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Original Article

Physiological and molecular characterization of atypical lipid-dependent *Malassezia* yeasts from a dog with skin lesions: adaptation to a new host?

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Three lipid-dependent *Malassezia* isolates (here named 114A, 114B and 114C) recovered from a dog with skin lesions were phenotypically and genotypically characterized. All presented ovoid cells and buds formed on a narrow base. Most of the results from physiological tests were consistent with those of *Malassezia furfur*. The phylogenetic analysis of ITS-1 and LSU nucleotide sequences was concordant in placing all three clinical Malassezia isolates close to *M. furfur*. However, the phylogenetic data on the *chs*-2 sequence revealed that clinical isolate 114A is distinct from *M. furfur* and was closely affiliated to the sequence of *M. pachydermatis* with high nodal support. In particular, lipid-dependent isolates 114A displayed *chs*-2 sequences similar (100%) to that of the non-lipid dependent species *Malassezia pachydermatis*. The presence of the genetic and physiological polymorphisms detected in these three isolates of *M. furfur* could have resulted from a process of adaptation of this anthropophilic species to a new host.

Keywords dog, lipid-dependent yeasts, *Malassezia furfur*, molecular characterization and physiological characterization

Introduction

Members of the genus *Malassezia* are lipophilic, non-mycelial, unipolar budding yeasts characterized by a thick cell wall [1]. They are common commensals of the skin of animals, but may cause infections under the influence of predisposing factors [2–5]. Currently, 14 species are included in the *Malassezia* genus of which 13 are lipid-dependent and one is non-lipid dependent (i.e., *Malassezia pachydermatis*) [3,6,7]. Nine of the 13 species (i.e., *Malassezia furfur, Malassezia sympodialis*, *Malassezia globosa*, *Malassezia obtusa*, *Malassezia slooffiae*, *Malassezia restricta*, *Malassezia dermatis*, *Malassezia japonica*, *Malassezia yamatoensis*) have been isolated from normal human skin in association with *pityriasis versicolor*, folliculitis, seborrhoeic dermatitis and, sometimes, atopic dermatitis [3,8]. Conversely, *M. pachydermatis*, *Malassezia nana*, *Malassezia caprae*, *Malassezia equina* and *Malassezia cuniculi* are closely associated with animals [5–11]. Lipid-dependent yeasts may be isolated from otitis externa in cats and cows (i.e., *M. nana*), from healthy skin sites or mucosa in goats (i.e., *M. caprae*) and horses (i.e., *M. equina*), or from rabbit skin (*M. cuniculi*) [6,7,11]. *M. pachydermatis* is frequently recovered from the skin and/or mucosa of dogs and cats, in which it can cause chronic dermatitis and external otitis [8,12,13]. The presence of *Malassezia* lipid-dependent yeasts in dogs has been rarely reported [12,14–17]. However, lipid-dependent strains from

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healthy skin sites of dogs have been molecularly characterized using DNA sequence analysis of the chitin synthase 2 gene (*chs*-2), the large subunit (LSU) and the internal transcribed spacer 1 (ITS-1) of nuclear ribosomal DNA [18]. The recent isolation of lipid-dependent *Malassezia* strains from a dog with severe skin lesions [19] stimulated us to perform the present study that aimed to investigate the physiological, morphological and molecular features of the above-mentioned lipid-dependent yeast strains.

Materials and methods

Malassezia isolates and their phenotypic identification

A 14-year-old female poodle suffering for a chronic pruritic skin disease was presented at the Veterinary Medical Teaching Hospital, University of Rio Grande do Sul, Porto Alegre, Brazil. A sample was collected from the diseased skin with a piece of fitted sterile carpet and cultured onto modified Dixon agar at 32°C for 7 days [20].

Eleven colonies were isolated and three of them (i.e., 114A, 114B, 114C), selected on the basis of their colony diameters (i.e., ranging from 2–4 mm), were sub-cultured onto modified Dixon agar (mDA). The isolates were sent to the Faculty of Veterinary Medicine at the University of Bari (Italy), preserved in 40% glycerol solution at −80°C and recovered using mDA by incubation for 4–5 days at 32°C.

The morphological characteristics of the colonies were recorded and gram staining was performed to assess the morphology of the cells. To evaluate their physiological characteristics we employed the following tests: growth on medium without lipid supplementation, i.e., Sabouraud Dextrose Agar (SAB; Liofilchem Diagnostici, Roseto degli Abruzzi, Italy) at 32°C for 10 days; enzyme production (β-glucosidase and catalase; Liofilchem Diagnostici, Roseto degli Abruzzi, Italy); assimilation of different Tweens (i.e., Tweens 20, 40, 60, 80; Sigma-Aldrich, Italy) and cremophor EL (PeG 35 castor oil, Sigma-Aldrich, Italy); pigment production on tryptophan-based medium; and growth on mDA at 32 and 40°C [20–22]. Yeasts were grown in 3 ml of modified Dixon broth at 32°C for 7 days before molecular processing. *M. furfur* CBS1878 and CBS7019 were used as reference strains and processed as described above. All isolates were subjected to molecular characterization. Isolates have been deposited in the fungal collection of the Faculty of Veterinary Medicine at the University of Bari (Italy), with the following code numbers CD864 (114A), CD865 (114B) and CD866 (114C).

PCR amplification and sequencing

Genomic DNA from each isolate and from the reference strains was extracted from 1 ml of the culture (containing ~1–2 × 10^8 cells), as described previously [18]. The *chs*-2 gene (~540 bp) was amplified from genomic DNA by the polymerase chain reaction (PCR) using the primers CED1 and CED2, the ITS-1 region (~282 bp) was amplified using the primers 18SF1 and 5.8SR1 and the D1/D2 regions of the LSU rRNA gene (~640 bp) were amplified using the primers F63 and LR3 [18]. Genomic DNA (4 µl) was added to the PCR mix (46 µl) containing 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3 and 50 mM KCl, 250 µM of each dNTP, 50 pmol of each primer and 1.25 U of Ampli *Taq* Gold (Applied Biosystems, Milan, Italy). The PCR was performed in a thermal cycler (Gene Amp PCR System 2700; Applied Biosystems, Milan, Italy) at 94°C for 12 min (polymerase activation), followed by 25–30 cycles of 94°C for 1 min (*chs*-2) or 30 sec (LSU and ITS-1) (denaturation), respectively; 55°C (LSU) and 60°C for 1 min (*chs*-2) or 15 sec (ITS-1) (annealing); 72°C for 2 min (*chs*-2) or 1 min (LSU) or 15 sec (ITS-1) (extension), followed by 7 min at 72°C (final extension). Amplicons were resolved in 2% w/v agarose (Gellyphor®, Euroclon, Milan, Italy) gels, stained with ethidium bromide (10 mg/ml) and then purified using Ultrafree-DA columns (Amicon, Millipore; Bedford, USA) and sequenced directly using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems, Milan, Italy) employing an automated sequencer (ABI-PRISM 377; Applied Biosystems, Milan, Italy). Sequences were determined from both strands (using the same primers as used for the PCR) and the electropherograms were checked visually. Sequences were aligned using the program Clustal X [23] and compared with *Malassezia* sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov) (Table 1).

Cloning and sequencing of chs-2 amplicons

Chitin synthase 2 amplicons of the three *Malassezia* isolates (114A, 114B and 114C) were cloned in order to confirm the results of the sequence analysis. In particular the PCR products of the *chs*-2 gene of each isolate were purified using a PCR Extract Mini Kit (5PRIME, Inc., Bucksfield Road, Gaithersburg, USA) and cloned into the pGME-T Easy Vector using the pGME-T Easy Vector System I (Promega Corporation, Woods Hollow Road, Madison, USA). For transformation, JM109 competent *Escherichia coli* cells were used. Recombinant clones containing an insert of the correct size were selected and confirmed by PCR using T7 (5'-TAA-TAC-GAC-TCA-CTA-TAG-GG-3') and SP6 (5'-ATT-TAG-GTG-ACA-CTA-TAGAA-3') primers and colonies as template. DNA of the recombinant clones (i.e., 20 for each isolate) were extracted using DNeasy Blood & Tissue Kit, protocol "Purification of Total DNA from Animal Tissues" (Qiagen, GmbH, Hilden, Germany). The *chs*-2 gene (~540 bp) was amplified and amplicons were resolved as above.

Table 1 Strains of *Malassezia* species and accession numbers available in the GenBank™ database (http://www.ncbi.nlm.nih.gov/Genbank/index.html), used to examine the phylogenetic relationships for chitin synthase 2 (*chs*-2) gene, first internal transcribed spacer (ITS-1) and large subunit (LSU).

	Strain*/Accession number		
Malassezia species	*chs*-2	ITS-1	LSU
Malassezia caprae	CBS10434/EF140653	CBS10434/AY743656	CBS10434/AY743616
	CBS9967/EF140649	CBS9967/AY743647	CBS9967/AY743618
	CBS9973/EF140650	CBS9973/AY743657	CBS9973/AY743615
Malassezia sympodialis	CBS9968/EF140659	CBS9968/AY743640	CBS9968/AY743627
	CBS9970/EF140660	CBS9970/AY743638	CBS9970/AY743620
	CBS7222/EF140661	CBS7222/AY387157	CBS7222/AY743626
	CBS8740/EF140662	CBS8740/EF140668	CBS8740/EF140670
Malassezia dermatis	CBS9169/EF140655	M9931/AB070360	CBS9169/AB070361
Malassezia equina	CBS9969/EF140645	CBS9969/AY743641	CBS9969/AY743621
	CBS9986/EF140647	CBS9986/AY743645	CBS9986/AY743622
Malassezia nana	CBS9557/EF140648	CBS9557/EF140666	CBS9557/EF140671
	CBS9558/EF140654	CBS9558/EF140667	CBS9558/EF140673
Malassezia globosa	CBS7966/EF140665	CBS7966/AY387132	CBS7966/AY743604
Malassezia slooffiae	CBS7956/EF140663	CBS7956/AY387146	CBS7956/AY743606
Malassezia restricta	CBS7877/EF140664	CBS7877/AY387143	CBS7877/AY743607
Malassezia yamatoensis	CBS9725/EF140658	CBS9725/AB125261	CBS9725/AB125263
Malassezia pachydermatis	CD74/DQ915507	CD74/DQ915503	CD74/DQ915500
	CD32/DQ915508	CD32/DQ915504	CD32/DQ915501
	CD50/DQ915509	CD50/DQ915505	CD50/DQ915502
		CD72/DQ915506	
		CD172/EU158829	
		CD177/EU158827	
		CD77/EU158826	
		CD89/EU158828	
Malassezia japonica	CBS9431/EF140642	CBS9431/EF140669	CBS9431/EF140672
Malassezia obtusa	CBS7876/EF140656	CBS7876/AY387137	CBS7876/AY743629
Malassezia furfur	CBS1878/EF140640	CBS7982/AB019335	CBS7982/AY387224
	CBS7019/EF140641	CBS7984/AB019334	CBS7984/AY387226
		CBS7985/AY387129	CBS7985/AY387225
		CBS7984/AY387130	CBS7710/AY387213
		CBS7982/AY387128	CBS6000/AY387209
		CBS6000/AY387113	CBS7867/AY387211
		CBS7969/AY387103	CBS4172/AY387198
		CBS7710/AY387117	CBS7865/AY387212
		CBS4172/AY387102	CBS7969/AY387199
		CBS7865/AY387116	CBS7860/AY387210
		CBS7860/AY387114	
		CBS7867/AY387115	

*CBS: CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; CD: *Malassezia* strains cultured at Faculty Veterinary Medicine University of Bari, Italy.

Phylogenetic analysis

To examine the phylogenetic relationships, the nucleotide sequences of each gene of the three *Malassezia* isolates (114A, 114B and 114C) and of *M. furfur* (CBS1878 and CBS7019) were analyzed by MEGA 3.0 [24] and compared with sequences of different *Malassezia* species available in GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) for each locus investigated (Table 1). Phylogenetic analysis was conducted by Neighbor-joining (NJ) and Maximum Parsimony (MP) methods using *Cryptococcus neoformans* (accession nos. XM569357 and AF444326) for the *chs*-2 and ITS-1 genes and *Filobasidiella neoformans* (accession no. AF075484) for the D1/D2 domains of the LSU rRNA gene, as outgroups. The length, consistency index, excluding uninformative characters, and the retention indices of each most parsimonious tree were recorded. Bootstrap analyses (1050 replicates) were conducted using heuristic searches and tree bisection reconnection (TBR) branch-swapping to determine the relative support for the clades in the consensus trees.

The nucleotide sequences reported in this paper are available in the GenBank™ database (http://www.ncbi.nlm.nih.gov) under the accession nos. HM177251-HM177265.

Results

All *Malassezia* colonies (i.e., 114A, 114B, 114C, *M. furfur* CBS1878 and CBS7019) were opaque, umbonate and had

entire margins. Microscopically, *M. furfur* CBS1878 and CBS7019 appeared as cylindrical cells (4.8–7.2 × 1.1–2.0 μm) and buds were formed on a broad base. Conversely, the clinical *Malassezia* isolates 114A, 114B and 114C appeared as ovoid cells (3.1–5.8 × 1.3–3.1 μm) and buds were formed on a narrow base. The results of physiological tests (except for cremophor EL and tryptophan assimilation) for the clinical isolates were consistent with those of *M. furfur*. In particular, isolates 114A and 114B did not use tryptophan as a nitrogen source. Isolates 114B and 114C assimilated cremophor EL showing an inhibition area around the wells, whereas no assimilation was recorded for isolate 114A.

The PCR amplification from individual samples resulted in amplicons of the expected size of about 540 bp for *chs*-2, 280 bp for ITS-1 and 640 bp for LSU. Sequencing of amplicons representing the three *Malassezia* lipid-dependent isolates revealed three different sequence types for *chs*-2 and LSU and 2 sequence types for ITS-1. LSU sequence types representing *Malassezia* 114A and 114C differed from *M. furfur* reference strains and 114B exclusively by two transitions at position 61 (T↔C) and 509 (A↔G), for 114C and 114A respectively.

By comparing sequence types of *Malassezia* isolates 114B and 114C with those of *M. furfur* CBS1878 and CBS7019, a similarity ranging from 90–96% was found for the *chs*-2 sequence, whereas the similarity ranged from 96–100% for ITS-1 and from 99–100% for LSU (data not shown). The *chs*-2 gene of *Malassezia* 114A showed 100% similarity with that of *M. pachydermatis* (accession number DQ915507), whereas it showed 99% and 100% similarity to the gene of *M. furfur* CBS1878 and CBS7019 for LSU and ITS-1, respectively. In particular, pairwise comparisons among the different clinical *Malassezia* isolates (i.e., 114A, 114B and 114C) and *M. furfur* (CBS1878 and CBS7019), revealed nucleotide variations ranging from 3.7–35.7% for *chs*-2, from 0 to 3.7% for ITS-1 and from 0 to 0.2% for LSU sequences (Table 2).

Table 2 Pairwise comparisons of sequence differences in percentages among sequence types representing clinical *Malassezia* isolates (i.e., 114A, 114B and 114C) and reference strains of *M. furfur* (CBS7019 and CBS1878) for each gene (*chs*-2, ITS-1 and LSU) examined.

chs-2	CBS1878 *M. furfur*	CBS7019 *M. furfur*	114A	114B
CBS1878 *M. furfur*	-----			
CBS7019 *M. furfur*	4.6	---		
114A	34.8	35.7	---	
114B	3.7	6.9	35.0	----
114C	10.4	7.1	35.7	11.7
ITS-1	CBS1878 *M. furfur*	CBS7019 *M. furfur*	114A	114B
CBS1878 *M. furfur*	-----			
CBS7019 *M. furfur*	0.0	----		
114A	0.0	0.0	----	
114B	0.0	0.0	0.0	----
114C	3.7	3.7	3.7	3.7
LSU	CBS1878 *M. furfur*	CBS7019 *M. furfur*	114A	114B
CBS1878 *M. furfur*	-----			
CBS7019 *M. furfur*	0.0	------		
114A	0.2	0.2	------	
114B	0.0	0.0	0.2	-----
114C	0.2	0.2	0.3	0.2

Twenty DNA clones for *chs*-2 were isolated and sequenced of each *Malassezia* strain (114A, 114B and 114C). No nucleotide differences among clone sequences of each strain were revealed and thus they were used for the molecular and phylogenetic analyses. For the *chs*-2, most of the nucleotide variability ($n = 108$; 62.4%) occurred at the third codon position, whereas the remainder ($n = 65$; 37.6%) was at the first and second position. While 80 nucleotide changes in the *chs*-2 were silent (i.e., did not lead to an amino acid alteration), 93 mutations (i.e., $n = 37$ transitions, $n = 56$ transversions) resulted in an alteration of the *chs*-2 amino acid sequence (Fig. 1). In particular, isolate 114A displayed the highest number of nucleotide changes (i.e., $n = 91$) in the *chs*-2 leading to 41 amino acidic variations (Fig. 1).

Phylogenetic analyses of the present sequence data sets revealed discordance in grouping *Malassezia* sequence types for all the three loci studied (Fig. 2). In particular, the *chs*-2 gene of *Malassezia* isolate 114A clustered with those from *M. pachydermatis* (accession number DQ915507), whereas the ITS-1 and LSU genes clustered with those from *M. furfur*. The phylogenetic analysis of the sequence data of 114B and 114C showed that they are closely related to *M. furfur*.

Discussion

The results of our phenotypical, physiological and molecular characterization of lipid-dependent *Malassezia* isolates obtained from a dog with skin lesions, suggest the occurrence of genetic variants of *M. furfur* on the skin of dogs. Indeed, the phenotypic characterization revealed that cells of isolates 114A, 114B, 114C differ from those of *M. furfur* reference strains (CBS1878 and CBS7019) appearing as ovoid cells with buds formed on a narrow base. Using physiological tests isolate 114C was identified as *M. furfur*, whereas isolates 114A and 114B did not assimilate tryptophan and/or cremophor EL, thus making them different from *M. furfur*. The occurrence of phenotypic and metabolic variants of *M. furfur*, as well as of *M. pachydermatis*, has already been reported [20,25–27]. Colonies may vary in size and form, thus showing a high degree of cellular pleomorphism, including oval, cylindrical and spherical

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CBS7019 M.furfur MQNIAYLCKL KNSRVWGPDG WKHITVCIVA DGRHKVSSRT LSVLATMGVY QEGVAKNTVR
CBS1878 M.furfur .......... .......... .......... .......... .......... ..........
114A             .R......S. ..HH...K.S .QKVV.V..C ...L.MNA.. .....A..I. ....G....Q
114B             .......... ........G. .......... .......... .......... ..........
114C             .......... .......... .......... .......... .......... .......S..

CBS7019 M.furfur GQPVEMHLYE YTAQISVDGM MRFRSKERGI VPVQIVLCIK EHNRKKINSH RWCFNAFGPV
CBS1878 M.furfur .......... .........R .......... .......... .......... ..........
114A             .T...A.M.. ..T...I.PS LK...A.... ....VL.... ...K...... ..A......L
114B             .......... .........R .......... .......... .......... ..........
114C             .......... ........R. .......... .......... .......... ..........

CBS7019 M.furfur LQPNVYLLLD VGTRPCSKSI YRLW [144]
CBS1878 M.furfur .......... ......A... .... [144]
114A             .....CM... ...M.TAR.. .... [144]
114B             .......... ......A... .... [144]
114C             .......... .......... .... [144]
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Fig. 1 Alignment of the amino acid sequences of clinical *Malassezia* 114A, 114B and 114C cultured from the skin of dog with those from the reference strains of *M. furfur* (CBS7019 and CBS1878).

cells depending to their pathogenicity [20]. Recently, an atypical Tween assimilation pattern was reported for *M. furfur* isolates recovered from patients with and without dermatologic lesions [27,28]. On the basis of the above findings, the atypical tryptophan and cremophor EL assimilation patterns, as well as the atypical morphology found with 114A, 114B and 114C isolates suggest that they might be linked to adaptation of the yeast to a new host species.

The molecular characterization of the clinical isolates was not concordant with the above phenotypic and physiological features. In particular, *Malassezia* isolate 114B might be genetically identified as *M. furfur* for each of the three loci examined since the nucleotide variations fell within the range of 'intraspecific variation' [29–31]. Conversely, *Malassezia* isolate 114A presented a high similarity with the *M. furfur* reference strains for ITS-1 and LSU sequences, but with *M. pachydermatis* for *chs*-2. Similarly, *Malassezia* isolate 114C was genetically identified as *M. furfur* for ITS-1 and LSU, but not for *chs*-2 since the sequence variations in the *chs*-2 region did not fall within the range of 'intraspecific variation' previously reported [6,29].

The phylogenetic analysis of ITS-1 and LSU nucleotide sequences was concordant and placed all three clinical *Malassezia* isolates close to *M. furfur*. However, the phylogenetic data of the *chs*-2 sequence revealed that clinical isolate 114A is distinct from *M. furfur* and was closely affiliated to the sequence of *M. pachydermatis* with high nodal support. The same results were obtained by phylogenetic analysis of the *chs*-2 amino acid sequences (data not shown).

The discrepancies observed in the sequencing of the *chs-2* gene suggest a low genetic resolution of this gene [32] or the occurrence of multiple copies of the *chs*-2 gene in the genome of *Malassezia* isolates as reported for other genes [33]. However, the latter hypothesis could not be confirmed by our cloning experiments since sequence analysis of 20 DNA clones for *chs*-2 of each *Malassezia* strain (114A, 114B and 114C) revealed no nucleotide differences among them. Finally, the lack of concordance between the phylogenetic patterns for each individual gene marker herein examined may indicate that a recombination or horizontal gene transfer may have occurred between this species. A further possibility may be that *Malassezia* strains on the skin of dogs might hybridize, although further studies are needed to confirm this hypothesis. This data is of particular interest considering that the mode(s) of *Malassezia* reproduction is not well known. However, the presence of two karyotypes with a different estimated genome size in *M. furfur* [34] and AFLP genotype heterogeneity of this species [35,36] suggested the possibility of a sexual process. Accordingly, a case of gene horizontal transfer was documented for *C. neoformans* [37]. The presence of a mating type locus in the genome of *M. globosa* [33] also suggested the possibility of sexual reproduction in these yeasts. The identification of multiple genetic variants of *M. furfur* on the skin of an individual host might support either the hypothesis that *Malassezia* spp. have a sexual reproductive phase, or the occurrence of multiple infections, either by different strains of the same pathogenic species or even different species [33,38].

The possibility that *M. furfur* colonized the skin of the dog cannot be ruled out, completely. Few reports described the occurrence of *M. furfur* [14,17] or *Malassezia* lipid-dependent yeasts [12,15,16] on dogs, but their identification was performed only by physiological and morphological assays [12,14,17]. Thus, this is the first report in which an atypical *M. furfur* isolate from a dog has been morphologically, physiologically and molecularly characterized. In particular, *M. furfur*, which is mainly of

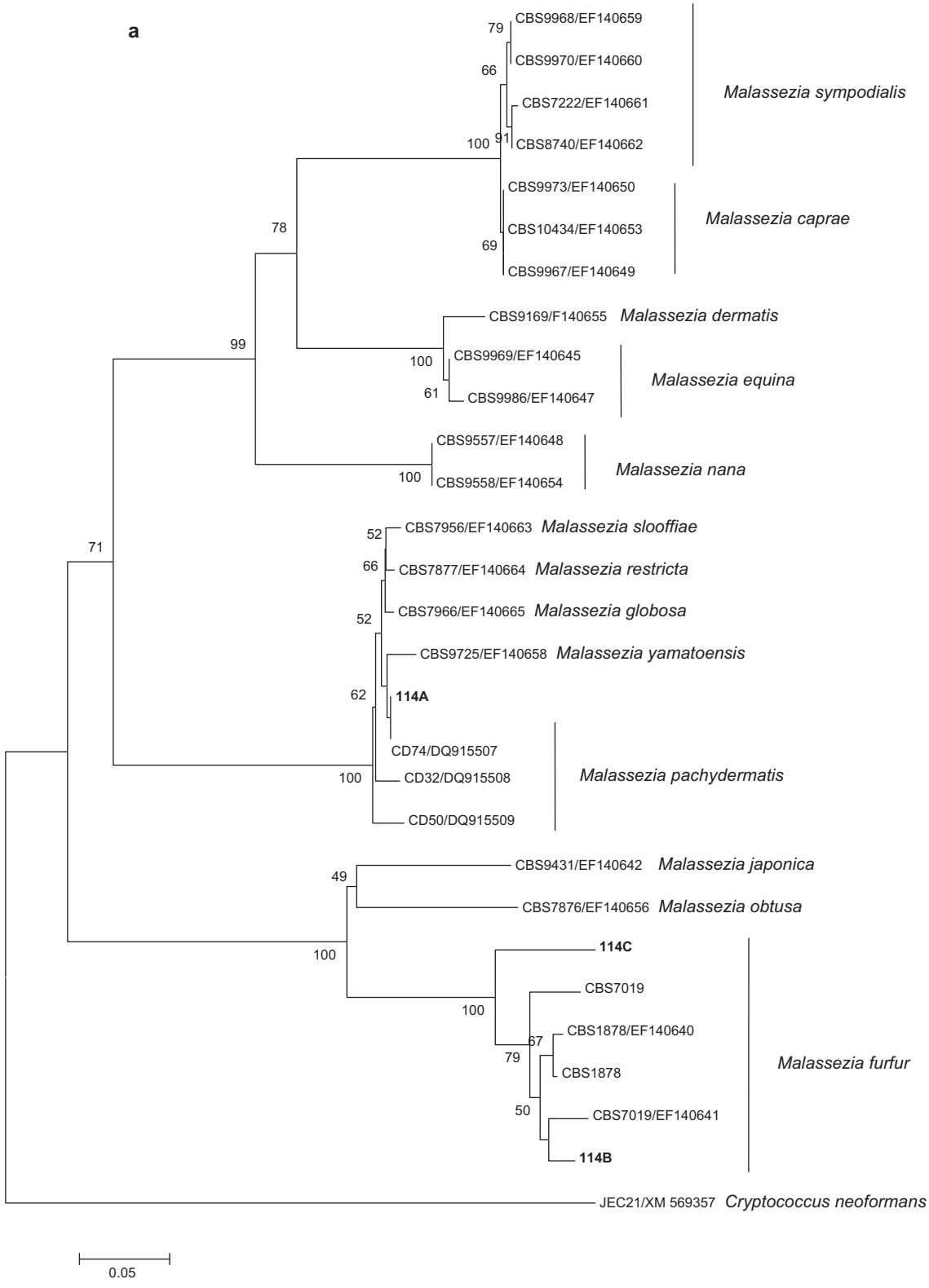

Fig. 2 Phylogenetic analysis of *chs*-2 (a), ITS-1 (b) and LSU (c) sequence data for clinical *Malassezia* isolates 114A, 114B, 114C and reference strains of *M. furfur* (CBS1878 and CBS7019), compared with recognized *Malassezia* species reported with GenBank accession numbers, NJ method (1050 replicates) and *Cryptococcus neoformans* (XM569357 and AF444326) for the *chs*-2 and ITS-1 genes respectively and *Filobasidiella neoformans* for LSU as outgroups were used.

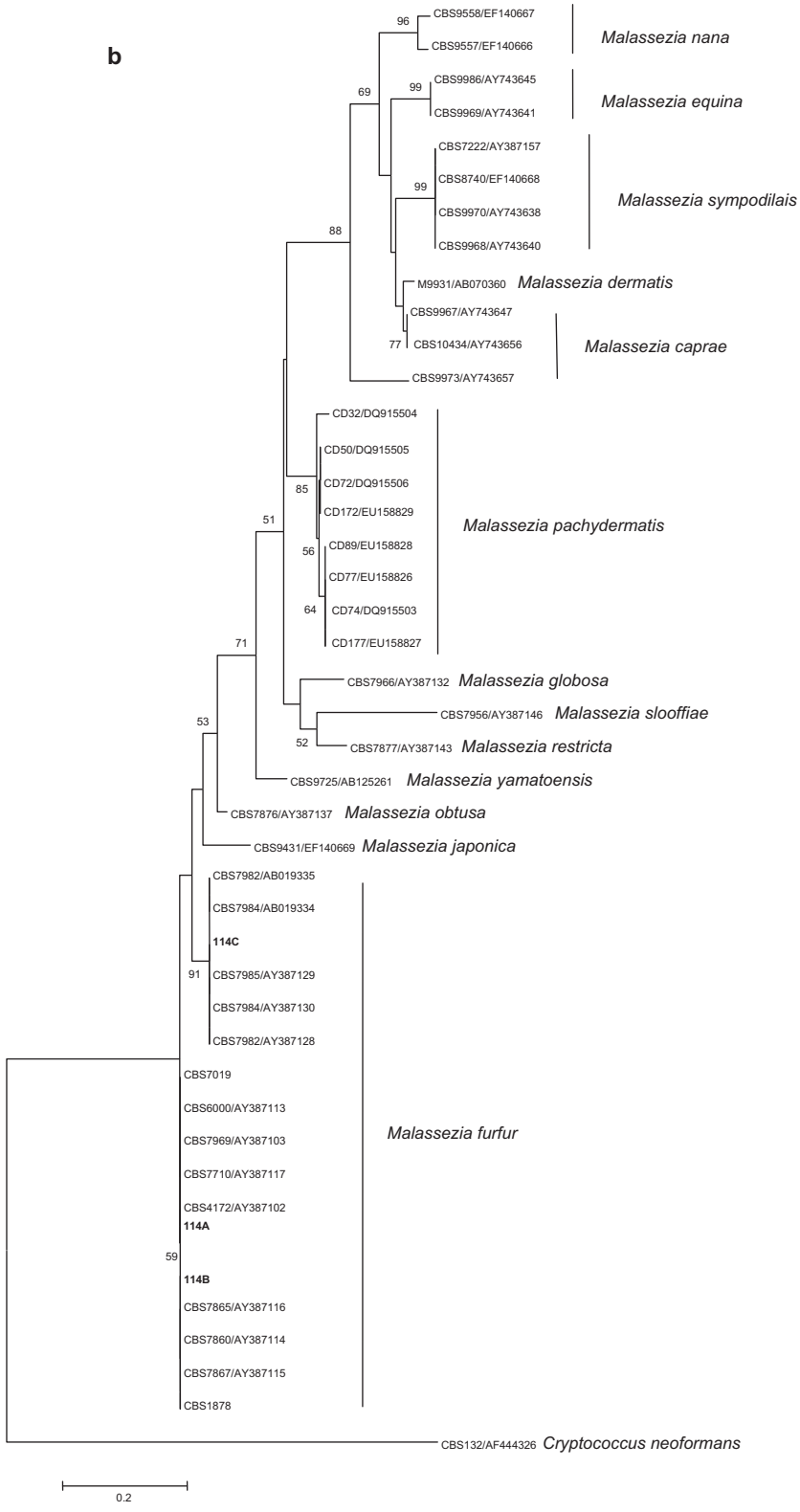

Fig. 2 *(Continued)*

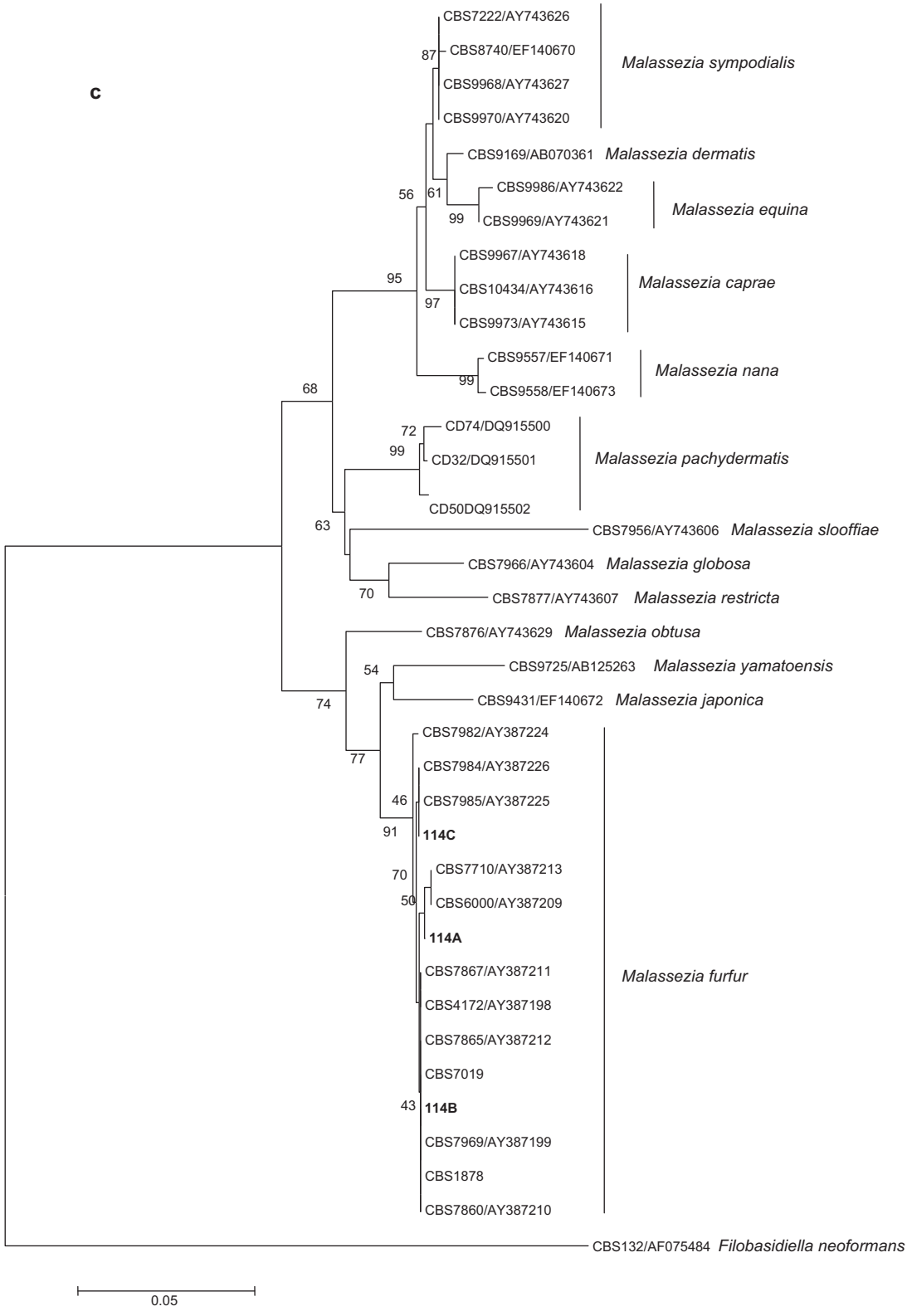

Fig. 2 *(Continued)*

anthropophilic origin, may undergo phenotypic and metabolic changes on the skin of dog as an adaptation strategy to live and to propagate in a new environment with a different chemical composition. These external factors may induce altered gene expression related to the availability of nutrients in certain body regions as has been previously documented for *Candida albicans* [39].

In conclusion, our results suggest that the skin of dogs may be colonized by different genetic variants of *M. furfur*. The presence of genetic and physiological polymorphism in *M. furfur* detected in this study may be due to a process of adaptation of anthropophilic *M. furfur* strains to a new host. The role of sexual or parasexual reproduction in the process of this adaptation, as well as the pathogenic role of these yeasts on the skin of dog should be further investigated.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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