

Microsatellite markers reveal geographic population differentiation in *Trichophyton rubrum*

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A worldwide selection of more than 200 isolates of the anthropophilic dermatophyte *Trichophyton rubrum* were analysed using seven microsatellite markers. Fifty-five multilocus genotypes were recognized, allowing a subdivision of the species into two populations. Both populations reproduced strictly clonally, showed a different predilection on the human host (scalp vs foot) and displayed geographic differentiation. Genotypes of one population originated predominantly from Africa, whilst the second population showed a worldwide distribution excluding the African continent. Genotypic diversity was highest in the African population, despite the lower number of strains analysed, suggesting that *T. rubrum* is likely to have evolved in Africa. No diagnostic correlation was observed between multilocus genotypes and any of the phenotypical characteristics of the strains. The involvement of multiple strains in a single patient detected by workers using other typing methods was not supported by these microsatellite markers. Four of the developed microsatellite markers may be applied for diagnostic purposes.

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INTRODUCTION

The complex of anthropophilic dermatophyte species *Trichophyton rubrum* s.l. comprises two anamorphic taxa, *Trichophyton rubrum* and *Trichophyton violaceum*. Whilst *T. rubrum* is the most frequently isolated agent of dermatophytosis worldwide with clinical manifestations ranging from onychomycosis to tinea capitis, *T. violaceum* is endemic to Africa and the Middle East and mainly causes tinea capitis and tinea corporis.

T. rubrum shows a wide variability in its phenotypic features, including the presence or absence of reflexively branching hyphae, micro- and macroconidia, red colony pigmentation and urease activity (Guoling *et al.*, 2006). The high degree of morphological diversity is in contrast to the homogeneity of the genome, as revealed by analyses of anonymous DNA markers (Zhong *et al.*, 1997; Gräser *et al.*, 1999a). However, recent studies using randomly amplified polymorphic DNA (RAPD) and repetitive elements of the non-transcribed spacer (NTS) region of the rRNA gene have demonstrated some genetic variability (Jackson *et al.*, 2000; Baeza *et al.*, 2006). Baeza *et al.* (2006) used two arbitrary primers and detected 11 and 12 genotypes,

respectively, among 67 clinical isolates from Brazil. NTS data revealed two genotypes in the same set of isolates (Baeza *et al.*, 2006). Jackson *et al.* (2000) used the NTS technique and revealed 21 genotypes in a set of 100 strains from continental Europe, Iceland and Japan. In these studies, large clonal spreads, involving 40% and nearly 90% of the isolates of a single genotype ('PCR pattern 1'), were observed. Yazdanparest *et al.* (2003) applied the NTS region to multiple isolates of ten patients and revealed the occurrence of infection by more than one strain in a single patient. None of the studies included any isolates from Africa, the continent of the suggested origin of *T. rubrum* (Ohst *et al.*, 2004).

Recently, Ohst *et al.* (2004) demonstrated the utility of microsatellite markers for the detection of variability among strains of *T. rubrum*. An association between genotype and geographic distribution (or origin, in the case of immigrants) was revealed. In other human pathogenic fungi, multilocus microsatellite typing (MLMT) has proved to be a promising tool for uncovering intraspecific diversity (Fisher *et al.*, 2004) due to the high mutation rate of these markers. In the present study, we developed and analysed seven microsatellite markers in a global set of isolates of *T. rubrum* from patients with diverse clinical pictures. The method was applied to an expanded collection of strains to:

- confirm that the distribution of genotypes in *T. rubrum* is associated with the geographic origin of the human host;
- determine whether there is a correlation between

Abbreviations: I_A , index of association; MCMC, Markov chain Monte Carlo; MLMT, multilocus microsatellite typing; NTS, non-transcribed spacer; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DBJ accession numbers for the microsatellite markers determined in this study are AM295312–AM295317.

particular genotypes and clinical manifestations; and (iii) determine whether multiple strains can be detected in a single patient.

METHODS

Fungal strains. A total of 236 isolates were analysed in this study, including 12 reference strains from the Centraalbureau voor Schimmelcultures (CBS 452.61, 289.86, 100081, 288.86, 360.62, 735.88, 100237, 100084, 100238, 287.86, 202.88 and 499.48). Most strains were obtained between 1995 and 2005. Three strains were morphologically identified as *T. violaceum* and 233 as *T. rubrum*; of strains attributed to the latter species, 19 showed reflexive branching hyphae and 14 had urease activity, whilst the remaining strains showed neither characteristic. The *T. violaceum* strains were used as an outgroup in the analysis. All but six *T. rubrum* strains were isolated from epidemiologically unrelated human hosts at geographically distant locations (22 from the USA, five from Canada, 41 from Greece, 16 from Bulgaria, 14 from Spain, 47 from Germany, 13 from Mexico, 31 from Japan and 21 from Nigeria). The following isolates were collected from immigrants: two strains each from Cuba, Morocco, Ecuador, Colombia, Guinea and Ethiopia, and single strains from Senegal, Romania, Bolivia, Angola, Algeria, Gambia, Togo, Zaire and Peru. The origin of the remaining strains was unknown. Onychomycosis or tinea pedis was diagnosed in 139 patients, whereas 55 patients were suffering from tinea capitis or tinea corporis. The remaining strains lacked clinical information. In six cases, two separate isolates were collected from different body sites of a single patient.

DNA extraction. DNA was extracted by the CTAB method (Gräser *et al.*, 1999b) after growing the fungus on Sabouraud glucose agar (Difco Laboratories).

Isolation of microsatellites. Microsatellites were developed as described by Ohst *et al.* (2004), with modifications. Briefly, genomic DNA was isolated from clinical isolates of *T. rubrum* (R 5, R 26 and R 28). About 10 µg of the DNA was digested with *DpnII* and cleaned by drop dialysis for 15 min. Linkers (Sau-A, 5'-GCGGTACCCGGG-AAGCTTGG-3'; and Sau-B, 5'-GATCCCAAGCTTCCCGGGTACC-GC-3') were ligated to both ends of the fragments using T4 DNA ligase (New England Biolabs). After purification via columns (Chroma spin columns; Clontech Laboratories), pre-hybridization PCR was performed with the Sau-A linker only (15 cycles of

denaturation for 50 s at 95 °C, annealing for 60 s at 56 °C and extension for 120 s at 72 °C). For enrichment, the PCR product was denatured and hybridized to biotinylated (GT)₁₂ and (GA)₁₂ probes in a solution of 6 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate) and 0.1 % SDS. The mixture was denatured at 95 °C and cooled slowly (over 15–20 min) to room temperature. The probe was then captured with avidin beads (VECTREX Avidin D; Vector Laboratories) in TBT buffer [100 mM Tris/HCl (pH 7.5), 0.1 % Tween 20] at 50 °C for 30 min, followed by several washing steps (three times with TBT plus 150 mM NaCl and three times with 0.2 × SSC/0.1 % SDS). Subsequently, the DNA was denatured from the beads in 10 mM Tris/HCl (pH 8) with 0.1 mM EDTA at 95 °C for 5 min and again PCR amplified using Sau-A. The resulting PCR product was cloned and transformed using the TOPO TA cloning kit (Invitrogen). White colonies were selected and checked for repeat inserts using M13 and (AC)₁₀ or (GA)₁₀ as primers. Inserts of 300–600 bp were chosen for sequencing using the M13 primer and an automated sequencing system (3130x Genetic Analyser). Subsequently, specific primers were designed to amplify PCR fragments in the range of 100–300 bp containing more than 12 GT or GA repeats. Amplification of each primer pair was tested on a panel of strains of *T. rubrum*, including the genomic DNA of the isolate that the library was generated from.

PCR amplification of microsatellite markers using specific primers. Standard PCRs were performed in 50 µl volumes containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 30 pmol each primer (see Table 1), 50 µM each dNTP, 2.5 U *Taq* polymerase (Applied Biosystems) and 50 ng template DNA. Samples were amplified by initial denaturation for 10 min at 95 °C, followed by 30 cycles of denaturation for 50 s at 95 °C, annealing for 60 s at 56–62 °C depending on the primers used (see Table 1) and extension for 60 s at 72 °C. This was followed by a final extension step of 10 min at 72 °C.

Screening for length polymorphisms. Fifteen microlitres of PCR product was loaded onto 12 % polyacrylamide gels (Rotiphorese Gel 29 : 1, 40 %; Carl Roth) and the microsatellites run for 18 h at 12 W (constant power). Gels were silver stained and dried for documentation. Repeat numbers in alleles were calculated visually using the sequenced allele as the reference with known repeat number.

Data analysis. Different approaches were used to assign strains to populations. Firstly, microsatellite genotype data were analysed by calculating genetic distances between individuals based on Dc (Cavalli-Sforza & Edwards, 1967), Dm and Ds (Saitou & Nei, 1987)

Table 1. Markers developed in this study and their corresponding primers

Marker	Primer (5'→3')	Size (bp)	T _a (°C)	GenBank accession no.
Tr (CT) ₂₀	GCGGTGGCGTCTTCTATC CAGCAGACAACATAGCAGTCG	136	60	AM295312
Tr (GA) ₂₅	GCGATGGTTGGAGGAGTTG GCCTGTCGCTGCTTACTTG	142	58	AM295313
Tr (T) ₁₇	CCCATCGAGGTCATATACGC ATTTTGGTTTCTTTTTTGAGTG	100	56	AM295314
Tr (GA) ₁₈	GACTCAACGGTTCCAGATTAC CTTATTTGTCCGCTTCTCTCG	155	58	AM295315
Tr (GA) ₁₇	CGTCGCTTGCTTTGGTACAC TATGGACTTGTTCCGGATGGG	95	62	AM295316
Tr (GA) ₁₈ (CA) ₁₃	CTCCGTCCGGTCCAGATG CGCTGCTGGTAAAGAGATGAC	137	62	AM295317
T1	From Ohst <i>et al.</i> (2004)	271	60	AJ745081–AJ745084

Table 2. Frequency of each genotype for the markers T2–T7

The most common genotype (GT) of each marker is highlighted in bold. T2, Tr T₍₁₇₎; T3, Tr GA₍₁₈₎CA₍₁₃₎; T4, Tr GA₍₁₇₎; T5, Tr GA₍₁₈₎; T6, Tr GA₍₂₅₎; T7, Tr CT₍₂₀₎.

T2		T3		T4		T5		T6		T7	
GT	Frequency	GT	Frequency	GT	Frequency	GT	Frequency	GT	Frequency	GT	Frequency
A	0.004237	A	0.004237	A	0.004237	A	0.004237	A	0.004255	A	0.004274
B	0.004237	B	0.885593	B	0.923729	B	0.881356	B	0.017021	B	0.025641
C	0.097458	C	0.097458	C	0.033898	C	0.114407	C	0.476596	C	0.760684
D	0.012712	D	0.004237	D	0.016949			D	0.148936	D	0.076923
E	0.813559	G	0.008475	F	0.012712			E	0.182979	E	0.012821
F	0.050847			G	0.004237			F	0.004255	F	0.004274
G	0.016949			H	0.004237			G	0.008511	H	0.098291
								H	0.004255	J	0.008547
								I	0.004255	K	0.004274
								J	0.021277	L	0.004274
								K	0.068085		
								L	0.004255		
								M	0.008511		
								N	0.008511		
								O	0.004255		
								P	0.021277		
								Q	0.008511		
								S	0.004255		

distances implemented in the software package POPULATIONS version 1.2.28 (<http://www.pge.cnrs-gif.fr/bioinfo/populations/index.ph-p?lang=en>). Neighbour-joining trees were constructed from the distance matrices and displayed using TREEVIEW (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>). As an alternative, a model-based method was used for delineating clusters of individual strains on the basis of their genotypes at multiple loci; this Bayesian approach is implemented in the program STRUCTURE version 2.1 (Pritchard *et al.*, 2000). A variety of models were used, including the no admixture model assuming a clonal population structure. One million Markov chain Monte Carlo (MCMC) replications and a burn-in period of 100 000 generations were used. The burn-in length defines how long to run the simulation before collecting the data to minimize the effect of the starting configuration. A burn-in of 10 000–100 000 is usually more than adequate (Pritchard *et al.*, 2000). In MCMC, the sample mean over a run of the Markov chain to approximate an expectation with respect to the equilibrium distribution of the chain is used. The probability of the data, assuming one to five populations (K), was estimated in three replicate analyses. The posterior probability and other values displaying the confidence of the number of populations were recorded. The analyses were performed on all isolates and also on subsets derived from previous analyses.

After structuring the populations, Wright's F statistics were applied to compute the variance in allele frequencies and to test for free gene flow versus population differentiation between the inferred populations. Theta (Weir, 1996) was calculated across loci and populations using the program MULTILOCUS version 1.3 (Agapow & Burt, 2001). Here, the null hypothesis was no population differentiation; 10 000 randomizations were used.

To analyse the genetic diversity of the sample and to test for clonality versus recombination in *T. rubrum*, the overall and 'in population separated' (based on both cluster methods, see below) index of association (I_A) was calculated using MULTILOCUS. Observed data were compared with the null hypothesis of random mating (random association of alleles from different DNA loci). When the null hypothesis is rejected, a clonal population structure is suggested.

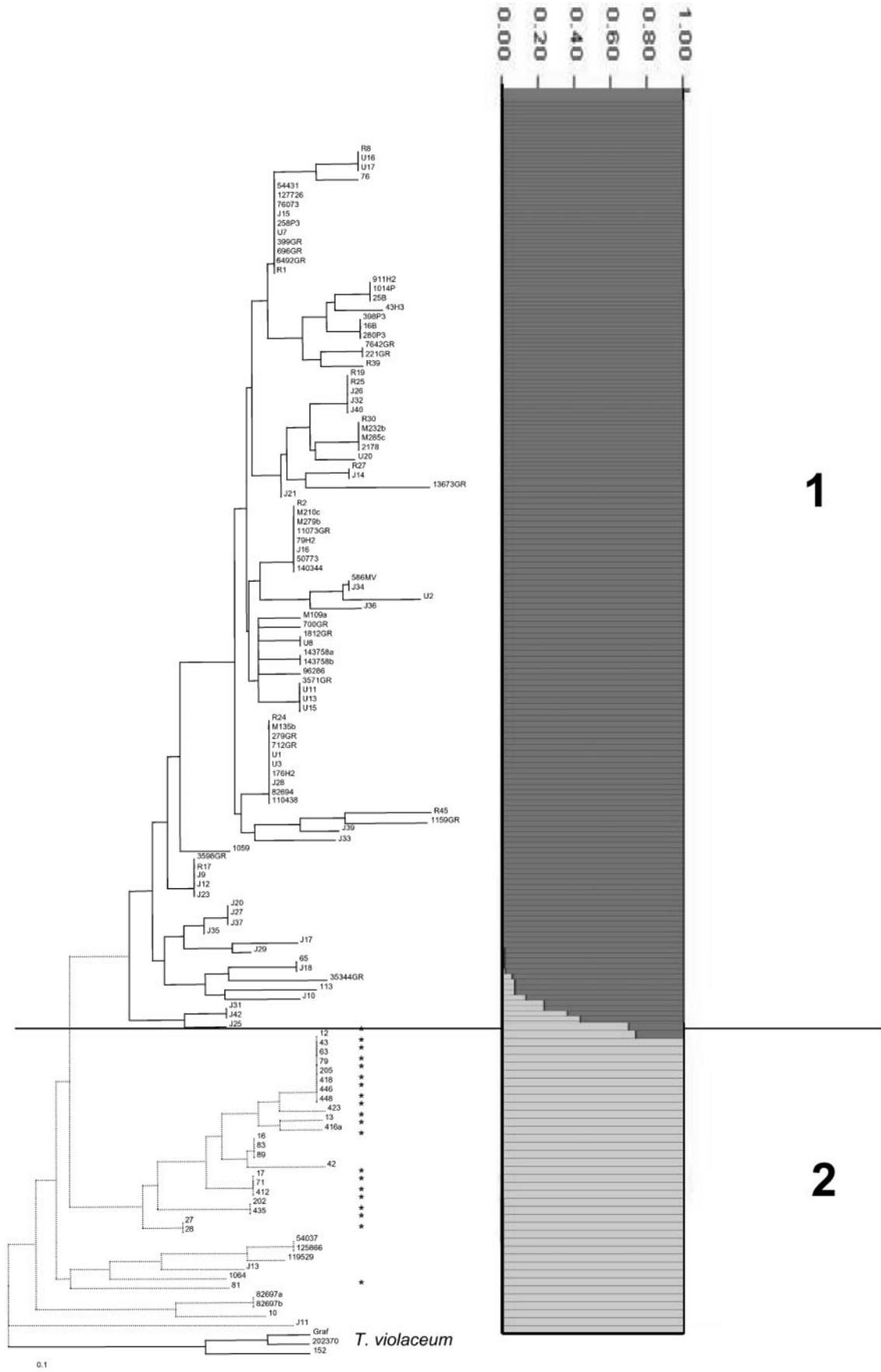
The genotype frequencies of each marker were calculated using the software MSA version 3.12 (Dieringer & Sclötterer, 2003).

RESULTS

Genetic diversity

Of the 22 typable microsatellite markers developed, we used the most polymorphic loci, Tr (CT)₂₀, Tr (GA)₂₅, Tr (T)₁₇, Tr (GA)₁₈, Tr (GA)₁₇, Tr (GA)₁₈(CA)₁₃ and T1, of which the last mentioned was developed in an earlier study (Ohst *et al.*, 2004). The number of alleles revealed by these seven markers ranged from two to 18. Loci T1 and Tr (GA)₂₅ were the least and most polymorphic (Table 2), respectively, within the *T. rubrum* sample. The alleles in these two loci varied by two and 26 dinucleotide repeats, respectively. With the exception of the marker Tr (GA)₂₅, the frequency of the most common genotype was always very high, larger than 0.75 (Table 2). A maximum of only

Fig. 1. Left, neighbour-joining tree based on Dc distance. Right, bar plot for $K=2$ using STRUCTURE, showing the same number of clusters as the distance tree. Every bar corresponds to a single strain. Strains marked by asterisks form reflexive branching hyphae. Isolates of population 1 (dark grey/black line) and population 2 (light grey/dotted line) are coded. Three strains of *T. violaceum* were used as the outgroup.



two alleles was found in the other 15 markers that were not developed further. In contrast to T1, different alleles at these loci were only represented by single strains within the sample set (data not shown). In *T. violaceum*, alleles were unique (species specific) with all markers when compared with *T. rubrum*. For this reason, the three *T. violaceum* strains were used as an outgroup in the distance tree (Fig. 1). Complete data for each strain and locus are available upon request.

Using several distance methods, analysis of the combined dataset of 55 multilocus genotypes among the 233 *T. rubrum* strains and three genotypes among *T. violaceum* strains generated identical results (Fig. 1). An indication for clonal reproduction in the overall *T. rubrum* sample was the observation that the three most common multilocus genotypes were shared by 76, 35 and 28 individual strains from unrelated hosts, respectively. This corresponds to a genotypic diversity in the dataset of 0.85. The linkage disequilibrium analysis of the overall sample and the clone-corrected sample rejected the null hypothesis of random mating ($I_A=1.92$; $P<0.001$).

Cluster analyses

Independent of the underlying STRUCTURE model and distance measurements used, both approaches revealed two clusters/populations comprising the same strains (Fig. 1). Populations 1 and 2 contained 201 and 32 strains, respectively. Phenetically, population 1 was dominated by strains that did not produce urease and lacked reflexively branching hyphae (97%), whereas all strains with reflexive hyphae were located in cluster 2. However, the association of urease and reflexive hyphae was not diagnostic, as urease was expressed in strains of both populations, with or without the presence of reflexive hyphae (Fig. 2). Within population 2, strains with reflexive branching did not form a monophyletic group (Fig. 1, strains marked with asterisks). Geographically, most strains of African origin

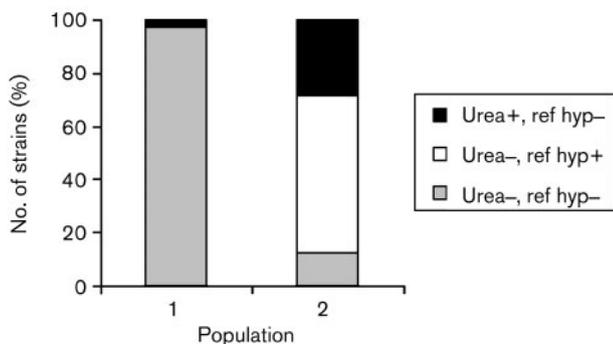


Fig. 2. Association between genotypes separated in the two populations and morphological features. Urea, production of urease; ref hyp, production of reflexive hyphae. The number of strains is shown as a percentage.

(isolated in Africa or from African immigrants; 89%) grouped in population 2, which was clearly separate from population 1 (Figs 1 and 3). Population 1 contained five strains (3%) from African immigrants who became symptomatic after leaving Africa (Fig. 3). Eighty-three per cent of the strains of population 1 were aetiological agents of onychomycosis or tinea pedis, whereas genotypes in population 2 predominantly had been isolated from patients with tinea capitis or tinea corporis (90%, Fig. 4).

In order to explore further any evidence of genetic substructuring among the strains in the two clusters, a second set of STRUCTURE analyses was conducted. This resulted in all isolates being placed with intermediate probabilities into each of K populations, where K varied from two to ten. The available data provided no indication of further genetic subdifferentiation among the two clusters.

Linkage disequilibrium

By pre-defining two populations in accordance with the analyses above, the repeated linkage disequilibrium test rejected the null hypothesis of random mating for both populations [$I_{A(\text{pop1})}=0.26$, $I_{A(\text{pop2})}=1.8$; $P<0.001$]. Support for population differentiation in *T. rubrum* was given by the statistics of θ ($\theta=0.64$; $P<0.001$).

Multiple-strain infections

In the six cases where two strains were recovered from a single patient, identical multilocus genotypes for both isolates were mostly obtained. In a single patient from Mexico suffering from onychomycosis as well as from tinea corporis, the two strains showed distinctive genotypes in one out of seven of the microsatellite markers (Table 3).

Specificity of microsatellite markers

Amplification of the seven microsatellite markers using genomic DNA of dermatophyte species outside *T. rubrum*

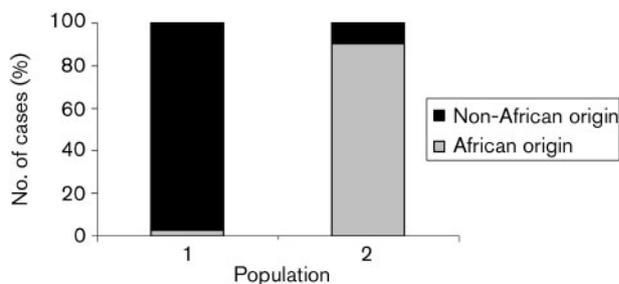


Fig. 3. Association between genotypes separated in the two populations and autochthonous geographic origin of the strains (Africa vs the rest of the world). The number of cases for populations 1 and 2 are given as a percentage.

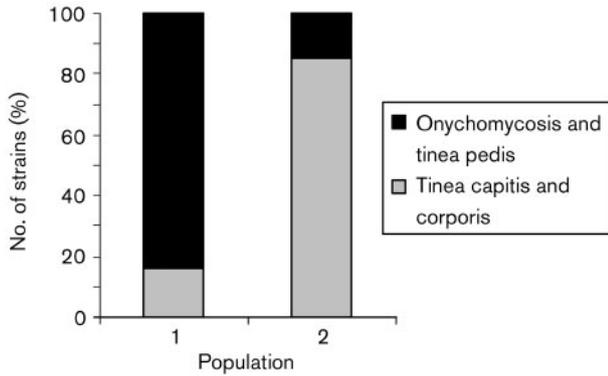


Fig. 4. Association between genotypes separated in the two populations and the clinical picture (onychomycosis plus tinea pedis, or tinea capitis plus tinea corporis). The number of strains for populations 1 and 2 are given as a percentage.

T. violaceum revealed that the following loci were specific (no PCR product was generated) for the complex: Tr (GA)₁₇, Tr (T)₁₇, Tr (CT)₂₀, Tr (GA)₂₅ and T1. For this purpose, 14 isolates were tested (*Trichophyton tonsurans* CBS 496.48, *Trichophyton interdigitale* CBS 165.66 and 558.66, *Trichophyton verrucosum* CBS 562.50, *Trichophyton schoenleinii* VKPGF 231/16 and 232/181, *Trichophyton mentagrophytes* CBS 388.58 and 318.56, *Trichophyton erinacei* CBS 677.86 and CBS 511.73, *Trichophyton immergens* CBS 338.37, *Trichophyton abyssinicum* CBS 126.34, *Microsporum canis* CBS 495.86 and *Microsporum vanbreuseghemii* CBS 243.66). The specificity of the markers was confirmed when non-dermatophytes causing similar clinical manifestations were compared (data not shown).

DISCUSSION

Using our recently developed MLMT system, the *T. rubrum* strains analysed could be divided into two populations. Support for the argument that the sample was heterogeneous was found in the statistically significant value of θ . Both populations reproduced strictly clonally. An entirely clonal mode of reproduction is believed to be quite unusual for fungi outside the dermatophytes (Taylor *et al.*, 1999). The requirement for sexuality apparently primarily applies to environmental fungi and less to fungi with life cycles that are completed on mammals and with host-to-host transmission (Gräser *et al.*, 2006).

As the interpretation of population structures is not always straightforward in clonal fungi, we applied two distinct models of analysis. Congruent findings were obtained when the number of populations was postulated as two. The possible existence of a third, preponderantly Asian, clade as suggested in our previous studies (Ohst *et al.*, 2004; Gräser *et al.*, 2006), partly corresponding to the previous concept of *Trichophyton raubitschekii* and associated with chronic tinea corporis, was not observed in our data. To establish a possible Asian origin for the rapid spread of *T. rubrum* in the 20th century, now belonging to the less diversified population 1, would require a larger sample from Asian countries other than Japan. Populations 1 and 2 showed geographic differentiation. Whilst the genotypes of population 2 originated predominantly from Africa, those of population 1 showed a worldwide distribution excluding the African continent. On the basis of this finding, we suggest that the 3% of isolates in population 1 that were isolated from Africans were collected from persons who very likely had acquired their infection in the countries to which they had immigrated recently. Thus our typing system enables the assignment of geographic origin of the patient's isolate. The identity of

Table 3. Genotypes of the seven microsatellite markers from the six cases where two strains were isolated from different body sites

The two strains from a single patient with differing genotypes in marker T7 are highlighted in bold. T2, Tr T₍₁₇₎; T3, Tr GA₍₁₈₎CA₍₁₃₎; T4, Tr GA₍₁₇₎; T5, Tr GA₍₁₈₎; T6, Tr GA₍₂₅₎; T7, Tr CT₍₂₀₎.

Lab./ref. no.	Origin	Clinical picture	T1	T2	T3	T4	T5	T6	T7
M196a	Mexico	Onychomycosis	B	E	B	B	B	E	C
M196b	Mexico	Tinea pedis	B	E	B	B	B	E	C
M278b	Mexico	Tinea pedis	B	E	B	B	B	D	C
M278c	Mexico	Tinea cruris	B	E	B	B	B	D	C
M279a	Mexico	Tinea pedis	B	E	B	B	B	D	C
M279b	Mexico	Onychomycosis	B	E	B	B	B	D	C
M285b	Mexico	Onychomycosis	B	E	B	B	B	D	C
M285c	Mexico	Tinea corporis	B	E	B	B	B	D	D
143758a	Columbia	Onychomycosis	B	E	B	B	B	M	C
143758b	Columbia	Tinea pedis	B	E	B	B	B	M	C
82697a	Ethiopia	Tinea capitis	A	G	B	F	C	E	J
82697b	Ethiopia	Tinea capitis	A	G	B	F	C	E	J

spatially separated strains exhibiting the same genotype demonstrates the high reproducibility of the MLMT technique used.

The genotypic diversity in the African population (cluster 2) was higher than in cluster 1 (0.92 vs 0.80), despite the larger number of strains collected in the latter clade. This finding is in line with our previous conclusion that *T. rubrum* is likely to have evolved in Africa (Ohst *et al.*, 2004).

No diagnostic correlation was observed between multilocus genotypes and the phenotypic characteristics of the strains, although all genotypes with the ability to produce reflexively branching hyphae belonged to the African population (population 2). This supports the view that the former subdivision of *T. rubrum* into several taxa (Kane *et al.*, 1997) is not warranted. Structuring of populations in *T. rubrum* is through geographic separation, in conjunction with their predilection on the human host (scalp vs foot). Isolates of population 2 predominantly caused tinea capitis or tinea corporis (>80%), and only rarely were involved in infection of the foot. Part of these approximate differences may be explained by (micro)climatic differences. In the rural communities of tropical West Africa, where most African isolates originated, the climate is warm and humid, but the habit of wearing closed footwear is less widely distributed and hence a low incidence of tinea pedis is expected. Conversely, African emigrants in temperate climates suffer from tinea pedis and are infected by the non-African genotypes of clade 1. The microclimates optimal for fungal growth are thus found on other body sites. We did not find any evidence of maintenance of genotypes in African communities outside Africa. Genotypes that are brought in from the countries of origin seem to disappear at the expense of local genotypes that apparently are better adapted to local environmental conditions. In areas with climatic conditions similar to those of equatorial Africa, such as South America and East Asia, infections of the scalp and hair are caused by dermatophytes other than *T. rubrum*, e.g. by *T. tonsurans*, *T. violaceum*, *M. canis* or *Microsporum ferrugineum* (Kane *et al.*, 1997). These species occupy the ecological niche that *T. rubrum* has in Africa. The suggestion that factors associated with types of human race might play a role (Rippon, 1988) cannot be confirmed by the present data.

In conclusion, this is the first proof on the basis of an extended set of molecular data that the evolution of populations in *T. rubrum* is due primarily to geographic barriers, and, as a result, the limited availability of ecological niches due to the presence of competing species. This matches with the observation that mixed infection by strains with identical predilections – genotypes as well as species – are uncommon (Gaedigk *et al.*, 2003). The involvement of multiple strains in a single patient detected by workers using a rRNA gene NTS region (Yazdanparast *et al.*, 2003) or RAPD analysis (Baeza *et al.*, 2006) was partly supported by our microsatellite markers. For

example, the RAPD analysis revealed that more than a single genotype was detected (60–80% similarity among types) in 46% of the patients investigated. In the present study, 16% (one case) of the patients carried two genotypes differing in one out of seven microsatellite markers. As the RAPD method is known to suffer from problems of reproducibility, it is possible that the greater diversity of strain types within specimens from a single patient found by Baeza *et al.* (2006) may be artefactual and that the greater reproducibility of the specific PCR of the MLMT method accurately represents the factual degree of strain diversity. The preponderance of a limited number of genotypes in the same geographic area interferes with the distinction of relapse from reinfection in the case of infections on different body sites. This type of question might be addressed with our methodology in a longitudinal study or applied to a dataset comprising larger numbers of strains from single patients.

Within geographic borderlines, different ecological conditions are preponderant and hence the clinical syndromes of *T. rubrum* tend to vary with continent. Earlier typing studies (Jackson *et al.*, 2000; Baeza *et al.*, 2006), although successful in discriminating individual strains, were unable to reveal a meaningful segregation of populations, due to the fact that isolates of African origin were not included.

The MLMT system, applied to epidemiological studies in the present paper, also has the potential to address questions of another nature. For example, it may be used to detect markers of virulence and drug resistance in specific genotypes. The loci under study are unlikely to be based on these genes, but due to the clonal mode of reproduction of populations of *T. rubrum*, genomes are transmitted to the next generation in unaltered condition and thus associated genes – such as virulence genes and microsatellite markers – are linked. The distribution of such virulence genes is expressed in the population structure. In addition, four of the developed microsatellite markers [Tr (GA)₁₇, Tr (T)₁₇, Tr (CT)₂₀ and Tr (GA)₂₅] may be applied for diagnostic purposes, whilst T1 has already been applied successfully for rapid and specific identification of species of the *T. rubrum* complex, either from culture or directly from clinical specimens (Kardjeva *et al.*, 2006). Using the marker Tr (GA)₂₅, it is even possible to discriminate *T. rubrum* from *T. violaceum* by performing a simple horizontal gel electrophoresis using MetaPhor agarose only. The repeat number discriminating the two species is large enough for this purpose, being more than six dinucleotide repeats (>12 bp).

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