Molecular Characterization of Pathogenic Members of the Genus *Fonsecaea* Using Multilocus Analysis

Jiufeng Sun¹²³, Mohammed J. Najafzadeh³⁴⁵, Albertus H. G. Gerrits van den Ende³, Vania A. Vicente⁶, Peiying Feng³⁷, Liyan Xi¹, Gerrit S. De Hoog¹³⁴⁸

¹ Department of Dermatology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China, ² Department of Parasitology, Zhongsahn School of Medicine, Key Laboratory for Tropical Disease Control, Ministry of Education, Sun Yat-sen University, Guangzhou, Guangdong, China, ³ CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, ⁴ Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands, ⁵ Department of Parasitology and Mycology, and Cancer Molecular Pathology Research Center, Ghaem Hospital, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran, ⁶ Department of Basic Pathology, Federal University of Paranaí, Curitiba, PR, Brazil, ⁷ Department of Dermatology, Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China, ⁸ Peking University Health Science Center, Research Center for Medical Mycology, Beijing, China

Abstract

Members of the fungal genus *Fonsecaea* causing human chromoblastomycosis show substantial geographic structuring. Genetic identity of clinical and environmental strains suggests transmission from plant debris, while the evolutionary processes that have led to spatially separated populations have remained unexplained. Sequences of ITS, BT2, ACT1, Cdc42, Lac and HmgA were analyzed, either by direct sequencing or by cloning. Thirty-seven clinical and environmental *Fonsecaea* strains from Central and South America, Asia, Africa and Europe were sequenced and possible recombination events were calculated. Phylogenetic trees of Cdc42, Lac and HmgA were statistically supported, but ITS, BT2 and ACT1 trees were not. The Standardized Index of Association (Iₐ) did not detect recombination (Iₐ = 0.4778), neither did the Phi-test for separate genes. In *Fonsecaea nubica* non-synonymous mutations causing functional changes were observed in Lac gene, even though no selection pressures were detected with the neutrality test (Tajima D test, p < 0.05). Genetic differentiation of populations for each gene showed separation of American, African and Asian populations. Strains of clinical vs. environmental origin showed genetic distances that were comparable or lower than found in geographic differentiation. In conclusion, here we demonstrated clonality of sibling species using multilocus data, geographic structuring of populations, and a low functional and structural selective constraint during evolution of the genus *Fonsecaea*.

Introduction

The genus *Fonsecaea* comprises etiologic agents of human chromoblastomycosis, a chronic (sub)cutaneous infection eventually leading to cauliflower-like eruptions on the skin [1,2]. The fungus is present in human tissue in the form of muriform cells. The disease has been reported worldwide, but mostly in tropical and subtropical climate zones, with high incidence in endemic areas [3–7].

Inoculation of contaminated thorns or wooden splinters has been hypothesized to be a main route of infection [8,9]. Thus far the etiologic agents within *Fonsecaea* are limited to three closely related siblings comprising a clearly delimited clade [10]: *Fonsecaea pedrosoi*, *F. monophora* and *F. nubica*. Environmental sampling to recover the species from their supposed natural habitat has been done [8,9]. *F. pedrosoi* and *F. monophora* were only rarely encountered. However, the majority of *Fonsecaea*-like strains concerned non-virulent species, which were not frequently isolated from on human infections [8]. Either the natural habitat of pathogenic *Fonsecaea* species has to be found somewhere else, or, alternatively, the species have some kind of advantage of being carried by a mammal host. The existence of evolutionary processes supporting the latter hypothesis may be revealed by comparing patterns of variability and distribution of potential etiologic agents.

The pathogenic strains form a well-supported clade in the Chaetothyriales [11], but specific delimitation within this clade is still a debated issue. Analysis of global genetic diversity using AFLP showed that five groups were distinguishable, which were considered to belong to three different species. *Fonsecaea pedrosoi* was relatively homogeneous and was found nearly exclusively in Central and South America, while *F. monophora* and *F. nubica* each comprised several AFLP groups and had worldwide distribution. Cases were found in a tropic climate zone around the equator, while the few clinical cases outside endemic areas were supposed to have been distributed by recent migration of the human host [11].

In the present study, we investigate patterns of variability of pathogenic *Fonsecaea* species using multilocus analysis of five functional genes with anonymous sequence and AFLP markers. The set of strains analyzed comprised clinical and environmental strains from three continents.
Materials and Methods

Ethical Standards

The present study has been fully reviewed and approved by Sun Yat-Sen University’s Academic Committee. All subjects provided written informed consent and the procedures have been approved by the Sun Yat-sen University Medical Ethics Committee.

Fungal Strains and Culture Conditions

Seventeen strains of F. pedrosoi, 12 of F. monophora, 8 of F. nubica (Table 1) and one of a neighbouring Cladophialophora species were obtained from the reference collection of the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (Utrecht, the Netherlands), in addition to fresh strains recovered from patients, and environmental isolates. Stock cultures were maintained on slants of potato dextrose agar (PDA, Difco Laboratories, Sparks, MD, U.S.A.).

DNA Extraction and Identification

DNA extraction and quality test were performed as previously reported [12,13]. DNA concentrations were measured with a nanoparticle DNA concentration detector at 260 nm (Thermo Scientific, U.S.A.). Sequence data were edited using the SeqMan of the DNASTar Inc., Madison, U.S.A.).

Degenerate Primer Design, Cloning and Specific Primer Design for Cdc42, Lac and HmgA

Degenerate and specific primers of Cdc42 refer to the study of Xie et al. [14]. The degenerate primers of HmgA and Lac were designed using a complete alignment of the amino acid sequences of species listed in Table 2. Multiple sequence alignments were generated with the software ClustalW [15] using the amino acid substitution matrix BLOSUM62 [16,17]. Highly conserved areas were chosen for degenerate primer design. Degenerate forward and reverse primers were designed with minimal degenerate degree using Primer 5.0 software (Table 2).

DNA of type strains of the genus Fonsecaea were used as the PCR amplification template. Optimal amplification condition was optimized by temperature gradient PCR amplification. Specific amplicons were purified using gel extraction kit (Qiagen, Germany), cloned using a cloning kit (Promega, Madison, WI, U.S.A.) and confirmed by direct PCR amplification with the primer set M13fw (5′-GTA AAA CGA CGG CGA GT-3′) and M13rv (5′-GGA AAG AGC TAT GAC CAT G-3′) according to the manufacturer’s instructions. PCR amplicons were then purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence data were edited using the SeqMan of the LaserGene software (DNASTar Inc., Madison, U.S.A.).

Phylogenetic Reconstruction and DNA Polymorphism

The Cipres Portal (http://www.phylo.org) was used to construct maximum likelihood trees with RAxML v. 7.2.6 for ITS, BT2, ACT1, Cdc42, Lac and Hmg1. Maximum likelihood searches for the best scoring tree were made after a bootstrap estimate of the proportion of invariant sites automatically determined the number of bootstrapping runs. RAxML will then automatically determine the point at which enough bootstrapping replicates have been produced [19]. Bootstrap values equal to or greater than 90% were considered significant. After repeated construction for all six markers, the combined single file was used to calculate the standardized Index of Association, I_A^2 [20] using the LIAN 3.5 webserver (http://pubmlst.org). The test options were set to Monte Carlo with 1,000 iterations/random resamplings. The same alignments were used to show split-decomposition trees using SPLITSTREE 4 v. 4.8. The same software package was used to apply Phi test (pairwise homoplaspy index) to distinguish recurrent mutations (or homoplasies) from recombination in generating genotypic diversity.

DNA polymorphism analyses were carried out using DNAsp 5.10.00 software. A subset of Fonsecaea strains and genotypes was used to calculate haplotype and nucleotide diversity, as well as Tajima’s D neutrality test that is based on the number of pairwise differences and the number of segregating sites in a sample of sequences and the number of parsimonious informative sites [21].

AFLP Genotyping Assay

AFLP genotyping data were taken from our previous study, where a detailed description of the methodology is provided [11].

Laccase and Homogentisate 1,2-dioxygenase Enzyme Activity Assays

All strains representing F. pedrosoi, F. monophora and F. nubica indicated in Table 1 were tested for laccase and homogentisate 1,2-dioxygenase enzyme activities. Tests were repeated three times for each strain. Laccase was tested according to Mander et al. [22]. Solid MM with a pH of 5 supplemented with 5 mM 2, 2-azino-di-(3-ethylbenzthiazolin sulfonate) (ABTS) which is oxidized by laccase and results in colored compounds. Cultures

Multilocus Analysis of Fonsecaea

The present study has been fully reviewed and approved by Sun Yat-Sen University’s Academic Committee. All subjects provided written informed consent and the procedures have been approved by the Sun Yat-sen University Medical Ethics Committee.
### Table 1. Detailed information of *Fonsecaea* isolates used in this study.

| Taxonomic name | CBS number | origin | Host/sex | Location | AFLP genotyping | Multilocus genotyping |
|----------------|------------|--------|----------|----------|------------------|-----------------------|
| *F. nubica*    | CBS 121733 | Chromoblastomycosis | Human/M | China, Guangdong | A | A | A | A |
|                | CBS 121720 | Chromoblastomycosis | Human/M | China, Guangdong | A | A | A | A |
|                | CBS 121734 | Chromoblastomycosis | Human/M | China, Guangdong | A | A | A | A |
|                | CBS 269.64 | Chromoblastomycosis | Human/F | South Africa | ND | B | B | B |
|                | CBS 444.62 | Chromoblastomycosis | Human/M | Surinam | ND | B | B | B |
| CBS 557.76     | Unknown | Unknown | Unknown | Unknown | B | B | B | B |
| CBS 270.37     | Unknown | Unknown | Unknown | France (from S. America) | B | B | B |
| CBS 277.29     | Chromoblastomycosis | Human/M | Brazil | B | B | B | B |
| *F. monophora* | CBS 102243 | Chromoblastomycosis | Human/M | Brazil, Parana, Ibituva | C | C | C | C |
| CBS 117236     | Brain | Human/M | United States | C | C | C | C |
| CBS 102246     | Chromoblastomycosis | Human/M | Brazil, Parana, Campo Largo | C | C | C | C |
| CBS 269.37     | Chromoblastomycosis | Human | South America | C | C | C | C |
| CBS 102238     | Soil | Soil | Brazil, Parana, Tibagi River | C | C | C | C |
| CBS 102229     | Decaying vegetable cover | Plant | Brazil, Parana, Piracuara | C | C | C | C |
| CBS 397.48     | Chromoblastomycosis | Human/M | South America | C | C | C | C |
| CBS 102248     | Chromoblastomycosis | Human/M | Brazil, Parana, Piracuara | C | C | C | C |
| CBS 121727     | Chromoblastomycosis | Human/M | China, Guangdong | D | D | D | C |
| CBS 121721     | Chromoblastomycosis | Human/M | China, Guangdong | D | D | D | C |
| CBS 117238     | Brain | Human | United Kingdom | D | D | D | C |
| CBS 121724     | Chromoblastomycosis | Human/M | China, Guangdong | D | D | D | C |
| *F. pedrosi*   | CBS 273.66 | Mouse passage | Soil | Venezuela | ND | E | E | D |
| CBS 271.37     | Chromoblastomycosis | Human/M | South America | E | E | E | D |
| CBS 671.66     | Mouse passage | Soil | Venezuela | E | E | E | D |
| CBS 274.66     | Mouse passage | Soil | Venezuela | E | E | E | D |
| CBS 102247     | Chromoblastomycosis | Human/M | Brazil, Parana | E | E | E | D |
| CBS 122740     | Chromoblastomycosis | Human/M | Mexico, Mexico City | E | E | E | D |
| CBS 122736     | Chromoblastomycosis | Human/M | Mexico, Mexico City | E | E | E | D |
| CBS 122849     | Chromoblastomycosis | Human/M | Mexico, Mexico City | E | E | E | D |
| CBS 285.47     | Chromoblastomycosis | Human/M | Puerto Rico | E | E | E | D |
| CBS 342.34     | Chromoblastomycosis | Human/M | Puerto Rico | E | E | E | D |
| CBS 122741     | Chromoblastomycosis | Human/M | Mexico, Mexico City | E | E | E | D |
| CBS 670.66     | Mouse passage | Soil | Venezuela | E | E | E | D |
| CBS 212.77     | Chromoblastomycosis | Human/M | Netherlands, Amsterdam | E | E | E | D |
| CBS 117910     | Chromoblastomycosis | Human/M | Venezuela, Coro, Falcón State | E | E | E | D |
| CBS 272.37     | Chromoblastomycosis | Human | Brazil | E | E | E | D |
| CBS 253.49     | Chromoblastomycosis | Human | Uruguay, Montevideo | E | E | E | D |
| CBS 201.31     | Gazelle, ear | Animal | Libya, Cyrenaica, Derna | E | E | E | D |
| *Cladophialophora* sp. | CBS 109631 | Unknown | Human | Uruguay | F | F | F | E |

CBS: Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, Netherlands.
ND: not determined.
doi:10.1371/journal.pone.0041512.t001
were pre-incubated at 25°C for 7 days. Subcultures were cut with a cork borer 2 mm diam and placed at the centre of the plate with three replicates. Diameters of colored metabolite halos were measured from day 1 to day 7. For the homogentisate 1,2-dioxygenase enzyme activity test, we followed Ye & Szaniszlo [23]. Solid MM was supplemented separately with 5 mM L-phenylalanine (Sigma, U.S.A.) and 5 mM L-tyrosine (Sigma) which served as artificial substrates to evaluate homogentisate 1,2-dioxygenase enzyme activity. Culture conditions were the same as in the laccase test. After two weeks of culture, colony diameters were measured.

Statistics
Metabolite diameters were analyzed by one way ANOVA using Prism 5.0 software, followed by Tukey’s HSD Post-hoc test. Mean diameters are the result of triplicate experiments. The error bars indicate standard error of the mean; p<0.05 was considered to indicate a significant difference.

Results
Primer Development for Cdc42, Lac and HmgA
Highly conserved domains were found in Cdc42, HmgA and Lac genes after comparison of sequences downloaded from GenBank (Table 3) and these were used for degenerate primer design. PCRs with degenerate primer pairs Cdc42-F and Cdc42-R, Lac-Ds and Lac-Das, HmgA-F2 and HmgA-R22 yielded multiple bands. After cloning and alignment analysis, the specific primers cdc42-SF1s and cdc42-SR1s, Lac-Is and Lac-Is, and HmgA-F2s, HmgA-R12s/HmgA-R22s were obtained (Table 2). The sets of specific primers each yielded single PCR products of about 0.85 kb, 1 kb and 0.9 kb, respectively (data not shown). The introns were taken out when used for further analysis. The primer sets proved to amplify all Fonsecaea agents of chromoblastomycosis successfully. To establish an outgroup, degenerate primers were used to amplify the target gene, and multiple bands were cloned and sequenced. BLAST searches using translated amino acid sequences in GenBank showed that the amplified fragments of Cdc42, Lac and HmgA had high homology with published target genes [24–26]. The conserved domain search revealed that Cdc42 contains a Ras-like GTPase superfamily (aa1–120) which involved a GTP/Mg2+ binding site (aa45–100) and switch I and II regions (aa20–25, aa40–80) [27]. Lac contained a Cu-oxidase superfamily which typically exists in the laccase family [27]. HmgA contained the HmgA superfamily (aa1–204), a hexamer arrangement consisting of a dimer of trimers with which the active site iron ion is coordinated [27].

Phylogeny
Six phylogenetic trees were constructed for 37 Fonsecaea strains distributed globally using sequenced ITS, BT2, ACT1, Cdc42, Lac and HmgA genes, and one Cladophialophora strain (CBS 109631) used as outgroup. Three clades corresponding to F. pedrosoi, F. monophora and F. nubica showed strong support in Cdc42, Lac and HmgA genes (bootstrap values >80%) (Fig. 1). F. pedrosoi showed limited variability within the species. Two subclades were distinguished within F. monophora with high bootstrap support in Cdc42 and Lac (Fig. 1), while within F. nubica, two subclades in Cdc42, Lac and HmgA (Fig. 1). The AFLP genotyping assay showed the similar tree topology (Fig. 2), with five subclades with high bootstrap support within the genus Fonsecaea. However, for ITS, BT2 and ACT1 genes, no

Table 2. Degenerate primers and specific primers used in this study.

| Gene  | Degenerate primers | Specific primers | Reference |
|-------|--------------------|-----------------|-----------|
| Cdc42 | Cdc42-SF1s         | Cdc42-Is        | Glass & Donaldson (1995) |
|       | Cdc42-SR1s         | Cdc42-Is        | Glass & Donaldson (1995) |
|       | HmgA-F2s           | HmgA-R12s       | Masclaux et al. (1995) |
|       | HmgA-R22s          | HmgA-R12s       | Masclaux et al. (1995) |
| Lac   | Lac-Is             | Lac-Is          | White et al. (1990) |
|       | HmgA-R12s          | HmgA-R12s       | White et al. (1990) |
| ITS   | V9G, L5266         | V9G, L5266      | Glass & Donaldson (1995) |
| BT2   | Bt2a, Bt2b         | Bt2a, Bt2b      | Glass & Donaldson (1995) |
| ACT1  | Actaw, Actfw       | Actaw, Actfw    | Glass & Donaldson (1995) |
significantly bootstrap support was obtained (Fig. 1). Fixed populations were observed throughout the six phylogenetic
and AFLP genotyping trees. The F. pedrosoi clade comprised 17 strains from patients and from the environment in South America and Europe. F. monophora genotype A comprised 17 clinical strains from South China, and genotype B comprised 5 clinical strains from in Europe and South America. Coccidioides immitis (0.4778 (0.61812) were not statistically supported (0.63696) and
laccase (Lac) references taken from GenBank.

Table 3. Homogentisate 1,2-dioxygenase (HmgA) and laccase (Lac) references taken from GenBank.

| Taxonomic name                  | Associated strain number | gene   | GenBank no. | protein |
|---------------------------------|--------------------------|--------|-------------|---------|
| Ajellomyces dermatitidis        | SLH14081                 | HmgA   | XP_002626277.1 |         |
| Trichophyton tonsurans          | CBS 112818               | HmgA   | EGD98945    |         |
| Coccidioides immitis            | RS                       | HmgA   | XP_001247541.1 |         |
| Paracoccidioides brasilienis    | Pb03                     | HmgA   | EEH17396.1  |         |
| Trichophyton tonsurans          | CBS 112818               | HmgA   | EGD98945.1  |         |
| Trichophyton equinum            | CBS 127.97               | HmgA   | EGE07801.1  |         |
| Aspergillus terreus             | NIH2624                  | HmgA   | XP_00218689.1 |         |
| Aspergillus niger               | CBS 513.88               | HmgA   | XP_00388730.2 |         |
| Aspergillus oryzae              | RIB40                    | HmgA   | XP_001727215.2 |         |
| Trichophyton rubrum             | CBS 118892               | HmgA   | XP_003238076.1 |       |
| Neurospora crassa               | OR7A                     | HmgA   | XP_960461.1  |         |
| Aspergillus fumigatus           | AF293                    | HmgA   | XP_750969.1  |         |
| Penicillium marneffei           | ATCC 18224               | HmgA   | XP_002150285.1 |       |
| Neurospora crassa               | Lac                      | Lac    | AAA33591.1  |         |
| Cryptococcus neoformans var. grubii | JEC21                | Lac    | ABI58272.1  |         |
| Cryptococcus neoformans var. neoformans | FGSC A4         | Lac    | AAW464742.1 |         |
| Aspergillus nidulans            | NRRL3357                 | Lac    | EED57644.1  |         |
| Aspergillus flavus              | NIH2624                  | Lac    | EAU34323.1  |         |
| Aspergillus terreus             | ATCC 10500               | Lac    | EED19078.1  |         |
| Talaromyces stipitatus          | ATCC 18224               | Lac    | EEA21273.1  |         |
| Ajellomyces dermatitidis        | SLH14081                 | Lac    | XP_002629368.1 |       |
| Penicillium marneffei           | ATCC 18224               | Lac    | EEA21273.1  |         |
| Aspergillus clavatus            | NRRL 1                   | Lac    | EAW07265.1  |         |
| Aspergillus fumigatus           | AF293                    | Lac    | XP_752933.1  |         |
| Trichophyton tonsurans          | CBS 112818               | Lac    | EGD95875.1  |         |
| Coccidioides immitis            | RS                       | Lac    | XP_001239516.1 |       |

CBS: Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, Netherlands.
NIH: The National Institute of Health, Bethesda, Maryland, USA.
ATCC: American Type Culture Collection, Maranvis, VA, USA.
FGSC: The Fungal Genetics Stock Center, Kansas City, Missouri, USA.
NRRL: ARS Culture Collection, Washington DC, USA.
doi:10.1371/journal.pone.0041512.t003

Multilocus Recombination Analyses

Standardized Index of Association $I^S$ [28] performed using LIAN 3.5 [20] confirmed clades according to the RasML trees in the different partitions. $I^S$ measures the degree of association between alleles at different loci based on the variance in genetic distance between genotypes and is expected to be 0 if populations are freely recombining and >0 if there is an association between alleles. The calculated index using 1000 Monte Carlo resamplings was $0.4778 (V_B = 3.8561, V = 0.9973)$, showing no evidence of recombination. The Phi-test [29] is performed for individual loci

for detection of recombination within sequences with expected recombination events the value is $p<0.05$, otherwise, homoplasy ($p>0.05$) is considered. The results based on six genes showed no significant statistical evidence of recombination at ITS ($p = 0.076$), BT2 ($p = 0.112$), ACT1 ($p = 1.0$), Cdc42 ($p = 1.0$), Lac ($p = 0.79$), or $HmgA$ ($p = 0.46$).

Strain Polymorphism Statistics

The calculated parsimonious informative sites, monomorphic sites, segregating sites and the total number of mutations are summarized in Table 4. In total 37 strains were used for all six genes. The haplotype diversity for ITS ($0.761$), BT2 ($0.824$), ACT1 ($0.725$), Cdc42 ($0.883$), Lac ($0.65057$), and $HmgA$ ($0.820$) were in comparable range. For the neutrality test, Tajima’s $D$ values for ITS ($1.23506$), BT2 ($0.27942$), ACT1 ($0.65057$), Cdc42 ($1.23506$), Lac ($0.63696$) and $HmgA$ ($0.61812$) were not statistically supported ($p>0.10$), no positive selection being detected within the tested genus (Table 4).

The values of $F_{ST}$ lie between 0 (panmictic) and 1 (total separation). The tested $F_{ST}$ values based on six genes by comparing geographic origins of the strains (Table 5A) show...
similar values between South and Central America, while those of Chinese and Africa strains were higher. The $F_{ST}$ values based on six combined genes from clinical (28 strains) and environmental origins (6 strains) (Table 1) showed a comparable or lower value (0.07567) than with comparisons of geographic origins between South America and Central America (0.10549), South America and Africa (0.33106), South America and Asia (0.25447), Central America and Africa (0.41542), Central America and Asia (0.425565), and Asia and Africa (0.17738) (Table 5B). The comparisons of geographic origins between continents low values were found (Table 5) suggesting separation of populations.

Figure 1. Consensus trees of *Fonsecaea* based on ITS ribosomal DNA, BT2, ACT1, Cdc42, HmgA and Lac of 37 strains, constructed with MEGA5.0 and 500 bootstrap replicates, CBS 109631 was taken as outgroup. doi:10.1371/journal.pone.0041512.g001

Synonymous and non-synonymous changes of the genus *Fonsecaea* in amino acid sequence in six genes are listed in Table 4. In total 787 amino acid codons were used for the comparison, and 81st base, 22nd base and 813rd base mutations were found within the three species. All 1st base mutations caused non-synonymous changes, but the 2nd base and 3rd base mutations caused synonymous changes. A further analysis showed that the non-synonymous changes in *ACT1* and *BT2* both did not occur in the functional domain (*ACT1*$aa_{135}$, $BT2$*$aa_{91}$), while non-synonymous changes in *Lac* and *HmgA* both occurred in functional domains (*Lac*$aa_{159}$, *HmgA*$aa_{38}$, $aa_{88}$, $aa_{164}$, $aa_{175}$). Most non-synonymous changes were observed in *F. nubica*, where all strains isolated to date originate from chromoblastomycosis patients (Table 6).

**Laccase and Homogentisate 1,2-dioxygenase Enzyme Activity Assay**

All strains tested yielded positive laccase activity. Colored metabolites were observed in all three species, but statistical analysis showed that *F. nubica* had higher enzyme activity than other species (*F. nubica* vs. *F. pedrosoi*, $p<0.001$, *F. nubica* vs. *F. monophora*, $p>0.05$, *F. monophora* vs. *F. pedrosoi*, $p<0.01$) (Fig. 3). The homogentisate 1,2-dioxygenase enzyme activity assay revealed that all strains are able to assimilate L-phenylalanine and L-tyrosine as sole carbon sources; no difference was observed within the three species (data not shown).
Figure 2. Clustering of amplified fragment-length polymorphism banding pattern of isolates of *Fonsecaea* spp. analyzed by using unweighted pair group method with arithmetic mean. Subclusters showed as in Figure.
doi:10.1371/journal.pone.0041512.g002

Table 4. Phylogenetic marker diversity and molecular evolutionary parameters for the gene segments examined.

| Parameters                        | Phylogenetic Marker | ITS      | Cdc42    | Lac      | HmgA     | BT2      | ACT1     |
|-----------------------------------|---------------------|----------|----------|----------|----------|----------|----------|
| Fragment features                 |                      | Exon/intron | Exon    | Exon    | Exon/intron | Exon/intron |
| No. of sequences                  |                      | 37       | 37       | 37       | 37       | 37       |
| No. of characters                 |                      | 572      | 360      | 708      | 612      | 303      | 486      |
| No. of codon                      |                      | n.a      | 120      | 236      | 204      | 83       | 144      |
| DNA polymorphism analysis         |                      |          |          |          |          |          |
| Gaps/missing data                 |                      | 572      | 360      | 708      | 612      | 278      | 485      |
| Segregating sites                 |                      | 22       | 8        | 37       | 32       | 18       | 10       |
| No. of mutations (\(\delta\))    |                      | 22       | 8        | 38       | 32       | 18       | 10       |
| No. of haplotypes                 |                      | 6        | 5        | 9        | 7        | 9        | 7        |
| Haplotype diversity               |                      | 0.761    | 0.725    | 0.883    | 0.820    | 0.743    | 0.824    |
| Nucleotide diversity              |                      | 0.01417  | 0.00662  | 0.01478  | 0.01374  | 0.01682  | 0.00600  |
| Neutrality analysis               |                      |          |          |          |          |          |
| Tajima's D test                   |                      | 1.21111  | 1.23506  | 0.63696  | 0.61812  | 0.27942  | 0.65057  |

doi:10.1371/journal.pone.0041512.t004
Table 5. Population differentiation index (FST) of 37 Fonsecaea strains based on separate (A) and combined (B) multilocus gene sequences clustered by geographical origin. Seven Fonsecaea strains from China, 3 from Africa, 17 from South America and 6 from Central America.

|       | South America | Central America | Africa |
|-------|---------------|-----------------|--------|
| ITS   | 0.0865       | 0.1304         | 0.1772 |
| ACT1  | 0.2658       | 0.2148         | 0.2524 |
| BT2   | 0.0807       | 0.0767         | 0.0748 |
| Cdc42 | 0.1334       | 0.2177         | 0.2224 |
| HmgA  | 0.2145       | 0.3874         | 0.4352 |
| Lac   | 0.1737       | 0.3360         | 0.3158 |
|       | 0.1909       | 0.2341         | 0.2156 |
|       | 0.1909       | 0.2341         | 0.2156 |
|       | 0.1638       | 0.2163         | 0.2286 |
|       | 0.1638       | 0.2163         | 0.2286 |

Discussion

In the evolution of black fungi (order Chaetothyriales) [30], we witness a functional change from a rock-inhabiting life style prevalent in ancestral Coniosporium (Knufia) and relatives to an increased ability to infect humans and other vertebrates in derived clades. Agents of chromoblastomycosis are particularly interesting because they exhibit a pathogenic phase in tissue, the muriform cell, which shows morphogenetic resemblance isodiametrically enlarging cell clumps of rock-inhabiting Coniosporium (Knufia) species. A functional change in the Cdc42 gene, involved in cellular polarity has been hypothesized [31]. The change of life style seems to have been quite successful in the F. pedrosoi clade, judging from the fact that three related species are nearly exclusively found on humans [32]. Nevertheless the shift was not seen to be reflected in the cytoskeleton-associated Cdc42 gene when compared over the order Chaetothyriales [33].

In the present study, six genes were compared in human-pathogenic Fonsecaea species. ITS was used as a standard for phylogenetic construction. ACT1, BT2 and Cdc42 play a role in cell cycle progression and actin cytoskeleton construction, and are involved in morphogenetic switching, leading to large spherical cells with subsequent cellular division giving rise to the infective muriform cell [34]. Lac and HmgA are well-documented virulence factors of black fungi, and participate in the synthesis of melanin. DHN melanin is negatively charged, hydrophobic and of high molecular weight, and arises by the oxidative polymerization of phenolic and/or indolic precursors [35]. Melanin enhances virulence in black fungi of the order Chaetothyriales [36–42]. We developed primers to amplify Cdc42, Lac and HmgA which proved to be specific for Fonsecaea. The sequenced genes were aligned and confirmed to be Cdc42, Lac and HmgA using BLAST one search in GenBank. The genes contained the gene-specific conserved domains when searched with translated amino acid sequences [27].

The phylogenetic trees reconstructed with Cdc42, Lac and HmgA (Fig. 1) yielded high bootstrap support for the three sibling Fonsecaea species, while the ITS, ACT1 and BT2 trees were not supported. The lack of support was probably caused by incomplete lineage sorting, several mutations not having reached fixation. Based on the Standardized Index of Association (IA) and Phi-test using six genes, no recombination events were detected among the three sibling species. This phenomenon is frequently observed in opportunistic members of Chaetothyriales, where clonality seems to be prevalent [43]. The neutrality test with Tajima’s D yielded no significant results, suggesting that no positive selection was detected in the sequenced genes indicating a low functional and structural selective constraint during evolution.

Relatively low haplotype diversity was observed within the six genes analyzed. A total of 91 fixed synonymous and non-synonymous changes were observed in coding regions. The non-synonymous changes in the cytoskeleton genes ACT1 and BT2 are not responsible for morphogenetic changes [7,44] among the three species because the mutations occurred outside functional domains. The non-synonymous changes in Lac and HmgA both occurred in functional domains (Lacaas, HmgAas, aa159, aa164, and aa173) (Table 6), but did not cause obvious functional changes when catalysis of substrates was tested in vitro. A possible explanation might be that the non-synonymous mutations did not cause any changes in the three-dimensional structure of the molecule. A systematic alignment of 223 plant and fungi laccase sequences showed that there are four signature sequence regions (L1-4) and 12 housekeeping amino acids [45], while the detected non-synonymous mutations (Lacaas, aa159) in this study occurred...
between L2 and L3 and do not belong to a conserved region. DNA sequence alignment of HmgA showed that HmgA aa38, aa88, aa164, and aa175 are not located in conserved regions either. Therefore we conclude that the non-synonymous changes within two genes are not linked to functional or structural selective constraints within the genus Fonsecaea. Subsequent studies may

| Gene  | Species          | Total codon | 1st base | 2nd base | 3rd base | Amino acid change | Strains                  |
|-------|------------------|-------------|----------|----------|----------|-------------------|--------------------------|
| ACT1  | F. pedrosoi      | 144         |          |          |          | CAT→TAT/H→Y       | All tested F. nubica     |
|       | F. monophora     | 144         |          |          |          |                   |                          |
|       | F. nubica        | 144         | 1        | 9        |          |                   |                          |
| BT2   | F. pedrosoi      | 83          | 1        | 3        |          | CAT→GAT/Y→D       | CBS 671.66, CBS 273.66, CBS 670.66 |
|       | F. monophora     | 83          |          | 1        |          |                   |                          |
|       | F. nubica        | 83          |          |          | 1        |                   |                          |
| Cdc42 | F. pedrosoi      | 120         |          |          |          | CAT→GAT/Y→D       | CBS 671.66, CBS 273.66, CBS 670.66 |
|       | F. monophora     | 120         |          |          |          |                   |                          |
|       | F. nubica        | 120         |          |          | 4        |                   |                          |
| Lac   | F. pedrosoi      | 236         | 1        | 5        |          |                   |                          |
|       | F. monophora     | 236         |          |          | 7        |                   |                          |
|       | F. nubica        | 236         | 1        |          | 22       | CCG→CTG/P→L       | All tested F. nubica     |
| HmgA  | F. pedrosoi      | 204         |          |          |          |                   |                          |
|       | F. monophora     | 204         |          |          | 3        |                   |                          |
|       | F. nubica        | 204         | 4        | 1        | 21       | AGC→GGC/S→G       | All tested F. nubica     |
|       |                   |             |          |          |          | GCC→ACC/A→T       | All tested F. nubica     |
|       |                   |             |          |          |          | AGC→AAC/S→N       | All tested F. nubica     |
|       |                   |             |          |          |          | GCT→ACT/A→T       | All tested F. nubica     |
| Total |                 | 787         | 8        | 2        | 81       |                   |                          |

H: Histidine, Y: Tyrosine, D: Arginine, P: Proline, L: Leucine, S: Serine, G: Glycine, A: Alanine, T: Threonine, N: Asparagine.

CBS: Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.
doi:10.1371/journal.pone.0041512.t006

**Figure 3. Laccase activity assay.** Colored metabolite diameters of tested strains were measured after 7-day culture at 25°C on solid MM medium with 5 mM ABTS. Statistical analysis shown as mean ± standard deviation (A). The plates show the generation of colored metabolite compound (B), F. pedrosoi (CBS 273.66), F. monophora (CBS 117236), F. nubica (CBS 121720).
doi:10.1371/journal.pone.0041512.g003
reveal whether such changes have occurred in the analyzed genes in ancestral clades, where dramatic changes in life style are supposed to have taken place.

Several studies reported on the molecular epidemiology of the sibling species *Fonsecaea* [45]. Ribosomal and mitochondrial DNA typing has been used to reveal the geographic origin of strains [46,47]. The molecular epidemiology of this genus showed substantial geographic structuring in all species with differences between American, African and Asian populations similar to what has been found by Kawasaki et al. [46] in mtDNA profiles. In conclusion, we demonstrated clonality of sibling species using multilocus data, geographic structuring of populations, and a detected low functional and structural selective constraint during evolution of the genus *Fonsecaea*.

Author Contributions

Conceived and designed the experiments: LX GSDH. Performed the experiments: JS MJN AHGG. Analyzed the data: JS AHGG. Contributed reagents/materials/analysis tools: VAV PF. Wrote the paper: JS GSDH.

References

1. Queiroz-Telles F, Esterre P, Perez-Blanco M, Vitale RG, Salgado CG, et al. (2009) Chromoblastomycosis: an overview of clinical manifestations, diagnosis and treatment. Med Mycol 47: 5–13.
2. Lopera Martinez R, Mendez Tovar LJ (2007) Chromoblastomycosis. Clin Dermatol 25: 188–194.
3. Esterre P, Abdallamhakavandy A, Ramarcel ER, Pecaree JL (1996) Forty years of chromoblastomycosis in Madagascar: a review. Am J Trop Med Hyg 55: 45–47.
4. Kombila M, Gomez de Diaz M, Richard-Lenoble D, Renders A, Walter P, et al. (1995) [Chromoblastomycosis in Gabon. Study of 64 cases]. Sante 5: 235–244.
5. Silva JP, de Souza W, Rozental S (1998) Chromoblastomycosis: a retrospective study of 325 cases on Amazonic Region (Brazil). Mycopathologia 143: 171–175.
6. Atapattu MC (1997) Chromoblastomycosis: a clinical and mycological study of 71 cases from Sri Lanka. Mycopathologia 137: 143–151.
7. Xi L, Sun J, Lu C, Liu H, Nie Z, et al. (2009) Molecular diversity of *Fonsecaea* (Chaetothyriales) causing chromoblastomycosis in southern China. Med Mycol 47: 27–33.
8. Vicente VA, Anti-Angelus D, Pie MB, Queiroz-Telles F, Cruz LM, et al. (2008) Environmental isolation of black yeast-like fungi involved in human infection. Stud Mycol 61: 137–144.
9. Salgado CG, da Silva JP, Diniz JA, da Silva MB, da Costa PF, et al. (2004) Isolation of *Fonsecaea* pedrosoi from thorns of Mimosa pudica, a probable natural source of chromoblastomycosis. Rev Inst Med Trop Sao Paulo 46: 33–36.
10. Najafzadeh MJ, Sun J, Vicente VA, Klaassen CH, Bonifaz A, et al. (2011) Molecular diversity of *Fonsecaea* species. Emerg Infect Dis 17: 464–469.
11. Sun J, Zhang J, Najafzadeh MJ, Badali H, Li X, et al. (2011) Melanization of a meristematic mutant of *Fonsecaea nubica* sp. nov, a new agent of human chromoblastomycosis revealed using molecular data. Med Mycol 49: 800–806.
12. Najafzadeh MJ, Sun J, Vicente VA, Klaassen CH, Bonifaz A, et al. (2010) Molecular epidemiology of *Fonsecaea* species. Emerg Infect Dis 14: 573–580.
13. Sun J, Zhang J, Najafzadeh MJ, Badali H, Li X, et al. (2011) Genetic diversity and species delimitation in the opportunistic genus *Fonsecaea*. Med Mycol 49: 17–25.
14. Xi Z, Feng P, Zhang J, Li X, Sun J, et al. (2012) Molecular cloning, characterization and differential expression of Cdc42 in *Fonsecaea nubica*. Med Biol Rep 39: 839–844.
15. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and matrix choice. Nucleic Acids Res 22: 4673–4680.
16. Henikoff S, Henikoff JG (1992) Amino acid substitution matrices from protein blocks. Proc Natl Acad Sci U S A 89: 10915–10919.
17. Najafzadeh MJ, Gueidan C, Badali H, Van Den Ende AH, Xi L, et al. (2009) Evolution of CDC42, a putative virulence factor triggering meristematic growth of meristematic fungi. Antonie Van Leeuwenhoek 74: 271–281.
18. Polercky B, Bissett A, Al-Najjar M, Faeber P, Osmers H, et al. (2009) Modular spectral imaging system for discrimination of pigments in cells and microbial communities. Appl Environ Microbiol 75: 758–771.
19. Haubold B, Hudson RR (2000) LIAN 3.0: detecting linkage disequilibrium in multilocus data. Linkage Analysis. Bioinformatics 16: 847–848.
20. Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585–595.
21. Mander GJ, Wang H, Bodie E, Wagner J, Vienken K, et al. (2006) Use of lifestyle as a novel virulence reporter system in filamentous fungi. Appl Environ Microbiol 72: 5767–5776.
22. Haubold B, Hudson RR (2000) LIAN 3.0: detecting linkage disequilibrium in multilocus data. Linkage Analysis. Bioinformatics 16: 847–848.
23. Arias Barrau E, Olivera ER, Luengo JM, Fernandez C, Galan B, et al. (2004) The homogentisate pathway: a central catabolic pathway involved in the biosynthesis of melanin in *Fonsecaea* species. Emerg Infect Dis 10: 2069–2081.
24. Dong F, Zhang Y, Arulananandam B, Zhong G (2005) Production of a proteolytically active protein, chamydial protease/protase-a-like activity factor, by five different Chlamydia species. Infect Immun 73: 1868–1872.
25. Nosanchuk JD, Casadevall A (2003) The contribution of melanin to microbial pathogenesis. Cell Microbiol 5: 203–223.
26. Lyons JJ, Newell SY, Buchanan A, Moran MA (2003) Diversity of ascomycete laccase genes in a southeastern US salt marsh. Microb Ecol 45: 270–281.
27. Mander GJ, Wang H, Bodie E, Wagner J, Vienken K, et al. (2006) Use of lifestyle as a novel virulence reporter system in filamentous fungi. Appl Environ Microbiol 72: 5767–5776.
28. Nosanchuk JD, Casadevall A (2003) The contribution of melanin to microbial pathogenesis. Cell Microbiol 5: 203–223.
29. Nosanchuk JD, van Duin D, Mandal P, Aisen P, Legendre AM, et al. (2004) Detection of melanin-like pigments in the dimorphic fungal pathogen *Fonsecaea pedrosoi* and strain typing of *Fonsecaea* agents of chromoblastomycosis. J Microbiol Methods 80: 19–24.
30. Sterflinger K (1998) Temperature and NaCl-tolerance of rock-inhabiting meristematic fungi. Antonie Van Leeuwenhoek 75: 235–244.
31. Cooper CR, Jr., Szaniszlo PJ (1993) Evidence for two cell division cycle (CDC) genes that govern yeast bud emergence in the pathogenic fungus *Wangella dermatitidis*. Infect Immun 61: 2069–2081.
32.добал Х, Gueidan C, Najafzadeh MJ, Bonifaz A, van den Ende AH, et al. (2008) Biodiversity of the genus *Cladosporium*. Stud Mycol 61: 175–191.
33. Norris-Jones R, Gomez BL, Deiz S, Ursan M, Morris-Jones SD, et al. (2005) Synthesis of melanin pigment by *Candida albicans* in vitro and during infection. Infect Immun 73: 6147–6150.
34. Nosanchuk JD, Casadevall A (2003) The contribution of melanin to microbial pathogenesis. Cell Microbiol 5: 203–223.
35. Nosanchuk JD, van Duin D, Mandal P, Aisen P, Legendre AM, et al. (2004) Blastosynus dermatitidis produces melanin in vitro and during infection. FEBS Lett 582: 549–553.
36. Nosanchuk JD, van Duin D, Mandal P, Aisen P, Legendre AM, et al. (2004) Blastosynus dermatitidis produces melanin in vitro and during infection. FEBS Lett 582: 107–109.
37. Nosanchuk JD, van Duin D, Mandal P, Aisen P, Legendre AM, et al. (2004) Blastosynus dermatitidis produces melanin in vitro and during infection. FEBS Lett 582: 549–553.
38. Nosanchuk JD, van Duin D, Mandal P, Aisen P, Legendre AM, et al. (2004) Blastosynus dermatitidis produces melanin in vitro and during infection. FEBS Lett 582: 107–109.
39. Nosanchuk JD, van Duin D, Mandal P, Aisen P, Legendre AM, et al. (2004) Blastosynus dermatitidis produces melanin in vitro and during infection. FEBS Lett 582: 107–109.
40. Nosanchuk JD, van Duin D, Mandal P, Aisen P, Legendre AM, et al. (2004) Blastosynus dermatitidis produces melanin in vitro and during infection. FEBS Lett 582: 107–109.
41. Nosanchuk JD, van Duin D, Mandal P, Aisen P, Legendre AM, et al. (2004) Blastosynus dermatitidis produces melanin in vitro and during infection. FEBS Lett 582: 107–109.