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Original Articles

Genetic diversity and species delimitation in the opportunistic genus *Fonsecaea*

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Genetic diversity and species delimitation were investigated among 39 isolates recovered from clinical and environmental sources in Central and South America, Africa, East Asia and Europe. All had been morphologically identified as *Fonsecaea* spp. Molecular analyses were based on sequences of the ribosomal internal transcribed spacers (ITS), β-tubulin (*TUB1*) and actin (*ACT1*) regions. A phylogenetic approach using haplotype networks was used to evaluate species delimitation and genetic diversity. The presence and the modes of reproductive isolation were tested by measuring the index of differentiation (ID) and the index of association (I_A). Based on the sequence data, 39 *Fonsecaea* strains were classified into three major entities: (i) a group representing *Fonsecaea pedrosoi*, (ii) a second composed of *F. monophora*, and (iii) a third group including mostly strains from South America. The two major, clinically relevant *Fonsecaea* species, *F. monophora* and *F. pedrosoi*, also differed in the pathological symptoms found in patients. Moreover, *F. pedrosoi* is mostly recovered in clinical settings, whereas *F. monophora* is commonly isolated from the environment. One environmental strain with *Fonsecaea*-like appearance was shown to belong to a different species, only distantly related to the core-group of *Fonsecaea*.

Keywords Chromoblastomycosis, *Fonsecaea pedrosoi, Fonsecaea monophora*, molecular phylogenetics

Introduction

Human chromoblastomycosis is a chronic cutaneous and subcutaneous infection caused by melanized moulds and characterized by verrucose skin lesions which eventually lead to emerging, cauliflower like eruptions that consist of muriform cells. The latter are produced in human skin and represent the supposed pathogenic invasive form of the fungi causing the disease. Chromoblastomycosis is found worldwide, but most clinical reports are from tropical and subtropical climates [1–4]. Proven causative agents include *Fonsecaea pedrosoi* [5], *F. monophora* [6–8], *Cladophialophora carrionii* [5], *Phialophora verrucosa* [9,10] and *Rhinocladiella aquaspersa* [11,12]. All these fungi are anamorphs of ascomycetes that belong to the family *Herpotrichiellaceae* (order *Chaetothyriales*). Cases of chromoblastomycosis caused by species outside the *Chaetothyriales*, such as *Chaetomium funicola* reported from western

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Panama in Central America [13] and *Catenulostroma chromoblastomycosum* from Zaire in Africa [14] have to be re-evaluated due to the fact that the characteristic muriform cells and host response with acanthosis were not conclusively established. The current hypothesis of infections caused by *Fonsecaea* species is that patients suffering from chromoblastomycosis are mainly rural workers that acquire the infection after being pricked by contaminated thorns or wood splinters. Recently, with the development of molecular tools for species identification, doubt has arisen about the correctness of this supposed route of infection [3].

The genus *Fonsecaea* is currently composed of two species, *F. pedrosoi* and *F. monophora,* based on the analysis of the ITS rDNA region. Morphologically, these species are very similar, but their pathology may be somewhat different from each other. *F. pedrosoi* is strictly associated with chromoblastomycosis, while *F. monophora* may also cause other types of infections [7]. Previously, an additional species was recognized, *F. compacta*, which was also described as an agent of chromoblastomycosis. This species is now, on the basis of sequence data, regarded to be a dysplastic mutant of *F. pedrosoi* [7], illustrating the limitations of traditional morphological species recognition (MSR). Biological species recognition (BSR) is widely applied to sexually reproducing organisms, including animals and plants, but cannot be used with *Fonsecaea* species because they seem to lack a sexual stage. In fungi, species previously diagnosed by MSR frequently appear to be composed of more than one species when applying phylogenetic species recognition (PSR) [15]. In PSR, individuals are grouped objectively, but the decision on the exact delimitation of species remains arbitrary. To avoid the subjectivity of determining the limits of a species, Taylor and his coworkers applied multilocus sequence typing to recognize fungal species to establish the priniciple of GCPSR, genealogical concordance phylogenetic species recognition [15,16]. The strength of GCPSR lies in a comparison of the congruency between several gene genealogies enabling the detection of recombination events [15]. Using GCPSR, multilocus sequence typing (MLST) schemes have been developed to investigate species delimitation in human or animal pathogenic fungi, such as *Coccidioides immitis* [17,18], *Coccidioides posadasii* [19], *Histoplasma capsulatum* [20], *Cryptococcus neoformans* [21], *Candida albicans* [22] and *Candida glabrata* [23], as well as in plant pathogenic fungi such as *Fusarium graminearum* [24,25] and *Mycosphaerella graminicola* [26]. In this study, we evaluate the genetic diversity and species delimitation in the genus *Fonsecaea* on the basis of three loci and temperature tests.

Material and methods

Fungal strains

Strains studied are listed in Table 1, which includes reference strains from the CBS collection as well as fresh isolates recovered from patients and environmental samples. Stock cultures were maintained on slants of 2% malt extract agar (MEA) and oatmeal agar (OA) at 24°C.

Physiology

Cardinal growth temperatures of all strains were determined on 2% MEA. Plates were incubated in the dark for 3 weeks at temperatures ranging at 3°C intervals from 21–36°C. In addition, growth was also examined at 37°C and at 40°C.

DNA extraction

Approximately 1 cm^2 of 14 to 21-day-old cultures was transferred to a 2 ml Eppendorf tube containing 400 µl TEx buffer (pH 9.0) and glass beads (Sigma G9143). The fungal material was homogenized with MoBio vortex for 1 min. Subsequently 120 µl SDS 10% and 10 µl proteinase K were added and incubated for 30 min at 55°C, the mixture was vortexed for 3 min. After addition of 120 µl of 5M NaCl and 1/10 vol CTAB 10% (cetyltrimethylammoniumbromide) solution, the material was incubated for 60 min at 55°C. Then the mixture was vortexed for 3 min. Subsequently 700 µl SEVAG (24:1, chloroform: isoamylalcohol) was mixed carefully by hand and centrifuged for 5 min at 4°C at 20400 *g* force value. The supernatant was transferred to a new Eppendorf tube with 225 µl 5 M NH_4-acetate, mixed carefully by inverting, incubated for 30 min on ice water, and centrifuged again for 5 min at 4°C at 20400 *g* force value. The supernatant was then transferred to another Eppendorf tube with 0.55 vol isopropanol and centrifuged for 5 min at 20400 *g* force value. Finally, the pellet was washed with 1000 µL ice cold 70% ethanol. After drying at room temperature, it was re-suspended in 100 µL TE buffer (Tris 0.12% w/v, Na-EDTA 0.04% w/v). For some strains, DNA was prepared with Ultra Clean™ Microbial DNA Isolation Kit (MoBio Laboratories).

DNA Amplification and sequencing

Three gene regions were chosen for the multilocus sequence typing, i.e., rDNA Internal Transcribed Spacers (ITS), partial genes and introns of actin (*ACT1*) and β-tubulin (*TUB1*) genes (primers are listed in Table 2). PCR was performed in a 25 µl volume of a

Table 1 Isolate and GenBank numbers and specimen information for the *Fonsecaea* investigated

CBS number	Other reference(s)	GenBank ITS, *TUB1, ACT1*	Source	Host	Geography
(B) F. monophora					
102243	dH 11607 = FP31I	EU938579, EU938542, EU938596	Chromoblastomycosis	Human	Brazil
102246	dH 11611	AY366928, EU938543, EU938597	Chromoblastomycosis	Human	Brazil
117237	dH 15331 = UTHSC 04-2631	EU938580, EU938544, EU938598	Chromoblastomycosis	Human	USA
102229	dH 11590 = 8DPIRA	EU938581, EU938545, EU938599	Decaying vegetable cover	Plant	Brazil
102238	dH 11602 = 1PLE	AY366927, EU938546, EU938600	Soil	Soil	Brazil
269.37	dH 12659 = Bonifaz 150-89	AY366906, EU938547, EU938601	Chromoblastomycosis	Human	South America
115830	dH 12978	EU938582, EU938548, EU938602	Brain	Human	Brazil
102242	dH 11606 = FP26III	EU938583, EU938549, EU938603	Chromoblastomycosis	Human	Brazil
102248	dH 11613	AY366926, EU938550, EU938604	Chromoblastomycosis	Human	Brazil
117236	dH 15330 = UTHSC 04-2904	AB240948, EU938551, EU938605	Brain abscess	Human	USA
117542	dH 14523 = Marty 2005226	EU938584, EU938552, EU938606	Brain, biopsy	Human	USA
117238	dH 13130 = UTHSC-R3486	AB240949, EU938553, EU938607	Brain	Human	UK
289.93	dH 15691	AY366925, EU938554, EU938608	Lymphnode, aspiration-biopsy	Seabear	Netherlands (zoo)
397.48	dH 15828	EU938585, EU938555, EU938609	Chromoblastomycosis	Man	South America
	SUMS0300	EU285273, EU938556, EU938610	Chromoblastomycosis	Human	China (Nanjing)
	SUMS0324	EU285270, EU938557, EU938611	Chromoblastomycosis	Human	China (Shanghai)
	SUMS0322	EU285268, EU938558, EU938612	Chromoblastomycosis	Human	China (Shanghai)
(A) F. pedrosoi					
271.37	dH 15659	AY366914, EU938559, EU938613	Chromoblastomycosis	Human	South America
671.66	dH 16159	EU938586, EU938560, EU938614	Mouse passage	Soil	Venezuela
274.66	dH 15665	EU938587, EU938561, EU938615	Mouse passage	Soil	Venezuela
102245	dH 11610 = FP63I	AY366918, EU938562, EU938616	Chromoblastomycosis	Human	Brazil
201.31	dH 15523	AY366913, EU938563, EU938617	Auditory canal	gazelle	Libiya
670.66	dH 16157	EU938588, EU938564, EU938618	Mouse passage	Soil	Venezuela
273.66	dH 15663	AY366916, EU938565, EU938619	Mouse passage	Soil	Venezuela
102247	dH 11612 = FP77II	AY366919, EU938566, EU938620	Chromoblastomycosis	Human	Brazil
659.76*	dH 16142	AY366920, EU938567, EU938621	Chromoblastomycosis	Human	Argentina
212.77*	dH 15549	AY366912, EU938568, EU938622	Chromoblastomycosis	Human	Netherlands
342.34	dH 15773	AY366915, EU938569, EU938623	Chromoblastomycosis	Human	Puerto Rico
117910	dH 14477 = UNEFM-0002-04		Chromoblastomycosis, hand	Human	Venezuela
122741	dH 18431 = Bonifaz 02300	EU938589, EU938570, EU938624	Chromoblastomycosis, foot	Human	Mexico
253.49	dH 15620	AY366921, EU938571, EU938625	Chromoblastomycosis	Human	Uruguay
122740	dH 18430 = Bonifaz 002200	EU938590, EU938572, EU938626	Chromoblastomycosis, foot	Human	Mexico
285.47*	dH 15680	EU938591, EU938573, EU938627	Chromoblastomycosis	Human	Puerto Rico
(C) F. species					
269.64	dH 15656	EU938592, EU938574, EU938628	Chromoblastomycosis	Human	Cameroon
444.62	dH 15886	AY366931, EU938575, EU938629	Chromoblastomycosis	Human	Surinam
557.76	ATCC 28174	EU938593, EU938576, EU938630		Unknown	Unknown
277.29	dH 15668	EU938594, EU938577, EU938631	Chromoblastomycosis	Human	Brazil
271.33	ATCC 18658 = IMI 134458	AB114127, EU938578, EU938632	Chromoblastomycosis	Human	South America
102224	dH 11584	EU938595	Wood, *Grevillea*	Plant	Brazil
Cladophialophora					
102237	dH 11601	AY366929	Decaying cover vegetable	Plant	Brazil
173.52			Brain abscess	Human	USA

Table 1 (*Continued*)

		GenBank			
Outgroup					
556.83	dH 16013 = ATCC 52853	AY251087	Decaying wood	Plant	Japan
306.94			Tracheal abscess	Human	Germany

Abbreviations used: ATCC = American Type Culture Collection, Manassas, USA; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; dH = G.S. de Hoog working collection; IMI = International Mycological Institute, London, U.K; UTHSC = Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, USA; mutant indicated by a star.

reaction mixture containing 7 µl GoTaq Green master mix (Promega) containing dNTPs, $MgCl_2$, reaction buffer, 1 µl of each primer (10 pmol) and 1 µl rDNA. If no amplicons were obtained, other primer combinations were tried. Amplification was performed in an ABI PRISM 2720 (Applied Biosystems, Foster City, USA) thermocycler as follows: 95°C for 4 min, followed by 35 cycles consisting of 95°C for 45 sec, 52°C for 30 sec and 72°C for 2 min, and a delay at 72°C for 7 min. Annealing temperature was changed to 58°C for the BT2 gene. Amplicons were cleaned with GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, U.K.). Concentrations of amplicons were estimated on gel, photographed and analyzed by the Gel Doc XR system (Biorad), with SmartLadder (Eurogentec, Seraing, Belgium) as size and concentration marker. Amplicons were then subjected to direct sequencing using ABI prism BigDye™ terminator cycle sequencing kit (Applied Biosystems, Foster City, USA) and analysed on an ABI Prism 3730XL Sequencer.

Alignment and phylogenetic reconstruction

Sequences were edited using Seq Man II in the Lasergene software (DNASTAR, Wisconsin, USA). Iterative alignment was performed by hand with MacClade 4.01. A phylogenetic approach was used to investigate relationships among the 39 strains of *Fonsecaea* and two related species of *Cladophialophora* (*C. bantiana* and *C. immunda*). For this approach, the three genes ITS, *TUB1* and *ACT1* were first analyzed separately. Conflicts were estimated using the partition homogeneity test [33] and by comparing topologies. As the three gene partitions were congruent (no supported conflicts), they were combined and analyzed using maximum parsimony (MP) as implemented in PAUP* v.4.0b10 [33]. A tree search of 100 random addition sequences (RAS) and a bootstrap analysis of 1000 replicates and two RAS were conducted after assessing congruence between the three genes. Two species of *Cladophialophora* (*C. arxii* and *C. minourae*) were chosen as members of an outgroup. Haplotype networks were also reconstructed for each gene in order to assess genetic variation within *Fonsecaea*. The networks were obtained using MP (tree search and bootstrap analysis as described above), and drawn by hand in PowerPoint. To detect recombination in each population, the index of association (I_A, measure of multilocus linkage disequilibrium) was calculated with Multilocus 1.2.2 (http://www.bio.ic.ac.uk/evolve/software/multilocus/). The null hypothesis for this analysis is complete panmixia (rejected when $P < 0.05$). To investigate population differentiation, an index of differentiation (ID: θ) was calculated using the same software. The null hypothesis for this analysis is no population differentiation (rejected when $P < 0.05$).

Table 2 List of the various primers used for amplification and sequencing

locus	Primer	´Primer sequence (5′- >3′)	Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGG	[27]
	ITS4	TCCTCCGCTTATTGATATGC	[27]
	ITS5	GGAAGTAAAAGTCGTAACAAGG	[27]
	V9G	TTACGTCCCTGCCCTTTGTA	[28]
	LS266	GCATTCCCAAACAACTCGACTC	[29]
TUB1	BT-2a	GGTAACCAAATCGGTGCTGCTT	[30]
	T2	TAGTGACCCTTGGCCCAGTTG	[31]
ACT1	Esp ACT fw	CACGTTGTCCCCATCTAC	[32]
	Esp ACT bw	ATGAAGGTCAAGATTATC (T) GC	[32]

Results

Physiology

Cardinal growth temperatures of voucher strains showed optimal development at 27-33°C (Fig. 1), while growth was observed in the entire range between 21-37°C. The maximum growth temperature of all strains analyzed was found to be 37°C. No growth was observed at 40°C, but when plates precultured at 40°C were returned to 30°C, some strains showed

notable growth (viz., CBS 671.66, dH 13277, CBS 277.29, CBS 274.66, CBS 271.37, CBS 270.37, CBS 102245, CBS 397.48 and CBS 269.37). Thus, 40°C is fungistatic for some *Fonsecaea* strains and fungicidic for others. These data were, however, not consistent with the molecular groupings (see below).

Phylogenetic approach

PHT did not detect conflict between loci. Topologies of trees from all three genes were concordant when occasional paralogues in *TUB1* were excluded from the analysis. Three clades corresponding to *F. monophora*, *F. pedrosoi* and group C showed strong support in the *TUB1* tree (data not shown). High support was also obtained for *F. pedrosoi* with ITS, and for group C with *ACT1*. Because the three markers used did not yield enough parsimony informative characters to resolve relationships within each of the three inferred clades, the GCPSR was not applicable. The genes (ITS, *TUB1*, *ACT1*) were therefore combined in order to investigate species delimitation using PSR. The resulting data set included 43 taxa and 1,368 characters, among which 181 were parsimony informative. The tree search yield 40 most parsimonious trees of 500 steps. Fig. 2 presents one of these trees with branch lengths and bootstrap values. Except for one strain (CBS 102224), which was here shown to be only distantly related to *Fonsecaea*, all isolates of *Fonsecaea* could be grouped into three major clades: (i) the well supported group A (100% bootstrap), representing *F. pedrosoi*, (ii) the moderately supported group B (71% bootstrap), representing *F. monophora*, and (iii) the well-supported group C (100% bootstrap). The isolates within group C could not be assigned to either *F. pedrosoi* or *F. monophora* because the relationship between group C and *F. monophora* was not supported. When the three loci were analyzed separately, the relationships among the three clades changed, and never obtained significant support. This lack of support was not caused by problematic taxa with incongruent gene trees. A study of the synapomorphies showed that, among each gene, there were some variable sites that supported the relationship *F. monophora*/group C, and some others that supported the relationship *F. pedrosoi*/group C (Table 3). For the three genes, these incongruent sites seemed to be randomly distributed along the locus, and they seemed to account for the low bootstrap value obtained for the relationship *F. monophora*/group C in our analyses.

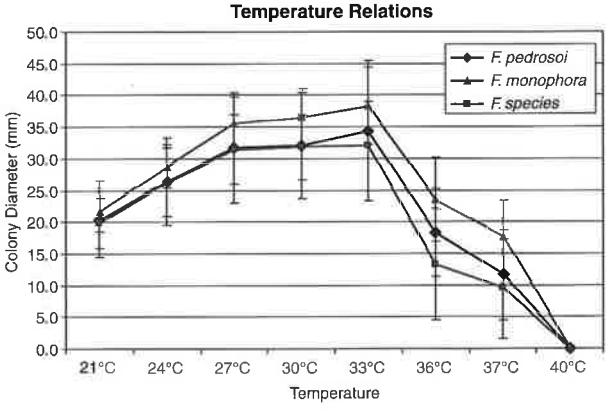

Fig. 1 Colony diameters at various temperatures with 3°C increments ranging from 21–40°C, measured after 3 weeks on 2% MEA were calculated for *Fonsecaea pedrosoi*, *F. monophora* and *Fonsecaea* spp.

Haplotype networks

Results of the analyses are presented in Table 4. One of the most parsimonious reconstructions (MPRs) was selected to draw the haplotype networks of ITS (Fig. 3A) and *TUB1* (Fig. 3B). For *ACT1*, the MPR was used to draw the haplotype network (Fig. 3C). These networks support the existence of three genetically differentiated groups: *F. pedrosoi*, *F. monophora* and group C.

Reproductive isolation

Index of association (I_A) showed that grouping was based on clonal evolution rather than on recombination ($I_A = 0.8249$ and $P = 0.01$) and ID (θ) showed that there was population differentiation between clades ($\theta = 0.91$ and $P < 0.01$).

Discussion

In the present study, we investigated the genetic diversity and species delimitation of *Fonsecaea* and now discuss the implications of a comparison of 39 clinical and environmental isolates. Based on the sequences of ITS, *TUB1* and *ACT1* genes, we classified strains of *Fonsecaea* into three major clades, which were supported by high bootstrap values (Fig. 2), i.e., Group A, which contains the ex-type strain of *F. pedrosoi*, Group B, which contains the ex-type strain of *F. monophora*, and a Group C, which did not include any type strain, and might correspond to a new species. In temperature tests no difference could be detected among these three group, that is the shape of the growth curves at different temperatures are identical (Fig. 1). However, the two described *Fonsecaea* species seem to be ecologically different from each other in that *F. pedrosoi* is consistently the cause in humans of

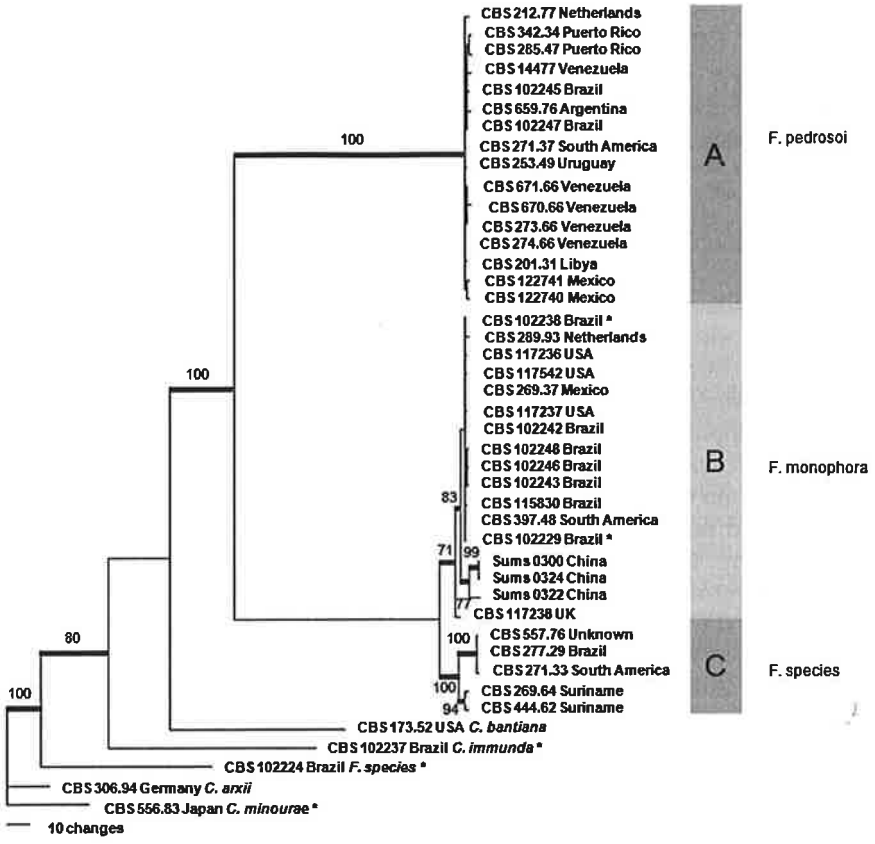

Fig. 2 Maximum parsimony tree based on the combined data of ITS and partial *TUB1* and *ACT1* genes of 39 members of *Fonsecaea*, and four members of *Cladophialophora* constructed with PAUP v.4.0b10, with 100 bootstrap replications (values >70 are shown at the branches in bold). CBS 306.96, CBS 556.83 are taken as outgroup (CI = 0.8040, HI = 0.1960, RI = 0.8722) (* = Environmental samples).

chromoblastomycosis, while *F. monophora* is more of a general opportunist [7,34]. Although in most regions *F. pedrosoi* is the prevalent agent of chromoblastomycosis, *F. monophora* or other *Fonsecaea*-like species are generally recovered from environmental source [35]. Environmental strains of *F. pedrosoi* thus far have only been isolated by the use of living mouse baits [36]. These clinical and ecological differences support the separation of these two groups at the species level. The environment, still only fragmentarily sampled, harbors more species that are morphologically *Fonsecaea*-like but have only occasionally, if ever been encountered as agents of human disease [36]. One such a species was included in our study (Fig. 2, CBS 102224) and proved to be significantly different from *F. pedrosoi*, *F. monophora* and group C. The morphological concept of *Fonsecaea vs. Cladophialophora*, i.e. short *versus* long conidial chains, is therefore not supported by molecular phylogeny [37].

Based on Index of Association (I_a), no recombination was detected among the three inferred groups. This touches on the problem of species concepts in black yeasts and relatives. *Fonsecaea* seems to exhibit high degrees of clonality, similar to some pathogenic and opportunistic chaetothyrialean anamorphs [32]. For *Exophiala jeanselmei* for example [32], in the apparent absence of sexuality, each branch is concordantly separated from any other one, and thus could be regarded as a separate molecular species. In this case, delimiting species based on GCPSR is therefore unpractical. Geographical data suggest a broad distribution for both *F. pedrosoi* and *F. monophora*. However, the origin of some strains (e.g., CBS 212.77 from the Netherlands, and CBS 117238 from the UK) is doubtful, as patients might have acquired their infections while traveling abroad. Although the infraspecific relationships are poorly resolved, some geographical structure can be observed in some cases. The three Chinese isolates of *F. monophora* cluster together, as do two strains from Puerto Rico and two strains of *F. pedrosoi* from Mexico, and two strains from Surinam in group C. This is suggestive of a low dispersal pace, and - given the small genetic distances between these geographical clusters - indicates that they are likely to represent populations of a single species.

Although group C has 12 unique positions in three investigated genes, it shares 9 positions with *F. pedrosoi* and 15 with *F. monophora* (Table 3). Due to conflicting synapomorphies, the bootstrap value is low for the

Table 3 Informative sites in ITS, *ACT1* and *TUB1* in members of Groups A, C and B; number of mutations given relative to length of the sequence are in brackets. Positions of ITS1 and 2 are counted after ATCATTA (SSU), in intron 5 of *ACT1* starting from CCCATA in exon 5, and in introns 4 and 5 of *TUB1* starting from GCAGAT in exon 4.

ITS (30/569)	22	23	48	54	69	76	86	95	106	107	111	112	116	120	142	148	155	158	205	391	418	419	427	468	491	493	516	517	551	558
A = F. pedrosoi	C	-	C	A	C/T	C	T	T	C	T	G	C	T	A	T	A	T	C	A	T	C	G	A	A	T	T	T	G	T/-	T
C = F. sp.	T	-	T	A	C	C	T	C	C	T	A	C/T	T	G	T	C	T/C	C/T	A/-	C	C	G	A	A	A	C	T	G	-	C
B = F. monophora	T	C/-	T	A/T	C	C/T	G/T	T	T	T/C	G	C	C	G	C/T	A	T	C	A	T	T	A	A/G	G/A	A	C	C	G/A	G/T	C

TUB1 (22/370)	19	28	37	49	68	77	80	86	136	157	159	166	183	221	227	239	287	329	346	361	365	368
A = F. pedrosoi	G	T	T	C	A	A	G	C	T	T	A	G	C	C	C	C	T	C	T	C	-	G
C = F. sp.	G/A	C	C/T	T	C	G	G/A	C/G	C	T/C	A	A	C	T	C	C/T	T	C/T	T	G	A	A
B = F. monophora	G	T	T	C	C	G	G	C	C	T	C	A	T	T	C/T	C	C	C	T/C	C	-	A

ACT1 (11/560)	224	242	275	299	380	422	430	453	460	467	496
A = F. pedrosoi	A	A/C	G	C/G	C	A	C	T	-	C	T/G
C = F. sp.	G	A	G	C	T	G	G	C	T	T	T
B = F. monophora	G	A	A	C	T	A	C	T	T	C	T

Table 4 Molecular data and result of the phylogenetic analyses for the haplotype networks

Loci	Total number of sites	Number of variable sites	Number of parsimony informative sites	Number of MPRs	Tree length (steps)
ITS	533	57	30	78	68
*tub*1	361	36	22 (inc. 9 in intron)	12	39
*act*1	525	12	11 (inc. 5 in intron)	1	12

relationship *F. monophora*/group C in the combined analysis. These nearly equal distances between all three groups might indicate that they have evolved in a relatively simultaneous way from a common ancestor. In these three species, ancestral polymorphic sites may have become fixed in different ways according to the site, resulting in these conflicting synapomorphies. In our data set, ITS and *TUB1* are significantly more variable than *ACT1* introns (Table 3). In other fungal orders, some authors, e.g., Hirata *et al.* [38] and Banke *et al.* [26] have shown that ITS and *TUB1* are insufficient to resolve the relationships among closely related taxa, while *ACT1* tends to be more variable and thus more sensitive at this taxonomic level. We found the opposite, suggesting that the molecular clock in comparable partial genes is not always consistent between groups of organisms.

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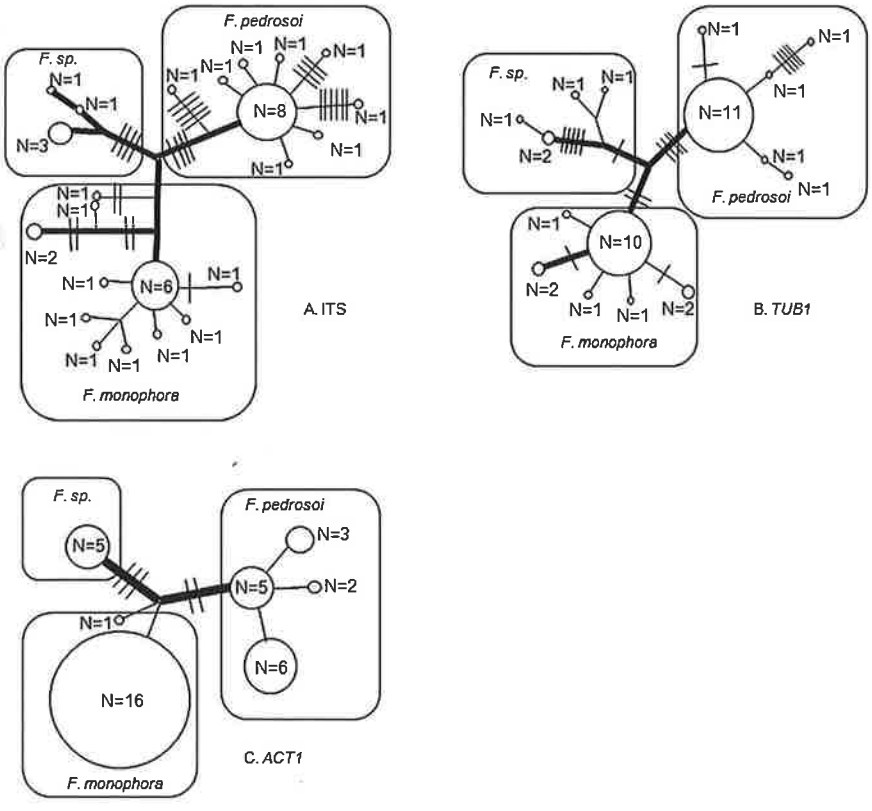

Fig. 3 Haplotype networks for *Fonsecaea* isolates. Each branch represents one mutation, and each hash mark signifies one additional mutation. Circle sizes and the numbers (N) reported next to or within the circles indicate the total number of times an allele was sampled. Bootstrap support values of 70% or greater for particular nodes are indicated by bolding of the internode.

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