isolates
Detection of *C. posadasii* in soil

<table>
<thead>
<tr>
<th>Soil samplea</th>
<th>PCR conditionsb</th>
<th>ITSc</th>
<th>CocciC</th>
<th>Nestede</th>
<th>20 ng DNA spiked ITSf</th>
<th>Soil plating resultsg</th>
<th>Mouse inoculation resultsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>407</td>
<td>20 µg BSA, 1:8 dilution</td>
<td>4/4</td>
<td>4/4</td>
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<td>2/4</td>
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<tr>
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<td>0/20</td>
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<td>0/20</td>
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<td>0</td>
<td>2/4</td>
<td>nd</td>
<td>nd</td>
<td>1/6</td>
</tr>
</tbody>
</table>

a Sample referenced by soil collection site.

b PCR conditions producing positive results. Quantity of BSA and DNA dilution endpoint is indicated when different from standard protocol; standard conditions were unsuccessful.

c Results for the four extractions made from each soil using ITS primers (White et al. 1990), which amplify any fungal DNA present.

d Results for the four extractions made from each soil using C1A and ITS C2 (Greene et al. 2000), which amplify *Coccidioides* DNA.

e Results for the four extractions made from each soil using the ITS PCR product as template for C1A and ITS C2 (Greene et al. 2000).

f Results for the four extractions made from each soil using ITS primers after spiking the extract with *C. posadasii* DNA, to test for PCR inhibitors.

g Soil plating results from colonies recovered from the mouse-positive soils. The denominator indicates the number of colonies analyzed per soil. The number of colonies positive per mouse-positive soil.

h Number of mice positive for *Coccidioides* per number tested.

obtained from mouse tissue were *Coccidioides*, 20 ng of genomic DNA was used for PCR with oligonucleotide primers ITS C1A and ITS C2 (Greene et al. 2000), which amplify a 228–237 bp fragment. PCR reactions were performed with 2 Master mix (Promega, Madison WI) under the following conditions: one cycle of 2 min at 96 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C, and one cycle of 5 min at 72 °C. Samples were visualized on agarose gels. Nine microsatellite primers developed for phylogenetic analysis and tested for concordance in *Coccidioides* (Fisher et al. 1999, 2000a) were used to genotype strains for comparison to laboratory strains. All microsatellite fragments were amplified using one cycle of 2 min at 96 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, and one cycle of 5 min at 72 °C with 2.5× Hotmaster mix (Eppendorf, USA) and 20 ng of genomic DNA. One primer from each set was end-labeled with a fluorescent tag (either NED dye from ABI, USA, or FAM or HEX dye from Eurogentec, USA).

Microsatellite fragments from each strain were grouped into three sets of three fragments for performing multiplex PCR for genotyping. One primer set in each grouping was labeled with HEX, FAM or NED, each corresponded to a different wavelength, so that no interference could confound the results. PCR products were separated on an ABI3730 at the University of Arizona GATC sequencing facility, using a ROX-labeled ladder for sizing. Chromatographs were read with GENOTYPER (ABI, USA), and highest peaks identified. Because *Coccidioides* is haploid, only one allele is present; thus only a single peak for each microsatellite was captured. Microsatellites were amplified and analyzed at least twice to verify their size. Additional online information was used to aid scoring *Coccidioides* microsatellites (http://nature.berkeley.edu/taylorlab/ftp/microsats.pdf).

To compare our strains to published data we analyzed the microsatellite sizes from a subset of previously analyzed isolates on our ABI3730 system (Fisher et al. 2002). Calibration was necessary to compare the published microsatellite sizes to our data (Table S1). Additionally, one microsatellite (GA1) did not amplify a band of the reported size, and it was investigated in greater detail. Analysis of sequence data for GA1 on the Broad website (http://www.broad.mit.edu/annotation/genome/coccidioides_group/MultiHome.html) indicated that the correct locus was identified. However, an incorrect primer sequence was published, resulting in a band of around 430 bp being amplified for GA1 instead of the published size of ~260 bp for *C. posadasii*. The correct primer sequence was no longer available from the authors. Thus, we utilized a different primer set to amplify this region, resulting in a ~245 bp fragment (Jewell et al. 2008).

Clone-corrected microsatellite data were analyzed with STRUCTURE 2.3.1 to determine if any population structure exists within *C. posadasii* (Hubisz et al. 2009). The running length of burnin period was 100,000 generations with 100,000 MCMC generations thereafter. The default settings for the STRUCTURE program were used such that it inferred alpha, which is the admixture parameter or the degree to which the populations exchange genes, and the same alpha was used for all populations. Allele frequencies are assumed to be correlated among populations, assuming different values of Fst for different subpopulations. Population values of K were set from one to ten, with ten replicates. Likelihood scores and AK were averaged for the ten replicates (Evanno et al. 2005). Probability assignments to populations were averaged across ten replicates. The same data were also analyzed using principal components analysis (PCA) in GENALEX 6.2 (Peakall & Smouse 2006). Three strains, Silveira, RS and an isolate from a Chinese patient H184 (Fisher et al. 2002), were removed from this analysis because GENALEX 6.2 was unable to assign them to any population with confidence.
Isolation and analysis of DNA from soils

One hundred seventeen soil samples were selected for DNA extraction for PCR analysis. DNA was extracted from the soils using the Ultra Clean® or UltraClean MegaSoil® DNA extraction kits (MoBio, Carlsbad, CA). Eighty-three of these samples were also tested in mice and 34 were not; soils were chosen from public sites near seropositive dog addresses, from yards of people participating in the epidemiologic study, from soil collected at a location positive in 1964, and from the intensively sampled trench/flood plain from which strains were isolated in the present study.

Soils were processed separately to avoid cross contamination. Soils were shaken in the storage containers to break up soil aggregates before DNA preparation. Four DNA isolations were performed for each soil sample with UltraClean® or UltraClean MegaSoil® kits. For UltraClean® kits, 1 g of soil was placed in the MoBio® bead tubes and for MegaSoil® kits, 5 g of soil was placed in the MoBio® bead tubes. Both sets were processed according to manufacturer’s instructions, with the following modifications. Tubes were vortexed briefly to mix and the Inhibitor Removal Solution (IRS) for PCR application was added to each tube. Tubes were heated at 65°C for 10 min before vortexing at maximum speed for 10 min using the vortex adapter (MoBio, Carlsbad, CA). Tubes were heated a second time at 65°C for 15 min. UltraClean® Soil DNA extracts were eluted with 50 μl of elution buffer (MoBio, Carlsbad CA), and 10 μl was used for visualization of DNA on an agarose gel. MegaSoil® soil DNA extracts were eluted with 8 ml of elution buffer (MoBio, Carlsbad CA), and 10 μl was used for visualization of DNA on an agarose gel.

To screen for the presence of Coccidioides DNA in extracted soil, extracts were diluted 1:2, 1:4, 1:6, 1:8 and 1:10 prior to PCR according to the manufacturer’s protocol (MoBio, Carlsbad, CA). PCRs were replicated three times using 2 μl of the dilutions of the DNA extracts, with bovine serum albumin (BSA) added (10 µg/reaction) to limit the impact of inhibitors found in some soils, as suggested by the study of Kreader (1996). In some reactions, BSA was increased to 20 or 30 µg/reaction as described (Table 1). Coccidioides sequences were screened either directly, using primers ITS C1A and ITS C2 (Greene et al. 2000), as described above, or in a nested reaction where first, a reaction was done with fungal rDNA primers ITS1 and ITS4 (White et al. 1990). In the nested reaction, a second PCR was done by diluting the PCR product 1:10 and using 2 μl of the ITS1–ITS4 product as a template with primers ITS C1A and ITS C2. Negative controls included water and nested water reactions to ensure no contamination had occurred. All reactions were set up in a UV hood to eliminate genomic DNA contamination.

To calibrate the sensitivity of our detection of Coccidioides DNA in soil, six samples representing common soil types in the Tucson area (Palos Verdes–Sahuarita complex, Mojave, Anthony, and Pinaleno soil series, Cochran & Richardson 2003) were sterilized and spiked with Coccidioides arthroconidia in three biological replicates. To sterilize the soils, 100 g were spread on aluminum foil in a metal pan, moistened with dH2O, covered with foil, and incubated at room temperature for 2 hr prior to autoclaving. The soil was then autoclaved for 30 min, cooled overnight and autoclaved again for 30 min. After cooling, the soil was stored in sterile 50 ml centrifuge tubes. Soils were tested for sterility by plating on GYE agar and no growth was observed. Sterile soils were inoculated with 1 ml suspensions of 101, 102, 5 x 102, 103, 104, 105 and 106 spores/g of soil. These soils were extracted and screened by PCR, as described above, with three replications for each reaction.

Results

Detection of Coccidioides in soil by inoculating mice with soil extracts

A total of 124 soils were extracted and screened in BALB/c mice for the presence of Coccidioides, resulting in the identification of 11 positive soils, or 8.9% of those tested (Table 1). C. posadasii was cultured from one or more tissues from 30 mice (Mandel et al. 2007). Of these, 16 died or were euthanized due to illness and weight loss between 8 and 21 d post-inoculation and all displayed granulomatous or abscess-like lesions consistent with coccidioidal infection. Portions of lungs and spleen from seven ill mice were submitted for histopathological evaluation, and disseminated coccidioidomycosis was confirmed. The other 14 culture-positive animals remained clinically healthy until their scheduled sacrifice at 21 d post-inoculation, though their lungs, spleens or abdomens had observable lesions that produced characteristic Coccidioides colonies in culture.

By preserving only a single colony from each plated mouse organ or lesion, sixty-six strains (Mandel et al. 2007) were recovered from the mice and verified as Coccidioides by PCR analysis with Coccidioides-specific primers ITS C1A and ITS C2 and as C. posadasii by microsatellite primers (Fisher et al. 1999; Greene et al. 2000). Only Coccidioides strains were recovered from lung tissue, although other fungi were occasionally isolated from the spleen and abdominal abscesses. Non-coccidioidal fungi were not further identified.

The estimated sensitivity of using BALB/c mice for detecting Coccidioides in soil samples per binomial probability was 0.483 per mouse (95% confidence interval: 0.355–0.614), 0.73 for two replicate tests, 0.86 for three replicates, 0.93 for four replicates, 0.96 for five replicates, 0.98 for six replicates, and 0.99 for seven replicates.

Detection of Coccidioides in soil by plating soil extracts

More than 200 soils (data not shown) were subjected to the spore extraction and double-pour plating method detailed above without recovering a single strain of Coccidioides by direct plating. A major reason for obtaining cultures directly from soil is that passing the extractions through mice selects for virulent strains and excludes recovery of possible avirulent or subvirulent strains in the environment. Following identification of soils that contained Coccidioides via mouse inoculation, those 11 samples were subjected to plating to determine whether strains could be recovered directly from known positive soils. Four hundred and forty colonies were subcultured from these plates and assessed for gross morphology similar to Coccidioides. Those appearing similar to Coccidioides...
were screened with Coccidioides-specific PCR primers. None of the colonies recovered were Coccidioides.

Plating of extracts from sterile soil to which arthroconidia had been added ($10^6$, $10^5$, $10^4$, $10^3$, $5 \times 10^2$ or $10^2$ spores/g of soil) resulted in a low rate of colony recovery (0.2 %) for all spore dilutions. No colonies grew when soils were spiked with 500 or fewer spores/g of soil. From these results, coupled with the low recovery rate of Greene et al. (2000), who directly plated more than 700 soils and recovered only 11 isolates from four separate soil samples, we conclude that directly plating soil with the methods currently available is a very low yield procedure and thus is a poor tool to survey soil for Coccidioides, either to determine soil that may have infected people or to perform ecological studies on Coccidioides spp.

Detection of Coccidioides in soils by PCR

To define the sensitivity of detection of Coccidioides in soil by PCR, six soils common in the Tucson area were spiked with different numbers of arthroconidia and extracts screened for Coccidioides rDNA sequences using Coccidioides-specific primers, ITS C1A and ITS C2, directly, or with a nested reaction as described above. When $10^3$ spores were added per gram of soil, Coccidioides DNA was detected in all samples using the Coccidioides-specific primers (Table 2). Sensitivity was increased when nested PCR was used to amplify the Coccidioides ITS sequences using the fungal-specific rDNA primer product as template. The nested reaction allowed detection of Coccidioides sequences in five of the six soils when only 500 spores had been added. The addition of BSA as well as dilution of the DNA improved PCR amplification.

To compare detection of Coccidioides in soils by PCR vs. the mouse assay, the 11 mouse-positive soils were screened for Coccidioides ITS sequences (Table 1). Only three of the 11 mouse-positive soils produced PCR products with the Coccidioides primers ITS C1A and ITS C2. Using nested PCR, coccidioidal DNA was detected in eight of the 11 soils, although when four replicate tests were performed, positive results occurred in two to four of the reactions. This is similar to the mouse results, in which mouse inoculations yielded from one to five infected animals out of six. Comparison of the mouse and PCR results did not show a correlation between the percentages of positive replicates in either assay. For example, soils 407, 485B and 500 all produced Coccidioides-specific products in 4/4 replicate extractions, whereas in mouse inoculations they produced positive results in 4/4, 5/6 and 1/4 mice respectively.

Mouse-positive soils 574, 578 and 582 produced negative PCR results even in nested reactions. For soils 574 and 578, this is likely due to co-extraction of high levels of inhibitors that the techniques used in these studies were unable to overcome, as both were PCR negative with fungal ITS primers, even when spiked with 20 ng of Coccidioides DNA. Soil 582 produced PCR results in only 1 of 4 ITS reactions and negative results with the nested reaction, but was positive in 4 of 4 reactions when spiked with 20 ng of Coccidioides DNA. Only 1 of 6 mice was positive for Coccidioides when inoculated with extracts from soil 582. This could indicate a low level of Coccidioides spores in the soil. The PCR results could indicate a low level of fungal matter in the soil and/or a low level of inhibitors that is overcome by the 20 ng of added DNA. Resolving the problem of enzyme inhibitors in soils is necessary before PCR can confidently be used to identify soil with coccidioidal DNA for environmental surveys.

Besides the 11 mouse-positive soils, an additional 106 soils were screened by PCR. Of these 106 soils, 72 were tested in mice with 16 being PCR-positive for coccidioidal DNA, but negative in the mouse assay. Direct plating of extracts of a number of these soils via the double-pour method, as with the 11 mouse-positive soils and others not subjected to PCR, resulted in no recovery of Coccidioides colonies (data not shown). Soils that are PCR-positive but mouse negative could indicate questions about the sensitivity of the mouse assay, the viability of putative Coccidioides in the soil, or the possibility of low virulence Coccidioides strains in the soil. The only definitive proof of Coccidioides in soils is the recovery of strains.

Physical and chemical characteristics of soils harboring Coccidioides

Chemical and physical analysis was performed on all positive and representative negative soils to determine if there were predictable characteristics associated with recovering Coccidioides from soil in the Tucson area. The data were analyzed using logistic regression (Table S2). Four variables (clay content, pH, chloride concentration and calcium concentration) were selected through stepwise analysis of all the variables at a 0.10 significance level with odds ratios of 9.450 ($p = 0.043$, 95% CI 1.3–6.63E+7) for each unit increase in pH, 0.48 ($p = 0.035$, 95% CI 0.24–0.95) for each percent increase in clay content, 0.98 ($p = 0.095$) for chloride ions (Cl$^-$ μg/g soil), and 1.03 ($p = 0.66$) for calcium ions (Ca$^{2+}$ μg/g soil). Two characteristics, higher soil pH, within the range found in Tucson soils of 6.9–8.0 and lower clay percentages among the Tucson soils which ranged from 3.8 % to 15.2 % were predictive for Coccidioides-positive soils. The 17 other soil characteristics measured did not show a strong association with Coccidioides-positive samples (sand, silt, total organic and total nitrogen content, conductivity, and levels of nitrate, nitrite,
phosphorus, sulphate, sodium, potassium, calcium, magnesium, boron and halogen ions other than chloride – Table S2).

Directed sampling along a 3 m trench (n = 8) dug at a site we identified as containing Coccidioides and the surrounding flood plain (n = 4) resulted in five positive soils out of 12 sampled. Positive samples were from the wall of a rodent tunnel, from soil collected around a 5 cm diameter live mesquite root (Prosopis velutina) as well as a 4 cm diameter dead mesquite root, and in the root zone of burroweed (Isocoma tenuisecta) just above a calcic horizon. Coccidioides was not detected from surface samples (0–2 cm) or below 25 cm deep. At another location, a positive soil was collected from a rodent hole at an animal pen site where Coccidioides had been isolated in 1964 (Converse & Reed 1966). Of the 11 soils from which the fungus was isolated, five were collected from rodent burrows. Coccidioides-positive sites are summarized in Table 3.

Population analysis

STRUCTURE 2.3.1 indicated that the Tucson soil isolates grouped with the overall Arizona population of C. posadasii, consistent with isolates previously characterized from Arizona patients by Fisher et al. (2002) (Fig 2). There was no observed population differentiation among Arizona isolates infecting humans and those found in soil in the Tucson area. Overall, Arizona C. posadasii formed one population, and Texas and South American isolates formed another population (Fisher et al. 2002). Isolates from Mexico are intermixed, containing signatures of both populations (Fig 2). Calculation of ΔK supports a more limited population structure within C. posadasii (Fig 3) than proposed previously (Fisher et al. 2001).

Principal components analysis performed in GENALEX 6.2 (Peakall & Smouse 2006) suggests that C. posadasii is divided into at least two subpopulations (Fig 4). Arizona patient and soil isolates grouped together, with Texas and South America forming the second group. A third subpopulation may exist, grouping Mexico and San Diego isolates separate from Texas and South America, but the sample size was not large enough to determine if this was a robust association. The exact boundaries of subpopulations in nature are unknown. Soil isolates analyzed from Tucson suggest that patient isolates mirror the environmental isolate genotypic profile; however, there is greater ambiguity in patient data because an exact location cannot be assigned to the strain origin.

Analysis revealed genetic diversity among the positive sites in the present study, similar to reports on previous population studies of clinical isolates (Burt et al. 1997; Fisher et al. 2002; Jewell et al. 2008). Interestingly, at site 409B, a diversity of genotypes was identified within the several isolates recovered from the three infected mice, with variation at six of nine microsatellite loci analyzed and the presence of both mating types in the recovered strains. Soil samples from sites 578 and 580 also contained strains with genetic variation, with differences at two microsatellite loci and both mating types present in the recovered strains (Mandel et al. 2007). In one mouse inoculated with soil from site 574, two genotypes were recovered, one from the site of injection, and another from the lung and spleen (Table S1, strains CPA0037, CPA0038 and CPA0039

### Table 3 – Site descriptions for locations with Coccidioides-positive soil samples, as determined by mouse biosensor

<table>
<thead>
<tr>
<th>Soil #</th>
<th>Site characteristics</th>
<th>Sample deptha (cm)</th>
<th>Soil textureb</th>
<th>Sample location in soil profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>407</td>
<td>Edge of wash, entrance to mouse hole, 30 % slope</td>
<td>0–20</td>
<td>Sandy loam</td>
<td>A horizon^c</td>
</tr>
<tr>
<td>409B</td>
<td>Native vegetation at end of divided road median, in mound of pocket mouse hole, 6 % slope, upland ridge</td>
<td>0–20</td>
<td>Sandy loam</td>
<td>A horizon</td>
</tr>
<tr>
<td>448</td>
<td>Upland, Harris antelope squirrel hole</td>
<td>0–10</td>
<td>Sandy loam</td>
<td>A horizon</td>
</tr>
<tr>
<td>485B</td>
<td>Imported sand, used as play area for child</td>
<td>0–20</td>
<td>Sand</td>
<td>A horizon</td>
</tr>
<tr>
<td>500</td>
<td>Catalina foothills, side slope, rocky soil approx. 20 % slope</td>
<td>0–15</td>
<td>Sandy loam</td>
<td>A horizon</td>
</tr>
<tr>
<td>573</td>
<td>Flood plain, edge of wash channel, edge of creosote limb drip zone</td>
<td>13–25</td>
<td>Sandy loam</td>
<td>Collected from wall of 5–10 cm diameter rodent tunnel</td>
</tr>
<tr>
<td>574</td>
<td>Flood plain, edge of wash channel, 50 cm from sample 573</td>
<td>12–23</td>
<td>Sandy loam</td>
<td>Collected from around a 5 cm diameter live mesquite root</td>
</tr>
<tr>
<td>578</td>
<td>Flood plain, edge of wash channel, approx. 1 m from sample 573</td>
<td>5–13</td>
<td>Sandy loam</td>
<td>Collected from around a 4 cm diameter dead mesquite root</td>
</tr>
<tr>
<td>580</td>
<td>Flood plain, edge of wash channel, in root zone of burroweed, approx. 1 m from sample 573</td>
<td>2–13</td>
<td>Sandy loam</td>
<td>Collected above a calcic soil horizon</td>
</tr>
<tr>
<td>582</td>
<td>Flood plain, edge of wash channel, entrance to 5 cm diameter rodent hole, approx. 11 m from sample 573</td>
<td>0–8</td>
<td>Sandy loam</td>
<td>A horizon</td>
</tr>
<tr>
<td>604</td>
<td>Flood plain of Santa Cruz, entrance to 7 cm diameter rodent hole, old animal pen #3 (Converse &amp; Reed 1966)</td>
<td>0–10</td>
<td>Sandy loam</td>
<td>A horizon</td>
</tr>
</tbody>
</table>

a Depth of soil sample in centimeters.
b US Department of Agriculture texture description based on composition of sand, silt, and clay.
c "A horizon" is the top soil, or surface soil horizon.
from site 574 mouse #1). A clonal population structure was observed at soil sites 407 and 485B, where multiple isolates were recovered from inoculated mice, with a single genotype recovered at each site (Table S1). At three sites (448, 500 and 604) only a single mouse was infected out of six mice inoculated and only the lung was infected, therefore only one strain was recovered for each soil sample. No conclusion regarding diversity at these three sites can be made. Finally, analysis of the samples recovered from the intensive sampling along the trench/flood plain (soils 572–583) revealed diversity within that location (less than 100 m²). Of the nine microsatellite loci analyzed, two (GAC2 and 621.2) had fixed allelic diversity in *C. posadasii* positive soils. Seven of the loci had allelic diversity rates of two to five alleles (Table 4).

**Discussion**

A major goal of this study was to identify soil sites that contain *Coccidioides* and recover environmental cultures of this fungus to use for comparative population analyses with clinical isolates. Initial efforts to isolate *Coccidioides* from soils by semi-selective plating, were unsuccessful. Therefore, to identify potential infected soils highly susceptible (BALB/c) mice were inoculated with extracts and coccidioidal strains were successfully recovered from several soils. The recovery rate of 8.9% from 124 soils was higher than reported rates for direct soil plating (Elconin et al. 1957; Maddy 1958; Swatek et al. 1967; Swatek & Omieczynski 1970 Greene et al. 2000) and the soil data are integrated with patient data. Population 1 (white bars) contains the entire Arizona patient and soil isolates and population 2 (black bars) contains the majority of Texas and South America isolates. Bars represent the percentage of times out of 100 000 that the isolate was placed in population 1 or 2. There is ambiguity with some strains from Mexico and Texas (STX–18TX), such that >20% of the replications placed those isolates in a different population. SA = South America, TX = Texas, AZ = Arizona, M1 = North Mexico (Sonora, Chihuahua, Coahuila), M2 = Central Mexico (Nuevo Leon, San Luis Potosi, Durango, Tamaulipas, Aguascalientes) M3 = South Mexico (Michoacan), SOIL = soil samples from Tucson area.
provided 66 isolates for genotypic analysis. By comparison, a recent study using direct plating, recovered 16 isolates from only four soils out of 720 sampled (Greene et al. 2000). The sixteen isolates were genotyped, but five were lost due to overgrowth by other fungi during subculture, thus becoming unavailable for further study. Recovery via mouse inoculation purifies Coccidioides from other environmental fungi that outgrow it in culture and provides a higher rate of success than selective plating.

We believe our successful rate of recovery of soil isolates was due in part to the choice of mouse model. The BALB/c mouse is reportedly the most sensitive mouse to coccidioidal infection, with approximately 50 arthroconidia injected intraperitoneally being a lethal dose for 90% of the animals inoculated (Kirkland & Fierer 1983; Abuodeh et al. 1999) and seven arthroconidia being a lethal dose during intranasal inoculation (Shubitiz et al. 2002). The estimated sensitivity of detection by mouse inoculation was calculated using binomial probability distribution based on results of four or six replicates in mice for the eleven positive soils. Although the sample size is small (30 positives from 62 mice tested), the results suggest a sensitivity of 0.98 for detecting Coccidioides in infected soil if six mice are tested. Even though inoculating more mice increases sensitivity, there are limited returns beyond six mice (7 mice = 0.99 sensitivity). The sensitivity of the mouse-method is likely overestimated due to the likelihood that some of the 113 soils determined to be negative contained Coccidioides.

Fig 3 – Analysis of the number of C. posadasii population clusters using $\Delta K$. The average and standard deviation of $\Delta K$ is represented for ten replications, where $K$ is the number of clusters defined for STRUCTURE. Estimation of two populations is supported because $\Delta K$ drops to nearly zero, and the standard deviation increases for any model above two populations, indicating that increasing the number does not improve the resolution of populations (Evanno et al. 2005).

Fig 4 – Principal component analysis shows strong division of C. immitis from C. posadasii (PC1 eigenvalue 1.08). There is one C. immitis (Mexico CI, SD and SJV) population, and three C. posadasii subpopulations, grouping the Mexico and San Diego patient isolates (Mexico CP, SD CP) together with the Texas/South America patient and environmental isolates (TX, SA), and the Arizona C. posadasii (AZ) patient isolates grouping with soil isolates (SOIL) collected from the Tucson area (PC2 eigenvalue 0.243).
Allelic diversity of positive sites

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>Size</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAC2</td>
<td>1 Alleles</td>
<td>206</td>
<td>1</td>
</tr>
<tr>
<td>621L</td>
<td>1 Alleles</td>
<td>398</td>
<td>1</td>
</tr>
<tr>
<td>GA37</td>
<td>4 Alleles</td>
<td>219 0.421</td>
<td>216 0.050</td>
</tr>
<tr>
<td></td>
<td></td>
<td>218 0.478</td>
<td>217 0.051</td>
</tr>
<tr>
<td>GA1</td>
<td>4 Alleles</td>
<td>244 0.061</td>
<td>245 0.777</td>
</tr>
<tr>
<td></td>
<td></td>
<td>246 0.050</td>
<td>247 0.112</td>
</tr>
<tr>
<td>ACJ</td>
<td>2 Alleles</td>
<td>187 0.664</td>
<td>185 0.336</td>
</tr>
<tr>
<td>K03</td>
<td>3 Alleles</td>
<td>236 0.695</td>
<td>238 0.274</td>
</tr>
<tr>
<td></td>
<td></td>
<td>256 0.031</td>
<td></td>
</tr>
<tr>
<td>K07</td>
<td>3 Alleles</td>
<td>299 0.677</td>
<td>293 0.269</td>
</tr>
<tr>
<td></td>
<td></td>
<td>301 0.054</td>
<td></td>
</tr>
<tr>
<td>K01</td>
<td>5 Alleles</td>
<td>236 0.480</td>
<td>234 0.397</td>
</tr>
<tr>
<td></td>
<td></td>
<td>244 0.056</td>
<td>238 0.030</td>
</tr>
<tr>
<td></td>
<td></td>
<td>246 0.036</td>
<td></td>
</tr>
<tr>
<td>K09</td>
<td>4 Alleles</td>
<td>148 0.906</td>
<td>146 0.070</td>
</tr>
<tr>
<td></td>
<td></td>
<td>140 0.024</td>
<td></td>
</tr>
</tbody>
</table>

Drawbacks to the use of animal inoculation include high cost, selection for pathogenic strains and length of time of experiments. Detection through a mouse requires fungal virulence, which may represent a subset of strains in the environment. In the studies reported here, several soils were PCR-positive for *Coccidioides* but did not result in recovery of strains via mouse inoculation, suggesting the possibility that those soils contained avirulent *Coccidioides*. Alternatively, the soils may have contained non-viable *Coccidioides* arthroconidia or hyphae, or *Coccidioides* DNA. Since we were also unable to recover any strains directly from selective plate culture, it remains unknown if all environmental strains of *Coccidioides* are pathogenic for mammals or if some may have limited host range. Murine infection by strains isolated from human patients suggests variable virulence (Friedman et al. 1955, 1956; Pappagianis et al. 1956; Cox & Magee 2004). Finally, recovery of strains via mouse injection takes up to 3 weeks from inoculation to plating of organ homogenates, followed by the time it takes to grow and purify strains for PCR and genotyping.

DNA extraction from soil followed by PCR using primers specific for *Coccidioides* is a procedure that would be very cost effective and allow rapid screening of many samples, thus allowing more extensive sampling of the environment. One challenge to this method is that many of the Tucson area desert soils tested have inhibitors for PCR that co-purify with the DNA. A second challenge was inconsistency between mouse and PCR detection: *Coccidioides* was detected by direct PCR in only three of the 11 mouse-positive soils, and by nested PCR in eight of those 11 soils. We do not know the concentration of *Coccidioides* arthroconidia in these soils, but preliminary experiments with nested PCR suggest that the pathogen was detected only when >500 arthroconidia per gram of soil were present in Tucson area soils. If hurdles such as soil inhibitors and inconsistent results could be overcome, it appears that nested PCR has potential for high-throughput screening of soils to identify putative *Coccidioides*-containing soils. The benefits to being able to widely map the presence of *Coccidioides* in the environment suggest that research into improving methods of DNA extraction from desert soil should be pursued despite the difficulties.

During revision of this paper, a report was published where soils around armadillo burrows in Brazil were screened for *Coccidioides* (de Macedo et al. 2011). They used both semi-nested PCR of 28S rDNA and inoculation into Swiss mice on 24 soils collected from two sites in burrows that were excavated by armadillo hunters who developed coccidioidomycosis 9–14 d after digging. Extracts of all 24 soils produced a *Coccidioides*-specific DNA fragment by semi-nested PCR, but only six of 24 soils were positive in the mouse assay. Sampling at sites where people had been digging prior to becoming ill likely contributed to their high rate of detection, in contrast to the majority of samples in the current studies that were based on proximity to addresses of seropositive dogs and people but not from specific locations associated with infection. The opposing sensitivity between PCR and mouse biosensor results in the two studies could be attributed to the relative resistance of Swiss mice compared to BALB/c mice to *Coccidioides* (Shubitz et al. 2008), or the presence of dead fungal material in the Brazilian soils. Additionally, it is possible that the armadillo burrow soils contained fewer PCR inhibitors than the Tucson area soils, allowing greater sensitivity in PCR detection. Alternatively there could be avirulent *Coccidioides* strains at these sites. Finally, the sensitivity of the semi-nested PCR assay using the 28S rDNA primers in terms of the limit of detection of *Coccidioides* arthroconidia in the Brazilian soils was not reported; 500 arthroconidia appeared to be the lower limit of detection using our PCR parameters on Tucson soil.

Direct semi-selective plating and analysis of fungal colonies from the 11 mouse-positive soils failed to yield any strains of *Coccidioides*. Despite the fact that *Coccidioides* cultures could not be isolated directly from soil in these studies, and this is historically a low yield process, the benefits to understanding both the ecology and the pathogenicity of this organism argue that research into reliable methods of recovering living isolates from soil should be pursued. Even with plating soil known to have *Coccidioides* via mouse infections, overgrowth by other fungal species on semi-selective medium resulted in a failure to isolate *Coccidioides* colonies. The 11 positive soils from these studies can be used to develop new methods of selective culture; modifying growth parameters and attempting to develop more selective media that give *Coccidioides* a growth advantage over other soil fungi are two obvious approaches.

The 66 *Coccidioides* strains isolated from mice in the present study were subjected to population genetic analysis. As anticipated, all environmental strains collected in the Tucson area were *C. posadasii* and grouped with other Arizona strains.
(Fig 2). Previously, all genotyped strains from Arizona were from patient cultures (Burt et al. 1997; Fisher et al. 2002; Jewell et al. 2008). Analysis of the strains isolated from the soil in the present study supports previous population designations, and shows that Arizona soils contain C. posadasii strains that are distinct from Texas, South America and Mexico strains (Fisher et al. 1999, 2001).

The present study shows that there is genetic variability among strains found at sites in the environment, with some sites yielding only clonal strains and others yielding a diversity of genotypes. Diverse strains were also found throughout a small area surrounding a positive site that was intensively sampled by digging a trench and collecting individual samples from several sites along or adjacent to the trench (samples 572–583, see Tables 1 and 3 for description of intensive sampling). Previous work also found two distinct genotypes in a single soil sample (Greene et al. 2000). One possible reason for this is that growth is not solely by clonal expansion but involves sexual reproduction as suggested by population genetic analysis of clinical strain genotypes (Burt et al. 1996; Fisher et al. 2000b). The presence of both mating types among strains from a single soil site (Mandel et al. 2007) demonstrated the proximity of mating partners in the environment.

A second possibility regarding the genetic variability is that arthroconidia are transported to a site by air, rainfall and runoff, rodent activity, or activity of humans and other animals. One of our samples, 485b, was isolated from soil that had been imported to the property. An argument for the possible role of rodents as either mechanical or biological transporters of fungal strains is the high rate of recovery from soil associated specifically with rodent habitation sites (Ashburn & Emmons 1942; Davis et al. 1942; Emmons 1942; Egeberg & Ely 1956; Elconin et al. 1957; Maddy 1958, 1959, 1965; Egeberg 1964; Reed & Converse 1965; Swatek et al. 1967; Smith 1972), including the recoveries in the study reported here. At the trench location where genetic diversity was identified, two of the five positive samples were acquired from the wall of a rodent tunnel, and overall, five of the 11 positive soil samples we identified were from rodent burrows (Table 3). No animal trapping was conducted at these positive sites, so there is no knowledge regarding rodent populations or communities. Further research could shed light on whether one or both of these mechanisms contribute to the diversity of strains and survival of C. posadasii throughout Arizona and within small sampling sites.

Although it is interesting that multiple genotypes were recovered within a single soil site, it is not entirely unexpected. However, we also observed variation within a single mouse, indicating multiple strains were inoculated. For soil 574, mouse #1, one genotype was recovered from the lung and spleen of the mouse, while a second genotype was obtained from the site of injection (Table S1). The injection site tissue produced a genotype that was similar to the strains recovered from this area in lung and spleen tissue of other mice, whereas the genotypes obtained from the spleen and lung tissue were unique and not present in any other soil tested. Some potential explanations for this observation are that there is: (1) a rare genotype present at the site, and it was recovered only once; (2) parasexual recombination; or (3) sexual recombination. Further investigation into the sexual life cycle of Coccidioides is needed to answer these questions.

In summary, these studies show that a susceptible mouse model is currently the most reliable tool for sampling the environment to identify Coccidioides, but that advantages of higher throughput and reduced cost are strong reasons to pursue direct soil PCR and improvements of direct plating methods. We confirmed genetic diversity at the level of the soil that has been previously reported from patient isolates, and found both mating types of Coccidioides at the same microsite. These findings suggest that investigation into the ecology of Coccidioides should continue, so that we may discover where and how it maintains itself and spreads in the environment. In endemic areas it would not be possible to completely avoid exposure as it is known that unusual soil disturbances and wind events, can lead to arthroconidial dispersal and infection (Flynn et al. 1979; Schneider et al. 1997). However, with greater knowledge of point sources of Coccidioides in the environment, it should be possible to describe areas where people are more likely to encounter high levels of the pathogen propagules in order for them to take appropriate precautions.

Acknowledgments

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Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.funeco.2011.07.010.

References


Detection of C. posadasii in soil


