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Molecular identification of *Sporothrix* species in a hyperendemic area in Peru

Max C. Ramírez-Soto[a,b,*,1], Elsa G. Aguilar-Ancori[c,d,1], María A. Quispe-Ricalde[c], Julia G. Muñiz-Duran[c], Mercedes M. Quispe-Florez[c,d], Aldo Chinen[c]

[a] *School of Public Health and Administration, Universidad Peruana Cayetano Heredia, Lima, Peru*
[b] *Facultad de Ciencias de la Salud, Universidad Tecnológica del Peru, Lima, Peru*
[c] *Facultad de Ciencias, Universidad Nacional de San Antonio Abad del Cusco (UNSAAC), Cusco, Peru*
[d] *Instituto Universitario de Enfermedades Tropicales y Biomedicina del Cusco–UNSAAC, Cusco, Peru*

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ABSTRACT

To date, there have been no molecular typing studies to identify the *Sporothrix* species circulating in Abancay, a hyperendemic area of sporotrichosis in Peru. To identify six clinical isolates of the *Sporothrix schenckii* complex from Abancay, Peru, we used PCR-sequencing of the calmodulin gene, and a phylogenetic analysis was conducted with these and additional sequences from GenBank. All clinical isolates were identified as *S. schenckii* (*sensu stricto*). Phylogenetic analysis revealed that the six clinical isolates from Abancay, Peru clustered in a clade along with sequences from Costa Rica, Iran, South Africa, and four other sequences from Peru. These findings reveal the presence of *S. schenckii* (*sensu stricto*) in Abancay, Peru.

Introduction

Sporotrichosis is a subcutaneous mycosis, caused by species of the genus *Sporothrix* [1]. Medically relevant species of *Sporothrix* include *S. schenckii* (*sensu stricto*), *S. globosa*, and the emerging species *S. brasiliensis* [2]. The plant material or felines are the main source of infection for *S. schenckii* and *S. brasiliensis*. *Sporothrix schenckii* (*sensu stricto*) has a wide geographical distribution worldwide [1,2]. This species has been isolated from the American Continent (USA, Mexico, Venezuela, Colombia, Argentina, Peru, and Brazil), Australia, Europe, and Asia (China, Iran, and Japan) [1,2]. Genome sequence analysis shows two highly supported clades associated with certain geographical regions [2]. Clade I includes the isolates from South America (Argentina, Bolivia, Colombia, Peru, and Venezuela) and the United States, while clade II includes isolates from Peru, Argentina, and South Africa [2]. In addition, the genetic diversity of *S. schenckii* (*sensu stricto*) strains varies from country to country and shows high rates of recombination [3–8].

Sporotrichosis is hyperendemic in Abancay, Peru, a poor area in the south-central highlands of Peru [9,10]. Since 1982, a steady increase in the number of cases has been reported, reaching a total of 1503 cases between 1985 and 2012 [10], with a mean annual incidence of 98 and 156 cases per 100,000 inhabitants among the general population and among children <15 years old [9]. Despite the high incidence rate of sporotrichosis in Abancay and the confirmed circulation of *S. schenckii* (*sensu stricto*) in Peru, to date, there are no data on the circulating species of *Sporothrix* in Abancay. To fill this research gap, in this study, we identified clinical isolates of the *S. schenckii* complex from Abancay, Peru using PCR sequencing of the calmodulin gene and performed a phylogenetic analysis with these and additional sequences from GenBank.

Materials and methods

Clinical samples

Six clinical isolates of the *S. schenckii* complex from Abancay, Peru were identified previously as *Sporothrix* spp. based on the macroscopic and microscopic characteristics of the culture in Sabouraud agar (Merk, Alemania). Conversion to the yeast-like form was performed on brain heart infusion (BHI) agar media for 7 days at 37 °C. Strains were stored in the culture collection of

* Corresponding author at: School of Public Health and Administration, Universidad Peruana Cayetano Heredia, Lima, Peru.
E-mail addresses: maxcrs22@gmail.com, max.ramirez@upch.pe (M.C. Ramírez-Soto).
[1] These authors contributed equally to this work.

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the Microbiology Laboratory of School of Sciences, Universidad Nacional de San Antonio Abad del Cusco, Cusco, Peru.

Amplification and sequencing

The total genomic DNA of six *Sporothrix* isolates was extracted and purified from a mushroom culture in an RPMI medium with an antibiotic (Gentamicin 50 mg) for 7 days at 25 °C under constant stirring of 110 rpm. Then, the culture was centrifuged at 8000 g for 20 min at 4 °C, the supernatant was removed, and the pellets were distributed into 1.5 mL microcentrifuge tubes and stored at −70 °C until use. DNA extraction and purification were performed using a combination of two extraction methods applied by van Burik et al. [11]. The extraction method used grinding and CTAB, as well as glass beads and vortexing. We placed 300 μL of the sample in a pre-cooled sterile mortar and pestle, froze the sample with liquid nitrogen, and then ground the sample to a fine powder. We then transferred the sample to a microcentrifuge tube and suspended the sample in 600 μL of a 1% CTAB extraction buffer and incubated the tube on ice for 1 h. We next added 400 μL of phenol/chloroform/isoamyl alcohol (24:24:1, v/v) and applied continuous vortexing for 30 min at the highest intensity setting. The aqueous layer was removed and placed in another microcentrifuge tube, followed by a second wash with phenol/chloroform/isomyl alcohol (24:24:1, v/v) with continuous vortexing for 30 min at the highest intensity setting. The aqueous layer was removed and placed in another microcentrifuge tube. Then, an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and vortexed for 30 min at the highest intensity setting. The aqueous layer was then removed and precipitated with 0.1 volume of ammonium acetate 3 M and 2.0 volume of 100% cold ethanol and incubated at −20 °C for two hours. Next, we centrifugated the mixture at 13,000 g for 10 min, discarded the supernatant, added 100 μL of T.E. buffer and 1 μL of 500 μg/mL Rnase, and then incubated the solution at 37 °C for one hour.

The isolated DNA was measured by a nanospectrophotometer DS-11 (Denovix), and 10 ηg was subjected to PCR amplification of the CAL gene fragment using CL1(5′- GA(GA)T(AT)C AAG GAG GCC TTC TC -3′) and CL2A (5′- TTT TTG CAT CAT GAG TTG GAC -3′) primers, as described by Oliveira et al. [12]. The PCR mix in a final volume of 50 μl contained 1.5 mM of $MgCl_2$, 10 pmol of each primer (Integrated DNA technologies -IDT), 200 μM dNTP (Promega), 1x buffer reaction, and 0.5U of Green Taq DNA Polymerase (GenScript). The annealing temperature was 60 °C in a Verity 96 thermal cycler (Applied biosystems). The 800 pb fragment was purified with a QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions. Next, sequencing on both strands, with the same primers used for PCR, was performed in Macrogen (Spain). All sequences obtained in this study were deposited in GenBank database. The results were compared with published sequences in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

The phylogenetic analysis was inferred using the Neighbor-Joining method [13]. The optimal tree with a sum of branch length = 0.56383135 is shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [14]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [15] and are presented in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 47 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option) for a total of 914 positions in the final dataset.

The evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model [15]. The tree with the highest log likelihood (−2201.45) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with the best log likelihood value. This analysis involved 47 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 669 positions in the final dataset.

The evolutionary history was inferred using the Maximum Parsimony (MP) method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed [14]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [14]. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in Ref. [17]) with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 47 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 669 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [16].

Results

The six sequences showed 98%–99% nucleotide sequence identity to CAL (CL1 and CL2A) from the *S. schenckii* (*sensu stricto*) type strain. Therefore, all clinical isolates were identified as *S. schenckii* (*sensu stricto*). We constructed a phylogenetic tree with the sequences of our study together with the reference sequences obtained from GenBank (Fig. 1). Our six sequences were grouped into a clade. This clade included sequences from Costa Rica, Iran, and South Africa, and four other sequences from Peru (Fig. 1). Two group sequences from Abancay (SPX5 and SPX6 and SPX25 and SPX3) formed a highly supported cluster, that was separate from the sequences from Costa Rica, Iran, South Africa, and the other sequences from Peru (Fig. 1).

Discussion

To date, molecular methods have been widely used for differentiating species within the *S. schenckii* complex [2–6]. In this study, we found that *S. schenckii* (*sensu stricto*) is circulating in Abancay province, Peru based on PCR-sequencing of the calmodulin gene. This finding is compatible with the studies showing that *S. schenckii* (*sensu stricto*) is prevalent in the American Continent (Colombia, Mexico, Argentina, Venezuela, USA, Bolivia, and Peru) and Europe (Portugal and Spain) but was also reported in Asia (Japan, China, Iran, and Malaysia), as well as South Africa [2–5,7,8,18].

Our findings also indicate that the six sequences of *S. schenckii* (*sensu stricto*) were grouped in a new clade along with the sequences from Costa Rica, Iran, and South Africa, and four other sequences from Peru. Unlike our findings, in the litera-

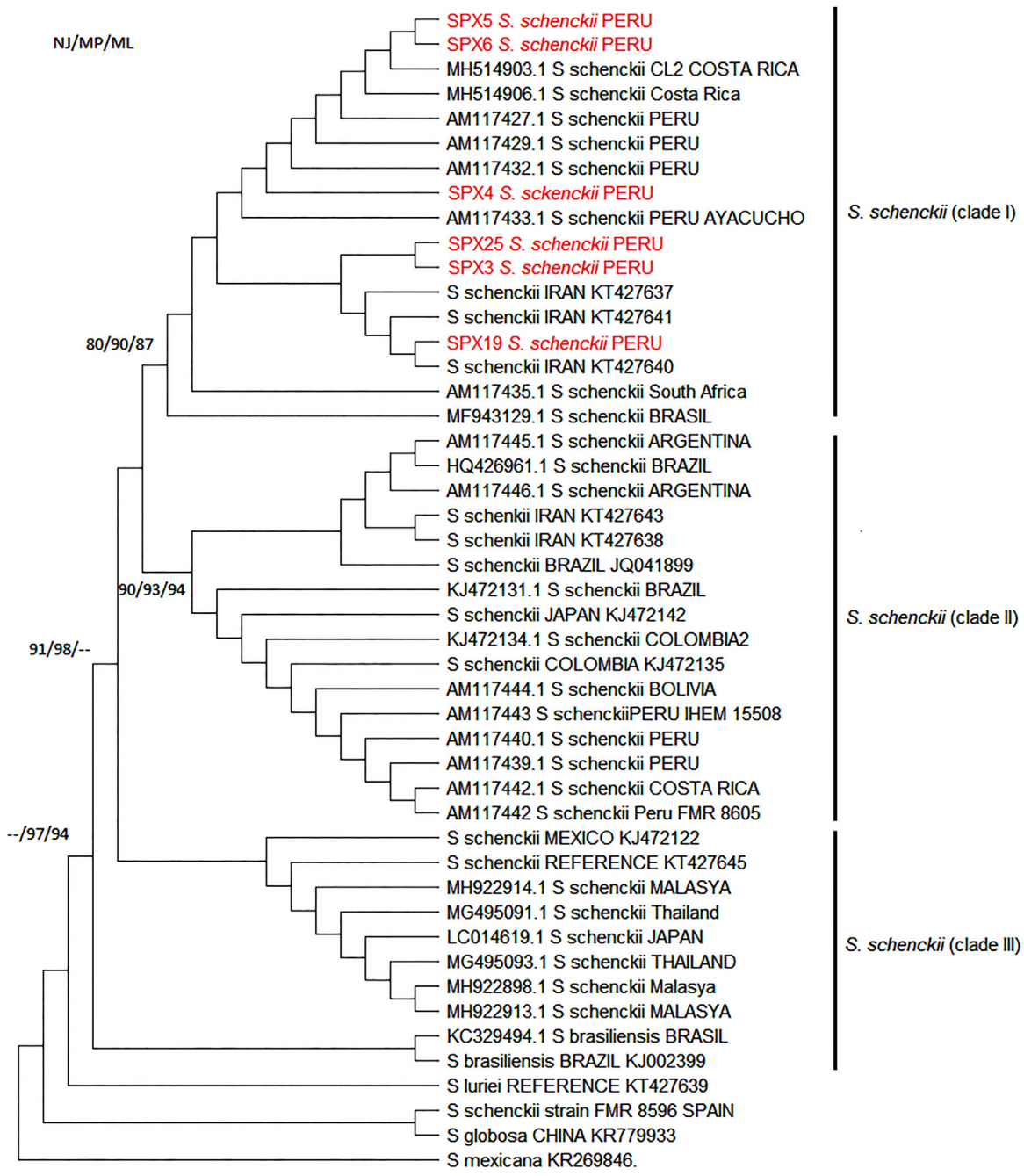

Fig. 1. Phylogenetic tree of *S. schenckii* (*sensu stricto*) in Abancay, Peru. Sequences included in the study (SPX3, SPX4, SPX5, SPX6, SPX19, and SPX25) are shown with the sequence references from GenBank. Letters to the right of the sequence identification indicate the species, followed by the country in the reference sequence. The MEGA software, version 6, was used with 1000 bootstrap replicates. Bootstrap values above >50% are shown above the branches.

ture, sequences of *S. schenckii* (*sensu stricto*) from Peru were grouped into clade I, along with sequences from Argentina, Bolivia, Colombia, and the United States, and in clade II, with sequences from Argentina and South Africa [2]. These clade differences are likely related to the genetic diversity of the *S. schenckii* (*sensu stricto*) sequences that we found in our study. Another explanation is that in hyperendemic areas such as Peru or Mexico, there is likely a high genetic diversity of *S. schenckii* (*sensu stricto*) [19,20], whereas in Brazil and China, there is a high genetic diversity of *S. brasiliensis* [6] and *S. globosa* [21,22], respectively. In addition, a study recently suggested that the *S. schenckii* complex has experienced a divergent evolution process, whereas *S. brasiliensis* could have been subject to a purifying selection or expansion process [20]. Therefore, more studies are required to determine if there is high genetic diversity of *S. schenckii* (*sensu stricto*) in Abancay.

In conclusion, our findings show that *S. schenckii* (*sensu stricto*) seems to be the predominant species in Abancay, Peru. However, due to the limited number of isolates studied, this result may not represent the true predominance of *S. schenckii* (*sensu stricto*) in Abancay province, Peru. Moreover, due to the epidemic of *S. brasiliensis* in Brazil [6] and the geographical distribution wide of *S. globosa* [21], the presence of these *Sporothrix* species in Abancay province cannot be ruled out. Therefore, further studies on clinical and environmental isolates are required to confirm this hypothesis.

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Competing interests

None declared.

Ethical approval

This study was approved by the Sciences School at Universidad Nacional de San Antonio Abad del Cusco, Peru.

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