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A virulent genotype of *Microsporum canis* is responsible for the majority of human infections

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The zoophilic dermatophyte species *Microsporum canis* belongs to the *Arthroderma otae* complex and is known to mate with tester strains of that teleomorph species, at least in the laboratory. Human infections are likely to be acquired from the fur of cats, dogs and horses. Epidemiological studies to reveal sources and routes of infection have been hampered by a lack of polymorphic molecular markers. Human cases mainly concern moderately inflammatory tinea corporis and tinea capitis, but, as cases of highly inflammatory ringworm are also observed, the question arises as to whether all lineages of *M. canis* are equally virulent to humans. In this study, two microsatellite markers were developed and used to analyse a global set of 101 *M. canis* strains to reveal patterns of genetic variation and dispersal. Using a Bayesian and a distance approach for structuring the *M. canis* samples, three populations could be distinguished, with evidence of recombination in one of them (III). This population contained 44 % of the animal isolates and only 9 % of the human strains. Population I, with strictly clonal reproduction (comprising a single multilocus genotype), contained 74 % of the global collection of strains from humans, but only 23 % of the animal strains. From these findings, it was concluded that population differentiation in *M. canis* is not allopatric, but rather is due to the emergence of a (virulent) genotype that has a high potential to infect the human host. Adaptation of genotypes resulting in a particular clinical manifestation was not evident. Furthermore, isolates from horses did not show a monophyletic clustering.

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INTRODUCTION

Dermatophytes belonging to the *Arthroderma otae* complex comprise three closely related anamorphic species: *Microsporum canis, Microsporum audouinii* and *Microsporum ferrugineum*. *M. canis* is known to mate with tester strains of *A. otae*, at least in the laboratory. These mating experiments, however, have detected only a handful of (+) mating type strains, whilst the great majority of isolates displays the opposite (–) type. This observation has led several workers (Hasegawa & Usui, 1975; Hironaga *et al.*, 1980; Weitzman & Padhye, 1978) to conclude that the (+) mating type may now be on the brink of extinction and that recombination no longer occurs in nature, or is at least highly reduced. Adaptation to carriage by furred mammals as primary hosts may explain the unbalanced distribution of mating types, reducing the probability of encountering a partner of the opposite sex.

Abbreviation: I_A, index of association.

All three species are able to cause human tinea capitis and tinea corporis, especially in prepubescent children. *M. audouinii* and *M. ferrugineum* are anthropophilic, generally being transmitted from human to human, and have evolved in Africa and Asia, respectively (Kaszubiak *et al.*, 2004). *M. canis* has a worldwide distribution and is zoophilic, with humans predominantly only being infected after contact with mammals. The natural habitat of this species is the furred skin of cats, dogs and horses, where it generally resides asymptomatically. The occasional human-to-human infections are self-limiting after a few transmissions.

Epidemiological studies of human and animal infections by contagious strains of *M. canis* have remained unresolved due to a lack of polymorphic molecular markers. The detection of sources and routes of infection, and identification of contaminated spaces in hospitals, kindergartens, schools and animal nurseries would contribute to optimized therapy, prophylaxis and a hygienic regimen, and thus save financial resources. In addition, human infections due to *M. canis* tend to be moderately inflammatory, but cases of severe kerion-like tinea capitis

or highly inflammatory ringworm infections do occur in similar patient populations (Ernst 1980; Pryce & Verbov, 1992; Stephens *et al.*, 1989; Terragni *et al.*, 1993). The question arises as to whether animal hosts harbour mixed genotypes of *M. canis* that differ in their degree of virulence to humans. In other words, do all lineages of *M. canis* have the same potential to infect humans, or is virulence limited to a subset of isolates within a genetically diverse population? Do these genotypes differ in predilection and pathogenicity? Do strains from particular animal host species compose monophyletic groups within *M. canis* with decreased gene flow? In order to address these questions, there is an urgent need for well-characterized neutral markers to analyse the population structure of *M. canis*.

In recent studies, several DNA markers (randomly amplified polymorphic DNA, sequencing of internal transcribed spacer and non-transcribed spacer regions of rRNA genes, intergenic spacers of nuclear DNA, and mitochondrial DNA genes) have been applied, but the degree of polymorphism was low within the species (Kaszubiak *et al.*, 2004; Yu *et al.*, 2004). Typing systems based on microsatellite markers have been shown to detect diversity at all levels from species down to individuals in pathogenic fungi such as *Histoplasma*, *Coccidioides* and *Penicillium* (Carter *et al.*, 2001; Fisher *et al.*, 2000, 2004). In the present study, we report on the application of microsatellite markers to a global set of *M. canis* strains to reveal patterns of genetic variation in this species.

METHODS

Fungal strains. A total of 137 strains was analysed, most of them acquired from 1996 to 2002 (Table 1). Of these, 101 strains were identified morphologically as *M. canis*, 29 were *M. audouinii* and 7 were *M. ferrugineum*. Strains of the latter two species were used as outgroups. Thirty-three *M. canis* strains were isolated from epidemiologically unrelated animals (mainly cats and dogs) in Germany, whilst fifteen strains originated from horses. Fifty-three strains were obtained from epidemiologically unrelated humans (mostly children) at geographically distant locations (Austria, Mexico, Turkey, Korea, The Netherlands, USA, New Zealand and the Dominican Republic). Tinea capitis and tinea corporis was diagnosed in 22 and 18 cases, respectively, in addition to 7 cases of tinea faciei. No clinical data were available on the remaining six patients.

DNA extraction. DNA was extracted using the CTAB (*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide) method (Gräser *et al.*, 1999) after growing the fungus on Sabouraud glucose agar (Difco Laboratories).

Isolation of microsatellites (enrichment methods). Microsatellite sequences were captured using biotinylated $(GT)_{12}$ and $(GA)_{12}$ probes and immobilized on avidin-coated beads. The captured DNA was subjected to washing steps, and then eluted, amplified and cloned to produce a library enriched for the target sequence. The method was modified slightly from the one used previously by Ohst *et al.* (2004). Briefly, genomic DNA was isolated from a clinical isolate of *M. canis* (H22). Approximately 10 μg DNA was digested with *Dpn*II and cleaned by drop dialysis for 15 min. Linkers (Sau-A, 5′-GCGGTACCCGGGAAGCTTGG-3′; Sau-B, 5′-GATCCCAAGCT-TCCCGGGTACCGC-3′) were ligated to both ends of the fragments using T4 DNA ligase (New England Biolabs). After purification via columns, pre-hybridization PCR was performed with the Sau-A linker only (annealing temperature of 56 °C, 15 cycles). For enrichment, the PCR product was denatured and hybridized to the biotinylated (CA or GA) probe in a solution of 6× SSC (1× SSC, 0.15 M NaCl, 0.015 M sodium citrate)/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 and washed three times with TBT plus 150 mM NaCl and three times with 0.2× SSC/0.1 % SDS. The DNA was then denatured from the beads in 10 mM Tris/HCl (pH 8)/0.1 mM EDTA at 95 °C for 5 min and again PCR amplified with Sau-A. The resulting PCR product was cloned and transformed using a TOPO TA cloning kit (Invitrogen). White selection colonies were picked and checked for the repeat insert using M13 primers and $(AC)_{10}$ or $(GA)_{10}$ primers. Inserts of 300–600 bp were chosen for sequencing using an M13 primer and an automated sequencing system (3130x Genetic Analyzer). Specific primers were then 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 *M. canis*, including the genomic DNA of the isolate the library was generated from.

PCR amplification of microsatellite markers using specific primers. Standard PCR conditions were as follows: reactions were performed in 50 μl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$ (1.5 mM for $McGT_{17}$), 20 pmol each primer ($McGT_{13}$ forward, 5′-GATCGGAGCATGCCATACAG-3′; $McGT_{13}$ reverse, 5′-TCTTCCCACCCTTCTCAATG-3′; $McGT_{17}$ forward, 5′-GCTCTGGGATAAGGTGTTTG-3′; and $McGT_{17}$ reverse, 5′-GTAGCAGTAAAGCCAAGAGGG-3′), 50 μM each dNTP, 2.5 U *Taq* polymerase (Applied Biosystems), and 50 ng template DNA. Samples were amplified through 30 cycles as follows: initial denaturation for 10 min at 95 °C, followed by denaturation for 50 s at 95 °C, annealing for 60 s at 60 °C 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. PCR products (15 μl) were loaded onto 12 % polyacrylamide gels (Rotiphorese gel 29 : 1, 40 %; Carl Roth) and the microsatellites were 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 a sequenced allele with known repeat number as the reference (EMPL accession nos AM295318 and AM295319).

Data analysis. Different approaches were used to assign strains to populations. Whilst distance-based methods proceed by calculating a pairwise distance matrix whose entries give the distance between every pair of individuals, model-based methods proceed by assuming that observations from each cluster are random draws from some parametric model. In the first case, this matrix is then displayed using a graphical presentation such as a tree. In the second case, inference for the parameters corresponding to each cluster is done jointly with inference for the cluster membership of each individual, using standard statistical methods such as the Bayesian method. The disadvantage of the distance-based method is that the identified clusters may be heavily dependent on the distance measurement and graphical presentation chosen (Pritchard *et al.*, 2000). Therefore, we calculated genetic distances between individuals based on three different measurements, Dc (Cavalli-Sforza & Edwards, 1967), Dm and Ds (Saitou & Nei, 1987) 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 were displayed using TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). As a model-based method, a Bayesian approach was used in

Table 1. Fungal strains analysed in this study

Strain no.	Reference no.	Source	Clinical picture	Geographical origin	Date	Microsatellite loci repeat no.	
						$Mc(GT)_{13}$ 106–138 bp	$Mc(GT)_{17}$ 133–167 bp
M. canis							
240	M 361	Human	–	Netherlands	2000	12	17
G4	4976	Child	Tinea capitis	Austria	2001	12	17
G6	5145	Child	Tinea corporis	Austria	2001	12	17
G7	5178	Child	Tinea corporis	Austria	2001	12	17
G8	5179	Child	Tinea corporis	Austria	2001	12	17
G9	5217	Child	Tinea corporis	Austria	2001	12	17
G10	5227	Child	Tinea faciei	Austria	2001	12	17
G12	5251	Adult	Tinea corporis	Austria	2001	12	17
G13	5308	Adult	Tinea capitis	Austria	2001	12	17
G16	5927	Child	Tinea capitis	Austria	2001	12	17
G17	5934	Child	Tinea faciei	Austria	2001	12	17
G18	6000	Child	Tinea corporis	Austria	2001	12	18
G19	6037	Child	Tinea corporis	Austria	2001	12	17
G22	6046	Child	Tinea capitis	Austria	2001	12	17
G24	6077	Adult	Tinea corporis	Austria	2001	12	17
G25	6129	Child	Tinea capitis	Austria	2001	12	17
G26	6158	Adult	Tinea capitis	Austria	2001	10	17
G27	6197	Child	Tinea faciei	Austria	2001	10	17
G31	6219	Adult	Tinea faciei	Austria	2001	12	17
G32	6221	Child	Tinea capitis	Austria	2001	12	15
G34	6324	Child	Tinea corporis	Austria	2001	12	17
G35	6349	Adult	Tinea corporis	Austria	2001	12	17
H21	T 033/97	Cat	Ringworm	Hildesheim/Germany	1997	12	17
H22	T 271/97	Cat/dog	Ringworm	Nienburg/Germany	1997	13	17
H24	T 203/97	Cat/dog	Ringworm	Ahaus/Germany	1997	13	18
H25	T 256/97	Cat/dog	Ringworm	Minden/Germany	1997	13	18
H27	E 1860/99	Cat	Ringworm	Nienburg/Germany	1999	12	19
H28	K 452/99	Cat	Ringworm	Hamburg/Germany	1999	13	17
H29	E 1142/99	Cat	Ringworm	Düsseldorf/Germany	1999	12	18
H30	K 436/99	Cat	Ringworm	Wuppertal/Germany	1999	12	18
H31	B 2/2.1/99	Cat	Ringworm	Köln/Germany	1999	12	18
H33	E 705/99	Cat	Ringworm	Hannover/Germany	1999	12	18
H34	K 1269/99	Cat	Ringworm	Ebstorf/Germany	1999	12	17
H35	E 456/99	Cat	Ringworm	Hannover/Germany	1999	13	17
H36	K 1188/99	Cat	Ringworm	Bünde/Germany	1999	12	17
H37	K 565/00	Dog	Ringworm	Düsseldorf/Germany	2000	12	14
H38	E 1233/99	Cat	Ringworm	Hannover/Germany	1999	12	15
H39	B 7/1.1/99	Cat	Ringworm	Hannover/Germany	1999	12	18
H40	K 603/00	Dog	Ringworm	Uthlede/Germany	2000	13	14
H41	K 1247/99	Cat	Ringworm	Celle/Germany	1999	13	14
H42	B 45/2.1/99	Cat	Ringworm	Essen/Germany	1999	12	17
H43	R 008/97	Cat	Ringworm	Hildesheim/Germany	1997	12	17
H46	K 54/99	Cat	Ringworm	Bünde/Germany	1999	12	18
H48	T 012/97	Cat/dog	Ringworm	Hannover/Germany	1997	12	17
H50	E 1335/99	Cat	Ringworm	Hannover/Germany	1999	12	18
H51	K 948/96	Cat	Ringworm	Hamburg/Germany	1996	12	18
H53	K 1514/98	Cat	Ringworm	Metzingen/Germany	1998	12	18
H54	K 1537/98	Cat	Ringworm	Bürstedt/Germany	1998	12	18
H55	K 304/99	Cat	Ringworm	Wuppertal/Germany	1999	12	18
H56	K 364/99	Cat	Ringworm	Hamburg/Germany	1999	12	17
H57	K 370/99	Cat	Ringworm	Metzingen/Germany	1999	12	17
H58	K 496/99	Cat	Ringworm	Duisburg/Germany	1999	12	17
H60	E 501/99	Cat	Ringworm	Hannover/Germany	1999	12	18

Table 1. cont.

Strain no.	Reference no.	Source	Clinical picture	Geographical origin	Date	Microsatellite loci repeat no.	
						$Mc(GT)_{13}$ 106–138 bp	$Mc(GT)_{17}$ 133–167 bp
H61	E 527/99	Cat	Ringworm	Göttingen/Germany	1999	12	19
51	CBS 495.86	Chicken	–	Japan	1986	17	17
K8	MC 8	Child	Tinea capitis	Kyungpook/Korea	2000	12	17
K9	MC 9	Child	Tinea capitis	Kyongki/Korea	2000	12	17
K10	MC 10	Child	Tinea capitis	Kyongki/Korea	2000	12	17
K11	MC 11	Adult	Tinea capitis	Kyungpook/Korea	2000	12	17
K12	MC 12	Child	Tinea capitis	Kyungpook /Korea	2000	12	17
K13	MC 13	Adult	Tinea capitis	Taegu/Korea	2000	12	17
K14	MC 14	Child	Tinea corporis	Kyungpook/Korea	2000	12	17
K15	MC 15	Child	Tinea capitis	Kyungpook/Korea	2000	12	17
K16	MC 16	Child	Tinea faciei	Kyungpook/Korea	2000	12	17
K17	MC 17	Child	Tinea faciei	Taegu/Korea	2000	12	17
K18	MC 18	Adult	Tinea corporis	Taegu/Korea	2000	12	17
K19	MC 19	Adult	Tinea corporis	Taegu/Korea	2000	12	17
K20	MC 20	Child	Tinea corporis	Taegu/Korea	2000	12	17
K21	MC 21	Child	Tinea faciei	Taegu/Korea	2000	12	17
K22	MC 22			Taegu/Korea	2000	12	17
K23	MC 23	Child	Tinea corporis	Taegu/Korea	2000	12	17
K24	MC 24	Child	Tinea capitis	Kwangju/Korea	2000	12	17
K25	MC 25	Child	Tinea capitis	Kwangju/Korea	2000	12	17
K26	MC 27	Child	Tinea capitis	Kyongki/Korea	2000	12	17
Mex10	A. Z.	Human	Tinea capitis	Dominican Republic	2002	12	17
Mex12	564-01	Human	Tinea capitis	Mexico City/Mexico	2002	12	17
Mex16	273-01	Human	Tinea capitis	Mexico City/Mexico	2002	12	18
Tu2	3561/2	Adult	Tinea corporis	Afyon/Turkey	2000	12	14
Tu5	3264	–	–	Turkey	2000	12	15
Tu7	33	Child	Tinea capitis	Agri/Turkey	2000	12	14
Tu12	58	Adult	Tinea corporis	Izmir/Turkey	2001	12	14
Tu15	3611/1	Adult	Tinea corporis	Afyon/Turkey	2000	12	14
Tu17	–	–	–	Turkey		12	18
Tu18	–	–	–	Turkey		13	15
70	CBS 277.62	Human	–	USA	1962	13	14
181	CBS 101514	Human	Tinea capitis	New Zealand		13	14
H1	E 979/96	Horse	Ringworm	Bamberg/Germany	1996	13	15
H2	K 1456/84	Horse	Ringworm	Mechen/Germany	1984	13	14
H7	K 1100/99	Horse	Ringworm	Bremerhaven/Germany	1999	13	14
H8	E 589/99	Horse	Ringworm	Ebstorf/Germany	1999	13	14
H9	E 1221/96	Horse	Ringworm	Hannover/Germany	1996	13	14
H10	E 1745/96	Horse	Ringworm	Bamberg/Germany	1996	13	14
H12	K 887/96	Horse	Ringworm	Ritterhude/Germany	1996	12	17
H13	K 1025/96	Horse	Ringworm	Vierhöfen/Germany	1996	12	18
H14	K 1151/99	Horse	Ringworm	Heiligenhaus/Germany	1996	13	14
H15	E 590/99	Horse	Ringworm	Uthlede/Germany	1999	13	14
H16	E 1338/98	Horse	Ringworm	Büren/Germany	1998	13	14
H17	E 681/91	Horse	Ringworm	Hannover/Germany	1991	13	14
H18	62623/00	Horse	Ringworm	Coppenbrügge/ Germany	2000	13	15
H19	K 925/99	Horse	Ringworm	Heiligenhaus/Germany	1999	13	14
H20	K 486/84	Horse	Ringworm	Remscheid/Germany	1984	13	17
M. audouinii							
171	CBS 215.47	Child	Tinea capitis		1947	9	2
172	CBS 344.50	–	–		1950	9	2
233	CBS 108932	Human	Tinea capitis	Africa	1994	9	2
234	CBS 108933	Human	Tinea capitis	Africa	1997	9	2

Table 1. cont.

Strain no.	Reference no.	Source	Clinical picture	Geographical origin	Date	Microsatellite loci repeat no.	
						$Mc(GT)_{13}$ 106–138 bp	$Mc(GT)_{17}$ 133–167 bp
235	CBS 108934	Human	Tinea capitis	Africa	1996	9	2
237	M 294	Human	Tinea capitis	Haarlem/Netherlands	2000	9	2
241	FR 414	Human	Tinea capitis	Canada	2001	9	2
242	FR 569	Human	Tinea capitis	Canada	2001	9	2
238	STJEK 4873	–	–	UK	2000	9	2
E33	SJ EM 8199	Child	Tinea capitis	UK	2001	8	2
E35	SJ equation 8560	Child	Tinea capitis	UK	2001	8	2
E36	SJ equation 9154	Child	Tinea capitis	UK	2001	8	2
E37	SJ equation 9777	Child	Tinea capitis	UK	2001	9	2
E38	SJ EK 161	Child	Tinea capitis	UK	2001	9	2
E39	SJ EK 3447	Child	Tinea capitis	UK	2001	9	2
E40	SJ EK 5724	Child	Tinea capitis	UK	2001	8	2
E41	SJ EN 7789	Child	Tinea capitis	UK	2001	9	2
E42	SJ EN 7875	Child	Tinea capitis	UK	2001	9	2
E43	SJ equation 8050	Child	Tinea capitis	UK	2001	9	2
E45	SJ equation 8749	Child	Tinea capitis	UK	2001	9	2
E46	SJ equation 8899	Child	Tinea capitis	UK	2001	9	2
E47	SJ equation 9476	Child	Tinea capitis	UK	2001	9	2
E48	SJ equation 9572	Child	Tinea capitis	UK	2001	9	2
E50	SJ equation 9906	Child	Tinea capitis	UK	2001	9	2
E51	SJ equation 6500	Child	Tinea capitis	UK	2001	9	2
E29	SJ EC 1304	Child	Tinea capitis	UK	2001	9	2
E30	SJ K 1293	Child	Tinea capitis	UK	2001	9	2
E31	SJ EK 5415	Child	Tinea capitis	UK	2001	9	2
E32	SJ EN 2804	Child	Tinea capitis	UK	2001	9	2
M. ferrugineum							
E63	No. 1	–	–	Thailand	2001	25	2
E64	No. 2	–	–	Thailand	2001	25	2
E65	2.1	–	–	Thailand	2001	25	2
E66	2.2	–	–	Thailand	2001	25	2
E67	2.3	–	–	Thailand	2001	25	2
E68	2.4	–	–	Thailand	2001	25	2
E72	2.8	–	–	Thailand	2001	25	2

the program STRUCTURE version 2.1 (Pritchard *et al.*, 2000). This method allows the assessment of confidence of the inferred clusters by fine statistical analysis, whilst genetic distance methods are more suited to exploratory data analysis. Various models were used with STRUCTURE, including the no-admixture model, which can deal with clonal reproduction. One million Markov chain Monte Carlo replications and a burn-in period of 100 000 generations were 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.

After structuring the populations, Wright's F statistics were applied to compute the variance in allele frequencies and test for free gene flow versus population differentiation between the inferred populations. Theta (Weir, 1996) was calculated across loci and populations using MULTILOCUS version 1.3 (Agapow & Burt, 2001). Here, the null hypothesis is no population differentiation; 400 000 randomizations were used.

To test for clonality versus recombination in the *M. canis* sample, the overall and the 'in population separated' (based on both cluster methods; see Results) index of association (I_A) was calculated using the software MULTILOCUS. In this test, the observed data are compared against 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.

RESULTS

Of the 19 typable microsatellite markers developed, we used the most polymorphic loci, $Mc(GT)_{17}$ and $Mc(GT)_{13}$, revealing four and five alleles within the *M. canis* set of strains, respectively. The alleles varied by seven and five dinucleotide repeats within each locus. Up to 3 alleles were found with the remaining 17 markers; however, these were represented only by single strains (data not shown). In *M. audouinii* and *M. ferrugineum*, the alleles were species specific. With marker $Mc(GT)_{13}$, two alleles with a single dinucleotide difference among *M. audouinii* strains were detected. Strains of *M. audouinii* and *M. ferrugineum* were

excluded from further analysis because of the low variability detected with both markers. Only one strain of each was used as outgroup for the distance tree in Fig. 1. The data for each strain and locus are presented in Table 1.

Analysis of the combined dataset of both markers detected a total of 11 multilocus genotypes among the 101 *M. canis* strains and 3 among the 36 *M. audouinii* and *M. ferrugineum* strains. The application of several distance methods revealed identical results (Table 1, Fig. 1). An indication of clonal reproduction within the *M. canis* sample set was the observation of three multilocus genotypes that were shared by multiple strains from unrelated hosts. Of the 11 multilocus genotypes, 1 was shared by 50 strains, whilst 2 genotypes comprised 14 and 15 strains . This corresponds to a genotypic diversity in this dataset of 0.71. The linkage disequilibrium analysis of the overall sample and the clone-corrected sample rejected the null hypothesis of random mating (I_A=0.19, P<0.001). The identity of spatially separated strains of the same genotype demonstrated the high degree of reproducibility of the technique used.

Independent from the underlying assumptions and using several distance measurements – the Bayesian approach and neighbour joining – always revealed trees with three

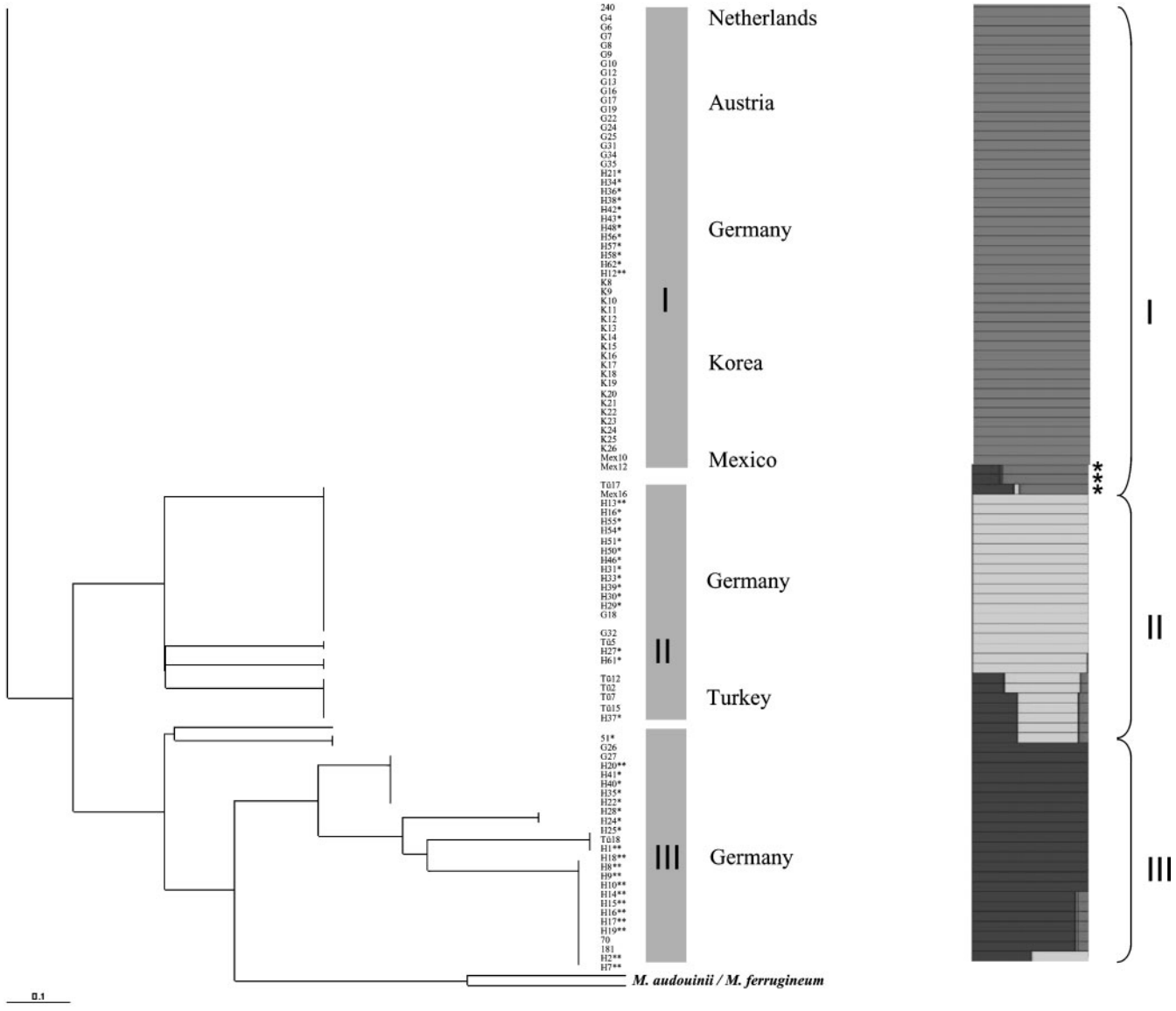

Fig. 1. Neighbour-joining tree based on Dc distance (on the left): no asterisks, humans isolates; *, cat or dog isolates; **, horse isolates. A bar plot for K=3 using STRUCTURE (on the right) shows the same three clusters as the distance tree. The three populations (I–III) are indicated by different shades of grey. The three strains marked by asterisks grouped with cluster I by STRUCTURE and with cluster III by the distance method.

clusters (I–III) within *M. canis*. Each method generated branches with nearly identical sets of strains, except for three strains that grouped with cluster I (Bayesian approach) or cluster III (distance approach), but with a slight affinity to cluster III (mixed genotype) using the Bayesian approach (Fig. 1). Isolates from cats and dogs were distributed evenly among the three clusters. The 6 multilocus genotypes in cluster III were shared by 13 isolates (87 %) from horses, 8 strains (24 %) from other animals and 5 human isolates (9 %) (Figs 1 and 2). Cluster II was less variable, although the total number of strains was comparable to that in cluster III (26 strains in cluster III and 24 strains in cluster II). Only 4 multilocus genotypes were shared by 15 animal strains (31 %), among which was 1 isolate from a horse, and 9 (17 %) of the human isolates (Figs 1 and 2). Six out of seven (86 %) human strains of Turkish origin were found in group II (Fig. 1). In group I, a single multilocus genotype was shared by most of the human isolates (74 %), independent of their distant geographical origins (Korea, Austria and Mexico), together with 11 animal strains (23 %) from Germany, including one isolate from a horse (Figs 1 and 2).

After pre-defining the three populations on the basis of the cluster analyses, the repeated linkage disequilibrium analysis did not reject the null hypothesis of random mating for population III (I_A=0.049; P=0.33). Population I did not recombine, as it consisted of a single clone. For population II, the I_A did not reveal a meaningful result as several genotypes were present in one of the loci. Support for population differentiation in *M. canis* was given by the statistics of theta (θ=0.733, P<0.001).

Data on clinical pictures were obtained from 47 of the 53 patients studied. In total, 22, 18 and 7 cases of tinea capitis, tinea corporis and tinea faciei were revealed, respectively. Whilst strains in cluster I were able to cause all three forms of tinea, cases of tinea faciei and tinea corporis were missing in clusters II and III, respectively (Fig. 3).

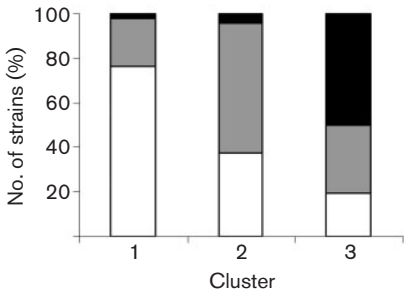

Fig. 2. Association between the three clusters of genotypes and the host animal. The number of strains is given as a percentage. Black bars, horse; grey bars, cat/dog; white bars, human.

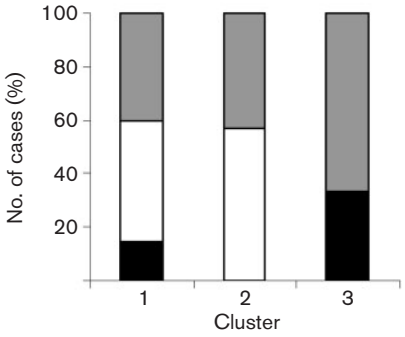

Fig. 3. Association between the three clusters of genotypes and the clinical picture [tinea capitis (grey bars), tinea corporis (white bars) and tinea faciei (black bars)]. The number of cases (37, 7 and 3) for clusters 1–3 are given as a percentage.

DISCUSSION

The *M. canis* sample set under study could be subdivided into three populations (I–III), of which I and II in particular had established largely clonal dimensions. By contrast, in population III comprising the majority of animal strains (44 %), the null hypothesis of random mating was not rejected. These results suggested that clonal and recombining population structures in *M. canis* exist concomitantly and that mating may occur. This finding is not unexpected, as the species is known to mate in the laboratory, but reproduces mitotically by conidia when transmitted between host individuals (Hironaga *et al.*, 1980). Support for the argument that *M. canis* is composed of heterogeneous populations comes from the statistically significant high values of theta. Low mating competence and a predominantly clonal mode of reproduction are evident for many fungal species (Taylor *et al.*, 1999). The differences between populations are meaningful when associations are found with particular virulence factors, such as the keratinolytic proteinases in dermatophytes (Giddey *et al.*, 2007; Monod *et al.*, 2002).

From our results, we could exclude the possibility that geographical differentiation and allopatric speciation played an important role in structuring the populations of *M. canis*. The strains from Germany (all animal isolates) did not show any monophyletic clustering, and the human isolates from Europe, South America and Asia could be found jointly as a single cluster.

M. canis is a zoophilic fungus that is only isolated rarely from soil. Hence, we anticipated an association of genotypes with particular host species. However, we revealed cat-associated genotypes in all three populations (I–III). Similarly, equine ringworm or colonization of horse fur could not be linked to a monophyletic group of strains. The set of 15 isolates from Germany comprised 5 multilocus genotypes belonging to all 3 populations of *M. canis*. Of the horse isolates 10 out of 15 had identical alleles

at both loci, indicating that a single clone was involved. It is evident that horse isolates have acquired the ability to cause superficial disease more than once in the course of their evolution. Maintenance of horse isolates as a separate species, *Microsporum equinum* (Delacroix & Bodin, 1896), is therefore not justified. A similar situation has been published with multilocus genotypes of *Histoplasma capsulatum* (Kasuga *et al.*, 2003). Molecular analysis of four genes of isolates causing equine histoplasmosis, formerly classified as a separate variety, *H. capsulatum* var. *farciminosum*, revealed that strains were distributed over three phylogenetic clades. The authors concluded that maintenance of the var. *farciminosum* was phylogenetically meaningless.

Despite the fact that all of the *M. canis* strains under study were isolated from epidemiologically unrelated individuals, 74 % of the isolates from human patients but only 23 % of the animal isolates shared a single genotype. The over-representation of this genotype 1 among the human isolates suggests that it may have a higher degree of virulence (possibly a proteinase adapted to human keratin) than the ten remaining genotypes, having a higher potential to infect humans when transmitted from animals. The genotype has a pandemic distribution. Strains harbouring genotype 1 were isolated from patients from three continents (Europe, South America and Asia). This seems to be in conflict with the data presented by Cano *et al.* (2005) using inter-simple-sequence repeat PCR (ISSR-PCR), who demonstrated that numerous strains from humans have a limited distribution, and may even be restricted to a single patient. The authors found a total of 21 genotypes among 24 mainly human isolates from Spain. With the exception of a single genotype (pattern 1), none of the animal genotypes occurred on humans, and vice versa. The conclusions derived from these data could not be explained by DNA loci located between single repeats, such as the flanking regions of microsatellites. Mutation rates of flanking and repeat regions have been analysed in detail in other fungal species, revealing a 2500-fold higher mutation rate for the latter loci (Dettman & Taylor, 2004). Thus, microsatellite loci are likely to be more variable than the flanking regions analysed using ISSR-PCR. The high variability among strains in the study of Cano *et al.* (2005) may partly be due to the low reproducibility (93 %) of the technique used. In contrast to Cano *et al.* (2005), the few other studies performing strain typing in *M. canis*, e.g. randomly amplified polymorphic DNA, reported a very low variability among epidemiologically unrelated strains from cats, dogs and humans, despite their morphological diversity (Brilhante *et al.*, 2005; Faggi *et al.*, 2001). Such results are in agreement with the extensive developmental work we have done while searching for polymorphic markers: 90 % of the microsatellite markers were unacceptable for the population analysis due to the low variability displayed. This is likely to be caused by the high portion of clonal reproduction within the species. In another clonal species, *Trichophyton rubrum*, only 30 % of the typable microsatellites were polymorphic, whereas in strongly recombining populations of *Microsporum persicolor*, almost 100 % of the loci were useful for a population genetic study (R. Sharma, S. de Hoog, W. Presber, Y. Gräser & R. C. Rajak, unpublished data). Although our findings suggest that some *M. canis* strains have an increased infective potential to humans, there was no indication of an association between genotypes and the type of tinea caused by this genotype, as tinea capitis was caused by genotypes of all three clusters. The missing manifestations of tinea corporis and tinea faciei by strains of clusters II and III, respectively, may have been due to the low number of human cases (nine and five) falling in these population groups.

In conclusion, our data cannot rule out the possibility that there are several asexual, separated lineages within *M. canis*. Systematic sampling needs to be undertaken, particularly in animal strains from geographically remote locations. Additional microsatellite markers need to be developed to support the indication of the presence of recombination events in this fungus.

In addition to epidemiological studies, the markers developed in this project can be applied to other studies. In addition to virulence traits, as shown here, drug resistance may also be associated with particular genotypes. Although the loci under study are probably not based on resistance genes, the largely clonal reproduction in populations of *M. canis* keeps genes and their associated traits together. The developed microsatellite markers can also be used as diagnostic tools for the rapid and specific identification of species of the *M. canis* complex directly from clinical specimens. This has been shown already by Kardjeva *et al.* (2006) for the detection of *T. rubrum*. $Mc(GT)_{13}$ is able to discriminate the species *M. canis, M. audouinii* and *M. ferrugineum* by agarose gel electrophoresis.

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