

Founder Effects Contribute to the Population Genetic Structure of the Major Dermatophytosis Pathogen *Trichophyton rubrum* on Hainan Island, China

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Background: Founder events have been observed among numerous plants and animal species living on oceanic islands due to the geographic separation of these islands and the small amount of original life they harbor. However, there has been little research on the ecological characteristics of pathogenic microorganisms on islands. *Trichophyton rubrum* ranks the most common isolated dermatophyte causing dermatophytosis in clinic and has become an epidemic strain worldwide in recent decades.

Objective: To study the phylogenetic characteristics and the distribution pattern of genetic polymorphism of *T. rubrum* in China, which further provide theoretical basis for the prevention and control of *T. rubrum*.

Methods: In the present study, we sequenced and analyzed the genetic characteristics of 204 *T. rubrum* isolates from Hainan Island and other sites in China. Phylogenetic analysis and genetic polymorphisms were studied based on a total of 41,409 high-quality whole-genome SNPs.

Results: The majority of the isolates from Hainan Island clustered together. Mixed *T. rubrum* population differentiation was observed among the strains of different geographical origins. In addition, the genetic diversity (π) of the Hainan isolates was low and showed no significant difference from that of isolates from other sites.

Conclusion: This study is the first to discuss general ecological and evolutionary principles related to pathogenic fungi. Our findings reveal a founder effect during the origination of *T. rubrum* on Hainan Island and provide guidance regarding prevention and treatment strategies.

Keywords: founder effects, *Trichophyton rubrum*, genetic diversity, population differentiation

Introduction

Founder effects occur mostly when a new population is formed initially by a few individuals (the founders) from the original population.^{1,2} The founders do not include all the genetics of the original population, resulting in the lack of genetic diversity of newly established population. This is usually the case in groups of many species living on islands, in lakes, and in isolated forests and habitats.²⁻⁴

Due to the limited area of oceanic islands and their varying degrees of isolation from nearby continents, they are considered ideal systems for investigating evolutionary patterns such as founder effects.^{5,6} Founder effects are observable in many plant and animal species; however, few studies have focused on the ecological

characteristics of pathogenic microorganisms.^{2–4,7} Hainan Island is the southernmost tropical island in China. It is a geographically independent island separated from the mainland to the north by the Qiongzhou Strait. The unique geographical features of Hainan Island make it an ideal model for the study of evolutionary processes.^{8,9}

According to a previous report, dermatophytes are the main superficial pathogenic fungi in Hainan Province, among which *Trichophyton rubrum* is the dominant species.¹⁰ Dermatophytes are the most common filamentous pathogenic fungi in the clinic, which tend to infect tissues rich in keratin, such as skin and nails,¹¹ leading to superficial infections, such as tinea cruris, tinea corporis, tinea manuum, etc. Dermatophytes are capable of infecting healthy individuals with competent immune system, which is different from most other pathogenic fungi. Dermatophyte infection is the most common human fungal infection, affecting 20–25% of the global population, and asymptomatic carriers account for 30–70% adult population,^{12,13} resulting in a great burden of disease and medical burden. According to epidemiological data, *T. rubrum* is the dominant superficial pathogen, accounting for more than 80% of dermatophyte infections.¹⁴ It survives in the environment for at least six months, capable to be universally infected,¹⁵ besides, its infections are mostly chronic and easy to relapse.^{16,17} Due to its biological characteristics and prevalence, *T. rubrum* has become a model organism for studying human pathogenic filamentous fungi. The identification of *T. rubrum* included morphological, physiological, and molecular methods, such as microscopy examination, urea hydrolysis, keratinase activity detection, internal transcribed spacer (ITS) polymerase-chain reaction (PCR), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), electron spray ionization mass spectrometry (ESI-MS), etc.^{18,19} ITS PCR was most used method for routine diagnostics.

Studies on the *T. rubrum* population structure suggested two populations, one population contains genotypes originated from Africa, while the other population distributed worldwide excluding Africa area.^{20,21} *T. rubrum* has been found to be the dominant epidemic species in recent decades.^{22,23} In the past few years, the technology of high-throughput sequencing makes great progress, providing information for in-depth understanding of fungi characteristics with the whole-genome data. In dermatophytes, sequencing and analysis of the genome data about some important species have been reported, including

Microsporium canis, *T. rubrum*, *T. violaceum*, etc., which provide insights into their population differentiation and potential pathogenic genes.^{24,25} Compared to the genetic variation of other fungal pathogens, the genetic diversity level within *T. rubrum* is significantly low. Zheng et al resequenced whole genome of 48 *T. rubrum* strains from China and analyzed their population differentiation and drug resistance based on the sequencing data.¹⁷

This study collected and sequenced 204 *T. rubrum* strains from different regions in China, aiming to describe the genetic characteristics of *T. rubrum* population in greater coverage, making the results more representative, and providing theoretical foundation for the formulation of treatment strategies in clinic. Besides, there were a large amount of strains from Hainan, which is a good geographic model for studying founder effect, the analysis of related strains can provide information for the evolutionary characteristics of *T. rubrum*.

Materials and Methods

T. rubrum Isolates

A total of 204 clinical *T. rubrum* isolates were collected from different cities of China between 2018 and 2019. Samples from patients were isolated in the laboratory using potato dextrose agar (PDA) medium (Land bridge, Beijing, China) containing 0.05% chloramphenicol (Sangon Biotech, Shanghai, China). After 7 days' cultivation at 28°C, the cultures were separated into monoclonal cultures and identified by the molecular sequencing of their ITS regions.^{26,27} The primer sequences were as follows:

ITS1 (forward): 5'-TCCGTAGGTGAACCTGCGG-3'

ITS4 (reverse): 5'-TCCTCCGCTTATTGATATGC-3'²⁸

ITS sequence alignment was conducted using national center for biotechnology information (NCBI) nucleotide basic local alignment search tool (BLAST). Strains identified as *Trichophyton rubrum* were preserved and stored long term at –80°C using 25% glycerol (v/v) at the National Institute for Communicable Disease Control and Prevention (ICDC, Beijing, China).

The sampling sites were recorded as the locations of the hospitals where the isolates were collected. The characteristics of the sampling sites were obtained from data for the cities where these hospitals were located. The population size was obtained from the yearbook published by the statistical bureau of each city. Meteorological information, including the average temperature, humidity and

atmospheric pressure, was collected from monthly data from surface meteorological stations in the China National Meteorological Information Center (http://data.cma.cn/data/cdcdetail/dataCode/SURF_CLI_CHN_MUL_MON.html). For cities without a weather station, the meteorological data were replaced by data from the nearest city with a weather station.

DNA Extraction and Whole-Genome Sequencing

All the *T. rubrum* isolates were cultivated on Sabouraud's agar (SDA) medium for 10–14 days. *T. rubrum* cells were first added to centrifuge tubes containing lysis solution (20 mM Tris, 5 mM EDTA, 400 mM NaCl) and zirconium dioxide beads (Φ 1 mm and Φ 3 mm). The cells were disrupted by shaking using a rapid biological sample preparation system at low temperature (Life Real, Hangzhou, China). After cell disruption, QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA in accordance with the operating instructions. Quality of extracted DNA, including concentration and purity, was controlled by sequencing company. Only samples passed the quality inspection will be used for the subsequent library construction and sequencing.

T. rubrum genomes were sequenced by Illumina technology (Novogene, Beijing, China) after library construction. In brief, genomic DNA was randomly fragmented using a Covaris ultrasonic disruptor. A paired-end library was prepared following the steps of end repair and phosphorylation, including A-tailing, index adapter ligation, purification and PCR amplification. After the library was qualified, the Illumina HiSeq X platform was used for high-throughput sequencing, and paired-end reads of 150 bp were obtained, resulting in approximately 3 G of sequencing data for each strain.

De Novo Assembly

Raw reads were first trimmed to improve data reliability. Reads with low-quality bases (Phred score ≤ 5) exceeding 50% of the base number and reads with an Ns content exceeding 10% were discarded. Clean data were employed for genome assembly using SPAdes v3.12.0.²⁹ The parameter “-cov_cutoff” was set to auto. Contigs shorter than 200 bp were filtered to make the assembled data more reliable.³⁰ Sequencing depth was calculated as follows: reads number*150*2/base number in the genome.

SNP Calling

The reference *T. rubrum* genome CMCC(F)Tli was downloaded from the NCBI Genome dataset. Burrows–Wheeler Alignment tool (version 0.7.17) was used to map the paired-end reads against CMCC(F)Tli genome, choosing the BWA-MEM algorithm. SAMtools (version 1.9) was used to filter, sort and convert files.³⁰

Genome Analysis Toolkit (version 4.1.4.1) was used for SNPs calling as described previously.^{30,31} Specifically, following conditions were employed to filter SNPs and indels: VariantFiltration, QD < 2.0, LowQD, ReadPosRankSum < -8.0, LowRankSum, FS > 60.0, HightFS, MQRankSum < -12.5, MQRankSum, MQ < 40.0, LowMQ, HaplotypeScore > 13.0, and HaploScore.

Phylogenomics, Structure, and Population Genetics Analyses

The phylogenetic relationships of the 204 *T. rubrum* isolates were analyzed by RAxML (version 8.2.12) based on the dataset composed of 41,409 filtered SNPs.^{30,32} A maximum likelihood phylogenetic tree was inferred with the GTRMMA model, and the bootstrap value was set to 1000. iTOL (<https://itol.embl.de/>) was used for visualization. ADMIXTURE (version 1.3.0) was used to analyze the population structure. K value was examined through the cross-validation (CV) process, and the best fit was selected with the minimum CV error.

Statistics of Population Genetics

Nucleotide diversity (π) was calculated by VCFtools³³ based on the data of all 204 isolates classified by geographic origin, respectively, with the site-pi option. Differentiation between isolates from Hainan Island and other collecting sites (F_{st}) was obtained using VCFtools. All the analysis was done genome-wide along with 5 kb sliding windows. Plots were done using the R package ggplot2.³⁴

Results

Independent Evolution of *T. rubrum* from Hainan Island

To examine the population genetic characteristics of *T. rubrum* in China, the genomes of 204 *T. rubrum* isolates collected from 7 provinces were sequenced and analyzed. To maximize the ecological and geographical breadth of their origins, this collection included 89 strains from Hainan Island that were geographically independent from inland areas and 115 strains from inland cities. Overall, the sampling sites

covered different climate zones and areas showing different ecological and social characteristics (Figure 1, Table 1).

The calculated sequence coverage ranged from 70x to 193x (average = 132.9x; median = 134.5x). A total of 41,049 single-nucleotide polymorphisms (SNPs) (Supplementary Data 1) were identified through the 204 genomes. Phylogenetic relationship of the 204 *T. rubrum* from different geographical regions was shown by a maximum likelihood phylogenetic tree, which resulted from the 41,049 confident SNPs. The evolutionary relationships of the strains from different regions were not completely separated according to the phylogenetic tree, but between the lineages, we observed geographical patterns in which the majority of isolates from Hainan Island clustered together (Figure 2, pink area), suggesting an independent evolutionary trend of these strains.

Genetic Diversity and Population Differentiation

To further verify the classification of *T. rubrum* strains, we analyzed their population structure. As shown in

Figure 3, genetic admixture was observed among *T. rubrum* from different regions. When K value varied from 2 to 10, the populations were all mixed with no obvious resolution. We further studied genetic diversity among 29,433 polymorphic sites, which were filtered from 41,049 SNPs using VCFtools with the “max missing” parameter set to 1. The average nucleotide diversity (π) calculated from all 204 *T. rubrum* was 0.22, while the π value of Hainan strains was 0.24. No significant difference was found when nucleotide diversity was compared according to the geographical classification (Figure 4). The low genetic polymorphism observed verified the low levels of variation previously reported among *T. rubrum*.^{17,24} In addition, no significant F_{st} values were obtained between different geographical populations (Figure 4). The results of the genetic polymorphism analysis suggested no clear genetic differentiation, which was consistent with the population structure analysis; thus, the differentiation of isolates from Hainan Island is only reflected in the phylogenetic tree.

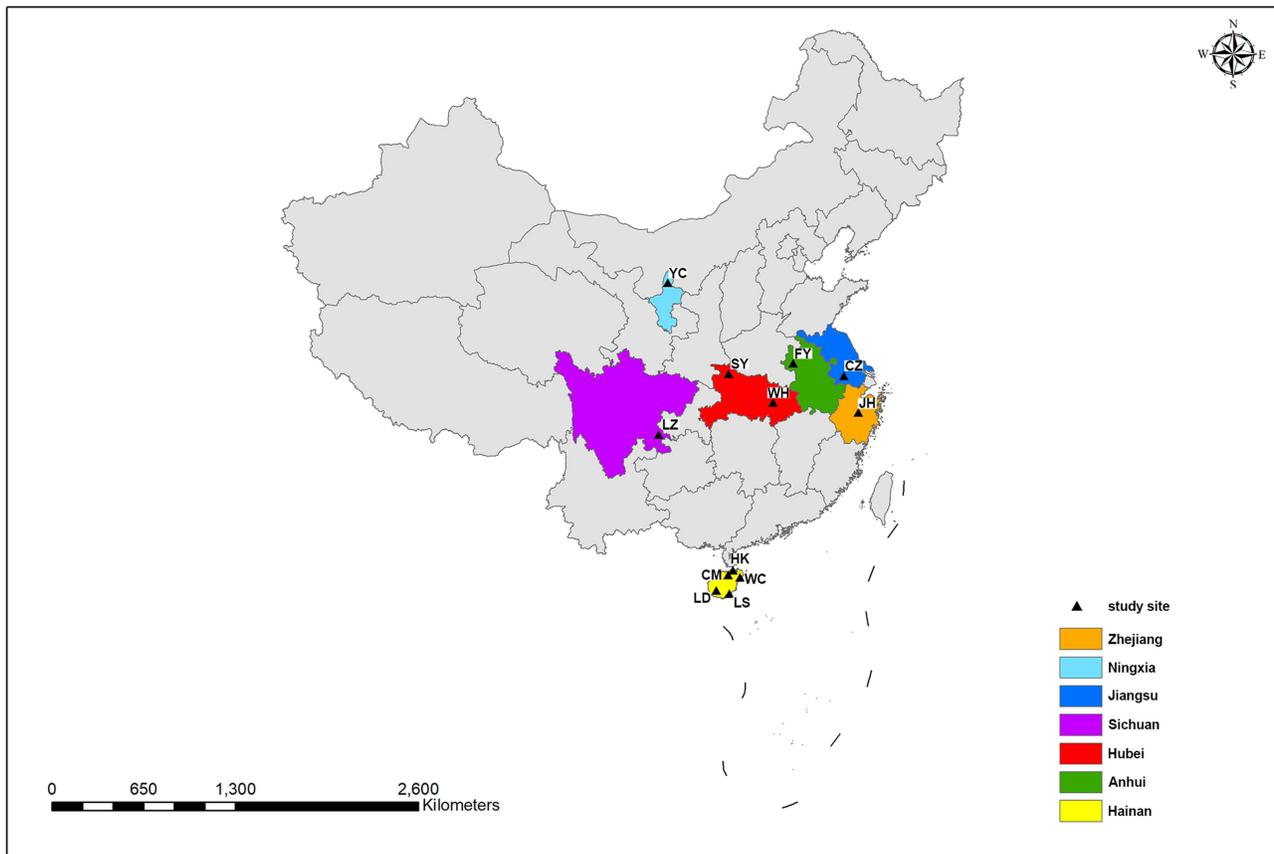


Figure 1 Geographical distributions of *T. rubrum* isolates collected in China. Sampling sites from different provinces were marked with different colors, and the cities were indicated by black triangle.

Table I Ecological and Geographic Information of *T. rubrum* Isolates in This Study

Province	Collection Site	No. of Strains	Longitude (E°)	Latitude (N°)	Climate Zone	Population Size (Ten Thousand)	Temperature (°C)	Humidity (%)	Atmospheric Pressure
Hainan	Haikou, HK	33	110.33	20.03	Tropical	224.83	24.96	81.50	1004.12
	Wenchang, WC	26	110.80	19.54	Tropical	55.94	25.33	83.44	1008.57
	Lingshui, LS	19	110.04	18.51	Tropical	32.97	23.96	83.05	983.06
	Chengmai, CM	7	110.00	19.74	Tropical	48.72	24.79	80.42	992.08
	Ledong, LD	4	109.17	18.75	Tropical	47.59	23.96	83.05	983.06
Hubei	Wuhan, WH	37	114.03	30.58	Subtropical	1073.71	17.20	79.41	1013.18
	Shiyan, SY	26	110.81	32.59	Subtropical	809.02	16.31	71.02	988.71
Anhui	Fuyang, FY	32	115.86	32.91	Temperate	339.77	16.08	75.66	1012.86
Sichuan	Luzhou, LZ	11	105.44	28.88	Subtropical	429.64	18.48	79.78	967.03
Jiangsu	Changzhou, CZ	4	119.60	31.72	Subtropical	471.04	17.09	76.08	1015.72
Ningxia	Yinchuan, YC	4	106.24	38.47	Temperate	219.20	10.75	48.21	890.66
Zhejiang	Jinhua, JH	1	120.24	29.29	Subtropical	481.75	18.92	73.75	1008.57

Discussion

T. rubrum ranks the most common isolated dermatophyte causing dermatophytosis in clinic and has become an epidemic strain worldwide since the 1950s.^{14,21,35} From ecological and evolutionary points of view, *T. rubrum* infection recurrence essentially occurs via the adaptation of a group of *T. rubrum* to the ecological environment and the host environment. Therefore, in-depth study of the population genetic characteristics of *T. rubrum*, especially its adaptive evolution, may provide guidance for the prevention and treatment of *T. rubrum* infection.

In this study, we collected *T. rubrum* from different geographical regions of China. The phylogenetic characteristics and genetic polymorphism distribution pattern of *T. rubrum* were studied by analyzing genomic sequences. A total number of 41,049 SNPs were identified among 204 isolates, which was much lower than other fungal genera (2,454,052 SNPs among 157 *Saccharomyces cerevisiae* isolates;³² 589,255 SNPs among 182 *Candida albicans* isolates,³⁰ or 300,116 SNPs among L- and S-type *Aspergillus flavus* isolates,³⁶ etc.). However, it's reasonable for *T. rubrum* to present less SNPs. Zheng et al detected 23,394 genomic variations through 48 sampled *T. rubrum* strains;¹⁷ in another study, an average of 8092 SNPs were identified for 10 *T. rubrum* isolates compared to reference genome.²⁴ The low number of SNPs reflected the low degree of variation of *T. rubrum*, which may result from the clonal reproduction of *T. rubrum*.³⁷

Based on the 41,049 SNPs, we found that the genetic differentiation structure of *T. rubrum* in China showed no

significant relationship with the geographical regions of the patients and that the genetic diversity of the studied isolates was low, which was consistent with previous studies. Numerous studies have confirmed the low genetic diversity of *T. rubrum* as it has spread around the world in recent decades, and its genetic differentiation remained low levels.^{17,20,24}

However, it is worth noting that the strains from Hainan Island clustered together in the phylogenetic tree, whereas no genetic differentiation was observed in relation to strains outside the island. Previous reports have indicated that *T. rubrum* is highly clonal. Its reproduction may be asexual, or its sexual reproduction occurs infrequently,^{24,38} which could be responsible for the low frequency of genetic differentiation observed. Due to the unique geographical location of Hainan Island, which was separated from the mainland, we inferred that the current distribution pattern of *T. rubrum* on Hainan Island was a result of a founder effect.

Founder effects occur mostly when a new population is formed initially and developed by a few individuals (the founders) from the original population, leading to a low genetic diversity of the new population.^{6,8,39} The *T. rubrum* population of Hainan Island experienced a founder effect when it was established. Due to personnel exchanges, people from outside of Hainan enter the island carrying *T. rubrum*, leading to its spreading and reproduction. Local transmission may occur among populations from contaminated skin flakes, either directly from humans to humans or via environment. The subsequent

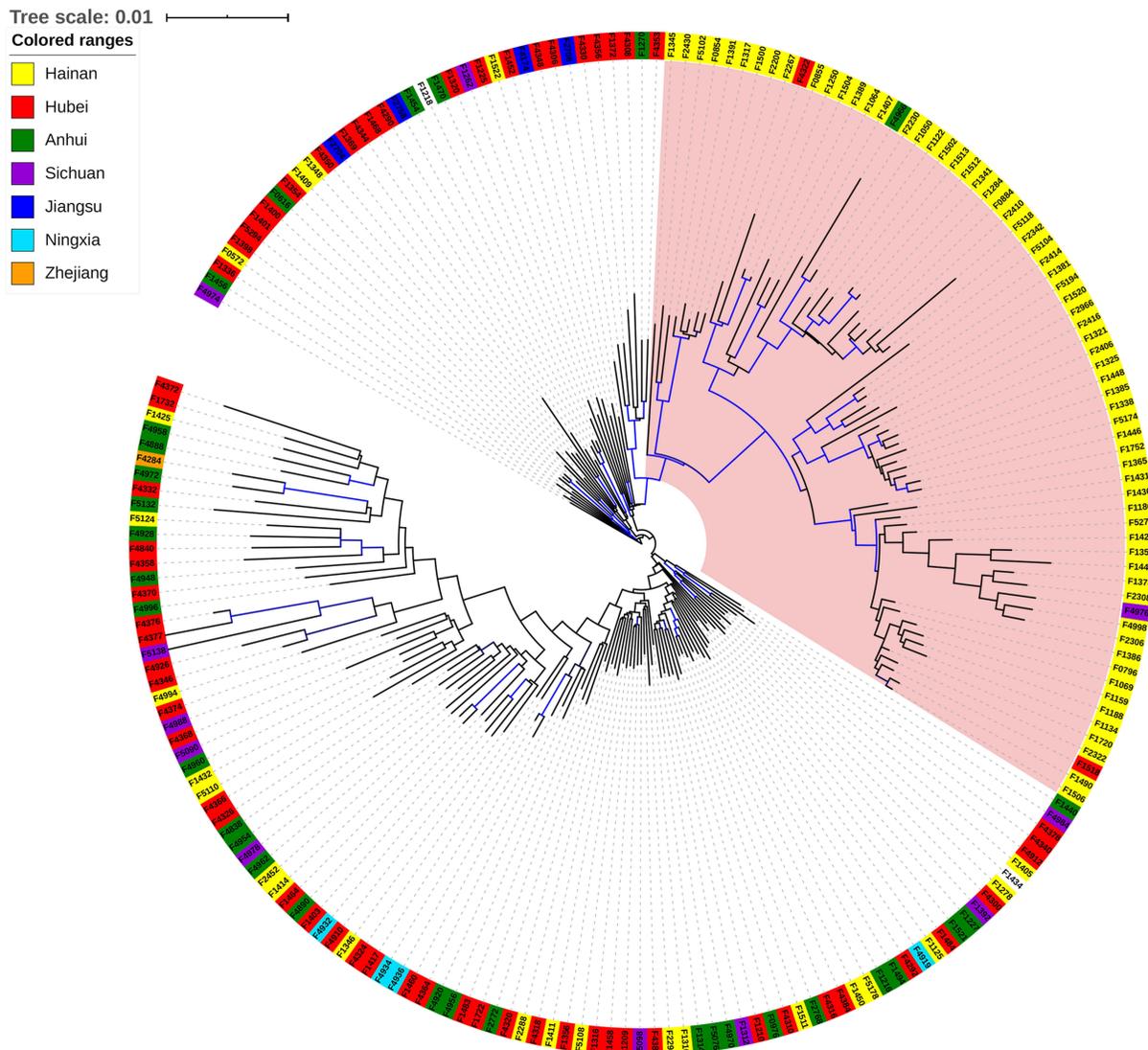


Figure 2 Phylogenetic relationships of *T. rubrum* isolates of China. Phylogeny among the 204 *T. rubrum* strains sequenced in this study was shown as a maximum likelihood phylogenetic tree. Isolates from different provinces were indicated by different color ranges. Blue Branch lines represented bootstrap supports >90%.

T. rubrum infections on the island were all caused by descendants of one or several ancestors.

The genetic variability level of island populations is usually lower than mainland populations of the same species.⁴ As the genetic diversity of *T. rubrum* is low overall within the species, the *T. rubrum* population on Hainan Island has maintained low genetic diversity during its evolution and shows no significant differentiation from populations at sites other than Hainan Island. The ecological influence of genetic diversity can be reflected at different levels, from the population to the whole ecosystem.⁴⁰ High genetic diversity means that populations can better adapt to the environment under selective pressure. In this case, since

stains from Hainan Island presented no differentiation from other sites, we suggested no specific modification for the prevention and treatment strategies against *T. rubrum*.

Based on our findings, we proposed that the *T. rubrum* population on Hainan Island originated from a specific historical event and reproduced after being introduced to the island. With the increase in cross-strait exchanges, a small number of *T. rubrum* from other regions have spread and survived on the island. Due to the short evolutionary time (being the epidemic strain worldwide since the 1950s), only a differentiation trend was observed between the population of Hainan Island and those of other regions.

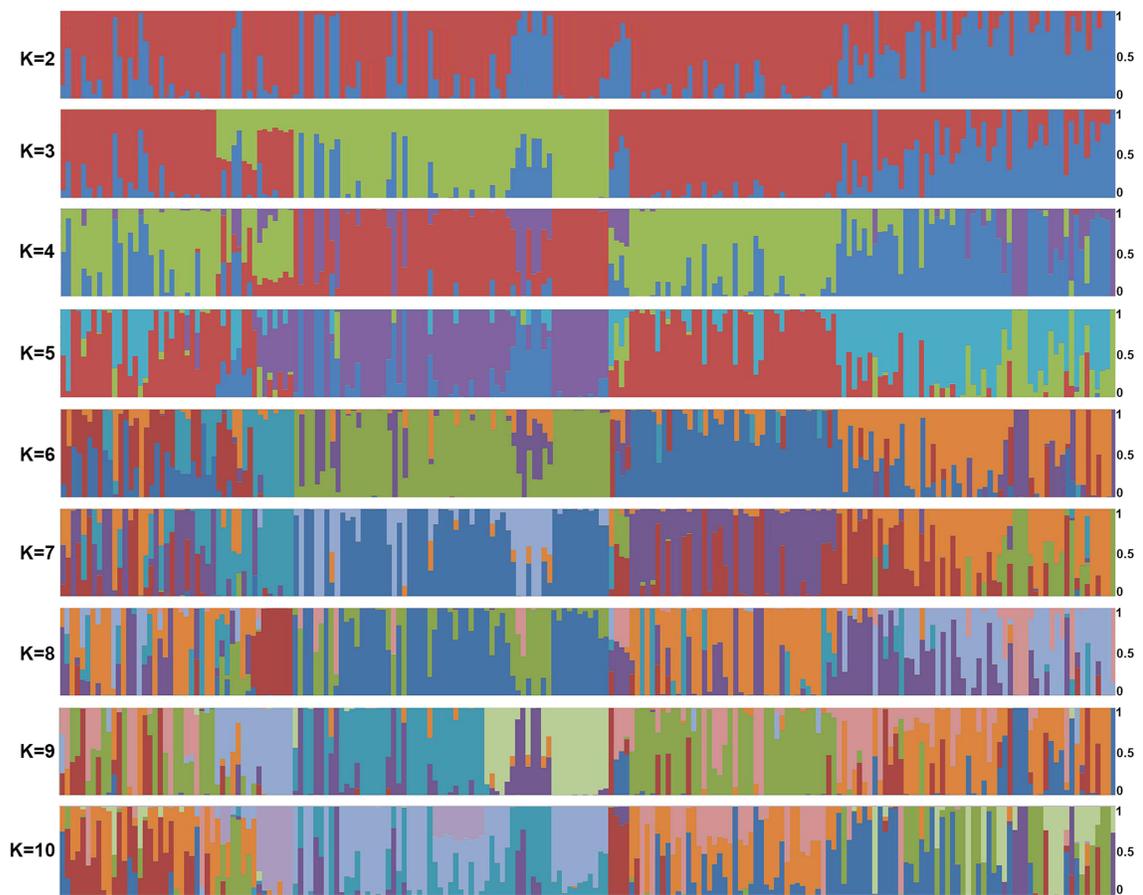


Figure 3 Population structure generated by the 204 sequenced *T. rubrum* strains. Horizontal axis: strains listed in [Supplementary Data 1](#); vertical axis: the fractional representation of populations derived from 204 strains when $K = 2-10$.

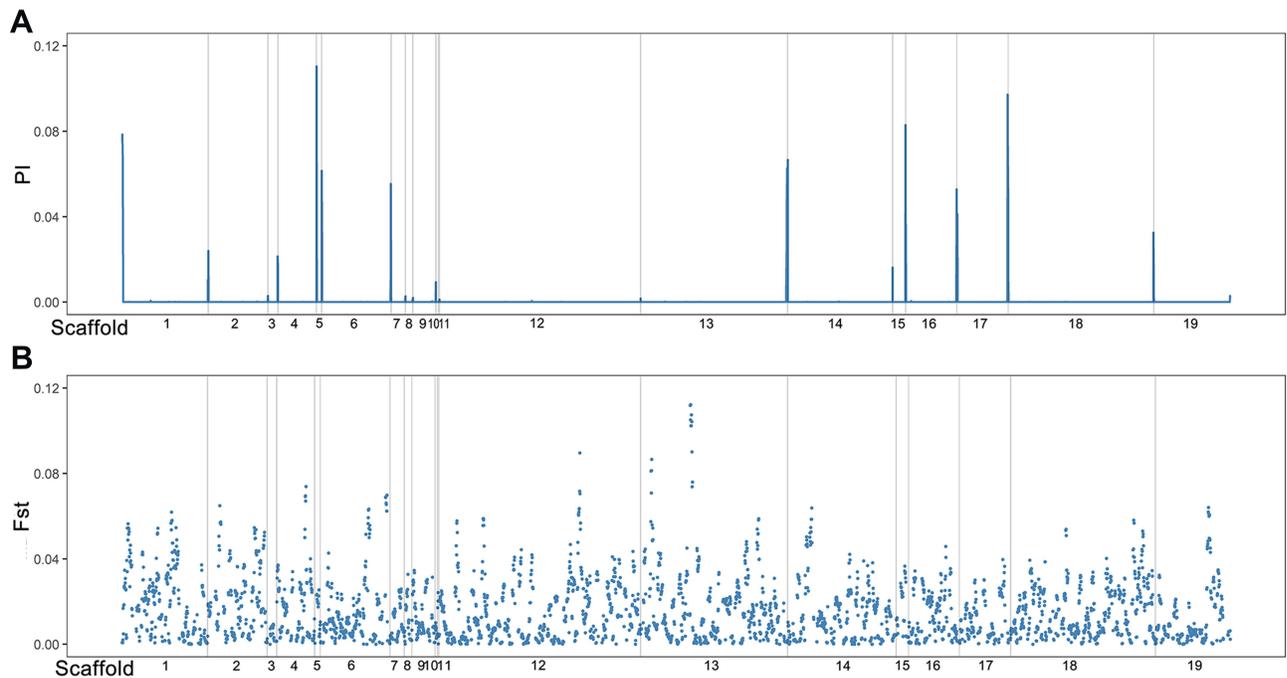


Figure 4 Genetic differentiation in the 204 surveyed *T. rubrum* strains. **(A)** Genome-wide (5-kb windows) pairwise F_{st} between stains from Hainan Island and other collecting sites; **(B)** genome-wide (5-kb windows) nucleotide diversity (π) of surveyed strains. Gray lines: scaffold boundaries. X-axis: scaffolds exhibit in order.

Taken together, the results of this research showed that *T. rubrum* was introduced into Hainan from off of the island, followed by its evolution for a short time, and there was a trend of differentiation observed in comparison with strains from sites other than Hainan Island. This study discussed general ecological and evolutionary principles with regard to pathogenic fungi. Due to the epidemiological characteristics of *T. rubrum* (high prevalence, worldwide dominant epidermal pathogens), it can be compared with the genome data of other dermatophytes, and reveals candidate genes related to its pathogenicity or invasion of the host. Besides, association studies with phenotype information such as drug resistance will provide a theoretical basis for development of new antifungal drug and further benefits clinical treatment.

Data and Code Availability

Assembled genomes have been uploaded to NCBI GenBank with BioProject ID PRJNA673795.

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. This study was approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention (ICDC) and is consistent with the guidelines of the Helsinki Declaration.

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Disclosure

The authors declare no conflicts of interest.

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