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Molecular diversity of *Fonsecaea* (*Chaetothyriales*) causing chromoblastomycosis in southern China

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Chromoblastomycosis is a chronic, cutaneous and subcutaneous infection caused by members of the order *Chaetothyriales* and commonly found in China. Among the etiologic agents, members of the genus *Cladophialophora* are predominant in northern China. Alternatively, *Fonsecaea* spp. are particularly common in southern China. However, the identification of *Fonsecaea* isolates recovered in China is difficult due to the fact that different species lack distinctive morphological characters. Therefore, the identification of 24 *Fonsecaea* isolates from symptomatic patients were re-evaluated by using morphology, ITS rDNA sequence diversity and partly through the use of randomly amplified polymorphic DNA (RAPD) typing. Twenty strains, including a morphological mutant were found to be *Fonsecaea monophora*, while four strains corresponded to *F. pedrosoi*. We have demonstrated that *Fonsecaea monophora* is the predominant etiologic agent of chromoblastomycosis in southern China and populations showed marked geographic structuring.

Keywords Chromoblastomycosis, *Fonsecaea*, taxonomy

Introduction

Chromoblastomycosis is a chronic, cutaneous and subcutaneous infection characterized by verrucose lesions and a tissue phase that consists of muriform cells. The disease is found world wide, but most reports are concerned with its presence in tropical and subtropical areas. The causative fungi include *Fonsecaea pedrosoi* [1,2], *F.monophora* [3–5], *Cladophialophora carrionii* [1], *Phialophora verrucosa* [6,7] and *Rhinocladiella aquaspersa* [8,9]. In China the disease is fairly common, especially in Shandong and Henan, with more than 500 cases reported from 20 provinces since the first case description in 1952 [10–22]. The predominant causative pathogen in northern parts of the country is *Cladophialophora carrionii*, while infections in the south are mostly ascribed to *Fonsecaea pedrosoi* [13,20].

Traditionally the genus *Fonsecaea* included two morphological species, i.e., *Fonsecaea pedrosoi* and *F. compacta* [23,24], but recent molecular analysis suggested that *F. compacta* is a mutant of *F. pedrosoi*, while another, morphologically nearly indistinguishable species, *Fonsecaea monophora*, was segregated from *F. pedrosoi* on the basis of ITS rDNA data [4]. This separation was recently confirmed by multilocus analysis [25]. Because the morphologic features of conidial formation in *Fonsecaea pedrosoi* and *F. monophora* is simple and relatively uncharacteristic, both species may be easily misidentified in clinical practice. However, separation of the two species is clinically significant because the spectrum of infection due to *Fonsecaea monophora* has been shown to be more variable than that of *F. pedrosoi*. *Fonsecaea pedrosoi* seems to be a highly specialized agent of chromoblastomycosis, whereas the opportunistic *F.monophora* has been involved in cerebral infections [3].

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We recently re-evaluated the identity of 24 isolates recovered from patients with chromoblastomycosis in our hospital and in others in China that were originally identified as *F. pedrosoi*. Strains were identified by ITS rDNA sequencing, while epidemiological questions were addressed using Randomly Amplified Polymorphic DNA (RAPD) typing.

Materials and methods

Strains and morphology

All 24 strains recovered from Chinese patients (23 male and 1 female) who were symptomatic for chromoblastomycosis (20 from southern, three from eastern and one from northern China), and 59 reference strains of *Fonsecaea* from the CBS collection are listed in Table 1. The predominant type of lesion was verrucous and was located on the lower limbs, with traumatic history. Strains were grown at 26°C on plates of Sabouraud's glucose agar (SGA), potato dextrose agar (PDA), cherry decoction agar (CDA) and cornmeal agar (CMA) for 2 wk and slide cultures were prepared for morphological observation.

Physiology

The hydrolysis of urea was tested using Christensen's urea agar. Inoculated tubes were incubated at 26°C and the results were considered positive if the medium turned pink within 4 days. Cycloheximide tolerance was investigated on SGA plates containing 0.1, 0.05 and 0.01% of the antifungal. Thermotolerance was explored with strains incubated on SGA for 2 weeks at 26, 37 and 40°C. Growth responses were scored as positive (+), negative (−), weak (w) or ambiguous (w/+ or w/−).

DNA analysis and sequencing

DNA was extracted using 6% InStaGene™ Matrix (BioRad, USA). Ribosomal DNA ITS domains were amplified in a Biometra T-Gradient Thermoblock (Germany) using primers ITS-4 (5′-TCCTCCGCTT ATTGATATGC-3′) and ITS-5 (5′-GGAAGTAAAAG TCGTAACAAGG-3′), with the following conditions: 95°C for 4 min, followed by 30 cycles of 94°C for 60 s, 55°C for 90 s, and 72°C for 90 s. Direct sequencing of PCR products was done with an ABI PRISM 3100 sequencer after labeling with BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, Calif.). ITS sequences were aligned using BioNumerics (Applied Maths, Kortrijk, Belgium). A consensus tree of 83 members of *Fonsecaea*, constructed with the Tree Finder algorithm (v. June 2007, by Gangolf Jobb) and 100 bootstrap replicates (values >90 are shown with the branches) and edited with FigTree v.1.0 (http://evolve.zoo.ox.ac.uk/software/figtree).

RAPD analysis

Primers ATGS (5′-ATGGATCSSC-3′) and UBC701 (5′-CCCAACAACCC-3′) were used for RAPD typing in which a 50 µl reaction system contained; 5 µl template DNA (20 ng), 4 µl dNTP (2.5 mM) mixture (Nippon Gene, Japan), 5 µl 5 pmol primer, 0.25 µl (5 U/µl) *Taq* polymerase (Nippon Gene, Japan), 5 µl 10 × reaction buffer (Nippon Gene, Japan), 30.75 µl deionized water. Forty PCR cycles were applied under the following conditions; 94°C for 2 min (delay), 94°C for 1 min (denaturation), 34°C for 1 min (annealing), 72°C for 2 min (extension), final extension at 72°C for 10 min. Products were electrophoresed in 2% agarose gel (Nusieve 3:1,100 v for 90 min) and strained with ethidium bromide. A tree was constructed using Ward's algorithm with Jaccard's coefficient.

Results

All 24 of the Chinese strains except SUMS0254 had the colony and microscopic appearance typical of the genus *Fonsecaea* (Fig. 1a, b). SUMS0254 had black, dry slow growing colonies with friable texture and meristematic growth was abundant in slide cultures. The maximum growth temperature of all strains analyzed was found to be 37°C but the results of urea hydrolysis studies were ambiguous or weakly positive. All 24 strains were tolerant to all concentrations of cycloheximide employed in this study.

Five groups were observed in the consensus tree of ITS sequence data (Fig. 2), with two main clusters, i.e., cluster A (matching *Fonsecaea pedrosoi*) and cluster B (*F.monophora*), with the latter also comprising a paraphyletic group (B-3). The species and genotypes were separated by consistent, phylogenetically informative polymorphisms based on ITS alignment (Table 2). Twenty of the Chinese strains that were formerly identified as *Fonsecaea pedrosoi* were found to be *F.monophora* based on ITS rDNA sequence analysis. The strain with meristematic growth, SUMS0254 was also found to be *Fonsecaea monophora* on the basis of molecular evidence, and hence was regarded to be a morphological mutant of this species. Four Chinese strains and a few reference strains of *F. pedrosoi* recovered from Africa, France and Japan were classified as genotype A-2, while reference strains, mostly

Table 1 The strains listed as *Fonsecaea pedrosoi* and *F. monophora* are the re-identified ones, grouped according to similarities in ITS sequence and RAPD studies

Strain number	GenBank	Geography	Source	ITS	RAPD
F. pedrosoi					
IFM 41705	AB091206	China	Bark	A1	
ATCC 46428	AF397132	Brazil	Chromoblastomycosis	A1	
dH 12664	AY366907	Mexico	Chromoblastomycosis	A1	
dH 12658	AY366908	Mexico	Chromoblastomycosis	A1	
dH 12661	AY366909	Mexico	Chromoblastomycosis	A1	
dH 12660	AY366911	Mexico	Chromoblastomycosis	A1	
CBS 102244		Brazil	Chromoblastomycosis	A1	
CBS 102245		Brazil	Chromoblastomycosis	A1	
CBS 201.31	AY366913	Libyia	Ear gazelle	A1	
CBS 212.77 'compacta'			Chromoblastomycosis	A1	
CBS 271.37 (NT)		South America	Human	A1	
CBS 272.37		Brazil	Chromoblastomycosis	A1	
CBS 274.66		Venezuela	Soil-mouse passage	A1	
CBS 285.47 'compacta'	AF361053	Puerto Rico	Chromoblastomycosis	A1	
CBS 342.34		Brazil	Chromoblastomycosis	A1	
IFM 41704	AB091209	Japan	Unknown	A1	
IFM 41931	AB091210	Japan	Unknown	A1	
IFM 4886	AB091208	Japan	Unknown	A1	
CBS 659.76 'compacta'	AY366920	Argentina	Chromoblastomycosis	A1	
CBS 670.66		Venezuela	Soil-mouse passage	A1	
CBS 671.66		Venezuela	Soil-mouse passage	A1	
Fp28III		Brazil	Chromoblastomycosis	A1	
FP63I		Brazil	Chromoblastomycosis	A1	
FP77II =CBS 102247		Brazil	Chromoblastomycosis	A1	
IMTSP 877	AY366922		Unknown	A1	
KMU3665	AB117983		Unknown	A1	
KMU3709	AB117984		Unknown	A1	
dH 13277	UNEFM 9807	Venezuela	Chromoblastomycosis	A1	
dH 13278	UNEFM 95009B	Venezuela	Chromoblastomycosis	A1	
dH 13279	UNEFM 9301	Venezuela	Chromoblastomycosis	A1	
dH 13280	UNEFM R4	Venezuela	Environment	A1	
dH 14477	UNEFM 0002-04	Venezuela	Choromoblastomycosis hand	A1	
dH 18431		Mexico	Choromoblastomycosis foot	A1	
CBS 269.64		Kameroen	Chromoblastomycosis	A2	
CBS 444.62		Surinam	Chromoblastomycosis	A2	
KMU3537	AB117980		Unknown	A2	
CBS 270.37		France	Unknown	A2	
CBS 271.33	AB114127	South America	Chromoblastomycosis	A2	
CBS 277.29		Brazil	Chromoblastomycosis	A2	
CBS 557.76			Unknown	A2	
IFM 46410	AB091207	Japan	Unknown	A2	
SUMS0251	EF513757.1	China (Guangdong)	Chromoblastomycosis knee	A2	A
SUMS0255	EF513758.1	China (Guangdong)	Chromoblastomycosis hand	A2	A
SUMS0011	EF513756.1	China (Guangdong)	Chromoblastomycosis	A2	A
SUMS0323	Eu285271	China (Shanghai)	Chromoblastomycosis	A2	
F. monophora					
KMU3780	AB117979		Unknown	B1	
SUMS0147	Eu285266	China (Shandong)	Chromoblastomycosis	B1	B1
SUMS0300	Eu285273	China (Nanjing)	Chromoblastomycosis	B1	B3
SUMS0324	Eu285270	China (Shanghai)	Chromoblastomycosis	B1	B3
SUMS0322	Eu285268	China (Shanghai)	Chromoblastomycosis	B1	
CBS 102229		Brazil	Litter	B2	
CBS 102238	AY366927	Brazil	Soil	B2	
CBS 102242		Brazil	Chromoblastomycosis	B2	
CBS 102243		Brazil	Chromoblastomycosis	B2	
CBS 102246	AY366928	Brazil	Chromoblastomycosis	B2	
CBS 102248	AY366926	Brazil	Chromoblastomycosis	B2	

Table 1 (*Continued*)

Strain number	GenBank	Geography	Source	ITS	RAPD
CBS 269.37 (T)	AY857511	South America	Chromoblastomycosis	B2	
Fp65		Brazil	Chromoblastomycosis	B2	
Fp82		Brazil	Chromoblastomycosis	B2	
KMU3666	AB117977		Unknown	B2	
KMU3713	AB117978		Unknown	B2	
dH 12978		Brazil	Brain	B2	
dH 14523		USA	Phaeohyphomycosis	B2	
dH 15331		USA	Chromoblastomycosis leg	B2	
dH 18209		USA	Brain	B2	
dH 18210		USA	Chromoblastomycosis leg	B2	
CBS 117238		England	Brain	B3	
SUMS0013	EF513760.1	China (Guangdong)	Chromoblastomycosis leg	B3	B2
SUMS0012	EF513759.1	China (Guangdong)	Chromoblastomycosis buttock	B3	B2
SUMS0034	EF513762.1	China (Guangdong)	Chromoblastomycosis ankle	B3	B2
SUMS0246	EF513768.1	China (Guangdong)	Chromoblastomycosis leg	B3	B2
SUMS0254 'meristematic'	EF513737.1	China (Guangdong)	Chromoblastomycosis hand	B3	B2
SUMS0247	EF513769.1	China (Guangdong)	Chromoblastomycosis foot	B3	B2
SUMS0228	EF513767.1	China (Guangdong)	Chromoblastomycosis face	B3	B2
SUMS0200	EF513766.1	China (Guangdong)	Chromoblastomycosis leg	B3	B2
SUMS0250	EF513770.1	China (Guangdong)	Chromoblastomycosis knee	B3	B2
SUMS0192	EF513770.1	China (Guangdong)	Chromoblastomycosis ankle	B3	B2
SUMS0190	EF513764.1	China (Guangdong)	Chromoblastomycosis leg	B3	B2
SUMS0158	EF513763.1	China (Guangdong)	Chromoblastomycosis leg	B3	B2
SUMS0014	EF513761.1	China (Guangdong)	Chromoblastomycosis leg	B3	B2
SUMS0301	Eu285272	China (Nanjing)	Chromoblastomycosis leg	B3	B2
SUMS0325	Eu285269	China (Wuhan)	Chromoblastomycosis leg	B3	B2
SUMS0295	Eu285267	China (Guangdong)	Chromoblastomycosis leg	B3	B2

from South America, were consistent with genotype A-1. Similarly, twenty strains of *F. monophora* from China were members of genotypes B-1 and B-3 and with the exception of strain CBS117238, all isolates for which geographic information was available provided to belong to B-2.

Epidemiological typing of the Chinese strains of clusters I (*F. pedrosoi*) and II (*F. monophora*) was obtained with RAPD. A limited degree of polymorphism was observed as for example presented in Fig. 3. Within cluster II, which included 19 of the 20 strains of *F. monophora*, there were three sub-clusters II-1, II-2 and II-3 representing one isolate northern, 15 strains from southern, and two from eastern China, respectively. Cluster I included 3 of 4 strains identified as *F. pedrosoi* recovered from patients (one exception) who lived in the same area of Guangdong were found to be 100% genetically similar with primer ATGS.

Discussion

Fonsecaea pedrosoi and *F. monophora* had similar morphological and physiological patterns and could not be distinguished phyotypically, but could be differentiated by consistent, phylogenetically informative polymorphism in ten bases using molecular data from the rDNA locus [3,4]. Clinically they seem to differ in that *Fonsecaea pedrosoi* was isolated from

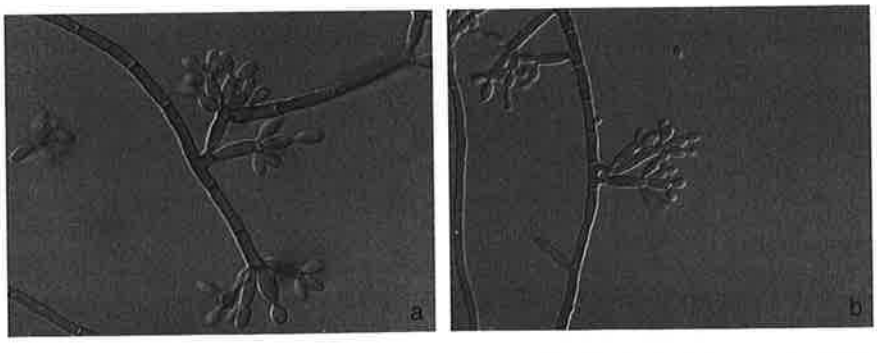

Fig. 1 Microscopic appearance of Chinese strains of *Fonsecaea pedrosoi* (left) and *Fonsecaea monophora*: SUMS0251(a), SUMS0014(b).

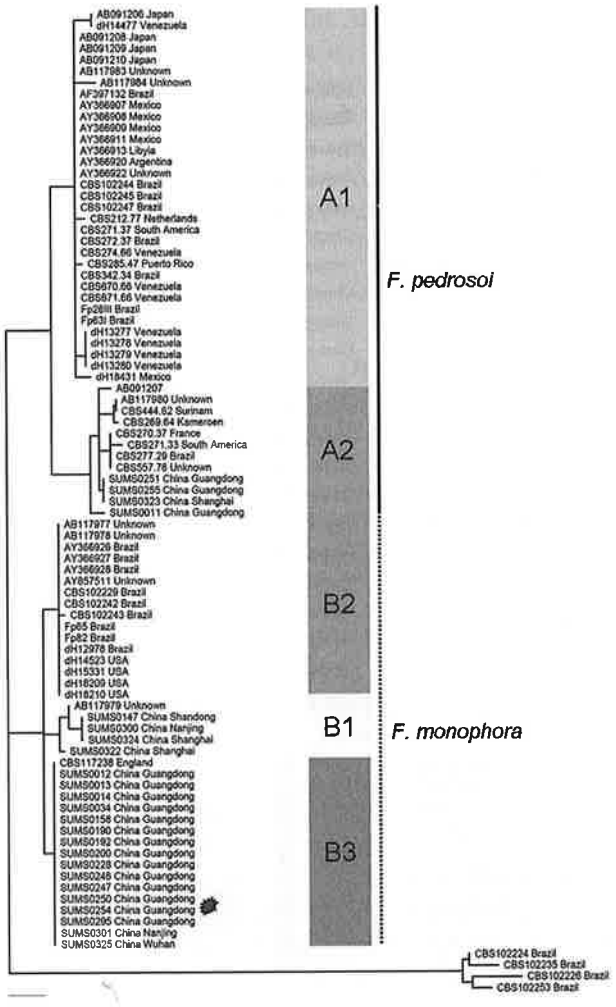

Fig. 2 Consensus tree of *Fonsecaea* based on Internal Transcribed Spacer (ITS) ribosomal DNA of 83 strains, constructed with the Tree Finder algorithm (v. June 2007) and 100 bootstrap replicates (values ≥80 are shown with the branches) and edited with Fig Tree v. 1.0. CBS 102224, CBS102235, CBS 102226 and CBS 102253 are taken as outgroup. Meristematic mutant indicated by a star.

cases of chromoblastomycosis, while *F. monophora* was involved in other types of infection, among which recurrent cases of cerebritis were the most striking [3]. In the present study, 19 out of 20 strains of *F. monophora* had similar phyenotypic features as four strains of *F. pedrosoi*. But the strain SUMS0254 was quite different from all of these by showing meristematic morphology, similar to *Botryomyces caespitosus* or *Exophiala phaeomuriformis* [26]. Despite this morphology, ITS rDNA data showed 100% identity with the type strain of *F. monophora*, CBS 269.37. All our isolates were obtained from patients suffering chromoblastomycosis.

Two genotypes were recognizable in *Fonsecaea pedrosoi* and three in *F. monophora* (Fig. 2). Three locations of mutation in ITS1 and ITS2 were found to be characteristic for each of the species, while 12 additional mutations were found to define genotypes A-1 and A-2 (*F. pedrosoi*), and ten defined genotypes B-1, B-2 and B-3 (*F. monophora*) (Table 2). Multilocus data are currently being analyzed in order to verify whether this entity should be classified as a separate species (unpublished data). Strains of the genotype *F. monophora* B-3, which were prevalent in southern China, were primarily recovered from cases of localized, subcutaneous chromoblastomycosis presenting as verrucous, nodular or plaque lesions (Fig. 4). These strains are identical to only a single non-Chinese strain, CBS 117238, which was the etiologic agent of a brain infection in a patient from UK [3]. It is interesting to note that there is no documentation that demonstrates that this patient ever lived or traveled in China. Strains of genotype *F. monophora* B-1 included a few strains that were prevalent in eastern and northern China. Genotype B-2 is encountered in South America and the USA. These results indicate that populations of *F. monophora* are highly structured, as was found for *F. pedrosoi* in Japan [5]. RAPD data demonstrated that local strains belonged to a limited number of identical populations (Fig. 3). Using mtDNA, Kawasaki *et al.* [27] reported geographical clustering of *F. pedrosoi* in 1999. These data show that *Fonsecaea* strains have local foci, and it may be assumed that they are distributed globally by the travels of the hosts. The RAPD technique can successfully be applied as a rapid and easy method to resolve epidemiological questions in analyses of strains from different sources and geography.

Table 2 Phylogenetically informative sites in *Fonsecaea pedrosoi* and *Fonsecaea monophora* by ITS comparing

		16	42	48	70	78	81	90	101	102	106	111	115	137	143	386	413	414	463	486	488	511	545	546	553
F. pedrosoi	A1	C	C	A	C	...	T	T	C	T	G	T	A	T	A	T	C	G	A	T	T	T	T/..	T/..	T
	A2	T	T	A	C	...	T	C	C	T	A	T	G	T	C	C	C	G	A	A	C	T	...	...	C
F. monophora	B1	T	T	A	C	...	T	T	T	T	G	C	G	C	A	T	T	A	G	A	C	C	G	T	C
	B2	T	T	T	C	...	G	T	T	C	G	C	G	T	A	T	T	A	A	A	C	C	...	T	C
	B3	T	T	A	T	A	T	T	T	C	G	C	G	T	A	T	C	A	A	A	C	T	...	T	C

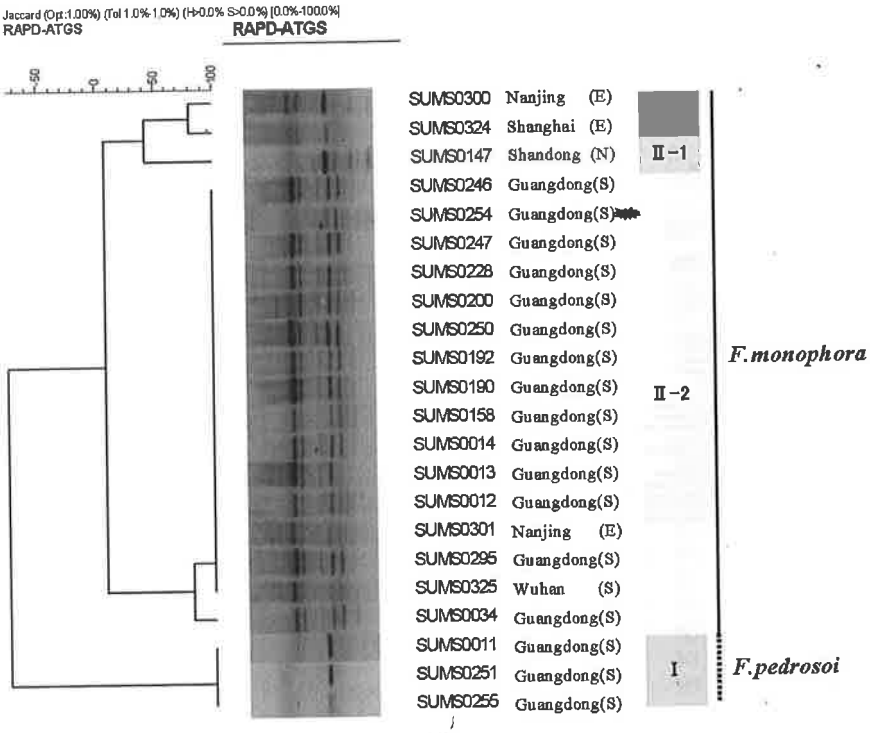

Fig. 3 Dendrogram based on pooled randomly amplified polymorphic DNA (RAPD) similarity among strains of *Fonsecaea* species of China using Jaccard's coefficient with 2000 bootstrap replicates (values in% are shown with the branches). Meristematic mutant indicated by a star.

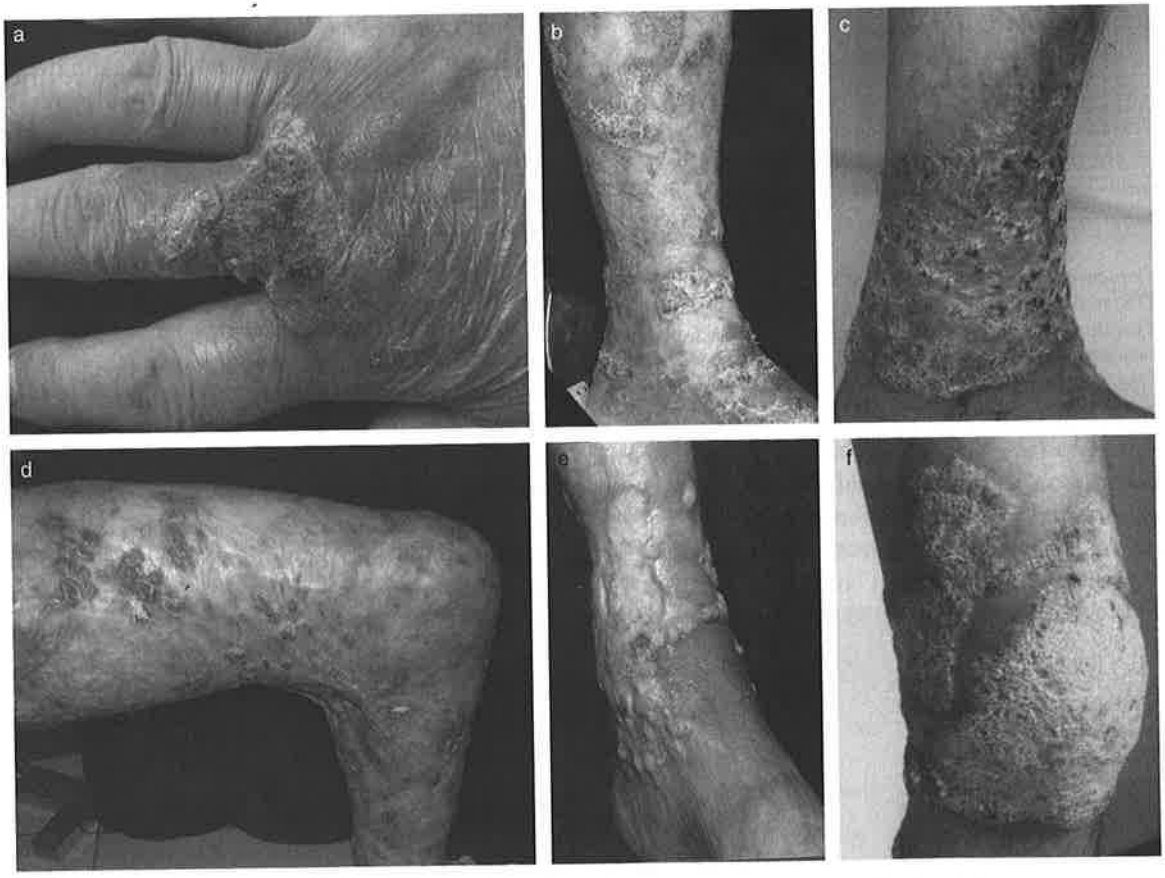

Fig. 4 Diversity of the lesions: reddish flat, scaly plaques (a), psoriasis-like (b), hyperkeratotic lesions (c), cicatrical atropic lesions (d), tumoral lesions (e), verrucose (f).

Our study suggests that the etiologic agent of chromoblastomycosis in China in most cases has been incorrectly identified as *Fonsecaea pedrosoi*, since our studies demonstrated that most of the strains belong to *F. monophora*. The fact that only two out of five known genotypes of *Fonsecaea* are represented in China shows that the species are geographically structured, suggesting a relatively slow vector of dispersal.

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