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Infection, Genetics and Evolution 4 (2004) 179–186
Infection, Genetics and Evolution
www.elsevier.com/locate/meegid

Population structure and evolutionary origins of *Microsporum canis*, *M. ferrugineum* and *M. audouinii*

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Received 28 August 2003; received in revised form 12 December 2003; accepted 26 December 2003

Available online 18 May 2004

Abstract

The recurrent evolutionary emergence of asexual lineages within sexual zoo- and anthropophilic dermatophyte species living in animal-frequented soil is likely to be triggered by changes in ecological niche, i.e., shifts of host animal. Subsequent adaptation to the new host species is noted. Sometimes geographic isolation or intrinsic host factors like human race may also play a role in speciation. In the present study, we elaborate concepts of speciation in dermatophytes using the *Microsporum canis* complex as an example. The group consists of a cluster of phylogenetically closely related anamorphs: the anthropophilic taxa *Microsporum audouinii* and *M. ferrugineum*, and the zoophilic taxon *M. canis*. The sexually reproducing species underlying this complex is *Arthroderma otae*. The study is done by an analysis of the population structure of about 200 isolates and using intergenic spacers, non-translated regions of genes as well as hypervariable microsatellite markers that are known to evolve at high mutation rates. The results suggest that sympatric speciation took place already during the period where mating ability was maintained and thus that strictly clonal fungal species emerged in Africa and led to genetically isolated clonal species elsewhere.

Keywords: Fungi; Dermatophytes; *Microsporum*; Population structure; Microsatellite; Clonality

1. Introduction

The human-associated dermatophytes encompass a number of organisms with a very recent phylogenetic history. Frequently, individual clades are discernible which contain mutually closely related, zoophilic next to anthropophilic species. Most of the entities are morphologically slightly different from each other (Kane et al., 1997). The clades are hypothesized to encompass an ancestral, sexually reproducing species, plus a number of derived, clonal, strictly asexual lineages (Summerbell, 2002) that have host-specificities different from that of the ancestral species. The exclusively mitosporic, human-associated *Trichophyton rubrum* complex is the only exception to this scenario. An example of a mixed anthropo- and zoophilic clade is the teleomorphic species *Arthroderma vanbreuseghemii*, with its derived mitosporic lineages of human-associated *T. interdigitale* and *T. tonsurans* and horse-associated *T. equinum*. The lineages may differ in phylogenetic age: *T. interdigitale* is considered to be the most recent of the three. Another example is the sexually reproducing species *A. simii* and its derived clonal lineages *T. mentagrophytes*, associated with the house mouse, and *T. schoenleinii*, which is anthropophilic (Probst et al., 2002).

The recurrent evolutionary emergence of asexual lineages in zoo- and anthropophilic dermatophytes is likely to be triggered by changes in ecological niche, i.e., shifts of host animal, with subsequent adaptation to the new host species. For *T. mentagrophytes* and *T. schoenleinii*, it is evident that speciation occurred due to a host shift from animals to humans (Probst et al., 2002). Additional conditions such as geographic isolation or intrinsic host factors like human race may also play a role in speciation. Otherwise, it is difficult to understand, for example, why several anthropophilic lineages may arise within the same group of related organisms, such as *M. ferrugineum*/*M. audouinii* from *Arthroderma otae*, or *T. interdigitale*/*T. tonsurans* from *A. vanbreuseghemii*.

With the frequent emergence of exclusively clonal lineages, the problem of species delimitation in dermatophytes becomes urgent. A wide diversity of theoretical and

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doi:10.1016/j.meegid.2003.12.004

operational approaches is available (Taylor et al., 2000). The biological species concept (Mayr, 1940), where associations of interfertile strains determine a species, is not applicable by definition. Phenetic-morphological concepts that have started with Sabouraud (1910) have been applied more or less successfully for over a century in dermatophyte taxonomy. Subsequent molecular and polyphasic approaches to species recognition (Cohan, 2001), being based on average molecular distances between defined DNA regions and averaged combinations of dissimilar data sets, respectively, have been applied (Gräser et al., 2000). Most of the phenotypically described taxa were thus confirmed, as they proved to be supported by reproducible distances in ITS rDNA and in fingerprint differences. In a number of cases, however, conflicts between the phenetic and molecular data sets remain. Application of phylogenetic species recognition, where fixed, concordant polymorphisms in molecular markers are shared by a number of isolates and not by others (Taylor et al., 2000), would reliably define borderlines of species, as it applies independent data sets indicating comparable degrees of genetic isolation. Essential difference with previous approaches is the application of fixed multiallelic polymorphisms proving separation of lineages, while mean distances may actually be quite small. However, distinction between speciation and meiotic recombination may be problematic in this approach. Operationally the method applied should be independent from sexuality. All current approaches have their limitations, and the extend to which they are applicable to strictly clonal lineages has not been defined. It is problematic, for example, that some clonal lineages are isolated, whereas in other groups speciation may already occur before mating is lost completely. Approaches in defining sexual as well as mitotic entities in dermatophytes have to be standardized for the sake of nomenclatural stability (Summerbell, 2002).

In the present paper, we will elaborate concepts of speciation in dermatophytes using the *Microsporum canis* complex as an example. The group consists of a cluster of phylogenetically closely related anamorphs: the anthropophilic taxa *M. audouinii* and *M. ferrugineum* and the zoophilic taxon *M. canis*. The sexually propagating species underlying this complex is *A. otae*. Further, phenotypically distinguishable species of the complex are the anthropophilic species *M. rivalieri* and *M. langeronii*, the zoophilic taxa *M. equinum* on horses and *M. distortum* on hedgehogs, but these were recently synonymized either with *M. canis* or *M. audouinii* on molecular grounds (Gräser et al., 2000). The latter conclusion involved data of the internal transcribed spacer (ITS) region of the rDNA and PCR fingerprinting analyses (Gräser et al., 2000). The aim of our study is to re-analyze the validity of morphological entities in the complex by revealing the evolutionary history of the *A. otae* clade. This is done by an analysis of the population structure of a larger number of isolates using intergenic spacer, non-translated regions of genes as well as hypervariable microsatellite markers that are known to evolve at mutation rates of up to 10^{-2} to 10^{-3}.

2. Materials and methods

2.1. Fungal strains

In total, 205 clinical and reference strains were analyzed. Of these, 14 human isolates identified as *M. ferrugineum* were from Kenya, Germany, France and Thailand collected between 1963 and 2001. The 50 human strains of *M. audouinii* (among them five *M. rivalieri* and two *M. langeronii* isolates) were collected in The Netherlands, Germany, UK, Canada, USA, Nigeria and the Dominican Republic between 1947 and 2002. The majority of isolates (human and animal) concerned *M. canis* (among them four *M. distortum* and 23 *M. equinum* strains) originating from The Netherlands, Germany, Austria, UK, Turkey, Russia Korea, Japan, Dominican Republic and Mexico, collected between 1932 and 2002. Whereas the clinical picture caused by *M. ferrugineum* and *M. audouinii* strains was always *Tinea capitis*, the symptoms caused by *M. canis* strains were variable (*Tinea corporis*, *T. capitis*, *Tinea faciei* in adults and children, and ringworm in horses, cats, dogs, chicken and a monkey). Further information on the strains is available on request from the corresponding author. Strains were cultured on Sabouraud's glucose agar (SGA; Difco Laboratories, Detroit, MI, USA), and genomic DNA was extracted as described previously (Gräser et al., 1999).

2.2. Development of markers

Microsatellite loci (AC and C) were isolated as described by Rassmann et al. (1991). Libraries containing 300–700 bp inserts in the vector pGEM 4Z (Promega, Mannheim, Germany) were made for *M. canis* strains T2 and Tü2–10. About 3500 inserts were amplified using M13 primers directly from the colonies. Amplicons were spotted to Nylon membranes (BIOBOND^{TM}, Sigma, USA) following the manufacturer's instructions. $(AC)_{10}$, $(GA)_{15}$, $(GACA)_4$ and $(GTG)_5$ oligonucleotides (Tib Molbiol, Berlin, Germany) were endlabeled using T4 polynucleotide kinase (MBI Fermentas, Vilnius, Lithuania) and [γ^{32}P]-ATP (3000 Ci/mmol, NEN, Boston, USA) and used to screen the libraries. Filters were hybridized in a solution containing 0.5 M NaH_2PO_4 (pH 7.2), 1 mM EDTA, 7% sodium dodecyl sulphate (SDS) at 55–60 °C and washed once in 5× SES (40 mM Na_2HPO_4 [pH 7.2], 1 mM EDTA, 5% SDS), twice in 1× SES and finally in 0.1× SES. Positive PCR products/clones were cycle sequenced using the same M13 primers as mentioned above. Primer pairs were designed on the basis of these sequences (Table 1).

The remaining markers were developed by screening the EMBL database for entries of gene sequences of dermatophytes. The following targets were used (Table 1): two DNA markers located within the spacer region between the ATPase 9 and cytochrome oxidase II genes (ATP9/COXII), an intergenic spacer region between the genes encoding the NADH dehydrogenase subunits 1 and 3 (N3/N1) and the 3′

Table 1
Markers developed in this study and their corresponding primers

Marker	Size (bp)	Primers	5′–3′	T_a (°C)	$(Motif)_{Repeat\text{-}No.}$	Enzyme
ATP9/COXII	630	ATP9.for ATP9.rev	AGAAACCCTTCACTTAGAGGTC TCTATACCTTCCATTTGAGGTG	62	–	*Apo*I
N3/N1	670	N3.for N3.rev	GGGTCAAAATAGAACACAGTTC CTTCTTTGCATACTAGCCATTG	56	–	*Taq*I
UB/VAR	260	UB.for UB.rev	CTGTTTCCTCTTATGCTTTTTC TTTTGCTCCAAAAGTATTGTG	55	$(T)_{3–5}$ $(CCT)_{2–3}$	
AC1	160	AC1.for AC1.rev	ACAGAAAATCCATCGCAACC GCCTTGCCTCTGACAGCC	63	–	
AC3	250	AC3.for AC3.rev	AATATGCCTCATGCGATCTC TGTTCACCACGACTTGCC	61	$(AAC)_{1–2}$	
AC4	220	AC4.for AC4.rev	GGAAAACTTGGTAAAGTCCTG CCGTTCTCACGAATGATGTC	60	–	
AC6	200	AC6.for AC6.rev	TGATTACGAGAAAGAGACTGGC TCATTGAGGCGTCAAAATTG	60	–	
AC8	520	AC8.for AC8.rev	GGTGTTTAGCGTCTGGAATG TGTCACCAGCATGTTCTTTC	58	–	
AC9	200	AC9.for AC9.rev	TAAATCTTTGGCTTCATCGG AGTTGTCTAATAACCTCCCGC	58		
AC11	400	AC11.for AC11.rev	CCCTCGTCTCTTCCAGGC TAGCAAAGTCGCCCAGTCTC	61	$(AGA)_{3–5}$	
AC12	350	AC12.for AC12.rev	GAGTCAAGGGTTCACATCAC GGTACAGTCTTTGTTTGGCTC	56	–	
C235	110	C235.for C235.rev	GACACGCTCCGACTTTTTAC GGCCTTCTCTGGAGTACATAC	62	$(GT)_{11–14}$	
C772	120	C772.for C772.rev	GACGGAGCCATGTGTATCTA ATAAAACCCCACCTCGTTAC	58	$(TGTC)_{3–4}$	

non-coding region of the ubiquitin gene (UB/VAR). Whereas ATP9/COXII and N3/N1 are located within the mtDNA the UB/VAR marker is part of the nDNA. The location of the remaining markers developed in this study is unknown, with the exception of AC8 showing 60% similarity to a part of the *mep*B gene encoding an 82 kDa intracellular metalloproteinase of *Aspergillus fumigatus* (Ibrahim-Granet and D'Enfert, 1997).

2.3. PCR and restriction analysis

DNA regions were amplified using corresponding primer pairs (Table 1). Amplification reactions were performed in 50 μl volumes containing 10 mM Tris–HCl, 50 mM KCl, 3 mM $MgCl_2$, 15–60 pmol of each primer, 50 μM of each deoxynucleotide triphosphate, 2.5 U *Taq* polymerase and 30–50 ng of template DNA. Samples were amplified through 30 cycles as follows: initial denaturation for 10 min at 95 °C; denaturation for 60 s at 95 °C; annealing for 60 s at the corresponding temperature and extension for 2–3 min at 72 °C. This was followed by a final extension step of 7 min at 72 °C.

The PCR products (22.5 μl) were digested using 6 U of the corresponding restriction enzyme (Table 1) for 3 h.

2.4. Screening for polymorphisms using single-strand conformation polymorphism (SSCP) and microsatellite analyses

Restriction products or PCR amplicons in 20 μl volumes were mixed with 2 μl 1% sodium dodecyl sulphate, 10 mM ethylenediamine tetraacetic acid (EDTA) (for denaturation) and 2 μl of loading buffer (98% formamide 10 mM EDTA, 0.15% bromophenol blue and 0.15% xylene cyanol) denatured at 95 °C for 15 min, followed by snap cooling on ice. For SSCP, 15–20 μl sample were loaded on 6% MDE Hydrolink acrylamide gels (FMC Bioproducts, Rockland, ME, USA) and run in 1x TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8]) for 4.5–16.5 h at constant power, 40 and 10 W, respectively.

For microsatellite analysis, only 2–5 μl of the PCR product without SDS and denaturation were loaded on 12% acrylamide gels (Rotiphorese® Gel 29:1, 40%, Carl Roth, Karlsruhe, Germany). Gels were run for 4–5 h at 40 W (constant power). Gels were silver-stained as follows: fixation in 1% HNO_3 (Merck, Darmstadt, Germany) for 10 min, incubation in 0.2% $AgNO_3$ (Merck, Darmstadt, Germany) for 20 min, then in 0.28 M $NaCO_3$ (Merck, Darmstadt, Germany) plus 1.5 ml formaldehyde (in 3 l) for 20–30 min.

The reaction was stopped by incubation in 10% CH_3COOH (Merck, Darmstadt, Germany). Between each step the gels were washed in Aqua-bidest. for 5–10 min and finally dried on GB 002 gel blotting paper (Schleicher & Schuell, Dassel, Germany) using a gel drier.

2.5. Alignment and analysis

Sequences were assembled and aligned using the STADEN package (Staden et al., 2000) and CLUSTAL V (DKFZ, Heidelberg, Germany), respectively. Since microsatellite repeats are known and indels were shown to mutate in a stepwise fashion, each was coded as present or absent in a 0/1 matrix and analyzed as single events. The phylogenetic tree of the combined data set (including and excluding the microsatellite loci) was obtained by parsimony analysis using Branch-and-bound as well as Heuristic search options implemented in PAUP 4.0b8 (Swofford, 2000). Only 5 (ATP9/CYTII, AC4, 6, 8, N3N1) out of 13 DNA markers yielded PCR products with the species *M. vanbreuseghemii* known to be the nearest neighbor of the *M. canis* complex (data not shown). However, since only an incomplete set of sequence data was available for this outgroup species, the tree was midpoint rooted.

3. Results

3.1. Development of markers

A large number of clones (110) with a rather low number of simple sequence repeat motifs were detected. Only 10 of the developed primer pairs performed well (Table 1). Coding and non-coding regions of genes (UB/VAR, N3/N1 and ATP9/COXII) were amplified using primers created on the basis of the known mRNA sequences of these genes in *M. canis* or in the related dermatophyte species *T. rubrum*. PCR products generated were in the range of 260–670 bp. Using SSCP and microsatellite analyses, the amplified fragments were screened for DNA polymorphisms. While the DNA marker UB/VAR was subjected to SSCP analysis in undigested form, the larger products of the remaining two DNA markers were digested with an appropriate restriction enzyme (Table 1) to generate smaller pieces ensuring the detection of single nucleotide polymorphisms (SNPs). The DNA marker ATP9/COXII consisted of 625 bp, corresponding to positions 2632–3256 of the 5248 bp locus spanning several genes and tRNAs of the mitochondrial DNA of *T. rubrum* (de Bièvre and Dujon, 1992; *T. rubrum* IP1817.89, accession no. X65223). The fragment covered the second exon and the group 1 intron of the ATPase 9 and initial exon of the cytochrome oxidase II gene. Most of the variability was found in the intron region (240 bp) of the ATPase 9 gene and the spacer (197 bp) between the two genes. N3/N1 was 670 bp in size and located at positions 6600–7270, covering the last 291 bp of the subunit 3 and the first 112 bp of the subunit 1 of the NADH dehydrogenase gene including a spacer region of about 270 bp (de Bièvre and Dujon, 1999; *T. rubrum* IP1817.89, accession no. Y18476). The two SNP's found were located in the subunit 3 gene and the spacer region, respectively. The UB/VAR marker was about 260 bp in length, spanning positions 467–724 of the 3′ non-translated region of the ubiquitin gene (Kano et al., 2001, *M. canis* VUT 77055, accession no. AB035543). The polymorphisms within this marker were due to two SNPs and two indels of respectively 10 and 1 nucleotide as well as one incomplete microsatellite repeat. All but one (intron variation) polymorphisms within the AC8 marker reflect substitutions within the third codon positions. The location of this marker was between 1463–1960 bp of the *mep*B gene encoding 149 amino acid and an intron of 66 nucleotides (Ibrahim-Granet and D'Enfert, 1997).

3.2. Analysis of alleles

Two hundred five strains were analyzed in this study using 13 molecular markers displaying different discriminatory levels, with two to six alleles per marker (Table 2) and seven genotypes for the combined data set of sequences (Fig. 1). Some of the polymorphisms were detected by sequencing only. Thus using analysis of SSCP types only less genotypes were present (2–4; Table 3). Therefore the following results refer to the sequencing data. Three markers (C772, AC1 and AC6) with the lowest discriminatory potential distinguished the strains of *M. canis* (including *M. equinum* and *M. distortum*) from the remaining strains. The markers N3/N1, AC3, AC4 and AC12 revealed three alleles/genotypes over the entire set of isolates investigated. While N3/N1 was unable to differentiate between *M. audouinii* and *M. ferrugineum* but did differentiate between *A. otae* CBS 495.86 (MT+) and *M. canis*, the remaining three markers showed different character states between the recognized main species *M. canis*, *M. audouinii* and *M. ferrugineum*, but not between *M. canis* and its teleomorph CBS 495.86 (MT+). Discrimination of the main species including separation of *A. otae* was possible with UB/VAR, ATP9/COXII, AC8 and AC9. Five alleles were present with AC11, where the fifth allele revealed an additional microsatellite polymorphism among three *M. audouinii* strains. The marker with the highest discriminatory power (six alleles) was C 235, harbouring a microsatellite with the largest number of repeat motifs (11–14 GT repeats) analyzed in this study. Although no polymorphism was observed within *M. audouinii*, an additional allele was detected with this marker in three *M. ferrugineum* strains and a further one in a *M. canis* strain. None of the additional alleles and genotypes corresponded to the morphologically entities described as *M. equinum*, *M. distortum*, *M. rivalieri* or *M. langeronii*.

3.3. Multilocus genotyping

Phylogenetic analysis of the multilocus genotypes including microsatellite and non-microsatellite regions based on

Table 2
Distribution of the allele frequencies among the main species of the *Microsporum canis* complex

Locus	∑ Allele	Frequencies of alleles						
		M. ferrugineum		*M. audouinii, M. rivalieri, M. langeronii*		*M. canis, M. equinum, M. distortum, A. otae* CBS 495.86		
		11[a]	3[a]	47[a]	3[a]	1[a]	139[a]	1[a]
C235	6	0.054	0.015	0.24		0.005	0.68	0.005
AC11	5	0.07		0.23	0.015	0.005	0.68	
UB/VAR	4	0.07		0.24		0.005	0.68	
ATP/COXII	4	0.07		0.24		0.005	0.68	
AC8	4	0.07		0.24		0.005	0.68	
AC9	4	0.07		0.24		0.005	0.68	
AC3	3	0.07		0.24		0.69		
AC4	3	0.07		0.24		0.69		
AC12	3	0.07		0.24		0.69		
N3/N1	3	0.31				0.005	0.68	
C772	2	0.32					0.68	
AC1	2	0.32					0.68	
AC6	2	0.31				0.69		

[a] Number of strains.

all 65 informative sites of the 13 polymorphic markers with both the branch-and-bound and heuristic search options of PAUP produced one parsimonious tree of 80 steps. The tree (Fig. 1) shows strongly concordant clustering distinguishing *M. canis* from *M. audouinii* and *M. ferrugineum*. No polymorphism proved to be available to distinguish strains phenetically identified as *M. equinum* and *M. distortum*, taxa that had been synonymized with *M. canis* on molecular grounds, nor for *M. rivalieri* and *M. langeronii*, purported synonyms of *M. audouinii*. However, the *M. audouinii*

Table 3
Multilocus genotype of polymorphic markers including SSCP and microsatellite patterns (MSG)

Marker	**UB/VAR**																	**N3/N1**			**ATP9/COXII**									**AC1**	**AC3**									**AC4**			
Position	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**124**	**128**	**168**	**191**	**192**	**193**	**SSCP**	**189**	**506**	**SSCP**	**96**	**134**	**160**	**189**	**239**	**291**	**419**	**473**	**SSCP**	**36**	**SSCP**	**124**	**133**	**134**	**154**	**169**	**174**	**175**	**176**	**184**	**SSCP**	**39**	**126**	**159**	**SSCP**
Mf	C	T	A	A	C	T	T	G	A	C	A	-	A	C	C	T	B	C	A	B	A	T	T	A	T	G	A	T	B	A	B	A	G	G	G	A	-	-	-	C	B	C	A	C	B
Ma / Ml / Mr	-	-	-	-	-	-	-	-	-	-	*	-	*	*	*	*	D	*	*	B	T	A	A	C	G	*	*	-	D	*	B	*	*	A	*	*	*	*	*	T	B	T	*	*	D
Mc / Md / Me	*	*	*	*	*	*	*	*	*	*	T	T	G	A	*	*	E	*	G	D	*	*	*	*	*	*	*	-	B	G	D	G	A	*	A	C	A	A	C	*	D	*	T	T	E
Ao CBS 495.86	*	*	*	*	*	*	*	*	*	*	*	-	*	-	-	-	F	T	*	B	*	*	*	*	*	T	T	-	E	*	B	G	A	*	A	C	A	A	C	*	D	*	T	T	E
	AC6	**AC8**										**AC9**				**AC11**															**AC12**														
	7	**SSCP**	**8**	**44**	**68**	**160**	**191**	**344**	**377**	**437**	**SSCP**	**266**	**100**	**119**	**SSCP**	**99**	**100**	**101**	**102**	**103**	**104**	**136**	**168**	**176**	**226**	**227**	**228**	**321**	**333**	**SSCP**	**16**	**17**	**43**	**87**	**95**	**134**	**180**	**203**	**227**	**246**	**247**	**281**	**289**	**SSCP**
Mf	A	B	C	C	T	T	T	C	T	G	B	G	G	T	B	A	G	A	A	G	A	C	A	T	A	G	A	A	T	B	C	C	T	C	C	C	C	C	G	C	G	T	G	B
Ma / Ml / Mr	*	B	*	T	*	*	*	T	*	*	D	*	A	C	D	*	*	*	-	-	-	A	C	G	*	*	*	*	C	B	T	*	C	T	T	T	T	A	T	*	*	*	D	
Mc / Md / Me	G	D	T	*	*	C	C	*	*	*	E	*	*	C	B	-	-	-	-	-	*	C	G	*	*	*	*	C	D	*	A	*	T	*	*	*	*	*	A	C	A	E	
Ao CBS 495.86	G	D	*	*	C	*	*	*	C	A	F	C	*	C	E	*	*	*	*	*	*	C	G	*	*	*	G	C	E	*	A	*	T	*	*	*	*	*	A	C	A	E	
Ma 133/Af4/E47																*	*	*	-	-	-	A	C	G	-	-	-	*	C	F														
	C772						**C235**																																					
	33	**49**	**50**	**51**	**52**	**MSG**	**19**	**32**	**36**	**41**	**43**	**55**	**56**	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**MSG**									
Mf	C	C	T	G	T	B	T	A	T	G	G	G	T	G	T	G	T	G	T	G	T	G	T	G	T	A	C	T	A	T	-	-	G	T	B									
Ma / Ml / Mr	*	*	*	*	*	B	*	*	*	*	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	E									
Mc /Md/ Me	T	-	-	-	-	D	-	C	C	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	F									
Ao CBS 495.86	*	*	*	*	*	B	*	*	*	A	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	-	-	G									
Mc G12							-	C	C	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	H									
Mf E64/E71/E72							*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	T	*	*	D									

Positions of the polymorphisms given correspond to the 3′ end of the forward primers. Vertical number columns in each top line give base positions (e.g., 14–193) of polymorphic sites of the 13 DNA markers (UB/VAR-C235). Symbols: Mc = *M. canis*, Md = *M. distortum*, Me = *M. equinum*, Ma = *M. audouinii*, Ml = *M. langeronii*, Mr = *M. rivalieri*, Mf = *M. ferrugineum*, Ao = *Arthroderma otae*. The letters in the SSCP/MSG columns show the different profiles which not always correspond to the polymorphisms found by sequencing. The letters of the remaining columns correspond to base positions A, C, G and T. (*) Same base as in the core sequence; (–) deletion.

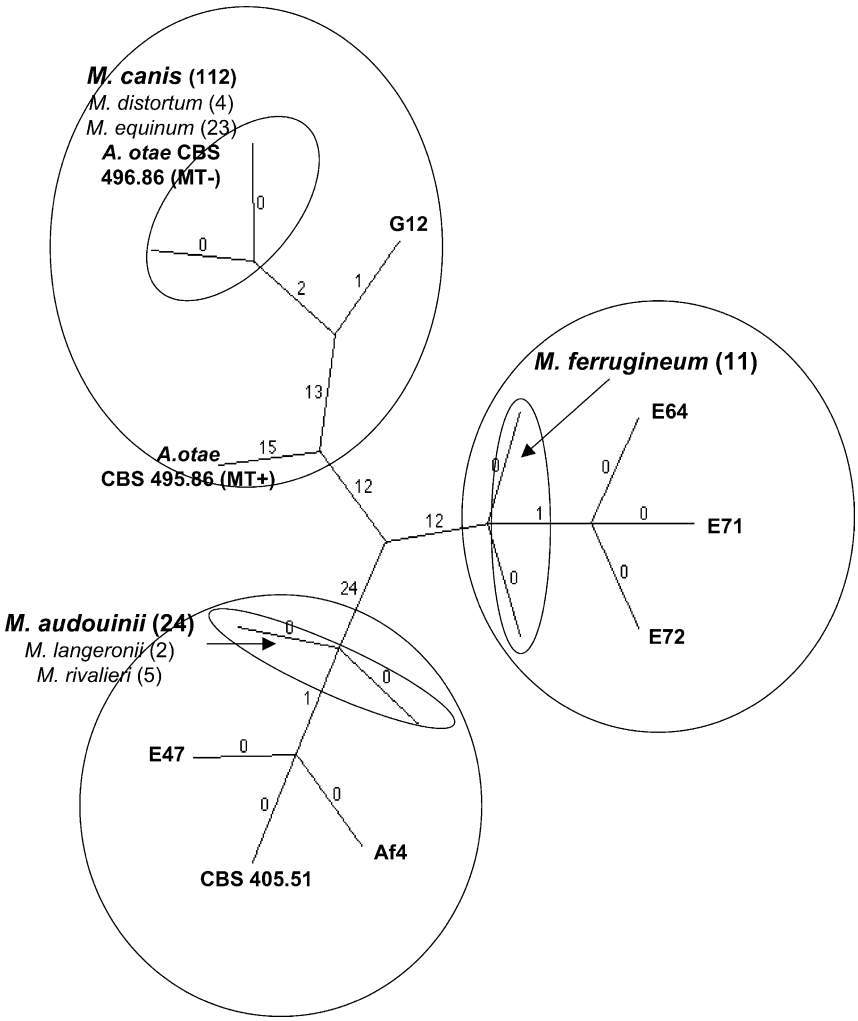

Fig. 1. Phylogenetic relationships of the species of the *M. canis* complex inferred from a combined data set (microsatellite and non-microsatellite regions) of 77 polymorphic characters of 13 DNA markers. The cladogram presented was generated by parsimony analysis using the branch-and-bound option of PAUP 4.0. Branch lengths are displayed by numbers which correspond to the total nucleotide changes assigned to each branch by PAUP. The homoplasy index was 0.0375. The tree was midpoint rooted. Numbers in brackets refer to the number of strains found in the cluster.

strains were subdivided into two groups (Fig. 1) by one microsatellite polymorphism (loss of an "AGA" repeat) in the AC11 marker. One of the three strains separated was an authentic strain of *M. langeronii*, CBS 408.51, which was isolated in 1951 in Zaire. A second authentic strain of *M. langeronii* (CBS 405.61) proved to be identical to *M. audouinii*. Of the two remaining strains of this AC11 subgroup, Af4 and E47, one originated from Nigeria and the other from the UK. Among the *M. canis* isolates, 10 out of 13 markers revealed a subgrouping separating the MT + strain (CBS 495.86) of *A. otae*. Subgrouping of strains of *M. ferrugineum* (two groups) and *M. canis* (two groups) was due to the microsatellite polymorphisms within the C235 marker. Three (E64, E71 and E72) out of 10 strains of *M. ferrugineum* originating from Thailand and one strain of *M. canis* (G12) from Austria were separated in different clades.

4. Discussion

The *A. otae*/*M. canis* complex forms a single clade within the dermatophytes (Gräser et al., 2000) and clearly consists of three distinct subgroups (Fig. 1). In the present study, this basic substructure was robust, recurring with different algorithms and including the use of repeats as individual mutations versus singular events. The structure corresponds to earlier data based on ITS rDNA sequences (Gräser et al., 2000). This suggests that ITS, despite its low resolution at the species level, is indeed predictive to detect main relationships within the dermatophytes. The slight morphological differences underlying the former species *M. langeronii* and *M. equinum* were again found to be taxonomically insignificant. Apparently a single molecular entity may encompass several morphospecies whose separation has not yet been expressed in general phylogenetic distance. The morphological

differences may have resulted from selective pressures acting on a small number of genes (Bruns et al., 1989), e.g. related to hair perforation in *M. equinum*. The occurrence in dermatophytes of different morphotypes with exactly the same genotype (so far explored) seems to be in conflict with most other fungal groups, where single morphological entities frequently comprise several cryptic species defined by molecular characters (Taylor et al., 2000). This contradiction may be due to the very recent evolution of the zoo- and anthropophilic dermatophytes, where the time passed after the emergence of often pleiotropic phenetic changes may have been too short to accumulate a sufficient number of random mutations to be detected. On the other hand, some molecular mavericks without distinct morphological features encountered in the present study show that the DNA markers used are sufficiently polymorphic. They each deviated in only a single locus.

Hypervariable domains were analyzed in order to reveal precise relationships between evolving entities and to allow a hypothetical reconstruction of the course of evolution. The remarkable molecular distance between + and − mating types of *A. otae* found in our study confirmed earlier ITS data (Gräser et al., 2000). In accordance with species concepts enfolded earlier in dermatophytes (Summerbell, 2002), we presume that the intermating community of *A. otae*/*M. canis* must represent an ancestral condition. The genotype of the + mating type is represented by only a single strain, whereas the − mating type is identical to the preponderant genotype, which is considerably different in several concordant markers (Table 3). This suggests that sympatric speciation takes place already during the period where mating ability is maintained. Clonal lineages thus can emerge within a single biological species. It may be supposed that actual mating events have diminished and that recombination no longer occurs in nature or is highly reduced. One of the mating types may subsequently have become rare due to random genetic drift or due to lower fitness on the new host. Loss of effective crossing suggests that a soil borne phase such as wild animal burrows, where mating partners could meet, is no longer required. As cats presently are the main reservoir for *M. canis* (Pier and Moriello, 1998), the suggestion is allowed that a host shift from burrowing animals (e.g., wild lions in Africa) to ranging felines may have accompanied this evolutionary step. The genotype of the + mating partner may now be on the brink of becoming extinct, as has been suggested earlier by several workers (Weitzman and Padhye, 1978; Hironaga et al., 1980; Hasegawa and Usui, 1975). Possible explanations for the overwhelming distribution of one of the mating types may be a higher degree of pathogenicity to the animal. World wide only a handful of strains with the + mating type were discovered by crossing experiments, all of them in Japan (Hironaga et al., 1980; Kubo et al., 1987; Hasegawa and Usui, 1975). However, since mating experiments with African isolates, the possible origin of one of the derived anamorphic species, *M. audouinii* were never performed, this does not give a clue to the origin of *A. otae*.

M. audouinii and *M. ferrugineum* are derived from one of the two mating partners of *A. otae* (11 apomorphic characters in five independent markers; UB/VAR, AC3, AC4, AC6, AC12; Fig. 1, Table 2), possibly from an intermediate genotype which has become extinct. Since that lineage was already associated with stray animals and thus mating was already becoming a rare phenomenon, the emergence of clonal lineages seems logical from that time onwards. Since the entire anamorph clade is anthropophilic, the event leading to the extant species may have been the host shift from stray animals to humans. The source of domesticated cats and dogs in Northern Africa and the Middle East seems to be a likely candidate.

This scenario matches with suggestions of Vanbreuseghem (1950) that the early evolution of the *M. canis* complex may have taken place in Africa. Wild large cats such as lions would then be a probable host for the ancestral, mating populations that should have accumulated relatively many mutations; the populations are predicted to be more variable than the presently analyzed strains. Domestication of cats and dogs originated in Northern Africa and the Middle East, suggesting a later evolution of the presently predominant genotype of *M. canis*, which now has become a common fungus on animals with loose furs and has a world wide distribution. *M. ferrugineum* is more or less endemic to Southeast Asia. It is sterile and does not seem to have changed after its first emergence. The origin of *M. audouinii* is puzzling. It is strongly represented in Africa, but its molecular similarity to *M. ferrugineum* suggests a common ancestor of the two species, which emerged after a host shift from domesticated animals to humans and now has become extinct. In order to explain the larger distance to *M. audouinii*, we have to assume accelerated evolution of *M. audouinii*, possibly as a result of the very small effective population size of the intermediate genotype being in a status of extinction.

Acknowledgements

We are very thankful to Richard Summerbell, Centraalbureau voor Schimmelcultures, Utrecht The Netherlands, for helpful discussion of the results. For providing the collection of strains and their morphological identification we kindly thank Mary Moore from the Department of Medical Mycology, King's College London, UK, Karl-Heinz Böhm and Ute Siesenop from the College of Veterinary Medicine, Hannover, Germany, Hans-Jürgen. Tietz from the Department of Dermatology (Charité-Virchow) of the Humboldt University, Berlin, Germany, Walter Buzina from the Laboratory for Mycology and Molecular Biology, ENT-University Hospital, Graz, Austria, Nuri Kiraz from the Department of Microbiology, Osmangazi University Faculty of Medicine, Eskisehir, Turkey, Roberto Arenas from the Department of Dermatology, General Hospital 'Dr Manuel Gea Gonzalez', Mexico City, Mexico, Jeong Aee Kim from the Department

of Dermatology, Seoul National University, Seoul, Korea, S.B. Suh from the Catholic Skin Clinic, Taegu, Korea, Y.S. Lee from the Mycology Department of the National Institute of Health, Korea, Y.H. Won from the Department of Dermatology, Chonnam National University, Kwangju, Korea and H.J. Yu from the department of Dermatology, Inha University, Kyongki, Korea.

We thank Riccarda Müller, Antje Konietzny and Mrs. Deutsch for excellent technical assistance. Funding was provided by the Deutsche Forschungsgemeinschaft, GR 1147/4-1 to Y. Gräser.

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