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CHARACTERIZATION OF TYPICAL AND ATYPICAL *MALASSEZIA* SPP. FROM CATTLE AND DOG BY RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

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ABSTRACT

There are few numbers of biochemical tests for specie classification in the genus *Malassezia* and these can to fail in the identification of the atypical isolates. In this study, typical and atypical isolates were analysed by random amplification of polymorphic DNA (RAPD) to compare with biochemical-physiological characteristics of the *Malassezia* species from bovine and canine ears. RAPD band patterns using OPA4 primer clustered all isolates according its biochemical-physiological characteristics in the species from cattle and dog. *Malassezia nana* and *M. sympodialis* isolates were sub-clustered in separated sub-branches and both were from a different branch of the other species. The DNA pattern of the two atypical lipid-dependent *M. pachydermatis* strains was similar with of other typical strains but it did not show the one specific band of 200bp. Future studies in the specific RAPD bands of genetic profiles can be important to corroborate the identification of typical and atypical isolates of the genus *Malassezia*.

KEY WORDS: *Malassezia* spp., identification, atypical isolates, RAPD analysis.

RESUMO

CARACTERIZAÇÃO DE ISOLADOS TÍPICOS E ATÍPICOS DE *MALASSEZIA* SPP. PROVENIENTES DE BOVINOS E CÃES UTILIZANDO A ANÁLISE DO DNA POLIMÓRFICO AMPLIFICADO ALEATORIAMENTE**.** Existem poucos testes para a identificação das espécies do gênero *Malassezia* e estes podem ser insuficientes para a correta identificação de isolados atípicos. O objetivo deste estudo foi caracterizar isolados típicos e atípicos dessas leveduras, comparando características bioquímicas e fisiológicas com a análise do DNA polimórfico amplificado aleatoriamente (RAPD). Foram analisados 30 isolados provenientes do ouvido de bovinos e de cão. Utilizando o iniciador OPA4, os padrões de RAPD foram agrupados de acordo com suas características bioquímicas e fisiológicas. Isolados da nova espécie *M. nana* e de *M. sympodialis* foram subagrupados em dois diferentes sub-ramos, pertencentes a um grupo distinto das outras espécies. Os padrões de RAPD de duas cepas atípicas lipo-dependentes de *M. pachydermatis* foram similares com os de outras cepas típicas dessa espécie, entretanto não apresentaram uma banda específica de 200 pb. Em futuros estudos a caracterização e a análise de sequências de bandas específicas podem corroborar na correta identificação de isolados atípicos e típicos pertencentes ao gênero *Malassezia*.

PALAVRAS-CLAVE: *Malassezia* spp., isolados atípicos, identificação, RAPD.

INTRODUCTION

The genus *Malassezia* is composed by thirteen species of yeasts of increasing importance in human and veterinary medicine. In human they have been associated with pityriasis versicolor (PV), seborreic dermatitis (SD), folliculitis, and systemic infection (Guého et al., 1998; Sugita et al., 2006). *M. pachydermatis*, *M. nana, M. equina* and *M. caprae* could be considered zoophilic (Guého et al., 1998; Sugita et al., 2003, Sugita et al., 2004; Hirai et al., 2004; Cabañes et al., 2007). *Malassezia pachydermatis* is zoophilic and associated with external otitis and dermatitis in dog and cat (Bond et al., 2000). The lipid dependent species *Malassezia globosa*, *M. sympodialis*, *M. furfur* and *M. slooffiae* have been associated with bovine parasitic otitis (Duarte et al., 2001; Duarte et al., 2003).

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The random amplification of polymorphic DNA (RAPD) has been employed to epidemiological studies. The intra-species subtypes obtained with RAPD from samples of *Malassezia* species suggest the presence of genetic population differences that may be an important tool for epidemiological investigation (Boekhout et al., 1998; Theelen et al., 2001). The most important argument to consider the RAPD-PCR as a clearly useful procedure for grouping *Malassezia* species according to their origin may be attributed to the nosocomial and occasionally fatal infections in neonates as reported by Belkum et al. (1994), Chang et al. (1998) e Chryssanthou et al. (2001).

Malassezia furfur isolates from of patients with PV, SD and SD of the HIV positive patients was characterized by RAPD-PCR analyses. A distinct differentiation between isolates from PV and SD patients with or without AIDS was observed (Gandra et al., 2006). Similar results with isolates obtained from patients presenting different dermatosis or systemic infections were observed by BoekhouT et al. (1998) suggesting that an intra-specific genetic variation determine the presence of the different populations of *M. furfur.*

In a recent study this technique was applied for genetic typing of *Malassezia pachydermatis* isolates from different domestic animals. Four different genetic types were distinguished and one predominant genotype was observed in isolates recovered from different anatomical locations in cats, horses, goats and pigs (Castellá et al., 2005).

The identification system based on differences in the ability of *Malassezia* species to grow in the presence of tweens or cremophor EL and to split esculin can fail to identify atypical isolates or new species. Lipid-dependent variants of *M. pachydermatis* and atypical *M. furfur* have needed complementary tests to achieve a correct identification (Duarte et al., 2002). *Malassezia* species can be distinguished by RAPD patterns. In the study of Boekhout et al. (1998), *Malassezia sympodialis* and *M. slooffiae* showed relative uniform banding patterns with OPA4 and OPA5 respectively.

The objective of the present study was to compare the random amplified polymorphic DNA (RAPD) profiles with specific biochemical-physiological characteristics of typical and atypical *Malassezia* isolates from bovine and canine ears in Minas Gerais State of Brazil.

MATERIAL AND METHODS

A total of 30 isolates from ear cattle and ear dog in Minas Gerais State of Brazil were identified based on morphological characteristics, growth on Dixon medium at different temperatures, growth on Sabouraud's medium supplemented with tweens and cremophor EL in diffusion test, esculin hydrolise and catalase screening (Mayser et al., 1997; Gueho et al., 1998).

The typical and atypical isolates of *Malassezia* spp. and two standard strains used in the RAPD genetic analysis were listed in the Table 1. As controls for the biochemical-physiological tests and genetic analysis the standard strains CBS-1878 (*Malassezia furfur*) and CBS-1879 (*Malassezia pachydermatis*) were simultaneously tested with each set of 10 to 18 isolates. The isolates of the novel species *M. nana* (now standard strains) were identified and characterized by DNA sequence analysis in a previous study (Hirai et al., 2004). The identification of the atypical lipid-dependent strain of *M. pachydermatis* (VG Luz 794,) two typical *M. furfur* strains from cattle with otitis (VG Ig 02 and VG RC 4349) and the atypical strain (C Lerik,) were confirmed by rDNA sequence analysis of D1/D2 domains in Duarte et al. (2002).

Following the biochemical-physiological tests of the species identification, the isolates were seeded on solid Dixon medium and incubated for 5 days at 32º C. DNA was prepared as described by Borges et al. (1990) after enzymatic digestion with glucanase (Glucanex- Novo Nordisk, USA).

The following primers were used: M13 F, M13 R, M13 FR, OPA 1, OPA 2, OPA 4, SOY, 8L AND 10L for RAPD reaction. The RAPD analyses were carried out as described by Williams et al. (1990). Reproducibility was checked by repeating PCR for at least three times. RAPD products were analyzed by electrophoresis on a 8% polyacrilamide gel in Tris borate-EDTA, pH 8.0 buffer and visualized by silver staining as described by Santos et al. (1993). Isolates were considered with similar genetic types varying up to two DNA bands of electrophoresis on polyacrilamide gel.

For RAPD data analysis the relative mobility position of all bands present in each analyzed *Malassezia* strain were calculated and transformed in a data matrix where the character one means the presence of a specific band and zero represents its absence. We used the Nei & Li algorithm contained in the TREECON computer package program to calculate the genetic distances between the strains (Van De Peer; Wachter, 1994). The Unrooted phenograms were then constructed by UPGMA (Unweighted Pair Group with Arithmetic Mean) method and the robustness of the tree topology was assessed 1,000 bootstrap resampling.

RESULTS AND DISCUSSION

The primers M13 F (5'-TGACCGGCAGAAAAA TG-3'), OPA 2 (5'-TGCCGAGCTG-3') and OPA 4 (5'-

AATCGGGCTG-3') produced more polymorphic and reproductive profiles. These oligonucleotides were selected based on high-intensity bands, hypervariability and good definition of DNA fragments. The OPA 2 and OPA 4 primers were also used by BOEKHOUT et al. (1998).

A total of 15 electrophoretic profiles on 8% polyacrilamide gels and their phenograms were selected. Their analysis, supported by bootstrap values higher than 80%, demonstrated that the RAPD band patterns using OPA4 primer clustered all isolates according their biochemical-physiological characteristics. Using the M13 F and OPA2 primers 83.4% of isolates were clustered according to their biochemical-physiological characteristics.

Table 1 - Typical and atypical isolates of *Malassezia* spp. from ear cattle and ear dogs in Minas Gerais State of Brazil and standard strains used in the RAPD genetic analysis.

Strains	Hosts	Origin
Malassezia furfur		
VH Fl Sueli	Holstein cow	Florestal/Centre
BH Fl Cóia	Holstein calf	Florestal/Centre
VH Pm Paloma	Holstein cow with otitis	Pará de Minas/Centre
VH Pm Duqueza	Holstein cow	Pará de Minas/Centre
VG Rc 4349	Gyr cow with otitis	Rio casca/east
VG Ig 02 *(AY 072789)	Gyr cow with otitis	Igarapé/Centre
C Lerik *(atypical strain, AY072790)	Ear dog	Belo Horizonte/Centre
CBS 1878	Pityriasis capitis (human)	R.W. Benham
Malassezia sympodialis		
VM Pom Morena	Hybrid cow	Pompéu/Centre
VM Pom Sortuda	Hybrid cow	Pompéu/Centre
VG Luz 188	Gyr cow	Luz/Centre
VG Luz 345	Gyr cow with otitis	Luz/Centre
VG Luz 808	Gyr cow with otitis	Luz/Centre
VG Luz 973	Gyr cow with otitis	Luz/Centre
VG C 13	Gyr cow	Caeté/Centre
NG C 05	Gyr Heifer	Caeté/Centre
Malassezia nana		
CBS 9558 (VG Luz 776)	Gyr cow with otitis	Luz/Centre
CBS 9559 (VG Luz 979)	Gyr cow with otitis	Luz/Centre
CBS 9560 (VSF Geléia)	European cow	Florestal/Centre
CBS 9561 (VM Pom Passarela)	Hydrid cow	Pompéu/Centre
Malassezia slooffiae		
VZ Jor 111	Zebu cow	Jordânia/North
NG C 06	Gyr cow	Caeté/Centre
VH L 22	Holstein cow	Lavras/South
VM Mu 344	Hybrid cow	Muriaé/East
VM Pom Palhaçada	Hybrid cow	Pompéu/Centre
TG Rc 01	Gyr bull	Rio Casca/East
VG Rc Oiana	Gyr cow	Rio Casca/East
Malassezia pachydermatis		
VH L Baliza	Holstein cow	Lavras/South
VH L Tiroleza	Holstein cow	Lavras/South
BM Mc 77 (atypical lipid-dependent strain)	Hybrid calf	Montes Claros/North
VG Luz 794 (atypical lipid-dependent strain)	Gyr cow with otitis	Luz/Centre
CBS 1879	Dog with external otitis	Sweden

*GenBank accession numbers are show in parentheses.

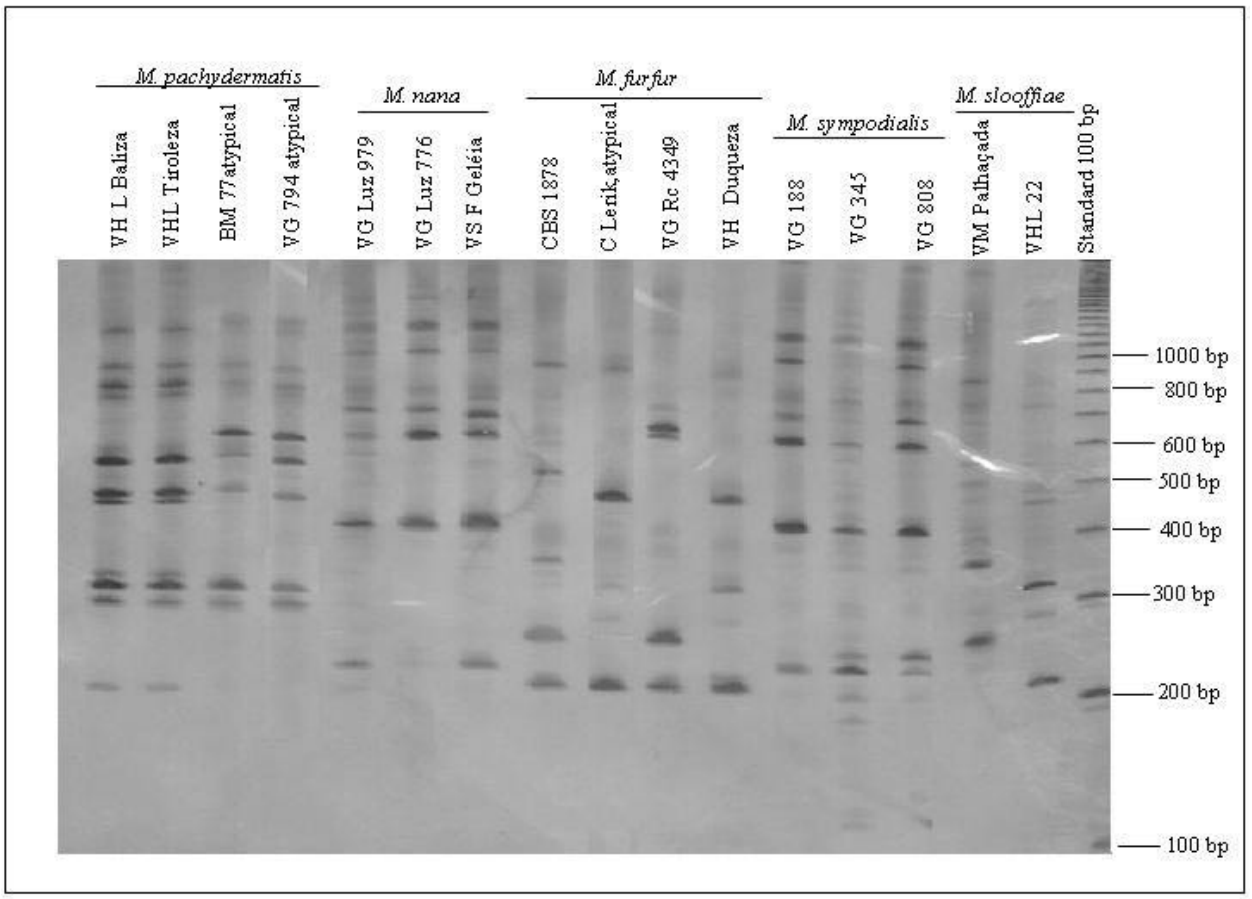

Fig. 1 - Eletrophoretic profiles in 8% polyacrylamide gel and phenogramas of different *Malassezia* species by RAPD-PCR using the OPA2 primer.

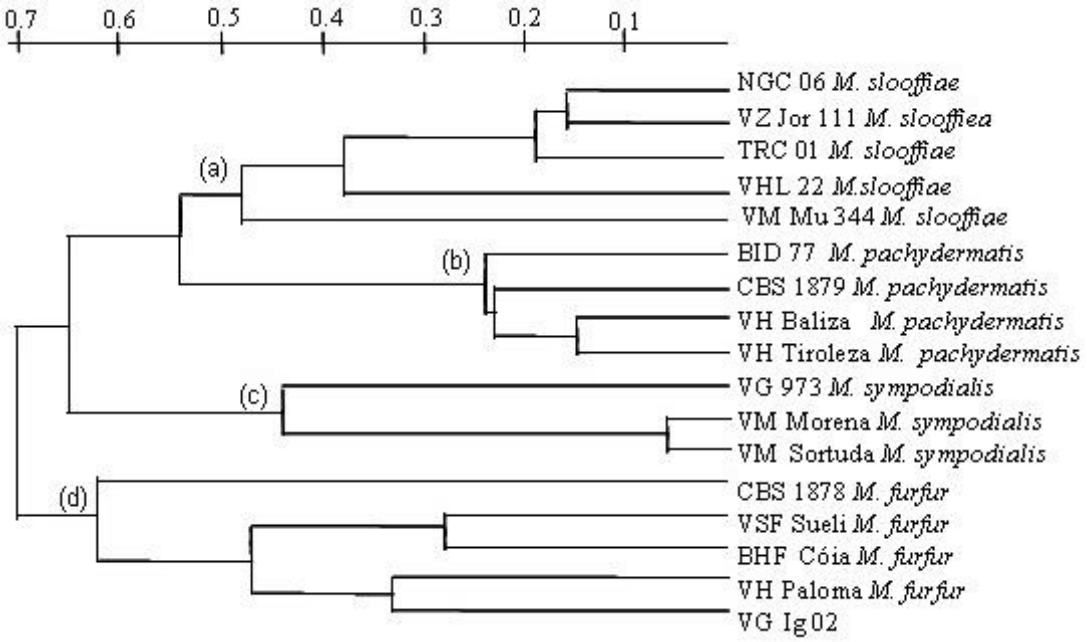

Fig. 2 - Phenogram constructed by UPGMA method of different *Malassezia* species derived from RAPD assays generated by using OPA2 primer.

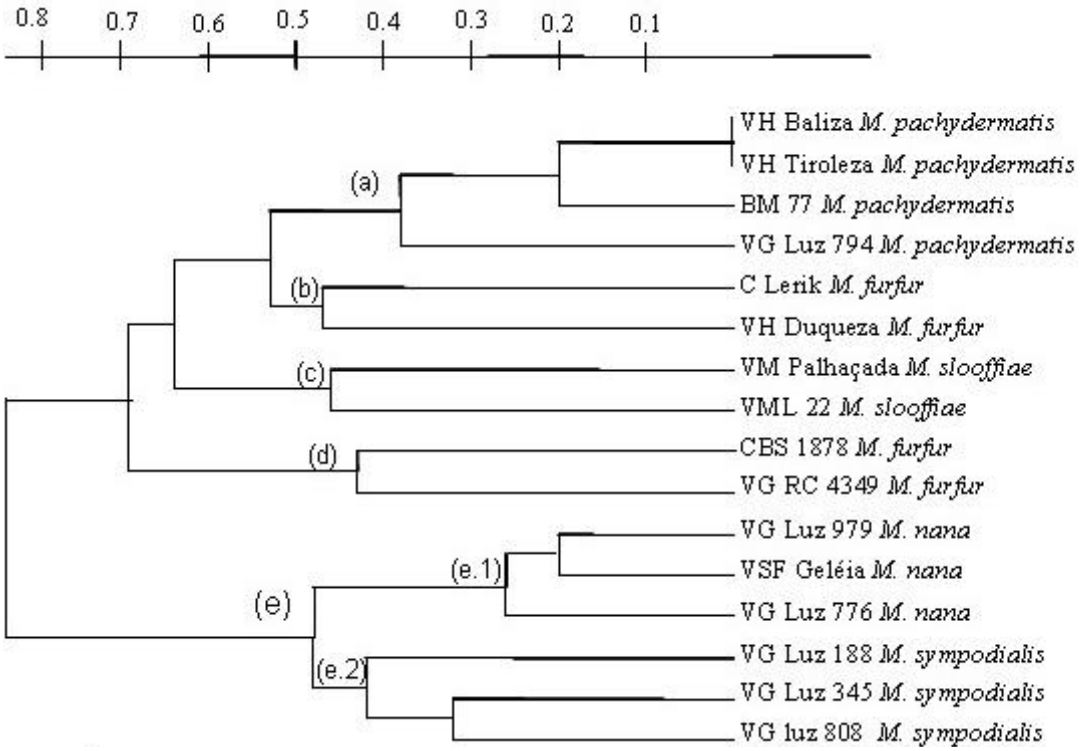

Fig. 3 - Phenogram of different *Malassezia* species based on UPGMA method derived from RAPD assays generated by using OPA4 primer.

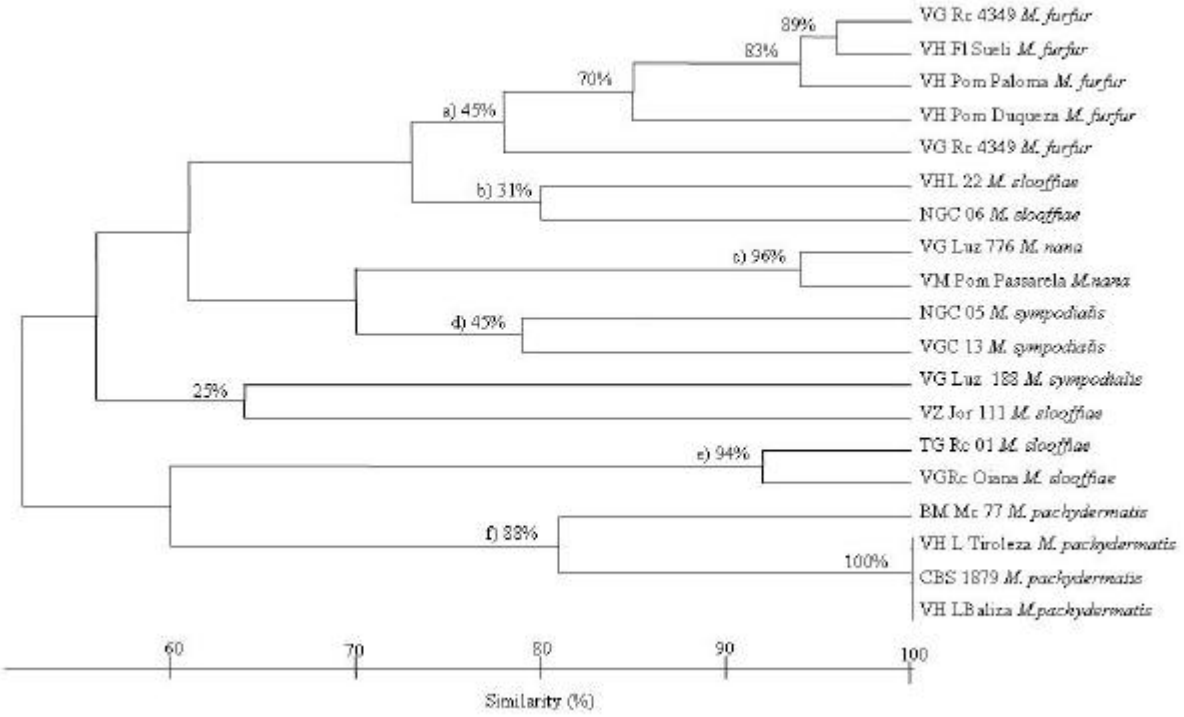

Fig. 4 - Phenogram constructed by UPGMA method of five *Malassezia* species derived from RAPD assays generated by using M13 primer.

Better DNA amplification of *M. furfur* and *M. sympodialis* isolates were observed using the OPA 2 primer while the DNA polymorphism of *M. slooffiae* strains was better demonstrated using the primer OPA4. We detected 13.22 +/- 3.191 (average +/- the standard deviation) bands for *M. furfur* strains varying

from 100 to 1200 bp using the OPA2 primer, 22 +/- 3.823 bands for *M. sympodialis* strains varying from 100 to 1400 bp using the OPA2 primer. Using the OPA4 primer we detected 17.59 +/- 3.726 bands for *M. slooffiae* strains varying from 100 to 1400 bp.

Figure 1 shows the electrophoretic profiles of five different *Malassezia* species generated by RAPD-PCR with OPA 2 primer. The Figures 2, 3 and 4 show phenograms of *Malassezia* species based on UPGMA method derived from RAPD assays and generated by using OPA2, OPA4 and M13 primers, respectively. All strains analyzed were demonstrated in these phenograms.

The atypical strains *Malassezia* were characterized in our previous study. The C Lerik strain (*M. furfur* from dog) did not grow on Sabouraud´s medium supplemented with cremophor EL. The atypical isolate VG Luz 794 of *M. pachydermatis* never grew on Sabouraud medium without lipids and BM Mc 77 atypical strain showed poor growth (Duarte et al., 2002). The DNA pattern of the two atypical lipid-dependent *M. pachydermatis* strains was similar with of other typical strains of this specie (Figs. 2, 3 and 4) but it did not show one band of 200bp present in typical strains. One specific band about 600 bp could be observed to these two atypical strains (Fig. 1). The four *M. furfur* strains in this figure showed different DNA patterns. However, all these strains had an identical 200 bp band and the atypical strain C Lerik was similar with the typical strain *M. furfur* VH Duqueza.

In this study, RAPD analysis is shown to be an important complementary procedure for the characterization and identification of atypical strains in the genus *Malassezia*. It was corroborated by rDNA sequence analysis of D1/D2 domains of these atypical isolates in our preliminary study (Duarte et al. 2002).

Using RAPD-PCR and UPGMA methods, the genetic profiles of the new specie *M. nana* and *M. sympodialis* strains were sub-clustered in separated sub-branches (Figs. 2, e.1 and e.2, respectively). Both were from a branch (e) different of the other species supported by a bootstrapped value of 88%. All *Malassezia sympodialis* isolates had a specific band about 400 bp while *M nana* isolates showed a band higher than 400 bp (Figs. 1 and 2). In our preliminary study, the physiological and morphological characteristics of the *M. nana* are similar in many ways to those of *M. sympodialis*. Phylogenetic trees based on the D1/D2 region of 26S rDNA and ITS1 sequences of *M. nana* isolates from cattle and cat showed that they were related closely to *M. sympodialis* (Hirai et al., 2004).

Using the OPA4 primer, the genetic profiles of *M. slooffiae* isolates were clustered in separated in the Figure 3 (cluster a). Some intra-species variations in *M. furfur, M. slooffiae* and *M. sympodialis isolates* from cattle have been observed using OP2 primer. In the Figure 2 *M. furfur* had two sub-types sub-clustered in separated sub-branches (b and d, respectively). The presence of intra-species RAPD subtypes suggesting different populations of *M. furfur* and *M. pachydermatis* have been related in some studies (Boekhout et al., 1998; Aizawa et al., 2001).

Intra-specific polymorphisms have been observed between DNA patterns of *Malassezia* isolates from different bovine herds in different regions of Brazil (Duarte et al., 2000). The RAPD analysis and phenograms showed the formation of analogous clusters among the isolates from cattle with or without otitis raised in the same herds. These data suggested that genetically similar *M. sympodialis* and *M. furfur* strains, found as members of the normal ear microbiota, could become opportunistically active in the inflammatory process in cattle (Duarte; Hamdan, 2008).

There are few numbers of tested characteristics to species identification in the genus *Malassezia* and these tests can fail to identify atypical isolates. In this study, the RAPD pattern of typical and atypical *Malassezia* isolates from bovine and dogs ears was associated with its biochemical-physiological and morphological characteristics, favouring the species identification. Future analyzes in specific bands of genetic profiles from typical and atypical isolates could corroborate the specie identification in the genus *Malassezia*.

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